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[K1]

Antiproliferative actions of estrogen receptor beta and liver X receptor

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Estrogens control many cellular processes including cellular growth, differentiation and function of the reproductive system. Estrogens interact with two estrogen receptors (ERs), ERα and ERβ, and exert their effects through diverse signaling pathways that mediate genomic and nongenomic events, resulting in tissue-specific responses. Estrogen-regulated gene expression is controlled by a complex array of factors such as ER ligand-binding, the DNA sequence bound by ERs, ER interacting cofactors and chromatin context. The discovery of a second ER, ERβ, in 1995 has prompted renewed efforts to investigate the mechanisms of action of estrogenic molecules. There is now compelling evidence that ERβ is involved in various types of cancer (breast, ovarian, colorectal, prostate, endometrial), in bone and brain physiology, and in the cardiovascular system and inflammation. Recently, global analysis of gene expression profiles and identification of protein-DNA interactions have begun to reveal the molecular architecture of ERβ binding to DNA and the subsequent effects on gene expression. This lecture will discuss the current knowledge of gene regulatory networks influenced through ERβ, as well as novel discoveries pertaining to roles of ERβ in **epithelial-mesenchymal transition (EMT)** and cancer.

Gallbladder cancer is a highly aggressive disease with poor prognosis that occurs more frequently in women than men and evolves from chronic inflammation to dysplasia/metaplasia, carcinoma *in situ* and invasive carcinoma. In female mice in which the oxysterol receptor Liver X Receptor β (LXRβ) has been inactivated, preneoplastic lesions of the gallbladder developed and evolved to cancer in old animals. LXRβ is a nuclear receptor involved in the control of lipid homeostasis, glucose metabolism, inflammation, proliferation and CNS development. No abnormalities were evident in male mice, neither in LXRα-/- nor in LXRα-/-β-/- animals of either gender. Interestingly, the elimination of estrogens with ovariectomy prevented development of preneoplastic lesions in LXRβ-/- mice. The process seems to involve TGF-β signaling since the precancerous lesions were characterized by strong nuclear reactivity of p-SMAD-2 and SMAD-4 and loss of E-cadherin expression. Upon ovariectomy, E-cadherin was re-expressed on the cell membranes and immunoreactivity of p-SMADs in the nuclei was reduced. These findings suggest that LXRβ in a complex interplay with estrogens and TGFβ could play a crucial role in the malignant transformation of the gallbladder epithelium.

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[K10]

Inhibitors for histone modifications as therapeutic intervention

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Steroid hormones are ligands for nuclear receptors and transcription factors that participate in organ development, reproduction, body homeostasis, and stress responses. The reversible -methylation of lysyl side-chains in histones, catalyzed by distinct classes of methyl transferases and demethylases, has emerged as an important mechanism in orchestration of chromatin state, gene regulation and DNA repair. The dynamic exchange of histone lysine methylation status by histone methyltransferases and demethylases has been previously implicated as an important factor in chromatin structure and transcriptional regulation by nuclear hormone receptors. Despite the progress achieved over the past years in understanding regulation and interactions of these enzymes, chemical tools to interrogate their biological functions, especially for the histone demethylases, are lacking.

We have formed an international public private partnership to generate freely available chemical tools to study epigenetic modification systems. For the histone demethylases we have thus far generated chemical leads for 3 subfamilies of Jmj-type demethylases.

Here we present a first attempt to understand kinetic features and active site variabilities in the JmjD2 (KDM4) subfamily of human H3K9 and H3K3 demethylases, members of the Fe2+ and 2-oxoglutarate dependent oxygenases. The combination of high-throughput and focussed library screening efforts in conjunction with x-ray crystallography allowed inhibitor identification of simple chemotypes that were used to map the active site of the JmjD2 subfamily. Differential patterns of crossreactivity across distinct subfamilies of human 2'OGs form the basis of understanding and developing further potent subfamily-specific chemical probes to interrogate functions in steroid hormone biology.

[K11]

Coupling of 11beta-hydroxysteroid dehydrogenase 1 functions to energy supply and NADPH generation in the endoplasmic reticulum

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In human the expression of up to 10% of all genes is modulated by glucocorticoids. The coordinated regulation of glucocorticoid-mediated gene expression requires a highly tissue- and time-dependent regulation. By controlling intracellular concentrations of active glucocorticoids 11beta-hydroxysteroid dehydrogenases (11bHSDs) represent a key mechanism to achieve tissue- and cell-specific sensitivity to glucocorticoids.

Previous studies revealed that the oxoreductase activity of 11bHSD1, and thus glucocorticoid activation, is dependent on the availability of NADPH in the endoplasmic reticulum (ER). The physical interaction with hexose-6-phosphate dehydrogenase (H6PDH) provides efficient delivery of NADPH and allows a functional coupling to glucose-6-phosphate (G6P) levels and thus to energy metabolism. Incubation of HEK-293 cells expressing 11bHSD1 or 11bHSD1 and H6PDH with increasing concentrations of glucose stimulated 11bHSD1 activity. An even more efficient stimulation of 11bHSD1 oxoreductase activity was obtained upon incubation with fructose. Using microsomal preparations, we found that fructose-6-phosphate (F6P) can substitute for G6P and that F6P is sufficient to maintain 11bHSD1 oxoreductase activity. Furthermore, experiments with isolated microsomes indicate that F6P is converted to G6P in the ER lumen. Because F6P is not a substrate of H6PDH and H6PDH does not act as a phosphohexose isomerase, we postulate the existence of a hexose-phosphate isomerase in the ER.

These recent findings reveal that the ER luminal orientation of 11bHSD1 allows fine-tuning of glucocorticoid activation depending on the availability of various hexose-phosphates. Furthermore, recent findings indicate that the ER luminal orientation of 11bHSD1 and NADPH supply by H6PDH are required for the efficient 11bHSD1-dependent conversion of the oxysterol 7-oxocholesterol to 7beta-hydroxycholesterol and of the secondary bile acid 7-oxolithocholic acid to chenodeoxycholic acid. These observations reveal functional interactions between glucocorticoid metabolism and the metabolism of oxysterols and bile acids, respectively.

In conclusion, the ER luminal orientation of 11bHSD1 is essential for the appropriate function of this enzyme. Both the coupling of glucocorticoid activation to energy metabolism and the metabolism of 7-oxysterols and 7-oxo bile acids are dependent on the functional interaction between 11bHSD1 and H6PDH.

[K12]

11ß-Hydroxysteroid Dehydrogenase Type 1 and its role in the hypothalamo-pituitary-adrenal axis, metabolic syndrome and inflammation

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The seminal studies of Harvey Cushing informed us of the deleterious consequences of circulating cortisol excess – hypertension, osteoporosis and obesity that contributes to diabetes and premature mortality. Conversely, Hench, Kendall and Reichstein were Nobel Laureates in Physiology 1950 for the discovery of cortisone and demonstrating efficacy in patients with Rheumatoid Arthritis – in effect the birth of the anti-inflammatory actions of glucocorticoids that has saved thousands of lives ever since.

The tissue-specific generation of cortisol, independent of circulating levels, can be catalysed by 11β-hydroxysteroid dehydrogenase (11β-HSD1) that converts cortisone (E) to cortisol (F). High levels are expressed in liver and omental adipose tissue where the enzyme amplifies glucocorticoid-mediated hepatic glucose output and adipocyte differentiation and obesity. The former effect occurs through induction of hepatic gluconeogenesis (GNG); recombinant mice with global deletion of 11β-HSD1 or liver-specific deletion of the GR demonstrate reduced GNG. Conversely over expression of 11β-HSD1 in both liver and fat reproduce features of the metabolic syndrome.

Selective 11β-HSD1 inhibitors have been developed by several pharmaceutical companies that, in animal models, lower blood glucose, improve insulin sensitivity and cause weight loss. Biomarkers have been validated to confirm target inhibition in primate and human studies. The first clinical trials have recently been published from Incyte and show reduction in HbA1C and blood pressure in obese patients with diabetes mellitus who have failed on metformin therapy. Filed clinical trial data indicates that many similar studies are underway by other companies. Potential the therapy offers a “magic bullet” for patients with Metabolic syndrome with reduced blood glucose accompanying improved insulin sensitivity, lower lipids and hepatic steatosis, and reduced blood pressure consequent upon reduced autocrine generation of cortisol in liver, adipose tissue, pancreas and muscle.

Liabilities include activation of the hypothalamo-pituitary-adrenal axis secondary to increased cortisol clearance with adrenal hyperplasia and hyperandrogenism, though the extent and significance of this is debated. Perhaps of more concern is the potential impact upon the inflammatory process. 11β-HSD1 expression is significantly induced at the site of inflammation where we believe it plays a crucial anti-inflammatory action. It remains to be seen whether 11β-HSD1 inhibitors will either increase susceptibility to or persistence of an inflammatory insult.

Modulation of glucocorticoid hormone action via selective 11β-HSD1 inhibitors represents a novel therapeutic advance to treat the global epidemic of Metabolic Syndrome.

[K13]

A combined genomic and enzymatic study demonstrating a special function of 17beta-HSD1

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Human 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) has been studied extensively since early 1950s due to its important role in the activation of estrogens, especially in the production of estradiol. Its structure was only determined in 1995, facilitating further study of its mechanism. More recently the second enzymatic function, the inactivation of DHT has been revealed at both molecular and cellular levels, based on the pseudo-symmetrical structure of DHT. Such dual function of the enzyme contributes critically to the proliferation of breast cancer cells.

Moreover, 17β-HSD1 can enhance the E2-induced expression of the endogenous estrogen-responsive genes, such as pS2. It also modulates the expression of other important genes and proteins that are relevant to cell growth control, such as PCNA and BCCIP, by DNA array and proteomic analyses on breast cancer cells. These genes and the corresponding proteins are up or down-regulated following 17β-HSD1 overexpression in MCF7 cells. The above results facilitate the improvement of breast cancer therapy, the most incidental cancer in western women.

[K14]

Estrogens and breast cancer in the post-menopausal woman: Importance of where they are made.

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After the menopausal transition, the ovaries cease to make estrogens, yet the incidence of breast cancer increases with aging and the majority of these tumours are ER positive. So, where is the estrogen driving this tumour development coming from? Several extra-gonadal sites synthesize estrogens from circulating C19 steroids, such as bone, brain and adipose. The largest of these depots is the adipose tissue, and increased BMI is associated with increased breast cancer risk as well as increased circulating estrogen levels. It is reasonable to suppose then that most of the estrogen in the circulation of the post-menopausal woman is derived from the adipose depots. So, given the global 'pandemic' of obesity, we are faced with the daunting prospect that tens of millions more women may be at risk of breast cancer in their later years than was previously thought. Yet the mechanisms linking obesity to breast cancer risk are not completely understood, although it is widely assumed that estrogens produced in the fat play a role. Factors increased in obesity such as leptin and insulin appear to increase breast cancer risk, whereas adiponectin, which is decreased in obesity, has been shown to decrease the risk in a number of studies. This appears to be due, in part, to activation of the LKB1/AMPK pathway. The anti-diabetic drug metformin has also been shown to decrease the risk of breast cancer, and it also acts to stimulate AMPK.

We have shown that aromatase expression in breast adipose stromal cells is inhibited by AMPK due to sequestration of the CREB coactivator CRTC2 in the cytoplasm. Moreover, leptin inhibits and adiponectin and metformin stimulate, the LKB1/AMPK pathway in these cells. This is associated with stimulation and inhibition of aromatase expression, respectively. Thus another mechanism is provided whereby obesity can increase breast cancer risk, by increasing local estrogen synthesis in the breast mediated by the actions of adipokines, and metformin can act to reduce this risk.

Keywords: estrogens, breast cancer, obesity, aromatase

[K15]

Estrogen and androgen measurements in breast and prostate tissues: What have we learned?

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Although circulating levels of androgens and estrogens have been measured in numerous studies of prostate and breast cancers, there are a limited number of studies on intratumoral hormone levels in these cancers and how the levels compare to corresponding levels in normal prostate and breast tissues. The limited studies on tissue hormone levels generally have deficiencies; in particular, they lack sufficient controls and have no reference ranges for the androgens and estrogens, due to the difficulty in obtaining the normal tissues. In addition, there is no standard methodology for extracting and purifying the hormones from prostate and breast tissues, and various assay methods have been used to quantify the androgens and estrogens. Nevertheless, certain conclusions can be drawn from some studies. In normal prostate tissue, levels of dihydrotestosterone (DHT) are 20-30 times higher than testosterone (T) levels. In contrast, T is 6-10 times higher than DHT in serum. Finasteride treatment of men with benign prostate hyperplasia results in at least a 6-fold decrease and 10-fold increase in intraprostatic DHT and T levels, respectively. In anorchid men with metastatic prostate cancer, intratumoral androgens are maintained at concentrations capable of activating androgen receptor target genes and maintaining tumor cell growth. Comparison between tumor and normal tissues of the breast show higher intratumoral E_2 levels independent of ER status. Also, lower levels of DHEA, DHEAS and 5-androstene-3β,17β-diol are found in tumor compared to glandular breast tissue, whereas there appears to be no difference in T or androstenedione levels. In both prostate and breast tissue studies, large inter-subject variability is observed in androgen and estrogen values. Also, correlations between tissue and serum levels of these hormones are not well established. More studies are needed to gain a better understanding of differences in androgen and estrogen levels between normal and intratumoral prostate or breast tissues, and how these levels relate to corresponding values in serum.

[K16]

Steady-state Hydroxysteroid/Ketosteroid ratios catalyzed by Hydroxysteroid Dehydrogenases: Can we force an oxidative enzyme to favor reduction?

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Hydroxysteroid dehydrogenases (HSDs) catalyze the interconversion of hydroxysteroids and their cognate ketosteroids, thus regulating intracellular hormone potency. Each HSD shows directional preference in intact cells, favoring oxidation or reduction based on its utilization of nicotinamide cofactors (NAD[P][H]) and existing intracellular cofactor concentrations. Using AKR1C9 and human 17βHSD1, we have shown that the reductive directional preference of these enzymes can be attenuated or reversed with changes in macronutrient supply, which alter intracellular cofactor concentrations, and/or by point mutations in the cofactor-binding domain, which changes the binding affinity for various cofactors. We have sought to explain the magnitude of these changes and the physical chemistry of enzyme-cofactor interactions by determining the kinetic constants for 17βHSD1 mutations and by solving the structures for these R38 mutations with bound $NAD(P)^+$. We find that very slight changes in enzyme structure and cofactor affinity produce marked alterations in steady-state estrone/estradiol ratios in intact cells. This result reflects the moderately high affinity of 17βHSD1 for both NAD(H) and NADP(H). We have constructed additional mutations designed to retain binding of NAD(H) yet preclude NADP(H).

Thomas et al also showed that mutagenesis of two adjacent residues in 3βHSD1, an oxidative HSD, reversed its cofactor preference from NAD(H) to NADP(H). We conducted similar experiments with human 17βHSD2 and 17βHSD6 (oxidative 3αHSD, RODH), but we were unable to reverse the strong oxidation preference in intact cells. Experiments with recombinant enzymes in yeast microsomes indicate that these enzymes have markedly higher affinity for NAD(H) than NADP(H), explaining their resistance to shifts in directional preference. We have designed experiments to probe the active sites of these enzymes, to reveal the biophysical basis for the strong exclusion of NADP(H) binding, which in turn maintains steroid flux favoring oxidation in intact cells.

Keywords: hydroxysteroid dehydrogenase, short-chain dehydrogenase/reductase, estrogen, structural biology

[K17]

New functionalities of steroid metabolizing SDR-enzymes revealed by animal models

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The amount and type of ligand available for receptor binding is one of the key detriments regulating the extent of sex steroid action. In addition to the traditional endocrine action of sex steroids, it is now evident that ligand metabolism in peripheral tissues, at the site of action, also plays a key role in regulating the action of sex steroids. Among these enzymes, we have recently focused on Hydroxysteroid (17-beta) dehydrogenases (HSD17Bs) catalyzing the reaction between the highly active 17β-hydroxy and less active 17-keto steroids. HSD17B-enzymes belong to the short-chain dehydrogenases/reductases (SDR) defined by a common sequence motif but constitute a functionally heterogenous superfamily of enzymes. Our hypothesis is that HSD17B-enzymes play a significant role in the regulating the availability of the highly active ligands for nuclear receptor at the target cells. Currently more than ten different HSD17B - enzymes having individual cell-specific expression profiles, substrate specificities, and regulatory mechanisms have been identified. Among these enzymes we have generated genetically modified loss-of-function and gain-of-function mouse models for HSD17B1, HSD17B2, HSD17B7 and HSD17B12. The data obtained *in vivo* suggest that HSD17B-enzymes are involved in various metabolic routes, thereby metabolizing sex steroid, lipids and sterols. HSD17B1 evidently catalyzes the activation of 17-keto steroids to 17-hydroxy forms, confirmed both in tumor xenograft studies carried out with MCF-7 breast cancer cells stably transfected with human HSD17B1, and with transgenic mice over expressing the human HSD17B1. In contrast, the central role for HSD17B2 in the inactivation of 17-hydroxy steroids to corresponding 17-keto is to be confirmed in vivo, while the data indicate a novel function for the HSD17B2 in the placental development. Identically, we have shown a central role for HSD17B7 in cholesterol biosynthesis and a role for HSD17B12 in the lipid metabolism, while their role in sex steroid metabolism in vivo is still only superficially known.

[K18]

Synthesis and biological evaluation of non-estrogenic steroidal inhibitors of 17β-Hydroxysteroid Dehydrogenase Type 1

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Estrogens play a crucial role in the development and regulation of estrogen-dependent breast cancer through their action on the estrogen receptor (ER). Different enzymes participate in the biosynthesis of estrogens; among these we have focused on 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1), which is involved in the conversion of estrone (E1) into estradiol (E2), the most potent estrogen, as well as in the conversion of dehydroepiandrosterone into 5-androstene-3β,17β-diol (5-diol), a less potent estrogen that becomes more important after menopause. To better control the formation of E2 as well as 5-diol we have focused over the past several years on designing inhibitors of 17β-HSD1. We succeeded by synthesizing C6-alkylamide derivatives of E2 and C16-bromoalkyl derivatives of E2 as competitive reversible and irreversible inhibitors, respectively, but they were unfortunately found to be estrogenic compounds. E2-adenosine (or partial mimic) hybrid compounds were also synthesized and found to be potent competitive inhibitors, but their efficiency dropped when tested in intact cells. Potent and non-estrogenic inhibitors of 17β-HSD1 were however recently obtained by adding an *m*-carbamoyl benzyl group at position 16β of E2 and by blocking the residual estrogenicity of the E2 nucleus by substituting the OH at position 3 by a bromoethyl group. The optimized inhibitor, named PBRM, inhibited the transformation of E1 (60 nM) into E2 by endogenous 17β-HSD1 present in intact T-47D (ER^+) breast cancer cell lines with IC_{50} = 68 nM. It also inhibited the proliferation of T-47D cells induced by 0.1 nM of E1. No estrogenic effect was observed on estrogen-sensitive cells (T-47D) and mouse tissues (uterus and vagina). When tested in T-47D breast cancer xenograft models (nude OVX mice), PBRM (250 µg/d, sc) fully blocked the tumor growth induced by E1 (0.1 µg/d, sc) thus demonstrating its ability to block the transformation of E1 into E2 by 17β-HSD1 *in vivo*.

[K19]

Anticancer agents incorporating the aryl sulfamate pharmacophore: Design, synthesis and clinical translation

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Many breast tumours are hormone-dependent (17ß-oestradiol) with estrogens playing a key role in their growth and development. There is increasing evidence that inhibition of steroid sulfatase (STS), which converts oestrone (E1) sulphate to E1 and also dehydroepiandrosterone (DHEA) sulfate to DHEA, will attenuate oestrogenic stimulation in hormone dependent breast cancer (HDBC). We designed E1-3-*O*-sulfamate (EMATE) as the first potent, orally active, irreversible active site-directed STS inhibitor (IC_{50} 10pM), that reached multiple phase I & II clinical trials for a non-oncology indication. Non-steroidal compounds had even superior activity and a series of highly potent and *in vitro* non-oestrogenic tricyclic sulfamate candidates led to the clinical drug candidate Irosustat [STX64, BN83495]. Early mechanistic work shows that irreversible sulfatase inhibition occurs by a completely novel mechanism.

In vivo, Irosustat is non-oestrogenic, inhibits STS potently and causes regression of E1S-stimulated tumour growth in nude mouse xenografts. Irosustat has 95% rodent oral bioavailability and is sequestered by carbonic anhydrase II (hCAII) in red blood cells. We solved the X-ray crystal structure of the drug bound to hCAII. Irosustat is the first STS inhibitor to enter human clinical trial and, in postmenopausal women with HDBC, is well tolerated orally and very potent, with *ca* 100% targeted tumour enzyme inhibition even at doses as low as 5-20mg and with an elimination half-life of *ca* 30h. A recently concluded Phase I/II clinical trial in women with locally advanced or metastatic breast cancer, who had already been heavily pre-treated with other clinically established agents including aromatase inhibitors, showed evidence of 5/8 evaluable women exhibiting stable disease for up to 7 months. Marked decreases in androstenediol and, more surprisingly, androstenedione were observed. Pre-clinical applicability of this approach to endometriosis, prostate and endometrial cancer has also been demonstrated. A further five phase I & II clinical trials are currently in progress with our drugs Irosustat and PGL2001, in breast, endometrial, prostate cancer and in endometriosis. Second generation agents have also been designed and evaluated. Extension of the concept to dual sulfatase-aromatase inhibition *via* a single molecule has the potential to be a new paradigm in oncology. The potential of other novel steroidal sulfamate-based drugs as anti-angiogenic and pro-apoptopic agents targeted against tubulin and now in preclinical development targeted at *hormone-independent* and particularly taxane-resistant cancer will be discussed. Such compounds are not substrates for efflux pumps implicated in drug resistance. The aryl sulfamate pharmacophore is a powerful new motif for anticancer drug design and steroid sulfatase is a novel and attractive oncology target for clinical intervention.

[K2]

Long-range transcriptional control of progesterone receptor gene expression

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Estrogen receptor α (ERα) binds to specific target DNA sequences, estrogen response elements (EREs), to regulate estrogen-responsive gene expression. The progesterone receptor (PR) gene has been used extensively as a marker of estrogen responsiveness. Although we previously identified cis elements within one kb of the PR-B transcription start site that are associated with ERα and help to confer estrogen responsiveness, the identification of ERα binding sites far removed from the transcription start site suggested that long-range regulation of this gene may occur. We now show that eight regions of the PR gene from 311 kb upstream to 4 kb downstream of the PR-B transcription start site interact with ERα and that coactivator proteins and acetylated histones are selectively associated with these gene regions. Specific PR gene regions confer estrogen responsiveness to a heterologous reporter plasmid and mutation of EREs within these regions diminishes estrogen-induced transactivation. Importantly, chromosome conformation capture assays reveal ERα-dependent interactions between proximal and distal PR gene regions. Taken together, our studies suggest that distal regions of the PR gene participate in the dynamic regulation of this gene and that the coordinated action of proximal and distal PR gene regions allows cells to respond to changes in hormone levels with extraordinary versatility and sensitivity.

[K20]
Efficient strategies for the development of potent and selective steroidogenic enzyme inhibitors

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In recent years, efficient methods for the discovery and development of novel drugs have been developed. Experimental screening of compound libraries with robotics proved appropriate in identifying substances which possess the desired *in vitro* properties. An alternative is the rational design of active compounds,which is either based on known ligands or the (3D-)structure of the protein. In any case, identified active compounds (hits) must be further optimized to lead compounds, i.e., compounds which not only show the desired *in vitro* activity, but also *in vivo* efficacy. Some of these methods will be exemplified by the discovery of active and selective inhibitors of steroidogenic enzymes. The review primarily emphasizes aldosterone synthase (CYP11B2) and 17β-hydroxysteroid dehydrogenase 1 (17β-HSD1). CYP11B2 was first propagated by our group as a target for the treatment of hyperaldosteronism, heart failure,and myocardial fibrosis. The most potent and selective compounds discovered so far will be presented showing IC_{50} values for CYP11B2 inhibition in the pM range, selectivity factors with regard to other CYP enzymes exceeding 1000 and *in vivo* activity in the rat. In contrast to CYP11B2 inhibitors, compounds inhibiting 17β-HSD1 do not unfold their activity on an endocrine level, but act within the target cell – they are potential therapeutics for the treatment of breast cancer and endometriosis. Very potent and selective, nonsteroidal 17β-HSD1 inhibitors will be introduced showing activity in the low nM range and selectivity toward 17β-HSD2 and the estrogen receptors α and β.

[K21]

Ent-Steroids: Novel reagents for studies of membrane receptors and membrane properties

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ent-Steroids are the non-naturally occurring mirror images (enantiomers) of naturally occurring steroids. They are prepared by chemical synthesis and they are useful tools for studying the way that steroids affect the function of membrane bound proteins (e.g., ion channels, G coupled proteins, receptor kinases) involved in cell signaling pathways. Steroid modulation of membrane receptors may result from direct binding of steroids to the receptors or may be caused indirectly by steroid perturbation of the membrane containing the receptor. How can these two types of steroid modulation be distinguished? Potentially, one way to make the distinction is by comparing the effects of the natural steroid enantiomer and its corresponding non-natural enantiomer, the *ent*-steroid. Because enantiomers have mirror image shapes and steroid receptors typically have well-defined binding pockets that discriminate between steroids of different shapes, it is likely that binding of a steroid to its receptor will be enantioselective (i.e., one enantiomer binds more effectively than the other enantiomer). By contrast, membrane lipids in the non-gel state constitute a dynamic fluid environment lacking well-defined binding sites for steroids. Hence, in the membrane, the physiochemical properties of the steroid, not its absolute configuration (one enantiomer or the other), will be the dominant factor affecting membrane properties. Since enantiomers have identical physicochemical properties, the steroid and *ent*-steroid effects on membrane properties will be essentially equivalent (non-enantioselective). Therefore, the direct and indirect effects of steroids on membrane receptor function could potentially be distinguished by differences in the observed actions of the steroid and *ent*-steroid (i.e., direct binding is enantioselective, membrane perturbation is not). Results from *ent*-steroid studies which illustrate the occurrence of both types of steroid modulation of membrane receptors will be presented. Particular emphasis will be given to *ent*-steroid modulation of $GABA_A$ receptors.

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[K22]

Obesogens, stem cells, and the maternal programming of obesity

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Obesity and metabolic syndrome diseases have exploded into an epidemic of global proportions. Consumption of calorie-dense food and diminished physical activity are generally accepted to be the causal factors for obesity. But could environmental factors expose preexisting genetic differences or exacerbate the root causes of diet and exercise? The environmental obesogen model proposes that chemical exposure during critical stages in development can influence subsequent adipogenesis, lipid balance and obesity. Obesogens are chemicals that inappropriately stimulate adipogenesis and fat storage. Tributyltin (TBT) is a high-affinity agonistic ligand for both the Retinoid X Receptor (RXR) and Peroxisome Proliferator Activated Receptor gamma (PPARγ). RXR-PPARγ signaling is a key component in adipogenesis and the function of adipocytes and activation of this receptor heterodimer can elevate adipose mass in rodents and humans. Thus, inappropriate activation of RXR-PPARγ can directly alter adipose tissue homeostasis. We previously showed that TBT promoted adipocyte differentiation, modulates adipogenic genes in vivo, and increased adiposity in mice after in utero exposure. These results are consistent with the environmental obesogen model and suggest that organotin exposure is a previously unappreciated risk factor for the development of obesity and related disorders. Prenatal and early postnatal events such as maternal nutrition, drug, and chemical exposure are received, remembered and then manifested in health consequences later in life. Based on the observed effects of TBT on adipogenesis, we hypothesized that organotin exposure during prenatal adipose tissue development alters the pool of multipotent progenitor cells to favor the subsequent development of adipocytes. We found that TBT exposure during early life alters the balance of progenitor types in the stem cell compartment to favor the production of adipocytes. These results provide a potential explanation for the obesogenic properties of prenatal TBT exposure and illustrate more generally how prenatal exposure to xenobiotic compounds could affect adipogenesis and obesity.

[K23]

Developmental origins of disease: Role of environmental exposures and epigenetics

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It is now clear that all complex diseases have both a genetic and an environmental component. Since the prevalence of many diseases has increased during the last 40yrs it is likely that the increase is due to changes in the environment. There is also increasing evidence that many complex diseases have their origins during development. This has led to a paradigm shift in toxicology called the developmental origins of disease. Indeed data from animal models has shown that developmental exposures in utero or neonatal) to environmental chemicals can lead to increased susceptibility to cancers, infertility, obesity and diabetes, asthma and learning an behavioral problems, and neurodegenerative diseases long after the chemical exposure is gone. The resulting disease is related both to the environmental chemical exposure and the window of exposure during development. So how can short term exposures to environmental chemicals lead to increased incidence of disease years later? The answer seems to be that developmental exposures interfere with epigenetic signaling (DNA methylation, chromatin remodeling or iRNA) resulting in altered gene expression during development that persists throughout life thereby increasing susceptibility to disease. In addition, there are data showing that depending on the chemical exposure and the timing of exposure effects of the exposure may be transmitted across generations. Indeed some effects have been shown to be transmitted via the germ line for 3-4 generations. The mechanism of this new phenomenon is unclear but seems to also involve alterations in the epigenetic signaling pathways. Examples of the developmental basis of disease will be discussed in the presentation along with a discussion of epigenetic pathways and marks and their role in disease pathology across generations.

[K24]

Neonatal exposure to estrogens or environmental estrogens induces aberrant promoter methylation and expression of nucleosomal binding protein (Nsbp1) in later life

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Although genetics plays a critical role in endocrine disorders, it cannot fully explain the great variability in susceptibility among individuals or populations. Recent literature suggests that epigenetics, defined as mitotically heritable but potentially reversible changes in gene function without alterations in nucleotide sequence, may provide a more complete explanation. Epigenetic mechanisms, including DNA methylation, histone modification, and microRNA expression, partition the genome into active and inactive domains based on endogenous and exogenous hormonal and metabolic cues and developmental stages, creating phenotype plasticity that can explain inter-individual and -population variability in physiological responses, and in disease susceptibility and progression. In this presentation, we will use rodent models to address the issue of whether early-life exposure to diethylstilbestrol, genistein, bisphenol A and estradiol can predispose to later-life risk in development of uterine or prostate malignancies. The methylation status of a 5' CpG-island located in the nucleosomal binding protein 1 (*Nsbp1*) gene was identified as an epigenetic mark altered in early-life through exposure to these estrogens. However, the phenotypic expression of this epigenetic mark significantly varied between male and female, and was found to be dependent on the presence or absence of ovaries in females. Although much remains to be learned, the concept that an early-life epigenetic mark, induced by hormonal exposure, appears to be critically dependent on the evolution of various later-life events, has emerged. This novel concept is expected to stimulate discussion in this conference with regard to the complex interactions between developmental reprogramming via epigenetics and the modifying effects of adult life events, in shaping the ultimate phenotype(s) governing disease susceptibility. Support in part by NIH grants ES013071, ES019480, ES006096, and CA015776.

[K25]

Children's toxicology "early exposure-delayed effects"

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"Children are not small adults". This is a well-known phrase, especially in the clinics. However, this issue has long been a challenge for toxicology. The knowledge has been limited to the differences in metabolism and other physiological factors. Toxicology test guidelines currently available are targeted for teratogenicity and reproductive toxicity studies. These tests look into essentially macroscopic organic changes occurring in a rather short period of time after perinatal exposure. However, recent advances in molecular toxicology allow identification of the target molecules and receptors in quantitative fashion and at the fine structure levels around and below the resolution of light microscopy. Such information lead us to consider an entity of "receptor mediated toxicity" or "signal toxicity". Such insults (non-cytotoxic *per se*) would merely induce transient effects on adults. However, there are growing evidences that such insults on the developing and maturating organisms can leave irreversible effects that become overt later in adulthood. Our initial targets in the developing brain were the neurotransmitter receptors, such as glutamate receptor (Tanemura et al. J Tox. Sci. 34: SP279, 2009); transplacental exposure to domoic acid resulted in anomaly of high brain function in adulthood. Now, we extend the target to other systems possibly including nuclear receptor family. Bisphenol A was perinatally exposed via drinking water to dams at dosage levels equivalent to the EPA maximum safe dose of 50 μg/kg/day, and F1 male mice at the age of 12-14 weeks were tested by Openfield, Hole-board, Light/Dark transition, Elevated plus maze, Fear conditioning, and Prepulse inhibition test. This test battery picked up significant alterations in brain high function. Transcriptomic, protein expression, as well as electrophysiological data on hippocampal slice will be added for the discussion on steroid receptor-mediated children's toxicology as a study field of the "early exposure- irreversible delayed effects". (Supported by MHLW Health Sciences Research Grants)

[K26]

Molecular basis of de novo and acquired aromatase inhibitor resistance

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Aromatase is the enzyme that converts androgen into estrogen. Aromatase inhibitors (AIs) have been quite effective and extensively used in the clinic for the treatment of estrogen receptor (ER) positive breast cancer in post-menopausal women. Unfortunately, development of resistance to these inhibitors can occur. In order to study the mechanisms of endocrine resistance and to explore ways to prevent/delay and treat endocrine resistance, our laboratory prepared a set of resistant cell lines with consideration of the physiological relevance of the models. There are two types of resistance: acquired and *de novo*. MCF7aro cell line was generated in our laboratory by over-expressing aromatase in MCF-7 cells, and is used to study AI responses. A series of MCF7aro cell lines that acquired resistance to each of three AIs (letrozole, anastrozole, exemestane) or tamoxifen were generated five years ago. These MCF7aro-derived cell lines have been extensively characterized and verified to be relevant models of acquired endocrine resistance. Furthermore, long term estrogen deprivation MCF7aro lines (LTEDaro) were generated and shown to represent a model of late stage acquired resistance that does not respond to treatment with any AI or tamoxifen. Results from this and other laboratories have revealed that, for acquired resistance, growth factor pathways are up-regulated after ER-dependent pathways are suppressed, and ER is then activated through different cross-talk mechanisms. Therefore, ER is still a key player in acquired resistance cancers.

Approximately 30% of ERα+/aromatase+ breast cancers do not respond to AIs at all, i.e., *de novo* resistance. The growth of such tumors can be driven by multiple growth factor pathways, although functional ER and aromatase are present. Several growth factor receptors, EGFR, HER2, IGF-1R, and kinases in the related pathways such as PI3K and Akt have been implicated in endocrine resistance. Our laboratory prepared two *de novo* resistant cell lines (MCF7Akt-aro and MCF7HER2-aro). These lines have functionally active aromatase and ER that can be inhibited by AIs and antiestrogens, respectively. However, these drugs cannot suppress the proliferation of the cells because their proliferation is driven by activated Akt or HER2. Results from our studies have demonstrated that our acquired and *de novo* endocrine resistant cell lines are important models to investigate the molecular mechanisms and to assess new strategies against AI resistance. Our findings will be reviewed and discussed.

[K27]

Pre-receptor regulation of the androgen receptor in prostate cancer

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Prostate cancer is a leading cause of cancer in the aging male and is an androgen dependent disease. With the emergence of castrate resistant prostate cancer (CRPC) and its effective response to CYP17 inhibitors (e.g. abiraterone acetate) attention has refocused on the intratumoral synthesis of androgens. Our group has elucidated the identity and role of steroid hormone transforming aldo-keto reductases (AKRs) in the pre-receptor regulation of androgens available to bind to the androgen receptor (AR) in human prostate. We find that *AKR1C2* [type 3 3α-hydroxysteroid dehydrogenase (HSD)] is responsible for elimination of 5α-dhydrotestosterone (5α-DHT) from the prostate while *HSD17B6* (RoDH like 3α-HSD) is responsible for the back conversion of 3α-androstanediol to 5α-DHT. Together these enzymes function as a molecular switch to regulate androgen access to the AR. Conversely, we find that AKR1C1 converts 5α-DHT to 3β-androstanediol (an endogenous pro-apoptotic ligand for ERβ). We and others have also shown that *AKR1C3* [type 5 17β-HSD] is the major enzyme responsible for the peripheral conversion of Δ^4-androstene-3,17-dione to testosterone and that is one of the most highly up-regulated genes in CRPC. AKR1C1-AKR1C3 share 86% sequence identity, and AKR1C1 and AKR1C2 differ by only a single amino acid at the active site. This makes the development of selective inhibitors of AKR1C3 for the treatment of CRPC challenging. AKR1C3 is potently inhibited by the nonsteroidal anti-inflammatory drugs indomethacin and N-phenylanthranilic (NPA) acid analogs. High-through put screening of libraries of indomethacin and NPA analogs have identified lead compounds with appropriate selectivity, potency and efficacy in cell-based models. [Supported by 1R01-CA90744, and a Prostate Cancer Foundation Challenge Grant].

[K28]

Human lung cancer is a novel estrogen target tissue

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Lung cancer is the leading cause of cancer mortality in both women and men worldwide but gender differences exist in their clinical and biological manifestations. In particular, among life time non-smoker, women are far more likely to develop lung carcinoma, mostly adenocarcinoma than male. Human lung cancers have been recently demonstrated to frequently express both estrogen receptor alpha and beta in carcinoma cells in both male and female patients. In addition, estrogen has been demonstrated to be synthesized *in situ* in both male and female lung cancers through aromataization of circulating androgens. Aromatase was also demonstrated to be predominantly expressed in carcinoma or parenchymal cells of lung cancer tissues in contrast to breast cancer as a result of recent study using laser capture microscopy and immunohistochemistry . These data all suggest that locally produced estrogen may contribute to the pathogenesis and development of lung carcinoma. In addition, a lower expression of aromatase was reported to be associated with better clinical outcome of the patients. Preclinical studies further demonstrated that cell lines derived from both genders respond to estrogens receptor blockers and aromatase inhibitors (AI). These findings indicate a vital role of intratumoral aromatase and estrogen receptors in carcinoma cells in biological and /or clinical behavior of non-small cell lung cancer (NSCLC), the most common type of human lung malignancy. Therefore, intratumoral aromatase expression and estrogen receptors in carcinoma cells may not only be an effective predictive biomarker for clinical outcome and AIs could become viable therapeutic options for disease management in NSCLC patients.

[K29]

Genomics of estrogen receptor signaling and actions in breast cancer

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Estrogens regulate the gene transcriptional programs and functions of many reproductive and non-reproductive tissues, and of hormone-responsive cancers, and the mechanisms by which estrogen hormones act are multi-faceted, involving two estrogen receptors (ERα and ERβ) and their dynamic interplay, and nuclear-initiated and extranuclear-initiated signaling pathways. Hormonal regulation of breast cancer involves cooperation and linkages between estrogen receptors (ERs) and protein kinases. The relative inputs from these two pathways are thought to determine whether the cancer is responsive vs. resistant to endocrine therapies, with the up-regulation of kinases being a hallmark of resistance, a major limitation to the treatment of ER-positive breast cancers. Estrogen and protein kinase signaling are interdependent and even convergent, and we have found that estrogen-stimulated cell proliferation and gene regulations require ERα action through nuclear and extranuclear-initiated pathways involving extracellular signal-regulated kinase (ERK2). We will present our findings on the genome-wide chromatin binding of ERs and ERK2, and show how the ER and kinase-mediated signaling pathways converge at the level of chromatin and contribute to the aggressive behavior of breast cancer and to endocrine resistance through regulation of gene expression, microRNA production, and changes in cell properties. Understanding the crucial interplay between ER and protein kinases in controlling cell regulatory programs should assist in enhancing human health and guide the development of novel therapeutic strategies for suppressing breast cancer progression and preventing or overcoming endocrine therapy resistance in breast cancer.

(Supported by grants from NIH, BCRF, and DOD)

Key Words: estrogen receptors, breast cancer, protein kinases, gene regulation

[K3]

Differential signaling mechanisms for ERa and ERb in response to diverse ligands

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Natural and synthetic ligands regulate diverse signaling and transcriptional networks via one or both of two estrogen receptor subtypes (ERα & ERβ). The structural events underlying ligand-specific coregulator recruitment and transcriptional activation/repression are still not well defined for the nuclear receptor superfamily. Subtype and ligand-specific recruitment of coregulators and modulation of target gene expression have important implications for understanding the specificity of nuclear receptor signaling, for the treatment or prevention of a variety of diseases, and for endocrine disruption. The development of diverse ER subtype-selective ligands (SERMs) provides molecular tools to study unresolved issues in the structural linkage between ligand and transcription. To better understand the relationship between nuclear receptor ligand positioning and the formation of cofactor-binding surfaces, we have solved multiple ERα/β LBD structures and investigated determinants of ligand selectivity between the two estrogen receptor subtypes. Structurally guided amino acid substitutions have identified amino acids required for selectivity. Residues within the ligand-binding pocket as well as distal secondary structural interactions contribute to subtype specific positioning of the ligand and coregulator selectivity. These data demonstrate the importance of both short- and long-range interactions in the allosteric transmission of information through the nuclear receptor ligand-binding domain, and in determining the specificity of closely related receptor subtypes, such as ERα & ERβ. Detailed structure-function information for the two ERs has proved useful both for understanding as well as designing ligands with tissue- and pathway-selective behaviors. Co-occupancy of receptor dimers by SERM/agonist mixtures as well as occupancy of a single monomer by ligands at low doses may contribute to unexpected behaviors of individual ligands and ligand mixtures.

[K30]

Obesity, aromatase and breast cancer: Classic concepts and a novel mechanism

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Obesity increases the risk for postmenopausal breast cancer. Although the underlying mechanism is unknown, estrogen produced by aromatase in adipose tissue has long been suspected as an underlying cause. Aromatase encoded by a single gene is essential for estrogen production and is the target of aromatase inhibitors, the most effective endocrine treatment of postmenopausal breast cancer. The brain and many peripheral tissues (e.g., fat in breast and other sites) in postmenopausal women express aromatase via a number of alternative promoters. Since female mice lack the promoters for aromatase expression in fat or other peripheral tissues, no suitable mouse models have been available for studying the link between adipose tissue aromatase, obesity and breast cancer risk. We recently generated transgenic humanized aromatase ($Arom^{hum}$) mouse lines containing a single copy of the entire human aromatase gene. These novel $Arom^{hum}$ mice mimic human physiology with respect to estrogen production and express the aromatase gene in many peripheral tissues—including breast fat—under the control of alternatively used human promoters via distinct signaling pathways. The mice develop excessive local estrogen production in breast tissue leading to hyperplasia at 24 weeks. In $Arom^{hum}$ mice, the human aromatase gene is expressed at basal levels via the TNF-regulated distal promoter I.4 in white adipose tissue of the breast and gonadal regions and in dorsal brown fat, whereas the PGE_2-Jun N-terminal kinase (JNK)-regulated proximal promoter I.3/II remains quiescent. Studies on this new mouse model that accurately represents estrogen production in peripheral tissues including fat will be discussed.

[K31]

Mechanisms linking glucocorticoids with cardiovascular risk

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In Cushing's syndrome, excessive activity of glucocorticoids causes obesity, type 2 diabetes, and cardiovascular disease. We have shown that variations in plasma cortisol in the normal population are also associated with cardiovascular risk factors, and have investigated the molecular basis for the underlying dysregulation of the HPA axis.

In addition, we demonstrated that the microsomal enzyme 11β-HSD1 converts inert cortisone into active cortisol, thereby amplifying intra-cellular cortisol concentrations (in liver, adipose tissue and vascular smooth muscle) and increasing glucocorticoid receptor activation, independently of circulating cortisol concentrations. We proposed that inhibition of 11β-HSD1 would reduce intracellular cortisol levels and hence glucocorticoid action without interfering with the normal stress response, providing a novel therapeutic approach in type 2 diabetes. Proof of concept was confirmed in a series of mouse transgenic models and in humans using a prototype 11β-HSD inhibitor, carbenoxolone. These confirmed the influence of 11β-HSD1 not only on obesity and insulin sensitivity, but also on the development of atheromatous lesions and the tissue response to inflammation and ischaemia.

Using novel stable isotope tracers and other tools to quantify 11β-HSD1 *in vivo* in humans, we showed that activity is increased in adipose tissue in obesity and type 2 diabetes. Selective inhibitors of 11β-HSD1 have been developed in several pharmaceutical companies, and also by an in-house drug discovery team created within the University of Edinburgh. These show efficacy in a wide range of pre-clinical models and encouraging efficacy in a Phase II study in type 2 diabetes.

Most recently, our studies have focused on the tissue-specific regulation of 11β-HSD1 activity and its physiological role as a sensor of the nutritional environment. These studies highlight that pre-receptor modulation of glucocorticoid action is more complex than previously appreciated, and is likely to offer further insights into determinants of cardiovascular risk and opportunities for therapy.

[K32]

Metabolic impact of sex hormones on obesity

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Females are protected against metabolic perturbations known to cause the Metabolic Syndrome and insulin resistance (IR) when compared to males. In the United States, approximately 4000 women enter menopause each day, and these individuals are at increased risk for developing obesity, IR, and diabetes. Estrogen replacement therapy is an established way to reduce these risks. However, due to ubiquitous expression of estrogen receptors (ERs), especially in peripheral tissues, the metabolic benefits provided by estrogen are often associated with increased risk of heart disease and breast cancer. Disruption of estrogen (E2) signaling by estrogen receptor (ER)-α ablation yields an obese, glucose intolerant phenotype in rodents and humans. Together these findings support a role for E2 and ERα in preventing obesity and in maintaining insulin sensitivity (IS); however, the molecular mechanisms and tissue specific sites conferring ERα activity are unknown. We have developed exciting and novel mouse models with which we can study the effects of ERα specifically in the brain and in adipocytes. To this end we have employed the ERα floxed mouse crossed to a mouse that expresses CRE recombinase under the control of the SF-1 (VMH), POMC, and adiponectin promoter. Using the SF-1 and the POMC promoters we have been able to delineate where estrogenic action in the brain regulates energy expenditure and food intake/glucose homeostasis. Using the adiponectin promoter, which is highly restricted to adipocytes and is not present in macrophages or in other components of the stromal vascular fraction of adipose tissue, we have been able to define estrogenic action in adipose tissue. Analyses of these mice demonstrate that female $ER\alpha^{lox/lox}$/Adipo-CRE mice differ in body weight but not food intake when compared to their $ER\alpha^{lox/lox}$ littermate controls. Furthermore, the male $ER\alpha^{lox/lox}$/Adipo-CRE do not differ with respect to food intake or body weight. However, both the male and female $ER\alpha^{lox/lox}$/Adipo-CRE mice show an increased triglyceride deposition in their visceral adipose depots. Analysis of adipose tissue histology from the male mice show enlarged adipocytes and enhanced crown-like structures in the $ER\alpha^{lox/lox}$/Adipo-CRE cohort, indicative of inflamed adipose tissue. Moreover, both males and female $ER\alpha^{lox/lox}$/Adipo-CRE mice have impaired glucose tolerance when compared to $ER\alpha^{lox/lox}$ littermates; this impairment being especially pronounced in the in the males. Following ovariectomy (OVX, to most closely mimic a menopausal state), the adipose tissue histology from female $ER\alpha^{lox/lox}$/Adipo-CRE more closely resembles that of the male $ER\alpha^{lox/lox}$/Adipo-CRE. Striking differences between the littermate controls and the $ER\alpha^{lox/lox}$/Adipo-CRE OVX mice were found with respect to adipocyte morphology in response to estrogen. We have complimented these studies with a technique whereby we can manipulate gene expression in a fat pad specific way. For these studies we injected an adeno-associated virus driving CRE expression (or a scrambled control virus) directly into the visceral adipose pads of female mice $ER\alpha^{lox/lox}$. These mice show a similar phenotype to the $ER\alpha^{lox/lox}$/Adipo-CRE mice – they too have larger fat pads, increased adipocyte size, and enhanced adipose inflammation. Finally, we have produced mouse embryo fibroblasts from these mice (both $ER\alpha^{lox/lox}$/Adipo-CRE and $ER\alpha^{lox/lox}$) of which we have differentiated to adipocytes. Data from these MEFs show striking similarities to those collected from the *in vivo* models, further suggesting the relevance of our data. These data together, strongly suggest that reduced ERα in specific brain regions regulates energy expenditure and food intake. Wereas ERα in adipose tissue, specifically in the adipocytes themselves, has profound effects on adipose tissue function. Moreover, the levels of ERα in adipocytes regulate hepatic glucose handling and systemic metabolic homeostasis. Therefore, our findings demonstrate the critical site of estrogen action for protecting women from the Metabolic Syndrome may be at the level of adipose tissue ERα.

[K33]

Familial phenotypes in Polycystic ovary syndrome

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Polycystic ovary syndrome (PCOS) is the most common endocrine disorder of premenopausal women, with the classic syndrome of anovulation and hyperandrogenism affecting ~7% of this population. It has major reproductive and metabolic morbidities across the lifespan, including markedly increased prevalence rates of obesity, type 2 diabetes (T2D), metabolic syndrome and other cardiovascular disease risk factors. These abnormalities cluster in PCOS families providing evidence that genetic variation contributes to their pathogenesis. Indeed, male as well as female first-degree relatives have reproductive and metabolic phenotypes. These familial phenotypes include hyperandrogenemia with regular menses in ~15% of reproductive age sisters, whereas ~20% of such sisters have the classic PCOS phenotype. Postmenopausal mothers of women with PCOS have hyperandrogenemia, insulin resistance, dyslipidemia and increased risk for metabolic syndrome. Male first-degree relatives also have hyperandrogenemia, dyslipidemia and an increased risk for metabolic syndrome; the latter risk is entirely accounted for by obesity in males. Prepubertal female first-degree relatives have hyperandrogenemia.

We have now mapped a genetic variant conferring PCOS susceptibility to an allele of a dinucleotide repeat (D19S884) within intron 55 of the fibrillin-3 gene (*FBN3)* that is both linked and associated with the reproductive phenotype. This allele is also associated with a metabolic phenotype in affected women and their brothers. However, it is clear that this allele does not account for all cases of PCOS since the frequency of women with the D19S884 susceptibility allele in our PCOS population is only 30%. There are likely other PCOS susceptibility genes. Indeed, we have found a novel transcription factor 7-like 2 (*TCF7L2*) locus associated with the reproductive phenotype, whereas *TCF7L2* T2D risk SNPs are associated with evidence of pancreatic β-cell dysfunction in women with PCOS analagous to the association seen in the general population.

Variation in these genes may contribute to PCOS by producing hyperandrogenemia. Since PCOS is a heterogeneous disorder, there may be genetic mechanisms other than hyperandrogenemia and non-genetic (e.g. environmental) factors that result in the PCOS phenotype. Some of the phenotypic heterogeneity of PCOS, however, appears to reflect variable expression of the same gene since several reproductive phenotypes can occur within family members who would be expected to share the same genetic basis for the disorder.

[K34]

Steroid receptors: Structure, function, ligand design, and in vivo imaging

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Estrogens are steroidal and non-steroidal hormones that have diverse actions in both reproductive and non-reproductive tissues, regulating normal physiological processes and pathologies, including breast cancer. These hormones act through the estrogen receptor, a ligand-modulated transcription factor. When estrogens bind to the estrogen receptor, they stabilize specific conformations that reflect their size and shape. The rigidified external surface features of the ligand-receptor complex then serve as specific docking sites for coregulators, thereby altering the rates of target gene transcription and controlling cell phenotypic properties.

Using X-ray crystallography and molecular modeling as guides, we have developed modular methods for the synthesis of non-steroidal estrogens, adaptable to combinatorial approaches, through which we have prepared a number of estrogens of novel structure that are highly selective for only one of the two estrogen receptor subtypes, ERα of ERβ. These selective ligands are proving to be useful as pharmacological probes of the functions of the two different ERs. We have also diversified the structure and elemental composition of ER ligands, introducing three-dimensional core elements and replacing a carbon-carbon double bond with a boron-nitrogen bond. We have designed estrogen conjugates that selectively activate extranuclear-initiated ER action and show selective cardiovascular protection. Other ligands have unexpected biological selectivities that could be medically important as neuroprotective and antitumor agents.

Elevated levels of steroid receptors are found in many tumors and serve as targets for endocrine therapies that can be effective, with minimal side effects. We have designed high affinity receptor ligands, labeled with the radionuclide fluorine-18, for positron emission tomographic (PET) imaging of these tumor receptors. This non-invasive determination of receptor levels in the tumors provides valuable information in selecting breast cancer patients most likely to benefit from endocrine therapy. In addition, a hormone challenge test which images hormone-induced changes in tumor metabolism is proving highly predictive of response to endocrine therapies in breast cancer.

[K4]

Androgen sensitivity present at all stages of prostate cancer

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Prostate cancer is the most hormone sensitive of all cancers. However, any therapeutic strategy related to androgen blockade must be based upon the fact that two almost equivalent sources of androgens are in operation in the prostate, namely testosterone of testicular origin and local androgen formation from the inactive precursor dehydroepiandrosterone (DHEA) of adrenal origin by the process of intracrinology. Accordingly, combined androgen blockade (CAB) (castration with a GnRH agonist or orchiectomy) plus a pure antiandrogen is the only first line treatment based upon the available scientific knowledge although castration alone or an antiandrogen alone is still chosen in the majority of cases, except in Japan. When androgen blockade is started in metastatic disease, the response is temporary and progression always occurs, while combined androgen blockade started in localized disease can provide cure in the vast majority of cases. Recent data explain resistance to androgen blockade by elevated androgen receptor (AR) levels which can increase the response to low androgen concentrations and modify the response to antiandrogens as well as by the local biosynthesis of androgens. These crucial data indicate the importance of obtaining new and more potent antiandrogens as well as blockers of androgen biosynthesis (abiraterone as example). The positive data obtained in castration-resistant prostate cancer (CRPC) with the 17α-hydroxylase inhibitor abiraterone plus prednisone versus abiraterone and placebo can suggest that the action of the compound could inhibit peripheral androgen formation in addition to the inhibition of DHEA secretion from the adrenals by prednisone.

[K5]

The K303R breast cancer estrogen receptor a mutation and hormone resistance

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Aromatase inhibitors (AIs) and antiestrogens, such as tamoxifen (Tam) are frequently used for hormonal therapy of estrogen receptor (ER)-positve breast cancers in postmenopausal women. However, many patients are initially refractory or acquire resistance to either treatment. A mutation which causes a single amino acid change in the ERα hinge domain (lysine 303 to arginine, called *K303R ERα*), has been reported and represents a somatic, gain-of-function mutation arising in the breast resulting in a receptor which is hypersensitive to the growth effects of estrogen. We discovered that the K303R ERα mutation confers resistance to the AI anastrazole via upregulation of the IGF/PI3K/AKT signaling pathways, and blocks Tam antagonist action when engaged in crosstalk with growth factor receptor signaling pathways. The AI-resistant phenotype associated with expression of the K303R ERα mutation is dependent on activation of phospho-S305 within the receptor, and blockade of S305 phosphorylation can restore hormone sensitivity and signaling to the IGF/PI3K/AKT pathways. We are currently using mass spectroscopy to detect the mutation in breast cancer patients treated with monotherapy Tam, along with detection of specific mutations in PI3K (E545K and H1047R) and AKT (E17K), and will correlate the presence of these mutations with clinical outcomes. An important consideration will be the appropriate selection of patients that may benefit from new targeted therapies to PI3K/AKT using sensitive methods for detection of these mutations.

[K6]

New activities of GPR30 and its selective ligands

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Responses to hormones are often divided into two categories based on the time frame in which they occur. The first includes genomic responses that represent changes in gene transcription and occur on the time frame of hours to days. The second includes rapid signaling events that occur within seconds to minutes of cell stimulation. Signaling events induced by estrogen, until a few years ago, were attributed solely to the classical estrogen receptors (ERα and ERβ). However, it is now clear that a member of the 7-transmembrane G protein-coupled receptor family, GPR30 (now also named GPER), is also capable of mediating both rapid and transcriptional events in response to estrogen. In addition to reproductive functions, estrogen-dependent signaling also plays key roles in the physiology of many non-reproductive systems, including the cardiovascular, skeletal, immune and neurological systems. GPR30 was first identified as a receptor required for estrogen-mediated rapid cell signaling in 2000. In the intervening 10 years, GPR30 has been demonstrated to play a critical role in cell-mediated responses to estrogen in a diverse array of cellular and physiological systems. Although estrogen is a non-specific agonist for both classical estrogen receptors and GPR30, compounds such as SERMs and ICI182,780 are largely antagonists of ER signaling but agonists of GPR30 signaling. To provide selective tools to study GPR30 function, we have identified both a selective agonist and antagonist of GPR30, named G-1 and G15, respectively. These highly selective ligands have been used extensively to begin to identify some of the physiological functions of GPR30. Roles for GPR30 have been identified in the reproductive, nervous, immune and vascular systems as well as in cancer/cell proliferation and metabolism/obesity. In many of these systems, G-1 has been shown to reproduce to a great extent the actions of estrogen. Based on the experimental results to date that suggest roles for GPR30 in several physiopathological processes and diseases, one can speculate that the regulation of GPR30 activity could be of therapeutic value in many areas of clinical medicine. In particular, since G-1/GPR30 lacks many of the activities that lead to complications in hormone/estrogen replacement therapy, GPR30 may represent a superior target to classical estrogen receptors in a number of diseases. With continued investigation into the functions and mechanisms of actions of GPR30, there is great promise that novel therapeutic approaches based on this receptor can be developed.

[K7]

Integration of progesterone receptor-mediated rapid signaling events with genomic actions in breast cancer models

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Progesterone receptors (PR) are critical for massive breast epithelial cell expansion during mammary gland development and contribute to breast cancer progression. Progestin-bound PRs induce rapid activation of cytoplasmic protein kinases, leading to regulation of growth-promoting genes by transcription complexes that include phospho-PR species. We propose that hormonal and growth factor signals converge at the level of PR-target gene promoter selection. PR undergoes significant post-translational modification in response to activation of mitogenic protein kinases, including the ubiquitously expressed cancer-associated kinase, ck2. Herein, we show that phosphorylation of PR Ser81 is ck2-dependent and progestin-regulated in intact cells, but also occurs in the absence of PR ligands, when cells enter the G1/S phase of the cell cycle. T47D breast cancer cells stably expressing a PR-B mutant that cannot be phosphorylated at Ser81 (S81A) showed significant defects in the ability to form (ligand independent) soft agar colonies. Regulation of selected PR target genes also required Ser81 phosphorylation for basal (BIRC3, HSD11b2) or progestin-regulated (HbEGF) expression; these genes were selectively regulated by PR-B but not PR-A. Additionally, wt, but not S81A mutant PR, was robustly recruited to a PRE-containing transcriptional enhancer region of BIRC3. Abundant ck2 also associated with this enhancer region in cells expressing wt but not phospho-mutant PR. We conclude that phospho-Ser81 PR provides a platform for ck2 recruitment and regulation of selected PR-B target genes. Understanding how PR functions in the context of high kinase activities characteristic of breast cancer is critical to understanding the basis of tumor-specific changes in gene expression and will speed the development of highly selective treatments that primarily target cancer but not normal tissues.

Keywords: breast cancer, progesterone receptor, ck2 (casein kinase 2), phosphorylation, cell cycle

[K8]

Targeting androgen receptors in androgen-dependent and androgen-independent prostate cancer

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Prostate cancer is androgen-dependent. Androgen blockade is the principal therapy for metastatic disease but many tumors become resistant to treatment relatively rapidly. Many of these recurrent tumors remain androgen receptor (AR) dependent and have developed alternate means for activating androgen receptor (AR). Recent studies suggest that one mechanism for androgen independence is alternate splicing of AR producing constitutively active splice variants lacking their carboxyl terminal hormone binding domain. Thus, treatments that target the activity of the amino-terminal coactivator binding sites or the DNA binding domain would be beneficial. An alternative approach is to identify a means to inhibit AR expression. Using a portion of the p160 coactivator SRC-1 that interacts with AR, we have found that this peptide specifically blocks androgen-dependent induction of AR target genes. Furthermore, the peptide inhibited proliferation of androgen-dependent LNCaP prostate cancer cells, but not AR negative PC-3 cells. Importantly, this peptide also inhibited the hormone-independent, but AR dependent prostate specific antigen (PSA) expression and growth of the hormone refractory C4-2 cell line. As a second approach, we have sought treatments that reduce AR expression. In examining the actions of kinase inhibitors, we found that short term treatment with the MEK inhibitor, U0126, caused a target gene specific reduction in AR activity, and that longer term treatment reduced overall AR expression in part through a reduction in the stability of AR. Thus, the coactivator binding site and cell signaling pathways are candidates to reduce AR activity regardless of the mode of AR activation.

[K9]

High throughput and standardized LC-MS/MS steroid-quantification for clinical metabolomics

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Mass spectrometry based clinical metabolomics needs standardization of analytical assays to improve future inter-laboratory comparability and to become a successful new and established technology in clinical routine laboratories such as mass spectrometry based therapeutic drug monitoring (TDM) over the past 10 years. Steroid hormones are one of the most interesting endogenous metabolite class in clinical metabolomics today. Standardized determination of concentrations of steroid hormones in serum may aid in improvements in the clinical environment, e.g. *in vitro* fertilisation, diagnosis and treatment of steroid-related diseases in children and adults. The application of this assay will be focused on analysis of premenopausal women, i.e. the quantitation range of steroids is optimised for this clientele.

We will present a newly developed high throughput and standardized steroid assay intending the quantitative determination of 16 steroids in human serum samples for clinical application. The steroid metabolites are testosterone, progesterone, cortisol, estradiol (E2), DHEAS androstenedione, 17-OH Progesterone, corticosterone, 11-Deoxycortisol, estrone (E1), DHEA, 11-Deoxycorticosterone, aldosterone, cortisone, etiocholanolone, androsterone.

The analysis is based on LC-MS/MS. The assay includes standardized sample preparation and LC-MS/MS analysis in 96-well plate format allowing high sample throughput. The sample preparation, needed to clean up and pre-concentrate the sample, is performed by a solid phase extraction (SPE) procedure in 96-well plate format. 500µL serum sample volume is needed. Two different elution steps are necessary (first fraction: all steroids except DHEAS, second fraction: DHEAS) for highly pure extraction fractions resulting in minimal matrix effects and improved accuracy. As the result there are two subsequent LC-MS/MS runs with a total run time of 22 minutes.

Validation data of the assay for human serum will be presented including inter-laboratory comparisons demonstrating the precision, accuracy, stability, and reproducibility of the developed assay.

[O1]

a critical role for the endogenous estrogen receptor (er) α monoubiquitination in the 17β-estradiol-dependent extranuclear signalling

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Besides being the signal to trigger 26S proteasome-mediated protein degradation, protein modification with ubiquitin (Ub) confers also non-degradative functions. In particular, protein monoubiquitination (*i.e.,* the attachment of one single Ub motif to the substrate) is a non-proteolytic signal involved in several different physiological processes. Recently, monoubiquitination of the estrogen receptor α (ERα), which has been discovered in *in vitro* ubiquitination assays, seems to contribute to the ERα nuclear effects. However, the ERα mediates the pleiotropic effects of the cognate hormone 17β-estradiol (E2) through the activation of both nuclear and extranuclear mechanisms. Nonetheless, at the present no information are available about the monoubiquitination-dependent regulation of the ERα extranuclear activities as well as on the ability of E2 to modulate this receptor post-translational modification. Therefore, we sought to determine the impact of monoubiquitination in the regulation of the E2-dependent ERα extranuclear mechanism. Here, we show that ERα monoubiquitination endogenously occurs in breast cancer cells and is negatively modulated by E2. Furthermore, mutation of the ERα monoubiquitination sites prevents the E2-induced extranuclear ERα-mediated activation of signalling pathways (*i.e.,* AKT activation and cyclin D1 promoter activity) and consequently cell proliferation. In addition, the interference with ERα monoubiquitination results in a deregulated E2-induced association of ERα to the insulin like growth factor receptor (IGF1-R). Moreover, lack of ERα monoubiquitination renders cells less sensitive to H2O2-induced apoptosis. Altogether these data demonstrate an inherent role for monoubiquitination in ERα extranuclear signalling and point to the physiological function of ERα monoubiquitination in the regulation of the E2-induced cell proliferation.

Keywords: estrogen, estrogen receptor alpha, monoubiquitination, signalling

[O2]

MicroRNA 124a modifies the expression pattern of androgen receptors in malignant and non-malignant human thyroid tumour tissues

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Gender bias in thyroid cancer incidence is known and androgens favour carcinogenesis. We hypothesized that androgen receptor (AR) gene expression in human thyroid tumour tissues may vary in a gender specific manner due to specific changes in its transcription and translation regulators. Human thyroid tumour tissues [PTC(17 men and 51 women); FTC(1male and 6 women); FTA (8 men and 43 women)] were subjected to real time PCR of AR, western blot of AR, CBP, Sp1 and P53 and AR nuclear ligand binding activity and compared to controls. AR and miRNA124a interaction were analysed by bioinformatics and luciferase analysis. Data were subjected to correlation analysis. Serum and tissue testosterone decreased in males, while increased in females. AR mRNA and protein were under-expressed in most PTC and FTA women but over-expressed or unaltered in men. CBP and Sp1 under-expressed in PTC and FTA women, while it over-expressed in FTA men and under-expressed in PTC men. AR protein and AR LBA positively correlated in females, while such correlation was not evident in males. P53 and miRNA124a were negatively correlated with AR protein expression. Bioinformatic analysis revealed a binding site for miRNA124a on 3' UTR region of AR and transfection analysis showed AR is negatively regulated by miRNA124a. The present study conclude that varied AR expression due to specific expression patterns of CBP, Sp1 or P53 and miRNA124a underlie the gender bias in thyroid cancer incidence.

Keywords: Androgen Receptor, microRNA124a, Thyroid cancer

[O3]

Estradiol synergizes with SSRIs to induce desensitization of serotonin 1A receptor signalling in rat paraventricular nucleus of the hypothalamus

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Decreased levels of estrogens are associated with various neuropsychiatric disorders such as depression, anxiety and panic disorders in women. One hallmark of these disorders is a change in serotonergic function, particularly serotonin1A (5-HT_{1A}) receptor function. Estradiol regulates 5-HT_{1A} receptor signalling. Since desensitization of 5-HT_{1A} receptor signalling may be an underlying mechanism by which selective serotonin reuptake inhibitors (SSRIs) mediate their therapeutic effects in neuropsychiatric disorders, we explored the effects of combining estradiol with SSRIs and found that estradiol enhances the efficacy of the SSRIs on desensitization of 5-HT_{1A} receptor signalling in rat hypothalamic paraventricular nucleus (PVN). We further explored which estrogen receptors are capable of desensitizating 5-HT_{1A} receptor function. Using the selective estrogen receptor (ER) β agonist, diarylpropionitrile (DPN), and recombinant adenovirus containing ERβ siRNAs to decrease ERβ expression, we found that desensitization of 5-HT_{1A} receptor signalling does not appear to be mediated by ERβ in PVN oxytocin cells. Treatment with G-1, a selective agonist for a membrane-associated ER, possibly either an ERα splice variant or GPR30, attenuated 5-HT_{1A} receptor signalling in the PVN. Reducing GPR30 via administration of recombinant adenovirus containing GPR30 siRNAs into the PVN blunts estradiol-induced desensitization of 5-HT_{1A} receptor signalling. We have identified several novel ERα splice variants expressed in rat PVN and will explore if these splice variants mediate the effect of estradiol on 5-HT_{1A} receptor signalling and are regulated by GPR30. Taken together, these studies suggest that a membrane ER may play a role in estradiol-mediated attenuation of 5-HT_{1A} receptor signalling, and potentially in accelerating the effects of SSRIs in treatment of mood disorders. Since estrogens and GPR30 itself may increase the probability of breast and endometrial cancer, identifying the ERs that underlie the positive effects of estrogens in desensitization of 5-HT_{1A} receptor signalling is of paramount importance.

Keywords: GPR30, estrogen receptors, hypothalamus, serotonin receptors

[O4]

Design, synthesis, and initial biological evaluation of a steroidal anti-estrogen-doxorubicin hybrid for targeting estrogen receptor-positive breast cancer cells

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As part of our program to develop breast cancer specific therapeutic agents we undertook the synthesis of a hybrid agent that combines a high affinity steroidal anti-estrogen with the potent cytotoxin doxorubicin. In this effort we employed a modular assembly approach to prepare a novel 11ß-substituted steroidal anti-estrogen functionalized with an azido-tetraethylene glycol moiety which could be coupled to a complementary doxorubicin benzoyl hydrazone functionalized with a propargyl tetraethylene glycol moiety. Huisgen [3+2] cycloaddition chemistry gave the final hybrid that was evaluated for receptor binding to demonstrate ER-affinity and for cytotoxicity in ER(+)-MCF-7 and ER(-)-MDA-MB-231 breast cancer cell lines. The presence of the linker or the linker-doxorubicin component had essentially no effect on the binding of the steroidal anti-estrogen to ER. The anti-estrogen-doxorubicin hybrid demonstrated enhanced (>100-fold) selectivity for ER(+)-cells versus ER(-)-cells and enhanced efficacy versus doxorubicin alone. The reversal of these effects by co-administration of estradiol demonstrated that the presence of the anti-estrogenic component was critical for selectivity and cytotoxicity in ER(+)-MCF-7 human breast cancer cells. The results suggest that this approach can serve as the basis for developing selective therapeutic agents for ER(+)-cancer cells with reduced effects on non-target tissues.

Keywords: anti-estrogen, doxorubicin, hybrids, drug delivery

[O5]

Long term nandrolone induced perturbations of endocrine homeostasis

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Background

The potential of serious and persisting health risks associated with the use of nandrolone is still largely unresolved. Its main urinary metabolite 19-norandrosterone (19NA) is used as a biomarker for the detection of nandrolone abuse, and may be detected several months after an intra-muscular injection of nandrolone. However, the endocrine effect of nandrolone and the slow release of 19NA is not known. In the present study we addressed this issue in a 1 year follow-up study in nandrolone abusers.

Experimental design:

Twenty four men tested positive for nandrolone and with a desire to give up their steroid abuse were recruited via our Anti-Doping Hotline and included after informed consent. Serum levels of LH, FSH and urinary concentration of 19NA were determined by immunoassays and gas chromatography-mass spectrometry, respectively. All men were genotyped for the uridine diphospho-glucuronosyltransferase (UGT) 2B7 His268Tyr polymorphism.

Results

In some individuals, urinary 19NA was detected up to one year after their last nandrolone injection. All individuals showed initial signs of compromised endocrine function as revealed by very low levels of LH (0.93 IU/L) and FSH (0.69 IU/L). There was a correlation between LH, FSH and 19NA ($R=0.6$-0.9, $p<0.001$) that persisted for four months. Individuals, homozygous for UGT2B7 His polymorphism, exhibited a faster 19NA excretion rate (90ng/day) than individuals expressing the Tyr allele (10 ng/day).

Conclusion

Nandrolone abuse leads to a long-standing impairment of the production of gonadotropins. Our results indicate that 19NA exerts a negative feed-back regulation on the hypothalamic-pituitary axis. The inter-individual variation in 19NA excretion rate was not predictable from intensity or duration of the abuse but could partly be explained by a polymorphism in UGTB7, the main enzyme responsible for 19NA inactivation.

Keywords: 19-nortestosterone, gonadotropine, UGT2B7, abusers

[O6]

Metformin inhibits aromatase in a promoter-specific manner in breast adipose stromal cells.

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There is a clear link between obesity, aging and breast cancer, and the majority of obesity- and age-related breast cancers are oestrogen-dependent. Aromatase converts androgens into oestrogens, and its importance in breast cancer is highlighted by the efficacy of aromatase inhibitors as endocrine therapy. These inhibitors inhibit oestrogen production not only in the breast but throughout the body leading to side-effects including arthralgia, bone loss and possible cognitive defects. The complex nature of the aromatase gene, *CYP19A1,* allows for tissue-specific regulation of aromatase via the use of tissue-specific promoters. In the case of breast cancer, tumour-derived factors such as PGE2 increase the activity of aromatase promoter PII and PI.3 - these promoters being silenced in other parts of the body in postmenopausal women. We have recently demonstrated that factors produced in obesity and breast cancer cause an increase in aromatase expression by inhibiting AMPK. This leads to the nuclear translocation of the CREB-coactivator CRTC2, where it can bind to aromatase PII/PI.3 and increase their activity and hence, aromatase expression. This study aimed to examine the effect of metformin, a commonly used anti-diabetic known to activate AMPK, on promoter-specific expression of aromatase in primary human breast adipose stromal cells. Our findings demonstrate that metformin inhibits PII/PI.3, thereby leading to a significant decrease in total aromatase in forskolin/PMA (to mimic PGE2) treated cells, leaving PI.4-specific transcripts unchanged. Moreover, metformin has no effect on the dexamethasone/TNFα-stimulated expression of PI.4-specific aromatase transcripts, where PII/PI.3-driven expression is low. These findings are the first to demonstrate the promoter-specific effects of metformin in human adipose stromal cells, and suggest that metformin may in fact be a breast-specific inhibitor of aromatase expression. Clinical trials are currently underway.

Keywords: aromatase, metformin, AMPK, breast cancer

[O7]

A novel 20beta-hydroxysteroid dehydrogenase from zebrafish is important for glucocorticoid catabolism

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Hydroxysteroid dehydrogenases (HSDs) are involved in biosynthesis of steroid hormones, in pre-receptor regulation of their biological activity, and in their catabolism. In mammals, many HSDs acting on different positions on the steroid backbone (3, 11, 17, 20) are well known. However, only few HSDs have been identified in non-mammalian species. In zebrafish (*Danio rerio*), a model for functional genetics, up to now three 17beta-HSDs have been characterized in detail, namely 17beta-HSD type 1, 2, and 3.

To search for further HSDs we performed phylogenetic analyses of the zebrafish genome and discovered an uncharacterized gene closely related to 17beta-HSD type 3 and 12. The novel enzyme catalyzes the conversion of cortisone to 4-pregnen-17alpha,20beta,21-triol-3,11-dione and is therefore named 20beta-HSD. The identity of reaction product was checked by LC-MS/MS. Although 20beta-HSD is similar to 17beta-HSD type 3 and 12, the enzyme did not convert androgens or estrogens. Zebrafish 20beta-HSD is expressed very early in embryonic development and shows a ubiquitous expression pattern in adults with strong signals in liver, kidney, and gills. This expression pattern is similar to that of 11beta-HSD type 2, which oxidizes cortisol to cortisone. In zebrafish embryos treated with cortisol, we observed a strong up-regulation of both 20beta-HSD and 11beta-HSD type 2. In addition, the product of the reaction catalyzed by 20beta-HSD, 4-pregnen-17alpha, 20beta,21-triol-3,11-dione, was found to be excreted by adult fish into their habitat water.

Based on our results, we hypothesize a role of novel 20beta-HSD in cortisol catabolism. Cortisol is inactivated by catalysis of 11beta-HSD type 2 to cortisone, which is further converted by 20beta-HSD to yield a hydrophilic steroid that can be easily excreted into the water. Therefore, we suggest 20beta-HSD to be important in glucocorticoid inactivation and stress control.

[O8]

Structural bases for disruption of nuclear hormone receptor signaling by environmental pollutants

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With industrialization, the production of chemicals and their introduction into the environment has increased rapidly. A number of these compounds act as endocrine disrupting chemicals (EDCs) that cause adverse effects in the endocrine system by interfering with hormone signaling. Epidemiological studies suggest a link between the exposure to these chemicals and the development of diseases like cancers, reproduction defects, or metabolic disorders. EDCs act via different pathways including interactions with nuclear hormone receptors (NHRs) which are primary targets of a variety of environmental contaminants. Early studies have focused on NHRs involved in reproductive processes, in particular estrogen (ER) and androgen (AR) receptors but it has been shown more recently that the activity of many other receptors including the retinoid X (RXRs) or peroxisome proliferator-activated (PPARs) receptors can also be affected by EDCs.

We have recently launched a research program to elucidate the mechanisms by which EDCs, which are generally structurally and chemically unrelated to natural hormones, interact with NHRs and impact their signaling pathways at concentrations within the micro- to nanomolar ranges. Our experimental approach involves a combination of structural, biochemical, cell-based and in vivo assays.

This presentation will review our most recent studies on various receptors and representative environmental disruptors like BPA derivatives, phthalates or organotins. We discovered that some EDCs mimic the natural hormones through conserved protein-ligand contacts while others employ radically different binding mechanisms.

To date, only a few EDC-bound NHRs have been characterized at the structural level as compared with the 150,000 synthetic chemicals used in consumer products. Our effort should contribute to increase our knowledge of the structural mechanisms and molecular interactions used by different NHRs and a wide range of structurally and chemically diverse compounds and help the development of computational tools for assessment of the toxic potential of large numbers of chemicals.

Keywords: Nuclear hormone receptors, Endocrine-disrupting chemicals, Environmental pollutants, Crystal structures

[P1.01]
Profiling of endogenous steroids by UHPLC-QTOF-MS^E

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Introduction: The urinary steroid profile is constituted by anabolic androgenic steroids (AAS), including testosterone and relatives. In the anti-doping field, their determination is commonly performed by GC-MS after hydrolysis of the glucuronide part and derivatization. The World Anti-Doping Agency (WADA) has determined criteria to consider for testosterone abuse, such as the testosterone to epitestosterone ratio (T/E). A ratio $\geq$ 4 is considered as suspicious of testosterone misuse. However, as these molecules are extensively metabolized in urine as phase II metabolites (glucuronide and sulfate conjugates), the phase II information is partially lost during the hydrolysis step. Therefore, the direct quantification of steroid glucuronides by means of an appropriate analytical technique remains challenging.

Methods: Ultra-high-pressure liquid chromatography (UHPLC) coupled to hybrid quadrupole time-of-flight (QTOF) mass spectrometry was selected for this purpose. UHPLC offers high chromatographic performance by using columns packed with small particles (i.e. sub-2µm), allowing high peak capacity within reasonable analysis time. QTOF mass analyzer enabled exact mass determination on molecular and fragment ions over the entire selected mass range. After a sample preparation by solid phase extraction (SPE), a highly selective chromatographic separation was performed in 36 min. The analytes were detected in the ESI negative mode and 2 functions were acquired simultaneously in the MS^E mode.

Results: This approach allows the quantification of the investigated metabolites by assessing the molecular ion obtained in the first function at low collision energy (5 eV). The second function acquired at ramped collision energy (5 to 70 eV) afforded a rich fragmentation pattern helping the identification of testosterone relatives.

Discussion: This development reveals the promising opportunity to supply a broader steroid profiling including an extensive monitoring of endogenous metabolites (steroidomics). The combination of a target quantitative steroid profile analysis and a steroid profiling approach can provide a deeper insight into the urinary excretion pattern after testosterone intake. Chemometric tools were used to highlight biomarkers of interest and to extend the detection window of testosterone doping.

Keywords: Testosterone phase II metabolism, Ultra-High Pressure Liquid Chromatography, Quadrupole Time-of-Flight, Profiling

[P1.02]

Evolution of human steroid dehydrogenases from actinobacteria and species specific inhibition

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The rational design of enzyme inhibitors that are substrate and species specific depends upon the precision and accuracy of alignment of members of the family. We have developed a novel technique to identify and align all members of any major family of proteins and applied it to the 20,000 members of the short chain oxido reductase (SCOR) enzyme family. The alignment is achieved by locating ten residue whose identity are 100% conserved in all SCORs and the four sites in the sequence where insertions and deletions have occurred in the three billion years of evolution from early actinobacteria to humans. Glycine, Alanine, Arginine and Proline (GARP) residues are critically important to the alignment process. The alignment is of sufficient accuracy to separate Gram positive from Gram negative bacteria, to identify the last universal common ancestor (LUCA) of all living species as an early actinobacteria, and to determine that the earliest SCOR enzymes probably existed prior to the appearance of any of the species currently on earth. The alignment allows us to trace the divergent evolution of human members of the family directly to actinobacteria (the phylum that includes *Mycobacterium tuberculosis*). Perfect alignment achieves unequivocal identification of the residues that form the substrate-binding pocket and allows cluster analysis to identify patterns of co-evolution of species, substrate, cofactor and function. This information can be used to design enzyme inhibitors that are substrate and species specific. We know of no other alignment program that can match the performance of GARP based alignment.

Keywords: Alignment, Evolution, Microbial phylogenetics

[P1.03]

Pre and post ACTH analysis of corticosteroid hormones in adrenal vein samples

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Introduction: Although the numerous steroid hormones produced by the adrenal gland play critical roles in human physiology, a detailed quantitative analysis of the steroid products has not been reported. Herein, we describe the use of liquid chromatography-tandem mass spectrometry (LC-MS/MS) to quantify 10 corticosteroids in human adrenal vein (AV) samples. **Methods:** AV sampling was done in nine subjects (3 men, 6 women) with a suspected diagnosis of adrenal aldosterone-producing tumor. For the current study, serum samples were collected from the iliac vein and the AV from the adjacent normal adrenal, before and after administration of ACTH (15 min). Iliac vein and AV samples were analyzed by LC-MS/MS for levels of 10 unconjugated 21 carbon (C_{21}) corticosteroids and precursors. The percentage of each steroid was defined as the percent of the molar net AV total of the 10 steroids measured. **Results:** Cortisol, cortisone and corticosterone were the most abundant steroids in the AV samples pre ACTH stimulation, whereas cortisol, corticosterone and pregnenolone were the major steroids post ACTH infusion. ACTH significantly increased the absolute adrenal output of all the 10 corticosteroids measured ($p<0.05$). Within 15 min, ACTH increased the mean concentration of cortisol by 23-fold, corticosterone by 124-fold, cortisone by 8-fold and pregnenolone by 300-fold. In the AV, cortisol was the most abundant of the steroids analyzed, representing 90±1% (982±228 nmol/L) prior to ACTH stimulation, and 79±1% (17,995 ± 4760 nmol/L) post ACTH infusion. **Discussion:** The current study defined the relative production of unconjugated corticosteroids in the human adrenal and their precursors pre and post ACTH stimulation. We found that the human adrenal secretes mainly two glucocorticoids, cortisol and corticosterone.

Keywords: adrenal gland, corticosteroid hormones, LC-MS/MS

[P1.04]

Capture compound mass spectrometry: Searching for novel cortisol-binding proteins as potential drug targets

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Cortisol is a very potent hormone that is important for the regulation of various physiological processes like glucose metabolism, immune system suppression, and stress reaction. Dysfunction of homeostasis of this hormone is overt in several diseases, e.g. obesity, hypertension and Cushing's Syndrome. Furthermore, cortisol and derivatives are used for treatment of allergic diseases like asthma, inflammatory and autoimmune diseases. However, continuous treatment with cortisol can cause undesirable side effects, which mimic the Cushing's Syndrome.

Cortisol regulates the transcription of glucocorticoid target genes upon binding to the glucocorticoid receptor (GR). Beside the GR, other yet unknown proteins might be involved in mediating cortisol dependent effects in a non-genomic manner. These unknown cortisol-binding proteins are potentially responsible for side effects in cortisol treatment and therefore interesting novel drug off-targets. To identify these proteins, we used the novel technology Capture Compound Mass Spectrometry (CCMS). This technology detects small molecule-protein-interactions. Capture Compounds are tri-functional small molecules containing a selectivity function (e.g. cortisol) for reversible, equilibrium-driven interaction with targeted proteins, a reactivity function for irreversible, covalent photo-crosslinking of bound proteins, and a sorting function for isolating the Capture Compound-protein conjugates. Capture Compound-protein conjugates isolated from biological samples can be further analyzed by standard biochemical methods such as SDS-PAGE or western blot and captured proteins can be identified by mass spectrometry after tryptic digestion.

We examined the functionality of a Capture Compound with cortisol as selectivity function by analyzing its inhibitory potential on 11beta-HSD type 2. The cortisol Capture Compound showed an IC_{50} of approximately 100 nM comparable to free cortisol (~ 70 nM). To prove if the cortisol Capture Compound is able to bind to a known interactor, we used human glucocorticoid binding globulin (hCBG) spiked into HepG2 cell lysates and were able to demonstrate specific binding. The method may therefore be suited to identify other cortisol binding proteins in complex samples like cell lysates or blood plasma.

[P1.05]

A simple and rapid quantitative method of bile acids in biological samples using liquid chromatography tandem electrospray mass spectrometry

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Bile acids are important metabolites in metabolism of cholesterol and lipids. They are synthesised in hepatocytes of liver and reach the colon via the gallbladder, bile ducts, and duodenum. Their concentrations in biofluids and tissues are important prognostic and diagnostic indicators of hepatobiliary and intestinal dysfunction. To monitor the concentration of bile acids in biological fluids and tissues, a sensitive and robust analytical method for different biological matrices is crucial. Major bile acids found in human and animals are cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid und ursodeoxycholic acids. They present either as free molecules or in conjugation with glycine or taurine. These amphiphilic metabolites are characterised by steroid scaffolding with a carboxyl group located in the side chain. Several analytical methods have been available with different sensitivity, specificity, accuracy and simplicity, but chromatographic technique represents an innovative tool in quantification of specific bile acids in biological samples. A robust and sensitive LC-MS/MS method with simple sample preparation has been developed based on high performance liquid chromatography separation couple with mass spectrometer to detect 12 bile acids in one step. Internal isotope-labeled standards allow for quantification. The separation is performed using a monolithic C18 column. The elution profiles are monitored in multiple reaction-monitoring mode identifying and quantifying the analytes according to their unique precursor to product pattern. For plasma samples, the correlation of concentration-response is linear for concentrations up to 500 ng/ml. The average recoveries of spiked free and conjugated bile acids are in range of 75 ± 2 % to 90 ± 3 %. In a daily routine 80 samples could be prepared and processed on LC-MS/MS within 24 hours.

This method has been already applied in our routine analyses to quantify the concentrations of bile acids in plasma samples from different strain of mice for metabolomics studies.

[P1.06]

Steroidal 5α-reductase inhibitors using 4-androstenedione as substrate

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Introduction: The aim of this study was to determine the capacity of some progesterone derivatives, to inhibit the conversion of labeled androstenedione ([^{3}H] 4-dione) to [^{3}H]dihydrotestosterone ([^{3}H]DHT) in prostate nuclear membrane fractions, where the 5α-reductase activity is present. The enzyme 5α-reductase catalyzes the 5α-reduction of 4-dione whereas the 17β-hydroxysteroid dehydrogenase catalyzes the transformation of 4-dione to T or 5α-dione to DHT. Moreover, we also investigated the role of unlabeled 5α-dione in these pathways.

Method: In order to determine the inhibitory effect of different concentrations of the progesterone derivatives in the conversion of [^{3}H] 4-dione to [^{3}H]DHT, homogenates of human prostate were incubated with [^{3}H] 4-dione, NADPH and increasing concentrations of non labeled 5α-dione. The incubating mixture was extracted and purified using TLC. The fraction of the chromatogram corresponding to the standard of DHT was separated and the radioactivity determined.

Results and discussion: The results showed that the presence of [^{3}H] 4-dione plus unlabelled 5α-dione produced similar levels of DHT as compared to [^{3}H] 4-dione. On the other hand, the results indicated that 17α- hydroxypregn-4-ene-3,20-dione **5** and 4-bromo-17α-hidroxypregn-4-ene-3,20-dione **7b**, were the most potent steroids to inhibit the conversion of [^{3}H] 4-dione to [^{3}H]DHT, showing IC_{50} values of 2 and 1.6 nM respectively.

Keywords: Androstenedione, Prostate, Progesterone derivatives, 5-alpha inhibitors

[P1.07]

Design and synthesis of molecular umbrellas based on steroidal backbone

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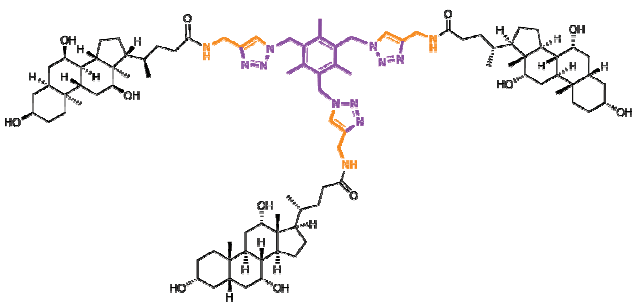
The idea of molecular umbrellas based on conection between steroidal backbone and central scaffold was first introduced by Janout et. al. Bile acids are nature compounds with characteristics including large, rigid and chiral steroidal skeleton and their unique amphipilicity have proved to be useful in the area of supramolecular chemistry. Estrone is marked with aromatic A-ring which can be usefull for fluorescence screening. Copper-catalyzed Huisgen 1,3-dipolar cycloadition reactions (azide-alkyne coupling) have been used for the synthesis of steroidal core containing molecular umbrellas. Transformation into 1,4-disubstituated 1,2,3-triazoles is based on *in situ* generated copper(I) acetylides. Steroid conjugates with natural products such as β-peptoid, porphyrin and some aromatic or heterocyclic foldamers were investigated. They can be studied as models of biological molecules. Capability to form secondary structures in presence of metal ions, anions, sugars or mimicking the ability to fold into well-defined conformations is now being sdudied. It have been proved that foldamers display a number of interesting supramolecular properties including molecular self-assambly, host-guest chemistry or molecular recognition. Our view is engaged in synthesis of various steroid containing molecular umbrellas using click chemistry and in study of their properties.



Keywords: molecular umbrella, click chemistry, synthesis, supramolecular properties

[P1.08]

Highly potent and selective inhibitors of 17beta-Hydroxysteroid Dehydrogenase Type 1 (17beta-HSD1) novel, potential therapeutics for breast cancer and endometriosis

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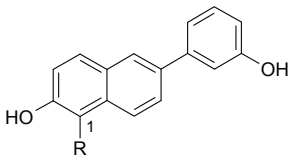
Breast cancer is the most frequent cancer type among women and the most common cause of cancer death. Endometriosis affects approximately 20 % of the female population and can lead to infertility. Both endometriosis and a high percentage of the breast tumours are estrogen-dependent, i.e. growth and proliferation are stimulated by estrogens. Present endocrine therapies - aromatase inhibitors, GnRH-analogues or anti-estrogens - result in a radical and unspecific reduction of estrogenic effects throughout the body leading to severe side-effects like osteoporosis or induction of endometrium carcinomas.

17β-HSD1 catalyses the reduction of the weak estrogen estrone (E1) to the highly potent estradiol (E2). The enzyme is expressed in different organs like ovaries, breast, endometrium and is present in breast cancer tissue as well as in endometriosis. Inhibition of 17β-HSD1 could selectively reduce the E2-level in specific tissues thus allowing for a novel, targeted approach in the treatment of these diseases resulting in less side-effects compared to established therapies.

As 17β-HSD2 catalyses the reverse reaction – decreasing the level of active E2 – it should not be affected by inhibitors of the type 1 enzyme. Furthermore, to avoid intrinsic estrogenic effects, 17β-HSD1 inhibitors should not bind to the estrogen receptors (ERs) α and β.

The combination of a structure- and ligand-based drug design approach led to the development of the previously described steroidomimetics hydroxyphenylnaphthols[1-3] as potential inhibitors of the target enzyme. Structural optimisations have been performed by introduction of susbtituents in the 1-position of the naphthalene core (compound **A**) and led to the identification of new highly potent inhibitors of 17β-HSD1 with IC_{50} values in the low nanomolar range displaying good selectivity toward 17β-HSD2 and ERs α and β.



Compound **A**

References:

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Keywords: 17beta-HSD1, Inhibitor, estrogen dependent diseases, steroidomimetic

[P1.09]

Synthesis of four pairs of perhydrochrysene enantiomers and their effects on $GABA_A$ receptor function

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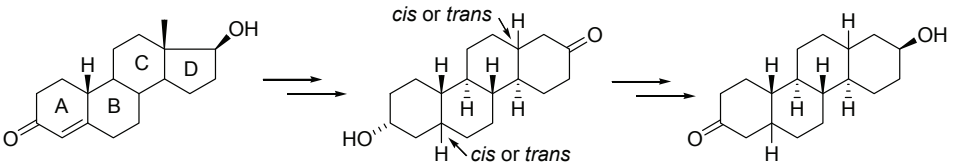

Endogenous steroids of the androgen and pregnane class augment neuronal inhibition by enhancing the actions of the inhibitory neurotransmitter γ-amino butyric acid. We have shown steroid effects to be enantioselective. To extend these enantioselectivity studies to related non-steroidal ring systems, we are studying the enantioselective actions of chrysenes. We have synthesized four difunctionalized perhydrochrysene diastereomers and their corresponding enantiomers from 19-nortestosterone. The four diastereomers differ from each other by the configurations of their A,B and C,D ring fusions. Their synthesis involves first establishing the stereochemistry of the steroid A,B ring fusion as either *cis* or *trans*, then incorporating the steroid 18-methyl group into the steroid D-ring to convert it into the six-membered ring found in the chrysenes, and finally establishing the stereochemistry of the steroid C,D-ring fusion in either the *cis* or *trans* configuration. Because of the symmetry of the chrysene ring system, the enantiomer of each diastereomer is also prepared by the same general strategy using reactions that switch the functional groups on the chrysene A and D rings while avoiding meso intermediates.

The binding interactions of the enantiomer pairs were determined by noncompetitive displacement of [^{35}S]-TBPS from the picrotoxin site found on the heterogeneous population of $GABA_A$ receptors of rat brain membranes. We also evaluated electrophysiological actions on rat $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus laevis* oocytes and anesthetic effects by measuring the loss of righting reflex and loss of swimming reflex in *Xenopus laevis* tadpoles. The activity of the compounds as well as the extent of observed enantioselectivity was dependent on the stereochemistry of the A,B and C,D ring fusions. Supported by NIH grant GM47969 and NSF grant (MRI, CHE-0420497).

Keywords: neurosteroids, chrysene, enantiomer, GABA receptor

[P1.10]
Simultaneous determination of kinetic constants of adrenal steroidogenic reactions

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The interplay of the steroidogenic enzymes yields rich systemic behavior with several substrate enzyme pairs, even though relatively few enzymes are involved. To quantitatively understand the regulatory interactions governing the control of steroid intermediate production by the adrenal steroid biosynthetic enzymes one should, as a first step, construct a kinetic model describing the system interactions. Systems biology tools such as metabolic control analysis can then be used to examine the constructed kinetic model.

The construction of a mathematical model of adrenal steroid biosynthesis requires the determination of substrate binding constants for each enzyme-substrate pair. It is also quintessential to determine how the substrate and product of each reaction indirectly affects the other reaction rates within the system.

This contribution shows how ordinary differential equations (ODEs) comprised of irreversible Michaelis-Menten kinetics, which allow for competitive binding by all steroid intermediates within the system under study, can be used to quantitatively describe an adrenal steroid biosynthetic system.

To illustrate our approach we constructed a kinetic model for the two-step cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17) catalyzed conversion of pregnenolone to 17-hydroxypregnenolone and subsequently DHEA. CYP17 was transiently expressed in COS-1 cells and incubated with pregnenolone. Time course data was used to determine kinetic constants for our ODE model by means of a random search parameter estimation routine. The kinetic constants were validated by showing that the ODE model could describe steroid conversion profiles obtained experimentally under different conditions.

Keywords: Adrenal, Steroidogenesis, enzymes, kinetics

[P1.11]

Dynamic model of how two key enzymes, 3β-HSD and P450c17, control the balance of steroid hormones synthesis

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Steroid hormone synthesis must maintain a proper balance of steroid absolute concentrations and relative ratios for normal development, homeostasis and reproduction. An understanding of steroid flux regulation and possible perturbations is necessary for detection and devising treatment strategies for disease associated with an imbalance of steroid concentrations.

This study is concentrated on a model of androstenedione synthesis from pregnenolone in a network that comprises two interconnected pathways (known as Δ^4 and Δ^5), catalyzed by two key steroidogenic enzymes, 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17α-hydroxylase/17,20-lyase (P450c17). The network utilizes recombinant human enzymes, but can be considered representative of other species such as bovine, ovine and canine, that exhibit similar catalytic specificities.

A mathematical model was developed based on known catalytic characteristics of the two enzymes and basic assumptions. The model can predict the impact of changes in the levels of enzymes activities and changes in biochemical characteristics of enzymes such as affinities and how that might affect pathway flux.

Simulations based on the developed model demonstrate that androstenedione output can vary somewhat paradoxically in response to changes in 3β-HSD activity. Partial inhibition of 3β-HSD can increase androstenedione output by favouring metabolism through the Δ^5 pathway. Specifically, the rate of synthesis and output of androstenedione at steady state consistently increased whenever 3β-HSD activity decreased to a certain level, after which androstenedione output eventually becomes inhibited.

The model allows the use of metabolic data for predicting changes in steroid flux through particular pathways that might be affected by disease or endocrine disrupting chemicals, thereby providing new hypotheses for subsequent investigations.

Keywords: steroid hormones synthesis, dynamic model, 3beta-HSD, P450c17

[P1.12]

Microwave-assisted synthesis of 3β-acethoxy-3'-cyano-6'-methylandrost-5-eno[17,16-c]pyridine-2'(1'H)-thione and its utility in a domino-type synthesis of annulated heterocycles

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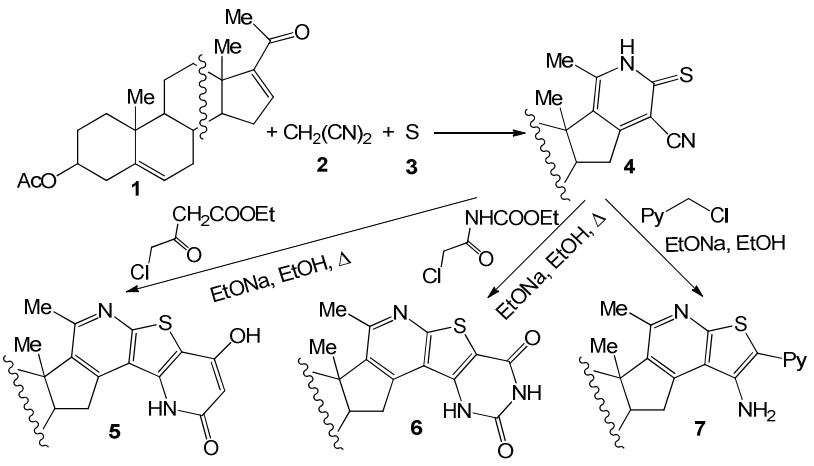
A three-component reaction of unsaturated carbonyl compounds, malnonitrile, and elemental sulfur is often used for the preparation of substituted 3-cyano-2(1H)-thiones – valuable building blocks in synthesis of polyannuated heterocycles [1]. This reaction was modified to synthesize a substituted androsten[17,16-*c*]pyridine-2'(1'*H*)-thione (**4**). The reaction of compounds (**1-3**) was conducted in ethanol in the presence of triethylamine in the Teflon reactor for 6 minutes at 300W. Under such conditions the reaction did not modify the steroid moiety and gave **4** in 76% yield.



Subsequently, compound **4** was used in S_N2 → Thorpe-Ziegler → Thorpe-Guareschi domino-type reactions. It was demonstrated that **4** can be used as a versatile building block in the synthesis of pollyannulated heterocycles (**5-7**) conjugated with steroid fragments.

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Keywords: Multicomponent synthesis, Pyridinethiones, androsten pyridinethione

[P1.13]

20α-Dihydrodydrogesterone: Synthesis, structure and biology

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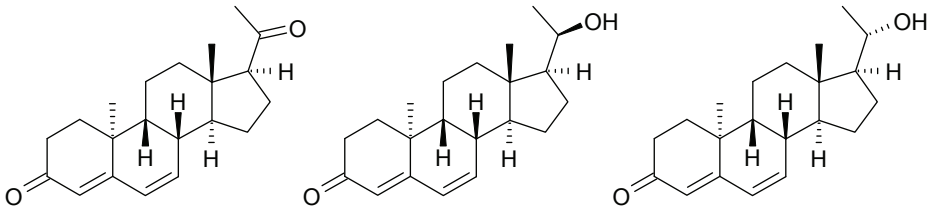
The human sex hormone progesterone plays an essential and complex role in a number of physiological processes. Progesterone deficiency is associated with menstrual disorders and infertility as well as premature birth and abortion. For progesterone replacement therapy the synthetic progestogen dydrogesterone is commonly used. In the human body this drug is stereo-specifically reduced by AKR1C1 (20α-hydroxysteroid dehydrogenase) to 20α-dihydrodydrogesterone (20α-DHD). 20α-DHD also displays extensive pharmacological effects.



Dydrogesterone 20β – Dihydrodydrogesterone 20α – Dihydrodydrogesterone

The original synthesis of 20α-DHD starts with ergosterol and leads to 20α-DHD in 12 steps including a photochemical rearrangement. The use of dydrogesterone as starting point should lead to a faster and easier access of 20α-DHD so different synthetic approaches were tested as well as biotransformation based on fission yeast Schizosaccharomyces pombe[2].

The stereochemistry of the position C20 was determined by new NMR techniques unambiguously, which was important as in the literature two different assignments for the stereochemistry can be found[3].

The poster shall give an overview of the synthesis, structure elucidation of 20α & 20β -dihydrodydrogesterone.

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Keywords: Dydrogesterone, 20-alpha-dihydrodydrogesterone, Schizosaccharomyces pombe

[P1.14]

Genotoxicity of nonylphenol in human mesangial cells

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Nonylphenol ethoxylate is widely used in industrial products and after discharge it is found in aquatic environments. In the environment it breaks down to 4-nonylphenol (NP), which is more stable and persistent. Despite environmental concerns, little research exists evaluating its genotoxicity to human cells. *In vitro* cell viability (lethal concentration - LC) was carried out using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); LC_{50} values were estimated as 0.7×10^{-4} µl/ml Thus, the concentrations of nonylphenol used for genotoxicity test were 0.28×10^{-3} µl/ml and 0.56×10^{-3} µl/ml, which means 40 and 80% of the LC_{50} respectively. Genotoxicity of NP was tested in human mesangial cells using comet assay while cell morphology was evaluated by light microscopy. Ability to induce apoptosis and necrosis was assessed by the acridine orange/ethidium bromide fluorescent-dyeing test. The data showed that NP at test concentrations induces DNA damage without inducing cell death. The comet assay showed that untreated cells had 1.32% of DNA damage while NP-treated cells showed DNA damage ranging between 61.27% and 55.19%. The quantification of cell death induced by NP was not statistically significant when compared with the control group. No differential morphological changes were observed in cells exposed to different concentrations of NP. This study increases the stock of information relevant on nonylphenol genotoxicity.

Keywords: Nonylphenol, DNA damage, comet assay, apoptosis

[P1.15]

Post-exercise heart rate recovery is impaired in anabolic steroids users

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Purpose: We hypothesized that heart rate recovery (HRR) would be lower in anabolic androgenic steroids users (AASU) compared with anabolic androgenic steroids nonusers (AASNU).

Methods: Eight AASU and 7 AASNU participated in the study. Anabolic steroid was tested by urine (chromatography-mass spectrometry). Cardiopulmonary exercise was performed on treadmill ramp protocol. Heart rate was evaluated by a 12 leads EKG. Muscle sympathetic nerve activity (MSNA) was measured by microneurography.

Results: Peak oxygen consumption was lower in AASU compared with AASNU (45±2.27 vs. 52±1.85 ml/kg/min, *P*=.04). Peak HR was not different between groups (189±3 vs. 184±2 bpm, *P*=.28). There were no difference in peak respiratory exchange ratio between groups (1.26±.05 vs. 1.26±.03, *P*=.96). The HRR at first and second minute of recovery was lower in AASU than AASNU (18±2 vs. 26±1 bpm, *P*=.003; 34±4 vs. 43±1 bpm, *P*=.04, respectively). MSNA was higher in AASU than AASNU (29±3 vs. 20±1 bursts/min, *P*=.01). Further analysis showed an inverse correlation between MSNA and HRR (r= .64, *P*=.02).

Conclusion: The impairment in HRR during the post-exercise period and the increased MSNA are consistent with autonomic dysfunction in AASU.

Keywords: Anabolic Steroids, Heart Rate Recovery

[P1.16]

Paraoxonase 1 Q192R polymorphism is associated with adverse cardiovascular risk factors at school age in children after prenatal pesticide exposure

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Introduction: Prenatal exposure to endocrine disrupting chemicals may contribute to development of obesity and metabolic syndrome, both of which predispose to cardiovascular disease. Paraoxonase 1 (PON1) has anti-oxidative functions that may protect against atherosclerosis, and it also hydrolyzes many substrates, including some pesticides. A common polymorphism, Q192R, seems to affect both properties of PON1. Several studies have indicated an increased risk of cardiovascular disease in R-allele carriers, but the significance of chemical exposure is unknown. The purpose of this study was to examine these issues in children of female greenhouse-workers exposed to pesticides early in pregnancy.

Methods: Pregnant greenhouse-workers were categorized as exposed or unexposed to pesticides. At age 6 to11 years their children underwent a standardized physical examination where blood pressure, skin folds, and other anthropometric parameters were measured. Exposure status was unknown to the examiner. Blood samples (non-fasting) were obtained from 145 out of 177 children. PON1-genotype was determined for 141 of these children (88 pesticide-exposed and 53 unexposed). Serum was analyzed for insulin-like growth factor I (IGF-1), insulin-like growth factor binding protein 3 (IGFBP3), insulin and leptin. Body fat percentage was calculated from skin fold thicknesses. BMI results were converted to age and sex specific Z-scores.

Results: Prenatally pesticide exposed children carrying the PON1 R-allele had higher blood pressure, BMI Z-scores, abdominal circumference and body fat percentage than did unexposed children. For children with the PON1 192 QQ genotype, none of these variables was significantly affected by prenatal pesticide exposure. Insulin and leptin concentrations were enhanced after prenatal pesticide exposure in both QQ homozygotes and R-allele carriers, even after adjustment for BMI. IGF-1 and IGFBP3 were enhanced only in R-allele carriers.

Conclusion: Our results indicate a gene-environment interaction between prenatal pesticide exposure and PON1 gene polymorphisms that affects cardiovascular risk markers already known to be associated with the PON1 192 R-allele.

Keywords: paraoxonase, pesticide, gene-environment, BMI

[P1.17]

Topical anti-inflammatory activity of Bauhinia purpurea linn and Moringa oleifera extract in the TPA model of mouse ear inflammation

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[1]NGSM Institute of Pharmaceutical Sciences, India, [2]Manipal College of Pharmaceutical Sciences, India

Background:

This study tested the ability of a characterized extract of Bauhinia purpurea linn and Moringa oleifera which used in traditional medicine, for many ailments viz; inflammations, rheumatism; were investigated for their anti-inflammatory potential to inhibit mouse ear inflammation in response to topical application of 12-Otetradecanoylphorbol-13-acetate (TPA).the steroidal moieties Cyloartenol of Bauhinia purpurea linn and Taraxerone of Moringa oleifera steroidal compounds showing the anti inflammatory action

Methods:

A 50% (wt:vol) ethanolic solution of Bauhinia purpurea linn and Moringa oleifera extracts, applied to both ears of female Swiss mice (n = 6) at 0.075, 0.15, 0.3, 1.25 and 2.5 mg/ear 30 min after TPA administration(2 μg/ear). For comparison, 3 other groups were treated with TPA and either 1) the vehicle (50%ethanol) alone, 2) indomethacin (0.5 mg/ear), or 3) trans-resveratrol (0.62 mg/ear). Ear thickness was measured before TPA and at 4 and 24 h post-TPA administration to assess ear edema. Ear punch biopsies were collected at 24 h and weighed as a second index of edema. Myeloperoxidase activity was measured in each ear punch biopsy to assess neutrophil infiltration.

Results:

Bauhinia purpurea linn and Moringa oleifera extracts,treatment at all doses significantly reduced ear edema compared to the TPA control.The Bauhinia purpurea linn and Moringa oliefera extracts, response was dose-dependent and 2.5 mg Bauhinia purpurea linn and Moringa oleifera significantly inhibited all markers of inflammation to a greater extent than indomethacin (0.5 mg). MPO activity was inhibited at Bauhinia purpurea linn and Moringa oliefera doses ≥ 1.25 mg/ear. Trans-resveratrol inhibited inflammation at comparable doses.

Conclusion:

Bauhinia purpurea linn and Moringa oleifera inhibits development of edema and neutrophil infiltration in the TPA-treated mouse ear model of topical inflammation. Cyloartenol of Bauhinia purpurea linn and Taraxerone of Moringa oleifera steroidal compounds showing the anti inflammatory action

Keywords: anti inflammatory activity, Bauhinia purpurea Linn., Moringa oleifera

[P1.18]

Estrogen receptor alpha signaling promotes Type I interferon and pro-inflammatory cytokine synthesis induced by toll-like receptor signaling in dendritic cells

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Estrogen receptors (ER) are ligand-dependent transcription factors that regulate gene expression by forming complexes with chromatin-modifying coregulators and other transcription factors. Indeed, ER alter the activity of transcription factors, such as NF-κB, C/EBPβ and AP-1, that are crucial regulators of cytokine gene expression. Dendritic cells (conventional or plasmactyoid) produce cytokines in response to signaling mediated by Toll-like receptors (TLR), which sense pathogen-associated molecules such as viral or bacterial nucleic acids. To test the hypothesis that ER alpha (ERα) signaling regulates pro-inflammatory cytokine and type I interferon production by $CD11c^+$ dendritic cells, we developed a novel line of mice, bearing CD11c-Cre and a conditional allele of ERα, in which ERα deficiency is restricted to $CD11c^+$ cells. Isolated $ER\alpha^+$ or $ER\alpha^{-/-}$ splenic dendritic cells were stimulated by TLR ligands in steroid hormone-deficient medium with physiological levels (1 nM) of estradiol, followed by assessment of cytokine RNA and protein synthesis. ERα deficiency significantly reduced the synthesis of *Ifna* and *Il6* RNA by plasmacytoid dendritic cells triggered via TLR7 with influenza virus or triggered via TLR9 with unmethylated CpG-rich DNA. $ER\alpha^{-/-}$ plasmacytoid dendritic cells also secreted reduced amounts of cytokines. In conventional dendritic cells stimulated via TLR9, ERα deficiency led to significantly reduced levels of *Il1a*, *Csf2*, *Ifnb, Nos2* and *Ifng* RNA. Taken together, these data show that cell autonomous ERα signaling in dendritic cells promotes type I interferon and pro-inflammatory cytokine synthesis induced by pathogen-specific nucleic acids. Thus, estradiol-bound ERα may interact with transcription factors activated by TLR 7 and 9 signaling to increase transcription of cytokine genes, thereby regulating the ability of dendritic cells to promote pathogen immunity.

Keywords: cytokine, estrogen receptor, dendritic cells, pathogen

[P1.19]
Sex steroids replacement prevent lymphocytic infiltration of the lacrimal glands in mouse model of Sjögren's Syndrome (SS)

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Sjogren's syndrome (SS) is a chronic autoimmune disease characterized by dry eye, affecting primarily postmenopausal women. A characteristic hallmark of SS is lymphocytic infiltration of the lacrimal glands, consisting mainly of $CD4^+$, $CD8^+$ T cells and $B220^+$ B cells. We have been studying disease progression using ovariectomized (OVX) NOD.B10.H2b mice, which provide a model of menopause combined with a genetic background predisposed to SS. Recently, we have shown that ovariectomy increases significantly the numbers of $CD4^+$, $CD8^+$ T cells and $B220^+$ B cells at 3, 7 and 21 post-OVX days compared to the sham operated groups. In this study we investigated whether replacement of the sex hormones dihydrotestosterone (DHT) or 17β estradiol (E_2) at physiological doses can prevent lymphocytic infiltration in these mice.

Six weeks old C57BL/10SnJ, control and NOD.B10-H2b, were subjected to 4 treatments: Sham, OVX, OVX+DHT or OVX+E_2 for 3, 7 or 21 days. Subcutaneous pellets providing physiological doses of DHT or E_2 were administered to DHT or E_2 treatment groups respectively. At the end of each experimental period, lacrimal glands were removed and processed for immunocytochemistry. The numbers of T and B cells were measured by using the Avidin-Biotin Complex method. Quantification of the stained positive cells was done using an Image Pro Plus analysis system.

Treatment with DHT or E_2 prevented the increase in the numbers of $CD4^+$, $CD8^+$ T cells and $B220^+$ B cells observed at 3, 7 and 21 days in the post-OVX NOD.B10-H2b and at 3 and 7 days in the control C57BL/10SnJ mice.

Our results suggest that sex hormones play a major role in the prevention of lymphocytic infiltration of the lacrimal glands and therefore dry eye in SS. The mechanisms by which decreased levels of sex hormones cause lymphocytic infiltration and interact with the genetic elements remain to be elucidated.

Keywords: Sjogren's Syndrome, Lymphocytic infiltration, Lacrimal gland, Sex steroids

[P1.20]

Spliceosome protein (SRp) regulation of glucocorticoid receptor isoforms and the glucocorticoid response in human trabecular meshwork cells

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Introduction:

Glaucoma is a leading cause of visual impairment and blindness, with elevated intraocular pressure (IOP) as a major causative risk factor. Glucocorticoid (GC) therapy causes morphological and biochemical changes in the trabecular meshwork (TM), an ocular tissue involved in regulating IOP, which can lead to the development of glaucoma in susceptible individuals (steroid responders). Steroid responders comprise 40% of the general population and are at higher risk of developing glaucoma. In addition, almost all glaucoma patients are steroid responders. Differential distribution of various isoforms of GC receptor (GR) may be responsible for this heterogeneity in the steroid response. The alternatively spliced GRβ isoform acts as dominant negative regulator of classical GRα transcriptional activity. mRNA splicing is mediated by spliceosomes, which include SR proteins (SRps). The purpose of our study was to determine whether specific SRps regulate levels of these isoforms and therebyGC response in TM.

Methods:

Quantitative RT-PCR was used to determine the differential expression of SRp20, SRp30c and SRp40 in human normal and glaucomatous TM cell strains. A peptide modulator of splicing (bombesin) and SRp expression vectors were used to modulate SRps levels and determine their effects on GRα/GRβ ratios as well as dexamethasone (DEX) responsiveness via GRE- luciferase reporter activity, fibronectin and myocilin induction in TM cells.

Results:

SRp20, SRp30c and SRp40 regulate GR splicing and the GC response in TM cells. Modulation of SRps levels altered the GRα/β ratio which correlated with DEX responsiveness. Bombesin increased SRp30c levels, decreased GRα/β ratio, and suppressed DEX response in TM cells.

Conclusion:

Relative levels of SRp20, SRp30c, and SRp40 in TM cells control differential expression of the two alternatively spliced isoforms of the GR and thereby regulate GC responsiveness. Different levels and/or activities of these SRps may account for differential GC sensitivity among the normal and glaucoma populations.

Keywords: Glaucoma, Glucocorticoids, Alternative Splicing, Trabecular Meshwork

[P1.21]

Ligand control of estrogen receptor rapid signals determine different cellular outcomes

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E2 induces profound and rapid effects on the conformation of ERs allowing them to dimerize and translocate into the nucleus where specific hormone response elements present in DNA are recognized. ER-E2 complexes can also function as a cytoplasmic signaling molecule eliciting extra-nuclear signaling pathways supposedly independent of transcription. In particular, 17β-estradiol (E2)-induced extra-nuclear signal transduction pathways (i.e., AKT and ERK), in the presence of ERα, are both necessary and sufficient to mediate proliferation in several target cells; whereas, in the presence of ERβ E2 rapidly induces cancer cell apoptosis via p38/MAPK. Besides E2, the crystal structures of ERs indicate that their binding pockets can accommodate many other ligands.

Here, the ability of the flavonoid naringenin (Nar), a natural compound present in human diet, and of the bisphenol A (BPA), a component of plastics, to activate specific extra-nuclear signal pathways and their impact on cell proliferation has been assessed in human cancer cells. The results show that either compounds bind to both ERs with less efficiency than E2. Nonetheless, the compound effects on signal transduction pathways and, in turn, on cell growth result completely different. In fact, in the presence of ERα, BPA and E2 enhance the kinase cascades that drive cancer cell to proliferation, whereas Nar-induced signals completely prevent both indirect transcriptional activity of ERα and cell growth. On the contrary, in the presence of ERβ, E2 and Nar induced the activation of p38/MAPK leading to the pro-apoptotic caspase-3 activation, whereas BPA acts as a pure E2 antagonists. Intriguingly, Nar maintains its effects even in the presence of a background of E2 or BPA. As a whole ER-mediated rapid/extra-nuclear signal pathways are necessary for BPA, Nar, and E2 effects in cancer cell lines. Furthermore, Nar could be considered as a natural and excellent candidate as chemo-preventive agent in E2-induced cancer.

Keywords: Estrogen, Estrogen receptors, Flavonoids, Bisphenol A

[P1.22]

The sexual dimorphic response of 17b-estradiol in female and male costochondral cartilage resting zone chondrocytes is mediated via ERa36

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Our previous studies have demonstrated that 17β-estradiol (E2) regulates rat costochondral cartilage chondrocyte differentiation in a sex-specific and cell maturation-dependent manner via classic nuclear receptors and membrane-associated signaling. E2 activates protein kinase C (PKC) and phospholipase A2 (PLA2) only in chondrocytes isolated from female animals. The aims of the present study were to: (1) identify the estrogen receptor responsible for the activation of rapid membrane signaling; (2) determine the pathway that mediates the membrane effect; and (3) determine the reason for the sexual dimorphism. Western blots and flow cytometry were used to assess expression levels and subcellular location of ERα in both male and female RC cells. Confluent, fourth passage resting zone chondrocytes from female rats were treated with E2 in the presence or absence specific mouse anti-estrogen receptor alpha (ERα) monoclonal antibody, and non-specific mouse IgG as well as with blocking antibodies to ERα36. To examine the potential signal pathways involved in PKC activation, female chondrocytes were treated with E2 in the presence or absence of methyl β cyclodextrin (MβCD), which disrupts caveolae; the cytosolic PLA2 inhibitor AACOCF3; the intracellular calcium channel inhibitor thapsigargin; and the calcium channel inhibitors nifedipine and verapamil.. Western blots and flow cytometry showed that female chondrocytes had 2 to 3 fold greater ERα on the plasma membrane compared to male chondrocytes. All three known ERα isoforms (ERα66, ERα46, and ERα36) were present in cells from both sexes but E2 activated PKC in female cells only and ERα36 was found in caveolae of female cells only. MβCD, AACOCF3, thapsigargin, and antibodies to ERα36 inhibited E2 stimulated PKC activity in female RC cells. In conclusion, the rapid membrane response to 17β-estradiol in female resting zone chondrocytes requires intact caveolae, is mediated via PLA2 and intracellular calcium, and is ERα36 dependent. Sexual dimorphism in response is due to the number of receptors and presence of ERα36 in caveolae.

Keywords: Estrogen, Estrogen Receptor 36, Chondrocytes, sexual Dimorphic

[P1.23]

Role of the phospholipase A_2 activating protein (PLAA) complex in the $1\alpha,25(OH)_2D_3$-induced rapid membrane response in osteoblasts

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1α,25-dihydroxyvitamin D_3 (1,25D3) regulates cells via two different mechanisms: VDR-dependent gene transcription and rapid membrane-signaling. In membrane-signaling, phospholipase-A2 (PLA2) is activated after 1,25D3 binds its membrane-associated receptor protein disulfide isomerase family A, member 3 (Pdia3), leading to release of arachidonic acid and protein kinase C (PKC) activation. Caveolae are required for 1,25D3-dependent PKC signaling. PLAA, which activates PLA2, exhibits homology with the G-protein beta subunit, suggesting it is also membrane-associated, but its presence in caveolae and its interaction with caveolin-1 (Cav-1) or Pdia3 is not known. Wild type and PLAA-silenced osteoblasts **(**MC3T3-E1) were treated with either vehicle or 1,25D3. Whole cell lysates were collected for immunoprecipitation and caveolae isolation and analyzed by Western blot. Subconfluent cultures of MC3T3-E1 cells were immunostained against PLAA, Cav-1 and Pdia3 imaged using confocal microscopy. The effect of 1,25D3 on PLAA silenced cells was examined by measuring PLA2 activity, PKC activity and PGE2 release. PLAA, PLA2, Pdia3, and Cav-1 were detected in plasma membranes, but only Pdia3 and Cav-1 were in caveolae. Pdia3-immunoprecipitated samples were positive for PLAA only after 1,25D3 treatment. Cav-1 was detected when immunoprecipitated with anti-Pdia3 and anti-PLAA in both vehicle and 1,25D3 treated cells. These observations were confirmed by immunofluorescence staining studies. 1,25D3 failed to activate PLA2 and PKC or cause PGE2 release in PLAA-silenced cells. **Conclusion:** Taken together, our results show that PLAA and PLA2 are present in plasma membranes from MC3T3-E1 cells. PLAA and Pdia3 interact with Cav-1 in both control and 1,25D3 groups. PLAA interacts with Pdia3 only in the presence of 1,25D3. PLAA-silenced MC3T3-E1 cells do not exhibit rapid 1,25D3-dependent changes in PLA2, PKC or PGE2. Thus, 1,25D3 initiates conformational changes bringing Pdia3 into proximity with PLAA and aiding in transducing the signal from caveolae to the plasma membrane.

Keywords: 1α,25(OH)2D3, Phospholipase A2 Activating Protein, Rapid Membrane Response, Osteoblasts

[P1.24]

Suppressor of cytokine signalling-3 (SOCS3) in human breast carcinoma

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Introduction: SOCS3 acts on STAT3 pathway in suppressing cellular proliferation and promoting apoptosis in various human malignancies. Results of previous studies indicated SOCS3 as a potential prognostic marker in human breast cancer. However, little has been known in the status of this protein in human malignancies such as the mechanisms of its induction. Recently SOCS3 has been reported to be associated with an androgenic effect in prostate cancer. Androgens are reported to act as an anti-proliferative agent in breast cancer but the relation between androgen actions and SOCS3 has not been studied in breast cancer. Therefore, in this study, we evaluated SOCS3 expression and its possible activators in human breast cancer with relation to androgen actions.

Materials and methods: SOCS3 expression was evaluated using immunohistochemistry (IHC) in 135 human breast carcinoma specimens obtained from Tohoku University Hospital, Sendai, Japan including 30 triple negative breast carcinoma tissues and correlated the findings with clinicopathological findings of the patients. Laser Capture Microdissection (LCM) was performed to further validate its localization with combination of RT-PCR.

Results: SOCS3 mRNA and protein were both detected in intratumoral stromal and carcinoma cells. The status of SOCS3 immunoreactivity in stromal cells was significantly correlated with ER-alpha, lymph node metastasis, and recurrence rate of the cases of human breast cancer and was significantly correlated with AR status in carcinoma cells only in triple negative breast cancer cases.

Conclusion: SOCS3 is expressed in both stromal and carcinoma cells in human breast cancer tissues but only that in stromal cells is considered to play important roles in biological behavior of breast cancer patients including androgen actions in triple negative carcinoma. Further studies are required to evaluate the clinical significance of this protein in human breast cancer.

Keywords: Breast cancer, SOCS3

[P1.25]

The non-benzodiazepine anxiolytic etifoxine stimulates neurosteroid biosynthesis in the central nervous system

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Etifoxine (Stresam®), a molecule structurally unrelated to benzodiazepines, has been shown to possess anxiolytic-like properties. These effects have been ascribed to the potentiating action of etifoxine on GABAergic transmission. However, the exact mechanism of action of etifoxine is not completely understood. It has been shown that etifoxine causes an increase in neurosteroid concentration in brain tissue. It is also known that neurosteroids can modulate the activity of $GABA_A$ receptor, and thus affect anxiety-like behaviors. However, a direct action of etifoxine on neurosteroid formation has never been reported. In the present study, we have investigated the effect of etifoxine on neurosteroid biosynthesis, using the brain of the frog *Rana esculenta* as an experimental model. Exposure of hypothalamic explants to graded concentrations of etifoxine ($3x10^{-7}$ – $3x10^{-5}$ M) produced a dose-dependent increase in the biosynthesis of 17-hydroxypregnenolone, progesterone, dehydroepiandrosterone and tetrahydroprogesterone, and concomitantly decreased the production of dihydroprogesterone. Time-course experiments revealed that a 15-min incubation of hypothalamic explants with etifoxine was sufficient to induce a robust increase in neurosteroid synthesis, and that the maximum effect was observed after a 3-h exposure to etifoxine, suggesting that etifoxine activates steroidogenic enzymes at a post-translational level. Etifoxine-evoked neurosteroid biosynthesis was not affected by the central-type benzodiazepine receptor (CBR) antagonist flumazenil and by the translocator protein (TSPO) antagonist PK11195. Similarly, the effect of etifoxine was not modified by the $GABA_A$ receptor antagonist bicuculline. In conclusion, the present study demonstrates that etifoxine exerts a stimulatory effect on the production of various neurosteroids. Our data also show that the action of etifoxine on neurosteroid synthesis is not mediated through activation of $GABA_A$/CBR or TSPO. These observations indicate that etifoxine may exert its anxiolytic effects either by an indirect action through a receptor different from $GABA_A$/CBR and TSPO, or by a direct action on the activity of neurosteroidogenic enzymes.

Keywords: Neurosteroids, Etifoxine, Brain, Anxiety

[P1.26]

Neurosteroids attenuate stress-induced anxiogenesis in rats : Possible role of free radicals

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Neurosteroids act as modulators in the CNS and there is growing evidence that they may play an important role as endogenous regulators of physiological and neurobehavioral responses. Stress is known to alter the neurobehavioral profile of the organism resulting in the precipitation of anxiety like states and complex neurochemical factors may act as predictors of stress susceptibility. The present study assessed the effects of the neurosteroid, dehydroepiandrosterone sulfate (DHEAS), on stress induced anxiogenesis in rats and evaluated the possible mechanisms involved in this effect. Restraint stress (RS) induced anxiogenic behavior in the elevated plus maze test, as evidenced by the reductions in open arm entries and open arm time. This was accompanied by elevations in plasma corticosterone levels. Pretreatments with DHEAS (5, 10, 20 and 40 mg/kg) dose dependently attenuated RS-induced anxiogenic behavior in the elevated plus maze, and these effects were comparable with diazepam. The nitric oxide (NO) precursor, L-arginine (100 mg/kg), potentiated the anxiolytic effects of DHEAS, whereas, the NO synthase inhibitor, L-NAME (50 mg/kg), showed opposite modulations of DHEAS effects. Restraint stress also induced elevations in malondialdehyde (MDA) and reductions in reduced glutathione (GSH) and NO metabolite (NOx) levels in brain homogenates of rats. These RS induced changes in brain biochemical markers were reversed by DHEAS and L-arginine pretreatments. In the interaction studies, L-arginine synergized with DHEAS in reversing the brain oxidative stress markers, whereas, L-NAME showed neutralizing effects when given together with the neurosteroid. These observations suggest that the neurosteroid exerts an attenuating effect on restraint stress induced alteration of neurobehavioral changes suggestive of anxiolysis and brain oxidative stress markers in rats, and that NO mediated mechanisms may be involved n these effects.

Keywords: neurosteroids, restraint stress, anxiogenesis, nitric oxide

[P1.27]

Effects of ovariectomy and 17B-estradiol replacement on apoptosis-inducing factor (AIF) and Endonuclease-G in the rat hippocampus.

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17β-estradiol plays a potent neurotrophic and neuroprotective role in the brain. The mechanisms underlying 17β-estradiol neuroprotection are not fully understood, several candidate targets have been identified. Recent studies from our laboratory have shown that 17β-estradiol may help maintain long-term neuronal viability in the rat hippocampus by regulating the expression of members of BCL2 family. Furthermore, the duration of the treatment with 17β-estradiol after ovariectomy is a key factor to restore the expression of BCL2 and BAX to control levels. Along with these proteins, other mitochondrial proteins, such as apoptosis-inducing factor (AIF) and Endonuclease-G, are known to play a key role in mammalian cell apoptosis. AIF and Endonuclease-G are released from mitochondria to the cytosol, translocate into the nucleus, and cause chromatin condensation and fragmentation of DNA when cells are exposed to an apoptosis-inducing stimulus.

In the present study, we examined the effects of different periods of ovariectomy and the regulatory effects of the administration of 17β-estradiol for different periods on the expression of AIF and Endonuclease-G in mitochondrial and cytosolic fractions obtained from adult female rat hippocampus.

Hippocampi were obtained from rats in proestrus (control), rats ovariectomized for 15, 21 and 36 days, rats ovariectomized for 15 days and then treated with 17β-estradiol benzoate (50 μg/kg, sc, every other day) for 7 or 21 days and rats ovariectomized and immediately treated with 17β-estradiol for 21 days. AIF and Endonuclease-G expression were determined by Western Blot. Both ovariectomy and ovariectomy and 17β-estradiol replacement did not affect AIF expression in the mitochondrial and cytosolic fractions of the hippocampus. On the other hand, Endonuclease-G expression decreased in the mitochondrial and increased in cytosolic fraction of the hippocampus obtained from ovariectomized rats. The treatment with 17β-estradiol for different periods was able to revert the effect of ovariectomy, suggesting that 17β-estradiol is involved in the regulation of Endonuclease-G. These data provide new understanding into the mechanisms involved in the neuroprotective role of 17β-estradiol.

Supported by FAPESP and CNPq.

Keywords: Ovariectomy, 17B-estradiol, Endonuclease-G, apoptosis-inducing factor

[P1.28]

Metabolism of the synthetic progestin norethynodrel by human ketosteroid reductases of the aldo-keto reductase superfamily

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Human ketosteroid reductases of the aldo-keto reductase (AKR) superfamily, i.e. AKR1C1-4 enzymes, are implicated in the biotransformation of many synthetic steroid hormones. Norethynodrel (17α-17-hydroxy-19-norpregn-5(10)-en-20-yn-3-one), which was the progestin component of the first marketed oral contraceptive, is known to undergo rapid and extensive metabolism to 3α- and 3β-hydroxy metabolites. Norethynodrel is structurally similar to the hormone replacement therapeutic tibolone ([7α,17α]-17-hydroxy-7-methyl-19-norpregn-5(10)-en-20-yn-3-one), with the difference being the presence of a 7-methyl group in the latter compound. Previous studies showed that tibolone is bioactivated to 3α- and 3β-hydroxy metabolites by human AKR1C enzymes. In this study, the ability of the four human AKR1C isoforms to catalyze the metabolism of norethynodrel has been characterized. Comparison of the product profile and kinetic parameters of the reduction of norethynodrel and tibolone catalyzed by each individual AKR1C isoform highlights the effect of the 7-methyl group on the stereochemical outcome and kinetic behavior of each enzyme. Norethynodrel is also structurally related to norethindrone (17α-17-hydroxy-19-norpregn-4-en-20-yn-3-one), with the difference being the position of the double bond. Norethindrone is the common progestin component in oral contraceptives and hormone replacement therapy and is not a substrate for AKR1C isoforms. Our results provide insights into the differential pharmacological properties of these three synthetic progestins.

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Keywords: steroid metabolism, synthetic progestin, oral contraceptives, hormone replacement therapy

[P1.29]

Natural compounds with estrogenic activity are potent inhibitors of TGF beta-induced prostate stromal metabolism of DHEA and androgen-induced PSA secretion by epithelial cells

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The reactive stromal phenotype in the prostate is mechanistically important for understanding prostate cancer progression and may be a novel target for prevention. A new experimental model reflects the interaction of endocrine, paracrine, and immune factors on induced androgen metabolism in prostate stroma. The model includes coculturing human primary prostate stromal cells and LAPC-4 prostatic adenocarcinoma cells. Conversion of DHEA (D) by the reactive stroma following TGF-β (T) stimulation is assessed by induced PSA secretion in LAPC-4 cells simulated by stromal production of androgens. Distinctions could be seen between direct androgenic effects on epithelial cells using the non-metabolizable androgen (R1881) and secondary androgen production by stromal metabolism of DHEA. Key findings were that D+T treatment upregulated androgen-production in the stroma resulted in increased PSA secretion by cocultured LAPC-4 cells. Further, estrogen receptor (ER) agonists PPT (ERα) and DPN (ERβ), as well as estrogenic natural compounds including isoflavones and naringenins attenuated increased PSA production. The soy isoflavones genistein and daidzein showed potent dose dependent inhibition (0.1 – 10 μM, 67-92% and 62-92% respectively) to the D+T-treated PSA induction and only mildly inhibited the response to R1881 treatment (40% and 60% at 10μM). The naringenins, 8-prenylnaringenin and 6-dimethylallylnaringenin had nearly equal potency in inhibiting stromal and epithelial response (around 95 % at 10 μM), whereas naringenin itself was very potent to inhibit DHEA conversion (90% at 1μM) but showed a mild effect (55% at 1μM) on the epithelial response. Studies with pure ER agonists showed that activating either receptor inhibited D+T-mediated PSA production with the ERb effect was more pronounced. In conclusion, natural compounds with estrogenic activity are very potent inhibitors of both stromal conversion of androgenic prohormones and androgen-induced PSA secretion by epithelial cells. More studies are needed to characterize how these compounds may be valuable substances in the prevention of prostate cancer progression.

Keywords: reative stroma, estrogen receptors, steroid metabolism, natural compounds

[P1.30]

Cortisol metabolism by rainbow trout ovarian follicles and the affect of in ovo cortisol exposure on the ability of embryos to metabolize cortisol

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In teleost fish, maternal cortisol is transferred to oocytes during gonadal recrudescence, and elevated maternal plasma cortisol levels increase oocyte cortisol content. *In vitro* studies have shown that cortisol suppresses thecal/granulosal cell steroidogenesis and *in vivo* studies have shown that cortisol affects several aspects of early embryogenesis. Moreover, glucocorticoid receptors (GRs) are present in both thecal and granulosal cells of the ovarian follicles, and in very early stage embryos; in addition, the follicles and early embryos express *GR1* and *GR2* genes. Thus, there is the potential for the exposure of the maternal stock to elevate oocyte cortisol content and thereby affecting embryogenesis. A series of studies was undertaken using *in vitro* incubations of ovarian follicles and embryos in the presence of [^{3}H]cortisol, followed by separation of ^{3}H-labelled metabolites to: a) examine the ability of ovarian follicles and embryos to metabolize cortisol, b) examine the affect of increasing *in ovo* cortisol content on the ability of embryos to metabolize the glucocorticoid (using HPLC). In addition studies were carried out to examine the effects of glucocorticoid receptor blockade with RU 486 on aspects of embryo metabolism and development. The oocytes and embryos have a limited ability to metabolize cortisol into corticosterone (CS) and deoxycorticosterone (DOC). A single i*n ovo* exposure to RU 486 prior to fertilization had no marked affect on the fertilization rates of the oocytes, or on hatching, deformity or mortality rates of the embryos; however, during the transition from late stage embryos (reliant on the yolk sac) to early stage juveniles (post-yolk sac absorption), there was marked mortalities, associated with delayed yolk sac absorption.

Keywords: glucocorticoid metabolism, ovarian follicles, early embryos, rainbow trout

[P1.31]

New coumarin derivatives as selective nonsteroidal inhibitors of 17β-Hydroxysteroid Dehydrogenase Type 1 (17β-HSD1)

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17β-Hydroxysteroid dehydrogenase type 1 (17β-HSD1) catalyzes NADPH-dependent reduction of estrone into estradiol, which exerts proliferative effects via the estrogen receptors. Overexpression of 17β-HSD1 in estrogen responsive tissues is related to the development of hormone-dependent diseases, such as breast cancer and endometriosis; thus, 17β-HSD1 represents an attractive target for the development of new therapies. Although clear structure–activity relationships (SARs) have been established for 17β-HSD1 inhibitors, no successful clinical drug candidates have been discovered yet.

We have synthesized a library of 41 coumarin derivatives which were designed as mimetics of steroidal substrate. All compounds were assayed for inhibition of 17β-HSD1. SAR study established that acetyl group and proper phenyl substituent at positions 3 and 6, respectively, are important for potent 17β-HSD1 inhibition. The most potent inhibitor (Figure 1) in series reversibly inhibits the 17β-HSD1 reductive activity with a K_i value of 53 nM in a purified recombinant enzyme assay. Potent 17β-HSD1 inhibitors of these series did not show any inhibition of 17β-HSD2, which catalyzes the oxidation of estrone into estradiol thereby having the protective physiological role.

None of the tested coumarins displayed detectable binding affinity towards ERα or ERβ. Their plausible estrogenic effects were also evaluated in T-47D cell proliferation assay where none of the coumarin derivatives increased proliferation of T-47D cells. The additional biological evaluation confirmed that the most promising coumarin derivative (Figure 1) inhibits endogenous 17β-HSD1 activity in the human breast cancer T-47D cells and also reduces the estrone dependent growth of T-47D cells.

Coumarin scaffold thus offers potential for development of potent and selective 17β-HSD1 inhibitors. Currently, we are performing advanced computational and medicinal chemistry approaches in order to optimize the selective 17β-HSD1 inhibitory activity of this series of compounds.

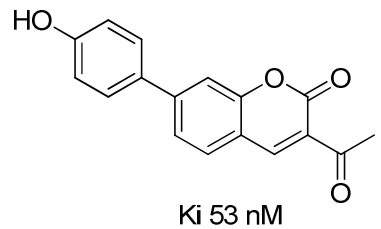

Figure 1. The most potent 17β-HSD1 inhibitor reported in this study

Keywords: 17beta-HSD1, breast cancer, coumarins, selective nonsteroidal inhibitors

[P1.32]

Carbenoxolone, a non selective 11beta-Hydroxysteroid Dehydrogenase Type 1 increased erythrocyte membrane fluidity and ouabain-sensitive ATPase activity in obesity: Studies on WNIN/Ob obese rat, a new rat model of genetic obesity

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Introduction

11beta-hydroxysteroid dehydrogenase type 1 (11beta-HSD1) inhibition in liver and adipose tissue decreases obesity and improves insulin sensitivity. Although majority of beneficial effects of 11beta-HSD1 inhibition are due to reduced active glucocorticoid levels in tissues, downstream cellular mechanisms involved are poorly known. Here, we studied the effect of carbenoxolone, a non-selective 11beta-HSD1 inhibitor on membrane fluidity and activities of membrane-bound enzymes of erythrocyte membranes from WNIN/Ob lean and obese rats.

Methods

Three month-old, male lean and obese rats (n=12 for each phenotype) were divided into control and experimental groups (n=6). Carbenoxolone (CBX) (50 mg/Kg body wt) was administered subcutaneously to experimental group of lean and obese rats for four weeks. Blood was collected, erythrocytes were isolated, and hemolysed. Membranes were isolated by ultracentrifugation. Membrane fluidity was determined by spectroflurimetry using 2, 5 diphenyl hexatriene. Total ATPase, ouabian sensitive and insensitive ATPase, Ca^{+2}-Mg^{+2}- ATPase and acetylcholine esterase enzyme activities were determined.

Results

Membrane fluidity was significantly less in control obese rats compared to control lean rats. CBX significantly increased membrane fluidity ($p \leq 0.01$) in obese rats reaching up to the level observed in lean control rats. Total ATPase activity significantly increased in CBX- treated obese rats ($p \leq 0.01$) but not in lean rats. Ouabain sensitive ATPase activity ($p \leq 0.01$) increased significantly in CBX-treated obese rats, however not altered activity in lean rats. Ouabain-insensitive ATPase, Ca^{+2}-Mg^{+2}- ATPase and acetylcholine-esterase activities were not altered by CBX treatment in lean and obese rats.

Discussion

This is the first study to report the effect of 11beta-HSD1 inhibitor on membrane fluidity and activity of membrane-bound enzymes. 11beta-HSD1 inhibition increased the membrane fluidity and activity of ouabain-sensitive ATpase in erythrocytes of WNIN/Ob obese rats. The observed amelioration of metabolic syndrome features by 11beta-HSD1 inhibition is possibly mediated through altered membrane fluidity, which in turn affects the activities of membrane-bound enzymes.

Keywords: 11beta-hydroxysteroid dehydrogenase type 1, Obesity, Membrane fluidity, WNIN/Ob obese rat

[P1.33]

11Beta-Hydroxysteroid Dehydrogenase Type 1 activity in liver and adipose tissue of WNIN/GR-Ob obese rats, a new genetic obese rat model with impaired glucose tolerance

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Introduction

11beta-hydroxysteroid dehydrogenase type I (11beta-HSD 1) generates active glucocorticoids in tissues from inactive forms. 11beta-HSD1 activity is elevated in adipose tissue and reduced in liver of obese rodent models and in humans. Here, we report 11beta-HSD1 activity in liver, adipose tissue and skeletal muscle of WNIN/GR-Ob obese rat model, a new genetically obese rat model with glucose intolerance.

Methods

Three month-old, male WNIN/GR-Ob lean and obese rats (n=6 for each phenotype) were taken and body composition was analyzed by total body electrical conductivity. Oral glucose tolerance test (OGTT) was carried to assess glucose intolerance. Plasma insulin, corticosterone and glucose, lipids were measured. 11beta-HSD1 activity was measured in liver, skeletal muscle and adipose tissue by HPLC method using 1, 2, 6, 7-^{3}H4 corticosterone as substrate.

Results

WNIN/GR-Ob obese rats have significantly increased body weight, fat percentage ($p \leq 0.001$) and heavier adrenals ($p \leq 0.05$). Fasting insulin ($p \leq 0.001$) and corticosterone ($p \leq 0.05$) levels were elevated in obese rats and they have glucose intolerance as evidenced by OGTT curve. 11beta-HSD1 activity was significantly decreased in omental adipose tissue of obese rats compared to lean rats ($p \leq 0.05$). 11beta-HSD1 activity was not altered in liver, subcutaneous adipose tissue and skeletal muscle of WNIN/GR-Ob obese rats compared to lean rats.

Discussion

WNIN/GR-Ob obese rats carry genetic mutations for obesity and glucose intolerance which are yet to be discovered. WNIN/Ob obese rats carrying mutation for obesity but not glucose intolerance had elevated 11beta-HSD 1 activity in adipose tissue and lowered enzyme activity in liver. In contrast to WNIN/Ob obese rats, WNIN/GR-Ob obese rats have reduced 11beta-HSD1 in omental adipose tissue and unaltered activity in liver and subcutaneous adipose tissue. Here we conclude that glucose intolerance alters the 11beta-HSD1 activity in liver and adipose tissue under obese conditions.

Keywords: 11beta-hydroxysteroid dehyrogenase type 1, Obesity, Impaired glucose tolerance, WNIN/GR-Ob obese rat

[P1.34]
Cognitive behaviors and pre-receptor metabolism of androgens in ovariectomized rats

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Female rats and women have surprisingly high levels of testosterone (TS) yet the effects of androgens on female behaviors are not well understood. Our interest is in the functional outcomes of testosterone metabolites binding to androgen or estrogen receptors in brain regions underlying non-reproductive neurophysiology and behavior. Here, we address the question of the steroid receptor responsible for the findings that elevated TS enhance some forms (hippocampal dependent spatial) but not other forms (cortical dependent non-spatial) of learning and memory.

An aromatase inhibitor, Letrozole, was used to block conversion of testosterone to estrogen in 2 of 4 groups female rats largely depleted of estrogen (E2) with ovariectomies. OVX females were administered daily either vehicle only, Letrozole only (2mg/ Kg bwt), TS only (400ug/ kg) or the combination of Letrozole + TS over 4 weeks. During the last 3 weeks of exposure, the animals were tested in two paradigms to examine different forms of learning and memory. An object recognition task was used to test for non-spatial, short-term working memory while a radial arm maze (RAM) was used to test for spatial learning and memory. The hypothesis was that blocking TS from being metabolized to E2 would have different influences on spatial vs. non-spatial working memories. Comparisons with TS only treatments revealed no differences with the Letrozole + TS group on the objective recognition task. However, the TS only group was superior to the Letrozole + TS group on the RAM task. The suggestion is that inhibiting aromatase metabolism in the brain of TS to E2 blocked the typical enhancement of performance on spatial tasks, but not of non-spatial performance. Implications are that some cognitive abilities require binding of the androgen receptor either by TS or DHT while other cognitions depend on TS converting to E2 and binding the estrogen receptor.

Keywords: testosterone, females, cognition, memory

[P1.35]

Characterization and estrogen modulation of human adult prostate progenitor cells

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Applying recent advances made in stem cell research, we have been able to isolate and expand human prostate progenitor cells (PPCs) from primary human prostate epithelial cells (PrECs) *in vitro*. Using a 3-D culture system, PPCs were differentially selected and increased with about 0.2% of PrECs forming spheroid structures referred to as prostaspheres (PS). By day 4, PS were solid and non-canalized with diameter averages of 30 μm. Day 7 PS increased to an average diameter of 80 μm and by day 10 between 100-150 μm. Immunohistochemical analyses of day 10 PS revealed double-layerd PS in which the periphery consisted primarily of p63-positive cells and the inner-core contained more differentiated CK8/18-positive cells. The outer layer p63-positive cells were also positive for the prostate stem cell markers CD117 and CD133 indicating higher self-renewal capacity. At day 30, ductal branch formation was induced from the PS buds by HGF. Steroid receptor status of early stage PS showed lack of androgen receptor (AR) but elevated expression of all known estrogen receptors (ER) including ERα, ERβall, ERβ1, GPR30 and progesterone receptor (PR) when compared to LnCaP cells. Investigating effects of estrogen on PS formation, PrECs were treated with 17β-estradiol (E2) at doses of 1 to 1000 nM for 7 days and there was a significant dose-dependent increase in both the number and size of formed PS. In summary, adult PPCs are a rare population that reside in human prostate tissue and by using an *in vitro* 3-D culture system PPCs can self-renew and form PS. Our lab has previously presented evidence for the increased predisposition of adult prostate to disease when neonatally exposed to estrogens and this new model of exposure to PPCs promises to offer better insight into the mechanisms of estrogen induced prostatic disease.

Keywords: estrogen, progenitor cell, prostate, prostasphere

[P1.36]

Detection of ER22/23EK and BclI polymorphisms in glucocorticoid receptor gene in blood donors from belgrade commuNITY

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Introduction: Glucocorticoid hormones (GCs) accomplish their effects through binding to glucocorticoid receptor (GR). Presence of GR gene polymorphisms can modulate GCs effects. The ER22/23EK polymorphism in hGR gene has been connected to decreased and *Bcl*I polymorphism to increased sensitivity to GCs.

Methods: The aim of this study was to detect frequency of ER22/23EK and *Bcl*I polymorphisms find possible association between *Bcl*I polymorphisms in 247 blood donors from Belgrade community (104 women, 143 men; mean age 44.47±11.76).

Healthy volunteers were recruited from the National Institute for blood Transfusion. All subjects underwent metabolic, genetic, biochemical and anthropometric testing. DNA was obtained from peripheral blood leucocytes. The ER22/23EK and *Bcl*I polymorphisms were detected by using PCR, RFLP and DNA sequencing.

Results: The ER22/23EK polymorphism was found in 3 (1.21%) and *Bcl*I polymorphism in 63 (25.51%) healthy subjects. Individuals with detected larger C allele of *Bcl*I polymorphism had elevated cholesterol levels, comparing to non-carriers ($p=0.000$). Also, older *Bcl*I polymorphism carriers had higher cholesterol levels, comparing to younger carriers ($p=0.000$). Because of rather small number of ER22/23EK carriers, there was unpossible to perform detail statistical analysis.

Discussion: The *Bcl*I polymorphism of the GR gene can bee associated with presence of metabolic risc factors, which could indicate that healthy subjects with *Bcl*I polymorphism can have increased risk for metabolic syndrome development, especially in elder period of life.

Keywords: Glucocorticoid receptor, ER22/23EK polymorphism, BclI polymorphism, Metabolic syndrome

[P1.37]

Role of metastasis tumor antigen 1 and estrogen receptor in rat placenta

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Despite the high levels of circulating maternal estrogens during pregnancy, placental growth arrest is spared. One mechanism maybe by down-regulation of the estrogen receptor (ER). Alternatively, this can be attributed to the interaction of metastasis tumor antigen (MTA1) and its truncated form (MTA1s) with the ER as it has been demonstrated that MTA1 inhibits the ligand induced transactivation effect of estradiol on ER and MTA1s sequesters ER in the cytoplasm inhibiting its nuclear functions and allowing estradiol to act via non-genomic pathways. In an attempt to understand the role of ERα, ERβ, MTA1 and MTA1s in placental growth we investigated mRNA expression of ERα, ERβ, MTA1 and MTA1s in whole rat placenta and protein expression of ERα, ERβ, and MTA1 in the homogenate, cytosolic and nuclear fractions during gestation. Maternal serum, amniotic fluid and placental samples were collected at 16, 19 and 21 days gestation (dg). Gene and protein expression were studied using Real-Time PCR (ReT-PCR) and Western blotting, respectively. Placental weights increased significantly between 16 and 21 dg. Maternal serum, amniotic fluid and placental estradiol levels increased with gestation. ERα mRNA and protein expression showed a trend to decrease and ERβ levels decreased significantly at the mRNA (16 vs 21 dg) and protein level in the nuclear fraction (19 vs 21). MTA1 and MTA1s levels increased, at the mRNA level only, with MTA1 expression being higher than MTA1s at all gestational ages. Estradiol, acting through its receptor, may play a direct role in inhibiting placental growth. However, expression of MTA1 and its truncated form in rat placenta suggests that they may play a role in this inhibition sparing the placenta from growth arrest. MTA1 expression may contribute to placental growth via other mechanisms including activation of β-catenin and cyclin D1 indicating an activation of Wnt pathway promoting cell growth.

Keywords: Estrogen receptor, Metastasis tumor antigen 1, Placenta

[P1.38]

Regulation of senescence by androgen receptor

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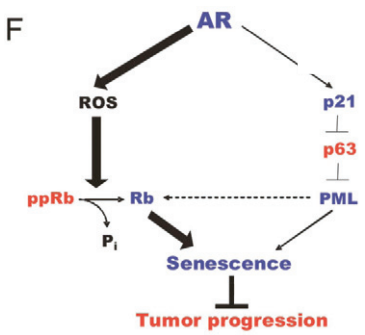
The cells in the prostate tumors can be classified as androgen-inducible, androgen-independent and androgen-repressible. Thus androgen receptor (AR) function in prostate tumors range from promoting proliferation, survival and cancer progression to growth suppression and opposing tumor growth. The growth-inhibitory and tumor-suppressive AR effects have been clearly documented but the underlying mechanisms have not been well elucidated. Here we, for the first time link AR anti-cancer action with cellular senescence *in vitro* and *in vivo* and identify its molecular mediators. We found that p53 was not involved in AR-driven Instead, AR directly induced p21 expression, which, in turn, reduced ΔN isoform of p63, a p53 family member. IN addition, AR activation increased reactive oxygen species (ROS) and thereby suppressed Rb phosphorylation causing senescence. Both pathways were critical as was demonstrated by p21 and Rb knock-down and by the use of ROS quencher N-Acetyl cysteine. Moreover, p63 knock-down also mimicked AR-induced senescence. The three pathways were engaged in a cross-talk, possibly via PML tumor suppressor, whose localization to the senescence-associated chromatin foci was dramatically increased by AR activation. Our results for the first time demonstrate senescence response caused by a nuclear hormone receptor and define the underlying molecular mechanisms.



Keywords: Androgen receptor, Senescence, Prostate cancer

[P1.39]

Mineralocorticoid receptor involves short term osmoregulation in Japanese eel, Anguilla japonica

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Aquatic teleosts have highly efficient osmoregulatory mechanisms in order to maintain their body fluid homeostasis. Euryhaline and migratory fishes such as Japanese eel adapts to wide salinity in their life cycle. In fresh water, they actively absorb salt through the gills from the environment, but drink seawater and actively secrete salt via the gills in sea water. An important factor modulating the balance of ions and water has been considered by corticosteroid hormone. It has generally been thought that a single hormone, cortisol, carries out both glucocorticoid and mineralocorticoid actions through glucocorticoid receptor (GR) in teleost fish (Uchida, 1998). The current study focuses on the newly cloned mineralocorticoid receptor (MR) in a migratory teleost, Japanese eels, *Anguilla japonica* relating to ion regulation. It is possible that MR in teleost execute same mineralocorticoid functions as mammals. MR sequences of several fishes were aligned to design primers in the conserved region and did blast after sequencing. Partial cloned eel MR is similar to MR of euryhaline fish than its GR (bootstrap value is 100). Tissue distribution showed MR expression in osmoregulatory organs. Eels were transferred from tap water to pure water within 96 hours in order to investigate the expression of MR in osmoregulation organs and construct fresh water model. The real-time quantitative PCR with absolute quantification showed massive expression in esophagus within 96 hours and a gradual up-regulation (nearly two fold every time-point) of MR in the rest osmoregulation organs. *In situ* hybridization results also found MR expression in chloride cells of gills. These data suggest the function of MR involving osmoregulation, and we need more experiments like antagonist treatment to support it.

Keywords: Japanese eel, mineralocorticoid receptor, glucocorticoid receptor, osmoregulation

[P1.40]
Nuclear trafficking signals of LRH-1
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Liver receptor homologue-1 (LRH-1) is a member of the nuclear receptor superfamily. We characterized two functional nuclear localization signals (NLSs) in LRH-1. NLS1 (residues 117–168) overlaps the second zinc finger in the DNAbinding domain. Mutagenesis showed that the zinc finger structure and two basic clusters on either side of the zinc finger loop are critical for nuclear import of NLS1. NLS2 (residues 169–204) is located in the Ftz-F1 box that contains a bipartite signal. In full-length LRH-1, mutation of either NLS1 or NLS2 had no effect on nuclear localization,

but disruption of both NLS1 and NLS2 resulted in the cytoplasmic accumulation of LRH-1. Either NLS1 or NLS2 alone was sufficient to target LRH-1 to the nucleus. Both NLS1 and NLS2 mediate nuclear transport by a mechanism involving importin a/b. Finally, we showed that three crucial basic clusters in the NLSs are involved in the DNA binding and transcriptional activities of LRH-1.

Keywords: LRH-1

[P1.41]

Combining ms-hrm and pyrosequencing for methylation status analysis of the estrogen receptor alpha gene in male growing piglets

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Male piglets exhibit very distinct endogenous levels of estradiol-17β over the first few weeks of lifespan. Since it is widely accepted that estrogen receptor alpha (ESR1) functions as the most important mediator of estrogenic action, we characterized the expression of *ESR1* in various tissues during three different time points and designed DNA methylation analyses attempting to explain differential gene expression.

Male siblings of German Landrace sows (n=6) were slaughtered at <1 h, 11 d and 56 d after birth. Samples were taken from reproductive (e.g. epididymis) and non-reproductive tissues (e.g. heart). RNA was extracted for quantitative real-time PCR of ESR1 and genomic DNA was isolated for global (Luminometric Methylation Assay) and local methylation analysis. For the latter, we combined methylation-sensitive high resolution melting (MS-HRM) with subsequent pyrosequencing.

Reproductive tissues exhibited distinctly higher *ESR1* expression levels than non-reproductive tissues (e.g. 250-fold epididymis vs. heart). *ESR1* expression varied in epididymis over the different time points showing a 2.1-fold and 1.7-fold higher expression on days 11 and 56, respectively, compared to newborns (*P=0.01*). Interestingly, mean global methylation was lower in heart (72%) compared to epididymis (75%). In testis, global methylation varied significantly over time (75 %, 73 % and 71 %, respectively, *P<0.05*). With our inventive approach for local methylation analysis, primer bias was effectively controlled by MS-HRM on gradually artificially methylated DNA prior to pyrosequencing, while MS-HRM, limited by low resolution and solely qualitative readout, was perfectly enhanced by subsequent pyrosequencing. Promoter, CpG-island and intragenic region in the 5' end of the ESR1 gene are currently investigated to explain expression variations.

In summary, tissues of growing piglets are distinctly regulated during pre-pubertal growth with respect to *ESR1*. We show that a combination of MS-HRM and pyrosequencing successfully resolves respective limitations inherent in both methods and highlight the necessity of both assays for reliable quantification of local DNA methylation at a single CpG-site resolution.

Keywords: Estrogen receptor alpha, DNA methylation, MS-HRM, Pyrosequencing

[P1.42]

Estrogen-dependent ER-alpha activation is required for normal branching morphogenesis of the mouse prostate gland

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Estrogens acting through estrogen receptors (ER) play an important role during prostate gland development and abnormal growth associated with prostatic diseases such as BPH and cancer. Studies using ER knock-out mice have shown that developmental effects of estrogens on the prostate gland are mediated through ER-alpha (Prins et al, *Can Res* 2001). Recent studies using ACTB-Cre/ER-alpha$^{-/-}$ mice demonstrated reduced branching morphogenesis of the prostate glands suggesting a critical role for ER-alpha in this process (Chen et al, *Endocrinology* 2001). ER-alpha actions can be mediated through both estrogen-dependent and estrogen-independent activation. **The present study sought to determine whether cell membrane estrogen-dependent and/or ligand-independent actions of ER-alpha are involved in prostate gland development.** To do so, we examined the prostate glands of peri-pubertal (day 30) male membrane only ERα (MOER) and estrogen nonresponsive ER-alpha knock-in (ENERKI) mice. Prostate lobes from day 30 male MOER and ENERKI compared to either *background* or *wild type* (*wt*) mice, respectively, were analyzed morphologically for branching and differentiation endpoints. Branch tip number in microdissected ventral prostates was significantly increased from 160.3 ± 8.4 in *background* mice to 194.0 ± 1.5 in MOER males ($p<0.05$). In contrast, branch tip number in microdissected ventral prostates in ENERKI mice was significantly reduced from 155.70 ± 8.7 in *wt* mice to 131.27 ± 3.08 ($p<0.05$). While histologic inspection in the ENERKI mice revealed normal epithelial cytodifferentiation, the lumens were markedly enlarged. In total, these findings suggest that estrogen dependent ER-alpha activation that includes membrane-initiated signaling is involved in branching morphogenesis and planar cell division controlling circumferential growth of the prostatic ducts. (Supported by DK40890 and CA89089)

Keywords: Estrogen receptor alpha, Prostate, ENERKI, MOER

[P1.43]

Progesterone and prolactin synergistically increase epithelial cell proliferation in the mouse mammary gland

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The principle reproductive hormones that regulate human breast development and tumorigenesis are estrogen (E), progesterone (P), and prolactin (PRL). Of these, the proliferative effects of E have been studied widely, where antiestrogens are an effective therapy for ER-positive breast cancers. The mitogenic effects of P, both in the normal breast and in breast cancer, remain less clear. Limited data also exist regarding the role of PRL in breast cancer, where PRL stimulates the proliferation of breast cancer cells. While P and PRL are both independently essential for normal mammary gland development, we now demonstrate the synergistic effect of these two hormones on epithelial cell proliferation in the mammary glands of adult female mice. The ER antagonist, ICI 182,780 almost completely blocked the proliferative effect of P+PRL, while the aromatase inhibitor, letrozole was without effect. Furthermore, P and PRL synergistically induced a 3-fold increase in the incidence of cyclin D1-positive mammary epithelial cells. The mechanism by which P and PRL cooperatively increase epithelial cell proliferation and cyclin D1 expression may depend on ER activation and/or increased Jak/STAT signaling, where both ER and PRL receptors have been shown to independently activate STAT signaling. Given roles for ER and cyclin D1 expression during normal mammary gland development and tumorigenesis, these data emphasize an important synergy between P and PRL in directing these processes during mammary epithelial cell proliferation.

Keywords: Progesterone, Prolactin, mammary gland, mouse

[P1.44]

Correlation of a Glucocorticoid Receptor (GR) polymorphism but not GR splice variant expression with maternal attitudes towards pregnancy and fetal weight.

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Pregnancy is associated with major physiological and future psychosocial changes and maternal adaptation to these changes is crucial for normal fetal development. Recently it has been suggested that pregnancy-specific stress may be a more powerful contributor to birth outcomes than general stress. The stress hormone, cortisol mediates its effects by binding and activating the glucocorticoid receptors (GRs). In this study, expression of placental GRs (n=23) as well as a GR polymorphism (n=83) were assessed. These data were correlated to self-reported maternal attitudes towards their pregnancy as well as fetal weight. Moreover, we have used two placental cell lines to asses further the effect of cortisol on GR expression *in vitro*. Quantitative PCR analysis revealed that “wild type” GR-α was the predominant transcript when compared to GR-β, GR-γ and GR-P. No apparent changes at mRNA level were evident in all other transcripts when compared with each other.BeWo and JEG-3 placental cell lines were treated with low (1nM) and high doses (100nM) of cortisol in order to resemble a stress environment *in vitro*. Q-PCR analysis revealed that cortisol did not affect expression of GR splice variants. Using a self-reported questionnaire, women with negative attitudes gave birth to infants with significantly lower birth weights than those with positive or neutral attitudes towards the pregnancy, and this was unrelated to age, BMI or physical activity of women tested. Interestingly, the group that were homozygous for the *Bcl* I polymorphism of the GR, maternal attitude towards the pregnancy was predictive of infant birth weight. In conclusion, this study demonstrates that maternal stress in combination with a GR polymorphism might be of prognostic value towards pregnancy outcome.

Keywords: Cortisol, Glucocorticoid receptors, human placenta

[P1.45]

B-1 Cell Lymphoma in Mice Lacking the Steroid and Xenobiotic Receptor

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The Steroid and Xenobiotic Receptor, SXR, also known as PXR and NR1I2, is a broad-specificity nuclear hormone receptor that is highly expressed in the liver and intestine, where its primary function is to regulate drug and xenobiotic metabolism. SXR is expressed at lower levels in other tissues, where little is known about its physiological functions. We previously linked SXR with immunity and inflammation by showing that SXR antagonizes the activity of NF-kB in vitro and in vivo. Mice deficient in SXR demonstrate aberrantly high NF-kB activity and over-expression of NF-kB target genes. Here we show that SXR-null mice develop chronic lymphoma and leukemia derived from B-1 B cells in an age-dependent manner. SXR-null mice develop multiple hyperplastic lymphoid foci composed of B-1a cells in the intestine, spleen, lymph nodes, and peritoneal cavity, together with increased circulating B-1a lymphocytes in the peripheral blood. In all circumstances these foci share cell surface and molecular characteristics of chronic lymphocytic leukemia and non-Hodgkins lymphoma. These results demonstrate a novel and unsuspected role for SXR signaling in the B-1 cell compartment. This work establishes SXR as a tumor suppressor in B-1 cells, and may provide a novel link between chronic exposure to environmental chemicals that inhibit SXR activity and lymphomagenesis.

[P1.46]
Detection of biologically active withasteroids in Withania somnifera (Ashwagandha) by NMR spectroscopy

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Withania somnifera L. Dunal (Solanaceae), popularly known as “Ashwagandha”, is one of the top rank medicinal plants of India and is highly valued for its medicinal and nutraceutical properties in herbal and Ayurvedic systems of medicine. It is an annual herb growing as a wild plant in dry and arid soil and commercially cultivated throughout India for its invariable use in the herbal formulations which are helpful in the re-juvenation of the human metabolic functions. The plant is known to synthesize withasteroids which have shown antioxidant, anti-tumour, adaptogenic, anti-stress, anti-convulsant, immuno-modulatory, aphrodisiac, neurological effects and selective COX-2 inhibitory activity.

The major source of withasteroids in W. somnifera has been reported to be in its leaves and roots. We have, recently, published the isolation and identification of several withasteroids from the roots, leaves and fruits of the polar and non-polar extracts. The withasteroides from W. somnifera, show immunomodulatory, rejuvenating, antiageing actions and is recommended as a powerful nutraceutical. That is why it is also known as “Indian ginseng”. Withaferin A is the chief component of leaves and minor component of the roots having 4β-hydroxy-5,6β-epoxy system in its AB ring. It has been found to be very active steroid of W. somnifera and shows several activities including the anticancer on various cell lines. On the other hand, the second major constituent of W. somnifera leaves is withanone having 5α-hydroxy-6,7α-epoxy system in its AB ring. Withanone is a steroid having little significance as far as the biological activities are concerned. The isolation, structure elucidation, structure activity relationship has been studied by us, in detail. Now, we have developed a method to detect the steroids and their type by NMR spectroscopy. The importance of this technique is that by employing NMR spectroscopy we would be able to economically ascertain the claim of herbal industries for the presence of W. somnifera in their preparations without losing any substance from the sample.

Keywords: Withasteroids, Withania somnifera, Ashwagandha, NMR

[P1.47]

Subchronic steroid administration induces long-term changes on cocaine-induced dopamine outflow in the rat nucleus accumbens

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Introduction: A large number of young adults abuse anabolic androgenic steroids (AASs). This type of use probably involves more than a desire to enhance the users' appearance or sports performance. It has been hypothesized that steroid hormones are important determinants of stimulant's effects on behavior by influencing neuronal activity and plasticity. Several studies have shown that chronic AAS use may cause dependence, but the underlying biochemical mechanisms of AASs are still poorly understood. The specific aims of this study were to assess if steroid exposure modulates the acute neurochemical effects of cocaine on dopamine (DA) system, and to investigate if the steroid-treatment induces long-term changes in brain reward circuitry, and how long these changes remains.

Methods: Nandrolone was administered at doses of 20 mg/kg (i.m.), five times in total. Microdialysis surgery was followed 1, 23, 30 or 44 days after the last steroid injection. Cocaine (20 mg/kg i.p.) was injected 6, 28, 35 or 49 days after steroid dosing and samples were collected from NAc by *in vivo* microdialysis. Concentration of DA was measured by HPLC.

Results: The results showed that steroid treatment attenuates the increase in extracellular DA evoked by cocaine in the NAc. Cocaine-induced changes in extracellular DA concentration after the recovery period of 28 days did not differ statistically from those obtained after the shorter recovery period of 6 days. Only after the 49 days recovery period, the DA system has returned to basal function.

Discussion: Our results support indirectly anecdotal and case studies suggesting that AASs may increase the amount of cocaine needed to achieve desired effects. Further, because cocaine-induced DA response was still attenuated in steroid pre-treated animals after long recovery period (more than three times longer recovery than dosing period), it seems that exogenic steroid induce long lasting changes in the brains of rats.

Keywords: anabolic steroids, addiction, dopamine

[P1.48]

Corticosteroid-binding globulin, cortisol, free cortisol and sex hormone-binding globulin responses following oral glucose challenge in spinal cord-injured and able-bodied men

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Introduction: Insulin resistance can be induced by hydrocortisone and is increased in patients with abdominal obesity. We therefore wondered whether cortisol responses to oral glucose could be a contributing factor to the propensity towards insulin resistance and truncal obesity in spinal cord injury patients.

Methods: Circulating cortisol, corticosteroid-binding globulin (CBG) and sex hormone-binding globulin (SHBG) were measured retrospectively by ELISA in plasma samples following the oral glucose tolerance test in 20 spinal cord-injured men and 20 able-bodied controls. Plasma free cortisol was calculated from cortisol and CBG levels.

Results: Plasma free cortisol responses attenuated more rapidly in the able-bodied men, compared to spinal cord-injured subjects, due to significant rise in circulating corticosteroid-binding globulin whereas changes in total plasma cortisol were similar in both groups. The changes in plasma free cortisol in both groups paralleled changes in insulin and glucose and show that spinal cord-injured men had heightened exposure to free cortisol (23% versus 5%) during this dynamic test.

Conclusion:This heightened exposure to free cortisol raises the possibility that the mechanism of abdominal obesity and the propensity towards insulin resistance in spinal cord-injured men could be subtly mediated by perturbations in free cortisol. There were no significant changes in plasma SHBG in either group.

Keywords: cortisol, glucose tolerance test, insulin resistance, corticosteroid binding globulin

[P1.49]
Hepatic dysfunctions in C57BL/6 mice after liver-based POMC over-expression

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The pro-opiomelanocortin (POMC) prohormone produces several biologically active peptides, including alpha-melanocyte-stimulating hormones (alpha-MSH, beta-MSH, gamma-MSH), corticotropin (ACTH) and beta-endorphin, in the central and the peripheral nervous systems. POMC-expressing neurons in the brain play a major role in the control of pain, energy homeostasis, pigmentation, adrenocortical function, and sebaceous gland lipid production. Recently, the peripheral POMC system is under active investigation to delineate their pathogenic roles in metabolic diseases such as Cushing's syndrome and obesity. In the present study, we employed adenovirus gene delivery system to achieve POMC over-expression in the livers of adult C57BL/6 mice for at least 30 days. Subsequently, the plasma and livers of mice were collected and analyzed by biochemical assays and histological analysis, respectively. It was found that hepatic POMC over-expression resulted in liver injuries that the ALT and AST levels of POMC-expressing mice were significantly higher than control groups. Histological studies using hematoxylin-eosin staining, PAS staining, Oil Red O staining revealed that the glycogen store in the livers of POMC-expressing mice diminished to nearly 1/4 of basal levels. Moreover, a dramatic accumulation of fat droplets was observed in the livers of POMC-expressing mice. These results suggested that liver-based POMC gene delivery induced inflammatory insults and fatty changes in the livers of mice. Future studies are undertaking to shed lights on the molecular mechanism underlying fatty livers.

Keywords: POMC, Gene delivery

[P1.50]

Steroid therapy and the health status in sarcoidosis patients

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Introduction

Sarcoidosis is a multisystem disease of unknown cause and unpredictable course.

Some patients with the chronic form of sarcoidosis require steroid therapy for years or even life time, or the combined immunosuppressive therapy with steroids and cytotoxic agents i.e. Methotrexate

The aim of this study is to provide the information about the influence of steroid therapy on health status in sarcoidosis patients

Methods

292 (207 female), biopsy positive sarcoidosis patients, were enrolled in the study. All pts were on steroid therapy included: patients on prednisone 246, and patients on combined therapy methotrexate and low doses of prednisone 46.

For assessing health status we used the Sarcoidosis Health Questionnaire (SHQ) - disease specific questionnaire for measuring health status (HS) in sarcoidosis. The SHQ covers the domains of: Daily Functioning, Physical Activity, and Emotional Activity. Higher scores represent better HS.

Independent variables, significant at $p<0.05$ level were assessed together as a single block in multivariate regression models, to evaluate the contribution of various risk factors on HS: gender, course of disease, therapy.

The stepwise (backward elimination of variables) model was used.

Results

Patients treated with prednisone had significantly higher SHQ scores for all domains than those treated with combined therapy (T-test $p<0.05$)

Multiregression analyses revealed: it was the course of sarcoidosis not the steroid therapy that predicted lower SHQ scores in the domains of Daily Functioning, and Physical Activity. Steroid therapy was an independent predictor of lower Emotional Activity in female patients only.

Discussion

The belief that steroids impaired the quality of life in patients with sarcoidosis is strongly present both between the health practitioners and patients..

However, it is not always steroid therapy the reason of impaired HS in patients requiring long term steroid treatment. It can very well be the course of chronic disease that contributes the HS impairments.

Multiple linear regression analyses for HS in patients on steroid therapy with various risk factors				
Included Variables	Multiple linear regression analysis			
	Daily Activity	Physical Functioning	Emotional Functioning	Total Score
	β(p-value)	β(p-value)	β(p-value)	β(p-value)
Gender	-	-	(0.003)	(0.010)
Course of disease	(0.004)	(0.000)	-	(0.000)
Therapy (prednisone/combined)	-	(0.008)	(0.031)	(0.012)
(-) not signifiant)				

Keywords: steroid therapy, sarcoidosis, quality of life, health status

[P1.51]

Estrogen action in suppressing inflammation-associated colon cancer: A tale of ERα and ERβ

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The risk for developing colon cancer increases significantly in persons with inflammatory bowel disease. Men also have an increased risk for colon cancer when compared to women. The protection in women is due, in part, to the presence of estrogens. Previous data from our laboratory and that presented here demonstrate that estradiol (E_2) and dietary phytoestrogens suppress colon tumor development by inducing apoptosis non-malignant colonocytes and that this is estrogen receptor (ER) β mediated. This study aimed to determine if E_2 protects against inflammation-associated colon cancer and if E_2 treatment is preventative against the disease if administered following initiation of DNA damage to the colon. E_2 containing pellets were implanted into the mice after co-treatment with azoxymethane (AOM) and two rounds of dextran sulfate sodium (DSS). Wild type (WT) mice treated with E_2 had nearly a 50% reduction in adenocarcinomas and a 30% decrease in average adenocarcinoma area compared to control. No differences were observed between treatments in ERβ knockout mice for tumor multiplicity or size. ERβ protein expression was significantly reduced in colon tumors of WT mice compared to uninvolved colon samples. Furthermore, tumors had increased ERα mRNA expression compared to samples from non-AOM/DSS treated mice. Surprisingly, E_2 treatment resulted in reduced apoptosis and increased proliferation in the tumors. We conclude that the change in the colonocyte response to E_2 is due, partly, to reduced ERβ expression accompanied by enhanced ERα expression. To our knowledge, this is the first example of a shift from an ERβ to an ERα-mediated response to E_2 as a disease develops. Reduced tumor number and size indicates E_2 is protective against the development of inflammation-associated colon cancer. These data also highlight the importance of understanding the timing of exposure to estrogenic compounds in determining meaningful preventative strategies against the disease.

Keywords: Estradiol, Colon Cancer, Estrogen Receptor, Apoptosis

[P1.52]

Steroid hormones and fat distribution in men

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Obese men and women still show their sex-specific fat accumulation. Not only does fat distribution differ between the sexes after puberty, but the dynamics of fat cell size and fat metabolism differ as well. While there is reliable evidence that pubertal sex steroids induce a sex-specific fat distribution with preferential abdominal/visceral fat accumulation in males and preferential gluteofemoral fat accumulation in females, later in life a number of paradoxes occur in the relationship between sex steroids and fat distribution. The difference between aromatizable and non-aromatizable androgens could explain this paradox.

In our study we will search for the relation between steroid hormones and antropometric parametres in group of 20 healthy men (mean age 33.5 years, mean BMI 23.6). In all individuals, we analyzed all steroids in delta 4 and delta 5 metabolic pathways and their polar conjugates, progesterone-reduced metabolites and their polar conjugates,5 alpha/beta reduced C_{-19} metabolites including polar conjugates, 7 alpha/beta hydroxymetabolites of delta 5 steroids, 20 alpha metabolites of C_{21} steroids, LH, FSH and SHBG. We measured BMI, waist, hip and waist-hip ratio. Multivariate regression with reduction of dimensionality, bidirectional orthogonal projection to latent structures, O2PLS was used for statistic comparison. The local Ethics Committee approved the study, and all patients signed an informed consent form before taking part in the study.

The hormonal levels and antropomeric data were compared. We found the most potent correlation between waist and DHEAS, androstanediol sulphate, isopregnalone sulphate, 5alpha-pregnan-3-beta-20alpha-diol sulphate, androsterone sulphate, epiandrosterone sulphate and 5alpha-androstane-3beta, 17-beta-diol sulphate ($p<0.05$), The most confidential correlation we found between these steroids and hipcircumference ($p<0.01$).

The final metabolites of sterods originated from adrenocortical zone could influence male type of fat distribution.

The study was supported by grant No.NT11 277 and NS10215-3 of the IGA MZCR.

Keywords: fat distribution, steroids, man, antropomeric data

[P1.53]

Steroidal conjugates with succinobucol as complex systems for treatment of atherosclerosis

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Succinobucol and its parental drug probucol have been intensively studied for impact on atherosclerosis and related disorders via a range of biological activities (affecting lipid metabolism, anti-inflammatory and antioxidant effects, etc.). Succinobucol has undergone Phase III clinical trials to determine its effect on atherosclerotic endpoints. Nevertheless, these results have not provided consistent data supporting strong cardio-protective effects (e.g. the lowering of HDL cholesterol level). Still, due to found interesting anti-hyperlipidemic effects, an additional Phase III clinical study evaluating its impact on Type 2 diabetes is going on.[1]

Steroids, namely phytosterols, bile acids and cholesterol, are naturally occurring compounds with wide spectrum of biological activity. Phytosterols or their esterified forms lower serum total and LDL cholesterol by up to 20 %, this cholesterol malabsorption in general does not consistently affect HDL cholesterol or triglyceride levels.[2] Cholesterol or bile acids are being used for conjugation with drugs to increase their absorption through ileal membrane and target a drug into liver, the place where the metabolism of lipoproteins happen. Conjugation of phytosterols with succinobucol connects biological activities of both into one entity and may result in positive impact on metabolism of lipoproteins leading to improved results in treatment or regression of atherosclerosis.

Prepared phytosterol and cholesterol conjugates have been fully characterized on their molecular and submolecular levels and tested for their toxicity on mouse fibroblasts, where they have shown insignificant toxicity. Conjugates, together with phytosterols, probucol and succinobucol were also tested for their antioxidant activity on scavenging of DPPH radical with positive results. Further biological studies are going on to reveal the potential of the conjugates.

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Keywords: phytosterol, succinbucol, atherosclerosis

[P1.54]

A CYP11A1 $(TTTTA)_n$ polymorphism is associated with prostate cancer risk but not with overall survival or duration of androgen-deprivation therapy

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CYP11A1 (P450scc) catalyzes the rate limiting step in androgen synthesis, the conversion of cholesterol to pregnenolone. Polymorphic variation in the gene encoding CYP11A1 has been poorly studied in relation to the development, clinical progression, and survival of men with prostate cancer. To this end, we genotyped the $(TTTTA)_n$ repeat polymorphism in 193 patients with castration-resistant prostate cancer (CRPC) who were treated at the National Cancer Institute. Data pertaining to the duration of androgen deprivation therapy (ADT) and stage at the initiation of ADT (either D0 or D2) were also available in 67 patients. We also genotyped 16 healthy Caucasian controls (all ≥50 years old) and compared our results these and a much larger cohort of historical controls (*n* = 478). No relationships were found between the $(TTTTA)_n$ repeat and age at diagnosis or Gleason score in patients with CRPC, nor was the initial stage at diagnosis (i.e., D0 or D2) associated with this polymorphism. The allele frequency of $(TTTTA)_4$ in our control cohort matched historical control data (~40% allele frequency, *P*=0.57). Risk analysis between CRPC cases and controls (all ≥50 years old) did not reveal any statistically significant relationships (*P*=0.12); however, the $(TTTTA)_4$ repeat was associated with an increased risk of prostate cancer when compared to our control cohort (OR(95%CI) = 3.4(1.6-7.1); P=0.0014) and historical controls (OR(95%CI) = 2.2(1.6-2.8); P<0.0001). No association was detected between *CYP11A1* $(TTTTA)_n$ status and duration of ADT or overall survival from diagnosis to death. The present results indicate that carriers of shorter $(TTTTA)_4$ repeats may be at higher risk for developing CRPC at an older age, although the repeat polymorphism is not related to clinical characteristics or progression of prostate cancer.

Keywords: CYP11A1, P450scc, Prostate Cancer

[P1.55]

Polymorphisms of the glucocorticoid receptor gene, as phenotype modifiers in patients with hormonally inactive adrenal adenomas

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Introduction: Altered sensitivity against glucocorticoids is influenced by polymorphisms (SNP) of the glucocorticoid receptor gene (GR). The aim of the present study was to explore whether SNPs or a certain combination of SNPs of the *GR* could be associated with adrenal tumor formation, hormonal activity and clinical parameters.

Methods: The study included 102 patients with hormonally inactive (HI) adrenal adenomas discovered incidentally (incidentalomas) and 129 healthy controls. Hormonal evaluation of the hypothalamo-pituitary-adrenal (HPA) axis and measurement of metabolic parameters were carried out in patients, and genetic analysis in all subjects. Polymorphisms of the *GR* were detected by allele-specific PCR methods for N363S and BclI polymorphism, with RFLP for ER22/23EK and with Taqman allele discrimination assay for A3669G.

Results: The prevalence of the N363S was higher and A3669G was lower in HI than in healthy controls, especially in patients with bilateral HI tumors: (N363S: 10.5% vs. 2.7% $p<0.05$; A3669G: 10.5% vs. 22.1% $p<0.05$). The ER22/23EK occurred together with A3669G both in patients and controls. Patients who had either unilateral or bilateral HI adrenal adenomas and carried the Bcl*I* polymorphism had higher body weight, body mass index (BMI) and higher ACTH levels measured after metyrapine test. The presence of the A3669G polymorphism aggravated the body weight and BMI in Bcl*I* carriers. Type 2 diabetes mellitus was observed more frequently in N363S carriers than in non carriers.

Conclusions: The increased prevalence of N363S and the decreased prevalence of A3669G variants of the *GR* by altered sensitivity against endogenous glucocorticoids may play a role in the pathogenesis of hormonally inactive adrenal incidentalomas and may contribute to increased morbidity observed in these patients.

Keywords: adrenal, glucocorticoid receptor, polymorphism

[P1.56]

Synthesis of the steroidal glycoside (25R)-3β,16β-diacetoxy-12,22-dioxo-5α-cholestan-26-yl β-D-glucopyranoside and its anticancer properties on cervicouterine HeLa, CaSki, and ViBo cells

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Introduction.

Cancer is responsible for about 25% of deaths in developed countries and for 15% of all deaths worldwide.[1] Antitumor research is a very active field, and a large amount of information dealing with clinical aspects of cancer chemotherapy is generated. Synthetic chemistry has been used extensively to modify drug targets, especially those of natural origin, and to solve the problem of the scarce supply of natural products by developing semi-synthetic or synthetic strategies.[2] Steroidal saponins possess varied biological activity, some steroidal glycosides exhibit high anticancer activities, Recently, it was demonstrated that the sapogenins, for example, hecogenin exhibit antiproliferative activity and induce apoptosis in several cell lines.[3]

Methods, results and discussion.

Compound **1** was synthesized starting from hecogenin; the results have shown that the glycoside **1** is very active against HeLa, Caski and ViBo cells with a dose-dependent activity and IC_{50} values from 42-65 μM.

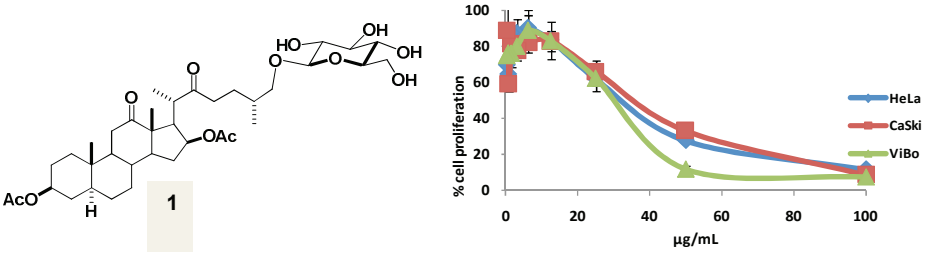

Figure 1. Dose-response curves of the antiproliferative effect of glycoside **1** on HeLa, CaSki, and ViBo cells.

Biological evaluations suggest that **1** induces HeLa, CaSki, and ViBo cells to stop their cycle in the S, G_2-M, and G_1 phases respectively, and that it is a potent apoptosis inducer with a null cytotoxic consequence. In addition, the proliferation of fibroblast cells and peripheral blood lymphocytes was not affected significantly. These *in vitro* results certainly augur well for *in vivo* assays in the next stage of the research.

Conclusion

Glycoside **1** induces HeLa, CaSki and ViBo cells to die by apoptosis with IC_{50} values from 42-65 μM. On the other hand, the same concentration does not affect the proliferation of human uterine cervical fibroblast cells. We believe, therefore, that glycoside **1** serves as a promising lead candidate for further evaluation.

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Keywords: Steroidal glycoside, Cervicouterine cancer cells, Antiproliferative activity, Apoptosis

[P1.57]

Association between blood pressure and CYP19 gene expression in subcutaneous adipose tissue of women with polycystic ovary syndrome

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Introduction: The activity of aromatase, an enzyme encoded by the CYP19 gene, affects androgen metabolism and estrogen synthesis, influencing the androgen to estrogen balance in different tissues (1,2). Our aim was to characterize CYP19 gene expression in subcutaneous adipose tissue from normotensive and hypertensive polycystic ovary syndrome (PCOS) patients and to establish whether subcutaneous fat CYP19 mRNA is associated with androgen and estrogen levels and blood pressure (BP) in PCOS.

Methods: This cross-sectional sudy included 31 women of reproductive age and 27 BMI-matched normotensive non-hirsute controls. Hypertension was defined as BP ≥130/85 mmHg (3). Metabolic and hormonal measurements were performed. Real-time quantitative PCR determined gene expression levels (delta delta C_T Method).

Results: Table 1 shows clinical and hormonal results. CYP19 mRNA in subcutaneous fat from PCOS patients correlated positively with systolic (r=0.509 p=0.006) and diastolic (r=0.485 p=0.009) BP.

Discussion: Our data suggest that androgen excess and hyperinsulinemia could play a role on the molecular mechanisms activating aromatase mRNA transcription in abdominal fat tissue in PCOS women.

Table 1: Clinical and hormonal features for control and PCOS women

		PCOS		
	Control group (*n*=27)	Normotensive (*n*=20)	Hypertensive (*n*=11)	p
Age (years)	30.1±4.5[a]	25.5±5.3[b]	21.1±6.1[b]	<0.001
Systolic BP (mmHg)	113±7[a]	109±10[a]	134±13[b]	<0.001
Diastolic BP (mmHg)	72±7[a]	69±9[a]	83±10[b]	<0.001
HOMA-IR	2.0 (1.2-2.7)[a]	2.1 (1.5-3.2)[a]	4.2 (3.2-7.0)[b]	0.001
Total testosterone (ng/mL)	0.6 (0.5-0.7)[a]	0.9 (0.8-1.2)[b]	1.5 (1.3-2.0)[c]	<0.001
Free androgen index	4.5 (3.9-6.9)[a]	7.9 (5.4-13.2)[b]	19.8 (11.8-41.9)[c]	<0.001
Estradiol (pg/mL)	45.7 (33.0-97.0)	53.7 (39.7-69.0)	55.0 (39.7-57.8)	0.889

Estrogen:androgen ratio	0.07 (0.06-0.17)[a]	0.05 (0.04-0.08)[ab]	0.04 (0.02-0.05)[b]	<0.001
CYP19 mRNA (n fold change)	2.891±0.995[a]	2.822±0.761[a]	3.839±1.010[b]	0.016

Values are expressed as mean±SD or median and interquartile range (25%-75%). Different symbols indicate statistical difference by One-way ANOVA+Tukey *post hoc* test. HOMA-IR: homeostasis model assessment; mRNA: messenger ribonucleic acid.

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Keywords: Blood pressure, CYP19 mRNA, Subcutaneous adipose tissue, PCOS

[P1.58]

Osteoprotective action of 17aβ-hydroxy-3-methoxy-D-homo-8alpha-estra-1,3,5(10)-trien-6-one acetate

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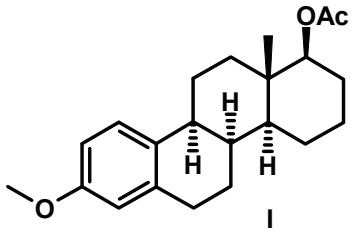
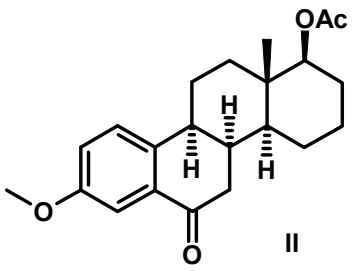
It is well known that the using of estrogens as hormone-replacement medications prevents the loss of bone mineral components. However, during the long-term application of estrogens the risk of breast cancer [1] and endometrial cancer [2] is increasing. This is the reason for the development of analogues of steroid estrogens with osteoprotective action and lowered hormonal activity. To reach this task it is necessary to investigate the influence of various substituents in steroid skeleton on biological properties of analogues. Because the introduction of keto-group at position 6 of natural and 8 alpha series of estrogens with the free hydroxyl group at position 3 leads to the decreasing of uterotropic action, the aim of this investigation was to obtain the additional information about the influence of such modification on biological properties of analogues with methoxy group at position 3.

Group of rats (dose in mg/kg of body weight per day, *per os*)	Body weight change, g	Uterine weight, mg/ 100 g body weight	Bone mineral density at zone L4 of femur, g/sm^2	Serum cholesterol, mg/dl
Sham-operated	38±6*	150±9**	0.255±0.005*	79.5±7.2*
Ovariectomized	115±9	19±1	0.231±0.007	95.1±6.8
Ovariectomized, treated with EE, (0.1)	25±4*	127±9**	0.255±0.004*	26.1±2.8**
Ovariectomized, treated with steroid I (5.0)	-7±5*	114±7**	0.254±0.007*	29.2±2.9**
Ovariectomized, treated with steroid II (5.0)	4±5**	136±5**	0.255±0.007*	33.5±3.7**

* - $p<0.05$, **- $p < 0.01$ (Student)



I



II

We have synthesized analogues **I** and **II** and investigated biological parameters in the experiments on ovariectomized rats using the modified model of Kalu (Table). We have shown that the influence of analogues **I** and **II** is equal on all parameters. This allows to assume that the influence of keto-group at position 6 on uterotropic activity of investigated earlier steroid estrogens is connected with pK of phenolic hydroxyl group.

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Keywords: osteoprotectors, uterotropic activity, hormone-replacement therapy

[P1.59]

Estradiol treatment enhances p53 activity in non-malignant colonocytes

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Colon cancer is the second leading cause of cancer related death in the US. Women have a decreased incidence of colon cancer suggesting that female hormones play a protective role against this disease state. In addition, numerous clinical and animal studies have shown that hormone replacement therapy or estrogen replacement therapy can decrease the risk of colon cancer development. However, little research into the mechanism of protection has been done. Our laboratory has demonstrated that estradiol (E_2) inhibits the development of pre-neoplastic lesions in mice. Our data also suggest that the primary protective role of E_2 treatment is increased apoptosis in non-malignant colonocytes that are damaged and at risk of becoming cancerous. The p53 protein plays a crucial role in the cellular response to stress by inducing cell cycle arrest, DNA repair mechanisms, and/or apoptosis. Because of observed induction of apoptosis, we are investigating the role p53 might have in this chemo-protective mechanism. To do so, we have employed molecular biology approaches to define how E_2 treatment alters p53 expression, transcriptional activity, and the resulting physiological changes in non-malignant colonocytes. E_2 suppressed growth of young adult mouse colonocytes (YAMCs) resulting from induction of apoptosis and these physiological responses were completely lost in YAMCs lacking a functional p53 protein. Western blot analysis demonstrated increases in p53 protein levels in YAMCs after treatment with E_2. Transient transfection with a reporter construct showed that E_2 also enhances the transcriptional activity of p53. In addition, expression levels of downstream targets of p53 have been analyzed. Finally, repair of DNA double stranded breaks was shown to be increased by E_2 treatment. Collectively, these data are the first to demonstrate that p53 is a primary mediator of the protective actions of E_2 in the colon, which may lead to novel targets for colon cancer prevention strategies.

Keywords: Estradiol, Colon Cancer, p53, Prevention

[P1.60]

Estrogen modulation of EZH2 in the prostate epithelial and progenitor cells

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Post-translational modifications of histones are known to regulate chromatin structure, genomic stability, and gene expression. Major epigenetic programming events take place during development that may have long-lasting consequences on adult health. Brief exposure of rodents to estradiol during the neonatal period results in permanent alterations of the prostate gland with severe epithelial cell differentiation defects in the ventral lobes. Although the mechanisms underlying such observations are not well elucidated, epigenetic alterations of histones might be of central importance. In the present study we sought to characterize the expression of *EZH2*, a polycomb group enzyme that methylates H3K27, in the prostate epithelium and progenitor cells, likely the primary target of estrogenic exposure. We then asked whether neonatal estrogen exposure affected its expression in microdissected epithelial cells from adult ventral prostate. Real-time RT-PCR revealed that *EZH2* is localized in epithelial cells and is markedly decreased by estradiol in day 90 ventral prostate ($p < 0.005$); but not in day 6 ($p = 0.5311$). Since epigenetic mechanisms are crucial in maintaining stem cell pluripotency and controlling their differentiation into epithelial cell lineages, we measured *EZH2* levels in rat prostate basal-like epithelial NRP-152 cells treated with 5-aza-2'-deoxycytidine (5-aza-2'-dC) or 17α-estradiol. While there was a significant decrease in *EZH2* expression with 5-aza-2'-dC treatment, E2-exposure had no effect on expression levels in sphere-forming epithelial NRP152 cells. In summary, these data indicate that neonatal estrogen down-regulates *EZH2* mRNA expression in the prostate epithelium which may in turn underpin epigenetic memory as a function of early life exposures.

Supported by NIDDK-40890

Keywords: estrogen, EZH2, prostate, progenitor cells

[P1.61]

GBE as a candidate of aromatase inhibitors for breast cancer therapy

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Breast cancer is the most common cancer of women in the world. There are many endocrine adjuvant therapies, which are categorized according to their mechanism– Tamoxifen (selective estrogen receptor modulator), Goserelin (LHRH-agonists), Herceptin (monoclonal antibody of HER2 receptor), and aromatase inhibitors. Many clinical trials compared the effects of tamoxifen to those of aromatase inhibitors. It showed Tamoxifen limits of its initial efficacy against breast cancer, and increasing attention has been paid to aromatase inhibitors that block the synthesis of estrogens.

But aromatase inhibitors also have their limits for their side effects – increasing in musculoskeletal complaints and potential for decreasing bone density. So we need a new candidate of aromatase inhibitors. GBE (*ginkgo biloba* extract) is one of the most potent candidates. It's a kind of phytochemical from tree. It was already proved that it has a biphasic effects for estrogenic effects and osteoporosis inhibiting activities as SERMs (Selective Estrogen Receptor Modulators), which is from previous articles in our lab. Also, previous study suggested the possibility of GBE as an aromatase inhibitor. From these results, we concluded that GBE could be a new candidate for adjuvant therapy of breast cancer – acting like SERM and aromatase inhibitors at the same time with less side-effects.

In this present study, GBE inhibited aromatase activity and gene expression (CDS, transcripts I.1, I.3 and II) in JEG-3 cells and MCF-7 cells. In addition, By assaying on MCF-7 cells transiently transfected with CYP19 promoter vector, GBE inhibited the luciferase activity. These results indicate that GBE could inhibit the transcriptional control of CYP19A. In conclusion, we suggested that pharmacological dosage of GBE could be a candidate for aromatase inhibitor at both the enzyme and mRNA levels.

Keywords: aromatase inhibitor, GBE, breast cancer, adjuvant therapy

[P1.62]

Estradiol-activated estrogen receptor α and its regulation of microRNAs in T47D breast cancer cells

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Breast cancers are sensitive to hormones such as estrogen, which activates the estrogen receptors (ERα and ERβ) and leads to significant changes in gene expressions. To enhance prognosis and diagnoses of breast cancer, a detailed study of the ER regulating pathways and identifying the microRNAs (miRNA) that are associated with normal or disrupted estrogen signaling is required. We have previously shown that 24 hour 17β-estradiol (E2) activation of ERα in T47D cells showed significant changes in the expression of genes involved in cell cycle, proliferation, and apoptosis. To identify miRNAs regulated by E2-activated ERα, we screened 24 hour E2-activated T47D parental cells (ER-positive) using dual-color microarray techniques. Microarray expression profiles were confirmed by real-time PCR using both SYBR Green and Taqman technologies. Although estrogen treatment renders a massive regulation of up to 900 genes, no significant changes in miRNA expressions could be confirmed. Comparison to previous studies done on miRNA in ER-positive breast cancers cell lines have revealed conflicting results due to time of exposure, technology used, and cell type and morphology. This study would contribute to narrowing the study area of miRNA in these cells.

Keywords: estrogen receptor alpha, miRNA, breast cancer cells, ER-positive

[P1.63]

The isoflavone luteolin and two oxysterols (25- and 27-hydroxycholesterol) and their binding selectivity to estrogen receptor beta over estrogen receptor alpha: Implications for hedgehog signaling in prostate cancer

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Introduction: Prostate cancer (PCa) risk is influenced by dietary factors and increases with age. It has been proposed that ERα and ERβ act in opposition in PCa. These findings suggest ERβ protects against PCa while ERα may promote it. Therefore, selective ERβ agonists and ERα antagonists are desired for PCa therapy and prevention. Interestingly, genistein is an ER agonist which binds ERβ ~20 fold better than ERα. It is also possible that an ERβ-specific antagonist may increase PCa incidence. Micromolar concentrations of 25- and 27-hydroxycholesterol (25- and 27-OHC) have been reported to be SERMs.

Hypotheses: The isoflavone luteolin, which shares structural similarity to genistein, will bind selectively to ERβ. 25- and 27-OHC will also bind ERβ better than ERα.

Results/Conclusions: Using a competitive ^{3}H-estradiol binding assay, we found that luteolin bound (>70 fold) selectively to ERβ (~15nM Kd) over ERα (Kd > 1μM). In our system 25- and 27-OHC bound ERβ (~25nM Kd) 200 fold better than ERα (~5μM Kd). Our binding assay confirms that ERβ binds both estradiol and genistein with their reported Kds. In conclusion, luteolin is an ERβ-selective ligand and its functional activity on ERs is currently being tested in our lab. Additionally, 25- and 27-OHC are endogenous, low Kd (~25nM), ERβ-selective compounds. 27-OHC has been reported to increase in concentration with age along with PCa risk. This is presumably due to oxysterols stimulating hedgehog signaling via LXR. However, we have found that estradiol and the ERβ-specific agonist DPN will inhibit hedgehog signaling but the ERα-specific agonist PPT will not. Based on this data, a diet rich in isoflavones, like luteolin and genistein, and low in oxysterols, such as 25- and 27-OHC, could decrease the risk of prostate cancer occurrence via an ER-Hedgehog interaction mechanism.

Keywords: Estrogen, Oxysterol, Prostate, Cancer

[P1.64]

Experimental and clinical studies of estrogen deprivation in breast cancer: The PDGF/Abl pathway as a novel therapeutic target to overcome resistance

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The majority of breast tumours at primary diagnosis are estrogen receptor positive (ER+). Estrogen (E) mediates its effects by binding to the ER. E-bound ER associates classically with estrogen response elements (EREs) on target genes controlling proliferation and cell survival. ER's ability to regulate gene transcription is also influenced by the activity of co-regulatory proteins such as AIB1. Therapies targeting the estrogenic stimulation of tumour growth have been a major success in reducing mortality from ER-positive breast cancer. However, resistance remains a major clinical problem. To identify the molecular mechanisms associated with resistance to E-deprivation, we assessed the temporal changes in gene expression during adaptation to long-term culture of MCF7 human breast cancer cells in the absence of E2 (LTED), modelling resistance to an aromatase inhibitor (AI). Strikingly, the expression of a proliferation metagene identified that resistance occurred as early as 9 weeks post E deprivation. This observation was validated by global assessment of gene expression, which showed a stabilisation of the gene signatures after this time-point. Based on these findings, a pair wise comparison of wt MCF7 versus week 9 showed 1909 genes were down-regulated and 1082 genes were up-regulated ($p<0.001$). Strikingly, all major up-regulated canonical pathways were involved in cell signalling including PDGF, actin cytoskeleton, integrin, PI3K/AKT, EGF, neuregulin, IGF1, ERK/MAPK and P70S6K. Analyses of canonical signalling pathways showed that platelet-derived growth factor (PDGF)/Abl was significantly elevated as early as one-week post E-deprivation ($p=1.94$ E-04). Of note this became the top adaptive pathway at the point of resistance ($p=1.15$ E-07). Clinical data from 81 patients treated with an AI in the neoadjuvant setting, showed increases in PDGFRβ expression after two weeks (1.25 fold, $p=0.003$). Low PDGFRβ at pre-treatment was associated with a better response. Assessment of the level of protein expression and activation status in these cell lines modelling both endocrine sensitive and resistant breast cancer showed PDGFRβ was elevated in LTED cells compared to wt MCF7. Moreover, the level of receptor phosphorylation was increased in the resistant cell line. The PDGFR/Abl tyrosine kinase inhibitor, nilotinib, suppressed proliferation in LTED cells in the presence or absence of E. These data suggest that PDGFRβ was associated with the endocrine resistant phenotype. Nilotinib also suppressed ER-mediated transcription by destabilising the ER and reducing recruitment of AIB1 and the CBP to the promoter of the E-responsive gene *GREB1*. Overall the results reveal cross-talk between ER and PDGF/Abl strongly suggesting that the PDGF signalling pathway merits clinical evaluation as a therapeutic target in endocrine resistant breast cancer.

[P1.65]

Effect of novel glucocorticoid receptor modulator, Compound A on inflammation and proliferation in skin.

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Glucocorticoids are important physiological and pharmacological regulators of skin development, maintenance and tumorigenesis. Their biological effects are mediated by the glucocorticoid receptor (GR), that regulates gene expression by (i) transactivation that requires GR binding to gene promoters and (ii) DNA-independent transrepression through negative GR interaction with other transcription factors including NF-kB. It was shown that therapeutic anti-inflammatory effects of glucocorticoids are mediated by GR transrepression. At the same time, GR transactivation underlies many metabolic side effects of glucocororticoids. We and others showed that GR/glucocorticoids exert strong tumor suppressor effect in skin. The analysis of GR effect on gene expression in Keratin5.GR mice resistant to skin carcinogenesis, indicated that gene transrepression plays a leading role in GR anti-cancer effects. This ties anti-cancer and anti-inflammatory effects of glucocorticoids. Thus, “dissociated” ligands that preferentially shift GR activity towards transrepression hold a great promise for the treatment of inflammatory skin diseases as well as for skin cancer prevention and treatment. Compound A (CpdA), a plant-derived phenyl aziridine precursor, was recently characterized by us and others as a novel non-steroidal ligand acting mostly via GR transrepression. Here we report that CpdA acted as GR dissociated ligand in mouse keratinocytes in vitro and in vivo. CpdA did not activate GR-dependent genes but mimicked inhibitory effect of glucocorticoids on expression of inflammatory cytokines and metalloproteinases. When applied topically to skin, CpdA inhibited skin hyperplasia and inflammation induced by tumor promoter TPA. In contrast to glucocorticoids, CpdA in single treatment did not induce skin hypoplasia which is considered one of the important adverse effects of glucocorticoids. These results suggest that CpdA and its derivatives represent a novel promising class of anti-inflammatory and anti-cancer compounds for the treatment of dermatological patients. This work was supported by NIH grant R01 CA118890 (IB).

Keywords: glucocorticoid receptor, non-steroidal modulator, skin, inflammation

[P1.66]

Pharmacophore-based virtual screening for identification of novel inhibitors of 17beta-Hydroxysteroid Dehydrogenases Type 3 and 5

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17beta-hydroxysteroid dehydrogenases type 3 and 5 (17beta-HSD3 and 17beta-HSD5) catalyze the synthesis of androstenedione to testosterone and thereby constitute therapeutic targets for androgen-related diseases or endocrine-disrupting chemicals. We developed ligand- and structure-based pharmacophore models as fast and efficient tools to identify potential ligands for both enzymes. A pharmacophore model collection for steroidal and non-steroidal 17beta-HSD3 inhibitors as well as 17beta-HSD5 inhibitors was developed using LigandScout 2.03, Catalyst 4.11 and DiscoveryStudio 2.1.The models were experimentally validated by *in silico* screening of commercial compound databases and subsequently by enzymatic efficacy tests of selected virtual hits with different recombinantly expressed 17beta-HSDs. We used HEK293 cells stably transfected with either 17beta-HSD3 or 17beta-HSD5 and bacteria expressing 17beta-HSD types 1, 2, 4, 5, and 7 for the inhibitor screen. Among the 35 tested compounds, 11 novel inhibitors with diverse chemical scaffolds, e.g. sulfonamides and triazoles, and with different selectivity properties were discovered. With this screen we provide several potential starting points and strategies for further development of selective 17beta-HSD3 and 17beta-HSD5 inhibitors.

[P1.67]

Biological activity of the designer supplement methyl-1-testosterone after oral and subcutaneous administration - characterization of anabolic-androgenic potency and metabolism

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Various products containing unapproved anabolic androgenic steroids have been marketed as dietary supplements in the recent years. Also a number of products containing methyl-1-testosterone (M1T, fig. 1) are available. It is advertised to be highly anabolic and moderately androgenic.

The aim of this study was to further describe the biological activity of M1T and its metabolism. In a yeast androgen receptor transactivation assay M1T was characterized as potent androgen (potency in the range of the endogenous AR ligand dihydrotestosterone). To determine its tissue specific androgenic and anabolic potency and to identify potential adverse effects M1T was studied in a rat animal model. Orchiectomized rats were treated with M1T for 12 days either s.c. or p.o. (0.03, 0.3 or 2 mg/kg BW/day).

Tissue wet weights determined the anabolic (*m. lev. ani*) and androgenic (prostate) activity. Additionally the expression of molecular and physiological markers in liver (TAT), prostate (proliferation), and *m. gastrocnemius* (IGF-I, AR) were determined and correlated to the serum concentrations of M1T. Analysis of prostate and lev. ani weight demonstrated that after s.c. administration M1T dose dependently stimulated the weight of these tissues while oral administration had no effect on the weight. However, proliferation in the prostate and IGF-I and AR expression in the *m. gastrocnemius* were modulated in a dose dependent manner in good agreement to the determined M1T serum levels in both administration routes.

This data clearly demonstrated that M1T is also a potent androgenic and anabolic steroid *in-vivo*, as well after oral as s.c. administration. Analysis of TAT expression provides evidence for liver toxicity especially after oral administration.

Following administration in man M1T was excreted in urine besides its main metabolites 17α-methyl-5α-androst-1-ene-3α,17β-diol and 17α-methyl-5α-androstane-3α,17β-diol.

This enables the detection of M1T abuse in urine samples, which was already applied recently in top level sports doping control.

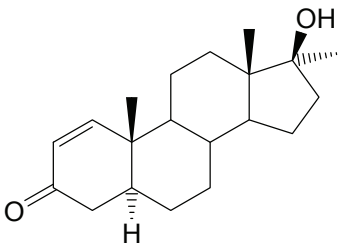

Fig 1: Chemical structure of methyl-1-testosterone (M1T)

Keywords: anabolic androgenic steroid, biological activity, metabolism, 17-methyl steroid

[P1.68]

Novel nanodimeric cyclodextrin ligands with high affinity to steroids

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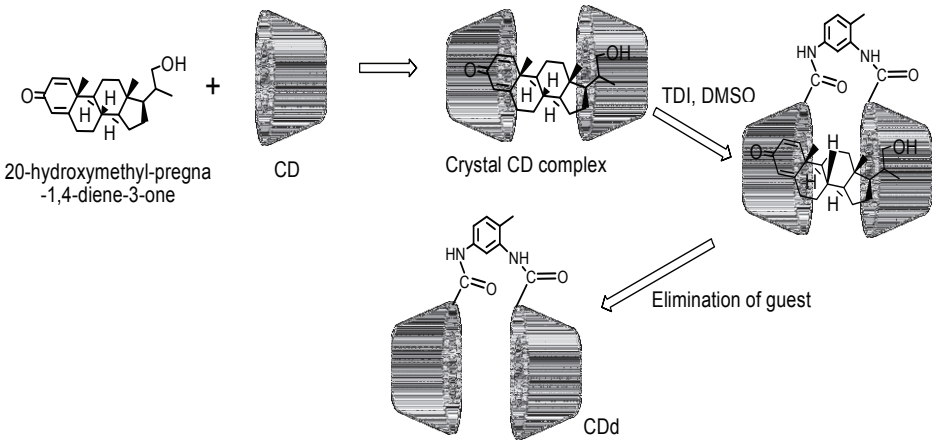
Molecular-imprinting for the synthesis of nanostructures forming the inclusion complexes of “guest-host” type is a progressive way to obtain complementary ligands to steroids. Such tailor-made structures can be of extraordinary affinity and meet the requirements of specific receptors.

Novel tailor-made cyclodextrin (CD) dimer was synthezised using original method of molecular-imprinting by cross-linking of ligands of β-cyclodextrin (CD) complex with steroids. The method is based on the obtaining of crystalline [β-CD-steroid] complex of (2:1) stoichiometry followed by toluene 2,4-diisocyanate (TDI) cross-linking.



Steroids of both androstane and pregnane series were applied as templates. The best results were obtained using steroids with hydrophobic side chain at C-17 (e.g. 20-hydroxymethyl pregna-1,4-dien-3-one). The dimer obtained was characterized by ^{1}H NMR and mass-spectrometry (ESI).

The linked CD dimer formed water-soluble inclusion complexes with steroids and their complex stability constants were extremely high for template HMPD. Method of equilibrium dialysis of steroid and that based on the nonlinear competitive spectrometry of dyes were used for independently evaluation of steroid affinity to tailor-made CD dimer.

The newly synthesized material can be potentially applied for specific recognition of steroids as target molecules, as well as at the creation of novel drug delivery system with adversely affect drug absorption.

Keywords: cyclodextrin ligand, steroid cyclodextrin inclusion complex, specific recognistion, target steroid molecule

[P1.69]

Pathophysiology in animal models of diet induced obesity: A critical role for insulin resistance.

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Introduction: Obesity is often but not always accompanied by insulin resistance (IR) in animal models. We have investigated two animal models of diet induced obesity: one with normal insulin sensitivity and the other with insulin resistance. This specific difference has dramatically divergent effects on pathophysiology in obesity.

Methods: Wistar rats were fed either a normal rat chow; High Carbohydrate/High Fat (HCHF); or High Carbohydrate/Low Fat (HCLF) diet for up to 30 weeks to establish control, obese (OB) and obese with insulin resistance (OBIR) cohorts respectively. In the current study, these groups were comprehensively profiled with respect to a range of biometric and metabolic parameters. Our study investigated the impact of isolated obesity on obesity accompanied by IR on the cardiovascular system.

Results: When compared to their respective internal control (IC) groups, both the OB and OBIR groups developed increased: 1) bodyweight (114% OB vs. 116% OBIR), 2) visceral fat weight % (corrected for bodyweight) (134% OB vs. 144% OBIR) and 3) serum triglyceride concentration (173% OB vs. 202% OBIR). Homeostatic model assessment of insulin resistance (HOMA-IR) confirmed insulin resistance in OBIR and normal insulin sensitivity in OB rats (HOMA-IR calculation = *fasting glucose (mmol)* x *fasting insulin (μIU.ml^{-1} / 22.5*).

Following ischemia reperfusion (I/R) experiments *ex vivo,* left ventricular developed pressure recoveries were increased in OB (140.1%) and decreased in OBIR (55.0%) when compared to IC rats. Cardiac infarct size assessment following I/R *in vivo* showed infarct size reductions in OB (54.6% of IC) and increases in OBIR (192% of IC) rats.

Conclusions: Despite both groups being obese, only the OBIR group was more susceptible to myocardial I/R injury. Conversely, isolated obesity may render the myocardium more tolerant to I/R. It is therefore essential that models of obesity used for intervention studies must be characterised by the presence or absence of IR.

Keywords: Obesity, Insulin Resistance, Rat Model, Cardioprotection

[P1.70]

Androgen receptor activity in urine after testosterone intake is not dependent on UGT2B17

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Background: The current test to detect doping of testosterone is based on determination of the urinary testosterone glucuronide/epitestosterone glucuronide ratio (T/E). The major enzyme responsible for testosterone glucuronidation is UGT2B17 and we have shown that the results of the testosterone doping test are highly dependent on UGT2B17 deletion genotype.

Androgen responsive reporter gene assays have been described as a promising screening method for detection of anabolic androgenic steroids. Here we have investigated if the androgen receptor (AR) activity is dependent on UGT2B17 genotype, and if there is a time dependency after testosterone administration.

Material and methods: Healthy volunteers were given 500 mg of testosterone enanthate im and testosterone excretion was monitored prior to (day 0), 2, 4 and 15 days after injection. The androgen receptor activity was determined using a yeast-based dual-color bioluminescence assay. The individuals were genotyped for UGT2B17 using real-time PCR.

Results: The androgenic activity, expressed as testosterone-equivalents, increased 4-5 fold two and four days after testosterone intake ($p<0.0001$) and was back to basal activity on day 15. The increase and the activity profile were independent of UGT2B17 deletion polymorphism. The concentration of free testosterone in the samples was significantly associated with AR activity on day 0 ($r^2=0.10$, $p=0.01$), day 2 ($r^2=0.38$, $p<0.0001$) and day 4 ($r^2=0.23$, $p=0.0002$).

Discussion: The results show that AR activity as assessed with the bioluminescence assay is related to free urinary testosterone (38%) which increases after testosterone administration. However, the genetic variation in UGT2B17 had no impact on these results. Further studies are required to identify the other androgen receptor active metabolites of exogenous testosterone.

Keywords: Testosterone, UGT2B17, Androgen receptor activity, Doping

[P1.71]

Biological assessment of novel glycyrrhetinic acid derivatives acting as selective inhibitors for 11β-hydroxysteroid dehydrogenase type 2

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Introduction: Availability of glucocorticoids and activation of glucocorticoid receptors (GR) is tightly regulated by 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) and 11β-HSD2. 11β-HSD2 catalyzes the conversion of active 11β-hydroxyglucocorticoids (cortisol, corticosterone) into inactive 11-ketoglucocorticoids (cortisone, 11-dehydrocorticosterone). Impaired local glucocorticoid metabolism has been associated with several disease states, and modulation of intracellular glucocorticoid availability is considered to be a promising strategy to treat glucocorticoid-dependent diseases. 18β-glycyrrhetinic acid (GA), the biologically active triterpenoid of licorice (*Glycyrrhiza*), represents a well-known but non-selective inhibitor of both 11β-HSD enzymes. Selective inhibitors are needed for mechanistic studies and future therapeutic applications. In the present study, we biologically characterized a set of novel and selective 11β-HSD2 inhibitors.

Methods: The activities of selected inhibitors were compared in assays using lysates and intact cells expressing recombinant human enzymes. Species-specific differences were considered by comparing inhibitory activities of the compounds on human and mouse 11β-HSD2. Further, the impact of the GA derivatives on 11β-HSD-dependent modulation of GR transactivation activity was assessed. In an attempt to understand the selectivity of the GA derivatives to inhibit 11β-HSD1 and 11β-HSD2, respectively, an 11β-HSD2 homology model based on structural information on the related 17β-HSD1 was generated and applied together with our recently constructed pharmacophore of 11β-HSD1.

Results: Modifications of functional groups on the triterpenoid backbone led to selective inhibitors. Specific modifications at the 3-hydroxyl and the carboxyl of GA rendered high selectivity for 11β-HSD2. The data generated significantly extend the current knowledge about structure activity relationships of GA derivatives as inhibitors of human 11β-HSDs.

Conclusions: The structural analyses provide an explanation for the differences in the selectivity and activity of the GA derivatives investigated. The most potent and selective 11β-HSD2 inhibitors should prove useful as mechanistic tools for further anti-inflammatory and anti-cancer *in vitro* and *in vivo* studies.

Keywords: 11beta-hydroxysteroid dehydrogenase, pharmacophore, inhibitor, glycyrrhetinic acid

[P2.01]

Screening for modulatory effects on steroidogenesis using the human H295R adrenocortical cell line: A metabolomics approach

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Non-receptor mediated effects of endocrine disruptors can be caused indirectly via alterations of common signal-transduction pathways or through direct (non-)competitive inhibition of enzymes involved in the steroidogenic pathways. The H295R steroidogenesis assay provides an *in vitro* cell-based assay to evaluate the potential interference of compounds with steroid hormone production [1] Current endpoints in this assay are the levels of several free steroid hormones, which are usually determined by targeted analytical methods such as LC- and GC-MS or ELISAs. Recent developments in LC-MS and bioinformatics however, allow more comprehensive approaches to evaluate changes in steroid profiles [2]. Therefore, the feasibility of an untargeted metabolomics approach to evaluate changes in steroid profile of H295R cells exposed to known steroidogenic modulating compounds was explored.

H295R cells were exposed for 48 h to aminoglutethimide, abiraterone, trilostane, fadrozole and metyrapone, which are known to inhibit specific enzymes in the steroidogenesis. After exposure of the cells to these reference compounds, the culture medium was subjected to a solid phase extraction clean-up procedure and analyzed by Ultra Performance Liquid Chromatography Time-Of-Flight Mass Spectrometry (UPLC-TOF/MS). Generated profiles were compared to profiles obtained with a DMSO blank using sophisticated preprocessing and alignment software (MetAlign^{TM}) [3]. Differential mass signals (p-value <0.05 and fold change >2) were selected and profiles typical for the selected reference compounds were constructed. The observed differences in metabolite profiles were mainly caused by alterations in free and sulfated steroids. These alterations were considered to be very relevant as the identity of the differentially abundant metabolites could be related to the expected effect of the compound under investigation.

In conclusion, it can be stated that application of a comprehensive metabolite profiling methodology provides a promising analytical approach to screen compounds for steroidogenic modulating properties as well as chemical class prediction.

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[3] Lommen, A. *Anal. Chem.* **2009**, 81, 3079-3086.

Keywords: Steroidgenesis, Endocrine disruptors, Metabolomics

[P2.02]

Development of stable isotope dilution liquid-chromatography mass spectrometry (LC/MS) methods for the determination of the androgen metabolome

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Metastatic prostate cancer is initially responsive to androgen ablation therapy, but eventually recurs as castrate resistant prostate cancer (CRPC). The success of the CYP17A1 inhibitor abiraterone acetate in the treatment of CRPC indicates that this disease remains hormonally driven. Determination of the androgen metabolome in both tumor and serum samples would elucidate which enzymatic steps contribute to androgen biosynthesis and are most likely to respond to pharmacotherapy for CRPC. Analysis of the androgens in serum samples from patients undergoing treatment for prostate cancer could also provide an early predictor of the success of the treatment regimen and aid in tailoring personalized treatments. To this end, we developed stable isotope dilution LC/MS methods for the determination of androgens in biospecimens. Direct detection of androgens by LC/MS lacks sensitivity, particularly for the 5α-reduced products, so derivatization of ketosteroids as Girard T oximes and hydroxysteroids as picolinic esters has been achieved. These derivatives are much more readily ionized and introduce reliable mass transitions for every analyte, allowing detection of pg or lower quantities of all of the androgens. The derivatized steroids are separated with reverse phase HPLC and quantified through comparison to deuterated internal standards using electrospray ionization-MS detection. Using Girard T derivatization for ketoandrogens, we analyzed androgen levels in samples from patients undergoing hormone suppressive therapy for prostate cancer. As expected, treatment with leuprolide significantly reduced levels of testosterone, but had a limited effect on other androgens, while combination therapy with leuprolide and abiraterone acetate drastically reduced the levels of all of the androgens analyzed. (Supported by 1R01-CA90744 to TMP).

Keywords: Prostate cancer, Androgen metabolome, Abiraterone, Leuprolide

[P2.03]

Steroid hormone profiling by mass spectrometry in rat blood collected via the Culex® automated in vivo sampling system

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Introduction: Impaired steroid hormone regulation has been implicated in the pathogenesis of a variety of drug-induced toxicities, especially those associated with pancreas, liver, kidney, adrenals, muscle, and central nervous system. A quantitative profile of steroid hormone metabolites can serve as a biomarker of altered endocrine functions as well as altered renal and hepatic metabolism. It holds great potential to help assessing drug safety, dissecting toxic mechanisms, understanding disease progress and providing rational therapeutic intervention.

Methods: An LC-MS/MS-based method has been established to quantify a panel of 12 steroid hormones: aldosterone, cortisol, cortisone, corticosterone, 11-dehydrocorticosterone, 11-deoxycorticosterone, 11-deoxycortisol, testosterone, dihydrotestosterone, androstenedione, 17α-hydroxyprogesterone and progesterone (comprising rodent and human metabolites). Steroid metabolites were resolved by liquid chromatography using an Allure Biphenyl (Restek) column and quantified by Agilent 6410 Triple-Q mass spectrometer. The method presented excellent dilution linearity for all 12 metabolites and the limits of quantitation were ranging from 730 pM for androstenedione to 12 nM for corticosterone. Method validation and measurements were performed in 50 µL of rat plasma spiked with calibrators and deuterized internal standards.

Results: The method was then applied to profile time-course changes of steroid hormones in rat plasma. Male Wistar rats were connected to the Culex® system through carotid artery or femoral vein. Blood collection was programmed every 3 hours during a 24-hour period. Interim results indicated that changes of corticosterone, 11-dehydrocorticosterone, and 11-deoxycorticosterone followed the known circadian rhythm, suggesting the non-disturbance of glucocorticoid profiles in catheterized rats. Levels of testosterone, androstenedione and progesterone were rather constant regardless of the day and night shift. The other steroid metabolites analyzed were below the detection limit.

Conclusions: This platform is currently being used to detect drug-induced disturbances in steroid hormone profiles and to elucidate mechanisms of toxicity of drug candidates.

Keywords: steroid hormone, mass spectrometry, automated in vivo sampling system, drug-induced toxicity

[P2.04]

Quantitative analysis of dietary sterols and ecdysteroids in the Silkworm by LC/APCI-MS/MS

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Insect molting hormones (ecdysteroids) are essential for insect development. Since the machinery of *de novo* sterol synthesis has been lost in insects, the ecdysteroids are synthesized from dietary sterols. This steroid metabolism, from dietary sterols to ecdysteroids, is not fully understood. To elucidate comprehensive mechanism of ecdysteroidgenesis, we first established the LC/APCI-MS/MS system for analyzing multiple steroids from an individual sample. The major sterols in the artificial diet were campesterol, stigmasterol and β-sitosterol. On the other hand, prothoracic gland cells, where the ecdysteroids are synthesized, accumulated high amount of cholesterol and 7-dehydrocholesterol. These results suggest insects have a unique mechanism of utilizing dietary sterols and a storage system of cholesterol and 7-dehydrocholesterol in prothoracic gland cells. Further analysis with the LC/APCI-MS/MS system for detecting multiple steroids will reveal the steroid metabolism in insects.

Keywords: ecdysteroid, insect, phytosterol, LC/APCI-MS/MS

[P2.05]

LC/MS based Steroid Screen: A new module for the comprehensive phenotyping of mice models in the German Mouse Clinic

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The goal of the German Mouse Clinic* (GMC) in the Helmholtz Zentrum München is to find and characterize mouse mutants that can serve as models for human disorders and for developing new therapies. The GMC develops and offers a large scale of standardized and comprehensive phenotypic analysis of mouse mutants. The primary screens in the GMC are designated to the areas of behaviour, bone and cartilage development, neurology, clinical chemistry, eye development, immunology, allergy, steroid metabolism, energy metabolism, lung function, vision and pain perception, molecular phenotyping, cardiovascular analyses and pathology. The steroid metabolism screen* is provided by the Genome Analysis Center. The Screen allows the simultaneous extraction and detection of key steroids (testosterone, corticosterone and androstenedione) from mouse plasma in only one step via online solid phase extraction (SPE) coupled with LC-MS/MS. The aim of the steroid screen is the identification of new animal models for human steroid-related diseases. Steroids are involved in the control of cell and tissue differentiation and proliferation processes, as well as in the regulation of apoptosis, bone remodelling and neuroregeneration. Disorders in the steroid metabolism contribute to the pathogenesis of different complex diseases, like cancer (e.g. breast or prostate cancer), diabetes, diseases of cartilage and bone (e.g. atherosclerosis or osteoporosis) or neurological diseases (e.g. Alzheimer). Therefore the measurement of steroids in tissues is the next step in the investigation of steroid-related diseases and can lead to new insights in their pathogenesis.

[P2.06]

Metabolomic platform at the helmholtz zentrum münchen

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Metabolomics is an emerging research field for phenotyping of biological samples with an unbiased approach of characterization. Minor changes in metabolism due to illness or treatment as well as phenotypes that are not much pronounced or subsidiary could be determined by metabolomic analysis if many different parameters are correlated. The two initial metabolomic approaches are targeted metabolomics (quantification of a chosen set of metabolites) and non-targeted metabolomics (profiling or search for biomarkers).

The Metabolomic Platform of the Genome Analysis Center at the Helmholtz Zentrum München is designed to mediate progress in science through development of new metabolomic methods and provision of analytical measurements applicable to man, animal models, plants, environmental samples and ex vivo systems. We already established several targeted metabolomics methods and started to implement a new method for analysis of plasma samples by non-targeted metabolomics.

In our activities related to targeted metabolomics, we pursue the quantification of a portfolio of metabolites including lipids, amino acids, steroids, and bile acids by LC-MS/MS. For this, we developed or adapted standardized high-throughput methods for rapid and reproducible quantification of metabolites. Our methods cover the analysis of these metabolites out of different matrices like human or animal plasma (serum), many different animal tissue types, or lung lavage.

Here, we describe the quantification of metabolites by the Biocrates Absolute*IDQ* kits p150 and p180. These kits cover each more than 150 or 180 endogenous metabolites, respectively, like lipids, amino acids, acylcarnitines, or carbohydrates. For this, a sample amount of e.g. only 10 µl plasma is needed. The measurements perform very well with high reproducibility. We successfully performed studies in the human cohorts KORA and UK Twins and in animal models in elucidating metabolomic effects in complex diseases or drug development, respectively (Gieger et al, 2008; Illig et al, 2010). To facilitate high quality standards and sample tracking we build up a tailor made LIMS.

[P2.07]
New ester derivatives of dehydroepiandrosterone as 5α-reductase inhibitors

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Introduction: The aim of these studies was to synthesize different ester derivatives of dehydroepiandrosterone with therapeutic potential as antiandrogens.

Methods: The biological effect of these steroids was demonstrated in *in vivo* as well as *in vitro* experiments. In the *in vivo* experiments, we measured the activity of seven steroids on the weight of the prostate and seminal vesicles of gonadectomized hamsters treated with testosterone. For the studies *in vitro*, we determined the IC_{50} values by measuring the concentration of the steroidal derivatives that inhibits 50% of the activity of 5α-reductase present in human prostate and its binding capacity to the androgen receptors (AR) obtained from rat's prostate cytosol.

Results: The results from these experiments indicated that compounds **8** 5α,6β-dibromo-3β[3'-oxapentanoyloxy]androstane-17-one, **9** 5α,6β-dibromo-3β-propanoyloxyandrostane-17-one and **10** 5α,6β-dibromo-3β-butanoyloxyandrostane-17-one significantly decreased the weight of the prostate and seminal vesicles as compared to testosterone treated animals and this reduction of the weight of these glands was comparable to that produced by finasteride. On the other hand, **1** 3β-acetoxyandrost-5-en-17-one, **2** 3β-acetoxyandrost-5-en-17-one, **3** 3β [3'-oxapentanoyloxy]androst-5-en-17-one inhibited the activity of human 5α-reductase enzyme; but **7** 3β-hexanoyloxyandost-5-en-17-one, **8** and **10** exhibited the most inhibitory activity with IC_{50} values of 10, 12.5, 6.6 nM respectively. However, none of these compounds binds to the AR.

Discussion: The compounds containing a bromine atom in their molecule (**8, 9, 10**) showed higher pharmacological activity. However, all the studied compounds inhibited the activity of 5α-reductase, but did not bind to the AR.

Keywords: Prostate illness, antiandrogens, dehydroepiandrosterone derivatives, 5-alpha reductase

[P2.08]

Steroids as chiral modifiers of oligopyrrole macrocycles

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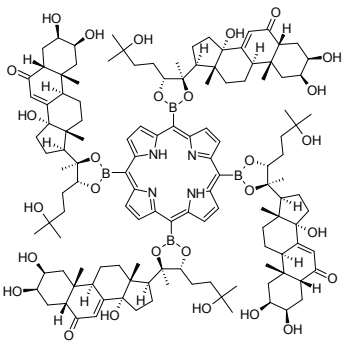
[1]ICT Prague, Czech Republic, [2]IBCH NAS, Belarus, [3]University Tor Vergata, Italy, [4]Charles University, Czech Republic

Steroids, as natural products are used as powerful chirality modifiers in self-assembly of oligopyrrole macrocycles modified in this way on *meso*-positions. The self-assembly, mainly involving the chirality, is one of the principal substantiations of life. Compounds with achiral "receptor", as porphyrin or calix[*n*]pyrrol could, in theory, form chirotopic assemblies. However, when connected to chiral part they are expected to have explicit properties of chiral receptor or chiral supramolecular synthon. Hence, their super-assembly could yield to suprachiral nanoscopic clusters or organised structures that may serve as chiral scaffoldings in synthetic organic chemistry or even biochemical processes.

The communication aims on the synthesis and study of behavior of oligopyrrole macrocycle conjugates in aqueous (polar, protic) media by spectroscopic methods. There were synthesized calix[4]pyrroles, -phyrins, and porphyrins modified by steroids and glycosylated steroids in *meso*-positions, and their self-assembly properties were studied. The potential of chiral natural compound conjugates will be described and several different types of conjugates presented for comparison.



Study of properties and solvent (matrix) driven self-assembly of synthons containing steroids is one possible way for the nature better understanding and modelling.

We found that steroidal oligopyrroles exhibit chiral superassembly properties and proved it by several physico-chemical measurements and also by a computed model.

This work was supported by the Ministry of Education, Youth and Sports of CR by projects No. P304/10/1951, P503/11/0616, MSM6046137305, and 2B06024.

Keywords: chirality, self-assembly, oligopyrrole macrocycle, steroid

[P2.09]

The surprising biological function(s) of 11-dehydro-glucocorticoids, the end-products of 11β-HSD2.

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Mechanisms governing the interplay between mineralocorticoids and glucocorticoids (GCs) in the kidney [1] are complex and not well understood. Historically, the first important observation was that Aldosterone (Aldo)-induced renal sodium retention could only be reliably measured in adrenalectomized (ADX) animals. Later, experiments conducted with isolated toad bladders, demonstrated that 11-dehydro- GC metabolites generated by 11β-HSD2 blunted the anti-natriuretic action of Aldo. These observations led investigators to consider the concept that adrenally produced and renally modified GCs could blunt Aldo action.11-dehydro-GCs have now been shown to play a previously un-recognized functional role, to decrease translocation of Aldo-bound mineralocorticoid receptors (MR) to the nucleus of target cells[2]. We have recently demonstrated[3] that when mouse inner medullary collecting duct (IMCD) cells, which contain both 11β-HSD2 and MR, are exposed to Aldo (10nM) for 48hrs, the cells display a marked increase in the expression of collagen, fibronectin, and connective tissue growth factor(CGTF). These effects in IMCD cells (which possess no reverse reductase activity) were attenuated by both the MR antagonist RU 28318 and the 11β-HSD2 end-product, 11-dehydro-corticosterone. Similar findings were observed in kidneys isolated from normotensive ADX mice following continuous infusion of Aldo (8ug/kg/day) for 1 wk. These early Aldo-induced fibrotic increases in collagen, fibronectin, and CTGF expression were attenuated in ADX mice treated with RU28318 or 11-dehydro-corticosterone. This Aldo infusion also produced significant pro-fibrotic changes in heart and aortic tissue in the absence of pre-existing hypertension or systemic disease. Again, RU 28318 and 11-dehydro-corticosterone attenuated these fibrogenic and pro-inflammatory effects of Aldo in vascular tissue.

Thus, a dual role for 11β-HSD2 is emerging; first as the putative “guardian” over the MR by reducing inappropriate GC binding to MR, and second as an endogenous source of 11-dehydro- GCs, which locally attenuate both Aldo-induced sodium transport and the pro-fibrotic effects of this hormone.

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Keywords: 11β-HSD2, Aldosterone, 11-Dehydro-Glucocorticoids, kidney, mineralocorticoid receptors mineralocorticoid receptors

[P2.10]

The synthesis and evaluation of specific oxysterol derivatives as potential regulators of the hedgehog signaling pathway

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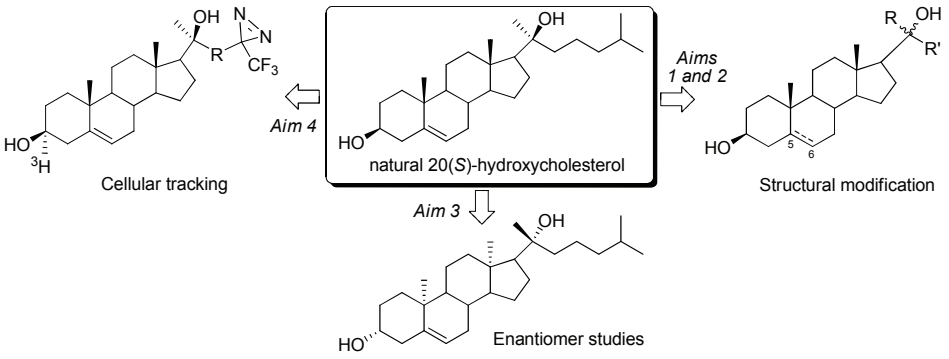
Hedgehog (Hh) signal transduction has been found to be essential for both embryonic development and organ repair and maintenance. Consequently, serious health complications can arise from the improper function of this biological pathway. For instance, defective Hh signaling can lead to severe birth defects (such as holoprosencephaly), whereas constitutive Hh signaling can cause Gorlin's syndrome (a familial cancer syndrome, characterized by basal cell carcinomas and medulloblastomas), and several other sporadic cancers of the pancreas, intestine, and lung. As such, the Hh signaling pathway represents an ideal therapeutic target for cancer-treatments and regenerative medicines. It is therefore critical to understand the intracellular mechanisms involved in this biological pathway. However, while there is a general understanding of the key biological players within the pathway, many of the specific interactions are not well understood at a biochemical level.

The focus of this investigation is to pinpoint the specific interactions responsible for Hh signaling, by exploring the known ability of oxysterols to activate the Hh pathway. After the evaluation of several oxysterols, natural-20(*S*)-hydroxycholesterol was found to be an extremely potent activator. Therefore, by derivatizing this lead compound, we aim to:

1. Elucidate what structural elements are essential for biological activity
2. Gain a better structural understanding of the target receptor
3. Characterize binding interactions (lipid perturbation vs. protein binding)
4. Locate the specific biological target of the oxysterol activation



Included herein, are the syntheses of several 20(*S*)-hydroxycholesterol derivatives, accompanied by preliminary corresponding biological results. Supported by NIH grants GM47969 (DFC) and 5-T32-HL07275 (LKM), and the Stand Up To Cancer Foundation (RR).

Keywords: structure activity relationship (SAR), oxysterol, hedgehog signaling pathway, photoaffinity label

[P2.11]

Synthesis of cyclopenta[b]anthracene analogues of the neuroprotective steroid 17β-estradiol

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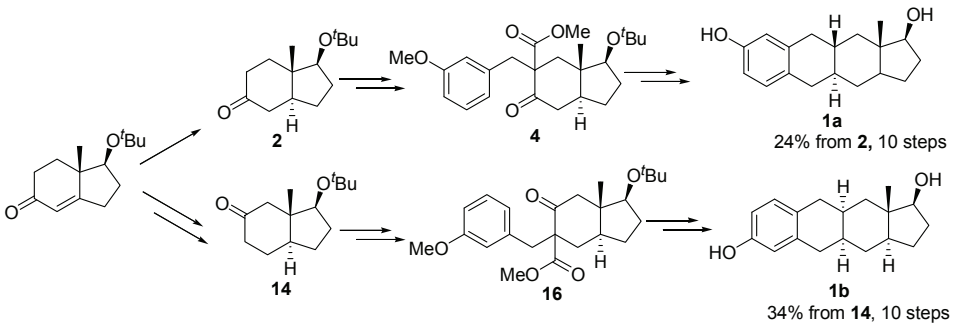
There are convincing data showing that 17β-estradiol is neuroprotective *in vitro* and *in vivo*. However, clinical development of 17β-estradiol as a therapy for human neurological diseases is limited by its hormonal effects, which include feminization and the potential stimulation of estrogen-sensitive malignancies. Therefore, drugs possessing the neuroprotective properties of 17β-estradiol, but lacking its hormonal properties, would be very attractive. In this regard, we are exploring the possibility that analogues of 17β-estradiol in which the four rings of the steroid ring system are arranged in the linear cyclopenta[*b*]anthracene ring system may be both neuroprotective and non-hormonal. Synthetic routes to the desired linear ring system have been developed using enantiopure Hajos-Parrish indenone as a starting material. The Hajos-Parrish indenone was then converted into either indenone **2** or **14** and these indenones were subsequently converted into cyclopenta[*b*]anthracenes **1a** and **1b**, respectively. 8-Hydroxy-*trans*-4a,10a-cyclopenta[*b*]anthracene (**1a**) was prepared from intermediate **2** in 10 steps in 24% yield and 7-hydroxy-*cis*-4a,10a-cyclopenta[*b*]anthracene (**1b**) was prepared from intermediate **14** in 10 steps in 34% yield.



Supported by NIH grants AG10485 and GM47969 and NSF grant **(**MRI, CHE-0420497).

Keywords: Indenones, Neuroprotective steroids, Synthesis, Estradiol analogues

[P2.12]

Microbial transformation of newly synthesized 6-aminomethyl androstenones

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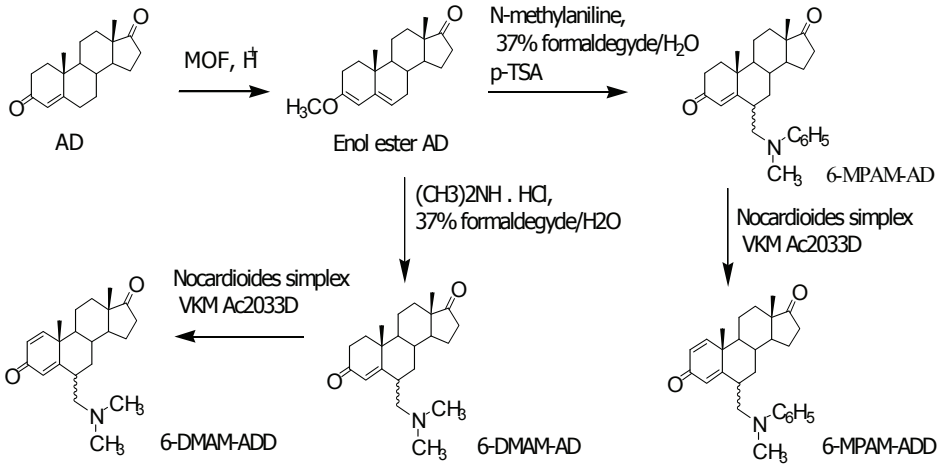
[2]*Moscow State University, Russia*

Microbial transformation of 6-aminomethyl substituted androstenones was firstly investigated. N-Methyl-N-phenyl- and N,N-dimethyl-substituted derivatives of 6-aminomethyl androstenedione (AD) were synthesized using Mannich reaction. The conditions of steroid obtaining and recovery were optimized. The strain of *Nocardioides simplex* VKM Ac-2033D was applied as an only biocatalyst at the study of the bioconvertability of these steroids.



1-Dehydroderivatives were formed as major bioconversion products. The structures of all steroids were confirmed using MS, 1H-NMR and element analysis data. When the mixture of diastereomers of 6-(N-methyl-N-phenyl-aminomethyl)-androst-4-ene-3,17-dione (6-MPAM-AD) was applied as bioconversion substrate, the preferable formation of alpha-isomer of the corresponding 1,4-diene steroid was observed. Similar results were obtained at the transformation of alpha- and beta-stereomer mixtures of N,N-dimethyl-substituted AD by *N.simplex*.

Along with 1-dehydrogenation, the desamination and desamynomethylation reactions of 6-(N,N-dimethylaminomethyl)-AD were observed. These types of steroid transforming activities by actinobacteria were not reported so far.

Novel steroids which were firstly obtained in this study by chemical-microbiological synthesis can combine therapeutic activity of steroid base with the potency of the amine constituent thus providing obtaining of high effective drugs against hormone-dependent cancers.

Keywords: microbial transformation, N-substituted androstenones, 1-dehydrogenation, novel steroids

[P2.13]
The influence of aspalathus linearis flavonoids on glucocorticoid biosynthesis

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Flavonoids exhibit a wide range of biological activities including the modulation of adrenal steroidogenic enzymes [1,2]. Flavonoids may inhibit or stimulate the catalytic activity of these enzymes, as well as influencing their expression [3]. This study aims to investigate the inhibitory effect of *Aspalathus linearis* (Rooibos), a dietary source rich in flavonoids, on glucocorticoid (cortisol and corticosterone) production.

P450 17α-hydroxylase/17, 20 lyase (CYP17), P450 11β-hydroxylase (CYP11B1) and 3β-hydroxysteroid dehydrogenase II (3βHSD) were expressed in COS1 cells and the catalytic activity assayed in the presence of two major flavonoid compounds, aspalathin, a dihydrochalcone unique to Rooibos, and orientin, a flavone. The effect of Rooibos on plasma cortisol levels was subsequently investigated in a human study - 42 subjects consumed Rooibos for six weeks. Subjects had two or more risk factors for coronary heart disease (hypercholesterolemia, hypertension/pre-hypertension or increased body mass index) and were not medicated [4]. A study was also conducted in male Wistar rats to determine the influence of Rooibos on corticosterone production. A control placebo group received two oral gavages placebo treatments (isotonic saline) daily for 10 consecutive days; a Rooibos group received Rooibos instead of saline.

CYP17 inhibition was significant ($P<0.001$) while the inhibition of CYP11B1 was markedly less, with only deoxycortisol conversion inhibited by orientin ($P<0.05$). Inhibition of 3βHSD II was significant, orientin ($P<0.001$) and aspalathin ($P<0.01$). In the human study, males showed a significant decrease in serum cortisol levels ($P<0.05$) while females showed a downward trend. Similar findings showed corticosterone concentrations were significantly decreased ($P<0.05$) in the rats receiving Rooibos compared to the placebo group.

Selective inhibition of adrenal enzymes will influence the flux within the mineralocorticoid, glucocorticoid and androgen steroidogenic pathways. Current investigations into steroid metabolites in these pathways using H295R cells will provide insights into steroid hormone homeostasis within the adrenal gland.

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Keywords: cytochrome P450, cortisol, flavonoid, Rooibos

[P2.14]

The steroidogenic cytochrome P450 enzymes CYP17A1 and CYP21A2 epoxidize progesterone analogs

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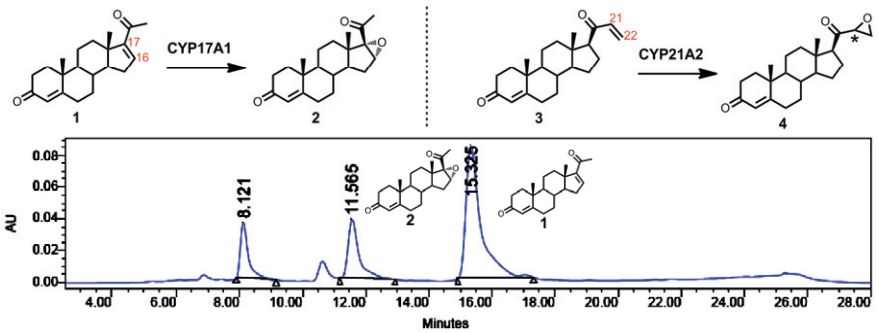
The prototypical cytochrome P450 reaction is oxygen insertion or hydroxylation of a C-H bond; however, P450 enzymes catalyze other reactions, including epoxidation, carbon-carbon bond cleavage and [2+2] cycloaddition (Paterno-Buchi type reaction). CYP17A1 (17α-hydroxylase/17,20-lyase, P450c17) hydroxylates the 17-position and less so the 16-position of 21-carbon steroids and cleaves the 17,20-bond of 17-hydroxy,21-carbon steroids to yield 19-carbon steroids. In contrast, CYP21A2 (21-hydroxylase, P450c21) hydroxylates only the 21-position of steroids. The activities of these enzymes give rise to important hormones responsible for development, stress response, carbohydrate metabolism and reproduction. Epoxidation chemistry for CYP17A1 and CYP21A2 has not been explored, as catalysis occurs near the saturated D-ring. We synthesized 16,17-dehydroprogesterone (**1**) and 21,22-dehydro-*homo*-progesterone (**3**) to determine if these steroid hydroxylases catalyze epoxidation of unsaturated progesterone analogs. As predicted, CYP17A1 metabolized **1** to 16α,17α-epoxyprogesterone (**2**), which co-chromatographs with synthetic **2** on reverse-phase HPLC, and one other product. CYP21A2 appears to epoxidize **3** to 21,22-epoxy-*homo*-progesterone (**4**), which we have also synthesized. We conclude that these two steroidogenic cytochrome P450 enzymes epoxidize unsaturated substrate analogs, demonstrating that their chemistry is more diverse and mechanistically complex than previously assumed.



Keywords: CYP17A1, CYP21A2, cytochrome P450, epoxidation

[P2.15]

Investigation of structure and conformational equilibrium of steroid estrogen analogues of 8alpha aeries and application for the prediction of biological properties

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Saint-Petersburg State University, Russia

It is well–known that the metabolic modifications of steroid estrogen analogues with unnatural ring junction could be significantly different in comparison with the same modifications of natural steroids. It leads to some advantages of synthetic analogues. For example, when metabolic hydroxylation at position C-16 (or C-17 in the case of D-homo analogues) is impossible, the risk of breast cancer and endometrial cancer appearing should be decreased.

Because the total synthesis of steroid estrogen analogues is complicated and hard task, the widely used method for the prediction of biological properties of new analogues is the docking of ligands into ligand-binding domain of various complexes of estrogen receptors. For this it is necessary to know the exact spatial structure of modified analogues.

The comparison of data obtained from X-Ray analysis of synthesized steroids and data derived from NMR spectroscopy methods in the solution as well as the calculated data of structures by MM+ and *ab initio* methods gave us the possibility to find the optimal criteria for the investigation of structure in the solution and choose the methods for the calculation of structure of new modified analogues.

In this presentation the investigation of structures of various steroid estrogen analogues in the solution by various NMR spectroscopy methods and criteria for the spatial structure definition will be considered and in the final part the practical application and experimental confirmations of results will be presented.

Keywords: steroid estrogen analogues, MNR spectroscopy, docking, estrogen receptors

[P2.16]

Effect of environmental pollutants on obesity parameters *in vitro*

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Recent data link development exposure to environmental endocrine disrupting chemicals with adverse human health effects, like obesity and diabetes. Data from animal as well as human studies show an association between development of obesity and exposure to endocrine disrupting chemicals, such as diethylstilbestrol, bisphenol A (BPA), polychlorinated bisphenyls (PCBs), and organotins.

Adipose tissue growth involves formation of new adipocytes from precursor cells, further leading to an increase in adipocyte size. The transition from undifferentiated fibroblast-like preadipocytes into mature adipocytes constitutes the adipocyte life cycle, and a better understanding of how and what regulates both size and number of adipocytes may provide a better approach for understanding and treating obesity. One of the transcription factors shown to play a key role in adipocyte differentiation is the peroxisome proliferator activated receptor (PPAR) family, specifically PPAPγ, the activation of which has been shown to induce the differentiation of preadipocytes into adipocytes.

In the current study the effect of different environmental pollutants including BPA, chlorpyrifos, mancozeb, prochloraz and, PBC 153 were tested for effects on adipocyte differentiation and subsequent lipid accumulation in 3T3-L1 adipocytes, and for their ability to activate PPARα and γ.

The results show that many of the tested environmental pollutants caused a decrease in the amount of lipid droplets formed in the 3T3-L1 adipogenesis assay. BPA showed an increase in the amount of lipid droplets formed, at the lowest concentrations tested while having a decreasing effect at the highest concentration. Studies on the mechanism of action are in progress. Overall the results support the hypothesis that environmental chemicals are able to affect adipocyte differentiation as well as other obesity parameters *in vitro*, although the exact mechanisms behind is not yet understood.

This work is supported by the Danish Agency for Science Technology and Innovation

Keywords: endocrine disrupters, in vitro, adipogenesis, obesity

[P2.17]

Influence of chronically ingested depleted uranium on hepatic cholesterol metabolism in ApoE-deficient mice

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Institut de Radioprotection et de Sûreté Nucléaire, France

Depleted uranium (DU) is an artificial radioelement and a waste product from the enrichment process of natural uranium. Because of its high density, it is used in industrial and military items. This may increase its deposition in some areas and lead to a possible exposure of the populations living on these territories through a chronic ingestion of contaminated foodstuff. Previous studies have shown that cholesterol metabolism was modulated at molecular level in the liver of rats contaminated for nine months with DU (Racine et al. 2010). However these animals did not display any pathological feature at organ or body level. In this regard, the use of a pathological model should be helpful in order to clarify the possible impact of this type of exposure on cholesterol metabolism.

Thus, the present study aims at assessing the effects of a chronic ingestion of a low level of DU on hypercholesterolemic apolipoprotein E-deficient mice, i.e. at ascertaining whether the physiopathology of these animals is worsened by the contamination. In this regard, mutant mice were given DU-supplemented water (20 mg/L) for three months. Cholesterol metabolism was then studied in the liver. Gene expression of cholesterol-catabolizing CYP7A1, CYP27A1 and CYP7B1 as well as associated nuclear receptors LXRα, FXR, PPARα, and SREBP 2 was analyzed. mRNA levels of ACAT 2, as well as HMGCoA Reductase and HMGCoA Synthase were also measured. The gene expression study was completed with SRB1 and LDLr, apolipoproteins A1 and B and membrane transporters ABC A1, ABC G5. The major effect induced by DU was a decrease of hepatic gene expression of enzyme CYP7B1 (-23 %) and of nuclear receptors LXRa (-24 %), RXR (-32 %), HNF4a (-21 %) compared to unexposed Apo E-deficient mice.

In conclusion, these modifications on cholesterol metabolisms did not lead to increased disturbances that are specific of apolipoprotein E-deficient mice, suggesting that chronic DU exposure did not worsen the pathology in this experimental model.

Racine R, Grandcolas L, Grison S, Stefani J, Delissen O, Gourmelon P, Veyssière G, Souidi M. Cholesterol 7alpha-hydroxylase (CYP7A1) activity is modified after chronic ingestion of depleted uranium in the rat. J Steroid Biochem Mol Biol **120**:60-6 (2010).

Keywords: cholesterol, uranium, contamination, chronic

[P2.18]

Development of a rapid yeast estrogen bioassay through homologous recombination

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[1]Research Center for Eco-Environment Science, Chinese Academy of Sciences, China, [2]Institute of High Energy Physics, Chinese Academy of Sciences, China

In this study we developed an estrogen transcription activation assay based on yeast. This assay was sensitive, fast and easy to use in the routine screening of estrogen activity in complex matrices. The designed strain, which was derived from *Saccharomyces cerevisiae* strain Y187 through homologous recombination, contains the luciferase reporter instead of LacZ reporter constructs. This assay made use of recombined human estrogen receptor（ER） gene and glutamate receptor interacting protein 1（GRIP1）gene plasmids in yeast, which specifically expressed luciferase when incubated with estrogen. Dose–response curves and EC50 of 0.07 nM for 17beta-estradiol (E2) obtained with the luciferase assay were similar to beta-galactosidase assay which was reported by us before. Compared with the beta-galactosidase assay, this assay could be detected simply after adding luciferin in to the exposure system which needn't cell wall disruption. Furthermore, this assay can be performed completely in 96 well plates within 3.5 h. This makes the test sensitive, rapid and convenient with high reproducibility and small variation. On the other hand, we provided a powerful strain which can be used for detecting protein interactions in all two-hybrid screen based GAL4 protein interactions.

Keywords: estrogen bioassay, luciferase reporter, yeast-two-hybrid, homologous recombination

[P2.19]

Estrogenic effects of red clover (trifolium pratense) extracts containing different isoflavone compositions

K.A. Power*[1], J. Lu[1], W. Wu[1], D. Lepp[1], W.E. Ward[2], R. Tsao[1] et al
[1]*Agriculture and Agri-food Canada, Canada,* [2]*University of Toronto, Canada*

Red clover (RC) isoflavone (e.g. biochanin A (BioA), formononetin, genistein, and daidzein) supplements are commercially available and used to reduce menopause-related symptoms and diseases, such as osteoporosis. Studies show however, that different RC supplements contain varying ratios of isoflavones, especially, BioA and formononetin, but it is not known if the health effects induced by RC are dependent on the isoflavone composition. The objective of this study was to compare the effects of RC extracts and purified isoflavones mixtures, differing in their ratios of isoflavones, on reproductive tract and mammary gland histomorphology, in a postmenopausal mouse model. Two RC extracts were obtained and characterized; Extract 1: 46.33 mg isoflavones/ g extract with 44% BioA, 45% formononetin, 6% genistein, and 4% daidzein; Extract 2: 35 mg isoflavones/g extract with 16% BioA, 62% formononetin, 12% genistein, and 9% daidzein. HeLa cells transfected with estrogen receptor (ER)α or β, and an ERE-driven luciferase plasmid, were treated with two RC extracts (0.1-1.0μM total isoflavones) and Extract 2 was a stronger inducer of ER-mediated gene transcription, suggesting that at equal total isoflavone concentrations, the biological effect was different depending on the isoflavone composition. In vivo, C57Bl/6 ovariectomized mice were fed basal diet with or without A) RC Extract 1 or 2 (standardized to contain equal total isoflavones) or B) varying ratios of purified BioA and formononetin, for 8 weeks. With regards to bone health, diets containing higher amounts of BioA induced the greatest increase in femur biomechanical strength, but also induced other mild estrogenic effects, as indicated by increased uterine luminal epithelial and mammary gland growth. These results indicate that different commercial RC isoflavone supplements, even if administered at equivalent total isoflavone doses, may not induce the same effects on estrogen-sensitive tissues in postmenopausal women.

Keywords: phytoestrogen, isoflavone, menopause, estrogen receptor

[P2.20]

Non-hormonal steroidal compounds are effective at reducing asthmatic inflammation

J.M. Damsker*[1], M.A. Balsley[2], A.M. Watson[3], E.J. Stemmy[2], D.M. Berman[2], R.A. Jurjus[2] et al

[1]Validus Biopharma, USA, [2]The George Washington University, USA, [3]Children's National Medical Center, USA

Asthma is a chronic inflammatory condition of the lower respiratory tract associated with airway hyperreactivity and mucus obstruction. Glucocorticoids (GCs) such as prednisone have been considered the standard of care treatment for asthma for the past 60 years. Despite their effectiveness, long-term treatment with GCs is limited by detrimental side effects believed to be due to the hormonal steroidal response involving glucocorticoid receptor (GR) binding and subsequent gene transcription. The beneficial anti-inflammatory effect of GCs is believed to be attributed to their protein-protein signaling activity (e.g. NFkB inhibition). We have developed a novel series of steroidal compounds (VBP) that lack GR-binding and do not induce GR-mediated transcription. The purpose of this study was to determine if these non-hormonal steroidal compounds still retain GC anti-inflammatory capabilities in the context of asthmatic inflammation. We demonstrate using a mouse model of allergic lung inflammation that VBP compounds are as effective as traditional glucocorticoids in terms of their ability to reduce inflammation of the lung. When benchmarked against traditional glucocorticoids, VBP compounds had equal or greater reduction of two major hallmarks of asthma pathogenesis-- leukocyte degranulation and proinflammatory cytokine release from asthmatic epithelial cells. These data suggest that VBP compounds represent an equally efficacious but less toxic alternative to traditional glucocorticoids in the treatment of asthma and other inflammatory diseases.

Keywords: Asthma, Inflammation

[P2.21]

Progesterone and prolactin increase inflammation in the mammary glands of STAT5b knockout mice

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Both progesterone (P) and prolactin (PRL) are essential for normal mammary gland development. Signal transducer and activation of transcription (STAT) 5b functions downstream of the PRL receptor, where loss of STAT5b impairs ductal branching and lactogenesis. In addition to its role during mammary gland development, PRL activates numerous cells of the immune system to mediate immune and inflammatory responses. We find that compared to age-matched wild type (WT) females, the mammary glands of female STAT5b$^{-/-}$ mice undergo precocious alveolar development in response to STAT5b$^{-/-}$-induced hyperprolactinemia. The role of STAT5b during P+PRL-induced mammary gland development was tested using heterologous transplants of WT and STAT5b$^{-/-}$ epithelium into the cleared fat pads of WT hosts. Transplants of STAT5b$^{-/-}$ epithelium exposed to P+PRL displayed thjckening of the mammary ducts, stromal fibrosis, and infiltration of T cells, B cells and macrophages. These inflammatory responses did not occur in the contralateral WT transplants, or in any transplants from vehicle-treated mice. No differences in the activation of STAT1, -3 or -5a was detected between WT and STAT5b$^{-/-}$ transplants following treatment with P+PRL, ruling out the possibility that loss of STAT5b was compensated for by other STAT family members. Collectively these results suggest that STAT5b functions to mediate stromal remodeling and immune cell recruitment to the epithelial microenvironment in response to the synergistic proliferative effects of P and PRL.

Keywords: STAT5b, mammary, immune, Prolactin

[P2.22]

Up-regulations of GILZ by hypoxia and glucocorticoid inhibit the expression of IL-1β under hypoxic condition

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Hypoxia and inflammation often develop concurrently in numerous diseases, and the influence of hypoxia on the natural evolution of inflammatory responses is widely accepted. Glucocorticoid-induced leucine zipper (GILZ) is thought to be an important mediator of the anti-inflammatory and immune suppressive action of GC. However, whether GILZ is involved in the hypoxic response is still unclear. In this study using real time PCR and western blot analysis we investigated the effects of hypoxic exposure or / and treatment of dexamethasone (Dex), a synthetic GC on the GILZ expressions both in vitro and in vivo, and further explored the relationship of expression between GILZ and IL-1β, a pro-inflammatory cytokine under normoxic and hypoxic conditions. We found that hypoxia not only remarkably up-regulated the expression of GILZ, but also significantly enhanced Dex-induced expression of GILZ in RAW264.7 cells and in spleen of rat. Inhibiting the expression of GILZ in RAW264.7 cells using specific GILZ small interfere RNA (siRNA) led to a significant increase in IL-1β mRNA in hypoxia, and abrogated the inhibitory effect of Dex on the expression of IL-1β in normoxia and hypoxia. We also found that the up-regulation the expression of GILZ by hypoxia in spleen is dependent on adrenocortical steroids. Taken together, the data presented here suggested that GILZ not only plays an important role in the adaptive response to hypoxia by negatively regulating activation of macrophages and expression of pro-inflammatory cytokine, but also mediates the anti-inflammatory action of GC under hypoxic condition.

This work was supported by grant from The National Basic Research Program “973” No. 2006CB504100.

[P2.23]

Role of progesterone (P) and estradiol 17 beta (E) in regulation of expression of secretory leucocyte protease inhibitor in bewo cells and rat uterus

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Secretory Leucocyte Protease Inhibitor (SLPI) was one of the several transcripts differentially expressed transcripts during Forskolin induced differentiation of BeWo cells. It is a 12 kD protein reported to exhibit a variety of activities which include, inhibition of proteases and elastase, in addition to antibacterial and antiinflammatory activities. Studies using RU486 a progesterone receptor antagonist and estrogen receptor antagonists and inhibitor of aromatase a key enzyme involved in biosynthesis of estrogen, revealed that SLPI expression is regulated by progesterone in BeWo cells. However, *in vivo* and *in vitro* studies with rat uterine minces in the presence or absence of estrogen as well as progesterone or estrogen receptor antagonists and ovariectomized rats revealed that in the uterus SLPI is regulated by estrogen. Analysis of the promoter sequences of both rat and human SLPI revealed the absence of a consensus progesterone responsive element (PRE) or estrogen responsive element (ERE) suggesting the possibility of a non-genomic action of progesterone and estrogen in induction of SLPI. This was confirmed by the observation that induction of SLPI expression could be blocked by addition of staurosporin an inhibitor of protein kinase along with P & E to either BeWo cells or rat uterine minces. These results suggest that the non-genomic action may be involved in induction of SLPI.

[Supported by Rajaramanna Fellowship, DST (Govt. of India) to A.J. Rao]

[P2.24]

17Beta-estradiol induces CREB phosphorylation through pathways involving ERK1/2 and AKT and increases cyclin D1 expression in rat sertoli cells.

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Estrogen receptors (ESRs) can mediate rapid signaling events via the activation of different downstream signaling pathways, such as mitogen-activated protein kinases (ERK1/2) and phosphatidylinositol 3-kinase (PI3K/AKT) pathways, which in turn can modulate nuclear transcriptional events in different cells (Mol Endocrinol 19:833, 2005). In rat Sertoli cells, 17β-estradiol (E2) activates translocation of ESRs to the plasma membrane mediated by SRC (SRC family of tyrosine kinases), which results in the activation of EGFR (epidermal growth factor receptor), ERK1/2 phosphorylation and cell proliferation (Biol Reprod 78:101, 2008). We now report the effect of E2 on AKT phosphorylation and cyclic AMP response element binding protein (CREB) activity and Cyclin D1 expression in these cells. Primary culture of Sertoli cells was obtained from 15-day old Wistar rats. AKT and CREB phosphorylation and Cyclin D1 expression were determined by Western Blot. 17β-Estradiol (E2, 0.1 nM, 35°C) induced a rapid and transient increase in the phosphorylation state of AKT and CREB. The peak of CREB phosphorylation occurred at 10 min of E2 treatment (4-fold increase). The activation of CREB induced by a 10 min-treatment with E2 was blocked by pretreatment with PI3K inhibitors, Wortmannin (1 μM, 30 min) and LY294002 (20 μM, 60 min), EGFR kinase inhibitor AG 1478 (50 μM, 15 min) and MEK1/2 inhibitor U0126 (20 μM, 30 min), indicating that AKT, EGFR and ERK1/2 are upstream components regulating CREB activity. E2 treatment for 24 hours (0.1 nM, 35°C) increased Cyclin D1 expression (3.4-fold). KG-501 (25 μM, 30 min), which disrupts the CREB:CBP complex, only partially blocked this effect, suggesting that CREB is involved in Cyclin D1 expression, but that other transcription factors may be involved. In conclusion, these results indicate that in Sertoli cells E2-ESR may regulate gene expression involved with cell proliferation. ESR may mediate E2 actions important for Sertoli cell function and maintenance of normal testis development and homeostasis.

Supported by FAPESP, CNPq.

Keywords: estrogen receptor, sertoli cells, AKT, CREB

[P2.25]

Oestrogen-nitric oxide (NO) interactions may regulate gender based differences in stress susceptibility and adaptation in rats

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Stress plays a significant role in the development of psychopathology and reports indicate that marked gender related differences in stress related neuropsychiatric and related disorders. Complex factors and mechanisms may be involved in these differential gender related effects and oestrogen dependent mechanisms could be involved. Further, most experimental studies on stress have focused on the male species, and data concerning stress responsiveness in females and their mechanisms are not clearly defined. Nitric oxide (NO), which is known to act as a signaling molecule in the CNS, has been shown to influence stress responsiveness, and the present study evaluated the possible role of oestrogen - NO interactions during gender based differences in stress induced modulation of anxiety in rats. Acute restraint stress (RS x1) induced anxiogenic responses in the elevated plus maze (EPM) test, and suppression of both open arm entries and time spent were greater in males as compared to females. These were accompanied by reductions in brain and plasma NO metabolites (NOx) and increases in ADMA (an endogenous NOS inhibitor) and malondialdehyde (MDA) levels. Pretreatments with L-arginine attenuated both neurobehavioral and biochemical changes after RS. Exposure of female rats to RS resulted in elevated levels of 17-β oestradiol and MDA and lowered NOx levels as compared to controls, and these effects were antagonized by formestane (but not tamoxifen) pretreatment. In the chronic restraint stress (RSx15) model, stress induced adaptation was apparent in female (and not in male) rats, as neurobehavioral effects in the EPM were attenuated towards control levels and brain NOx was also augmented. The chronic RS induced effects were, however, antagonized by tamoxifen pretreatment. These results suggest that female rats were more resistant to stressful experiences as evidenced by the nature of their acute and chronic RS responsiveness in a complex oestrogen dependent manner and that oestrogen–NO interactions were involved in such differential stress susceptibility and adaptation.

Keywords: Oestrogen-Nitric Oxide Interactions, Gender specificity, Stress susceptibility, Stress adaptation

[P2.26]

R5020 rescues Interleukin 1-beta (IL-1β) induced cell death via a MAPK induced pathway in BeWo and JEG3 cells.

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Proliferation, differentiaion and invasion of the trophoblast cells is a crucial step in the healthy progression of pregnancy. Aetiophathogenesis of pre-eclpampsia and intra-uterine growth retardation include impairment in the invasion and proliferation of trophoblasts and are characterised by elevated levels of the proinflammatory cytokine IL-1β. Indeed, abnormal pathologies during pregnancy can have detrimental effects not only on the fetus but on the mother as well. We have used a well established cell line (BeWo) as a model to study the effects of progestagen R5020 (promegestone) and IL-1β on cell death. Moreover changes in the phosphorylation status of ERK1/2 were assessed following treatment with R5020.

ImageStream analysis confirmed at single cell level the presence of progesterone receptors in the cytoplasm and the nucleus. This was further confirmed with Western blotting. Cells were treated with IL-1β (10ng/ml), R5020 (30nM) and UO126 (MAPK inhibitor) for 24 hrs and trypan blue exclusion assay was used to assess the cell death. Treatment of BeWo cells with IL-1β induced cell death in 21.6% of total cells, whereas treatment with R5020 induced a modest (4.4% cell death) response. Interestingly, treatment with IL-1β in the presence of R5020 significantly inhibited the cytokine's effect (10.4% dead cells). Pre-incubating cells with UO126 and performing the combined treatment of IL-1β and R5020 increased the cell death to 33%.

It is attractive to speculate that R5020 rescues placental cells from cell death and this effect is mediated via a MAPK pathway. As a result, the phosphorylation status of $ERK_{1/2}$ was assessed following treatment with R5020 over a period of 1 hour. A biphasic response was seen in BeWo syncytiotrophoblast cells whereas in BeWo cytotrophoblast cells phosphorylation was seen at 3 minutes only. Long and short term responses of R5020 are indicative of a higher order of complexity in this placental cell line.

Keywords: Promegestone (R5020), placental cells, Il-1beta, MAPK

[P2.27]

Corticotropin-releasing hormone and related neuropeptides stimulate neurosteroid biosynthesis

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Neurosteroids are known to exert a wide variety of pathophysiological, behavioral and neuroendocrine activities. Notably, certain neurosteroids mimic the anxiogenic, depressive-like and anorexigenic effects of corticotropin-releasing hormone (CRH) and its paralogs urocortins (UCNs). However, the possible role of CRH and UCNs in the regulation of neurosteroid production has never been investigated. We have thus studied the possible effect of CRH and related peptides on the biosynthesis of neurosteroids, using the brain of the frog *Rana esculenta* as an experimental model. Double immunohistochemical labeling of frog brain sections, showed that 3β-hydroxysteroid dehydrogenase (3β-HSD)-, cytochrome $P450_{C17}$ ($P450_{C17}$)- and 5α-reductase (5α-R)-containing neurons are often apposed by CRH-immunoreactive fibers. 3β-HSD- and 5α-R-expressing neurons are also surrounded by UCN-positive processes. In addition, diencephalic regions, where most 3β-HSD-, $P450_{C17}$- and 5α-R-positive neurons are located, are enriched with CRH receptor-like immunoreactivity. Exposure of frog hypothalamic explants to graded concentrations of CRH or urocortin-I (UCN-I) (10^{-12} – 10^{-5} M) produced a dose-dependent increase in the formation of P, 17OH-Δ^5P, DHEA and THP. The stimulatory effect of CRH and UCN-I on neurosteroid production was mimicked by sauvagin and urotensin-I. Time-course experiments revealed that a 15-min incubation of hypothalamic explants with CRH or UCN-I was sufficient to induce a robust increase in neurosteroid production and that the maximum effect was observed after a 1-h exposure to CRH or a 2-h exposure to UCN-I, suggesting that CRH and UCN-I activate steroidogenic enzymes at a post-translational level. In conclusion, the present study provides the first evidence that, in the brain of vertebrates, CRH and UCNs regulate the activity of neurosteroid-producing neurons. Since neurosteroids have been implicated in the control of a number of behavioral and metabolic activities, our data strongly suggest that some of the neurophysiological and behavioral effects of CRH and related peptides may be mediated via the regulation of neurosteroid production.

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Keywords: Neurosteroids, CRH, Urocortin, Stress

[P2.28]

Azido analogs of neuroactive steroids

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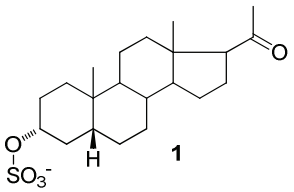
The presented work is part of a broader study of interactions of neuroactive steroids with the NMDA receptors. The 20-oxo-5beta-pregnan-3alpha-yl sulfate (**1**) is an use-dependent allosteric inhibitor of NMDA receptors[1], thus its derivatives could be potentially useful neuroprotectives for the treatment of disorders caused by hyper excitation of the NMDA receptors (e.g. traumatic brain injury etc.).

We have substituted the acetyl side chain in position 17 of the endogenous derivative **1** with an azido group placed on the skeleton or on one- or two-carbon containing side chains. The S_N2 substitution of sulfonates with sodium azide or Mitsunobu reaction of corresponding hydroxyderivatives with azoimide were used for the synthesis. The last step of the reaction sequences was an introduction of sulfate group to the position 3alpha.

Inhibitory activity of newly prepared compounds (**2-6**) on the NMDA receptor was evaluated by the patch-clamp technique and imaging recordings of HEK293 cells expressing NR1/NR2B receptors. Steroids (5 – 100 μM) were applied simultaneously with 1 mM glutamate[1].

Azidosteroid sulfates had inhibitory effect at response to glutamate mediated by NR1/NR2B receptors with IC_{50} varying in the range from 2.4 ± 0.5 to 154.8 ± 21.9 μM. Most of them exert a higher activity than endogenous 20-oxo-5beta-pregnan-3alpha-yl sulfate (IC_{50} = 47.2 ± 9.9).

Newly prepared steroid azides could serve as a leading structure for the development of pharmacologically useful analogs.



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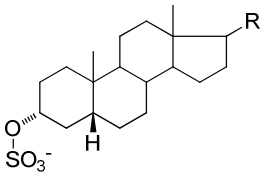

2. R = -βN_3
3. R = -αN_3
4. R = -β[*R*]-CHN_3CH_3
5. R = -β[*S*]-CHN_3CH_3
6. R = -β-CH_2N_3

*Acknowledgements:*The work was supported by the IGA Ministry of Health CR NS10365 and the Research Projects of the AS CR Z4 055 0506 and AV0Z 50110509.

References:

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Keywords: Neuroactive steroids, NMDA receptor, steroid azide

[P2.29]

Circulating steroids in premenopausal smokers

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Introduction: Chronic smoking alters the circulating levels of sex hormones and possibly also the neuroactive steroids, however, the data available is limited.

Methods: Therefore, a broad spectrum of free and conjugated steroids and related substances was quantified by GC-MS and RIA in premenopausal smokers and in age-matched (38.9 ± 7.3 years of age) non-smokers in the follicular (FP) and luteal phases (LP) of menstrual cycle (10 non-smokers and 10 smokers, in the FP, and 10 non-smokers and 8 smokers in the LP).

Results: Smokers in both phases of the menstrual cycle showed higher levels of conjugated 17-hydroxypregnenolone, 5α-dihydroprogesterone, conjugated isopregnanolone, conjugated 5α-pregnane-3β,20α-diol, conjugated androstenediol, androstenedione, testosterone, free testosterone, conjugated 5α-androstane-3α/β,17β-diols, and higher free testosterone index. In the FP, the smokers exhibited higher levels of conjugated pregnenolone, progesterone, conjugated pregnanolone, lutropin and higher lutropin/follitropin ratio, but lower levels of cortisol, allopregnanolone, and pregnanolone. In the LP, the smokers exhibited higher levels of free and conjugated 20α-dihydropregnenolone, free and conjugated dehydroepiandrosterone, free androstenediol, 5α-dihydrotestosterone, free and conjugated androsterone, free and conjugated epiandrosterone, free and conjugated etiocholanolone, 7α/β-hydroxy-dehydroepiandrosterone isomers, and follitropin but lower levels of estradiol and sex hormone binding globulin (SHBG) and lower values of lutropin/follitropin ratio.

Discussion: Chronic cigarette smoking augments serum androgens and their 5α/β-reduced metabolites (including GABAergic substances) but suppresses levels of estradiol in the LP and SHBG and may induce hyperandrogenism in female smokers. The female smokers had pronouncedly increased serum progestogens but paradoxically suppressed levels of their GABA-ergic metabolites. Further investigation is needed concerning the machinery of these effects.

Grants NS/9834-4, NS/9790-4, and NT 11513-5 from the Internal Grant Agency of the Czech Ministry of Health (IGA MZCR) supported the study.

Keywords: smoking, sex hormones, neuroactive steroids, menstrual cycle

[P2.30]

7-Dehydrocholesterol reductase does not require cytochrome P450 reductase for activity

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7-Dehydrocholesterol reductase (DHCR7) catalyzes the final step in cholesterol synthesis. The enzyme utilizes NADPH as a source of electrons, and it has been reported that cytochrome P450 reductase (POR), an electron transfer protein that utilizes NADPH, is required for this reaction. To test this hypothesis, we prepared microsomes from the livers of mice in which hepatic POR expression was extinguished during maturation and examined DHCR7 activity, measured as ergosterol reduction to brassicasterol, in these preparations. Liver microsomes from these mice contained negligible levels of POR (<5% of wild-type), but had DHCR7 activity equal to that of wild-type mice and DHCR7 protein levels that were not different from wild-type mice. Because the original observation that POR was necessary for DHCR7 activity was based in part on antibody inhibition studies with POR antibody, we evaluated the ability of a polyclonal antibody to POR to inhibit DHCR7 activity. This antibody had no effect on DHCR7 activity, but effectively inhibited POR-catalyzed reduction of cytochrome c. The POR antibody did not cross-react with DHCR7, and a DHCR7 antibody did not recognize POR. We conclude that cytochrome P450 reductase is not required for DHCR7 activity; because the previous report utilized a substrate-disappearance assay for 7-dehydrocholesterol, we suspect that POR was contributing to alternative enzymatic pathways for 7-dehydrocholesterol metabolism. We are currently testing this hypothesis.

Keywords: 7-dehydrocholesterol reductase, cholesterol, cytochrome P450 reductase, Smith-Lemli-Opitz syndrome

[P2.31]
Overexpression of AKR1C3 (Type 5 17beta-Hydroxysteroid Dehydrogenase) in LNCaP cells as a model of androgen metabolism in castration-resistant prostate cancer

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Adaptive intratumoral androgen biosynthesis using adrenal dehydroepiandrosterone as a precursor contributes to the development of castration resistant prostate cancer (CRPC). Upregulation of 17β-hydroxysteroid dehydrogenase type 5 (AKR1C3) mRNA has been detected in CRPC. We hypothesize that overexpression of AKR1C3 increases cancer cell responsiveness to adrenal androgens by converting 4-androstene-3,17-dione to testosterone. To test this theory, we generated a LNCaP cell line stably expressing AKR1C3 (LNCaP-AKR1C3) and investigated metabolism of [^{3}H]-4-androstene-3,17-dione in both cell lines. We observed strong glucuronidation activity so that many metabolites were only detectable following β-glucuronidase treatment. In LNCaP cells, 4-androstene-3,17-dione was primarily reduced to 5α-androstane-3,17-dione and subsequently to (epi)androsterone. A small amount of testosterone formation was also observed but was significantly increased in LNCaP-AKR1C3 cells. Addition of indomethacin, a competitive AKR1C3 inhibitor, had limited effect on 4-androstene-3,17-dione metabolism in LNCaP cells, but in LNCaP-AKR1C3 cells formation of testosterone was blocked and the metabolic profile reverted to that observed in the parental cell line. Addition of finasteride, a 5α-reductase inhibitor, eliminated formation of 5α-reduced metabolites. In LNCaP cells, testosterone levels were similar to untreated cells while in LNCaP-AKR1C3 cells, inhibition of 5α-reductase markedly increased testosterone levels. When both inhibitors were applied simultaneously, testosterone production in LNCaP cells resembled that in cells treated with finasteride only. In LNCaP-AKR1C3 cells, testosterone production was reduced with both inhibitors but was higher than in cells treated with indomethacin only. Our findings indicate that AKR1C3 expression in a prostate cancer cell line can lead to significant production of testosterone from 4-androstene-3,17-dione, which could increase androgen receptor activation and cancer growth. In addition, we show that finasteride treatment will increase testosterone formation in LNCaP-AKR1C3 cells through redirection of the metabolic pathway and therefore may not be suitable to ablate androgen receptor activation in CRPC when AKR1C3 is overexpressed. [Supported by 1R01-CA90744 awarded to TMP].

Keywords: AKR1C3, HSD17B5, prostate cancer

[P2.32]

Development of murine models to investigate the role of glucuronidation in androgen homeostasis in vivo

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In their target tissues, androgens control cell proliferation via activation of the androgen receptor (AR) pathway. However, an androgen excess can lead to the initiation and development of prostate cancer. In human prostate cancer cells, the UDP-glucuronosyltransferase (UGT)2B15 and 2B17 enzymes regulate the androgen signalling pathway by inactivating these hormones. Indeed, *ex vivo* experiments with prostate cancer cell lines identified glucuronidation as a major determinant of androgen activity. However, the lack of animal model prevents the validation of this hypothesis *in vivo*. In order to overcome this limitation, two types of transgenic mice expressing the human UGT2B15 enzyme have been constructed and are currently being characterized. The two constructions include 6 exons and the 1^{st} intron 1of the human *UGT2B15* gene. The first transgene, Tg-p2B15 also contains the active proximal promoter of UGT2B15, and aims at providing animals with similar tissue-distribution of the transgene as the humans. The second transgene, Tg-pProba, used the composite probasin promoter ARR_2PB to obtain a prostate-specific expression in transgenic animals. After successful pronuclear injection, 9 Tg-p2B15 and 6 Tg-pProba strains were identified through genotyping. However, sequencing of the complete transgenes identified the presence of multiple mutations in 5 strains, and 1 strain had lost the transgene in subsequent generation, leaving 5 strains (numbered 1 to 5) for each type of transgenic animals. Furthermore, genomic localization of the transgene by TAIL-PCR analyses indicated possible alterations of endogenous gene expression in two other transgenic mouse strains. Nevertheless, RT-PCR experiments revealed expression of the transgene into at least 1 strain of each transgenic type. Overall, these preliminary observations indicate that 2 novel transgenic mouse models have been generated and will allow for the first time the study of the physio-pathological impact of androgen glucuronidation, at both the systemic (Tg-p2B15) and local (Tg-pProba) levels.

Keywords: Glucuronidation, Androgens, Prostate, Transgenic mice

[P2.33]
Phosphodiesterase 7B is a determinant of testosterone and nandrolone bioavailability

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Background

Injectable anabolic androgenic steroid are generally esterified in order to retard their release from the injection site. The steroids are activated by esterase catalysed hydrolysis. The esterases involved in this activation remain unknown. Previous Affymetrix analysis has shown that genetic variation in phospohodiesterase 7B (PDE7B) is associated with the serum testosterone level two days after testosterone administration in healthy volunteers.

The aim of this study was to determine if PDE7B is involved in the hydrolysis of testosterone enanthate (TE) and 19-nortestosterone decanoate (19-NTD) and to which degree. These androgens are commonly used and abused. Moreover, we investigated if these drugs affect the gene expression of PDE7B.

Materials and Methods

HepG2 liver cells were incubated with TE or 19-NTD for 2, 8, 24 and 48h. The effect on the PDE7B specific mRNA was determined after cDNA synthesis using quantitative rtPCR analysis. The inhibition potential of a PDE7B inhibitor (BL50481) against ester hydrolysis was investigated in human liver homogenate samples from Caucasian donors. The production of esterified steroid was followed by liquid-chromatography coupled to ultra-violet detection.

Results and discussion

The PDE7B mRNA in HepG2 cells was significantly increased 4-fold after 2h incubation with TE or 19-NTD, but not after 8, 24 and 48h. Furthermore, BRL50481 was found to partially inhibit the hydrolysis of the steroid ester both for TE (40%) and 19-NTD (80%). These results are suggestive of a role of PDE7B in steroid activation.

Conclusion

Polymorphism in the PDE7B gene partially explains the inter-individual variation in testosterone and nandrolone bioavailability in the human body after intra-muscular injection.

Keywords: testosterone, 19-nortestosterone, phosphodiesterase 7B, bioavailability

[P2.34]

11Beta-Hydroxysteroid Dehydrogenase Type 1 inhibition ameliorates metabolic syndrome in WNIN/Ob obese rats but induced glucose intolerance in lean rats

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Introduction

11beta-hydroxysteroid dehydrogenase type 1 (11beta-HSD1) converts inactive glucocorticoids to active ones and plays a major role in the development of metabolic syndrome. WNIN/Ob obese rat, a novel genetically obese rodent model exhibits all major features of metabolic syndrome and had elevated 11beta-HSD1 activity in adipose tissue. Here, we studied the effect of carbenoxolone (CBX), a non-selective 11beta-HSD inhibitor on the various physiological components of metabolic syndrome in WNIN/Ob obese rats.

Methods

Three month-old, male lean and obese rats (n=12 for each phenotype) were divided into control and experimental groups (n=6). CBX (50 mg/Kg body wt) was administered subcutaneously to experimental group rats for four weeks. Oral glucose tolerance test was performed and body composition was analyzed by total body electrical conductivity. 11beta-HSD1 activity was measured in liver, skeletal muscle, adipose tissue [4]. Plasma insulin, corticosterone, glucose, and lipids were assayed.

Results

CBX significantly inhibited 11beta-HSD1 activity in liver and subcutaneous adipose tissue of both lean ($p \leq 0.001$) and obese rats ($p \leq 0.05$). Fat percentage significantly decreased in CBX-treated lean and obese rats ($p \leq 0.01$). CBX significantly decreased plasma insulin ($p \leq 0.01$), corticosterone ($p \leq 0.05$), triglycerides ($p \leq 0.05$) and cholesterol ($p \leq 0.001$) in obese rats but not in lean rats. Insulin resistance as calculated by homeostasis model assessment of insulin resistance was significantly decreased by CBX in obese rats ($p \leq 0.05$). CBX did not altered glucose tolerance in obese rats, but induced glucose intolerance in lean rats.

Discussion

11beta-HSD1 inhibition by CBX decreased fat mass, hypertriglyceridemia, hypercholesterolemia, and insulin resistance in WNIN/Ob obese rats. Interestingly, 11beta-HSD1 inhibition decreased fat percentage and induced glucose intolerance in lean rats. We conclude that 11beta-HSD1 inhibition ameliorates metabolic syndrome in WNIN/Ob obese rats. 11beta-HSD1 inhibition is a good strategy to treat metabolic syndrome, but its inhibition under normal conditions may result in fat loss and glucose intolerance.

Keywords: 11beta-hydroxysteroid dehydrogenase type1, Obesity, Metabolic syndrome, WNIN/Ob obese rat

[P2.35]

Progestins affect the pre-receptor regulatory enzymes akr1c1, akr1c2 and akr1c3

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The human aldo-keto reductases 1C1, 1C2 and 1C3 (AKR1C1, AKR1C2 and AKR1C3) function *in vitro* as 3-keto-, 17-keto- and 20-ketosteroid reductases, or as 3α/β-, 17β- and 20α-hydroxysteroid oxidases, to varying extents. *In vivo* these enzymes act as reductases and have important roles in the pre-receptor regulation of estrogen, androgen and progesterone actions. Recently, we reported significant up-regulation of AKR1C1, AKR1C2 and AKR1C3 in ovarian endometriosis at the mRNA level. Endometriosis is a complex estrogen-dependent disease with increased local formation of estradiol, which stimulates proliferation of endometriotic tissue, and disturbed action of the protective progesterone. Although progestins have been used for treatment of endometriosis since the 1960s, their detailed mechanisms of action are still not completely understood. In the present study, we evaluated the potential inhibitory effects of progestins on the pre-receptor regulatory enzymes AKR1C1, AKR1C2 and AKR1C3. We examined the following progestins: progesterone derivatives (dydrogesterone, its metabolite, 20α-hydroxydydrogesterone; and medroxyprogesterone acetate), 19-nortestosterone derivatives (desogestrel, noretinodrone and levonorgestrel), and the androgen danazol. Dydrogesterone, medroxyprogesterone acetate, and 20α-hydroxydydrogesterone inhibited AKR1C1, AKR1C2 and AKR1C3 with low μM K_i values. Norethinodrone inhibited AKR1C1 and AKR1C3, while levonorgestrel and desogestrel preferentially inhibited AKR1C3; all with μM K_i values. Our data thus revealed that dydrogesterone, medroxyprogesterone acetate, and 20α-hydroxydydrogesterone inhibit all three AKR1C isozymes *in vitro*, although their physiological inhibitory effects still need to be evaluated further. Docking simulations of dydrogesterone, medroxyprogesterone acetate and 20α-hydroxydydrogesterore into AKR1C1, AKR1C2 and AKR1C3 also support these experimental data.

Dydrogesterone and 20α-hydroxydydrogesterone were kindly provided by Abbott Products GmbH, Hannover, Germany. The pcDNA3-AKR1C1, pcDNA3-AKR1C2 and pGex-AKR1C3 constructs were kind gifts of Dr. T.M.Penning (University of Pennsylvania, Philadelphia, USA) and Dr. J.Adamski (Helmholtz Zentrum München, Germany).

Keywords: aldo-keto reductases, progestins, endometriosis, dydrogesterone

[P2.36]

The effect of intracellular location on hydroxysteroid dehydrogenase activity: Implications for 11βHSD1 catalytic mechanism and beyond

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Hydroxysteroid dehydrogenases (HSDs) catalyze the interconversion of inactive steroids and active hormones. HSDs use nicotinamide cofactors in the cytosol and ER lumen to either reduce or oxidize their steroid substrates. Our lab has extensively studied the 17βHSDs types 1, 2 and 3 of the short-chain dehydrogenase-reductase family, particularly human 17β-HSD1, which favors estrone reduction to estradiol. Rat AKR1C9 has also been thoroughly studied as a model HSD of the aldo-keto reductase family; AKR1C9 catalyzes the reduction of dihydrotestosterone to androstanediol. These two enzymes provide a basis for comparative studies with 11βHSD1, which catalyzes the reduction of cortisone to cortisol.

Most mammalian cells supplied with adequate glucose and oxygen maintain high cytoplasmic nicotinamide concentration gradients, [NADPH] >> [$NADP^+$] and [NAD^+] >> [NADH], and in the strongly oxidizing environment of the endoplasmic reticulum (ER) lumen, both these gradients are shifted to more oxidized cofactor. Whereas 17βHSD types 1, 2, 3 and AKR1C9 catalyze their respective reactions in a thermodynamically predictable manner based on cofactor gradients, 11βHSD1 does the opposite. 17βHSD1, 17βHSD3, and AKR1C9 favor reduction in the cytosol using NADPH, and 17βHSD2 favors oxidation in the ER lumen using NAD^+. In contrast, 11βHSD1 reduces cortisone to cortisol in the highly oxidative ER lumen but requires hexose-6-phosphate-dehydrogenase (H6PDH) to regenerate NADPH in the ER lumen. We hypothesize that H6PDH directly channels NADPH to 11βHSD1 through specific interactions.

To test this hypothesis, we have targeted 17βHSD1 and AKR1C9 to the ER lumen rather than the cytosol. Conversely, we have targeted 11βHSD1 to the cytoplasmic surface of the ER. In addition, we have engineered point mutations in 17βHSD1, AKR1C9, 11βHSD1 and H6PDH, designed to attenuate the directional preferences by altering cofactor binding. Unexpectedly, some enzymes targeted to the ER lumen appear to maintain similar steroid distribution patterns as their cytosolic counterparts, while others vary significantly.

Keywords: hydroxysteroid dehydrogenase, redox state, endoplasmic reticulum, nicotinamide cofactors

[P2.37]

Effect of diet-rich in n-6 polyunsaturated fatty acids on 11beta-Hydroxysteroid Dehydrogenase Type 1 activity in liver and adipose tissue of WNIN/Ob obese rats

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Introduction

11beta-hydroxysteroid dehydrogenase type 1 (11beta-HSD1) amplifies tissue glucocorticoid action by converting inactive glucocorticoids to active ones. 11beta-HSD1 activity is elevated in adipose tissue of obese rodents and obese humans. 11beta-HSD1 inhibition decreases obesity and improves insulin resistance. Studies on the role of nutrients on 11beta-HSD1 activity are very few. Here, we studied the effect of diet-rich in n-6 polyunsaturated fattyacids (n-6 PUFA) on hepatic and adipose tissue 11beta-HSD1 activity in obesity, using WNIN/Ob obese rat model.

Methods

210-day old male WNIN/Ob lean and obese rats (n=16 for each phenotype) were equally divided in to control and experimental groups. Control group of rats was fed with stock diet (4%ground nut oil) and experimental group of rats was fed on n-6 PUFA rich-diet (4% sunflower oil) for two months. Animals were sacrificed, blood and tissues were collected. Plasma corticosterone levels were measured. 11beta-HSD1 activity was measured in liver and omental adipose tissue by HPLC method using 1, 2, 6, 7-^{3}H (4) corticosterone as substrate.

Results

Hepatic 11beta-HSD1 activity decreased significantly in n-6 PUFA-rich diet fed lean rats compared to control lean rats ($p \leq 0.05$), but not altered the activity in obese rats. n-6 PUFA-rich diet significantly increased 11beta-HSD1 activity in omental adipose tissue of lean rats ($p \leq 0.01$), however, a strong tendency towards decrease ($p=0.16$) in enzyme activity was observed in n-6 PUFA-rich diet fed obese rats. Plasma corticosterone levels significantly decreased in n-6 PUFA rich diet fed-lean rats ($p \leq 0.05$) but not in obese rats.

Discussion

This is the first study to report the effect of n-6 polyunsaturated fattyacids on 11beta-HSD1 activity in obesity. In lean rats, n-6 PUFA decreased plasma corticosterone levels, hepatic 11β-HSD1 activity, and increased adipose tissue 11beta-HSD1 activity. In obese rats, PUFA did not alter the circulatory corticosterone, hepatic and adipose 11beta-HSD1 activities. We conclude that dietary n-6 PUFA alters the 11beta-HSD1 activity under normal conditions but not in obese condition.

Keywords: 11beta-hydroxysteroid dehydrogenase type 1, Polyunsaturated fattyacids, Obesity, WNIN/Ob obese rat

[P2.38]

Study of the multi-specificity of the estrogenic 17beta-Hydroxysteroid Dehydrogenase Type 1 in HEK-293 and T47D breast cancer cells

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17β-hydroxysteroid dehydrogenases (17β-HSDs) are key enzymes in the synthesis of sex steroids or their degradation. 17β-HSD1 catalyses the reversible 17β oxido-reduction of steroids *in vitro*. In spite of the enzyme's narrow binding tunnel leading to its estrogen specificity, the pseudo-symmetry of C19 steroids leads to the multispecificity of the enzyme via the alternative binding orientation of such steroids. However, the enzyme's multispecificity in cells remains unclear. In the present study, 17β-HSD1 activity toward various C-19 steroids have been examined to reveal the function of the critical enzyme at the cell level. The steady-state kinetic studies were carried out in HEK-293 cells stably transformed with 17β-HSD1(HEK-293-1), as well as in T47D cells. Enzymatic assay was assessed using [^{14}C]-labelled androgen/estrogen as substrates. Molecular Docking has been executed using Lamarckian Genetic algorithm with the help of AutoDock4.1 software. The results showed that when 17β-HSD1 catalyzed 3β-keto reduction of dihydrotestosterone(DHT) into 5α-androstane-3β,17β-diol(3β-diol) , there is a significant difference between kinetics with purified enzyme using NADPH as the cofactor (Km of NADPH=32μM; V_{max}=0.035U/mg) and that carried out in HEK-293-1 cells (Km=256μM; V_{max}=7.86μM/hour·millon cells) without extra cofactor added. We also found that the Km for estrone (E1) is significantly higher in HEK-293-1 than that with purified enzyme. Another kinetic study performed with either Androstenedione(4-dione) or Testosterone(T) as substrate indicated that even for these non-cognate substrates, the steroid reduction is more facilitated than their oxidation in the cell environments, the reduction having 38 times higher specificity over that of oxidation. The modelling of four cofactors with complex structure of 17β-HSD1/DHT (PDBid: 3KLM) agrees with the steady-state kinetics, which showed that the Km of 3β-keto reduction of DHT into 3β-diol is lower when using NADH as a cofactor than when using NADPH, indicating that different cofactors may be used for reductive reactions for 17β-HSD1depending on substrates. Further investigation is needed.

Keywords: 17beta-hydroxysteroid dehydrogenase, Enzyme kinetics, Multispecificity, Androgen inactivation

[P2.39]

A transgenic reporter mouse model for human aromatase gene reveals seminal vesicles as a source of estrogens in men

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There are major differences in the regulatory regions between the human and rodent aromatase (*CYP19A1*) genes, and hence, rodents as such cannot serve as a model to study the regulation of the human *CYP19A1* gene expression. Therefore, we have generated a reporter mouse model with a 100-kbp-long region of the human *CYP19A1* gene. A luciferase reporter gene was subcloned into a BAC clone containing the 5' region of the *CYP19A1* promoter, and transgenic mice were generated. Luciferase activity was measured *in vivo* using a CCD camera or *ex vivo* using a luminometer. For studying the hormonal regulation of the reporter gene, we analyzed the reporter gene expression in prepubertal and adult mice, and in gonadectomized mice injected with 5 mg/kg of testosterone. Immunohistochemical and qRT-PCR analyses were also performed to study the aromatase expression in a set of human tissue samples.

The data revealed that the human *CYP19A1* promoter was functional in mouse tissues. The reporter gene activity was detected, as expected, in the ovary, placenta, brain, bone, mammary gland, testis, adipose tissue and uterus. Furthermore, luciferase activity in the gonads was up-regulated during sexual maturation. Unexpectedly, the reporter gene was highly expressed in the mouse seminal vesicles, indicating a role for seminal vesicles as a source of estrogen in men. The aromatase expression in the human seminal vesicles was confirmed with human samples. Moreover, the expression of the reporter gene in the seminal vesicles was regulated by androgens. In conclusion, we have generated a mouse model for studying the tissue-specific regulation of the human aromatase gene *in vivo* in transgenic mice. The mouse model reveals the seminal vesicles as previously unknown sites of estrogen production in men.

Keywords: aromatase, reporter mouse, luciferase, seminal vesicles

[P2.40]

Paradoxical cellular response by MCS-C3, a novel pyrrolo-pyrimidine analog, via AR-mediated up-regulation of $p21^{CIP1}$ in androgen-sensitive prostate cancer cells

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Prostate cancer is the most frequently diagnosed cancer and is the leading cause of cancer death in men in the US. Despite the initial efficacy of androgen deprivation therapy, the advanced prostate cancer patients eventually develop resistance to this therapy and progress to hormone-refractory prostate cancer (HRPC), for which there is no curative therapy. Androgen is essential for prostate development and homeostasis, and exerts its biological effects by binding to androgen receptor (AR). Androgen regulates not only a series of androgen target genes, but also genes for cell cycle- and apoptosis-regulatory molecules within prostate epithelial cells, such as $p21^{CIP1}$, which induces cell cycle arrest in response to DNA damage and protects cancer cells against p53-mediated apoptosis.

In the course of screening for novel modulators on apoptotic induction, we generated MCS-C3, a pyrrolo-pyrimidine derivative, which induced cell growth inhibition and apoptosis in a time- and dose-dependent manner in androgen-independent prostate cancer cells (DU145, PC3) and also in various non-prostate cancer cells. However, MCS-C3 paradoxically induced apoptosis at specific drug concentration (5 μM) in androgen-dependent prostate cancer cells, while weakly inducing apoptosis at lower and higher drug concentrations.

To investigate the molecular mechanisms underlying this paradoxical cellular response by MCS-C3 in LNCaP and its Subline cells, we performed real time-CES, Western blots, real time-PCR, confocal microscopic analysis, blocking AR activation using AR antagonist. Interestingly, paradoxical apoptotic induction of 5 μM MCS-C3 is associated with dramatic up-regulation (46-fold) of p53-independent, AR-dependent $p21^{CIP1}$.

Androgen, in general, up-regulates expression of $p21^{CIP1}$ gene in stimulating prostate cancer cell proliferation. However, in contrast, we conclude that up-regulation of 5 μM MCS-C3-mediated, p53-independent $p21^{CIP1}$, which is activated by AR via a canonical androgen response element (ARE) in its proximal promoter region, plays pivotal role in the cellular signaling pathways that control apoptosis of androgen-dependent prostate cancer cells.

Keywords: Apoptotic induction, Androgen receptor, Prostate cancer

[P2.41]

The possible androgen actions in ovarian development of female Japanese eel (Anguilla japonica)

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The Japanese eel (*Anguilla japonica*) is one of the major aquaculture species in East Asia. The cultivated eel further gonadal development is successfully induced by injection of salmon pituitary homogenate (SPH). However, according to our previous *in vivo* data, we use SPH with 17α-methyltestosterone for gonadal maturation, and found that more ovarian follicles can be maintained to survive and develop synchronously. This phenomenon show that androgens may modulate ovarian follicular development. Two major androgens has been detected in adult teleost. One is Testosterone (T) to be believed as an aromatizable precursor of Estradiol. The other is non-aromatizable 11-Ketotestosterone (11-KT). Rohr et al., (2001) detects high levels of serum 11-KT in migratory female eel. Recently, we detected 11β-Hydroxysteroid Dehydrogenase (11β-HSD) mRNA in the female ovary. The deduced eel 11β-HSD amino acid sequences of cDNA homolog show 60-70% similarity to teleost fishes and show about 40-50% similarity to mammalian 11β-HSD type 2. In teleost, 11-KT is known to produced by 11β-HSD in the male testis. Thereby, these evidences may show the female ovary has potential to synthesize 11-KT as the male does. The mRNA expression level in the female after injection of SPH+MT is higher than control group under seawater acclimation. Histological data shows the lipid droplets more accumulate and the diameter increase in oocyte. We hypothesized that 11-KT and T are both functionally participate in different androgen actions through receptors in ovarian development. As to T, its action is supposed to support more ovarian follicle to survive. 11-KT may be synthesized by 11β-HSD in ovary and act directly on the oocyte to increase lipid droplets accumulation. This supposition needs to be verified by further study.

Keywords: eel, 11-Ketotestosterone, 11beta-Hydroxysteroid Dehydrogenase, androgen action

[P2.42]

Antiestrogen fulvestrant regulates the expression of androgen receptor in rat epididymis.

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The role of estrogen on epididymal function is still unclear, although estrogen receptors (ESR1 and ESR2) have been described in the epididymis. The antiestrogen fulvestrant (ICI 182,780) impairs estrogen action on ESR1 and ESR2, and regulates the expression of the androgen receptor (AR) and ESR1 in the male reproductive tract. To further investigate the role of E2 during pubertal development, this study analyzed the effect of fulvestrant on testosterone and estradiol levels, and on the expression of steroid receptors in the different segments of the epididymis: initial segment and caput (IS/CAP), corpus (CO) and cauda (CA).

Thirty-day-old rats were treated once a week for 2 months with corn oil (control group) or fulvestrant (10 mg/rat, s.c.). Testosterone and estradiol levels were measured by radioimmunoassay. Expression of the steroid receptors AR and ESR1 was measured by real time PCR and Western blot.

In control animals, testosterone levels were similar in IS/CAP and CA, and higher in CO, and were not modified by fulvestrant. E2 levels were similar in IS/CAP and CA, and lower in CO, and fulvestrant increased E2 levels in CO and CA. The mRNA levels for ESR1 were lower in IS/CAP, intermediate in CO, and higher in CA, whereas the protein content was higher in CO than in CA and IS/CAP. Treatment with fulvestrant did not affect ESR1 mRNA or protein levels. The mRNA levels for AR were higher in CA than in IS/CAP and CO, whereas the expression of the protein was similar in CO and CA, and lower in IS/CAP. Treatment with fulvestrant did not affect the mRNA levels for AR, but markedly increased the AR protein levels in IS/CAP, and to a lesser extent in CO and CA.

In conclusion, fulvestrant up-regulates AR expression in the epididymis possibly by post-transcriptional mechanisms. The different segments of the epididymis seem to display differential sensitivity to the estrogen action. This regulatory diversity is probably important to control region-specific sperm-related functions.

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Keywords: estradiol, epididymis, androgen receptor, estrogen receptors

[P2.43]

Thermodynamic characterization of the human vitamin D receptor binding to 1,25-dihydroxyvitamin D3 and analogs

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The hormonal form of vitamin D_3, 1,25 dihydroxy vitamin D3 [$1,25(OH)_2D_3$], generates biological responses in many tissues including bone and intestine, the immune system, pancreas, heart, skeletal muscle and brain through binding to the nuclear vitamin D receptor (VDR). Ligand binding induces conformational changes in the VDR that enable the receptor to interact with other coactivators to modulate gene transcription. Thermodynamic characterization of the vitamin D receptor (VDR) and variant receptors is needed in order to better define the signal transduction pathway and to aid in the design of therapeutic agents. In order to investigate the thermodynamic parameters for the binding of the VDR to $1,25(OH)_2D_3$ and other analogs, we have cloned the cDNA for the full-length wild-type human VDR receptor (1-427), a polymorphic form of the receptor (4-427), and the C-terminal ligand binding domain of the receptor (118-427) into the pE-SUMOstar expression vector (Lifesensors). The resulting plasmids were transformed into BL21(DE3)pLysS cells, and used for protein expression. The expressed proteins were purified with Ni-NTA affinity chromatography, and protein identity was confirmed by mass spectrometry. Saturation binding analysis confirmed that the receptors bind to $1,25(OH)_2D_3$ with an affinity of ~0.7 nM, consistent with literature values. We are currently characterizing the thermodynamic parameters of hormone and analog binding using micro isothermal calorimetry.

Keywords: vitamin D receptor, 1,25-dihydroxyvitamin D

[P2.44]

The Na/K-ATPase-caveolin complex as the progesterone surface receptor in the vertebrate Oocyte

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Progesterone triggers the resumption of meiosis in the amphibian oocyte through a signaling system at the plasma membrane. Analysis of progesterone binding to isolated plasma membranes of the *Rana pipiens* oocyte indicates that the initial progesterone binding site is located in the external loop (23 amino acids) between transmembrane helices M-1 and M-2 of the α1-subunit of the Na/K-ATPase (112 kDa, 10 transmembrane domains). Studies in other laboratories have shown that Na/K-ATPase is associated with caveolin-1 (CAV-1, a 22 kDa caveolar protein with two transmembrane domains) and that CAV-1-Na/K-ATPase-Src complexes are involved in cardiotonic steroid (ouabain)-initiated signal transduction. We find that, following a 4 - 5 h uptake of exogenous progesterone by the oocyte plasma membrane, more than 95% of both bound progesterone and Na/K-ATPase as well as 60% of the plasma membrane surface area are rapidly internalized by a net inward movement of caveolae. The α-subunit of Na/K-ATPase contains a CAV-1 binding motif within the M-1 helix. We propose that progesterone binding to the M-1/M-2 loop of the α-subunit causes a change in helix-helix interactions which initiates the internalization of the progesterone-α-subunit-CAV-1-Src complex as well as both depolarization of the oocyte plasma membrane and formation of energy-rich protein enol-phosphates. A depolarized (excitable) plasma membrane and an increased availability of high energy phosphates are essential for fertilization and early cleavage. We find that Na/K-ATPase-bound progesterone must be further metabolized to form polar steroids in order for meiosis (nuclear breakdown) to continue. The data suggest that the signaling peptides attached to CAV-1-Na/K-ATPase plus newly formed polar steroids trigger activation of “maturation promoting factor”, a complex of Cdc2 and cyclin B, which induces changes that accompany nuclear membrane breakdown and arrest at second meiotic metaphase.

[P2.45]

Hepatic reduction of the secondary bile acid 7-oxolithocholic acid is mediated by 11beta-hydroxysteroid dehydrogenase 1

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Introduction: The oxidized bile acid 7-oxolithocholic acid (7-oxoLCA), formed primarily by gut microorganisms, is reduced in the human liver to chenodeoxycholic acid (CDCA) and to a lesser extent to ursodeoxycholic acid (UDCA). However, the enzyme(s) responsible for this biotransformation reaction remained unknown.

Methods: Using liver microsomal preparations and LC-MS/MS-based detection of bile acids we characterized the hepatic 7-oxo bile acid reductase activity. Applying recombinant enzymes and transient transfection of HEK-293 cells, we tested the hypothesis whether 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) catalyzes the reduction of 7-oxoLCA. Finally, we applied 3D structural modelling to study the binding of bile acids by 11β-HSD1.

Results: Experiments with human liver microsomes revealed that reduction of 7-oxoLCA is stimulated by NADPH. 7-oxo bile acid reductase activity was latent and stimulated by glucose-6-phosphate, suggesting localization in the endoplasmic reticulum (ER). Using recombinant human 11β-HSD1, we found that this enzyme efficiently converts 7-oxoLCA to CDCA and, to a much lesser extent, UDCA, in addition to its role in the interconversion of glucocorticoids. In contrast to metabolism of glucocorticoids and other substrates, 11β-HSD1 mediated solely 7-oxo reduction of 7-oxoLCA and its taurine and glycine conjugates. Furthermore, 7-oxoLCA and its conjugates preferentially inhibited cortisone reduction, while CDCA and its conjugates preferentially inhibited cortisol oxidation. 3D modelling provided an explanation for the binding mode and selectivity of the bile acids studied.

Conclusions: Our results reveal that 11β-HSD1 is responsible for the observed reduction of 7-oxoLCA in humans and provide a further link between hepatic glucocorticoid activation and bile acid metabolism.

[P2.46]

A potent steroid sulfatase inhibitor blocks the DHEAS-stimulated growth of androgen-sensitive tissues and human prostate cancer xenografts (LNCaP cells) in nude mice

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The transformation of dehydroepiandrosterone sulfate (DHEAS) by steroid sulfatase (STS) is an important step in the synthesis of androgens. Consequently, the use of an STS inhibitor is expected to be a useful strategy for prostate cancer therapy. Since we previously identified 3-*O*-sulfamate-2-methoxy-17α-benzyl-estradiol, namely EM-1913, as a potent inhibitor of STS *in vitro*, we are now interested in evaluating its *in vivo* efficacy. We first demonstrated that EM-1913 did not increase the weight of androgen-sensitive prostate and seminal vesicles when tested subcutaneously (sc) on male castrated rats, thus suggesting a suitable non-androgenic profile. We next determined that EM-1913 (5 µg/mouse/day, sc) inhibited 48%/81% of the effect of DHEAS (10 mg, BID, sc) on ventral prostate and seminal vesicles, respectively. Similarly, in castrated rats, EM-1913 (50 µg/rat/day, sc) partly inhibited (35-51%) and almost entirely inhibited (79-94%) the effect of DHEAS (40 mg, BID, sc) on ventral prostate and seminal vesicles, respectively. These two tissues were completely deprived of STS activity following a treatment with EM-1913. This inhibitory effect of EM-1913 on STS is also reflected in the blood circulation since the plasma level of DHEAS was increased from 770 to 5410 ng/mL in rats treated with STS inhibitor (50 µg/rat/day) and meanwhile the levels of DHEA (from 5.1 to 0.7 ng/mL) and potent androgen dihydrotestosterone (0.01 ng/mL to BLQ) decreased. In this experiment, the concentration of EM-1913 was found to be higher in ventral vesicles (28.3 ng/mL) than in ventral prostate (2.5 ng/mL). Finally, we demonstrated that EM-1913 was able to block the STS activity in homogenated prostate cancer cells LNCaP (>92% at 0.01 µM) and to decrease the DHEAS-stimulated tumor growth in intact male nude mice (LNCaP xenograft). In summary, all these results indicate that the STS inhibitor EM-1913 may have therapeutic utility for the treatment of androgen-sensitive prostate cancer.

[P2.47]

Genomic (via MR) and nongenomic (via GR) effects of aldosterone on H^+-ATPase in proximal tubule

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The mineralocorticoid receptor (MR) is the receptor for aldosterone, however, aldosterone can bind to glucocorticoid receptor (GR), with low affinity. The genomic and nongenomic effects of aldosterone on the intracellular pH recovery rate (pHirr) via H^+-ATPase and on cytosolic free calcium concentration ($[Ca^{2+}]i$) were investigated in isolated proximal S3 segment of rat, during superfusion with Na^+-free solution, by using the fluorescent probes BCECF-AM and FLUO-4-AM, respectively. The pHirr, after cellular acidification with an NH_4Cl pulse, was 0.064 $\pm$ 0.003 pH units/min (n = 74) and was abolished by concanamycin. Aldosterone [10^{-12}, 10^{-10}, 10^{-8} or 10^{-6}M with 1h or 15 or 2 min preincubation (pi)] increased the pHirr. The baseline $[Ca^{2+}]i$ was 103 $\pm$ 2nM (n = 58). After 1 min of aldosterone pi there was a transient and dose-dependent increase of $[Ca^{2+}]i$ and after 6 min pi there was a new increase of $[Ca^{2+}]i$ that persisted after 1h. Spironolactone (MR antagonist), actinomycin D or cycloheximide did not affect the effects of aldosterone (15 or 2 min pi) on pHirr and on $[Ca^{2+}]i$, but inhibited the effects of aldosterone (1 h pi) on these parameters. RU 486 (GR antagonist) prevented the effect of aldosterone (10^{-12} or 10^{-6}M, 15 or 2 min pi) on both parameters. The data indicate a hormonal genomic (1h, via MR) and a nongenomic action (15 or 2 min, probably via GR) on the H^+-ATPase and on $[Ca^{2+}]i$ and are in accordance with our finding showing expression of these receptors in the proximal S3 segment. The results are compatible with stimulation of the H^+-ATPase by increases in $[Ca^{2+}]i$ (at 10^{-12}-10^{-6}M aldosterone) and inhibition of the H^+-ATPase by decreases in $[Ca^{2+}]i$ (at 10^{-12} or 10^{-6}M aldosterone plus RU 486). These Aldosterone effects may represent physiologically relevant regulation of proximal tubular acidification in the intact animal.

Keywords: Mineralocorticoid stimulatory action, renal vacuolar H+-ATPase, aldosterone receptor, pHi

[p2.48]

Ethanol interferes with glucocorticoid receptor regulation of the Corticotrophin-Releasing Hormone (CRH) promoter in PVN-derived neuronal cells

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Alcohol (EtOH) abuse during adolescence can lead to permanent changes in brain function that often manifest in adulthood as psychological disorders, such as depression and anxiety. We have shown previously that binge alcohol exposure in peri-pubertal male rats increased circulating plasma corticosterone levels, as well as corticotropin-releasing hormone (CRH) mRNA levels in the paraventriclular nucleus of the hypothalamus (PVN), a brain region responsible for coordinating stress and anxiety responses in mammals. In this study we investigated the molecular mechanisms involved in mediating these effects by examining the direct effects of EtOH on CRH promoter activity in a neuronal cell line derived from the PVN (IVB cells). In addition, we tested the potential interactions of alcohol and glucocorticoids on CRH promoter activity by concomitantly treating cells with EtOH and the glucocorticoid receptor (GR) antagonist RU486 (100nM) and by sequentially deleting GR binding sites within glucocorticoid response element on the CRH promoter. Cells were transiently transfected with a firefly luciferase reporter construct containing 2.5 kb of the rat wild type or mutated CRH promoter. Cells were treated 24 hours after transfection with 12.5mM concentration of EtOH for 2h. Our results showed that EtOH significantly increased CRH promoter activity and that treatment with RU486, or deletion of the GR binding sites, abolished the EtOH-induced increase in promoter activity. Overall, our data suggest that alcohol exposure directly regulates CRH promoter activity by interfering with the normal feedback mechanisms of glucocorticoids mediated by GR signalling.

Keywords: CRH, alcohol, GR, PVN

[P2.49]
Long-range chromatin interaction in ERα -based regulation of Kruppel-like Factor 9

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Estrogens act by binding to estrogen receptors (ERα and ERβ). Estrogen bound receptors bind to DNA response elements associated with the genes they regulate. However, less is known about the role of chromatin structure in this activity. The genome-wide MCF-7 ERα binding site interactome [1] strongly supports a chromatin looping hypothesis that brings distant ERα binding sites together with promoters. We are examining the role of chromatin looping on ERα mediated transcriptional regulation using a model based upon the estrogen responsive transcription factor kruppel-like factor 9 (KLF9).

KLF9 is a nuclear hormone receptor co-regulator with an established role in the uterine endometrial cell proliferation, adhesion and differentiation in pregnancy and tumorigenesis [2], as well as, repression of ERα transcription [3]. KLF9 has not yet been studied in breast cancer. We detect early KLF9 transcriptional response to estradiol (E2) and have validated previously identified ERα binding sites in the regulatory regions of this gene [1, 4, 5]. A single ERα chromatin loop has been identified for this gene [1]. We are currently developing a BAC-based reporter model to use in RNAi assays to identify the factors that are required for the E2-modulated activity of this loop in KLF9. It is likely that many of the factors we identify will also mediate the E2 response at other genes. Future chromatin immunoprecipitation (ChIP) sequencing experiments will test for ERα cistrome-wide activity of the factors we discover with our model. We hypothesize that for many estrogen-responsive genes chromatin looping is essential for ERα activity, and that the proteins required to initiate and to maintain these loops present a novel category of drug targets for the treatment of anti-endocrine refractory tumors.

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[P2.50]

Cell Cycle Dependent Regulation of Progesterone Receptor Transcriptional Activity in Breast Cancer Cells

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Recent clinical studies have highlighted a role for progesterone/progesterone receptors (PR) in breast cancer development and progression, as demonstrated by an increased risk in breast tumor incidence and size in women taking hormone replacement therapy that includes progestins. PR activity is tightly coupled to post-translational modification by kinases including mitogen activated (MAPK) and cell cycle regulated cyclin dependent kinases (cdks). We hypothesized that PR action is coupled to breast cancer cell cycle progression via direct interactions of phosphorylated PR species with cell cycle regulatory molecules. Our recent data using T47D breast cancer cells, demonstrates that PR displays distinct cell cycle-regulated patterns of phosphorylation in the absence of ligand, particularly during S phase (phosphorylation on PR Ser81, Ser190, Ser400) or G_2M phase (Ser190, Ser294, Ser345, Ser400). While PR activity is primarily driven by ligand binding, PR also has unliganded transcriptional and cytoplasmic signaling capabilities which are most pronounced in the high kinase, low cell cycle inhibitor environment of cancer cells (i.e. when PR is heavily phosphorylated). We synchronized populations (G_2M) of T47D cells to examine cell cycle-regulated PR activity (unliganded and liganded) on specific genes. Quantitative PCR performed on synchronized cell populations revealed a subset of genes that are regulated by PR, both basally and in a ligand-dependent manner, but only during G_2M phase. We mapped PR/G_2M-dependent regulation of one such gene, HSPB-8 (known to be proliferative and promote anchorage independent breast cancer cell survival) to phosphorylation of PR Ser345 and detected phospho-PR (Ser345) dependent SP-1 interaction on the HSPB-8 promoter using PR Ser345 phospho-mutants and chromatin immunoprecipitation (ChIP). We next examined the physical interaction between PR and cyclin D1 in order to further characterize PR regulation of HSPB-8 (a cyclin D1-induced gene). We observed constitutive (in the absence or presence of ligand) co-immunoprecipitation of cyclin D1 with PR. Ours is the first evidence that cyclin D modifies PR transcriptional activity. ChIP experiments confirmed the co-recruitment of cyclin D and PR at SP1 sites of the HSPB-8 promoter in breast cancer cells during G_2M. Our data suggests that PR and cyclin D1, both commonly co-upregulated in breast tumors, may functionally cooperate to drive transcription of previously uncharacterized PR genes important for tumor cell biology and provide a rationale for targeting PR as part of endocrine-based breast cancer therapies.

[P2.52]

Role of estrogen receptor alpha (Esr1) in Hepatocellular Carcinoma

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Hepatocellular carcinoma is a predominantly male disease. Animal models using potent carcinogens reflect this gender bias. Previous work showed that in mice neonatally treated with diethylnitrosamine (DEN), males develop 8-10 times more liver tumors than females; ovariectomy increased the number of tumors in females and treatment with estrogen reduced tumorigenesis in ovariectomized females and in males. We examined DEN-induced tumorigenesis in the ERα knockout (ERαKO), C57Bl/6J mouse. Neonatal (PND12-14) mice were injected with 20 mg/kg DEN and at 52 weeks of age their livers were examined. As expected, we found that wild type (WT) males developed more tumors than WT females, 73 ± 5.66 vs 10 ± 1.67 tumor nodules. Surprisingly, tumorigenesis remained low in ovariectomized females, either ERαKO (9.4 ± 2.99) or WT (14 ± 2.37). In a mixed strain of mice, B6;129, ovariectomy did increase tumorigenesis (8.3 ± 1.71 and 64 ± 25.42, intact and ovariectomized, respectively), indicating that the role of ovarian factors may be strain dependent. ERαKO males had significantly fewer tumor nodules (39 ± 4.59) than WT males ($p < 0.01$). Others had suggested that the male pattern of growth hormone (GH) secretion was responsible for the higher incidence of tumors in males. However, the reduced tumorigenesis in ERαKO males was not due to altered GH pattern, as demonstrated by the pattern of liver enzyme expression. Continuous treatment of males with estradiol (E2) starting at PND28 protected against tumorigenesis only in WT (2.5 ± 1.02 vs. 37 ± 5.23 tumors, WT vs ERαKO, $p < 0.01$). These studies show that ERα is required for the protective effects of exogenous E2 but they call into question the notion that estradiol is the ovarian factor responsible for lower tumorigenesis in females. Furthermore, the results suggest that ERα plays a role in promotion of liver tumors in males. (supported by NIH R21 ES014367).

Keywords: estrogen, liver, cancer

[P2.53]

Neuromodulatory effects of estrogen and SERMs in age related cognitive decline: A proteomic and neurobehavioral approach

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Nearly four decades of steroid hormone research have given ample data to understand the actions of estrogen (E2) in brain areas traditionally not involved in reproductive functions and attention is now largely focused on finding novel proteins associated with estrogen signaling. Our previous findings have revealed that long-term estrogen therapy modulates the synaptic plasticity, apoptotic proteins and affords neuroprotection to the hippocampal neurons through pCREB and MAPK (Mehra et al., 2007,2008,2010). However, a quest for other non-steroidal estrogenic compounds is necessary to avoid undesirable side-effects of hormone on certain other estrogen sensitive tissues. Selective estrogen receptor modulators (SERMs) may offer an alternate to reduce the risks associated with ERT. A clear understanding of the effects of SERMs in brain is still underway and requires comprehensive studies. To elucidate the role and mechanism of estrogen in brain areas related to memory and cognition, and possible use of SERMs as alternate therapy, studies were conducted in conditions of estrogen depletion (with ovariectomy or natural aging) and replenishment with estrogen or SERM (tamoxifen, raloxifene) therapy in female rats. Hormone depletion with surgical or natural estropause adversely affected neuronal cytoarchitecture, decreased synaptic activity and increased apoptosis in brain areas related to learning and memory (hippocampus, subiculum and cerebellum). Proteomic analyses indicated downregulation of estrogen receptor subtypes, synaptic, apoptotic and transcription factors/proteins. These detrimental changes following ovariectomy or aging were reversible with ERT which was substantiated by functional improvement of learning and recall tasks. Proteomic profile comparison of ovx and E2 primed animals revealed identification of some new protein densities (which may further be characterized with mass spectrometric analysis). Both the SERMs used in this study improved synaptic activity, afforded neuroprotection and bettered spatial reference memory. These studies suggest that estrogen or SERM therapy may favorably avert estrogen deficit effects on the neurodegenerative aging process.

Keywords: Estrogen, SERM, Neuroprotection

[P2.54]

The impact of steroid therapy on osteoporosis in patients with sarcoidosis

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Introduction

Risk factors for development of osteoporosis are numerous, and among them is prolonged use of corticosteroids (6 months or more). The patients have enhanced risk of development secondary osteoporosis.

The aim of this study is to enlighten the impact of steroid therapy on developing osteoporosis in patients with different clinical course of sarcoidosis.

Sarcoidosis is granulomatous multisystem disease of unknown etiology. Granuloma formations frequently require steroid therapy. Depending on the course of disease patients are treated with high doses of steroid therapy (30-20mg/ daily) or morbostatic doses of 10-5mg /daily or alternatively.

Methods

Due to the higher risk for osteoporosis 63 female , biopsy positive sarcoidosis patients were analysed. Mean age 48,43±10,91 (19years-72years). 14 pts were menopausal women, mean duration of menopause 8 years. Patients were theated with high doses of prednisone 6 months up to 1 year, due to an acute form of sarcoidosis 43pts, while 20pts were treated for more than 2 years due to the chronic form of sarcoidosis with low doses of prednisone.

Osteoporosis was measured using bone densitometry (DXA).

Results

DXA scores of the analysed patients are presented at the table.

DXA scores in sarcoidosis patients treated with steroids

DXA	Doses of prednisone	Mean DXA score	STD
T Score	High doses (30-20mg/ daily)	- 1.33	1.24
	Low doses (10-5mg /daily)	- 1.51	0.87
Z Sscore	High doses (30-20mg/ daily)	- 0.54	0.83
	Low doses (10-5mg /daily)	- 0.57	1.27

Independent samples T test was used to analyse statistical significance between patients on high and low doses of prednisone comparing to their DXA scores.

No statistical signicance was found beetween the group of female sarcoidosis patiens treated with high and low doses of steroid therapy.

(p = 0.09)

Conclusion

Steroid therapy does not obviosly mean serious side effects.

Keywords: steroid therapy, sarcoidosis, osteoporosis, relationship

[P2.55]

Development of novel structural steroid analogues for the treatment of duchenne muscular dystrophy

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Glucocorticoids are the only effective treatment for the mitigation of disease progression in DMD but their acceptance and long term use is limited due to toxicity. As a result, many patients are unable to benefit from these drugs over the long term. Our hypothesis is that the efficacy of glucocorticoids in DMD is via NF-κB inhibition and modulation of cell membrane physicochemical properties, whereas side effects are due to receptor-ligand interactions (GRE-mediated transcriptional activity). We have developed a series of delta 9,11 steroids that retain myogenic NF-κB inhibition and membrane stability activities while showing no GR-GRE activity. Transcriptome studies showed that delta-9,11 drugs retained little or no transcriptional changes similar to those induced by prednisone, and that they did not exhibit the well-characterized negative feedback loop of down-regulation of the glucocorticoid receptor. Delta-9,11 analogues also showed inhibition of TNFα-induced NF-κB signalling and showed superior activity in plasma membrane stability assays compared to prednisone. To test for *in vivo* efficacy, we treated dystrophin-deficient *mdx* mice with both acute and chronic regimens. For the sub-acute dosing experiment, daily oral administration (3 wks) of prednisone and delta-9,11 analogues in a mouse model of muscular dystrophy (*mdx*) showed that both drugs reduced muscle inflammation (cathepsin B imaging). This indicates that delta 9,11 compounds have anti-inflammatory activities that are similar to prednisone. To test for side effect profiles and efficacy, a four month chronic study was done with VBP compounds compared to prednisone. This study showed prednisone to induce significant loss in body weight and spleen size, whereas delta-9,11 analogues did not. We have performed ADME, PK, safety studies on our lead candidate, VBP15, with favourable NME properties. Taken together, these data suggests VBP compounds maintain the efficacy of traditional glucocorticoids without the toxicity thus potentially providing a better alternative to current treatment.

Keywords: therapeutic, drug development, steroidal analogues, glucocorticoids

[P2.56]

Steroid therapy as predictor of metabolic syndrome in patients with sarcoidosis.

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Introduction

Sarcoidosis is granulomatous multisystem disease of unknown etiology. Granuloma formations frequently require steroid therapy. The aim of this study is to analyse the influence of steroid therapy on metabolic derangements i.e metabolic syndrome.

Methods

We analyzed the impact of duration and doses of steroid therapy on parameters of metabolic syndrome : 33 patients with biopsy positive sarcoidosis.. The mean age of patients population was 48,3 ± 13,05 years.Prevalence of the metabolic syndrome as defined by ATP III (≥ 3 of the following abnormalities): waist circumference greater than 102 cm in men and 88 cm in women; serum triglycerides level of at least 150 mg/dL (1.69 mmol/L); high-density lipoprotein cholesterol level of less than 40 mg/dL (1.04 mmol/L) in men and 50 mg/dL (1.29 mmol/L) in women; blood pressure of at least 130/85 mm Hg; or serum glucose level of at least 110 mg/dL (6.1 mmol/L).

Statistical analyzes were done using Cramer`s V Correlation and MACNOVA. According to the therapy regimes patients were devided into 3 groups: without therapy 4 pts, patients treated with high doses prednisone (30-20mg) 18 pts, less than two years, and 11 pts treated with low doses of steroid therapy (5-10mg) -more than two years.

Results

Out of the analyzed group 10 patients fulfield at least 3 criteria for metabolic syndrome. Only 4 patients were without a single criterium for developing metabolic syndrome.The analyses revealed positive correlation between duration of therapy and waist circumference .

(Cramer's V=0.46;p=0.031). However there was not statististcal significance between the preasence of metabolic syndrome in patients treated with high doses of prednisone and low doses.

Conclusion

Not doses of prednisone but the therapy duration (long lasting steroid therapy) significantly correlates with waist circumference, glicemia, arterial blood preasure , triglycerides level and high-density lipoprotein cholesterol level.

Keywords: steroid therapy, metabolic syndrome, sarcoidosis, duration of therapy

[P2.57]

Breast cancer initiation, tumor induction and growth are promoted by 5alpha-dihydroprogesterone (5alphaP) and inhibited by 3alpha-dihydroprogesterone in the microenvironment.

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Our previous studies showed that human breast tissues convert progesterone (P) to 5α-dihydroprogesterone (5αP) and 3α-dihydroprogesterone (3αHP) and that tumorous tissues produce more 5αP and less 3αHP than normal tissues, due to differences in expression of P metabolizing enzymes. Numerous in vitro studies on various breast cell lines have shown that 5αP stimulates, whereas 3αHP suppresses, proliferation and detachment of human breast cells, due to opposing actions on mitosis, apoptosis, actin polymerization, adhesion plaque formation, and Bcl-2, Bax and p21 expression. The opposing actions of 5αP and 3αHP were observed on all human breast cell lines studied, regardless of estrogen and P receptor levels, and whether cells are tumorigenic or 'normal'. The objective of the current studies was to determine if 5αP and 3αHP affect human breast cell tumor induction and growth in xenografts and to measure the levels of these hormones in matched serum and tumor tissues. Breast cancer (MDA-MB-231) cells, were surgically implanted into the mammary fat pads of 6 week old SCID mice which received two sc injections of vehicle without (control), or with either 5αP, 3αHP, or 5αP+3αHP. Three experiments were conducted with essentially similar results. In comparison with the controls, tumors developed significantly earlier, in more mice and more rapidly (19.8-fold in volume) in the 5αP group, significantly later, in fewer mice and more slowly (0.54-fold in volume) in the 3αHP group, and the stimulatory effects of 5αP were significantly abrogated by 3αHP in the 5αP+3αHP group. Measurements of 5αP and 3αHP (by RIA and mass spectrometry) showed that in tumors, 5αP levels were about 4-fold greater, and 3αHP levels were less than half those in serum. In all tumors, regardless of treatment, the levels of 5αP were, on average, 10-fold higher than those of 3αHP. The concentrations of progesterone did not vary significantly. The findings provide the first in vivo evidence that the P metabolites, 5αP and 3αHP, are potent endogenous mammary cancer regulating hormones and that elevated 5αP:3αHP ratios in the microenvironment of the breast promote neoplasia.

Keywords: breast cancer, progesterone metabolites, microenvironment, pro-cancer & anti-cancer steroids

[P2.58]

The detection of adrenal hypofunction in patients with diabetes mellitus Type 1

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Detailed information on adrenal function in autoimmune Type 1 diabetes with onset in adults is still lacking. This work aimed at gathering own data on adrenal response to low-dose Synacthen test, CRH test and about periferal metabolism of cortisol.

Seventy diabetics were investigated; age 44±10 yr(mean ±SD), age at diagnosis 28.5±10 yr, disease duration 15±8yr, BMI 24.5±2.7kg/cm2, HbA1c7.2±1.2%.

The study was approved by the Ethical Committee.

Adrenal reserve was tested by low-dose Synacthen test, pituitary function was tested by CRH test and periferal metabolism of cortisol was evaluated by suppresion of cortisol endogenous production by dexametasone administered orally and followed by cortisone acetate (25mg) administration.

We evaluated serum ACTH, serum cortisol, salivary cortisol aldosterone, DHEA, cortisone during these tests, cortisol binding globulin, adrenal autoantibodies, thyroid function and metabolic paramerts of diabetics.

We have found a subnormal response in 25% of patients (<500 nmol/L) of the serum cortisol during low-dose Synacthen test, accompanied by significantly decreased stimulated values of aldosterone and salivary cortisol. Basal serum cortisol, aldosterone, were significantly reduced, while ACTH, cortisol binding globulin and salivary cortisol did not differ. The CRH test displayed the low response in serum cortisol and ACTH as well in group of these patients.

As compared with group of patients with sufficient response to Synacthen, the course of cortisol after cortisone acetate administration was delayed and significantly different from cortisol response in diabetics with hypocorticalism.

The results indicate that the disorder of adrenocortical function occurs in all adrenocortical zones, on pituitary level and periferal adrenal metabolism could be change as well. These result may contribute to better understanding latent adrenal insuficiency adaptation in diabetics type 1.

The study was supported by grant No.NT11 277 of the IGA MZCR.

Keywords: diabetes mellitus, adrenal function, cortisone, pituitary

[P2.59]

New analogues of steroid estrogens with cardioprotective properties

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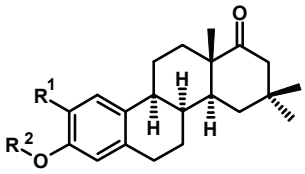
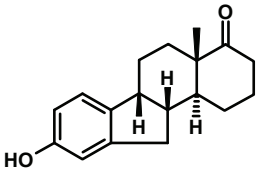
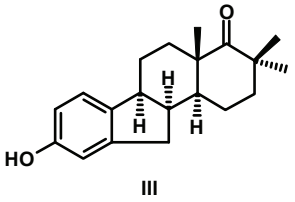
Earlier we have synthesized analogues of steroid estrogens with cardioprotective action and without uterotropic and hypertriglyceridemic activities. Such properties have for example analogues **Ia** [1] and **II** [2]. Being in the progress for the obtaining of steroid analogues with improved biological properties we prepared new steroids **Ib,c** and **III**.



I a) R^1**= H,** R^2**=** CH_3
b) R^1**= F,** R^2**=** CH_3
c) R^1 **= F,** R^2**= H**



II



III

These compounds have been chosen owing to the expected lowered carcinogenicity due to the presence of fluorine at position 2 [3], and compound **III** was chosen because its synthesis is easier in comparison with 9 beta analogues. All model compounds have been synthesized according to Torgov-Ananchenko scheme, and their structures have been confirmed by NMR spectroscopy methods DQF-COSY, HSQC, COLOC and NOESY.

We have confirmed experimentally that steroids **Ib,c** and **III** have cardioprotective action and they have not uterotropic activity.

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Keywords: cardioprotectors, total steroid synthesis, hormonal activity, carcinogenicity

[P2.60]

Estrogen therapy improves synaptic plasticity and postural balance of ovariectomized female rats

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Age related deterioration in postural balance is associated with an increased propensity for falling and peripheral bone mass measurements have indicated that more than 80% of postmenopausal women with fractures do not have osteoporosis, while more than 90% of all hip fractures result from a simple fall (De Laet CE et al., 1997). Beneficial effects of estrogen (E2) on cerebellar structure and functions have not been widely studied despite the available claims of improved postural balance in postmenopausal women receiving estrogen therapy. In continuation of our efforts to elucidate the neuromodulatory mechanisms of action of estrogen in cerebellum, studies were conducted on estrogen deficient (with ovariectomy or natural aging) and estrogen replenished female rats. Ovariectomy ensued in widespread structural changes in cerebellum including decrease in width of granule and molecular cell layers, distortion of Purkinje cell layer and significant reduction in neuronal size. Ovariectomy also led to decreased synaptic activity and a shift towards apoptotic mechanisms as observed with altered synaptophysin, Bcl2 and Bax expression. Chronic estrogen therapy to E2 deficient rats led to the reversal of ovariectomy induced anatomico-chemical changes and restored synaptic activity with a healthy neuronal status. These beneficial estrogenic actions are mediated by interaction of hormone with its receptors and also via activation of MAPK pathway and phosphorylation of CREB. Proteomic analysis of various molecules involved in this process further confirmed these findings. Rotarod performance also confirmed improved postural balance and motor coordination following estrogen therapy. These findings suggest that estrogen has a potential of improving neuronal health in cerebellum and thus may support the view that increased risk of fall fractures postmenopause could also be related to cerebellar involvement, besides osteoporosis.

Keywords: Estrogen, cerebellum, postural balance

[P2.61]

Metabolite signatures of apoptotic human cancer cell lines

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Steroid-related breast/prostate cancers are the second most common cause of death in western countries. Currently, agents that promote apoptosis in tumours are used as powerful tools for cancer therapies. The present study aimed to find metabolic biomarkers of apoptotic processes in cancer cells. For that purpose steroids, bile acids, amino acids, acylcarnitines, phospholipids, hexoses, and spingomyelines were measured by high-throughput LC-MS/MS-based methods in breast or prostate derived cancer cell lines MCF7 and PC3, respectively. We further employed hepatoma HepG2 cells as well as a control cell line HEK293. We induced apoptosis by staurosporine for 4, 12, and 24 h. Apoptosis was monitored by MTT viability and caspase 3/7 assays. Metabolite measurements were performed with cell culture supernatants as well as in homogenates of untreated, apoptotic, and necrotic cells (the latter taken as non-apoptotic control). Several metabolites indicative for apoptotic processes have been observed in our screen. In some cell lines metabolite changes were visible as early as 4 h after apoptosis induction and were preceding the detection of apoptosis by caspase 3/7 assay. We demonstrate that the cell specific signatures for apoptosis can be seen by metabolite analysis and some metabolites may even be valuable biomarker candidates for further cancer therapy developments.

[P2.62]

Assessing in vitro estrogenic properties of party pill drugs - BZP and TFMPP

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Party pill drugs are one of the new classes of drugs of abuse and gained popularity as 'rave drugs'. The main ingredient in most party pill drugs is benzyliperazine (BZP) which is one of the psychoactive piperazine derivaties. BZP has been shown to have amphetamine-like effects, approximately one-tenth the potency of dexamphetamine. Trifluoromethylphenylpiperazine (TFMPP) is also contained in party pill drugs as a major active component and reported to have lysergic acid diethylamide (LSD)-like effects. BZP's CNS stimulant effects are due to its effects on dopaminergic neuronal transmission while TFMPP acts on a number of serotonin (5-HT) receptors. Feelings of euphoria and energy obtained from party pill drugs are resulted in their recreational use.

BZP and TFMPP have proven central effects that mimic those of other illicit drugs; however, the endocrine disruptive effects are not fully understood. Since party pill drugs are particularly popular among young people, toxicological evaluation of their effects on reproductive function is becoming more important.

In this study, we identified estrogenic effects of BZP and TFMPP using in vitro bioassay. BZP and TFMPP stimulated cell proliferation in a dose-dependent manner, while co-treatment with tamoxifen and BZP or TFMPP showed a decrease of E2-induced cell proliferation. And also we found transcriptional activity of party pill drugs by estrogen sensitive reporter gene assay. These results indicate that the BZP and TFMPP have estrogenicity related to the ER-mediated pathway.

Keywords: Party pill drugs, E-screen, Reporter gene assay

[P2.63]

A new syndrome of arterial hypertension, from adrenal origin, associated to chimeric CYP11B1/CYP11B2 gene

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Familial Hyperaldosteronism type I (FH-I) is an autosomal dominant disorder causing hypertension, cardiac hypertrophy and cerebrovascular abnormalities. FH-I is caused by an unequal cross-over of the gene encoding steroid 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2), giving rise to a chimeric CYP11B1/CYP11B2 gene that displays aldosterone synthase activity. **Aim**: To study a family carrying a new subtype of FH-I in relation to its inheritance and clinical-biochemical parameters. **Subjects and Methods**: We studied 4 generations of a family from hispanic-american ethnicity, being the index case a teenager of 15 years-old (III-10), who debuted with hypertension, hyperaldosteronism and normokalemia. The rest of the family consists in 27 patients from generation (Gen) I, II, III and IV. In all of them the arterial blood pressure (ABP) was measured and then classified by hypertension (HT) stages (JNC-VII). We measured in all of them, the serum aldosterone and plasma renin activity, and we calculated the Aldo to renin ratio (ARR). Also, we identified the chimeric gene (CG) CYP11B1/CYP11B2 by XL-PCR and further analyses by sequencing. **Results**: In this family, we have studied 28 patients where 16/28 were adults, 9/28 were pediatrics (<16 years-old), 3/28 were infants (<2 years-old). In this family the hypertension, primary aldosteronism (PA) prevalence and CG CYP11B1/CYP11B2 were presented as follows: Gen-II HT: 100% (5/5), PA: 20% (1/5), CG: 100% (5/5); Gen-III HT: 64% (9/14), PA: 64% (9/14), CG: 100% (14/14); Gen-IV HT: 25% (2/8), PA: 38% (3/8), CG: 62% (5/8). Sequence analysis showed the unequal cross-over of CYP11B1/CYP11B2 comprises a region of 700 bp between at exon 2 and the intron 4 (CYP11B2). **Conclusion**: We describe a new familial syndrome of hypertension, of adrenal origin associated to chimeric gene CYP11B1/CYP11B2, showing differences in its genotipic and phenotypic presentation, commonly observed in FH-I. Supported by FONDECYT 1100356, FONDEF D08i1087 and NMII P07/088-F Chilean Grants.

Keywords: essential hypertension, aldosteronism, chimeric gene, CYP11b2

[P2.64]

The effects of melamine, cyanuric acid and atrazine on testosterone biosynthesis in MLTC-1 and fresh Balb/C Leydig cells.

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The unscrupulous use of nitrogen-rich melamine to surreptitiously boost "protein" content of milk caused morbidity and mortality from renal damage in pets and infants fed the adulterated food products. Melamine is structurally similar to atrazine, a herbicide with endocrine disruptive properties e.g. feminization of male frogs; perhaps mediated via blockage of androgen receptors or biosynthesis. We studied the effects of melamine, its deaminated product - cyanuric acid (CA) and atrazine on testosterone biosynthesis and gene expression of steroidogenic enzymes in mouse Leydig tumor cells (MLTC)-1 and Balb/C Leydig cells (BLC) in vitro.

Melamine and CA weakly inhibited 10IU/L hCG stimulated testosterone production in MLTC-1 (BLC untested) at 1mM, with CA displaying higher potency. In contrast atrazine, dose (50-200μM) dependently stimulated testosterone in MLTC-1, an effect further enhanced with hCG. But atrazine inhibited testosterone production in BLC, without or with hCG stimulation. Only StAR protein and 3β-HSD type I were down-regulated in hCG stimulated MLTC-1 with 1mM melamine or CA. 200uM atrazine up-regulated the former in hCG stimulated MLTC-1 and BLC, but 3β-HSD type I was down-regulated in BLC.

Melamine and CA only slightly inhibited testosterone biosynthesis in MLTC-1. Atrazine stimulated testosterone production in immortalised MLTC-1, without or with hCG, but inhibited it in fresh BLC. Atrazine's stimulatory effect in MLTC-1 without hCG suggests the herbicide affected path downstream of cAMP generation, perhaps prolonging the latter's life by inhibiting phosphodiesterase[1]. The in vitro inhibitory effects of atrazine on testosterone synthesis in BLC agree with responses of Leydig cells from in vivo treated rats[2], but disagree with study using only in vitro treated cells[1]. The latter discrepancy may be species related or due to different incubation periods, 1h (present study) vs 24h. In conclusion, only atrazine significantly affected steroidogenesis.

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Keywords: Testosterone biosynthesis, Melamine, Cyanuric acid, Atrazine

[P2.65]

Structure-activity relationship study of a series of 3-substituted derivatives of 16 beta-(m-carbamoylbenzyl)-estradiol leading to a new potent non estrogenic steroidal inhibitor of 17beta-hydroxysteroid dehydrogenase Type 1

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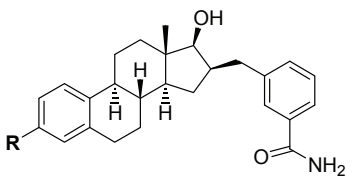
17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) transforms estrone (E1) into estradiol (E2), the most potent ligand for the estrogen receptor (ER). This enzyme also catalyses the transformation of dehydroepiandrosterone into 5-androstene-3β,17β-diol, a weak estrogen. Inhibitors of 17β-HSD1 are thus interesting potential therapeutic agents for the control of estrogen-dependent diseases such as breast cancer and endometriosis. The 16β-(*m*-carbamoylbenzyl)-E2 (compound **1**) has already been reported by our group as a strong inhibitor of 17β-HSD1 (IC_{50} = 27 nM). However, despite its good inhibitory activity in intact T-47D cells, this compound showed an undesirable estrogenic activity *in vitro* and *in vivo*. In order to remove its residual estrogenic activity, we synthesized a series of 3-substituted derivatives of 16β-(*m*-carbamoylbenzyl)-E2 (general structure **2**). More than 25 estrane derivatives were prepared with various substituents at position 3 (3-amino, 3-amido, 3-alkyl, 3-alkylhalogenated, 3-carboxy, 3-carbamide, 3-boronic acid, 3-fluoro or 3-alkylphenyl). These compounds were tested for their potential to inhibit the transformation of E1 (60 nM) into E2 by T-47D cells expressing 17β-HSD1. The strongest inhibitors of the series were also tested on estrogen-sensitive (ER^+) T-47D cells to assess their proliferative (estrogenic) activity. This structure-activity relationship study led to the conclusion that adding a bromoethyl at position 3 of a 16β-(*m*-carbamoylbenzyl)-17β-hydroxy-estrane scaffold generates a potent inhibitor of 17β-HSD1 (IC_{50} = 68 nM) and most importantly a compound without estrogenic activity. This inhibitor, named PBRM (**3**), was then tested on the T-47D xenograft tumor model in nude ovariectomized mice to evaluate its *in vivo* potential. As a result, a complete inhibition of tumor growth induced by E1 at the control group level (no E1) was observed after 32 days of treatment. This compound represents a promising preclinical lead candidate for future studies on human subjects.



(**1**) R = OH
(**2**) R = F, $B(OH)_2$, $CONHR_1$, NH_2, NHR, $(CH_2)_nX$, CH_2R_1
(**3**) CH_2CH_2Br

Keywords: 17 beta-Hydroxysteroid dehyrogenase type 1, Inhibitor, breast cancer, chemical synthesis

[P2.66]

Novel concept of prostate cancer therapy by differential regulation of androgen and glucocorticoid receptors.

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Androgen (AR) and glucocorticoid (GR) receptor signaling play opposing roles in prostate tumorigenesis: in prostate carcinoma (PC) AR acts as an oncogene and GR is a tumor suppressor. Recently, we found that non-steroidal phyto-chemical Compound A (CpdA) is AR/GR modulator acting as anti-inflammatory anti-androgen. We and others showed that CpdA inhibits AR and prevents GR transactivation, while enhancing GR transrepression. We also found that CpdA strongly inhibits proliferation and viability of PC cells in AR/GR-dependent fashion.

Expression and functions of GR and AR are controlled by proteasome. We show here that prolonged exposure of PC cells to proteasome inhibitor Bortezomib (BZ) caused AR degradation and GR accumulation. BZ enhanced CpdA ability to inhibit AR and to augment GR transrepression. We also found that CpdA+BZ differentially regulated GR/AR to cooperatively suppress PC cell growth and survival and to induce endoplasmic reticulum stress (ERS). Importantly, CpdA+BZ differentially regulated GR-responsive genes: they blocked activation of glucocorticoid-responsive pro-survival genes including SGK1, but activated BZ-induced ERS-related genes BIP and CHOP/GADD153. Using ChIP we showed that differential SGK1 and CHOP regulation was due to the effects of CpdA and CpdA+BZ on GR loading on their promoters.

To validate our proposed concept of PC treatment by dual AR/GR targeting we evaluated AR and GR expression in advanced PCs from 45 patients after androgen ablation or chemotherapy. We report that GR was well expressed and had nuclear localization in ~ 60% of treated tumors. 57% of PCs obtained from treated patients expressed both receptors; the other 26% expressed either GR or AR. This finding strongly supports the feasibility of proposed AR/GR targeted therapy using BZ combination with dual-target steroid receptor modulators for advanced PC. Work is supported by RO1CA118890 and SPORE in Prostate Cancer, P30 CA090386 (to IB), and ACS IL grant #160185 (to AY).

Keywords: prostate cancer, steroid receptor modulator, proteasome inhibitor, endoplasmic reticulum stress

[P2.67]
A novel selective inhibitor of 11β-HSD1 lowers blood glucose in obese mice

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Introduction

11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) inhibitors improve many of the metabolic disturbances associated with metabolic syndrome by reducing intracellular glucocorticoid levels. In this study our aim was to investigate the pharmacological efficacy of a new class of 11β-HSD1-selective inhibitor in the leptin deficient ob/ob mouse, a model that replicates the up-regulated adipose 11β-HSD1 expression that occurs in human obesity.

Methods

Compound UE2054 or vehicle was administered for 13 days to obese (ob/ob) mice via osmotic pump at a daily dose of 10mg/kg (n=8). Insulin sensitivity was assessed by a glucose tolerance test on day 10.

Results

UE2054 selectively inhibits 11β-HSD1 activity and is potent against both primate and rodent 11β-HSD1 enzymes in intact cells. When administered to ob/ob mice UE2054 inhibited 11β-HSD1 activity in liver and fat by 42 % (±14) and 36 % (±14) respectively. This was accompanied by a 49 % (±3) (p=0.001) reduction in fasted blood glucose and a 35 % (±8) (p=0.01) reduction in fed blood glucose levels. Plasma insulin levels were also found to reduced by 31% (±6) (p=0.04). No changes in body weight, cumulative food intake or circulating lipids were recorded. The activity of the liver enzyme phosphoenolpyruvate kinase (PEPCK), a key activity of the gluconeogenic pathway, was found to reduced by 36% (±9) (p=0.03) in UE2054 treated mice.

Discussion

UE2054 inhibits both liver and adipose 11β-HSD1 activity in obese (ob/ob) mice. This inhibition is associated with a reduction in both fed and fasted hyperglycaemia in the absence of weight loss, changes in food intake or circulating lipids. A 36% reduction the activity of the gluconeogenic enzyme PEPCK in UE2054 treated mice suggests the compound reduces hyperglycaemia in this model by reducing endogenous glucose production. This study provides further evidence of the therapeutic potential of selective 11β-HSD1 inhibitors in the treatment of metabolic syndrome.

Keywords: hyperglycaemia, obesity, ob/ob, inhibitor

[P2.68]

Dynamic motion investigation of 17β-HSD1 provides insights in its enzyme kinetics and ligand binding

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Bisubstrate enzymes, such as 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1), exist in solution as an ensemble of conformations. 17β-HSD1 catalyzes the last step of the biosynthesis of estradiol and, thus, it is a potentially attractive target for breast cancer treatment.

Based on a structural analysis of the available crystal structures, different enzyme conformations were assigned to the putative five steps of the random bi-bi kinetic cycle of 17β-HSD1. Further, in order to validate the designed catalytic cycle all-atom molecular dynamic simulations were performed using the four threedimensional structures best describing apoform, opened, occluded and closed state of 17β-HSD1 as starting structures. With three of them binary and ternary complexes were built with NADPH and NADPH-estrone, respectively, while two were investigated as apoform. Free energy calculations followed up with the aim to judge more accurately which of the MD complexes describes a specific kinetic step. The analysis of these eight long range MDs revealed an essential role played by backbone and side chain motions, especially of the βFαG'-loop, in cofactor and substrate binding. Thus, a selected-fit mechanism is suggested for 17β-HSD1, where ligand-binding induced concerted motions of the FG-segment and the C-terminal part guide the enzyme along its preferred catalytic pathway.[1]

The elucidation of the kinetic mechanism and of the role of the flexible βFαG'-loop laid the basis for the identification of a novel binding mode for the bis(hydroxyphenyl)arene derivatives, potent inhibitors of 17β-HSD1. When considering the opened and the occluded enzyme conformers this class seems to bind in a synergic manner to the nicotinamide moiety of NADPH via π-π stacking and H-bond formation, freezing the enzyme in a „half-switching" state and inducing a dynamic disruption of the enzyme's kinetics. The binding mode was further validated by multiple MD simulations and free binding energy calculations.

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Keywords: Molecular dynamic simulation, bi-bi kinetic mechanism, 17beta-HSD1, free binding energy

[P2.69]

Estrogen receptor-selectivity of phytoestrogens from traditional chinese medicine

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Estrogen replacement therapy (HRT) markedly reduces the risk of cardiovascular disease in postmenopausal women. However, the use of HRT as a cardioprotective strategy is greatly limited owing to carcinogenic effects of estrogens on the endometrium and breast in women. Recently, interest has focused on phytoestrogens, which are natural dietary plant compounds with estrogenic activity. Epidemiological studies also suggest that phytoestrogens are associated with a lower risk of breast and prostate cancer and cardiovascular disease. We found some phytoestrogens come from Traditional Chinese Medicine(TCM) with estrogen-like activity, which can increases ERE-luciferase activity in an ER subtype-dependent manner, but with different affinity to ER. We got seven compounds with estrogen-like activities from Psoralea corylifolia L. by chromatographic purification which are the two coumarins isopsoralen and psoralen, the four flavonoids isobavachalcone, bavachin, corylifol A and neobavaisoflavone, and the meroterpene phenol, bakuchiol. In reporter gene assay, the two coumarins acted as ER8-selective agonists while the other compounds activated both ER8 and ER8with more activation to ER8. From Salvia miltiorrhiza BUNGE we found Tan IIA can activate ER8 and ER8 transcription in transient transfected assay, with more activation to ER8. From *Eucommia ulmoides* Oliv., there were seven compounds including two lignans, one iridoids aucubin and four flavonoids(wogonin,baicalein,oroxylinA,α-O-β-D-glucopyranosyl-4,2′,4′ trihydroxydihydrochalcone), activated ER-dependent reporter gene on both ER subtypes. While, baicalein and wogonin had less potent on transactivation through ERα and exhibited ERβ selectivity. From the study we found phytoestrogens from TCM showed different effection to ER and with cell specificity. The reasous can be summarized as follows: 1) different ER ligands (e.g., SERMs) may bind ER8 and ER8selectively; 2) the nature of the ligand and of the ER subtype determine the conformation of the ER-ligand complex; 3) the structure of the ER-ligand complex determines its ability to interact with other molecules, et al.

Keywords: Estrogen receptor-selectivity, phytoestrogens, Traditional Chinese Medicine

[P2.70]

Aldosterone production and aldosterone synthase (CYP11B2) expression are blocked by steroidogenic factor 1 (SF-1)

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Introduction: CYP11B2 (aldosterone synthase) is responsible for the final step in the biosynthesis of aldosterone. This enzyme is expressed almost solely in the adrenal glomerulosa and in adrenal tumors that produce aldosterone. Because of the key role of aldosterone in the regulation of sodium balance and blood pressure regulation, we have studied the molecular mechanisms regulating transcription of this gene. We have recently demonstrated that the nuclear receptor steroidogenic factor 1 (SF-1) represses CYP11B2 expression. Herein, we attempt to better define the mechanism for SF-1 repression of CYP11B2. **Methods:** H295R-TR/SF-1 (TR/SF-1) adrenocortical cells expressing SF-1 transgene under the control of doxycyclin (Doxy) were used. Quantitative RTPCR (qPCR) was used to study expression of CYP11B2 and NURR1 in cells treated with or without Doxy. RNA was also used for microarray analysis to study the effect of elevated SF-1 on Ang II stimulated adrenal gene expression. **Results:** Doxy increased SF-1 protein and mRNA in a time-dependent induction manner that plateaued after 6 h. Microarray analysis indicated that elevated SF-1 inhibited Ang II stimulated CYP11B2 and NURR1 expression. To further study SF-1 effects on CYP11B2 and one of its key regulators (NURR1), cells with/without Doxy treatment were stimulated for 1 h (for NURR1) and 6 h (for CYP11B2) with Ang II, K^+ and forskolin. Preliminary experiments also indicate that SF-1 represses CYP11B2 promoter activity even in response to constitutive NURR1 and/or ATF2. **Discussion:** SF-1 inhibition of CYP11B2 expression occurs at multiple levels including blockade of NURR1 expression and CYP11B2 transcription. Defining the mechanisms regulating CYP11B2 will add to our understanding of adrenal zonation, aldosterone biosynthesis and diseases associated with aldosterone excess.

Keywords: Aldosterone synthase (CYP11B2), Aldosterone, Steroidogenic Factor 1 (SF-1), Gene expression

[P2.71]

Evidence for cytochrome b_5 complex formation in vivo

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Cytochrome b_5 (cyt b_5) is a ubiquitous hemoprotein also associated with microsomal cytochromes P450. Cyt b_5 attenuates the 17,20 lyase reaction catalysed by cytochrome P450 17α-hydroxylase/17,20 lyase (CYP17) through direct protein–protein interactions without affecting the 17α-hydroxylation catalysed by the same enzyme. The dual activity of CYP17 forms an important branch point in steroidogenesis and the regulation of the lyase reaction is critical in determining the steroidogenic output of the pathway. In this study multimeric complex formation by cyt b_5 and the possible regulatory role of these complexes were investigated.

Cyt b_5 was isolated from ovine liver and analysed by SDS- polyacrylamide gel electrophoresis, Western blotting, mass spectrometry and HPGPC. The cDNA encoding the N-terminal globular head domain or the C-terminal membrane anchoring tail of cyt b_5 were cloned into a mammalian expression vector together with the sequences coding for CFP or YFP. The constructs were transiently transfected in COS-1 cells and assayed for Fluorescent Resonance Energy Transfer (FRET).

Even after stringent detergent and denaturing conditions, Western blotting using anti b_5 antibodies indicated multimeric forms of the protein, the most prominent being a tetramer. Tetramer formation was abolished by the removal of the C-terminal membrane anchoring tail. FRET confirmed that cyt b_5 forms complexes in COS-1 cells and that complex formation is dependent on the C-terminal tail region of the protein. Site-directed mutagenesis of the tail domain significantly reduced FRET, without effecting ER targeting and membrane binding. The introduction of the same mutations into wild type cyt b_5 had no effect on the ability of cyt b_5 to augment the lyase activity in COS-1 cells. Furthermore, truncated cyt b_5 was also able to augment lyase activity.

This study has shown that cyt b_5 multimeric complexes *in vivo*, implicating complex formation as a possible regulatory mechanism in adrenal steroidogenesis.

Keywords: Cytochrome b5, Cytochrome P450 17alpha-hydroxylase/17,20-lyase

[P2.72]

Long-term estradiol treatment influences growth hormone-regulated liver transcriptome in male rats: Relevance to drug metabolism

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GH is a major regulator of growth and metabolism. Estrogens may modulate GH-regulated endocrine and metabolic functions in liver. To test this hypothesis, we used adult hypothyroid-castrated (TX-OX) male rats to minimize the influence of internal hormones on treatment. TX-OX rats were treated with E2 benzoate (50 μg/kg; sb; 5 days/week) for 20 days before GH replacement (0.3 mg/kg/day; sb; two daily injections) during seven days. Hypothyroidism reduced body weight gain, circulating IGF-I, and mRNA levels of IGF-I and male-specific CYP2C11 gene in liver, which were restored by GH. However, in the presence of E2, GH was not able to restore the changes induced by hypothyroidism. CYP2C12, a female differentiated gene, was induced by E2. To obtain comprehensive information on effects of E2 treatment on GH-regulated gene expression, we performed microarray analysis of liver transcriptome. In the absence of E2, we identified 218 genes that were up-regulated by more than 50% by GH treatment, while 139 were down-regulated to the same extent. In the presence of E2, 172 genes were up-regulated by GH treatment, while 243 were down-regulated. Administration of E2 to hypothyroid rats, provoked drastic changes (up-regulated genes=382; down-regulated genes=290) in liver transcriptome. A set of 84 genes were regulated in common by GH and E2. In the presence of E2, the number of Biological Processes with significant representation in our set of genes that were up-regulated by GH treatment was drastically reduced. This work highlights the influence of estradiol on male liver which has relevance for drug metabolism and several diseases. [This research has been supported by grants from the MICIIN-SAF2006-07824, ACIISI-PI2007/033, and ULPGC-ACIISI-ULPAPD-08/01-4].

Keywords: estradiol, GH, liver, pharmacogenomic

[P2.73]

ANP impairs the nongenomic effect of aldosterone on Na^+/H^+ exchanger in proximal tubule

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Considering that ANP inhibits the proximal [1] and distal reabsorption [3] of sodium and fluid and aldosterone has nongenomic effect on the Na^+/H^+ exchanger and on cytosolic free calcium concentration ($[Ca^{2+}]i$) in isolated proximal S3 segment of rat [2], the objective of the present study was to examine, in this segment, the influence of ANP on these actions of aldosterone. The effects of aldosterone [10^{-12} and 10^{-6} M, with 2 min preincubation (pi)] and ANP (10^{-6} M, 2 min pi) on the intracellular pH recovery rate (pHirr) mediated by Na^+/H^+ exchanger, after the acid load by NH_4Cl, and on $[Ca^{2+}]i$ were investigated by the fluorescent probes BCECF-AM and FLUO-4-AM, respectively. The results, mentioned below, confirm the rapid biphasic effect of aldosterone on the Na^+/H^+ exchanger. ANP has no effect on the exchanger, however, prevents both the effects of aldosterone on it. Additionally, ANP decreases the $[Ca^{2+}]i$ and inhibits the stimulatory effect of both doses of aldosterone on this parameter. Since the Na^+/H^+ exchanger in proximal tubule plays an important role in maintaining fluid and electrolyte homeostasis, the aldosterone and ANP interaction, that we observed, may represent a rapid physiologically relevant regulation in conditions of volume depletion or expansion.

	pHirr (pH units/min)	**$[Ca^{2+}]$** (nM)
Control	0.195 ± 0.012	104 ± 3
Aldosterone 10^{-12} M	0.310 ± 0.026 [a]	156 ± 9 [a]
Aldosterone 10^{-6} M	0.096 ± 0.009 [a]	233 ± 9 [a]
ANP	0.218 ± 0.012	58 ± 2 [a]
ANP + Aldosterone 10^{-12} M	0.20 ± 0.027 [b]	80 ± 3 [a b e]
ANP + Aldosterone 10^{-6} M	0.211 ± 0.022 [c]	143 ± 7 [a c e]

Means ± SEM; number of tubules = 5-16. a vs control; b vs Aldo 10^{-12}M; c vs Aldo 10^{-6}M; e vs ANP 10^{-6}M.

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Keywords: Mineralocorticoid stimulatory action, volume regulation, NHE1, calcium

[P2.74]

Dehydroepiandrosterone's antiepileptic action in iron-induced animal model of posttraumatic epilepsy

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Introduction: Neuroactive steroids (estrogen, progesterone, and testosterone) are important in the physiology and pharmacology of epileptic disorders. There is an increased incidence of epilepsy in elderly. The level of Dehydroepiandrosterone (DHEA) declines with age. DHEA is an antioxidative agent, involved in neuronal excitability as it affects GABA/glutamate receptors. The purpose of the present study was to determine whether DHEA has an antiepileptic effect. In the present experiments the antiepileptic effect of DHEA was studied in the iron-induced animal model of posttraumatic epilepsy.

Method: To determine the antiepileptic action of DHEA, observations were made on epileptic seizure activity in iron-induced chronic epileptogenic foci in rat brain. Antiepileptic influence was ascertained on the basis of epileptiform electrical activity, biochemical parameters, gene expression and cognitive-behavioral parameters.

Result: DHEA (30 mg/kg/day) administered for 21 days to epileptic rats prevented the epileptiform electrophysiological activity. DHEA also prevented epileptiform activity-related behavioral alterations studied by Morris water maze and open field tests in epileptic animals. DHEA also augmented the expression of CREB in epileptic rats. Intracerebroventricularly administered DHEA (200 n mole/day for 5 days) also attenuated seizure activity. Alterations in epileptogenesis-related biochemical parameters: lipid peroxidation, protein oxidation, membrane fluidity, acetylcholinesterase and Na^+, K^+- ATPase activities were also countered by DHEA.

Discussion: Our study demonstrated that DHEA suppressed iron-induced experimental seizure activity, and countered epileptogenesis-related cognitive deficits. The results points to that DHEA can be an antiseizure compound clinically.

Keywords: Dehydroepiandrosterone, Iron-induced epilepsy, posttraumatic epilepsy, Morris water maze

[P2.76]

LC-MSMS method development for steroids analysis at Low Detection Limits

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Steroid analysis is critical for research into a number of common endocrine disorders and also applied as biomarker for numbers of diseases. Major challenges of steroids analysis include sensitivity, selectivity, and large sample volume. Using liquid chromatography and mass spectrometry to analyze steroids at low level has been becoming a new trend to overcome these challenges.

Three major steroid methods will be presented including: testosterone for low level detection; estrogens for pg/mL detection; steroids panel including 11 major steroids for general screening.

Testosterone, the major androgenic hormone in humans, is commonly measured for its excess or deficiency. The concentration of testosterone in women, children and men undergoing anti-androgen therapy are typically less than 500 pg/mL. A simple one step liquid-liquid extraction method was developed using 200µL serum with 10 pg/mL detection limit and less than 10% CV. Good correlation was observed between the results obtained from this method and other reference results.

Estrogens are a group of steroid compounds functioning as the primary female sex hormone. Accurate low pg/mL level detection is required for estrogen analysis often obtainable through multiple complex sample preparation including derivatization. We developed a two step liquid-liquid extraction without derivatization method, coupling with mobile phase modifier to improve the ionization, achieving less than 5pg/mL quantification limit; less than 10%CV.

Steroidogenesis is the process wherein desired forms of steroids are generated by transformation of other steroids. An LC-MSMS method for analysis of 11 major steroids products covering the wide concentration range from µg/mL to pg/mL using simple protein precipitation sample preparation was developed and verified. The cross interference from high concentration steroid analytes to low concentration analytes and isomer separation are evaluated and considered in the method.

Keywords: LC/MS/MS, Low level testosterone, Steroid panel,

[P2.78]

17Beta-Hydroxysteroid Dehydrogenase Type 1 expression is determinant in the estradiol-estrone ratio in breast cancer cells

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Estradiol (E2), the most potent estrogen, stimulates the development of breast cancer cells (BCCs), and the cellular E2/E1 ratio is considered important to the proliferation of these cancer cells. Reductive 17β-hydroxysteroid dehydrogenases (17β-HSDs) are involved in the last step of E2 synthesis. Several breast cancer cell lines were chosen to evaluate E2/E1 ratio, including ER positive cell lines (T47D, MCF-7 and ZR75-1), and ER negative cell line (BT-20), and choriocarcinoma cell line (JEG-3) as the controls. Four different concentrations of E1 and E2 was added in the cell culture to be used as the substrates, the E2/E1 ratio was always 9:1 after incubation for 24 hour in all the chosen cell lines with high expression of 17β-HSD1, while E2/E1 ratio was less than 1:5 in cells with low expression of 17β-HSD1 (e.g., MCF-7). In order to know whether the intracellular environment or 17β-HSD1 expression level was determinate for the high E2/E1 ratio, the conversion of E1 to E2 was tested in MCF-7 over expressing 17β-HSD1 (MCF-7-1), HEK 293 over expressing 17β-HSD1 (HEK 293-1) were used as control. E2/E1 ratio was modified to 9:1 in both HEK 293-1 and MCF-7-1 cells. E2/E1 ratio was decreased to 5:95 after treatment with specific siRNAs for 17β-HSD1 in ER positive cell line (T47D) and ER negative cell line (BT-20). It demonstrates that 17β-HSD1 expression level determines the high ratio of E2/E1. Further, some kinetic study was carried out in HEK 293-1 and breast cancer cell line (T47D). The Km value were respectively 0.25 uM and 0.12 uM for E1 to E2 conversion in HEK 293-1 and T47D cells, demonstrating a strong apparent affinity of 17β-HSD1 to the estrone substrate.

Keywords: 17β-hydroxysteroid dehydrogenases 1, estradiol/estrone ratio, breast cancer cell, intracellular kinetics