249 DNA adduct recognition by proteins: identification, binding specificity and molecular consequences for S23906−1/DNA adduct

G. Lenglet[1], S. Depauw[1], H. Drobecq[2], D. Mendy[1], M.H. David-Cordonnier[1]. [1]*INSERM U837, Centre de Recherche Jean-Pierre Aubert and IMPRT-IFR114, IRCL, Lille, France,* [2]*CNRS UMR 8161, Institut Pasteur, Lille, France*

Background: S23906−1 is an acronycine derivative presenting DNA alkylation properties and being original for its covalent bonding to the ***minor groove of the DNA*** leading to a ***local opening of the double helix***. Based on high antitumour activities, S23906−1 entered clinical trial in 2006–2007. However, the exact mechanism by which S23906−1 achieves its anti-tumour activity is not fully known. Interaction of proteins to DNA adduct is well known for cisplatin and ET-743 which are recognized by HMG-B1 and the nucleotide excision repair protein XPG, respectively, increasing cell death potency. Therefore, we wanted to identify the proteins that could interact with S23906/DNA adduct and understand the cellular consequences of such recognition.
Methods and Results: Using a proteomic approach we have identified HMG-B1 and GAPDH as proteins interacting with S23906-alkylated DNA. Direct binding of GAPDH, but not HMG-B1, to the S23906−1/DNA adduct was validated using EMSA. Such GAPDH interaction depends both on adduct and DNA sequence specificities. Indeed, GAPDH also recognizes Saframycin A (SafA) but not ET-743 adducts, both being DNA minor groove alkylating agents. GAPDH and S23906 are known to bind to both double- (ds-) and single-stranded (ss-) DNA. We showed that GAPDH interacts strongly with both S23906-alkylated ds- and ss-DNA.
Only little is known about the nuclear roles of GAPDH (DNA repair, transcription and replication) and its DNA binding potency in particular. To identify the possible consensus for GAPDH/DNA interaction we used a CASTing method to select the best recognition sequences from random oligonucleotides.
At the cellular level, it was already observed a nuclear translocation of the GAPDH followed by cell apoptosis, under several stresses (heat shock, H_2O_2, SafA treatment...). However, even if siRNA and cytotoxicity approaches showed a small effect of cellular GAPDH in S23906−1 cytotoxic effect, no nuclear translocation of GAPDH was observed after S23906 cell treatment by contrast with published results that used SafA.
Conclusion: GAPDH binds to S23906−1/DNA adduct in some specific way. The possible sequence-specificity of GAPDH to DNA will be discussed. GAPDH may be implicated in S23906−1 cytotoxicity through its role in DNA repair processes.

250 Small molecule inhibitors of cellular stress response pathways can modulate viability of malignant melanoma cells

S. Uldrijan[1], T. Vaclova[1], V. Oralova[1], J. Hammerova[1], M. Sedlackova[2], H. Hribkova[1], K. Cetkovska[1], I. Slaninova[1]. [1]*Masaryk University Faculty of Medicine, Department of Biology, Brno, Czech Republic,* [2]*Masaryk University Faculty of Medicine, Department of Histology and Embryology, Brno, Czech Republic*

Background: The advanced metastatic form of malignant melanoma is usually refractory to all available treatments and has very poor prognosis. The 5-year survival rate has been estimated at only 6%, with median survival time of 6 months.
New molecular targets suitable for the development of more efficient therapies could be found through better understanding of the biology of malignant melanoma cells.
Constitutive activation of the extracellular signal-regulated kinase (ERK) pathway by somatic mutations of *N-Ras* and *B-RAF* proto-oncogenes seems to be critically important for melanoma development and survival, and a number of small molecule inhibitors of this pathway have already entered clinical trials. In contrast to the ERK pathway, the role of other mitogen-activated protein kinase (MAPK) pathways in melanoma cell biology still remains unclear.
Material and Methods: We have tested, in viability and proliferation assays, the response of several melanoma cell lines to SB202190, a small molecule inhibitor of the p38α/β MAPK pathways. At the same time, the morphology of cells was studied using light and electron microscopy. In addition to that, the effects of combined treatments with p38 inhibitors and small molecule inhibitors of other cellular signaling pathways were also tested.
Results: On its own, the pharmacological inhibition of the p38 MAPK had little impact on the viability of malignant melanoma cells. However, we have observed a strong induction of autophagy in p38 inhibitor-treated cells. Autophagy is a coordinated dynamic process of cytoplasmic material degradation in lysosomes which can promote both cell survival and cell death. Importantly, a simultaneous inhibition of autophagy using chemical inhibitors 3-methyladenine or Bafilomycin A1 significantly decreased the viability of several SB202190-treated melanoma cell lines, suggesting that autophagy might be required for the survival of melanoma cells that are lacking active p38 MAPK. In addition to that, p38 inhibitor-treated cells became extremely sensitive to thapsigargin, a small molecule inhibitor of endoplasmic reticulum (ER) calcium pump and ER stress inducer. These results indicate that p38 MAPK signalling might be necessary for the proper response of melanoma cells to ER stress.
Conclusions: Taken together, our results suggest that p38 MAPK signaling can play an important role in the biology of melanoma cells and together with the ER stress response and autophagy pathways might be a suitable target for the development of novel therapeutic strategies for malignant melanoma.
This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic (Grant No. LC06077) and the Ministry of Health of the Czech Republic (Grant No. NS10236−3/2009).

251 Potent and efficient testosterone suppression by chronic administration of novel metastin analogues, TAK-448 and TAK-683, in male rats

H. Matsui[1], Y. Takatsu[2], A. Tanaka[2], T. Asami[2], N. Nishizawa[2], A. Kiba[2], S. Kumano[2], A. Suzuki[2], M. Kusaka[2], T. Ohtaki[2]. [1]*Millennium: The Takeda Oncology Company, Takeda Development Research, Cambridge, USA,* [2]*Takeda Pharmaceutical Company Ltd., Pharmaceutical Research Division, Tsukuba, Japan*

Background: Metastin/kisspeptin is the cognate ligand for a G-protein-coupled receptor, GPR54, and functions as a key regulator in the control of hypothalamic gonadotoropin-releasing hormone (GnRH) neurons. TAK-448 and TAK-683 are metastin analogues. We evaluated their *in vitro* receptor activities as well as the effect of chronic administration on plasma testosterone (T) levels, weights of genital organs, and pituitary and GnRH neuronal responsiveness using male rats.
Material and Methods: Agonistic or receptor-binding activities of TAK-448 and TAK-683 were evaluated using GPR54-expressing Chinese hamster ovary cells or their membrane fraction. For *in vivo* studies these compounds were chronically administered subcutaneously (sc) in male rats. Plasma T levels, and weights of the prostate, seminal vesicles (SV), and testes were evaluated. These effects were compared with those of leuprolide acetate (LA), a GnRH analogue, or bilateral orchiectomy (ORX). As mechanism of action studies, we assessed the pituitary responsiveness to LA by evaluating luteinizing hormone (LH) release, and the responsiveness of GnRH neurons to TAK-683 by evaluating c-fos immunoreactivity (ir), a marker of neuronal activity. We also determined GnRH peptide contents in the hypothalamus.
Results: TAK-448 and TAK-683 both were observed to be potent receptor agonists *in vitro*. Chronic administration of TAK-448 ($\geqslant$10 pmol/h) or TAK-683 ($\geqslant$30 pmol/h) reduced T levels to undetectable levels (<0.04 ng/mL) within 3−7 days, while LA (300 pmol/h) produced slower and less dramatic T reduction than metastin analogues. The weights of the prostate and SV were reduced by ~90% by metastin analogues or ORX, or by ~70% (prostate) and 80% (SV) by LA vs controls. The pituitary maintained LA responsiveness. Surprisingly, the GnRH neurons also maintained responsiveness via GPR54 since ~90% of GnRH neurons expressed c-fos-ir in response to TAK-683. However, hypothalamic GnRH contents were reduced after chronic TAK-683 administration.
Conclusions: TAK-448 and TAK-683 showed more efficient T suppression compared with LA in male rats, and TAK-448 possessed superior *in vivo* activity vs TAK-683. Chronic administration of metastin analogues appears to deplete GnRH from the hypothalamus, resulting in potent T suppression. Consequently, these compounds hold promise as therapeutic agents in hormone-related disorders, and their potential utilities in prostate cancer therapy will be discussed.

252 Withdrawn

253 Autophagy triggered by ursolic acid synergistically enhances 5-fluorouracil induced cell death in HCT15 (MSI p53 mutant) colorectal cancer cells

C. Pinto Ribeiro Xavier[1], C. Lima[2], M. Rohde[3], C. Pereira-Wilson[1]. [1]*University of Minho, CBMA Department of Biology, Braga, Portugal,* [2]*University of Minho, CITAB Department of Biology, Braga, Portugal,* [3]*Danish Cancer Society, Apoptosis Laboratory, Copenhagen, Denmark*

Colorectal carcinoma (CRC) is a common cause of cancer-related death and tumours with microsatellite instability (MSI) and p53 mutations have been shown to be resistant to chemotherapy with 5-fluorouracil (5-FU). Therefore, it is essential to find compounds that could contribute to treatment efficacy by increasing the sensitivity to 5-FU. HCT15, a MSI human CRC derived cell line that harbours a p53 mutation, was incubated with the triterpenoid ursolic acid (UA) at a concentration that induces approximately 50% cell death (measured by PI stainning) after 48 h. A synergistic enhancement of apoptosis was observed when co-incubating 5-FU with UA (measured by TUNEL assay). UA induction of apoptosis was totally abolished by the JNK inhibitor SP600125 (SP), but not by the caspase inhibitor zVAD-fmk. Apoptosis did not account for all the observed cell death induced by UA. Thus, we asked whether UA was also inducing autophagy. We observed that UA induced

accumulation of autophagossomes (using fluorescent dyes) as well as of LC3-II (assessed by western blot), which was also significantly inhibited by SP. These results suggest that UA induction of apoptosis and autophagy is JNK dependent. A decrease in mutated p53 and phospho mTOR, which are associated with an induction of autophagy, were also observed. In conclusion, UA showed anticancer activity by inducing apoptosis and autophagy, which was JNK-dependent in HCT15 cells. In addition, in these resistant cells, UA synergistically cooperate with 5-FU to induce cell death.

Acknowledgements: Cristina Xavier was supported by the Foundation for Science and Technology (FCT), Portugal, through the grant SFRH/BD/27524/2006 and the work was supported by the FCT research grant PTDC/AGR-AAM/70418/2006.

254 Down-regulation of P-gp and drug resistance to csiplatin and VP-16 in human lung cancer cell lines

Q. Wang[1], J. Wang[1], J. Zhang[1], L.O.N.G. Zhao[1], L. Zhang[2]. [1]*The Second affiliated Hospital, Respiratory Medicine, Dalian, China,* [2]*The Zhongshan affiliated Hospital of Dalian University, Respiratory Medicine, Dalian, China*

Background: The aim of this study is to investigate whether the expression of P-glycoprotein is correlated to chemotherapy resistance to cisplatin and VP-16 in four different subtypes of lung cancer cells.

Material and Methods: The expression of P-gp with or without pretreatment of verapamil in four different subtypes of lung cancer cell lines was analyzed with RT-PCR and immunofluorescence. Cell survival to cisplatin and VP-16 was determined by MTT assay.

Results: Our study shows that the expression of P-gp can be inhibited by verapamil in SPCA-1, NCI-H-460 and NCI-H-446 cell line, but not in SK-MES-1 cell line. With the pretreatment of verapamil, NCI-H-446 was more sensitiveto cisplatin (IC_{50}: 67.39±4.3 vs 50.69±2.25).; NCI-H-460, SPCA-1 and NCI-H-446 were more sensitive to VP-16 (IC_{50}: 67.39±4.3 vs 50.69±2.25, 62.37±2.88 vs 45.79±4.47 and 56.35±3.15 vs 43.61±1.64, respectively, $p < 0.05$) as well compared to the control group.

Conclusions: Verapamil can inhibit the expression of P-gp both at mRNA and protein level in NCI-H-460, SPCA-1 and NCI-H-446 lung cancer cell lines. The down-regulation of P-gp is associated with the intrinsic resistance to cisplatin in NCI-H-446 cell line and to VP-16 in NCI-H-460, SPCA-1 and NCI-H-446 cell lines. All these indicated that selecting appropriate mediator to inhibit the expression of P-gp maybe helpful for the reversion of drug resistance in some subtypes of lung cancer cell lines.

255 Withdrawn

256 A dichloromethane fraction of Strobilanthes crispus induces apoptosis and promotes the effect of tamoxifen in MCF-7 and MDA-MB-231 cells

N.S. Yaacob[1], N.N. Nik Mohd Kamal[1], C.S. Lai[2], V. Navaratnam[2], M.N. Norazmi[3]. [1]*University Sains Malaysia, Department of Chemical Pathology School of Medical Sciences, Kubang Kerian, Malaysia,* [2]*University Sains Malaysia, Centre for Drug Research, Pulau Pinang, Malaysia,* [3]*University Sains Malaysia, School of Health Sciences, Kubang Kerian, Malaysia*

Background: The leaves of *Strobilanthes crispus* (*S. crispus*) which is native to the Malay Archipelago, are used in folk medicine for their antidiabetic, diuretic, anticancer and blood pressure lowering properties. Crude extracts of this plant have been found to be cytotoxic to cancer cell lines. In this study, an active fraction of the dichloromethane extract of *S. crispus* (SC/D-F9) was isolated and analysed for its anticancer activities in MCF-7 and MDA-MB-231 breast cancer cell lines.

Materials and Methods: The dichloromethane extract of *S. crispus* was chromatographed on a silica gel and SC/D-F9 was isolated by gradient step elution using a combination of hexane, DCM and MeOH. Cytotoxicity was measured using the LDH assay and apoptosis was determined using Annexin V antibody and analysed by flow cytometry and fluorescence microscopy. Alterations in the mitochondrial membrane potential were also determined by flow cytometry. Modulation of specific gene expression was determined using PCR array and analysed by real-time PCR.

Results: SC/D-F9 is relatively more cytotoxic to the MCF-7 and MDA-MB-231 cells compared to tamoxifen, paclitaxel and doxorubicin, while is non-cytotoxic to the normal breast epithelial cell line, MCF-10A. Cell death occurs by apoptosis via depolarization of the mitochondrial membrane potential and transcriptional modulation of specific signaling molecules. In addition, SC/D-F9 promotes the apoptotic effects of low dose tamoxifen on both breast cancer cell lines.

Conclusion: These findings suggest the potential of SC/D-F9 as a cancer therapeutic agent.

257 Effects of Drug-X on cisplatin-resistant and cisplatin-sensitive ovarian cancer cells

J. Nordquist[1], S.C. Chauhan[1], D. Maher[1], M. Ebeling[1], M. Bell[2], M. Jaggi[1]. [1]*Sanford Research/USD, Cancer Biology Research Center, Sioux Falls SD, USA,* [2]*Sanford School of Medicine, Department of OB/GYN, Sioux Falls SD, USA*

Background: With 204,000 new cases and 125,000 deaths annually worldwide, ovarian cancer is the most lethal gynecological malignancy. Using current treatment options, recurrence is very common. The median survival rate for relapsing women is two to three years with only 30% surviving five years from diagnosis. Resistance to existing treatments (such as cisplatin) attributes to this poor survival rate. Consequently, our laboratory has identified a potentially new drug for treating ovarian cancer, Drug-X (name of the drug can not be disclosed at this point due to proprietary reasons). For decades, Drug-X has been widely used for other clinical indications. Herein, we investigated the effects of Drug-X on cisplatin-sensitive and cisplatin-resistant ovarian cancer cells.

Material and Methods: Three ovarian cancer cell lines (SKOV-3 [cisplatin-resistant], A2780-CP [cisplatin-resistant], and A2780 [cisplatin-sensitive]) were cultured and treated with Drug-X. To determine colony forming effects, 1000 cells were plated overnight, treated for 14 days, stained, and colonies were counted. To test mitochondrial effects, cells were treated (10−20 μM) at three different time points (6, 12, and 18 hours), incubated in 100 nM tetramethylrhodamine (TMRE) for 20 minutes, and analyzed by flow cytometry. In addition, after 48-hour treatment (1−27 μM), cells were analyzed using these methods: (1) Cell proliferation data was gathered by counting cells with an automated cell counter. (2) Apoptotic cells were identified using Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) and propidium iodide (PI) staining assays. (3) For immuno-blotting, cell lysates were prepared using RIPA buffer, electrophoretically resolved on SDS-PAGE, transferred to PVDF membrane, and probed with various apoptosis-related antibodies. Experiments were performed in triplicate, and statistical significance was determined using a two-tailed student t-test with equal variance.

Results: In all three cell lines, Drug-X inhibited cell proliferation and reduced colonogenic potential in a dose-dependent manner. Further analysis using TUNEL and PI staining revealed a dose-dependent increase in percent of apoptotic cells. Immuno-blotting assays showed a dose-dependent decrease in full-length caspase-9 and caspase-3 (indicating an increase in activity), and a marked increase in cleaved Poly (ADP-ribose) polymerase (PARP). The TMRE assay also revealed a dose-and time-dependent decrease in mitochondrial membrane potential ($\Delta\Psi m$) which is an early sign of the intrinsic apoptotic pathway.

Conclusions: Drug-X effectively inhibits growth of ovarian cancer cells *via* induction of caspase-mediated apoptosis. Our data suggest that Drug-X induces an intrinsic apoptotic pathway by altering mitochondrial membrane potential, triggering caspase-9 activity, and subsequently increasing caspase-3 activity and PARP cleavage. Therefore, Drug-X may be a potential treatment modality for cisplatin-resistant and cisplatin-sensitive ovarian cancer.

258 Anticancer activity of a novel kaempferol glucoside, Tac, is mediated by a mechanism involving RSK inhibition

C. Tsimplouli[1], L. Mahaira[1], G. Rassidakis[2], K. Dimas[1]. [1]*Biomedical Research Founation Academy of Athens (BRFAA), Pharmacology-Pharmacotechnology, Athens, Greece,* [2]*National and Kapodistrian University of Athens, First Department of Pathology, Athens, Greece*

Background: The Ras/mitogen-activated protein kinase (MAPK) pathway regulates diverse cellular processes such as proliferation, survival, growth and motility. p90 ribosomal S6 kinase (RSK) constitutes a family of protein kinases downstream of the MAP kinase cascade and has been shown to regulate cancer progression by controlling cell proliferation. Recent studies on RSK's involvement in cancer pathways have shown that some flavonoids can act as specific inhibitors against members of the RSK family. In this regard, we have further investigated the mechanism of action of the semisynthetic kaempferol glusoside, named Tac, and whether this compound acts downstream to MAPK pathway via RSK inhibition. Finally, we have evaluated the antitumour activity of *Tac* in the human tumour xenograft/immunodeficient mouse model.

Materials and Methods: The antiproliferative effect of *Tac* was examined using the SRB assay. COMPARE algorithm was further employed for an initial evaluation of the mechanism of action. *Tac*-induced cell death perturbations on cell cycle were further examined on the HCT116 human colon cancer cell line using FACS analysis. Western blot was utilized for the detection of caspase activation, PARP cleavage and changes in the levels of MARCKS, p-MARCKS, ERK2, p-ERK1/2, RSK, p-RSK, total EF2, eEf2 and p-eEf2. The *in vivo* antitumour activity of *Tac* was also evaluated against HCT116 xenografts.

Results: COMPARE analysis revealed great similarities with DNA damaging agents. FACS analysis with PI stain indicated that *Tac* treatment resulted in