147P THE TOXICOLOGY OF TAMOXIFEN

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The antioestrogenic drug tamoxifen is used successfully as an adjuvant therapy in the treatment of breast cancer. This drug has been approved by the FDA for prophylactic use in healthy women at increased risk of this disease. However, the use of tamoxifen is not without risk. Tamoxifen is a liver carcinogen in rats and increases the risk of endometrial cancer in women. Tamoxifen induces liver DNA damage in rodents as detected by the ^{32}P-postlabelling assay, demonstrating that tamoxifen is a genotoxic carcinogen *in vivo*.

Toremifene, a structural analogue of tamoxifen, does not induce significant DNA damage or liver tumours in rats. In rats, neither drug results in uterine DNA damage detectable by ^{32}P-postlabelling. In women taking tamoxifen therapeutically, little or no DNA damage is detected in the liver or uterine tissues. Classical *in vitro* mutagenicity tests give negative results for tamoxifen. However, tamoxifen causes dose related increases in the mutation frequency of the *lacI* gene in liver of transgenic Big Blue rats. Uterine DNA shows no change in mutation frequencies. Toremifene is negative for both uterus and liver.

Phase 1 detoxication of tamoxifen produces N-desmethyltamoxifen, 4-hydroxytamoxifen and tamoxifen N-oxide as main metabolites. Tamoxifen itself is not genotoxic but can be metabolised by CYP to more reactive species. a-Hydroxytamoxifen, a minor phase 1 metabolite, is further activated to a sulphate-ester which reacts with DNA via a carbocation to form the major adducts in rat liver.

The therapeutic dose of tamoxifen is 20 mg daily ~ 0.3 mg/kg. To detect DNA damage and develop liver tumours, rats receive 30 mg/kg/day. Metabolism of tamoxifen to a-hydroxytamoxifen in rat liver microsomes is 3 fold higher than in human and subsequent bioactivation (sulphation) is ~5 faster in rats. Detoxication via glucuronidation of a-hydroxytamoxifen in human liver microsomes is ~100 fold faster than in rats. This gives a ~15000 fold safety factor for women.

Thus from our understanding of the mechanisms of toxicity and metabolism of tamoxifen, it is highly unlikely to be an hepatic carcinogen in humans. It is unclear whether the mechanism of uterine carcinogenicity is associated with genotoxicity. Even if tamoxifen undergoes uterine metabolic activation resulting in DNA damage, it is unlikely to be causally related to the mechanism of carcinogenesis.

Despite the risks, the prophylactic use of tamoxifen in women at risk of breast cancer provides considerable benefit in reducing mortality from this disease.

148P BIOCHEMICAL AND BIOPHYSICAL EVIDENCE OF G PROTEIN-COUPLED RECEPTOR DIMERIZATION

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A growing body of evidence suggests that G protein-coupled receptors (GPCRs) may form functionally relevant homo- and hetero-dimers. However, most biochemical approaches used to study such dimerization are based on co-immuno-precipitation techniques that require solubilization of these hydrophobic proteins that could lead to artefactual aggregation.

In an effort to directly assess the existence of GPCR dimers in whole cells, we used a newly developed assay known as Bioluminescence Resonance Energy Transfer (BRET). BRET is the non-radiative transfer of energy between a luminescent donor, e.g. luciferase (luc) and a fluorescent acceptor, e.g. the Green Fluorescent Protein (GFP). Upon di-merization of the donor and acceptor, the luminescence resulting from the catalytic degradation of coelanterazine by luc is transferred to the GFP that, in turn, emits fluorescence at its characteristic wavelength.

To apply this approach to the study of β-adrenergic receptor (βAR) dimerization, fusion βAR-luc and βAR-GFP constructs were co-expressed in HEK-293 cells and the occurrence of BRET assessed.

The detection of BRET under basal conditions unambiguously demonstrated that βARs form constitutive complexes that are minimally dimeric. The selectivity of these interactions was confirmed by showing that no BRET occurred between the βARs and other unrelated GPCRs.

Stimulation with the agonist isoproterenol led to an increase in BRET, indicating that receptor dimers play a role in signal transduction. This notion is further supported by the observation that a peptide that blocks dimerization also inhibits receptor function. Using the V2 vasopressin receptor (V2R) as another GPCR model, we found that the formation of dimers occurs very early following receptor biosynthesis suggesting that it could play a role in proper folding and maturation. Indeed, both immature (precursor) and mature forms of receptor were observed as dimers. Furthermore, mutant forms of the V2R that cannot reach the cell surface are also observed as dimeric complexes.

Taken together, our data demonstrate that GPCRs exist as dimers in whole cells, and suggest that dimerization could play important roles in both receptor ontogeny and function.

149P MOLECULAR INSIGHTS INTO $GABA_B$ RECEPTOR PHYSIOLOGY

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$GABA_B$ receptors were first recognized 18 years ago and it is 25 years since the $GABA_B$ receptor agonist baclofen was introduced into the treatment of spasticity. Despite these early achievements, the molecular structure of $GABA_B$ receptors has remained elusive. Considerable therapeutic benefit is expected of $GABA_B$ receptor drugs in conditions that affect the central nervous system and mental health. However, the lack of a molecular understanding of the $GABA_B$ receptor system made it impossible to fully exploit its therapeutic potential.

It was not until 1997 that the development of the high-affinity antagonist [^{125}I]CGP 64213 allowed the isolation of $GABA_BR1\alpha$ using an expression cloning approach. Subsequently the $GABA_BR1\beta$ and $GABA_BR2$ cDNA were isolated. The cloned $GABA_B$ receptors showed many of the expected properties in terms of structure and pharmacology, however they only reluctantly reproduced the signalling properties of native receptors when expressed individually in heterologous cells.

The distribution of $GABA_BR1$ and $GABA_BR2$ transcripts in the brain, as studied by *in situ* hybridization, is largely overlapping. The strong overlap of the *in situ* hybridization patterns indicated that $GABA_BR1$ and $GABA_BR2$ are co-expressed in many neuronal populations and that a co-expression was possibly needed for robust functional activity. Indeed while neither $GABA_BR1\alpha/\text{-}\beta$ nor $GABA_BR2$ alone efficiently activated Kir3 channels their co-expression in HEK293 cells and *Xenopus* oocytes yielded robust GABA evoked currents.

This finding represented the first demonstration of heteromerization among G-protein coupled receptors.

The use of functional assay systems to study signalling and pharmacological properties of recombinant $GABA_B$ receptors will be discussed.

150P MODULATION OF FUNCTION BY HETERODIMERIZATION OF OPIOID RECEPTORS

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Protein-protein interactions govern a large number of biological processes. It is well established that a variety of cell surface receptors interact with each other to form dimers and that this is essential for their activation.

A number of G protein-coupled receptors including members of the rhodopsin, secretin and metabotropic glutamate receptor family have been recently shown to exist as dimers. In some cases dimerization has been found to alter ligand binding, signaling and receptor trafficking properties.

We have examined the dimerization of opioid receptors, which are members of the rhodopsin subfamily of the G-protein coupled receptor superfamily. Delta and kappa opioid receptors exist as homodimers, and agonist treatment differentially modulates the level of dimers. These two opioid receptor types are also able to hetero-dimerize with each other and hetero-dimerization leads to changes in agonist affinity, efficacy and potency.

Recently we have examined dimerization between delta and mu opioid receptors. When co-expressed in heterologous cells, these receptors interact with each other to form dimers; mu-delta heterodimerization leads the generation of binding sites with unique biochemical, pharmacological and signaling properties. Thus, dimerization of opioid receptors appears to be a universal phenomenon that provides a mechanism for cross-talk between members of this family.

These findings have important clinical ramifications and may provide new foundations for more effective therapies.

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151P RAMPs (RECEPTOR ACTIVITY MODIFYING PROTEINS) AS DETERMINANTS OF GPCR LIGAND SELECTIVITY

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Receptor Activity Modifying Proteins (RAMPs) are a family of three single transmembrane proteins that control the pharmacology of the G protein coupled receptors activated by the calcitonin family of peptides.

The Calcitonin Receptor Like Receptor (CRLR) is non functional when expressed in most recombinant systems due to a lack of cell surface expression. When co-expressed with RAMPs, CRLR is transported to the cell surface where its ligand speci-ficity and pharmacology is determined by the individual RAMP. RAMP1 with CRLR reconstitutes a CGRP receptor. When CRLR is expressed with RAMP2 or RAMP3, an adrenomedullin (ADM) receptor is formed.

Characterisation of the RAMP2/CRLR and RAMP3/CRLR receptors by either radioligand binding (^{125}I]ADM as radioligand), functional assays (cAMP measurement) or biochemical analysis (SDS-PAGE) revealed them to be indistinguishable. Chimeric RAMP proteins were created in which the transmembrane and cytosolic portions of RAMP2 were linked to the extracellular amino terminus of RAMP1 (RAMP1/2) and vice versa (RAMP2/1). RAMP1/2 and RAMP2/1 formed CGRP or ADM receptors respectively when co-expressed with CRLR, suggesting that the amino termini of RAMPs are key to their distinct biological activities.

RAMP1 and 3 can also alter the pharmacology of the calcitonin receptor to produce different amylin receptors. Co-expression of RAMP1 with the calcitonin receptor produces a receptor where both CGRP and amylin are potent agonists, whilst co-expression of RAMP3 produces an amylin receptor with a lower affinity for CGRP.

The discovery of RAMPs has demonstrated that heterodimerisation of a GPCR with a 1 TM protein can regulate the pharmacology of the receptor. The exact mechanism for this remains to be determined but it seems likely that RAMPs will either directly contribute to the ligand binding of the receptor and/or alter the receptor conformation. RAMPs provide a novel mechanism whereby a cell could change its sensitivity from one ligand to another.

152P EVIDENCE FOR LIGAND-INDUCED CHANGES IN THE DIMERISATION OF CLASS 1 GPCRs

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There is abundant evidence from studies of solubilised GPCRs for their coassociation. For example, studies using SDS PAGE, normally under non-reducing conditions, often show higher M_r bands corresponding to dimers and higher oligomers. It is often also possible to co-immunoprecipitate one receptor species using an antibody against another species.

In membranes or whole cells, coexpression studies of two appropriately 'disabled' receptors can result in a reconstitution of function, possibly by a process of 'domain swapping', although some of the studies have not excluded the alternative possibility of proteolytic cleavage of the two receptors and reconstitution of a functional monomer.

$GABA_B$ receptors are clearly heterodimeric, and heterodimers are also suggested in studies of both closely and more distantly related receptors of Class I (rhodopsin-like) GPCRs (e.g. δ and μ opioid receptors and SSTR5 somatostatin and D_2 dopamine receptors, respectively). Resonance energy transfer techniques have also shown the close proximity of GPCRs on a cell surface, with agonists promoting a closer interaction of the probes.

Obvious questions are whether some or all GPCRs exist as homo- or heterodimers (or higher oligomers), do they exist as both monomers and oligomers, is the oligomeric state of a receptor important for function and is that state changed by ligand binding?

The aim of this talk is to examine the evidence from primarily ligand binding studies of Class 1 GPCRs that receptor-receptor interactions can occur within membranes and that ligands may change the nature of the receptor-receptor interactions, either by favouring formation or disruption of these interactions or just by changing the nature of the interactions. Data from published studies by others on receptor heterodimers will be discussed, as well as some of our recent studies using what is, in principle, a simple method of detecting receptor-receptor interactions.