355P TREATMENT OF ANXIETY AND PANIC: FUTURE DIRECTIONS

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There is now ample evidence, both preclinically and clinically, that the $GABA_A$-benzodiazepine receptor complex and the serotonergic (5-HT) system, are crucial in the modulation of anxiety and fear.

Agonists of the 5-HT_{1A} receptor (e.g. 8-OH-DPAT, flesinoxan, buspirone) have anxiolytic activity in most animal models of anxiety (e.g. conflict procedures, elevated plus maze, light-dark box, stress-induced hyperthermia, distress vocalizations). Recently, 5-HT_{1A} receptor antagonists have become available (WAY 100,635, S-UH 301, DU 125530) which were initially qualified as "silent". We tested several 5 HT_{1A} receptor antagonists in various animal paradigms of anxiety, including ultrasonic distress calls in adult rats and the fear-potentiated startle test in rats.

Surprisingly, WAY 100,635 had an anxiogenic effect at lower doses, which faded away at higher doses, in the ultrasonic distress test (Groenink *et al*, 1996). Conversely, three 5-HT_{1A} receptor antagonists (WAY 100,635; (+)pindolol and DU125630) had anxiolytic activity in the fear-potentiated startle test (Joordens *et al* 1997). In a number of other anxiety paradigms (stress-induced hyperthermia, ultrasonic vocalizations in pups, Geller-Seifter conflict procedure), 5-HT_{1A} receptor antagonists were devoid of intrinsic activity. It can be hypothesized that, depending on the tone of the serotonergic system, 5-HT_{1A} receptor antagonists may exert anxiety-modulating effects.

It seems worthwhile to study such compounds in a variety of human anxiety disorders, because the intrinsic activity of the 5-HT systems may vary considerable under different conditions.

Genetic manipulations of the serotonergic system may be of further help to unravel the role of (parts of) this system in anxiety and fear processes.

Recently, the 5-HT_{1B} receptor knock-out (KO) mouse has been introduced (Sau-dou *et al* 1994). The 5-HT_{1B} receptor contributes an important feedback system on the release of 5-HT from serotonergic terminals (autoreceptor) and also as an heteroreceptor on other neurons (eg ACh, NA).

5 HT_{1B} KO (compared to u/wildtype (WT) mice) showed a deviant circadian rhythm (telemetrically measured) of heart rate, body temperature and activity. In the stress-induced hyperthermia paradigm, KO's had higher basal body temperatures than WTs, but were equally sensitive to the temperature-lowering effects of the 5-HT_{1A} receptor agonist flesinoxan. KO's were very sensitive to mild stressors and can be characterized as more "impulsive" than WT's. Apparently, the permanent loss of the "inhibitory" 5-HT receptor has led to permanent and rather drastic changes in behaviour.

Whether the 5-HT_{1B} KO mouse can be used as an animal model of impulsiveness (OCD?) is a matter of further investigations.

It can be concluded however, that 5-HT_{1A} and 5-HT_{1B} receptors are of high interest in the area of anxiety, aggression, stress and depression.

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356P MOLECULAR MODELLING OF P-450 SUBSTRATE INTERACTIONS

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Development of three-dimensional models of human cytochromes P450 involved in drug metabolism, using sequence homology with a unique bacterial P450 template, is discussed.

With the inclusion of experimental information from site-directed mutagenesis and other data, it is found that the homology models generated are consistent with results on specific P450 substrate metabolism.

Key determinants of P450 substrate specificity appear to be associated with the spatial disposition of certain amino acid residues lining the respective P450 binding sites which are complementary to similar groupings on the substrate molecules. These key interactions orientate P450 substrates for metabolism at the experimentally observed positions.

357P SITE-DIRECTED MUTAGENESIS OF PUTATIVE ACTIVE-SITE RESIDUES OF CYP2D6

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Cytochrome P450 2D6 (CYP2D6) is one of the most important human drug metabolising enzymes, not only for its role in the metabolism of a wide range of clinically important drugs, but also because of its polymorphic nature which also varies between different ethnic groups.

The capability to predict substrates and/or inhibitors of CYP2D6 from candidate drugs at an early stage in drug discovery and development is an important goal of the pharmaceutical industry. As no crystal structure of a eukaryotic P450 is available to date, let alone one of a human drug-metabolising P450, the topology of the active sites of such enzymes is currently determined by protein homology modelling.

A number of homology models of the active site of CYP2D6 have been published (Koymans *et al.*, 1992; Ellis *et al.*, 1996; Modi *et al.*, 1996; Lewis *et al.*, 1997; de Groot *et al.*, 1997) from which candidate amino acid residues bordering the active site cavity and/or determining substrate/inhibitor specificity have been putatively identified. Natural allelic variants of CYP2D6, with mutations affecting catalytic activity, as determined from in *vivo* phenotyping/genotyping studies, can also help to identify candidate residues of the active site.

This presentation will focus on our current knowledge of the active site of CYP2D6 based on the functional analysis of a number of mutant forms of the enzyme, generated by site-directed mutagenesis and expressed heterologously in yeast, and nominated by allelic variants and homology models. Residues that influence ligand binding, substrate selectivity, chiral selectivity and protein folding/haem incorporation will be discussed.

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358P THE USE OF NMR METHODS TO STUDY SUBSTRATE BINDING TO CYTOCHROMES P450

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As yet, no structure is available for any mammalian cytochrome P450, but there are now crystal structures of six microbial P450s, and these have been widely used as the basis for building models of the human enzymes.

In carrying out this modelling, we are faced with a relatively low sequence similarity, coupled with the fact that the part of the structure in which we are most interested, the active site, is generally the least similar in the different enzymes. We have used NMR methods to provide experimental data on the location and orientation of the substrate in the active site of bacterial and human P450s. The methods are based on the paramagnetic relaxation effects of the haem iron, and allow us to make accurate measurements of distances between the iron and atoms of the bound substrate. They have been used, for example, to demonstrate substantial movements of the substrate during catalysis by *Bacillus* P450 BM3 (Modi *et al.* 1996a).

In studying human P450s, the experimental information from NMR can be combined with homology modelling; the constraints on the substrate position relative to the haem in turn introduce valuable constraints on the positions of amino-acid side-chains in the active site. The method has been applied to a number of complexes of P450 2D6 and 3A4 (e.g. Modi *et al.* 1996b), and the resulting models have been used to design mutants with altered specificity. We have also used the NMR approach to show that the interaction of NADPHcytochrome P450 reductase with P450 2D6 can have allosteric effects on substrate binding (Modi *et al.* 1997).

The prospects of using these approaches to develop models of human P450 which will allow one to predict the metabolism of new compounds will be discussed.

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359P STRUCTURAL FEATURES OF P450 AND ITS ANTIGENIC DETERMINANTS REVEALED BY DESIGNER ANTIBODY STUDIES

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We are investigating the role of surface regions of P450 in enzyme function using antibodies that bind to pre-determined sites.

A series of antibodies has been produced by immunising rabbits with short synthetic peptides that mimic target regions of P450. The selection of regions suitable for antibody targeting is based upon considerations of primary structure, to identify surface regions, secondary structure, to reveal loop and turn regions, and tertiary structure, to locate likely functional regions. The specificity and utility of such antibodies is demonstrated by the ability to produce selectively binding antibodies to all of the major xenobiotic metabolising P450 enzymes in families 1, 2, 3 and 4 in human and rat.

Amongst the antibodies produced, some have been shown to inhibit enzyme activity. This property appears to be due to the position of the antibody binding site on the surface of P450. Initially, a pro-inhibitory antibody was produced by targeting region 290-296 of rat CYP 1 A2. The equivalent region in rat CYP1A1 (294-301) was identified through sequence alignment and antibodies targeted against this region were also inhibitory. Although the pro-inhibitory effect of these antibodies was specific to each form, in both cases, the maximum inhibition achieved was 65% and suggested that the antibodies may not have been targeted optimally.

This was investigated further by producing a series of antibodies against human CYP1A2. Antibodies that completely inhibited enzyme activity were produced by targeting residues 291-302 of this P450 enzyme.

Other workers have also produced inhibitory anti-peptide antibodies against CYP1A1, CYP2D6 and CYP3A4 that bind to a similar or adjacent region. However, one problem regularly encountered is the variability of the inhibitory activity of the antibodies. This may be related to the difference between the antigenicity of the relatively conformationally constrained protein target compared with the flexible peptide immunogen.

Our preliminary data suggest that constraining the flexibility of the peptide immunogen through cyclisation results in antibodies with improved protein antigenicity. We have investigated the relationship between the structure of P450 and the function of the various regions by locating the epitopes of the antibodies on a model of the 3-dimensional structure of eukaryotic P450.

These studies have defined an area on the surface of P450 where antibody binding interferes with enzyme activity. The function of this region is not yet known, but may involve the transfer of electrons from P450 reductase to the active site.

360P STRUCTURE AND QSARs OF SELECTED GLUTATHIONE S-TRANSFERASES

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Glutathione S-transferases (GSTs) constitute an important class of biotransformation enzyme systems catalysing metabolic reactions of structurally greatly differing endogenous and xenobiotic chemicals. They are catalyzing the conjugation of the endogenous tripeptide glutathione (GSH) to electrophilic species.

The primary function of these enzymes is generally considered to be detoxification of xenobiotic compounds. GST- mediated toxication reactions of xeno-biotics, however, are also well known. Moreover, GSTs are known to play a role in other important phenomena, such as multi-drug resistance and interindividual susceptibility to certain diseases and chemically induced toxicities. In recent years a number of cytosolic GSTs were crystallized and thus a great body of information has been obtained on the structure of the respective proteins as well as on the structure of their active sites. Combined with data from site specific mutation and expression experiments many new insights have been obtained on the molecular mechanism of actions of these GSTs as well as on their substrate selectivities.

We have used molecular modelling techniques to derive a protein model for the rat class μ GST-4-4, starting from the crystal structure of class μ GST 3-3. GST 4-4 distinguishes itself from GST 3-3 as GST4-4 is much more efficient and stereoselective in the addition of GSH to arene and alkene oxides and α,β-unsaturated ketones compared to GST 3-3, which is better at catalyzing nucleophilic substitution reactions.

Moreover, using molecular modelling techniques we have derived a substrate model for class μ GST 4-4. Information on region and stereoselective product formation of 20 substrates was used to construct a substrate model containing three interaction sites responsible for Lewis acid, Lewis base interactions as well as a region responsible for aromatic interactions. The predictive value of the substrate model has been evaluated by rationalizing the conjugation to GSH of 1 1 substrates of GST 4-4, which were not used to construct the model. The data acquired from the substrate model were incorporated in the protein model of GST 4-4 by docking the substrate model into the derived protein model. The amino acids corresponding to the protein interaction sites in the substrate model have been identified by the derived protein model.

Apart from the use of the three-dimensional structure of GSTs, chemical modification and site-specific mutagenesis techniques and substrate models, also the determination of structure-activity relationships can be used to help explaining the substrate selectivity and the catalytic mechanisms GSTs. We therefore focused attention as well on three different classes of rat GST isoenzymes (i.e. class α, μ and π GSTs) and their structure-activity relationships with a series of 2-substituted 1chloro-4-nitrobenzene substrate and product derivatives. Remarkable differences were found between the enzyme kinetic parameters (Km, Ki, kcat and kcat/Km) of the various GST isoenzymes and between the different substrates. The experimentally determined kinetic parameters were correlated with classical physico-chemical features (notably electronic, steric, lipophilic) of the R-substituents and with several computational molecular parameters, such as HOMOs, LUMOs and charge distributions (in substrates and intermediates).

In this contribution computer graphics and molecular modelling approaches and of active site- and mechanism- based (Q)SAR studies on selected GSTs will be discussed, as well as some of the possibilities and limitations in this regard.