## 287P PHARMACOLOGY *IN SILICO*: REDUCTION TO PRACTICE?

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Our understanding of pharmacology will be revolutionised by advances in genomics, computation structural biology and new processes for handling this tidal wave of information. When the giant robot sequencers have completed their task and all of the human genome has been catalogued, pharmacologists will be left with the task of making sense of the data so that it can be utilised for new approaches to desirable therapies.

Single nucleotide polymorphisms (SNPs) offer the promise of tailoring therapies to patient genotypes. SNP databases will become much larger than the simple human genome collection. The challenge will be to identify the role of SNPs in disease and account for their influence on clinical trials. Current estimates suggest that at the moment there are around 300 molecular targets available for drug design; over the next three years, the number of targets is expected to rise 10-fold.

Clearly, new technologies for structure determination and drug design are needed to keep up with the data that will come onstream. The development of gene expression microarrays, whereby it is possible to put the genome on a chip, herald a new form of large-scale, highly parallel experiments to monitor gene expression, even in a single cell.

Complex gene expression networks are now being identified by reverse engineering using signalling network theory. Where a time element is present in the analysis, we have, in principle, the capability of understanding the dynamics of pharmacogenomics on single cells, tissues and the body.

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## 288P ABC TRANSPORTERS, CHANNELS, CHANNEL REGULATORS AND DRUG RESISTANCE

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The ABC superfamily of transporters and channels includes over 100 examples from bacteria, protozoa, fungi, insects, plants and man. ABC transporters play a wide variety of physiological roles and many are of consideable medical relevance, including the cystic fibrosis gene product CFTR, the pfmdr protein which contributes to chloroquine resistance of the malaria parasite, and P-glycoprotein and MRP which confer resistance of tumours to chemotherapeutic drugs.

The substrates handled by ABC transporters can vary from small molecules such as ions, amino acids and sugars, to large molecules such as polysaccharides and proteins. It has recently become apparent that several ABC proteins, in addition to their intrinsic transporter/channel activity, have regulatory functions modulating the activity of heterologous ion channels.

The structure and function of one ABC transporter, the multidrug resistance P-glycoprotein, will be described. This protein is responsible for the resistance of many cells and tumours to chemotherapy. Recently we have obtained the first structural data, through electron microscopy, of P-gp. The mechanisms by which it acts as a transporter, and by which it modulates cell volume-regulated chloride channels will be discussed.

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## 289P REGULATION OF CFTR BY BICARBONATE IONS.

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Bicarbonate secretion is a feature of the epithelia affected by cystic fibrosis and CFTR plays a key role in this process. Recently, using pancreatic duct cells, we have discovered that extracellular $HCO_3^-$ causes a marked inhibition of whole cell CFTR currents (70% inhibition at 100 mM $HCO_3^-$). This inhibitory effect of $HCO_3^-$ is rapid, dose-dependent (Ki = 8.3 mM), and fully reversible.

Extracellular $HCO_3^-$ inhibits both the inward ($Cl^-$ efflux) and outward ($Cl^-$ influx) currents through CFTR. The reduced inward current indicates that external $HCO_3^-$ is causing ëtransí inhibition of $Cl^-$ efflux through the channel. Our data suggest that a single site is involved in $HCO_3^-$ inhibition and, since inhibition was only weakly voltage-dependent, that this site does not sense the voltage-drop across the channel. The inhibitory effect of $HCO_3^-$ was not replicated by aspartate, or by changes in pHI, pH0, and the $PCO_2$ of extracellular fluid.

Our hypothesis is that extracellular $HCO_3^-$ ions inhibit CFTR by a direct interaction with the channel. Moreover, because the inhibitory effect of $HCO_3^-$ was not markedly voltage-dependent, the anion probably does not exert its effect by blocking the CFTR pore (i.e. by reducing the size of the single channel currents). More likely, $HCO_3^-$ either inhibits CFTR gating or reduces the number of active channels in the plasma membrane.

Interestingly, there are a number of positively charged residues in the extracellular loops (EL) of human CFTR (e.g. K114, R104, R117 in EL1; K329 in EL 3; H897, K892, R899 in EL 4; and R1128 in EL6) that could form a $HCO_3^-$ binding site and we plan to test for their involvement using site-directed mutagenesis. We propose that the inhibitory effect of $HCO_3^-$ ions on CFTR is a negative feedback mechanism for the regulation of luminal surface pH in epithelia.

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## 290P CONTROL OF EPITHELIAL SODIUM CHANNELS IN MOUSE SALIVARY DUCTS

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Salivary ducts, like other electrically tight, absorptive epithelia, transport sodium across their apical membranes via amiloride-sensitive sodium channels. Using whole-cell patch-clamp techniques, we have previously shown that the amiloride-sensitive sodium channels in these cells are inhibited by increased intracellular concentrations of sodium and chloride.

Increased cytosolic sodium acts via an intracellular receptor for sodium, the G protein, Go, and the ubiquitin-protein ligase, Nedd4, which binds to and ubiquitinates the sodium channels. Regulation by intracellular chloride involves the G protein, Gi2, and is not mediated by Nedd4 or by ubiquitination of the sodium channels. Nedd4 is known to bind through its WW domains to the PY motifs in the C-terminals of sodium channel subunits and loss of these PY motifs leads to the autosomal dominant form of hypertension, Liddle's syndrome.

In the present study, we have examined the binding of Nedd4 to sodium channels in response to raised cytosolic sodium. We produced in E. coli GST-fusion proteins containing the C-terminals of mouse alpha-, beta- and gamma-ENaC subunits and GST-fusion proteins containing each of the 3 WW domains of mouse Nedd4.

We then used far-Western blotting to show that domains WW2 and WW3 of Nedd4, but not WW1, interacted with the C-terminals of alpha-, beta- and gamma-ENaC. Whole-cell patch-clamp studies showed, however, that all three WW domains of Nedd4 were involved in inhibiting sodium channel activity. Furthermore, we found that inclusion of a GST-fusion protein of the beta-ENaC C-terminal inhibited Nedd4 action on the channels, whereas a GST-fusion protein of the alpha-ENaC C-terminal was without effect.

These results indicate that Nedd4 binds to sodium channels through two of its three WW domains. The third domain, WW1, binds to an unknown protein essential for channel inhibition. Furthermore, Nedd4 binds to the C-terminal of the beta-, but not the alpha-subunit of ENaC.

## 291P ROLE OF EPITHELIAL SODIUM CHANNELS FOR LUNG FLUID CLEARANCE

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Amiloride-sensitive electrogenic sodium transport mediated by the epithelial sodium channel (ENaC) is the rate-limiting step for sodium reabsorption by epithelial cells that line the distal renal tubule, the distal colon, the ducts of salivary and sweat glands and lung epithelium. The aldosterone-dependent expression of ENaC in the principal cells of the renal collecting ducts is critical for sodium homeostasis and the control of blood volume and blood pressure.

Genetic evidence for direct involvement of ENaC in sodium balance comes from observations that mutations in the subunits of ENaC cause two human monogenic diseases. Liddle's syndrome is characterized by a gain of function mutations leading to early onset of salt retention, hypervolaemia, low plasma aldosterone and hypertension whereas PHA-1 is a salt wasting syndrome with hypovolaemia, high plama aldosterone and hypotension.

In contrast to the kidney and colon, ENaC mediated sodium transport in the lung is glucocorticoid-dependent. Independent of salt diet and plasma aldosterone, a basal constitutive level of sodium transport appears to be required for proper lung fluid clearance from birth to adulthood.

Two models are presently proposed for ion and water transport in airway surface liquid (ASL): one predicts an hypotonic and the other an isotonic ASL composition. A direct or indirect pathophysiological role of ENaC-mediated sodium tranport in lung fluid clearance has been proposed in respiratory distress syndrome in very premature newborn and in cystic fibrosis.

The role of ENaC in various forms of lung edema is now under intense investigation. The amiloride-sensitive ENaC is a heterotetrameric protein composed of three homologous subunits with an arrangement around the channel pore, consisting of two $\alpha$ subunits separated by $\beta$ and $\gamma$ subunits.

In this presentation, I will briefly describe the basic biophysical and biochemical properties of the channel, its tissue distribution, and its functions *in vitro* and *in vivo*. I will describe mouse models engineered by homologous recombination where the $\alpha$, the $\beta$ or the $\gamma$ subunits of ENaC genes have been inactivated and I will address the question on how these animal models may contribute to our understanding of the physiology and pathophysiology of blood pressure control and lung fluid clearance.

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## 292P CFTR GENE THERAPY

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We have undertaken a programme of work for cystic fibrosis gene therapy. This began with proof-of-principle studies of gene transfer in CF mice and continued into a phase I study in man.

In preparation for this we developed an assay to measure nasal bioelectric properties, which could distinguish CF from non-CF subjects. Subsequently we undertook a nasal trial in 15 CF subjects, which showed no safety problems. Restoration of approximately 20% of the basic chloride defect could be demonstrated with 2 subjects correcting into the normal range.

Since the eventual target is the lungs, we undertook a number of preparatory studies for such a trial. We selected the current optimal liposome (Lipid 67, Genzyme Corporation) and checked that nebulisation did not destroy the DNA-liposome complex. We showed that CF sputum was a potent barrier to gene transfer, and hence selected the fittest CF subjects to take part in the trial. For safety reasons we nebulised lipid 67 into the lungs of 15 normal volunteers, showing no toxicity problems. To allow the effects of gene transfer to be detected in the lungs we developed three new assays.

Firstly, *in vivo* lower airway potential difference measurements via a bronchoscope. Secondly, epithelial cells obtained by brushing, were loaded with a fluorescent indicator SPQ which responds to intracellular chloride levels. This distinguished between CF and non CF cells, and demonstrated correction of the chloride defect following *in vitro* gene transfer.

Finally, we developed an assay of adherence of Pseudomonas aeruginosa to epithelial cells using scanning electron microscopy. CF cells showed significantly higher levels of adherence compared to non-CF cells, and this could be reduced towards normal values by in vitro gene transfer.

We undertook the first controlled lung trial of CFTR gene transfer in 16 CF subjects. Both groups underwent bronchoscopy under general anaesthesia prior to, and following, a single application of treatment. Seven days later they also received a nasal instillation of the same treatment.

Each of the active group showed mild flu-like symptoms not requiring specific treatment. This group showed a significant change of approximately 25% in the chloride response towards normal values. Bacterial adherence reduced, as did neutrophils and interleukin-8 levels in sputum. Nasal bioelectric parameters showed a greater and more sustained change towards normal values than in the first trial.

These data provide the first proof-of-principle in the target organ in man for gene transfer. Clearly, transfection efficiency needs to be increased and we have identified non-infected mucus as an important biological barrier. Two clinically used drugs have been shown to produce marked improvements in gene transfer efficiency in an *ex vivo* model. We now need to assess these *in vivo*.