AuPS/ASB Meeting - Adelaide 2010

Symposium: Mechanisms of multidrug resistance - the role of transporters in human disease

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Chair: Megan O'Mara & Chris McDevitt

Perspectives on multidrug resistance in membrane transporters

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Membrane proteins constitute about 30% of all proteins and among these are a large cohort of transporters, receptors, and enzymes. Membrane transporters have become the subject of intense research over the past two decades, chiefly because many of them are responsible for a process known as drug efflux in which the drug is captured at the cell membrane and extruded to the outside, thereby protecting the cell from the action of the drug. This is best and most simply illustrated by the resistance of many bacteria to antibiotics by drug efflux. Though this is one of many mechanisms of cellular resistance to drugs, it is perhaps the most perplexing as it most often appears in the guise of multidrug resistance. In all, there are five major classes or families of membrane drug transporters, namely, the Major Facilitator Superfamily (MFS), Small Multidrug Resistance (SMR), Multidrug and Toxin Extrusion (MATE), Resistance Nodulation Division (RND, and ATP-Binding Cassette Transporter (ABC) families.

The failure of cancer chemotherapy in humans is a noted example of efflux-mediated resistance. A most insidious feature of multidrug resistance is that it is often triggered by exposure to a single drug. For example, if human tumours are treated with a cytotoxic drug, the drug will bring about unbridled expression of an ABC transporter called P-glycoprotein. Though this protein has seen only one drug, it elaborates an efflux mechanism for all known cytotoxic drugs thereby rendering chemotherapy irrelevant as a curative treatment for cancerous tumours. A single membrane transporter is capable of extruding a large number of unrelated drugs, making the cells multidrug resistant. That this mechanism is ubiquitous among all phyla makes it a major investigative topic. It was thought that recent resolved structures of some of these transporters would quickly yield the mechanistic details of multidrug resistance, but the reality is less promising. However, some important studies have given clues about the way in which specific transporters are able to extrude a diverse range of drugs.

Molecular determinants of MRP1/ABCC1 expression and transport

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The 190-kDa Multidrug Resistance Protein 1 (MRP1/ABCC1), first cloned by our group in 1992 from a drug resistant lung cancer cell line (Cole *et al.*, 1992), is a member of the 'C' branch of the ATP-binding cassette (ABC) superfamily of polytopic membrane proteins. MRP1 belongs to a subset of ABCC proteins comprising five domains: 3 membrane spanning domains (MSDs), containing 5, 6, and 6 transmembrane (TM) α-helices, respectively, and two cytoplasmic nucleotide binding domains (NBDs) (Leslie, Deeley & Cole, 2005). In addition to its drug efflux properties, MRP1 mediates the ATP-dependent transport of a broad array of exogenous and endogenous organic anions, including the cysteinyl leukotriene C4 (LTC4), an eicosanoid derivative that mediates inflammation and is involved in human bronchial asthma (Leslie, Deeley & Cole, 2005; Cole & Deeley, 2006). Thus MRP1 plays important roles in the cellular efflux of physiologically important signalling molecules as well as participating in the tissue disposition and elimination of drugs and their conjugated metabolites (Leslie, Deeley & Cole, 2005; Cole & Deeley, 2006). A substantial number of individual amino acids in different regions of MRP1 have been demonstrated to be critical for its substrate specificity and transport activities, as well as its stable expression in the plasma membrane. While some mutation-sensitive residues are found in the cytoplasmic loops (CLs) of MRP1, many are in, or are closely associated with, the TM helices of the core region of the transporter that likely forms part of a substrate/inhibitor binding pocket and/or substrate translocation pathway through the membrane. Particularly crucial for LTC4 binding and transport are Lys332 in TM6 (Haimeur, Deeley & Cole, 2002; Haimeur *et al.*, 2004; Maeno *et al.*, 2009). Current evidence suggests that TM6-Lys332 is involved in the recognition of the γ-Glu portion of LTC4 and other substrates/modulators containing GSH or GSH-like moieties (Maeno *et al.*, 2009). These and other data indicate that MRP1 has at least three substrate binding pockets. In contrast, residues involved in the proper assembly and/or structural stability of MRP1 have thus far been found in the CLs that mediate the coupling of the ATPase activity at the NBDs to the substrate translocation through the MSDs. Thus non-conservative substitutions of several ionizable amino acids in CL7 in MSD1 (Conseil, Deeley & Cole, 2006) and CL5 in MSD1 (Iram & Cole, 2010) profoundly diminish the levels of MRP1 at the plasma membrane of mammalian cells. Many (but not all) of these residues are predicted by homology models of MRP1 to be located in an environment where they could form bonding interactions with residues in the opposing NBD. We propose that these and other interdomain interactions are critical to the proper assembly and trafficking of MRP1 to the plasma membrane of mammalian cells.

Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AM, Deeley RG. (1992) *Science* **258,** 1650-4.

Cole SP, Deeley RG. (2006) *Trends Pharmacological Sciences* **27,** 438-46.

Conseil G, Deeley RG, Cole SP. (2006) *Journal of Biological Chemistry* **281,** 43-50.

Leslie EM, Deeley RG, Cole SP. (2005) *Toxicology and Applied Pharmacology* **204,** 216-37.

Haimeur A, Conseil G, Deeley RG, Cole SP. (2004) *Molecular Pharmacology* **65,** 1375-85.

Haimeur A, Deeley RG, Cole SP.(2002) *Journal of Biological Chemistry* **277,** 41326-33.

Iram S, Cole SPC. (2010) unpublished observations

Maeno K, Nakajima A, Conseil G, Rothnie A, Deeley RG, Cole SP. (2009) *Drug Metabolism and Disposition* **37,** 1411-20.

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Drug translocation by P-glycoprotein: how do topographical changes in transmembrane helices assist?

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Background: Resistance to chemotherapy is a major causative factor in the frustratingly poor success rate of chemotherapeutic management in cancer. The resistant phenotype is multifactorial involving both cellular and tissue architectural factors. One of the most prevalent, and effective, resistance pathways involves drug efflux pumps from the ATP-Binding Cassette (ABC) superfamily of transport proteins. The efflux proteins confer resistance by ensuring that cancer cells accumulate insufficient concentrations of anti-cancer drugs and thereby evade their cytotoxic actions. P-glycoprotein (a.k.a P-gp or ABCB1) is an archetypal ABC drug efflux pump and is known to confer resistance in a variety of cancer types including colon, liver, breast and a host of haematological disorders. P-gp is known to bind and transport over 200 pharmacological agents; hence it is referred to as a multi-drug transporter. This property remains a biological enigma and remains poorly understood. P-gp comprises four domains, two intramembranous (TMD) and two cytoplasmic (NBD). The TMDs are known to bind drug substrates and provide the translocation pore whilst the NBDs hydrolyse ATP to power the active transport. The TMD and NBDs are linked during translocation to ensure that drug binding and ATP hydrolytic events are efficiently coupled.

Objective: The process of coupling remains undefined, although two transmembrane helices (TM6 & TM12) appear to be intimately involved. As part of an ongoing investigation we aim to provide molecular details on the coupling process and the involvement of these two TM helices. The present investigation describes the role of TM12 in mediating coupling and the conformational changes it undergoes during the translocation process.

Strategy: The investigative strategy involves the mutagenesis based insertion of cysteine residues into various positions along TM12 using the fully functional cysteine-less isoform of P-gp as a template. The single cysteine mutant isoforms were expressed in insect cells with a recombinant baculovirus and purified using affinity chromatography. The purified, reconstituted mutant isoforms were assessed for function to ascertain the functional involvement of the targeted residue positions. In the second phase of investigation, the introduced cysteine residues were measured for their accessibility to covalent modification by a variety of thiol-reactive probes. Using probes with varying biophysical properties, differential labelling would reveal the local environment at each position. Finally, the isoforms were "trapped" in different conformations to assess the changes in local environment and thereby reveal the topography alterations during the translocation process.

Results: This cytosolic region undergoes a shift from a hydrophilic to hydrophobic environment during ATP hydrolysis. Overall the carboxy-proximal region of TM12 appears more responsive to changes in the catalytic state of the protein compared to its amino-proximal region. Thus, the carboxy-proximal region is suggested to be responsive to nucleotide binding and hydrolysis at the NBDs and therefore directly involved in inter-domain communication. These data can be reconciled with an atomic scale model of human ABCB1.

Conclusion: Taken together, these results indicate that TM12 plays a key role in the progression of the ATP hydrolytic cycle in ABCB1, in particular coordinating conformational changes between the NBDs and TMDs.

The malaria parasite's chloroquine resistance transporter: a multidrug resistance carrier?

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Malaria remains a major infectious disease in many parts of the world; an effective vaccine is not yet available and the parasite has developed resistance to most of the antimalarial drugs currently in use. When it was introduced in the 1940s, the affordability, low toxicity, and effectiveness of chloroquine (CQ) caused a revolution in the control of the disease. Around 15 years later, however, chloroquine-resistant (CQR) parasites had emerged and by the 1990s resistant strains were prevalent in most regions where malaria is endemic. CQR parasites accumulate much less CQ than do their CQ-sensitive (CQS) counterparts and it is this marked decrease in drug accumulation that underlies the phenomenon of CQ resistance. CQ resistance has been attributed primarily to mutations in the chloroquine resistance transporter (PfCRT), an integral membrane protein localised to the parasite's internal digestive vacuole (believed to be the site of CQ action) and a member of the Drug/Metabolite Transporter (DMT) Superfamily. Furthermore, mutations in this protein also modulate the parasite's susceptibility to a number of other clinically important drugs. However, the mechanism by which mutant PfCRT confers reduced drug accumulation within the digestive vacuole, and hence resistance, has been unclear.

We have expressed PfCRT in *Xenopus* oocytes, achieving a robust heterologous system for the functional characterization of this protein. Achieving expression was not straightforward; the coding sequence was codon-harmonised to facilitate correct folding of the protein and a number of putative trafficking motifs were removed to prevent its retention at internal membranes. Without these changes, PfCRT was not expressed at significant levels in the oocyte plasma membrane. Using this system, we undertook direct measurements of [^{3}H]CQ transport *via* PfCRT and provided a clear demonstration that the resistance-conferring form of PfCRT ($PfCRT^{CQR}$) has the ability to transport CQ out of the digestive vacuole whereas the sensitive form of the protein ($PfCRT^{CQS}$) does not (Martin *et al.*, 2009). We also showed that the transport of CQ *via* $PfCRT^{CQR}$ is inhibited by verapamil, a drug long-recognised for its ability to reverse CQ-resistance *in vitro*. Moreover, CQ uptake was inhibited by a number of quinoline antimalarials (including quinine and amodiaquine) as well as the antiviral agent amantadine (which exhibits some antimalarial activity *in vitro*, particularly against CQR parasites). By contrast, piperaquine and artemisinin (both clinically effective against CQS and CQR strains) were without effect. A focus of our recent work has been on determining whether verapamil, quinine, or amodiaquine are also transported *via* mutant forms of PfCRT.

There has been some conjecture as to whether PfCRT behaves as a channel or a carrier (*e.g.* Sanchez *et al.*, 2007). We found that the transport of CQ *via* $PfCRT^{CQR}$ is saturated by low (clinically relevant) concentrations of the drug (the apparent K_m(CQ) was 245 μM; Martin *et al.*, 2009). To place this in context, the addition of 100 nM CQ to the extracellular solution is estimated to result in a CQ concentration of around 2 mM in the digestive vacuole of CQS parasites and 200 to 500 μM in the digestive vacuole of CQR parasites - due to the accumulation of the drug *via* 'weak-base trapping' in this acidic compartment. A characteristic of saturable transport is the ability of unlabelled substrate to *cis*-inhibit the uptake of a radiolabelled substrate. We observed *cis*-inhibition of [^{3}H]CQ transport by unlabelled CQ in oocytes expressing $PfCRT^{CQR}$ and likewise found that the inhibition of $PfCRT^{CQR}$ by a number of different compounds, including quinine, verapamil, and amantadine, was concentration-dependent. Furthermore, we have recently shown that CQ uptake in oocytes expressing $PfCRT^{CQR}$ displays another hallmark of carrier-mediated transport - a marked dependence on temperature (Summers & Martin, 2010). Taken together, the saturability and strong temperature-dependence of CQ transport *via* $PfCRT^{CQR}$, along with the placement of the protein within the DMT superfamily of carrier proteins, support the view that PfCRT is a carrier. Moreover, the interaction of mutant forms of the protein with a number of different drugs suggests that PfCRT can function as a multidrug resistance carrier. The finding that $PfCRT^{CQR}$ behaves as a carrier has significant implications for the treatment of CQR parasites with CQ or CQ-like drugs. In particular we relate this to the example of Guinea-Bissau, where high doses of CQ are routinely used to cure CQR malaria (Ursing *et al.*, 2007).

Martin RE, Marchetti RV, Cowan AI, Howitt SM, Bröer S & Kirk K (2009) *Science* **325**, 1680-2.
Sanchez CP, Stein WD & Lanzer M (2007). *Trends in Parasitology* **23**, 332-9.
Summers RL & Martin RE (2010) *Virulence* **1**, 304-308.
Ursing J, Schmidt BA, Lebbad M, Kofoed PE, Dias F, Gil JP & Rombo L (2007) *Infection, Genetics, and Evolution* **7**, 555-61.

Antimicrobial resistance in staphylococci: Molecular architecture of a multidrug binding site

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A significant mechanism of bacterial resistance is the active export of antimicrobials from cells by broad specificity multidrug efflux systems. The export of antiseptics and disinfectants in staphylococci, mediated by the plasmid-encoded determinant *qacA*, is an example of such a multidrug efflux system (Brown & Skurray, 2001). QacA is a 514-amino acid membrane protein, containing 14 transmembrane segments (TMS), and is a member of the Major Facilitator Superfamily (MFS) of transport proteins. QacA confers resistance to more than 30 different monovalent and bivalent cationic, lipophilic antimicrobial compounds from 12 different chemical families *via* a proton motive force-dependent efflux mechanism. Transport and competition studies have indicated that QacA interacts with monovalent and bivalent compounds at two distinct substrate-binding sites (Mitchell *et al.*, 1999). Mutagenesis of the aspartic acid residue at position 323 in TMS 10 of the QacA protein has been shown to radically alter the substrate specificity of the transporter. Cysteine-scanning mutagenesis of 35 residues within and surrounding TMS 10 delineated the extents of the TMS and identified residues within the substrate-binding site; TMS 10 appears to play an integral role in the formation of the substrate-binding site of QacA (Xu *et al.*, 2006). Interestingly, the location of key negatively-charged residues in QacA has an influence on the subset of bivalent cations recognised (Hassan *et al.*, 2007).

To date there is no high resolution structure of a 14-TMS transport protein. However, since QacA possesses a number of amino acid sequence motifs conserved within the MFS protein family, is it practical to extrapolate the known structures of related 12-TMS transport proteins, such as GlpT and LacY? An alternative way of obtaining information on the structure of multidrug binding exporters, in particular their binding pockets, is to analyse other multidrug-binding proteins that may be more amenable to crystal structure analyses (Grkovic *et al.*, 2002). One such protein, the QacR transcriptional repressor, negatively regulates the expression of *qacA* and is induced by interaction with antimicrobials which are substrates of QacA; binding of these compounds conformationally modifies QacR such that it can not bind to the *qacA* DNA operator sequence (Schumacher *et al.*, 2002). Crystal structures of QacR complexed to a number of chemically- and structurally-different cationic compounds have shed light on the induction mechanism of QacR and also illustrate the versatility of the substrate-binding domain of this protein, revealing separate, but linked ligand-binding sites within a single protein (Schumacher *et al.*, 2001; Brooks *et al.*, 2007). Recent mutagenic studies have focused on the four glutamic acid residues, identified from these structures, that line and surround the QacR ligand binding pocket (Peters *et al.*, 2008). Biochemical analyses and examination of crystal structures have revealed that these acidic resides do not appear to play a role in charge neutralisation of cationic substrates but may be involved in substrate discrimination through affecting the positioning of the drugs within the binding pocket. This only serves to provide further evidence of the promiscuous nature of the binding pocket of multidrug binding proteins.

Brooks BE, Piro KM & Brennan RG. (2007) *Journal of the American Chemical Society* **129**: 8389-8395.
Brown MH & Skurray RA. (2001) *Journal of Molecular Microbiology and Biotechnology* **3**: 163-170.
Grkovic S, Brown MH & Skurray RA. (2002) *Microbiology and Molecular Biology Reviews* **66**: 671-701.
Hassan KA, Skurray RA & Brown MH. (2007) *Journal of Bacteriology* **189**: 9131-9134.
Mitchell BA, Paulsen IT, Brown MH & Skurray RA. (1999) *Journal of Biological Chemistry* **274**: 3541-3548.
Peters KM, Schuman JT, Skurray RA, Brown MH, Brennan RG & Schumacher MA. (2008) *Biochemistry* **47**: 8122-8129.
Schumacher MA, Miller MC, Grkovic S, Brown MH, Skurray RA & Brennan RG. (2001) *Science* **294**: 2158-2163.
Schumacher MA, Miller MC, Grkovic S, Brown MH, Skurray RA & Brennan RG. (2002) *EMBO Journal* **21**: 1210-1218.
Xu Z, ORourke BA, Skurray RA & Brown MH. (2006) *Journal of Biological Chemistry* **281**: 792-799.