Introduction
1. INTRODUCTION

The incidence of fungal infections has increased dramatically over the past few decades due to increase in the members of population susceptible to such infections. This population includes individuals undergoing chemotherapy for cancer, those enduring long-term treatment with antibacterial agents, those receiving immunosuppressive drugs following transplantation or those immunosuppressed due to diseases, such as AIDS, or malignancies (Frosco and Barrett, 1998). The severity of the fungal infections varies from superficial infections to life-threatening systemic infections. Most of the infections are opportunistic because the infecting agent is found as a commensal or is ubiquitous in the environment and can easily gain access to debilitated patients. While infections due to Candida and Aspergillus species are most common, previously rarely encountered opportunistic fungi have emerged recently as significant pathogens (Prasad, 1991). As example Trichosporon beigelii, Fusarium species, Pseudallescheria boydii and moulds of the class zygomycetes can cause invasive infections. Candida spp., Cryptococcus neoformans, and Aspergillus spp. are among the leading fungi responsible for these invasive infections. In addition to the opportunistic pathogenic fungi, a limited number of fungi exist with a true pathogenic potential for healthy hosts and cause life threatening infections. These pathogens, e.g., Histoplasma capsulatum, Paracoccidioides brasiliensis, Pencillium marneffei and Coccidioides immittis, are therefore classified as biohazard class 3 (Marichal, 1999).

1.1 Candida albicans and Candidiasis

Candida albicans (Figure 1) is the species most frequently associated with fungal infections.
infections in humans (Vanden Bossche et al., 1998). The history of Candidiasis
dates back to the fourth century BC when Hippocrates described oral apthae in
two patients with severe underlying disease in his book Epidemics. Although a
potential pathogen, *Candida albicans* is present as a commensal organism in
many, if not most, healthy individuals, where it may be found on the skin and in
the oral cavity, gastrointestinal tract and vagina. When something perturbs the
balance between the host and this yeast, notable when the host's immune system
becomes compromised or when the microenvironment shifts to favor the growth
of the yeast, this commensal organism really becomes pathogenic. For example,
when the immune system of the HIV infected individuals begins to deteriorate,
they frequently develop oral Candidiasis.

This condition also often develops among individuals with normal immune
systems who are taking antibiotics because such drugs eliminate the normal,
local bacteria and allow *C. albicans* to grow in that microenvironment. Similarly
changes in the hormone levels that affect the microenvironment of the vagina
lead certain women to experience frequent episodes of vaginal Candidiasis.
*Candida* vulvovaginitis is found in 10% women of childbearing age: Its
prevalence increases to up to 30% during pregnancy. In AIDS patients, 90% have
at least one episode of mycosis during their illness. Pfalller et al reported that
*Candida* species have become the fourth leading causes of nosocomial infections
in the USA. About 50% of these infections are due to *C. albicans*. Although *C.
albicans* is the most common cause of human infection, the genus *Candida*
includes about 200 species. *C. albicans* is now listed as one of the top five most
frequently isolated organisms from blood infections, as it is recognized as a
major contributor to morbidity and mortality worldwide (Frosco and Barrett,
1998).
C. albicans, the principal infectious agent of human infection, is an oval yeast of 2-6 μM in diameter. It exists as diploid, asexual polymorphic yeast with various biochemical abilities, both assimilative and fermentative, but lacks any proper sexual stage as well as carotenoid pigments. This medically significant fungus has the ability to undergo phenotypic switching and has 8 chromosomes (Odds, 1988; Prasad, 1991). One of the most remarkable aspects of C. albicans biology is its ability to assume a variety of cell morphologies (Figure 2). These range from yeast like cells to a variety of elongated growth forms, including a thread like hyphal growth form, germ tubes during the transition between yeast and

![Figure 2: Candida albicans cells in various morphological forms.](image)

hyphae and pseudohyphae, which vary in shape from attached strings of yeast like cells to long filaments with constrictions at septae. The yeast form is the one most commonly found in the laboratory. The transition to elongated growth is promoted by a wide range of environmental conditions, including growth at 37°C or exposure to serum (Ernst and Schmidt, 2000).

Although both bud and hyphae are found in infected tissues, it seems likely that the elongated hyphae penetrate tissues, leaving its path in lateral colonies of budding cells that in turn give rise to new penetrating hyphae (Shepherd, 1985; Odds, 1985; Soll et al., 1988; Scherer and Magee, 1990; Berger et al., 1990). The etiological, biochemical and morphological attributes of Candida makes it a unique eukaryote.
1.2 Therapy of Candidiasis

The therapeutic options for treating fungal infections, often caused by the emerging new pathogens whose incidence has increased due to the AIDS pandemic and use of immunosuppressive drugs in transplant and cancer patients, are limited by the relatively low number and structural variety of antifungals (Kolaczkowski and Goffeau, 1997). Only a few classes of antifungal agents are available to treat these infections. Most commonly used antifungals inhibit the ergosterol biosynthetic pathway and chiefly includes azoles, allylamines and morpholines and others such as polyenes and 5-FC, impair membrane barrier function and macromolecule synthesis respectively (Vanden Bossche, 1997).

1.3 Multidrug resistance

Multi Drug Resistance (MDR) is defined as the resistance of an organism towards a number of structurally and functionally unrelated compounds and is not a result of one mechanism but is caused by the synergistic action of a number of mechanisms. MDR can develop after sequential or simultaneous exposure to all the different drugs to which the cell or microorganism is resistant. MDR is a major concern in medical and agricultural developments. In medicine, the emergence of resistance to multiple drugs commonly used in the therapy is a major obstacle in the treatment of several tumors as well as of diverse diseases such as malaria (Chow and Volkman, 1998), tuberculosis and various bacterial and fungal infections (Gottesman and Pastan, 1993a; Balzi and Goffeau, 1995) which often complicate major debilitating syndromes like AIDS. In agriculture, the control of resistance to plant pathogens towards natural plant defense toxins
and towards common fungicides as well as the development of parasite-toxins resistant crops, are of major economic importance.

It generally involves a network of membrane-associated transporters acting as multidrug efflux pumps and transcription factors regulating the expression of these pumps. These multidrug efflux systems present a disturbing clinical threat, since the acquisition of such a single system by a cell may decrease its susceptibility to a broad spectrum of chemotherapeutic drugs. Multidrug resistance is one of the major obstacles for cancer therapy. One well documented mechanism underlying drug resistance in cancer cells implicates the over-expression of a membrane protein, the P-glycoprotein (P-gp), functioning as an ATP-dependent extrusion pump for drugs and physiological substrates (Balzi and Goffeau, 1991).

In another important field of health sciences, the resistance to drugs developed by Plasmodium falciparum is becoming a major obstacle for the treatment of malaria. The mechanism responsible for drug resistance in malaria seems analogous to that evoked for mammalian tumor cells. It involves the amplification and over-expression of a family of genes highly homologous to the mammalian P-gp encoding genes (Wilson et al., 1989).

Finally an understanding of a fatal and widespread hereditary disease was obtained by the isolation of the gene responsible for cystic fibrosis, and by the discovery that it encodes a putative membrane transport protein, remarkably similar to the mammalian multidrug resistance pump, and to its counterpart from other species (Schwiebert et al., 1998).

In yeast studies on multidrug resistance received a recent impetus, not only because of the involvement of some yeast species in pathogenicity for men and plants, but also because yeast is a universal, easy-to-manipulate model system for the study of higher eukaryotic cells. Therefore mechanisms of fundamental importance for mammalian cells may sometimes be approached
more efficiently by the study of similar yeast functions. In this respect, the recent identification in yeast of genes homologous to mammalian multidrug resistance genes opens new prospects (Goffeau et al., 1997; Decottignies and Goffeau, 1997; Paulsen et al., 1998).

1.4 Antifungal resistance

1.4.1 The emerging fungal threat

During the last three decades or so, the incidence of fungal infections has increased dramatically. Deep-seated mycoses are creating serious problems for clinicians working with large populations of immunocompromised patients with underlying diseases, such as hematological malignancies and acquired immunodeficiency syndrome (AIDS), or patients undergoing cancer chemotherapy or immuno suppressive therapy.

The need for effective antifungal drugs has been felt more and more acutely with the emergence of the AIDS pandemic and the AIDS related complex (ARC), which is nearly always associated with opportunistic fungal infections.

1.4.2 Factors contributing to clinical resistance

Factors which may contribute to clinical resistance, can be classified in to three groups namely, host-related, antifungal drug related and factors related to colonizing pathogen (Figure 3). The immune competence status of the host is an important factor in the occurrence of resistance. Indeed, until the late 1980s,azole resistance was only sporadically reported and always in patients suffering from chronic mucocutaneous candidiasis, who received antifungal therapy over an unusually long period of time (Vanden Bossche et al., 1994). It was the increase in the number of immunocompromised patients, particularly as a result of the AIDS pandemic, that preceded the sudden increase of reports on clinical failure to
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Fungal Factors

- Initial MIC
- Cell type
- Yeast/hyphae
- Switch phenotype
- Serotype
- Genomic stability of strain
- Size of population
- Biofilms

Clinical Resistance

Antifungal Drug
- Fungistatic nature of drug
- Dosing
  - Frequency
  - Quantity
  - Schedule (intermittent vs. continuous)
  - Cumulative dose
- Pharmacokinetics
  - Absorption, Distribution, Metabolism
  - Drug-drug interactions

Host factors
- Immune status
- Site of infection
- Severity of infection
- Presence of foreign materials
- Abscess formation
- Patient noncompliance with drug regimen

Figure 3: Factors which may contribute to clinical resistance.
antifungal therapy. The crucial importance of an intact immune system can be related to fungistatic mode of action of azole antifungals, which implies that part of the clearance of the infection is accomplished by host-related factors. A defect in this system allows residual fungal growth and could provide ideal circumstances for the selection or induction of resistant clinical isolates. The site and the severity of infection could also favor residual colonization because the pharmacokinetics of the azoles is such that not all organs reach the same level of antifungal concentration. Patient compliance with the prescribed drug regimen is another reason why insufficient concentrations of antifungal agents may reach the focus of infection. Insufficient dosage, decreased absorption from the gut, abnormal distribution or increased metabolic degradation of the antifungal agent in the patient, all result in an insufficient concentration at the site of infection. Bio-film formation on catheters, prosthetic equipments or dentures has also been shown to be a cause of persistent infection, which is difficult to treat.

The precise identification to species level, of the fungus causing clinical symptoms is also of importance because of differences in the antifungal susceptibility spectrum, even within a single class of antifungals. As mentioned, fluconazole is regarded as intrinsically inactive against a number of Candida species; infections caused by these organisms will not be cleared with this azole, but could be improved by changing to an azole compound that does include these species in the antifungal spectrum.

1.5 MDR in Candida albicans

Dimorphic, opportunistic and the most predominant human pathogenic yeast C. albicans is naturally more resistant to several drugs than S. cerevisiae. In addition, the incidence of C. albicans cells acquiring resistance to antifungals like azoles has increased considerably in recent years which have posed serious
problems towards the successful chemotherapy. The incidence of antifungal resistance has also increased in the non-\textit{albicans} species such as \textit{C. glabrata}, \textit{C. parapsilosis}, \textit{C. tropicalis} and \textit{C. krusei}. \textit{Candida} infections are treated with antifungal agents, particularly with the triazole derivatives fluconazole, itraconazole, ketoconazole, and voriconazole. In order to combat the attack of antifungals, evolution has equipped \textit{Candida} with an exodus of protecting systems. The earliest known protecting system in \textit{C. albicans} involved in the alteration or over-expression of the target enzymes P450_{14adm}. Recently the characterization of efflux pumps which throw the drugs out of the cell in \textit{C. albicans}, \textit{C. dubliniensis} and \textit{C. glabrata} has opened newer avenues and has provided impetus to dissect the mechanism of resistance employed by this pathogenic fungi to evade the toxic effects of the drugs.

\subsection*{1.6 Mechanism of antifungal drug resistance}

Although the molecular basis of antifungal resistance in fungi are not very clear evidence accumulated so far suggests that MDR is a multifactorial phenomenon comprising a combination of several mechanisms (White, 1997a; White \textit{et al.}, 1998; Ghannoum and Rice, 1999). A few of the well known molecular mechanisms of antifungal drug resistance in \textit{C. albicans} are discussed below and shown in Figure 4.

\subsubsection*{1.6.1 Target alteration or over-expression}

Amongst the known antifungals,azole derivatives like fluconazole, ketoconazole and itraconazole have been the most widely used triazoles for combating fungal infections. Azoles specifically inhibit P450_{14adm} enzyme (Erg11p) of the ergosterol biosynthetic pathway (Wilkinson \textit{et al.}, 1972; Wilkinson \textit{et al.}, 1974; Vanden Bossche \textit{et al.}, 1989). Once the azole drug enters the cell its
interaction with Erg11p can be modified in two ways; target alteration and over-expression. Both are discussed in the following sections.

1.6.2 Alterations in Erg11p

Erg11p is the target of azole derivative as explained above and it can be expected that amino acid substitutions could affect optimal binding to azole derivatives. Several point mutations in the ERG11 gene coding for P450\(\text{h}_{4\text{adm}}\) enzyme which reduce its affinity for azoles have been identified (Lamb et al., 1997; Loffler et al., 1997; White, 1997c; Sanglard et al., 1998; Kelly et al., 1999; Marichal et al., 1999; Favre et al., 1999). Reduced affinity of Erg11p to azole seems to be responsible for intrinsic resistance to azoles in C. krusei. ERG11 sequences from matched pairs of azole-susceptible and azole-resistant C. albicans isolates have shown mutations resulting in amino acid changes. Functional expression of PCR-amplified ERG11 genes in S. cerevisiae followed by susceptibility assay showed mutations coupled with development of azole resistance (Sanglard et al., 1998). While some ERG11 alleles contained a single mutation responsible for azole resistance, others ERG11 alleles were seen to contain several mutations with potential additive effects (Sanglard et al., 1998). White (White, 1997b) investigated the target enzyme (Erg11p) in the C. albicans series (which consist of 17 isolates obtained from the same patient over a 2-year period) by using biochemical and molecular techniques. Testing the susceptibility of Erg11p to fluconazole in cell extracts revealed that a substantial decrease occurred in isolate 13, corresponding to resistance development. To determine whether the ERG11 gene acquired any alterations in response to drug pressure, this gene was sequenced. Sequence analysis identified a single point mutation that resulted in a single amino acid substitution (R467K) (White, 1997c). This substitution resides between two residues known to be involved in interactions with the heme moiety in the active site of the enzyme. A similar
mutation (T315A) that alters the susceptibility of the target enzyme has been observed in close proximity to the active site of this enzyme in *C. albicans* (Lamb *et al.*, 1997). A compilation to show the frequency and positions of the so far

![Diagram](image)

**Figure 4: Mechanism by which microbial cells might develop resistance.** 1. The entry of the drug is prevented at the cell membrane/cell wall level. 2. The drug target is altered so that the drug cannot bind to the target. 3. The target enzyme is overproduced, so that the drug does not inhibit the biochemical reaction completely. 4. Some fungal "enzymes" that convert an inactive drug to its active form are inhibited. 5. The drug is pumped out by an efflux pump. 6. The cell secretes some enzymes to the extracellular medium which degrade the drug. 7. The cell has a bypass pathway that compensates for the loss of function inhibition due to the drug activity.
identified amino acid substitutions shows that the mutations D116E, K128T, E266D and G464S occur with highest frequency of which only the G464S mutation is exclusively present in azole-resistant isolates. The other three mutations are also found in azole-sensitive isolates (Marichal et al., 1999). The recently reported crystal structure of a cytochrome P450 from Mycobacterium tuberculosis, which is a soluble orthologue of Erg11p, can help to establish the effect of mutations on the protein conformation and on interference with azole binding (Ludovico et al., 2001).

1.6.3 Upregulation of ERG11

Over-expression of P450<sub>14adm</sub> (ERG11) has also been implicated as a mechanism of resistance to azole antifungals. Vanden Bossche et al (Vanden Bossche et al., 1992) characterized an azole-resistant C. glabrata strain and showed that its ergosterol content was increased compared with that of pretreatment isolate. This increase was accompanied by decrease in the susceptibility to both azoles and amphotericin B. The increase in the ergosterol synthesis was attributed to an elevated microsomal P-450 content in the resistant strain, suggesting an over-expression of the enzyme. Although the intracellular content of the fluconazole in the resistant strain was 1.5 to 3-fold lower than that in the pretreatment isolate, suggesting active efflux of this antifungal, the amount of itraconazole retained by the resistant strain did not differ from that found in the pretreatment isolate (Vanden Bossche et al., 1992). This finding suggests that the increased P-450 levels were responsible for the cross-resistance of these two triazoles.

1.6.4 Chromosome alterations

With the possibility of undergoing karyotype variability, fungi have an advantage of being flexible with their genome, which enables them in adapting
to environmental variations (Fierro and Martin, 1999). An alteration in the copy number of chromosomes as a mechanism of regulating gene expression in *C. albicans* has been observed (Janbon et al., 1998) wherein nondisjunction of two specific chromosomes has been proposed as the cause of in vitro development of fluconazole resistance. It appears that chromosomal nondisjunction, which awaits further proof, points to a new mechanism of drug resistance in the pathogenic yeasts (Perepnikhatka et al., 1999).

1.6.5 Modification or degradation of drugs

The detoxification of drugs by enzymes like the cytochrome P450s, which is a common method of rendering the drugs non-toxic in prokaryotes and higher eukaryotes, has not been observed in yeasts (Kappeli, 1986; Omura, 1999). Failure to detect any metabolite ofazole antifungals in *Candida* cells has further reaffirmed the above notion (Hitchcock, 1993; White et al., 1998). *Candida* species possess a unique class of cytochrome which comprises a family of proteins called the alkane-inducible cytochrome P450s (CYP52 gene family or P450alk) (Seghezzi et al., 1992; Ohkuma et al., 1995). Unlike the P45014DM cytochrome of yeast, the P450alk genes are involved in the hydroxylation of fatty acids and alkanes, thereby catalyzing a step that precedes their further metabolism (Kappeli, 1986; Seghezzi et al., 1992; Ohkuma et al., 1995). Recently, the over-expression of one of the members of the P450alk gene family viz. *CaALK8* in a hypersensitive *C. albicans* host (disrupted in *CDR1* and *CDR2*, the two drug extrusion pump encoding genes) (Sanglard et al., 1997) has been shown to confer resistance to at least three drugs including azoles (Panwar et al. 2001). Interestingly, the hydroxylation of lauric acid mediated by *CaALK8* is competed out with these drugs implying a direct interaction of drugs with this P450alk protein. This suggests that *CaALK8* mediated hydroxylation of drugs could be a possible mechanism by which drugs could be rendered non-toxic in *C. albicans*. However,
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no metabolic product of azoles has so far been detected in CaALK8 expressing cells. It also remains to be established how a P450alk mediated drug resistance mechanism fits in with the overall known and established contributors of MDR. Of note Δ^{22}-desaturase of *S. cerevisiae*, which appears to be a low activity hydroxylase has already been shown to metabolize xenobiotics. (Kelly *et al.*, 1993; Kelly *et al.*, 1997). Taken together, the modification of drugs could well be a mechanism, which might contribute to the overall drug resistance of *C. albicans* in some selected scenario.

1.6.6 Drug import

It is presumed that the hydrophobic nature of drugs permits easy import of these compounds by passive diffusion. However, the contribution of drug import to the overall scenario of MDR is not well established since technically it has not been possible to separate efflux of drug from their import. Nonetheless, there are a few studies particularly with mammalian cells where passive diffusion of drugs through lipid bilayer has been shown to be an important determinant of MDR (Ferte, 2000). The fluctuations in membrane fluidity are thus expected to affect passive diffusion and sensitivity to drugs. Precisely this was observed in a study wherein erg mutants of *S. cerevisiae* defective in different steps of ergosterol biosynthesis were used. These mutants were shown to possess high membrane fluidity and were hypersensitive to several drugs. The enhanced fluidity was linked to enhanced diffusion of drugs (Kaur and Bachhawat, 1999). Of note there are factors other than membrane fluidity, which can also influence passive diffusion of drugs across the membrane bilayer and thus can affect drug susceptibilities. As an example, the deletion of *PDR16* and *PDR17*- homologues of *SEC14* which are involved in regulating lipid synthesis in *S. cerevisiae*, results in changes in the lipid composition without affecting the membrane fluidity (Van Den Hazel *et al.*, 1999). As a consequence of the changes in the lipid composition,
the uptake (passive diffusion) of rhodamine 6-G into de-energized cells was found to be increased in a hypersensitive ∆pdr16∆pdr17 double mutant as compared to the wild type strain (Van Den Hazel et al., 1999). In this context, the role of import of drugs needs to be analyzed more carefully. It is expected that with better experimental design and more studies, the contribution of import of drugs in MDR can be established.

1.6.7 Failure of drug accumulation
Considerable evidence has now been accumulated to suggest that active efflux is an important mechanism of resistance to azole antifungals (Sanglard et al., 1997; Walsh et al., 1997). Efflux pumps, which are generally present in the plasma membranes, have now emerged as one of the important components of multidrug resistance. An over-expression of P-glycoprotein (P-gp)/human MDR1 remains the most documented and well-characterized example of a drug

Figure 5: Schematic representation of the two major classes of multidrug transporters: (A) ABC-type multidrug transporters utilize the free energy of ATP hydrolysis to pump drugs out of the cell. (B) Secondary multidrug transporters mediate the diffusion of structurally unrelated drugs in a coupled exchange with protons or sodium ions.
extrusion pump belonging to the ABC superfamily that results in the failure of chemotherapy in tumor cells (Ambudkar et al., 1999; Ueda et al., 1999). The presence of proteins homologous to P-gp in all the organisms ranging from the prokaryotes to eukaryotes portrays extrusion of drugs as a general theme of mechanism of MDR (Michaelis and Berkower, 1995; Smart and Fleming, 1996; Tommasini et al., 1997; van Veen and Konings, 1997; Urban et al., 1999; Theodoulou, 2000). MDR in S. cerevisiae is designated as PDR network, which comprises genes encoding for the drug extrusion pumps and their regulators. Homologues of the PDR genes, which contribute to antifungal resistance, are also present in the pathogenic yeast C. albicans (Prasad et al., 1995; Sanglard et al., 1997; Walsh et al., 1997). Recent studies indicate that fungi possess at least two efflux systems: (i) Protein belonging to major facilitator superfamily (MFS) (Marger and Saier, 1993; Michaelis and Berkower, 1995) and (ii) ATP-binding cassette (ABC) superfamily of proteins (Figure 5). The MFS drug efflux proteins are associated with the transport of structurally diverse compound and account for a range of resistance to toxic compounds in microorganisms (Jenkinson, 1996). An example of MFS protein associated with the drug resistance in Candida is BENr (CaMDR1), which is implicated in resistance to several drugs, including benomyl, methotrexate, and fluconazole. The ABC superfamily of proteins bind ATP, which is essential for substrate transport, through a highly conserved amino acid sequence (known as the binding cassette) (Jenkinson, 1996). Four families of ABC transporters have been identified in S. cerevisiae (MDR, CFTR, YEF, and PDR). These transporters have a common four-core domain structure (Higgins, 1992) consisting of two integral membrane domains.
1.7 MFS transporters

MFS has originally been defined as a superfamily of permeases that are characterized by two structural units of six transmembrane spanning α-helical segments connected by a cytoplasmic loop. The MFS consists of over 50 transporters membrane protein form bacteria to higher eukaryotes involved in symport, antiport, or uniport of various substrates (Marger and Saier, 1993; Pao et al., 1998) which have been classified into five distinct clusters or families of membrane transport proteins. MFS proteins are shown to be involved in (i) drug resistance, (ii) sugar uptake, (iii) uptake of Krebs cycle intermediates, (iv) phosphate ester/phosphate antiport, and (v) oligosaccharide uptake (Marger and Saier, 1993; Paulsen et al., 1996b). The drug resistance proteins are PMF-dependent antiporters which efflux out drugs exchanging one or more H⁺ ions with a substrate molecule (Paulsen and Skurray, 1993). On the basis of hydropathy and phylogenetic analysis, drug efflux proteins can be divided into two distinct types with 12 and 14 transmembrane segments (TMS) which contain more than 100 members (Paulsen et al., 1996a). The first report of an MDR gene, which acts as a pump in bacteria, came from studies of Qac locus in Staphylococcus for resistance to quaternary ammonium compounds (Rouch et al., 1990). Multidrug efflux protein Bmr from Bacillus subtilis mediates resistance to structurally diverse compounds, including rhodamine 6G and acridine dyes, ethidium bromide, TPP, puromycin, chloramphenicol, doxorubicin and fluoroquinolones. Drug transport by Bmr is sensitive to inhibitors of the mammalian P-gp pump such as reserpine and verapamil (Neyfakh et al., 1991).

S. aureus protein NorA was identified as a chromosomal fluoroquinolones resistance gene but was subsequently shown to confer resistance to similar range of substrates as that of Bmr. In S. cerevisiae FLR1 gene product, an MFS, has been shown to cause resistance to cycloheximide, fluconazole, cadmium and H₂O₂.
IntrOlfuction
(Alarco et al., 1997). Another MFS, HXT11 gene product, which is a sugar transporter of S. cerevisiae is involved in pleotropic drug resistance. Loss of HXT11 and/or HXT9 confers cycloheximide, sulphometuron methyl and 4-NQO resistance.

1.8 ABC transporters

ABC Transporters constitute one of the largest and highly conserved superfamilies and is found in large numbers in all organisms. ABC protein family with currently more than 1000 members represents the largest protein family known to date (Higgins, 1992; Higgins, 1993). ABC transporters have been identified till now in species including bacteria, yeasts, insects, protozoa, plants and humans (Higgins, 1992; Fath and Kolter, 1993; van Veen and Konings, 1997) (Figure 6). They fulfill a remarkable variety of cellular functions. While most ABC proteins are purely ATP-driven membrane transporters, some of them act as ion channels, channel regulators, receptors, proteases and even sensing proteins (Higgins, 1995). As far as the transport of substrates is concerned, ABC transporters mediate translocation of ions, heavy metals, carbohydrates, anticancer drugs, amino acids, phospholipids, steroids, glucocorticoids, bile acids, mycotoxins, antibiotics, pigments, peptides through membranes an in some cases even whole proteins (Higgins, 1992; Dean and Allikmets, 1995; Ambudkar and Gottesman, 1998; Bevers et al., 1999). However, each ABC transporter transports a large variety and size of substrates, but at the same time maintains selectivity for its particular substrate. This represents an intriguing and yet unsolved mystery. Several mammalian ABC proteins are medically important because mutations in corresponding genes cause severe genetic diseases such as cystic fibrosis (Harris and Argent, 1993), adrenoleukodystrophy (Mosser et al., 1993) and zeweller syndrome (Gartner and Valle, 1993), dubin-
johnson syndrome (Paulusma et al., 1996), familial hyperinsulinemic hypoglycemia of infancy, hepatic choleostasis and Stargardt’s muscular dystrophy of the eye (Klein et al., 1999).

Figure 6: Topology of bacterial, lower eukaryote and human ABC proteins: For comparison, the use of same color indicates the homologous sequences in different MDR proteins.
1.8.1 Domain structure and organization

All ABC proteins share a similar molecular architecture with a hallmark domain organization that includes the presence of at least one evolutionary conserved ATP-binding cassette (ABC), also known as NBD (nucleotide binding domain), as well as several predicted α-helical membrane-spanning segments (TMS) (Klein et al., 1999). The TMS and NBDs are normally arranged in duplicated forward (TMS6-NBD)$_2$ or reverse (NBD-TMS6)$_2$ configuration, but numerous half-size transporters with various topologies are also known as shown in figure 7 (Higgins, 1992; Dean and Allikmets, 1995). Full size ABC proteins usually have six predicted TMS in each half, connected by a charged linker region. Additional TMS proximal to N-terminus also exist in some ABC proteins (Cole and Deeley, 1998). Finally the hydrophilic NBD domains encompass approximately 250 residues with five conserved protein motifs. The Walker A and Walker B motifs, found in all other nucleotide binding proteins (Walker et al., 1982) and the ABC signature or C motif with the consensus sequence LSGGQ are diagnostic hallmarks for all ABC proteins. In addition, there are two less conserved regions, the so-called center region between Walker
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A and Walker B and another downstream of Walker B motif (Berkover and Michaelis, 1996). Many ABC proteins are located in plasma membrane (Higgins, 1992; Decottignies and Goffeau, 1997; Kolaczkowski and Goffeau, 1997). In bacteria, where there are no intracellular membranes, all ABC transporters are situated on the cytoplasmic membrane mediating the movement of solutes in and out of the cells (George, 1996; Neyfakh, 1998).

A typical ABC transporter consists of four units, viz., two membrane domains comprising of six transmembrane segments and two nucleotide-binding domains (NBDs), which bind and hydrolyze ATP. These four modules can be expressed as separate polypeptides (Higgins, 1993). In E. coli histidine permease, four genes express these polypeptides whereas there are three genes for the
ribose transporter, two encoding membrane bound domains and one encoding
the two fused NBDs (Higgins, 1993). Among eukaryotic members of the ABC
superfamily, the TAP1 and TAP2 peptide transporter is encoded by two genes,
each encoding a membrane bound domain fused to an NBD whereas for human
MDR1 (P-gp), a single gene encodes all the four modules (Higgins, 1992; Ames et

1.9 ABC proteins of *Saccharomyces cerevisiae*

From the genome-sequencing project, it has been revealed that there exist
at least 33 distinct ABC transporters in *S. cerevisiae*. This budding yeast is a
powerful model organism for higher eukaryotes as it allows the combination of
classical genetics and biochemistry with recombinant technology. Almost each
cellular compartment harbors at least one ABC transporter except endoplasmic
reticulum and nuclear membrane. It has been found that 25% of human genes
significantly match yeast proteins. In some cases even human genes are capable
to complement yeast function, facilitating structure / function studies in yeast.
Based on phylogenetic classification tree analysis, yeast ABC proteins are
classified into six distinct subfamilies (Taglicht and Michaelis, 1998) . These
families are defined as the MDR, the PDR, the MRP/CFTR, the ALDP, the YEF3
and the RLI, PDR5, SNQ2, YOR1 are some of the well known ABC drug
transporters (Figure 8).

1.10 ABC proteins in antifungal resistance in *C. albicans*

It has been observed that treatment of fungal infections with triazoles can
result in severe antifungal resistance (Sanglard *et al*., 1995; White *et al*., 1998; Ha
and White, 1999). The discovery that Pdr5p mediates pronounced resistance to
mycotoxins and antifungal azoles prompted the hunt for Pdr5p homologues in
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pathogenic fungi. Intracellular levels of drug in resistant isolates were increased by the addition of sodium azide suggesting that an energy dependent efflux.

Figure 8: Phylogenetic unrooted tree of the yeast ABC protein
pump was associated with resistance (Sanglard et al., 1995; Albertson et al., 1996). As a result CDR1 (Candida drug resistance) gene was first cloned by Prasad et al, by functional complementation of a null mutant of PDR5 of S. cerevisiae (Prasad et al., 1995). The expression of CDR1 even in low copies in S. cerevisiae conferred drug resistance, while multiple copies of PDR5 are required to confer resistance.

The levels of resistance to drugs like cycloheximide was much stronger as compared to PDR5 which suggested it is intrinsically much more effective against drugs. Reduced accumulation of radiolabeled fluconazole has been observed in a variety of fluconazole resistant clinical isolates of C. albicans when compared with fluconazole sensitive isolates (Albertson et al., 1996). Later on another gene from C. albicans was isolated, named as CDR2 which also conferred resistance to different drugs. The characterization of ABC proteins, e.g., CDR1 (Krishnamurthy et al., 1998a; Krishnamurthy et al., 1998b; Krishnamurthy et al., 1998c; Smriti et al., 1999), CDR2 (Sanglard et al., 1997) from C. albicans and their over-expression in certain instances of azole resistant clinical isolates has been confirmed that these transporters are involved in MDR scenario of C. albicans (Maesaki et al., 1998; Cannon et al., 1998). This led to the isolation of other ABC transporters of Candida.

The homologues Cdr3p and Cdr4p show highest homology to Cdr1p and Cdr2p, however, as compared to Cdr1p and Cdr2p, which are more than 90% similar, Cdr3p and Cdr4p are only 75% similar to Cdr1p and Cdr2p. Interestingly, over expression of CDR3 and deletion of CDR3 and CDR4 could not affect drug susceptibilities (Franz et al., 1998). It is not yet known why some ABC proteins show drug resistance phenomenon whereas others do not, in spite of their close homology. Both the proteins have similar topological arrangements where hydrophilic domain containing nucleotide binding motif precedes hydrophobic transmembrane stretches. The only apparent difference between the two proteins appears in C-terminal where Cdr3p has an extended loop
connecting TM11 and TM12. In addition, the last 21 amino acids in the C-terminal of Cdr3p are totally different from Cdr1p. The molecular basis of drug transport by these transporters will have to await a high-resolution 3D protein structure analysis; however, analyses of mutations of homologous and non-homologous regions between different Cdrps can soon provide valuable information. This situation is similar to the presence of two types of P-glycoproteins in mammals: one, which transports hydrophobic drugs while the other, which does not transport drugs. Mouse mdr1 and mdr3 and human MDR1 belong to the Type I, which can transport and confer drug resistance. Mouse
Introduction

mdr2 and human MDR2 (also known as MDR3) belong to the type II (Ueda et al., 1987; Buschman et al., 1992; Smith et al., 1994). Studies employing MDR1-MDR2 chimeric proteins led to the identification of some amino acid residues in the TM6 of MDR1 which are sufficient to allow an MDR2 backbone in the N-terminal half of P-gp to transport several MDR1 substrates (Zhou et al., 1999). These studies indicate a close relationship between MDR1, a multidrug transporter and MDR2, a phosphatidylcholine flippase. Since Cdr1p, Cdr2p and Cdr3p have similar domain structure, their substrate preferences are most likely to be determined by some non-identical amino acid residues. However this remains to be investigated. The current inventory, based on the sequence similarities with ABC systems in other living organisms, indicate that C. albicans harbors 28 putative ABC proteins which cluster into six subfamilies (Figure 9).

1.11 CDR1, an ABC transporter from C. albicans

Among ABC transporters in C. albicans, CDR1 has been shown to play a key role in azole resistance in C. albicans as deduced from its high level of expression found in several azole resistance clinical isolates recovered from patients receiving long term antifungal therapy (Sanglard et al., 1995; Sanglard et al., 1996). Additionally, high level of expression of CDR1 invariably contributes to an increased efflux of fluconazole and thus corroborating its direct involvement in drug efflux (Krishnamurthy et al., 1998c; Sanglard et al., 1999). Cdr1p thus has not only acquired significant clinical importance but is considered an important player in any design of strategies to combat antifungal resistance.

The CDR1 gene encodes for an integral plasma membrane protein of 1501 amino acids, with a predicted molecular weight of 169.9 kDa. On the basis of its amino acid sequence, Cdr1p is predicted to consist of two homologous halves, each comprising of one N-terminal hydrophilic domain followed by a C-terminal hydrophobic domain (Figure 10). The hydrophilic domain comprised of a
conserved ABC region, including the ATP-binding motifs known as the Walker A and Walker B (Walker et al., 1982) and another highly conserved motif, ABC signature, preceding the Walker B motif (Dudani and Prasad, 1984). Cdr1p has a similar topology to its close homologues Pdr5p and Snq2p of *S. cerevisiae* (Prasad et al., 1995) (NBD-TMS6)2 but it has a reverse topology than STE6 of *S. cerevisiae* and *MDR1* from *Homo sapiens* which has a (TMS6-NBD)2 domain organization (Figure 7).

![Predicted two dimensional topology model of Cdr1p](image)

**Figure 10:** A hypothetical two-dimensional model of Cdr1p: The model is based on the hydrophobicity profiles of amino acid sequences and functional domains. Small circles represent amino acid residues, which are filled with single letter code of amino acid. The numbers indicate the beginning and the end of the transmembrane segments.
1.12 Functional and Structural aspects of the ABC transporters

ABC transporters are integral membrane proteins that typically utilise cellular energy to translocate solutes across cellular membranes in all phyla. These transporters have been conserved across the three kingdoms of archaea, eubacteria and eukarya (Ames et al., 1990), (Higgins, 1992). Their ubiquitous distribution and primordial origin reflect the fundamental requirement of cellular homeostasis to import and concentrate essential nutrients, and expel toxins acquired from the environment or produced as metabolic by-products (Schneider and Hunke, 1998). Prokaryotes and eukaryotes also contain an additional large group of ABC proteins - non transporters - located in the cytosol and employed mostly for maintenance and repair of DNA and for gene regulation (Aravind et al., 1999). These two major families of ABC proteins are known collectively as the ABC-ATPase superfamily (Aravind et al., 1999), (Holland and Blight, 1999). In a 1997 report of the complete genome sequence of Escherichia coli K-12, at least 80 ABC proteins ~5% of the genome were identified (Blattner et al., 1997). ABC transporters make up one of the four major gene families in humans (Tatusov et al., 1997); and it is now believed that ABC proteins may be found in all cells of all species.

The signposting of ABC transporters as a Superfamily with a core structure of four domains was made in 1986 (Higgins et al., 1986) and was followed in 1992 by an encyclopaedic review on ABC transporters (Higgins, 1992). Despite the plenitude of solute types and processes with which they are involved, ABC transporters comprise a conserved core structure of two transmembrane domains (TMDs) and two cytosolic ABCs, also commonly known as nucleotide-binding domains (NBDs). The acronym ‘ABC’ derives from ATP binding cassette (Hyde et al., 1990). The TMDs contain multiple hydrophobic segments, which span the membrane and form the transmembrane (TM) channel. The primary sequences of ABC transporter TMDs are markedly
variable compared with those of the NBDs, which contain the highly conserved Walker A and B consensus motifs for nucleotide binding (Walker et al., 1982) and the 'LSGGQ' motif (Bianchet et al., 1997), the diagnostic signature sequence of ABC proteins. While the TMDs form the TM channel and are thought to contain the substrate binding sites, the NBDs are molecular motors that transform the chemical potential energy of ATP into protein conformational changes. Prokaryote importers or permeases generally deploy four separate subunits in the core configuration together with accessory periplasmic-binding proteins, which collect and present solute to the membrane-bound transporters. Eukaryotes only have export ABC transporters, which commonly have a single polypeptide for the core structure with each NBD being C-terminal to each TMD. There are exceptions to this scheme, with the most usual of these being half-transporters found in both prokaryotes and eukaryotes, in which each TMD is fused covalently to a C-terminal NBD, or with an N-terminal NBD followed by a TMD. The complete core structures for half-transporters can be homo- or heterodimers.

Some ABC transporters are involved in multidrug resistance in bacteria, fungi, yeasts, parasites and mammals (van Veen and Konings, 1997), (Borst and Elferink, 2002), (Gottesman, 2002), (Holland et al., 2003), with resistance to anticancer agents being a major concern in humans and, in particular, that elicited by the human multidrug transporter P-glycoprotein (P-gp; MDR1; or ABCB1). Human P-gp is the archetypal ABC transporter and has earned its reputation by being the first discovered, the most important medically, the most studied and the one having arguably the broadest portfolio of substrates and reversing agents (Gottesman and Pastan, 1993b), (Germann, 1996).

Though research interest in the ABC superfamily is widespread, the stimulus for much of this research may be traced to P-gp, discovered by Victor Ling's group nearly 30 years ago as an integral plasma membrane glycoprotein.
This group also established the experimental basis of P-gp's role in multidrug resistance in cancer cells; that it was a near perfect tandem duplication of the haemolysin transporter from *E. coli*; and its similarity with bacterial permease subunits HisP, MalK and OppD. They also made the prescient predictions that these proteins arose from a common ancestor; that transport was coupled to energy production; that P-gp could bind and export structurally diverse drugs; that its chief physiological role might be to protect cells from lipophilic toxins; and that its multidrug resistance property simply reflects gene amplification or over-expression.

On the eve of the new millennium, it was widely considered that significant further progress in ABC transporter research would require high-resolution structural data. Since that time, a wealth of structural information has been generated concerning ABC transporters and related proteins, beginning in 1998 with the landmark crystal structure of HisP, the NBD subunit of the well-characterized bacterial histidine permease complex (Hung et al., 1998); a structure of the remotely related ABC ATPase DNA repair enzyme, the Rad50cd catalytic domain (Rad50cd) (Hopfner et al., 2000); through to recent crystal structures of complete ABC transporters (Chang and Roth, 2001), (Locher et al., 2002). One of the most significant questions answered by these structural studies concerns the role of the ABC signature sequence and the mode of interaction of the two NBDs. The dimeric structure of the Rad50 showed the signature sequence completing the active site of the opposite monomer in an NBD dimer (Hopfner et al., 2000), as we had first predicted for ABC transporters (Jones and George, 1999). Since then two crystal structures of ABC transporter NBDs (Locher et al., 2002), (Smith et al., 2002) together with a number of experimental studies have confirmed this mode of interaction of the NBDs. Notwithstanding these advances, however, many fundamental questions remain unanswered. In particular, the signal routes and mechanisms by which the TMDs control ATPase
activity in the NBD in response to substrate binding, and the way in which the NBDs harness the energy of ATP to drive conformational changes in the TMDs that drive substrate translocation, remain to be elucidated.

1.12.1 Transmembrane domains: is it crystal clear?

The first crystal structure of a complete ABC transporter appeared in 2001, the lipid A transporter from E. coli, Eco-MsbA (Chang and Roth, 2001); it was followed in 2002 by the E. coli vitamin B12 transporter, BtuCD (Locher et al., 2002), and in 2003 by a structure of the Vibrio cholera MsbA transporter (VC-MsbA) (Chang, 2003). MsbA is a homodimer of two fused TMD-NBD halves and is a close homologue of human P-gp. As originally predicted for P-gp on the basis of hydropathy analysis of the primary sequences, and later substantially corroborated by experimental approaches, the TMDs of the MsbA monomer form an arc of six a helices, which in dimers could form a TM channel (Figure 11A). Indeed, such a channel-like configuration was observed in the recent VC-MsbA dimer structure (Chang, 2003). In view of the close homology between P-gp and MsbA, the crystal structures of MsbA should provide a fruitful template for interpreting the extensive experimental data concerning.

Perhaps the most puzzling feature of the two MsbA structures is that in neither case can the monomers be oriented in such a way that they form a TM channel and a Rad50cd-like NBD dimer at the same time. In the case of the Eco-MsbA structure, while the N-terminal regions of the NBDs are disordered, their approximate locations can readily be predicted and indeed have been homology modelled by ourselves (Jones and George, 2002) and others (Thomas and Hunt, 2001). When this exercise is performed and two Eco-MsbA monomers are arranged such that their NBDs form a Rad50cd-like dimer, the two TM arcs are inverted back to back relative to their orientation in the Eco-MsbA crystal dimer, and cannot form a channel without rotating 180 degrees
Figure 11: Schematic representations of MsbA dimer configurations viewed from the extracellular side, along the bilayer normal (upper panel), with the corresponding view within the plane of the membrane (lower panel). In the upper panels the NBDs are depicted as 'L'-shaped boxes with the signature sequences indicated by the letters 'SS'. (A) Eco-MsbA crystallographic dimer with two arcs of six helices closed at the extracellular surface. (B) Eco-MsbA homology modelled to the Rad50cd NBD dimer. (C) VC-MsbA dimer.


Figure 12: ABC-ATPase catalytic site. Representative ABC transporter active site derived from the MJ0796 E171Q dimer (1L2T) showing deployment of conserved residues, water and catalytic magnesium ion in relation to nucleotide triphosphate moiety. The P-loop of one monomer is shown in a 'worm' representation and is coloured grey. Residue side chains and the nucleotide triphosphate group are shown in stick form with oxygen atoms coloured light red, nitrogen atoms blue and phosphorus orange. Carbon atoms of the monomer containing the P-loop are coloured yellow, while those of the (L)SGG of the opposite monomer are coloured green. The catalytic magnesium is cyan, and its two coordinating water molecules are light red. The nucleophilic water is situated at the centre of the figure and is dark red. Residues depicted are: a, Walker A 3 (serine); b, Walker A 8 (serine); c, Walker B aspartate; e, conserved glutamate at C-terminus of b-strand 6; f, C-motif second glycine; h, conserved histidine at C-terminus of b-strand 7; k, Walker A lysine; q, conserved glutamine at C-terminus of b-strand 5; s, C-motif serine; g, nucleotide g-phosphate.
about the bilayer normal relative to the NBD, or radical rearrangement of the putative TM helices (Figure 11B). Most astonishingly, the putative substrate sites within the interior of the TM channel would now be on the outside.

The TM structure of the VC-MsbA monomer is very close to that of Eco-MsbA, with homologous residues forming the concave surface of the 6 TM arc, and thus the interior of the putative TM channel formed by the VC-MsbA dimer (Figure 11C). While the NBDs are fully resolved, in comparing the VC-MsbA NBD to the consensus NBD fold, it appears that about one-half of the NBD is rotated about 120 degrees relative to the rest of the NBD. The two halves of the NBDs have approximately preserved their structure in this rotation, based on sequence and structural comparisons between VC-MsbA and other ABC transporters for which structures are known. The relative rotation of the two VC-MsbA NBD halves was likened to the rotation of a helical subdomain observed among the various NBD crystal structures (Chang, 2003).

However, whilst the NBD helical subdomain rotates about flexible loops at its N- and C-termini, the rotation of the VC-MsbA NBD halves breaks the structurally conserved central $b$ sheet of the NBD core, rupturing the catalytic site and moving highly conserved active site residues, including the Walker B motif, over 25'Å from the Walker A nucleotide-binding region (Figure 11C). This configuration has not been observed in any other related nucleotide-binding protein and, rather than representing a natural mechanistic transition, may be an artefact of the crystallisation process. Nonetheless, if the N-terminal half of each VC-MsbA NBD is rotated 120 degrees to form a consensus NBD fold, the NBD dimer thus formed indeed approximates the mode of interaction observed in Rad50 and other ABC transporter structures. Incredibly, however, in this configuration, each complete NBD is rotated 180 degrees relative to the TMDs when compared with the orientation observed in the Eco-MsbA monomer structure (Figure 11A, C; top panels), and the NBD interacts with the TMD in a
completely different way in the two structures. Rather than representing a mechanistic transition, which to our knowledge is without precedent, this may indicate that either or both MsbA structures are artifactual to some extent.

1.12.2 The P-loop ATPase family

ABC transporters NBDs contain the Walker A and Walker B consensus sequences for nucleotide binding (Walker et al., 1982). These sequence motifs have been identified in a range of proteins engaged in diverse cellular processes such as: receptor signalling, phosphoryl transfer reactions, motility, ATP synthesis/proton efflux, membrane transport, DNA translation and DNA maintenance/repair. The Walker A motif (G-X-X-G-X-G-K-S/T) embodies a structure known as the phosphate-binding loop or P-loop, a glycine rich loop followed by an uncapped α helix (Figure 12). This structure functions to bind the nucleotide through electrostatic interactions with the triphosphate moiety. The Walker B motif is F-F-F-F-D, where ‘F’ is a hydrophobic residue. In P-loop ATPases, this sequence constitutes a buried β strand within the core of the nucleotide-binding fold. The Walker B aspartate hydrogen bonds to coordinating ligands of the catalytic Mg²⁺ ion, thereby assisting in establishing and maintaining the geometry of the active site.

The core fold of P-loop ATPases is characterised by a central, mostly parallel β sheet flanked by α helices. ABC transporter NBDs belong to a broad subclass of topologically homologous P-loop ATPases which includes RecA, F1-ATP synthase, UvrB, helicases and a number of more closely related proteins involved in DNA maintenance and repair such as Rad50, MutS and SMC. These latter proteins form a superfamily together with ABC transporter NBDs, known as ABC ATPases (Hopfner and Tainer, 2003). The RecA-like ATPase subclass is distinguished from other P-loop ATPases by the pattern of interdigitation of the parallel β strands, which form the backbone β sheet of the nucleotide-binding
fold. In ras-like proteins, adenylate kinase and myosin, the Walker B $b$ strand is located immediately adjacent to the $b$ strand preceding the P-loop, while in the F1-ATPase, RecA and ABC ATPases, a third strand, derived from the peptide C-terminal to the Walker B, is inserted between the P-loop $b$ strand and the Walker B $b$ strand (Jones and George, 1999).

1.12.3 The ABC ATPase: the adaptable engine

Notwithstanding the striking functional divergence of the transmembrane transport ABC ATPases and the DNA maintenance/repair ABC ATPases, it is becoming increasingly clear that these multisubunit enzymes are unified through a conserved global architecture and conformational mechanism of the ATPase domains (Hopfner and Tainer, 2003). The ABC ATPase consists of two lobes or arms, lobe I constituting a characteristic conserved ATP-binding subdomain and

Figure 13: (A) Topology diagram of the consensus ABC transporter NBD fold. $b$ strands are depicted as arrows and $a$ helices as rectangles. Loops connecting secondary structural elements are depicted as thick lines. (B) Three-dimensional structure of ABC transporter NBD. Ribbon diagram of HisP (1B0U) colour coded and numbered as in (A). $b$ strands are numbered and depicted as arrows and $a$ helices as coiled ribbons. ATP is shown in stick form with carbon (yellow), nitrogen (blue), oxygen (orange) and phosphorous (magenta). Regions proposed to form interfaces with the TMDs, as discussed in the text, are labelled IR1 and IR2.
lobe II comprising a more structurally diverse ‘helical’ subdomain. The central functional unit of ABC proteins consists of a heterotetramer comprising one NBD dimer and one substrate/function-specific dimer, this latter corresponding to the two TM domains in ABC transporters. X-ray crystal analysis reveals that the orientation of the substrate-specific domains with respect to the NBD dimer, and the regions of the NBDs which form the oligomeric interfaces, are analogous in all ABC proteins. In addition to this conserved quaternary structure, the region of the NBDs implicated in the transmission of conformational changes to the substrate-specific dimer, although structurally divergent, maps to the equivalent lobe II subdomain of the NBD. Thus, important insights into ABC transporter function are to be gained from the mechanistic and architectural parallels between the ABC ATPases.

1.12.4 The ABC transporter NBD: a bilobal design

The NBDs of ABC transporters can be divided into three structurally and functionally distinct subdomains, and these are colour coded in Figure 13, which depicts a generic topology and numbering system for the secondary structural elements of the NBD. The central, mostly parallel $\beta$ sheet forming the binding site for the nucleotide phosphates, and the $\alpha$ helices flanking and joining these $\beta$ strands, are referred to collectively as the ABC core subdomain (Karpowich et al., 2001). The core subdomain contains the Walker A and B consensus motifs. The antiparallel $\beta$ sheet which functions in binding the ribose and adenine moieties of the bound nucleotide is designated the ABC $\beta$ antiparallel subdomain (Karpowich et al., 2001) or $\beta$ subdomain. The $\beta$-subdomain $\beta$ sheet is characteristic of ABC ATPases and is deployed approximately at right angles to the core subdomain $\beta$ sheet, with helix 1 situated in the angle thus formed, held in place by extensive hydrophobic contacts which thus constitute the interior of lobe I. The core subdomain and the ABC $\beta$ subdomain together constitute lobe/arm I of
the ABC ATPase. The ABC transporter NBD also contains a third subdomain with a structurally conserved core comprising a bundle of three α helices (Figure 13A, B), variously known as arm/lobe II, the helical domain (Ames et al., 1992), the ABC This condition subdomain or α subdomain (Karpowich et al., 2001). The N-terminal halves of helix 3 and helix 5 within lobe II comprise of residues which are, for the most part, highly conserved amongst ABC transporters (Jones and George, 2002). The (L)SGGQ signature sequence forms the N-terminus of helix 5 within the bundle, with the serine side chain capping the helix. Helix 4 and the two loops at its N- and C-termini, which join the two conserved regions in the α subdomain, are highly variable in sequence, length and structure, and the loop regions are the sites of transporter-specific insertions (Figure 13) (Jones and George, 2002), (Smith et al., 2002). Some non-transporter ABC ATPases, such as Rad50, also contain the LSGG sequence, while others which do not, such as MutS, nevertheless contain structural homologues of the capped helix structure.

1.12.5 The ABC dimer

Amongst the crystal structures of ABC ATPases, several different conformations of the ATPase dimer have been observed (Hung et al., 1998), (Smith et al.,...
2002), (Diederichs et al., 2000). However, only dimers in which the LSGG sequence, or the structurally homologous region, completes the ATP binding site in the opposite monomer have been observed more than once, namely in Rad50cd (Hopfner et al., 2000), MutS, BtuCD (Locher et al., 2002) and MJ0796 E171Q (Smith et al., 2002); and these are all akin to our earlier modelled HisP dimer (Jones and George, 1999). This dimer is also most consistent with biochemical and sequence data (Jones and George, 1999). The MJ0796 E171Q dimer structure (Figure 14) represents the only atomic level structure of the complete ABC transporter NBD active site with bound nucleotide - the BtuD dimer contains cyclo-vanadate and the details of the BtuCD dimer interface are unlikely to represent the native structure. Although the catalytic sites in the Rad50cd and MJ0796 E171Q dimers are closely similar and likely represent the natural interaction of the LSGGQ with the ATP-bound active site, it is important to note that neither structure represents the wild-type protein and cannot therefore be taken to prove that the observed 'dimer', with two bound ATP or ATP-analogue molecules, represents a natural physiological state of the NBDs.

1.12.6 ATP: central organizing force of the NBD

Binding of ATP by the P-loop mediates NBD: NBD interactions by altering the surface of the monomer and this is achieved in two ways. First, ATP is sandwiched between the monomers in the NBD dimer and forms a significant part of the dimer interface (Figure 14), acting as the 'glue' between the monomers, with NBD residues making direct contacts with the bound nucleotide rather than with residues from the opposite monomer. Second, comparative analysis of ABC
NBDs suggests that ATP binding, and in particular the g-phosphate, produces an 'induced fit' effect, altering the conformation of the Q-, D-, H- and P-loops in the vicinity of the g-phosphate, thereby altering or ordering the surface of the monomer (Karpowich et al., 2001). Since the P-, H- and D-loops form part of the NBD-NBD interface, as shown in the MJ0796 E171Q and Rad50cd dimer structures, sharing the binding of the g-phosphate with the LSGG region of the opposite monomer, this nucleotide binding-induced fit effect is also likely to be important for NBD 'dimer' formation (Karpowich et al., 2001).

1.12.7 The Q-Loop: A conserved glutamine: the signaller?

A phylogenetically invariant glutamine residue (Q100 in HisP) is located at the C-terminus of b strand 5 (Figure 13). This residue is followed by a flexible loop known as the Q-loop (Hopfner et al., 2000) or g phosphate linker (Jones et al., 1995), which joins the ATP-binding core (lobe I) to the a subdomain (lobe II). Comparative analysis of the NBD crystal structures suggests that the conserved glutamine 'switches' in and out of the active site during the catalytic cycle, engaging the Mg$^{2+}$ATP-bound active site, and disengaging and moving away subsequent to ATP hydrolysis (Hopfner et al., 2000), Jones et al., 1995). This idea is consistent with the mechanism of other P-loop proteins, such as G proteins and the F1-ATPase, in which the Mg$^{2+}$ coordinating protein ligand equivalent to the conserved glutamine is situated on a switch region that mediates oligomeric interactions in response to nucleotide binding and hydrolysis.
Our MD simulations of HisP (Jones and George, 2002) suggest that the Q-loop may undergo conformational switching transitions, and this idea is supported by the structural variability of this region amongst the ABC crystal structures. The structural data also indicate that the Q-loop forms part of the NBD: TMD interface, making contacts with the ICDs in both the Eco-MsbA and BtuCD structures. The high sequence variability amongst ABC transporters of the N- and C-termini of the Q-loop is consistent with our notion that these segments are involved in subunit-subunit interactions (Jones and George, 2002), and there is also biochemical evidence that the conserved glutamine and the Q-loop are involved in interdomain communication (Hunke et al., 2000b),(Hunke et al., 2000a),(Urbatsch et al., 2000). In summary, it appears that through its interactions with the nucleotide and catalytic Mg\(^2+\), the conserved glutamine may signal between the active site and the TMDs, possibly by moderating conformational transitions of the Q-loop, and thereby TMD:NBD interactions (Jones and George, 2002). Yuan et al. (Yuan et al., 2001a) have suggested that interaction of the conserved glutamine with the g-phosphate of the bound

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Figure 15: Model of NBD dimer interactions. Each NBD monomer is represented by two rectangular boxes, corresponding to lobes I and II connected by a thick bar, and the associated Lobe II is indicated by the symbol 'α' and also contains the letters 'LSGG' to indicate the signature sequence. Lobe I contains an oval representing the catalytic site; the letters 'ATP' within this oval indicate bound trinucleotide. The letter 'Q' indicates the conserved glutamine thick bar represents the Q-loop. Schematic representation illustrating a model in which binding of ATP to the NBD induces NBD dimer formation: (i) two nucleotide-free NBD monomers with their respective α subdomains rotated outward from the catalytic site; (ii) ATP has bound to each NBD monomer, and interaction of the conserved glutamine with the g-phosphate has induced the inward rotation of their respective α subdomains; (iii) formation of NBD 'sandwich' dimer with two ATP molecules.
nucleotide mediates the rotation of the α subdomain into the 'closed' conformation, in which its LSGGQ is correctly oriented to engage the opposite catalytic site within the dimer. Since it seems clear that both the conserved glutamine and the LSGGQ must engage an ATP-bound catalytic site, these ideas suggest that ATP binding may mediate formation of a dimer such as that observed for MJ0796 (Smith et al., 2002) and Rad50cd (Hopfner et al., 2000), with ATP bound in both active sites. This idea is illustrated schematically in Figure 15. Interestingly, a recent crystal structure of the GcV monomer with bound ATP analogue (Verdon et al., 2003) shows that the conserved glutamine can engage the catalytic Mg\(^{2+}\) when the α subdomain is rotated outward, and it was suggested that the glutamine may not mediate α subdomain rotation (Verdon et al., 2003). The GcV structure suggests to us that the flexibility of the Q-loop may function, at least in part, to accommodate the outward rotation of the α subdomain while the glutamine remains engaged in the active site. Thus, the nature of the relationship between rotation of the α subdomain and Q-loop transitions is uncertain at present.

1.12.8 ATP hydrolysis: processive or alternating?

In order to mediate the active translocation of substrates across cellular membranes, ABC transporters must couple conformational changes in the NBDs, powered by the free energy released by ATP hydrolysis, to conformational changes in the TMDs. However, the manner in which the energy of ATP hydrolysis is coupled to substrate transport is as yet unclear. Recently a model for this process was proposed in which the energy of ATP binding drives the formation of an NBD 'sandwich' dimer with two ATP molecules bound at the dimer interface, similar to that observed in the MJ0796 E171Q dimer structure (Hopfner et al., 2000),(Smith et al., 2002), thus representing the 'powerstroke' of the transport cycle. In this scheme, hydrolysis of ATP and release of products
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generates electrostatic and conformational changes that drive the monomers apart (Smith et al., 2002). Both dimer formation and separation processes are proposed to provide opportunities to couple free-energy changes to solute transport (Smith et al., 2002). Recent crystal structures have further supported the existence of an NBD sandwich dimer and the notion that the NBDs dimerise and subsequently move apart in the catalytic cycle. Crystal structures of nucleotide-free and ATP bound forms of the maltose permease NBD MalK suggest a tweezers-like movement of the NBDs in which ATP induces formation of a double ATP-bound sandwich dimer and nucleotide hydrolysis and product release results in separation of the NBDs (Chen et al., 2003). An NBD sandwich dimer containing two ATP molecules, structurally similar to the MJ0796 E171Q dimer, has also recently been observed in a crystallographic analysis of the GlcV E166Q mutant, in which the conserved glutamate following the Walker B aspartate, the putative catalytic base, was also changed to glutamine (equivalent to MJ0796 E171Q). An interesting consequence of the sandwich dimer mechanism, however, lies with respect to the ATP hydrolysis cycle of the transporter. Either the NBD monomers must move apart subsequent to ATP hydrolysis in one site only, thus wasting the binding energy of the nonhydrolysed ATP, or there is a processive, asymmetric pattern of ATP hydrolysis, in which the first ATP is hydrolysed with ATP in the second site, and the second ATP is hydrolysed while the first active site either contains hydrolysis products or is empty (Figure 16A, B). Indeed, a processive ‘clamp’ model for the ATPase cycle was recently proposed, based on biochemical data from the isolated NBD of MD11p, a mitochondrial TAP-like half-transporter from Saccharomyces cerevisiae (Janas et al., 2003). Incubation of the wild-type MD11p NBD with orthovanadate or beryllium fluoride and Mg-ATP induced the formation of stable dimers, which contained two ADP molecules, shown to be produced exclusively by ATP hydrolysis in the NBD active site. MD11p NBD
mutant E599Q, in which the conserved glutamate following the Walker B aspartate was changed to glutamine, similarly to MJ0796 E171Q formed stable dimers containing two ATP molecules in the presence of Mg-ATP. Interestingly, the E599Q mutant slowly hydrolysed ATP at 30°C, and a stable dimer containing one ADP and one ATP molecule was identified under limiting concentrations of Mg-ATP. Together these data were interpreted as indicating that binding of ATP to two NBDs induces NBD dimer formation and that ATP hydrolysis occurs in a sequential processive mode (Figure 16; panel 1 & 2). We believe, however, that the central role that the γ-phosphate appears to have in determining the global conformation of the NBD (Karpowich et al., 2001), and its likely influence on the activity of the opposite catalytic site, argues against the notion of an asymmetric sequential hydrolysis mode, and thus the processive clamp model of NBD dimer formation and ATP hydrolysis. In addition, we note that the NBD sandwich dimer has only been observed for mutant or incomplete NBDs or with wild type NBDs in presence of transition-state analogues, or in the case of MalK (Chen et al., 2003), in the absence of magnesium. Significantly, ATP analogues such as AMP-PNP and ATP γS do not induce NBD dimer formation (Janas et al., 2003). Whilst it has been suggested that these analogues may distort the dimer interface, thus prohibiting dimer formation, the crystal structure of the Rad50cd dimer, which has a dimer interface identical in its essential elements to that of the ABC transporter NBD dimer, contains AMP-PNP (Hopfner et al., 2000). Thus, we suggest, the physiological existence of the double ATP-bound NBD sandwich dimer is as yet uncertain.

An alternative scenario is that the NBDs remain in close proximity and ATP hydrolysis occurs in a continuous alternating cycle in which ATP is hydrolysed when the opposite site contains products or is empty. This idea is illustrated in figure 16; panel 1 & 2. There is biochemical evidence that ATP is
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Model 1: ATP binding provides the power-stroke

Model 2: ATP Hydrolysis provides the power-stroke

Figure 16: Proposed models for Drug transport and ATP hydrolysis by P-gp.
Introduction

bound in one active site while ADP is bound in the opposite site for P-gp (Qu et al., 2003), Mdl1p (Janas et al., 2003) and MutS. Moreover, in P-gp, one active site is empty in the vanadate-trapped transition state, consistent, we believe, with the idea that products are expelled from the active site upon hydrolysis in the opposite site. In the alternating mechanism, nucleotide exchange could occur upon outward rotation of the \( \alpha \) subdomain and disengagement of the LSGGQ with the active site, as suggested by Yuan et al. (Yuan et al., 2001a). This leaves the problem of how the NBD dimer could be stabilized with only one nucleotide bound, although it is possible that this could occur through interactions with the TMDs (Yuan et al., 2001a). This alternating mode also appears to better accommodate the expected negative cooperativity between the NBDs, since in this mechanism there is always an asymmetry in the conformation of the two monomers in the NBD dimer (Figure 16B).

1.12.9 The D-loop: central switch of the NBD mechanism?

\( b \)-strand 6 forms part of the core subdomain \( b \) sheet (Figure 13) and corresponds to the Walker B consensus motif. A highly conserved aspartate residue at its C-terminus is involved in the coordination of the catalytic magnesium ion (Figure 11). In ABC ATPases, the Walker B aspartate is followed immediately by a highly conserved glutamic acid residue (Figure 12), postulated to act as the catalytic base of the hydrolysis reaction (Hung et al., 1998), and thence by a conserved six-residue loop known as the D-loop (Hopfner et al., 2000). While the conserved glutamate at the N-terminus of the D-loop interacts with the active site within the monomer, a backbone oxygen atom near the C-terminus of the D-loop interacts with the putative nucleophilic water in the active site of the opposite monomer in both the Rad50cd and MJ0796 E171Q dimers. The D-loop corresponds, in terms of its position in the protein fold, to an important switch region in other P-loop ATPases such as myosin and G proteins.
Like the corresponding switch regions in other P-loop ATPases, the ABC D-loop shows significant structural variation among the crystal structures, and this implied hyperflexibility is supported by MD simulations, which revealed heightened torsion angle and positional fluctuations for residues within this region in HisP (Jones and George, 2002). In view of its potential ability to influence hydrolysis in both catalytic sites of the dimer, we and others have suggested that the D-loop may be involved in communication between the catalytic sites (Hopfner et al., 2000), (Jones and George, 1999). Changes in the conformation of the D-loop are able to affect the orientation of key residues in the catalytic sites, particularly the putative catalytic base, thereby enabling and/or preventing hydrolysis in each active site, and we thus suggest that the D-loop appears well suited to mediate negative allostery between the ABC NBD monomers.

1.12.10 The H-Loop: What is the role of the conserved active site histidine?

In all ABC transporter NBD crystal structures, with the exception of TAP1, a conserved histidine residue occurs at the C-terminus of b strand 7, and this residue is followed by a short, approximately six-residue a helix (Figure 13). This region is known collectively as the H-loop (Hopfner et al., 2000) and corresponds approximately to a region earlier referred to as the switch region (Schneider and Hunke, 1998). Comparison of the X-ray structures of ABC NBDs reveals that the position of the histidine, and of the immediately downstream helix 7, is quite variable with respect to the central b sheet of the nucleotide-binding core, with this region appearing to pivot about C a atoms at its N- and C-termini, a notion supported by our MD simulations of HisP, which reveal hinges at these points. The function of the conserved histidine is not known. Mutation of this residue to arginine in both HisP and in MalK resulted in complete loss of ATPase activity of
the purified mutant NBD subunits, and loss of transport function in the complete transporter. The location of the histidine within the catalytic site, its high conservation and the effects of its mutation indicate clearly that it has a crucial role in transporter function. Indeed, we observe that in the catalytic site of the MJ0796 E171Q dimer, the histidine is well positioned to shield the attacking nucleophile from the electronegative charge of the g-phosphate oxygen atoms, possibly indicating a role in catalysis for this residue.

1.13 Summary and overview: the big picture

The g-phosphate of the bound nucleotide is the central focus of the ABC transporter NBD both structurally and functionally. The g-phosphate plays a crucial organizing role, altering and stabilising the conformation of the key P, Q-, D- and H-loop regions and thereby influencing subunit-subunit interactions within the transporter complex. While their exact roles are unknown, the Q-, D- and H-loops appear to be important switch regions of the NBD mechanism, with the Q-loop likely to mediate signaling between the TMDs and the NBD active sites, and the D-loop to influence the catalytic activity and intercommunication of the active sites. The α subdomain (lobe II) appears to undergo a mechanistically important rotational movement in relation to lobe I, and this rotation may influence ATP hydrolysis by correctly orienting the LSGGQ signature sequence to enable NBD ‘dimer formation’. The engagement of the LSGGQ within the α subdomain with the ATP-bound catalytic site in the opposite monomer is essential for ATP hydrolysis and appears to be determined by the TMDs, thus representing a possible allosteric mechanism by which the TMDs control ATP hydrolysis. It has been suggested that the conserved glutamine and the Q-loop mediate the rotation of the α subdomain in response to
ATP binding, but the exact nature of Q-loop transitions and their relationship to lobe II rotation is unclear.

Two models exist for the transduction of the free energy of ATP to conformational work in ABC transporters. In the first of these, ATP binding in both NBDs promotes dimer formation, and the energy of ATP binding ultimately drives substrate translocation. In the second model (Jones and George, 2002), the NBDs remain pre-oriented with one or both catalytic sites being engaged and completed by the LSGGQ of the opposite monomer at all times. The α subdomains alternately rotate during cycles of engagement/disengagement with the catalytic site and concomitant ATP hydrolysis, in response to signals from the TMDs. The free energy of ATP is harnessed by conformational gearing to active site geometry during the formation and collapse of the transition state of ATP hydrolysis.

1.14 AIMS AND OBJECTIVES: The big questions in Cdr1p NBD research

Cdr1p, a major ABC transporters of Candida albicans is not only involved in efflux of drugs, but also in steroid efflux, and plays a role in maintaining the phospholipid asymmetry across the two monolayers of the plasma membranes (Krishnamurthy et al., 1998b; Krishnamurthy et al., 1998c; Smriti et al., 1999; Dogra et al., 1999). Cdr1p, like any other member of the ABC super family of membrane proteins, has four distinct modules: two transmembrane domains (TMDs) consisting of six transmembrane segments (TMSs) and two nucleotide binding domains (NBDs) located on the cytosolic side of the membrane. The NBDs, which couple energy of ATP hydrolysis to power drug export are highly conserved throughout the evolutionary scale (Holland et al., 2003), (Walmsley et al., 2003). Each NBD contains three characteristic sequence motifs: the Walker A and Walker B motifs, which form the nucleotide binding site (Walker et al., 1982), and an ABC
signature sequence, or C motif, for which several functions have been proposed, including communication between the TMDs and NBDs during the transport cycle (Schneider and Hunke, 1998).

In general, in ABC transporters, loop regions immediately C-terminal to the Walker B and other core b strands make contacts with the g-phosphate of the bound nucleotide, and constitute important switch regions, which undergo conformational changes during the catalytic cycle. In ABC transporters, three highly conserved active site residues have been observed to make contact with the g-phosphate in various crystal structures, which are known as the Q-, D- and H-loops, respectively. The role of these active site residues and their associated loops in the mechanism of the NBD is a key question in current research of Cdr1p, an ABC transporter of C. albicans.

In contrast to most ABC transporters, the NBDs of all other fungal transporters of the super family including Cdr1p, have unique positioning of a typical amino acid Cys193 in the Walker A, Glu238 in the Q-loop, Trp326 and Asn328 in and adjacent to Walker B motifs of N-terminal NBDs, respectively (Decottignies and Goffeau, 1997). Thus the otherwise much conserved N-terminal NBD domains of fungal transporters have the distinction of unique positioning of typical amino acid residues. On the other hand, the C-terminal NBD of Cdr1p and other ABC fungal transporters possess perfectly conserved motifs which are essentially identical to those in non fungal transporters (Decottignies and Goffeau, 1997). Based on sequence analyses, it was earlier suggested that the N-terminal NBD of Pdr5p (a close homologue of Cdr1p) of S. cerevisiae is probably unable to perform ATP hydrolysis and that the transporter might function with only one of its two NBDs (Decottignies and Goffeau, 1997). The functional asymmetry of NBDs in Cdr1p was illustrated in our recent study where swapping of NBDs resulted in non-functional Cdr1p chimeras and thus suggested that the two NBDs are non exchangeable (Saini et al., 2006).
together, our results confirm that the two potential ATP binding sites of Cdr1p as well as of other fungal ABC transporters are not identical yet are functional. But other important questions which still remain unanswered include: Why conserved residues are uniquely replaced in N-terminal NBD of Cdr1p? What is their functional significance? How is ATP binding and hydrolysis in the NBDs coordinated and controlled by the TMDs? How is the free energy of ATP harnessed to produce conformational changes that result in substrate translocation? How is ATP hydrolysed - processively or alternately? In the catalytic cycle, do the NBDs come together to form a dimer and subsequently separate or do they remain in direct contact? Present work centers on to address some of these questions.

The first chapter of thesis deals with critical but a typical substitution, Trp326 of NBD-512, which is uniquely positioned in Walker B preceding a well-conserved Asp327. We show that out of the five tryptophan residues, only the uniquely positioned Trp326 of Walker B is important for ATP binding without being directly implicated in the catalytic activity of the purified NBD-512 domain. Further, we show that this effect is independent of the adjacent conserved Asp327 in the Walker B of NBD-512.

In the next section (Chapter II), the precise role of Asp327 of N-terminal NBD in ATP binding and hydrolysis is further explored and is compared with its counterpart Asp1026 of C-terminal NBD. Our results show that because of replacement of conserved Glu with Asn at 328 position in N-terminal NBD of Cdr1p and as well as other fungal ABC transporters, the conserved Asp327 of the Walker B motif of N-terminal NBD has acquired a new role to act as a catalytic base for ATP hydrolysis.

In the Chapter III of the thesis, we have employed Förster Resonance Energy Transfer (FRET) to get a deeper insight into the spatial proximity of the atypical residues such as Cys193 of Walker A, Trp326, Asp327 and Asn328 of
Walker B and Glu238 of Q loop of N-terminal NBD of Cdr1p. We show that FRET is enhanced in the presence of Mg$^{2+}$ and thus for the first time confirm that Cys193 and Trp326 draw closer in the presence of Mg-ATP within the nucleotide binding pocket. Our FRET results establish that Mg$^{2+}$ is coordinated by Trp326 and Glu238 and not by the conserved Asp327 of the Walker B motif. This study also highlights the role of uniquely placed Asn328 that acts as a γ-phosphate sensor and is important for subsequent conformational changes. Taken together, it appears that the uniquely placed residues in N-terminal NBD of Cdr1p have acquired critical role in ATP catalysis which drive drug extrusion.