Review Article

Efflux Pump-Mediated Resistance in Chemotherapy

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Abstract

Efflux pump mechanisms perform important physiological functions such as prevention of toxin absorption from the gastrointestinal tract, elimination of bile from the hepatocytes, effective functioning of the blood–brain barrier and placental barrier, and renal excretion of drugs. They exist in all living cells, but those in the bacterial and mammalian cells are more important to the clinician and pharmacologist, as they constitute an important cause of antimicrobial drug resistance, which contributes to treatment failure, high medical bills, and increased mortality / morbidity. This review was aimed at highlighting the role of efflux pump mechanisms in microbial resistance to chemotherapeutic agents. It was also aimed to elucidate their structure and mechanisms of action so as to integrate the efflux pump mechanisms in the design and development of novel antimicrobial agents.Findings from previous studies and research on this subject assessed through Google search, Pubmed, Hinari websites, as well as standard textbooks on chemotherapy, provided the needed information in the process of this review. Efflux pump inhibitors are promising strategies for preventing and reverting efflux-mediated resistance to chemotherapeutic agents. They are usually employed as adjuncts in antimicrobial and cancer chemotherapy. Toxicity, more common with the older-generation inhibitors such as verapamil and reserpine, constitutes the greatest impediment to their clinical applications. No efflux pump inhibitor has been approved for routine clinical use, as a result of doubtful clinical efficacy and unacceptably high incidence of adverse effects, particularly inhibition of the P-450 drug metabolizing enzyme. At present, their applications are mainly restricted to epidemiological studies. Nonetheless, the search for efficacious and tolerable efflux pump inhibitors continues because of the potential benefits. There is a need to consider efflux pump substrate selectivity in the design and development of novel chemotherapeutic agents.

Keywords: Efflux pump, Chemotherapy, Drug resistance

Introduction

The problem of resistance to chemotherapeutic agents is perhaps one of the greatest challenges in clinical medicine. Drug resistance in malaria, Human Immunodeficiency Virus (HIV)/Acquired Immune Deficiency Syndrome (AIDS), bacterial infections, and cancer has continued to challenge the ingenuity of pharmacologists and physicians alike. Causes of drug resistance include irrational drug prescription and usage, abuse of antimicrobials, and substandard pharmaceuticals.[1-3] Implications of drug resistance include increased mortality and morbidity, increased cost of medical treatment, diagnostic uncertainties, and loss of faith in orthodox medicines. There is a need, therefore, for a continued search for more efficacious and more tolerable antimicrobial agents. This review helps in elucidating the mechanisms of action and chemical structures of efflux pumps, and thus helps in the design of novel antimicrobial agents.

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The Methods of Literature Search

The information needed in this review was sourced from Google search, Pubmed, and Hinari websites, as well as from standard textbooks on chemotherapy. Key words used for the search include, efflux pump, resistance, chemotherapy and antimicrobials.

Mechanisms of Resistance to Chemotherapy

Antimicrobial challenge to microorganisms is a stress to which the organisms respond by developing resistance. Resistance in an organism can be acquired vertically from an organism to its offspring or horizontally between organisms by conjugation, transduction, and transformation. Genes for resistance are usually carried on plasmids. There are four main mechanisms by which an organism or a cell exhibits resistance to chemotherapeutic agents:

Drug inactivation

a. Inactivation of penicillin G by β-lactamases in penicillin-resistant bacteria.
b. Inactivation of chloramphenicol by the production of chloramphenicol acetyltransferase in resistant organisms.
c. Inactivation of aminoglycosides through phosphorylation, adenylation, or acetylation via aminoglycoside modifying enzymes.

Modification of drug binding sites

a. Alteration of the 30s subunits of the ribosome in aminoglycoside resistance.
b. Alteration of the DNA gyrase protein in fluoroquinolone resistance.
c. Alteration of the penicillin binding protein (PBP) in penicillin-resistant bacteria.

Alteration of the metabolic pathway

Alteration of dihydropteroate synthase in sulfonamide resistance.

Reduced intracellular drug accumulation

a. By decreasing drug influx as a result of mutations involving polysaccharide outer membrane (porins) in Gram-negative organisms.[4]
b. By increasing efflux of drugs from the intracellular compartment via energy-dependent efflux pumps. This mechanism is very common in resistance to tetracyclines, erythromycin, and fluoroquinolones. It is also the main mechanism of resistance in cancer chemotherapy.[5] This efflux pump-mediated resistance to chemotherapy is the focus of this review.

Efflux Pump Mechanisms

Efflux pumps, expressed in all living cells, protect the cells from the toxic effects of organic chemicals. An individual pump recognizes a large number of compounds as substrates because recognition is based on physical properties rather than on defined chemical structures, as in enzyme–substrate recognition. The efflux pump systems may be broadly divided into two:

a. Prokaryotic efflux pumps that mediate resistance in bacteria and viruses.
b. Eukaryotic efflux pumps that mediate drug resistance in fungi, protozoa, and cancer cells. The division is incomplete as some pumps mediate resistance in both prokaryotic and eukaryotic cells.

Bacterial efflux pumps

The prokaryotic (bacterial) efflux pumps are divided into six classes:[6]

a. Major facilitator superfamily (MFS)
b. ATP-binding cassette (ABC) superfamily
c. Small multidrug resistance (SMR) family
d. Resistance-nodulation cell division (RND) superfamily
e. Multi-antimicrobial extension (MATE)
f. Drug metabolite transporter (DMT) superfamily

Antibiotics can act as inducers and regulators of expression of efflux pumps.[7] Several efflux pumps can be expressed on a given bacterial species, thus conferring on it resistance to many antimicrobials. The ABC efflux pumps are *adenosine-5′-triphosphate* (ATP)-dependent (primary transporters) and others are drug-proton antiporters and are the major efflux pumps involved in multidrug resistance.[8]

Efflux pumps in eukaryotes

These are divided into five groups:

a. Monocarboxylate transporter (MCT)
b. Multidrug resistance protein (MDR, P-glycoprotein)
c. Multidrug resistance-associated proteins (MRPs)
d. Peptide transporters (PEPTs)
e. Na^+ phosphate transporters (NPTs)

Structure of Efflux Pumps

In Gram-negative bacteria, characterized by a protective double membrane system, a typical efflux pump consists of the following four components:

a. Outer membrane proteins
b. Middle periplasmic protein
c. Inner membrane protein
d. Transmembrane duct

The periplasmic membrane protein interacts with the outer and inner membranes to stabilize the duct (channel) in a closed state. Opening of the duct is triggered by binding of the drug to the inner membrane protein and the energy-dependent, protein–protein interaction between the outer membrane protein and periplasmic membrane protein. The inner membrane transporter provides energy by exchanging the substrate (drug) with H^+. Figure 1 shows schematic drawing of the structure of an efflux pump.[9]

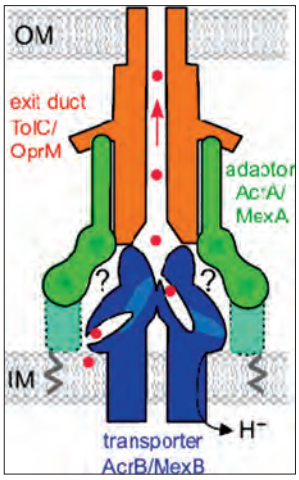

Figure 1: Schematic of the tripartite multidrug efflux pump. The pumps comprise an exit duct (shown in orange; TolC in *E. coli*, OprM in *P. aeruginosa*) anchored in the outer membrane (OM), an integral inner membrane (IM) transporter (in blue; AcrB and MexB) and a periplasmic adaptor protein (in green; AcrA and MexA) linked to the inner membrane by a fatty acid (zigzagline). The adaptor binds the exit duct α-helical domain via it's α-hairpin (23) and thre transporter via it's α-hairpin (23) and the transporter via unknown interactions (indicated by ?). The adaptor linear multidomain structure is characterized by interdomain flexibility, but it is incompletye, missing the MP domain indicated by the dotted outline. Red dots indicate antibacterial drugs bound to putative pockets in the transporter, passing through TolC (arrowed), and out of the cell[9]

Physiological Functions of Efflux Pumps

Under normal physiological conditions, efflux pumps are expressed in organs involved in elimination of endogenous waste and xenobiotics, such as the kidney, liver, and epithelial tissues that protect important organs like the small intestine, placenta, blood–brain barrier, and the testes.[10] In the small intestine, the efflux pump-mediated mechanism limits the absorption of several drugs.[11]

The brain is well protected against toxins and drugs. In addition to the anatomical modification of cerebral endothelial cells in the blood–brain barrier, the efflux pumps ensure that harmful substances that enter the brain cells are pumped out.[10] Wang *et al.* demonstrated that knock-out mice lacking P-glycoprotein exhibited higher brain concentrations of peripherally administered vincristine compared to normal mice.[12]

The active efflux of drugs by placental efflux pumps help to maintain their barrier function. These pumps, mainly the ABC type, are located on the maternal surface of the syncytial membrane of placental microvilli.[13]

The blood–testis barrier (BTB), formed by the adjacent membranes of Sertoli cells, protects the spermatozoa from the effect of harmful substances and drugs as well as forms immunological sanctuaries so that antibodies are not formed against sperm cells during spermatogenesis. Efflux pumps located in the Sertoli cell membranes help in this protection by pumping out drugs and other harmful substances.[14]

P-glycoprotein and MRP_2 secrete metabolites such as conjugated bilirubin out of the hepatocytes into the bile canaliculi;[15,16] genetic absence of MRP_2 causes decreased excretion of bilirubin in the Dubin–Johnson syndrome.[17] P-glycoprotein mediates the tubular secretion of cholesterol and uric acid, thereby protecting the proximal tubular epithelial cells from cellular injury.[18,19] P-glycoprotein located on the luminal membrane of renal epithelial cells actively secretes digoxin, ceimetidine, and many other drugs, and the efflux pump inhibitors such as verapamil, reserpine, vinblastin, and daunorubicin inhibit the tubular secretion of digoxin.[20]

Overcoming Efflux-mediated Resistance

The strategies to overcome efflux-mediated drug resistance include the following:

Bypassing the efflux pump

Structural analogs of an antimicrobial agent show differences as substrates for efflux pumps. The newer generation agents are less susceptible to efflux pumps than the older generation agents of the same class. For example, the glycyclines are less susceptible than tetracyclines, and ketolides are less susceptible than macrolides.[21,22]

Biological inhibition of the efflux pump

Being proteins, the efflux pumps could be neutralized by antibodies. Alternatively, translation of the genes coding for these efflux pumps could be prevented by using antisense oligonucleotides. Oethinger *et al.* demonstrated that deletion of the AcrB gene in *E. coli* restored its sensitivity to fluoroquinolones.[23]

Pharmacological inhibition of efflux pumps

Drugs that are competitive and non-competitive inhibitors of efflux pumps are used to reverse or prevent the development of efflux-mediated drug resistance. These efflux pump inhibitors are usually used as adjuncts in therapy.

Efflux Pump Inhibitors

Efflux pump inhibitors prevent the energy-dependent efflux of drugs and some endogenous metabolites from the cells. They are a promising strategy for restoring the activity of existing antimicrobial agents. A majority of the efflux pump inhibitors are not used as pump inhibitors in routine clinical practice because concentrations that achieve efflux inhibition *in vitro* are rarely achieved *in vivo* without serious toxicities.[24] At present, many are used for epidemiological surveys of drug-resistant organisms. In this direction, ethidium bromide is an efficient substrate for many MDR pumps and is used to assess the effect of inhibition on such pumps.

An ideal efflux pump inhibitor should:

a. restore the activity of an antimicrobial in both intrinsic and acquired resistance;
b. have a wide range of activity against Gram-positive and Gram-negative bacterial pumps; and
c. not affect the physiological efflux pumps so as to minimize adverse effects.

Mechanism of action of efflux pump inhibitors

Minimum inhibitory concentrations (MIC) are very important in assessing the inhibitory actions of efflux pumps. Couto*et al*. (2008) demonstrated that reduction of a drug's MIC by at least a quarter of its original value by addition of an efflux pump inhibitor was indicative of efflux activity.[25]

a. Mechanisms of action of efflux pump inhibitors
b. Non-competitive blocking of the drug-binding site on the efflux pump
c. Dissipating the energy source of the efflux pump[26]

Classification of efflux pump inhibitors

Some efflux pump inhibitors are naturally occurring lipophilic alkaloids, terpenoids, and flavonoids, while others are rationally designed by manipulation of molecular structures of pump substrates.[27] Classification of efflux pump inhibitors along the line of efflux pumps is difficult because just like the pumps, some inhibitors are pump specific, while others are not. The closest attempt at classification is based on the effect on bacterial and mammalian efflux pumps:

a. Microbial efflux pump inhibitors
b. Mammalian efflux pump inhibitors

Some efflux pump inhibitors such as verapamil and reserpine inhibit both microbial and mammalian efflux pumps.

Microbial efflux pump inhibitors

As Gram-negative bacteria usually have a thick outer wall protection, which does not allow easy access of substances, the effects of efflux pumps and consequently efflux pump inhibitors are more pronounced in them as compared to the Gram-positive organisms. Microbial efflux pump inhibitors include:

- Analogs of antimicrobial agents such as tetracyclines, aminoglycosides, and fluoroquinolones. Modifications in these analogs enable them to escape efflux pumps.[22]
- Peptidomimetics. The prototype here is phenylalanine arginyl-β-naphthylamide (PAβN).
- Amide derivatives
- Quinolone derivatives, for example, quinazolinones
- Phenothiazines, for example, chlorpromazine
- Selective serotonin reuptake inhibitors (SSRI), for example, paroxetine
- Protein pump inhibitors, for example, omepraole, pantoprazole

Mammalian efflux pump inhibitors

A broad division of mammalian efflux pump inhibitors includes:

a. P-glycoprotein inhibitors
b. First-generation P-gp inhibitors, for example, verapamil, nifedipine, lovastatin, simvastatin, cyclosporine A, tamoxifen, ketoconazole, erythromycin, progesterone
c. Second-generation P-gp inhibitors, for example, valspodar, biricodar, timicodar
d. Third-generation P-gp inhibitors, for example, elacridar, zosupidar, tariquidar
e. MRP inhibitors, for example, argosterol A, ralozitene
f. Breast cancer receptor protein (BCRP) inhibitors, for example, elacridar, reserpine

Previous Works on Efflux Pump Inhibitors

Efflux pump inhibitors for microbial infections

Most reports on bacterial efflux pump inhibitors are based on *in vitro* laboratory experiments. One major impediment for the use of these inhibitors in treating clinical infections is the high plasma concentrations of these drugs needed to achieve efflux pump inhibition *in vivo*.

Zhang *et al.* demonstrated that reserpine reversed the resistance of *Mycobacterium tuberculosis* to isonicotinylhydrazine (INH) and pyrazinamide.[28] Also, Cui *et al.* observed decreased MIC of anti-TB agents to resistant *M. tuberculosis* strains in a liquid culture, after addition of verapamil and reserpine.

Overexpression of the efflux pump genes *RV2459* and *RV3728* was induced by administration of combined INH and ethambutol Gupta, and Ramon-Garcia *et al.* demonstrated that deletion of the *RV1410C* gene (which encodes the P55 efflux pump in the mycobacteria) made the organisms more susceptible to first- and second-line anti-TB drugs.[30,31]Farnesol significantly enhanced accumulation of ethidium bromide in *Mycobacterium smegmatis* and showed significant synergism when combined with rifampicin.[32]

Omeprazole inhibited the NorA pump of Gram-positive bacteria, while pantoprazole restored the antibiotic susceptibilities of the multidrug-resistant strains of *Helicobacter pylori,* as they significantly reduced the MIC of these antibiotics.[28,33] Reserpine enhanced the activity of fluoroquinolones on the multidrug-resistant, Gram-positive bacteria, decreased the emergence of resistant strains in *Staphylococcus aureus* and *Streptococcus pneumoniae*, and improved the susceptibility to tetracycline in methicillin-resistant *Sta. aureus*.[22,34]

Chlorpromazine enhanced the antimicrobial activity of aminoglycosides and macrolides, and also had a synergistic effect in combination with penicillin G against *E. coli* by inhibition of bacterial efflux pumps.[35,36]

Plasmodium falciparum (causative organism of malignant malaria) developed resistance to chloroquine, proguanil, and pyrimethamine, which was partly traced to a decreased accumulation of these drugs by the falciparum organism due to development of the *P. falciparum* multidrug-resistance gene.[37] In other *in vitro* studies, verapamil restored the chloroquine concentrating ability and sensitivity in *P. falciparum.* Bray *et al*. had earlier demonstrated that verapamil reversed chloroquine resistance in *P. falciparum* in a dose-dependent manner, suggesting competition between chloroquine and verapamil for the organism's MDR pump.[38]

Lee *et al.* observed that milbemycins were potent inhibitors of the CDR1 pump in *Candida albicans* and these drugs potentiated the antifungal activity of fluconazole against a wide variety of *C. albicans* clinical isolates.[39] Similarly, quinazolinones have been identified as inhibitors of fungal efflux pumps.[40]

Drug resistance is becoming a big challenge in the fight against HIV / AIDS. An efflux-mediated mechanism contributes to this resistance. It has been demonstrated that long-term administration of anti-retroviral drugs contribute to efflux-mediated resistance by inducing expression and function of P-glycoproteins.[41,42]Khaliq*et al.* demonstrated that ketoconazole (P-glycoprotein inhibitor) increased the *cerebrospinal fluid* (CSF) levels of ritonavir and saquinavir.[43]

Efflux pump inhibitors for cancers

P-glycoprotein is encoded by the *MDR1 g*ene and its overexpression in cancer cells contributes to resistance in cancer chemotherapy. The P-glycoprotein inhibitors are competitive substrates for P-glycoproteins; therefore, they increase the intracellular concentration of co-administered anticancer drugs. Many of the agents entered clinical trials, but none was successful due to an unacceptably high incidence of adverse effects. The observed toxicities were mainly a result of the competitive inhibition of cytochrome P-450 enzymes, which increased the plasma concentration of the co-administered anticancer agents.[44] The physiological efflux pumps were also inhibited.

This high incidence of adverse effects was very common with the first- and second-generation P-glycoprotein inhibitors, such as verapamil, cyclosporine A, valspodar, and biricodar. Third-generation P-glycoprotein inhibitors were designed to specifically inhibit only P-glycoprotein, and thus reduce the adverse effects. However, clinical trials using the third-generation P-glycoprotein inhibitors produced conflicting results.[45] In a phase I clinical trial, van Zuylen *et al*. found that a combination of R101933 (laniquidar, a third-generation P-glycoprotein) with docetaxel, in the treatment of solid tumors, did not alter the plasma pharmacokinetics of the latter, suggesting that R101933 did not influence the disposition of docetaxel.[46] Some newer products, for example, R-verapamil had minimal side effects when combined with anticancer agents, but exhibited poor clinical response.[47,48] Toppmeyer *et al.* demonstrated the efficacy of biricodar (Incel) when combined with paclitaxel in the treatment of advanced breast cancer, as compared to paclitaxel alone.[49] However, in a phase II clinical trial in which biricodar was combined with doxorubicin and vincristine, in patients with recurrent small cell lung cancer, Gandhi and his colleagues did not observe enhanced antitumor activity.[50]

Flavonoids as efflux pump inhibitors

Flavonoids (herbal constituents) also exhibited P-gp inhibitory and direct antitumor activity, thereby acting synergistically with taxanes, vinca alkaloids, and campothecins, in cancer chemotherapy.[51] Table 1 shows some efflux pump substrates and their inhibitors.

Adverse Effects of Efflux Pump Inhibitors

Available laboratory data indicate that many drugs are

Table 1: Efflux pump substrates and inhibitors in some microorganisms and cancer cells

Bacteria (or cell)	Substrate	Efflux inhibitor	References
Str. pneumoniae, Sta. aureus	Fluoroquinolones	Reserpine	[52]
E. coli	Tetracycline	Chlorpromazine	[53]
E. coli, M. smegmatis	Tetracycline	Verapamil	[54]
E. coli, Sta. aureus	Norfloxacin, tetracycline	Paroxetine	[55]
Sta. aureus	Fluoroquinolones	Omeprazole	[56]
Sta. aureus	Norfloxacin	Flavonoids	[57]
M. tuberculosis	Fuoroquinolones	Verapamil, reserpine	[29]
M. tuberculosis	Rifampicin	Farnesol	[32]
P. falciparum	Chloroquine	Verapamil	[38]
C. albicans	Fluconazole	Melbemycins	[39]
Human	Ritonavir,	Quinazolinones	[40]
Immunodeficiency virus	Saquinavir	Ketoconazole	[43]
Cancer cells	Docetaxel	R-verapamil	[48]
			[47]
Cancer cells	Taxanes, vinca alkaloids	Flavonoids	[51]

substrates for both efflux pumps and the cytochrome p-450 A34 metabolizing enzyme. P-glycoprotein inhibitors also inhibit cytochrome P-450 enzymes that metabolize anticancer agents, thus leading to increased toxicity when they are co-administered with these agents.

This is particularly so with the first- and second-generation P-glycoprotein inhibitors. For example, Ebert *et al.* demonstrated that the co-administration of the P-glycoprotein inhibitor erythromycin and cardiac glycosides (digoxin) in hospitalized patients was associated with increased serum concentration of the latter.[58]Wakasugi*et al*. had earlier shown that clarithromycin increased the plasma concentration of co-administered digoxin by inhibition of P-glycoprotein–mediated renal excretion.[59] Verapamil and reserpine increased the cytotoxicity of taxols, anthracyclines, and vinca alkaloids by inhibition of P-glycoprotein–mediated efflux.[60]

The second-generation P-glycoprotein inhibitor valspodar inhibited the P-450 3A4-mediated metabolism of paclitaxel and vinblastine, resulting in an increased serum concentration of these agents.[61] This often necessitated a reduction in doses of anticancer agents with attendant reduction in clinical response.

These older-generation P-glycoprotein inhibitors also inhibit physiological efflux pumps such as those involved in blood–brain barrier, BTB, and placental functions.[62]

Generally, third-generation P-glycoprotein inhibitors exhibit a decreased incidence of toxicity when co-administered with other drugs.[49,63] Thus, tariquidar, laniquidar, and zosuquidar do not affect cytochrome P-450 3A4 at relevant concentrations; also, they do not affect physiological efflux pumps.[64]

Efflux pump inhibitors also exhibit adverse effects not related to efflux pump or the cytochrome P-450 enzyme function. Such effects include arrythmias (verapamil), immunosuppression (cyclosporin A), vaginal bleeding (tamoxifen), allergic hepatitis (ketoconazole), and cholestatic hepatitis (erythromycin).

Conclusion

Efflux pump-mediated mechanisms contribute to resistance in chemotherapy. As promising as efflux pump inhibitors appear to be, none has been approved for routine clinical use as a result of doubtful clinical efficacy and unacceptably high incidence of adverse effects. At present, their applications are mainly restricted to epidemiological studies. These drawbacks, notwithstanding, the search for efficacious and tolerable pump inhibitors continues because of the potential benefits. With such an agent, most chemotherapeutic agents rendered useless by efflux-mediated resistance will become useful again.

Way forward

a. Consider efflux pump substrate selectivity in the design and development of novel chemotherapeutic agents.
b. Structural elucidation of efflux pumps will help to develop more effective / specific inhibitors.
c. There is a need to screen natural herbs for efflux pump inhibitory activity.

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