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SECONDARY METABOLITES FROM PLANTS AS A SOURCE OF EFFLUX PUMP INHIBITERS OF BACTERIA

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ABSTRACT:

Antibiotic resistance has become a major clinical problem nowadays. Multidrug resistance efflux pumps are an important cause of antibiotic resistance in bacteria. Antibiotic efflux is one of the major mechanisms by which bacteria pump out the antibiotics from their cellular interior to the external environment with the help of special transporter proteins called efflux pumps. Inhibiting these pumps seems to be a good strategy at a time when novel antibiotic supplies are diminishing. Molecules capable of inhibiting these pumps, known as efflux pump inhibitors (EPIs), have been viewed as potential therapeutic agents that can revitalize the activity of antibiotics that are no longer effective against bacterial pathogens. These pumps are responsible for the intrinsic resistance of bacteria to antibiotics. EPIs, both chemically and plant derived have been discovered till date against many important bacteria. Initially discovered EPIs have a drawback that they are toxic at the concentrations required for their EPI activity. Some plant extracts have the potential for containing such compounds based on their substantial synergistic activity with the antibiotics. Many of these EPIs are well studied for their action on the specific pumps and the drug so effluxes. Some of the EPIs are broad spectrum while some narrow-spectrum acting only against one drug/EP family.

KEYWORDS: Antibiotic resistance, Efflux pumps, EPIs, Bacteria, Broad-spectrum.

INTRODUCTION:

In today's era we are facing ever increasing numbers of antibiotic Resistance. According to World Health Organization the post-antibiotic era has reached global life becoming serious life-threatening. This can be traced back to an extensive misuse of antibiotics in farming and noncompliance in humans. Resistances are naturally occurring in bacteria but the uncontrolled use of Antibiotics increases the selective pressure and therefore further promotes the spread of resistances (WHO 2014). In the 1950s, the phenomenon of multidrug-resistant (MDR) strains was first reported in enterobacteria (Fernández and Hancock 2012). Nowadays, even total drug-resistant strains are reported (Velayati et al. 2009).

Linezolid, daptomycin and bedaquiline are the newest Antibiotics which have entered the market against MDR strains since 2000, but even some years later first resistances are reported against these back-up cure (U.S. Food and Drug Administration 2015; Tsiodras et al. 2001; Lewis et al. 2005; Andries et 2014).

Therefore, alternatives to treat MDR strains are desperate in need. There are some promising compounds in the pipeline (Ling et al. 2015; Gavrish et al.

2014; Roche 2015), but it can be assumed that these substances will most likely not meet the public health needs (Freire-Moran et al. 2011).

There is a plenty of ways how bacteria can become resistant to antibiotics. Intrinsic resistance of bacteria substantially contributes to the development and implementation of resistances. This is mediated by reduced permeability due to lipophilic cell wall, porins and efflux pumps (EP) present at the cell walls. In combination with drug degrading enzymes these tools ensure survival of bacterial cells (Fernández and Hancock 2012).

Efflux Pumps in Bacteria

The first incident of resistance due to efflux was observed in *E. coli* against tetracycline. (McMurry LM et al., 1980). In bacteria there are five major families of efflux transporters:

1. MF (major facilitator), 2. MATE (multidrug and toxic efflux), 3.RND (resistance nodulation-division), 4. SMR (small multidrug resistance), and 5.ABC (ATP binding cassette).

All these families utilize the proton motive force as an energy source, apart from the ABC family, which utilizes ATP hydrolysis for the export of substrates. MFS and RND are the most abundant pumps. MFS is found in both Gram positive and Gram-negative bacteria while RND is found only in Gram negative bacteria. (Han XY et al., 2007). In Gram-negative bacteria, most of the efflux pumps that contribute to resistance to most antibiotics are three component structures that traverse both inner membrane & outer membrane. This structural organization allows extrusion of substrates directly into the external medium bypassing the periplasmic space and makes efflux pumps more efficient. (Zgurskaya H et al.,1999).

There are several modes of action of efflux pumps: a) There are several modes of action of efflux pumps: a) The EPI may bind directly to the pump in a competitive or noncompetitive manner with the substrate, causing the blocking of the efflux pump; b) EPI may also cause a depletion of energy, through the inhibition of the binding of ATP or them disturbance of the proton gradient across the membrane; c) EPI may have affinity for substrates, and bind them, forming a complex that facilitates the entry of the drug in the cell and prevents its efflux.

Efflux pump inhibitors:

The use of efflux pump inhibitors can ease the re-introduction of therapeutically ineffective antibiotics back into clinical use and might even suppress the emergence of MDR strains. (Stavri M, Piddock LJV, 2007)

Synthetic efflux pump inhibitors:

Synthetic compounds characterized as major efflux pump inhibitors.

Phenylalanine arginyl β-naphthylamide-PAβN (MC-207,110)

It is a dipeptide amide and had potentiated the activity of levofloxacin up to 8 folds at10 µg/mL against P. aeruginosa (Barrett, 2001). It enhances the susceptibility of (1) Phenylalanine arginyl β-naphthylamide-PAβN (MC-207,110) It is a dipeptide amide and had potentiated the activity of levofloxacin up to 8 folds at10 µg/mL against P. aeruginosa (Barrett, 2001). It enhances the susceptibility of Chapter 2 Review of Literature Faculty of Applied Sciences & Biotechnology 30 erythromycin 8-32-fold and rifampicin 8-64 folds against Campylobacter jejuni and Campylobacter coli (Hannula et al., 2008). It also inhibited the efflux pump in E. coli and also reduced the susceptibility of rifaximin (Gomes et al., 2013). PAβN converts ciprofloxacin resistant strains of P. aeruginosa, Acinetobacter baumannii and E. coli to susceptible ones (Cetinkaya et al., 2008). In combination fluoroquinolones, it showed inhibitory activity against the MexCD-OprJ, AcrABTolC efflux pump and MexEF OprN pumps of P. aeruginosa, and against other efflux pumps of Gram-negative bacteria (Zechini and Versace, 2009; Rana et al., 2014). It is also toxic in nature. Its toxicity prevents its use in clinical applications. However, it is widely used to experimentally determine and evaluate the efflux mechanisms in different bacterial pathogens. In addition, it is used to measure the efflux efficacy and to determine the level of inhibitor-sensitive efflux for specific antibiotics in case of various bacteria (Mahamoud et al., 2007).

Arylpiperidines and arypiperazines: These efflux pump inhibitors have the ability to lower the multidrug resistance in case of RND efflux

pump of E. coli bacteria (Bohnert and Winfried, 2005).

Nocardamines: These efflux pump inhibitors are known as iron chelator and they show inhibitory activity against TetB and TetK efflux pumps of Staphylococcus aureus (Rothstein et al., 1993).

Arylated benzothiophenes and tiophenes: These efflux pump inhibitors show inhibitory activity against NorA efflux pump of Staphylococcus aureus (Chabert et al., 2007).

Quinoline derivatives: Quinoline compound showed inhibitory activity against multidrug resistant Enterobacter aerogenes. Different quinoline compounds subsequently enhance the intracellular concentration of chloramphenicol and inhibit the transportation of drug by AcrAB – TolC efflux pump (Mahamoud et al., 2006)

Indole derivatives: Indole derivatives such as, INF- 55 and INF-271 showed efflux pump inhibitory activity against NorA efflux pump of Staphylococcus aureus (Ambrus et al., 2008). 3-amino-6- carboxyl- indole and nitro-6-amino-indole enhanced the antimicrobial efficacy of ciprofloxacin, tetracycline, chloramphenicol and erythromycin against some strains of E. coli (Zeng et al., 2010).

Amide derivatives: 4, 8- trienoic acid amides, 5, 9- dimethyl- deca-2 and 9- formyl-5- methyl-deca-2, 4, 8- trienoic acid are compounds of amide family and they possess the ability to potentiate the activity of ciprofloxacin against Staphylococcus aureus (Sumithra et al., 2012).

Sodium orthovanadate: In biological systems, vanadium compounds have proved to inhibit several enzymes like ATP-phosphohydrolases, glyceraldehyde-3-phosphate dehydrogenase, adenylate kinase, phosphofructokinase, ribonuclease, squalene synthetase, glucose-6-phosphatase and phosphotyrosyl-protein phosphatase (Nechay, 1984). Sodium orthovanadate proves to be a promising inhibitor of ABC efflux pump of Streptococcus pneumonia (Garvey and Piddock, 2008). The activity of vanadate compounds as efflux pump inhibitors was studied and demonstrated in M. smegmatis (Choudhuri et al, 1999).

Phenothiazines: Thioridazine an efflux pump inhibitor belongs to Phenothiazines is a neuroleptic drug. Thioridazine showed efflux pump inhibitory activity against multidrug resistant bacteria such as, S. aureus, E. coli, M.tuberculosis, P. aeruginosa and S. typhimurium (Thorsing et al., 2013; Amaral and Viveiros, 2012; Chan and Chua, 2005; Martins et al., 2008).

Carbonyl cyanide m- chlorophenyl hydrazone (CCCP):

CCCP is a protonophore. It remarkably affects the energy level of the membrane and cell viability by causing a recreation of the proton motive force of the membrane, affecting the transporters that depend on this mechanism. Besides its high toxicity for the cell, it is also characterized as a substrate of bacterial efflux pumps (Mahamoud et al., 2007). It has shown inhibitory activity in Mycobacterium smegmatis and in Mycobacterium fortuitum by inhibition of the MFS efflux pump (Rana et al., 2014). This is a strong uncoupler that disturbs electrochemistry gradient to inhibit RND, MATE, MFS and SMR efflux pumps. CCCP increase the intracellular concentrations of antibiotics and become a necessary tool for studying efflux systems MICs (Poole, 2002).

Alkoxyquinolone derivatives:

The derivatives of Alkoxyquinolone, 2, 8-dimethyl- 4- (2, pyrrolidinoethyl) oxyquinolone, inhibits the activity of efflux pump of E. aerogenes and K. pneumonia. This efflux pump inhibitor elaborated the effect of chloramphenicol, norfloxacin and tetracycline up to 8-fold (Chevalier et al., 2004).

Substituted polyamines:

These compounds show efflux pump inhibitory activity against Hemophilus influenza (Sumithra et al., 2012).

Verapamil:

Verapamil is an efflux pump inhibitor currently used to evaluate the efflux pump activity in Gram-positive bacteria, and it can be also used in Gram-negative bacteria, like E. coli (Viveiros, 2008; Martins, et al., 2008). This drug is classified as an antiarrhythmic drug. Verapmil is also used in the treatment of hypertension and cluster headaches. It shows efflux pump inhibitory activity against Mycobacterium tuberculosis. It also intensifies the activity of isoniazid and rifampin (Gupta et al., 2013). Verapamil also shows EPI activity in case of Lactococcus lactis (Vanveen et al., 1996).

Thioridazine:

Belonging to the family of phenotiazines, thioridazine is an antipsychotic drug which is widely used to treat schizophrenia and psychosis. It has been suggested that this class of drugs affect the transporter proteins that depend on the proton-motive force, and like chloropromazine, another phenotyazine, it affects the flux of calcium and potassium across the membrane (Kaatz et al. 2003) and inhibits the binding of calcium ions (Ca2+) to enzymes and proteins (Amaral and Viveiros, 2012; Martins, 2008).

Omeprazole: Omeprazole is an anti-ulcer drug recurrently used in several gastric pathologies. The mechanism of action of this drug involves the inhibition of the H+-K+-ATPase (Wishart, 2011, Aeschlimann et al., 1999). Omeprazole has been described as an effective efflux pump inhibitor of the MFS efflux pump NorA of S. aureus (Aeschlimann et al., 1999), and its activity has already been studied using Mycobacterium (Suzuki et al., 2000).

Natural Inhibitors of efflux pumps of Gram negative and Gram-positive bacteria:

Several EPIs from plant sources have been discovered lately but most of them characterized with few pathogens like Staphylococcus aureus. The studies conducted by Gibbons et al. (2003); Dickson et al. (2006) and Braga et al. (2005) reported that plant extracts can enhance the in vitro activity of resistant antibiotics against MDR strains of S. aureus, B. subtilis and S. pneumoniae. Reserpine is the most common EPI discovered against S. aureus and B. subtilis. The antihypertensive plant alkaloid reserpine was isolated from roots of Rauwolfia vomitoria (Poison et al., 1954) and reported as an EPI against B. subtilis (Rana et al., 2014). It is a broad-spectrum natural EPI and active against different efflux pumps viz. NorA, TetK, Bmr present in S. pneumoniae, S. aureus, B. subtilis respectively (Neyfakh et al., 1991). It is assumed that plants should be further utilized for their potential to produce compounds which are capable of blocking the activity of efflux pumps. There is an ecological rationale for the production of natural products that modify bacterial resistance as plants have evolved compounds which evade MDR mechanisms and that bioactive molecules might be developed into broad-spectrum antibiotics in combination with inhibitors of MDR (Tegos et al., 2002). Currently there are no EPI and antibiotics combinations available, although researches are going on to identify a potential EPIs (Guz et al., 2001; Lomovskaya et al., 2001) which would be able to enhance the sensitivity of resistant antibiotics.

Efflux pump inhibitors against Gram-negative bacteria:

Multidrug resistance in gram - negative bacteria is very usual problem. So far very less efflux pump inhibitors have been detected against Gram - negative bacteria, this is due the efflux pumps present in gram - negative bacteria are comprises of an inner membrane pump, an outer- membrane channel, and periplasmic adaptor protein, which helps in transportation of structurally unrelated drugs (Pages et al., 2005). It is important to search new compounds which have the ability to make the reuse of previous

antibiotics against gram negative bacteria, as there is a slight decrease in number of new agents and development of antibiotics. Very few compounds have been discovered so far which shows efflux pump inhibitory activity against gram-negative bacteria (Garvey et al., 2010). Derivatives of isopimarane shows efflux pump inhibitory activity against efflux pumps of Enterobacter aerogenes (Gibbons et al., 2003). Cathinone is a monoamine alkaloid which shows efflux pump inhibitory activity against Salmonella Typhimurium along with ciprofloxacin (Piddock et al., 2010). Only few plants have reported so far which shows efflux pump inhibitory activity against gram - negative bacteria in combination with different antibiotics. The plants which show EPI activity against gram - negative bacteria are Helichrysum italicum, Thymus maroccanus, Thymus broussonetii, Centella asiatica, Callistemon citrinus, Daucus carota, Commiphora molmol, Citrus aurantium and Glycyrrhiza glabra. Extracts of these plants shows EPI activity against Pseudomonas aeruginosa and Salmonella enteric (Piddock, 2006; Lorenzi et al., 2009; Fadli et al., 2011). The extract of Berberis aetnensis along with ciprofloxacin shows efflux pump inhibitory activity against E. coli. The ethanolic extracts of Vernonia adoenis, Mangifera indica and Callistemon citrinus shows efflux pump inhibitory activity against Pseudomonas aeruginosa and E. coli (Chitemerere and Mukanganyama, 2011). During the last few years, different bioactive compounds which alter the function of the efflux pumps have been identified. Efflux pump inhibitors (EPIs) have been investigated against both gram negative and gram-positive bacteria. The sources of these EPIs were both natural (Plant derived) and synthetic (Chemicals). The majority of EPIs investigated are putative inhibitors of efflux pumps of the highly problematic gram- positive bacteria like S. pneumonia, B. subtilis, S. aureus etc (Stermitz et al., 2002). Various abietane diterpenes such as carnosic acid and carnosol, isolated from Rosemary officinalis acts as potentiators of tetracycline and erythromycin against S. aureus strains (Oluwatuyiet et al., 2004). Plant extracts of Lycopus europaeus and Camellia sinensis enhances the activity of antibiotics against TetK or MsrA, TetB or TetK efflux pumps of S. aureus (Gibbons et al., 2003; Fujita et al., 2005).

(a) Baicalein: Baicalein is isolated from Thymus vulgaris (Fujita et al., 2005). Baicalein, a well-known efflux pump inhibitor which shows the activity against efflux pump of E. coli and enhances the activity of Tetracycline. (Rana et al., 2014).

(b) Pheophorbide a: Pheophorbide-a is isolated from Berberis aetnensis and shows efflux pump inhibitory activity against E. coli and P. aeruginosa by enhancing the activity of ciprofloxacin (Rana et al., 2014).

(c) Theobromine: Theobromine is isolated from Theobroma cacao and acts as an efflux pump inhibitor by enhancing the activity of ciprofloxacin against MexAB-OprM and AcrAB–TolC efflux pumps of P. aeruginosa and K. pneumonia. Its EPI activity has also reported against Salmonella typhimurium (Piddock et al., 2010).

(d) Cathinone: Cathinone is isolated from Catha edullis and it enhances the activity of ciprofloxacin against Salmonella typhimurium by reducing the function of AcrABTolC efflux pump (Piddock et al., 2010).

Efflux pump inhibitors against Gram positive bacteria:

1) Carnosic acid: Carnosic acid is a natural compound which is isolated from Rosmarinus officinalis, it is structurally unrelated to known antibiotics and function as an efflux pump modulator. Therefore, Carnosic acid would be employed as a novel therapeutic agent which

can be used in combination therapies against multi drug-resistant S. aureus infections (Piddock et al., 2010).

2) Carnosol: Carnosol is isolated from herb Rosemary (Rosmarinus officinalis) it potentiated the activity of tetracycline and erythromycin against some strains of S. aureus possessing the Msr (A) efflux pumps (Rana et al., 2014).

3) Baicalein: Baicalein is isolated from Thymus vulgaris (Fujita et al., 2005). It synergistically restored the antibacterial actions of ciprofloxacin against the NorA efflux pump over expressed SA-1199B strain of S. aureus (Chan et al., 2011).

4) 2, 6-Dimethyl-1, 4-phenyl-pyridine-3, 5-dicarboxylic acid diethyl ester: It is isolated from Jatropha elliptica and acts as an EPI against S. aureus (Rana et al., 2014).

5) Piperine: Piperine is isolated from Piper nigrum and it showed EPI activity against S.aureus (Rana et al., 2014). Piperine is also involved in the inhibition of clinically over expressed mycobacterial putative efflux pump (Rv1258c). It is useful in augmenting the anti-mycobacterial activity of rifampicin in the clinical settings (Sharma et al., 2010).

6) Reserpine: Reserpine is isolated from Rauwolfia vomitoria and it is the most common EPI reported against S. aureus and B. subtilis (Neyfakh et al., 1993; Rana et al., 2014). Reserpine is an indole alkaloid which inhibits efflux pumps of gram-positive bacteria and is mainly involved in fluoroquinolone efflux but it is neurotoxic at the concentration required to inhibit pumps. It is also known as a broad-spectrum natural EPI and active against different efflux pumps i.e., NorA, TetK, Bmr in S. pneumoniae, S. aureus, B. subtilis respectively. A major limitation of combining this EPI with drugs is its use at higher concentrations which may prove toxic at clinical levels (Rana et al., 2014).

7) Chalcone: Chalcone is isolated from Nicotiana tobacum and it acts as an efflux pump inhibitor against Bacillus cereus by enhancing the activity of Berberine, Erythromycin and Tetracycline (Hiramatsu et al, 2001). Efflux pump inhibitory activity of Chalcone is also reported against Staphylococcus aureus (Belofsky et al, 2004).

8) Orobol: Orobol is isolated from Lupinus argenteus. It acts as efflux pump inhibitor against Staphylococcus aureus by enhancing the activity of Berberine against NorA efflux pump (Kourtesi et al, 2013).

9) Quercetin: Quercetin a flavonoid which is isolated from Allium cepa showed efflux pump inhibitory activity against Mycobacterium (Lechner et al, 2008).

10) Fernasol: Fernasol isolated from Cymbopogon citratus enhances the activity of Ethidium bromide against TetK efflux pump of Mycobacteria (Jing et al, 2010).

To date, no efflux pumps inhibitors has been used in the treatment of bacterial infections in human or animals, although research continues.

The rich chemical diversity in plants promises to be a potential source of antibiotic resistance modifying compounds and has yet to be adequately explored for EPIs which are nontoxic at higher concentrations. Most of these EPIs had only demonstrated their activity in vitro, so further investigations are needed for evaluation of their clinical potential. As large number of synthetic and natural EPIs has been discovered, none have been approved for routine clinical use as a result of doubtful clinical efficacy and high incidence of adverse effects.

CONCLUSION :

To date, no efflux pumps inhibitors has been licensed for use in the treatment of bacterial infections in human or veterinary settings, although research continues. In the treatment of bacterial disease cystic fibrosis, one drug development program involving co-administration of an EPI with an antibiotic agent

has reached human clinical trials. In this trial, an aerosolized formulation of the EPI compound MC-601, 205 is being combined with ciprofloxacin for the treatment of pulmonary exacerbations in cystic fibrosis patients in a phase II trial being conducted by Mpex Pharmaceuticals. In this disease, the most serious symptoms are observed in lungs, increasing the risk of bacterial infection of the bacteria like *B. Cepacia, P. aeruginosa* and *S. aureus*. Even if a glance is given on the literature of secondary metabolites of plants, they also show activity against Gram positive bacteria and not against Gram negative bacteria because Gram negative bacteria have evolved effective barriers for all amphipathic compounds (cationic, neutral & anionic). In Gram negative bacteria presence of extra outer membrane inhibits the entry of amphipathic compounds. While in case of Gram-positive bacteria only a single membrane is present. So, the entry of amphipathic compounds is easy in Gram positive bacteria Therefore there are a great need to explore novel plant sources for EPIs against Gram- negative bacteria.

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