Review

Inhibitors of Bacterial Efflux Pumps that also Inhibit Efflux Pumps of Cancer Cells

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Abstract. *Bacteria and cancer cells frequently increase their resistance to chemotherapeutics as a consequence of therapy. Whenever studied, refractory response to chemotherapy is due to the over-expression of efflux pumps that render the bacterium or cancer cell resistant not only to the agent used for therapy, but to many, if not all other agents as well. Control over the efflux pump that bestows multidrug resistance has been a goal of research during the past decade. As a consequence of this search for inhibitors of efflux pumps, it has been noted that many agents which affect the efflux pump system of bacteria also have similar activity against efflux pumps of drug-resistant cancer cells. This review aims to identify such agents.*

Phenothiazines: Inhibitors of Bacterial Efflux Pumps

The phenothiazines are heterocyclic compounds whose origins lie in the middle of the 19th century when Bernthsen in 1883 reacted diphenylamine with sulfur (1). The phenothiazine dye methylene blue was developed soon thereafter and became a focus of the studies of the German physician Paul Ehrlich for almost 20 years, during which time he was able to show that the dye had antibacterial and antimalarial properties, and malaria could be cured with administration of methylene blue (2). However, because the patient who received the dye was noted to become calm, interest in the dye for possible therapy of psychosis took preference over that of its antimicrobial properties, and after almost 50 years of study, a colourless phenothiazine with neuroleptic properties was synthesised by Rhone Polenc in the 1950s and introduced in the USA as chlorpromazine (CPZ) and elsewhere as largactil (3). The wide use of CPZ worldwide resulted in a large number of observations indicating that the compound had strong anti-tuberculosis activity (1, 4, 5) but because at that time, isoniazid and rifampicin were very effective for therapy of tuberculosis, little interest in CPZ as an anti-tuberculosis drug developed. Nevertheless, some interest in CPZ as an anti-tubercular agent remained and a number of *in vitro* studies showed that CPZ indeed had anti-tubercular activity (6, 7). However, the *in vitro* activities took place at concentrations of the compound which exceeded by far any that could be clinically reached (8). Nevertheless, the demonstration by Crowle *et al.* that CPZ could promote the killing of intracellular *Mycobacterium tuberculosis* (*Mtb*) with concentrations in the medium that correspond to those clinically achievable (9), prompted Amaral *et al.* to study another phenothiazine, thioridazine, which is as effective as CPZ for therapy of psychosis but produces fewer serious negative side-effects. The *in vitro* activity of thioridazine was shown to be as effective as that of CPZ against all antibiotic-resistant strains of *Mtb* (10), although, as was the case for CPZ, the *in vitro* activities were clinically irrelevant. Nevertheless, spurred by the work of Crowle *et al.* (9), thioridazine was shown by Amaral's group to promote the

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Key Words: Bacteria, cancer, multidrug resistance, efflux pumps, inhibition of efflux pumps, phenothiazines, chlorpromazine. thioridazine, hydantoins, phenytoin, SILA 421, SILA 409, trifluoromethyl ketones, review.

killing of multidrug-resistant strains of *Mtb* that had been phagocytosed by non-killing human macrophages at concentrations that were below those that are used for chronic therapy of psychosis (11). Soon thereafter this same group cured an *Mtb* infected mouse of an antibiotic-susceptible infection of *Mtb* (12) and later cured a mouse infected with a multidrug-resistant strain of *Mtb* (MDR *Mtb*) (13). Using protocols developed by Amaral *et al*. (14, 15), Abbate and his group successfully treated patients infected with extensively drug-resistant strains of *Mtb* (XDR *Mtb*) with combinations of thioridazine and three antibiotics to which the *Mtb* strains were resistant (16). Given the mechanisms by which thioridazine promotes the killing of intracellular antibiotic-susceptible, MDR and XDR *Mtb*, which will be discussed in sections to follow, it is reasonably expected that thioridazine will cure patients infected with strains of XDR *Mtb* (17, 18).

Mechanism of Action by which TZ Promotes the Killing of Intracellular *Mtb* Regardless of its Antibiotic Resistance

Tuberculosis is mainly an intracellular infection caused by the steadfast human pathogen *Mycobacterium tuberculosis*. The bacterium is not killed when ingested by the pulmonary macrophage soon after it finds its way *via* inhaled microdroplets of *Mtb*-containing sputum expelled someone with active tuberculosis. Consequently, the individual remains infected for many decades, and only about 5 to 10% of such infectious progress to active disease, the infectious stage of the infection.

Human pulmonary macrophage does not kill the ingested organism due to the efflux of K^+ and Ca^{2+} from the phagolysosome containing the trapped organism. With efflux of K^+ and Ca^{2+}, the required fall in phagolysosomal pH does not take place and, consequently, the activation of hydrolases that would normally degrade and kill the bacterium does not take place (19, 20). Phenothiazines are well known to inhibit efflux pumps of eukaryotes (21, 22). They are also inhibitors of calcium binding to proteins and enzymes (23). Verapamil, an inhibitor of calcium binding, also inhibits efflux activities of mammalian cells mediated by the transporter ABCB1 (24) as well as K^+ transport (25). Ouabain, also an inhibitor of K^+ transport, and verapamil both promote the killing of intracellular MDR *Mtb* (26). Hence, the mechanism by which thioridazine promotes killing of intracellular *Mtb* by non-killing macrophages has been postulated to be due to the inhibition of K^+ efflux from the phagolysosome containing the ingested bacterium (26-28). In addition, another mechanism is also affected by thioridazine, namely, thioridazine is an inhibitor of efflux pumps of bacteria (29-32) as well as those of mycobacteria (5, 33-36). Because MDR phenotypes of bacteria are mediated by over-expressed efflux pumps (5, 29-36), thioridazine probably inhibits the efflux pumps of MDR, XDR and TDR *Mtb* strains, thereby rendering these strains susceptible to antibiotics to which they were initially resistant (17, 18).

Inhibition of over-expressed efflux pumps of bacteria that bestow the organism with an MDR phenotype when inhibited by a phenothiazine become susceptible to the antibiotics to which they were initially resistant (37-46). With respect to *Salmonella*, the response to a phenothiazine is quite different because of its efflux pump. During the first 6 to 8 h of exposure to a phenothiazine, whereas initially susceptible to the phenothiazine, after this period, resistance builds to the point that the organism is now resistant to concentrations as high 125 mg/l (47). During the initial 6 to 8 h the phenothiazine induces expression of the genes that regulate and code for the main efflux pump of the organism (48). The synthesis of the main efflux pump AcrAB-Tol resulting from exposure to the phenothiazine actually promotes resistance to the phenothiazine. Therefore, one has to be careful making a generalization that exposure to a phenothiazine results in the inhibition of an efflux pump system.

The efflux pumps of bacteria perform functions not associated with the extrusion of an antibiotic that has penetrated the cell envelope directly, as may be the case for lipid-like compounds (49), or *via* porins (50). Efflux pumps provide the conduits for elimination of toxins produced from metabolism (51, 52), enzymes that increase the virulence of the bacterium (53), quorum sensing signals (54), biofilm (55-57), and probably many other products of secretion (58). Consequently, because these efflux pump functions render the bacterial organism more virulent, the inhibition of the efflux pump by a phenothiazine would be expected to reduce, if not obviate the virulence of the organism. Coupled to the phenothiazine-promoted reversal of resistance of the organism to antibiotics, the use of phenothiazines as adjuncts for therapy of a problematic bacterial infection, such as that produced by MDR bacteria, make these compounds significant for future use as anti-infectious agents.

Phenothiazines: Inhibitors of Efflux Pumps of Cancer Cells

The first demonstrations that phenothiazines could inhibit cancer growth were reported during the early 1950s (59, 60). Since those early years, many reports have been published showing that indeed, phenothiazines can inhibit the growth of some types of cancers (61-69). Among the most studied phenothiazines are benzo-phenothiazines (64, 68-78), chlorpromazine and its derivatives (22, 79-89), methylene blue (90-95) and toluene blue (96). Methylene blue in combination with light is now receiving increasing attention for therapy of tumors (97, 98).

All of these phenothiazine anticancer compounds have activity against bacteria (99-107) and their mechanism of

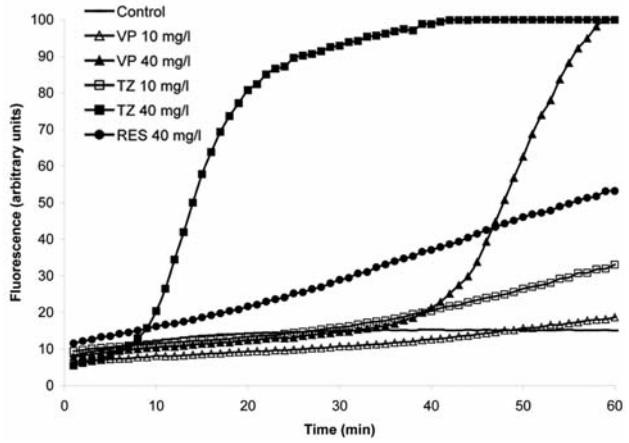


Figure 1. *The effect of thioridazine (TZ) concentrations on the retention of the universal efflux pump substrate ethidium bromide. Ethidium bromide accumulation by mouse T-lymphoma cells overexpressing the ABCB1 transporter in the presence of verapamil (VP), thioridazine (TZ) and reserpine (RES). Cells (2×10^6 cells/ml) were suspended in 100 µl of phosphate-buffered saline solutions (pH 7.4) supplemented with 1 mg/l of EB with and without agent at the given concentrations, then the fluorescence was continuously monitored (108).*

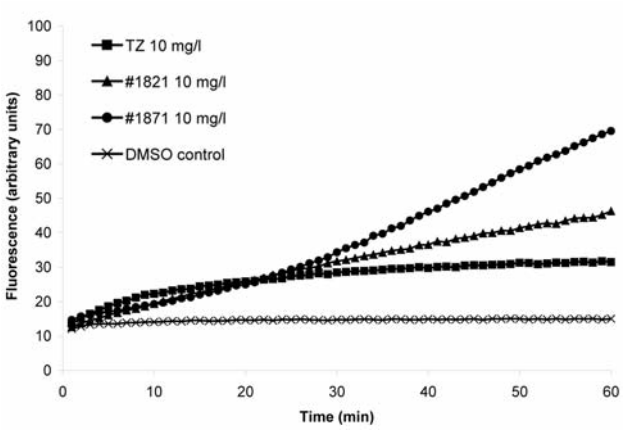


Figure 2. *The activity of derivatives of thioridazine. Ethidium bromide (EB) accumulation by mouse T-lymphoma cells overexpressing the ABCB1 transporter in the presence of thioridazine (TZ), and thioridazine derivative #1821 and thioridazine derivative #1871. Cells (2×10^6 cells/ml) were suspended in 100 µl of phosphate-buffered saline solution (pH 7.4) and supplemented with 1 mg/l of EB with and without agent at the given concentrations, then the fluorescence was continuously monitored.*

action as anticancer agents is different for many cancer tissues. Nevertheless, when studied, these phenothiazine anticancer agents have activity against the ABC efflux pump ABCB1 of cancer cells (21, 22, 108-120), thereby rendering these cells susceptible to anticancer drugs to which they were initially resistant (108, 112, 114, 116).

ABCB1 is a member of the ATP binding cassette genetic family of ABC transporters and is present in many of the cells of the human body and the gene that codes for it is the *ABCB1* gene. ABC transporters are proteins that are intimately associated with the plasma membrane of the cell. ABC transporters contain a pair of ATP-binding domains, also known as nucleotide-binding folds (NBF), a pair of substrate binding sites and two sets of transmembrane (TM) domains, typically containing six membrane-spanning α-helices. The ATP and the substrate binding sites are located in the cytoplasmic side of the plasma membrane. The NBF sites bind ATP when the substrate site binds the agent that is to be extruded. This is followed by the hydrolysis of the ATP, and the energy released promotes the conformational change needed in the transporter for translocation of the agent to the environmental side of the plasma membrane (120). Certain cancer cells over-express the ABCB1 transporter when the patient is under chemotherapy, and when over-expression of ABCB1 takes place, the cancer becomes resistant not only to the agent that promoted the over-expression of ABCB1, but also to other anticancer drugs (108). Consequently, for the past decade, efforts to obtain agents that will selectively inhibit ABCB1 activity have intensified.

Of the phenothiazines that affect the activity of ABCB1, thioridazine has been shown to be very effective (108). Usually, the use of a flow cytometer is employed for the evaluation of an agent against the ABCB1 transporter (121, 122). However, a more effective way to demonstrate the activity of an agent on the ABCB1 transporter of a cancer cell has been developed (123, 124). This method evaluates the activity of ABCB1 on a real time basis and under any physiological condition required for a given assay. An example of the assay is provided in Figure 1 for the evaluation of thioridazine for inhibitory activity against ABCB1 of the mouse lymphoma cells transfected with the multidrug resistant gene *ABCB1*.

The method is identical to that used for the evaluation of efflux pumps of bacteria and yields similar real-time data for the accumulation of ethidium bromide (41). Forty derivatives of thioridazine that had been shown to have activity against efflux pumps of bacteria (45) have been evaluated for activity against cancer cells and an example of these results is presented in Figure 2. Therefore, one may conclude that phenothiazines that inhibit efflux pumps of bacteria have similar properties towards the efflux pumps of cancer cells. This relationship has been previously noted and reviewed (125).

Phenothiazines, in general, are well known electron donors, where they bind by charge transfer complex formation to target molecules when an electron goes from the highest filled molecular orbital to the lowest empty orbital of the acceptor molecule on the target. If the

phenothiazine acts as an electron donor at the surface of the plasma membrane of the cell or within the lipid bilayer of the plasma membrane, then the electron transfer on the outside will result in depolarization of the membrane. Because this depolarization reduces the activity of the plasma membrane (conductivity, *etc*.), phenothiazines have been referred to as membrane-stabilizing agents. However, when the phenothazine acts as an electron donor on the cytoplasmic side of the plasma membrane, hyperpolarization results and membrane-linked processes are inhibited. If the biological activity is actually due to charge transfer complex formation, pharmacological activity resulting from electron donation by the phenothiazine should be expected (there are some exceptions to this rule: CPZ-, sulfon- or, sulphoxides and methylene blue, where the asymmetric distribution of charge is a main cause of ineffective activity).

The electron levels of the classic phenothiazine, CPZ, were calculated by Karemann, Isenberg and Szent-Györgyi many years ago (126). These workers obtained K values of 0.217 for the highest filled orbital and –1.000 for the lowest empty orbitals. These negative values were found also in leuco-methylene blue (similar results were found in case of D-lysergic acid diethylamine) and reduced flavin-mononucleotide (127).

Hydantoins: Activity Against the Efflux Pump of Bacteria

Hydantoins play an important role in the purine catabolic pathway that regulates the purine pool in cells to provide precursors for nucleic acid synthesis (128). In addition, hydantoinases have essential metabolic function because they hydrolyse hydantoin and 5'-monosubstituted hydantoin derivatives, and for this reason their biotechnological application is valuable in the production of optically pure amino acids (129). The nucleobase cation symport-1 (NCS1) transporters are essential components of salvage pathways for nucleobases and related metabolites, *e.g*. NCS1 benzylhydantoin transporter, Mhp1, from *Microbacterium liquefaciens* (130). Besides these biochemical processes, hydantoins have pharmacological properties and are used to treat many human diseases. A well known example of a drug featuring a hydantoin is phenytoin (5,5-diphenylhydantoin, Dilantin), which has been used for decades to treat epilepsy (131, 132). Hydantoins have different pharmacological properties depending on the nature of substitution on the hydantoin ring, *e.g*. fungicidal, herbicidal, antitumor, anti-inflammatory, anti-HIV, hypolipidemic, antiarrhythmic and antihypertensive activities, have also been identified (133-136). Furthermore, it has been demonstrated that 5-arylidene-2-thiohydantoins have *in vitro* antimycobacterial activity (136).

The activity of 39 hydantoin compounds on the efflux pumps of bacteria (137, 138) has been evaluated by the real-time ethidium bromide fluorometric method developed in our laboratory (41). Many of these compounds had exceptional activity against Gram-positive and Gram-negative bacteria. However, although nothing is yet known as to their mechanism of action, the fact that these hydantoin compounds were shown to be non-toxic (139, 140), makes them attractive for therapy of selected bacterial infections whose MDR phenotype is mediated by an over-expressed efflux pump system.

Aromatic hydantoins, such as 5-benzylhydantoin presented in Figure 3a, display structural similarities to the most promising inhibitors of MDR efflux pumps of Gram-negative bacteria from the peptidomimetics family (141). Various chemical modifications of the aromatic hydantoin can be considered as cyclic analogs of efflux pump inhibitors (EPIs), PAβN and MC-04,124 (Figure 3a). Therefore, their EPI action in Gram-negative bacteria overproducing tripartite efflux pump AcrAB-TolC has been investigated. The first study performed for a series of N1-aminealkyl derivatives of phenytoin (142) allowed identification of compounds with moderate potency to increase antibiotic effectiveness in strains of *Enterobacter aerogenes* overproducing AcrAB-TolC. Further chemical modifications of the aromatic hydantoins gave compounds with higher EPI properties. The compounds have been intensively examined in microbiological studies using various MDR strains of *E. aerogenes* and *E. coli* (142). They give new hope to finding nontoxic potent bacterial efflux pump inhibitors useful for improving antibiotic therapy.

The Effect of Hydantoins on the Efflux Pump of Cancer Cells

Hydantoins shown to be active against the efflux pump of bacteria (137, 138, 142) have also been shown to have activity against the efflux pumps of cancer cells (139-141). Moreover, hydantoins have also been shown to have anticancer properties (142-144). Perturbation of the lipid membrane may be one of the targets in the inactivation of ABCB1 or other transmembrane efflux pumps by some type of intercalation of flavonoids into the phosphatidyl bilayer (145).

Although the hydantoin moiety seems to promote activity against cancer efflux pumps, the main role for the activity is carried out at substituent at positions 1, 3 and 5 of the hydantoin ring. It should be noted that the hydantoin phenytoin used for therapy of epilepsy (146-149) has been shown not to have activity against efflux pumps (150). However, chemical modifications of phenytoin to give derivatives substituted with methyl or aromatic aminealkyl

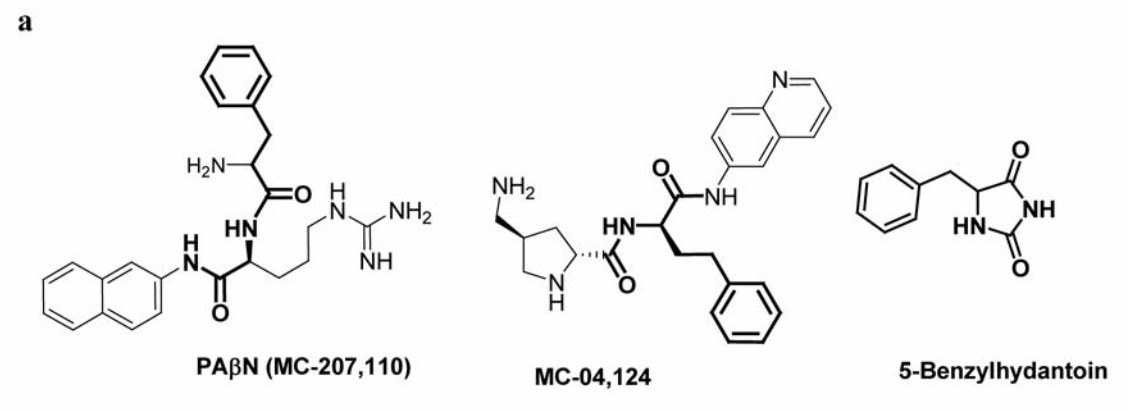


Figure 3. *a: Structural similarities of 5-benzylhydantoin to potent bacterial efflux pump inhibitor peptidomimetics PAβN and MC-04,124. Analogous ring (hydantoin) and open (peptidomimetics) structural fragments are shown in bold. b: The effect of the hydantoin HY84 on the accumulation of ethidium bromide by mouse lymphoma cells transfected with the human ABCB1 gene that codes for the ABCB1 transporter. Mouse lymphoma cells were incubated at 37°C in saline containing 0.6% glucose plus 1 mg/l of EB, without and with 20 mg/l of hydantoin HY84 (144).*

at positions 1 or 3, significantly increased the compound's potency at inhibiting ABCB1 in T-lymphoma cells within 123 rhodamine accumulation assays (141). The effect of 5-arylidenehydantoin HY84, a very active efflux pump inhibitor on the real-time retention of the ethidium bromide substrated by cancer cells is presented in Figure 3b.

The Activity of SILA Compounds on Bacteria and Cancer

SILA 421 (1,3-dimethyl-1,3-*bis*(4-fluorophenyl)-1,3-*bis*{3-[1(4-butylpiperazinyl)]-propyl}-disiloxan-tetrahydrochloride) is a silicon compound (Figure 4) that was developed as a modulator of ABCB1 (151-154). Furthermore, it exerts anti-mycobacterial activity (155) and has the ability to cure bacteria of plasmids (156). SILA 409 increased the apoptotic activity of drug resistant pancreatic cancer cells and exhibited some tumor growth delay (153). Because SILA 421 promotes killing of intracellular XDR *Mtb* it is currently being evaluated for its ability to cure mice of a drug-resistant *Mtb* infection. SILA 409 has little activity against intracellular *Mtb* (155).

Trifluoromethyl Ketones: Activity Against Bacterial Efflux Pumps

Trifluoromethyl ketones (TFKs) have been studied over a number of years and bioactive derivatives, such as those shown in Figure 5 have a variety of antimicrobial and antimotility effects on various bacterial species (157-161). Some of these TFKs inhibit only the growth of various Gram-positive bacteria, while others exhibit antimicrobial activity against Gram-negative bacteria and yeasts. The combination of certain derivatives of TFKs with promethazine results in a synergistic antibacterial effect (157). Recently, twelve TFKs (Figure 5) were shown to inhibit quorum sensing of bacteria, as well as inhibiting the efflux pumps of *E. coli* (54). Their mode of action appears to be due to their having a negative effect on the proton motive force of bacteria (158-161).

TFKs also have activity against cancer cells (162-166). However, they have not been studied for any specific activity against an over-expressed efflux pump of a cancer cell. Nevertheless, given that inhibitors of efflux pumps of bacteria may also have similar properties against over-expressed efflux pumps of cancer cells, they are good candidates for evaluation for such activity.

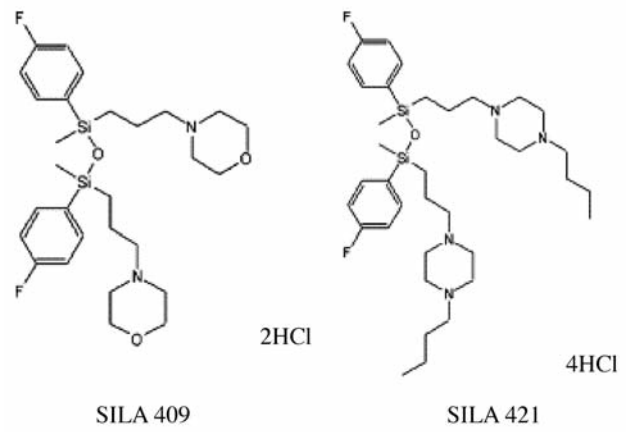
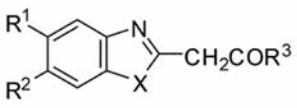


Figure 4. *Structure of two SILA compounds: SILA 409 and 421. SILA compounds 1,3-dimethyl-1,3-bis(4-fluorophenyl)-1,3-bis(3-morpholino-propyl) disiloxan-dihydrochloride (SILA 409) and 1,3-dimethyl-1,3-bis(4-fluorophenyl)-1,3-bis{3-[1(4-buthyl-piperazinyl)]-propyl}-disiloxan-tetrahydrochloride (SILA 421) were synthesised by Hegyes et al. (151). These compounds have received patents (Brevet European n. 0099150.6, PCT/DE00/04110).*



TF	R^1	R^2	R^3	X
1	H	H	CF_3	O
2	Cl	H	CF_3	O
3	F	H	CF_3	O
4	CH_3	H	CF_3	O
5	CH_3O	H	CF_3	O
6	Ph	H	CF_3	O
7	$COOC_2H_5$	H	CF_3	O
8	H	$COOC_2H_5$	CF_3	O
9	H	H	$CClF_2$	O
10	H	H	CH_3	O
11	H	H	CF_3	NH
12	H	H	CF_3	S

Figure 5. *Structure of the parent and derivatives of the trifluoromethyl ketone compounds (From reference 54).*

Conclusion

Phenothiazines, hydantoins and TFK compounds that inhibit the efflux pump of bacteria have also been shown to inhibit the efflux of cancer cells which when over-expressed mediate the multidrug resistance of these cells. SILA compounds that are non-toxic, such as SILA 421, have strong activity against intracellular mycobacteria and modulate the activity of resistant cancer cells.

These observations suggest that any inhibitor of a bacterial efflux pump is a promising candidate for evaluation for similar activity against the over-expressed efflux pumps of cancer cells.

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Received April 23, 2012
Revised June 11, 2012
Accepted June 11, 2012