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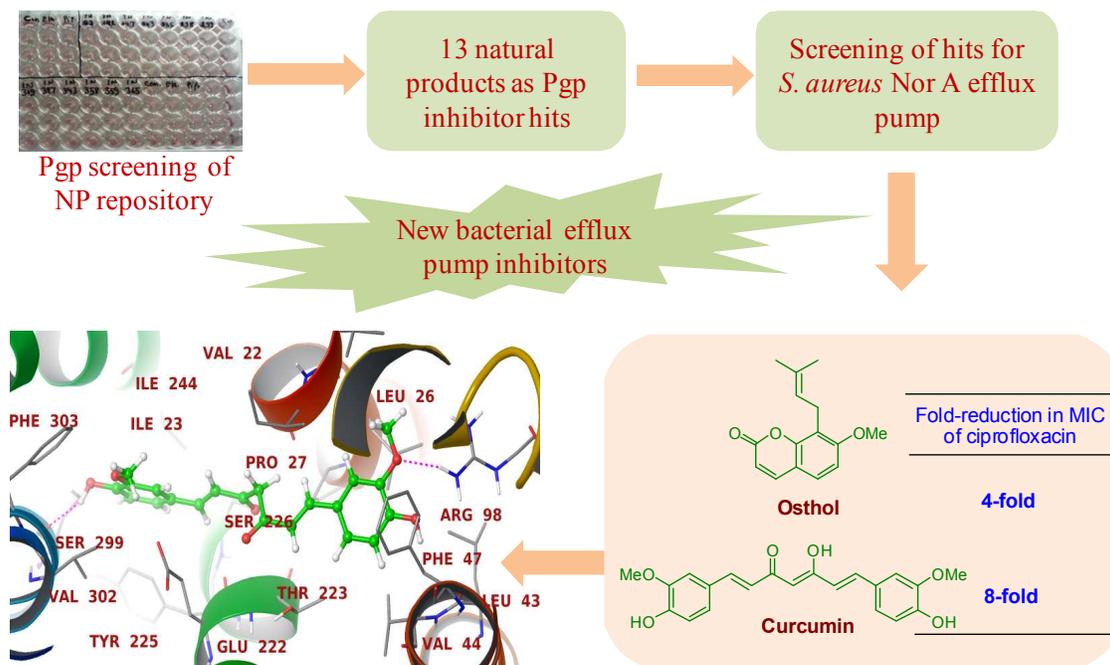
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GRAPHICAL ABSTRACT



Natural products curcumin and osthol have been identified as new Nor A efflux pump inhibitor leads.

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Concise Article

Osthol and curcumin as inhibitors of human Pgp and multidrug efflux pumps of *Staphylococcus aureus*: Reversing the resistance against frontline antibacterial drugs

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The in-house IIM natural product repository of 302 small molecules was screened for their ability to inhibit p-glycoprotein (Pgp) in Pgp-overexpressing human adenocarcinoma LS-180 cells. The screening has identified 13 natural products displaying significant Pgp-inhibition activity which include praeruptorin B, curcumin, imperatorin, osthol, 5,7-diacetoxy-8-(3-methyl-2-butenyl)-coumarin, 5,7-dihydroxy-8-(3-methyl-2-butenyl) coumarin, pongamol, phellopterin, tangeretin, 3-(2-methyl but-3-en-2-yl) xanthyletin, 7-demethyl osthol, allorottlerin and tetrahydroangeolide. These natural products were then screened for their effect on bacterial efflux pump inhibition activity against Nor A (*Staphylococcus aureus*), Mde A (*S. aureus* Mup^r-1), Tet K (*S. aureus* SA-K2192), and Msr A (*S. aureus* SA-K2191) efflux pumps. The curcumin and osthol showed significant inhibition of *S. aureus* NorA efflux pump with 8- and 4-fold reductions in the MIC of ciprofloxacin at 25 μ M. The molecular docking studies of curcumin and osthol with the human Pgp and *S. aureus* Nor A efflux pump identified plausible binding mode and binding site for these natural products.

Introduction

The development of multi-drug resistance (MDR) upon chronic exposure to chemotherapeutics has been considered to be the major reason for failure of chemotherapy in cancer and infectious diseases.¹ The defiant nature of the target cells to the anticancer (*viz.* colchicine, doxorubicin, paclitaxel, imatinib) and antibacterial (*viz.* ciprofloxacin, mupirocin, and erythromycin) drugs is mostly associated with the liability of their active efflux. Therefore, inhibition of such efflux pumps is viewed as a promising strategy to overcome MDR liability.² P-glycoprotein (Pgp, mol. wt. 170 kd)³ is a multidrug-resistance protein (also called as MDR1) responsible for development of resistance to

variety of drugs⁴ as well as for the transport of endogenous and exogenous substrate ligands such as various toxins (amyloid- β), hormones, and drugs (phenytoin, saquinavir, imatinib, doxorubicin).⁵ Under physiological conditions, the Pgp is involved in normal ADME process of a cell⁶ while in resistant-cancer cells, it contributes to the cellular accumulation of chemotherapeutic drugs by decreasing influx and increasing 'active efflux'.⁷

Despite the lack of structural and mechanistic information on efflux pumps, more than a dozen of Pgp inhibitors have entered in clinical trials in last two decades, starting from cyclosporin to the third generation inhibitors elacridar and tusquidar. Further efforts for developing better and efficacious efflux pump inhibitors are continuing worldwide. Natural products from plants and microbes provide unique source of the discovery of efflux pump inhibitors with potential MDR reversal activity. Several natural products scaffolds (such as polyprenylated coumarins from *Mesua ferrea*,⁸ reserpine,⁸ piperine^{1b,9} and capsaicin)¹⁰ are reported to reverse MDR in infectious diseases by inhibiting the Nor A efflux pump in clinical isolates of *S. aureus*. Recently, Sabatini and coworkers rationally designed and developed quinoline derivatives based on in-silico analysis of chromen-4-ones as potent Nor A EPIs.¹¹ Interestingly, several known inhibitors of human Pgp are also reported to inhibit efflux pumps of pathogenic bacteria, fungi and protozoa. Examples of such dual inhibitors includes piperine and capsaicin (bacteria),^{1b,9a,9c,10} plagiocchin E (fungi),¹² and dihydro- β -agarofuran sesquiterpenes (leishmania).¹³

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Our earlier efforts in this area^{1b, 9a, 9b, 14} led to the identification of piperine and its structural analog SK-20 as potent inhibitor of Nor A efflux pump of *S. aureus*. The SK-20 showed bio-enhancement of ciprofloxacin activity in both *S. aureus* resistant isolates over-expressing Nor A and wild isolates *in-vitro* and *in-vivo*.^{9b} Moreover, not only natural products but synthetic Pgp inhibitor clinical candidates such as verapamil,¹⁵ elacridar and tariquidar¹⁶ also displayed bacterial efflux pump inhibition activity.¹⁷ This inspired us to investigate further the effects of natural product Pgp inhibitor hits for bacterial efflux pump inhibition activity in *S. aureus*. The chemical structures of dual Pgp and bacterial efflux pump inhibitors are shown in Figure 1.

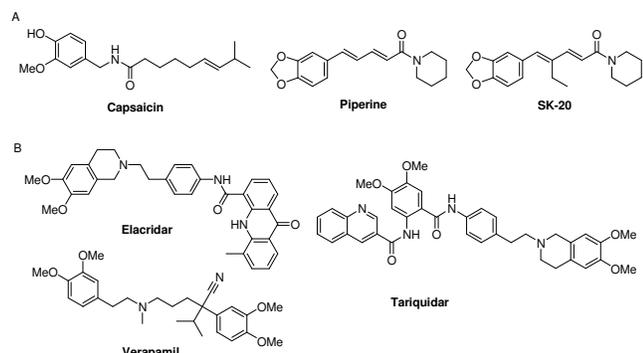


Figure 1. Structure of dual Pgp and bacterial efflux pump inhibitors. (A)

15 Natural products (B) Clinical candidates

Herein, we report screening of in-house natural product repository of 302 compounds for Pgp inhibition activity in LS180 cells using Rh123 as fluorescent substrate. The identified hits were explored for their ability to modify bacterial resistance to various classes of antibacterial drugs by efflux pump inhibition. Further, the molecular modeling studies were carried out for these natural products in order to understand the interaction of identified inhibitors with the efflux pumps.

Results and discussion

The in-house natural products repository was screened for Pgp-inhibition activity in Pgp-overexpressing human adenocarcinoma LS-180 cells using rhodamine123 (Rh123) cell exclusion method and capsaicin, piperine, and elacridar were used as positive controls. The test concentration of 50 μM was used for preliminary screening. This concentration was selected in order to avoid chance of missing any possible Pgp inhibitor in the first step. In order to rule out the false-positive hits due to toxic effect of any compound, all samples were normalized by dividing the fluorescence with total protein of each sample. The preliminary screening identified 13 natural products which displayed significant Pgp-inhibition activity as indicated by the increase in the % intracellular accumulation of Rh123 in LS180 cells compared to the control (100%) as depicted in Figure 2.

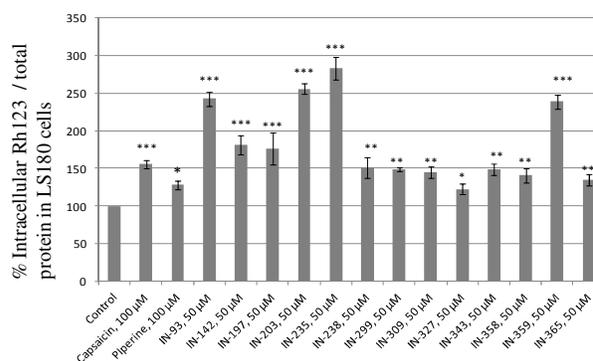


Figure 2. Screening of in-house natural products repository for Pgp-inhibition studies in LS180 cells. Statistical comparisons were made between control vs inhibitors by using Bonferroni method. The p value <0.05 was considered to be significant. p value * < 0.5, ** < 0.01, *** < 0.001.

These natural products were identified to be praeruptorin B (IN-93), curcumin (IN-142), imperatorin (IN-197), osthol (IN-203), 5,7-diacetoxy-8-(3-methyl-2-butenyl)-coumarin (IN-235), 5,7-dihydroxy-8-(3-methyl-2-butenyl) coumarin (IN-238), pongamol (IN-299), phellopterin (IN-309), tangeretin (IN-327), 3-(2-methyl but-3-en-2-yl)xanthyletten (IN-343), allotretlerin (IN-359) and tetrahydroangeolide (IN-365). Out of these 13 hits, curcumin¹⁸ and tangeretin¹⁹ were reported earlier as MDR reversal agents in cancer cells, however remaining natural product hits have never been reported as Pgp inhibitors. The IC₅₀ values were then determined for identified hits. Among identified natural product hits, the most potent IN-93 and IN-343 were found to show IC₅₀ of 16 and 15 μM in Pgp inhibition assay. Other compounds IN-142, IN-197, IN-203, IN-235, IN-299 and IN-309 inhibited Pgp with IC₅₀ values of 62, 28, 34, 44, 88 and 32 μM , respectively. The chemical structures of 13 identified hits are shown in Figure 3. The results of Pgp screening for 302 natural products are provided in the ESI.

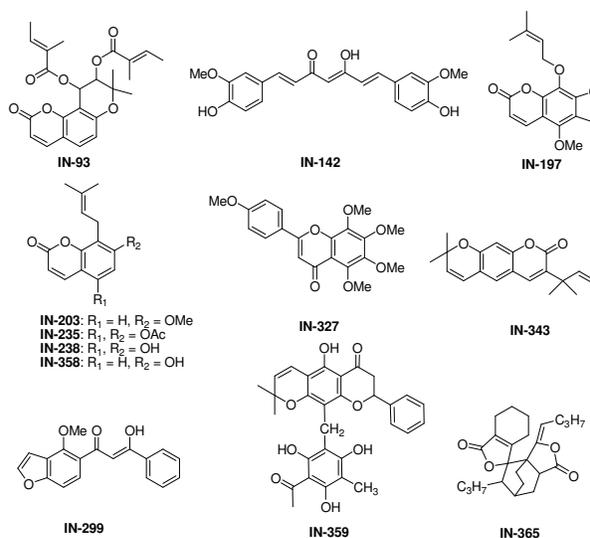


Figure 3. Chemical structures of identified Pgp inhibitor hits.

Table 1. Pgp substrate properties and hrCYP P450 isoenzyme assay profile (at 10 μ M) of potent Pgp inhibitor

Entry	P _{app} (10 ⁻⁶ cm/sec) ^a		Efflux ratio ^b	hrCYP P450 isoenzyme assay (% inhibition)			
	A-B	B-A		CYP3A4	CYP2D6	CYP2C9	CYP2C19
IN-203	26	35	1.3	27.3	60.5	39.8	56.5
Digoxin	0.3	12.4	41.5	nd	nd	nd	nd
Ketoconazole	nd	nd	nd	95.2	nd	nd	nd
Paroxetine	nd	nd	nd	nd	96.5	nd	nd
Sulphaphenazole	nd	nd	nd	nd	nd	93.7	nd
Alpha Naphthoflavone	nd	nd	nd	nd	nd	nd	nd
Ticlopidine	nd	nd	nd	nd	nd	nd	104.4

^a P_{app}, absorptive permeability coefficient; ^b Efflux ratio = P_{app} (B-A)/P_{app} (A-B); nd: not determined.

Table 3. Bacterial efflux pump inhibition and human Pgp inhibition activity of identified natural product hits

Test compound name	Test compound code (conc. μ M)	MIC (μ g/ml) of indicated drug for indicated strain without/ with test compound (fold inhibition)				Pgp inhibition (IC ₅₀ , μ M)
		Ciprofloxacin SA1199B (NorA)	Tetracycline SA-K2192 (TetK)	Erythromycin SA-K2191 (MsrA)	Mupirocin Mup-1 (MdeA)	
Praeurtorin B	IN-93 (25)	8/4 (2)	32/32 (0)	64/64 (0)	256/256 (0)	16
Curcumin	IN-142 (25)	8/1 (8)	32/32 (0)	64/64 (0)	256/256 (0)	62
Imperatorin	IN-197 (25)^a	8/4 (2)	32/32 (0)	64/64 (0)	256/128 (2)	28
Osthol	IN-203 (25)^a	8/2 (4)	32/16 (2)	64/64 (0)	256/256 (0)	34
5,7-Diacetoxy-8-(3-methyl-2-butenyl)-coumarin	IN-235 (25)^{ab}	8/4 (2)	32/16 (2)	64/16 (4)	256/256 (0)	44
5,7-Dihydroxy-8-(3-methyl-2-butenyl)-coumarin	IN-238 (25) ^b	8/4 (2)	32/32 (0)	64/64 (0)	256/256 (0)	nd
Pongomol	IN-299 (25)	8/4 (2)	32/32 (0)	64/64 (0)	256/256 (0)	88
Phellopterin	IN-309 (25)^a	8/4 (2)	32/32 (0)	64/32 (2)	256/256 (0)	32
Tangeretin	IN-327 (12.5)^{ac}	8/4 (2)	32/32 (0)	64/32 (2)	256/128 (2)	nd
3-(2-Methyl but-3-en-2-yl) xanthyletin	IN-343 (25)^a	8/2 (4)	32/16 (2)	64/64 (0)	256/256 (0)	15
7-Demethyl osthol	IN-358 (50)	8/2 (4)	32/32 (0)	64/64 (0)	256/256 (0)	nd
Allorottlerin	IN-359 (3.12)	8/2 (4)	32/32 (0)	64/64 (0)	256/256 (0)	nd
Tetrahydroangeolide	IN-365 (50)	8/8 (0)	32/32 (0)	64/64 (0)	256/256 (0)	nd
Piperine (25)		8/4 (2)	32/32 (0)	64/64 (0)	256/256 (0)	nd

nd, not determined due to low quantity; ^a Compounds marked in boldface are multidrug efflux pump inhibitors; ^b IN-235 and IN-238 are analogs of natural products; ^c IN-327 also showed 2-fold reduction in MIC of ciprofloxacin at 25 μ M.

In addition to the resistance phenomenon, efflux pumps and metabolizing enzymes constitutes the vital xenobiotic metabolic system in humans, where inhibition of Pgp could alter the pharmacokinetic properties of drugs and food ingredients.

Therefore, in order to determine whether osthol is a non-substrate Pgp inhibitor or substrate Pgp inhibitor, it was tested in caco-2 permeability assay for efflux pump substrate behavior. It was observed that osthol is not a substrate inhibitor of Pgp as indicated by the lower efflux ratio of 1.3 as depicted in Table 1. Furthermore, in Cyp screening, it did not show effect on most of the CYP enzymes at 10 μ M) (data shown in Table 1).

The identified 13 natural product hits were then screened for their inhibitory effect on various bacterial efflux pumps including *S. aureus* SA-1199B (Nor A over-expressed), Mup^f-1 (Mde A over-expressed), SA-K2192 (Tet K over-expressed), and SA-K2191 (Msr A over-expressed) (listed in Table 2).

Table 2. Study strains of *S. aureus*

Strain	Relevant characteristic(s)	Drug substrate
SA-1199B (NorA)	NorA overexpressing derivative of a clinical isolate (SA-1199)	Ciprofloxacin ²⁰
XU212 (TetK)	Clinical isolate, has TetK efflux pump overexpressed	Tetracycline ²¹
RN4220 (MsrA)	Transformed with pSK265 into which the gene for MsrA has been cloned	Erythromycin ²²

Mup^f-1 (MdeA) Lab generated resistant mutant of mupirocin overexpressing MdeA efflux pump Mupirocin^{1b}

The results of bacterial efflux pump inhibition are shown in Table 3. The most potent compound IN-142 showed reduction in MIC of ciprofloxacin by 8-fold. Compounds IN-203, IN-343, IN-358 and IN-359 showed reduction in MIC of ciprofloxacin by 4-fold, while IN-93, IN-197, IN-235, IN-238, IN-299, IN-309, IN-327 and piperine at the same concentration reduced MIC of ciprofloxacin by 2-fold. Interestingly, out of 13 compounds, 8 compounds belong to coumarin class. Amongst these, six coumarins inhibited multiple *S. aureus* efflux pumps and led to enhancement in potency of respective anti-infective agents by resensitization. These multiple efflux pump inhibitors include IN-197 (inhibits Mde A and Nor A), IN-203 (inhibits Nor A, Tet K), IN-235 (inhibits Nor A, Tet K and Msr A), IN-309 (inhibits Nor A and Msr A), IN-327 (inhibits Nor A, Msr A and Mde A) and IN-343 (inhibits Nor A and Tet K).

It is noteworthy to mention that the substitution of 7-hydroxy group of 7-hydroxy coumarin nucleus seems to be important for inhibitory activity against Tet K efflux pump. The 7-hydroxy coumarins containing protected 7-OH group (7-OME in IN-203 and 7-OAc in IN-235) showed inhibition of Tet K pump, with 2-fold reduction in MIC of tetracycline. However, 7-hydroxy coumarins without any substitution on 7-OH (IN-238 and IN-

358) were inactive against Tet K. Similar observation was also noticed in case of MsrA efflux pump inhibition. For example, the IN-235 (5,7-di-OAc) showed 4-fold reduction in MIC of erythromycin, whereas IN-238 (5,7-di-OH) is inactive.

5 The process of substrate transport across the biological membranes by Pgp and NorA efflux pump involves binding of the ligand to the efflux pump, followed by conformational change in efflux pump structure and then translocation of the substrate. The energy for this active efflux process is compensated by two
10 ATP molecules in Pgp and H⁺ gradient for Nor A in order to bring these efflux pumps in native form, to make it further ready for another transport.²³ The problems in computational simulation of this process are flexible transmembrane loops, multiple ligand binding sites, binding of more than one ligand at a time larger,
15 variable size of substrate/inhibitor binding cavity and dynamic nature of the substrate-translocation process. Pgp displays poly-specificity in ligand recognition, which further makes it extremely difficult to establish an efficient structure-based design protocol for accurate prediction of the binding affinity for Pgp
20 substrate, inhibitors and inducers.²⁴ Based on the docking score and ΔG of binding to the human Pgp homology model, plausible Pgp binding site is proposed for identified natural products.²⁵ The

phenolic hydroxyl group of IN-142 interacts with the Leu975 backbone by H-bonding while its flexible spacer bends the IN-142 structure so that it orients in such a way that its second aromatic ring interacts with multiple phenylalanine and other hydrophobic residues of the verapamil binding site. Similarly in case of IN-203, carbonyl group of coumarin ring interacts with the Ser773 residue by H-bonding and Phe72, Tyr307, Tyr310,
30 Leu332, Phe336, Phe728, Phe732, Phe759, Leu975, Phe978, Val982, Phe983 and Met986 by hydrophobic interactions.

Nor A efflux pump is a transmembrane pump in which ciprofloxacin optimally binds to the site 1 through strong H-bonding of carboxyl functionality with Arg98 cationic guanido
35 group (Figure 5a and 5b). The hydrophobic interactions as well as H-bonding interactions of ciprofloxacin were mimicked by compounds IN-142 and IN-203 which probably blocks entry of ciprofloxacin (a Nor A pump substrate) to enter site 1. However, the higher potency of IN-142 might be due to its additional
40 interaction with Ser299 residue. In case of IN-343, it binds deeper in Nor A binding cavity by hydrophobic interactions only, which does not allow Nor A efflux pump to translocate ciprofloxacin substrate.

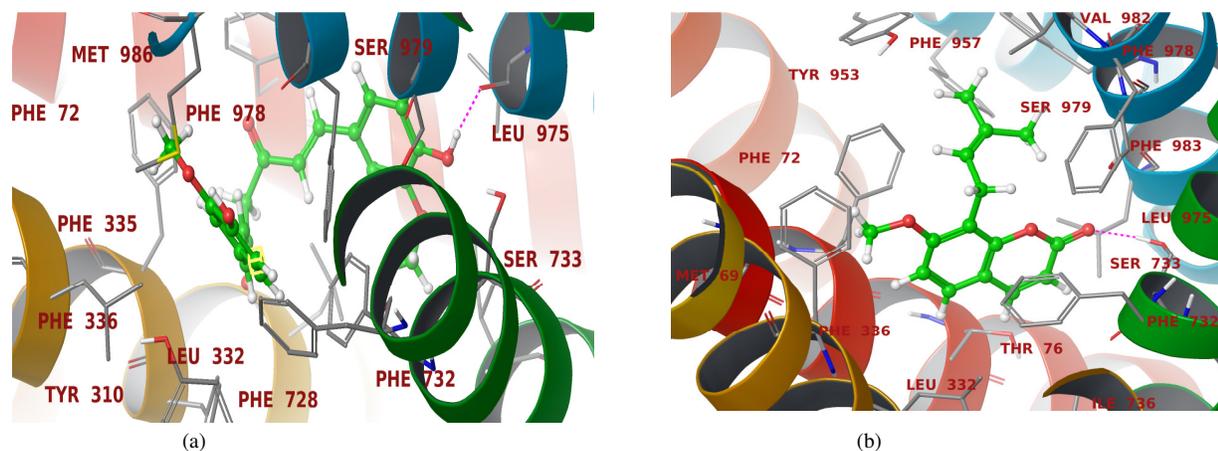


Figure 4. Proposed hypothetical binding site for Pgp inhibitor natural products. (a) Pgp binding interactions of IN-142 (b) Pgp binding interactions of IN-203.

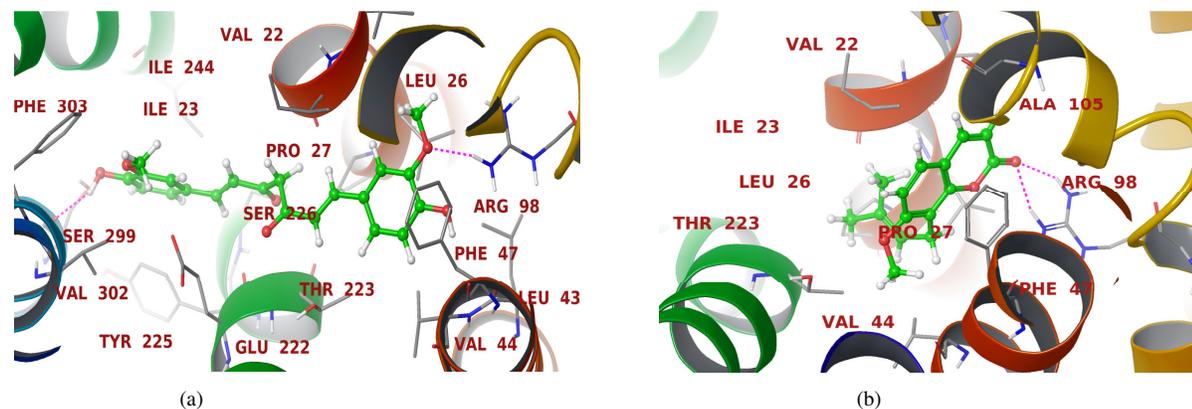


Figure 5. Proposed hypothetical binding site for Nor A inhibitor natural products. (a) Nor A binding interactions of IN-142 at site 1; (b) Nor A binding interactions of IN-203 at site 1.

Although there is no clear understanding of structural similarity between human Pgp and bacterial Nor A efflux pump, there exists overlapping of their substrates/ inhibitor (e.g. ciprofloxacin, elacridar and tariquidar).¹⁶ The possible reason for this substrate overlapping could be due to the presence of dynamic and flexible hydrophobic transmembrane domains at active site of both these pumps and presence of hydrophobic cores in most of these substrates.

10 Conclusion

In conclusion, the screening of in-house natural product repository led to identification of several natural products displaying promising Pgp inhibition activity. Interestingly, many of the identified Pgp inhibitors also inhibited *S. aureus* Nor A efflux pump. These compounds have been identified as Nor A efflux pump inhibitors for the first time. Natural products osthol and curcumin showed 4- and 8-fold reduction in the MIC of ciprofloxacin. In addition to the adjuvant use of obtained Nor A efflux pump inhibitors in combination with regular antibacterial drugs; their interesting ability to inhibit both human Pgp and Nor A pumps, may be further exploited clinically in treatment of cancer patients suffering with sepsis (*S. aureus* infections), where it could potentiate the action of both anticancer and anti-infective agent.

25 Experimental section

Natural product repository. The natural product repository (302 compounds) generated from compounds isolated from our in-house phytochemical investigation efforts on Indian medicinal plants was used for screening. The repository consisted of structures belonging to diverse chemotypes, including phenolics (flavonoids, coumarins), alkaloids, terpenoids, lignans, glycosides and polysaccharides. The structural details of the repository with the Pgp inhibition activity data (Table S1). The literature references for spectral data of identified 13 Pgp inhibitor hits are: praeruptorine B (IN-93),²⁶ curcumin (IN-142),²⁷ imperatorin (IN-197),²⁸ osthol (IN-203),^{28b, 29} 5,7-diacetoxy-8-(3-methyl-2-butenyl)-coumarin (IN-235),³⁰ 5,7-dihydroxy-8-(3-methyl-2-butenyl) coumarin (IN-238),³⁰ pongamol (IN-299),³¹ phellopterin (IN-309),³² tangeretin (IN-327),¹⁹ 3-(2-methyl but-3-en-2-yl)xanthylettin (IN-343)³³ allorottlerin (IN-359)³⁴ and tetrahydroangelide (IN-365).³⁵

In-vitro screening of natural product repository for Pgp inhibitory activity. Colorectal LS180 cells were seeded at a density of 2×10⁴ per well of 96 well plate and allowed to grow for next 24 h. Cells were further incubated with HANKS salt solution for 40 minutes before incubation with the test compounds, and diluted to a final concentration of 50 μM and elacridar (standard) to a final concentration of 10 μM in HANKS buffer containing 10 μM of Rh123 as a Pgp substrate for 90 minutes. The final concentration of DMSO was kept at 0.1%. Test compounds were removed and cells were washed four times with cold PBS followed by cell lysis for 1 h using 200 μl of lysis buffer (0.1% Triton X 100 and 0.2 N NaOH). A total of 100 μl of lysate was used for reading fluorescence of Rh123 at 485/529

nm. All samples were normalized by dividing fluorescence of each sample with total protein present in the lysate. IC₅₀ value for each of the selected compound was calculated by treating the cells at different concentrations (1, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μM) and analyzing the data by using Graphpad Prism software. Data expressed as mean ± SD or representative of one of three similar experiments unless otherwise indicated. Comparisons were made between control and treated groups or the entire intra group using one way ANOVA with post Bonferroni test through GraphPad Prism 5.00.288 statistical analysis software. p-values *<0.05 were considered significant.

In-vitro screening of identified Pgp inhibitor hits in bacterial efflux pump inhibition assay. The strains of *S. aureus* and their substrate drugs are listed in Table 2. The combination studies were performed by a broth checkerboard method.³⁶ A series of two fold dilutions of drug substrates in Muller Hinton Broth (pH 7.0) were tested in combination with 2 fold dilutions of compounds in 96-well micro titer plates. The final concentrations of antibacterial drugs ranged from 12 μg/ml to 256 μg/ml and for test compounds from 0.7 μM to 50 μM. Piperine, earlier reported efflux pump inhibitor by us was used as positive control in this study. Bacterial inocula were prepared by adjusting the inoculum density of the overnight grown bacterial cultures to 0.5 McFarland (~ 1.5 × 10⁸ CFU/mL of *Escherichia coli*). These inocula were diluted 1:100 in sterile normal saline and 100 μl of these diluted inocula was dispensed in each well. The final bacterial inoculum reached in each well was equal 5 × 10⁵ CFU/mL. The plates were incubated at 37 °C for 24 hrs. The MIC was read visually as the lowest concentration of drug inhibiting the growth of bacteria as evident from the absence of turbidity.

Molecular modeling of identified Pgp inhibitors with Pgp. The human Pgp is a 170 kD, transmembrane protein belong to ABC transporter family, whose structure has not been solved. Therefore, molecular modeling studies were carried out using Pgp homology model. The Pgp homology model developed using *C. elegans* crystal structure (PDB: 4AZF), was kindly provided by the Prof. Jue Chen.³⁷ the obtained homology model was subjected to protein preparation wizard facility under default conditions implemented in Maestro v9.0 and Impact program v5.5 (Schrodinger, Inc., New York, NY, 2009). The prepared protein was further utilized to construct grid file by selecting verapamil interacting residues to murine Pgp.³⁸ All chemical structures were sketched, minimized and docked using GLIDE XP, minimized using macromodel and free energy of the binding (ΔG) was calculated using Prime MMGB/SA function.

Molecular modeling of identified Pgp inhibitors with Nor A efflux pump. *S. aureus* efflux pump Nor A, is a member of major facilitator superfamily group, whose crystal structure has not been solved, however, homology based prediction of its transmembrane structure and ligand binding domain was carried out using glycerol-3-phosphate transporter pump.¹⁰ Homology modeled Nor A protein structure was minimized by protein preparation wizard using OPLS 2005 force field. As the exact binding site of substrate and inhibitor to Nor A efflux pump is not available, the sitemap analysis was performed, in which two major binding cavities (site 1 and site 2) were observed in Nor A efflux pump. The site 1 is surrounded by large number of

transmembrane loops and located deeper in efflux pump while site 2 is quiet open and it is located on surface. Therefore, ligand binding site was defined based on the optimization of docking protocol using XP docking score and free energy of the binding (ΔG) of ciprofloxacin, capsaicin, piperine and reserpine, where receptor vander waal radius, size of grid box and residue lining the cavity were varied. Optimized grid box have size of 25 \AA^0 and receptor vander waal radius of 1.0 \AA^0 . All natural compounds were docked using optimized protocol, which includes, grid box size of 25 \AA^0 and vanderwaal radii of 1.0 \AA^0 . Further docked poses were minimized by macromodel to optimize receptor-ligand binding interactions.

Caco-2 permeability assay. Caco-2 cells were seeded at a density of 5×10^4 cells/well of 24-well Millicell plate (apical chamber, A) and 1.8 mL media (MEM with 20% FBS) was added to the receiver chamber (basolateral, B). The cells were grown at $37 \text{ }^\circ\text{C}$ and 5% CO_2 for the next 21 days and the culture media was replaced every 2 days. The permeability studies were initiated in the wells showing TEER values $>500 \text{ } \Omega/\text{cm}^2$. Briefly, each well containing Caco-2 monolayer was washed twice with HBSS buffer and the cell filter plate was transferred to a 24-well transporter analysis plate containing 1 mL HBSS buffer in each basolateral well. The permeability studies from apical to basolateral chamber were initiated by adding 0.3 mL of various concentrations of drug to apical chamber ($n=2$). The plates were further incubated on a shaking incubator at $37 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$ and 100 μL of samples were collected from both the chambers at 0 and 90 minutes for analysis by HPLC utilizing mass spectrometry detection. Similarly, basolateral to apical chamber transport was measured by adding the test compounds to basolateral chamber. The efflux ratio was calculated from transport rate of test compounds across Caco-2 cell monolayer from apical to basolateral (A to B) and basolateral to apical (B to A) side. Dexamethasone was used as a positive control for transport from A to B, while, digoxin was used as a standard for transport from B to A.³⁹

Human cytochrome P450 (CYP450) isoenzymes assay. The CYP P450 isoenzymes were aliquoted as per the total concentration required to conduct the study and stored at $-70 \text{ }^\circ\text{C}$ until use. Total assay volume was adjusted to 200 μL containing three components: cofactors, inhibitor/vehicle and enzyme-substrate (ES) mix. The 50 μL of working cofactor stock solution was dispensed to all the specified wells in a black nunc microtiter polypropylene plate. The 50 μL of diluted working concentrations of NCE's/ positive control inhibitors/vehicle were dispensed in triplicates to the specified wells as per the plate map design. Reaction plate with cofactor and test item was pre incubated at $37 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$ shaking incubator for 10 minutes. Simultaneously, ES mix was prepared by mixing the CYP P450 isoenzyme. Remaining volume was made up with the buffer and pre incubated for 10 minutes at $37 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$. 100 μL of ES mix was dispensed per well as per the plate map design and incubated at $37 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$ shaking incubator for predetermined time. A set of controls were incubated with CYP P450 isoenzymes and substrate without test or reference item. A set of blanks were incubated with substrate and test or reference item, in the absence of CYP P450 isoenzymes. Reaction was terminated by adding

specific quenching solutions (For CYP2C19 and CYP3A4 – 75 μL of 100% acetonitrile; For CYP2C9 – 20 μL of 0.25 M Tris in 60% methanol; For CYP2D6 – 75 μL of 0.25 M Tris in 60% methanol). The reaction was quenched by thoroughly mixing the final contents of the wells by repeated pipetting using multichannel pipette. The product fluorescence per well was measured using a fluorimeter at excitation and emission wavelength for respective CYP P450 isoenzyme fluorogenic metabolites. Data was analyzed by using Excel spreadsheet and the % inhibition was calculated.⁴⁰

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Abbreviations

MDR: Multi-drug resistance, Pgp: P-glycoprotein, *S. aureus*: *Staphylococcus aureus*, MDR: multi drug resistance, Rh123: Rhodamine 123, MIC: minimum inhibitory concentration, CFU/mL: colony forming unit/mL, hrCYP: human cytochrome P450.

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