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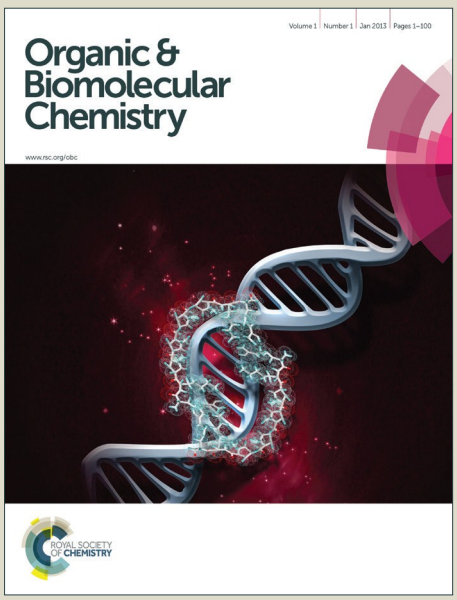

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Paper

Discovery of 4-acetyl-3-(4-fluorophenyl)-1-(p-tolyl)-5-methylpyrrole as a dual inhibitor of human P-glycoprotein and *Staphylococcus aureus* Nor A efflux pump¤‡

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Polysubstituted pyrrole natural products lamellarins are known to overcome multi-drug resistance in cancer via inhibition of p-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) efflux pumps. Herein, a series of simplied polysubstituted pyrroles, prepared via one-pot domino protocol, were screened for P-gp inhibition in P-gp overexpressing human adenocarcinoma LS-180 cells using rhodamine 123 efflux assay. Several compounds showed significant inhibition of P-gp at 50 µM, as indicated by increase in intracellular accumulation of Rh123 in LS-180 cells. Furthermore, pyrrole 5i decreased the efflux of digoxin, a FDA approved P-gp substrate in MDCK-MDR1 cells with IC_{50} of 11.2 µM. In in-vivo studies, following oral administration of a P-gp substrate drug rifampicin along with compound 5i, the C_{max} and $AUC_{0-\infty}$ of rifampicin was enhanced by 31 and 46%. All compounds were then screened for their ability to potentiate ciprofloxacin activity via inhibition of *Staphylococcus aureus* Nor A efflux pump. Pyrrole 5i showed significant inhibition of *S. aureus* Nor A efflux pump with 8- and 4-fold reductions in the MIC of ciprofloxacin at 50 and 6.25 µM, respectively. The molecular docking studies of compound 5i with the human P-gp and *S. aureus* Nor A efflux pump identified its plausible binding site and key interactions. Thus, the results presented herein strongly indicate the potential of this scaffold for use as multi-drug resistance reversal agents or bioavailability enhancers.

Introduction

Pyrrole heterocycle widely occur among marine natural products. One of the largest group of pyrrole alkaloids is lamellarins (> 30 alkaloids) which are isolated from marine invertebrates such as sponges, molluscs and tunicates.[1, 2] This group of alkaloids are known to possess promising cytotoxicity and inhibit number of protein kinases involved in the cancer progression.[3] Apart from this bioactivity, several lamellarins possess ability to overcome the drug-resistance in cancer cells. Lamellarin I (**1**)[4] is reported to potentiate the cytotoxic effect of doxorubicin, vinblastine and daunorubicin. Similar effect was also reported for other lamellarins such as lammelarin D (**2**)[5] and synthetic analog **3**.[6] Chen and Capon's group have identified simplified and less complex lamellarin O (**4**) possessing ability to reverse the breast cancer resistance protein (BCRP) mediated drug resistance in cancer cells.[7] The chemical structures of pyrroles **1-4** are shown in Figure 1.

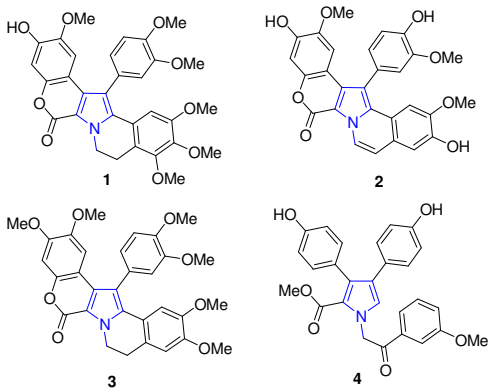

Figure 1. Structures of lamellarins **1-4** showing P-gp/BCRP inhibition activity

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As a part of our efforts towards development of domino one-pot protocols for synthesis of medicinally important scaffolds, one-pot domino four-component protocol was established for synthesis of polysubstituted pyrroles and a library of 17 compounds was prepared.[8] Apart from the development of new synthetic protocol, the purpose of making molecular libraries was also to explore their biological potential. Based on the literature precedence on substituted pyrroles as multi-drug resistance reversal agents,[4-7] and in continuation to our recent efforts in this area,[9] a prepared series of polysubstituted pyrroles was screened for P-gp inhibition activity in P-gp overexpressing human adenocarcinoma LS-180 cells using rhodamine123 (Rh123) cell exclusion method. The effect of key compound on the efflux of FDA approved P-gp substrate digoxin was also investigated in MCDK-MDR1 cells. The potential of identified lead as a bioavailability enhancer of P-gp substrate drugs was then investigated. Based on the literature precedence,[9] these compounds were further investigated for bacterial efflux pump inhibition activity.

Results and discussion

Synthesis. A library of multi-substituted pyrroles **5a-q** was prepared via a montmorillonite clay catalyzed domino four-component reaction of amines **6**, aldehydes **7**, 1,3-dicarbonyl compounds **8** and nitroalkanes **9**[10] (Figure 2a). Total 17 pyrroles varying in the substitution pattern at three different positions were synthesized. Chemical structures of compounds **5a-q** are shown in Figure 2b.

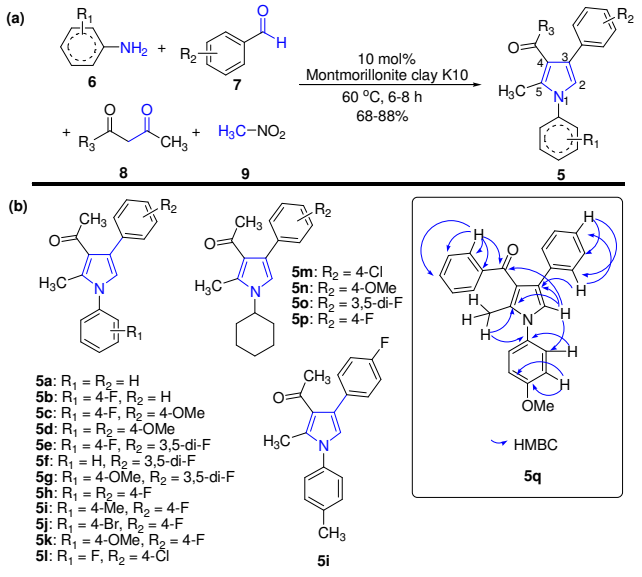

Figure 2. Synthesis of pyrroles **5a-q**. (a) Scheme for preparation of pyrroles **5a-q**. The amine **6** is either arylamine or cyclohexyl amine; (b) structures of synthesized pyrroles. A separate structure of active compound **5i**, which is studied in detail in this paper, and the key HMBC correlations for **5q** are also shown.

In case of product **5q**, due to the bulkier benzoyl group, there is a possibility of formation of two positional isomers differing in the position of benzoyl and methyl groups (interchange of their positions). The distinct ^{1}H NMR chemical shift value of methyl and ^{13}C NMR chemical shift value of carbonyl group indicated the obtained product is **5q** (4-benzoyl isomer) and not the other isomer. In the case of 5-benzoyl isomer, the expected ^{1}H NMR chemical shift value for methyl protons is ~2.1 ppm, whereas in case of 4-benzoyl isomer **5q**, C$\underline{H}_3$ protons are expected to have slight downfield shift because of adjacent ring nitrogen. The observed C$\underline{H}_3$ chemical shift value of 2.27 ppm pointed towards formation of 4-benzoyl isomer **5q**. Next, the ^{13}C NMR chemical shift value of –$\underline{C}$=O group in 4-benzoyl isomer is expected at 195 ppm, whereas in 5-benzoyl isomer, it should be shifted upfield (~180 ppm) because of the adjacent ring nitrogen. The observed value of 194.2 ppm, again indicated formation of 4-benzoyl isomer **5q**. The final confirmation and structure assignments were made by 2D NMR experiments (assignments are shown in section S5 of ESI). Furthermore, in case of acetyl pyrroles **5a-p**, similar trend of chemical shift values of –$\underline{C}$=O and C$\underline{H}_3$ groups was observed, indicating the formation of 4-acetyl pyrroles (and not the 5-acetyl pyrrole).

P-gp inhibition activity. All compounds **5a-q** were screened for P-gp inhibition activity in P-gp overexpressing human adenocarcinoma LS-180 cells using rhodamine123 (Rh123) cell exclusion method and elacridar was used as positive control. The test concentration of 50 µM was used for preliminary screening. This concentration was selected in order to avoid chance of missing any possible P-gp 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 of 17 compounds, have identified 6 compounds which displayed significant P-gp inhibition activity as indicated by the increase in the % intracellular accumulation of Rh123 in LS-180 cells compared to the control (100%) as depicted in Figure 3. Compounds **5a** and **5i** were better than others showing 180% and 172% intracellular levels of Rh123 in LS-180 cells in comparison to the control (100%). The compound **5i** was not cytotoxic in LS-180 cells (IC_{50}>100 µM).

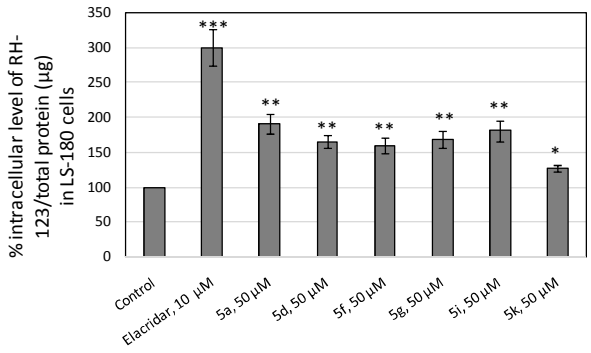

Figure 3. P-gp inhibition activity of substituted pyrroles in LS-180 cells at 50 µM. Statistical comparisons were made between control vs inhibitors by using Bonferroni method. The p value <0.05 was considered to be significant. p value *< 0.5, **<0.01, ***<0.001.

Digoxin is a FDA approved P-gp substrate. The efflux ratio of digoxin in the presence of compound **5i** was measured in the MDCK-MDR1 cells. The efflux ratio (B2A/A2B) of digoxin in these cells was found to be 39.0, which was reduced to 12.7 in presence of compound **5i** (100 µM). The IC_{50} for P-gp inhibition

was determined by co-treatment of digoxin with compound **5i** at different concentrations ranging from 1 to 100 μM. It was observed that co-treatment of digoxin with compound **5i** resulted in dose-dependent decrease in the efflux ratio of digoxin. The IC_{50} was determined from the change in efflux ratio's at different concentrations, and this value was found to be 11.2 μM.

Further, one of the representative compound **5i** was selected for validating the P-gp inhibition property of this scaffold in in-vivo system. The strategy for validating the P-gp inhibition ability in in-vivo system was to investigate the effect of our identified P-gp inhibitor **5i** on the enhancement in the pharmacokinetic properties of a P-gp substrate. The antitubercular drug rifampicin, is a potent substrate and inducer of P-gp. Therefore, as a model drug for understanding the behavior of compound **5i** on pharmacokinetics of a P-gp substrate drugs, we selected rifampicin. Compound **5i** being a promising P-gp inhibitor, it was hypothesized that on oral administration along with a P-gp substrate drug rifampicin, it will improve the bioavailability of a drug via inhibition of gut P-gp.

The $AUC_{0-\infty}$ and C_{max} of rifampicin alone on oral administration (10 mg/kg) were found to be 74865 ng·h/mL and 5838 ng/mL with the half life of 5.44 h. On co-administration of compound **5i** (10 mg/kg) and rifampicin (10 mg/kg), the $AUC_{0-\infty}$ value of rifampicin was enhanced to 109220 ng·h/mL (1.46 fold increase). Similarly, the C_{max} of rifampicin was also enhanced (7661 ng/mL) by 1.31 fold. The half life of the rifampicin was also increased by 1.5-fold in presence of compound **5i**. The significant enhancement (46 and 31%) in the $AUC_{0-\infty}$ and C_{max} of rifampicin (Table 1) indicates the modulatory effect of compound **5i** on the gut P-gp, resulting in decreased efflux of rifampicin from the gut, an enhanced level in the blood.

Table 1. Pharmacokinetic parameters of rifampicin alone (10 mg/kg, oral) and after co-administration with compound **5i** (10 mg/kg, oral) post oral dose of rifampicin at 10 mg/kg to BALB/c mice.

Parameter	Unit	Rifampicin alone	Rifampicin + **5i**	Fold change[a]
$t_{1/2,\beta}$	(h)	5.44	8.24	1.51
AUC_{0-t}	(ng·h/mL)	70366	94118	1.34
$AUC_{0-\infty}$	(ng·h/mL)	74865	109220	1.46
C_{max}	(ng/mL)	5838	7661	1.31
t_{max}	(h)	2.00	0.25	0.125
Time points considered for $t_{1/2,\beta}$ calculation:		8 – 24 h	8 – 24 h	

[a] fold change in the pharmacokinetic parameters of rifampicin on co-administration with compound **5i** by p.o. route.

In order to know whether compound **5i** is a substrate or non-substrate P-gp inhibitor, it was tested in Caco-2 permeability assay for efflux pump substrate behavior. It was observed that compound **5i** is a weak substrate inhibitor of efflux pumps as indicated by the efflux ratio of 2.3, which is at the borderline (the efflux ratio >2.0 is considered as a substrate of efflux pump). Furthermore, the effect of compound **5i** on drug metabolizing cytochrome p450 isozymes CYP3A4, CYP2D6, CYP2C19 and CYP1A2 (using isolated CYPs as microsomes) was checked at 10 μM. At this concentration, compound **5i** showed 0, 8, 23 and 51% inhibition of these enzymes, respectively, indicating no CYP liability of this compound.

Nor A efflux pump inhibition activity. Literature reports show that several P-gp inhibitors also inhibit efflux pumps of pathogenic bacteria, fungi and protozoa. These includes piperine and capsaicin (inhibits bacterial efflux pump),[11-14] plagiochin E (inhibits efflux pump in fungi),[15, 16] and dihydro-*β*-agarofuran sesquiterpenes (inhibits leishmania efflux pump).[17, 18] Furthermore, synthetic P-gp inhibitor clinical candidates such as verapamil,[19-21] elacridar and tariquidar[22] also displayed bacterial efflux pump inhibition activity.[23] Although there is no clear understanding of structural similarity between these two pumps (human Pgp and bacterial Nor A), there exists overlapping of their substrates/ inhibitors as mentioned above. This inspired us to investigate these pyrroles for bacterial efflux pump inhibition activity in *Staphylococcus aureus* SA-1199B (Nor A over-expressed) strain.

Before performing the antibacterial activity potentiation experiment, all test compounds **5a-q** were first checked for their own antibacterial effect, wherein none of the compound showed any antibacterial activity. Next, for antibacterial activity potentiation study, first we selected ethidium bromide. The screening was carried out at 7 different concentrations (ranging from 50 to 0.7 μM) of test compounds in combination with ethidium bromide having serial two-fold dilution (concentration ranging from 128-0.25 μg/ml). From this screening, 13 compounds displayed ability to potentiate the antibacterial activity of EtBr. Compounds **5i** and **5k** were the most active, which led to 4-fold improvement in the MIC of EtBr at 6.25 μM. MEC_4 values (the concentration of a test compound at which MIC of EtBr was reduced by 4-fold) for all compounds were also determined (Table 2). Since efflux is the only known mechanism for ethidium bromide resistance,[24] the reversal of its MIC against *Staphylococcus aureus* SA-1199B (Nor A over-expressed) strain clearly indicates the role of these compounds as bacterial efflux pump inhibitors.

Compounds with 2 or more than 2-fold improvement in MIC of EtBr were selected for resistance reversal using ciprofloxacin. The screening was carried out exactly in similar way like EtBr using the same concentration range. Screening results are shown in Table 3. Among all the compounds tested, two compounds **5i** and **5k** were the most potent, showing MEC_4 values of 6.25 μM. Furthermore, compounds **5b**, **5d**, **5e**, **5f**, **5g**, and **5j** showed 4-fold reduction in MIC of ciprofloxacin at 12.5 μM. It is noteworthy to mention that compounds **5d**, **5g** and **5i** displayed significant P-gp inhibition as well as Nor A efflux pump inhibition activity.

Molecular modeling studies for P-gp and Nor A. Next, in order to have insights on the plausible interactions between the efflux pump and inhibitor, molecular docking studies were carried out for compound **5i** with human P-gp and *S. aureus* Nor A efflux pumps. Due to the ambiguity and dynamic instability of these pumps, the substrate transport process is energy dependent complex phenomenon. P-gp requires ATP whereas Nor A requires H^+ gradient for substrate transport. The consumption of

Table 2. Potentiation of ethidium bromide by compounds **5a-q**

Entry	MIC of EtBr (µg/ml) alone	MIC (µg/ml) of EtBr in presence of compounds							*MEC_4 (µM)
		Compound concentration (µM)							
		50	25	12.5	6.25	3.12	1.5	0.7	
5a	16	4	8	16	16	16	16	16	50
5b	16	4	4	8	16	16	16	16	25
5d	16	4	4	8	8	16	16	16	25
5e	16	4	4	8	16	16	16	16	25
5f	16	16	16	16	16	16	16	16	>50
5g	16	4	4	8	16	16	16	16	50
5i	16	2	2	4	4	8	16	16	6.25
5j	16	4	4	8	16	16	16	16	50
5k	16	2	4	4	4	8	16	16	6.25
5l	16	8	8	16	16	16	16	16	25
5m	16	16	16	16	16	16	16	16	50
5n	16	8	8	16	16	16	16	16	16
Piperine	16	8	16	16	16	16	16	16	>50

*MEC_4 = The minimum concentration of a compound that exhibits at least 4 folds reduction in the MIC of EtBr (16 µg/ml) against the test organism (*S. aureus* 1199B) overexpressing the Nor A bacterial efflux pump.

Table 3. Potentiation of ciprofloxacin by compounds **5a-q**

Entry	MIC of ciprofloxacin (µg/ml) alone	MIC (µg/ml) of ciprofloxacin in presence of compounds							*MEC_4 (µM)
		Compound concentration (µM)							
		50	25	12.5	6.25	3.12	1.5	0.7	
5a	8	2	4	4	8	8	8	8	50
5b	8	1	2	2	4	nd	nd	nd	12.5
5d	8	2	2	2	4	4	8	8	12.5
5e	8	1	2	2	4	nd	nd	nd	12.5
5f	8	8	8	8	8	8	8	8	>50
5g	8	2	2	2	4	4	8	8	12.5
5i	8	1	2	2	2	4	4	8	6.25
5j	8	1	2	2	4	nd	nd	nd	12.5
5k	8	1	1	2	2	4	4	4	6.25
5l	8	2	2	4	4	nd	nd	nd	25
5m	8	2	4	8	8	nd	nd	nd	50
5n	8	2	4	8	8	nd	nd	nd	50
Piperine	8	4	8	8	8	8	8	8	>50

*MEC_4 = The minimum concentration of a compound that exhibits at least 4 folds reduction in the MIC of ciprofloxacin (8 µg/ml) against the test organism (*S. aureus* 1199B) overexpressing the Nor A bacterial efflux pump.

(a) (b)

Figure 4. Proposed hypothetical binding sites and interaction pattern of compound **5i** with P-gp and Nor A. (a) Compound **5i** interactions with P-gp efflux pump at verapamil site; (b) Compound **5i** interactions with Nor A efflux pump at site 1.

energy leads to conformational changes in the structure and shape of these pumps.[25, 26] Therefore, computational simulation of this process requires consideration of flexible trans-membrane loops, multiple ligand binding sites, binding of more than one ligand at a time, variable size of substrate/inhibitor binding cavity, dynamic nature of the substrate-translocation process and poly-specificity of ligands.[27-30] Homology models of P-gp and Nor A,[9] were used to understand the interaction pattern of compound **5i**.

P-gp substrates digoxin and Rh123 interacts with P-gp via hydrophobic interactions, as suggested by vacuum cleaner model.[31-33] Compound **5i** was also found to interact with P-gp by purely hydrophobic interactions, and thereby was able to inhibit

the transport of digoxin and Rh123. Compound **5i** displayed Vander waal and π-π interactions with the hydrophobic residues like Tyr310, Phe335, Leu339, Ile736, Phe957, Phe978 and Phe983 of P-gp as shown in Figure 4a. Furthermore, similar to P-gp, compound **5i** also showed interactions with the hydrophobic residues (Val22, Ile23, Leu26, Pro27, Val44, Phe47, Ala48, Arg98, Phe216, Ser219, Thr223 and Asn340) of Nor A efflux pump by hydrophobic Vander waal interactions as shown in Figure 4b. Interestingly, polar interactions like H-bonding were completely missing in case of both P-gp and Nor A efflux pumps.

Conclusion

In conclusion, herein we have identified a simplified pyrrole alkaloid **5i** possessing dual inhibition of human P-gp and *S. aureus* Nor A efflux pumps. The compound **5i** decreased the efflux of P-gp substrate digoxin with IC_{50} of 11.2 μM. It potentiated the antibacterial activity of ethidium bromide and ciprofloxacin which are substrates of *S. aureus* Nor A efflux pump. The compound **5i** also displayed significant bioenhancement of P-gp substrate drug rifampicin in BALB/c mice. Results presented herein strongly indicate the promise of this scaffold as a potential multi-drug reversal agent or bioavailability enhancer. Furthermore, the dual inhibition ability of these compounds could be beneficial in case of antibiotics with bioavailability issues, wherein these dual inhibitors will be useful to overcome bioavailability issue (by inhibiting gut P-gp) and to overcome the drug-resistance (by inhibiting bacterial efflux pump). Furthermore, in addition to the adjuvant use of obtained Nor A efflux pump inhibitors in combination with regular antibacterial drugs; their ability to inhibit both human Pgp and Nor A pumps, may be further exploited clinically in treatment of cancer patients suffering with *S. aureus* infections (sepsis), where it could potentiate the action of both anticancer and anti-infective agent.

Experimental section

General information. All chemicals were obtained from Sigma-Aldrich Company and used as received. ^{1}H, ^{13}C and DEPT NMR spectra were recorded on Brucker-Avance DPX FT-NMR 500 and 400 MHz instruments. Chemical data for protons are reported in parts per million (ppm) downfield from tetramethylsilane and are referenced to the residual proton in the NMR solvent ($CDCl_3$, 7.26 ppm). Carbon nuclear magnetic resonance spectra (^{13}C NMR) were recorded at 125 MHz or 100 MHz: chemical data for carbons are reported in parts per million (ppm, δ scale) downfield from referenced to the carbon resonance of the solvent ($CDCl_3$, 77 ppm). ESI-MS and HRMS spectra were recorded on Agilent 1100 LC-Q-TOF and HRMS-6540-UHD machines. IR spectra were recorded on Perkin-Elmer IR spectrophotometer. Melting points were recorded on digital melting point apparatus. XRD spectra and Scanning electron micrograph were obtained on XRD Mini-Flex Rigaku Model and Jeol.JEM100C-XII electron microscope respectively.

The Caco-2 permeability, CYP liability, MDCK-MDR1 permeability and pharmacokinetic studies were carried out at Jubilant Biosys Bangalore on commercial basis. All animal experiments were approved by the Jubilant Biosys Institutional Animal Ethics Committee, Bangalore, India (IAEC/JDC/2012/27) and were in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Environment, Government of India.

General procedure for synthesis of pyrroles 5a-q: To the stirred solution of aniline (**6**, 0.132 g, 1.42 mmol), benzaldehyde (**7**, 0.1 g, 0.94 mmol), and acetylacetone (**8**, 0.094 g, 0.94 mmol) in nitromethane (**9**, 1.1 ml, 20 mmol) was added 10 mol% montmorillonite clay K10 catalyst. The mixture was refluxed for 5-8 h and then cooled to room temperature. The excess solvent was removed under vacuum, and the residue was purified by silica gel (#100-200) column chromatography using EtOAc: n-hexane (95:5) to get pyrroles **5a-q** in 68-88% yield. Spectral data of all compounds is provided in our earlier paper.[10]

In-vitro screening for P-gp inhibitory activity. Colorectal LS-180 cells were seeded at a density of 2×10^4 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 elacaridar (standard) to a final concentration of 10 μM in HANKS buffer containing 10 μM of Rh123 as a P-gp 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. 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.

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 °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 Ω/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 °C ± 1 °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.[34]

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 °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 test compounds/ 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 °C ± 1 °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 °C ± 1 °C. 100 µL of ES mix was dispensed per well as per the plate map design and incubated at 37 °C ± 1 °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. 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.[35]

MDR1 transfected MDCK permeability assay for P-gp IC_{50} determination. The experimental protocol used in this assay, was similar to the Caco-2 permeability assay as described above. For P-gp inhibition studies, the transport buffer contained 10 µM digoxin along with desired concentration of test compound. The efflux ratio of digoxin in presence of various test concentrations of compound **5i** (ranging from 1 to 100 µM) was determined. IC_{50} was determined by plotting the concentration versus percent inhibition of P-gp using GraphPad Prism software.

Pharmacokinetics.

Dose preparation: 10.52 mg of rifampicin was dissolved in 53 µL of DMSO and to it was added 5.26 mL of PEG-200. Finally normal saline was added to make up the final volume up to 10.52 mL (in portions with the help of 1 mL pipette) and mixed well. For compound **5i**, 5.85 mg of the compound was wetted with ~30 µL of tween-80 and triturated in a mortar and pestle. Then slowly, 0.5% of methyl cellulose was added to make up the final volume up to 5.83 mL with trituration.

Treatment: The pharmacokinetic study of compound **5i** and rifampicin, alone and in combination was carried out in BALB/c male mice (12 mice for each study) of age 4-6 weeks, by administering compound orally at dose of 10 mg/kg. Plasma samples were collected at appropriate time points between the range of 0 hours to 24 hours (0.25, 0.5, 1, 2, 4, 8, 10 and 24 h time intervals) and analyzed by LC-MS-MS. Mean plasma concentration was calculated and data was further analyzed for PK parameters evaluation using WinNonlin 5.3 software package. Pharmacokinetic paramaters of rifampicin with and without compound **5i** were determined and are shown in Table 3. The pharmacokinetic parameters of compound **5i**, alone are showin in Section S6 of ESI.

***In-vitro* screening of identified P-gp inhibitor hits in bacterial efflux pump inhibition assay**. Preliminary screening of compounds was accomplished by ethidium bromide and the compounds showing two or more than two-fold reduction in MIC were selected and assessed for resistance reversal of ciprofloxacin. The combination studies were performed by a broth checkerboard method.[36] A series of two fold dilutions of ethidium bromide or ciprofloxacin in Muller Hinton Broth (pH 7.0) were tested in combination with 2 fold dilutions of compounds in 96-well micro titer plates in individual experiments respectively. The final concentrations of antibacterial drugs ranged from 0.12 µg/ml to 64 µg/ml and for test compounds from 0.7 µM to 50 µM. Piperine, earlier reported[24] 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 x 10^8 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× 10^5 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 P-gp inhibitors with P-gp. The human P-gp 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 P-gp homology model. The P-gp 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 P-gp.[38] Chemical structure of compound **5i** was 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 P-gp 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.[11] 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 site-map 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 defind 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 Å and receptor vander waal radius of 1.0 Å. All natural compounds were docked using optimized protocol, which includes grid box size of 25 Å and vander waal radii of 1.0 Å. Further docked poses were minimized by macromodel to optimize receptor-ligand binding interactions.

Abbreviations.

AUC, area under the curve; BCRP, breast cancer resistance protein; 2D-NMR, Two-dimentional nuclear magnetic resonance; EtBr, ethidium bromide; FDA, United States Food and Drug Administration; HMBC, Heteronuclear Multiple-Bond Correlation; HSQC, Heteronuclear Single Quantum Coherence; LS-180, human adenocarcinoma cell line; MDCK-MDR1, Madin-Darby canine kidney cells transfected with the human MDR1 gene; MIC, minimum inhibitory concentration; P-gp, P-glycoprotein; Rh123, rhodamine 123; V_d, volume of distribution; C_{max}, maximum concentration of the compound in plasma.

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