Journal of Medicinal Chemistry

Brief Article

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J. Med. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jmedchem.6b01281 • Publication Date (Web): 02 Dec 2016

ACS Publications

Journal of Medicinal Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Improved potency of indole-based NorA efflux pump inhibitors: from serendipity towards rational design and development.

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KEYWORDS NorA inhibitors; scaffold hopping approach; SAR study; indole

ABSTRACT: The NorA efflux pump is a potential drug target for reversal of resistance to selected antibacterial agents, and recently we described indole-based inhibitor candidates. Herein we report a second class of inhibitors derived from them, but with significant differences in shape and size. In particular, compounds **13** and **14** were very potent inhibitors in that they demonstrated the lowest IC_{50} values (2 μM) ever observed among all indole-based compounds we have evaluated.

INTRODUCTION

The decreasing effectiveness of antibiotics in the treatment of common infections has quickened in recent years with worldwide consequences. Of particular concern are globally disseminated methicillin-resistant *Staphylococcus aureus* (MRSA) clones that cause healthcare-associated infections such as pneumonia, endocarditis, sepsis and complicated skin structure infections. A recent report by the US Centers for Disease Control and Prevention has estimated 80461 severe MRSA infections and 11285 deaths from MRSA per year.[1] Several agents including vancomycin, linezolid, daptomycin, fluoroquinolones, and tigecycline are recommended to treat this Gram-positive bacterium,[2] although it often expresses concomitant resistance to many of them.

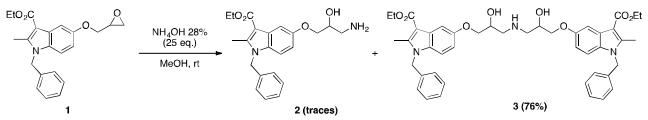
Among factors triggering the resistance response, overexpression of efflux pumps is one of the most studied as it leads to sub lethal drug concentrations at the active site predisposing to the development of high-level target-based resistance.[3]

Therefore, the susceptibility of resistant strains might be restored by co-administration of efflux pump inhibitors (EPIs) and antibacterials (efflux pump substrates); thus, efflux pumps are viable antibacterial targets and identification of potent EPIs is a promising and valid strategy to fight antibiotic resistance.[4] The most studied efflux system in *S. aureus* is NorA, which is able to extrude multiple structurally dissimilar substrates such as hydrophilic fluoroquinolones (ciprofloxacin, norfloxacin), biocides (acriflavine, cetrimide, benzalkonium chloride), and dyes (ethidium bromide [EtBr]), through an antiport mechanism driven by the proton-motive force.[5-7]

To date, no crystal structure of NorA has been determined. Nevertheless, sequence homology and sharing of several substrates with other MDR pumps have suggested that NorA may have a large hydrophobic binding site which may explain the broad substrate specificity of MDR pumps.[8] In the past, several NorA inhibitors belonging to different chemical classes have been identified including natural and synthetic ones.[3] In particular, among heterocyclic compounds, the indole scaffold is typical of some potent NorA inhibitors such as the alkaloid reserpine, 5-nitro-2-phenyl-1*H*-indole (INF55)[9] and analogues,[10] and aldonitrones.[11]

Starting from these literature data about known indole-based NorA inhibitors, we have recently identified a novel class of NorA EPIs with a polysubstituted indole moiety; their activity was evaluated in terms of EtBr efflux inhibition and intrinsic antimicrobial activity determination (MIC).[12] Twenty of them were highly active, inhibiting EtBr efflux by more than 90% (obtained results are briefly summarized in Figure S1). During the development of the our first class of indole-based NorA inhibitors, the reaction of the epoxide intermediate **1**[12] with 28% NH_4OH aqueous solution, did not give the desired compound **2**,[12] while the symmetrical compound **3** was obtained instead (Scheme 1).

Scheme 1.[a]



[a]Reagents and conditions: aq. NH_4OH 28% 25 eq., MeOH, r.t.

Testing **3** as a NorA inhibitor, we found it to be very active with 78% inhibition of EtBr efflux and an IC_{50} value of 19.9 μM, comparable with compound **2** (100% EtBr efflux inhibition; IC_{50} of 17.1 μM). This finding prompted us to investigate not only larger compounds characterized by a symmetrical indole scaffold, but also unsymmetrical compounds similar in size, shape and ability to form chemical interactions with the

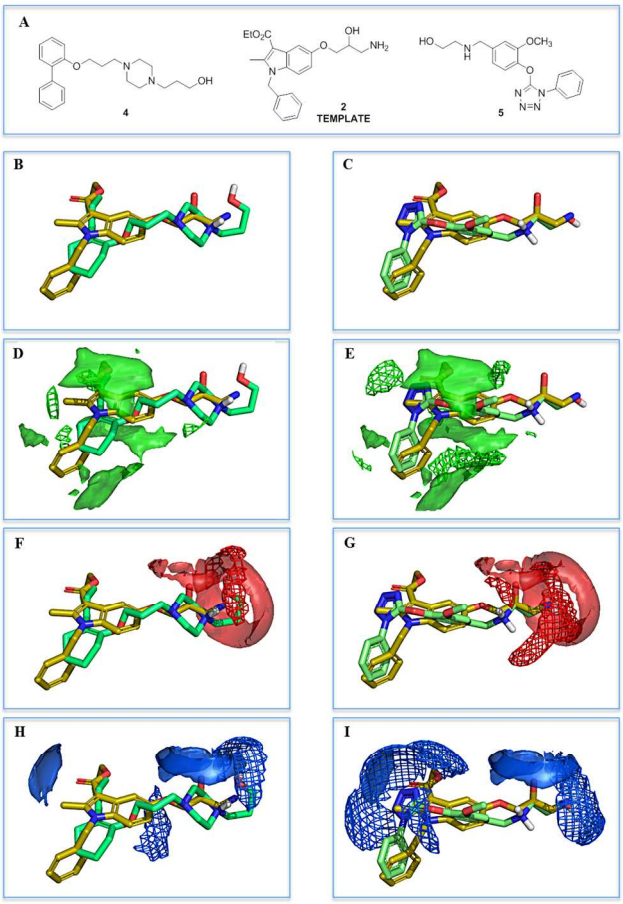
NorA protein, as predicted by *in silico* scaffold hopping analysis. As a result, four out of eleven compounds tested showed improved potency ($IC_{50} \leq 4.9$ μM).

RESULTS AND DISCUSSION

Design. On the basis of the unexpected but exciting potency of compound **3**, we were encouraged to further examine structure-activity relationships. Therefore, we were interested to observe how the activity may change in case of unsymmetrical derivatives of compound **3**, where one of the two indoles was replaced with a molecular fragment similar in shape, size and chemical properties.[12]

Thus, a virtual screening campaign on the SPECS database was performed to search for novel scaffolds using the FLAP software.[13,14] An extensive description of the FLAP algorithm has been reported elsewhere.[13,15] Briefly, FLAP provides a common reference framework for comparing molecules, using GRID Molecular Interaction Fields (MIFs),[16] and the FLAP fingerprints can be used for ligand-ligand,[17,18] ligand-pharmacophore,[19,20] ligand-receptor,[21-23] and receptor-receptor comparison.[24,25] In particular, the FLAP ligand-based virtual screening approach has already proved to be a good strategy for the discovery of novel NorA EPIs,[18] and for scaffold-hopping applications.[26] Thus, in this study, a ligand-based approach was applied to identify compounds highly similar to compound **2** (template), to be used to design and synthesize unsymmetrical analogues of **3**. Details of the virtual screening procedure are reported in the Supporting Information. Starting from more than 16500 structures, a final list of the top-ranked 44 compounds with high global similarity to the template **2** was generated. Compounds **4** and **5** (Figure 1) were selected as possible scaffolds to replace the indole moiety, based on the high FLAP similarity score, the good synthetic accessibility, and commercial availability. The optimal superimpositions, according to FLAP, of compounds **4** and **5** on the template **2** are reported in Figure 1 (B and C, respectively). The phenylphenol (**4**) and the phenyltetrazole (**5**) pose on the benzylindole ring (Figure 1B and Figure 1C). Concerning the terminal amino group of **2**, one of the two piperazine nitrogens of **4** perfectly matches it, while for compound **5** the hydroxyl group instead of the amino group are aligned. However, this preliminary analysis can be misleading. Indeed, the power of using a GRID-MIFs-based similarity software is that the concept of similarity is translated from chemical groups to chemical interactions. Thus, the GRID fields for the aligned structures are reported in Figure 2D-I. Concerning the hydrophobic interactions, both compounds **4** and **5** seem to display a reduced hydrophobicity with respect to the template **2**, but compound **5** shows a more efficient overlap of the hydrophobic portions (Figure 1D-E). Also for polar interactions (HBA and HBD, Figures 1F-G and 1H-I, respectively) a higher similarity was found between **5** and **2**. In summary, according to GRID MIFs analysis, compound **5** was a better candidate than **4** in mimicking the chemical features of **2**. Thus, we decided to design the new unsymmetrical analogues **7** and **12**, where one of the indole moieties was replaced with **4** (best match of the amino group) or **5** (best match of GRID-MIFs), respectively (Table 1). In particular, in compound **7** both the spacers were preserved (with respect to **3**) with the piperazine being the new asymmetric center. From our previous paper,[12] a terminal benzylamino moiety (as in compound **6a**, Table 1) was shown to be a valid scaffold to obtain reasonable NorA inhibitors (EtBr efflux inhibition = 45%); thus compound **12** was synthesized accordingly (Table 1, Figure S2). Finally, being that the linker is an important feature for potency, we focused our attention on the synthesis of novel derivatives of compounds **7** (**8-11**) and **12** (**13-16**) (Table 1).

Figure 1. Structures 2, 4, 5 (A) and superimposition of 4 or 5 on 2 in the FLAP ligand based virtual screening (B-I).[a]

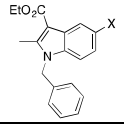


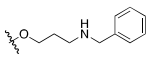
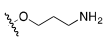
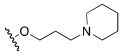
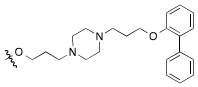
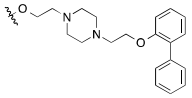
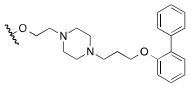
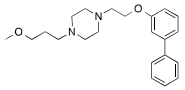
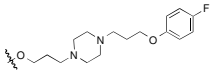
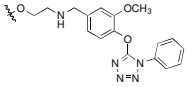
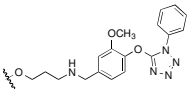
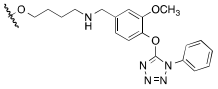
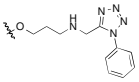
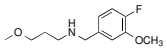
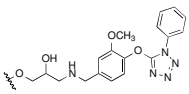
[a]In FLAP poses (B-I) the template **2** is depicted by yellow sticks, while the candidates **4-5** are shown in green sticks. The simple structure overlays of candidates **4** and **5** are shown in panels B and C, respectively. A candidate-template comparison of the GRID hydrophobic (green), HBA (red) and HBD (blue) fields is reported for **4** and for **5** in panels D-F-H and E-G-I, respectively. MIFs for the template are reported in mesh style, while candidate MIFs are shown in wireframe style.

Chemistry. The compounds reported herein were obtained through different chemical synthetic approaches (Schemes 1-4). The synthesis of epoxide **1**, amino derivatives **6a-e**, and bromo-indoles **20a-b** have been described previously.[12]

As shown in Scheme 2, refluxing 2-phenylphenol with an excess of 1,2-dibromoethane or 1,3-dibromopropane in the presence of potassium carbonate in absolute ethanol gave halo-derivatives **18a-b**, that were used to give piperazine ethers **19a-b**. Final compounds **7-10** were prepared analogously by combining halo-ethers **20a-b** with piperazine **19a-b** (Scheme 2). Similarly, compound **11** was obtained from 4-fluorophenol and 1-bromo-3-chloropropane to prepare 1-(3-chloropropoxy)-

Table 1. Biological activity of compound **3** analogues.

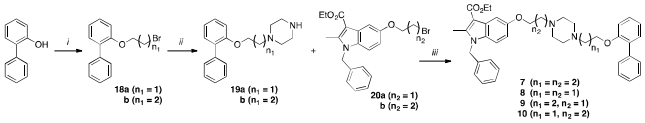


Comp.	X	LogP[a]	pK$_a$[a]	% EtBr efflux inhibition @50 µM[b]	MIC (µg/mL)[c]	IC$_{50}$ (µM)	FIC index[d]
6a		6.0	8.81 (N)	45.5	>100	_	_
6c		4.0	10.1 (N)	100.0	>100	12.5	_
6f		6.0	9.6 (N)	98.5	>100	6.0	0.28
7		8.6	3.82 (N) 8.39 (N)	12.1 ± 2.1	_	_	_
8		7.6	7.62 (N) 3.05 (N)	24.3 ± 6.5	_	_	_
9		8.1	3.82 (N) 8.39 (N)	27.1 ± 7.9	_	_	_
10		8.1	8.39 (N) 3.82 (N)	23.9 ± 5.5	_	_	_
11		7.1	3.82 (N) 8.39 (N)	53.7 ± 1.6	_	_	_
12		5.8	7.00	50.3 ± 2.0	_	_	_
13		6.3	7.21	96.5 ± 0.3	>100	1.82 ± 0.21	0.27
14		6.8	8.20	100.8 ± 2.1	>100	2.02 ± 0.27	0.25
15		4.6	6.93	22.8 ± 3.4	_	_	_
16		6.0	8.54	67.0 ± 1.4	6.3	3.83 ± 0.45	_
17		5.2	6.73 (N) 14.83 (OH)	96.9 ± 2.5	>100	4.93 ± 0.82	0.13

a) predicted by MoKa;[27,28] Biological data were generated employing SA-1199B, which overexpresses *norA*; b) a compound was considered inhibitor if showing an EtBr efflux inhibition > 60%; c) inhibitors with a MIC >100 do not have intrinsic antimicrobial activity; d) FIC indices were for the combination of ciprofloxacin (CPX) with NorA inhibitors by using strain SA1199B. A FIC index of 0.5 was considered to be an indicator of synergistic activity; more details are given in Supplementary Information.

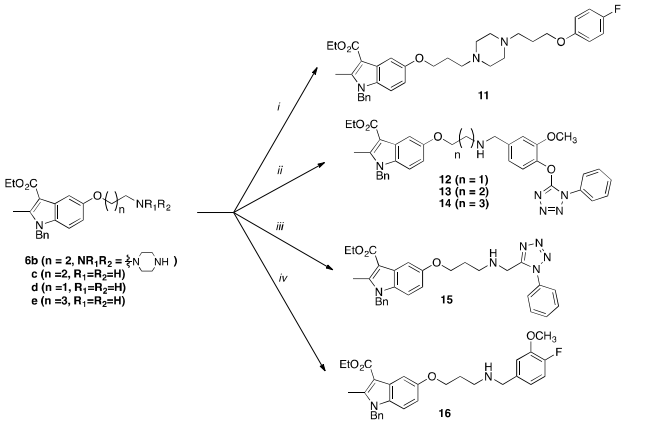
4-fluorobenzene, further condensed with the piperazine derivative **6b** (Scheme 3). According to Scheme 3, tetrazole derivatives **12-14** were prepared from the suitable amino intermediate **6c-e** and 3-methoxy-4-((1-phenyl-1*H*-tetrazol-5-yl)oxy)benzaldehyde (**21**), in turn prepared by *O*-alkylation of vanillin with 1-phenyl-5-chloro-1*H*-tetrazole in the presence of potassium *tert*-butoxyde. The amine **22** was obtained by conversion of the aldehyde **21** into the corresponding oxime, followed by reduction with zinc and hydrochloric acid. Amine **22** was converted into final compound **17** by reaction with the epoxide **1** (Scheme 4). Amine **6c** was used as starting material to give compounds **15-16** upon reaction with 5-(chloromethyl)-1-phenyl-1*H*-tetrazole and 4-fluoro-3-methoxybenzaldehyde, respectively (Scheme 3).

Scheme 2.[a]



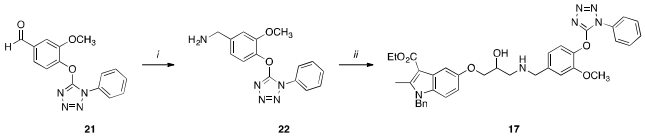
[a]Reagents and conditions: (*i*) 1,3-dibromopropane or 1,2-dibromoethane, K_2CO_3, ethanol reflux; (*ii*) *1.* NaI, acetonitrile reflux; *2.* K_2CO_3, piperazine; (*iii*) *1.* NaI, acetonitrile reflux; *2.* K_2CO_3.[29]

Scheme 3.[a]



[a]Reagents and conditions: *i*) *1.* 1-(3-chloropropoxy)-4-fluorobenzene, NaI, acetonitrile reflux; *2.* K_2CO_3;[29] *ii*) **21**, $NaBH_4$, MeOH, r.t.; *iii*) *1.* 5-(chloromethyl)-1-phenyl-1*H*-tetrazole, NaI, acetonitrile reflux; *2.* K_2CO_3;[29] *iv*) *1.* 4-fluoro-3-methoxybenzaldehyde, Ti(O*i*-Pr)$_4$, EtOH r.t.; *2.* $NaBH_4$, r.t., 5 h.[30]

Scheme 4.[a]



[a]Reagents and conditions: (i) *1*) Na_2CO_3, $NH_2OH \cdot HCl$, EtOH, H_2O, r.t., 24 h;[31] *2*) Zn, H_2O, 10% HCl, EtOH reflux, 12 h;[31] (ii) **1**, $LiClO_4$, CH_3CN, r.t., 12 h.[32]

Biological evaluation. The biological activity of compounds was evaluated by use of EtBr efflux inhibition assays and MIC determinations, as described in Supporting Information.

Compounds **4** and **5** were inactive as EPIs (EtBr efflux Inhibition < 10%), suggesting the fundamental role of the indole scaffold to achieve satisfactory activity. Like fragment **4**, also compounds **7-10** were inactive. Interestingly, compound **11** having 4-fluorophenoxy group instead of a 2-phenylphenoxy group, as for compounds **7-10**, showed an improved inhibitory effect with the percentage of efflux inhibition between 10 and 30%. This finding might suggest that the hydrophobicity and the rigidity of the 2-phenylphenoxy group were detrimental for the activity and a more polar moiety was preferable. Overall according to the data reported in Table 1, the NorA binding site seems to be sensitive to how hydrophobic the inhibitor is: compounds with a LogP value > 8 were not active (e.g. **7**, **9-10**). On the contrary, the lack of inhibition of **5** is probably related to its very high polarity (LogP = 0.9). Inhibitor **12** containing fragment **5** inhibited EtBr efflux by 50%. Between the two derivatives with three (**13**) and four (**14**) carbon atoms chain, the first was a very strong inhibitor (% inhibition = 96%), having the best IC_{50} value ever observed (1.8 μM) among all indole-based compounds that we have reported.[12] Indeed, comparing the biological profile of **13** with those previously published, it exhibited a 2-fold lower IC_{50} compared to the best inhibitor found in that series (compound **6f**) and a 6-fold lower IC_{50} compared to **6c**, that has the same alkyl chain.[12] Moreover **13** had a better activity than that of the symmetrical compound **3**, being 10-fold more active at the 50 μM screening concentration.

Concerning the length of the alkyl spacer, the analogue **14,** having a four carbon atoms chain, exhibited the same % inhibition and IC_{50} value of **13**, 100% and 2 μM respectively; the shorter chain (2 carbon atoms) of **12** produced a 2-fold activity decrease (50% efflux inhibition). Thus, three or four carbon atoms seem to confer the right distance between the indole scaffold and the basic center to obtain the best inhibitory effect. The insertion of the hydroxyl groups in **13** to give **17** was detrimental producing a 2-fold activity decrease with respect to the IC_{50}.

Compounds **15** and **16** have been synthetized as analogues of **13** to study the importance of the 3-methoxybenzyl moiety bound to the amino group: compound **15** that lacks this group was inactive, while compound **16** that preserves the 3-methoxybenzyl but lacks the tetrazole moiety had a lower inhibitory activity (67% efflux inhibition) and an intrinsic antimicrobial activity (MIC = 6.3 μg/mL).

Although the promiscuous activity of **16** as NorA inhibitor and antibacterial compound might be interesting, to avoid misleading results we preferred to further investigate only compounds **13** and **14**, whose activity was exclusively due to NorA inhibition contribution. Indeed, synergistic studies were performed to evaluate their ability to restore antibiotic activity versus SA-1199B, a strain that overexpresses *norA*. As a result, compounds **13** and **14** were also able to restore ciprofloxacin (a commonly used antibiotic) activity according to the FIC index values observed (Table 1).

ADME studies. Metabolic stability (in HLM), water solubility (by NMR)[33] and permeability in CaCo2 cells (predicted *in silico* by VolSurf+ and reported as value of CACO2 descriptor),[34] have been evaluated for the two best compounds **13** and **14** (Table 2). They are metabolically stable, but characterized by a quite low solubility and permeability.

Table 2. ADME study of the best compounds.

Comp.	pK_a	Solub. (mM)[a]	Met analysis[b]	CACO2 Permeability[c]
13	7.21	< 0.002	stable	0.73

14	8.20	< 0.002	stable	0.70

a) evaluated by ^{1}H-NMR in D_2O solution (containing 0.4% of DMSO-d_6) at 25 °C; b) see experimental section for further details;[12] c) predicted by VolSurf+ (the value +1 is assigned to permeable compounds, with $P_{app.} \geq 8 \times 10^{-6}$ cm s^{-1}).[34]

CONCLUSIONS

In this study, we conducted an investigation on the anti-efflux pump properties of a series of indole-based derivatives against a NorA-overexpressing *S. aureus* strain. During the development of our first class of NorA inhibitors, we identified a symmetrical compound that even though being a very large molecule was able to inhibit EtBr efflux by more than 80%. This unexpected discovery suggested that the NorA efflux pump can bind molecules bigger than the ones we had previously developed.[12] Therefore, on the basis of a virtual screening with FLAP, we selected two molecular moieties **4** and **5** to bind to the indole scaffold and to obtain asymmetrical derivatives similar in size, shape and chemical properties to the symmetrical compound **3**. The combination of **5**, disclosing the best GRID-MIFs, with the indole moiety, allowed to identified compounds **13** and **14** as very strong NorA efflux pump inhibitors, both with IC_{50} values of ≤ 2 μM. To conclude, within this study we confirmed that the indole is a promising scaffold for the development of new EPIs and we have identified two very potent NorA multidrug EPIs with unexpected features in shape and size.

EXPERIMENTAL SECTION

Chemistry. Starting materials were purchased from Aldrich-Fluka and Apollo Scientific Ltd and used without further purification. Chloroacetanilide was prepared in 95% yield according to literature procedure.[35] ^{1}H, ^{13}C and ^{19}F NMR spectra were recorded with a Bruker Avance III 400 MHz. Solubility was determined by ^{1}H NMR experiments in D_2O solution at 25 °C using 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP) as the internal standard.[33] HRMS spectra were registered on Agilent Technologies 6540 UHD Accurate Mass Q-TOF LC/MS, HPLC 1290 Infinity. Purities of the final compounds were ≥98% as determined by UHPLC.

General procedure A. The suitable halo intermediate (176 mmol) was added to a stirred mixture of phenol derivative (59 mmol) and K_2CO_3 (8.1 g, 59 mmol) in ethanol (100 mL) and refluxed for 6 h. After cooling, precipitated salt was filtered off and the filtrate was concentrated under reduced pressure. The residue was diluted with H_2O and extracted with AcOEt. The combined AcOEt layers were washed with brine, dried over anhydrous Na_2SO_4, concentrated under vacuum and purified by column chromatography on silica gel (eluent PE/AcOEt 95:5).

General procedure B. A mixture of the suitable halo intermediate (11 mmol) and NaI (3.3 g, 22 mmol) in CH_3CN (30 mL) refluxed for 30 min. After cooling to room temperature, piperazine or the suitable amino derivative (33 mmol) and anhydrous K_2CO_3 (44 mmol) were added and the resulting mixture was refluxed again for 6-12 h. The reaction mixture was diluted with H_2O and extracted with CH_2Cl_2. The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under vacuum. If not stated otherwise, the crude was purified by chromatography on silica gel.

General procedure C.[36,37] Aldehyde **21** (243 mg, 0.82 mmol) was added to a MeOH (10 mL) solution of the suitable amine **6c-e** (0.82 mmol). The resulting suspension was stirred at room temperature overnight. $NaBH_4$ (187 mg, 4.92 mmol) was added and the reaction mixture was heated for 4 h at 50 °C. After cooling, the mixture was diluted with H_2O and extracted with CH_2Cl_2. The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude was purified by column chromatography on silica gel (eluent, CH_2Cl_2/MeOH/Et_3N 95:4:1).

Diethyl 5,5'-((azanediylbis(2-hydroxypropane-3,1-diyl))bis(oxy))bis(1-benzyl-2-methyl-1*H*-indole-3-carboxylate) (3). 28% aquous NH_4OH (4 mL) was added to a stirred solution of epoxyde **1** (515 mg, 1.41 mmol) in methanol (20 mL) and the mixture was reacted at room temperature overnight. All the volatile materials were evaporated under reduced pressure and the residue was taken up with chloroform, washed with water and brine, dryed under sodium sulfate anydrous and concentrated under vacuum. The residue was purified by column cromatography on silica gel eluting with AcOEt/MeOH/Et_3N (8/1.5/0.5) to obtain 400 mg of a white solid (76% yield).

Ethyl 5-(3-(4-(3-([1,1'-biphenyl]-2-yloxy)propyl)piperazin-1-yl)propoxy)-1-benzyl-2-methylindole-3-carboxylate (7) was obtained from the monoalkylated piperazine **19b** and the bromoether **20b** according to the general procedure B. Trituration of the crude residue into a 90:10 Et_2O/PE mixture gave a white solid (64% yield).

Ethyl 5-(3-(4-(3-([1,1'-biphenyl]-2-yloxy)ethyl)piperazin-1-yl)ethoxy)-1-benzyl-2-methylindole-3-carboxylate (8) was obtained from compounds **19a** and **20a**. The crude was purified by column chromatography eluting with a mixture CH_2Cl_2/MeOH 95:5 affording 200 mg of a white solid (46% yield).

Ethyl 5-(3-(4-(3-([1,1'-biphenyl]-2-yloxy)propyl)piperazin-1-yl)ethoxy)-1-benzyl-2-methylindole-3-carboxylate (9) was obtained from compounds **19b** and **20a** according to the general procedure B. Trituration of the crude residue into a 90:10 Et_2O/PE mixture gave a white solid (80% yield).

Ethyl 5-(3-(4-(3-([1,1'-biphenyl]-2-yloxy)ethyl)piperazin-1-yl)propoxy)-1-benzyl-2-methylindole-3-carboxylate (10) was obtained from compounds **19a** and **20b** according to the general procedure B. After trituration of the crude into a 90:10 Et_2O/hexane mixture, a white solid (75% yield) was obtained.

Ethyl 1-benzyl-5-(3-(4-(3-(4-fluorophenoxy)propyl)piperazin-1-yl)propoxy)-2-methyl-1*H*-indole-3-carboxylate (11) was obtained from chloro derivative **18** and amine **19** according to the general procedure B. Chromatography of the crude eluting with a mixture CH_2Cl_2/MeOH/Et_3N 95:4:1 allowed to recover 102 mg of a white solid (74% yield).

Ethyl 1-benzyl-5-(2-((3-methoxy-4-((1-phenyl-1H-tetrazol-5-yl)oxy)benzyl)amino)ethoxy)-2-methyl-1H-indole-3-carboxylate (12) was obtained according to general procedure C from amino derivative **6d** as a pale yellow solid (69% yield).

Ethyl 1-benzyl-5-(3-((3-methoxy-4-((1-phenyl-1*H*-tetrazol-5-yl)oxy)benzyl)amino)propoxy)-2-methyl-1*H*-indole-3-carboxylate (13) was obtained according to general procedure C from amino derivative **6c** as a pale yellow solid (68% yield).

Ethyl 1-benzyl-5-(4-((3-methoxy-4-((1-phenyl-1*H*-tetrazol-5-yl)oxy)benzyl)amino)butoxy)-2-methyl-1*H*-indole-3-carboxylate (14) was obtained from the amino indole **6e** according to the general procedure C as a pale yellow solid (74% yield).

Ethyl 1-benzyl-2-methyl-5-(3-(((1-phenyl-1*H*-tetrazol-5-yl)methyl)amino)propoxy)-1*H*-indole-3-carboxylate (15) was obtained from amine **6c** and 5-(chloromethyl)-1-phenyl-1*H*-tetrazole according to the general procedure B. After chromatography using as eluent CH_2Cl_2/AcOEt/Et_3N 70:29:1, a pale yellow solid (123 mg) was collected (46% yield).

Ethyl 1-benzyl-5-(3-((4-fluoro-3-methoxybenzyl)amino)propoxy)-2-methyl-1*H*-indole-3-carboxylate (16).[30] A mixture of 4-fluoro-3-methoxybenzaldehyde (43 mg, 0.28 mmol), Ti(O*i*-Pr)$_4$ (166 μl, 0.59 mmol) and amine **6c** (50 mg, 0.14 mmol) in EtOH (1 mL) was stirred under nitrogen atmosphere at r.t. overnight. $NaBH_4$ (8 mg, 0.21 mmol) was then added and the resulting mixture was stirred at room temperature for additional 5 h. The reaction was quenched with 2 M NH_4OH aq. solution and extracted with AcOEt. The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under vacuum. Chromatography of the crude on silica gel (eluent CH_2Cl_2/MeOH 95:5) afforded 50 mg of a colourless oil (71% yield).

Ethyl 1-benzyl-5-(2-hydroxy-3-((3-methoxy-4-((1-phenyl-1*H*-tetrazol-5-yl)oxy)benzyl)amino)propoxy)-2-methyl-1*H*-indole-3-carboxylate (17). $LiClO_4$ (44 mg, 0.41 mmol) was added to a solu-

tion of epoxide **1** (150 mg, 0.41 mmol) in dry CH_3CN (2 mL) under nitrogen atmosphere and the mixture was stirred at r.t. for 1 h. Then the amine **22** (122 mg, 0.41 mmol) was added and the reaction mixture was stirred overnight. The mixture was diluted with H_2O and extracted with AcOEt. The combined organic layers were dried over anhydrous Na_2SO_4. After solvent evaporation under reduced pressure, the crude was purified by column chromatography on Silica gel (eluent CH_2Cl_2/MeOH/Et_3N 97:2:1) affording 60 mg (22%) of a white solid.

2-(2-Bromoethoxy)-1,1'-biphenyl (18a) was prepared as a white solid (6.3 g, 38% yield) according to the general procedure A from 1,2-dibromoethane and 2-phenylphenol.

2-(3-Bromopropoxy)-1,1'-biphenyl (18b) was prepared as a white solid according to the general procedure A from 1,3-dibromopropane and 2-phenylphenol (15.7 g, 90% yield).

1-(2-[(1,1'-Biphenyl)-2-yloxy)ethyl]piperazine (19a). The title intermediate was obtained from **18a** and piperazine according to the general procedure B. The oily crude residue was acidified with 5 N HCl. The resulting precipitate was filtered off, washed with Et_2O and then dissolved in 5 N NaOH. The aqueous phase was extracted with AcOEt and the combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under vacuum affording a white solid (58% yield) used without further purification.

1-(3-[(1,1'-Biphenyl)-2-yloxy)propyl]piperazine (19b) was obtained from **18b** and piperazine according to the general procedure B. Purified as described for compound **19a**, a white solid (62% yield) was obtained.

3-Methoxy-4-((1-phenyl-1*H*-tetrazol-5-yl)oxy)benzaldehyde (21). *t*-BuOK (3.0 g, 26.6 mmol) was added to a solution of vanillin (3.40 g, 22.1 mmol) in dry DMF (50 mL) under nitrogen atmosphere. Upon complete dissolution of the base, 5-chloro-1-phenyltetrazole (4.00 g, 22.1 mmol) was added and the mixture was stirred at r.t. for 18 h. The reaction mixture was poured into ice water and the formed precipitate was filtered off under vacuum, washed with H_2O and dried under vacuum obtaining 4.8 g of a white solid (73% yield).

(3-Methoxy-4-((1-phenyl-1*H*-tetrazol-5-yl)oxy)phenyl)methanamine (22). A solution of Na_2CO_3 (207 mg, 1.95 mmol) in H_2O (1.2 mL) was added to a mixture of aldehyde **21** (500 mg, 1.86 mmol) in EtOH (4 mL). After a solution of $NH_2OH \cdot HCl$ (181 mg, 2.60 mmol) in H_2O (1.8 mL) was added slowly to the mixture and the resulting solution was stirred at r.t. for 24 h. H_2O was added and the mixture was extracted with AcOEt; the organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Flash chromatography of the crude on silica gel (eluent CH_2Cl_2/MeOH 95:5) afforded 3-methoxy-4-((1-phenyl-1*H*-tetrazol-5-yl)oxy)benzaldehyde oxime as a white solid (420 mg, 72% yield). The oxime (420 mg, 1.35 mmol) was suspended in EtOH (28 mL) and a suspension of zinc (980 mg, 15 mmol) in H_2O (5.6 mL) was added. 10% aqueous hydrochloric acid (5.6 mL) was added dropwise and the mixture was refluxed for 12 h. After cooling at r.t., 20% NaOH aqueous solution (5.6 mL) was added and the reaction mixture was extracted with CH_2Cl_2. The organic layer was dried over anhydrous Na_2SO_4 and concentrated under vacuum. The crude was purified by flash chromatography on silica gel eluting with CH_2Cl_2/MeOH/Et_3N 90:9:1 affording a light brown solid (311 mg, 78% yield).

1-(3-Chloropropoxy)-4-fluorobenzene was prepared according to general procedure A from 1-bromo-3-chloropropane and 4-fluorophenol, obtaining a pale yellow oil (1.2 g, 71% yield).

5-(Chloromethyl)-1-phenyl-1*H*-tetrazole. PCl_5 was added slowly to a solution of chloroacetanilide (4.00 g, 23.6 mmol) in dry benzene (26 mL) cooled to 0 °C. The mixture was stirred under nitrogen atmosphere at room temperature for 2 h, NaN_3 was added and after 30 min, H_2O was also added. The reaction mixture was refluxed for 5 h and, after cooling, was diluted with H_2O and extracted with CH_2Cl_2. The combined organic layers were washed with 5% NaOH aqueous solution and brine. After drying over anhydrous Na_2SO_4, the solvent was eveporated under reduce pressure and the residue was purified by column chromatography on Silica gel eluting with PE/AcOEt 7:3 to give a pale orange solid (2.7 g, 59% yield).

ASSOCIATED CONTENT

Supporting Information

More experimental details (e.g. all compounds characterization, metabolism assays procedure and computational methods) are given in the Supporting Information.

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Funding Sources

Work performed in the Kaatz laboratory was supported in part by a VA Biomedical Laboratory Research and Development grant (grant number BX000465-01A2). Work performed by S.L. was supported by a Molecular Discovery (London, UK) grant (grant number MD011-2014).

ABBREVIATIONS

MRSA, methicillin-resistant *Staphylococcus aureus*; EPI(s), efflux pump inhibitor(s); MDR, multiple drug resistance; EtBr, ethidium bromide; MIC, minimum inhibitory concentration; SAR, structure-activity relationship; FLAP, fingerprints for ligands and proteins; MIFs, Molecular Interaction Fields; HBD, hydrogen Bond donor; HBA, hydrogen Bond acceptor; HLM, human liver microsomes; DMF, N,N-dimethylformamide; TSP, 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt; LBVS, ligand-based virtual screening.

REFERENCES

1. Antibiotic Resistance Threats in the United States. http://www.cdc.gov/drugresistance/threat-report-2013 (accessed July 17, 2014).
2. Thai, K.-M.; Ngo, T.-D.; Phan, T.-V.; Tran, T.-D.; Nguyen, N.-V.; Nguyen, T.-H.; Le, M.-T. Virtual Screening for Novel Staphylococcus Aureus Nora Efflux Pump Inhibitors from Natural Products. *Med. Chem.* **2015**, *11*, 135-155.
3. Handzlik, J.; Matys, A.; Kieć-Kononowicz, K. Recent Advances in Multi-Drug Resistance (Mdr) Efflux Pump Inhibitors of Gram-Positive Bacteria S. Aureus. *Antibiotics* **2013**, *2*, 28-45.
4. Poole, K.; Lomovskaya, O. Can Efflux Inhibitors Really Counter Resistance? *Drug Discovery Today: Ther. Strategies* **2006**, *3*, 145-152.
5. Yoshida, H.; Bogaki, M.; Nakamura, S.; Ubukata, K.; Konno, M. Nucleotide Sequence and Characterization of the Staphylococcus Aureus Nora Gene, Which Confers Resistance to Quinolones. *J. Bacteriol.* **1990**, *172*, 6942-6949.
6. Kaatz, G. W.; Seo, S. M. Mechanisms of Fluoroquinolone Resistance in Genetically Related Strains of Staphylococcus Aureus. *Antimicrob. Agents Chemother.* **1997**, *41*, 2733-2737.
7. Pao, S. S.; Paulsen, I. T.; Saier, M. H. Major Facilitator Superfamily. *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 1-34.
8. Neyfakh, A. A. Mystery of Multidrug Transporters: The Answer Can Be Simple. *Mol. Microbiol.* **2002**, *44*, 1123-1130.
9. Samosorn, S.; Bremner, J. B.; Ball, A.; Lewis, K. Synthesis of Functionalized 2-Aryl-5-Nitro-1*H*-Indoles and Their Activity as Bacterial Nora Efflux Pump Inhibitors. *Bioorg. Med. Chem.* **2006**, *14*, 857-865.

10. Ambrus, J. I.; Kelso, M. J.; Bremner, J. B.; Ball, A. R.; Casadei, G.; Lewis, K. Structure–Activity Relationships of 2-Aryl-1*H*-Indole Inhibitors of the Nora Efflux Pump in Staphylococcus Aureus. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 4294-4297.
11. Hequet, A.; Burchak, O. N.; Jeanty, M.; Guinchard, X.; Le Pihive, E.; Maigre, L.; Bouhours, P.; Schneider, D.; Maurin, M.; Paris, J. M. 1-(1h-Indol-3-Yl) Ethanamine Derivatives as Potent Staphylococcus Aureus Nora Efflux Pump Inhibitors. *ChemMedChem* **2014**, *9*, 1534-1545.
12. Lepri, S.; Buonerba, F.; Goracci, L.; Velilla, I.; Ruzziconi, R.; Schindler, B. D.; Seo, S. M.; Kaatz, G. W.; Cruciani, G. Indole Based Weapons to Fight Antibiotic Resistance: A Structure-Activity Relationship Study. *J. Med. Chem.* **2016**, *59*, 867-891.
13. Baroni, M.; Cruciani, G.; Sciabola, S.; Perruccio, F.; Mason, J. S. A Common Reference Framework for Analyzing/Comparing Proteins and Ligands. Fingerprints for Ligands and Proteins (FLAP): Theory and Application. *J. Chem. Inf. Model.* **2007**, *47*, 279-294.
14. Molecular Discovery Ltd. http://www.moldiscovery.com/ (accessed February 11, 2016).
15. Cross, S.; Baroni, M.; Carosati, E.; Benedetti, P.; Clementi, S. Flap: Grid Molecular Interaction Fields in Virtual Screening. Validation Using the Dud Data Set. *J. Chem. Inf. Model.* **2010**, *50*, 1442-1450.
16. Goodford, P. J. Drug Design by the Method of Receptor Fit. *J. Med. Chem.* **1984**, *27*, 558-564.
17. Muratore, G.; Goracci, L.; Mercorelli, B.; Foeglein, A.; Digard, P.; Cruciani, G.; Palu, G.; Loregian, A. Small Molecule Inhibitors of Influenza a and B Viruses That Act by Disrupting Subunit Interactions of the Viral Polymerase. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 6247-6252.
18. Brincat, J. P.; Carosati, E.; Sabatini, S.; Manfroni, G.; Fravolini, A.; Raygada, J. L.; Patel, D.; Kaatz, G. W.; Cruciani, G. Discovery of Novel Inhibitors of the Nora Multidrug Transporter of Staphylococcus Aureus. *J. Med. Chem.* **2011**, *54*, 354-365.
19. Chapy, H.; Goracci, L.; Vayer, P.; Parmentier, Y.; Carrupt, P. A.; Decleves, X.; Scherrmann, J. M.; Cisternino, S.; Cruciani, G. Pharmacophore-Based Discovery of Inhibitors of a Novel Drug/Proton Antiporter in Human Brain Endothelial Hcmec/D3 Cell Line. *Br. J. Pharmacol.* **2015**, *172*, 4888-4904.
20. Goracci, L. B., S.; Urbanelli, L.; Ferrara, G.; Di Guida, R.; Emiliani, C.; Cross, S. Evaluating the Risk of Phospholipidosis Using a New Multidisciplinary Pipeline Approach. *Eur. J. Med Chem.* **2015**, *92*, 49-63.
21. Spyrakis, F.; Singh, R.; Cozzini, P.; Campanini, B.; Salsi, E.; Felici, P.; Raboni, S.; Benedetti, P.; Cruciani, G.; Kellogg, G. E.; Cook, P. F.; Mozzarelli, A. Isozyme-Specific Ligands for O-Acetylserine Sulfhydrylase, a Novel Antibiotic Target. *PLoS One* **2013**, *8*, e77558.
22. Spyrakis, F.; Benedetti, P.; Decherchi, S.; Rocchia, W.; Cavalli, A.; Alcaro, S.; Ortuso, F.; Baroni, M.; Cruciani, G. A Pipeline to Enhance Ligand Virtual Screening: Integrating Molecular Dynamics and Fingerprints for Ligand and Proteins. *J. Chem. Inf. Model.* **2015**, *55*, 2256-2274.
23. Sirci, F.; Istyastono, E. P.; Vischer, H. F.; Kooistra, A. J.; Nijmeijer, S.; Kuijer, M.; Wijtmans, M.; Mannhold, R.; Leurs, R.; de Esch, I. J.; de Graaf, C. Virtual Fragment Screening: Discovery of Histamine H3 Receptor Ligands Using Ligand-Based and Protein-Based Molecular Fingerprints. *J. Chem. Inf. Model.* **2012**, *52*, 3308-3324.
24. Siragusa, L.; Cross, S.; Baroni, M.; Goracci, L.; Cruciani, G. Biogps: Navigating Biological Space to Predict Polypharmacology, Off-Targeting, and Selectivity. *Proteins* **2015**, *83*, 517-532.
25. Siragusa, L.; Spyrakis, F.; Goracci, L.; Cross, S.; Cruciani, G. Biogps: The Music for the Chemo- and Bioinformatics Walzer. *Mol. Inf.* **2014**, *33*, 446-453.
26. Lepri, S.; Nannetti, G.; Muratore, G.; Cruciani, G.; Ruzziconi, R.; Mercorelli, B.; Palu, G.; Loregian, A.; Goracci, L. Optimization of Small-Molecule Inhibitors of Influenza Virus Polymerase: From Thiophene-3-Carboxamide to Polyamido Scaffolds. *J. Med. Chem.* **2014**, *57*, 4337-4350.
27. Cruciani, G.; Milletti, F.; Storchi, L.; Sforna, G.; Goracci, L. In Silico Pka Prediction and ADME Profiling. *Chem. Biodiversity* **2009**, *6*, 1812-1821.
28. Milletti, F.; Storchi, L.; Sforna, G.; Cruciani, G. New and Original Pk(a) Prediction Method Using Grid Molecular Interaction Fields. *J. Chem. Inf. Model.* **2007**, *47*, 2172-2181.
29. Chen, X.; Sassano, M. F.; Zheng, L.; Setola, V.; Chen, M.; Bai, X.; Frye, S. V.; Wetsel, W. C.; Roth, B. L.; Jin, J. Structure–Functional Selectivity Relationship Studies of B-Arrestin-Biased Dopamine D2 Receptor Agonists. *J. Med. Chem.* **2012**, *55*, 7141-7153.
30. Miriyala, B.; Bhattacharyya, S.; Williamson, J. S. Chemoselective Reductive Alkylation of Ammonia with Carbonyl Compounds: Synthesis of Primary and Symmetrical Secondary Amines. *Tetrahedron* **2004**, *60*, 1463-1471.
31. Moreno, E.; Plano, D.; Lamberto, I.; Font, M.; Encío, I.; Palop, J. A.; Sanmartín, C. Sulfur and Selenium Derivatives of Quinazoline and Pyrido [2, 3-D] Pyrimidine: Synthesis and Study of Their Potential Cytotoxic Activity in Vitro. *Eur. J. Med. Chem.* **2012**, *47*, 283-298.
32. Beeley, L. J.; Berge, J. M. 2-Benzoheterocyclyloxy or Thiopropanolamine Derivatives with Adreno Receptor Agonist Activity. WO1995004047 A1, February 9, 1995.
33. Lin, M.; Tesconi, M.; Tischler, M. Use of (1)H Nmr to Facilitate Solubility Measurement for Drug Discovery Compounds. *Int. J. Pharm.* **2009**, *369*, 47-52.
34. Cruciani, G.; Pastor, M.; Guba, W. Volsurf: A New Tool for the Pharmacokinetic Optimization of Lead Compounds. *Eur. J. Pharm. Sci.* **2000**, *11 Suppl 2*, S29-39.
35. Guo, X.; Yang, Q.; Xu, J.; Zhang, L.; Chu, H.; Yu, P.; Zhu, Y.; Wei, J.; Chen, W.; Zhang, Y. Design and Bio-Evaluation of Indole Derivatives as Potent Kv1. 5 Inhibitors. *Bioorg. Med. Chem.* **2013**, *21*, 6466-6476.
36. Yamazaki, Y. A., T.; Koura, M.; Shibuya, K. Enantioselective Synthesis of the Ppara Agonist (R)-K-13675 Via (S)-2-Hydroxybutyrolactone. *Synthesis* **2008**, 1017-1022.
37. Yamazaki, Y.; Ogawa, S.-i.; Shibuya, K. Synthesis of Highly Deuterium-Labeled (R)-K-13675, Ppar A Agonist, for Use as an Internal Standard for Low-Level Quantification of Drugs in Plasma. *Bioorg. Med. Chem.* **2009**, *17*, 1911-1917.

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