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Ligand Promiscuity between the Efflux Pumps Human P-Glycoprotein and *S. aureus* NorA

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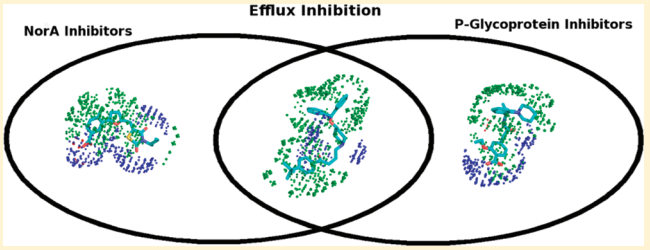
Supporting Information

ABSTRACT: Thirty-two diverse compounds were evaluated for their ability to inhibit both Pgp-mediated efflux in mouse T-lymphoma L5178 MDR1 and NorA-mediated efflux in *S. aureus* SA-1199B. Only four compounds were strong inhibitors of both efflux pumps. Three compounds were found to inhibit Pgp exclusively and strongly, while seven compounds inhibited only NorA. These results demonstrate that Pgp and NorA inhibitors do not necessarily overlap, opening the way to safer therapeutic use of effective NorA inhibitors.



KEYWORDS: *Multiple drug resistance, NorA, P-glycoprotein, promiscuous activity, efflux pump inhibitor, S. aureus*

Human P-glycoprotein (Pgp), also known as ABCB1 or MDR1, is one of the most studied human transporters, since it influences the metabolism and the distribution of a high number of marketed drugs in gut, liver, brain, heart, and many other tissues.[1] The *S. aureus* efflux pump NorA is a bacterial membrane transport protein which is of great clinical importance, since it is known to play a major role in the development of resistance to the fluoroquinolone drugs.[2] Both these membrane transporters reduce the concentration of a number of structurally diverse and apparently unrelated xenobiotics, including drugs, from inside their host cells without alteration or degradation.[3,4] However, they differ in their mechanism, since they belong to different protein families: Pgp is an ATP Binding Cassette (ABC) type pump and utilizes the energy of ATP hydrolysis directly, while NorA is a Major Facilitator Superfamily (MFS) type pump and utilizes the H^+ gradient for active efflux.[5,6]

While Pgp inhibition is generally considered to be an unwanted effect, in oncology it is a long sought-after goal, since multidrug resistance (MDR) in cancer cells is often associated with Pgp overexpression.[7,8] However, due to the key role played in the elimination and distribution of its substrates, Pgp inhibition is generally an unwanted property for therapeutics not employed in the oncologic field, since it might alter the pharmacokinetics parameters of coadministered drugs (for example transporter−enzyme interplay).[9] NorA is responsible for the phenomenon of MDR in some pathogenic strains *S. aureus* and is not considered to be an antitarget. Its inhibition is potentially beneficial, since when certain antimicrobials, including for example most fluoroquinolones, are being used as antibacterials against pump-related resistant strains, the inhibition of NorA by efflux pump inhibitors (EPIs) may restore the original efficacy of the compounds, unless some other resistance mechanism is also present.[10,11]

Recent studies have revealed four compounds which inhibit both efflux pumps: biricodar and timcodar,[12] elacridar[13] and tariquidar.[14] Few other compounds are known to inhibit both pumps, such as reserpine (**1**) and verapamil.[15] This study takes into consideration both pumps together in order to investigate whether the activities of Pgp and NorA are correlated or not. Results presented here show that most of the recently discovered novel NorA inhibitors do not significantly inhibit the human Pgp pump at a concentration of 10^{-4} M. Furthermore, few compounds have been shown to inhibit Pgp activity while being noninhibitors of the NorA efflux pump. In conclusion, results show that in a significant number of cases these promiscuous targets do not necessarily share common inhibitors. This supports the investigation and development of effective NorA inhibitors which are nontoxic to humans.

Our group has been involved in both NorA[16,17] and Pgp[18] in silico modeling. The entire set of compounds in the NorA data set have been projected into the Pgp in silico model,[18] and a number of compounds for which NorA inhibitory activity is already available have been selected and tested for their activity against Pgp. Similarly, the entire Pgp data set was virtually screened using the NorA in silico model, and a number of

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compounds have been selected and tested for their NorA inhibitory activity. This preliminary analysis guaranteed an optimal selection of compounds for the experimental study of the selectivity between the pumps. Five compounds which were untested in both experiments were also acquired in order to balance the data set.

A total of 32 compounds are presented here (Table 1): 21 compounds for which NorA inhibition experimental data were available which were tested for Pgp inhibition, six compounds for which Pgp inhibition experimental data were available which were tested for NorA inhibition, and five compounds which were tested in both experiments. The latter set of compounds is composed entirely of marketed or previously marketed drugs: amlodipine (**2**), astemizole (**3**), dipyridamole (**4**), loperamide (**5**), and quinidine (**6**).

Table 1. Inhibition of the NorA-Mediated Efflux of EtBr in SA-1199B Cells and of the Pgp-Mediated Cell Efflux of R123 in Mouse T Lymphoma L5178 MDR1 Cells

Cpd ID[a]	common name	% inhibition of EtBr efflux	% inhibition of R123 efflux
1[b]	reserpine	84.8[c]	N.A.
2	amlodipine	60.6	52.8[d]
3	astemizole	80.6	12.8[d]
4	dipyridamole	42.3	4.7[e]
5	loperamide	77.7	4.5[d]
6	quinidine	31.5	21.1[e]
7	aripiprazole	92.3	99[e,f]
8	ebastine	88.3	99[e,f]
9	sertindole	81.8	74[e,f]
10	ziprasidone	86.6	19[e,f]
11	aprindine	51.6	16[e,f]
12	repaglinide	14.0	35[e,f]
13[g]	cyclosporine A	N.A.	92[e,f]
14[h]	alprenolol	N.A.	5.0[e,f]
15		22.8[i]	44.1[e]
16		23.6[i]	64.7[e]
17		33.9[j]	1.9[e]
18		1.7[j]	2.6[d]
19		4.4[j]	3.2[e]
20		91.2[k]	40.6[e]
21		19.7[f]	16.8[e]
22		88.2[c]	4.3[e]
23		88.4[c]	1.6[e]
24		0[c]	2.3[e]
25		30.6[c]	21.7[e]
26		86.6[c]	7.8[d]
27		0[c]	2.2[e]
28		2.8[c]	2.1[e]
29		71.7[c]	0.6[d]
30		41.8[c]	2.6[e]
31		41.3[c]	20.4[e]
32		81.0[c]	0.7[d]
33		15.5[c]	2.6[e]
34		74.7[c]	1.7[d]
35		0[c]	11.3[e]

[a]Structures for compounds **1**–**35**, IUPAC names for compounds **15**–**35**, and SMILES are given as Supporting Information. [b]Reserpine was used as a positive control for EtBr efflux inhibition. [c]From ref 16. [d]Tested at 10^{-5} M concentration. [e]Tested at 10^{-4} M concentration. [f]From ref 18. [g]Cyclosporine A was used as a positive control for R123 efflux inhibition. [h]Alprenolol was used as a negative control for R123 efflux inhibition. [i]From ref 21. [j]From ref 22. [k]From ref 17.

Eleven compounds were evaluated for their ability to inhibit the efflux of ethidium bromide (EtBr). Tests were performed at a concentration of 50 μM against SA-1199B using **1** as a positive control. The SA-1199B strain contains a point mutation in *grlA* (topoisomerase IV A subunit gene) resulting in an amino acid substitution in GrlA (A116E), and it also overexpresses the NorA efflux pump (*norA*++) by way of a promoter up-mutation.[19,20]

Compounds **3**, **5**, aripiprazole (**7**), ebastine (**8**), sertindole (**9**), and ziprasidone (**10**) demonstrate an inhibition of EtBr efflux in the SA-1199B strain of >70%, which is comparable to that of the reference compound, **1** (≥80%) (Table 1). These six compounds were tested for their intrinsic antibacterial activity. Only **9** displayed weak intrinsic antibacterial activity (25 μg/mL, Table 2). Aprindine (**11**) and repaglinide (**12**) did not inhibit EtBr efflux.

Table 2. Minimum Inhibitory Concentration (MIC) of the Compounds That Have an EtBr Efflux Inhibition >70% against the *S. aureus* Strain SA-1199B

compd	common name	MIC (μg/mL)
3	astemizole	100
5	loperamide	>100
7	aripiprazole	>100
8	ebastine	>100
9	sertindole	25
10	ziprasidone	>100

Twenty-seven compounds were subjected to Pgp inhibition experiments, carried out by measuring the ability of these compounds to inhibit Pgp-mediated cell efflux of rhodamine 123 (R123) in mouse T lymphoma L5178 MDR1 cells. Cyclosporine A (**13**) was used as a positive control, and alprenolol (**14**) was used as a negative control.

As can be seen in Table 1, most NorA inhibitors were not effective Pgp inhibitors. In particular, compounds **5**, **22**, **23**, **26**, **29**, **32**, and **34** are inhibitors of NorA, but not Pgp, while compounds **4**, **17**, **18**, **19**, **24**, **27**, **28**, **30**, and **33** are clearly inhibitors of neither NorA nor Pgp efflux. Compounds **12**, **15**, and **16**, and to a lesser extent compounds **6**, **21**, **25**, **31**, and **35**, are inhibitors of Pgp but not NorA. Only compounds **7**, **8**, **9**, and **20**, and to a lesser extent compounds **2**, **3**, **10**, and **11**, are inhibitors of both NorA and Pgp efflux.

Of the 12 marketed drugs tested, **6** (quinidine) is known in the literature to be one of the least potent drugs reported to affect the pharmacokinetics of other coadministered drugs by inhibiting Pgp activity.[23] This is also confirmed in a list of clinically relevant drug–drug interactions due to the alteration of transporters' activity which has recently been made available by the FDA.[24] Hence, we used the percent of inhibition of Pgp mediated R123 efflux caused by 100 μM of compound **6** as a threshold for defining Pgp inhibitors and noninhibitors. This value was 21.1%. The threshold for NorA inhibition was set at 70%, while molecules with a percentage inhibition <50% were considered to be noninhibitors, as in previous publications.[16,17,21]

At least three compounds which were measured to be NorA inhibitors do not inhibit Pgp. Six other compounds tested showed similar results but cannot be directly compared to

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compound **6**, since they were tested at lower concentration due to solubility problems. These results seem to be mostly comparable to those obtained by others.[25,26] In fact, compounds **2** and **6** are known effective Pgp inhibitors. Compounds **3** and **5**, that at 100 μM are better Pgp inhibitors than compound **6**, were less potent in this study due to the lower concentration at which they were tested (10 vs 100 μM).[27] Compound **4**, that was previously reported to inhibit over 90% of Pgp mediated calcein-AM efflux,[25] did not affect R123 efflux from L5173 MDR1 cells. However, a significant degree of variability in the IC_{50} values depending on the different probe substrates used (generally within 5-fold, in few cases over 10-fold) has already been observed by Rautio et al.[23] In this case, the use of different cell lines adds a further variable.

Several fluoroquinolones used to treat bacterial infections are known in vivo Pgp substrates. By inactivating Pgp in mice, several authors showed a marked influence of Pgp mediated transport in sparfloxacin and grepafloxacin brain penetration and in grepafloxacin heart disposition and renal excretion.[28,29] In particular, Sasabe and co-workers suggested a possible involvement of Pgp inactivation in grepafloxacin cardiac side effects.[28]

Pgp is also known to share a large number of inhibitors with CYP3A4, and this lack of selectivity is a major problem in the research of effective human chemosensitizers.[30] The results presented here show that this sort of promiscuity does not necessarily exist between Pgp and NorA and, hence, does not bar the development of clinically useful NorA inhibitors.

A simple qualitative analysis of the compounds was carried out by means of multivariate statistics (Principal Component Analysis, PCA) on VolSurf+[31,32] molecular descriptors in order to obtain clear indications for the design of safer NorA inhibitor compounds. Only the compounds with a marked inhibition of either or both pumps and the compounds which were clearly noninhibitors of both pumps were used in the analysis. This study revealed the following:

(1) A significant apolarity (hydrophobicity) is necessary to guarantee affinity with both pumps, since the compounds inhibiting both pumps are markedly more hydrophobic.
(2) The "size" of the molecule is determinant in the distinction between noninhibitors (smaller-size) and strong inhibitors (larger-size) of the two proteins. Interestingly, medium-size compounds are able to inhibit only NorA.
(3) A previous analysis[18] concluded that at least one polar feature (HB accepting group) is necessary for binding with Pgp. Here we confirm this hypothesis for both the proteins. The only compound with the "right" size and apolarity that occurs in this study among the inhibitors of both pumps is compound **27**, which lacks polar groups. Contrarily, too many polar groups make the molecule unable to interact with the pumps, most likely because of a low permeability (for example, compound **4**).
(4) A significant "polar capacity",[31,32] namely the ratio of polar volumes with respect to overall molecular volume, can play a critical role in the selectivity in favor of NorA. In particular, acid groups are present in compounds **29**, **32**, and **34**. These observations are highlighted in Figure 1.

Compound **34**, which is an inhibitor of NorA but not Pgp, has a more pronounced GRID N1 field (blue dots) than the other two molecules. This highlights the importance of a hydrophilic hydrogen-bond acceptor atom in the inhibitor molecules. In all three cases, the DRY field (green dots) is relatively strong, while the O field (red dots) is practically not present.

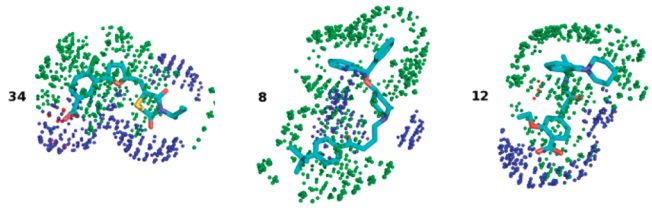

Figure 1. Compound **34** is an inhibitor of NorA, but not Pgp. Compound **12** is an inhibitor of Pgp, but not NorA. Compound **8** is an inhibitor of both NorA and Pgp. The green dots represent the GRID DRY field at −0.5 kcal mol^{-1}, and the blue dots represent the N1 field at −3.5 kcal mol^{-1}. The O field is not visible at −3.5 kcal mol^{-1}. The polar capacity values (CW5, energy = −3.0 kcal mol^{-1}) are 0.24 for compound **34**, 0.07 for compound **8**, and 0.12 for compound **12**. The capacity values at the same energy level for the entire data set and, for comparison, for the compounds biricodar, elacridar, tariquidar, and timcodar are given in the Supporting Information.

In conclusion, the design of selective inhibitors of the NorA bacterial efflux pump should focus on medium-size molecules with a marked polar surface area and, preferably but not necessarily, a benzoic acid group.

■ ASSOCIATED CONTENT

Supporting Information

Compound molecular structures, SMILES codes, and polar capacity values; common or IUPAC names; score plot and loading plot of the PCA model; detailed graphical comparison of NorA and Pgp inhibitors; detailed experimental methods; and experimental data demonstrating the purity of the active compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

Pgp, P-glycoprotein, also known as ABCB1 and MDR1; ABC, ATP-binding cassette; MFS, major facilitator superfamily; MDR, multidrug resistance; EPI, efflux pump inhibitor; EtBr, ethidium bromide; R123, Rhodamine 123; MIC, minimum inhibitory concentration; FDA, Food and Drug Administration; CYP3A4, cytochrome P450 3A4; PCA, principal components analysis

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