354 J. Med. Chem. 2011, 54, 354–365
DOI: 10.1021/jm1011963

Journal of Medicinal Chemistry Article

Discovery of Novel Inhibitors of the NorA Multidrug Transporter of *Staphylococcus aureus*

Jean Pierre Brincat,[†] Emanuele Carosati,[†] Stefano Sabatini,[‡] Giuseppe Manfroni,[‡] Arnaldo Fravolini,[‡] Jose L. Raygada,[§] Diixa Patel,[§] Glenn W. Kaatz,[§] and Gabriele Cruciani*[,†]

[†]*Dipartimento di Chimica, and* [‡]*Dipartimento di Chimica e Tecnologia del Farmaco, Università degli Studi di Perugia, 06123 Perugia, Italy, and* [§]*Department of Internal Medicine, Division of Infectious Diseases, School of Medicine, Wayne State University and the John D. Dingell Department of Veterans Affairs Medical Center, Detroit, Michigan 48201, United States*

Received September 14, 2010

Four novel inhibitors of the NorA efflux pump of *Staphylococcus aureus*, discovered through a virtual screening process, are reported. The four compounds belong to different chemical classes and were tested for their in vitro ability to block the efflux of a well-known NorA substrate, as well as for their ability to potentiate the effect of ciprofloxacin (CPX) on several strains of *S. aureus*, including a NorA overexpressing strain. Additionally, the MIC values of each of the compounds individually are reported. A structure−activity relationship study was also performed on these novel chemotypes, revealing three new compounds that are also potent NorA inhibitors. The virtual screening procedure employed FLAP, a new methodology based on GRID force field descriptors.

Introduction

The emergence of drug resistance in different species of bacteria is a growing cause of concern.[1,2] These bacteria include strains of *S. aureus* that are resistant to vancomycin[3,4] and to the recently discovered linezolid,[5−7] often considered "the last line of defense" against *S. aureus* strains demonstrating multidrug resistance (MDR[a]). Even though new methods of treatment have been developed or are about to be made available,[8] the restoration of clinical efficacy of antibacterials against which resistance has developed remains an important goal.

Antibiotic resistance mechanisms include alteration or modification of the target site,[9] degradation of the antibiotic molecule,[9] and reduction of effective intracellular antibiotic concentration via changes in membrane permeability[10] and/or membrane-based efflux pumps.[11,12] The latter mechanism is of special interest because, much like the widely studied human P-glycoprotein (Pgp, also known as ABCB1), bacterial MDR efflux pumps have been shown to transport a wide variety of structurally unrelated compounds without alteration or degradation.[13]

Of particular concern among resistant microorganisms is the alarming rise of methicillin-resistant *Staphylococcus aureus* (MRSA) strains that are highly virulent.[14] The proportion of healthcare-associated staphylococcal infections that are due to MRSA has been increasing: 2% of *S. aureus* infections in U.S. intensive care units were MRSA in 1974, 22% in 1995, and 64% in 2004.[15] Invasive MRSA infections occur in approximately 94 000 persons each year and are associated with about 19 000 deaths. Approximately 86% of these infections are healthcare-associated, while the remainder are community-associated.[16]

The *S. aureus* NorA efflux pump, which is a member of the major facilitator superfamily (MFS), is known to play a major role in the development of resistance to the quinolone drugs[17] by reducing their concentration inside the target pathogens.[11,12] This leads to a decrease in efficacy and a greater chance for the emergence of high-level target-based resistance.[18−20] When quinolones 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. Different classes of compounds have already been shown to be capable of doing this, including 1,4-benzothiazine derivatives,[21] *N*-caffeoylphenalkylamide derivatives,[22] piperine analogues,[23,24] flavonolignan and flavone compounds,[13] 2-aryl-5-nitro-1*H*-indoles,[25] omeprazole analogues,[26] benzo[*b*]thiophenes,[27,28] fluoroquinolone[29] and 6-amino-8-methylquinolone ester derivatives,[30] fractions of plant extracts,[31−34] and some mammalian Pgp inhibitors.[35,36] Kaatz and co-workers have been studying the inhibition of various bacterial efflux pumps for several years. They have identified a substantial number of diverse compounds with a marked NorA inhibitory activity including phenothiazines, thioxanthenes, selective serotonin reuptake inhibitors, and a variety of phytochemicals.[24,29,41−43] For a more in-depth review see ref 37.

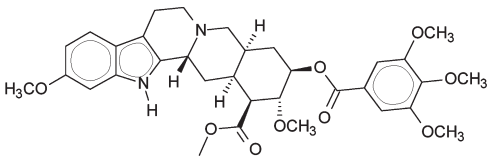
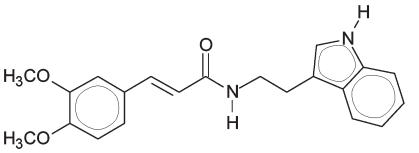
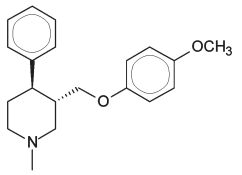
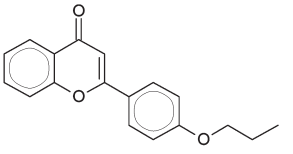
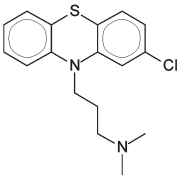
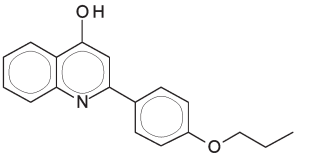
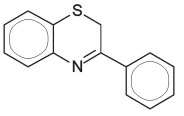
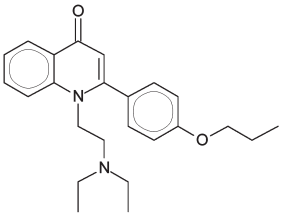
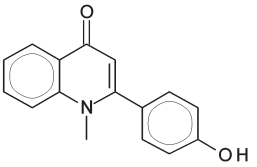
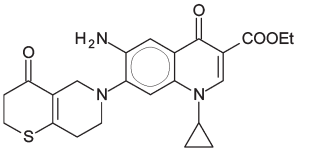
Only one high-throughput screening study[38] and a limited number of QSAR studies concerning the inhibition of NorA are reported in the literature.[23,39,40] These models, generated using different methodologies, are mostly local in terms of chemical diversity and explain how slight modifications in

*To whom correspondence should be addressed. Phone: +39 075 5855629. Fax: +39 075 45646. E-mail: gabri@chemiome.chm.unipg.it.

[a] Abbreviations: CPX, ciprofloxacin; *S. aureus*, *Staphylococcus aureus*; MIC, minimum inhibitory concentration; FLAP, fingerprints for ligands and proteins; MDR, multiple drug resistance; SAR, structure−activity relationship; Pgp, P-glycoprotein, also known as ABCB1; MRSA, methicillin-resistant *Staphylococcus aureus*; MFS, major facilitator superfamily; EPI, efflux pump inhibitor; QSAR, quantitative structure−activity relationship; EtBr, ethidium bromide; MIF, molecular interaction field; sdf, structure-data file; LDA, linear discriminant analysis; WT, wild type; LC−MS, liquid chromatography−mass spectrometry; CLSI, Clinical Laboratory Standards Institute.

 Published on Web 12/08/2010

Table 1. Representative Molecules from the Training Set[a]

 **1:** Reserpine: the reference molecule for NorA inhibition.[22,27,36,41,42,45]	 **2**: represents one active molecule in a class of its own.[22]
 **3**: represents 2 active and 2 inactive molecules.[42]	 **4**: represents 2 inactive molecules.[44]
 **5**: represents 6 active phenothiazine molecules.[41]	 **6**: represents 1 inactive molecule.[44]
 **7**: represents 1 active, 12 inactive and 1 uncertain benzothiazine.[21]	 **8**: represents 3 active and 2 uncertain molecules.[44]
 **9**: represents 15 inactive molecules.[44]	 **10**: represents 3 active, 9 inactive and 4 uncertain molecules.[30]

[a] The full list of molecules is available as Supporting Information.

chemical structure can be correlated with the observed activity within the homologous series. Our group has also attempted to model NorA inhibition for a limited series of molecules in the past, with varying degrees of success and a limited applicability domain.[21,30] This previous work has convinced us that a more global model for NorA inhibition is required.

The aim of the work presented here is to bring together this large and chemically diverse data set (Table 1) into a single, global in silico model for NorA inhibition with a large validity domain. The model was used to select a number of compounds from a public database that were subsequently tested for their NorA-inhibitory activity.

Results

Data Set. The data set used in this work was built using compounds that have been tested against the same strain, with the same experimental procedure, yet reported in different publications.[21,22,27,30,36,41,42,44,45] Ethidium bromide (EtBr)[45] efflux assays were performed and published for all the molecules using *S. aureus* 1199B (SA-1199B) as the test strain and employing identical experimental protocols. The same experimental methods have been used for the in vitro experiments presented in this work. Details are given in the Experimental Section.

Since the computational method being used is discriminant while the percentage of efflux inhibition obtained from

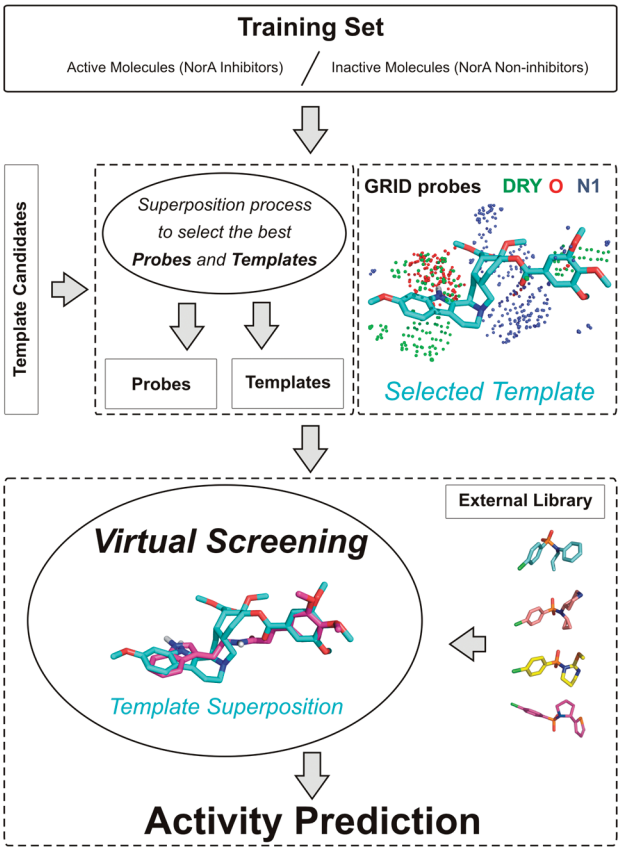


Figure 1. Scheme for the Flap process (refer to the section Computational Methods for details).

the experimental data is continuous, in order to define a training set, each compound was classified into one of three classes based on its experimental percentage inhibition value. In order to ensure that the classification was homogeneous, all percentage inhibition values were taken from experiments performed using 50 μM test compound. Molecules producing a reduction in EtBr efflux of 50% or less were considered as noninhibitors of NorA, while those with an observed reduction of 70% or greater were considered as efflux pump inhibitors. Since none of the compounds for which data are available at different concentrations show reduced efflux inhibition at higher concentrations, or vice versa, molecules with an observed reduction of 70% or more at a concentration lower than 50 μM were also considered as inhibitors, while molecules with an observed reduction of 50% or less at a concentration higher than 50 μM were also considered as noninhibitors. Molecules with intermediate efflux reduction were not used in the training set.

The data set is summarized in Table 1, while the full list of compounds is given in the Supporting Information. A total of 17 active molecules and 41 inactive molecules formed the training set of the model. It is noteworthy that the molecules represented by **4**, **6**, and **9** are all noninhibitors. The presence of such inactive molecules within the training set is important, as they form part of the criteria upon which the in silico model is built.

Model. The computational procedure applied in this work makes use of the most recent advances of the FLAP software,[46,47] which has been previously used successfully for ligand-based virtual screening projects,[48−50] pharmacophore hypothesis generation,[51,52] and high-throughput virtual screening of proteins.[53] FLAP is now a virtual screening and model-development program based on 3D molecular similarity, measured through common GRID[54−56] molecular interaction field (MIF) volumes.

FLAP functions by selecting a user-specified number of molecular structures as "templates" from a list of candidates, then superimposing the test molecules onto these templates and comparing the resulting MIF volume overlaps. Scoring functions are used to assign numerical values to the extent of MIF volume overlap of the two molecules. These scores represent the chemical differences between the template molecule being used and the screening molecule being tested and are hence a measure of the likelihood of the molecule being tested having an activity similar to that of the template compound. The procedure is schematized in Figure 1, and details are given in the Experimental Section.

Virtual Screening. Using the training set summarized in Table 1, a NorA inhibition model with an r^2 value of 0.95[57] was developed using FLAP. This model was then used to screen a large commercial database in an attempt to find novel inhibitor chemotypes.

A database of about 300 000 compounds was prepared from the Specs catalogue.[58] All structures present in the catalogue were downloaded as sdf files from the Specs database and then cleaned by removing all salts, noncovalent complexes, and nondruglike molecules. A total of 295 182 compounds were retained. These compounds were then passed to FLAP in order to generate a database upon which the virtual screening was performed.

In the first selection step, all compounds in this database were subjected to a quick screening using FLAP in order to evaluate whether their molecular structure was roughly similar to that of any of the template molecules. This step was necessary to eliminate compounds that would otherwise have been obvious outliers and more importantly to enrich the selected fraction in molecules that have a good possibility of being NorA inhibitors.

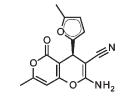
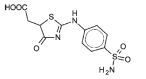
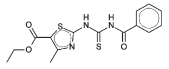
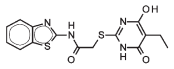
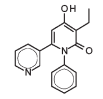
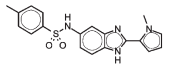
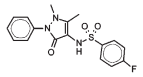
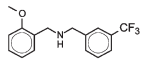
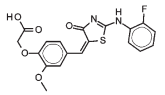
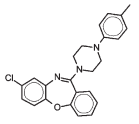
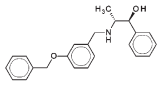
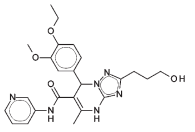
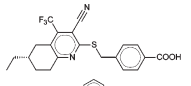
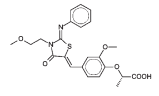
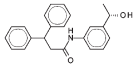
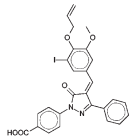
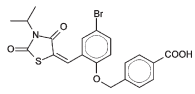
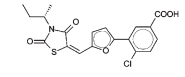
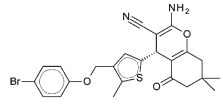
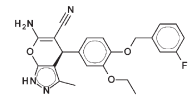
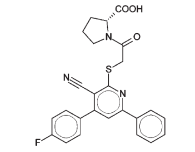
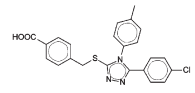
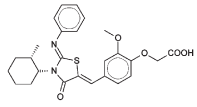
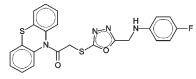
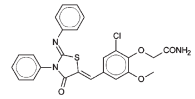
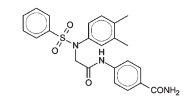
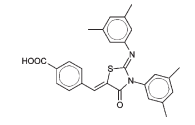
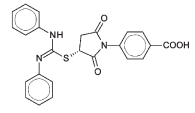
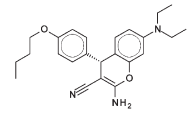
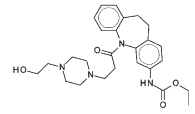
All molecules with a calculated distance score lower than 0.925 toward any one of the two templates (3348 structures) in the first selection step were then projected onto the FLAP model a second time. This round of calculations, while employing the same FLAP algorithm, made use of linear discriminant analysis (LDA) modeling, which implies that the types of interactions that the molecules being screened are able to have were evaluated with more precision.

Thirty molecules that were predicted to be the strongest inhibitors were then selected. This selection was done on the basis of which molecules were commercially available at the time in the amounts required for the biological assays while at the same time attempting to keep as high a chemical diversity as possible. These 30 compounds, which are shown in Table 2, formed the so-called "candidates" set. Note that, as shown in Figure 2, in each of the two selection steps performed in order to arrive at this final selection of compounds, only the top ~1% of the available compounds were selected.

To the best of our knowledge, no activity studies have been published for any of the 30 selected compounds with the exceptions of **15**, **16**, **34**, and **38** which are patented as a method for altering the lifespan of eukaryotic organisms,[59] **20** which is patented as an antipsychotic agent,[60] **28** which is a patented antiviral agent,[61] and **40** which is an antiarrhythmic.[62]

EtBr Efflux Inhibition Results. All candidate compounds were first evaluated for their ability to inhibit the efflux of EtBr. These tests were performed at 50 μM against SA-1199B using

Table 2. The 30 Candidate Molecules

Code	Specs Code	Structure	Reduction of EtBr Efflux (%)	MIC (μM)
11	AG-205/11317135		0.0	-[a]
12	AP-906/42288162		0.0	-
13	AF-886/31411042		23.2	-
14	AG-205/13056088		19.1	-
15	AE-406/41056622		30.7	-
16	AE-848/42434549		88.2	> 273
17	AG-205/36565028		3.2	-
18	AG-690/11822559		30.0	-
19	AP-828/41001984		11.9	-
20	AQ-360/41402277		35.1	-
21	AN-465/42885978		86.6	> 288
22	AP-906/41638445		14.8	-
23	AM-807/41624543		71.7	15
24	AH-487/41138989		10.6	-
25	AO-548/43242435		31.4	-
26	AP-970/41128510		3.8	-
27	AH-487/41184581		3.1	-
28	AH-487/41139966		81.0	123
29	AK-968/41016976		0.0	-
30	AP-964/40915300		4.9	-
31	AM-807/42946590		36.3	-
32	AG-690/40751424		1.3	-
33	AH-487/15277106		32.6	-
34	AG-690/40702076		11.5	-
35	AN-465/15401006		18.3	-
36	AN-988/41721006		0.6	-
37	AN-655/13869026		55.0	-
38	AG-205/36712046		0.0	-
39	AG-205/33140042		20.5	-
40	AI-020/37162014		0.0	-

[a] MIC values were only measured when the % EtBr efflux inhibition was > 70%.

reserpine (**1**) as the reference compound (Table 3). 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 also overexpresses the NorA efflux pump (*norA*++) by way of a promoter up-mutation.[63,64]

Thirteen of the tested compounds demonstrate > 20% inhibition of EtBr efflux in the SA-1199B strain. Four compounds, including 4-methyl-*N*-[2-(1-methyl-1*H*-pyrrol-2-yl)-1*H*-benzimidazol-5-yl]benzenesulfonamide (**16**), 2-{[3-(benzyloxy)benzyl]-amino}-1-phenylpropan-1-ol (**21**), 4-({[3-cyano-6-ethyl-4-(trifluoromethyl)-5,6,7,8-tetrahydroquinolin-2-yl]thio}methyl)-benzoic acid (**23**), and 3-{5-[(*Z*)-(3-*sec*-butyl-2,4-dioxo-1,3-thiazolidin-5-ylidene)methyl]-2-furyl}-4-chlorobenzoic acid (**28**), exhibit a strong inhibitory activity (> 70%). These four

molecules were considered as "hits". In three cases (**16**, **21**, and **28**) this inhibition is comparable to that of the reference compound reserpine (≥80%).

Two of these compounds (**23** and **28**) display variable intrinsic antibacterial activity, whereas such activity is totally lacking in the other two compounds (**16** and **21**). In the latter cases, the observed inhibition of EtBr efflux can be attributed solely to the inhibition of the NorA efflux pump (Table 3).

For those compounds with a percentage of inhibition of EtBr efflux greater than the threshold of 70%, a dose-response curve has been built (Figure 3). The dose-response curves confirm that compounds **16**, **21**, **23**, and **28** are as potent inhibitors of EtBr efflux as the reference compound reserpine. Furthermore, compounds **16** and **21**, which are devoid of intrinsic antibacterial activity, are slightly stronger inhibitors than reserpine at concentrations greater than 30 μM.

Ciprofloxacin Synergistic Activity Results. Further studies were performed on the four active hits in order to evaluate whether the observed inhibition of EtBr efflux, and hence the inhibition of the NorA efflux pump of *S. aureus*, can actually restore the original antibacterial activity of ciprofloxacin (CPX) against both wild-type and resistant *S. aureus* strains. For this purpose checkerboard assays were performed using two pairs of *S. aureus* strains, SA-K1902 (*norA*-)/SA-K2378 (*norA*++) and SA-1199 (*norA* wild-type)/SA-1199B (*norA*++ and A116E GrlA), as well as *S. aureus* ATCC25923 (control, wild-type strain) (Figure 4).

The intrinsic antibacterial activity of each of the compounds being investigated on each test strain was evaluated with the aim of highlighting any possible antibacterial activity that could interfere with the assessment of synergistic activity with CPX based on NorA inhibition (Table 3).

Among the tested compounds **23** displayed the best antibacterial activity. The other compounds have less or no antibacterial activity, with MICs of around 50 μg/mL for **28**, 100 μg/mL for **21**, and >100 μg/mL for **16**. The last can be considered to be devoid of any antibacterial activity.

As can be seen in Figure 4, three of the four hits (**21**, **23**, and **28**) do not exhibit a significant reduction of the MIC of CPX (2- to 4-fold reduction) in the strains with a basal expression of the NorA pump (SA-K1902 and SA-1199) and in the wild-type *S. aureus* ATCC25923 strain. Only compound **16** displays a synergic activity with CPX against *S. aureus* ATCC25923 and SA-1199, with an 8-fold reduction of the antibacterial MICs at 3.13 and 0.78 μg/mL, respectively. Considering that compound **16** is devoid of any intrinsic antibacterial activity and that these strains have a basal expression of NorA efflux pump, it could be proposed that other non-NorA efflux pumps are involved in the synergistic activity of **16** with CPX (Figure 4).

Compound **16** is the most interesting of the four hits because, besides being devoid of any intrinsic antibacterial activity against all the tested strains (>100 μg/mL), it is more potent than compounds **21** and **28** against SA-K2378 (32-fold reduction of CPX MIC at 3.13 μg/mL) and slightly better than **21** against SA-1199B, with a 4-fold MIC reduction at 6.25 μg/mL and an 8-fold reduction at 25 μg/mL (Figure 4).

The intrinsic antibacterial activity of compound **21** is not significant (≥100 μg/mL in most strains, 50 μg/mL in SA-K2378), while it displays a good synergistic activity. In fact, compound **21** was shown to reduce the MIC of CPX on SA-K2378 by 4-fold at only 3.13 μg/mL and by 16-fold at 12.50 μg/mL. With respect to SA-1199B, compound **21** reduces the MIC of CPX by 4-fold at 12.50 μg/mL and by 16-fold at 25 μg/mL (Figure 4).

The synergic activity with CPX of compound **23** is heavily influenced by its evident intrinsic antibacterial activity (6.25 μg/mL in most strains, 12.5 μg/mL on SA-K1199) (Table 3). It is still possible to consider the synergic activity with CPX of compound **23** at a concentration that is $\leq {}^1/_4$ of its respective MIC against the strains included in the assays and in doing so probably to reduce this interference. **23** reduces the MIC of CPX 16-fold in the SA-K2378 strain at 1.56 μg/mL. However, against SA-1199B the 2-fold MIC reduction at this concentration is negligible (Figure 4).

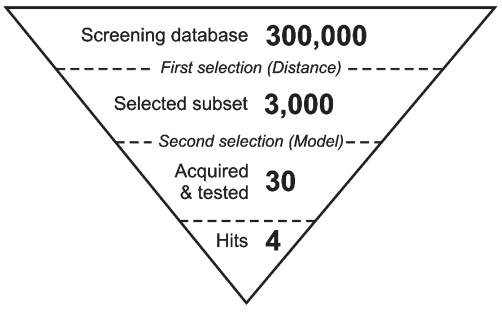


Figure 2. Scheme summarizing the entire virtual screening process, from the initial database to the final hits discovered.

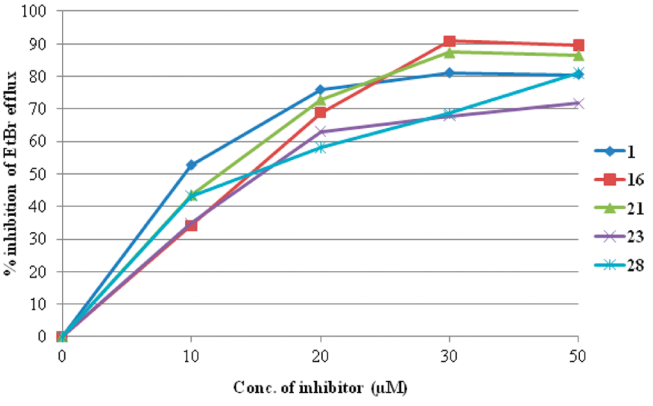


Figure 3. Dose-response curve of EtBr efflux inhibition in SA-1199B for **16**, **21**, **23**, and **28** and the reference compound reserpine (**1**).

Table 3. MICs of Hit Compounds against *S. aureus* Strains Included in the Tests

	S. aureus strains, MIC (μg/mL)				
compd	ATCC25923 (WT)	SA-K1902 (norA-)	SA-K2378 (norA++)	SA-1199 (norA WT)	SA-1199B (norA++/A116E GrlA)
16	>100	>100	>100	>100	>100
21	100	100	50	100	>100
23	6.25	6.25	6.25	12.50	6.25
28	50	50	50	25	50

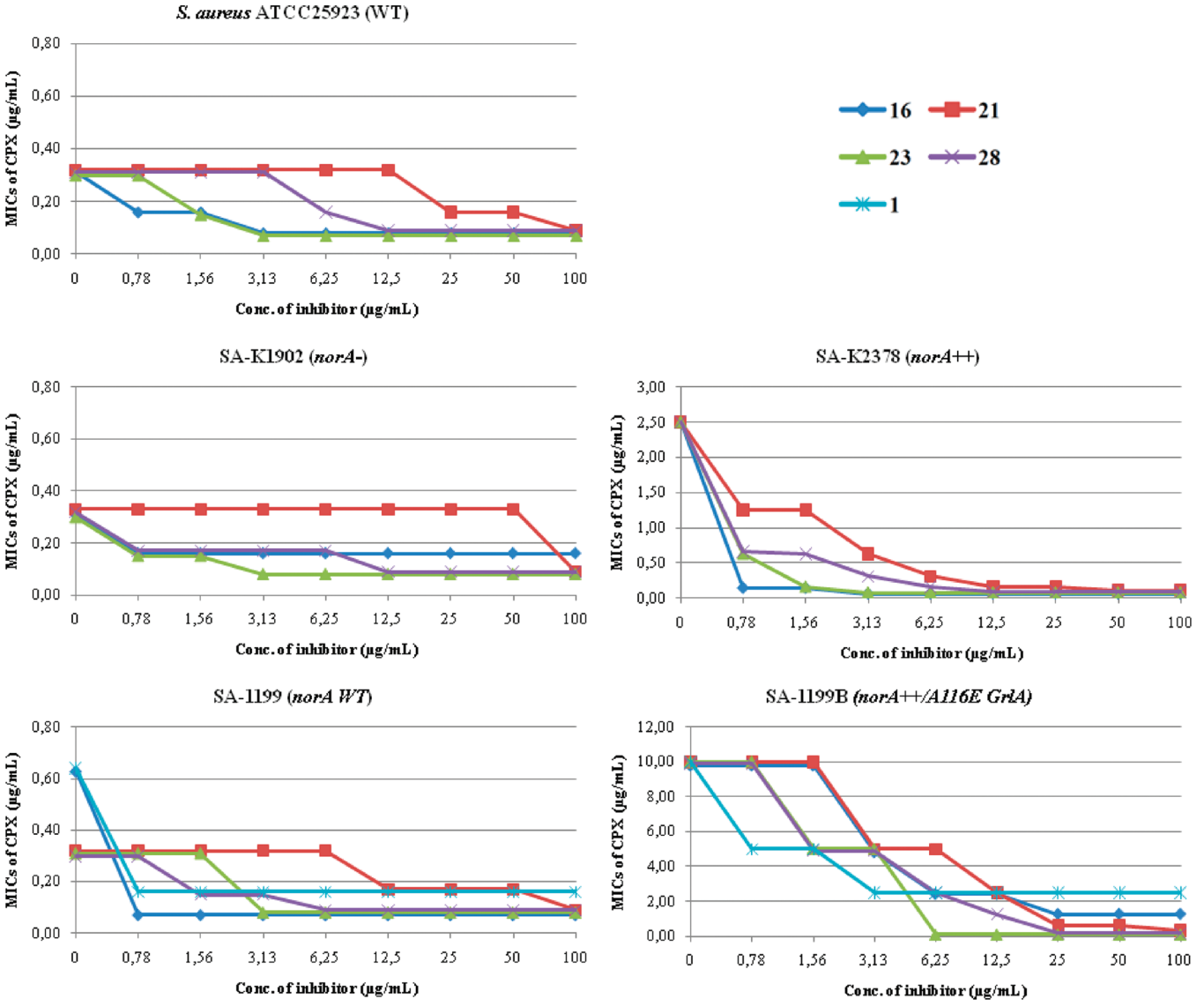


Figure 4. Effect of compounds **16**, **21**, **23**, and **28** on the MIC of ciprofloxacin against *S. aureus* ATCC25923, SA-K1902, SA-K2378, SA-1199, and SA-1199B. Reserpine (**1**) was included as a reference compound for tests against SA-1199 and SA-1199B.

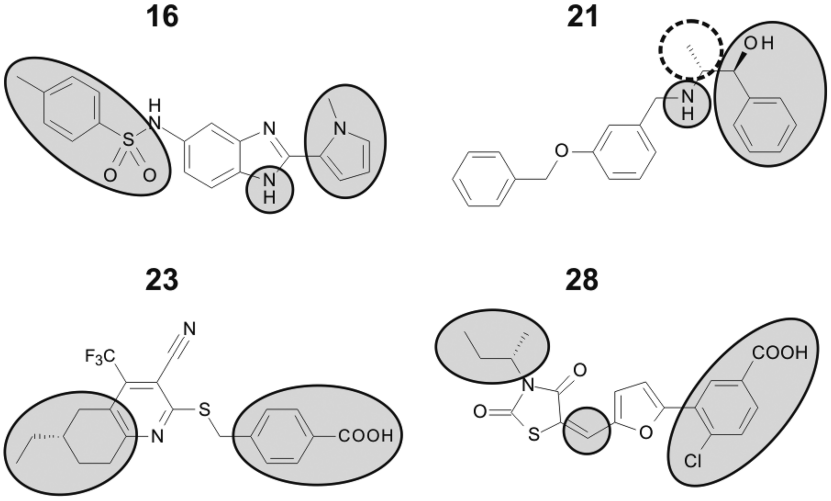
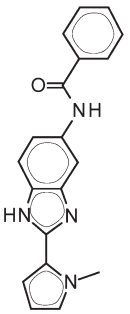
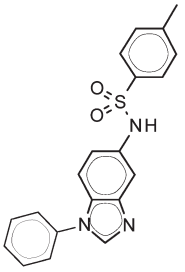
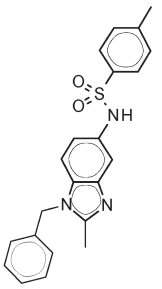
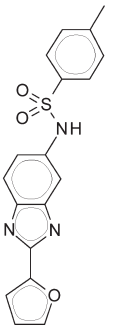
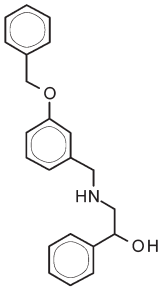
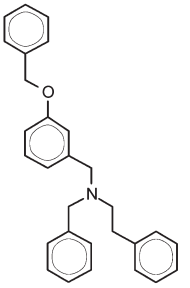
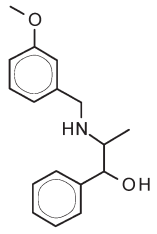
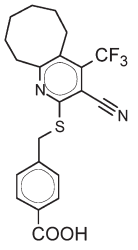
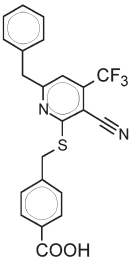
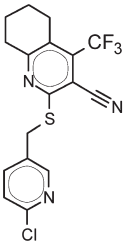
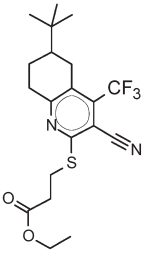
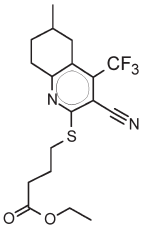
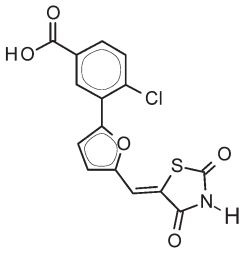
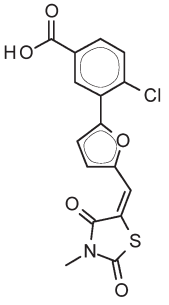
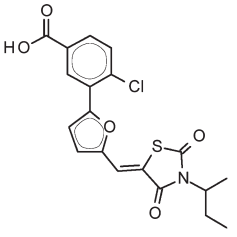
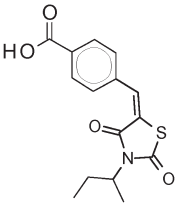
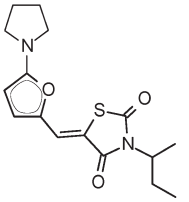


Figure 5. Structure−activity relationship study of the four hits. The highlighted areas represent the regions in which modifications have been made.

Compound **28** reduces the MIC of CPX by up to 32-fold against SA-K2378 at 12.5 μg/mL, which is $^1/_4$ of the MIC of the same compound on that strain. A synergic activity with CPX due to the intrinsic antibacterial activity of **28** is hence excluded,

Table 4. Structures and Data for the "Analogues"

					
41	**42**	**43**	**44**	**45**	**46**
Red.[a]: 88.4%	Red.[a]: 0 %	Red.[a]: 30.6 %	Red.[a]: 67.5 %	Red.[a]: 92.4 %	Red.[a]: 0 %
MIC: >316	MIC: -[b]	MIC: -[b]	MIC: -[b]	MIC: >300	MIC: -[b]
					
47	**48**	**49**	**50**	**51**	**52**
Red.[a]: 2.8 %	Red.[a]: 67.1 %	Red.[a]: 41.8 %	Red.[a]: 41.3 %	Red.[a]: 25.2 %	Red.[a]: 48.6 %
MIC: -[b]	MIC: -[b]	MIC: -[b]	MIC: -[b]	MIC: -[b]	MIC: -[b]
					
53	**54**	**55**	**56**	**57**	
Red.[a]: 15.5%	Red.[a]: 20.9 %	Red.[a]: 74.7 %	Red.[a]: 0 %	Red.[a]: 0 %	
MIC: -[b]	MIC: -[b]	MIC: 123	MIC: -[b]	MIC: -[b]	

[a] Percent reduction of EtBr efflux in SA-1199B by 50 μM test compound. [b] Not tested.

and the potentiation of the MIC of CPX should therefore be solely due to the inhibition of the NorA pump. Even in the SA-1199B strain, which is characterized by the overexpression of the NorA pump and a substitution mutation in topoisomerase IV, **28** is capable of reducing the MIC of CPX by 8-fold (from 10 to 1.25 μg/mL) at 12.5 μg/mL, restoring the susceptibility of the strain to the quinolone antibacterial (Figure 4).

Considering the synergistic antibacterial activity with CPX against SA-1199B, all four tested compounds (**16**, **21**, **23**, and **28**) are more potent than the reference compound reserpine, at least at concentrations higher than 6.25 μg/mL. From the data obtained (reported in Figure 4) it is possible to confirm that for compounds **16**, **21**, **23**, and **28**, the observed inhibition of EtBr efflux leads to a potent synergic effect with

the quinolone antibacterial CPX, which is caused by the inhibition of the NorA efflux pump.

Validation of the Hits. It was deemed necessary to investigate these hits further, in particular to decide whether these compounds were spike actives or else members of a series of potentially active compounds. Structures and substructures similar to those of the four discovered hits were searched for in the ZINC database.[65,66] These searches resulted in a large number of "analogue" compounds for each of the hits, of which a small sample of three to five molecules were selected for each hit. The selected compounds all differed from the "parent" compound in definite, but relatively minor, ways (see Figure 5). The molecules acquired and tested are listed in Table 4, together with the results of the biological assays performed.

Almost all the analogue compounds exhibit a slight to moderate inhibitory effect on the efflux of EtBr in SA-1199B, with the exceptions of **42**, **46**, and **47** which are completely inactive, while compounds **41** and **45** demonstrate impressive 94.3% and 88.4% reductions of EtBr efflux, respectively, without any intrinsic antibacterial activity. These two compounds are more potent than any of the original hits discovered and of reserpine itself.

Dose-response curves (Figure 6) and CPX synergistic activity assays in the SA-1199 and SA-1199B strains (Figure 7) were also created for each of the three "analogue" compounds which surpassed the 70% efflux inhibition threshold (**41**, **45**, and **55**). The dose-response curves for the three "analogue" compounds (**41**, **45**, and **55**) confirm that all these structures are good inhibitors of EtBr efflux.

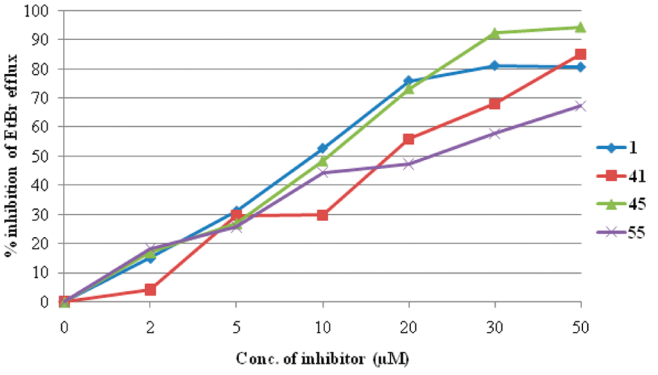


Figure 6. Dose-response curve of EtBr efflux inhibition for compounds **41**, **45**, and **55** against SA-1199B. For a comparison with their respective hits and reserpine (**1**), refer to Figure 3.

Only compound **45** is slightly more active than the parent hit compound and reserpine (Figure 6).

From the isobologram of the synergistic activity for the three compounds against SA-1199 (NorA WT) strain, it can be observed that compounds **41** and **45** show a slight synergistic activity with CPX (from 2- to 4-fold MIC reduction), while **55** reduces the MIC of CPX by more than 8-fold at 3.13 μg/mL, which is less than $^1/_4$ of its MIC (25 μg/mL) against the strain. Compounds **41**, **45**, and **55** are slightly less potent than their "parent" hits **16**, **21**, and **28** in reducing the MIC of CPX against SA-1199B and more potent than the reference compound reserpine at concentrations higher than 25 μg/mL. This confirms that the inhibition of the *S. aureus* NorA efflux pump can actually restore the original antibacterial activity of CPX.

Discussion and Conclusions

Using the novel FLAP procedure and starting from a large and chemically diverse data set, four novel inhibitors of NorA have been discovered. These four compounds were the hits obtained from a total of 30 tested compounds, implying a hit rate of ~13%. Three of these compounds (**16**, **21**, and **28**) are as potent as reserpine at 50 μM, with compounds **16** and **21** being stronger EtBr efflux inhibitors at concentrations above 30 μM.

Compound **23** and, to a lesser extent, compound **28** exhibit intrinsic antibacterial activity, while compounds **16** and **21** can be considered to be devoid of such activity. Compounds with a high intrinsic activity might be interesting for further development, since they might result in compounds that exhibit antibacterial activity and are capable of inhibiting efflux pumps.

All four compounds exhibit a better synergistic activity than reserpine with CPX in the bacterial strains overexpressing the NorA efflux pump at concentrations greater than 6.25 μg/mL. Because of its intrinsic antibacterial activity, it is not known whether or not the CPX activity potentiation observed for compound **23** is due to its inhibition of the NorA efflux pump. For the other three hits, it is presumed that the inhibition of NorA is the main mechanism through which the observed increase in efficacy of CPX is obtained.

The synergistic results for compound **16** show that, unexpectedly, this compound also potentiates the effect of CPX against *S. aureus* ATCC25923 and SA-1199. A possible reason for this could be that **16** is capable of blocking efflux pumps other than NorA which also transport CPX, thus

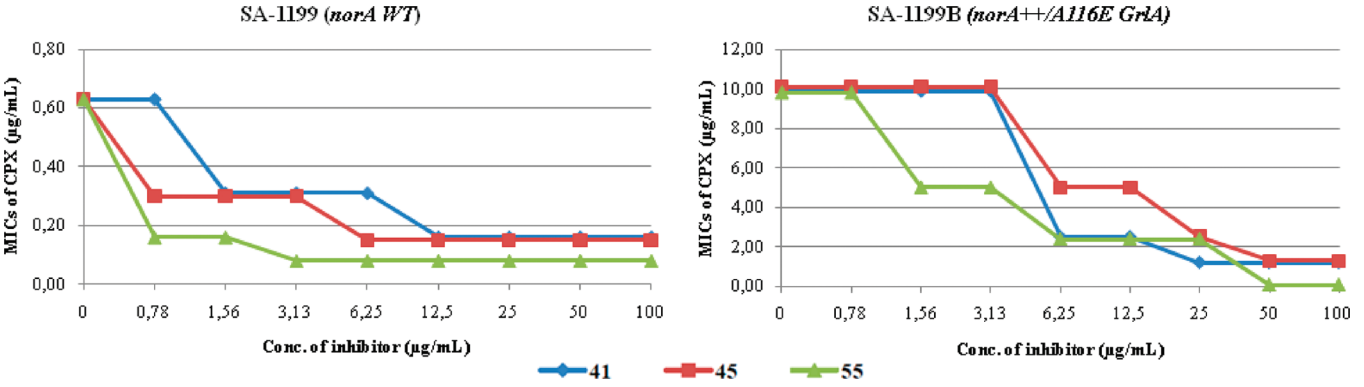


Figure 7. Isobolograms of synergistic activity with CPX for "analogue" compounds **41**, **45**, and **55** compared to their respective hits and reserpine, against the SA-1199 and SA-1199B *S. aureus* strains.

bringing about the observed effect. This hypothesis could be tested when *S. aureus* strains overexpressing pumps other than NorA are made available.

The testing of compounds analogous to the four active hits has allowed us to speculate on which parts of their structures are most important in causing inhibition of NorA. From the compound **16** analogues tested, it appears that limited modifications to the sulfone and amide group (**41**) do not cause a significant change in inhibitory activity, whereas modifications to the substituents of the imidazole ring (compounds **42** and **43**) drastically reduce the observed activity. Changing the 1-methylpyrrole ring with a furan ring (compound **44**) only brings about a slight decrease in inhibitory power.

Molecule **45** shows that the methyl group of the 2-(1-phenyl-1-propanol) moiety is unnecessary for the observed activity in molecule **21** and can be replaced with a 2-(1-phenyl-1-ethanol) scaffold without a decrease of activity, while molecule **47** shows that the *O*-benzyl group is essential. The introduction of a benzyl group onto the secondary nitrogen causes a complete loss of activity.

All analogues of compound **23**, which had a modification in either the fused ethylcyclohexane or the benzoic acid, produced structures with poor in vitro activity. This implies that both parts of the original compound are essential for the observed activity and that the inhibition effect in vitro is probably brought about through a very specific binding mode.

Compound **55** demonstrates that cis−trans tautomerism at the only bond in which this is possible in the parent molecule does not seem to affect the observed activity of the molecule. Compounds **53** and **54** show that modifying the *sec*-butyl chain on the imidic nitrogen causes a complete loss of activity. Compound **57** shows that replacing the substituted phenyl ring with a pyrridolidine ring also causes a complete loss of activity. The central furan ring appears to be also essential to retain the inhibitory activity.

In conclusion, a NorA model for EtBr efflux inhibition has been created. By use of the predictions of this model, four hits from a total of 30 tested compounds have been discovered. Dose-response curves, synergistic activity studies, and preliminary SAR studies performed on these hits have shown that compound **16** and to a lesser extent compound **21** seem to be ideal candidates for further investigation.

Experimental Section

Purity of Tested Compounds. Purity of the active compounds (**16**, **21**, **23**, **28**, **41**, **44**, **45**, and **55**) was determined by ^{1}H NMR and LC−MS according to the UV trace at 230 and 254 nm and is reported in the Supporting Information. Purity was found to be over 95% for all compounds tested. Screening compounds **11**−**40** were all purchased from Specs.[58] Derivative compounds **41**−**43** and **47**, **49**, **51**, and **52** were also purchased from Specs. Compounds **44**, **45**, **56**, and **57** were purchased from ChemDiv.[67] Compound **45** was purchased from ChemBridge.[68] Compounds **46** and **53**−**55** were purchased from Vitas-M.[69] Compounds **48** and **50** were purchased from KeyOrganics.[70] Compound codes from the vendors are available as Supporting Information.

Bacterial Strains. The strains of *S. aureus* employed were ATCC 25923 (wild-type), SA-1902 (*norA*-deleted), and SA-1199B (overexpressing *norA* and also possesses an A116E GrlA substitution).[63,64] In addition SA-K2378, which overexpresses *norA* from a multicopy plasmid, also was used. This strain was produced by cloning *norA* and its promoter into plasmid pCU1 and then introducing the construct into SA-K1902.[71]

Microbiologic Procedures. MICs were determined in duplicate by microdilution techniques according to CLSI guidelines.[72] The effect of combining reserpine and chlorpromazine or scalar dilutions of freshly prepared solutions of each test compound on the MICs of CPX also was determined. Checkerboard combination studies were performed as described previously.[73]

EtBr Efflux. The loss of EtBr from *S. aureus* SA-1199B was determined fluorometrically as previously described.[74] Experiments were performed in duplicate, and the results were expressed as the mean total efflux over a 5 min time course. EtBr efflux of SA-1199B in the presence of test compounds was compared to that determined in their absence, and percent reduction in efflux was calculated. The effect of increasing concentrations of test compounds on EtBr efflux was determined to generate dose-response profiles.

Computational Methods. The computational tools employed in this work all form part of the FLAP package, which is a tool designed principally for model creation and virtual screening. As with other GRID-based methods applied to virtual screening,[49,75−78] FLAP makes use of reference molecules, also called "templates", as common targets for structural comparison of all the molecules in the database. What makes it almost unique is that the templates can be chosen by the program in an automatic manner. This is only possible because FLAP is also a model-development program: the choice of templates is based on which molecules would produce the best superpositions for the molecules in the data set which exhibit the desired activity, as described below. These template structures serve as the base upon which molecular comparison is performed for other molecules.

In order to perform template selection, FLAP requires a training set of "active" and "inactive" molecules, as well as a list of candidate template molecules, which are usually the active molecules of the training set. The number of molecules used in the training set and the number of "uncertain" molecules that were excluded from this set are highlighted in Table 1. The full list of molecules can be found in the Supporting Information. Each of the training set molecules is first superimposed onto each of the templates, and their pairwise similarities are evaluated on the basis of the common volumes of their corresponding MIFs.

FLAP performs superposition by comparing the structures of ligand and template using the atoms of the structures themselves. The individual atoms are combined into all possible groups of four points, called quadruplets. This is a computationally very efficient short-cut. The approach adopted is to use the quadruplets to search for as large a number of superpositions as possible, yet proceed with the orientation and scoring of the resulting structures only when their superposition produces a potentially favorable result. This allows for a larger number of superpositions to be sampled from all those mathematically possible, without making the computational time required impractical.

A potentially favorable superposition (also known as a "solution") is said to be found when each of the points of one quadruplet is within a given distance of an individual point in the quadruplet it is being compared to. When this occurs, FLAP will orient the two structures and their MIFs to fit onto the superimposed quadruplets and then proceed to score the overlap of the MIF volumes.

A score is assigned to the overlap of each of the probes being used and to the overlap of several probe combinations. These scores are the "probe-scores". An entire set of probe-scores are produced for each of the solutions found, yet for each molecule, only the best score is saved for each of the probes used and for each of the probe combinations. Of course, these saved scores do not necessarily refer to the same solution.

The final probe-scores saved for each molecule are then combined into a single score value, referred to as a "distance-score". This distance-score represents the overall similarity

between the molecule being investigated and the template being employed and can be used as a rough estimate of how similar two structures are in terms of the interactions they are capable of having.

Finally, the templates chosen from the list of candidates are those that give the best distance-scores for the active molecules and the worst distance-scores for the inactive molecules. The templates chosen by the algorithm as the most representative molecules of the training set are molecules **1**, **63**, and **98** (refer to the Supporting Information).

FLAP was then used to produce a linear discriminant analysis (LDA) model. In general, LDA model scores are superior to distance-scores, since they represent the similarity of the molecule being studied to an entire set of molecules rather than to a single template molecule. In order to create such a model, FLAP therefore needs to refer to the training set again (Table 1).

The LDA models were implemented in the FLAP function by performing a linear combination of the probe-scores and their combinations. "LDA-scores" are calculated for each of the molecules in the set by combining a fixed number of the various scores produced previously. All possible probe-score combinations are attempted. The combinations of probe-scores that produce the highest LDA-scores for the active molecules and the lowest LDA-scores for the inactive molecules are then saved into the LDA model, since they best describe the observed inhibitory activity. The original probes used in this analysis were the default probes DRY, O, N1, and H. The probe-scores selected in the created LDA model were H, H*N1*H, H*DRY*H, and the Global Product.

The prediction of external compounds is performed by superimposing the test molecules onto the optimally selected templates and evaluating their probe-scores using the procedure just described. The selected probe-scores are then combined into the LDA-score according to the rules saved in the LDA model. In the virtual screening scenario, where hundreds or thousands of molecules are being studied, this LDA-score represents the likelihood of the individual screening molecules of being active. The standard procedure is therefore to rank the list of screened molecules by their LDA-score, then selecting the top fraction.

Supporting Information Available: Compound name, molecular structure, SMILES code and biological data for the molecules tested; experimental data demonstrating the purity of the active compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Spellberg, B.; Guidos, R.; Gilbert, D.; Bradley, J.; Boucher, H. W.; Scheld, W. M.; Bartlett, J. G.; Edwards, J., Jr. The epidemic of antibiotic-resistant infections: a call to action for the medical community from the Infectious Diseases Society of America. *Clin. Infect. Dis.* **2008**, *46*, 155–164.

(2) Kumarasamy, K. K.; Toleman, M. A.; Walsh, T. R.; Bagaria, J.; Butt, F.; Balakrishnan, R.; Chaudhary, U.; Doumith, M.; Giske, C. G.; Irfan, S.; Krishnan, P.; Kumar, A. V.; Maharjan, S.; Mushtaq, S.; Noorie, T.; Paterson, D. L.; Pearson, A.; Perry, C.; Pike, R.; Rao, B.; Ray, U.; Sarma, J. B.; Sharma, M.; Sheridan, E.; Thirunarayan, M. A.; Turton, J.; Upadhyay, S.; Warner, M.; Welfare, W.; Livermore, D. M.; Woodford, N. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infect. Dis.* **2010**, *10*, 597–602.

(3) Finks, J.; Wells, E.; Dyke, T. L.; Husain, N.; Plizga, L.; Heddurshetti, R.; Wilkins, M.; Rudrik, J.; Hageman, J.; Patel, J.; Miller, C. Vancomycin-resistant *Staphylococcus aureus*, Michigan, USA, 2007. *Emerging Infect. Dis.* **2009**, *15*, 943–945.

(4) Sievert, D. M.; Rudrik, J. T.; Patel, J. B.; McDonald, L. C.; Wilkins, M. J.; Hageman, J. C. Vancomycin-resistant *Staphylococcus aureus* in the United States, 2002−2006. *Clin. Infect. Dis.* **2008**, *46*, 668–674.

(5) Diekema, D. J.; Jones, R. N. Oxazolidinone antibiotics. *Lancet* **2001**, *358*, 1975–1982.

(6) Meka, V. G.; Gold, H. S. Antimicrobial resistance to linezolid. *Clin. Infect. Dis.* **2004**, *39*, 1010–1015.

(7) Tsiodras, S.; Gold, H. S.; Sakoulas, G.; Eliopoulos, G. M.; Wennersten, C.; Venkataraman, L.; Moellering, R. C.; Ferraro, M. J. Linezolid resistance in a clinical isolate of *Staphylococcus aureus*. *Lancet* **2001**, *358*, 207–208.

(8) Livermore, D. M. Has the era of untreatable infections arrived? *J. Antimicrob. Chemother.* **2009**, *64*, 29–36.

(9) Lomovskaya, O.; Zgurskaya, H. I.; Bostian, K. Bacterial Multidrug Transporters: Molecular and Clinical Aspects. In *Transporters as Drug Carriers*; Ecker, G. F., Chiba, P., Eds.; Methods and Principles in Medicinal Chemistry, Vol. 44; Wiley-VCH: Weinheim, Germany, 2009; pp 121−157.

(10) Nikaido, H. Molecular basis of bacterial outer membrane permeability revisited. *Microbiol. Mol. Biol. Rev.* **2003**, *67*, 593–656.

(11) Piddock, L. J. Multidrug-resistance efflux pumps—not just for resistance. *Nat. Rev. Microbiol.* **2006**, *4*, 629–636.

(12) Poole, K. Efflux pumps as antimicrobial resistance mechanisms. *Ann. Med.* **2007**, *39*, 162–176.

(13) Guz, N. R.; Stermitz, F. R.; Johnson, J. B.; Beeson, T. D.; Willen, S.; Hsiang, J.; Lewis, K. Flavonolignan and flavone inhibitors of a *Staphylococcus aureus* multidrug resistance pump: structure−activity relationships. *J. Med. Chem.* **2001**, *44*, 261–268.

(14) Payne, D. J. Desperately seeking new antibiotics. *Science* **2008**, *321*, 1644–1645.

(15) Klein, E.; Smith, D. L.; Laxminarayan, R. Hospitalizations and deaths caused by methicillin-resistant *Staphylococcus aureus*, United States, 1999−2005. *Emerging Infect. Dis.* **2007**, *13*, 1840–1846.

(16) Klevens, R. M.; Morrison, M. A.; Nadle, J.; Petit, S.; Gershman, K.; Ray, S.; Harrison, L. H.; Lynfield, R.; Dumyati, G.; Townes, J. M.; Craig, A. S.; Zell, E. R.; Fosheim, G. E.; McDougal, L. K.; Carey, R. B.; Fridkin, S. K. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA, J. Am. Med. Assoc.* **2007**, *298*, 1763–1771.

(17) 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.

(18) Lomovskaya, O.; Lee, A.; Hoshino, K.; Ishida, H.; Mistry, A.; Warren, M. S.; Boyer, E.; Chamberland, S.; Lee, V. J. Use of a genetic approach to evaluate the consequences of inhibition of efflux pumps in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **1999**, *43*, 1340–1346.

(19) Markham, P. N. Inhibition of the emergence of ciprofloxacin resistance in *Streptococcus pneumoniae* by the multidrug efflux inhibitor reserpine. *Antimicrob. Agents Chemother.* **1999**, *43*, 988–989.

(20) Ricci, V.; Tzakas, P.; Buckley, A.; Piddock, L. J. Ciprofloxacin-resistant *Salmonella enterica* serovar typhimurium strains are difficult to select in the absence of AcrB and TolC. *Antimicrob. Agents Chemother.* **2006**, *50*, 38–42.

(21) Sabatini, S.; Kaatz, G. W.; Rossolini, G. M.; Brandini, D.; Fravolini, A. From phenothiazine to 3-phenyl-1,4-benzothiazine derivatives as inhibitors of the *Staphylococcus aureus* NorA multidrug efflux pump. *J. Med. Chem.* **2008**, *51*, 4321–4330.

(22) Michalet, S.; Cartier, G.; David, B.; Mariotte, A. M.; Dijoux-franca, M. G.; Kaatz, G. W.; Stavrid, M.; Gibbons, S. *N*-Caffeoylphenalkylamide derivatives as bacterial efflux pump inhibitors. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 1755–1758.

(23) Nargotra, A.; Sharma, S.; Koul, J. L.; Sangwan, P. L.; Khan, I. A.; Kumar, A.; Taneja, S. C.; Koul, S. Quantitative structure activity relationship (QSAR) of piperine analogs for bacterial NorA efflux pump inhibitors. *Eur. J. Med. Chem.* **2009**, *44*, 4128–4135.

(24) German, N.; Kaatz, G. W.; Kernsa, R. J. Synthesis and evaluation of PSSRI-based inhibitors of *Staphylococcus aureus* multidrug efflux pumps. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 1368–1373.

(25) 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.

(26) Vidaillac, C.; Guillon, J.; Arpin, C.; Forfar-Bares, I.; Ba, B. B.; Grellet, J.; Moreau, S.; Caignard, D. H.; Jarry, C.; Quentin, C. Synthesis of omeprazole analogues and evaluation of these as potential inhibitors of the multidrug efflux pump NorA of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **2007**, *51*, 831–838.

(27) Chabert, J. F. d.; Marquez, B.; Neville, L.; Joucla, L.; Broussous, S.; Bouhours, P.; David, E.; Pellet-Rostaing, S.; Marquet, B.; Moreaub, N.; Lemaire, M. Synthesis and evaluation of new arylbenzo[*b*]thiophene and diarylthiophene derivatives as inhibitors of the NorA multidrug transporter of *Staphylococcus aureus*. *Bioorg. Med. Chem.* **2007**, *15*, 4482–4497.

(28) Marquez, B.; Neuville, L.; Moreau, N. J.; Genet, J. P.; Santos, A. F. d.; Andrade, M. C. C. d.; Sant'Ana, A. E. G. Multidrug resistance reversal agent from *Jatropha elliptica*. *Phytochemistry* **2005**, *66*, 1804–1811.

(29) German, N.; Wei, P.; Kaatz, G. W.; Kerns, R. J. Synthesis and evaluation of fluoroquinolone derivatives as substrate-based inhibitors of bacterial efflux pumps. *Eur. J. Med. Chem.* **2008**, *43*, 2453–2463.
(30) Pieroni, M.; Dimovska, M.; Brincat, J. P.; Sabatini, S.; Carosati, E.; Massari, S.; Kaatz, G. W.; Fravolini, A. From 6-aminoquinolone antibacterials to 6-amino-7-thiopyranopyridinylquinolone ethyl esters as inhibitors of *Staphylococcus aureus* multidrug efflux pumps. *J. Med. Chem.* **2010**, *53*, 4466–4480.
(31) Musumeci, R.; Speciale, A.; Costanzo, R.; Annino, A.; Ragusa, S.; Rapisarda, A.; Pappalardo, M. S.; Iauk, L. *Berberis aetnensis* C. Presl. extracts: antimicrobial properties and interaction with ciprofloxacin. *Int. J. Antimicrob. Agents* **2003**, *22*, 48–53.
(32) Chérigo, L.; Pereda-Miranda, R.; Fragoso-Serrano, M.; Jacobo-Herrera, N.; Kaatz, G. W.; Gibbons, S. Inhibitors of bacterial multidrug efflux pumps from the resin glycosides of *Ipomoea murucoides*. *J. Nat. Prod.* **2008**, *71*, 1037–1045.
(33) Stermitz, F. R.; Lorenz, P.; Tawara, J. N.; Zenewicz, L. A.; Lewis, K. Synergy in a medicinal plant: antimicrobial action of berberine potentiated by 5*-methoxyhydnocarpin, a multidrug pump inhibitor. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 1433–1437.
(34) Pereda-Miranda, R.; Kaatz, G. W.; Gibbons, S. Polyacylated oligosaccharides from medicinal mexican morning glory species as antibacterials and inhibitors of multidrug resistance in *Staphylococcus aureus*. *J. Nat. Prod.* **2006**, *69*, 406–409.
(35) Mullin, S.; Mani, N.; Grossman, T. H. Inhibition of antibiotic efflux in bacteria by the novel multidrug resistance inhibitors biricodar (VX-710) and timcodar (VX-853). *Antimicrob. Agents Chemother.* **2004**, *48*, 4171–4176.
(36) Gibbons, S.; Oluwatuyi, M.; Kaatz, G. W. A novel inhibitor of multidrug efflux pumps in *Staphylococcus aureus*. *J. Antimicrob. Chemother.* **2003**, *51*, 13–17.
(37) Zhang, L.; Ma, S. Efflux pump inhibitors: a strategy to combat P-glycoprotein and the NorA multidrug resistance pump. *ChemMedChem.* **2010**, *5*, 811–822.
(38) Markham, P. N.; Westhaus, E.; Klyachko, K.; Johnson, M. E.; Neyfakh, A. A. Multiple novel inhibitors of the NorA multidrug transporter of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **1999**, *43*, 2404–2408.
(39) Takenouchi, T.; Tabata, F.; Iwata, Y.; Hanzawa, H.; Sugawara, M.; Ohya, S. Hydrophilicity of quinolones is not an exclusive factor for decreased activity in efflux-mediated mutants of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **1996**, *40*, 1835–1842.
(40) Nargotra, A.; Koul, S.; Sharma, S.; Khan, I. A.; Kumar, A.; Thota, N.; Koul, J. L.; Taneja, S. C.; Qazi, G. N. Quantitative structure−activity relationship (QSAR) of aryl alkenyl amides/imines for bacterial efflux pump inhibitors. *Eur. J. Med. Chem.* **2009**, *44*, 229–238.
(41) Kaatz, G. W.; Moudgal, V.; Seo, S.; Kristiansen, J. Phenothiazines and thioxanthenes inhibit multidrug efflux pump activity in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **2003**, *47*, 719–726.
(42) Kaatz, G. W.; Moudgal, V.; Seo, S.; Hansen, J.; Kristiansen, J. Phenylpiperadine selective serotonin reuptake inhibitors interfere with multidrug efflux pump activity in *Staphylococcus aureus*. *Int. J. Antimicrob. Agents* **2003**, *22*, 254–261.
(43) Smith, E.; Kaatz, G.; Seo, S.; Wareman, N.; Williamson, E.; Gibbons, S. The phenolic diterpene totarol inhibitors multidrug efflux pump activity in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **2007**, *51*, 4480–4483.
(44) Sabatini, S.; Gosetto, F.; Manfroni, G.; Tabarrini, O.; Kaatz, G. W.; Cecchetti, V. Unpublished results.
(45) Neyfakh, A. A. The multidrug efflux transporter of *Bacillus subtilis* is a structural and functional homolog of the Staphylococcus NorA protein. *Antimicrob. Agents Chemother.* **1992**, *36*, 484–485.
(46) 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.
(47) Cross, S.; Baroni, M.; Carosati, E.; Benedetti, P.; Clementi, S. FLAP: GRID molecular interaction fields in virtual screening. Validation using the DUD sata set. *J. Chem. Inf. Model.* **2010**, *50*, 1442–1450.
(48) Carosati, E.; Cruciani, G.; Chiarini, A.; Budriesi, R.; Ioan, P.; Spisani, R.; Spinelli, D.; Cosimelli, B.; Fusi, F.; Frosini, M.; Matucci, R.; Gasparrini, F.; Ciogli, A.; Stephens, P. J.; Devlin, F. J. New calcium channel antagonists discovered by a multidisciplinary approach. *J. Med. Chem.* **2006**, *49*, 5206–5216.
(49) Carosati, E.; Mannhold, R.; Wahl, P.; Hansen, J. B.; Fremming, T.; Zamora, I.; Cianchetta, G.; Baroni, M. Virtual screening for novel openers of pancreatic K_{ATP} channels. *J. Med. Chem.* **2007**, *50*, 2117–2126.
(50) Carosati, E.; Sforna, G.; Pippi, M.; Marverti, G.; Ligabue, A.; Guerrieri, D.; Piras, S.; Guaitoli, G.; Luciani, R.; Costi, M. P.; Cruciani, G. Ligand-based virtual screening and ADME-tox guided approach to identify triazolo-quinoxalines as folate cycle inhibitors. *Bioorg. Med. Chem.* **2010**, *18*, 7773–7785.
(51) Carosati, E.; Budriesi, R.; Ioan, P.; Cruciani, G.; Fusi, F.; Frosini, M.; Saponara, S.; Gasparrini, F.; Ciogli, A.; Villani, C.; Stephens, P. J.; Devlin, F. J.; Spinelli, D.; Chiarini, A. Stereoselective behaviour of the functional diltiazem analogue 1-[(4-chlorophenyl)sulfonyl]-2-(2-thienyl)pyrrolidine, a new L-type calcium channel blocker. *J. Med. Chem.* **2009**, *52*, 6637–6648.
(52) Budriesi, R.; Cosimelli, B.; Ioan, P.; Ugenti, M. P.; Carosati, E.; Frosini, M.; Fusi, F.; Spisani, R.; Saponara, S.; Cruciani, G.; Novellino, E.; Spinelli, D.; Chiarini, A. L-type calcium channel blockers: from diltiazem to 1,2,4-oxadiazol-5-ones via thiazinooxadiazol-3-one derivatives. *J. Med. Chem.* **2009**, *52*, 2352–2362.
(53) Sciabola, S.; Stanton, R. V.; Mills, J. E.; Flocco, M. M.; Baroni, M.; Cruciani, G.; Perruccio, F.; Mason, J. S. High-throughput virtual screening of proteins using GRID molecular interaction fields. *J. Chem. Inf. Model.* **2010**, *50*, 155–169.
(54) Goodford, P. J. A computational procedure for determining energetically favourable binding sites on biologically important macromolecules. *J. Med. Chem.* **1985**, *28*, 849–857.
(55) Carosati, E.; Sciabola, S.; Cruciani, G. Hydrogen bonding interactions of covalently bonded fluorine atoms: from crystallographic data to a new angular function in the GRID force field. *J. Med. Chem.* **2004**, *47*, 5114–5125.
(56) The GRID package, version 22, is distributed from Molecular Discovery Ltd., http://www.moldiscovery.com.
(57) r^2 was calculated as the fraction of the total number of predicted molecules that were predicted in the correct activity class using the binary LDA model.
(58) http://www.specs.net/.
(59) Goldfarb, D. S. Method Using Lifespan-Altering Compounds for Altering the Lifespan of Eukaryotic Organisms, and Screening for Such Compounds. U.S. Pat. Appl. Publ. US2009163545 A1 20090625, 2009.
(60) Wagh, S. B.; Yerande, S.; Patil, B. Preparation of 8-Chloro-11-(1-piperazinyl)-dibenz[*b*,*f*][1,4]oxazepines as Antipsychotic Agents. Indian Pat. Appl. 2005MU00365 A 20070420, 2007.
(61) Shibo, J.; Debnath A. S.; Lu, H. Anti-Viral Compositions Compromising Heterocyclic Substituted Phenyl Furans and Related Compounds. U.S. Pat. Appl. Publ. US 2006287319 A1 20061221, 2006.
(62) Kaverina, N. V.; Lyskovtsev, V. V.; Senova, Z. P.; Gritsenko, A. N.; Ermakova, Z. I.; Skoldinov, A. P.; Carstens, E.; Wunderlich, H.; Stark, A. 5-(ω-Aminoacyl)-3-carbalkoxyamino-10,11-dihydro-5*H*-dibenz[*b*,*f*]azepine Derivatives and Their Therapeutic Use. Fr. Demande, FR 2493314 A1 19820507, 1982.
(63) Kaatz, G. W.; Seo, S. M. Mechanisms of fluoroquinolone resistance in genetically related strains of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **1997**, *41*, 2733–2737.
(64) Price, C. T. D.; Kaatz, G. W.; Gustafson, J. E. The multidrug efflux pump NorA is not required for salicylate-induced reduction in drug accumulation by *Staphylococcus aureus*. *Int. J. Antimicrob. Agents* **2002**, *20*, 206–213.
(65) Irwin, J. J.; Shoichet, B. K. ZINC—a free database of commercially available compounds for virtual screening. *J. Chem. Inf. Model.* **2005**, *45*, 177–182.
(66) http://zinc.docking.org.
(67) http://www.chemdiv.com.
(68) http://www.chembridge.com.
(69) http://www.vitasmlab.com.
(70) http://www.keyorganics.ltd.uk.
(71) Augustin, J.; Rosenstein, R.; Weiland, B.; Schneider, U.; Schnell, N.; Engelke, G.; Entian, K. D.; Götz, F. Genetic analysis of epidermin biosynthetic genes and epidermin-negative mutants of *Staphylococcus epidermidis*. *Eur. J. Biochem.* **1992**, *204*, 1149–1154.
(72) Clinical Laboratory Standards Institute. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*. *Approved Standard M7-A7*, 7th ed.; Clinical and Laboratory Standards Institute: Wayne, PA, 2006.
(73) Eliopoulos, G. M.; Moellering, R. C. J. Antimicrobial Combinations. In *Antibiotics in Laboratory Medicine*; Lorian, V., Ed.; Williams and Wilkins: Baltimore, MD, 1991; pp 432−492.
(74) Kaatz, G. W.; Seo, S. M.; O'Brien, L.; Wahiduzzaman, M.; Foster, T. J. Evidence for the existence of a multidrug efflux transporter distinct from *norA* in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **2000**, *44*, 1404–1406.
(75) Ahlström, M. M.; Ridderström, M.; Luthman, K.; Zamora, I. Virtual screening and scaffold hopping based on GRID molecular interaction fields. *J. Chem. Inf. Model.* **2005**, *45*, 1313–1323.

(76) Lo Piparo, E.; Koehler, K.; Chana, A.; Benfenati, E. Virtual screening for aryl hydrocarbon receptor binding prediction. *J. Med. Chem.* **2006**, *49*, 5702–5709.
(77) Carosati, E.; Budriesi, R.; Ioan, P.; Ugenti, M. P.; Frosini, M.; Fusi, F.; Corda, G.; Cosimelli, B.; Spinelli, D.; Chiarini, A.; Cruciani, G. Discovery of novel and cardioselective diltiazem-like calcium channel blockers via virtual screening. *J. Med. Chem.* **2008**, *51*, 5552–5565.
(78) Durán, A.; Zamora, I.; Pastor, M. Suitability of GRIND-based principal properties for the description of molecular similarity and ligand-based virtual screening. *J. Chem. Inf. Model.* **2009**, *49*, 2129–2138.