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ORIGINAL PAPER

In silico screening for antibiotic escort molecules to overcome efflux

Sheikh S. Rahman · Ivana Simovic · Simon Gibbons · Mire Zloh

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Abstract Resistance to antibiotics is a growing problem worldwide and occurs in part due to the overexpression of efflux pumps responsible for the removal of antibiotics from bacterial cells. The current study examines complex formation between efflux pump substrates and escort molecules as a criterion for an in silico screening method for molecules that are able to potentiate antibiotic activities. Initially, the SUPERDRUG database was queried to select molecules that were similar to known multidrug resistance (MDR) modulators. Molecular interaction fields generated by GRID and the docking module GLUE were used to calculate the interaction energies between the selected molecules and the antibiotic norfloxacin. Ten compounds forming the most stable complexes with favourable changes to the norfloxacin molecular properties were tested for their potentiation ability by efflux pump modulation assays. Encouragingly, two molecules were proven to act as efflux pump modulators, and hence provide evidence that complex formation between a substrate and a drug can be used for in silico screening for novel escort molecules.

Keywords Multidrug resistance · MDR · Efflux pumps · Molecular interaction fields · Escort molecules · In silico screening

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S. S. Rahman · I. Simovic · S. Gibbons · M. Zloh (✉)
The School of Pharmacy, University of London,
29–39 Brunswick Square,
London WC1N 1AX, UK
e-mail: mire.zloh@pharmacy.ac.uk

Introduction

Multidrug resistance (MDR) to antibiotics is an ever-increasing problem worldwide and occurs due to a number of mechanisms: (i) receptor alteration, where the target site may become altered, resulting in a less efficient interaction between the binding site and drug; (ii) antibiotic modification, where the bacteria may produce novel enzymes that inactivate or alter the drug; or (iii) the removal of the drug from the bacterial cell by efflux pumps (the major mechanism of resistance) [1]. These pumps are membrane-bound proteins that are found in both eukaryotes and prokaryotes, and can be either specific or nonspecific. Efflux pumps that are specific assist the removal of only one compound or a class of compounds, whereas nonspecific efflux pumps assist the removal of a broad range of compounds that are structurally unrelated. It is these nonspecific efflux pumps that lead to MDR.

Bacterial efflux pumps are divided into two major classes based on their energy source. The first are primary transporters that obtain their energy for efflux by hydrolysis of ATP and belong to the ATP-binding cassette (ABC) superfamily. The second are secondary transporters that obtain their energy for efflux in a coupled exchange with H^+ (or Na^+) ions [1]. These secondary transporters are then subdivided into various families based on the size and similarity of their structures: the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, the resistance nodule cell division (RND) family, and the multidrug and toxic compound extrusion (MATE) family.

There are various ways of combating MDR to restore the antibiotic activity by preventing efflux using a range of structurally unrelated molecules. These efflux pump modulators can come from different sources (natural

products, drugs and synthetic analogues) [2], and their structural diversity indicates that various mechanisms are involved in restoring the action of antibiotics. These mechanisms can be based on affecting the pump function either by removing the energy source using inhibitors such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), by abolishing the membrane potential using inhibitors such as valinomycin, or by preventing efflux pump assembly or efflux pump expression. Alternatively, drug efflux can be prevented by intermolecular interactions that efflux pump modulators can form, either through the binding of a molecule to the hydrophobic regions of the binding site [3], or through the formation of a complex between a drug and a modulator. The latter involves two molecules forming a noncovalent complex which is not recognized by the efflux pump [4, 5]. A modulator of MDR complexed with a drug could act as an "escort molecule" to deliver the drug into the bacteria [6].

Small molecule–small molecule interactions may play an important role in biological processes and few experimental studies have confirmed interactions between drugs in solution [7–9]. These interactions can be studied and predicted computationally using different levels of theory; however, these methods do not have the capacity for high-throughput in silico screening where the target is a small molecule. Previously, we demonstrated the use of GRID software and its module GLUE to predict the interaction energies between two small molecules, and reported the link between interaction energies and efflux pump modulation [4, 6]. Furthermore, we have shown the high similarity between MDR inhibitors in terms of shape, lipophilicity and orientation of aromatic moieties [10].

Here, we report the in silico screening process to detect potential escort molecules that could restore the activity of an antibiotic, in this case norfloxacin. The similarity, complex formation, interaction energies and physicochemical properties of complexes between norfloxacin and a small molecule were considered as criteria in this in silico screening process to find suitable escort molecules, where escort molecules may be selected from approved drugs, natural products or nutrients.

Materials and methods

Examination of drug–drug interactions

The GRID22 package [11] consists of six programs including a graphical interface called GREATER and a GRID-based docking program, GLUE. To validate the use of GLUE as a method for detecting complex formation where the target was a small molecule, we used three previously published studies, where the small molecule–small molecule interactions between drug pairs were confirmed using experimental methods [7–9]. The experimental evidence and key interactions were given for complexes of atorvastatin with three antibiotics (ciprofloxacin, gatifloxacin, and ofloxacin), indomethacin with lidocaine, and cocaine with a salt of morphine.

Ionization states of studied molecules were predicted using LigPrep [11] or Avogadro [12]. The molecular mechanics software Macromodel [13, 14] was used to perform a 1000-step energy minimization, followed by a conformational search using the Monte Carlo multiple minima (MCMM) method and the MMFF94s force field [15] for each of the molecules in order to obtain the five most stable representative conformers. These were used as input files for GLUE [16]. GREATER was used to compute the molecular interaction fields (MIF) and to obtain the GRID .kout file required for docking using GLUE. Each of the conformers was used as the target and its corresponding drug pair was defined as the ligand. The eight default GRID probes (H, OH_2, DRY, N1, N+, O, O::, O1) were used, and the box defining the binding site was set to contain whole molecules as a target. To allow flexibility of the ligand, the number of rotatable bonds was set to 5 as the maximum number allowed within GLUE, and the binding energies were calculated without and with considering electrostatic interaction contributions. The docking experiments were repeated by reversing the roles of the ligand and target; i.e. the molecules that were targets in the first docking experiment were set as the ligand in the second docking experiment, and the molecules that were used as the ligands in the first docking experiment were used as the target in the second docking experiment in order to allow for ligand flexibility of both drugs. Vega ZZ [17–20] was used to visualise and analyse the interactions between the two drugs when complexed.

In silico screening strategy and docking experiments

To demonstrate the use of the GRID software for in silico screening, we used the online database of 2,396 already FDA approved and readily available drug molecules, SUPERDRUG [21]. Initially and to minimize the computational time required for computing interaction energies, we screened the database for drug molecules that contain the moieties of known efflux pump inhibitors (reserpine, verapamil, epicatechin gallate, epigallocatechin gallate, biricodar, timcodar, pheophorbide A, 5′-methoxyhydnocarpin, NNC 20-7052, INF55, INF240, INF271, INF277 and INF392). The "build your own structure" feature of the SUPERDRUG database was utilized by evaluating similarity and Tanimoto coefficients [21]. A set of drug molecules with the highest similarity were selected as potential escort molecules for norfloxacin (NOR). The

Table 1 The binding energies of the drug–drug interactions predicted by GLUE

Drug 1	Drug 2	Binding energy (kcal/mol)[a]	Binding energy (kcal/mol)[b]
Ciprofloxacin	Atorvastatin	−20.309	−38.357
Gatifloxacin	Atorvastatin	−14.42	−32.531
Ofloxacin	Atorvastatin	−17.336	−39.459
Indomethacin	Lidocaine	−12.805	−12.742
Morphine	Cocaine	−8.363	−14.343

[a] The binding energies as calculated using GLUE without electrostatic contributions; [b] the binding energies as calculated using GLUE taking into account electrostatic contributions

ionizable groups of 3D structures of these compounds downloaded from the SUPERDRUG database were adjusted to the correct ionized state at pH 7.4 using Avogadro [13]. Final conformations were obtained by VegaZZ using the AM1BCC force field, Gasteiger charges, and a 3000-step energy minimization using the AMMP module.

GRID and GREATER were used as described above to carry out the docking between norfloxacin and selected drug molecules from the SUPERDRUG database. The structures of the drugs identified in SUPERDRUG were used as ligands and subjected to docking protocols using norfloxacin as the target to compute their binding energies.

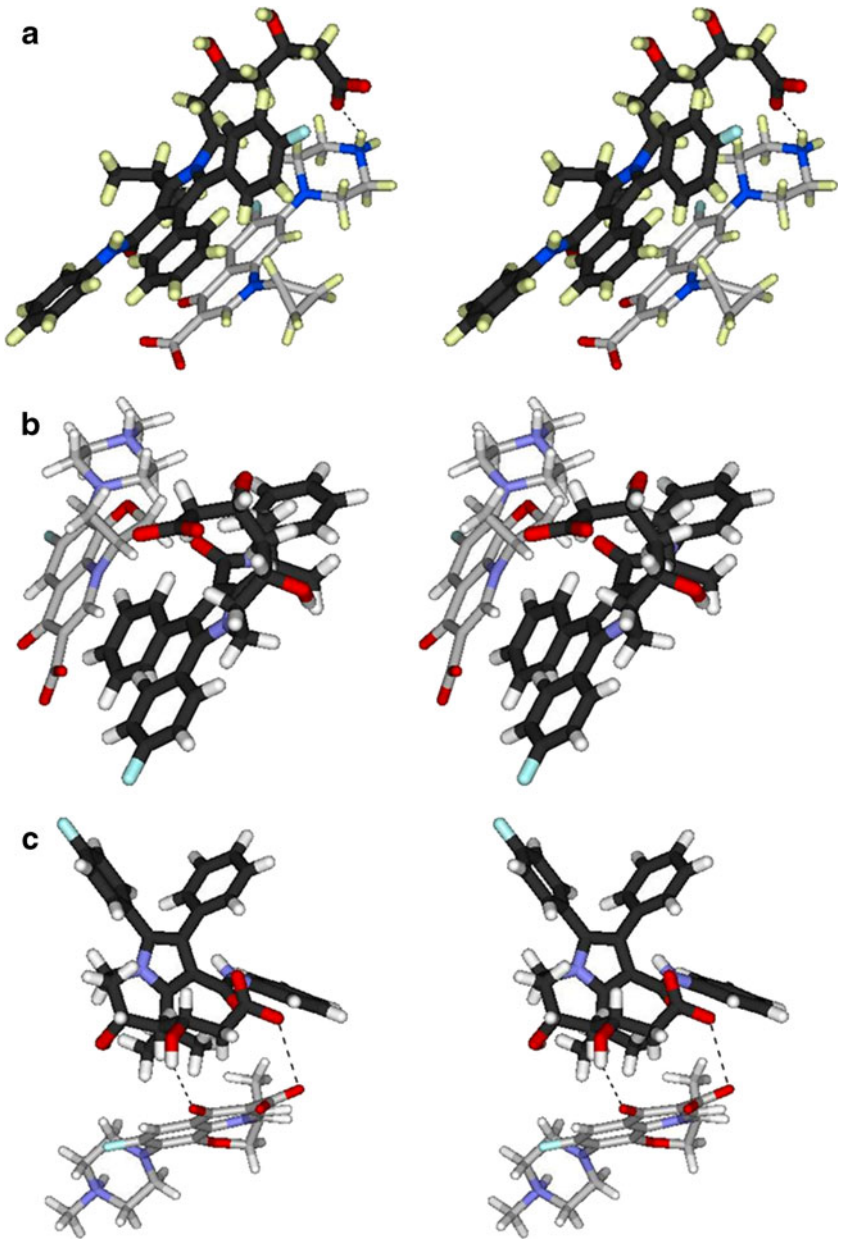

Fig. 1a–c The stereoview representations of noncovalent complexes predicted by GLUE: **a** atorvastatin–ciprofloxacin (conformer with the best binding energy); **b** atorvastatin–gatifloxacin (conformer with the best binding energy); **c** atorvastatin–ofloxacin (conformer with a less favourable binding energy). Atorvastatin is depicted in the *darker shade of grey* and with *thicker sticks*

The results of docking were saved as ligands in noncovalent complexes with norfloxacin, using VegaZZ [17–20], and employed for the prediction of various physicochemical properties of the single molecules as well as their docked complexes, including their surface area (SA), polar surface area (PSA), log*P*, lipole and virtual log*P* properties [22, 23]. The lipophilic surface of each single molecule as well as its docked complex was also calculated using the "surface management" option, where the surface chosen was MLP (molecular lipophilicity potential), the color of the gradient was set to 6, and the probe radius was the default value [24]. Ten drugs with a range of interaction energies, favourable physicochemical properties, and availability for purchase were selected to biologically evaluate their ability to restore the action of norfloxacin against a norfloxacin-effluxing strain of *Staphylococcus aureus*.

Biological evaluation

Alprenolol hydrochloride, apomorphine hydrochloride hemihydrate, bergapten, betaxolol hydrochloride, chlorpromazine hydrochloride, demecolcine, hydroxyzine dihydrochloride, naproxen, paroxetine hydrochloride, pridinol methanesulfonate, and norfloxacin were purchased from Sigma and used without further purification. The assays to test the intrinsic antibacterial activities and potentiating abilities of these molecules are described elsewhere [4]. The assays to test the potentiating activity of these molecules were then repeated as described previously [4], but the mixtures of norfloxacin and the test compounds were left to incubate for 24 h at 37 °C to allow more time for complexation between the two molecules before the addition of 125 μL of the bacterial inoculum (5×10^5 cfu/mL) to wells 1–11.

Results and discussion

A major mechanism of resistance to antibiotics occurs due to the removal of the drug from the bacterial cell by efflux pumps. Here, an in silico screening process to find potential escort molecules was examined by evaluating the complex formation between norfloxacin and chosen small molecules, their interaction energies and their physicochemical properties.

GLUE identifies favourable binding modes between a target and ligands using all of the options and capabilities of the GRID force field and proposes several lower energy poses. The binding energy is expressed by a energy-scoring function which takes into account the steric-repulsion contributions, electrostatic contributions, the hydrophobic contributions and the hydrogen-bonding contributions. Although GLUE suffers from some limitations in the prediction of binding energies, we have previously shown empirically that the ability to restore the activity of antibiotics and anticancer cytotoxics can be qualitatively correlated to binding energies between drugs and known MDR modulators that are lower than −9 kcal mol^{-1}, as predicted by GLUE [5, 6]. In order to examine the use of GLUE for the docking experiments for in silico screening, we have compared the resulting docking complexes to the experimentally predicted complexes for selected pairs of drugs (Table 1). Additionally, we have included two molecules in our study: a known MDR modulator (GG918) [25] and a non-MDR potentiator (aspartame).

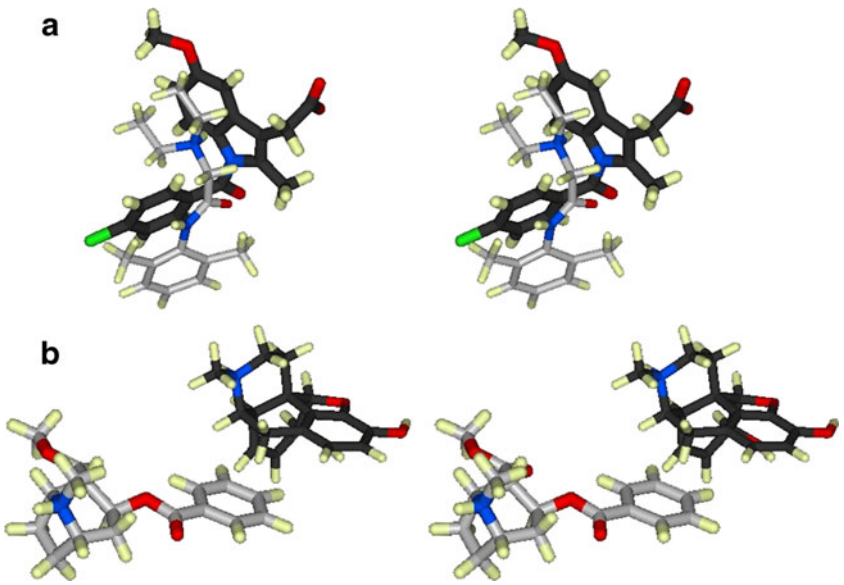

Fig. 2a–b The stereoview representations of predicted noncovalent complexes of **a** lidocaine and indomethacin (indomethacin is shown in a *lighter shade of grey* and with *thicker sticks*) and **b** morphine and cocaine (morphine is depicted in a *darker shade of grey* and with *thicker sticks*)

Can a small molecule be used as a docking target?

The dominant interactions reported in the atorvastatin–ciprofloxacin, atorvastatin–gatifloxacin and atorvastatin–ofloxacin complexes were H-bonding between the carboxylic acid group of atorvastatin and the carboxylic acid group of the fluoroquinolone drugs or the piperazine ring of the fluoroquinolone drugs [7]. The docking experiments performed using GLUE predicted complexation with favourable binding energies, with those that included electrostatic interaction generally being higher (Table 1). However, upon closer inspection of the formed complexes, it was found that results obtained by docking without considering the electrostatic contribution correlated better with the experimental observations.

The most stable ciprofloxacin–atorvastatin complex, obtained without taking into account the electrostatic contributions, exhibited H-bonding interactions between the carboxylic acid group of atorvastatin and the N–H group of the piperazine ring of ciprofloxacin, which is in good agreement with that determined experimentally. For the ofloxacin–atorvastatin and gatifloxacin–atorvastatin complexes, the conformers with the highest binding energies (without taking into account electrostatic contributions) did not exhibit the experimentally determined interactions. However, examining all of the predicted complexes by GLUE, it is found that the experimentally determined interactions are observed but in complexes with less favourable binding energies (Fig. 1).

Umeda et al. suggested that the dominant intermolecular interaction in the case of the lidocaine–indomethacin complex occurs between the carboxylic acid group of indomethacin and the diethyl amino group of lidocaine [9]. The GLUE docking experiments found that the complex formed would be stable, as it exhibits a favourable binding energy, with the most dominant interaction between

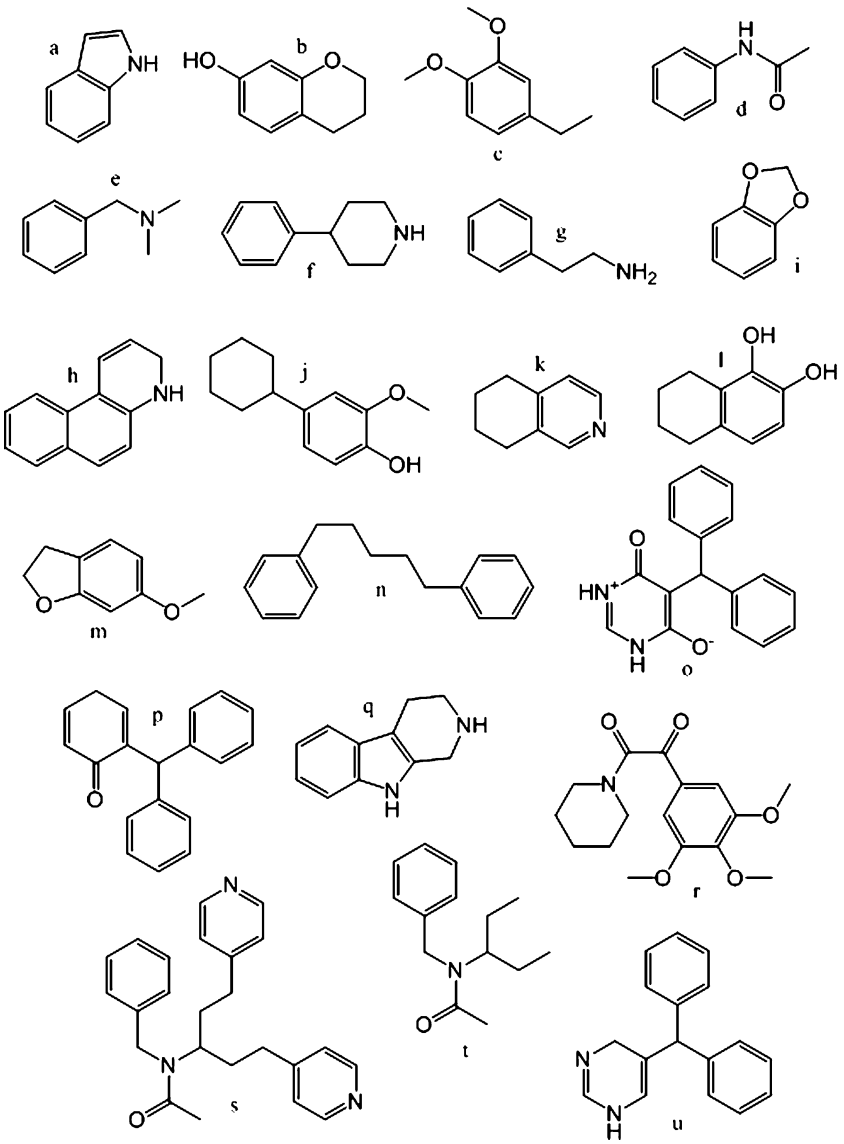

Fig. 3 The moieties extracted from known efflux pump inhibitors (reserpine, verapamil, epicatechin gallate, epigallocatechin gallate, biricodar, timcodar, pheophorbide A, 5′-methoxyhydnocarpin, NNC 20-7052, INF55, INF240, INF271, INF277 and INF392) that were used to screen for potential efflux pump modulator–escort molecules

lidocaine and indomethacin being π–π stacking. Although the carboxylic group and amino group are in proximity, the hydrogen bond is not observed in the complex, which may be a result of the limitations of GLUE in relation to carrying out flexible target docking (Fig. 2a).

With respect to the complex formed between cocaine and morphine, an interaction predicted by DFT calculations occurred between the morphine cavity defined by the two rings containing hydroxyl groups and the cocaine $COOCH_3$ [8]. The results from GLUE for docking indicated a weaker binding energy between cocaine and morphine when excluding electrostatic contributions, and the strongest interactions between the two compounds were aromatic π–π interactions. It can therefore be concluded that weak interactions are not reproduced when computing small molecule–small molecule interactions using GLUE (Fig. 2b). Overall, these computations have shown that interactions seen experimentally can be predicted using GLUE, and that the stronger interactions are more accurately predicted. Despite the observed limitations, it was deemed viable to use GLUE to carry out in silico screening where the target was a small molecule, and in this case an antibiotic.

In silico screening and docking experiments

The SUPERDRUG database [21] is an online source that contains 2,396 3D structures of drug molecules with 108,198 different conformers. This database was used to search for drugs that are similar in structure to known efflux pump inhibitors in order to find drugs that might potentially complex with norfloxacin and therefore act as modulators in vitro (Fig. 3). Eighty-nine drugs with the highest percentage similarities (Tanimoto coefficients) with known efflux pump inhibitors and the moieties of known efflux pump inhibitors were chosen for further studies (see the "Electronic supplementary material", ESM). This set of 89 selected drugs and two control molecules were subjected to docking studies using GLUE with norfloxacin as a target, and it was found that the majority of the drug molecules exhibited favourable interactions with norfloxacin, presenting binding energies of ≤ -10.0 kcal mol^{-1} (see the ESM). As seen earlier, the complex with the lowest most favourable binding energy is not necessarily the complex detected experimentally, so the average binding energy as well as the standard deviation of the GLUE data was also calculated (see the ESM). Two molecules included as a positive control (GG918) and a negative control (aspartame) had the highest and lowest average binding energies, respectively.

To get a better understanding of these docked complexes, VegaZZ was used to assess various physicochemical properties of the single molecules as well as their docked complexes. The interaction energy is an important criterion for the selection of escort molecules [5], but the availability for purchase in pure form and the toxicities of the drugs were considered during the selection process, as these would obviously affect the future use of these drugs as escort molecules. After applying all of the abovementioned criteria, we chose ten molecules for further analysis and biological testing (Table 2).

Table 2 Properties of the ten drugs

Complex (compound and NOR)	Tanimoto coefficient	Most favourable binding energy (kcal/mol)[a]	Average binding energy (kcal/mol)[b]	Change of virtual log*P* (%)	Change of PSA coverage (%)	MIC (μg/ml)	Potentiation[c]
Alprenolol	68.52	−10.237	−8.820	−12.0	−22.8	>512	32 (NC)
Apomorphine	63.27	−15.671	−10.581	−19.5	−3.8	>512	16 (2)
Aspartame*	–	−6.893	−6.277	−64.6	12.2	>512	32 (NC)
Bergapten	39.60	−13.421	−10.136	0.6	−3.8	>512	32 (NC)
Betaxolol	48.15	−10.734	−8.531	14.9	−35.4	>256	32 (NC)
Chlorpromazine	47.69	−14.819	−12.279	49.4	−31.1	64	8 (4)[d]
Demecolcine	53.29	−14.184	−10.446	−27.0	−11.0	>256	32 (NC)
GG918* [29]	–	−15.815	−12.252	64.8	−32.2	>512	4 (8)
Hydroxyzine	50.47	−14.188	−9.601	−25.5	−23.0	>512	32 (NC)
Naproxen	55.77	−12.987	−10.628	64.5	−22.3	>512	32 (NC)
Paroxetine	53.06	−13.881	−11.617	12.2	−18.3	64	32 (NC)
Pridinol	77.08	−12.675	−8.820	71.9	−41.5	>512	32 (NC)

[a] Binding energies without electrostatic forces

[b] The average binding energy without taking into account electrostatic contributions

[c] Decrease in the MIC of norfloxacin (-fold), *NC* no change

[d] Potentiation increased twofold upon incubating chlorpromazine with norfloxacin overnight

The example of the MDR modulator

Three-dimensional structures of the ten chosen drugs complexed with norfloxacin were visualized using VegaZZ. It was apparent that aromatic face-to-face interactions were dominant in most complexes (Fig. 4). In some complexes, these interactions were further stabilized by intermolecular hydrogen bonds. Complexes with hydrogen bonding have a less polar surface exposed to the solvent, as the polar groups tend to interact inside the complex and are thus hidden from the surface.

It was previously reported that the physicochemical properties of an antibiotic change upon complexation, which in turn might enhance the permeability of a drug-escort molecule [6]. The change of virtual log*P*, i.e. (Vlog$P_{(complex)}$ − Vlog$P_{(NOR)}$) / Vlog$P_{(NOR)}$, was used as a measure of the change in lipophilicity of the complex in comparison to norfloxacin on its own. The Vlog*P* of norfloxacin was -3.073, which suggested that it is a hydrophilic antibiotic drug that has a higher affinity for the aqueous phase. The biggest change was observed for a known MDR modulator, GG918, which exhibits the most favourable binding energies and the greatest increase in Vlog*P* (64.8%), while aspartame made the complex even more hydrophilic, with Vlog*P* decreasing (−64.4%).

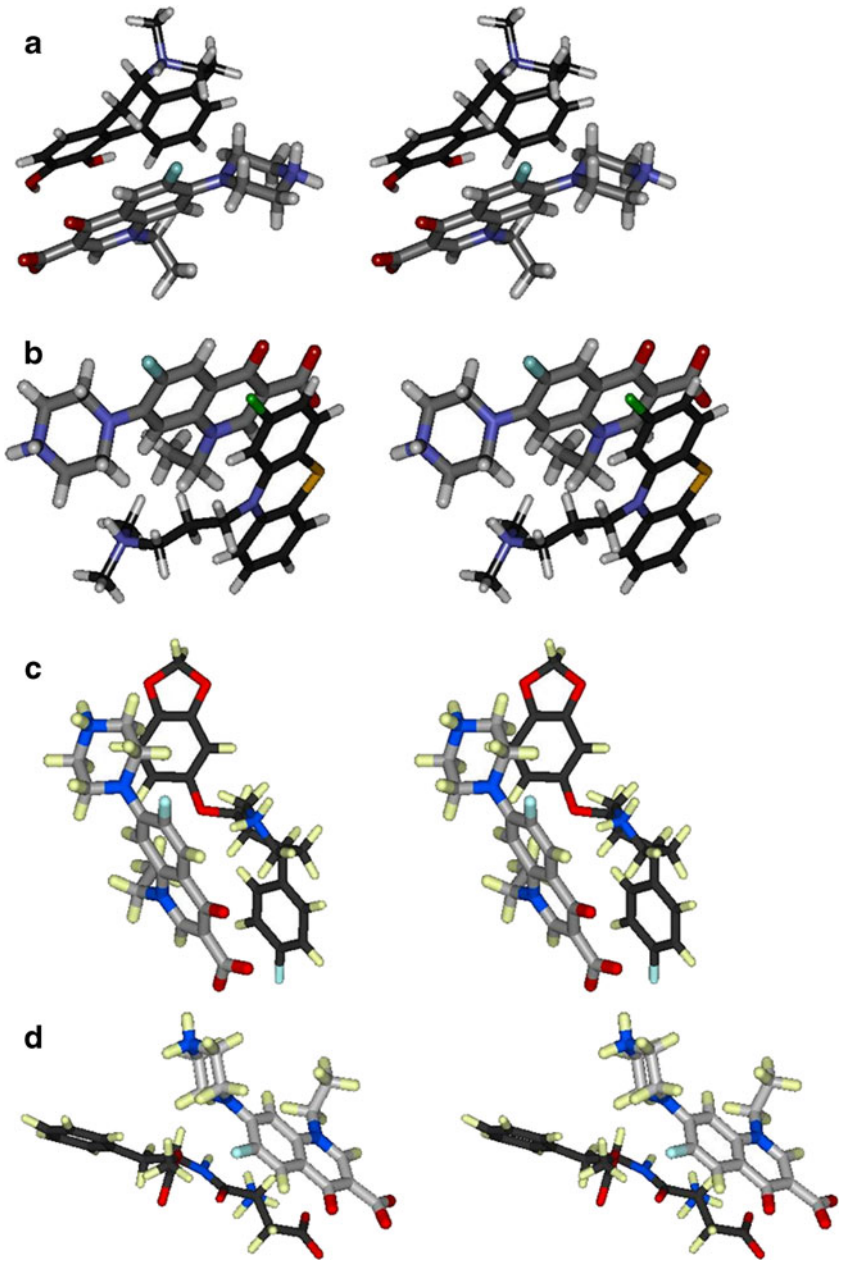

Fig. 4a–d The stereoview representations of noncovalent complexes of **a** apomorphine–norfloxacin, **b** chlorpromazine–norfloxacin, and **c** paroxetine–norfloxacin, which exhibit face-to-face aromatic interactions, and **d** the aspartame–norfloxacin complex (norfloxacin is depicted in a *lighter shade of grey* and with *thicker sticks*)

It was found that after complexation the change in VlogP was positive for the majority of the studied molecules, suggesting that the complexes were less hydrophilic. Hence, complexes have surfaces that are more lipophilic (the average increase in lipophilicity was 19%). Specifically, for chlorpromazine and paroxetine, which exhibited the most favourable average binding energies of the ten studied complexes, we observed increases of 61.4% and 49.4%, respectively. Interestingly, apomorphine exhibited the third most favourable average binding energy, and showed a decrease in lipophilicity of 19.5%.

Furthermore, the polarity of the surface decreased upon complexation, as it was apparent that the percentage of PSA coverage (PSA/SA×100) decreased by 21.8% on average after complexation when compared to the %PSA coverage of norfloxacin (Fig. 5). This indicated a possibility that complexation between the two molecules would enable the antibiotic (norfloxacin) to pass through the membrane with greater ease, as it is well documented in various studies that there is a promising inverse relationship between the polar surface area of a drug molecule and its permeability through the membrane [26, 27].

Evaluation of biological activity

Following in silico screening and analysis of the physicochemical properties, the antibacterial activities and potentiation abilities of the ten chosen molecules were experimentally determined to test whether these molecules potentiated the activity of norfloxacin (Table 2). MIC assays were performed and it was found that chlorpromazine and paroxetine exhibited intrinsic antibacterial activity (MIC=64 μg/ml). The modulation activity assays performed established that chlorpromazine and apomorphine both possessed weak potentiating activity, as they caused the MIC of norfloxacin to decrease two- and fourfold, respectively. This was promising, as apomorphine and chlorpromazine exhibited the most favourable maximum and average binding energies among of the ten tested molecules when docked with norfloxacin (Table 2). Interestingly, chlorpromazine potentiated the activity of norfloxacin twofold when the modulation assay was carried out immediately after mixing the two compounds, but when the two compounds were left in solution overnight before the assay was carried out, the potentiation was fourfold. This has further confirmed our hypothesis that two molecules can form a complex and improve the activity of norfloxacin. However, care needs to be taken when interpreting these results with regards to complex formation between norfloxacin and chlorpromazine, as chlorpromazine is known to change the expression of efflux pumps [28]; nevertheless, an increase of potentiation from twofold to fourfold after incubation suggests that complex formation may also play a role in overcoming resistance.

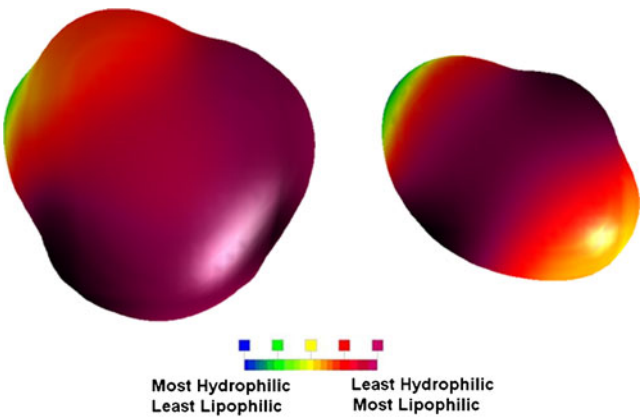

Fig. 5 The molecular lipophilicity (MLP) surface of the norfloxacin–chlorpromazine complex (*left*) and norfloxacin (*right*). The size and position of norfloxacin is the same in both cases. It can be seen that when complexed, the VlogP changes by 49.4%, indicating an increase in lipophilicity

This result agrees well with that computed by GLUE, as chlorpromazine exhibited the best average binding energy and favourable changes in physicochemical properties, and hence would be expected to potentiate the activity of norfloxacin, although not to the same extent as GG918. Apomorphine also exhibited a favourable average binding energy, but the small change in the physicochemical properties suggests that the complex would not increase the permeability of norfloxacin, so this may explain the weaker potentiating activity of the compound compared to GG918 and chlorpromazine.

Paroxetine, bergapten, naproxen and demecolcine were found to have favourable average binding energies, but they did not exhibit any potentiating activity. There are a number of speculations that can be made to explain this. Paroxetine has exhibited intrinsic antibacterial activity, and the modulation assay had to be carried out at a much lower concentration compared to the apomorphine and chlorpromazine concentrations. Demecolcine induces a decrease in VlogP (−27%), as well as a lower change in percentage PSA coverage when complexed with norfloxacin (11% decrease) compared to the change induced by chlorpromazine (31.1% decrease), thus suggesting that the complex formed between demecolcine and norfloxacin would not be able to pass through the membrane as easily, as less polar surface areas yield higher permeability [19, 20]. Bergapten and naproxen lack a nitrogen at the centres of their structures, which is essential for efflux modulation [29].

It was not possible to establish a quantitative correlation between these calculated binding energy values for the studied molecules and their levels of potentiation. It has to be noted that no other interactions were considered in this work, such as interactions between potential escort molecules and membranes. Furthermore, we should bear in mind that the interaction energy and the conformation of a complex depend

on the medium in which the complex is formed. These complexes between a drug and an escort molecule may be formed in water, in a membrane or within a binding site of the efflux pump, and these different environments may affect the binding energies. This will be the subject of further study.

This method of performing a similarity search followed by docking using GLUE was able to identify two new escort molecules (apomorphine and chlorpromazine), as well as a number of molecules that are already known to act in synergy with antibacterial drugs (methdilazine, oxyfedrine and trimeprazine) [30, 31]. It appears that it is possible to discriminate molecules that will not have potentiation ability, such as aspartame. However, this method requires further development and refinement of the criteria to qualitatively predict their ability to restore the activity of an antibiotic.

Conclusions

This study demonstrates that GRID and GLUE can be used in a qualitative way to predict complex formation between two small molecules, and as such it has the potential to be used for in silico screening of modulators of efflux pumps. A combination of similarity search and assessment of interaction energies and physicochemical properties of complexes formed between norfloxacin and potential escort molecules was utilized to search for escort molecules that are able to potentiate the activity of norfloxacin against multidrug-resistant *S. aureus*. Using our approach, among the 2396 molecules available in the SUPERDRUG database, we distinguished three molecules that have aleady demonstrated the ability to act in synergy with antibiotics, and discovered two molecules that modulate the efflux pumps. This study suggests that complexes exhibiting good binding energies and favourable changes to the molecular properties of norfloxacin should restore the activity of this antibiotic. Although our predictions are successful to some extent, the method requires further refinement of the selection criteria. This study could be expanded by querying other databases of molecules, and it has the potential to be utilized for the discovery of escort molecules for other drugs that are effluxed by multidrug-resistant cells.

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