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A molecular model of the enantioselective liquid chromatographic separation of (R,S)-ifosfamide and its N-dechloroethylated metabolites on a teicoplanin aglycon chiral stationary phase

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Abstract

The enantioselective separations of the chiral oxazaphosphorines (R,S)-ifosfamide (IF), (R,S)-2-N-dechloroethyl-IF (2-DCE-IF) and (R,S)-3-N-dechloroethyl-IF (3-DCE-IF) were achieved on teicoplanin-based chiral stationary phase using isopropanol:methanol (60:40, v/v) as the mobile phase. Computational models of the teicoplanin and teicoplanin aglycon (TAG) chiral selectors were constructed and used in docking experiments to examine the chiral recognition mechanism associated with the observed resolutions. Initial data showed no significant differences between the simulated selector-selectand complexes using teicoplanin and TAG, and the full study was conducted using TAG. The data from the study indicate that hydrophobic interactions arise between the chlorine atom present in the dechloroethyl moieties of the oxazaphosphorine molecules and hydrophobic pockets within the TAG basket and that these interactions anchored and positioned the selectands within the selector-selectand complexes. The complexes were stabilized through the formation of a network of hydrogen bond and cation - π interactions, in which the latter involved the phosphorous atom of the phosphoramidate moiety and aromatic components of the TAG aglycon basket. The chirality of the oxazaphosphorine molecule determined the number and strength of the stabilizing interactions which resulted in significant differences in the relative mean binding energies between the complexes formed by the (R) and (S) enantiomers of the selectands. These differences were consistent with the observed chromatographic enantioselectivity and suggest a multi-step chiral recognition mechanism involving the tethering of the selectand to the selector followed by conformational adjustments and stabilization of the selectand-selector complex.

1. Introduction

(R,S)-Ifosfamide ((R,S)-IF; Fig. 1) is a chiral oxazaphosphorine anticancer agent that is used in the treatment of a variety of pediatric and adult tumors including soft tissue sarcomas and lymphomas [1,2]. IF is extensively metabolized by hepatic cytochrome P450 (CYP)

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enzymes via two major pathways. One pathway involves the 4-hydroxylation of IF and eventually leads to the production of the cytotoxic isophosphoramidate mustard and the second proceeds via the oxidation of either the exocyclic-N2- or endocyclic-N3-chloroethyl moieties producing 2-N-dechloroethyl-ifosfamide [(R)- and (S)-2-DCE-IF] and 3-N-dechloroethylifosfamide [(R)- and (S)-3-DCE-IF], respectively, Fig. 1 [3,4]. About 50% of the administered IF is N-dechloroethylated and this pathway has been associated with the neurotoxicity observed with the clinical use of (R,S)-IF [3]. In vivo and in vitro studies of IF N-dechloroethylation have demonstrated that the transformation is enantioselective with a >2-fold selectivity for (S)-IF, suggesting that (R)-IF would be the preferred formulation [4–7].

As part of our continuing studies of the clinical use of (R,S)-IF and its single enantiomers, we recently reported the development and validation of an enantioselective LC-MS assay for the measurement of the enantiomers of IF, 2-DCE-IF and 3-DCE-IF in human plasma [8]. The separations were achieved on a teicoplanin-based chiral stationary phase, Chirobiotic T2 CSP, using a mobile phase composed of 2-propanol:methanol (60:40). During the optimization of the assay, it was observed that the enantioselectivity and resolution, α and R_S , of the separation of (R)- and (S)- IF increased by 53% and 27%, respectively when the mobile phase was changed from methanol to 2-propanol and the retention, k , also increased by 89%. It was assumed that these changes reflected an increase in the stabilities of the solute-CSP complexes produced by the decrease in mobile phase polarity. However, when the mobile phase changed to 100% acetonitrile, the observed enantioselectivity was lost, even though the retention increased by 161% relative to methanol. Based upon earlier studies of the effect of acetonitrile on retention and enantioselectivity on the teicoplanin CSP [9], the data suggested that for (R,S)-IF, (R,S)-2-DCE-IF and (R,S)-3-DCE-IF, hydrophobic, π - π , ion-dipole or dipole-dipole interactions play a key role in the chiral recognition mechanism, while hydrogen bond interactions contribute to the observed retention. This hypothesis is consistent with previous descriptions of enantioselective resolution and chromatographic retention on the Chirobiotic T CSP [9–12] in which the enantioselective separation of neutral solutes in the polar organic mode are the result of a mixture of hydrogen bonding, π - π or dipole-dipole interactions and hydrophobic inclusion while chromatographic retention is affected by the polarity of the solvent with increasing polarity reducing retention.

Since IF, 2-DCE-IF and 3-DCE-IF have relatively simple and rigid molecular structures with a unique asymmetric phosphorous atom as part of the central phosphoramidate moiety, we felt that the observed enantioselective separations and the effect of mobile phase composition presented an opportunity to construct a chemometric model of the chiral recognition mechanism. Previous docking studies using “Quasi Flexible” method for stereoselective recognition had been reported [13–15]. The Authors found that conformational flexibility and H-bond interactions play an important role in the chiral recognition of glycopeptide antibiotics. As far as we know this was the first docking work that studied in detail chiral separation mechanisms of chiral antibiotics using atomistic models and compared the results to experimental NMR results.

This manuscript reports the results of this study and includes the construction and application of computational models of teicoplanin and its aglycon (TAG), which were based upon a model of vancomycin. The teicoplanin models were used in AutoDock-based (ver. 4.2.1; autodock.scripps.edu) binding studies of the enantiomers of IF, 2-DCE-IF and 3-DCR-IF. Additional docking simulations were carried out for the enantiomers of propranolol and cyclophosphamide (Fig. 1). The results indicate that the observed enantioselectivity is based upon the steric fit of the selectands into the rigid environments of the macrobiotic selector with hydrophobic interactions involving the chloroethyl moieties as the anchoring

and directing interactions. The selector-selectand complexes are stabilized by cation- π and hydrogen bond interactions with enantiospecific differences in the hydrogen bond interactions forming the basis of the observed chiral recognition.

2. Methods

2.1 Chromatographic Experiments

The sources of (R,S)-IF, (R)-IF, (S)-IF, (R,S)-2-DCE-IF and (R,S)-3-DCE-IF have been previously reported, as have the conditions for their enantioselective separations [8]. In brief, the compounds were chromatographed on a Chirobiotic T2 chiral stationary phase (250 mm $\times$ 4.6 mm i.d., 5 μ m particle size) with a Chirobiotic T guard column (20 mm $\times$ 4 mm i.d.) purchased from Advanced Separations Technologies (Supelco/Sigma-Aldrich, St. Louis, MO). The chromatographic retention data used in this study was obtained with a mobile phase composed of 2-propanol:methanol (60:40) and is reported in Table 1. The enantioselective separation of (R,S)-propranolol (purchased from Sigma Aldrich) was achieved on the same column using a mobile phase composed of 2-propanol:methanol (95:5).

2.2. Construction of the teicoplanin model

The structure of vancomycin was obtained from RCSB Protein Data Bank [PDB ID:1aa5] [16] and imported into Catalyst (Ver. 4.11, Accelrys Inc, San Diego, CA), where the structure was optimized using the 3D Minimize module to the nearest energy minimum state and saved out as mol2 file. The molecule was then read into Sybyl [ver. 7.0, Tripos, St. Louis, MO, USA], all hydrogens were added, and partial charges were computed using MMFF94 method [17]. The all-atom structure was energy minimized using MMFF94 force field. The model of the teicoplanin molecule was built by modifying the vancomycin structure though the addition of the appropriate aromatic and associated moieties as has been previously discussed [9]. The aglycon model of teicoplanin (TAG) was built from the glycon model.

2.3 Eanantiomers of IF, 2-DCE-IF, 3-DCE-IF, propranolol and CP

The enantiomers of IF and CP were extracted from the Cambridge Structural Database (CSD, version 5.27) [18,19] and the enantiomers of 2DCE-IF and 3DCE-IF were built by modifying the corresponding IF isomer molecules and close analogs from CSD [18]. Propranolol isomers were downloaded from Pubchem database (<http://pubchem.ncbi.nlm.nih.gov/>). All the structures were read in Discovery Studio software (versions 2.55, 3.0 or 3.1; Accelrys Inc, San Diego, CA) for further modification or analysis. Additional information is available from Supplementary pages. Taking into account the chemistry of the small molecules, preliminary docking results and the correlation between the experimental results, we decided to use the protonated forms for propranolol, while IF, 2-DCE-IF, 3-DCE-IF and CP were used as their non-protonated forms.

2.4 Docking simulations

Selector-selectand binding simulations were carried out using AutoDock (ver. 4.2.1) [20]. AutoDockTools (ver. 1.5.0) [21,22] an autodock-friendly graphical-user-interface (GUI) software was used for preparing input files for the AutoDock simulations. Discovery Studio [versions 2.0, 2.55 and 3.1, Accelrys Inc, San Diego, CA] was used for the analysis.

AutoDock employs Lamarckian Genetic Algorithm (LGA) to identify binding conformation(s) of a flexible ligand (or small molecule) to a target receptor. The basic ideas for Genetic algorithm [23] based methods were derived from the principles of genetics and biological evolution, and have been successfully applied to understand several complex

problems in multi-disciplinary areas. LGA is based on the assumption that the phenotypic traits gained during one's lifetime can become heritable traits [24]. Genetic algorithm methods describe the 3D arrangement of the molecules involved in the docking using state variables (or genes). In docking, the state variables are the distance of ligand molecule (selectand) from the receptor, the orientation of the ligand with respect to the receptor and the torsional degrees of freedom (number of rotatable bonds) of the ligand. The atomic coordinates corresponding to the ligand are called phenotype, and the genotype refers to the collection of the following three genes (identified as chromosome): i) translation gene: the three coordinates defining the ligand position with respect to the receptor ii) orientation gene (4 quaternion coordinates) iii) torsion gene (one for each torsion angle).

An approach using a combination of local search (energy minimization) method with genetic algorithm had been found to improve docking results and was chosen as the default method [20,21] in our study. AutoDock uses a simplified form of AMBER force field for the energy calculations, and an empirical functional form for computing the free energy of binding [20,21]. As a preliminary preparation step to docking, AutoGrid (module in AutoDock) creates 3D grid boxes (maps) to generate a simplified representation for target receptor (ex. TAG). Each atom type (called probe) of the ligand will be placed at the grid points and its interaction energy with all the atoms of the receptor will be computed and assigned to the corresponding grid point. The collection of such grid points for a particular atom-type (Ex. C-Atomtype) is what constitutes a map (ex. C Map). For each atom-type in ligand, a corresponding map will be created and stored. Finally two additional maps, one for electrostatics (electron map) and the other for desolvation will also be created. During docking, the maps will be used as a look-up table for extracting interaction energies of the ligand with the receptor. In this study, a grid box ($60 \times 60 \times 60$ grid points; equal spacing of $0.375 \text{ \AA}$) which was large enough to cover the entire TAG molecule was constructed and used for docking. TAG is mostly made up of interconnected rings and hence was modeled as a rigid structure in the docking simulations. Information regarding the parameters used in AutoGrid (Grid Parameter File, GPF) and AutoDock (Docking Parameter File, DPF) is available from Supplementary pages (see S2). At the end of docking calculations, AutoDock was used to group the solutions (binding modes of ligands) into clusters based on structural Root Mean Squared Deviation (RMSD) tolerance of $2 \text{ \AA}$. Mean binding energy of the cluster solutions along with the number of members in the cluster are shown in Supplementary pages (section S2.4).

3. Results and Discussion

3.1 The structural model of the teicoplanin aglycon (TAG) selector

In their initial studies of the use of macrocyclic antibiotics as chiral selectors, Armstrong and co-workers described the basic structure of vancomycin, teicoplanin and ristocetin A and provided both structural figures and space filling models of these compounds [9,10]. The aglycon cores of these molecules are basket-like rigid structures rich in aromatic rings with multiple amide bonds, ionic sites and hydrogen-bonding groups. The multiple fused ring arrangement along with functional groups gives rise to a rigid frame-work of charged and hydrophobic centers that play a key role in the enantioselective separation of the chiral compounds. In this study, the structure of vancomycin, obtained from RCSB Protein Data Bank [16], was used to construct a computational model of the molecule, Fig. S-1. As teicoplanin contains extra aromatic rings relative to vancomycin, the computational model of vancomycin was appropriately altered to produce models of teicoplanin and its aglycon (TAG; see Fig 2a). The teicoplanin and TAG are distinctly different structures relative to vancomycin with greater rigidity and a pronounced cavity. The initial docking simulations of (R)- and (S)-IF using teicoplanin and TAG models showed that the interaction and binding takes place predominantly in the core cavity away from the sugar moieties with the

IF-protein bound conformations being very similar. Taking this into account, we restricted our simulations to the TAG model, which is presented in Figs. 2a and b.

In the working model of TAG presented in Fig. 2a, the 4 macrocyclic rings that compose the aglycon “basket” are designated by labels **A** to **D**. The model demonstrates that there are multiple potential interaction sites within the molecule as a whole and within each of the components of the “basket”. In order to develop a method for the designation of the potential selectand interaction sites within each of the macrocyclic rings, the moieties involved in the interaction have been identified as **A** indicating an aromatic ring, **H** indicating a hydrogen atom and **O** indicating an oxygen atom. Using this approach, the designations **BH1**, **B02** and **BA4** refer to the TAG atoms and part of macrocyclic ring designated as **B** and describe potential sites for hydrogen bond donation, hydrogen bond acceptance and aromatic-based interactions, respectively.

3.2 The simulated docking of (R)-IF and (S)-IF to the TAG model

The binding modes of (R)-IF and (S)-IF to the TAG model were simulated using AutoDock [20,21]. The mean docking energy of the (R)-IF-TAG complex was -4.95 kcal/mol compared to -4.71 kcal/mol for (S)-IF-TAG complex and the relative stabilities are consistent with the chromatographically determined elution times, Table 1.

When (R)-IF and (S)-IF were docked into the TAG model, both molecules placed the chlorine atom present in the chloroethyl moiety on the exocyclic nitrogen (*N2*, see Fig 1) in an energetically favorable hydrophobic pocket formed by the **A** and **D** rings of the TAG molecule (designated as *N2-Cl-AA4/DA1* in Table 2). The complex is depicted using simple (schematic model of TAG) and detailed atomistic models in Figs. 3a–c. The *N2-Cl-AA4/DA1* interaction appears to position the IF enantiomers relative to the TAG aglycon basket and the chirality of the phosphorous appears to direct the chlorine atom of the chloroethyl moiety of the endocyclic nitrogen (*N3*, see Fig 1) into different hydrophobic environments. The chloroethyl chain of the endocyclic moiety (*N3*) on (R)-IF is directed to the pocket created by the **A** and **B** rings of the TAG molecule and the corresponding chloroethyl moiety (*N3*) on (S)-IF is directed to the pocket created by **B**, **C** and **D** rings, designated as *N3-Cl-AA3/BA3* and *N3-Cl-BA4/CA4/DA4*, respectively, in Table 2. The relative positioning of (R)-IF and (S)-IF is shown in Fig. 3a in which the components of the TAG aglycon basket are represented by outlines in the plane of the figure.

Additional interactions between (R)-IF and the components of the TAG aglycon basket are presented in Fig. 3b, and, although only the top ranked conformation is shown, 80% of the docking solutions did not significantly differ from this model ($\text{RMSD} < 2 \text{ \AA}$; see Supplemental Information section S2.4). In this conformation, the phosphoramidate oxygen is pointing out and the ring oxygen is buried within the TAG molecule, which results in additional hydrogen bonding interactions between the (R)-IF ring oxygen atom and an amide hydrogen within the **C** ring of the TAG molecule (*O1-CH1*) and the exocyclic phosphoramidate hydrogen and a carbonyl oxygen on the **B** ring of the TAG molecule (*N2-H-BO2*) and cation - π interactions between the phosphorus atom and aromatic moieties present in the **A**, **B** and **C** rings of the TAG molecule (*P-AA4/BA4/CA4*), Table 2. One of the attractive interactions within the selectand-selector complex occurs between a partial positive charge located on the P atom and the pi electrons of the aromatic rings (**A**, **B** and **C**; see Fig 2a) located within the TAG basket. The charge on the P atom reflects the electron withdrawing effects of the oxygen and nitrogen substituents attached to this atom and can be viewed as similar to the charge distribution observed within a dipole. Based upon this understanding, we have labeled this interaction as a “cation-pi” interaction and used this nomenclature in the rest of the manuscript. When (S)-IF was docked in the TAG molecule,

Fig. 3c, the phosphoramidate oxygen is pointing towards the **D** ring and the ring oxygen is pointing out of the aglycon basket. The result is that the hydrogen bonding interactions between the (S)-IF ring oxygen atom and an amide hydrogen within the **C** ring of the TAG molecule was not observed nor was one of the cation - π interactions between the phosphorus atom on (S)-IF and an aromatic moieties present in **C** (**CA4**) ring of the TAG molecule, while the hydrogen bond interaction between the exocyclic phosphoramidate hydrogen and a carbonyl oxygen on the **B** ring of the TAG molecule was preserved, Table 2. It is interesting to note that (S)-IF bound conformations due to its phosphoramidate oxygen orientation switches its cation - π interactions from AA4 ring (deeper in the cavity) to the ring, AA3 (Table 1) present in the outer ring of the basket. It is assumed that these variations are the primary source of the difference in the mean binding energies between the (R)-IF – TAG complex and the (S)-IF – TAG complex.

3.3. The simulated docking of (R)-2-DCE-IF and (S)-2-DCE-IF to the TAG model

The binding modes of (R)-2-DCE-IF and (S)-2-DCE-IF to the TAG model were simulated using AutoDock, Figs. 4a–c. The mean docking energy of the (R)-2-DCE-IF-TAG complex was -4.54 kcal/mol compared to -4.15 kcal/mol for (S)-2-DCE-IF-TAG complex and the relative stabilities are consistent with the chromatographically determined elution times, Table 1. In the simulations, the chlorine atom present in the cholorethyl moiety on the *N3* endocyclic nitrogen in both enantiomers was placed in the hydrophobic environment between rings **A** and **B** (*N3-Cl* – **AA3/BA3**), Table 2, producing two different environments for the (R)- and (S)-enantiomers, Fig. 4a. The positioning of the (R)-2-DCE-IF molecule model permits additional hydrogen bonding interactions between the ring oxygen atom and an amide hydrogen within the **C** ring of the TAG molecule (*O1*–**CH1**) and the exocyclic phosphoramidate hydrogen and carbonyl oxygens on the **B** and **D** rings of the TAG molecule (*N2-H*– **BO2/DO2**) and cation - π interactions between the phosphorus atom and aromatic moieties present in the **A**, **B** and **C** rings of the TAG molecule (**P** – **AA4/BA4/CA4**), Table 2, Fig. 4b. When (S)-2-DCE-IF was docked in the TAG molecule, Fig. 4c, the hydrogen bonding interactions between the (S)-2-DCE-IF ring oxygen atom (*O1*) and an amide hydrogen within the **C** ring (**CH1**) of the TAG molecule was not observed nor was the hydrogen bonding interaction between the exocyclic phosphoramidate hydrogen and a carbonyl oxygen on the **B** ring of the TAG molecule, Table 2. It is assumed that these differences are the primary source of the variation in the mean binding energies between the (R)-2-DCE-IF – TAG complex and the (S)-2-DCE-IF – TAG complex.

3.4. The simulated docking of (R)-3-DCE-IF and (S)-3-DCE-IF to the TAG model

The binding modes of (R)-3-DCE-IF and (S)-3-DCE-IF to the TAG model were simulated using AutoDock, Figs. 5a–d. The mean docking energy of the (R)-3-DCE-IF-TAG complex was -4.83 kcal/mol compared to -4.59 kcal/mol for (S)-3-DCE-IF-TAG complex and the relative stabilities are consistent with the chromatographically determined elution times, Table 1. In the simulations, the chlorine atom present in the cholorethyl moiety on the *N2* exocyclic nitrogen in both enantiomers was placed in the hydrophobic environment between rings **A** and **D** (*N2-Cl* – **AA4/DA1**), similar to the positioning of the same moiety in the parent IF molecule, Table 2, *D*Figure 5a. In the simulated docking conformations, the phosphoramidate oxygen moieties of both enantiomers align with each other resulting in identical hydrogen bonding interactions with an amide hydrogen in ring of the TAG molecule (**P=O** – **DH3**), the hydrogen atom on *N2* exocyclic nitrogen in both enantiomers interact with a carbonyl oxygen on the **D** ring of the TAG molecule (*N2-H* – **DO2**) and cation - π interactions between the phosphorus atom and aromatic moieties present in the **B** and **C** rings of the TAG molecule (**P** – **BA4/CA4**), Table 2, Figs. 5b,c. The chirality of the molecule is expressed in the positioning of the ring oxygen (*O1*) and endocyclic nitrogen (*N3*) relative to the TAG molecule. When the (R)-3-DCE-IF is docked, *N3* points in towards

the TAG aglycon basket and is in the position to create a hydrogen bond with a carbonyl moiety on ring **B** (*N3-H* – **BO2**), Table 2, Fig 5b, while in the (S)-3-DCE-IF – TAG complex, ring oxygen (*O1*) swaps places with *N3* and as a result the hydrogen bond interaction is lost, Fig. 5c. It is assumed that this difference is the primary source of the variation in the mean binding energies between the (R)-3-DCE-IF – TAG complex and the (S)-3-DCE-IF – TAG complex. It is interesting to note that the second best ranked docking solution for (S)-3-DCE-IF (shown in Fig 5d) is similar to its corresponding parent isomer, (R)-IF (see Fig 3b) and also completely different from the top ranked conformations of both isomers (Figs 5a–c). In this binding mode N2-CL, ring oxygen (*O1*) and phosphoramidate oxygen moieties are interacting with TAG like (R)-IF. Previous study by D'Acquarica et al [15] modeled the binding association of N-acetyl-D-Ala-D-Ala to TAG and identified binding modes that formed strong H-bonds between TAG atoms, BO2 (carbonyl oxygen), amide hydrogens (CH1, CH3 and DH3), and ligand molecules. Furthermore, these authors [15] also reported additional docking simulations that modeled the chiral discrimination of N-acetyl-D/L-phenylglycine isomers to identify similar H-bonding interactions as before with the three central amide hydrogens present in B and C rings of TAG (see Fig 2a) and the ligand phenyl group from N-APG interacting with the aromatic groups of TAG. Even though the ligand systems (IF isomers and metabolites etc.) that we have reported in this study are very different, the binding mode interactions especially involving BO2, CH1, CH3 and the aromatic rings from TAG are the same.

3.5. Discussion

Macrocyclic glycoprotein selectors such as teicoplanin have been described as complex molecules capable of all of the molecular interactions associated with chromatographic retention and enantioselectivity [11,25]. The computational models of the teicoplanin and TAG CSPs developed in this study support this observation, as does the data from the simulated docking studies with (R,S)-IF and its N-dechloroethylated metabolites. The results indicate that the TAG basket provides a rich environment with multiple charge centers, hydrophobic pockets and functional groups that are available to participate in hydrophobic, hydrogen bond, dipole-dipole and cation - π interactions. In addition, the relative rigidity of the TAG molecule produces a stable chiral environment in which the molecular recognition of the selectand occurs.

The data from the current study suggest a multi-step chiral recognition process involving the tethering of the oxazaphosphorine molecules IF, 2-DCE-IF and 3-DCE-IF within the aglycon basket of the TAG CSP via hydrophobic interactions between the chlorine atom present in the cholorethyl moieties of the oxazaphosphorine molecules and hydrophobic subpockets within the TAG basket. It is important to note that the hydrophobic interactions are often predominant in purely aqueous media and in case of alcohol medium these interactions are known to be diminished. However, in this study, the top-ranking docked conformations of selectands consistently show that parts of molecules (ex. chloroethyl moieties) clearly prefer placement in local hydrophobic pockets (ex. between the phenyl rings, AA4 and DA1). These interactions anchor the selectand within the TAG basket. Since the oxazaphosphorine molecules are also relatively rigid, the resulting complexes express the molecular asymmetry of the phosphoramidate moiety through the differential positioning of the interactive substituents of the oxazaphosphorine molecule within the selector-selectand complexes, c.f. Figs. 3a, 4a, 5a. The complexes are then stabilized through the formation of a network of hydrogen bond and cation - π interactions, in which the chirality of the oxazaphosphorine molecule determines the number and strength of the stabilizing interactions, Table 2. This, in turn, produces the enantioselective differences in the relative mean binding energies and the chiral discrimination of the (R) and (S) enantiomers of the selectands.

The data from this study identifies the key atoms and functional groups within the TAG basket associated with the chiral discrimination of IF, 2-DCE-IF and 3-DCE-IF as the hydrophobic clefts created by aromatic rings **AA4** and **DA1** and **BA4** and **CA4**, hydrogen bond formations involving the ether oxygen (**DO2**) and the backbone amide groups **BH1**, **BO2**, **CH1**, **CO2**, and cation- π interactions between the phosphoryl group and the aromatic rings **AA3**, **AA4**, **BA3**, **BA4** and **CA4**, Fig. 2a. The key binding interactions involving these moieties are summarized in Table 2. When (R,S)-IF and (R,S)-2-DCE-IF are the selectands, the results suggest that hydrogen bonding interactions involving the oxazaphosphorine ring oxygen (*OI*) and the TAG **CH1** moiety is the chiral discriminating interaction, Figs. 3b,c and 4b,c. The ability to achieve the *OI*-**CH1** interaction is a result of the hydrophobic interactions involving the N2-chloroethyl moiety and the hydrophobic pocket formed by **AA4** and **BA3**. In the case of 3-DCE-IF, the hydrophobic interaction involves the N2-chloroethyl moiety and the hydrophobic pocket created by **AA4** and **DA1**, Fig. 5a, which places the oxazaphosphorine ring oxygen (*OI*) in a position where it is unable to make the *OI*-**CH1** interaction observed with the other selectands. For this compound, chiral recognition appears to be the ability of the hydrogen atom at the N3 position on (R)-3-DCE-IF to create a hydrogen bond with the carbonyl oxygen designated as **BO2** on the TAG molecule, Figs. 5b,c, Table 2.

The data from this study indicate that the docking conformations and the resulting binding energies associated with the oxazaphosphorine molecules used in this study closely mimic the chromatographic results. Thus, the observed binding patterns and trends from docking simulations provide an insight into the mechanisms associated with chiral recognition by the TAG CSP. However, the data used in the study was derived using six small, rigid and closely related compounds and a single polar organic mobile phase. Thus, the broader applicability of the TAG CSP model is untested.

While the extensive testing of the TAG CSP model was outside of the scope of the current study, the enantioselective separation of (R,S)-propranolol was examined on the Chirobiotic T2 CSP. Previous data indicated that the compound could be resolved on the Chirobiotic TAG column (sigmaaldrich.com/catalog/analytical/supelco) although the mobile phase was composed of methanol modified with 0.1% ammonium formate. In the current study, a mobile phase composed of 2-propanol:methanol (60:40, v/v) produced an asymmetric peak at 11.3 min (Fig. S-2A) and changing the polarity of the mobile phase by reducing the methanol content to 5% increased the retention and produced an observable enantioselective separation of (R)-propranolol (23.9 min) and (S)-propranolol (22.6 min), Fig S-2B. When the protonated forms of the propranolol enantiomers were docked into the TAG model, the naphthalene moiety on both isomers made π - π interactions with the TAG aromatic groups **AA4** and **DA1** and the hydroxyl moiety at the chiral center formed a hydrogen bond with either **BO2** carbonyl or **DO2** ether oxygen atoms, Figs. S-3A,B. It is interesting to note that in propranolol, protonated nitrogen center seems to mimic the role of IF phosphorous by retaining similar cation- π interactions with TAG aromatic ring moieties in **B/C** (for R-) or **A/B/C** (for S-). These interactions anchor and position the propranolol molecule relative to the TAG basket such that the hydrogen atoms on the aminopropyl substituent of (R)-propranolol are able to hydrogen bond with the **BO2** oxygen moiety. The well-balanced cation- π interactions along with energetically favorable hydrogen bonded interactions (C=O rather than ether oxygen) seem to create favorable binding pocket when (R)-propranolol is docked in the TAG basket, Figs. S-3A,B. Using this model, the calculated mean binding energy of (S)-propranolol is relatively weaker than R-isomer, -5.75 Kcal/mol and -5.81 Kcal/mol, respectively, which is consistent with the observed enantioselectivity on the TAG CSP. The enantioselective separation of (R)- and (S)-cyclophosphamide (CP) on the teicoplanin CSP has also been reported using a mobile phase composed of water : ethanol : THF (95:5:5) (sigmaaldrich.com/catalog/analytical/supelco). Since CP and IF are closely

related, see Fig. 1, (R)- and (S)-CP were docked into the TAG model. In spite of missing the endocyclic chloroethyl chain (N3-CL), the CP enantiomers continue to use the second chloroethyl chain present on N2 (Fig 1) in the same role as endocyclic chloroethyl chain originating from N3 in IF isomers. This along with the key interactions involving ring oxygen (O1), additional cation- π interactions involving P, create a stronger binding mode for (S)-CP (-4.55 Kcal/mol) relative to the complex determined using (R)-CP (-4.42 Kcal/mol). The top ranked binding mode of (R)-CP and (S)-CP to TAG identified from docking simulations are shown in Figs. S4A and S5A. The relative stabilities of the (S)-CP and (R)-CP complexes are consistent with the enantiomeric elution order the elution order in which (R)-CP elutes before (S)-CP, established using the optical rotation of the eluted peaks obtained with an online polarimeter (personal communication, Tomas Beesley, Advanced Separations Technologies).

4. Conclusions

The results indicate that the TAG CSP model developed in this model is a reasonable starting point for the development of a descriptive and perhaps predictive model for enantioselective separations on this column in both polar organic and reversed phase modes. The refinement and validation of the model awaits the results of ongoing studies utilizing different families of chiral compounds chromatographed using a variety mobile phase conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- 3D models of teicoplanin and vancomycin chiral stationary phases reported
- A molecular model that describes experimentally observed chiral separation
- Initial model to predict enantiomeric elution order on a teicoplanin CSP

Fig. 1. Chemical structures of ifosfamide (IF) isomers, their dechloroethyl (DCE) metabolites, isomers of cyclophosphamide and propranolol. The arrows identify ifosfamides and their corresponding metabolites. Cytochrome P-450 enzymes (CYP3A4, CYP2B6) that function in each IF metabolic pathways are also shown.

Fig. 2.

Fig 2a. Key interaction sites (indicated by spheres) in aglycon form of teicoplanin (TAG). Green sphere mark the centroid position of aromatic ring. Red and white small spheres indicate the position of oxygen and hydrogen atoms. Rings are indicated by bold-font alphabets **A** to **D**. Each marked atom or position (in case of aromatic centroid) can be referenced by ring label followed by atom element type and then atom number. For example, **AH1** indicates that the atom is part of ring **A**, of element type hydrogen, H, and has an atom number 1.

Fig. 2b. Schematic simplified top-view of TAG with key interaction sites identified by labels.

Fig. 3.

Fig. 3a. Schematic top-view highlighting the hydrophobic interactions between IF isomers and TAG. The TAG is drawn in schematic form and the ifosfamide isomers are shown in stick form. The top ranked (R)/(S) isomers shown together in the bound state with TAG (same conformations are shown separately in Figs 3b and c). The locations of aromatic substituent rings in TAG are indicated by labels (Ex. AA3, BA3 etc.) in the schematic drawing. Please refer to Fig. 2a for atom labeling convention. Note in this conformation both

IF isomers place their chloroethyl moieties from exocyclic nitrogen (*N*2; see fig 1) in a hydrophobic cavity created by aromatic rings **AA4** and **DA4**. The other chlorine atoms from endocyclic nitrogen (*N*3; see fig 1) are placed near **AA3/BA3** [(R)-IF] or **BA4/CA4/DA4** [(S)-IF].

Fig. 3b. Top ranked docking conformation of (R)-IF. Atoms of chiral selector, TAG and the ligand are drawn in line and stick representations. Figure shows the key interactions between TAG and ligands [H-bonded and Cation-PI interactions are marked in green dotted and solid lines].

Fig. 3c. Top ranked docking conformation of (S)-IF. Atoms of chiral selector, TAG and the ligand are drawn in line and stick representations. Figure shows the key interactions between TAG and (S)-IF [H-bonded and Cation-PI interactions are marked in green dotted and solid lines].

Fig. 4.

Fig. 4a. Schematic top-view highlighting the hydrophobic interactions between 2-DCE-IF isomers and TAG. The TAG is drawn in schematic form and the ifosfamide isomers are shown in stick form. The top ranked (R)-2/(S)-2-DCE-IF isomers shown together in the bound state with TAG (same conformations are shown separately in Figs 4b and c). The locations of aromatic substituent rings in TAG are indicated by labels (Ex. AA3, BA3 etc.) in the schematic drawing. Please refer to Fig. 2a for atom labeling convention. Note in this conformation both 2-DCE-IF isomers place their chloroethyl moiety from endocyclic nitrogen (*N*3; see fig 1) in a hydrophobic cavity created by aromatic rings **AA3** and **BA3**. For clarity, only (R)-2-DCE isomer is identified by label.

Fig. 4b. Top rank docked complex of (R)-2-DCE-IF with TAG. TAG atoms are shown in line mode and IF in stick form. Cation-Pi and H-bonded interactions are marked by dotted and solid green line respectively.

Fig. 4c. Top rank docked complex of (S)-2-DCE-IF with TAG. TAG atoms are shown in line mode and IF atoms in stick. Cation-Pi and H-bonded interactions are shown in dotted and solid green line respectively.

Fig. 5.

Fig. 5a. Schematic top-view highlighting the hydrophobic interactions between 3-DCE-IF isomers and TAG. The TAG is drawn in schematic form and the ifosfamide isomers are shown in stick form. The top ranked (R)-3/(S)-3-DCE-IF isomers shown together in the bound state with TAG (same conformations are shown separately in Figs 5b and c). The locations of aromatic substituent rings in TAG are indicated by labels (Ex. AA3, BA3 etc.) in the schematic drawing. Please refer to Fig. 2a for atom labeling convention. Note in this aligned mode both 3-DCE-IF isomers align nicely their phosphoryl oxygen along with chloroethyl moiety from exocyclic nitrogen (*N*2; see fig 1). Also this chloroethyl moiety is placed in an energetically favorable hydrophobic cavity created by aromatic rings **A** and **D**. This alignment and the chirality forces the ring oxygen (O1) and endocyclic nitrogen (N3) to swap places between the isomers. For clarity, only (R)-3-DCE isomer (Phosphorous atom and N3) is identified by label.

Fig 5b. Top rank docked complex of (R)-3-DCE-IF (stick) with TAG (line mode). H-bonded and Cation-Pi interactions are shown in dotted and solid green lines respectively.

Fig 5c. Top rank docked complex of (S)-3-DCE-IF (stick) with TAG (line mode). H-bonded and Cation-Pi interactions are shown in dotted and solid green lines respectively.

Fig 5d. Second rank docked complex of (S)-3-DCE-IF (stick) with TAG (line mode). H-bonded and Cation-Pi interactions are shown in dotted and solid green lines respectively. Note this conformation is very similar to the (R)-IF except for the N3-endocyclic chloroethyl group which is not present in this molecule.

Table 1

Experimental elution times, computational binding energy values for isomers of ifosfamide along with their metabolites on teicoplanin aglycon (TAG) column. Docking simulations were carried out using AutoDock software (ver. 4.2.1; autodock.scripps.edu). Mean binding energy values are presented along with the number of members (shown in parenthesis) for the cluster. Please refer to Supplementary file for additional information.

Compounds	Elution Times	Mean Binding Energy (Kcal/mol)
(R)-IF	13.0	-4.95 (81)
<i>(S)-IF</i>	<i>10.0</i>	<i>-4.71 (70)</i>
(R)-2-DCE-IF	16.0	-4.54 (100)
<i>(S)-2-DCE-IF</i>	<i>14.0</i>	<i>-4.15 (67)</i>
(R)-3-DCE-IF	20.0	-4.83 (99)
<i>(S)-3-DCE-IF</i>	<i>17.0</i>	<i>-4.59 (71)</i>

Table 2

Key interactions [H-bonds, Cation- π , Sigma- π and hydrophobic (HYP)] between Ifosfamide (IF) isomers, their metabolites with **TAG** observed from docking simulations are summarized in a table. Atoms of IF isomers and their metabolites are *shown in italics* and the atoms of the separator, **TAG**, are shown in **bold face**. The atom names of the small molecules and column separator (**TAG**) involved in the interactions are based on the convention detailed in Figures 1 and 2a. For example, the interaction, *P-AA4/BA4/CA4*, shown under cation- π column, indicates phosphorous atom (*P*) from the ligand forming cation- π interactions with aromatic rings (indicated by centroid sphere in Fig. 2a), **AA4**, **BA4** and **CA4** located on **TAG**. Note *N3-CL* indicates the chlorine atom (*CL*) that belongs to the chain arising from *N3*. H-bonded interactions are indicated by dotted lines (--).

Compounds	H-bond		Cation- π	HYP	
(R)-IF	N2-H-BO2	<i>O</i> / <i>-</i> CHI	<i>P-AA4/BA4/CA4</i>	<i>N2-CL-AA4/DA1</i>	<i>N3-CL-AA3/BA3</i>
(S)-IF	N2-H-BO2		<i>P-AA3/BA4</i>	<i>N2-CL-AA4/DA1</i>	<i>N3-CL-BA4/CA4/DA4</i>
(R)-2-DCE-IF	N2-H-BO2/DO2	<i>O</i> / <i>-</i> CHI	<i>P-AA4/BA4/CA4</i>		<i>N3-CL-AA3/BA3</i>
(S)-2-DCE-IF	N2-H-DO2 <i>P=O- CHI</i>		<i>P-AA4/BA4/CA4</i>		<i>N3-CL-AA3/BA3</i>
(R)-3-DCE-IF	N2-H-DO2 <i>N3-H-BO2</i> <i>P=O-DH3</i>		<i>P-BA4/CA4</i>	<i>N2-CL-AA4/DA1</i>	
(S)-3-DCE-IF	N2-H-DO2 <i>P=O-DH3</i>		<i>P-BA4/CA4</i>	<i>N2-CL-AA4/DA1</i>	