Interactions of some commonly used drugs with human α-thrombin

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Adverse side effects of drugs are often caused by the interaction of drug molecules to targets other than the intended ones. In this study, we investigated the off-target interactions of some commercially available drugs with human α-thrombin. The drugs used in the study were selected from Super Drug Database based on the structural similarity to a known thrombin inhibitor argatroban. Interactions of these drugs with thrombin were initially checked by in silico docking studies and then confirmed by thrombin inhibition assay using a fluorescence microplate-based method. Results show that the three commonly used drugs piperacillin (anti-bacterial), azlocillin (anti-bacterial), and metolazone (anti-hypertensive and diuretic) have thrombin inhibitory activity almost similar to that of argatroban. The $K_i$ values of piperacillin, azlocillin, and metolazone with thrombin are .55, .95, and .62 nM, respectively. The $IC_{50}$ values of piperacillin, azlocillin, and metolazone with thrombin are 1.7, 2.9, and 1.92 nM, respectively. This thrombin inhibitory activity might be a reason for the observed side effects of these drugs related to blood coagulation and other thrombin activities. Furthermore, these compounds (drugs) may be used as anti-coagulants as such or with structural modifications.

Keywords: drug side effects; off-target interactions; thrombin inhibition; argatroban; docking

Introduction

Basis of the medicinal activity of drug molecules resides in the mode of interaction with their specific target molecules (Pattabiraman, Levitt, Ferrin, & Langridge, 1985). The drug molecules can interact with unpredicted targets and this lack of specificity may lead to unexpected side effects (Whitebread, Hamon, Bojanic, & Urban, 2005). This off-target interaction is a major problem in the field of drug discovery and development. Understanding these processes may greatly impact the field of drug discovery through the development of safer drugs, and the identification of new uses of existing drugs.

Knowledge of the side effects has been contributed in the development of novel drugs (Stockwell, 2004). Very first examples were organomercurials (now being obsolete), which were originally used for the treatment of syphilis, but turned out to act as diuretics (Sneader, 1985). Another drug is sildenafile which is used for the treatment of male sexual disorder. Sildenafil was resulted from the optimization and development of antiallergic, antihypertensive, and antiangiinal drug candidates (Kling, 1998). Hence, interactions with an unintended target might suggest new uses for an existing drug (Ekins, 2004; Keiser et al., 2009).

In this study, we are discussing about the possible reason for the observed side effects, related to blood coagulation and other thrombin activities, of some commonly used drugs. The present study fixed thrombin as the target examined due to its importance in hemostatic and non-hemostatic processes (Bode, Turk, & Karshikov, 1991; Xie et al., 2005). The modes of interactions of 50 drugs, which have different therapeutic uses and mechanism of action, with human α-thrombin, are discussed here. These drugs have been selected based on the structural similarity with the known thrombin inhibitor argatroban (Chen, 2001). The main objective of this study is to get information regarding the thrombin-mediated side effects of the selected drugs. Another objective is to get information of the lead compounds which can be used for the development of novel anti-thrombotic drugs as such or with some structural modification.

The selection of the drug molecules used in this study was done based on their structural similarity with a well-known thrombin inhibitor, argatroban. The initial screening of the selected molecules, which can interact with thrombin, was done by in silico docking methods. Further confirmation was done by fluorescence-based in vitro assay method.
Materials and methods

In silico docking studies

In silico docking was employed to examine the binding possibility of the selected drugs to thrombin active site. Docking was carried out using the well accepted Autodock4.0 (Scripps.edu) program (Morris et al., 1998).

Ligands (some commonly used drugs)

Fifty commonly used drug molecules were selected, for this study, based on their structural similarity with a well-known thrombin inhibitor, argatroban. For this selection, a structural similarity search in the Super Drug Database (http://bioinf.charite.de/superdrug; Goede, Dunkel, Mester, Frommel, & Preissner, 2005) using argatroban as the scaffold structure was carried out. A conformer-based superposition algorithm in the SuperDrug database enables generation of different conformers of the molecules and superimposing them to find the root mean square distance. Based on the results of the 3D superposition of the most similar conformations of the query structures and argatroban, the fifty compounds were selected for the present study. The basis of this selection is that the compounds having structural similarity may show related biological activity (Eckert & Bajorath, 2007; Fliri, Loging, Thadeio, & Volkman, 2005). Both peptide and non-peptide molecules were included in the list. Non-peptide compounds showed structural similarity with argatroban as a whole. But, the peptide compounds showed similarity only in the thrombin active site binding region. Atomic coordinates of the selected drug molecules were taken from the PubChem database of NCBI (http://pubchem.ncbi.nlm.nih.gov). The missing hydrogen atoms were added and charges on the molecules were kept to zero.

Receptor (human α-thrombin)

Human α-thrombin was used as the receptor in this docking study. The atomic coordinates of human α-thrombin was downloaded from Protein Data Bank (PDB ID: 1DWC). The downloaded thrombin structure existed as a complex with argatroban (Banner &

| Sl. No. | Compound name | Therapeutic class       | Binding energy (kcal/mol) |
|--------|---------------|-------------------------|---------------------------|
| 1      | Argatroban    | Anti-coagulant           | -16.07                    |
| 2      | Piperacillin  | Anti-bacterial           | -16.69                    |
| 3      | Azlocillin    |                         | -16.35                    |
| 4      | Mezlocillin   |                         | -15.84                    |
| 5      | Cefonicid     |                         | -15.42                    |
| 6      | Cefsulodin    |                         | -14.73                    |
| 7      | Cefpirome     |                         | -12.83                    |
| 8      | Metolazone    | Anti-hypertensive and Diuretic | -16.36                  |
| 9      | Indapamide    |                         | -15.12                    |
| 10     | Cyclophenthiazide |                   | -12.06                    |
| 11     | Bendroflumethiazide |                | -13.92                    |
| 12     | Polythiazide  |                         | -13.48                    |
| 13     | Cyclothiazide |                         | -12.75                    |
| 14     | Methylthiazide|                         | -12.73                    |
| 15     | Trichlormethiazide |                | -12.46                    |
| 16     | Hydroflumethiazide |                      | -11.88                    |
| 17     | Methbutizide  |                         | -12.69                    |
| 18     | Vardenafil    | Anti-Impotence          | -16.22                    |
| 19     | Sildenafil    |                         | -16.04                    |
| 20     | Bromocriptine | Anti-parkinson          | -14.75                    |
| 21     | Ergoloid Mesylate |                  | -10.62                    |
| 22     | Zafirlukast   | Anti-Asthmatic          | -16.02                    |
| 23     | Nelfinavir    | Anti-HIV Agents         | -15.91                    |
| 24     | Delavirdine   |                         | -14.53                    |
| 25     | Gimeprivide   | Hypoglycemic            | -15.71                    |
| 26     | Gliquidone    |                         | -15.67                    |
| 27     | Tirofiban     | Anti-coagulant          | -11.32                    |
| 28     | Vindesine     | Anti-neoplastic         | -10.47                    |
| 29     | Goserelin     | Anti-neoplastic         | -99.85                    |
| 30     | Leuprolide    |                         | -24.89                    |
| 31     | Cereuletide   | Diagnostic              | -25.02                    |
| 32     | Epitifibatide | Anti-coagulant          | -10.64                    |

Table 1. Binding energies of argatroban and other 31 drug molecules with thrombin obtained from docking using Autodock4.0 program.
Hadvary, 1991). Prior to docking the bound ligand and water molecules were removed from the structure. The missing hydrogen atoms were added and charges on the molecule were kept to zero.

**Docking**

The Lamarckian genetic algorithm of Autodock4.0 software package was used (Morris, Huey, & Olson, 2008). Grid maps were prepared using the AutoGrid with $62 \times 82 \times 58$ points and grid spacing was set to .375. AMBER force field was used. The distant dependent dielectric constant was set to +80. Thrombin was first docked with argatroban, and the resulting structure was compared with the crystal structure of thrombin–argatroban complex. The docking parameters for the other molecules were standardized based on these results.

Docking parameters were modified from the default values as follows: number of individuals in the population (set to 300), maximum number of energy evaluations (set to 2,500,000), maximum number of generations (set to 27,000), and number of hybrid GA-LS runs (set to 100). The same receptor grid was used for the docking of all 50 ligands for the better comparison of the binding energy values. Flexible docking strategy was applied in which all the binding site residues and ligand were set flexible (Morris et al., 2008). For each drug–thrombin complexes, 100 independent docking poses were calculated and the lowest energy pose with acceptable geometry was selected for further analysis.

**In vitro thrombin inhibition assay**

Four compounds were then experimentally validated by using a fluorescence-based thrombin inhibition assay. These compounds are piperacillin, azlocillin, metolazone, and tirofiban. Piperacillin and azlocillin are anti-bacterial agents. Metolazone is an anti-hypertensive and diuretic agent, and tirofiban is an anti-coagulant. The three drugs piperacillin, azlocillin, and metolazone showed binding energies almost comparable to that of argatroban. Tirofiban showed comparatively low binding energy. No previous reports were available regarding the affinity of these compounds towards human α-thrombin.

*In vitro* fluorescence assay was used to confirm the thrombin inhibitory activity of the above compounds. Compounds were purchased from Sigma. The SensoLyte AFC Thrombin Assay Kit was purchased from AnaSpec. This kit contains a fluorogenic substrate with the $K_m$ value of 12 μM. Thrombin cleaves the substrate resulting in release of AFC (7-amido-4-trifluoromethylcoumarin) fluorophore. The assay run was carried out in a 96-well
black plate in a Spectromax M5e Microplate Reader using Xenon flash lamp as the light source. All kit components were thawed to room temperature before starting the experiments. The instrument was calibrated first by using the AFC fluorescence reference standard. The thrombin inhibitor NAPAP was used as the reference inhibitor instead of argatroban, as NAPAP was the default component of the kit. It is also a strong inhibitor of thrombin with $K_i$ value in nanomolar range (Bode, Turk, & Stürzebeche, 1990).

Piperacillin and azlocillin were dissolved in deionized water; and metolazone and tirofiban in 1% DMSO. The 10 μL of test compound and 40 μL of enzyme solution were added to each of the microplate test wells. Each well contains 1 nM thrombin. The concentration dependence of thrombin inhibition by the test compounds was obtained by adding different concentrations in the ratios 1:1, 1:2, 1:3, 1:4, and 1:5 with respect to that of thrombin concentration. There were five control wells also. A positive control containing the thrombin without test compound, an inhibitor control containing thrombin and inhibitor (NAPAP), a vehicle control containing thrombin and vehicle used in delivering test compound (e.g. DMSO), test compound control containing assay buffer and test compound. A test control well was also there to test whether the test compounds had strong auto fluorescence (and if so it would have given false results). The substrate control contained assay buffer. The total volume of each control was made up to 50 μL using assay buffer. The assay temperature was 20 °C and the plate had been pre-incubated for 10 min at the assay temperature.

For running the enzymatic reaction, 50 μL of thrombin substrate solution was added into each well. In order to ensure best accuracy, the substrate solution was equilibrated to the assay temperature. The reagents were mixed completely by shaking the plate gently for 30 s. The reaction was monitored using a microplate fluorometer setting at an excitation wavelength of 380 nm and emission wavelength of 500 nm. Measurements of the fluorescent intensity were started immediately after mixing the solution in every 5 min for a total time of 60 min.

**Results**

**In silico docking study**

The binding affinity of the selected drug molecules to human α-thrombin is described in terms of their binding energy, hydrogen bonding, and van der Waals interactions. The binding energies of these compounds are summarized

| Ligands   | Binding energy (kcal/mol) | No. of Bonds | Hydrogen bonding                  | No. of van der Waals’ interactions (≤4 Å) |
|-----------|---------------------------|--------------|-----------------------------------|------------------------------------------|
| Goserelin  | −99.8                     | 6            | Trp60D NE1, Lys60F NZ, Glu192 OE2, Ser195 OG, Gly216 N, Gly219 N | 142                                      |
| Leuprolide | −24.89                    | 4            | Lys60F NZ, Glu192 OE2, Trp215 NE1, Tyr60A OH, Glu97A O, Ser214 N, Gly216 N | 198                                      |
| Ceruletide | −25.02                    | 4            | Tyr94 OH, Gly216 N, His57 NE2, Gly216 O, His57 O, Tyr60A OH, Gly216 O | 149                                      |
| Piperacillin | −16.69                 | 2            | Gly216 N, Gly216 N, Asp189OD2, Gly193 N, Gly213 N, Gly216 N | 97                                       |
| Metolazone | −16.36                    | 2            | Try94 OH, Gly216 N, His57 O, Gly216 O, Asp189OD2, Gly193 N | 66                                       |
| Azlocillin | −16.35                    | 3            | His57 NE2, Gly216 N, Asp189OD2, Gly193 N, Gly213 N, Gly216 N | 152                                      |
| Vardenafil | −16.22                    | 1            | Gly219 O, Gly216 O, Gly213 N, Gly216 N | 101                                      |
| Argatroban | −16.07                    | 4            | Gly219 O, Gly216 O, Gly193 N, Gly213 N, Gly216 N | 99                                       |
The binding energy of argatroban is $-16.07$ kcal/mol. Superposition of the docked structure on the crystal structure showed that both position and orientation of the ligand in crystal and docked structures are very similar (Figure 1). Out of the 50 compounds used in this study 31 compounds have shown interactions with thrombin. The selection is based on two criteria: mode of binding and binding energy. Only the compounds that can bind to the active site of thrombin and the compounds having free energy of binding less than or equal to $-10$ kcal/mol ($\Delta G \leq -10$ kcal/mol) are selected. They include 4 peptides and 27 non-peptide compounds. The binding properties of compounds having comparable binding affinity, towards thrombin, as that of argatroban are listed in Table 2.

**In vitro thrombin inhibition assay**

The three drugs piperacillin, azlocillin, and metolazone showed binding energies almost comparable to that of argatroban. Tirofiban showed comparatively low binding energy. The mode of binding of piperacillin, azlocillin, and metolazone in the active site of thrombin are shown in Figure 2. These four compounds, piperacillin, azlocillin, metolazone, and tirofiban, were then experimentally validated by using a fluorescence-based thrombin inhibition assay. In vitro thrombin inhibition assay shows that the three compounds piperacillin, azlocillin, and metolazone have thrombin inhibitory activity, but tirofiban has no inhibitory activity.

The fluorescence reading from the substrate control well was used as the background fluorescence. This background reading was subtracted from the readings of the other wells containing substrate. All fluorescence readings were expressed in relative fluorescence units (RFU). For kinetic analysis, the RFU data were plotted as a function of time for each concentration of test compounds using the Origin 6.0 program. These RFU vs. time plots of piperacillin, azlocillin, and metolazone are shown in Figures S1, S2, and S3, respectively (in the Supplementary Material). The thrombin inhibitory activity of these compounds was qualitatively proved by the significant reduction in the fluorescence signal intensity when compared to that of the control. The initial reaction
velocity (Vo), in RFU/min, was obtained from the slope of the plots. As concentration of inhibitor increased, the rate of formation of the fluorescent product decreased. Hence, the rate of reaction decreased with the increase in the concentration of the test compounds.

The best fit Morrison Ki values were calculated using GraphPad Prism software (Robort, 2000) and the graphs are shown in Figure 3. The Ki values obtained for piperacillin, azlocillin, and metolazone are .55, .95, and .62 nM, respectively. The IC50 values were calculated using the formula $K_i = IC_{50}/(S/K_m + 1)$, where, $S$ is the substrate concentration. The IC50 values for piperacillin, azlocillin, and metolazone are 1.7, 2.9, and 1.92 nM, respectively.

Overall, the binding energies, $K_i$ and IC50 values have shown that the three drugs piperacillin, azlocillin, and metolazone have interactions with thrombin and they may be inhibitors of thrombin. Since, these compounds have been using clinically for a long time, further in vivo studies are needed to fully understand the effect on thrombin.

**Discussion**

Among the non-peptide compounds selected for docking studies azlocillin, piperacillin, metolazone, vardenafil, cefonicid, and sildenafil showed strong binding interactions with thrombin. All these compounds bind to the active site of thrombin in a binding mode similar to that of argatroban. The three peptide derivatives goserelin, leuprolide, and ceruletide also possess good binding affinity to thrombin. Four non-peptide compounds azlocillin, piperacillin, metolazone, and tirofiban were selected for experimental validation of their thrombin inhibition. The fluorescence-based inhibition assay confirmed that the three compounds azlocillin, piperacillin, and metolazone have thrombin inhibitory activity. These drugs have van der Waals and hydrogen bonding interactions with the active site residues of thrombin. But, they do not have a long carboxyl tail which can bind to Asp189 of thrombin. However, highly potent and selective thrombin inhibitors without long carboxyl tail were reported with modifications of the P1 and P3 residues (St-Denis et al., 2002). By combining the results of docking studies and the enzyme inhibition assay, these compounds can be proposed as inhibitor analogs of thrombin.

Piperacillin, azlocillin, and metolazone have also been docked with their known targets. The known target of azlocillin and piperacillin is specific penicillin binding proteins (PBP) located inside the bacterial cell wall (Chen, Ji, & Chen, 2002; Williamson, Hakenbeck, & Tomasz, 1980). The binding energy of azlocillin with PBP-1A is –10.03 kcal/mol. Similarly, the binding...
energy of piperacillin with its known target PBP-1B is $-13.27 \text{kcal/mol}$. Metolazone also was docked with its known target Na–Cl symporter (Chen et al., 2002). The binding energy obtained is $-12.87 \text{kcal/mol}$. So, the binding affinities of these drugs towards thrombin may be higher than that to their known targets.

It has been observed that the anticoagulant effect of warfarin increased, when used along with piperacillin, azlocillin, and metolazone drugs (Bayes, Rabasseda, & Prous, 2002). Warfarin acts by inhibiting the synthesis of vitamin-K dependent coagulation factors. This increase in anticoagulation might be due to the inhibition of thrombin by these drugs. Also, a clinical study had proven that piperacillin cause prolongation of the bleeding time (Gentry, Jemsek, & Natelson, 1981).

It has been reported that the PDE5 inhibitors such as vardenafil and sildenafil improve coronary patency in a canine model of platelet-mediated coronary artery thrombosis (Lewis et al., 2006; Schmidt et al., 2001). It is believed to be achieved via the inhibition of platelet aggregation. But, the docking studies explain that the PDE5 inhibitors vardenafil and sildenafil can also perform as thrombin inhibitors and such a way delimit the thrombus formation.

The peptide drugs goserelin and leuprolide may inhibit fibrinolytic system by decreasing tissue-type plasminogen activator (t-PA) levels (Agirbasli et al., 2009). The synthesis of t-PA and plasminogen activator inhibitor 1 (PAI-1) is known to be correlated with human alpha thrombin. Besides its procoagulant activity, thrombin has been shown to stimulate cell proliferation and to regulate the fibrinolytic pathway. Thrombin (0–2.5 U/ml) increased the production of t-PA and PAI-1 to two- to three-fold in a time- and dose-dependent manner (Villamediana et al., 1990). The inhibition of thrombin by GnRH agonists may decrease the production of t-PA levels. Hence, the effect of GnRH agonists on plasma fibrinolytic balance may depend on their interaction with thrombin. These results lead to the observation that the peptide drugs goserelin and leuprolide inhibit thrombin activity.

**Conclusion**

Adverse side effects of drugs are often caused by the interaction of drug molecules to sites other than the intended targets. Docking as well as *in vitro* inhibition assay studies showed that the three commonly used drugs piperacillin (anti-bacterial), azlocillin (anti-bacterial), and metolazone (anti-hypertensive and diuretic) have thrombin inhibitory activity almost similar to that of a well-known thrombin inhibitor argatroban. The $K_i$ values of piperacillin, azlocillin, and metolazone with thrombin are .55, .95, and .62 nM, respectively. The IC$_{50}$ values of piperacillin, azlocillin, and metolazone with thrombin are 1.7, 2.9, and 1.92 nM, respectively. This thrombin inhibitory activity might be a reason for the observed side effects of these drugs related to blood coagulation and other thrombin activities. Furthermore, these compounds (drugs) may also be used as anti-coagulants as such or with structural modifications.

**Supplementary material**

The supplementary material for this paper is available online at http://dx.doi.org/10.1080/07391102.2014.923329.

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