Cassia tora (Senna tora), a member of Leguminosae (subfamily Caesalpinoideae), is found as weed throughout the India. The herb is reportedly beneficial in skin diseases, possesses anthelmintic properties and at times recommended in liver complaints and gastrointestinal disorders.

Proteinaceous protease inhibitors are important defense molecules expressed in various plants, animals and microbes. The evidence of protease inhibitors’ involvement in plant defense was demonstrated as early in 1947 by Mickel and Standish, when they observed that the larvae of certain insects were unable to develop normally on soybean products. Subsequently, the trypsin inhibitors present in soybean were shown to be toxic to the larvae of flour beetle, tribolium confusum.

These findings were further substantiated by expression of the cowpea trypsin inhibitor gene in tobacco, which increased its resistance against herbivorous insects. The plant protease inhibitors are generally small proteins, which regulate significant physiological processes, and are also induced upon attack by insects or pathogens. Protease inhibitors exhibit a peculiar property of forming complexes with proteolytic enzymes and promote inhibition of their activity by competing for the catalytic site. Majority of proteinase inhibitors studied in plant kingdom originate from three main families namely leguminosae, solanaceae and gramineae.
Experimental:

To measure the binding affinity and thermodynamic parameters, Iso thermal Titration Calorimetry (ITC) experiments were carried out. The purified protease inhibitor was analyzed for its Themodynamical parameters with Bovine Trypsin.

Thermodynamics of CVTI-Trypsin interaction (Isothermal Titration Calorimetry)

The Nano ITC instrument (TA Instruments, Lindon, Utah, USA) was used to determine the enthalpy and entropy changes resulting from the titration of CVTI (20 µM) with Bovine trypsin (200 µM) in titration buffer 50 mM Tris pH 7.8. All the solutions used were degassed for about 60 Minutes with 270 rpm under176 Hg vacuum. 300 µl buffer was injected in both the cells and the base line correction was carried out. To confirm the absence of dilution factor, various concentrations of buffer to protein (trypsin) and ligand (Cassia tora trypsin inhibitor) to buffer experiments were performed. A quantity of 2.02 µl of Cassia tora trypsin inhibitor was injected sequentially into a 170 µl titration cell initially containing bovine trypsin. The temperature of solutions in the titration cell was 37 °C. Between all successive injections, 250 seconds of time interval was maintained. A rotating Hamilton micro-syringe (50 µl) ensure homogeneous phase by constant stirring of the solution at a speed of 200 rpm. The heat of dilution from the blank titration of ‘buffer to buffer’ was measured and these heats of dilution were subtracted from the raw data. The results were analyzed using Nano ITC (7.0) software.
The variation of Gibbs free energy of mixing was calculated using the well-known relationship:

\[ \Delta G^{\circ}_b = -RT \ln K_b, \]

Changes in entropy was calculated using:

\[ \Delta G^{\circ}_b = \Delta H_b - T \Delta S_b. \]

Data acquisition and analyses were performed using Nano Analyze. The “Independent Binding Model” module was used in the “data fitting” options for data analysis.

The interaction of bovine pancreatic trypsin with Cassia tora trypsin inhibitor was studied with help of isothermal calorimeter at 37 °C. When ITC experiments were carried out with Cassia tora (0.0135 mM) and Trypsin (0.235 mM) for 24 injections, the binding was evidenced as enthalpy driven and the binding constant (K_a) was found (Table 5.1) to be \(5.13 \times 10^5\) (1/M) (Ladbury and Chowdhry, 1996). ITC results (Fig.5.1) also clearly show an endothermic mode of binding where heat exchange decreases for successive injections until saturation.

**Result and Discussion:**

Nano analyzer module was used to determine change in enthalpy (\(\Delta H = 77.11\) K.Joule/mol), entropy (\(\Delta S = 359.9\) Joule/Mole. K) and from the relationship equation from (\(\Delta G = \Delta H - T \Delta S\)), change in Gibb’s free energy (\(\Delta G = -34.459\) K. Joule/mol. K) was calculated. Binding stoichiometry (n) was determined to be 1.486 indicating one to one binding mode of Cassia tor trypsin inhibitor against the Trypsin.
Fig. 5.1: Endothermic Trypsin-Cassia tora trypsin inhibitor binding as evidenced from Thermogram of ITC experiments.
Table 5.1: Thermodynamic parameters determined for Trypsin-Cassia tora binding by ITC experiment.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Association Constant ($K_a$) (1/M)</td>
<td>$6.512 \times 10^5$</td>
</tr>
<tr>
<td>Change in Enthalpy ($\Delta H$) (K. Joule/mol)</td>
<td>77.11</td>
</tr>
<tr>
<td>Stoichiometry (N)</td>
<td>1.486</td>
</tr>
<tr>
<td>Dissociation Constant ($K_d$) (M)</td>
<td>$1.536 \times 10^{-6}$</td>
</tr>
<tr>
<td>Change in Entropy ($\Delta S$) (Joule/mol. K)</td>
<td>359.9</td>
</tr>
<tr>
<td>Gibb’s Free Energy ($\Delta G$) (K. Joule/mol. K)</td>
<td>-34.459</td>
</tr>
</tbody>
</table>

With the Stoichiometry (N) of 1.4, $\Delta G$ was measured as -34.459 kJ/mol shows a better binding. The association constant values of $6.512 \times 10^5$ and dissociation constant $1.536 \times 10^{-6}$ suggest the tight binding of cassia tora inhibitor with trypsin. Since its binding with trypsin is strong, protease inhibition action can be projected as the major factor for the pesticidal activity of the Cassia tora seeds.
A.1. INTRODUCTION

CA (carbonic anhydrase; EC 4.2.1.1) is a Zn(II)-dependent enzyme that catalyses the reversible hydration of carbon dioxide into hydrogen carbonate and a proton, and plays an important role in physiological anion-exchange processes and fluid balance (Maren, et al., 1988). This enzyme has been drug target for cancer (Ho, Y.T. et al., 2003), (Abbate et al., 2004). CA isoforms can also catalyse a series of other hydration and dehydration reactions. Atleast 14 isoforms of CA have been identified in vertebrates with different physiological and pathological roles, and they can be localized in the cytosol (CA I–III and VII) or mitochondria (CA V), whereas others are secreted (CA VI) or are membrane-bound and have extracellular active-site domains (CA IV, IX, XII and XIV)

There is convincing evidence for the over expression of CA isoforms IX and XII in cancer and this is supposed to promote growth of the tumour by acidification of the extracellular environment. Expression is up-regulated under hypoxic conditions, which is common in solid tumours. Among 144,937 cases detected with breast cancer, 70,218 women died, roughly it can be said that for every 2 women newly diagnosed with breast cancer, and one lady is dying of it.
A.1.2 MATERIAL AND METHODS

Preparation of protein Structures

Experimentally determined crystal structures of Human carbonic anhydrase (PDB.ID: 3HLJ) was retrieved from the Protein Data Bank. The small molecules and all the water molecules present were removed from the co-ordinates file. The resultant PDB co-ordinates were taken for docking studies.

Preparation of Ligand

The crystal structures of various sulfonamide derivatives determined using X-ray crystallography were taken as inhibitors against the receptors. i.e drug targets.

Determination of active site residues

As structures of the enzyme Human carbonic anhydrase is crystallographically determined, the active sites information for the above proteins were retrieved from the literature published.

Docking

Docking was carried out using Schrödinger’s Glide. Glide (Grid based Ligand Docking with Energetics) (Halgren et al., 2004) uses a hierarchical series of filters to search for possible locations of the ligand in the active site region of the receptor. The prepared protein is docked using Induced fit protocol. Initially softened potential filter (van der Waals radii scaling) was used to retain 20 geometrically possible poses per ligand and then further evaluated for the coulombic-vdW scores. The structures whose coulombic-vdW scores less than 100 and H-bond score less than -0.005 were retained.

The retained structures enter one round of prime side chain prediction for each of the protein-ligand complex with the given distance of 5Å. Poses that pass these initial screens
enter the final stage of the algorithm, which involves evaluation and minimization of a grid approximation to the OPLS-AA non-bonded ligand–receptor interaction energy. Each of the protein-ligand complexes was specified with the lowest energy (30Kcal/mol) and then the ligand was docked with the receptor molecule. Final scoring is then carried out on the energy-minimized poses. Schrödinger’s proprietary GLIDE Score multi ligand scoring function is used to score the poses.

**A.1.3 RESULTS AND DISCUSSION**

The three dimensional structures of the enzyme Human carbonic anhydrase was retrieved from the Protein Data Bank were shown in Fig.6.1 along with its active site residues which have been selected as the receptor for the binding of the ligand molecules.

Sulfonamide derivatives which have been solved in our lab were subjected to docking analysis with the defined active site using Schrodinger’s GLIDE.

![](image.png)

**A.1 Three Dimensional structures of the enzyme Human Carbonic Anhydrase**

**A. 1.4 RESULTS AND DISCUSSION:**

In assessment with these results, the ligands, which show interaction with the active site residues of Human carbonic anhydrase were considered and those ligands that do not form interaction with the defined active site residues were omitted and their corresponding
score are tabulated (Table 1a ). The best interaction obtained with the active site residues are discussed hereunder.

**Molecular docking studies of the Sulfonamide compounds with Human Carbonic Anhydrase as anti-cancer target.**

The results of Induced Fit docking for Sulfonamides as anti-cancer target (PDB id: 3HLJ) are presented in Table 1a, showing hydrogen bonding interactions, docking score and glide energy for the Sulfo 6, Topotecan (Reference drug) and co-crystallised ligand in the best pose. Induced Fit docking studies reveal that Sulfo 6 bind well at the active site and have hydrogen bonding cum hydrophobic interactions with the active site.

The Docking Score and the Glide energy of Sulfo 6 is -5.72 and -54.00 Kcal/mol, and Topotecan (reference drug) is -5.52 and -51.6 where as the co-crystallised ligand (3-Methylthiobenzimidazo [1,2-c][1,2,3] thiadiazol-7-sulfonamide) exhibits a glide score of -4.6 and glide energy of -49.82 kcal/mol. The compound Sulfo 6 had hydrogen bonding interactions with residues like Asn 67, Asn 62, at a distance of 2.7Å, 2.9Å respectively. The reference drug Topotecan had hydrogen bonded with Thr 200 at a distance of 1.9Å, while as the cocystal interacts had hydrogen bonding interactions with residues with Thr 199, Thr 200 at a distance of 3.0Å and 2.7Å respectively. Interactions of Sulfonamide derivatives can be depicted in fig. 1c
Table 1a: Induced fit Docking results of Sulfonamide against Human Carbonic Anhydrase (PDB ID: 3HLJ)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Docking Score</th>
<th>Glide energy</th>
<th>Hydrogen Bonding Interaction</th>
<th>Distance</th>
<th>Hydrophobic residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfo 3</td>
<td>-3.25</td>
<td>-38.86</td>
<td>(Asn 62) N-H…O</td>
<td>2.9</td>
<td>Phe 131, Phe 20, His 94, His 64, Leu 198, Tyr 7</td>
</tr>
<tr>
<td>Sulfo 5</td>
<td>-3.11</td>
<td>-33.80</td>
<td>O-H…O(Asn 67) O-H…N(Asn 62)</td>
<td>2.7 3.0</td>
<td>Phe 131, Val 143, Leu 141, Val 143, Leu 203</td>
</tr>
<tr>
<td>Sulfo 6</td>
<td>-5.72</td>
<td>-54.00</td>
<td>Asn 67(N-H…O) Asn62 N-H…O</td>
<td>2.7 2.9</td>
<td>Phe 131, Trp 5, Val 121, Tyr 7</td>
</tr>
<tr>
<td>Topotecan</td>
<td>-5.52</td>
<td>-51.06</td>
<td>(Thr200)O-H…O</td>
<td>1.9</td>
<td>Ile 91, Leu 141, Val 143, Val121, Leu 203</td>
</tr>
<tr>
<td>Cocrystal</td>
<td>-4.6</td>
<td>-49.82</td>
<td>Thr199N-H…O N-H…OThr 200</td>
<td>3.0 2.7</td>
<td>Phe 131, Ile 91, Leu 141, Val 143, Val121, Leu 203</td>
</tr>
</tbody>
</table>
Fig. 1c Interactions of best Sulfonamide derivatives against Human carbonic anhydrase
A.2 INTRODUCTION

HIV-1 Protease:

HIV-1 protease being a retroviral aspartyl protease is important for the lifecycle of HIV (Davies et al., 1990). HIV-1 protease (HIV-1PR) is probably the most extensively investigated enzyme for therapeutic intervention in the short history of structure-based drug design. The development of HIV protease inhibitors is regarded as one of the most successful examples of structure-based drug design efforts to date. This enzyme is a critical component in the replicative cycle of the human immunodeficiency virus (HIV) that cleaves the polyproteins transcribed from the gag and pol genes into enzymes and structural proteins essential for the assembly and maturation of infectious virions (Brik et al., 2003), (Henderson et al., 1988), (Loeb et al., 1989).

The finding that inactivation of this viral encoded aspartyl protease produces a progeny of virions that are immature and noninfectious, elicited intense efforts in the development of specific and potent inhibitors targeted against this enzyme, as a novel therapy for AIDS. Since then, HIV-1PR has become a prime target for therapeutic intervention in this disease. Moreover, availability of several X-ray crystal structures of HIV-1 protease has guided structure-based searches for specific inhibitors. We have taken the Pyrrolidine derivatives to target proteases.
The ability of the virus to circumvent the inhibitors via mutation clearly signifies the need for improved therapeutic agents. To facilitate the design of more specific and potent HIV-1 protease inhibitors, we must improve our understanding of the principles of molecular recognition for this enzyme. The dynamic aspects of ligand-enzyme interactions may indicate an important role of conformational variability in HIV-1 PR inhibitor/drug design.

**Endothiapepsin:**

Endothiapepsin belongs to the family of pepsin-like aspartic proteases. Pepsin-like aspartic proteases are active as monomers, which comprise two similar domains, each of which contributes an aspartic acid residue to the catalytic dyad (D35 and D219 for endothiapepsin) that hydrolyzes the substrate’s peptide bond utilizing a catalytic water molecule.

Aspartic proteases are a class of enzymes widely found in fungi, plants, vertebrates, as well as, e.g., in HIV retro-viruses. These enzymes are involved in diseases, such as AIDS, malaria, fungal infections etc. In HIV, the aspartic protease has an essential role in maturation of the HIV virus, making it a validated target for the treatment of AIDS (Cooper, 2002). In eukaryotes, the aspartic protease renin plays a role in hypertensive action, cathepsin D in tumorigenesis, plasmepsins in the degradation of human hemoglobin, which is required by Plasmodium falciparum, the causative agent of malaria and pepsin in the hydrolysis of acid-denatured proteins. Therefore, the enzymes of this class of aspartic proteases are considered as a rich source of therapeutic targets. As endothiapepsin model enzyme has been used to elucidate the mechanism of aspartic proteases (Coates et al., 2001; Coates et al., 2008), we have chosen Pyrrolidines for the design of a new series of inhibitors of endothiapepsin.
A.2.2 MATERIAL AND METHODS

Preparation of protein Structures

Experimentally determined crystal structures of HIV-1 protease (PDB.ID: 2PQZ) and Endothiapepsin (PDB ID: 3Q6Y) was retrieved from the Protein Data Bank. The small molecules and all the water molecules present were removed from the co-ordinates file. The resultant PDB co-ordinates were taken for docking studies.

Preparation of Ligand

The crystal structures of various Pyrrolidine derivatives determined using X-ray crystallography was taken as inhibitors against the receptors. i.e drug targets.

Determination of active site residues

As structures of the enzyme Human HIV-1 protease and Endothiapepsin has been crystallographically determined, the active sites information for the above proteins were retrieved from the literature published.

Fig.6.2(a) and (b) Three Dimensional structures of the enzyme HIV-1 PROTEASE and Endothiapepsin
A.2.3 RESULTS AND DISCUSSION

The three dimensional structures of the enzyme HIV-1 Protease and Endothiapepsin were retrieved from the Protein Data Bank were shown in Fig.6.2 (a) and (b) respectively along with its active site residues which have been selected as the receptor for the binding of the ligand molecules. The Pyrrolidine derivatives which have been solved in our lab were subjected to docking analysis with the defined active site using Schrodinger’s GLIDE.

In assessment with these results, the ligands, which show interaction with the active site residues of HIV-1 Proteases and Endothiapepsins were considered and those ligands that do not form interaction with the defined active site residues were omitted and their corresponding score are tabulated (Table 2b). The best interaction obtained with the active site residues are discussed hereunder.

Molecular docking studies of the Pyrrolidine compounds with HIV-1 Proteases and Endothiapepsin as anti-HIV targets.

The cocrystal ligands were redocked with the target Hiv-1 protease (PDB id: 2PQZ) and Endothiapepsins (PDB id: 3Q6Y). The pyrrolidine compounds 1 and 2 were too put for Induced fit Docking and finally, the best docked receptor complex were taken into consideration. Results of the best induced fit docking of Pyrrolidines as anti-HIV-1 protease targets are presented in table 2b, showing hydrogen bonding interaction, docking score and glide energy for the pyrrolidine.
Table 2b: Induced fit Docking results of Pyrrolidine against HIV-1 Proteases (PDB ID: 2PQZ)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Docking Score</th>
<th>Glide energy</th>
<th>Hydrogen Bonding Interaction</th>
<th>Distance</th>
<th>Hydrophobic residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrrolidine 1</td>
<td>-7.08</td>
<td>-51.62</td>
<td>O-H…O(Asp 25)</td>
<td>3.0</td>
<td>Leu 23, Ala 28, Val 32, Ile 84, Ile 47, Pro 81</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>O-H…O(Asp 25)</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ile 50(N-H…O)</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>Pyrrolidine 2</td>
<td>-3.54</td>
<td>-28.31</td>
<td>Ile 50(N-H…O)</td>
<td>2.7</td>
<td>Val 32, Asp 29</td>
</tr>
<tr>
<td>Cocrystal</td>
<td>-5.08</td>
<td>-47.08</td>
<td>Ile 50(N-H…O)</td>
<td>2.7</td>
<td>Asp 29, Asp 30, Arg 8</td>
</tr>
<tr>
<td>Abacavir (Reference Drug)</td>
<td>-5.76</td>
<td>-41.38</td>
<td>Ile 50(N-H…O)</td>
<td>2.8</td>
<td>Arg 8, Asp 29, Asp 30, Ile 50, The 80,</td>
</tr>
</tbody>
</table>

Pyrrolidine 1  

Abacavir (Reference Drug)  

Co-cystal
Interaction Diagrams of Pyrrolidine, Cocrytal and Reference drug with HIV-1 Protease

The docking score and glide energy of Pyrrolidine 1 with HIV-1 protease target is -7.08 and -51.62 kcal/mol, and abacavir (Reference drug) is -5.76 and -41.38 where as the co-crystallised ligand N,N’-(3S,4S)-Pyrrolidine-3,4-diylbis(N-Benzylbenzenesulfonamide) exhibits a docking score of -5.76 and glide energy of -41.38 kcal/mol.

Table 2b: Induced fit Docking results of Pyrrolidine with HIV-1 Proteases (PDB ID:3Q6Y)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Docking Score</th>
<th>Glide energy</th>
<th>Hydrogen Bonding Interaction</th>
<th>Distance</th>
<th>Hydrophobic residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrrolidine 1</td>
<td>-6.92</td>
<td>-51.97</td>
<td>N-H…O(Asp 35) O-H…O(Asp 35) O-H…O(Asp 219)</td>
<td>2.9</td>
<td>Trp32, Ser38, Phe 194, Ile217, Ile 300, Ile302</td>
</tr>
<tr>
<td>Abasavir</td>
<td>-5.68</td>
<td>-50.02</td>
<td>(Asp 35) O-H…N (Asp 219) O-H…N O-H…O(Ile 300)</td>
<td>2.8</td>
<td>Phe 194, Asp 219, Ash 219, Asp 35, Leu 133</td>
</tr>
<tr>
<td>Pyrrolidine 2</td>
<td>-5.94</td>
<td>-49.18</td>
<td>_______</td>
<td>______</td>
<td>_______</td>
</tr>
<tr>
<td>Cocryystal</td>
<td>-6.05</td>
<td>-48.94</td>
<td>O-H---O(Thr 222 O-H…O(Asp 219) (Asp 35) O-H…O (Trp 42) N-H…O</td>
<td>3.5</td>
<td>Asp 219, Leu 133, Ile 300</td>
</tr>
</tbody>
</table>
Pymol Interaction Diagrams of Pyrrolidine, Abacavir and Co-crystal with Endothiapepsin Target.
A. 4. CONCLUSION

Docking analysis of all the Sulfonamide derivatives with the protein targets Human Carbonic anhydrase and Pyrrolidine derivatives against HIV-1 Proteases brought important interactions operating at the molecular level. Hence targeting the above proteins is necessary for the design of broad spectrum of antibiotics. The docking studies with the structure of Sulfonamide derivatives determined from our lab shows that the compound sulfo 6 binded very well at the active site of Human carbonic anhydrase, so showing its anti-cancer potential and on the other hand the Pyrrolidine 1 was tight binder to active site of HIV-1 proteases and Endothiapepsins. In order to study the binding mode and enzyme-inhibitor interaction with the active site residues of the target proteins, the well established inhibitor like Topotecan and abacavir are included in our docking studies for Carbonic anhydrases and HIV-1 Proteases respectively. The docking studies with the Sulfonamide derivatives showed that our derivatives also established a similar way of binding with the target protein which is explained by their comparable binding energy and interaction with the active site residues of the enzymes. Thus in-short, this study opens the window optimizing the Sulfo 6 as anti-cancer potential and Pyrrolidine 1 as anti-HIV agent.