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ORIGINAL PAPER

Structural analysis of secretory phospholipase A_2 from *Clonorchis sinensis*: therapeutic implications for hepatic fibrosis

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Abstract Hepatic fibrosis is a common complication of the infection by the parasite, *Clonorchis sinensis*. There is a high incidence of this disease in the Asian countries with an increased risk of conversion to cancer. A secretory phospholipase A_2 (PLA_2) enzyme from the parasite is implicated in the pathology. This is an attractive drug target in the light of extensive structural characterization of this class of enzyme. In this study, the structure of the enzyme was modeled based on its sequence homology to the group III bee venom PLA_2. On analysis, the overall structure essentially is comprised of three helices, two sets of β-wings and an elongated C-terminal extension. The structure is stabilized by four disulfide bonds. The structure is comprised of a calcium binding loop, active site and a substrate binding hydrophobic channel. The active site of the enzyme shows the classical features of PLA_2 with the participation of the three residues: histidine-aspartic acid-tyrosine in hydrogen bond formation. This is an interesting variation from the house keeping group III PLA_2 enzyme of human which has a histidine-aspartic acid and phenylalanine arrangement at the active site. This difference is therefore an important structural parameter that can be exploited to design specific inhibitor molecules against the pathogen PLA_2. Likewise, there are certain unique structural features in the hydrophobic channel and the putative membrane binding surface of the PLA_2 from *Clonorchis sinensis* that not only help understand the mechanism of action but also provide knowledge for a targeted therapy of liver fibrosis caused by the parasite.

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Keywords *Clonorchis sinensis* · Drug target · Group III phospholipase A_2 · Hepatic fibrosis

Abbreviations

PLA_2	Phospholipase A_2
ExPASy	Expert protein analysis system
PROCHECK	Protein structure check
CHARMM	Chemistry at HARvard Macromolecular Mechanics

Introduction

Clonorchis sinensis, the Chinese liver fluke, is a parasite in the phylum *Platyhelminthes,* class *Trematoda*. The parasites live in the liver of humans and are the third most prevalent worm parasite in the world. Though endemic in southern Korea, China (including Taiwan), Japan, northern Vietnam and the far eastern part of Russia, they are known to occur in all parts of the world with Asian immigrants from endemic areas [1]. The infection causes hepatic stellate cell activation and causes hepatic fibrosis [2]. Clonorchiasis induces biliary epithelial hyperplasia and metaplasia which in turn could lead to cholangiocarcinoma [3].

Liver fibrosis is one of the complications of clonorchiasis and requires a targeted drug therapy [4]. There have been several attempts to identify potential drug targets by genomic [5] and proteomic approach [6]. Recently, a secretory protein from the parasite *Clonorchis sinensis* has been established to be the causative agent of hepatic fibrosis and is being advocated as a potential drug target [7]. It has been shown that the protein binds to LX-2 cell membranes and causes an up regulation of collagen. This secretory protein is identified as a group III PLA_2 [8]. Group III PLA_2 enzymes

have been known to be present in bees [9] and scorpions [10, 11] as a constituent of the venom and found in humans for house keeping function [12]. Structural characterization of group III PLA_2 has resulted in discovery of potential drug molecules [13]. We present in this report, the detailed model structure of the secretory group III PLA_2 enzyme from the parasite *Clonorchis sinensis*. The study provides details of the structural parameters of the protein that is essential in the design of potent inhibitory molecules. The comparative analysis of the target with the human enzyme is particularly important for the development of targeted therapy for liver fibrosis caused by the parasite.

Materials and methods

Sequence analysis

Homology search for the *Clonorchis sinensis* PLA_2 (*Cs*IIIPLA$_2$) enzyme was carried out using BLAST search tool at ExPASy proteomics server (http://expasy.org/). The homologous protein sequences of the group whose three dimensional structures are known were aligned with *Cs*IIIPLA$_2$ using ClustalW [14]. In another experiment, the sequence variations at the positions of Arg82 and Phe91 of A^{80}TRVGNLYFNVFK^{92} of *Clonorchis sinensis* PLA_2 were analyzed by aligning it with other sequences in the family.

Modeling studies

Homology models of the *Cs*IIIPLA$_2$ was done based on the crystal structure of bee venom PLA_2 (PDB ID: 1POC), the only available structure in the group III PLA_2, using Geno 3D [15], SWISS-MODEL [16], ESyPred3D [17], PHYRE [18] and CPHmodel [19] softwares. The coordinates obtained were visualized using the program PyMOL [20] available at http://www.pymol.org/. The analysis of the conformational correctness and reliability was carried out using PROCHECK [21]. The calcium coordinate was taken from the template structure and fitted into the best validated model. Hydrogen atoms were added and the model was minimized in the presence of calcium keeping the backbone of the model fixed using steepest descent method followed by conjugate gradient method in the presence of distance dependent dielectric implicit solvent model with the convergence criteria of$\leq$0.05 kcal mol^{-1} Å^{-1} using CHARMm (version c32b1) forcefield [22, 23] in Discovery Studio (DS) 1.7 (Accelrys Software Inc., San Diego) [24]. This model was then taken for a detailed structural analysis.

Results and discussion

Sequence analysis

The sequence of *Cs*IIIPLA$_2$ was used for a homology search and aligned with the group III PLA_2 sequences whose three dimensional structures were known. This primary structure analysis was carried out to provide information regarding the residue content and positioning of the residues. The sequence alignment of *Cs*IIIPLA$_2$ sequences with the other two proteins is shown in Fig. 1. The enzymatic domain of CsIIIPLA$_2$ has a sequence identity of 46% and 40% with the enzymatic domains of human group III PLA_2 (hIIIPLA$_2$) [25] and bee venom PLA_2 respectively [9]. All three sequences have varying stretches of sequence preceding the enzymatic PLA_2 domain and therefore the N-terminal extensions have very low sequence identity amongst themselves. In the following discussion, the enzymatic domain will henceforth be referred to by the term PLA_2. The calcium binding motif is present at the N-terminal region with a consensus sequence of P^5GTLWCGXG^{13} (X is any amino acid) and the calcium binding residues Trp9, Gly11 and Gly13 are conserved in all three PLA_2 sequences. The catalytic residues His35 and Asp65 forming the classical 'dyad' are conserved. However, the catalytic tyrosine of the bee venom PLA_2 is replaced by phenylalanine at position 87 in the hIIIPLA$_2$. This replacement of tyrosine with phenylalanine is a significant variation. Nine of the ten cysteines in the PLA_2 domain are conserved. There are three potential N-glycosylation sites in *Cs*IIIPLA$_2$ and hIIIPLA$_2$ sequences and one in the bee venom PLA_2. The C-terminal extension of the *Cs*IIIPLA$_2$, after the tenth cysteine, has no sequence identity with the other two sequences. It may be noted that the enzymatic domain of *Cs*IIIPLA$_2$ without the C-terminal extension has a shorter sequence (95 residues) compared to the group I (119 residues) [26] and group II (121 residues) [27] PLA_2s. A C-terminal extension deleted mutant of group IIFPLA$_2$ had enzyme activity, membrane binding properties and monolayer preparation that was comparable with that of the wild type [28]. The conservation of all functions in a smaller sequence implies a more compact three dimensional structure. This, therefore, suggests that the group III enzymes have evolved with more efficient machinery at the genomic and proteomic level.

Structural analysis of the CsIIIPLA$_2$

The conformation of the enzymatic subunit of *Cs*IIIPLA$_2$ was modeled based on the crystal structure of bee venom PLA_2 (1POC), the only experimental structure in group III PLA_2. The structure model statistics establish the structural and biological plausibility of the model. The model with the tightest geometrical restraints on the C_α positions was

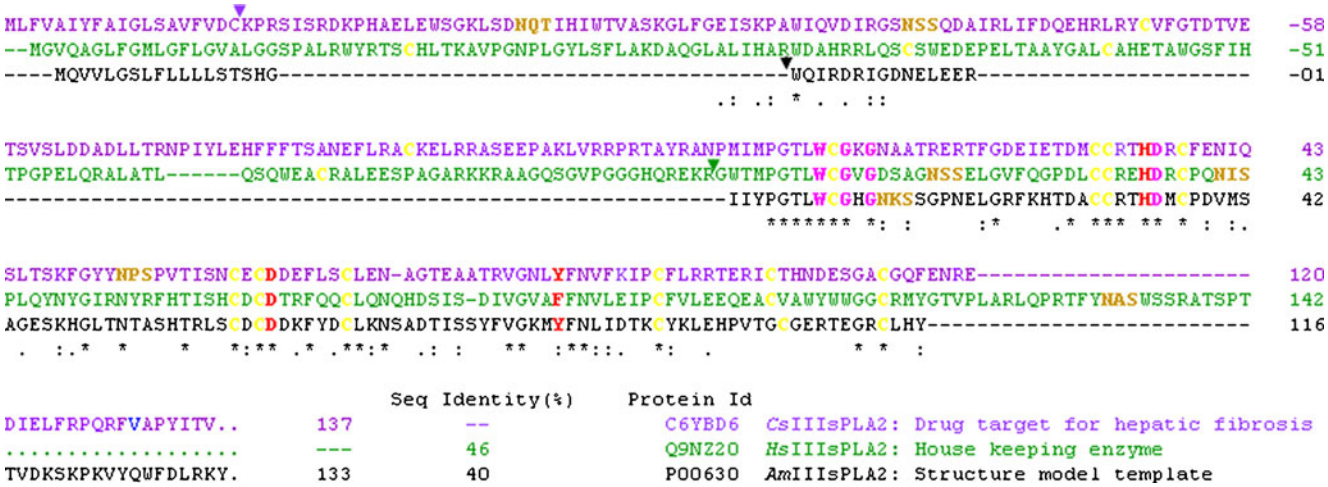

Fig. 1 Amino acid sequence homology of *Cs*IIIPLA$_2$. The sequence of *Clorenchis sinensis* PLA$_2$ is shown in purple, the sequence of human group III PLA$_2$ is shown in green and the sequence of bee venom PLA$_2$ is shown in black. The active site residues are shown in bold red. The calcium binding residues, cysteines and potential glycosylation are shown in bold pink, yellow and brown respectively. A few dashes have been inserted to enhance similarities. The symbol "*" indicates identical residues, ":" indicates conserved substitution and "." indicates semi-conserved substitution. Residue numbers indicated at the end of the lines are in accordance with the human group III PLA$_2$. Filled triangles indicate the signal sequence cleavage. The percentage homology with the *Cs*IIIPLA$_2$, the protein Swiss Prot identity number and usage in this work are given at the end of the respective sequences

obtained by the SWISS-MODEL software. Ramachandran plot for the model shows 99.1% of the residues in either the core region or allowed region and remaining 0.9% of the residues in the generously allowed region with no residue in the disallowed region. The averaged 3D-1D score is 76.2 as compared to the 97.8 of the template. This is understandable because of the relatively higher percentage of hydrophobic residues on the *Cs*IIIPLA$_2$ surface exposed to the solvent as compared to the template and is indicative of its membrane binding function [7]. The backbone r.m.s.d of 0.7 Å between the model and the template also establishes the correctness of the model conformations. The overall structure of *Cs*IIIPLA$_2$ comprises a N-terminal calcium binding loop, three helices and two sets of β-wings. The significant deviations of the CsIIIPLA$_2$ structure from other characterized secretory groupI/II PLA$_2$s include: a short N-terminus preceding the calcium binding loop, the presence of a second β-wing and a long C-terminal extension. The conformation of the C-terminal extension which includes the second β-wing is reminiscent of the structure of the non-enzymatic subunit of the heterodimeric scorpion sPLA$_2$ [29]. The last 16 residues in this C-terminal extension show a loop conformation which is stabilized in *Cs*PLA$_2$ by a number of hydrogen bonded (···) and hydrophobic (·····) interactions. The interactions within the loop include: O Ala112 ··· Nδ2 Asn107=3.3Å and N Gly111 ··· O Glu109=2.8 Å. The interactions between the loop and the C-terminal region are: CA Gly111 ··· Cε1 Phe130=3.0Å, C Gly111 ····· Cε1 Phe130=3.0Å, CG Glu109 ····· Cε2 Phe130=3.8Å, CG Arg102 ····· Cε1 Phe125=3.8Å, O Cys113 ··· N Arg126=3.5 Å, and CA Cys113 ····· CG Arg126=3.2Å. The interactions between the C-terminus and the core of the enzyme are N Val131 ··· Oδ1 Asn89 (H3)=2.7Å and O Phe125 ··· N Cys95 (β-wing 2)=3.1Å. The conformation, surface area and the number of interactions stabilizing it seem to suggest that the C-terminal extension may have a functional role such as in plasma membrane localization and optimal cellular functions [30].

The N-terminal region of the enzyme has a loop (nine residues) preceding the first helix and has a calcium ion at its center. This is called the calcium binding loop which is canonical for most of the PLA$_2$s. The calcium ion in *Cs*IIIPLA$_2$ is coordinated with three main chain carbonyl oxygen atoms of Trp9, Gly11, Gly13 residues from the calcium binding loop and the carboxyl oxygens of Asp36 (Fig. 2). The calcium binding loop in *Cs*IIIPLA$_2$ is stabilized in three important ways: (1) participation of three backbone carbonyl oxygen atoms of residues in coordination of the calcium ion; (2) presence of covalent disulfide bond between the Cys8 on the loop and Cys30 on helix H1 and (3) hydrogen bonds Nε1 Trp9 ··· O Thr21 (2.7 Å) and NZ Lys12 ··· O Pro56 (3.1Å). These interactions in the calcium binding loop appear to optimize the local conformation for possible substrate binding function and subsequent catalysis. Secretory PLA$_2$ catalysis starts with the enzyme binding to calcium and substrate followed by base mediated attack, formation and collapse of the intermediate state and finally the release of the product [9]. The calcium directs the stereospecific positioning of the substrate in the active site of the enzyme and is known to serve as an electrophile during the

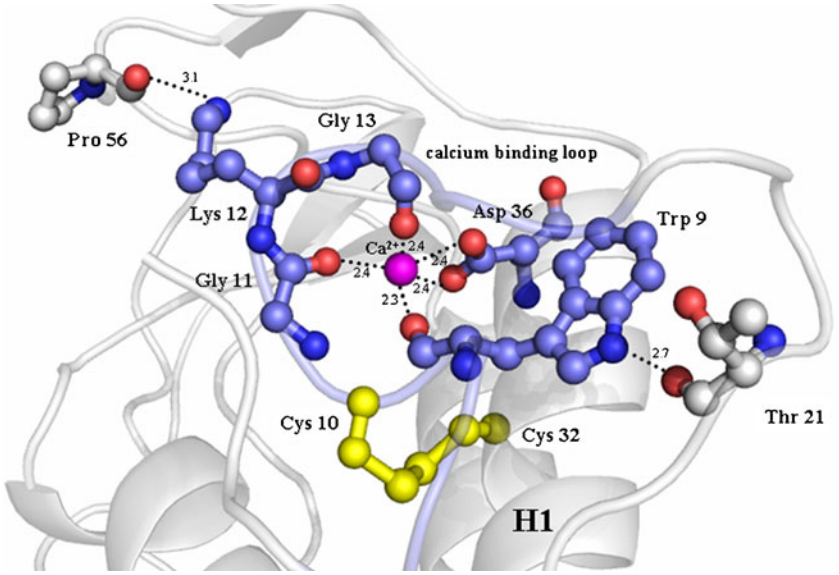

Fig. 2 A view of the calcium binding loop of *Cs*IIIPLA$_2$. The calcium binding loop is shown in light purple while the remaining secondary structural motifs are shown in white. The residues Trp7, Gly9 and Gly11 of the loop along with the Asp34 that make a co-ordinate cage around the calcium ion (pink sphere) are shown along with the main chain atoms. The co-ordinate bonds are indicated by dotted lines and the distances in Å are shown adjacent to it. The disulfide bond between the Cys10 on the calcium binding loop and Cys32 on helix 1 is shown in yellow. The residues Pro56 and Thr21 are shown in white and their hydrogen bonded interactions with the residues Lys12 and Trp9 on the loop are shown in dotted lines and the distances in Å are shown adjacent to it

general base mediated catalysis. The loop also forms the wall of the hydrophobic channel. Superimposition of the enzymatic subunit of *Cs*IIIPLA$_2$ with the human group III PLA$_2$ shows the calcium binding loop does not vary in its conformation. The only notable difference is the interaction of the lysine side chain in the loop with the main chain oxygen atom of Pro56 which has been discussed previously. Therefore calcium ion is an essential cofactor for the functioning of the *Cs*IIIPLA$_2$ which is shown to hydrolyse phosphotidylcholine in the presence of calcium [7].

The phospholipase A$_2$ enzyme hydrolyses the *sn*-2 ester bond of the phospholipids by the interactions of the residues at the active site of the enzyme [31]. A His-Asp dyad along with the conserved water molecule is known to act as a catalytic triad in the hydrolysis of phospholipids. The His35 from helix H1 is at 2.6 Å from Asp65 on helix H2. The function of aspartate in this interaction is to reduce the pKa of histidine and enable it to activate the nucleophile water molecule [32]. The positioning and orientation of the aspartate is, therefore, very important in the catalytic reaction just as it is in serine proteases [33]. In *Cs*IIIPLA$_2$, the aspartate is stabilized by a hydrogen bond between Oδ1 with OH of Tyr87 (Fig. 3a). Thus, the tyrosine at the active site has more structural role than a functional participation in the catalysis. Nevertheless, all three residues at the active site are termed catalytic residues. The three residues along with spatial coupling of the catalytic His35 with the calcium binding Asp36 provide a perfect geometry at the active site. Interestingly, this tripartite arrangement between the three residues His-Asp-Tyr is not seen in the house keeping

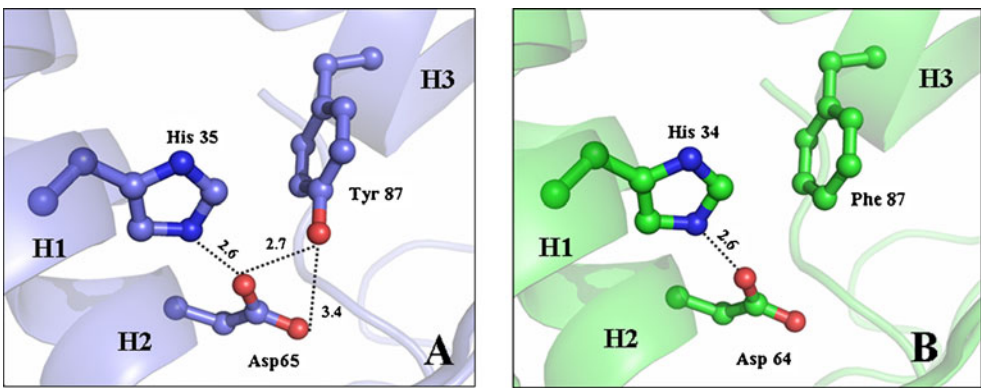

Fig. 3 Comparative analysis of the residues at the active site of (**a**) drug target, *Cs*IIIPLA$_2$ and (**b**) house keeping enzyme, human group III PLA$_2$. The residues along with their position numbers are shown. The hydrogen bonded interactions are shown as dotted lines and the distances in Å are given adjacent to the bonds

human group III PLA_2, wherein phenylalanine replaces tyrosine at position 87 (Fig. 3b) [12]. This replacement results in the loss of the hydroxyl moiety and consequently a potential hydrogen bond with Asp65 is lost. The fact that the enzyme is active despite the change in one of the active site residues demonstrates the minor role of the Tyr87 in this type of PLA_2s. The possibility of a conserved water molecule stabilizing the Asp65 in the human PLA_2 enzyme cannot be completely ruled out. However, the variation of the aromatic residue could be exploited to a good effect in targeted drug designing. Potential drug candidate molecules have the scope to interact with the residues at the active site and make hydrogen bonds with the Tyr87 in *Cs*IIIPLA$_2$. The void of a hydrogen bonded interaction between the drug molecule and human group III PLA_2 would decrease the binding affinity of the molecule to the enzyme. This would enable it to retain its house keeping function relating to the growth of neurons [12].

The hydrophobic channel in *Cs*IIIPLA$_2$ is a cavity within the enzyme whose walls are lined by side chains of non-polar amino acids. The volume of the channel is sufficient to accommodate the alkane chains of phospholipids. The channel is continuous with the catalytic site. The residues forming the channel in *Cs*IIIPLA$_2$ are Met2, Met4, Gly11, Gly13, Val57, Val83, Leu86, Tyr87 and Phe91 (Fig. 4). In hIIIPLA$_2$, Trp1, Lys10, Val12, His57, Phe87 and Leu91 are involved in the channel formation. It is evident that the channel environment is less polar in the pathogen compared with the host. The presence of lysine and histidine enable more polar interactions in hIIIPLA$_2$. The absence of tyrosine and presence of phenylalanine in the host can also differentiate the host and pathogen in PLA_2s.

Interfacial membrane binding surface is that face of the structure that anchors the enzyme to the membrane interface. This event is crucial to the enzymatic function [34] and is subsequently followed by the occupation of the hydrophobic channel by the substrate for the final catalysis. Based on the detailed description of the putative membrane binding surface (i-face) of homologous group III bee venom PLA_2 [9], the putative membrane binding surfaces of CsIIIPLA$_2$ and hIIIPLA$_2$ were structurally modeled (Fig. 5). The i-face in all these enzymes is formed by the third helix, calcium binding loop and the N-terminal region. The residues of these regions provide a heterogeneous surface for interaction with the membrane. Hydrogen bonded interactions and hydrophobic interactions have been shown to provide the major portion of the interfacial binding energy [35]. The contribution of the electrostatic interactions of the cationic residues with the anionic vesicles appears to be significant. There are six cationic residues distributed evenly across the putative membrane binding surface

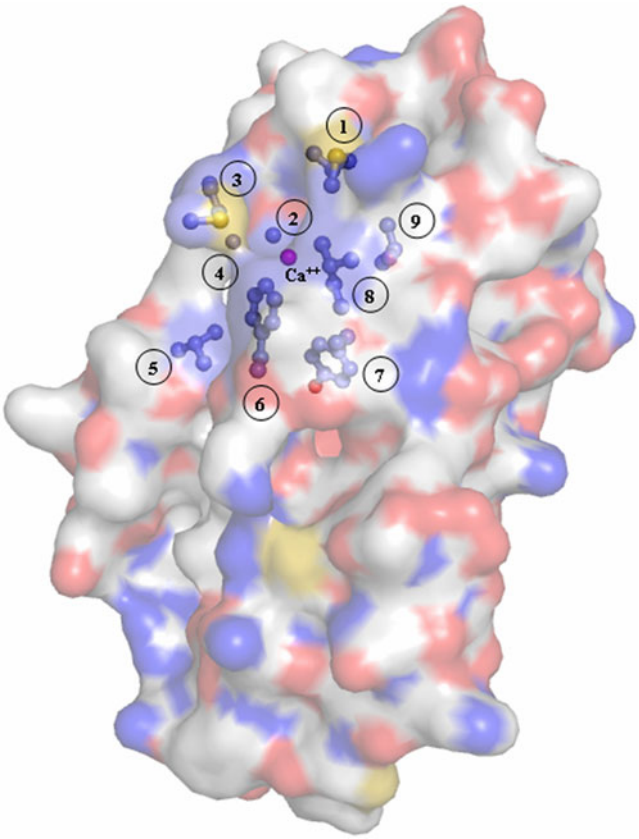

Fig. 4 A GRASP model of the *Cs*IIIPLA$_2$ showing the residues of the hydrophobic channel. The residues are: (1) Met4, (2) Gly11, (3) Met2, (4) Gly13, (5) Val 57, (6) Phe91, (7) Tyr87, (8) Leu86 and (9) Val83.

of *Cs*IIIPLA$_2$. The hIIIPLA$_2$ in comparison has only three cationic residues at the i-face [13]. Of particular significance, is the presence of Phe91 with its aromatic side chain fully exposed at the center of the putative membrane binding surface. Thermodynamic analysis has suggested that the side chain of the phenylalanine is fully inserted into the hydrophobic core of the membrane during the interfacial catalysis of a group II secretory PLA_2 [36]. This residue is conspicuously absent in the human enzyme implying the need of substrate specificity in the enzymes. Also, sequence comparison at the positions 82 and 91 revealed interesting features. Arg82 and the Phe91 were unique to *Cs*IIIPLA$_2$ enzyme. While the residues at position 91 were either non-aromatic residues or tyrosine in all the other PLA_2 sequences, the residues at position 82 were mostly hydrophobic residues (Fig. 6). These variations in the putative membrane binding surface may in part explain certain aspects of the pathology at the molecular level. While *Cs*IIIPLA$_2$ is clearly implicated in hepatic fibrosis, hIIIPLA$_2$ has not been known to cause any pathology despite its high abundance in the liver [25]. It is, therefore, clear that the *Cs*IIIPLA$_2$ has more

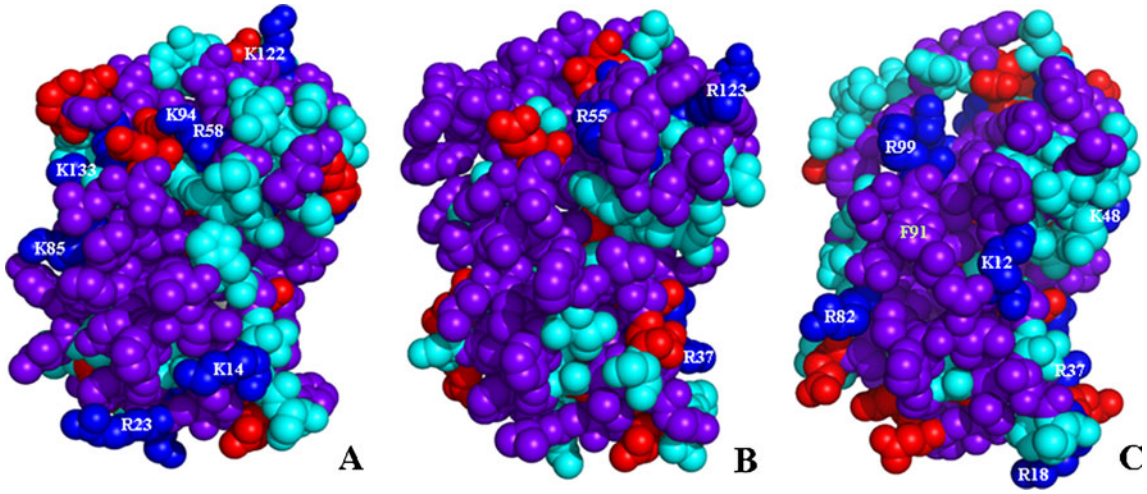

Fig. 5 Comparative analysis of the substrate interacting i-face of different group III PLA_2 isoforms. (**a**) Bee venom PLA_2 [PDB Id 1POC]; (**b**) Human group III PLA_2 model [29] and (**c**) Secretary PLA_2 from the liver fluke *Clorencis sinensis* [This study]. The acidic residues are shown in red, the basic residues are shown in blue, the hydrophilic residues are shown in cyan and the hydrophobic residues are shown in magenta. The basic residues are shown in blue and the Phe91 in *Cs*III PLA_2 is labeled in light green

predilections to bind to the membrane liver cells than the other group III PLA_2s.

Swiss Prot Id|Organism	Sequence				
C6YBD6|*Cs*	AT	R	VGNLYFNV	F	K
P80003|*Hs*	AD	L	VGMTYFTV	L	K
D3ZGN6|*Rn*	AD	I	MGVAFFNV	L	E
Q7M4I6|*Mp*	AA	F	VGRTYFTI	L	G
Q9NZ20|*Hs*	SD	I	VGVAFFNV	L	E
Q8BZT7|*Mm*	SD	I	MGVAFFNV	L	E
Q1JPB9|*Bt*	SD	I	MGVAFFNV	L	A
E1JJG5|*Dm*	AN	T	LGAIFYNV	V	Q
Q7QGJ7|*Ag*	AN	L	VGKLFFNV	M	Q
P00630|*Am*	SY	F	VGKMYFNL	I	D
Q3YAU5|*Hf*	AF	T	IRKLYFGL	Y	G
Q6T178|*Mt*	AA	A	VRKTYFDL	Y	G
P59888|*Pi*	AG	F	VRKTYFDL	Y	G
P0C8L9|*Hg*	VS	A	VKFTYFTL	Y	G
Q6PXP0|*Ap*	AE	N	VWRFYFQW	Y	N

Fig. 6 Part of the sequence alignment of PLA_2 of *Cs, Clonorchis sinensis* (Chinese liver fluke) sequence (shown in purple) with other group III PLA_2 sequences of *Hsu, Heloderma suspectum*; (Gila monster), *Rn, Rattus norvegicus* (Rat); *Hsa, Homo sapiens* (Human); *Mm, Mus musculus* (Mouse); *Bt, Bos taurus* (Cow); *Dm, Drosophila melanogaster* (Fruit fly); *Ag, Anopheles gambiae* (African malaria mosquito); *Mp, Megabombus pennsylvanicus* (American common bumblebee); *Am, Apis mellifera* (Honeybee); *Hf, Heterometrus fulvipes* (Indian black scorpion); *Mt, Mesobuthus tamulus* (Eastern Indian scorpion); *Pi, Pandinus imperator* (Emperor scorpion); *Hg, Hadrurus gertschi* (Mexican scorpion) and *Ap, Anuroctonus phaiodactylus* (Mafia scorpion). The Swiss Prot Ids and the name of the organism are given preceeding the respective sequence. The putative interfacial membrane binding residues lysine and phenylalanine are shown in blue and light green

Structural details of CsIIIPLA$_2$ to aid drug design

Based on the structural analysis of *Cs*III PLA_2, we propose the important determinants for an ideal inhibitory molecule of *Cs*IIIPLA_2. The molecule must (a) make hydrogen bonded interactions with the catalytic residues His35 and Asp65; (b) make hydrogen bonded interactions with the Tyr87 and Lys12 which are unique to the target enzyme; (c) make hydrophobic interactions with the hydrophobic residues: Met4, Gly11, Met2, Gly13, Val 57, Phe91, Leu86 and Val83 lining the channel and (d) make coordinate bond with the calcium ion.

Conclusions

The secretory PLA_2 from the liver fluke, *Clorenchis sinensis* is a possible target for the treatment of hepatic fibrosis. The structural details of this protein provided here give insights into the enzyme's functionality. The observed variations between this protein and its human counterpart with respect to the amino acid composition, active site geometry, substrate binding channel, and the putative membrane binding surface provide valuable inputs for the design of molecules for targeted inhibition of the parasite enzyme. This is the only available structural model of secretory PLA_2 from the liver fluke parasite that is currently available for structure based drug designing.

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