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Finding off-targets, biological pathways, and target diseases for chymase inhibitors via structure-based systems biology approach

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ABSTRACT

Off-target binding connotes the binding of a small molecule of therapeutic significance to a protein target in addition to the primary target for which it was proposed. Progressively such off-targeting is emerging to be regular practice to reveal side effects. Chymase is an enzyme of hydrolase class that catalyzes hydrolysis of peptide bonds. A link between heart failure and chymase is ascribed, and a chymase inhibitor is in clinical phase II for treatment of heart failure. However, the underlying mechanisms of the off-target effects of human chymase inhibitors are still unclear. Here, we develop a robust computational strategy that is applicable to any enzyme system and that allows the prediction of drug effects on biological processes. Putative off-targets for chymase inhibitors were identified through various structural and functional similarity analyses along with molecular docking studies. Finally, literature survey was performed to incorporate these off-targets into biological pathways and to establish links between pathways and particular adverse effects. Off-targets of chymase inhibitors are linked to various biological pathways such as classical and lectin pathways of complement system, intrinsic and extrinsic pathways of coagulation cascade, and fibrinolytic system. Tissue kallikreins, granzyme M, neutrophil elastase, and mesotrypsin are also identified as off-targets. These off-targets and their associated pathways are elucidated for the effects of inflammation, cancer, hemorrhage, thrombosis, and central nervous system diseases (Alzheimer's disease). Prospectively, our approach is helpful not only to better understand the mechanisms of chymase inhibitors but also for drug repurposing exercises to find novel uses for these inhibitors.

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Key words: off-targets; biological pathways; cardiovascular diseases; chymase; systems biology, cancer; Alzheimer's disease; complement system; coagulation cascade.

INTRODUCTION

Complex biological networks regulate diseases like cancers, cardiovascular diseases, and neurodegenerative disorders which are further influenced by a number of environmental and genetic factors.[1] Therefore, single-target drug mediation cannot efficiently battle the complex pathologies of these systemic diseases. Due to interactions between various pathways in the disease network, mono-target drugs may not show the same effect in clinical treatment.[2] The diversity of biological functions that a protein assumes, depend on the molecular interactions that it makes. Each protein selectively binds to a number of other proteins, small molecules, lipids or nucleic acids. The explication of protein interactions would be helpful in a number of ways such as construction of protein–protein interaction networks, identification of multimolecular assemblies, and eventually in drug design.[3] Therefore, detecting protein–ligand interaction networks on a proteome-wide scale is essential to deal with a wide

Additional Supporting Information may be found in the online version of this article.

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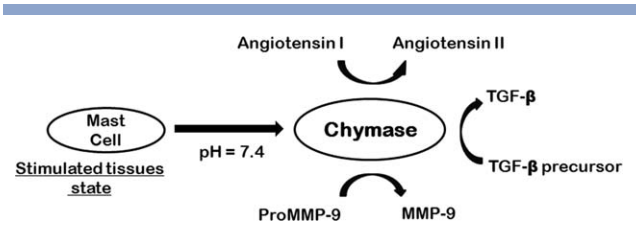

Figure 1

Catalytic activity of human chymase at pH 7.4 leading to the chymase-dependent conversion of various substrates such as angiotensin I, precursor of TGF-β, and proMMP-9 to their active forms.

range of biological problems such as associating molecular functions to physiological processes and designing safe and efficient therapeutics. In order to boost clinical efficacy, the interest in the identification of multiple targets associated with a phenotype and in developing combinatorial therapies is escalating. These facts highlight the critical importance of studying polypharmacology in a systems level context.[4] The developing field of systems biology, might not have delivered the simple solution for development of multitarget drugs, however, it has clarified the intricacy of the problem. Thus, this filed of systems biology may pave the way to a new approach for drug design and discovery. Polypharmacology focuses on searching for multitarget drugs to perturb disease-associated networks rather than designing selective ligands to target individual proteins. Examples of such strategy can be found in the combinatorial therapy of AIDS, atherosclerosis, cancer, and depression.[5] Thus, in principle, multitarget drugs can demonstrate greater efficacy to treat complex diseases than mono-target drug therapeutics.

Chymase (EC 3.4.21.39) is an enzyme of the hydrolase class that catalyzes the hydrolysis of peptide bonds and it is abundant in secretory granules of mast cells. Chymase is stored in mast cells in an inactive form and is released as an active enzyme when mast cells are stimulated by injury or inflammation. Chymase shows enzymatic activity immediately after its release into the interstitial tissues at pH 7.4 following various stimuli in tissues. Since chymase has no enzymatic activity in normal tissues, chymase inhibitors have the potential to be safe/nontoxic because specific chymase inhibitors may not have effects on any other targets in normal tissues.[6] Chymase is the major extravascular source of vasoactive angiotensin II (Ang II), which is generated with exceptional efficiency by human chymase via hydrolysis of the Phe-8–His-9 bond of angiotensin I (Ang I) (Fig. 1). Chymase also activates matrix metalloproteinase (MMP)−9 by cleaving a specific site of the catalytic domain of MMP-9. MMP-9 is correlated with an increase in infarct size and left ventricle (LV) fibrosis following experimental AMI.[7] Chymase also converts precursor of transforming growth factor-β (TGF-β) to its active form thus contributing to vascular response to injury (Fig. 1). Both TGF-β and MMP-9 are involved in tissue inflammation and fibrosis, resulting in organ damage.[8] Previous studies have demonstrated the involvement of chymase in the escalation of dermatitis and chronic inflammation pursuing cardiac and pulmonary fibrosis.[9] A connection between heart failure and chymase has also been recognized and a study revealed the importance of developing a specific chymase inhibitor as a new therapeutic treatment for the disease.[10] The multiple functions of chymase may play an important role in the development and promotion of various diseases. Therefore, inhibition of chymase is likely to divulge therapeutic ways for the treatment of cardiovascular diseases, allergic inflammation, and fibrotic disorders. Chymase inhibition may also be useful for preventing the progression of type 2 diabetes, along with the prevention of diabetic retinopathy.[11] Moreover, role of chymase in inflammation has prompted its restorative value in diseases such as chronic obstructive pulmonary disease (COPD) and asthma.[12]

In order to evaluate the role of a ligand in targeting multiple proteins of a biological system, we should know about the specific proteins which are inhibited/activated by the ligand. It is also significant to find out the associations of these proteins to the biological pathways and their effects on overall physiological process? Keeping these quires in view, various computational approaches have been applied to predict novel drug repositioning candidates via methods such as protein structural and functional similarity, chemical similarity, and molecular docking.[13–16] We have also developed a system biology-based computational strategy in the present study to identify off-target binding networks for human chymase enzyme (Fig. 2). In comparison with the existing methods, our system biology approach not only includes structural and functional similarity of the proteins; nevertheless, it also refines the results using multiple protein–ligand docking scores. Moreover, this computational endeavor includes significant information regarding protein–protein interaction networks as well. The computational strategy proceeds as follows: (1) In the first step, the binding pocket of the enzyme is extracted from a 3D structure of the target enzyme. (2) This binding pocket is further used to search against 5985 PDB structures of human proteins or homologs of human proteins using the SMAP software which is based on a sensitive and robust ligand binding site comparison algorithm. (3) The resulting list of putative off-targets is subjected to structural and functional cluster analysis. (4) This study has also incorporated molecular docking studies to help filter interactions predicted through protein binding site similarity. Molecular docking analysis of experimentally known inhibitors of the target enzyme against off-targets was performed to predict ligand–protein interactions. Our method emphasizes removing false positive predictions using scoring and ranking thresholds, and retaining

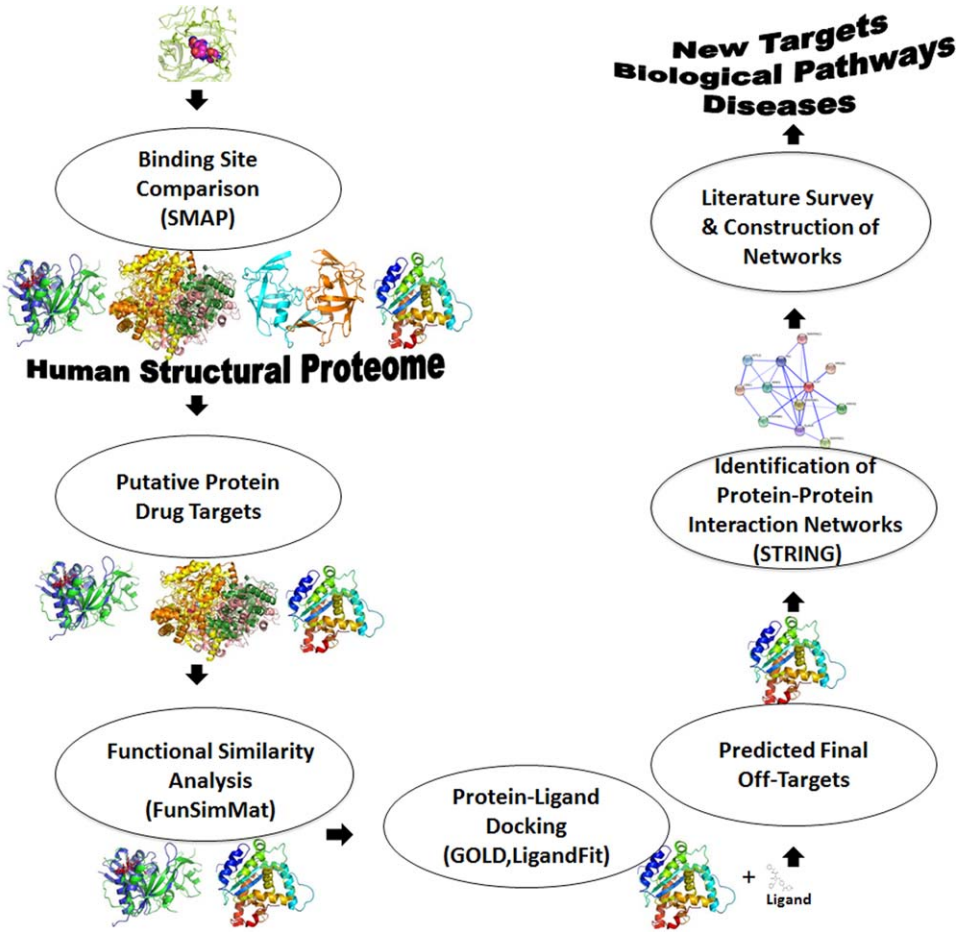

Figure 2

Computational strategy for identification of new off-targets, biological pathways and associated diseases for human chymase inhibitors employed in this study. This strategy comprises of structural and functional similarity analysis along with binding site comparison via multiple protein–ligand docking scores. Protein–protein interaction networks were identified and off-targets were linked to biological pathways and associated diseases via literature survey. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

only the highest confidence interactions of putative off-targets candidates. (5) To reveal protein–protein interaction networks, an interaction analysis was performed on chymase and its off-targets. (6) In the final step, target list predicted from the off-target pipeline is incorporated into biological pathways using a survey of literature.

We have identified a panel of off-targets for chymase inhibitors from the human structural genome and mapped those targets to biological pathways via the literature survey. Novel targets discovered may lead to the chymase inhibitors being repositioned as therapeutic treatment for its off-target's associated diseases. This study has proposed several opportunities to experimentally evaluate auspicious hypotheses that may lead to significant advancements in developing chymase inhibitors for treatment of off-target's associated diseases. It also demonstrates the significance of computational strategies for efficacy prediction and the role that systems biology may play in multitarget therapeutics.

MATERIALS AND METHODS

Binding site similarity search on a structural proteome scale

The SMAP program, which implements the Sequence Order Independent Profile-Profile Alignment (SOIPPA) algorithm to identify significant structural similarity to a given ligand-binding site was applied to predict off-target binding sites for chymase inhibitors across the proteome.[17] The chymase structure (PDB Id: 3N7O) was used to search against 5985 PDB structures of human proteins or homologous of human proteins (sequence identify *Homo sapiens*, alignment coverage larger than 90%) using the SMAP which can be downloaded from http://funsite.sdsc.edu. The SOIPPA algorithm is based on finding the maximum-weight common sub-graph (MWCS) between two encoded protein graphs. A weight is assigned according to the chemical similarity or evolutionary correlation of the associated sites. In brief, two proteins are aligned to identify

similar local binding sites using SOIPPA algorithm.[18] The statistical significance of the binding site similarity is estimated using an extreme value distribution model.

$$(s > S) = 1 - \exp(-\exp(-Z)) \quad (1)$$

where

$$Z = (S^2 - \mu)/\sigma \quad (2)$$

where S is the raw SOIPPA similarity score. μ and σ are fitted to the logarithm of *N*, which is alignment length between two proteins:

$$\mu = a^*\ln(N)^2 + b^*\ln(N) + c \quad (3)$$

$$\sigma = d^*\ln(N)^2 + e^*\ln(N) + f \quad (4)$$

Six parameters *a*, *b*, *c*, *d*, *e*, and *f* are 5.963, 215.523, 21.690, 3.122, 29.449, and 18.252 for the McLachlan similarity matrix are used in this study, respectively. With the use of this statistical model, 65 off-targets were identified with *P* values less than $1.0e^{-3}$.

Functional similarity calculation

The functional similarity between chymase and the off-targets is further quantitatively measured using gene ontology (GO) relationships found with the Functional Similarity Matrix (FunSimMat) web server (http://funsimmat.bioinf.mpi-inf.mpg.de/index.php).[19] The FunSimMat is a comprehensive database providing various precomputed functional similarity values for proteins in UniProtKB and for protein families in Pfam and SMART. FunSimMat offers several different types of queries that are available through the web front-end and the XML-RPC server. For this study, "Comparing one protein / protein family with a list of proteins / protein families" query was used to find functional similarity between chymase and off-targets. The results table returns different scores. The BPscore measures the similarity of the biological processes annotated to the two proteins, protein families or diseases. Likewise, MFscore and CCscore measure the similarity of the molecular functions and the cellular components, respectively. The funSim scores are computed from BPscore and MFscore. The funSimAll scores combine all three BPscore, MFscore, and CCscore measuring the overall functional similarity of the two proteins or protein families using the following equation:

$$funSimAll(p,q) = \frac{1}{3}\left[\left(\frac{BPscore(p,q)}{\max_{BPscore}}\right)^2 + \left(\frac{MFscore(p,q)}{\max_{MFscore}}\right)^2 + \left(\frac{CCscore(p,q)}{\max_{CCscore}}\right)^2\right] \quad (5)$$

While, the rfunSimAll score is calculated as the square root of the funSimAll score of a pair of proteins or protein families. It ranges from 0 for no functional similarity to 1 for maximal functional similarity. From 65 off-targets, 32 had detectable similarities with rfunSimAll score above 0.5, thus, were selected for further analysis.

Filtering of putative off-targets via protein–ligand docking

After structural and functional similarity analysis, the resulting list of putative off-targets was further filtered via more computationally intensive protein–ligand docking. A set of four compounds reported as chymase inhibitors with their diverse experimentally known inhibitory activity (IC_{50}) data was compiled from the literature.[20–22] This set includes TPC-806 which is the first reported noncovalent chymase inhibitor and has been in phase II clinical trials for heart failure since 2007.[23] The bioactive binding poses of these chymase inhibitors in the active sites of putative off-targets were generated and analyzed to determine their affinity for these off-targets. In the first step of protein–ligand docking, Genetic Optimization for Ligand Docking (GOLD) 5.1 program from Cambridge Crystallographic Data Center, UK, software was used to determine the ligand binding pose and affinity for putative off-targets. GOLD uses a genetic algorithm for docking ligands into protein binding sites to explore the full range of ligand conformational flexibility with partial flexibility of protein. Goldscore function is a molecular mechanics–like function with four terms:

$$\text{GOLD Fitness} = S_{hb\_ext} + S_{vdw\_ext} + S_{hb\_int} + S_{vdw\_int} \quad (6)$$

where S_{hb_ext} is the protein–ligand hydrogen-bond score and S_{vdw_ext} is the protein–ligand van der Waals score. S_{hb_int} is the contribution to the fitness due to intramolecular hydrogen bonds in the ligand; while, S_{vdw_int} represents the contribution to the fitness score due to intramolecular strain in the ligand. Thus, the interacting ability of a compound depends on the fitness score, greater the GOLD fitness score better the binding affinity. Ten docking runs were performed per structure unless five of the 10 poses were within 1.5 Å RMSD of each other. All other parameters were kept at their default values. Protein structures complexed with their corresponding inhibitors were downloaded from the PDB (http://www.pdb.org) and were subjected to docking process. The active site was defined with a 10 Å radius around the bound inhibitor. In case of proteins with no complex crystal structures available, docking site was defined with a 10 Å radius around the key active site residues. The protein–ligand interactions were examined by Accelrys Discovery Studio v3.0 (DS), Accelrys, San Diego, USA, program. The best poses of the chymase inhibitors with highest GOLD fitness score were selected for further docking analysis.

In the next step of protein–ligand docking, molecular docking calculation was performed using LigandFit,

which can perform either rigid or flexible docking referring to the conformational state of the ligand. LigandFit docks the small molecules into the protein active site by considering its shape complementary.[24] There are three stages in LigandFit protocol: (i) docking: attempt is made to dock a ligand into a user-defined binding site (ii) *in situ* ligand minimization and (iii) scoring: various scoring functions were calculated for each pose of the ligands. Maximum 10 poses were retained based on the root mean square deviation (RMSD) threshold for diversity (1.5 Å) and score threshold for diversity 20 kcal/mol. Consensus scoring function implemented in the LigandFit module was used to select the best leads from molecular docking study. Consensus scoring implemented in the LigandFit,[25] combination of multiple scoring functions, might dramatically reduce the number of false positives by its distinct scoring functions. In our study, consensus scoring method was based on different scoring functions such as LigScore1, LigScore2, piecewise linear potential 1 (PLP1),[26] PLP2, potential mean force (PMF), Jain, Ludi,[27] and Dock score. Thus, based on the Consensus scoring function, best orientation of chymase inhibitors at active sites were selected and analyzed for their affinity to the putative off-targets.

Use of STRING for known and predicted protein–protein interactions of off-targets

A STRING interaction analysis was also performed on the chymase and its off-targets to find protein–protein interaction networks in this study. STRING is a database of known and predicted protein interactions. It is the most comprehensive resource of protein–protein interactions as it incorporates information from other databases such as BioGRID,[28] HPRD,[29] IntAct,[30] MINT,[31] and KEGG. In STRING database, interactions include direct (physical) and indirect (functional) associations and they are derived from four sources: genomic context, high-throughput experiments, (conserved) coexpression, and previous knowledge (from the literature).[30] The PPIs from STRING http://string.embl.de were classified into four categories: highest, high, medium, and low confidence, using STRING scores of 0.900, 0.700, 0.400, and 0.150, respectively. In this study, we used STRING score of 0.700 with high confidence category. Moreover, *Homo sapiens* was selected as search option to find protein–protein interaction network for chymase and its off-targets.

Integration of the off-targets into biological pathways via literature survey

Final step of the applied computational methodology was to integrate identified off targets with their corresponding biological pathways. For this purpose, various resources were utilized. Research papers about the off-targets were surveyed to reveal their link to biological pathways and associated diseases. In order to further validate the findings from literature survey, identified biological pathways such as complement system, coagulation cascade, and fibrinolytic systems were also analyzed using KEGG pathway maps.[32] These pathways also showed the links of identified off-targets to the same biological pathways which were identified via literature survey.

RESULTS

Binding site similarity search on a structural proteome scale

Detecting the binding regions of proteins is extremely important for elucidating the functions of complexes or designing inhibitors for known specific complexes. The inhibitor binding region in human chymase is defined by residues K40, H57, Y94, N95, T96, L99, D102, A190, F191, K192, G193, S195, V213, S214, Y215, G216, and R217. The region is mostly polar because of main chain carbonyls that are arranged along the surface of the region. Human chymase is folded into two six-stranded β-barrels, which are connected by three trans-domain segments (Fig. 3). Human chymase contains 13 arginine and 15 lysine residues, but only eight glutamate and eight aspartate residues, resulting in a rather high overall basicity. The catalytic residues Ser195, Asp102, and His57 are located at the junction of the two β-barrels, while the active-site cleft runs perpendicular to this junction. The crystal structure of human chymase was used to detect common binding sites for chymase inhibitors on human structural proteome scale by SMAP. The off-target search by SMAP returned 3402 hits. The hits from database search were sorted by the similarity score of the match, along with P-values of the match, their PDB structure ids, chain ids and biological descriptions. The PDB id is linked to the structure summary page of the RCSB PDB. For each of the hits, detailed information on the ligand-binding site similarity was analyzed (*P* value, raw alignment score, RMSD and Tanimoto coefficient of overlap). The amino-acid residue alignment between two ligand-binding sites and the transformation matrix to superpose them were also evaluated. Among 3402 hits, 66 structures were found to have good binding site similarity with SMAP p-value less than $1.0e^{-3}$ (Supporting Information Table 1). The 3D structure of human pro-chymase was also found in these 66 hits and was removed from the list. The most of remaining 65 putative off-targets were part of the immune system, blood coagulation pathways, and complement system. The cathepsin G was at the top of list with lowest *P* value and hepatocyte growth factor was at the bottom with *P* value $9.79e^{-03}$. The panel of these 65 potential off-targets was subjected to further computational studies for their functional correlations and binding affinity analysis with chymase and its inhibitors, respectively.

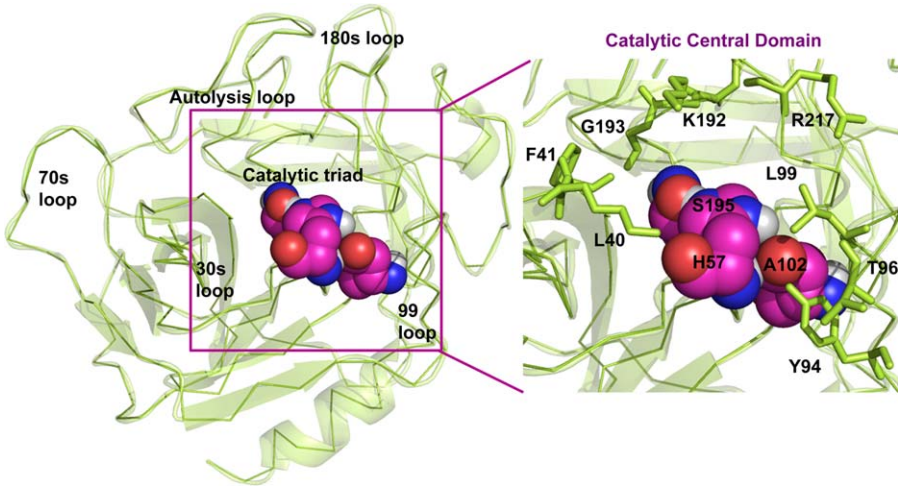

Figure 3

Crystal structure of human chymase and the zoomed view of its active site which clearly shows the catalytic triad and important binding site residues such as G193, K192, and R217. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Functional similarity between chymase and putative off-targets

In order to find out possible functional correlation between chymase and its potential off-targets, FunSimMat web server was used. The results table returned different scores including BPscore, MFscore, CCscore, funSimAll, and rfunSimAll. From 65 off-targets, 32 had detectable similarities with a rfunSimAll score above 0.5 (Supporting Information Table 2). Among these 32 structures, kallikrein-4 exhibited highest functional similarity with rfunSimAll score of 0.93. Moreover, kallikrein-7, complement c1s component, mannan-binding (MB) lectin serine protease 2 (MASP2) also ranked top showing rfunSimAll score above 0.80. While dipeptidyl aminopeptidase-like protein 6 with rfunSimAll score of 0.53 was at the bottom of putative off-targets list. It was observed that most of 32 structures which showed good values for functional similarity with chymase also exhibited high binding site similarity from SMAP calculation. This observation compelled us to find out whether these structures having functional association and similar binding site correlation with chymase form an interconnected off-target network. Thus, these 32 putative off-targets were subjected to computationally intensive protein–ligand docking studies.

Filtering of putative off-targets via protein–ligand docking

The binding of a small molecule drug to its target protein in a cell is much more complex than a single docking calculation. For example, an ATP-competitive kinase drug would have hundreds of ATP-binding sites to choose from due to the large size of the kinome. Cancer drugs such as sunitinib are now identified to potently inhibit many more kinase targets than anticipated earlier.[33] Large-scale docking of many targets to many drugs is now feasible when run on powerful computer clusters. However, limitations in scoring methods result in high false positive prediction rates,[34] and large-scale studies amplify these low prediction accuracies. In this study, various scoring and ranking thresholds were applied to remove false positive predictions. A computational pipeline for large-scale molecular docking of chymase inhibitors to off-targets was developed. In the first step of protein–ligand docking, GOLD software was used to determine the ligand binding pose and affinity for putative off-targets. The best poses of the chymase inhibitors with highest GOLD fitness score were further filtered using another protein–ligand docking program LigandFit. The consensus scoring method which was based on different scoring functions was used to analyze orientation of chymase inhibitors at active sites of putative off-targets for their binding affinity. Briefly, we collected 3D structures available for each off-target, and docked a set of structurally diverse chymase inhibitors (Supporting Information Fig. 1) to each pocket. Results were collected and thresholds were applied to select the top predicted interactions, which were then visually inspected. Finally, 13 off-targets for chymase inhibitors were predicted through protein–ligand docking process (Fig. 4).

Use of STRING for known and predicted protein–protein interactions of off-targets

The next phase in data analysis is the identification of protein–protein interaction network maps and their visualization for chymase and its off-targets predicted in this study. For this purpose, STRING database was used. The interaction analysis showed that chymase and several of its off-targets proteins are connected to each other

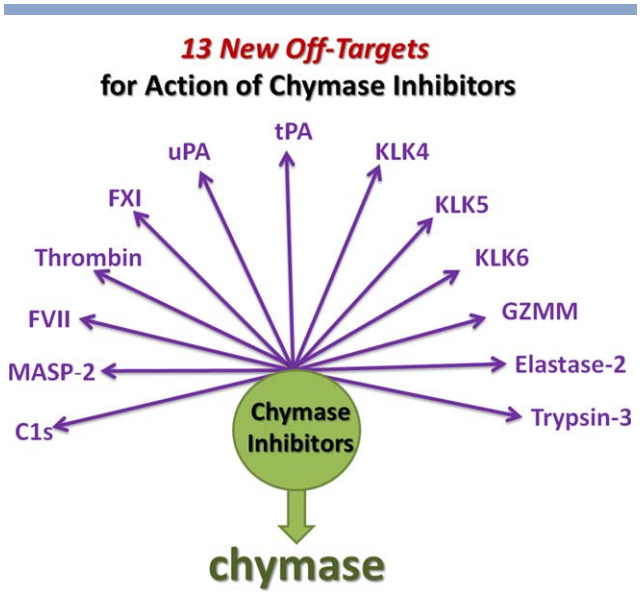

Figure 4

Illustration of 13 new off-targets for chymase inhibitors identified in this study. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

through various indirect ways thus supporting the GO functional analysis performed by FunSimMat (Supporting Information Figs. 2–9). The tissue-type plasminogen activator (tPA) which is encoded by PLAT gene is connected with chymase through various indirect ways (Supporting Information Fig. 2). The interaction analysis performed by STRING for chymase and tPA showed that PLAT was strongly associated with MMP9 and SERPINB1 with STRING scores of 0.982 and 0.985, respectively. MMP9 and SERPINB1 were further linked with chymase (CMA1) showing STRING scores of 0.764 and 0.815, respectively thus exhibiting high level of association. KLK5 is linked with CMA1 through SPINK9 and SPINK5 which are serine peptidase inhibitors (Supporting Information Fig. 3). While, urokinase-type plasminogen activator (uPA) which is encoded by PLAU gene was found to have various interaction partners like PLAUR, SERPINB2, and VTN which further showed direct or indirect interactions with CMA1(Supporting Information Fig. 4). Leukocyte elastase encoded by ELANE gene exhibited indirect associations to CMA1 via SLPI, and SERPINB1 with high STRING scores of 0.823 and 0.815, respectively (Supporting Information Fig. 5). Analysis of protein–protein interaction network of coagulation factor XI (F11) indicated that F11 was indirectly connected with CMA1 through APP and EDN1 with very high STRING score of 0.910 (Supporting Information Fig. 6). Similarly, analysis of interaction maps of Granzyme M(GZMM), Trypsin-3 (PRSS3), and MASP2 also revealed a number of indirect associations between chymase and its off-targets (Supporting Information Figs. 7–9).

Integration of the off-targets into biological pathways via literature survey

Finally, literature survey was performed to incorporate these off-targets into various biological pathways. Literature review for off-targets of chymase inhibitors revealed their link to various biological pathways such as complement system, intrinsic, and extrinsic pathways of coagulation cascade, fibrinolytic system, and tissue kallikreins (Fig. 5). These biological pathways were further examined using KEGG pathway maps. These maps also showed the association of off-targets to complement system, coagulation cascade, and fibrinolysis. Thus, exploration of Kegg pathways validated the results from literature review. This phase helped us to explore the effects of using chymase inhibitors on different pathways through their interactions with corresponding off-targets which are as following:

Role of MASP-2 and C1s receptors in complement system and associated diseases

The panel of off-targets for chymase inhibitors includes MASP-2 also known as mannose-binding protein-associated serine protease 2 which is an enzyme that in humans is encoded by the MASP2 gene. MASP-2 showed high functional correlation with chymase enzyme among other off-targets of chymase inhibitors identified in this study. MASP-2 is involved in the complement system which is a complex series of more than 30 proteins (soluble and membrane bound) that has a pivotal role in innate immunity. Complement system has three initiating mechanisms known as the classical, lectin, and

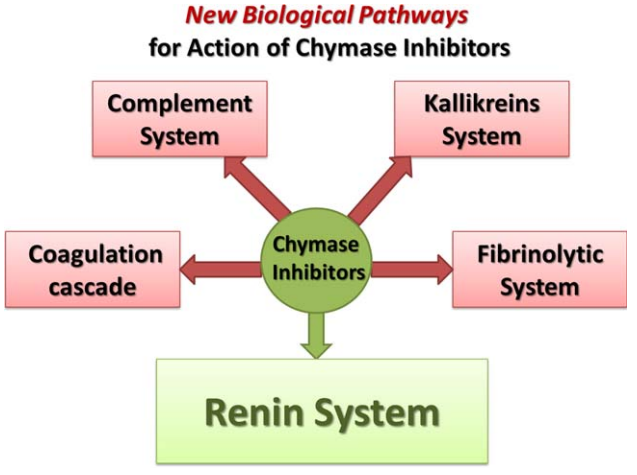

Figure 5

Depiction of biological pathways for human chymase inhibitors. Red arrows indicate new biological pathways for human chymase inhibitors revealed from literature survey of off-targets. While green color arrow is depicting target biological pathway already known for human chymase inhibitors. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

alternative pathways. Activation of MB lectin pathway of the complement system occcurs when MB lectin binds to carbohydrate structures on microorganisms. Due to autoactivation of the MASP-2, which then cleaves complement factors C4 and C2, C3 convertase C4bC2b is generated. Due to activation of C3, alternative pathway is initiated and membrane-attack complex is formed.[35] Complement C1s component (C1s) is another off-target of chymase inhibitors which belongs to the complement system initiating the first step of the classical pathway of complement activation to form C1. C3-convertase plays a vital role in pathways of complement system by triggering a cascade of cleavage and activation events. Defects in C1s leads to complement component C1s deficiency thus causing severe immune complex disease with features of systemic lupus erythematosus and glomerulonephritis.[36,37] MASP2 deficiency causes autoimmune manifestations, recurrent severe infections, and chronic inflammatory disease. MASP-2 inhibitors completely block the activation of the lectin pathway which is the initiating mechanism of complement system.[38] Therefore, chymase inhibitors may play important role in treatment of MASP-2 and C1s's associated diseases along with the diseases caused by biological systems to which these off-targets are linked such as complement system.

Role of coagulation factor VII (FVII), thrombin, and coagulation factor XI (FXI), in coagulation cascade and associated diseases

Literature survey showed that three of the off-targets for chymase inhibitors identified in this study are linked to coagulation cascade. Clot formation is a complex process which is performed by blood coagulation involving many proteins. Coagulation cascade can be initiated by two separate initiation mechanisms known as the intrinsic and extrinsic pathway. All functional components of the intrinsic pathway are blood borne, while the tissue factor which activates extrinsic pathway is found in extravascular tissue. The off-target FVII initiates the extrinsic pathway of blood coagulation. FVII is found as the inactive zymogen, however a very small amount of activated form FVIIa is also present in circulation.[39] As damage to the blood vessel occurs, FVII forms an activated complex (TF-FVIIa) with tissue factor (TF), which further activates FX to form FXa. In extrinsic pathway, FXa and its co-factor FVa form the prothrombinase complex, which activates prothrombin to thrombin which is also an off-target of chymase inhibitors. Thrombin then activates other components of the coagulation cascade, including FV and FVIII. The main function of thrombin is the conversion of fibrinogen to fibrin, the building block of a hemostatic plug.[40] Factor XI which is another off-target of chymase inhibitors triggers the middle phase of the intrinsic pathway of blood coagulation by activating factor IX. The lesser part that the intrinsic pathway has in instigating clot formation can be demonstrated by the fact that patients with severe deficiencies of FXII do not suffer from bleeding disorder.[41] In contrast, this system shows more participation in inflammation. The whole coagulation pathway, consisted of several steps of zymogen to enzyme conversions are certainly very nested and complex. Nevertheless, FX activation which is the point of convergence for the extrinsic and intrinsic pathways, has been regard as the rate limiting step for thrombin generation.[42] The formation of an insoluble network of fibrin is the final endpoint of both coagulation pathways. Disorders of coagulation can steer the risk of bleeding (hemorrhage) or obstructive clotting (thrombosis).[43] All three off-targets of chymase inhibitors including FVII, thrombin, and FXI are important in coagulation cascade. FVII initiates the extrinsic pathway of blood coagulation, therefore, inhibitors of FVIIa could be used to reduce bleeding and for the treatment of thrombosis.[44] Due to the key role of thrombin in arterial thrombogenesis, the aim of most treatment procedures is to block thrombin generation or inhibit its activity. Thrombin inhibitors block its interaction with its substrates thus inhibiting both soluble thrombin and fibrin-bound thrombin.[45] While the selective inhibitors of FXI cause disruption in the intrinsic coagulation pathway thus making FXI a therapeutic target thromboembolic disease.[46] Identification of FVII, thrombin, and FXI as off-targets and their link to coagulation cascade signifies that chymase inhibitors may have a role in treatment of hemorrhage and thrombosis diseases.

Role of uPA, and tPA in fibrinolysis and associated diseases

Fibrinolysis regulates the degradation of the fibrin network into soluble products by the function of the key enzyme plasmin. Plasmin cuts the fibrin mesh at diverse points thus instigating the generation of circulating fragments that are cleared by other proteases or by the kidney and liver.[47] Initiation of the fibrinolytic system can be accomplished by either tPA or uPA (Fig. 6). Activation of plasmin initiates a proteolysis cascade that play role in the degradation of extracellular matrix and thrombolysis. High expression levels of uPA and several other components of the plasminogen activation system are determined to be associated with tumor malignancy. This links uPA to vascular diseases and cancer as well.[48] It is believed that the tissue degradation following plasminogen activation assists in tissue invasion hence contributing to metastasis. This makes uPA an attractive drug target and, so, inhibitors have been required to be employed as anticancer agents.[49] A previous study also showed that binding of small molecular inhibitors to the uPA considerably inhibited the migration and invasion of pancreatic cancer cells *in vitro*.[50] tPA plays participates

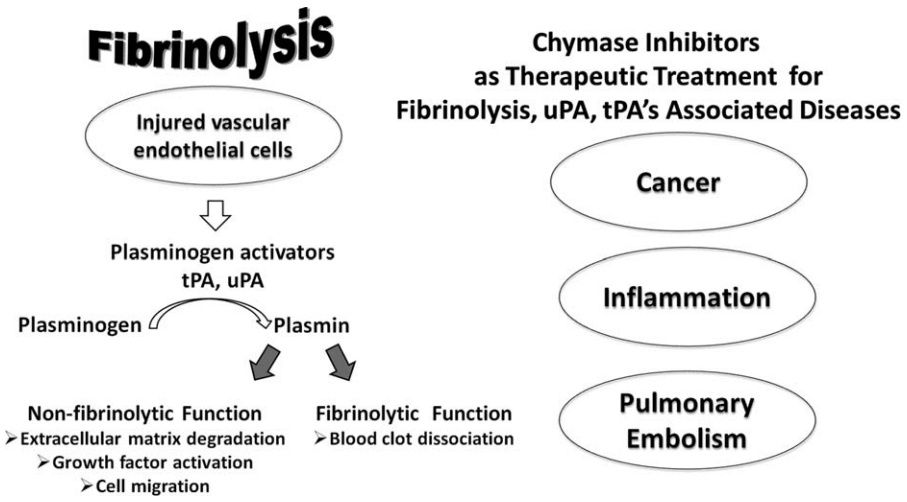

Figure 6

Role of uPA, and tPA in activation mechanism of fibrinolytic system and associated diseases such as cancer, inflammation and pulmonary embolism.

in tissue remodeling and degradation, in cell migration and many other physiopathological events. tPA is used in diseases that characterize blood clots, for instance pulmonary embolism, myocardial infarction, and stroke, in a medical treatment called thrombolysis.[51] As fibrinolytic system is activated by tPA and uPA, therefore, inhibitors of these enzymes can affect the cell migration and tissue remodeling processes. Detection of uPA, and tPA as off-targets and their role in fibrinolysis advocates the potential role of chymase inhibitors in the pathways associated with these off-targets.

Kallikreins (KLKs) and associated diseases

Tissue kallikreins are the major group of serine proteases in the human genome and are known to participate in proteolytic cascades. Proteolytic cascades are implicated in many physiological processes, such as blood coagulation, food digestion, apoptosis and others.[52] Three tissue kallikreins KLK4, KLK5, and KLK6 are identified in this study as off-targets of chymase inhibitors. KLK4 and KLK5 are expressed in the prostate and are considered important for regulating semen liquefaction through hydrolysis of seminogelin.[53] Literature survey revealed that proteinase activated receptors (PARs) are activated by KLK5, KLK6, and KLK14.[54] Activation of PARs by KLKs may contribute to prostate cancer progression. The use of KLKs inhibitor compounds may be helpful to down regulate the activity of KLKs in the cancer cells.[55] The predominant expression of KLK6 in brain cells suggested a potential involvement of this kallikrein in the development and progression of Alzheimer's disease (AD)as well.[56]

Other identified off-targets and associated diseases.

Another identified off-target Granzyme M (GZMM) is a novel serine protease whose expression is highly restricted to natural killer (NK) cells, $CD3^{+}CD56^{+}$ T cells, and γδ T cells. It has been suggested that this enzyme may play a role in the effector phase of innate immune responses.[57] Purified human GM was reported to induce cell death in Jurkat cells with ROS production, caspase activation, ICAD cleavage and DNA fragmentation[58] or in the absence of any apoptotic feature. Leukocyte elastase or neutrophil elastase (Elastase-2) is another type of off-target which has broad substrate specificity. It is secreted by neutrophils and macrophages during inflammation and destroys bacteria and host tissue.[59] Elastase 2 may play a role in degenerative and inflammatory diseases by its proteolysis of collagen-IV and elastin of the extracellular matrix. Aberrant expression of this important protease enzyme can cause emphysema or emphysematous changes.[60] Emphysema is called an obstructive lung disease because the destruction of lung tissue around smaller sacs, called alveoli, makes these air sacs unable to hold their functional shape upon exhalation. Experimental models have shown the role of elastase inhibitors in suppressing the formation of atherosclerotic plaques.[61] Trypsin-3 which is also known as mesotrypsin is an enigmatic minor human trypsin isoform showing resistance to natural trypsin inhibitors such as soybean trypsin inhibitor (SBTI) or human pancreatic secretory trypsin inhibitor (SPINK1).[62] Mesotrypsin is upregulated in advanced prostate cancer and other cancers including nonsmall cell lung cancer, colon cancer, and breast cancer.[63] Mesotrypsin expression is

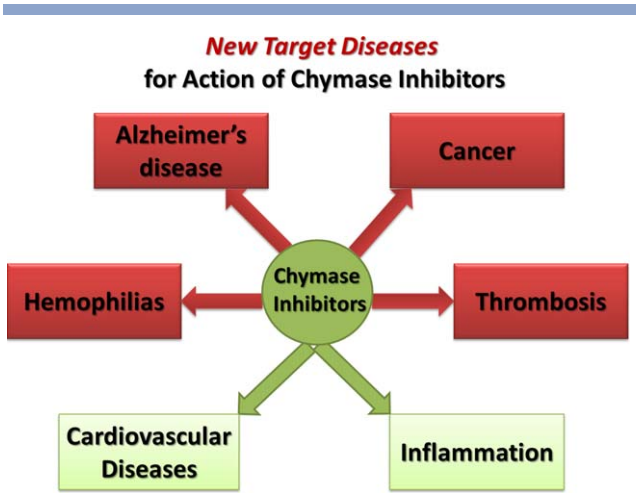

Figure 7

Target diseases for human chymase inhibitors. Red arrows indicate new target diseases for human chymase inhibitors revealed from literature survey of off-targets and their linked biological pathways. While green color arrows are showing target diseases already known for human chymase inhibitors. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

predictive of prostate cancer recurrence and promotes malignant morphology, invasion, and metastasis of prostate cancer in 3D culture assays and mice.[64] Reducing mesotrypsin expression in human prostate cancer tumors implanted into mice dramatically reduces tumor metastasis in the animals.[65] These novel off-targets including tissue kallikreins (KLK4, KLK5, and KLK6), neutrophil elastase, and trypsin-3 discovered may lead to the chymase inhibitors being repositioned as a therapeutic treatment for its off-target's associated diseases such as emphysema, prostate cancer, and AD.

Finally, 13 off-targets and their biological pathways were elucidated meticulously and their link to various diseases was explored (Fig. 7). We have built a hierarchical biological network that connects chymase inhibitors to their predicted off-targets, biological pathways and associated diseases (Fig. 8). Previous experimental studies have also shown the direct or indirect influence of chymase and its inhibitors on the identified biological systems and their associated diseases. A study on heparin-deficient mice exhibited that MC chymase:heparin system controlled the main mechanism of thrombin and plasmin regulation in this cellular system thus playing a key role in regulation of extravascular coagulation and fibrinolysis.[66] Another work reported by Gunnar *et al.* showed the inactivation of thrombin by mast cell secretory granule chymase thus suggesting the potential role of chymase inhibitors in thrombosis.[67] Like the predicted pathways including complement, Kallikrenins, fibrinolytic systems and blood coagulation cascade, chymase enzyme also play a key role in the inflammatory diseases. A study on the main tissue factor pathway inhibitor (TFPI) of blood coagulation exhibited the chymase-mediated proteolysis of TFPI.[68] The results from this research work clearly show the functional role of chymase in inflammation. Moreover, activation of MMP-9 by chymase suggests the importance of chymase inhibitors in degradation and remodeling of extracellular

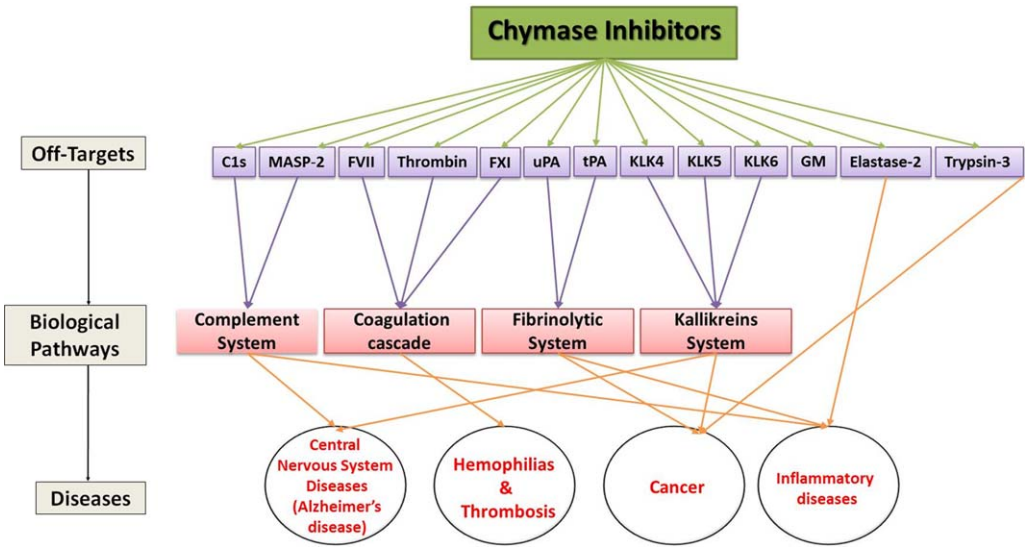

Figure 8

A hierarchical biological network connecting chymase inhibitors to their identified off-targets, biological pathways and associated diseases. Green color arrows indicate binding of human chymase inhibitors to their off-targets. Purple color arrows have shown associations of these off-targets to their corresponding biological pathways. While, brown color arrows depict linkage of biological pathways to the diseases in which these pathways are involved.

matrix during inflammation. Kakimoto *et al.* evaluated the impact of a chymase inhibitor on the indomethacin-induced small intestinal damage in rats and demonstrated the significance of chymase inhibitors in the process of intestinal damage via down regulation of MMP-9.[69] Inhibition of cathepsin G and chymase also affected the processes of platelet aggregation and inflammation in a mouse model.[70] Moreover, a previous study reported how degranulation of chymase facilitated the activation of complement pathway after acid aspiration.[71] Chymase also contribute to the early innate immune response via inactivation of bioactive chemerin.[72] All these experimental findings support the predictions of the computational approach applied in this study. Thus, the predicted network can help us in exploring the impact of administering chymase inhibitors on different biological pathways through their interactions with identified off-targets.

DISCUSSION

Overview of computational strategy for off-targets identification

A systematic approach to predict novel drug targets of human chymase inhibitors has been introduced that integrates both *in silico* modeling and experimental data. The structural proteome-wide computational strategy applied in this study for off-targets identification is depicted in Figure 2. Firstly, the chymase binding pocket was used to search against proteins using the SMAP software. The resulting list of putative off-targets is subjected to the functional similarity measured between chymase and the off-targets using GO relationships found with the FunSimMat web server. Furthermore, docking studies were carried out on the ligand binding sites of off-targets that bind chymase inhibitors using GOLD and LigandFit Docking softwares. In order to find protein–protein interaction networks, an interaction analysis was also performed on the chymase and its off-targets in this study using STRING database. Finally, literature survey was performed to incorporate these off-targets into various biological pathways. The results suggested that the systems biology-based approach may facilitate identification of on-target and off-targets effects of a new drug which can be determined in the early stages of the drug development cycle. Analysis of adverse drug reactions (ADRs) of a drug on a human subject is very important and has become matter of attention in recent years. Wide-ranging *in vitro* Safety Pharmacology Profiling can be very costly (in the order of about $3000–$10,000 for a panel of 50–100 targets).[73] To reduce this, there is growing interest in *in silico* methods, which do not need any underlying functional knowledge, to predict possible ADRs. Approach applied in this study will also improve understanding of drug efficacies and assist in forecasting safety leading to reduced costs of drug development and drug attrition rates. We escalate the scale of the chemical genomics approach beyond sequence and fold similarity by searching for similar ligand binding sites in this study. Thus a ligand binding site-based strategy will present an ever improving way to develop a candidate list of proteins contributing in interconnected biochemical pathways and to determine their relationships to biological processes. It is expected that this approach will ultimately offer the basis for the *in silico* simulation of the effect of small molecules on biological systems. It represents significant ideas for preclinical research and may have imperative applications for development of novel drug discovery strategies including drug combinations.

Discovering off-targets

Proteins function in networks and generate complex cellular and larger-scale phenomena. Consequently, if a drug is applied to a system it instigates many different processes. Therefore, drugs with efficacy predicted only from their specific target-binding experiment may not have the same outcome in clinical treatment owing to interactions between pathways in the disease network. In general, there is huge amount of information available associated with genes and proteins and their interactions. Moreover, there is a multitude of chemogenomics databases that describe modulation of proteins by small molecules. When used collectively, these data sources facilitate sophisticated systems biology and data analysis approaches. To predict novel drug off-targets, current computational endeavors have used ways including protein structural similarity, chemical similarity,[14] or side effect similarity.[74] Here we present a computational strategy which incorporates ligand binding site and functional similarities along with large-scale molecular docking analysis of putative off-targets and chymase inhibitors for the prediction of novel targets for chymase inhibitors. In first step, the crystal structure of chymase was used to detect common binding sites for chymase inhibitors on human structural proteome scale by SMAP. Although, several web servers for ligand binding site search are available. In comparison with these web servers, SMAP has a number of distinctive features which make it particularly appropriate to distinguish off-targets on a structural genome-wide level. For instance, SOIPPA algorithm of SMAP web server aligns two structures in the spirit of local sequence alignment, but independent of the sequence order.[13] Consequently, the location and boundary of the ligand-binding site does not need to be pre-defined. This feature is essential for real world applications as information on the ligand-binding site maybe unidentified. SMAP can compare two biological units that may include multiple chains. This is significant as binding sites maybe positioned in the homo- or hetero-dimer interface. Lastly, SMAP detects the similarity

between two binding sites through the combination of geometrical fit, residue conservation and physiochemical similarity.[13] The statistical importance of the similarity is predicted using an extreme value distribution model.[75] Summing-up all above-mentioned features collectively within a parallel computing environment means that SMAP-WS is competent enough to perform an all-by-all comparison of binding sites for a complete structural proteome. Several predictions resulting from SMAP-WS have also been validated experimentally. Among 3402 hits, 65 structures were found to have good binding site similarity with SMAP p-value less than $1.0e^{-3}$ in this study.

In order to further improve the quality of our SMAP results, FunSimMat web server was used in the next step to detect possible functional correlation between chymase and its potential off-targets. FunSimMat is a comprehensive database providing various precomputed functional similarity values for proteins in UniProtKB and for protein families in Pfam and SMART. The current FunSimMat release 3.1 contains almost 8.4-million proteins from UniProtKB (release 15.3) and 26.9-million GO annotations of proteins extracted from UniProtKB and from GOA (release of May 2009). Different methods like GO Graph, DynGO, FSST, Gene Functional Similarity Search Tool (GFSST), and FuSSiMeG have been suggested for computing the functional similarity between gene products. However, these tools either require the user to build a local database or to download a large database before the functional similarity can be computed on the own computer. Furthermore, some databases allow functional similarity searches but restrict the user to either the human or the mouse proteome.[76] Another web service reports the functional similarities between two GO terms annotated to each of them, but the results lack a combined score.[77] However, the results table from FunSimMat returns different scores, thus, FunSimMat provides a novel way of performing rapid functional similarity searches within large protein databases. From 65 putative off-targets which were short-listed form results of SMAP were given as input to FunSimMat. The 32 of-targets had detectable similarities with a rfunSimAll score above 0.5 (Supporting Information Table 2).

In the third step of this work, these 32 putative off-targets were finally filtered by molecular docking studies. Discovering potential small-molecule drugs by assessing if, where, and how well they fit to a target receptor has become increasingly important over the years. However, reliable predictions of ligand binding modes with biological targets, the so-called docking problem, are of major importance in drug design. Molecular docking can evaluate any protein with a solved structure due to its virtual nature, without the need for tailoring enzymatic assays or collecting drugs in solutions. Currently, many molecular docking programs are available including DOCK,[78] AutoDock,[79] LigandFit,[24] GOLD,[80] FlexX,[81] Glide,[82] ICM,[83] and Surflex.[84] Among these, two protein–ligand docking programs including GOLD and LigandFit were applied in the docking studies of off-targets. GOLD is popular commercial software which has been evaluated for docking pose predictions with many target proteins. In a comparison study between various docking programs, GOLD showed best performance in predicting binding pose among the tested programs. The program could consider side chain flexibility and local backbone movement of protein with the full range of ligand conformational flexibility during docking. The best poses of the chymase inhibitors at the active sites of off-targets with highest GOLD fitness score were selected for further docking analysis using docking software LigandFit. LigandFit gives the best poses at the binding site by a stochastic conformational search and the energy of the ligand–protein complex. It uses a grid method when evaluating interactions between the protein and the ligand. Scoring of the docked ligand conformations is done with a variety of different scoring functions implemented in the Cerius2 program (see Materials and Methods). Consensus scoring,[85] combination of multiple scoring functions, might dramatically reduce the number of false positives by its distinct scoring functions. Thus, the consensus scoring method of LigandFit was used to filter putative off-targets for their binding affinity and finally off-targets with best binding affinity for human chymase inhibitors were selected. Thus, various scoring and ranking thresholds were applied to remove false positive predictions through molecular docking studies.

Finally, literature survey was carried out to incorporate the identified off-targets into various biological pathways and to establish links between pathways and particular adverse effects. Off-targets of chymase inhibitors are linked to various biological pathways such as classical and lectin pathways of complement system, intrinsic and extrinsic pathways of coagulation cascade, and fibrinolytic system. Kegg pathway analysis also showed the involvement of the identified off-targets in these biological pathways thus validated the outcomes from literature survey. All these identified biological pathways perform a wide variety of biological functions in the human body. For instance, complement pathways hold a key relation between infection and diverse local or systemic inflammatory or autoimmune diseases.[86] The complement system is also becoming more and more connected with diseases of the central nervous system such as Alzheimer's disease and other neurodegenerative conditions for instance spinal cord injuries.[87] While, the primary function of coagulation cascade is to promote hemostasis and limit blood loss in response to tissue injury.[88] Tissue kallikreins, granzyme M, neutrophil elastase, and mesotrypsin are also identified as off-targets. As, all the potential off-targets and their associated pathways are revealed for the effects of diseases like inflammation, cancer, hemorrhage, and thrombosis. Therefore, this study can be very helpful to reposition chymase inhibitors for treatment of off-target's

associated diseases and to develop novel therapeutic strategies including drug combinations. Although the current study focused on a specific enzyme, the general methodology is extensible to other systems as well.

Towards multitarget drug design at system level

Drugs are often multitargeted, and a recent study proposed that the average number of target proteins per drug is 6.3.[89] It was observed that cancer drug therapy might change the behavior of nearly 1000 different proteins, suggesting that the disturbance of a signaling network through a single protein may affect other proteins. Recently, system wide approaches are increasingly being considered. Combination therapy showed success in diseases such as AIDS, atherosclerosis, cancer, and depression.[90] Although attacking more than one "strategic" point of the system might be a useful approach, however, off-target effects may result in adverse drug reactions that account for around one-third of drug failures during development and may contribute to idiosyncratic drug-induced damage to tissues. Putative off-targets have been recognized through diverse computational techniques, such as docking, ligand structure similarity, pharmacophore mapping, ligand binding site similarity, side effect similarity, and text mining.[91] Proteins with similar binding sites often recognize similar ligands. Earlier works suggested that even weak binding to multiple targets may have profound effects on the biological system.[92] The effects of drugs on known or unsuspected targets present both opportunities and challenges for modern drug discovery. Keeping this in view, we also incorporated off-targets identified in this study into various biological pathways to explore the implications of administering chymase inhibitors on different pathways through their interactions with corresponding off-targets. On the whole, this study represents the imperative role of systems biology approaches in drug design.

CONCLUSIONS

We see systems biology approaches as the decisive part of future development in drug design. The vision would be to assimilate all predictive methods with data from various biological databases. Particularly, the ever escalating public accessibility of data on drugs and drug-like molecules can make analyses similar to that described in this research exertion possible for scientists in the drug design sector. Here, we develop a robust computational strategy that is applicable to any enzyme system and that allows the prediction of drug effects on biological processes. First, binding pocket of the target enzyme chymase was used to search against 5985 PDB structures of human proteins or homologous of human proteins using the SMAP software. The resulting list of putative off-targets is subjected to the functional similarity measured between chymase and the putative off-targets. Protein–ligand docking studies were carried out on the ligand binding sites of off-targets using structurally diverse chymase inhibitors with their varied experimentally known IC_{50} data. A STRING interaction analysis was also performed on the chymase and its off-targets to find protein–protein interaction networks in this study. Finally, literature survey was carried out to incorporate these off-targets into various biological pathways. By analyzing a set of off-targets that share a common binding active site and by incorporating them into the pathways they affect we are able to establish links between pathways and particular adverse effects. We further link these predictive results with literature data in order to explain why a certain pathway is predicted. Off-targets of chymase inhibitors are linked to various biological pathways such as classical and lectin pathways of complement system, intrinsic and extrinsic pathways of coagulation cascade, and fibrinolytic system. Tissue kallikreins, granzyme M, neutrophil elastase, and mesotrypsin are also identified as off-targets of chymase inhibitors. These off-targets and their associated pathways are elucidated for the affects of inflammation, cancer, hemorrhage, thrombosis, and central nervous system diseases (AD). Prospectively, our approach is valuable not only to better understand the mechanisms of chymase inhibitors but also for drug repurposing exercises to find novel uses for these inhibitors. This strategy may also have important applications and implications for preclinical research and for development of novel therapeutic strategies including drug combinations.

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