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Identification of FDA-approved drugs as novel allosteric inhibitors of human executioner caspases

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ABSTRACT

The regulation of apoptosis is a tightly-coordinated process and caspases are its chief regulators. Of special importance are the executioner caspases, caspase-3/7, the activation of which irreversibly sets the cell on the path of death. Dysregulation of apoptosis, particularly an increased rate of cell death lies at the root of numerous human diseases. Although several peptide-based inhibitors targeting the homologous active site region of caspases have been developed, owing to their non-specific activity and poor pharmacological properties their use has largely been restricted. Thus, we sought to identify FDA-approved drugs that could be repurposed as novel allosteric inhibitors of caspase-3/7. In this study, we virtually screened a catalog of FDA-approved drugs targeting an allosteric pocket located at the dimerization interface of caspase-3/7. From among the top-scoring hits we short-listed five compounds for experimental validation. Our enzymatic assays using recombinant caspase-3 suggested that four out of the five drugs effectively inhibited caspase-3 enzymatic activity *in vitro* with IC_{50} values ranging ~10-55 μM. Structural analysis of the docking poses show the four compounds forming specific non-covalent interactions at the allosteric pocket suggesting that these molecules could disrupt the adjacently-located active site. In summary, we report the identification of four novel non-peptide allosteric inhibitors of caspase-3/7 from among FDA-approved drugs.

INTRODUCTION

Apoptosis or programmed cell death is a vital biological process essential for the elimination of unwanted cells, and maintenance of tissue homeostasis in multicellular organisms. Physiological processes such as embryonic development, normal cell turnover and adult tissue maintenance, immune system function, are closely regulated by apoptosis. Consequently, defects in regulation of apoptosis is commonly associated with various medical conditions such as neurodegeneration, ischemic damage, autoimmune diseases, and certain types of cancers [1,2]. Critical regulators of apoptosis are a family of **c**ysteine-dependent **asp**artic-specific endoprote**ases** called *caspases*. According to their recognized roles in apoptosis, caspases are further sub-classified into initiators (caspase-2, -8 and -9) and executioners (caspase-3, -6, and -7). Among the executioners, caspase-3 is regarded as the primary effector of apoptosis acting as a global mediator essential for multiple proteolytic events [3,4]. Active caspase-3 cleaves an array of protein/peptide substrates triggering apoptosis-associated events such as chromatin condensation and margination, DNA fragmentation, and demolition of structural proteins eventually leading to the death of the cell [5]. Owing to its central role in driving apoptotic pathways, inhibition of caspase-3 has been looked upon as a potential therapeutic strategy to halt progression of a number of diseases including Alzheimer's disease, Parkinson's disease, and stroke [6-8].

Within cells caspase-3 exists as a stable but inactive dimeric zymogen or procaspase and is activated via proteolytic cleavage by initiator caspases resulting in a small and a large subunit. Mature caspase-3 molecules are dimers of heterodimers comprising two copies each of the small and large subunits. The substrate-binding region, highly-conserved among caspases, is formed by rearrangement of the active site loops L1-L4 from one heterodimer and L2′ from the other, forming two distinct active sites in each molecule [9]. Several peptide-based caspase inhibitors have been developed which target the substrate-binding/catalytic site

(orthosteric) [10]. However, the orthosteric mode of inhibition has the inherent disadvantage of high competition from caspase-3's diverse indigenous substrates [11]. Most peptide-based inhibitors have also been regarded as mediocre drug candidates for use as pharmacological modulators owing to their poor metabolic stability and cell membrane penetration, thus largely restricting their use to research [12]. On the other hand, exploring an allosteric mode of inhibition presents the possibility of overcoming competitive interference from substrates, and presents with opportunities for the development of small-molecule-based inhibitors with better pharmacological characteristics [13,14].

The first allosteric site in caspases was discovered by Hardy et al. in caspase-3 employing a site-directed fragment-based ligand discovery approach referred to as disulfide tethering [15]. Using said method two classes of thiol-containing molecules namely, 2-(2,4-dichlorophenoxy)-*N*-(2-mercapto-ethyl)-acetamide (DICA) and 5-fluoro-1*H*-indole-2-carboxylic acid (2-mercapto-ethyl)-amide (FICA) were found to irreversibly inhibit enzymatic activity of caspase-3. Mutational studies, mass spectrometry and peptide mapping studies univocally showed that both DICA and FICA formed a disulfide bridge with a single non-catalytic, surface-exposed cysteine residue (Cys264) located in the central cavity at the dimer interface. Both molecules were also shown to inhibit caspase-7 which shares 53% sequence identity with caspase-3. Structural studies clearly demonstrated the binding of DICA and FICA to the allosteric central cavity *via* covalent interaction with Cys290 in caspase-7 (structurally-equivalent to caspase-3 Cys264) in a two ligands per dimer ratio. The allosteric site now referred to as exosite A, with bound DICA, along with the active site in capsase-7 are illustrated in Figure 1A. In the crystal structure, two molecules of DICA are symmetrically bound to the allosteric central cavity anchored to the site via specific intermolecular disulfide bridges formed with Cys290 (one from each monomer/chain). Binding of DICA breaks an important cation-π interaction (Figure 1B) between Arg187

(large subunit) and Tyr223 (small subunit) which in turn disrupts the organization of the adjacent substrate-binding pocket. Consequently, substrate binding to the active site is affected rendering the enzyme inactive. Structurally, with respect to the conformation of Tyr223 and L2′, the allosterically inhibited caspase structure closely resembles that of the inactive procaspase (Figure 1C) [16].

Although several compounds have exhibited the ability to inhibit caspase-3 activity *in vitro* and in animal models, very few molecules have successfully managed to cross pre-clinical and clinical trials due to their inherent toxicity [17,18]. Therefore, it seems worthwhile to look for potential caspase-3 inhibitors among FDA-approved compounds and understand the mechanism of inhibition with the objective of repurposing them for alternative therapeutic approaches. Thus, we virtually screened a catalog of FDA-approved drugs by docking for their ability to bind to the allosteric site in caspase-7, which is practically identical to the one found in caspase-3 (Figure 1B & C). From among the top hits identified in the virtual screening we selected five compounds for experimental testing their ability to inhibit caspase-3 activity. Four out of the five compounds, namely diflunisal, flubendazole, fenoprofen, and pranoprofen, showed remarkable inhibition of caspase-3 activity *in vitro* both in recombinant caspase-3 activity assay and cell-based assay. In summary, in the present study we demonstrate the value of *in silico* structure-based virtual screening augmented with *in vitro* experimental validation for repurposing FDA-approved drugs as novel allosteric inhibitors for a pharmacologically important but poorly druggable target, namely caspase-3/7.

MATERIALS AND METHODS

Preparation of Target Structure for Docking. No structure of caspase-3 with an allosteric ligand bound in the central cavity is currently available. However, caspase-3 and -7 share high sequence identity (53%) and structural similarity with all 16 residues projecting into the

central cavity being identical or conservatively-substituted (Figure S1). Thus, we reasoned that ligands binding to caspase-7 would interact with caspase-3 in a similar fashion and be able to break the important Arg-Tyr cation-π interaction. Thus, in this study we targeted the allosteric site in the crystal structure of caspase-7 solved in dimeric form irreversibly complexed with DICA [PDB ID: 1SHJ[15]] in virtual screening. We also screened the same chemical library against the orthosteric site in the caspase-7 structure solved in complex with the peptide inhibitor AC-DEVD-CHO [PDB ID: 1F1J] [19]. Prior to virtual screening, the target structures were prepared by adjusting the side-chain rotamer of Cys290 in chain A of 1SHJ (allosteric site) and Cys186 in chain A of 1F1J (orthosteric site) based on the Dunbrack backbone-dependent rotamer library [20] breaking the Cys290-DICA and Cys186-AC-DEVD-CHO disulfide bridges, respectively, so that steric clashes between their side-chain sulfur atoms and ligands being sampled could be avoided. Subsequently, all non-protein atoms from the coordinate file the coordinates were removed.

Virtual Screening. The ZINC database is a freely available collection of over 400 compound catalogs (e.g., DrugBank[21], Therapeutic Target Database (TTD)[22]) sourced from over 300 vendors. We screened an annotated catalog of FDA-approved drugs (updated in Feb, 2013) obtained from the ZINC 12 database [23,24] against the allosteric exosite A in caspase-7 (PDB ID: 1SHJ, chain A) and the orthosteric site (PDB ID: 1F1J, chain A) using DOCK 3.6 [25]. We aimed to identify drug molecules that bind preferentially to the allosteric dimer interface of caspase-3/7 instead of the orthosteric site. For the allosteric site we considered approximately half the molecular surface of exosite A (Figure 1A), formed by residues primarily from chain A of caspase-7, for docking. A total of 45 matching spheres were generated based on the crystallographic coordinates of DICA (PDB ID: 1SHJ, chain A) for the allosteric site and AC-DEVD-CHO (PDB ID: 1F1J, chain A) for the orthosteric site, which were used to guide the docking of ligands to the site.

Docking using SwissDock. The four compounds listed in Table 2 were docked to monomeric caspase-3 [PDB ID: 2DKO] and dimeric caspase-7 [PDB ID: 1SHJ] structures using the SwissDock webserver (http://www.swissdock.ch/docking) [26,27]. The entire surface of the proteins was considered for docking. Favorable binding mode (BMs) clusters were interpreted on the basis of their reported Full Fitness scores which are calculated based on CHARMM force field.

Cell Culture and Reagents. HEK293 cells were obtained from NCCS, Pune, India and cultured in DMEM-containing 10% FBS, 1x antibiotic and antimycotic solution, and 2mM L-glutamine at 37°C and 5% CO_2 in humid atmosphere. All the test compounds used in this study were procured from Sigma-Aldrich (India). We purchased staurosporine from Alfa Aesar (India) and Z-VAD-FMK from MedChem Express (USA).

Caspase-3/7 Activity Assay. For the caspase 3/7 activity assay $10x10^3$ HEK293 cells were seeded on a 96 wells plate (opaque, white) on day 0. Next day, medium was removed from each well and then pre-treated with test compounds for 90 minutes followed by treating the cells with staurosporine. Cells were incubated for 24 h and caspase 3/7 activity assay was performed as per manufacturer's instruction. Readings were measured using luminometer (Berthold).

Caspase-3 Enzyme Inhibition Studies. Recombinant human caspase-3 enzyme was obtained from Enzo Life Sciences (BIOMOL cat. # SE-169), and Caspase-Glo® 3/7 assay reagent from Promega, USA. 2U of enzyme was used per well in solid bottom and opaque 96-well-plate containing a reaction mixture formed with 10mM HEPES and 0.1% BSA. Reduction in the luminescence reveals the magnitude of inhibition caused by the test compounds from cleaving Z-DEVD-aminoluciferin by recombinant caspase-3 enzyme.

Statistical Analysis. All data are presented as the standard error of mean (S.E.M) of at least two independent experiments. Statistical comparisons of apolar and polar desolvation values, and curve-fitting for sigmoidal dose-response were carried out using unpaired *t*-test, and one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test, respectively, using GraphPad Prism, version 5.0. p-value < 0.05 was considered statistically significant.

RESULTS

Virtual Screening. Crystal structures of caspase-7 with DICA and FICA [PDB ID: 1SHJ and 1SHL, respectively] [15] and Comp-A, a Cu-containing pyridinyl compound [PDB ID: 4FEA] [28] revealed the existence of an allosteric site in caspases symmetrically occupied by two ligand molecules. We selected the structure of caspase-7 dimer inactivated by the binding of DICA as the target protein in our virtual screening strategy for identification of allosteric inhibitors. We wanted to identify ligands that could bind reversibly to the dimer interface in caspase-3/7 in a two ligands per dimer ratio similar to the binding of DICA, FICA, and Comp-A to caspase-7. Thus, we considered a sub-region of the allosteric central cavity (highlighted in Figure 1A) that surrounds one of the two bound DICA molecules (from chain A) and docked a catalog of FDA-approved drugs obtained from ZINC 12 database [23,24] to this site using DOCK 3.6 [25]. To find compounds with preference to the allosteric site over the orthosteric site, we also docked the FDA catalog to the orthosteric site in the caspase-7 structure solved in the presence of the orthosteric peptide-inhibitor AC-DEVD-CHO [PDB ID: 1F1J] [19]. The docked conformations of the FDA compounds were scored based on an energy function incorporating Poisson-Boltzmann electrostatic, van der Waals, and ligand desolvation penalty terms. Following virtual screening, we sorted the docked compounds based on their docking scores and compared the compounds docked to the orthosteric and allosteric sites to identify any distinguishing features the compounds might possess that might

play a role in their preference for either site. We observed that the top 25 orthosteric site-bound ligand candidates had an acidic group (carboxylate, sulfoxylate, sulfonate or equivalent) that formed salt-bridges simultaneously with Arg87 and Arg233. The acidic group of a ligand candidate could potentially mimic the aspartate residue of the peptide sequence recognized by caspases and thus maybe essential for recognition and binding to the orthosteric site. In contrast, although the allosteric site has two basic residues (Arg187 and Lys212'), only five instances were observed where ligand candidates with an acidic or equivalent group interacted with either residue. The observation suggests that the presence of an acidic moiety in the ligand candidate could potentially be critical for binding to the orthosteric site. Such a conclusion is further supported by the observation that, on average, at pH 7 the top 25 orthosteric ligand candidates had a net charge of -1.16 ± 0.47 compared to only -0.28 ± 0.67 for the allosteric ligand candidates. Another interesting distinguishing feature emerged between the two classes of ligand candidates when we compared their respective apolar and polar desolvation values. While the top 25 orthosteric ligand candidates had apolar and polar desolvation values of 0.84 ± 6.47 kcal/mol and -70.89 ± 30.47 kcal/mol, respectively, the allosteric ligand candidates had values of 4.25 ± 2.91 kcal/mol and -35.12 ± 20.98 kcal/mol, respectively. The differences observed between the two groups of ligand candidates are statistically significant (two-tailed P value = 0.0202) for apolar desolvation and extremely statistically significant (two-tailed P value = 0.0001) for polar desolvation values.

Considering the findings discussed above, from among the top 100 hits (sorted based on docking scores) for the allosteric site we considered only those compounds that did not appear in the top 100 hits for the orthosteric site. In total, five such compounds were selected from the virtual screening for experimental testing via a hit-picking process. Hit-picking involves critical observation, review, and evaluation of the docked poses by multiple

investigators following which a number of ligands from the top-scoring hits are prioritized for purchase and testing. This candidate ligand selection was done by considering their docking ranks, intermolecular polar interactions, and their chemical similarity with respect to DICA and FICA measured by Tanimoto coefficient (Tc) values. The five compounds are flubendazole, L-tryptophan, fenoprofen, diflunisal, and pranoprofen, all dissimilar from DICA and FICA with Tc values less than 0.4. We also did not consider those FDA-approved compounds that have been previously reported to possess caspase-inhibitory activity [29]. Details of five selected compounds along with their docking ranks for the allosteric and orthosteric sites are summarized in Table 1, and the 2D-structures of the same are provided in the supplementary information (Figure S2).

Inhibition Studies on Recombinant Human Caspase-3 Enzyme.

To test the caspase-3 inhibitory activity of screened hit compounds, Caspase Glo-3/7 assay (Promega) was performed using purified recombinant human caspase-3 enzyme. Since previous studies have ruled out the possibility of NSAIDs inhibiting the activity of luciferase, we did not perform further studies on that prospect [29]. Initial screening was performed using 50 μM concentration of each compound and tested against caspase-3 enzyme activity. Diflunisal and pranoprofen showed maximal inhibition when compared to fenoprofen and flubendazole. L-tryptophan did not show any significant inhibitory activity. AC-DEVD-CHO, a pan-caspase inhibitor, was used as positive control (Figure 2A). The inhibitory activity of the five compounds was studied for up to 60 minutes to estimate their time-dependent inhibitory activity (Figure 2B).

Further studies on dose-dependent inhibition of compounds also showed a maximum inhibition exhibited by diflunisal compared to pranoprofen, flubendazole, and fenoprofen (Figure 3A-D).

To ascertain the IC_{50} values of the inhibitors, compounds were again tested with various concentrations ranging from 1 nM to 500 µM. All the four compounds showed caspase-3 inhibition in time- and concentration-dependent manner (Figure 4). However, we did not observe any decreasing enzymatic activity with increase in the concentration of flubendazole beyond 10 µM concentration. The IC_{50} value of all the compounds have been tabularized in Table 2.

Structural Analysis of Docked Caspase-Ligand Complexes

From Table 2 it is clear that diflunisal exhibits the best inhibition profile against caspase-3 *in vitro* followed by flubendazole, fenoprofen and pranoprofen. In order to speculate on the structural basis of the inhibitory capacity of the tested compounds, we analyzed the various non-covalent interactions formed between the docked compounds and the residues of the allosteric pocket. The docking poses of the four compounds showing caspase-3 inhibitory activity are illustrated in Figure 5. From the figure it is evident that all four compounds form extensive intermolecular non-covalent interactions within the allosteric binding site of caspase-7 and could potentially interact with caspase-3 in a similar fashion.

Our analysis revealed the presence of various intermolecular interactions such as hydrophobic contacts, π-π, cation-π, salt-bridge, and hydrogen bonds in the docked complexes (Figure 5). Overall the allosteric pocket in each monomer can be considered as having an interior hydrophobic and an exterior polar region. The hydrophobic region comprises Tyr211, Ile213, Tyr223, Cys290, Val292, Val215, and Val292, whereas the polar region includes Glu146, Glu147, Arg187 and Lys212. All four compounds, in their docked conformations, are aligned such that one of their aromatic moieties is buried in the hydrophobic region of the allosteric pocket while their polar moieties are favorably positioned towards the exterior polar region. Based on this observation it could be speculated

that L-tryptophan did not show any inhibitory activity owing to its smaller size preventing it from fully occupying the allosteric pocket.

All four compounds showing inhibitory activity have two aromatic moieties both of which form π-stacking interactions with Tyr211 and Tyr223. Except flubendazole, docked conformations of the other three compounds show the presence of a cation-π interaction with Arg187 suggesting that an ability to engage with Tyr223 while displacing Arg187 could be crucial for effecting caspase-3 inhibition. Although the docked conformation of flubendazole doesn't form the cation-π interaction with Arg187, it forms multiple H-bonds with Glu147, which in turn could stabilize its interaction with caspase-3. It could thus be speculated that binding of the inhibitory compounds could break the intramolecular cation-π interaction between Arg187 and Tyr223 observed in active caspase-3/7 (Figure 1B) and engage the residues in intermolecular interactions (Figure 5), thereby disrupting the organization of the active site similar to DICA (Figure 1C). The binding of the four compounds could also be further favored by the formation of salt-bridge/hydrogen bonds with residues such as Glu146, Glu147, and Lys212′. In summary, it could be suggested that small-molecules that possess the features to disrupt the Arg187-Tyr223 interaction and engage these residues in favorable interactions (*via* aromatic moieties) along with Glu147 (*via* hydrogen bond donors), and Lys212′ could act as allosteric inhibitors of caspace-3/7.

NSAIDs as Caspase Inhibitors

It is interesting to note that three out of the four FDA compounds capable of inhibiting caspase-3 activity, namely diflunisal, pranoprofen, and fenoprofen, are non-steroidal anti-inflammatory drugs (NSAIDs). We also observed other NSAIDs among the top 100 hits from our virtual screening, such as fenbufen (allosteric site rank: 11, orthosteric site rank: 76), and flufenamic acid (78, 272) which have been shown to have anti-caspase activity

by Smith et al [30] but suggested that the NSAIDs could bind to the orthosteric S1 subsite. In order to ascertain whether the compounds identified in our study could also bind to the orthosteric site, employing an identical docking strategy as Smith et al, we carried out docking calculations with the four compounds against monomeric caspase-3 [PDB ID: 2DKO] and dimeric caspase-7 [PDB ID: 1SHJ] structures using SwissDock [26,27]. Analysis of the docking results showed the top-ranked binding mode (BM) cluster (Cluster0) with the best Full Fitness score located at a novel site (other than allosteric or orthosteric) for diflunisal and fenoprofen, and at the orthosteric site for flubendazole and pranoprofen when the monomeric caspase-3 structure was used as the receptor (Figure S3A, B, C, & D). With caspase-3, the BM clusters for all four ligands were located predominantly in orthosteric site. Some BM clusters were also observed at the partially obscured allosteric site in the case of fenoprofen and pranoprofen (Figure S3C & D). With the dimeric caspase-7, the top-ranking BM cluster (Cluster0) was located in the allosteric pocket for all four ligands. Overall, BM clusters were located in both subsites of the allosteric pocket, the orthosteric site, and various other novel sites (Figure S3E, F, G, & H). The location of the top five BM clusters (Cluster0-4) observed when using the monomeric caspase-3 and dimeric caspase-7 structures as receptors are summarized in Table S1.

Analysis of Hit Compounds for Caspase-3/7 Inhibition Activity in HEK293 cell line.

To confirm the caspase-3 inhibitory activity of the four compounds *in* vitro, we assayed the compounds against HEK293 cells treated with staurosporine. Staurosporine is a pan-kinase inhibitor and a well-known activator of caspase-3 activity. Our results showed that while all four compounds exhibited inhibition of staurosporine-induced caspase-3 activity in HEK293 cells, diflunisal at 50 µM concentration exhibited maximal inhibition (Figure 6).

DISCUSSION

Structure-guided virtual screening is a well-established approach and has been used successfully in numerous earlier studies for the identification of allosteric modulators of important pharmacological targets [31,32]. In the present study, we prospected a catalog of FDA-approved drugs using a structure-based virtual screening approach and identified novel reversible allosteric inhibitors targeting the human caspase-3/7. We adopted a computational docking/screening strategy that specifically identified compounds that exhibit a preference for the allosteric exosite A on the dimer interface over the orthosteric site of caspase-3/7. We observed differences in structure, net charge, desolvation values suggesting that the orthosteric site prefers highly polar and relatively less hydrophobic ligands compared to the allosteric site. *In vitro* functional assays of capsase-3 enzyme activity showed that four out of the five short-listed FDA-approved compounds, namely diflunisal, pranoprofen, flubendazole, and fenoprofen exhibited inhibitory capabilities, with diflunisal and flubendazole showing remarkable potency. Based on the docking poses observed for the compounds, it could be suggested that intermolecular π-π stacking interactions with Tyr211 and Tyr223 and salt-bridge/polar interactions with Glu147, Arg187 and Lys212 of exosite A could favor ligand binding. The essential role of π-π stacking, cation-π interactions and other non-covalent interactions in ligand binding has been extensively studied in numerous protein-ligand systems [33-37]. Based on these insights gleaned from the docking calculations performed using SwissDock we posit that the ligands identified in our study could interact differently with caspase-3/7 depending upon their chemical nature and the oligomeric state of the protein. We also suggest that when caspase-3/7 molecules exist in their dimeric form, the ligands could preferentially bind to the allosteric site followed by the orthosteric site. The observation of BM clusters occupying both subsites of the allosteric pocket when using a dimeric caspase-7 structure as the receptor further strengthens our assertion that the compounds could bind in a two molecules per dimer fashion (Figure S3, Table S1). The

differences in the chemical nature of diflunisal and flubendazole versus fenoprofen and pranoprofen in terms of presence/absence of halogen atoms or carboxylate moiety (discussed in the Results section) could shed light why the former have relatively superior inhibitory effect on caspase-3 and exhibit greater preference for the allosteric site over the orthosteric site as compared to the latter (Table 2 & Table S1).

Since the reports on DICA and FICA the dimerization sites of executioner caspases have been targeted for designing non-peptide caspase inhibitors, but no small-molecules have been approved for use as drugs against caspases yet [38]. Unlike DICA and FICA, which binds irreversibly to caspase-3/7, compound-A reported by Feldman et al interacts reversibly with the dimerization site of executioner caspase-7 [39]. Recently, Spies and co-workers [40], using a fragment-based drug discovery approach, identified a series of compounds exhibiting allosteric inhibitory capabilities against caspase-7 (IC_{50} values 637-8520 μM). Two of these compounds share good structural similarity with three compounds tested in our study (Tc value 0.4-0.7) and are found to occupy part of the allosteric pocket at the dimer interface in caspase-7. Results from the study support our conclusion that the compounds identified in our study could indeed bring about reversible allosteric inhibition by binding to the dimer interface of caspase-3/7 and, and that these FDA-approved drugs can be taken further as therapeutic leads. However, we acknowledge the possibility that some of compounds could also act on the orthosteric site, and robust biochemical and crystallographic evidence is needed to unambiguously ascertain the binding site preferences and mode of action of these compounds.

To our knowledge this is the first study to reporting the ability of FDA-approved drugs to act as allosteric inhibitors of caspase-3 targeting exosite A. The combined *in silico-in vitro* approach presented here could help circumvent the limitations of traditional peptide-based inhibitors such as poor pharmacological properties and metabolic stability. The

pharmacophores of the newly-identified inhibitors could also help in the identification/design of specific and more potent caspase inhibitors. For example, a search of the ZINC 12 database for compounds with 70% structural similarity to diflunisal lists 176 compounds that are commercially available. Screening of these compounds could bring to fore new molecules that have better potency and selectivity. In summary, our study reports the identification of four FDA-approved drugs that could potentially inhibit caspase-3/7 activity allosterically and establishes a new direction for researchers to explore for rational drug design and development.

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Author Contributions

P.R., H.F., P.T., A.A. and K.D. conceived and designed the experiments. K.D. and A.A. performed the computational and experimental studies, respectively, and are co-first-authors contributing equally to the work. A.A., K.D., P.R., and H.F. analyzed and interpreted the data, and wrote the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

DICA, 2-(2,4-Dichlorophenoxy)-*N*-(2-mercapto-ethyl)-acetamide; FICA, 5-Fluoro-1*H*-indole-2-carboxylic acid (2-mercapto-ethyl)-amide; NSAID, non-steroidal anti-inflammatory drugs.

SUPPORTING INFORMATION

Supporting information available: Multiple sequence alignment of caspases (Figure S1); 2D structures of five compounds tested (Figure S2); Summary of SwissDock docking results (Figure S3 & Table S1)

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Figure legends:

Figure 1. (A) Structure of caspase-7 dimer solved in the presence of DICA (yellow). The allosteric central cavity/exosite A and orthosteric active site are depicted using translucent blue and green surface representations, respectively. In our virtual screening, we targeted the region of the allosteric pocket occupied by a single DICA molecule, highlighted with a black dotted line. **(B)** Superposed structures of substrate-mimic DEVD-bound active state caspase-3 (pink, PDB ID: 2DKO[41]) and caspase-7 (green, PDB ID: 1F1J) allosteric sites showing the presence of a conserved cation-π interaction between Arg164 and Tyr197 in caspase-3 and Arg187 and Tyr223 in caspase-7. Residue numbers corresponding to only caspase-7 are shown for brevity. **(C)** Superposition of structures of inactive zymogen states of procaspase-3 (pink, PDB ID: 4JQY[42]), procaspase-7 (green, PDB ID: 1GQF[43]), and DICA-bound inactive caspase-7 (orange, PDB ID: 1SHJ) allosteric site showing breakage of Arg-Tyr cation-π interaction. Binding of DICA breaks the cation-π interaction between Arg187 and Tyr223 resulting displacement of Arg and Tyr residues resembling inactive procaspase-3 and procaspase-7 zymogens. Residue numbers corresponding to only caspase-7 are shown for brevity.

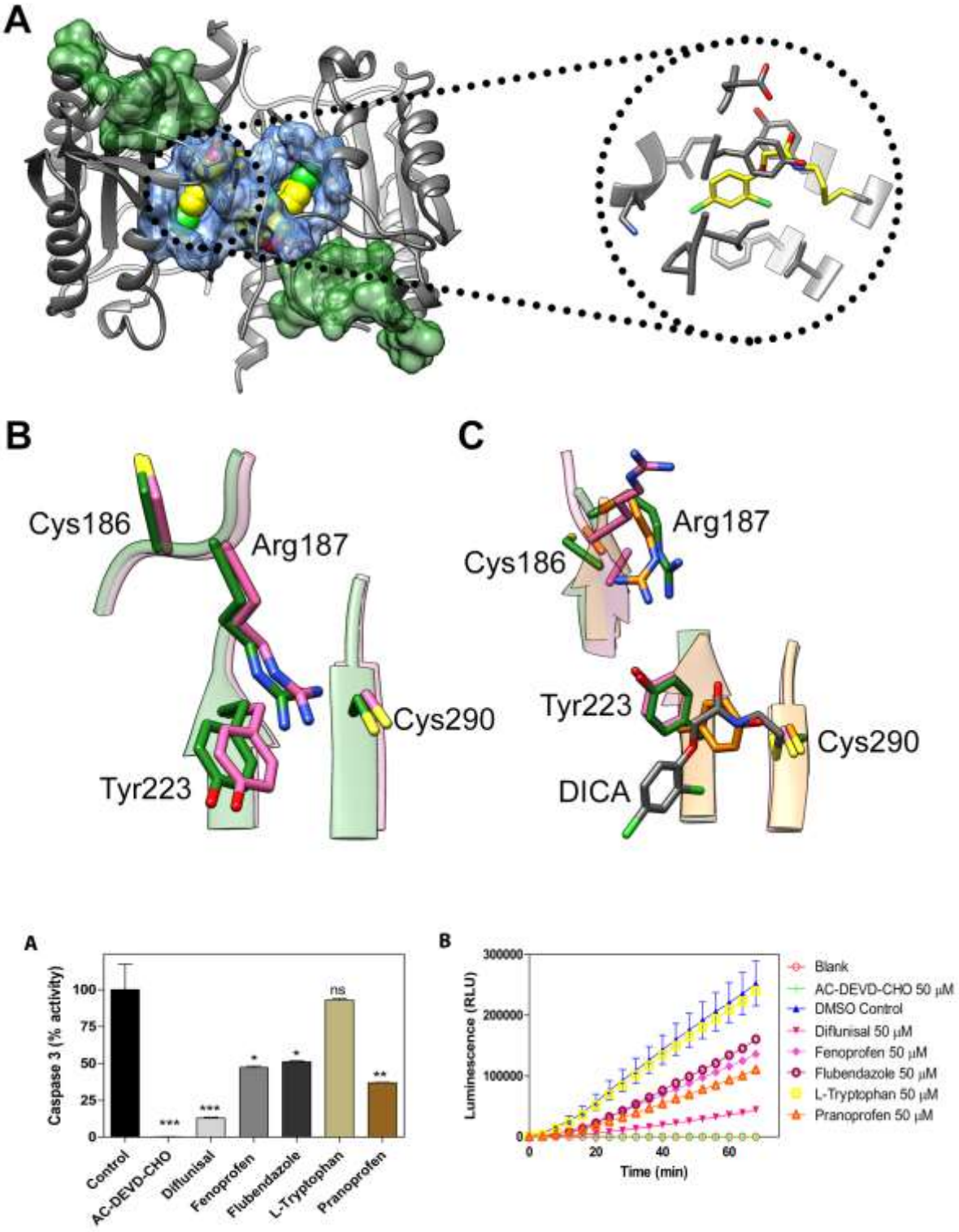
Figure 2. (A) Caspase 3/7 activity assay performed with recombinant human caspase-3 enzyme against the five selected compounds for inhibitory activity. **(B)** Time-dependent inhibitory activity of the test compounds. (AC-DEVD-CHO was used as a positive control). *represents significance between compound added wells compared to control well at $p<0.05$ (One-way ANOVA).

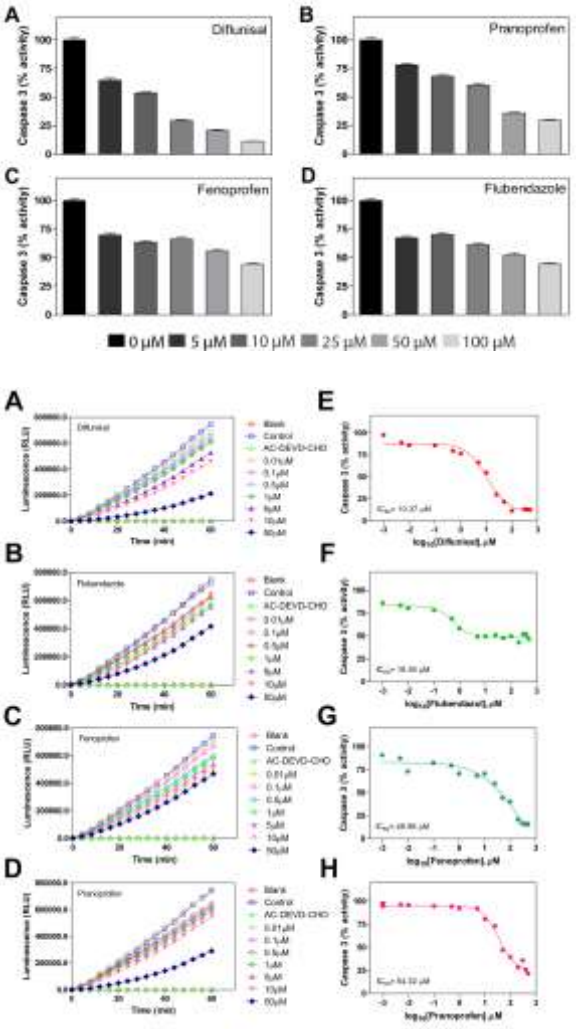
Figure 3. Dose-dependent activity of **(A)** diflunisal, **(B)** pranoprofen, **(C)** fenoprofen, and **(D)** flubendazole showing inhibition of caspase-3 enzymatic activity. Data represented as average S.E.M of experiment performed in triplicate.

Figure 4: Graphs showing the time- and dose-dependent inhibition by **(A)** diflunisal, **(B)** flubendazole, **(C)** fenoprofen, and **(D)** pranoprofen for a period of 60 minutes. Dose-response curves used for determination of IC_{50} values for **(E)** diflunisal, **(F)** flubendazole, **(G)** fenoprofen, and **(H)** pranoprofen.

Figure 5: Docking poses of the four compounds showing caspase-3 inhibitory activity, namely (**A)** diflunisal, (**B)** flubendazole, (**C)** fenoprofen, and (**D)** pranoprofen within the allosteric pocket in caspase-7 (PDB ID: 1SHJ, chain A). We superposed a modeled caspase-3 structure (template PDB ID: 1SHJ) onto the caspase-7 structure to show the structurally-equivalent residues from caspase-3 forming the allosteric pocket. Most residues of the pocket are identical or conservatively-substituted (Tyr211 to His185). Non-covalent interactions such as π-π stacking and cation-π are shown using dotted lines while salt-bridges and hydrogen bonds are shown using dashed lines. The geometric criteria for identifying the interactions have been adopted from Jain et al[44]. The active site C186 and allosteric site C290 are shown for reference. Residue numbers corresponding to only caspase-7 are shown for brevity.

Figure 6. Caspase 3/7 activity assay showing the fold increase of caspase 3/7 activity in Staurosporine (STS 1 µM) alone treated HEK293 cells and inhibition showed by pretreatment with screened compounds after 24h of incubation. * represents the significance between staurosporine alone treated cells to compounds pretreated cells at $p < 0.05$ (One way ANOVA).





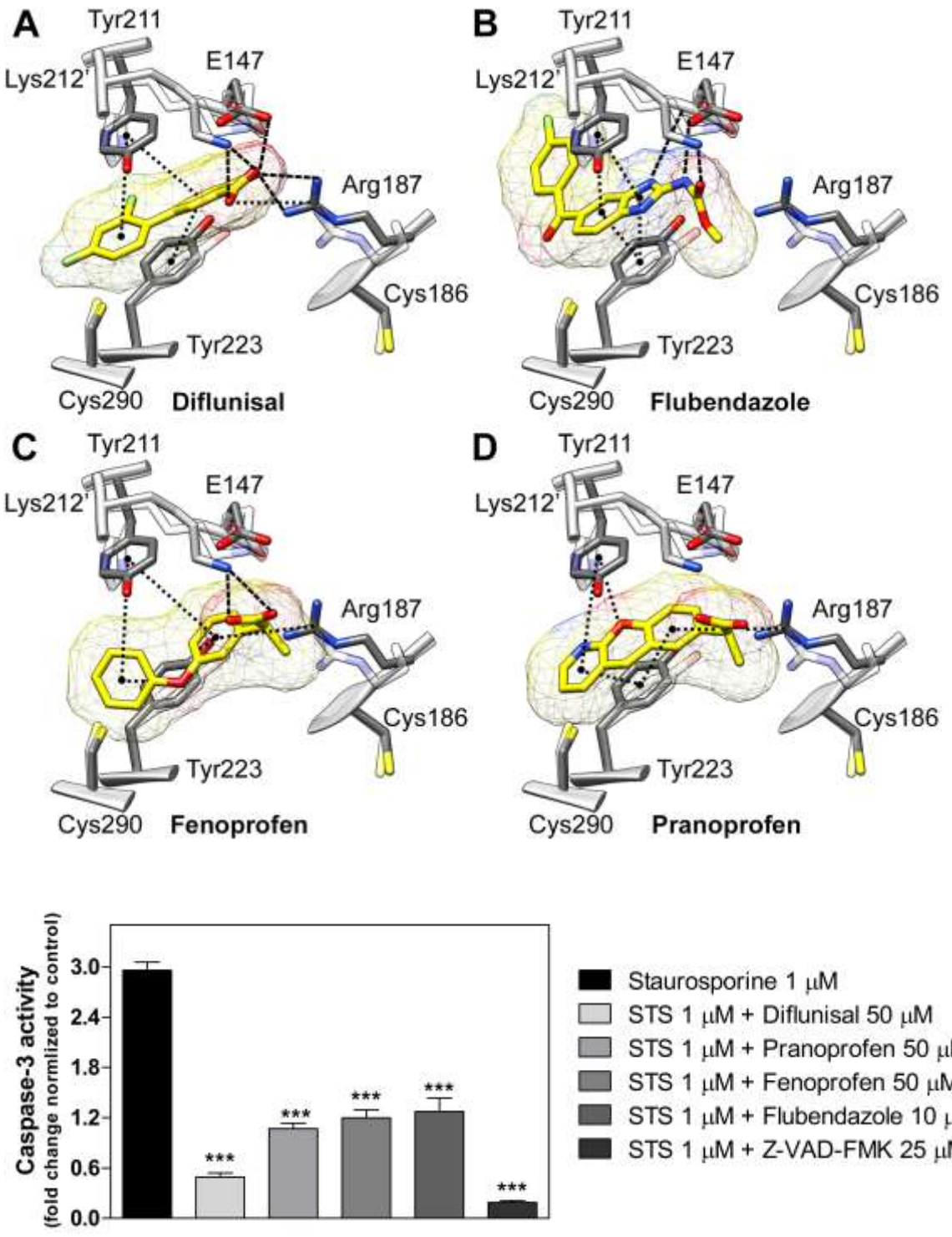


Table 1: Summary of the five FDA-approved compounds picked for experimental validation of caspase-3 inhibitory activity

S. No:	Compound Name	ZINC ID	Rank (Allosteric site)	Rank (Orthosteric site)
1.	Flubendazole	C03830847	1	459
2.	L-tryptophan	C00083315	44	-na-
3.	Fenoprofen	C00402909	62	139

4.	Diflunisal	C00020243	68	106
5.	Pranoprofen	C00000654	92	237

Table 2: IC_{50} values determined for the four hit compounds for caspase-3 inhibition activity

S. No:	Compound Name	Inhibition of caspase-3	
		IC_{50} (µM)	R^2
1.	Diflunisal	10.37 ± 0.5	0.9845
2.	Flubendazole	18.36 ± 4.23	0.9654
3.	Fenoprofen	46.98 ± 6.07	0.9657
4.	Pranoprofen	54.32 ± 0.6	0.9838