Identification and Characterization of Novel Caspase Inhibitors: The Unforeseen Effects of NSAIDs

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Abstract

Proper regulation of cell death and the inflammatory mechanism leading to cell death is vital for the development and homeostasis of living organisms. Dysregulation of cell death leads to cancer, neurodegenerative disorders, and inflammatory conditions such as sepsis. Caspases are major players of cell death. In gram-negative bacteria induced sepsis, LPS directly binds to caspase-4 and 5 to induce pyroptosis. With the hypothesis that inhibition of caspase-4 may be required to provide protection from sepsis, the first goal of this research is to identify and characterize novel small molecules that inhibit caspase-4. Additionally, nonsteriodal anti-inflammatory drugs (NSAIDs) provide analgesic and antipyretic effects, but the mechanism of their adverse side effects such as the gastrointestinal bleeding and development of ulcers is unknown. With the hypothesis that NSAIDs mechanism may involve caspases, the second goal of this research is to study the effect of NSAIDs on caspases. 3700 compounds from the Soft Focus Protease/Kinase Library (P/K) and Prestwick Chemical Library (FDA) were screened against caspase-4 activity using a Caspase-Glo enzymatic activity assay. Two compounds from P/K were identified as promising caspase-4 specific inhibitors. FDA screen revealed that 56% of the top caspase-4 inhibitors were NSAIDs. Further characterization of six diverse NSAIDs revealed NSAIDs are pan-caspase inhibitors and that their IC_{50} values are lower than the average concentration present in blood. Considering that the knockout of mice-homologous caspase-4 increased susceptibility of bacterial infection in mice gut and knockout of caspase-1 increased tumorogenesis in mice, NSAIDs being pan-caspase inhibitors has serious implications for NSAIDs' side effects. This study supports the hypothesis that caspases may be involved in the mechanism of side effects of NSAIDs.

Background

Caspases

Caspases are a family of cysteine proteases that are vital in programmed cell death. An extrinsic or intrinsic pathway, depending on the origin of the stimulus, can activate the caspase cascade. In the extrinsic pathway, extracellular signals such as tumor necrosis factors bind to their specific receptors on the cell membrane, triggering the oligomerization of a family of transmembrane adapter proteins called death proteins.[1] Death adapter oligomers further oligomerize procaspases, leading to auto-proteolytic activation of the initiator caspase-8 (figure 1). Activated initiator caspase cleaves and activates effector caspase-3, which activates cytokine-activating caspases, signaling cell death (figure 1).[2] On the other hand, the intrinsic pathway is mediated by mitochondria, which releases signaling molecules such as cytochrome c, in response to intracellular environment such as stress.[3] The signaling molecules form the apoptosome, which activates initiator caspase-9 and intersects the extrinsic pathway of caspase activation to lead to apoptosis (figure 1).

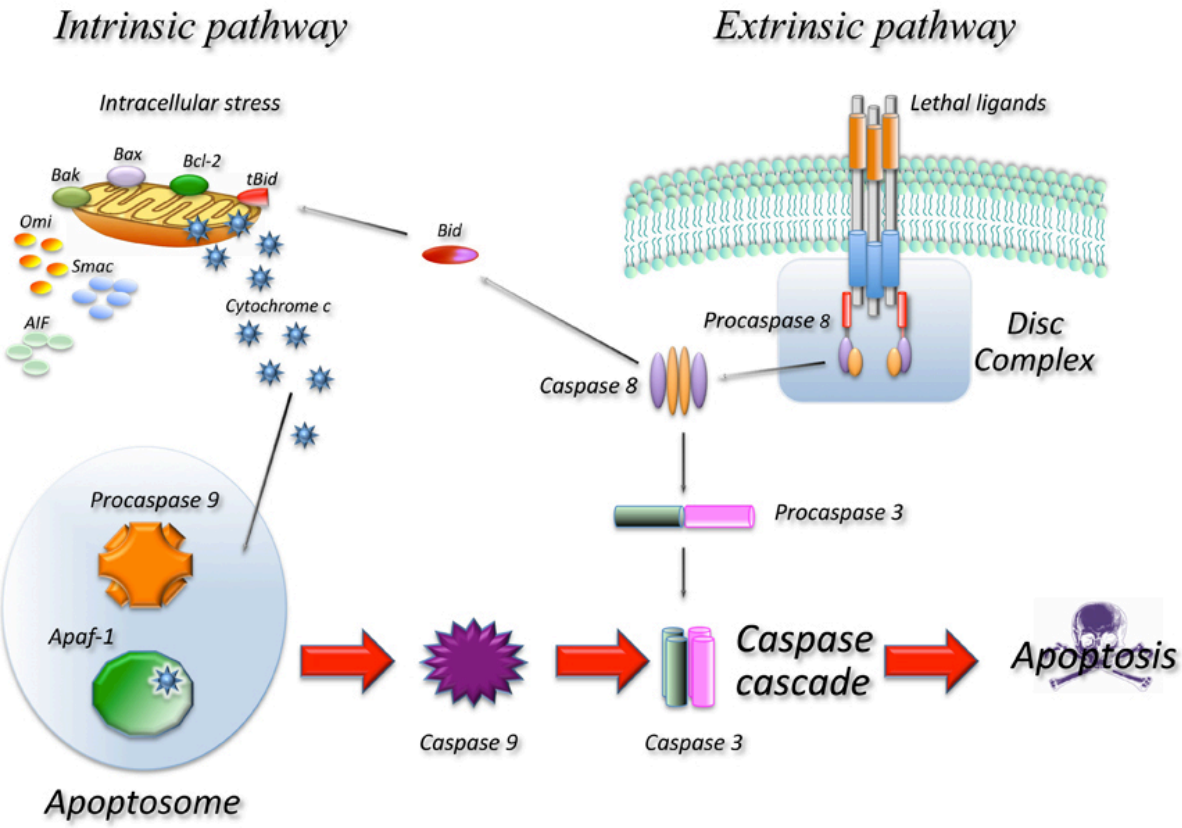


Figure 1: Schematic representation of the main molecular pathways leading to apoptosis. In the intrinsic pathway, mitochondrial release of cytochrome c forms apoptosome and activates caspase 9. In the extrinsic pathway, ligand binding to specific receptors forms DISC complex, activating capase-8. Both pathways converge to activate caspse-3 and trigger apoptosis. [4]

Fourteen distinct caspases have been identified in mammals, eleven of which are present in humans[5]. All caspases cleave their substrates after an aspartate residue, but are divided into families and subgroups based on their function and the amino acid sequence of the substrates they recognize, respectively (figure 2). This study closely studies caspase-9, which is an initiator caspase, caspase-3, which is an effector caspase, and caspases- 4 and 5, which are inflammatory

caspases. Despite being in different functional families, caspases-4 and 5 recognize the same substrate as 9 and 1 (figure 2).[6]

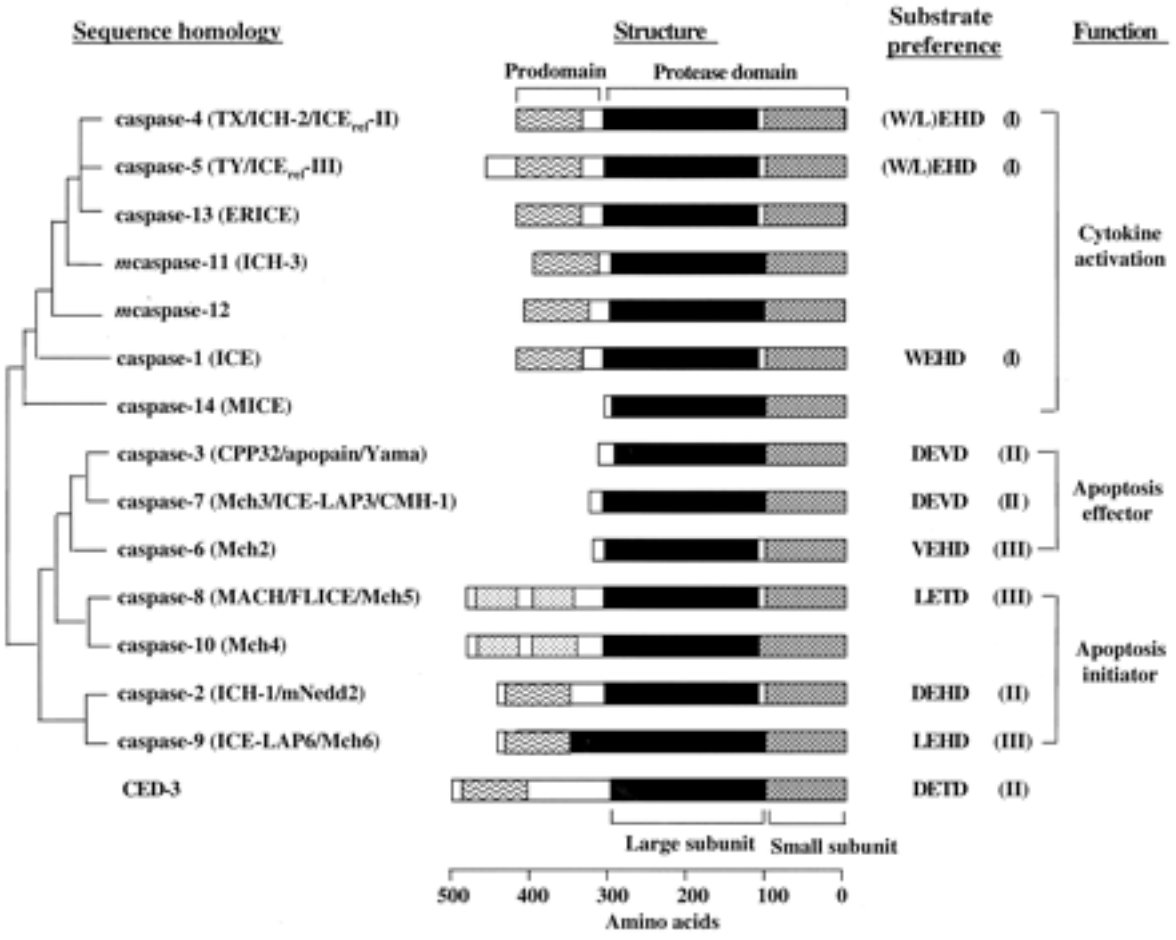


Figure 2: All mammalian caspases are of human origin, except for caspase-11 and 12, for which human counterparts do not exist. The structure of each caspase and its substrate preference at the P1 to P4 position are given. Caspases are also divided into families based on their functions.[7]

Caspases are an important area of study because dysregulation of cell death leads to cancer, neurodegenerative disorders, and autoimmune diseases.[8] Proper regulation of cell death and the inflammatory mechanism leading to cell death is vital for the development and homeostasis of living organism.

Sepsis

One of the conditions caused by disruption of the homeostasis of inflammatory mechanism is Sepsis. Sepsis is a serious medical condition caused by an excessive immune response to severe infections most common in the lungs, abdomen, and urinary tract.[9] It is common in immunocompromised individuals, elders, children, as well as in patients after invasive medical procedures. Even though fungi and viruses can also cause sepsis, gram-negative bacteria are the most common inducers.[10] Over-stimulation of the inflammatory response to the bacteria in the blood can cause blood clots and leaky vessels, preventing the transport of nutrients and oxygen, thus damaging the organs.[11] Sepsis affects 1.2 million people in the U.S. each year and costs $14.6 billion per year. It is the 10th leading cause of death in many western nations and the most common cause of death in the intensive care units.[12]

Toll-like Receptor and Sepsis

Toll-like Receptors (TLRs) are transmembrane proteins that recognize pathogen associated molecular patterns (PAMPs) such as molecular motifs conserved within microbes and endogenously expressed danger associated molecular pattern (DAMPs) such as heat shock proteins.[13] TLRs combat the presence of PAMPs or DAMPs by releasing a cascade of inflammatory molecules, however, over-active TLRs can lead to over-inflammation resulting in autoimmune diseases and various forms of allergies.[14] Human cells have 10 different TLRs, and each receptor recognizes unique molecular patterns that induce TLR specific inflammatory response.[15]Toll-like Receptor 4 (TLR4) recognizes lipopolysaccharide (LPS), present on the surface of gram-negative bacteria, and activates the production of inflammatory molecules necessary to fight off infection.[16]

The pathogenesis of sepsis has not been clearly elucidated, but the prominence of TLR4 in sepsis propagation is well established. The usage of anti-TLR4 antibodies significantly increases the survival rate of mice with lethal gram-negative sepsis.[17] Additionally, TLR4 expression increases on human monocytes in volunteers undergoing LPS challenge[18] and in patients with sepsis.[19]

Inhibitors for TLR4 such as TAK242[20] and Eritoran[21] exist, but they failed in phase III clinical trials due to lack of efficacy. This leads us to believe that other signaling pathways, in addition to TLRs, are also involved in sepsis propagation.

Caspases and Sepsis

Normally, when LPS binds to TLR4, it activates a signaling cascade leading to downstream activation of transcription factor, NF-κB, [22] which up-regulates the expression of caspase-4.[23] Recent studies have shown that LPS not only binds to TLR4 on the cell surface, but also enters the cytoplasm through cytoplasmic GTPase mediated lysis of pathogen containing vacuoles.[24, 25] Once in the cytoplasm, LPS directly binds to caspase-4/5.[26] This binding oligomerizes caspase-4 and leads to the activation of caspase-1 leading to pyroptosis.[27] Pyroptotic cell death produces inflammatory cytokines, which in the presence of high concentration of LPS, can surmount to be lethal. The massive host response through inflammatory mediators and procoagulant factors in the systemic circulation can lead to endothelial injury, tissue hypo-perfusion, intravascular coagulation, and refractory shock.[28] The breakthrough that LPS directly binds to caspase-4 and induces inflammation provides reasons to discover inhibitors of caspase-4 that would provide protection from sepsis in the presence of gram-negative bacteria.

Nonsteroidal Anti-Inflammatory Drugs and Caspases

Salicylic acid and salicylates, derived from nature, have long been used in medicine for antiseptic purposes. In 1900, aspirin was created as a more palatable form of salicylates. Since then, several aspirin-like drugs have been discovered and were categorized as non-steroidal anti-inflammatory drugs (NSAIDs).[29] Mechanistically, NSAIDs act by inhibiting cyclo-oxygenase-1 (COX-1) and cyclo-oxygenase-2 (COX-2), which reduces the synthesis of prostaglandins and is believed to provide pain-killing and fever-reducing effects.[30] In addition to aspirin, naproxen and ibuprofen are common NSAIDs.

NSAIDs are effective at reducing inflammation, but are also known for having adverse side effects, such as gastrointestinal bleeding[31] and increased risk of myocardial infarction and stroke.[32] However, the mechanism of side effects is unknown. NSAIDs have been shown to increase apoptosis[33] as well as inhibit cell death. Given the crucial role caspases play in cell death and considering that the knockout of caspase-11, the mice homologous to human caspase-4, disrupts gastrointestinal homeostasis similar to the side effects of NSAIDs in mice,[34] the mechanism of NSAIDs may involve caspases. While in vivo studies indicate inconclusive effects of NSAIDs on caspase-3 activity,[35,36] in vitro experiments to study the sole interaction between caspases and NSAIDs have never been performed. Therefore, with the aim of elucidating the mechanism of the side effects of NSAIDs, the second goal of this study is to examine the direct interaction between NSAIDs and caspases by characterizing their specificity and IC_{50}.

Summary and Objectives

Caspases are major players of cell death. Failure to maintain homeostasis of cell death results in various forms of cancer and inflammatory diseases such as sepsis. With the hypothesis that inhibition of caspase-4 may be required to provide protection from sepsis, the first goal of this research is to identify and characterize novel small molecules that inhibit caspase-4. Additionally, given the mechanistically unexplained side effects of NSAIDs and the hypothesis that NSAIDs mechanism may involve caspases, the second goal of this research is to study the effect of NSAIDs on caspases activity by characterizing their specificity and IC_{50}.

Methods

Big Picture

The Soft Focus Protease/Kinase Library (P/K) and Prestwick Chemical Library (FDA) of small molecules were screened against caspase-4 activity using a Caspase-Glo enzymatic assay in the University of Colorado high throughput screening facility. The compounds that reduced caspase-4 activity to 25% of the untreated control were considered to be top hits from each library. Top antagonists from each library were tested for caspase-4 specificity by performing Caspase-Glo enzymatic assay on caspase-3 and caspase-9. Compounds from P/K that specifically inhibited caspase-4 were further characterized to find the IC_{50} value with caspase-4. However, because top antagonists from FDA also happened to be NSAIDs that do not inhibit caspase-4 specifically, IC_{50} values of each NSAID with caspase-3, caspase-4, caspase-5, and caspase-9 were determined. In order to perform a comprehensive study of the effect of NSAIDs on caspases, at least one caspase from each caspase activity family was tested. While caspase-9

is an initiator caspase, caspase-3 is an effector caspase and caspase-4 is an inflammatory caspase. Additionally, caspase-4, caspase-5, and caspase-9 cleave the same substrate, thus ensuring that the top compounds do not also inhibit caspase-5, and caspase-9 is vital in discovering a caspase-4 specific inhibitor.

Expressing and Purifying Caspases

In order to prepare for high throughput screening, specificity test, and IC_{50} characterization, wild type human caspase-3, caspase-4, and caspase-9 were purified using the his-tag purification process; caspase-5 was bought from Enzo. A catalytically inactive caspase-4, with point mutation from cysteine to alanine on the 258^{th} residue, was also purified as an enzymatic control as well as a control for the purification process.

All constructs contained a C-terminal 6x His-tag, and were expressed in the *E. coli* strain BL21 pLysS using the pET expression system from Novagen. BL21 colonies carrying the plasmid of interest were expanded to 500 ml 2x TY culture volume, and induced to express the recombinant protein using IPTG. After protein expression, bacterial cells were sonicated to collect the lysate, which was then run through a column containing Ni^{+2} chelating resin. The His-tag on the C-terminus of the protein bound to the Ni^{+2} beads and eluting the column with buffers containing 50 mM Tris.Cl, 100 mM NaCl, and up to 200 nM imidazole purified the protein (figure 3). SDS-PAGE, such as in figure 3, was used to verify the purification of each caspase. For more details, the protocol outlined by Denault and Salvesen in Expression, Purification, and characterization of Caspases was used to express and purify all the caspases[37].

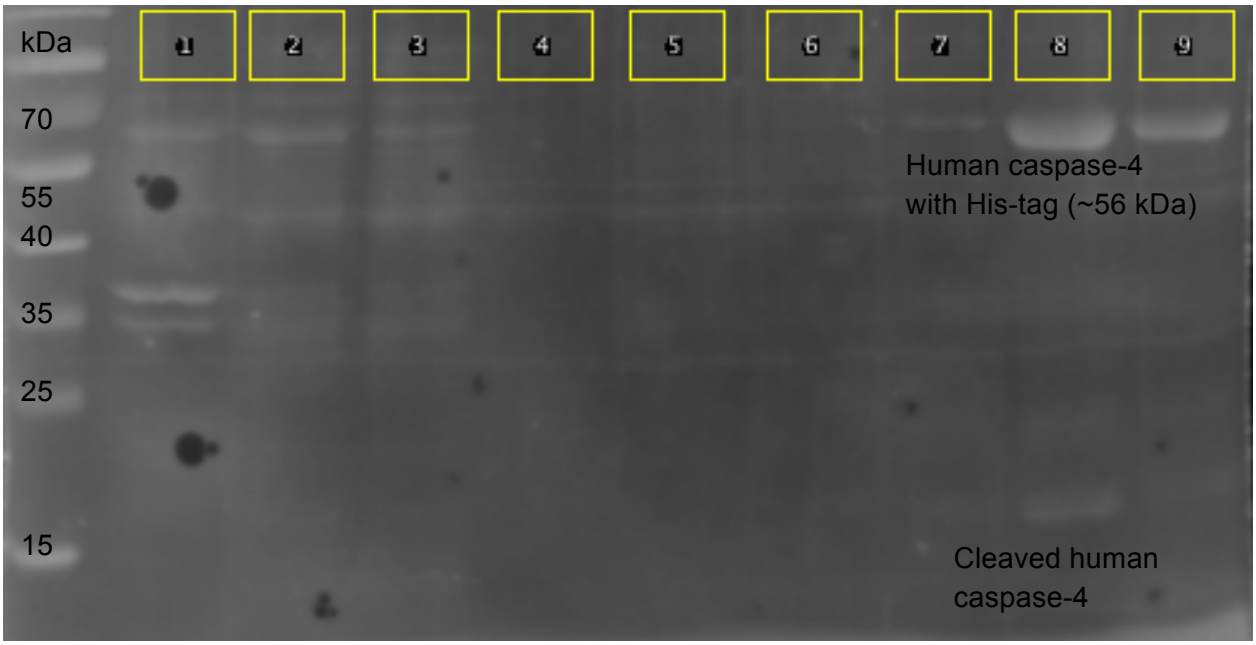


Figure 3: SDS-PAGE shows that 100 mM, 250 mM, and 1 M imidazole successfully elutes 56 kDa of mutant caspase-4 with histidine tag. 250 mM also elutes 20 kDa of activated caspase-4. 1) Pellet after centrifuging bacterial lysate 2) Supernatant after centrifugation 3). Supernatant unbound to the nickel column 4) Wash #1 of the protein-bound column 5) Wash #2 of the protein-bound column 6) Protein elution with 10 mM Tris, 25 mM NaCl, and 50 mM Imidazole 7) Protein elution with 10 mM Tris, 25 mM NaCl, and 100 mM Imidazole 8) Protein elution with 10 mM Tris, 25 mM NaCl, and 250 mM Imidazole 9) Protein elution with 10 mM Tris, 25 mM NaCl, and 1 M Imidazole

High Throughput Screening

In order to identify caspase-4 inhibitors, the Soft Focus Protease/Kinase Library (P/K) containing 2500 compounds and Prestwick Chemical Library (FDA) containing 1200 compounds were screened against caspase-4 activity. Measuring the luminescence produced in the Caspase-Glo assay assessed Caspase activity. In Caspase-Glo assay, a known substrate,

Promega CaspGlog LEHD, is added to caspase-4, which in its activated form cleaves the substrate and produces a molecule that luminesces in the presence of ATP and oxygen (figure 2). Thus, the strength of luminescence is directly proportional to the activity of the caspase.

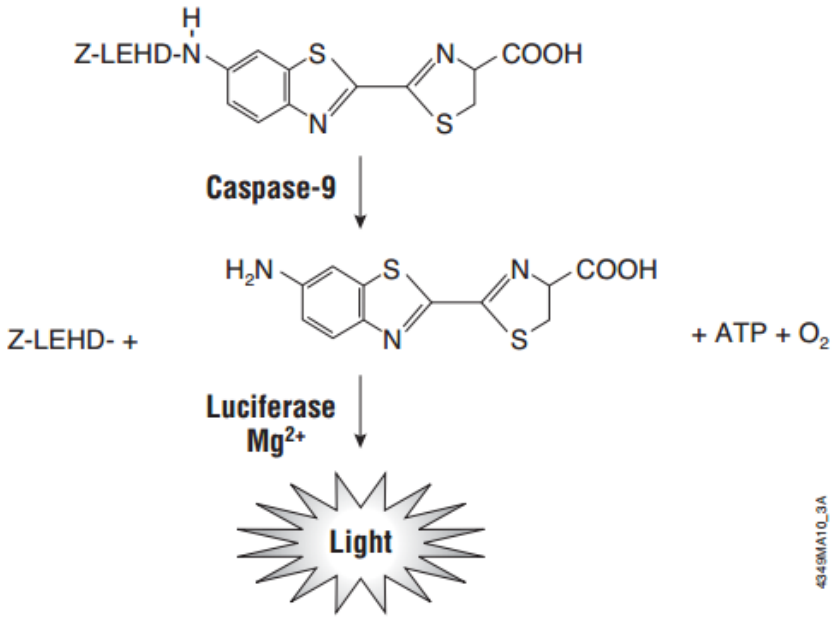


Figure 4: Mechanism of Caspase-Glo Assay: When Promega Caspase-Glo 9 LEHD substrate is added to caspase-9, the caspase cleaves the substrate, which reacts with ATP and oxygen to generate a luminescent signal. The signal emitted is proportional to the activity of the caspase. Although the figure is labeled caspase-9, it is also applicable to caspase-4 and caspse-5 because they also cleave the same substrate as caspase-9. Assays with caspases that recognize different substrates, such as caspase-3 and caspase-7, undergo the same mechanism except the substrate would contain DEVD sequence instead of LEHD[38].

In order to ensure the activity is measured at the maximum enzyme efficiency, the concentration of substrate must exceed the concentration of the enzyme. The activity of 10 nM caspase-4 with 3 μM substrate in the presence of 30 μM of different compounds in DMSO was

measured on white 384-well plates. Controls of the experiment included caspase-4 with and without substrate and with substrate and DMSO, substrate plus buffer, pan-caspase inhibitor z-VAD-FMK with substrate, and catalytically inactive mutant human caspase-4 with substrate. Z' factor of the screen was calculated using

$$\text{Z-factor} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}.$$

where p stands for positive control, and n stands for negative control. Z' factor of 0.66 indicated that the difference between the positive and negative control was high enough that this experimental setup was valid (Figure 2). All results were duplicated and compounds that reduced caspase-4 activity to 25% of the activity of caspase-4 with substrate and DMSO control were considered top hits.

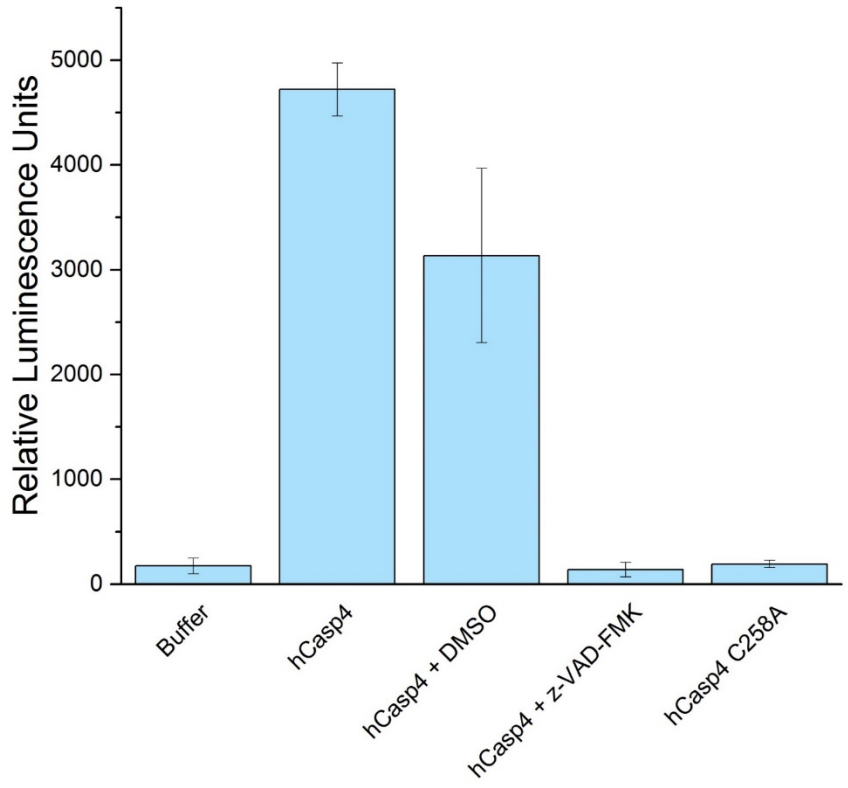


Figure 5: Relative luminescence units corresponding to activity of substrate plus buffer, caspase-4 with substrate, with substrate and DMSO, with pan-caspase inhibitor z-VAD and substrate, and catalytically inactive mutant human caspase 4 with substrate controls. Almost 3000 fold activity of caspase-4 with substrate and DMSO compared to buffer, z-VAD or mutant caspase-4 controls determine the Z' factor of 0.66, validating the Caspase-Glo enzymatic assay.

Specificity Assay

The compounds that resulted in 25% reduction of caspase-4 activity from both libraries were tested for their inhibition of caspase-3, caspase-4, and caspase-9. Therefore, Caspase-Glo assay with similar experimental setup as with caspase-4 in high throughput screening (3 μM substrate, 10 nM caspase, and 30 μM compounds) was used to measure caspases-3 and caspase-9 activities. The scaffold that resulted in the highest difference between caspase-4 activity and the activities of caspase-3 and caspase-9 was considered a specific inhibitor of caspase-4.

IC_{50}

Three caspase-4-specific compounds from P/K and six compounds from FDA were tested to find the concentration of the compounds that would inhibit caspase activity by 50%. The P/K drugs shared a six-member and five-member fused heterocyclic scaffold, and demonstrated the most specificity for caspase-4 during initial validation. The FDA drugs were selected to represent a diverse class of NSAIDs with a range of potencies. The activity of caspases-3, 4, 5, and 9 in the presence of compound concentration ranging from 52 nM to 660 μM were tested for each compound to develop a full dose-response curve. In order to measure the activity during the maximum efficiency of each enzyme, the substrate concentration was three hundred-fold higher than the enzyme concentration, and the enzyme concentration was kept well below IC_{50}.

In order to analyze the data, a graph of enzyme activity over time was plotted for each concentration. Average slope of the linear portion of the activity versus time graph was normalized to the average slope of DMSO control containing enzyme, substrate, and DMSO equal to the solvent of each drug concentration. Although higher concentrations of compounds were tested, the data analysis only included up to 66 μM because the DMSO control lowered enzyme activity beyond 66 μM, thus experiments performed wither higher concentrations were not valid. The normalized slope was plotted against log of compound concentration to create an IC50 curve. Using the equation,

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(\text{LogEC50}-X)\cdot\text{HillSlope}}}$$

a four-point dose response curve was fitted on the experimental data to determine the IC50 values as well as R-squared values. The top asymptote of the curve represents the DMSO control and the bottom asymptote represents the pan caspase inhibitor control. Top and bottom are fixed to 100% and 0, respectively. The enzyme activity in DMSO control was required to be at least ten-fold higher than the z-VAD control in order to consider each plate valid. The aim for each curve was to obtain the R-squared value greater than 0.95.

Results

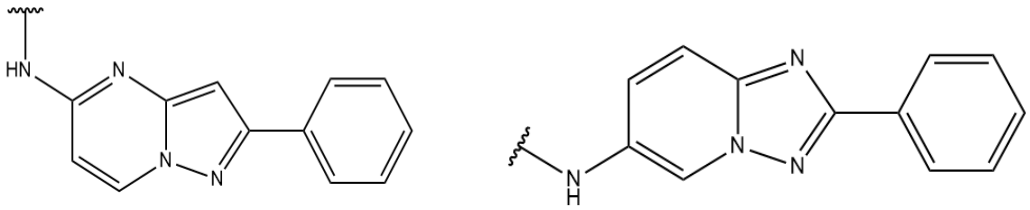
High throughput screening

The high throughput screening identified 75 total compounds from P/K and FDA as hits, which are compounds that reduced caspase-4 signal to less than 25% relative to the DMSO control. The entire screen had a hit rate of 2%. Figure 6 is a representative 384 well- plate for the high throughput screening.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1.4743036	1.6312279	1.5527658	1.5998431	1.5496273	1.4491958	1.3393488	1.5307964	1.1039623	1.4429188	1.2514712	1.4994115	1.2659945	1.4711652	1.289133	1.5213809	0.0400157	1.3832876	0.6645743	1.4743036	1.2608866	0.5923892	1.5559043	0.0274617
B	1.3519027	1.5370734	1.4554727	1.4303648	1.4429188	1.4460573	1.5530428	1.5276579	1.516104	1.5684582	1.5496273	1.5778737	1.612397	1.3739721	1.5213809	1.5056885	1.4272264	1.508827	1.4680267	1.5998431	1.4617497	1.4366418	1.5747352	0.0180463
C	1.4240879	1.4178109	1.508827	1.4805606	1.4178109	1.392703	1.4303648	0.3256179	0.9093762	1.4146724	1.2932715	1.3895645	0.6990377	1.3644566	0.1686936	1.5872691	1.0035308	1.4774421	0.1090624	1.2043939	0.1561397	1.3111024	1.4178109	0.0117693
D	1.1698705	1.2765791	1.3299333	1.4083954	1.4566112	1.2797175	1.3519027	1.3079639	1.2577481	1.3330718	1.2640251	1.2765791	1.3424872	1.39898	1.3956415	1.0976854	1.3581797	1.1071008	1.39898	1.2932715	1.5810122	1.4115339	1.392703	0.0117693
E	1.2797175	1.289133	1.392703	1.3644566	1.3393488	1.3801451	1.2420557	0.8779914	1.2483327	1.2608866	1.1887015	1.2295018	1.3142409	1.1573166	0.0431542	1.173286	0.0619851	1.2075324	0.0851238	1.2389172	0.1847731	1.4475527	1.4178109	0.0180463
F	0.9376226	1.2106709	1.3205179	1.1687321	1.2075324	1.2357787	1.1761475	1.1039623	1.1918399	1.2232248	1.1102393	1.3016869	1.3550412	1.2985485	1.2420557	1.2263633	1.1510396	0.4731267	1.1887015	1.3236563	1.2043939	1.1353472	1.2295018	-0.003923
G	1.0976854	1.2232248	1.2263633	1.063162	1.2326403	1.0976854	0.9815614	1.0192232	0.9909769	1.1604551	0.9909769	1.1384857	0.9972538	1.0286387	1.1290702	1.0882699	1.0537466	1.0349157	0.416634	1.1547781	0.0401157	0.9846399	1.0123463	-0.007062
H	0.8528835	1.1196548	1.1416242	1.069439	0.9941153	1.0411626	1.0474696	1.0537466	1.0098078	1.0192232	0.8779914	1.1008239	1.039623	1.1071008	1.1447627	1.1102393	1.2012554	1.1071008	1.0506081	1.0663005	1.0600235	1.1636705	1.2322715	-0.013339
I	0.9941153	1.0914084	0.9752844	1.0286387	0.9941153	0.8811299	0.9030993	0.9721459	0.9187317	0.8779914	0.8654374	1.063162	0.8748529	1.0663005	1.1039623	1.0568851	1.0380541	0.953315	0.3475873	0.8717144	1.0223617	0.9846399	1.0255002	-0.003923
J	0.8183601	0.965869	0.9282071	0.9630075	0.9187317	0.8968223	0.9376226	0.8622989	0.8654374	0.9344841	0.9630075	1.0443311	0.9630075	0.9062377	1.0003923	0.9187917	1.0003923	1.1196548	1.0003923	0.8591605	0.7995292	0.9156532	0.8214986	0.0023539
K	0.7367595	0.8968223	0.9093762	0.8308141	0.8089447	0.7901138	0.7179286	0.9407611	0.6394685	0.856022	0.7367595	0.8379311	0.8120832	0.8874068	0.2659867	0.9125147	0.7901138	0.843468	0.184388	0.9062377	0.8999608	0.7681444	0.7430365	-0.000785
L	0.7587289	0.8968223	0.8685759	0.8277756	0.8466065	0.8340526	0.8622989	0.8528835	0.7242056	0.8685759	0.9313456	0.8717144	0.8528835	0.8717144	0.8058062	0.8152217	0.9815614	0.8748529	0.739898	0.8152217	0.8654374	0.9187917	0.7430365	-0.016477
M	0.746175	0.8246371	0.8779914	0.8591605	0.8277756	0.8591605	0.8089447	0.843468	0.8717144	0.8591605	0.3381718	0.8591605	0.8277756	0.9470381	0.0525896	0.8528835	0.9470381	0.953315	0.8811299	0.9564535	0.7430365	0.0619851	0.7963907	-0.016477
N	0.8403295	0.8936838	0.9501765	0.9093762	0.8403295	0.8654374	0.8936838	0.9030993	0.8999608	0.9564535	0.8528835	0.9846399	0.9564535	0.9125147	0.8905453	1.0223617	1.0411926	0.8779914	0.9878384	0.8528835	0.7597289	0.7775598	0.843745	0.0023539
O	0.7555904	0.7806983	0.9219302	0.8528835	0.7901138	0.8874068	0.4888191	0.7901138	0.7932523	0.9630075	0.739898	0.8152217	0.8622989	0.9214966	0.0243233	0.8089447	0.2679561	0.8968223	0.843745	1.0003923	0.8717144	0.8874068	0.6582974	-0.0102
P	0.7618674	1.1259317	0.9062377	0.9941153	0.7618674	0.9376226	1.0003923	0.9093762	0.9250687	0.8811299	0.8748529	0.9909769	0.8968223	0.9187917	0.8591605	0.9438996	1.0600235	0.9219302	0.953315	0.8622989	0.9438996	0.8779914	0.9752844	-0.019616

Figure 6: A representative 384 well- plate for high throughput screening. DMSO control lies in column 1 while the z-VAD control is in column 24. Each well represents a compound tested. Compounds in red wells reduced caspase-4 activity to less than 25%, yellow wells reduced activity to 25%-50%, and green reduced activity to greater than 50% of DMSO control. All compounds were tested at 30 µM and each plate was read for 20 minutes after substrate addition.

The screening of PK identified 46 hits, which were retested for caspase-4 activity and validated. All hits were six-member and five-member fused heterocyles containing two or more nitrogen heteroatom, some of which are highlighted in figure 7.



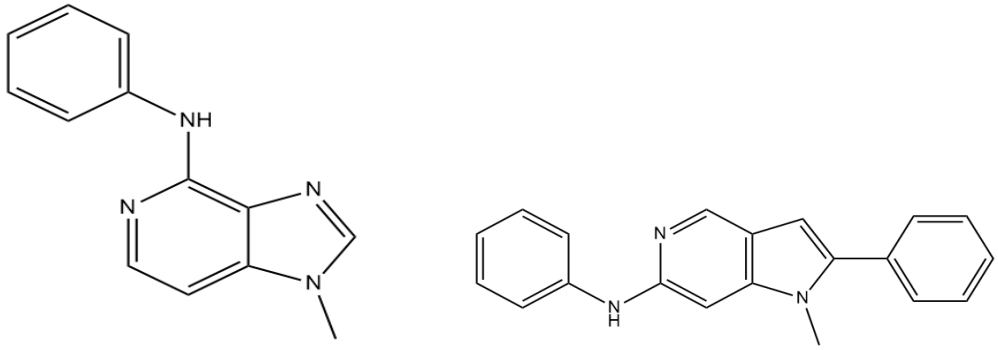
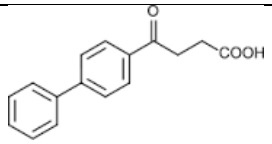
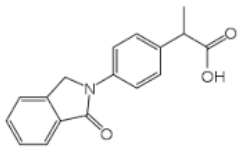
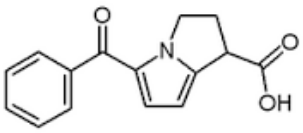
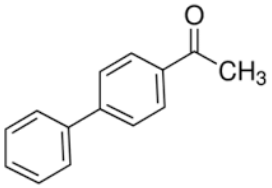
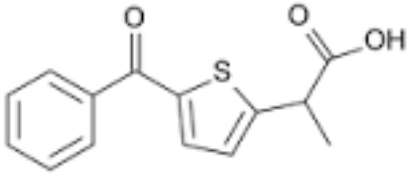
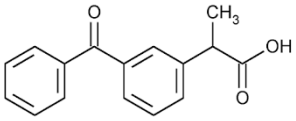


Figure 7: Sample structures of top hits indicate a common six-member and five-member fused heterocycles containing nitrogen linked to aromatic ring as a common scaffold.

The screening of FDA identified 26 hits, some of which were retested for caspase-4 activity and validated. Interestingly, 7 out of top 10 high throughput screening hits and 56% of the total FDA hits were NSAIDs. Six different NSAIDs listed in table 1 were chosen for further characterization based on the wide arrays of structures and activity.

Table 1: Summary of the structures of the six chosen NSAIDs, their inhibition mechanism, class of molecules, availability status, and corresponding caspase activities. All activities are results from the original screening data at 30 μM.

Name	Structure	COX-2 specific	Class	Availability Status	Caspase-4 Activity
Fenbufen		yes	Propionic Acid	Withdrawn	~2%
Indoprofen		yes	Phenylpropionic Acid	Withdrawn	~5%

Ketorolac		No	Hetroaryl Acetic Acid	Active in the US	~5%
Felbinac		No	Arylpropionic Acid	Active in the US	~9%
Tiaprofenic Acid		No	Arylpropionic Acid	Withdrawn in the US; Active in developing countries	~5%
Ketoprofen		No	Propionic Acid	Active in the US	~60%

Specificity Test

All hits from the P/K Library were tested at 30 μM for caspase-3, caspase-4, and caspase-9 activities. In addition to having a low caspase-4 activity, a compound was considered specific if the caspase-4 activity was significantly lower than the activities of caspase-3 and caspase-9. Figure 8 shows the specificity results of the top ten compounds. Compounds 18-G9 and 25-E2 had the highest difference in caspase activities. 22-H8 did not have high difference in caspase activities, but shared a similar scaffold as other two top compounds as demonstrated in figure 8. 18-G9, 22-H8, and 25-E2 are further characterized for IC_{50}. The compound designations arise from the plate and well number from the compound plates in high throughput screening.

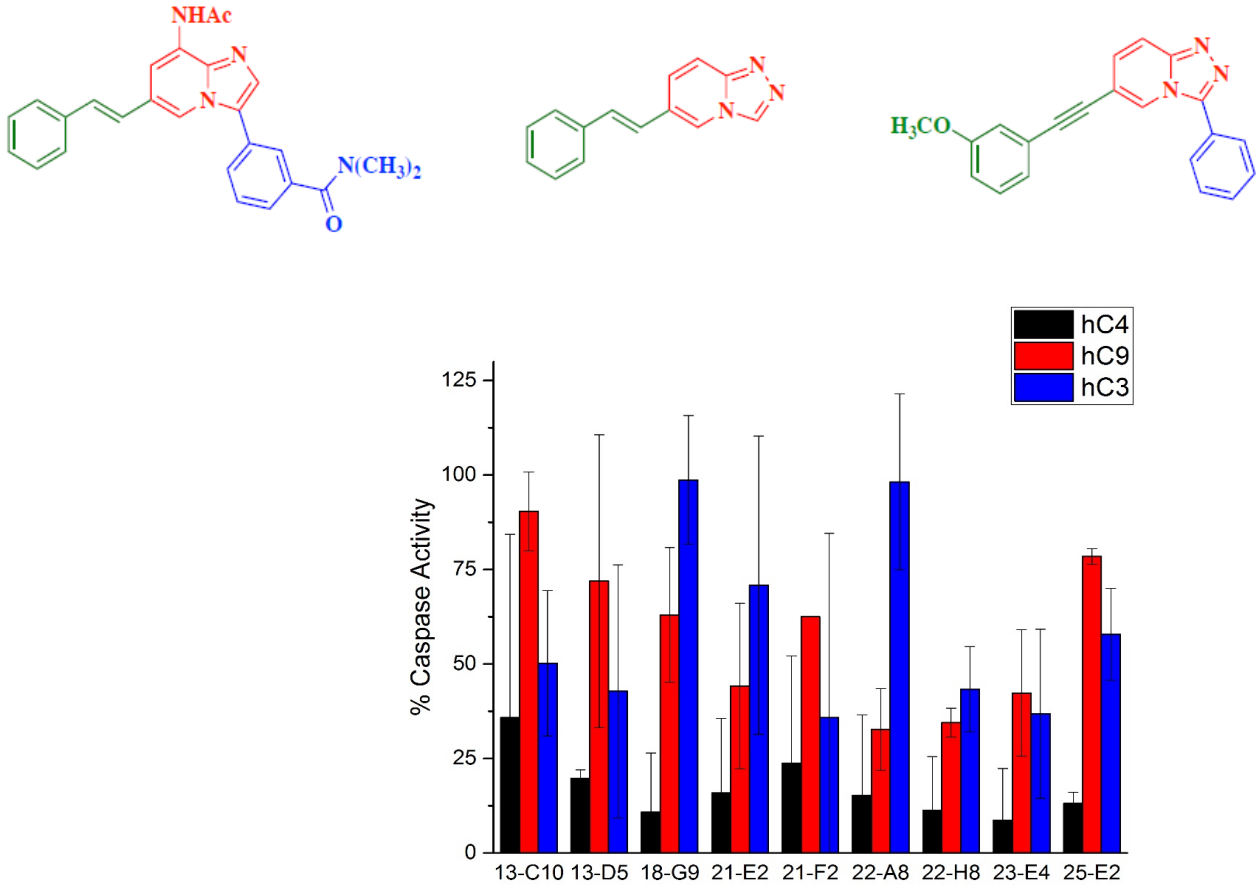


Figure 8: Activity of caspase-4, 9, and 3 in the presence of top 10 hits from the P/K Library. 18-G9 and 25-E2 were determined to be the most specific to caspase-4 based on the difference in activity between caspase-4 and other caspases. 22-H8 was also chosen for its similarity in structure with 18-G9 and 25-E2. The compound on the left is 18-G9, the middle compound is 22-H8 and the compound on the right is 25-E2; all of the three compounds highlight a common scaffold. All compounds were tested at 30 μM in biological triplicates.

Six inhibitors, which also happened to be NSAIDS, from FDA were tested for specificity at 30 μM. Measuring activities of caspase-3, caspase-4, and caspase-9 in the presence of six inhibitors (data not shown) indicate that the NSAIDs are pan-caspase inhibitors.

IC_{50}

Adam Csakai synthesized 22-H8 and IC_{50} of the compound with human caspase-3 and caspase-4 indicated that 22-H8 is not specific to caspase-4. IC_{50} with caspase-3 is 6306.5 ± 416.5 nM while with caspase-4 is 2042.4 ± 351.3 nM (figure 9). 18-G9 and 25-E2 are being synthesized and their IC_{50} values are yet to be determined.

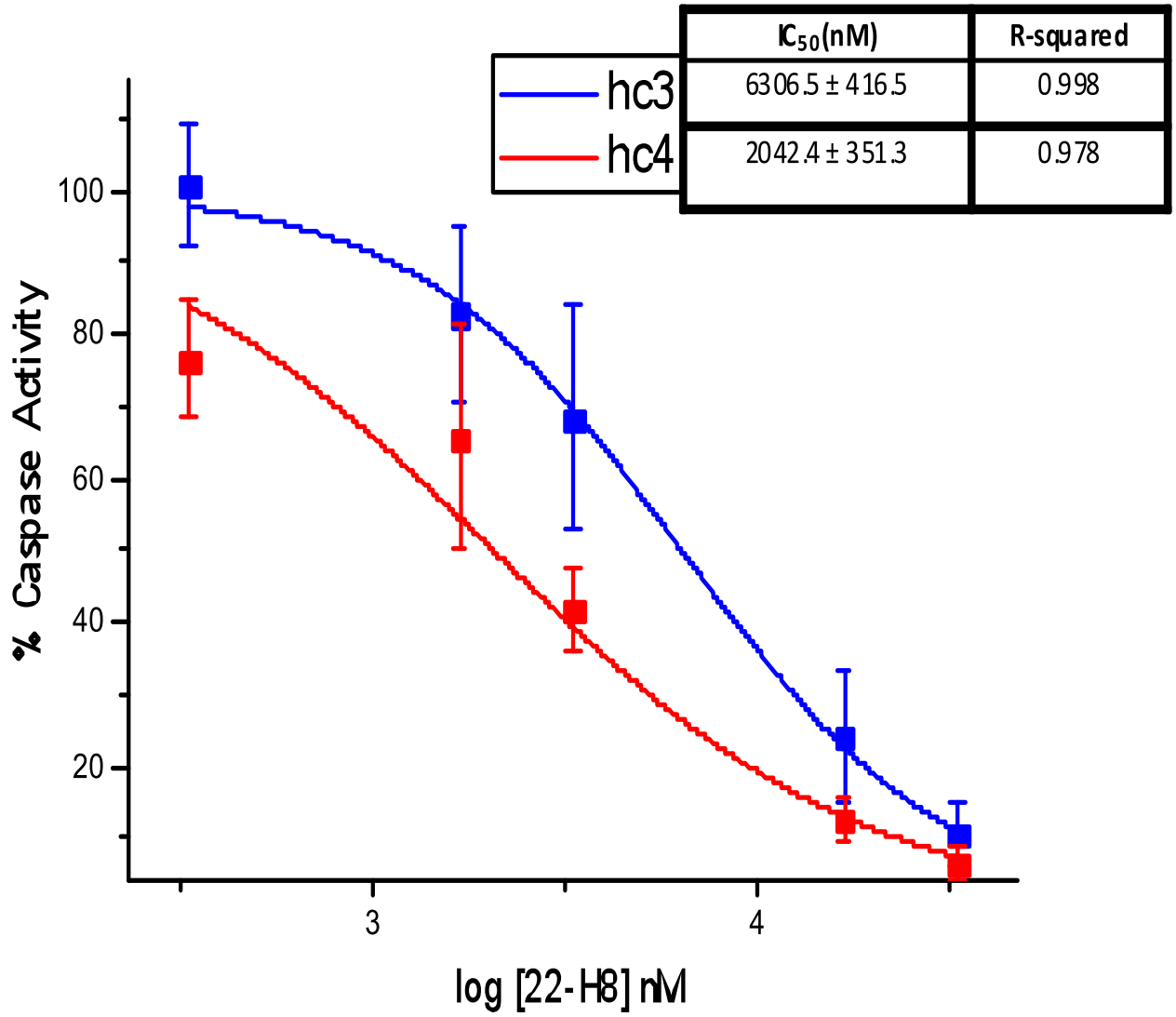


Figure 9: IC_{50} curve of human caspase-3 and human caspase-4 in the presence of 22-H8. % Caspase Activity is normalized to DMSO control containing enzyme, substrate, and same

concentration of DMSO as the compound tested. The top asymptote, representing DMSO control, and the bottom asymptote, representing total inhibition by z-VAD, are fixed at 100% and 0%, respectively. All activities are measured in biological duplicates, each in technical triplicates.

IC_{50} values of six NSAIDs with human caspase-3, caspase-4, caspase-5, and caspase-9 were determined using the data in figures 10-15. All curves have an R-squared value greater than 0.95, except for Felbinac with caspase-4 whose value is 0.917 (table 2). The IC_{50} curves for Ketorolac (figure 10), Ketoprofen (figure 11), Fenbufen (figure 12), Indoprofen (figure 13), Felbinac (figure 14), and Tiaprofenic Acid (figure 15) indicated that the IC_{50} values of caspase-4, 5 and 9 generally are below 2000 nM but IC_{50} values of caspase-3 are in the range of tens of thousands of nM. All NSAIDs inhibited all caspases tested.

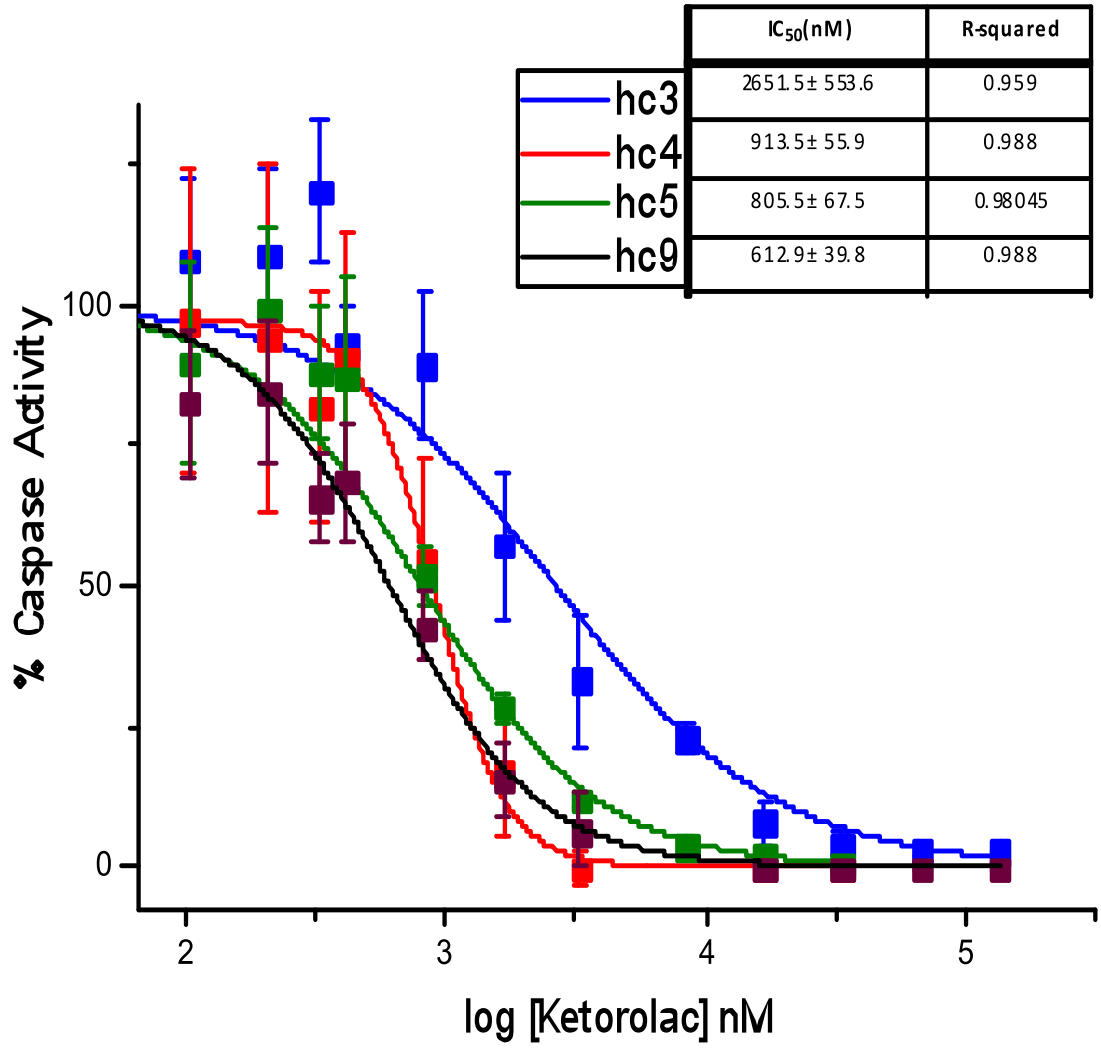


Figure 10: IC50 curve of human caspase-3, human caspase-4, human caspase-5, and human caspase-9 in the presence of Ketorolac. % Caspase Activity is normalized to DMSO control containing enzyme, substrate, and same concentration of DMSO as the compound tested. The top asymptote, representing DMSO control, and the bottom asymptote, representing total inhibition by z-VAD, are fixed at 100% and 0%, respectively. All activities are measured in biological duplicates, each in technical triplicates.

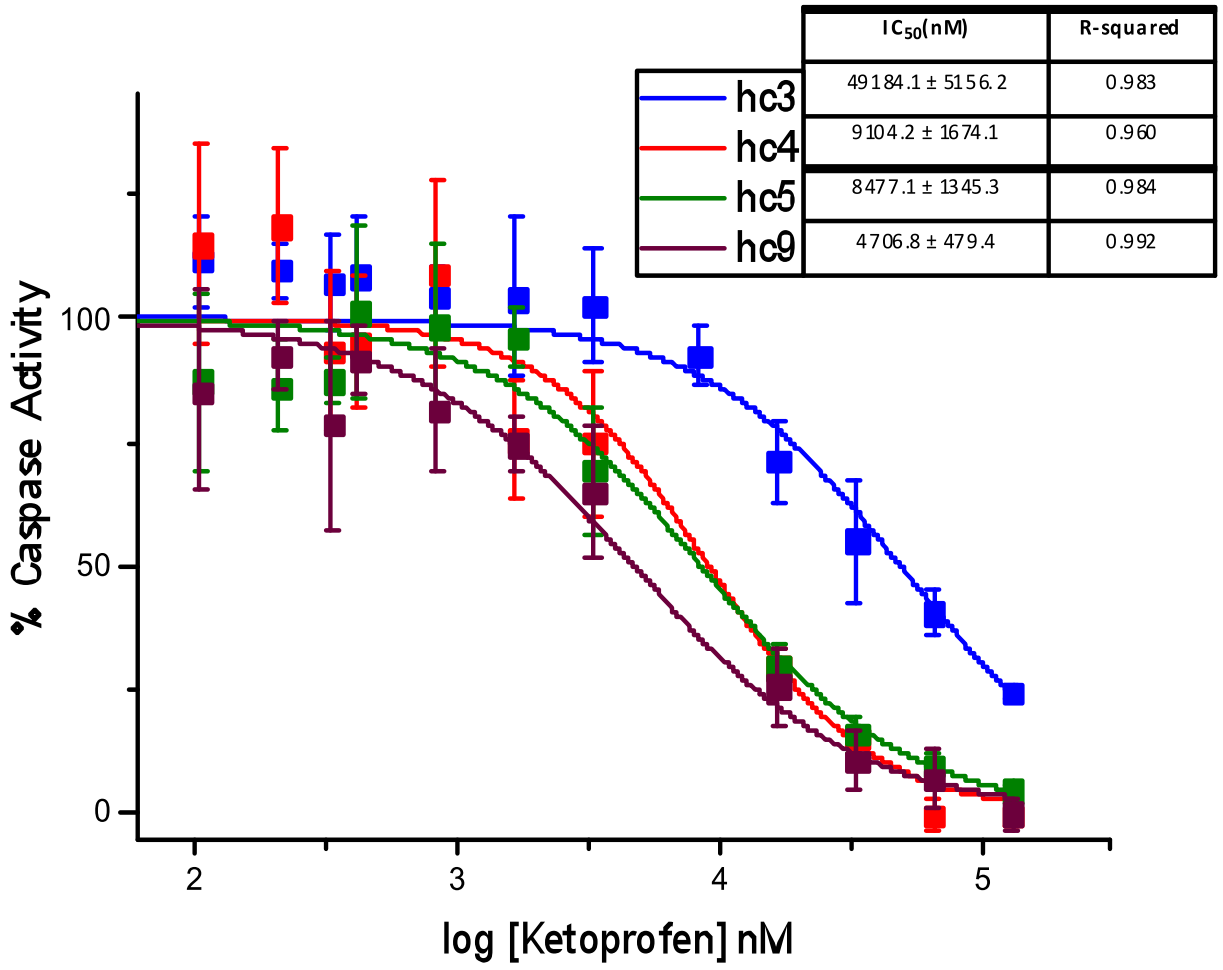


Figure 11: IC50 curve of human caspase-3, human caspase-4, human caspase-5, and human caspase-9 in the presence of Ketoprofen. % Caspase Activity is normalized to DMSO control containing enzyme, substrate, and same concentration of DMSO as the compound tested. The top asymptote, representing DMSO control, and the bottom asymptote, representing total inhibition by z-VAD, are fixed at 100% and 0%, respectively. All activities are measured in biological duplicates, each in technical triplicates.

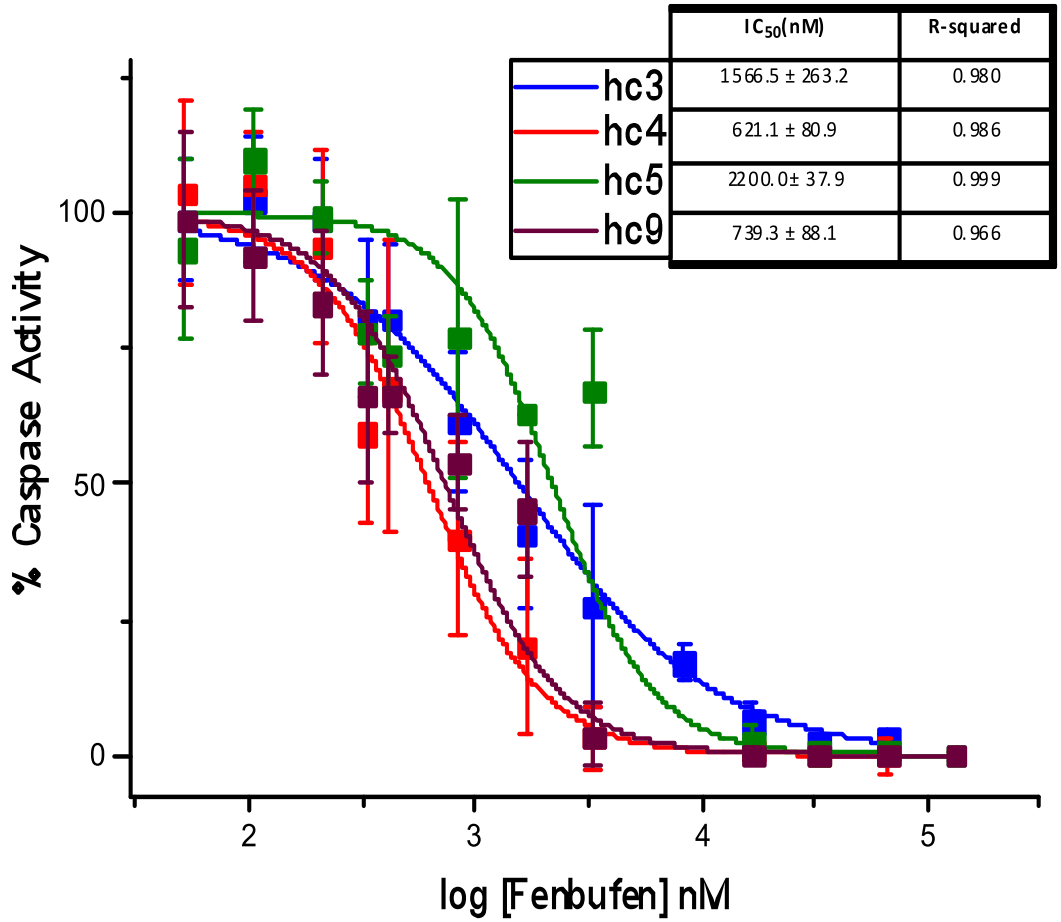


Figure 12: IC50 curve of human caspase-3, human caspase-4, human caspase-5, and human caspase-9 in the presence of Fenbufen. % Caspase Activity is normalized to DMSO control containing enzyme, substrate, and same concentration of DMSO as the compound tested. The top asymptote, representing DMSO control, and the bottom asymptote, representing total inhibition by z-VAD, are fixed at 100% and 0%, respectively. All activities are measured in biological duplicates, each in technical triplicates.

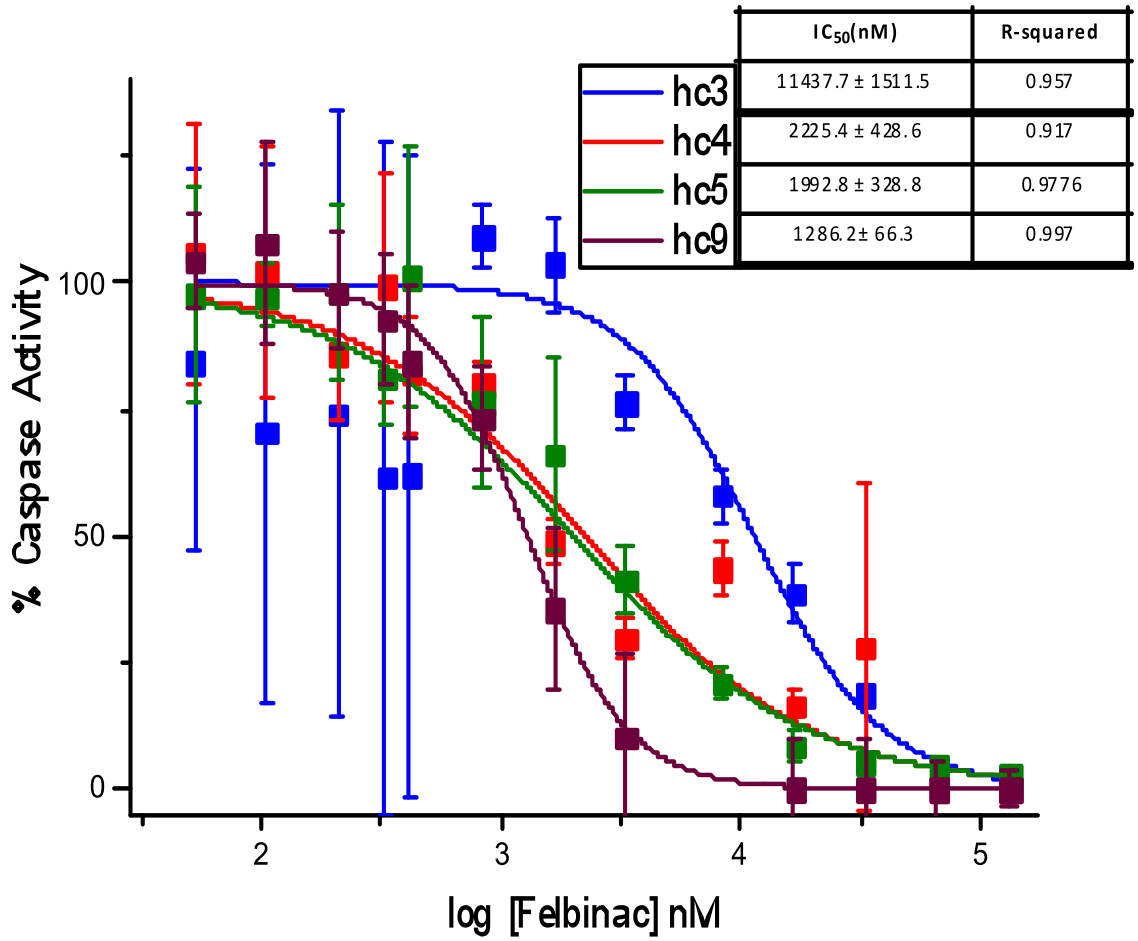


Figure 13: IC50 curve of human caspase-3, human caspase-4, human caspase-5, and human caspase-9 in the presence of Felbinac. % Caspase Activity is normalized to DMSO control containing enzyme, substrate, and same concentration of DMSO as the compound tested. The top asymptote, representing DMSO control, and the bottom asymptote, representing total inhibition by z-VAD, are fixed at 100% and 0%, respectively. All activities are measured in biological duplicates, each in technical triplicates.

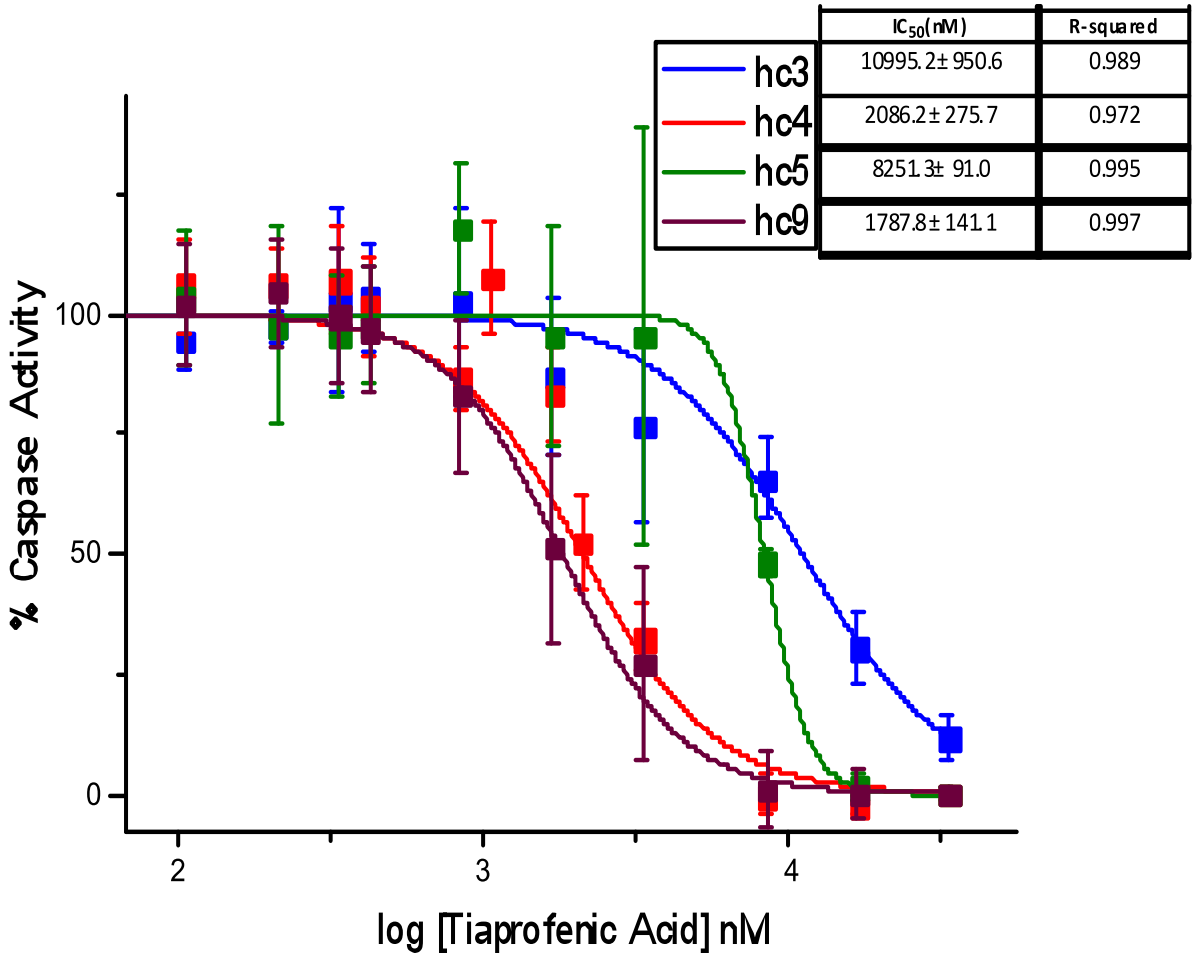


Figure 14: IC50 curve of human caspase-3, human caspase-4, human caspase-5, and human caspase-9 in the presence of Tiaprofenic Acid. % Caspase Activity is normalized to DMSO control containing enzyme, substrate, and same concentration of DMSO as the compound tested. The top asymptote, representing DMSO control, and the bottom asymptote, representing total inhibition by z-VAD, are fixed at 100% and 0%, respectively. All activities are measured in biological duplicates, each in technical triplicates.

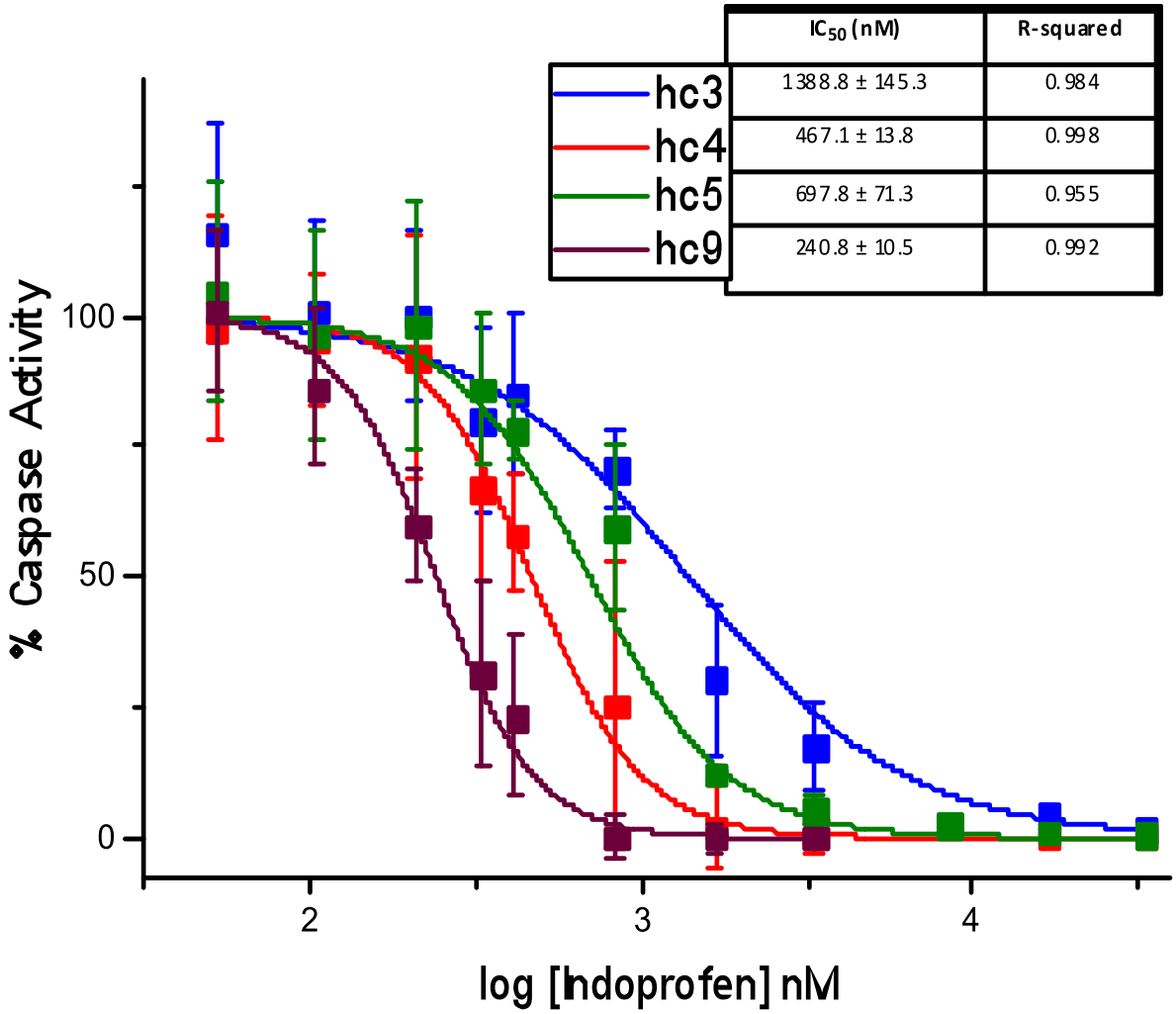


Figure 15: IC50 curve of human caspase-3, human caspase-4, human caspase-5, and human caspase-9 in the presence of Indoprofen. % Caspase Activity is normalized to DMSO control containing enzyme, substrate, and same concentration of DMSO as the compound tested. The top asymptote, representing DMSO control, and the bottom asymptote, representing total inhibition by z-VAD, are fixed at 100% and 0%, respectively. All activities are measured in biological duplicates, each in technical triplicates.

Table 2: Summary of IC_{50} values of all six NSAIDs with caspase-3, caspase-4, caspase-5, and caspase-9 calculated from their four-point dose response curved as well as their R-squared values

indicating how well the data fits into the regression. All values are determined from biological duplicates, each in technical triplicates.

Compound	Caspase	IC50 (nM)	RSQ
Ketorolac	3	2651.5 ± 553.6	0.959
	4	913.5 ± 55.9	0.988
	5	805.5 ± 67.5	0.980
	9	612.9 ± 39.8	0.988
Ketoprofen	3	49184.1 ± 5156.2	0.983
	4	9104.2 ± 1674.1	0.960
	5	8477.1 ± 1345.3	0.984
	9	4706.8 ± 479.4	0.992
Fenbufen	3	1566.5 ± 263.2	0.98
	4	621.1 ± 80.9	0.986
	5	2200.0 ± 37.9	0.999
	9	739.3 ± 88.1	0.966
Felbinac	3	11437.7 ± 1511.5	0.957
	4	2225.4 ± 428.6	0.917
	5	1992.8 ± 328.8	0.978
	9	1286.2 ± 66.3	0.997
Indoprofen	3	1388.8 ± 145.3	0.984
	4	467.1 ± 13.8	0.998
	5	697.8 ± 71.3	0.955
	9	240.8 ± 10.5	0.992
Tiaprofenic Acid	3	10995.2 ± 950.6	0.989
	4	2086.2 ± 275.7	0.972
	5	8251.3 ± 91.0	0.995
	9	1787.8 ± 141.1	0.997

Discussion

Implications of P/K Library Results

The P/K Library was screened with the purpose of discovering a novel small molecule that specifically inhibits caspase-4 for therapeutic purposes. 46 compounds were identified for reducing caspase-4 activity to less than 25% activity and all the compounds were six-member and five-member fused heterocycles containing two or more nitrogen linked to an aromatic ring. The conserved structural motifs among hits suggest consistent chemical effect and promising outcome of high throughput screening.

Out of 46 compounds, only one scaffold, common in 18-G9, 22-H8, and 25-E2, was identified as being specific to caspase-4. The high throughput screening facility had problems with mis-ordered compounds that they were repeatedly freeze-thawed, which could have led to cross contamination or degradation. Therefore, Adam Cskai, a graduate student at the Yin Lab, re-synthesized the compounds to ensure the hit was as advertised and still worked.

While 18-G9 and 25-E2 are being synthesized, IC_{50} characterization of 22-H8 was performed. The R-squared values of 0.998 for caspase-3 and 0.978 for caspase-4 for four-point dose response curve indicate that 22-H8 inhibits caspase-3 in addition to caspase-4. The similarity in IC_{50} values of about 6 μM for caspase-3 and 2 μM for caspase-4 indicates that there will be no appreciable difference in a biological system. Even though 22-H8 is not specific to caspase-4, 18-G9 and 25-E2 are still promising caspase-4 specific inhibitor because unlike 22-H8, whose caspase-3 and caspse-9 activities were less than 25% different from its caspase-4 activity, the activities of caspase-3 and caspase-9 are more than 50% greater than caspase-4 activity for18-G9 and 25-E2. Even though the difference between caspase-4 activity and other

activities was not high for 22-H8, it was chosen for IC_{50} analysis because it is structurally similar to the two most specific compounds. The fact that IC_{50} results indicate non-specific inhibition of caspase-4 with 22-H8 is consistent with the specificity results. Therefore, once they are synthesized, 18-G9 and 25-E2 IC_{50} analysis must be pursued to find a caspase-4 specific inhibitor.

Implications of FDA Library Results

In addition to finding a novel caspase-4 inhibitor, the FDA Library was screened to study the effect of NSAIDs on caspase activity. The results of the high throughput screening indicated that 56% of the top caspase-4 inhibitors and seven out of the top ten combined library hits were NSAIDs. IC_{50} characterization of six NSAIDs, chosen based on their diversity in structure and potency, indicated that NSAIDs are pan-caspase inhibitors. All the curves have R-squared value greater than 0.95, except for one outlier that lies between 0.91 and 0.95. High R-squared values indicate the strength with which the IC_{50} curves justify the experimental data points and indicate the confidence with which NSAIDs inhibit caspases. Interestingly, while the active sites of caspases are similar, the active sites of COX-1 and COX-2 are different from the active sites of caspases, adding to the mystery that NSAIDs are pan-caspase inhibitors.

Among all the caspases, caspase-3 inhibition generally requires the highest concentration of NSAIDs and caspase-9 inhibition requires the lowest (table 2). Caspase-9 is an initiator caspase and appears earlier in the caspase activation pathway than other caspases tested, thus small amount of NSAID may be sufficient to inhibit caspase-9. That being said, caspases-4,5 and 9 have similar IC_{50} values and are much lower than the value for caspase-3 for each NSAID. Such similarity may be explained by the fact that caspases-4, 5, and 9 share the LEHD substrate, indicating similarity in active sites of caspases- 4, 5, and 9 as well.

Based on their IC_{50} values, Indoprofen is the best inhibitor and Ketoprofen is the worst inhibitor of all the caspases tested (table 2). Coincidentally, Indoprofen was withdrawn by the US government for severe gastrointestinal bleeding and carcinogenicity while Ketoprofen is still actively available in the US despite displaying gastrointestinal upset and ulceration.[39] Perhaps the severity of the side effect is correlated with the potency in caspase inhibition. If such is the case, top inhibitors of the study, Ketorolac and Felbinac, are still available in the United States and further study must be performed to assess their side effects. On average, patients in taking the recommended dosage of 10-40 mg/day have 39 μM of Ketorolac circulating in their blood. However, IC_{50} of Ketorolac is less than 1μM for caspases-4,5, and 9 and less than 3μM for caspase-3.[40] Thus, even though *in vivo* values would be greater than *in vitro*, IC_{50} determined from this study is biologically relevant to patients using Ketorolac. Such is the case for all the NSAIDs tested, except for Ketoprofen, which has higher IC_{50} value than average concentration it the blood. The relevance is especially high since caspase-11, the mice homologous to human caspase-4, is crucial in maintaining gut homeostasis[41] and the knockdown of caspase-1 enhanced tumor formation in a mouse model of colorectal cancer,[42] both of which are also common side effects of NSAIDs. Thus, further studies into adjusting the recommended dosage of the drugs is recommended.

Parallel experiments

Based on the results of the in vitro experiments, caspase-1 and caspase-3 activities in cells are measured in the presence of the six NSAIDs. The cell culture experiments mirror the in vitro results, with Ketoprofen being the worst inhibitor of caspase-1 and caspase-3. IC_{50} characterization of two additional NSAIDs, Ibuprofen and Naproxen, shows they also inhibit

caspases at lower concentration than their average circulation level. This data is performed by a graduate student in the Yin Lab, Tina Smith, and the results further validate and strengthen the conclusion that NSAIDs are pan-caspase inhibitors.

Future Directions

Once 18-G9 and 25-E2 are synthesized, the IC_{50} values of 18-G9 and 25-E2 need to be determined to assess whether the P/K Library contain caspase-4 specific inhibitor that could be used to treat sepsis.

This study concludes that NSAIDs are pan-caspase inhibitors in vitro, however, more experiments in vivo need to be performed to confirm the results and increase the relevance of the results presented in this study. Some ideas for future work could be using mass spectrophotometer to analyze the interaction between NSAIDs and the binding sites of caspases or using *C. Elegans* as a model to test the in vivo effect of NSAIDs on caspase-1 activity.

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