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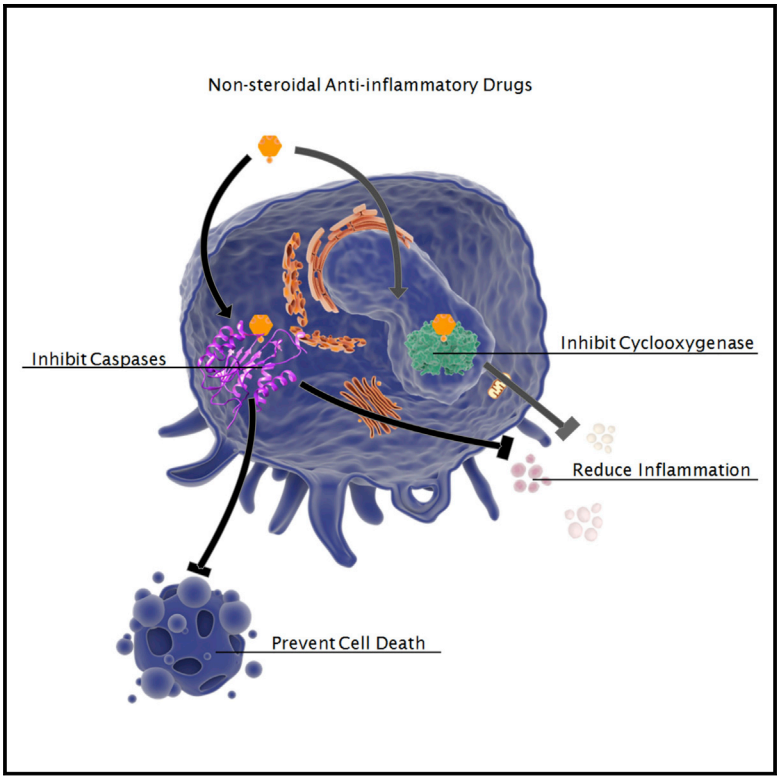
Cell Chemical Biology

Non-steroidal Anti-inflammatory Drugs Are Caspase Inhibitors

Graphical Abstract



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In Brief

Smith et al. report the identification and characterization of caspases as general NSAID pharmacological targets. Inhibition occurs at physiologically relevant concentrations both in vitro and in vivo, and expands the NSAID anti-inflammatory mechanism.

Highlights

- Caspases are identified as a novel target for a subset of NSAIDs
- Inhibition occurs at physiologically relevant concentrations in vitro and in vivo
- New molecular insight expands anti-inflammatory drug mechanism

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Article

Non-steroidal Anti-inflammatory Drugs Are Caspase Inhibitors

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SUMMARY

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most commonly used drugs in the world. While the role of NSAIDs as cyclooxygenase (COX) inhibitors is well established, other targets may contribute to anti-inflammation. Here we report caspases as a new pharmacological target for NSAID family drugs such as ibuprofen, naproxen, and ketorolac at physiologic concentrations both in vitro and in vivo. We characterize caspase activity in both in vitro and in cell culture, and combine computational modeling and biophysical analysis to determine the mechanism of action. We observe that inhibition of caspase catalysis reduces cell death and the generation of pro-inflammatory cytokines. Further, NSAID inhibition of caspases is COX independent, representing a new anti-inflammatory mechanism. This finding expands upon existing NSAID anti-inflammatory behaviors, with implications for patient safety and next-generation drug design.

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most prevalent pharmaceuticals in the world, with an estimated 30 billion doses taken annually in the United States alone (Green, 2001; Ong et al., 2007). The anti-inflammatory properties of NSAIDs have been segued into analgesic and anti-pyretic treatments targeting conditions ranging from mild aches and pains to rheumatoid arthritis to cancer (Ricciotti and FitzGerald, 2011). The best-characterized anti-inflammatory properties of NSAIDs stem from inhibition of cyclooxygenase (COX) isoenzymes (Gierse et al., 1999; Vane, 1971). COX inhibition reduces the production of prostaglandins, which are lipid autacoids involved in diverse cellular processes such as angiogenesis, apoptosis, and cell migration (Ricciotti and FitzGerald, 2011). However, much remains unclear about the existence and contributions of additional NSAID targets (Lounkine et al., 2012). These targets may contribute to NSAID anti-inflammatory mechanisms, applications, and the occurrence of adverse drug reactions (Lounkine et al., 2012). Reported NSAID functions include inhibition of nuclear factor κB, inhibition of proteasome function, activation of intrinsic and extrinsic pathways of apoptosis, cell-cycle arrest, and activation of stress kinases (Jana, 2008; Leibowitz et al., 2014). However, many of these effects are seen only at superphysiological concentrations, limiting their biological relevance (Ghosh et al., 2015; Mehlisch and Sykes, 2013). Here, we have identified cysteine-aspartic proteases (caspases) as novel targets for some NSAIDs such as ibuprofen, naproxen, and ketorolac at physiologic concentrations. During inflammation, these NSAIDs inhibit caspase catalytic activity, reducing cell death and the induction of inflammatory cytokines.

Caspases are a family of cysteine proteases that cleave peptide substrates after aspartic acid. Conserved from metazoans to humans, caspases play crucial roles in cell death, differentiation, and inflammation (Lamkanfi and Dixit, 2012). Initiator caspases (caspase-2, -8, -9, and -10) contain large prodomains for recruitment into multi-protein complexes, such as inflammasomes and apoptosomes. Upon activation, initiator caspases will be proteolytically activated and subsequently activate executioner caspases (caspase-3, -6, and -7), resulting in immunologically silent cell death through apoptosis (Lamkanfi, 2011). The aptly named inflammatory caspases (caspase-1, -4, and -5) generate biologically active cytokines such as IL-1β and IL-18, and induce pyroptotic cell death (Kayagaki et al., 2011; Shi et al., 2014; Thornberry and Molineaux, 1995). Due to the important role of caspases in inflammation and disease, it is essential to understand the contributions of caspase inhibition in NSAID pharmacology.

While previous research has observed pro-apoptotic and chemoprotective NSAID behaviors, these studies were not conducted under inflammatory conditions, which are more relevant to patient physiology (Jana, 2008; Leibowitz et al., 2014). This approach masks the independent contributions of caspases, which are upregulated under inflammatory conditions, and suggests that environment may affect the NSAID anti-inflammatory mechanism. In the presence of inflammatory stimuli, caspase activation propagates inflammation through the release of pro-inflammatory cytokines and danger-associated molecular patterns (DAMPs) from dying cells (Creagh, 2014). Consequentially, caspase inhibition by NSAIDs will reduce inflammation. However, this behavior is a double-edged sword, as the reduction of cytokines and DAMPs is paired with prevention of beneficial apoptosis. Armed with an expanded understanding of

how caspase inhibition affects NSAID pharmacology, it may be possible to improve patient safety and account for the occurrence of negative side effects. As caspases are an attractive therapeutic target in their own right, it is also feasible that drug repurposing could expand applications of some NSAIDs. Caspases have known roles in cancer, cardiovascular disease, rheumatoid arthritis, inflammation, and neurodegenerative diseases (McIlwain et al., 2013).

Here, we identify a novel role for NSAID family drugs such as ibuprofen, naproxen, and ketorolac as caspase inhibitors. At physiologically relevant concentrations, numerous NSAIDs impede caspase catalysis, reducing inflammation and cell death. Through study of COX-deficient cells and a *Caenorhabditis elegans* model we have determined that caspase inhibition is COX independent, and represents a new NSAID anti-inflammatory target. As caspases play a pivotal role in inflammation and cell death, inhibition could contribute to the NSAID anti-inflammatory mechanism and inform patient safety.

RESULTS

High-Throughput Screening Identifies NSAIDs as Caspase-4 Inhibitors

Caspase-4 is an exciting new innate immune receptor because, unlike other caspases, caspase-4 is able to directly bind its cognate ligand, lipopolysaccharide (Shi et al., 2014). Caspase-4 inhibitors have promising anti-inflammatory applications in septic shock treatment; however, to date no caspase-4 small-molecule inhibitors have been developed (Hagar et al., 2013; Smith et al., 2015). Further, recent efforts in drug repurposing and computational analysis have identified a myriad of previously unknown targets for Food and Drug Administration (FDA)-approved pharmaceuticals (Ashburn and Thor, 2004; Lounkine et al., 2012). To this end, we aimed to use high-throughput screening to identify therapeutic inhibitors of caspase-4 enzymatic activity. We screened the 1,280 compound Prestwick Chemical Library, which is comprised of FDA-approved bioavailable drugs selected for chemical and pharmacological diversity (Figure S1). Screening at 33 μM yielded 27 compounds that reduced caspase-4 catalytic activity to less than 25%, a 2% hit rate (Table 1). Due to the nature of the Promega Caspase-Glo-coupled enzyme screen, these hits included known luciferase inhibitors, such as resveratrol and AMP. Interestingly, NSAIDs comprised half of the hits, and eight of the top ten most potent inhibitors. Hits were not limited to one NSAID class (e.g., propionic acid class), and are structurally diverse. This was intriguing because, while caspases and COX share interrelated signaling, the enzymes themselves are largely dissimilar. Further, earlier studies aiming to observe NSAID off-target effects would not detect contributions from caspases, which are upregulated only in an inflammatory environment (Hagar et al., 2013; Jana, 2008).

Despite the prevalence of NSAIDs as caspase-4 inhibitors, not all of the NSAIDs screened reduced enzymatic activity (Table S1). To ascertain the chemical properties that contribute to caspase inhibitory activity, we performed a principal-component analysis (PCA) using ChemGPS. This tool uses t scores from PCA to compare NSAIDs in terms of physical-chemical properties such as size, aromaticity, polarity, and flexibility (Larsson et al., 2007). From this evaluation of chemical space and its relationship to caspase inhibition, several trends emerged (Figures S2A–S2D). The primary determinants were observed to be compound size, shape, and polarizability (correlation coefficient 0.72). While aromaticity was not a factor, extremes in polarity or hydrophobicity were not tolerated, and increased flexibility discouraged binding (correlation coefficient 0.39). For example, exceptionally polar compounds such as oxicam-class NSAIDs or lipophilic compounds such as coxib-class NSAIDs are unable to inhibit caspase-4. These factors are consistent with known caspase active-site inhibitors. The NSAID size restriction mirrors existing caspase inhibitors that often contain small core scaffolds (Poreba et al., 2015). It may be that larger NSAIDs are incapable of fitting into the S1 pocket, and are unable to compensate by forming sufficient contacts with the rest of the active site (Figure S2E). Further, at the P1 position, there is a nearly inalienable preference for aspartic acid, and the S1 pocket is narrow and extremely electropositive (Wei et al., 2000). Thus, extremely hydrophobic or charged NSAIDs would be unfavorable binding partners. Additionally, many NSAIDs contain a carboxylic acid that could form hydrogen bonds with the S1 pocket, mimicking aspartic acid.

From the numerous NSAIDs considered thus far, nine compounds (fenbufen, indoprofen, ketoprofen, ketorolac, felbinac, tiaprofenic, aspirin, ibuprofen, and naproxen) were selected to further examine the effect of NSAIDs on caspase catalytic activity. These compounds represent diverse NSAID classes, scaffolds, sizes, polarities, rigidities, and caspase-4 inhibitory potencies in order to provide a broad-scope assessment.

NSAIDs Are Multi-caspase Inhibitors

The caspase enzyme family is subdivided by their function as inflammatory, initiator, or executioner caspases, or segregated by recognition of particular tetrapeptide substrates (Garcia-Calvo et al., 1998; Thornberry, 1998). Despite these functional differences, caspases are structurally analogous, posing a challenge for chemical targeting of individual caspases (Poreba et al., 2015; Wei et al., 2000). To explore NSAID specificity, we assayed the catalytic activity of caspases-1, -3, -4, -5, and -9 (Figure 1). These caspases were selected to represent inflammatory, initiator, and executioner caspases with diverse tetrapeptide substrates. Inhibition was not specific to caspase-4, as NSAIDs reduced catalysis of multiple caspases (Table 2). Aspirin is a notable exception, with no inhibitory properties. However, aspirin has a unique acetylation mechanism that will not affect caspase catalysis (Lecomte et al., 1994).

Multi-caspase inhibition suggests that NSAIDs are primarily binding to a conserved subsite such as S1. In general, NSAID inhibition was more pronounced for caspases-4, -5, and -9, with weaker activity against caspases-3 and -1. This may be due to differences in the substrate recognition pocket, as caspases-4, -5, and -9 all recognize the LEHD tetrapeptide, whereas caspase-1 recognizes WEHD and caspase-3 DEVD. It is feasible that interactions with the adjacent but less-conserved S2 and S1′ subsites account for the small differences in activity observed (Poreba et al., 2013; Talanian et al., 1997).

We next expanded specificity testing to encompass cathepsin B, a lysosomal cysteine protease (Barrett, 1980; Turk et al., 2012). No inhibition of cathepsin B was observed, demonstrating specificity for caspases over other cysteine

Table 1. Total Hits from Caspase-4 Screening with the Prestwick Chemical Library

Name	CAS Number	Therapeutic Category	Caspase-4 Activity (%)
Terazosin	63590-64-7	α-adrenergic blocker	3.25
Fenbufen	36330-85-5	NSAID	3.71
Ketorolac tromethamine	74103-07-4	NSAID	4.09
Indoprofen	31842-01-0	NSAID	4.23
Tiaprofenic acid	33005-95-7	NSAID	4.32
Flurbiprofen	5104-49-4	NSAID	5.78
Ebselen	60940-34-3	NSAID	5.95
Ketoprofen	22071-15-4	NSAID	6.50
Resveratrol*	501-36-0	dietary supplement	7.48
Felbinac	5728-52-9	NSAID	8.11
Tolmetin	64490-92-2	NSAID	8.64
Suprofen	40828-46-4	NSAID	8.74
Luteolin	491-70-3	flavonoid	9.74
Niclosamide	50-65-7	anthelmintic	11.3
Tranilast	53902-12-8	H_1-antihistaminic	11.4
Carprofen	53716-49-7	NSAID	12.2
Diacerein	13739-02-1	interleukin-1 receptor antagonist	12.5
Tiabendazole	148-79-8	anthelmintic	13.5
Monobenzone	103-16-2	demelanizing agent	15.1
Prazosin	19237-84-4	α-adrenergic blocker	13.3
Leflunomide	75706-12-6	disease-modifying anti-rheumatic drug	16.3
Flunixin	42461-84-7	NSAID	17.9
Gemfibrozil	25812-30-0	fibrate	23.8
Adeonsine 5′-monophosphate*	18422-05-4	nucleotide	20.9
Flufenamic acid	530-78-9	NSAID	23.4
Phenazopyridine	136-40-3	analgesic	23.0
Anethole trithione	532-11-6	sialagogue	24.5

Compounds identified by high-throughput screening (HTS) that reduced caspase-4 catalytic activity to less than 25% were considered to be hits. Information on the therapeutic categories for each compound was obtained from drugs.com. Values shown represent the average of two independent replicates. Due to the nature of the Promega Caspase-Glo 9 coupled-enzyme system, luciferase inhibitors will also appear as false positives. Known luciferase inhibitors are denoted with asterisks, including resveratrol and AMP. Data are reported to three significant figures. See also Figure S1 for HTS Z′ determination, and Table S1 for additional screened NSAIDs.

proteases and ruling out the presence of redox cycling compounds (RCCs) (Figure S3A). RCCs generate hydrogen peroxide in the presence of strong reducing agents, and indirectly inhibit enzyme activity through oxidation of the catalytic cysteine (Johnston, 2011). We also accounted for luciferase inhibition, although NSAIDs have been widely tested in luciferase-based assays (Figure S3B). We did not observe inhibition of luciferase or cathepsin B, suggesting that NSAID inhibition is specific to the caspase family. This finding links NSAIDs to a class of enzymes with known roles in cancer, cardiovascular disease, rheumatoid arthritis, and neurodegenerative diseases (McIlwain et al., 2013).

NSAIDs Are Competitive Inhibitors

We next sought to further characterize the interaction between NSAIDs and various caspases. To assess binding, we observed caspase-3 tryptophan fluorescence. Caspase-3 was selected because it has two active-site tryptophan residues (Trp206 and Trp214) that provide information on the active-site microenvironment (Kyoung et al., 2002). We observed quenching of caspase-3 tryptophan fluorescence with indoprofen titration, giving an apparent K_D of 30 ± 2 μM (Figure 2A). Aspirin quenched with a substantially weaker apparent K_D of 1290 ± 10 μM. The disparate binding affinities of these two NSAIDs corroborates the rank order observed in our enzymatic data.

The hypothesized binding interaction was further explored with computational modeling and enzymatic competition assays. Computational docking over the entire caspase-3 surface places indoprofen and fenbufen in the caspase-3 S1 subsite, obscuring the substrate binding pocket at the catalytic cysteine (Figures 2B–2D) (Ganesan et al., 2006; Grosdidier et al., 2011a). This localization would suggest that NSAIDs act as competitive inhibitors. The S1 subsite is the most conserved, reflecting the strict requirement for aspartic acid at the P1 position. NSAIDs binding to this highly conserved site would not confer caspase specificity, in agreement with the multi-caspase activity observed. The carboxylic acid moiety of indoprofen and fenbufen is able to form hydrogen bonds (Arg64, Arg207, and Gln161) mimicking the aspartic acid substrate. This hypothesis

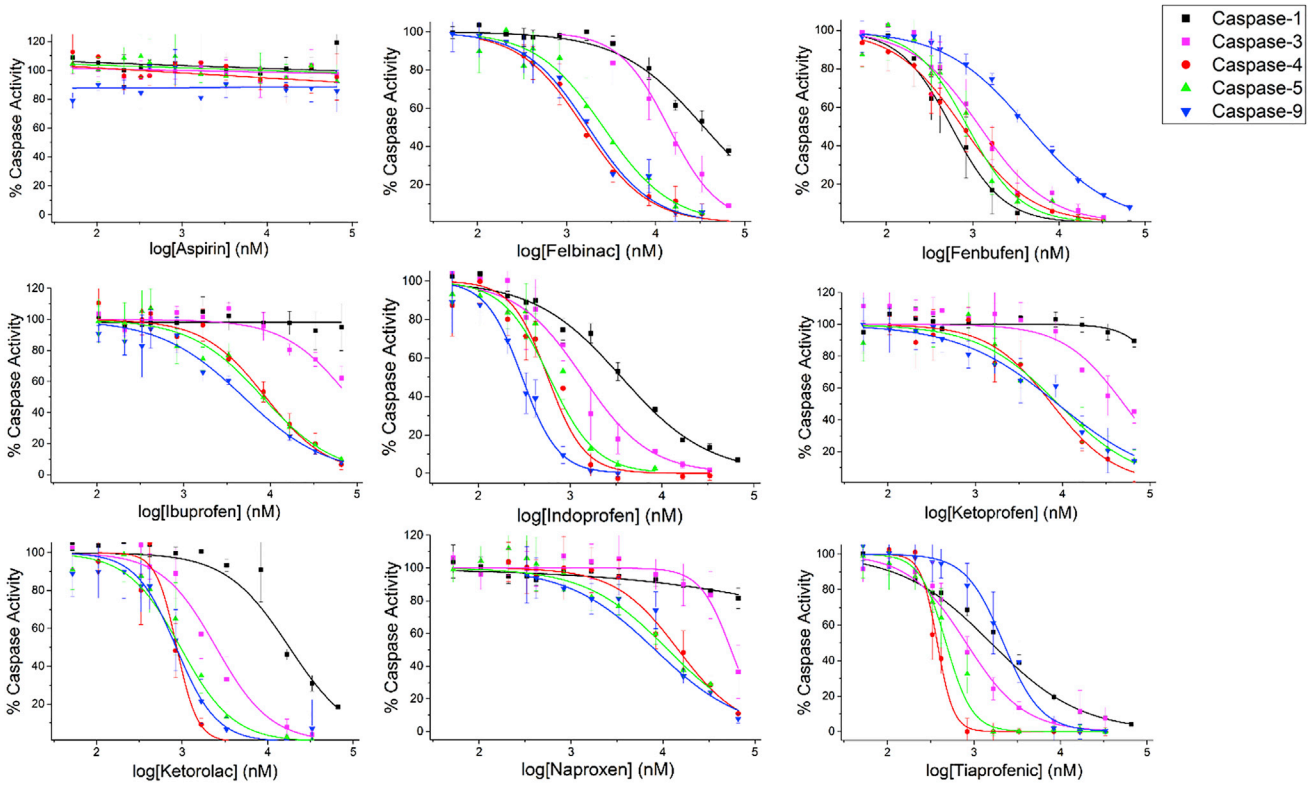

Figure 1. NSAIDs Are Multi-Caspase Inhibitors

Dose-response curves of five caspases treated with up to 66 μM of the indicated NSAIDs. Each caspase is denoted with a different color: caspase-1 in black, caspase-3 in pink, caspase-4 in red, caspase-5 in green, and caspase-9 in blue. The associated IC_{50} values are available in Table 2. 100% = substrate with solvent, 0% = z-VAD-FMK. Data are representative of the average and SD of at least two independent replicates.

was experimentally validated with a substrate competition assay. At increasing substrate concentrations, the observed half maximal inhibitory concentration (IC_{50}) value increases, consistent with inhibitor and substrate competing for the same site (Table S2).

Conversely, despite the presence of a carboxylic acid, aspirin does not preferentially dock to the active site (Figure 2E). Taken together with the observed caspase inhibition, these results suggest that NSAIDs may be competitive caspase inhibitors recognizing the active site.

NSAIDs Are Physiologically Relevant Caspase Inhibitors

We next studied inhibition of caspases by NSAIDs in a cellular context. With NSAID treatment between 0 and 500 μM, we observed dose-dependent inhibition of caspase-3 catalytic activity in HeLa cells (Figures 3A and S3C). Consistent with the in vitro observation of multi-caspase targeting, we observed multi-caspase inhibition with caspases-1, -3, and -9 when HeLa and THP-1 cells were treated with 100 μM of NSAIDs (Figures 3B–3D). In addition to these direct measurements of catalysis, we also assessed cell viability and IL-1β production as hallmarks of caspase signaling. THP-1 cells treated with up to 500 μM of NSAIDs demonstrated improved viability when challenged with the apoptosis inducers nigericin or staurosporine (Figures 4A and S3D). As caspase activity culminates in cell death, this is consistent with a reduction of caspase catalysis. In further support of caspase-1 inhibition, we also observed a decrease in IL-1β production in NSAID-treated cells (Figure 4B). Caspase-1 cleaves pro-IL-1β to its mature secreted form, thus caspase-1 inhibition halts cytokine release and subsequent inflammation. Importantly, across all the different assays the NSAID rank order is maintained, with fenbufen and indoprofen displaying substantial inhibitory capabilities, while naproxen and ibuprofen are weak inhibitors, and aspirin is ineffective.

Unlike previously identified NSAID targets observed only at high doses (mM), caspase inhibition occurs at micromolar concentrations in biological systems, consistent with physiological levels of NSAIDs (5–500 μM), and comparable with the IC_{50} values for COX (Ghosh et al., 2015; Gierse et al., 1999; Kato et al., 2001; Piazza et al., 1997). While inhibition is not comparable with that of potent, irreversible pan-caspase inhibitors such as z-VAD-FMK, at physiological concentrations caspase inhibition by NSAIDs may still have unforeseen effects on patients.

Caspase Inhibition by NSAIDs Is COX Independent

As inflammatory signaling has extensive crosstalk, it is difficult to parse out the individual contributions of caspase and COX inhibition by NSAIDs in a biological system. We explored the COX dependence of caspase inhibition in HCT116 cells, which lack COX-2. HCT116 cells treated with 10 μM staurosporine and 100 μM of NSAIDs demonstrated comparable inhibition with

Table 2. NSAID Inhibition of Caspases-1, -3, -4, -5, and -9

NSAID	Caspase	IC_{50} (μM)	R^2
Aspirin	1	no activity	NA
	3	no activity	NA
	4	no activity	NA
	5	no activity	NA
	9	no activity	NA
Felbinac	1	35 ± 2	0.984
	3	14 ± 2	0.970
	4	2.0 ± 0.1	0.988
	5	2.6 ± 0.3	0.987
	9	1.8 ± 0.2	0.989
Fenbufen	1	4.4 ± 0.2	0.996
	3	1.2 ± 0.2	0.972
	4	0.57 ± 0.04	0.991
	5	0.87 ± 0.09	0.982
	9	0.76 ± 0.08	0.987
Ibuprofen	1	no activity	NA
	3	>66	NA
	4	8.8 ± 0.6	0.991
	5	8.1 ± 0.5	0.991
	9	4.9 ± 0.3	0.991
Indoprofen	1	3.6 ± 0.2	0.996
	3	1.4 ± 0.2	0.983
	4	0.57 ± 0.07	0.971
	5	0.59 ± 0.08	0.959
	9	0.30 ± 0.01	0.994
Ketorolac	1	18 ± 2	0.991
	3	2.5 ± 0.3	0.991
	4	0.86 ± 0.07	0.988
	5	1.0 ± 0.1	0.982
	9	0.85 ± 0.07	0.990
Ketoprofen	1	>66	NA
	3	50 ± 10	0.856
	4	10 ± 1	0.989
	5	10 ± 2	0.972
	9	10 ± 1	0.960
Naproxen	1	>66	NA
	3	55 ± 7	0.798
	4	15 ± 1	0.988
	5	11 ± 1	0.977
	9	10 ± 1	0.983
Tiaprofenic acid	1	1.6 ± 0.1	0.999
	3	0.81 ± 0.07	0.991
	4	0.38 ± 0.01	0.993
	5	0.47 ± 0.03	0.986
	9	2.2 ± 0.2	0.997

IC_{50} values were determined for each NSAID and caspase combination. NSAIDs were tested from 66 μM to 26.1 nM to obtain a dose-response curve. Aspirin shows no inhibition against any caspase at the concentrations tested; however, all other tested NSAIDs demonstrate caspase inhibition. NSAIDs with no inhibition up to 66 μM are denoted as no activity, while weak inhibitors have an IC_{50} value > 66 μM. The associated dose-response curves are available in the supplemental information. NA, not applicable.

that observed in HeLa and THP-1 cells (Figure 5A). This finding is supported by small interfering RNA (siRNA) knock down of COX-2 and caspase-3 in HeLa cells. Knock down of COX-2 did not alter the caspase-3 response, indicating that the caspase catalysis assay is not contingent on COX-2 activity (Figure S4).

We further investigated NSAID treatments in the model organism *C. elegans*, in which the caspase homolog cell death proteins play a central role in apoptosis. *C. elegans* is an excellent model as apoptotic machinery is highly conserved, but lacks expression of either COX isoform (Lesa et al., 2003; Yuan et al., 1993). We performed cell death analysis using the *ced-1(e1735)* mutant that is defective in the cell corpse engulfment process, which sensitized the cell death assay (Conradt et al., 2016; Nakagawa et al., 2010). With 100 μM fenbufen or indoprofen treatment, we observed a statistically significant decrease of cell death starting at 2-fold, whereas 0.5% DMSO did not cause any effect (Figures 5B and S4C). This assay presents an in vivo COX-independent model for NSAID inhibition of caspases. While both pathways will be simultaneously affected in patients, caspase inhibition is a previously unacknowledged avenue that likely contributes to NSAID pharmacology.

DISCUSSION

Here we report a novel anti-inflammatory mechanism for NSAIDs such as ibuprofen, naproxen, and ketorolac. We observe that these NSAIDs are competitive caspase inhibitors, binding to the S1 subsite and obscuring the catalytic cysteine. Unlike previously identified non-COX NSAID targets, caspase inhibition is observed at physiological concentrations up to 500 μM. The caspase inhibitory profile is comparable in potency to COX, with micromolar IC_{50} values (Gierse et al., 1999; Kato et al., 2001; Piazza et al., 1997). We also observe that caspase inhibition is COX independent, implicating a novel pathway for NSAID pharmacology in patients. At physiological concentrations, it is likely that both COX and caspase pathways are simultaneously modulated, each contributing to the anti-inflammatory mechanism.

We propose that caspase contributions are relevant to the NSAID anti-inflammatory mechanism of action by reducing cell death and the generation of pro-inflammatory cytokines. However, despite the beneficial anti-inflammatory results of caspase inhibition, the possible detriments should not be discounted. Reducing healthy caspase signaling increases the incidence of viral and bacterial infection, and deregulates inflammation and cell proliferation (Guo et al., 2015; McIlwain et al., 2013). Interestingly, we observed a trend between the potency of caspase inhibition and drug recall and incidence of side effects. For example, fenbufen and indoprofen have been largely recalled from global markets due to liver and gastrointestinal toxicity (Fung et al., 2001; Lewis and Stine, 2013). However, aspirin, ibuprofen, and naproxen remain prevalent, and demonstrate weaker caspase inhibition. Future efforts should look to further explore how caspase inhibition by NSAIDs affects patients. Drug repurposing may expand NSAID applicability to caspase-focused pharmacological

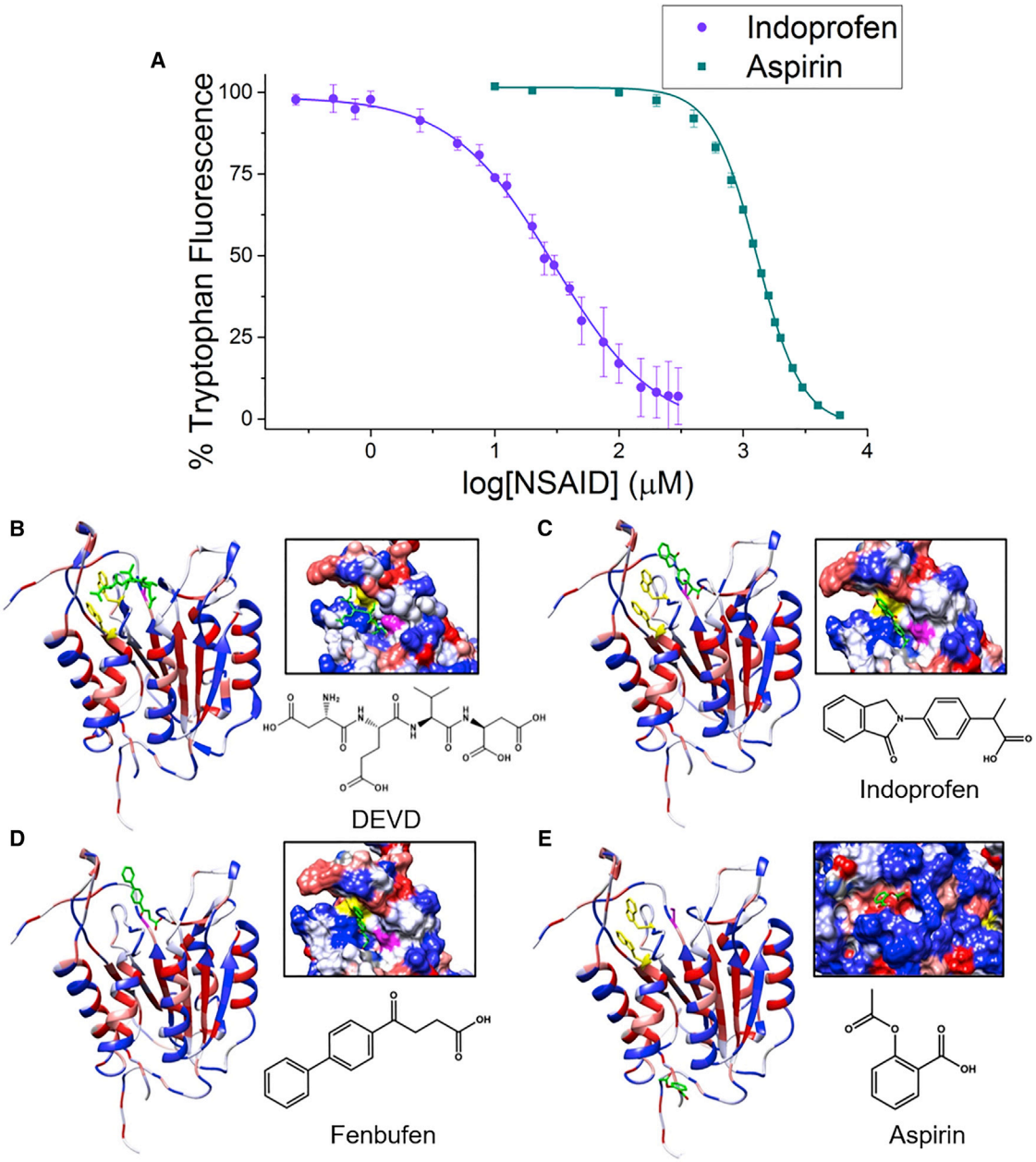

Figure 2. A Biophysical and Computational Model of Caspase Binding Interactions

(A) Titration with indoprofen or aspirin quenches caspase-3 tryptophan fluorescence. The apparent K_D for indoprofen (purple circles) is 30 ± 2 μM. The apparent K_D for aspirin (green squares) is 1,290 ± 10 μM. Data are representative of the average and SD of at least two independent experiments, normalized with the titrated solvent control as 100%, and buffer titrated with compound as 0%.

(B) Caspase-3 (PDB: 2DKO) is shown with a tetrapeptide substrate or NSAID (green). The catalytic cysteine denoted in pink, tryptophans are yellow, and other residues depict hydrophobicity (blue more polar, red more hydrophobic). The inset shows the surface of the binding pocket.

(C) A representation of the top computationally predicted cluster of indoprofen bound to caspase-3. Predictions place indoprofen in the S1 pocket of the active site.

(D) A representation of the top computationally predicted cluster of fenbufen bound to caspase-3. Predictions place indoprofen in the S1 pocket of the active site.

(E) A representation of the top computationally predicted cluster of aspirin bound to caspase-3. Predictions place aspirin away from the active site.

targets such as cancer, cardiovascular disease, rheumatoid arthritis, and neurodegenerative diseases. An improved understanding of how caspases control the NSAID anti-inflammatory mechanism may lead to the design of more specific drugs, and improve patient safety.

SIGNIFICANCE

While NSAIDs are commonly used drugs, the pharmacological contributions of non-canonical cellular targets remain unclear. Through high-throughput screening of FDA-approved

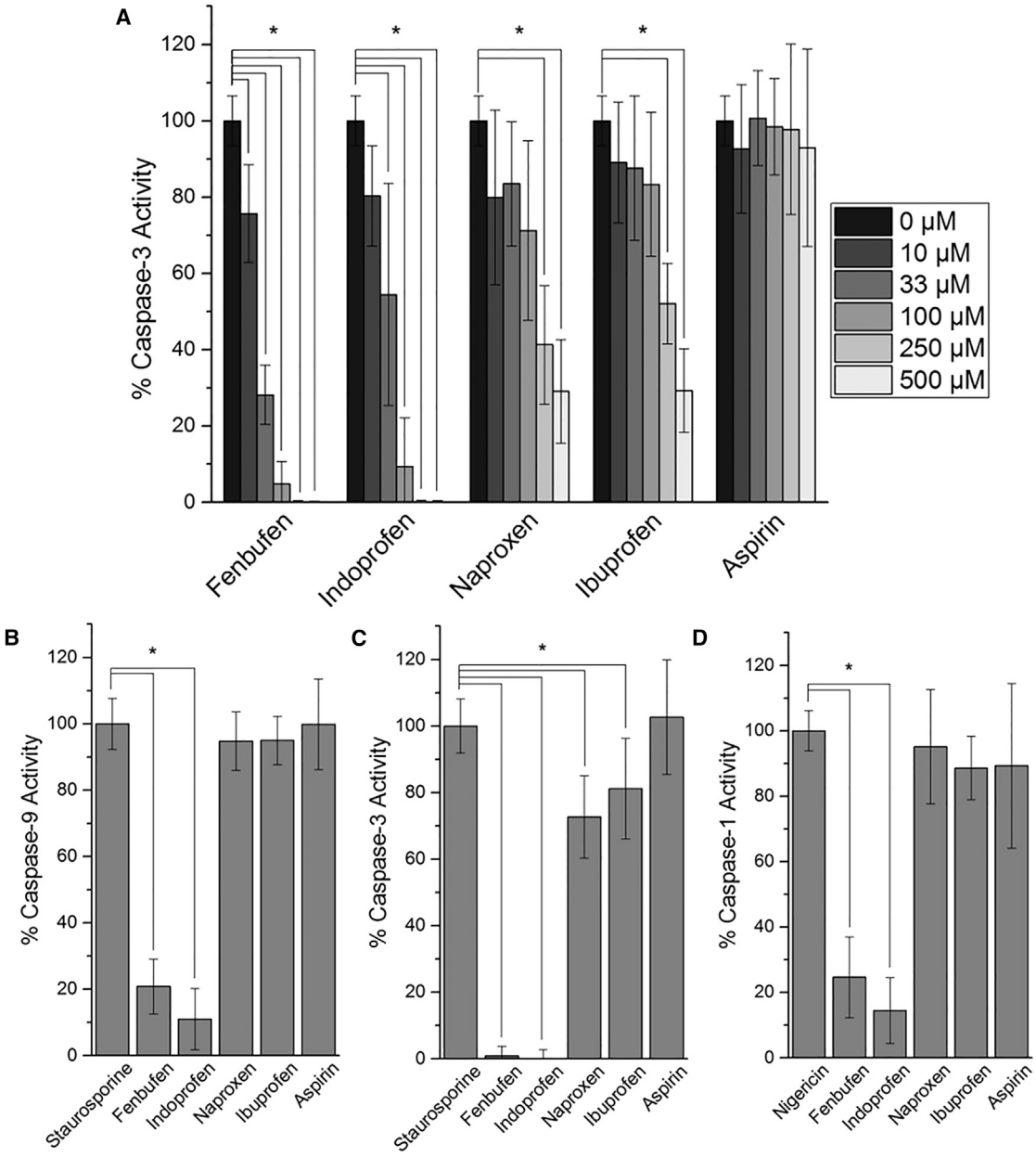

Figure 3. NSAIDs Inhibit Catalysis of Multiple Caspases in Cell Culture

(A) HeLa cells treated with 1 μM staurosporine show dose-dependent caspase-3 inhibition when treated with 0–500 μM NSAIDs. 100% = staurosporine, 0% = z-VAD-FMK. *$p \leq 0.05$ compared with 100% control.

(B) HeLa cells treated with 1 μM staurosporine and 100 μM NSAIDs show a reduction in caspase-9 activity. 100% = staurosporine, 0% = z-VAD-FMK. *$p \leq 0.05$ compared with 100% control.

(C and D) THP-1 cells treated with 100 μM of selected NSAIDs in the presence of (C) staurosporine (caspase-3) or (D) 1 μM nigericin (caspase-1) show inhibited caspase catalytic activity. 100% = staurosporine or nigericin, 0% = z-VAD-FMK. *$p \leq 0.05$ compared with 100% control.

All data are the average and standard deviation of three biological replicates. See also Figure S3C.

drugs, we have identified a subset of NSAIDs capable of inhibiting caspases at physiological concentrations. In vitro characterization obtained IC_{50} values with multiple caspases, representing wide applicability against diverse biological functions and substrates. Principal-component analysis examined NSAID chemical space, and identified compound size as the discerning factor for inhibition. We explored active-site binding interactions with tryptophan quenching, substrate competition, and computational modeling. Further, we examined the biological context of this work through cell culture and *C. elegans* animal models. Here we observed a non-canonical NSAIDs mechanism that is COX independent, establishing caspases as a novel pharmacological target.

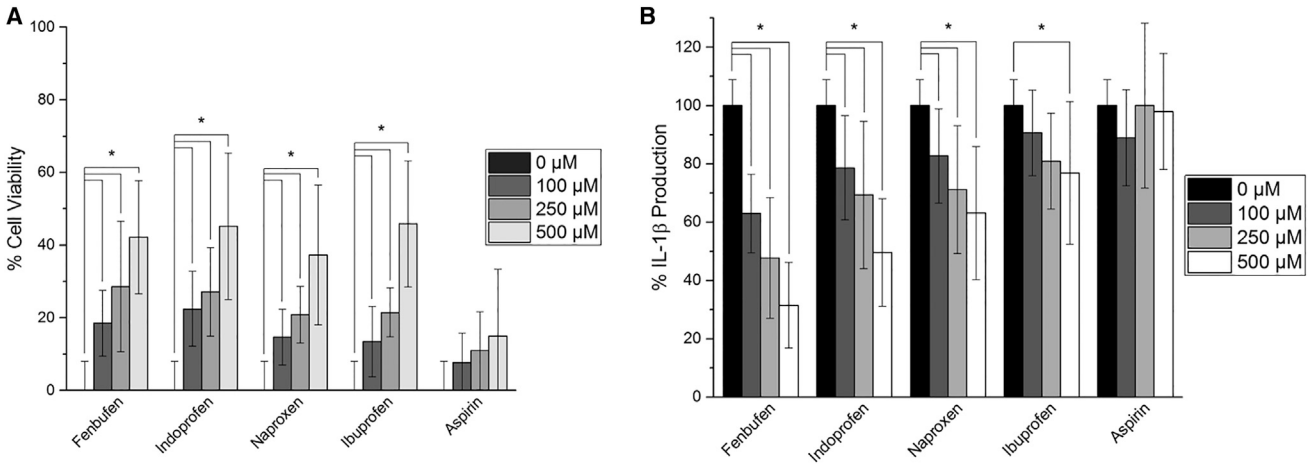

Figure 4. NSAIDs Inhibit Caspase Signaling in Cell Culture

(A) Treatment with 0–500 μM of NSAIDs improves the viability of apoptotic THP-1 cells treated with 25 μM nigericin. 100% = untreated cells, 0% = nigericin. *$p \leq 0.05$ compared with 0% control.

(B) THP-1 cells were treated with nigericin to activate caspase-1 and promote the inflammatory cytokine IL-1β. NSAID treatment from 0 to 500 μM demonstrates a dose-dependent reduction of IL-1β release. 100% = staurosporine, 0% = untreated cells. *$p \leq 0.05$ compared with 100% control. All data are the average and SD of three biological replicates. The statistical significance was determined using a one-way ANOVA followed by Tukey's test. See also Figure S3D.

While extensive effort has been invested in the study of NSAIDs, previous research linking NSAIDs to apoptosis was not conducted under inflammatory conditions. In these circumstances, the contribution of caspases would not be apparent, as these important enzymes are upregulated only under inflammatory conditions. Further, many studies are conducted at superphysiologic NSAID concentrations, and may not be relevant to patients. By measuring caspase signaling at physiologic concentrations under inflammatory conditions, we were able to observe a novel role for NSAIDs. Understanding how additional targets may contribute to NSAID pharmacology could clarify the anti-inflammatory mechanism, improve patient safety, and facilitate the design of next-generation therapeutics.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification

For high-throughput screening, a caspase-4 C258A catalytically inactive mutant was expressed using a modified pET vector, which was generously gifted by Dr. Feng Shao (Shi et al., 2014). To obtain the catalytically active mutant, a New England Biolabs Q5 Site-Directed Mutagenesis Kit was used to revert the catalytically inactive caspase-4 to an active form with cysteine at site 258. Caspase-3 (pET23b-Casp3-His Addgene no. 11821) and caspase-9 (pET23b-Casp9-His Addgene no. 11829) were gifts from Dr. Guy Salvesen (Stennicke et al., 1999; Zhou et al., 1997). Proteins were expressed and purified using the protocol described previously by Denault and Salvesen (2002). Briefly, plasmids were expressed in *E. coli* BL21 pLysS cells and grown in 2× tryptone yeast culture medium at 18°C overnight after induction with 50 μM isopropyl β-D-1-thiogalactopyranoside. Cells were then pelleted and sonicated to collect the lysate, and purified using Nickel NTA Agarose Beads (Gold Bio). Fractions were analyzed by SDS-PAGE. For IC_{50} determination, caspase-1, -4, and -5 were purchased from Enzo Life Sciences.

High-Throughput Screening

High-throughput screening for inhibitors of human caspase-4 was performed using the 1280 compound Prestwick Chemical Library at the University of Colorado High-Throughput Screening Core Facility. The screen was designed in a 384-well format (Greiner no. 781207), using 33 μM compound, 1 μM recombinant human caspase-4 in high-citrate buffer (50 mM Tris-HCl [pH 7.5], 1 M sodium citrate, 10 mM DTT, and 10% sucrose), and 20 μM Promega Caspase-Glo 9 Assay substrate (O'Brien et al., 2005; Roschitzki-Voser et al., 2012). While designed for use with caspase-9, the LEHD substrate is also recognized by caspase-4 (Roschitzki-Voser et al., 2012; Talanian et al., 1997). The assay Z′ factor is 0.66, recommending this method for high-throughput experimentation (Figure S1) (Zhang et al., 1999). Plates were read with endpoint luminescent analysis 20 and 60 min after substrate addition. Compounds were considered to be hits if caspase-4 activity was inhibited to less than 25% relative to the solvent control. Using these criteria, we identified 27 hits, which are summarized in Table 1. Fenbufen, indoprofen, ketoprofen, ketorolac, felbinac, naproxen, ibuprofen, and tiaprofenic were purchased from Sigma-Aldrich. Aspirin was purchased from TCI America. z-VAD-FMK was obtained from InvivoGen.

Caspase Catalytic Activity, IC_{50} Determination, and Specificity

To assess the potency and specificity of each NSAID, IC_{50} values were determined against caspases-1, -3, -4, -5, and -9. Experiments were performed in a 384-well format (Greiner no. 781207) as per the conditions noted here.

Caspase-1: 2.5 nM enzyme, 6.5 μM WEHD substrate, ECB
Caspase-3: 200 nM enzyme, 3.3 μM DEVD substrate, SCB
Caspase-4: 1 nM enzyme, 10 μM LEHD substrate, HCB
Caspase-5: 20 nM enzyme, 10 μM LEHD substrate, HCB
Caspase-9: 200 nM enzyme, 6.5 μM LEHD substrate, HCB
ECB (Enzo Caspase Buffer): 50 mM HEPES (pH 7.4), 100 mM NaCl, 0.5% Tween 20, 10 mM DTT, and 10% glycerol
SCB (Standard Caspase Buffer): 20 mM PIPES (pH 7.5), 100 mM NaCl, 1 mM EDTA, 10 mM DTT, and 10% sucrose
HCB (High-Citrate Buffer): 50 mM Tris-HCl (pH 7.5), 1 M sodium citrate, 10 mM DTT, and 10% sucrose

Substrate was present in at least 16-fold excess, and enzyme concentrations were kept well below IC_{50}. Activity was measured as the change in luminescent signal for at least 30 min. Analysis was performed using the linear portion of the curve; however, endpoint analysis yielded values within error. Percent inhibition was determined compared with the DMSO-only control. Each assay included solvent and z-VAD-FMK controls. Compounds were tested between 26.1 nM and 66 μM to obtain a dose-response curve. Higher

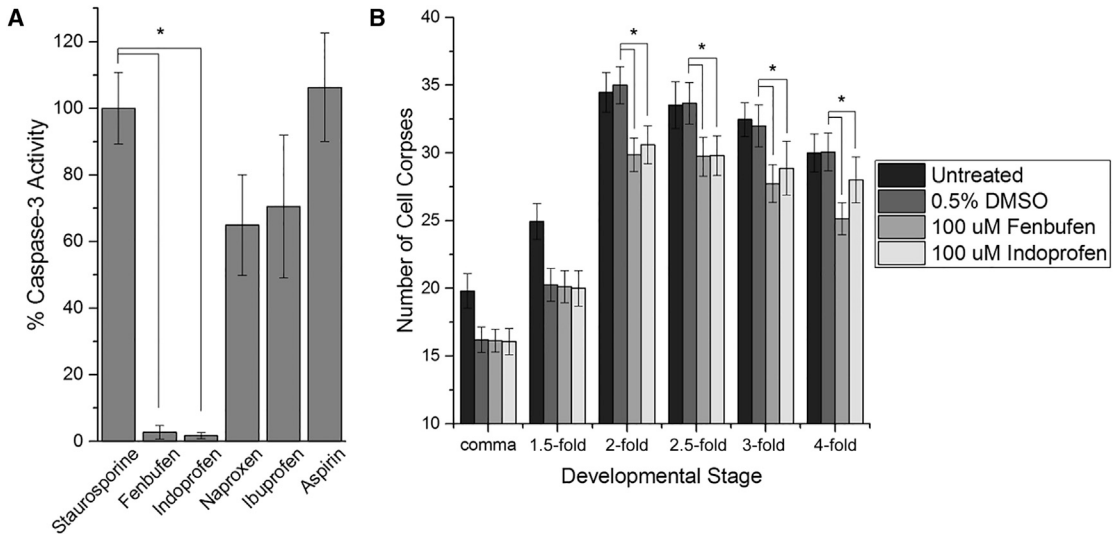

Figure 5. Caspase Inhibition by NSAIDs Is COX Independent

(A) HCT116 cells treated with 10 μM staurosporine and 100 μM NSAIDs demonstrate inhibition of caspase-3 catalysis. HCT116 cells do not express COX-2, indicating that caspase inhibition is not contingent on canonical COX2 pharmacology. Data are representative of the average and SD of three biological replicates. 100% = staurosporine, 0% = z-VAD-FMK. *$p \leq 0.05$ compared with 100% control.

(B) Cell death assays in *C. elegans* embryos treated with fenbufen or indoprofen. L4-stage *ced-1(e1735)* animals were exposed to 100 μM fenbufen or indoprofen in 0.5% DMSO on NGM agar plates, and their progenies were analyzed. Embryonic cell corpses were scored at 1.5-, 2-, 2.5-, 3-, and 4-fold stages. The y axis represents the mean and SD of cell corpses scored (n = 15). The statistical significance was determined using a one-way ANOVA followed by Tukey's test. *$p \leq 0.05$ compared with 0.5% DMSO control. See also Figure S4.

concentrations were not tested since higher DMSO concentrations (>1%) decreased luminescence. IC_{50} values were calculated using OriginPro 2016 using a dose-response curve with a variable Hill slope, where A1 and A2 represent the asymptotes, p is the Hill slope, and Logx0 is the center.

$$y = A1 + \frac{A2 - A1}{1 + 10^{(\log x0 - x)p}}$$

The asymptotes were fixed at 100% and 0% to represent the solvent and z-VAD-FMK controls, respectively. Data shown are the average and SD of at least two technical replicates repeated in two independent assays.

Cathepsin B Catalytic Activity

Recombinant human liver cathepsin B was obtained from Enzo Life Sciences. NSAID inhibition of cathepsin B was tested in a 384-well format (Greiner no. 781209) in buffer containing 352 mM KH_2PO_4, 48 mM Na_2HPO_4, 4 mM EDTA, 8 mM cysteine, and 0.1% Triton X at pH 6.0 with 66 μM of compound (Barrett, 1980). Cathepsin B was assayed at 75 nM with 2.5 μM z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride (Z-RR-AMC) (Sigma-Aldrich). N-Acetyl-Leu-Leu-Methional (ALLM) from BioVision Technologies was used at 1 μM as an inhibitory control. Data shown are the average and SD of at least three technical replicates repeated in three independent assays (n = 9). Data were normalized such that cathepsin B with solvent is 100%, and 0% as ALLM inhibitor.

Caspase Catalytic Activity in Cultured Cells

HeLa (human epithelial) and HCT 116 (human colon) cells from ATCC were cultured in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin were plated at 10,000 cells/well in a white 96-well plate (Greiner no. 655083) and grown overnight. THP-1 (human peripheral blood monocyte) cells from ATCC were grown in RPMI with 10% FBS, 1% penicillin/streptomycin, and 0.05 mM 2-mercaptoethanol. THP-1 cells were plated at 25,000 cells/well in a white 96-well plate and differentiated with 10 ng/mL phorbol myristate acetate (PMA) overnight. The day after plating the cell culture medium was replaced. THP-1 cells were primed for 4 hr with 1 μg/mL *E.* coli O111:B4 lipopolysaccharide (InvivoGen). HeLa and HCT 116 cells were not primed. Cells were then treated with 1 or 10 μM staurosporine (Sigma-Aldrich) or 1 μM nigericin (InvivoGen) and the indicated concentration of inhibitor (Locovei et al., 2007; Mariathasan et al., 2006; Omura et al., 1977; Pelegrin and Surprenant, 2007; Tamaoki et al., 1986). After incubation, caspase-1 (2 hr incubation, Promega Caspase-Glo 1 Inflammasome Assay), caspase-3 (4 hr incubation, Promega Caspase-Glo 3/7 Assay), or caspase-9 (4 hr incubation, or Promega Caspase-Glo 9 Assay) activity was assayed per the manufacturer's recommendations. Data were normalized with 10 μg/mL z-VAD-FMK as 0% caspase activity, and staurosporine or nigericin as 100% activation. Data shown are the average and SD of at least three biological replicates. Statistical significance was determined using a one-way ANOVA test followed by Tukey's test to compare compound treated with nigericin or staurosporine only.

Cell Viability Determination

THP-1 cells were cultured and plated as above in a 96-well black plate (Greiner no. 655086). THP-1 cells were differentiated for 3 days with PMA. Apoptosis was induced using 10 μM staurosporine or 25 μM nigericin, and viability was measured after 2 hr using the Promega CellTiter-Fluor Viability Assay as per the manufacturer's recommendations. Additionally, the Promega CellTiter-Glo Luminescent Cell Viability Assay was used to assess compound toxicity and observe luciferase inhibition. Data were normalized using untreated cells as 100% viability and staurosporine or nigericin cells as 0%. For CellTiter-Glo, 0% is lysed cells. Data shown are the average and SD of at least three biological replicates. Statistical significance was determined using a one-way ANOVA test followed by Tukey's test to compare the compound treated with nigericin or staurosporine only.

ELISA

THP-1 cells were plated in a 96-well plate (Costar no. 3596), differentiated for 3 days with PMA, and treated with 20 μM nigericin. IL-1β cytokine production was determined using the BD Biosciences Human IL-1β ELISA Set II as per the manufacturer's instructions. Data were normalized with 100% as nigericin treated, and untreated cells as 0%. Data shown are the average and SD of at least three biological replicates. Statistical significance was determined using a one-way ANOVA test followed by Tukey's test to compare compound treated with nigericin only.

siRNA Knock Down and Western Blot

HeLa cells were plated at 300,000 cells/well in a 6-well tissue-culture treated plate (Costar no. 3516) and grown in supplemented DMEM (1% penicillin/streptomycin, 10% FBS). Wells were transfected using 1.2 μL Lipofectamine RNAiMAX (Thermo Fisher Scientific) and 25 nM of siRNA. COX2 siRNA was obtained from Dharmacon (ON-TARGETplus Human PTGS2 SMARTpool 5743). Silencer Select CASP3 (no. 4290824) and GAPDH (no. 4390849) siRNAs were obtained from Thermo Fisher Scientific. For each experiment, a mock transfection control was performed without siRNA. Cells were maintained for 48 hr, then transferred into a 96-well plate at 10,000 cells/well and grown overnight. The following day, 72 hr post transfection, cells were assayed for caspase-3 activity as described above, or collected for protein expression analysis by western blot. For western blot, cell pellets were collected, lysed in radioimmunoprecipitation assay buffer (Thermo Fisher Scientific) and assayed for total protein content using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) as per the manufacturer's instructions. For caspase-3 and GAPDH detection, 20 μg of total protein was loaded per lane. Due to the lower expression levels of COX2, 50 μg of total protein was loaded per lane. COX2 (no. 4842), GAPDH (no. 2118), and caspase-3 (no. 9662) primary antibodies were obtained from Cell Signaling Technologies. Peroxidase-conjugated Affinity Pure Goat Anti-rabbit IgG secondary antibody was obtained from Jackson ImmunoResearch Laboratories (111-035-003). Knock down was quantified using Fiji (Schindelin et al., 2012; Schneider et al., 2012). Data shown are the average and SD of at least two biological replicates. Data were normalized with mock staurosporine treated as 100%, and 0% as z-VAD-FMK treated.

Tryptophan Fluorescence

Caspase-3 tryptophan fluorescence was detected on a HORIBA Jobin Yvon Fluorolog using FluorEssence software. Fluorescence of 250 nM caspase-3 was measured with excitation at 295 nm and emission from 300 to 450 nm. Indoprofen or aspirin was titrated into the sample and the change in fluorescence at 342 nm was quantified. Samples were incubated for 5 min after titration in order to ensure equilibrium. Data were normalized as caspase-3 titrated with DMSO as 100% and buffer titrated with compound as 0%. This accounts for quenching by DMSO, and for the intrinsic fluorescence of the NSAID. Data shown are the average and SD of at least two independent trials.

COX-Independent Apoptosis in *C. elegans*

The phenotypic analysis in animals was conducted using a *C. elegans* mutant, *ced-1(e1735),* which is defective in the cell corpse clearance process. Fenbufen or indoprofen (100 μL of 100 μM) with 0.5% DMSO were added to the nematode growth medium plates seeded with bacteria (OP-50), and incubated for 3 min at room temperature for absorption. One hundred L4 animals were placed on the plates and incubated for 1 day at 20°C to allow them to lay embryos. The live embryos were then subjected to cell corpse counting as described previously (Parrish et al., 2003). Statistical significance was determined using a one-way ANOVA test followed by Tukey's test to compare compound treated with the 0.5% DMSO control.

Computational Docking Analysis

Docking of NSAIDs with caspase-3 was performed using SwissDock (Grosdidier et al., 2011a, 2011b). Compounds (indoprofen: ZINC 391; aspirin: ZINC 53; fenbufen: ZINC 1427) were docked against caspase-3 (PDB: 2DKO) (Ganesan et al., 2006). Drug structures were obtained from ZINC, provided by the Irwin and Shoichet Laboratories in the Department of Pharmaceutical Chemistry at the University of California, San Francisco (UCSF) (Irwin and Shoichet, 2005; Irwin et al., 2012; Sterling and Irwin, 2015). Clusters were scored using their FullFitness, and the top hit was visualized. Molecular graphics and analyses were performed with the UCSF Chimera package. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the UCSF (Pettersen et al., 2004).

Chemical Space Analysis

ChemGPS-NP was used to explore the chemical space of screening hits (Larsson et al., 2007). ChemGPS-NP was developed and made available by the Backlund group at Uppsala University. Using ChemGPS-NP PCA analysis, compounds were compared based on factors such as size, aromaticity, polarity, and hydrophobicity. Compound size, shape, and polarizability is determined by molecular weight, number of atoms, and Ghose-Crippen molar refractivity. Aromaticity and conjugation is based on the number of aromatic bonds, aromatic carbons, aromatic hydroxyl groups, and the aromatic ratio. Lipophilicity, polarity, and hydrogen-bond capacity are determined by the number of hydrogen-bond donors and acceptors, the sum of atomic polarizabilities, and the Ghose-Crippen ALogP. Flexibility and rigidity are calculated with the rotatable bond fraction based on the number of bonds, rings, and rotatable bonds.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2017.02.003.

AUTHOR CONTRIBUTIONS

Conceptualization, Methodology, Visualization, and Formal Analysis: C.S.; Investigation and Validation: C.S., S.S., T.J., and A.N.; Writing-Original Draft: C.S. and S.S.; Writing-Review and Editing: C.S., S.S., T.J., A.N., D.X., and H.Y.; Supervision and Project Administration: C.S., H.Y., and D.X.; Funding acquisition: H.Y. and D.X.

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