Potent and Selective Nonpeptide Inhibitors of Caspases 3 and 7

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5-Dialkylaminosulfonylisatins have been identified as potent, nonpeptide inhibitors of caspases 3 and 7. The most active compound within this series (**34**) inhibited caspases 3 and 7 in the 2−6 nM range and exhibited approximately 1000-fold selectivity for caspases 3 and 7 versus a panel of five other caspases (1, 2, 4, 6, and 8) and was at least 20-fold more selective versus caspase 9. Sequence alignments of the active site residues of the caspases strongly suggest that the basis of this selectivity is due to binding in the S_2 subsite comprised of residues Tyr204, Trp206, and Phe256 which are unique to caspases 3 and 7. These compounds inhibit apoptosis in three cell-based models: human Jurkat T cells, human chondrocytes, and mouse bone marrow neutrophils.

Introduction

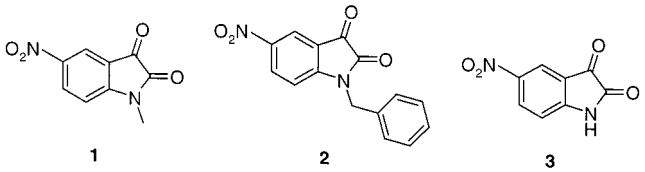
Dysregulated apoptosis has been intensely studied in recent years and is believed to play a role in several diseases of therapeutic interest.[1,2] The molecular components of the signaling pathway can vary depending on cell type and apoptotic inducing agent. However, caspases are proposed to play a critical role in the majority of cell death pathways characterized thus far.

Caspases were first characterized as cysteinyl aspartyl proteinases via the homology of the *Caenorhabditise elegans* cell death gene *ced-3* and the interleukin-1β converting enzyme (ICE). To date, a total of 11 human caspases have been characterized (ICE and 10 family members).[3] They can be subdivided into three groups based on homology and substrate specificity: (1) caspases involved in inflammation (caspases 1, 4, 5, and 13), (2) initiator caspases which are found at the top of the signaling cascade (caspases 6 and 8−10), and (3) effector caspases which are activated further downstream (caspases 2, 3, and 7).[4] The study of the relative importance of different caspases in apoptosis has been hampered by the lack of availability of selective inhibitors of individual caspases.[5−7] Potent peptide inhibitors are only moderately selective at best and possess poor cell permeabilities. To overcome this latter problem, they are often dosed as prodrugs, but this complicates interpretation of results further because the inhibiting species, and hence its selectivity, is not well characterized. The delineation of the roles of individual caspases in apoptosis would be facilitated with caspase-selective inhibitors.

Caspase 3 is a member of the class of effector caspases and has been found to be activated in nearly every model of apoptosis encompassing several different signaling pathways.[8] As such, caspase 3 is a potential therapeutic target for the treatment of diseases involving dysregulated apoptosis. Our strategy was to identify a potent and selective inhibitor for this enzyme and evaluate its efficacy using human chondrocytes undergoing apoptosis, a cell-based model for osteoarthritis.

Results and Discussion

Identification of Isatin Sulfonamides as Inhibitors of Caspase 3. A high-throughput screen of the SmithKline Beecham compound collection for inhibitors of caspase 3 resulted in the identification of 5-nitroisatins **1** and **2** which possessed IC_{50}'s of 1 and 0.25 μM, respectively. The closely related unalkylated analogue **3** was only slightly less active (IC_{50} = 3 μM).



The importance of the 5-nitro functionality was evaluated by screening several 5-substituted isatins as inhibitors of caspase 3 (Table 1). A correlation between the electron-withdrawing ability of the 5-substituent (σ_m)[9] and the potency of inhibition was observed. Nitro (**1**), methoxycarbonyl (**5**), iodo (**6**), and cyano (**7**) substitution all led to low micromolar inhibition potencies, while carboxylate (**4**) (predominant form at pH 7.5) and protio (**8**) compounds exhibited no activity at 50 μM. These results suggested that the electrophilicity of the isatin carbonyl was critical for activity and that the

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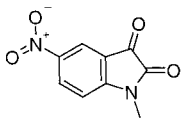
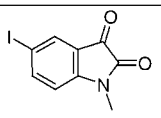
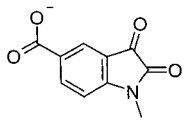
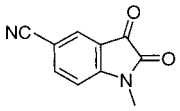
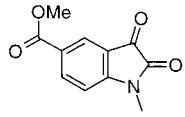
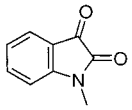
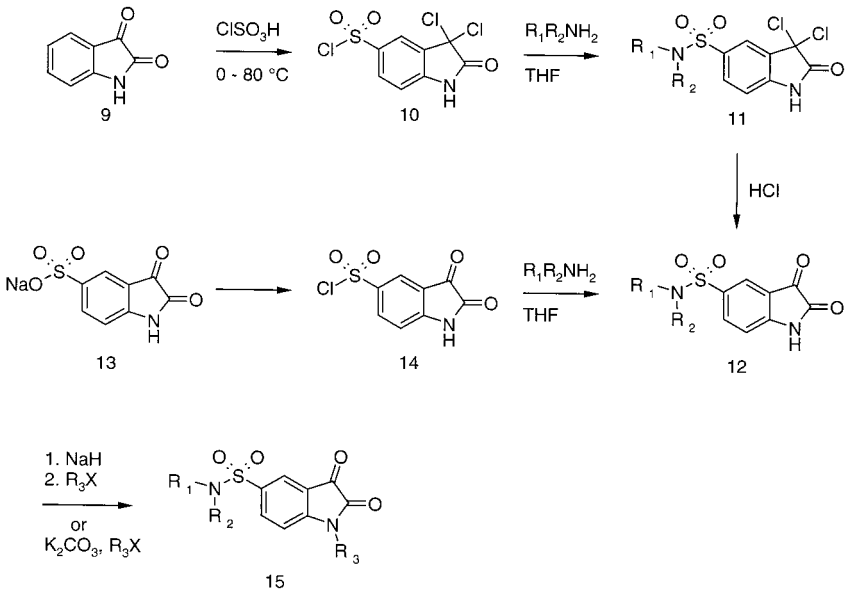
Table 1. Importance of the 5-Nitro Group

Compd	Structure	$IC_{50}(\mu M)$	σ_m	Compd	Structure	$IC_{50}(\mu M)$	σ_m
1		1	0.71	**6**		7	0.35
4		>50	-0.1	**7**		0.6	0.56
5		15	0.36	**8**		>50	0

Scheme 1



mechanism of action involved addition of the catalytic cysteine residue of the enzyme to this functionality. In addition, the diversity of groups which led to active compounds indicated that they were not involved in specific interactions with the enzyme.

Isatins had previously been reported to be inhibitors of chymotrypsin,[10] and after the initiation of our studies, potent isatin-based inhibitors of rhinovirus 3C protease were disclosed.[11] In this latter study, a 5-carboxamido functionality was critical for recognition by the S_1 subsite of the protease.

Our initial binding model was based on the previously determined X-ray structures[12,13] and postulated the formation of a tetrahedral intermediate between the catalytic cysteine thiol of caspase 3 and the isatin carbonyl group. The positioning of the inhibitor suggested that access to the extended binding regions of the active site (S2−S4) would be attainable by elaborating groups off of the 5- or 6-positions of the isatin core. We chose to investigate the 5-position with two goals in mind: (1) replacement of the nitro group which may be subject to metabolic reduction[14] and (2) identification of a replacement functionality with the appropriate electronic properties which would also allow for the incorporation of molecular diversity. An examination of σ_m values for a variety of functional groups[9] led to the identification of 5-*N*,*N*-dialkylisatin sulfonamides as potential inhibitors of caspase 3.

Syntheses of 5-*N*,*N*-Dialkylisatin Sulfonamides. *N*,*N*-Dialkylisatin sulfonamides were prepared by condensing secondary amines with 5-chlorosulfonylisatin **14** (Scheme 1). Our first attempts at securing the sulfonyl chloride involved heating isatin **9** in chlorosulfonic acid at 70 °C.[15] However, in contrast to previously published results, only the gem-dichloro derivative **10** was isolated in high yield. Lowering the temperature and adding methylene chloride as a cosolvent did not result in isolation of any desired keto product **14**.[16] However, the desired 5-isatin sulfonamide could be obtained via reaction of gem-dichloro **10** with an amine to afford **11**, which upon hydrolysis (3 N HCl, 50 °C) afforded **12**. A more straightforward route involved the preparation of 5-chlorosulfonylisatin by treating sodium 5-isatin sulfonate **13** with phosphorus oxychloride in sulfolane at 80 °C.[17] Reaction of the sulfonyl chloride with 1 equiv of amine in the presence of 1 equiv of diisopropylethylamine in tetrahydrofuran yielded 5-isatin sulfonamide **12**. This product was alkylated by treatment with sodium hydride in dimethylformamide, and the resulting salt reacted with an alkyl halide at

Table 2. Importance of the Chiral Methoxymethylpyrrolidine Group and Ring Size

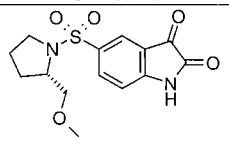
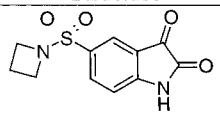
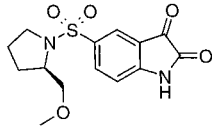
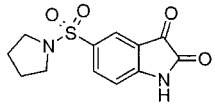
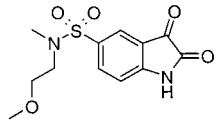
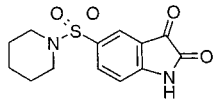
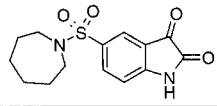
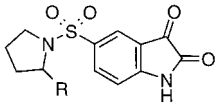
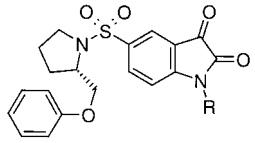
Compd	Structure	IC_{50} (nM)	Compd	Structure	IC_{50} (nM)
16		120	**19**		170
17		18,000	**20**		2,800
18		910	**21**		2,200
			22		1,900

Table 3. Substitution of the Pyrrolidine Ring



compd	structure	IC_{50} (nM)	compd	structure	IC_{50} (nM)
23	(*S*)-MeO_2C	170	**27**	(*R*)-$PhNMCH_2$	5500
24	(*S*)-$^tBuO_2C$	70	**28**	(*S*)-PhNH(O)C	140
25	(*S*)-$Me_2N(O)C$	410	**29**	(*S*)-$PhOCH_2$	44
26	(*S*)-$PhNHCH_2$	31	**30**	(*S*)-$PhSCH_2$	44

Table 4. Alkylation of the Isatin Nitrogen



compd	structure	IC_{50} (nM)	compd	structure	IC_{50} (nM)
31	CH_3	30	**35**	$^tBuO_2CCH_2$	3.1
32	$CH_2CH{=}CH_2$	4.6	**36**	HO_2CCH_2	170
33	$CH_2C_6H_{11}$	5.2	**37**	4-Pyr-CH_2	4.2
34	$PhCH_2$	2.5			

ambient temperature to give 1-alkyl-5-alkylaminosulfonylisatin **15**.[18] Alternatively, the alkylation may also be conducted in the presence of the alkyl halide and potassium carbonate in DMF.

Inhibition of Caspase 3 by the Isatin Sulfonamides. Inhibition of recombinant human caspase 3 by the compounds was assessed using a standard fluorometric assay.[19] The preparation of a diverse array of sulfonamides initially led to the discovery of isatin sulfonamide **16** which was a 120 nM (IC_{50}) inhibitor of caspase 3 (Table 2).

The importance of the chiral methoxymethylpyrrolidine group for recognition was initially examined (Table 2). Antipode **17** and the open-ring derivative **18** were significantly less active. The effect of ring size on activity was evaluated with compounds **19**−**22**. Increasing the ring size from five to seven atoms had little effect on activity (cf. **20** with **21** and **22**), but decreasing it to an azetidine resulted in a 10-fold increase in potency relative to that of **20**. The SAR around the pyrrolidine ring system was further pursued because of the commercial availability of substituted pyrrolidines.

The methyl prolinate **23** and dimethylamide **25** were slightly less active than compound **16** (Table 3). However, the *tert*-butyl prolinate **24** exhibited a 2-fold enhancement in activity and is suggestive of a hydrophobic interaction between the *tert*-butyl group and the enzyme surface. Encouraged by this result, we prepared the anilinomethyl derivative **26** and found it to be a 31 nM inhibitor of caspase 3. Its antipode **27** was 100-fold less active, a result similar to that observed with the methoxymethyl pair, **16** and **17**. The amide **28** was 4-fold less active than **26**. Oxygen (**29**) and sulfur (**30**) heteroatom replacements for the anilino nitrogen of **26** yielded compounds of similar potency.

The potencies of 5-nitroisatins **1**−**3** suggested that another order of magnitude in affinity might be obtainable by alkylating the isatin nitrogen. Compound **31** exhibited a minor improvement in activity relative to its precursor **29** (Table 4), but alkylation with larger hydrophobic groups (allyl **32**, cyclohexyl **33**, and benzyl **34**) yielded compounds with activities as low as 2.5 nM. The importance of a hydrophobic interaction is highlighted by the difference in activities between *tert*-butyl acetate **35** and the corresponding acid **36**. If positioned correctly, polar functionalities can be accommodated as evidenced by the activity of the 4-pyridylmethyl derivative **37**.

Mechanistic Characterization of the Isatins. The inhibition profiles of the initial screening hit **1** and isatin sulfonamide **16** were investigated (Figure 1). Kinetic patterns indicating simple linear competitive inhibition versus Ac-AspGluValAsp-AMC were obtained with estimated K_i's of 1.3 μM and 83 nM, respectively. These results are consistent with our initial binding model in which the inhibitor is binding at the catalytic site. Our model also predicted the formation of a covalent, tetrahedral adduct between the isatin carbonyl and the active site cysteine. The formation of this adduct was recently confirmed upon X-ray analysis of compound **31** bound to caspase 3.[20] Because of this, we extended our

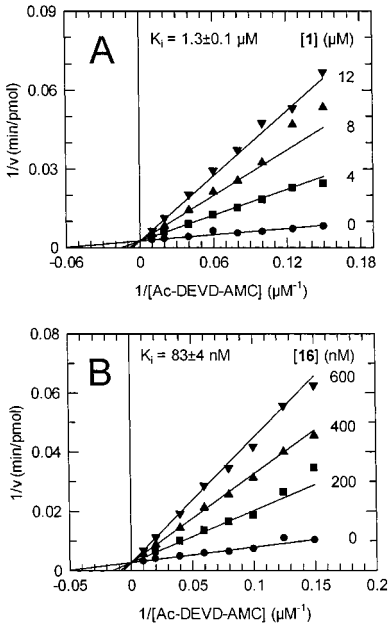


Figure 1. Competitive inhibition of caspase 3 by **1** (A) and **16** (B).

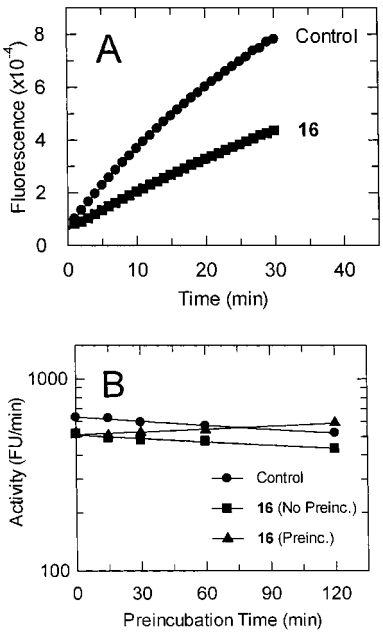


Figure 2. Time dependence and reversibility studies with compound **16**. (A) Progress curves (change in fluorescence vs time) for the hydrolysis of Ac-DEVD-AMC in the absence and presence of 200 nM **16**. Data were fit to eq 2 to estimate the observed inactivation rate constant k. (B) Reversibility of compound **16** following preincubation with caspase 3. In the sample labeled "**16** (No Preinc.)", caspase 3 was preincubated in the absence of compound, similar to control. At the end of the preincubation, the enzyme was added to assay mixtures containing **16** at the same final concentration as the sample preincubated with the inhibitor.

mechanistic studies to include measurements of time dependence and reversibility.

Fitting of the nonlinear progress curves typical of caspase 3 to eq 2, describing time-dependent inactivation, demonstrated that compound **16** is not a time-dependent inhibitor; in addition, it significantly protected caspase 3 from its normal, spontaneous inactivation during the assay (Figure 2A). This was shown to be evident by comparing the inactivation rate constant k from the samples containing inhibitor ($k = 0.0136 \pm 0.0009$ min^{-1}) to that of the control samples containing no inhibitor ($k = 0.0249 \pm 0.0002$ min^{-1}). In addition, compound **16** was rapidly reversible as demonstrated by an experiment in which a high concentration of the compound was preincubated with caspase 3 for up to 2 h followed by dilution into a standard assay to a final concentration well below its IC_{50} (Figure 2B). In this experiment, the measured activity was independent of preincubation time and was identical to the level of inhibition obtained in the absence of preincubation. Not only this, but compound **16** effectively protected the enzyme from the time-dependent loss of activity observed in the control sample during the 2 h preincubation [$k_{obs} = -0.0016$ min^{-1} for "Control"; $k_{obs} = 0.0011$ min^{-1} for "**16** (Preinc.)"], an effect often observed with simple, reversible inhibitors. We conclude from these studies that the formation and breakdown of the thiohemiketal adduct is rapid and readily reversible within the time scale of our assay.

Selectivity. The selectivities of a subset of the inhibitors among eight caspases were determined (Table 5). The initial screening leads **1** and **2** were most potent versus caspases 3 and 7, but were also relatively active against caspases 1, 4, 6, and 9. Introduction of the 5-pyrrolidinylsulfonyl functionality yielded compounds with much improved selectivity for caspases 3 and 7. Compound **16** inhibited caspases 3 and 7 with modest selectivity versus caspase 9, but with greater than 100-fold selectivity versus the remaining caspases. The methoxymethyl side chain is important for imparting

Table 5. Selectivity of Inhibitors among Various Caspases

	K_{iapp} (μM)							
inhibitor	casp 1	casp 2	casp 3	casp 4	casp 6	casp 7	casp 8	casp 9
1	12	>50	0.50	4.0	1.7	0.29	>50	7.2
2	18	>25	0.13	5.0	1.0	0.17	31	5.0
16	>25	>25	0.060	>25	>25	0.17	>25	1.1
20	9.0	>50	1.4	>50	>50	3.0	>50	12
26	21	6.5	0.019	30	7.5	0.047	80	0.85
27	>50	11	12	>50	57	10	>50	>50
29	17	4.9	0.015	33	29	0.047	49	1.2
34	>5	4.0	0.0012	>5	>5	0.006	>5	0.12

selectivity versus caspase 1 (cf. **16** with **20**). The selectivity improves slightly with the more potent caspase 3 inhibitors **26** and **29**, while **27**, the less active enantiomer of **26**, inhibits caspases 2, 3, 6, and 7 at similar micromolar potencies. An approximately 1000-fold selectivity for caspases 3 and 7 versus caspases 1, 2, 4, 6, and 8, and ≥20-fold selectivity versus caspase 9 is achieved with the most potent inhibitor of caspases 3 and 7 (**34**).

The basis of this selectivity was investigated by carrying out a sequence alignment of the active site residues. On the basis of our initial binding model, the amino acid side chains which comprise subsites S_1-S_3 were examined. Those which align the S_1 pocket are invariant among all caspases, and thus do not account for the observed selectivity. On the other hand, high variability in the S_2 and S_3 subsites is found between caspases. Examination of these residues reveals that those which make up the S_2 subsite are likely responsible for the observed inhibitor selectivity. Table 6 shows the alignments of the S_2 residues of all 11 human

Table 6. Sequence Alignments of Active Site Residues about the S_2 Subsite

	residue[a]				residue[a]		
caspase	204	206	256[b]	caspase	204	206	256[b]
1	V	W	?	7	***Y***	***W***	***F***
2	A	M	F	8	V	Y	?
3	***Y***	***W***	***F***	9	V	W	F
4	V	W	?	10	Y	F	?
5	V	W	?	13	V	W	?
6	Y	H	A				

[a] Residue numbers correspond to those of caspase 3. [b] "–" indicates that there is no corresponding residue, and "?" indicates that the identity of the amino acid at this position is ambiguous from the alignment.

caspases, and clearly shows that hydrophobic residues Tyr204, Trp206, and Phe256 are unique to caspases 3 and 7. The X-ray cocrystal structure of caspase 3 in complex with a tetrapeptide aldehyde[13] shows a hydrophobic pocket formed by these three residues. On the basis of the selectivity data for the inhibitors, it is likely the pyrrolidinylsulfonamide group interacts with this pocket. The sequence alignments also suggest that caspases 5, 10, and 13 would be less potently inhibited by these compounds.

The S_1 subsite of the caspases confers high selectivity for the cleavage of substrates possessing a P_1 aspartic acid. However, since the isatin sulfonamides do not interact at that site, we investigated whether selectivity would be maintained among other, non-caspase cysteine proteases. A representative set of isatin sulfonamides (compounds **16**, **26**, and **34**) did not inhibit recombinant human cathepsins B, K, L, and S ($IC_{50} \gg 5\ \mu M$).[21] In addition, these compounds exhibited little inhibition of human recombinant calpain I (<20% at 50 μM),[20] a protease implicated in apoptosis.[22]

At the outset of this effort, our goal was to identify selective inhibitors of caspase 3. Instead, we have identified potent inhibitors which are selective for both caspases 3 and 7. However, studies have shown that caspases 3 and 7 possess similar substrate specificities[23] and inhibitor profiles,[6] and are often activated at similar time points during the apoptosis signaling cascade.[24−27] This may be indicative of redundant or parallel divergent signaling cascades in the cell death pathway. Thus, there may actually be advantages to developing inhibitors with dual selectivity for caspases 3 and 7.

Jurkat Cell Apoptosis. The activities of these compounds in cell-based models of apoptosis were initially evaluated in human Jurkat T cells. Cells were treated with camptothecin to induce cell death, and the ability of compounds to inhibit cell death was assessed by FACS analysis.

A good correlation exists between relative cell-based activities of the compounds with their in vitro isolated caspase 3 or 7 inhibition activities (Table 7). Inhibitor **16**, a 120 nM inhibitor of caspase 3, exhibited 54% inhibition of Jurkat cell apoptosis at 50 μM, while its antipode **17** showed little activity. The analogues with more potent in vitro activities (**26**, **29**, **31**, and **32**) were most active in cells (except for **34** which possessed limited solubility).

Chondrocyte Apoptosis. Osteoarthritis (OA) is characterized by the progressive erosion of articular cartilage and an elevated level of chondrocyte apoptosis;[28−30] therefore, inhibition of apoptosis may have substantial therapeutic benefit in this disease. The activities of several isatin sulfonamides were determined in a cell-based chondrocyte model of apoptosis as a means of evaluating the role of caspases 3 and 7 in OA.

Table 7. Activity of Selected Compounds in the Jurkat Cell Apoptosis Assay

compd	% inhibition at 10 μM	% inhibition at 50 μM
16	22	54
17	15	22
26	8	61
29	51	82
31	41	92
32	60	89
34	27	47

Table 8. Activity of Selected Compounds in the Chondrocyte Apoptosis Assay

compd	% inhibition at 10 μM	% inhibition at 25 μM
16[a]	44	NT
17	NA	NA
26	NT	96
27	NA	NA
29	NT	83
31	48	85
32	71	95
34	48	84
37	64	89

[a] Ninety-eight percent at 50 μM.

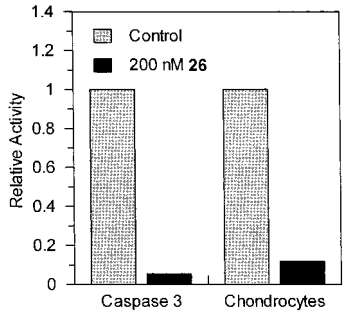


Figure 3. Inhibition of caspase activity in apoptotic chondrocyte cell extracts by the caspase 3-selective inhibitor **26**.

The immortalized human chondrocyte cell line C20/A4[31] was used to investigate the effect of caspase 3- or 7-selective inhibitors on chondrocyte apoptosis as measured by cell death ELISA. Compounds **16** and **26** exhibited activities similar to those observed in the Jurkat cell system, while their corresponding antipodes (**17** and **27**), with attenuated enzyme inhibitory potencies, had no detectable activity at either 10 or 25 μM (Table 8). Compounds with more potent in vitro enzyme activities (**29**, **31**, **32**, and **37**) were the most active in chondrocytes. The most potent compound, **32**, possessed an IC_{50} of 1.7 μM.

Camptothecin-induced apoptosis of chondrocytes results in a 19-fold increase in caspase 3-like enzyme activity as measured by hydrolysis of Ac-DEVD-AMC (data not shown). To determine whether this activity is due to caspases 3 and/or 7 rather than other caspase 3-like family members, extracts from activated chondrocytes were assayed in the presence of inhibitor **26** (200 nM). Figure 3 shows that 95% of enzyme activity is blocked in an isolated enzyme assay with inhibitor present. In activated chondrocyte extracts, 88% of

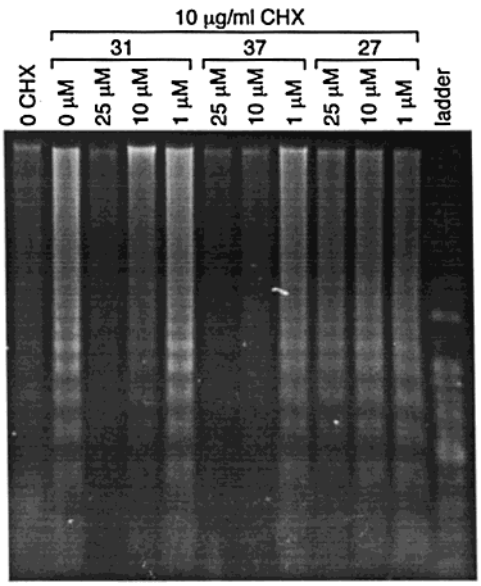


Figure 4. Neutrophil apoptosis with cycloheximide. Mouse bone marrow was treated with 0 or 10 μg/mL cycloheximide (CHX) at a density of 2×10^6 cells/mL in RPMI containing 10% FCS in 5 mL round-bottomed polypropylene tubes for 3 h at 37 °C. Inhibitors were added at the same time as the CHX. Cells were pelleted, and the DNA that was released into the cytoplasm was extracted and run on a 1% agarose gel and stained with ethidium bromide: lane 1, control cells, without CHX; lanes 2−11: cells treated with 10 μg/mL CHX and varying concentrations of inhibitors; lane 12, 100 bp DNA ladder as a standard.

caspase 3-like activity is inhibited. These results suggest that the majority of caspase activity in these apoptotic cells is due to caspases 3 and/or 7.

Mouse Bone Marrow Neutrophils. Results from studies in both Jurkat cells and chondrocytes reveal a significant shift in activity when comparing in vitro isolated enzyme activities of the inhibitors to the corresponding cell-based activities. Such shifts in activity are normally associated with factors which limit the ability of a compound to reach its intracellular target (e.g., cell permeability, protein binding, etc.). However, it was possible that high inhibitor concentrations were required for activity in cells because inhibition of a caspase(s) other than 3 or 7 (which the compounds would inhibit much less potently relative to caspase 3 or 7) was required for anti-apoptotic activity.

To demonstrate that caspases 3 and 7 are the relevant targets in the Jurkat cell and chondrocyte apoptosis systems, compounds were evaluated in mouse bone marrow neutrophils. These cells had previously been shown to be dependent upon caspase 3 for cycloheximide-induced apoptosis.[20,32] Mouse bone marrow was treated with cycloheximide for 3 h with or without caspase inhibitors, and the soluble DNA was extracted from the cells and run on a gel (Figure 4). Significant DNA laddering was observed in the absence of any caspase inhibitor or with the addition of a weakly active compound (**27**). Compounds **31** and **37** demonstrated a dose-dependent inhibition of DNA laddering, with compound **37** being the most potent, completely preventing DNA laddering at 10 μM. The inhibitory activities for compounds **31** and **37** were similar to those observed in the Jurkat and chondrocyte cell-based apoptosis assays. Since these cells are critically dependent upon caspase 3 for apoptosis, these results support the mechanism of action of this class of compounds as inhibitors of caspases 3 and/or 7 in the cell-based models of apoptosis described herein.

Table 9. IC_{50}'s for Compound **31** in Chondrocyte Cell Cytosol

cytosol[a]	caspase 3	IC_{50} (nM)	fold shift
none	yes	47 ± 2	–
naive[b]	yes	7500 ± 3500	160
induced[c]	no	1300 ± 300	28
induced	yes	840 ± 210	18

[a] Cytosol was included at 90% of the total assay volume. [b] For naive, cytosol was prepared from uninduced (nonapoptotic) cells. [c] For induced, cytosol was prepared from apoptotic cells induced with camptothecin.

Attenuation of in Vitro Caspase 3 Inhibitory Activity in the Presence of Chondrocyte Cytosol. Having confirmed that caspases 3 and/or 7 are the intracellular targets in the cells described, we investigated the cause of the observed activity shifts in cell-based models by studying the effect of the cellular medium on the enzyme inhibitory activities of the compounds. The level of inhibition of recombinant caspase 3 by inhibitor **31** decreased when assayed in the presence of increasing proportions of naive chondrocyte cytosol (data not shown). In addition, the IC_{50} for this compound in 90% naive cytosol containing exogenous, recombinant caspase 3 was shifted 160-fold relative to that in buffer alone (Table 9). Large upward shifts in IC_{50} were also observed in apoptotic cytosol when measuring either the endogenous caspase 3-like activity or the activity of a sample spiked with recombinant caspase 3. These results suggest that an interaction of the inhibitor with cytosolic constituents contributes significantly to the activity shifts observed in cell-based assays. The exact nature of this effect is unknown. It may be the result of protein binding, or the electrophilic isatin carbonyl group may be involved in nonspecific interactions with nucleophiles such as thiols and amines. We have demonstrated that this effect is reversible (data not shown). Similar shifts in activity have been reported with 5-carboxamidoisatin inhibitors of rhinovirus 3C protease,[11] and may be a general phenomenon associated with this class of compounds.

Conclusion

The first reported examples of potent and selective inhibitors of caspases 3 and 7 have been described. 5-Pyrrolidinylsulfonyl isatins represent a unique direction in the design of selective inhibitors for caspases 3 and 7. In contrast to previously reported inhibitors of caspases 3 and 7, these structures do not possess an acidic functionality which may bind in the primary aspartic acid binding pocket, S_1.[33−35] In addition, previous studies have shown that binding within the S_3 and S_4 subsites is most important for deriving selectivity between caspases. The data described herein strongly suggest that selectivity can be achieved from an interaction between the pyrrolidinylsulfonyl group of the inhibitor and the hydrophobic S_2 pocket of caspase 3.

Despite the fact that distinguishing the roles of caspases 3 and 7 has been difficult, there has been recent evidence for the differential activation[36,37] and subcellular distribution[38] of these two proteases in cells. A compound which selectively inhibits only caspase 3

or caspase 7 would aid in characterizing the role that each protease plays during apoptosis. Since the isatin sulfonamides derive their selectivity by binding in the S_2 subsite, caspase 3- or 7-selective inhibitors may be obtainable through modification of the isatin sulfonamide core. Elaboration with chemical groups which bind in the extended binding sites (S_3 and S_4) where caspases 3 and 7 diverge may enhance selectivity, and perhaps affinity for one caspase over the other.

The isatin sulfonamides block apoptosis in several cell-based systems, including human chondrocytes, which are used as a model for osteoarthritis. There is an attenuation in cell-based activity relative to in vitro isolated caspase 3 activity for this class of inhibitors. Our studies demonstrate that interaction of the inhibitor with cytosolic constituents can contribute significantly to the observed shifts in activity.

Experimental Section

5-Nitroisatin (**3**), *N*-methylisatin (**8**), isatin (**9**), and 5-isatinsulfonic acid (sodium salt, **13**) are commercially available from Aldrich Chemical Co. The preparations of compounds **1**, **2**, and **4**−**7** have been described.[11,39,40] Proton magnetic resonance spectra (NMR) were recorded using a 400 MHz Bruker spectrometer. Chemical shifts are reported in parts per million (δ) with references set such that in $CDCl_3$ the $CHCl_3$ is at 7.26 ppm. Mass spectra were recorded on a Fisons VG Platform II hands-on electrospray mass spectrometer. Elemental microanalyses were determined by Quantitative Technologies (Whitehouse, NJ) and gave results for the elements stated within 0.4% of the theoretical values unless otherwise listed.

5-Chlorosulfonylisatin (14).[17] To a mixture of 5-isatinsulfonic acid (**13**), sodium salt dihydrate (10 g, 35.1 mmol), and 50 mL of tetramethylene sulfone was added phosphorus oxychloride (16.5 mL, 177 mmol). The resulting mixture was heated at 60 °C for 3 h. The mixture was cooled to 0 °C, and 120 mL of water was cautiously added. The resulting green solid was filtered and washed with water. The solid was dissolved in 100 mL of EtOAc and washed three times with 50 mL of water. The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give a yellow solid. The solid was recrystallized from an EtOAc/hexanes mixture to give the title compound as an orange solid (5.2 g, 60.5%): ^{1}H NMR (1:1 $CD_3CN/CDCl_3$) δ 9.26 (s, 1H), 8.01 (d, J = 8.5 Hz, 1H), 7.95 (s, 1H), 6.95 (d, J = 8.5 Hz, 1H); ES (−) MS *m/e* 344 (M − H).

(*S*)-5-[1-(2-Methoxymethyl)pyrrolidinylsulfonyl]isatin (16). To a solution of 5-chlorosulfonylisatin (0.5 g, 2.04 mmol) in 24 mL of a 1:1 THF/$CHCl_3$ mixture at 0 °C was added dropwise, via syringe pump, a solution of (*S*)-(+)-2-(methoxymethyl)pyrrolidine (0.305 g, 2.65 mmol) and *N*,*N*-diisopropylethylamine (0.526 g, 4.08 mmol) in 4 mL of $CHCl_3$. The reaction was followed by TLC until complete (∼20 min). The solution was concentrated under reduced pressure to a small volume and purified by silica gel chromatography with a 2−3% CH_3OH/CH_2Cl_2 mixture to give a yellow solid. The solid was then recrystallized from an EtOAc/hexanes mixture to give the title compound as a yellow solid (0.205 g, 31%): ^{1}H NMR ($CDCl_3$) δ 9.23 (s, 1H), 8.06−8.09 (m, 2H), 7.27 (d, J = 8.1 Hz, 1H), 3.76 (m, 1H), 3.58 (dd, J = 9.4, 3.7 Hz, 1H), 3.36−3.44 (m, 2H), 3.36 (s, 3H), 3.13 (m, 1H), 1.91 (m, 2H), 1.68 (m, 2H); ES (−) MS *m/e* 323 (M − H). Anal. ($C_{14}H_{16}N_2O_5S$) C, H, N.

(*R*)-5-[1-(2-Methoxymethyl)pyrrolidinylsulfonyl]isatin (17) was prepared according to the procedure for compound **16** except using (*R*)-(−)-2-(methoxymethyl)pyrrolidine and purification by silica gel chromatography with 2−3% CH_3OH/CH_2Cl_2 mixture to give a yellow solid. The solid was then recrystallized from EtOAc to give the title compound as a yellow solid in 29% yield: ^{1}H NMR (1:1 $CD_3CN/CDCl_3$) δ 9.07 (s, 1H), 7.77 (d, J = 8.5 Hz, 1H), 7.71 (s, 1H), 6.90 (d, J = 8.5 Hz, 1H), 3.45 (m, 1H), 3.27 (dd, J = 9.3, 3.8 Hz, 1H), 3.09−3.15 (m, 2H), 3.09 (s, 3H), 2.88 (m, 1H), 1.56 (m, 2H), 1.35 (m, 2H); ES (−) MS *m/e* 323 (M − H). Anal. ($C_{14}H_{16}N_2O_5S$) C, H, N.

5-{1-[(2-Methoxyethyl)methylamino]sulfonyl}isatin (18) was prepared according to the procedure for compound **16** except using (2-methoxyethyl)methylamine, and purification by silica gel chromatography with a 3% CH_3OH/CH_2Cl_2 mixture followed by recrystallization from EtOAc afforded the title compound as a red solid in 65% yield: ^{1}H NMR (1:1 $CD_3CN/CDCl_3$) δ 8.99 (s, 1H), 7.72 (d, J = 8.2 Hz, 1H), 7.65 (s, 1H), 6.87 (d, J = 8.2 Hz, 1H), 3.25 (t, J = 5.4 Hz, 2H), 3.03 (s, 3H), 2.98 (t, J = 5.4 Hz, 2H), 2.55 (s, 3H); ES (−) MS *m/e* 297 (M − H). Anal. ($C_{12}H_{14}N_2O_5S$) C, H, N.

5-[1-(Azetidinyl)sulfonyl]isatin (19) was prepared according to the procedure for compound **16** except using azetidine, and purification by silica gel chromatography with a 2% CH_3OH/CH_2Cl_2 mixture followed by recrystallization from an EtOAc/hexanes mixture afforded the title compound as yellow needles: ^{1}H NMR (1:1 $CD_3CN/CDCl_3$) δ 9.11 (s, 1H), 7.78 (d, J = 8.4 Hz, 1H), 7.70 (s, 1H), 6.96 (d, J = 8.4 Hz, 1H), 3.53 (t, J = 7.7 Hz, 4H), 1.83−1.94 (m, 2H); ES(−) MS *m/e* 265 (M − H). Anal. ($C_{11}H_{10}N_2O_4S$) C, H, N.

5-[1-(Pyrrolidinyl)sulfonyl]isatin (20) was prepared according to the procedure for compound **16** except using pyrrolidine, which afforded the title compound as an orange solid in 8% yield: ^{1}H NMR (1:1 $CD_3CN/CDCl_3$) δ 9.02 (s, 1H), 7.76 (d, J = 8.5 Hz, 1H), 7.67 (s, 1H), 6.89 (d, J = 8.5 Hz, 1H), 2.97 (m, 4H), 1.52 (m, 4H); ES (−) MS *m/e* 279 (M − H). Anal. ($C_{12}H_{12}N_2O_4S$) C, H, N.

5-[1-(Piperidinyl)sulfonyl]isatin (21) was prepared according to the procedure for compound **16** except using piperidine, which afforded the title compound as an orange solid: ^{1}H NMR ($CDCl_3$) δ 8.73 (s, 1H), 8.00 (m, 2H), 7.16 (d, J = 8.5 Hz, 1H), 3.05 (m, 4H), 1.68 (m, 4H), 1.48 (m, 2H); ES (−) MS *m/e* 293.2 (M − H). Anal. ($C_{12}H_{12}N_2O_4S \cdot 0.10CH_2Cl_2$) C, H, N.

5-[1-(Hexamethyleneimino)sulfonyl]isatin (22) was prepared according to the procedure for compound **16** except using hexamethyleneimine, which afforded the title compound as an orange solid in 23% yield: ^{1}H NMR (1:1 $CD_3CN/CDCl_3$) δ 9.07 (s, 1H), 7.76 (d, J = 8.1 Hz, 1H), 7.68 (s, 1H), 6.89 (d, J = 8.1 Hz, 1H), 3.05 (t, J = 5.95 Hz, 4H), 1.38 (m, 4H), 1.50 (m, 4H); ES (−) MS *m/e* 307.2 (M − H). Anal. ($C_{14}H_{16}N_2O_4S$) H, N; C: calcd, 54.53; found, 54.03.

(*S*)-5-{1-[(2-Methoxycarbonyl)pyrrolidinyl]sulfonyl}isatin (23) was prepared according to the procedure for compound **16** except using L-proline methyl ester hydrochloride, which afforded the title compound as a yellow foam in 18% yield: ^{1}H NMR (1:1 $CD_3CN/CDCl_3$) δ 9.07 (s, 1H), 7.86 (d, J = 8.3 Hz, 1H), 7.80 (s, 1H), 6.95 (d, J = 8.3 Hz, 1H), 4.11 (dd, J = 8.7, 3.9 Hz, 1H), 3.54 (s, 3H), 3.26 (m, 1H), 3.10 (m, 1H), 1.62−1.95 (m, 4H); ES (−) MS *m/e* 337 (M − H). Anal. ($C_{14}H_{14}N_2O_6S$) C, H, N.

(*S*)-5-{1-[(2-*tert*-Butoxycarbonyl)pyrrolidinyl]sulfonyl}isatin (24) was prepared according to the procedure for compound **16** except using L-proline *tert*-butyl ester, and purification by silica gel chromatography with a 1−2% CH_3OH/CH_2Cl_2 mixture afforded the title compound as an orange-yellow solid in 74% yield: ^{1}H NMR (1:1 $CD_3CN/CDCl_3$) δ 9.03 (s, 1H), 7.79 (d, J = 8.5 Hz, 1H), 7.71 (s, 1H), 6.88 (d, J = 8.5 Hz, 1H), 3.89 (dd, J = 8.6, 3.6 Hz, 1H), 3.18 (m, 1H), 3.05 (m, 1H), 1.53−1.85 (m, 4H), 1.22 (s, 9H); ES (−) MS *m/e* 379 (M − H). Anal. ($C_{17}H_{20}N_2O_6S$) C, H, N.

(*S*)-5-(1-{[2-(Dimethylamino)carbonyl]pyrrolidinyl}sulfonyl)isatin (25) was prepared according to the procedure for compound **16** except using *N*,*N*-dimethyl-L-prolinamide, and purification by silica gel chromatography with a 1−2% CH_3OH/CH_2Cl_2 mixture. Recrystallization from acetonitrile afforded the title compound as yellow needles in 45% yield: ^{1}H NMR (1:1 $CD_3CN/CDCl_3$) δ 9.14 (s, 1H), 7.81 (d, J = 8.3 Hz, 1H), 7.73 (s, 1H), 6.84 (d, J = 8.3 Hz, 1H), 4.56 (dd, J = 8.8, 3.9 Hz, 1H), 3.17 (m, 2H), 2.87 (s, 3H), 2.69 (s, 3H), 1.60−1.93 (m, 4H); ES (−) MS *m/e* 350 (M − H). Anal. ($C_{15}H_{17}N_3O_5S$) C, H, N.

(*S*)-5-{1-[2-(Anilinomethyl)pyrrolidinyl]sulfonyl}isatin (26) was prepared according to the procedure for compound **16** except using (*S*)-(+)-2-anilinomethylpyrrolidine, and purification by silica gel chromatography with a 2−3% CH_3OH/CH_2Cl_2 mixture. Recrystallization from EtOAc afforded the title compound as red crystals in 16% yield: ^{1}H NMR (1:1 $CD_3CN/CDCl_3$) δ 9.01 (s, 1H), 7.78 (d, J = 8.1 Hz, 1H), 7.70 (s, 1H), 6.93 (t, J = 7.9 Hz, 2H), 6.87 (d, J = 8.1 Hz, 1H), 6.41−6.47 (m, 3H), 3.56 (m, 1H), 3.14−3.22 (m, 2H), 2.86−2.96 (m, 2H), 1.38−1.66 (m, 4H); ES (−) MS *m/e* 384 (M − H). Anal. ($C_{19}H_{19}N_3O_4S$) C, H, N.

(*R*)-5-{1-[2-(Anilinomethyl)pyrrolidinyl]sulfonyl}isatin (27). (a) (*S*)-*N*-Boc-2-Phenylaminocarbonylpyrrolidine. To a solution of *N*-Boc-D-proline (2.0 g, 9.3 mmol), aniline (0.87 g, 9.4 mmol), and 1-hydroxybenzotriazole hydrate (1.5 g, 11 mmol) in 20 mL of methylene chloride at 0 °C was added 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (2.1 g, 11 mmol), and the resulting solution was stirred at room temperature overnight. The solution was washed with 100 mL of 3 N HCl, and the aqueous layer was extracted with methylene chloride. The combined organic layers were dried over magnesium sulfate, filtered, and concentrated under reduced pressure to give the title compound as a white solid: ES (+) MS *m/e* 291 (M + H).

(b) (*R*)-2-Phenylaminocarbonylpyrrolidine. To a solution of (*S*)-*N*-Boc-2-phenylaminocarbonylpyrrolidine (2.8 g, 10 mmol) in 25 mL of CH_2Cl_2 at 0 °C was added 25 mL of TFA. The solution was warmed to room temperature and stirred for 2 h. The organic layer was concentrated under reduced pressure. The residue was redissolved in ethyl acetate and washed with 10% NaOH. The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The resulting oil was purified by silica gel chromatography with a 90:9:1 EA/$CH_3OH/CH_2Cl_2/NH_4OH$ mixture to give the title compound as a white solid (1.2 g, 68% yield): ^{1}H NMR ($CDCl_3$) δ 9.74 (br s, 1H), 7.61 (d, J = 8.7 Hz, 2H), 7.31 (t, J = 7.0 Hz, 2H), 7.09 (t, J = 7.3 Hz, 1H), 3.87 (dd, J = 9.2, 5.1 Hz, 1H), 2.96−3.13 (m, 2H), 2.02−2.27 (m, 2H), 1.73−1.82 (m, 2H); ES (+) MS *m/e* 191 (M + H).

(c) (*R*)-2-(Anilinomethyl)pyrrolidine. To a solution of D-proline anilide (1.15 g, 6.0 mmol) in 20 mL of THF at 0 °C was added a 1 M solution of lithium aluminum hydride (14 mL, 14 mmol) in THF. The resulting solution was stirred at 0 °C for 11 h. The reaction was carefully quenched with a saturated solution of Na_2SO_4 and the mixture extracted with EtOAc. The organic layer was dried over sodium sulfate, filtered, and concentrated under reduced pressure to give the title compound as an oil. The oil was purified by silica gel chromatography with a 3% CH_3OH/CH_2Cl_2 mixture and 1% triethylamine to give the title compound as a yellow oil (0.67 g, 63%): ^{1}H NMR ($CDCl_3$) δ 7.18 (d, J = 8.4 Hz, 2H), 6.71 (t, J = 7.4 Hz, 2H), 6.62 (d, J = 9.5 Hz, 1H), 4.2 (br s, 1H), 2.7−3.5 (m, 5H), 1.6−2.0 (m, 4H); ES (+) MS *m/e* 177 (M + H).

(d) (*R*)-5-{1-[2-(Anilinomethyl)pyrrolidinyl]sulfonyl}isatin was prepared according to the procedure for compound **16** except using (*R*)-2-(anilinomethyl)pyrrolidine, and purification by silica gel chromatography with a 2−3% CH_3OH/CH_2Cl_2 mixture afforded the title compound as an orange solid in 78% yield: ^{1}H NMR (1:1 $CD_3CN/CDCl_3$) δ 9.02 (br s, 1H), 7.79 (d, J = 8.4 Hz, 1H), 7.72 (s, 1H), 6.94 (t, J = 7.9 Hz, 2H), 6.88 (d, J = 8.4 Hz, 1H), 6.41−6.46 (m, 3H), 4.33 (br s, 1H), 3.56 (m, 1H), 3.16−3.26 (m, 2H), 2.91−2.99 (m, 2H), 1.37−1.66 (m, 4H); ES (+) MS *m/e* 386 (M + H). Anal. ($C_{19}H_{19}N_3O_4S\cdot0.15CH_2Cl_2$) C, H, N.

(*S*)-5-{1-[2-(Phenylaminocarbonyl)pyrrolidinyl]sulfonyl}isatin (28). (a) (*S*)-*N*-Boc-2-(Phenylaminocarbonyl)pyrrolidine was prepared according to the procedure for compound **27** (part a) except using *N*-Boc-L-proline (89%): ES (+) MS *m/e* 291 (M + H).

(b) (*S*)-2-(Phenylaminocarbonyl)pyrrolidine was prepared according to the procedure for compound **27** (part b) except using (*S*)-*N*-Boc-2-(phenylaminocarbonyl)pyrrolidine (56%): ^{1}H NMR ($CDCl_3$) δ 9.85 (br s, 1H), 7.62 (d, J = 8.0 Hz, 2H), 7.33 (t, J = 7.9 Hz, 2H), 7.10 (t, J = 7.3 Hz, 1H), 3.98 (dd, J = 9.2, 5.4 Hz, 1H), 3.12 (m, 1H), 3.05 (m, 1H), 1.78−2.26 (m, 5H); ES (+) MS *m/e* 191 (M + H).

(c) (*S*)-5-{1-[2-(Phenylaminocarbonyl)pyrrolidinyl]sulfonyl}isatin was prepared according to the procedure for compound **16** except using (*S*)-2-(phenylaminocarbonyl)pyrrolidine, and purification by silica gel chromatography with a 4% CH_3OH/CH_2Cl_2 mixture afforded the title compound as a yellow solid in 56% yield: ^{1}H NMR (1:1 $CD_3CN/CDCl_3$) δ 9.14 (br s, 1H), 8.46 (br s, 1H), 7.83 (d, J = 8.2 Hz, 1H), 7.77 (s, 1H), 7.35 (d, J = 7.9 Hz, 2H), 7.10 (t, J = 7.9 Hz, 2H), 6.87−6.92 (m, 2H), 3.90 (dd, J = 7.8, 3.5 Hz, 1H), 3.38 (m, 1H), 3.04 (m, 1H), 1.47−1.91 (m, 4H); ES (−) MS *m/e* 398 (M − H). Anal. ($C_{19}H_{17}N_3O_5S$) H; C: calcd, 57.13; found, 56.54; N: calcd, 10.52; found, 10.01.

(*S*)-5-{1-[2-(Phenoxymethyl)pyrrolidinyl]sulfonyl}isatin (29). (a) (*S*)-*N*-Boc-2-(4-Toluenesulfonyloxymethyl)-pyrrolidine. To a solution of (*S*)-*N*-Boc-2-prolinol (3.51 g, 17.4 mmol) and pyridine (9.87 mL, 122 mmol) in 18 mL of CH_2Cl_2 at 0 °C was added dropwise a solution of *p*-toluenesulfonyl chloride in 20 mL of CH_2Cl_2. The solution was warmed to room temperature and stirred overnight. The solution was treated with 140 mL of water and extracted twice with 20 mL of CH_2Cl_2. The organic layer was then dried over Na_2SO_4, filtered, and concentrated under reduced pressure to give an oil. The oil was purified by silica gel chromatography with a 20−25% EtOAc/hexanes mixture to give the title compound as a colorless oil (5.6 g, 90%): ^{1}H NMR ($CDCl_3$) δ 7.76 (d, J = 8.1 Hz, 2H), 7.33 (br s, 2H), 3.88−4.09 (m, 3H), 3.28 (m, 2H), 2.43 (s, 3H), 1.78−1.95 (m, 4H), 1.35−1.39 (m, 9H); ES (+) MS *m/e* 256 (M + H).

(b) (*S*)-*N*-Boc-2-(Phenoxymethyl)pyrrolidine. To a solution of phenol (0.40 g, 4.23 mmol) in 10 mL of THF at 0 °C was added sodium hydride (0.226 g, 5.65 mmol), and the mixture was warmed to room temperature. The mixture was stirred for 10 min until evolution of hydrogen ceased and cooled to 0 °C. A solution of (*S*)-*N*-Boc-2-(4-toluenesulfonyloxymethyl)-pyrrolidine was added dropwise to the mixture, and the resulting mixture was refluxed overnight. To the mixture was added 5 mL of DMF, and the mixture was heated at 100 °C overnight. To the mixture was added EtOAc, and the organic layer was washed thrice with water, thrice with 1 N NaOH, and thrice with water. The organic layer was then dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give an oil. The oil was purified by silica gel chromatography with a 7% EtOAc/hexanes mixture to give the title compound as a colorless oil (0.55 g, 71%): ^{1}H NMR ($CDCl_3$) δ 7.26−7.30 (m, 2H), 6.93−6.96 (m, 2H), 4.14 (m, 2H), 3.86 (br s, 1H), 3.40 (m, 2H), 1.88−2.07 (m, 4H), 1.48 (s, 9H); ES (+) MS *m/e* 278 (M + H).

(c) (*S*)-2-(Phenoxymethyl)pyrrolidine. To a solution of (*S*)-*N*-Boc-2-(phenoxymethyl)pyrrolidine (0.81 g, 2.9 mmol) in 5 mL of CH_2Cl_2 at 0 °C was added dropwise 5 mL of TFA over the course of 1 h. The solution was warmed to room temperature and stirred for 1.5 h. The reaction mixture was slowly poured into 30 mL of 10% NaOH and extracted thrice with 20 mL of CH_2Cl_2. The organic layer was then dried over Na_2SO_4, filtered, and concentrated under reduced pressure to give a light yellow oil (0.41 g, 79%): ^{1}H NMR ($CDCl_3$) δ 7.27 (d, J = 8.2 Hz, 2H), 6.91 (m, 3H), 3.88 (m, 2H), 3.52 (m, 1H), 2.94−3.05 (m, 2H), 2.36 (br s, 1H), 1.55−1.95 (m, 4H); ES (+) MS *m/e* 178 (M + H).

(d) (*S*)-5-{1-[2-(Phenoxymethyl)pyrrolidinyl]sulfonyl}isatin was prepared according to the procedure for compound **16** except using (*S*)-2-(phenoxymethyl)pyrrolidine, which afforded the title compound as a yellow solid in 46% yield: ^{1}H NMR (1:1 $CD_3CN/CDCl_3$) δ 8.98 (br s, 1H), 7.79 (d, J = 8.4 Hz, 1H), 7.05 (t, J = 8.0 Hz, 2H), 6.86 (d, J = 8.4 Hz, 1H), 6.71 (t, J = 7.3 Hz, 1H), 6.66 (d, J = 8.1 Hz, 2H), 3.92 (dd, J = 8.9, 2.8 Hz, 1H), 3.73 (m, 3H), 3.25 (m, 1H), 2.98 (m, 1H), 1.46−1.76 (m, 4H); ES (+) MS *m/e* 387 (M + H). Anal. ($C_{19}H_{17}N_3O_5S$) C, H, N.

(*S*)-(+)-5-{1-[2-(Thiophenoxymethyl)pyrrolidinyl]sulfonyl}isatin (30). (a) (*S*)-*N*-Boc-2-(Thiophenoxymethyl)-pyrrolidine. To a solution of thiophenol (0.465 g, 4.23 mmol)

in 10 mL of THF at 0 °C was added sodium hydride (0.203 g, 5.08 mmol), and the mixture was warmed to room temperature. The mixture was stirred for 10 min until evolution of hydrogen ceased and cooled to 0 °C. A solution of (*S*)-*N*-Boc-2-(4-toluenesulfonyloxymethyl)pyrrolidine was added dropwise to the mixture, and the resulting mixture was stirred overnight. To the mixture was added EtOAc, and the organic layer was washed twice with water, twice with 1 N NaOH, and twice with brine. The organic layer was then dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give an oil. The oil was purified by silica gel chromatography with a 7% EtOAc/hexanes mixture to give the title compound as a colorless oil (0.67 g, 81%): ^{1}H NMR ($CDCl_3$) δ 7.41 (d, J = 7.3 Hz, 2H), 7.27 (t, J = 7.8 Hz, 2H), 7.18 (d, J = 7.5 Hz, 1H), 3.98 (m, 1H), 3.38 (m, 3H), 2.73 (m, 1H), 1.79−2.00 (m, 4H), 1.45 (s, 9H); ES (+) MS *m/e* 294 (M + H).

(b) (*S*)-2-(Thiophenoxymethyl)pyrrolidine was prepared according to the procedure for compound **29** (c) except using (*S*)-*N*-Boc-2-(thiophenoxymethyl)pyrrolidine, which afforded the title compound as a light yellow oil (0.4 g, 91%): ^{1}H NMR ($CDCl_3$) δ 7.36 (d, J = 7.8 Hz, 2H), 7.28 (t, J = 7.8 Hz, 2H), 7.17 (d, J = 7.3 Hz, 1H), 3.27 (m, 1H), 3.01 (m, 3H), 2.90 (m, 1H), 1.45−1.95 (m, 4H); ES (+) MS *m/e* 194 (M + H).

(c) (*S*)-5-{1-[2-(Thiophenoxymethyl)pyrrolidinyl]sulfonyl}isatin was prepared according to the procedure for compound **16** except using (*S*)-2-(thiophenoxymethyl)pyrrolidine, which afforded the title compound as a yellow solid in 32% yield: ^{1}H NMR (1:1 $CD_3CN/CDCl_3$) δ 8.98 (br s, 1H), 7.59 (d, J = 8.5 Hz, 1H), 7.54 (s, 1H), 7.20 (d, J = 7.2 Hz, 2H), 7.14 (t, J = 7.7 Hz, 2H), 7.04 (t, J = 7.3 Hz, 1H), 6.80 (d, J = 8.4 Hz, 1H), 3.20−3.34 (m, 3H), 2.90 (m, 1H), 2.63 (m, 1H), 1.3−1.7 (m, 4H); ES (+) MS *m/e* 403 (M + H). Anal. ($C_{19}H_{18}N_2O_4S$) C, H, N.

(*S*)-1-Methyl-5-{1-[2-(phenoxymethyl)pyrrolidinyl]sulfonyl}isatin (31). To a mixture of (*S*)-5-{1-[2-(phenoxymethyl)-pyrrolidinyl]sulfonyl}isatin (0.050 g, 0.13 mmol) and K_2CO_3 (0.045 g, 0.32 mmol) in 1.5 mL of DMF was added iodomethane (0.038 g, 0.26 mmol). The mixture was allowed to stir overnight. Ethyl ether was added, and the mixture was washed with water and acidified with 3 N HCl. The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give an orange oil. Purification by silica gel chromatography with a 0−1% CH_3OH/CH_2Cl_2 mixture afforded the title compound as an orange solid (0.052 g, 81%): ^{1}H NMR ($CDCl_3$) δ 8.08 (d, J = 8.3 Hz, 1H), 8.02 (s, 1H), 7.25 (m, 3H), 6.94 (t, J = 7.2 Hz, 2H), 6.81 (d, J = 8.1 Hz, 2H), 4.16 (dd, J = 9.3, 3.0 Hz, 1H), 4.00 (m, 1H), 3.93 (t, J = 8.3 Hz, 1H), 3.53 (m, 1H), 3.30 (m, 1H), 3.27 (s, 3H), 2.05 (m, 2H), 1.81 (m, 2H); ES (+) MS *m/e* 401 (M + H). Anal. ($C_{20}H_{20}N_2O_5S$) C, H, N.

(*S*)-1-Allyl-5-{1-[2-(phenoxymethyl)pyrrolidinyl]sulfonyl}isatin (32) was prepared according to the procedure for compound **31** except using allyl bromide, which afforded the title compound as an orange oil in 99% yield: ^{1}H NMR ($CDCl_3$) δ 8.01 (m, 2H), 7.24 (m, 2H), 6.92 (m, 2H), 6.81 (d, J = 8.0 Hz, 2H), 5.81 (m, 1H), 5.33 (m, 2H), 4.36 (dd, J = 5.4, 0.9 Hz, 2H), 4.15 (dd, J = 9.2, 2.9 Hz, 1H), 3.91 (m, 2H), 3.50 (m, 1H), 3.23 (m, 1H), 2.03 (m, 2H), 1.80 (m, 2H); ES (+) MS *m/e* 427 (M + H). Anal. ($C_{22}H_{22}N_2O_5S$) C, H, N.

(*S*)-1-(Cyclohexylmethyl)-5-{1-[2-(phenoxymethyl)pyrrolidinyl]sulfonyl}isatin (33). To a solution of (*S*)-5-{1-[2-(phenoxymethyl)pyrrolidinyl]sulfonyl}isatin (0.066 g, 0.17 mmol) in 1 mL of DMF at 0 °C was added 60% sodium hydride (0.010 g, 0.25 mmol), and the solution was warmed to room temperature. The resulting solution was stirred for 20 min. Cyclohexylmethyl bromide (0.048 mL, 1.3 mmol) and a catalytic amount of tetrabutylammonium iodide were added, and the solution was heated at 70 °C overnight. The reaction was quenched with water and the mixture extracted twice with CH_2Cl_2. The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give an oil. Purification by silica gel chromatography with CH_2Cl_2 afforded the title compound as a yellow solid in 32% yield: ^{1}H NMR ($CDCl_3$) δ 8.05 (d, J = 8.2 Hz, 1H), 8.02 (s, 1H), 7.27 (m, 2H), 6.94 (m, 2H), 6.85 (d, J = 8.1 Hz, 2H), 4.19 (dd, J = 9.2, 2.9 Hz, 1H), 3.91−4.01 (m, 2H), 3.55 (m, 3H), 3.26 (m, 1H), 1.5−2.2 (m, 14H), 1.0−1.4 (m, 4H); ES (+) MS *m/e* 483 (M + H). Anal. ($C_{26}H_{30}N_2O_5S$) H, N; C: calcd, 64.71; found, 64.24.

(*S*)-1-Benzyl-5-{1-[2-(phenoxymethyl)pyrrolidinyl]sulfonyl}isatin (34) was prepared according to the procedure for compound **33** except without heating and using benzyl bromide in the absence of tetrabutylammonium iodide. Purification by silica gel chromatography with a 0−0.5% CH_3OH/CH_2Cl_2 mixture afforded the title compound as an orange oil in 81% yield: ^{1}H NMR ($CDCl_3$) δ 7.80 (s, 1H), 7.94 (d, J = 8.5 Hz, 1H), 7.17−7.38 (m, 7H), 6.75−6.94 (m, 4H), 4.92 (s, 2H), 4.14 (m, 1H), 3.92 (m, 2H), 3.49 (m, 1H), 3.19 (m, 1H), 2.02 (m, 1H), 1.72 (m, 1H); ES (+) MS *m/e* 477 (M + H). Anal. ($C_{26}H_{24}N_2O_5S\cdot0.10CH_2Cl_2$) C, H, N.

(*S*)-1-[(*tert*-Butoxycarbonyl)methyl]-5-{1-[2-(phenoxymethyl)pyrrolidinyl]sulfonyl}isatin (35) was prepared according to the procedure for compound **31** except using *tert*-butyl bromoacetate, which afforded the title compound as an orange oil in 79% yield: ^{1}H NMR ($CDCl_3$) δ 8.07 (m, 2H), 7.27 (t, J = 8.0 Hz, 2H), 6.95 (t, J = 7.2 Hz, 1H), 6.86 (m, 3H), 4.39 (d, J = 17.7 Hz, 1H), 4.40 (d, J = 17.7 Hz, 1H), 4.21 (dd, J = 9.1, 2.9 Hz, 1H), 3.90−4.00 (m, 2H), 3.53 (m, 1H), 3.23 (m, 1H), 2.05 (m, 2H), 1.80 (m, 2H), 1.49 (s, 9H); ES (+) MS *m/e* 546 (M + HCO_2H). Anal. ($C_{25}H_{28}N_2O_7S$) C, H, N.

(*S*)-1-(Carboxymethyl)-5-{1-[2-(phenoxymethyl)pyrrolidinyl]sulfonyl}isatin (36). To a solution of (*S*)-1-[(*tert*-butoxycarbonyl)methyl]-5-{1-[2-(phenoxymethyl)pyrrolidinyl]-sulfonyl}isatin (0.052 g, 0.104 mmol) in 5 mL of CH_2Cl_2 at 0 °C was added 5 mL of TFA. The solution was warmed to room temperature and stirred for 1.5 h. The organic layer was concentrated under reduced pressure and redissolved in CH_2Cl_2, and toluene was added. The organic layer was concentrated under reduced pressure to give an oil. The oil was purified by silica gel chromatography with a 3% CH_3OH/CH_2Cl_2 mixture and 1% acetic acid to give the title compound as an orange oil (0.029 g, 63%): ^{1}H NMR (10:1 $CDCl_3/CD_3OD$) δ 8.01 (m, 2H), 6.7−7.3 (m, 6H), 4.44 (s, 2H), 3.1−4.2 (m, 5H), 1.97 (m, 2H), 1.76 (m, 2H); ES (+) MS *m/e* (M + H). Anal. ($C_{21}H_{20}N_2O_7S$) C, H, N.

(*S*)-1-(4-Pyridinylmethyl)-5-{1-[2-(phenoxymethyl)pyrrolidinyl]sulfonyl}isatin (37). To a solution of (*S*)-5-{1-[2-(phenoxymethyl)pyrrolidinyl]sulfonyl}isatin (0.305 g, 0.80 mmol) in 5 mL of DMF at 0 °C was added 60% sodium hydride (0.035 g, 0.88 mmol), and the solution was stirred for 10 min. 4-(Bromomethyl)pyridine hydrobromide[41] (0.10 mg, 0.4 mmol) was added, and the solution was stirred at room temperature for 2 h. The reaction was quenched with water and the mixture extracted twice with CH_2Cl_2. The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give an oil. Purification by silica gel chromatography with a 50−80% ethyl acetate/hexanes mixture afforded the title compound as yellow solid (35 mg, 18%): ^{1}H NMR ($CDCl_3$) δ 8.65 (d, J = 5.4 Hz, 2H), 8.07 (s, 1H), 7.99 (d, J = 8.2 Hz, 1H), 7.22−7.28 (m, 4H), 6.94 (t, J = 7.3 Hz, 1H), 6.81 (d, J = 8.3 Hz, 2H), 6.76 (d, J = 8.4 Hz, 1H), 4.95 (s, 3H), 4.14 (m, 1H), 4.00 (m, 1H), 3.92 (t, J = 8.3 Hz, 1H), 3.52 (m, 1H), 3.28 (m, 1H), 2.05 (m, 1H), 1.82 (m, 1H); ES (+) MS *m/e* 478 (M + H). Anal. ($C_{25}H_{23}N_3O_5S\cdot0.15CH_2Cl_2$) C, H, N.

Protein Supply and Enzyme Inhibition Assays. Recombinant full-length human caspases (1−4 and 6−8) were expressed and purified as previously described.[20] Caspase 9 and its substrate (Ac-LEHD-AFC) were purchased from Chemicon International (Temecula, CA). Other caspase substrates were obtained from the following sources: Ac-YVAD-AMC from BACHEM Bioscience (King of Prussia, PA), Ac-VDVAD-AFC from Enzyme Systems Products (Dublin, CA), Ac-DEVD-AMC and Ac-LEED-AMC from California Peptide Research (Napa, CA), and Ac-IETD-AMC from BIOMOL Research Laboratories (Plymouth Meeting, PA). Enzyme assays were run in 200 μL volumes and contained the following: 25 mM K^+HEPES (pH 7.5), 10% sucrose, 0.1% CHAPS, 5 mM β-mercaptoethanol, and 10 μM Ac-YVAD-AMC (caspase 1); 50 mM sodium acetate (pH 6.2), 10% glycerol, 0.25 mM EDTA, 5 mM

β-mercaptoethanol, and 25 μM Ac-VDVAD-AMC (caspase 2); 25 mM K^+HEPES (pH 7.5), 0.1% CHAPS, 50 mM KCl, 5 mM β-mercaptoethanol, and 10 μM Ac-DEVD-AMC (caspases 3 and 7); potassium acetate (pH 5.8), 1 mM EDTA, 10% sucrose, 0.1% CHAPS, 5 mM β-mercaptoethanol, and 100 μM Ac-LEED-AMC (caspase 4); 50 mM Tris-HCl (pH 7.4), 0.1% CHAPS, 1.5 mM $MgCl_2$, 1 mM EDTA, 5 mM β-mercaptoethanol, and 10 μM Ac-DEVD-AMC (caspase 6); or Na^+MOPS (pH 7.5), 10% glycerol, 0.25 mM EDTA, 5 mM β-mercaptoethanol, and 10 μM Ac-IETD-AMC (caspase 8). Caspase 9 was assayed as previously described.[42] Recombinant caspases were diluted into the appropriate buffer to a concentration of ~10 units/assay (1 unit = 1 pmol of AMC/AFC product formed per min) and were added to the above incubation mixtures. Accumulation of AMC/AFC was assessed at 30 °C with a Cytofluor 4000 fluorescent plate reader (Perseptive Biosystems) at excitation and emission wavelengths of 360 and 460 nm (AMC) and 395 and 530 nm (AFC), respectively.

All the inhibitors that were tested were dissolved and diluted in DMSO prior to addition to the assay mixture; the final DMSO concentration was 5%. Compounds were tested at a single dose (typically 5−50 μM), and full IC_{50} curves were run for all compounds expressing measurable inhibitory activity. The IC_{50}'s for compounds demonstrating unexpected or potent activity were repeated with CVs of typically <30%.

Mechanistic Studies. The inhibition profiles for compounds **1** and **16** were determined for caspase 3 using buffer conditions described above. The concentration of Ac-DEVD-AMC was varied from 6.25 to 100 μM, and the concentration of the inhibitor was varied from 0 to 12 μM and from 0 to 600 nM for compounds **1** and **16**, respectively. Initial velocities were fit using GraFit 4.0 (Erithacus Software, Staines, U.K.) to eq 1 describing linear competitive inhibition:

$$v = \frac{V_m S}{K_m\left(1 + \frac{I}{K_i}\right) + S} \quad (1)$$

where v is the observed velocity, S is the substrate concentration, V_m is the velocity at saturating substrate, K_m is the Michaelis constant of the substrate, I is the inhibitor concentration, and K_i is the dissociation constant of the inhibitor from the E·I complex.

Compound **16** was tested for both time dependence and reversibility. To test for time dependence, the nonlinear progress curves typical of caspase 3 from a 30 min assay were fit to eq 2 in the presence and absence of an inhibitory concentration (200 nM) of the compound. Equation 2 is a modification of the model for slow binding inhibition originally described by Morrison and Stone:[43]

$$F = \frac{v_0(1 - e^{kt})}{k} + y_0 \quad (2)$$

where F is the observed fluorescence, t is the time, v_0 is the velocity at time zero, y_0 is the y-intercept at time zero, and k is the observed rate constant for inactivation. In this model, the steady state velocity is assumed to be zero (complete inactivation at $t = \infty$). Time-dependent inhibition would be identified as an increase in magnitude of the rate constant for inactivation k in the inhibited sample relative to the uninhibited control.

In a related experiment, caspase 3 (4 nM) was preincubated at 22 °C with 200 nM compound **16** for up to 2 h. At various times, residual activity was determined in assays initiated by the addition of a final Ac-DEVD-AMC concentration of 6.25 μM.

To test for reversibility, compound **16** (2 μM, approximately 17 × IC_{50}) was preincubated at 22 °C in assay buffer containing 67 nM caspase 3 for up to 2 h. Under these conditions, caspase 3 activity should be completely inhibited. At various times, the preincubation mixture was diluted 40-fold into a standard assay mixture containing 25 μM Ac-DEVD-AMC to assess activity. The final concentration of **16** in the assay was 50 nM, which is 2.4-fold below its IC_{50}.

Apoptosis in Jurkat Cells. The activities of compounds in cell-based models of apoptosis were evaluated in human Jurkat T cells using a method for measuring the level of apoptosis, the quantitation of the amount of broken DNA fragments using a fluorescent end-labeling method.[44,45] This is the method used in the ApoTag kit from Oncor (Gaithersburg, MD). In brief, the enzyme terminal deoxynucleotidyl transferase extends the DNA fragments with digoxigenin-containing nucleotides, which are then detected with an anti-digoxigenin antibody carrying fluorescein to allow detection by fluorescence (494 nm excitation, 523 nm emission). Propidium iodide was used as a counter stain to measure the total DNA content. Flow cytometric analysis was carried out on a Becton-Dickinson FACScan instrument using CellQuest software. We used camptothecin for induction of apoptosis in these studies.[46] The cells were treated with test compounds, induced to apoptosis for the indicated times, and then fixed. The DNA strand breaks which occurred during apoptosis were quantified.

Chondrocyte Cell Death ELISA. C20/A4 cells were grown in 24-well plates at a density of 20 000 cells/well overnight and then treated with 4 μg/mL camptothecin (Biomol), either alone or in combination with the nonselective caspase inhibitor Z-VAD-FMK (50 μM) or isatin sulfonamides, for 24 h. Cell lysates were prepared by combining the cells from the monolayer with the cells which detached during the treatment period. Cells floating in the medium were pelleted by centrifugation for 5 min at 1000 rpm, resuspended in the manufacturer's lysis buffer, and then added back to the monolayer, and the total cell population was brought to 500 μL with lysis buffer. Samples were lysed for 30 min at 4 °C and then centrifuged for 10 min at 14000*g* to clarify the lysates. Samples (100 μL) were evaluated for mono- and oligonucleosome DNA fragment formation in the cell death ELISA following the manufacturer's protocol (Roche Molecular Biochemicals, Indianapolis, IN).

Neutrophil Apoptosis Assay. Mouse bone marrow cells were treated with 10 μg/mL cycloheximide (CHX) at a density of 2 × 10^6 cells/mL in RPMI containing 10% FCS in 5 mL round-bottomed polypropylene tubes for 3 h at 37 °C; inhibitors were added at the same time as CHX. For DNA laddering, treated cells were pelleted, and the DNA released into the cytoplasm was extracted, run on a 1% agarose gel, and stained with ethidium bromide.[47]

Inhibition of Caspase 3 by Compound 31 in the Presence of Chondrocyte Cytosol. Recombinant caspase 3 activity was measured as described above in the presence of 2 μM **31**, 10 μM Ac-DEVD-AMC, and varying amounts of naive (uninduced) chondrocyte cytosol ranging from 0 to 80% (prepared as described above). IC_{50}'s were also estimated as described above except that chondrocyte cytosol was included at 90% of the assay volume. Various conditions were compared, including buffer alone (no cytosol) with added recombinant caspase 3, naive (uninduced) cytosol from nonapoptotic chondrocytes with caspase 3, cytosol from camptothecin-treated (apoptotic) cells without exogenous caspase 3 (to measure the IC_{50} of the endogenous caspase 3-like activity), and cytosol from camptothecin-treated cells with added recombinant caspase 3.

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