Discovery of a Novel Class of Reversible Non-Peptide Caspase Inhibitors via a Structure-Based Approach

Roberto Fattorusso, Dawoon Jung, Kevin J. Crowell, Martino Forino, and Maurizio Pellecchia*

The Burnham Institute, 10901 North Torrey Pines Rd., La Jolla, California 92037

Received August 17, 2004

In this paper, we report a simple structure-based iterative optimizations (SUBITO) strategy to identify and optimize new protein ligands and inhibitors. The approach is based on a combination of NMR-based screening and computational docking methods and enabled the identification of novel chemical leads among hundreds of thousands of commercially available compounds by screening only a few hundred compounds from a scaffold library followed by iterative screening steps where only few dozen compounds are tested. As an application, we report on the discovery of a novel class of non-peptide reversible caspase inhibitors, with IC_{50} values in the low micromolar range.

Introduction

The rapid identification of initial hit compounds against a given protein target is a first, crucial step in a drug discovery program. The current most common strategy makes use of high-throughput screening and automation. When the three-dimensional structure of the target protein is known, it is also possible to perform virtual docking up to millions of compounds and to select those that exhibit the best fit to be tested experimentally. Alternative routes to these approaches are the NMR-based strategies in which an initial weak binder is found among a small library of scaffolds and subsequently optimized either by the derivation of focused libraries or by linking two weak binders together.[1,2] The latter approach can also be aided by the use of X-ray crystallography.[3] While each strategy has proven quite effective in several cases, we sought here to develop a simple strategy that combines the advantages of NMR-based screening methods with virtual docking techniques. As a test example we selected caspases, a family of highly homologous cysteine proteases that specifically cleave their substrates at an aspartic acid residue. A common feature of all the caspases is the presence of a catalytic diad constituted by a nucleophilic cysteine and a histidine imidazole ring. Caspases are accumulated in the cell as inactive proenzymes (zymogens) and become fully functional only upon proteolytic cleavage at specific sites.[4,5] To date, at least 14 human caspase members have been identified. On the basis of their sequence homology, substrate specificity, and structural similarities, the caspases can be divided in two major subfamilies. Those related to ICE (interleukin-β-converting enzyme), caspase-1, -4, -5, and -13, are involved in inflammation (cytokine maturation). The second subfamily is comprised by caspases mediating programmed cell death (apoptosis).[6] In apoptosis, activation takes place in the form of a cascade, in which effector caspases (such as caspases-3, -6, and -7) are cleaved by initiator caspases (such as caspase-2 and -8−10). Initiator caspases are activated in response to a proapoptotic signals, and effector caspases are involved in the final stages of cell disassembly. Altered regulation of apoptosis is implicated in many human malignancies; in particular, enhanced levels of apoptosis are observed in many acute and chronic conditions, such as myocardial infarction, stroke, sepsis, traumatic brain injury, liver failure, spinal cord injury, Alzheimer 's disease, Huntington 's disease, and Parkinson 's disease.[7] Therefore, reduction of the apoptotic response may be of therapeutic benefit. Potent peptide inhibitors have been extensively used to validate the role of caspases in many diseases, but they are only moderately selective and possess poor cell permeability; moreover, converting peptides into drugs can be difficult. The use of nonpeptidic small-molecule inhibitors has been reported in a few cases.[8−12] In this paper, we show that by combining experimental NMR techniques and computational methods we were able to derive a novel class of non-peptide, reversible caspase inhibitors with a relatively small effort.

Results

Keeping in mind "drug-like-ness" criteria, such as molecular weight, solubility, number of rotatable bonds, and hydrogen-bond donors and acceptors, as well as availability of several hundreds of analogues, we have assembled a small but diverse library containing 300 compounds representing the most common scaffolds frequently found in drugs.[13,14] About each of the chemical moieties of our library is representative of a family of hundreds of analogues, having similar chemical properties. Each family of chemical analogues may share with other families some little chemical subspace. The screening of such a representative and small library is expected to result in only few weak inhibitors (millimolar to high micromolar range). As the detection of weak inhibitors is usually difficult to attain with conventional spectrophometric assays,[15] we made use of NMR-based assays, which are well-suited for the identification of weak binders. In particular, for the screening of caspase inhibitors, we exploited a simple NMR-based enzymatic assay based on ^{19}F NMR spectroscopy, which allows the unambiguous detection of

* Corresponding author. Tel: (858) 646-3159. Fax: (858) 646-3159. E-mail: mpellecchia@burnham.org.

10.1021/jm0493212 CCC: $30.25
Published on Web 02/15/2005

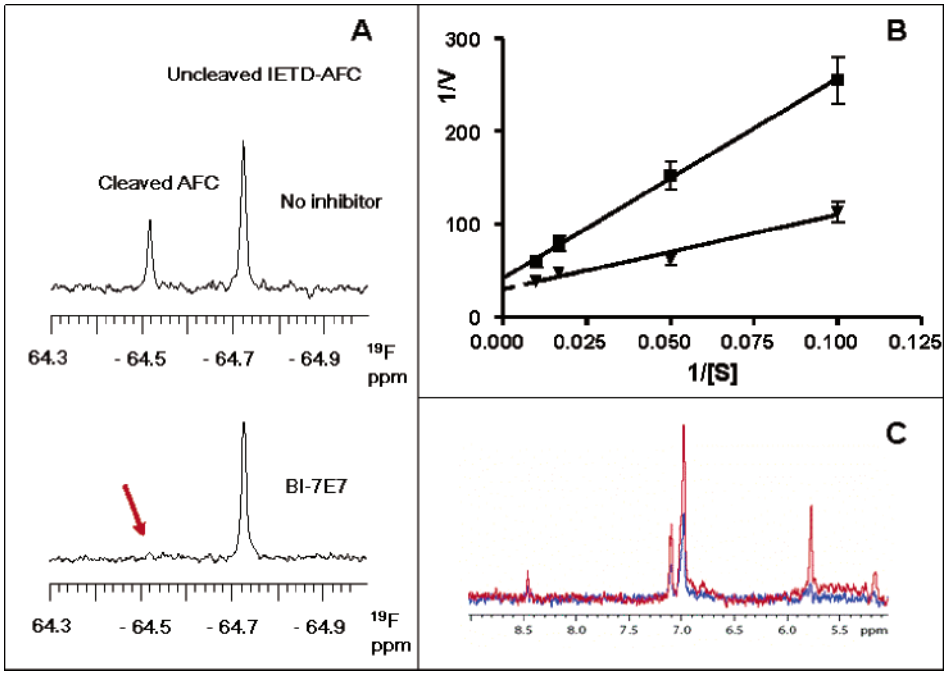


Figure 1. (A) ^{19}F NMR spectra of the caspase-8-mediated cleavage of IETD-AFC, quenched after 12 min upon Z-VAD addition, without inhibitor (above) and in the presence of 200 μM BI-7E7 (below). The assignments of the two trifluoromethyl groups are indicated. (B) Lineweaver−Burk plot of IETD-AFC (10, 20 60, and 100 μM) enzymatic cleavage by caspase-8 (50 nM) in the absence (below) and presence of 100 μM BI-7E7. The reaction velocity is given in nanomoles/second. (C) Comparison of ^{1}H spectrum of BI-7E7 (100 μM) without (red) and with (blue) water presaturation, in the presence of 4 μM C285A catalytic caspase-8 mutant.

even millimolar enzyme inhibitors. In this assay, caspase-8 (caspase-3 and caspase-7) mediated cleavage of the tetrapeptide IETD-AFC (DEVD-AFC) was followed by ^{19}F NMR, exploiting the difference in chemical shift of the trifluoromethyl substituent on the coumarin moiety after hydrolysis (Figure 1A). A similar approach was recently reported for the detection of kinases and phosphatases kinetics by ^{19}F NMR.[16] We first focused our attention on caspase-8 and were able to screen our library by using this method. The results of the screening indicated that a benzodioxane derivative compound (BI-7E7) is able to inhibit IETD-AFC cleavage by caspase-8 with an IC_{50} in the micromolar range. This scaffold appears particularly interesting due to its ready availability and shape characteristics, and it has been found in other inhibitors (e.g. ref 39). The Lineweaver−Burk analysis (Figure 1B) showed that the K_m of the caspase-8 enzymatic reaction under these conditions is 30 ± 5 μM and the K_i of BI-7E7 is 30 ± 5 μM, furthermore indicating that the BI-7E7 competes reversibly with the substrate. The binding of BI-7E7 to caspase-8 was also confirmed by a WaterLOGSY experiment,[17] in which after the adding of 5 μM caspase-8 a strong decrease of the 100 μM BI-7E7 ^{1}H NMR spectrum after water presaturation could be observed (Figure 1C). Therefore, by screening our scaffold library a potential relatively weak hit compound was identified. The most logical subsequent step to improve the inhibitory properties of this scaffold would consist of the selection and testing of all the 500 possible analogues that are commercially available (ChemNavigator, San Diego, CA; Asinex, Moscow, Russia; Maybridge, Cornwall, U.K.; Chembridge, San Diego, CA, etc.). While this route is certainly possible, it is neither rapid nor inexpensive. However, given the availability of the three-dimensional structure of the enzyme, it should be possible to select among the available derivatives only those compounds that are most likely to exhibit improved affinity for the enzyme. To this end, we have used FlexX (Sybyl, TRIPOS)[18] and GOLD[19] to dock the compound BI-7E7 in the catalytic pocket of caspase-8. As a test, we included all the compounds present in our scaffold library. The result of this computational analysis confirmed that BI-7E7 ranked first, according to several different scoring functions (CSCORE, ref 20) among all the compounds and nicely fits into the caspase-8 catalytic pocket (Figure 2C). On the basis of these results, we subsequently performed a docking analysis of 500 BI-7E7 analogues that are commercially available. Because of the nice fit of the benzodioxane moiety in the "aspartic acid hole" of caspase-8, a structural feature conserved in most caspases, we first focused on compounds containing this moiety with different derivatizations on the thiazole ring. Initially, we selected the 10 derivatives that are listed in Table 1 that have been tested for caspase-8, -7, and -3 inhibition, with the hypothesis that different substitutions on the thiazole ring would confer improved affinity and selectivity for each enzyme. In this phase of inhibitor optimization, classical fluorescence enzymatic assays have been preferred to the NMR assays. In Table 1, the results of the fluorescence assays are reported for each of the caspases tested; one of the analogues, BI-9B12, is 10-fold more active than the BI-7E7 against caspase-8. The result revealed that BI-9B12 can also inhibit caspase-3 and -7 enzymatic activities, with slightly higher K_i values; a similar behavior is observed for BI-7E7, while BI-9C4 and BI-9C1 show some selectivity for caspase-3 and -8, respectively.

Docking studies with GOLD[19] and the available three-dimensional structures of caspase-8, -3, and -7[21−23] reveal a similar binding mode of the benzodioxane

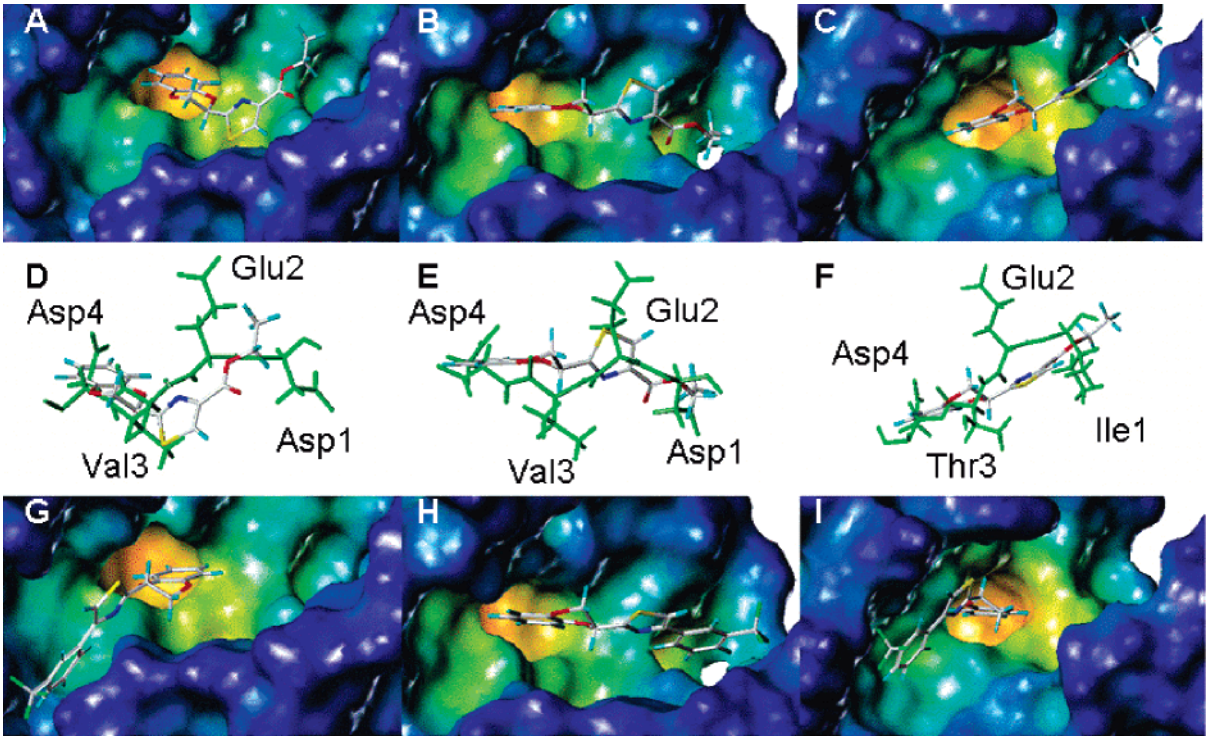


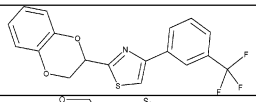
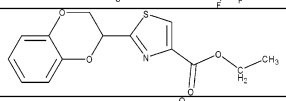
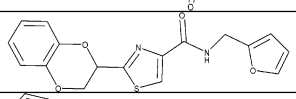
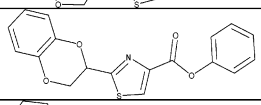
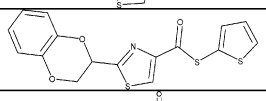
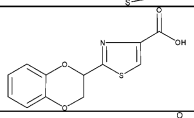
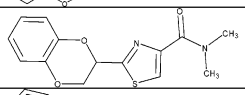
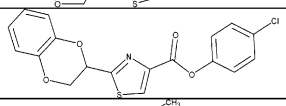
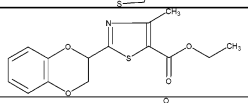
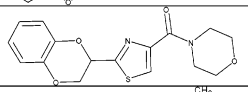
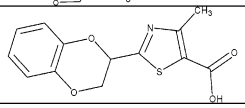
Figure 2. Docking studies with compounds BI-7E7 and the three-dimensional structures of caspases-3, -7, and -8 (panels A, B, and C). The superposition of BI-7E7 with the peptides taken from their respective X-ray structures is also shown in panels D, E, and F for the three caspases. Panels G, H, and I report the docked structure of BI-9B12 in the binding pockets of caspases-3, -7, and -8, respectively.

moiety in the "aspartate hole" of each enzyme. However, given the symmetric nature of such a moiety, the remainder of the molecule can assume two possible orientations (Figure 2A–I). In particular, while in BI-7E7 the ester group points toward the subpocket occupied by the amino acid in position 1 in the natural substrate (also called S4 subpocket) (Figure 2C,F), compound BI-9B12 appears to be flipped toward an additional subpocket in caspase-3 and -8 (Figure 2G,I). To further validate these models, we evaluated the binding energies of each docked structure and correlated them to the experimental inhibition data. We focused on caspase-3, for which all compounds but BI-9C1 gave measurable IC_{50} values. By using compounds BI-9B12, BI-7E7, and BI-9C3, we have derived binding energy scoring function coefficients similar to what was recently proposed (Fresno).[24] We subsequently used them to calculate the binding energies of the remaining compounds according to the equation $\Delta E_{bind} = \alpha(HB) + \beta(Lipo) + \gamma(Rot)$, where α, β, and γ are the coefficient and HB, Lipo, and Rot values represent the external hydrogen bonding score, the external van der Waals energy, and the internal van der Waals term, respectively, calculated by using GOLD (see also Table S1 in Supporting Information). The data reported in Table 2 clearly demonstrate the good correlation between the predicted and the experimental values, further validating our models (see also Supporting Information).

A good correlation is therefore found between the docked structures of the derivatives shown in Table 2, all derivatized on the thiazolidinyl ring, with the experimental IC_{50} values. However, in absence of experimental structural data, it is still possible, though unlikely, that the compounds would bind to a different allosteric site[40] and that the correlation observed with our models could be just coincidental, especially because of some conformational mobility of caspases' binding pockets.[44] While a definitive answer to this question can only be obtained by determining the structure of the complex by X-ray crystallography, we could attempt here to study additional analogues, particularly those with substitutions of the benzodioxane moiety. If the binding mode of the benzodioxane is correct (or close to reality) one would expect that related compounds bearing structural similarities with this moiety should retain some inhibitory activity. For example, we could find a related series of compounds in which the benzodioxane ring is replaced by a coumarin ring. The IC_{50} data obtained with some of such derivatives, measured by using the NMR-based assay, are reported in Table 3. As can be seen, the compounds are still able to inhibit caspase-3, though at higher concentrations. In addition, in the same series it can be noted that compounds with substitutions on the benzene ring, that are predicted not to fit as well in the "Asp-hole", show a further increase in IC_{50} values (Table 3). We are confident that these additional data corroborate even further our working hypotheses.

As in all lead discovery approaches, it is always possible that compounds inhibit a particular enzyme simply due to nonspecific interactions with either the peptide substrate or the protein itself. While the NMR-based assays ensured that problems of interactions between compounds and peptide can be excluded, it is still possible that the compounds interact in a nonspecific manner with the proteins. While the data obtained and the correlation with the docking models and the NMR-based binding assays seem to suggest that this is not the case, it is still imperative that additional experiments are conducted to eliminate the possibility

Table 1. Inhibition Activities against Caspase-8, -3, and -7 of BI-7E7 and Its Ten Selected Analogues as Calculated with Fluorometric Assays

Name	Structure	Caspase-8 IC_{50} (μM)	Caspase-7 IC_{50} (μM)	Caspase-3 IC_{50} (μM)
BI-9B12		2.7± 0.5 (**Ki**)[a]	6.2 ± 0.6 (**Ki**)	4.3 ±0.5 (**Ki**)
BI-7E7		30±5 (**Ki**)	46 ± 8	40 ± 8
BI-9C8		60 ± 10	> 200	133 ± 15
BI-9C3		60 ± 10	> 200	40 ± 8
BI-9B11		62 ± 10	> 200	55 ± 10
BI-9C1		75 ± 10	170 ± 15	>200
BI-9C7		>200	>200	129 ±15
BI-9C4		>200	>200	74 ± 12
BI-9C5		>200	>200	82 ± 13
BI-9C2		>200	>200	150 ± 15
BI-9C6		>200	>200	128 ± 15

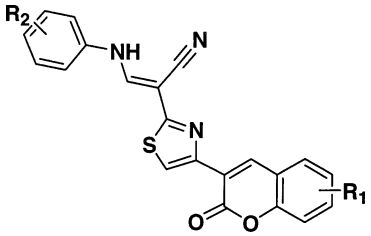
[a] K_i values were calculated through Lineweaver−Burk analysis, which indicated the presence of a competitive inhibition.

Table 2. Binding Energies and Predicted IC_{50} Values for Caspase-3

name	expl IC_{50}	calcd ΔE_{bind}[a]	pred[b] IC_{50}
BI-9B12*	8.6 ± 1.0	29.09	8.6
BI-7E7*	40 ± 8	25.26	40
BI-9C8	133 ± 15	24.67	50.54
BI-9C3*	40 ± 8	25.26	40
BI-9B11	55 ± 10	25.57	35.28
BI-9C1	>200	13.36	4720.6
BI-9C7	129 ± 15	22.56	118.06
BI-9C4	74 ± 12	26.29	26.48
BI-9C5	82 ± 13	23.07	96.01
BI-9C2	150 ± 15	22.43	124.18
BI-9C6	128 ± 15	24.75	49

[a] Modified Fresno equation: BE = 33.614 − 1.273(HB) − 0.199(Lipo) + 1.975(Rot). The asterisk (*) indicates the inhibitors that were used to derive constants. [b] Predicted IC_{50} values calculated using $\Delta E_{bind} = -RT \ln(K_i)$. The correlation coefficient between experimental and calculated IC_{50} values is 0.72.

Table 3. IC_{50} Values Measured against Caspase-3 (NMR) for a Related Series of Compounds



compd	R_1	R_2	IC_{50} (μM)
BI-9C9	H	*p*-$COOC_2H_5$	30
BI-9C10	6-NO_2	*p*-$COOC_2H_5$	>100
BI-9C11	H	*o*,*p*-OCH_3	30
BI-9C12	6-NO_2	*p*-OCH_3	>1000

of artifacts. To this end, a simple test to ensure that the compounds inhibit in a stoichiometric fashion is to measure the IC_{50} values of a given compound at different protein concentrations.[41,42] Accordingly, we performed an additional experiment in which IC_{50} value for compound BI-9B12 was measured at two different concentrations of caspase-3 (25 and 250 nM). In agreement with our other data, we did not detect an appreciable change in IC_{50} values when the concentration was increased 10-fold, thus suggesting a specific interaction.[41,42] In addition, we have also tested the ability of BI-9B12 to inhibit a metalloprotease (LF) under investigation in our laboratory.[43] When tested at con-

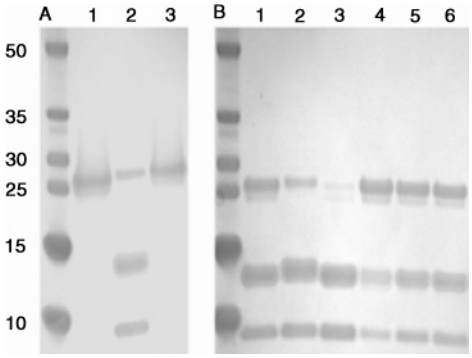


Figure 3. BID cleavage by caspase-8 monitored via SDS−PAGE. Panel A shows the progress of the reaction and inhibition by zVAD. The first numbered lanes represent BID in the absence (lane 1) and presence of caspase-8 (lane 2) and in the presence of caspase-8 and zVAD (lane 3). Panel B shows the progress of the reaction at 5, 15, and 30 min in the absence (lanes 1−3) and presence (lanes 4−6) of BI-9B12 (10 μM). As is particularly evident at 15 and 30 min time points, BI-9B12 has a significant impact on the rate of the cleavage reaction.

centrations up to 20 μM, compound BI-9B12 did not exhibit appreciable inhibition of LF both in a NMR-based assay and in a fluorescence-quenched assay, again suggesting a specific interaction with caspases.

Finally, to evaluate the ability of BI-9B12 to inhibit the cellular caspase-8 enzymatic activity, an in vitro assay of the caspase-8-mediated cleavage of the pro-apoptotic protein BID, which is the natural substrate of caspase-8,[25] was utilized. As is clearly shown in Figure 3, BI-9B12 inhibits BID cleavage by caspase-8 with an IC_{50} that is comparable with that obtained by the fluorescence assays, carried out with the IETD-AFC tetrapeptide as substrate.

Discussion

The interest of caspases as drug targets has encouraged a great deal of effort to find potent and selective inhibitors with pharmaceutically acceptable properties. Over the past decade, a family of peptide caspase inhibitors has been developed, and proof of concept data have been obtained in several animal models that show substantial protection in rodent models of stroke, myocardial infarction, hepatic injury, sepsis, amyotrophic lateral sclerosis (ALS), and several other diseases. However, the presence of electrophilic groups in those caspase inhibitors is an obstacle in the development of clinically safe drugs, since they are susceptible to reactions in vivo such as Schiff base formation or nonspecific reaction with cysteine. Very few cases of non-peptide caspase inhibitors have been described in the literature so far;[8−12] in particular, potent non-peptide caspase-3 and -7 inhibitors have been recently reported to inhibit apoptosis in three cell-based models.[8,9] The extended tethering strategy allowed the finding of new aspartyl derivatives that inhibit caspase-3 activities in vitro and in vivo assays,[11] and an HTS study led to the definition of a caspase-8 inhibitor in the low micromolar range.[12]

In such a contest, the series shown in Tables 1 and 3 represents a new class of caspase inhibitors that are based on neither irreversible inhibition of the enzyme nor on aspartyl derivatives, where the compound BI-9B12 proved to inhibit in vitro the natural enzymatic activity of caspase-8, i.e., the cleavage of the proapototic protein BID, suggesting its potential use as in vivo marker of caspase activity.

This novel class of reversible caspase inhibitors has been derived through a structure-based iterative optimization approach. Our simple approach is based on the sensitivity of NMR assays to monitor enzyme inhibition and ligand binding to select initial weak hits from a scaffold library. Subsequently, taking advantage of virtual docking, only a subset of the possible analogues is selected and tested either by NMR or by a traditional flourimetric assay. The advantage of such an approach, which we named SUBITO (for *stru*cture-*b*ased *it*erative *o*ptimizations approach), is that it enables the identification of novel hit compounds among hundreds of thousands of commercially available compounds without relying on costly high-throughput screening techniques. In fact, the procedure includes an initial screening of only a few hundred compounds from a scaffold library followed by iterative screening steps where only few dozen compounds are screened, at the most. The clear advantage of such a strategy resides not only in the reduced number of compounds to be purchased and tested but also in the fact that intrinsic structural information on mode of binding can be obtained. In fact, if the docking strategy does lead to compounds with improved affinity or, in other words, if there is a general agreement between the computed ranking and the experimental inhibition constants, there is also a good reason to believe that the structural models obtained are quite close to reality. Hence, when the route of commercially available compounds is exhausted, the docked structures can then be used to guide medicinal chemistry on the most potent compounds. Without any doubts, the greatest advantage of this strategy is that the process is inexpensive and very fast, hence the name SUBITO, the Italian translation of the expression "right now!".

The caspase-8 inhibition activity of the BI-7E7 family of analogues is clearly dictated by the benzodioxane moiety that precisely fits into the enzyme catalytic pocket, which hosts the aspartate residue during the reaction (S1 pocket[26]) (Figures 2 and 3). The aromatic ring of the benzodioxane moiety can form a cation−π interaction with two arginine residues[26] which are located in S1 pocket and are appointed to interact with the substrate aspartate residue during the enzymatic cleavage. The cation−π effect arises from favorable electrostatic interactions between the electron-rich π system of an aromatic molecule and a positively charged species such as a metal ion or quaternary amine.[27,28] Interactions between aromatic amino acid residues and positively charged side chains, attributed to the cation−π effect, are commonly observed in proteins[29,30] and also appear to be important in the binding of positively charged substrates in enzymes.[31] In our case, an opposite situation is observed, where an electron-rich π system of the benzodioxane aromatic ring is able to fit precisely into a catalytic cavity and make a cation−π interaction with the positively charged side chain of the enzyme. The aptitude to bend the dioxane ring appears to have a crucial role in orienting the aromatic ring to reach the S1 pocket correctly and at the same time in allowing the other part of the molecule to occupy

adjacent pockets. While the "aspartic hole" is extremely well conserved in nature, these adjacent pockets exhibit some differences and could be further exploited to improve affinity and selectivity. Although not spectacular, some selectivity could be observed for the compounds in Table 1. Appropriate further chemical modifications of this class of compounds (based on the models of the docked structure) could result in novel derivatives with increased caspase inhibition activity and selectivity. Such compounds would result in very useful chemical tools in deciphering the complex role of each caspase in the cell and could be translated in potential therapeutic compounds for those human diseases that are attributable to caspase-dependent uncontrolled cell death.

Conclusion

We have proposed a simple *s*tru*c*ture-*b*ased *it*erative *o*ptimizations (SUBITO) strategy to identify and optimize new protein ligands and inhibitors. The SUBITO approach, which is based on a combination of experimental, NMR-based screening, and computational docking methods, enables the identification of novel chemical leads among hundreds of thousands of commercially available compounds by screening only a few hundred compounds from a scaffold library followed by iterative screening steps where only few dozen additional compounds are purchased and evaluated. As a test, we have applied this approach to derive novel non-peptide reversible caspase inhibitors that have been shown to inhibit the BID cleavage by caspase-8 in vitro, with IC_{50} values in the low micromolar range. The docked structures of these initial hit compounds should facilitate their further conversion into more potent and selective inhibitors, that may provide novel insights in the role of caspases in apoptosis and could possibly represent potential therapeutic agents.

Experimental Section

Materials. Active caspase-3, -7, and -8 were expressed in *Escherichia coli* and isolated in the full active form as previously described.[32] The active concentrations of each purified enzymes were determined by titration using benzyloxarbonyl-Val-Ala-DL-Asp-fluoromethyl ketone (Z-VAD-FMK).[32] Z-VAD-FMK was purchased from Enzyme System Products (Livermore, CA), acetyl-Ile-Glu-Thr-Asp-7-amino-4-trifluoromethyl coumarin (Ac-IETD-AFC) and acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC) were purchased from Biosource International, Inc. (Camarillo, CA). Compounds BI-7E7, BI-9B11, BI-9B12, BI9-C1, BI-9C2, BI-9C3, BI-9C4, BI-9C5, BI-9C6, BI-9C7, and BI-9C8 were purchased from Maybridge (Cornwall, U.K). Elements of the scaffold library were selected on the basis of the work of Bemis and Murko.[13,14] Other criteria includes MW < 300; number of hydrogen-bond donor and acceptors < 5; number or rotatable bonds between 0 and 2; and calculated partition coefficient octanol/water, clogP < 2. Generally, for each scaffold selected several hundred analogues were commercially available. We used the compounds collections listed in Chemnavigator (www.chemnavigator.com) and used the software Chemfinder (Cambridge) for 2D compound substructure and similarity searches.

Enzyme Inhibition Assays. NMR and fluorescent enzymatic assays were run in 500 and 100 μL volumes of buffer solutions, respectively, and contained the following: 40 μM Ac-IETD-AFC (Ac-DEVD-AFC), 25−50 nM caspase-8 (caspase-3 or -7), 10 mM phosphate buffer (pH 7.5), and 10 mM NaCl. For the estimation of K_i (see below), different concentrations of substrate have been used under the same experimental conditions. All the inhibitors that were tested were dissolved and diluted in DMSO-d_6 prior to addition to assay mixture; the final concentration of DMSO was always less than 5%.

The enzymatic reactions followed by NMR were performed at room temperature in Eppendorf vials and then quenched after a defined delay with the addition of Z-VAD-FMK. To avoid unwanted interactions of the screened compounds with the high concentrations of chemicals, which are generally used to stabilize the catalytic activity of the caspases,[9] we set up simpler experimental conditions for the caspase enzymatic reactions that allow the complete peptide cleavage to occur with K_m values in reasonable ranges (see Results). ^{19}F NMR spectra were run at 23 °C with a Bruker500 NMR spectrometer, operating at a ^{19}F Larmor frequency of 470 MHz using a 5 mm ^{19}F probe. The data were acquired with proton decoupling, with an acquisition time of 0.8 s and a relaxation delay of 1.5 s. The WaterLOGSY experiment[17] was acquired using the water irradiation scheme previously published[33] and adding to BI-7E7 (100 μM) the [Cys285Ala]caspase catalytic mutant (4 μM), prepared as previously described.[34] This protein was used as it was available at higher concentrations needed for the NMR-based binding assay.

Fluorescent assays were carried out in black 96-well flat-bottom polystyrene plates. Caspase activity was monitored using a Labsystem Fluoroskan II spectrofluorometer with an excitation wavelength of 405 nm and an emission wavelength of 505 nm. Kinetic data were collected over a 30-min assay run at room temperature. Inhibition IC_{50} values were calculated as previously reported.[26] K_i and K_m values were calculated using the Lineweaver−Burk double reciprocal equation: $1/v = (K_m V_{max})(1/[\text{substrate}]) + (1/V_{max})$.

IC_{50} values for compounds BI-9B12 where also measured at two different concentrations of caspase-3 (25 nM and 250 nM).

LF inhibition assay was performed by preparing a 50 nM solution of protein in 20 mM HEPES, pH 7.4, including 4 μM labeled substrate MAPKKide and 20 μM of compound BI-9b12. Kinetic measurements were carried out every minute for 30 min using a fluorescent plate reader (Finstruments Fluoroskan II). Excitation and emission maxima were 485 and 590 nm, respectively.

Molecular Modeling. Molecular models were built by using Sybyl 6.9 (TRIPOS) and energy minimized by using the routine MAXIMIN. Initial docking studies with the entire scaffold library and BI-7E7 derivatives were performed with FlexX[18] as implemented in Sybyl 6.9 by using the three-dimensional structure of caspase-8 in complex with a tetrapeptide IETD (PDB ID 1QTN). For each compound, 10 solutions were generated and compounds were ranked according to CSCORE,[19] which included drug score,[35] chemscore[36] and Gold score[18] functions. Further studies on the 10 compounds reported in Tables 1 and 2 were performed with Gold[18] by suing the three-dimensional structure of caspase-8, caspase-3 (PDB ID 1PAU) and caspase-7 (PDB ID 1FLJ). The docked structures of compounds BI-7E7 and BI-9B12 are reported in Figure 3 together their superposition with the peptide substrates (IETD for caspase-8 and DEVD for caspases-3 and -7). Surface representation of caspases was generated with MOLCAD[37] and color-coded according to cavity dept (blue, shallow; yellow, buried). Molecular modeling studies were conducted on several R12000 SGI Octane workstations with the software package Sybyl version 6.9 (TRIPOS) or on a Linux workstation (for Gold calculations). The binding energies of the docked compounds were correlated with the experimental inhibition constants by using an empirical binding energy scoring function similar to what recently reported (by Fresno[23]). The correlation between the observed and predicted inhibition constants is reported in Figure S1 of the Supporting Information.

Expression and Purification of Caspase-8 and BID. Caspase-8 was expressed in *E. coli*. BL21(DE3) and purified as previously described,[31] and BID was expressed in *E. coli*. BL21(DE3) as previously described.[38] BID was purified using

an Akta-Prime with a combination of Ni-affinity chromatography using a 5 mL Hi-TRAP HP column, followed by cation exchange chromatography using a Hi-Trap 16/10 SP FF column (GE-Amersham Biosciences). BID was dialyzed into PBS, pH 7.5, and concentrated to approximately 1 mM prior to use.

In Vitro BID Cleavage Assays. Cleavage reactions were carried out in a total volume of 400 μL in PBS, with shaking at room temperature. Typical concentrations of the reaction components were as follows: BID, 40 μM; caspase-8, 100 nM; zVAD, 10 μM; BI-9B12, 1−25 μM; and BI-7E7, 150 μM. Samples for SDS−PAGE were taken at 5, 15, and 30 min of reaction time by adding an aliquot of each reaction directly to SDS−PAGE sample buffer, followed immediately by vortexing, flash freezing, and heating to 100 °C for approximately 20 min.

Acknowledgment. We thank Dr. Sina Sareth for initial docking studies, Dr. Guy Salvesen for the kind gift of recombinant caspase-3, -7, and -8, and NIH for financial support (grant CA107875-01 to M.P.).

Supporting Information Available: Tables S1−S3 reporting the GOLD scores of our potential inhibitors used for caspases-3, -7, and -8. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Pellecchia, M.; Sem, D.; Wuthrich, K. NMR in drug discovery. *Nat. Rev. Drug Discovery* **2002**, *1*, 211−219.
(2) Pellecchia, M.; Becattini, B., Crowell, K. J.; Fattorusso, R.; Forino, M.; Fragai, M.; Jung, D.; Mustelin, M.; Tautz, L. NMR in the hit identification and optimization processes. *Expert Opin. Ther. Tar*. **2004**, *8*, 1−15.
(3) Rees D. C.; Congreve M.; Murray C. W.; Carr R. Fragment-based drug discovery. *Nat. Rev. Drug Discovery* **2004**, *3*, 660−672.
(4) Denault, J. B.; Salvesen, G. S. Caspases: Keys in the ignition of cell death. *Chem Rev*. **2002**, *102*, 4489−4500.
(5) Grutter, M. G. Caspase: Key players in programmed cell death. *Curr. Opin. Struct. Biol.* **2000**, *10*, 649−655.
(6) Reed, J. C. Apoptosis-based therapies. *Nat. Rev. Drug Discovery* **2002**, *1*, 111−121.
(7) Hengartner, M. O. The biochemistry of apoptosis. *Nature* **2000**, *407*, 770−776.
(8) Lee, D.; Long, S. A.; Adams, J. L.; Chan, G.; Vaidya, K. S.; Francis, T. A.; Kikly, K.; Winkler, J. D.; Sung, C. M.; Debouck, C.; Richardson, S.; Levy, M. A.; DeWolf, W. E., Jr; Keller, P. M.; Tomaszek, T.; Head, M. S.; Ryan, M. D.; Haltiwanger, R. C.; Liang, P. H.; Janson, C. A.; McDevitt, P. J.; Johanson, K.; Concha, N. O.; Chan, W.; Abdel-Meguid, S. S.; Badger, A. M.; Lark, M. W.; Nadeau, D. P.; Suva, L. J.; Gowen, M.; Nuttall, M. E. Potent and selective nonpeptide inhibitors of caspases 3 and 7 inhibit apoptosis and maintain cell functionality. *J. Biol. Chem.* **2000**, *275*, 16007−16014.
(9) Lee, D.; Long, S. A.; Murray, J. H.; Adams, J. L., Nuttal, M. E.; Nadeau, D. P.; Kikly, K.; Winkler, J. D.; Sung, C. M.; Ryan, D.; DeWolf, Jr W. E. Potent and selective nonpeptide inhibitors of caspase 3 and 7. *J. Med. Chem.* **2001**, *44*, 2015−2026.
(10) Choong, I. C.; Lew, W.; Lee, D.; Pham, P.; Burdett, M. T.; Lam, J. W.; Wiesmann, C.; Luong, T. N.; Fahr, B.; DeLano, W. L.; McDowell, R. S.; Allen, D. A.; Erlanson, D. A.; Gordon, E. M.; O'Brien, T. Identification of potent and selective small-molecule inhibitors of caspase-3 through the use of extended tethering and structure-based drug design. *J. Med. Chem.* **2002**, *45*, 5005−5022.
(11) Erlanson, D. A.; Lam, J. W.; Wiesmann, C.; Luong, T. N.; Simmons, R. L.; DeLano, W. L.; Choong, I. C.; Burdett, M. T.; Flanagan, W. M.; Lee, D.; Gordon, E. M.; O'Brien, T. In situ assembly of enzyme inhibitors using extended tethering. *Nat. Biotechnol.* **2003**, *21*, 308−314
(12) Allen, D. A.; Pham, P.; Choong, I. C.; Fahr, B.; Burdett, M. T.; Lew, W.; DeLano, W. L.; Gordon, E. M.; Lam, J. W.; O'Brien, T.; Lee, D. Identification of potent and novel small-molecule inhibitors of caspase-3. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3651−3655.
(13) Bemis, G. W.; Murcko, M. A. The properties of known drugs. 1. Molecular frameworks. *J. Med. Chem.* **1996**, *39*, 2887−2893.
(14) Bemis, G. W.; Murcko, M. A. The properties of known drugs. 2. Side chains. *J. Med. Chem.* **1999**, *42*, 5095−5099.
(15) Dove, A. Drug screening—Beyond the bottleneck. *Nat. Biotechnol.* **1999**, *17*, 859−863.
(16) Dalvit, C.; Ardini, E.; Flocco, M.; Fogliatto, G. P.; Mongelli, N.; Veronesi, M. A general NMR method for rapid, efficient, and reliable biochemical screening. *J. Am. Chem. Soc.* **2003**, *125*, 14620−14625.
(17) Dalvit, C.; Fogliatto, G.; Stewart A.; Veronesi, M.; Stockman, B. WaterLOGSY as a method for primary NMR screening: Practical aspects and range of applicability. *J. Biomol. NMR* **2001**, *21*, 349−359.
(18) Rarey, M.; Kramer, B.; Lengauer, T.; Klebe, G. A fast flexible docking method using an incremental construction algorithm. *J. Mol. Biol* **1996**, *261*, 470−489.
(19) Verdonk, M. L.; Cole, J. C.; Hartshorn, M. J.; Murray C. W.; R. D. Taylor Improved protein−ligand docking using GOLD. *Proteins* **2003**, *52*, 609−623.
(20) Clark, R. D.; Strizhev, A.; Leonard, J. M.; Blake, J. F.; Matthew, J. B. Consensus scoring for ligand/protein interactions. *J. Mol. Graph. Model*. **2002**, *20*, 281−295.
(21) Watt, W.; Koeplinger, K. A.; Mildner, A. M.; Heinrikson, R. L.,; Tomasselli, A. G.; Watenpaugh, K. D. The atomic-resolution structure of human caspase-8, a key activator of apoptosis. *Struct. Folding Des.* **1999**, *7*, 1135−1143.
(22) Rotonda, J.; Nicholson, D. W.; Fazil, K. M.; Gallant, M.; Gareau, Y.; Labelle, M.; Peterson, E. P.; Rasper, D. M.; Ruel, R.; Vaillancourt, J. P.; Thornberry, N. A.; Becker, J. W. The three-dimensional structure of apopain/CPP32, a key mediator of apoptosis. *Nat. Struct. Biol.* **1996**, *3*, 619−625.
(23) Wei, Y.; Fox, T.; Chambers, S. P.; Sintchak, J.; Coll, J. T.; Golec, J. M.; Swenson, L.; Wilson, K. P.; Charifson, P. S. The structures of caspases-1, -3, -7 and -8 reveal the basis for substrate and inhibitor selectivity. *Chem. Biol*. **2000**, *7*, 423−432.
(24) Rognan, D.; Lauemoller, S. L.; Holm, A.; Buus, S.; Tschinke, V. Predicting binding affinities of protein ligands from three-dimensional models: Application to peptide binding to class I major histocompatibility proteins. *J. Med. Chem.* **1999**, *42*, 4650−4658.
(25) Li, H.; Zhu, H.; Xu, C. J.; Yuan, J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **1998**, *94*, 491−501.
(26) Chereau, D.; Kodandapani, L.; Tomaselli, K. J.; Spada, A. P.; Wu, J. C. Structural and functional analysis of caspase active sites. *Biochemistry* **2003**, *42*, 4151−4160.
(27) Ma, J. C.; Dougherty, D. A. The cation−π interaction. *Chem. Rev.* **1997**, *97*, 1303−1324.
(28) Waters, M. L. Aromatic interactions in model systems. *Curr. Opin. Chem. Biol.* **2002**, *6*, 736−741.
(29) Burley, S. K.; Petsko, G. A. Aromatic−aromatic interaction—A mechanism of protein-structure stabilization. *Science* **1985**, *229*, 23−28.
(30) Dougherty, D. A. Cation−π interactions in chemistry and biology: A new view of benzene, Phe, Tyr, and Trp. *Science* **1996**, *271*, 163−168.
(31) Slutsky, M. M.; Marsh, E. N. Cation−π interactions studied in a model coiled-coil peptide. *Protein Sci.* **2004**, *13*, 2244−2251.
(32) Stennicke, H. R.; Salvesen, G. S. Caspases: Preparation and characterization. *Methods* **1999**, *17*, 313−319.
(33) Pellecchia, M.; Meininger, D.; Dong, Q.; Chang, E.; Jack, R.; Sem, D. S. NMR-based structural characterization of large protein−ligand interactions. *J. Biomol. NMR* **2002**, *22*, 165−73.
(34) Boatright, K. M.; Renatus, M.; Scott, F. L.; Sperandio, S.; Shin, H.; Pedersen, I. M.; Ricci, J. E.; Edris, W. A.; Sutherlin, D. P.; Green, D. R.; Salvesen, G. S. A unified model for apical caspase activation. *Mol Cell.* **2003**, *11*, 529−541.
(35) Gohlke, H.; Hendlich, M.; Klebe, G. Knowledge-based scoring function to predict protein−ligand interactions. *J. Mol. Biol*. **2000**, *295*, 337−56.
(36) Eldridge, M.; Murray, C. W.; Auton, T. A.; Paolini, G. V.; Lee, R. P. Empirical scoring functions: I. The development of a fast empirical scoring function to estimate the binding affinity of ligands in receptor complexes. *J. Comput.-Aided Mol. Des.* **1997**, *11*, 425−445.
(37) Teschner, M.; Henn, C.; Vollhardt, H.; Reiling, S.; Brickmann, J. Texture mapping: A new tool for molecular graphics. *J. Mol. Graph*. **1994**, *12*, 98−105.
(38) Becattini, B.; Sareth, S.; Zhai, D.; Crowell, K. J.; Leone, M.; Reed J. C.; Pellecchia, M. Targeting apoptosis via chemical design: Inhibition of Bid-induced cell-death by small organic molecules. *Chem. Biol*. **2004**, *11*, 1107−17.
(39) Koide, Y.; Tatsui, A.; Hasegawa, T.; Murakami, A.; Satho, S.; Yamada, H.; Kazayama, S.; Takahashi, A. Identification of a stable chymase inhibitor using a pharmacophore based database search. *Bioor. Med. Chem*. **2003**, *13*, 25−29.
(40) Hardy, J. A.; Lam, J.; Nguyen, J. T.; O'Brien, T.; Wells, J. A.Discovery of an allosteric site in the caspases. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 12461−6.
(41) McGovern, S. L.; Helfand, B. T.; Feng, B.; Shoichet, B. K.A specific mechanism of nonspecific inhibition. *J. Med. Chem*. **2003**, *46*, 4265−72.

(42) McGovern, S. L.; Caselli, E.; Grigorieff, N.; Shoichet, B. K. A common mechanism underlying promiscuous inhibitors from virtual and high-throughput screening. *J. Med. Chem*. **2002**, *45*, 1712−22.
(43) Moayeri, M.; Leppla, S. H. The roles of anthrax toxin in pathogenesis. *Curr. Opin. Microbiol*. **2004**, *7*, 19−24.
(44) Romanowski, M. J.; Scheer, J. M.; O'Brien, T.; McDowell, R. S. Crystal structures of a ligand-free and malonate-bound human caspase-1: Implications for the mechanism of substrate binding. *Structure* **2004**, *12*, 1361−71.

JM0493212