Cell Death and Differentiation (2003) 10, 881–888

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Inhibition of papain-like cysteine proteases and legumain by caspase-specific inhibitors: when reaction mechanism is more important than specificity

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Received 17.11.02; revised 3.2.03; accepted 13.2.03
Edited by G Salvesen

Abstract

We report here that a number of commonly used small peptide caspase inhibitors consisting of a caspase recognition sequence linked to chloromethylketone, fluoromethylketone or aldehyde reactive group efficiently inhibit other cysteine proteases than caspases. The *in vitro* studies included cathepsins B, H, L, S, K, F, V, X and C, papain and legumain. Z-DEVD-cmk was shown to be the preferred irreversible inhibitor of most of the cathepsins *in vitro*, followed by Z-DEVD-fmk, Ac-YVAD-cmk, Z-YVAD-fmk and Z-VAD-fmk. Inactivation of legumain by all the inhibitors investigated was moderate, whereas cathepsins H and C were poorly inhibited or not inhibited at all. Inhibition by aldehydes was not very potent. All the three fluoromethylketones efficiently inhibited cathepsins in Jurkat and human embryonic kidney 293 cells at concentrations of 100 μM. Furthermore, they completely inhibited cathepsins B and X activity in tissue extracts at concentrations as low as 1 μM. These results suggest that data based on the use of these inhibitors should be taken with caution and that other proteases may be implicated in the processes previously ascribed solely to caspases.

Cell Death and Differentiation (2003) **10,** 881–888. doi:10.1038/sj.cdd.4401247

Keywords: cathepsin; caspase; apoptosis; specificity; inhibition; legumain; cysteine protease

Abbreviations: Ac, acetyl; AFC, 7-amido-4-trifluoromethylcoumarin; AMC, 7-amido-4-methylcoumarin; Bz, benzoyl; CA-074, *n*-propyl-epoxysuccinyl-Ile-Pro; Dnp, 2,4-dinitrophenyl; E-64, L-*trans*-epoxysuccinyl-Leu-amido-(4-guanidino) butane; E-64d, L-*trans*-epoxysuccinyl(OEt) -Leu-3-methylbutylamide; β NA, β -naphthylamide; pNA, *p*-nitroanilide; Z, benzyloxycarbonyl.

Introduction

Apoptosis is the major way by which multicellular organisms eliminate excessive, damaged and potentially dangerous cells (reviewed in Hengartner[1]). The cysteinyl aspartate-specific proteinases (caspases) fulfill a central role in the process of apoptosis. Based on phylogenetic analysis of the catalytic domains, mammalian caspases can be divided into three primary groups.[2] The first group comprises a dense cluster of caspases involved in the control of inflammation (caspases 1, 4, 5, 11, 12). Caspases 2 and 14 are loosely associated with this cluster. The next cluster consists of the apoptotic executioner caspases (caspases 3, 7, 6), whereas caspases involved in initiation of apoptosis constitute the third cluster group (caspases 8, 9, 10).

An extensive study by combinatorial approach revealed that in addition to the absolute requirement of all caspases for an Asp residue in P1 position, the P4 position is a critical specificity determinant.[3] Caspases can be divided into three different families based on extended P4 substrate specificity. Group I accepts primarily large aromatic residues in this position, group II requires an Asp residue and group III prefers residues with larger aliphatic side chains. This classification also largely agrees with the phylogenetic classification and physiological roles of these enzymes. Proinflammatory caspases belong to group I, whereas the proapoptotic caspases fall into groups II (executioner caspases 3 and 7, and caspase 2) and III (initiator caspases 8, 9 and 10, and caspase 6).[2,4]

In order to block caspase activity, several substrate-mimetic inhibitors have been synthesized either as reversible aldehydes, such as Ac-YVAD-CHO, Ac-DEVD-CHO or Ac-WEHD-CHO,[5] or irreversible fluoro- or chloromethylketones, such as Z-VAD-fmk, Z-YVAD- fmk/cmk and Z-DEVD-fmk/cmk (reviewed in Nicholson[6]) (Ac=acetyl, Z=benzyloxycarbonyl). These inhibitors have been extensively used to address the functions of caspases in cellular systems. A few of these inhibitors have even entered preclinical studies with animal models of human diseases.[6] However, despite structural differences between the protease families, design of truly specific inhibitors is often a problem.[7,8] Caspases are no exception and several of caspase inhibitors have been recently shown to block efficiently the activity of another cysteine protease, cathepsin B.[9–13] In addition, cathepsin B

inhibition by Ac-YVAD-cmk rescued neuronal cells against glucose/oxygen deprivation-induced apoptosis,[12] suggesting that other proteases might contribute to the processes previously ascribed solely to caspases.

Cathepsin B, a target of caspase inhibitors, is a lysosomal protease which, together with 10 other related human enzymes, belongs to the papain family of cysteine proteases.[14] In contrast to caspases, this group of enzymes exhibits no strict S1 substrate-binding site specificity. In addition, there is no common S4 substrate-binding site in papain-like proteases.[14] Furthermore, several lysosomal cysteine proteases were shown to be involved in various apoptosis models, although the mechanisms of their involvement are not yet clear (reviewed in Leist and Jäätelä[15], Turk *et al*.[16]). Therefore, a thorough screening of substrates and inhibitors of caspases and papain-like proteases was performed to examine the possible crossreactivities of these reagents and to give us hints about the biological role of specific enzymes. Studies included caspases 1, 3, 6, 7, 8 and 11, cathepsins B, C, F, H, K, L, S, V and X, and papain as the archetype of the family. In addition, legumain, an Asn-specific lysosomal cysteine protease related to caspases,[17] was included.

Results

Activity of caspases towards the substrates and inhibitors of papain-like cysteine proteases

In the initial experiments, activities of caspases 1, 11, 3, 6, 7 and 8, representing all the three caspase-specificity subgroups,[3] were assayed using the most common substrates for papain- like cysteine proteases, Z-FR-AMC, Z-RR-AMC and R-AMC (AMC=7-amido-4-methylcoumarin).[14] As expected, none of the caspases displayed any activity towards any of the three substrates (data not shown), which is in agreement with previous studies (reviewed in Nicholson[4]). We next investigated the interaction between the same caspases (1, 3, 6, 7, 8 and 11) and the two inhibitors of papain-like cysteine proteases: L-*trans*-epoxysuccinyl-Leu-amido-(4-guanidino) butane (E-64), a potent, general inhibitor of papain-like cysteine proteases,[18] and *n*-propyl-epoxysuccinyl-Ile-Pro (CA-074), a specific inhibitor of cathepsin B. [19] No inhibition of caspase activities was observed for either of the two inhibitors even at concentrations as high as 200 μM, indicating that E-64 and CA- 074 are not crossreactive with any of the caspases.

Activity of papain-like cysteine proteases and legumain towards the caspase substrates

Activities of papain-like cysteine proteases cathepsins B, C, F, H, K, L, S, V, X, papain and legumain were tested using the caspase substrates Ac-DEVD-AFC and Ac-WEHD-AMC (AFC=7-amido-4-trifluoromethylcoumarin). Under the conditions used, almost no activity towards tested substrates of the enzymes investigated was observed (data not shown). A slight activity was observed for legumain. However, this activity was negligible when compared to the activity of the enzyme, as measured using its preferred substrate.

Inhibition of papain-like cysteine protease and legumain by caspase inhibitors

The last group of reagents investigated were the commonly used caspase inhibitors: Z- DEVD-fmk/cmk, Ac/Z-YVAD-cmk/fmk, Z-VAD-fmk, Ac-YVAD-CHO and Z-DEVD-CHO (reviewed in Nicholson[6]). Each was tested for activity against the papain-like cysteine proteases cathepsins B, C, F, H, K, L, S, V, X, papain and legumain. Initial screening with Z-DEVD-cmk and Z-VAD-fmk indicated that most of the enzymes tested were at least partially inhibited by both inhibitors at relatively low inhibitor concentration ($\leqslant 1$ μM; not shown).

Inhibition kinetics of papain-like cysteine proteases and legumain by the irreversible caspase inhibitors were also determined. Initially, fully active cathepsin B, titrated with E-64 (Figure 1a), was treated with increasing concentrations of Z-DEVD-cmk. As can be seen in Figure 1b, the inhibitor bound to cathepsin B with an apparent 1 : 1 stoichiometry, suggesting that papain-like cysteine proteases are irreversibly inactivated by Z-DEVD-cmk (Figure 1b). Similar results were obtained for papain and cathepsin L. In an additional experiment, cathepsin B was allowed to react with a stoichiometric amount

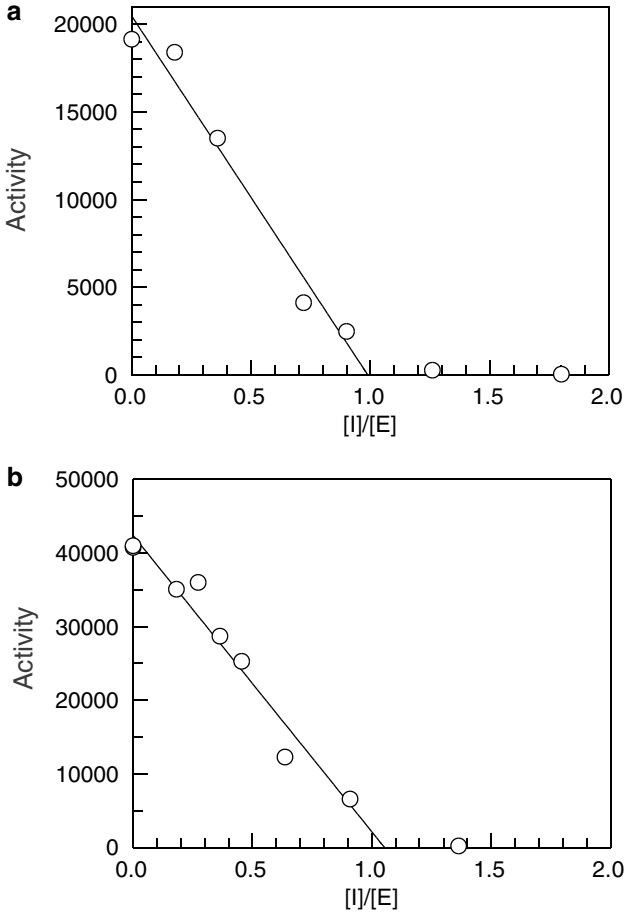

Figure 1 Titration of cathepsin B with E-64 (**a**) and Ac-DEVD-cmk (**b**) at pH 6. 0 and 25°C. Cathepsin B concentration was 250 nM in (**a**) and 500 nM in (**b**). Other experimental conditions were as described in Materials and Methods. The solid lines were generated by linear regression analysis

of Z-DEVD-cmk in a small volume and then diluted 100-fold into the buffer solution, containing 20 μM substrate. No substrate hydrolysis was observed even after prolonged incubation (120 min, not shown), indicating that the reaction is indeed irreversible.

Next, the rate constants for inactivation of papain-like cathepsins and legumain by cmk/fmk-type inhibitors were determined. All the progress curves recorded in the presence of substrate reached a plateau, confirming the irreversible mechanism of inhibition. Curves were fitted to single exponentials,[20] yielding k_{obs} values for each inhibitor concentration (data not shown). The rate constants of inactivation, k_2/K_1, were obtained from the slopes of the plots of k_{obs} *versus* $[I_0]$, which were corrected for substrate competition. However, several enzymes were less stable (cathepsin L, cathepsin K) or inhibition was too slow (all Z-VAD-fmk-cathepsin interactions, cathepsin H, cathepsin C, legumain) to allow determination of the k_2/K_1 values by the progress curve method owing to substrate consumption. Some of these interactions were monitored by the discontinuous method. The curves obtained could be best analyzed by single exponential decays. The k_{obs} values obtained were plotted *versus* $[I_0]$ and the inactivation rate constants were calculated from the slopes of those plots. The k_2/K_1 values, obtained by either of the two methods, are listed in Table 1. Several endopeptidases (papain, cathepsins B, L, V, S) and carboxypeptidase cathepsin X were inactivated by Z-DEVD-cmk, Ac-YVAD-cmk and Z-DEVD-fmk with the k_2/K_1 values ranging from $\sim$1000/M/s (cathepsin X) to as high as >350 000/M/s (cathepsin V). With the exception of papain ($k_2/K_1 \geqslant$ 10 000/M/s), these enzymes were less potently inhibited by Z-YVAD-fmk ($k_2/K_1 <$ 1000/M/s). Moreover, the rate constants for Z-DEVD-cmk inhibition of cathepsins S and L (k_2/K_1 28 000–36 000/M/s) are almost comparable with the rate constants for inhibition of cathepsins L and B by E-64 (k_2/K_1=96 000 and 89 400/M/s, respectively;[18]). The cathepsin V inhibition was substantially faster, indicating that Z-DEVD-cmk is a potent inhibitor of the enzyme. Cathepsin K was poorly inhibited and aminopeptidases were inactivated only very slowly (cathepsin H) or not at all (cathepsin C) by all the inhibitors studied. Z-VAD-fmk was an exception and inhibited all of the papain-like enzymes extremely slowly ($k_2/K_1 \leqslant$ 160/M/s). In contrast, the rate constant of inactivation of legumain by Z-VAD-fmk was $\sim$10-fold higher. Moreover, Z-VAD-fmk inhibited legumain with similar efficiency as the other two inhibitors investigated (Z-DEVD-cmk and Ac-YVAD-cmk). However, legumain, although a lysosomal protease, belongs to clan CD proteases similar to caspases and is not related to cathepsins.[17] Therefore, any further conclusions seem too speculative.

In a set of control experiments, caspase 3 interaction with the same inhibitors was studied (Table 1). Z-DEVD-cmk was by far the preferred inhibitor with the k_2/K_1 value of $\sim$1 000 000 $M^{-1}s^{-1}$, which is substantially higher than for any of the cathepsins. Esterification of the acidic side chains of the inhibitor, however, had considerably larger effect ($\sim$500-fold) on caspase 3 than on the cathepsins, in agreement with the requirement for an unmodified Asp residue in the P1 position.[3] Z-VAD-fmk was a moderate inhibitor of caspase 3. Inhibition by Ac-YVAD-cmk was negligible and therefore inhibition of caspase 3 by Z-YVAD-fmk was not studied.

The K_i values for inhibition of cathepsins B, H, V and papain by the reversible inhibitors Z-DEVD-CHO and Ac-YVAD-CHO were determined by the method of Greco and Hakala.[21] As can be seen from Table 2, all the proteases were only weakly inhibited by the two inhibitors (K_i=0.24–40 μM), especially when compared with caspase-1 and -3 inhibition ($K_i \leqslant$ 1 nM for the preferred inhibitor).

Inhibition of cathepsins in cell cultures

All of the caspase inhibitors were tested for their ability to inhibit papain-like cysteine proteases at 100 μM, a concentration widely used in studies of apoptosis to block caspase activity. Initial experiments were performed in Jurkat T cells, which are often used in apoptosis studies. After 24 h incubation in the presence of inhibitors, both Z-FR-AMC and Z-RR-AMC activities, typical for cathepsins, were inhibited 50–85%in total cell extracts in the presence of cell-permeable

Table 1 Inactivation kinetics of papain-like cysteine proteases and legumain by caspase inhibitors

	Inhibitor				
Enzyme	Z-DEVD-cmk	Ac-YVAD-cmk	Z-DEVD-fmk k_2/K_1 (1/M/s)	Z-YVAD-fmk	Z-VAD-fmk
Papain	1750	8100	15 100	10 250	18
Cathepsin B	5200	10 200	3 800	810	56
Cathepsin L	36 000	1 900	ND	ND	<50
Cathepsin V	355 000	15 000	22 000	180	28
Cathepsin K	290	26	ND	ND	<50
Cathepsin F	22 100	11 900	ND	ND	170
Cathepsin S	28 100	6 900	ND	ND	160
Cathepsin X	850	1000	ND	600	34
Cathepsin H	<10	13	57	43	1.5
Cathepsin C	<10	<10	ND	ND	<10
Legumain	660	1300	ND	ND	1200
Caspase 3	1 000 000	<100	2000	ND	6500

The best estimates for the inactivation rate constants, k_2/K_1, were obtained by linear regression analysis from the plots of k_{obs} *versus* $[>I_0]$. Other experimental conditions were as described in Materials and Methods. ND, not determined.

fmk inhibitors (Figure 2a). Ac-YVAD-cmk treatment resulted in ~20–25% inhibition, whereas Z-DEVD-cmk and Z-DEVD-CHO treatment did not result in any effect on cathepsin activity. As a positive control L-*trans*-eponysuccinyl (OEE)-leu-3-methylbutylamide (E-64d), a cell-permeable form of E-64, was used at a concentration of 5 μM, which was sufficient to block completely any cathepsin activity. Jurkat T cells were also incubated with the fmk-type inhibitors for 6 h with approximately the same effect on the cathepsin activity (not shown). However, incubation with 20 μM fmk-type inhibitors for 24 h resulted in decreased inhibition (15–40%; data not shown).

In order to test whether this is a general phenomenon, the experiment was repeated using human embryonic kidney (HEK)-293 cells. Cells were incubated with fmk inhibitors that showed the greatest effect on the Jurkat T cells. Treatment of 293 cells with 100 μM inhibitors virtually abolished cathepsin activity in the extracts (88–96% inhibition; Figure 2b).

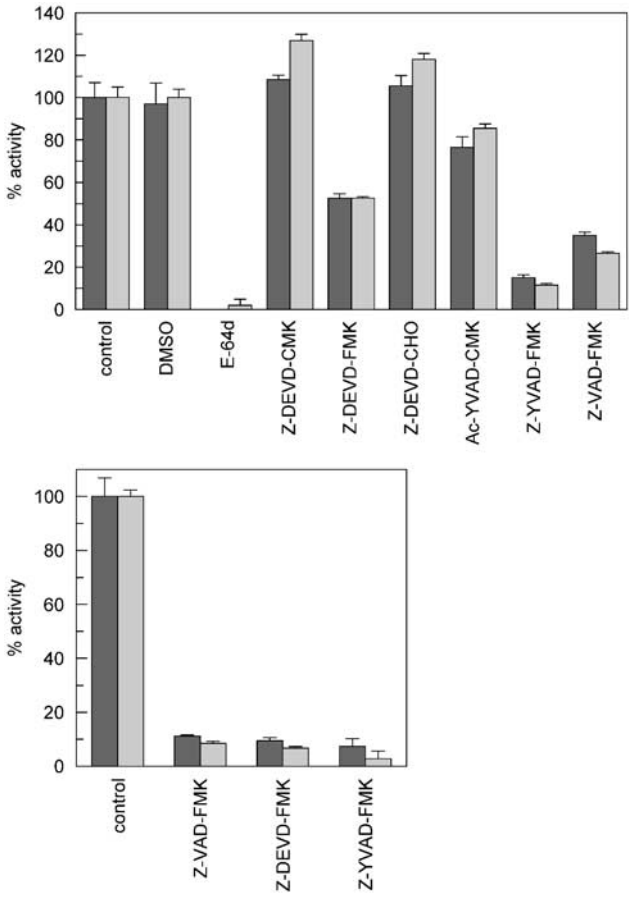

Figure 2 Inhibition of lysosomal cathepsins by caspase inhibitors in Jurkat-T cells (**a**) and HEK-293 cells (**b**). Filled column, Z-FR-AMC; hatched column, Z-RR-AMC. Error bars represent standard errors calculated on the basis of three different experiments. Experimental conditions were described in the Materials and Methods section. Concentration of caspase inhibitors was 100 μM, whereas E-64 was 5 μM

Selective inhibition of cathepsins in tissue extracts

Experiments using Z-FR-AMC and Z-RR-AMC suffer from the fact that these substrates are nonselective and can potentially be cleaved by multiple cathepsins as well as other proteases within the cell.[22] Therefore, an additional experiment was performed with ^{125}I-DCG-04, an active site-directed probe that can be used to label specific cathepsin proteases in crude protein extracts. This reagent has been used previously to identify several cathepsins (cathepsins B, X, H and C/J) in whole-cell extracts from rat liver.[23,24] Furthermore, this reagent covalently tags papain family cysteine proteases in an activity-dependent manner and can be used to assess potency of inhibitors for specific protease targets using competition experiments.[23–25] Z-DEVD-fmk, Z-YVAD-fmk, Z-VAD-fmk and E-64 were added to crude liver homogenates over a range of concentrations. Residual active cathepsins (identified based on the previously reported labeling profile; Greenbaum *et al*.[23]) were then labeled by the addition of ^{125}I-DCG-04. Loss of labeling of a protease by ^{125}I-DCG-04 upon addition of capase inhibitor indicates prior inhibition of that protease by the caspase reagent. As can be seen from Figure 3, the *bona fide* cathepsin inhibitor E-64 completely inhibited all cathepsins at concentrations as low as 1 μM. The caspase inhibitors Z- DEVD-fmk and Z-YVAD-fmk proved to be very efficient inhibitors of cathepsin X (IC_{50}=0.63 and 0.65 μM) and cathepsin B (IC_{50}=0.62 and 1.4 μM). In contrast with other *in vitro* studies, Z-VAD-fmk also inhibited both cathepsins X and B very efficiently with IC_{50} values of 1.9 (cathepsin X) and 3.1 μM (cathepsin B). Cathepsins C and J were only poorly inhibited by Z-DEVD-fmk and Z-YVAD-fmk (IC_{50} ~ 100–170 μM), whereas almost no inhibition of cathepsin H by any of the two inhibitors was observed (IC_{50}>200 μM).

Discussion

Since caspases exhibit exquisite specificity for P1 Asp-containing substrates,[3] several classes of 'caspase-specific' inhibitors have been developed.[6] These reagents are all designed based upon a specific tetrapeptide recognition sequence fused to the cmk or fmk reactive groups, resulting in inhibitors that were initially thought to be nonreactive towards other classes of proteases. However, we have shown that not only cathepsin B,[10–13] but also most other cathepsins (L, V, S, K, F, X), as well as papain and legumain, can be inhibited by common 'caspase-specific' inhibitors. With the exception of papain, all other enzymes studied were much more potently inhibited by chloromethylketones than by fluoromethylketones (Table 1), in agreement with previous results on other halomethylketone inhibitors (reviewed in Shaw[7]). Among chloromethylketones, Z-DEVD-cmk was preferred over Ac-YVAD-cmk (2–15-fold) by most of the enzymes except cathepsin B and papain. Although the data for the esterified fluoromethylketones are less complete, the same pattern was observed: Z-DEVD-fmk was preferred over Z-YVAD-fmk. On the other hand, nonesterified Z-VAD-fmk was the worst inhibitor of all the enzymes investigated. However, all the inactivation rate constants are substantially lower for the cathepsins than for the caspases (Table 1,[5,26]), indicating a significant preference for the caspases.

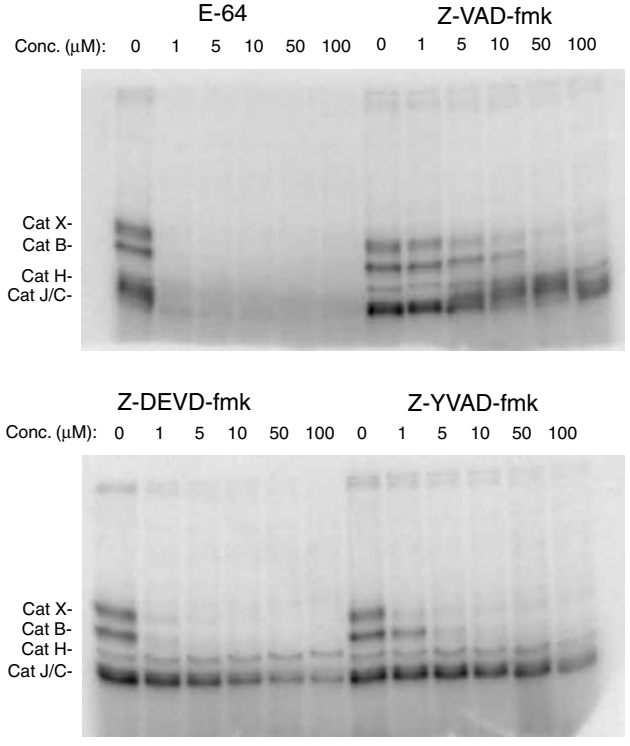

Figure 3 Inhibition of cathepsins B, X, H and C in rat liver extracts by E-64, Z-DEVD-fmk, Z-YVAD-fmk and Z-VAD-fmk. Caspase inhibitors were added to total cell extracts followed by addition of the radiolabeled probe ^{125}I-DCG-04. The ability of each caspase inhibitor to bind specific cathepsin targets was reflected in its ability to block labeling by the active site-directed probe. Loss of labeling therefore directly correlates with inhibition by a given caspase inhibitor. IC_{50} values were determined by quantitation of labeled bands and calculation of the concentration at which labeling was reduced by half

Structural comparison showed that the only common feature of the active sites of cathepsins and the caspases is the reactive site cysteine residue. Even the reactive site histidine residues play different roles: in papain-like proteases, the catalytic histidine residue forms an ion pair with the catalytic cysteine residue,[14] whereas in caspases it forms a part of the oxyanion hole.[27] This is consistent with the inability of papain-like proteases to degrade caspase-specific substrates and indicate that for the interaction of the 'caspase' inhibitors with the papain-like enzymes the reactivity of the cmk, fmk and aldehyde groups was crucial.

Studies on cell cultures confirmed the *in vitro* results. All the three fluoromethylketone inhibitors used (Z-DEVD-fmk, Z-DEVD-fmk and Z-VAD-fmk) efficiently inhibited most of the cathepsin activity in two different cell lines (Jurkat and HEK-293) at 100 μM concentrations. Lowering the concentration of fmk inhibitors to 20 μM resulted in a decreased inhibition of papain-like proteases, whereas shortening the incubation time to 6 h had no effect on inhibition (data not shown). Despite poor *in vitro* efficacy, Z-VAD-fmk was found to be a potent inhibitor of the papain-like proteases in intact cells, almost comparable to Z-YVAD-fmk. However, inhibition of papain-like proteases in the two cell lines used was slightly different, in agreement with previous results showing complete inhibition of Z-RR-ase activity in WEHI-164 cells already

Table 2 Kinetics of inhibition of papapin-like cysteine proteases by Z-DEVD-CHO and Ac-YVAD-CHO

Ki (μM)	Z-DEVD-CHO	Ac-YVAD-CHO
Papain	0.53	0.24
Cathepsin B	16.0	4.8
Cathepsin V	1.5	1.7
Cathepsin H	–	40.0

The K_i values were determined by the method of Greco and Hakala.[27] Experimental conditions were as described in Materials and Methods

at 50 μM concentration of Z-VAD-fmk.[10] This is also consistent with different expression profiles of various cathepsins in different cell types.[22,28] One should consider that Z-FR-ase and Z-RR-ase activities in cells mainly reflect cathepsin B activity because of its abundance, ubiquitous distribution, lower susceptibility to endogenous cystatin inhibitors and increased pH stability when compared to cathepsin L. This can also explain why Z-DEVD-fmk was a less efficient inhibitor than Z-YVAD-fmk in the cells.

Despite much better *in vitro* characteristics, all the -cmk inhibitors failed to inhibit papain-like cysteine proteases in whole cells, probably due to limited cell permeability, consistent with previous results on WEHI-164 cells and lack of cathepsin B inhibition by Ac-YVAD-cmk.[10] The lack of inhibition by the aldehydes was probably a result of a much lower affinity of this type of inhibitors for papain-like proteases (Table 2).

Studies in tissue extracts also corroborated *in vitro* results. Cathepsins B and X, which were efficiently inactivated *in vitro* (Table 1), were also very efficiently inactivated in the mixtures containing the inhibitors at concentrations below 1–10 μM, depending on the inhibitor (Z-DEVD-fmk, Z-YVAD-fmk, Z-VAD-fmk). In contrast, aminopeptidases cathepsins H and C activities were virtually unaffected even at the highest concentration of inhibitor used (100 μM).

In order to inhibit caspases more selectively, or to exclude the involvement of papain- like proteases in various models of cell death, one should modify the current strategies. One such approach is to design more specific inhibitors of caspases. Alternatively, existing inhibitors, preferentially aldehydes, can be used at a lower concentration. Both approaches can benefit from additional control experiments in which involvement of papain-like proteases in the specific cell death pathway could be excluded or confirmed by the use of more specific inhibitors of papain-like proteases, such as E-64, CA-074 or Z-FA-fmk, which do not crossreact with caspases. The latter approach has already been successfully applied in two different models of cell death.[10,13] However, even these inhibitors are not as specific as generally believed. E-64, originally discovered as an inhibitor of papain-like cysteine proteases,[18,29] was also shown to inhibit calpain, although with an order of magnitude lower rate constants of inactivation ($k_2/K_1 \geq 80\,000$/M/s *versus* $k_2/K_1 \sim 8000$/M/s;[8,30,31]), and even a serine protease trypsin, although by a different, reversible mechanism with a K_i value of 0.3 μM.[32] CA-074, a cathepsin B-specific E-64 analogue,[19] failed when it came to the specificity in *in vivo* studies. Namely, its esterified cell-

permeable analog, CA-074-OMe, was shown to inhibit several cysteine proteases including cathepsin S, probably because of incomplete removal of the ester group within the cells.[33] This also suggests that the esterified caspase inhibitors such as Z-DEVD-fmk may still contain partially esterified carboxylic groups of aspartic and glutamic acid side chains and may as such not inhibit caspases but some other proteases. On the other hand, Z-FA-fmk does not inhibit aminopeptidases such as cathepsin H, which are also only poorly inhibited by E-64. The role of papain-like cysteine proteases can also be addressed by overexpressing their endogenous intracellular protein inhibitor, stefin A.[34] Stefin A is preferred over stefin B because of its more efficient inhibition of cathepsin B (reviewed in Turk *et al*.[35]), although the lack of the latter was shown to be responsible for cerebellar apoptosis in stefin B knockout animals.[36] In any case, results obtained using small peptidyl inhibitors should be interpreted with extreme care, preferentially including control experiments with cathepsin inhibitors.

Materials and Methods

Substrates and inhibitors

Most of the compounds were obtained from Bachem (Bubbendorf, Switzerland). These included cathepsin substrates Z-RR-AMC, Z-FR-AMC, R-AMC and GF-4MβNA, caspase substrates Ac-DEVD-AFC and Ac-WEHD-AMC, legumain substrate Bz-N-pNA, caspase inhibitors Z-DEVD-cmk, Ac-YVAD-cmk, Z-DEVD-CHO, Ac-YVAD-CHO and Z-VAD-fmk. (Bz=benzoyl, pNA= *p*-nitroanilide) Caspase inhibitors Z-DEVD-fmk and Z-YVAD-fmk, purchased from Enzyme Systems Products (Dublin, CA, USA), have all the acidic side chains methylated in order to enhance their cell permeability. Cathepsin inhibitors E-64, E-64d and CA-074 were from Peptide Research Institute (Osaka, Japan). The specific cathepsin X substrate Dnp-GFFW-OH[37] was a kind gift of Dr. Luiz Juliano (University of Sao Paulo, Brazil)(Dnp=2,4-dinitrophenyl). ^{125}I-DCG-04 was prepared as described previously.[23] The stock solutions of substrates and inhibitors were made in dimethylsulfoxide (Merck, Darmstadt, Germany).

Enzymes

Papain (2 $\times$ crystallized) was purchased from Sigma (St. Louis, USA) and further purified by affinity chromatography as described by Blumberg *et al*.[38] Other enzymes were either purified from various sources (human cathepsin C,[39] porcine cathepsin H,[40] human cathepsin X[37]and porcine legumain[41]), or expressed in *E. coli* (human cathepsin B,[42] human cathepsin L[43] and murine caspases 1, 3, 6, 7, 8 and 11[44,45]) or in *P. pastoris* (human cathepsin F,[46] human cathepsin K[47] and human cathepsin V).[48] Papain-like cysteine proteases were active-site titrated by E-64, stefin A[49] or cystatin C[39] and caspases by Z- VAD-fmk.[50] All the enzymes were 20 (cathepsin L) to 100% active (papain, cathepsin B) and active concentrations of the enzymes were used throughout.

Buffers

0.1 M phosphate buffer, pH 6.0, was used for cathepsins B, C, K and papain, 0.1 M acetate buffer pH 5.5, for cathepsins F, L, V and X, and 0.1 M phosphate buffer, pH 6.5, for cathepsin H. All buffers contained also 1 mM EDTA, 1 mM dithiothreitole and 0.1% (w/v) polyethyleneglycol. In addition, cathepsin C buffer contained 0.1 M NaCl. Legumain was assayed in citrate/phosphate buffer (39.5 mM citric acid/121 mM Na_2HPO_4), pH 5.8, containing 1 mM EDTA and 0.1% (w/v) CHAPS.[41] All the caspases (caspases 1, 3, 6, 7, 8 and 11) were assayed in 50 mM HEPES buffer, pH 7.4, containing 0.1 M NaCl, 0.1% (w/v) CHAPS, 10% (w/v) sucrose, 1 mM EDTA and 10 mM dithiothreitole.[50] Activating buffers for all the enzymes consisted of 10 mM dithiothreitole in the assay buffer, except for caspases where the assay buffer was also used as the activating buffer. Dimethylsulfoxide concentration was less than 5% throughout all assays.

Cells

Jurkat T cells (ATCC, CCL TIB 152) were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). HEK-293 cells (ATCC CRL-1573) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (50 μg/ml).

Substrate hydrolysis

All kinetic measurements were performed at 25°C. Reactions were started by the addition of 10–30 μl of each enzyme, activated for 5 min in the activating buffer, to the 20 μM substrate solution in the assay buffer (final volume 2.5 ml). Final enzyme concentration was 0.1–300 nM, depending on the enzyme and type of experiment. Fluorescence of the released product was monitored continuously for 10 min in the C-61 fluorimeter (Photon Technology International, USA). In the case of AMC substrates, the excitation and emission wavelengths were set to 370 and 460 nm, respectively, whereas in the case of AFC substrates excitation and emission wavelengths were set to 400 and 505 nm, respectively. Measurements of 4-methoxy-βNA were performed at an excitation wavelength of 335 nm and emission wavelength of 415 nm, and measurements of the liberated tryptophane from Dnp-GFFW were performed at excitation and emission wavelengths of 280 and 340 nm, respectively (βNA=β-naphthylamide).

Inhibitor screening

In the initial inhibitor screening, activated enzymes (final concentrations 0.01–5 μM) were incubated with the inhibitors (final concentration 0.1–200 μM, depending on the inhibitor) in a final reaction volume of 50 μl. After 40–120 min of incubation, reaction mixtures were added to 2.45 ml of 20 μM appropriate substrate solution (Ac-DEVD-AFC for caspases 3, 6, 7 and 8, Ac-WEHD-AMC for caspases 1 and 11, Z-FR-AMC for papain and cathepsins L, S, K, V and F, Z-RR-AMC for cathepsin B, R-AMC for cathepsin H, Dnp-GFFW for cathepsin X, GF-4-methoxy-βNA for cathepsin C). Fluorescence of the released product was then measured for 2 min as described above. In the case of legumain, 100 μM Bz-N-pNA was used as a substrate and the absorbance of the released product was measured spectrophotometrically at 410 nm in a Perkin-Elmer λ-18 spectrophotometer (USA). The residual activities were calculated from the slopes of the lines. In the control experiments inhibitors were omitted.

Kinetics of inhibition

The kinetics of the reaction between irreversible caspase inhibitors Z-DEVD-cmk, Ac-YVAD-cmk, Z-VAD-fmk, Z-DEVD-fmk and Z-YVAD-fmk, and cathepsins B, C, F, H, K, L, S, V, X, papain and legumain were analyzed by continuous measurements of the loss of enzymatic activity in

the presence of substrate under pseudo-first-order conditions with at least 10-fold molar excess of inhibitor. Inhibitor in increasing concentrations (0.03–100 μM final concentration) and the appropriate fluorogenic or chromogenic substrate (20 or 100 μM; see above) were mixed in a cuvette with appropriate buffer (see above) to a final volume of 2.47 ml. The reaction was started by the addition of 30 μl of activated enzyme solution and the release of product was monitored continuously by a C-61 spectrofluorimeter (Photon Technology Instruments, USA) or by a Perkin-Elmer λ-18 spectrophotometer (USA). Less than 3% of the substrate was hydrolyzed during the experiments throughout. In all, 6–10 different inhibitor concentrations were used with each enzyme.

When the reaction was too slow to be measured by continuous measurements, a different approach was used. A volume of 10–20 μl of activated enzyme were added to 480–490 μl of appropriate buffer, containing increasing concentration of inhibitor (5–300 μM), to a final concentration of 44 nM–1.5 μM (depending on the enzyme), ensuring that the enzyme : inhibitor molar ratio never exceeded 1 : 10. After appropriate time intervals, 20 μl aliquots were taken for the determination of residual activity. The aliquots were added to 2.48 ml of the same buffer solution, containing also 10 μM substrate (see above for the list). Product release was then continuously measured for 1 min as described above. The residual activities were calculated from the slopes of the lines. In a set of separate experiments, enzymes were tested to be stable for the duration of measurement. Each enzyme was tested with at least six different concentrations of inhibitors.

Interaction between an enzyme and its irreversible inactivator is normally a two-step reaction. The first step is typically reversible and diffusion controlled, whereas the second (slow) step is the irreversible (covalent) modification.[7] Therefore, all the inactivation rate constants are represented as k_2/K_1.

Equilibrium kinetics

Interaction between reversible caspase inhibitors Z-DEVD-CHO and Ac-YVAD-CHO, and cathepsins B, H, V and papain were evaluated by equilibrium measurements. Briefly, activated enzyme (1–7.2 nM final concentration) was mixed with increasing concentration of inhibitor (0–10 μM) and incubated in a final volume of 1.25 ml for 45 min, which was shown to be sufficient for >98% completion of the reaction. Reaction mixtures were then added to 1.25 ml of 20 μM substrate solutions and the release of product was measured fluorimetrically for 1 min (see above). All experiments were performed in triplicate.

Activity measurements in cell extracts

Cells were incubated with a set of inhibitors (E-64d, Z-DEVD-cmk, Z-DEVD-fmk, Z- DEVD-CHO, Ac-YVAD-cmk, Z-YVAD-fmk, Ac-YVAD-CHO and Z-VAD-fmk) at a final concentration of 100 μM for 24 h if not otherwise specified. The only exception was E-64d, the cell-permeable form of E-64, which was used at a final concentration of 5 μM. Dimethylsulfoxide never exceeded 1% (v/v). Cells were then harvested as follows: HEK-293 cells were trypsinized, followed by centrifugation at 250 $\times$ g for 10 min, whereas Jurkat-T cells were only centrifuged under the same conditions. Pellet was rinsed with cold PBS buffer. Excessive PBS buffer was removed, followed by the addition of 500 μl cold 50 mM HEPES buffer, pH 7.0, containing 0.25 M NaCl and 0.1% (v/v) NP-40. After 5 min of incubation on ice, samples were centrifuged for 2 min. Supernatant was then transferred to a fresh Eppendorf tube and pellet discarded.

In all, 100 μl of cell extract (supernatant) was mixed with 10 μl 100 mM DTT, incubated for 5 min at 37°C and then added to 2.4 ml of 30 μM substrate Z-Phe-Arg-AMC or Z-Arg -Arg-AMC in 100 mM phosphate buffer, pH 6.0. Release of AMC was monitored continuously in a C-61 fluorimeter for 2 min as described above. Control experiments were performed in the absence of inhibitors and in the absence and presence of 1% (v/v) dimethylsulfoxide, which served as a negative control. All the experiments at the same inhibitor concentration were performed in triplicate.

Competitive inhibition assays

Rat liver homogenates (100 μg in 100 μl buffer 50 mM acetate pH 5.5, 5 mM $MgCl_2$, 2 mM DTT) were incubated with increasing amounts of inhibitors (E-64, Z-VAD-fmk, Z-DEVD- fmk and Z-YVAD-fmk; 0–100 μM final concentration) for 30 min at 25°C. ^{125}I-DCG-04 (1×10^6 c.p.m./sample) was then added followed by an additional 30 min incubation at 25°C, samples were separated by SDS-PAGE and analyzed by autoradiography as described previously.[23]

Acknowledgments

This work was supported by the Ministry of School, Science and Sports of the Republic of Slovenia (BT, DT, VT), by the Interuniversitaire Attractiepolen V, the Fonds voor Wetenschappelijk Onderzoek Vlaanderen (Grant 3G.0006.01 to PV), the Bijzonder Onderzoeksfonds (PV), and an EC-RTD Grant QLRT-1999-00739 (PV), by Sandler Program in Basic Sciences (MB) and by the NIH Grant AR 46182 (DB).

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