# Antiviral activity of caspase inhibitors: effect on picornaviral 2A proteinase

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**Abstract Peptide-based fluoromethyl ketones have been considered for many years to be highly specific caspase inhibitors distinctly blocking the progress of apoptosis in a variety of systems. Here we demonstrate that these compounds can significantly reduce rhinovirus multiplication in cell culture. In their methylated forms they block eIF4GI cleavage in vivo and in vitro and inhibit the activity of picornaviral 2A proteinases.**



*Key words:* Rhinovirus; Picornavirus; 2A proteinase; eIF4GI cleavage; Caspase inhibitor; Apoptosis

## 1. Introduction

Human rhinoviruses (HRVs) and enteroviruses are small, (+)-strand RNA viruses which belong to the Picornaviridae family. A crucial step in the life cycle of picornaviruses is the processing of the polyprotein precursor synthesized from a single open reading frame. HRVs and enteroviruses encode two proteinases designated 2A and 3C which are responsible for polyprotein processing. The first cleavage is catalyzed by the 2A proteinase ($2A^{pro}$) separating the viral capsid proteins from the non-capsid proteins [1,2]. In terms of structure these viral proteinases are unique as they resemble serine proteinases with a typical chymotrypsin-like fold but harbor Cys rather than Ser as an active site residue [3].

Cells frequently undergo apoptosis when infected with representatives of Picornaviridae such as poliovirus and coxsackievirus (CV) [4–9]. Caspases act as central mediators in apoptosis participating in both signaling and effector pathways [10,11]. They are typical cysteine proteinases cleaving the substrate usually at specific aspartate residues in the P1 position [12,13]. Caspases are highly conserved throughout evolution and are structurally unrelated to viral 2A and 3C proteinases [14,15].

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*Abbreviations:* HRV2, human rhinovirus serotype 2; CV, coxsackievirus; $2A^{pro}$, 2A proteinase; pNA, *para*-nitroanilide; eIF, eukaryotic initiation factor; zVAD.fmk, benzyloxycarbonyl-Val-Ala-Asp(OMe) fluoromethyl ketone; zIETD.fmk, benzyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp (OMe) fluoromethyl ketone; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; p.i., post infection; $TCID_{50}$, 50% tissue culture infectious dose

Several fluoromethyl ketone (fmk)-derivatized peptides have been developed as inhibitors of caspases [16,17]. Fmk acts as a trapping group which upon covalent binding to –SH of an adjacent cysteine residue causes irreversible inhibition. These inhibitors are known to prevent apoptosis in various cell lines without major cytotoxic side effects [18]. In this study we demonstrate that caspase inhibitors benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone (zVAD.fmk) and benzyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone (zIETD.fmk) inhibit HRV multiplication in HeLa cells. These compounds also block eukaryotic initiation factor (eIF) 4GI cleavage in vivo and in vitro and significantly affect the viral $2A^{pro}$ activity in vitro.

## 2. Materials and methods

### 2.1. Media, reagents, chemicals

The caspase inhibitors zVAD.fmk, unmethylated zVAD.fmk, and zIETD.fmk were purchased from Enzyme Systems Products (Livermore, CA, USA) and from Calbiochem (La Jolla, CA, USA). Rabbit anti-eIF4GI was supplied by Dr. R. Rhoads (Louisiana State University, Shreveport, LA, USA). TRPIITTA-*para*-nitroanilide (pNA) was purchased from piChem (Graz, Austria) [19]. Human cervix carcinoma HeLa cells (strain Ohio, European Collection of Cell Cultures, Salisbury, UK) were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Eggersheim, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% $CO_2$.

### 2.2. Infection of cells with HRV2

HRV2 (American Type Culture Collection) was grown in suspension cultures of HeLa cells and purified as described previously [20]. Virus titers were determined as 50% tissue culture infectious doses ($TCID_{50}$/ml) according to Reed and Muench [21]. HeLa cells were infected at 80–90% confluence in minimal essential medium (MEM) (Gibco) containing 2% FCS, 2 mM L-glutamine, 30 mM $MgCl_2$, 100 U/ml penicillin, and 100 μg/ml streptomycin with purified HRV2 at a multiplicity of infection of 100 [20]. One hour post infection (p.i.) non-incorporated virus was removed by washing three times with MEM. Infected HeLa cell cultures were incubated for a total of 12 h and the $TCID_{50}$ was determined.

### 2.3. Treatment of cells with caspase inhibitors

Stock solutions of caspase inhibitors (100 mM in dimethyl sulfoxide, DMSO) were diluted in MEM so that the final content of DMSO never exceeded 0.2%. 200 μM zVAD.fmk and 100 μM zIETD.fmk were added to cells at the times indicated.

### 2.4. Labeling of viral proteins

HeLa cells were infected with HRV2 as above in DMEM (lacking L-methionine and L-cysteine) in the presence of 200 μM zVAD.fmk and 100 μM zIETD.fmk. Four hours p.i. medium was supplemented with fresh solutions of inhibitors and 5 h p.i. [$^{35}$S]methionine was added to a final concentration of 100 μCi/ml. Twelve hours p.i. cells

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were harvested and solubilized directly in Laemmli sample buffer. Samples were subjected to 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and analyzed by autoradiography.

*2.5. Inhibition of eIF4GI cleavage in vivo and in vitro*

For in vivo eIF4GI cleavage HeLa cells were infected with HRV2 as above in the presence of 200 μM zVAD.fmk and 100 μM zIETD.fmk. Fresh inhibitors were added after 4 h. Eight hours p.i. cells were solubilized in Laemmli sample buffer, samples were subjected to 6% SDS–PAGE and analyzed by immunoblotting using a polyclonal antibody against the N-terminus of eIF4GI. Detection of inhibition of in vitro eIF4GI cleavage was performed by incubation of 9 μg of HeLa cell cytoplasmic protein extracts [22] with 20 ng of purified HRV2 $2A^{pro}$ in buffer A (100 mM NaCl, 25 mM Tris–HCl, 1 mM EDTA, 5 mM dithiothreitol, pH 8). Cleavage reaction was carried out at 30°C in the presence and in the absence of 15 μM zVAD.fmk, 15 μM of unmethylated zVAD.fmk or 15 μM zIETD.fmk. After 4 h the incubation was terminated by adding Laemmli sample buffer. Samples were analyzed by 6% SDS–PAGE and Western blotting.

*2.6. In vitro analysis of the inhibition of HRV2 VP1-$2A^{pro}$ self-processing activity*

Plasmid preparation of pHRV2 VP1-$2A^{pro}$, two-step in vitro transcription and in vitro translation were employed as described [23]. After preincubation for 2 min at 30°C and 10 min of protein synthesis, inhibitors were added to a final concentration of 200 μM. Control reactions contained DMSO (0.2% v/v) only.

*2.7. Inhibition of HRV2 $2A^{pro}$ activity*

Recombinant HRV2 $2A^{pro}$ was purified as described previously [24]. Activity of the enzyme was detected in the colorimetric assay using the HRV2 $2A^{pro}$-specific chromogenic peptide substrate TRPIITTA-pNA derived from the C-terminus of VP1 of the cleavage site in the viral polyprotein precursor [19,25]. The HRV2 $2A^{pro}$ activity assay was performed at 25°C in 100 μl of buffer A with 1 μg of $2A^{pro}$ and was initiated by the addition of 0.25 μM TRPIITTA-pNA. Absorbance was continuously monitored for 20 min at $\lambda = 405$ nm against a blank containing no enzyme. To test inhibitory activities 50 μM each of zVAD.fmk, unmethylated zVAD.fmk and zIETD.fmk were added to the reaction mixture just before addition of the substrate.

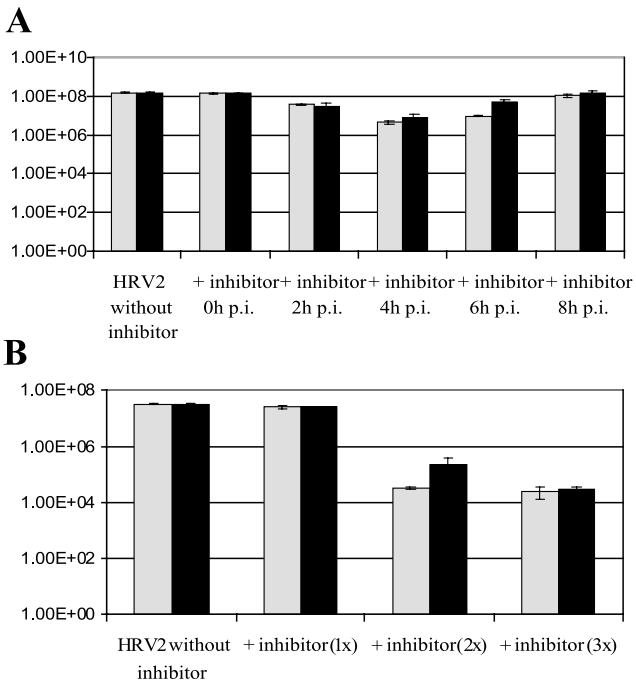


Fig. 1. Effect of zVAD.fmk and zIETD.fmk on HRV2 multiplication. A: Virus titers were determined 12 h p.i. of HeLa cells infected with HRV2 in the absence or in the presence of 200 μM zVAD.fmk (gray bars) or 100 μM zIETD.fmk (black bars) added either at the time of infection (0 h p.i.) or 2 h, 4 h, 6 h, 8 h p.i. as indicated. For the determination of the standard deviations the experiment was repeated three times under the same conditions. B: Virus titers were determined 12 h p.i. of HeLa cells infected with HRV2 in the absence or in the presence of 200 μM zVAD.fmk (gray bars) or 100 μM zIETD.fmk (black bars) supplemented either once, twice or three times during infection (at 0, 4 and 8 h p.i., respectively) as indicated. For the determination of the standard deviations the experiment was repeated three times under the same conditions.

## 3. Results

*3.1. Caspase inhibitors affect HRV2 multiplication*

The influence of caspase inhibitors on apoptosis induced by poliovirus and CV has been described previously [4–9]. Similarly apoptosis was blocked by caspase inhibitors when HeLa cells were infected with HRVs (data not shown). To investigate the effect on HRV2 multiplication, HeLa cells were infected in the presence and in the absence of the caspase inhibitors (Fig. 1A). 200 μM zVAD.fmk and 100 μM zIETD.fmk were added at the indicated times of infection and the total virus titers were determined 12 h p.i. It was observed that addition of the caspase inhibitors not only reduced progress of apoptosis (data not shown), but also affected HRV multiplication. The extent of suppression of virus production was dependent on the time of addition of inhibitors. Virus titers were not changed significantly when the caspase inhibitors were added at the time of infection. However, there was a progressive inhibition of virus multiplication when the substances were added up to 4 h p.i. Under these conditions virus titers were reduced by about 50-fold at 200 μM zVAD.fmk and by 25-fold at 100 μM zIETD.fmk (Fig. 1A). Addition of inhibitors at later times had only a very moderate effect. Adding inhibitors at 8 h p.i. did not cause any significant reduction in virus titers.

In order to further investigate the inhibitory effect of zVAD.fmk and zIETD.fmk, a similar experiment was performed in which caspase inhibitors were added once, twice and three times at 4-h intervals during HRV2 infection (Fig. 1B). When inhibitors were added at the time of infection and again at 4 h p.i., the inhibitory effect on virus multiplication was much more pronounced. Further addition of inhibitors at 8 h p.i. decreased virus titers by about 2000-fold for zVAD.fmk and 1000-fold for zIETD.fmk as determined 12 h p.i. However, without further addition of caspase inhibitors there was almost full recovery of virus yield after 24 h of incubation (data not shown). Thus, inhibition of HRV multiplication by these caspase inhibitors is obviously a transient phenomenon.

*3.2. zVAD.fmk and zIETD.fmk inhibit HRV protein synthesis*

To check whether the reduction in HRV production in HeLa cells was caused by inhibition of viral synthesis or by a block in viral maturation, we performed $^{35}S$-labeling experiments of viral proteins in the presence and in the absence of the caspase inhibitors. It was found that both caspase inhibitors blocked synthesis of HRV2 capsid proteins whereas cellular protein synthesis was not affected significantly (Fig. 2A). In the presence of these inhibitors there was obviously no host cell shut-off of cellular protein synthesis.

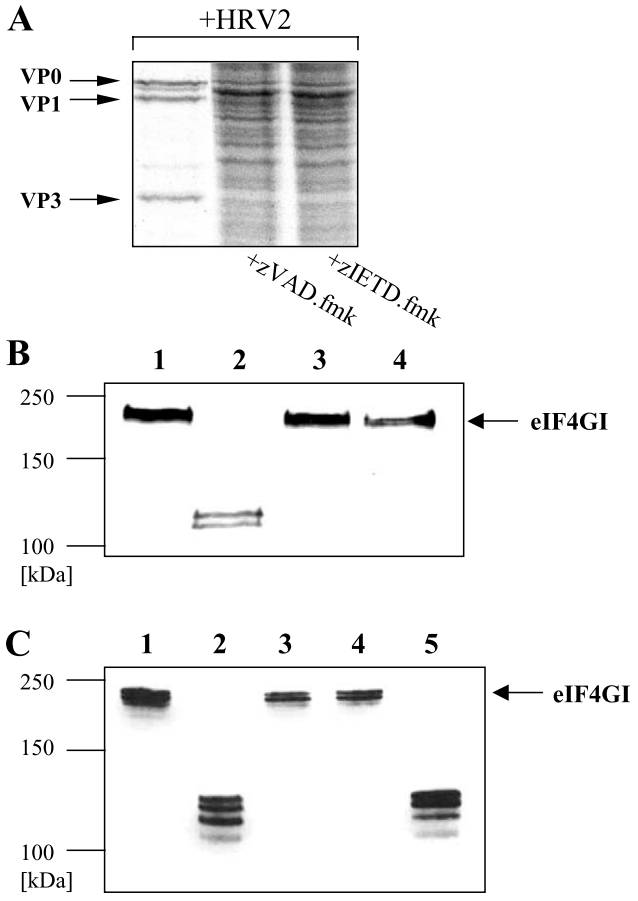


Fig. 2. A: Influence of caspase inhibitors on synthesis of rhinovirus structural proteins. HeLa cells were infected with HRV2 for 12 h in the absence or in the presence of 200 μM zVAD.fmk or 100 μM zIETD.fmk. The inhibitors were added at the time of infection and 4 h p.i. Autoradiogram of 12.5% SDS-PAGE showing synthesis of $^{35}$S-labeled proteins. (B) In vivo and (C) in vitro inhibition of eIF4-GI cleavage by caspase inhibitors. B: HeLa cells were mock-infected (lane 1) or were infected with HRV2 for 8 h in the absence (lane 2) or in the presence of 200 μM zVAD.fmk (lane 3) or 100 μM zIETD.fmk (lane 4). The inhibitors were added at the time of infection and 4 h p.i. C: HeLa cell protein extract (lane 1) was incubated at 30°C for 4 h with HRV2 2A$^{pro}$ in the absence (lane 2) or in the presence of 15 μM zIETD.fmk (lane 3), 15 μM of the methylated form of zVAD.fmk (lane 4) or 15 μM of the unmethylated form of zVAD.fmk (lane 5). Samples were analyzed in 6% SDS–PAGE and by Western blotting using polyclonal antibody against eIF4GI.

### 3.3. *Caspase inhibitors block eIF4GI processing in vivo and in vitro*

Shut-off of cellular protein synthesis is caused by direct 2A$^{pro}$ cleavage of eIF4GI and II which leads to a stop in the cap-dependent initiation of translation [26,27]. eIF4GI and II act as scaffolding proteins in the eIF4F initiation factor complex which comprises among others the cap binding protein eIF4E, the helicase eIF4A and eIF3, which mediates small ribosomal subunit binding [28,29]. In addition, eIF4G also provides binding sites for poly(A) binding protein and for mnk-1 kinase [30]. Cleavage of eIF4G by 2A$^{pro}$ releases the eIF4E cap binding domain from the eIF4F complex thus preventing recognition of capped mRNAs. In contrast to cellular mRNAs, picornaviral RNAs do not contain a cap but instead protein synthesis is initiated at an internal ribosome entry site. Consequently, picornaviral translation is not inhibited by eIF4G cleavage, but is in fact stimulated [31].

In order to test whether there was a causal relationship between the specific inhibition of viral protein synthesis and eIF4G, the influence of the two caspase inhibitors on in vivo eIF4GI cleavage was examined. HeLa cells were infected with HRV2 in the presence of 200 μM zVAD.fmk or of 100 μM zIETD.fmk. Western blot analysis of the protein extracts revealed that by 8 h p.i. eIF4GI was completely cleaved in control cells whereas in inhibitor-treated cells cleavage of eIF4GI was completely blocked (Fig. 2B).

As 2A$^{pro}$ is responsible for eIF4G cleavage, the effect of zVAD.fmk and zIETD.fmk on in vitro eIF4GI cleavage in HeLa cell extracts was tested using recombinant HRV2 2A$^{pro}$. Indeed eIF4GI cleavage by HRV2 2A$^{pro}$ was already inhibited by 15 μM zVAD.fmk and by 15 μM zIETD.fmk (Fig. 2C). A similar degree of inhibition was observed for CV B4 2A$^{pro}$ indicating that the effect of caspase inhibitors is not restricted to 2A$^{pro}$ of HRV2 (data not shown).

In order to increase stability and cell permeability, zVAD.fmk and zIETD.fmk are generally applied as methyl esters, i.e. as benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone and as benzyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone, respectively [17,32]. After entering the cell the methyl groups are removed by endogenous esterases. However, the kinetics of the demethylation reactions are not known. In order to examine the influence of the presence of the methyl groups on the specificity, the inhibitory potential of zVAD.fmk (benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone) was compared with that of the unmethylated form (benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone) in the in vitro eIF4GI cleavage reaction using recombinant HRV2 2A$^{pro}$. In contrast to the methylated form of zVAD.fmk, the unmethylated form had no inhibitory effect on 2A$^{pro}$ cleavage of eIF4GI in vitro (Fig. 2C). This suggests that under in vivo conditions the methylated form of zVAD.fmk interacts with viral 2A$^{pro}$ which results in inhibition of virus multiplication. As zVAD.fmk becomes gradually demethylated by cellular esterases, the inhibitor loses its affinity for 2A$^{pro}$. As newly synthesized 2A$^{pro}$ is no longer inhibited, viral synthesis is initiated and proceeds normally thereafter.

### 3.4. *zVAD.fmk and zIETD.fmk delay 2A$^{pro}$ self-cleavage*

The first step in the picornaviral polyprotein maturation is catalyzed by 2A$^{pro}$ which cleaves between the C-terminus of VP1 and its own N-terminus. We therefore checked the influence of caspase inhibitors on self-processing of HRV2 2A$^{pro}$ during translation in vitro in rabbit reticulocyte lysates. As indicated in Fig. 3A, self-processing of 2A$^{pro}$ is clearly delayed in the presence of 200 μM of the methylated form of zVAD.fmk as compared to the control. 200 μM zIETD.fmk exhibited a similar inhibitory influence on HRV2 2A$^{pro}$ activity as zVAD.fmk (data not shown). In contrast the unmethylated form of zVAD.fmk did not impair cleavage at all.

### 3.5. *Caspase inhibitors affect activity of HRV2 2A$^{pro}$ as determined with a synthetic substrate*

In order to exclude potential effects of endogenous cellular proteinases which might be present in HeLa cell extracts, the in vitro cleavage experiments were repeated using purified recombinant 2A$^{pro}$ of HRV2 and the chromogenic peptide TRPIITTA-pNA as substrate [19,24]. As shown in Fig. 3B, again a strong inhibition by the methylated forms of

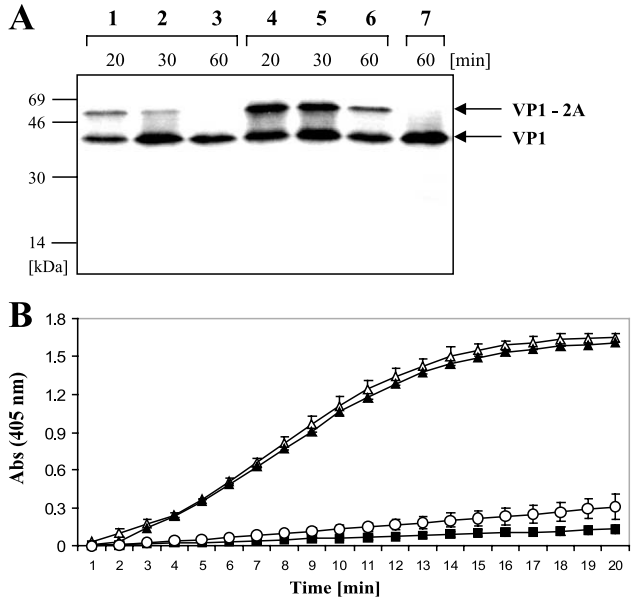


Fig. 3. A: Caspase inhibitors impair HRV2 $2A^{pro}$ self-processing activity. Autoradiogram of in vitro translation product of RNA from pHRV2 VP1-$2A^{pro}$. Ten minutes after the start of protein synthesis inhibitors were added to a final concentration of 200 μM. Subsequently samples were taken at the times indicated and $2A^{pro}$ self-cleavage from VP1 was examined. The reaction of self-processing was performed either in the presence of DMSO alone (0.2%) (lanes 1–3) or in the presence of zVAD.fmk [zVal-Ala-Asp(OMe).fmk] (lanes 4–6) or in the presence of the unmethylated form of zVAD.fmk [zVal-Ala-Asp.fmk] (lane 7). B: Inhibition of HRV2 $2A^{pro}$ in vitro by caspase inhibitors. HRV2 $2A^{pro}$ activity was measured using 0.25 mM TRPIITTA-pNA in the absence (△) or presence of 50 μM zVAD.fmk [zVal-Ala-Asp(OMe).fmk] (■), 50 μM unmethylated zVAD.fmk [zVal-Ala-Asp.fmk] (▲) and 50 μM zIETD.fmk [zIle-Glu(OMe)-Thr-Asp(OMe).fmk] (○). Each time point represents the average of three experiments.

zVAD.fmk and zIETD.fmk on $2A^{pro}$ cleavage activity was observed. The 50% inhibitory concentration values were determined as $5.6\times10^{-6}$ mol/l for zVAD.fmk and $7.7\times10^{-6}$ mol/l for zIETD.fmk, respectively [25]. In contrast, the unmethylated form of zVAD.fmk did not inhibit the cleavage reaction (Fig. 3B). Similar results were obtained for $2A^{pro}$ of CVB4 (data not shown).

## 4. Discussion

Cells infected with viruses frequently respond by induction of apoptosis, which is characterized by activation of caspases. In order to investigate whether there is an influence of apoptotic events on HRV2 multiplication, the caspase inhibitors zVAD.fmk and zIETD.fmk were employed. zVAD.fmk was chosen since it is a general inhibitor of most caspases and zIETD.fmk was employed as an inhibitor of high specificity for caspase 8. Indeed it was observed that depending on the time of addition of these inhibitors a reduction in virus yield was obtained. Similar results were obtained with HRV14 (data not shown).

Fmk-derivatized peptides used as inhibitors of caspases are usually employed as methyl esters. Inside the cell endogenous esterases cause the demethylation of the inhibitors. As demonstrated in this paper the methylated forms of the caspase inhibitors show a significant inhibitory effect on $2A^{pro}$ both in eIF4GI cleavage and in the self-cleavage assay. Apparently the properties of the methylated peptide moieties of zVAD.fmk and zIETD.fmk are not sufficient to confer exclusive specificity for caspases. The cleavage specificity of $2A^{pro}$ of HRV2 has been investigated extensively [33]. The enzyme tolerates a number of different amino acids in the P1 position [13] including large side chains such as in Thr, Leu, Phe, Tyr and Met and basic residues such as in Arg and Lys. However, there is absolutely no cleavage of peptides harboring a negatively charged residue such as Asp or Glu in P1, even when all the other amino acids correspond to the viral polypeptide cleavage site sequence [33]. As seen in the three-dimensional structure, the site on the enzyme binding the P1 residue of the inhibitor is fairly large but contains the carboxylate side chain of Glu102 at the bottom of the pocket [3]. This negative charge probably prevents the Asp side chain of the unmethylated form of zVAD.fmk from entering the pocket due to unfavorable electrostatic interactions. However, in the methylated form of zVAD.fmk the inhibitor can bind to $2A^{pro}$ as the additional methyl residue can be easily accommodated in the binding pocket.

We therefore postulate that the transient nature of the inhibitory effect on virus multiplication can be explained by the gradual decrease in the concentration of methylated zVAD.fmk. As addition of fresh zVAD.fmk results in effective inhibition of newly synthesized $2A^{pro}$, the antiviral effect is strongly enhanced upon repeated addition of the inhibitors. Presently it is not clear whether zVAD.fmk and zIETD.fmk block only $2A^{pro}$ or whether both $2A^{pro}$ and $3C^{pro}$ are inhibited.

The inhibitory activity of zVAD.fmk and zIETD.fmk is presumably not restricted to the HRV system. Indeed a transient inhibition of zVAD.fmk on CV B3 multiplication has been observed recently [7]. Furthermore, an inhibition of the release of CV B3 upon zVAD.fmk treatment was reported [8]. This was attributed to the inhibition of apoptosis by zVAD.fmk as the titer of intracellular CV B3 was increased while that of extracellular virus was decreased. In contrast, in our experimental system total HRV2 yields were determined comprising both intracellular and extracellular virus.

The results of this investigation are not only important for the relationship between viral infection and apoptosis, they are of more general significance. Considering the widespread use of zVAD.fmk and similar fmk peptide derivatives in apoptosis research, it is important to consider that the methylated forms of the inhibitors may exhibit significant reactivity towards –SH-containing enzymes other than caspases. Although specificity for caspases is increased upon demethylation of the inhibitors, the kinetics of demethylation is unknown and may vary between different cells and tissues. Thus, inhibitory effects caused by fmk-based peptide inhibitors do not necessarily reflect an involvement of caspases and can therefore not be considered as proof of apoptosis.

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