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# Q-VD-OPh, a broad spectrum caspase inhibitor with potent antiapoptotic properties

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**In recent years, several inhibitors that prevent caspase activation and apoptosis have emerged. At high doses, however, these inhibitors can have nonspecific effects and/or become cytotoxic. In this study, we determined the effectiveness of broad spectrum caspase inhibitors to prevent apoptosis. A carboxy terminal phenoxy group conjugated to the amino acids valine and aspartate (Q-VD-OPh) potently inhibited apoptosis. Q-VD-OPh was significantly more effective in preventing apoptosis than the widely used inhibitors, ZVAD-fmk and Boc-D-fmk, and was also equally effective in preventing apoptosis mediated by the three major apoptotic pathways, caspase 9/3, caspase 8/10, and caspase 12. In addition to the increased effectiveness, Q-VD-OPh was not toxic to cells even at extremely high concentrations. Our data indicate that the specificity, effectiveness, and reduced toxicity of caspase inhibitors can be significantly enhanced using carboxyterminal o-phenoxy groups and may have important uses *in vivo*.**

*Keywords:* apoptosis; caspase; inhibitors

## Introduction

Apoptosis is an active process of cell death characterized by the lack of an inflammatory response, caspase activation, substrate cleavage, DNA laddering, and the formation of apoptotic bodies.[1] Apoptosis is mediated by specific initiating and effector cysteine proteases (caspases) that are unique in cleaving substrates specifically following aspartate residues.[2–5] The activation of specific caspases has defined three major pathways that can carry out the apoptotic process. Caspase 9 and 3 can be activated by the release of cytochrome c from the mitochondria into the cytosol and this can be triggered by the addition of actinomycin D, etoposide or indirectly by anti-Fas antibody.[6–9] The caspase 8/10 pathway is activated via ligand binding to death receptor systems of the Fas/CD95 and tumor necrosis factor alpha families.[7] Caspase 12 has been shown to become activated in response to thapsigaragin and other endoplasmic reticulum stressors in rodent cells; however, a recent report suggests that caspase 12 may not be functional in human cells.[10–13]

Recent advances have led to commercially available inhibitors that prevent caspase activation. Specific, as well as broad spectrum caspase inhibitors consist of o-methylated monopeptide to tetrapeptide amino acids which enhance cell permeability and are conjugated to carboxyterminal groups such as chloromethyl ketone (cmk), fluoromethyl ketone (fmk), or aldehyde (cho) that enable them to act as competitive inhibitors. These cell permeable inhibitors alkylate the active site cysteine of caspases and irreversibly block apoptosis by preventing caspase activation, substrate cleavage, and DNA ladder formation; however, at high doses these inhibitors have nonspecific or toxic effects.[14–17]

The broad spectrum inhibitor, ZVAD-fmk, can prevent apoptosis of the major pathways at high concentrations and has a preference for the caspase 3 pathway at somewhat lower doses. Boc-D-fmk consists of a single aspartate residue and is capable of preventing apoptosis mediated by any of the three pathways at about one half the effective concentration of ZVAD-fmk. Although these broad spectrum inhibitors have been effective in identifying caspase-mediated events, the relatively high doses required can limit their usefulness in some systems.

In this study, we determined the effectiveness of several, broad spectrum caspase inhibitors to prevent DNA laddering and caspase activation during apoptosis induced via several stimuli. Actinomycin D rapidly induced apoptosis and this was dramatically inhibited by the caspase inhibitor, Q-VD-OPh (quinolyl-valyl-O-methylaspartyl-[-2,6-difluorophenoxy]-methyl ketone). Q-VD-OPh was significantly more effective in preventing apoptosis than the widely used inhibitors, ZVAD-fmk and Boc-D-fmk.

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Q-VD-OPh was also equally effective in preventing apoptosis mediated by the three major apoptotic pathways, caspase 9/3, caspase 8/10, and caspase 12. In addition to the increased effectiveness, Q-VD-OPh was not toxic to cells, even at high concentrations. Our data indicate that the specificity, effectiveness, and reduced toxicity of caspase inhibitors can be significantly enhanced using carboxyterminal o-phenoxy groups.

## Materials and methods

### Materials

The caspase inhibitors, Boc-D-fmk, Z-VAD-fmk, Q-VD-OPh (Quinolyl-valyl-O-methylaspartyl-[2,6-difluorophenoxy]-methyl ketone), Boc-VD-OPh, and Z-VD-OPh were obtained from Enzyme Systems Products, Inc. and resuspended in DMSO as per the manufacturer's instructions. Apoptosis activating anti-Fas, clone CH-11, was purchased from Upstate Biotechnology. TUNEL assays were performed using the APO-BRDU kit (Pharmingen, Inc.) Actinomycin D, thapsigargin, and PARP monoclonal (AM30) antibody were purchased from Calbiochem, Inc. Etoposide was purchased from Sigma, Inc. Alpha II spectrin (mAB 1622) monoclonal antibody was obtained from Chemicon, Inc. Supersignal chemiluminescence reagent was purchased from Pierce, Inc. TGF beta was obtained from Genzyme, Inc and Caspase 3 (CSP3) polyclonal antibody from Idun Pharmaceuticals, Inc. Caspase 8, 9 and 12 polyclonal antibodies were purchased from Santa Cruz Biotechnologies. Beta actin monoclonal antibody was kindly provided by Dr. J. Lessard, Cincinnati Childrens Hospital Medical Center.

### Cell culture

The mouse immature B cell line, WEHI 231 was obtained from ATCC. Human Jurkat T cell lines were kindly provided by Dr. Andrew Larner, Cleveland Clinic Foundation and Dr. F. Monty Hughes, University of North Carolina-Charlotte. The rat placental trophoblast cell line, HRP-1, was kindly provided by Dr. Michael Soares, Kansas University Medical Center. WEHI 231 cells were cultured in RPMI 1640 containing 10% FBS and 29 $\mu$M 2-mercaptoethanol. Jurkat T cells were cultured in RPMI 1640 containing 10% FBS. HRP-1 cells were cultured in 2.5% fetal bovine serum in RPMI 1640. All cells were cultured at 37°C and 95% $O_2$/5% $CO_2$.

### Apoptosis assays

Apoptosis was measured by analysis of an oligonucleosomal ladder in agarose gels as previously reported[5,15,16,18] Vehicle treated or cells induced to undergo apoptosis ($1 \times 10^6$) were collected and lysed in HL buffer (10 mM Tris, pH 8.0, 1 mM EDTA, and 0.1% Triton X-100) for 15 min. The lysate was extracted with an equal volume of phenol and then phenol:chloroform: isoamyl alcohol (25:24:1) and precipitated with an equal volume of isopropanol and 0.1 volume of 5 M NaCl. The precipitated DNA was resuspended in Tris/EDTA, pH 8.0, containing DNase-free RNase A and incubated at 37°C for 30 min. The DNA was separated on a 1.2% agarose gel in 1X TBE containing ethidium bromide.

Apoptosis was quantitated by TUNEL assay using the APO-BRDU kit. Briefly, at the end of each treatment, cells were washed and resuspended for 15 min in ice-cold 1% paraformaldehyde. The cells were further fixed in 70% ethanol and TUNEL was performed according to the manufacturer's instructions prior to analysis by flow cytometry on a BD FacScan. The Cell Quest software program was used to analyze flow cytometry data. Caspase inhibitors were added at the indicated concentrations 30 minutes prior to the addition of apoptotic stimuli. Viability and cell number were determined by trypan blue exclusion from three random fields of greater than 200 cells/field. All experiments were performed a minimum of three times.

### Western blotting

Protein concentrations and Western blotting were performed as described previously.[5,15,16,18] Briefly, whole cell lysates were separated by SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride membrane. Protein transfer was empirically determined by staining with 1.0% Ponceau S in 5% glacial acetic acid.[5,16] The membrane was incubated in blocking buffer (60 mM Tris, 200 mM NaCl with 0.05% Tween 20 containing 5% nonfat dry milk, pH 7.4) and subsequently incubated with a 1/1000 dilution of primary antibody for 2 h at room temperature. Primary antibodies consisted of a caspase 3 polyclonal and a PARP monoclonal. The blot was washed extensively and incubated with a 1/20,000 dilution of rabbit anti-mouse or goat anti-rabbit horseradish peroxidase (Transduction Laboratories) for 1 h at room temperature and processed using the Supersignal chemiluminescence reagent according to manufacturer's instructions.

## Results

Caspase activation is the catalyst needed to carry out the apoptotic process. Caspase inhibitors have been routinely used to identify specific caspases and analyze particular mechanisms involved in different apoptotic pathways. Two of the most widely used caspase inhibitors are the

broad spectrum fluoromethyl ketone caspase inhibitors, Boc-D-fmk (BD) and Z-VAD-fmk. The amino terminal Boc and Z groups serve to block the amino acids D (aspartate) or VAD (Val-Ala-Asp) while the carboxy terminal fluoromethyl ketone facilitates cell permeability.

Actinomycin D has previously been shown to induce caspase activation in WEHI 231 immature cells and this could be prevented by the caspase 3 inhibitor, DEVD-fmk.[16] Actinomycin D has also been reported to release cytochrome c in HeLa cells, implicating activation of the caspase 9/3 apoptotic pathway.[6] To analyze the effects of broad spectrum caspase inhibitors on actinomycin D-induced apoptosis in WEHI 231 cells, DNA fragmentation was analyzed after 4 h, when substantial apoptosis, in the absence of caspase inhibitors, had occurred. Incubation with decreasing doses of ZVAD-fmk, Boc-D-fmk, or Q-VD-OPh in the presence of 1 $\mu$g/ml actinomycin D indicated that each compound exhibited a dose dependent inhibition of apoptosis (Figure 1). ZVAD-fmk, however, was only partially effective at inhibiting DNA laddering at 50 $\mu$M, consistent with what has been observed in other systems (Figure 1A). This result was also confirmed by TUNEL assay and flow cytometry (data not shown). The broad spectrum caspase inhibitor, Boc-D-fmk, completely prevented apoptosis in WEHI 231 cells at 50 $\mu$M, but was ineffective at lower doses (Figure 1B). In striking contrast to ZVAD-fmk and BD-fmk, the caspase inhibitor Q-VD-O-phenoxy (Q-VD-OPh) dramatically prevented DNA fragmentation at concentrations as low as 5 $\mu$M (Figure 1C). Q-VD-OPh uses an aminoterminal quinoline group conjugated to the amino acids valine and aspartate and a carboxyl ester attached to a phenoxy ring. The effect of Q-VD-OPh was also analyzed by TUNEL assay and quantitated by flow cytometry. Actinomycin D alone induced 92% apoptosis in 4 h (Figure 2); whereas, the addition of vehicle had no effect. As little as 1 $\mu$M Q-VD-OPh reduced apoptosis by 22%; whereas, 2.5 $\mu$M reduced actinomycin D-induced apoptosis by 48%. From 5 $\mu$M to 100 $\mu$M, Q-VD-OPh completely protected WEHI

**Figure 1**. Caspase inhibitors inhibit Actinomycin D-induced DNA laddering. Panel A = Z-VAD-fmk. Panel B = Boc-D-fmk. Panel C = Q-VD-OPh. Mouse WEHI-231 immature B cells ($1 \times 10^5$ cells/ml) were treated for 4 hrs with (V) vehicle, (Inh) 50 $\mu$M caspase inhibitor alone, (Act.D) 1 $\mu$g/ml actinomycin D, or caspase inhibitor (at either 1 $\mu$M, 2 $\mu$M, 5 $\mu$M, 10 $\mu$M, 25 $\mu$M, or 50 $\mu$M as indicated) preincubated 1 hr prior to addition of actinomycin. DNA was isolated, separated on a 1.2% agarose gel and stained with ethidium bromide to determine the effective dose of each inhibitor. (M) indicates a 100 bp DNA molecular weight marker.

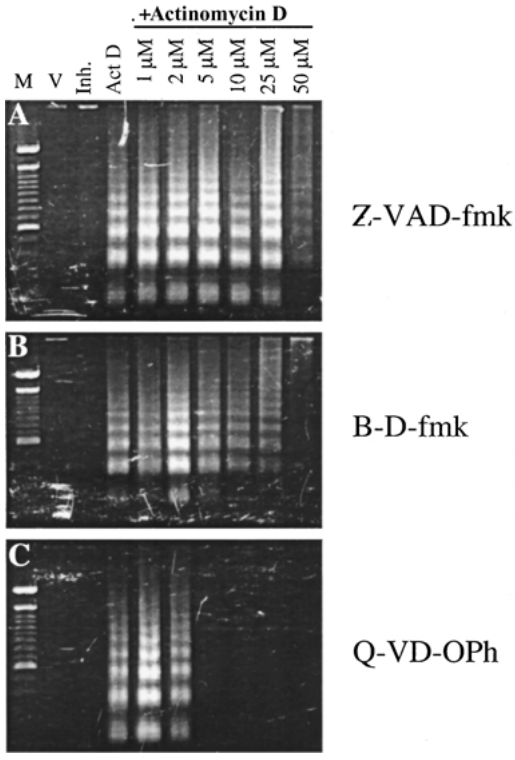



**Figure 2**. Q-VD-OPh potently inhibits Actinomycin D-induced apoptosis. WEHI-231 cells ($1 \times 10^5$ cells/ml) were treated for 4 hrs with (V) vehicle, (Act.D) 1 $\mu$g/ml actinomycin D, or Q-VD-OPh (at either 1 $\mu$M, 2 $\mu$M, 5 $\mu$M, 10 $\mu$M, 25 $\mu$M, 50 $\mu$M, or 100 $\mu$M as indicated) preincubated 1 hr prior to addition of actinomycin. Cells were fixed in 1% paraformaldehyde for 15 min and stored in 70% ethanol. Tunel assay was performed as indicated in the materials and methods and data analyzed with a BD FacScan flow cytometer. The percentage of apoptosis was determined using CellQuest software and is indicated adjacent to the treatment regime.

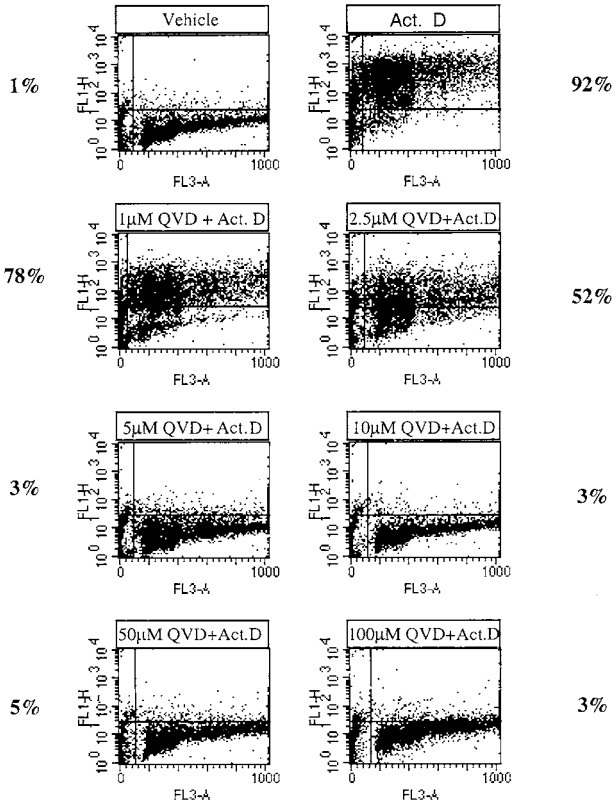
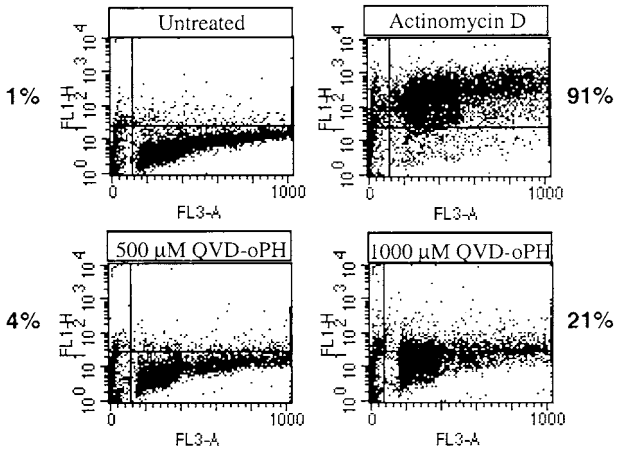


**Figure 3**. Q-VD-OPh exhibits minimal toxicity in WEHI 231 cells. WEHI-231 cells ($7.5 \times 10^4$ cells/ml) were treated for 4 hrs with (V) vehicle, (Act.D) 1 $\mu$g/ml actinomycin D, or Q-VD-OPh (at with either 500 $\mu$M or 1000 $\mu$M) preincubated 1 hr prior to addition of actinomycin. Cell were fixed in 1% paraformaldehyde and fixed in 70% ethanol. Tunel assay was performed as indicated in the materials and methods and data analyzed with a BD FacScan flow cytometer. The percentage of apoptosis was determined using CellQuest software and is indicated adjacent to the treatment regime.




231 cells from apoptosis induced by actinomycin D. The TUNEL data support the effective inhibitory concentrations of Q-VD-OPh, as determined by DNA laddering, at a maximally effective dose of 5 $\mu$M.

The effects of Q-VD-OPh on cellular toxicity were also determined in WEHI 231 cells. Concentrations of dimethylsulfoxide greater than 0.33% can induce apoptosis in WEHI 231 cells (data not shown). The presence of 500 $\mu$M Q-VD-OPh, which results in a DMSO vehicle concentration of 5%, resulted in 4% apoptosis; whereas, 1 mM Q-VD-OPh (10% DMSO concentration) resulted in only 21% apoptosis (Figure 3). Chemical breakdown is often a limiting factor in the efficacy of small peptide inhibitors for use in long term studies. BD-fmk has previously been shown to be stable for 48 h in cultured cells treated continuously with actinomycin D.[13] Analysis of 5 $\mu$M Q-VD-OPh in the presence of actinomycin D in WEHI 231 cells for at least 48 h resulted in no change in the cell number and complete cellular viability as determined by cell number and trypan blue exclusion (data not shown).

In addition to the WEHI 231 mouse B cell line, the human Jurkat T cell line and the rat placental trophoblast cell line, HRP-1, were also analyzed to determine the effective apoptotic inhibitory concentration of Q-VD-OPh in other cell types. Jurkat T cells, incubated for 4 h with actinomycin D, displayed a significant amount of apoptosis as indicated by DNA laddering. Similar to WEHI 231 cells, the presence of 5 $\mu$M Q-VD-OPh completely prevented the actinomycin D-induced DNA fragmentation and apoptosis from occurring in Jurkat T cells (Figure 4). WEHI 231 or Jurkat cells in the presence of Q-VD-OPh and actinomycin D were completely viable, as determined by trypan blue exclusion , but were strongly growth inhibited (data not shown). Q-VD-OPh alone did not interfere with cell growth or viability. To determine if receptor mediated apoptosis would also be inhibited by Q-VD-OPh, the multifunctional cytokine, TGF beta, was also used to induce apoptosis in a rat trophoblast cell line, HRP-1. TGF beta induced apoptosis in HRP-1 cells within 24 h, as determined by DNA laddering, and this was completely inhibited by 5 $\mu$M Q-VD-OPh, consistent with other cell lines (Figure 5).

**Figure 4**. Q-VD-OPh inhibits Actinomycin D-induced DNA laddering in Jurkat T cells. Human Jurkat T cells ($7.5 \times 10^5$ cells/ml) were treated with (V) vehicle or 1 $\mu$g/ml actinomycin D, 5 uM Q-VD-OPh, or 5 uM Q-VD-OPh in conjunction with 1 ug/ml actinomycin D for 4 h, as indicated. DNA was isolated, separated on a 1.2% agarose gel and stained with ethidium bromide to determine DNA laddering. A 100 bp DNA molecular weight marker is present in the far left lane.

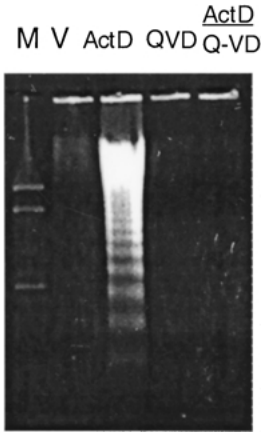



**Figure 5**. Q-VD-OPh inhibits TGF beta inducible DNA laddering in rat trophoblast cells. HRP-1 rat trophoblast cells ($1 \times 10^5$ cells/ml) were treated 5 ng/ml TGF beta for 24 hrs in the absence or presence of 50 uM B-D-fmk or 5 uM Q-VD-Oph as indicated. DNA was isolated, separated on a 1.2% agarose gel and stained with ethidium bromide to determine DNA laddering. A 100 bp DNA molecular weight marker is present in lane 5.

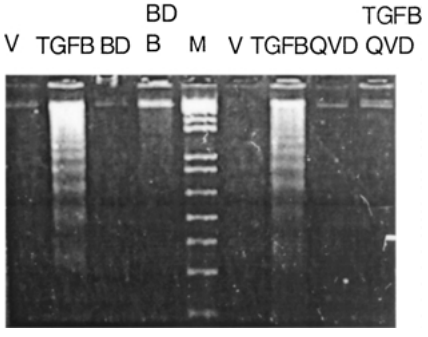
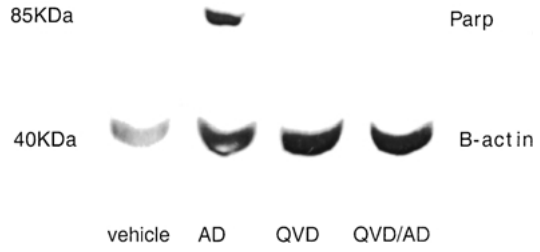


**Figure 6**. Q-VD-OPh prevents caspase mediated cleavage of PARP. Jurkat T-cells were treated for 4 hrs with (V) vehicle, (AD) 1 ug/ml actinomycin D, (QVD) 5 $\mu$M Q-VD-OPh, or (QVD/AD) 5 $\mu$M Q-VD-OPh preincubated 1 hr prior to addition of 1 $\mu$g/ml actinomycin D. 100 $\mu$g of whole cell lysate was separated by SDS-PAGE and Western blotting was performed using a PARP monoclonal antibody as described in the Materials and Methods. The blot was reprobed with beta actin as a loading control.




Actinomycin D has been shown to activate caspase 3 in some cell types.[6,14] To determine the effects of Q-VD-OPh on the activation of caspases during apoptosis, Jurkat T cells were treated with actinomycin D for 4 h and analyzed for the cleavage of poly-(ADP ribose) polymerase and alpha II spectrin, characteristic caspase 3 substrates. Treatment of Jurkat cells with actinomycin D resulted in the characteristic cleavage of PARP as demonstrated by the 89 kDa fragment and this is completely abrogated in the presence of 5 $\mu$M Q-VD-OPh (Figure 6). Similarly, actinomycin D induced alpha II spectrin cleavage into 150 and 120 kDa protein fragments, as expected for caspase 3 cleavage (data not shown).

In addition to the caspase 3 pathway, we also determined if Q-VD-OPh was capable of inhibiting the other known major pathways of apoptosis at this low concentration. DNA laddering, representative of apoptosis, occurred in WEHI 231 cells by the caspase 9/3 pathway activated by actinomycin D (Figure 7A) and the caspase 12 pathway after treatment with thapsigargin (Figure 7B) as well as the caspase 8 pathway which was induced in Jurkat T cells after stimulation with anti-Fas (Figure 7C). All three apoptotic pathways were completely inhibited in the presence of 5 $\mu$M Q-VD-OPh. To demonstrate that Q-VD-OPh is capable of inhibiting caspase activation, Jurkat T cells were treated with anti-fas antibody, or etoposide for 6 hours and Western blotting was performed for caspase 3, caspase 8, and caspase 9. Q-VD-OPh (5 $\mu$M) abolished the caspase cleavage indicative of activity (Figure 8). Although several attempts were made, caspase 12 was not detectable by Western blotting in Thapsigargin treated Jurkats or WEHI 231 cells (data not shown).

The development of Q-VD-OPh relies on carboxy and amino-terminal modifications to increase cell permeability, stability and efficacy. To determine if the carboxyterminal blocking group contributes to the high level of effectiveness of Q-VD-OPh, WEHI 231 cells were treated with actinomycin D for 4 h in the presence or absence of

**Figure 7**. Q-VD-OPh inhibits DNA laddering in all major apoptotic pathways. Panel 1. WEHI-231 cells ($1 \times 10^5$ cells/ml) were treated for 4 hrs with (**B**) vehicle, (**C**) 1 $\mu$g/ml actinomycin D, (**D**) DMSO + 1 $\mu$g/ml actinomycin D, or (**E**) 5 $\mu$M Q-VD-OPh preincubated 1 hr prior to actinomycin D addition. (**A**) indicates a 100 bp DNA molecular weight marker. Panel 2: WEHI-231 cells ($1 \times 10^5$ cells/ml) were treated for 4 hrs with (**B**) vehicle, (**C**) 1 $\mu$M Thapsigargin, (**D**) DMSO + 1 $\mu$M thapsigargin, or (**E**) 5 $\mu$M Q-VD-OPh preincubated 1 hr prior to thapsigargin addition. (**A**) indicates a 100 bp DNA molecular weight marker. Panel 3: Jurkat cells were treated with (**B**) vehicle, (**C**) 100 ng/ml anti Fas (Clone CH-11), (**D**) DMSO + 100 ng/ml anti Fas or (E) 5 $\mu$M Q-VD-OPh preincubated 1 hr prior to anti Fas addition. (**A**) indicates a 100 bp DNA molecular weight marker. DNA was isolated from treated cells, separated on a 1.2% agarose gel and stained with ethidium bromide to determine DNA laddering.

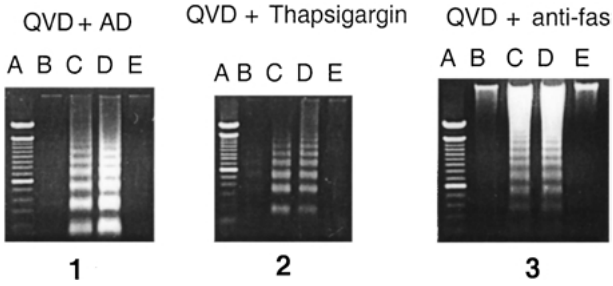
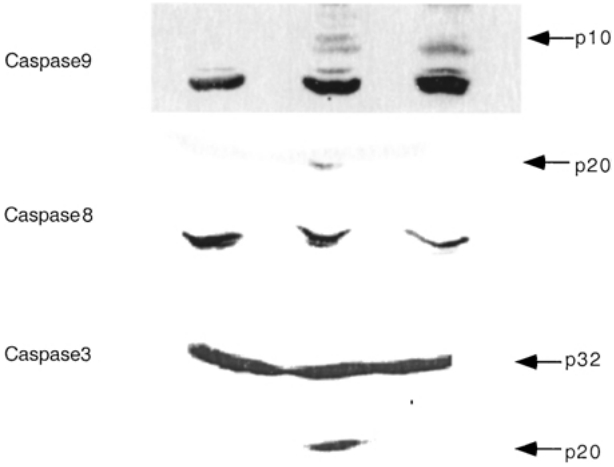
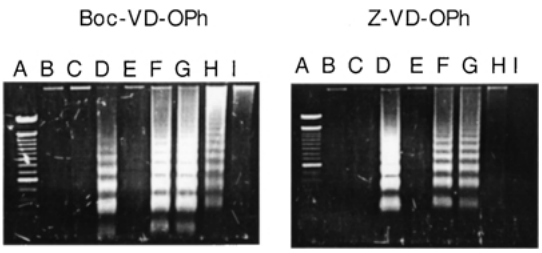



**Figure 8**. Q-VD-OPh prevents activation of the major initiator and effector caspases. Jurkat T cells cells were treated with 100 ng/ml anti fas clone CH-11 for 4 h or 25 $\mu$M etoposide for 6 h in the absence or presence of Q-VD-OPh. Whole cell lysates (200 $\mu$g) was separated by SDS-PAGE and Western blotting was performed using caspase 3 proform and p20, caspase 8 p20 or caspase 9 p10 monoclonal or polyclonal antibodies.




decreasing concentrations of Boc-VD-OPh, Z-VD-OPh, or Q-VD-OPh and analyzed by DNA laddering. As shown in Figure 9, the increased ability to protect against apoptosis induced by actinomycin D is directly related to the carboxyterminal modification contributed by the -OPh group. Substitution of the -OPh group for the -fmk group

**Figure 9**. C-terminal O-phenoxy is responsible for increased apoptotic inhibition. WEHI-231 cells ($1 \times 10^5$ cells/ml) were treated with (**B**) 10 $\mu$M inhibitor alone, (**C**) vehicle (**D**) 1 $\mu$g/ml Actinomycin D, (**E**) 0.1% DMSO; Samples (**F–I**) were pretreated with either 1 $\mu$M, 2 $\mu$M, 5 $\mu$M, 10 $\mu$M inhibitor (Boc-VD-OPh or Z-VD-OPh) one hour prior to 1 $\mu$g/ml actinomycin D treatment for 4 hours. DNA was isolated, separated on a 1.2% agarose gel and stained with ethidium bromide to determine the effective dose of each inhibitor. (**A**) indicates a 100 bp DNA molecular weight marker. Comparative Q-VD-OPh panel is Figure 1, Panel C.




decreased the effective concentration of Boc-VD-OPh to 10 $\mu$M (compared to 50 $\mu$M Boc-D-fmk) and Z-VD-OPh to 5 $\mu$M. Suprisingly, the substitution of an amino terminal "Mu" blocking group conjugated to VD-OPh, in an attempt to increase water solubility, was completely ineffective at preventing actinomycin D induced apoptosis in WEHI 231 cells at concentrations as high as 10 $\mu$M (data not shown).

## Discussion

The present study has determined that broad spectrum caspase inhibitors can have dramatic differences in effectiveness depending upon specific amino and carboxy-terminal modifications. The broad spectrum caspase inhibitor, Q-VD-OPh, was found to be maximal at one tenth the concentration of the most currently effective caspase inhibitor, Boc-D-fmk, in cell culture. Q-VD-OPh was equally effective at inhibiting the three major apoptotic pathways, was functional in different cell types and species (human, mouse, and rat) and prevented terminal caspase activation, substrate cleavage, and DNA ladder formation associated with apoptosis.

The effective concentration of Q-VD-OPh (5 $\mu$M) is ten fold lower than Boc-D-fmk, suggesting that the addition of a quinolyl and phenoxy moieties may greatly enhance cellular permeability and/or substrate access. Alternatively, the phenoxy moiety may provide a much more active leaving group and the quinolyl may act as a peptidomimic thereby elevating the active intracellular concentration of inhibitor present. Q-VD-OPh can inhibit recombinant caspases 1,3,8, and 9 with IC50 values ranging from 25 to 400 nM.[17] The effectiveness of Q-VD-OPh as an apoptotic inhibitor is evidenced by the complete suppression of an apoptotic inducer capable of inducing substantial cell death in less than 4 hours. In addition, Q-VD-OPh prevented Fas-activated caspase 8/10, intracellular calcium-activated caspase 12 (endoplasmic reticulum-specific), and cytochrome c-activated caspase 9/3 DNA laddering at the same concentration. The specificity of Q-VD-OPh in inhibiting apoptosis was also indicated in that the inhibitor did not effect the growth arrest function of the RNA synthesis inhibitor, actinomycin D, but did prevent caspase 3 specific cleavage of a target substrate, poly ADP-ribose polymerase (PARP). Addition of the carboxy terminal O-phenoxy (-OPh) group was primarily responsible for the increased effectiveness as an apoptotic inhibitor as indicated by the similar results obtained using the amino terminal Z or Boc groups conjugated to -VD-OPh. Aminoterminal modifications; however, can also alter the effectiveness of the inhibitor as indicated by the slight less effective Boc blocking group when compared to Z- or Q-blocking groups conjugated to -VD-Oph and the lost of effectiveness by Mu-VD-OPh.

Most commercial caspase inhibitors are hydrophobic and as such require suspension in DMSO to solubilize them. This can present particular problems in DMSO sensitive cells such as lymphocytes since inhibitors such as Z-VAD-fmk require a dose of greater than 50 $\mu$M to be effective. High concentrations of caspase inhibitors may also lead to some nonspecificity and binding to other cellular proteins not involved in the apoptotic pathway further compounding analysis.[13,14,17] The ability to use a caspase inhibitor at such a low effective dose eliminates the problems associated with vehicle concentrations or nonspecificity associated with the widely used fluoromethyl ketone caspase inhibitors, not to mention the increased cost effectiveness.

Q-VD-OPh protected against the substantial apoptosis induced by actinomycin D. In addition, Q-VD-OPh alone exhibited little or no toxicity, even at extremely high concentrations. WEHI 231 cells treated with 500 $\mu$M Q-VD-OPh in a 5% DMSO solution did not undergo apoptosis. A DMSO concentration this high would normally be expected to induce significant apoptosis in lymphocytes. Even at 1 mM concentrations, where the DMSO concentration was 10%, only a minimal increase in apoptosis was detected. These results indicate that Q-VD-OPh provides dramatic protection against apoptotic inducers without significant toxicity.

The effective concentration of Q-VD-OPh may provide a unique reagent when trying to revive hard to propagate cell lines from liquid nitrogen. The addition of this inhibitor to thawed cells would give the cells adequate time to recover, even in the presence of standard DMSO concentrations (10%), from the stress of thawing and begin to proliferate in the absence of toxicity. Q-VD-OPh is stable in solution for several months and is effective in culture for at least 2.5 days. This would provide an ideal time frame for cell recovery; whereas, the subsequent decrease

in effectiveness over time would be fortuitous in that the cells would return to standard culture conditions with minimal manipulation. It is likely that the decreased inhibitory effect on apoptosis in cell culture over time is likely due to uptake and cellular depletion of the inhibitor rather than degradation.

In conclusion, the broad spectrum caspase inhibitor, Q-VD-OPh, provides a cost effective, non toxic, and highly specific means of apoptotic inhibition and provides new insight into the design of new inhibitors. Our data indicate that the specificity and effectiveness of O-phenoxy caspase inhibitors will be significantly enhanced by incorporating conjugated aminoterminal quinolyl and carboxyterminal O-phenoxy groups. A major disadvantage of fluoromethyl ketone and other carboxyterminal-conjugated caspase inhibitors has been the resultant toxicity *in vivo* and this has hampered their use. Future studies examining other amino terminal modifications to 0-phenoxy conjugates to decrease hydrophobicity as well as nonpeptide, selective caspase inhibitors should provide even greater effectiveness. Studies assessing *in vivo* specificity, clearance, and toxicity of Q-VD-OPh will determine the potential use of this new generation of O-phenoxy caspase inhibitor conjugates as promising new therapeutics.

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