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Dose-Dependent Effects of the Caspase Inhibitor Q-VD-OPh on Different Apoptosis-Related Processes

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ABSTRACT

The effects of the pan-caspase inhibitor Q-VD-OPh on caspase activity, DNA fragmentation, PARP cleavage, 7A6 exposition, and cellular adhesivity to fibronectin were analyzed in detail in three different apoptotic systems involving two cell lines (JURL-MK1 and HL60) and two apoptosis inducers (imatinib mesylate and suberoylanilide hydroxamic acid). Q-VD-OPh fully inhibited caspase-3 and -7 activity at 0.05 μM concentration as indicated both by the measurement of the rate of Ac-DEVD-AFC cleavage and anti-caspase immunoblots. Caspase-8 was also inhibited at low Q-VD-OPh concentrations. On the other hand, significantly higher Q-VD-OPh dose (10 μM) was required to fully prevent the cleavage of PARP-1. DNA fragmentation and disruption of the cell membrane functionality (Trypan blue exclusion test) were both prevented at 2 μM Q-VD-OPh while 10 μM inhibitor was needed to inhibit the drug-induced loss of cellular adhesivity to fibronectin which was observed in JURL-MK1 cells. The exposition of the mitochondrial antigen 7A6 occurred independently of Q-VD-OPh addition and may serve to the detection of cumulative incidence of the cells which have initiated the apoptosis. Our results show that Q-VD-OPh efficiency in the inhibition of caspase-3 activity and DNA fragmentation in the whole-cell environment is about two orders of magnitude higher than that of z-VAD-fmk. This difference is not due to a slow permeability of the latter through the cytoplasmic membrane. J. Cell. Biochem. 112: 3334–3342, 2011.

KEY WORDS: Q-VD-OPh; z-VAD-fmk; APOPTOSIS; IMATINIB; SAHA

The proteases of the caspase family are on the basis of the central system operating during the programmed cell death and are required for initiation and execution of many mutually interconnected processes starting from disruption of contacts between the early apoptotic cell and its surroundings to the safe cell decomposition into apoptotic bodies [see, e.g., Kurokawa and Kornbluth, 2009; Pop and Salvesen, 2009; Schrader et al., 2010 for recent reviews]. One of the most important and probably the best explored member of the family is the effector caspase-3 which integrates pro-apoptotic signals from different stimuli and coordinates the many different apoptotic processes.

Caspase activity can be in vitro assessed using synthetic substrates which contain an oligopeptide matching the caspase cleavage site and a fluorogenic group (AFC or AMC). According to the amino acid sequence of the peptide, the substrate can be relatively specific for a given caspase or a group of caspases (e.g., DEVD-based substrates are preferentially cleaved by caspases-3 and -7). Nevertheless, the specificity is limited and the substrate cleavage can never be exclusively attributed to a particular caspase [McStay et al., 2008].

The same peptides form the basis of irreversible caspase inhibitors which are covalently bound to caspase active sites using, for example, fluoromethylketone (fmk) or difluorophenoxymethyl (OPh) group. The inhibitor z-DEVD-fmk is relatively specific for caspases-3, -7, and -10, but it also inhibits caspases-6 and -8 with lower affinity. On the other hand, z-VAD-fmk is considered to be a broad-range caspase inhibitor. Later introduced, the pan-caspase inhibitor Q-VD-OPh is claimed to be more potent, more specific to the caspase family of proteases and less cytotoxic [Caserta et al., 2003]. Chauvier et al. [2007] compared the ability of z-VAD-fmk and Q-VD-OPh to inhibit the recombinant human caspases 1–10. According to these results, the efficiency of Q-VD-OPh in the individual caspase inhibition only slightly surpasses that of z-VAD-fmk, except for caspase-2 where the difference is somewhat more marked. On the other hand, several works including Chauvier et al. [2007] brought evidence of the superiority of Q-VD-OPh over

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z-VAD-fmk in the apoptosis inhibition in the whole-cell environment [Caserta et al., 2003; Yang et al., 2004; Chen et al., 2005].

We have recently shown that Q-VD-OPh suppresses the apoptosis induced by imatinib mesylate in the chronic myelogenous leukemia (CML) cell line JURL-MK1 [Kuzelova et al., 2010b]. Imatinib treatment of JURL-MK1 cells triggers the activation of caspases and DNA fragmentation, but also induces changes in cellular adhesivity to fibronectin-coated surfaces, F-actin decomposition, and changes in the expression level or phosphorylation of proteins involved in focal adhesion signaling. Q-VD-OPh was able to prevent the majority of these processes while other caspase inhibitors were ineffective (z-DEVD-fmk) or markedly less effective (z-VAD-fmk). In this work, we studied in detail the effects of Q-VD-OPh at different concentrations on caspase-3 activity and other apoptosis-related processes, in three different experimental systems involving two cell lines (JURL-MK1 cells derived from CML and HL-60 cells derived from acute myelogenous leukemia) and two unrelated apoptosis-inducing drugs (imatinib mesylate and suberoylanilide hydroxamic acid—SAHA).

MATERIALS AND METHODS

CELL LINES

JURL-MK1 cells were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and HL-60 cells from the European Collection of Animal Cell Cultures (Salisbury, UK). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 4 mM L-alanyl L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2 humidified atmosphere.

CHEMICALS

Imatinib mesylate was kindly provided by Novartis (Basel, Switzerland). The stock solution (10 mM) was prepared by dissolving imatinib mesylate in sterile distilled water and stored at −20°C. SAHA was supplied by Alexis (San Diego). Caspase inhibitors z-DEVD-fmk and z-VAD-fmk and the caspase-3 substrate Ac-DEVD-AFC were from Sigma (Prague, Czech Republic), Q-VD-OPh from R&D Systems (Minneapolis). Human fibronectin (alpha-chymotryptic fragment, 120K) was purchased from Chemicon International (CA). The antibodies against caspase-3, -7, and poly-ADP ribose polymerase-1 (PARP-1) were from Santa Cruz, Inc.

ASSESSMENT OF CASPASE ACTIVITY

Caspase-3-like activity was determined by fluorometric measurement of the kinetics of 7-amino-4-trifluoromethyl coumarin (AFC) release from the fluorogenic substrate Ac-DEVD-AFC in the presence of cell lysates. The method was described in detail in Kuzelova et al. [2007]. After the incubation with the effectors, the cells were washed and lysed and aliquots of cytosolic fractions were incubated for 30 min at 37°C with the fluorogenic substrate. The linear increase of fluorescence intensity at 520 nm was monitored during this incubation time using Fluostar Galaxy microplate reader (BMG Labtechnologies, Germany). Caspase-8-like activity was determined using the fluorogenic substrate Ac-IETD-AFC and following the same procedure except for an additional 30 min incubation at 37°C after the substrate addition and prior to the measurement of fluorescence intensity (this was necessary to obtain linear increase of fluorescence in time).

ELECTROPHORESIS AND WESTERN BLOTTING

Control and treated cells (3×10^6) were washed twice in PBS, suspended in 150 μl lysis buffer (50 mM HEPES, 0.15 M NaCl, 2 mM EDTA, 0.1% NP-40, 0.05% sodium deoxycholate, pH 7.2, 1 mM PMSF, phosphatase inhibitor coctail 5 μl per 2×10^7 cells, protease inhibitor coctail 4 μl per 2×10^7 cells), kept for 30 min on ice and then centrifuged for 30 min at 20,000*g*. The protein concentration was measured using a BioRad protein assay (Bio-Rad Laboratories, CA). Protein samples were heated to 100°C for 4 min in the presence of 2.5% 2-mercaptoethanol, chilled and subjected to one-dimensional electrophoresis (PAGE) in 12% or 15% gel with SDS according to Laemmli [1970]. As a rule, 15 μg total protein was applied to each well. Proteins were transferred to Hybond-ECL membrane at 90 V for 1–2 h (wet transfer). Nitrocellulose membranes were blocked with 5% non-fat milk in TBS-T (Tris-buffered saline, 0.1% Tween-20) for 1 h at room temperature, incubated with the appropriate antibody in TBS-T solution (1 h at room temperature or overnight at 4°C), washed in TBS-T and incubated with the horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody. The antigens were detected using SuperSignal® West Pico Chemiluminiscent Substrate (Pierce Biotechnology, IL) according to the manufacturer's instructions and visualized by autoradiography on X-ray film. Immunoblots were always reacted in parallel with anti-β-actin as a control of the equal protein loading. The protein bands were evaluated by densitometry using AIDA version 4.08 (Raytest, Germany). The precise position of the fragments was determined by multiplex scanning of chemiluminiscent bands and pre-stained MW markers (Precision Plus Protein Standards, Bio-Rad Laboratories) using G-box iChemi XT4 digital imaging device (Syngene Europe, Cambridge).

DETECTION OF APOPTOTIC DNA BREAKS

The fraction of cells containing apoptotic DNA breaks was measured by TUNEL assay using the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics GmbH, Mannheim, Germany) following the standard manufacturer's protocol. Flow cytometry measurements were performed using Coulter Epics XL flow cytometer.

DNA CONTENT ANALYSIS (PI ASSAY)

For DNA content analysis, the cells (5×10^5) were harvested, resuspended in 4.5 ml of cold 70% ethanol, incubated for 30 min at 10°C and kept for 5–7 days at −20°C. The sample was washed twice in PBS and incubated for 2 h at 10°C in 0.5 ml of the modified Vindelovs propidium iodide (PI) buffer (10 mM Tris, pH 8, 1 mM NaCl, 0.1% Triton X-100, 20 μg/ml PI, and 10K units ribonuclease A). The fraction of cells with low DNA content was determined from sub-G1 region of red fluorescence histograms.

APO2.7 STAINING

The cells (5×10^5) were washed in PBS and permeabilized with 100 μl cold digitonin (100 μg/ml in 2.5% BSA/PBS) for 20 min on

ice. Then, they were washed in 2.5% BSA/PBS and stained with PE-conjugated APO2.7 antibody (Coulter Immunotech) for 15 min at room temperature, following the manufacturer's instructions. The red fluorescence was analyzed using Coulter Epics XL flow cytometer.

CELL ADHESION ASSAY

JURL-MK1 cell adhesivity to fibronectin was measured using the previously described protocol [Kuzelova et al., 2010a]. Briefly, the cells (1×10^4) were applied to fibronectin-coated wells of a microtitration plate and incubated for 1 h at 37°C. Then, the wells were washed with PBS/Ca2+/Mg2+ using a multichannel adaptor to the succion pump and the remaining cells were quantified by means of fluorescent labeling (Cy-Quant Cell Proliferation Assay Kit, Molecular Probes). The adherent cell fraction (ACF) was calculated using the fluorescence signal from fibronectin-coated plate and that from reference plate which contained the total cell number.

RESULTS

We have previously shown that the caspase inhibitors z-DEVD-fmk, z-VAD-fmk, and Q-VD-OPh prevent caspase-3 activation resulting from imatinib mesylate treatment of JURL-MK1 cell line [Kuzelova et al., 2010b]. On the other hand, we found substantial differences in the ability of the individual inhibitors to prevent DNA fragmentation induced by imatinib treatment: the most marked effect was achieved using Q-VD-OPh while z-VAD-fmk was efficient only at high concentration (50 μM) and z-DEVD-fmk did not affect the apoptotic DNA fragmentation at all. In the present work, we analyzed in detail the effects of Q-VD-OPh on different apoptosis-related processes in JURL-MK1 cells treated either with imatinib mesylate or with SAHA and HL-60 cells treated with SAHA. The treatment with 2 μM SAHA (but not with 0.5 μM SAHA) also triggers the apoptosis in both JURL-MK1 and HL-60 cells [Kuzelova et al., 2010a].

Figure 1 shows the effect of caspase inhibitors on caspase-3-like activity which was measured as the rate of cleavage of the fluorogenic substrate Ac-DEVD-AFC. Cell treatment with imatinib or SAHA resulted in 7- to 15-fold increase in DEVDase activity after 42 h (resp. 48 h) for JURL-MK1, resp. HL60 cells. Simultanous addition of 50 nM Q-VD-OPh was sufficient to reduce caspase-3-like activity below the value obtained from untreated cells in all the studied experimental systems. In contrast, about 100-fold higher concentration of z-VAD-fmk was required to achieve comparable effect in JURL-MK1 cells and even more for HL60 cells.

To test the hypothesis that the lower efficiency of z-VAD-fmk compared to Q-VD-OPh is due to lower z-VAD-fmk permeability through the cell membrane [Chauvier et al., 2007], we analyzed the effect of variable incubation time. In the majority of our experiments, the caspase inhibitors were added simultaneously with the apoptosis inducers. However, the observed effects did not essentially change if the inhibitors were added with a delay. Two examples are shown in Figure 2 for JURL-MK1 cells treated with imatinib. In the absence of the inhibitor, DEVD-ase activity started

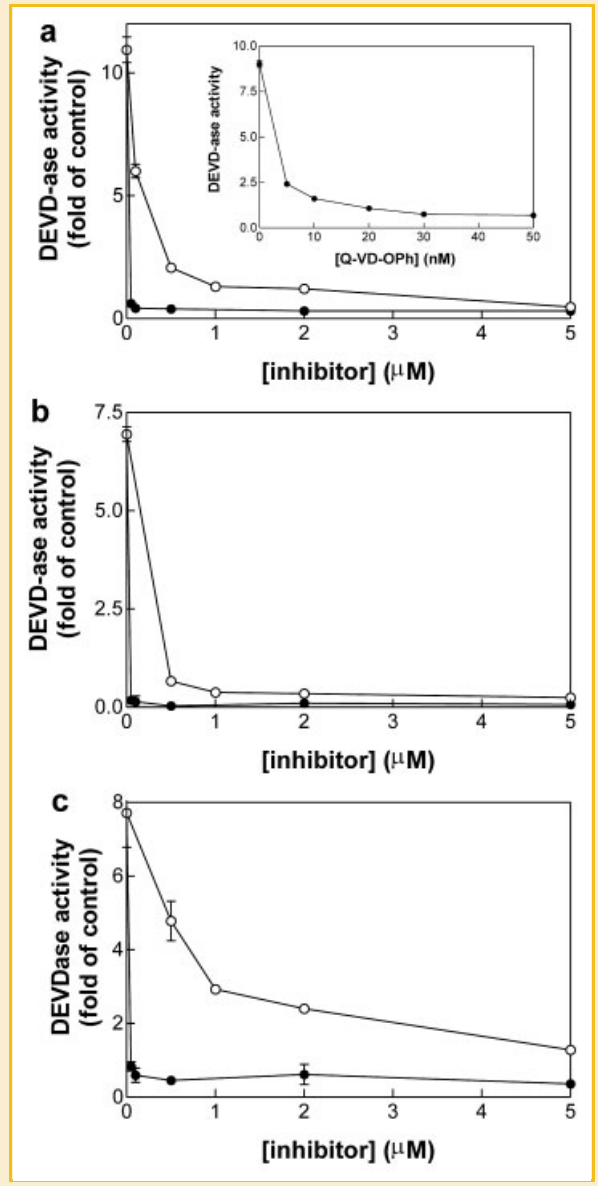

Fig. 1. Inhibition of caspase-3-like activity by Q-VD-OPh and z-VAD-fmk. JURL-MK1 or HL60 cells were treated with 1 μM imatinib or 2 μM SAHA in the presence or in the absence of Q-VD-OPh (closed symbols) or z-VAD-fmk (open symbols) at different concentrations. After 42–48 h incubation, the cells were harvested, the cytosolic proteins were extracted and incubated with fluorogenic caspase substrate Ac-DEVD-AFC. The value of DEVDase activity is given as the ratio of cleavage rates from treated/control cells. a: JURL-MK1 + imatinib, 42 h, (b) JURL-MK1 + SAHA, 42 h, (c) HL60 + SAHA, 48 h. The figure shows representative experiments, the error bars correspond to the standard deviation of triplicates. All experiments were repeated (N = 3) with closely similar results.

to increase at about 16 h after imatinib addition and reached the maximum at about 30 h (open circles and dashed line in Fig. 2b). The rate of Ac-DEVD-AFC cleavage was significantly reduced by 5 μM z-VAD-fmk or 2 μM Q-VD-OPh even when the inhibitors were added for the last 2 h of imatinib treatment (Fig. 2a). The effect of z-VAD-fmk (50 μM) on DNA fragmentation (Fig. 2b, squares) was maintained when the inhibitor was added after up to 16 h delay from

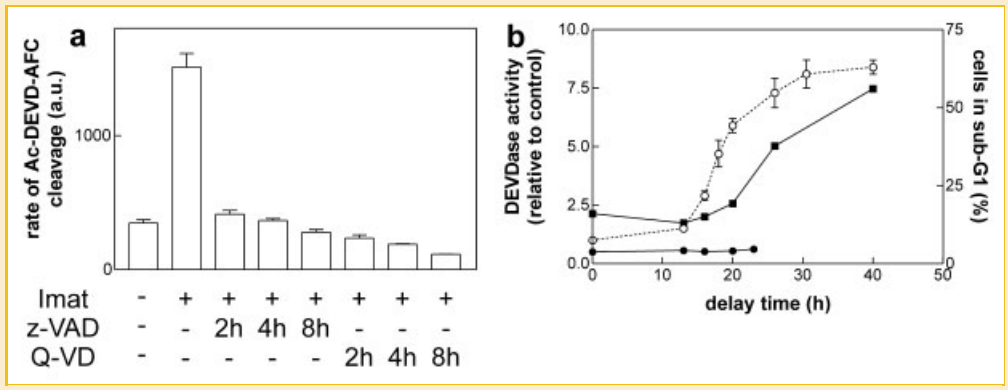

Fig. 2. Effect of delay in inhibitor addition on its efficiency. JURL-MK1 cells were treated with 1 μM imatinib mesylate and splitted into aliquots. Subsequently, z-VAD-fmk was added with variable time delay. At the end of incubation time, the cells from all samples were harvested and assayed for caspase-3-like activity or for apoptotic DNA fragmentation. The kinetics of caspase activation in samples treated with imatinib only is shown for comparison by open circles and dashed line in the panel b. a: The cells were treated with imatinib for 32 h and z-VAD-fmk (5 μM) or Q-VD-OPh (2 μM) were added for the last 2, 4, or 8 h of the incubation time as indicated. The caspase-3-like activity was measured in vitro using the fluorogenic substrate Ac-DEVD-AFC. b: Closed circles (left axis): DEVD-ase activity in lysates from cells treated with imatinib and subsequently with 5 μM z-VAD-fmk with variable delay time as indicated, the activity was assessed at 40 h from imatinib addition. Squares: cell fraction in sub-G1 region of DNA content histograms, cells were treated with imatinib and subsequently with 50 μM z-VAD-fmk with variable delay time as indicated, the apoptotic cell fraction was determined at 40 h after imatinib addition.

imatinib. Larger delay in z-VAD-fmk addition resulted in progressive loss of its effect, simultaneously with the progression of caspase activation.

The effect of Q-VD-OPh at different concentrations on procaspase-3 processing was studied by Western blotting using a monoclonal anti-caspase-3 antibody (Fig. 3). We have previously shown that the covalent binding of z-DEVD-fmk to the active caspase-3 results in a small shift of the 17 kDa band corresponding to the large caspase-3 subunit towards higher MW [Kuzelova et al., 2007]. In the case of Q-VD-OPh, the shift is hardly discernable (about 0.3 kDa), but still present (in Fig. 3, cf. the strong bands at 17 kDa in second lanes with the corresponding weak bands from third lanes) and it indicates that virtually all large caspase-3 subunits are linked to Q-VD-OPh already at the lowest concentration used (0.05–0.1 μM). In addition, Q-VD-OPh clearly interferes with caspase-3 processing, as the 17 kDa band disappears and is replaced by bands at higher molecular weight (about 18.5 and 20 kDa). We observed similar effects of z-VAD-fmk on the procaspase-3 processing [Fig. 3a, Kuzelova et al., 2007], but at higher inhibitor concentrations.

We also tested the effect of Q-VD-OPh on the activity of caspases-7 and -8 for selected experimental systems. As it is shown in Figure 4a, imatinib mesylate treatment of JURL-MK1 cells induces cleavage of procaspase-7 and the active caspase-7 subunit (at 17 kDa) appears in Western blots. Simultaneous addition of 50 nM Q-VD-OPh results in a small shift (about 0.4 kDa) of the 17 kDa band towards higher MW indicating that the subunit is covalently bound to the inhibitor and thereby not active. Moreover, the subunit progressively disappers when higher Q-VD-OPh concentrations are used indicating that procaspase-7 processing is hampered up from 2 μM Q-VD-OPh.

Although imatinib mesylate has been reported to activate caspase-8, we detected no increase in the cleavage rate of Ac-IETD-AFC substrate following JURL-MK1 cell treatment with imatinib or SAHA (multiple time points up to 48 h were tested,

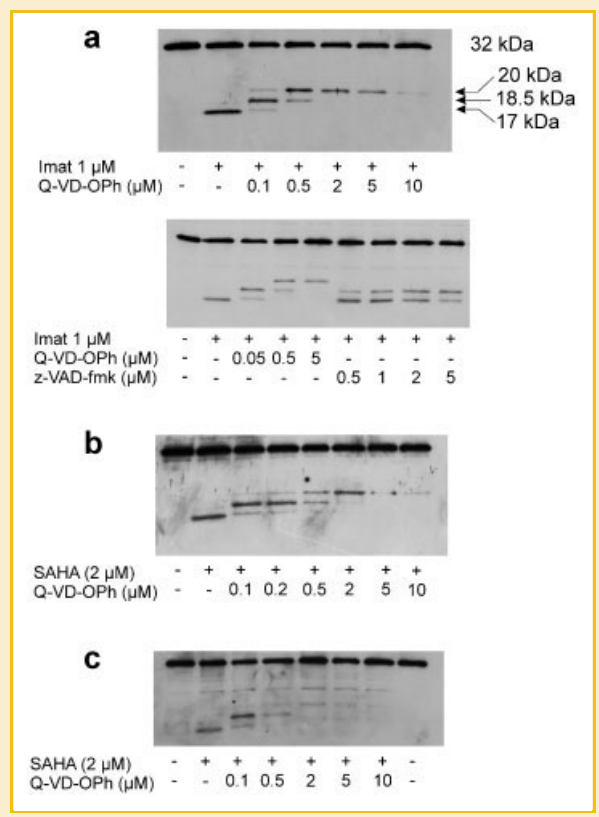

Fig. 3. Procaspase-3 processing in cells treated with apoptosis inducer and caspase inhibitors. The cells were treated with the drug and, simultaneously, with Q-VD-OPh or z-VAD-fmk at different concentrations and incubated for 42 or 48 h. The procaspase-3 (32 kDa), the mature large caspase-3 subunit (17 kDa) and intermediate caspase-3 fragments (about 18.5 and 20 kDa) were detected by Western blotting using anti-caspase-3 antibody. a: JURL-MK1 + imatinib 1 μM, 42 h, the lower blot shows the comparison of Q-VD-OPh with z-VAD-fmk, (b) JURL-MK1 + SAHA 2 μM, 42 h, (c) HL60 + SAHA 2 μM, 48 h. The figure shows representative experiments. Repeated experiments (N = 3) yielded similar results.

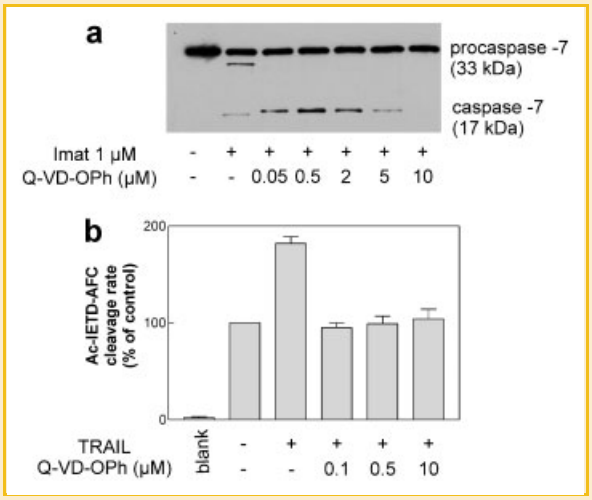

Fig. 4. Effect of Q-VD-OPh on caspases-7 and -8. a: JURL-MK1 cells were treated for 42 h with 1 μM imatinib alone or in combination with Q-VD-OPh as indicated and procaspase-7 (33 kDa) and the mature caspase-7 subunit (17 kDa) were detected by Western blotting using anti-caspase-7 antibody. b: JURL-MK1 cells were treated for 5 h with 200 ng/ml TRAIL alone or in combination with Q-VD-OPh (the inhibitor was added 2 h prior to TRAIL). The caspase-8-like activity was measured using the fluorogenic substrate Ac-IETD-AFC. The results are given as the mean and standard deviation from three experiments.

data not shown). To analyze the effect of Q-VD-OPh on caspase-8 activity, we treated JURL-MK1 cells with TRAIL (TNF-related apoptosis inducing ligand) which is known to trigger the extrinsic apoptotic pathway and to activate caspase-8. Indeed, in this case, we could observed moderate increase in Ac-IETD-AFC cleavage rate reaching the maximum at 4–6 h of the treatment. This increase was prevented by pre-treatment with Q-VD-OPh (0.1 μM concentration was sufficient to reduce the signal to the level obtained from untreated samples) (Fig. 4b).

As we reported previously [Kuzelova et al., 2009], imatinib treatment also induces about twofold increase in caspase-9 activity as measured by the cleavage rate of Ac-LEHD-AFC substrate. This increase was inhibited by 5 μM z-DEVD-fmk as well as by 0.1 μM Q-VD-OPh (data not shown).

The apoptotic DNA fragmentation induced by imatinib or SAHA treatment was analyzed both using the TUNEL method (enzymatic fluorescent labeling of DNA breaks which are typical for the apoptosis) and the analysis of DNA content using flow-cytometry (PI assay). The results of both these methods showed that the caspase inhibitor Q-VD-OPh is able to prevent the apoptotic DNA cleavage in all the studied cellular systems (Fig. 5). However, while caspase-3 and -7 activity was suppressed at 50 nM Q-VD-OPh, no statistically significant effect on the DNA fragmentation was observed at 50–100 nM concentration (highlighted points in Fig. 5) and markedly higher Q-VD-OPh dose (2 μM) was required to completely inhibit the apoptotic DNA fragmentation. The inhibitors z-DEVD-fmk and z-VAD-fmk were much less efficient than Q-VD-OPh in inhibiting the apoptotic DNA fragmentation in both JURL-MK1 and HL-60 cells. The effect of z-VAD-fmk on sub-G1 cell fraction is shown in Figures 2 (50 μM z-VAD-fmk) and 5 (up to 10 μM z-VAD-fmk),

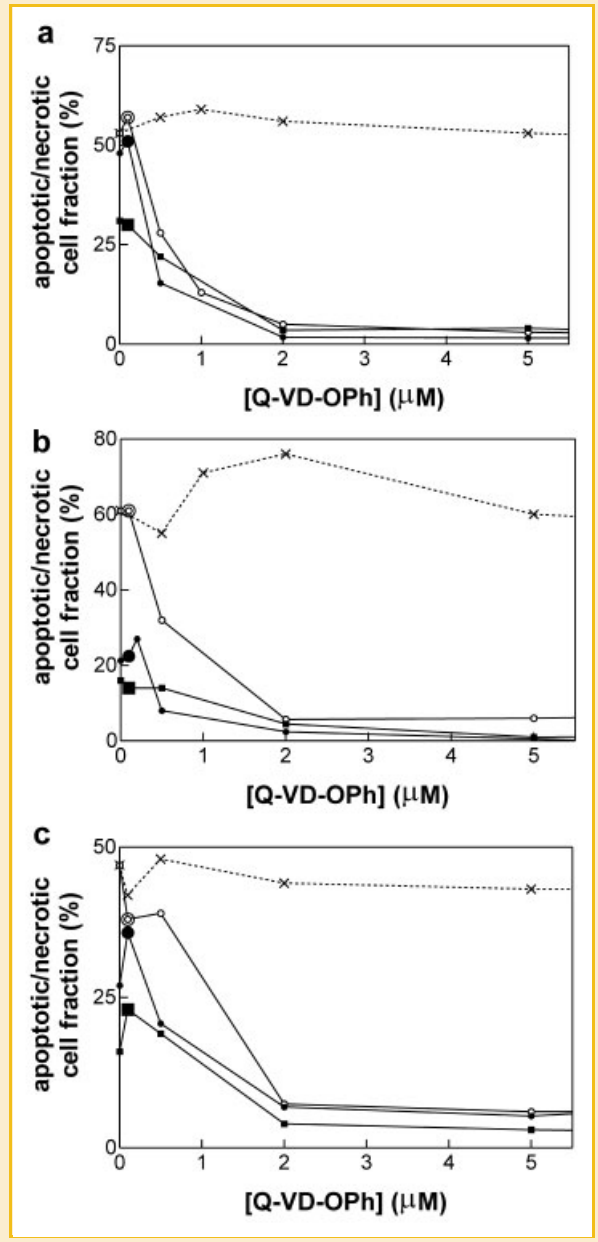

Fig. 5. Effect of Q-VD-OPh on DNA fragmentation and cell death. The cells were treated with 1 μM imatinib or 2 μM SAHA in the presence or in the absence of Q-VD-OPh at different concentrations. After 48 h incubation, the cells were harvested and the extent of DNA fragmentation was assessed using either TUNEL staining (closed circles) or DNA content analysis (cells in sub-G1 region, open circles). The fraction of late apoptotic + necrotic cells was determined by Trypan blue exclusion test (squares). The effect of 0.1 μM Q-VD-OPh is highlighted using larger symbols. For comparison, the effect of z-VAD-fmk (instead of Q-VD-OPh) on the cell fraction in sub-G1 under the same experimental conditions is shown by cross symbols and dashed lines. a: JURL-MK1 + imatinib, (b) JURL-MK1 + SAHA, (c) HL60 + SAHA. Repeated experiments (at least three) yielded similar results—the maximal effect is always achieved at 2 μM Q-VD-OPh.

z-DEVD-fmk had no effect at up to 50 μM concentration (data not shown).

Concurrently with the inhibition of DNA fragmentation, Q-VD-OPh inhibited drug-induced cell death (assessed using the Trypan

blue or PI exclusion test) in all the studied experimental systems, the maximal effect was also achieved at about 2 μM Q-VD-OPh concentration (Fig. 5). However, although Q-VD-OPh stopped the apoptotic cell death, the cell proliferation was not renewed and the cells finally died (Trypan blue-positive cells usually started to appear after about 5 day incubation), presumably through the senescence or other form of cell death.

One of the prominent targets of caspase-3 is the protein PARP-1 which is involved in DNA repair. PARP-1 cleavage is even used as an internal marker of caspase activation. We indeed observed a reduction of full-length PARP level and the emergence of the characteristic 89 kDa PARP fragment upon drug treatment of both JURL-MK1 and HL-60 cells. Surprisingly, the inhibitor z-DEVD-fmk was completely inefficient in inhibiting PARP cleavage (up to 50 μM, data not shown). Q-VD-OPh inhibited PARP cleavage only partially when it was used at concentrations up to 2 μM (Fig. 6) while complete inhibition was achieved using 10 μM Q-VD-OPh (not shown in Fig. 6 due to the limited number of lanes per experiment). The inhibitor z-VAD-fmk was again less efficient than Q-VD-OPh (Fig. 6).

The expression of the mitochondrial antigen 7A6, which is recognized by APO2.7 antibody, is another specific marker of the proceeding apoptosis [Koester et al., 1997; Nagahara et al., 2007]. Both imatinib mesylate and SAHA at the toxic dose (2 μM) markedly increased the fraction of APO2.7-positive cells. Q-VD-OPh did not prevent 7A6 staining at concentrations up to 50 μM (Fig. 7).

During the early stages of apoptosis, the cells become round and detache from its environment. In our previous work [Kuzelova et al., 2010b], we described a decrease of JURL-MK1 cell adhesivity to fibronectin-coated surface as a part of imatinib-induced apoptosis and we showed that the loss of cellular adhesivity can be prevented by 10 μM but not by 2 μM Q-VD-OPh. We found similar dose dependency in JURL-MK1 cells treated with 2 μM SAHA (Fig. 8). While SAHA at subtoxic concentration (0.5 μM) increases the cell adhesivity to fibronectin [Kuzelova et al., 2010a], toxic SAHA dose induces the apoptosis and, concurrently, a loss of cellular adhesivity, which can be reversed by 10 μM Q-VD-OPh. On the other hand, neither z-DEVD-fmk nor z-VAD-fmk (up to 50 μM) were able to prevent the loss of JURL-MK1 cell adhesivity to fibronectin due to cell treatment with imatinib or SAHA (data not shown).

We detected no significant changes in any studied process which would be induced by caspase inhibitors themselves (Q-VD-OPh, z-DEVD-fmk, z-VAD-fmk up to 50 μM).

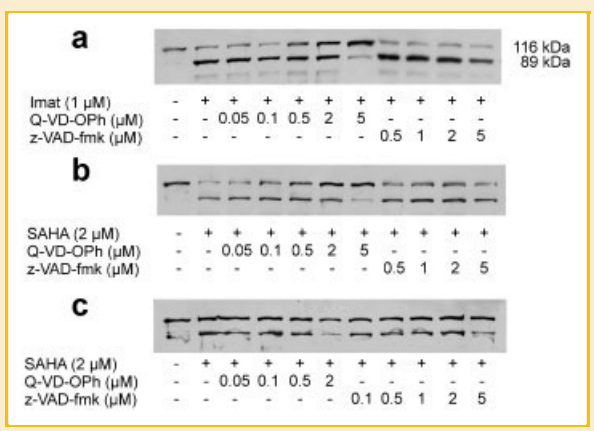

Fig. 6. Effect of caspase inhibitors on PARP cleavage. The cells were treated with the drug and, simultaneously, with a caspase inhibitor at different concentrations as indicated. The level of the full-length PARP-1 and of the 89 kDa PARP fragment was assessed by Western blotting using anti-PARP-1 antibody. a: JURL-MK1 + imatinib 1 μM, 42 h incubation, (b) JURL-MK1 + SAHA 2 μM, 42 h incubation, (c) HL60 + SAHA 2 μM, 48 h incubation. The figure shows representative experiments. Repeated experiments (N = 3) yielded similar results.

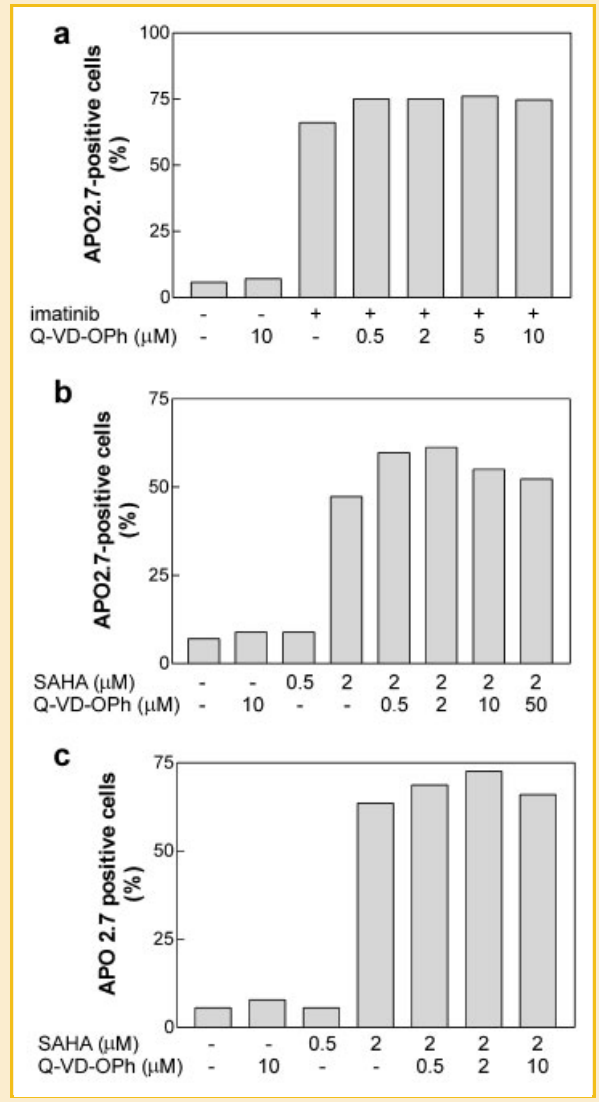

Fig. 7. Effect of Q-VD-OPh on 7A6 exposition. The cells were incubated for 48 h with imatinib (1 μM) or SAHA (0.5 or 2 μM) in the absence or in the presence of Q-VD-OPh at different concentrations as indicated. Then, they were permeabilized with digitonin and the mitochondrial antigen 7A6 was stained using APO2.7 antibody. a: JURL-MK1 + imatinib, (b) JURL-MK1 + SAHA, (c) HL60 + SAHA. The figure shows representative experiments. Similar results (no significant difference between samples incubated in the presence and in the absence of Q-VD-OPh) were obtained in all repeated experiments (N = 2–3).

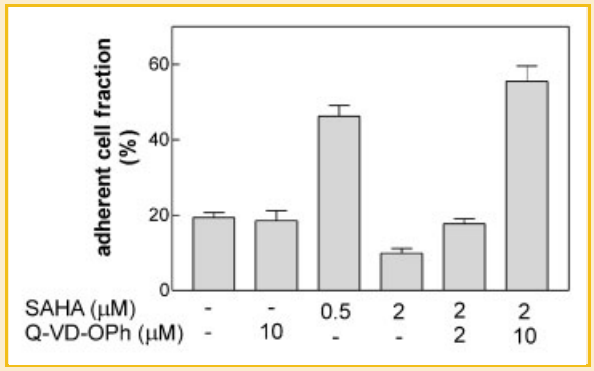

Fig. 8. Effect of Q-VD-OPh on SAHA-induced decrease of JURL-MK1 cell adhesivity to fibronectin. JURL-MK1 cells were treated for 48 h with SAHA (0.5 or 2 μM as indicated) in the presence or in the absence of Q-VD-OPh (2 or 10 μM as indicated) and then incubated for 1 h in fibronectin-coated wells. The wells were gently washed and the attached cells were quantified using a fluorescent dye. The figure shows a representative experiment, the error bars correspond to the standard deviation of quadruplicates. Statistical analysis of repeated experiments shows significant difference between controls and 0.5 μM SAHA-treated samples (15 experiments, $P < 0.0001$) as well as between 2 μM SAHA alone and 2 μM SAHA combined with 10 μM Q-VD-OPh (3 experiments, $P < 0.05$).

DISCUSSION

We tested the effect of Q-VD-OPh on the activity of caspase-3 in three different systems where the apoptosis of leukemic cell lines was induced by drug treatment. Simultaneous addition of Q-VD-OPh at <50 nM concentration was sufficient to fully inhibit caspase-3 and -7 activity which was assessed using the fluorogenic substrate Ac-DEVD-AFC (Fig. 1). Anti-caspase-3 immunoblots (Fig. 3) showed that Q-VD-OPh binds to the large caspase-3 subunit and even prevents the procaspase-3 processing. The active caspase-3 is produced by proteolytic cleavage of the proenzyme (32 kDa) at Asp175 into the 12 kDa subunit and a 20 kDa fragment and subsequent two-step autoprocessing of the latter fragment into the mature 17 kDa subunit [Fernandes-Alnemri et al., 1996; Han et al., 1997]. The antibody we used detects an epitope within the 17 kDa subunit which contains the reactive cysteine. Covalent binding of the inhibitor Q-VD-OPh to the cysteine results in a very slight shift (about 0.3 kDa) of the detected band to the higher MW. As shown in Figure 3, Q-VD-OPh also prevents the autoprocessing of the 20 and 18.5 kDa fragments into the 17 kDa subunit. Moreover, at 10 μM concentration, Q-VD-OPh inhibits the protease responsible for the initial procaspase3 cleavage (possibly one of the initiator caspases or granzyme B).

IC50 value for recombinant caspase-3 inhibition by Q-VD-OPh in a cell-free system is <25 nM (product datasheet, Calbiochem). The comparison of this value with our experimental data (Fig. 1a, inset) indicates that the effective Q-VD-OPh concentration in the whole-cell environment is not very different from that for the cell-free system which implies that the inhibitor can easily penetrate the cell membrane and that the presence of cell structures does not significantly limit its activity. This should be true for other cellular Q-VD-OPh targets as well. Indeed, we found that Q-VD-OPh inhibits caspases-7 and -8 at low concentrations (Fig. 4).

Although the inhibition constants relative to the individual recombinant caspases are quite similar when comparing Q-VD-OPh with z-VAD-fmk [Chauvier et al., 2007], about 100-fold higher dose of z-VAD-fmk is required to achieve similar caspase-3 inhibition in the whole cells (Fig. 1). Larger cellular effectiveness of Q-VD-OP compared to z-VAD-fmk was suggested to be related to the replacement of the aminoterminal benzyloxycarbonyl (z) into quinoline moiety (Q) that renders peptides more hydrophobic and enhances cell membrane permeability. Nevertheless, the inhibitors z-VAD-fmk and z-DEVD-fmk are designed as methylesters to facilitate cell permeability and their ability to penetrate the cell membrane is expected to be rather high. We addressed this point experimentally by varying the time delay between JURL-MK1 cell treatment with the apoptosis inducer (imatinib mesylate) and the addition of z-VAD-fmk. A short (2 h) incubation with z-VAD-fmk was sufficient to substantially reduce the in vitro measured caspase-3-like activity (Fig. 2a). Similarly, the ability of z-VAD-fmk at 50 μM concentration to inhibit the apoptotic DNA fragmentation was maintained when the inhibitor was added after a delay provided that caspases were still not activated (Fig. 2b). A slow penetration of z-VAD-fmk through the cell membrane is thus not the reason for the lower z-VAD-fmk efficacy in the whole-cell environment, at least in our experimental systems. However, it is possible that z-VAD-fmk is sequestred in the intracellular environment. Interestingly, McStay et al. [2008] analyzed the effect of caspase inhibitors on the cleavage of different fluorogenic caspase substrates induced by cytochrome c and dATP addition to cytosolic extracts from JURKAT cells. In these membrane-free experiments, addition of z-VAD-fmk at 5 μM reduced to about 15% the rate of cleavage of DEVD-based substrate while almost complete inhibition of the cleavage was achieved using 25 μM z-VAD-fmk. This dose dependency is in good agreement with the results obtained in our experiments.

The regulation of the apoptotic DNA cleavage can be both caspase-dependent and caspase-independent [Lecoeur, 2002]. In our experimental systems, drug-induced alteration of nuclear DNA was clearly detectable in the TUNEL assay as well as in PI assay. The observed influence of caspase inhibitors suggests that caspase-3 is not required for DNA cleavage, neither in JURL-MK1 nor in HL-60 cells. Indeed, despite the inhibition of caspase-3 activity by 50 nM Q-VD-OPh, the DNA fragmentation is not blocked at this inhibitor concentration (Fig. 5). At least 2 μM Q-VD-OPh was required to fully inhibit this process, consistently in all the three systems we examined and very similar value was also found by others for mouse B-cell line WEHI 231 treated with actinomycin D [Caserta et al., 2003]. In addition, DNA fragmentation was completely insensitive to z-DEVD-fmk (up to 50 μM) which fully inhibits caspase-3 activity at 0.5 μM concentration (see data in Kuzelova et al. [2010b] for JURL-MK1 cells treated with imatinib mesylate, we obtained similar data for SAHA-treated JURL-MK1 and HL-60 cells, not shown). The marked effect of 2 μM Q-VD-OPh suggests that another caspase could be involved in the regulation of DNA cleavage.

Poly(ADP-ribosylation) is a post-translational modification of proteins that plays a crucial role in DNA repair and replication and represents a cellular emergency reaction. However, these energy-

consuming processes become wasteful when the cell is committed to the apoptosis and this may be the reason for caspase-mediated proteolytic PARP-1 inactivation. In addition, PARP-1 fragments may have a positive role in the apoptosis progression [Soldani and Scovassi, 2002; Koh et al., 2005]. PARP-1 cleavage into 24 and 89 kDa fragments is a known hallmark of apoptosis and is even used as an intracellular marker of caspase activation. The preferred cleavage site within human PARP-1 amino acid sequence is DEVD (210–213) and caspase-3 and -7 are considered to be the main actors in the proteolytic PARP inactivation. However, we observed no effect on PARP cleavage in imatinib-treated JURL-MK1 cells using z-DEVD-fmk at up to 50 μM concentration (data not shown), despite the inhibition of caspase-3 activity. Moreover, as it follows from Figure 6, PARP is still cleaved at 2 μM Q-VD-OPh concentration. This infers that, at least in our experimental systems, PARP-1 is preferentially cleaved by a protease different from caspase-3. Several previous reports also indicate that PARP-1 can be cleaved even in the absence of caspase-3 [Soldani and Scovassi, 2002]. Our results also show that caspase-7 and -8, which are known to be involved in PARP-1 cleavage, are inhibited at low Q-VD-OPh concentrations (0.1 μM, Fig. 4).

It is quite surprising that caspase inhibitors are able to completely inhibit the cleavage of the synthetic substrate Ac-DEVD-AFC (Fig. 1) while they have no or limited effect on the cleavage of the intracellular substrate PARP bearing the same cleavage site. We speculate that the difference could be due to the different conditions of the reaction—PARP-1 is cleaved in the whole-cell environment while the test using Ac-DEVD-AFC substrate is performed under controlled conditions (pH, composition of the reaction buffer) which are optimized for the activity of caspases and may not be suitable for the activity of another protease. Alternatively, the conformation of the DEVD peptide may be different and more apt to the cleavage when it forms part of a larger amino acid chain.

It was reported that Q-VD-OPh enables sensitive measurement of low-level apoptosis in cell culture models [Goldstein et al., 2005]. Q-VD-OPh preserves the apoptotic cell integrity, but does not prevent cytochrome *c* release from mitochondria into the cytoplasm which can be used for the assessment of the cumulative incidence of apoptotic cells. Our results show that the fraction of cells which have initiated the apoptosis can also be detected by flow-cytometry using APO2.7 antibody as the unmasking of the mitochondrial antigen 7A6 occurs independently of Q-VD-OPh addition as well (Fig. 7). The absence of Q-VD-OPh (up to 50 μM) effect on APO2.7 staining strongly suggest that 7A6 unmasking is a caspase-independent process. This is apparently contradictory to the results of Nagahara et al. [2007] who studied 7A6 exposure in JURKAT cells after two different apoptosis-inducing treatments and found a reduction of APO2.7 staining using the caspase inhibitor z-VAD-fmk. However, the inhibitor was used at rather high concentration (40 μM) and its effect thus may have been unspecific. Similarly, Greco et al. [2006] reported that both z-VAD-fmk and Q-VD-OPh were able to reduce the expression of APO2.7 reactivity caused by cryopreservation/thawing in umbilical cord CD34+ blood cells, but the inhibitors were again added at high concentration (100 μM). This raises a little doubt as to the strict Q-VD-OPh specificity for the caspase family of proteases.

Caspases were shown to modulate the activity of several proteins involved in focal adhesion pathways, such as ROCK1, PAK2, or FAK [Kurokawa and Kornbluth, 2009] and it is thus not surprising that caspase inhibitors affect also the cellular adhesivity. We found a decrease in JURL-MK1 cell binding to fibronectin after treatment with imatinib or 2 μM SAHA. This was prevented by 10 μM (but not 2 μM) Q-VD-OPh [Fig. 8 for SAHA and Kuzelova et al., 2010b for imatinib] while z-VAD-fmk (up to 50 μM) had no effect. Caspases-3 and -7 are thus not essentially involved in this process. The loss of cellular adhesivity can be due to other member of the caspase family, but the difference in the dose dependency suggests that it is regulated independently of the DNA fragmentation. The lack of effect of z-VAD-fmk even at 50 μM concentration also indicates that Q-VD-OPh could have other targets in addition to caspases.

Caspase inhibitors are widely used to document caspase involvement in different programmed cell death systems. However, although the recommended working concentration for the use in cell cultures is usually 10–20 μM, the inhibitors are often added at higher doses (10 out of 19 randomly chosen papers reported the use of Q-VD-OPh at 40–100 μM concentration). The limited specificity has already been warranted for other caspase inhibitors than Q-VD-OPh [Ekert et al., 1999; Martin et al., 2007; Lidsky et al., 2009]. The latter is supposed to be more selective for the caspase family as it does not cross-react with cathepsins (except for cathepsin H), granzyme B, and calpains [Chauvier et al., 2007] but the hypothesis that even this inhibitor may have non-caspase targets cannot be ruled out. Thus, the results obtained using caspase inhibitors including Q-VD-OPh must always be interpreted with caution. On the other hand, these compounds can be useful in the analysis of processes which are triggered simultaneously with the apoptosis, such as other forms of cell death (e.g., cellular senescence [Rebbaa et al., 2003] or autophagy [Vandenabeele et al., 2006]) or changes in cell signaling which are directly due to an intervention [e.g., drug treatment, Kuzelova et al., 2010b]. In these cases, suppression of the marked manifestation of the apoptosis highlights the non-apoptotic processes.

Caspase inhibitors are also tested for use as protective agents to decrease the degradation of stored tissue samples or to limit tissue damage following an injury. It is not clear if the apoptosis inhibition is enough to fully preserve the cell functionality. At least in experiments performed using cell lines, the inhibitor often blocks the apoptosis but the cell cycle progression is not maintained and the cells are finally eliminated by other form of cell death [Rebbaa et al., 2003; Brown et al., 2007 as well as our own experiments]. However, this may not be the case for terminally differentiated, non-proliferating cells and several works indeed documented an improvement in the outcome following an experimental injury which was achieved by animal pre-treatment with Q-VD-OPh [DeBiasi et al., 2004; Braun et al., 2007; Renolleau et al., 2007; Colak et al., 2009; Liu et al., 2009; Psotka et al., 2009]. Interestingly, it was also reported that simultaneous inhibition of the apoptosis by Q-VD-OPh and of the back-up caspase-independent form of cell death by GAPDH overexpression, CML cell lines could maintain the proliferation despite the presence of imatinib mesylate [Lavallard et al., 2009].

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