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Caspase-3 is not essential for DNA fragmentation in MCF-7 cells during apoptosis induced by the pyrrolo-1,5-benzoxazepine, PBOX-6

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Abstract Effector caspases-3, -6 and -7 are responsible for producing the morphological features associated with apoptosis, such as DNA fragmentation. The present study demonstrates that a member of a novel series of pyrrolo-1,5-benzoxazepines, PBOX-6, induces apoptosis in MCF-7 cells, which lack caspase-3. Apoptosis was accompanied by DNA fragmentation and the activation of caspase-7, but not caspases-3 and -6. Inhibition of caspase-7 activity reduced the extent of apoptosis induced, indicating that activation of caspase-7 is involved in the mechanism by which PBOX-6 induces apoptosis in MCF-7 cells. This study suggests that caspase-3 is not necessarily essential for DNA fragmentation and the morphological changes associated with apoptosis.

Key words: Apoptosis; Caspase; Pyrrolo-1,5-benzoxazepine; DNA fragmentation; MCF-7

1. Introduction

Apoptosis is a cell suicide mechanism invoked in disparate situations to remove redundant, damaged, or infected cells. An essential component of the apoptotic machinery, the caspase family, consists of a group of intracellular cysteine proteases with at least 14 members [1]. They can be divided into initiator or upstream caspases (caspases-2, -8, -9, and -10), which go on to activate the downstream or effector caspases (caspases-3, -6 and -7). Cleavage of a select group of substrates by effector caspases is responsible for dismantling of essential cell components, which results in morphological and biochemical changes that characterise apoptotic cell death: cytoskeletal rearrangement, cell membrane blebbing, nuclear condensation and DNA fragmentation [2]. For example, in in vitro studies caspase-3 cleaves ICAD (inhibitor of caspase-activated DNase), promoting release of active CAD (caspase-activated DNase), which then cleaves DNA and promotes chromatin condensation [3,4].

Previous work has demonstrated that MCF-7 breast carcinoma cells, which lack caspase-3 owing to a functional deletion in the CASP-3 gene, can be killed by apoptotic stimuli, such as tumour necrosis factor (TNF) and staurosporine, without DNA fragmentation and many of the other hallmarks of apoptosis [5]. These results indicated that caspase-3 was likely to be essential for such hallmarks of apoptotic cell death. In contrast to this, a recent report has revealed that release of mitochondrial endonuclease G results in DNA fragmentation in murine embryonic fibroblast cells following UV irradiation, which occurs independently of caspase activation [6]. In addition, apoptosis-inducing factor has been shown to induce apoptosis in a caspase-independent manner [7]. In a recent study we have shown that some members of a series of novel pyrrolo-1,5-benzoxazepines (PBOXs) induce apoptosis as shown by cell shrinkage, chromatin condensation, and DNA fragmentation in three human cell lines, HL-60 promyelocytic, Jurkat T lymphoma and Hut-78 lymphoma cells [8]. This indicated the potential of these compounds for use as anti-cancer drugs and prompted us to examine both the effect of these compounds on other cancerous cell lines and the mechanism by which these novel compounds induce apoptosis. In the present study we determined whether a potent member of this series, PBOX-6, could induce apoptosis in MCF-7 cells, which of the remaining effector caspases (-6 and -7) were activated in response to PBOX-6 treatment and whether apoptotic cell death in this case was accompanied by the usual morphological features such as DNA fragmentation. This study enabled us to determine whether caspase-3 is really an absolute requirement for DNA fragmentation associated with apoptosis in intact cells or whether another effector caspase such as caspase-6 or -7 may mediate this apoptotic hallmark in caspase-3-deficient MCF-7 cells.

2. Materials and methods

2.1. Cell culture and induction of apoptosis

MCF-7 breast carcinoma cells were obtained from the European Collection of Animal Cell Culture (Salisbury, UK) and were grown in minimum essential medium supplemented with 1% non-essential amino acids, 10% foetal calf serum, gentamicin (0.1 mg/ml), and L-glutamate (final concentration 2 mM), all obtained from Sigma (Poole, Dorset, UK). The pyrrolobenzoxazepine 7-[(dimethylcarbamoyl)-oxy]-6-(2-naphthyl)pyrrolo-[2,1-*d*][1,5]-benzoxazepine (PBOX-6) was synthesised as described previously [8]. Cells were seeded at a density of 3×10^5 cells/ml and following treatment with the PBOX-6 or the caspase-7 inhibitor, DEVD-cmk (Calbiochem-Novabiochem, Nottingham, UK), an aliquot (100 μl) was cytocentrifuged onto glass slides precoated with poly-L-lysine. They were then stained with the Rapi-Diff kit (Diagnostic Developments, Burscough, Lancashire, UK)

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Abbreviations: PBOX, pyrrolo-1,5-benzoxazepine; AMC, amino-4-methyl coumarin; CAD, caspase-activated DNase; ICAD, inhibitor of caspase-activated DNase

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under conditions described by the manufacturer. The degree of apoptosis and necrosis was determined by counting approximately 300 cells under a light microscope. At least three fields of view per slide, with an average of approximately 100 cells per field, were counted and the percent apoptosis and necrosis were determined. TUNEL staining, characteristic of DNA fragmentation, was performed as described previously [9]. Cells were fixed in 4% paraformaldehyde, permeabilised in 0.1% Triton X-100 solution and labelled using terminal transferase and horseradish peroxidase (Boehringer Mannheim). Colorimetric detection was performed using diaminobenzidine.

2.2. Caspase assay

Cells (5×10^6 cells) were harvested by trypsinisation, centrifugation, washing in ice-cold phosphate-buffered saline and the pellets resuspended in 200 μl of harvesting buffer (20 mM HEPES, pH 7.5, 10% (w/v) sucrose, 0.1% (w/v) CHAPS, 2 mM dithiothreitol, 0.1% (v/v) Nonidet NP40, 1 mM sodium EDTA and 1 mM phenylmethylsulfonyl fluoride) supplemented with protease inhibitors (1 μg/ml pepstatin A and 1 μg/ml leupeptin). Following incubation on ice for 15 min, samples were centrifuged at $20\,000\times g$ for 20 min and the resulting supernatants used to measure caspase-6 or -7 activity. This enzyme activity was determined by a fluorometric assay using either the substrate Ac-DEID-AMC or Ac-DEVD-AMC (Alexis, Nottingham, UK), which is cleaved by caspase-6 or -7, respectively, to release the fluorescent leaving group, amino-4-methyl coumarin (AMC). Enzyme extracts (50 μg) were incubated with 100 mM HEPES, pH 7.5, containing 10% (w/v) sucrose, 0.1% (w/v) CHAPS, 10 mM dithiothreitol and 20 μM substrate in a total reaction volume of 3 ml. Following incubation for 60 min at 25°C, fluorescence was monitored continuously using a spectrofluorimeter (excitation wavelength 380 nm, emission wavelength 460 nm). The amount of AMC released was determined by comparison with a standard curve generated with known amounts of AMC. Western blotting to measure expression of procaspase-3 in MCF-7 cell extracts was performed as previously described [7] using an anti-pro-caspase-3 antibody (Pharmingen, San Diego, CA, USA).

2.3. Fluorescence-activated cell sorting (FACS) analysis

FACS analysis was performed as described previously [10] with a FACScalibur flow cytometer (Becton Dickinson). Analysis of data was performed using the computer programme Cell Quest.

3. Results and discussion

3.1. DNA fragmentation can be induced in MCF-7 cells during PBOX-6-mediated apoptosis

MCF-7 cells were shown to undergo apoptosis in response to PBOX-6 treatment in a dose- and time-dependent manner (Fig. 1A,B). This induction of apoptosis was accompanied by many of the typical morphological features associated with apoptotic cell death, including cell shrinkage, chromatin condensation and nuclear fragmentation as observed by light microscopy of cytospin preparations (Fig. 2A,B). In contrast to this result other reports have shown that MCF-7 cells treated with various apoptotic stimuli (e.g. transforming growth factor-β1, etoposide and TNF) undergo cell death in the absence of DNA fragmentation [5,11]. In agreement with these reports, we have shown that TNF/cycloheximide (Chx) treatment of MCF-7 cells resulted in cell death but that nuclear fragmentation was not observed (Fig. 2C). TUNEL staining (Fig. 3A,B) of PBOX-6-treated MCF-7 cells confirmed that apoptotic cell death in response to this death stimuli was accompanied by DNA fragmentation. In addition cell cycle analysis with propidium iodide-stained cells (Fig. 3C) confirmed these results, because PBOX-6 induced the appearance of a sub-G_1 apoptotic peak (22% of cells in PBOX-6-treated cells compared to less than 3% in control cells) characteristic of DNA fragmentation. These results indicate that only some death stimuli (e.g. TNF), that induce apoptosis in MCF-7 cells, do so without the accompaniment of DNA fragmentation.

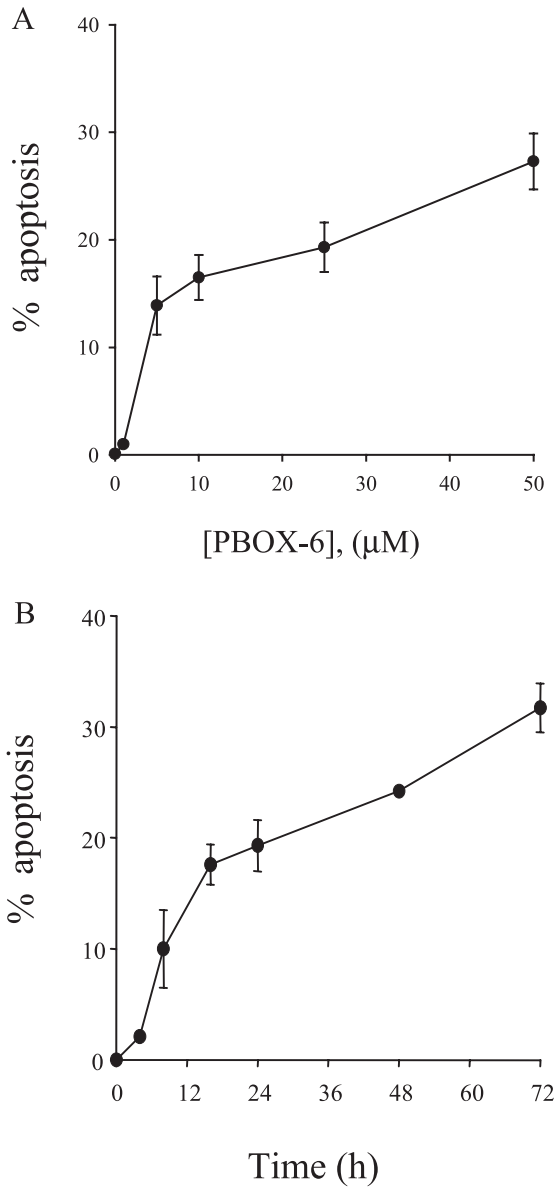


Fig. 1. PBOX-6 induces apoptosis in MCF-7 cells in a dose- and time-dependent manner. MCF-7 cells were seeded at a density of 2×10^5 cells/ml and were treated with (A) either a range (0–50 μM) of concentrations of PBOX-6 for 16 h or (B) one concentration of PBOX-6 (25 μM) for a period of 4, 8, 16, 24, 48 and 72 h. The percentage of apoptosis was determined by cytospinning and staining the cells with the RapiDiff kit. Values represent the means ± S.E.M. for three separate experiments.

3.2. Activation of caspase-7 accompanies the induction of apoptosis by PBOX-6 in MCF-7 cells

It has been widely reported that MCF-7 cells do not express detectable levels of caspase-3, a result that we have confirmed by Western blotting (results not shown). Caspase-3 has been reported to initiate DNA fragmentation by proteolytically inactivating ICAD, which releases the active endonuclease CAD. This endonuclease then cleaves DNA. It has thus been suggested that caspase-3 is essential for DNA fragmentation during apoptosis [5]. In contrast to this, the results

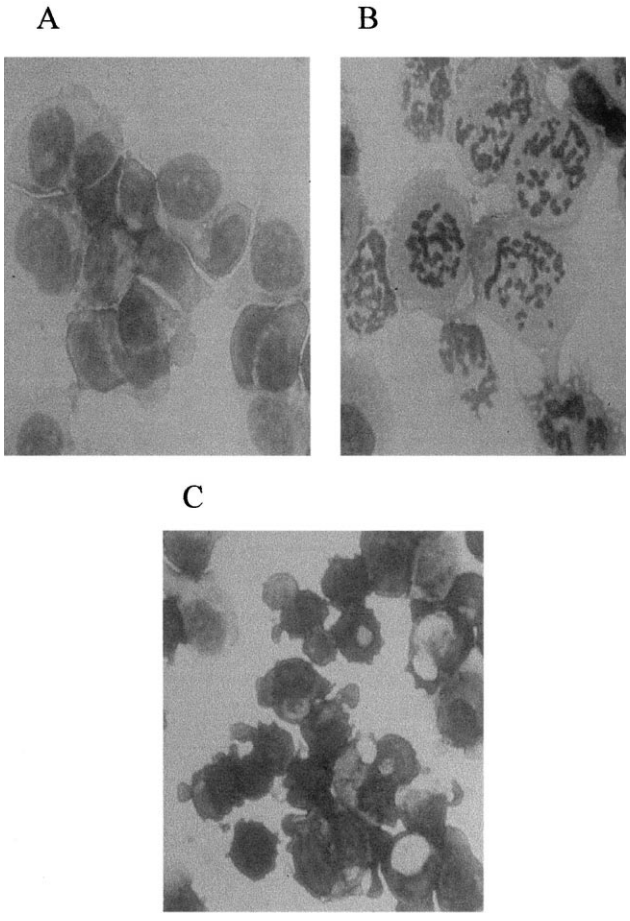


Fig. 2. Morphological assessment of chromatin condensation and nuclear fragmentation in MCF-7 cells undergoing apoptosis in response to treatment with PBOX-6. Microscopic analysis of MCF-7 cells was performed on cytospin samples which were fixed in methanol. The nucleus was stained pink with eosin-Y and the cytoplasm was stained blue with methylene blue. Vehicle (1% ethanol)-treated cells (A) are characterised by a continuous plasma membrane and an intact nucleus. PBOX-6-treated cells (B) display the morphological features of apoptosis that include chromatin condensation and nuclear fragmentation. TNF/Chx-treated cells (C) display a lack of nuclear fragmentation.

presented here suggest that another effector caspase may take over the role of caspase-3 in mediating DNA fragmentation in caspase-3-deficient MCF-7 cells. We thus examined whether the other effector caspases-6 and/or -7 become activated in MCF-7 cells in response to PBOX-6 treatment. Like caspase-3, caspase-7 cleaves a DEVD-based peptide substrate, whereas caspase-6 cleaves a DEID-based peptide substrate [1]. Cytosolic extracts from MCF-7 cells treated with PBOX-6 were incubated with either the fluorogenic caspase-6 substrate DEID-AMC or the caspase-7 substrate DEVD-AMC. HL-60 cells treated with etoposide (50 μM for 6 h) were used as a positive control for caspase-6 activation. As shown in Fig. 4A, treatment of MCF-7 cells with PBOX-6 did not cause activation of caspase-6 but did result in caspase-7 activation (Fig. 4B).

3.3. *Caspase-7 activity is involved in PBOX-6-mediated apoptosis*

Caspases are specifically inhibited in vitro and in vivo by cell permeable tetrapeptides designed to mimic cleavage sites of their respective substrates [12]. Pretreatment of MCF-7 cells with the caspase-7 inhibitor, z-DEVD-cmk, followed by treatment for a further 24 h with PBOX-6 significantly reduced both the activity of caspase-7 (Fig. 4B) and the appearance of the morphological signs of apoptosis (Fig. 4C). Inhibition of caspase-7 activity correlated directly with the level of protection observed against apoptosis. Pretreatment of MCF-7 cells for 1 h with the general caspase inhibitor, z-VAD-fmk (200 μM), inhibited the appearance of the morphological signs of apoptosis to the same extent as that shown with z-DEVD-fmk, however, no protective effect against apoptosis was observed when cells were treated with the caspase-1-like inhibitor, YVAD-fmk (results not shown). This would suggest that caspase-7 is involved in the mechanism by which PBOX-6 induces apoptosis in MCF-7 cells.

3.4. *Caspase-7 may be responsible for DNA fragmentation in apoptotic MCF-7 cells*

Because neither caspase-3 (due to its lack of expression in these cells) nor caspase-6 becomes activated in MCF-7 cells in

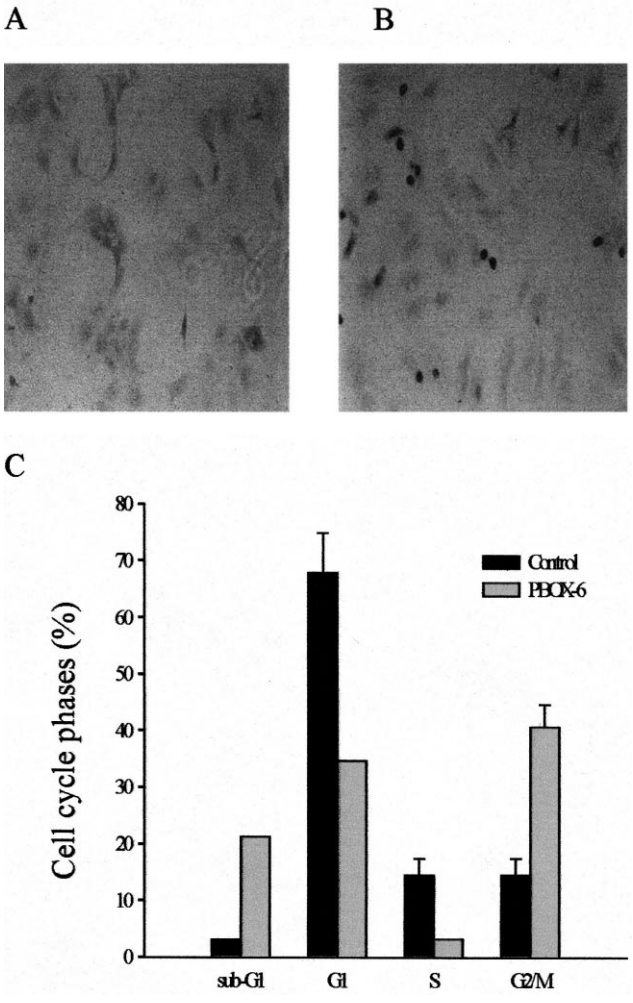
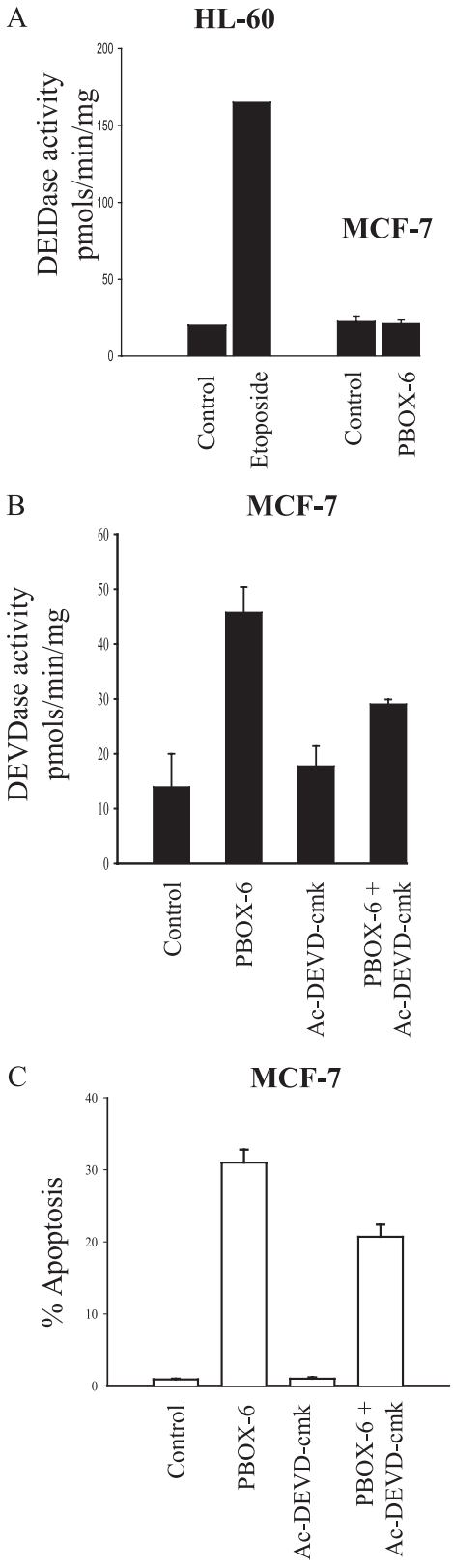


Fig. 3. Confirmation of DNA fragmentation in MCF-7 cells, in response to PBOX-6 treatment, by TUNEL staining and FACS analysis. TUNEL staining of vehicle- (A) or PBOX-6-treated (48 h) MCF-7 cells (B). C: Changes in cell cycle phase distribution following PBOX-6 treatment of MCF cells. The cell cycle phases G_1, S and G_2/M as well as the sub-G_1 apoptotic peak are indicated. FACS analysis was performed with propidium iodide-stained cells as described in Section 2.

Fig. 4. PBOX-6 induces apoptosis in MCF-7 cells through activation of caspase-7. A: Cytosolic extracts (50 μg of protein) prepared from either HL-60 cells treated with etoposide (50 μM) for 6 h or from MCF-7 cells treated with PBOX-6 (25 μM) for 24 h were incubated with DEID-AMC to assess caspase-6 activation. The amount of fluorochrome released was determined by comparison to an AMC standard curve. B and C: MCF-7 cells were treated with PBOX-6 (25 μM) for 24 h, in the absence or presence of the inhibitor DEVD-cmk (200 μM). Cytosolic extracts (50 μg of protein) were prepared and were incubated with DEVD-AMC to assess caspase-7 activation (B). The percentage of apoptosis was determined by cytospinning and staining the cells with the RapiDiff kit (C). Values represent the means ± S.E.M. of three separate experiments. →



response to PBOX-6 treatment this would suggest that the other remaining effector caspase, caspase-7, which becomes activated, may be responsible for the DNA fragmentation exhibited by MCF-7 cells treated with PBOX-6. This suggestion is supported by a recent report which demonstrated that in an in vitro CAD assay, of all the proteinases suggested to participate in apoptotic DNA fragmentation, only caspase-3 and caspase-7 induced DNA fragmentation by inactivating ICAD [1]. These workers also showed that in vitro, however, caspase-3 inactivated ICAD and promoted DNA fragmentation more effectively than caspase-7. In agreement with this report, we suggest that although caspase-3 may be the primary regulator of apoptotic DNA fragmentation via proteolysis of ICAD, caspase-7 may mediate this apoptotic hallmark in response to certain death stimuli such as PBOX-6, in caspase-3-deficient cells. As we were unable to completely inhibit apoptosis using the general caspase inhibitor, z-VAD-fmk, or the caspase-7 inhibitor, z-DEVD-fmk, we cannot exclude the possibility that in addition to caspase-7, non-caspase proteases such as cathepsins, or endonuclease G, may also participate in apoptosis induced by PBOX-6. We also show in the present study that apoptosis induction in MCF-7 cells in response to TNF/Chx treatment does not result in caspase-7

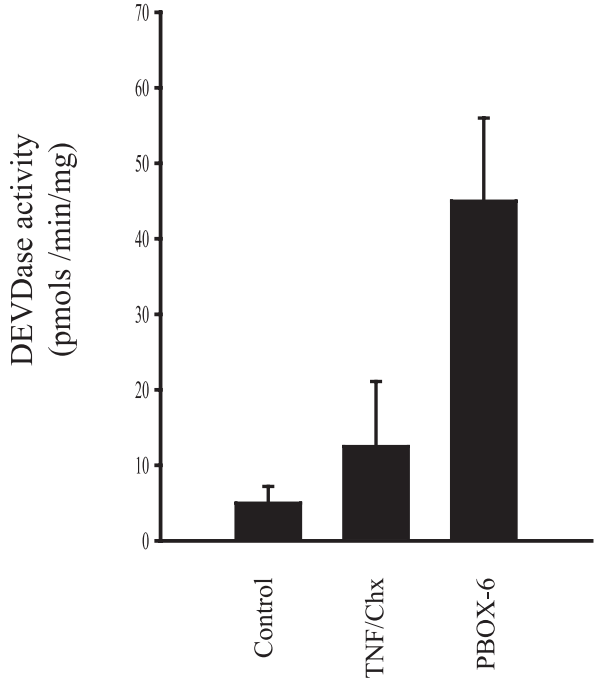


Fig. 5. Lack of activation of caspase-7 in MCF-7 cells in response to treatment with TNF/Chx. Cytosolic extracts (50 μg of protein) prepared from MCF-7 cells treated with either TNF/Chx for 20 h or PBOX-6 for 24 h were incubated with DEVD-AMC to assess caspase-7 activation. The amount of fluorochrome released was determined by comparison to an AMC standard curve. Values represent the means ± S.E.M. of four separate experiments.

activation (Fig. 5). This may explain why DNA fragmentation does not occur in MCF-7 cells in response to TNF/Chx.

In this study we report the detection of caspase-7-like DEVDase activity in extracts from MCF-7 apoptotic cells. These results are in agreement with Germain et al. [13] who reported that staurosporine treatment of MCF-7 cells resulted in caspase-7 activation with concomitant cleavage of Parp. Apoptosis induced by PBOX-6 in MCF-7 cells was associated with DNA fragmentation, as demonstrated by light microscopy, TUNEL staining and propidium iodide staining with FACS analysis. This is in contrast to the recent study showing that apoptotic MCF-7 cells, despite caspase-7 activation, failed to fragment DNA [1]. However the stimulus used to induce apoptosis in MCF-7 cells was not reported. Perhaps in that particular instance caspase-7 activation did not reach the level required to initiate DNA fragmentation.

In conclusion, induction of apoptosis in MCF-7 cells by a novel pyrrolobenzoxazepine, PBOX-6, appears to be mediated in part by activation of one of the effector caspases, caspase-7. Apoptosis is accompanied by DNA fragmentation, suggesting that in these caspase-3-deficient cells, caspase-7 may mediate this apoptotic hallmark. It would also suggest that caspase-3 is not necessarily essential for DNA fragmentation associated with apoptosis.

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