ANTICANCER RESEARCH *25*: 4197-4202 (2005)

Caspase-3 Activation and Induction of PARP Cleavage by Cyclic Dipeptide Cyclo(Phe-Pro) in HT-29 Cells

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Abstract. *Background: Cyclo(Phe-Pro) has been shown to inhibit cancer cell growth and induce apoptosis in HT-29 colon cancer cells. Materials and Methods: The molecular mechanisms mediating cyclo(Phe-Pro)-induced apoptosis in HT-29 cells were investigated. Cells were treated with 5 mM or 10 mM cyclo(Phe-Pro) for varying times. Immunoblot analysis was used to detect poly(ADP-ribose)polymerase (PARP) cleavage. A fluorescence-based enzymatic assay was used to measure caspase-3 activity. Results: Cyclo(Phe-Pro) (10 mM) induced time-dependent cleavage of PARP, detected as early as 8 hours post treatment. PARP cleavage was blocked by co-administration with the broad-range caspase inhibitor Z-VAD-FMK. Cyclo(Phe-Pro) also induced a time-dependent increase ($p<0.01$) in caspase-3 activity. This increase in activity was blocked in the presence of the caspase-3 inhibitor Ac-DEVD-CHO. Conclusion: These results provide evidence that cyclo(Phe-Pro)-induced apoptosis in HT-29 cells is mediated by a caspase cascade. These findings warrant further investigation into the potential antitumour activity of cyclo(Phe-Pro) and its related cyclic dipeptide derivatives.*

An increasing number of cyclic dipeptides (CDPs) and other diketopiperazine derivatives have been shown to modulate important physiological activities *in vitro* and *in vivo* (1-6). In particular, the CDP cyclo(Phe-Pro) has been shown to exhibit antifungal activity (4), antibacterial activity (7) and the potential to induce differentiation in HT-29 colon cancer cells (3). We have previously shown that cyclo(Phe-Pro) inhibits cell proliferation and induces apoptosis in HT-29 colon cancer cells (8). However, the molecular mechanisms mediating cyclo(Phe-Pro)-induced apoptosis have not been studied.

Apoptosis or programmed cell death is a distinct, intrinsic cell death programme that occurs in various homeostatic or pathological conditions in which the affected cell orchestrates its own demise. Apoptosis has been described as the physiological counterpart of necrosis or "accidental" cell death, which is an uncontrolled, catabolic and degenerative process that often leads to tissue damage (9). The induction of tumour cell death by various approaches such as anticancer drugs, gamma-irradiation, suicide genes or immunotherapy has been shown to be mediated through induction of apoptosis in the target cells (10, 11). A family of cysteine proteases, the caspases, are known to play a central role in various models of cell death (12-14). The activation of effector caspases such as caspase-3 leads to downstream cleavage of various cytoplasmic or nuclear substrates including PARP. These downstream cleavage events mark many of the morphological features of apoptotic cell death (15). Given the important role of caspases as effector molecules in various forms of cell death including drug-induced apoptosis, the ability of anticancer agents to trigger caspase activation appears to be a critical determinant of sensitivity or resistance to cytotoxic therapies (16, 17). The aim of the present study was to investigate the potential of cyclo(Phe-Pro) to induce caspase-3 activation and PARP cleavage in HT-29 colon cancer cells.

Materials and Methods

Chemicals. Cyclo(Phe-Pro) was synthesized from the corresponding linear dipeptide (Bachem, Budendorf, Switzerland) by phenol cyclization (18). Structural elucidation of the synthetic product was performed with the aid of infrared spectroscopy, mass spectrometry and NMR spectroscopy.

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Key Words: Cyclic dipeptides, cancer cells, HT-29, poly(ADP-ribose)polymerase (PARP), caspase-3, apoptosis.

Cell culture conditions. HT-29 cells were cultured in 10-cm culture dishes without antibiotics in RPMI 1640 medium (Sigma, Steinheim, Germany) containing 10% heat-inactivated foetal bovine serum, 25 mM HEPES and 2 mM glutamine (Highveld Biological, Johannesburg, South Africa), in a humidified 5% CO_2 incubator at 37˚C.

Detection of PARP cleavage. Exponentially growing HT-29 cells were seeded into 60-mm culture dishes at a density of $4x10^4$ cells/cm^2 and allowed a 24-hour recovery period. In one set of experiments, cells were treated with 5 and 10 mM cyclo(Phe-Pro) for 24 and 48 hours. In a second set of experiments, cells were treated with 10 mM cyclo(Phe-Pro) for 1, 4, 8 and 24 hours to examine the time-course of PARP cleavage over 24 hours. Control cells were incubated with growth medium containing an equivalent percentage of DMSO (0.5% final concentration) used to dissolve cyclo (Phe-Pro).

The effect of Z-VAD-FMK and calpain inhibitor protein on PARP cleavage. To investigate the potential role of caspases in cyclo(Phe-Pro)-induced apoptosis, HT-29 cells were treated with 10 mM cyclo(Phe-Pro) for 8, 12, 16 and 24 hours, with or without 50 μM pre-treatment of the broad-range caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp-(OMe)-fluoromethylketone (Z-VAD-FMK). The calpain inhibitor peptide (Sigma) was used to determine whether calpain activation plays a role in cyclo(Phe-Pro)-induced apoptosis in HT-29 cells. The cells were treated with 10 mM cyclo(Phe-Pro) for 8 and 12 hours, with or without 50 μM of the calpain inhibitor peptide.

Protein extraction and Western blotting for PARP. All protein extraction steps were carried out at 4˚C or on ice. Both adherent cells and detached cells released into the growth medium were collected, washed twice in phosphate-buffered saline (PBS) and centrifuged at 500 x g for 10 minutes. For the extraction of total cellular proteins, the cell pellets were resuspended in PARP extraction buffer [6 M urea, 2% (w/v) SDS, 10% (v/v) glycerol, 62.5 mM Tris-HCl (pH 6.8), 5% (v/v) β-mercaptoethanol (freshly added) and 0.3% (w/v) bromophenol blue]. The protein concentration was determined by the BCA protein assay (Sigma) in the absence of β-mercaptoethanol. The cell extracts were sonicated for 15 seconds (Sonoplus, Bandelin, HD 2200, MS 73 probe), incubated at 65˚C for 15 minutes, and protein samples of 35 or 50 μg (as indicated) were separated by SDS-PAGE.

The proteins were transferred to a PVDF membrane and non-specific binding sites were blocked by incubating the membrane for 60 minutes in TBS-T buffer [20 mM Tris (pH 7.6), 137 mM NaCl and 0.015% Tween 20] containing 5% skim milk powder. For the immunoassay, the membrane was incubated with a mouse monoclonal antibody (diluted 1:1000) (Chemicon International, Hofheim, Germany) that recognizes the N-terminal region of PARP. Immunodetection was performed according to the manufacturer's instructions for the enhanced chemiluminescence (ECL) detection kit (Amersham, Upsala, Sweden), using a secondary rabbit anti-mouse immunoglobulin antibody (diluted 1:2000) conjugated to horseradish peroxidase. The membranes were exposed to X-ray film (Amersham) for 1-5 minutes.

Caspase-3 activity assay. HT-29 cells were seeded into 60-mm culture dishes ($4x10^4$ cells/cm^2) and treated with 10 mM cyclo(Phe-Pro) for 8 and 12 hours, with or without the caspase-3 inhibitor Ac-DEVD-CHO (Promega, Madison, USA). Following treatment, the cells were washed once with PBS (4˚C) and centrifuged at 500 x g for 10 minutes at 4˚C. The cells were resuspended in hypotonic cell lysis buffer [25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 5 mM EDTA, 5 mM DTT, 2 mM PMSF, 10 μg/ml pepstatin A and 10 μg/ml leupeptin] at a density of 10^7 cells/ml and subjected to four cycles of freezing and thawing. The cell lysates were centrifuged at 16,000 x g for 20 minutes at 4˚C and the supernatant fraction was collected and assayed for caspase-3 activity. The protein concentration was estimated by the Bradford assay (Sigma). Caspase-3 activity was measured by using a fluorometric caspase assay kit (Promega), according to the manufacturer's instructions. Ac-DEVD-AMC was used as substrate and the specific activity was calculated as pmol AMC liberated/minute at 37˚C/μg protein.

Toxicity testing in Caco-2 cells. Differentiated Caco-2 cells are considered to express a normal phenotype of the gastrointestinal epithelium (19) and have been proposed as a model for screening of substance toxicity (20). In order to investigate whether cyclo(Phe-Pro) was toxic to cells of a normal phenotype, differentiated Caco-2 cells were treated with 10 mM cyclo(Phe-Pro) for 72 hours. Cell viability was measured using the MTT assay, as previously described (8).

Results

Induction of PARP cleavage by cyclo(Phe-Pro). HT-29 cells were treated with 5 and 10 mM cyclo(Phe-Pro) for 24 and 48 hours. Figure 1 shows the resultant immunoblot for the detection of PARP cleavage. In control cells (lane 1), PARP remained uncleaved (M_r 113 kDa). No PARP cleavage was detected in cells treated with 5 mM cyclo(Phe-Pro) for 24 or 48 hours (lanes 2 and 3, respectively). The appearance of a distinct M_r 24 kDa band in cells treated with 10 mM cyclo(Phe-Pro) for 24 hours (lane 4) was indicative of caspase-dependent PARP cleavage (21). Forty-eight hours after treatment with 10 mM cyclo(Phe-Pro) (lane 5), the levels of the cleavage fragment (M_r 24 kDa) decreased, returning to those comparable with untreated cells. However, the concomitant decrease in the level of uncleaved PARP in these cells (M_r 113 kDa, lane 5) demonstrated that PARP cleavage did occur. It is reasonable to speculate that the absence of the M_r 24 kDa band in these cells (lane 5) could be attributed to further degradation by cellular proteases. Heerdt *et al.* (22) reported a similar "loss" of PARP cleavage in MCF-7 cells after 24 hours of treatment with tributyrin, a triglyceride analogue of butyrate. The presence of an unexpected $M_r \sim 45$ kDa band (lanes 1-4) was initially thought to be an artefact of non-specific binding to a protein that may have been abundant in the cell extracts.

In a subsequent set of experiments, HT-29 cells were treated with 10 mM cyclo(Phe-Pro) to investigate the progression of PARP cleavage up to 24 hours, and to establish a time-point for the onset of PARP cleavage. Cell

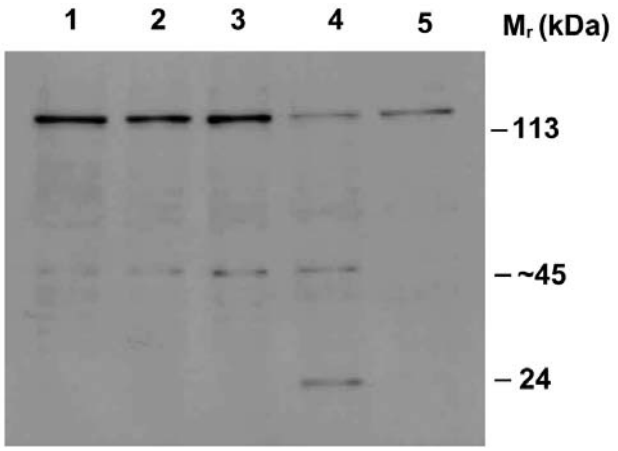

Figure 1. *Immunoblot showing the detection of PARP cleavage in HT-29 cells treated with 5 and 10 mM cyclo(Phe-Pro) for 24 and 48 hours. Lanes: 1, untreated control cells; 2 and 3, 5 mM cyclo(Phe-Pro) for 24 and 48 hours, respectively; 4 and 5, 10 mM cyclo(Phe-Pro) for 24 and 48 hours, respectively.*

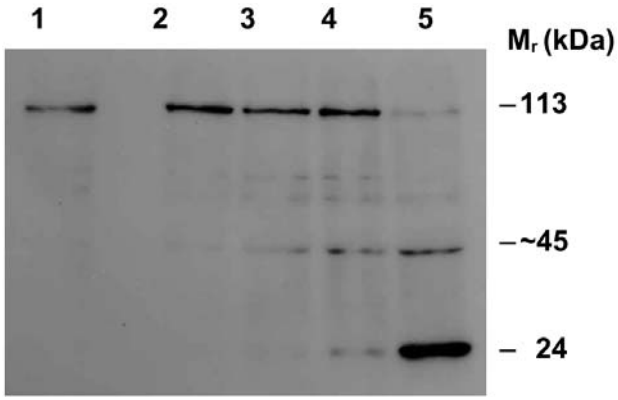

Figure 2. *Immunoblot showing the time-course study of PARP cleavage in cyclo(Phe-Pro)-treated HT-29 cells up to 24 hours. Lanes: 1, untreated control cells; 2, 3, 4 and 5, cells treated with 10 mM cyclo(Phe-Pro) for 1, 4, 8 and 24 hours, respectively. Thirty-five μg of protein was loaded in lanes 1-4; 50 μg of protein was loaded in lane 5 in order to compare this to the results shown in Figure 3.*

extracts were obtained at 1, 4, 8 and 24 hours after exposure to 10 mM cyclo(Phe-Pro). PARP cleavage was detectable as early as 8 hours following cyclo(Phe-Pro) treatment, demonstrated by the appearance of a faint band corresponding to the M_r 24 kDa cleavage fragment (Figure 2, lane 4). After 24 hours, PARP cleavage had progressed further (lane 5), demonstrated by a marked decrease in the level of the M_r 113 kDa fragment, concomitant with an increase in the M_r 24 kDa cleavage fragment. Lanes 1-4 were loaded with 35 μg of protein, whereas lane 5 was loaded with 50 μg of protein to be comparable to results shown in Figure 3. Interestingly, the intensity of the unexpected $M_r \sim 45$ kDa band appeared to increase with time, from 1 hour to 8 hours of treatment with cyclo(Phe-Pro) (Figure 2, lanes 2-4) and was still prominent in lane 5. Thus, it was suspected that the presence of the $M_r \sim 45$ kDa band was more than simply an artefact of non-specific binding.

The effect of Z-VAD-FMK on PARP cleavage. The broad-range caspase inhibitor, Z-VAD-FMK, was used to evaluate the role of caspases in cyclo(Phe-Pro)-induced apoptosis. The resultant immunoblot is shown in Figure 3, demonstrating that 10 mM cyclo(Phe-Pro) induced PARP cleavage after 8, 12, 16 and 24 hours of treatment (lanes 2, 4, 6 and 8, respectively). Co-administration of Z-VAD-FMK inhibited the cyclo(Phe-Pro)-induced PARP cleavage in HT-29 cells at each time-point, namely 8, 12, 16 and 24 hours (lanes 3, 5, 7 and 9, respectively). These results strongly suggest that cyclo(Phe-Pro) treatment of HT-29 cells triggers the induction of apoptosis involving caspase-3-like activity.

The $M_r \sim 45$ kDa fragment observed during the course of PARP cleavage was clearly shown to be a specific cleavage product by its inhibition in the presence of Z-VAD-FMK (Figure 3, lanes 3, 5, 7, and 9), compared to cells that received cyclo(Phe-Pro) in the absence of inhibitor (lanes 2, 4, 6 and 8). These results demonstrated that the formation and variability of the $M_r \sim 45$ kDa fragment was not simply due to non-specific binding. Instead, this anomaly was related to cyclo(Phe-Pro)-induced, caspase-dependent PARP cleavage in HT-29 cells. A possible explanation for this outcome was the simultaneous activation of the calcium-dependent protease, calpain, in these cells. It has been shown that the cleavage of PARP by calpain (specifically m-calpain), *in vitro*, yields fragments that range in M_r from 70 to 40 kDa (23). In addition, it is known that calpain and caspase-3 share a number of dual substrates (23), and that both calpain and caspases can be activated by the same apoptotic stimulus in various cells (24-26). The possibility of calpain activation was, therefore, explored in this study by investigating the effects of the calpain inhibitor peptide on PARP cleavage. Treatment of HT-29 cells with 50 μM calpain inhibitor peptide had no detectable effect on cyclo(Phe-Pro)-induced PARP cleavage after 8 or 12 hours of co-administration with 10 mM cyclo(Phe-Pro) (Figure 4).

Cyclo(Phe-Pro) induces caspase-3 activation. To confirm the participation of effector caspases, HT-29 cells, treated with cyclo(Phe-Pro) for 0, 8 and 12 hours, were analysed for caspase-3 activity. The results are shown in Figure 5, illustrating a time-dependent activation of caspase-3. Since the fluorogenic substrate Ac-DEVD-AMC may be

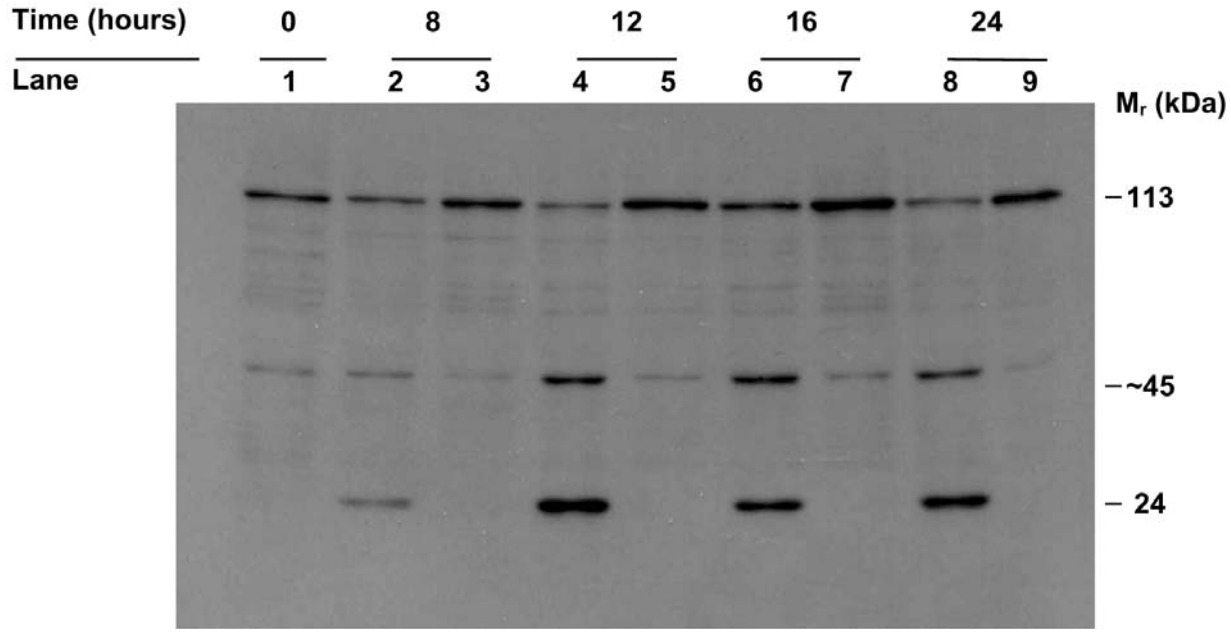

Figure 3. *Immunoblot showing the effect of the caspase inhibitor Z-VAD-FMK on cyclo(Phe-Pro)-induced PARP cleavage in HT-29 cells. Lane 1: untreated control cells; lanes 2, 4, 6 and 8: cells treated with 10 mM cyclo(Phe-Pro) for 8, 12, 16 and 24 hours, respectively; lanes 3, 5, 7 and 9: cells treated with 10 mM cyclo(Phe-Pro) in the presence of inhibitor (50 μM) for 8, 12, 16 and 24 hours, respectively. Fifty μg of protein was loaded in each lane to clearly demonstrate the change in the $M_r \sim 45$ kDa fragment.*

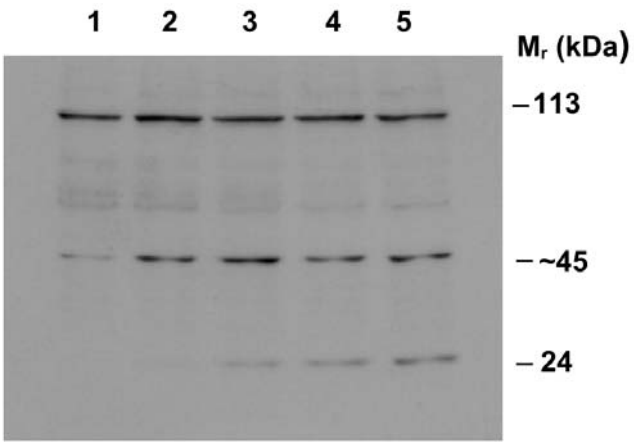

Figure 4. *Immunoblot showing the effect of calpain inhibitor peptide on cyclo(Phe-Pro)-induced PARP cleavage in HT-29 cells. Lanes: 1, untreated control cells; 2 and 4, cells treated with 10 mM cyclo(Phe-Pro) for 8 and 12 hours, respectively; 3 and 5, cells treated with 10 mM cyclo(Phe-Pro) in the presence of calpain inhibitor (50 μM) for 8 and 12 hours, respectively. Thirty-five μg of protein was loaded in each lane.*

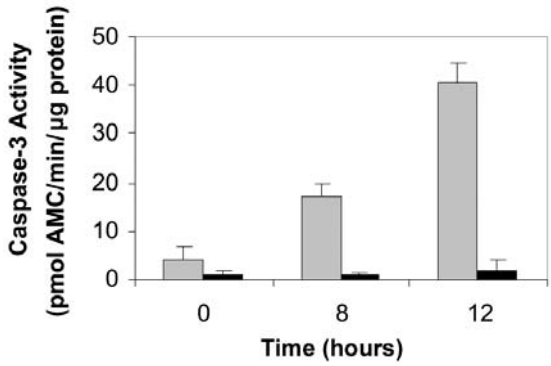

Figure 5. *Increase in caspase-3 activity during cyclo(Phe-Pro)-induced apoptosis in HT-29 cells. Cells were treated with cyclo(Phe-Pro) for 0, 8 and 12 hours with (black) or without (grey) the caspase-3 inhibitor Ac-DEVD-CHO. The results are expressed as the mean±SD of triplicate determinations, representative of three independent experiments.*

susceptible to cleavage by other related proteinases, the specific contribution of caspase-3 activity was confirmed by using the inhibitor Ac-DEVD-CHO. These results are consistent with previous reports of butyrate-induced apoptosis in LIM 1215 colorectal cancer cells, which showed caspase-3 activation within 16 hours of treatment (27).

Toxicity testing in Caco-2 cells. Cells treated with 10 mM cyclo(Phe-Pro) for 72 hours showed no significant difference in viability (94.14±3.62 %, n=2) compared to untreated control cells. However, the selective toxicity of

dietary factors, such as glucosinolate hydrolysis products, has previously been reported in HT-29 *versus* differentiated Caco-2 cells (28).

Discussion

The biological diversity of CDPs is exemplified by their ubiquitous nature, physicochemical properties and the wide range of biological processes affected by this unique group of compounds. Cyclo(Phe-Pro), in particular, has been shown to exhibit significant biological activity *in vitro* including antibacterial (7) and antifungal activity (4), as well as the potential to induce differentiation in HT-29 colon cancer cells (3). Furthermore, we have previously shown that cyclo(Phe-Pro) induces apoptosis in HT-29 colon cancer cells with morphological features indicative of caspase activation (8). In the present study, the roles of caspase-3 activation and PARP cleavage were investigated as part of the molecular events mediating cyclo(Phe-Pro)-induced apoptosis in HT-29 cells.

We report, here, that cyclo(Phe-Pro) induced time-dependent cleavage of PARP, one of the hallmark events of apoptosis (29). The use of the broad-range caspase inhibitor Z-VAD-FMK, which inhibited PARP cleavage, demonstrated the involvement of the caspase cascade. These results are consistent with previous reports of Z-VAD-FMK inhibition of caspase-dependent PARP cleavage in drug-induced apoptosis (30-32) and indicate that caspases are key components of cyclo(Phe-Pro)-induced apoptosis in HT-29 cells.

We also report a novel finding compared to previous reports of typical PARP cleavage (21); *i.e.* the appearance of an unexpected $M_r \sim 45$ kDa fragment in addition to the characteristic M_r 24 kDa fragment (Figures 1-3). These results raise a number of interesting possibilities. First, the atypical pattern of PARP cleavage observed here could be attributed to HT-29 cells undergoing late stage apoptosis (secondary necrosis). A similar result was obtained by Shah *et al*. (33), who reported the appearance of a minor degradation fragment of PARP at $M_r \sim 47$ kDa in drug-induced apoptosis in HL-60 human promyelomonocytic leukemia cells undergoing secondary necrosis. Alternatively, there have been reports of the calcium-dependent protease, calpain, being activated in various models of apoptotic cell death, resulting in atypical PARP cleavage (23, 30). Z-VAD-FMK is known to inhibit both calpains and caspases in drug-induced apoptosis (24, 26, 34), therefore, it is possible that both calpains and caspases may be activated by cyclo(Phe-Pro)-induced apoptosis in HT-29 cells. To examine these possibilities, the specific calpain inhibitor peptide was co-administered with cyclo(Phe-Pro). This failed to inhibit the appearance of the atypical $M_r \sim 45$ kDa fragment, suggesting that this fragment is not a product of calpain activity. However, it should be noted that the calpain inhibitor peptide is derived from the calpain inhibitory domain of calpastatin, the endogenous inhibitor of calpain (35), and caspase-3 has been shown to cleave calpastatin in some cell death models (23), thus perpetuating calpain activation. It is conceivable that the activation of caspase-3 in cyclo(Phe-Pro)-induced apoptosis could have blocked or bypassed the inhibitory effect of the calpain inhibitor peptide. Thus, further investigation is needed to probe the possibility of calpain activation in cyclo(Phe-Pro)-induced apoptosis. Although cyclo(Phe-Pro) induced an atypical pattern of PARP cleavage, the activation of caspase-3 in this system confirmed the participation of effector caspases.

The lack of significant growth inhibition by 10 mM cyclo(Phe-Pro) in differentiated Caco-2 cells indicated a selective toxicity of these CDPs towards cancer cells while having little effect on normal cells, as also suggested by Gamet-Payrastre *et al*. (28). Future work should include toxicity studies on a broad range of normal human cells to confirm the selectivity of these CDPs towards cancerous cells. Nevertheless, the data presented here demonstrate a potential antitumour activity for cyclo(Phe-Pro), that may be harnessed by its use as a lead compound in the development of a cyclic dipeptide derivative with potent and selective antitumour activity.

Acknowledgements

This study was supported by the National Research Foundation of South Africa.

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Received June 10, 2005
Accepted July 26, 2005