

Selected Cyclic Dipeptides Inhibit Cancer Cell Growth and Induce Apoptosis in HT-29 Colon Cancer Cells

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Abstract. *Background:* An increasing number of cyclic dipeptides (CDPs), particularly those containing proline, have been shown to exhibit important biological activity. *Materials and Methods:* We investigated the potential of seven proline-based CDPs to inhibit cancer cell growth in HT-29, HeLa and MCF-7 cell lines. We also tested whether any of the CDPs were able to induce apoptosis in HT-29 cells. *Results:* The SRB assay showed that only cyclo(Phe-Pro) (10 mM) exhibited more than 50% growth inhibition ($p < 0.01$). The MTT assay was used to demonstrate a dose-dependent (0.008-10 mM) growth inhibition by cyclo(Phe-Pro). Hoechst 33342 staining showed that 5 mM cyclo(Phe-Pro) induced chromatin condensation in $18.3 \pm 2.8\%$ ($p < 0.01$) of HT-29 cells after 72 hours. Furthermore, annexin V binding revealed phosphatidylserine externalisation in cyclo(Phe-Pro)-treated HT-29 cells. *Conclusion:* Our findings demonstrate that cyclo(Phe-Pro) inhibits the growth of HT-29, MCF-7 and HeLa cells and induces apoptosis in HT-29 colon cancer cells, suggesting a potential antitumour activity.

Peptides and proteins constitute a diverse family of endogenous compounds that regulate a wide range of important biological functions. In addition to the vital role that endogenous peptides play in normal tissue homeostasis, some peptides produced by microorganisms have pharmacological significance, functioning as antimicrobial agents, immunomodulators, or antioxidants (1,2). Cyclic peptides have inherent physiological advantages, including stability (resistance to enzymatic degradation) compared to their linear counterparts, conformational rigidity and improved receptor site selectivity and pharmacological specificity (3,4). The synthesis and study of cyclic peptide analogues thus presents a powerful advance in the design and discovery of novel peptide drugs (2).

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Key Words: Cyclic dipeptides, HT-29, apoptosis.

The smallest cyclic peptides are dipeptides called diketopiperazines or cyclic dipeptides (CDPs). CDPs are simple and abundant in nature, common to synthetic, spontaneous and biological formation pathways (5). They are detected naturally in marine sponges (6), microorganisms (7,8) and mammals (5). CDPs are also found in processed foods such as various cereal grains, cocoa, cheese and beverages including beer (9) and roasted coffee (10).

Although CDPs have been detected and studied as far back as the early 1900s, it is only in the last decade that their biological activity has attracted considerable interest (5). Proline appears to be prevalent as a constituent amino acid among these biologically active CDPs. For example, there have been reports of phytotoxic activity exhibited by cyclo(Tyr-Pro) (11), immunomodulation by cyclo(His-Pro) (5,12), modulation of dietary fat intake by cyclo(Asp-Pro) (13) and antifungal activity of cyclo(Phe-Pro) (8). Cyclo(Phe-Pro) and cyclo(Tyr-Pro) have also been implicated in the induction of differentiation in colon cancer cells (14).

Kanoh *et al.* (7) reported potent *in vitro* and *in vivo* antitumour activity of a cyclic dipeptide derivative, phenylahistin, a fungal metabolite composed of phenylalanine and isoprenylated dehydrohistidine. The aim of the present study was to investigate the potential *in vitro* anticancer activity of seven proline-based CDPs (Figure 1) against three human cancer cell lines, namely HT-29 (colon), HeLa (cervical) and MCF-7 (breast).

The CDP that exhibited the highest growth inhibitory activity was used to investigate its potential to induce apoptosis in HT-29 colon cancer cells. Most cancer therapies are known to induce cell death in tumour cells *via* apoptotic pathways (15-19). Apoptosis, as opposed to necrosis, has attracted attention as a favourable pharmacodynamic end point of anticancer drug action (20,21). The HT-29 cell line has previously been described as a model system for the study of apoptosis in an adherent cell line (22,23). In the current investigation HT-29 cells were used to study markers of apoptosis such as chromatin condensation and phosphatidylserine (PS) translocation.

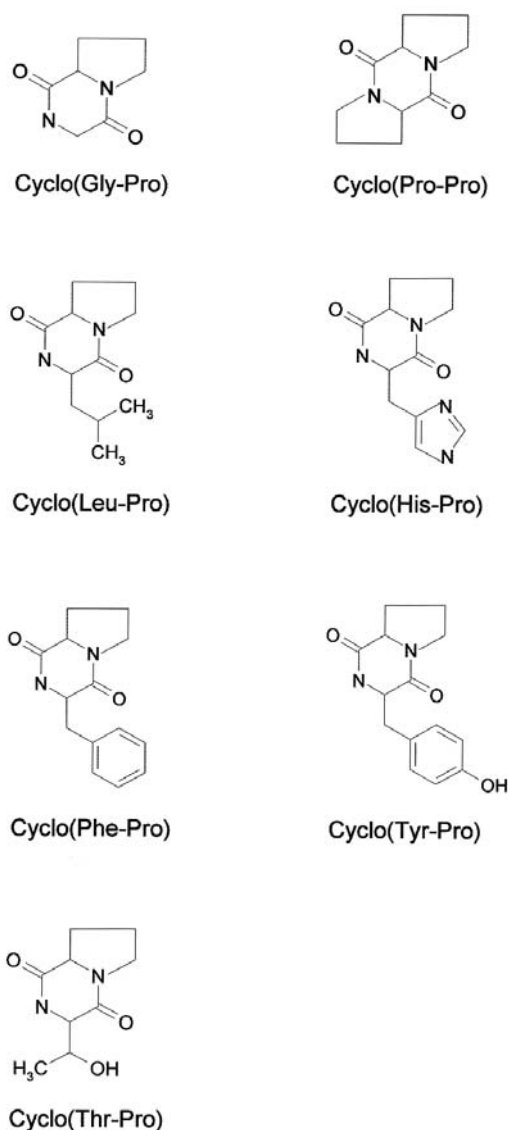


Figure 1. Chemical structures of the proline-based CDPs used in the present study.

Materials and Methods

Cyclic dipeptides. Cyclo(Gly-Pro), cyclo(Pro-Pro), cyclo(Leu-Pro), cyclo(Thr-Pro) and cyclo(His-Pro) were purchased from Bachem (Germany). Cyclo(Phe-Pro) and cyclo(Tyr-Pro) were synthesized from the corresponding linear dipeptides (Bachem) by the phenol cyclization (24). The crystalline products of cyclo(Phe-Pro) and cyclo(Tyr-Pro) were shown to be free of phenol contamination by using high-performance liquid chromatography (Waters TSK ODS 120T column, 4.6 x 250mm, 40:60 acetonitrile:water). Structural elucidation of the synthetic products was performed with the aid of infrared spectroscopy, mass spectrometry (MS) and NMR spectroscopy.

Cell lines and culture conditions. HT-29, HeLa and MCF-7 cell lines (Highveld Biological, Kelvin, South Africa), were cultured in 10-cm culture dishes without antibiotics in RPMI 1640 medium (Sigma, Germany) containing 10% heat-inactivated fetal bovine serum, 25 mM HEPES and 2 mM glutamine (Highveld Biological) in a humidified 5% CO₂ incubator at 37°C. The medium was replaced every 48 hours and cells were passaged at 70-80% confluence.

Assays of cell growth and viability

Sulforhodamine B assay. SRB (Sigma) is a protein-binding dye that binds to the basic amino acids of cellular macromolecules and is used in the end-point determination of cell growth or viability (25). Cells were seeded into 96-well microtiter plates at a density of 5000 cells per well. CDPs were dissolved in DMSO, diluted in RPMI 1640 medium and 200µl of the appropriate CDP was added to the cells. Melfalan (Sigma) was included as a positive control at a final concentration of 0.1 mM (26). After 48 hours of exposure to CDPs, the SRB assay was performed as previously described (25). Briefly, cells were fixed *in situ* with 10% trichloroacetic acid (TCA) and stained with SRB (0.4% w/v in 1% acetic acid) for 10 minutes at room temperature. Bound stain was solubilised with 10 mM Tris base, and the optical densities were read at 540 nm using a plate reader (Multiskan MS, Labsystems, Finland). A measure was also made of the cell density at time zero from an extra reference plate of cells fixed with TCA immediately prior to drug addition to the test plates.

MTT viability assay. HT-29, MCF-7 and HeLa cells were exposed to cyclo(Phe-Pro) and cyclo(Tyr-Pro) at a concentration range of 0.008-10 mM for 72 hours. The effects of these CDPs were also tested on differentiated Caco-2 cells. The MTT assay was performed as previously described (27). Briefly, cells were incubated with 200µl MTT (Sigma) (0.5mg/ml in growth medium) for 4 hours at 37°C. After this time the formazan product was dissolved in DMSO and the response was read at 540 nm (Multiskan MS, Labsystems).

Assessment of apoptosis

Analysis of chromatin condensation. Cells were plated at a density of 2 x 10⁴ cells/cm² on 13-mm glass coverslips (BDH Laboratory Supplies, England) in a 24-well plate and treated with 1mM or 5 mM cyclo(Phe-Pro) for 4, 12, 24, 48 and 72 hours. At the end of each treatment, cells were washed twice with PBS at room temperature and stained with Hoechst 33342 (Sigma) (10µg/ml) in PBS for 15 minutes at room temperature in the dark. Cells were washed with PBS, placed on a microscope slide and analysed immediately using a fluorescence microscope (Olympus BX 60). Quantitative assessment was performed by counting 300 cells for each treatment and evaluating the percentage of apoptotic cells.

Annexin V-fluorescein and Hoechst 33342 dual-labelling. Simultaneous analysis of the presence of PS on the outer leaflet of the cell membrane as well as chromatin condensation were performed using a double-labelling experiment with annexin V-fluorescein and Hoechst 33342 according to Martin *et al.* (28). HT-29 cells were grown on coverslips as described above and treated with 5 mM cyclo(Phe-Pro) for 48 hours. Cells were simultaneously stained with

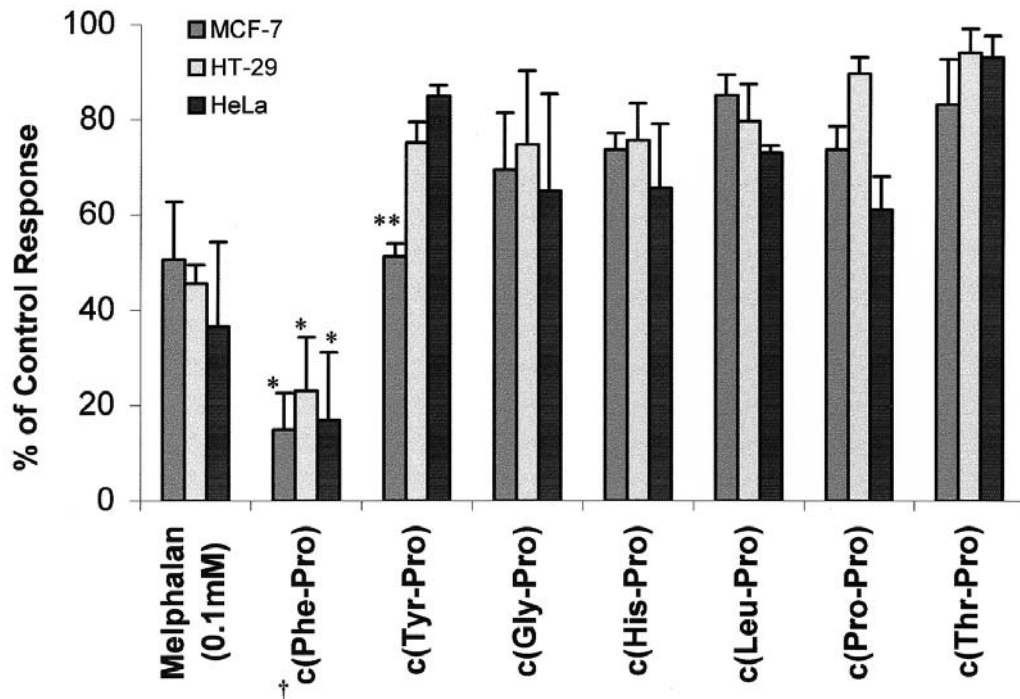


Figure 2. Effects of proline-based CDPs (10 mM) on the growth of HT-29, HeLa and MCF-7 cells. Exponentially growing cells were incubated with CDPs for 48 hours and the cellular response was measured by the SRB assay. Melphalan (0.1 mM) was included as a positive control. Results are expressed as means \pm standard deviation of triplicate experiments, in which each treatment was performed in quadruplicate. *, $p < 0.01$ (compared to control cells); **, $p < 0.01$ (compared to HT-29 and HeLa cells); †, $p < 0.01$ (compared to HT-29 and HeLa cells).

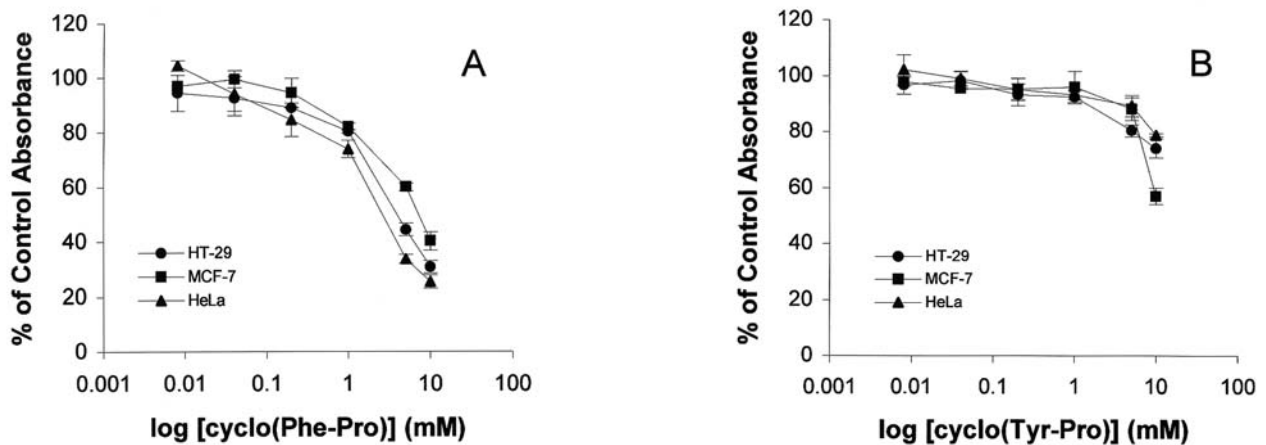


Figure 3. Effects of cyclo(Phe-Pro) (A) and cyclo(Tyr-Pro) (B) on the growth of HT-29 (●), MCF-7 (■) and HeLa (▲) cells as measured by the MTT assay after a 72-hour exposure to the CDPs. Results are expressed as means \pm standard deviation of quadruplicate assays in one experiment, which is representative of three independent experiments.

Hoechst 33342 and annexin V-fluorescein (Annexin-V-FLUOS staining kit, Roche Diagnostics) and analysed with a fluorescent microscope (Olympus BX 60) equipped with Hoechst/DAPI and FITC filters (Olympus BX FLA).

To discriminate apoptotic from necrotic cells, the annexin V staining kit (Roche Diagnostics) was also used, according to the manufacturer's instructions, for dual-labelling of treated cells with propidium iodide (PI) and annexin V-fluorescein.

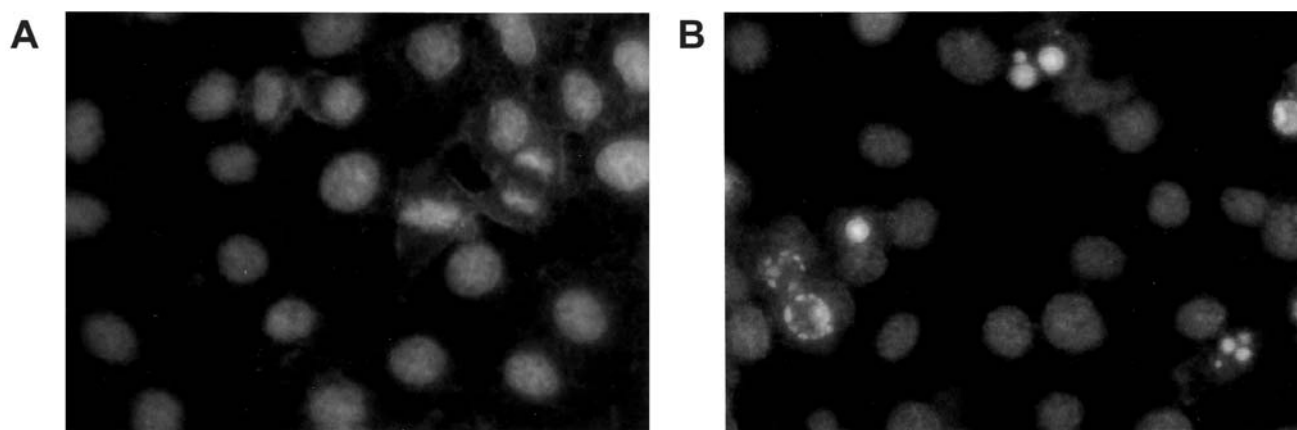


Figure 4. Photomicrographs showing HT-29 cells stained with Hoechst 33342 (original magnification = 400x). Cells treated with 5 mM cyclo(Phe-Pro) for 48 hours display chromatin condensation and nuclear fragmentation (B) compared to normal nuclei staining in untreated control cells (A).

Results

Effects of CDPs on cell growth

Sulforhodamine B assay. The comparative effects of seven proline-based CDPs (10 mM) on the growth of three cancer cell lines HT-29, MCF-7 and HeLa are shown in Figure 2. Cellular responses were calculated for the growth of treated cells as a percentage of control cell growth relative to the time zero measurements. Cyclo(Phe-Pro) exhibited the greatest growth inhibitory effect ($p < 0.01$) on the three cell lines. MCF-7 cells were more sensitive than HT-29 and HeLa cells to treatment with cyclo(Tyr-Pro) ($p < 0.01$). Cyclo(Gly-Pro), cyclo(His-Pro), cyclo(Leu-Pro), cyclo(Pro-Pro) and cyclo(Thr-Pro) showed only marginal growth inhibitory effects in the three cell lines. The growth inhibition exhibited by cyclo(Phe-Pro) and cyclo(Tyr-Pro) was subsequently confirmed by using the MTT assay as recommended by Freshney (29). It should be noted that for the MTT assay, the cells were exposed to the CDPs for 72 hours as opposed to 48 hours for the SRB assay.

MTT assay. HT-29, HeLa and MCF-7 cells were exposed to a concentration range of 0.008-10 mM of either cyclo(Phe-Pro) or cyclo(Tyr-Pro) for a period of 72 hours. At the end of this exposure the cellular response was measured by the MTT assay and compared to that of untreated control cells. Figure 3A shows a dose-dependent growth inhibition of HT-29, HeLa and MCF-7 cells after treatment with cyclo(Phe-Pro). The IC_{50} values for cyclo(Phe-Pro) were 2.92 ± 1.55 mM for HeLa, 4.04 ± 1.15 mM for HT-29, and 6.53 ± 1.26 mM for MCF-7 cells. Cyclo(Tyr-Pro) exhibited a greater growth inhibitory effect in MCF-7 cells ($p < 0.01$) compared to HT-29 and HeLa cells (Figure 3B). Preliminary results

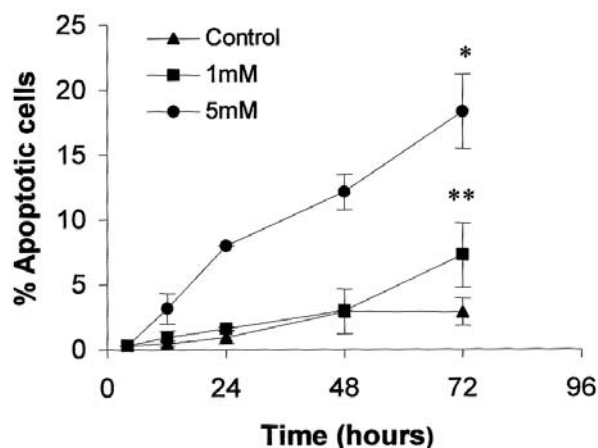


Figure 5. Percentage of apoptosis in cells treated with cyclo(Phe-Pro). Fluorescence microscopy of Hoechst 33342-stained cells was used to evaluate the number of cells with condensed chromatin and fragmented nuclei. Results are expressed as means \pm standard deviation of triplicate assays in one experiment, which is representative of three independent experiments. *, $p < 0.01$; **, $p < 0.05$ (compared to control cells).

indicate that the CDPs have no significant effect on the viability of differentiated Caco-2 cells (data not shown). Differentiated Caco-2 cells are considered to express a normal phenotype of the gastrointestinal epithelium (30).

Induction of apoptosis

Analysis of apoptotic morphology. Nuclear chromatin condensation is considered to be one of the hallmark events in apoptosis. Hoechst 33342 staining of HT-29 cells showed

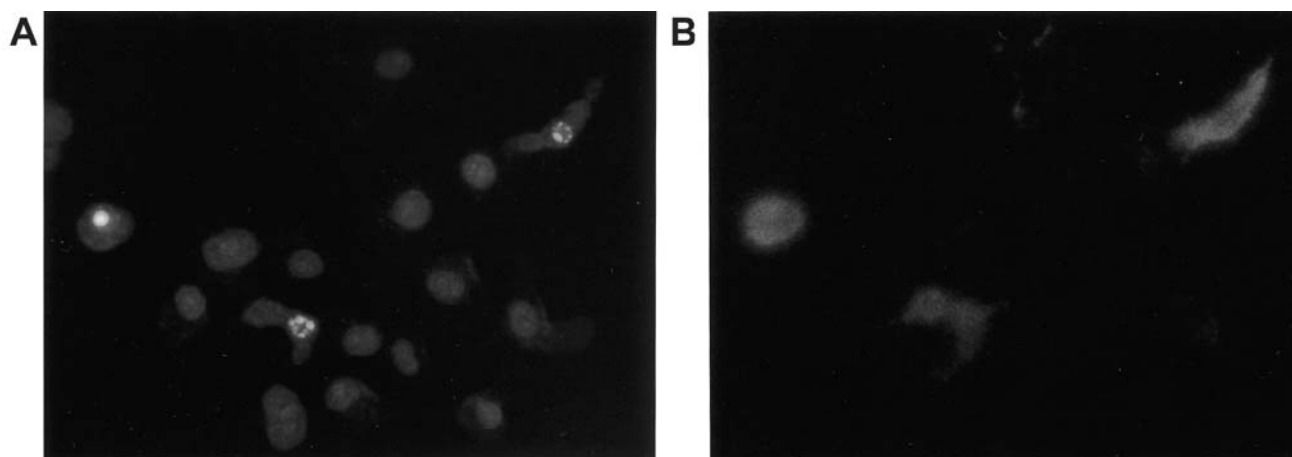


Figure 6. Photomicrographs showing dual staining of HT-29 cells after treatment with 5 mM cyclo(Phe-Pro) for 48 hours (original magnification = 400x). Hoechst 33342 staining (A) shows three cells with markedly condensed chromatin (indicative of apoptosis). Annexin V-fluorescein staining (B) shows that only these three apoptotic cells display significant binding of annexin V (indicative of PS externalization).

that untreated control cells displayed normal staining of chromatin (Figure 4A). By contrast, cells treated with 5 mM cyclo(Phe-Pro) for 48 hours displayed hyperfluorescence of condensed chromatin and fragmented nuclei (Figure 4B). In addition, within the cyclo(Phe-Pro)-treated cells, some appeared rounded and detached from surrounding cells compared to the uniformity in control cells when observed using phase-contrast microscopy (photographs not shown). Desjardins and MacManus (23) describe the "rounding" of cells as a feature of the apoptotic process in HT-29 cells. Results for the quantitative assessment of apoptosis are shown in Figure 5. For the 5 mM cyclo(Phe-Pro) treatment, apoptotic cells were first detected from 12 hours at less than 4% and this increased to $18.3 \pm 2.85\%$ ($p < 0.01$) after 72 hours, compared to control cells in which $2.9 \pm 1.03\%$ of the cells were counted as apoptotic.

Annexin V-fluorescein and Hoechst 33342 dual-labelling. During apoptosis, one of the classical alterations observed at the plasma membrane level is the translocation of phosphatidylserine (PS) from the inner layer to the outer layer, exposing PS at the external surface of the cell. In the present study Hoechst 33342 was used in conjunction with annexin V-fluorescein to investigate membrane changes and nuclear changes simultaneously. The results are shown in Figure 6. Hoechst 33342 staining (Figure 6A) shows three cells with markedly condensed chromatin (indicative of apoptosis). Annexin V-fluorescein staining (Figure 6B) shows that only these three apoptotic cells display significant binding of annexin V (indicative of phosphatidylserine translocation) (31).

Necrotic or lysed cells may also expose PS according to the loss of membrane integrity (32). To distinguish between

apoptosis and potential necrosis, treated cells were concomitantly stained with annexin V-fluorescein and propidium iodide (PI). We did not observe PI staining among the annexin V-positive cells (data not shown), excluding the presence of necrotic or lysed cells in the population.

Discussion

CDPs have attracted considerable attention in terms of their stability, limited conformational freedom and the diverse biological activities that have been ascribed to this group of compounds (5). The CDP derivative phenylahistin has been shown to have potent *in vitro* antitumour activity as assessed in several human cancer cell lines (7). Furthermore, cyclo(Phe-Pro) and cyclo(Tyr-Pro) have been implicated in the induction of differentiation in HT-29 colon cancer cells (14).

The present study investigated whether selected proline-based CDPs could inhibit the growth of three cancer cell lines, namely HT-29, MCF-7 and HeLa; and whether any of these CDPs could induce apoptosis in HT-29 colon cancer cells. Our results showed that, out of seven CDPs, cyclo(Phe-Pro) exhibited the highest growth inhibitory effect on the three cell lines (Figure 2). The growth inhibition exhibited by cyclo(Phe-Pro) was shown to be dose-dependent (Figure 3A). Interestingly cyclo(Tyr-Pro), which has a very similar structure to cyclo(Phe-Pro), exhibited significantly less of an effect on cell growth (Figure 3B). Subtle structural differences are known to play a major role in the biological activity of some compounds. For example, Kanoh *et al.* (7) reported a 33- to 100-fold difference in antitumour activity between the (+) and (−) enantiomers of

phenylahistin, demonstrating that the stereochemistry of the α -carbon of the phenylalanine residue was important for the antitumour activity.

Our results also show, for the first time, that cyclo(Phe-Pro) induces apoptotic cell death in HT-29 colon cancer cells. The apoptotic programme is characterised by particular morphological features such as chromatin condensation and nuclear fragmentation (33). As much as 18% of cells treated with 5 mM cyclo(Phe-Pro) for 72 hours showed condensed chromatin compared to less than 3% in untreated control cells (Figure 5). Leist and Jäätelä (34) presented an extensive review of four different forms of cell death from apoptosis to necrosis. In this review (34) the authors describe apoptosis as being defined by stereotypic morphological changes, particularly in the nucleus where chromatin condenses to compact geometric (globular or crescent-shaped) figures. These features were clearly observed in the present study (Figure 4B). Leist and Jäätelä (34) go further to associate these morphological features with that of apoptosis observed when caspases, particularly caspase-3, are activated, *i.e.* caspase-dependent programmed cell death or apoptosis in its most classic form. The induction of apoptosis was confirmed by showing that the treatment with 5 mM cyclo(Phe-Pro) resulted in the translocation of PS to the outer layer of the plasma membrane in HT-29 cells. Necrotic effects were excluded by the lack of plasma membrane permeability as assessed by the absence of PI uptake.

The IC₅₀ values for cyclo(Phe-Pro) are much higher than those reported for most anticancer drugs, which are effective in the sub-micromolar to micromolar range (15-19,26). The high CDP concentrations required for significant growth inhibition could be due to a number of factors, including the possibility of CDPs binding to serum proteins in the growth medium, drug efflux by P-glycoprotein (35), or the lack of sufficient absorption of peptide into the cells. Tamura *et al.* (36) have reported that CDPs permeate *in vitro* intestinal models primarily *via* a paracellular route with minimal absorption of peptide into the cells. The concentration of cyclo(Phe-Pro) (1mM or 5 mM) that induced apoptosis in HT-29 cells in the present study is, however, comparable to that of a few compounds that show similar activity in the millimolar range. For example, sodium butyrate and aspirin have been shown to induce apoptosis in HT-29 colon cancer cells at concentrations of 5 mM (37) and 3 mM (38), respectively. Furthermore, the apoptotic index ($7.3 \pm 2.5\%$) [$p < 0.05$] for the 1mM cyclo(Phe-Pro) treatment is also of biological relevance. According to Bursch *et al.*, (39) a measurable level of 3% apoptotic cells can result in tissue regression of 25% over several days if not balanced by proliferation.

In conclusion, our results showed that cyclo(Phe-Pro) significantly inhibits the growth of HT-29, MCF-7 and HeLa cells. Furthermore cyclo(Phe-Pro) induced apoptotic cell death

in HT-29 cells, suggesting the potential to inhibit the growth of tumours *in vivo*. Our findings warrant further investigation into the effects of cyclo(Phe-Pro) and related CDPs in the context of cancer chemoprevention or chemotherapy in humans. Further assessment is required to determine precise intracellular or extracellular targets and the mechanism of action by which cyclo(Phe-Pro) induces apoptosis.

Acknowledgements

This study was supported by the National Research Foundation of South Africa. We also thank Dr Michael Graz for original ideas on cyclic dipeptide research.

References

- 1 Rang HP, Dale MM and Ritter JM: Pharmacology, 4th ed., Churchill Livingstone:Edinburgh, pp.184-187, 1999.
- 2 Rizo J and Gierasch LM: Constrained peptides: Models of bioactive peptides and protein substructures. *Ann Rev Biochem* 61: 387-418, 1992.
- 3 McDowell RS and Gadek TR: Structural studies of potent constrained RGD peptides. *J Am Chem Soc* 114: 9245-9253, 1992.
- 4 Smith DD, Slaninova J and Hruby VJ: Structure-activity studies of a novel bicyclic oxytocin antagonist. *J Med Chem* 35: 1558-1563, 1992.
- 5 Prasad C: Bioactive cyclic dipeptides. *Peptides* 16: 151-164, 1995.
- 6 Fu X, Ferraira MLG, Schmitz FJ and Kelly-Borges M: New diketopiperazines from the sponge *Disidea chlorea*. *J Nat Prod* 61: 1226-1231, 1998.
- 7 Kanoh K, Kohno S, Katada J, Hayashi Y, Muramatsu M and Uno I: Antitumor activity of phenylahistin *in vitro* and *in vivo*. *Biosc Biotechnol Biochem* 63: 1130-1133, 1999.
- 8 Ström K, Sjögrén J, Broberg A and Schnürer J: *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. *Appl Environ Microbiol* 68: 4322-4327, 2002.
- 9 Gautschi M and Schmid JP: Chemical characterization of diketopiperazines in beer. *J Agric Food Chem* 45: 3183-3189, 1997.
- 10 Ginz M and Engelhardt UH: Identification of proline-based diketopiperazines in roasted coffee. *J Agric Food Chem* 48: 3528-3532, 2000.
- 11 Stierle AC, Cardellina JH (III) and Stroebel GA: Maculosin, a host-specific phytotoxin for spotted knapweed from *Alternaria alternata*. *Proc Natl Acad Sci USA* 85: 8008-8011, 1988.
- 12 Fragner P, Presset O, Bernard N, Martinez J, Roze C and Aratan-Spire S: A new biological contribution of cyclo His-Pro to the peripheral inhibition of pancreatic secretion. *Am J Physiol* 273(*Endocrinol Metab* 36): E1127-E1132, 1997.
- 13 Lin L, Okada S, York DA and Bray GA: Structural requirements for the biological activity of enterostatin. *Peptides* 15: 849-854, 1994.
- 14 Graz CJM, Grant GD, Brauns SCA, Hunt A, Jamie H and Milne PJ: CDPs in the induction of maturation for cancer therapy. *J Pharm Pharmacol* 52: 75-82, 2000.
- 15 Huschtscha LI, Bartier WA, Ross CEA and Tattersall MHN: Characteristics of cancer cell death after exposure to cytotoxic drugs *in vitro*. *Br J Cancer* 73: 54, 1996.

- 16 Havrilesky LJ, Elbendary A, Hurteau JA, Whitaker RS, Rodriquez GC and Berchuck A: Chemotherapy-induced apoptosis in epithelial ovarian cancers. *Obstet Gynecol* 85: 1007, 1995.
- 17 Shinomiya N, Shinomiya M, Wakiyama H, Katsura Y and Rokutanda M: Enhancement of CDDP cytotoxicity by caffeine is characterized by apoptotic cell death. *Exp Cell Res* 210: 236, 1994.
- 18 Walker PR, Smith C, Youdale T, Leblanc J, Whitfield JF and Sikorska M: Topoisomerase II-reactive chemotherapeutic drugs induce apoptosis in thymocytes. *Cancer Res* 51: 1078, 1991.
- 19 Kaufmann S: Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: A cautionary note. *Cancer Res* 49: 5870, 1989.
- 20 Au JL-S, Panchal N, Li D and Gan Y: Apoptosis: a new pharmacodynamic endpoint. *Pharm Res* 14: 1659-1671, 1997.
- 21 Sellers WR and Fisher DE: Apoptosis and cancer drug targeting. *J Clin Inv* 104: 1655- 1661, 1999.
- 22 Clarke RG, Lund EK, Johnson IT and Pinder AC: Apoptosis can be detected in attached colonic adenocarcinoma HT29 cells using annexin V binding, but not by TUNEL or sub-G0 DNA content. *Cytometry* 39: 141-150, 2000.
- 23 Desjardins LM and MacManus JP: An adherent cell model to study different stages of apoptosis. *Exp Cell Res* 216: 380-387, 1995.
- 24 Kopple DK and Ghazarian HG: A convenient synthesis of 2,5-piperazinediones. *J Org Chem* 33: 862-864, 1968.
- 25 Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S and Boyd MR: *J Natl Cancer Inst* 82: 1107-1112, 1990.
- 26 Monks A, Scudiero D, Skehan P, Shoemaker R, Paul K, Vistica D, Hose C, Langley J, Cronise P, Vaigro-Wolf A, Gray-Goodrich M, Campbell H, Mayo J and Boyd M: Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumour cell lines. *J Natl Cancer Inst* 83: 757-766, 1991.
- 27 Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski MJ, Fine DL, Abbott BJ, Mayo JG, Shoemaker RH and Boyd MR: Feasibility of drug screening with panels of human tumour cell lines using a microculture tetrazolium assay. *Cancer Res* 48: 589-601, 1988.
- 28 Martin SJ, Reutelingsperger CPM and Green DR: Annexin V: a specific probe for apoptotic cells. *In: Techniques in Apoptosis* (Cotter TG and Martin SJ, eds). London, Portland Press, 1996, pp 116-119.
- 29 Freshney RI: *Culture of Animal Cells: A Manual of Basic Technique*. 4th edition. New York, Wiley-Liss, 2000, pp 329-340.
- 30 Pinto M, Robine-Leon S, Appay MD, Keding M, Triadou N, Dussaulx E and Lacroix B: Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol Cell* 47: 323-330, 1983.
- 31 Verhoven B, Schlegel RA and Williamson P: Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes. *J Exp Med* 182: 1597-1601, 1995.
- 32 Darzynkiewicz Z, Juan G, Li X, Gorczyca W, Murakami T and Traganos F: Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). *Cytometry* 27: 1-20, 1997.
- 33 Kerr JFR, Winterford CM and Harmon BV: Apoptosis. Its significance in cancer and cancer therapy. *Cancer* 73: 2013-2026, 1994.
- 34 Leist M and Jäätelä M: Four deaths and a funeral: From caspases to alternative mechanisms. *Nature Reviews (Molecular Cell Biology)* 2: 589-598, 2001.
- 35 Bellamy WT: P-glycoproteins and multidrug resistance. *Ann Rev Pharmacol Toxicol* 36: 161-183, 1996.
- 36 Tamura K, Lee C, Smith PL and Borchardt RT: Effect of charge on oligopeptides transporter-mediated permeation of cyclic dipeptides across Caco-2 cell monolayers. *Pharm Res* 13: 1752-1754, 1996.
- 37 Heerdt BG, Houston MA and Augenlicht LH: Potentiation by specific short-chain fatty acids of differentiation and apoptosis in human colonic carcinoma cells. *Cancer Research* 54: 3288-3294, 1994.
- 38 Qiao L, Hanif R, Sphicas E, Shiff SJ and Rigas B: Effect of aspirin on induction of apoptosis in HT-29 colon adenocarcinoma cells. *Biochem Pharmacol* 55: 53-64, 1998.
- 39 Bursch W, Paffe S, Putz B, Barthel G and Schulte-Hermann R: Determination of the length of the histological stages of apoptosis in normal liver and in altered hepatic foci of rats. *Carcinogenesis* 11: 847-853, 1990.

Received November 12, 2003
Accepted February 25, 2004