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Cyclic Dipeptides in the Induction of Maturation for Cancer Therapy

C. J. M. GRAZ, G. D. GRANT, S. C. BRAUNS, A. HUNT, H. JAMIE AND P. J. MILNE

Cyclic Peptide Research Unit, University of Port Elizabeth, Box 1600, Port Elizabeth 6000, Republic of South Africa

Abstract

Studies have suggested a possible form of therapy based on the use of maturation-inducing compounds to induce differentiation of neoplastic cells and stimulate faster recovery of the normal cell population. The study of the effects of nine cyclic dipeptides on biochemical markers of differentiation implicated their potential to induce differentiation. Studies were undertaken to determine the specificity of these agents for HT-29 cell cultures as well as the identification of the signal transduction pathways affected by these agents inducing the differential gene expression observed in the cells.

The cyclic dipeptides studied showed a high degree of specificity, having no significant effect on Caco-2 cells ($P > 0{\cdot}05$), representing the normal gastrointestinal mucosa. All inducers administered were shown to affect the total energy state of HT-29 cells, an effect which increased the probability of HT-29 cell differentiation. Results indicated that those agents which induced differential gene expression acted at different steps in the isolated signal transduction pathway. Cyclo(Trp-Trp) and cyclo(Phe-Pro) induced a high degree of acetylation of histones ($P < 0{\cdot}05$), while the remaining cyclic dipeptides induced a high degree of phosphorylation of histones ($P < 0{\cdot}05$) (cyclo(Trp-Trp) induced a moderate degree of histone phosphorylation). The results from histone phosphorylation and acetylation and cyclic AMP responsive element binding protein phosphorylation studies suggest that the cyclic dipeptides activate a chromatin switch, which leads to the increase in accessibility of lineage-specific genes for transcription.

One of the most intriguing possibilities in the treatment of neoplasia, is the use of maturation induction therapy (Paul 1978; Dexter & Hagen 1980; Zweibaum 1993). It was shown by Paul (1978) that some compounds, including certain short-chain fatty acids (butyrate), could induce the expression of normal cell markers in myeloid leukaemia cells. Butyrate specifically has been shown to have numerous effects involving differentiation and alteration of malignant to more benign phenotypes of various tumour cell type cultures (Scheppach 1994). Furthermore, in the presence of butyrate, murine leukaemia L1210 cells cease proliferation and become arrested in the G1A component of the G2 phase of the cell cycle (Darzynkiewicz et al 1981). Recent studies have shown that mixtures of short-chain fatty acids are able to induce cell cycle inhibitors in colonocytes, inhibiting cell growth (Wang & Friedman 1998). These results suggest a possible form of therapy based on the use of maturation-inducing compounds to induce differentiation of neoplastic cells and stimulate faster recovery of the normal cell population.

The history of cyclic dipeptides shows that these compounds possess the potential to effect a large number of pertinent biological processes (Ovchinnikov & Ivanov 1975). The therapeutic potential of these compounds ranges from antibiotic, antiviral, anti-tumour, tenso-active to muscle relaxant activity (Milne et al 1998).

The study of the effects of nine cyclic dipeptides on biochemical markers and energy-related metabolic markers was undertaken. Carbonic anhydrase I, alkaline phosphatase and isomaltase dehydrogenase have for a long time served as biochemical markers of differentiation; however recent evidence has suggested that they are not

Correspondence: P. J. Milne, School of Pharmacy, University of Port Elizabeth, Box 1600, Port Elizabeth 6000, Republic of South Africa.

sufficiently reliable to be exclusively used as markers for differentiation.

Graz & Cowley (1997) have shown that the energy state of HT-29 cells is linked to their differentiation. HT-29 cells treated with appropriate inducers (including certain short-chain fatty acids) undergo differential gene expression in conjunction with a rise in the total energy state of the cell. It is proposed that the use of energy-related metabolic markers together with the biochemical markers would serve as a more accurate indicator of differentiation. The screening was undertaken to identify compounds with activity equal to, and in excess of, that shown by short-chain fatty acids, specifically inducing maturation in HT-29 cells, whilst having no substantial effect on the differentiation of the Caco-2 colonic carcinoma cell line.

HT-29 cells are undifferentiated in their normal state whilst Caco-2 cells, a representative model of the normal gastrointestinal cell mucosa, spontaneously differentiate upon spatial inhibition (Rousset 1986). The effects that these non-physiological inducers of maturation induction (Figure 1) have on Caco-2 cells would serve as an indication of their effects on the normal differentiating gastrointestinal cell. Specificity for HT-29 cells would be exemplary of a selective approach to cancer therapy.

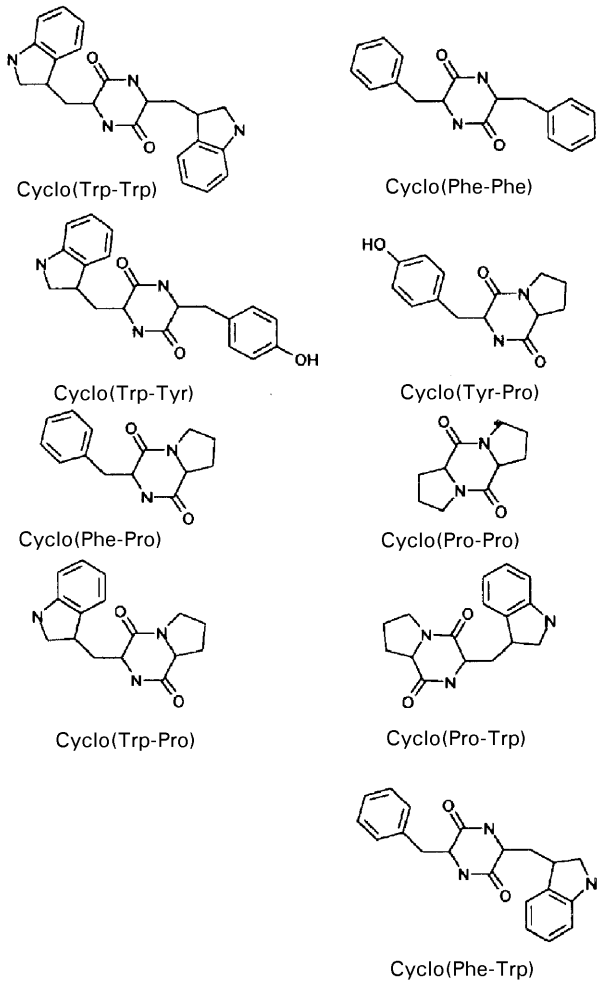

Figure 1. Structural representation of nine cyclic dipeptides.

Materials and Methods

Synthesis of cyclic dipeptides

All cyclic dipeptides were synthesized according to the methods of Milne et al (1992) and Grant et al (1999). Cyclo(Trp-Pro) and cyclo(Pro-Trp) were synthesized and tested for activity due to the abundance of data that suggests the existence of two chemical species depending on the order of amino acid coupling. Very evident is the difference in the angles of donor–hydrogen–acceptor and the lengths of the hydrogen bonds between cyclo(Trp-Pro) and cyclo(Pro-Trp) (Grant et al 1999).

Cell culture

The cell lines, HT-29 (donated by Miss P. Sommer, Department of Anatomical Science, Medical School, University of Witwaterstrand) and Caco-2 cells (obtained from Highveld Biologicals, Kelvin, South Africa), were routinely maintained in 25-cm^2 culture flasks (Corning Incorporated, Corning, NY) in Dulbecco's modification of Eagles Minimal Essential Medium (DMEM) (Highveld Biologicals, Kelvin, South Africa), supplemented with 10% heat-inactivated foetal calf serum (Delta, Johannesburg, South Africa). Cells were incubated at 37°C (5% CO_2). Cells were subcultured at 70–80% confluence.

Preparation of media

The cyclic dipeptides cyclo(Trp-Trp), cyclo(Trp-Pro), cyclo(Trp-Tyr), cyclo(Pro-Trp), cyclo(Phe-Phe), cyclo(Phe-Pro), cyclo(Tyr-Pro), cyclo(Phe-Trp) and cyclo(Pro-Pro) were each dissolved in 1 mL preheated (80°C) glycerol to facilitate dissolution (cyclic dipeptides are stable at this temperature), and then dissolved in DMEM. The solution was filter-sterilized through a 0·2-μm syringe filter. Each compound was added to the cells at a concentration of 125 mg mL^{-1} on the appropriate assay day.

Differentiation marker assay, carbonic anhydrase I

The CO_2–veronal indicator method of Vasseur (1989) was used. Modifications were made to the

assay to suit the requirements of a discontinuous microtitre plate assay. Bromothymol blue was added to veronal buffer (equal volumes of 0·022 M veronal and 0·022 M sodium salt of veronal giving a pH of 7·95) in a ratio of 200 μL deionized water to 15 μL enzyme (carbonic anhydrase I; Boehringer Mannheim GmbH, Germany). For the test samples, the cells in the 96-well plates (5×10^4 cells/well) were washed twice with a solution containing 8 g NaCl, 0·2 g KH_2PO_4 (Protea Laboratory Services, Johannesburg, South Africa), 1·44 g $Na_2HPO_4.12H_2O$ (Unilab Saarchem, Chamdor, Krugersdorp, South Africa), 0·2 g KCl (Unilab Saarchem), 0·2 g EDTA in 1 L, pH adjusted to 7·4 (phosphate-buffered saline (PBS)) and 15 μL deionized water was added to the cells in place of enzyme solution. The microtitre plate containing the reaction mixtures was placed on ice in a sealed container for 15 min. An inlet was made in the sealed container to flush CO_2 through the system for 1 min, thus saturating the atmosphere of the container with CO_2. The drop in pH produced a colour change due to the increase in CO_2. The absorbance was read immediately at 412 nm against a blank. The enzyme activity was determined from a standard curve ($r^2 = 0{\cdot}99$) prepared using commercial carbonic anhydrase I (Boehringer Mannheim, Mannheim, Germany). Cell counts were performed in triplicate and the results were expressed as the activity per 10^5 cells.

Assessment of the effects of cyclic dipeptides on energy-related metabolism in HT-29 and Caco-2 cells

At confluence HT-29 and Caco-2 cells were treated with 125 μg mL^{-1} of the appropriate cyclic dipeptide. The cells were incubated at 37°C for 24 h after which the media was collected and analysed for the uptake of glucose from the growth medium and the release of acetoacetate and lactic acid into the growth medium. The cell mass was determined and divided equally into three samples for the determination of adenosine 5′-triphosphate levels, glucose, isocitrate dehydrogenase activity and pyruvate levels within the cells (chosen as markers of the glycolytic and tricarboxylic acid cycles). Adenosine 5′-triphosphate, isocitrate dehydrogenase and pyruvate levels were determined with the aid of diagnostic kits (Sigma, St Louis, MO). The tests were performed according to the manufacturer's instructions. The presence of ketones was assayed using Ketodiastix (Bayer Diagnostics, Hampshire, UK).

Immunocytochemical detection of CREB (cyclic AMP responsive element binding protein) phosphorylated at Ser-133

On days 4 and 12 (determined as stable culture days), HT-29 cells and Caco-2 cells were washed with 1 mM PBS (pH 7·4) to remove any traces of media in the flasks. The cells were fixed to the flasks by incubating the cells in 1 mM PBS (pH 7·4) containing 3% paraformaldehyde (BDH Chemicals, Poole, UK) for 20 min at 4°C. The cells were washed three times with 1 mL TBST (2·42 g L^{-1} Tris base, 8 g L^{-1} NaCl, 0·1% Tween-20 (pH 7·6)). One millilitre of blocking buffer (10 mL 10 × TBS, 90 mL H_2O, 100 μL Tween-20, 5 g non-fat dry milk) was then added to each flask. The cells were incubated in the blocking buffer for 1 h at room temperature. Once the blocking buffer was removed, the bottom of the flask was cut, producing small squares of the flask bottoms, to which the cells were fixed. The primary antibody (Phospho-specific CREB antibody; New England Biolabs), was added to the cells for 24 h at 4°C. The cells were washed three times with TBST, after which the secondary antibody (anti-rabbit IgG-AP conjugate; Sigma) was added as substrate at room temperature. Three minutes was allowed for the reaction to occur. The presence of a blue precipitate indicated a positive result. The percentage activity of CREB was graded by visually determining the amount of phosphorylated CREB present in the sample, using an Olympus BX60 microscope with camera attachment.

Histone modification: histone phosphorylation and acetylation studies

At confluence HT-29 and Caco-2 cells were incubated with either 5 μL 2 mCi mL^{-1} ^{32}P-orthophosphate (ICN Pharmaceuticals, Irvine, CA) or with 5 μL 0·25 mCi [^{14}C]acetate (NEN, Boston) together with the appropriate inducers predissolved in DMEM.

Extraction of nuclei

After 24 h in the presence of inducers and the radionuclide, extraction of the nuclei was as follows: the cells were trypsonized then centrifuged at 750 rev min^{-1} for 10 min. The cell pellets were washed twice in Ca^{2+}, Mg^{2+}-free saline water, containing 0·01% EDTA (BDH Chemicals Ltd, Poole, UK). The pellet was resuspended in 1 mL 0·5 M sucrose (NT Laboratories, Excom, Johannesburg, SA), 10 mM Tris (Merck, Germany), pH 7·5, to swell the cells. The cells were dissociated by the loose plunge of a Dounce homo-

genizer, after which the concentration was adjusted to 1 : 20 relative to packed cell volume. The cells were lysed with an equal volume of buffer containing 20 mM NaCl (NT Laboratories, Excom, Johannesburg, SA), 10 mM $MgCl_2$ (Saarchem, Chamdor, SA), 20 mM Tris, pH 7·5; 1% Triton X-100 (BDH Chemicals Ltd, Poole, UK) with mixing by vortexing. The nuclei were resuspended in 0·5 mL buffer containing 0·25 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, pH 7·5. The suspension was then centrifuged at 1000 rev min^{-1} for 10 min. The pellet was retained for extraction of histones.

Histone extraction

Histones were extracted according to Wiekowski & DePamphilis (1993). The nuclear pellet was resuspended in 200 μL nuclear disruption buffer (10 μL mL^{-1} 1 M Tris–HCl (pH 7·5), 26 μL mL^{-1} 0·5 M EDTA, 5·2 mg mL^{-1} sodium bisulphite, 20 μL mL^{-1} 1 M sodium butyrate) using a vortex device. The nuclei were recovered by centrifugation at 12 000 rev min^{-1} for 10 min at 4°C. The pellet was resuspended in 200 μL chromatin wash buffer (10 μL mL^{-1} 1 M Tris–HCl (pH 7·5), 28 μL mL^{-1} 5 M NaCl, 26 μL mL^{-1} 0·5 M EDTA, 5·2 mg mL^{-1} sodium bisulphite, 20 μL mL^{-1} 1 M sodium butyrate) and centrifuged to remove any proteins. The pellet was resuspended in 50 μL nuclear disruption buffer, which was then sonicated for 7 s using a sonicator bath (50–60 Hz) (Bandelin Sonorex NK51). The histones were extracted into acid for 1 h by adding 5·5 μL 3·6 M H_2SO_4 (BDH Chemicals, Poole, UK) to each sample. Cell debris was then removed by centrifugation at 12 000 rev min^{-1} at 4°C for 30 min. The supernatant, which contained the histones, was recovered. Histone proteins were precipitated with 10 vol acetone (Associated Chemical Enterprises, Glenvista, SA) at −20°C overnight. The precipitate was recovered by centrifugation at 12 000 rev min^{-1} at 4°C for 30 min. The supernatant was discarded and the pellet dried under vacuum for 10 min. The pellet was then resuspended in 500 μL deionized water. The Folin–Lowry protein assay (Plummer 1987) was used to determine the amount of protein in each sample. Liquid scintillation analysis was used to assay the radioactive product. Protein content of samples was determined from the standard curve.

Liquid scintillation analysis

Radiolabelled histone extracts (10 μL) were added to 3 mL scintillation fluid (Ultlima Gold XR, Packard) in each vial. The histones were gently mixed into the scintillation fluid, after which they were equilibrated in the dark for 4 h, allowing the phosphorescence induced in the scintillant by sunlight to decay before counting (Graz & Cowley 1997). The vials, as well as controls, were counted twice for 5 min in a Liquid Scintillation Counter (Tri Carb 2300 TR Liquid Scintillation Analyzer, Packard). Counts min^{-1} were recorded to allow the determination of the acetylation and phosphorylation of the samples in relation to the control sample.

Statistical analysis

Results for energy related markers and biochemical markers are expressed as means ± s.d. Differences between effects of cyclic dipeptides on the expression of biochemical markers, induction of histone acetylation and phosphorylation, induction of CREB phosphorylation, and energy related markers in Caco-2 and HT-29 cells were assessed by analysis of variance. P values of $< 0{\cdot}05$ were considered to be indicative of significance.

Results

Effect of cyclic dipeptides on the energy metabolism of HT-29 and Caco-2 cells

Caco-2 and HT-29 cells were exposed to 125 μg mL^{-1} of the appropriate inducers. All cyclic dipeptides induced a significant decrease in the glucose absorption from the culture medium of HT-29 cells, when compared with control, showing no significant effect in absorption in Caco-2 cells ($P > 0{\cdot}05$). The maximal inhibitory effect in glucose absorption (Table 1) was presented by those agents bearing a tryptophan amino acid residue, suggesting that tryptophan may be the essential pharmacophore involved in inducing maturation induction (cyclo(Trp-Trp) and cyclo(Trp-Tyr)). Concomitant decreases in pyruvate and lactate levels were recorded, similar to results obtained for the positive control butyrate and acetoacetate. No significant changes were induced in the activity of isocitrate dehydrogenase ($P > 0{\cdot}05$) (results not shown). In addition to the effects that cyclic dipeptides had on glycolysis and the tricarboxylic acid cycle, most of the cyclic dipeptides exhibited a ketogenic potential (the exceptions were cyclo(Phe-Phe), cyclo(Trp-Tyr), and cyclo(Phe-Trp)). Cyclic dipeptides are able to effect basal adenosine 5′-triphosphate levels. Decreased adenosine 5′-triphosphate utilization, as expected (Cooper 1997) from the results of glycolysis and tricarboxylic acid

Table 1. Effect of cyclic dipeptides on energy related metabolites (glucose, adenosine 5′-triphosphate, ketogenesis, lactate and pyruvate) in HT-29 cells.

Treatment of HT-29 cells 24 h	Lactate production (mM)[a]	Glucose utilization (%)[b]	Increase adenosine 5′-triphosphate utilization (%)[c]	Keto-genesis[d]	Pyruvate production ((mM)/g cells)[e]
Cyclo(Pro-Trp)	11·0 ± 0·3	46·7 ± 0·8	82·0 ± 0·4	++	29·0 ± 1·4
Cyclo(Trp-Pro)	8·3 ± 1·8	53·2 ± 4·4	5·5 ± 7·8	+++	107·4 ± 6·8
Cyclo(Tyr-Pro)	9·5 ± 2·1	34·3 ± 2·5	68·0 ± 8·3	++	29·1 ± 6·8
Cyclo(Phe-Pro)	9·4 ± 0·2	27·9 ± 7·3	95·9 ± 0·9	++	9·9 ± 5·6
Cyclo(Trp-Trp)	9·0 ± 1·0	19·8 ± 8·8	0	++	31·7 ± 9·8
Cyclo(Phe-Phe)	9·1 ± 0·1	31·7 ± 4·9	19·8 ± 2·9	+	41·1 ± 4·9
Cyclo(Trp-Tyr)	8·4 ± 0·5	23·3 ± 0·6	80·2 ± 0·9	+	33·8 ± 9·9
Cyclo(Phe-Trp)	8·1 ± 0·1	25·0 ± 2·7	82·6 ± 2·7	+	11·7 ± 2·0
Cyclo(Pro-Pro)	10·7 ± 0·2	30·3 ± 2·3	58·0 ± 7·7	++	24·0 ± 1·0
Control	10·9 ± 0·1	59·9 ± 3·0	NA	+	47·3 ± 2·9

Values are mean ± s.d. [a]Lactate concentration in culture medium. [b]Percentage of available glucose utilized. [c]% adenosine 5′-triphosphate utilization is reported relative to control values. [d]Ketone levels represented as: +, 0·5 mM; ++, 1·5 mM; +++, 4 mM. [e]Pyruvate concentration produced within the cell.

cycle, were not seen in cultures treated with cyclic dipeptides. Paradoxically the adenosine 5′-triphosphate concentration decreased (Table 1) ($P < 0·05$) with all cyclic dipeptides with the exception of cyclo(Trp-Trp). Results indicate that cyclic dipeptides, amongst other effects, induced the conversion of adenosine 5′-triphosphate to cyclic adenosine 5′-monophosphate (cAMP), opening the possibility that specific protein phosphorylation cascades are activated, thereby eliciting a variety of functional responses presented by the inducers (Crespo et al 1998).

Effect of cyclic dipeptides on the degree of CREB phosphorylation

Forskolin (0·1 mg mL^{-1}) (ICN Biomedicals, Aurora, OH), a known activator of cAMP and CREB, was used as a positive control. Table 2 suggests a stimulated increase in the degree of phosphorylation of CREB by cyclic dipeptides, suggesting phenotype specificity towards the induction of differential gene expression. It is proposed that CREB is a definitive indicator of lineage-specific differential gene expression and could be used as a differentiation marker. This result was supported by the expression of biochemical markers (Table 3).

Effect of cyclic dipeptides on phosphorylation and acetylation patterns of histones

The results obtained (Table 4) from the histone phosphorylation studies confirm that it is in part responsible for the increase in the adenosine 5′-triphosphate requirement of those cells treated with cyclic dipeptides. The results indicate a correlation with differentiation, which from a statistical point of view could be significant. The study confirmed the specificity of the effects of cyclic dipeptides on

Table 2. Effect of cyclic dipeptides on the degree of CREB phosphorylation in HT-29 cell cultures. On days 4 and 12 of treatment cells were washed with 1 mMPBS to remove traces of media and the degree of phosphorylated CREB was determined through immunocytochemical detection.

Treatment 24 h	HT29 CREB (%)		Caco-2 CREB (%)	
	Day 4	Day 12	Day 4	Day 12
Cyclo(Pro-Trp)	80	90	0	1–5
Cyclo(Trp-Pro)	5	95	5	5
Cyclo(Tyr-Pro)	0	90	1	1–5
Cyclo(Phe-Pro)	30	75	10	50
Cyclo(Trp-Trp)	10	80	0	1–5
Cyclo(Phe-Phe)	40	95	0	1–5
Cyclo(Trp-Tyr)	0	60	0	1–5
Cyclo(Phe-Trp)	0	30	0	1–5
Cyclo(Pro-Pro)	0	90	0	1–5
Control	0	0	0	1–5

Table 3. Effect of cyclic dipeptides on the biochemical marker of differentiation, carbonic anhydrase I, in HT-29 cells and Caco-2 cells.

Treatment administered	Carbonic anhydrase (units mL^{-1})	
	Day 4	Day 12
Cyclo(Pro-Trp)	2 ± 0·1	12 ± 0·2
Cyclo(Trp-Pro)	3 ± 0·3	21 ± 0·1
Cyclo(Tyr-Pro)	1 ± 0·1	23 ± 0·3
Cyclo(Phe-Pro)	2 ± 0·1	26 ± 0·1
Cyclo(Trp-Trp)	2 ± 0·4	22 ± 0·1
Cyclo(Phe-Phe)	1 ± 0·1	22 ± 1·0
Cyclo(Trp-Tyr)	3 ± 0·4	25 ± 0·2
Cyclo(Phe-Trp)	1 ± 0·1	21 ± 1·1
Cyclo(Pro-Pro)	2 ± 0·1	20 ± 0·4
Control	1 ± 0·1	4 ± 0·2

Results are reported as the average ± s.d.

Table 4. Effect of cyclic dipeptides on the degree of histone phosphorylation and/or acetylation in HT-29 and Caco-2 cultures. Statistically significant induction of histone phosphorylation and acetylation, as compared with the untreated control, by analysis of variance.

Treatment 24 h	Histone phosphorylation		Histone acetylation	
	HT-29[a] (counts min^{-1} mg^{-1})	Caco2[b] (counts min^{-1} mg^{-1})	HT-29[c] (counts min^{-1} mg^{-1})	Caco2[d] (counts min^{-1} mg^{-1})
Cyclo(Pro-Trp)	19511·7 ± 468·8	1631·9 ± 234·4	2557·9 ± 296·0	1281·4 ± 149·6
Cyclo(Trp-Pro)	12438·9 ± 256·4	861·3 ± 128·2	603·8 ± 7·7	1835·9 ± 90·7
Cyclo(Tyr-Pro)	8249·4 ± 907·8	2917·1 ± 173·1	964·3 ± 104·9	2238·1 ± 123·6
Cyclo(Phe-Pro)	2519·3 ± 649·1	647·7 ± 21·6	61679·6 ± 3829·0	2855·8 ± 86·5
Cyclo(Trp-Trp)	10519·2 ± 240·1	2413·9 ± 96·2	33546·37 ± 302·9	9383·6 ± 395·6
Cyclo(Phe-Phe)	1153·2 ± 230·8	4297·4 ± 423·1	218·56 ± 28·8	688·5 ± 115·4
Cyclo(Trp-Tyr)	3396·5 ± 288·5	1555·4 ± 198·3	821·4 ± 84·6	4895·8 ± 365·4
Cyclo(Phe-Trp)	7425·3 ± 524·5	2206·8 ± 209·8	254·5 ± 24·7	650·2 ± 14·4
Cyclo(Pro-Pro)	3537·8 ± 453·3	695·5 ± 46·7	3855·4 ± 358·5	303·2 ± 25·0
Control	1960·7 ± 17·0	708·1 ± 33·9	854·4 ± 149·3	2912·4 ± 221·9

[a]Degree of induced histone phosphorylation in HT-29 cultures monitored through ^{32}P-orthophosphate detection. [b]Degree of induced histone phosphorylation in Caco-2 cultures monitored through ^{32}P-orthophosphate detection. [c]Degree of induced histone acetylation in HT-29 cultures, monitored through [^{14}C]acetate detection. [d]Degree of induced histone acetylation in Caco-2 cultures, monitored through [^{14}C]acetate detection.

HT-29 cells in inducing differential gene expression. Cyclo(Trp-Trp) and cyclo(Phe-Pro) showed that the mode in which they activated differential gene expression was by high levels of acetylation of histones (Table 4) as opposed to the predominant phosphoralative effect that the other agents induced on histones (with the exception of cyclo(Phe-Phe) which failed to induce similar phosphorylation of histones). Both acetylation (primary modifier of histones) and phosphorylation (secondary modifier of histones) cause an increase in the unwinding of the chromatin from around the nucleosome, making the gene more accessible for transcription. The evidence suggests that cyclic dipeptides activate a chromatin switch (Bodnar & Bradley 1996).

Discussion

The hypothesis that a freely available carbon and energy source such as acetoacetate increases the probability of HT-29 differentiation, suggests that the energy metabolism of the cell could be used as a marker for differential gene expression (Graz & Cowley 1997). From the study of the effects of the cyclic dipeptides (Figure 1) on glycolysis it was evident that there was a decrease in the glucose utilization when compared with the control (Table 1). The effects of the cyclic dipeptides on the energy metabolism of the cell showed that these agents possess ketogenic potential (Table 1). The ketones, which are primarily stored as acetoacetate (also a ketone) in HT-29 cells, may result in an increase of the total energy state of the cell and this could affect its state of terminal differentiation (Graz & Cowley 1997). It is proposed that certain ketones provide acetate to the cell, which can act as an energy source by complexing with CoA and entering the tricarboxylic acid cycle as acetyl–CoA (Tsanev 1975). The acetyl–CoA can be used by the tricarboxylic acid cycle to produce either adenosine 5′-triphosphate or acetoacetate (McGarry & Foster 1980). The increased adenosine 5′-triphosphate requirement of the cell is substantiated by the fact that the ketogenic potential of the cyclic dipeptides may result in increases in the levels of acetoacetate which may influence the differential gene expression in HT-29 cells (Graz & Cowley 1997).

The decrease in adenosine 5′-triphosphate concentration in the treated cells would be expected to cause a consequent increase in cAMP concentration. cAMP could serve as a source of phosphates for the specific protein phosphorylation cascades involved in differential gene expression. The increase in the induced histone phosphorylation (Table 4), and the increased degree of phosphorylated CREB (Table 2) substantiates the above. The results of this study indicate that cyclic dipeptides affect cAMP-dependent pathways, and it is hypothesized that these agents can act by either mimicking cAMP at protein kinase A, or they may inhibit the breakdown of cAMP by inhibiting phosphodiesterase. These speculations were made due to the high degree of structural similarity between these compounds and cAMP, which was elucidated by computational chemistry and molecular modelling (results not shown).

Histone phosphorylation and acetylation may cause an increase in the unwinding of the chro-

matin from around the nucleosome, making genes previously inaccessible for transcription, accessible (Darzynkiewicz et al 1981). It is proposed that such changes in phosphorylation and acetylation patterns on histone proteins could result in a conformational change of the chromatin material from a condensed conformation to an active form, referred to as the ''chromatin switch'' (Bodnar & Bradley 1996).

Mizzen & Allis (1998) outline the relation between histone acetylation and gene activation and confirm the notion that histone acetylation is part of the mechanism responsible for unfolding condensed chromatin, rendering it accessible for transcription.

Histone acetylation therefore represents a major pathway for transcriptional regulation. It is evident from the results obtained that the sites of action of the cyclic dipeptides vary in inducing differential gene expression. The results suggest that either an acetylation effect and/or phosphorylation effect be involved in the activation of the chromatin switch, which would lead to the induction of lineage-specific gene expression.

Cyclo(Trp-Trp) and cyclo(Phe-Pro) were able to induce a high level of acetylation of histones (Table 4), an effect that could be linked to the high level of ketogenic potential of these agents. Graz & Cowley (1997) showed that certain short-chain fatty acids through their ketogenic potential could increase the total energy state of HT-29 cells, an effect which ultimately leads to terminal differentiation. The ketones may provide acetyl groups for direct acetylation of the histones, the effect of which would be the activation of the chromatin switch (Bodnar & Bradley 1996).

The results obtained from the histone acetylation and phosphorylation studies suggest that cyclo(Trp-Trp) and cyclo(Pro-Trp) induce differential gene expression via different signal transduction pathways. Cyclo(Trp-Trp) induced the highest level of acetylation (Table 4) of histones whereas cyclo-(Phe-Pro) induced high levels of phosphorylation of histones (Table 4). Studies of the use of combinations of cyclo(Phe-Pro) and cyclo(Pro-Trp) are necessary to determine whether a synergistic affect on the induction of differential gene expression can be obtained through inducing both acetylation and phosphorylation of histones and the phosphorylation of CREB. The results of this study suggest that the lineage specific gene expression observed in HT-29 cells was related to the administration of the cyclic dipeptides. The differences in activity of cyclo(Pro-Trp) and cyclo(Trp-Pro) would suggest that the remaining cyclic dipeptides be synthesized in reverse order to determine which form exhibits greatest activity.

Although all cyclic dipeptides induced differential gene expression, they all showed selectivity towards the HT-29 phenotype rather than Caco-2 (data not shown). In HT-29 cells it has been shown that growth and intestinal differentiation are independently regulated (Shroy et al 1994). Throughout the study the effects of the cyclic dipeptides were significant in HT-29 cells ($P < 0{\cdot}05$), whereas their effect on Caco-2 cells was insignificant ($P > 0{\cdot}05$). Therefore the results reported (Table 4) suggest that the cyclic dipeptides would selectively inhibit the growth of a carcinoma, whilst allowing the normal cell population to recover.

The results from this study would suggest that cyclic dipeptides are exemplary of a highly selective agent offering a radically new direction in cancer therapy.

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