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The synthesis and anticancer activity of selected diketopiperazines

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ABSTRACT

Six selected diketopiperazines, cyclo(Gly-Val), cyclo(Gly-D-Val), cyclo(Gly-Leu), cyclo(Gly-Ile), cyclo(Phe-Cys) and cyclo(Tyr-Cys), were synthesized via various synthetic routes. Their potential to inhibit cancer cell growth in HT-29, HeLa and MCF-7 cells was determined. Cyclo(Tyr-Cys) caused the greatest inhibition in cervical carcinoma cells with near equivalent activity against HT-29 and MCF-7 cells. The other cyclic dipeptides tested were effective in the inhibition of colon, cervical and breast carcinoma cells, respectively, but the percentage inhibition was lower than for cyclo(Tyr-Cys).

1. Introduction

Cyclic dipeptides (CDPs) are more than simple curiosities [15] and are well known for their economically beneficial biological activities [25]. CDPs are relatively simple compounds, and therefore are among the most common peptide derivatives found in nature [25]. A host of other CDPs is reportedly endogenous to different living organisms or exists as either by-products of manufacturing processes or simply degradation products of larger proteins and peptides [25]. The history of cyclic peptides shows that they possess a large number of biological activities as antibiotics, toxins, hormones and ion transport regulators [22]. These compounds are easily formed from unprotected linear dipeptides under basic conditions, especially if the necessary head-to-tail folding is not prevented by steric constraints [7]. Once formed these CDPs behave as relatively stable molecules [25]. For that reason many of the diketopiperazines are now regarded as important metabolic intermediates rather than as protein artifacts [25]. Due to their relative simplicity [1] and stability [25] diketopiperazines provide excellent models for theoretical studies as well as the development of pharmaceutical compounds.

Cysteine is an important thio-containing compound and involved in a variety of important cellular functions, such as protein synthesis, detoxification and metabolism [16]. Information about diketopiperazines, containing cysteine, is limited. A possible reason for the lack of information is the complex preparations, purification and low yields obtained by conventional synthesis schemes [8,28].

Cancer is one of the major causes of deaths in developed countries with figures for the past 100 years indicating the incidence of the disease is increasing in these countries [26]. Worldwide there were 10.9 million new cases, 6.7 million deaths and 24.6 million persons living with cancer in the year 2002, with the most common cancers affecting the lungs, breast, prostate

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and colorectal areas [24]. Cancer chemotherapy remains the only modality with curative activity against different forms of metastatic malignancy. Over the past decade, cytotoxic and anti-endocrine drugs have been supplemented by targeted therapies that seek to exploit the molecular lesions that underlie the carcinogenic process or maintain the cancer phenotype [21]. Despite the significant progress made in the development of anticancer chemotherapy, there is still no cure for patients with malignant diseases. In addition to the long standing problem of chemotherapy is the absence of tumor specific treatments. Traditional chemotherapy relies on the premise that rapidly proliferating neoplastic cells are more likely to be killed by a cytotoxic agent. In actual fact cytotoxic agents have very little or no specificity, which leads to systemic toxicity, resulting in undesirable adverse drug effects such as alopecia, hepatotoxicity, nephrotoxicity and bone marrow suppression [11]. Ideal anticancer drugs would eradicate cancer cells without harming normal tissues, while evading the mechanisms of drug resistance. With no currently available agents meeting the aforementioned criteria, and the continued elucidation of new biochemical mechanisms that contribute to tumor progression, the search for new novel agents and therapies, with the aid of rational drug design, remains a necessity [10].

The identification and validation of the drug target is however the starting point for the optimal route to the development of active drugs [21]. The search for new anticancer drugs over the past 20 years has proved to be difficult, where few cytotoxic drugs have been specifically designed to attack particular cellular targets. Fascinating alternative sources of new cytotoxic compounds are continually under assessment [5]. The roles of many proteins and peptides have been identified as novel targets in cancer therapy allowing for the design of more selective agents [6]. Milne et al. [18] observed the effects of cyclo(Phe-Pro), cyclo(Tyr-Pro) and cyclo(Trp-Trp) on undifferentiated human colon carcinoma cells (HT-29). These compounds significantly enhanced the expression of the biochemical differentiation markers and retarded the growth of these cells. Graz et al. [10] observed the ability of the CDPs to induce differentiation of neoplastic cells to non-neoplastic cells as well as stimulating faster recovery of these cells through histone acetylation and phosphorylation while allowing the recovery of normal cells. Kanzaki et al. [12] showed that the enzymatic dehydrogenation of cyclo(L-Phe-L-Leu) to its dehydro derivative, albonoursin, resulted in tumor-suppressor activity, as a possible means of tumor cell inhibition and therefore cyclo(L-Phe-L-Leu) may be considered as a prodrug. The results of the study indicated that cyclo(L-Phe-L-Leu) and its didehydro derivatives failed to show any cell growth inhibitory activity, in contrast to the high cell growth inhibitory activity exhibited by its dehydro derivative, albonoursin [12,17]. The study of the potential of CDPs as anticancer agents is a promising field of study with many other CDPs demonstrating anticancer potential [18].

2. Materials and methods

2.1. Materials

The protected amino acids, N-t-Boc-L-Phe, N-t-Boc-L-Tyr and L-Cys-OMe HCl, were obtained from Fluka (Buchs, Switzerland) while the linear dipeptides, Gly-Val, Gly-D-Val, Gly-Leu and Gly-Ile were obtained from Bachem (Bubendorf, Switzerland). Silica gel TLC plates were obtained from Merck (Darmstadt, Germany). Cell lines and growth media for cell culture were obtained from Highveld Biological (Johannesburg, South Africa). Sterile cell culture plasticware was obtained from Corning (Lowell, MA, USA). Cisplatin was a kind donation from Prof. JGH du Preez of the Metal Ions Separation Unit, NMMU. All other chemicals were of reagent grade and obtained from Sigma (St. Louis, MO, USA).

2.2. Synthesis, qualitative analysis and structural elucidation

2.2.1. Synthesis

Cyclo(Phe-Cys) (cFC) and cyclo(Tyr-Cys) (cYC) were synthesized by modifying the method of Grant et al. [9] from protected amino acids. Briefly, triethylamine and diethylphosphoryl cyanide were added to a stirred solution of either N-t-Boc-L-Phe and L-Cys-OMe HCl or N-t-Boc-L-Tyr and L-Cys-OMe HCl in 1,2 dimethoxyethane and stirred at 0 °C for 3 h, followed by constant stirring for 3 h at room temperature. The reactions were diluted with chloroform and washed successively with 5% hydrochloric acid, 5% aqueous sodium hydrogen carbonate and saturated brine. Removal of the solvent *in vacuo* furnished the protected products, N-t-Boc-Phe-Cys methyl ester or N-t-Boc-Tyr-Cys methyl ester, as white crystals. These products was then dissolved in formic acid containing 1% (v/v) anisole and stirred for 3 h at room temperature. After removal of the excess formic acid *in vacuo*, the residue containing the crude dipeptide esters (formate salts) were refluxed in *sec*-butanol and toluene (4:1) for 3 h at 120 °C. After concentration and cooling of the solutions, the products were filtered and recrystallized from methanol and water, yielding pure cyclic dipeptides.

Cyclo(Gly-Val) (cGV), cyclo(Gly-D-Val) (cG(D)V), cyclo(Gly-Leu) (cGL) and cyclo(Gly-Ile) (cGI) were synthesized using a phenol-induced cyclization procedure from their linear dipeptide counterparts, adapted from Kopple and Ghazarian [13].

2.2.2. Thin layer chromatography (TLC)

Purity was initially confirmed by means of thin layer chromatography on silica gel plates, Silica gel 60 F_{254}, as the stationary phase with four separate mobile phases. The mobile phases used were the following: mobile phase 1 [chloroform (SMM Instruments, SA)–methanol (BDH Laboratory, Supplies, England)–acetic acid (Saarchem, SA)–water (Millipore MilliQ^{TM} (45:13:1:2)]; mobile phase 2 [*n*-butanol (Associated Chemical Enterprises, SA)–acetic acid (Saarchem, SA)–water (Millipore MilliQ^{TM} (2:1:1)]; mobile phase 3 [*n*-isopropanol (Associated Chemical Enterprises, SA) and ammonium hydroxide (Merck Laboratory Supplies (Pty)., SA) (1:1)] and mobile phase 4 [methanol (BDH Laboratory, Supplies, England)–acetic acid (Saarchem, SA)–water (Millipore MilliQ^{TM} (2:1:1)]. Chromatograms were visualized by short-wave UV light or by developing in an iodine chamber.

2.2.3. High performance liquid chromatography (HPLC)

Reversed-phase HPLC analyses of the cyclic dipeptides were performed using an HPLC instrument fitted with Thermo

Separations Products Spectraseries P100 Isocratic Solvent Delivery Module, Thermo Separations Products UV-150 variable wavelength detector and Rheodyne manual injector. The analysis was performed on an Atlantis® dC18 5 μm spherical packing column with dimensions 4.6 mm × 150 mm. The analysis was performed at ambient temperature at a flow rate of 1.0 ml/min for superior quality resolution and an injection volume of 20 μl. All the data and results were recorded using Delta Chromatography Software (Version 5.0).

Three mobile phases were used: mobile phase 1 [methanol (HPLC grade, Sigma®–Aldrich, Germany)–0.1% trifluoracetic acid (Fluka, Germany) (35:65) in double distilled, de-ionized milliQ™ water]; mobile phase 2 [30% methanol–70% milliQ™ water] and mobile phase 3 [methanol 10%–milliQ™ water 90%].

2.2.4. *Scanning electron microscopy (SEM)*

A Philips XL30 scanning electron microscope was used to record the scanning electron micrographs of the diketopiperazines. Samples were attached to stubbs using silver nitrate paste and coated with a thin layer of gold using a Novotech Semprep 2-sputter coater.

2.2.5. *Mass spectrometry (MS)*

The mass spectra of the CDPs were recorded on a VG-7070E mass spectrometer (IF, Biotech, Altricham, United Kingdom) equipped with a VG2035 data system. The CDPs were run under positive ion operating conditions. The FAB mass spectra of the CDPs were recorded with an Ion Tech saddle-field gun employing xenon as the bombardment gas (8 kV, 1 mA). The mass spectrometer was operated at 6 kV and the magnetic analyser was scanned at a rate of five seconds per decade over the range 500–100 Da. The solvent used was deuterated dimethyl sulphoxide (DMSO-d_6), and the sample matrix used was 3-nitrobenzyl alcohol. A small amount of each cyclic dipeptide was mixed on the probe tip with the solvent.

2.2.6. *Nuclear magnetic resonance spectroscopy (NMR)*

The ^{1}H (300.13 MHz) and ^{13}C (75.48 MHz) spectra for the CDPs were recorded on a Bruker AMS 300 NMR spectrometer. HMQC and COSY spectra were used to allow unambiguous assignment of proton and carbon resonances of the CDPs and to indicate couplings of protons to carbons and protons to protons, respectively. Tetramethylsilane (TMS) was used as the internal standard for the determination of chemical shifts. Deuterated dimethyl sulphoxide (DMSO-d_6) was the solvent used to prepare the solutions of the CDPs.

2.2.7. *Infrared spectroscopy*

Infrared spectra were recorded using a PerkinElmer 1600 Fourier transform infrared (FTIR) spectrophotometer with CDPs compressed within potassium bromide disks according to standard sample preparation guidelines.

2.2.8. *Differential scanning calorimetry*

A DSC Q100 differential scanning calorimeter (TA Instruments, Wilmington, DE, USA) was used to record the DSC scans for all compounds which facilitated accurate melting point determination.

2.2.9. *Computer modelling*

The molecular modelling and computational nuclear magnetic resonance studies were computed using the molecular modelling system Hyperchem® Professional (release 7.04 for Windows). The 3D structures of the CDPs were built and the geometry of all the structures was optimized using the steepest descent (SD) algorithm followed by the Polak-Ribiere Conjugate gradient (CG) method. The optimization calculation is usually terminated by Hyperchem® when a specified root mean square (RMS) or maximum cycle value is reached. This specified RMS value was set to 10^{-3} kcal/(Å mol), and the maximum cycles were computed as 200 and 32,650, for the SD and CG methods, respectively. The molecular mechanics force field was calibrated according to the assisted model building with an energy refinement (AMBER) parameter for the optimization calculations. The torsional angles chosen were then used to identify the respective lowest most stable energy conformations of the respective CDPs. The atoms of each structure were aligned, calculating the best superposition of atoms such that the RMS deviation between the set of atomic co-ordinates is minimized. The RMS deviation value of zero can be seen as a perfect fit whereas a value below 0.1 represents good alignment. Values greater than 1 represent poor alignments.

2.3. *Cytotoxicity assay*

All culture manipulations were performed under aseptic conditions employing sterile culture techniques. HeLa, HT-29 and MCF-7 carcinoma cell lines were routinely maintained in RPMI 1640 growth medium (pH 7.4) supplemented with 10% heat inactivated fetal calf serum (FCS) at 37 °C in 10 cm^2 cell culture dishes in a humidified atmosphere with 5% CO_2.

Cytotoxicity was determined by using a modified MTT assay previously described by Mosmann [20] and Carmichael et al. [4]. Cells in exponential growth were trypsinized (0.25% trypsin), counted and diluted to 30 000 cells/ml. Aliquots of 200 μl of the cell suspensions were added to each well of a 96-well culture plate and incubated at 37 °C for 24 h prior to the addition of the drug solutions. The CDPs and cisplatin (positive control) were initially solubilized in 0.5% DMSO and stock concentrations of 200 μM were prepared in growth medium. Aliquots of 200 μl of the drug solutions were added to the wells such that the final drug concentration per well was 10 and 100 μM, and the cells incubated at 37 °C (5% CO_2 humidified atmosphere) for 48 h. Drug solutions were then aspirated and replaced with 200 μl of 0.5 mg/ml MTT in growth medium and incubated for a further 3 h at 37 °C. The MTT solution was aspirated and replaced with 200 μl of DMSO to dissolve the MTT formazan precipitate. The plates were then agitated on a shaker for 5 min, before the absorbance was read at 540 nm on a Multiscan MS multiwell scanning spectrophotometer (Labsystem, Helsinki, Finland).

3. Results

3.1. *Synthesis, qualitative analysis and structural elucidation*

Synthesis of the CDPs via the two synthetic pathways was shown to be successful and the products were of a high purity

Table 1 – Synthesis and physicochemical characterization of selected CDPs

Cyclic dipeptide	Yield (%)	Molecular formula	Molecular mass (g/mol)		TLC	HPLC (t_r, min)	SEM (crystal habit) [2]	Melting point (°C)
			Calculated	Observed[a]				
Cyclo(Phe-Cys)	78.0	$C_{12}H_{14}N_2O_2S$	250.32	251.09	Mobile phase 1: Rf = 0.51 Mobile phase 2: Rf = 0.61	5.97	Needle-like porous crystal	245.37
Cyclo(Tyr-Cys)	70.0	$C_{12}H_{14}N_2O_3S$	266.32	267.08	Mobile phase 1: Rf = 0.42 Mobile phase 2: Rf = 0.76	5.13	Solid flakes	279.84
Cyclo(Gly-L-Val)	89.0	$C_7H_{12}N_2O_2$	156.02	157.09	Mobile phase 3: Rf = 0.74 Mobile phase 2: Rf = 0.69	5.16	Acicular crystal habit	263.42
Cyclo(Gly-D-Val)	88.7	$C_7H_{12}N_2O_2$	156.02	157.08	Mobile phase 3: Rf = 0.73 Mobile phase 2: Rf = 0.68	5.17	Tabular crystal habit	261.29
Cyclo(Gly-Leu)	75.0	$C_8H_{14}N_2O_2$	170.10	171.11	Mobile phase 2: Rf = 0.91 Mobile phase 4: Rf = 0.85	4.65	Bladed/flat acicular crystal habit	236.94
Cyclo(Gly-Ile)	87.4	$C_8H_{14}N_2O_2$	170.10	171.11	Mobile phase 2: Rf = 0.90 Mobile phase 4: Rf = 0.78	4.29	Needle like, acicular crystal habit	240.71

[a] Mass observed for the protonated forms of the CDPs, as determined by FAB-MS.

as shown in Table 1. Structural elucidation by means of MS (Table 1), IR (Table 2) and NMR (data not shown) showed that the products formed corresponded with the structures shown in Fig. 1.

Results obtained for the lowest energy conformer for cFC (−49.89 kcal/mol) and cYC (−95.03 kcal/mol) indicate that the cysteine and phenylalanine/tyrosine side chains are in the unfolded conformation with a twisted-boat form for the diketopiperazine (DKP) ring. This is in contrast to the results obtained from the NMR spectra in DMSO-d_6. The $^3JH_\alpha$-H_β vicinal coupling constants of the cysteine, phenylalanine and tyrosine residues for cFC and cYC that were observed from the NMR spectra are 4.21; 3.67 and 3.54; 3.44 Hz, respectively. According to Kopple and Marr [14] equal couplings near 3 Hz may be expected for a folded conformation, whereas one large (14 Hz) and one small (3 Hz) coupling would present an unfolded conformation. If the two unfolded conformations are equally populated and the folded form is negligibly so, the two couplings should be equal and approximately 8 Hz. If, however, all three conformations are equally populated, the couplings should be equal and near 6.5 Hz. These NMR results obtained indicated that the aromatic side chains of the cysteine-containing diketopiperazines are in the folded conformation with a conformer population of 75.57 and 83.79% for cFC and cYC, respectively [23]. The cysteine side chain for cFC and cYC is also in the folded conformation with a conformer population of 92.19 and 94.43%, respectively.

The lowest energy conformer of cG(D)V (15.85746 kcal/mol) is in a slightly higher energy state than the conformer of cGV (15.85743 kcal/mol). The DKP ring of both CDPs exits pre-

Table 2 – IR absorption bands (cm^{-1}) observed for the diketopiperazines

Band description	Cyclo(Phe-Cys)	Cyclo(Tyr-Cys	Cyclo(Gly-L-Val)	Cyclo(Gly-D-Val)	Cyclo(Gly-Leu)	Cyclo(Gly-Ile)
Amide 1 (CO stretch), *cis* 1670–1690 [19], *trans* 1650	1668.1	1675.3	1695.2	1657.4	1678.9	1670.1
Amide 11 (NH-in-plane vibration), *cis* 1420–1460 [22], *trans* 1480–1575	1455.7	1461.9	1454.2	1550.2	1466.0	1461.0
Amide 111 (*cis* CONH), *cis* 1300–1350 [3]	1341.6	1333.4	1345.5	Not present	1325.7	1335.9
NH stretching, *cis* 3180–3195 [27], *trans* 3350	3189.3	3206.4	3194.4	3350.9	3194.2	3193.7
CN stretching *cis*-Amide or *trans*-amide bond	1341.6 *cis*	1333.4 *cis*	1370.1 *cis*	1369.9 *trans*	1325.7 *cis*	1335.9 *cis*

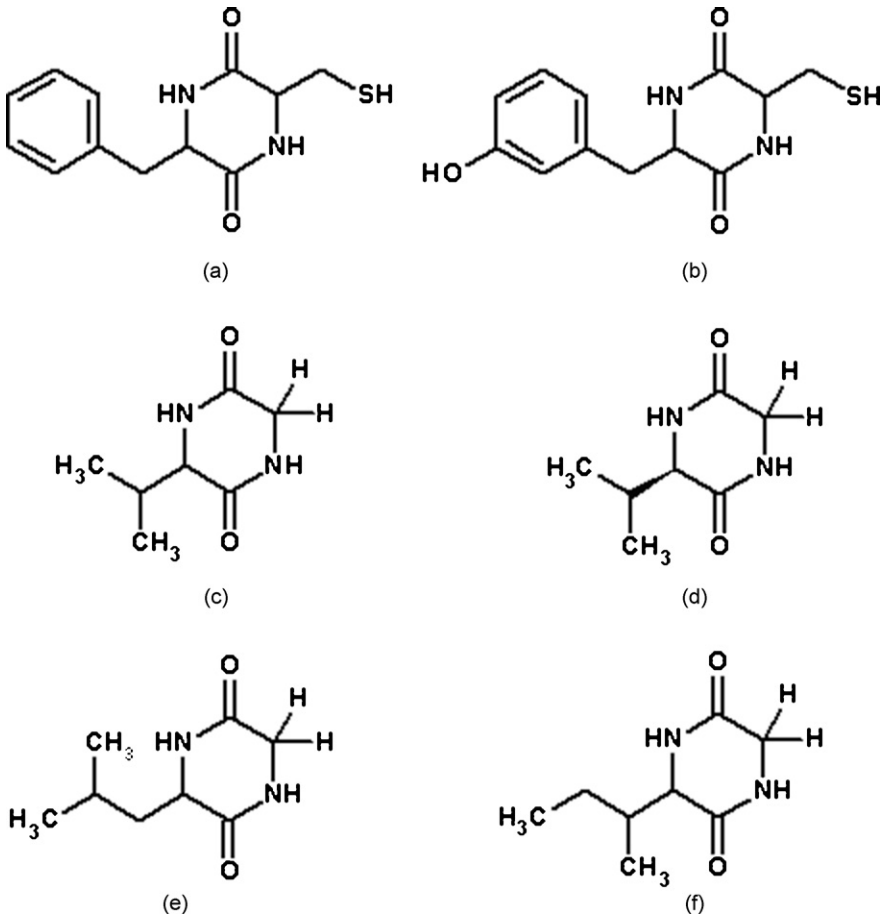

Fig. 1 – Structures of diketopiperazines: (a) cyclo(Phe-Cys), (b) cyclo(Tyr-Cys), (c) cyclo(Gly-Val), (d) cyclo(Gly-D-Val), (e) cyclo(Gly-Leu) and (f) cyclo(Gly-Ile).

dominantly in the boat conformation with an extended valine side chain. The $^{3}JH_{\alpha}$-H_{β} vicinal coupling constants of the valine residue for cGV that were observed from the NMR spectra are 3.4 and 3.4 Hz, respectively. These results indicated that the valine side chain is in a folded conformation with a conformer population of 86% [23].

The lowest energy conformer of cGI (6.356 kcal/mol) is in a higher energy state compared to the lowest energy conformer of cGL (5.588 kcal/mol). Both side chains are in the unfolded conformation. The DKP ring of both CDPs exits predominantly in the boat conformation. The $^{3}JH_{\alpha}$-H_{β} vicinal coupling constants of the leucine residue for cGL that were observed from the NMR spectra are 6.8 and 6.8 Hz, respectively. These results indicated that the leucine side chain is in the unfolded conformations (extended to NH or C═O) with a conformer populations of 38 and 38%, respectively [23].

3.2. *Effects of diketopiperazines on tumor cells*

cFC and cYC significantly reduced the growth of HeLa cells as shown in Table 3. Screening concentrations of 10 and 100 μM cFC lead to a reduction in viable cells of 43.92 ± 0.02% ($P < 0.0001$) and 57.79 ± 0.02% ($P < 0.0001$), respectively, while the 10 and 100 μM screening concentrations of cYC also reduced viable HeLa cell numbers by 54.61 ± 0.02% ($P < 0.0001$) and a 61.36 ± 0.02% ($P < 0.0001$). 10 μM cFC resulted in a 30.56 ± 0.03% reduction in HT-29 cell viability, increasing to 51.29 ± 0.01% inhibition at a concentration of 100 μM while cYC showed inhibition of 32.41 ± 0.03 and 52.27 ± 0.02% at 10 and 100 μM, respectively, indicating a close similarity between the inhibitory potential of the two cysteine-containing CDPs. cFC inhibited the growth of MCF-7 cells, producing a 36.49 ± 0.02% inhibition at 10 μM and a 56.08 ± 0.01% inhibition at 100 μM, while cYC inhibited cell viability by 35.15 ± 0.01% at 10 μM and 52.42 ± 0.02% at 100 μM, once again, showing similar results between the two cysteine-containing CDPs.

10 μM cGV inhibited the growth of HeLa cells by 12.58 ± 1.23% ($P = 0.0219$), while no significant inhibitory effect on HT-29 ($P = 0.0617$) and MCF-7 cell lines ($P = 0.0529$) was observed. At 100 μM, cGV inhibited the growth of all three cell lines with the greatest activity being noted for HT-29 at 23.90 ± 2.25% inhibition ($P = 0.0031$). 10 μM cG(D)V resulted in a significant inhibition of all three cell lines, with the most pronounced activity being noted for MCF-7 cells with an inhibition of 19.78 ± 1.86% while an inhibition of 19.61 ± 0.82% ($P = 0.0231$) and 14.29 ± 1.40% ($P = 0.0392$) was noted for HeLa and HT-29 cells, respectively. The greatest activity noted for cells exposed to cG(D)V at 100 μM was against MCF-7 with an inhibition of 21.94 ± 2.17% ($P = 0.0014$).

Both cGL and cGI at 10 and 100 μM produced moderate inhibitory effects against HeLa, HT-29 and MCF-7 cancer cell lines, except for cGI which showed very little activity against

Table 3 – Effects of diketopiperazines on cancer cell lines

Cyclic dipeptide	Concentration (μM)	HeLa	HT-29	MCF-7
Cyclo(Gly-Leu)	10	22.39 ± 1.98 ($P = 0.0146$)	21.55 ± 1.93 ($P = 0.0097$)	19.50 ± 1.34 ($P = 0.0168$)
	100	28.64 ± 1.51 ($P = 0.0018$)	17.25 ± 1.66 ($P = 0.019$)	27.49 ± 2.68 ($P = 0.0026$)
Cyclo(Gly-Ile)	10	21.33 ± 1.83 ($P = 0.0195$)	8.62 ± 0.78 ($P = 0.133$)	12.56 ± 1.03 ($P = 0.0284$)
	100	28.45 ± 2.05 ($P = 0.0012$)	26.74 ± 1.38 ($P = 0.0028$)	23.58 ± 1.70 ($P = 0.0041$)
Cyclo(Gly-L-Val)	10	12.58 ± 1.23 ($P = 0.0219$)	6.87 ± 0.67 ($P = 0.0617$)	7.00 ± 0.56 ($P = 0.0529$)
	100	23.46 ± 2.04 ($P = 0.0049$)	23.90 ± 2.25 ($P = 0.0031$)	21.19 ± 2.04 ($P = 0.0107$)
Cyclo(Gly-D-Val)	10	19.61 ± 0.82 ($P = 0.0231$)	14.29 ± 1.40 ($P = 0.0392$)	19.78 ± 1.86 ($P = 0.0015$)
	100	20.55 ± 2.03 ($P = 0.0043$)	16.78 ± 1.65 ($P = 0.0157$)	21.94 ± 2.17 ($P = 0.0014$)
Cyclo(Phe-Cys)	10	43.92 ± 0.02 ($P < 0.0001$)	30.56 ± 0.03 ($P < 0.0001$)	36.49 ± 0.02 ($P < 0.0001$)
	100	57.79 ± 0.02 ($P < 0.0001$)	51.29 ± 0.01 ($P < 0.0001$)	56.08 ± 0.01 ($P < 0.0001$)
Cyclo(Tyr-Cys)	10	54.61 ± 0.02 ($P < 0.0001$)	32.41 ± 0.03 ($P < 0.0001$)	35.15 ± 0.01 ($P < 0.0001$)
	100	61.36 ± 0.02 ($P < 0.0001$)	52.27 ± 0.02 ($P < 0.0001$)	52.42 ± 0.02 ($P < 0.0001$)
Cisplatin	10	75.50 ± 0.68 ($P = 0.00034$)	42.20 ± 3.23 ($P = 0.00161$)	63.81 ± 2.74 ($P = 0.00048$)
	100	88.90 ± 0.26 ($P = 0.00028$)	83.77 ± 1.27 ($P = 0.00021$)	77.04 ± 1.94 ($P = 0.00012$)

Values represent mean percentage inhibition ± S.D. ($n = 6$).

HT-29 cells at a concentration of 10 μM with the greatest activity being noted for 100 μM cGL against HeLa cells (28.64 ± 1.51% inhibition, $P = 0.0018$).

The results of the study clearly indicate the positive control, cisplatin, to have the most significant inhibition of tumor cell growth for all three cell lines in comparison to the CDPs. Cisplatin at 100 μM produced the most significant inhibitory effect on the HeLa cell line resulting in an 88.90 ± 0.26% inhibition ($P = 0.00028$).

4. Discussion

Cysteine-containing diketopiperazines require time-consuming preparations. This may be the reason why, to our knowledge, they have not been used extensively in biological studies. The synthesis of cysteine-containing peptides requires the heavy use of protecting groups, which may result in many reaction steps. As many as eight steps may be required to synthesize any cysteine-containing diketopiperazines [28]. A successful route of synthesis for cysteine-containing CDPs was established showing good yields (>70%). The synthesis of the other four CDPs was also successfully accomplished by the method of Kopple and Ghazarian [13].

Results from the anticancer studies (Table 3), suggest that cFC and cYC have the potential as antitumor agents against HeLa (cervical carcinoma), HT-29 (colon carcinoma) and MCF-7 (breast carcinoma). Moderate inhibition of all three cell lines (ranging from 12.56 to 28.64%) was observed for cGV, cG(D)V, cGL and cGI against the three cell lines at the concentrations tested except for cGI at a concentration of 10 μM against HT-29 cells and 10 μM cGV against HT-29 and MCF-7 cells, respectively.

The results, when compared to an established tumor suppressive agent such as the positive control, cisplatin, indicate only a mild tumor suppressive action for the CDPs, with the highest concentration of cisplatin resulting in more than a 20% higher inhibitory action for the best performing CDP. This indicates that the clinical potential of these agents is limited in their current form and it is recommended that modification of the structures should be investigated in order to optimize their tumor suppressive actions with special reference to the cysteine-containing CDPs.

5. Conclusion

From the qualitative analysis performed, the synthesized cyclic dipeptides were found to be free of any contaminants, which enabled them to be used in various biological studies with the aim of being possible novel drug candidates. Structural elucidation allowed for the verification of the respective cyclic dipeptide structures, and determined their possible conformations and degradation patterns.

cYC showed significant antitumor activity, causing the greatest inhibition in cervical carcinoma cells. cGV is more active in terms of its ability to inhibit colon and cervical carcinoma cell lines. cG(D)V can be described as being most active against breast carcinomas with near equivalent activity against cervical carcinomas. The two diketopiperazines should be tested in combination to determine whether any possible synergistic effects are present.

Although this study serves primarily to screen selected CDPs as potential tumor suppressive agents, it does, however, highlight the potentially useful effects of this group of molecules in the treatment of cancer.

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