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The biological activity of the histidine-containing diketopiperazines cyclo(His-Ala) and cyclo(His-Gly)

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ARTICLE INFO

Article history:
Received 2 February 2006
Received in revised form
25 April 2006
Accepted 25 April 2006
Published on line 21 June 2006

Keywords:
Cyclo(His-Ala)
Cyclo(His-Gly)
Antimicrobial
Anticancer
Cardiac activity
Antithrombotic effects

ABSTRACT

Two cyclic dipeptides, cyclo(His-Ala) and cyclo(His-Gly,) were synthesized from their linear counterparts and their structures elucidated using standard elucidation techniques. Molecular modeling and predictive NMR results indicated that the majority of energetically favourable conformers adopted a boat conformation with respect to the diketopiperazine ring. Cyclo(His-Ala), at concentrations of 100 μM inhibited the growth, in vitro, of various cancer cell lines, including HT-29, MCF-7 and HeLa carcinoma cells while cyclo(His-Gly) inhibited the growth of MCF-7 cells. While the antibacterial potential of these two compounds was limited, both cyclic dipeptides significantly inhibited the growth of *C. albicans*. Both compounds at a concentration of 100 μM resulted in a decrease in heart rate, coronary flow rate and left ventricular systolic pressure in the isolated rat heart. Inhibition of thrombin, amounting to a 63.3% and 36.7% reduction in the rate of fibrin formation, was noted for cyclo(His-Ala) and cyclo(His-Gly), respectively. While cyclo(His-Ala) showed no notable effects on platelet aggregation, cyclo(His-Gly) significantly inhibited both pathways tested with greatest effects on thrombin-induced platelet aggregation, yielding an IC_{50} of 0.0662 mM ($R^2 = 0.989$). The results of the anticancer and hematological studies indicate that histidine-containing diketopiperazines have potential as a novel group of cytotoxic agents with antithrombotic effects.

1. Introduction

Initially cyclic dipeptides were synthesized for the sole purpose of examining their particularly interesting physicochemical properties [27]. Originally thought to be inert, their metabolic fate was paid little attention [2]. However with a growing awareness of the diversity and biological roles played by many of the diketopiperazines found in nature, interest in these compounds has grown. Although cyclic dipeptides are omnipresent in nature, only a handful have known biological activity in mammals. These include cyclo(His-Pro), cyclo(Leu-Gly), cyclo(Tyr-Arg) and cyclo(Asp-Pro) [24]. Previously, dipeptides were thought merely to be undesirable by-products of polypeptide synthesis, but they have since been shown to possess remarkable biological activities [2,9].

Derivatives of compounds with diketopiperazine rings, such as bicyclomycin, have shown antitumor properties. Gliotoxins too have shown antiviral properties [10]. Diketopi-

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0196-9781/$ – see front matter
doi:10.1016/j.peptides.2006.04.023

perazines have been identified in nature as playing an important role as links between small peptides such as the growth factor rhodotorulic acid [5]. Restrained analogues of cholecystokinin-4 (CCK-4) containing a diketopiperazine nucleus were prepared to provide rigid templates for the eventual design of peptidomimetics as the diketopiperazine was believed, in all likelihood, to improve central nervous system (CNS) permeability [4].

The literature suggests cyclic dipeptides exist as fairly stable entities. However, it has been shown that either of the carbonyl groups on the diketopiperazine ring can in certain circumstances undergo hydrolysis resulting in two different peptides [15] which suggests their possible use as pro-drugs. Studies reported by Mizuma et al. [25] indicated that the cyclic form of peptides are stable against peptidases in the intestine, retaining the structural parameters required for active transport [24,25]. The stability of cyclo(His-Gly) is illustrated by the experiments conducted by Sheehan and McGregor [30], which shows the decomposition of cyclo-glycyl-L-histadyl-L-serylglycyl-L-histadyl-L-seryl into the diketopiperazine of glycine and histidine together with serine. It is reasonable to assume that the serine hydroxyl group may catalyse the decomposition of the histidine amide bond if the two moeties are in the appropriate steric positions [30].

The linear form of the dipeptide is often less stable in vivo than its cyclic counterpart, therefore making the latter variety far more promising in terms of drug candidacy. Added to this is their markedly reduced conformational mobility and relative simplicity that make diketopiperazines model compounds for studying the conformations of larger peptides [18]. Cyclic dipeptides may have a higher activity and in addition the selectivity in receptor binding may be enhanced by the aforementioned simplicity. The lack of a free terminal carboxylic acid or nitro group and higher rigidity of the peptide backbone may be reasons for the prolonged in vivo lifetime often observed with cyclic versions [19]. Diketopiperazines are readily formed from dipeptide esters (due to the presence of a good leaving group) and dipeptides (with alternate chirality). This is owing to the new species displaying increased stability in comparison with its precursors [12].

It is a well-documented fact that dipeptide esters and amides readily undergo cyclization to the diketopiperazine and that the rate of cyclization competes favorably with hydrolysis [15]. Cyclization is the simplest and most efficient means of producing a conformationally constrained peptide. This cyclic nature frequently leads to superior receptor specificity [8]. The presence of one 4–6 membered ring annealed to the 2,5-diketopiperazine ring results in a considerable rigidity of the cyclic dipeptide as well as some degree of deformation with decreases in C–C–N bond angles [33]. Higher linear peptides containing imino acid residues have been shown to spontaneously cyclisize forming a diketopiperazine ring. The biological implication of this is intriguing [5]. It has been shown that in the majority of cases the presence of an imidazole ring, such as that found as part of the histidine residue, is located above the 2,5-diketopiperazine ring with possibilities of interaction with both cis amides, further contributing to the structural rigidity [2].

Diketopiperazines present a unique class of compounds, as their six-membered ring is able to adopt a multitude of conformations, such as chairs, boats and twists. This is in contrast to the fact that non-aromatic six-membered rings invariably assume chair-like conformations when unconstrained [18]. Conformational rigidity enhances cyclic dipeptides' ability to serve as ideal lead compounds for rational drug design. Modification of the primary structure of the cyclic dipeptide and the resultant alteration in the side chain, or lack thereof, attached to the diketopiperazine ring may be utilized to manipulate the physicochemical and biological activity of the agent [31]. Physicochemical properties such as hydrophilicity, may be modified by altering the R substituents attached to the diketopiperazine ring [13,31]. Diketopiperazines appear attractive in thermodynamic studies on non-ionic compounds in water due to the fact that they share the capability of establishing hydrogen bonds with the solvent via the two cis-amide groups of the DKP ring, while still giving rise to the possibility of hydrophobic interactions as determined by the R substituents. The histidine moiety is a well recognized enzymatic entity proving to be a powerful hydrolytic catalyst when combined with leucine in a cyclic dipeptide form [17]. Furthermore diketopiperazines possess the necessary characteristics to act as denaturing agents, by bringing about a weakening of secondary structures of globular proteins by competing with inter-chain hydrogen bonds [13]. There has been an increased interest in studying diketopiperazine derivatives which are designed to substitute a dipeptide unit within a peptide chain and thus induce certain conformational restrictions. These resultant restrictions would serve to either increase or decrease biological response to an agent, cause antagonism or inhibition with regards to an enzyme target or indeed induce receptor subtype selectivity, thereby potentially reducing side-effects [3].

2. Materials and methods

2.1. *Synthesis, chemicals, solutions and structural elucidation*

The diketopiperazines cyclo(His-Ala) and cyclo(His-Gly) were synthesized by means of a modified method of Kopple and Ghazarian [20]. Sterically pure linear dipeptides were cyclisized using heated phenol (140–150 °C). Purity was determined by chromatographic (HPLC & TLC) and thermal analysis while structural elucidation was achieved using mass spectrometry, infrared spectroscopy and nuclear magnetic resonance spectroscopy. The synthesis and structures of the diketopiperazines are shown in Fig. 1. After synthesis, cyclo(His-Ala), cyclo(His-Gly) and all solutions were stored at 4 °C prior to use.

2.2. *Ethical approval*

The use of human blood samples in the hematological studies was approved by the Human Ethics Committee of the NMMU (formerly University of Port Elizabeth) (approved January 2003). The use of experimental animals was approved by the Animal Ethics Committee of the NMMU (Formerly University of Port Elizabeth) (approved January 2003).

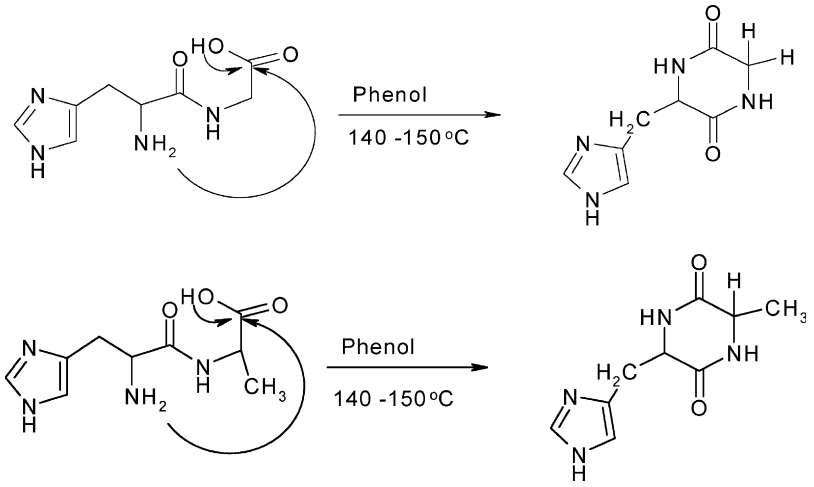

Fig. 1 – Synthesis and structures of the histidine-containing diketopiperazines, cyclo(His-Gly) (top) and cyclo(His-Ala) (bottom).

2.3. *Hematological methods*

2.3.1. *Thrombin assay*

The method for the thrombin-substrate assay was adapted from Rob et al. [28] and involves the reaction of the enzyme thrombin with the chromogenic thrombin specific substrate S2238 (chromogenix). A Labsystems Multiskan microtiter plate reader was used to measure absorbance at 410 nm every 10 s for 5 min. Solutions of cyclo(His-Ala) and cyclo(His-Gly) were prepared using 1% dimethyl sulphoxide (DMSO) in buffer A containing 145 mM NaCl, 5 mM KC1, 10 mM HEPES, 0.5 mM Na_2HPO_4, 6 mM glucose with 0.2% w/v bovine serum albumin in double distilled, de-ionized water. Final concentrations of cyclo(His-Ala) and cyclo(His-Gly) ranged from 0.0125 mM to 0.2 mM. A control was used consisting of buffer A, 1% DMSO and 30 U/ml of thrombin. The experiments were repeated in triplicate and expressed as a percentage absorbance versus time.

2.3.2. *Platelet aggregation*

Platelet aggregation studies were prepared from methods adapted from Walkowiak et al. [34] and Bednar et al. [6] on the basis that there is an increase of light transmittance through a suspension of individual platelets as it is converted into a suspension of aggregates following stimulation by ADP or thrombin [6]. Approximately 8 ml of blood was drawn by venipuncture from a healthy volunteer who had abstained from any medication, alcohol or cigarettes for two weeks and fasted for 8 h prior to the procedure. Blood was collected in 4.5 ml tubes containing 0.105 mM trisodium citrate at a ratio of 9 parts blood to 1 part trisodium citrate. The blood was then centrifuged at $300 \times g$ for 10 min to obtain platelet rich plasma (PRP) and an aliquot was re-centrifuged at $900 \times g$ for 20 min to obtain platelet poor plasma (PPP). The platelet suspension was then diluted to yield the desired concentration of 45×10^6 platelets/ml. The PRP was used within 1 h and pre-warmed to 37 °C. The cyclic dipeptides cyclo(His-Ala) and cyclo(His-Gly) were prepared by dissolving them in 1% DMSO in buffer A to provide the final concentrations ranging from 0.0104 mM to 0.3330 mM. The change in turbidity at 600 nm was measured every 4 min for 1 h. Two methods of platelet aggregation were monitored. Thrombin-induced platelet aggregation used 2.5 U/ml thrombin and ADP-induced platelet aggregation used 20 μM ADP. Triplicate results were obtained and represented as a percentage transmission versus time.

2.3.3. *Fibrinolysis*

Fibrinolysis was adapted from the methods outlined in Schatteman et al. [29]. The cyclic dipeptides, cyclo(His-Ala) and cyclo(His-Gly), were tested at final concentrations of 0.0078–0.25 mM. The final concentration of streptokinase used was 400 units/ml. The absorbance was measured at 410 nm every 5 min for the first 30 min, and then every 10 min for a further 190 min. The positive control consisted of 80 μl citrated plasma, 20 μl streptokinase, 40 μl 0.9% (m/v) sodium chloride solution and 20 μl of 160 mM calcium chloride solution. All experiments were conducted in triplicate.

2.4. *Cardiac methods*

Male Long Evans rats weighing between 250 g and 300 g were anesthetized in an ether saturated desiccator to loss of pain and blink reflexes. 20 IU of Heparin Sodium (Fresenius-Kabi, South Africa) was injected into the left femoral vein to prevent blood coagulation in coronary vessels. Once the heart was removed it was arrested in an ice-cold solution (4 °C) of a modified Krebs-Henseleit Buffer (KHB), consisting of 118 mM Na^+, 1.2 mM $H_2PO_4^-$, 1.2 mM Mg^{2+}, 2.5 mM Ca^{2+}, 25 mM HCO_3^-, 123 mM Cl^-, 1.2 mM SO_4^{2-}, 5.9 mM K^+ and 11 mM glucose. In order to replace cold and desaturated medium with warm carbogenated medium, 5–10 ml of medium were run through the cannula before the heart was mounted. The aorta was then cannulated, the cannula tied in place with a silk thread and the perfusion medium allowed to flow freely through the coronary vessels. A platinum ECG electrode was carefully inserted into the myocardium of the right ventricle, while the anode of the bipolar ECG leads was clamped to the cannula. The temperature of the beating heart was main-

tained at 37 °C by surrounding it with a warming jacket. An original Langendorff perfusion system was modified from that described by Lubbe et al. [23] to enable dual perfusion of physiological buffers and to alternate the perfusion medium from Krebs-Henseleit physiological buffer (KHB) to drug-containing KHB instantly. A Watson–Marlow multihead peristaltic pump maintained the one-meter of constant water pressure required. Carbogen (95% O_2, 5% CO_2) (Afrox, South Africa) was constantly bubbled through the perfusion medium which was maintained at 37 °C. The perfusion medium flowing out of the heart was collected at 5 min intervals in a measuring cylinder as a measure of coronary flow rate.

Two experimental protocols were used, one investigating the effects of the cyclic dipeptides on heart rate and coronary flow rate over a 30 min infusion period and the other investigating the drugs' effects on ventricular pressure over a 30 min infusion period, independent of heart rate. The second protocol was achieved by placing a small PVC balloon connected to a pressure transducer inside the left ventricle through an incision made in the left atrial wall and the hearts paced at 300 bpm.

2.5. Microbiological methods

The test organisms included Gram-positive bacteria (*Bacillus* spp., *Staphylococcus aureus*, *Streptococcus pyogenes*), Gram-negative bacteria (*Klebsiella pneumoniae*, *Salmonella typhimurium*, *Escherichia coli*), and a yeast fungus (*Candida albicans*). A microplate method adapted from Eloff [14] and Thom et al. [32], using *p*-iodonitrotetrazolium (INT) violet (Sigma–Aldrich) was used. Substrate-dependent reduction of INT, a water-soluble dye, indicates active metabolism by such cells through induction of a color change within the sample. This change can then be measured using spectrophotometric analysis. Cyclo(His-Ala) and cyclo(His-Gly) were dissolved in 1% dimethyl sulphoxide (SAARCHEM) to yield a stock concentration of 1 mM in single strength nutrient broth (for bacteria) or Sabouraud broth (for *C. albicans*). Fifty microlitres of peptide was added to the well and made up to volume by adding 50 μl of the standardized culture. The final concentration in each well was 0.5 mM of cyclic dipeptide. Fifty microlitres of control drug was added to respective wells together with 50 μl of specific test organism. The bacterial or fungal control wells were made up of 50 μl of single strength nutrient broth or Sabouraud broth together with 50 μl of the specific micro-organisms being tested. All controls and experiments were conducted in quadruplicate. The 96-well plate was then incubated at 37 °C for 24 h in an orbital shaker at 150 rpm. After incubation, 40 μl of *p*-iodonitrotetrazolium violet (0.1 μg/μl) was added to each well and the zero time read on an automated spectrophotometric microplate reader at 540 nm (bacteria) or 600 nm (fungi). Thereafter, for bacteria, the 96-well plate was incubated at 37 °C for 1 h at 150 rpm to allow the INT to be metabolized by viable bacteria. A second reading was taken at 540 nm. The t_0 values were subtracted from the second microtiter reading and percentage viability calculated relative to the controls. For fungi, the plate was incubated at 28 °C for 4 h and the second reading was also taken at 600 nm.

2.6. Anticancer methods

A primary screen for anticancer potential of the cyclic dipeptides cyclo(His-Ala) and cyclo(His-Gly) was conducted using three cell lines of different origin, namely HT-29 (colon carcinoma), MCF-7 (breast carcinoma) and HeLa (cervical carcinoma). RPMI 1640 (Highveld Biological, South Africa) supplemented with 10% heat inactivated fetal calf serum (Highveld Biological) and 2 mM glutamine was used to maintain all three cell lines. The cells were cultured in 10 cm culture dishes (Nunclon) at 37 °C in a humidified 5% CO_2 incubator. The medium was replaced every 48 h and the cells sub-cultured by trypsinization at approximately 80% confluency. For determination of cell viability, cells were seeded into 96-well culture plates (Nunclon) at a density of 6000 cells/well in 200 μl aliquots. Cells were left for 24 h before the medium was replaced with culture medium containing 10 μM or 100 μM concentrations of cyclo(His-Ala), cyclo(His-Gly) and cisplatin (generously donated by Prof JGH du Preez from the Unit for Metal Ion Separation, Nelson Mandela Metropolitan University). The samples were solubilized in DMSO before further dilution with culture medium to give a final DMSO concentration of 0.25%. Control wells received culture medium with 0.25% DMSO. Both samples and control were prepared in quadruplicate for each cell line. The plates were incubated for a further 48 h at 37 °C. Immediately following the 48-h incubation period, cell numbers were determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay as previously described [1,11].

2.7. Statistical analysis

The average for all replicates was determined and represented as a mean ± standard deviation. Differences in means were deemed statistically significant, if, after performing a Student's *t*-test, a *p*-value of less than 0.05 was obtained. Graphical representation of data and statistical analysis was performed using the software package Graphpad Prism version 4.02 (Graphpad Software Inc., San Diego, CA).

3. Results

3.1. Synthesis and structural elucidation

The synthesis of cyclo(His-Ala) and cyclo(His-Gly) showed relatively good yields of 91 and 84%, respectively. Purity was confirmed by HPLC and TLC. HPLC analysis, using an Aztec chirobiotic column (250 mm × 46 mm) and a mobile phase of methanol–acetate buffer (30:70) yielded a single peak with retention times of 5.74 and 5.58 min for cyclo(His-Ala) and cyclo(His-Gly), respectively. TLC analysis, with a Silica gel 60 F_{254} (Merck, SA) stationary phase and two separate mobile phases (mobile phase 1: isopropyl ether–NH_4OH (1:1); mobile phase 2: methanol–chloroform–ammonia solution 17% (2:2:1)) showed single pure spots with R_f values of 0.7917 (mobile phase 1) and 0.8752 (mobile phase 2) for cyclo(His-Ala) and 0.6520 (mobile phase 1) and 0.9116 (mobile phase 2) for cyclo(His-Gly). Mass spectra showed that the parent peaks $(M+H]^+$) of both cyclo(His-Ala) and cyclo(His-Gly) correspond

to the exact molecular mass of each respective protonated molecule namely 209 *m/e* and 195 *m/e*. Both parent peaks have a saturation index of 6, confirming their presence. The saturation index is calculated from the formula $C_xH_yN_zO_n$, where the total number of rings plus double bonds is equal to *x* minus half of *y* plus half of *z* plus 1. In other words the oxygen is not included when calculating saturation indices [22]. NMR analysis confirmed the structures of the synthesized compounds corresponding to those proposed in Fig. 1.

3.2. *Effects of cyclo(His-Ala) and cyclo(His-Gly) on thrombin inhibition*

Both cyclo(His-Ala) and cyclo(His-Gly) showed promising results in the inhibition of the rate of fibrin formation. Cyclo(His-Ala), at higher concentrations, showed no significant effects but did show a 43.3% and 63.3% reduction ($p < 0.001$ for both) in clotting rate at 0.025 and 0.0125 mM, respectively, indicating that lower concentrations yielded a greater effect (Fig. 2a). No noticeable difference was seen between the highest and lowest concentrations of cyclo(His-Gly), with even the lowest concentration tested showing a 36.7% reduction ($p < 0.01$) in the rate of fibrin formation (Fig. 2b).

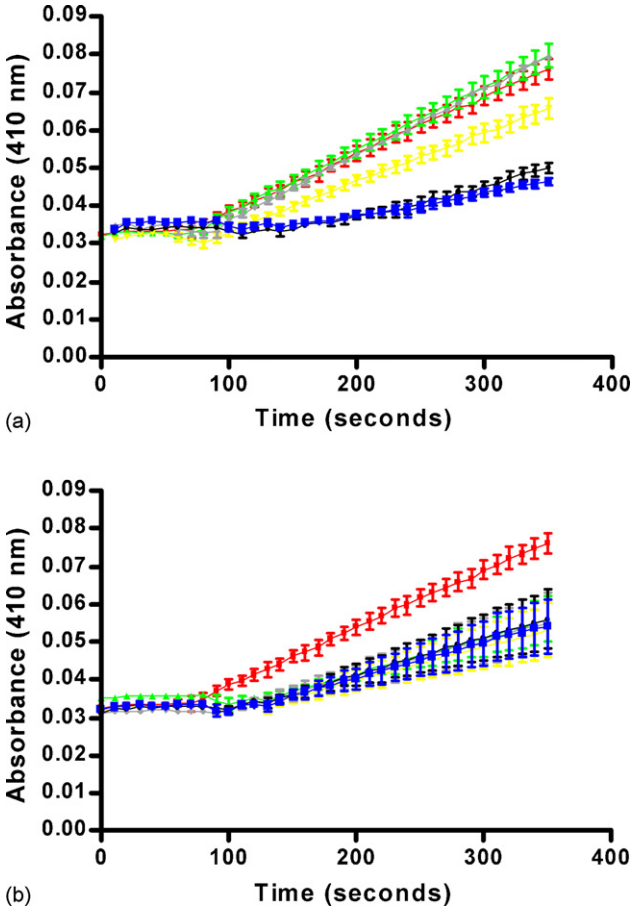

Fig. 2 – The effect of (a) cyclo(His-Ala) and (b) cyclo(His-Gly) on thrombin-substrate binding. Results indicated that both cyclic dipeptides caused a reduction in the rate of fibrin formation with cyclo(His-Ala) having greater effects at lower concentrations. Data points represent means ± standard deviation of triplicate determinations at concentrations of 0.2 (), 0.1 (), 0.05 (), 0.025 (), and 0.0125 () mM when compared with the control ().

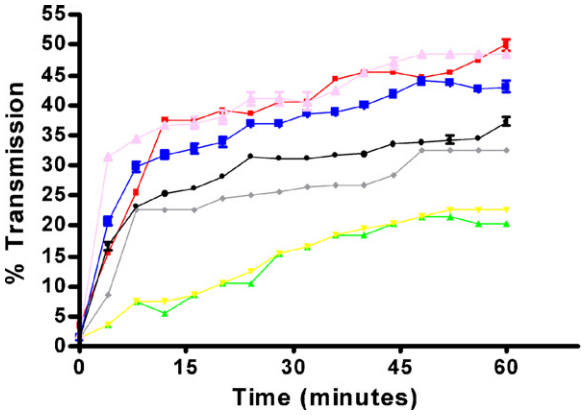

Fig. 3 – The effect of cyclo(His-Gly) on thrombin-induced platelet aggregation. Non-linear regression analysis revealed that, after 60 min exposure, the IC_{50} for cyclo(His-Gly) was 0.0662 mM ($R^2 = 0.989$). Data points are represented as means ± standard deviation of triplicate determinations at concentrations of 0.33 (), 0.167 (), 0.083 (), 0.042 (), 0.021 () and 0.010 () mM when compared with the control ().

3.3. *Effects of cyclo(His-Ala) and cyclo(His-Gly) on platelet aggregation*

No notable inhibition of thrombin-induced or ADP-induced platelet aggregation occurred with cyclo(His-Ala) (data not shown). Cyclo(His-Gly), however, produced significant inhibition of thrombin-induced platelet aggregation (Fig. 3). Analysis of the results using non-linear regression investigating the dose-response relationship for cyclo(His-Gly) showed positive results, yielding an IC_{50} of 0.0662 mM with a Hillslope factor of 0.8607 ($R^2 = 0.989$). Cyclo(His-Gly) also showed a dose-dependent inhibition of ADP-induced platelet aggregation (Fig. 4). Non-linear regression analysis revealed an IC_{50} of 0.1636 mM with a Hillslope factor of 2.618 ($R^2 = 0.998$).

3.4. *Effects of cyclo(His-Ala) and cyclo(His-Gly) on cardiac activity*

Hundred micromolars of cyclo(His-Ala) and cyclo(His-Gly) had marked effects on coronary flow rate, heart rate and ventricular pressure in the retrogradely perfused, isolated rat heart.

Hearts exposed to 100 μM cyclo(His-Ala) exhibited a 38.2 ± 4.8 bpm reduction in heart rate ($p < 0.001$) after 30 min exposure as compared with the control group while 100 μM cyclo(His-Gly) also resulted in a decrease in heart rate, amounting to 48.0 ± 7.4 bpm after 30 min exposure (Fig. 5). Cyclo(His-Ala) and cyclo(His-Gly) both resulted in a decrease in coronary flow rate of 1.9 ± 0.25 ml/min ($p < 0.001$) and

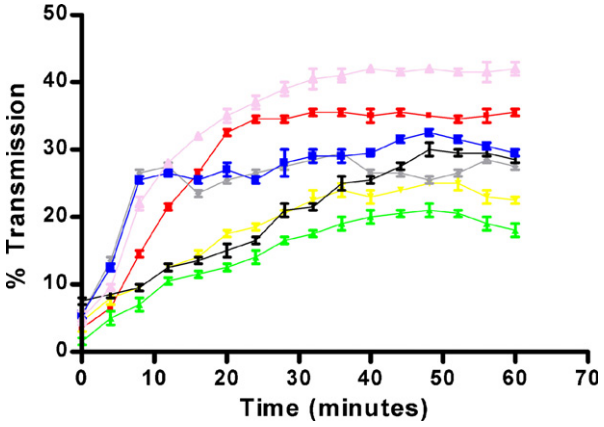

Fig. 4 – The effect of cyclo(His-Gly) on ADP-induced platelet aggregation. Non-linear regression analysis revealed that, after 60 min exposure, the IC_{50} for cyclo(His-Gly) was 0.1636 mM ($R^2 = 0.998$). Data points are means ± standard deviation of triplicate determinations at concentrations of 0.33 (▲), 0.167 (▼), 0.083 (●), 0.042 (■), 0.021 (■) and 0.010 (▲) mM when compared with the control (■).

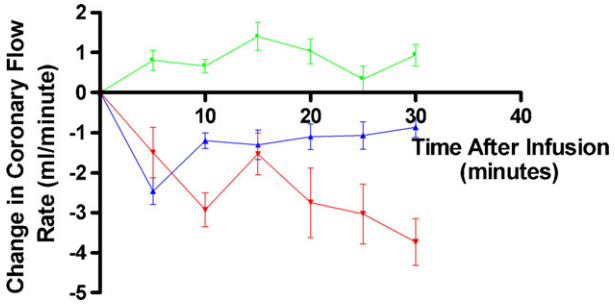

Fig. 6 – Effects of 100 μM cyclo(His-Ala) and cyclo(His-Gly) on coronary flow rate. Both cyclo(His-Ala) (▲) and cyclo(His-Gly) (▼) showed significant ($p < 0.001$) changes in coronary flow rate over the experimental period when compared to the control (■). The cyclic dipeptides also differed significantly form each other with respect to their effect on coronary flow rate ($p < 0.05$). Data points are means ± standard deviation ($n = 6$ for each data point).

4.7 ± 0.57 ml/min ($p < 0.001$) during the first experimental protocol (Fig. 6). The same cardiac depressive effects were noted for investigations into the effects of cyclo(His-Ala) and cyclo(His-Gly) on left systolic ventricular pressure (LSVP). Cyclo(His-Ala) and cyclo(His-Gly) resulted in a decrease in the LSVP of 50.4 ± 3.8 mmHg ($p < 0.001$) and 42.3 ± 7.7 mmHg ($p < 0.001$), respectively in paced rat hearts (Fig. 7).

3.5. *Effects of cyclo(His-Ala) and cyclo(His-Gly) on microbial cultures*

Both cyclic dipeptides cyclo(His-Ala) and cyclo(His-Gly) were effective against two of the three Gram-positive bacteria, namely *Bacillus* and *S. aureus* at concentrations of 0.5 mM (data not shown). Cyclo(His-Ala) was more effective against the above mentioned bacteria than cyclo(His-Gly) ($p < 0.05$). Cyclo(His-Gly), however, was more effective against *K. pneumoniae* (Gram-negative) than cyclo(His-Ala). Both cyclo(-His-Gly) and cyclo(His-Ala) showed viabilities of greater than 100% when compared to the negative control and therefore exhibited growth stimulation of *Pseudomonas aeruginosa* ($p < 0.05$).

The positive antibiotic controls all showed an expected trend, causing, for the most part, significant inhibition of growth. An interesting result occurred when *Salmonella* spp. was exposed to chloramphenicol. This species was usually chloramphenicol-sensitive, however, as reported by Kucers and Bennett [21], drastic changes in the sensitivity of these organisms have occurred previously.

The antifungal activity of cyclo(His-Ala) and cyclo(His-Gly) appeared to be promising, with both exhibiting a tendency to prevent growth (data not shown). Both cyclo(His-Ala) and cyclo(His-Gly) inhibited the growth of *C. albicans* by 66.3% and 47.0%, respectively ($p < 0.05$ for both).

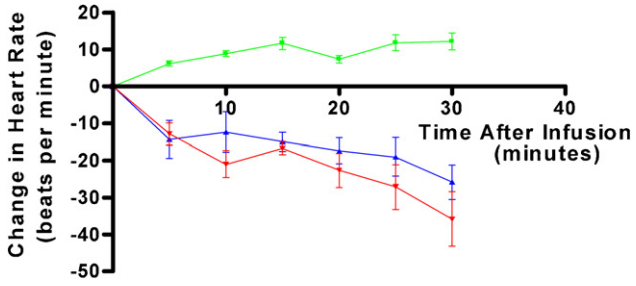

Fig. 5 – Effects of 100 μM cyclo(His-Ala) and cyclo(His-Gly) on heart rate in the isolated rat heart. Results showed a significant decrease in heart rate ($p < 0.001$) for both cyclo(His-Ala) (▲) and cyclo(His-Gly) (▼) with respect to the control experiments (■), from 5 min through to the end of the experiment. Data points are means ± standard deviation ($n = 6$ for each data point).

3.6. *Effects of cyclo(His-Ala) and cyclo(His-Gly) on tumor cells*

Cyclo(His-Ala), except for MCF-7 cancer cells, yielded better percentage inhibition than cyclo(His-Gly) (Fig. 8). Cyclo(His-Ala) showed no significant ($p > 0.05$) difference in percentage inhibition at 100 μM when compared with cyclo(His-Gly) at the same dose against HT-29 colon cancer cells. Furthermore the percentage inhibition between cyclo(His-Ala) at the above mentioned dose was also not significantly different ($p > 0.05$) to that observed for the same drug at a dose of 10 μM (Fig. 8). Perhaps one of the most promising results of this study was the fact that cyclo(His-Ala) at a dose of 100 μM was statistically comparable with cisplatin at the same dose ($p > 0.05$) against HeLa carcinoma cells (Fig. 8). Against both MCF-7 and HT-29 cell lines there was no significant difference ($p > 0.05$) between cyclo(His-Ala) at a dose of 100 μM and cisplatin at a dose of 10 μM, indicating that the cancer cell inhibition of cyclo(His-Ala) in these two lines is comparable with that of cisplatin, albeit at doses 10 times higher.

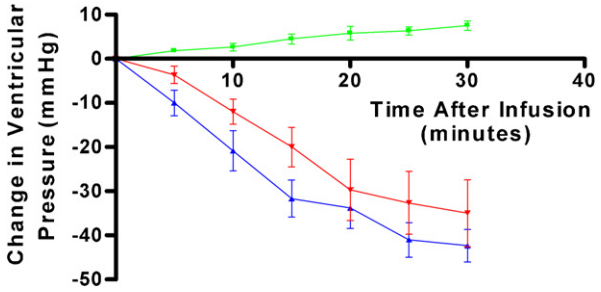

Fig. 7 – Effects of 100 μM cyclo(His-Ala) and cyclo(His-Gly) on ventricular pressure. Heart rate was maintained at 300 beats per minute for the duration of the experiment. Both cyclo(His-Ala) (▲) and cyclo(His-Gly) (▼) showed significant decreases ($p < 0.001$) in left ventricular systolic pressure after 30 min when compared to the control (■). Data points are means ± standard deviation ($n = 6$ for each data point).

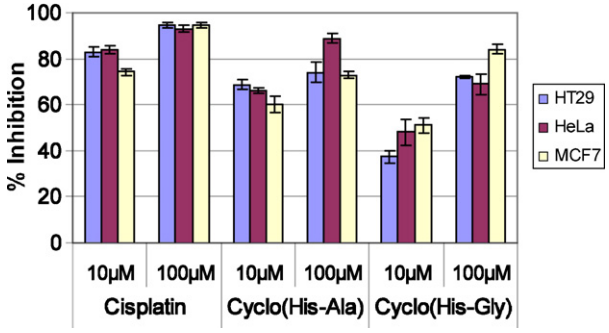

Fig. 8 – Inhibition of growth by cyclo(His-Ala), cyclo(His-Gly) and cisplatin on HT-29, HeLa and MCF-7 cells. Cyclo(His-Ala) showed the greatest inhibition against HeLa cells, with the percentage inhibition being comparable to cisplatin at the same concentration. Data points are represented as means ± standard deviation of quadruplicate determinations.

4. Discussion

4.1. *Antithrombotic and anticancer activity of cyclo(His-Ala) and cyclo(His-Gly)*

The association between tumor development and thrombosis is widely known, where tumor-associated thrombosis may often lead to death via disseminated intravascular coagulation [26]. It has also been proposed that thrombin plays another role in the development of tumors, in that thrombin may also activate tumor cells to become more adhesive, promoting metastatic progression of the disease [26]. The link between coagulation and neoplasia has therefore lead to the need for treatments that inhibit tumor growth as well as thrombin-associated coagulation. Both compounds tested showed inhibition of cancer cells in vitro with cyclo(Gly-Ala) showing the greatest activity. Both agents also resulted in significant inhibition of thrombin induced clot formation, with cyclo(Gly-Ala) causing the greatest inhibition (63.3%) at lower concentrations. Cyclo(His-Gly), however, showed promise as an anticoagulant, as it also inhibited platelet aggregation, showing greatest activity against the thrombin-induced platelet aggregation pathway. None of the two compounds tested had any notable effects as fibrinolytics, indicating that they would be useful to reduce a hypercoagulable state but would have no effects on already-formed blood clots. When looking at the structure of these two cyclic dipeptides, it is interesting to note the similarity, in that the only structural difference between cyclo(His-Ala) and cyclo(-His-Gly) is the methyl group on the diketopiperazine ring in cyclo(His-Ala). The difference in activity between these two molecules, especially with respect to platelet aggregation, provides useful insight into the role of the methyl group in the structure-activity relationship of anticoagulants. This perhaps indicates that the presence of a methyl group such as that found on cyclo(His-Ala) may be an essential pharmacophore for the inhibition of the three lines tested, but particularly with respect to HeLa cervical cancer cells. The additional methyl group of cyclo(His-Ala) increases the lipophilicity of the molecule, facilitating better permeation into cells, enhancing its effects. It is therefore recommended that substitution of the methyl group on the Alanine moiety be investigated to improve the anticoagulant potential, while still maintaining the good tumor inhibitory properties of cyclo(His-Ala).

4.2. *Cardiac activity of cyclo(His-Ala) and cyclo(His-Gly)*

Both cyclo(His-Gly) and cyclo(His-Ala) resulted in significant changes in heart rate, coronary flow rate and ventricular pressure. A marked decrease in heart rate was noted for cyclo(His-Ala) and cyclo(His-Gly) when compared to control experiments, while significant decreases in coronary flow rate were also noted for both drugs, with cyclo(His-Gly) showing a far greater reduction in coronary flow rate. When analyzing the data, it was noted that the coronary flow rate after 10 min of exposure to 100 μM cyclo(His-Ala) seemed to have recovered markedly from the initial drop. This upward trend continues throughout the experiment. It is interesting to note that an increase in heart rate at 5 min also occurred. Cyclo(His-Gly) caused a similar increase in heart rate and coronary flow rate after 10 min exposure, however, a sharp drop towards the end of the experiment was also noted. Compensatory mechanisms, whereby neurotransmitters, such as catecholamines, are released may result in this observed recovery of heart rate and coronary flow rate, the extent of which is determined by a number of factors, including the pathways that are blocked to result in the initial cardiac depression as well as the nature of the receptor-drug interaction [7]. Also, it is unsure as to whether the initial reduction in heart rate led to the reduction in coronary flow rate or if a reduction in tissue perfusion resulted in a decrease in heart rate. A recommendation would be to investigate the effects of the two agents under constant coronary flow rate conditions to fully understand the mechanisms at hand.

Ventricular pressure is directly related to coronary flow rate when heart rate is kept constant [7]. Thus it is expected, as is the case, that the cyclic dipeptide which most affects ventricular pressure also most affects coronary flow rate in the

experiment. Cyclo(His-Ala) significantly changes ventricular pressure and coronary flow rate when compared with the control, as does cyclo(His-Gly). The decrease in coronary flow rate may be due to lower ventricular pressure resulting in less venous emptying. The converse is also possible, whereby a decrease in coronary flow rate may result in a lower ventricular pressure due to less tissue perfusion and thus compromised functioning.

Further investigations with respect to the effects of cyclo(His-Gly) and cyclo(His-Ala) on ion channel activity would be beneficial in further elucidating the mechanism by which the cardiac depressant effects occur.

4.3. *Antimicrobial activity of cyclo(His-Ala) and cyclo(His-Gly)*

While, statistically, the inhibition of bacteria as described in the results was significant, the clinical relevance thereof is questionable. Concentrations of 0.5 mM resulted in a maximum inhibition of 31.3% for any of the bacteria tested (0.5 mM cyclo(His-Ala) against *Bacillus*). Such high serum concentrations are practically unobtainable clinically and further development of these compounds as antibacterial agents would be unjustified. In some cases, microbial growth was stimulated when compared to the control group, especially against *Pseudomonas* spp. This could be explained by the discovery of a number of endogenous cyclic dipeptide, such as cyclo(Ala-Val) and cyclo(Phe-Pro), in *Pseudomonas aeruginosa*, therefore, exposure to cyclo(His-Ala) and cyclo(His-Gly) may have resulted in nutritional supplementation and improved growth for the bacteria [16].

The antifungal activity of cyclo(His-Ala) was notable, resulting in a 66.3% inhibition at 0.5 mM.

5. Conclusion

Results from this study have shown that cyclo(His-Ala) and cyclo(His-Gly) exert many biological effects that have potential in the pharmaceutical industry. The biological activity shown by the cyclic dipeptides cyclo(His-Ala) and cyclo(His-Gly) are of importance to the understanding of the structure-activity relationship of both the histidine moiety as well as the Alanine methyl group within the cyclic dipeptide structure. The anticancer activity demonstrated by these histidine-containing cyclic dipeptides is of particular interest, and together with the potential antithrombotic activity has potentially opened a new avenue into the development of novel chemotherapeutic agents. Although effects on growth inhibition of cancer cell lines have been published before, the effects of cyclo(His-Ala) and cyclo(His-Gly) appear to be much more significant than that of cyclo(Phe-Pro) and cyclo(Tyr-Pro) at comparable concentrations [11]. Interestingly, in the study by Brauns et al. [11], cyclo(His-Pro) had no significant effect on growth inhibition of HT-29, MCF-7 or HeLa cells despite the presence of the histidine moiety.

The antimicrobial activity of cyclo(His-Ala) and cyclo(His-Gly) was not of therapeutic significance, although the inhibition of *C. albicans* by cyclo(His-Ala) could possibly be exploited further.

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