JPP Journal of Pharmacy and Pharmacology

JPP 2004, 56: 1143–1153

Received March 30, 2004
Accepted June 15, 2004
DOI 10.1211/0022357044139
ISSN 0022-3573

An investigation into the biological activity of the selected histidine-containing diketopiperazines cyclo(His-Phe) and cyclo(His-Tyr)

K. McCleland, P. J. Milne, F. R. Lucieto, C. Frost, S. C. Brauns, M. Van De Venter, J. Du Plessis and K. Dyason

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

K. McCleland, P. J. Milne, F. R. Lucieto

Department of Biochemistry and Microbiology, University of Port Elizabeth, Box 1600, Port Elizabeth 6000, South Africa

C. Frost, S. C. Brauns, M. Van De Venter

School of Physiology, Nutrition and Consumer Sciences, North-West University, Potchefstroom Campus, Private Bag X6001, Potchefstroom, 2520, South Africa

J. Du Plessis, K. Dyason

Correspondence: P. J. Milne, Department of Pharmacy, University of Port Elizabeth, P.O. Box 1600, Port Elizabeth, 6001, South Africa.
E-mail: pmapjm@upe.ac.za

Acknowledgement and funding: Thank you to Prof. P. J. Milne, Dr C. Frost, Mr C. Brauns, Mr F. R. Lucietto, Mr G. Kilian, Mrs A. Erasmus and Dr K. Dyason for all their contributions and assistance. The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at are those of the authors and are not necessarily attributable to the NRF.

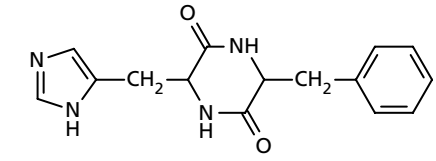
Abstract

Although cyclic diketopiperazines have been known since the beginning of the century, only now have they attracted considerable interest with respect to their biological activity. The aim of this study was to determine if the diketopiperazines cyclo(L-histidyl-L-phenylalanyl) (cyclo(His-Phe)) and cyclo(L-histidyl-L-tyrosyl) (cyclo(His-Tyr)) have significant biological activity relevant to the treatment of cardiovascular-related disease states, cancer and infectious diseases. Haematological studies were performed, including thrombin substrate binding, blood clotting time, platelet adhesion, platelet aggregation and fibrinolysis assays. A cytotoxicity screening utilizing a tetrazolium-based assay on the cell lines HeLa, WHCO3, and MCF-7 was performed. The whole-cell patch-clamp technique was used to investigate ion-channel activity in ventricular myocytes of rats, and isolated rat heart studies were performed to investigate the cardiac effects involving heart rate and coronary flow rate. Cyclo(His-Tyr) produced a significant prolongation of blood clotting time, slowing of clot lysis and inhibition of ADP-induced platelet adhesion and aggregation ($P < 0.05$). Cyclo(His-Phe) showed significant ($P < 0.05$) anti-tumour activity, causing greatest reduction of cell viability in cervical carcinoma cells. Preliminary results from patch-clamp studies indicate that both diketopiperazines caused blocking of sodium and calcium ion channels, but opening of inward rectifying potassium ion channels. In the rat isolated heart studies, cyclo(His-Phe) caused a gradual reduction in heart rate ($P = 0.0027$) and a decrease in coronary flow rate ($P = 0.0017$). Cyclo(His-Tyr) significantly increased the heart rate ($P = 0.0016$) but did not cause any significant change of coronary flow rate ($P > 0.05$). Cyclo(His-Tyr) showed notable ($P < 0.05$) antibacterial activity and both diketopiperazines showed excellent antifungal activity ($P < 0.05$). These observations reveal diketopiperazines to be ideal lead compounds for the rational design of an agent capable of preventing metastasis, inhibiting tumour growth, and as potential chemotherapeutic, antiarrhythmic and antihypertensive agents, as well as potential antibacterial and antifungal agents.

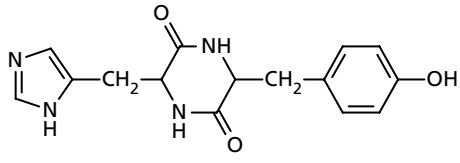
Introduction

In recent years there has been a growing awareness of the diversity and biological roles played by many of over one-hundred diketopiperazines found in nature. Many derivatives have antiviral (e.g., the gliotoxins and sporidesmins), phytotoxic (e.g., cyclo(Pro-Tyr)) and antibiotic (e.g., bicyclomycin) properties, whereas simple members like cyclo(His-Pro), cyclo(Pro-Leu), cyclo(Asp-Pro), cyclo(Pro-Val) and cyclo(Pro-Phe) show various biological activities (Prasad 1995). For this reason, many of these diketopiperazines are now regarded as important metabolic intermediates rather than as protein artifacts. A host of other diketopiperazines, or cyclic dipeptides, are reportedly endogenous in different living organisms or exist as either by-products of manufacturing processes or simply degradation products of larger proteins and peptides. Although there is no doubt that many of these cyclic dipeptides may possess interesting and possibly economically beneficial biological activity, evaluations on a small scale have only begun in 1995 (Prasad 1995).

It has been shown that these agents can be used as templates from which peptidomimetic analogues with enhanced activity can be designed (Henczi & Weaver 1995). The



Cyclo(His-Phe)
284.1270 g mol^{-1}



Cyclo(His-Tyr)
300.1220 g mol^{-1}

Figure 1 Structures of the histidine-containing diketopiperazines cyclo(His-Phe) and cyclo(His-Tyr).

general structure of homodetic diketopiperazines bears a diketopiperazine nucleus with two side chains of amino acid residues. Modification of the primary structure of the diketopiperazine and the resultant alteration in the R substituents attached to the diketopiperazine ring (Figure 1) may be utilized to manipulate the hydrophilicity and other physicochemical properties, thereby affecting the biological activity (Crescenzi et al 1973; Sijpkes et al 1994).

The search for new anti-cancer drugs over the past 20 years has proved to be difficult, with few cytotoxic drugs having been specifically designed to attack particular cellular targets. Fascinating alternative sources of new cytotoxic compounds are continually under assessment (Carmichael 1994). As solid tumours rely upon the bloodstream to deliver nutrients necessary for growth, it follows that interference with that supply may compromise tumour growth (Murray 1991). The procoagulant activity of tumour cells has been thought to enhance metastasis by encasing the cells in a fibrin clot, thereby allowing them to escape detection and clearance by the host immune system. Some of the molecules involved in blood coagulation might therefore represent novel targets for a new generation of therapeutic agents designed to inhibit tumour growth and prevent metastasis (Bromberg & Cappello 1999). Drugs directed against new targets such as angiogenesis, tumour invasion and metastasis, as well as signal transduction, have great potential as comparatively selective anti-cancer agents (Carmichael 1994). Various diketopiperazines have been demonstrated to possess anti-cancer properties with studies by Graz et al (2000) suggesting selective inhibition of carcinoma cells (Milne et al 1998; Graz et al 2000).

Cardiac cellular injury leads first to reversible alterations in electrical and contractile properties followed by irreversible alterations and cell death (cardionecrosis). The three main causes of cardionecrosis are ischaemia (reduction of blood supply), anoxia (reduction of oxygen supply) and direct toxic action on the heart (Godfraind et al 1986). The action potential and electrophysiology of the heart depends greatly on the movement of ions, namely Na^+, Ca^{2+} and K^+, across cell membranes facilitated by ion channels, passive exchangers and active transport pumps (Marban et al 1998). Calcium-channel-blocking agents, such as verapamil, nifedipine and diltiazem, have long been studied for potential application in cardiovascular disorders, including angina, paroxysmal supraventricular tachyarrhythmias, atrial fibrillation, hypertension and cardioplegia (Cook 1988; Narahashi & Herman 1992). The potential calcium-channel-blocking effect of diketopiperazines was demonstrated in studies by Milne et al (1998), where cyclo(Trp-Trp) and cyclo(Trp-Pro) were shown to block calcium channels in rat ventricular myocytes. The cellular site of action of a number of drugs used therapeutically seems to involve the modulation of membrane potassium ion channels (e.g., blockers of K^+ channels are able to promote the secretion of insulin from the pancreas and to suppress dysrrhythmias of the heart, whereas opening of K^+ channels can play a role in the relaxation of a variety of smooth muscles, thereby having potential antihypertensive effects (Cook 1988)). Developing selective K^+-channel modulators should indeed be plausible since these channels represent novel sites for therapeutic drug action in the near future (Cook 1988). The diketopiperazines have recently shown potential for use in the treatment of cardiovascular dysfunction as demonstrated by isolated perfused rat heart experiments where isomers of cyclo(Trp-Pro) showed antiarrhythmic potential (Jamie et al 2002). A decrease in ventricular fibrillation may reduce the mortality rates in acute myocardial infarction (Jamie et al 2002).

The increased resistance of various bacteria toward available antibiotic drugs has initiated intensive research efforts into identifying new sources of antimicrobial substances (Kolusheva et al 2000). Considering this rapid escalation of bacterial resistance to antibiotics, there is a profound need for novel antimicrobial agents but few new drugs are appearing on the horizon. The pharmaceutical industry is moving away from drug discovery by chemical alteration of existing compounds and the screening of new compounds, but is looking towards rational drug design to provide new candidates for the antimicrobial drug market (Graz et al 1999). Short antimicrobial peptides (10–30 residues) are prevalent in nature as part of the intrinsic defence mechanisms of most organisms and have been proposed as a blueprint for the design of novel antimicrobial agents. Antimicrobial peptides are generally believed to kill bacteria through membrane permeabilization and extensive pore formation (Hancock 1999). Antimicrobial peptides have a great potential to be the next breakthrough class of antimicrobials and the first truly novel class of antibiotics in 30 years (Hancock & Lehrer 1998). Thus, research into the antimicrobial activity of cyclic dipeptides is not only justified but also essential for the discovery of new antimicrobial agents (Graz et al 1999). Six- and eight-residue cyclic D,L-α-peptides possess unique structural features that are not found in the natural class of peptide antibiotics or their derivatives. They act preferentially on Gram-positive or Gram-negative bacterial membranes compared with mammalian cells, increase membrane permeability,

collapse transmembrane ion potentials and cause rapid cell death. The effectiveness of this class of materials as selective antibacterial agents is highlighted by the high efficacy observed against lethal methicillin-resistant *Staphylococcus aureus* infections in mice. The unique abiotic structure of the cyclic peptides and their quick bactericidal action may also contribute to limit temporal acquirement of drug resistant bacteria. The low-molecular-weight D,L-α-peptides hold considerable potential in combating a variety of existing and emerging infectious diseases (Fernandez-Lopez et al 2001).

This study aimed to investigate the biological activity of the selected histidine-containing cyclic diketopiperazines, cyclo(L-histidyl-L-phenylalanyl) (cyclo(His-Phe)) and cyclo(L-histidyl-L-tyrosyl) (cyclo(His-Tyr)). In continuation of our research to identify diketopiperazines with biological activity, a primary screen was performed on the above mentioned diketopiperazines. The next step, once the most promising diketopiperazines are identified, is structural modification to increase the biological activity.

Materials and Methods

Chemicals and solutions

The diketopiperazines were synthesized from their corresponding sterically pure linear precursors (Bachem), using a modified phenol-induced cyclization procedure, adapted from Kopple & Ghazarian (1968). All other chemicals and solutions used were of analytical grade and purchased from Sigma (St Louis, MO). Structural elucidation was accomplished by infrared spectroscopy, mass spectrometry, nuclear magnetic resonance spectroscopy and melting-point determination. The structures of the diketopiperazines are shown in Figure 1. The diketopiperazines and solutions were stored at 4°C.

Ethical approval

The use of human blood samples in the haematological studies was approved by the Human Ethics Committee of the University of Port Elizabeth. The use of experimental animals was approved by the Animal Ethics Committee of the University of Port Elizabeth. Ethical approval was obtained from the ethical committee of North West University, Potchefstroom Campus, for the ion channel studies (ethics number: FLG-02D01).

Cell cultures and microorganisms

All manipulations were performed using sterile culture techniques. HeLa, WHCO3 and MCF-7 carcinoma cell lines were routinely maintained as monolayers in multiple, 25 cm^2 culture flasks (Corningware, Cambridge, MA) in RPMI 1640 growth medium (pH 7.4) (Highveld Biological, Johannesburg, South Africa), supplemented with 10% heat-inactivated fetal calf serum (FCS) (Highveld Biological) at 37°C (5% CO_2) and 2 mM glutamine (Highveld Biological) in a humidified atmosphere. This antibiotic-free medium allowed for early detection of microbial contamination and was replaced every 48 h. To standardize the culture conditions with respect to the cytotoxicity assay, cell lines were sub-cultured 24 h before the day of the experiment. The bacterial cultures used were obtained from the culture collection in the Department of Biochemistry and Microbiology at the University of Port Elizabeth.

Effect of cyclo(His-Phe) and cyclo(His-Tyr) on thrombin substrate, thrombin time, platelet adhesion, platelet aggregation and fibrinolysis

A modified method adapted from Rob et al (1997) was utilized for the thrombin substrate assay. The method involved the reaction of the enzyme thrombin with a chromogenic thrombin specific substrate known as S2238 (α-Phe-L-pipecolyl-L-Arg-*p*-nitroanilide) (Chromogenix). The progression of this colour reaction was measured in terms of the change in absorbance in a 96-well flat-bottom microplate reader (Costar, Corning).

The thrombin time assay involved the measurement of the time taken for normal human plasma to clot, detected by the first sight of a white fibrin thread, induced by thrombin. Heparin was used as a positive control to establish a standard curve. This was followed by measurement of the prolongation of clotting time by the two diketopiperazines. The method utilized for this assay was modified and adapted from Tanaka et al (1999).

Methods utilized for the platelet adhesion assay were adapted from Bellavite et al (1994). A photometric method for platelet count in plasma or in suspension by means of a spectrophotometer and a microplate reader was utilized, as developed by Walkowiak et al (1997). Blood was drawn by venipuncture from a healthy subject who had abstained from any medication, alcohol and nicotine for two weeks and had fasted for at least 8 h. The blood was collected in 4.5-mL tubes containing 0.5 mL of 0.105 mM trisodium citrate. The blood was then centrifuged at 300 *g* for 10 min to obtain platelet-rich plasma (PRP), which was then re-centrifuged at 900 *g* for 20 min to obtain platelet-poor plasma (PPP). Linearity of acid phosphatase versus platelet number was determined before the assays. Concentration ranges of 0.0125–0.1250 U mL^{-1} (final concentration) for thrombin and 3.125–25 μM for adenosine diphosphate (ADP) (final concentration) were utilized to obtain standard curves. Thrombin at a final concentration of 0.0625 U mL^{-1} and ADP at 25 μM were then utilized in the assays. Both agonists were made up in a buffer supplemented with 4 mM $CaCl_2$ and 4 mM $MgSO_4$. Platelets were at a concentration of 4.5×10^6/well for the thrombin-induced assay and 2.25×10^6/well for the ADP-induced assay. The diketopiperazines were dissolved in the buffer to give a final concentration of 0.125–1 mM. The absorbance was read at 412 nm in the microplate reader and the number of cells adhering was calculated on the basis of a standard curve obtained from the known number of platelets (linearity of acid phosphatase). The percentage inhibition of agonist-induced adhesion was then calculated.

The platelet aggregation assay was performed using the agonists thrombin and ADP, to determine platelet aggregation responses in the presence of the two diketopiperazines, by means of a method adapted from Walkowiak et al (1997) and Bednar et al (1995). A platelet-free control for 100% transmission, platelet suspension control for 0% transmission, a thrombin/ADP control and a positive control (acetylsalicylic acid) were used.

The method utilized for the fibrinolysis assay was modified and adapted from Schatteman et al (1999). A 160 mM $CaCl_2$ solution was utilized to initiate clotting of the plasma, and two controls, tissue-type plasminogen activator (t-PA) and streptokinase, were used. A diketopiperazine-free control (containing only t-PA/streptokinase), t-PA-/streptokinase-free and diketopiperazine-free positive control and blank were used. The effect of the diketopiperazines on t-PA- and streptokinase-induced clot lysis could therefore be illustrated graphically.

Anti-cancer activity of cyclo(His-Phe) and cyclo(His-Tyr)

HeLa (cervical), WHCO3 (oesophageal) and MCF-7 (breast) carcinoma cell lines were routinely maintained in RPMI 1640 growth medium (pH 7.4) (Highveld Biological, Johannesburg, South Africa) supplemented with 10% heat-inactivated FCS at 37°C (5% CO_2) and 2 mM glutamine (Highveld Biological) in a humidified atmosphere. Each cell line was seeded at a density of 25 000 cells/mL. The cells were allowed 24 h for attachment and on the day of the assay, cells were lifted by trypsinization and diluted to 1×10^5 cells/mL with RPMI 1640 growth medium. One-hundred microlitres of each respective cell suspension was then seeded into the appropriate wells of 96-well microtitre plates. Diketopiperazines and melphalan (positive control) were initially solubilized in 0.5% dimethyl sulfoxide (DMSO) and stock concentrations of 0.04–1 mM were prepared. One-hundred microlitres of each were added to the appropriate wells. The samples were incubated at 37°C (5% CO_2, humidified atmosphere) for 72 h and cells were subsequently processed for 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) reductivity. The number of viable cells was assessed using the modified MTT assay as described previously (Mosmann 1983; Carmichael et al 1987).

Effect of cyclo(His-Phe) and cyclo(His-Tyr) on sodium, calcium and delayed-rectifier potassium channels in ventricular myocytes of rats

Ventricular myocytes from the hearts of Sprague Dawley rats, 200–300 g, were isolated by enzymatic dispersion of the cells with 9 mg collagenase type II (Sigma, St Louis, MO) and 3 mg protease type XIV (Sigma, St Louis, MO) on a Langendorff apparatus as previously described by Mitra & Morad (1985) and adjusted by Tytgat (1994). The rats were anaesthetized with pentobarbitone before removing the heart.

A Dagan 8800 total clamp amplifier (DAGAN Corporation, Minneapolis, MN) was used for patch clamping and electrodes were made from borosilicate glass using a Flaming/Brown model P-27 micropipette puller (Sutter Instrument Company, Novato, USA). Electrodes (2–4 mΩ) were heat polished before seal formation. A GΩ seal was formed by light suction. The patch was ruptured and internal dialysis was allowed to reach equilibrium (10 min). A multibarrel superfusion system with micro valves, which allowed changes in the bath solutions in less than 1 s, was used. The cells were exposed to each diketopiperazine individually at a concentration of 100 μM. The pclamp 5.5 program (Axon Instruments Inc., 1989) was used to control the experiments and to store the acquired data.

Extracellular and intracellular solutions were made up and used for the ion channel experiments. Sodium ion channel experiments: extracellular solution (composition in mM) (pH 7.4, made up in milli-Q water, NaCl 137, KCl 5.4, $MgCl_2$ 0.5, HEPES-NaOH 11.6, $CaCl_2$ 1.8, glucose 10); intracellular solution (composition in mM) (pH adjusted to 7.2 with NaOH, made up in milli-Q water, CsCl 125, $MgCl_2$ 5, EGTA 15, HEPES 10, TEA-Cl 20, Na_2-ATP 5). Calcium ion channel experiments: extracellular solution (composition in mM) (pH adjusted to 7.4 with HCl, made up in milli-Q water, $MgCl_2$ 0.5, HEPES 10, Tris 138, $CaCl_2$ 5.4, CsCl 20, glucose 5); intracellular solution (composition in mM) (pH adjusted to 7.2 with CsOH, made up in milli-Q water, CsCl 125, $MgCl_2$ 5, EGTA 15, HEPES 10, TEA-Cl 20, Mg-ATP 5). Potassium ion channel experiments: extracellular solution (composition in mM) (pH adjusted to 7.4 with HCl, made up in milli-Q water, NaCl 130, KCl 4, $MgCl_2$ 1, HEPES-NaOH 10, $CaCl_2$ 1.8, glucose 10, 0.2×10^{-6} M nisoldipine); intracellular solution (composition in mM) (pH adjusted to 7.2 with NaOH, made up in milli-Q water, $MgCl_2$ 2, EGTA 11, HEPES 10, $CaCl_2$ 1, Na_2-ATP 5).

Effect of cyclo(His-Phe) and cyclo(His-Tyr) on heart rate and coronary flow rate of rat hearts

Male Long Evans rats, 250–400 g, were anaesthetized and maintained under ether in a dessicator until they lost pain and blink reflexes. Heparin sodium (Fresenius-Kabi, SA) was injected intravenously into the femoral vein. The heart was removed and arrested in ice-cold oxygenated Krebs-Henseleit buffer solution (pH 7.4, 4°C) containing (in mM): Na^+ 118, $H_2PO_4^-$ 1.2, Mg^{2+} 1.2, Ca^{2+} 2.5, HCO_3^- 25, Cl^- 123, SO_4^{2-} 1.2, K^+ 5.9 and glucose 11.1 (Bova et al 1997; Wu et al 1997). It was then mounted as described previously (Lubbe et al 1978). A dual-perfusion Langendorff system was used (Langendorff 1895) and hearts were perfused with Krebs-Henseleit buffer aerated with carbogen (5% CO_2, 95% O_2) (Afrox Pty, Ltd). The hearts were allowed to stabilize for 15 min before exposure to 100 μM diketopiperazine dissolved in 2% DMSO in Krebs-Henseleit buffer. Diketopiperazine-free controls were used containing Krebs-Henseleit buffer and 2% DMSO. Heart rate was recorded and coronary flow was measured at 5-min intervals for 30 min.

Antibacterial and antifungal activity of cyclo(His-Phe) and cyclo(His-Tyr)

The test organisms included Gram-positive bacteria (*Staphylococcus aureus*, *Streptococcus pyogenes, Bacillus subtilis*), Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae, Salmonella typhimurium*) and yeast fungus (*Candida albicans*).

The antimicrobial activity of the diketopiperazines was firstly assayed according to the Kirby-Bauer disc-diffusion method. Cyclo(His-Phe) and cyclo(His-Tyr) were dissolved in DMSO and diluted in milli-Q water to give test solutions of $10\,mg\,mL^{-1}$ and $1\,mg\,mL^{-1}$. Chloramphenicol was used as the control at a concentration of $30\,\mu g\,mL^{-1}$. Pour-plate overlays from overnight cultures of the test organisms in nutrient broth (Biolab) were prepared on standard nutrient agar. Agar plates were prepared using Antibiotic Agar No 1 (Merck, Darmstadt, Germany) for the bacterial specimens and Sabourauds 2%-Dextrose Agar (Merck) for the fungal specimens. The nutrient agar plates were inoculated uniformly with $100\,\mu L$ of the overnight broth culture of test organism (1×10^9 colonies/mL). Holes (6 mm) were then sucked out of the media with a sterile disposable glass pipette and filled with $25\,\mu L$ of the test sample. At a concentration of $10\,mg\,mL^{-1}$ of cyclic dipeptide, the quantity per hole was $250\,\mu g$, and at $1\,mg\,mL^{-1}$, the amount of cyclic dipeptide was $25\,\mu g$ per hole. The final concentration of chloramphenicol used was $750\,\mu g$ per hole. The plates were incubated at 37°C for 24 h. Antibacterial and antifungal activity was determined by measuring the zone of inhibition (mm) around the holes.

Secondly, a method adapted from Eloff (1998) and Thom et al (1993), using the tetrazolium salt *p*-iodonitrotetrazolium (INT) violet, was used to test the antimicrobial activity of cyclo(His-Phe) and cyclo(His-Tyr). Final concentrations utilized in the assay were as follows: test organisms 2×10^8 colonies/well, diketopiperazines $0.50\,mg\,mL^{-1}$ and $0.25\,mg\,mL^{-1}$, chloramphenicol $0.005\,mg\,mL^{-1}$ as a positive control, and INT $0.057\,mg\,mL^{-1}$. After addition of INT, the microtitre plates were incubated at 37°C for 1 h and the absorbance was measured at 540 nm (for the bacteria) or 600 nm (for *C. albicans*). The percentage of the inhibition of each sample was then calculated, based on the positive bacterial growth control.

Statistical analysis

Results were calculated as means ± standard deviation (s.d.) and plotted graphically. For haematological studies, significance was examined using analysis of variance and significant differences in means were tested using the paired *t*-test and defined as $P < 0.05$. For anti-cancer studies, significance in activity was determined using a one-way analysis of variance. In calcium ion channel studies, deviation in $V_{\frac{1}{2}}$ was determined graphically using an activation curve obtained from the Boltzman equation. For cardiac studies, significant differences in means were also examined using the paired *t*-test and defined as $P < 0.05$. For antimicrobial studies, analysis of variance was carried out to determine whether the reduction in cell viability by the compounds being tested was statistically significant from each other (i.e., if the analysis of variance test showed $P < 0.05$, separate *t*-tests were performed on each concentration of cyclic dipeptide). Values of $P < 0.05$ were considered statistically significant.

Results

Effect of cyclo(His-Phe) and cyclo(His-Tyr) on thrombin substrate, thrombin time, platelet adhesion, platelet aggregation and fibrinolysis

No statistically significant inhibition of thrombin substrate binding was observed for either diketopiperazine. However, in the thrombin time assay, a concentration-dependent prolongation of blood clotting time of normal human plasma, induced by thrombin, was observed for both diketopiperazines, suggesting an inhibitory effect of thrombin. Cyclo(His-Tyr) produced more pronounced effects causing doubling of clotting time at a concentration of 0.0929 mM. However, compared with heparin, cyclo(His-Tyr) prolonged clotting time to a much lesser extent, since heparin doubled clotting time at a considerably lower concentration of $0.1029\,\mu M$. Both diketopiperazines concentration-dependently inhibited the adhesiveness of platelets stimulated by ADP and thrombin, with cyclo(His-Tyr) at 1 mM showing the greatest inhibition of ADP-induced platelet adhesion (60.2 ± 2.9%). The diketopiperazines caused significant inhibition of thrombin- and ADP-induced platelet aggregation, with maximum inhibition being observed for 0.25 mM cyclo(His-Tyr) ($P = 2.0600 \times 10^{-9}$) against ADP. Cyclo(His-Tyr) (1 mM) caused significant slowing of clot lysis stimulated by t-PA and streptokinase ($P < 0.05$).

Anti-cancer activity

At 1 mM, cyclo(His-Phe) significantly reduced the growth of all three cell lines ($P < 0.05$) after a 72-h exposure period. Cyclo(His-Phe) reduced the growth of HeLa cells the most, to 43.2 ± 6.2% ($P < 0.05$) cell viability, suggesting cyclo(His-Phe) to be most active in terms of ability to induce cell death in HeLa (cervical carcinoma) cells. This anti-tumour activity in comparison with melphalan, the positive control used, is illustrated graphically in Figure 2. Cyclo(His-Tyr) showed less anti-cancer activity than cyclo(His-Phe). Cyclo(His-Tyr), however, did cause significant concentration-dependent reduction of WHCO3 cell growth, with the most significant effect at 1 mM (cell viability 76.1 ± 4.1%).

Effect of cyclo(His-Phe) and cyclo(His-Tyr) on sodium, calcium and delayed-rectifier potassium channels in rat ventricular myocytes

Both diketopiperazines had an effect on sodium, calcium and delayed-rectifier potassium channels. For each channel type, the diketopiperazines were tested on two different cells. Due to the low number of replicates the results are interpreted as a preliminary indication of the ion channel activity

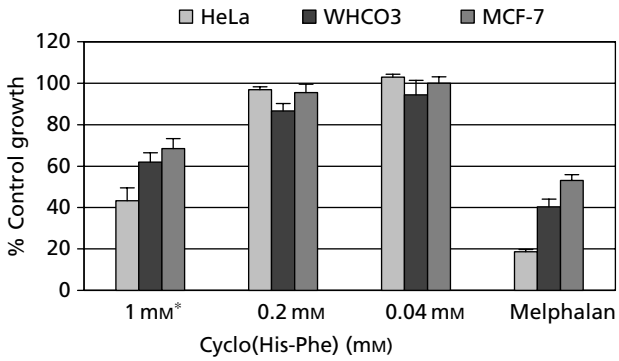


Figure 2 Effect of cyclo(His-Phe) on HeLa, WHCO3 and MCF-7 growth after a 72-h exposure period (error bars represent the s.d. of quadruplicate measurements). *$P < 0.05$ vs drug-free control for all 3 cell lines. Melphalan 0.1 mM was included as positive control.

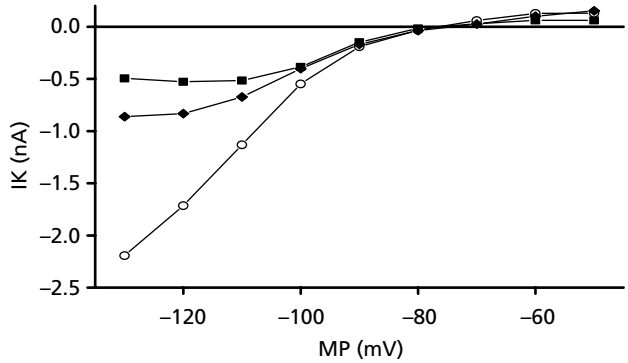


Figure 4 Effect of 100 μM cyclo(His-Tyr) (circles) on the current–voltage relationship of inward rectifying potassium currents in rat ventricular myocytes. Squares and diamonds represent the current–voltage relationship in the absence of cyclo(His-Tyr). IK, potassium ion current; MP, applied potential to myocytes.

of the diketopiperazines tested in this study and justification for the use of experimental animals in isolated heart studies. Both diketopiperazines blocked sodium ion channels, with cyclo(His-Phe) reducing the control current at −40 mV by 61% and cyclo(His-Tyr) by 63% after a 5-min exposure. The maximum inward sodium current measured at −40 mV in the control was decreased by 57% in the presence of cyclo(His-Phe) and by 63% in the presence of cyclo(His-Tyr). This sodium channel antagonistic activity (results not shown) was almost completely reversible for cyclo(His-Tyr) but only weakly reversible for cyclo(His-Phe).

In the calcium channel studies, the maximum inward current was decreased by 58% by cyclo(His-Phe) (Figure 3) and 52% by cyclo(His-Tyr), suggesting that cyclo(His-Phe) could have a greater blocking effect on calcium ion channels. Both diketopiperazines influenced the voltage dependence of activation by shifting it to a more negative potential. This indicates that their effect on calcium channels is voltage dependent. Due to the similarities in effects, only the effect of cyclo(His-Phe) on calcium ion channels is shown in Figure 3.

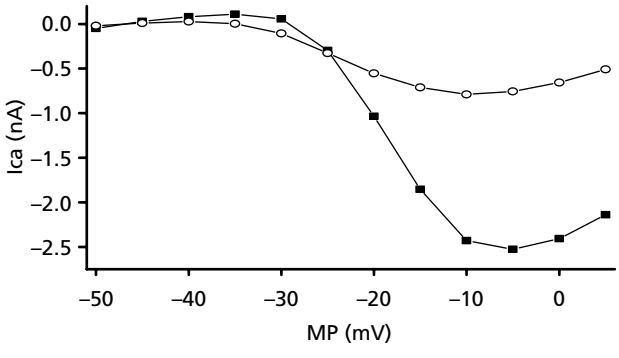


Figure 3 Influence of 100 μM cyclo(His-Phe) (circles) on calcium channels in rat ventricular myocytes. Squares represent the current–voltage relationship in the absence of cyclo(His-Phe). ICa, calcium ion current; MP, applied potential to myocytes.

The inward potassium current was increased by 50% at −100 mV and by 185% at −130 mV for cyclo(His-Phe) and by 40% at −100 mV and by 350% at −130 mV for cyclo (His-Tyr) (Figure 4). The outward potassium currents were not significantly influenced by either diketopiperazine. Both diketopiperazines, particularly cyclo(His-Tyr), enhanced the inward rectifying potassium currents by keeping these channels open for longer, with effects being only partially reversible.

Effect of cyclo(His-Phe) and cyclo(His-Tyr) on heart rate and coronary flow rate

When compared with the control, cyclo(His-Phe) significantly slowed heart rate over the experimental time period ($P = 0.0027$) (Figure 5). Cyclo(His-Phe) gradually reduced

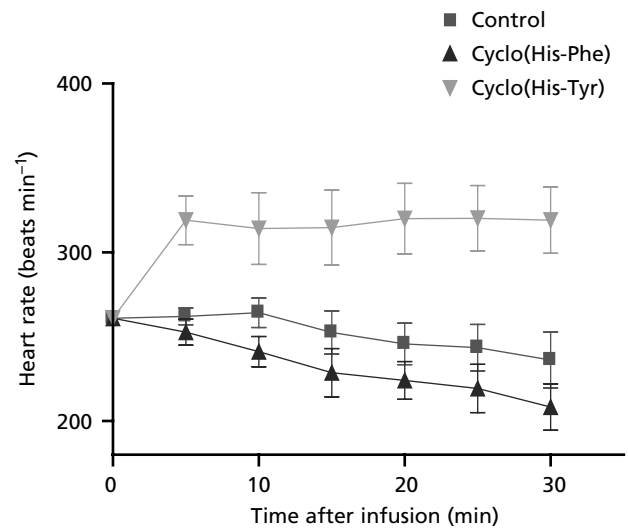


Figure 5 Effect of 100 μM cyclo(His-Phe) ($P < 0.05$) and cyclo(His-Tyr) ($P < 0.05$) on heart rate in the rat isolated heart. Values are means ± s.d., n = 6 experiments.

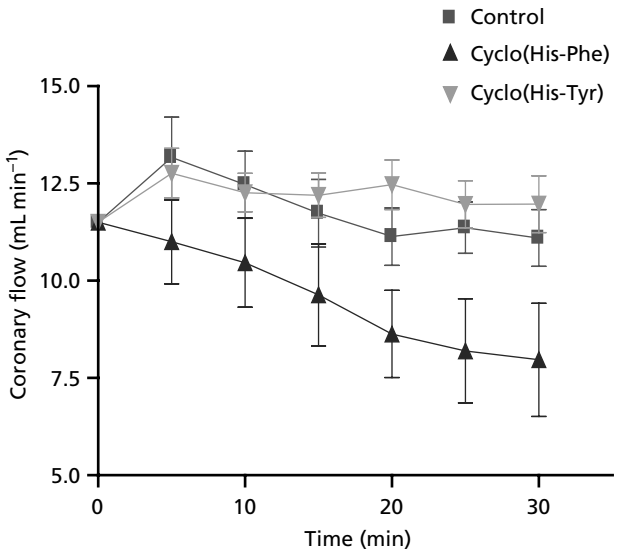


Figure 6 Effect of 100 μM cyclo(His-Phe) ($P < 0.05$) and cyclo(His-Tyr) on coronary flow rate in rat isolated heart. Values are means ± s.d., n = 6 experiments.

the heart rate over time. Cyclo(His-Phe) caused an initial 3.2% reduction of heart rate after 5 min of infusion and thereafter caused a gradual reduction of heart rate of around 8% when compared with the control. On the other hand, cyclo(His-Tyr) significantly increased the heart rate in relation to the control ($P = 0.0016$). The effect of cyclo(His-Tyr) on heart rate was significantly greater than that caused by cyclo(His-Phe) since the paired *t*-test resulted in a smaller *P*-value. Figure 5 illustrates how cyclo(His-Tyr) caused a sudden initial 18.2% increase in heart rate after 5 min of infusion, thereby showing cyclo(His-Tyr) to be faster acting than cyclo(His-Phe). The increased heart rate thereafter remained elevated compared with the control at a fairly constant rate of approximately 320 beats min^{-1}.

Cyclo(His-Phe) caused a significant decrease ($P = 0.0017$) in coronary flow rate when compared with the control (Figure 6), resulting in a 16.5% reduction after 5 min of perfusion and a 28.2% reduction after 30 min exposure. The reduction in coronary flow rate may be as a result of the reduced heart rate observed. On the other hand, cyclo(His-Tyr) failed to cause any significant change in coronary flow rate when compared with the control ($P = 0.1540$).

Antibacterial and antifungal activity

In the Kirby-Bauer disc-diffusion assay, the zones of inhibition (not shown) showed cyclo(His-Phe) to be effective against Gram-positive bacteria but less effective against Gram-negative bacteria. It also showed antifungal activity. Cyclo(His-Tyr) exhibited greater activity against the Gram-positive and Gram-negative bacteria than cyclo(His-Phe) (Table 1), and also showed higher activity against Gram-positive bacteria than against Gram-negative bacteria when considering the zones of inhibition. Cyclo(His-Tyr) showed greater antifungal activity since it was more effective than cyclo(His-Phe) against *Candida albicans*.

Table 1 Antibacterial and antifungal activity of cyclo(His-Phe) and cyclo(His-Tyr) as determined by the Kirby-Bauer disc-diffusion method

Diketopiperazine	Minimum inhibitory concentration (μg/hole)		
	Gram-positive bacteria	Gram-negative bacteria	Fungi
Cyclo(His-Phe)	250	250	250
Cyclo(His-Tyr)	25	25	25

In the INT assay, the diketopiperazines failed to display very broad antibacterial activity, having activity against only specific organisms. The inhibitory effects are illustrated in Table 2. Cyclo(His-Tyr), in particular, proved to be a potential antibacterial agent against the Gram-positive bacteria *S. pyogenes* and the Gram-negative bacteria *E. coli* and *P. aeruginosa*. Both cyclo(His-Phe) and cyclo(His-Tyr) displayed antifungal activity against *C. albicans*, with cyclo(His-Phe) in particular displaying great potential as an antifungal agent.

Discussion

The microtitre-plate-based assay for thrombin substrate binding, platelet adhesion, platelet aggregation and fibrinolysis has been shown to be sensitive enough for determining the effects of the diketopiperazines on ADP and thrombin activity. Both the diketopiperazines under study were inherently more active in inhibiting the effects of ADP than those of thrombin. ADP is therefore the molecule involved in blood coagulation under focus, and represents a novel target for a new generation of therapeutic agents designed to inhibit tumour growth and prevent metastasis. Cyclo(His-Tyr) had a greater activity than cyclo(His-Phe) in terms of effects on thrombin time, platelet adhesion and aggregation and fibrinolysis. This activity, as well as that of greater activity against ADP rather than against thrombin, can possibly be attributed to the presence of the hydroxyl group on the tyrosine moiety, contributing to enhanced hydrophilicity and electronegativity. Another proposed explanation is the enhanced ability for hydrogen bonding to ADP molecules. Cyclo(His-Tyr) produced a greater prolongation of clotting time than cyclo(His-Phe). This can be seen as beneficial in terms of preventing metastasis, since it demonstrates potential to inhibit the fibrin clot formation in metastasis, thus allowing detection and clearance of the cancer cells by the host immune system.

From the results of the MTT assay, it is clear that cyclo(His-Phe) can be considered a potential anti-tumour

Table 2 Inhibition of microorganisms by cyclo(His-Phe) and cyclo(His-Tyr) expressed as a percentage of the control using the INT method

Microorganism	Agent concn (mg mL^{-1})	Cyclo(His-Phe)	Cyclo(His-Tyr)	Chloramphenicol
Gram-positive bacteria				
S. aureus	0.5	16.74 ± 2.48*	9.63 ± 9.52	—
	0.25	NI	1.13 ± 4.01	—
	0.005	—	—	77.62 ± 1.04*
S. pyogenes	0.5	66.67 ± 26.94	82.35 ± 11.35*	—
	0.25	33.73 ± 4.75*	63.92 ± 11.43*	—
	0.005	—	—	66.67 ± 6.48*
B. subtilis	0.5	1.89 ± 3.33	11.95 ± 6.92	—
	0.25	NI	NI	—
	0.005	—	—	NI
Gram-negative bacteria				
E.coli	0.5	57.28 ± 6.60*	85.99 ± 0.60*	—
	0.25	36.17 ± 12.38*	45.00 ± 10.01*	—
	0.005	—	—	31.54 ± 9.73*
P. aeruginosa	0.5	32.30 ± 9.36*	46.57 ± 13.31*	—
	0.25	16.91 ± 9.43	41.68 ± 3.03*	—
	0.005	—	—	38.38 ± 15.45*
K. pneumoniae	0.5	NI	NI	—
	0.25	NI	0.51 ± 2.16	—
	0.005	—	—	76.43 ± 3.21*
S. typhimurium	0.5	16.76 ± 4.45*	10.47 ± 9.13	—
	0.25	NI	1.91 ± 7.36	—
	0.005	—	—	NI
Fungi				
C. albicans	0.5	71.83 ± 4.99*	50.35 ± 17.50*	—
	0.25	72.46 ± 8.24*	38.38 ± 3.70*	—
	0.005	—	—	50.49 ± 8.94*

NI = no inhibition; *$P < 0.05$.

agent against HeLa, WHCO3 and MCF-7 cells, but in particular against HeLa, where more than 50% reduction in viability occurred (43.2 ± 6.2%). Cyclo(His-Phe), however, caused less than 50% reduction in cell viability in WHCO3 and MCF-7 cells and would therefore have to be used in conjuction with other therapeutic agents in a combination chemotherapy strategy. In comparison with cyclo(His-Phe), cyclo(His-Tyr) demonstrated far less potential as an anti-tumour agent, but did show some significant reduction in cell viability in WHCO3 cells. This greater anti-tumour activity demonstrated by cyclo(His-Phe) could be attributed to its greater lipophilicity and greater ease of tumour cell penetration. Cyclo(His-Phe) may potentially play a vital role in chemotherapy in the future.

The growth-inhibitory action of the diketopiperazines was shown to be concentration dependent and is proposed to be due to effects such as interaction with nucleotides, effects on energy-linked reactions, ion fluxes such as calcium or potassium, or protein phosphorylation (Milne et al 1998). The histidine-containing diketopiperazines have the potential to become lead compounds for the development of highly potent agents, capable of inhibiting the growth and proliferation of cervical, oesophageal and breast carcinomas, since they have been shown in this study to have both tumour inhibitory effects as well as beneficial effects on tumour-induced haemostatic responses.

Cyclo(Trp-Trp) has been shown to affect selected haemostatic responses that have been directly linked to the metastatic process and this diketopiperazine was proposed to be a suitable agent for further development. Cyclo(Trp-Trp) is structurally similar to the anti-tumour agent compound 593 A (Sammes 1975), and it can be proposed that similar substitution on the aromatic rings by means of halogenation of cyclo(His-Phe) and cyclo(His-Tyr) would enhance the inherent chemotherapeutic effect and possibly produce the desired activity of concomitantly inhibiting tumour growth and metastasis. Cyclo(His-Phe) and cyclo(His-Tyr) therefore present possibilities to become suitable agents for further investigation and development in terms of their ability to inhibit both the proliferation and metastasis of tumours.

Preliminary results demonstrate promising antagonistic activity towards sodium and calcium ion channels for both cyclo(His-Phe) and cyclo(His-Tyr). The diketopiperazines

show potential as sodium- and calcium-channel blockers, which possibly indicates therapeutic usefulness in various clinical disease states. Cyclo(His-Tyr) displayed more potent blocking activity of sodium ion channels in particular. This can probably be attributed to the presence of the hydroxyl group on the tyrosine moiety, allowing longer attachment and blocking effect of the ion channels. Previous studies by Milne et al (1998) have shown that other diketopiperazines block cation channels in ventricular myocytes, namely cyclo(Trp-Pro), cyclo(Tyr-Pro), cyclo(Trp-Trp) and cyclo(Phe-Pro). Cyclo(Trp-Trp) and cyclo(Trp-Pro) were shown to block calcium channels in the ventricular myocytes of rats, whereas cyclo(Trp-Pro) and cyclo(Tyr-Pro) blocked delayed-rectifier potassium channels in the ventricular myocytes from guinea-pig (Milne et al 1998).

Drugs that block sodium channels have been used for many years as local anaesthetics and antiarrhythmics (Cook 1988). The class I antiarrhythmic agents used therapeutically in the treatment of cardiac arrhythmias are sodium-channel-blocking drugs. Since cyclo(His-Phe) and cyclo(His-Tyr) showed potential sodium-channel-blocking activity, they therefore could potentially have local anaesthetic or antiarrhythmic activity. However, more replicates need to be done and numerous other factors need to be investigated (e.g., the frequency- and use-dependence of channel state block) before coming to this conclusion. Calcium-channel blockers have shown promise in the treatment of cardiovascular disorders (Cook 1988). Many are being used in the treatment of angina, paroxysmal supraventricular tachyarrhythmias, atrial fibrillation, hypertension and cardioplegia (Narahashi & Herman 1992). The potential L-type calcium-channel-blocking activity of cyclo(His-Phe) and cyclo(His-Tyr), particularly of cyclo(His-Phe), shown in these studies, indicates their potential therapeutic usefulness in these disease states.

Several types of chemicals have been shown to open potassium channels, and this opening effect would lead to relaxation of the smooth muscle in blood vessels, resulting in vasodilatation, and an antihypertensive effect (Narahashi & Herman 1992). As potent peripheral vasodilators, smooth muscle potassium-channel openers find use in the treatment of cardiovascular disorders, such as hypertension, angina pectoris and congestive heart failure (Cook 1988). There are currently relatively few potassium-channel openers available for widespread clinical application (Robertson & Steinberg 1990). Both the diketopiperazines under study demonstrated an opening effect of inward rectifying potassium channels and therefore might possess therapeutic potential as vasodilators to be used in various cardiovascular disease states.

Hearts exposed to 100 μM cyclo(His-Phe) showed a significant gradual reduction in heart rate in the isolated rat heart experiments. Cyclo(His-Phe) caused a significant decrease ($P = 0.0017$) in coronary flow rate when compared with the control. Cyclo(His-Tyr) significantly increased the heart rate in relation to the control. This change of heart rate was significantly greater than that caused by cyclo(His-Phe). Cyclo(His-Tyr) failed to cause any significant change of coronary flow rate when compared with the control experiments, and demonstrated a profile very close to that of the control. It caused an initial increase in coronary flow rate and thereafter a gradual reduction in coronary flow rate over time which, however, remained elevated for the rest of the experimental period compared with the control, albeit not statistically significant in terms of increased coronary flow. Although cyclo(His-Tyr) did not have as much calcium-channel-blocking activity as cyclo(His-Phe) in the ion channel experiments on ventricular myocytes, it did demonstrate some voltage-dependent calcium-channel antagonism. Voltage-dependent calcium-channel blockage is characteristic of dihydropyridines such as nifedipine, which show a greater selectivity for vascular smooth muscle – dilating coronary vessels, increasing cardiac perfusion and hence increasing heart rate (Ferrari et al 1994).

The micro-dilution technique, using 96-well microplates and the tetrazolium salt *p*-iodonitrotetrazolium (INT) violet to indicate bacterial and fungal growth, proved to be a quick method and more sensitive than the agar diffusion technique. This method was robust, inexpensive, gave reproducible results, required only a small quantity of sample, did not require high levels of skill, left a permanent record and required little time (Eloff 1998). This technique proved suitable for giving an indication of the antimicrobial effects of the cyclic dipeptides and results from the INT assay could be taken as more reliable than those from the Kirby-Bauer disc-diffusion technique for reasons previously highlighted. It is possible that antimicrobial activity may not be demonstrated using the Kirby-Bauer technique and techniques such as the colorimetric tetrazolium-based assays may find evidence of activity where none is reported in the Kirby-Bauer disc-diffusion assay. However, the Kirby-Bauer assay is, to date, still the most important acceptable form of screening used and should always remain the first technique chosen when assessing the antimicrobial properties of a compound.

The antibacterial activity displayed by cyclo(His-Tyr) can be explained by its greater ability to attach to these bacterial cells by means of hydrogen bonding, causing disruption of cell membranes and the complex cell wall of Gram-negative bacteria. This hydrogen bonding would occur at the hydrophilic ends of the phospholipid units of the cytoplasmic membrane, which point outwards in the membrane (Jenson et al 1997). Concentration-dependent antibacterial effects were observed for the cyclic dipeptides studied by Milne et al (1998), including phenylalanine-containing cyclo(Phe-Pro) and tyrosine-containing cyclo(Tyr-Pro). Graz et al (1999) explained the activity of the cyclic dipeptides as a function of the amino acids used in their synthesis. The aromatic amino acids, phenylalanine and tyrosine, can participate in hydrophobic interactions that would affect the cell membrane as has been shown for other antimicrobial peptides such as melittin, magainin, gramicidin and cecropin (Hancock & Lehrer 1998). The effects of these cyclic dipeptides, as well as that of cyclo(His-Phe) and cyclo(His-Tyr), can therefore be explained by the hydrophobic nature of the compounds. Their hydrophobicity would enable the cyclic dipeptide to interfere with outer-membrane (Gram-negative) and plasma membrane (Gram-positive) function. The loss of function could result in a loss

of cellular integrity leading to cell death (Milne et al 1998). The antifungal activity displayed by both diketopiperazines could well be due to the fact that cyclo(His-Phe) is more lipophilic and therefore can pass through the lipophilic cell walls of fungi, which are rich in cellulose, thereby interfering with fungal growth.

S. pyogenes causes diseases such as scarlet fever, acute tonsillitis, wound infections, impetigo, puerperal sepsis and toxic shock-like syndrome (Hugo & Russell 1992; Volk et al 1996). *E. coli* is the most common Gram-negative pathogen responsible for causing septic shock, meningitis in neonates, cystitis and pyelonephritis in women, and several distinct forms of diarrhoeal disease and dysentery (Volk et al 1996). *P. aeruginosa* is a causative organism of a wide range of infections and severe diseases, including pneumonia, eye infections and infections of the urinary tract and meninges (Hugo & Russell 1992; Volk et al 1996). It is responsible for over 90% of deaths from cystic fibrosis following colonization and infection of the respiratory tract (Volk et al 1996). Difficulty in penetrating the complex outer layer of Gram-negative bacteria is probably the reason why some antibiotics are less active against Gram-negative than Gram-positive bacteria. This is the basis of the extraordinary non-susceptibility to most antibiotic drugs of *P. aeruginosa*, a pathogen that causes life-threatening infections in neutropenic patients and patients with burns and wounds. The lipopolysaccharide of the cell wall of Gram-negative bacteria is also a major barrier to penetration (Rang et al 1999). Cyclo(His-Tyr) can therefore play a key role in these disease states.

Increasing numbers of fungal strains are becoming resistant to the currently used antifungal drugs. Fortunately, drug resistance is not transferable in fungi, though this is small comfort to a patient infected with a resistant strain. An additional problem is that new strains of commensal-turned-pathogenic fungi have emerged. New and better antifungal agents are therefore being sought (Rang et al 1999). The antifungal activity against *C. albicans* displayed by cyclo(His-Phe) therefore makes it a suitable potential agent for treatment of candidal infections of the oral cavity, vagina, intestinal tract and skin, and for prevention of more serious diseases such as endocarditis, septicemia and kidney and lung infections (Volk et al 1996; Jenson et al 1997).

Conclusion

Cyclo(His-Phe) and cyclo(His-Tyr) demonstrated a wide variety of activity in this study, and were therefore not clearly selective in a specific area of biological activity. The effects of the diketopiperazines on the heart rate and coronary flow rate of rat isolated hearts in conjunction with the potential effects observed on sodium, calcium and potassium ion channels of ventricular myocytes therefore suggests their potential usefulness in cardiac disease states, including hypertension, arrhythmias, angina and congestive heart failure. The effect of the diketopiperazines on bacterial and fungal growth demonstrates their potential usefulness as antimicrobial agents, and their tumour-inhibitory effects in conjunction with their beneficial effects on tumour-induced haemostatic responses demonstrates their potential to inhibit the growth, proliferation and metastasis of tumours.

References

Bednar, B., Condra, C., Gould, R. J., Connolly, T. M. (1995) Platelet aggregation monitored in a 96 well microplate reader is useful for evaluation of platelet agonists and antagonists. *Thrombosis Res.* **77**: 453–463

Bellavite, P., Andriolo, G., Guzzo, P., Arigliano, P., Chirumbolo, S., Manzato, F., Santonastaso, C. (1994) A colorimetric method for the measurement of platelet adhesion in microtiter plates. *Anal. Biochem.* **216**: 444–450

Bova, S., Cargnelli, G., D'Amato, E., Forti, S., Yang, Q., Trevisi, L., Debetto, P., Cima, L., Luciani, S., Padrini, R. (1997) Calcium-antagonist effects of norbormide on isolated perfused heart and cardiac myocytes of guinea-pig: a comparison with verapamil. *Br. J. Pharmacol.* **120**: 19–24

Bromberg, M. E., Cappello, M. (1999) Cancer and blood coagulation: molecular aspects. *Cancer J.* **5**: 132–133

Carmichael, J. (1994) Current issues in cancer: cancer chemotherapy: identifying novel anticancer drugs. *Br. Med. J.* **308**: 1288–1290

Carmichael, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D., Mitchell, J. B. (1987) Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res.* **47**: 936–942

Cook, N. S. (1988) The pharmacology of potassium channels and their therapeutic potential. *Trends Pharmacol. Sci.* **9**: 21–28

Crescenzi, V., Cesaro, A., Russo, E. (1973) On some physicochemical properties of diketopiperazines in water. *Int. J. Peptide Protein Res.* **5**: 427–434

Eloff, J. N. (1998) A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Med.* **64**: 711–713

Fernandez-Lopez, S., Kim, H.-S., Choi, E. C., Delgado, M., Granja, J. R., Khasanov, A., Kraehenbuehl, K., Long, G., Weinberger, D. A., Wilcoxen, K. M., Ghadiri, M. R. (2001) Antibacterial agents based on the cyclic D,L-α-peptide architecture. *Nature* **412**: 452–455

Ferrari, R., Cucchini, F., Bolognesi, R., Bachetti, T., Baraso, A., Bernocchi, P., Gaia, G., Visioli, O. (1994) How do calcium antagonists differ in clinical practice. *Cardiovasc. Drugs. Ther.* **8**: 565–575

Godfraind, T., Miller, R., Wibo, M. (1986) Calcium antagonism and calcium entry blockade. *Pharmacol. Rev.* **38**: 321–416

Graz, M., Hunt, A., Jamie, H., Grant, G., Milne, P. (1999) Antimicrobial activity of selected cyclic dipeptides. *Pharmazie* **54**: 772–775

Graz, C. J. M., Grant, G. D., Brauns, S. C., Hunt, A., Jamie, H., Milne, P. J. (2000) Cyclic dipeptides in the induction of maturation for cancer therapy. *J. Pharm. Pharmacol.* **52**: 75–82

Hancock, R. E. W. (1999) Host defense cationic peptides. *Drugs* **57**: 469–483

Hancock, R. E. W., Lehrer, R. (1998) Cationic peptides: a new source of antibiotics. *Tibtech* **16**: 82–87

Henczi, M., Weaver, D. F. (1995) Fragmentation of a series of cyclic dipeptides in fast-atom bombardment mass spectrometry. *Rapid Commun. Mass Spectrom.* **9**: 800–803

Hugo, W. B., Russell, A. D. (1992) *Pharmaceutical microbiology*. 5th edn, Blackwell Science, Oxford, pp 33–38, 50–51, 57, 117, 119, 120, 128, 134, 136, 139, 199, 221

Jamie, H., Kilian, G., Dyason, K., Milne, P. J. (2002) The effect of the isomers of cyclo(Trp-Pro) on heart and ion-channel activity. *J. Pharm. Pharmacol.* **54**: 1659–1665

Jenson, M. M., Wright, D. N., Robinson, R. A. (1997) *Microbiology for the health sciences*. 4th edn, Prentice-Hall, New Jersey, pp 38, 44, 201, 203, 215, 273, 301, 302, 307, 489

Kolusheva, S., Boyer, L., Jelinek, R. (2000) A colorimetric assay for rapid screening of antimicrobial peptides. *Nature Biotechnol.* **18**: 225–227

Kopple, K. D., Ghazarian, H. G. (1968) A convenient synthesis of 2,5-dioxopiperazines. *J. Org. Chem.* **33**: 862–863

Langendorff, O. (1895) Untersuchungen am uberlebenden saugethierhergen. *Arch. Geo. Physiol.* **61**: 291–332

Lubbe, W. F., Daries, P. S., Opie, L. H. (1978) Ventricular arrhythmias associated with coronary artery occlusion and reperfusion in the isolated perfused rat heart: a model for assessment of antifibrillatory action of antiarrhythmic agents. *Cardiovasc. Res.* **12**: 237–247

Marban, E., Yamagishi, T., Tomaselli, G. F. (1998) Structure and function of voltage-gated sodium channels. *J. Physiol.* **508**: 647–657

Milne, P. J., Hunt, A. L., Rostoll, K., van der Walt, J. J., Graz, C. J. M. (1998) The biological activity of selected cyclic dipeptides. *J. Pharm. Pharmacol.* **50**: 1331–1335

Mitra, R., Morad, M. (1995) A uniform enzymatic method for dissociation of myocytes from hearts and stomachs of vertebrates. *Am. J. Physiol.* **249**: H1056–H1060

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**: 55–63

Murray, J. C. (1991) Coagulation and cancer. *Br. J. Cancer* **64**: 422–424

Narahashi, T., Herman, M. D. (1992) Overview of toxins and drugs as tools to study excitable membrane ion channels: I. Voltage-activated channels. *Methods Enzymol.* **207**: 620–643

Prasad, C. (1995) Bioactive cyclic dipeptides. *Pergamon* **16**: 151–164

Rang, H. P., Dale, M. M., Ritter, J. M. (1999) *Pharmacology*. 3rd edn, Churchill Livingstone, USA, pp 657, 688, 707

Rob, J. A., Tollefsen, S., Helgeland, L. (1997) A rapid and highly sensitive chromogenic microplate assay for quantification of rat and human prothrombin. *Anal. Biochem.* **245**: 222–225

Robertson, D. W., Steinberg, M. I. (1990) Potassium channel modulators: scientific applications and therapeutic promise. *J. Med. Chem.* **33**: 1529–1541

Sammes, P. G. (1975) Naturally occurring 2,5-diketopiperazines and related compounds. *Fortschr. Chem. Org. Naturst.* **32**: 51–118

Schatteman, K. A., Goossens, F. J., Scharpe, S. L., Hendriks, D. F. (1999) Activation of plasma procarboxypeptidase U in different mammalian species points to a conserved pathway of inhibition of fibrinolysis. *Thromb. Haemost.* **82**: 1718–1721

Sijpkes, A. H., van der Kleut, G. J., Gill, S. C. (1994) *Biophys. Chem.* **52**: 75–82

Tanaka, A. S., Silva, M. M., Torquato, R. J. S., Noguti, M. A. E., Sampaio, C. A. M., Fritz, H., Auerswald, E. A. (1999) Functional phage display of leech-derived tryptase inhibitor (LDTI): construction of a library and selection of thrombin inhibitors. *Fed. Euro. Biochem. Soc. Let.* **458**: 11–16

Thom, S. M., Horobin, R. W., Seidler, E., Barer, M. R. (1993) Factors affecting the selection and use of tetrazolium salts as cytochemical indicators of microbial viability and activity. *J. Appl. Bacteriol.* **74**: 433–443

Tytgat, J. (1994) How to isolate cardiac myocytes. *Cardiovasc. Res.* **28**: 280–283

Volk, W. A., Gebhardt, B. M., Hammarskjöld, M.-L., Kadner, R. J. (1996) *Essentials of medical microbiology*. 5th edn, Lippincott-Raven Publishers, New York, pp 273, 331–333, 342, 345, 346, 362, 363, 366, 367, 379, 380, 483

Walkowiak, B., Kesy, A., Michalec, L. (1997) Microplate reader – a convenient tool in studies of blood coagulation. *Thromb. Res.* **87**: 95–103

Wu, S., Chen, W., Murphy, E., Gabel, S., Tomers, K. B., Foley, J., Steenbergen, C., Falck, J. R., Moomaw, C. R., Zeldin, D. C. (1997) Molecular cloning, expression, and functional significance of a cytochrome P450 highly expressed in rat heart myocytes. *J. Biol. Chem.* **272**: 12551–12559