Cyclic Dipeptide Research Unit, University of Port Elizabeth, South Africa

Biological activity of selected tyrosine-containing 2,5-diketopiperazines

G. Kilian, H. Jamie, S. C. A. Brauns, K. Dyason, P. J. Milne

Received July 28, 2003, accepted June 16, 2004

P. J. Milne, Cyclic Peptide Research Unit, University of Port Elizabeth, P.O. Box 1600, Port Elizabeth 6000, South Africa
pmapjm@upe.ac.za

Pharmazie 60: 305–309 (2005)

The study investigates two cyclic dipeptides, cyclo(Tyr-Tyr) (cTT) and cyclo(Phe-Tyr) (cPT) with respect to their biological activity. Investigations using the whole-cell patch-clamp technique testing the effects of 100 μM cyclic dipeptide on ion channels, revealed reversible voltage-dependant blockade for cTT, while cPT exhibited irreversible time-dependant blockade of L-type calcium channels. The isolated retrogradely-perfused rat heart was used to determine the effects of 100 μM of either cTT or cPT on heart rate (HR), coronary flow rate (CFR), left ventricular systolic pressure (LVSP) and cardiac conduction speed. Results indicated opposing effects for the two compounds, where cTT increased HR and CF while cPT decreased HR, CF, LVSP as well as conduction speed. Other biological investigations included opioid binding and anti-neoplastic assays. Competitive binding curves, using tritiated DAMGO, revealed significant binding to μ-opioid receptors with IC_{50} values for cTT and cPT being 0.82 μM and 69.7 μM respectively. Anti-neoplastic activity was tested using three cultured cell lines: HT-29, MCF-7 and HeLa which were exposed to 2.5 mM cyclic dipeptide or 0.1 mM melphalan as a positive control. While cTT showed little activity against these lines, cPT resulted in as much as a 75.6% growth inhibition of MCF-7 cells, while also being active against HeLa (73.4% inhibition) and HT-29 (60.6%). The results indicate potential biological activity, showing a need for more investigation into tyrosine containing cyclic dipeptides and their analogues as potential bioactive compounds.

1. Introduction

Cyclic dipeptides (CDP's), comprising a diketopiperazine ring structure, were first characterized in the early 1900's. A greater degree of emphasis has now been placed on their biological activity since the discovery of naturally occurring cyclic dipeptides in both humans and micro-organisms (Ström et al. 2002). These small and conformationally rigid molecules allow a somewhat simplified study of their structure-activity characteristics as well as give insight into the conformations and structure-activity relationships of larger peptides and proteins (Graz et al. 2000). A recent study (Milne et al. 1998) investigated the biological activity of selected cyclic dipeptides and the results indicated significant cardiac, anticancer and antibacterial activity. Cyclic dipeptides containing amino acids with aromatic side chains are even more interesting, as aromatic rings can be found in many bioactive molecules, often being the moieties that result in receptor interaction (Yamazaki et al. 1991). One of the more important reasons for the interest in the biological activity of cyclic dipeptides is due to their limited conformational freedom, enabling the investigator to gain valuable insight into the conformational characteristics required for receptor interaction in order to produce a physiological response (Anteunis, 1978). This study takes a look at the biological activity of the tyrosine-containing cyclic dipeptides cyclo(L-Phenylalanyl-L-Tyrosine) (cPT) and cyclo(L-Tyrosyl-L-Tyrosine) (cTT) with respect to their cardiovascular and anti-tumour activity as an ongoing screening process undertaken by the research unit for identifying cardioactive and antineoplastic peptides. The study also investigates the μ-opioid receptor binding characteristics of these two compounds, justified by the presence of a tyrosine hydroxyl group on both compounds, an essential element for opioid receptor interaction in naturally occurring opioid peptides (Chang and Fong, 1976).

2. Investigations, results and discussion

2.1. Ion channel activity

The whole-cell patch-clamp method was utilized to determine the effects of cTT and cPT on potassium and L-type calcium channel current in cardiac myocytes. Investigation into the effects of 100 μM of each CDP on inward-rectifier potassium channels revealed little to no difference in potassium currents (Fig. 1). Results obtained from tests on L-type calcium channels revealed that 100 μM cTT exposure resulted in an increase in calcium current at test potentials ranging from −5 to −50 mV, but decreasing calcium currents by 22.2% between test potential of −5 to 30 mV (Fig. 2a). This voltage-dependent block is also observed with dihydropyridines, resulting in their selectivity for depolarized cells in vascular smooth muscle rather than cardiac muscle tissue (Ferrari et al. 1994). There was also a shift in peak inward current from 10 mV to 0 mV, which was reversed 5 min after washout, indicating vol-

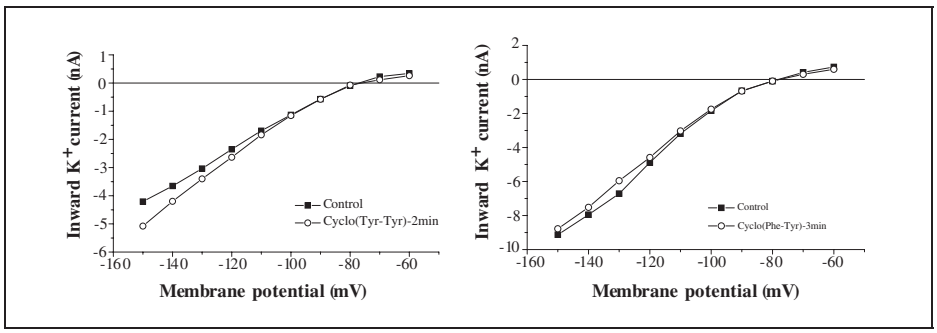

Fig. 1:
Inward-rectifier K^+ current. No significant effects were noted for either cTT (n = 1) nor cPT (n = 1) when compared with the control experiment (n = 1)

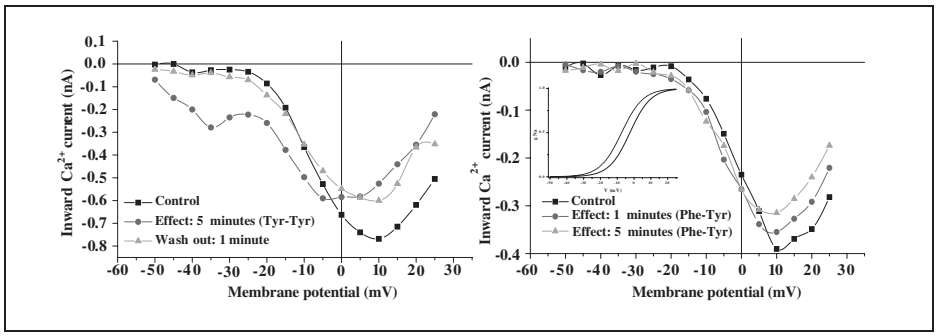

Fig. 2:
L-type Ca^{2+} current. (a) Voltage-dependent blockade was noted for cTT (n = 1), with increasing calcium current between −5 and −50 mV. (b) cPT (n = 1) showed time-dependent irreversible blockade amounting to a decrease of 20.3% in Ca^{2+} current after five minutes exposure. No washout was obtained (data not shown). (Inset) Boltzman activation plot showing a shift to the left of $V_{1/2}$

tage-dependent reversible blockade of L-type calcium channels. Antagonistic properties were also noted for cPT (Fig. 2b), were calcium currents were decreased by 8.5% after 1 min exposure and further decreased to 20.3% after 5 min exposure, indicating a time-dependent blockade. A Boltzman activation plot also clearly shows a shift in $V_{1/2}$ to the left. No washout was obtained (data not shown) and this fact, along with the surmountable effects with time, would indicate an irreversible blockade of L-type calcium channels. The initial screening performed using the whole-cell patch-clamp method indicated potential cardiac activity for both cTT and cPT, justifying further investigation using the isolated rat heart model.

2.2. Effects on the isolated rat heart

Effects of cTT and cPT on the isolated rat heart confirmed the results obtained in ion channel studies. Heart rate was increased significantly ($p < 0.005$) by 5.7% after 5 min exposure to 100 μM cTT. cPT resulted in a large decrease (27.2%, $p < 0.005$) in heart rate (Fig. 3a). Coronary flow rate significantly decreased as a result of 100 μM cPT exposure ($p < 0.007$), decreasing flow by 52.9% after 30 min (Fig. 3b). cTT increased coronary flow significantly ($p < 0.001$) by 74.1% after 30 min exposure. Ventricular pressure was not significantly affected by cTT but was decreased by 35% after 30 min exposure to 100 μM cPT ($p < 0.001$) (Fig. 3c). Measurement of QT interval, an indication of the time taken from initial excitation until the end of ventricular systole (Sperelakis 1989), revealed a slightly significant decrease in QT duration ($p < 0.05$) for hearts exposed to cTT, however a significantly large increase ($p < 0.01$) in QT interval was noted for cPT exposed hearts (Fig. 3d). This increase in QT duration indicated slowing of cardiac conduction speed, which may predispose or induce fatal arrhythmias such as *torsades des pointes.*

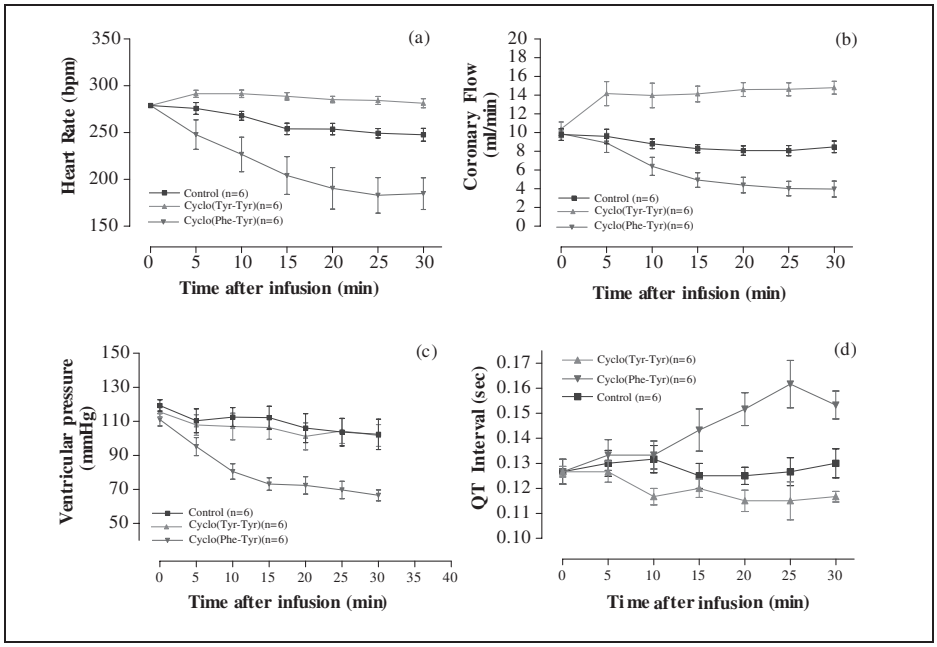

Fig. 3: The effects of cTT and cPT on the retrogradely-perfused isolated rat heart. Graphical representation of the effects of 100 μM of each cyclic dipeptide on (a) heart rate, (b) coronary flow rate, (c) ventricular pressure and (d) conduction speed

The effects on heart rate for cTT may be explained by the increase in coronary flow rate, as vasodilators have a tendency to increase heart rate as a result of increased cardiac perfusion (Fallen et al. 1967). The effects of cTT on calcium channels confirms the results obtained in the isolated rat heart, as voltage-dependent blockade of calcium channels tends to be more selective for vascular smooth muscle due to a high degree of depolarization, as observed with dihydropyridines such as nifedipine (Ferrari et al. 1994). Clinical evidence suggests that a decrease in vascular tone is beneficial for the treatment of disease states such as congestive heart failure (CHF) (Hondeghem and Mason 1989). Therefore, the vascular effects of cTT as well as the findings that it has no significant effects on ventricular systolic pressure may prove useful in the development of therapeutic agents for the treatment of CHF.

The results from channel studies and isolated heart experiments also showed correlation. Irreversible blockade of L-type calcium channels was noted using the whole-cell patch-clamp method. This finding was further confirmed when looking at the effects of cPT on heart rate, coronary flow rate, left ventricular systolic pressure and conduction speed. Heart rate continued to decrease with time indicating irreversible blockade as a result of slow dissociation of the drug from the channel receptor. The reduction in coronary flow rate may not be a result of vasoconstriction, but rather due to the decrease in both ventricular systolic pressure and heart rate.

2.3. μ-Opioid receptor binding characteristics

DAMGO ([D−Ala^2, N−Me−Phe^4, Gly(ol)^5]enkephalin) has been shown to exhibit high affinity for μ-opioid receptors, those associated with pain, and is widely used in competition binding experiments for determination of μ-opioid receptor binding (Blake et al. 1997). Saturation plots for [^{3}H]DAMGO (Fig. 4) indicated an affinity (K_D) of 2.90 nM which is comparable with the literature range of 0.45 to 5.5 nM (Lagane et al. 2000; Chen et al. 1997; Blake et al. 1997; Chalecka-Franaszek et al. 2000). Non-specific binding in the presence of 10 μM naloxone was approximately 60% which is also within normal limits for crude extracts (Lambert et al. 1993). The B_{max} for the membrane preparation was 350.9 fmol/mg protein. A Scratchard plot (inset of Fig. 4), showed a linear relationship ($R^2 = 0.96$) between free and bound enkephalin, confirming the specificity of DAMGO for a single receptor (Blake et al. 1997). Competition binding curves (Fig. 5) showed promising results, indicating receptor interaction at concentrations much lower than expected from previous biological screening (Milne et al. 1998). Data from competitive binding curves was analyzed using the sigmoidal dose response (variable slope) equation (eq. 1)

$$Y = \text{BOTTOM} + \frac{(\text{TOP} - \text{BOTTOM})}{(1 + 10^{(\text{EC}_{50} - X) \times \text{Hill Slope}})} \qquad (1)$$

also known as the four-parameter logistic equation. IC_{50} values derived from this equation were 0.82 μM and 69.7 μM for cPT and cTT respectively. Hill slope factors indicated slightly shallow slopes where $n_H = -0.53 \pm 0.07$ for cPT and $n_H = -0.65 \pm 0.08$ for cTT. Using the Cheng and Prusoff equation (eq. 2) (Chang and Cuatrecasas 1979)

$$K_i = \frac{IC_{50}}{1 + [L]/K_D} \qquad (2)$$

to calculate affinity showed K_i for cPT and cTT to be 0.3 μM and 25.6 μM respectively. However, little confidence can be placed in the calculation of the affinities as the Hill slope factor (n_H) for both curves differ slightly from unity.

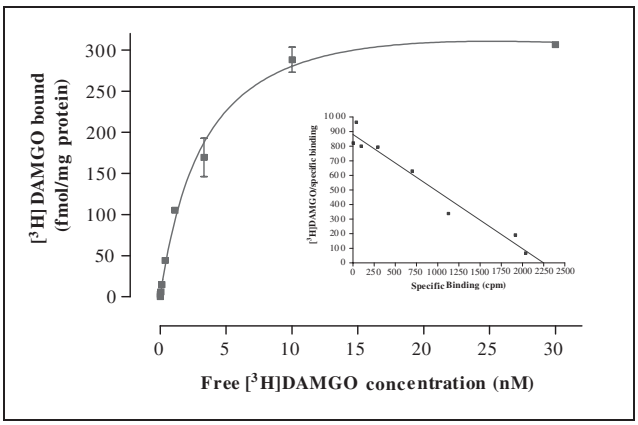

Fig. 4: Saturation plot for [^{3}H]DAMGO showing specific binding in fmol bound enkephalin per mg of protein. K_D for [^{3}H]DAMGO = 2.90 ± 0.4 nM. Inset is a Scratchard Plot indicating linearity with an R^2 value of 0.96 (n = 2)

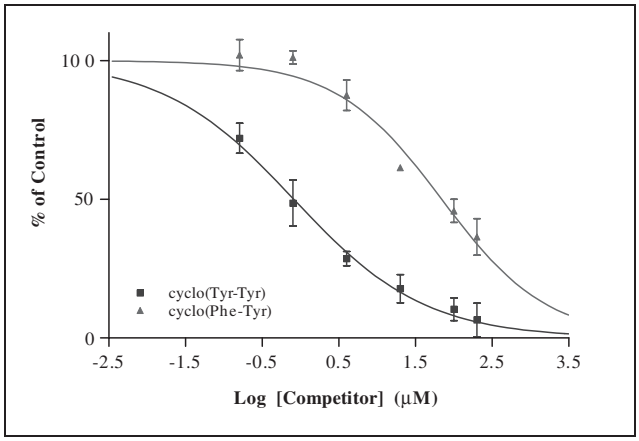

Fig. 5: Competitive binding curves in relation to [^{3}H]DAMGO. IC_{50} values for cTT and cPT were 0.82 μM and 69.7 μM respectively (n = 2)

2.4. Anti-neoplastic activity

cTT showed little tumour inhibition potential when compared to 0.1 mM melphalan, a known tumour suppressor.

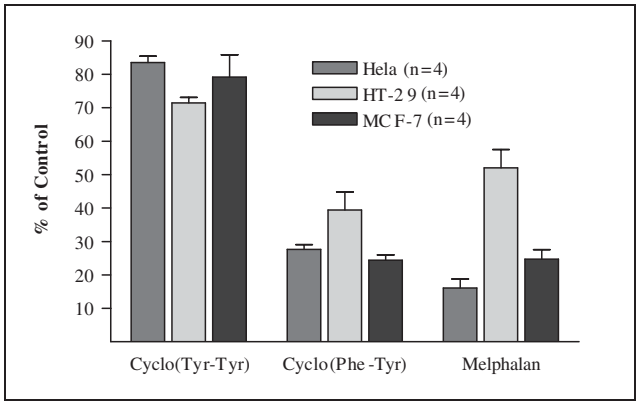

Fig. 6: Cytotoxic activity of cTT and cPT. Graph indicates little inhibitory activity for cTT but tumour inhibition of 2.5 mM cPT was comparable to that of melphalan at concentrations of 0.1 mM

2.5 mM cPT showed significant inhibition of all three cell lines, comparable to that of 0.1 mM melphalan. A 73.4% growth inhibition was noted for HeLa cells, 75.6% inhibition for MCF-7 cells and a lower inhibition of 60.6% for HT-29 cells (Fig. 6). This trend was also noted for melphalan, where HT-29 colon carcinoma cells were also more resistant than other cell lines. Colon cells, while being extremely permeable to many molecules in order to facilitate absorption, are also able to eliminate molecules by active transport facilitated by the membrane bound ATPase transporter, P-glycoprotein, which plays a major role in resistance to chemotherapeutic agents (Lavie et al. 1998). The positive results obtained in the primary screening of cPT would require further analysis of this compound as a potential chemotherapeutic agent.

3. Experimental

The use of experimental animals in this study was approved by the University of Port Elizabeth Animal Ethics Committee (date of approval: 20 February 2001).

3.1. Synthesis and elucidation of CDP's

cTT and cPT were both synthesized in our laboratory from their linear counterparts (Bachem, Switzerland) using the method of Kopple and Ghazarian (1968). Structures were elucidated using standard drug elucidation methods, namely mass spectrometry, IR spectroscopy, DSC and TGA analysis, TLC, HPLC and NMR spectrometry. MS confirmed the molecular masses of cTT and cPT to be 326 g/mol and 310 g/mol respectively. TLC, using silica gel 60 F_{254} plates (Merck, USA) and two mobile phases: (1) chloroform:methanol (7 : 1) and (2) chloroform : methanol : acetic acid (14 : 2 : 1), was used to determine purity of the products. Rf values for cTT were 0.5862 (mobile phase 1) and 0.6753 (mobile phase 2) while Rf values for cPT were 0.6437 (mobile phase 1) and 0.8182 (mobile phase 2).

3.2. Whole-cell patch-clamp method

3.2.1. Calcium channel activity

The whole-cell patch-clamp technique was used according to Hamil et al. (1981). Ventricular cells were isolated from guinea-pigs as described previously (Mitra and Morad 1985, modified by Tytgat 1994). Patch-clamping was performed using a Dagan 8800 Total Clamp amplifier and heat polished electrodes were made from borosilicate glass using a Narishige PP 83-model puller. Currents were recorded using Clampex software (Labmaster TM40, version 5.5.1). The intracellular solution consisted of (in mM): 125 CsCl, 2 $MgCl_2$, 5 EGTA, 10 HEPES, 1 $CaCl_2$, 3 Mg-ATP and 10 glucose and the pH adjusted to 7.2 with NaOH, while the extracellular solution consisted of (in mM): 138 Tris, 0.5 $MgCl_2$, 10 HEPES, 5.4 $CaCl_2$, 20 CsCl and 5 glucose. Cells were exposed to 100 µM of either cTT- or cPT in 2% DMSO or a control solution containing 2% DMSO (pH 7.4). Calcium currents were recorded at hyperpolarizing steps from a holding potential of −90 mV to test potentials of between −50 and 25 mV at steps of 5 mV. Pulses were given at 5 s intervals for duration of 100 ms. All experiments were carried out at room temperature.

3.2.2. Potassium channel activity

Inward rectifier K^+ currents were recorded under voltage clamp conditions using the whole-cell patch-clamp technique utilizing single cells isolated by enzymatic dispersion from guinea-pig ventricles, as described in calcium channel activity. Intracellular solutions consisted of (in mM): 140 KCl, 2 $MgCl_2$, 11 EGTA, 10 HEPES, 1 $CaCl_2$ and 5 Na_2-ATP (pH adjusted to 7.2 with KOH), while the extracellular solution consisted of (in mM): 130 NaCl, 4 KCl. 1 $MgCl_2$, 10 HEPES-NaOH, 1.8 $CaCl_2$ and 10 glucose. Potassium currents were recorded from a holding potential of −80 mV to test potentials of −140 mV to −50 mV during 500 ms hyperpolarizing steps. Single experiments were performed for both calcium and potassium ion channel experiments as a primary screening in order to justify the use of further experimental animals for isolated organ experiments.

3.3. Isolated rat heart model

3.3.1. Anaesthesia, dissection and mounting

Male Long Evans rats (250–350 g) were anaesthetized under ether to loss of pain and blink reflexes and maintained as such during dissection. 40 IU of heparin sodium (Fresenius-Kabi, South Africa) were injected into the left femoral vein. The heart was removed and arrested in ice-cold (4 °C) Krebs-Henseleit Physiological Buffer (KHB) containing (in mM): 118 Na^+, 1.2 $H_2PO_4^-$, 1.2 Mg^{2+}, 2.5 Ca^{2+}, 25 HCO_3^-, 123 Cl^-, 1.2 SO_4^{2+}, 5.9 K^+ and 11 glucose. The heart was mounted as described previously (Lubbe et al. 1978)

3.3.2. Langendorff perfusion system

A dual-perfusion Langendorff system was used in order to facilitate a rapid switch in perfusion media. The heart was perfused with a modified KHB solution as described above, which was aerated with carbogen (95% O_2, 5% CO_2) (Afrox (Pty) Ltd., South Africa). Hearts were allowed a 15 min stabilization period prior to drug exposure. Any abnormalities in the ECG during this period resulted in the experiment being aborted.

3.3.3. Determination of heart rate, coronary flow rate and conduction speed

Hearts mounted as described above, were exposed to 100 µM of cTT or cPT dissolved in 5% dimethylsulfoxide (DMSO) in KHB or a control solution containing only 5% DMSO in KHB. Heart rates were recorded at 5 min intervals and a constant ECG trace was recorded for the detection of conduction speed and conduction abnormalities. Coronary flow was measured at 5 min intervals. Hearts were exposed to test solutions for 30 min, after which the experiment was terminated. Heart rates, derived from ECG traces, were recorded at 5 min intervals. Measurement of QT intervals was performed using Grass Polyview Data Acquisition and Analysis software (Version 2.0). Measurements were made at 5 min intervals on three successive ECG traces, the means being recorded for analysis.

3.3.4. Determination of left ventricular systolic pressure

Hearts were mounted as described above. A PVC balloon attached to a pressure transducer was inserted into the left ventricle and inflated to a pre-load pressure of 10 mmHg. Hearts were then electronically paced at 300 bpm. Maximum ventricular pressure (systolic) was measure at 5 min intervals. Hearts were exposed to test solutions for 30 min.

3.4. Opioid binding assay

3.4.1. Preparation of crude brain extracts

Male Long Evans Rats, weighing between 250 and 350 g were anaesthetized with diethyl ether (Merck, SA) and their whole brains were excised and washed in ice-cold (4 °C) isotonic sucrose solution (0.32 M). The cerebellum was removed and the brain sliced into smaller portions and immersed in 10 volumes of the isotonic sucrose solution. The method of differential centrifugation (modified from Chang and Cuatrecasas, 1979) was used. Briefly, the brain was homogenized for 1 min with a Janke and Kunkel Model RM 18 homogenizer. The homogenate was centrifuged at 6000 × g for 15 min at 4 °C. The nuclei and mitochondrial rich pellet was discarded and the supernatant centrifuged at 40000 × g for a further 30 min at 4 °C. Two distinguishable pellets formed, the top, whiter pellet was separated from the bottom brownish pellet and re-suspended in 10 volumes of the isotonic sucrose solution. The centrifugation steps were repeated and the final white pellet was re-suspended in 5 volumes of 50 mM Tris-HCl (pH 7.4). Total protein of the crude extract was determined using the bicinchoninic acid (BCA) method of Lowry et al. (1951) (modified by Smith et al. 1985) and the protein concentration adjusted to 1 mg/ml by dilution with 50 mM Tris-HCl. Extracts were stored at −20 °C until used.

3.4.2. Determination of K_D for [^{3}H]DAMGO

Tritiated DAMGO (Perkin-Elmer Life Sciences) was used and a saturation curve determined according to the rapid filtration method of Chen et al. (1997). Briefly, 100 µL of varying concentrations of [^{3}H]DAMGO (0.8×10^{-2} nM to 150 nM) in 50 mM Tris-HCl were diluted with 200 µL 50 mM Tris-HCl. The reaction was initiated by the addition of 200 µL crude brain extract and incubated for 60 min at 24 °C. The incubated samples were filtered rapidly through 25 mm Whatman GF/C filters and washed twice with 3 ml ice-cold 50 mM Tris-HCl. Filters were placed in scintillation vials and dried for 24 h in a dessicator at 37 °C. 10 ml Ultima Gold scintillation cocktail was added to the vials and vortexed for 30 s and left in the dark for 3 h prior to reading on a Packard Liquid Scintillation Counter. Non-specific binding was defined in the presence of 2 µM naloxone (Fresenius-Kabi, SA).

3.4.3. Determination of competitive binding

200 µL of the crude brain extract was incubated with 200 µL of varying concentrations of either cTT or cPT (500, 250, 50, 10, 2 and 0.4 µM) in 50 mM Tris-HCl along with 100 µL of [^{3}H]DAMGO to a final concentration of 5 nM. Incubation, filtration and reading was then performed as described above. Once again, non-specific binding was defined in the pre-

sence of 2 μM naloxone. IC_{50} values for each drug were then determined using Graphpad Prism® (Version 3.1) and receptor affinity calculated using the Cheng and Prusoff equation (Chang & Cuatrecasas, 1979).

3.5. Anti-cancer screening

HT-29, HeLa and MCF-7 (Highveld Biologicals) cell cultures were maintained in Dulbeco's Modified Eagles Medium (DMEM) (Highveld Biologicals) supplemented with 5% bovine foetal calf serum (FCS). Cells were harvested, diluted to 250 000 cells/ml and 200 μL seeded into 96-well microtiter plates. cTT (3.3 mg) and cPT (3.1 mg) were dissolved in 50 μL DMSO and made to 1 ml with DMEM and 5% FCS. 50 μL of the cyclic dipeptide solution was added to the well and incubated at 37 °C for 24 h. Final concentrations of the CDP's amounted to 2.5 mM. Melphalan (0.1 mM) was used as a positive control. After incubation, the cells were fixed with 50% trichloracetic acid (TCA) and viable cells determined using the Sulforhodamine B assay (Skehan et al. 1990).

3.6. Statistical analysis

Results were calculated as means ± standard deviation and plotted graphically using the software package Graphpad Prism Version 4 (Graphpad, San Diego). For cardiac studies, significant differences in means were calculated using the paired t-test and significance defined as a p value of less than 0.05. In calcium channel studies, deviation in $V_{1/2}$ was determined graphically using an activation curve utilizing the Boltzman equation. Ligand binding curves were also analyzed using Prism 4, where curve fitting was performed by non-linear regression analysis using the sigmoidal dose-response curve (variable slope) equation (1) from which the IC_{50} values and Hill slope factors were derived. Affinity (K_i) of cTT and cPT was calculated using the Cheng and Prusoff equation (2). Significance in anti-cancer activity was determined using a one-way ANOVA analysis.

References

Anteunis MJO (1978) The cyclic dipeptides. Proper model compounds in protein research. Bull Soc Chim Belg 87: 627–650.

Blake AD, Bot G, Freeman JC, Reisine T (1997) Differential opioid agonist regulation of the mouse μ opioid receptor. J Biol Chem 272: 782–790.

Chalecka-Franaszek E, Weems HB, Crowder AT, Cox BM, Côté TE (2000) Immunoprecipitation of high-affinity, guanine nucleotide-sensitive, solubilized μ-opioid receptors from rat brain. J Neurochem 74: 1068–1078.

Chang K-J, Cuatrecasas P (1979) Multiple opiate receptors. J Biol Chem 254: 2610–2618.

Chang J-K, Fong BTW (1976) Opiate receptor affinities and behavioural effects of enkephalin: structure-activity relationship of ten synthetic peptide analogues. Life Sci 18: 1473–1482.

Chen JJ, Dymshitz J, Vasko MR (1997) Regulation of opioid receptors in rat sensory neurons in culture. Mol Pharmacol 51: 666–673.

Fallen EL, Elliott WC, Gorlin R (1967) Apparatus for study of ventricular function and metabolism in the isolated perfused rat heart. J Appl Physiol 22: 836–839.

Ferrari R, Cucchini F, Bolognesi R, Bachetti T, Boraso A, Bernocchi P, Gaia G, Visioli O (1994) How do calcium antagonists differ in clinical practice. Cardiovasc Drugs Ther 8 (supp 3): 565–575.

Graz MGC, Grant GD, Brauns, SC, Hunt, A, Jamie, H., Milne PJ (2000) Cyclic dipeptides in the induction of maturation for cancer therapy. J Pharm Pharmacol 52: 75–82.

Hamil OP, Marty A, Neher E, Sakman B, Sigworth FJ (1981) Improved patch clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch 391: 85–100.

Hondeghem LM, Mason JW (1989) Agents used in cardiac arrhythmias, in: Katzung BG (ed) Basic and Clinical Pharmacology. 4th Edn, Prentice-Hall International, USA, p. 165.

Kopple KD, Ghazarian HG (1968) A convenient synthesis of 2,5-piperazinediones. J Org Chem 33: 862–864.

Lagane B, Gaibelet G, Meilhoc E, Masson J-M, Cézanne L, Lopez A (2000) Role of sterols in modulating the human μ-opioid receptor in *Saccaromyces cerevisiae.* J Biol Chem 275: 33197–33200.

Lambert DG, Atcheson R, Hirst RA, Rowbotham DJ (1993) Effects of morphine and its metabolites on opiate receptor binding, cAMP formation and [^{3}H]noradrenaline release from SH-SY5Y cells. Biochem Pharmacol 46: 1145–1150.

Lavie Y, Fiucci G, Liscovitch M (1998) Up-regulation of caveolae and caveolar constituents in multidrug-resistant cancer cells. J Biol Chem 243: 32380–32383.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193: 265–275.

Lubbe WF, Daries PS, Opie LH (1978) Ventricular arryhthmias associated with coronary artery occlusion and reperfusion in the isolated perfused rat heart: a model for assessment of antifibrillatory action of antiarrhythmic agents. Cardiovascular Res 12: 237–247.

Milne PJ, Hunt AL, Rostoll K, van der Walt JJ, Graz CJM (1998) The biological activity of selected cyclic dipeptides. J Pharm Pharmacol 50: 1331–1337.

Mitra R, Morad R (1985) A uniform enzymatic method for dissociation of myocytes from hearts and stomachs of vertebrates. Am J Physiol 249: 1056–1060.

Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR (1990) New colorimetric assay for anticancer-drug screening. J Natl Cancer Inst 82: 1107–1112.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke, NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150: 76–85.

Sperelakis (ed.) (1989) Physiology and Pathophysiology of the Heart – 2nd Edition. Kluwer Adademic Publishers, Norwell. P. 1009.

Ström K, Sjogren J, Broberg A, Schnurer J (2002) *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. Appl Environ Microbiol 68: 4322–4327.

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

Yamazaki T, Nunami K-I, Goodman M (1991) Cyclic retro-inverso dipeptides with two aromatic side chains. II. Conformational Analysis. Biopolymers 31: 1513–1528.