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

Hepatotoxicity of the isomers of cyclo(Trp-Pro)

H. JAMIE, G. KILIAN and P. J. MILNE

The hepatotoxicity of the novel cyclic dipeptide cyclo(Trp-Pro), which has shown potential usage in various pharmacological fields, had not been assessed. Further studies on the isomers of this cyclic dipeptide (cyclo(L-Trp-L-Pro), cyclo(L-Trp-D-Pro), cyclo(D-Trp-L-Pro) and cyclo(D-Trp-D-Pro)) revealed further biological activities. The assessment of hepatotoxicity of these isomers was thus warranted. *In vitro* screens were performed on primary isolated rat hepatocytes, the Chang liver and N-2-alpha cell lines. *In vivo* screening involved the assessment of serum levels of lactate dehydrogenase, aspartate transaminase, ATP, Ca^{2+} and albumin after intraperitoneal injection over a 1 and 5 day period in the rat model. Liver samples were also obtained for the assessment of lipid peroxidation. It was found that only cyclo(D-Trp-L-Pro) was hepato-specific in its action, while the other isomers were not. The greatest effect on any biochemical or physiological parameter was noted after 5 days. LDH secretion was greatly increased in the presence of cyclo(L-Trp-L-Pro) and cyclo(L-Trp-D-Pro) ($p < 0.05$). Significantly increased levels of lipid peroxidation were observed in all the isomer-treated samples ($p < 0.05$), while Ca^{2+} concentrations were decreased at day 5. Decreased protein synthesis was noted in the presence of all the isomers at day 1. These results indicate the potential harm involved in the administration of the isomers, which may limit their potential usage in the treatment of various diseases.

1. Introduction

Research in our laboratories has shown the potential of the novel cyclic dipeptide cyclo(Trp-Pro) as an antimicrobial substance, as well as its potential use in the treatment of cardiovascular dysfunction [1].

The safety evaluation of novel compounds of potential medical applicability must involve a number of tests using *in vitro* and *in vivo* methods to determine their general toxicity and mutagenicity. A primary function of the liver is the metabolism of xenobiotics. In assessing the toxicological potential of new drugs or in environmental studies, the assessment of hepatotoxicity by both *in vivo* and *in vitro* methods plays a major role [2, 3].

In vitro methods used in assessing hepatotoxicity include concentration-toxicity curves, which should be assessed in primary cultured hepatocytes, non-hepatic cells and in non-metabolising hepatocytes. This would give an indication as to whether the drug is toxic preferentially on hepatocytes, or whether bioactivation of the drug is necessary to cause cellular damage [4].

In vivo injury to the liver can be evaluated through the determination of a number of parameters of the cell, including synthesis and secretion of albumin, ureogenesis, glutathione levels, ATP levels, concentration of Ca^{2+}, membrane leakage of cytosolic enzymes such as lactate dehydrogenase, protein synthesis and morphological changes [5]. In the detection of liver disease, the most commonly assayed enzymes are aspartate transaminase (AST) and alanine transaminase (ALT). In acute liver disease, higher values of ALT are found in plasma as compared to AST. A continuing rise in the plasma transaminase concentration may be detected as a result of hypersensitivity hepatocellular damage caused by drugs. Measurement of AST levels is 'however' preferred as this enzyme, is used primarily in the management of liver disease. Here, a raised level indicates hepatocellular damage [6, 7].

The energetic balance of the cells may be indirectly altered by many hepatotoxins by increasing the energy demand, reducing ATP production, or both. A common event in cellular damage is the depletion of ATP. Alternatively, hepatocyte ATP production may be altered by the xenobiotic or its metabolites. ATP is largely produced from acetyl CoA via the Krebs cycle and oxidative phosphorylation of NADPH in mitochondria. Any substance that interferes with these processes may result in decreased ATP production. All cellular anabolic processes are thus disrupted, as well as hepatocyte functions [4].

Lipid peroxidation levels may also increase due to the depletion of normal cellular protective mechanisms (eg. glutathione). Lipid peroxidation, a free radical-mediated process, results in oxidative degradation of the component lipids found in the cell membranes. Several cardiovascular, pulmonary and hepatic diseases are affected by peroxidised lipids present in animal tissues [8].

In order to determine the hepatotoxic potential of the isomers of cyclo(Trp-Pro) (cyclo(L-Trp-L-Pro), cyclo(L-Trp-D-Pro), cyclo(D-Trp-L-Pro) and cyclo(D-Trp-D-Pro)), the effects of the isomers on cell viability were assessed on primary isolated hepatocytes, as well as on two different cell lines, N-2-alpha and Chang liver cells. The metabolic and physiological functioning of the liver was assessed by using various biochemical assays in order to determine serum levels of LDH, AST, ATP, Ca^{2+}, and albumin. The amount of lipid peroxidation was assessed on liver samples.

2. Investigations, results and discussion

2.1. In vitro toxicity screen

In the past decade, the use of cellular models as a partial alternative to whole animal experiments in toxicity testing has increased. Cell cultures present a simplified, valid biological model for *in vitro* screening of the hepatotoxicity of different compounds and their respective metabolites. In the assessment of cytotoxicity of the isomers and isoniazid, three different cell types were chosen in such a manner as to determine the specificity of toxicity, i.e. if any toxicity is detected, whether it is hepatocyte-specific or not. The primary isolated hepatocytes from rats, Chang liver cells, a hepatoma cell line (transfected with a cervical carcinoma) and N-2-alpha cells (a neuronal cell line) were used. Results are depicted as % viability of the control cultures [10].

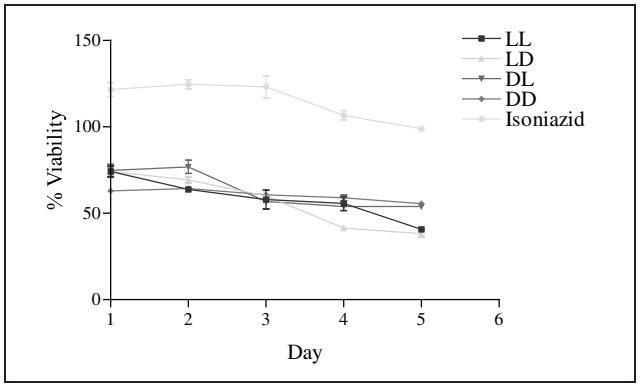

Fig. 1: Effects of the compounds on the viability of isolated rat hepatocytes. LL = cyclo(L-Trp-L-Pro); LD = cyclo(L-Trp-D-Pro); DL = cyclo(D-Trp-L-Pro); DD = cyclo(D-Trp-D-Pro). Values indicated are the mean ± s.d. of 6 experiments per day per treatment

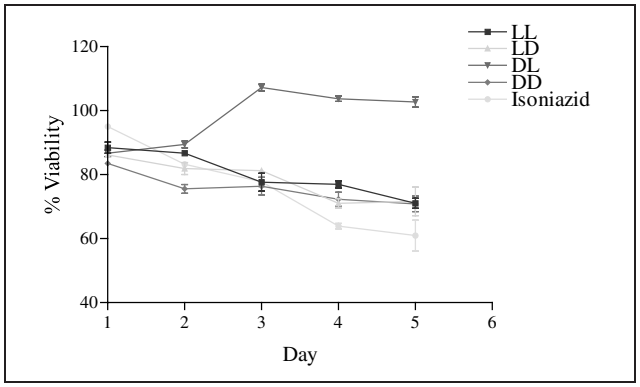

Fig. 3: Effects of the compounds on the viability of N-2-alpha cells. LL = cyclo(L-Trp-L-Pro); LD = cyclo(L-Trp-D-Pro); DL = cyclo(D-Trp-L-Pro); DD = cyclo(D-Trp-D-Pro). Values indicated are the mean ± s.d. of 6 experiments per day per treatment

In the isolated hepatocytes (Fig. 1), isoniazid was the only treatment that favoured the growth of the cells, and it thus differed significantly from the other treatments ($p < 0.05$). All isomers resulted in decreased growth in comparison to the control culture over the 5 day period and did not differ significantly from each other ($p > 0.05$). The greatest inhibition of growth was noted for cyclo(L-Trp-L-Pro) and cyclo(L-Trp-D-Pro) (60% and 62% inhibition, respectively), while cyclo(D-Trp-L-Pro) and cyclo(D-Trp-D-Pro) resulted in a decrease of only ~55%, in comparison to the control.

When the isomers were applied to the Chang liver cells (Fig. 2), isoniazid resulted in decreased growth over a 2 day period (~30% inhibition of growth). After that, growth appeared to increase slightly (from 70 to 78%). Cyclo(D-Trp-L-Pro) also resulted in an initial decrease in cell growth, but not to the same extent as with isoniazid. This was also followed by a slight increase in cell growth. Cyclo(L-Trp-L-Pro), cyclo(L-Trp-D-Pro) and cyclo(D-Trp-D-Pro) greatly inhibited cell growth over the 5 day period ($p < 0.05$) (Fig. 2), with cyclo(L-Trp-L-Pro) being the most cytotoxic isomer, resulting in a 50% inhibition of cell growth.

Cyclo(D-Trp-L-Pro) did not adversely affect the growth of N-2-alpha cells (Fig. 3), indicating that any hepatoxic effects noted in *in vivo* studies were hepato-specific. However, in the presence of cyclo(L-Trp-L-Pro), cyclo(L-Trp-D-Pro), cyclo(D-Trp-D-Pro) and isoniazid, the growth of N-2-alpha decreased over the 5 day period to approximately 75% of the control growth. This indicated that the cytotoxicities noted for these compounds were not hepatocyte-specific, and they may be capable of exerting their cytotoxic effects on a number of different cell types.

2.2. LDH concentrations

One of the most common assays used in the assessment of hepatotoxicity of a drug is LDH activity, which gives an indication of cell membrane integrity. Increased LDH activity is associated with disruption of cell membrane structure. Significantly increased ($p < 0.05$) levels of LDH were determined for all the compounds in comparison to the control after 24 h (Fig. 4). These levels increased significantly for cyclo(L-Trp-L-Pro) and cyclo(L-Trp-D-Pro) ($p < 0.05$), and decreased for cyclo(D-Trp-L-Pro) and isoniazid ($p < 0.05$), with no changes noted for the control and cyclo(D-Trp-D-Pro) groups ($p = 0.5602$ and $p = 0.6857$, respectively), when day 1 and day 5 levels were compared. At day 5, levels detected for cyclo(L-Trp-L-Pro) and cyclo(L-Trp-D-Pro) increased in comparison to the control group ($p < 0.05$) while no differences were noted for any other compound in comparison to the control group. It appears that cyclo(L-Trp-L-Pro) and cyclo(L-Trp-D-Pro) induced the greatest disruption in cell membrane integrity, which resulted in the largest leakage of LDH from the cell [5].

2.3. AST serum levels

AST serum levels are normally low (2–20 IU/l in humans). An increase in serum activity is resultant of da-

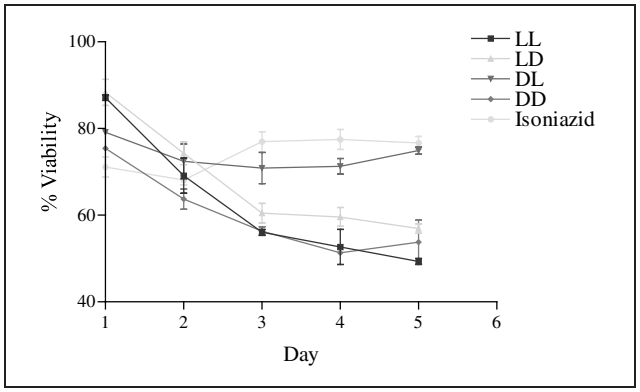

Fig. 2: Effects of the compounds on the viability of Chang liver cells. LL = cyclo(L-Trp-L-Pro); LD = cyclo(L-Trp-D-Pro); DL = cyclo(D-Trp-L-Pro); DD = cyclo(D-Trp-D-Pro). Values indicated are the mean ± s.d. of 6 experiments per day per treatment

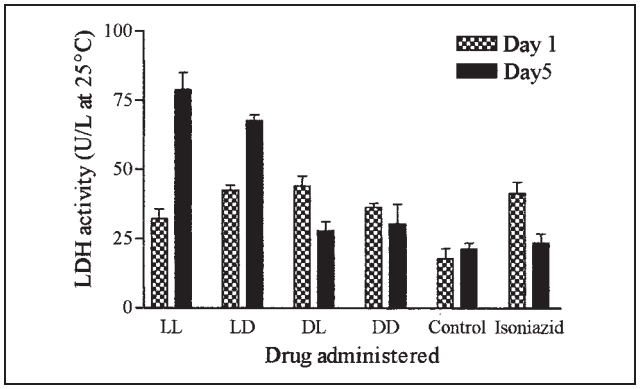

Fig. 4: Effects of the compounds on lactate dehydrogenase activity in the blood stream. LL = cyclo(L-Trp-L-Pro); LD = cyclo(L-Trp-D-Pro); DL = cyclo(D-Trp-L-Pro); DD = cyclo(D-Trp-D-Pro). Values indicated are the mean ± s.d. of 6 experiments per day per treatment

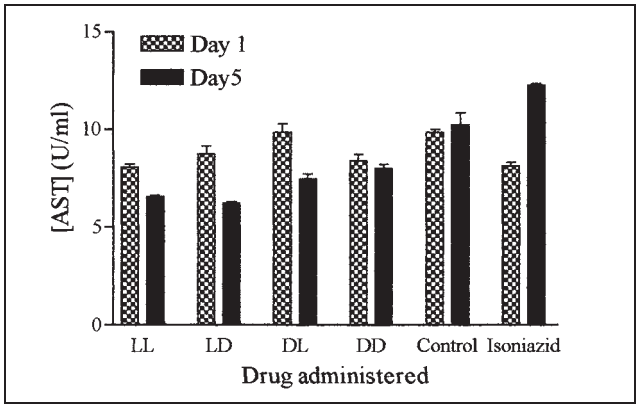

Fig. 5: Effects of the compounds on the concentration of aspartate transaminase in the blood stream. LL = cyclo(L-Trp-L-Pro); LD = cyclo(L-Trp-D-Pro); DL = cyclo(D-Trp-L-Pro); DD = cyclo(D-Trp-D-Pro). Values indicated are the mean ± s.d. of 6 experiments per day per treatment

mage to organs that involve necrosis of cells or increased cell permeability. For this reason, it was expected that AST levels in the cyclo(L-Trp-L-Pro) and cyclo(L-Trp-D-Pro) groups would be elevated (LDH leakage, Fig. 4). This was, however, not observed with cyclo(L-Trp-L-Pro) showing significantly lowered levels ($p = 0.0286$) and cyclo(L-Trp-D-Pro) showing no difference from the control group ($p = 0.1143$) (Fig. 5). On day 1, no drug produced any significantly increased AST concentration, while significantly decreased levels were noted on day 5 for all the compounds (with the exception of isoniazid) in comparison to the control group ($p < 0.05$). After 5 days, significantly elevated levels of AST were noted only in the isoniazid-treated group ($p = 0.0286$) [13].

2.4. Energy state as measured by serum ATP concentrations

In the presence of hepatotoxins, ATP levels may be decreased by an increase in energy demand, a common event in cell injury. The energy-metabolism, in terms of ATP levels in serum, was also assessed. Similar levels were noted for the control and isoniazid groups on both days 1 and 5 ($p > 0.05$). After 24 h (Fig. 6), significantly decreased levels of ATP were observed in the cyclo(L-Trp-D-Pro), cyclo(D-Trp-L-Pro) and cyclo(D-Trp-D-Pro) groups ($p < 0.05$), while no difference was noted for cyclo(L-Trp-

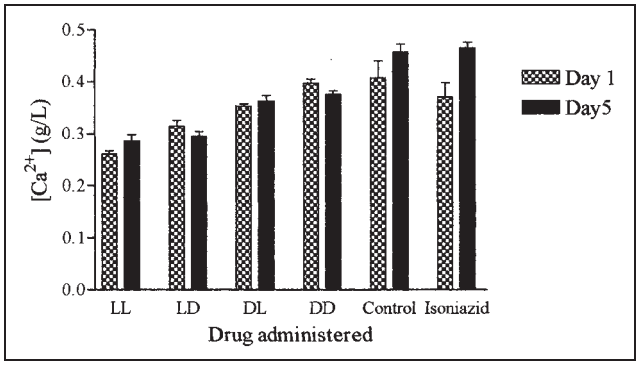

Fig. 7: Effects of the compounds on Ca^{2+}-levels in the blood stream. LL = cyclo(L-Trp-L-Pro); LD = cyclo(L-Trp-D-Pro); DL = cyclo(D-Trp-L-Pro); DD = cyclo(D-Trp-D-Pro). Values indicated are the mean ± s.d. of 6 experiments per day per treatment

L-Pro) ($p = 0.6857$) in relation to the control group. This decrease in ATP would thus indicate a greater energy demand in those cells treated with cyclo(L-Trp-D-Pro), cyclo(D-Trp-L-Pro) and cyclo(D-Trp-D-Pro), or mitochondrial dysfunction. This was however ruled out as, at day 5 (Fig. 6), significantly increased levels were observed for all the isomers ($p < 0.05$) when compared to the respective day 1 levels. At day 5, the highest level was noted for cyclo(D-Trp-L-Pro) ($p < 0.01$), followed by cyclo(D-Trp-D-Pro), cyclo(L-Trp-L-Pro) and cyclo(L-Trp-D-Pro). This increase may be as a result of increased rates of glycolysis. However, no significant difference was noted between the day 5 cyclo(L-Trp-L-Pro) and cyclo(L-Trp-D-Pro) levels ($p = 0.2469$) [4, 15].

2.5. Serum Ca^{2+} concentrations

Ca^{2+} accumulates in dying cells and is thus involved in toxicological processes. Significantly decreased levels of Ca^{2+} were obtained in the cyclo(L-Trp-L-Pro) and cyclo-(L-Trp-D-Pro) samples ($p < 0.05$) (Fig. 7), whereas cyclo-(D-Trp-L-Pro), cyclo(D-Trp-D-Pro) and isoniazid did not show any significantly different levels ($p > 0.05$) in comparison to the control group. The only group to show any significant difference in Ca^{2+} levels from day 1 to day 5 was the isoniazid-treated group ($p = 0.0286$). The isomer-treated groups showed significantly diminished Ca^{2+} levels in comparison to the control on day 5 ($p < 0.05$) with no significant difference between the control and isoniazid-trea-

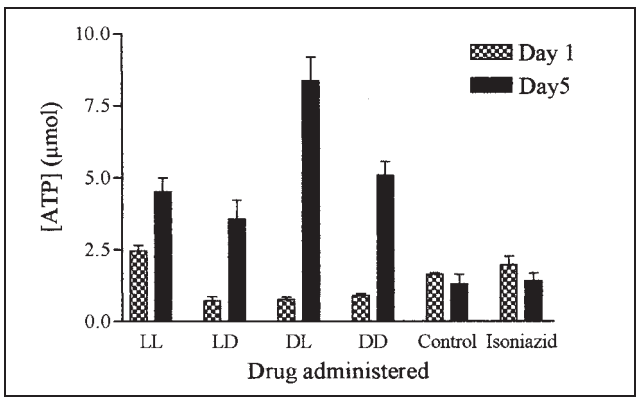

Fig. 6: Effects of the compounds on energy metabolism in rat hepatocytes, measured in terms of ATP concentrations in the blood stream. LL = cyclo(L-Trp-L-Pro); LD = cyclo(L-Trp-D-Pro); DL = cyclo(D-Trp-L-Pro); DD = cyclo(D-Trp-D-Pro). Values indicated are the mean ± s.d. of 6 experiments per day per treatment

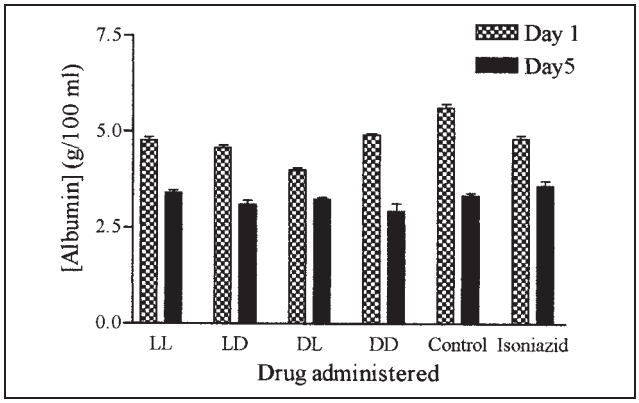

Fig. 8: Effects of the compounds on protein synthesis, as measured by albumin concentrations in the blood stream. LL = cyclo(L-Trp-L-Pro); LD = cyclo(L-Trp-D-Pro); DL = cyclo(D-Trp-L-Pro); DD = cyclo(D-Trp-D-Pro). Values indicated are the mean ± s.d. of 6 experiments per day per treatment

ted groups ($p = 0.6857$). These changes in Ca^{2+} homeostasis would adversely affect signal transduction of the cell, as Ca^{2+} plays a vital role as a second messenger [16].

2.6. Protein synthesis

Free albumin concentration may decrease due to binding to drugs or as a result of decreased protein synthesis. As a result, free drug concentration may increase which may increase the risk of toxicity. In addition, Ca^{2+} homeostasis may be disrupted by changes in protein synthesis. Decreased protein synthesis is one of the earliest and most sensitive signs of cellular hepatocyte damage. After 24 h exposure (Fig. 8) to the compounds and isoniazid, the levels of albumin in the serum were significantly decreased in comparison to the control value ($p < 0.05$), indicating decreased protein synthesis and hepatocyte damage. Cyclo(D-Trp-L-Pro) produced the largest decrease in albumin synthesis ($p = 0.0286$). However, after 5 days (Fig. 8), all the levels of albumin had decreased, including that of the control group, with only cyclo(D-Trp-L-Pro) and cyclo(D-Trp-D-Pro) differing significantly from the control sample ($p = 0.0286$ for both compounds). From the results, it is clear that the compounds resulted in decreased protein synthesis, with maximal effects noted over a longer period [4, 7, 16].

2.7. Lipid peroxidation

Oxidative degradation of lipid components of the cell membrane is caused by lipid peroxidation. This process would obviously disrupt the integrity of the cell membrane. The thiobarbituric acid (TBA) reaction of liver homogenates in rats showed significant elevations of malondialdehyde concentrations after a single intraperitoneal injection of cyclo(L-Trp-L-Pro), cyclo(D-Trp-L-Pro) and cyclo(D-Trp-D-Pro), indicating the occurrence of lipid peroxidation ($p < 0.05$) (Fig. 9). No significant elevation in malondialdehyde levels was observed for cyclo(L-Trp-D-Pro) ($p = 0.667$) and isoniazid ($p = 0.333$). However, after 5 days, malondialdehyde levels increased dramatically for the cyclo(L-Trp-D-Pro) and isoniazid-treated groups ($p < 0.05$). This significant increase was also noted for the other compounds in relation to the control group ($p < 0.05$), indicating lipid peroxidation [14].

Only cyclo(D-Trp-L-Pro) is hepatocyte-specific in its cytotoxicity while cyclo(L-Trp-L-Pro), cyclo(L-Trp-D-Pro) and cyclo(D-Trp-D-Pro) are cytotoxic for other cell types, too. As can be seen from the results, it is clear that isoniazid exerts its cytotoxic effect by producing lipid peroxidation. Various effects on biochemical and physiological parameters are apparent in the presence of the isomers. Cyclo(L-Trp-L-Pro) and cyclo(L-Trp-D-Pro) are able to disrupt cell membrane integrity, as can be seen from the LDH results. However, no great elevation in AST concentrations was observed for these isomers. After a short exposure period, cyclo(L-Trp-D-Pro), cyclo(D-Trp-L-Pro) and cyclo(D-Trp-D-Pro) resulted in decreased ATP concentrations, which increased to day 5. This indicates an increase in ATP production, which may result from an increased rate of glycolysis. Decreases in Ca^{2+} concentration occurred in the presence of cyclo(L-Trp-L-Pro) and cyclo(L-Trp-D-Pro) after 1 day, which indicates a disruption in Ca^{2+} homeostasis, which can lead to various forms of interference with signal transduction. Furthermore, depletion of the cell's protective mechanism is indicated by the elevated lipid peroxidation levels in the presence of all the isomers. This suggests that the isomers disrupt glutathione levels, leading to cells with an increased vulnerability to damage by the foreign compounds. This may limit the use of these isomers in the treatment of various diseases.

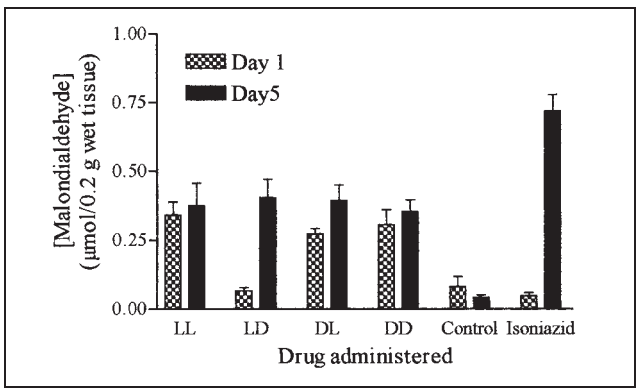

Fig. 9: Effects of the compounds on lipid peroxidation, as measured in terms of malondialdehyde concentrations in the serum of the rats. LL = cyclo(L-Trp-L-Pro); LD = cyclo(L-Trp-D-Pro); DL = cyclo(D-Trp-L-Pro); DD = cyclo(D-Trp-D-Pro). Values indicated are the mean ± s.d. of 6 experiments per day per treatment

3. Experimental

The use of rats for this study was approved by the Animal Ethics Committee, University of Port Elizabeth.

3.1. Isolation of primary hepatocytes

A modified collagenase method was used as previously described [9].

3.1.1. Routine cell culture

Hepatocytes were seeded in 96-well cell culture plates (Corningware, Cambridge, U.S.A.) at 75 000 cells per well and incubated at 37 °C in an atmosphere of 95% O_2 and 5% CO_2 (Fedgas, South Africa). Prior to seeding, the wells were coated with newborn calf serum (BioWhittaker, Walkersville) to facilitate attachment of the cells to the plate. For the first 24 hr of incubation, serum-supplemented Hams F-12 medium was used. This medium was replaced after 24 hr, with Hams F-12 medium supplemented with 10^{-8} M dexamethasone (Sigma, St. Louis). After the 24-h incubation period, the cells were exposed to 200 µM of each isomer (pH 7.4), respectively. The control solution consisted of Hams F-12 medium containing 0.5% glycerol. As a positive control, the cells were exposed to 1 mg/ml isoniazid dissolved in Hams F-12 medium containing 0.5% glycerol. Isoniazid has also been associated with hepatotoxicity over long periods of administration. Liver functions become impaired, jaundice may result and multilobular necrosis may occur. The cells were incubated in the presence of the compounds for a total of 5 days; on each day the MTT assay was used to determine viability of the cells of the treated cells, as well as of the controls [10, 11].

3.1.2. Cell culture of N-2-alpha and Chang liver cells

In order to determine whether any effects exerted on the isolated hepatocytes were hepatocyte-specific or not, Chang Liver cells and N-2-alpha cells were also exposed to 200 µM of each compound, made up in DMEM (0.5% glycerol). These cells were treated in the same manner as were the isolated hepatocytes, with the exception that these cells were seeded at a density of 25 000 cells per well, as these cell lines undergo proliferation in culture. No coating of the plates was necessary as these cells readily adhere to the wells.

3.1.3. MTT assay

On the assay day, 50 µl 0.5% MTT (Sigma, St. Louis) solution was added to each well, and incubated at 37 °C for 2 h. After the incubation period, the solution was aspirated from the cells, and 200 µl DMSO was added to each well. A 5 min reaction period was allowed with mild agitation. The resultant extracted formazan products were removed from the plate and put into a clean 96-well plate. The absorbance at 600 nm was read against a DMSO blank using Labsystems Multiskan MS (Multiskan Transmit Program, Rev. 1.3. (1995)). Results are reported as a percentage of those of the control group, which was taken as 100% viability.

3.2. Treatment of rats and sample collection

Male Long Evans rats (250–350 g) were housed in a well ventilated room at 24 °C, with 12 h light. Fasting and starvation may adversely affect biotransformation of oxidising agents as well as the antioxidant state of the liver, and therefore, animals were fed ad lib. and not fasted prior to experimentation.The rats were injected intraperitonealy with a 7.1 mg/50 ml saline solution of the respective compounds, representing a final concentration of 500 μM. 0.5% glycerol was used to facilitate dissolution of the isomers in saline. All solutions were filter sterilized with a 0.45 μm filter unit (Millipore) before use. The rats were separated into 2 sets of 6 groups of rats. Rats in set 1 were injected only once and killed after 24 h, while the rats in set 2 were subjected to injections every alternate day for a period of 5 days before being killed. Group 1 of each set served as the control group, receiving normal saline injections containing 0.05% glycerol. Groups 2–5 received injections of the 500 μM solution of the respective cyclic dipeptide in saline containing 0.05% glycerol. Group 6, the positive control group, received saline injections containing isoniazid at a concentration of 1 mg/ml [12].
Rats were subjected to light ether anesthesia to a loss of blink and pain reflexes. The entire liver was removed and rinsed in 0.15 M KCl solution. The livers were placed in 0.15 M KCl solution and frozen at −20 °C until lipid peroxidation was assessed. Blood was collected by cardiac puncture into empty Vacutainer tubes and held at 4 °C to allow for clot formation. Serum was obtained by centrifugation at 3000 rpm for 15 min. The serum was retained for the assessment of hepatotoxicity by various enzymatic and biochemical assays.

3.3. Enzymatic and biochemical assays

3.3.1. Lactate dehydrogenase

The lactate dehydrogenase kit (Sigma, St. Louis, U.S.A.) was used according to manufacturer's specifications. Briefly, 10 μl of the serum sample was placed into a 96-well plate to which 200 μl LDH reagent was added. An intial absorbance at 340 nm was read using a Labsystems Multiskan MS (Multiskan Transmit Program, Rev. 1.3. (1995)), after which the reaction was followed at 340 nm for 1 min. The change in absorbance/min was determined. Results of the control group of rats are included.

3.3.2. Aspartate transaminase

A modified version of the aspartate transaminase assay as described previously was used to determine the levels of AST in serum [13].
To each set of tubes, 250 μl AST substrate (37 °C) was added. To the rat serum tubes, 50 μl serum was added, whilst to the blank tube, 50 μl H_2O was added. The tubes were mixed and incubated at 37 °C for 1 hr. 250 μl 1 mM 2,4-dinitrophenylhydrazine was added and mixed and the solution was incubated at RT for 20 min. 2.5 ml 0.4 N NaOH was then added and allowed to incubate at RT for 5 min. The solution was diluted 2X with 0.4 N NaOH and the absorbance was read at 505 nm using a Shimadzu UV-160A UV-Visible recording spectrophotometer, against a blank consisting of reaction mixture.

3.3.3. Lipid peroxidation

The method to determine lipid peroxidation was used as described previously [14].

3.3.4. ATP

An adenosine-5′-triphosphate kit from Sigma Diagnostics was used according to manufacturer's specifications.

3.3.5. Ca^{2+} concentrations

Ca^{2+} content in the serum samples was determined by using a serum Ca^{2+} kit from Sigma Diagnostics according to manufacturer's specifications.

3.3.6. Albumin levels

A modification of the albumin assay as described previously was used. To a set of test tubes, 5 ml working reagent solution was added, to which 25 μl sample/albumin standard was added, mixed and left to incubate at RT for 10 min. The absorbance was read at 630 nm using a Shimadzu UV-160A UV-Visible recording spectrophotometer against a blank consisting of working reagent. A 10 g/100 ml albumin standard was used. Albumin content in serum samples was calculated using the following formula:

$$\text{Abs sample/Abs std} \times \text{conc. std} = \text{conc. sample g/100 ml [13].}$$

3.3.7. Statistical analysis

Results are expressed as mean ± s.d. for the indicated number of experiments. Results were analysed using the software packages GraphPad Prism Version 2.0 and GraphPad InStat (GraphPad Software, Inc., San Diego, U.S.A.). All tests were performed on raw data obtained from the experiments ($n = 6$). The effect of a single qualitative factor on a single response variable was determined by univariate ANOVA using the Mann-Whitney test. P values < 0.05 were accepted as evidence of a statistically significant difference.

References

1 Milne, P. J.; Hunt, A. L.; Rostoll, K.; van der Walt, J. J.; Graz, C. J. M.: J. Pharm. Pharmacol. **50**, 1331 (1998)
2 Castell, J. V. M.; Montoya, A.; Larrauri, A.; Lopez, P.; Gomez-L. J.: Xenobiotica **15**, 743 (1985)
3 Bhattacharya, R.; Lakshmana Rao, P. V.; Bhaskar, A. S. B.; Pant, S. C.; Dube, S. N.: Human and Experimental Toxicology **15**, 105 (1996)
4 Castell, J. V.; Gomez-Lechon, M.; Ponsoda, X.; Bort, R.: in: In Vitro methods in pharmaceutical research. p. 375, Academic Press, San Diego 1997
5 Vickers, A. E.: in: In Vitro methods in pharmaceutical research. p.103, Academic Press, San Diego 1997
6 Baron, D. N.: A short textbook of Chemical Pathology, 4th Ed, p. 198, Hodder and Stoughton, London 1982
7 Marshall, W.: Clinical Chemistry, 3rd Ed., p. 231; Mosby, Great Britain 1997
8 Uchiyama, M.; Mihara, M.: Anal Biochem. **86**, 271 (1978)
9 Seglen, P. O.: in: Cell Biology: A laboratory handbook, p. 96, Academic Press, London 1994
10 Jover, R.; Ponsoda, X.; Castell, J. V.; Gomez-Lechon, M. J.: Toxic. in vitro **6**, 47 (1992)
11 Jawetz, E.:, in: Katzung, B. G. (Ed.): Basic and Clinical Pharmacology, 4th Ed, p. 579., Prentice-Hall International, U.S.A. 1989
12 Tanaka, K.; Sato, M.; Tomita, Y.; Ichichara, A.: J. Biochem. **84**, 937 (1978)
13 Bauer, J. D.; Ackermann, P. G.; Toro, G.: Clinical Laboratory methods, 8th Ed, p. 393–4, CV Mosby Co., St. Louis, U.S.A. 1974
14 Souza, M. F.; Tome, A. R.; Rao, V. S. N.: J. Pharm. Pharmacol. **51**, 125 (1999)
15 Ross, D.: J. Pharm. Pharmacol. **41**, 505 (1989)
16 Orrenius, S.; McConkey, D.; Bellomo, G.; Nicotera, P.: Trends Pharmacol. Sci. **10**, 281 (1989)

Received March 8, 2002
Accepted April 13, 2002

Prof. Dr. Pieter J. Milne
Department of Pharmacy
PO Box 1600
Port Elizabeth 6000
Republic of South Africa
pmapjm@upe.ac.za