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Original research paper

Peptide chirality and opioid receptor modulation: Hepatoprotective effect of D-Met-enkephalin in acetaminophen-induced liver injury

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ABSTRACT

L-Met-enkephalin is a neuropeptide known to exert protective effects in various experimental models of autoimmune and inflammatory diseases. These effects are mediated through opioid receptors and can be abolished by the opioid receptor antagonist naltrexone. Investigation of peptide enantiomerism and the incorporation of D-amino acids are crucial for designing novel peptides with altered structural and biological properties compared with their native L-forms. Since no data are currently available on the properties or biological activity of the D-Met-enkephalin enantiomer, we evaluated its hepatoprotective potential in a mouse model of acetaminophen-induced hepatotoxicity. Male CBA mice were treated with D-Met-enkephalin, and hepatoprotection was assessed by measuring plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, along with histological liver necrosis scores. The peptide's secondary structure and antisense peptide binding were analysed using circular dichroism and fluorescence spectroscopy, respectively.

D-Met-enkephalin demonstrated dose-dependent hepatoprotective effects within the range of 0.5–20 mg kg^{-1}, with maximal protection observed at 5 mg kg^{-1}, a dose comparable to that of the L-enantiomer (7.5 mg kg^{-1}). This preservation of biological activity may be attributed to the presence of the achiral amino acid glycine at positions 2 and 3, which maintains the functional conformation of the D-enantiomer. The role of opioid receptor involvement was further examined through direct receptor blockade using naltrexone and indirect inhibition with the antisense peptide IPPKY.

Keywords: D-Met-enkephalin, hepatoprotection, opioid receptor, antisense peptide, fluorescence spectroscopy, circular dichroism

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INTRODUCTION

A fundamental characteristic of mammalian proteins and peptides is that they are composed predominantly of L-amino acids, with incorporation of D-amino acids occurring only rarely under physiological conditions (1–5). Recent advances in analytical chemistry and peptide-synthesis technologies have enabled systematic investigation and comparison of the properties of enantiomeric peptides, those containing the mirror-image configurations of amino acids (1, 5–9). For instance, the use of D-amino acid substitution offers an attractive strategy for modulating peptide and protein biological function, enhancing proteolytic stability, altering receptor affinity or signalling bias, and potentially improving therapeutic performance. Research in this area includes examples such as D-amino acid-containing polymer-peptide conjugates, which show markedly reduced immunogenicity compared to their L-counterparts (2–13).

Met-enkephalin is an ancient and evolutionarily conserved endogenous opioid pentapeptide with the amino acid sequence Tyr-Gly-Gly-Phe-Met (YGGFM) (14–17).

Its natural L-enantiomer (Fig. 1a) exhibits tissue and organ protective effects in different animal disease models, *e.g.*, experimental allergic encephalomyelitis, histamine-induced bronchoconstriction, Arthus skin reaction, delayed skin reaction, adjuvant arthritis, cancer, allograft rejection, anaphylactic shock, and acetaminophen (APAP) induced hepatotoxicity (14–26). Mostly, the protective effects of L-Met-enkephalin are mediated *via* δ and ζ opioid receptors, and could be abolished with naltrexone, a competitive antagonist of the opioid receptors (15–17, 20, 21). The protective effects of L-Met-enkephalin could also be blocked with the antisense peptide IPPKY, which prevents its interaction with the opioid receptors (20, 25, 28).

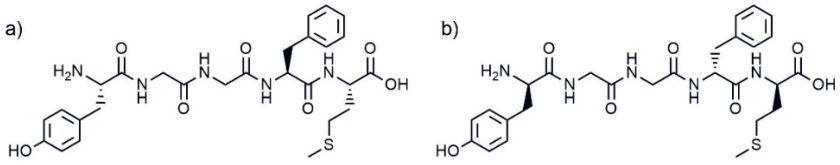

Fig. 1. Structure of: a) L-Met-enkephalin; b) D-Met-enkephalin.

In contrast, the D-enantiomer structure of Met-enkephalin is characterized by D-amino acid substitution (chiral inversion) at the positions 1, 4 and 5 of the L-amino acid sequence (Fig. 1b). It is important to observe that amino acid glycine (G) at positions 2 and 3 is not a chiral amino acid, so this part of the molecule, including the glycine-glycine bond, is identical for both enantiomers (Fig. 1).

In this study we therefore investigated three key questions: (*i*) whether D-Met-enkephalin exhibits hepatoprotective activity in a model of acetaminophen-induced liver injury, (*ii*) whether its protective effect is modulated by naltrexone (to probe opioid receptor involvement) or by the antisense peptide IPPKY (to assess receptor-interaction specificity), and (*iii*) how its effect compares to the previously reported hepatoprotection of the L-enantiomer (20).

Met-enkephalin hepatoprotection has been examined previously only for the natural L-enantiomer in the same experimental model, where a 7.5 mg kg^{-1} dose produced the maximal effect (20). However, no biological or biophysical data exist for the D-enantiomer

of Met-enkephalin, and the consequences of chiral inversion at positions 1, 4, and 5 on receptor-mediated hepatoprotection have not been addressed. The present work, therefore, represents a stereochemical extension of earlier studies: it examines whether D-Met-enkephalin retains hepatoprotective activity, whether receptor-mediated mechanisms are preserved, and whether its interaction with the cognate antisense peptide differs from that of the L-form. Answers to these questions are necessary to define the structure-function relationship between peptide chirality and opioid receptor-dependent cytoprotection.

EXPERIMENTAL

Treatment regimen and experimental model

The effects of D-Met-enkephalin were evaluated using the APAP-induced hepatotoxicity in male CBA mice. This murine model is useful for testing substances with potential hepatoprotective and anti-inflammatory effects (29, 30). The APAP reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI) is responsible for damage to the centrilobular regions of the liver after overdose (30, 31). NAPQI reacts with sulfhydryl (thiol) groups of important intracellular proteins – particularly mitochondrial proteins involved in energy production, antioxidant defence-related proteins containing critical cysteine residues, and structural proteins that support cellular integrity-leading to mitochondrial dysfunction and cell death with the release of intracellular content (29). Damage-associated molecular patterns (DAMPs), *e.g.*, nuclear protein HMGB1, nuclear DNA fragments, mitochondrial DNA, uric acid, ATP, and others, cause the transcriptional activation of pro-inflammatory cytokines in macrophages *via* DAMP receptors (29). Released proinflammatory mediators activate and recruit neutrophils and monocytes to the liver. Consequently, APAP overdose is accompanied by strong sterile inflammation of the liver (29). For clarity, all subsequent references to D-Met-enkephalin effects refer to our own experimental results.

Hepatotoxicity was induced following the procedure described by Guarner *et al*. with slight modifications (30–32). The experimental animals in the hepatotoxicity model were male CBA mice, 12–16 weeks old, weighing 20–25 g. The animals were bred at the Ruđer Bošković Institute and were kept under standard laboratory conditions (dark-light cycle (12/12 h), constant temperature (22 ± 1 °C) and humidity 55 %) with free access to water and standard food pellets (4 RF 21 GLP Mucedola srl, Italy). The experimental animals (n = 48) were randomly divided into five experimental groups (n = 8 animals per group), and a control group where animals were treated with saline (0.9 % NaCl).

For seven days, mice were given 300 mg L^{-1} phenobarbital (Phenobarbiton, PLIVA, Croatia) in drinking water. Prior to inducing liver damage by APAP, the animals were fasted overnight with free access to water. Acetaminophen (> 99 % purity; Sigma-Aldrich, USA) was given intragastrically (*i.g.*), in a dose of 150 mg kg^{-1} of body mass, dissolved in 0.9 % NaCl (37 °C) *via* a gastric tube, in a volume of 0.5 mL. Mice were re-fed after 4 h. Control animals were treated with physiological saline (0.9 % NaCl, Croatian Institute for Transfusion Medicine, Zagreb, Croatia).

The following test substances were given intraperitoneally (*i.p.*) 1 h before APAP administration, in a volume of 0.2 mL:

1. D-Met-enkephalin (M_r = 573.66, > 99 % purity; GenScript, USA);
2. Naltrexone hydrochloride (M_r = 377.86, > 99 % purity; Sigma-Aldrich;

3. Antisense peptides (IPPKY, M_r = 616.75, 98.5 % purity, and IPPKYW, M_r = 802.96, > 99 % purity, GenScript, USA);
4. Administration of naltrexone and D-Met-enkephalin (naltrexone was given 30 min prior to D-Met-enkephalin);
5. Mixture of D-Met-enkephalin and antisense peptide IPPKY (mixed together 30 minutes prior to the administration).

Twenty-four hours after APAP administration, the experimental animals were sacrificed. Fifteen minutes before sacrifice, 250 IU heparin was given *i.p.* to each animal, and trunk blood was collected into heparinised tubes. Plasma was separated by centrifugation for 5 minutes at 8000 g and was stored at –20 °C for 24 h before transaminase activity determination. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity were determined from plasma on an Olympus AU® 400 analyser using standard reagents.

Sections of the liver were fixed in 10 % phosphate-buffered formalin and embedded in paraffin for histopathological analysis. Three specimens of the liver tissue were analysed. Each specimen was cut into six sections of 3 μm and stained with hemalum-eosin. Liver lesions were graded using light microscope (×100) on 0–5 scale: 0 = no lesions; 1 = minimal lesions (individual necrotic cells); 2 = mild lesions (10 to 25 % of necrotic cells or mild diffuse degenerative changes); 3 = moderate lesions (25 to 40 % of necrotic cells); 4 = marked lesions (40 to 50 % of necrotic cells); and 5 = severe lesions (> 50 % of necrotic cells) (33). The final score for each liver was the consensus score of all examined sections.

All the experiments were performed according to the ethical guidelines of the International Council for Laboratory Animal Science (ICLAS), Council Directive 2010/63/EU, and Croatian Animal Protection Act (Official Gazette 135/06). All animal experimentation protocols were approved by the Ruđer Bošković Institute and by the Croatian Ministry of Agriculture (ethics approval: No. 525-10/0255-13-2).

Circular dichroism spectroscopy

The circular dichroism (CD) spectra of L- and D-Met-enkephalin enantiomers were acquired at room temperature with a Jasco J-815 CD spectropolarimeter (Jasco Inc., USA), in a 0.2 mm optical path quartz precision cell. The peptides were dissolved in 10 mmol L^{-1} phosphate buffer at pH = 7.4 and 25 °C. Concentration of the peptides was 2 mg mL^{-1}. Circular dichroism spectra show a random coil structure of both Met-enkephalin peptides, and a typical mirror image CD spectra of L- and D-enantiomers due to L/D isomerisation (Fig. 2).

Peptide binding assay using tryptophan fluorescence spectroscopy

The binding of D-Met-enkephalin and its antisense peptide ligand, modified by the C-terminal tryptophan fluorophore (IPPKYW), was measured by OLIS RSM 1000F spectrofluorometer (Olis, Inc., USA) equipped with thermostated cell at 25 °C (34, 35). Both reactants (D-Met-enkephalin and IPPKYW) were fluorophores, and the third spectrally active species was attributed to the complex of two reactants. D-phenylalanine, present in D-Met-enkephalin, which was in excess, had a much smaller quantum yield than the tryptophan present in IPPKYW (21). The excitation wavelength at 290 nm was chosen to diminish the fluorescence of D-phenylalanine and maximise the fluorescence of antisense tryptophan (Fig. 3) (20).

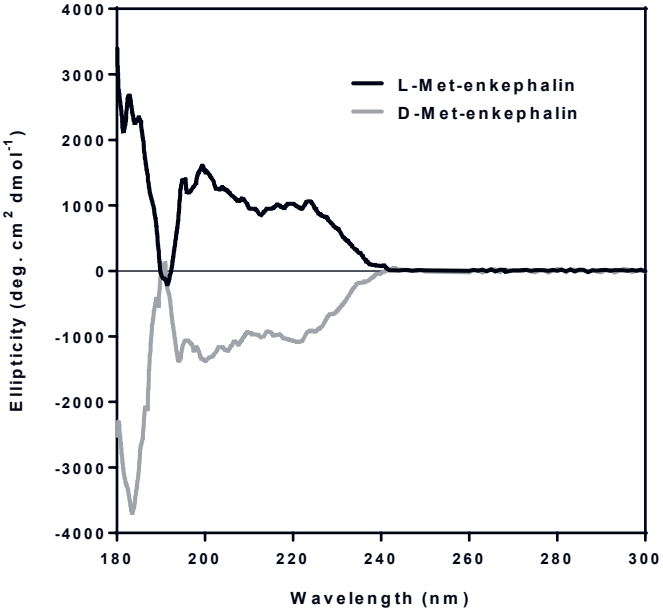

Fig. 2. Circular dichroism spectroscopy analysis of L- and D-Met-enkephalin structures.

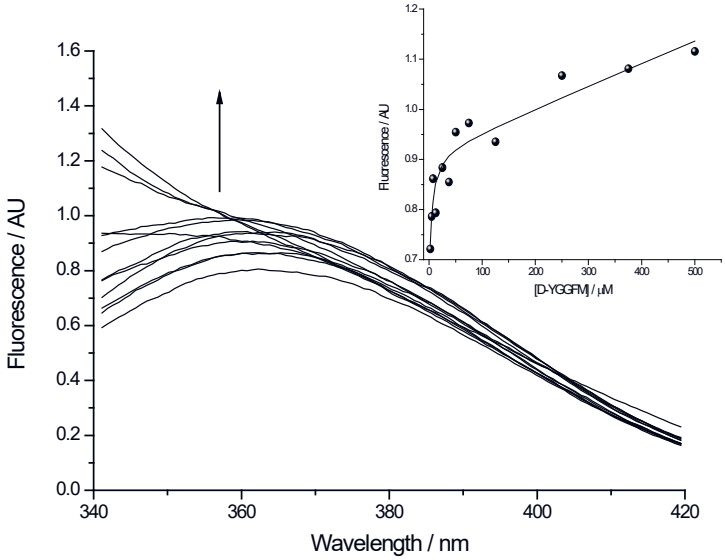

Fig. 3. Titration of 2.5 μmol L^{-1} solution of IPPKYW with D-Met-enkephalin at 25 °C and pH = 7.4 in 10 mmol L^{-1} phosphate buffer. The concentration of D-Met-enkephalin varied from 2.5 to 500 μmol L^{-1}. Fluorescence in arbitrary units (AU) is given as a ratio of signals obtained from sample and reference PMTs. Inset: Fitting curve at 350 nm.

SPECFIT software was used to analyse all spectra in fluorescence titrations (Fig. 3). Three spectrally active species were suggested by single value decomposition (SVD) statistical analysis (20, 35–37). Data analysis suggested 1 to 1 complex formation and did not indicate the presence of higher-order complexes. Dissociation constant of the complex (K_d = 3.7 ± 0.9 μmol L^{-1}) was calculated according to the model given by Equation *1* and Equation *2*:

$$\text{IPPKYW-D-YGGFM} \leftrightarrow \text{IPPKYW} + \text{D-YGGFM} \quad (1)$$

$$K_d = \frac{|\text{IPPKYW}||\text{D-YGGFM}|}{|\text{IPPKYW-D-YGGFM}|} \quad (2)$$

Statistical analysis

GraphPad Prism for Windows (version 10) and KyPlot (version 6) were used for data plotting and statistical analysis. Descriptive statistics for AST and ALT activities were based on means, medians, and standard deviations. Minimum, first quartile (Q1), median, third quartile (Q3) and maximum were used to describe liver necrosis scores. Data shown in plots represent medians and interquartile ranges. Differences between the groups were analysed using Steel's test, a non-parametric multiple comparison test that compares treatment groups with control. All applied tests were two-tailed, and the critical *p*-value for accepting or rejecting the null hypothesis was 0.05.

RESULTS AND DISCUSSION

Hepatoprotective effects of D-Met-enkephalin

The hepatoprotective effects of D-Met-enkephalin were assessed using two standard criteria: (*i*) plasma activities of the liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and (*ii*) the histopathological necrosis score (scale 0–5). D-Met-enkephalin produced dose-dependent hepatoprotective effects in the acetaminophen-induced liver injury model, as reflected by plasma AST and ALT activities and histopathological necrosis score (Figs. 4–6). Control animals treated with 0.9 % NaCl showed markedly elevated AST (mean 7500 U L^{-1}) and ALT (mean 3606 U L^{-1}) levels and high necrosis scores (median 3.5), indicating severe liver injury. Treatment with D-Met-enkephalin at 0.5, 5, and 20 mg kg^{-1} significantly reduced AST (means 486.3, 285.0, and 430.7 U L^{-1}; p = 0.0065), ALT (means 458.4, 158.3, and 196.6 U L^{-1}; p = 0.0065), and necrosis scores (medians 2.0, 1.5, and 2.0; p = 0.0151, 0.0115, and 0.0265, respectively), with maximal protection observed at 5 mg kg^{-1}. Although this optimal dose is lower than the 7.5 mg kg^{-1} reported for the natural L-enantiomer (mean ALT = 794 U L^{-1}, p = 0.0072; AST = 1313 U L^{-1}, p = 0.0039 and median necrosis score = 2.5, p = 0.0094) (20), both values fall within a similar effective range. The difference may reflect enantiomer-specific pharmacodynamic properties as well as methodological differences among studies. These doses are also consistent with the optimal protective range (4–10 mg kg^{-1}) described in other models of inflammatory and autoimmune disease in mice, rats, and guinea pigs (21, 23–26). The highest dose of D-Met-enkephalin (50 mg kg^{-1}), did not confer hepatoprotection, as AST (mean 11,884 U L^{-1}; p = 0.262), ALT (mean 5406 U L^{-1}; p = 0.678), and necrosis scores (median 4.0; p = 0.446) were comparable to control values (Fig. 4–6).

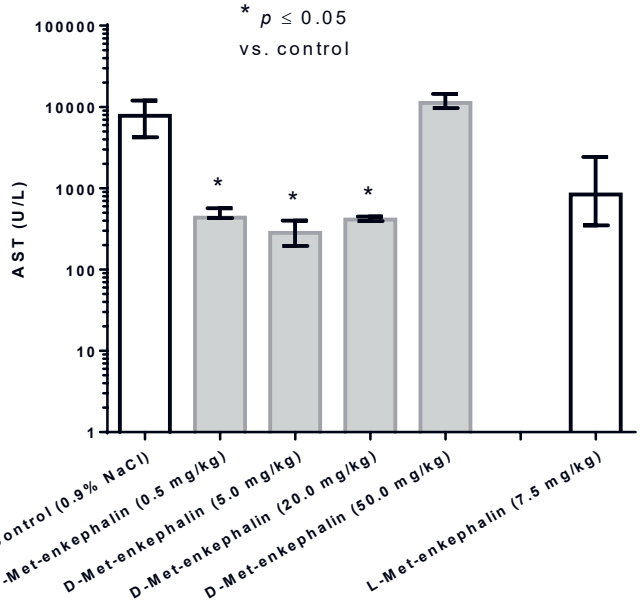

Fig. 4. Dose-dependent effects of D-Met-enkephalin on plasma AST activity 24 h after acetaminophen administration. Data are shown as medians and interquartile ranges.

Similarly to the results for L-enantiomer (20), the effects of D-Met-enkephalin in the model of acetaminophen-induced liver toxicity were also characterised by a U-shaped curve relationship between the applied doses and observed protective effects. This was valid for all three parameters relevant for this experimental model – AST, ALT, and the liver necrosis score (Fig. 4–6). The U-shaped curve is often observed with peptide ligands, including opioid system ligands characterised by low-dose stimulation and high-dose inhibition (20, 41).

Interestingly, D-Met-enkephalin doses in the range of 0.5–5 mg kg^{-1} have somewhat better protective potency than the similar range of L-enantiomer doses (20). The most probable explanation is that the substitution of L-amino acid with D-amino acid at positions 1, 4, and 5 influences the enzymatic breakdown of the molecule, prolonging its life and consequently enhancing its effects (3, 42). However, additional studies of the D-Met-enkephalin degradation with specific enzymes, *e.g.*, aminopeptidase N, enkephalinase A, enkephalinase B and carboxypeptidase A6 are needed (Fig. 7).

To date, no published data have described the biological effects of the D-Met-enkephalin enantiomer. In this study, we demonstrated for the first time that D-Met-enkephalin exhibits hepatoprotective activity in a model of acetaminophen (APAP)-induced liver injury, a well-established system for screening compounds with hepatoprotective and anti-inflammatory properties (7, 20, 31, 33, 38). This model is particularly suitable for evaluating pharmacologically active agents with anti-inflammatory and anti-necrotic potential (28, 39–40).

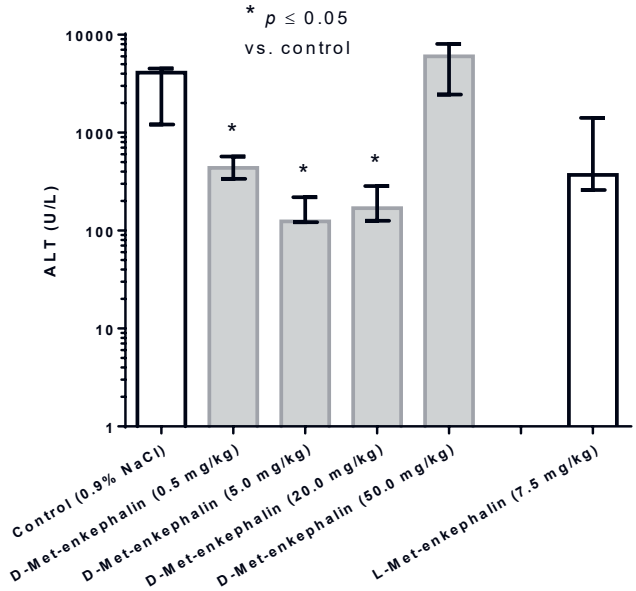

Fig. 5. Dose-dependent effects of D-Met-enkephalin on plasma ALT activity 24 h after acetaminophen administration. Data are shown as medians and interquartile ranges.

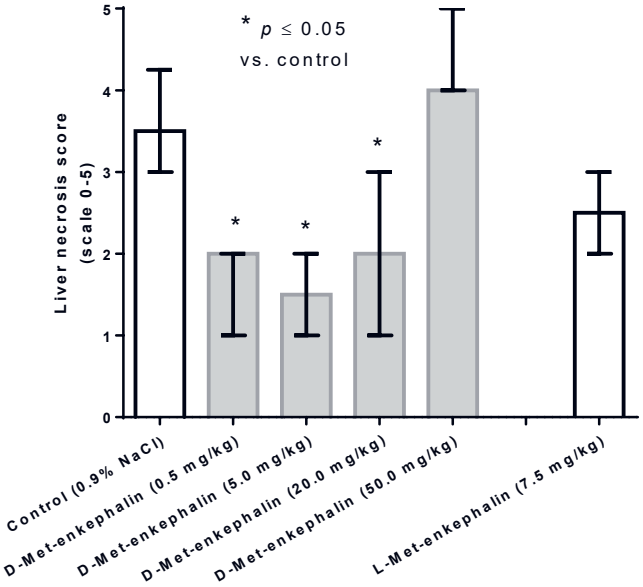

Fig. 6. Dose-dependent effects of D-Met-enkephalin on liver necrosis (scale 0–5) 24 h after acetaminophen administration. Data are shown as medians and interquartile ranges.

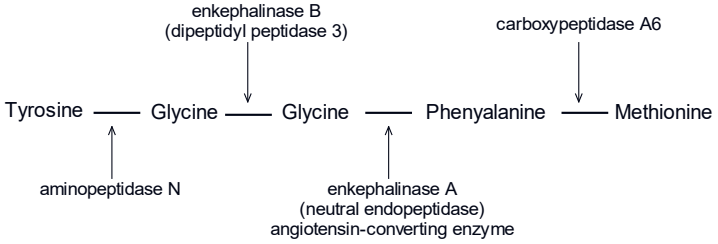

Fig. 7. Enzymes involved in the breakdown of the Met-enkephalin molecule.

Modulation of D-Met-enkephalin hepatoprotection with receptor and peptide blockade

The effects of L-Met-enkephalin are mediated *via* δ and ζ opioid receptors and could be abolished by the opioid receptor antagonist naltrexone (15, 17). Martinić *et al*. showed that naltrexone, a competitive and long-acting antagonist of the opioid receptors, blocks the hepatoprotective effects of L-Met-enkephalin (20). The same phenomenon was observed with L-Met-enkephalin antisense peptide (IPPKY) (20). Consequently, we investigated D-Met-enkephalin hepatoprotection from the standpoint of the opioid receptor blockade (with naltrexone) and D-Met-enkephalin blockade (with antisense peptide IPPKY).

The effects of opioid receptor blockade with naltrexone were investigated in group 2 and group 3 (Table I). The administration of naltrexone 30 minutes prior to D-Met-enkephalin completely abolished its protective effects. The mortality rate of animals due to acetaminophen toxicity in the groups treated with naltrexone (6/8, 75 %) and naltrexone + D-Met-enkephalin (5/8, 62.5 %) was high. This high mortality rate probably results from the blockade of both pharmacologically applied D-Met-enkephalin, as well as the blockade of endogenous L-Met-enkephalin (20). Similar mortality was observed when antisense peptide was applied alone (5/8, 62.5 %) or together with D-Met-enkephalin (6/8, 75 %). Sense-antisense peptide complex, naltrexone and antisense peptide could also contribute to the hepatotoxicity, but this could not be evaluated due to the high number of deceased animals (Table I).

Table I. Mortality in different experimental groups when the D-Met-enkephalin effects were blocked with naltrexone and antisense peptide

Group	Mortality
Control (0.9 % NaCl)	2/8
D-Met-enkephalin (5 mg kg^{-1})	0/8
Naltrexone (10 mg kg^{-1})	6/8
Naltrexone + D-Met-enkephalin	5/8
Antisense peptide (15 mg kg^{-1})	5/8
Antisense peptide + D-Met-enkephalin	6/8

The blockade of the best protective dose of D-Met-enkephalin (5 mg kg^{-1}) with naltrexone suggests that hepatoprotection induced by D-enantiomer is mediated *via* opioid receptors, similarly to the situation with L-enantiomer. In addition, the blockade of D-Met-enkephalin with antisense peptide allowed us to observe its biological effect in the state of the preserved receptor function, enabling other endogenous substances to act concomitantly (31).

The effects of Met-enkephalin blockade with an antisense peptide were investigated in groups 4 and 5 (Table I). A mixture of D-Met-enkephalin and antisense peptide (mixed together 30 minutes prior to the administration) blocked the hepatoprotective effects of D-Met-enkephalin and confirmed the results of the *in vitro* binding assay (Fig. 3).

It is known that D-amino acid substitution in the peptide sequence may improve its binding and preserve function and/or structure. Dissociation constant for the complexes of D-Met-enkephalin with antisense peptide was 3.7 ± 0.9 μmol L^{-1} (mean ± SD). Martinić *et al*. showed that the dissociation constant for the complex of L-Met-enkephalin with antisense peptide was 19 ± 3 μmol L^{-1} (mean ± SD) (20). Those results indicate a slightly higher affinity of the D-enantiomer for the antisense peptide. It also supports the concept of the sense-antisense peptide interaction, stating that antisense peptides specified by the complementary RNAs bind to sense peptides, with enhanced specificity and affinity (27, 33, 34, 43, 44, 46). In this way, antisense peptides could abolish the biological activity of the peptide hormones, a fact experimentally verified for more than 40 different ligand-receptor systems (20, 27, 33, 34, 43, 44, 46).

Preclinical and early-phase clinical trials of L-Met-enkephalin, also considered to be an opioid growth factor (OGF) (17) and cytokine (15), showed that its administration is safe and non-toxic (14–19). The obtained data suggest that both L- and D-Met-enkephalin enantiomers might possess therapeutic potential in inflammatory diseases of the liver and in acetaminophen overdose. Although these results are limited to an experimental model, the hepatoprotective activity of D-Met-enkephalin suggests that D-amino-acid-modified enkephalins may merit further preclinical investigation. Additional studies on pharmacokinetics, safety, and the mechanism of action will be needed before any potential clinical application can be considered.

CONCLUSIONS

The earlier study on L-Met-enkephalin demonstrated receptor-dependent hepatoprotection but did not address how stereochemical inversion influences biological activity, receptor engagement, or antisense peptide recognition. Although the same APAP-induced hepatotoxicity model was used in our earlier study of the L-enantiomer, the present study demonstrates that the D-enantiomer of Met-enkephalin possesses distinct yet comparable hepatoprotective activity to its natural L-form in acetaminophen-induced hepatotoxicity in male CBA mice. D-Met-enkephalin exhibited dose-dependent protection within the 0.5–20 mg kg^{-1} range, with maximal efficacy at 5.0 mg kg^{-1}. The observed effects were both peptide- and receptor-specific, mediated *via* opioid receptor activation and abolished by the opioid antagonist naltrexone. These findings indicate that partial chiral inversion does not abolish the biological activity of Met-enkephalin, likely owing to the presence of achiral glycine residues preserving the peptide's functional conformation. The results further

suggest that strategic incorporation of D-amino acids can yield bioactive peptides with retained receptor affinity and improved stability, offering new perspectives for the rational design of peptide-based hepatoprotective and cytoprotective therapeutics.

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Conflict of interest. – The authors declare that they have no conflicts of interest.

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Authors contributions. – Conceptualisation, P.T. and N.Š.; design of D-Met-enkephalin enantiomer and antisense peptides, N.Š. and R.M., investigation, P.T., R.S., A.B.-B., and T.W.; statistical analyses, M.M. and P.T.; spectroscopic studies and transaminase measurement, T.W., M.M., and P.T.; histopathology analysis and scoring, M.K. All authors have read and agreed to the published version of the manuscript.

REFERENCES

1. M. Li, M. Liu, Y. Shang, C. Ren, J. Liu, H. Jin and Z. Wang, The substitution of a single amino acid with its enantiomer for control over the adjuvant activity of self-assembling peptides, *RSC Adv.* **10**(23) (2020) 13900–13906; https://doi.org/10.1039/C9RA10325B
2. M. Abdulbagi, L. Wang, O. Siddig, B. Di and B. Li, D-amino acids and D-amino acid-containing peptides: Potential disease biomarkers and therapeutic targets?, Biomolecules **11**(11) (2021) Article ID 1716 (14 pages); https://doi.org/10.3390/biom11111716
3. A. A. Ageeva, A. B. Doktorov, N. E. Polyakov and T. V. Leshina, Chiral linked systems as a model for understanding D-amino acids influence on the structure and properties of amyloid peptides, *Int. J. Mol. Sci.* **23**(6) (2022) Article ID 3060 (18 pages); https://doi.org/10.3390/ijms23063060
4. A. Jilek and G. Kreil, D-amino acids in animal peptides, *Monatsh. Chem.* **139** (2008) 1–5; https://doi.org/10.1007/s00706-007-0780-5
5. P. Conti, L. Tamborini and A. Pinto, Drug discovery targeting amino acid racemases, *Chem. Rev.* **111**(11) (2011) 6919–6946; https://doi.org/10.1021/cr2000702
6. E. Rosini, P. D'Antona and L.Pollegioni, Biosensors for D-amino acids: Detection methods and applications, *Int. J. Mol. Sci.* **21**(13) (2020) Article ID 4574 (16 pages); https://doi.org/10.3390/ijms21134574
7. P. Turcic, M. Bradamante, K. Houra, N. Stambuk, T. Kelava, P. Konjevoda, S. Kazazic, D. Vikic-Topic and B. Pokric, Effects of alpha-melanocortin enantiomers on acetaminophen-induced hepatotoxicity in CBA mice, *Molecules* **14**(12) (2009) 5017–5026; https://doi.org/10.3390/molecules14125017
8. C. Jia, C. B. Lietz, Q. Yu and L. Li, Site-specific characterization of D-amino acid containing peptide epimers by ion mobility spectrometry, *Anal. Chem.* **86** (2014) 2972–2981; https://doi.org/10.1021/ac4033824
9. M. V. Humpola, R. Spinelli, M. Erben, V. Perdomo, G. G. Tonarelli, F. Albericio and A. S. Siano, D- and *N*-methyl amino acids for modulating the therapeutic properties of antimicrobial peptides and lipopeptides, *Antibiotics* **12**(5) (2023) Article ID 821 (15 pages); https://doi.org/10.3390/antibiotics12050821
10. G. Murtas and L. Pollegioni, D-amino acids as novel blood-based biomarkers, *Curr. Med. Chem.* **29**(24) (2022) 4202–4215; https://dx.doi.org/10.2174/0929867328666211125092438

11. Y. Shi, Z. Hussain and Y. Zhao, Promising application of D-amino acids toward clinical therapy, *Int. J. Mol. Sci.* **23**(18) (2022) Article ID 10794 (18 pages); https://doi.org/10.3390/ijms231810794
12. G. Murtas and L. Pollegioni, D-Amino acids and cancer: Friends or foes?, *Int. J. Mol. Sci.* **24**(4) (2023) Article ID 3274 (12 pages); https://doi.org/10.3390/ijms24043274
13. R. P. Millar, Y. F. Zhu, C. Chen and R. S. Struthers, Progress towards the development of non-peptide orally-active gonadotropin-releasing hormone (GnRH) antagonists: Therapeutic implications, *Br. Med. Bull.* **56**(3) (2000) 761–772; https://doi.org/10.1258/0007142001903346
14. J. M. Cullen and M. Cascella, *Physiology, Enkephalin*, in *StatPearls* [Internet], StatPearls Publishing, Treasure Island 2025; https://www.ncbi.nlm.nih.gov/books/NBK557764/N
15. J. Tian, W. Fu, Z. Xie, Y. Zhao, H. Yang and J. Zhao, Methionine enkephalin (MENK) protected macrophages from ferroptosis by downregulating HMOX1 and ferritin, *Proteome Sci.* **22** (2024) Article ID 2 (15 pages); https://doi.org/10.1186/s12953-024-00228-x
16. X. Wang, S. Li, S. Yan, Y. Shan, X. Wang, Z. Jingbo, Y. Wang, F. Shan, N. Griffin and X. Sun, Methionine enkephalin inhibits colorectal cancer by remodeling the immune status of the tumor microenvironment, *Int. Immunopharmacol.* **111** (2022) Article ID 109125 (12 pages); https://doi.org/10.1016/j.intimp.2022.109125
17. V. Asokan, A. M. Koundinya and V. Aranganathan, An overview of opioid peptides: Their sources and molecular sequences, *J. Opioid Management* **21** (2025) 439–459; https://doi.org/10.5055/jom.0954Y
18. Y. Tuo, C. Tian, L. Lu and M. Xiang, The paradoxical role of methionine enkephalin in tumor responses, *Eur. J. Pharmacol.* **882** (2020) Article ID 173253 (10 pages); https://doi.org/10.1016/j.ejphar.2020.173253
19. N. Qu, R. Wang, Y. Meng, N. Liu, J. Zhai and F. Shan, Methionine enkephalin inhibited cervical carcinoma via apoptosis promotion and reduction of myeloid derived suppressor cell infiltrated in tumor, *Int. Immunopharmacol.* **110** (2022) Article ID 108933 (10 pages); https://doi.org/10.1016/j.intimp.2022.108933
20. R. Martinić, H. Šošić, P. Turčić, P. Konjevoda, A. Fučić, R. Stojković, G. Aralica, M. Gabričević, T. Weitner and N. Štambuk, Hepatoprotective effects of Met-enkephalin on acetaminophen-induced liver lesions in male CBA mice, *Molecules* **19**(8) (2014) 11833–11845; https://doi.org/10.3390/molecules190811833
21. B. D. Janković and D. Marić, *Enkephalins as Regulators of Inflammatory Immune Reactions*, in *Neuropeptides and Immunoregulation* (Ed. B. Scharrer, E. M. Smith and G. B. Stefano) Springer-Verlag, Berlin 1994, pp. 76–100.
22. X. Bai, F. Shan, N. Qu, H. Huang, M. Handley, N. Griffin, S. Zhang and X. Cao, Regulatory role of methionine enkephalin in myeloid-derived suppressor cells and macrophages in human cutaneous squamous cell carcinoma, *Int. Immunopharmacol.* **99** (2021) Article ID 107996 (6 pages); https://doi.org/10.1016/j.intimp.2021.107996
23. P. Konjevoda, N. Štambuk, G. Aralica and B. Pokrić, Cytoprotective effects of met-enkephalin and α-MSH on ethanol induced gastric lesions in rats, *J. Physiol. (Paris)* **95**(1–6) (2001) 277–281; https://doi.org/10.1016/s0928-4257(01)00038-9
24. N. Štambuk, N. Kopjar, K. Šentija, V. Garaj-Vrhovac, D. Vikić-Topić, B. Marušić-Della Marina, V. Brinar, M. Trbojević-Čepe, N. Žarković, B. Ćurković, Đ. Babić-Naglić, M. Hadžija, N. Zurak, Z. Brzović, R. Martinić, V. Štambuk, P. Konjevoda, N. Ugrinović, I. Pavlić-Renar, Z. Biđin and B. Pokrić, Cytogenetic effects of Met-enkephalin (peptid-M) on human lymphocytes, *Croat. Chem. Acta* **71**(3) (1998) 591–605; https://hrcak.srce.hr/132370
25. D. Tjesić-Drinković, N. Štambuk, D. Tjesić-Drinković, P. Konjevoda, N. Gotovac, T. Ćurović and A. Votava Raić, Met-enkephalin effects on histamine-induced bronhoconstriction in guinea pigs, *Coll. Antropol.* **29**(2) (2005) 689–692; https://hrcak.srce.hr/5330

26. P. Konjevoda, N. Štambuk, D. Vikić-Topić, A. Boban-Blagaić, S. Vikić-Topić, V. Mrljak, J. Pavan, P. Ramadan and Z. Biđin, Protective effects of met-enkephalin on alcohol induced gastric lesions, *Croat. Chem. Acta* **73**(4) (2000) 1111–1121; https://hrcak.srce.hr/131992
27. A. D. Miller, Sense-antisense (complementary) peptide interactions and the proteomic code; potential opportunities in biology and pharmaceutical science, *Expert Opin. Biol. Ther.* **15**(2) (2015) 245–267; https://doi.org/10.1517/14712598.2015.983069
28. K. Hinz, M. Niu, H. M. Ni and W. X. Ding, Targeting autophagy for acetaminophen-induced liver injury: An update, *Livers* **4**(3) (2024) 377–387; https://doi.org/10.3390/livers4030027
29. F. Guarner, N. K. Boughton-Smith, G. J. Blackwell and S. Moncada, Reduction by prostacyclin of acetaminophen-induced liver toxicity in the mouse, *Hepatology* **8**(2) (1988) 248–253; https://doi.org/10.1002/hep.1840080210
30. H. Jaeschke and A. Ramachandran, Acetaminophen hepatotoxicity: Paradigm for understanding mechanisms of drug-induced liver injury, *Annu. Rev. Pathol.* **19** (2024) 453–478; https://doi.org/10.1146/annurev-pathmechdis-051122-094016
31. P. Turcic, N. Stambuk, P. Konjevoda, T. Kelava, M. Gabricevic, R. Stojkovic and G. Aralica, Modulation of γ_2-MSH hepatoprotection by antisense peptides and melanocortin subtype 3 and 4 receptor antagonists, *Med. Chem.* **11**(3) (2015) 286–295; https://dx.doi.org/10.2174/1573406410666140914161421
32. V. M. Silva, C. Chen, G. E. Hennig, H. E. Whiteley and E. J. Manautou, Changes in susceptibility to acetaminophen-induced liver injury by the organic anion indocyanine green, *Food Chem. Tox.* **39**(3) (2001) 271–278; https://doi.org/10.1016/s0278-6915(00)00138-1
33. K. Houra, P. Turčić, M. Gabričević, T. Weitner, P. Konjevoda and N. Štambuk, Interaction of α-melanocortin and its pentapeptide antisense LVKAT: Effects on hepatoprotection in male CBA mice, *Molecules* **16**(9) (2011) 7331–7343; https://doi.org/10.3390/molecules16097331
34. N. Štambuk, Z. Manojlović, P. Turčić, R. Martinić, P. Konjevoda, T. Weitner, P. Wardega and M. Gabričević, A simple three-step method for design and affinity testing of new antisense peptides: An example of erythropoietin, *Int. J. Mol. Sci.* **15**(6) (2014) 9209–9223; https://doi.org/10.3390/ijms15069209
35. H. Gampp, M. Maeder, C. J. Meyer and A. D. Zuberbühler, Calculation of equilibrium constants from multiwavelength spectroscopic data-I: Mathematical considerations, *Talanta* **32**(2) (1985) 95–101; https://doi.org/10.1016/0039-9140(85)80035-7
36. H. Gampp, M. Maeder, C. J. Meyer and A. D. Zuberbühler, Calculation of equilibrium constants from multiwavelength spectroscopic data-II[1]32, 95.: Specfit: Two user-friendly programs in basic and standard FORTRAN 77, *Talanta* **32**(4) (1985) 257–264; https://doi.org/10.1016/0039-9140(85)80077-1
37. H. Gampp, M. Maeder, C. J. Meyer and A. D. Zuberbühler, Calculation of equilibrium constants from multiwavelength spectroscopic data-IV: Model-free least-squares refinement by use of evolving factor analysis, *Talanta* **33**(12) (1986) 943–951; https://doi.org/10.1016/0039-9140(86)80233-8
38. V. Blagaić, K. Houra, P. Turčić, N. Stambuk, P. Konjevoda, A. Boban-Blagaić, T. Kelava, M. Kos, G. Aralica and F. Culo, The influence of α-, β-, and γ-melanocyte stimulating hormone on acetaminophen induced liver lesions in male CBA mice, *Molecules* **15**(3) (2010) 1232–1241; https://doi.org/10.3390/molecules15031232
39. H. Jaeschke, Role of inflammation in the mechanism of acetaminophen-induced hepatotoxicity, *Expert Opin. Drug Metab. Toxicol.* **1**(3) (2005) 389–397; https://doi.org/10.1517/17425255.1.3.389
40. S. D. Nelson and S. A. Bruschi, *Mechanisms of Acetaminophen-induced Liver Disease*, in *Drug-Induced Liver Disease* (Ed. N. Kaplowitz and L. D. DeLeve), Informa Healthcare, New York 2007, pp. 353–388.
41. E. J. Calabrese and L. A. Baldwin, Hormesis: The dose-response revolution, *Annu. Rev. Pharmacol. Toxicol.* **43** (2003) 175–197; https://doi.org/10.1146/annurev.pharmtox.43.100901.140223

42. T. P. Khaket, J. Singh, P. Attri and S. Dhanda, Enkephalin degrading enzymes: Metalloproteases with high potential for drug development, *Curr. Pharm. Des.* **18**(2) (2012) 220–230; https://doi.org/10.2174/138161212799040547

43. N. Štambuk, P. Konjevoda, A. Boban-Blagaić and B. Pokrić, Molecular recognition theory of the complementary (antisense) peptide interactions, *Theory Biosci.* **123**(4) (2005) 265–275; https://doi.org/10.1016/j.thbio.2005.02.001

44. J. C. Biro, The proteomic code: A molecular recognition code for proteins, *Theor. Biol. Med. Model.* **4** (2007) 1–45; https://doi.org/10.1186/1742-4682-4-45

45. M. García-Domínguez, Enkephalins and pain modulation: Mechanisms of action and therapeutic perspectives, *Biomolecules* **14** (2024) Article ID 926 (23 pages); https://doi.org/10.3390/biom14080926

46. N. Štambuk, P. Konjevoda, P. Turčić, H. Šošić, G. Aralica, D. Babić, S. Seiwerth, Ž. Kaštelan, R. Novak Kujundžić, P. Wardega, J. Barać Žutelija, A. Gudelj Gračanin and M. Gabričević, Targeting tumor markers with antisense peptides: An example of human prostate specific antigen, *Int. Mol. Sci.* **20**(9) (2019) Article ID 2090 (20 pages); https://doi.org/10.3390/ijms20092090