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Oral absorption and in vivo biodistribution of α-conotoxin MII and a lipidic analogue

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Abstract

Conotoxins are highly constrained peptide toxins that exhibit pharmaceutically relevant biological activities. We herein report the extent of absorption and profile of distribution of a native α-conotoxin, MII and a lipophilic analogue of MII (N-LaaMII) after intravenous (iv) and oral administration to male Sprague–Dawley rats. N-LaaMII is formed by coupling 2-amino-D,L-dodecanoic acid (Laa) to the N-terminus of MII and has previously been shown to exhibit significantly improved permeability across Caco-2 cell monolayers compared to the native MII while maintaining the potency in inhibition of nAChRs of the parent peptide. Both peptides crossed the GI tract after oral administration (~6% after 30 m). While Laa conjugation did not significantly improve absorption, it did greatly increase the accumulation of the compound in the liver after iv administration. Neither peptide crossed the blood–brain barrier to any significant extent. This is the first study of the in vivo biodistribution of an α-conotoxin after oral administration.

Keywords: α-Conotoxin MII; α-Aminododecanoic; Biodistribution; Lipoamino acid; Oral absorption

Predatory marine snails belonging to the species *Conus* use spectacularly complex cocktails of bioactive peptide toxins to immobilise their prey [1]. These conotoxins are small peptides (12–19 residues) constrained into their active conformer by disulfide bonds. Several classes of these toxins have been studied at length due to their high affinity and selectivity for certain neuronal receptors [2]. There are several conotoxins that are in clinical trials for pharmaceutical applications including chronic pain [3,4]. The α-conotoxins have shown remarkable selectivity for different subtypes of nicotinic acetylcholine receptors (nAChRs) [5–10]. This property, in addition to the peptides' remarkable stability in biological systems, [11] make conotoxins interesting drug candidates, however like most peptides, they are not expected to pass easily through biological barriers such as the GI tract and blood–brain barrier (BBB). Our study aimed to quantify the absorption of a model conotoxin, MII across the GI tract and investigate the distribution of the peptide throughout the organs of a rat after administration directly into the circulatory system (iv administration). We compare these results to those of a synthetic, lipophilic analogue of MII (N-Laa-MII) to determine if significantly increasing the lipophilicity of the peptide can enhance its absorption or alter the distribution profile in vivo. We have previously reported the synthesis, structure elucidation and in vitro biological evaluation of lipidic analogues of MII [12], and we now report the first in vivo biodistribution study in male rats after both oral and intravenous administration of a native α-conotoxin and the lead analogue identified in the in vitro studies.

α-Conotoxin MII is a potent and highly selective competitive antagonist of the α3β2 subtype of the nAChR.

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The toxin was first isolated from *Conus magus* by Cartier et al. [13] and the three-dimensional solution structure described initially by Shon et al. [14] and later by Hill et al. [15]. The 16 amino acid peptide (Fig. 1) belongs to the α4/7 subclass and possesses Cys2–Cys8 and Cys3–Cys16 disulfide bonds. The toxin exhibits an IC50 value of 0.5 nM towards Xenopus oocytes expressing α3β2 nAChRs and is several orders of magnitude less potent towards other subunit combinations [13].

In order for any peptide to progress to the clinic, the issues of bioavailability and membrane permeability must be addressed. Although MII is relatively stable under biological conditions with a recent study showing that over 60% of the peptide remained intact after 24 h exposure to human serum [11], it would not be expected to readily cross intestinal mucosal membranes or the blood–brain barrier. Increasing the lipid solubility of a hydrophilic compound has been established as an important factor in improving absorption via passive transport across intestinal mucosal membranes [16,17]. Our own strategy relies on the use of lipoamino acids (Laas) to introduce lipidic groups into peptides. These α-amino acids bearing a variable alkyl side-chain allow the conjugation of one or more of these groups via standard peptide coupling techniques and for them to be incorporated into the solid phase synthesis of a peptide. To investigate the effect of lipidation on MII, we have previously synthesised two lipidic analogues of MII, the first by coupling 2-amino-D,L-dodecanoic acid to the N-terminus (N-LaaMII) and the second by replacing Asn5 in the MII sequence with this lipoamino acid (5LaaMII) [12]. Both lipidic linear peptides were then oxidised under standard conditions [15]. ^{1}H NMR shift analysis of these peptides and comparison with the native MII peptide showed that the secondary structure of the N-conjugated analogue, N-LaaMII, was consistent with the native conotoxin while the 5LaaMII analogue formed the correct disulfide bridges but failed to adopt the native helical secondary structure [12]. The N-terminus conjugate was also found to inhibit nAChRs of the subtype α3β2 with equal potency to the parent peptide whereas the 5LaaMII analogue showed no inhibitory activity [12]. The active N-LaaMII analogue was found to exhibit significantly improved permeability across Caco-2 cell monolayers (an in vitro model for intestinal absorption) compared to the native MII and showed negligible toxicity [12]. This current study investigated whether this in vitro improvement in absorption would translate into enhanced oral uptake of the peptide or improved permeability across the BBB compared to the native peptide. The biodistribution of tritium labelled samples of both native MII and N-LaaMII was determined using male Sprague–Dawley rats.

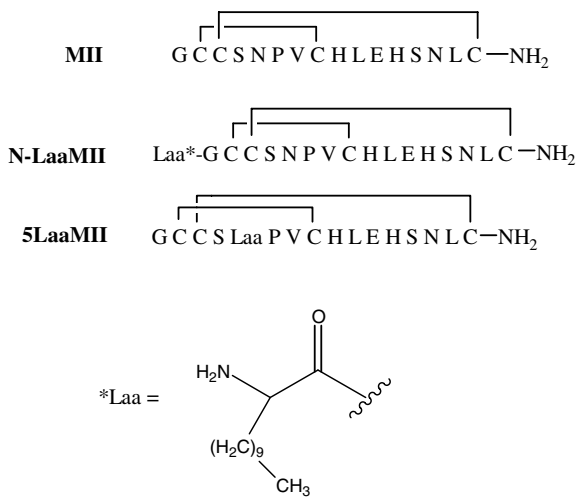

Fig. 1. Structure of MII, N-LaaMII and 5LaaMII.

Results and discussion

The synthesis and purification of both native MII and N-LaaMII was performed as described in our previous paper where the structures of these peptides were fully characterised by NMR and mass spectrometry [12]. In order to follow the fate of the peptides after administration, it was necessary to radiolabel them at the N-terminus with ^{3}H-acetic anhydride (($^{3}H_3CCO)_2O$). The products of the acetylation were lyophilised several times in order to remove any residual tritium labelled acetic acid and were not further purified before stability or feeding studies.

The biodistribution studies described herein rely on assessing the level of radioactivity in each organ. This assumes that the tritiated acetate group is still associated with the intact peptide. In order to validate this assumption, non-radioactive N-acetylated MII was treated with plasma and the solution monitored for peptide levels over 2.5 h by analytical RP-HPLC. These results showed that there was minimal breakdown of N-Ac-MII by plasma enzymes with the peptide still >90% intact after 2.5 h (Fig. 2). This result indicates that the peptides are delivered to the organs with the radiolabel intact.

The assessment of the biodistribution of MII and N-LaaMII was achieved by administering 1 mg of the tritium labelled peptides by iv injection into the jugular vein or by oral gavage to male rats and then examining the radiation levels found in various organs over time. The radiation

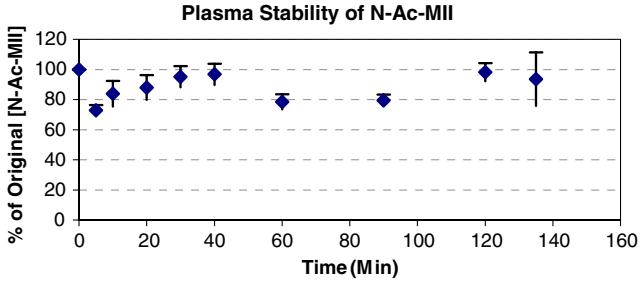

Fig. 2. The stability of the N-acetylated MII peptide in plasma over 2.5 h. The data points are expressed as the percentage of the original concentration of the tested peptide remaining in the solution and are an average of 3 experiments ± SD.

levels were expressed as a percentage of the total amount of radioactive material administered to the animal (measure in dpm). The radioactivity of the organs was measured by liquid scintillation counting. The organs were removed and washed thoroughly with PBS before being homogenised in a minimum amount of buffer. It is important to note that the stomach and intestines of the rats are cleaned of all food or waste material and thus the levels measured for these organs correspond to the levels of the actual tissue.

Access to the CNS continues to be a great challenge for most peptide based drugs. We were, therefore particularly interested in the blood–brain barrier permeability of MII and N-LaaMII. The experiment employing intravenous administration provided the most direct route of assessing this. After iv injection, whole blood, the brain and liver of each animal were removed and analysed for levels of radioactivity. Fig. 3 shows the graph of the radioactivity levels in these organs at 10, 20 and 40 m after injection. The N-LaaMII derivative appeared to accumulate very quickly in the liver and high levels were detected in this organ even after 40 m. We also noted that by 40 m post injection, the level of MII detected in the blood and liver was much lower than for N-LaaMII. These results suggest that the N-LaaMII derivative was cleared much more slowly from the body of the rats than the native MII peptide. This may be a result of increased resistance to peptidase enzymes of the of the Laa derivative or an increase in this more lipophilic compound's ability to bind to plasma proteins. Lipophilicity has been established as a major determining factor in a compound's plasma protein binding affinity [18,19]. Thus, by the addition of a long lipid moiety to the peptide we may have increased its binding to proteins, slowing its digestion by proteases in the liver.

The levels of radioactive compound detected in the brains of the rats were similar for both peptides. The levels reached between 0.12% and 0.16% of the total radioactivity administered after 10 min for the native peptide and the Laa derivative, respectively. These levels were maintained at the 20 min time point and were decreasing by the 40 min point. The delivery of peptides across the BBB remains a major hurdle in the development of CNS active

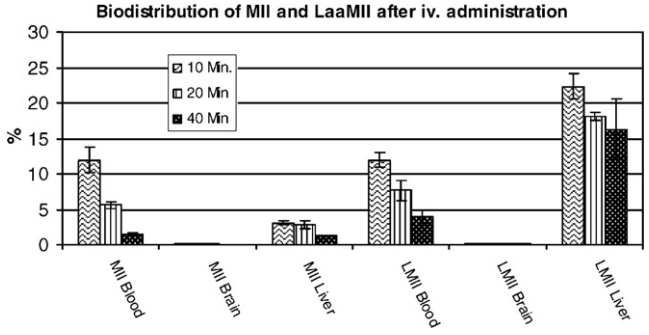

Fig. 3. Graph of the biodistribution of MII and N-LaaMII after intravenous injection. The level of radioactivity observed in each organ is the average of five rats and is expressed as a percentage of the radioactivity administered to the rats ± SD.

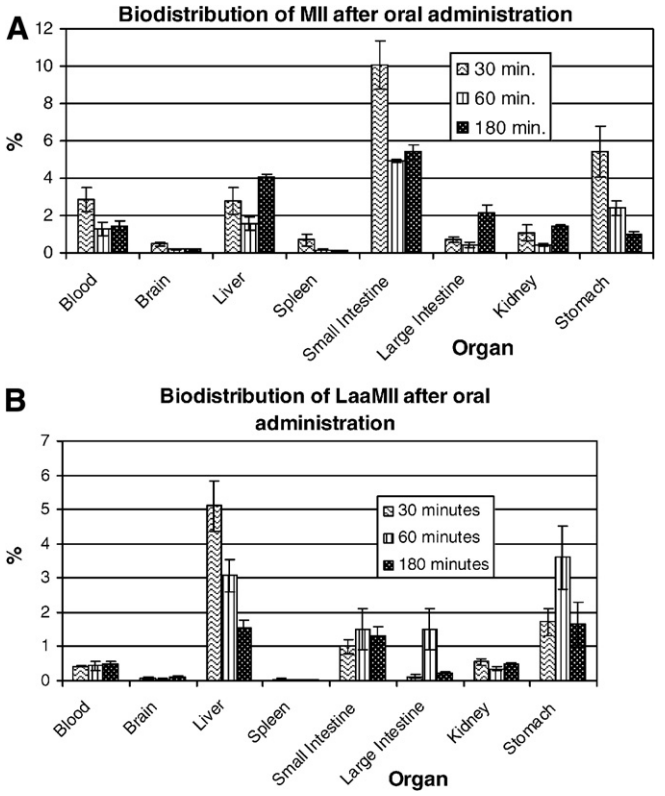

Fig. 4. Graphs of the biodistribution of (A) MII and (B) N-LaaMII after oral administration. The level of radioactivity observed in each organ is the average of five rats and is expressed as a percentage of the radioactivity administered to the rats ± SD.

peptide drugs [20] and these levels are far from ideal. However, they are higher than those reported in a similar study on the brain influx of an ω-conotoxin, Ziconotide that was in late stage clinical trials [21]. After iv injection of radiolabelled Ziconotide, 0.0096% of the injected compound per gram of tissue was detected in the brain (cortex + subcortex) after 20 min. Given that the average weight of the rat brains we harvested was ~2 g, our highest levels correspond to 0.08% per gram of tissue, almost 10 times higher than the BBB absorption of Ziconotide. Clearly, the passage of conotoxin peptides from the blood into the brain is not efficient and the addition of a single lipoamino acid is not sufficient to induce significant passive diffusion of the peptide across this tight barrier. Passive diffusion may therefore not be the best mode of absorption to target conotoxins to the CNS.

The oral bioavailability of native conotoxins has not previously been described. In this study a 1 mg/kg dose of MII or the lipid analogue, N-LaaMII was delivered via gavage to male rats (Fig. 4). After 30 min, a significant amount of both peptides had been absorbed through the gut with the concentration of MII in whole blood being 2.85% of the total radioactivity administered, much higher than that of the lipidic analogue. The sum of the radioactivity levels in the blood, brain, liver, spleen and kidney represents that portion that can be considered as having crossed the GI tract. At 30 min these levels were 7.86%

for MII and 6.22% for N-LaaMII (Fig. 4). These levels are remarkable considering the size of the peptides (16–17 amino acids) and represent the first evidence that conotoxin peptides can be absorbed across the GI tract.

Achieving oral bioavailability of peptides is notoriously difficult and the subject of research efforts across the world [22]. Octreotide is an eight amino acid analogue of the naturally occurring tetradecapeptide, Somatostain which is of great clinical significance with applications in the treatment of hormonal hypersecretion. An N-terminus maltose conjugate of Octreotide, SDZCO611 exhibited clinically significant oral activity in rhesus monkeys [23] and in humans [24]. The actual oral bioavailability of the conjugated peptide after intrajejunal administration was only 1.09% in monkeys compared to 0.28% for the parent octapeptide [23]. Oral delivery of insulin is, of course, of great clinical and commercial interest. The first oral insulin derivative is currently in late clinical trials [25] and consists of an amphiphilic polyethylene glycol oligomer conjugated to a single lysine unit through a six carbon linker [26]. The bioavailability of this so called hexyl-insulin (HIM2) was found to be approximately 8% in fasting dogs [26] and while it has not been directly measured in humans, the results of the clinical trials indicate an oral absorption of 5% in humans. This is a remarkable oral absorption for a peptide of over 50 amino acids in length.

Lipidation strategies such as that employed in this study have also been successful in improving the oral bioavailability of short peptides. Radioactively labelled lipoamino acid analogues of a tripeptide, thyrotropin-releasing hormone, and a decapeptide, luteinizing hormone-releasing hormone, showed analogous absorption profiles to those of the MII analogues when administered to Wistar rats [27]. The level of the smaller peptide, Laa-TRH in the liver, kidney and blood of the animals was ~16% of that originally administered and this dropped to 6% for the larger decapeptide [27]. Reversible lipidization is a technique used successfully to improve the oral absorption of the pentapeptide Leu-enkephalin [28] and the 32 amino acid peptide calcitonin [29] in rats. While no direct indication of percentage absorption was given, the authors quote increases of approximately 20 times the oral absorption compared to the parent peptides.

In the case of 16–17 amino acids MII and N-LaaMII, it is clear that the lipid attachment had no effect on the total peptide uptake from the GI tract. As in the iv experiment, N-LaaMII accumulated quickly in the liver and may explain the lower levels of this compound in the blood compared to the native peptide. This accumulation in the liver is a phenomenon observed in other lipidated peptides [29,30]. We also noted a small increase in the level of MII in the kidneys as compared to LaaMII which could be another indication of the more rapid clearance of the more water soluble MII that was suggested in the iv results. The levels of the peptides in the brain tissue were similar to those in the iv experiments.

Conclusions

Our results clearly indicate that there is significant absorption of two conotoxin MII-related peptides across the GI tract (~7%) of Sprague–Dawley rats. Interestingly, their biodistribution was influenced by the attachment of a lipid moiety with the lipophilic compound, N-LaaMII, accumulating in the liver. This accumulation of the peptide in the liver of the rats has important implications for the future development of drug delivery strategies involving these Laas. Our results also confirm that the BBB remains a major challenge to the delivery of peptides to the CNS. Research will continue into alternative modification strategies and alternative routes of administration in an attempt to optimise the delivery of conotoxins to the CNS.

Our study is the first to show that an α-conotoxin, MII, can be absorbed through the GI tract into the circulation and thus has exciting implications for the development of conotoxin based drugs. We have also confirmed the stability of MII in plasma and have demonstrated that it is not significantly permeable to the BBB.

Experimental

Synthesis

2-(*t*-Butoxycarbonylamino)-D,L-dodecanoic acid (Laa) was synthesised from diethyl acetomido malonate and 1-bromodecane as described in the literature [12,31]. The peptides were synthesised and characterised as described in the previous paper concerning their in vitro evaluation [12]. The solid phase synthesis of the peptides used HBTU/DIEA activation and the in situ neutralisation protocol for Boc-chemistry [32]. MBHA resin and protected amino acids were obtained from Novabiochem (Melbourne, Australia). DMF and TFA of peptide synthesis grade were purchased from Auspep (Parkville, Australia). HPLC grade acetonitrile was purchased from Labscan Asia Co. Ltd. (Bangkok, Thailand). Mass spectrometric measurements were performed using a triple quadrupole, PE Sciex API 3000 mass spectrometer with positive ion electrospray (ES).

Acetylation of peptides

The peptide (15 mg, 8.7 μmol MII, 7.8 μmol N-LaaMII) was dissolved in a minimum volume of DMF and $^{3}H_3$-acetic anhydride (50 mCi/mmol) (346 μL, 450 μCi, 9 μmol) or acetic anhydride (600 μL) was added followed by DIEA (3 eq). The solution was then allowed to stand at room temperature overnight. In the case of the $^{3}H_3$-acetic anhydride reactions, a further 300 μL of non-radiolabelled acetic anhydride was added and the solution stirred for a further 4 h. The volatiles were removed under nitrogen and the reaction mixture lyophilised to dryness. The residue was redissolved in water and lyophilised to dryness. This was repeated three times until a white solid was produced for both MII and N-LaaMII peptides.

Plasma stability assay

The assay was performed by mixing 300 μL of a solution of N-acetylated Conotoxin (approximately 1 mg/mL) with 300 μL of plasma prewarmed at 37.5 °C. At chosen time points, 50 μL of the plasma mixture was sampled, placed on ice, and 75 μL acetonitrile added and the samples were centrifuged at 10,000 rpm for 10 m. The supernatant was analysed by analytical RP-HPLC using a Shimazu SCL-10AVP system controller, FCV-10ALVP pump and SPD-6A UV detector (214 nm) on a Vydac C18 Column. An elution gradient from 100% solvent A (0.1% formic acid in water) to 100% solvent B (90% acetonitrile, 0.1% formic acid in water) over 20 m, ($R_t = 15.1$ min) was used. The peak areas were calculated and compared to the area of the same peak in the time zero samples. The assay was performed in triplicate with the data expressed as an average of these experiments ± standard deviation.

In vivo biodistribution studies

Oral feeding experiment

The radiolabelled peptide was dissolved in 50 mM phosphate buffer (pH 3, containing 2% DMF) (15 mL) to a final concentration of ~1 mg/mL and 1 mL aliquots administered to each male Sprague–Dawley rat via oral gavage using a blunt tipped feeding needle. To one group of four rats, only 50 mM phosphate buffer was delivered in order to obtain baseline radiation levels for each organ. After 30 m, four rats were anesthetised to unconsciousness by inhalation of a mixture of 50% CO_2 and 50% O_2 gas. Blood samples (~2 mL) were taken from each rat by cardiac puncture and then the rats were euthanised by cervical dislocation. The rats were then dissected and the brain, liver, spleen, small intestine, large intestine, kidneys and stomach were removed, washed extensively to remove all waste and food products, weighed and suspended in a measured volume of PBS. A mechanical homogeniser was then used to homogenise the organs and 0.5 mL of each homogenate and of the blood was added to 2 mL of Amersham Biosciences NCSII Tissue Solubilizer. This solution was then allowed to stand for 3–4 days, after which 0.2 mL of the solution was added to 0.2 mL of hydrogen peroxide solution (30% w/w) and allowed to stand for several hours for the purpose of decolourisation. The solvent was then removed by a steady stream of air overnight. Perkin-Elmer Optiphase HiSafe 3 liquid scintillation cocktail (4 mL) was added to the dried material, and the sample allowed to stand overnight in order to solubilise. Finally, the radiation level of each sample was determined using a Tri-Carb 2700 TR liquid scintillation spectrometer. After 60 and 180 min this process was repeated on another five rats per time point.

Intravenous biodistribution

The dried, radiolabelled peptides were dissolved in 7.5 mL of 50 mM phosphate buffer (pH 3, containing 2% DMF) to a final concentration of 2 mg/mL. The jugular vein of each male Sprague–Dawley rat was surgically exposed and a small cannula inserted, through which the peptide solution (0.5 mL, 1 mg) was injected. In this experiment the time points were 10, 20 and 40 m post injection and five rats were examined at each time point. The rats were treated exactly as described above except that only the blood, brain and liver were removed. The organs were treated as described above.

Table 1
Summarises the average ($n = 4$) level of radioactivity observed in each organ after iv administration

Organ	10 min (± SD)		20 min (± SD)		40 min (± SD)	
	MII	N-LaaMII	MII	N-LaaMII	MII	N-LaaMII
Blood	11.96 ± 1.86	11.91 ± 1.02	5.62 ± 0.5	7.69 ± 1.43	1.52 ± 0.22	3.93 ± 0.95
Brain	0.12 ± 0.01	0.16 ± 0.02	0.14 ± 0.02	0.15 ± 0.02	0.06 ± 0.01	0.10 ± 0.01
Liver	3.07 ± 0.29	22.33 ± 1.79	2.89 ± 0.58	18.14 ± 0.60	1.24 ± 0.16	16.31 ± 4.21

This is the data presented graphically in Fig. 3. Administration expressed as a percentage of the initial amount of radioactive material administered.

Table 2
Summarises the average ($n = 4$) level of radioactivity observed in each organ after oral administration

Organ	30 min (± SD)		60 min (± SD)		180 min (± SD)	
	MII	N-LaaMII	MII	N-LaaMII	MII	N-LaaMII
Blood	2.85 ± 0.62	0.43 ± 0.03	1.29 ± 0.37	0.44 ± 0.12	1.45 ± 0.25	0.49 ± 0.08
Brain	0.47 ± 0.12	0.08 ± 0.02	0.19 ± 0.04	0.05 ± 0.01	0.18 ± 0.02	0.12 ± 0.02
Liver	2.80 ± 0.71	5.11 ± 0.73	1.58 ± 0.33	3.07 ± 0.47	4.04 ± 0.17	1.53 ± 0.24
Spleen	0.68 ± 0.33	0.05 ± 0.01	0.15 ± 0.03	0.05 ± 0.01	0.14 ± 0.01	0.04 ± 0.01
Small intestine	10.08 ± 1.26	1.00 ± 0.22	4.94 ± 0.08	1.52 ± 0.60	5.40 ± 0.38	1.32 ± 0.25
Large intestine	0.71 ± 0.17	0.13 ± 0.06	0.43 ± 0.11	1.52 ± 0.60	2.14 ± 0.4	0.23 ± 0.02
Kidney	1.06 ± 0.42	0.55 ± 0.09	0.43 ± 0.08	0.35 ± 0.06	1.40 ± 0.13	0.48 ± 0.06
Stomach	5.43 ± 1.38	1.72 ± 0.39	2.4 ± 0.41	3.6 ± 0.91	1.00 ± 0.15	1.64 ± 0.67

This is the data presented graphically in Fig. 4. Administration expressed as a percentage of the initial amount of radioactive material administered.

Calculations

The total level of radioactive compound delivered to each rat was calculated by first measuring the radioactivity, in dpm of 10 μL of the acetylated peptide solutions used in the experiment. This value was then multiplied by 1000 for the oral experiments where 1 mL of solution was administered per rat and 500 for the intravenous experiments where 0.5 mL of solution was administered.

For each organ sample, the value of radioactivity measured in dpm converted to a level of radioactivity in the entire organ via the following calculation and the values appear in Tables 1 and 2:

$\text{dpm} \times 12.5 \times \text{vol. of organ homogenate}/0.5$

For blood samples, an average total blood volume for a rat of 6 mL/100 g was used and so the total radioactivity in the blood was calculated by:

$\text{dpm} \times 12.5 \times 0.06 \times \text{weight of rat (g)}/0.5.$

Acknowledgments

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References

[1] B.M. Olivera, J.E. Rivier, C. Clark, C.A. Ramilo, G.P. Corpuz, F.C. Abogadie, E. Mena, S.R. Woodward, D.R. Hillyard, L.J. Cruz, Diversity of *Conus* neuropeptides, Science 249 (1990) 257–263.

[2] L.J. Cruz, J.M. McIntosh, J.S. Imperial, W. Gray, in: H. Rochat, Martin-Eauclaire (Eds.), Methods and Tools in Biosciences and Medicine, Birkhauser Verlag, Basel, 2000, pp. 74–89.

[3] G.P. Miljanich, Ziconotide: Neuronal calcium channel blocker for treating severe chronic pain, Curr. Med. Chem. 11 (2004).

[4] B.G. Livett, K.R. Gayler, Z. Khalil, Drugs from the sea: Conopeptides as potential therapeutics, Curr. Med. Chem. 11 (2004) 1715–1723.

[5] J.M. Ward, V.B. Crockcroft, G.G. Lunt, F.S. Smillie, S. Wonnacott, Methyllycaconitine: a selective probe for neuronal α-bungarotoxin binding sites, FEBS Lett. 270 (1990) 45–48.

[6] P. Seguela, J. Wadiche, K. Dineley-Miller, J.A. Dani, J.W. Patrick, Molecular cloning, functional properties, and distribution of rat brain alpha 7: a nicotinic cation channel highly permeable to calcium, J. Neurosci. 13 (1993) 596–604.

[7] C.W. Luetje, K. Wanda, S. Rogers, S.N. Abramson, K. Tsuji, S. Heinemann, J.W. Patrick, Neurotoxins distinguish between different neuronal nicotinic acetylcholine receptor subunit combinations, J. Neurochem. 55 (1990) 632–640.

[8] J.M. McIntosh, A.D. Santos, B.M. Olivera, Conus peptides targeted to specific nicotinic acetylcholine receptor subtypes, Ann. Rev. Biochem. 68 (1999) 59–88.

[9] R.W. Janes, a-Conotoxins as selective probes for nicotinic acetylchholine receptor subclasses, Curr. Opin. .Pharmacol. 5 (2005) 280–292.

[10] B.G. Livett, D.W. Sandall, D. Keays, J. Down, K.R. Gayler, N. Satkunanathan, Z. Khalil, Therapeutic applications of conotoxins that target the neuronal nicotinic acetylcholine receptor, Toxicon 48 (2006) 810–829.

[11] R. Clark, J.H. Fischer, L. Dempster, N.L. Daly, K.J. Rosengren, S.T. Nevin, F.A. Meunier, D.J. Adams, D.J. Craik, Engineering stable peptide toxins by means of backbone cyclization: Stabilization of the α-conotoxin MII, PNAS 102 (2005) 13767–13772.

[12] J. Blanchfield, J. Dutton, R. Hogg, O. Gallagher, D. Craik, A. Jones, D. Adams, R. Lewis, P. Alewood, I. Toth, Synthesis, structure elucidation, in vitro biological activity, toxicity, and Caco-2 cell permeability of lipophilic analogues of alpha-conotoxin MII, J. Med. Chem. 46 (2003) 1266–1272.

[13] E.G. Cartier, D. Yoshikami, W. Gray, R.S. Luo, B.M. Olivera, J.M. McIntosh, A new α-conotoxin which targets α3β2 nicotinic acetylcholine receptors, J. Biol. Chem. 271 (1996) 7522–7528.

[14] K.-J. Shon, S.C. Koerber, J.E. Rivier, B.M. Olivera, J.M. McIntosh, Three-dimensional solution structure of α-conotoxin MII, an α3β2 neuronal nicotinic acetylcholine receptor-targeted ligand, Biochem. 36 (1997) 15693–15700.

[15] J.M. Hill, C.J. Oomen, L.P. Miranda, J.-P. Bingham, P.F. Alewood, D.J. Craik, Three-dimensional solution structure of α-conotoxin MII by NMR spectroscopy: Effects of solution environment on helicity, Biochem. 37 (1998) 15621–15630.

[16] J.T. Blanchfield, I. Toth, Lipid, sugar and liposaccharide based delivery systems 2, Curr. Med. Chem. 11 (2004) 2375–2382.

[17] H. Bundgaard, B.B. Hansen, Prodrugs as drug delivery systems, Pharmacy Int. (1981) 136–140.

[18] P.M. Gleeson, Plasma protein binding affinity and its relationship to molecualr structure: An in-silico analysis, J. Med. Chem. 50 (2007) 101–112.

[19] L. Li, Y. Xu, Z. Su, The correlation of serum protein association and cellular uptake with lipophilicity and polarity of the backbone substituent of the technitium-99m-amine-oxime chelates, J. Label. Comp. Radiopharm. 50 (2007) 91–95.

[20] K.A. Witt, T.P. Davis, CNS drug delivery: opioid peptides and the blood-brain barrier, AAPS Journal 8 (2006) E76–E88.

[21] R. Newcomb, T.J. Abbruscata, T. Singh, L. Nadaski, T.P. Davis, G. Miljanich, Bioavailablity of Ziconotide in brain: influx from blood, stability and diffusion, Peptides 21 (2000) 491–501.

[22] J.H. Hamman, G.M. Enslin, A.F. Kotze, Oral delivery of peptide drugs, Biodrugs 19 (2005) 165–177.

[23] R. Albert, P. Marbach, W. Bauer, U. Briner, G. Fricker, C. Bruns, J. Pless, SDZCO611: A highly potent glycated analog of somatostatin with improved oral activity, Life Sciences 53 (1993).

[24] C. Nelson-Piercy, P.J. Hammond, M.E. Gwilliam, N. Khandan-Nia, M.J. Myers, M.A. Ghatei, S.R. Bloom, Effect of a new oral somatostatin analog (SDZCO611) on gastric emptying, mouth to cecum transit time, and pancreatic and gut hormone release in normal male subjects, J. Clin. Endocrin. Metab. 78 (1994) 329–336.

[25] S. Clement, P. Dandona, G.J. Still, G. Kosutic, Oral modified insulin (HIM2) in patients with type 1 diabetes mellitus: Results from a Phase I/II clinical trial, Metabolism 53 (2004) 54–58.

[26] G.J. Still, Development of oral insulin: progress and current status, Diabetes Metab. Res. Rev. 18 (2002) S29–S37.

[27] N. Flinn, S. Coppard, I. Toth, Oral absorption studies of lipidic conjugates of thyrotropin releasing hormone (TRH) and luteinizing hormone-releasing hormone (LHRH), Inter. J. Pharm. (1996) 33–39.

[28] J. Wang, D.J. Hogenkamp, M. Tran, W.-Y. Li, R.F. Yoshimura, T.B.C. Johnstone, W.-C. Shen, K.W. Gee, Reversible lipidization for the oral delivery of leu-enkephalin, J. Drug Target. 14 (2006) 127–136.

[29] J. Wang, D. Chow, H. Heiati, W.-C. Shen, Reversible lipidization for the oral delivery of salmon calcitonin, J. Controll. Rel. 88 (2003) 369–380.

[30] J. Wang, D. Shen, W.-C. Shen, Preparation, Purification and Characterization of a reversibaly lipidized desmopressin with potentiated anti-diuretic activity, Pharma. Res. 16 (1999) 1674–1679.

[31] J.T. Blanchfield, I. Toth, in: J. Howl (Ed.), Peptide Synthesis and Applications, The Humana Press Inc, Totowa, 2005, pp. 45–61.

[32] M. Schnolzer, P.F. Alewood, A. Jones, D. Alewood, S.B.H. Kent, *In situ* neutralization in Boc-chemistry solid phase peptide synthesis, Inter. J. Pep. Pro. Res. 40 (1992) 180–193.