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Comparison of the in vitro apparent permeability and stability of opioid mimetic compounds with that of the native peptide

Yasuko Koda,[a] Kimitaka Shiotani,[b] Istvan Toth,[a,c] Yuko Tsuda,[b] Yoshio Okada[b] and Joanne T. Blanchfield[c,*]

[a]*School of Pharmacy, University of Queensland, St. Lucia, Qld 4072, Australia*
[b]*Department of Medicinal Chemistry, Faculty of Pharmaceutical Sciences, and The Graduate School of Food and Medicinal Sciences, Kobe Gakuin University, Nishi-ku, Kobe 651-2180, Japan*
[c]*School of Molecular and Microbial Sciences, University of Queensland, St. Lucia, Qld 4072, Australia*

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Abstract—Three dimethyl-L-tyrosine (Dmt) based peptide analogues were identified in a previous study as excellent agonists for the μ-opioid receptor showing very low K_i values and good in vivo antinociceptive activity upon intracerebroventricular administration to mice. This activity decreased markedly when the compounds were delivered subcutaneously or orally. To establish the cause of this decrease of activity the apparent permeability across Caco-2 cell monolayers of each compound and their relative stability to the digestive enzymes present in the cell line has been determined and compared to that of the native peptide endomorphin 2. The compounds' permeabilities clearly correlate with their increasing lipophilicity suggesting that the analogues cross the monolayer via passive diffusion and the results show that the compound with high K_i value for the μ-receptor ($K_i\mu$ = 0.114 nM) exhibited the highest permeability suggesting that this may be the better lead compound despite the lower binding affinity than that of compound **2** or **3**.

The last several years have seen significant advances in identification and synthesis of short peptides which exhibit high affinity and selectivity for the various opioid receptors (μ-, δ-, κ-).[1] These opioid peptides, such as endomorphin 2 (Endo2: H-Tyr-Pro-Phe-Phe-NH_2), are potential therapeutic agents; however peptides suffer from poor bioavailability due to their size and hydrophilic nature which lead to poor membrane permeability and their susceptibility to enzymatic degradation in the gastrointestinal (GI) tract. As opioid peptides largely act in the central nervous system (CNS), the blood–brain barrier (BBB) is an additional significant barrier to their effectiveness as drug candidates. Peptides are unable to cross this barrier and therefore require modification or conjugation to delivery agents which facilitate transport across the BBB. There are many strategies employed to improve the bioavailability of peptides and many of them involve reducing the number of peptide bonds in the molecule while retaining the chemical characteristics and spatial orientation of the functional groups important for receptor binding.[2]

Compounds **1**–**3** have emerged as important lead compounds in a study aimed at developing simple opioid mimetics incorporating the minimal functionality required for opioid receptor recognition and activation (Fig. 1).[3,4] The compounds were developed as mimetics of the endogenous opioid peptides endomorphin 1 and 2. Compound **3** consists of two 2′,6′-dimethyl-L-tyrosine (Dmt) residues that are essential for receptor binding, separated by a four-carbon alkyldiamine linker. This compound exhibited very high-affinity for the μ-opioid receptor ($K_i\mu$ = 0.041 nM)[3] and excellent selectivity for this receptor subtype over the δ-receptor (Table 1). Compound **3** rapidly produced centrally mediated analgesia after intracerebroventricular (icv) injection in mice and was 1.5–2.2 times more potent than morphine when assessed by the tail-flick test (spinal nociception) and equivalent in potency to morphine in the hot-plate test (supraspinal nociception). This antinociceptive activity was evident when the compound was administered subcutaneously (sc), although decreased considerably (Table 1), suggesting that BBB permeability is a major issue for this compound.[3] Compounds **1** and **2** consist

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* Corresponding author. Tel.: +61 7 33653622; fax: +61 7 33654299; e-mail: j.blanchfield@uq.edu.au

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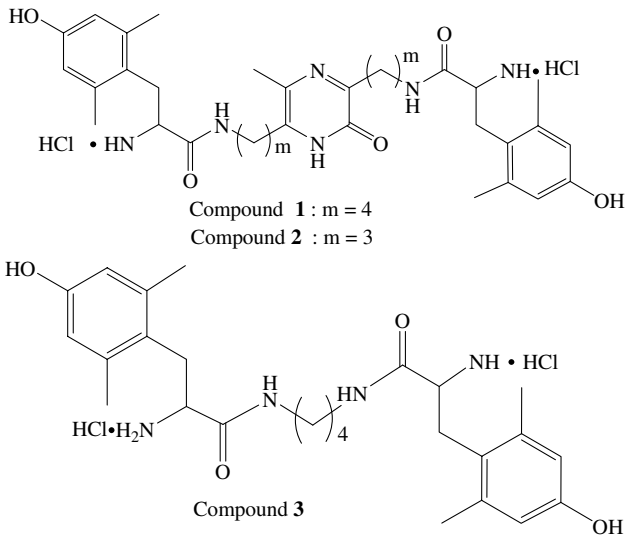

Figure 1. The structures of opioid peptide mimetics, compounds **1**–**3**. Compound **4**, endomorphin 2 H-Tyr-Pro-Phe-Phe-NH_2.

of the Dmt residues separated by the more conformationally rigid 3,6-bis-(aminoalkyl)-2(1*H*)-pyrazinone linker. Compound **1** exhibited good μ-opioid binding affinity with reasonable selectivity over the δ-receptor but was not assessed for in vivo activity. Compound **2**, however, exhibited excellent μ-opioid affinity, the same as that of compound **3** though with slightly lower selectivity (Table 1). Compound **2** was found to be 50–63 times more potent than morphine after icv injection in the tail-flick test and ~20 times more potent in the hot-plate test. After subcutaneous injection, the compound exhibited 63% and 55% potency compared to morphine in the two tests. Even after oral administration, compound **2** was found to be 42% as potent an analgesic as morphine in the tail-flick test and ~20% as potent in the hot-plate test.[4] These results indicated that compound **2**, in addition to having very high receptor affinity, is able to cross both the BBB and the GI tract.

The identification of these lead compounds (**1**–**3**) highlighted the fact that, while we can achieve excellent opioid receptor binding efficiencies compared to the opiate alkaloids.[5] with pseudopeptides and mimetics based on the endogenous opioid peptides, BBB permeability, and/or stability remain significant hurdles for the development of these compounds as pharmaceutics. In this current study we looked more closely at the relative permeability and stability of these three compounds across an epithelial barrier and compared these directly to the permeability of the peptidic analogue endomorphin 2 (**4**) (H-Tyr-Pro-Phe-Phe-NH_2), which exhibited less than 5% antinociceptive activity compared with morphine by icv adimistration to mice in the tail-flick test, although it exhibited $K_i\mu$ value of 0.69 nM.[6] From the Caco-2 cell monolayer permeability results the factors effecting the permeability and possible mechanism of passage across the barrier can be determined as well as the compounds' stability to the digestive enzymes present in the monolayers.

We chose to use the Caco-2 cell monolayer system to examine our compounds in this study. The Caco-2 cell monolayer system has become a standard in vitro method for the prediction of oral bioavailability over 20 years,[7–10] and has also been used as an initial BBB screening model.[11–14] Caco-2 cells are derived from a human colorectal carcinoma and, when cultured under the correct conditions, will form highly polarized monolayers with tight junctions between individual cells.[15] They express most of the active and facilitative transporters present in the small intestine epithelial barrier including the peptide transporters, glucose transporters, and efflux pumps such as *p*-glycoprotein.[16–19] The Caco-2 cell monolayer forms a well-developed brush border on the apical side and they express the associated digestive enzymes.[20,21] including dipeptidyl peptidase IV (DPPIV; EC 3.4.14.5).[22] They are considered an excellent model of the epithelial barrier of the small intestine.

The experiments discussed herein were performed on compounds **1**–**4** (Fig. 1) synthesized and purified as previously described.[3,4] with 2′,6′-dimethyl-L-tyrosine being prepared according to the procedure of Dygos et al.[23] Endo2 was synthesized by solution-phase synthesis as described by Okada et al.[24] The opioid agonists **1**–**4** were soluble and stable in HBSS–25 mM-Hepes buffer (pH 7.4) at 37 °C. Their apparent permeability coefficients (P_{app}; cm/s) are detailed in Table 2 and Figure 2. These values were generated from Caco-2 cell monolayer permeability assays performed according to standard procedures described by Wu et al.[25,26] with sample quantification achieved by LC-MS.

Table 1. Previously reported opioid receptor binding affinity and in vivo analgesia produced by compounds **1**–**3** compared to morphine[3,4]

Compound	$K_i\mu$ (nM)	$K_{i\delta}$ (nM)	$K_i\mu/K_i\delta$	Tail-flick test[a]	Hot-plate test[a]
1[4]	0.114	23.2	204	Not tested	Not tested
2[4]	0.042	13.7	326	50–63 (icv)[b]	18–21 (icv)
				0.63 (sc)[c]	0.55 (sc)
				0.42(po)[d]	0.16–0.24 (po)
3[3]	0.041	53.4	1302	1.5–2.2 (icv)	~1 (icv)
				0.1–0.12 (sc)	5% (sc)
Morphine[5]	14			1	1

[a] x potency of morphine, derived from concentrations of the test compound that produced an equivalent analgesia as morphine.
[b] icv, intracerebroventricular administration.
[c] sc, subcutaneous administration.
[d] po, oral administration.

Table 2. P_{app} values for compounds **1**–**4**

	1	2	3	4
P_{app} (cm/s)	8.45×10^{-7}	5.47×10^{-7}	1.39×10^{-7}	6.43×10^{-8}
SD	2.11×10^{-7}	7.75×10^{-8}	2.22×10^{-8}	6.35×10^{-9}

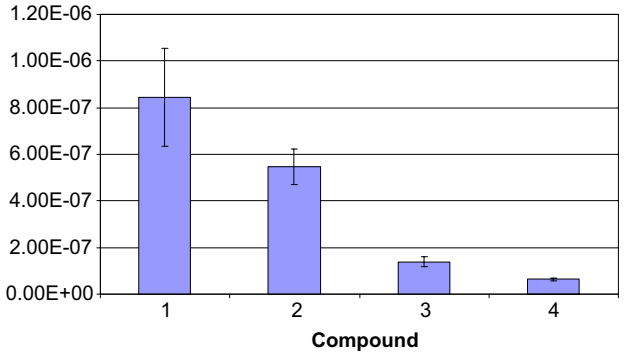

Figure 2. A graphical comparison of the P_{app} values obtained for compounds **1**–**4**.

Compounds **1** and **2**, bearing the more lipophilic linker between the Dmt moieties, are clearly significantly more permeable than the simple alkyldiamine-linked compound **3**. The observed P_{app} for compound **3** is very low (1.39×10^{-7} cm/s), even lower than the peptidic compound it is intended to mimic. The permeability of **3** is in the range considered to correspond to a compound with no oral bioavailability or BBB permeability.[7] This is consistent with the observed decrease in this compound's in vivo activity when administered subcutaneously compared to icv administration.

All three of the mimetic compounds exhibit significantly higher P_{app} values than the peptide **4**. This is primarily due to the rapid degradation of the native peptide by the digestive enzymes expressed by the Caco-2 cells. When a sample was removed from the apical chamber of the Caco-2 cell monolayer at the end of the experiment (2.5 h), the concentration of intact peptide remaining was only 0.81 μM from an original concentration of 200 μM. This almost complete destruction of the peptide is expected for a small native peptide such as Endo 2.

Compounds **1** and **2** both bear the central pyrazinone group with Dmt residues attached through linkers to each side of the core. Compound **1** has an extra methylene group on each of the appended alkyl chains making it more lipophilic than compound **2**. It has already been demonstrated that the presence of these longer alkyl chains causes a decrease in opioid receptor binding affinity; however they also appear to provide sufficient increase in lipophilicity to facilitate an increase in the permeability across the Caco-2 cell monolayer ($P_{app} = 8.45 \times 10^{-7}$ cm/s). The P_{app} of **2** (Table 2) is indicative of a compound that would exhibit some oral bioavailability though would not be considered completely orally available.[7] This is in keeping with the in vivo results for compound **2** which showed both BBB and GI tract permeability. From these new results it would be expected that compound **1** would exhibit greater permeability across both the BBB and GI tract which may, in part, compensate for the drop in opioid receptor binding affinity.

While the mimetic compounds do not appear to provide considerable improvement to the permeability of the compounds compared to the peptidic form, they are significantly more metabolically stable than the peptide **4**. On completion of the permeability assays, a sample from the apical chamber of each transwell was examined to determine if the test compound had been degraded over the 2.5 h of the experiment. For compounds **1**–**3**, the compounds were found intact and there appeared to be no loss due to protein binding, absorption into the membranes of the cells or enzymatic degradation. As previously mentioned, peptide **4** was almost completely destroyed in this time. This indicates that the all three mimetic compounds are much more stable to the digestive enzymes present in the Caco-2 cell monolayer than the analogous peptide.[20,21]

The monolayer integrity was evaluated both at the beginning and the end of the experiment by monitoring the transepithelial electrical resistance (TEER) values of each monolayer and the determination of the permeability of ^{14}C-mannitol. The TEER values for each well were between 2.5 and 3.6 kΩ cm^2 at the commencement of the assay, indicating confluent monolayers with well-established tight junctions.[27] and by the end of the assay the values had not changed by more than ±0.5 kΩ cm^2. This result shows that the compounds are not toxic to the cells in any way and do not disrupt the tight junctions in the monolayer. Mannitol is a compound known to have no oral bioavailability and pass through the Caco-2 cell monolayer via paracellular absorption and so it exhibits a very low P_{app}. Our value of 5.85×10^{-8} cm/s ($\pm 4.62 \times 10^{-9}$) is well within those reported in the literature and indicates healthy and tight monolayers.[7]

The relative permeability of compounds **1**–**4** clearly correlates to their increasing lipophilicity which suggests that they cross the monolayer via passive diffusion and not via the use of a transporter. Increasing lipophilicity increases the permeability of the compound in the lipid bilayer of the cell membranes and thus, the opportunity for passive diffusion. The involvement of a transporter or of efflux pumps such as *p*-glycoprotein can be eliminated entirely by future experiments using the same Caco-2 cell monolayer system. Compounds **1**–**3** were designed as mimetics of peptide **4** and our results clearly indicate that the mimetic compounds exhibit greatly improved metabolic stability compared to **4**, with compound **1** showing the greatest increase in permeability across Caco-2 cell monolayers. Though compound **1** was not tested in vivo, these permeability results suggest that, perhaps an improved permeability may be able to compensate for the lower opioid receptor binding which was, after all, still higher than morphine.

The results of these permeability assays, in conjunction with the previously reported functional assays, provide key data to direct the further development of these opioid mimetics. We have identified the pyrazinone moiety as an

important central unit and identified the key alkyl chain length by which to attach the vital Dmt residues. We have also established that enzyme stability is not a factor in the compounds' poor bioavailability but that lipophilicity is the overriding factor. Future efforts should be directed towards increasing the lipophilicity of compound **2** without altering the spatial orientation or distance between the two Dmt residues. This may be done by incorporating a lipid moiety via a prodrug linkage that can be cleaved in vivo or by cyclisation of the compound.[28,29] We could also investigate the incorporation of sugar moieties in an attempt to increase the BBB permeability of the compound by the use of sugar transporters.

References and notes

1. Bryant, S. D.; Jinsmaa, Y.; Salvadori, S.; Okada, Y.; Lazarus, L. H. *Biopolymers* **2003**, *71*, 86.
2. Adessi, C.; Soto, C. *Curr. Med. Chem.* **2002**, *9*, 963.
3. Okada, Y.; Tsuda, Y.; Fujita, Y.; Yokoi, T.; Sasaki, Y.; Ambo, A.; Konishi, R.; Nagata, M.; Salvadori, S.; Jinsmaa, Y.; Bryant, S. D.; Lazarus, L. H. *J. Med. Chem.* **2003**, *46*, 3201.
4. Jinsmaa, Y.; Miyazaki, A.; Fujita, Y.; Li, T. Y.; Fujisawa, Y.; Shiotani, K.; Tsuda, Y.; Yokoi, T.; Ambo, A.; Sasaki, Y.; Bryant, S. D.; Lazarus, L. H.; Okada, Y. *J. Med. Chem.* **2004**, *47*, 2599.
5. Poisnel, G.; Quentin, T.; Barre, L.; Debruyne, D. *J. Neurosci. Methods* **2006**, *154*, 60.
6. Li, T. Y.; Fujita, Y.; Tsuda, Y.; Miyazaki, A.; Ambo, A.; Sasaki, Y.; Jinsmaa, Y.; Bryant, S. D.; Lazarus, L. H.; Okada, Y. *J. Med. Chem.* **2005**, *48*, 586.
7. Artursson, P.; Karlsson, J. *Biochem. Biophys. Res. Commun.* **1991**, *175*, 880.
8. Wong, A. K.; Ross, B. P.; Chan, Y. N.; Artursson, P.; Lazorova, L.; Jones, A.; Toth, I. *Eur. J. Pharm. Sci.* **2002**, *16*, 113.
9. He, X.; Sugawara, M.; Kobayashi, M.; Takekuma, Y.; Miyazaki, K. *Int. J. Pharm.* **2003**, *263*, 35.
10. Zornoza, T.; Cano-Cebrian, M. J.; Nalda-Molina, R.; Guerri, C.; Granero, L.; Polache, A. *Eur. J. Pharm. Sci.* **2004**, *22*, 347.
11. Cruciani, G.; Pastor, M.; Guba, W. *Eur. J. Pharm. Sci.* **2000**, *11*, S29.
12. van der Sandt, I. C. J.; Vos, C. M. P.; Nabulsi, L.; Blom-Roosemalen, M. C. M.; Voorwinden, H. H.; de Boer, A. G.; Breimer, D. D. *Aids* **2001**, *15*, 483.
13. Lohmann, C.; Huwel, S.; Galla, H. J. *J. Drug Target* **2002**, *10*, 263.
14. Garberg, P.; Ball, M.; Borg, N.; Cecchelli, R.; Fenart, L.; Hurst, R. D.; Lindmark, T.; Mabondzo, A.; Nilsson, J. E.; Raub, T. J.; Stanimirovic, D.; Terasaki, T.; Oberg, J. O.; Osterberg, T. *Toxicol. In Vitro* **2005**, *19*, 299.
15. Artursson, P.; Palm, K.; Luthman, K. *Adv. Drug Delivery Rev.* **2001**, *46*, 27.
16. Mahraoui, L.; Rodolosse, A.; Barbat, A.; Dussaulx, E.; Zweibaum, A.; Rousset, M.; Brotlaroche, E. *Biochem. J.* **1994**, *298*, 629.
17. Suzuki, H.; Sugiyama, Y. *Eur. J. Pharm. Sci.* **2000**, *12*, 3.
18. Goto, M.; Masuda, S.; Saito, H.; Inui, K. *Biochem. Pharmacol.* **2003**, *66*, 163.
19. Kipp, H.; Khoursandi, S.; Scharlau, D.; Kinne, R. K. H. *Am. J. Physiol-Cell Physiol.* **2003**, *285*, C737.
20. Howell, S.; Kenny, A. J.; Turner, A. J. *Biochem. J.* **1992**, *284*, 595.
21. Howell, S.; Brewis, I. A.; Hooper, N. M.; Kenny, A. J.; Turner, A. J. *FEBS Lett.* **1993**, *317*, 109.
22. Erickson, R. H.; Lai, R. S.; Kim, Y. S. *Biochem. Biophys. Res. Commun.* **2000**, *270*, 235.
23. Dygos, J. H.; Yonan, E. E.; Scaros, M. G.; Goodmonson, O. J.; Getman, D. P.; Periana, R. A.; Beck, G. R. *Synthesis—Stuttgart* **1992**, 741.
24. Okada, Y.; Fukumizu, A.; Takahashi, M.; Shimizu, Y.; Tsuda, Y.; Bryant, S. D.; Lazarus, L. H. *Biochem. Biophys. Res. Commun.* **2000**, *7*, 276.
25. Wu, S.; Campbell, C.; Koda, Y.; Blanchfield, J. T.; Toth, I. *Med. Chem.* **2006**, *2*, 203.
26. Permeability assays were performed in the apical to basolateral direction in the absence of inhibitors. Cells were used 25 days after being subcultured onto Transwell® plates (0.45 μm × 6.5 mm diameter). Samples taken from the basolateral chamber were analysed by LCMS and the apparent permeability (P_{app}) calculated using the following formula:

$$P_{app} = dC/dt \times V_r/(A \times C_0)$$

dC/dt, steady-state rate of change in the chemical concentration (mol/s) or radiochemical concentration (dpm/mL/s) in the receiver chamber, V_r, volume of the receiver chamber (mL), A, surface area of the cell monolayers, and C_0, initial concentration in the donor chamber (mol or dpm/mL).
27. Konsoula, R.; Barile, F. A. *Toxicol. In Vitro* **2005**, *19*, 675.
28. Pauletti, G. M.; Gangwar, S.; Siahaan, T. J.; Aube, J.; Borchardt, R. T. *Adv. Drug Delivery Rev.* **1997**, *27*, 235.
29. Borchardt, R. T. *J. Controlled Release* **1999**, *62*, 231.