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In Vivo Pharmacokinetics of Selective μ-Opioid Peptide Agonists

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ABSTRACT

Recent evidence suggests that highly selective μ-opioid agonists may provide good analgesia with less development of tolerance and dependence. H-Tyr-D-Arg-Phe-Lys-NH_2 (DALDA) and H-Dmt-D-Arg-Phe-Lys-NH_2 ([Dmt[1]]DALDA) were found to display high binding affinity and much greater selectivity for the μ-opioid receptor ($K_i^{\delta}/K_i^{\mu} > 10{,}000$) compared with H-Tyr-D-Ala-Gly-MePhe-Gly-ol (DAMGO). In addition, [Dmt[1]]DALDA was 3000-fold more potent than morphine when administered intrathecally. A potential problem with peptide analogs as therapeutic agents is their susceptibility to enzymatic degradation in vivo and short elimination half-lives. In this study, we compared the stability of DAMGO, DALDA, and [Dmt[1]]DALDA after systemic administration in sheep. Peptide concentrations were measured using high performance liquid chromatography-mass spectrometry. When incubated in sheep blood at 37°C, DAMGO, DALDA, and [Dmt[1]]DALDA were stable over 2 h. When given intravenously to sheep, the apparent volume of distribution was 50 to 80 ml/kg for all three peptides, suggesting that distribution was limited to blood volume. Plasma clearance of DAMGO (223 ml/kg/h) was 10-fold faster than DALDA and [Dmt[1]]DALDA (24 ml/kg/h), and their elimination half-lives were 0.24, 1.5, and 1.8 h, respectively. The half-lives of DALDA and [Dmt[1]]DALDA are even longer than morphine or meperidine in sheep. These favorable pharmacokinetic properties of DALDA and [Dmt[1]]DALDA, together with their μ-selectivity, potency, and long duration of action, make them ideal candidates as opioid analgesics.

There is accumulating evidence that the μ-opioid receptor plays a more important role in analgesia than the δ-opioid receptor. Although δ-selective opioid agonists mediate spinal and supraspinal analgesia, more recent data with μ-opioid receptor knockout animals suggest that the presence of μ-opioid receptors is essential for antinociceptive action of δ-selective opioid agonists (Sora et al., 1997; Matthes et al., 1998). In addition, there is evidence that δ-selective opioid agonists produce more respiratory depression than μ-selective opioid agonists (Szeto et al., 1999). These results led to the proposal that highly selective μ-opioid agonists may have an advantage over existing opiate analgesics such as morphine.

Early attempts at development of highly selective ligands for the μ-opioid receptor were based on modifications of the enkephalin peptides (H-Tyr-Gly-Gly-Phe-Met(or Leu)-OH). One major drawback of the natural opioid peptides is their susceptibility to rapid enzymatic degradation. It was found that the stability of the enkephalin peptides could be significantly improved by substitution of D-amino acids in position 2 of the peptide sequence and through amidation of the C-terminal carboxyl group (Pert et al., 1976). This finding led to the development of H-Tyr-D-Ala-Gly-MePhe-Gly-ol (DAMGO), which remains the most popular μ-selective enkephalin analog (Handa et al., 1981). DAMGO has high affinity for the μ-opioid receptor and is 1000-fold more selective for the μ-opioid receptor compared with the δ-opioid receptor (Schiller et al., 1989, 1990).

More recent developments of μ-selective opioid agonists have been based on modifications of the dermorphin sequence. Dermorphin (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) is a natural peptide found in amphibian skin (Montecucchi et al., 1981). This heptapeptide shows moderate μ-selectivity (~300-fold) (Sagan et al., 1989) and is relatively more resistant to enzymatic hydrolysis in plasma (Scalia et al., 1986). However, degradation of dermorphin by tissue peptidases resulted in the N-terminal tetrapeptide H-Tyr-D-Ala-Phe-Gly-OH, which has a μ-selectivity similar to the

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ABBREVIATIONS: DAMGO, H-Tyr-D-Ala-Gly-MePhe-Gly-ol; DALDA, H-Tyr-D-Arg-Phe-Lys-NH_2; [Dmt[1]]DALDA, H-Dmt-D-Arg-Phe-Lys-NH_2; Dmt, 2′,6′-dimethyltyrosine; DPDPE, H-Tyr-c[D-Pen-Gly-Phe-D-Pen]-OH; MS, mass spectrometry; CL, clearance.

parent peptide (Sasaki et al., 1985; Scalia et al., 1986). Amino acid substitutions of this tetrapeptide have led to the development of the two most selective μ-opioid agonists, DALDA (H-Tyr-D-Arg-Phe-Lys-NH_2) and [Dmt^1]DALDA (H-Dmt-D-Arg-Phe-Lys-NH_2) (Schiller et al., 1989, 2000). DALDA and [Dmt^1]DALDA both displayed high binding affinity and extraordinary selectivity ($K_i^{\delta}/K_i^{\mu} > 10{,}000$) for the μ-opioid receptor (Schiller et al., 2000). Replacement of the Tyr^1 in DALDA with Dmt produced a 180-fold potency enhancement in the guinea pig ileum assay (Schiller et al., 2000). When administered intrathecally to rats, DALDA and [Dmt^1]DALDA were found to have 14- and 3000-times, respectively, higher analgesic potency compared with morphine in the rat tail-flick test (Shimoyama et al., 2001). In addition, the duration of analgesia was significantly longer after DALDA and [Dmt^1]DALDA administration when compared at equieffective doses.

The incorporation of D-Arg^2 and amidation of Lys^4 in DALDA and [Dmt^1]DALDA are likely to enhance the stability of these two peptide analogs against enzymatic hydrolysis. However, D-amino acid substitutions do not necessarily protect against in vitro enzymatic degradation (Darlak et al., 1988). Furthermore, in vitro degradation studies may not reveal the pharmacokinetics of these peptide analogs in vivo. For example, although H-Tyr-c[D-Pen-Gly-Phe-D-Pen]-OH (DPDPE) was relatively stable when incubated with purified enkephalinase, its elimination half-life was only 10 min after intravenous administration to rats (Chen and Pollack, 1996). The short half-life in vivo was apparently due to extensive biliary excretion (Chen and Pollack, 1997). We previously found that the elimination half-life of DALDA in sheep after intravenous administration was 1.4 h (Szeto et al., 1998), suggesting that this substituted dermorphin analog is much more stable in vivo compared with the enkephalin analog. In this study, we compared the stability of DAMGO, DALDA, and [Dmt^1]DALDA when incubated in plasma, and their in vivo pharmacokinetics in sheep after intravenous administration.

Materials and Methods

Animal Preparation. Chronic indwelling catheters were surgically placed in the descending aorta and inferior vena cava of adult female sheep under epidural anesthesia, according to guidelines approved by the Institution for the Care and Use of Animals at Weill Medical College of Cornell University. Five or more days was allowed for recovery from surgery prior to experimental studies.

Compounds. DAMGO and DALDA were provided by the National Institute on Drug Abuse (Rockville, MD). [Dmt^1]DALDA were prepared by solid-phase synthesis as described previously (Schiller et al., 2000). For the synthesis of the deuterated peptide analogs, Boc-Phe(d_5)-OH was used in place of Boc-Phe-OH. Pentadeuterophenylalanine was purchased from C/D/N Isotope, Vaudreuil, Quebec, Canada, and was converted to Boc-Phe(d_5)-OH by reacting with di-*tert*-butyldicarbonate. The deuterated peptides were purified by semipreparative reversed-phase high performance liquid chromatography (Schiller et al., 1989). The purity of all peptides was verified by fast atom bombardment-MS, and the correct amino acid sequence was confirmed by tandem mass spectrometry (Schiller et al., 1989).

In Vitro Degradation Studies. To study the degradation of the three peptide analogs in blood, DAMGO (50 μg), DALDA (10 μg), or [Dmt^1]DALDA (10 μg) was added to 25 ml of freshly collected sheep blood, and the mixture was incubated in a water bath at 37°C. Three milliliters of blood was removed from the blood sample at 15 s and at 5, 15, 30, 60, and 120 min after peptide addition. The blood sample was gently mixed throughout the entire incubation period.

In Vivo Pharmacokinetic Studies. The peptides were administered as constant rate intravenous infusions to sheep. Based on the known elimination half-life of ~1.5 h for DALDA (Szeto et al., 1998), it was estimated that a 4-h infusion would allow plasma drug levels to approach steady-state levels. DALDA (0.6 mg/kg/h) and [Dmt^1]DALDA (0.06 mg/kg/h) were, therefore, infused via the venous catheter for a period of 4 h. [Dmt^1]DALDA was infused at a lower dose because it was found to be 200-fold more potent than DALDA after intrathecal administration in the rat tail-flick test (Shimoyama et al., 2001) and 100-fold more potent than DALDA in increasing blood pressure in sheep (Szeto et al., 2001). The current limit of sensitivity of the analytical method prevented the use of an even lower dose of [Dmt^1]DALDA. Blood samples (5 ml) were collected from the arterial catheter at 0, 1, 2, 3, 3.5, 4, 4.25, 4.5, 5, 6, and 7 h. Because preliminary data showed much more rapid elimination of DAMGO in sheep, DAMGO (0.6 mg/kg/h) was only infused for 3 h, and blood samples were collected at 0, 0.5, 1, 2, 2.5, 3, 3.25, 3.5, 3.75, 4, and 4.5 h.

Quantitative Analysis of DAMGO, DALDA, and [Dmt^1]DALDA. All blood samples were collected into chilled borosilicate glass tubes that contained EDTA, and were centrifuged; the plasma was stored in glass containers with Teflon-lined caps and frozen at −80°C. All three peptides were separated by high performance liquid chromatography and quantified with mass spectrometry. Details of the quantitative method for DALDA and DAMGO have been published (Grigoriants et al., 1997; Desiderio et al., 2000) and will only be presented briefly here. All plasma samples (300 μl) were deproteinated and eluted through a solid phase extraction cartridge (Sep-Pak C18; Millipore Corporation, Milford, MA) with CH_3CN. An internal standard, the respective deuterated peptide analog [H-Tyr-D-Ala-Gly-MePhe(d_5)-Gly-ol, H-Tyr-D-Arg-Phe(d_5)-Lys-NH_2, or H-Dmt-D-Arg-Phe(d_5)-Lys-NH_2], was added to each plasma sample before deproteinization. The filtered plasma sample was chromatographed on an RP-analytical column (Delta Pak, 5 μm, C18, 100 129, 150 × 3.9 mm; Waters, Milford, MA) at a flow rate of 1.5 ml min^{-1}, and UV absorption was monitored at 200 nm (Varian, Walnut Creek, CA). Gradient elution (7 → 30% acetonitrile in 0.1% trifluoroacetic acid; 30 min) was used. One-minute fractions were collected, and each fraction was lyophilized for MS analysis. A matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Voyager-DE RP Biospectrometry Workstation; PerSeptive Biosystems Inc., Framingham, MA) was used to quantify the peptide in each plasma sample. The $(M + H)^+$ ion current for each peptide was compared with the ion current from the d_5-peptide. $(M + H)^+$ data were used to quantify DALDA and [Dmt^1]DALDA because no intense signal that included the d_5 label was available. A postsource decay fragment was available for DAMGO. The lower limit of detection was 0.05 μg/ml for DALDA and [Dmt^1]DALDA, and 0.02 μg/ml for DAMGO.

Pharmacokinetic Analyses. Plasma levels of DAMGO, DALDA, and [Dmt^1]DALDA during and after the constant rate infusion were subjected to compartmental analysis using nonlinear regression (WinNonlin, version 1.1; Scientific Consulting Inc., Apex, NC). A one-compartment open model with constant input and first order output (model 2; WinNonlin Library) was used for fitting pooled data from all animals. Uniform weighting was used based on the assumption that the observed concentration ranges were relatively small. Initial parameter estimates for apparent volume of distribution (Vd) and the elimination rate constant (k_{10}) were computed by WinNonlin using curve stripping. Secondary parameters were derived from the final parameter estimates and included elimination half-life ($t_{1/2}$) and clearance (CL). For the secondary parameters, standard errors were obtained by computing the linear term of a Taylor series expansion of the secondary parameters (WinNonlin).

Results

Figure 1A illustrates the plasma concentrations of DAMGO at various times after incubation in sheep blood at 37°C (n = 3). There was no significant change in DAMGO concentration over the 2-h interval. Figure 1B shows plasma DAMGO concentrations in sheep during and after infusion of DAMGO at 0.6 mg/kg/h for 3 h (n = 4). Plasma DAMGO levels rose rapidly and approached steady state approximately 2 h after the start of infusion. Upon termination of drug infusion, plasma DAMGO concentrations declined rapidly and were below our detection limit after 1 h. The plasma concentration data were fitted to a one-compartment open model and the calculated pharmacokinetic parameters are summarized in Table 1.

When DALDA was incubated in vitro in sheep blood, plasma concentration did not change significantly over the 2-h interval (n = 3) (Fig. 2A). Figure 2B shows plasma DALDA concentrations in sheep during and after the 4-h intravenous infusion (n = 5). Plasma DALDA concentrations increased much more slowly than DAMGO and did not approach steady state until 3 h after the start of infusion. The decline in plasma DALDA concentrations was also much slower than DAMGO, and detectable peptide levels were found in all animals up to 3 h after termination of peptide infusion. The estimated and derived pharmacokinetic parameters for DALDA are summarized in Table 1.

[Dmt^1]DALDA was also very stable when incubated in blood in vitro (n = 3) (Fig. 3A). When infused to sheep, plasma [Dmt^1]DALDA concentrations continued to increase throughout the infusion period and the decline in plasma [Dmt^1]DALDA concentrations was much slower than DAMGO (Fig. 3B; n = 5). The results of the pharmacokinetic analysis are summarized in Table 1.

Discussion

Although synthetic opioid peptide analogs have been used extensively to study the functional roles of opioid receptor subtypes, little information is available on the pharmacokinetics of these peptide analogs in vivo. The endogenous opioid peptides are known to be highly susceptible to peptidases, and their elimination half-lives are very short (<2 min) when administered systemically. Amino acid modifications such as substitution with D-amino acids are thought to improve stability, but there is little evidence that these substituted peptides have significantly longer elimination half-lives when administered systemically. Few studies have been conducted to characterize the pharmacokinetics of peptides in vivo because of the lack of specific and sensitive analytical methods. In this article, we used state-of-the-art mass spectrometric methods to compare the stability of three opioid peptide analogs in vitro and in vivo. The combination of MS and a stable isotope-incorporated internal standard provided the highest level of molecular specificity for the quantification of these peptides.

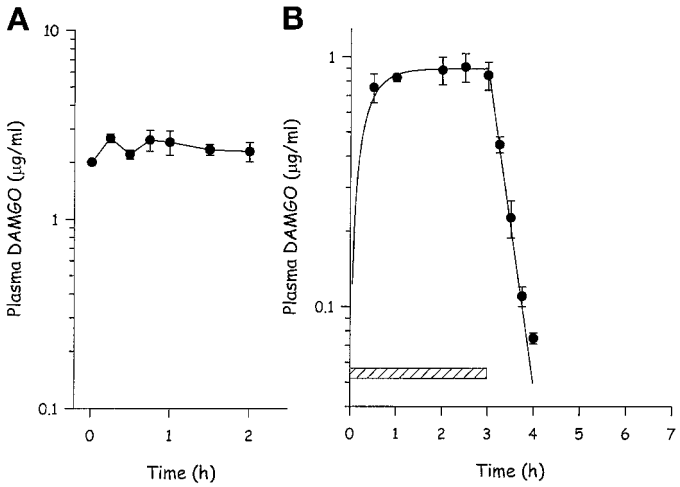

Fig. 1. A, plasma DAMGO concentration at various times after 50 μg of DAMGO was incubated in sheep blood at 37°C (n = 3). B, plasma DAMGO concentrations during and after the infusion of DAMGO (0.6 mg/kg/h) to sheep (n = 4). Data shown are mean ± S.E. Symbols represent observed data and the line represents predicted data according to the one-compartment open model as described under *Materials and Methods*.

Our results show that all three D-amino acid-substituted peptide analogs are resistant to peptidases when incubated in blood in vitro. This stability is likely to be due to the presence of a D-amino acid in position 2 of the peptide sequence in all three peptides, an *N*-methylated Phe^4 residue and C-terminal alcohol function in DAMGO, and a C-terminal carboxamide group in DALDA and [Dmt^1]DALDA.

The plasma concentration-time curves after intravenous infusion to sheep were different for the three peptides but were all adequately described by a one-compartment open model, with correlation being >0.99. The apparent volume of distribution values for DAMGO, DALDA, and [Dmt^1]DALDA were all in the range of 50 to 80 ml/kg, which is equivalent to blood volume in sheep. The limited distribution of DALDA and [Dmt^1]DALDA is consistent with the polar character of these two highly charged (3+) peptides at physiological pH. The presence of the two additional methyl groups in the Dmt^1 residue makes [Dmt^1]DALDA somewhat less polar than DALDA, but it is still quite hydrophilic (Schiller et al., 2000). The limited distribution of these peptides is consistent with previous findings of a very restrictive distribution of DALDA across the blood-brain barrier (Schiller et al., 1990; Samii et al., 1994) and placental barrier (Szeto et al., 1998). It is interesting that the distribution of DAMGO was also quite limited; it is 4-fold smaller than the volume of distribution for DPDPE (76 versus 296 ml/kg) (Chen and Pollack, 1996). The cyclic [D-Pen^2-D-Pen^5] structure may reduce hydrogen bonding and enhance the ability of DPDPE to distribute across lipid membranes.

Our results show that in vitro stability was not predictive of in vivo pharmacokinetics of these peptide analogs. When administered intravenously, DAMGO was cleared very rapidly, with an elimination half-life of only 15 min. The clearance for DAMGO was calculated to be 223 ml/kg/h, which is similar to creatinine clearance in sheep (~210 ml/kg/h) (English et al., 1977). This finding might suggest that DAMGO is eliminated via renal glomerular filtration, provided that DAMGO is not significantly bound to plasma proteins and is excreted unchanged in urine. This possibility will be addressed in future studies. The half-life estimated for DAMGO in sheep is similar to the half-life reported for DPDPE, another D-amino acid-substituted enkephalin analog, in rats (Chen and Pollack, 1996). The clearance of DPDPE was reported to be ~22.5 ml/kg/min or ~1350 ml/kg/h, and this high clearance was attributed to extensive biliary excretion (Chen and Pollack, 1997). The 10-fold lower clearance of DAMGO

TABLE 1
Pharmacokinetic parameters derived from compartmental analysis in sheep

Peptide	n	Vd	k_{10}	$t_{1/2}$	CL	MRT	Correlation
		ml/kg	h^{-1}	h	$ml/kg/h$	h	r
DAMGO	4	76.4 ± 4.2	2.92 ± 0.16	0.24 ± 0.01	223 ± 3.7	0.34 ± 0.02	0.9974
DALDA	5	50.2 ± 2.7	0.47 ± 0.03	1.48 ± 0.10	23.5 ± 0.7	2.14 ± 0.15	0.9918
[Dmt[1]]DALDA	5	61.0 ± 2.9	0.39 ± 0.03	1.77 ± 0.12	23.9 ± 0.7	2.55 ± 0.17	0.9919

MRT, mean residence time.

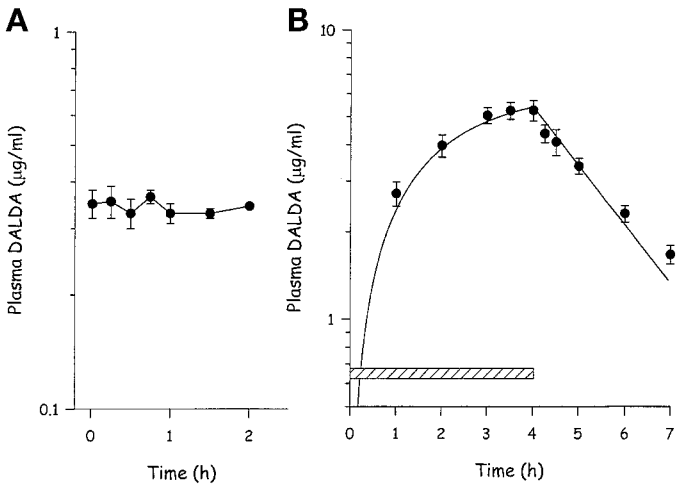

Fig. 2. A, plasma DALDA concentration at various times after 10 μg of DAMGO was incubated in sheep blood at 37°C (n = 3). B, plasma DALDA concentrations during and after the infusion of DALDA (0.6 mg/kg/h) to sheep (n = 5). Data shown are mean ± S.E. Symbols represent observed data and the line represents predicted data according to a one-compartment open model as described under *Materials and Methods*.

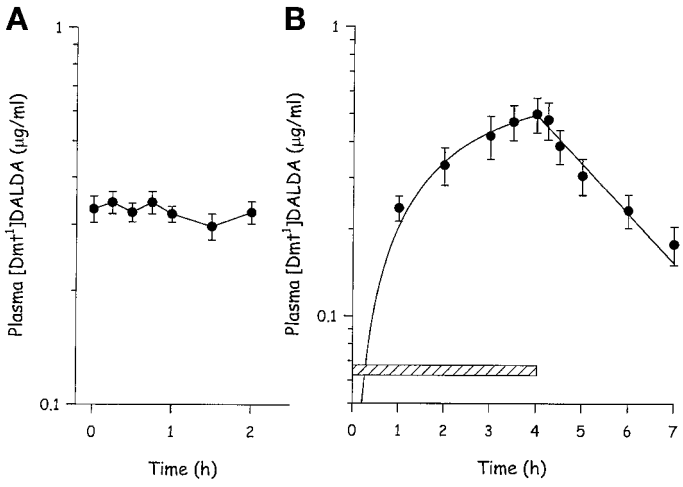

Fig. 3. A, plasma [Dmt[1]]DALDA concentration at various times after 10 μg of [Dmt[1]]DALDA was incubated in sheep blood at 37°C (n = 3). B, plasma [Dmt[1]]DALDA concentrations during and after the infusion of [Dmt[1]]DALDA (0.06 mg/kg/h) to sheep (n = 5). Data shown are mean ± S.E. Symbols represent observed data and the line represents predicted data according to a one-compartment open model as described under *Materials and Methods*.

suggests that it is unlikely to undergo extensive biliary excretion.

In contrast to DAMGO and DPDPE, the clearance of DALDA and [Dmt[1]]DALDA were much slower, and their elimination half-lives were 6 and 7 times longer, respectively. Given that these peptides carry a 3+ charge at pH 7.4, it is rather surprising that their clearances (23 ml/kg/h) are so much less than creatinine clearance. The slower clearance and longer half-life for DALDA and [Dmt[1]]DALDA might suggest tubular reabsorption of these peptides. Proximal tubular cells of the kidney possess a specific transport system for small peptides that is H^+-dependent and electrogenic (Ganapathy and Leibach, 1986). This renal peptide transporter has been cloned, and is designated PEPT2 (Liu et al., 1995). Although generally thought to transport di- and tripeptides, PEPT2 has been shown to transport tetrapeptides, although at a much slower rate. Future studies will determine whether positively charged tetrapeptides such as DALDA and [Dmt[1]]DALDA are substrates for PEPT2.

Although substitutions with D-amino acids can protect against peptidase activity, the hydrophilic nature of small peptides makes them susceptible to glomerular filtration at the kidneys, and can account for the rapid elimination of the peptide from the circulation unless there is significant plasma protein binding or substantial tubular reabsorption. In the case of DPDPE, this peptide is removed rapidly from the circulation by biliary excretion (Chen and Pollack, 1997). Although all the opioid peptide analogs were highly stable when incubated in blood in vitro, the substituted dermorphin analogs (DALDA and [Dmt[1]]DALDA) were significantly more stable in vivo than substituted enkephalin analogs (DAMGO and DPDPE). Elimination half-lives of 1 to 2 h in sheep are considered to be very long because metabolic rates tend to be much faster in sheep compared with humans. For comparison, the elimination half-lives of opiate alkaloids such as morphine, methadone, and meperidine are only in the order of 20 to 30 min in sheep (Szeto et al., 1978, 1981, 1982). These favorable pharmacokinetic properties, coupled with their high affinity and selectivity for the μ-opioid receptor, make DALDA and [Dmt[1]]DALDA very promising as opioid analgesics. DALDA and [Dmt[1]]DALDA were found to be 14- and 3000-fold more potent than morphine after intrathecal administration in rats (Shimoyama et al., 2001). The analgesic action of [Dmt[1]]DALDA lasted 4 times longer than morphine, and [Dmt[1]]DALDA had a much lower propensity to produce respiratory depression. When administered to pregnant animals, DALDA and [Dmt[1]]DALDA only produced a transient increase in maternal blood pressure with no significant effect on other maternal or fetal hemodynamic, respiratory, or metabolic functions (Clapp et al., 1998). Thus, DALDA and [Dmt[1]]DALDA are both promising analgesic drug candidates.

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