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RESEARCH PAPER

Sustained Analgesia Achieved Through Esterase-Activated Morphine Prodrugs Complexed with PAMAM Dendrimer

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ABSTRACT

Purpose Design and evaluate the *in vitro and in vivo* efficacy of two extended release morphine formulations developed for IV administration by complexing esterase activated morphine prodrugs to surface-modified, generation 5 (G5) poly(amidoamine) (PAMAM) dendrimer.

Methods Prodrugs were synthesized, complexed with PAMAM dendrimer, characterized via ultra performance liquid chromatography (UPLC), nuclear magnatic resonance (NMR), and tested *in vitro* using rat plasma *vs*. saline control and in an *in vivo* rat and guinea pig pain model (modified Randall and Selitto test).

Results We demonstrated that complexation with dendrimer allowed the solubilization of the prodrugs for *in vivo* applications without the need for salt, and that the structural design of the morphine prodrugs allowed the controlled release of morphine which extended the action of morphine-induced analgesia in an animal pain model from 2 h (control) to 6 h (Morphine Prodrug A).

Conclusion The concept of complexing/solubilizing appropriately designed esterase-sensitive prodrugs with dendrimer to enhance the sustained release of these drugs may be a useful pharmacokinetic strategy for a range of therapeutics.

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INTRODUCTION

Safe and effective pain relief is an important medical need. This is particularly relevant when injured individuals have no immediate access to medical care, such as the battlefield setting (1). Injured patients forced to wait extended periods of time prior to evacuation to medical facilities are often given suboptimal dosages of analgesics due to concerns for side effects including respiratory depression and hypotension. Unfortunately, recent evidence suggests that when challenged with severe acute pain, the central nervous system responds by pathologic remodeling, which can lead to the development of chronic pain conditions (2). In addition, acute pain experienced on the battlefield has been associated with long-term psychological effects that are thought to contribute to post-traumatic stress disorder, as well as depression, anxiety, and substance abuse (3).

Parental administration of drugs is often the preferred, most reliable, efficient and effective route. Sustained-release formulations of morphine (e.g. MS Contin®) for oral use are only appropriate for sub-acute and chronic pain provided the alimentary tract is functioning, therefore limiting their utility in the acute setting. While parenteral narcotics such as morphine and fentanyl can provide analgesia for up to 4 h in humans (4), potential side effects such as respiratory depression and hypotension make it difficult to dose them at appropriate levels, particularly during emergency situations. Given this, there is a need for a drug formulation that can provide both safer and more prolonged pain relief than what is currently available.

Dendrimers are a class of highly branched, monodispersed, synthetic macromolecules with a well-defined

composition and architecture (5). Generally spherical in shape, the core, interior structure and surface groups of these macromolecules can be tailored for specific biomedical applications. For example, the surface groups can be used as covalent attachment sites for a variety of molecules (6–10), while the interior structure can be used to encapsulate or complex molecules through electrostatic and/or hydrophobic/hydrophilic interactions, enhancing their solubility and bioavailability (11–17). This versatility has made dendrimer particularly attractive for use in therapeutic applications (11,18,19). The hydrophobic interior of dendrimer makes them an ideal "carrier" for encapsulation or complexation of hydrophobic drugs thereby improving the water solubility and biocompatibility of such drugs (20).

Prodrug approaches have been applied to morphine to improve solubility, absorption, tissue selectivity, and other drug delivery properties. Generally, this involves either one or both 3- and 6- hydroxyl groups being converted to an ester. Because the 3- OH-substitution of morphine results in the loss of its biological activity, the synthesis and esterase release kinetics of morphine from various 3-substituted morphine prodrugs have been previously studied (20–23). Our previous work has shown PAMAM dendrimer to be a non-toxic polymer similar in diameter to small proteins. In addition, dendrimer is capable of encapsulating large payloads of hydrophobic small molecule drugs such as methotrexate (24). In this study, we examine the hypothesis that sustained pain control can be achieved with extended release intravenous morphine using a dendrimer-based prodrug approach.

MATERIALS AND METHODS

Materials

Amine-terminated Generation 5 PAMAM dendrimer with an ethylenediamine core (G5′NH_2) in methanol were purchased from Dendritech (Midland, MI). The G5 PAMAM dendrimer with mixed acetyl and carboxyl groups (G5′$NHAc_{80}$-$COOH_{35}$) were synthesized and characterized according to published methods (25). Briefly, the surfaces of G5′NH_2dendrimer were partially acetylated by reacting with acetic anhydride (on average, 80 amine groups per G5 dendrimer were converted to acetamide groups) (25–27). The remaining amine groups of the partially acetylated G5.$NHAc_{80}$ dendrimer were then converted to carboxyl groups by reacting them with succinic anhydride (20,28–30). The molecular weight of G5.$NHAc_{80}$-$COOH_{35}$ dendrimer was determined by MALDI-TOF mass spectrometry to be 33,014. Morphine prodrug A was synthesized from morphine (Mallinckrodt-Covidien, St. Louis, MO) using a linear aliphatic ester linker. Morphine prodrug B was synthesized from morphine using an aromatic linker following standard N,N'-Dicyclohexylcarbodiimide (DCC) catalyzed coupling reactions and the appropriate protecting corresponding acids (20,21,31). All other chemicals were obtained from Sigma-Aldrich (Sigma-Aldrich Corp. St. Louis, MO, USA) and used unaltered. All water was passed through a Millipore Milli-Q Plus 185 purification system resulting in a resistivity exceeding 18.2 MΩcm. All ^{1}H and ^{13}C NMR spectra were measured on a 500 MHz Varian NMR systems equipped with multinuclear 5-mm probes (Fig. 1). ^{1}H chemical shifts are reported in parts per million from TMS. Mass spectra were performed on a Waters 1,525 mass spectrometer. MALDI-TOF mass spectra were obtained with a Waters Tofspec-2E run in linear mode. The instrument was mass calibrated with a mixture of cytochrome-C and bovine insulin in a CHCA matrix. The data was acquired and processed using MassLynx 3.5 software. A thermometer was used without further calibration. Thin layer chromatography (TLC) was performed using Whatman Adsorption plates, 60 Å silica gel, 250 μm layer thickness.

The Synthesis and Characterization of Morphine Prodrugs A and B

The synthesis of morphine prodrug A and B is demonstrated in Scheme **1** Morphine prodrug A contains an aliphatic ester, while morphine prodrug B contains an aromatic ester. The amine groups are Boc protected to enhance the hydrophobicity of the prodrugs.

Morphine Prodrug A

Morphine (400 mg, 1.40 mmol), mono-tert-butyl succinate **1** (488 mg, 2.80 mmol), and DMAP (trace) were dissolved in 20 mL of THF.DCC (1.16 g, 5.60 mmol) in 10 mL of THF was added with stirring. The mixed solution was stirred at room temperature overnight. The mixture was concentrated and the resulting residue was purified by column chromatography on silica gel (eluent MeOH /CH_2Cl_2=2:98, MeOH/CH_2Cl_2=5/95) to give Morphine Prodrug A as a white solid (440 mg, 71%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.41 (s, 9H), 2.03 (td, J_1=7.5 Hz, J_2=5.0 Hz, 1H), 2.27 (dd, J_1=19.0 Hz, J_2=6.5 Hz, 1H), 2.34 (td, J_1=12.0 Hz, J_2= 3.5 Hz, 1H), 2.40 (s, 3H), 2.55 (m, 1H), 2.59 (m, 2H), 2.66 (m, 1H), 2.76 (td, J_1=7.0 Hz, J_2=1.5 Hz, 2H), 3.01 (d, J= 19.0 Hz, 1H), 3.32 (m, 2H), 4.10 (m, 1H), 4.86 (dd, J_1= 6.5 Hz, J_2=1.0 Hz, 1H), 5.22 (dt, J_1=7.5 Hz, J_2=2.5 Hz, 1H), 5.67 (m, 1H), 6.55 (d, J=8.5 Hz, 1H), 6.69 (d, J=

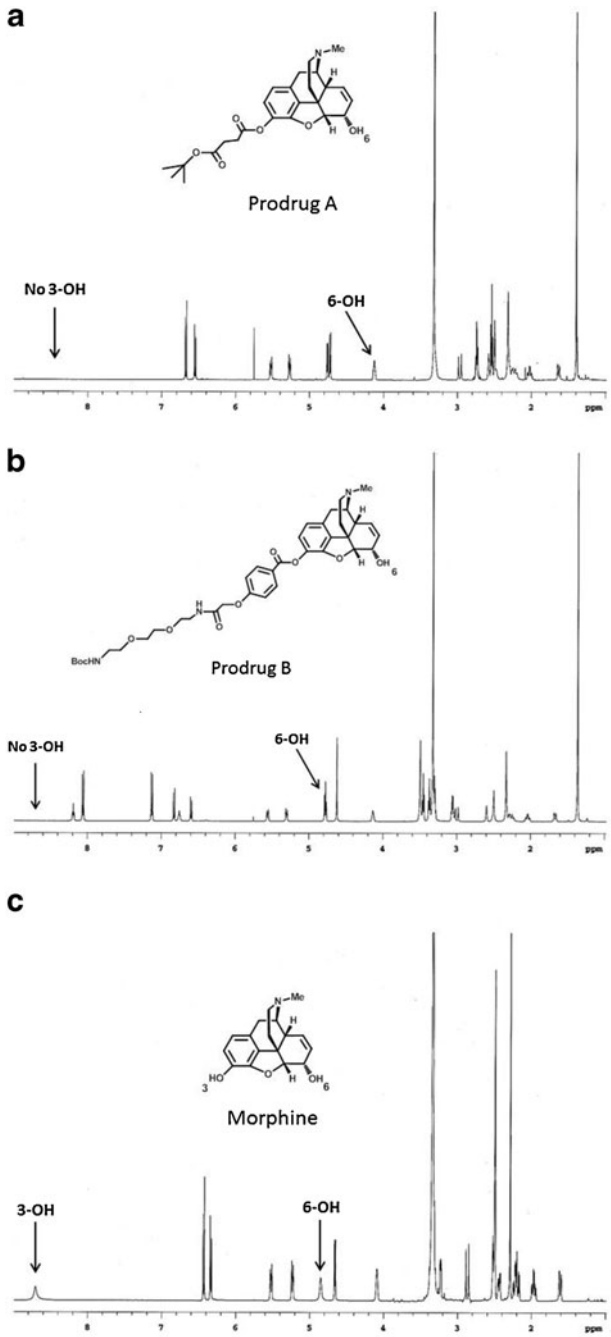


Fig. 1 NMR of (**a**) morphine prodrug A, (**b**) morphine prodrug B, (**c**) morphine.

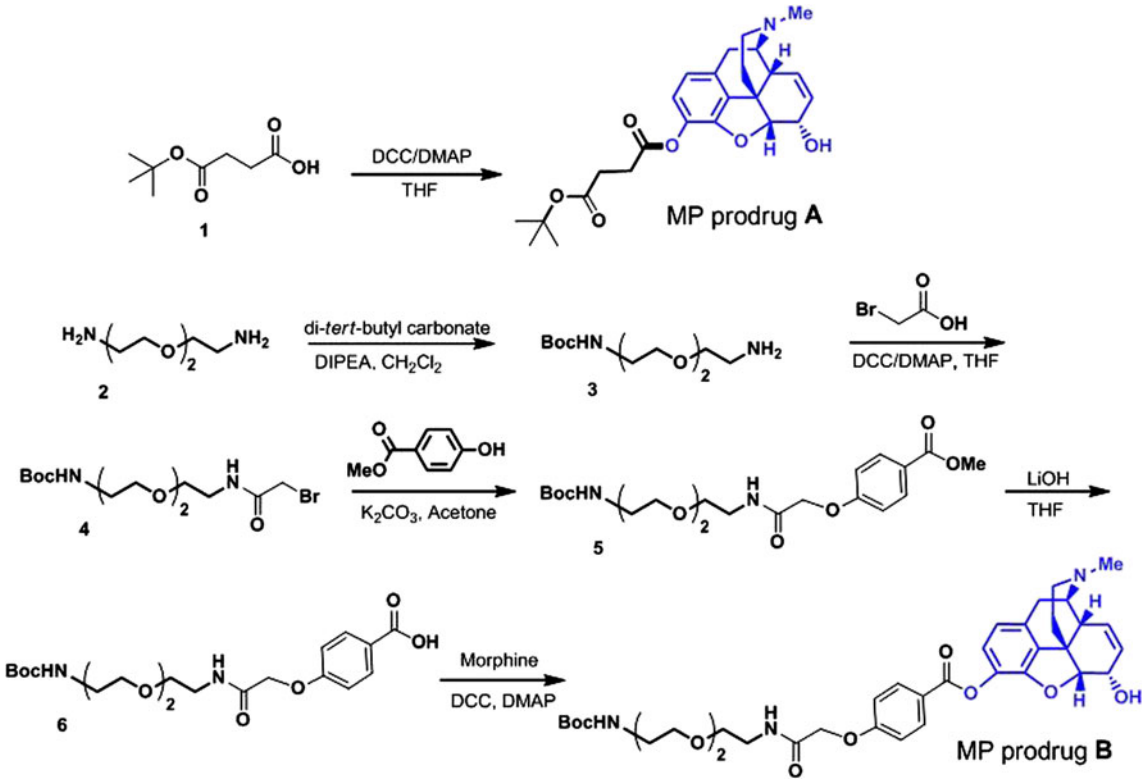
8.0 Hz, 1H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 20.8, 28.1, 29.1, 30.2, 30.9, 35.2, 40.4, 42.7, 43.0, 46.4, 53.6, 58.9, 65.9, 80.9, 92.3, 119.9, 121.1, 127.7, 131.8, 132.3, 132.8, 134.2, 148.9, 170.3, 171.1; ESI-MS m/z 442.2 ($M + H^+$) calcd for $C_{25}H_{32}NO_6$ 442.2.

Tert-butyl2-(2-(2-aminoethoxy)ethoxy)ethylcarbamate ***3*** was prepared as reported (36). All spectra obtained for this compound were in agreement with published results.

For *tert*-butyl2-(2-(2-(2-bromoacetamido)ethoxy)ethoxy) ethylcarbamate **4** *tert*-butyl2-(2-(2-aminoethoxy)ethoxy) ethylcarbamate (1.98 g, 7.97 mmol), bromoacetic acid (1.33 g, 9.57 mmol), and 4-(dimethylamino)pyridine (DMAP) (trace) were dissolved in 30 mL of tetrahydrofuran (THF). N,N'-dicyclohexylcarbodiimide (DCC) (1.97 g, 9.55 mmol) in 20 mL of THF was added with stirring. The mixed solution was stirred at room temperature overnight. The mixture was concentrated and the resulting residue was purified by column chromatography on silica gel (eluent $MeOH/CH_2Cl_2$=5/95) to give **4** as a white solid (2.25 g, 76%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.33 (s, 9H), 3.20 (m, 2H), 3.37 (m, 2H), 3.44 (t, J_1= 5.0 Hz, 2H), 3.47 (t, J=5.0 Hz, 2H), 3.51 (m, 4H), 3.77 (s, 2H), 5.09 (m, 1H), 7.07 (m, 1H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 28.3, 28.9, 29.5, 39.8, 40.2, 69.2, 70.1, 70.2, 79.1, 155.9, 165.9; ESI-MS m/z 391.1 ($M+Na^+$) calcd for $C_{13}H_{25}BrN_2NaO_5$ 391.1.

For methyl4-(2,2-dimethyl-4,15-dioxo-3,8,11-trioxa-5,14-diazahexade- can-16-yloxy)benzoate **5**, to a well stirred slurry of compound **4** (1.94 g, 5.25 mmol) and K_2CO_3 (1.45 g, 10.5 mmol) in acetone (50 mL) was added methyl 4-hydroxybenzoate (0.88 g, 5.78 mmol), and the solution was heated at 60°C under N_2 for 24 h. The reaction contents were filtered and washed with water. The organic layers were dried over Na_2SO_4 and concentrated. The resulting residue was chromatographed on silica gel (eluent CH_2Cl_2, then $AcOEt/CH_2Cl_2$=2/98) to yield a white solid **5** (2.10 g, 91%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.44 (s, 9H), 3.31 (m, 2H), 3.53 (t, J_1=5.0 Hz, 2H), 3.58 (m, 8H), 3.90 (s, 3H), 4.56 (s, 2H), 5.00 (m, 1H), 6.96 (m, 3H), 8.03 (d, J= 8.5 Hz, 2H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 18.18, 18.80, 28.6, 31.1, 39.1, 40.5, 52.2, 66.5, 66.6, 67.5, 69.9, 70.3, 70.4, 70.5, 114.5, 132.0, 156.1, 166.7; ESI-MS m/z 463.2 ($M+Na^+$) calcd for $C_{21}H_{32}N_2NaO_8$ 463.2.

For 4-(2,2-dimethyl-4,15-dioxo-3,8,11-trioxa-5,14-diazahexadecan-16-yloxy)benzoic acid **6** 20 mL of 0.4 M LiOH solution (aq) was added to a solution of compound **5** (1.82 g, 4.13 mmol) in 20 mL THF. The mixed solution was stirred at room temperature for 2 days. The THF was removed by rotary evaporation. The mixture was washed with 20 mL CH_2Cl_2. Then 0.1 M HCl was added dropwise to the aqueous phase until the solution became acidic. The mixture was extracted with 20 mL CH_2Cl_2 twice. The organic phase was dried (Na_2SO_4) and rotary evaporated. Compound **6** was obtained as a white solid (1.02 g, 58%). The product is used for next step without further purification. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.44 (m, 9H), 3.32 (m, 2H), 3.54 (t, J=5.0 Hz, 2H), 3.60 (m, 8H), 4.60 (s, 2H), 5.04 (m, 1H), 6.98 (m, 2H), 7.04 (m, 1H), 8.07 (d, J=9.0 Hz,



Scheme I Synthesis of morphine prodrug A and morphine prodrug B.

2H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 28.4, 38.9, 40.3, 65.7, 65.8, 67.3, 69.7, 70.1, 70.3, 114.4, 123.4, 132.5, 161.2, 167.7, 170.3; ESI-MS m/z 425.3 ($M - H^+$) calculated for $C_{20}H_{29}N_2O_8$ 425.2.

Morphine Prodrug B

Morphine (513 mg, 1.80 mmol), compound **6** (1.15 g, 2.70 mmol), and DMAP (trace) were dissolved in 15 mL of THF. DCC (770 mg, 3.73 mmol) in 10 mL of THF was added with stirring. The mixed solution was stirred at room temperature overnight. The mixture was concentrated and the resulting residue was purified by column chromatography on silica gel (eluent MeOH /CH_2Cl_2=2:98, then MeOH/CH_2Cl_2=6/94) to give Morphine Prodrug B as a white solid (827 mg, 66%): ^{1}H NMR (500 MHz, CD_3OD) δ 1.42 (s, 9H), 2.02 (m, 1H), 2.37 (m, 1H), 2.80 (m, 4H), 3.00 (m, 1H), 3.07 (m, 1H), 3.17 (m, 6H), 3.25 (m, 1H), 3.45 (t, J=5.5 Hz, 2H), 3.54 (m, 6H), 3.93 (m, 1H), 4.23 (m, 1H), 4.60 (m, 2H), 4.94 (m, 1H), 5.34 (d, J= 9.0 Hz, 1H), 5.71 (d, J=9.5 Hz, 1H), 6.69 (d, J=8.5 Hz, 1H), 6.88 (d, J=8.0 Hz, 1H), 7.08 (d, J=8.0 Hz, 2H), 8.07 (d, J=8.0 Hz, 2H); ^{13}C NMR (500 MHz, CD_3OD) δ 9.30, 14.8, 17.1, 23.2, 28.8, 34.1, 39.5, 40.0, 41.2, 42.1, 43.5, 47.8, 61.4, 67.3, 68.2, 70.4, 71.0, 71.3, 80.0, 93.2, 116.0, 121.0, 123.4, 123.6, 127.1, 131.7, 132.1, 133.5, 135.6, 151.2, 157.0, 163.6, 165.3; ESI-MS m/z 694.3 ($M + H^+$) calculated for $C_{37}H_{48}N_3O_{10}$ 694.3.

Complexation of Morphine and Morphine Prodrugs with Dendrimers

A surface modified G5 PAMAM dendrimer (G5.$NHAc_{80}$-$COOH_{35}$) was used to complex either morphine base, morphine prodrug A or morphine prodrug B. Briefly, a known amount of (~30–40 mg) of G5.$NHAc_{80}$-$COOH_{35}$ was dissolved in 3.6 mL of DI water. Similarly, a known amount of either a drug or a prodrug (~20–40 mg) was dissolved in (0.6–0.9 mL) methanol. The methanol solution of either the drug or a prodrug was added drop wise to the aqueous solution of the dendrimer. The sample was stirred overnight with gentle flow of nitrogen gas. At the end of the experiment, methanol was completely evaporated and the remaining sample was reconstituted in DI water to a final volume of 3.6 mL. The sample was then filtered through an Anotop 10, 0.02 μm filter to yield a clear solution of dendrimer-drug OR dendrimer-prodrug complex. The aqueous sample was stored in a refrigerator at 4°C until use. The association of the drug/prodrugs to the dendrimer scaffold is due to weak interactions (ionic, hydrogen bond). When the complex is incubated in ionic solvent, due to the exchange of ions morphine is dissociated from the dendrimer scaffold, however, in non ionic solvents it remains bound to the dendrimer scaffold. Unlike morphine, the prodrugs have the ester cleavable linker that is stable in buffer conditions. We utilize this difference in order to calculate the amount of

drug/prodrug complexed within the dendrimer. The complexed drug/prodrug is incubated in a buffer system which dissociates the drug/prodrug from the dendrimer scaffold. The sample is then run on UPLC to quantitate the amount of free drug/prodrug. Using an independent calibration curve method, we quantitate the amount of dissociated drug/prodrug and hence calculate the amount of complexation. Based on the calibration curve, the complexation efficiency was calculated to be close to 90% (e.g., In one of the experiments, starting with a concentration of 16.75 mg/mL of the morphine prodrug A, the final concentration of the prodrug complexed was calculated to be 14.86 mg/mL). The average number of moles of morphine, morphine prodrug A or morphine prodrug B, complexed to each mole of dendrimer is shown in Table I.

UPLC Analysis of Morphine and Morphine Prodrug Content

UPLC, a recent advancement in the field of chromatography, can perform analysis in a matter of a few minutes (~10–15 min) with low equilibration times as compared to the required 35 min to complete a conventional high performance liquid chromatography. UPLC analysis was carried out on a Waters Acquity Peptide Mapping System equipped with a Waters photodiode array detector, a column manager that facilitates 4 column housing, and a sample manager. The instrument was controlled by Empower 2 software. For our calibration and release kinetics studies, morphine and its prodrugs were run on an Acquity UPLC HSS T3 column (100×2.1 mm, 1.8 mm). The analysis was carried out using an elution gradient beginning with 98:2 (v/v) water/acetonitrile (ACN) reaching 40:60 water/acetonitrile (ACN) in 6.0 min. The gradient was then ramped up to 2:98 water/ACN in the next 0.5 min and was maintained at that composition until 10 min. The system was then re-equilibrated to the original starting conditions. Flow rate was maintained at 0.5 mL/min. Note that trifluoroacetic acid (TFA) (0.14 wt.%) was added to the water as a counter ion. A 3 μL of sample was injected using a "partial loop with needle overfill," an inbuilt sample loop option in the software. The column temperature was maintained at 45°C. A calibration curve was generated for each of the samples by making duplicate 3 μl injections of each standard solution. The calibration curve was generated by plotting the appropriate peak area *versus* the corresponding concentration of the drug (morphine, morphine prodrug A and morphine prodrug B).

Table I Complexation Capacity of Morphine and Morphine Prodrugs with G5 Dendrimer (in Molar Ratio)

Dendrimer ($G5.NHAc_{80}$-$COOH_{35}$)	Moles of drug/prodrug complexed
Morphine loading[a]	53 ± 4
Morphine prodrug A loading	79 ± 8
Morphine prodrug B loading	56 ± 3

[a] Number of drug molecules per dendrimer

Release of Morphine from Morphine Complex and Morphine Prodrug Complexes

To study the release kinetic profiles for each of the samples, a similar protocol was followed for each of the complexes as well as the morphine control. The release kinetics for each of the samples were studied in plasma taken from rats (n=3) and guinea pigs (n=3); samples in PBS buffer served as controls. Briefly, working standard solutions of the complexes (morphine base complex and morphine prodrug complexes) were created by diluting them in DI water to yield a concentration of 500 μM, based on the dendrimer. For release kinetics in plasma, 75 μL of the working standard was incubated with 75 μL of plasma (rat or guinea pig) or PBS at 37°C and 20 μL Aliquots were taken at various time points (0′, 5′, 15′, 30′, 60′, 180′, 360′) spanning up to 6 h, as we have described previously (32). These aliquots were treated with 40 μL of 10% DMSO in acetonitrile to remove the plasma proteins. The samples were then centrifuged at 16,000 rpm for 10 min and the supernatant was subjected to UPLC to determine the content of free morphine in the sample. The '0' time points for the plasma-incubated samples were obtained by initially precipitating the plasma by DMSO/acetonitrile solution followed by adding the prodrugs, under similar conditions. To evaluate our recovery efficiency, morphine sulfate was incubated with plasma and subjected to the same precipitation protocol described above.

Animals

Male Hartley Guinea pigs (375–475 g) supplied by Elm Hill laboratories (Chelmsford, MA) and male Sprague–Dawley rats (325–375 g) from Charles River laboratories (Portage, MI) were used for all pain testing. Animals were under a 12:12 h light–dark cycle, had free access to standard laboratory food and tap water while housed in animal facilities at the University of Michigan, Ann Arbor, MI. Care and use of the animals and the experimental protocol conformed to, and were approved by the University Committee on Use and Care of Animals (UCUCA) and United States Animal Care and Use Review Office (ACURO). The protocols adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised in 1985).

Pain Testing

A modified Randall and Selitto test was used to measure pain in all animals (32). The mechanical nociceptive threshold was measured using an analgesimeter apparatus (Harvard Instruments, Holliston, MA). The device was used to generate a mechanical force that increased linearly over time. The force was applied to the dorsal surface of the hind paw via a conical-shaped stylus that was 1 mm in diameter. The nociceptive threshold was the terminal force recorded (in grams) upon withdrawal of the hind limb from the testing platform. Five repeated measurements of pain threshold were recorded for each time point. Time points for morphine sulfate, complexed morphine, and prodrugs were chosen prospectively given release kinetics anticipated from our *in vitro* work. In addition, consideration was given to limit the overall number of time points over a 6 h period due to potential systematic errors attributable to hyperalgesia from multiple measurements on a single limb. All animals were administered drug intravenously through a carotid cut-down before pain testing. Pain measurements for rats were: morphine sulfate: pre-drug control, 1, 2.5, 4, and 6 h.; morphine prodrug A complex: pre-drug control, 1, 2.5, 4, and 6 h.; morphine prodrug B complex: pre-drug control, 1, 2.5, 4, and 6 h.; morphine complex: pre-drug control, 1, 2.5, 4, 6 h. after drug administration (Fig. 5). Pain measurements for guinea pigs were: morphine sulfate: pre-drug control, 1, 2, 4, and 6 h.; morphine complex: pre-drug control, 1, 2, 4, and 6 h.;morphine prodrug A: pre-drug control, 1, 4, 5, 6 h. after drug administration (Fig. 6). All values were normalized to control and the mean ± SEM for each time point were recorded. To avoid permanent tissue damage a weight limit 900 g was used, and each animals individual baseline response was normalized to itself. Statistical analysis for nociceptive pressure thresholds was carried out using one-way ANOVA for each time point followed by a two-tail Student's *t*-test. The level of significance was set at $P<0.05$.

Pathologic Evaluation

In order to evaluate toxicity from the dendrimer prodrug formulation , 3 rats and 3 guinea pigs were administered a single dose of 5 mg/kg IV morphine prodrug A complex. At 2 weeks, all animals were sacrificed with harvest of blood from cardiac puncture and urine from bladder puncture for comprehensive metabolic panels including hematologic and blood chemistry markers, urinalysis, and 10 organs for histopathologic evaluation.

RESULTS

Drug and Prodrug Loading Capacity of the Dendrimer

Table I lists the complexing capacity of the G5 PAMAM dendrimer for either morphine or the morphine prodrugs. As presented in Table I, 1 mole $G5.NHAc_{80}$-$COOH_{35}$ dendrimer can complex 79 moles of morphine prodrug A. The formed complexes were found to be stable, either as a lyophilized powder or in DI water at 4°C, for the length of study.

Release of Morphine and Prodrugs from Dendrimer Complex

As shown in Fig. 2, morphine was completely released from the complex within 5 min of incubation with the pbs. While complexing morphine with the dendrimer enhanced its solubility it did not appear to alter the bioavailability of the complexed drug. The release of complexed morphine prodrug A and B in rat and guinea pig plasma was rapid. The secondary release of free morphine from prodrug A and B (Figs. 3 and 4) moieties showed prodrug and species specific differences. In rats, free morphine was available from morphine prodrug A rapidly while prodrug B reached plateau by 1 h. In guinea pigs, free morphine was available from morphine prodrug A in a similar rapid fashion; however, release of free morphine from morphine prodrug B demonstrated a more gradual release over the 6-h monitoring period.

To clarify the role of dendrimer disassociation and esterase activity on the release of morphine from prodrugs, we incubated complexed prodrugs with PBS buffer. As the prodrug association to the dendrimer scaffold is via weak interaction, when incubated in an ionic solvent (e.g. PBS, plasma), due to

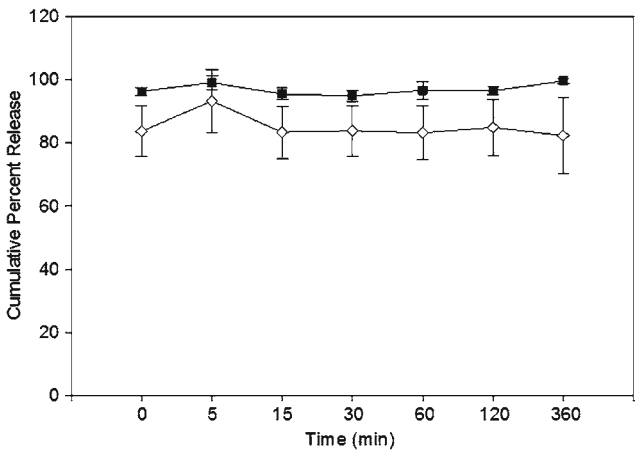


Fig. 2 Release profile for morphine when complexed with surface modified PAMAM dendrimer (*white diamond*) *vs*. release of morphine sulfate in PBS (*black square*). The results indicate that complexing morphine with dendrimer does not significantly affect the release profile for morphine and dendrimer aids in solubilizing the morphine (base) in aqueous medium.

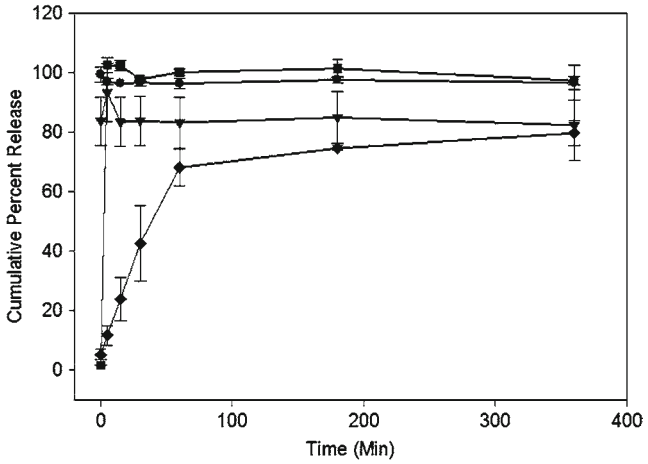


Fig. 3 Release of morphine from morphine prodrug A dendrimer complex (*black square*), morphine prodrug B dendrimer complex (*black diamond suit*), morphine dendrimer complex (*black down-pointing triangle*), in rat plasma with (*black circle*) showing recovery of uncomplexed morphine incubated with rat plasma under identical conditions.

the exchange of ions, the prodrug is dissociated. As proof of concept, we incubated the Morphine Prodrug complexed with the dendrimer in PBS buffer and injected the aliquots in UPLC over 6 h. The prodrugs did not hydrolyze over the period of study (6 h). As seen in the Fig. 5, the peak at 3.4 min represents the dendrimer scaffold whereas a peak at 5.3 min represent the Morphine Prodrug peak. On comparison of the peak areas at 5 min and 6 h after incubation in PBS, the prodrug peak showed no change for the prodrug peak. This further confirmed our result that the ester linkage in the prodrug are only esterase susceptible and will hydrolyze only in presence of plasma esterases.

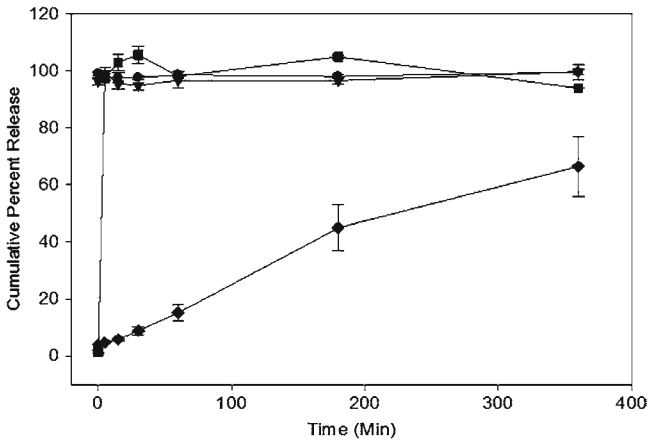


Fig. 4 Release of morphine from morphine prodrug A dendrimer complex (*black square*), morphine prodrug B dendrimer complex (*black diamond suit*), morphine dendrimer complex (*black down-pointing triangle*), in guinea pig plasma with (*black circle*) showing recovery of uncomplexed morphine incubated with guinea pig plasma under identical conditions.

Analgesic Efficacy *In Vivo*

Using the modified Randall Selitto test in rats, morphine sulfate peak analgesia was seen at 1 h. after administration. A rapid decrease in analgesic effect was noted thereafter, resulting in analgesia values below baseline due to a hyperalgesic response at the 4 and 6 h marks. The efficacy curve for morphine dendrimer complex was similar in shape to morphine sulfate with a minimal decrease in comparable efficacy after 1 h. In contrast, while the dendrimer morphine prodrug A complex also peaked at 1 h, the analgesic effect obtained was maintained for 4 h. At 6 h there remained a 50% reduction in pain control (Fig. 6). Morphine prodrug B complex demonstrated minimal efficacy in this model, consistent with the slow release profile that was shown *in vitro*

Building upon our experience in rats, we modified the guinea pig model to include a 5 h time point and focused only on the differences between morphine sulfate and morphine prodrug A complex. Morphine prodrug A maintained peak efficacy for 5 h with a greater than 60% pain control at the 6 h time point (Fig. 7).

Pathology

Blood and urine were submitted from all animals for comprehensive metabolic panels. There were no significant findings among the test animals and controls that received only morphine sulfate. Histopathology from ten harvested organs was evaluated with standard H&E staining. There were no significant drug related findings in either rats or guinea pigs for animals that received morphine prodrugs complexed with PAMAM dendrimer as compared to controls.

DISCUSSION

In this study, we demonstrated that morphine prodrugs can be complexed with and solubilized by dendrimers for *in vivo* therapeutic applications. The structural design of the morphine prodrugs allowed the controlled release of morphine that extended the action of morphine-induced analgesia in an animal pain model from 2 h (control) to 6 h (Morphine Prodrug A), and this was not altered by complexing the drugs with dendrimers. Figure 2 shows that the amount of free morphine released following the dissociation from the dendrimer scaffold was nearly complete after 5 min of incubation with guinea pig plasma, indicating that complexation with the dendrimer did not alter the pharmacokinetics of morphine. We also have experience testing uncomplexed prodrugs for their *in vitro* efficacy against various enzymes prior to *in vivo* studies (32). For *in vivo* studies, we employed a solubilization protocol that used a surface modified G5 PAMAM dendrimer. To verify

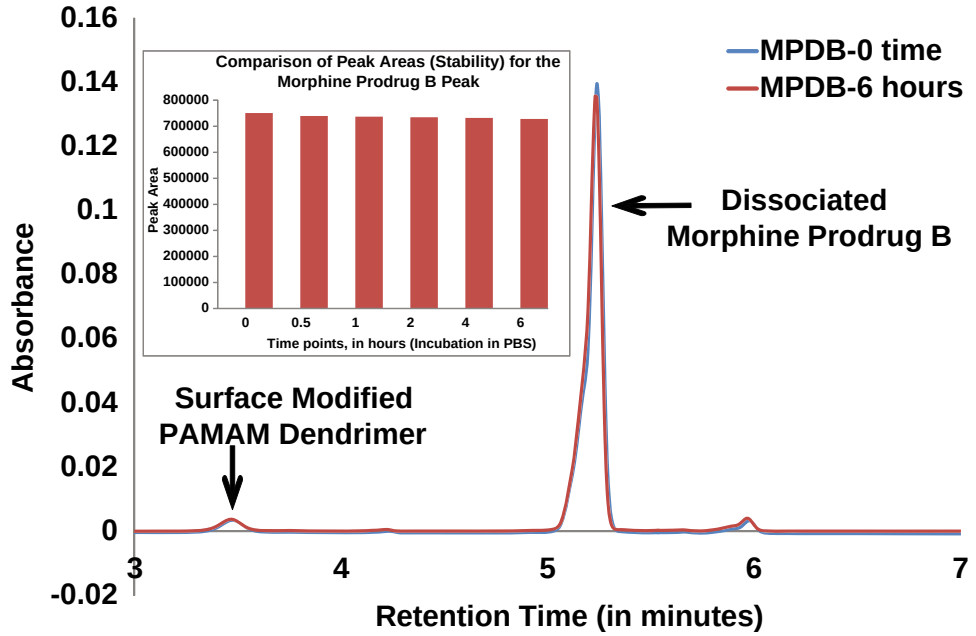


Fig. 5 Chromatogram for Morphine Prodrug B dissociated from Dendrimer Scaffold after incubation in PBS buffer over 6 h with inset comparison of Peak Areas (Stability) for Morphine Prodrug B Peak after Dissociation from the Dendrimer Scaffold (incubation in PBS Buffer 0–6 h).

that the process of complexation/solubilization does not significantly affect the prodrug efficacy, we incubated the complexed prodrugs in PBS buffer and analyzed them by HPLC. The results indicated the prodrugs were intact after dissociation from the dendrimer scaffold. Therefore, the primary role that the dendrimer played in this formulation was to increase the solubility of the morphine prodrugs.

Figures 3 and 4 show the release profile of morphine from the morphine prodrug dendrimer complexes following incubation with rat and guinea pig plasma, respectively. Immediately evident is that morphine prodrug B complex shows a substantially different release profile than the morphine prodrug A complex, suggesting that the linker of morphine prodrug A is more readily hydrolyzed in both rat and guinea pig plasma. This is consistent with previous studies that suggested that the aromatic ester bond present in our morphine prodrug B is more

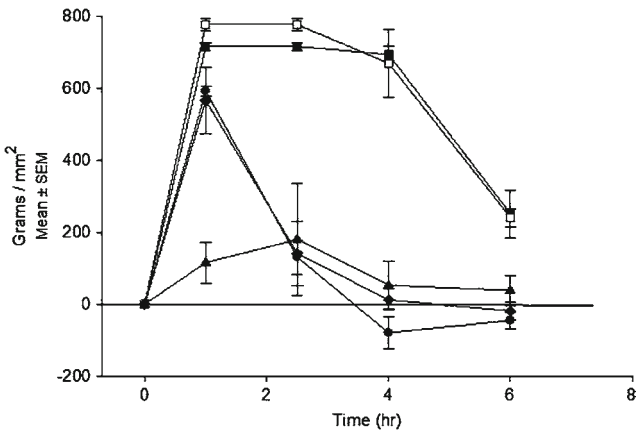


Fig. 6 Analgesic effect of (*black circle*) morphine sulfate (5 mg/kg iv), (*black square*) morphine prodrug A complex (5 mg/kg iv), (*white square*) morphine prodrug A complex (10 mg/kg iv), (*black up-pointing triangle*) morphine prodrug B complex (5 mg/kg iv), and (*black diamond suit*) morphine complex (5 mg/kg iv) in the modified Randall Selitto test with rats. *N*=3 for each group.

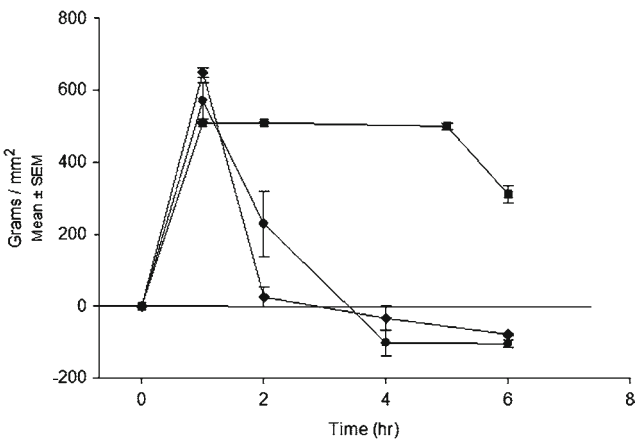


Fig. 7 Analgesic effect of (*black circle*) morphine sulfate (5 mg/kg iv), (*black square*) morphine prodrug A complex (5 mg/kg iv), (*black diamond suit*) morphine complex (5 mg/kg iv) in the modified Randall Selitto test with guinea pigs. *N*=3 for each group.

stable than the aliphatic ester present in the morphine prodrug A (33). The difference in the release profiles for a given compound between plasma samples is attributed to the difference in plasma esterase activity measured in rats and guinea pigs (34).

Despite similar *in vitro* release kinetics of morphine from dendrimer complexes of either morphine or the morphine prodrug A (< 5 min), there is a clear difference in the analgesic effect of the two formulations (Figs. 6 and 7). While morphine provided less than 4 h of analgesia, morphine prodrug A at an equimolar concentration provided 6 h. A number of hypotheses for this finding exist, including differences in volume of distribution, half-life, or elimination rates. However, further testing is needed to delineate the contribution of these theories to our findings and would be important to an overall understanding of the pharmacokinetics of the compound.

CONCLUSION

We have shown that parentally administered morphine prodrugs complexed to dendrimer can provide 6 h of sustained analgesic relief without acute toxicity. These formulations could have a number of applications for standard patient care where parenteral medications are desired or required. The sustained efficacy of our morphine prodrugs could overcome limitations of current intravenous opioid therapeutics that require continuous administration, often with complex dosing mechanisms. Mixtures of these prodrugs complexed together with dendrimers could provide unique pain therapeutics. For example, in our model, morphine prodrug B alone did not deliver adequate analgesia but could be used as part of a combination compound for greater extended effects given its release profile. This designer approach to analgesic therapy could have a variety of clinical applications where desired pharmacologic effects vary significantly dependent on need. This platform also should be useful for a variety of other pharmaceuticals when parenteral administration and sustained release is desired and is currently unattainable.

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