

Antinociceptive Structure-Activity Studies with Enkephalin-Based Opioid Glycopeptides

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Nonstandard abbreviations: Fmoc, 9-Fluorenylmethyloxy carbamate; ^1H NMR, Proton Nuclear Magnetic Resonance Spectroscopy; COSY, ^1H - ^1H Correlation Spectroscopy; TOCSY, Total Correlation Spectroscopy; FAB, Fast Atom Bombardment Mass Spectroscopy; TFA, Trifluoroacetic acid

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Abstract

Development of opioid peptides as therapeutic agents has historically been limited due to pharmacokinetic issues including stability and blood-brain barrier (BBB) permeability. Glycosylation of opioid peptides can increase peptide serum stability and BBB penetration. To further define the requirements for optimizing *in vivo* antinociceptive potency following intravenous administration, we synthesized a series of enkephalin-based glycopeptides using solid phase Fmoc methods. The compounds differed in the 6th and subsequent amino acid residues (Ser or Thr), and in the attached carbohydrate moiety. *In vitro* binding and functional smooth muscle bioassays indicated that the addition of mono- or disaccharides did not significantly affect the opioid receptor affinity or agonist activity of the glycopeptides compared to their unglycosylated parent peptides. All of the glycopeptides tested produced potent antinociceptive effects in male ICR mice following intracerebroventricular injection in the 55°C tail-flick test. The calculated A_{50} values for the Ser/Thr and monosaccharide combinations were all very similar, with values ranging from 0.02-0.09 nmol. Selected compounds were administered to mice intravenously and tested for antinociception to indirectly assess serum stability and BBB penetration. All compounds tested produced full antinociceptive effects with calculated A_{50} values ranging from 2.2-46.4 $\mu\text{mol/kg}$, with the disaccharides having potencies that equaled or exceeded that of morphine on a $\mu\text{mol/kg}$ basis. Substitution of a trisaccharide or bis- and tris-monosaccharides resulted in a decrease in antinociceptive potency. These results provide additional support for the utility of glycosylation to increase CNS bioavailability of small peptides and compliment our ongoing stability and blood-brain barrier penetration studies.

The addition of carbohydrate moieties to a protein (glycoprotein) or peptide (glycopeptide) produces changes in the molecular structure that, in turn, can have dramatic effects on the pharmacodynamic and/or pharmacokinetic properties of proteins and peptide hormones (Lis and Sharon, 1993). The glycoprotein follicle stimulating hormone (FSH), for example, has multiple glycosylation sites and exists as a family of isoforms that differ with respect to their oligosaccharide structure (Stanton et al., 1996; Ulloa-Aguirre et al., 1995). The isoforms can differ substantially with respect to their in vitro and in vivo potency and efficacy (Barrios-De-Tomasi et al., 2002). These observations have led to the modification of hFSH and other glycoproteins in an effort to improve pharmacokinetic properties while maintaining optimal activity. Site-directed mutations and the extension of the N-terminus subunits with additional glycosylation sites are two approaches that have been used to increase the half-life of FSH (Perlman et al., 2003). This strategy has also been used to increase the half-life of erythropoietin, another glycoprotein, with at least one variant (darbepoetin- α) being approved for the treatment of anemia (Macdougall et al., 1999). Surprisingly, few studies have been done to explore glycosylation strategies in small peptides for therapeutic applications.

The development of neuropeptide drugs has been hampered by instability and poor BBB penetration. Several strategies have been used to overcome these obstacles, including substitution of unnatural amino acids (Mosberg et al., 1982; Hruby and Mosberg, 1982), the use of conformational constraints (Hruby, 1989), the design of highly potent peptide analogs (Handa et al., 1981; Horan et al., 1993), and the addition of lipophilic side groups or other transport vectors (Bodor et al., 1992; Rousselle et al., 2000). While stability and binding issues have been successfully addressed, problems with transport and BBB penetration remain. Making the compound more lipophilic, for example, has proven to be self-limiting due to the aqueous

environment of the transport medium (*i.e.* blood, CSF) (Bodor et al., 1992). The addition of bulky transport vectors may also have adverse effects on peptide bioactivity, necessitating the incorporation of an enzymatic cleavage site into the transport vector in order to release the active peptide after transport (Rousselle et al., 2000).

It is reasonable to hypothesize that glycosylation of a small peptide will increase the hydrophilicity, stability and bioavailability of peptides (Albert et al., 1993; Polt et al., 1994; Negri et al., 1998; Bilsky et al., 2000). In studies investigating peptides and proteins which had been glycosylated non-specifically via the Amidori reaction, an increase in delivery to both the peripheral and central nervous system was observed (Podulso et al., 1994). BBB penetration studies of glycopeptides have indicated up to a three-fold increase in the rate of brain delivery of these compounds compared to the unglycosylated parent peptides (Bilsky et al., 2000; Eggleton et al., 2000; Eggleton et al., 2001). Recent studies with glycopeptides in artificial membrane systems indicate that the amphipathicity of the glycopeptides may be an important factor in BBB penetration (Palian et al., 2003). In addition, there is evidence that suggests that the type of glycosylation can alter tissue distribution patterns (Susaki et al., 1999, Suzuki et al., 1999a,b), BBB penetration (Eggleton et al., 2000, Eggleton et al., unpublished results) and peptide/receptor interactions (Palian and Polt, 2001; Gysin and Schwyzer, 1983; Sargent et al., 1988).

The subtle modifications of a glycopeptide may alter tissue and receptor specificity, potentially decreasing drug toxicity and side effects. In the opioid field, for example, there is considerable interest in developing compounds that have agonist actions at both delta and mu opioid receptors, with the optimal ratio of selectivity still needing to be determined in preclinical and clinical models (Gengo and Chang, 2004; Lipkowski et al., 2004). This prospect prompted our exploration of opioid glycopeptide chemistry and pharmacology using a linear hexapeptide

(Tyr-D-Thr-Gly-Phe-Leu-Ser-CONH₂) that has affinity for delta and mu receptors. The current study is an extension of previous work showing that the glycosylation of the 6th residue of a linear opioid hexapeptide significantly increased the *i.v.* potency and *in situ* BBB permeability of the compound (Bilsky et al., 2000). We hypothesized that further modification of the attachment site and the carbohydrate-moiety would alter pharmacokinetic and/or pharmacodynamic properties of the molecule. To test this hypothesis we characterized a series of novel opioid glycopeptides (Figure 1) in *in vitro* and *in vivo* bioassays.

Methods

Animals. A total of 960 male, ICR mice (25-30 g) (Harlan Industries, Cleveland, OH) were housed in groups of 5 in Plexiglas chambers with food and water available *ad libitum* prior to any procedures. Animals were maintained on a 12 hr light/dark cycle (lights on at 07:00) in a temperature and humidity controlled animal colony. All testing was carried out between 10:00 and 15:00 hrs. A total of Studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Injections. All compounds were dissolved in distilled water (*i.c.v.* injections) or physiological saline (*i.v.* injections). For *i.c.v.* injections, mice were lightly anesthetized with ether and an incision was made in the scalp. Injections were performed using a 10 μ l Hamilton microsyringe at a point 2 mm caudal and 2 mm lateral from the bregma. Compounds were injected at a depth of 3 mm in a volume of 5 μ l. Intravenous (*i.v.*) injections were performed by restraining the mouse in a Plexiglas holder, dipping the tail for 10 sec in 42°C warm-water to dilate the tail vein, and subsequent injection into the vein with a 30 gauge needle.

Antinociceptive testing. Antinociception was assessed using the 55°C warm-water tail-flick test. The latency to the first sign of a rapid tail-flick was taken as the behavioral endpoint. Each mouse was first tested for baseline latency by immersing its tail in the water and recording the time to response. Mice not responding within 5 sec were excluded from further testing (average latency = 1.95 ± 0.08 seconds). Mice were then administered the test compound and tested for antinociception at 10, 20, 30, 45, 60, 90 and 120 min post-injection. A maximum score was assigned (100%) to animals not responding within 10 seconds. This cutoff gives us almost identical calculated A_{50} results compared to the 15 second cutoff when the data is expressed as % antinociception while producing less tissue damage when animals are tested repeatedly. We

ran separate groups of mice to assess the effects of repeated testing in the tail-flick assay to rule out any time-related changes in response latency that might affect antinociceptive potency determinations. From baseline through the 120 min time point, mean latencies were very consistent for uninjected controls (1.82-1.90 sec), i.v. saline (1.62-1.70 sec) and i.c.v. distilled water (1.72-2.02 sec).

Antinociception was calculated by the following formula: % antinociception = $100 \times (\text{test latency} - \text{control latency}) / (10 - \text{control latency})$. Dose-response lines were constructed at times of peak agonist effect, and analyzed by linear regression using FlashCalc software (Tallarida and Murray, 1987). All A_{50} values (95% confidence limits) shown are calculated from the linear portion of the dose-response curve. A minimum of 3 doses/curve and 10 mice were used at each dose level.

Synthesis and purification of glycopeptides. The required acetate-protected glycosyl Fmoc-amino acids were synthesized using published methods (Polt *et al.*, 1994; Szabó *et al.*, 1995; Mitchell, *et al.*, 2001). Deprotection of the Schiff base glycoside esters and reprotection afforded the FMOC-amino acid glycosides in excellent yield. Peptide synthesis utilized deprotection with 30% piperidine in N,N-Dimethylformamide, and amino acids coupling with Benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluoro-phosphate/N-Hydroxybenzotriazole mono-hydrate in N,N-Dimethylformamide. Couplings were sequential and proceeded in over 98% yield per step. The acetate protecting groups from the carbohydrate were removed while on the resin *via* treatment with $\text{H}_2\text{N}-\text{NH}_2 \cdot \text{H}_2\text{O}$ in MeOH. Cleavage from the resin was accomplished with 95% TFA in CH_2Cl_2 and appropriate scavengers over 2 hours. This also affected cleavage of the tert-butyl side chain protecting groups from the Tyr¹ and D-Thr² residues, but did not affect the glycosidic linkage. Precipitation of the crude peptide with

ice-cold diethyl ether, followed by purification on a Vydak preparative C₁₈ reversed-phase HPLC column provided the pure compounds, which were characterized by ¹H-NMR, ¹³C-NMR, COSY, TOCSY, ROESY, and FAB⁺ high resolution mass spectroscopy.

Radioligand binding studies. Membranes were prepared from whole brains taken from adult male Sprague-Dawley rats (250-300 g). All radioligand displacement experiments were run against the [³H]-labeled ligands [D-Pen², Phe(p-Cl)⁴, D-Pen⁵]enkephalin and D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP) as described previously (Hawkins et al., 1989; Vaughn et al., 1989). At least three experiments were done for each radioligand. Statistical comparisons between 1-2 site fits were made using the F-ratio test with a P value of 0.05 as the cut-off for significance (Shook et al., 1987). Data that were best fitted by a one-site model were reanalyzed with the logistic equation.

Mouse vas deferens (MVD) and guinea pig ileum (GPI) bioassays. Electrically induced smooth muscle contractions of isolated MVD and strips of GPI longitudinal muscle-myenteric plexus were used as a bioassay (Shook et al., 1987). Percent inhibition was calculated as the average contraction height for 1 min preceding the addition of the agonist divided by the contraction height 3 min after the addition of the agonist. IC₅₀ values represent the mean of not less than four tissue samples. IC₅₀ values, relative potency estimates, and their associated standard errors were determined by fitting the mean data to the Hill equation with a nonlinear least-squares method.

Results

Figure 1 summarizes the opioid receptor binding affinity and agonist potency of the compounds in the GPI and MVD bioassays, along with the i.c.v. and i.v. potency determinations in the 55°C tail-flick assay. The antinociceptive potencies are also represented graphically in Figure 2 as a plot of the calculated i.c.v. versus i.v. A_{50} values along with 95% confidence intervals.

Changes in the 6th amino acid residue attachment site (Ser versus Thr) did not affect the pharmacophore portion of the parent peptides (**1** and **2**) to any appreciable extent. Both peptides had high affinity for delta and mu opioid receptors, with a slightly higher affinity for delta over mu receptors, and negligible affinity for kappa receptors (>500 nM, data not shown). Both compounds potently inhibited electrically stimulated contractions in the smooth muscle tissue assays, being approximately 10-fold more potent in the MVD bioassay. The compounds produced dose- and time-related antinociceptive effects with full efficacy in the 55°C tail-flick assay following i.c.v. and i.v. administration (Figures 3 and 4). The L-Thr compound was marginally more potent than the L-Ser compound following i.v. administration on a $\mu\text{mol/kg}$ basis, while the i.c.v. potencies almost met criteria for statistical significance.

In general, the glycopeptides retained high affinity and modest delta receptor selectivity characteristic of the parent peptides. Attachment of a single β -glucose to L-Ser (**3**) or L-Thr (**4**) resulted in only minor (2-3-fold) changes in opioid receptor affinity and potency *in vitro*. There was a modest but significant increase in i.c.v. antinociceptive potency from compound **1** to **3**, and a trend for increased potency from compound **2** to **4**. Accompanied by these changes in potency was an increased duration of action with the glycopeptides, with compound **3** producing significant analgesia for over 120 minutes after i.c.v. administration and 90 minutes after i.v.

administration of an approximate A_{90} dose (Figures 3 and 4). Most significantly, the addition of a β -glucose group to **1** increased the i.v. potency of peptide by over 4-fold.

Compounds **5** and **6** were synthesized to determine if the orientation of the 6th amino acid (L- versus D-isomer) affected the overall potency and/or efficacy of the glycopeptides. A modest decrease in μ and δ binding affinity, along with a decrease in potency in the MVD bioassay, was observed going from a L-Ser to D-Ser (compound **3** versus **5**). There was not, however, a decrease in antinociceptive potency following i.c.v. administration. Similar effects were seen with the L-Thr to D-Thr substitution (compound **4** versus **6**). All of the compounds produced a full antinociceptive effect in the tail-flick assay.

Two additional glycopeptides with monosaccharides attached to 6th amino acid (L-Ser) were synthesized to determine if the increased i.v. potency observed with compound **3** was due to the β -glucose group or was more generally the result of a monosaccharide addition. The α -mannose (**7**) and β -xylose (**8**) compounds had in vitro profiles in line with compound **3**. Compound **7** was equipotent to **3** following i.c.v. administration while compound **8** was approximately 4-fold less potent. Interestingly, both compounds were equipotent to **3** following i.v. administration, with **8** being 4.9 times more potent than **1**.

Based on the results with the monosaccharide, several di- and trisaccharides were synthesized (compounds **9-11** and **12**, respectively) to determine if a larger carbohydrate group would further increase or decrease antinociceptive potency. Similar to the monosaccharides, all of the L-Ser attached disaccharides had similar binding affinities and IC_{50} potencies in the in vitro assays. The disaccharides were between 0.8 and 2.7-fold less potent than the monosaccharide **3** following i.c.v. administration. When administered i.v., all three disaccharides were significantly more potent than compound **3**, with **11** (β -Melibiose) being 5.3-

fold more potent than **3** and 21.5-fold more potent than the unglycosylated parent peptide **1**. A decrease in potency with the trisaccharide **12** was observed in the in vitro assays and the compound was less potent compared to any of the disaccharides tested i.v..

A series of bis-monosaccharides (**13-16**) and one tris-monosaccharide (**17**) were synthesized to further explore the structure-activity relationships of the glycopeptides. These compounds maintained low nM potency in the MVD bioassay but had a decrease in potency in the GPI bioassay (2.0-6.6 fold less potent than **1**). As a group these compounds were 3.9-16.5 times less potent than the monosaccharide **3** following i.c.v. administration. The one bis-monosaccharide tested i.v. (**13**) was 12.4 times less potent than compound **3**.

Discussion

In vivo glycosylation has been shown to alter the stability, intracellular transport, secretion, distribution and overall activity of a variety of proteins (Hilgenfeldt, 1988; Mann et al., 1996; Gimenez-Roqueplo et al., 1998; Barrios-De-Tomasi et al., 2002). We, along with others, have hypothesized that glycosylation of smaller peptides may have desirable effects on the pharmacokinetic and/or pharmacodynamic properties of the parent peptide (Albert et al., 1993; Polt et al., 1994; Negri et al., 1998; Susaki et al., 1999; Suzuki et al., 1999a,b; Bilsky et al., 2000). More specifically, we have shown that O-linked glycosylation of a potent opioid hexapeptide does not interfere with the pharmacophore portion of the molecule if the carbohydrate moiety is placed on the 6th amino acid residue (current results and Bilsky et al., 2000). This observation is consistent with Schwyzer's concept of message and address segments in opioid receptor activation (Schwyzer, 1986). The glycosylation does, however, increase peptide stability, BBB penetration and antinociceptive potency following *i.v.*, *s.c.* and *i.p.* routes of administration (Bilsky et al., 2000). The current experiments further define the structure-activity relationship of a series of opioid glycopeptides with varied glyco-address segments with a constant pharmacophore region.

From the *in vitro* and *in vivo* data, the use of either an L-Ser or L-Thr amino acid in the 6th position on the C-terminus appears to be a viable attachment point for carbohydrate groups. Glycopeptides with either amino acid displayed high affinity for mu and delta receptors, and potently inhibited electrically-stimulated contractions in the MVD and GPI bioassays. Furthermore, the stereochemical configuration of the 6th amino acid (L versus D) had minimal effects on the agonist potency of the unglycosylated and monoglycosylated compounds in smooth muscle bioassays and in the 55°C tail-flick assay following *i.c.v.* administration. These

data further support the hypothesis that minor modifications of the address segment of opioid peptides have minimal effects on the pharmacophore message. With additional glycosylation sites (bis and tris-monosaccharides) we observed a consistent and significant decrease in IC_{50} values and i.c.v. antinociceptive potency. This suggests multiple glycosylations of the parent peptide results in conformational changes that affect the pharmacophore (message segment). This hypothesis requires additional experimental studies and molecular modeling.

Substitution of α -Man or β -Xyl for β -Glc decreased the affinity of the peptide at both mu and delta receptors, with a loss of delta-receptor selectivity. These changes were not observed in the smooth muscle bioassays, which may reflect differences in the membrane component of brain homogenate versus the complete membrane in intact tissues. The β -Xyl compound was approximately 4x less potent than the β -Glc compound when administered i.c.v., though they were equipotent when administered i.v. Collectively, these data suggest that the carbohydrate group may have more profound effects on pharmacokinetics which ultimately plays a greater role in determining i.v. potency (see discussion below).

The disaccharides as a group were significantly more potent following i.v. administration than any of the monosaccharides tested. Based on these results, it was hypothesized that a greater carbohydrate surface area (increased hydrodynamic volume, Molineux, 2002) might be responsible for increased BBB transport and/or peptide half-life. To test this hypothesis further, a trisaccharide and a series of bis- and tris-monosaccharides were synthesized and tested. In general, we observed a decreased potency in vitro and a decrease in i.c.v. and i.v. potency.

From our data we can conclude that disaccharides provide the optimal improvement in BBB transport and/or pharmacokinetic factors, to maximize i.v. antinociceptive potency for hexapeptides. While there were some variations in the disaccharides in terms of receptor affinity

and bioassay IC₅₀ values, these can not fully explain the higher antinociception induced by these peptides. There was no obvious correlation between the i.c.v. and i.v. potencies of these compounds, nor between the binding affinities, IC₅₀ values or selectivity ratios and the measured i.v. potencies (data not shown). It is more likely that the changes in the carbohydrate moiety had effects on the stability and biodistribution of the compounds *in vivo*. Previous studies by our group have compared both the metabolic stability and BBB penetration for a number of monosaccharide glycopeptides and their parent peptides. These studies showed an increase in metabolic stability in both brain and blood for glycopeptides (Egleton et al., 2001) coupled with an enhanced BBB penetration (Bilsky et al., 2000; Egleton et al., 2000; Egleton et al., 2001). Studies investigating BBB penetration of compounds **1**, **3**, **10**, and **12** indicate that a disaccharide (compound **10**) had both the best brain delivery and metabolic stability, while the trisaccharide **12** had reduced BBB transport and stability compared to the disaccharide (Egleton et al., in preparation). This decrease in both BBB penetration and stability of the trisaccharide, is paralleled by the reduction in antinociception observed in this study. This indicates that the type of sugar used can modulate the bioavailability of opioid peptides to the brain.

Groups also have observed an effect of glycosylation on the distribution of peptides. Suzuki and colleagues investigated the effects of glycosylation on the pharmacokinetics of arginine-vasopressin and oxytocin analogs following i.v. administration (Susaki et al., 1999; Suzuki et al., 1999a,b). Specific tissue uptake was dependent on both peptide sequence and the carbohydrate moiety added (Suzuki et al., 1999b). The uptake into the kidney was increased while clearance via the liver was decreased (Suzuki et al., 1999b), indicating a shift from liver to renal clearance. Distribution studies of opioid peptides have shown a clearance predominantly via the liver-fecal route (Weber et al., 1992; Witt et al., 2001). Shifting the clearance from the

liver-fecal route to the kidney-urine route of excretion, has shown a good correlation in increasing both plasma half-lives and antinociception (Witt et al., 2001). It is thus possible that the addition of carbohydrate moieties to our peptide leads to an increase plasma half-life due to a change in excretion profile. This increase in plasma half-life then results in a higher uptake of peptide into the brain and thus an enhanced antinociceptive profile.

The glycosylation strategy, along with other approaches, for improving the pharmacokinetic profile of peptides has reignited interest in developing peptide-based pharmacotherapies. Advances in synthetic peptide chemistry and manufacturing processes have driven down the costs associated with large scale synthesis of these molecules. From our perspective, opioid peptides may offer several advantages over alkaloid based compounds. The availability of a number of unique pharmacophores based on naturally occurring neuropeptides that preferentially bind to delta opioid receptors may offer efficacy and tolerability advantages over mu selective analgesics in both acute and chronic pain management (Bilsky et al., 2000; Gengo and Chang, 2004; Lipkowski et al., 2004). The probability of accumulation of active metabolites in renal and liver disease is also presumably less with peptide based drugs versus alkaloids. The metabolites of the opioid glycopeptides, for example, would be small di- and tri-peptides and sugars. Furthermore, the glycosylation approach may be applicable to any number of peptide molecules, though further testing will have to determine the effects of these modifications on a peptide by peptide basis (see, for example, Susaki et al., 1999; Suzuki et al., 1999a,b).

In conclusion, we have provided further evidence that glycosylation of a small opioid peptide can significantly increase i.v. antinociceptive potency of the compound compared to the parent peptide. These changes in i.v. potency are likely due to changes in pharmacokinetic factors rather than changes in binding affinity and mu/delta receptor selectivity. For the opioid peptide

tested, the attachment of the carbohydrate groups can be accomplished with either a Ser or Thr in the 6th position, with the stereochemical configuration of that amino acid not being critical for activity. Finally, glycosylation with disaccharides resulted in the most potent compounds, with potencies on a $\mu\text{mol/kg}$ basis exceeding that of the prototypical opioid analgesic morphine.

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Footnotes

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Figure Legends

Figure 1. Chemical structures of the tested glycopeptides and the unglycosylated parent peptides, along with in vitro and in vivo bioassay data. The primary structure of the peptides is drawn at the top of the figure with the individual transport sequences drawn out and listed in the table below. Binding affinities at delta and mu opioid receptors are listed for each compound along with IC₅₀ values to inhibit electrically-evoked smooth muscle contraction in the mouse vas deferens and guinea pig ileum bioassays (described in detail in the methods section). The i.c.v. and i.v. A₅₀ values (and 95% confidence intervals) are calculated from dose-response curves in the 55°C tail-flick assay. Footnotes for the i.c.v. and i.v. A₅₀ values refer to the time of peak effect (1=10 minutes, 2=20 minutes, 3=30 minutes) for each individual compound.

Figure 2. A plot of the calculated i.c.v. versus i.v. antinociceptive A₅₀ values (and 95% confidence intervals) for representative glycopeptides and the unglycosylated parent peptides in the 55°C tail-flick assay.

Figure 3. I.c.v. Antinociceptive dose- and time-response curves for selected compounds in the mouse 55°C warm-water tail-flick test. Compounds 1 and 2 are the parent peptides while compounds 3 and 4 are the glycosylated (β-glucose) analogs. Bars represent the standard error of the mean for each dose and the various time points after injection.

Figure 4. I.v. antinociceptive dose- and time-response curves for the prototypic opioid alkaloid morphine, the parent peptide (compound 1) and its glycosylated analogs (compound 3, β-glucose and compound 11, β-melibiose). Bars represent the standard error of the mean for each dose and the various time points after injection.

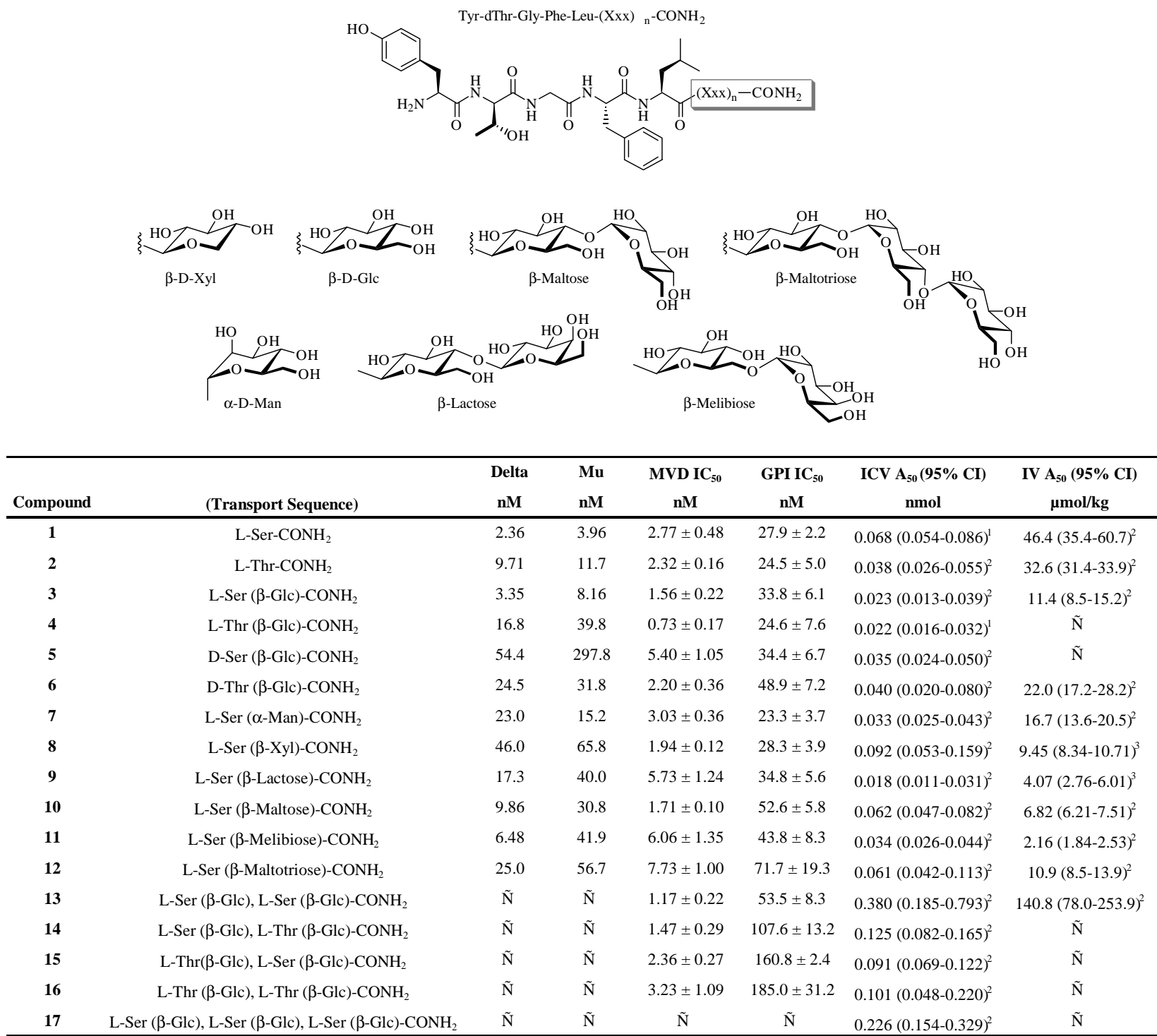


Figure 1.

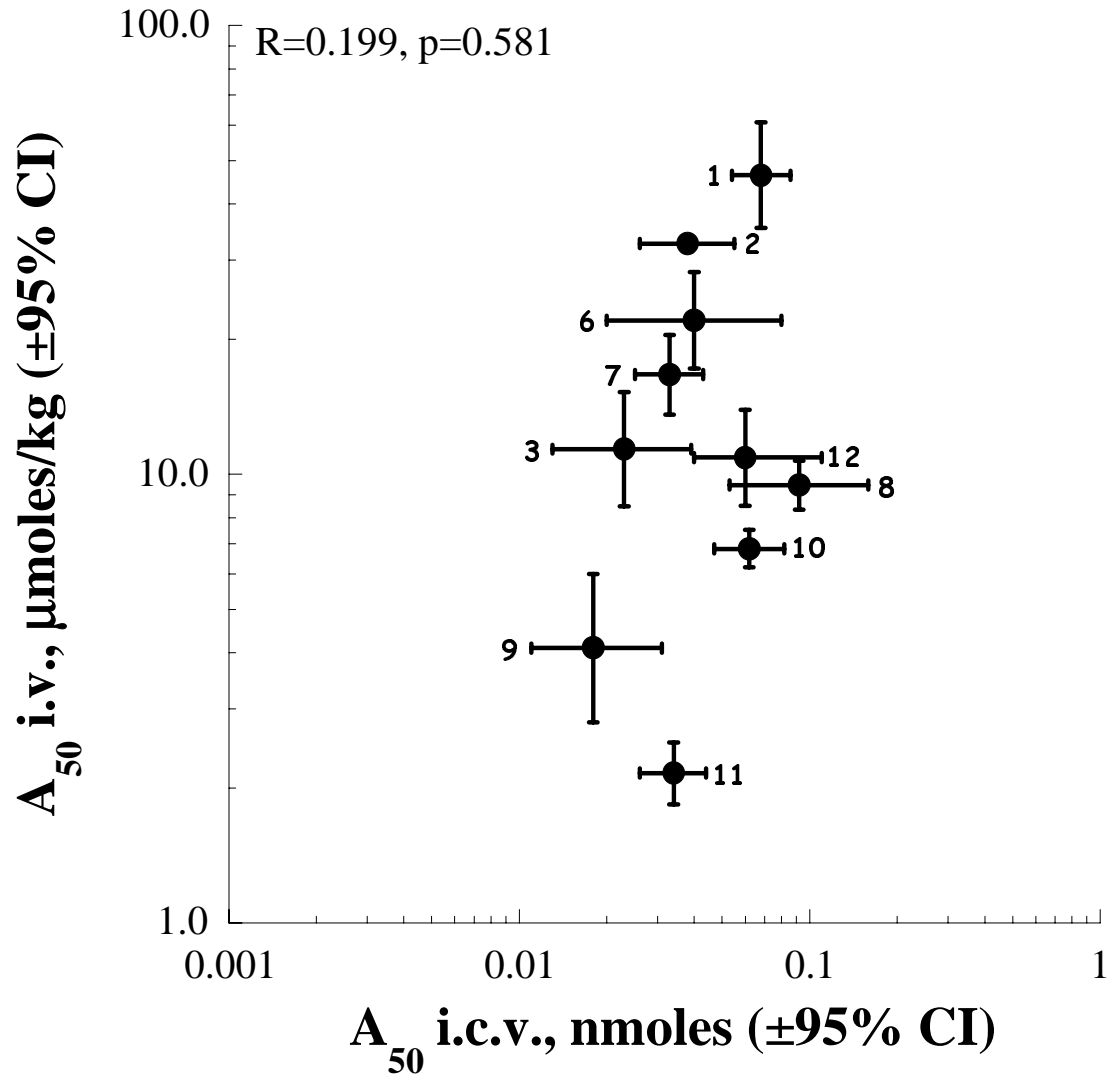


Figure 2.

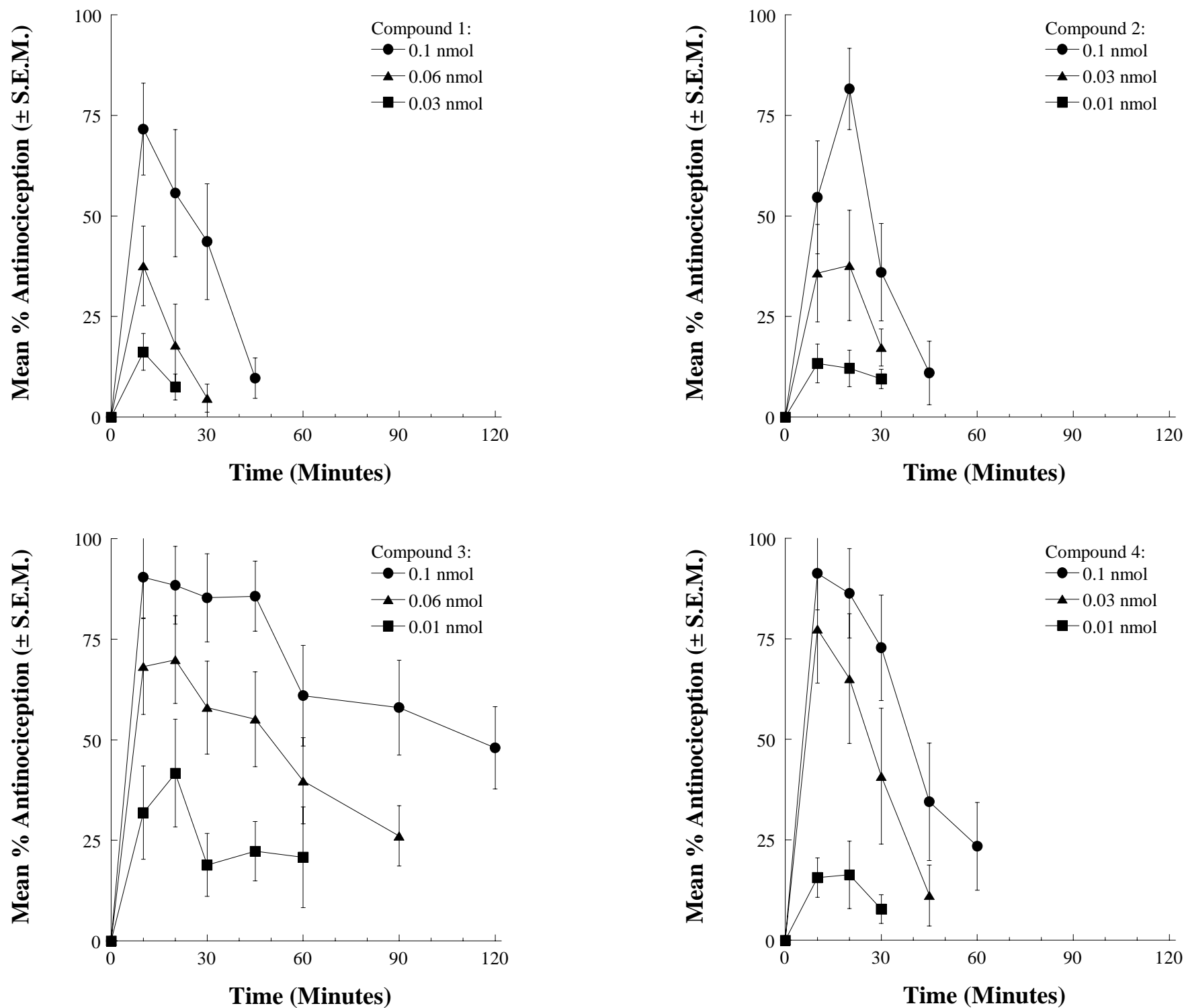


Figure 3.

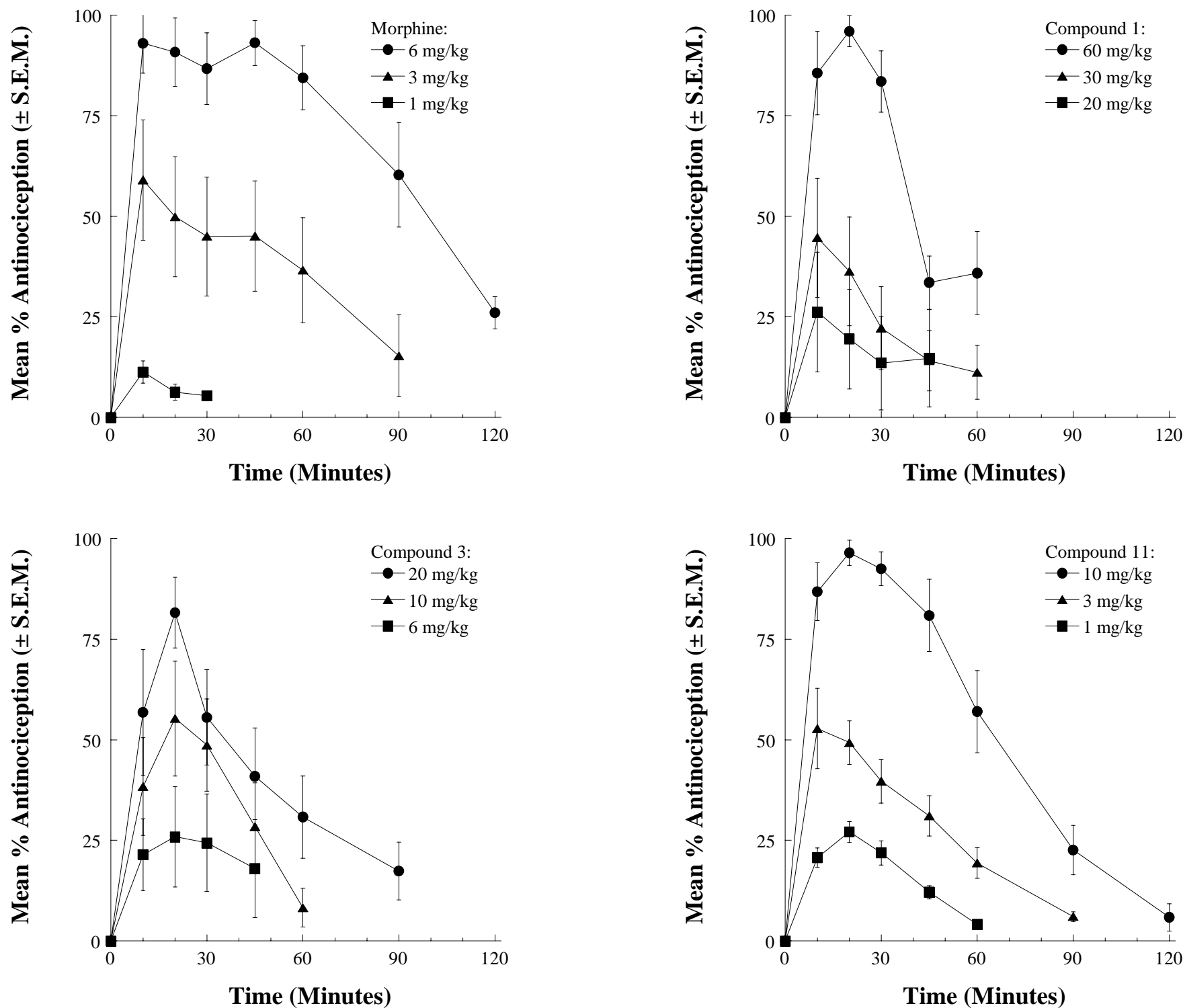


Figure 4.