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Evaluation of the P-glycoprotein (Abcb1) affinity status of a series of morphine analogs: Comparative study with meperidine analogs to identify opioids with minimal P-glycoprotein interactions

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ABSTRACT

One of the major shortcomings of many commonly used opioids is the fact that they are P-gp substrates, which represents a major obstacle towards effective pain management. P-gp can affect opioids' oral absorption, CNS accumulation, systemic clearance, antinociceptive activity, and tolerance development to their analgesic effects. Moreover, P-gp can be the locus of drug–drug interactions between opioids and other concomitantly administered drugs that are P-gp substrates/inhibitors. The objective of this study was to identify opioids that are non-P-gp substrates to overcome some of the mentioned shortcomings. We evaluated the P-gp affinity status (substrate, non-substrate, or inhibitor) of a series of morphine analogs (10 opioid agonist and 2 opioid antagonists) and compared them to previously reported meperidine analogs. The fold stimulation of the morphine analogs ranged from 1.01 to 1.54 while for the meperidine analogs the fold stimulation ranged from 1.10 to 3.66. From each series (morphine and meperidine analogs) we selected potential candidate opioids that are non-P-gp substrates and conducted *in vivo* assessments of their antinociceptive effects using P-gp knockout and P-gp competent mice. 6-Desoxymorphine, meperidine and *N*-phenylbutyl normeperidine did not significantly ($p > 0.05$) stimulate the basal P-gp ATPase activity, where, the fold stimulations of the basal P-gp ATPase activity were 1.01 ± 0.11, 1.51 ± 0.29 and 1.10 ± 0.23, respectively. Evaluation of the influence of P-gp ablation on their antinociceptive effects indicated that P-gp did not significantly ($p > 0.05$) affect their antinociceptive effects. Among the evaluated opioids *in vivo*, 6-desoxymorphine showed high potency and induced no apparent toxicity upon low- and high-dose administration. 6-Desoxymorphine is therefore an ideal lead compound to create a library of opioids that have negligible P-gp affinity for better management of pain.
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1. Introduction

Opioid agonists are potent analgesics that are commonly used for management of moderate to severe pain (Glare and Walsh, 1993). In order for opioids to exert their central action they need to bypass a formidable barrier, the blood brain barrier (BBB), to bind to the opioid receptors (μ, δ, κ) located in the CNS. The BBB is composed of endothelial cells that express a number of efflux transporters such as P-glycoprotein (P-gp, Abcb1), multidrug resistance associated proteins (Mrp, Abcc) and breast cancer related proteins (Bcrp, Abcg2). These efflux transporters act as a defense mechanism that protects the CNS from various xenobiotics such as opioids. Among the efflux transporters, P-gp was the first described and the most extensively studied efflux transporter. P-gp is encoded by *mdr1a* and *mdr1b* genes in rodents and MDR1 and MDR3 genes in humans and is well known to play a pivotal role in modulating the PK/PD of many therapeutic agents including opioids (Lin and Yamazaki, 2003; Dagenais et al., 2004).

Extensive studies indicated that P-gp can modulate the permeability, uptake, disposition and antinociceptive activities of opioids. For example, chemical and genetic disruption of P-gp using P-gp

Abbreviations: MDR, multidrug resistance proteins; P-gp, P-glycoprotein; MRP, multidrug resistance-associated proteins; Caco-2, epithelial human colon adenocarcinoma cell line; %MPE, percentage of maximal possible effect; AUEC, area under the percentage maximum possible effect vs. time curve; BBMECs, bovine brain microvessel endothelial cells.

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inhibitors and P-gp knockout mice suggested that P-gp had a significant impact on the cellular accumulation and the antinociceptive activity of many opioids (e.g., morphine, oxycodone, methadone, fentanyl, loperamide and DPDPE) (Chen and Pollack, 1999; Letrent et al., 1999a; Wandel et al., 2002; Skarke et al., 2003; Dagenais et al., 2004; Hoffmaster et al., 2004; Hassan et al., 2007). Overexpression of P-gp in cultured cells minimized the cellular accumulation of both synthetic and natural opioids (Callaghan and Riordan, 1993). One extreme example that manifests the adverse effects of P-gp on opioids is the active efflux of loperamide from the CNS by P-gp. As a result, loperamide, the potent opioid agonist *in vitro*, had no centrally mediated antinociceptive effect but rather a gastrointestinal effect (anti-diarrheal effect). These differential effects are believed to be due to the high affinity of loperamide to P-gp which actively extrudes loperamide out of the CNS and negates its antinociceptive activity. Interestingly, when P-gp was inhibited either chemically or genetically, central antinociceptive effects were observed for loperamide (Schinkel et al., 1996; Kalvass et al., 2004).

P-gp is also believed to be involved in tolerance development to opioids where P-gp expression was induced by 2-fold in brain tissues of morphine tolerant rats (Aquilante et al., 2000). In our laboratory we demonstrated that many opioids are P-gp substrates. For example, we demonstrated that oxycodone is a P-gp substrate and when repeatedly administered, it induced the expression of P-gp in brain, liver, intestine and kidney tissues of rats. This P-gp induction resulted in a P-gp-mediated drug–drug interaction when paclitaxel, the P-gp substrate, was administered to oxycodone treated rats (Hassan et al., 2007). Using microarray analysis, we observed that the expression levels of many efflux transporters were significantly regulated in brain and liver tissues of oxycodone treated rats (unpublished data). In addition, we demonstrated that many novel and known meperidine analogs were P-gp substrates (Mercer et al., 2007). Finally, we evaluated the P-gp affinity status (substrate, non-substrate, or inhibitor) of a representative opioid agonist (methadone), opioid agonist/antagonist (buprenorphine) and opioid antagonist (diprenorphine) using two *in vitro* (P-gp ATPase activity and monolayer efflux assays) and two *in vivo* (tissue distribution and antinociceptive monitoring in *mdr1a/b* (+/+) and *mdr1a/b* (−/−) mice (Hassan et al., 2009) and there was a good agreement among the four assays. The CNS distribution and the antinociceptive activity of methadone but not buprenorphine or diprenorphine were significantly ($p < 0.05$) dependant on P-gp. Based on these studies it is clear that one of the major shortcomings of the currently used opioid agonists is the fact that they are P-gp substrates. P-gp affects their (1) oral absorption, (2) CNS accumulation, (3) systemic clearance, (4) antinociceptive effects, and (5) tolerance development to their analgesic effects. In addition, P-gp can be the locus of drug–drug interactions between opioids and other concomitantly administered therapeutic agents that are P-gp substrates. It is therefore of great therapeutic importance to develop opioids that are not P-gp substrates. These new opioids are expected to have better BBB permeability, better antinociceptive activity, delayed development of tolerance and minimal P-gp-mediated drug–drug interactions. In this regard, we previously synthesized and tested the P-gp affinity status of a series of meperidine analogs ($n = 11$), searching for potent and specific opioids that have minimal P-gp affinity (Mercer et al., 2007). In expansion of our work we evaluated the P-gp ATPase activity of another series of morphine analogs ($n = 12$) and compared them to the previously synthesized meperidine analogs. From each series (morphine and meperidine analogs) we selected potential candidate opioids that are non-P-gp substrates and conducted *in vivo* assessments of their antinociceptive effects using P-gp knockout and P-gp competent mice.

2. Materials and methods

2.1. Drug-stimulated P-gp ATPase activity

Drug stimulated P-gp ATPase activity was estimated by Pgp-GIo assay system (Promega, Madison, WI). This method relies on the ATP dependence of the light-generating reaction of firefly luciferase. ATP consumption is detected as a decrease in luminescence. In a 96-well plate, recombinant human P-gp (25 μg) was incubated with P-gp-GIo assay buffer™ (20 μl) (control, $n = 4$), verapamil (200 μM) ($n = 4$), methadone (100 μM) ($n = 4$), sodium orthovanadate (100 μM) ($n = 4$), and morphine analogs listed in Table 1 (200 μM/analog) ($n = 3$/analog). All morphine analogs (Table 1) were purchased/supplied as gifts from Mallinckrodt, Inc (St. Louis, MO) or synthesized according to known procedures (Rapoport and Bonner, 1951; Koczka and Bernath, 1958; Bentley et al., 1967; Iijima et al., 1978). For each synthesized compound, product identity was determined by ^{1}H NMR (500 MHz Varian NMR) while the purity was determined by combustion analysis (Atlantic Microlab, Inc., Norcross, GA). Both verapamil and the opioid agonist, methadone (Hassan et al., 2009) served as positive controls while sodium orthovanadate was used as a P-gp ATPase inhibitor. In the presence of sodium orthovanadate ATP consumption by P-gp is negligible and without sodium orthovanadate, P-gp consumes ATP to a greater or lesser extent than the control, dependent

Table 1
Fold stimulation of basal P-gp-ATPase activity by a series of morphine and meperidine analogs.

Compound	*n*	Fold stimulation
Morphine analogs		
Non-treated (control)	4	1.00 ± 0.04
Morphine[a]	4	1.54 ± 0.08[b]
N-Benzylcodeine[c]	4	1.01 ± 0.14
6-Desoxymorphine[d]	4	1.01 ± 0.11
Oxymorphone[e]	4	1.12 ± 0.12
Thevinone[f]	4	1.28 ± 0.05[b]
Methyl thevinol[f]	4	1.28 ± 0.04[b]
Etorphine-3-methyl ether[f]	4	1.29 ± 0.10
6-Desoxycodeine[d]	4	1.31 ± 0.15
Etorphine[f]	4	1.34 ± 0.08[b]
Codeine[a]	4	1.36 ± 0.07[b]
Naloxone[a]	4	1.25 ± 0.11
Naltrexone[a]	4	1.13 ± 0.05
Meperidine analogs[g]		
Non-treated (control)	4	1.00 ± 0.03
Meperidine	3	1.51 ± 0.29
N-Phenylbutyl normeperidine	3	1.10 ± 0.23
N-Propyl normeperidine	3	1.81 ± 0.23[b]
N-2-Methylpropyl normeperidine	3	1.88 ± 0.22[b]
N-Methylallyl normeperidine	3	2.00 ± 0.41[b]
N-Butyl normeperidine	3	2.06 ± 0.19[b]
N-Phenylpropyl normeperidine	3	2.38 ± 0.12[b]
N-Crotyl normeperidine	3	2.80 ± 0.13[b]
N-Benzyl normeperidine	3	2.94 ± 0.07[b]
N-Allyl normeperidine	3	2.95 ± 0.29[b]
N-Phenylethyl normeperidine	3	3.66 ± 0.15[b]

Results are represented as mean ± S.E.M. The positive controls, verapamil and methadone stimulated the P-gp basal activity by 5.41 ± 1.52 and 2.45 ± 0.27-folds (Hassan et al., 2009), respectively.

[a] Indicates compounds purchased or supplied as gifts from Mallinckrodt, Inc. (St. Louis, MO).

[b] Indicates significant differences ($p < 0.05$) from corresponding control as determined by Student's *t*-test.

[c] Indicates a compound synthesized according to Koczka and Bernath (1958).

[d] Indicates compounds synthesized according to Rapoport and Bonner (1951).

[e] Indicates a compound synthesized according to Iijima et al. (1978).

[f] Indicates compounds synthesized according to Bentley et al. (1967).

[g] Meperidine analogs data were previously reported as change in luminescence (Mercer et al., 2007) and represented in the table for comparison with the morphine analogs.

on the effect of the test compound. The reaction was initiated by addition of MgATP (10 mM), stopped 40 min later by addition of 50 μl of firefly luciferase reaction mixture (ATP detection reagent) that initiated an ATP-dependent luminescence reaction. Signals were measured 60 min later and integrated for 10 s using Lmax® luminometer (Molecular Devices Corporation, Sunnyvale, CA) and converted to ATP concentrations by interpolation from a luminescent ATP standard curve. The rate of ATP consumption (pmol/min/μg protein) was determined as the difference between the amount of ATP in absence and presence of sodium orthovanadate (Eqs. (1) and (2)). Drug-stimulated P-gp ATPase activity was reported as fold-stimulation relative to the basal P-gp ATPase activity in the absence of drug (control) (Eq. (3)).

2.2. ATPase assay data analysis

Basal P-gp activity, test compound stimulated P-gp activity and fold stimulation by a test compound were calculated according to the following equations.

Basal P-gp activity (pmol ATP consumed/μg P-gp/min)

$$\frac{ATP_{vanadate} - ATP_{control}}{25_{\mu g\,Pgp} \times 40_{min}} \quad (1)$$

Test compound stimulated P-gp activity (pmol ATP consumed/μg P-gp/min)

$$\frac{ATP_{vanadate} - ATP_{compound}}{25_{\mu g\,Pgp} \times 40_{min}} \quad (2)$$

Fold stimulation by a test compound

$$\frac{\text{Test compound stimulated Pgp activity}}{\text{Basal Pgp activity}} \quad (3)$$

where, $ATP_{vanadate}$ is the number of non-consumed (total) pmol of ATP in the presence of sodium orthovanadate. $ATP_{control}$ is the number of non-consumed pmol of ATP in presence of the assay buffer. $ATP_{compound}$ is the number of non-consumed pmol of ATP in presence of a test compound. Two-tail Student's *t*-test (SigmaStat™ 2.03 statistical package, V2.03, Systat Software Inc., San Jose, CA) was used to determine the statistical significance of the difference between groups. The 0.05 level of probability was used as the criterion of significance. S.E.M of the fold stimulation was calculated using the delta-method (Polli et al., 1997).

2.3. Comparison of morphine and meperidine analogs

We have previously published a study (Mercer et al., 2007) in which the P-gp ATPase activity of a series of meperidine analogs was determined. In this previously reported study, we used the same experimental conditions as those employed in the present study. We concluded that meperidine and *N*-phenylbutyl normeperidine were not P-gp substrates. In the current study we compared morphine analogs with meperidine analogs in terms of the fold stimulation of the basal P-gp ATPase activity. Due to batch-to-batch variability of the basal activities of the recombinant human P-gp, the rates of ATP consumption of the morphine and the meperidine analogs were each compared to the corresponding control which was run on the same day under the same experimental conditions. The fold stimulation (Eq. (3)) was used as the main criterion of comparison between the two sets of analogs. Fold stimulation is a common tool for comparison purposes (Polli et al., 2001). In which the rates of ATP consumption due to a test compound is normalized to the rate of ATP consumption due to the basal P-gp ATPase activity which is determined in the same plate under the same experimental conditions (Eq. (3)).

2.4. Experimental animals

Male *mdr1a/b* (−/−) and male FVB *mdr1a/b* (+/+) mice weighing 28 ± 5 g (20–24 weeks of age) were purchased from Taconic Laboratories (Germantown, NY). The mice were housed individually and allowed to acclimate at least 1 week before the experiment was conducted. Animals were fed chow and water "*ad libitum*" and maintained on a 12-h light/dark cycle. The protocol for the animal studies was approved by the School of Pharmacy, University of Maryland IACUC.

2.5. Assessment of the antinociceptive effect in mdr1a/b (−/−) and mdr1a/b (+/+) mice

The tail-flick test was used to determine the antinociceptive effect of a representative morphine analog, 6-desoxymorphine, which is also known as 6-deoxymorphine (Orahovats et al., 1955; Reden et al., 1979) while from the meperidine series, both meperidine and its analog (*N*-phenylbutyl normeperidine) were evaluated. These three opioids were selected because they showed no significant ($p < 0.05$) stimulation of P-gp ATPase activity. The opioid agonist, methadone, was used as a positive control since previous studies in our laboratory (Hassan et al., 2009) indicated that its analgesic activity was significantly ($p < 0.05$) enhanced by 2.73 ± 0.55-fold in P-gp knockout mice versus wild type mice. Each opioid was administered s.c. to two groups of mice [*mdr1a/b* (+/+) and *mdr1a/b* (−/−)] ($n = 5$–6/group). No mouse in any group received more than one single s.c. dose of any opioid. The doses of the tested opioids were as follows: 0.2 and 2 mg/kg 6-desoxymorphine, 50 mg/kg meperidine and 3 and 60 mg/kg *N*-phenylbutyl normeperidine. Antinociceptive effect was monitored at 5, 15, 30, 45, 60, 90, 120, and 150 min post-dosing using a tail-flick analgesia meter (Pamotor, Burlingame, CA) (D'amour and Smith, 1941). The test was carried out using tail-flick analgesia meter. Briefly, each mouse was placed on the surface of the analgesia meter and heat was applied to the ventral surface of its tail (2–3 cm from the base of the tail). The intensity of the radiant heat was adjusted so that baseline tail-flick occurred within 1–2 s. Tail-flick latency responses were measured in duplicate. A cut-off time of 9 s was used to prevent tail damage. Mice that failed to respond within the respective cut-off time were defined as "analgesic". The percentages of the maximum possible effect (%MPE) were calculated using Eq. (4).

2.6. Tail-flick test data analysis

The tail-flick latency values were converted to a percentage of the maximum possible effect (%MPE) and plotted against time (Nielsen et al., 2000; Ross et al., 2000). The area under the %MPE vs. time curve (AUEC) was calculated using the trapezoidal method.

$$\%MPE = \frac{\text{post drug latency} - \text{pre drug latency}}{\text{cutoff} - \text{pre drug latency}} \times 100 \quad (4)$$

All data were presented as mean ± S.E.M. ANOVA with repeated measures (SigmaStat™ 2.03 statistical package) was used to determine the statistical significance between groups. The 0.05 level of probability was used as the criterion of significance.

3. Results

3.1. Effect of opioids on P-gp ATPase activity

Morphine analogs (Fig. 1A) were examined to determine their effects on the P-gp ATPase activity. Each opioid together with a known excess of ATP was incubated with recombinant human P-gp. ATP consumption due to P-gp stimulation by each opioid

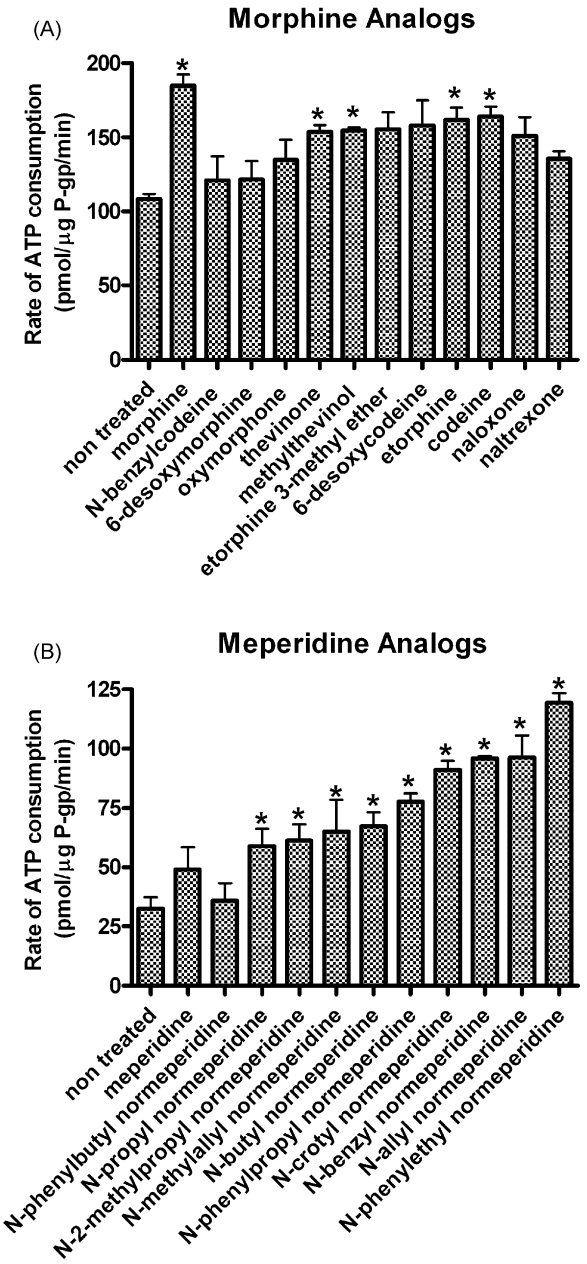

Fig. 1. Influence of different (A) morphine analogs and (B) meperidine analogs on the rate of ATP consumption by recombinant human P-gp. Recombinant human P-gp (25 μg) samples were incubated with GIO assay buffer™ (20 μl) (control, $n = 4$), morphine analogs (200 μM, $n = 4$/analog) and meperidine analogs (200 μM, $n = 3$/analog). Data are expressed as the mean ± S.E.M. Student's *t*-test (SigmaStat™ 2.03 statistical package) was used to determine significant differences from non-treated wells (controls). *Significant difference at $p < 0.05$. Data for meperidine analogs were previously reported as change in luminescence (Mercer et al., 2007) and represented in the figure for comparison with the morphine analogs.

was detected as a decrease in luminescence (i.e., the higher the potency of a compound to stimulate the P-gp ATPase activity, the lower the luminescence signal). The positive controls, verapamil and methadone stimulated the P-gp basal activity by 5.41 ± 1.52 and 2.45 ± 0.27-folds (Hassan et al., 2009), respectively (data not shown). The rates of ATP consumption for morphine, thevinone, methylthevinol, etorphine and codeine were significantly different ($p < 0.05$) from the non-treated (control) whereas the rates of ATP consumption for the rest of the morphine analogs were not significantly different ($p > 0.05$) from the non-treated (control) (Fig. 1A). The rates of ATP consumption for meperidine series were significantly different for all analogs with the exception of the parent opioid, meperidine and its analog, *N*-phenylbutyl normeperidine (Fig. 1B). The data presented here for the meperidine analogs differ slightly from those published earlier (Mercer et al., 2007), because they are presented as rates of ATP consumption (pmol ATP consumed/μg P-gp/min), whereas those published earlier were reported as relative luminescence (RLU).

3.2. Comparison of morphine and meperidine analogs in terms of their P-gp affinity status

Based on fold stimulation of the basal P-gp ATPase activity, meperidine analogs seem to stimulate the basal P-gp ATPase activity more than the morphine analogs (Table 1). The fold stimulation by the meperidine analogs ranged from (1.10 ± 0.23–3.66 ± 0.15) while those for the morphine analogs ranged from (1.01 ± 0.11–1.54 ± 0.08) indicating that meperidine analogs are greater of P-gp substrates as compared with the morphine analogs. For comparison purposes we compared the fold stimulation rather than the rates of ATP consumption because it seems that the basal activity of the recombinant human P-gp used in this study for testing morphine analogs is different (Fig. 1A and B) from the one reported in the previous meperidine analogs study (Mercer et al., 2007).

3.3. Assessment of the antinociceptive effect in mdr1a/b (−/−) and mdr1a/b (+/+) mice

The antinociceptive effects associated with single dose administration of 0.2 and 2 mg/kg 6-desoxymorphine, 50 mg/kg meperidine or, 3 and 60 mg/kg *N*-phenylbutyl normeperidine were monitored for 150 min in P-gp knockout and P-gp competent mice using tail-flick test (Fig. 2). For all the three opioids, P-gp had no significant effect ($p > 0.05$) on their antinociceptive activity with the exception of the early time points of the higher dose of *N*-phenylbutyl normeperidine (Fig. 2). In addition, no significant ($p > 0.05$) increase in the areas under the effect curve (AUECs) was observed for any tested dose of any opioid in *mdr1a/b* (−/−) mice vs. *mdr1a/b* (+/+) mice (Table 2). For all opioids, no signs of any toxicity were observed after administration of any dose with the exception of the higher dose (60 mg/kg) of *N*-phenylbutyl normeperidine, where 35 min post-dosing, some mice in both groups ($n = 4$) experienced episodes of convulsions that lasts for 1–2 min.

Table 2
Area under the effect curve (AUEC) (%MPE × min) of opioids after single s.c. administration in *mdr1 a/b* (+/+) and *mdr1 a/b* (−/−) mice.

Opioid	Dose (mg/kg)	*mdr1a/b* (+/+) (AUEC) (%MPE × min)	*mdr1a/b* (−/−) (AUEC) (%MPE × min)	AUEC ratio *mdr1a/b* (−/−)/*mdr1a/b* (+/+)
6-Desoxymorphine	0.2	367.30 ± 204.35	419.33 ± 322.59	1.14 ± 1.08
	2	6266.13 ± 700.5	7309.87 ± 680.77	1.17 ± 0.17
Meperidine	50	6752.14 ± 917.94	7678.40 ± 851.59	1.14 ± 0.2
N-Phenylbutyl normeperidine	3	473.91 ± 180	789.82 ± 177.41	1.67 ± 0.74
	60	7102.93 ± 2241.96	9407.39 ± 2195.22	1.32 ± 0.55

AUEC values were calculated from 0 to 150 min and expressed as mean ± S.E.M ($n = 5$–6). AUEC ratio for a single s.c dose of 3 mg/kg methadone (positive control) was 2.73 ± 0.55-fold ($p < 0.05$) (Hassan et al., 2009).

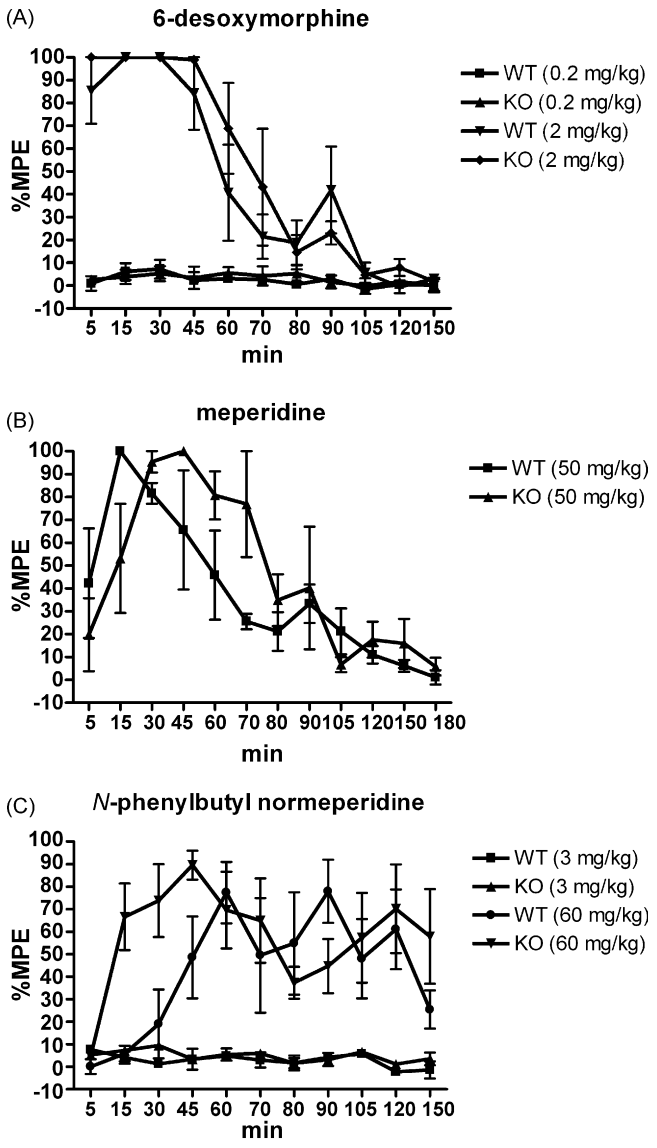

Fig. 2. Tail-flick latencies expressed as %MPE versus time for *mdr1a/b* (+/+) mice (WT) and *mdr1a/b* (−/−) mice (KO) that received single s.c. dose of (A) 0.2 mg/kg or 2 mg/kg 6-desoxymorphine, (B) 50 mg/kg meperidine or C) 3 mg/kg or 60 mg/kg *N*-phenylbutyl normeperidine. Data are expressed as the mean ± S.E.M ($n = 5$).

4. Discussion

The objective of this study was to evaluate the P-gp affinity status of a series of morphine and meperidine analogs to identify opioids with minimal P-gp interaction for better and effective management of pain. Since the majority of opioids have been reported to be potential P-gp substrates, P-gp can significantly affect their pharmacokinetics, pharmacodynamics and their therapeutic effects (Letrent et al., 1998, 1999a,b; Aquilante et al., 2000; Dagenais et al., 2004; Hassan et al., 2007). Moreover, P-gp can be the locus of drug–drug interactions (Lin, 2003; Lin and Yamazaki, 2003; Hassan et al., 2007), where, concomitant administration of opioids with other therapeutic agents that are P-gp substrates or inhibitors may render toxic or subtherapeutic doses of either the opioid itself or the concomitantly administered therapeutic agents (depending on the relative affinity of each to the binding sites on P-gp). With that in mind, our ultimate goal is to develop a library of potent opioids that are non-P-gp substrates to avoid some of the mentioned shortcomings. We combined both *in vitro* (P-gp ATPase assay) and *in vivo* (antinociceptive monitoring in P-gp deficient and P-gp competent mice) assays to obtain a better assessment of the impact of P-gp on the tested opioids.

The P-gp ATPase assay demonstrated that several morphine analogs are P-gp substrates (Fig. 1A, Table 1). The parent opioid, morphine significantly ($p < 0.05$) stimulated the P-gp ATPase activity indicating that it is a P-gp substrate (Fig. 1A, Table 1). Consistent with our *in vitro* study, previous P-gp knockout mice studies indicated that morphine is a P-gp substrate (Schinkel et al., 1995; Zong and Pollack, 2000). Other morphine analogs (thevinone, methylthevinol, etorphine and codeine) were also observed to significantly ($p < 0.05$) stimulate the basal P-gp ATPase activity, albeit to a lesser extent than morphine (Fig. 1A, Table 1). On the other hand, many morphine analogs were shown not to be P-gp substrates (e.g., *N*-benzylcodeine, 6-desoxymorphine, oxymorphone, etorphine-3-methyl ether and 6-desoxycodeine) (Fig. 1A, Table 1).

Oxymorphone, an active metabolite of oxycodone did not significantly ($p > 0.05$) stimulate the P-gp ATPase activity. Oxymorphone is a very potent μ opioid receptor agonist which is approximately 10 times as potent as morphine (Beaver et al., 1977). We have reported previously (Hassan et al., 2007) that oxycodone is a P-gp substrate that stimulates the P-gp ATPase activity in a concentration-dependant manner. Consistent with other P-gp substrates (e.g., morphine, dexamethasone and cyclosporin A) (Jette et al., 1996; Aquilante et al., 2000), chronic administration of oxycodone upregulated P-gp in various tissues (e.g., brain, intestine, kidney and liver). Upregulation of P-gp is proposed to be a protective mechanism by which cells restricts the influx of P-gp substrates across cell membranes, and it is mainly due to chronic administration of P-gp substrates (Pastan and Gottesman, 1991; Thorgeirsson et al., 1991; Gottesman and Pastan, 1993). Since oxymorphone is not a P-gp substrate (Fig. 1A, Table 1), upregulation of P-gp observed after chronic oxycodone administration is unlikely to be mediated by oxymorphone. However, further *in vivo* evaluation of the P-gp expression after repeated oxymorphone administration is needed to confirm this assumption.

The orvinols (e.g., thevinone, methylthevinol, etorphine-3-methyl ether and etorphine) showed minimal interactions with P-gp evidenced by fold stimulations that ranged from 1.28 to 1.34-fold (Fig. 1A, Table 1) although it was significantly different from the control for some of them (Table 1). Consistently, buprenorphine and diprenorphine, other orvinols, showed negligible interactions with P-gp when tested under similar experimental conditions (~1-fold stimulation of P-gp ATPase activity) (Hassan et al., 2009). Also, when buprenorphine and diprenorphine were tested in P-gp knockout mice, P-gp ablation had no influence of their tissue distribution or antinociceptive activity (Hassan et al., 2009). As such, studying the pharmacophore responsible for these orvinols not being potential P-gp substrates can help in developing novel μ-opioid agonists that do not interact with P-gp.

The commonly used opioid antagonists naloxone and naltrexone were also evaluated in this study and they did not significantly ($p > 0.05$) stimulate the P-gp ATPase activity (Fig. 1A, Table 1). Likewise, when the P-gp affinity status of both naloxone and naltrexone was tested using calcein inhibitory assay and monolayer efflux assay in absence and presence of the P-gp inhibitor GF120918, neither of these opioid antagonists was observed to be a P-gp substrate (Mahar Doan et al., 2002). Consistently, we demonstrated that diprenorphine like other opioid antagonists is not a P-gp substrate (Hassan et al., 2009).

While *in vitro* assays (e.g., P-gp ATPase assay, monolayer efflux assay and calcein inhibitory assay) are commonly used to characterize the P-gp affinity status of many compounds including opioids, the ultimate determination of the impact of P-gp on opioids requires *in vivo* evaluation. In this regard, P-gp deficient and P-gp competent mice were used to elucidate the role of P-gp on modulating

the antinociceptive effects of selected morphine and meperidine analogs that showed no affinity to P-gp *in vitro*. 6-Desoxymorphine, meperidine and *N*-phenylbutyl normeperidine were selected for further *in vivo* assessments. 6-Desoxymorphine (the major metabolite of 6-desoxycodeine similar to morphine which is the major metabolite of codeine) was selected as a representative morphine analog because: firstly, it induced no significant ($p > 0.05$) stimulation of the basal P-gp ATPase activity (Fig. 1A, Table 1). Secondly, it is ~10 times as potent as morphine as determined by tail-flick, hotplate, and paraphenylquinone assays conducted by the Drug Evaluation Committee (DEC) at the National Institute on Drug Abuse (NIDA). Finally, it is a highly specific μ opioid agonist (Ki at μ receptor = 2.9 nM) as determined by DEC, NIDA. These factors together made it a suitable candidate opioid for further *in vivo* evaluation. Testing low (0.2 mg/kg) and high (2 mg/kg) doses of 6-desoxymorphine in P-gp competent and P-gp deficient mice indicated that lack of P-gp had no significant influence on the antinociceptive activity of 6-desoxymorphine (Fig. 2A, Table 2). This suggests that it is not a P-gp substrate *in vivo* which is in agreement with the P-gp ATPase assay findings (Fig. 1A, Table 1). Consistent with DEC evaluation, after administration of high dose (2 mg/kg) of 6-desoxymorphine, the %MPE reached its maximum (100%) for 45 min in both P-gp knockout and wild type mice (Fig. 2A) while, in an earlier study (Thompson et al., 2000) a 10 times higher dose of morphine (20 mg/kg) was needed to reach ~95%MPE for 45 min in wild type mice. These results confirm that 6-desoxymorphine is a potent opioid relative to morphine but it has the advantage of being not a P-gp substrate (Figs. 1A and 2A and Table 1).

Previously, we synthesized and evaluated the P-gp affinity status of a series of meperidine analogs (Mercer et al., 2007) using the P-gp ATPase assay under the same experimental conditions described in Section 2. All meperidine analogs were P-gp substrates with the exception of the parent opioid, meperidine, and its analog *N*-phenylbutyl normeperidine (Fig. 1B, Table 1). Consequently, in this report, we evaluated the antinociceptive activity of meperidine and *N*-phenylbutyl normeperidine using P-gp knockout and P-gp competent mice. Similar to 6-desoxymorphine, the genetic disruption of P-gp had no significant influence on the antinociceptive activity of meperidine indicating that meperidine is not a P-gp substrate (Fig. 2B, Table 2). Consistently, previous brain uptake studies in P-gp competent and P-gp deficient mice indicated that meperidine is not a P-gp substrate (Dagenais et al., 2004). Collectively, these *in vitro* and *in vivo* findings give strong evidence that meperidine is not a P-gp substrate. Meperidine is well known to have greater CNS permeability than morphine. The intravenous to the intraventricular ED_{10} ratio for meperidine was only 8.5 while that for morphine was 910 (~107-fold that of meperidine), demonstrating the ease by which meperidine penetrates the BBB (Casy and Parfitt, 1986). One hypothesis that explains the higher BBB permeability of meperidine relative to morphine is the greater lipophilicity of meperidine than morphine (Casy and Parfitt, 1986). Another hypothesis comes from the differential interaction of P-gp with morphine and meperidine. P-gp can actively efflux morphine from the CNS, minimizing its BBB permeability and affecting its antinociceptive activity (Letrent et al., 1998, 1999a). However, for meperidine, P-gp does not seem to have any effect on its BBB permeability or antinociceptive activity (Fig. 2B) (Dagenais et al., 2004). These studies demonstrate that the pharmacokinetics as well as the pharmacodynamics of meperidine are most likely not mediated by P-gp. Meperidine displays characteristics of an opioid agonist that lacks P-gp interaction. However, it has low potency and short duration of action (Janssen and Eddy, 1960).

N-Phenylbutyl normeperidine was one of the selected analogs for *in vivo* evaluation due to lack of significant stimulation of the basal P-gp ATPase activity (Fig. 1B, Table 1), and due to its high potency (2 times as potent as meperidine) (Casy and Parfitt, 1986). Testing low and high doses of *N*-phenylbutyl normeperidine in P-gp competent and P-gp deficient mice indicated that P-gp had no significant effect ($p > 0.05$) on its antinociceptive activity with the exception of the early time points of the higher dose (Fig. 2C, Table 2). The %MPE after administration of 3 mg/kg (Fig. 2C) remained close to the baseline for a period of 150 min. However, when a higher dose (60 mg/kg) of *N*-phenylbutyl normeperidine was tested, some analgesic effects were observed (Fig. 2C), but several mice experienced signs of toxicity 30 min post-dosing. The toxicity was manifested in the form of convulsive episodes that lasted for a period of 1–2 min and may possibly be due to generation of the toxic metabolite normeperidine (Umans and Inturrisi, 1982). In contrast to the previous report (Casy and Parfitt, 1986) which indicated that *N*-phenylbutyl normeperidine is 2 times as potent as meperidine, our data indicated that it is a weak opioid analgesic requiring a high dose of 60 mg/kg to induce some analgesic activity (Fig. 2C, Table 2). As a result, to validate its potency, the analgesic activity of *N*-phenylbutyl normeperidine was re-evaluated by DEC using the tail-flick assay. The data indicated that *N*-phenylbutyl normeperidine was inactive at 1, 10 and 30 mg/kg doses. These data are consistent with ours and confirm that *N*-phenylbutyl normeperidine is a weak opioid analgesic contrary to the early report by (Casy and Parfitt, 1986). A possible explanation of these differences may be due to the advancement of the currently used instrumentation coupled with the better automated quantitation methodology used these days. Taken together, both our *in vitro* and *in vivo* evaluation of *N*-phenylbutyl normeperidine showed that it is not a P-gp substrate. However, it is not a suitable lead compound due to its dose dependant toxicity and weak potency.

In conclusion, based on the fold stimulation of the basal P-gp ATPase activity (Table 1), the morphine analogs have minimal interactions with P-gp relative to the meperidine analogs which is evidenced by minimal influence on the basal P-gp ATPase activity. The fold stimulation of the morphine analogs ranged from 1.01 to 1.54 while for the meperidine analogs the fold stimulation ranged from 1.10 to 3.66. In contrast to the parent opioid, morphine, the morphine analog 6-desoxymorphine lacks any interaction with P-gp *in vitro* and *in vivo,* it is a specific μ-opioid agonist, 10 times as potent as morphine and appears to be a suitable lead compound for developing new opioids that are specific, potent and non-P-gp substrates for better and effective management of pain.

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