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Opioids and efflux transporters. Part 4: Influence of *N*-substitution on P-glycoprotein substrate activity of noroxymorphone analogues

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ABSTRACT

The efflux transporter protein P-glycoprotein (P-gp) is capable of affecting the central distribution of diverse neurotherapeutics, including opioid analgesics, through their active removal from the brain. P-gp located at the blood brain barrier has been implicated in the development of tolerance to opioids and demonstrated to be up-regulated in rats tolerant to morphine and oxycodone. We have previously examined the influence of hydrogen-bonding oxo-substitutents on the P-gp-mediated efflux of 4,5-epoxymorphinan analgesics, as well as that of *N*-substituted analogues of meperidine. Structure–activity relationships (SAR) governing *N*-substituent effects on opioid efficacy is well-established, however the influence of such structural modifications on P-gp-mediated efflux is unknown. Here, we present SAR describing P-gp recognition of a short series of *N*-modified 4,5-epoxymorphinans. Oxymorphone, naloxone, naltrexone, and nalmexone all failed to demonstrate P-gp substrate activity, indicating these opioid scaffolds contain structural features that preclude recognition by the transporter. These results are examined using mathematical molecular modeling and discussed in comparison to other opioid scaffolds bearing similar *N*-substituents.

P-glycoprotein (P-gp) is an efflux transport protein coded in the multidrug resistance (MDR) genes, and a member of the ATP-binding cassette (ABC) superfamily of proteins.[1–3] P-gp is capable of transporting diverse xenobiotic substrates of various drug classes including but not limited to analgesics, antipsychotics, antiemetics, antineoplastics, antihypertensives, antibiotics, sedative hypnotics, and antihistamines.[4,5] Up-regulation or overexpression of P-gp may result in MDR, and has been implicated in reducing the effectiveness of various clinical drug therapies including cancer chemotherapy,[6] antibiotic and antiretroviral therapy,[7] and opioid mediated analgesia.[8] These known pharmacokinetic effects of P-gp on clinically used drugs illustrate the importance of identifying new lead compounds that are P-gp substrates early in their development. This identification is important for developing lead compounds optimized for either increased or decreased central penetration, and for decreased potential interactions with other pharmaceuticals.

Clinically useful opioid analgesics are subject to the development of tolerance and require escalating dosage regimens to maintain an acceptable level of analgesia. Side effects, such as constipation and respiratory depression, are increased at higher doses and complicate therapeutic regimens for providing efficacious analgesia.[9] Many mechanisms have been proposed for the development of tolerance to opioid analgesia, [10] however blood brain barrier efflux of opioids has been increasingly implicated in the development of central tolerance to opioids.[11] P-gp up-regulation has been demonstrated in rats tolerant to morphine, and rats tolerant to oxycodone.[11–13] P-gp knockout mice show increased magnitude and duration of antinociception induced by morphine, methadone, and fentanyl.[14] Conceptually, opioid analgesics that are P-gp substrates may require increased dosages to provide effective analgesia due to P-gp exacerbating the overall development of tolerance to these opioid analgesics. Diverse opioid receptor agonists have been found to be P-gp substrates to varying degrees, including morphine, oxycodone, fentanyl, U-69,593,

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bremazocine, methadone, loperamide, and SNC-121.[14–19] Fewer investigations have been undertaken to determine the P-gp substrate activity of opioid antagonists. Opioid receptor antagonists naloxone and naltrexone have been reported as non- substrates for P-gp,[5,20] whereas buprenorphine, nalbuphine, and naltrindole have each been identified as P-gp substrates.[5,14,19,21]

The *N*-substituent in opioids plays a significant role in the efficacy of the compound at opioid receptors in several opioid classes.[22] Previously, we examined the role of the *N*-substituent upon the P-gp substrate activity of a series of normeperidine analogues and found greater P-gp substrate activity in analogues bearing alkenyl and short phenylalkyl *N*-substituents.[23] As a continuation of our efforts studying the structure–activity relationships (SAR) of opioids as P-gp substrates,[23–26] here we examine a short series of *N*-substituted noroxymorphone analogues for their P-gp substrate activity. We also describe for the first time efforts toward correlating opioid ligand structure with P-gp function using a recently described model of the transporter.[27]

Analogues were prepared from noroxymorphone (**1**) by alkylation with the appropriate alkyl halide while stirring in DMF for 24 h in the presence of sodium carbonate (Fig. 1) to produce **2**–**7** (Table 1). Compounds were assayed as freebases except as noted.[28] Naloxone and naltrexone were obtained from Mallinckrodt, Inc. (St. Louis, MO) and reagents from Sigma–Aldrich, Inc. (Milwaukee, WI). Oxymorphone was synthesized from oxycodone according to literature procedure.[29] Calculation of molecular properties (*c*Log*P*, interacting surface area) and P-gp substrate prediction was carried out in silico using known procedures.[27] Results of this study are shown in Table 2 and Figure 3.

P-gp ATPase activity in the presence of the compounds was assessed using the Pgp-Glo assay system (Promega, Madison, WI) as described previously.[23,30] The results are presented in Figure 2. Briefly, the assay measures the relative luminescence units (RLU) generated by firefly luciferase when stimulated by ATP. Compounds are incubated in the assay buffer system containing recombinant human P-gp and MgATP, quenched with firefly luciferase, and RLU measured using the L_{max} luminometer (Molecular Devices, Sunnyvale, CA). The effects of the ligands on RLU are compared to control and evaluated for either their ability to stimulate P-gp ATPase activity (substrates, decrease in RLU), decrease P-gp ATPase activity (inhibitors, increased RLU), or lack of significant change from control (indicating the ligand is neither a substrate nor inhibitor of P-gp). The P-gp substrate verapamil was employed as a positive control and sodium orthovanadate, a P-gp inhibitor, as a negative control.

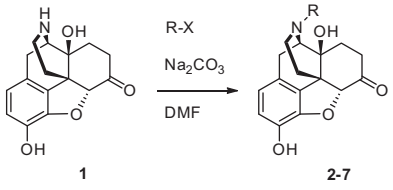

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Figure 1. Synthetic scheme of oxymorphone analogues.

Table 1
Compounds assayed

Compound	*N*-R	Salt form, mp (°C)	Opioid efficacy
Oxymorphone	CH_3	Oxalate, 120–125	Agonist
Naloxone	CH_2CHCH_2	HCl	Antagonist
Naltrexone	$CH_2C_3H_5$	HCl	Antagonist
2, Nalmexone	$CH_2CHC(CH_3)_2$	fb, >250	Mixed
3	$CH_2CHCHCH_3$	fb, >250	ND
4	$CH_2C(CH_3)CH_2$	fb, 182–184	ND
5	$CH_2(C_6H_5)$	fb, >220	Antagonist
6	$(CH_2)_2(C_6H_5)$	Oxalate, >250	Agonist
7	$(CH_2)_3(C_6H_5)$	fb, 100–101	ND

fb = freebase.
ND = Not determined.

The results of the assays demonstrate correlations between P-gp substrate activity and *N*-substitution. Naloxone, naltrexone, nalmexone (**2**), and oxymorphone were all found in this assay to be neither P-gp substrates nor inhibitors. The findings here that naloxone, naltrexone, and oxymorphone are not P-gp substrates are in agreement with previous reports.[5,20,26] Additionally, nalmexone (**2**), an opioid antagonist with analgesic properties,[31,32] is reported here also to be neither a P-gp substrate nor inhibitor. However, most oxymorphone analogues examined in this study were substrates of P-gp. Compounds **3**, **4**, **5**, **6**, and **7** were all found to be P-gp substrates. These analogues included the crotyl, 2-methallyl, and all three short chain phenylalkyl *N*-substituted compounds.

Toward describing the observed SAR, we employed a recently-described predictive mathematical model of P-gp substrates.[27] This model calculates common physiochemical descriptors for each compound (e.g., *c*Log*P*) and utilizes AutoDock Vina[33] to predict putative molecular modes of interaction with P-gp. A mathematical combination of physiochemical descriptors with the results of automated docking simulations within the consensus active site of the protein results in a prediction of P-gp activity. The results of this screen are shown in Table 2. The model accurately identified 66% of compounds tested in this study. In all cases of incorrect prediction, the model proposed P-gp substrate activity for compounds experimentally determined as non-substrates (false positive). Generally, compounds with lower Interacting Surface Area and lower lipophilicity were non-substrates in vitro.

Figure 3 shows the results of automated docking (AutoDock Vina)[33] of noroxymorphone analogues within the P-gp active site.[34,35] *N*-substituted noroxymorphone analogues are predicted to bind to P-gp in a consensus binding site that recognizes the cyclic peptide inhibitor, QZ59-RRR. This is different to oxymorphone, which was found to bind weakly to a region of the central pore containing Gly868, Glu871, and Met872. Significantly, oxymorphone was found to engage only in an ion/ion interaction with Glu871 and was determined to be a non-substrate in silico. *N*-substituted analogues were all projected to bind in a similar orientation that allows opioids to donate a phenolic hydrogen bond to the backbone carbonyl of Gln986 and maximize lipophilic interactions between *N*-substituent and hydrophobic side chains of Phe299, Tyr303, and Phe339.

Our results demonstrate the potential of this mathematical model as a tool for drug discovery. As described,[27] this tool combines two distinct aspects of computational chemistry: calculation of physiochemical descriptors and prediction of molecular mode of action. Independently, these two approaches aid our understanding of the actions of the opioid ligands produced here with the P-gp transporter. Our results suggest that while this model trended toward accurate prediction of opioids as P-gp substrates, the low overall hit rate (66%) supports the need for evaluation or generation of alternate, refined models of opioids as P-gp substrates.

The results presented here are in general agreement with results found in the literature. We have shown previously that short chain *N*-phenylalkyl analogues of normeperidine are substrates of P-gp. The findings reported here that *N*-benzyl, *N*-phenylethyl, and *N*-phenylpropyl substituents in the noroxymorphone series all are P-gp substrates support these previous findings. Examined together with the reported P-gp substrate activity of fentanyl (*N*-phenylethyl),[36] these data indicate that short chain *N*-phenylalkyl substituents may confer P-gp substrate activity across opioid chemical classes. The previously reported findings that *N*-allyl, *N*-crotyl, and *N*-methallyl normeperidine

Table 2
Molecular docking and physiochemical properties for standards and compounds **2–7**

Compound	Docking energy (kcal/mol)	Interacting surface area (Å^2)	*c*Log*P*	Theoretical substrate	Experimental substrate	Determination
Oxymorphone	−8.7	308.381	−0.783	No	No	Accurate
Naloxone	−8.0	336.542	−0.166	Yes	No	False positive
Naltrexone	−9.5	355.365	0.038	Yes	No	False positive
2	−9.8	365.227	0.683	Yes	No	False positive
3	−8.3	376.033	0.237	Yes	Yes	Accurate
4	−8.6	354.852	0.281	Yes	Yes	Accurate
5	−10.5	386.956	0.801	Yes	Yes	Accurate
6	−12.4	385.597	1.122	Yes	Yes	Accurate
7	−10.2	385.120	1.578	Yes	Yes	Accurate

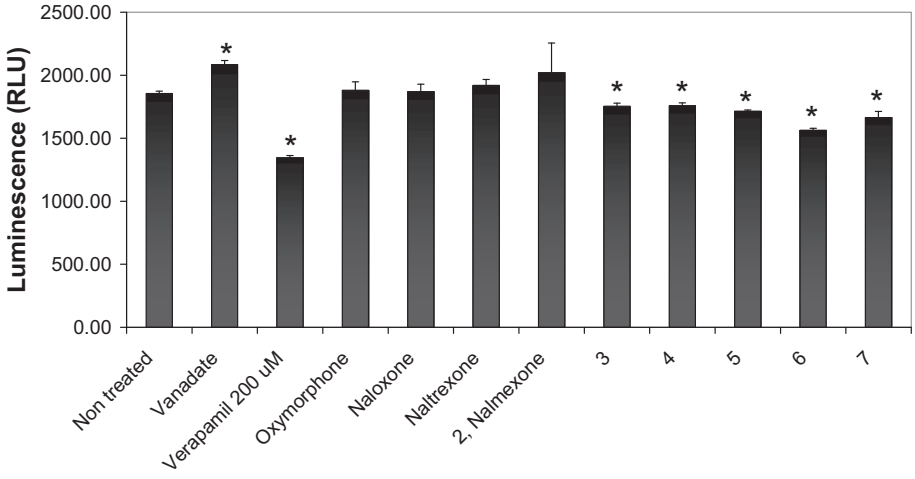

Figure 2. Results of compounds and standards in the Pgp-Glo assay system. All compounds assayed at 200 μM. P-gp activation is measured by relative luminescence units (RLU). Data are represented as mean ± SEM ($n = 4$). *Indicates significant difference from the control at $p < 0.05$ as determined by *t*-test.

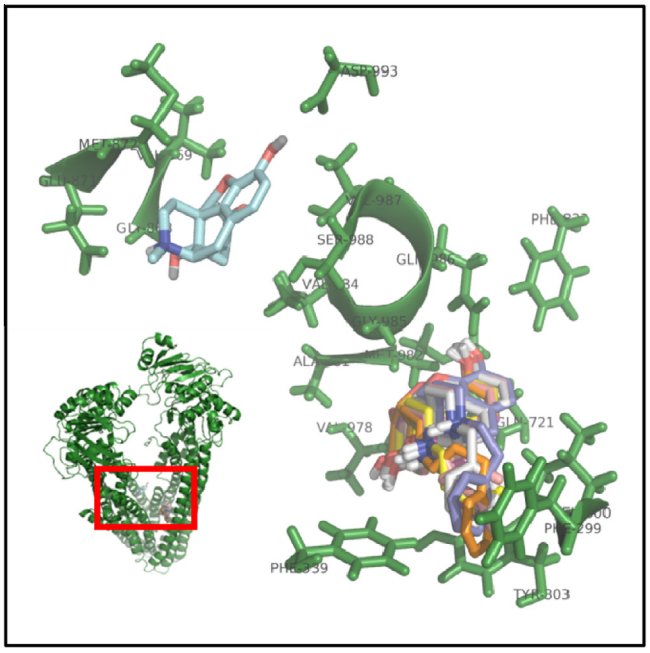

Figure 3. Results of automated docking of compounds tested in the present study. The global orientation of compounds within P-gp is shown in the inset (bottom-left). The close-up view of oxymorphone (cyan, upper-left) and noroxymorphone analogues (right) interacting with amino acid side chains within 4.0 Å of docked compounds is shown in detail. With the exception of oxymorphone (cyan, upper-left), all compounds were predicted to bind within a consensus hydrophobic binding site located within the hinge region. P-gp crystal structure 3G60 (pdb.org). Image produced using Pymol.

analogues are all P-gp substrates[23] do not overlap with the data presented here for the corresponding analogues in the noroxymorphone series which show varying P-gp substrate activity. Combined, these data suggest that *N*-alkenyl substituents likely do not confer a cross opioid class effect on P-gp substrate activity. Aggregated with the reported P-gp substrates pentazocine (*N*-prenyl-normetazocine),[34] naltrindole (*N*-cyclopropylmethyl, CPM),[15] nalbuphine (*N*-cyclobutylmethyl),[5] and buprenophine (*N*-CPM),[21] the data suggest that P-gp activity is not solely determined by the opioid *N*-substituent for short chain *N*-cycloalkyl and *N*-alkenyl substituted opioids. The P-gp substrate activity for non-aryl *N*-substituted opioids is likely determined by additional factors such as opioid class and binding modes.

Additionally, oxymorphone, unlike bemidone, morphine, and etorphine which all are P-gp substrates possessing a phenol moiety, contains a 3-phenol moiety and was not identified as a substrate of P-gp. Oxymorphone instead demonstrated similar activity as 6-desoxymorphone, which we previously identified as a P-gp non-substrate.[24,26] This represents variance between opioid classes where meperidine analogues display greater P-gp substrate activity when possessing a phenol moiety.[25] This pattern of variance across opioid classes indicates a complex relationship between P-gp affinity and opioid receptor pharmacophores. Opioids appear to possess a SAR profile at the P-gp transporter independent from SAR for opioid receptor efficacy and affinity. These findings indicate little correlation between (1) opioid intrinsic activity, and (2) physiochemical descriptors such as *c*Log*P* and polar surface area, and P-gp substrate activity in the 4,5-epoxymorphinan series of oxycodone analogues presented here.

The P-gp substrate activity of noroxymorphone analogues is affected by their *N*-substituent. The results show that short chain

N-phenylalkyl-*N*-noroxymorphones are substrates of P-gp, while *N*-alkenyl substituents do not show a common pattern of P-gp substrate activity. There appeared to be no correlation between P-gp substrate activity and opioid receptor efficacy. The conferring of P-gp substrate affinity by *N*-phenylalkyl substituents found across opioid classes may be generalized in scope.

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References and notes

1. Schinkel, A. H. *Adv. Drug Deliv. Rev.* **1999**, *36*, 179.
2. Higgins, C. F. *Annu. Rev. Cell Biol.* **1992**, *8*, 67.
3. Higgins, C. F. *Nature* **2007**, *446*, 749.
4. Seelig, A. *Eur. J. Biochem.* **1998**, *251*, 252.
5. MaharDoan, K. M.; Humphreys, J. E.; Webster, L. O.; Wring, S. A.; Shampine, L. J.; Serabjit-Singh, C. J.; Adkison, K. K.; Polli, J. W. *J. Pharmacol. Exp. Ther.* **2002**, *303*, 1029.
6. Blagosklonny, M. V. *Leukemia* **2001**, *15*, 936.
7. Kim, R. B.; Fromm, M. F.; Wandel, C.; Leake, B.; Wood, A. J.; Roden, D. M.; Wilkinson, G. R. *J. Clin. Invest.* **1998**, *101*, 289.
8. Mercer, S. L.; Coop, A. *Curr. Top. Med. Chem.* **2011**, *11*, 1157.
9. McNicol, E.; Horowicz-Mehler, N.; Fisk, R. A.; Bennett, K.; Gialeli-Goudas, M.; Chew, P. W.; Lau, J.; Carr, D. *J. Pain* **2003**, *4*, 231.
10. Hutchinson, M. R.; Shavit, Y.; Grace, P. M.; Rice, K. C.; Maier, S. F.; Watkins, L. R. *Pharmacol. Rev.* **2011**, *63*, 772.
11. Ambudkar, S. V.; Kimchi-Sarfaty, C.; Sauna, Z. E.; Gottesman, M. M. *Oncogene* **2003**, *22*, 7468.
12. Aquilante, C. L.; Letrent, S. P.; Pollack, G. M.; Brouwer, K. L. *Life Sci.* **2000**, *66*, 7.
13. Hassan, H. E.; Myers, A. L.; Lee, I. J.; Coop, A.; Eddington, N. D. *J. Pharm. Sci.* **2007**, *96*, 2494.
14. Thompson, S. J.; Koszdin, K.; Bernards, C. M. *Anesthesiology* **2000**, *92*, 1392.
15. Dagenais, C.; Graff, C. L.; Pollack, G. M. *Biochem. Pharmacol.* **2004**, *67*, 269.
16. Xie, R.; Hammarlund-Udenaes, M.; de Boer, A. G.; de Lange, E. C. *Br. J. Pharmacol.* **1999**, *128*, 563.
17. Schinkel, A. H.; Wagenaar, E.; Mol, C. A.; van Deemter, L. *J. Clin. Invest.* **1996**, *97*, 2517.
18. Ford, J. M. *Eur. J. Cancer* **1996**, *32A*, 991.
19. Moriki, Y.; Suzuki, T.; Fukami, T.; Hanano, M.; Tomono, K.; Watanabe, J. *Biol. Pharm. Bull.* **2004**, *27*, 932.
20. Callaghan, R.; Riordan, J. R. *Eur. J. Pharmacol.* **1995**, *291*, 183.
21. Suzuki, T.; Zaima, C.; Moriki, Y.; Fukami, T.; Tomono, K. *J. Drug Targeting* **2007**, *15*, 67.
22. Casy, A. F.; Parfitt, R. T. *Opioid Analgesics*; Plenum Press: New York and London, 1986.
23. Mercer, S. L.; Hassan, H. E.; Cunningham, C. W.; Eddington, N. D.; Coop, A. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 1160.
24. Cunningham, C. W.; Mercer, S. L.; Hassan, H. E.; Traynor, J. R.; Eddington, N. D.; Coop, A. *J. Med. Chem.* **2008**, *51*, 2316.
25. Mercer, S. L.; Cunningham, C. W.; Eddington, N. D.; Coop, A. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 3638.
26. Hassan, H. E.; Mercer, S. L.; Cunningham, C. W.; Coop, A.; Eddington, N. D. *Int. J. Pharm.* **2009**, *375*, 48.
27. Bikadi, Z.; Hazai, I.; Malik, D.; Jemnitz, K.; Veres, Z.; Hari, P.; Ni, Z.; Loo, T. W.; Clarke, D. M.; Hazai, E.; Mao, Q. *PLoS One* **2011**, *6*, e25815.
28. Spectra were consistent with the assigned structures; the salts gave satisfactory microanalyses (±0.4%).
29. Iijima, I.; Minamikawa, J.; Jacobson, A. E.; Brossi, A.; Rice, K. C. *J. Med. Chem.* **1978**, *21*, 398.
30. Ambudkar, S. V.; Dey, S.; Hrycyna, C. A.; Ramachandra, M.; Pastan, I.; Gottesman, M. M. *Annu. Rev. Pharmacol. Toxicol.* **1999**, *39*, 361.
31. Forrest, W. H., Jr.; Shroff, P. F.; Mahler, D. L. *Clin. Pharmacol. Ther.* **1972**, *13*, 520.
32. Loew, G. H.; Berkowitz, D. S. *J. Med. Chem.* **1978**, *21*, 101.
33. Trott, O.; Olson, A. J. *J. Comput. Chem.* **2010**, *31*, 455.
34. Aller, S. G.; Yu, J.; Ward, A.; Weng, Y.; Chittaboina, S.; Zhuo, R.; Harrell, P. M.; Trinh, Y. T.; Zhang, Q.; Urbatsch, I. L.; Chang, G. *Science* **2009**, *323*, 1718.
35. *Procedure for automated docking:* 3D structures were generated using Chem3DPro 12.0 (CambridgeSoft) and energy minimized. Compounds were then subjected to molecular dynamics to identify a global energy minimum (conditions: 10,000 steps, step interval 2.0 fs, frame interval 10 fs, heating/cooling rate 1.000 kcal/atom/ps, temperature 300 K) and energy minimized again. The resulting 3D structures were used for targeted docking using mouse P-gp (3G60, pdb.org) using conditions described in Ref. 27. A grid box of $22 \times 22 \times 22$ Å was centered on the cavity surrounding QZ59-RRR, also in accordance with Ref. 27. Appropriate protonation states were applied for all amines, and Gasteiger–Hückel charges calculated. The resulting lowest-energy binding modes for all compounds were selected, visualized using Pymol, and shown in Figure 3.
36. Henthorn, T. K.; Liu, Y.; Mahapatro, M.; Ng, K. Y. *J. Pharmacol. Exp. Ther.* **1999**, *289*, 1084.