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ORIGINAL ARTICLE

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Inhibition of transport function and desipramine binding at the human noradrenaline transporter by *N*-ethylmaleimide and protection by substrate analogs

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Abstract *N*-ethylmaleimide (NEM) inhibits [^{3}H]desipramine binding and [^{3}H]noradrenaline uptake at the rat noradrenaline transporter (rNET) by covalently modifying cysteine residues. We report here that NEM also inhibits [^{3}H]desipramine binding and [^{3}H]noradrenaline uptake at the cloned human noradrenaline transporter (hNET) stably expressed in C6 glial cells. The IC_{50} for NEM inhibition of [^{3}H]noradrenaline uptake was 43.6±5.5 μM. We tested several compounds for their abilities to inhibit [^{3}H]noradrenaline uptake via the hNET and for their abilities to protect against NEM inactivation of [^{3}H]desipramine binding. We found that the substrate analogs bupropion, 3-bromomethcathinone, and 4-bromomethcathinone all inhibit uptake at the hNET with IC_{50} values of 1370± 140, 158±20, and 453±30 nM, respectively. These compounds as well as methamphetamine, methcathinone, and desipramine also protected the hNET from NEM inactivation of [^{3}H]desipramine binding. The ability of substrate analogs and desipramine to protect the [^{3}H]desipramine binding site is consistent with the hypothesis that the desipramine binding site and the substrate binding site are mutually exclusive. It also supports the use of structure-activity relationships derived from substrate analogs in the rational design of hNET uptake inhibitors. The hNET contains 10 cysteine residues whereas the rNET contains 12 cysteine residues. Since the hNET and the rNET are both inhibited by NEM, and because the NEM inhibition is protectable by desipramine and substrate analogs, we conclude that the two additional cysteine residues (C28 and C447) present in the rNET are not likely to be involved in desipramine binding or uptake function.

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Introduction

Noradrenaline, a catecholamine neurotransmitter, plays an important role in mood and behavior (Ressler and Nemeroff 1999). Aberrant noradrenaline levels in the brain may account for pathological conditions such as depression and attention deficit hyperactivity disorder (ADHD; Maas 1975; Comings et al. 2000). The action of noradrenaline in the CNS is regulated in part by the noradrenaline uptake transporter (NET). This carrier protein couples the neuronal uptake of noradrenaline with the influx of Na^+ and Cl^- and is the primary mechanism by which noradrenaline signaling is terminated in the synaptic cleft. The human noradrenaline transporter (hNET) contains 12 transmembrane spanning domains as predicted by hydrophobicity analysis (Pacholczyk et al. 1991).

Drugs that act at the hNET have found many medical uses. These include treatments for depression, ADHD, obesity, and PET imaging (Bray 1993; DeGrado et al. 1993; Iversen 2000). The psychostimulant methcathinone and the entactogen 3,4-methylenedioxymethamphetamine (MDMA) also have actions at the NET (Nichols 1986; Wall et al. 1995; Cozzi et al. 1999). Drugs acting at the NET can be broadly classified as nonsubstrate uptake inhibitors such as desipramine or substrate analogs such as methamphetamine. The former compounds prevent noradrenaline uptake but are not themselves transported into the cell. Substrate analogs, on the other hand, inhibit noradrenaline transport by being transported in lieu of noradrenaline.

It has previously been shown that the rat noradrenaline transporter (rNET) is sensitive to alkylation by *N*-ethylmaleimide (NEM; Schömig et al. 1988). In this report, NEM treatment inhibited noradrenaline uptake and [^{3}H]desipramine binding. The substrate analog amezinium and the nonsubstrate inhibitor cocaine were shown to

protect against this NEM inactivation of [^{3}H]desipramine binding. NEM selectively modifies cysteine residues by forming covalent bonds with sulfhydryl groups at pH 6.5–7.5 (Smyth et al. 1964). The work by Schömig et al. (1988) thus implies that one or more cysteine residues is involved in rNET uptake function and [^{3}H]desipramine binding. The amino acid composition of the hNET is 93% identical and 97% homologous to its rat counterpart (Paczkowski et al. 1999). With respect to sulfhydryl modification by NEM, the rNET and hNET differ in the number of cysteine residues present in each transporter: the hNET contains 10 cysteine residues whereas the rNET contains 12 (Pörzgen et al. 1995; Brüss et al. 1997). The ten cysteine residues in the hNET are found in identical positions within the rNET but the rNET contains two additional cysteines at positions 28 and 447. In the hNET, these positions contain an arginine and phenylalanine, respectively. Thanks to the sequence divergence of cysteine residues between the rNET and the hNET, we have the opportunity to determine if the loss of two cysteine residues in the hNET confers resistance to NEM inactivation.

To determine whether C28 and C447 are crucial for NEM inactivation of the NET, we tested whether the hNET, like the rNET, was sensitive to inactivation by NEM. We also tested whether the substrate analogs methamphetamine, methcathinone, and bupropion, and the nonsubstrate uptake inhibitor desipramine could protect the hNET from inactivation by NEM. In addition, we investigated two novel analogs of methcathinone that we had previously reported as inhibitors of the human serotonin transporter (Cozzi and Foley 1999). These two compounds, 3-bromomethcathinone (3-BMAP) and 4-bromomethcathinone (4-BMAP), were examined for their abilities to inhibit [^{3}H]noradrenaline uptake and to protect against NEM inactivation of [^{3}H]desipramine binding. Protection by substrate analogs and nonsubstrate inhibitors would be consistent with the hypothesis that both classes of drugs share a common, cysteine-containing binding site within the NET.

Materials and methods

Drugs and reagents. [^{3}H]Noradrenaline (specific activity 51.8 Ci/mmol) and [^{3}H]desipramine (specific activity 25.5 Ci/mmol) were purchased from New England Nuclear (Boston, Mass., USA). Bupropion was donated by Dr. William Glassco (Virginia Commonwealth University, Richmond, Va., USA). Methcathinone, methamphetamine, 3-BMAP and 4-BMAP were synthesized in racemic form in our laboratory; chemical structures were confirmed by standard analytic methods. Desipramine, pargyline, NEM, and buffer reagents were purchased from Aldrich Chemical (Milwaukee, Wis., USA). Cell culture medium and antibiotics were obtained from Life Technologies (Gaithersburg, Md., USA). Fetal bovine serum was purchased from Hyclone (Logan, Utah, USA).

Inhibition of [^{3}H]noradrenaline uptake by substrate analogs. The abilities of 3-BMAP, 4-BMAP, and bupropion to inhibit hNET-mediated [^{3}H]noradrenaline uptake were determined in rat C6 glial cells stably expressing the human norepinephrine transporter (C6NET; obtained from Susan Amara, Oregon Health Sciences University) as previously reported, with minor modifications (Cozzi et al. 1999). Briefly, C6NET cells were grown to confluency on 24-well plates containing 1 ml per well of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The ability of the drugs to inhibit [^{3}H]noradrenaline uptake was measured as follows: the DMEM was aspirated from the 24-well plates and the cells were washed with 3×2 ml ice-cold Krebs-Ringer-HEPES buffer (KRH) containing (mM): NaCl (124), KCl (2.9), $MgSO_4$ (1.3), KH_2PO_4 (1.2), D-glucose (5.2), $CaCl_2$ (2.4) and HEPES (25), pH=7.4, adjusted with 5 N NaOH. The monoamine oxidase inhibitor pargyline (final concentration, 100 μM) and the antioxidant l-ascorbic acid (final concentration, 100 μM) were also added to the KRH buffer. After washing, 490 μl of KRH was added to each well. This was followed by the addition of either 5 μl of KRH (for total determinations), 5 μl of 10 mM desipramine (for nonspecific determinations; final concentration, 100 μM), or 5 μl of test drug solution (various concentrations, dissolved in KRH). The plates were preincubated at 37°C for 10 min. [^{3}H]Noradrenaline (5 μl; final concentration, 15 nM) was then added to each well to initiate uptake. [^{3}H]Noradrenaline uptake was allowed to proceed for 10 min at 37°C, the incubation buffer was then discarded, and the cells were washed with 3×2 ml ice-cold KRH. After washing, the cells were solubilized in 700 μl of 37°C 1% sodium dodecyl sulfate. Aliquots (500 μl) of the solubilized well contents were transferred to liquid scintillation vials containing 3.5 ml of scintillation cocktail. Vials were capped, vortexed, and counted using a Packard Tri-Carb 2200CA scintillation counter. Data were transformed from dpm to % specific uptake and fitted to a four-parameter logistic curve from which IC_{50} values were calculated. Specific uptake was defined as uptake at 37°C minus uptake in the presence of 100 μM desipramine. Six concentrations of each compound were tested. IC_{50} values were calculated as the means ± SEM of 3–6 experiments, each performed in triplicate. Results were plotted using Prism software (GraphPad, San Diego, Calif., USA).

Inhibition of [^{3}H]noradrenaline uptake by NEM. The procedure for determining the IC_{50} of NEM at the hNET was identical to that described above for drug inhibition of [^{3}H]noradrenaline uptake with the following exceptions: 6-well plates were used instead of 24-well plates and 10 μM nisoxetine was used to define nonspecific uptake. The cells were preincubated with various concentrations of NEM for 120 min at room temperature as described for the rNET (Schömig et al. 1988). The NEM solutions were then removed and the cells were washed with 5×2 ml ice-cold KRH before assessing [^{3}H]noradrenaline uptake. The IC_{50} value is reported as the mean ± SEM of three experiments, each performed in triplicate.

NEM protection experiments. To test whether substrate analogs and desipramine could protect against NEM inactivation of the hNET, C6NET cells were grown to approximately 80% confluency on 6-well plates over 4–5 days. The culture medium was removed and the cells were washed twice with 2 ml of room-temperature KRH, then 495 μl of KRH was added to each well. Substrate analogs were added at 1000 times their respective IC_{50} concentrations to ensure that at least 99.9% of the transporters would be occupied. Final concentrations of substrate analogs were: bupropion, 1.37 mM; methcathinone, 511 μM; methamphetamine, 647 μM; 3-BMAP, 158 μM; 4-BMAP, 453 μM. The uptake inhibitor desipramine was used at 100 μM. High concentrations of reversibly-binding protecting agents are required to ensure that protection from inactivation is not obscured by the irreversible NEM alkylation reaction over the time course of the experiment. Even if only a small fraction of binding sites remains accessible to an irreversible ligand at any moment, given enough time, covalently modified sites will accumulate and protection will be masked. The cells were preincubated with protecting drugs for 20 min at 37°C. NEM (5 μl; final concentration, 100 μM) was then added to each well except those wells used to determine total and nonspe-

cific [3H]desipramine binding. The cells were incubated for 120 min at room temperature in darkness. After NEM treatment, each well was washed with 5×2 ml ice-cold KRH and 490 µl of KRH was added to each well. This was followed by the addition of either 5 µl of KRH (for total determinations) or 5 µl of 1 mM nisoxetine (for nonspecific determinations; final concentration, 10 µM). The cells were kept at 37°C for 10 min, then 5 µl of [3H]desipramine was added to all wells (final concentration, 1 nM) and the incubation was continued at 37°C for an additional 15 min. The incubation buffer was then discarded and each well was gently washed with 3×2 ml ice-cold isotonic saline. After washing, the cells were solubilized and radioactivity was measured as described above. Specific [3H]desipramine binding was calculated as total [3H]desipramine binding minus [3H]desipramine binding in the presence of 10 µM nisoxetine. Nonspecific counts were subtracted from all wells and data were transformed from dpm to percent specific [3H]desipramine binding. Specific binding values are the means ± SEM of 3–13 experiments, each done in triplicate. Wells receiving only NEM and no protecting drugs were compared to control wells using Student's *t*-test with $P<0.05$ considered significant. Protector-treated wells were compared to wells that received NEM only using ANOVA followed by Dunnett's multiple comparisons test. Protection by substrate analogs was compared to protection by desipramine by ANOVA followed by Dunnett's multiple comparisons test.

Results

[3H]Noradrenaline uptake into C6NET cells was greater than 90% specific. Curves for inhibition of [3H]noradrenaline uptake by substrate analogs are shown in Fig. 1. All drug inhibition curves were best fitted with a slope coefficient of unity, indicating that the compounds bound to a single site on the hNET. Bupropion, 3-BMAP, and 4-BMAP exhibited IC_{50} values of 1370±140, 158±20, and 453±30 nM, respectively (Table 1). For comparison, the IC_{50} values for methcathinone and methamphetamine were 511±100 nM and 647±30 nM, respectively (Cozzi et al. 1999).

Table 1 IC_{50} values for drug inhibition of [3H]noradrenaline uptake into C6NET cells. Six concentrations of test drugs were used to generate IC_{50} curves (Fig. 1). Each IC_{50} value is the mean ± SEM of 3–6 experiments, each performed in triplicate

Drug	IC_{50}, nM (*n*)
Bupropion	1370±140 (3)
3-BMAP	158±20 (6)
4-BMAP	453±30 (6)
Methcathinone	511±100 (3)[a]
Methamphetamine	647±30 (3)[a]

[a]Values are from Cozzi et al. (1999)

Preincubation of the cells with NEM resulted in an inhibition of [3H]noradrenaline uptake with an IC_{50} for NEM of 43.6±5.5 µM and a slope coefficient of 1.7 (Fig. 2). Pretreatment with NEM also blocked [3H]desipramine binding. In C6NET control cells, specific [3H]desipramine binding was 268±39 fmol/well. When the cells were treated with 100 µM NEM, specific [3H]desipramine binding was reduced to 6.1±3.8% of the control value ($P<0.01$, $n=13$; Fig. 3). Desipramine ($n=5$) and the substrate analogs bupropion ($n=11$), methcathinone ($n=3$), methamphetamine ($n=3$), 3-BMAP ($n=8$), and 4-BMAP ($n=5$) all protected against NEM inactivation of [3H]de-

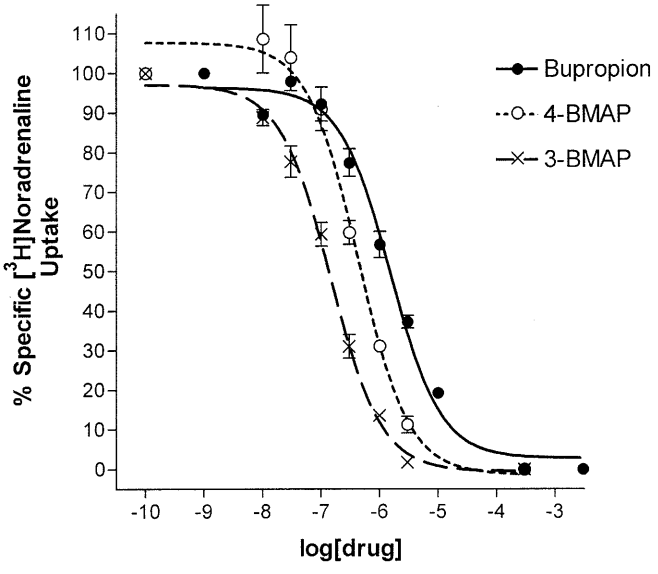

Fig. 1 Drug inhibition of [3H]noradrenaline uptake into C6NET cells. Drugs were tested for their ability to inhibit [3H]noradrenaline uptake into cells expressing the cloned human noradrenaline transporter (C6NET). *Points* represent the means ± SEM of 3–6 experiments performed in triplicate. Data were fitted to four-parameter logistic curves from which IC_{50} values were calculated. IC_{50} values are listed in Table 1

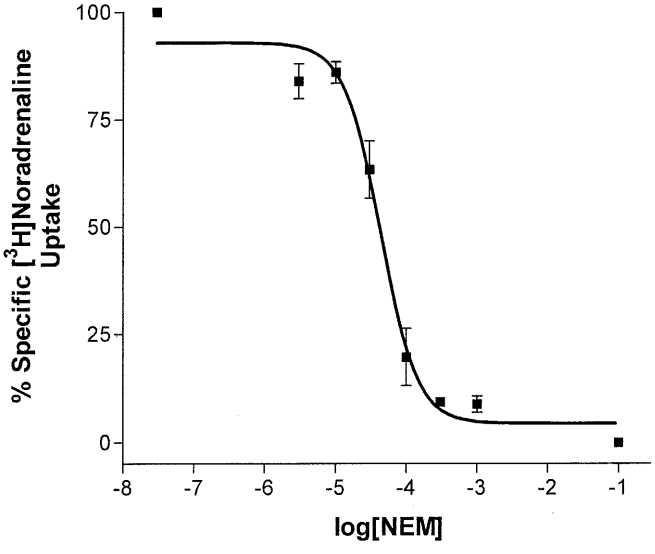

Fig. 2 Inhibition of [3H]noradrenaline uptake by *N*-ethylmaleimide (*NEM*) in C6NET cells. Cells expressing the cloned human noradrenaline transporter were preincubated with various concentrations of NEM for 120 min as described under Materials and methods. NEM displayed a concentration-dependent inhibition of [3H]noradrenaline uptake. *Points* represent the means ± SEM of three experiments, each performed in triplicate. Data were fitted to a four-parameter logistic curve for IC_{50} and Hill slope determination. IC_{50} for uptake inhibition is 43.6±5.5 µM; Hill slope coefficient is 1.7

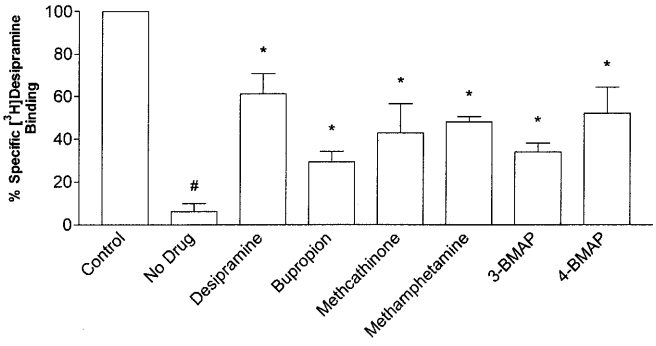

Fig. 3 Protection of [^{3}H]desipramine binding from *N*-ethylmaleimide (NEM) inactivation. C6NET cells were treated with 100 μM NEM for 120 min in the absence and presence of protecting drugs as described under Materials and methods. Control cells did not receive NEM. Following NEM treatment, cells were extensively washed and [^{3}H]desipramine binding (1 nM, 15 min at 37°C) was assessed. Nonspecific binding was defined by 10 μM nisoxetine. *Bars* represent the means ± SEM of 3–13 experiments, each performed in triplicate (see Results). Specific [^{3}H]desipramine binding was inhibited by 94% in cells that received NEM only. All of the test drugs protected against NEM inactivation of [^{3}H]desipramine binding. $^{\#}P<0.01$ vs. control; $^{*}P<0.01$ vs. no drug

sipramine binding ($P<0.01$; Fig. 3). The substrate analogs, with the exception of bupropion ($P<0.05$ vs. desipramine), were as effective as desipramine in the degree of protection afforded. When protecting drugs were added prior to NEM, specific [^{3}H]desipramine binding, expressed as percent of control binding, was as follows: desipramine, 61.2±9.5%; bupropion, 29.3±4.8%; methcathinone, 42.8± 13.8%; methamphetamine, 47.9±2.5%; 3-BMAP, 33.9± 4.1%; 4-BMAP, 52.0±12.3%.

Discussion

NEM is a sulfhydryl group alkylating agent which has been used to gain insights into monoamine transporter function and antidepressant inhibitor binding properties (Meyerson et al. 1987; Bönisch et al. 1990; Xu et al. 1997). In this study, we examined the effects of NEM on hNET transport function and on [^{3}H]desipramine binding and we tested the abilities of various compounds to protect the hNET from NEM inactivation of [^{3}H]desipramine binding. We found that NEM inactivates the ability of the hNET to transport [^{3}H]noradrenaline with an IC_{50} value of 43.6 μM and a Hill slope of 1.7 (Fig. 2). These values are similar to those reported by Schömig et al. (1988) for NEM inactivation of the rNET. A Hill slope greater than unity suggests multiple binding sites and positive cooperativity. This may be the result of covalent sulfhydryl bond modifications that expose additional NEM binding sites (i.e. cysteine residues). Associated with the NEM inactivation of [^{3}H]noradrenaline uptake was a 94% inhibition of specific [^{3}H]desipramine binding (Fig. 3). Consistent with the ability of NEM to form covalent bonds, [^{3}H]desipramine binding could not be restored after exposure to NEM, even after multiple washes with buffer. The sensitivity of the hNET to NEM demonstrates the importance of cysteine residues in transport function as well as in ligand binding. The hNET contains ten cysteine residues at positions 44, 86, 131, 185, 176, 240, 339, 351, 460, and 520 (Pacholczyk et al. 1991). The rNET contains cysteines in these same locations and contains two additional cysteines at positions 28 and 447. Our data show that despite the absence of C28 and C447, the hNET remains sensitive to NEM inactivation of transport and [^{3}H]desipramine binding. Therefore, C28 and C447 are not likely to be involved in NET uptake function or [^{3}H]desipramine binding.

The substrate analogs bupropion, methcathinone, methamphetamine, 3-BMAP, and 4-BMAP were tested for their abilities to inhibit [^{3}H]noradrenaline uptake into C6NET cells and to protect against the NEM inactivation of [^{3}H]desipramine binding. These drugs inhibited [^{3}H]noradrenaline uptake with IC_{50} values in the nanomolar-to-low micromolar range (Table 1). Of particular interest is the finding that bupropion inhibited [^{3}H]noradrenaline uptake at the hNET with an IC_{50} of about 1 μM. This value is in excellent agreement with the value reported by Eshleman and colleagues for bupropion inhibition of [^{3}H]noradrenaline uptake via the hNET expressed in human embryonic kidney 293 cells (Eshleman et al. 1999). Bupropion is known to inhibit the rNET, however it is considered only a weak inhibitor of the rNET when compared to the rat dopamine and serotonin transporters (Ferris 1993; Ascher et al. 1995). Although bupropion is considered a weak noradrenaline uptake inhibitor in humans, our data suggest that inhibition of the hNET is likely to be a significant factor in the mechanism of action of bupropion since it inhibits [^{3}H]noradrenaline uptake at therapeutically relevant concentrations.

The ability of a ligand to confer protection against NEM inactivation of [^{3}H]desipramine binding suggests that desipramine and that ligand either share a common binding site or that the ligand produces a nonlocal effect on the NEM-sensitive desipramine binding site such that NEM is unable to access and alkylate the site. As expected, desipramine itself protected against NEM inactivation of [^{3}H]desipramine binding (Fig. 3). If desipramine and substrates share a common binding site, then a substrate or substrate analog would also be expected to protect against NEM inactivation of [^{3}H]desipramine binding. We therefore hypothesized that the known hNET substrate methamphetamine (Wall et al. 1995) and the substrate analogs methcathinone, bupropion, 3-BMAP, and 4-BMAP would also protect against NEM inactivation of [^{3}H]desipramine binding. The data in Fig. 3 show that this is the case; all of the substrate analogs reversed NEM inactivation of [^{3}H]desipramine binding. This finding parallels that of Schömig et al. (1988) that amezinium, a NET substrate, protected the rNET from NEM inactivation of [^{3}H]desipramine binding. However, we cannot exclude the possibility that the substrate analogs bind to some other site that does not overlap the desipramine binding site and that this interaction produces a conformational change in the hNET such that the desipramine binding site

is no longer susceptible to NEM inactivation. Nevertheless, the fact that desipramine itself protected to approximately the same degree as did the other compounds suggests that the protection is due to a simple occupation of the desipramine binding site.

Our results demonstrate that the hNET is inhibited by NEM and suggest that the hNET desipramine binding site may be the substrate recognition site. Since the desipramine binding site is intimately linked to the substrate recognition site, structure-activity data obtained from inhibition of [^{3}H]noradrenaline uptake by substrate analogs may be useful in the rational design of nonsubstrate uptake inhibitors.

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