ENANTIOSELECTIVE EFFECTS OF HYDROXYMETABOLITES OF BUPROPION ON BEHAVIOR AND ON FUNCTION OF MONOAMINE TRANSPORTERS AND NICOTINIC RECEPTORS

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ABBREVIATIONS: nAChR, Nicotinic acetylcholine receptor(s); %MPE, maximum possible effect; CL, confidence limit; s.c., subcutaneous injection; AD₅₀, antagonist dose 50%; IC₅₀, drug concentration producing 50% inhibition of maximal response; DA, dopamineergic; hα₄β₂-, hα₄β₄-, hα₁*- and hα₃*-nAChR, human nicotinic acetylcholine receptor(s) composed of α₄ and β₂ subunits, α₄ and β₄ subunits, α₁, β₁, γ and δ subunits, or α₃, β₄ +/- β₂ and α₅ subunits, respectively; NE, norepinephrine (noradrenergic); PBS, phosphate-buffered saline.
Abstract  Bupropion is an atypical antidepressant that also has utility as a smoking cessation aid. Since hydroxybupropion, a major metabolite of bupropion, is believed to contribute to its antidepressant activity, this metabolite may also contribute to the smoking cessation properties of bupropion. The present study investigated the effects of hydrobupropion enantiomers on monoamine transporters and nAChR subtypes. Racemic bupropion and hydroxybupropion inhibit [3H]NE uptake with similar potency (IC$_{50}$ values of 1.9 and 1.7 µM, respectively), but most of the latter activity resides in the (2S,3S)-hydroxy isomer (IC$_{50}$ = 520 nM) rather than (2R,3R)-hydroxybupropion (IC50 > 10,000 nM). Similar results were found with [3H]DA uptake. The effects of bupropion and enantiomers of hydroxybupropion on human nAChR subtypes indicate that the (2S,3S)-isomer is more potent than the (2R,3R)-isomer or racemic bupropion as an antagonist of $\alpha_{4}\beta_{2}$ (functional IC$_{50}$ = 3.3 µM). In addition, (2S,3S)-hydroxybupropion and bupropion were considerably more potent than 2R, -3R)-hydroxybupropion in a mouse depression model (forced swimming test) and in antagonism of acute nicotine effects in mice. Collectively, our results suggest that clinical and behavioral effects of bupropion arise from actions at nAChR as well as DA and NE transporters. Further, our data suggest that the (2S,3S)-hydroxybupropion isomer may be a better drug candidate for smoking cessation than bupropion because of its higher potency at the relevant targets.
Tobacco use is the leading cause of premature death in the United States. The vast majority of smokers (70%) report a desire to quit smoking, but poor smoking cessation results indicate a need to explore innovative approaches to treating nicotine addiction. In addition to nicotine replacement therapy, the atypical antidepressant, bupropion, is now recognized as an effective aid to smoking cessation. The efficacy of bupropion in the treatment of nicotine dependence was thought to involve modulation of dopaminergic (DA) and noradrenergic (NE) systems. Indeed, bupropion is a relatively weak DA-reuptake inhibitor and inhibits the firing of locus coeruleus NE neurons at high concentrations (Cooper et al., 1994). Its inhibition of transporter function is associated with increases in extracellular DA and NE concentrations, which may substitute for nicotine-evoked neurotransmitter release during smoking, mimicking nicotine reinforcement and alleviating withdrawal symptoms stemming from the absence of nicotine. No other neuronal sites were thought to play a role in bupropion’s due to its lack binding affinity for almost all of the major classes of neuronal receptors (Ascher et al., 1995).

However, findings from our laboratories that bupropion acted as a relatively potent, non-competitive nAChR antagonist suggested that actions of bupropion at nAChR were of possible relevance for smoking cessation, especially given the hypothesis that chronic nicotine exposure acts to inhibit function of nAChR rather than to sustain nAChR activity (Gentry et al., 2003). Moreover, bupropion blocks several of nicotine’s behavioral effects at doses similar to or lower than those having activity in antidepressant behavioral tests (Martin et al., 1990; Slemmer et al., 2000) and blocking in vivo striatal DA uptake in mice (Stathis et al., 1995). Bupropion was shown to differentially block the function of various nAChR in oocytes (Slemmer et al., 2000), cell lines (Fryer and Lukas, 1999) and nicotine-evoked DA and NE release in striatal slices (Miller et al., 2002). Reported plasma levels of bupropion and its hydroxymetabolite are in the range of bupropion concentrations that antagonize diverse nAChR subtypes (Golden et al., 1988).

It is thought that the effects of bupropion’s major metabolites may be critical to its antidepressant activity, because bupropion is extensively metabolized to (2R,3R)- and (2S,3S)-
hydroxybupropion, (R, -R)- and (S, -S)-threohydrobupropion, and (R, -S)- and (S, -R)-
erthrohydrobupropion in humans (Cooper et al., 1994). The concentrations of
hydroxybupropion isomers present in CSF are six times greater than those of the parent
bupropion (Cooper et al., 1994). Although it has weak NE uptake properties, the high levels of
the metabolite in brain may be sufficient to produce clinically meaningful blockade of NE
reuptake and thereby account for much of the drug's activity. Indeed, plasma levels of
hydroxybupropion greatly exceed those of the parent drug, reaching 10 to 100 times the
concentration of bupropion (Findlay et al., 1981; Golden et al., 1988; Hysu et al., 1997; Welch et
al., 1987). Furthermore, hydroxybupropion shows stronger anti-tetrabenazine activity
(indicative of an antidepressant activity in animals) and has a lower LD$_{50}$ value than the erythro
and threo metabolites, suggesting that hydroxybupropion is the most important active metabolite
in vivo for its antidepressant activity (Martin et al., 1990). In addition, (2S,3S)- but not (2R,3R)-
hydroxybupropion partially substituted for nicotine in rat drug discrimination procedure
(Bondarev et al., 2003). Given the extensive metabolism of bupropion in humans and the
apparent clinical activity of hydroxybupropion (Martin et al., 1990) as well as its long-half life,
bupropion metabolites may play an important part in the mechanism of action of this medication.
The goals of the current studies were to compare the pharmacological properties of bupropion
and its hydroxy metabolites to determine the extent the latter were contributing to bupropion’s
antidepressant effects and its interaction with nicotine. Furthermore, by examining the
enantiomers of the hydroxy metabolites, we sought to establish the specificity of their actions.
Materials and Methods


**Synaptosomal preparation:** Bupropion analogs were evaluated in neurotransmitter uptake assays using synaptosomes prepared from rat brain (adult male Sprague-Dawley rats – 250 g). Since no significant differences between rat and human synaptosomes were observed in studies of monoamine uptake (Kuhar et al., 1999; Paczkowski et al., 1999), data obtained using rat tissue should predict the behavior of these compounds at the corresponding human transporter. Synaptosomal uptake of [^3]H]NE was performed as described elsewhere (Bennett et al., 1995; Eshleman et al., 2001). Briefly, rat brain was rapidly removed after decapitation and the cerebral cortex was dissected, weighed, and placed immediately in a Teflon-to-glass homogenizer pre-filled with 10 mL ice-cold oxygenated 5 mM HEPES buffer (pH 7.4 @ 4 °C) containing 0.32 M sucrose. Additional buffer was added to achieve a 1:45 tissue:buffer dilution. The tissue was then gently homogenized using four up-down strokes of the Teflon homogenizer. The homogenate was centrifuged at 1000 x g (10 min @ 4 °C). The supernatant was collected and centrifuged at 12,000 x g (10 min @ 4 °C). After this step, the supernatant was discarded and the pellet resuspended (Teflon-to-glass) in the original volume of an oxygenated 25 mM HEPES assay buffer (7.4 @ 37 °C) containing 128 mM NaCl, 2.4 mM KCl, 3.2 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, and 10 mM dextrose. Ascorbate (1 mM) and iproniazid (10 µM, MAO inhibitor) were added to the buffer on the day of the assay. In a final volume of 500 µL, each assay contains 100 µL of synaptosome preparation, one of at least eight different concentrations of test compound pre-incubated with the tissue (30 min @ 37 °C) and 5 nM [^3]H]NE (added last). Maximal uptake and nonspecific uptake are determined in samples containing buffer instead of test compound or 10 µM nisoxetine, respectively. After 5 min, the samples were harvested by vacuum filtration onto GF/B filter papers (presoaked for 30 min with 0.1% polyethylenimine), and washed twice with ten volumes of ice-cold assay buffer. The filter disks were incubated with 0.5 mL 10% SDS for 1 h before the addition of scintillation fluid and trapped radioactivity determined using standard liquid scintillation counting techniques. An
aliquot of each tissue preparation was taken for protein determination to monitor consistency of the tissue preparations. The procedure for preparing the synaptosomal uptake of $[\text{^3}H]\text{DA}$ was the same as described above for $[\text{^3}H]\text{NE}$ uptake except: the caudate was harvested and diluted 1:70, 5 nM $[\text{^3}H]\text{DA}$ was added to the assay, and nonspecific binding was determined in the presence of 5 µM mazindol.

**Data Analysis:** The IC$_{50}$ values for inhibition of $[\text{^3}H]\text{NE}$, and $[\text{^3}H]\text{DA}$ uptake were determined from a plot of the specific uptake vs. log concentration data fit to a four-parameter logistic equation (Prism v3.0, GraphPad, San Diego, CA). The IC$_{50}$ data was expressed as mean ± SD from at least two independent experiments.

**nAChR Functional Studies**

**Cell culture:** Cells of the TE671/RD human clone (Lukas, 1989) or of the SH-SY5Y human neuroblastoma (Lukas, 1993) naturally expressing muscle-type ($\alpha_1\beta_1\gamma\delta$-nAChR) or $\alpha_3^*$-nAChR, respectively, or cells of the SH-EP1-h$\alpha_4\beta_2$ (Eaton et al., 2003) or -h$\alpha_4\beta_4$ cell lines heterologously expressing human $\alpha_4\beta_2$- or $\alpha_4\beta_4$-nAChR, respectively, were used for nAChR functional studies. Cells were maintained in Dulbecco’s modified Eagle’s medium (high glucose, bicarbonate-buffered, with 1 mM sodium pyruvate and 8 mM L-glutamine) supplemented with 10% horse serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (all from Invitrogen, Carlsbad, CA) plus 5% fetal bovine serum (Hyclone, Logan, UT) on 100-mm diameter plates in a humidified atmosphere containing 5% CO2 in air at 37oC (Lukas, 1986; Lukas et al., 1993). Media for maintenance of transfected SH-EP1 cells also contained 0.25 µg/ml zeocin (Invitrogen) and 0.4 mg/ml hygromycin B (130 µg/ml biologically-active hygromycin; Calbiochem, San Diego, CA) to maintain positive selection for cells expressing dual drug resistance as well as nAChR $\alpha_4$ and $\beta_2/\beta_4$ subunit cDNA. All cell lines were maintained as low passage number (1-26 from our frozen stocks) cultures to ensure stable expression of phenotype, and they were passaged once weekly by splitting just-confluent
cultures 1/5 (SH-SY5Y), 1/20-1/40 (SH-EP1-\(h\alpha4\beta2/\beta4\)) or 1/300 (TE671/RD) to maintain cells in proliferative growth ((Lukas et al., 2002)).

\(^{86}\text{Rb}^+\) efflux assays of nAChR function: Cells were harvested at confluence from 100-mm plates by mild trypsinization (Irvine Scientific, Santa Ana, CA) before being resuspended in complete medium and evenly seeded at a density of one confluent 100-mm plate per 24-well plate (Falcon Plastics, Oxnard, CA; ~100-125 µg of total cell protein per well in a 500 µl volume). After cells had adhered (generally overnight, but no sooner than 4 hr later), medium was removed and replaced with 250 ml per well of complete medium supplemented with ~300,000 cpm of \(^{86}\text{Rb}^+\) (PerkinElmer Life Sciences; counted at 40% efficiency using Cerenkov counting (TriCarb 1900 liquid scintillation analyzer, PerkinElmer Life Sciences; 59% efficiency). After at least 4 hr and typically overnight, \(^{86}\text{Rb}^+\) efflux was measured using the “flip-plate” technique (Lukas et al., 2002). Briefly, after aspiration of the bulk of \(^{86}\text{Rb}^+\) loading medium from each well of the “cell plate,” each well containing cells was rinsed three times with 2 ml of fresh \(^{86}\text{Rb}^+\) efflux buffer (130 mM NaCl, 5.4 mM KCl, 2 mM CaCl2, 5 mM glucose, 50 mM HEPES, pH 7.4) to remove extracellular \(^{86}\text{Rb}^+\). Following removal of residual rinse buffer by aspiration, the flip-plate technique was used again to simultaneously introduce fresh efflux buffer containing drugs of choice at indicated final concentrations from a 24-well “efflux/drug plate” into the wells of the cell plate. After a 3-min incubation, the solution was “flipped” back into the efflux/drug plate for Cerenkov counting (PerkinElmer Wallac Micobeta Trilux 1450; 25% efficiency) after placement of inserts (PerkinElmer Wallac 1450-109) into each well to minimize cross-talk between wells. Any remaining medium in the cell plate was removed by aspiration, and cells in the cell plate were lysed and suspended by addition of 2 ml of 0.1M NaOH, 0.1% sodium dodecyl sulfate to each well. Suspensions in each well were then subjected to Cerenkov counting. \(^{86}\text{Rb}^+\) in both cell plates and efflux/drug plates was determined to ensure material balance (i.e., that the sum of \(^{86}\text{Rb}^+\) released into the efflux/drug plate and \(^{86}\text{Rb}^+\) remaining in the cell plate were the same for each well) and to determine efficiency of \(^{86}\text{Rb}^+\) loading (the percentage of applied \(^{86}\text{Rb}^+\) actually loaded into cells). For each experiment,
normalization and quality control measurements were made of total $^{86}\text{Rb}^+$ efflux in samples containing a fully efficacious dose of 1 mM carbamylcholine and of non-specific $^{86}\text{Rb}^+$ efflux measured using either samples containing 1 mM carbamylcholine plus 100 µM d-tubocurarine, which gave full block of agonist-induced or spontaneous, nAChR-mediated ion flux. Total minus non-specific ion flux equated to specific ion flux, and values of ion flux in test samples were normalized as percentage of specific, carbamylcholine-activated, d-tubocurarine-sensitive ion flux. Depending on cell density and the concentration of $^{86}\text{Rb}^+$ in the loading medium, SH-EP1-hα4β2 or -hα4β4 cells typically display specific efflux of 5,000-15,000 cpm of $^{86}\text{Rb}^+$ per sample with a ratio of total to non-specific efflux of 10:1 and with total efflux being about one-half of loaded $^{86}\text{Rb}^+$; SH-SY5Y cells display specific efflux of ~5,000 cpm of $^{86}\text{Rb}^+$ per sample with a ratio of total to non-specific efflux of 3:1 and with total efflux being about one-quarter of loaded $^{86}\text{Rb}^+$; and TE671/RD cells display specific efflux of ~20,000 cpm of $^{86}\text{Rb}^+$ per sample with a ratio of total to non-specific efflux of ~10:1 and with total efflux being about one-half of loaded $^{86}\text{Rb}^+$.

Effects of bupropion or its analogs on nAChR function were tested, first assessing whether those agents had intrinsic agonist activity, and then determining abilities of those agents to inhibit function of nAChR stimulated by 10 X EC50 value concentrations of carbamylcholine (1 mM for SH-SY5Y cells, 500 µM for TE671/RD cells, 200 µM for SH-EP1-hα4β4 or -hα4β4 cells). Carbamylcholine dose-response curves for nAChR function in the absence of added inhibitor or in the presence of bupropion or its metabolites at concentrations near to their IC50 values were also obtained to ascertain whether block occurred by competitive (functional blockade surmountable by increasing agonist concentration) or non-competitive (insurmountable block) mechanisms.

**Data analysis:** Ion flux assay results were fit to the Hill equation $[F = F_{max} / (1 + (Y/X)^n)]$ for specific ion flux, F, as a percentage of control, $F_{max}$, for EC50/IC50 value, Y, at ligand concentration, X, and for Hill coefficient, n (n>0 for agonists and EC50 determinations, n<0 for antagonists and IC50 determinations; Prism, GraphPad, San Diego, CA). Most ion flux data was fit allowing maximum and minimum ion flux values to be determined by curve fitting,
but in some cases where antagonists had weak functional potency, minimum ion flux was set at 0% of control. Statistically significant differences between IC\textsubscript{50} values for ion flux assays were determined by assessing overlap in 95% confidence intervals.

**Materials:** All other techniques and commercial sources for reagents were as indicated earlier (Bencherif and Lukas, 1993).

**In vivo Studies**

**Animals.** Male ICR mice (20-25g) obtained from Harlan Laboratories (Indianapolis, IN) were used throughout the study. Animals were housed in an AALAC approved facility and were placed in groups of six and had free access to food and water. Studies were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

**Drugs:** (-)-Nicotine was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI) and converted to the ditartrate salt as described by Aceto et al. (1979). Bupropion HCl was purchased from RBI (Natick, MA). (+)-(2S,3S)– and (-)-(2R,3R)- hydroxybupropion tartrates were synthesized using reported methods (Fang et al., 2000). All drugs were dissolved in physiological saline (0.9% sodium chloride) and given in a total volume of 1 ml/100 g body weight for s.c. injections. All doses are expressed as the free base of the drug.

**Antinociceptive tests:**

**Tail-flick test.** Antinociception was assessed by the tail-flick method of D'Amour and Smith (1941). Briefly, mice were lightly restrained while a radiant heat source was shone onto the upper portion of the tail. Latency to remove the tail from the heat source was recorded for each animal. A control response (2-4 sec) was determined for each mouse before treatment, and a test latency was determined after drug administration. In order to minimize tissue damage, a maximum latency of 10 sec was imposed. Antinociceptive response was calculated as percent maximum possible effect (% MPE), where \%MPE = [(test-control)/(10-control)] x 100.

**Hot-plate Test.** Mice were placed into a 10 cm wide glass cylinder on a hot plate (Thermojust Apparatus) maintained at 55.0 °C. Two control latencies at least ten min apart were
determined for each mouse. The normal latency (reaction time) was 8 to 12 seconds. Antinociceptive response was calculated as percent maximum possible effect (% MPE), where 

\[ \% \text{MPE} = \frac{\text{test-control}}{\text{40-control}} \times 100 \]

The reaction time was scored when the animal jumped or licked its paws. In order to minimize tissue damage, a maximum latency of 40 sec was imposed. Groups of eight to twelve animals were used for each dose and for each treatment. Antagonism studies in the tail-flick and hot-plate tests were carried out by pretreating the mice with either saline or bupropion metabolites 15 min before nicotine. The animals were then tested 5 min after administration of a s.c. dose of 2.5 mg/kg nicotine.

**Locomotor activity.** Mice were placed into individual Omnitech photocell activity cages (28 x 16.5 cm) 5 min after s.c. administration of either 0.9% saline or nicotine. Interruptions of the photocell beams (two banks of eight cells each) were then recorded for the next 10 min. Data were expressed as number of photocell interruptions. Antagonism studies were carried out by pretreating the mice with either saline or bupropion metabolites 15 min before nicotine. The animals were then tested 5 min after administration of a s.c. dose of 1.5 mg/kg nicotine.

**Body temperature.** Rectal temperature was measured by a thermistor probe (inserted 24 mm) and digital thermometer (Yellow Springs Instrument Co., Yellow Springs, OH). Readings were taken just before and at 30 min after the s.c. injection of either saline or nicotine. The difference in rectal temperature before and after treatment was calculated for each mouse. The ambient temperature of the laboratory varied from 21-24°C from day to day. Antagonism studies were carried out by pretreating the mice with either saline or bupropion metabolites 15 min before nicotine. The animals were then tested 5 min after administration of a s.c. dose of 2.5 mg/kg nicotine.

**Forced swimming test in mice.** The test was performed as described earlier (Porsolt et al., 1977). Briefly, mice were gently placed individually into glass cylinders (25x10 cm) containing 10 cm of water, maintained at 24°C, and left there for 6 min. Immobility was recorded during the last 4 min. A mouse was considered to be immobile when it floated in an upright position, and made only small movements to keep its head above water but do not
produce displacements. For ED$_{50}$ values calculation, a % decrease in immobility time after drug
treatment was determined as follow: \[ \text{%decrease} = \frac{(\text{Time post-drug}/\text{Time post-saline})-1}{1} \times 100. \]

**Statistical analysis.** Statistical analysis of all analgesic studies was performed using
either t-test or analysis of variance (ANOVA) with Tukey’s test post hoc test when appropriate.
All differences were considered significant at $p < 0.05$. ED$_{50}$ and AD$_{50}$ values with 95% CL
for behavioral data were calculated by unweighted least-squares linear regression, as described
by Tallarida and Murray (1987). Statistically significant differences between ED$_{50}$ and AD$_{50}$
values were determined by assessing overlap in 95% confidence intervals.
Results

In Vitro Studies

DA and NE Transporter Uptake Studies. Bupropion and hydroxymetabolites of bupropion were evaluated for their effects on monoamine uptake. Bupropion is relatively a weak inhibitor of DA uptake with an IC$_{50}$ of 550 nM, and it is even less potent as an inhibitor of NE reuptake (Table 1) with an IC$_{50}$ of 1.9 µM. Compared to bupropion, its racemic hydroxymetabolite produces equal inhibition of NE reuptake and much weaker inhibition of DA uptake (IC$_{50}$ > 10 µM). The (2S,3S) isomer is the active form (with IC$_{50}$ values of 790 and 520 nM for blockade of DA and NE uptake, respectively), while the (2R,3R) isomer shows no significant inhibition of either DA or NE uptake (IC$_{50}$ > 10 µM). When compared with bupropion, (2S,3S)-hydroxybupropion produces similar or stronger inhibition of DA and NE uptake, respectively.

nAChR Functional Studies. Initial studies of possible intrinsic activity of bupropion, (2S,3S)-hydroxybupropion, or (2R,3R)-hydroxybupropion clearly showed no ability of any of these agents to activate $^{86}$Rb$^+$ efflux (data not shown) under conditions where agonist activity at 1-2% of carbamylcholine efficacy could be reliably determined. By contrast, bupropion, (2S,3S)-hydroxybupropion, or (2R,3R)-hydroxybupropion had activity as functional antagonists at each human nAChR subtype tested (Fig. 1; Table 2). Antagonist log dose-response profiles showed full inhibition of nAChR function stimulated by carbamylcholine at a concentration 10-time higher than its EC$_{50}$ value. Bupropion (7.9 µM IC$_{50}$) and (2R,3R)-hydroxybupropion (7.6 µM IC$_{50}$) have similar functional inhibitory potency, significantly (p<0.05) exceeding that of the (2S,3S)-hydroxymetabolite (28 µM IC$_{50}$), at human $\alpha_1\beta_1\gamma\delta$-muscle-type nAChR, thereby showing evidence of enantioselectivity for the (2R,3R)-hydroxy isomer. Racemic bupropion has its highest functional antagonist potency (1.8 µM IC$_{50}$) at human $\alpha_3^*$-nAChR, whereas 2R,3R- (6.5 µM IC$_{50}$) and (2S,3S)-hydroxybupropion (10 µM IC$_{50}$) were significantly less potent.
(2S,3S)-Hydroxybupropion (3.3 µM IC₅₀) has significantly higher functional inhibitory potency at human α₄β₂-nAChR than either racemic bupropion (12 µM IC₅₀) or the (2R,3R)-hydroxymetabolite (31 µM IC₅₀). By contrast, racemic bupropion (14 µM IC₅₀) has significantly higher functional inhibitory potency than either its (2R,3R) or (2S,3S) hydroxymetabolites (41 and 30 µM IC₅₀, respectively), which show little-to-no enantioselectivity, at human α₄β₄-nAChR. Functional inhibitory potency is significantly higher for bupropion at α₃*-nAChR, for (2S, 3S)-hydroxybupropion at α₄β₂ nAChR and for (2R, 3R)-hydroxybupropion at α₃*- and α₁β₁γδ-nAChR than at the other nAChR subtypes.

When agonist dose-response studies were done alone or in the presence of antagonists at concentrations near to their IC₅₀ values (data not shown), block by bupropion or its hydroxy metabolites is noncompetitive as determined by insurmountability of functional inhibition with higher doses of agonist.

**In Vivo Studies**

**Mouse Forced Swimming Studies.** Bupropion and its hydroxymetabolites were evaluated for their ability to reduce immobility time in the forced swimming test. Time-course for immobility time for these analogs was determined after an acute s.c. dose of 10 mg/kg. As shown in Fig. 2A, the onset of action for bupropion and its hydroxymetabolites was rapid with maximum effect occurring between 0 and 10 min. The duration of effect was relatively brief in that the effect had disappeared completely within 60 min after bupropion administration in mice. The duration of effect was even shorter (30 min) for racemic hydroxybupropion and (2S,3S)-hydroxybupropion. In addition, no significant effect on immobility time was found after injection of the (2R,3R) isomer at different time points. Dose-response relationships were established for bupropion and its hydroxymetabolites by measuring immobility at the time of maximal effect (Fig. 2B). Ten minutes after injection, bupropion significantly reduced the immobility time of mice in a dose-related manner with an ED₅₀ value (± CL) of 4.2 (3.8-4.8) mg/kg. Racemic hydroxybupropion
was 1.5-fold less potent than bupropion in the swimming test with a potency of 6.5 (5.0-6.8) mg/kg. When compared with bupropion, (2S,3S)-hydroxybupropion was equally potent in the swimming test with an ED$_{50}$ value (± CL) of 4.4 (3.3-5.8) mg/kg, whereas the (2R,3R) isomer showed no significant inhibition of immobility time of mice. These results demonstrate that (2S,3S)-hydroxybupropion is the active isomer.

Antagonism of Nicotine’s Pharmacological Effects by Bupropion Hydroxymetabolites after Acute Administration. Bupropion and its hydroxymetabolites were evaluated for their ability to antagonize nicotine’s effects in the following procedures: antinociception using the tail-flick and hot-plate tests, decrease in locomotor activity and hypothermia. Table 3 summarizes the potency of the different bupropion analogs in blocking these different effects of nicotine.

1- Antinociception: Nicotine-induced antinociception in the tail-flick and hot-plate tests after systemic administration in mice (2.5 mg/kg) was blocked by bupropion and its hydroxymetabolites in a dose-dependent manner (Fig. 3A & B). Calculation of the AD$_{50}$ values (see Table 3) showed that (2S,3S) hydroxybupropion was 7- to 12-fold more potent than bupropion, (2RS,3RS)-hydroxybupropion and (2R,3R)-hydroxybupropion) in blocking the antinociceptive effect of nicotine. Similarly to the tail-flick results, (2S,3S)-hydroxybupropion isomer was the most potent nicotinic antagonist in the hot-plate test. By themselves, bupropion analogs did not cause antinociception at the indicated doses and times.

2- Body temperature and Spontaneous Activity: Hypothermia induced by systemic administration of nicotine (2.5 mg/kg) was blocked by bupropion and its hydroxymetabolites with differential potency (Fig. 3C & D). Bupropion’s AD$_{50}$ was 7.5 mg/kg and that of racemic hydroxybupropion was 19.4 mg/kg. As with blockade of nicotine-induced antinociception, (2S,3S)-hydroxybupropion (AD$_{50}$ = 1.5 mg/kg) was considerably more potent than the other compounds in antagonizing nicotine’s hypothermic effects. The (2R,3R) isomer was inactive at a dose of 20 mg/kg. Similar findings were obtained with blockade of hypomotility produced by nicotine (1.5 mg/kg). Bupropion significantly blocked nicotine’s hypomotility effects with AD$_{50}$
of 3 mg/kg. (2S,3S)-hydroxybupropion was the most potent blocker with an AD50 of 0.9 mg/kg. In contrast, the (2R,3R) isomer showed no significant inhibition at the highest dose tested (20 mg/kg). By themselves, none of these analogs had a significant effect on the body temperature or locomotor activity at the indicated doses and times.
Discussion

Nicotine is a powerful pharmacologic agent that has both stimulant and depressant effects on the central nervous system (Henningfield et al., 1991). The very high prevalence of smoking among patients with depression and other psychiatric disorders may be due to nicotine’s effect on mood. Major depression is more common among smokers than nonsmokers, further suggesting an important relationship between nicotine dependence and mood disorders. The relationship between nicotine dependence and mood disorders led researchers to consider antidepressants as potential treatments for smoking cessation. Thus far, the most useful antidepressant evaluated is bupropion.

Much research has been directed toward studies to identify the mechanism of antidepressant activity of bupropion. However, the specific sites that are responsible for its biological activity are still not fully understood. It is well recognized that bupropion exhibits both noradrenergic and dopaminergic activity. Thus, the effectiveness of bupropion as a smoking cessation treatment may be related to its effects on mood via enhancement of noradrenergic and dopaminergic signals. In addition, recent studies reported that bupropion is a non-competitive antagonist at various nAChR subtypes (Fryer et al., 1999; Slemmer et al., 2000) thereby suggesting another possible component in bupropion’s utility as an aid to smoking cessation. The fact that bupropion is extensively converted to biologically active metabolites raises the possibility that the latter may contribute to the mechanism of bupropion’s actions. However, to our knowledge, no studies have been reported on this subject.

The results of the current study indicate that bupropion and its hydroxymetabolites are non-competitive antagonists of nAChR at concentrations similar to those inhibiting [3H]NE and [3H]DA uptake. The in vitro concentrations for bupropion action at $\alpha_3\beta_4^*$-nAChR (1.8 µM IC$_{50}$) are comparable to those needed to inhibit DA (0.55 µM IC$_{50}$) or NE transporter (1.9 µM IC$_{50}$) function. In addition, the action of (2S, 3S)-hydroxybupropion at $\alpha_4\beta_2$-nAChR (3.3 µM IC$_{50}$) occurs in the concentration range needed to inhibit DA (0.79 µM IC$_{50}$) or NE transporter (0.52 µM IC$_{50}$) function. These findings suggests that blockade of nAChR, in addition to blockade of
monamine transporter function, may be involved in the effectiveness of bupropion as a treatment for smoking cessation. Not only are the effects of the hydroxymetabolites enantioselective, (2S,3S)-hydroxybupropion displays the same or better activity than the parent compound, at endpoints associated with blockade of nicotine-stimulated behaviors. Thus, the (2S,3S) metabolite may play a critical role in the effectiveness of bupropion as a smoking cessation pharmacotherapy. It would be interesting to determine if levels of the (2S,3S) metabolite are relatively low in patients for which bupropion is an ineffective treatment for smoking cessation. In contrast, the other enantiomer (2R,3R) exhibited greater functional antagonism at muscle-type nAChR, and no enantioselectivity was seen for nAChR containing β4 subunits (e.g., α4β4*- or α3β4*-nAChR).

Our in vivo data support a role for the (2S,3S) isomer in the actions of bupropion, since this isomer was the most potent compound in blocking nicotine’s behavioral effects. The in vitro data indicate these effects are mediated by α4β2-nAChR, because racemic bupropion is rapidly converted to its hydroxymetabolites and the (2S,3S) hydroxymetabolite preferentially blocks the α4β2-nAChR subtype. Although bupropion has higher affinity for α3β4*-nAChR and muscle-type nAChR than for α4*-nAChR, rapid metabolism of the compound would minimize its effects on autonomic ganglia. Moreover, enhancement of sympathetic tone via blockage of NE reuptake would counter toxic effects due to autonomic nAChR blockade. Indeed tachycardia is one of the most frequent side effects of bupropion treatment. Nevertheless, any α3β4* nAChR expressed in the brain also are potential clinically relevant targets for more actions of bupropion itself. With regard to targeted design of drugs useful in smoking cessation, depression, or both, the present findings indicate that (2S,3S)-hydroxybupropion would have a smaller peripheral nAChR-mediated side effect profile than would bupropion, assuming that central nAChR are the desired target.

The strong enantioselectivity for hydroxybupropion action at DA transporters, NE transporters, and α4β2-nAChR is consistent with the enantioselectivity seen in vivo for actions in a mouse depression model (forced swimming test). However, the 10-20-fold higher potency of
(2S,3S)-hydroxybupropion over racemic bupropion in blockade of nicotine’s behavioral effects more closely parallels their potency differences for α4β2-nAChR antagonism than in their actions at DA or NE transporters. The same can be said for comparisons between potency ratios for bupropion and (2R,3R)-hydroxybupropion. By contrast, the comparable potencies of racemic bupropion and (2S,3S)-hydroxybupropion in the forced swim test most closely matches effects of comparable potencies of these compounds in inhibition of DA uptake, suggesting dominance of dopaminergic mechanisms in behavioral tests employing the depression model. Nevertheless, of interest for future work are effects of nicotine on models of depression and sensitivity of any effects to bupropion.

It is interesting to note that racemic hydroxybupropion lacks potency at DAT relative to the activity of the (2S,3S)- isomer. It is generally accepted that when one isomer has much higher activity than the other, the activity of the mixture is normally closer to the active isomer. This was the case of the NET but not the DAT transporter inhibition by hydroxybupropion and its isomers. Although no clear explanation can be proposed for this discrepancy, it is possible that the inactive isomer is having a negative allosteric effect on binding to the DA transporter which suggest a differential interaction of hydroxybupropion stereoisomers at DAT that could invoke an allosteric site/multimer models.

Collectively, our findings support the hypothesis that bupropion’s utility as both an antidepressant and an aid to treatment of nicotine dependence reflects actions of bupropion and/or its hydroxymetabolites on a combination of targets including the DA transporter, the NE transporter, and members of the diverse family of nAChR. These findings suggest that it may be desirable to synthesize compounds with multiple biological activities. Thus, a successful smokingcessation pharmacotherapy would at least include activity at monoamine transporters and α4β2-nAChR. That the (2S,3S) isomer possess the most desirable pharmacodynamic properties of the compounds tested suggests that the efficacy of bupropion in the treatment of depression and smoking cessation may be linked to how it is metabolized. Further, our data
suggest that the (2S,3S) isomer may be a better drug candidate for smoking cessation than bupropion because of its higher potency at the relevant targets and low activity at α3*-nAChR.

Acknowledgments. The authors greatly appreciate the technical assistance of Tie Han.
References


FOOTNOTES

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FIGURE LEGENDS

**Fig. 1** Antagonist dose-response profiles for blockade of nAChR function. Specific $^{86}$Rb$^+$ efflux (ordinate; percentage of control) was determined in the presence of carbamylcholine at the concentration and as described in Materials and Methods alone or in the presence of the indicated concentrations (abscissa; log molar scale) of racemic bupropion ( ), (2S,3S)-hydroxybupropion ( ), or (2R,3R)-hydroxybupropion ( ) acting at $\alpha_1$*-nAChR (TE671/RD cells; upper left), $\alpha_3\beta_4$*-nAChR (SH-SY5Y cells; upper right), $\alpha_4\beta_2$-nAChR (SH-EP1-h$\alpha_4\beta_2$ cells; lower left), or $\alpha_4\beta_4$-nAChR (SH-EP1-h$\alpha_4\beta_4$ cells; lower right). IC$_{50}$ values and Hill coefficients (± S.E.M.) are provided in Table 2. Maximum and minimum values for specific $^{86}$Rb$^+$ efflux obtained from curve fitting were 100 ± 5% or 0 ± 5%, respectively, of control values except for the (2S,3S)-hydroxy isomer at $\alpha_3\beta_4$*-nAChR (maximum of 94 ± 5% of control) and at $\alpha_1$*-nAChR (maximum of 94 ± 2% of control) and for (2R,3R)-hydroxy isomer at $\alpha_4\beta_2$-nAChR (maximum of 92 ± 3% of control), except for the (2S,3S)-hydroxy isomer at $\alpha_3\beta_4$*-nAChR (minimum of -7 ± 16% of control) and at $\alpha_1$*-nAChR (minimum of -9 ± 19% of control), and except for fits to the Hill equation with minimum specific efflux values fixed at 0% of control for effects on $\alpha_4\beta_2$- and $\alpha_4\beta_4$-nAChR.

**Fig. 2** Effects of bupropion ( ), (2RS,3RS)-hydroxybupropion ( ), (2R,3R)-hydroxybupropion ( ) and (2S,3S)-hydroxybupropion ( ) in the forced swimming test after s.c. injection in mice. (A) Time-course of bupropion and its hydroxymetabolites effects on immobility time (sec) after s.c. administration of 10 mg/kg of each of the analogs in mice. (B) Dose-response curves of bupropion and its hydroxymetabolites in forced swimming test after s.c. administration in mice. Bupropion analogs at different doses were administered s.c. 10 min before the test.
Each point represents the mean ± SE of 8 to 12 mice. *p < 0.05 compared to correspondent zero time point (saline control).

Fig. 3  Blockade of nicotine’s behavioral effects by bupropion (Δ), (2RS,3RS)-hydroxybupropion (●), ((2R,3R)-hydroxybupropion ○) and (2S,3S)-hydroxybupropion (■) after s.c. injection in mice.  (A) Nicotine-induced antinociception in the tail-flick test (2.5 mg/kg, s.c.)  (B) Nicotine-induced antinociception in the hot-plate test (2.5 mg/kg, s.c.);  (C) Nicotine-induced hypothermia (2.5 mg/kg, s.c.) and  (D) Nicotine-induced hypomotility (1.5 mg/kg, s.c.).  Bupropion and its hydroxymetabolites at different doses were administered s.c. 15 min before nicotine and mice were tested 5 min later except for hypothermia (measure after 30 min). Each point represents the mean ± SE of 8 to 12 mice.
**Table 1:** Blockade of DA and NE transporters uptake in rat cortical synaptosomes by bupropion and its hydroxymetabolites.

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Bupropion</td>
<td>550 ± 65</td>
<td>1900 ± 12</td>
</tr>
<tr>
<td>(2RS,3RS)-Hydroxybupropion</td>
<td>&gt; 10,000</td>
<td>1700 ± 830</td>
</tr>
<tr>
<td>(2S,3S)-Hydroxybupropion</td>
<td>790 ± 11</td>
<td>520 ± 35</td>
</tr>
<tr>
<td>(2R,3R)-Hydroxybupropion</td>
<td>&gt; 10,000</td>
<td>&gt; 10,000</td>
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Table 2: Bupropion and its hydroxymetabolites as functional antagonists toward nAChR subtypes in cell lines. $^{86}$Rb$^+$ efflux assays were conducted as described in Methods and in the legend to Fig. 1. Results were fit to the logistic equation to determine molar IC$_{50}$ values (upper value; µM ± S.E.M.) and Hill coefficients (lower value in parentheses) presented as ± S.E.M.

<table>
<thead>
<tr>
<th></th>
<th>SH-SY5Y $\alpha_3\beta_4^*$</th>
<th>SH-EP1-h$\alpha_4\beta_2$</th>
<th>SH-EP1-h$\alpha_4\beta_4$</th>
<th>TE671/RD $\alpha_1^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bupropion</td>
<td>1.8 ± 1.1# (#1.24 ± 0.19)</td>
<td>12 ± 1.1# (#1.24 ± 0.18)</td>
<td>14 ± 1.1# (#1.19 ± 0.09)</td>
<td>7.9 ± 1.1# (#1.25 ± 0.14)</td>
</tr>
<tr>
<td>(2S,3S) OH-</td>
<td>10 ± 1.5 (-1.08 ± 0.35)</td>
<td>3.3 ± 1.1#@ (-1.31 ± 0.11)</td>
<td>30 ± 1.1@ (-1.10 ± 0.12)</td>
<td>28 ± 1.4 (-1.25 ± 0.32)</td>
</tr>
<tr>
<td>bupropion</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>(2R,3R)-OH-</td>
<td>6.5 ± 1.2#@ (-1.08 ± 0.18)</td>
<td>31 ± 1.1</td>
<td>41 ± 1.1@ (-1.65 ± 0.19)</td>
<td>7.6 ± 1.1# (-1.23 ± 0.13)</td>
</tr>
<tr>
<td>bupropion</td>
<td></td>
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</tbody>
</table>

# p<0.05 relative to other subtypes for the indicated drug.

# p<0.05 within subtype relative to 2S,3S,-hydroxybupropion.

@ p<0.05 within subtype relative to bupropion.
**Table 3:** Summary of the antagonistic potency of bupropion and bupropion hydroxymetabolites on different pharmacological actions of nicotine after s.c. administration in mice. Results are presented as AD$_{50}$ values (±CL) in terms of mg/kg. They were calculated from the dose-response and expressed as mg/kg. Each dose group included 8 to 12 animals.

<table>
<thead>
<tr>
<th>Pharmacological Effect</th>
<th>Bupropion (2RS,3RS)-Hydroxy-bupropion</th>
<th>(2S,3S)-Hydroxy-bupropion</th>
<th>(2R,3R)-Hydroxy-bupropion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tail-flick</td>
<td>2.0 (1.8-3.6)</td>
<td>1.5 (0.6-3.8)</td>
<td>0.2 (0.1-0.3)</td>
</tr>
<tr>
<td>Hot-plate</td>
<td>7.0 (5.9-9.5)</td>
<td>5.5 (3.6-16)</td>
<td>1.0 (0.2-5.2)</td>
</tr>
<tr>
<td>Hypomotility</td>
<td>3.0 (2.5-6.3)</td>
<td>8.2 (4-17.3)</td>
<td>0.9 (0.3-3.0)</td>
</tr>
<tr>
<td>Hypothermia</td>
<td>7.5 (3-15.4)</td>
<td>19.4 (10-35)</td>
<td>1.5 (0.8-3.0)</td>
</tr>
</tbody>
</table>

† p<0.05 relative to (2RS,3RS)-Hydroxy-bupropion.
bupropion and hydroxymetabolite antagonism of nAChR function

![Graphs showing specific %Rb⁺ efflux (% of control) versus log [drug] (M) for different cell lines.](molpharm.aspetjournals.org)