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Enantioselective Effects of Hydroxy Metabolites of Bupropion on Behavior and on Function of Monoamine Transporters and Nicotinic Receptors

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ABSTRACT

Bupropion is an atypical antidepressant that also has usefulness as a smoking-cessation aid. Because 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. This study investigated the effects of hydrobupropion enantiomers on monoamine transporters and nicotinic acetylcholine receptor (nAChR) subtypes. Racemic bupropion and hydroxybupropion inhibit [^{3}H]norepinephrine (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 (2*S*,3*S*)-hydroxy isomer (IC_{50} = 520 nM) rather than (2*S*,3*R*)-hydroxybupropion (IC_{50} > 10,000 nM). Similar results were found with [^{3}H]dopamine (DA) uptake. The effects of bupropion and enantiomers of hydroxybupropion on human nAChR subtypes indicate that the (2*S*,3*S*) isomer is more potent than the (2*S*,3*R*) isomer or racemic bupropion as an antagonist of $\alpha_4\beta_2$ (functional IC_{50} = 3.3 μM). In addition, (2*S*,3*S*)-hyroxybupropion and bupropion were considerably more potent than (2*R*, −3*R*)-hydroxybupropion in a mouse depression model (forced swimming test) and in antagonism of acute nicotine effects in mice. Together, our results suggest that clinical and behavioral effects of bupropion arise from actions at nAChR as well as DA and NE transporters. Furthermore, our data suggest that the (2*S*,3*S*)-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 believed to involve the modulation of dopaminergic (dopamine, DA) and noradrenergic (norepinephrine, 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 believed to play a role in bupropion's because of its lack of 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, noncompetitive nAChR antagonist suggested that actions of bupropion at nAChR were of possible relevance for smoking cessation, especially given the

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ABBREVIATIONS: NE, norepinephrine (noradrenergic); nAChR, nicotinic acetylcholine receptor; %MPE, maximum possible effect; CL, confidence limit; AD_{50}, antagonist dose 50%; DA, dopamine or dopaminergic; h $\alpha_4\beta_2$-, h $\alpha_4\beta_4$-, h α_1*-, and h α_3*-nAChR, human nicotinic acetylcholine receptor(s) composed of α_4 and β_2 subunits, α_4 and β_4 subunits, α_1, β_1, γ, and δ subunits, or α_3, $\beta_4\pm$ β_2, and α_5 subunits, respectively; DAT, dopamine transporter.

hypothesis that long-term 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 nAChRs 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 hydroxy metabolite are in the range of bupropion concentrations that antagonize diverse nAChR subtypes (Golden et al., 1988).

It is believed that the effects of bupropion's major metabolites may be critical to its antidepressant activity, because bupropion is extensively metabolized to (2*S*,3*R*)- and (2*S*,3*S*)-hydroxybupropion, (*R*, −*R*)- and (*S*, −*S*)-threohydrobupropion, and (*R*, −*S*)-, and (*S*, −*R*)-erythrohydrobupropion in humans (Cooper et al., 1994). The concentrations of hydroxybupropion isomers present in cerebrospinal fluid 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; Welch et al., 1987; Golden et al., 1988; Hysu et al., 1997). Furthermore, hydroxybupropion shows stronger antitetrabenazine 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, (2*S*,3*S*)- but not (2*S*,3*R*)-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 it 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

[^{3}H]NE and DA Uptake Studies

Synaptosomal Preparation. Bupropion analogs were evaluated in neurotransmitter uptake assays using synaptosomes prepared from rat brain (adult male Sprague-Dawley rats weighing 250 g). Because 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 (Eshleman et al., 2001). In brief, rat brain was rapidly removed after decapitation, and the cerebral cortex was dissected, weighed, and placed immediately in a Teflon-to-glass homogenizer prefilled with 10 ml of 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*g* (for 10 min at 4°C). The supernatant was collected and centrifuged at 12,000*g* (for 10 min at 4°C). After this step, the supernatant was discarded, and the pellet was resuspended (Teflon-to-glass) in the original volume of an oxygenated 25 mM HEPES assay buffer, pH 7.4 (37°C) containing 128 mM NaCl, 2.4 nM KCl, 3.2 mM CaCl2, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 10 mM dextrose. Ascorbate (1 mM) and iproniazid (10 μM, monoamine oxidase 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 preincubated with the tissue (for 30 min at 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 10 volumes of ice-cold assay buffer. The filter disks were incubated with 0.5 ml of 10% SDS for 1 h before the addition of scintillation fluid, and trapped radioactivity was 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 [^{3}H]DA was the same as described above for [^{3}H]NE uptake, except the caudate was harvested and diluted 1:70, 5 nM [^{3}H]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 [^{3}H]NE and [^{3}H]DA uptake were determined from a plot of the specific uptake versus log concentration data fit to a four-parameter logistic equation (Prism version 3.0; GraphPad Software Inc., San Diego, CA). The IC_{50} data were expressed as mean ± S.D. from at least two independent experiments.

nAChR Functional Studies

Cell Culture. Cells of the TE671/RD human clone (Lukas, 1989) or the SH-SY5Y human neuroblastoma (Lukas, 1993) naturally expressing muscle-type ($\alpha1\beta1\gamma\delta$-nAChR) or $\alpha3$*-nAChR, respectively, or cells of the SH-EP1-h$\alpha4\beta2$ (Eaton et al., 2003) or -h$\alpha4\beta4$ cell lines heterologously expressing human $\alpha4\beta2$- or $\alpha4\beta4$-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 Laboratories, Logan, UT) on 100-mm diameter plates in a humidified atmosphere containing 5% CO_2 in air at 37°C (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 $\alpha4$ and $\beta2/\beta4$ 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 to 1/40 (SH-EP1-h$\alpha4\beta2/\beta4$) or 1/300 (TE671/RD) to maintain cells in proliferative growth (Lukas et al., 2002).

$^{86}Rb^+$ 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 h later), medium was removed and replaced with 250 ml per well of complete medium supplemented with ~300,000 cpm of $^{86}Rb^+$ (PerkinElmer Life and Analytical Sciences, Boston, MA) and counted at 40% efficiency using Cerenkov counting (TriCarb 1900 liquid scintillation analyzer, 59% efficiency; PerkinElmer Life Sciences). After at least 4 h and typically overnight, $^{86}Rb^+$ efflux was measured using the "flip-plate" technique (Lukas et al., 2002). In brief, after aspiration of the bulk of $^{86}Rb^+$ loading medium from each well of the "cell plate", each well containing cells was rinsed three times with 2 ml of fresh $^{86}Rb^+$ efflux buffer (130 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 5 mM glucose, and 50 mM HEPES, pH 7.4) to remove extracellular $^{86}Rb^+$. After 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 (Micobeta Trilux 1450, 25% efficiency; PerkinElmer Wallac, Gaithersburg, MD) 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 the addition of 2 ml of 0.1 M NaOH and 0.1% sodium dodecyl sulfate to each well. Suspensions in each well were then subjected to Cerenkov counting. $^{86}Rb^+$ in both cell plates and efflux/drug plates was determined to ensure material balance (i.e., that the sum of $^{86}Rb^+$ released into the efflux/drug plate and $^{86}Rb^+$ remaining in the cell plate were the same for each well) and to determine the efficiency of $^{86}Rb^+$ loading (the percentage of applied $^{86}Rb^+$ actually loaded into cells). For each experiment, normalization and quality-control measurements were made of total $^{86}Rb^+$ efflux in samples containing a fully effective dose of 1 mM carbamylcholine and of nonspecific $^{86}Rb^+$ 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 nonspecific ion flux equated to specific ion flux, and values of ion flux in test samples were normalized as the percentage of specific, carbamylcholine-activated, *d*-tubocurarine–sensitive ion flux. Depending on cell density and the concentration of $^{86}Rb^+$ in the loading medium, SH-EP1-h$\alpha4\beta2$ or -h$\alpha4\beta4$ cells typically display specific efflux of 5000 to 15,000 cpm of $^{86}Rb^+$ per sample with a ratio of total-to-nonspecific efflux of 10:1 and with total efflux being approximately one-half of loaded $^{86}Rb^+$; SH-SY5Y cells display specific efflux of ~5000 cpm of $^{86}Rb^+$ per sample, with a ratio of total-to-nonspecific efflux of 3:1 and with total efflux being approximately one-quarter of loaded $^{86}Rb^+$; and TE671/RD cells display specific efflux of ~20,000 cpm of $^{86}Rb^+$ per sample with a ratio of total-to-nonspecific efflux of ~10:1 and with total efflux being approximately one-half of loaded $^{86}Rb^+$. Effects of bupropion or its analogs on nAChR function were tested, first assessing whether those agents had intrinsic agonist activity, and then determining the abilities of those agents to inhibit the function of nAChR stimulated by 10× EC_{50} value concentrations of carbamylcholine (1 mM for SH-SY5Y cells, 500 μM for TE671/RD cells, and 200 μM for SH-EP1-h$\alpha4\beta4$ or -h$\alpha4\beta4$ 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 IC_{50} values were also obtained to ascertain whether block occurred by competitive (functional blockade surmountable by increasing agonist concentration) or noncompetitive (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 EC_{50}/IC_{50} value (Y) at ligand concentration (X) and for Hill coefficient n ($n > 0$ for agonists and EC_{50} determinations, $n < 0$ for antagonists and IC_{50} determinations) (Prism, GraphPad Software). Most ion flux data were fit allowing maximum and minimum ion flux values to be determined by curve-fitting, but in some cases in which antagonists had weak functional potency, minimum ion flux was set at 0% of control. Statistically significant differences between IC_{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 described previously (Bencherif and Lukas, 1993).

In Vivo Studies

Animals. Male Institute of Cancer Research (ICR) mice (weighing 20–25 g) obtained from Harlan (Indianapolis, IN) were used throughout the study. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-approved facility, 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 Sigma/RBI (Natick, MA). (+)-(2*S*,3*S*)- and (−)-(2*S*,3*R*)- hydroxybupropion tartrates were synthesized using methods reported previously (Fang et al., 2000). All drugs were dissolved in physiological saline (0.9% sodium chloride) and given in a total volume of 1 ml per 100 g body weight for subcutaneous 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). In brief, 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 s) was determined for each mouse before treatment, and a test latency was determined after drug administration. To minimize tissue damage, a maximum latency of 10 s was imposed. Antinociceptive response was calculated as the percentage of maximum possible effect (%MPE), where %MPE = [(test − control)/(10 − control)] × 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 10 min apart were determined for each mouse. The normal latency (reaction time) was 8 to 12 s. Antinociceptive response was calculated as the %MPE, where %MPE = [(test − control)/(40 − control) × 100]. The reaction time was scored when the animal jumped or licked its paws. To minimize tissue damage, a maximum latency of 40 s was imposed. Groups of 8 to 12 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 subcutaneous dose of 2.5 mg/kg nicotine.

Locomotor Activity. Mice were placed into individual Omnitech photocell activity cages (28 × 16.5 cm; Omnitech Electronics, Columbus, OH) 5 min after subcutaneous 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 the 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 subcutaneous dose of 1.5 mg/kg nicotine.

Body Temperature. Rectal temperature was measured by a thermistor probe (inserted 24 mm) and digital thermometer (YSI Inc., Yellow Springs, OH). Readings were taken just before and 30 min after the subcutaneous 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 labora-

tory varied from 21 to 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 subcutaneous dose of 2.5 mg/kg nicotine.

Forced Swimming Test in Mice. The test was performed as described previously (Porsolt et al., 1977). In brief, mice were gently placed individually into glass cylinders (25 × 10 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 did not produce displacements. For the calculation of ED_{50} values, a percentage decrease in immobility time after drug treatment was determined as follows: % decrease = [(time postdrug/time postsaline) − 1] × 100.

Statistical Analysis. Statistical analysis of all analgesic studies was performed using either *t* test or analysis of variance with Tukey's 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 hydroxy metabolites of bupropion were evaluated for their effects on monoamine uptake. Bupropion is a relatively 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 with bupropion, its racemic hydroxy metabolite produces equal inhibition of NE reuptake and much weaker inhibition of DA uptake ($IC_{50} > 10$ μM). The (2*S*,3*S*) isomer is the active form (with IC_{50} values of 790 and 520 nM for blockade of DA and NE uptake, respectively), whereas the (2*S*,3*R*) isomer shows no significant inhibition of either DA or NE uptake ($IC_{50} > 10$ μM). Compared with bupropion, (2*S*,3*S*)-hydroxybupropion produces similar or stronger inhibition of DA and NE uptake, respectively.

nAChR Functional Studies. Initial studies of possible intrinsic activity of bupropion, (2*S*,3*S*)-hydroxybupropion, or (2*S*,3*R*)-hydroxybupropion clearly showed no ability of any of these agents to activate $^{86}Rb^{+}$ efflux (data not shown) under conditions in which agonist activity at 1 to 2% of carbamylcholine efficacy could be reliably determined. In contrast, bupropion, (2*S*,3*S*)-hydroxybupropion, or (2*S*,3*R*)-hydroxybupropion had activity as functional antagonists at each human nAChR subtype tested (Fig. 1 and Table 2). Antagonist log dose-response profiles showed full inhibition of nAChR function stimulated by carbamylcholine at a concentration 10 times higher than its EC_{50} value. Bupropion (IC_{50} = 7.9 μM) and (2*S*,3*R*)-hydroxybupropion (IC_{50} = 7.6 μM) have similar functional inhibitory potency, significantly ($p < 0.05$) exceeding that of the (2*S*,3*S*)-hydroxy metabolite (IC_{50} = 28 μM), at human $\alpha_1\beta_1\gamma\delta$ muscle-type nAChR, thereby showing evidence of enantioselectivity for the (2*S*,3*R*)-hydroxy isomer. Racemic bupropion has its highest functional antagonist potency (IC_{50} = 1.8 μM) at human α_3*-nAChR, whereas 2*R*,3*R*- (IC_{50} = 6.5 μM) and (2*S*,3*S*)-hydroxybupropion (IC_{50} = 10 μM) were significantly less potent. (2*S*,3*S*)-Hydroxybupropion (IC_{50} = 3.3 μM) has significantly higher functional inhibitory potency at human $\alpha_4\beta_2$-nAChR than either racemic bupropion (IC_{50} = 12 μM) or the (2*S*,3*R*)-hydroxy metabolite (IC_{50} = 31 μM). In contrast, racemic bupropion (IC_{50} = 14 μM) has significantly higher functional inhibitory potency than either its (2*S*,3*R*)- or (2*S*,3*S*)-hydroxy metabolites (IC_{50} = 41 and 30 μM, respectively), which show little to no enantioselectivity at human $\alpha_4\beta_4$-nAChR. Functional inhibitory potency is significantly higher for bupropion at α_3*-nAChR, for (2*S*,3*S*)-hydroxybupropion at $\alpha_4\beta_2$ nAChR, and for (2*S*,3*R*)-hydroxybupropion at α_3*- and $\alpha_1\beta_1\gamma\delta$-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 IC_{50} 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.

TABLE 1
Blockade of DA and NE transporters uptake in rat cortical synaptosomes by bupropion and its hydroxy metabolites
Values given are mean ± S.D.

Drug	IC_{50}	
	[^{3}H]DA	[^{3}H]NE
	nM	
Bupropion	550 ± 65	1900 ± 12
(2*RS*,3*RS*)-Hydroxybupropion	>10,000	1700 ± 830
(2*S*,3*S*)-Hydroxybupropion	790 ± 11	520 ± 35
(2*R*,3*R*)-Hydroxybupropion	>10,000	>10,000

In Vivo Studies

Mouse Forced Swimming Studies. Bupropion and its hydroxy metabolites 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 immediate dose of 10 mg/kg s.c. As shown in Fig. 2A, the onset of action for bupropion and its hydroxy metabolites 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 (2*S*,3*S*)-hydroxybupropion. In addition, no significant effect on immobility time was found after injection of the (2*S*,3*R*) isomer at different time points. Dose-response relationships were established for bupropion and its hydroxy metabolites 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 ED_{50} 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. Compared with bupropion, (2*S*,3*S*)-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 (2*S*,3*R*) isomer showed no significant inhibition of immobility time of mice. These results demonstrate that (2*S*,3*S*)-hydroxybupropion is the active isomer.

Antagonism of Nicotine's Pharmacological Effects by Bupropion Hydroxy Metabolites after Immediate Administration. Bupropion and its hydroxy metabolites were evaluated for their ability to antagonize nicotine's ef-

fects 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.

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 hydroxy metabolites in a dose-dependent manner (Fig. 3, A and B). Calculation of the AD_{50} values (see Table 3) showed that (2*S*,3*S*)-hydroxybupropion was 7- to12-fold more potent than bupropion, (2*RS*,3*RS*)-hydroxybupropion, and (2*S*,3*R*)-hydroxybupropion) in blocking the antinociceptive effect of nicotine. Similarly to the tail-flick results, (2*S*,3*S*)-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.

Body temperature and Spontaneous Activity. Hypothermia induced by systemic administration of nicotine (2.5 mg/kg) was blocked by bupropion and its hydroxy metabolites with differential potency (Fig. 3, C and 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, (2*S*,3*S*)-hydroxybupropion (AD_{50} = 1.5 mg/kg) was considerably more potent than the other compounds in antagonizing nicotine's hypothermic effects. The (2*S*,3*R*) 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. (2*S*,3*S*)-hydroxybupropion was the most potent blocker with an AD_{50} of 0.9 mg/kg. In contrast, the (2*S*,3*R*) 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 pharmacological 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 caused by 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 treat-

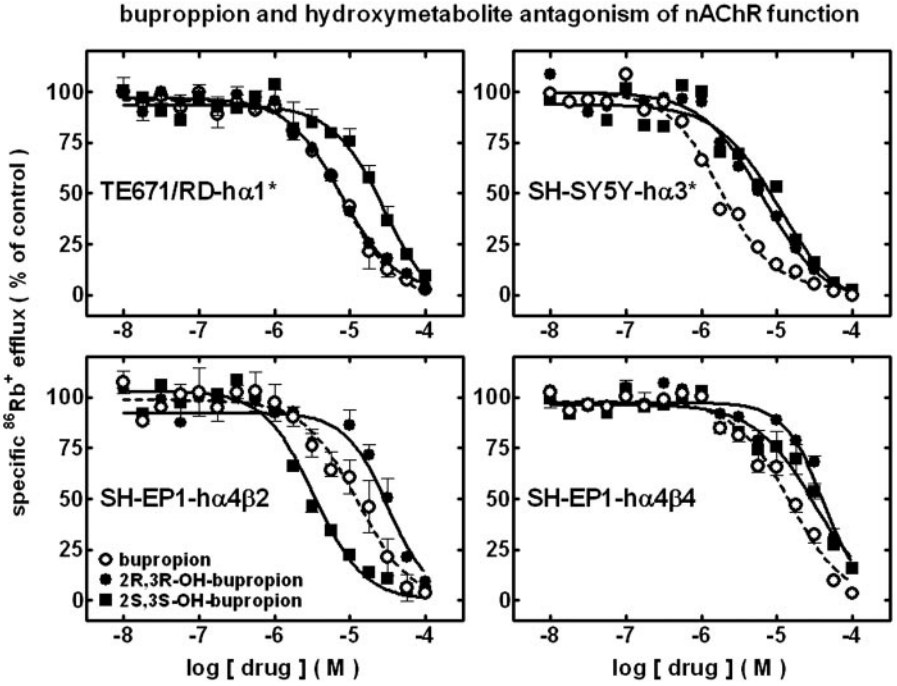

Fig. 1. Antagonist dose-response profiles for blockade of nAChR function. Specific $^{86}Rb^+$ efflux (ordinate; percentage of control) was determined in the presence of carbamylcholine at the concentration shown and as described under *Materials and Methods* alone or in the presence of the indicated concentrations (abscissa; log molar scale) of racemic bupropion (○), (2*S*,3*S*)-hyroxybupropion (■), or (2*S*,3*R*)-hydroxybupropion (●) acting at α_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 (2*S*,3*S*)-hydroxy isomer at $\alpha_3\beta_4$*-nAChR (maximum of 94 ± 5% of control) and at α_1*-nAChR (maximum of 94 ± 2% of control) and for (2*S*,3*R*)-hydroxy isomer at $\alpha_4\beta_2$-nAChR (maximum of 92 ± 3% of control), except for the (2*S*,3*S*)-hydroxy isomer at $\alpha_3\beta_4$*-nAChR (minimum of −7 ± 16% of control) and at α_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.

TABLE 2

Bupropion and its hydroxy metabolites as functional antagonists toward nAChR subtypes in cell lines

$^{86}Rb^+$ efflux assays were conducted as described under *Materials and Methods* and in the legend to Fig. 1. Results were fit to the logistic equation to determine molar IC_{50} values and Hill coefficients (values given in parentheses) presented as ± S.E.M.

	SH-SY5Y $\alpha_3\beta_4$*	SH-EP1-h$\alpha_4\beta_{2\ \alpha4}\beta_2$	SH-EP1-h$\alpha_4\beta_{4\ \alpha4}\beta_4$	TE671/RD α_1*
		μM		
Bupropion	1.8 ± 1.1[a,b] (−1.24 ± 0.19)	12 ± 1.1[b] (−1.24 ± 0.18)	14 ± 1.1[b] (−1.19 ± 0.09)	7.9 ± 1.1[b] (−1.25 ± 0.14)
(2S,3S) OH-bupropion	10 ± 1.5 (−1.08 ± 0.35)	3.3 ± 1.1[a,c] (−1.31 ± 0.11)	30 ± 1.1[c] (−1.10 ± 0.12)	28 ± 1.4 (−1.25 ± 0.32)
(2R,3R)-OH-bupropion	6.5 ± 1.2[a,b] (−1.08 ± 0.18)	31 ± 1.1 (−1.53 ± 0.27)	41 ± 1.1[c] (−1.65 ± 0.19)	7.6 ± 1.1[a,b] (−1.23 ± 0.13)

[a] $P < 0.05$ relative to other subtypes for the indicated drug.
[b] $P < 0.05$ within subtype relative to (2*S*, 3*S*)-hydroxybupropion.
[c] $P < 0.05$ within subtype relative to bupropion.

ments 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 noncompetitive antagonist at various nAChR subtypes (Fryer and Lukas, 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.

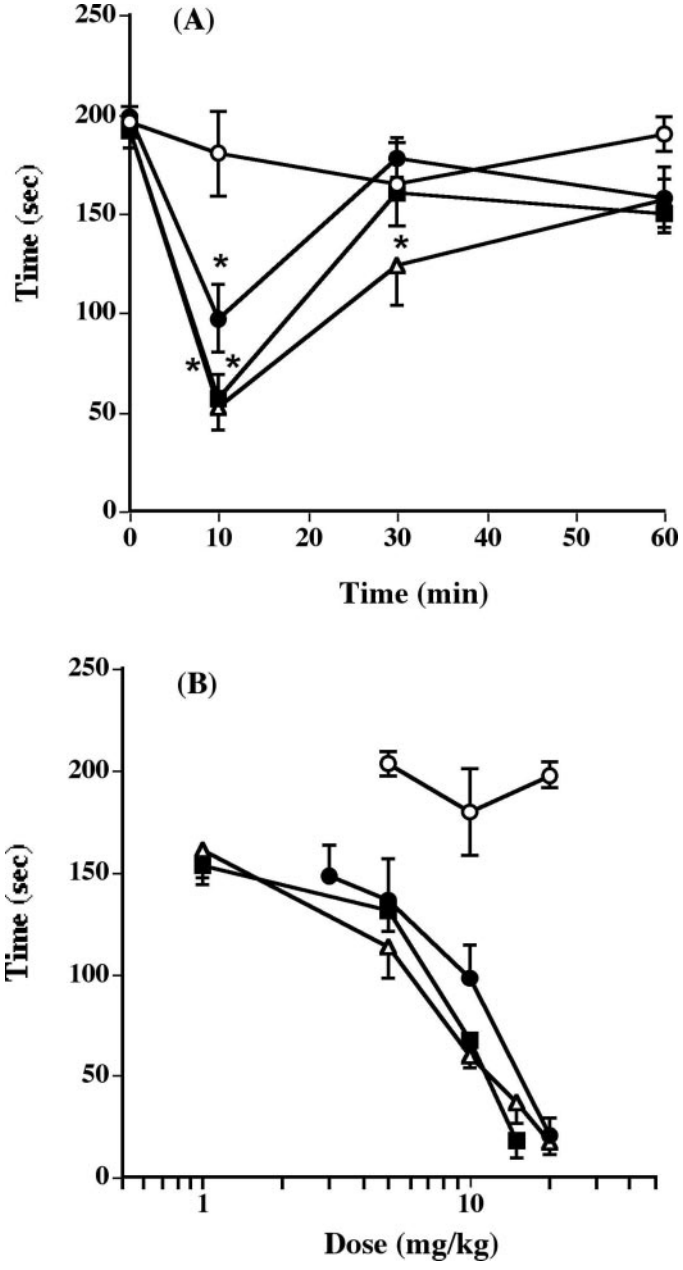

Fig. 2. Effects of bupropion (△), (2*RS*,3*RS*)-hydroxybupropion (●), (2*S*,3*R*)-hydroxybupropion (○), and (2*S*,3*S*)-hydroxybupropion (■) in the forced swimming test after subcutaneous injection in mice. A, time course of bupropion and its hydroxy metabolites effects on immobility time (in seconds) after subcutaneous administration of 10 mg/kg of each of the analogs in mice. B, dose-response curves of bupropion and its hydroxy metabolites in forced swimming test after subcutaneous administration in mice. Bupropion analogs at different doses were administered subcutaneously 10 min before the test. Each point represents the mean ± S.E. of 8 to 12 mice; ⋆, $p < 0.05$ compared with correspondent 0 time point (saline control).

The results of the current study indicate that bupropion and its hydroxy metabolites are noncompetitive antagonists of nAChR at concentrations similar to those inhibiting [^{3}H]NE and [^{3}H]DA uptake. The in vitro concentrations for bupropion action at $\alpha_3\beta_4$*-nAChR (IC_{50} = 1.8 μM) are comparable with those needed to inhibit DA (IC_{50} = 0.55 μM) or NE transporter (IC_{50} = 1.9 μM) function. In addition, the action of (2*S*,3*S*)-hydroxybupropion at $\alpha_4\beta_2$-nAChR (IC_{50} = 3.3 μM) occurs in the concentration range needed to inhibit DA (IC_{50} = 0.79 μM) or NE transporter (IC50 = 0.52 μM) function. These findings suggest that blockade of nAChR, in addition to blockade of monoamine transporter function, may be involved in the effectiveness of bupropion as a treatment for smoking cessation. Not only are the effects of the hydroxy metabolites enantioselective, (2*S*,3*S*)-hydroxybupropion displays the same or better activity than the parent compound at endpoints associated with blockade of nicotine-stimulated behaviors. Thus, the (2*S*,3*S*)-metabolite may play a critical role in the effectiveness of bupropion as a smoking-cessation pharmacotherapy. It would be interesting to determine whether levels of the (2*S*,3*S*) metabolite are relatively low in patients for whom bupropion is an ineffective treatment for smoking cessation. In contrast, the other enantiomer (2*S*,3*R*) exhibited greater functional antagonism at muscle-type nAChR, and no enantioselectivity was seen for nAChR containing β_4 subunits (e.g., $\alpha_4\beta_4$*- or $\alpha_3\beta_4$*-nAChR).

Our in vivo data support a role for the (2*S*,3*S*) isomer in the actions of bupropion, because this isomer was the most potent compound in blocking nicotine's behavioral effects. The in vitro data indicate that these effects are mediated by $\alpha_4\beta_2$-nAChR, because racemic bupropion is rapidly converted to its hydroxy metabolites, and the (2*S*,3*S*) hydroxy metabolite preferentially blocks the $\alpha_4\beta_2$-nAChR subtype. Although bupropion has higher affinity for $\alpha_3\beta_4$*-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 caused by autonomic nAChR blockade. Indeed tachycardia is one of the most frequent side effects of bupropion treatment. Nevertheless, any $\alpha_3\beta_4$* 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 (2*S*,3*S*)-hydroxybupropion would have a smaller peripheral nAChR-mediated side-effect profile than would bupropion, assuming that central nAChRs are the desired target.

The strong enantioselectivity for hydroxybupropion action at DA transporters, NE transporters, and $\alpha_4\beta_2$-nAChR is consistent with the enantioselectivity seen in vivo for actions in a mouse depression model (forced swimming test). However, the 10- to 20-fold higher potency of (2*S*,3*S*)-hydroxybu-

TABLE 3

Summary of the antagonistic potency of bupropion and bupropion hydroxy metabolites on different pharmacological actions of nicotine after subcutaneous administration in mice

Results are presented as AD_{50} values (±CL) calculated from the dose response. Each dose group included 8 to 12 animals.

Pharmacological Effect	Bupropion	(2*RS*,3*RS*)-Hydroxybupropion	(2*S*,3*S*)-Hydroxybupropion	(2*R*,3*R*)-Hydroxybupropion
			mg/kg	
Tail-flick	2.0 (1.8–3.6)	1.5 (0.6–3.8)	0.2 (0.1–0.3)[a]	2.5 (0.8–3.5)
Hot-plate	7.0 (5.9–9.5)	5.5 (3.6–16)	1.0 (0.2–5.2)	10.3 (8.0–12)
Hypomotility	3.0 (2.5–6.3)	8.2 (4–17.3)	0.9 (0.3–3.0)[a]	>20
Hypothermia	7.5 (3–15.4)	19.4 (10–35)	1.5 (0.8–3.0)[a]	>20

[a] $P < 0.05$ relative to (2*RS*,3*RS*)-hydroxybupropion.

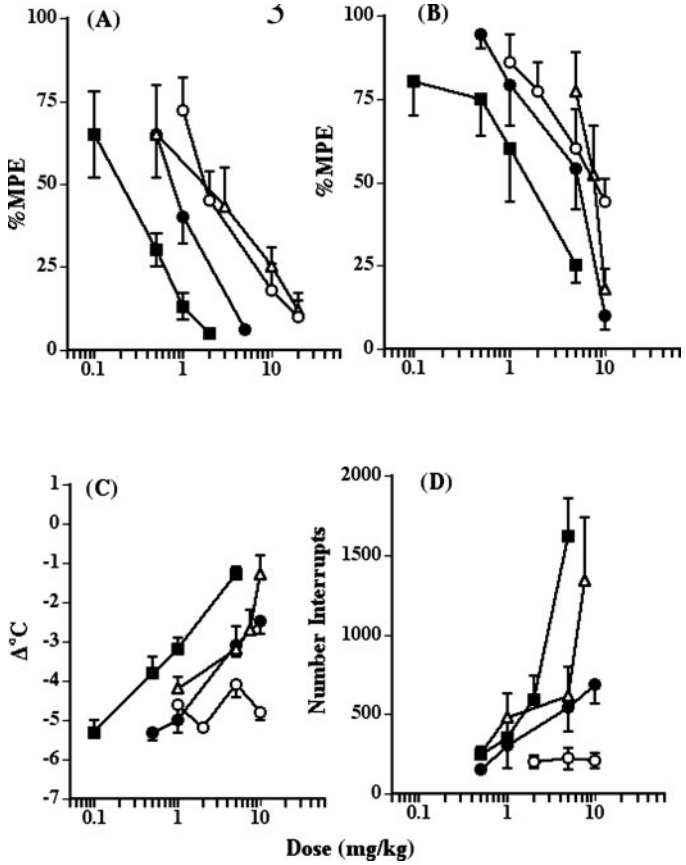

Fig. 3. Blockade of nicotine's behavioral effects by bupropion (△), (2*RS*,3*RS*)-hydroxybupropion (●), (2*S*,3*R*)-hydroxybupropion (○), and (2*S*,3*S*)-hydroxybupropion (■) after subcutaneous injection in mice. A, nicotine-induced antinociception in the tail-flick test (2.5 mg/kg s.c.). B, nicotine-induced antinociception in the hot-plate test (2.5 mg/kg s.c.). C, nicotine-induced hypothermia (2.5 mg/kg s.c.). D, nicotine-induced hypomotility (1.5 mg/kg s.c.). Bupropion and its hydroxymetabolites at different doses were administered subcutaneously 15 min before nicotine, and mice were tested 5 min later except for hypothermia (measured after 30 min). Each point represents the mean ± S.E. of 8 to 12 mice.

propion over racemic bupropion in blockade of nicotine's behavioral effects more closely parallels their potency differences for $\alpha_4\beta_2$-nAChR antagonism than in their actions at DA or NE transporters. The same can be said for comparisons between potency ratios for bupropion and (2*S*,3*R*)-hydroxybupropion. In contrast, the comparable potencies of racemic bupropion and (2*S*,3*S*)-hydroxybupropion in the forced swim test most closely match the effects of comparable potencies of these compounds in inhibition of DA uptake, suggesting dominance of dopaminergic mechanisms in behavioral tests using 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 hydroxy bupropion lacks potency at DAT relative to the activity of the (2*S*,3*S*) 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 NE transporter but not the DAT inhibition by hydroxybupropion and its isomers. Although no clear explanation can be proposed for this discrepancy, it is possible that the inactive isomer has a negative allosteric effect on binding to the DA transporter, which suggests a differential interaction of hydroxybupropion stereoisomers at DAT that could invoke an allosteric site/multimer models.

Our findings collectively support the hypothesis that bupropion's usefulness as both an antidepressant and an aid in the treatment of nicotine dependence reflects actions of bupropion and/or its hydroxy metabolites on a combination of targets including the DA transporter, the NE transporter, and members of the diverse family of nAChRs. These findings suggest that it may be desirable to synthesize compounds with multiple biological activities. Thus, a successful smoking-cessation pharmacotherapy would at least include activity at monoamine transporters and $\alpha_4\beta_2$-nAChR. That the (2*S*,3*S*) 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. Furthermore, our data suggest that the (2*S*,3*S*) 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 α_3*-nAChR.

Acknowledgments

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