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NO-SSRIs: Nitric Oxide Chimera Drugs Incorporating a Selective Serotonin Reuptake Inhibitor

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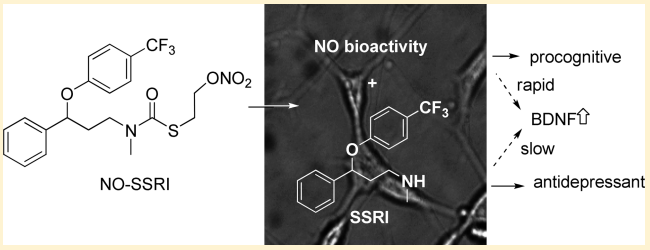
Supporting Information

ABSTRACT: Hybrid nitrate drugs have been reported to provide NO bioactivity to ameliorate side effects or to provide ancillary therapeutic activity. Hybrid nitrate selective serotonin reuptake inhibitors (NO-SSRIs) were prepared to improve the therapeutic profile of this drug class. A synthetic strategy for use of a thiocarbamate linker was developed, which in the case of NO-fluoxetine facilitated hydrolysis to fluoxetine at pH 7.4 within 7 h. In cell culture, NO-SSRIs were weak inhibitors of the serotonin transporter; however, in the forced swimming task (FST) in rats, NO-fluoxetine demonstrated classical antidepressant activity. Comparison of NO-fluoxetine, with fluoxetine, and an NO-chimera nitrate developed for Alzheimer's disease (GT-1061) were made in the step through passive avoidance (STPA) test of learning and memory in rats treated with scopolamine as an amnesic agent. Fluoxetine was inactive, whereas NO-fluoxetine and GT-1061 both restored long-term memory. GT-1061 also produced antidepressant behavior in FST. These data support the potential for NO-SSRIs to overcome the lag in onset of therapeutic action and provide cotherapy of neuropathologies concomitant with depression.



KEYWORDS: SSRI, nitric oxide, antidepressant, cognition, hybrid drug, nitrate

This paper is the first report on hybrid nitrate selective serotonin reuptake inhibitors (nitrooxy-SSRIs or NO-SSRIs; Figure 1). Akin to an approach taken for NO chimera hybrid drugs in Alzheimer's disease (AD)[1] and NO-donor NSAIDs for a variety of indications,[2,3] the NO bioactivity resulting from these drug hybrids is proposed to enhance and ameliorate the actions of the primary pharmacophore. (1) The clinical lag in onset of antidepressant action is a key source of noncompliance in SSRI pharmacotherapy, which is linked to the delay in elevation of brain-derived neurotrophic factor (BDNF).[4,5] NO is known to regulate BDNF production via positive feedback, since BDNF itself induces neuronal NO synthase (NOS) expression.[6,7] (2) Hybrid nitrates and nitrate NO-chimera drugs have been shown to reverse cognition deficits in animal models of dementia and AD,[1,8,9] and it has been suggested that therapeutic strategies for AD should target depression.[10,11] Several theories have been postulated for the comorbidity of depression and dementia, and patients with lifetime depression have a 50% higher risk of developing AD and more advanced AD pathophysiology.[12,13]

In pursuit of NO-SSRI hybrid drugs, two initial hurdles were presented as follows: (1) to develop a general prodrug strategy for amine SSRI drugs, since most hybrid nitrates reported rely on linker modification of hydroxyl and carboxylate groups in the drug itself, and (2) to ascertain whether the NO-SSRI retained the antidepressant activity of the primary pharmacophore. To determine the antidepressant activity of NO-fluoxetine, we used the modified forced swim test (FST), a model of "learned helplessness" that is used extensively as an antidepressant screen in industry.[14] Criticisms of the FST include the immediate induction of antidepressant activity in the FST by drugs that clinically show a therapeutic lag of several weeks. Despite the imperfections of the FST, antidepressant drugs almost universally decrease the "immobility" time of the animal in FST; therefore, a drug that is ineffective in the FST is unlikely to progress as a therapy for neurological disorders in which depression is comorbid. Because NOS inhibitors have been reported to decrease immobility in the FST,[15,16] it was essential to determine the influence on immobility of NO-SSRIs.

A NO chimera that incorporates an antidepressant pharmacophore, for example, fluoxetine (**2**) or paroxetine (**4**), represents a novel antidepressant therapeutic strategy that might be of general use in depression or of specialized use when cognition enhancement is required or beneficial. The appropriate commercially available SSRI drugs were modified at the secondary nitrogen position via formation of a thiocarbamate bond. Preliminary treatment of the parent SSRI molecules with phosgene followed by mercaptoethanol (Scheme 1, route A) gave a mixture of the desired thiocarbamate-containing compounds, either **2a** or **4a**, as well as approximately equimolar quantities of the

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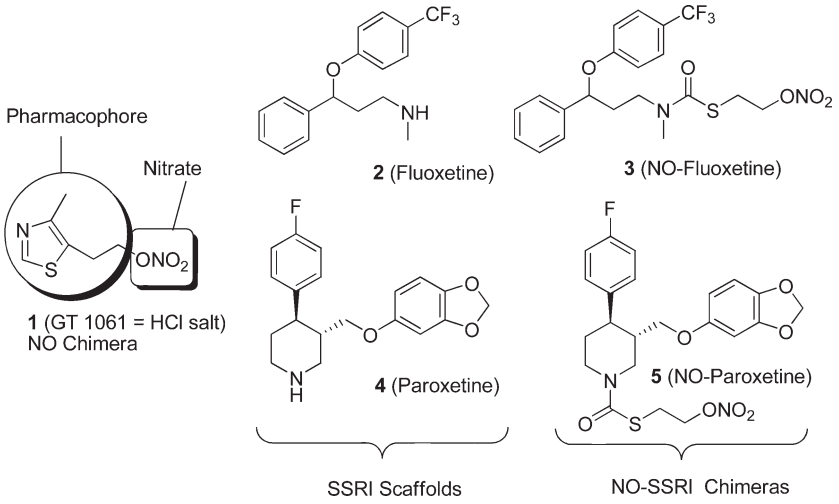
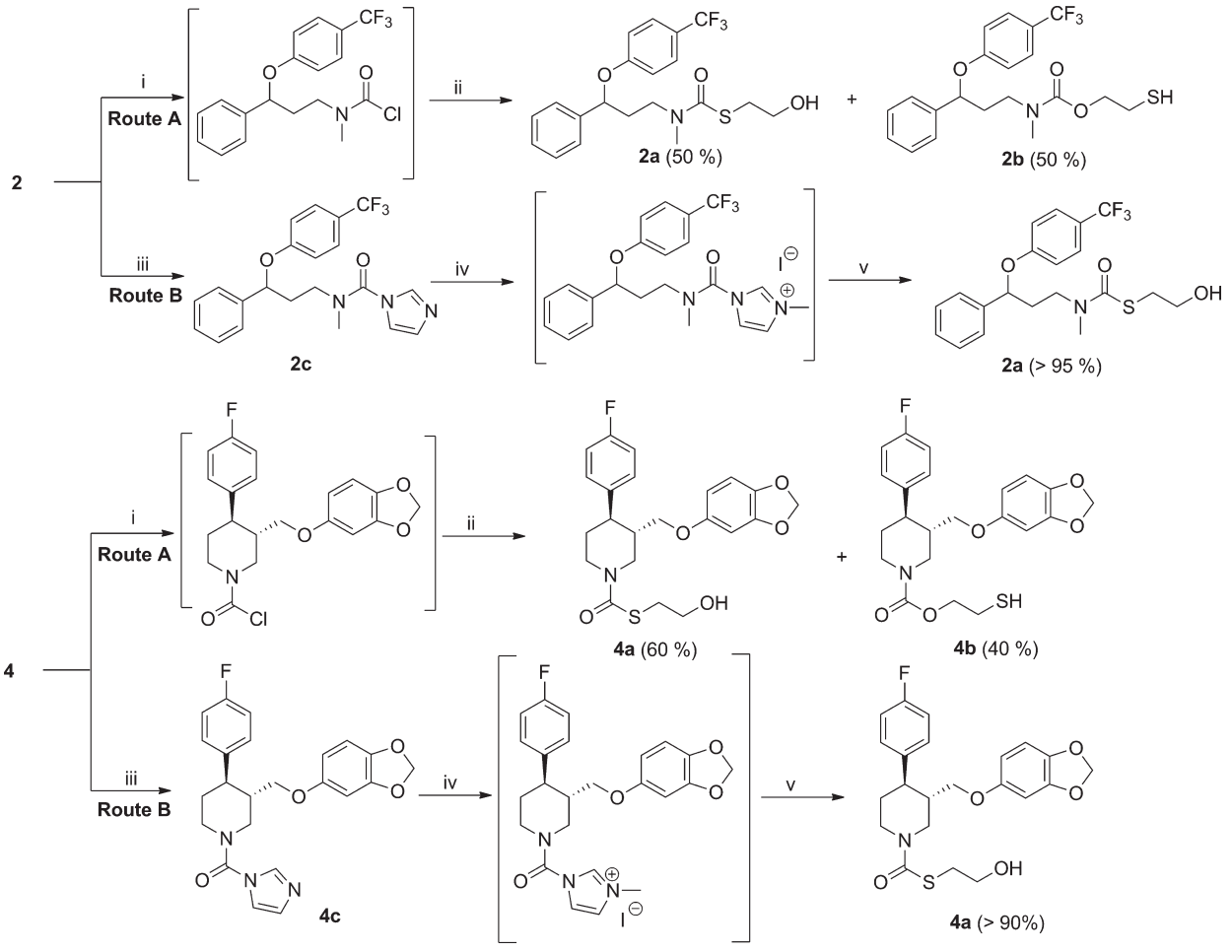


Figure 1. Hybrid nitrate NO chimeras and NO-SSRIs.

Scheme 1[a]



[a] Reagents and conditions. (i) Phosgene, TEA, CH_2Cl_2, 0 °C, 1 h. (ii) Mercaptoethanol, TEA, CH_2Cl_2, room temperature, 8 h. (iii) CDI, TEA, CH_2Cl_2, room temperature, 8 h. (iv) CH_3I, THF, room temperature, 12 h. (v) Mercaptoethanol, TEA, CH_2Cl_2, 0 °C, 8 h.

undesired carbamate derivatives, **2b** and **4b**, respectively. An alternative synthetic approach was taken entailing the treatment of the SSRI with CDI to give intermediates **2c** or **4c** (Scheme 1, route B), followed by methylation using iodomethane, and subsequent treatment with mercaptoethanol to preferentially yield the SSRI thiocarbamates **2a** and **4a**, respectively.

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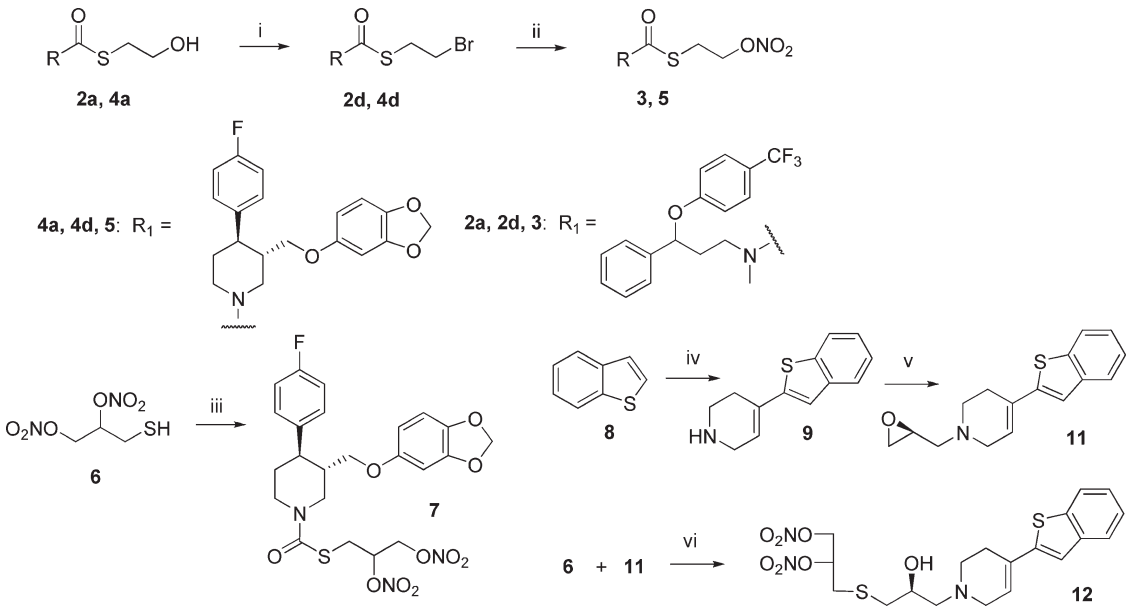
Scheme 2[a]



[a] Reagents and conditions. (i) PPh_3, CBr_4, $CHCl_3$, 0 °C. (ii) $AgNO_3$, MeCN, 80 °C. (iii) $C(O)Cl_2$, TEA, CH_2Cl_2, 0 °C, **4**. (iv) *t*-BuLi, *tert*-buty 4-oxopiperidine-1-carboxylate. (v) CF_3CO_2H. (vi) (*R*)-oxiran-2-ylmethyl 3- nitrobenzenesulfonate.

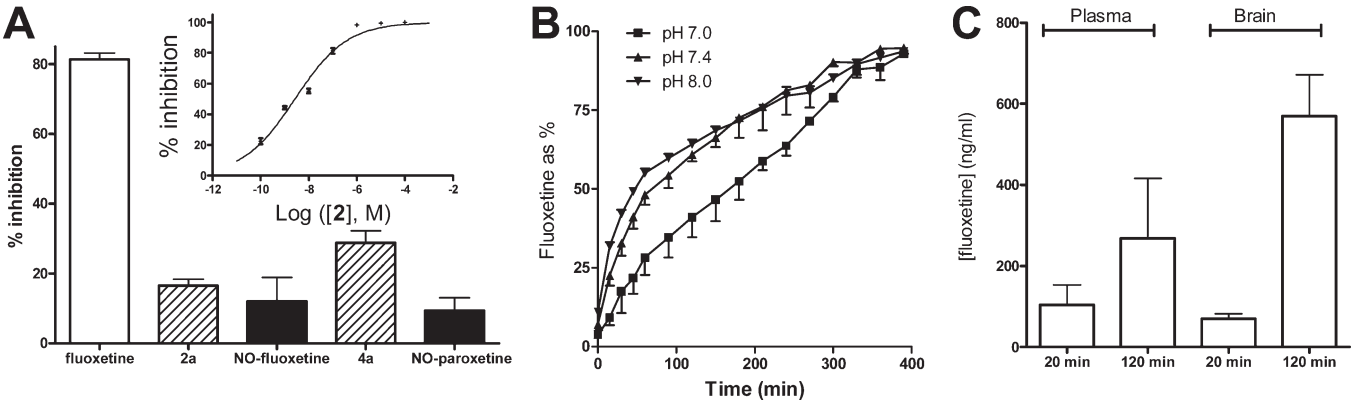


Figure 2. (A) Efficacy of NO-SSRIs and metabolites as compared to fluoxetine. HEK 293 cells stably transfected with the human serotonin transporter (SERT) were treated with fluoxetine (varied concentrations; inset figure) or test compounds (100 nM). SERT inhibition data were normalized to fluoxetine (100 μM) as 100% inhibition of reuptake and vehicle control as 0%. The data show means and SEMs from three separate experiments. (B) Hydrolysis of NO-fluoxetine to fluoxetine. Breakdown of NO-fluoxetine (200 μM) to fluoxetine measured by HPLC-UV in PBS (50 mM PBS; pH 7.0, 7.4, 8.0). The peak area of fluoxetine is expressed as a ratio against total product and substrate; data show means and SEMs. (C) Bioavailability of fluoxetine after administration of NO-fluoxetine. Quantitation of fluoxetine (**2**) by LC-MS/MS using MRM analysis of the m/z transition 310 → 44 after ip administration of NO-fluoxetine (**3**; 15 mg/kg ip) at two time points before sacrifice. Data (N = 2) show means and SDs.

Conversion of alcohols to nitrate was readily achieved in two steps by use of triphenylphosphine and carbon tetrabromide, followed by treatment with silver nitrate, yielding the mononitrate NO-SSRIs, **3** and **5** (Scheme 2). This procedure represents a general approach toward hybrid nitrate derivatives of CNS-active amines, using a thiocarbamate linker. The method was adapted for the synthesis of one example of a dinitrate NO-SSRI (**7**) using a nitrate-mercaptan synthon reported in 2001.[17] A further example of a dinitrate NO-SSRI (**12**) was prepared, making use of a pharmacophore explored by Eli Lilly in hybrid SSRI/$5HT_{1A}$ antagonist drugs designed to overcome antidepressant therapeutic lag.[18]

To determine the SSRI activity of NO-fluoxetine (**3**), NO-paroxetine (**5**), and their potential metabolic denitration products, **2a** and **4a**, inhibition of serotonin reuptake was measured in human HEK-293 cells stably transfected with the serotonin transporter (SERT). The affinity of fluoxetine itself for SERT in this assay was reflected by the EC_{50} = 2.7 nM (Figure 2A). At 100 nM, fluoxetine efficacy was 81%, whereas at this concentration, the NO-SSRIs tested showed <15% efficacy. Although the putative denitration metabolite, **4a**, appeared to show higher activity, the data demonstrate that the hybrid prodrugs and metabolites are weak inhibitors of SERT.

To determine the stability of the thiocarbamate linker selected for these hybrid nitrate prodrugs, the lability of NO-fluoxetine was assayed at three pH values spanning physiological pH (Figure 2B). LC-MS/MS was used to determine that fluoxetine

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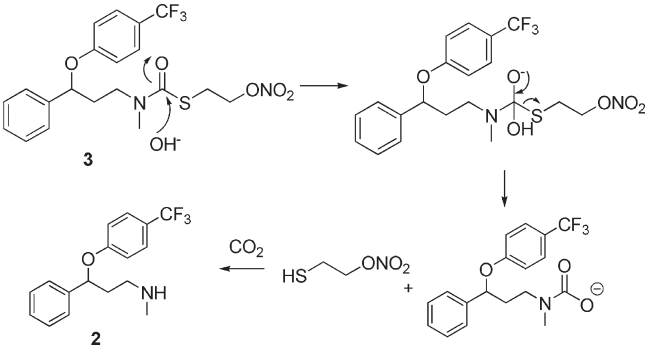
Scheme 3



was the sole hydrolysis product observed. The denitration product **2a** was not observed under these conditions, because denitration requires reductive conditions or alkaline pH. The fluoxetine concentration was determined using UV detection at multiple time points, showing that the decomposition of NO-fluoxetine reached completion at only 7 h, with a half-life at physiological pH of approximately 100 min (Figure 2B). The rate of hydrolysis increased with pH, compatible with a base-catalyzed mechanism of thiocarbamate cleavage and consequent decarboxylation (Scheme 3). Chemiluminescence detection of NO from the sealed headspace above a mixture of NO-fluoxetine (500 μM) in PBS-containing glutathione revealed production of low amounts of NO (8.9 $\pm$ 1.9 nM) after 30 min of reaction.

NO-SSRIs are designed as prodrugs; therefore, detection of drug at the site of action was seen as important. Administration to mice of **3** (15 mg/kg ip) 20 or 120 min prior to sacrifice, followed by quantitation of **2** by LC-MS/MS, demonstrated bioactivation of NO-fluoxetine to fluoxetine (**2**) in both plasma and brain at a favorable brain/plasma ratio (Figure 2C).

In the FST, the rat is acclimatized to a water-filled glass cylinder, in which the animal may display movements consistent with struggle, swimming, and immobility. Immobility is associated with the minimum movement required to maintain the animal at the surface of the water. Antidepressant drugs almost universally decrease the "immobility" time of the animal in FST. In addition, this animal model is reported to respond differently to serotonergic versus adrenergic and catecholaminergic drugs: Whereas all reduce immobility, serotonergic drugs do not increase struggle behavior.[14] NO-fluoxetine (**3**; 26.5 mg/kg equivalent to 20 mg/kg fluoxetine) was delivered in three ip injections 1, 5, and 24 h before the test phase of FST, a dose and delivery regime based upon literature reports for fluoxetine.[19] NO-fluoxetine was observed to reduce the immobility time significantly in the FST relative to the vehicle control and to increase swimming time significantly with no significant effect on struggle time (Figure 3A). These characteristics are compatible with antidepressant activity delivered by serotonergic drugs.

The positive antidepressant data obtained with NO-fluoxetine dispelled the hypothesis, based upon reports on NOS inhibitors,[15,20] that nitrate NO donors would de facto be pro-depressant in the FST. It was of further interest to test in FST the NO chimera, GT-1061, an experimental AD drug. An administration protocol was used identical to that for NO-fluoxetine, but dosing at 10 mg/kg (comparable to reported procognitive doses of this NO chimera[8,21]).

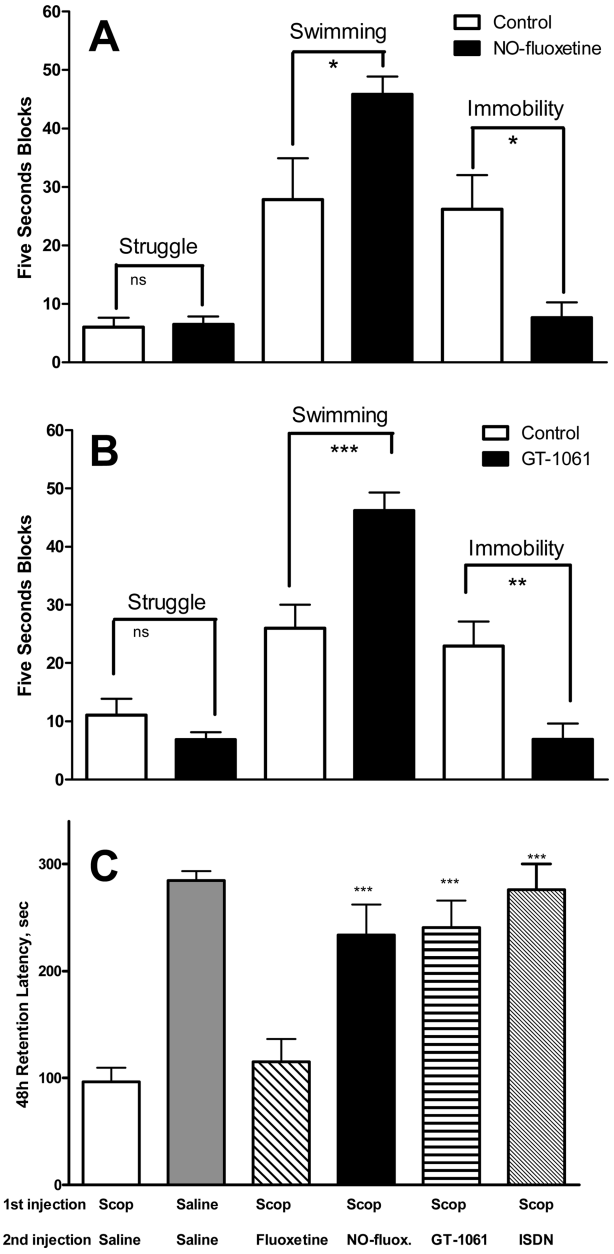


Figure 3. (A and B) Antidepressant activity of NO-fluoxetine and GT-1061. Effect of NO-fluoxetine (A) and GT-1061 (B) on the duration of struggle, swimming, and immobility in the FST. Rats received ip injections of drug or vehicle before testing. Data represent the number of 5 s blocks of each type of behavioral activity presented as means and SEMs ($N = 6$). (C) Pro-cognitive effects of NO-fluoxetine. The effect of NO-fluoxetine on long-term memory retention in the step through passive avoidance (STPA) task as compared to equimolar fluoxetine, GT-1061, and the clinical nitrate vasodilator, ISDN. Memory was tested 48 h after training. Data show latency to as means and SEMs ($N = 9-11$; for fluoxetine $N = 7$, since mice did not learn the task in training). The data were evaluated by ANOVA, followed by Tukey's posthoc test: ns, not significant; $p > 0.05$; $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$.

The nitrate GT-1061 was observed to display an "antidepressant profile" comparable to that of NO-fluoxetine (Figure 3B).

Given the comparable activity of NO-fluoxetine and GT-1061 in the FST and the demonstrated ability of GT-1061 to reverse cognition deficits in animal models using a variety of paradigms,

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the two drugs were compared in a simple animal model of learning and memory. The step-through passive avoidance (STPA) task was used to measure the reversal of a stimulus-response spatial memory deficit induced by the amnesic agent scopolamine that blocks muscarinic acetylcholine receptor activation. The STPA task tests learned aversion memory, relying on the natural preference of rats for dark areas over brightly lit and open areas. Following a period of habituation, the rat is trained. The animal is placed in the light compartment of the apparatus and inevitably crosses to the dark compartment of the apparatus at which time an electrical shock is delivered. The animals rapidly learn to avoid moving into the dark compartment. Retention of this newly learned behavior can be tested on subsequent days to assess the strength of the aversive memory, by measuring the latency to enter the dark area. NO-fluoxetine and GT-1061 were compared with fluoxetine and the clinical nitrate, isosorbide dinitrate (ISDN) administered at equimolar doses (NO-fluoxetine, 2.05 mg/kg; GT-1061, 1.0 mg/kg; and fluoxetine, 1.54 mg/kg). Long-term memory retention was measured 48 h after training in the absence of drugs (Figure 3C). No significant effect was apparent for fluoxetine relative to saline vehicle control. Interestingly, 5 of 12 animals receiving fluoxetine failed to learn the STPA task during the training phase, as compared to zero in the NO-fluoxetine treatment group. Regardless of cause, NO-fluoxetine significantly improved long-term memory, demonstrating that the hybrid nitrate prodrug possesses biological activity in vivo additional to that of the parent SSRI and in accord with observations on GT-1061.[8]

Current guidelines for pharmacotherapy of depression propose SSRIs as a first line therapy; however, the 2−6 week therapeutic lag before onset of behavioral improvement presents an impediment to compliance and therapy. The neurotrophin hypothesis posits a causative role for reduced activity of CREB (cAMP response element binding protein) and BDNF in pathogenesis of depression.[22,23] Downstream of neurotransmitter systems, neurotrophin-mediated long-term adaptation including enhancement of neurogenesis and neuronal plasticity is thought essential for clinical antidepressant efficacy and compatible with the observed antidepressant therapeutic lag.

Many drug classes would benefit from improvement of side effect profiles, which is a key incentive for prodrug and hybrid drug design. There are significant problems specifically associated with antidepressant therapy that might be overcome by new hybrid therapeutic agents and prodrugs that overcome the lag in onset of therapeutic action and provide cotherapy of at least one of the associated symptoms or neuropathologies concomitant with depression. Enhancement of CREB/BDNF signaling represents a therapeutic target for such hybrid and prodrug strategies.

Activation of CREB/BDNF can be elicited by enhancement of NO/cGMP signaling, and because organic nitrates are proven clinically, hybrid nitrates represent a reasonable therapeutic approach.[1,24] A simple, general synthesis of thiocarbamate-linked hybrid nitrates is described, yielding NO-SSRI prodrugs that release the parent SSRI and NO bioactivity on bioactivation. NO-fluoxetine retained the antidepressant activity of fluoxetine and the procognitive actions of the NO chimera nitrate, GT-1061, in animal behavioral models.

EXPERIMENTAL PROCEDURES

See the Supporting Information for specific reaction quantities, full product characterization, and detailed methodologies. Reaction of **3** (2 mM) in phosphate buffer at the appropriate pH was monitored by reverse phase HPLC using UV detection at 227 nm to monitor fluoxetine formation, which was quantified using peak area ratios with respect to the total UV absorbance. NO-fluoxetine prodrug was administered to mice 20 or 120 min prior to sacrifice and analysis by LC-MS/MS.

Human embryonal kidney cells (HEK-293) stably transfected with serotonin transporter (SERT) cDNA (a kind gift from Dr. Barker, Purdue University) were used to determine the serotonin reuptake inhibitory activity of test compounds.

The test was performed using male Sprague−Dawley rats weighing 200−250 g. Rats were trained to swim in the water tank 24 h prior to testing for 15 min. Drug was administered by ip injections 24, 5, and 1 h before the test at a dose of 26.5 mg/kg, which is equimolar to 20 mg/kg fluoxetine. The 5 min test was divided into 60 blocks of 5 s each. The major behavior in each 5 s block was assigned for the whole block by two observers blinded to the treatment: climbing, swimming, or immobility. The number of blocks representing each behavior was counted.

STPA was performed on male C57BL/6 mice of 8−10 weeks of age and weighing 22−27 g. The avoidance experiment was divided into three phases: habituation, training, and retention. The training phase, performed 2 h after habituation, consisted of individually placing animals in the illuminated compartment, with an electric shock (0.6 mA for 2 s) delivered immediately after the mouse entered the dark compartment, accompanied by the door closure. The time (latency) to enter the dark chamber was recorded for each mouse. Trials were repeated for a maximum of five trials or until the mouse remained in the light compartment for 300 s in one trial. The retention test was conducted 48 h after training, consisting of a single repeat of the training protocol without foot shock. The retention trial ended when the mouse entered the dark box or 300 s had elapsed. Scopolamine (1 mg/kg) dissolved in physiological saline was administered by ip injection 30 min before the training phase, and drugs dissolved in 25% DMSO were administered by ip injection 20 min before the training.

ASSOCIATED CONTENT

Supporting Information. Detailed experimental procedures, LC-MS/MS data, and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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