Novel p53 Inactivators with Neuroprotective Action: Syntheses and Pharmacological Evaluation of 2-Imino-2,3,4,5,6,7-hexahydrobenzothiazole and 2-Imino-2,3,4,5,6,7-hexahydrobenzoxazole Derivatives[⊥]

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Received January 28, 2002

Tumor suppressor protein, p53, is an intracellular protein that is critical within the biochemical cascade that leads to cell death via apoptosis. Recent studies identified the tetrahydrobenzothiazole analogue, pifithrin-α (**2**), as a p53 inhibitor that was effective in protecting neuronal cells against a variety of lethal insults and reducing the side effects of anticancer drugs. As up-regulation of p53 has been described as a common feature of several neurodegenerative disorders, including Alzheimer's disease, **2** and novel analogues (**3**−**16**) were synthesized to (i) assess the value of tetrahydrobenzothiazole analogues as neuroprotective agents and (ii) define the structural requirements for p53 inactivation. Not only did **2** exhibit neuroprotective activity in both tissue culture and in vivo stroke models but also compounds **6**, **7**, **10**, **13**, **15**, and **16** proved to be highly potent in protecting PC12 cells and compounds **3**, **4**, and **6** were highly potent in protecting primary hippocampal cells against death induced by the DNA-damaging agent, camptothecin.

Introduction

Because of improved preventative, diagnostic, and therapeutic measures for cardiovascular disease and a variety of cancers, the average age of the U. S. population continues to gradually rise. Regrettably, accompanying this increase in life span, there has been a progressive escalation in the number of individuals afflicted with age-related neurodegenerative disorders, which includes Alzheimer's disease (AD), Parkinson's disease (PD), and stroke. Whereas different brain areas are primarily affected in each of these diseases, specifically, hippocampal and cortical neurons in AD,[1] substantia nigral and midbrain dopaminergic neurons in PD,[2] and cortical and striatal neurons in stroke,[3] each disorder likely develops from activation of a common final cascade of biochemical and cellular events that eventually lead to neuronal dysfunction and death.[1] In this regard, different triggers, including oxidative damage to DNA, overactivation of glutamate receptors, and disruption of cellular calcium homeostasis, can initiate a cascade of intracellular events that proceed via p53 activation of a death program termed apoptosis.[1,4]

While considerable evidence implicates a central role for the transcription factor p53 in the neuronal deaths that occur in AD,[5] PD,[6] stroke,[7] traumatic brain injury,[8] and a variety of ischemic and excitotoxic insults,[9−12] no therapeutic agents have been specifically designed to target and inactivate p53 as an interventive strategy. Tissue culture studies have established strong correlations between p53 expression and neuronal death induced by DNA-damaging agents and glutamate,[7,8] and studies of p53 deficient mice have confirmed an essential role for p53 in neuronal apoptosis resulting from ischemic and excitotoxic insults.[9,10] The involvement of p53 in neurodegenerative disorders raises the possibility that chemical inhibitors of p53 activation might prove effective in suppressing the neurodegenerative process in such disorders.

Relating to this, we recently demonstrated that the antihelminthic (antiparasitic) compound, pifithrin-α (PFTα) **2** (Figure 1), proved effective in protecting cultured hippocampal neurons against death induced by the DNA-damaging topoisomerase I and II inhibitors, camptothecin and etoposide,[13,14] respectively.[11] These agents induce neuronal apoptosis via a mechanism that involves p53 induction and caspase activation,[12,13] which, parenthetically, were suppressed in the presence of **2**.[11] Compound **2**, additionally, protected neuronal cells against fatality induced by glutamate and the potentially toxic AD peptide, *β*-amyloid,[11] two insults that similarly induce p53-mediated neuronal death.[1,15] However, it was ineffective in preventing cell death caused by trophic factor withdrawal,[11] which is known to kill cells in a p53-independent manner.[16,17] With regard to *β*-amyloid, it would be interesting to examine the effect of **2** on the levels of its precursor protein and its C-terminal fragments, which form a transcriptionally active complex with Fe65.[18] These studies confirmed the activity of compound **2** as a p53 inhibitor[19] and provided a lead compound to optimize in the development of potentially useful neuroprotective agents for a variety of common debilitating neurological disorders. The current study describes a series of novel PFTα ana-

[⊥] Dedicated to Dr. Arnold Brossi, Scientist Emeritus, National Institutes of Health, Bethesda, MD, on the occasion of his 80th birthday.

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10.1021/jm020044d CCC: $22.00
Published on Web 10/03/2002

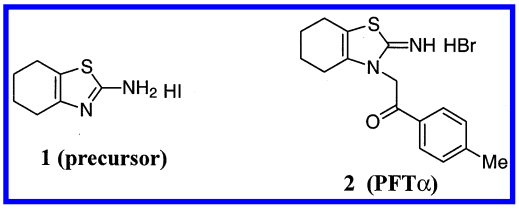


Figure 1. Chemical structures of **1** and of **2**.

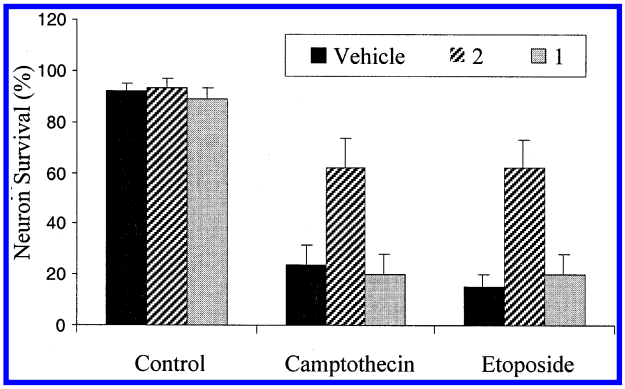


Figure 2. PFTα protects cultured hippocampal neurons against death induced by DNA-damaging agent. Hippocampal cell cultures were pretreated for 1 h with 0.5% DMSO (vehicle), 200 nM **2**, or 200 nM **2** precursor. Cultures were then exposed for 24 h to the DNA-damaging agents camptothecin (5 μM) and etoposide (2.5 μM). Neuron survival in each culture was quantified (n = 4−6 cultures + the standard deviation). Survival following administration of **2** was significantly different from that resulting from vehicle and precursor **1** (p < 0.01 ANOVA with Scheffe posthoc tests).

logues synthesized to both maximize and elucidate the p53 inactivation associated with **2**.

Results

Synthesis and Neuroprotective Activity for 2 and Its Precursor (1) in Tissue Culture. Synthesis of the precursor, 2-amino-4,5,6,7-tetrahydrobenzothiazole hydrogen iodide (**1**) was carried out following the method of King et al.[20] The crude product from the reaction of cyclohexanone with thiourea in the presence of iodine was dissolved in boiling water, extracted with ether, and made basic with solid $NaHCO_3$ to give pale yellow crystals as compound **1**. Compound **2** was synthesized from compound **1** according to a published procedure.[21] Both agents then were tested for their activity to protect rat hippocampal neurons and PC12 cells against the toxic agents, camptothecin and etoposide (Figures 2 and 3).

At concentrations of 500 nM and lower, compounds **1** and **2** were not toxic when administered alone to neurons but were toxic to neurons at higher concentrations. We therefore employed concentrations of 400 nM and lower in subsequent cell culture experiments. As shown in Figure 2, both camptothecin (5 μM) and etoposide (2.5 μM), known to induce neuronal apoptosis by a mechanism involving DNA damage and p53 induction, killed approximately 75 and 85% of neurons, respectively. Pretreatment of cells with **2**, but not **1** (200 μM), resulted in improved cell survival (60−65%) as compared to vehicle controls, demonstrating the potent neuroprotective activity of compound **2** in vitro. Likewise, and as illustrated in Figure 3 (initial two blocks) in a concentration-dependent manner, **2** but not **1** protected PC12 cells from camptothecin (40 μM)-induced cell death.

Design and Synthesis of Novel p53 Inactivators. The inactivity of precursor **1** indicates that the *N*-substituent group of compound **2** is important for its neuroprotective action. Modifications were therefore primarily made to this moiety to elucidate structure/activity relations for neuroprotective activity. In this regard, analogues were synthesized to assess the importance of the carbonyl and aryl groups, together with substitution of electron-donating and -withdrawing moieties on the latter (Scheme 1). In addition, the heterocycle was also modified to assess the influence of S to O atom replacement on neuroprotective activity (Scheme 2).

As shown in Scheme 1, compounds **3**−**5** and **7**−**14** were synthesized using cyclohexanone and thiourea as starting materials. 2-Amino-4,5,6,7-tetrahydrobenzothi-

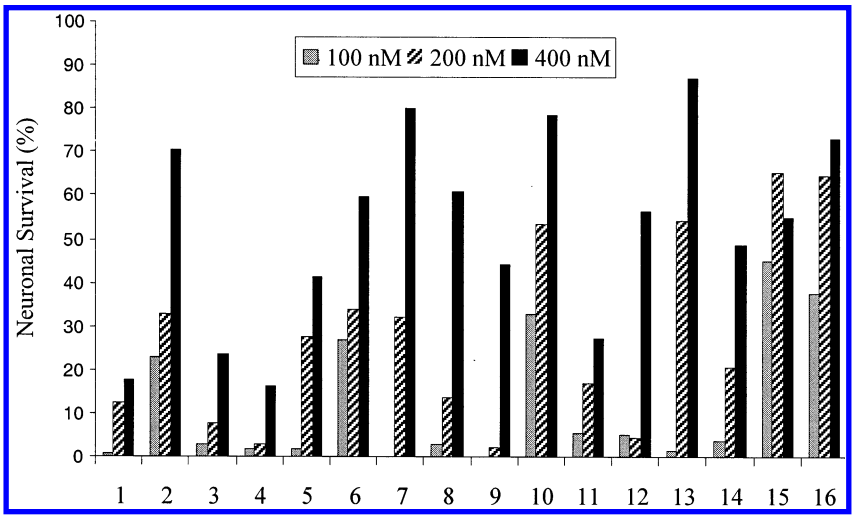
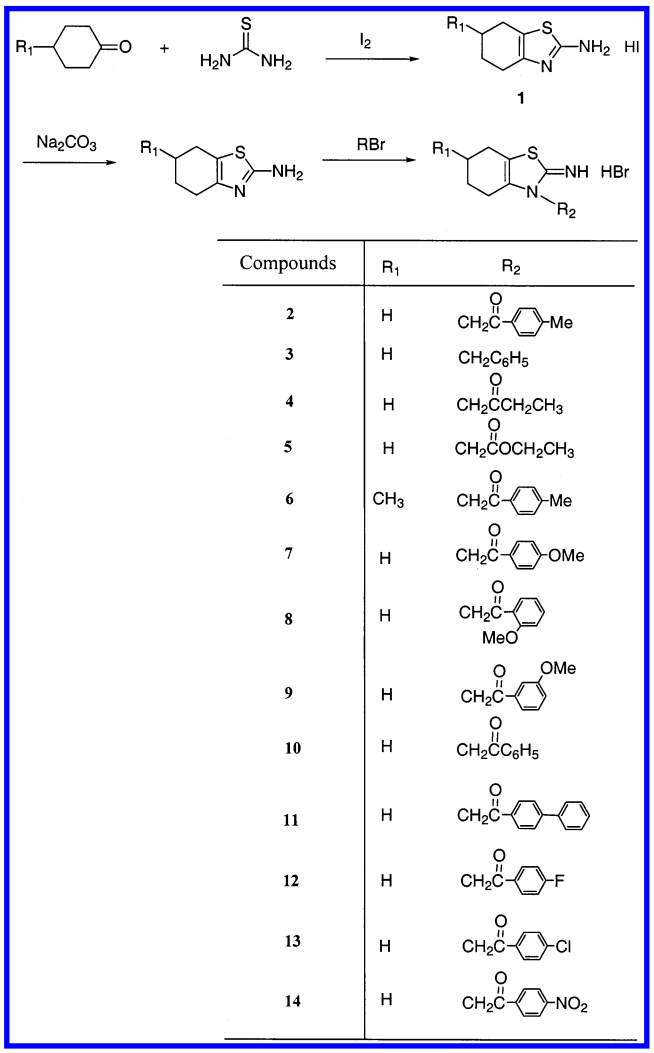


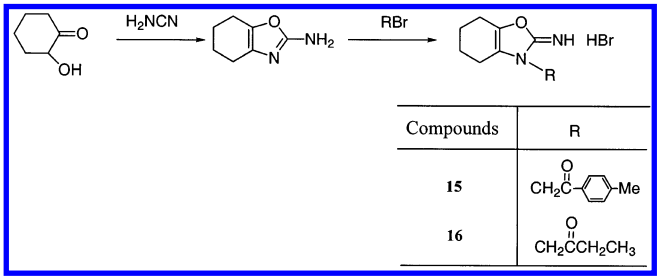
Figure 3. PFTα analogues protect cultured PC12 cells against death induced by the DNA-damaging agent camptothecin. PC12 cells were pretreated for 6 h with compounds **1**−**16** (prepared in 0.5% DMSO at concentrations between 100 and 400 nM) and were then exposed for 24 h to the DNA-damaging agents camptothecin (40 μM). Neuron survival in each culture was quantified (n = 3 cultures), and the mean is presented. In each case, the standard deviation was 5% or less and is not shown in the figure. For some compounds, studies were repeated at higher or lower concentrations to obtain an EC_{50} value. No toxicity was associated with the use of the PFTα analogues alone, as assessed by MTT and LDH assays (data not shown).

Scheme 1



Compounds	R_1	R_2
2	H	$CH_2C(=O)$-C$_6$H$_4$-Me
3	H	$CH_2C_6H_5$
4	H	$CH_2C(=O)CH_2CH_3$
5	H	$CH_2C(=O)OCH_2CH_3$
6	CH_3	$CH_2C(=O)$-C$_6$H$_4$-Me
7	H	$CH_2C(=O)$-C$_6$H$_4$-OMe
8	H	$CH_2C(=O)$-C$_6$H$_4$-OMe (MeO)
9	H	$CH_2C(=O)$-C$_6$H$_4$-OMe
10	H	$CH_2C(=O)C_6H_5$
11	H	$CH_2C(=O)$-C$_6$H$_5$-C$_6$H$_5$
12	H	$CH_2C(=O)$-C$_6$H$_4$-F
13	H	$CH_2C(=O)$-C$_6$H$_4$-Cl
14	H	$CH_2C(=O)$-C$_6$H$_4$-NO_2

Scheme 2



Compounds	R
15	$CH_2C(=O)$-C$_6$H$_4$-Me
16	$CH_2C(=O)CH_2CH_3$

azole, prepared from the neutralization of compound **1** with Na_2CO_3, was stirred with α-bromomethyl ketone in benzene at room temperature for 24 h to give the crude compounds **4**, **5**, and **7**−**14**, which were recrystallized from MeOH/EtOAc or EtOH/EtOAc to afford compounds **4**, **5**, and **7**−**14** as crystals. Furthermore, 2-amino-4,5,6,7-tetrahydrobenzothiazole was refluxed with benzyl bromide for 2 days, and the precipitate was collected by filtration and recrystallized from EtOH/Et_2O to obtain compound **3** as a white crystal. Compound **6** was synthesized from 4-methylcyclohaxanone and thiourea according to a published procedure.[21] Compounds **15** and **16** were prepared from 2-hydroxycyclohexanone dimer and cyanamide. Specifically, 2-hydroxycyclohexanone dimer was reacted with cyanamide in a 95 °C oil bath to give 2-amino-4,5,6,7-tetrahydrobenzoxazole according to the known procedure.[22] 2-Amino-4,5,6,7-tetrahydrobenzoxazole then was stirred with α-bromomethyl ketone at room temperature for 2 days, and the precipitate was collected by filtration and recrystallized from MeOH/EtOAC to give compounds **15** and **16** in yields of 51 and 57%, respectively.

Table 1. Concentration of Compounds Required to Induce 25% Protection (EC_{25}) and 50% Protection (EC_{50}) of PC12 Cells from Camptothecin-Induced Cell Death and Clog D Value[a]

compds	EC_{25} (nM)	EC_{50} (nM)	ClogD	compds	EC_{25} (nM)	EC_{50} (nM)	ClogD
1	855	8750		**9**	340	420	1.33
2	120	250	1.75	**10**	80	170	1.11
3	415	740	0.86	**11**	340	770	3.01
4	410	570	0.26	**12**	300	380	1.47
5	230	595	0.16	**13**	120	190	1.79
6	100	305	2.05	**14**	230	410	0.99
7	150	180	1.28	**15**	35	70	−0.30
8	250	350	1.26	**16**		145	−1.15

[a] EC_{50} and EC_{25} values were determined from logit plots of cellular survival vs compound concentration and were calculated from mean values ($n = 3$, standard error of the mean ≤5%) at three concentrations (in each, correlation coefficients $r^2 \geq 0.95$). The experimentally determined log D values of compounds **2** and **15** were 1.49 and 0.48.

Assays for Neuroprotective Activity in PC12 Cells. Compounds **3**−**16** were then assayed for neuroprotective activity in PC12 cells to which a lethal dose of camptothecin (40 μM) had been added. Neuronal survival, expressed as a percent of untreated cells (100% survival) and cells administered camptothecin alone (0% survival), is shown in Figure 3. The concentration of each analogue required to protect 25 and 50% of cells (determined as a EC_{25} and EC_{50} value, respectively) is shown in Table 1. Both values are included as in our prior studies involving constitutively expressed proteins; between 25 and 50% inactivation was required for clinical activity. With regard to Table 1, compounds **6**−**8**, **10**, and **13**−**15** proved to be highly potent.

Parenthetically, we initially investigated analogues with substituted phenyl groups to assess how such substituents affected activity. For example, analogue **10** has an unsubstituted phenyl moiety as its aryl group and is more potent (EC_{50} 170 nM) than the parent compound **2**, which additionally possesses a para-substituted methyl group (EC_{50} 250 nM). In contrast, **11**, which has a particularly bulky (biphenyl) aryl group, with a phenyl group in the para position of the phenyl ring (EC_{50} 770 nM), is less potent than **2**. Alternatively, for compounds with an aryl ring having an electron-donating substituent in the para position, such as compound **7** with a methoxy group (EC_{50} 180 nM), neuroprotective potency was elevated vs parent compound **2**. However, when the methoxy group was moved to the ortho position (**8**) or meta position (**9**), the resulting compounds possessed a reduced potency (EC_{50} 350 and 420 nM, respectively) vs **2**. Then again, for the compounds with an electron-withdrawing group on their aryl ring, analogue **13** proved to be more potent (EC_{50} 185 nM), whereas **12** and **14** were less potent (EC_{50} 380 and 410 nM, respectively) than **2**, indicating that a chloro group was favored and nitro and fluoro ones were detrimental to activity.

In the event that the aryl group was replaced with an aliphatic one, such as by an ethyl moiety (**4**) or an ethoxy (**5**), potency was decreased by some 2−3-fold

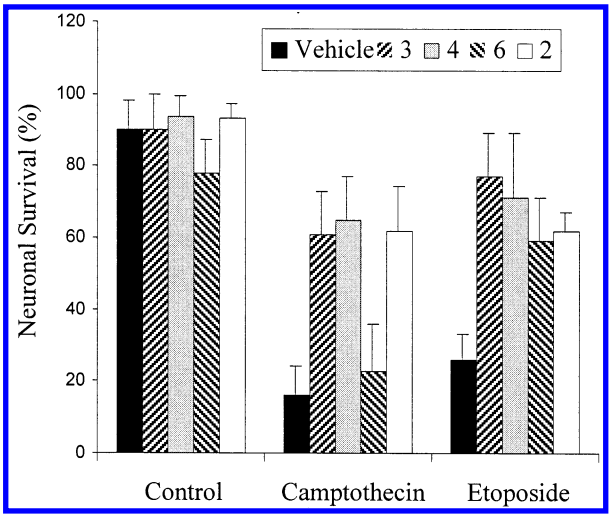


Figure 4. Protective effect of PFT-α derivatives against neuronal apoptosis induced by DNA damage. Compounds **2**−**4** and **6** (100 nM) were added 1 h before exposure of primary hippocampal neurons to the DNA-damaging compounds (a) camptothecin (5 μM) or (b) etoposide (2.5 μM). The percentage of neuronal survival 24 h after the treatment is given for each group as a mean value (± the standard deviation). The survivals associated with all agents were significantly greater than that associated with vehicle ($p < 0.05$ ANOVA with Scheffe posthoc tests). Cellular survival associated with exposure to compounds alone was no different from vehicle alone, indicating a lack of toxicity (shown in control column).

(EC_{50} 570 and 595 nM, respectively). Absence of the carbonyl moiety, as in **3**, resulted in a 4-fold loss of activity as compared to related analogue **10** (EC_{50} 740 vs 170 nM, respectively). Finally, modifications in the heterocyclic structure (Scheme 2) were undertaken to assess effects on potency. In this regard, **6**, a 6-methyl-substituted derivative of compound **2**, proved to be equipotent to the parent, **2**. In contrast, for compounds **15** and **16,** replacement of S by O resulted in a 3-fold greater potency than the related thiazole derivatives, **2** and **4**, respectively.

Assays for Neuroprotective Activity in Hippocampal Cells. Selected compounds then were assessed for their ability to protect hippocampal cells against camptothecin (5 μM) or etoposide (2.5 μM) alongside compounds **1** and **2**, which were used as external controls. As illustrated in Figure 4, none of the compounds were toxic at concentrations of 100 nM following administration to cells alone. In response to camptothecin challenge (inducing 84% cell death), neuronal survival for **2**−**4** and **6** was 62, 61, 65, and 29%, respectively, with **3** and **4** equipotent to parent, **2**. In response to etoposide challenge (inducing 74% cell death), all compounds possessed compelling neuroprotective action at the concentration assessed, with **3** and **4** proving to be more potent and **6** equipotent to **2** (78, 71, 60, and 60%, respectively).

As compounds **3** and **4** possessed neuroprotective action against camptothecin and etoposide, they were selected for further study against glutamate-induced cell death. In response to an 18% cell survival after glutamate (20μM) administration, **3** significantly elevated survival rate to 43% (Figure 5).

In Vivo Studies. To assess whether potency in the described tissue culture models translated into in vivo action, compound **2** was assessed for neuroprotective

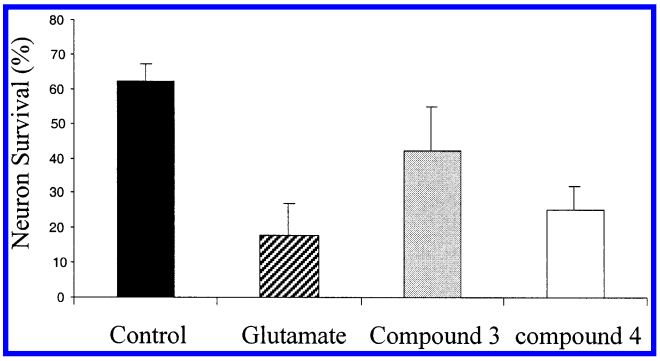


Figure 5. Primary hippocampal neurons were pretreated with compounds **3** and **4** (0.04 μM) 1 h before exposure to glutamate (20 μM) in Locke's medium, respectively. The percentage of neuronal survival 24 h after the onset of insult is given as a mean value (± the standard deviation). The survival associated with **3** was significantly greater than that of the vehicle ($p < 0.05$ ANOVA with Scheffe posthoc tests). Cellular survival associated with exposure to **3** and **4** alone was no different from untreated controls, indicating a lack of toxicity (data not shown).

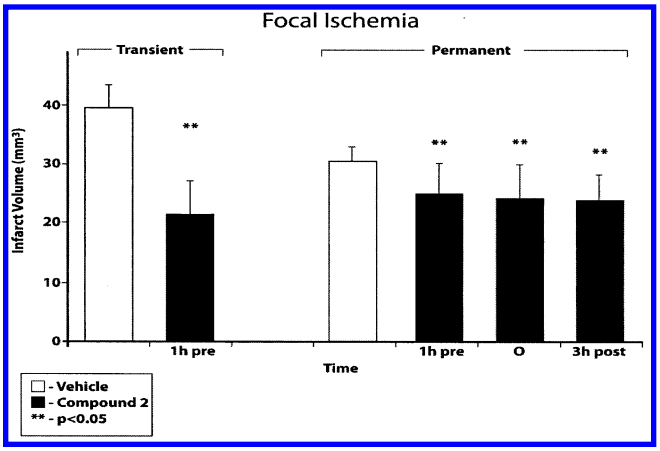


Figure 6. Protective effect of **2** in a model of transient and permanent focal cerebral ischemia in mice. Compound **2** (2 mg/kg) was administered intraperitoneally 1 h before transient and between 1 h before and 3 h after permanent middle cerebral artery occlusion in C57BL/6 mice. Twenty-four hours later, mice were euthanized and the infarct size was quantified after TTC staining of 2 mm brain sections. Values are the mean (± the standard deviation) of 12 animals per group. *$p < 0.05$ as compared to controls (Student's *t*-test).

activity in a rodent model of stroke (Figure 6). In models of transient and permanent focal ischemia, the infarct volume for animals pretreated with **2** systemically (2 mg/kg, ip) an hour prior to middle cerebral artery occlusion was significantly smaller ($p < 0.05$) than vehicle-treated controls by approximately 50 and 25%, respectively. When mice were treated with **2** either at the time of artery occlusion or 3 h after, infarct volume was similarly reduced ($p < 0.05$).

Conclusion

Several recent studies suggest that the DNA binding phosphoprotein, p53, plays multiple roles in cells, particularly with regard to cell cycle regulation.[1,23] Expression of high levels of endogenous wild-type (but not mutant) p53 has two primary outcomes, specifically, G1 cell cycle arrest or apoptosis.[1,23] The observation that DNA-damaging agents, such as etoposide and radiation, induce levels of p53 in cells led to the characterization

of p53 as a checkpoint factor. While dispensable for viability, in that p53 disrupted transgenic mice develop normally,[24] in response to genotoxic stress, p53 acts as an "emergency brake" causing either arrest of proliferation or apoptosis, ultimately to protect the genome from accumulating excess mutations. Consistent with this notion, cells that lack p53 are typically genetically variable and thus more prone to tumors.[24,25] Conversely, overexpression of p53 results in premature aging in mice.[26] Clearly, the value of p53-regulated apoptosis in protecting progenitor cells with proliferative potential (e.g., precursor B cells) from genetic instability must be counterbalanced by the disadvantage of the same machinery in end-differentiated cells (e.g., neurons) that lack proliferative ability and cannot be replaced.

The development of reversible inhibitors of p53 activity can fine-tune this balance and potentially restore it in clinical conditions, such as stroke, where cell death would be detrimental to survival. In this regard, our synthetic and pharmacological studies have thus far demonstrated that specific tetrahydrobenzothiazole analogues possess the ability to protect neuronal cells against toxic insults that induce cell death via p53-dependent apoptosis. Our understanding of this series of compounds leads to the identification of several highly potent analogues, some of which are more potent than the parent compound **2**.

Specifically, analogues **6**, **7**, **10**, **13**, **15**, and **16** proved to be highly effective in protecting PC12 cells. In this regard, the *N*-substituent group of the parent compound, **2**, is essential for biological action. Substitution on the distal aryl ring exerts a remarkable influence on activity, preferring *p*-chloro, *p*-methoxy, or a lack of substitution. Methyl substitution in position 6 (compound **6**) was well-tolerated and provided an agent that proved active in all assays. In addition, an oxazole vs a thiazole ring was preferred in the modification of the heterocyclic portion of the primary pharmacophore, as the tetrahydrobenzoxazole derivatives, **15** and **16**, proved to be remarkably more potent than their corresponding tetrahydrobenzothiazole derivatives (**2** and **4**). Interestingly, compounds **3** and **4**, which were not particularly potent neuroprotective agents in the PC12 cell assay, proved to be highly potent in protecting primary hippocampal cells from apoptosis induced by the same insult, camptothecin, as well as by etoposide. Of further interest and shown in Figure 4, compound **6**, although equipotent to parent **2** and more potent than **3** and **4** in protecting PC12 cells against camptothecin, proved less active than equimolar amounts of each in protecting primary hippocampal cells from the same insult. This suggests that different cell lines likely possess disparate vulnerabilities to apoptosis-inducing agents and express dissimilar levels of p53. Equally intriguing, in primary hippocampal cells, **6** proved to be more protective against apoptosis induced by etoposide, a topoisomerase II inhibitor, vs camptothecin, a topoisomerase I inhibitor. This suggests that within the same cells, the common biochemical pathway leading to cell death via p53 is differentially stimulated by different apoptotic insults with divergent pharmacological targets. Such caveats clearly would have to be considered and further examined when choosing specific compounds to take forward for potential clinical assessment.

Our current molecular studies are focused on elucidating the interaction of **2** and analogues with p53 and on the individual components within the biochemical cascade downstream of its activation. In this regard, X-ray crystallographic and NMR analyses have been undertaken on p53 to define its tertiary structure and elucidate potential ligand binding sites.[27−32] For biological function, p53 is active as a tetramer and must, therefore, self-associate to elicit interactions with other cellular proteins. Each p53 protein is comprised of a transactivating region, a DNA binding region, and an oligomerization region. Ligand binding or interaction to any of these would potentially result in a loss of action. For tetramerization to occur, individual p53 monomers form relatively stable dimers via specific amino acid interactions within their oligomerization domains;[27−29] thereafter, dimers associate entirely via hydrophobic interactions, which are particularly susceptible to disruption by small molecule compounds.[32] Parenthetically, our studies thus far have demonstrated that incubation of **2** with camptothecin-treated neuronal cells reduces their intracellular levels of p53, as assessed by Western blot utilizing an antigoat p53 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). In addition, nuclear extracts of cells subjected to gel-shift analysis demonstrated that **2** suppresses camptothecin-induced increases in the level of p53 DNA binding activity.[11] The precise mechanism through which these effects are achieved, either by direct interaction with p53 or via a binding protein,[33] remains to be elucidated. In this regard, structure/activity analysis, as described in the current study, may allow appropriate labeling of the pharmacophore to define whether, as well as how, **2** and analogues bind to p53 to modulate its function. Irrespective of this, downstream elements of p53 inactivation by **2** and analogues are clearly affected, with suppression in the levels of the death protein, Bax, as well as of caspase-3.[11,34]

From a pharmacokinetic perspective, compound **2** and analogues are sufficiently lipophilic to allow high brain penetration. In this regard, their computed log D values (octanol/water partition coefficient at physiological pH), which correlate with permeability at the blood−brain barrier in the absence of an inward or outward transporter,[35] are shown in Table 1 and cover the same approximate range as caffeine and haloperidol that readily enter brain.[36] These computed values favorably compare with experimentally determined values (Table 1 legend). Although the tetrahydrobenzoxazole derivatives, **15** and **16**, are more water soluble than their thiazole counterparts, they clearly are sufficiently permeable at the cell membrane to provide potent neuroprotective activity, and their brain uptake is the focus of current investigation. As substitution in the 6-position, as in **6**, was well-tolerated, it could provide a site to increase the lipophilicity of oxazole analogues to optimize their brain penetration. The high lipophilicity of **2** (ClogD 1.75, measured 1.49) is in accord with its in vivo activity, after systemic administration, to reduce stroke volume by 50 and 25% in a classical mouse model of transient and permanent focal cerebral ischemia, respectively. The magnitude of this reduction is consistent with that, ≈40%, found in p53 deficient mice, vs their normal littermates, in a focal ischemia stroke

model[12] and is in accord with p53 inactivation being the mechanism of action of compound **2** and analogues in vivo. The lower, but nevertheless significant, activity of **2** in the permanent vs transient focal ischemia model is in accord with the greater amount of necrosis- vs apoptosis-induced cell death associated with the former. Even so, the reduction in infarct volume determined at 3 h post permanent artery occlusion is suggestive of therapeutic value, and longer time intervals prior to treatment are the focus of current studies to define the window of treatment opportunity.

In summary, we describe herein a novel series of potent neuroprotective agents that may aid in providing a better understanding of the involvement in p53 in the molecular mechanisms underlying neuronal cell death associated with nervous system development, aging, injury, and disease. In addition, depending on the results of further work to characterize any adverse effects associated with either acute or chromic administration, the targeted inactivation of p53 function through the use of specific analogues of **2** may provide an innovative strategy to reduce or prevent neurodegeneration and neurological disabilities as well as to protect against the side effects associated with cancer therapy.[37]

Experimental Section

Chemistry. Melting points were determined with a Fisher-Johns apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR were recorded on a Bruker AC-300 spectrometer. Mass spectra and high-resolution mass spectra (HRMS) were recorded on VG 7070 mass spectrometer and Finnigan-1015D mass spectrometer. All exact mass measurements show an error of less than 5 ppm. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA.

2-Amino-4,5,6,7-tetrahydrobenzothiazole Hydrogen Iodide (1). A mixture of cyclohexanone (1.96 g, 0.02 mmol), thiourea (3.04 g, 0.04 mmol), and iodine (5.08 g, 0.02 mmol) was stirred in a 110 °C oil bath for 12 h. The reaction mixture then was cooled, dissolved in boiling water, and extracted with ether to remove ketone and iodine. The solution was neutralized with solid $NaHCO_3$, and pale yellow crystals thereafter precipitated and were collected by filter to give **1** (3.2 g, 57%); mp 185−187 °C. ^{1}H NMR (DMSO-d_6): δ 2.50−2.48 (m, 4 H), 1.78−1.75 (m, 4 H). ^{13}C NMR (DMSO-d_6): δ 168.6, 140.7, 116.3, 26.1, 24.1, 24.0, 23.6.

General Procedure I. Preparation of 2-Imino-2,3,4,5,6,7-hexahydrobenzothiazole Derivatives by Alkylation of 2-Amino-4,5,6,7-tetrahydrobenzothiazole with α-Bromomethyl Ketone. Compound **1** was dissolved in hot saturated aqueous Na_2CO_3, and after it was cooled, 2-amino-4,5,6,7-tetrahydrobenzothiazole was gained as white needle crystals (93%); mp 87−88 °C (lit.[20] 87−88 °C). A mixture of 2-amino-4,5,6,7-tetrahydrobenzothiazole (1 mmol) and α-bromomethyl ketone (1 mmol) in dry benzene (20 mL) was stirred at room temperature for 2 days. Thereafter, the precipitate was filtered off from the reaction mixture, washed with a small amount of benzene, and recrystallized from MeOH/EtOAc or EtOH/EtOAc.

Compounds **2**, **4**, **5**, and **7**−**14** were prepared according to general procedure I.

1-(4-Methylphenyl)-2-(4,5,6,7-tetrahydro-2-imino-3(2*H*)-benzothiazolyl)ethanone Hydrobromide (2). Yield (67%); mp 180 °C (EtOH/EtOAc) (lit.[21] 182 °C). ^{1}H NMR (DMSO-d_6): δ 8.85 (s, 1 H), 7.96 (d, J = 8.1 Hz, 2 H), 7.45 (d, J = 8.1 Hz, 2 H), 5.70 (s, 2 H), 2.55−2.30 (m, 7 H), 1.73 (m, 4 H).

3-(Phenylmethyl)-4,5,6,7-tetrahydro-2(3*H*)-benzothiazolimine Hydrobromide (3). A mixture of 2-amino-4,5,6,7-tetrahydrobenzothiazole (101 mg, 0.66 mmol) and benzyl bromide (115 mg, 0.67 mmol) in tetrahydrofuran (THF, 5 mL) was refluxed for 2 days. The precipitate was collected by filter, washed with a small amount of THF, and recrystallized from ethanol/ethyl ether to afford compound **3** (75 mg, 75%) as pale yellow crystals; mp 271 °C. ^{1}H NMR (DMSO-d_6): δ 9.63 (s, 1 H), 7.45−7.35 (m, 3 H), 7.15 (d, J = 7.1 Hz, 2 H), 5.29 (s, 2 H), 2.50−2.30 (m, 4 H), 1.76 (br, 4 H). HRMS *m*/*z* calcd for $C_{14}H_{17}N_2S$, 245.1112; found, 245.1104. Anal. ($C_{14}H_{17}BrN_2S$) C, H, N.

1-(4,5,6,7-Tetrahydro-2-imino-3(2*H*)-benzothiazolyl)-2-butanone Hydrobromide (4). Yield (50%); mp 124−125 °C (EtOH/EtOAc). ^{1}H NMR (DMSO-d_6): δ 9.45 (s, 1 H), 5.06 (s, 2 H), 2.64 (q, J = 7.2 Hz, 2 H), 2.41−2.52 (m, 2 H), 2.29 (br, 2H), 1.73 (br, 4H), 0.99 (t, J = 7.2 Hz, 3 H). HRMS *m*/*z* calcd for $C_{11}H_{17}N_2OS$, 225.1062; found, 225.1071. Anal. ($C_{11}H_{17}BrN_2OS$) C, H, N.

Ethyl 2-(4,5,6,7-Tetrahydro-2-imino-3(2*H*)-benzothiazolyl)acetate Hydrobromide (5). Yield (52%); mp 224 °C (MeOH/EtOAc). ^{1}H NMR (DMSO-d_6): δ 9.63 (s, 1H), 4.93 (s, 2H), 4.21 (q, J = 7.1 Hz, 2H), 2.38 (m, 2H), 1.73 (m, 4H), 1.24 (t, J = 7.1 Hz, 3H). Anal. ($C_{11}H_{17}BrN_2O_2S$) C, H, N.

1-(4-Methylphenyl)-2-(4,5,6,7-tetrahydro-2-imino-6-methyl-3(2*H*)-benzothiazolyl)ethanone Hydrobromide (6). Following a procedure similar to the one described in general procedure I, the product was obtained as white crystals in the yield of 54%; mp 256−257 °C (MeOH/EtOAc) (lit.[21] 278 °C). ^{1}H NMR (DMSO-d_6): δ 9.60 (s, 1 H), 7.95 (d, J = 8.2 Hz, 2 H), 7.45 (d, J = 8.2 Hz, 2 H), 5.70 (s, 2 H), 2.71−2.64 (m, 1 H), 2.51−2.31 (m, 5 H), 2.21−2.12 (m, 1 H), 1.83−1.79 (m, 2 H), 1.36 (br, 1 H), 1.02 (d, J = 6.5 Hz, 3 H). Anal. ($C_{17}H_{21}BrN_2OS$) C, H, N.

1-(4-Methoxylphenyl)-2-(4,5,6,7-tetrahydro-2-imino-3(2*H*)-benzothiazolyl)ethanone Hydrobromide (7). Yield (61%); mp 205 °C (MeOH/EtOAc). ^{1}H NMR (DMSO-d_6): δ 9.47 (s, 1 H), 8.03 (d, J = 8.9 Hz, 2 H), 7.16 (d, J = 8.9 Hz, 2 H), 5.67 (s, 2 H), 3.89 (s, 3 H), 2.55 (br, 2 H), 2.32 (br, 2 H), 1.73 (br, 4 H). HRMS *m*/*z* calcd for $C_{16}H_{19}N_2O_2S$, 303.1167; found, 303.1158. Anal. ($C_{16}H_{19}BrN_2O_2S$) C, H, N.

1-(2-Methoxylphenyl)-2-(4,5,6,7-tetrahydro-2-imino-3(2*H*)-benzothiazolyl)ethanone Hydrobromide (8). Yield (17%); mp 160−161 °C (MeOH/EtOAc). ^{1}H NMR (DMSO-d_6): δ 7.70−7.12 (m, 4 H), 5.42 (s, 2 H), 3.99 (s, 3 H), 2.50−2.39 (m, 4 H), 1.81 (br, 4 H). HRMS *m*/*z* calcd for $C_{16}H_{19}N_2O_2S$, 303.1167; found, 303.1173. Anal. ($C_{16}H_{19}BrN_2O_2S$) C, H, N.

1-(3-Methoxylphenyl)-2-(4,5,6,7-tetrahydro-2-imino-3(2*H*)-benzothiazolyl)ethanone Hydrobromide (9). Yield (52%); mp 143−145 °C. ^{1}H NMR (DMSO-d_6): δ 9.50 (s, 1 H), 7.67−7.37 (m, 4 H), 5.74 (s, 2 H), 3.86 (s, 3 H), 2.55−2.33 (m, 4H), 1.73 (br, 4 H). HRMS *m*/*z* calcd for $C_{16}H_{19}N_2O_2S$, 303.1167; found, 303.1171. Anal. ($C_{16}H_{19}BrN_2O_2S$) C, H, N.

1-Phenyl-2-(4,5,6,7-tetrahydro-2-imino-3(2*H*)-benzothiazolyl)ethanone Hydrobromide (10).[38] Yield (58%); mp 151−152 °C (MeOH/EtOAc). ^{1}H NMR (DMSO-d_6): δ 9.53 (s, 1 H), 8.07−8.04 (m, 2 H), 7.79−7.74 (m, 1 H), 7.66−7.62 (m, 2 H), 5.75 (s, 2 H), 2.55−2.33 (m, 4H), 1.72 (br, 4 H). HRMS *m*/*z* calcd for $C_{15}H_{17}N_2OS$, 273.1162; found, 273.1058. Anal. ($C_{15}H_{17}BrN_2OS$) C, H, N.

1-(4-Phenylphenyl)-2-(4,5,6,7-tetrahydro-2-imino-3(2*H*)-benzothiazolyl)ethanone Hydrobromide (11). Yield (58%); mp 188−189 °C (MeOH/EtOAc) (lit.[21] 195 °C). ^{1}H NMR (DMSO-d_6): δ 9.55 (s, 1 H), 8.15(d, J = 8.5 Hz, 2 H), 7.81 (d, J = 7.0 Hz, 2 H), 7.57−7.44 (m, 4 H), 5.80 (s, 2 H), 2.56−2.36 (m, 4 H), 1.74 (br, 4 H).

1-(4-Fluorophenyl)-2-(4,5,6,7-tetrahydro-2-imino-3(2*H*)-benzothiazolyl)ethanone Hydrobromide (12). Yield (44%); mp 255 °C (MeOH/EtOAc). ^{1}H NMR (DMSO-d_6): δ 9.51 (s, 1 H), 8.14 (d, J = 5.5 Hz and J = 8.7 Hz, 2 H), 7.49 (t, J = 8.7 Hz, 2 H), 5.74 (s, 2 H), 2.55−2.34 (m, 4 H), 1.73 (m, 4 H). HRMS *m*/*z* calcd for $C_{15}H_{16}FN_2OS$, 291.0967; found, 291.0964. Anal. ($C_{15}H_{16}BrFN_2OS$) C, H, N.

1-(4-Chlorophenyl)-2-(4,5,6,7-tetrahydro-2-imino-3(2*H*)-benzothiazolyl)ethanone Hydrobromide (13). Yield (70%); mp 248−249 °C (MeOH/EtOAc) (lit.[21] 140 °C). ^{1}H NMR

(DMSO-d_6): δ 9.52 (s, 1 H), 8.06 (d, J = 8.6 Hz, 2 H), 7.73 (d, J = 8.6 Hz, 2 H), 5.74 (s, 2 H), 2.55−2.33 (m, 4 H), 1.72 (br, 4 H).

1-(4-Nitrophenyl)-2-(4,5,6,7-tetrahydro-2-imino-3(2*H*)-benzothiazolyl)ethanone Hydrobromide (14). Yield (61%); mp 239−240 °C (MeOH/EtOAc). ^{1}H NMR (DMSO-d_6): δ 9.54 (s, 1 H), 8.46 (d, J = 8.9 Hz, 2 H), 8.28 (d, J = 8.9 Hz, 2 H), 5.80 (s, 2 H), 2.56−2.37 (m, 4 H), 1.74 (br, 4 H). HRMS m/z calcd for $C_{15}H_{16}N_3O_3S$, 318.0912; found, 318.0903. Anal. ($C_{15}H_{16}BrN_3O_3S\cdot0.5H_2O$) H, N; C: calcd, 44.23; found, 44.67.

General Procedure II. Preparation of 2-Imino-2,3,4,5,6,7-hexahydrobenzoxazole Derivatives.[39] 2-Amino-4,5,6,7-tetrahydrobenzoxazole (1 mmol) prepared from 2-hydroxycyclohexanone and cyanamide was stirred with α-bromomethyl ketone (1 mmol) in dry benzene (20 mL) at room temperature for 2 days. Thereafter, the precipitate was filtered off from the reaction mixture and recrystallized from MeOH/EtOAc.

Compounds **15** and **16** were prepared according to general procedure II.

1-(4-Methylphenyl)-2-(4,5,6,7-tetrahydro-2-imino-3(2*H*)-benzoxazolyl)ethanone Hydrobromide (15). Yield (51%); mp 237 °C (MeOH/EtOAc). ^{1}H NMR (DMSO-d_6): δ 9.64 (br, 1H), 7.95 (d, J = 8.0 Hz, 2H), 7.46 (d, J = 8.9 Hz, 2H), 5.78 (s, 2H), 2.52 (br, 2H), 2.44 (s, 3H), 2.33 (m, 2H), 1.77 (m, 4H). MS (DEI) m/z 270 $[M]^+$. HRMS (DEI) m/z calcd for $C_{16}H_{18}N_2O_2$, 270.1368; found, 270.1366. Anal. ($C_{16}H_{19}BrN_2O_2$) C, H, N.

1-(4,5,6,7-Tetrahydro-2-imino-3(2*H*)-benzoxazolyl)-2-butanone Hydrobromide (16). Yield (57%); mp 206 °C (MeOH/EtOAc). ^{1}H NMR (DMSO-d_6): δ 9.56 (s, 1H), 4.95 (s, 2H), 2.59 (q, J = 7.2 Hz, 2H), 2.27 (m, 2H), 1.78−1.69 (m, 4H), 0.99 (t, J = 7.2 Hz, 3H). HRMS (DEI) m/z calcd for $C_{11}H_{16}N_2O_2$, 208.1212; found, 208.1205. Anal. ($C_{11}H_{17}BrN_2O_2$) C, H, N.

Biological Activity. PC12 Cell Apoptosis Protection. PC12 cells were cultured in RPMI1640 media containing 10% horse serum and 5% fetal calf serum (37 °C, pH 7.4) until 70−80% confluent. Thereafter, 2.2×10^4 cells per mL was added to each trough of a 96 well plate (approximately 6270 cells per well 285 μL) and were left to grow for 12 h. The media then was removed and was replaced by media with vehicle or with one of compounds **1**−**16** (concentration 100−400 nM, prepared in dimethyl sulfoxide (DMSO) to a final dilution of 0.4%, eight wells per concentration). Following 6 h of incubation, camptothecin (40 μM) was added to four of each eight wells, and the cells were incubated for a further 24 h. Cells then were gently washed twice with phosphate-buffered saline (PBS: 0.1 M, 37 °C, pH 7.4) and were incubated in PBS containing the live-cell indicator dye, 2′,7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein AM ester (BCECF AM: 5 μM) (Molecular Probes, CA) for 45 min in minimal light. This fluorescent dye was taken up and retained by live cells. Finally, the cells were washed twice in PBS and their fluorescence then was quantified (excitation, 490 nm; emission, 535 nm; Perkin-Elmer HTS 7000).

One hundred percent cell survival was determined from cells incubated in the absence of both any compound and camptothecin. One hundred percent cellular death was determined from cells incubated in the absence of any compound and presence of camptothecin, which in prior studies was found to occur at a minimum concentration of 40 μM. Compound-induced toxicity was assessed by comparing cells treated with compound at various concentrations in the absence of camptothecin with cells incubated in the absence of both compound and camptothecin. No compound was found to induce PC12 cell toxicity at the maximal concentration assessed (400 nM). Compound-induced cellular protection was assessed by comparing cells incubated with camptothecin with cells incubated with camptothecin in the presence of compound, in a concentration-dependent manner. Finally, the natural fluorescence of each compound was quantified in the absence of cells and BCECF AM and was found to be negligible. A 50% effective concentration for neuroprotection (EC_{50}), commensurate with an IC_{50} (concentration required to inhibit 50% of p53 activity), was calculated by transforming the data into a logit format (logit = ln(% protection/100% − % protection)) and calculating the value from a correlation between a plot of the log concentration of compound vs logit activity. A lack of cellular toxicity was confirmed in separate studies in which PC12 cells (2.0×10^6 per 60 mm dish) were incubated with a selected group of analogues for 24 h and subjected to a sensitive lactate dehydrogenase (LDH) assay. The LDH results were consistent with the MTT assay that was used to determine cell viability.[40]

Hippocampal Cultures, Experimental Treatment, and Quantification of Cell Survival. Dissociated hippocampal cell cultures were established from embryonic day 18 Sprague−Dawley rats (Harlan, Inc.), as described previously.[11] Cells were grown in polyethyleneimine-coated plastic dishes or 22 mm^2 glass coverslips and incubated in Neurobasal medium containing B-27 supplements, 2 mM L-glutamine, 25 mg/mL gentamycin, 1 mM Hepes (Gibco BRL), and 0.001% gentamicin sulfate. All experiments were performed using 9−10 day old cultures. Camptothecin and etoposide (Sigma) were prepared as 500× stocks in DMSO. Glutamate was freshly prepared as a 200× stock in saline (pH 7.2). Neuron survival was quantified by established methods.[41] Briefly, viable neurons in premarked fields (10× objective) were counted before experimental treatment and at specified time points thereafter. Neurons with intact neurites of uniform diameter and soma with a smooth round appearance were considered viable. In contrast, neurons with fragmented neurites and vacuolated soma were considered nonviable.

Focal Cerebral Ischemia. Surgery was performed in anesthetized (xylazine 5 mg/kg, chloral hydrate 350 mg/kg) mice (3 month old C57Bl/6) in accord with a NIH Animal Care and Use Committee-approved protocol. The focal ischemia/reperfusion model has been described previously.[42] Briefly, the method involves occluding the middle cerebral artery for 1 h with a nylon thread and then removing it to allow reperfusion. In the permanent focal ischemia model, the same procedure was followed without allowing reperfusion. Compound **2** (2 mg/kg ip) and vehicle (saline controls) were administered either prior to (1 h), during, or after (3 h) the induction of ischemia. During the procedure, mice were maintained at 37 °C and blood gases, flow, and pressure were monitored. At 24 h after ischemia, mice were anesthetized and decapitated, and their brains were cut into 2 mm coronal sections, which then were stained with triphenyltetrazolium for 30 min and 37 °C. Images of the stained brains were captured by digital camera for quantitative analysis of infarct area and volume.

Octanol−Water Partition Coefficient. Five milliliters of 0.2 mM octanol solutions of compound **2** was prepared, and their UV absorbencies, A_1, were determined by spectrophotometer at 254 nm wavelength. The octanol solutions then were vigorously mixed with an equal volume of water for 15 min. Following separation, the absorbency of the octanol was again measured, A_2. An octanol−water partition coefficient, P, was calculated from the formula: $P = A_2/A_1 - A_2$.

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JM020044D