Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

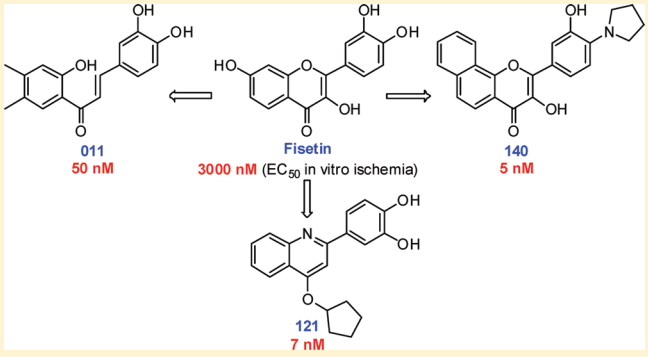
Chemical Modification of the Multitarget Neuroprotective Compound Fisetin

Chandramouli Chiruta, David Schubert, Richard Dargusch, and Pamela Maher*

The Salk Institute for Biological Studies, 10010 N. Torrey Pines Road, La Jolla, California 92037, United States

Supporting Information

ABSTRACT: Many factors are implicated in age-related central nervous system (CNS) disorders, making it unlikely that modulating only a single factor will provide effective treatment. Perhaps a better approach is to identify small molecules that have multiple biological activities relevant to the maintenance of brain function. Recently, we identified an orally active, neuroprotective, and cognition-enhancing molecule, the flavonoid fisetin, that is effective in several animal models of CNS disorders. Fisetin has direct antioxidant activity and can also increase the intracellular levels of glutathione (GSH), the major endogenous antioxidant. In addition, fisetin has both neurotrophic and anti-inflammatory activity. However, its relatively high EC_{50} in cell based assays, low lipophilicity, high topological polar surface area (tPSA), and poor bioavailability suggest that there is room for medicinal chemical improvement. Here we describe a multitiered approach to screening that has allowed us to identify fisetin derivatives with significantly enhanced activity in an in vitro neuroprotection model while at the same time maintaining other key activities.



INTRODUCTION

There are currently no drugs available that prevent the nerve cell death associated with the majority of age-related disorders of the CNS. A prime example of this problem is ischemic stroke, which is the leading cause of adult disability and the third leading cause of death in the U.S.[1] Worldwide, approximately 5 million people die each year of stroke, and the mortality rates are estimated to double by the year 2020.[2] The nerve cell death associated with cerebral ischemia is due to multiple factors resulting from the lack of oxygen to support respiration and ATP synthesis, acidosis due to the buildup of the glycolytic product lactic acid, the loss of neurotrophic support, multiple metabolic stresses, and inflammation.[3a,b] While the focus of current drug discovery paradigms is primarily on the development of high affinity, single target ligands, a drug directed against a single molecular target may not be effective in treating the nerve cell death associated with conditions such as stroke because of the multitude of insults that contribute to the cell's demise. This conclusion is supported by the lack of drugs for the treatment of stroke. Indeed, the only FDA-approved treatment to date is recombinant tissue-type plasminogen activator (rt-PA),[4] which is a vascular agent. An alternative approach is to identify small molecules that have multiple biological activities relevant to the maintenance of neurological function.

Over the past few years, we have identified an orally active, neuroprotective, and cognition-enhancing molecule, the flavonol fisetin.[5] Fisetin not only has direct antioxidant activity but can also increase the intracellular levels of glutathione (GSH), the major intracellular antioxidant, via the activation of transcription factors such as Nrf2.[5] Fisetin can also maintain mitochondrial function in the presence of oxidative stress. In addition, it has anti-inflammatory activity against immune cells and inhibits the activity of 5-lipoxygenase, thereby reducing the production of lipid peroxides and their proinflammatory byproducts.[5] This wide range of actions suggests that fisetin has the ability to reduce the loss of neurological function associated with multiple disorders, including stroke.

Although fisetin has been shown to be effective in the rabbit small clot embolism model of stroke,[6] its relatively high EC_{50} in cell based assays (2−5 μM) and its low lipophilicity (CLogP = 1.24), high tPSA (107 Å), high number of hydrogen bond donors (HBD = 5), and poor bioavailability[28] suggest that there is room for medicinal chemical improvement if fisetin is to be used therapeutically for treating neurological disorders such as stroke. However, given its ability to activate multiple target pathways related to neuroprotection, screening for improvements is significantly more complicated than with the current classical approach to the development of a single target drug. In this paper, we describe a multitiered approach to screening that has allowed us to identify fisetin derivatives with significantly enhanced neuroprotective activity in an in vitro ischemia model while at the same time maintaining other key actions including anti-inflammatory and neurotrophic activity as well as the ability to maintain GSH under conditions of oxidative stress.

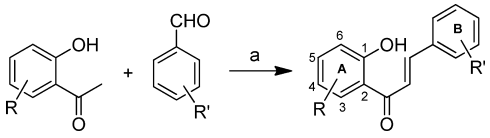
Received: September 20, 2011
Published: December 20, 2011

dx.doi.org/10.1021/jm2012563 | J. Med. Chem. 2012, 55, 378−389

CHEMISTRY

The synthesis of substituted chalcones **013**, **032**, **033**, **057**, **063**, **085**, **086**, **086A**, **105**−**108**, and **137** was carried out by condensation of 2′-hydroxyacetophenones with appropriately substituted aldehydes using $Ba(OH)_2$ in methanol[7] (Scheme 1).

Scheme 1. Synthesis of Chalcone Derivatives[a]



b
- **011**: R = 4, 5-di-Me; R' = *m, p*-di-OH
- **013**: R = 4, 5-di-Me; R' = *m, p*-di-OEt

b
- **032**: R = *α*-naphtha; R' = *m, p*-di-OEt
- **033**: R = *β*-naphtha; R' = *m, p*-di-OEt
- **034**: R = *α*-naphtha; R' = *m, p*-di-OH

- **057**: R = 4, 5-di-Me; R' = *m*-OMe, *p*-OBn
- **063**: R = *α*-naphtha; R' = *m*-OMe, *p*-OBn

b
- **085**: R = 4, 5-di-Me; R' = *o, p*-di-OMe
- **086**: R = 4, 5-di-Me; R' = *m*-OBn, *p*-OMe
- **086A**: R = 4, 5-di-Me; R' = *m*-OTHP, *p*-OMe
- **087**: R = 4, 5-di-Me; R' = *o, p*-di-OH
- **088**: R = 4, 5-di-Me; R' = *m*-OH, *p*-OMe

c

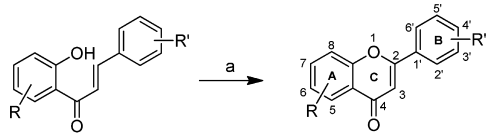
- **105**: R = *α*-naphtha; R' = *p*-pyrrolidine
- **106**: R = 4, 5-di-Me; R' = *p*-pyrrolidine
- **107**: R = 4, 5-di-Me; R' = *p*-NMe_2
- **108**: R = *α*-naphtha; R' = *p*-NMe_2
- **137**: R = *α*-naphtha; R' = *m*-OMe, *p*-pyrrolidine
- **138**: R = 4, 5-di-Me; R' = *m*-OMe, *p*-pyrrolidine

[a]Reagents and conditions: (a) $Ba(OH)_2$, MeOH, 40 °C, overnight, 30−90%; (b) BBr_3, CH_2Cl_2, 0 °C to room temp, overnight, 30−70%; (c) *p*TSA, MeOH, room temp, 94%.

The trihydroxy chalcones **011**, **034**, and **087** were prepared from the corresponding chalcones by treatment with BBr_3 in dicloromethane[8] and the dihydroxychalcone **088** was synthesized by THP deprotection using *p*TSA in methanol[7] from the corresponding chalcone. The suitably substituted flavones **018**, **038**, **058**, **068**, **089**, **115**, **116**, **119**, and **120** were synthesized from the corresponding chalcones using I_2 in DMSO[9] (Scheme 2). The hydroxyflavones **002**, **028**, **064**, **072**, and **094** were obtained from the corresponding chalcones by demethylation/deethylation or debenzylation using BBr_3 in dicloromethane[8] or H_2, Pd/C in EtOAc/methanol,[10] respectively.

Substituted flavonols **025**, **036**, **037**, **059**, **065**, **090**, **091**, **114**, **117**, **118**, **122**, and **139** were prepared (Scheme 3) using 5.4% NaOH, 30% H_2O_2 in methanol[11] from the corresponding chalcones. The known compounds fisetin, **002**, and **04P** were purchased from Indofine Chemicals, and the other hydroxyflavonols **027**, **040**, **041**, **069**, **070**, **092**, **093**, and **140** were obtained from their corresponding flavonols (Scheme 3) by demethylation/deethylation (BBr_3 in dicloromethane)[8] or debenzylation (H_2, Pd/C in EtOAc/methanol)[10] methods. Finally the substituted quinolines **001**, **004**, **007**, **017**, **021**−**024**, **083**, **084**, **109**−**113**, and **121** were synthesized (Scheme 4) by condensation of 2′-aminoacetophenones with appropriately substituted aldehydes using H_2SO_4 in methanol.[12] Experimental procedures and data for all of the compounds are reported below or in the Supporting Information.

Scheme 2. Synthesis of Flavone Derivatives[a]



Chalcones:
- **013**: R = 4, 5-di-Me; R' = *m, p*-di-OEt
- **032**: R = *α*-naphtha; R' = *m, p*-di-OEt
- **057**: R = 4, 5-di-Me; R' = *m*-OMe, *p*-OBn
- **063**: R = *α*-naphtha; R' = *m*-OMe, *p*-OBn
- **085**: R = 4, 5-di-Me; R' = *o, p*-di-OMe
- **105**: R = *α*-naphtha; R' = *p*-pyrrolidine
- **106**: R = 4, 5-di-Me; R' = *p*-pyrrolidine
- **107**: R = 4, 5-di-Me; R' = *p*-NMe_2
- **108**: R = *α*-naphtha; R' = *p*-NMe_2

Flavones:

b
- **002**: R = *α*-naphtha; R' = 3', 4'-di-OH
- **018**: R = 6, 7-di-Me; R' = 3', 4'-di-OEt
- **028**: R = 6, 7-di-Me; R' = 3', 4'-di-OH

b

- **038**: R = *α*-naphtha; R' = 3', 4'-di-OEt

c
- **058**: R = 6, 7-di-Me; R' = 3'-OMe, 4'-OBn
- **064**: R = 6, 7-di-Me; R' = 3'-OMe, 4'-OH

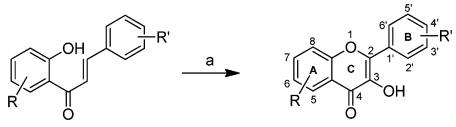
c
- **068**: R = *α*-naphtha; R' = 3'-OMe, 4'-OBn
- **072**: R = *α*-naphtha; R' = 3'-OMe, 4'-OH

b
- **089**: R = 6, 7-di-Me; R' = 2', 4'-di-OMe
- **094**: R = 6, 7-di-Me; R' = 2', 4'-di-OH

- **115**: R = *α*-naphtha; R' = 4'-pyrrolidine
- **116**: R = 6, 7-di-Me; R' = 4'-pyrrolidine
- **119**: R = 6, 7-di-Me; R' = 4'-NMe_2
- **120**: R = *α*-naphtha; R' = 4'-NMe_2

[a]Reagents and conditions: (a) I_2, DMSO, 130 °C, 6 h, 50−95%; (b) BBr_3, CH_2Cl_2, 0 °C to room temp, overnight, 30−70%; (c) H_2, Pd/C, 1:1, EtOAc/MeOH, overnight, 60%.

Scheme 3. Synthesis of Flavonol Derivatives[a]



Chalcones:
- **013**: R = 4, 5-di-Me; R' = *m, p*-di-OEt
- **032**: R = *α*-naphtha; R' = *m, p*-di-OEt
- **033**: R = *β*-naphtha; R' = *m, p*-di-OEt
- **057**: R = 4, 5-di-Me; R' = *m*-OMe, p-OBn
- **063**: R = *α*-naphtha; R' = *m*-OMe, p-OBn
- **085**: R = 4, 5-di-Me; R' = *o, p*-di-OMe
- **086**: R = 4, 5-di-Me; R' = *m*-OBn, *p*-OMe
- **105**: R = *α*-naphtha; R' = *p*-pyrrolidine
- **106**: R = 4, 5-di-Me; R' = *p*-pyrrolidine
- **107**: R = 4, 5-di-Me; R' = *p*-NMe2
- **108**: R = *α*-naphtha; R' = *p*-NMe2
- **137**: R = *α*-naphtha; R' = *m*-OMe, *p*-pyrrolidine
- **138**: R = 4, 5-di-Me; R' = *m*-OMe, *p*-pyrrolidine

Flavonols:

b
- **025**: R = 6, 7-di-Me; R' = 3', 4'-di-OEt
- **027**: R = 6, 7-di-Me; R' = 3', 4'-di-OH

b
- **036**: R = *α*-naphtha; R' = 3', 4'-di-OEt
- **037**: R = *β*-naphtha; R' = 3', 4'-di-OEt
- **040**: R = *α*-naphtha; R' = 3', 4'-di-OH

b

- **041**: R = *β*-naphtha; R' = 3', 4'-di-OH

c
- **059**: R = 6, 7-di-Me; R' = 3'-OMe, 4'-OBn
- **065**: R = *α*-naphtha; R' = 3'-OMe, 4'-OBn
- **069**: R = 6, 7-di-Me; R' = 3'-OMe,4'-OH

c

- **070**: R = *α*-naphtha; R' = 3'-OMe, 4'-OH
- **090**: R = 6, 7-di-Me; R' = 2', 4'-di-OMe

c
- **091**: R = 6, 7-di-Me; R' = 3'-OBn, 4'-OMe
- **092**: R = 6, 7-di-Me; R' = 3'-OH, 4'-OMe

b

- **093**: R = 6, 7-di-Me; R' = 2', 4'-di-OH
- **114**: R = *α*-naphtha; R' = 4'-pyrrolidine
- **117**: R = 6, 7-di-Me; R' = 4'-NMe_2
- **118**: R = *α*-naphtha; R' = 4'-NMe_2
- **122**: R = 6, 7-di-Me; R' = 4'-pyrrolidine
- **139**: R = *α*-naphtha; R' = 3'-OMe, 4'-pyrrolidine

b
- **140**: R = *α*-naphtha; R' = 3'-OH, 4'-pyrrolidine

b
- **141**: R = 6, 7-di-Me; R' = 3'-OMe, *p*-pyrrolidine
- **142**: R = 6, 7-di-Me; R' = 3'-OH, *p*-pyrrolidine

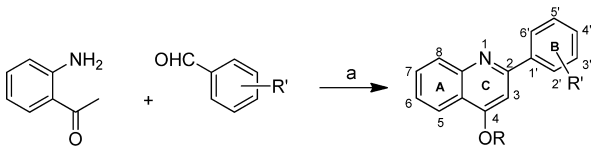
[a]Reagents and conditions: (a) 5.4% NaOH, 30% H_2O_2, MeOH, 0 °C to room temp, overnight, 40−90%; (b) BBr_3, CH_2Cl_2, 0 °C to room temp, overnight, 30−70%; (c) H_2, Pd/C, 1:1, EtOAc/MeOH, overnight, 60%.

RESULTS

The goals of this study were to improve the potency of fisetin based upon the activation of multiple neuroprotective pathways while at the same time altering its physicochemical properties to be more consistent with those of successful CNS drugs (molecular weight ≤ 400, CLogP ≤ 5, tPSA ≤ 90, HBD ≤ 3, HBA ≤ 7)[26,33] in order to increase the possibility of efficient brain penetration and to better understand its SAR. We took two different approaches to the improvement of fisetin. In the first, we removed/modified/replaced the different hydroxyl groups in a systematic manner. In the second approach, we

dx.doi.org/10.1021/jm2012563 | J. Med. Chem. 2012, 55, 378−389

Scheme 4. Synthesis of Quinoline Derivatives[a]



001: R = Me; R' = *m*-OH, *p*-OMe
004: R = Me; R' = *m*, *p*-di-OEt
007: R = Me; R' = *m*, *p*-di-OH
017: R = Me; R' = *m*-OMe, *p*-OH
021: R = Me; R' = H
022: R = Me; R' = *p*-OH
023: R = Et; R' = *m*, *p*-di-OH
024: R = *i*pr; R' = *m*, *p*-di-OH
083: R = Me; R' = *o*, *p*-di-OH
084: R = *i*pr; R' = *o*, *p*-di-OH
109: R = Me; R' = *p*-NMe_2
110: R = Me; R' = *p*-pyrrolidine
111: R = Me; R' = *m*-OH *p*-NO_2
112: R = *i*pr; R' = *p*-NMe_2
113: R = *i*pr; R' = *p*-pyrrolidine
121: R = cyclopentyl; R' = *m*, *p*-di-OH

[a]Reagents and conditions: (a) ROH, H_2SO_4, reflux, overnight, 15–40%.

modified the flavone scaffold by changing it to a quinoline while at the same time maintaining key structural elements.

We chose to use for our primary screen an in vitro ischemia model in combination with the HT22 hippocampal nerve cell line.[6] For this screen, we set a cutoff for the EC_{50} of 1 μM. To induce ischemia in the HT22 cells, we used iodoacetic acid (IAA), a well-known, irreversible inhibitor of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (G3PDH).[14] IAA has been used in a number of other studies to induce ischemia in nerve cells,[15a−e] and we have used it in several recent screens for neuroprotective molecules.[16a,b] The changes following IAA treatment of neural cells are very similar to those seen in animal models of ischemic stroke[17] and include alterations in membrane potential,[18] breakdown of phospholipids,[19] loss of ATP,[20a,b] and an increase in reactive oxygen species (ROS).[21,19]

We used three secondary screens that allowed us to assess three key activities of fisetin that are highly relevant to stroke, as well as other neurological disorders: maintenance of GSH, the major endogenous cellular antioxidant, inhibition of bacterial lipopolysaccharide (LPS) induced microglial activation, an indicator of anti-inflammatory activity, and PC12 cell differentiation, a measure of neurotrophic activity. All of these activities are relevant to the nerve cell loss seen in stroke.[22a−c] Previous and ongoing studies suggest that these activities of fisetin are mediated via distinct pathways but that all three may be important for the neuroprotective effects of fisetin in vivo.[5] To assess GSH maintenance, we looked at total intracellular GSH levels after a 24 h treatment with the compound both in the absence and in the presence of glutamate, an inducer of GSH loss and oxidative stress.[23a,b] Inhibition of LPS-induced microglial activation was determined by treating N9 mouse microglial cells with LPS alone and in the presence of the compounds and assaying nitrite (spontaneously produced by air oxidation of nitric oxide) levels in the medium 24 h later.[24] PC12 cell differentiation was determined by treating PC12 cells with the compounds and looking at neurite outgrowth after 24 h. In all cases, fisetin was used as a positive control.[25]

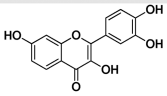
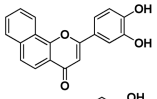
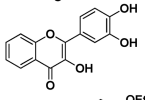
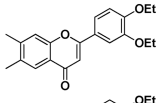
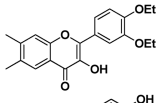
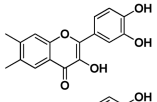
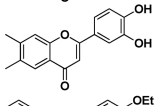
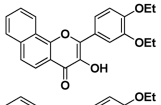
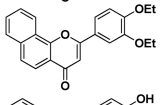
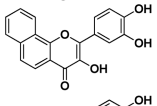
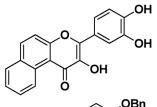
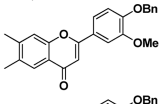
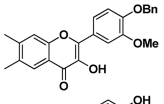
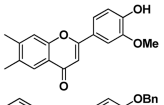
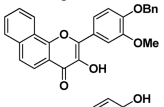
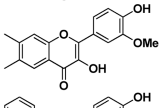
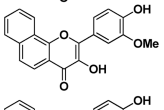
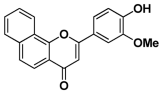
Structure–Activity Relationship. We began by assessing the roles of the four different hydroxyl groups in the activity of fisetin. We found that removal of the 7 hydroxyl (**04P**) not only improved the neuroprotective activity ∼6-fold over fisetin in our primary screen of in vitro ischemia without loss of either the GSH maintaining activity or PC12 cell differentiation but also enhanced lipophilicity increasing the CLogP from 1.24 to 1.82 (Table 1). Furthermore, this modification did not alter the anti-inflammatory activity of fisetin (Table 1). This finding allowed us to replace the 7 hydroxyl with hydrophobic groups in order to further improve the lipophilicity and tPSA to values more consistent with typical CNS drugs.[26,33] The addition of a benzene ring (**040**) to the A ring further enhanced neuroprotective activity ∼5.5-fold with a much more pronounced effect seen with the α-naphtha derivative (**040**) as opposed to the β-naphtha (**041**) derivative (Table 1). However, this modification eliminated the ability of the derivative to maintain GSH under conditions of oxidative stress. For this derivative, the 3-hydroxyl was not important for neuroprotective activity (**040** vs **002**) but did enhance anti-inflammatory activity.

We also examined the role of the B ring hydroxyls in neuroprotection as well as the other key activities of the α-naphtha derivative. Changing both hydroxyls to ethoxy groups (**036**, **038**) not only greatly reduced neuroprotective activity but also eliminated both the anti-inflammatory activity and the ability to induce PC12 cell differentiation. Changing only one of the hydroxyls to a methoxy group enhanced neuroprotective activity ∼2-fold over **040** in the absence of the 3-hydroxyl group (**072**) but greatly reduced neuroprotective activity relative to **040** in the presence of the 3-hydroxyl (**070**). Furthermore, this modification did not restore the ability to maintain GSH under conditions of oxidative stress and the derivative without the 3 hydroxyl (**072**) also lacked anti-inflammatory activity and the ability to induce PC12 cell differentiation. Surprisingly, changing the 4′-hydroxyl to a benzyloxy group (**065**) restored neuroprotective activity in the presence of the 3-hydroxyl. Compounds possessing tertiary nitrogen, a feature of many CNS drugs, show a higher degree of brain permeation.[26,33,34] With this observation in mind, both hydroxyls on the B ring were replaced with a single dimethylamino group at the 4′ position, resulting in a highly neuroprotective compound in the presence of the 3-hydroxyl group (**118**) and a somewhat less effective compound in its absence (**120**). This modification also eliminated two hydrogen bond donors. Although **118** regained the ability to maintain GSH levels, it lacked both anti-inflammatory and neurotrophic activity. Modification of the dimethylamine to a pyrrolidine group at the 4′ position gave a compound that had excellent neuroprotective activity in the presence of the 3-hydroxyl (**114**) and could also induce PC12 cell differentiation but had poor anti-inflammatory activity and did not maintain GSH levels. However, addition of a 3′-hydroxyl to this derivative resulted in a compound with outstanding neuroprotective activity (EC_{50} = 5 nM) (**140**) that could also maintain GSH under conditions of oxidative stress, induce PC12 differentiation and had reasonably good anti-inflammatory activity.

As a second approach, we replaced the benzene ring with two methyl groups (**027**) in order to generate a derivative with a similar CLogP and tPSA as **040** but with a less bulky addition to the A ring (Table 1). Surprisingly, this derivative not only showed significantly decreased neuroprotective activity compared with **040** but also lost the ability to induce PC12 cell differentiation along with the continued failure to maintain GSH levels. Removal of the 3-hydroxyl enhanced neuroprotective activity 2-fold (**028**) but did not restore the induction of PC12 cell differentiation or the maintenance of

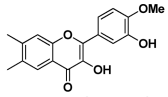
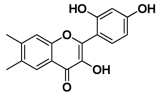
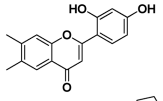
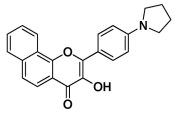
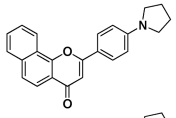
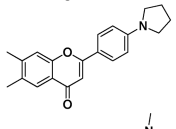
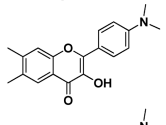
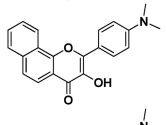
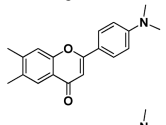
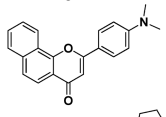
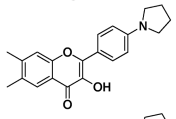
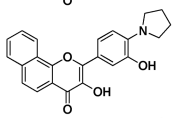
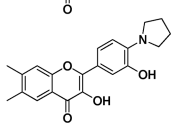
dx.doi.org/10.1021/jm2012563 | J. Med. Chem. 2012, 55, 378–389

Table 1[a]

Compound	M.Wt	tPSA	CLogP	Structure	EC_{50} in vitro ischemia (μM)	GSH	PC12 diff'n	microglia	TEAC
Fisetin	286	107	1.24		3	yes	yes	80%	3
002	304	67	3.52		0.08	no	yes	55%	0.18
04P	270	87	1.82		0.5	yes	yes	80%	2
018	338	45	5.16		no	no	no	2%	0.15
025	354	65	4.71		0.5	no	no	11%	0.84
027	298	87	2.77		0.5	no	no	82%	1.89
028	282	67	3.30		0.25	no	no	93%	0.27
036	376	65	4.94		0.3	no	no	13%	0.15
038	360	45	5.39		no	no	no	2%	0.2
040	320	87	2.99		0.09	no	yes	91%	2.4
041	320	87	2.99		0.25	no	yes	87%	1.26
058	386	45	5.87		0.5	no	no	83%	0.09
059	402	65	5.42		0.17	no	no	14%	0.27
064	296	56	3.66		0.03	no	no	41%	0.12
065	424	65	5.65		0.08	no	yes	88%	0
069	312	76	3.19		0.04	no	no	77%	1.89
070	334	76	3.41		>0.5	no	yes	78%	0.63
072	318	56	3.88		0.04	no	no	19%	0.2

dx.doi.org/10.1021/jm2012563 | *J. Med. Chem.* 2012, 55, 378–389

Table 1. continued

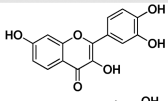
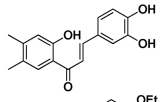
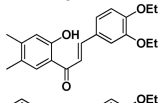
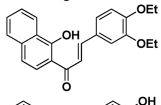
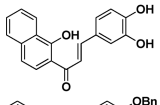
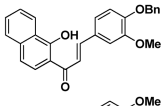
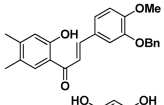
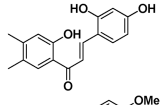
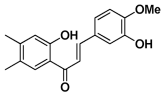
Compound	M.Wt	tPSA	CLogP	Structure	EC_{50} in vitro ischemia (μM)	GSH	PC12 diff'n	microglia	TEAC
092	312	76.00	3.19		0.02	no	no	72%	1.74
093	298	87.00	2.40		>0.5	no	no	0%	1.56
094	282	66.76	2.93		>0.5	no	no	5%	0.15
114	357	49.17	4.51		0.07	no	yes	26%	1.44
115	341	29.54	4.99		0.2	no	no	23%	0.03
116	319	29.54	4.70		>0.5	no	no	2%	0.12
117	309	49.77	4.17		0.02	yes	no	11%	3
118	331	49.77	4.40		0.04	yes	no	0%	0.93
119	293	29.54	4.65		>0.5	no	no	8%	0
120	315	29.54	4.88		0.25	no	no	35%	0.24
122	335	49.77	4.28		0.09	yes	yes	0%	2.4
140	372	70.00	4.09		0.005	yes	yes	50%	2.1
142	351	70.00	3.64		0.015	yes	yes	21%	2.79

[a]Half maximal effective concentrations (EC_{50}) for protection in the in vitro ischemia assay were determined by exposing HT22 cells to different doses of each derivative in the presence of 20 μM IAA for 2 h (HT22/IAA). Cell viability was determined after 24 h by the MTT assay. The ability to maintain GSH was determined by treating HT22 cells with different doses of each derivative (1−10 μM) in the presence of 5 mM glutamate. After 24 h cell extracts were prepared and analyzed for total GSH. Fisetin (10 μM) was used as a positive control. The ability to induce PC12 cell differentiation (PC12 diff'n) was determined by treating PC12 cells in N_2 medium with different doses of each derivative (1−10 μM) for 24 h. Differentiation was assessed by visual inspection with fisetin (10 μM) as a positive control. Anti-inflammatory activity (microglia) was assessed in N9 microglial cells treated with bacterial lipopolysaccharide alone or in the presence different doses of each derivative (1−10 μM) for 24 h. Fisetin was used as a positive control. TEAC values, a measure of direct antioxidant activity, were determined using the $ABTS^+$ decolorization assay.

GSH. Modification of the B ring hydroxyls produced mixed results. Modification of one hydroxyl to a methoxy (**064**, **069**, **092**) improved neuroprotective activity approximately 10- to 20-fold but slightly reduced anti-inflammatory activity. Similar

dx.doi.org/10.1021/jm2012563 | J. Med. Chem. 2012, 55, 378−389

Table 2[a]

Compound	M.Wt	tPSA	CLogP	Structure	EC_{50} in vitro ischemia (μM)	GSH	PC12 diff'n	microglia	TEAC
Fisetin	286	107	1.24		3	yes	yes	80%	3
011	284	78	3.64		0.05	yes	yes	94%	2.7
013	340	56	5.62		no	no	no	56%	0.09
032	362	56	5.84		no	no	no	5%	0.12
034	306	78	3.86		0.08	yes	yes	75%	2.8
063	410	56	6.55		0.5	no	no	9%	0.15
086	388	55.76	6.33		no	yes	yes	70%	0.12
087	284	77.76	3.57		0.05	no	yes	53%	0.93
088	298	66.76	4.08		0.2	no	no	56%	0.12

[a]Half maximal effective concentrations (EC_{50}) for protection in the in vitro ischemia assay were determined by exposing HT22 cells to different doses of each derivative in the presence of 20 μM IAA for 2 h (HT22/IAA). Cell viability was determined after 24 h by the MTT assay. The ability to maintain GSH was determined by treating HT22 cells with different doses of each derivative (1−10 μM) in the presence of 5 mM glutamate. After 24 h cell extracts were prepared and analyzed for total GSH. Fisetin (10 μM) was used as a positive control. The ability to induce PC12 cell differentiation (PC12 diff'n) was determined by treating PC12 cells in N_2 medium with different doses of each derivative (1−10 μM) for 24 h. Differentiation was assessed by visual inspection with fisetin (10 μM) as a positive control. Anti-inflammatory activity (microglia) was assessed in N9 microglial cells treated with bacterial lipopolysaccharide alone or in the presence different doses of each derivative (1−10 μM) for 24 h. Fisetin was used as a positive control. TEAC values, a measure of direct antioxidant activity, were determined using the $ABTS^+$ decolorization assay.

to the results with the derivatives of **040**, modification of both the B ring hydroxyl groups to ethoxy groups (**018**, **025**) did not improve neuroprotective activity. Furthermore, none of these derivatives regained the ability to maintain GSH or induce PC12 cell differentiation and they also showed reduced anti-inflammatory activity. While the methoxy, benzyloxydimethyl derivative did have somewhat enhanced neuroprotective activity relative to **027** in the presence of the 3-hydroxyl (**059**), it was deficient in anti-inflammatory activity. Furthermore, separation of the B ring hydroxyls (**093**, **094**) eliminated not only neuroprotective activity but the other key activities as well. However, similar to the results with the derivatives of **040**, replacement of the hydroxyls with a single dimethylamino group at the 4′ position produced a compound with excellent neuroprotective activity but only in the presence of the 3-hydroxyl (**117** vs **119**). This compound also regained the ability to maintain GSH but lacked both neurotrophic and anti-inflammatory activity. Addition of a single pyrrolidine group to the 4′ position instead gave a compound that had excellent neuroprotective activity only in the presence of the 3-hydroxyl (**122** vs **116**) and could also maintain GSH levels and induce PC12 cell differentiation but still had poor anti-inflammatory activity. Addition of a 3′-hydroxyl to this derivative resulted in a compound with outstanding neuroprotective activity (**142**) that could also maintain GSH under conditions of oxidative stress and induce PC12 differentiation but had lower anti-inflammatory activity than **140**.

Chalcones are intermediates in the synthesis of flavonoids and were used to determine the effect of opening up the C-ring on activity (Table 2). Surprisingly, the chalcones of both the naphtha (**034**) and dimethyl derivatives (**011**) had similar (**034**) or enhanced (**011**) neuroprotective activity compared to their flavone counterparts and also regained all of the key activities including the ability to maintain GSH under conditions of oxidative stress. In contrast, the chalcones where both the B ring hydroxyls were modified had either no (**032**, **013**, **086**) or greatly reduced (**063**) neuroprotective activity. Furthermore, splitting the B ring hydroxyls of **011** (**087**) eliminated the ability to maintain GSH under conditions of oxidative stress. The conversion of a hydroxyl to a methoxy (**088**) also eliminated the ability to promote PC12 cell differentiation. Thus, of the chalcones tested **011** and **034** are

dx.doi.org/10.1021/jm2012563 | J. Med. Chem. 2012, 55, 378−389

superior to fisetin by both the selection criteria and medicinal chemistry properties.

As an alternative approach to improving fisetin, we modified the flavone scaffold, changing it to a quinoline scaffold (Table 3) in an attempt to further improve potency and physiochemical properties while retaining the key structural elements of the flavone in the quinoline scaffold. The simplest version, **007**, showed a ~75-fold increase in neuroprotective activity relative to fisetin, maintained GSH under conditions of oxidative stress, and had strong anti-inflammatory activity. However, it did not induce PC12 cell differentiation. We explored a number of modifications to see if we could enhance neuroprotective activity and/or restore the PC12 cell differentiating activity. Interestingly, the substitution of an ethoxy (**023**) or an isopropoxy (**024**) for the methoxy group on the C ring did restore the differentiating activity while also slightly improving (~2-fold) the neuroprotective activity relative to **007**. Importantly, replacement of the *O*-methyl group with an *O*-cyclopentyl ring resulted in a compound with a >400-fold decrease in EC_{50} relative to fisetin for neuroprotective activity (**121**) and maintenance of all of the key activities. For all forms of the quinoline-based derivative, removal of one (**022**) or both (**021**) of the B ring hydroxyls or conversion of one or both of these hydroxyls to methoxy (**001**, **017**), ethoxy (**004**), nitro (**111**), or chlorine or fluorine (not shown) greatly reduced or eliminated neuroprotective activity. All of these changes also reduced or eliminated all of the other key activities. Splitting the two ring hydroxyls (**083**, **084**) also reduced neuroprotective activity and eliminated the ability to maintain GSH and induce PC12 cell differentiation but did not impact anti-inflammatory activity. In contrast to the derivatives based on the flavone scaffold, the addition of a single dimethylamino (**109**, **112**) or pyrrolidine group (**110**, **113**) to the 4′ position of the B ring did not enhance neuroprotective activity relative to the 3′,4′-dihydroxy derivative and generally resulted in a reduction or elimination of the other key activities. Thus, in the presence of the quinoline scaffold the catechol group on the B ring is essential for activity.

The transcription factor Nrf2 plays a key role in regulating GSH metabolism in many different cell types.[27] We have shown that fisetin can induce Nrf2 and that this correlates with its ability to enhance GSH levels.[5] To determine if the derivatives that can maintain GSH levels do so by increasing Nrf2, we looked at Nrf2 levels in the nuclei of derivative-treated cells using fisetin as a positive control (Table 4). Surprisingly, not all of the derivatives that maintain GSH levels induce Nrf2. This was particularly true for the derivatives based on the quinoline scaffold where none of them increased Nrf2 despite being very effective at maintaining GSH levels.

DISCUSSION AND CONCLUSIONS

Several important findings emerge from this study. First, within the flavone scaffold we were able to demonstrate SARs with respect to four distinct biological activities and to improve neuroprotective activity up to 600-fold (**140**). We also show that while it is possible to maintain all of the biological activities that are likely to be important for in vivo efficacy, each of these activities has specific and unique structural requirements. Thus, it is possible to balance enhanced neuroprotective activity with the other key activities as well as the physical characteristics of the compounds in order to arrive at compounds that have the best chance for efficacy in vivo. An additional key finding is that neither the neuroprotective activity nor any of the other three key activities of the fisetin derivatives show any correlation with antioxidant activity as defined by the TEAC value (Table 1).

Each of the key activities of the fisetin derivatives shows distinct structural requirements. For example, within the flavone structure (Table 1), the maintenance of GSH poses the strictest structural requirements. It is highly sensitive to modification of the A ring (**040**, **027**). However, substitution of the B ring hydroxyls with a single tertiary amino group is compatible with the maintenance of GSH even in the presence of A ring modifications (**117**, **118**) as long as a 3-hydroxyl group is present. In contrast, the anti-inflammatory activity of the flavone-based derivatives is not particularly sensitive to modification of the A ring, especially in the presence of a 3-hydroxyl group (e.g., **040** vs **04P**). The anti-inflammatory activity of the flavone-based derivatives, however, is not very tolerant of modification of the B ring hydroxyls (e.g., **036**, **072**) and is also not tolerant of the substitution of the tertiary amino groups regardless of the presence of a 3-hydroxyl group (e.g., **117**, **119**). However, the anti-inflammatory dampening effect of the tertiary amino groups can be reduced by the readdition of a hydroxyl group to the 3′ position (**140**). The PC12 differentiation promoting activity of the flavone-based derivatives shows a similar but less demanding set of structural requirements as the GSH maintaining activity for it is somewhat more tolerant of modifications to the A ring (e.g., **040** but not **027**). In addition, while this activity is sensitive to modifications of the B ring hydroxyls, it tolerates limited modifications that eliminate the GSH maintaining activity (e.g., **065**).

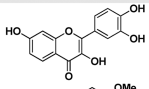
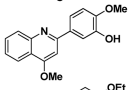
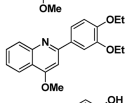
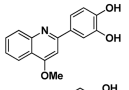
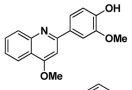
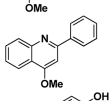
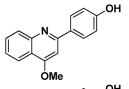
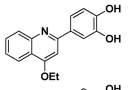
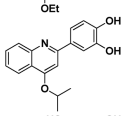
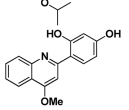
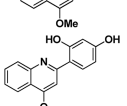
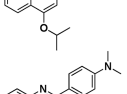
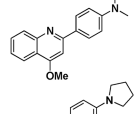
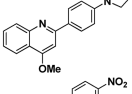
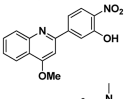
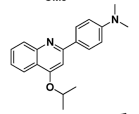
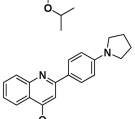
Once the flavone structure is opened up to give the chalcone (Table 2), only modification of the B ring hydroxyls affects the GSH maintaining activity of the fisetin derivatives. The one exception is **086** which has a methoxy and a benzyloxy group on the B ring. The PC12 differentiation promoting activity of the chalcone-based derivatives shows similar structural requirements as the GSH maintaining activity. Interestingly, while the anti-inflammatory activity of the α-naphtha chalcone based derivatives is eliminated by modification of the B ring hydroxyls, the anti-inflammatory activity of the dimethyl chalcone based derivatives is much more tolerant of this type of modification.

We have also identified a new quinoline scaffold that reserves the key structural elements of the flavone but results in enhanced neuroprotective activity (to >400×) while maintaining the other key activities. Although these derivatives have greatly reduced free radical scavenging activity relative to fisetin based on TEAC values (Table 3), several are highly neuroprotective in our in vitro assay. In addition, while the most neuroprotective compounds with this scaffold do have hydroxyl groups, they are not polyphenols. Interestingly, within the context of this scaffold, the structural requirements for each key activity are somewhat sharper. For the maintenance of GSH, a catechol group on the B ring is essential. PC12 differentiation promoting activity requires both a catechol group on the B ring and a hydrophobic group on the 4-position of the C ring. The requirements for anti-inflammatory activity are somewhat less stringent but are sensitive to modifications of the B ring hydroxyls in a manner similar to that of the flavone-based derivatives.

We have also found that it is possible to separate neuroprotective activity from the three other key activities of fisetin. This result suggests that none of the three key activities play a role in neuroprotection in our in vitro ischemia assay. Both the differentiation-promoting and anti-inflammatory

dx.doi.org/10.1021/jm2012563 | J. Med. Chem. 2012, 55, 378–389

Table 3[a]

Compound	M.Wt	tPSA	CLogP	Structure	EC_{50} in vitro ischemia (μM)	GSH	PC12 diff'n	microglia	TEAC
Fisetin	286	107	1.24		3	yes	yes	80%	3
001	281	51	3.89		no	no	no	69%	0.24
004	323	40	5.33		no	no	no	76%	0.12
007	267	62	3.66		0.04	yes	no	85%	0.36
017	281	51	3.89		0.5	no	no	90%	0.15
021	235	21	4.55		0.75	no	no	6%	0.12
022	251	42	4.07		no	no	no	4%	0.81
023	281	62	4.20		0.02	yes	yes	90%	0.90
024	295	62	4.50		0.02	yes	yes	80%	0.18
083	267	62.06	3.30		>0.5	no	no	84%	0.05
084	295	62.05	4.13		0.21	no	no	64%	0.27
109	278	24.83	4.82		0.06	no	no	62%	0.06
110	304	24.83	4.93		no	no	no	67%	0.27
111	296	93.63	4.33		no	no	no	25%	0.18
112	306	24.83	5.66		0.05	no	no	71%	0.15
113	332	24.83	5.77		0.5	no	no	67%	0.27
121	321	62.05	5.14		0.007	yes	yes	82%	0.40

[a]Half maximal effective concentrations (EC_{50}) for protection in the in vitro ischemia assay were determined by exposing HT22 cells to different doses of each derivative in the presence of 20 μM IAA for 2 h (HT22/IAA). Cell viability was determined after 24 h by the MTT assay. The ability to maintain GSH was determined by treating HT22 cells with different doses of each derivative (1−10 μM) in the presence of 5 mM glutamate. After 24 h cell extracts were prepared and analyzed for total GSH. Fisetin (10 μM) was used as a positive control. The ability to induce PC12 cell differentiation (PC12 diff'n) was determined by treating PC12 cells in N_2 medium with different doses of each derivative (1−10 μM) for 24 h. Differentiation was assessed by visual inspection with fisetin (10 μM) as a positive control. Anti-inflammatory activity (microglia) was assessed in N9 microglial cells treated with bacterial lipopolysaccharide alone or in the presence different doses of each derivative (1−10 μM) for 24 h. Fisetin was used as a positive control. TEAC values, a measure of direct antioxidant activity, were determined using the $ABTS^+$ decolorization assay.

dx.doi.org/10.1021/jm2012563 | J. Med. Chem. 2012, 55, 378−389

Table 4[a]

compd	Nrf2
fisetin	yes
04P	yes
007	no
011	yes
034	yes
086	yes
117	no
118	no
121	no
122	no
140	no
142	no

[a]The ability of the derivatives that maintain GSH levels to induce the transcription factor Nrf2 was assayed by SDS−PAGE and Western blotting of nuclear extracts of untreated and derivative-treated cells. Fisetin treatment was used as a positive control.

activities could have critical roles in maintaining CNS function in vivo but are less likely to be relevant in an in vitro assay with a single cell type. What is more surprising is that the ability to maintain GSH is not essential for neuroprotection in the in vitro ischemia assay, as GSH loss is a component of this cell death paradigm.[6] However, the compounds with the lowest EC_{50} values for neuroprotection are all effective at maintaining GSH levels. Furthermore, many of the effective neuroprotective compounds that do not maintain GSH are also not good antioxidants as defined by the TEAC assay, an in vitro assay for antioxidant activity. Together, these results suggest that the neuroprotection by these compounds is mediated by some other, as yet undefined, action that is independent of GSH. This is currently under investigation.

In addition, we also found that the ability to maintain GSH levels did not correlate with the induction of Nrf2 by the compounds. There are a number of other mechanisms for maintaining GSH levels that could be modulated by these compounds including reduction of GSH utilization or inhibition of GSH export.[27] These possibilities will be explored in future studies.

Many of our most effective fisetin derivatives also have improved medicinal chemical properties in terms of HBD, CLogP, and tPSA, falling within the criteria for CNS drugs.[26,33] Hydrogen bonding properties of drugs can significantly influence their CNS uptake profiles. Polar molecules are generally poor CNS agents, and low lipophilicity (CLogP) and high hydrogen bonding decrease BBB penetration.[33] Another important aspect of our fisetin derivatives is that they all lack A ring hydroxyl groups which are known to be subject to modification following oral administration.[28] Thus, they are less likely to be metabolized in this way, leading to enhanced bioavailability and brain penetration.

Recent studies in our laboratory have shown that fisetin is effective in multiple animal models of neurological disorders including stroke[6] and Huntington's disease.[29] Furthermore, fisetin can reduce both the kidney and CNS complications of diabetes in the Akita model of type 1 diabetes.[30] Thus, it shows promise for the treatment of multiple diseases for which there are currently no good treatments. The identification and characterization of more efficacious derivatives are the first steps in moving this lead compound toward the clinic.

In summary, starting with the multitarget polyphenol fisetin, we have generated a number of derivatives with greatly enhanced neuroprotective activity (e.g., **011**, 50 nM; **121**, 7 nM; **140**, 5 nM) in a cell culture-based model of ischemia. Many of the more potent fisetin derivatives also have good CNS medicinal chemical properties. In addition, some of these derivatives maintain the other three key activities of fisetin including anti-inflammatory, neurotrophic, and GSH-maintaining activities, making them good candidates for further testing in animal models of stroke as well as other neurological diseases. In creating these derivatives, we have shown that it is possible to enhance a primary activity of a polyphenol such as fisetin while at the same time maintaining other key activities that are not necessarily directly related to this primary activity. Thus, we are able to maintain the multitarget qualities while improving both the physiochemical and pharmacological properties of the compound.

EXPERIMENTAL SECTION

Biology. Cell Culture. Fetal calf serum (FCS) and dialyzed FCS (DFCS) were from Hyclone (Logan, UT). Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen (Carlsbad, CA). HT22 cells[6] were grown in DMEM supplemented with 10% FCS and antibiotics. PC12 cells were grown in DMEM supplemented with 10% FCS, 5% horse serum, and antibiotics. N9 microglial cells were grown in DMEM supplemented with 10% FCS, 1× nonessential amino acids, 1× essential amino acids, and antibiotics.

Cytotoxicity Assay. Cell viability was determined by a modified version of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay based on the standard procedure.[6] Cells were seeded onto 96-well microtiter plates at a density of 5×10^3 cells per well. For the in vitro ischemia assay, the next day, the medium was replaced with DMEM supplemented with 7.5% DFCS and the cells were treated with 20 μM iodoacetic acid (IAA) alone or in the presence of the different derivatives. After 2 h the medium in each well was aspirated and replaced with fresh medium without IAA but containing the derivatives. At 20 h later, the medium in each well was aspirated and replaced with fresh medium containing 2.5 μg/mL MTT. After 4 h of incubation at 37 °C, the cells were solubilized with 100 μL of a solution containing 50% dimethylformamide and 20% SDS (pH 4.7). The absorbance at 570 nm was measured on the following day with a microplate reader (Molecular Devices). Results were confirmed by visual inspection of the wells. Controls included compound alone to test for toxicity and compound with no cells to test for interference with the assay chemistry. All of the derivatives were tested twice in this assay, and those that showed a strongly positive response (EC_{50} < 1 μM) were tested a third time for confirmation.

Differentiation Assay. PC12 cells in N_2 medium were treated with the derivatives (1−10 μM) or fisetin (10 μM) as a positive control for 24 h, at which time the cells were scored for the presence of neurites. PC12 cells produce neurites much more rapidly when treated in N_2 medium than when treated in regular growth medium. For each treatment, 100 cells in each of three separate fields were counted. Cells were scored positive if one or more neurites with >1 cell body diameter in length were observed. All of the derivatives were tested twice in this assay, and those that showed a positive response were tested a third time for confirmation.

Anti-Inflammatory Assay. Mouse N9 microglial cells plated in DME with 7.5% DFCS were treated with 10 μg/mL bacterial lipopolysaccharide (Sigma) alone or in the presence of the fisetin derivatives (1−10 μM) or fisetin (10 μM) as a positive control. After 24 h the medium was removed, spun briefly to remove floating cells and 100 μL assayed for nitrite using 100 μL of the Griess reagent (Sigma) in a 96-well plate. After incubation for 10 min at room temperature the absorbance at 550 nm was read on a microplate reader. All of the derivatives were tested twice in this assay, and those

that showed a positive response were tested a third time for confirmation.

Total Glutathione. Total intracellular glutathione was determined by a chemical assay as described.[31] All of the derivatives were tested twice in this assay, and those that showed a positive response were tested a third time for confirmation.

SDS−PAGE and Immunoblotting. For immunoblotting of Nrf2, nuclear extracts were prepared as described[32] from untreated cells and cells treated with the fisetin derivatives for 1, 2, and 4 h. Fisetin was used as a positive control. For each derivative, the concentration that was most effective at preventing cell death was used. Protein concentrations were determined using the BCA protein assay (Pierce). Equal amounts of protein were solubilized in 2.5× SDS sample buffer, separated on 10% SDS−polyacrylamide gels, and transferred to nitrocellulose. Equal loading and transfer of the samples were confirmed by staining the nitrocellulose with Ponceau-S. Transfers were blocked for 1 h at room temperature with 5% nonfat milk in TBS/0.1% Tween 20 and then incubated overnight at 4 °C in the primary antibody diluted in 5% BSA in TBS/0.05% Tween 20. The primary antibodies used were anti-Nrf2 (no. SC13032, $^1/_{1000}$) from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-β-actin (no. 5125, $^1/_{20000}$) from Cell Signaling (Beverly, MA). The transfers were rinsed with TBS/0.05% Tween 20 and incubated for 1 h at room temperature in horseradish peroxidase−goat anti-rabbit or goat anti-mouse (Biorad, Hercules, CA) diluted $^1/_{5000}$ in 5% nonfat milk in TBS/0.1% Tween 20. The immunoblots were developed with the Super Signal reagent (Pierce, Rockford, IL). All of the derivatives were tested twice in this assay, and those that showed a positive response were tested a third time for confirmation.

Determination of the Trolox Equivalent Activity Concentration (TEAC). TEAC values for the flavonoids were determined as described.[31] Briefly, 250 μL of 2,2′-azinobis(3-ethylbenzothiazoline 6-sulfonate) (ABTS) treated overnight with potassium persulfate and diluted to an OD of ∼0.7 at 734 nm was added to 2.5 μL of a derivative solution in ethanol. The change in absorbance due to the reduction of the ABTS radical cation was measured at 734 nm for 4 min. To calculate the TEAC, the gradient of the plot of the percentage inhibition of absorbance vs concentration for the derivative in question was divided by the gradient of the plot for Trolox.

Chemistry: General Methods. All reagents and anhydrous solvents were obtained from commercial sources and used as received. ^{1}H NMR and ^{13}C NMR were recorded at 500 and 125 MHz, respectively, on a Varian VNMRS-500 spectrometer using the indicated solvents. Chemical shift (δ) is given in parts per million (ppm) relative to tetramethylsilane (TMS) as an internal standard. Coupling constants (J) are expressed in hertz (Hz), and conventional abbreviations used for signal shape are as follows: s = singlet; d = doublet; t = triplet; m = multiplet; dd, doublet of doublets; brs = broad singlet. Liquid chromatography−mass spectrometry (LCMS) was carried out using a Shimadzu LC-20AD spectrometer and electrospray ionization (ESI) mass analysis with a Thermo Scientific LTQ Orbitrap-XL spectrometer. Melting points were determined with a Thomas-Hoover capillary melting point apparatus and are uncorrected. All final compounds were characterized by LCMS and ^{1}H NMR and gave satisfactory results in agreement with the proposed structure. All of the tested compounds have a purity of at least 95% which was determined by analysis on a C18 reverse phase HPLC column [Phenomenex Luna (50 mm × 4.60 mm, 3 μm)] using 10−90% CH_3CN/H_2O containing 0.02% AcOH with a flow rate of 1 mL/min (5 min gradient) and monitoring by a UV detector operating at 254 nm. Mass spectra were acquired in the positive mode scanning over the mass range of 50−1000. LCMS M + H signals were consistent with the expected molecular weights for all of the reported compounds. Thin layer chromatography (TLC) used EMD silica gel F-254 plates (thickness of 0.25 mm). Flash chromatography used EMD silica gel 60, 230−400 mesh.

General Procedure A for the Synthesis of Chalcone Derivatives 013, 032, 033, 057, 063, 085, 086, 105−108, 137, and 138. A mixture of 2′-hydroxyacetophenone (1 equiv), arylaldehyde (1 equiv), and $Ba(OH)_2$ (1 equiv) in MeOH (3 mL/mmol) was stirred for 12 h at 40 °C. Methanol was evaporated, and the residue was diluted with water, neutralized with 1 N HCl, and extracted with ethyl acetate. The organic layer was washed with brine solution, dried (Na_2SO_4), and evaporated. Solid residues were recrystallized from CH_2Cl_2/hexane and liquid residues were purified by flash chromatography using silica gel (230−400 mesh) with 10−30% EtOAc/hexane, giving chalcones with 30−90% yield.

General Procedure B (Methyl/Ethyl Deprotection) for the Synthesis of Compounds 002, 011, 027, 028, 034, 041, 087, 093, 094, 140, and 142. To a stirred and cooled 0 °C solution of suitably protected starting material (1 equiv) in CH_2Cl_2 (5 mL/mmol) was added BBr_3 (2 equiv/alkoxy group), and the mixture was stirred overnight at room temperature under nitrogen atmosphere. The reaction mixture was quenched by adding 5% Na_2HPO_4 solution and extracted with CH_2Cl_2. The combined organic extracts were washed with saturated $NaHCO_3$, brine, dried (Na_2SO_4), and evaporated. The resulting solids were recrystallized from methanol.

Method C for the Synthesis of Chalcone 088. To a stirred solution of chalcone **086A** (74.7 mg, 0.203 mmol) in MeOH (2 mL) was added *p*-toluenesulfonic acid (77.3 mg, 0.407 mmol). The reaction mixture was stirred for 3 h at room temperature. After completion of the reaction, the solvent was evaporated and the residue was diluted with water (20 mL), then neutralized with saturated $NaHCO_3$, and extracted with EtOAc. Combined extracts were washed with brine, dried (Na_2SO_4), and evaporated. The residue was purified by flash chromatography using silica gel (230−400 mesh) with 20% EtOAc/hexane, giving **088** on 94% yield as a yellow solid.

General Procedure D (Debenzylation) for the Synthesis of Compounds 64, 069, 070, 072, and 092. The benzyl protected flavones and flavonols were dissolved in 1:1 EtOAc/methanol (10 mL/mmol) and then treated with 5% palladium on charcoal (5% w/w). The mixture was stirred under hydrogen atmosphere (balloon pressure) overnight. The reaction mixture was filtered and the solvent was evaporated. The resulting solids were recrystallized from dichloromethane/methanol.

General Procedure E for the Synthesis of Flavone Derivatives 018, 038, 058, 068, 089, 115, 116, 119, and 120. A solution of chalcone (1 equiv) and iodine (0.01 equiv) in DMSO (1 mL/mmol) was heated at 130 °C for 3−6 h. The reaction mixture was cooled and diluted with water, extracted with CH_2Cl_2, washed with aqueous saturated $Na_2S_2O_3$, dried (Na_2SO_4), and evaporated. Solid residues were recrystallized from CH_2Cl_2/hexane and liquid residues were purified by flash chromatography using silica gel (230−400 mesh) with 30−80% EtOAc/hexane, giving flavones with 50−95% yield.

General Procedure F for the Synthesis of Flavonol Derivatives 025, 036, 037, 059, 065, 090, 091, 114, 117, 118, 122, 139, and 141. To a stirred and cooled 0 °C solution of chalcone in MeOH (5 mL/mmol) was added 5.4% NaOH (3.2 mL/mmol) followed by 30% H_2O_2 (0.37 mL/mmol) dropwise, and the mixture was stirred for 3 h at 0 °C. Then the ice bath was left in place but not recharged, and stirring was continued overnight. The reaction mixture was neutralized with 2 M HCl, and the resulting precipitate was collected by filtration and washed with water and recrystallized from dichloromethane to give flavonols with 40−90% yield.

General Procedure G for the Synthesis of Quinoline Derivatives 001, 004, 007, 017, 021−024, 083, 084, 109−113, and 121. To a stirred solution of 2′-aminoacetophenone (1 equiv) and aromatic aldehyde (1 to 3 equiv) in alcohol (3 mL/mmol) was added H_2SO_4 (0.75 equiv), and the mixture was refluxed for 12−24 h. The reaction mixture was cooled, and the solvent was evaporated. The residue was diluted with water, neutralized with 5% $NaHCO_3$ solution, and extracted with ethyl acetate. The organic layer was washed with brine, dried (Na_2SO_4), and evaporated. Flash chromatography of the residue over silica gel using 10−50% EtOAc/hexane gave 4-alkoxy-2-arylquinolines with 15−50% yield.

Analytical Data for Selective Compounds (011, 121, and 140). (*E*)-3-(3,4-Dihydroxyphenyl)-1-(2-hydroxy-4,5-dimethylphenyl)prop-2-en-1-one (011). Following general procedure B, **011** was obtained from chalcone **013** as an orange solid (95% yield): mp 174−177 °C; LCMS purity 99%; ^{1}H NMR (DMSO-d_6,

dx.doi.org/10.1021/jm2012563 | J. Med. Chem. 2012, 55, 378−389

500 MHz) δ ppm 2.23 (s, 3H), 2.24 (s, 3H), 6.78 (s, 1H) 6.82 (d, J = 8.0 Hz, 1H), 7.23 (dd, J = 8.5, 2.0 Hz, 1H), 7.31 (d, J = 2.0 Hz, 1H), 7.72 (q, J = 15.5 Hz, 2H), 8.02 (s, 1H), 9.11 (br s, OH), 9.81 (br s, OH), 12.79 (s, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ ppm 18.72, 20.46, 116.18, 116.36, 117.94, 118.45, 118.69, 123.19, 126.65, 127.59, 130.91, 146.04, 146.12, 146.92, 149.59, 161.23, 193.36. LCMS: m/z 285 ([M + H]$^+$). MS (ESI): m/z calcd for $C_{17}H_{16}NO_4$ ([M + H]$^+$) 285.1082; found 285.1001 ([M + H]$^+$).

4-(4-(Cyclopentyloxy)quinolin-2-yl)benzene-1,2-diol (121). Following general procedure G, **121** was obtained as a dark yellow solid (16% yield): mp 199−201 °C; LCMS purity 98%; ^{1}H NMR (DMSO-d_6, 500 MHz) δ ppm 1.66 (m, 2H), 1.79 (m, 2H), 1.89 (m, 2H), 2.07 (m, 2H), 5.30 (m, 2H), 6.85 (d, J = 8.5 Hz, 1H), 7.31 (s, 1H), 7.44 (t, J = 8.0 Hz, 1H), 7.54 (dd, J = 8.5, 2.0 Hz, 1H), 7.67 (t, J = 8.0 Hz, 1H), 7.75 (d, J = 2.0 Hz, 1H), 7.88 (d, J = 8.0 Hz, 1H), 8.04 (d, J = 7.5 Hz, 1H), 9.21 (brs, OH); ^{13}C NMR (DMSO-d_6, 125 MHz) δ ppm 24.19, 32.71, 80.12, 99.28, 115.02, 115.99, 119.41, 120.60, 121.97, 125.21, 128.93, 130.30, 131.11, 145.88, 147.71, 149.11, 157.92, 160.73. LCMS: m/z 322 ([M + H]$^+$). MS (ESI): m/z calcd for $C_{20}H_{19}NO_3$ ([M + H]$^+$) 322.1437; found 322.1412 ([M + H]$^+$).

3-Hydroxy-2-(3-hydroxy-4-(pyrrolidin-1-yl)phenyl)-4*H*-benzo[*h*]chromen-4-one (140). Following general procedure B, **140** was obtained from compound **139** as an orange red solid (50% yield): mp 223−225 °C; LCMS purity 98%; ^{1}H NMR (DMSO-d_6, 500 MHz) δ ppm 1.88 (s, 4H), 3.45 (s, 4H), 6.74 (d, J = 8.5 Hz, 1H), 7.85 (m, 5H), 8.04 (d, J = 8.5 Hz, 1H), 8.11 (d, J = 8.5 Hz, 1H), 8.68 (d, J = 7.5 Hz, 1H), 9.37 (s, 1H); ^{13}C NMR (DMSO-d_6, 125 MHz) δ ppm 25.16, 50.19, 114.38, 114.69, 117.94, 120.61, 120.74, 120.99, 122.54, 124.10, 124.87, 128.06, 128.87, 129.77, 135.37, 139.19, 140.29, 146.34, 146.44, 151.65, 172.19. LCMS: m/z 374 ([M + H]$^+$). MS (ESI): m/z calcd for $C_{23}H_{19}NO_4$ ([M + H]$^+$) 374.1386; found 374.1402 ([M + H]$^+$).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed spectral analysis for all other compounds, structures, tables, schemes, mass spectral analysis, ^{1}H NMR and ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 1 858 453-4100, extension 1932. E-mail: pmaher@salk.edu.

■ ACKNOWLEDGMENTS

This research was supported by grants from the not-for-profit Fritz B. Burns Foundation, NIH (Grant U01 NS060685), and the Alzheimer's Association (Grant IRG-07-58874). We thank Professor Edward Roberts at the Scripps Research Institute for his advice and LCMS analysis. We also thank members of NMR and mass spectral group at the Salk Institute.

■ ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); BCA, bicinchoninic acid assay; BSA, bovine serum albumin; CNS, central nervous system; DFCS, dialyzed fetal calf serum; DME, Dulbecco's modified Eagle's; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; EC_{50}, half maximal effective concentration; EtOAc, ethyl acetate; ESI, electrospray ionization; FCS, fetal calf serum; FDA, Food and Drug Administration; G3PDH, glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione; HBA, hydrogen bond acceptor; HBD, hydrogen bond donor; IAA, iodoacetic acid; LPS, lipopolysaccharide; LCMS, liquid chromatography−mass spectrometry; MS, mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMR, nuclear magnetic resonance; Nrf2, NF-E2 related factor 2; p-TSA, p-toluenesulfonic acid; rtPA, recombinant tissue-type plasminogen activator; ROS, reactive oxygen species; SAR, structure−activity relationship; SDS, sodium dodecyl sulfate; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS, Tris buffered saline; TMS, tetramethylsilane; THP, tetrahydropyran; tPSA, topological polar surface area; TEAC, Trolox equivalent activity concentration

■ REFERENCES

(1) Véronique, L.; Roger, V. L.; Go, A. S.; Lloyd-Jones, d. M.; Adams, R. J.; Berry, J. D.; Brown, T. M.; Carnethon, M. R.; Dai, S.; Simone, G.; Ford, E. S.; Fox, C. S.; Fullerton, H. J.; Gillespie, C.; Greenlund, K. J.; Hailpern, S. M.; Heit, J. A.; Ho, P. M.; Howard, V. J.; Kissela, B. M.; Kittner, S. J.; Lackland, D. T.; Lichtman, J. H.; Lisabeth, L. D.; Makuc, D. M.; Marcus, G. M.; Marelli, A.; Matchar, D. B.; McDermott, M. M.; Meigs, J. B.; Moy, C. S.; Mozaffarian, D.; Mussolino, M. E.; Nichol, G.; Paynter, N. P.; Rosamond, W. D.; Sorlie, P. D.; Stafford, R. S.; Turan, T. N.; Turner, M. B.; Wong, N. D.; Wylie-Rosett, J. Heart disease and stroke statistics—2011 update: a report from the American Heart Association. *Circulation* **2011**, *123*, e18−e209.

(2) Donnan, G. A.; Fisher, M.; Macleod, M.; Davis, S. M. Stroke. *Lancet* **2008**, *371*, 1612−1623.

(3) (a) Lipton, P. Ischemic death in brain neurons. *Physiol. Rev.* **1999**, *79*, 1431−1568. (b) Pandya, R. S.; Mao, L.; Zhou, H.; Zhou, S.; Zeng, J.; Popp, A. J.; Wang, X. Central nervous system agents for ischemic stroke: neuroprotection mechanisms. *Cent. Nerv. Syst. Agents Med. Chem.* [Online early access]. DOI: 10.2174/187152411796011321, Apr 27, **2011**.

(4) Green, A. R.; Shuaib, A. Therapeutic strategies for the treatment of stroke. *Drug Discovery Today* **2006**, *11*, 681−693.

(5) Maher, P. Modulation of multiple pathways involved in the maintenance of neuronal function during aging by fisetin. *Genes Nutr.* **2009**, *Sep*, 10.

(6) Maher, P.; Salgado, K. F.; Zivin, J. A.; Lapchak, P. A. A novel approach for new neuroprotective compounds for the treatment of dtroke. *Brain Res.* **2007**, *1173*, 117−125.

(7) Sogawa, S.; Nihro, Y.; Ueda, H.; Izumi, A.; Miki, T.; Matsumoto, H.; Satoh, T. 3,4-Dihydroxychalcones as potent 5-lipoxygenase and cyclooxygenase inhibitors. *J. Med. Chem.* **1993**, *36*, 3904−3909.

(8) Chu, H. W.; Wu, H. T.; Lee, J. Y. Regioselective hydroxylation of 2-hydroxychalcones by dimethyldioxirane towards polymethoxylated flavonoids. *Tetrahedron* **2004**, *60*, 2647−2655.

(9) Cabrera, M.; Simoens, M.; Falchi, G.; Lavaggi, M. L.; Piro, O. E.; Castellano, E. E.; Vidal, A.; Azqueta, A.; Monge, A.; López de Ceráin, A.; Sagrera, G.; Seoane, G.; Cerecettoa, H.; Gonzáleza, M. Synthetic chalcones, flavanones, and flavones as antitumoral agents: biological evaluation and structure−activity relationships. *Bioorg. Med. Chem.* **2007**, *15*, 3356−3367.

(10) Horie, T.; Tsukayama, M.; Kourai, H.; Yokoyama, C.; Furukawa, M.; Yoshimoto, T.; Yamamoto, S.; Watanabe-Kohno, S.; Ohataf, K. Syntheses of 5,6,7- and 5,7,8-trioxygenated 3′,4′-dihydroxyflavones having alkoxy groups and their inhibitory activities against arachidonate 5-lipoxygenase. *J. Med. Chem.* **1986**, *29*, 2256−2262.

(11) Qin, C. X.; Chen, X.; Hughes, R. A.; Williams, S. J.; Woodman, O. L. Understanding the cardioprotective effects of flavonols: discovery of relaxant flavonols without antioxidant activity. *J. Med. Chem.* **2008**, *51*, 1874−1884.

(12) Wang, Y.; Peng, C.; Liu, L.; Zhao, J.; Su, L.; Zhu, Q. Sulfuric acid promoted condensation cyclization of 2-(2-(trimethylsilyl) ethynyl) anilines with arylaldehydes in alcoholic solvents: an efficient one-pot synthesis of 4-alkoxy-2-arylquinolines. *Tetrahedron Lett.* **2009**, *50*, 2261−2265.

(13) Pangalos, M. N.; Schechter, L. E.; Hurko., O. Drug development for CNS disorders: strategies for balancing risk and reducing attrition. *Nat. Rev. Drug Discovery* **2007**, *6*, 521−532.

(14) Winkler, B. S.; Sauer, M. W.; Starnes, C. A. Modulation of the Pasteur effect in retinal cells: implications for understanding compensatory metabolic mechanisms. *Exp. Eye Res.* **2003**, *76*, 715−723.
(15) (a) Reshef, A.; Sperling, O.; Zoref-Shani, E. Activation and inhibition of protein kinase C protect rat neuronal cultures against ischemia−reperfusion insult. *Neurosci. Lett.* **1997**, *238*, 37−40. (b) Sperling, O.; Bromberg, Y.; Oelsner, H.; Zoref-Shani, E. Reactive oxygen species play an important role in iodoacetate-induced neurotoxicity in primary rat neuronal cultures and in differentiated PC12 cells. *Neursci. Lett.* **2003**, *351*, 137−140. (c) Rego, A. C.; Areias, F. M.; Santos, M. S.; Oliveira, C. R. Distinct glycolysis inhibitors determine retinal cell sensitivity to glutamate-mediated injury. *Neurochem. Res.* **1999**, *24*, 351−358. (d) Sigalov, E.; Fridkin, M.; Brenneman, D. E.; Gozes, I. VIP-related protection against iodoacetate toxicity in pheochromocytoma (PC12) cells: a model for ischemic/hypoxic injury. *J. Mol. Neurosci.* **2000**, *15*, 147−154. (e) Reiner, P. B.; Laycock, A. G.; Doll, C. J. A pharmacological model of ischemia in the hippocampal slice. *Neurosci. Lett.* **1990**, *119*, 175−178.
(16) (a) Biraboneye, A. C.; Madonna, S.; Laras, Y.; Maher, P.; Kraus, J. L. Potential neuroprotective drugs in cerebral ischemia: new saturated and polyunsaturated lipids coupled to hydrophilic moieties: synthesis and biological activity. *J. Med. Chem.* **2009**, *52*, 4358−4369. (b) Biraboneye, A. C.; Madonna, S.; Maher, P.; Kraus, J. L. Neuroprotective effects of *N*-alkyl-1,2,4-oxadiazolidine-3,5-diones and their corresponding synthetic intermediates *N*-alkylhydroxylamines and *N*-1-alkyl-3-carbonyl-1-hydroxyureas against in vitro cerebral ischemia. *Chem. Med. Chem.* **2010**, *5*, 79−85.
(17) Lipton, P. Ischemic death in brain neurons. *Physiol. Rev.* **1999**, *79*, 1431−1568.
(18) Reiner, P. B.; Laycock, A. G.; Doll, C. J. A pharmacological model of ischemia in the hippocampal slice. *Neurosci. Lett.* **1990**, *119*, 175−178.
(19) Taylor, B. M.; Fleming, W. E.; Benjamin, C. W.; Wu, Y.; Mathews, R.; Sun, F. F. The mechanism of cytoprotective action of lazaroids: I. Inhibition of reactive oxygen species formation and lethal cell injury during periods of energy depletion. *J. Pharmacol. Exp. Ther.* **1996**, *276*, 1224−1231.
(20) (a) Winkler, B. S.; Sauer, M. W.; Starnes, C. A. Modulation of the Pasteur effect in retinal cells: implications for understanding compensatory metabolic mechanisms. *Exp. Eye Res.* **2003**, *76*, 715−723. (b) Sperling, O.; Bromberg, Y.; Oelsner, H.; Zoref-Shani, E. Reactive oxygen species play an important role in iodoacetate-induced neurotoxicity in primary rat neuronal cultures and in differentiated PC12 cells. *Neursci. Lett.* **2003**, *351*, 137−140.
(21) Sperling, O.; Bromberg, Y.; Oelsner, H.; Zoref-Shani, E. Reactive oxygen species play an important role in iodoacetate-induced neurotoxicity in primary rat neuronal cultures and in differentiated PC12 cells. *Neursci. Lett.* **2003**, *351*, 137−140.
(22) (a) Gelderblom, M.; Leypoldt, F.; Steinbach, K.; Behrens, D.; Choe, C. U.; Siler, D. A.; Arumugam, T. V.; Orthey, E.; Gerloff, C.; Tolosa, E.; Magnus, T. Temporal and spatial dynamics of cerebral immune cell accumulation in stroke. *Stroke* **2009**, *40*, 1849−1857. (b) Pandya, R. S.; Mao, L.; Zhou, H.; Zhou, S.; Zeng, J.; Popp, A. J.; Wang, X. Central nervous system agents for ischemic stroke: neuroprotection mechanisms. *Cent. Nerv. Syst. Agents Med. Chem.* [Online early access]. DOI: 10.2174/187152411796011321, Apr 27, **2011**. (c) Lewerenz, J.; Dargusch, R.; Maher, P. Lactacidosis modulates glutathione metabolism and oxidative glutamate toxicity. *J. Neurochem.* **2010**, *113*, 502−504.
(23) (a) Tan, S.; Schubert, D.; Maher, P. Oxytosis: a novel form of programmed cell death. *Curr. Top. Med. Chem.* **2001**, *1*, 497−506. (b) Maher, P. A comparison of the neurotrophic activities of the flavonoid fisetin and some of its derivatives. *Free Radical Res.* **2006**, *40*, 1105−1111.
(24) Zheng, L. T.; Ock, J.; Kwon, B. M.; Suk, K. Suppressive effects of flavonoid fisetin on lipopolysaccharide-induced microglial activation and neurotoxicity. *Int. Immunopharmacol.* **2008**, *8*, 484−494.
(25) Sagara, Y.; Vanhnasy, J.; Maher, P. Induction of PC12 cell differentiation by flavonoids is dependent upon extracellular signal-regulated kinase activation. *J. Neurochem.* **2004**, *90*, 1144−1155.
(26) Hitchcock, S. A.; Pennington, L. D. Structure−brain exposure relationships. *J. Med. Chem.* **2006**, *49*, 7559−7583.
(27) Lewerenz, J.; Maher, P. Control of redox state and redox signaling by neural antioxidant systems. *Antioxid. Redox Signaling* **2011**, *14*, 1449−1465.
(28) Shia, C. S.; Tsai, S, Y.; Kuo, S. C.; Hou, Y, C.; Chao, P. D. Metabolism and pharmacokinetics of 3,3′,4′,7-tetrahydroxyflavone (fisetin), 5-hydroxyflavone, and 7-hydroxyflavone and antihemolysis effects of fisetin and its serum metabolites. *J. Agric. Food Chem.* **2009**, *57*, 83−89.
(29) Maher, P.; Dargusch, R.; Bodai, L.; Gerard, P. E.; Purcell, J. M.; Marsh, J. L. ERK activation by the polyphenols fisetin and resveratrol provides neuroprotection in multiple models of Huntington's disease. *Hum. Mol. Genet.* **2011**, *20*, 261−270.
(30) Maher, P.; Dargusch, R.; Ehren, J. L.; Okada, S.; Sharma, K.; Schubert, D. Fisetin lowers methylglyoxal dependent protein glycation and limits the complications of diabetes. *PLoS One* **2011**, *6*, e21226.
(31) Maher, P. A comparison of the neurotrophic activities of the flavonoid fisetin and some of its derivatives. *Free Radical Res.* **2006**, *40*, 1105−1111.
(32) Schreiber, E.; Matthias, P.; Muller, M. M.; Schaffner, W. Rapid detection of octamer binding proteins with "mini-extracts", prepared from a small number of cells. *Nucleic Acids Res.* **1989**, *17*, 6419.
(33) Pajouhesh, H.; Lenz, G. R. Medicinal chemical properties of successful central nervous system drugs. *NeuroRx* **2005**, *2*, 541−553.
(34) Lloyed, E. J.; Andrews, P. R. A common structural model for central nervous system drugs and their receptors. *J. Med. Chem.* **1986**, *29*, 453−462.