Synthesis and Structure-Activity Relationship Studies of 3-Substituted Indolin-2-ones as Effective Neuroprotective Agents

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Neurodegenerative diseases are a major health problem particularly among the elderly. Drugs to prevent or slow down the death of neurons are urgently needed but are currently unavailable. We previously reported that the c-Raf inhibitor, GW5074 {5-iodo-3-[(3′,5′-dibromo-4′-hydroxyphenyl) methylene]-2-indolinone}, is protective in tissue culture and in vivo paradigms of neurodegeneration. However, at doses slightly higher than those at which it is protective, GW5074 displays toxicity when tested in neuronal cultures. We report herein the synthesis, biological evaluation, and structure-activity relationship (SAR) studies of novel 3-substituted indolin-2-one compounds that are highly neuroprotective but lack the toxicity of GW5074. Of the 45 analogs tested in this study, compounds 7, 37, 39, and 45 were found to be the most potent neuroprotective and thus represent promising lead compounds for preclinical development for the treatment of neurodegenerative disorders.
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Key words: neuroprotection; indolone; neurons; neurodegenerative disease

Introduction

Neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Huntington's disease are a highly prevalent class of disorders that afflict the elderly. With the increasing average life span of humans, the incidence of individuals afflicted with neurodegenerative disorders is on the rise representing a major health problem. A commonality shared among this diverse set of disorders is the abnormal and progressive degeneration affecting selective neuronal population. It is this loss of neurons that unleashes the debilitating neurological deficits characterizing the disease. While treatments to ameliorate the symptoms associated with some of these diseases are available, they do not affect disease progression because they are incapable of slowing down neuronal death. The development of strategies to stop or reduce the pathological neuronal death is thus of great urgency. There is general agreement that neuronal death occurring in many neurodegenerative diseases is due to the aberrant activation of apoptosis. Hence, identifying inhibitors of the apoptotic process in neurons could have therapeutic value in the treatment of neurodegenerative pathologies.

Research over the last decade has led to the identification of a number of chemical and small-molecule biological molecules that inhibit apoptosis in tissue culture paradigms of neuronal apoptosis. Among these are inhibitors of a variety of pro-apoptotic signaling molecules including kinases such as c-Jun N-terminal kinases (JNKs), glycogen synthase kinase-3 (GSK-3) and cyclin-dependent kinases (CDKs), the p53 tumor-suppressor and the Bcl2 family member BAD [reviewed in 1]. Many of these pharmacological inhibitors have also been tested in animal models of various neurodegenerative diseases and shown to be efficacious [1].

We recently demonstrated that a cell-permeable chem-

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ical inhibitor of c-Raf called GW5074 {5-iodo-3-[(3′,5′-dibromo-4′-hydroxyphenyl)methylene]-2-indolinone}, completely inhibits the death of cultured cerebellar granule and cortical neurons induced by a variety of different apoptotic stimuli [2]. GW5074 also prevents striatal degeneration and improves behavioral performance in mice administered with 3-nitropropionic acid, a commonly used *in vivo* paradigm of Huntington's disease. Although GW5074 is a versatile and potent neuroprotective agent, it does have a major drawback in that it is neurotoxic at concentrations that are not much higher than those at which it is protective [2]. In this study we have performed a structure activity relationship using GW5074 as the starting compound. The objective was to identify substituent groups that confer neuroprotective efficacy, as well as chemical groups that contribute to toxicity.

Materials and Methods

Materials. All cell culture media and reagents were from Invitrogen (Carlsbad, CA). Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and anhydrous solvents were from Fisher Scientific (Pittsburgh, PA). Antibody against active caspase-3 was from Cell Signaling Technology (Beverly, MA).

Chemistry. All products were obtained as crude solids which were purified by recrystallization from ethanol. NMR spectra were recorded by Brucker-400MHz and JEOL-500MHz Spectrometer. Chemical shifts are reported in parts per million (δ) downfield from TMS. Coupling constants are reported in hertz (Hz). Elemental analyses were performed on a Thermo Finnigan CE Elantech Model Flash EA1112 elemental analyzer with a Model MAS200R auto sampler. Observed C, H, and N elemental analysis of all compounds were within ± 0.4% of calculated values.

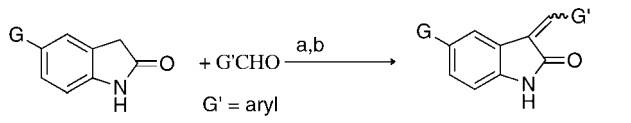
Chemical Synthesis of 3-(Benzylidene)indolin-2-ones. As shown in Table 1, a wide variety of 3-benzylidenes (**1**–**6**) and 5-substituted (3-benzylidene)indolin-2-ones (**7**–**34**) were prepared according to Scheme 1.

The 3-benzylidene derivatives **1**, **7**, **16**, **21**, **30**, and **34** were prepared according to the method (A) of Andreani *et al*. [3]. The remaining compounds **2**–**6**, **8**–**15**, **17**–**20**, **22**–**29**, and **31**–**33**, were prepared by the method (B) of Sun *et al*. [4].

Also listed in Table 1 are the % of *Z* isomer and chemical shift of the 2′,6′-H of the *Z* and *E* isomers. The ^{1}H NMR spectra are in agreement with assigned structures as determined by NOE experiments for all compounds having a 2′-H or 2′6′-H. As shown in Table 1, the chemical shifts of the 2′,6′-H in the *Z* configuration ranged from 7.76 to 8.78 and from 7.59–8.80 for the *E* configuration. In the case of the 2′,6′dichloro derivative (**11**), X-ray analysis confirmed that **11** existed in the *E* configuration.

Interestingly, with the exception of the 5-nitro, 5-aceto and 3-(2′,6′)-dichloro derivatives, the other benzylidenes were unstable in DMSO-d_6 changing slowly to give

Scheme-1



G = H, Cl, Br, F, NO_2, MeCO

Compounds 1-34

a. Method A = NaOAc / HOAc, reflux 3 h
b. Methyl B = piperidine, EtOH, refux 3-5 h

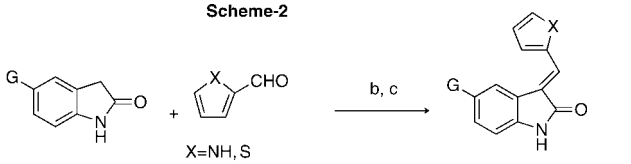
mixtures of *Z* and *E* isomers. To obtain some information on the role of 5-substituents on the isomerization we studied the 3′,5′-dibromo-4′-hydroxy derivatives (**1**, **7**, **16**, **21**, **30** and **34**) which were readily soluble in DMSO-d_6. With the exception of the 5-nitro (**21**) and the 5-aceto (**34**) derivatives, the initial NMR spectra (taken within 5 min of preparation) were mainly in the *E* configuration but slowly changed to give mixtures of *E* and *Z* isomers mainly in favor of the *Z* isomer. Thus, the % of Z configuration of **1** changed from 10% to 50% after 6 h from mixing, whereas the % of *Z* configuration of **7** and **16** changed from 5% to 70% and from 5% to 80%, respectively. In the exceptional cases, **21** existed as a 70:30 mixture of *Z* and *E* isomers, respectively, even after standing for 6 h.

Chemical Synthesis of 3-(Hetarylmethylene)indolin-2-ones. As shown in Table 2, the six 3-(1 *H*-pyrrol-2-yl) (**35**–**40**) and 3-(thiophene-2-yl) (**41**–**44**) derivatives were prepared by method B [4] and 3-(furan-2-yl) (**45**) were prepared by method C of Xiong *et al*. [5] shown in Scheme 2.

Additionally, the proposed structures listed in Table 2 (compounds **35**–**45**) were confirmed by ^{1}H- and ^{13}C-NMR spectroscopy and elemental analyses. Also listed in Table 2 are the percentages of *Z* isomers and chemical shift of the vinyl hydrogen of the *Z* and *E* isomers which were obtained by NOE experiments. As shown, the pyrrol-2-yl and 2-thienyl analogs exist exclusively in the *Z* configuration. Previous workers have observed similar results [4]. This is most likely due to stabilizing intramolecular H-bonding between the NH and C=O groups. However, the furan-2-yl analogs exist exclusively in the *E* configuration presumably due to unfavorable electronic pair interactions of the furan oxygen atom and C=O group that would exist in the *Z* configuration.

Culturing of Neurons and Treatments. Granule neuron cultures were obtained from dissociated cerebella of

Scheme-2



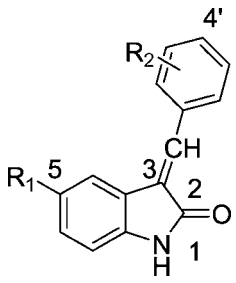
G = H, Cl, Br, F, NO_2, MeCO

Compounds **35-44**

b. Method B = piperidine, EtOH, refux 3-5 h

c. Method C = piperidine/MeOH, rt to O^0C/ 18 h

Table 1. Neuroprotective Effects of 3-(Substituted Benzylidenyl)-2-ones



Control	% neuronal survival Mean ± SD
HK	100.00 ± 1.47
LK	47.23 ± 7.585

Substituents			NMR data Chemical shift, ppm			% neuronal survival Mean ± SD		
ID	R_1	R_2	%, *Z* isomer	*Z* isomer 2′,6′-H	*E* isomer 2′,6′-H	1 μM	5 μM	25 μM
1	H	3′,5′-Br-4′-OH	10[a]	8.78	7.87	69.7 ± 2.19*	88.4 ± 11.74*	84.6 ± 7.57*
2	H	3′,5′-Br	10	8.69	7.85	54.7 ± 5.32	45.4 ± 4.32	46.9 ± 12.30
3	H	3′,4′,5′-OMe	90	7.98	7.03	50.8 ± 7.50	59.9 ± 7.98	61.3 ± 11.35
4	H	CH=CH-C_6H_4	50	7.75	7.40	86.0 ± 2.03*	83.6 ± 18.88	81.1 ± 6.22*
5	H	2′,6′-Cl	0	-	-	54.6 ± 10.27	52.9 ± 3.94	24.5 ± 12.38
6	H	2-NO_2	80	7.76	6.98	75.8 ± 3.20*	76.6 ± 7.14	81.5 ± 13.72
7	Cl	3′,5′-Br-4′-OH	5[b]	8.74	7.87	84.3 ± 1.04*	91.9 ± 5.31*	81.8 ± 14.94*
8	Cl	3′,5′-Br	90	8.59	7.79	77.7 ± 17.51	68.5 ± 11.86	64.4 ± 9.65
9	Cl	3′,5′-Br-4′-OAc	20	8.49	7.84	73.0 ± 5.59*	84.6 ± 16.19*	75.2 ± 31.54
10	Cl	3′,4′,5′-OMe	80	7.97	7.02	81.1 ± 9.81*	71.0 ± 7.26*	57.1 ± 10.13
11	Cl	2′,6′-Cl	0	-	-	56.4 ± 14.41	63.8 ± 3.46	45.7 ± 24.05
12	Cl	H	6	8.30	7.69	58.6 ± 3.27	63.8 ± 8.63	41.1 ± 21.41
13	Cl	4′-CH_3	20	8.49	7.56	70.0 ± 17.50	70.6 ± 13.86	67.4 ± 17.40
14	Cl	4′-OMe	4	8.55	7.69	84.9 ± 6.28*	85.6 ± 4.49*	81.6 ± 9.09*
15	Cl	4′-NMe_2	4	-	7.63	51.8 ± 15.66	55.8 ± 16.94	79.7 ± 4.69*
16	Br	3′,5′-Br-4′-OH	5[c]	8.74	7.55	88.4 ± 0.71*	98.7 ± 3.85*	*e*
17	Br	3′,5′-Br	90	8.59	7.57	54.7 ± 8.56	45.4 ± 3.05	46.9 ± 7.74
18	Br	3′,4′,5′-OMe	90	8.01	7.06	50.8 ± 7.67	59.9 ± 12.94	61.3 ± 1.20
19	Br	4′-OMe	10	8.45	7.66	57.1 ± 4.51	59.5 ± 9.10	60.5 ± 9.56
20	Br	4′-NMe_2	10	8.43	7.59	56.5 ± 2.28	54.9 ± 9.18	68.6 ± 9.38
21	NO_2	3′,5′-Br-4′-OH	70[d]	8.77	8.01	79.9 ± 12.81	78.4 ± 31.66	71.3 ± 19.80
22	NO_2	3′,5′-Br	90	8.63	7.72	78.1 ± 5.82*	78.7 ± 15.86	74.5 ± 9.12
23	NO_2	3′,4′,5′-OMe	90	8.07	7.16	76.6 ± 15.98	61.9 ± 5.87	69.0 ± 11.26
24	NO_2	2′,6′-Cl	0	-	-	76.4 ± 9.84*	69.9 ± 11.25	70.7 ± 8.50
25	NO_2	H	90	8.40	7.33	80.1 ± 18.01	78.4 ± 17.54	54.3 ± 3.63
26	NO_2	CH=CH-C_6H_5	-	7.79	7.59	80.1 ± 4.21*	78.6 ± 13.04	83.9 ± 9.28*
27	NO_2	CH=CH-C_6-H_4-2′NO_2	0	-	-	78.6 ± 6.84*	51.9 ± 7.50	78.6 ± 16.33
28	NO_2	4′-Me	90	8.33	7.67	54.6 ± 7.48	60.0 ± 1.87	54.3 ± 0.49
29	NO_2	4′-NMe_2	90	8.48	7.68	70.4 ± 9.37	67.6 ± 10.31	66.9 ± 9.86
30	F	3′,5′-Br-4′-OH	5[c]	8.74	7.87	49.3 ± 10.34	69.6 ± 8.96	18.1 ± 11.00*
31	F	3′,5′-Br	30	8.59	7.88	38.4 ± 6.24	46.1 ± 4.00	48.1 ± 14.65
32	F	3′,4′,5′-OMe	70	7.98	7.02	59.2 ± 1.77	51.9 ± 7.14	31.9 ± 1.77
33	F	H	10	8.35	7.65	53.4 ± 2.12	51.9 ± 15.56	31.5 ± 17.51
34	COMe	3′,5′-Br-4′-OH	90[d]	8.80	7.96	96.8 ± 6.15*	110.3 ± 8.50*	2.2 ± 1.63*

Shown are preferred configurations determined by NMR experiments along with the effects of each of the compounds on neuronal survival in LK medium. Each compound was tested at 3 concentrations (1, 5 and 25 μM) and added to LK medium. Survival is represented as % of survival in control cultures which received HK medium. In LK medium without any additives, survival was about 50%. Data represent mean values from at least 3 experiments each of which was performed in duplicate.

[a] Changed to 50% Z after 6 h. [b] Changed to 70% Z after 6 h. [c] Changed to 80% Z after 6 h. [d] No change after 6 h.

[e] Could not be accurately evaluated because of precipitation of the compound in the culture medium.

* $P < 0.05$ compared with viability of culture receiving LK.

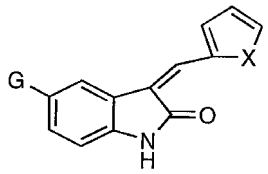
7–8 day old Wistar rats as described previously [6]. Cells were plated in Basal Eagle's Medium with Earles salts (BME) supplemented with 10% fetal calf serum (FCS), 25 mM KCl, 2 mM glutamine (Invitrogen), and 100 μg/ml gentamycin on dishes (Nunc) coated with poly-L-lysine in 24-well dishes at a density 1×10^6 cells/well. Cytosine arabinofuranoside (10 μM) was added to the culture medium 18–22 h after plating to prevent replication of non-neuronal cells. Previous immunocytochemical analyses by our lab and other investigators have shown that these cultures have high purity containing over 95% granule neurons [7–9].

The neuronal cultures were maintained for 7–8 days prior to experimental treatments. For this, the cells were rinsed once and then maintained in low K+ medium (serum-free BME medium, 5 mM KCl; referred to as LK), or in the case of control cultures, in high K+ medium (serum-free BME medium, supplemented with 20 mM KCl; referred to as HK). For treatments, the chemical compounds (dissolved in dimethylsulfoxide) were added directly to LK medium at the time of the switch from HK at concentrations of 1, 5, or 25 μM. Viability was assessed 24 hours later. Each compound was tested in duplicate (at each of the concentrations) and the experiment repeated at least 3 times. Although all compounds were solubilized in dimethylsulfoxide (DMSO), the amount of DMSO in the cultures never exceeded 0.1% (v/v). We have previously confirmed that DMSO itself has no effect on neuronal viability when used at dilutions of over 1:1000.

Evaluation of Neuroprotection by DAPI Staining. The viability status of neuronal cultures treated with HK, LK, or LK medium supplemented with various compounds was evaluated by phase contrast microscopy and quantified by staining cell nuclei with 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) as previously described [10, 11]. Briefly, the cells were fixed in 4% paraformaldehyde for 20 min at 4°C. After washing in phosphate buffered saline, DAPI (1 μg/ml in phosphate buffered saline) was added for 15 min at room-temperature and viewed under ultraviolet light (260 nm). Cells with condensed or fragmented nuclei were scored as dead. Viability has been expressed as percent of control cultures, which were switched to HK medium. Statistical analysis was performed using an unpaired, two-tailed Student's *t* test, compared to mean neuronal survival of control cultures receiving LK treatment.

TUNEL Staining and Active Caspase-3 Immunocytochemistry to Evaluate Neuronal Viability. The TUNEL assay of neuronal cultures was performed 24 h after treatment of the cultures using DeadEnd™ Fluorometric TUNEL System from Promega (Madison, WI) according to the manufacturer's instructions. For immunocytochemical analysis of active caspase 3, neuronal cultures cells were fixed and treated with 0.2% Triton for 5 minutes. After blocking with PBS containing 5% BSA and 5% goat

Table 2. Neuroprotective Effects of Substituted 3(2-Hetaryl)-2-ones[a]



Control	% neuronal survival Mean ± SD
HK	100.00 ± 1.47
LK	47.23 ± 7.585

Substituents			NMR data Chemical shift, ppm			% neuronal survival Mean ± SD		
ID	G	X	% Z	1-vinyl H	H-4	1 μM	5 μM	25 μM
35	H	NH	100	7.70	7.59	56.0 ± 17.21	51.1 ± 7.06	3.88 ± 9.58*
36	Cl	NH	100	7.85	7.74	71.2 ± 0.02*	84.0 ± 16.97*	82.6 ± 10.18*
37	Br	NH	100	7.86	7.84	75.5 ± 14.58	94.2 ± 0.15*	93.0 ± 1.38*
38	COMe	NH	100	7.95	8.26	69.5 ± 12.61	87.3 ± 22.50	87.1 ± 24.50
39	NO_2	NH	100	8.13	8.56	84.4 ± 3.41*	90.1 ± 6.55*	87.9 ± 1.40*
40	F	NH	100	7.85	7.70	58.4 ± 3.98	65.7 ± 0.07	96.6 ± 1.91*
41	H	S	100	7.96	8.13	68.3 ± 13.16	77.1 ± 13.16	86.2 ± 4.03*
42	NO_2	S	100	8.63	8.52	75.1 ± 9.11*	67.6 ± 2.29	66.9 ± 10.30
43	Br	S	100	8.22	7.90	56.8 ± 5.62	58.3 ± 9.82	66.4 ± 4.56
44	F	S	100	8.15	7.56	53.6 ± 14.24	60.1 ± 10.28	68.7 ± 9.35
45	H	O	0	7.31	8.35	70.4 ± 8.35	82.8 ± 5.22*	90.2 ± 3.91*

[a] Shown are preferred configurations determined by NMR experiments along with the effects of each of the compounds on neuronal survival in LK medium. Each compound was tested at 3 concentrations (1, 5 and 25 μM) and added to LK medium. Survival is represented as % of survival in control cultures which received HK medium. In LK medium without any additives, survival was about 50%. Data represent mean values from at least 3 experiments, each of which was performed in duplicate.

* $P < 0.05$ compared with viability of culture receiving LK.

serum in PBS for 30 minutes, the coverslips were incubated with the active capase-3 primary antibody overnight at 4°C. After three washes with phosphate-buffered saline (PBS), the cells were incubated with secondary antibodies for 45 minutes at 25°C after which the cells were washed with PBS. To visualize nuclei, cells were stained with DAPI for 15 minutes at 25°C.

Results

Granule neurons can be cultured from the postnatal rodent cerebellum at a high level of homogeneity and maintain their biochemical and electrophysiological properties *in vitro* [7–9]. When switched from HK medium (containing 25 mM KCl) in which they are normally maintained *in vitro*, to LK medium (containing 5 mM KCl), these neurons die by apoptosis, killing about 50% of the neurons within 24 hours [6, 12–15] (also see Fig. 1). This experimental model of neuronal apoptosis has been used widely to understand the molecular mechanisms underlying neurodegeneration and to identify biological and chemical agents with neuroprotective efficacy [16–18]. We tested all compounds synthesized as part of the present study in this paradigm. Each compound was tested at three different concentrations—1, 5, and 25 μM. The 25 μM concentration was included to evaluate neurotoxicity. As we have previously reported, GW5074, a compound that is neuroprotective at 1 μM starts displaying toxicity at doses of $\geq$ 5 μM and is highly toxic at 25 μM [2]. Neuronal viability was quantified by DAPI-staining, a common and reliable assay of apoptotic cell death (Fig. 1). In some cases, the results obtained from DAPI-staining were confirmed by two other assays of apoptosis—TUNEL staining and active caspase-3 immunocytochemistry (Fig. 1). Our goal in this study was to identify 3-substituted indolones that are protective at 1–5 μM but that displayed no toxicity when used at 25 μM.

We first looked at substituent effects on the neuroprotective ability of 3-(3′,5′-dibromo-4′-hydroxybenzylidene)indolin-2-one (**1**). As shown in Table 1, unlike GW5074, compound **1** is moderately effective and not toxic. Further, the importance of an acidic phenol was determined by observing that the absence of a 4′-phenolic group as in 3′,5′-dibromo (**2**) and 3′,4′,5′-trimethoxy derivatives (**3**) are slightly toxic at 5 and 25 μM but not protective at any of the three concentrations tested. The results in Table 1 show also that the 3-phenylallylideneindolin-2-one (**4**) was moderately protective and non-toxic and with the exception of the 2′-nitro derivative (**6**) which was moderately active at 25 μM, the 2′,6′-dichloro derivative (**5**) was ineffective and toxic.

We next looked at the influence of substitution at the 5-position on the activity of the indolin-2-one. As shown in Table 1 and Figure 1, the electronegative groups, *i.e.*, 5-acetyl (**34**), 5-bromo (**16**), 5-chloro (**7**), and 5-nitro (**21**) analogs are superior to **1**. For example, at 1μM and 5 μM **34** has the highest survival rate (96.8% and 110.3 % survival, respectively). However, this compound is highly toxic at 25 μM. At 5 μM, both 5-bromo (**16**) and 5-chloro (**7**) (98.7% and 91.9% survival, respectively) are highly effective and are not toxic. Unfortunately, **16** precipitated from DMSO at 25 μM precluding evaluation of its efficacy or toxicity at this concentration. Finally, the 5-fluoro derivative (**30**) is not effective and toxic. Given that the 5-iodo compound GW5074 is highly protective at 1 μM but toxic at 10 μm, our results indicate that electronegative substituents that are strong –I groups (electron-withdrawing by induction) but weak +R groups (electron-releasing by delocalization), such as Cl, Br, and NO_2 decrease the electron-density of the core indoline-2-one thus increasing the acidity of the 4′-hydroxy group. On the other hand, the 5-fluoro group being not only a strong –I but a strong +R increases the electron density of the core indoline-2-one as compared to the aforementioned strong –I groups and weak +R groups, which results in a less acidic 4′-hydroxy group thus rendering the 5-F derivative less neuroprotective.

We turned our attention to the preparation of the commercially available drug oxindole-1 (**35**) and some of its analogs. We have previously shown that **35** is quite effective at 10 μM, but highly toxic at higher concentrations. We were particularly interested in preparing the 5-Br (**37**), 5-Cl (**36**), 5-NO_2 (**39**), 5-acetyl (**38**) and the 5-F (**40**) derivatives to examine if these groups would similarly improve the neuroprotective efficacy of **35**.

The data in Table 2 show that the pyrrole and thiophene derivatives are non-toxic and, more importantly, that the 5-bromo analog (**37**) is one of the most potent effective neuroprotective agents discovered in this study. The remaining pyrrole derivatives have significant neuroprotective properties. Interestingly, furan (**45**) is highly effective at 25 μM and the fluoro derivative (**40**) is the most effective one at 25 μM.

Discussion

When cerebellar granule neuron cultures are switched to LK medium (lacking any neuroprotective chemical) about 50% of the cells die within 24 hours [6]. Several of the compounds tested in our analyses displayed a significant level of protection against LK-mediated neuronal death. Among these, **7**, **16**, **34**, **37**, **39**, and **45** were deemed to be highly neuroprotective ($\geq$ 90% survival in LK medium) at least one of the three doses (Fig. 1, Table 1, and Table 2). In the case of **34,** however, while exhibiting impressive neuroprotection at 1 μM and 5 μM concentrations, the drug is highly toxic at 25 μM (Fig. 1). Its high level of toxicity renders it unsuitable for consideration as a potential therapeutic agent. The efficacy of **16** which is highly protective at the two lower concentrations could not be evaluated at 25 μM because it was not completely soluble at this dose in the culture medium. In contrast, **4**, **7**, **14**, and **39** show solid neuroprotective activity providing at least 80% survival at all three concentrations utilized in this study. A

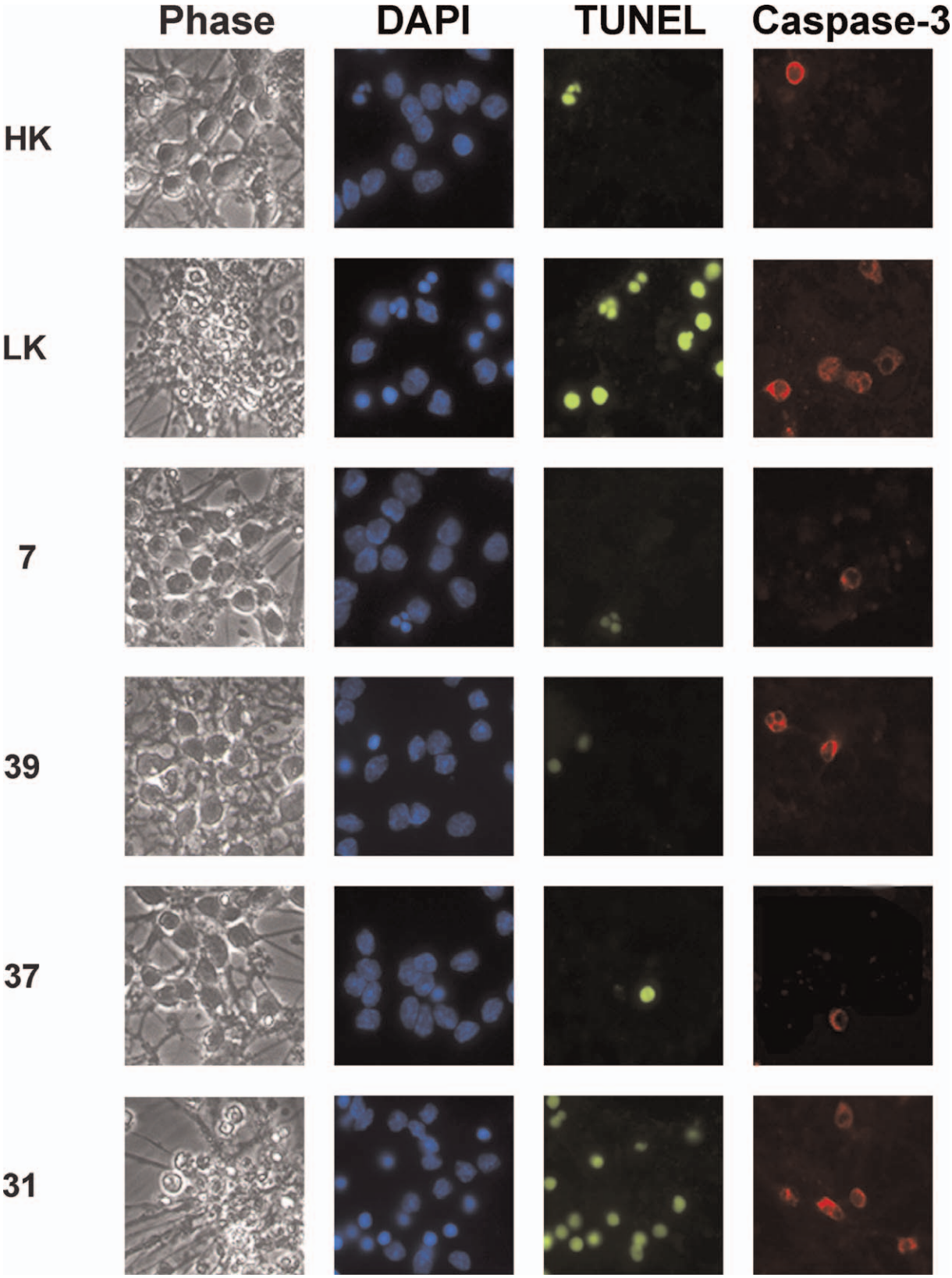


Figure 1. Neuroprotective effects of select compounds. Seven day-old cultures of cerebellar granule neurons were treated for with HK medium (HK), LK medium (LK), or LK medium containing various compounds at indicated doses. Results using compounds **7** (5 μm), **31** (1 μm), **37** (25 μm) and **39** (25 μm) are presented in the figure. From left to right columns show phase-contrast micrographs of neuronal morphology, DAPI-staining of nuclei (which stains condensed or fragmented nuclei), TUNEL staining (which stains nuclei with fragmented DNA), and active caspase-3 immunoreactivity (a marker of apoptosis). DAPI and TUNEL panels are taken from the same fields. In contrast to compounds **7**, **37**, and **39**, compound **31** is not protective. A color version of the figure is available in the online journal.

number of compounds displayed protection of over 70% survival at all three concentrations at which they were tested. These include **6**, **9**, **21**, **22**, **26**, **36**, and **45**. These compounds also have promise as neurotherapeutic agents. Although DAPI-staining, a reliable and extensively used assay of apoptosis, was utilized for quantification of neuroprotection, key results were confirmed using two independent viability assays of apoptosis—TUNEL staining and active caspase-3 immunoreactivity.

Based on our structure-activity studies the following conclusions or trends can be drawn:

4-OH is absolutely necessary for high activity of the 3′,5′-dibromophenyl compounds. For example, removal of the 4′-hydroxy group activity from **1** gives the 3,5-dibromo derivative (**2**) which is non-effective. Also, removal of the 4′-OH group from the 5-chloro derivative (**7**) gives **8**, which is substantially less effective. Also conversion of the 4-OH to acetyl ester (**9**) and and the 3′,4′,5′-trimethoxy derivative (**3**) have reduced activity as compared to the moderately active 5-chloro analog (**7**) and the 5-bromo (**18**) and 5-fluoro (**32**) are not effective.

The activity of CH=CH-C_6H_4 (**4**) and 5-nitrophenyl derivative (**25**) are moderately efficacious at all three concentrations used; however, the 5-F (**33**) and 5-Cl (**12**) derivatives are not effective. Substituents on the 4-position of the phenyl ring gave mixed results. For example, the 5-bromo derivative (**19**) was not effective. However, the 5-Cl derivative (**14**) was moderately active and non-toxic. Lastly, the introduction of a 4-dimethylamino group phenyl ring on the 4-dimethylamino group on the 5-bromo position (**20**) is not effective. However, substitution of a 2-nitro group on the benzene ring (**6**) results in moderate activity at high doses.

5-Substituents that are strongly electron withdrawing by induction increase the activity of the 3′,5′-dibromo-4′-hydroxyphenyl (**1**) compounds with Br (**16**) and Cl (**7**) increasing to over 90%, NO_2 (**21**) has modest neuroprotective activity and the 5-acetyl group (CH_3CO) in **34** is highly protective at lower dosages but also highly toxic at 25 μM. These compounds probably increase the acidity of the 4-OH group. However, the F (**30**) derivative is inactive and very toxic presumably due to the strong electron-releasing by resonance effect of F which decreases the acidity of the 4-OH group.

3-(1*H*-Pyrrol-2-yl) (**35**) has no protective effect and is highly toxic at 25 μM. Protective activity is provided by electron-withdrawing groups on the 5 position with 5-Br (**37**) and 5-NO_2 (**39**) having activity over 90% and, interestingly F (**40**) has highest neuroprotective activity among these compounds. 5-Cl (**36**) and COMe (**38**) have moderate protective activity.

Other 5-membered heterocycles on the 3-position have interesting effects. The thiophene derivative (**41**) is moderately active at high doses whereas the 5-fluoro-3-(thiophen-2-yl) (**44**) offers low protection. Interestingly the 3-(furan-2–yl) derivative (**45**) is highly protective at high dosages.

The configuration of the 5-membered heterocyclics does not appear to be important since both compound **35**, which exist as the Z-isomer, and **45**, which exists as the *E*-isomer, have excellent activity.

Taken together, our SAR analyses have yielded a number of compounds that display impressive protection but that lack toxicity at any of the concentrations used in this study. Substantial progress has thus been made over GW5074, which is neurotoxic at doses that are 5 times higher than the protective concentration [2]. It remains to be seen if the neuroprotective compounds identified in this are efficacious *in vivo*. If they are effective in animal models, they would be candidate therapeutic agents for pre-clinical testing against human neuropathological conditions.

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