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Flavaglines as Potent Anticancer and Cytoprotective Agents

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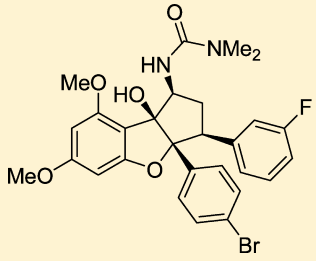
Supporting Information



cytotoxicicity on cancer cells: IC_{50} = 2-4 nM
cardio- and neuroprotective effects at 1-10 nM

ABSTRACT: Flavaglines represent a family of plant natural products that display potent anticancer, cardioprotective, and neuroprotective activities. Novel flavagline derivatives were synthesized and examined for their cytotoxicity on a panel of human cancer cell lines, their cardioprotection against doxorubicin-induced apoptosis in cardiomyocytes, and their neuroprotection in culture models of Parkinson's disease and cisplatin-induced neurotoxicity. The structural requirements of flavaglines for cardio- and neuroprotection were for the first time unraveled and appeared to be slightly different from those for cytotoxicity on cancer cells. We provide also the first evidence that flavaglines may alleviate cisplatin-induced neurotoxicity, suggesting a prophylactic potential of these compounds to prevent this frequently encountered adverse effect of cancer chemotherapies.

INTRODUCTION

Found in Asian medicinal plants, flavaglines are cyclopenta[*b*]-benzofurans that exhibit a unique profile of pharmacological activities.[1,2] In addition to potent cardioprotective[3] and neuroprotective activities,[4] flavaglines exhibit impressive and selective anticancer activities,[1,2,5−10] their cytotoxicity affecting only cancer cells. Flavaglines also potentiate the efficacy of chemotherapies in several mouse models of cancers. Importantly, these compounds show no sign of toxicity in vivo. To date, the molecular target and the detailed mechanism of action of these compounds remain largely unknown even though Li-Weber and colleagues recently demonstrated that flavaglines inhibit the MEK-ERK-eIF4E signaling pathway and consequently the cap-dependent synthesis of many proteins involved in cell division and resistance to apoptosis.[5] During the course of our research on the structure−activity relationship (SAR) of flavaglines, we identified FL3 (**2**) and FL23 (**4**) that inhibit cell proliferation and viability (IC50 ≈ 1 nM) at lower doses than did the parent natural compound, rocaglaol (**1**) (Figure 1).[11]

We also demonstrated that flavaglines may induce apoptosis in cancer cells through the Apoptosis Inducing Factor (AIF) and caspase-12, which suggest that these drugs might be of interest to treat tumors that are resistant to the classical intrinsic and extrinsic pathways of apoptosis. We also recently established that flavaglines protect mice against the cardiotoxicity of anthracylines.[3] Despite their effectiveness in the treatment of cancers, the use of anthracyclines is limited by a potentially fatal cardiomyopathy which remains a major problem in the clinical management of cancers.[12] Compound **2** efficiently protected not only cardiomyocytes in culture but also mice from the cardiotoxicity of the most widely used anthracycline, doxorubicin. Administration of flavagline **2** to mice attenuated cardiomyocyte apoptosis and cardiac fibrosis, as reflected by a 50% decrease of mortality, suggesting that flavaglines may enhance the efficacy and safety of anthracyclin-based chemotherapies.

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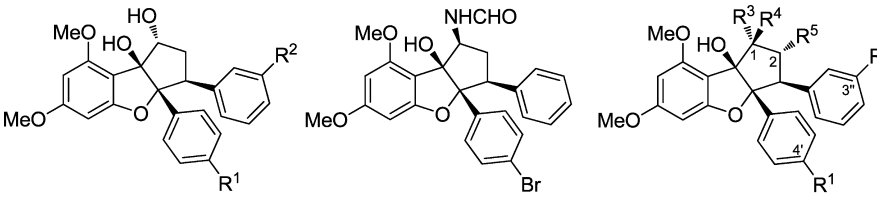
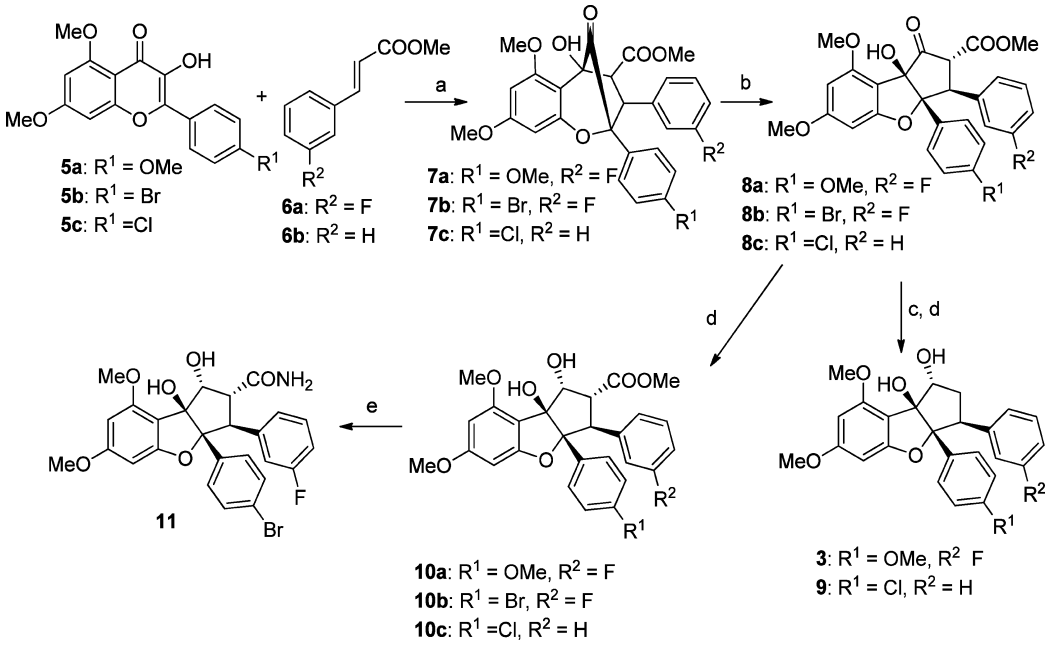


Figure 1. Structures of naturally occurring rocaglaol (**1**), FL3 (**2**), Bayer's flavagline (**3**), FL23 (**4**), and general structure of synthesized analogues (**A**).

Scheme 1[a]



[a]Reactants and conditions: (a) hν, CH_2Cl_2–MeOH, 0 °C, 15 h; (b) MeONa, MeOH, 60 °C, 20 min; (c) LiCl, H_2O, DMSO, 100 °C, 12 h; (d) $Me_4NBH(OAc)_3$, AcOH, CH_3CN; (e) NH_3, MeOH, 100 °C, 36 h.

Bayer's scientists also demonstrated the in vivo neuroprotective potential of flavagline **3** in cellular and animal models of stroke and Parkinson disease.[4] Yet, up to now, no flavagline other than **1**, **2**, or **3** has been described for its cardio- and neuroprotective effects.

Herein, we report the results of the first structure–activity relationship (SAR) trends for cardioprotection against doxorubicin-induced cardiotoxicity. Subsequent evaluation of the neuroprotective effects was performed using an in vitro models of Parkinson's disease as well. Furthermore, we demonstrated for the first time that flavaglines protect also neurons from cisplatin-induced apoptosis. Altogether, this set of data reinforces the view that flavaglines may enhance the efficacy of cancer chemotherapies and may also reduce in the same time their cardiac and neurological adverse effects.

CHEMICAL SYNTHESIS

To investigate the importance of the fluorine in position 3″ and the effect of a chlorine in position 4′, we first synthesized the reference compound **3** described by Bayer, and its analogues **9**–**11** following the biomimetic approach developed by John Porco (Scheme 1).[13,14] Photocycloaddition [3 + 2] of hydroxyflavone **5** with a cinnamic ester **6** afforded adduct **7** that underwent an acyloin rearrangement to give a diastereomeric mixture of ketoesters **8**. Reference compound **3** and its analogue **9** were then obtained by a decarboxylation followed by reduction with $Me_4NBH(OAc)_3$.[15] Reduction of **8** afforded the expected hydroxyesters **10**. Aminolysis of **10b** gave access to amide **11**.

The other flavaglines unsubstituted in position 2 were synthesized by the method originally developed by Taylor and optimized by Dobler (Scheme 2).[16] Condensation of benzofuranone **12** with 3-fluorocinnamaldehyde **13** afforded **14**. Formation of cyanohydrins, ring closure, and deprotection afforded the ketone **15**, which was reduced into alcohol **16** or condensed with *O*-methylhydroxylamine. The reduction of the oxime methyl ether **17** with borane, followed by an acylation step, afforded adducts **18a**–**j**.

BIOLOGICAL RESULTS

Protection of Cardiomyocytes against Doxorubicin-Induced Toxicity. The cardioprotective activity of newly synthesized flavaglines was examined on a rat H9c2 cardiomyoblast cell line that represents an established in vitro

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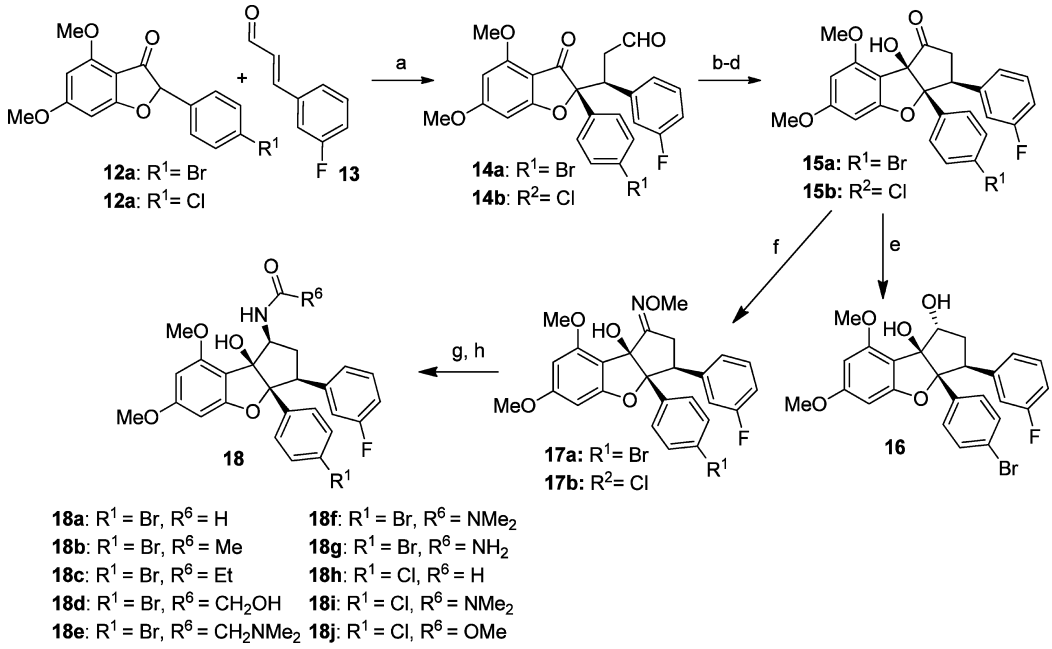
Scheme 2[a]



[a]Reactants and conditions: (a) Triton B, THF, rt, 1 h; (b) TMSCN, ZnI_2, MeCN, benzene, rt, 12 h; (c) LDA, THF, −78 °C, 1 h; (d) K_2CO_3, MeOH, rt, 15 min; (e) $Me_4NBH(OAc)_3$, AcOH, CH_3CN; (f) $H_2NOMe{\cdot}HCl$, pyr, EtOH, 70 °C, 4 h; (g) BH_3, THF, 66 °C, 12 h; (h) HCOOEt, CH_3COOH, THF, reflux, 12 h, or Ac_2O (or $(EtC(O))_2O$), DMAP, pyr, rt, or $HOCH_2COOH$ (or Me_2NCH_2COOH), EDCI, HOBt, DIPEA, DMF, rt, or Me_2NCOCl (or MeOCOCl), DIPEA, DMAP, CH_2Cl_2, rt, or TMSNCO, CH_2Cl_2, iPrOH, rt.

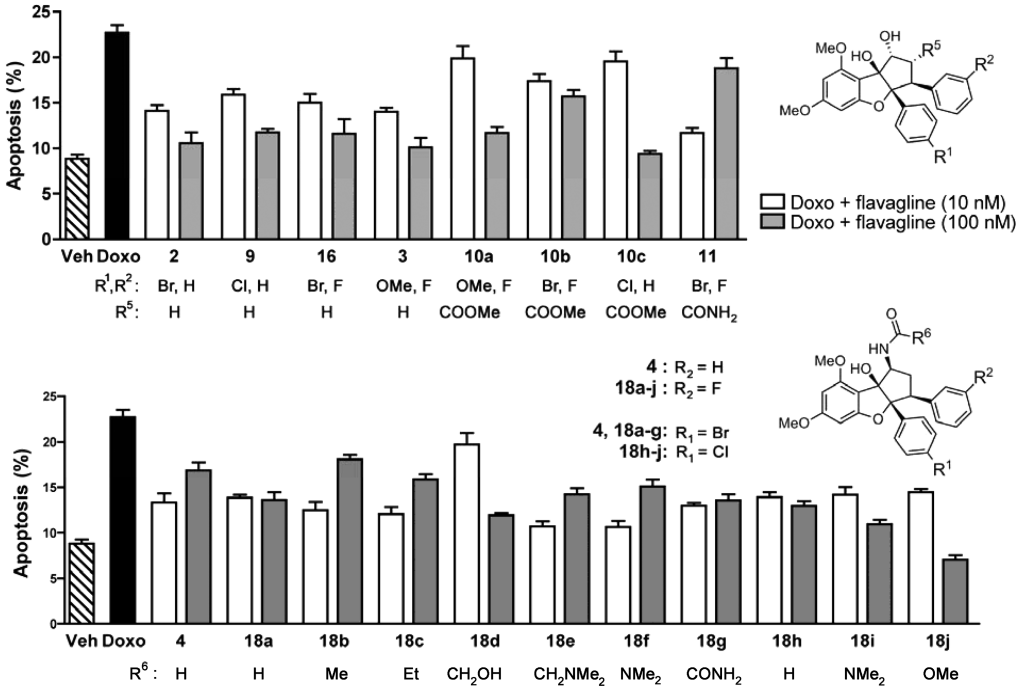


Figure 2. Cardioprotection. H9c2 cardiomyocytes were pretreated with flavaglines (10 or 10 nM) or their vehicle for 10 h and then treated with doxorubicin for additional 14 h (acute model of cardiotoxicity). Apoptosis was quantified by FACS analysis (annexin-V/propidium iodide, $n = 3$).

model of doxorubicin-induced cardiotoxicity (Figure 2).[3] Because bromoarenes are known to be potentially toxic, we examined whether the bromide in position 4′ could be replaced by a chloride (**9**). We also examined what is the effect of a fluorine in position 3″ (**16**), as in Bayer's compound **3**. We observed that **9** and **16** displayed a cardioprotection similar to that of reference compounds **2** and **3**, suggesting that, indeed, the 4-bromine may be replaced by a chlorine and also that the 3″-fluorine does not have a major influence on cardioprotection.

Next, we examined the effect of a substitution in position 2. Methyl esters **10a**, **10b**, and **10c** were less pronounced cardioprotectants than the cognate compounds **9**, **2**, and **16**. Surprisingly, amide **11** was more cardioprotectant at 10 than at

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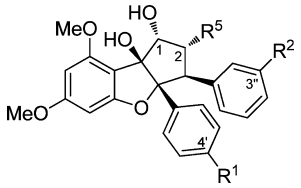
Table 1. Cytotoxicity of Flavaglines Analogues against Human Cancer Cell Lines (IC_{50}, nM)[a]



				cytotoxicity on cancer cell lines			
compd	R^1	R^2	R^5	HL60	HL60R	KB	HCT116
2	Br	H	H	3.8 ± 0.5	4.5 ± 0.4	2.4 ± 0.2	5.8 ± 0.5
9	Cl	H	H	5.9 ± 0.5	2.4 ± 0.4	6.6 ± 0.6	4.6 ± 0.6
16	Br	F	H	8.9 ± 1.1	3.1 ± 0.4	10 ± 0.9	5.3 ± 0.6
3	OMe	F	H	94 ± 4.5	60 ± 2.9	41 ± 6.4	23 ± 2.2
10a	OMe	F	COOMe	20 ± 3	28 ± 10	9.9 ± 1.5	5.2 ± 0.5
10b	Br	F	COOMe	3.2 ± 0.2	14 ± 4	2.8 ± 0.1	2.4 ± 0.2
10c	Cl	H	COOMe	29 ± 6	13 ± 0.7	2.4 ± 2.8	19 ± 1.4
11	Br	F	$CONH_2$	1.4 ± 0.1	3.3 ± 0.7	1.2 ± 0.1	1.2 ± 0.2
	doxorubicine			30	990	40	22
	taxotere			0.5	531	0.17	0.52
	vinblastine			1.11	>100	0.8	1.42

[a]Data are the average of two independent IC_{50} value determinations.[a]

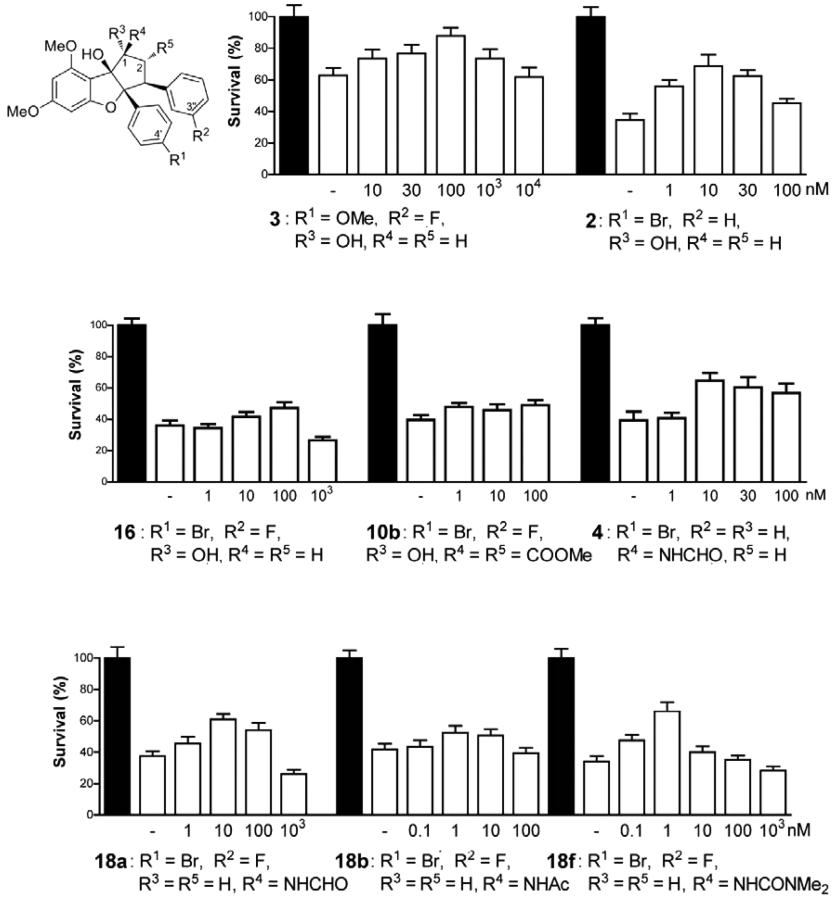


Figure 3. Effects of flavagline derivatives **2**, **3**, **4**, **10**, **16**, and **18a,b,f** in MPP^+-treated primary mesencephalic cultures. Cultures were exposed to 3 μM MPP^+ in the presence or absence of flavagline. The survival of dopaminergic neurons was evaluated by counting tyrosine hydroxylase (TH) immunopositive cells. Results are expressed in numbers of TH^+ cells per culture well (n = 3).

100 nM, suggesting a biphasic effect for this compound, the optimal concentration being around 10 nM, with equal but lesser inhibitions at 1 and 100 nM. Next, we examined the cardioprotective effect of epi-amides **4**, **18a–l**. At 10 nM, **4** induced the same cardioprotection as reference compound **2**, but this effect was lowered at a higher concentration (100 nM). Introduction of a fluoride in position 3″ maintained a good level of cardioprotection at 100 nM, without any repercussion

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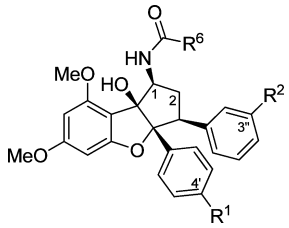
Table 2. Cytotoxicity of Flavaglines Analogues Against Human Cancer Cell Lines (IC_{50}, nM)[a]



compd	R^1	R^2	R^6	cytotoxicity on cancer cell lines			
				HL60	HL60R	KB	HCT116
4	Br	H	H	2.3 ± 0.1	1.7 ± 0.1	1.9 ± 0.1	1.8 ± 0.2
18a	Br	F	H	3.4 ± 0.4	1.4 ± 0.9	3.5 ± 0.2	1.9 ± 0.4
18b	Br	F	Me	10 ± 0.2	7.1 ± 0.6	8.4 ± 0.1	7.5 ± 0.6
18c	Br	F	Et	6.2 ± 0.7	4.9 ± 0.6	7.8 ± 0.5	5.3 ± 0.1
18d	Br	F	CH_2OH	31 ± 4	42 ± 3	37 ± 3	22 ± 0.7
18e	Br	F	CH_2NMe_2	4.8 ± 0.1	5.6 ± 0.1	5.8 ± 0.1	5.0 ± 0.3
18f	Br	F	NMe_2	4.1 ± 1	2.2 ± 0.1	3.6 ± 0.2	2.4 ± 0.5
18g	Br	F	NH_2	3.3 ± 0.1		2.9 ± 0.1	2.1 ± 0.1
18h	Cl	F	H	13 ± 0.8		10 ± 0.5	8.2 ± 0.4
18i	Cl	F	NMe_2	3.2 ± 0.5		2.1 ± 0.3	1.9 ± 0.1
18j	Cl	F	OMe	71 ± 2.9		50 ± 5.2	38 ± 2.5

[a]Data are the average of two independent IC_{50} value determinations.

at 10 nM (compare **18a** with **4**). Replacement of the formamide by an acetamide (**18b**) (Figure 4) or propionamide (**18c**) slightly increased cardioprotection at 10 nM but had the opposite effect at 100 nM. The introduction of a hydroxyacetamide (**18d**) was detrimental at 10 nM but well tolerated at 100 nM, while the substitution by a more polar dimethylaminoacetamide (**18e**) or dimethylurea (**18f**) significantly enhanced the cardioprotection. Urea **18g** was less cardioprotectant than dimethylurea **18f**, suggesting that the ability of the urea to give extra H-bonds is detrimental to the cardioprotective activity. Again, the replacement of the bromine in 4′-position was well tolerated (compare **18h** and **18i** with **18a** and **18f**). Interestingly, functionalization by a methylcarbamate greatly enhanced the cardioprotective efficacy: at the dose of 100 nM, **18j** totally abolished the cardiotoxicity induced by doxorubicin. This observation supports the preference for the substitution of the carbonyl by an electron-donating substituent that does not form additional H-bonds.

Cytotoxicity on Cancer Cells. The in vitro cytotoxicity was evaluated on a variety of human cancer cell lines from promyocytic neutrophils (HL60 and HL60R), nasopharynx (KB), and colon (HCT116) by the MTS assay after a 72 h treatment. Results are summarized in Table 1. Neither the replacement of the bromine in 4′-position in **2** by a chlorine (**9**) nor the introduction of a fluorine in position 3″ (**16**) significantly modified the cytotoxicity. Surprisingly, the introduction of a methyl ester moiety gave inconsistent results according to the nature of the substituent R^1 in position 4′: it improved cytotoxicity and decreased the sensitivity toward multidrug resistance when R^1 is a methoxy and had the opposite effects when R^1 is a bromine or a chlorine (compare **10a** with **3**, **10b** with **16**, and **10c** with **9**). Next, we pursued our SAR investigation with the study of analogues of formamide **4**, which displayed a cytotoxicity similar to that of the cognate alcohol **2**. Replacement of the formamide by a bulkier acetamide or propionamide slightly reduced cytotoxicity (compare **18b** and **18c** with **18a**). This deleterious effect was more pronounced with a hydroxyacetamide that may donate H bonds (**18d**). In contrast, the introduction of H-bond acceptors, such as a dimethylaminoacetamide (**18f**) or dimethylurea (**18f**), restored the activity observed with formamide **18a**. The more compact urea **18g** also retained an excellent activity. Again, in this series, the replacement of the bromine in position 4′ by a chlorine was well tolerated (compare **18h** with **18a** and **18i** with **18f**). The bioisosteric substitution of the dimethylurea moiety of **18i** with a less basic carbamate (**18j**) resulted in a significant loss of cytotoxicity.

Neuroprotection of Dopaminergic Neurons Using an in Vitro Model of Parkinson's Disease. Compound **3** had been shown by Bayer scientists to exert neuroprotective effects on dopaminergic neurons from primary mesencephalic cultures of rat embryos treated by MPP^+,[4] which represents an established in vitro model of Parkinson's disease. We confirmed that indeed **3** protects dopaminergic neurons, but surprisingly, this effect was biphasic, an optimal level of neuroprotection being observed at 100 nM (Figure 3). At 10 μM, this neuroprotective effect was totally abolished, which is in sharp contrast with the previous report. Interestingly, **2** induced a more pronounced neuroprotective effect at a lower concentration than **3**, with an optimal dose of 10 nM. As observed for cardioprotection, the substitution by a methyl ester moiety in C-2 was well tolerated (**10b**). Similar to **2**, epi-formamide **4** and its fluorinated derivative **18a** displayed a significant neuroprotective effect at an optimal dose of 10 nM (Table 2). Replacement of the formamide by bulkier substituents, such as an acetamide (**18b**), a propionamide (**18c**), a hydroxyacetamide (**18d**), or a dimethylaminoacetamide (**18e**), lowered the cytoprotective activity in a more drastic manner than what was observed for cardioprotection. Gratifyingly, the introduction of dimethylurea (**18f**) increased again significantly the cytoprotective activity, with an optimal effect at a concentration of 1 nM only. Furthermore, replacement of the bromine in position 4′ by a chlorine combined with the introduction of a fluorine on position 3″ (**18i**), attenuated the drop of activity observed at 10 nM. As previously observed with cardioprotection,

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carbamate **18j** displayed the strongest neuroprotection in this series of compounds.

Protection of Neurons against Cisplatin-Induced Toxicity. Peripheral neuropathy is a common side effect of many chemotherapeutic agents that affects the quality of life of patients and interferes with treatments.[17] The clinical use of platinum-based antineoplastic agents for example is limited by the development of severe peripheral neurotoxic effects reported in up to 90% of patients receiving a cumulative dose higher than 300 mg/m^2[18] (alternative less neurotoxic drugs forming a complex with another metal are currently under investigation).[19,20] The neuroprotective effect of flavaglines prompted us to examine whether these compounds could also protect primary cultures of mouse cerebellar granular neurons in an established in vitro model of cisplatin-induced neurotoxicity.[21] Incubation of neurons for 48 h with cisplatin (2.5 μM) and Bayer's reference compound **3** at the dose of 5 nM significantly promoted survival against an acute cisplatin insult (Figure 4). Unfortunately, this neuroprotective effect was abolished at the dose of 10 nM, which suggests that the potential therapeutic window for this agent is relatively narrow.

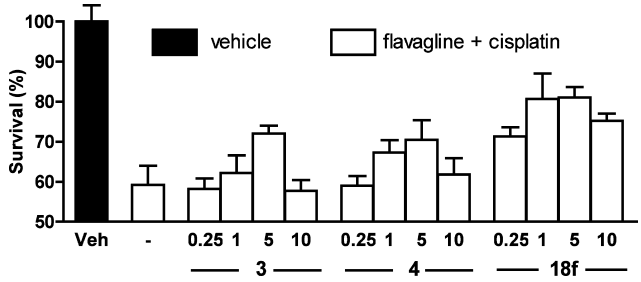

Figure 4. Neuroprotective activity of **3**, **4**, and **18f**. Primary cultures of mouse cerebellar neurons were treated with cisplatin (2.5 μM) and flavaglines or their vehicle for 48 h (acute model of neurotoxicity). Neuronal survival was measured by MTT assay. Bars indicate number of live cells relative to the vehicle (n = 6).

While **4** displayed a similar activity, **18f** displayed again a more pronounced cytoprotection, which reached a maximum between 1 and 5 nM. None of these compounds alone had any perceptible proapoptotic effects at those concentrations (data not shown).

DISCUSSION

We have extended the characterization of the structural requirements for cytotoxic and cytoprotective activities of flavaglines substituted in position 1 either with the "natural" hydroxy or with an *N*-acyl moiety with the opposite configuration (Figure 1). In both series, the replacement of the bromine by a chlorine in position 4′ or the introduction of a fluorine in position 3″ did not modify greatly the cytotoxicity and the cardioprotection (Figure 5). Members of the first series displayed similar SAR for both cytotoxicity and cardioprotection. The introduction of a primary amide in position 2 significantly enhanced the pharmacological activities, while the introduction of a methyl ester had a deleterious effect on cardioprotection and induced a sensitivity toward multidrug resistance as previously observed.[22] Regarding the second series of compounds, we found that changing the acyl moiety of the amide modifies the cytotoxic potential toward cancer cells and the cytoprotective potency toward cardiomyocytes and neurons differentially. The most striking example is illustrated by methyl carbamate **18j**, which displayed the strongest cardioprotection and the weakest cytotoxicity in this series. Except for this difference, the structural requirements of flavaglines for their cardioprotectant and cytotoxic activities are similar, suggesting that the same molecular target is likely involved in both types of action. The observed discrepancy might be due to a divergence in the cellular events that are activated downstream of the binding to the molecular target, leading either to survival promotion in cardiomyocytes or cell death in cancer cells.

Although not all of the compounds were tested in the labor-intensive cellular model of Parkinson's disease, our data strongly suggest that the structural requirements for neuro-

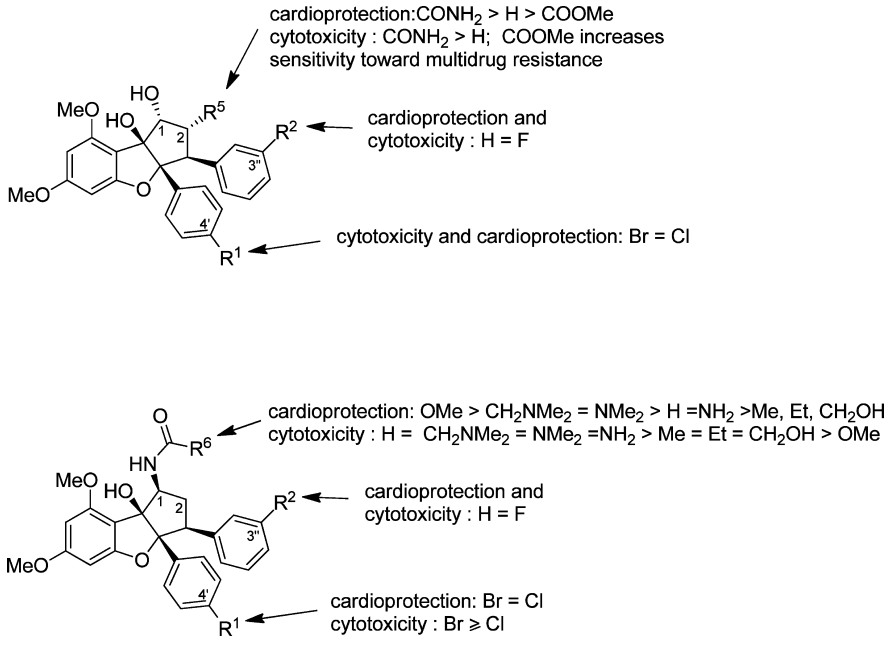

Figure 5. Summary of the structural requirements of flavaglines for cardioprotection and cytotoxicity on cancer cells.

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protection and cardioprotection are identical, suggesting that a common mechanism of action is involved in both processes.

This study is the first report on the neuroprotective potential of flavaglines since the initial work from Bayer's scientists in 2005.[4] We found therein that flavaglines neuroprotection is biphasic: beyond an optimal dose, this effect vanishes in a dose-dependent manner, suggesting that the therapeutic window of flavaglines for neuroprotection is a critical issue that needs to be considered with attention for the development of antiparkinsonian drugs. Hopefully, the therapeutic window is adjustable according to the structure of the flavagline. In particular, compound **18i** displayed an excellent neuroprotective profile over a 0.1−10 nM range.

While the anticancer properties of flavaglines are well described, only reports from Bayer's scientists and from us describe the potent cytoprotective activities of these compounds on neurons and cardiomyocytes.[3,4] Albeit this profile of pharmacological activities is original, it is not unique: rapamycin derivatives are currently used to treat cancer, and this class of compounds exerts also potent neuroprotective effects in animal models of neurodegeneration and traumatic brain injury.[23,24] Similarly, GW8510, an inhibitor of cyclin-dependent kinase 5 (CDK5), displays both cytostatic effects on cancer cells[25] and strong neuroprotection in models of cerebral ischemia and neurodegenerative diseases.[26,27] Moreover the clinically used anticancer drug SAHA, which inhibits histone deacetylases, reduces ischemic injury in the brain.[28] This class of drugs, which were originally developed to treat cancers, have emerged as candidate drugs for the treatment of heart failure[29] and neurodegenerative disorders such as Huntington's disease, Alzheimer's disease, Parkinson disease, amyotrophic lateral sclerosis, spinal muscular atrophy, or Friedreich's ataxia.[30,31]

■ CONCLUSION

Our study broadens the structure−activity data reported for flavaglines cytotoxicity and describes for the first time the SAR for cardio- and neuroprotection. Although the structural requirements for these activities were globally similar, some differences were observed between the cytotoxicity on cancer cells and the protection on cardiomyocytes and neurons. This indicates that it may be possible to shift the biological activity toward either cytotoxicity or cytoprotection by modifying substituents on the flavagline skeleton. This study also suggests for the first time that flavaglines may protect neurons against the adverse effects of cisplatin. Considering that flavaglines were shown to sensitize leukemic T cells to cisplatin-induced apoptosis,[32] a treatment that combines a flavagline and cisplatin might be doubly beneficial: the flavagline could enhance the anticancer effects of cisplatin and alleviate its neuronal adverse effects. Considering the paramount importance of cisplatin in oncology, further studies to validate this hypothesis are warranted.

■ EXPERIMENTAL SECTION

Chemistry. *General Methods.* All reagents and solvents for syntheses were purchased from Sigma-Aldrich, Fluka, or Acros and used without further purification. Reagent-grade solvents were purified and dried using standard methods. Reactions were carried out under an argon atmosphere using flame-dried glassware with magnetic stirring and degassed solvents. Column chromatography was carried out on silica gel 60 (Merck, 70−230 mesh). ^{1}H NMR spectra at 300 MHz and ^{13}C NMR spectra at 75 MHz were recorded with DPX 300 SY Brüker spectrometers with the deuterated solvent as the lock and residual solvent as the internal reference. Infrared spectra were recorded using a Perkin-Elmer 881. Purity of target compounds were over 95% based on reversed-phase HPLC analyses (Hypersil Gold column 30 mm × 1 mm, C18) under the following conditions: flow rate, 0.3 mL/min; buffer A, CH_3CN; buffer B, 0.01% aqueous TFA; gradient, 98−10% buffer B over 8 min (detection: λ = 220/254 nm).

*Methyl-3-(3-fluorophenyl)-5-hydroxy-6,8-dimethoxy-2-(4-methoxyphenyl)-10-oxo-2,3,4,5-tetrahydro-2,5-methanobenzo[b]-oxepine-4-carboxylate (**7a**).* A solution of hydroxyflavone **5a** (1.0 g, 3 mmol) and cinnamate **6a** (4.39 g, 24 mmol) in 90 mL of CH_2Cl_2/MeOH (3:1) was degassed with argon for 10 min in a pyrex tube. This mixture was then irradiated (450 W Iwasaki UV lamp) for 35 h at 0 °C under an argon atmosphere. The solution was concentrated in vacuo, purified by silica gel chromatography (pentane/AcOEt 8:2 to 4:6), and then heated to reflux in EtOAc (20 mL) for 4 h and concentrated in vacuo to give 403 mg (26%) of adduct **7a** as a white solid. ^{1}H NMR ($CDCl_3$): δ 3.57 (3H, s), 3.63 (1H, d, J = 9.2 Hz), 3.68 (3H, s), 3.74 (3H, s), 3.82 (3H, s), 4.18 (1H, d, J = 9.2 Hz), 6.09 (1H, d, J = 2.2 Hz), 6.18 (1H, d, J = 2.2 Hz), 6.66 (3H, m), 6.98 (3H, m), 7.58 (2H, d, J = 8.9 Hz). ^{13}C NMR ($CDCl_3$): δ 52.0, 54.3, 55.1, 55.4, 56.0, 62.8, 87.7, 92.8, 94.6, 98.1, 103.6, 112.9, 113.6 (d, J = 21.0 Hz), 116.7 (d, J = 22.1 Hz), 125.8, 127.7 (2C), 129.2 (d, J = 7.7 Hz), 130.2 (2C), 142.9 (d, J = 6.6 Hz), 153.7, 158.6, 158.9, 162.5 (d, J = 244.4 Hz), 161.4, 171.3, 171.8.

*Methyl-3-(3-fluorophenyl)-8b-hydroxy-6,8-dimethoxy-3a-(4-methoxyphenyl)-1-oxo-2,3,3a,8b-tetrahydro-1H-cyclopenta[b]-benzofuran-2-carboxylate (**8a**).* To a solution of aglain **7a** (300 mg, 0.6 mmol) in MeOH (30 mL) was added a solution of NaOMe in MeOH (0.4 M, 5 mL) at 0 °C. The resulting solution was stirred for 20 min at 60 °C. Then the reaction was cooled to room temperature, quenched with saturated NH_4Cl, and extracted with AcOEt (40 mL). The organic layer was washed with water (2 × 20 mL) and brine (20 mL), dried over $MgSO_4$, and concentrated in vacuo to afford 297 mg of crude β-ketoester **8a** as a white solid which was used without further purification. ^{1}H NMR ($CDCl_3$): δ 3.63 (3H, s), 3.64 (3H, s), 3.74 (3H, s), 3.79 (3H, s), 3.99 (1H, d, J = 13.3 Hz), 4.19 (1H, d, J = 13.3 Hz), 6.06 (1H, d, J = 1.9 Hz), 6.19 (1H, d, J = 1.9 Hz), 6.51 (2H, d, J = 8.9 Hz), 6.66 (3H, m), 6.80 (1H, m), 6.93 (2H, d, J = 8.9 Hz). ^{13}C NMR ($CDCl_3$): δ 51.8, 53.1, 55.2, 55.7, 55.8, 56.4, 88.6, 89.3, 90.0, 93.1, 99.3, 112.3, 113.4 (2C), 114.2 (d, J = 21.6 Hz), 115.1 (d, J = 22.4 Hz), 125.3, 127.9 (2C), 128.2 (d, J = 9.5 Hz), 129.6 (d, J = 9.2 Hz), 138.4 (d, J = 6.6 Hz), 158.7, 160.7 (d, J = 244.7 Hz), 161.0, 165.1, 167.2, 202.7.

*Methyl-3-(3-fluorophenyl)-1,8b-dihydroxy-6,8-dimethoxy-3a-(4-methoxyphenyl)-2,3,3a,8b-tetrahydro-1H-cyclopenta[b]-benzofuran-2-carboxylate (**10a**).* Glacial acetic acid (110 μL, 2.0 mmol) was added to a solution of $Me_4NBH(OAc)_3$ (310 mg, 1.2 mmol) in CH_3CN (10 mL). After stirring for 5 min at room temperature, a solution of ketone **8a** (100 mg, 0.2 mmol) in CH_3CN (1 mL) was added dropwise. The resulting mixture was stirred for 3 h at room temperature, successively quenched with saturated aqueous NH_4Cl (20 mL) and a 3 M aqueous solution of sodium/potassium tartrate (10 mL), and stirred for an additional 30 min. The aqueous solution was extracted with CH_2Cl_2 (2 × 30 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated in vacuo. Crude was purified by silica gel chromatography (CH_2Cl_2/Et_2O 95:5) to afford the title diol **10a** (86 mg, 86%) as a white solid. ^{1}H NMR ($CDCl_3$): δ 3.63 (3H, s), 3.66 (3H, s), 3.79 (3H, s), 3.82 (4H, m), 4.26 (1H, d, J = 14.0 Hz), 4.99 (1H, d, J = 6.6 Hz), 6.09 (1H, d, J = 1.9 Hz), 6.25 (1H, d, J = 1.9 Hz), 6.63 (4H, m), 6.73 (1H, m), 6.99 (1H, m), 7.08 (2H, d, J = 8.9 Hz). ^{13}C NMR ($CDCl_3$): δ 50.6, 52.2, 54.9, 55.3, 55.8, 55.9, 79.7, 89.7, 92.9, 93.8, 101.8, 107.8, 113.6 (d, J = 20.9 Hz), 115.2 (d, J = 22.0 Hz), 123.5, 126.3, 129.0 (2C), 129.2 (2C), 129.3 (d, J = 8.4 Hz), 140.3 (d, J = 7.3 Hz), 157.1, 159.0, 160.9, 162.6 (d, J = 244.7 Hz), 164.3, 170.4. IR (thin film): 3469, 2950, 2840, 1741, 1145, 702 cm^{-1}. LC-MS: calculated, 510.2; found, 511.1 (M+H)+.

*General Procedure for the Michael Reaction of Benzofuranone **12** with **13**.* A suspension of benzofuranone **12** (1 equiv) in *t*-BuOH (~0.045M) was heated to 50 °C under argon. Benzyltrimethylammo-

nium hydroxide in MeOH (40%, 0.05 equiv) was added, immediately followed by the addition of cinnamaldehyde **13** (2 equiv). After 2 h at 50 °C, the reaction was cooled to room temperature, concentrated, and acidified with 1 M HCl (4 equiv), then extracted with CH_2Cl_2, dried over $MgSO_4$, and concentrated to dryness. The resulting solid was purified by silica gel chromatography to afford expected adduct **14**.

*General Procedure for the Synthesis of Ketone **15**.* Trimethylsilylcyanide (3 equiv) was added dropwise to a solution of aldehyde **14** (1 equiv) in CH_3CN (1M) at room temperature under argon. Immediately after, zinc iodide (0.01 equiv) was added, and the resulting mixture was stirred for 1 h, filtered, and concentrated to afford crude cyanohydrin, which was used in the next step without purification. LDA (1.1 equiv, 1 M in THF) was added dropwise at −78 °C under argon to a solution of previous cyanohydrin (1 equiv) in dry THF (0.2M). After stirring for 2 h at −78 °C, the solution was heated to −50 °C for 30 min. The reaction was quenched by the addition of saturated aqueous ammonium chloride. Standard extractive workup (CH_2Cl_2) gave a yellow solid. This solid was directly treated with tetra-*n*-butylammonium fluoride (1.1 equiv, 1 M in THF), which was added dropwise at room temperature in dry THF (0.2M). The solution was stirred overnight and quenched by addition of methanol. Standard extractive workup (AcOEt) and purification by silica gel chromatography afforded tricyclic ketone **15** as a solid.

*3-(2-(4-Bromophenyl)-4,6-dimethoxy-3-oxo-2,3-dihydrobenzofuran-2-yl)-3-(3-fluorophenyl)propanal (**14a**).* See the general procedure for the Michael reaction: Benzofuranone **12a** (8.7 g, 24.9 mmol), *t*-BuOH (700 mL), benzyltrimethylammonium hydroxide (triton B) in MeOH (40%, 1.11 mL, 3.2 mmol) and 3-fluorocinnamaldehyde **13** (6.37 mL, 50.0 mmol). Purification of the resulting yellow solid residue by silica gel chromatography (Et_2O/pentane 6:4) yielded 3.95 g (33%) of ketoaldehyde **14a** as a white solid. ^{1}H NMR ($CDCl_3$): δ 2.59 (1H, dd, J = 3.8, 17.6 Hz), 3.02 (1H, ddd, J = 2.1, 10.9, 17.6 Hz), 3.70 (3H, s), 3.84 (3H, s), 4.17 (1H, dd, J = 3.8, 10.9 Hz), 5.82 (1H, d, J = 1.9 Hz), 6.21 (1H, d, J = 1.9 Hz), 6.71 (1H, m), 7.00 (1H, m), 7.09 (2H, m), 7.48 (2H, d, J = 8.7 Hz), 7.60 (2H, d, J = 8.8 Hz), 9.41 (1H, s). ^{13}C NMR ($CDCl_3$): δ 44.3, 46.5, 56.1, 56.2, 88.7, 93.2, 93.4, 103.8, 114.7 (d, J = 20.9 Hz), 116.2 (d, J = 22.0 Hz), 123.0, 125.9, 127.0 (2C), 129.9 (d, J = 8.4 Hz), 132.1 (2C), 135.6, 139.2 (d, J = 7.0 Hz), 159.4, 162.6 (d, J = 245.8 Hz), 170.3, 174.2, 194.0, 199.5. IR (thin film): 3435, 2936, 1720, 1699, 1147, 747 cm^{-1}.

*3a-(4-Bromophenyl)-3-(3-fluorophenyl)-8b-hydroxy-6,8-dimethoxy-2,3,3a,8b-tetrahydro-1H-cyclopenta[b]benzofuran-1-one (**15a**).* See the general procedure for the ring closing reaction: (i) Trimethylsilylcyanide (2.41 g, 24.3 mmol), aldehyde **14a** (3.90 g, 8.1 mmol), CH_3CN (50 mL), zinc iodide (30 mg); (ii) LDA (10.5 mmol, 1 M in THF), THF (40 mL); (iii) tetra-*n*-butylammoniumfluoride (2.33 mL, 1 M in THF), THF (30 mL). Purification by silica gel chromatography (Et_2O/pentane 6:4) afforded tricyclic ketone **15a** (1.98 g, 49%) as a light-orange solid. ^{1}H NMR ($CDCl_3$): δ 2.99 (2H, m), 3.80 (3H, s), 3.86 (4H, m), 6.09 (1H, d, J = 2.0 Hz), 6.32 (1H, d, J = 2.0 Hz), 6.70 (2H, m), 6.79 (1H, m), 6.91 (2H, d, J = 8.7 Hz), 7.06 (1H, m), 7.27 (2H, d, J = 8.7 Hz). ^{13}C NMR ($CDCl_3$): δ 39.6, 48.5, 55.8, 56.0, 88.9, 89.9, 93.2, 100.9, 106.2, 114.2 (d, J = 20.9 Hz), 115.2 (d, J = 22.0 Hz), 122.2, 123.4, 128.4 (2C), 129.7 (d, J = 8.4 Hz), 131.1 (2C), 132.9, 139.6 (d, J = 7.7 Hz), 158.6, 161.0, 162.8 (d, J = 245.0 Hz), 165.2, 209.9. IR (thin film): 3477, 2942, 2841, 1750, 1149 cm^{-1}.

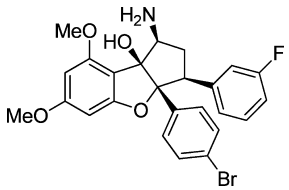
*3a-(4-Bromophenyl)-3-(3-fluorophenyl)-6,8-dimethoxy-2,3,3a,8b-tetrahydro-1H-cyclopenta[b]benzofuran-1,8b-diol (**16**).* Glacial acetic acid (30 μL, 0.60 mmol) was added to a solution of $Me_4NBH(OAc)_3$ (95 mg, 0.36 mmol) in CH_3CN (3 mL). After stirring for 5 min at room temperature, a solution of the compound **15a** (30 mg, 0.06 mmol) in CH_3CN (2 mL) was added dropwise. The resulting mixture was stirred for 3 h at rt, successively quenched with saturated aqueous NH_4Cl (5 mL) and a 3 M aqueous solution of sodium/potassium tartrate (3 mL), and stirred for 30 min. The aqueous solution was extracted with AcOEt (2 × 30 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated in vacuo. Purification by silica gel chromatography (CH_2Cl_2/Et_2O 95:5) afforded the desired alcohol **16** (17 mg, 55%) as a white solid. ^{1}H NMR ($CDCl_3$): δ 2.16 (1H, ddd, J = 1.1, 6.7, 13.8 Hz), 2.66 (1H, dt, J = 6.4, 13.7 Hz), 3.82 (3H, s), 3.87 (3H, s), 3.98 (1H, dd, J = 6.5, 14.1 Hz), 4.75 (1H, d, J = 5.9 Hz), 6.12 (1H, d, J = 1.9 Hz), 6.26 (1H, d, J = 1.9 Hz), 6.74 (3H, m), 7.05 (3H, m), 7.25 (2H, m). ^{13}C NMR ($CDCl_3$): δ 36.4, 53.3, 55.9, 56.0, 79.1, 89.6, 92.9, 95.1, 103.3, 107.4, 123.6 (d, J = 21.3 Hz), 115.1 (d, J = 21.6 Hz), 121.9, 123.7, 129.4 (d, J = 8.1 Hz), 129.5 (2C), 130.6 (2C), 134.1, 141.1 (d, J = 7.3 Hz), 157.1, 160.9, 162.7 (d, J = 245.0 Hz), 164.3. IR (thin film): 3477, 2943, 2842, 1597, 729, 503 cm^{-1}. LC-MS: calculated: 500.1, found: 501.0 $(M + H)^+$.

*General Procedure for the Formation of Oxime Ethers **17**.* Ketone **15** (1 equiv) and *O*-methylhydroxylamine hydrochloride (5 equiv) were diluted in absolute ethanol and distilled pyridine (ratio 1/1, 0.025M). The reaction was heated up to reflux for 3 h, and then the volatiles were removed and the crude product was extracted with EtOAc. The organic phase was washed twice with HCl (1M), a saturated solution of Na_2CO_3, and brine. Then, the organic phase was dried over $MgSO_4$ and concentrated to dryness to give quantitatively the desired oxime ether **17** as a white solid, which was used in the next step without purification.

*3a-(4-Bromophenyl)-6,8-dimethoxy-(3-fluorophenyl)-8b-hydroxy-3−2,3,3a,8b-tetrahydro-1H-cyclopenta[b]benzofuran-1-one O-methyl oxime (**17a**).* See the general procedure for the formation of the oxime ether: Ketone **15a** (500 mg, 1.00 mmol), *O*-methylhydroxylamine hydrochloride (418 mg, 5.00 mmol), ethanol (20 mL), and pyridine (20 mL). ^{1}H NMR ($CDCl_3$): δ 3.05 (2H, m), 3.69 (1H, m), 3.82 (3H, s), 3.83 (3H, s), 4.06 (3H, s), 6.10 (1H, d, J = 2.0 Hz), 6.27 (1H, d, J = 2.0 Hz), 6.76 (3H, m), 7.00 (2H, d, J = 8.5 Hz), 7.05 (1H, m), 7.29 (2H, d, J = 8.5 Hz). ^{13}C NMR ($CDCl_3$): δ 29.7, 49.6, 55.8, 55.9, 62.8, 87.5, 89.8, 93.2, 101.5, 108.0, 113.9 (d, J = 20.9 Hz), 115.1 (d, J = 20.9 Hz), 121.9, 123.5, 128.5 (2C), 129.5 (d, J = 8.4 Hz), 131.1 (2C), 133.5, 140.2 (d, J = 7.0 Hz), 158.9, 159.7, 160.3, 162.7 (d, J = 245.4 Hz), 164.5.

*General Procedure for the Formation of the Amine **19**.* BH_3−THF complex (14 equiv, 1M) was added dropwise to a solution of methylether oxime **17** (1 equiv) in dry THF (0.028M) at 0 °C. After addition, the reaction was heated to 65 °C overnight. Again BH_3−THF complex (4 equiv, 1M) was added at room temperature, then heated up to 65 °C for 8 h. A solution of 5 M NaOH was added to stop the reaction, and the aqueous phase was extracted twice with EtOAc. The cumulated organic phase was washed with brine, dried over $MgSO_4$, and concentrated to dryness.

1-Amino-3a-(4-bromophenyl)-6,8-dimethoxy-3-(3-fluorophenyl)-8b-hydroxy-2,3,3a,8b-tetrahydro-1H-cyclopenta[b]benzofuran-8b-



*ol (**19a**).* See the general procedure for the formation of the amine: BH_3−THF complex (15 mL, 1M), methylether oxime **17a** (1.60 g, 3.03 mmol), and THF (15 mL). The crude product was purified by silica gel chromatography (Et_2O/MeOH 70/30) to give 645 mg (41%) of the desired amine **19a** as a mixture of diastereomers (ratio 95/5). ^{1}H NMR ($CDCl_3$): δ 2.05 (1H, m), 2.45 (1H, m), 3.57 (1H, m), 3.77 (7H, m), 6.03 (1H, d, J = 1.9 Hz), 6.17 (1H, d, J = 1.9 Hz), 6.74 (3H, m), 7.01 (3H, m), 7.18 (2H, d, J = 8.7 Hz). ^{13}C NMR ($CDCl_3$): δ 36.5, 51.8, 55.7, 55.8, 57.1, 86.4, 88.9, 92.5, 101.8, 111.6, 113.4 (d, J = 21.3 Hz), 115.0 (d, J = 21.3 Hz), 121.2, 123.6, 129.3 (2C), 129.4, 130.3 (2C), 135.4, 140.9 (d, J = 7.3 Hz), 157.6, 159.6, 162.6 (d, J = 245.4 Hz), 163.5. IR (thin film): 3306, 2943, 2939, 1602, 1146, 691, 501 cm^{-1}.

*N-(3a-(4-Bromophenyl)-3-(3-fluorophenyl)-8b-hydroxy-6,8-dimethoxy-2,3,3a,8b-tetrahydro-1H-cyclopenta[b]benzofuran-1-yl)-2-(dimethylamino)-1,1-dimethylurea (**18f**).* DIPEA (66 μL, 0.38 mmol), DMAP (3 mg), and dimethylcarbamoyl chloride (32 μL, 0.35 mmol) were successively added to a solution of amine **19a** (160

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mg, 0.32 mmol) in dry CH_2Cl_2 (2 mL) at 0 °C. The reaction was stirred for 24 h at room temperature, and then CH_2Cl_2 (10 mL) was added. The organic phase was washed with HCl (10 mL, 1M), brine (10 mL), dried over $MgSO_4$, and concentrated to dryness. The crude product was purified by silica gel chromatography (EtOAc/MeOH 95/5) to afford 154 mg (84%) of **18f**. ^{1}H NMR ($CDCl_3$): δ 2.19 (1H, m), 2.65 (1H, m), 2.87 (6H, s), 3.58 (1H, m), 3.64 (3H, s), 3.72 (3H, s), 4.40 (1H, m), 5.94 (1H, d, J = 2.0 Hz), 6.13 (1H, d, J = 2.0 Hz), 6.74 (3H, m), 7.02 (3H, m), 7.17 (1H, d, J = 8.8 Hz). ^{13}C NMR ($CDCl_3$): δ 36.2 (2C), 36.7, 51.7, 55.5, 55.7, 57.3, 87.8, 88.8, 92.5, 102.1, 110.4, 113.3 (d, J = 20.9 Hz), 115.1 (d, J = 21.6 Hz), 121.3, 123.7, 129.2, 129.3 (2C), 130.3 (2C), 135.2, 141.1 (d, J = 7.3 Hz), 157.7, 158.4, 159.7, 162.7 (d, J = 245.4 Hz), 163.7. IR (thin film): 3233, 2937, 1627, 1147, 724 cm^{-1}. LC-MS: calculated, 570.1, found, 571.0 $(M + H)^+$.

Biological Assay Methods. *Materials.* TdT-mediated dUTP nick end-labeling (TUNEL) assay was purchased from Millipore (Billerica, MA, USA).

Cell Culture. The human cell lines KB, HCT116 and HCT15 were purchased from ECACC (Salisbury, UK), HL60 cells from ATCC, and HL60R-R10 overexpressing P-gp was originated from Oncodesign. KB cells were grown in D-MEM medium supplemented with 10% fetal calf serum, in the presence of penicillin, streptomycin, and fungizone in 75 cm^2 flask under 5% CO_2, whereas HCT116 and HL60 were grown in RPMI medium. H9c2 cells (rat heart myoblast) were obtained from ATCC and grown in D-MEM medium supplemented with 10% fetal calf serum in 5% CO_2. The medium was changed every 2−3 days.

Cell Proliferation Assay. Cells were plated in 96-well tissue culture plates in 200 μL of medium and treated 24 h later with compounds dissolved in DMSO; compound concentrations ranged 0.5 nM to 10 μM and were prepared by use of a Biomek 2000 (Beckman Coulter). Control cells received the same volume of DMSO (1% final volume). After 72 h exposure to the drug, MTS reagent (Promega) was added and incubated for 3 h at 37 °C: the absorbance was monitored at 490 nm and results are expressed as the inhibition of cell proliferation calculated as the ratio $[(OD_{490}$ treated/OD_{490} control) × 100]. For IC_{50} determinations (50% inhibition of cell proliferation) experiments were performed in duplicate.

In Vitro Cardioprotection Assay. H9c2 cells were plated for 24 h in 100 mm Petri dishes at 7 × 10^3 cells/cm^2. Next, the cells were washed and cultured for 12 h in glucose-free medium (Gibco, DMEM w L-glutamine, w/o D-glucose, sodium pyruvate) supplemented with only 1% fetal calf serum. Cells were then treated with flavaglines or the vehicle for 10 h and then were treated with doxorubicin for an additional 14 h. Cells were washed, and apoptosis was determined by FACS analyses. Cells (7 ×10^5) were harvested and washed with Annexin binding buffer (0.01 M HEPES, 0.14 M NaCl, 2.5 mM $CaCl_2$) and labeled with annexin-V (dilution 1:50) and propidium iodide (6.7 μg/mL). All assays were performed at least in triplicate, and the results were analyzed by BD Cell Quest Pro software.

In Vitro Neuroprotection against MPP$^+$-Induced Degeneration. Dopaminergic neurons from the ventral mesencephalon of embryonic day 15.5 Wistar rat embryos (CERJ, Le Genest St. Isle, France) were dissociated mechanically, plated onto polyethylenimine-precoated 24-well culture plates, and grown in N5 medium supplemented with 25 mM K$^+$, 1 μM MK-801, 5 mM glucose, 5% horse serum, and 2.5% fetal calf serum, as described previously.[33] After 3 days, fetal calf serum was reduced to 0.5% to limit astrocyte proliferation. Treatments with MPP$^+$ (3 μM) initiated at day 5 in vitro, were terminated 48 h later. Flavaglines added to the culture medium one day before MPP$^+$ were then left throughout the incubation period with the toxin. The cultures were fixed at day 7 in vitro with 4% formaldehyde and taken for immunocytochemical detection of tyrosine hydroxylase (TH).[33] Numbers of TH$^+$ cells/16 mm culture well were estimated by counting positive cells distributed along x and y axes using a ×20 objective.

In Vitro Neuroprotection against Cisplatin-Induced Apoptosis. Cerebellar granular neurons were dissected from 7-day old mice and cultured as previously described.[21] Neurons were considered as immature up to 2 days after plating and as mature 5 days after plating, when they develop a dense neuritis network. Mature neurons were cultured in 96-well culture dishes (Costar) and incubated for 48 h in presence of cisplatin (2.5 μM) and flavagline derivative (1 nM) (which were both added into the medium at the same time). Mitochondrial activity (MTT assay) was measured as described previously.[20] Briefly, cultures were rinsed with DMEM and incubated for 1 h at 37 °C in freshly prepared culture medium containing 0.5 mg/mL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma). Medium was then removed and dark-blue crystals formed during reaction were dissolved by adding 100 μL/well of 0.04 M HCl in isopropyl alcohol. Plates were stirred at room temperature to ensure that all crystals were dissolved and read on a Bio-Rad 680 micro-ELISA plate reader, using a test wavelength of 490 nm and a nonspecific wavelength of 650 nm for background absorbency. Results are given as percentage of survival, taking cultures grown in control (vehicle) medium as 100%.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary biological data (neuroprotective effect of **10a** and **18c−e**), synthesis of compounds **3**, **7b−c**, **8b−c**, **9**, **10b−c**, **11**, **14b**, **15b**, **17b**, **18a−e**, and **18g−j**. This material is available free of charge via the Internet at http://pubs.acs.org.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AIF, apoptosis inducing factor; eIF4E, eukaryotic translation initiation factor 4E; ERK, extracellular-signal-regulated kinase; MEK, mitogen-activated protein kinase kinase; MPP$^+$, 1-methyl-4-phenylpyridium; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium); TH, tyrosine hydroxylase

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