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ORIGINAL ARTICLE

Drug-Like Property Profiling of Novel Neuroprotective Compounds to Treat Acute Ischemic Stroke: Guidelines to Develop Pleiotropic Molecules

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Abstract The development of novel neuroprotective compounds to treat acute ischemic stroke (AIS) has been problematic and quite complicated, since many candidates that have been tested clinically lacked significant pleiotropic activity, were unable to effectively cross the blood brain barrier (BBB), had poor bioavailability, or were toxic. Moreover, the compounds did not confer significant neuroprotection or clinical efficacy measured using standard behavioral endpoints, when studied in clinical trials in a heterogeneous population of stroke patients. To circumvent some of the drug development problems describe above, we have used a rational funnel approach to identify and develop promising candidates. Using a step-wise approach, we have identified a series of compounds based upon two different neuroprotection assays. We have then taken the candidates and determined their "drug-like" properties. This guidelines article details in vitro screening assays used to show pleiotropic activity of a series of novel compounds; including enhanced neuroprotective activity compared to the parent compound fisetin. Moreover, for preliminary drug de-risking or risk reduction during development, we used compound assessment in the CeeTox assay, ADME toxicity using the AMES test for genotoxicity, and interaction with Cytochrome P450 using CYP450 inhibition analysis against a spectrum of CYP450 enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4) as a measure of drug interaction. Moreover, the compounds have been studied using a transfected Madin Darby canine kidney (MDCK) cell assay to assess blood brain barrier penetration (BBB). Using this series of assays, we have identified four novel molecules to be developed as an AIS treatment.

Keywords Ames test · Flavonoid · Chalcone · Flavone · MDCK · Screening · Pleiotropic · ADME · Toxicity

Abbreviations

AIS	acute ischemic stroke
CLogP	partition coefficient
MDCK	Madin Darby canine kidney (MDCK) cell assay
MW	molecular weight
tPA	tissue plasminogen activator
tPSA	polar surface area
SAR	structure activity relationship

Introduction

There is substantial risk associated with drug development for acute ischemic stroke (AIS), but the benefit to patients associated with a Food and Drug Administration (FDA) approved neuroprotective therapy is enormous [5]. Currently, tissue plasminogen activator (tPA) is the only FDA approved treatment for AIS based upon the original National Institute of Neurological Disorders and Stroke (NINDS) rt-PA Stroke Study [6]. Even though there is no doubt that tPA is quite useful and effective in stroke patients when given 3–4.5 h after an AIS [7–9], there are three important shortcomings to the drug. First, with tPA treatment, there is significant risk of intracerebral hemorrhage (ICH) in approximately 5–8 % of patients treated within 3–4.5 h of a stroke [9] and the odds ratio for mortality rate following the treatment of stroke patients increases after 4 h [9]. Second, even though tPA increases cerebral perfusion, it is not neuroprotective or neurotrophic and does not promote cell survival. Third, because of the inherent difficulties with

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administering tPA, and the short therapeutic window, it is estimated that 45–50 % of patients presenting within 4.5 h are treated with tPA [10]; however, this still represents less than 10 % of all stroke patients [10–12]. There remains a critical medical need for new therapeutics to be used an a monotherapy or in combination with tPA, to halt the debilitating neurodegenerative effects of AIS, the fourth leading cause of mortality and leading cause of adult morbidity in the USA. Annually, in the USA, approximately 0.8 million people suffer a stroke with 1 mortality every 3 min, and 15 million people worldwide suffer a stroke [5].

An efficacious FDA-approved neuroprotective treatment or neurorestorative treatment that promotes brain repair is not currently available to AIS patients in the USA. There is an urgent need for a new, safe, pleiotropic drug treatment that exhibits a long therapeutic time window that could be provided to the majority of AIS patients as a treatment alternative. With the huge number of therapy failures that have been documented in the literature [13–19], it has been recommended that researchers provide substantial rationale and efficacy data to justify the development of novel drug classes [15, 20–23] and de-risk (reduce the risk of) drug development to enhance the possibility of success in randomized and blinded clinical trials. Moreover, since measuring Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) parameters during the early phases of drug development can be done economically [24, 25] and with great utility toward the development of both translational and clinical programs, preclinical development plans should incorporate ADMET measures [24–31].

The activation of a cascade of deleterious events [32] following vascular occlusion is normally characterized by rapid oxygen deprivation, energy failure, and excitotoxicity [33–38]. The temporal profile of cascade activation has recently been reviewed [32]. The excitotoxin, glutamate, is released in abundance early after vascular occlusion and can propagate the ischemic response [39–43]. An important aspect of the cascade is the production of free radicals that can cause cellular damage, protein, and lipid peroxidation and can mediate ischemic necrosis and vascular and blood brain barrier (BBB) damage [44–49]. There are also a series of other mechanisms which are activated in parallel or simultaneously, pathways deleterious to tissues at risk within the penumbra [15, 20, 50], including neurons, glial cells, and the vasculature, now commonly known as the vascular compartment. Since a diverse cascade is activated, it is more than reasonable to hypothesize or suggest that clinically efficacious compounds may require molecules with pleiotropic activity or combination therapy [13, 15, 16, 20, 38, 51, 52].

In this report, we document a systematic screening approach using the selective use of neuroprotection assays in combination with ADMET assays to optimize new drug candidates at the in vitro screening level prior to initiating extensive, expensive drug development in animal models of stroke. Our approach to develop an efficacious stroke treatment is based upon the use of a moderately lipophilic molecule, fisetin, with multiple beneficial activities, a pleiotropic molecule to attenuate or simply block multiple aspects of the ischemic cascade. In this study, we have characterized and systematically de-risked a series of novel drug candidates using criteria necessary to pursue the development of a drug candidate in vivo.

Methods

A funnel approach was used which consisted of the following efficacy and de-risking assays:

(A) Efficacy in vitro using HT22 cells and cortical cultures
(B) CeeTox analysis
(C) Genotoxicity analysis (modified Ames test)
(D) CYP450 inhibition analysis
(E) BBB penetration (MDR1-MDCK assay)

In Vitro Neuroprotection Analysis: Two-Tiered Analysis for Neuroprotective Properties

1. In vitro ischemia assay: The in vitro ischemia assay was performed using HT-22 hippocampal neurons as described previously [4, 53]. HT22 cells were treated with iodoacetic acid (IAA), an irreversible inhibitor of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) to block the glycolytic pathway and deplete cellular energy stores. Fetal calf serum (FCS) and dialyzed FCS (DFCS) were from Hyclone Inc. (Logan, UT). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Invitrogen (Carlsbad, CA). HT-22 cells [54, 55] were grown in DMEM supplemented with 10 % fetal calf serum (FCS). Briefly, cells were seeded onto 96-well microtiter plates at a density of 5×10^3 cells per well. The next day, the medium was replaced with DMEM supplemented with 7.5 % DFCS and the cells were treated with 20 μM iodoacetic acid (SIGMA Inc, MO; IAA) alone or in the presence of drug. After 2 h, the medium in each well was aspirated and replaced with fresh medium without IAA but containing drug. Twenty hours later, the medium in each well was aspirated and replaced with fresh medium containing 5 μg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). After 4 h of incubation at 37 °C, 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 [4, 53].

2. Oxidative glutamate toxicity assay: As described previously [53], HT22 cells were treated with the excitotoxic amino acid glutamate (5 mM), which induces cell death mediated by the depletion of intracellular glutathione (GSH) via inhibition of the cystine/glutamate antiporter [56, 57]. It is noteworthy to mention that HT22 cells do not express functional NMDA receptors, thus the toxicity observed with glutamate in not excitotoxicity [56, 57]. Thirty minutes after drug addition, 5 mM glutamate was added to initiate the cell death cascade and 24 h later, cell survival was measured [53].
3. Statistical analysis: The in vitro cell death assays were repeated at least three times in triplicate each time and analyzed using Instat software. The data are presented as the mean±SD. Statistical analysis was done by ANOVA followed by Bonferroni's test ($P<0.05$ was considered statistically significant).

CeeTox Analysis

The CeeTox analyses were done in a blinded manner according to methodology described previously [27, 28, 58]. Since the drugs being developed are lipophilic compounds, dimethylsulfoxide (DMSO) was used to prepare stock solution. All experiments that used DMSO as the drug solvent also included a DMSO negative control [58]. A C_{Tox} value was generated by CeeTox Inc. using a patented proprietary algorithm [59].

H4IIE Cell Line Rat hepatoma-derived H4IIE cells were used to determine a C_{Tox} value because the cell has a rapid doubling time in culture (i.e., 22 h) [58]. The culture medium used for these cells was Eagles Minimum Essential Medium (MEM) with 10 % bovine serum and 10 % calf serum (Invitrogen Inc). H4IIE cells were seeded into 96-well plates and allowed to equilibrate for approximately 48 hr before drug assay to allow cells to move into a stable growth phase prior to treatment. Following the equilibration period, the cells were exposed to drug t at concentrations of 1–300 μM. Three to seven replicates were done for each assay to construct concentration–response curves. Solubility was determined by Nephalometry techniques immediately after dosing and prior to harvesting the cells at 6 or 24 h. Following the incubation of cells with drug, the cells or their supernatant (culture medium) were analyzed for changes in cell proliferation (cell mass), membrane leakage, mitochondrial function, oxidative stress, and apoptosis. The resultant exposure concentration response curves were graphed and analyzed for determining the concentration that produced a half maximal response or TC_{50} [58].

General Cellular Measures of Toxicity

Cell Mass Cell mass in each well was measured with a modified propidium iodide (PI) [60], a specific nucleic acid binding dye that fluoresces when intercalated within the nucleic acids. The 15-nm shift enhances PI fluorescence approximately 20 times while the excitation maxima are shifted 30–40 nm. Triton-X-100 was used to permeabilize the H4IIE cells thereby allowing the PI access to intracellular RNA and DNA. Fluorescence was measured using a Packard Fusion plate reader at 540 nm excitation and 610 nm emission [58]. Data was collected as relative fluorescent units (RFU) and expressed as percent change relative to control.

Membrane Toxicity The presence of α-glutathione S-transferase (α-GST), an enzyme leakage marker, was measured in the culture medium using an ELISA assay purchased from Argutus Medical [58, 61]. At the end of the exposure period, the medium covering the cells in each well was removed and stored at −80 °C until assayed. Absorbance values were measured with a Packard SpectraCount™ reader at 450 nm and reference absorbance at 650 nm. Leakage of α-GST from the cell into the culture medium was determined by collecting the culture medium at the end of the exposure period. Thus, the values measured represent total enzyme leakage lost over the exposure period.

3-[4,5-Dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT): After the medium was removed from a plate for α-GST analysis, the cells remaining in each well were evaluated for their ability to reduce soluble-MTT (yellow) to formazan-MTT (purple) [1, 2, 58]. An MTT stock solution was prepared in complete medium just prior to use and warmed to 37 °C. Once the medium was removed from all wells, MTT solution was added to each well and the plate was allowed to incubate at 37 °C for 3–4 h. Following incubation, all medium was removed and the purple formazan product was extracted using anhydrous isopropanol. Sample absorbance was read at 570 nm and reference absorbance at 650 nm with a Packard Fusion reader. The control for 100 % dead or maximum enzyme release was based on cells treated with 1 mM digitonin at the time of dosing. Percent dead cells relative to digitonin treated cells was determined and then subtracted from 100 % to yield the percent live cells.

Cellular ATP Content Adenosine triphosphate (ATP) content was determined using a modification of a standard luciferin/luciferase luminescence assay [62] based on a reaction between ATP + Dluciferin + oxygen catalyzed by luciferase to yield Oxyluciferin + AMP + PPi + CO2 + light. The emitted light is proportional to the amount of ATP present [58]. At the end of the 24-h exposure period, the

medium was removed from the cells and the ATP cell lysis buffer added to each well. Plates were analyzed immediately or stored at −20 °C until needed. On the day of analysis, the plates were thawed and calibration curve prepared with ATP in the same liquid matrix as samples. ATP was quantified by adding ATP substrate solution and then reading luminescence on a Packard Fusion Luminescence reader. ATP levels (picomoles ATP per million cells) in treated cells were extrapolated using the regression coefficients obtained from the linear regression analysis of the calibration curve. Background corrected luminescence was used to determine percent change relative to controls by dividing treated values by control values and multiplying by 100.

Oxidative Stress Intracellular glutathione (GSH) levels: Intracellular glutathione levels were determined using a modification of the procedure published by Griffith [63]. Briefly, the sulfhydryl group of GSH reacts with DTNB (5,5′-dithiobis-2-nitrobenzoic acid, Ellman's reagent) and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB (between GSH and TNB) that is concomitantly produced, is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to the concentration of GSH in the sample. Measurement of the absorbance of TNB at 405 or 412 nm provides an accurate estimation of GSH in the sample. At the end of the exposure period, the medium was removed from the cells and metaphosphoric acid (MPA) was added to each well. Plates were then shaken for 5 min at room temperature and stored at −20 °C until needed. The sample plates were thawed just prior to analysis and centrifuged at>2,000×*g* for 2 min. Sample aliquots were removed and transferred to a clean 96-well plate along with appropriate standard curve controls. Sample pH was neutralized just prior to analysis and each well received an aliquot of PBS reaction buffer containing Ellman's reagent, NADPH, and glutathione reductase. The plates were shaken for 15–30 min at room temperature and glutathione content was determined colorimetrically with a Packard Fusion reader at 415 nm. The assay is based on the concept that all GSH is oxidized to GSSG by DTNB reagent. Two molecules of GSH are required to make one molecule of GSSG (oxidized glutathione). Total GSH was determined by reducing GSSG to 2GSH with glutathione reductase. A standard curve was prepared with oxidized glutathione (GSSG) over a range of concentrations. These concentrations are then converted to glutathione equivalents (GSX) essentially by multiplying the GSSG standard concentrations by 2. The amount of GSX expressed as picomoles per well was determined using the standard curve and regression analysis and are expressed as percent of control;

Lipid Peroxidation Measured as 8-Isoprostane (8-ISO or 8-epi PGF2α): 8-ISO levels were determined using an ELISA (Cayman Chemical Inc). 8-ISO is a member of a family of eicosanoids produced nonenzymatically by random oxidation of tissue phospholipids by oxygen radicals. Therefore, an increase in 8-ISO is an indirect measure of increased lipid peroxidation [64]. At the end of the exposure period, plates were either analyzed immediately or stored at −80 °C until needed for analysis. Color development, which is indirectly proportional to the amount of 8-ISO present in the sample, was read on a Packard Fusion or equivalent plate reader at 415 nm [58]. Background absorbance produced from Ellman's reagent is subtracted from all wells. Non-specific binding is subtracted from the maximum binding wells to give a corrected maximum binding expressed as B_o. The percent of bound (B) relative to maximum binding capacity (Bo) for all unknown samples and for standards was determined an expressed as (%B/Bo). The %B/Bo for standards was plotted against the log of 8-ISO added to yield the final standard curve. This curve was used to convert %B/Bo to pg 8-ISO/mL of sample.

Apoptosis Caspase 3 activity was determined using a caspase substrate (DEVD, Asp-Glu-Val-Asp) labeled with a fluorescent molecule, 7-Amino-4-methylcoumarin (AMC). Caspase 3 cleaves the tetrapeptide between D and AMC, thus releasing the fluorogenic green AMC [58]. Following the test article exposure to cells in 96-well plates, the medium was aspirated from plates and PBS added to each well. Plates were stored at −80 °C to lyse cells and store samples until further analysis. On the day of analysis, plates were removed from freezer and thawed. Caspase buffer with fluorescent substrate was added to each well and incubated at room temperature for 1 h. AMC release was measured in a spectrofluorometer at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Values are expressed as relative fluorescent units (RFU). After sample plates were completely thawed, the caspase substrate buffer mix was added to each plate. Plates were incubated at room temperature for 1 h, shielded from light. Plates were read using a in a spectrofluorometer at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Values were expressed as relative fluorescent units (RFU).

P-glycoprotein (PgP) Binding Using the MTT Assay The H4IIE cells possess high levels of PgP protein in the outer membrane and be used effectively for evaluation of drug binding to PgP [58, 65–67]. For this assay, cells are incubated with and without cyclosporin A (CSA; a PgP inhibitor) at a single exposure concentration (20 or 50 μM) and the difference in toxicity determined with the MTT assay. Compounds with increased toxicity in the presence of CSA have a high probability of binding to PgP proteins. However, compounds of low toxicity will typically not show a difference relative to the addition of CSA, regardless of

whether they bind to PgP. At the end of the 24-hr exposure period the culture medium was removed and the remaining attached cells were assayed for their ability to reduce MTT. Viable cells will have the greatest amount of MTT reduction and highest absorbance values. Percent control values were determined by dividing the mean absorbance/fluorescence of the treatment group by the mean absorbance of the control group and multiplying by 100.

Genotoxicity Analysis

The genotoxicity studies were done in a blinded manner using 2 strains of histidine incompetent cells (±) S9 liver fraction in order to measure the effects of metabolic activation of STAZN on genotoxicity. Thus, either *S. typhimurium* TA989: *hisD3052*, *rfa*, *uvrB/*pKM101, which detects frameshift mutations or *S. typhimurium* TA100*: hisG45*, *rfa*, *uvrB*/pKM101, which detects base-pair substitutions, were incubated in the presence of drug, with colony appearance on histidine negative medium indicating point mutation reversal as originally described by Ames [68–70] and subsequently by various investigators [29, 71, 72].

Briefly, approximately ten million bacteria are exposed in triplicate to test agent (six concentrations between 0.001953 and 0.0625 mg/ml), a negative control (DMSO) and a positive control (2-aminoanthracene [73–76]) for 90 min medium containing a low concentration of histidine (sufficient for about two doublings). The cultures are then diluted into indicator medium lacking histidine, and dispensed into a 48-well plate. The plate is incubated for 48 h, and cells that have undergone a reversion will grow in a well, resulting in a color change in wells with growth. The number of wells showing growth are counted and compared to the vehicle control. An increase in the number of colonies of at least 2-fold over baseline (mean + SD of the vehicle control) indicates a positive response. An unpaired, one-sided Student's *t* test is used to identify conditions that are significantly different from the vehicle control. When S9 fraction is included in the experiment, S9 fraction from the livers of Aroclor 1254-treated rats [77, 78] is included in the incubation at a final concentration of 4.5 %. An NADPH-regenerating system is included as well to ensure a steady supply of reducing equivalents.

Cytochrome P450 (CYP) Inhibition Analysis

Inhibition profiles of five main cytochrome P450 isoforms (CYP1A, CYP2C9, CYP2C19, CYP2D6, and CYP3A4) were investigated in a blinded manner using the Cloe Screen Cytochrome P450 Inhibition assay. Isoform-specific substrates were incubated individually with human liver microsomes and a range of test compound concentrations (typically 0.1–25 μM, when possible, unless solubility was low). At the end of the incubation, the formation of the appropriate metabolite was monitored by LC-MS/MS (or fluorescence in the case of CYP1A using ethoxyresorufin as substrate) at each of the test compound concentrations. In the inhibition assay, a decrease in the formation of the metabolites compared to the vehicle control is used to calculate an IC_{50} value (test compound concentration which produces 50 % inhibition). For analysis, a specific positive control inhibitors for each of the isoform assays.

Isoform	Substrate Reaction	Positive Control Inhibitor
CYP1A	Ethoxyresorufin O-deethylation	α-Naphthoflavone
CYP2C9	Tolbutamide 4-hydroxylation	Sulphaphenazole
CYP2C19	S-mephenytoin 4-hydroxylation	Tranylcypromine
CYP2D6	Dextromethorphan O-demethylation	Quinidine
CYP3A4	Midazolam 1-hydroxylation	Ketoconazole

Typically the compounds can be categorized into the following classification based upon inhibition characteristics:

Potent inhibition $IC_{50}<1$ μM
Moderate inhibition IC_{50} between 1 and 10 μM
No or weak inhibition $IC_{50}>10$ μM

For compound advancement, inhibition potency for each isoform-specific enzyme was calculated. Potent inhibition is considered unfavorable and may preclude the further development of a compound.

Blood Brain Barrier Assessment

MDR1-MDCK cells originate from the transfection of Madin Darby canine kidney (MDCK) cells with the *MDR1* gene, the gene encoding for the efflux protein, P-glycoprotein (P-gp) [79]. The cells were seeded on a Multiscreen™ plate (Millipore, MA, USA) and formed a confluent monolayer over 4 days prior to the experiment. On day 4, the test compound was added to the apical side of the membrane and the transport of the compound across the monolayer was monitored over a 60 min time period. To study drug efflux, it was also necessary to investigate transport of the compound from the basolateral compartment to the apical compartment and calculate an efflux ratio. GC/MS was used to identify the compound on both sides of the membrane, and if necessary, elution peaks were integrated in order to quantify an efflux ratio. The apparent permeability coefficients (Papp) for both directions are calculated along with the efflux ratio (Papp B → A/Papp A → B).

The potential for BBB penetration was viewed according to the following standards:

(1) high if Papp A → B$\geq 3.0\times10^{-6}$ cm/s and efflux<3.0

(2) moderate if Papp A → B≥3.0×10^{-6} cm/s and 10> efflux≥3.0×10^{-6} cm/s.
(3) low if Papp A → B≥3.0×10^{-6} cm/s and efflux≥10 or Papp A → B<3.0×10^{-6} cm/s.

Results

This study documents a series of in vitro screening assays that can applied to the development of a wide variety of small molecules, and it not specific for flavonoids. In this program, a chemical library of new flavonoid molecules was synthesized based upon the parent compound fisetin [80, 81]. To ensure the development of compounds superior to that of fisetin, the baseline pharmacological properties of fisetin were characterized. Fisetin had a dose-dependent effect on HT22 cell survival with a calculated EC_{50} of 4 μM (Table 1). In the second cell screening assay, where neuronal degeneration of mouse cortical neurons is induced by administration of the excitotoxin glutamate, fisetin did not confer neuroprotection when tested at 0.1 μM (Table 1).

Neuroprotection Studies

Because of the high EC_{50} of 4 μM for fisetin in the HT22 cell based assay, low lipophilicity of fisetin (i.e., poor BBB penetration evaluated using the MDCK-MR1 cell assay. Papp A → B of 2.96×10^{-6} and efflux=0.7, placing it in the category of low BBB penetration), high topological polar surface area (tPSA), and poor bioavailability, we chemically modified the structure of fisetin to not only improve neuroprotective properties in cell culture assays, but to also improve BBB penetration. The primary goal was to develop a low MW compound with beneficial characteristics, including and improved EC_{50} in the primary HT22 cell assay, a CLogP value less than 5, a tPSA less than 90 [82, 83], and moderate to high BBB penetration in the MDCK assay. Taken together, these properties should increase lipophilicity and possibly brain penetration in vivo.

In this development program, we used two different screening assays to develop a neuroprotection profile for all newly synthesized compounds, so that the candidates chosen for further development are pleiotropic [15, 20, 38]. Table 1 presents a series of 10 novel flavonoid-based molecules that were chosen based upon extensive screening for neuroprotective activity in the HT22 IAA cell assay and using the cortical excitotoxicity neuroprotection assay. In this program, stringent, but achievable criteria were set for compound advancement including greater than 80 % neuroprotection with an EC_{50} of <100 nM in the HT22 cell IAA assay and >35 % cell survival against glutamate toxicity in the cortical cell assay using a drug concentration of 100 nM.

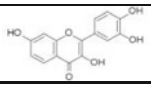
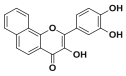
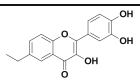
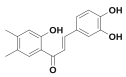
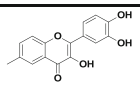
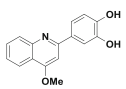
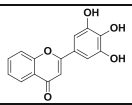
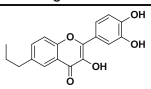
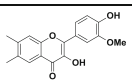
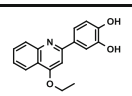
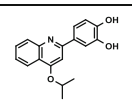
In Table 1, the compounds are sorted by decreasing EC_{50} value in the HT22 assay (Column 8), as a measure of enhanced neuroprotective potential. The data shows that the 10 new compounds have 45–190-fold the neuroprotection potential of the parent compound. It should be noted that hundreds of compounds (not shown) were excluded from further de-risking analysis because they did not meet the basic inclusion criteria described above. The data also shows that neuroprotective activity or potential (Column 8) is not directly correlated with either CLogP (Column 7) or tPSA (Column 6), as measures of estimated lipophilicity and polar surface area, respectively. In addition, there was no specific correlation between efficacy in the cortical neurons assay (Table 1 column 9) and either CLogP or tPSA (Column 7 and 6, respectively). Thus, this data set clearly shows that even with this rational approach, basic chemical characteristics are not effective predictors of "efficacy." On the basis of neuroprotective activity in two assays, the ten new compounds were further screened for drug-like properties using a series of in vitro assays for ADME [29], toxicity [28, 29], and BBB penetration [24–26, 79, 84].

CeeTox Analysis

Tables 2, 3, and 4 present data from CeeTox analysis. Fig. 2 shows the dose-response profiles for the effects of Compound CSMC-6 on cellular toxicity using a 24-h analysis endpoint. Similar dose–response curves were constructed for all ten compounds under investigation in the study. In general, for CSMC-6 (Fig. 2 and Tables 2, 3, 4, and 5), the concentration that produced a half-maximal response or TC_{50} (μM) for all general toxicity measures differed depending upon the specific endpoint assessed (range 71–282 μM); with a minimum TC_{50} was 71 μM. Moreover, as shown in Tables 2, 3, and 5, rotenone was highly toxic and significantly reduced TC_{50} values to 0.05–3.88 μM. The same toxic response was noted for camptothecin (TC_{50} values of 3.0–5.0) on all parameters except for membrane toxicity, which was not affected by topoisomerase inhibition.

The results from CeeTox analysis are to be viewed as a composite, since the strength of CeeTox analysis is the use of an algorithm [59] that integrates the findings from a series of assays to predict the overall toxicity of a drug candidate [27, 28, 58]. Data from all analytical endpoints shown in Tables 2, 3, and 4 are used to generate a C_{tox} value for each individual compound based upon a proprietary CeeTox Inc. algorithm [59]. The C_{tox} value was calculated from the TC_{50} values documented in Tables 2, 3, and 4. The C_{tox} ranking for each drug, which is an estimate of a sustained concentration expected or necessary to produce toxicity in a rat 14-day repeat dose study was 28–70 μM. Per standard CeeTox criteria, all drugs are thus considered to have a low to moderate probability of in vivo toxicity effects. We have rank ordered the 10 drug

Table 1 Primary drug candidates

CSMC ID#	Chemical Catalog #	Structure	Name	MW	tPSA	CLogP	HT22 EC_{50} (nM)	Cortical Protection @100 nM
Parent	Fisetin		3,3',4',7-tetrahydroxyflavone	286	107	1.24	4000	0
CSMC-6	CSM040		3,3',4'-trihydroxy alpha-naphthoflavone	320	87	2.99	88	86
CSMC-9	PM-013		6-ethyl-3,3',4'-trihydroxy flavone	298	87	2.84	61	51
CSMC-10	CMS011		4',5'-dimethyl-2',3,4-trihydroxychalcone	284	78	3.64	53	37
CSMC-11	PM-010		6-methyl-3,3',4'-trihydroxy flavone	284	87	2.31	46	65
CSMC-12	CMS007		4-methoxy-2-(3,4-dihydroxyphenyl) quinoline	267	62	3.66	43	43
CSMC-19	PM-008		3',4',5'-trihydroxy flavone	270	87	1.70	42	100
CSMC-13	PM-012		6-propyl-3,3',4'-trihydroxy flavone	312	87	3.37	38	82
CSMC-15	CMS069		6,7-dimethyl-3,4'-dihydroxy-3'-methoxyflavone	312	76	3.19	36	86
CSMC-16	CMS023		4-ethoxy-2-(3,4-dihydroxyphenyl) quinoline	281	62	4.20	21	100
CSMC-17	CMS024		4-isopropoxy-2-(3,4-dihydroxyphenyl) quinoline	295	62	4.50	21	58

candidates into 1 of 3 different C_{tox} ranking groups: 1–20 high probability, 21–50 moderate probability, and 51–300 low probability. Compounds that fit into the high probability of toxic effect include rotenone, a mitochondrial toxin and camptothecin, a topoisomerase inhibitor, the positive controls used in the analysis. However, none of the new drug candidates were characterized as having a high probability of in vivo toxic effects (Table 5).

Mutagenicity Tests

Genotoxicity studies were done using 2 strains of bacteria, specifically *S. typhimurium* TA989: *hisD3052*, *rfa*, *uvrB*/pKM101, which detects frameshift mutations and *S. typhimurium* TA100*: hisG45*, *rfa*, *uvrB*/pKM101, which detects base-pair substitutions either in the presence or absence of an S9 fraction from the livers of Aroclor 1254-treated rats clearly showed that none of the drug candidates were mutagenic. As shown in Table 6, under all conditions tested, there was no significant (≥2-fold) increase in the number of colonies formed in the presence of the drug candidates up to and including a concentration of 10 μM.

Cytochrome P450 Inhibition Analysis

The results of CYP450 inhibition analysis are presented in Table 7. In the Cloe Screen CYP450 Inhibition assay, a decrease in the formation of the metabolites compared to the vehicle control is used to calculate an IC_{50} value (test compound concentration which produces 50 % inhibition). For each enzyme isotype, a specific substrate was used for analysis [30, 31].

Table 2 CeeTox analysis—general cellular toxicity

Compound	Cell Mass TC_{50} (μM)	MemTox TC_{50} (μM)	MTT TC_{50} (μM)	ATP TC_{50} (μM)
CSMC-6	80	282	71	81
CSMC-9	82	>300	81	80
CSMC-10	86	>300	83	75
CSMC-11	>300	>300	>300	52
CSMC-12	208	>300	293	223
CSMC-19	>300	>300	>300	256
CSMC-13	78	>300	83	58
CSMC-15	79	>300	74	78
CSMC-16	215	>300	170	162
CSMC-17	241	>300	298	217
ROTENONE	0.09	3.88	0.19	0.05
CAMPTOTHECIN	3.0	>300	3.2	5.0

TC_{50} = concentration that produced a half-maximal response.TC_{50} values were estimated from the graphs similar to that presented in Fig. 2a–c

MemTox membrane permeability, *GST* α-glutathione S-transferase (membrane leakage), *MTT* 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide, *ATP* adenosine triphosphate

Table 3 CeeTox analysis—summary of oxidative stress and apoptosis

Compound	Total GSH TC_{50} (μM)	Membrane lipid peroxidation	Caspase 3 activity (index/dose)
CSMC-6	87	0	NC
CSMC-9	149	0	1/300
CSMC-10	144	2	1/100
CSMC-11	>300	0	1/300
CSMC-12	>300	1	NC
CSMC-19	>300	2	1/300
CSMC-13	252	2	1/100
CSMC-15	76	0	1/300
CSMC-16	243	0	NC
CSMC-17	295	0	NC
ROTENONE	0.03	2	1/1
CAMPTOTHECIN	5.0	1	3/100

Membrane lipid peroxidation data:

0 = No Change,

1 = Modest increase with maximum values≤15 pg/mL,

2 = Concentration related increase with maximum values≥15 pg/mL,

3 = Concentration related increase with a maximum value≥30 pg/mL

Caspase 3 data

0–200=1, 200–400=2, 400–600=3, 600–800=4, 800–1,000=5, 1,000–1,200=6, 1,200–1,400=7, 1,400–1,600=8, 1,600–1,800=9, 1,800–2,000=10

NC no change

TC_{50} = concentration that produced a half-maximal response

Table 4 CeeTox analysis—summary of P-glycoprotein (PgP) binding

Compound	% Control (Compound)	% Control (Compound + CSA)	% Difference
CSMC-6	68.3	65.6	NC
CSMC-9	97.0	68.2	29.7
CSMC-10	73.4	63.1	14.1
CSMC-11	80.3	67.8	15.7
CSMC-12	85.9	78.4	NC
CSMC-19	88.4	88.1	NC
CSMC-13	87.9	40.2	54.3
CSMC-15	70.8	61.8	12.6
CSMC-16	81.3	82.7	NC
CSMC-17	80.8	78.1	NC

The H4IIE cells possess high levels of PgP protein in the outer membrane. As a result compounds submitted for toxicity evaluations are also evaluated for their potential binding to PgP. Cells are incubated with and without cyclosporin A (CSA) (a PgP inhibitor) at a single exposure concentration (20 or 50 μM) and the difference in toxicity determined with the MTT assay. Compounds with increased toxicity in the presence of CSA have a high probability of binding to PgP proteins. PgP interaction ranking (based on % difference): 1–20 %= low interaction, 20–50 %=moderate interaction, and 50–100 % high interaction

NC no change

Of the five isotypes studies, the drug candidates did not significantly inhibit CYP2C9, CYP3A4, CYP2D6, or CYP2C10 at concentration up to and including 10 μM. In addition, five of the candidates also did not inhibit CYP1A2. However, as shown in the table, CSMC-6, -12, -10, -16, and -17 all inhibited this CYP isotype with IC_{50}'s between 1.14 and 7.49 μM. This is classified as moderate inhibition and may be important for chronic drug administration, but not acute neuroprotection.

BBB Penetration Analysis

For BBB penetration assessment of flavonoids, we have extensively utilized Madin Darby canine kidney (MDCK) cell assays [84, 85]. MDCK cells are plated under standard culture conditions, where they develop tight junctions and form monolayers of polarized cells. Since MDCK cells have low expression levels of transporters and low metabolic activity, we have utilized an MDCK cell line that is transfected with the human MDR1 gene encoding for the efflux protein, P-glycoprotein (P-gp) [28, 29].

The apparent BBB penetration of fisetin was evaluated using the MDCK-MR1 cell assay. Fisetin, has a Papp A → B of 2.96×10^{-6} and efflux=0.7, placing it in the category of low BBB penetration. By our current evolving criteria for candidate selection, fisetin would be excluded from further analysis.

Table 8 provides BBB penetration data for 5 of the 10 original candidates plus fisetin for comparison purposes.

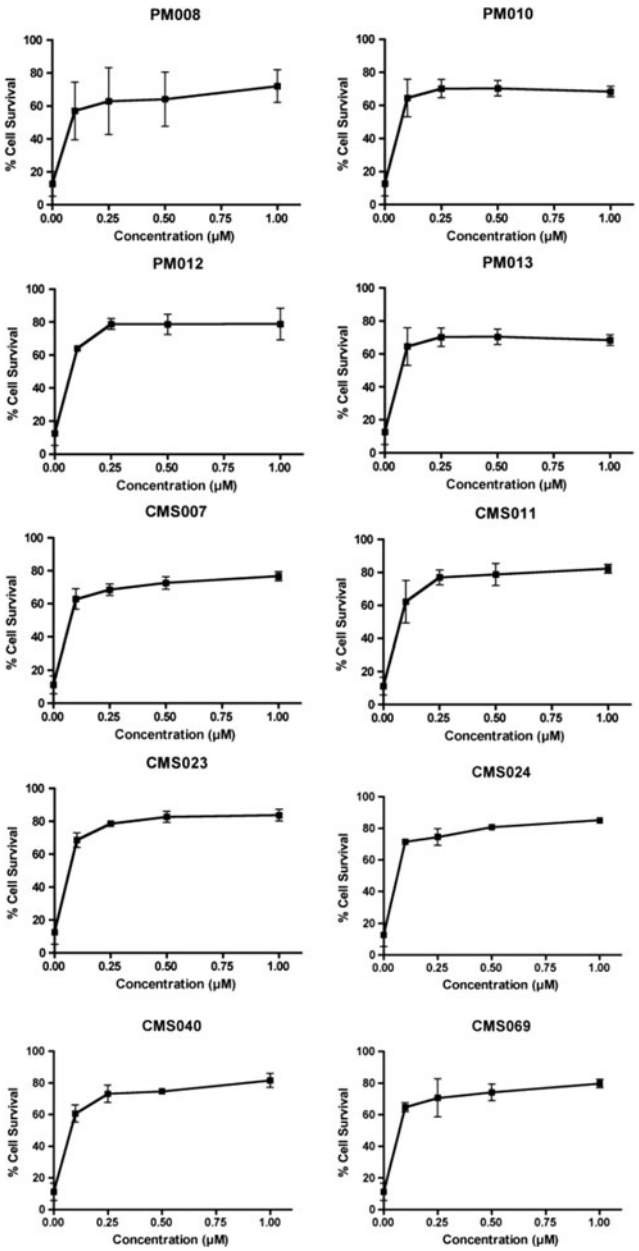


Fig. 1 HT22 IAA neuroprotection profiles. Flavones promote cell survival following IAA challenge. Pharmacological effects of the 10 primary candidates on cultured HT22 mouse hippocampal cells treated with 20 μM iodoacetic acid (IAA) for 2 h alone or in the presence of varying concentrations of drug. At 24 h, cell survival was measured using a standard MTT assay (1, 2). In the absence of a neuroprotective, >95 % of the cell population dies off within 24 h (3, 4). The graph shows that the candidates are neuroprotective up to 1 μM, the maximum concentration used in the assay and cell survival was maximally increased to 75–85 % of control

Only compounds classified as moderate to high BBB penetration are further considered for development. Of the 6 compounds included in the analysis, CSMC-13 is in the low BBB penetration category and is excluded from further analysis and 4 are in the high penetration category and will be pursued as drug candidates.

Discussion and Conclusion

Using a specific series of in vitro neuroprotection and de-risking assays, we have been able to determine some of the pharmacological properties necessary to advance novel fisetin analogs to the level of animal model efficacy assessment. In this study, we have modified the ring structure of fisetin to improve potency and physicochemical properties. Fisetin was identified as a weak lead neuroprotective compound through screening a library of small molecules using a mouse HT22 hippocampal chemical ischemia (IAA

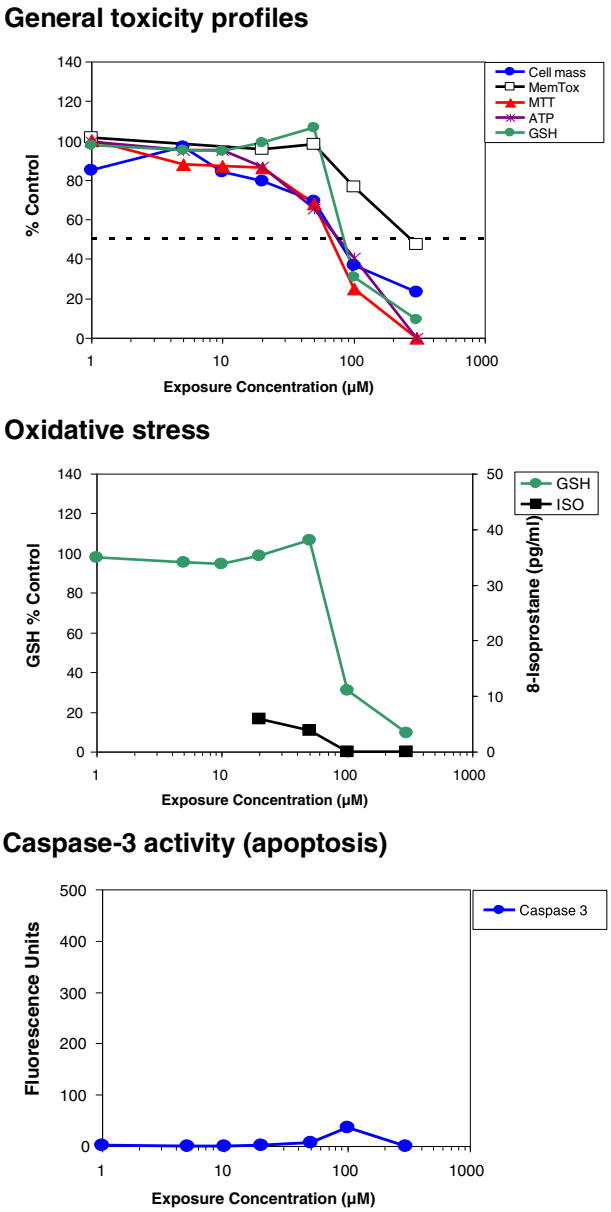


Fig. 2 Representative CeeTox analysis curves (Compound CSMC-6). **a** General toxicity profiles. **b** Oxidative stress. **c** Caspase-3 activity (apoptosis)

Table 5 CeeTox analysis—summary of general cytotoxicity

Ctox ranking (μM)—probability of in vivo effects		
High 1–20	Moderate 21–50	Low 51–300
Rotenone (0.03)	CSMC-6 (28)	CSMC-11 (52)
Camptothecin (0.1)	CSMC-9 (32)	CSMC-16 (56)
	CSMC-15 (32)	CSMC-12 (63)
	CSMC-10 (34)	CSMC-19 (64)
	CSMC-13 (37)	CSMC-17 (70)

Ctox = estimated sustained plasma concentration where toxicity would be expected to occur in vivo. Note: Compound listed under the high category, which are cytotoxic control molecules assayed in the Ceetox assay in parallel to test compound and are not being developed to treat stroke. Numbers in brackets indicate the Ctox value for each individual compound

toxicity) assay that mimics several important aspects of ischemia and stroke [3, 4, 56, 57] and in vivo activity [4, 86].

Based upon compelling correlative information reviewed by Pajouhesh and Lenz [83], which indicates that currently marketed CNS active drugs have a ClogP of approximately 2.5, we attempted to alter ClogP by chemically modifying fisetin. Pajouhesh and Lenz [83] have suggested that there is a good correlation between increasing ClogP and LogBBB, where increasing lipophilicity is correlated with increasing brain penetration. Moreover, Pajouhesh and Lenz [83] suggested that CNS active drugs have a lower polar surface area than other drug classes, and the upper limit of PSA for CNS penetration is approximately 90. In an initial attempt to enhance brain penetration, the CLogP of fisetin needed to be increased above 1.24, but must be maintained below 5 [82, 83] and the tPSA was reduced below 107 (Table 1). Table 1 represents a subset of the molecules that were

Table 6 Ames test for mutagenicity

Compound	TA98-S9	TA98+S9	TA98-S9	TA98+S9
CSMC-19	Negative	Negative	Negative	Negative
CSMC-11	Negative	Negative	Negative	Negative
CSMC-9	Negative	Negative	Negative	Negative
CSMC-6	Negative	Negative	Negative	Negative
CSMC-15	Negative	Negative	Negative	Negative
CSMC-13	Negative	Negative	Negative	Negative
CSMC-12	Negative	Negative	Negative	Negative
CSMC-10	Negative	Negative	Negative	Negative
CSMC-16	Negative	Negative	Negative	Negative
CSMC-17	Negative	Negative	Negative	Negative

Target criteria: not mutagenic<10 μM (negative)

Table 7 Cytochrome P450 isotype inhibition analysis

	CYP (IC_{50} ≥ 10 uM)				
	CYP1A2	CYP2C9	CYP3A4	CYP2D6	CYP2C19
Compound	Phenacetin	Tolbutamide	Mephenytoin	DextroM	Midazolam
CSMC-19	>25	>10	>10	>10	>10
CSMC-11	>25	>10	>10	>10	>10
CSMC-9	11.6	>10	>10	>10	>10
CSMC-6	1.14	>10	>10	>10	>10
CSMC-15	>25	>10	>10	>10	>10
CSMC-13	>25	>10	>10	>10	>10
CSMC-12	3.7	>10	>10	>10	>10
CSMC-10	6.99	>10	>10	>10	>10
CSMC-16	3.99	>10	>10	>10	>10
CSMC-17	7.49	>10	>10	>10	>10

Gray highlighted compounds do not meet minimum criteria for advancement. Target criteria, IC_{50}≥10 μM

synthesized and screened for in vitro efficacy; compounds that were advanced to this developmental stage based upon efficacy in HT22 cells. As shown in Table 1, we have identified a series of compounds with ClogP values below 5 and tPSA below 90, suggesting that the compounds may be lipophilic and cross the BBB. As shown in Table 1, with this specific selection of compounds, there was no correlation between the calculated CLogP value and the ability of the compound to confer neuroprotection in either the HT22 cell assay or the cortical cell assay.

Although we chose to use a HT22 cell IAA assay in combination with a cortical cell neurotoxicity assay for drug development, there are multiple alternative in vitro assays that can be utilized for the initial screening process. For example, many researchers believe that standard oxygen-glucose deprivation (OGD) cell culture assays reproduce some component on stroke and prefer that assay [87]. Others believe that an excitotoxicity assay is necessary during neuroprotectant development; however, since NMDA antagonists have failed in clinical trials [88, 89], excitotoxicity screening assays should not be used as primary screening assays and if they are used, they should be used with caution. The oxytosis assay using HT22 cells has also been described in the literature and has been shown to be useful as a screen for novel neuroprotective compounds [90]. Even though the assay utilizes HT22 cells incubated in the presence of 5 mM glutamate, since this particular hippocampal cell line does not have NMDA receptors, cell death is not mediated by an excitotoxic mechanism, rather cell death is mediated by the depletion of glutathione [56]. A review of the literature also shows that many laboratories prefer to use PC12 cells as the in vitro test system [91–93]. PC12 cells have been used to determine both neuroprotective and neurotrophic activity profiles of novel compounds. Given the fact that there is still no FDA-approved neuroprotective agent for stroke, the "best" strategy remains to be defined no matter what the bias is of the investigator. The strategy can only be defined and implemented when a neuroprotective

Table 8 BBB penetration potential of select drug candidates

Compound	Papp A→B	Efflux Ratio	BBB Potential	CLogP
Fisetin	2.96	0.7	low	1.24
CSMC-13	0.10	1.0	low	3.37
CSMC-15	4.08	0.4	HIGH	3.19
CSMC-11	6.67	0.12	HIGH	2.31
CSMC-9	40.9	0.19	HIGH	2.84
CSMC-19	105	0.01	HIGH	1.70

Gray highlighted compounds do not meet minimum criteria for advancement. Gold highlighted compounds are selected for advancement

Target criteria: high or moderate potential for BBB penetration

1) high if Papp A → B≥3.0×10^{-6} cm/s and efflux<3.0,

2) moderate if Papp A → B≥3.0×10^{-6} cm/s and 10>efflux≥3.0×10^{-6} cm/s

3) low if Papp A → B≥3.0×10^{-6} cm/s and efflux≥10 or Papp A → B<3.0×10^{-6} cm/s

compound is screened using in vitro assays and then proves to be positive in clinical trials.

To de-risk the development of novel flavonoids, a sequential series of in vitro assays were utilized, some of which have been described previously [28, 29, 58]. The tiered approach uses CeeTox analysis as the first assessment for general cellular toxicity of new drug candidates. In Table 4, data is provided for PgP binding characteristics of all compounds. The last column in the Table shows that all but 1 compound (i.e., CSMC-13) does not have a significant interaction with PgP, suggesting that flavonoid binding to PgP is not problematic. In Table 5, the C_{tox} ranking values are documented for the 10 drug candidates. Based upon the minimum criteria of moderate probability of in vivo effects, all 10 compounds meet Ctox criteria for continued development. There were differential effects of some of the compounds on the parameters measured, however, only the controls, rotenone and camptothecin proved to be excessively toxic. The inclusion of positive "toxic" controls demonstrates that the assay was within previously established parameters [28, 29, 58]. Moreover, the observation that all ten compounds were "negative" in the CeeTox assay justified their continued development and screening in additional de-risking assays.

Historically, the Ames test has been used as a useful screen for compounds that may be used chronically [29, 68–72]. Even though the neuroprotective compounds being developed for AIS are for acute use, primarily in elderly patients [13–15], for comprehensive risk assessment we included mutagenicity testing as the second tier of de-risking to ensure safe administration of a novel neuroprotective compound. In this study, we assessed the genotoxicity of all ten compounds using two strains of bacteria, TA989 and TA100, both in the absence or presence of an S9 liver fraction [73] from Aroclor 1254-treated rats; Aroclor 1254 was used a potent metabolic activator or inducer [74, 77, 78, 94]. The S9 fraction, which is a post-mitochondrial supernatant fraction, is a mixture of microsomes and cytosol that contains a wide variety of phase I and phase II metabolism enzymes including (alphabetically) acetyltransferases, carboxylesterases, cytochrome P450's (CYP 450), epoxide hydrolase(EH), flavin-monooxygenases, glutathione S-transferases, methyltransferases, sulfo-transferases, and uridine 5′-diphospho-glucuronosyl-transferase. Thus, in the presence of the S9 fraction, the study drug will be highly metabolized and transformed. The rationale for using two strains is based upon the sensitivity of each bacterial strain to toxins. Together, the use of two bacterial strains can detect frame-shift mutations or base-pair substitutions [68–70]. However, as shown using this thorough screen, none of the compounds were genotoxic. Thus, they were further de-risked using multiple concentration CYP 450 inhibition analysis to determine IC_{50} values.

For drug development, there is significant value is assessing possible drug-drug interactions mediated by CYP450, if drugs are to be administered orally. First pass hepatic metabolism [95] of orally administered drugs is orchestrated by a family of approximately 60 or more enzymes (CYP450). This type of metabolism is avoided by alternative drug administration routes such as intraveous (IV) administration, a route that is clinically relevant and appropriate for the treatment of stroke. Assessment of the potential of a compound to inhibit a specific CYP450 enzyme is important as co-administration of compounds may result in one or both inhibiting the other's metabolism potentially leading to adverse drug reactions or toxicity due to altered plasma levels in vivo. In vitro CYP450 inhibition analysis can be useful in designing strategies for developing new drugs for eventual testing in vivo [30, 96]. In this program, we used the CYP450 Cloe Screen Inhibition analysis assay, which meets criteria set out in FDA guidelines [30, 96]. In this study, we concentrated on 5 main CYP450 isotypes

[CYP1A2 (5–10 %), CYP2C9 (15 %), CYP3A4 (50 %), CYP2D6 (25 %) and CYP2C19 (5–10 %)], the most common isoforms studied, which together metabolize approximately 100 % of all drugs on the market (estimated percent for each isotype is shown in parentheses) [97–99]. As shown in Table 7, there was no inhibition of CYP2C9, CYP3A4, CYP2D6 or CYP2C19 by the 10 drug candidates. However, five of the ten compounds, CSMC-6, -12, -10, -16, and -17 produced modest inhibition of CYP1A2 with concentrations in the range of 1.14–7.49 μM. Because our target criteria was for no inhibition at ≥10 μM (see also [83]), the 5 compounds indicated above were excluded from further de-risking.

With recent notable cardiotoxicity of many FDA-approved drugs [100], it may be prudent to include a human ether-a-go-go related gene (hERG) assay in the development plan. hERG encodes the inward rectifying voltage gated potassium channel in the heart (I_{Kr}) which is involved in cardiac repolarization. It is known that inhibition of the hERG current causes QT interval prolongation resulting in potentially fatal ventricular tachyarrhythmia [101]. The assay is cell based using Chinese hamster ovary (CHO)-hERG cells in combination with the measurement of electrical currents through the channel (Cyprotex.com). Alternative hERG assay methods have been developed which can be utilized in a high through-put format [102].

The final phase of de-risking for the selected candidates was to determine if the molecules could effectively cross a simulated BBB in vitro using a standard MDCK assay [79]. CSMC-13 which had a moderate CLogP value of 3.37 was unable to penetrate the BBB. However, CSMC-15, -11, -9, and -19 were all classified as having high BBB penetration characteristics and are candidates for further development. It is interesting to note that calculated CLogP values, which are estimations of lipophilicity, do not necessarily correlate with the ability of a drug to cross the BBB in vitro. Nevertheless, the CLogP values of the drug candidates are in the range of 1.70–3.19, which is consistent with values described by Pajouhesh and Lenz [83].

In conclusion, using a series of established neuroprotection and AMDE assays in combination with the MDCK assay, we have systematically characterized a series of novel neuroprotective compounds. With specific chemical modification of the basic fisetin molecule at rings A, B, or C, we the studies have yielded novel compounds, all of which had neuroprotective activities superior to fisetin, at least using the HT22 cell IAA assay and the cortical neuron glutamate assay. The subsequent use of a tiered or funnel approach to de-risking novel flavonoids resulted in four flavones that were not cytotoxic or genotoxic and did not inhibit any of the major CYP450's. The four compounds also proved to be excellent drug candidates based upon their ability to cross the BBB in vitro (MDCK assay).

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Conflict of Interest Disclosure There are no conflicts of interest to disclose.

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