

High-Throughput Phenotypic Screening of Human Astrocytes to Identify Compounds That Protect Against Oxidative Stress

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ABSTRACT

Astrocytes are the predominant cell type in the nervous system and play a significant role in maintaining neuronal health and homeostasis. Recently, astrocyte dysfunction has been implicated in the pathogenesis of many neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis. Astrocytes are thus an attractive new target for drug discovery for neurological disorders. Using astrocytes differentiated from human embryonic stem cells, we have developed an assay to identify compounds that protect against oxidative stress, a condition associated with many neurodegenerative diseases. This phenotypic oxidative stress assay has been optimized for high-throughput screening in a 1,536-well plate format. From a screen of approximately 4,100 bioactive tool compounds and approved drugs, we identified a set of 22 that acutely protect human astrocytes from the consequences of hydrogen peroxide-induced oxidative stress. Nine of these compounds were also found to be protective of induced pluripotent stem cell-differentiated astrocytes in a related assay. These compounds are thought to confer protection through hormesis, activating stress-response pathways and preconditioning astrocytes to handle subsequent exposure to hydrogen peroxide. In fact, four of these compounds were found to activate the antioxidant response element/nuclear factor-E2-related factor 2 pathway, a protective pathway induced by toxic insults. Our results demonstrate the relevancy and utility of using astrocytes differentiated from human stem cells as a disease model for drug discovery and development. *STEM CELLS TRANSLATIONAL MEDICINE* 2016;5:613–627

SIGNIFICANCE

Astrocytes play a key role in neurological diseases. Drug discovery efforts that target astrocytes can identify novel therapeutics. Human astrocytes are difficult to obtain and thus are challenging to use for high-throughput screening, which requires large numbers of cells. Using human embryonic stem cell-derived astrocytes and an optimized astrocyte differentiation protocol, it was possible to screen approximately 4,100 compounds in titration to identify 22 that are cytoprotective of astrocytes. This study is the largest-scale high-throughput screen conducted using human astrocytes, with a total of 17,536 data points collected in the primary screen. The results demonstrate the relevancy and utility of using astrocytes differentiated from human stem cells as a disease model for drug discovery and development.

INTRODUCTION

Induced pluripotent stem cells (iPSCs) from patients can be used to elucidate the molecular and cellular pathology associated with a disease and serve as a cell-based disease model for screening chemical libraries [1, 2]. Pluripotent stem cells (PSCs), of both induced and embryonic origin, are self-renewable and can be a source for the large number of cells needed for chemical library screening. With the potential of being

differentiated into various cell types, human cells that used to be difficult to obtain, such as neuronal cells and cardiomyocytes, are now accessible for use as cell-based models for compound screening and drug discovery [3].

Human PSCs are a particularly valuable tool for studying neurodegenerative diseases (reviewed in [3]). In vitro and in vivo studies of neurological diseases are challenging for a number of reasons. Cells of the human nervous system—neurons, astrocytes, and glia—are nonproliferative and difficult

to obtain in large quantities. In addition, many neurological diseases lack a disease-relevant *in vivo* model. In some cases where there is an *in vivo* model, the model may not accurately represent the human disease or predict the human response to potential therapeutics. Application of human PSCs can help address these significant issues, because human PSCs can be differentiated into various cell types of the nervous system, enabling a “disease-in-a-dish” modeling system. iPSCs have been generated for a number of different neurological diseases, including Parkinson’s disease, amyotrophic lateral sclerosis (ALS), spinal muscular atrophy, familial dysautonomia, Alzheimer’s disease, fragile X syndrome, and Rett syndrome, among others (reviewed in [3–5]).

PSCs technically provide an unlimited number of cells that can be used for differentiation into more mature cell types for drug discovery, which generally requires large numbers of cells. These differentiated cells may provide an alternative model system for evaluation of compound efficacy and toxicity in humans [6, 7] that may be more relevant biologically and in terms of disease pathology than animal models. However, challenges remain in using PSC-derived cells for screening, including the relatively long time needed to generate differentiated cells, the potential for high batch-to-batch variation, and the significant cost for reagents required to generate large quantities of these cells (reviewed in [8]). Despite these challenges, progress is being made to overcome these difficulties and PSC-derived cells are increasingly used in screening campaigns [9–14]. Of particular relevance, Pei et al. recently published a study in which they tested 80 potentially neurotoxic compounds in human iPSCs and iPSC-derived neural stem cells (NSCs), neurons, and astrocytes [7].

Astrocytes are distributed throughout the brain and spinal cord, and make up the greatest percentage of cells in the mammalian brain [15]. The essential and multifaceted roles of astrocytes in maintaining normal neuronal function have implications for their role in neurodegenerative diseases (reviewed in [15, 16]). In fact, many diseases once thought to be associated only with neurons involve astrocytes (reviewed in [17]). Astrocyte dysfunction has been implicated in Rett syndrome, fragile X syndrome, Huntington’s disease, and ALS (reviewed in [15, 18]). An increase in reactive oxygen species (ROS) and oxidative damage is associated with the pathology of many neurological diseases (reviewed in [16, 19–21]). Preventing ROS-related astrocyte dysfunction and death may, in turn, help prevent damage and death of neurons. Targeting astrocytes may thus be a viable alternative approach to discovering new therapeutics for various neurodegenerative diseases.

Although astrocytes can be harvested from brain tissue, it is difficult to obtain large quantities of cells using this method and the cells obtained have limited proliferative capacity. Recently, astrocytes that grow rapidly and can be propagated for prolonged periods in culture have been differentiated from ESCs and iPSCs [7, 22, 23]. Whereas previous methods to generate astrocytes from PSCs took 180 days or longer [24], this new protocol takes only 5–6 weeks and stocks of these differentiated astrocytes can be cryopreserved with minimal loss of viability. These differentiated astrocytes have gene expression and cell function characteristic of primary astrocytes [22, 23], thus making these cells an excellent resource for chemical library screening.

Here we describe a high-throughput screen of approximately 4,100 bioactive tool compounds and approved drugs that uses human astrocytes. After generating and cryopreserving a large batch of differentiated astrocytes from human embryonic stem cells (hESCs), these astrocytes were used in a chemical library screen

in a 1,536-well plate format to identify compounds that protect astrocytes from acute cell death induced by oxidative stress. In this study, we demonstrate that astrocytes differentiated from human PSCs can successfully be used for compound screening and can serve as a viable target for drug discovery. We also identified 22 compounds acutely protective against oxidative stress in astrocytes and that may be useful in studies targeting neurodegenerative diseases.

MATERIALS AND METHODS

Generation of Astrocytes From hESCs

Human neural stem cells (hNSCs) derived from H9 (WA09) human embryonic stem cells (N7800-100; Thermo Fisher Scientific, Carlsbad, CA, <https://www.thermofisher.com>) were cultured on Geltrex substrate in StemPro NSC serum-free medium (SFM) (A10509-01; Thermo Fisher Scientific) consisting of KnockOut Dulbecco’s modified Eagle’s medium (DMEM)/F-12, StemPro Neural Supplement, GlutaMAX-I, basic fibroblast growth factor (20 ng/ml; PHG0021; Thermo Fisher Scientific), and epidermal growth factor (20 ng/ml; PHG0315; Thermo Fisher Scientific). The hNSCs were expanded for 1 week, and passaged every 2–3 days. After 1 week, the NSCs were differentiated into astrocytes as described recently [22, 23]. Briefly, the medium was changed to astrocyte differentiation medium consisting of StemPro hESC SFM (A1000701 [Thermo Fisher Scientific]; DMEM/F-12, GlutaMAX-I supplement, bovine serum albumin 25%, and StemPro hESC supplement), in addition to 1% fetal bovine serum, FGF2 (8 ng/ml; PHG0264; Thermo Fisher Scientific), activin A (10 ng/ml; 120-14; PeproTech, Quebec, Canada, <https://www.peprotech.com>), heregulin β (10 ng/ml; 100-03; PeproTech), and insulin-like growth factor-1 analog (200 ng/ml; 100-11; PeproTech) to promote astrocyte differentiation. The culture was designated as “Astro P0” at this stage and expanded by passaging (1:6 split) every 4–5 days for approximately 1 month (Fig. 1A). Cells were cryopreserved at various time points during the differentiation process. Cell viability upon thaw was generally >75%. Immunocytochemistry (ICC) was used to confirm the differentiation status of hESC-derived astrocytes and gene expression was analyzed using protocols described previously [23]; more detail is provided in the supplemental online data. All hESC-differentiated astrocytes used for experiments were from days 35–45 (Fig. 1A).

Compound Plates

Detailed information on the generation and content of control compound plates can be found in the supplemental online data. Generally, control compound plates were used to deliver dimethyl sulfoxide (DMSO; vehicle control; basal/neutral signal) or terfenadine (apoptotic/cytotoxic control [25]; inhibitory signal) controls. The high-content oxidative stress primary screen was performed against the Library of Pharmacologically Active Compounds (LOPAC1280; Sigma-Aldrich, St. Louis, MO, <http://www.sigmaaldrich.com>) and the NIH Chemical Genomics Center Pharmaceutical Collection (NPC [26]). We screened a 6-point interplate titration of the 1,280 compounds that make up the LOPAC1280 library (10 mM to 3.2 μ M in the compound plate; final concentrations screened in the assay: 46 μ M to 14.7 nM) and a 3- (FDA B) or 4-point (FDA A) interplate titration of a total of 2,816 compounds in the NPC (FDA A: between approximately 9.4 mM and approximately 76.1 μ M in the compound plate, final

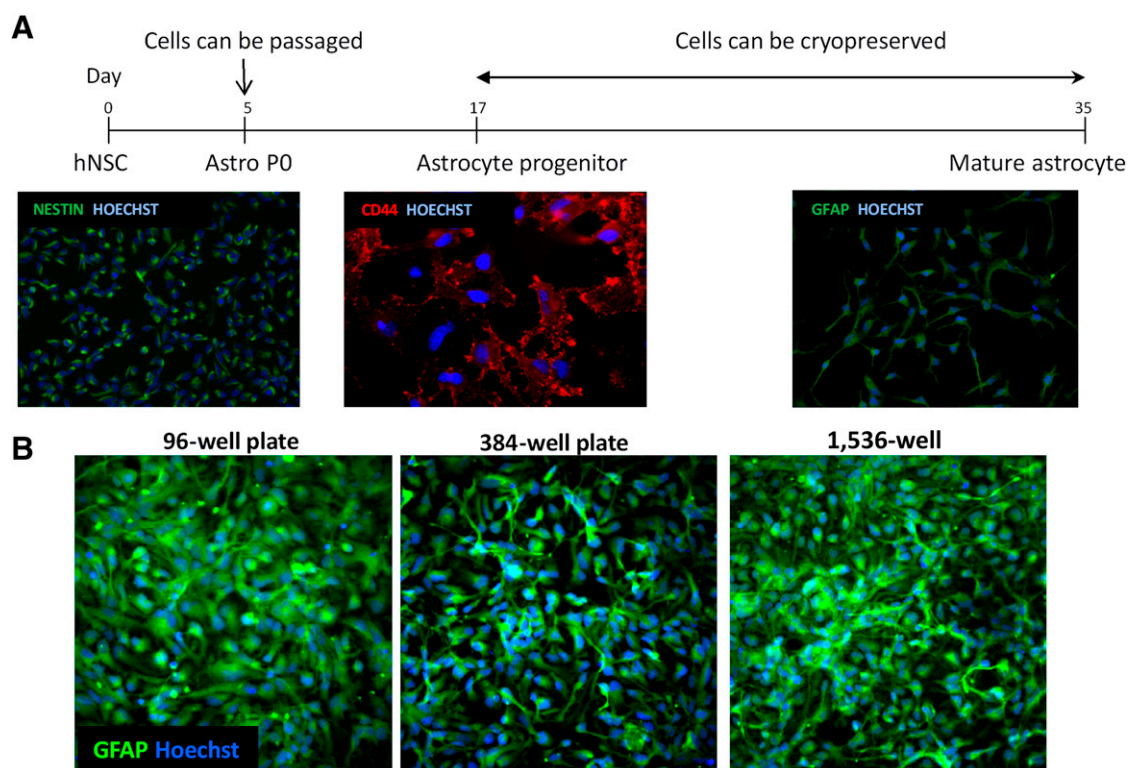


Figure 1. Differentiation of pluripotent stem cells to mature astrocytes and maintenance in 96-, 384-, and 1,536-well plates. **(A):** A differentiation timeline along with staining for nestin (an NSC marker), CD44 (marker of astrocyte precursor cells), and GFAP (a mature astrocyte marker) at critical time points in the differentiation protocol. The cells are proliferative, thus requiring passaging, and large stocks of cells can be cryopreserved after day 17. Later, when cells are brought up for use, differentiation into mature astrocytes is continued. **(B):** Human embryonic stem cell (hESC)-differentiated astrocytes adhered to, and were successfully maintained for >24-hours on PureCoat amine-coated microtiter plates. Astrocytes were healthy, stained positive for GFAP, and exhibited typical astrocyte morphology. Images from microtiter plates were acquired on an IN Cell Analyzer 2000. Magnification, $\times 40$. Abbreviations: GFAP, glial fibrillary acidic protein; hNSC, human neural stem cell.

concentrations screened in assay: approximately 43.2 to 0.35 μM ; FDA B: between approximately 22.4 mM and approximately 0.9 μM in the compound plate, final concentrations screened in assay: 103.2 μM to 4.13 μM). Each compound concentration was screened in duplicate for two assessments of activity for each compound at each concentration.

LOPAC1280 or NPC compounds identified as active in the primary screen ("hit/follow-up compounds"; $n = 66$) were replated in an 8-point 1:4 titration with a concentration range of 10 mM to 0.61 μM , for a final concentration range of 46 μM to 2.8 nM in a 5 μl per well assay volume. Follow-up compound plates were used for additional assays.

High-Content 1,536-Well Oxidative Stress Assay of hESC-Differentiated Astrocytes

To develop a high-throughput screening assay and screen chemical libraries using hESC-differentiated astrocytes, culturing conditions in the 1,536-well format had to be optimized for these cells, and cell viability in this format confirmed (supplemental online data). A detailed protocol for the oxidative stress assay to identify potentially cytoprotective compounds by screening the LOPAC1280 and NPC compound libraries can be found in supplemental online Table 1. The supplemental online data contains additional information on the development and optimization of this assay. The optimal concentration of, and incubation time with, hydrogen peroxide (H_2O_2), which was used to induce

oxidative stress, was experimentally determined to be 12 mM for 1 hour, which caused approximately 50%–80% of the hESC-differentiated astrocytes to display an apoptotic nuclear profile. Although this level of H_2O_2 is likely not physiologically relevant (postischemia concentrations of H_2O_2 are 50–100 μM [27]), treatment of astrocytes with more physiological concentrations of H_2O_2 did not induce levels of apoptosis significant enough to allow for the generation of a reliable and robust assay that is necessary for compound library screening. A compound tested in the assay that was found to reduce the number of apoptotic astrocytes after treatment with H_2O_2 , as assessed by nuclear characteristics, was considered active in the assay and of interest (Fig. 2A).

We used nuclear parameters to define astrocytes that were apoptotic. Nuclear characteristics have been demonstrated in previous studies to be reliable indicators of apoptosis and cell death [28–31]. Although some nuclear changes, such as chromatin condensation and pyknosis (nuclear shrinkage), are commonly attributed to apoptosis [32, 33], they can also be associated with necrosis [34, 35]. For this reason, with the assay format used in this study, there is the possibility that the increased nuclear condensation and nuclear shrinkage that we see upon H_2O_2 or compound treatment may also include cells undergoing not only apoptosis but also necrosis.

Terfenadine, which is neurotoxic [25] and has been shown to induce apoptosis in a number of cell lines [36, 37], was used as a positive control to define nuclear characteristics of cells undergoing apoptosis and cell death processes. At 100 μM terfenadine, significant loss of astrocytes occurs, and the remaining cells in

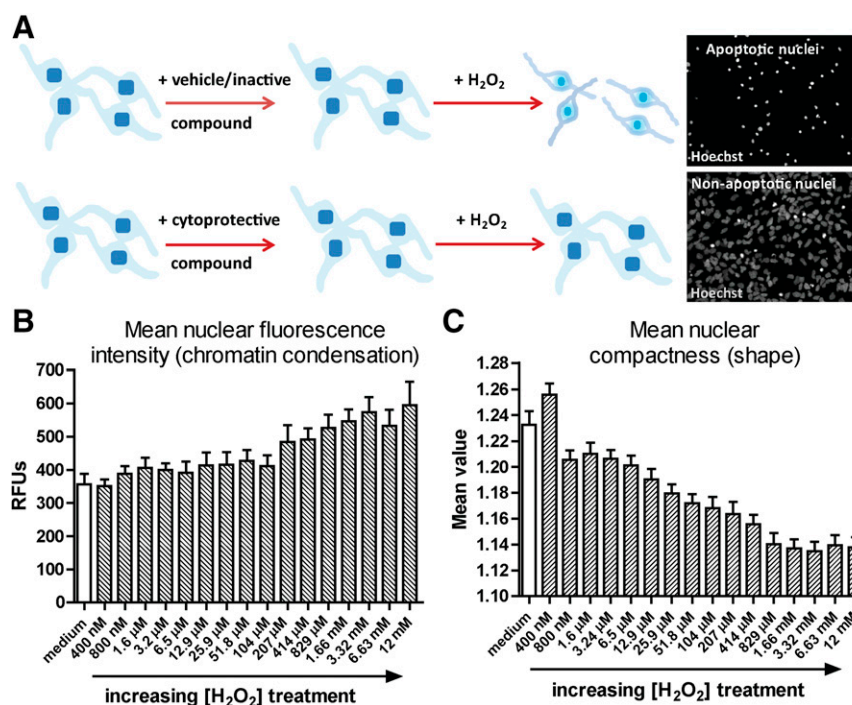


Figure 2. The high-content oxidative stress assay to identify cytoprotective compounds. **(A):** Human embryonic stem cell (hESC)-differentiated astrocytes are treated with compounds from a chemical library for 24 hours before treatment with 12 mM H₂O₂ for 1 hour. Treatment with 12 mM H₂O₂ induces cell death processes, characterized by cell and nuclear shrinkage and chromatin condensation. Compounds active in the assay prevent or reduce apoptotic events. Shown are images of astrocytes after a 24-hour treatment with 100 μM terfenadine, demonstrating nuclei of cells undergoing cell death, or DMSO, the vehicle control. Images were taken using the IN Cell Analyzer 2000 and 4',6-diamidino-2-phenylindole (DAPI) filters. Magnification, ×20. **(B, C):** hESC-differentiated astrocytes in a 1,536-well plate format were incubated with H₂O₂ up to a concentration of 12 mM for 1 hour. Assay media treatment is the far left bar in the graphs, followed by the increasing concentrations of H₂O₂ tested: 400 nM to 12 mM. **(B):** Mean nuclear fluorescence intensity of the DNA dye Hoechst 33342 shows a H₂O₂ concentration-dependent increase, indicative of chromatin condensation that is a characteristic feature of apoptosis. **(C):** The shape of nuclei also changes with increasing concentrations of H₂O₂, as demonstrated by a concentration-dependent decrease in mean nuclear compactness. Abbreviation: RFU, relative fluorescence unit.

the well exhibit features of apoptosis, including cell shrinkage, chromatin condensation (as evidenced by increased nuclear fluorescent intensity), and nuclear shrinkage [38] (Fig. 2A, inset). A nuclear profile for astrocytes treated with 100 μM terfenadine and indicative of cells undergoing cell death processes was characterized by a decrease in nuclear compactness values and an increase in nuclear intensity (supplemental online Fig. 1). Additional nuclear parameters that were found to change with 100 μM terfenadine treatment were an increase in nuclear elongation and a decrease in 1/(nuclear form factor). Changes in nuclear form factor have been previously found to significantly correlate with cells expressing caspase-9, a marker of apoptosis [28].

Because each compound from the LOPAC1280 and NPC libraries was screened in titration, concentration response curves (CRCs) were generated from the primary screen. Compounds were selected as primary hits and cytoprotective if they generated high-quality CRCs (supplemental online data) [39] and had a maximum response in the assay of at least 30% activity relative to the H₂O₂-treated control for at least two of the four nuclear parameters measured [i.e., nuclear intensity, compactness, elongation, or 1/(form factor)]. A hit compound that generates a “protective nuclear profile” gives values for nuclear parameters that are thus similar to DMSO-treated wells (supplemental online Fig. 2), characterized by a concentration-dependent increase in nuclear compactness and 1/(form factor) and a decrease in nuclear elongation and intensity compared with H₂O₂-treated wells and the 100-μM terfenadine control.

Data Analysis for the 1,536-Well Oxidative Stress Assay and LOPAC1280/NPC Screens

After acquiring images with the IN Cell Analyzer 2000 (GE Healthcare, Marlborough, MA, <http://www.gelifesciences.com>), data in the images were analyzed and quantified using IN Cell Analyzer Workstation software (GE Healthcare). Multitarget analysis was used to analyze the fluorescence-based images, and the Top-hat segmentation method was used to analyze nuclei, with nuclei defined as having a minimum area of 20 μm² with software sensitivity set at 50. Measures associated with analyzed nuclei were nuclear elongation, 1/(form factor), intensity, and compactness. Cell count, as determined by nuclei count, was also analyzed. Data from screening the LOPAC1280 and NPC compound libraries were processed using in-house-developed software, as described previously [40]. Formulas used to assess screening performance are as follows:

% Coefficient of variation:

$$\% CV = \left(\frac{SD}{mean} \right) \times 100$$

Z factor (or Z' value):

$$Z = \frac{[(mean_{max\ signal} - 3SD_{max\ signal}) - (mean_{min\ signal} + 3SD_{min\ signal})]}{(mean_{max\ signal} - mean_{min\ signal})}$$

Additional information regarding the high-content measurement definitions and quantitative high-throughput

screening data analysis can be found in the supplemental online data.

Secondary Assay to Eliminate False Positives in Primary Screening Assay

To help identify false-positive compounds among the 66 hit compounds identified in the primary assay, a luminescence-based adenosine triphosphate (ATP)-content cell viability assay was performed (supplemental online data). Possible false positives in the primary screening assay are compounds that are colored, autofluorescent, or cytotoxic. Data from this secondary assay, along with data from the primary screen and ARE- β -lactamase secondary assay (see below), were used to identify these potential false positives.

High-Content Assay to Assess Nuclear Parameters in the Presence of Compounds Without H₂O₂

To determine if any of the active compounds produced a nuclear phenotype in the absence of H₂O₂ treatment, the high-content assay was repeated without H₂O₂. Compound activity was normalized to the neutral control (vehicle-treated; 0% activity) and negative control (100 μ M terfenadine). Compounds were identified as active in the assay if they had activity for at least two specific nuclear parameters (i.e., CRCs characteristic of a protective compound), with efficacy \geq 20%.

Multiplexed ARE-*bla* and CellTiter Glo Assays in HepG2 cells

To determine if any of the 66 follow-up compounds activated a transcriptional response through the ARE, the compounds were tested in a β -lactamase (*bla*) reporter gene assay in HepG2 cells (CellSensor ARE-*bla* HepG2 cell line; K1633; Thermo Fisher Scientific) [41]. This assay was multiplexed with the CellTiter Glo assay (G7570; Promega, Madison, WI, <http://www.promega.com>) to assess compound cytotoxicity in this cell line. The assay protocol is provided in supplemental online Table 2 and details can be found in the supplemental online data.

High-Content Assay to Confirm Protective Activity of Compounds in an iPSC-Differentiated Astrocyte Cell Line

We performed the high-content nuclear morphology assay using iPSC-differentiated astrocytes (#AK-001-1V; XCell Science, Novato, CA, <http://www.xcell2.com>) challenged with 6 mM H₂O₂ to determine if the 22 prioritized compounds demonstrated protective activity in a different cell line (supplemental online data). A lower concentration of H₂O₂ was used in this assay because these astrocytes appeared more sensitive to H₂O₂ in an H₂O₂-titration experiment.

RESULTS

Generation and Characterization of Human Astrocytes From hESCs

hESC-Derived Astrocytes Express Appropriate Markers and Have the Gene Expression Profile of Mature Astrocytes

Astrocytes were differentiated from human ESC-derived NSCs over the course of 35 days. They were passaged at least twice a week and cryopreserved starting at day 17 to establish a stock of astrocyte progenitors that could later be thawed and matured

(Fig. 1A) [22]. Whereas NSCs expressed the NSC-marker nestin, differentiated progenitors were positive for the astrocyte progenitor marker CD44 by day 17 (Fig. 1A) [22]. On day 35, the purity of the differentiated astrocyte cultures was assessed by determining the expression of markers associated with NSCs, neurons, oligodendrocytes, and mature astrocytes using ICC and reverse transcription-polymerase chain reaction (RT-PCR) (supplemental online Table 3). The differentiated astrocyte cultures did not express Sox1, MAP2, or OLIG2, indicating that there were no detectable NSCs, neurons, or oligodendrocytes within this population. The differentiated cells were also found to express the mature astrocyte markers GFAP, S100 β , GLAST, and GLT-1. These data indicate that this method generates a population of mature astrocytes that can be used for further experiments.

To compare the maturity of differentiated astrocytes with the parental NSCs and commercially purchased fetal astrocytes, the gene expression profiles of these cell lines were determined by microarray analysis. Although the hESC-differentiated astrocytes clustered more closely with NSCs than fetal astrocytes (supplemental online Fig. 3), the correlation coefficients between the samples were all very similar (supplemental online Table 4) [23]. A high degree of correlation between hESC-differentiated astrocytes and NSCs is not unexpected and a number of studies have shown that a high degree of similarity in expression profiles exists between NSCs and NSC-derived astrocytes (hESC-differentiated astrocytes were NSC derived) [42, 43]. However, when the array data were examined at higher resolution for genes known to be highly expressed in astrocytes, such as GFAP and S100 β , the fetal and differentiated astrocyte samples showed very similar expression profiles. The microarray data were interrogated against lists of genes known to be important for astrocyte function, such as those involved in glutamate biology, iron metabolism, and cytokine signaling. Many of the genes that are important in these biological processes were expressed in both fetal and differentiated astrocytes in similar numbers (supplemental online Table 5). Some of the specific genes that were expressed in the differentiated astrocytes are listed in supplemental online Table 6 and include secreted factors, receptors, transporters, and downstream signaling components. Genes important for blood-brain barrier function and myelination, as well as genes associated with neurological diseases, were expressed in the differentiated astrocytes (supplemental online Table 6). The gene expression data reported here indicate that these astrocytes differentiated from pluripotent stem cells can be used for functional assays and as disease models.

Development of a 1,536-Well High-Throughput Oxidative Stress Assay Using hESC-Differentiated Astrocytes

Optimizing Cell Culture of Differentiated Astrocytes in 1,536-Well Plates

First, we compared the attachment and cell health of astrocytes grown in 6-, 96-, 384-, and 1,536-well formats (Fig. 1B) on 3 types of synthetic plate-surface coatings (supplemental online data). Astrocyte morphology appeared similar when grown in these plate formats, all cells stained positive for GFAP, and cells appeared healthy with projections to neighboring astrocytes. We chose to continue our experiments using the PureCoat amine-coated plates (354771; Corning, Corning, NY, <https://www.corning.com>) with a cell seeding density of 4,000 cells per well in a 1,536-well plate format for assays that lasted 24 hours.

Assessing Cell Viability of hESC-Differentiated Astrocytes in 1,536-Well Plates

To assess well-to-well variability in cell viability of hESC-differentiated astrocytes cultured for 24 hours in 1,536-well plates, we used the ATP content assay (CellTiter-Glo [CTG]; Promega). This assay measures the amount of ATP present in cells, which directly correlates with the number of viable and metabolically active cells in the well. We found that there was very little well-to-well variability in cell viability or cell number (supplemental online Fig. 4A). Terfenadine, which induces apoptosis in numerous cell lines [36, 37], also causes apoptosis in astrocytes and is cytotoxic (half maximal inhibitory concentration [IC₅₀]: 6.7 μ M) (supplemental online Fig. 4B).

Developing and Optimizing a 1,536-Well Format Oxidative Stress Assay Using hESC-Differentiated Astrocytes

Hydrogen peroxide can induce oxidative stress that ultimately results in apoptosis or necrosis [44, 45]. We used Hoechst 33342 DNA dye to assess these cell death processes, which are characterized by chromatin condensation and a decrease in nuclear size (pyknosis) [34, 35, 38]. Multiple studies have found that changes in nuclear morphology are a reliable indicator of apoptosis and cell death [29–31] and one study found that caspase-3 expression, a marker of apoptosis, correlates with certain nuclear changes, including nuclear form factor [28]. During apoptosis, as the chromatin in the nucleus condenses, the fluorescent Hoechst 33342 dye (H3570; Thermo Fisher Scientific) associated with the DNA also becomes more concentrated, such that nuclei have greater fluorescent intensity in a smaller nuclear area (Fig. 2A, inset). This DNA dye-based high-content imaging assay has several advantages: It does not require the use of expensive antibodies to measure apoptotic and cell death events, and the number of washes (that could cause cell loss) is kept to a minimum. We also found that this assay is more sensitive to compound toxicity than an ATP content assay (discussed below).

In addition, this DNA dye-based assay readout is impervious to H₂O₂, unlike the enzymatic reporter in the ATP content assay, which is inhibited by H₂O₂ (supplemental online Fig. 5). High-throughput screening for compounds that are antiapoptotic lends itself to a high-content, phenotypic screen [1, 46] because this type of screening assay is cell based and allows the collection and quantification of data on numerous nuclear characteristics that may be altered upon H₂O₂ and/or compound treatment. We found that after treatment of H₂O₂ for 1 hour, the majority of astrocytes initiated cell death processes, as characterized by an alteration in nuclear phenotype. When astrocytes are treated with a putative cytoprotective compound before the H₂O₂ challenge, immediate cell death is prevented, as indicated by normal nuclear characteristics (Fig. 2A).

Both the concentration of H₂O₂ and the duration of exposure to H₂O₂ required to induce apoptosis or necrosis vary depending on cell type, general cell health, and cell density [47]. Astrocytes are reportedly more resistant to oxidative stress than neurons [48], and thus may require higher concentrations of, and/or longer incubation times with, H₂O₂. We found that astrocytes had a concentration-dependent nuclear condensation response to H₂O₂, seen as an increase in fluorescence intensity (Fig. 2B). The mean nuclear compactness (a measurement of nuclear shape) of astrocytes treated with H₂O₂ for 1 hour was reduced significantly compared with untreated astrocytes (Fig. 2C). Thus, incubation with H₂O₂ for 1 hour was sufficient time to induce cell

death processes and cause nuclear condensation in astrocytes. With increasing incubation times with H₂O₂ (4 and 6 hours), we found that the cell count per well decreased (supplemental online Fig. 6) due to cell death, and there was an increase in signal variability (Z factors for vehicle-treated vs. 12 mM H₂O₂-treated astrocytes were 0.42 for 1-hour, -0.45 for 4-hour, and 0.18 for 6-hour incubation times with H₂O₂). We thus decided to conduct further experiments with a 1-hour incubation time with 12 mM H₂O₂, a condition in which 50%–80% of cells were undergoing cell death processes and which produced the best signal-to-basal (S/B) ratio with the least well-to-well variability.

Terfenadine also induced apoptosis and necrosis in astrocytes, causing a decrease in nuclear compactness and an increase in nuclear intensity (supplemental online Figs. 1A, 1B, 2A, 2B), similar to what was seen for astrocytes treated with H₂O₂ (supplemental online Fig. 2A, 2B). Additionally, terfenadine and H₂O₂ increased nuclear elongation and decreased 1/(nuclear form factor) (supplemental online Figs. 1C, 1D, 2C, 2D). Nuclear form factor has previously been found to significantly change with caspase-3 expression in apoptotic cells [28]. Thus, a compound that is potentially cytoprotective against oxidative stress in astrocytes is expected to increase the number of cells with nuclear characteristics more similar to vehicle-treated cells: decreased elongation, increased nuclear compactness, and increased 1/(nuclear form factor) values.

High-Throughput Screening of 4,096 Compounds With hESC-Differentiated Astrocytes

Screening Assay Performance

Once the high-content oxidative stress assay using hESC-differentiated astrocytes was optimized, we screened a set of 4,096 compounds that consisted of bioactive tool compounds and approved drugs. The performance for these two compound screens was similar (Fig. 3; supplemental online Fig. 7). Although the S/B ratio comparing the DMSO/vehicle-treated cells to H₂O₂-treated cells was low for most nuclear parameters (Fig. 3A; supplemental online Fig. 7A), the well-to-well variability, as measured by the percent coefficient of variation (%CV) was also extremely low—approximately 1% for most parameters in both screens (Fig. 3C; supplemental online Fig. 7C), resulting in Z' values of approximately 0.5 for some nuclear parameters (Fig. 3B; supplemental online Fig. 7B). The supplemental online data defines Z' value. The low S/B ratio when comparing the DMSO group with the H₂O₂-treated group was partially the result of using assay conditions in which only a fraction of the astrocytes underwent cell death processes. A noticeably larger S/B ratio was seen when astrocytes were treated with 100 μ M terfenadine for 24 hours (Figs. 2A [inset], 3D; supplemental online Fig. 7D). This generated Z' values greater than 0.5 for many of the nuclear parameters in both screens (Fig. 3E; supplemental online Fig. 7E).

The response of hESC-differentiated astrocytes to a titration of terfenadine was tested on each of the assay plates for both the LOPAC1280 and NPC screens to provide an additional assessment of assay performance and reproducibility. The terfenadine CRCs were similar for both screens (concentration causing apoptosis in 50% of the cells: approximately 10 μ M; Fig. 3F; supplemental online Fig. 7F). In addition, the well-to-well variability across the entire plate for the different nuclear parameters was low, with few outliers (supplemental online Fig. 8). This is consistent with the very low average %CV calculated for the compound fields for each of the plates in the LOPAC1280 and NPC screens (Fig. 3C; supplemental online Fig. 7C).

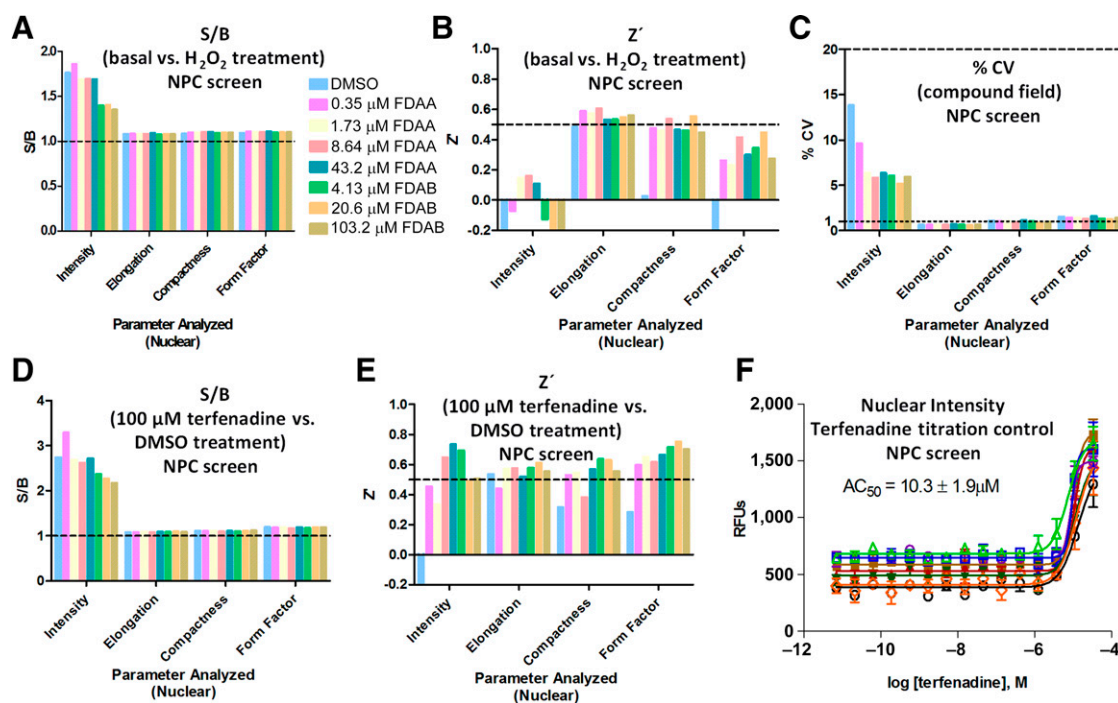


Figure 3. Performance of the high-content oxidative stress assay with human embryonic stem cell (hESC)-differentiated astrocytes in a screen against the NPC chemical library. A total of eight 1,536-well assay plates with hESC-differentiated astrocytes were screened against 2,816 compounds in titration in the NPC chemical library. Although the S/B was low for the screen (A, D), the variability was also very low for most parameters (C), which yields decent Z' values for many parameters (B, E). (F): The positive control, terfenadine, was tested in titration on each assay plate as a measure to assess the performance and reproducibility of the assay in the screen. Shown are nuclear intensity concentration response curves for the terfenadine titration on each of the eight assay plates screened. Abbreviations: %CV, coefficient of variation; AC₅₀, concentration of compound that gives half-maximal response; DMSO, dimethyl sulfoxide; FDA A and FDA B, subsets of the NPC compound library; NPC, NIH Chemical Genomics Center Pharmaceutical Collection; RFU, relative fluorescence unit; S/B, signal/basal.

Hit Identification From LOPAC1280 and NPC Chemical Library Screens

We identified 42 compounds in the NPC library and 24 compounds in the LOPAC1280 library that produced protective nuclear profiles and were considered hits in the primary screen (as discussed in Materials and Methods). Eliminating duplicated compounds found in both libraries resulted in a total of 66 hit compounds (supplemental online Tables 7, 8). An example of the nuclear profile of a hit compound is shown in Figure 4A (compare with the nuclear profile of an apoptotic compound in Fig. 4B) and supplemental online Figure 9. Generally, the concentrations at which we saw maximal changes in the nuclear profile were in the high nanomolar to midmicromolar range (approximately 100 nM to 50 μM).

Cytotoxic compounds were also identified in this screen by their nuclear profile. We found that cytotoxic compounds produced nuclear profiles similar to protective compounds but had a reduction in cell count (supplemental online Fig. 10A, 10B). These findings are presented in greater detail in the supplemental online data. We also found compounds that produced nuclear profiles similar to terfenadine, the control compound, and indicative of cells undergoing cell death processes (compare CRCs in Fig. 4B and supplemental online Fig. 1).

Secondary Assays to Prioritize Hit Compounds

Additional secondary assays were performed to prioritize hit compounds and to eliminate false positives due to assay artifacts (Fig. 5). We used the luminescence-based ATP content assay (CTG

assay) to eliminate autofluorescent [49], colored, or cytotoxic hit compounds in an assay involving astrocytes not treated with H₂O₂ (supplemental online data). Many of the 66 hits identified in the primary screen have been reported to cause apoptosis or cytotoxicity in various cell lines (e.g., melphalan and mycophenolic acid [50, 51]). We found that 33 compounds decreased cell viability or were autofluorescent or colored (supplemental online Table 9), leaving 34 hit compounds (including nitrovin) to evaluate in additional assays. An example of the activity of a colored compound that is considered a false positive in the assay is shown in supplemental online Fig. 11A. In addition, data from this CTG assay confirmed what we saw from the primary screening assay—that many compounds were cytotoxic at the highest concentration and were cytoprotective against oxidative stress at lower concentrations (supplemental online Fig. 11B), thus yielding biphasic CRCs for some parameters in the primary and secondary assays (e.g., nitrovin activity in the ARE-*bla* assay described below).

To determine if the compound activity in the primary screening assay was specific to oxidative stress induced by H₂O₂, we repeated the high-content assay in the absence of H₂O₂. Of the 34 compounds tested, 15 produced an altered nuclear profile in the absence of H₂O₂ and 15 did not (supplemental online Table 10). The nuclear phenotype generated by compounds active without H₂O₂ was different than that seen for terfenadine- or H₂O₂-treated astrocytes, with nuclei having an increase in size and change in shape beyond that of DMSO-treated astrocytes [i.e., nuclear compactness and 1/(nuclear form factor) were increased and nuclear elongation decreased relative to DMSO-treated astrocytes]

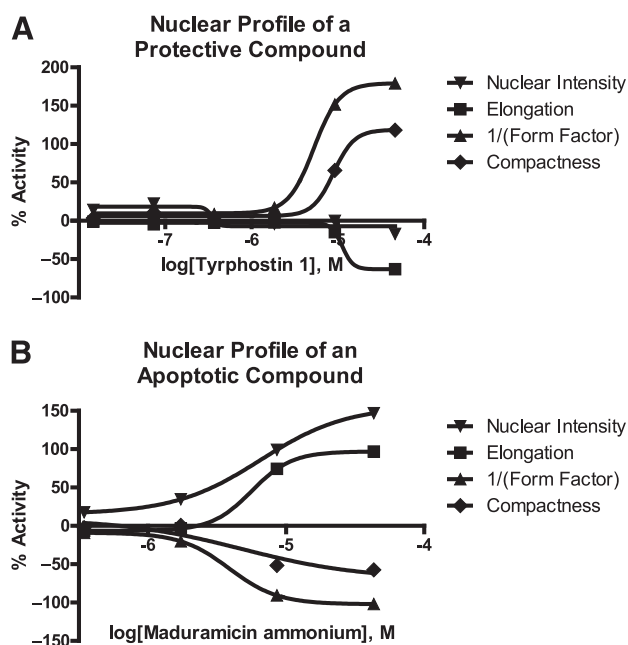


Figure 4. Concentration response curves depicting the distinctive nuclear profiles of a cytoprotective and an apoptotic compound. **(A):** A compound from the Library of Pharmacologically Active Compounds screen whose nuclear profile is characteristic of a protective compound, in which nuclear parameters trend toward that of the dimethyl sulfoxide (DMSO)/vehicle control, with concentration-dependent increases in nuclear compactness and 1/(nuclear form factor), and a decreased nuclear elongation. For this compound, nuclear intensity decreases only slightly. This is not unexpected, because the difference in nuclear intensity between DMSO/vehicle-treated astrocytes and H₂O₂-treated astrocytes was not as significant as was seen for terfenadine treatment (supplemental online Fig. 2A). **(B):** Nuclear characteristics of an apoptotic compound are similar to that seen for terfenadine (supplemental online Fig. 1), with a concentration-dependent increase in nuclear intensity and elongation, and decrease in compactness and 1/(nuclear form factor). Normalized data are shown with percent activity relative to controls (0% activity = basal condition, DMSO + 12 mM H₂O₂-treated astrocytes).

(supplemental online Fig. 12). The remaining four compounds were inconclusive. An example of a compound that demonstrated a protective nuclear profile in the presence of H₂O₂ and an altered nuclear profile in the absence of H₂O₂ is tyrphostin 1 (supplemental online Fig. 13), in which nuclear compactness and 1/(form factor) increased and nuclear elongation decreased in the concentration-dependent manner.

To begin to understand the mechanism of action behind the cytoprotection of the active compounds, we evaluated the activity of the compounds in an ARE- β -lactamase (ARE-*bla*) reporter gene cell line. Activation of the ARE/nuclear factor-E2-related factor 2 (ARE/Nrf2) pathway has been found to be cytoprotective in astrocytes under oxidative stress [52]. Activation of this Nrf2 pathway may also explain the protective nuclear phenotype induced by some compounds in the absence of H₂O₂, because xenobiotics induce Nrf2 [53]. Of the 34 compounds, we found 4 that directly activated ARE- β -lactamase expression in HepG2 cells: idazoxan hydrochloride, aurothioglucose, metrazoline oxalate, and nitrovin. Whereas idazoxan hydrochloride and metrazoline oxalate had a simple increase in ARE activation (BLA expression), the activity of both aurothioglucose and nitrovin in the ARE-*bla* assay was bell shaped, with cytotoxicity at higher concentrations of compounds (Fig. 6).

To determine if the activity in the ARE-*bla* assay was specific, we compared the reported activities of these compounds in other β -lactamase reporter assays previously performed. Nitrovin appeared to demonstrate similar bell-shaped activity in β -lactamase reporter gene assays for activator protein 1 (AP1), heat shock element (HSE) (PubChem AID 743228), and p53 (PubChem AID 720552). Idazoxan hydrochloride was not active in any of these assays and aurothioglucose had only been tested in the p53 assay, and was not active. In addition, previous screens confirmed that idazoxan hydrochloride, aurothioglucose, and nitrovin are not autofluorescent (PubChem AIDs 720684, 720685, 720686, 720687). This β -lactamase assay in HepG2 cells was multiplexed to an ATP content assay. Generally, we found that compounds that were cytotoxic to hESC-differentiated astrocytes were also cytotoxic to HepG2 cells (supplemental online Table 11), with the exceptions of thapsigargin, ebastine, PD 184352, 6-thioguanine, and nemorubicin, which were cytotoxic to HepG2 cells but not to astrocytes.

To generate a more complete picture of how the hit compounds themselves affected the cell health of the astrocytes (when not in the presence of H₂O₂), the microscopic images of astrocytes treated with each of the 34 compounds at the highest concentration were visually evaluated. Although there were some compounds that did not demonstrate any toxicity (supplemental online Fig. 14A; supplemental online Table 12), one trend that emerged was that many of the compounds that demonstrated a “protective” nuclear phenotype without H₂O₂ appeared to affect the cell health at the same compound concentrations. We noticed that these compounds reduced astrocytic processes/projections (supplemental online Fig. 14B; supplemental online Table 12). This effect on astrocytes was not detected by the ATP content assay for most of the compounds. Almost all of the compounds that did not generate a protective profile in the absence of H₂O₂ also did not appear to affect cell health as assessed by cellular morphology (supplemental online Table 12). Through this analysis, we also identified two compounds (D-delta-tocopherol and ebastine) that appeared to increase the number of astrocytes at compound concentrations that had a protective nuclear profile, both in the presence and absence of H₂O₂.

Based on the activity of a compound in the primary oxidative stress assay and secondary assays (i.e., ATP content assay, nuclear morphology assay without H₂O₂, ARE-*bla*, and ATP content assays in HepG2 cells), as well as the morphology of astrocytes in the presence of compound, we prioritized 22 compounds as cytoprotective against oxidative stress (Table 1; supplemental online data). Twelve of the 34 compounds were deprioritized because of lower relative efficacy or single-point activity (activity at only a single concentration of compound) in the primary screen and activity in subsequent secondary assays. The cytoprotective activity of the prioritized 22 compounds can be further evaluated in neurodegenerative disease models.

To determine if cytoprotection of these 22 compounds could be confirmed in a different cell line, we tested these compounds in a high-content oxidative stress assay with iPSC-differentiated astrocytes. Because H₂O₂ sensitivity and toxicity is cell-line dependent, we performed an H₂O₂ titration experiment with these iPSC-differentiated astrocytes and found that these cells were more sensitive to H₂O₂ treatment. Thus, we used 6 mM H₂O₂ in the assay with these astrocytes instead of 12 mM H₂O₂. We confirmed the protective activity of nine of the compounds found protective of hESC-differentiated astrocytes challenged with H₂O₂. These results are also shown in Table 1.

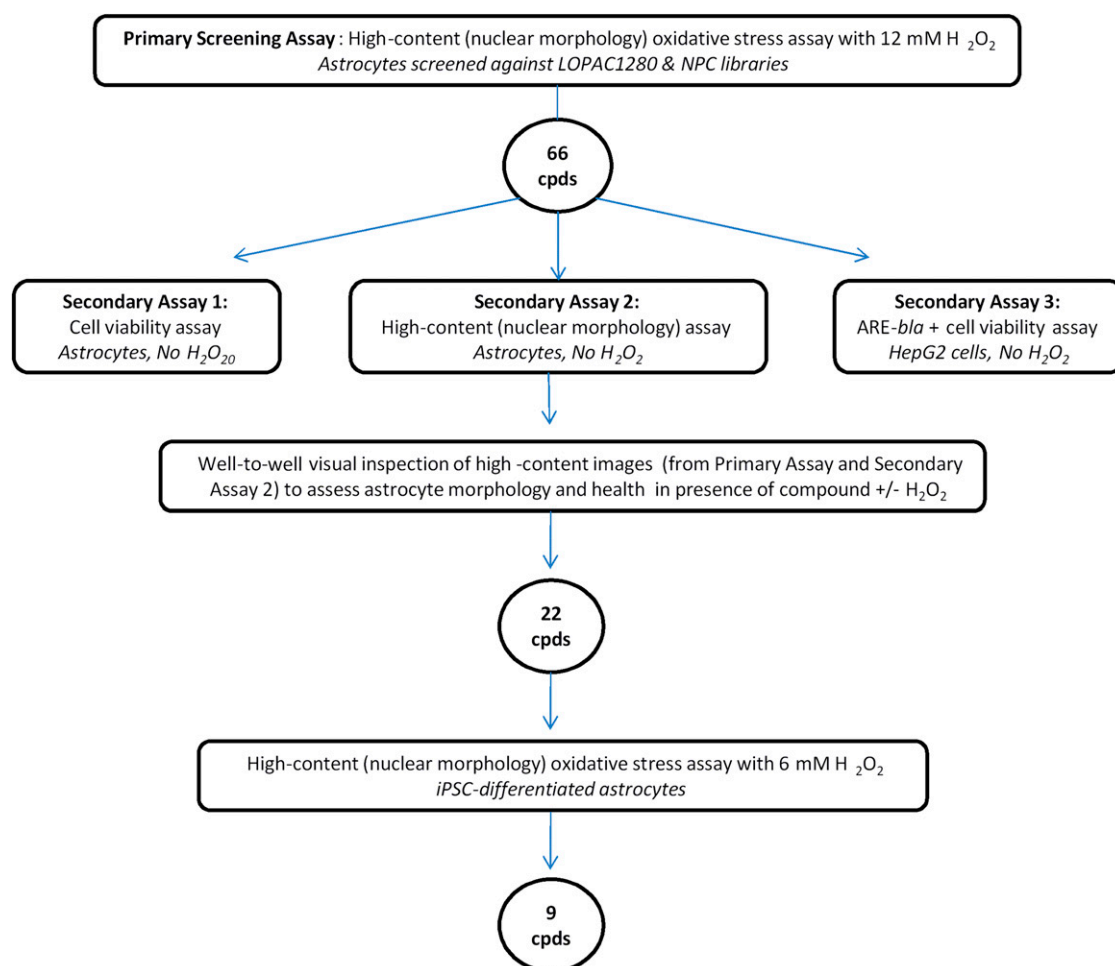


Figure 5. Flowchart of assays to assess the activity of 66 follow-up compounds found active in the primary high-content oxidative stress screening assay. Abbreviations: ARE-*bla*, antioxidant response element β -lactamase assay; cpds, compounds; iPSC, induced pluripotent stem cells; LOPAC1280, Library of Pharmacologically Active Compounds; NPC, NIH Chemical Genomics Center Pharmaceutical Collection.

DISCUSSION

Recent advances in stem cell technologies have enabled the use of hESC- and iPSC-derived cells as disease model systems for drug discovery. However, it is still a challenge to produce the large number of differentiated cells needed for high-throughput screening, and the process can be time consuming and costly. Although stem cells have been successfully differentiated into cell types such as cardiomyocytes, endothelial cells, neurons, and hepatocytes, they are not readily used in compound screens because of issues associated with the generation of large numbers of cells, including potentially high reagent cost and variable reproducibility in the purity, maturity, and function of the desired cell type [8]. These challenges may explain why current compound screening efforts using PSC-differentiated cells have been limited to relatively small libraries of up to 7,000 compounds [1, 2, 7, 9–14].

To overcome the challenges of generating a large population of mature astrocytes, and to reduce the batch-to-batch variability in the state of differentiation of cells used for screening, we optimized a protocol for human astrocyte differentiation such that it is reproducible, scalable, and generates differentiated astrocytes with high purity [22, 23]. We were able to do this by cryopreserving a large, single batch of proliferating astrocyte progenitor cells

(Fig. 1A). This allowed us to use cells from the same originating batch for all the screens and assays performed in this study, thus minimizing batch-to-batch variability. To further reduce variability in the state of differentiation, all cells used for experiments in this study were within the same age range (i.e., days 35–40). By cryopreserving a large quantity of cells at a proliferating stage, we were able to ensure an adequate supply of cells for screening. Although for this study, only relatively small compound libraries were screened, we effectively collected 17,536 data points in the primary screen, because each of the 4,096 compounds was screened in titration. Astrocytes were seeded into 15 1,536-well assay plates ($n = 23,040$ wells), and their response to compound or DMSO was measured. For this study, a total of approximately 1.4×10^8 hESC-differentiated astrocytes were required for the primary screen of 4,096 compounds in titration and for the secondary assays of 66 compounds in titration. Cryopreserved astrocyte progenitor cells were further differentiated and expanded for 2 weeks in flasks before seeding in assay plates for compound screening (at approximately day 35). By harvesting approximately 8×10^6 astrocytes per T175 flask, 17 T175 flasks of hESC-differentiated astrocytes were needed to produce 1.4×10^8 cells. Although this optimized protocol for astrocyte differentiation can accommodate the generation of greater numbers of astrocytes

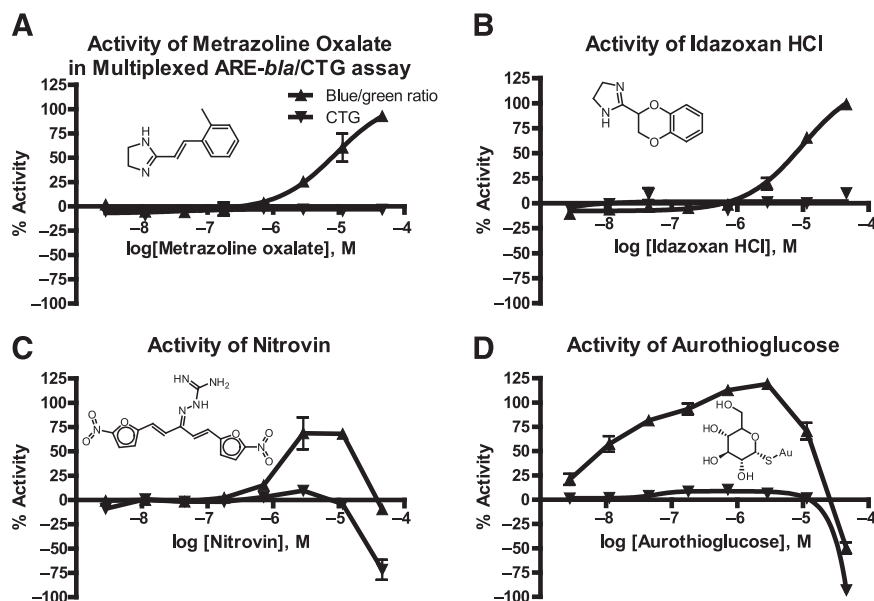


Figure 6. Concentration response curves (CRCs) of compounds active in the multiplexed ARE-*bla*/CTG assay in HepG2 cells. Metrazoline oxalate, idazoxan hydrochloride, nitrovin, and aurothioglucose activate the ARE/NF-E2-related factor 2 (Nrf2) pathway in HepG2 cells, as visualized by the blue/green ratio CRC (supplemental online data). **(A, B):** Little or no activity is seen in the CTG cell viability assay for metrazoline oxalate and idazoxan hydrochloride, indicating that these compounds are not toxic to HepG2 cells at the concentrations tested. Nonlinear regression analysis, with three-parameters least-squares fit. **(C, D):** Nitrovin and aurothioglucose are toxic at the highest concentrations, but activate the ARE/Nrf2 pathway at lower concentrations. Abbreviations: ARE-*bla*, antioxidant response element β -lactamase; CTG, CellTiter-Glo assay.

for significantly larger screens; in our case, to reduce the number of astrocytes used for screening as much as possible, we miniaturized the screening assay to a 1,536-well format. If the primary screen and secondary screens were performed in a 96- rather than 1,536-well format, instead of requiring approximately 1.4×10^8 hESC-differentiated astrocytes, we estimate that 10-fold more astrocytes (approximately 1.4×10^9), or approximately 175 T175 flasks of astrocytes, would have been required.

The purity of differentiated cells can be a significant issue in screening, where a robust, invariable cellular response to compound treatment is needed to identify a compound as active in an assay. We found that at day 35 and later, the majority of cells in culture were GFAP positive and all exhibited morphological characteristics of astrocytes. To confirm the astrocytic identity of cells used for experiments, RT-PCR analysis of hESC-differentiated astrocytes was performed periodically on cells before their use. In addition, all cells appeared to stain positive for GFAP when maintained in the assay plates used for screening assays. In our screen, we saw very low variability in response of cells to control compound treatment (low %CV) (Fig. 3; supplemental online Fig. 7), indicating that if there was a low level of contaminating cell types, it did not influence assay performance. In cases where heterogeneity of a differentiated cell population is an issue, cells may still successfully be used for screening by using a high-content screening assay that includes immunostaining for a cell-type specific marker. In this way, the response of the cell type of interest can be distinguished from that of contaminating cell types.

Another consideration when using cells differentiated from hESCs for experiments is that these cells may be developmentally less mature than most cells found in adult tissues [6], and thus the gene and protein expression profiles of these cells compared with adult primary cells of the same tissue may be different. Previous whole-genome microarray and gene clustering analyses of hESC-differentiated astrocytes generated by the method used here

demonstrated that these differentiated astrocytes are distinct from the originating neural stem cells [22]. Microarray analysis indicates that the hESC-differentiated astrocytes used in our studies have similar expression profiles to that of commercially available fetal astrocytes, which are considered more developmentally mature (supplemental online Table 5). In addition, our astrocytes expressed numerous genes characteristic of functionally mature astrocytes (supplemental online Table 6). Thus, based on our analysis, the differentiated astrocytes we used for screening are mature astrocytes.

It is estimated that astrocytes make up the majority of cells in the nervous system [15], and that a single astrocyte of the human cortex is associated with more than 1 million synapses [54]. Astrocytes are actually a heterogeneous cell population, with specific subpopulations defined by their developmental lineage and characterized by their morphology, gene expression and niche functional properties [55, 56]. Despite this heterogeneity, astrocytes are generally considered to play key roles during neurodevelopment, including aiding in neuronal migration, influencing dendritic and axonal growth, modulating synapse formation and elimination, and assisting in myelination [15]. In addition, mature astrocytes support neural homeostasis and function by their involvement in extracellular ion buffering and control of pH, clearance of extracellular neurotransmitters, modulation of synaptic strength, control of blood flow through vasculature of the CNS and maintenance of the blood-brain barrier, neutralization of ROS, and response to and repair of CNS injury (reactive astrogliosis) [16, 17, 57]. Because human astrocytes are unique in their complexity, gene expression profiles, and function compared with astrocytes of other species [58–60], studies such as ours, which use human PSC-derived astrocytes, may help identify more clinically relevant therapeutics for neurodegenerative diseases than studies performed with rodent astrocytes.

With astrocytes playing such key roles in maintaining a suitable environment for neuronal function, it is no surprise that

Table 1. Activity of 22 prioritized compounds in assays

Compound Name	“Protective” nuclear phenotype with H ₂ O ₂	Altered nuclear phenotype without H ₂ O ₂	Significant cell loss at top concentration without H ₂ O ₂	Morphological change at active concentrations without H ₂ O ₂ ^a	Increased cell number	Activates ARE	Active in CTG assay	“Protective” nuclear phenotype with iPSC-derived astrocytes (with H ₂ O ₂) ^b
Aurothioglucose	X	X	X	XX		X		X _B
Nitrovin	X	X	X	XX		X	X	X _B
4-Chloro-mercuribenzoic acid	X	X		XX				X _B
Norcantharidin	X	X		XX				X
Thapsigargin	X	X		XX				X _B
Oxyphenbutazone	X	X		XX				X
Tyrphostin A1	X	X		X				X
Melphalan	X	X		X				
CCG-2046	X	X		X				
Mycophenolic acid	X	X						
Thiophenamil	X	X						
JFD00244	X	X						
D-Delta-Tocopherol	X	X			X			
Ebastine	X	X			X			X _B
6-Thioguanine	X			X				
Vincristine	X			X				
Idazoxan hydrochloride	X					X		
Metrazoline oxalate	X					X		
Ammonium pyrrolidinedithiocarbamate	X							
SB 216763	X							
PD 184352/CI-1040	X							
Enzastaurin	X							X

^aXX indicates that the compound reduced processes/projections; X indicates that although the astrocytes do not morphologically look as healthy as dimethyl sulfoxide/vehicle control-treated cells, the astrocytes do have projections.

^bX_B indicates the compound has a protective profile at midrange concentrations and may be cytotoxic at higher concentrations (B, biphasic). Abbreviations: ARE, antioxidant response element; CTG, CellTiter Glo assay; iPSC, induced pluripotent stem cell.

astrocyte dysfunction is associated with neurodegenerative disease [15, 61]. Maintaining beneficial astrocytic function in the presence of toxic insults associated with neuropathology, such as ROS, is a desirable outcome and new therapeutic target. We developed a high-content phenotypic assay that used H₂O₂ to induce oxidative stress in astrocytes and screened for compounds that prevented induction of cell death processes by this oxidative stress. For screening purposes, it was necessary to treat astrocytes with relatively high concentrations of H₂O₂ for short periods of time to keep the %CV low. To produce the S/B values required of a successful screen, the majority of astrocytes had to be actively undergoing cell death, and this was only achieved with a high concentration of H₂O₂. It is known that astrocytes generate very high levels of glutathione—approximately 3.8 mM—which is released into the extracellular environment [57], perhaps explaining why astrocytes can accommodate high levels of exogenous H₂O₂. In addition, it is worth mentioning that cell culture medium itself contains components that can act as antioxidants (like phenol red) [62]. It is thus likely that components of the cell medium neutralized the H₂O₂ to some extent, so that although 12 mM is the estimated delivered concentration of H₂O₂, it is not the concentration that the majority of astrocytes experienced during the experiment.

High-content screening (HCS), which was used in this screen to identify compounds that prevented apoptosis, is an extremely powerful method that uses high-throughput technologies with quantification of phenotypic cellular phenomena [46]. Using immunocytochemistry techniques to look at cellular and molecular changes upon perturbation, such as after H₂O₂ or compound treatments, one has almost limitless phenotypic parameters that can be analyzed. HCS is becoming more popular as interest in phenotypic screening has increased. Phenotypic screening is thought to be more physiologically relevant and less artificial than screening that focuses on a specific molecular target, because it involves intact cells in a native cellular environment [1]. In the case of high-content, phenotypic screening, the target and mechanism of action of the active compound may not be known and additional experiments will be needed to determine this. This is true of the compounds we identified as active in our screen. In addition, a high-content, phenotypic screen is less hypothesis restricted. For example, in this screen we identified compounds that prevented apoptosis based on a nuclear phenotype. This allowed us to identify compounds that actually seemed to have a toxic effect on the cells, even though they prevented acute apoptosis by H₂O₂ challenge, presumably through preconditioning.

In our screen, active, “protective” compounds were defined as those that generated a nuclear profile that was more similar to vehicle/DMSO-treated astrocytes (Fig. 4A; supplemental online Fig. 9) and the opposite of a nuclear profile generated by 100 μ M terfenadine, which clearly caused apoptosis and necrosis (Fig. 2A; supplemental online Figs. 1, 2). The exact mechanism by which protective compounds alter the nuclear phenotype is not clear. However, we believe that many of the compounds activate stress response signaling pathways that are protective to the astrocytes upon subsequent stress by H_2O_2 challenge. Many of the compounds generated a nuclear phenotype without the presence of H_2O_2 , and many caused morphological changes to the astrocytes (such as reduced processes, indicative of cell stress) at the same concentrations at which the protective nuclear phenotype were seen (Table 1). In addition, many of the compounds identified as active and protective in this assay are known to induce oxidative stress, are characterized as redox-active compounds (RACs), or induce other forms of cellular stress (supplemental online Table 13). Many of these compounds are anticancer therapies and cause apoptosis in cancer cell lines [63–65]. It is possible that these compounds are largely toxic in many cell lines but can instigate protective pathways in astrocytes at low concentrations because astrocytes function to protect and promote neuronal health.

The phenomenon of sublethal concentrations of a compound inducing a protective effect upon subsequent challenge by a stressor (in this case, H_2O_2 and oxidative stress), is called hormesis and is well documented [66, 67]. At sublethal concentrations of a stressor (in this case, the compounds), protective pathways are induced so that cells are “preconditioned” to be able to handle a subsequent stressor event or agent. The potential antioxidant effect of redox-active compounds has been called the “the antioxidant paradox” [68–70]. As an example, pyrrolidine dithiocarbamate (PDTC; a hit compound in our study) is a member of a class of compounds, dithiocarbamates, that are metal chelators thought to influence the redox state of the cell [71]. Although found to inhibit apoptosis after acute treatment of cell lines in vitro [71–73], PDTC has also been found to induce necrosis after longer incubation times [71]. Protective pathways that are activated could include, but are not limited to, pathways that induce APE1 (by melphalan [74, 75]), increase intracellular glutathione (by tyrphostin A1 [76]; supplemental online Table 13), or activate the ARE/Nrf2 pathway (aurothioglucose, nitrovin, idazoxan, metrazoline oxalate; Table 1).

The identification of compounds that are themselves stressors and active without H_2O_2 challenge is not surprising if you consider how the screening assay protocol was executed: Astrocytes were incubated with compounds for 24 hours before H_2O_2 challenge (Fig. 2). This would select for compounds that induce protective activity before this oxidative challenge. If cells are not stressed and unchallenged, there would be little reason for the induction of cell survival pathways. The induction of cell survival pathways is initiated when cells are challenged with stressors like the compounds found to be active in this assay. With this assay, it seems we are capturing the activation of stress response pathways by changes in nuclear morphology.

To begin to explore the mechanisms by which these compounds exert their protective influence, we tested the activity of the compounds in an ARE/Nrf2 assay. The transcription factor Nrf2 plays an important role in activating the transcription of cytoprotective genes through the ARE in response to oxidative stress and other cellular stressors [52, 77]. Nrf2 has been found to be cytoprotective of astrocytes treated with H_2O_2 , with

activation of Nrf2 and the ARE pathway in astrocytes resulting in the expression of enzymes with key roles in detoxification, such as catalase [52, 78]. Interestingly, these studies have found that Nrf2 induction by *tert*-butyl hydroperoxide (tBHQ) increases the viability of astrocytes subsequently exposed to H_2O_2 . Additionally, Dowell and Johnson [52] found that astrocytes were able to tolerate significantly higher concentrations of H_2O_2 after treatment with tBHQ, which was achieved by astrocytes neutralizing extracellular H_2O_2 , thus lowering ROS levels. In addition, other studies have found that Nrf2 activation in astrocytes has non-cell autonomous effects, protecting cocultured neurons from H_2O_2 [48, 79]. We found that at least four of the active compounds identified in the primary screen activate the ARE/Nrf2 pathway in HepG2 cells (Fig. 6). Aurothioglucose, nitrovin, metrazoline oxalate, and idazoxan hydrochloride likely activate this pathway before H_2O_2 treatment (astrocytes were treated with these compounds for 24 hours before H_2O_2 treatment), offering protection against H_2O_2 -induced oxidative stress. Aurothioglucose, a gold-containing derivative of glucose, has previously been shown to activate transcription from the Maf-recognition element, which is related to the ARE and is activated by Nrf2 [80]. Idazoxan is an α 2-adrenergic agonist and imidazoline 2 receptor (I_2R) ligand that has been found to be neuroprotective in an animal model of multiple sclerosis, reducing demyelination of the spinal cord [81]. Idazoxan has also been found to reduce neuronal loss after ischemic events [82–84]. Interestingly, metrazoline, another I_2R ligand [85], was also found to activate Nrf2 in our assay. I_2R expression is enriched in astrocytes, with activation of the receptor associated with increases in GFAP expression [86].

It is possible that there are additional active compounds in the primary screen that offer cytoprotection via activation of the Nrf2/ARE pathway in astrocytes, and that these compounds do not robustly activate the ARE pathway in HepG2 cells. Additionally, we identified some compounds that were active in only one repetition of the ARE-*bla* assay in HepG2 cells (norcantharidin, mycophenolic acid, JFD00244) that could potentially be more efficacious as activators of Nrf2 in astrocytes.

In addition to these compounds, our screen identified other small molecules that have previously been found to be cytoprotective. SB 216763, a glycogen synthase kinase-3 β (GSK-3 β) inhibitor, has been found to decrease cell death caused by apoptosis and autophagy in rat astrocytes [87], and prevent death of cerebellar granule neurons [88]. GSK-3 β has been shown to be activated by H_2O_2 [89] and is implicated in various neuropathies [90]. Also of interest, the related analog SB 415286 was found to be cytoprotective for a neuroblastoma cell line treated with H_2O_2 , reducing ROS levels [91]. D-delta tocopherol, a member of the vitamin E family, is well known as an antioxidant and for its anti-inflammatory properties [92]. Studies have found that pyrrolidine dithiocarbamate (an NF- κ B inhibitor and also known as PDTC, here the ammonium form) is an antioxidant (reviewed in [93]) and neuroprotective during brain ischemia [94]. The identification of these known cytoprotective compounds in our screening assay validates our assay as one that is able to identify cytoprotective and antioxidant compounds.

Additional mechanisms by which the active compounds exert protection will need to be further explored. Although we believe that many of these compounds are protective due to activation of stress response pathways, some of these compounds are known to influence multiple signaling pathways (e.g., enzastaurin [63] and norcantharidin [95]) and, as such, their exact mechanism of cytoprotection may be not be straightforward. The ultimate

cellular consequences of oxidative stress by cytoprotective compounds—induction or inhibition of apoptosis/necrosis and potential apoptotic recovery—is complicated, as is the pathological consequences of oxidative stress [70]. In addition, hormetic responses, which occur at low to mid doses of a chemical stressor, are characteristically less reproducible, less predictable, and more variable than pharmacological activity (e.g., cell death) associated with high doses [96]. Future studies using any of the compounds identified in this study will need to carefully assess the specific antioxidant or cytoprotective activity of the compound for that given experimental condition or model.

We found 9 of the 22 prioritized compounds that were active in the hESC-differentiated astrocyte assay were active in the iPSC-differentiated astrocyte assay. It is important to note that the assay with the iPSC-differentiated astrocytes was not completely identical to that with hESC-differentiated astrocytes (a lower concentration of H₂O₂ was used and astrocytes were maintained in different medium). It is not entirely clear why not all compound activity confirmed, but there could be a number of explanations. First is that the compounds not active in the assay with iPSC-differentiated astrocytes are not protective, and that these compounds are false positives. While this explanation is possible, it is not likely that this is the case for all these compounds, particularly because of the reported association of these compounds with ROS and/or their potential roles in cytoprotection. In addition, a couple of the compounds are active in the ARE/Nrf2 assay. This type of compound activity seems similar to compounds that confirmed in the iPSC-based assay (Table 1; supplemental online Table 13).

A second explanation is that the iPSC-differentiated astrocytes were more sensitive to cellular stressors than the hESC-differentiated cell line. We found that the iPSC-differentiated astrocytes were more sensitive to H₂O₂, a characteristic intrinsic to the cell line, or perhaps due to different components in the assay medium (which can contain antioxidants and other components that can neutralize H₂O₂) compared with medium used for hESC-differentiated astrocytes. It would make sense, then, that these astrocytes would also be more sensitive to the toxic effects of some compounds when combined with H₂O₂ treatment. Thus the protective effects of these compounds could have been lost on this assay.

A third alternative is that some of these compounds are selectively active in hESC-differentiated astrocytes for unknown reasons. While we are confident in the protective effect of the 9 compounds that confirmed in the iPSC-based assay, we do want to make the identities of the other 13 compounds known, as it is likely that these compounds confer protection via similar mechanisms. Identifying the protective effect of compounds is challenging: We found that the high-content oxidative stress assay was very sensitive to astrocyte health. If astrocytes were stressed in any way during, or even before, the assay, the protective effect of some of the active compounds was not seen. This is likely because any additional stress on cell health, on top of the stress from the compound itself and treatment with H₂O₂, proved too toxic for the cells.

It is important that future studies that examine the protective potential of these compounds take into consideration that many or most of these compounds are cytotoxic at high concentrations or with long treatment times, and that cell health can significantly affect whether a compound is seen as protective in a given model. Inhibition or activation of apoptosis by RACs, in particular, is known to be dependent on assay conditions and the point in time at which data are captured [71]. As previously mentioned, hormetic responses can be quite variable [96] and thus

cytoprotection by these compounds may not be reproducible in all models tested. It will be interesting to see whether many of these compounds are able to serve a protective function in other cell lines (or in astrocytes differentiated from patient iPSCs), or whether these compounds are protective of neurons co-cultured with astrocytes. It will also be interesting to see if the protective capacity of some of these compounds is related to the fact that they are stem cell derived [97].

With this proof-of-concept study, our focus was to demonstrate that human stem cell-derived cells, specifically astrocytes, can successfully be used for compound screening and in drug discovery. With the identification of active compounds with potentially interesting pharmacological activity, we want to make the identity of these compounds available to the scientific community to serve as a basis for potential future studies.

CONCLUSION

Using an optimized protocol for differentiating human astrocytes from PSCs, we were able to generate a large quantity of sufficiently pure astrocytes for use in compound library screening. We then developed and validated a high-throughput screening assay using these differentiated human astrocytes, which identified 22 compounds that prevented induction of cell death processes induced by oxidative stress. Our approach, which can be directly applied to patient-derived astrocytes, thus provides a novel platform for neurodegenerative drug discovery.

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AUTHOR CONTRIBUTIONS

N.T.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; N.M.: conception and design, provision of study material or patients, collection and/or assembly of data, data analysis and interpretation, manuscript writing; S.S.: provision of study material or patients, collection and/or assembly of data, data analysis and interpretation; J.Z. and B.C.: collection and/or assembly of data; F.A. and N.S.: data analysis and interpretation; M.X.: conception and design, data analysis and interpretation; J.C.M.: conception and design, administrative support, final approval of manuscript; M.R.: conception and design, data

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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