Integrating Image-Based High-Content Screening with Mouse Models Identifies 5-Hydroxydecanoate as a Neuroprotective Drug for Paclitaxel-Induced Neuropathy

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Abstract

Chemotherapy-induced neurotoxicity is a common adverse effect of cancer treatment. No medication has been shown to be effective in the prevention or treatment of chemotherapy-induced neurotoxicity. This study aimed to discover potential neuroprotective drugs for paclitaxel-induced neurotoxicity. An image-based high-content platform was first developed to screen for potential neuroprotective drugs. The screening system comprised of automated image acquisition and multiparameter analysis, including neuronal viability, neurite outgrowth, and synaptic genesis. By this platform, we obtained a candidate list from compound libraries. In the drug screening from compound libraries of ion channel ligands, REDOX and GABAergic ligands, 5-hydroxydecanoate (5-HD) exhibited the most significant neuroprotective effects against paclitaxel-induced neurotoxicity in both cortical and dorsal root ganglion (DRG) neurons. In mouse behavioral tests, 5-HD restored the thermal sensitivity and alleviated mechanical allodynia induced by paclitaxel. Electron micrographs of sciatic nerve revealed that 5-HD reduced the damages caused by paclitaxel in the nonmyelinated and smaller myelinated fibers. The mechanistic study on DRG neurons suggested that 5-HD rescued the dysregulation of intracellular calcium homeostasis provoked by paclitaxel. Importantly, 5-HD did not jeopardize the antitumor effect of paclitaxel in tumor xenograft models. In conclusion, we established an image-based high-content screening platform and a protocol for verifying the neuroprotective effect in vivo, by which 5-HD was identified and validated as a potential neuroprotective drug for paclitaxel-induced neuropathy.

Introduction

Advances in cancer therapy have increased the number of cancer patients who live disease free for long periods. However, a considerable proportion of cancer survivors experience the side effects of chemotherapy. Neurotoxicity is a common complication in cancer patients receiving chemotherapy. The clinical symptoms are in a wide range, including numbness, unsteadiness, and chronic pain. These symptoms not only jeopardize the quality of life but also lead to early discontinuation of anticancer treatment (1–3). Effective neuroprotective drugs against chemotherapy are not available so far. The development of appropriate preclinical assay models and objective assessments of chemotherapy-associated neurotoxicity are thus critical steps to test potential therapies for toxic reduction or prevention.

Current strategies for evaluating chemical-induced neurotoxicity include in vitro examination of morphologic and functional changes in neurons, and in vivo assessment of neuropathologic and behavioral endpoints in rodent species (4). As animal behavioral studies are high-priced, time-consuming, labor-intensive, and low-throughput processes, the cell-based in vitro neurotoxicity assays are much easier to screen out toxic compounds in a high-throughput manner at the early stage of drug development. However, several shortages are noted in most in vitro studies, including irrelevant cell models, insufficient analytic parameters, and subjective image selections. For example, the easily handled and rapidly growing cancerous neuroblastoma or nonneuronal pheochromocytoma cells are usually used to study neuron survival and neurite outgrowth (1). These cancer cell lines cannot truly represent normal neurons due to their distinct signal pathways, gene expressions, and cellular responses to stimuli (5, 6). Although human stem cell–derived neural hN2™ cells have been reported to be more sensitive to neurite outgrowth inhibitors, they showed a wide range of variability, compared with primary neuron cultures (7, 8).
Regarding the analytic parameters, previous studies mostly applied neuron viability and neurite outgrowth for neurotoxicity and paid less attention to synapses (8, 9). However, the synapse is the basic element for neural network formation and is worth being included in a complete assessment of neural functions because it represents a specialized axon–dendrite contact site mediating neurotransmitter-based communication (10). Synaptic plasticity and integrity in the central nervous system (CNS) are important in pain perception and cognitive function (11, 12). The loss of synaptic function and the neural network have been reported in the presence of chemotherapy agents. For example, paclitaxel-induced hyperalgesia negatively modulates affective pain and synaptic function in the rat frontal cortex (13). Moreover, the throughput of image acquisition and analysis is the key to study cellular or subcellular changes in neurons, but to date, most studies have depended on the manual selection of representative images and lack standardized quantitative analyses, which leads to selection bias. Therefore, the high-content screening strategies combined with automated image acquisition and analyses are essential for the establishment of efficient in vitro neurotoxic assays.

This study aims to develop an image-based high-content platform and a mouse model for the discovery of potential neuroprotective drugs against chemotherapy. Using this high-content platform to screen compounds from drug libraries of ion channel ligands, REDOX (portmaneau of reduction and oxidation), and GABAergic ligands, we identified 5-hydroxydecanoate (5-HD) as a novel neuroprotective drug against paclitaxel-induced neurotoxicity. More importantly, the mouse behavioral tests validated the effectiveness of 5-HD in preventing and treating paclitaxel-induced neuropathy.

Materials and Methods

Compounds

Compounds listed in Supplementary Table S1 were from ENZO Life Sciences. Paclitaxel and liposomal doxorubicin were gifts from Bristol-Myers Squibb and TTY Biopharm, respectively. Cisplatin and vincristine were purchased from Sigma-Aldrich.

Primary cortical neuron and dorsal root ganglion cultures

Brain cortices were microdissected from postnatal day 0 C57BL/6J mouse pups, incubated with trypsin (Caisson) for 5 minutes. Minced cortical neuron mixtures were filtered, centrifuged, and resuspended in neurobasal medium designed for long-term viability of neurons, supplemented with B-27 supplement (Gibco), 0.5 mmol/L L-glutamine, and 0.5% penicillin–streptomycin (Caisson). Dorsal root ganglion (DRG) was removed from adult female C57BL/6J mice at 7 weeks old and the tissues digested with 0.1% collagenase (Sigma) for 1 hour, followed by 0.25% trypsin for 25 minutes. The dissociated mixed neuron cultures were plated at a density of 2.8 × 10⁴ cells per well in a 96-well thin-bottom optical microplate (Costar) precoated with 1 mg/mL poly-d-lysine (Sigma–Aldrich) for the indicated time period.

Immunofluorescence and automated image acquisition

Cells were fixed with 4% paraformaldehyde for 15 minutes, washed with 0.05% Triton X-100 in PBS for 30 minutes, and blocked with 3% bovine serum albumin (Sigma–Aldrich) at room temperature for 1 hour. Then, cells were stained with primary antibody at 4°C overnight, washed by PBS and stained with secondary antibodies at room temperature for 1 hour. Information on antibodies is presented in Supplementary Table S2. Cell images were automatically acquired using a 20 × objective with 0.75 numerical aperture and Hi-resolution mode (1 × 1 pixel binning) by an ImageXpress Micro automated wide-field fluorescent microscope (Molecular Devices).

Animal behavioral tests and drug administration

Female C57BL/6J mice, ages 7 weeks at the beginning of the experiment, were used for studying paclitaxel-induced neuropathy. Paclitaxel (4.5 mg/kg), vehicle (saline), or 5-HD (5 or 10 mg/kg) was injected intraperitoneally on four alternative days (days 0, 2, 4, and 6), as previously described (14). The baseline measurement of each test was taken prior to paclitaxel or vehicle administration, and five additional sessions of tests were recorded weekly for 5 weeks. Paw withdrawal by the von Frey filament test was assessed by electronic von Frey hairs (Part #2390; IITC Instruments). Heat hypersensitivity of tail withdrawal was measured by the tail immersion assay. Motor function impairment was measured by the grip strength test. The detailed information on animal tests is described in Supplementary Materials and Methods.

Ultrastructure of the sciatic nerve

Following mouse behavioral tests at the last time point, sciatic nerve samples were collected, fixed in 4% glutaraldehyde and post-fixed in 1% osmium tetroxide. The samples were dehydrated through a graded ethanol series, embedded in EMbed 812 (EMS; #14120), and sliced into 90 nm thick sections. After ultrathin sectioning, the sciatic nerve samples were observed and imaged with a transmission electron microscope (H7650, Hitachi).

Intracellular [Ca²⁺], measurement

DRGs were isolated at the sixth week after behavioral tests and cultured for 4 days for the measurement of intracellular Ca²⁺ concentration ([Ca²⁺]i) at 37°C with the Fura-2 fluorescence ratio method on a single-cell fluorescence method on a single-cell fluorescence fluorimeter as previously described (15). The Fura-2 was excited alternatively between 340 and 380 nm using the Polychrome IV monochromator (Till Photonics). The fluorescence intensity at 510 nm was analyzed, and [Ca²⁺]i was calculated using TILLvisION 4.0 (Till Photonics; ref. 15). Detailed information on the [Ca²⁺]i measurement is described in Supplementary Materials and Methods.

Tumor xenograft model

The cervical cancer SiHa cell line was prepared as previously described (15). The cell line was authenticated by the short-tandem repeats analysis using the Promega StemElite ID System (GeneLabs Life Science Corp). In the animal model, 2 × 10⁴ cervical cancer SiHa cells were inoculated subcutaneously into the basolateral flank of 10-week-old female NOD/SCID mice. Two weeks after implantation, mice were randomized to receive the different therapies. Body weight and tumor size were measured every other day. Tumor volume (mm³) was calculated by using the formula: 1/2 × (the shortest tumor diameter)² × (the longest tumor diameter) (15). Two weeks later, mice were sacrificed and tumors were isolated for weighing.

Supplementary material

Published OnlineFirst August 20, 2015; DOI: 10.1158/1535-7163.MCT-15-0268

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Statistical analysis

Data are expressed as mean ± SEM. Differences between groups were compared using the Student t test or one-way ANOVA followed by the Bonferroni post-test. The criterion for statistical significance was P < 0.05.

Results

The strategies of drug discovery

We first designed the protocol for drug development to include 5 steps (Supplementary Fig. S1). Step 1, establish a high-content image-based screening platform to study the central nervous system (CNS) and peripheral nervous system (PNS), respectively; step 2, screen lead compounds for neuroprotection; step 3, validate the neuroprotective effects in a mouse model of chemotherapy-induced neurotoxicity; step 4, study the effect of lead compounds on anticancer therapy; step 5, determine the mechanism of the protective effect.

For the cell model, cortical neurons from postnatal day 0 pups or DRG neurons from adult female mice at 7 weeks old were cultured in 96-well plates, fixed and stained for the automated image acquisition and analyses (Supplementary Fig. S2). About 200 micrographs in each well were taken to create a montage image showing the entire neural network. The neuron-specific microtubule-associated protein 2 (MAP2) antibody, a somatodendritic marker, was applied to visualize the cell body and extending processes of a cortical neuron, enabling the generation of a binary mask of neuronal morphology for the automated image analyses. To pick out a cortical neuron accurately from the mixed culture, the expected nuclear size of MAP2-positive cells was entered into the computer program. The specificity of MAP2 antibody for neurons was validated in the primary mixed cultures (Supplementary Fig. S3). Morphologic parameters, such as cell body, processes, branches, and synaptic puncta in cortical neuronal culture were defined (Supplementary Fig. S2B). These measurements represent the vitality, growth, and formation of functional units of cortical neurons. To optimize the in vitro culture period, the postnatal mouse cortical neurons were cultured for 7, 10, 14, and 21 days in vitro (DIV) and a binary mask of neuronal cell body and processes was obtained (Fig. 1A, a2–d2).

Synaptic puncta were obtained through the detection of synaptophysin staining (Fig. 1A, a3–d3). Quantified data shown in Fig. 1B display the control cell neural viability (neuron numbers) and synaptogenesis (synaptic puncta number per neuron) at 10 DIV. Morphologic parameters associated with neurite outgrowth, such as process number, process length, neurite length, and branch number also reached a plateau at 10 DIV (Fig. 1C). Therefore, 10 DIV is the optimal time point to assess CNS neuronal function in this image-based system. In the cell model of PNS, anti-NeuN and anti-beta III tubulin antibodies were applied to visualize the cell body and extending processes of DRG neurons, enabling the generation of a binary mask of neuronal morphology for the automated image analysis (Supplementary Fig. S2C).

Neurotoxic profiles of paclitaxel

By the screening platform, we studied the in vitro neurotoxicity of paclitaxel (Fig. 2A and B). Paclitaxel was incubated with primary cultures of cortical or DRG neurons for 24 hours at therapeutic ranges (16). Figure 2 shows the dose–response curves of neurotoxicity in terms of neuron viability, synaptic puncta formation, and neurite outgrowth. Paclitaxel at the therapeutic concentration of 1 μmol/L (17) inhibited about 75% and 30% of neurite outgrowth in DRG and cortical neurons, respectively (Fig. 2A and B). This implies that paclitaxel has a prominent neurotoxicity on PNS, compared with a lesser toxic effect on CNS neurons. This is consistent with clinical observations on paclitaxel-induced peripheral neuropathy (16).

After testing the neurotoxic effect of paclitaxel on primary cortical neurons, we also used this neuron-based screening platform to study the neurotoxicity of commonly prescribed chemotherapeutic drugs including cisplatin, vincristine, and liposomal doxorubicin. Among these drugs, vincristine showed the prominent toxicity on neuron viability, neurite outgrowth, and synaptic
formation (Fig. 2C–E). In contrast, liposomal doxorubicin displayed less neurotoxic profiles of these parameters (Fig. 2C–E).

High-content screening of potential neuroprotective agents against paclitaxel-induced neurotoxicity

We further used paclitaxel as a neurotoxic drug to screen for potential neuroprotective agents from compound libraries (Supplementary Table S1). To test neuroprotective effects, primary cortical neurons isolated from P0 mouse pups were pretreated with various concentrations of compounds for 24 hours and then exposed to paclitaxel for another 24 hours. In the pilot screening, 8 candidates, including 5-hydroxydecanoate (5-HD), amiloride, glyburide, gingerol, minoxidil sulfate, chlormezanone, hinokitiol, and propyl gallate showed a significant neuroprotective effect against paclitaxel (1 μmol/L) with regard to neurite outgrowth, synaptogenesis, and viability in cortical neurons (Fig. 3A and B). Each value represents the mean ± SEM from at least three different experiments.

Figure 2.
Neurotoxicity of paclitaxel. Dose–response curves of neurotoxic effects of paclitaxel on primary cortical neurons (A) and DRG neurons (B). Primary neurons were incubated with paclitaxel at therapeutic concentrations for 24 hours. All data were normalized to the control group. Y axis, the normalized ratio; x axis, the concentration of paclitaxel. Dose–response curves of neurotoxic effects of cisplatin, vincristine, and liposomal doxorubicin on viability (C), synaptogenesis (D), and neurite outgrowth of primary cortical neurons (E). Primary neurons were incubated with chemotherapy drugs at the therapeutic concentrations for 24 hours. All data were normalized to the control group. Y axis, the normalized ratio; x axis, the concentration of drugs. Data, mean ± SEM from at least three different experiments.

Figure 3.
Neuroprotective drugs screened from the image-based high-content platform. A, to test neuroprotective effects, primary cortical neurons isolated from P0 mouse pups were pretreated with various concentrations of compounds for 24 hours and then exposed to 1.0 μmol/L paclitaxel for another 24 hours. In the pilot screening, 8 candidates, including 5-hydroxydecanoate (5-HD, 1 μmol/L), amiloride (0.01 μmol/L), glyburide (1 μmol/L), gingerol (0.01 μmol/L), minoxidil sulfate (1 μmol/L), chlormezanone (1 μmol/L), hinokitiol (1 μmol/L), and propyl gallate (1 μmol/L) showed a significant neuroprotective effect against paclitaxel (1 μmol/L) with regard to neurite outgrowth, synaptogenesis, and viability in cortical neurons. Each value represents the mean ± SEM from at least three different experiments. #, P < 0.05; *, P < 0.01; **, P < 0.001, compared with the paclitaxel-treated group, by unpaired t test. B, representative images show that 5-HD is a potential neuroprotective drug in the cell model of cortical neurons. Green, MAP2; red, synaptophysin; blue, Hoechst. Scale bar, 50 μm. #, neuron body; arrow head, neurite outgrowth.

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Mol Cancer Ther; 14(10) October 2015

Molecular Cancer Therapeutics

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the data from central and peripheral neurons together, 1 μmol/L 5-HD exhibited the most significant neuroprotective effects against paclitaxel-induced neurotoxicity.

The neuroprotective effects of 5-HD in vivo

To validate the neuroprotective effects of 5-HD, a mouse model of paclitaxel-induced neuropathy was applied (14, 18). At first, we did a preliminary experiment to determine the effective concentrations of 5-HD in the mouse model, and 10 mg/kg 5-HD was shown to be the maximum safe dose. Subsequently, C57BL/6J female mice were pretreated with a safe dose of 5-HD (5 or 10 mg/kg) 1 hour prior to each injection of paclitaxel (4.5 mg/kg) over 7 days. Afterwards, the mouse behavioral tests, including tail immersion study, von Frey filament test, and grip test were conducted weekly to assess the thermal threshold, mechanical allodynia, and motor function, respectively (Fig. 4A). The paclitaxel-treated mice exhibited an increase of thermal sensitivity (Fig. 4B), and a dramatic drop of mechanical threshold (Fig. 4C), consistent with previous studies in mouse models (14). Pretreatment with 5-HD enabled the mice to regain their mechanical sensitivity (Fig. 4B) and alleviated mechanical allodynia (Fig. 4C). The grip strength was not affected by paclitaxel (Supplementary Fig. S5), consistent with clinical observations that paclitaxel predominantly affects sensory fibers instead of motor function (19). The post hoc analysis of data obtained at the post-treatment 5th week showed significant protective effects of 5-HD on paclitaxel-induced sensory deficits (Fig. 4B and C, right plane).
We also designed experiments to study whether 5-HD could reverse paclitaxel-induced neurotoxicity. 5-HD was administered after neuropathy was induced and mouse behavior tests were performed (Supplementary Fig. S6). Two weeks after paclitaxel injection, the mice exhibited an increase of thermal threshold (Supplementary Fig. S6B) and a significant decrease of mechanical threshold (Supplementary Fig. S6C). 5-HD partially alleviated mechanical allodynia (Supplementary Fig. S6C) but showed no effect on thermal sensitivity (Supplementary Fig. S6B).

**No adverse effect of 5-HD on antitumor treatment**

To test whether 5-HD alters tumor growth in vivo, we inoculated NOD/SCID mice subcutaneously with human cervical cancer SiHa cells. Paclitaxel and 5-HD were administered according to the protocol shown in Fig. 4A. As depicted in Fig. 4D, rapid tumor growth was obvious in the control groups. In contrast, treatment with paclitaxel inhibited tumor growth. 5-HD itself did not change the tumor growth and it also did not affect the antitumor effect of paclitaxel (Fig. 4D–F).

**Histology of sciatic nerve**

The ultrastructure of nerve tissues was studied at the 6th week after paclitaxel treatment (protocol as Fig. 4A). Paclitaxel causes nerve damage both to myelinated and nonmyelinated fibers (Fig. 5A), in which nerve tissues show a varying degree of dying axons (black arrow in Fig. 5A), vacuolated Schwann cells, detached compact myelin with accumulated myelin debris (white dashed arrow in Fig. 5A and B), and multiple swollen and vacuolated mitochondria in the axons (black dashed arrow in Fig. 5A and C). In contrast, 5-HD treatment displayed similar pathologies of nerve but with less severity (Fig. 5A–D).

We also assessed the integrity of myelination by the G-ratio, a ratio of axon circumference to myelin circumference. The micrographs (Fig. 5B are representative) show representative images of myelinated fibers in which the axon (white circle) and myelin circumference (white dashed line) are indicated. Axons were further grouped by axon sizes. In small fibers (axon diameter < 5 μm), G-ratio was decreased by paclitaxel, but could be significantly rescued by 5-HD treatment ($P < 0.001$, Fig. 5B). On the other hand, the G-ratio of large fibers was not significantly affected by paclitaxel (Fig. 5B). This result indicates that paclitaxel tends to affect smaller fibers, consistent with clinical observation indicating that paclitaxel-induced neurotoxicity predominantly affects sensory fibers (20).
5-HD protects DRG neurons by remodeling \([\text{Ca}^{2+}]_{i}\) homeostasis.

We further studied the protective mechanism of 5-HD. Paclitaxel has been shown to disturb intracellular \(\text{Ca}^{2+}\) (\([\text{Ca}^{2+}]_{i}\) homeostasis, which contributes to peripheral neuropathy (21). The process of store-operated \(\text{Ca}^{2+}\) entry (SOCE), whereby \(\text{Ca}^{2+}\) influx across the plasma membrane is activated in response to depletion of intracellular \(\text{Ca}^{2+}\) stores in the endoplasmic reticulum, actively participates in \([\text{Ca}^{2+}]_{i}\) homeostasis and potentially plays a significant role in pain pathologies (22). Accordingly, we measured the SOCE of DRG neurons in various conditions. DRG neurons from mice that have undergone behavioral tests with or without 5-HD treatment were isolated and cultured for \([\text{Ca}^{2+}]_{i}\) homeostasis and potentially plays a significant role in pain pathologies (22).

**Discussion**

Here, we established an optimal protocol for drug discovery to prevent paclitaxel-induced neurotoxicity, including a drug screening platform, validation experiments, mouse models for behavioral tests and tumor xenographs. A mixed cortical neuron-dominant culture and a DRG culture were used in our cell-based screening platform. These primary neuron cultures mimic the true environment of the nervous system and solve the complicated dedifferentiation issue of immortalized cell lines. The image-based high-content screening platform with automatic image acquisition and analysis overcomes the shortages of insufficient analytic parameters and image selection bias noted in previous studies.

Paclitaxel is a standard treatment for breast, cervical, ovarian, and lung cancer (23). The incidence of paclitaxel-induced neuropathy ranges from 20% to 70% of patients (24, 25). Mechanisms include disruption of axonal transport (26, 27), mitochondrial damage (28–30), altered ion channel activities (31), and neuronal inflammation (32, 33). Here we screened three compound libraries, including ion channel ligands, GABA and REDOX libraries, for the discovery of potential neuroprotective drugs. In the pilot screening, 5-HD showed potential protective effects on paclitaxel-induced neurotoxicity. This conclusion was supported by the following evidence: (i) 5-HD rescued paclitaxel-induced damage to neurite outgrowth in DRG neurons and the viability, neurite outgrowth and synaptogenesis in cortical neurons; (ii) 5-HD restored the thermal sensitivity and alleviated mechanical allodynia in paclitaxel-treated mice; (iii) the histopathology and quantified G ratio of myelin integrity of nerve tissue isolated from 5-HD/paclitaxel-treated mice supported the observations in mouse behavioral tests; (iv) 5-HD rescued paclitaxel-induced DRG damage in vitro and in vivo.

5-HD is a mitochondrial ATP-sensitive K\(^+\) channel (mitoK\(_{\text{ATP}}\) channel) antagonist. The mitoK\(_{\text{ATP}}\) channel regulates both intracellular \(\text{Ca}^{2+}\) concentration and the inner membrane potential upon the onset of cellular energy crisis (34). Chronic paclitaxel treatments have been reported to decrease \(\text{Ca}^{2+}\) signaling (14, 35) and impair SOCE (36), which is related to peripheral neuropathy (37). Our data showing that pretreatment with 5-HD significantly reversed the paclitaxel-induced decreases in SOCE implying that 5-HD may prevent chemotherapy-induced peripheral neuropathy by restoring \([\text{Ca}^{2+}]_{i}\) homeostasis in DRG neurons. The neuroprotective effect of 5-HD was shown previously in models of aging-related neurodegenerative disease, in which 5-HD reduced...
angiotensin II-induced cell death in dopaminergic neurons (38). In the vascular system, 5-HD prevents the development of pulmonary hypertension by inhibiting TGF-β1 or MCP-1 signaling pathway (39). Fatty acids are abundant in the brain and are important in neuronal membrane biosynthesis, energy metabolism and even nociceptive transmission (40–42). 5-HD has been reported to be a substrate for acyl-CoA synthetase, which activates fatty acids (43). This raises the possibility of another neuroprotective mechanism of 5-HD in paclitaxel-induced neurotoxicity, that of regulating fatty acid metabolism.

Conclusion

This study describes a feasible, reliable modular image-based high-content screening platform. Combined with in vivo validation studies in mouse models, we found a promising lead, 5-HD, that can potentially prevent and alleviate paclitaxel-induced neuropathy. It ameliorated paclitaxel-induced neurotoxicity by restoring [Ca2+], homeostasis in DRG neurons, and did not jeopardize the antitumor effect of paclitaxel. Early pharmacokinetic and metabolic evaluations in animal models are the immediate next steps planned for preclinical studies. Future efforts will be made to discover other neuroprotective agents by this well-established screening platform.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Authors’ Contributions

Conception and design: Y.-T. Sun, M.-R. Shen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L.-H. Chen, Y.-F. Chen, M.-Y. Lee, L.-Y. Chang, M.-R. Shen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.-H. Chen, Y.-T. Sun, M.-Y. Lee, M.-R. Shen
Writing, review, and/or revision of the manuscript: Y.-T. Sun, Y.-F. Chen, M.-Y. Lee, M.-R. Shen
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L.-H. Chen, Y.-F. Chen, L.-Y. Chang, J.-Y. Chang, M.-R. Shen
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Acknowledgments

The authors thank the Bio-image Core Facility of the National Core Facility Program for Biotechnology, Ministry of Science and Technology, Taiwan, for technical services.

Grant Support

This work was supported by the Ministry of Science and Technology, Taiwan (to J.Y. Chang and M.R. Shen), and the National Cheng Kung University, Taiwan (to J.Y. Chang and M.R. Shen).

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Received April 1, 2015; revised July 29, 2015; accepted July 30, 2015; published OnlineFirst August 20, 2015; DOI: 10.1158/1535-7163.MCT-15-0268

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Molecular Cancer Therapeutics

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