Supplementary methods

In vivo methods

Middle cerebral artery occlusion of rats

After a 7-day adaptation and pre-training period for neurological examination, the rats were subjected to left middle cerebral artery occlusion (MCAO) for 2 hours using the intraluminal filament technique. The rats were anesthetized with isoflurane (3% for induction and 2% for surgical procedure) in a mixture of oxygen/nitrous oxide (30%/70%). Body temperature was maintained at 36.6°C±0.5°C with a thermistor-controlled heating pad. Arterial pH, pCO_2, pO_2, and hematocrit were measured using a blood analysis system (International Technidyne, Edison, NJ, USA) in 0.1 mL of arterial blood obtained from a right femoral catheter. Arterial pressure was monitored from the femoral catheter with a strain-gauge transducer (LIFE KIT DX-360, Nihon Kohden, Tokyo, Japan) and amplifier (MacLab Bridge Amplifier, AD Instruments Pty Ltd., Castle Hill, Australia). Phasic pressure, mean arterial pressure, and heart rate were recorded at a sampling rate of 200/seconds using a data acquisition system and a laboratory computer (MacLab 8 analog-to-digital converter and Macintosh computer). After 2 hours of occlusion, reperfusion was performed.

For the sham surgery, a needle was introduced into the left common carotid artery of the rats and was immediately withdrawn. Regional cerebral blood flow was measured using a laser Doppler flowmeter (ALF21, Advance, Tokyo, Japan) and a wire-type probe (0.3 mm diameter; Unique Medical, Tokyo, Japan). These flowmeters and probes were inserted through a small burr hole 2 mm lateral to the bregma at the surface of the cortex to evaluate the ischemic core in the caudate and putamen.

Transferase-mediated deoxyuridine triphosphate nick end labeling assay

After sacrificing the rats, they were transcardially perfused with 0.9% sodium chloride. Brains were swiftly removed, fixed overnight in 4% paraformaldehyde solution, and then cryoprotected in a 30% sucrose solution for 3 days. Each brain was mounted on a cold metal block using an optimal cutting temperature compound (Sakura Tissue Tek, Torrance, CA, USA). Coronal sections (20 μm thickness) were cut using a motorized cryostat (Leica, Richmond, IL, USA) at –20°C. Slides were stored at –4°C and processed for transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining. After washing the fixed tissues with phosphate-buffered saline (PBS) for 30 minutes, the tissues were incubated with 3% H_2O_2 in methanol for 10 minutes. The tissues were rinsed with PBS and placed in 0.1% Triton X- 100 in 0.1% sodium citrate for 2 minutes on ice. After washing the tissues three more times, the TUNEL reaction mixture was added, and the tissues were finally incubated at 37°C without light for 1 hour. After washing three times with PBS, the tissues were mounted with 4',6- diamidino- 2- phenylindole (DAPI) mounting medium (Vector Laboratories, Burlingame, CA, USA), and the number of TUNEL-positive cells was counted.

Histology and immunohistochemistry

All rats used for the evaluation of the infarct volume with magnetic resonance imaging (MRI) were sacrificed 48 hours after MCAO for histological and immunohistochemistry analyses. The rats were transcardially perfused with PBS. The brains were quickly removed and cooled in ice-cold artificial cerebrospinal fluid for 5 minutes. Subsequently, 2 mm thick slices of the brain matrix were prepared by sectioning along the coronal plane approximately 0.4 mm from the bregma point on the skull. The slices were fixed overnight in 4% paraformaldehyde solution and then cryoprotected in a 30% sucrose solution for 3 days. The remaining brain tissues were stored at –80°C for Western blotting. Each brain specimen was mounted on a cold metal block using an optimal cutting temperature compound (Leica, Wetzlar, Germany), and coronal brain sections (20 μm in thickness) were cut on a motorized cryostat (Leica, Wetzlar, Germany) at –20°C. Three sections were placed on one "Muto" glass slide by pressing the slide onto the section as it came off the cryostat blade. Slides were stored at –80°C and processed for hematoxylin and eosin (H&E) and TUNEL staining. H&E staining distinguished the peri-infarct region. TUNEL-positive cells were counted in both the GV1001 and control groups. Immunohistochemical staining was performed using antibodies against phosphorylated Akt (pAkt; Ser473; 1:100, 9271; Cell Signaling Technology, Beverly, MA, USA), phospho-glycogen synthase kinase (pGSK-3β, 1:1,000, 9336, Cell Signaling Technology), phosphorylated-extracellular signal-regulated kinase (pERK1/2, Thr202/Tyr204; 1:1,000, 9101, Cell Signaling Technology), phosphorylated p38 (Thr180/182; 1:1,000, 9211, Cell Signaling Technology), B-cell lymphoma 2 (Bcl-2; 1:100, 2876, Cell Signaling Technology), nestin (1:200, ab6142, Abcam, Cambridge, MA, USA), neuronal nuclei (NeuN; 1:100; MAB377, Millipore, Bedford, MA, USA), and doublecortin (DCX; 1:100, ab28941, Abcam). Immunohistochemically positive cells were identified using a fluorescence microscope (Olympus, Tokyo, Japan). The number of positive cells in the peri-infarct region of the brain of the control group and in the corresponding area of the brain in the GV1001-treated group was expressed as the

average number of positive cells per high-power field (×200). As a negative control, the above procedures were repeated without a primary antibody; this sample showed no stained cells.

Western blotting

Frozen brain tissues of rats were rapidly micro-dissected on an ice-chilled plate. The peri-infarct region in the control group and the corresponding area in the GV1001-treated group, which were confirmed based on the MRI findings, were used in the Western blot analysis. The micro-dissected tissues were homogenized using a T 10 basic homogenizer (IKA Laboratory, Wilmington, NC, USA) and a type B pestle in 10:1 volume/weight buffer containing RIPA II cell lysis buffer 1× EDTA-free Triton, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium fluoride, 1 mM sodium orthovanadate (Na_3VO_4), and 0.5% 1× protease inhibitor cocktail. The protein concentrations of the tissue lysates were determined using a protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts (20 μg) of protein were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membranes were blocked with 5% skim milk and sequentially incubated with the same antibodies against phospho-insulin receptor substrate-1 (pIRS-1) versus neural stem cells (NSCs) only treated with OGD (Ser636/639) (1:1,000, 2388, Cell Signaling Technology), pAkt (ser473; 1:500, 9271, Cell Signaling Technology), Akt (1:2,000, 9272, Cell Signaling Technology), pGSK-3β (1:1,000, 9336, Cell Signaling Technology), GSK-3β (1:2,000, sc-9166, Santa Cruz Biotechnology, Dallas, TX, USA), pERK1/2 (Thr202/Tyr204; 1:1,000, 9101, Cell Signaling Technology), phosphorylated p38 (Thr180/182; 1:1,000, 9211, Cell Signaling Technology), Bcl-2 (1:1,000, 2876, Cell Signaling Technology), Bcl-2 associated X (Bax; 1:1,000, 2772, Cell Signaling Technology), and β-tubulin (1:2,000, 2146, Cell Signaling Technology). The membranes were washed with Tris-buffered saline containing 0.1% Tween-20 (TBST) and processed using horseradish peroxidase-conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA), followed by enhanced chemiluminescence detection (GenDEPOT, Katy, TX, USA). The Western blot results were quantified using an image analyzer (ImageQuant LAS 4000, GE Healthcare, Little Chalfont, UK).

In vitro methods

Culturing of neural stem cells

Rat embryos were decapitated on embryonic day 13. Their brains were rapidly removed and placed in a Petri dish half-full of ice-cold Hank's balanced salt solution (137 mM NaCl, 5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 5.6 mM glucose, and 2.5 mM HEPES; GIBCO BRL, Carlsbad, CA, USA). Single cells were dissociated from the whole cerebral cortex, lateral ganglionic eminence, and ventral midbrain of rat embryos. The resulting cells were plated at a density of 2×10^4 cells/cm^2 in culture dishes pre-coated with poly-L-ornithine/fibronectin in Ca^{2+}/Mg^{2+}-free PBS (GIBCO BRL) and cultured in N2 medium (Dulbecco's modified Eagle's medium [DMEM]/nutrient mixture F-12, 25 mg/L insulin, 100 mg/L transferrin, 30 nM selenite, 100 μM putrescine, 20 nM progesterone, 0.2 mM ascorbic acid, 2 nM L-glutamine, 8.6 mM D[+] glucose, and 20 nM $NaHCO_3$; Sigma-Aldrich, St. Louis, MO, USA) supplemented with basic fibroblast growth factor (10 ng/mL, R&D Systems, Minneapolis, MN, USA). Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere for 4 to 6 days.

Culturing of primary cortical neurons

Primary cultured cortical neurons were obtained from the cerebral cortex of fetal Sprague-Dawley rats (Orient Bio, Seongnam, Korea) on E16. The embryos were decapitated, and the brains were rapidly removed and placed in a Petri dish half-filled with ice-cold Hank's balanced salt solution (137 mM NaCl, 5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 5.6 mM glucose, and 2.5 mM HEPES; GIBCO BRL). Single cells dissociated from the whole cerebral cortex of the rat embryos were plated on a poly-L-lysine (Sigma-Aldrich) pre-coated 100 mm Corning dish (5×10^6 cells/cm^2) or glass coverslips placed in 6- or 24-well plates (Nunc, Roskilde, Denmark) at a cell density of 5×10^5 or 2.5×10^6 cells/cm^2. Cultures were incubated in DMEM (high concentration of glucose) supplemented with 10% heat-inactivated fetal bovine serum (1.7 days after plating), 1% penicillin-streptomycin, 3.7 g/L $NaHCO_3$, 0.5 μg/mL insulin, and p-aminobenzoic acid. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Two days after plating, non-neuronal cells were removed by adding 5 μM cytosine arabinoside for 24 hours. Only mature cultures (7 days *in vitro*) were used for the experiments. The population of neuronal cells was approximately 79.7% in cultured primary cortical neurons.

Trypan blue staining and lactate dehydrogenase release assay

Trypan blue staining and lactate dehydrogenase release assay were performed to assess cell viability and cytotoxicity, respectively. For trypan blue staining, 10 μL samples of cells were incubated with 10 μL of trypan blue solution for 2 minutes. Un-

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stained live cells were counted using a hemocytometer. A colorimetric assay kit (Roche Boehringer Mannheim, Indianapolis, IN, USA) was used to quantify the amount of lactate dehydrogenase released from cultured NSCs according to the manufacturer's instructions. Cell viability was assessed using a Synergy H1 Hybrid enzyme-linked immunosorbent assay (ELISA) plate reader (BioTek Instruments, Winooski, VT, USA) by measuring the absorbance at 490 nm at a reference wavelength of 690 nm. All results were normalized to the optical density (OD) of an identical well without cells.

Bromodeoxyuridine cell proliferation assay

NSCs were incubated in bromodeoxyuridine (BrdU) labeling medium (10 μM BrdU) for 5 hours. Cell proliferation was measured using a BrdU Labeling and Detection Kit (Roche Boehringer Mannheim, Grenzach-Wyhlen, Germany) according to the manufacturer's instructions. Cell proliferation was assessed by measuring the absorbance of each well at 370 nm (reference wavelength, 492 nm) using a Synergy H1 spectrophotometer plate reader (BioTek Instruments). All results were adjusted by subtracting the OD of an identical well without cells.

Colony-forming unit assay

The proliferation of NSCs was assessed using a colony-forming unit assay. Approximately 0.5×10^4 cells were seeded in a 60-mm grid plate and treated with oxygen-glucose deprivation/reoxygenation (OGD/R) and GV1001 (10 and 50 μM) for 8 hours. The cells were washed with Dulbecco's PBS (DPBS) and incubated with a fresh culture medium. After 14 days, the cells were washed again with DPBS and stained with 0.5% crystal violet (Sigma-Aldrich) in methanol for 30 minutes at room temperature. After staining, the plates were washed with DPBS and allowed to dry. Colonies were counted using a dissecting microscope. Colonies <2 mm in diameter or those that were faintly stained were excluded.

Annexin V/propidium iodide apoptosis assay

Apoptosis was measured using the fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit I (Beckton Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Briefly, NSCs were exposed to OGD with GV1001 (10 and 50 μM) and incubated for 8 hours at 37°C. Thereafter, the cells were rinsed twice with cold PBS and then resuspended in 1× binding buffer. Cells were then transferred (100 μL of the solution [1×10^5 cells]) to a 1.5 mL culture tube followed by application of 5 μL of FITC Annexin V and 10 μL propidium iodide. Samples were gently mixed and incubated for 15 minutes at room temperature in the dark. Finally, 400 μL of 1× binding buffer was added to each tube, and samples were analyzed using flow cytometry (Accuri C6 Flow cytometer, BD Biosciences, San Jose, CA, USA). Data were acquired and analyzed using BD Accuri C6 software.

Evaluation of apoptosis

NSCs were seeded on a 4-well chamber slide containing removable wells (Thermo Fisher Scientific, Waltham, MA, USA). The cells were exposed to OGD and GV1001 (10 or 50 μM) or OGD/R (exposed to OGD for 8 hours and subsequently reoxygenated for 8 hours) and 10 μM GV1001. The cells were then rinsed twice with PBS, air-dried, and fixed with 4% paraformaldehyde in PBS for 1 hour at room temperature. Apoptotic cell death was assessed using the TUNEL labeling assay (Roche Boehringer Mannheim, Indianapolis, IN, USA). To monitor intact, condensed, or fragmented nuclei, TUNEL-stained cells were counterstained with DAPI (Vector Laboratories, Burlingame, CA, USA) for 20 minutes, washed several times with PBS, and mounted on glass slides with mounting medium (Merck, Kenilworth, NJ, USA). The cells were then observed under a Bx53 microscope (Olympus).

Migration assay

The assay was performed using a QCM 24-well colorimetric cell migration assay kit (Chemicon, Temecula, CA, USA) according to the manufacturer's instructions. NSCs (3×10^5 cells/mL) treated with OGD and GV1001 (10 or 50 μM) or OGD/R and 10 μM GV1001 were added to the upper chamber of the plate and incubated for 24 hours at 37°C. The cells that migrated through the membrane were stained and enumerated by measuring the absorbance at 560 nm using a Synergy H1 spectrophotometer plate reader (BioTek Instruments), as previously described.[16]

Determination of free radical production

Cells were exposed to OGD and GV1001 (10 or 50 μM) or OGD/R and 10 μM GV1001 and incubated with the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Molecular Probes, Eugene, OR, USA) for 15 minutes to measure free radical production. After incubation with 10 μM H_2DCF-DA at 37°C for 15 minutes, the cells were washed three times with PBS. In the assay, H_2DCF-DA freely crosses cell membranes and is hydrolyzed by cellular esterase to H_2DCF, which is subsequently oxidized to the fluorescent molecule 2'7'-dichlorofluorescein (DCF) in the presence of peroxides. Therefore, DCF fluorescence is indicative of the level of intracellular hydrogen peroxide, but not of superoxide levels. The intracellular accumulation of DCF was assessed by measuring the fluorescence (exci-

tation at 488 nm; emission at 530 nm) of each well using a Synergy H1 spectrophotometer plate reader (BioTek Instruments). All results were adjusted by subtracting the OD of an identical well without cells.

Malondialdehyde assay of lipid peroxidation

The assay was performed using a lipid peroxidation (malondialdehyde [MDA]) assay kit (Sigma-Aldrich) according to the manufacturer's instructions. In brief, cells (1×10^6/mL) were homogenized on ice in 300 μL of MDA lysis buffer containing 3 μL of butylated hydroxytoluene. The cells were then incubated with 600 μL of thiobarbituric acid solution at 95°C for 1 hour. The MDA level of each sample was assessed by measuring the absorbance at 532 nm using a Synergy H1 spectrophotometer plate reader (BioTek Instruments).

Determination of Ca^{2+} level

NSCs were treated for 8 hours, and the culture medium was replaced with a fresh medium containing 5 μM fluo-4 acetoxymethyl (AM) (Life Technologies, Carlsbad, IL, USA). The cells were then incubated at 37°C for 1 hour. The cells were washed in PBS and incubated for another 30 minutes to allow complete de-esterification of intracellular AM esters for fluorescence analysis. The accumulation of fluo-4 AM in the cells was assessed using a Synergy H1 ELISA plate reader (BioTek Instruments) by measuring the fluorescence (excitation 505 nm; emission 530 nm). Fluo-4 AM intensity was quantified within a region of interest for each cell and was expressed as the relative change in fluorescence.

$\Delta F/F_0 = (F - F_0)/F_0$

Where F_0 is the fluorescence level at the start of the experiment, after subtracting the background fluorescence. Peak amplitudes of $\Delta F/F_0$ were determined after stimulation. Cumulative increases in $\Delta F/F_0$ were calculated for 420 seconds following stimulation using Excel software (Microsoft, Redmond, WA, USA).

Oxidative mitochondrial DNA damage assay

Oxidative mitochondrial DNA damage was measured using an OxiSelect Oxidative DNA Damage ELISA kit for 8-hydroxy-deoxyguanine (8-OHdG; Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions. Mitochondrial DNA was extracted using the Purelink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA). Samples/standards were added to the pre-adsorbed 8-OHdG/bovine serum albumin conjugate. Subsequently, an anti-8-OHdG monoclonal antibody was added, followed by a horseradish peroxidase-conjugated secondary antibody, and quantified with authentic 8-OHdG (Cell Biolabs) as a standard. Measurements were performed using a Synergy H1 ELISA plate reader (BioTek Instruments) with absorbance at 450 nm.

Adenosine triphosphate assay

Adenosine triphosphate (ATP) concentrations were measured using an ATP assay kit (Abcam) according to the manufacturer's instructions. The fluorometric assessment was performed using a Synergy H1 ELISA plate reader (BioTek Instruments). ATP concentrations were detected at fluorescence excitation and emission wavelengths of 535 and 587 nm, respectively.

Mitochondrial membrane potential assay

NSCs were seeded at a density of 5×10^5/mL in a 96-well culture plate. The cells were exposed to OGD and GV1001 (1, 10, or 50 μM) or OGD/R with 10 μM GV1001 and incubated for 8 hours at 37°C.

Mitochondrial membrane potential was measured using the JC-1 Mitochondrial Membrane Potential Assay Kit (Abnova, Taipei, Taiwan) according to the manufacturer's instructions using a Synergy H1 ELISA plate reader (BioTek Instruments). Healthy cells mainly comprising JC-1 J-aggregates were detected with fluorescence settings usually designed to detect rhodamine (excitation/emission wavelength=540/570 nm) or Texas Red (excitation/emission wavelength=590/610 nm). Apoptotic or unhealthy cells that mainly comprised JC-1 monomers were detected with settings designed to detect FITC (excitation/emission wavelength=485/535 nm).

Western blot analysis

The levels of several intracellular signaling proteins, including Ki67, p85α phosphoinositide 3-kinase (PI3K), pAkt (Ser 473), Akt, pGSK-3β (Ser 9), GSK-3β, high mobility group box protein 1 (HMGB-1), Bax, cytosolic cytochrome c, procaspase-9, cleaved caspase-3 (Asp 175), phospho-β-catenin (Ser33/37/Thr41), nonphospho (active) β-catenin (Ser33/37/Thr41), c-Myc, cyclin D1, and beta-tubulin (β-tubulin), were analyzed by Western blotting immediately after 24 hours of treatment. Cells (5×10^6) were washed twice in cold PBS and incubated for 30 minutes on ice in lysis buffer (RIPA II cell lysis buffer 1× with Triton, without EDTA, 1 mM PMSF, 1 mM sodium fluoride, 1 mM Na_3VO_4, and 0.5% 1× protease inhibitor cocktail). To evaluate cytosolic cytochrome c levels, cytosolic fractions were isolated using the mitochondria/cytosol fractionation kit (Abcam) according to the manufacturer's instructions. Briefly, after OGD for 8 hours and exposure to different concentrations of GV1001, NSCs were harvested, washed once with ice-cold PBS, and resuspended in 1.0 mL of 1× cytosol extraction

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buffer mix containing dithiothreitol (DTT) and protease inhibitors. After incubation on ice for 10 minutes, the cell suspension was sonicated using a Sonoplus apparatus 5 to 10 times on ice. The samples were centrifuged at 3,000 rpm at 4°C for 10 minutes. The supernatants were centrifuged again at 13,000 rpm for 30 minutes to separate the mitochondrial fraction (pellets) and the cytosolic fraction (supernatants). The mitochondrial pellet was washed once with the isolation buffer and then lysed in mitochondrial extraction buffer containing DTT and protease inhibitors. Samples containing equal amounts (30 μg) of protein were resolved by 4% to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech). The membranes were blocked with 2% skim milk and incubated with the following specific primary antibodies: anti-Ki67 (1:100, ab16667, Abcam), phospho-phosphoinositide 3-kinase (pPI3K) p85(Tyr458)/p55(Tyr199) (p85α PI3K; 1:1,000, 4228, Cell Signaling Technology), pAkt (ser473; 1:500, 9271, Cell Signaling Technology), Akt (1:2,000, 9272, Cell Signaling Technology), pGSK-3β (1:1,000, 9336, Cell Signaling Technology), GSK-3β (1:2,000, sc-9166, Santa Cruz Biotechnology), HMGB-1 (1:1,000, 3935, Cell Signaling Technology), Bax (1:1,000, 2772, Cell Signaling Technology), cytochrome c (1:500, 4272, Cell Signaling Technology), caspase-9 (1:500, 9506, Cell Signaling Technology), cleaved caspase-3 (Asp175; 1:500, 9661, Cell Signaling Technology), phospho-β-catenin (Ser33/37/Thr41; 1:1,000, 9561, Cell Signaling Technology), non-phospho (active) β-catenin (Ser33/37/Thr41; 1:1,000, 8814, Cell Signaling Technology), c-Myc (1:1,000, 5605, Cell Signaling Technology), cyclin D1 (1:2,000, 2926, Cell Signaling Technology), and β-tubulin (1:2,000, 2146, Cell Signaling Technology). The membranes were washed with TBST and incubated with the appropriate horseradish peroxidase-conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratories Inc.), followed by enhanced chemiluminescence detection (GenDEPOT). Band intensities were quantified using an image analyzer (ImageQuant LAS 4000, GE Healthcare, Buckinghamshire, UK).

Proteomics

1) Protein sample preparation

Cells were washed twice with ice-cold PBS and sonicated for 10 seconds with a Sonoplus (Bandelin Electronics, Berlin, Germany) in a sample lysis solution composed of 7 M urea and 2 M thiourea with 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 1% (w/v) DTT, 2% (v/v) pharmalyte, and 1 mM benzamidine. Proteins were extracted for 1 hour at room temperature by vortexing. After centrifugation at 15,000 ×g for 1 hour at 15°C, the insoluble material was discarded, and the soluble fraction was used for two-dimensional (2D) gel electrophoresis. Protein concentrations were determined using the Bradford method.

2) 2D PAGE

Immobiline DryStrip gel (IPG) dry strips (4 to 10 NL IPG, 13 cm, GE Healthcare) were re-swollen for 9.5 hours with Destreak rehydration solution and 0.5% IPG buffer and loaded with 150 μg of the sample. Isoelectric focusing (IEF) was performed at 20°C using an Ettan IPGphor 3 (GE Healthcare) following the manufacturer's instructions. For IEF, the voltage was linearly increased from 100 to 8,000 V over 7 hours for sample entry, followed by maintenance at a constant 8,000 V. Focusing was complete after 55 kVh. Before the second dimension separation, the strips were incubated for 15 minutes in equilibration buffer (75 mM Tris-Cl, pH 8.8, containing 6 M urea, 2% SDS, 0.002% 1% bromophenol blue stock solution, and 29.3% glycerol), first with 1% DTT and then with 2.5% iodoacetamide. Equilibrated strips were inserted into sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (13×18 cm, 12%), and SDS-PAGE was performed using a SE600 2D system (GE Healthcare) following the manufacturer's instructions. The 2D gels were run at 20°C for 1,700 Vh.

3) Image analysis

Quantitative analysis of digitized images was performed using ImageMaster 2D Platinum 7.0 software (GE Healthcare) according to the manufacturer's protocols. The intensity of each spot was normalized to the total valid spot intensity. The protein spots selected showed at least a two-fold difference in expression compared to those of the control or normal samples.

4) Peptide mass fingerprinting

For protein identification by peptide mass fingerprinting (PMF), protein spots were excised, digested with trypsin (Promega, Madison, WI, USA), and mixed with α cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid. The samples were analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry using a Microflex LRF 20 device (Bruker Daltonics, Billerica, MA, USA). Spectra were collected from 300 shots per spectrum over an m/z range of 600 to 3,000 and were calibrated by a two-point internal calibration using trypsin auto-digestion peaks (m/z 842.5099, 2211.1046). The peak list was generated using Flex Analysis 3.0 software. The threshold used for peak selection was 500 for a minimum resolution of monoisotopic mass and 5 for signal-to-

noise. The Mascot search program developed by Matrix Science (http://www.matrixscience.com/) was used for protein identification by PMF. The following parameters were used for the database search: trypsin as the cleaving enzyme, a maximum of one missed cleavage, iodoacetamide (Cys) as a complete modification, oxidation (Met) as a partial modification, monoisotopic mass, and a mass tolerance of ±0.1 Da. The PMF acceptance criteria were based on probability scoring.

Antibody microarray

The antibody microarray analysis was performed using a Panorama Antibody Microarray-Cell Signaling kit (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, 1.5×10^7 cells were seeded in 100 mm dishes and grown in the presence of OGD with GV1001 (1, 10, or 50 μM) or OGD/R with 10 μM GV1001 for 8 hours. Cells were collected, and protein samples were prepared according to the manufacturer's protocol. The protein samples were labeled with Cy3 or Cy5 (Amersham Biosciences) and subjected to antibody microarray analysis (Sigma-Aldrich). The array slides were scanned using a GenePix Personal 4100A scanner (Molecular Devices, San Jose, CA, USA), and the data were analyzed using GenePix Pro 5.0 (Molecular Devices).

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