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Characterization of Novel Synthesized Small Molecular Compounds Against Non-Small Cell Lung Cancer

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Abstract

Background—With the exception of surgery, the standard platinum-based chemotherapeutic agents are the preferred treatment for non-small cell lung cancer (NSCLC); however little improvement (5-year survival) has been made. Therefore it is highly desirable to develop innovative therapeutic agents for NSCLC treatment.

Methods—Highly enantioselective synthetic methods were developed and a broad compound library was established. Cell toxicity, cell sensitivity, cell proliferation, cell invasion, and three-dimensional colony formation assays were used to assess the anticancer potential of these compounds in non-small-cell lung cancer (NSCLC) cell lines.

Results—We found that the S-form of compound PL54 (PL54S, 5–20 μ M) exhibited strong anticancer activity in 5 tested NSCLC cell lines. We further synthesized a highly pure R-form enantiomer of PL54 (PL54R) and its race-mate (PL54Rac) and characterized their anticancer activities. The results showed that PL54S is more potent than PL54R and PL54Rac against the tested cell lines. Furthermore, less cellular toxicity was observed in the normal human lung fibroblasts. Similarly, PL54S displayed greater anti-colony formation activity compared with PL54R and PL54Rac. The cellular sensitivity assay revealed that PL54S and PL54Rac significantly suppressed cologenic formation compared with PL54R and dimethyl sulfoxide controls ($p < 0.01$). All PL54 compounds (5 to 20 μ M) significantly inhibited cell proliferation and invasion of the A549 cell line ($p < 0.01$). A soft agar colony formation assay revealed that PL54S and PL54Rac (10 mM), but not PL54R, significantly inhibited colony formation of tested NSCLC cells ($p < 0.01$).

Conclusions—The stereospecific compounds may prove to be a novel technique for the treatment of NSCLC.

Cancer is a leading cause of death worldwide and accounted for 7.6 million deaths (approximately 13% of all deaths) in 2008; it is projected to account for more than 11 million deaths by 2030 [1]. Lung cancer is the deadliest of all cancers in both men (299,200 cases [29%]) and women (270,290 [26%]) [2] according to the report of the Cancer Facts and Figures 2010. Non-small cell lung cancer (NSCLC)—including adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma—accounts for 80% to 85% of all lung cancer cases [3]. Surgical resection remains the best option for treatment of early-stage disease. Adjuvant chemotherapy is given to prevent recurrences, but the overall survival

benefit with the current regimens is marginal. Unfortunately, close to 70% to 80% of patients with lung cancer already have advanced or metastatic disease at the time of diagnosis. At present, platinum-based regimens are the standard of care for patients with advanced NSCLC. However these regimens may be associated with severe and cumulative hematologic and nonhematologic toxicities, which limit dose intensity. Therefore conventional therapy for NSCLC has reached a plateau for improving patient survival and there is a great need to develop novel therapeutic agents that are more effective with fewer side effects than the current chemotherapeutic drugs.

With a better understanding of tumor biological characteristics and molecular events governing oncogenesis, many “molecularly targeted” agents have emerged as potential therapeutic agents for various malignancies [4, 5]. Multiple novel agents targeting angiogenesis pathways have the potential to improve outcomes for patients with NSCLC. Apart from the use of monoclonal antibodies directly targeting the endothelial growth factor receptor (EGFR) extracellular binding site, small molecules designed to attack the intracellular adenosine triphosphate binding site of EGFR tyrosine kinase have been studied extensively, many of which have already been tested unsuccessfully in clinical settings. Other chemical agents targeting different pathways, DNA-damaging agents, antitumor agents, and compounds with potent antitumor activity through unknown mechanisms have been investigated [6–9]. However no potential molecule has been approved for clinical treatment of NSCLC so far.

In this study we developed a novel enantioselective synthetic method for synthesizing a broad compound library containing chiral molecules of high enantiomeric purity. Furthermore we characterized their antitumor activities using cell toxicity, sensitivity, cologenic formation, proliferation, invasion, and soft agar colony formation assays. The results show that compound PL54 is a stereoselective molecule with anticancer activities in 2 of the tested lung carcinoma cell lines, suggesting that PL54 is a potential novel therapeutic agent for NSCLC.

Material and Methods

Cell Culture

Non-small cell lung cancer lines (A549, H23, H1975, H1650, H4226) and the human lung fibroblast cell line WI-38 were obtained from the University of Virginia Tissue Culture Core Facility (Charlottesville, VA). The NSCLC cells were grown in Roswell Park Memorial Institute (RPMI) 1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified incubator containing 5% CO₂ at 37°C. WI-38 cells were grown in Minimum Essential Medium (MEM) supplemented with 10% FBS, 1 mM sodium pyruvate, and 1% MEM nonessential amino acids in a humidified incubator containing 5% CO₂ at 37°C.

Synthesis of the Enantiomerically Pure Compounds and the Racemic Compounds

Highly enantiomeric (mirror image S-form and R-form isomers) pure compounds as well as the racemic compounds for the anti-NSCLC studies were synthesized as previously described [10, 11]. We proposed that PL54 has a hydrophobic group, which could easily penetrate the cell membrane, and an activated unsaturated group, to which the nucleophilic guanine base of DNA, at the number 7 nitrogen atom of the imidazole ring, could be added and covalently linked. The R-form (PL54R), S-form (PL54S), and a 1:1 racemic mixture of both the R-form and S-form of PL54 (PL54Rac) were synthesized using a novel catalytic system, conducted at room temperature with excellent enantioselectivity (89% to 97%). After synthesis these compounds were purified and confirmed with mass spectroscopy.

Cytotoxicity Assay: Screening of Potential Antitumor Cell Compounds

To test the cytotoxicity of these compounds, cells (A549, H1975, H23, H1650, H4226, and WI-38) were seeded into 96 well plates (1.5×10^3 cells/well) and incubated for 24 hours in a humidified incubator containing 5% CO₂ at 37°C. The 70 compounds, including PL54R, PL54S, and PL54Rac, were added at varying concentrations from 5 to 40 μM and incubated for 24 hours in 5% CO₂ at 37°C. The cells were stained with 0.1% crystal violet for 1 minute and dried. The absorption was measured at 595 nm using the BIO-TEK multiple plate reader (Bio-TEK Instruments, Inc, Winooski, VT). The photographs were taken under an Olympus BX51 microscope equipped with an Olympus DP70 digital camera (Olympus America Inc, Center Valley, PA) and colonies were counted for statistical analysis.

Cytotoxicity Assay Based on Colony Formation

To test the inhibitory effects of these compounds, the cytotoxicity assay based on colony formation was performed according to the published method with modification [12]. Briefly, the cells were subcultured into 6 well plates (2.7×10^4 cell/well) and incubated for 18 hours in 5% CO₂ at 37°C. Cells were then cultured in media fortified with 10% serum and varying concentrations (0 to 20 μM) of PL54S, PL54R, PL54Rac, and dimethyl sulfoxide (DMSO) and incubated for 10 days in a humidified incubator containing 5% CO₂ at 37°C. The cells were fixed and stained with 0.1% crystal violet and dried. All assays were performed in triplicate.

Cell Sensitivity Assay/Clonogenic Assay

To further determine the sensitivities of lung cancer cells to these compounds, the “gold standard” cellular sensitivity assay/clonogenic assay was used. The assay was performed according to the published method with modification [12]. Briefly, the cells were subcultured into 6 well plates (2.7×10^4 cell/well) and incubated for 2 days in 5% CO₂ at 37°C. Cells were then cultured in media fortified with 10% serum and varying concentrations of PL54S, PL54R, PL54Rac, and DMSO as already described and incubated for an additional 24 hours. The treated cell lines were then subcultured and seeded into new 6 well plates at a concentration of 350 cells/well and incubated for 10 days in a humidified incubator containing 5% CO₂ at 37°C. The cells were fixed and stained with 0.1% crystal violet and dried. All assays were performed in triplicate.

Trypan Blue Staining and Cell Counting

To identify dead cells after treatment with these compounds, trypan blue was used to stain them [13]. Cells were plated onto 24-well cell culture plates in 10% serum medium at 1×10^4 cells/well and incubated at 37°C for 1 day. Cells were sequentially treated with PL54R, PL54S, and PL54Rac (2.5, 5, 10, and 20 μM) for an additional 24 hours. DMSO was used as the control. Treated cells were rinsed with cold PBS and stained with 0.4% wt/vol trypan blue (Sigma-Aldrich, St. Louis, MO) for 5 minutes. Stained cells were counted as dead cells and unstained cells were counted as viable cells. All assays were performed in triplicate.

Cell Proliferation Assays

Cellular proliferation of A549 cells was measured by Cell Counting Kit-8 Assay (Dojindo Molecular Technologies, Inc, Rockville, MD) according to the manufacturer's instructions. This assay uses a tetrazolium salt (WST-8 colorless substrate), which can be converted into the water-soluble WST-8 formazan by the reduced form of nicotinamide adenine dinucleotide (NADH)/reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) dehydrogenase. Briefly, cells in 10% FBS media were seeded in 96-well tissue culture plates at a density of 1.5×10^4 cells/well for 24 hours. Cell Counting Kit-8 solution, 100 μL, was added to each well for another 24 hours. The absorption was measured at 450

nm in a μ Quant enzyme-linked immunosorbent assay (ELISA) reader (Bio-TEK Instruments, Inc).

Collagen Type IV Invasion Assay

The cell invasion assay was performed as described previously [13, 14]. A Transwell insert with 8- μ m pores (Becton Dickinson, Boston, MA) was coated with 30 μ L of different concentrations of collagen type IV (0.25 mg/mL; Sigma Aldrich) and dried overnight. Cell suspension (0.5×10^5 cells/insert) in serum-free RPMI 1640 medium was added to the upper chamber in the presence or absence of PL54 compounds (10 μ M). The lower chamber contained 500 μ L of RPMI 1640 media with 0.1% FBS. After 24-hour incubation, the cells in the upper chamber that had not been invaded or had not migrated were gently scraped away, and adherent cells present on the lower surface of the insert were stained with 0.1% crystal violet solution and photographed. The invaded cells were labeled with the brush tool in red with Photoshop software (Adobe Systems Incorporated, San Jose, CA) and manually counted. DMSO was used as the control. Three independent experiments were performed.

Soft Agar Colony Formation

The soft agar colony formation assay was performed according to our previous publication [13]. Briefly, a heterogeneous mixture of 5×10^3 cells in 0.3 mL of 0.4% agar medium was layered on top of 0.5 mL of 0.8% agar medium in a 24-well plate. PL54R, PL54S, and PL54Rac were each added to the top agar at the desired concentration. RPMI 1640 media (Invitrogen, Inc) supplemented with 10% FBS, 0.5 mM sodium pyruvate, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), and 1% penicillin/streptomycin was added to the top agar the day after plating. After 22 days, the photographs of colonies were taken with microscopes at 5 $\times$ magnification. The area and number of colonies were determined with Image-Pro 4.5 (Media Cybernetics, Inc, Bethesda, MD) and Photoshop (Adobe Systems, Incorporated) software. The data are means $\pm$ standard error (SE) of 3 independent wells.

Statistical Analyses

Statistical differences of multiple groups were determined using 1-way analysis of variance followed by a Newman-Keuls comparison test from Prism software (GraphPad Software, Inc, La Jolla, CA). A student's *t* test was used to compare the 2 groups. Data were represented as the mean $\pm$ standard error of the mean (SEM) from at least triplicate experiments. A *p* value equal to or less than 0.05 was considered significant.

Results

Compound PL54 Displayed Strong Antitumor Activity

To select potential anti-lung carcinoma cell compounds, we tested 70 highly pure S-form compounds uniquely synthesized in our laboratory. Several compounds demonstrated antitumor-cell potential in human lung carcinoma cell lines; however PL54S was selected for further investigation because of its strong tumor cell toxicity in NSCLC (A549, H23, H1975, and H4226 [Fig 1A]) and H1650 cells (data not shown).

PL54 Shows Stereoselectivity in Its Anticancer Activities in A549 and H23 Cells but Not in H1975, H4226, and H1650 Cells

To further determine whether PL54 is stereoselective, we synthesized the pure S- or R-form of the enantiomers using a novel synthetic method and tested them in our in vitro cellular assays with 5 NSCLC cell lines. The results demonstrated that PL54S (5 and 10 μ M), but not PL54R, induced killing of H23 (Fig 1B) and A549 cells (data not shown). PL54R (20

μM) showed only slight killing activity. The other 3 NSCLC cell lines (H1975, H1650, and H4226) were sensitive to all the enantiomers of PL54 (data not shown). To compare the cell-killing activity of PL54 enantiomers against nonneoplastic human lung fibroblasts with the lung carcinoma cells, human lung fibroblast cells WI-38 were treated with PL54R, PL54S, and PL54Rac at the same concentration as the lung cancer cells. The result showed that PL54S (20 μM) exhibited strong killing activity, but not at 10 or 5 μM . PL54Rac showed only moderate killing activity at 20 μM , whereas the PL54R had no obvious effect on WI-38 cells (data not shown). These observations indicate that lung carcinoma cells may be more sensitive to these compounds than are normal human fibroblasts.

We then detected the number of dead cells still attached to the enantiomer-treated wells using trypan blue staining. The results demonstrated that PL54S-treated A549 and H23 cells showed significant cell mortality, with increased cell death in the H23 cell line compared with the A549 cell line at the same concentration ($p < 0.01$) (Fig 2). PL54Rac also displayed significant tumor-cell toxicity ($p < 0.05$) (Fig 2). These data indicate that PL54 is a stereoselective molecule for anticancer activities in certain lung cancer cells.

PL54S but Not PL54R Significantly Blocked Colony Formation in H23 and A549 Cells

A cytotoxicity assay based on colony formation was performed to explore the effect of PL54 enantiomers on lung cancer cell cytotoxicity. The results demonstrated that the PL54 enantiomers had different effects on A549 (data not shown) and H23 colony formation in a dose-dependent manner. PL54S significantly reduced colony formation at 5 and 10 μM and abolished colony formation at 15 and 20 μM ($p < 0.01$) (Fig 3A). PL54Rac also significantly inhibited colony formation at concentrations greater than 5 μM , whereas PL54R significantly affected colony formation only at 20 μM ($p < 0.05$) (Fig 3A). The cell sensitivity assay revealed dose-dependent anticolony formation activity similar to that seen with the cologenic formation assay (Fig 3B), especially at high concentration (more than 10 μM). There was no significant difference in colony formation at the concentration of less than 10 μM in any of the enantiomers and the racemer-treated cells. PL54S and PL54Rac (15 and 20 μM), but not PL54R, significantly inhibited colony formation of A549 and H23 cells (Fig 3B). These data further support PL54 having stereoselective tumor-cell toxicity against NSCLC A549 and H23 cells.

PL54 Enantiomers Suppressed Cell Proliferation and Cell Invasion of A945 Cells

To assess the effect of PL54 enantiomers on cell proliferation and invasion, cell proliferation and invasion assays were performed using A549 cells. Cell proliferation of A549 was significantly reduced after treatment with PL54R, PL54S, and PL54Rac (5, 10, and 20 μM) compared with DMSO controls (Fig 4A). The invasion assay revealed that all the PL54 enantiomers (10 μM) markedly blocked invasion of A549 cells when compared with DMSO controls ($p < 0.01$) (Fig 4B).

PL54S but Not PL54 Inhibited Soft Agar Colony Formation in A549 and H23 Cells

Since anchorage-independent growth is 1 of the hallmarks of tumor-cell transformation, we used the soft agar colony formation assay, the most accurate and stringent in vitro assay for detecting malignant transformation in cells, to monitor anchorage-independent growth in the 5 lung carcinoma cell lines in the presence or absence of the PL54 compounds. In the absence of PL54 in any form, all 5 cell lines formed colonies after 22 days. Among them, A549 cells formed the most colonies. The addition of both PL54S and PL54Rac (10 μM) to the culture medium significantly inhibited colony formation of A549, H23 (Fig 5A), H1975, and H4226 cells (data not shown) compared with PL54R and DMSO controls at the same concentration. PL54R had no significant effect on colony formation in either A549 cells ($p = 0.40$) or H23 cells ($p = 0.15$) at 10 μM when compared with DMSO control groups (Fig 5A).

The results clearly demonstrate that PL54S, but not PL54R, blocked colony formation in A549 and H23 cells. In contrast, PL54R significantly reduced colony formation of H1975 ($p = 0.012$) and H4226 ($p = 0.006$) cells when compared with DMSO controls (Fig 5B). No colony formation was observed in the human lung fibroblast (WI-38, data not shown).

Comment

Non-small cell lung cancer accounts for 80% to 85% of all lung cancer cases and is the leading cause of cancer mortality for men and women [3]. A number of novel therapeutic agents targeting specific pathways of NSCLC, including EGFR, vascular endothelial growth factor, the RAS/RAF/MAP kinase pathway, and the phosphoinositide 3-kinase pathway, have been investigated [15–18]. Despite all this work, there continues to be a lack of improvement in the treatment of NSCLC clinically.

We developed 70 enantiomerically pure compounds and screened them for their anti-NSCLC activity. One particular compound, PL54, had strong anti-NSCLC cell activity, including effects on cytotoxicity, cell death, cell colony formation and anchorage-independent growth in a three-dimensional environment. Intriguingly the 2 enantiomers of this compound acted differently against NSCLC in our in vitro cell culture assays. We propose that PL54 has a hydrophobic group enabling it to easily penetrate the cell membrane and an activated unsaturated group susceptible to nucleophilic attack by the guanine base of DNA at the number 7 nitrogen of the imidazole ring, allowing covalent linkage. This hypothesis was further proved by our recent study, which showed that the cell killing activity disappeared after replacing the hydrophobic group in PL54 with a hydrophilic group. Our data have provided evidence that PL54S has anticancer activities against NSCLC A549, H23, H1975, H4226, and H1650 cell lines. Notably, less toxicity against normal human fibroblasts (WI-38) was found compared with NSCLC cells.

Many antitumor agents are DNA-directed alkylating agents that use the aziridine alkylating center as a functional group [18]. Xing and colleagues synthesized aziridinyl quinone antitumor agents using indoles and cyclopent indoles [19–21]. The most lethal compounds are the indole-based systems with a leaving group in the 3 position. PL54 was developed in this fashion and we demonstrated that the 2 enantiomers of this compound acted differently against NSCLC in our in vitro cell culture assays. PL54S demonstrated a strong anticancer effect, whereas PL54R had no effect on NSCLC (A549 and H23) cell growth at the same concentration. In addition PL54Rac showed a moderate effect on the tested NSCLC cells. Interestingly, PL54R showed killing activities against the other 3 cell lines (H1975, H1650, and H4226) at a concentration of 10 μ M but almost no killing activity against A549 and H23 cells. Therefore PL54 is a stereoselective molecule and shows tumor cell toxicity in certain types of cancer cells, indicating that it may be a potential therapeutic agent for personalized lung cancer treatment.

We further evaluated the PL54 compounds using clonogenic assays (a cytotoxicity assay and a cell sensitivity assay), which are thought to be the gold standard for cellular sensitivity assays [12]. The cytotoxicity assay based on colony formation analyzes the “longer effects” of “potential drugs” on colony formation, whereas the sensitivity assay determines the ability of colony formation after 24 hours of treatment with the “potential drugs.” Our results revealed that PL54S and PL54Rac significantly reduced colony formation in a dose-dependent manner (5 to 20 μ M) in both assays; in contrast, PL54R showed no significant effect up to 15 μ M. PL54S and PL54Rac displayed more killing in the cytotoxicity assay than in the sensitivity assay, which suggests that the PL54 enantiomers are stable and effective in cell culture systems for more than 24 hours. Most importantly, the soft agar colony formation assay demonstrated that PL54S and PL54Rac completely blocked colony

formation, whereas PL54R had no significant effect on A549 and H23 cell colony formation.

In addition to increasing cytotoxicity/cell-killing activity and inhibiting colony formation of H23 and A549 cells, we further tested the effect of PL54 compounds on cell proliferation and invasive ability. The results confirmed that PL54S and PL54Rac significantly inhibited cell proliferation as well as cell invasion of A549 cells. Surprisingly PL54R also significantly inhibited cell proliferation as well as cell invasion of A549 cells. These results indicated that the less cytotoxic PL54R compound could be a potential drug to confine or suppress tumor cell proliferation and cell invasion in patients before operation or could be used after operation to prevent tumor recurrence and operation-related metastasis. Anchorage-independent growth is 1 of the hallmarks of tumor-cell transformation and is considered the most accurate and stringent in vitro assay for detecting malignant transformation of cells [13]. We have shown that the tumor cells are not tumorigenic in vivo if they are unable to form colonies in the anchorage-independent growth assay. Cisplatin, a well-established chemotherapy drug, binds to and causes cross-linking of DNA, which ultimately triggers apoptosis. It should be noted that although cisplatin is frequently designated as an alkylating agent, it has no alkyl group and thus cannot carry out alkylating reactions. It is correctly classified as alkylating-like. Recently it was shown that the apoptosis induced by cisplatin on human colon cancer cells depends on the mitochondrial serine protease Omi/HtrA2 [22]. Because this was demonstrated only for human colon carcinoma cells, it remains an open question if the Omi/HtrA2 protein participates in the cisplatin-induced apoptosis in other cancerous tissues. Based on our in vitro cell culture results, these compounds significantly inhibited the growth of NSCLC when compared with the DMSO control and the human lung fibroblast cells (WI-38). We are planning to further test these compounds in a preclinical mouse lung cancer model to further assess their toxicities and inhibition of tumor growth in vivo to provide a solid foundation from which to advance the development of human clinical trials using these compounds. It is highly possible that PL54S or PL54Rac could be used to treat NSCLC in vivo in the future.

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Fig 1.

Antitumor activities of our newly synthesized compounds. (A) The anti-lung carcinoma cell activities of PL54S (5 to 20 μ M) in A549, H23, H1975, and H4226. Mean $\pm$ SEM values from 3 separate experiments for each group are shown. (B) Comparison of H23 cells treated with PL54S, PL54R, and PL54Rac. H23 cells were grown in 96 well plates with RPMI media fortified with 10% FBS for 24 hours and then treated with 5, 10, and 20 μ M of PL54S, PL54R, and PL54Rac for another 24 hours. The cells were stained with 0.1% crystal violet. Images were taken under microscope with 60 $\times$ magnification. DMSO was used as the control. (DMSO = dimethyl sulfoxide; FBS = fetal bovine serum; RPMI = Roswell Park Memorial Institute; SEM = standard error of the mean.)

Fig 2.

Determination of dead cells after treatment with PL54 enantiomers in A549 and H23 cells. The cells were stained with 0.4% wt/vol trypan blue for 5 minutes. The number of cells per high-power (HP) field were counted. Data shown are the mean $\pm$ SEM values from 3 separate experiments for each group. (DMSO = dimethyl sulfoxide; SEM = standard error of the mean.)

Fig 3. Effects of PL54 enantiomers on colony formation in H23 cells. (A) Colony numbers per well after treatment with PL54 compounds for 10 days. (B) Colony number per well after treatment with PL54 compound for 24 hours and then grew for another 10 days without compounds. Data shown are the mean $\pm$ SEM values from 3 separate experiments for each group. (DMSO = dimethyl sulfoxide; SEM = standard error of the mean.)

Fig 4.

Effects of PL54 enantiomers on cell proliferation and cell invasion of A549. (A) PL54 enantiomers (5 to 20 μ M) inhibited A549 cell proliferation. DMSO was used as the control. Data shown are the mean $\pm$ SEM values from 3 separate experiments for each group. (B) PL54 enantiomers (10 μ M) blocked A549 cell invasion. DMSO was used as the control. Data shown are the mean $\pm$ SEM values from 3 separate experiments for each group. (DMSO = dimethyl sulfoxide; SEM = standard error of the mean.)

Fig 5. Effects of PL54 enantiomers on soft agar colony formation of lung carcinoma cells. (A) Colony formation in A549 cells and H23 cells treated with PL54 compounds. PL54 enantiomers (10 μ M) were added to the top agar. DMSO was used as the control. Images photographed under microscopes at 10 $\times$ magnification. (B) Colony number in A549, H23, H1975, and H4226 cells after PL54 treatment with enantiomers. The number of colonies per well was counted. Data shown are the mean $\pm$ SEM values from three separate experiments for each group. (DMSO = dimethyl sulfoxide; SEM = standard error of the mean.)