

A Novel Selenadiazole Derivative Induces Apoptosis in Human Glioma Cells by Dephosphorylation of AKT

Yikai Zhang,^{a,#} Shanyuan Zheng,^{b,#} Sai-Ming Ngai,^b Wenjie Zheng,^{*c} Jinying Li,^a Tianfeng Chen,^c and Xueyun Zhong^{*a}

^aGuangdong Province Key Laboratory of Molecule Immunology and Antibody Engineering, Department of Pathology, Jinan University; ^cDepartment of Chemistry, Jinan University; Guangzhou 510632, China; and ^bSchool of Life Sciences and State Key Laboratory of Agrobiotechnology, The Chinese University of Hong Kong; Shatin, Hong Kong SAR, China.

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Selenadiazole derivatives are synthetic organoselenium compounds with improved anticancer activity and greater selectivity than inorganic selenium. In this study, 4-(benzo[*c*][1,2,5]selenadiazol-6-yl)-benzene-1,2-diamine (BSBD) was shown to induce time- and dose-dependent apoptosis in SWO-38 human glioma cells by accumulation of a sub-G1 cell population, DNA fragmentation, nuclear condensation, caspase activation and poly(ADP-ribose) polymerase (PARP) cleavage. Further mechanistic investigation showed that BSBD treatment induced dephosphorylation of AKT and DNA damage-mediated activation of p53, leading to extensive apoptosis through the mitochondrial pathway. Our findings suggest that BSBD represents a potential human glioma therapeutic.

Key words selenium; apoptosis; signal transduction; organoselenium compound; glioma

Cancer is a major challenge in public health,¹⁾ and many naturally occurring^{2,3)} and synthetic agents⁴⁾ exhibit chemopreventive activity against cancer. Human glioma is the most common type of brain tumour, with high invasiveness, malignancy and recurrence⁵⁾; as a result, the prognosis of patients with glioma is poor.⁶⁾ Thus, the identification of new agents capable of selectively killing glioma cells is an urgent priority in cancer research.

Selenium (Se) is an essential micronutrient for mammals.²⁾ In recent decades, Se has been extensively researched as a chemopreventive agent for cancer.^{7,8)} Indeed, several trials have found that supplementation with Se at supranutritional levels may represent a safe and effective way to prevent cancers.^{9,10)} However, the anticancer activity of Se is highly dependent on which chemical form is used.^{11,12)} The most-studied forms of Se are inorganic and include selenite and selenate, although these compounds exhibit narrow margins between effective and cytotoxic dosages.¹³⁾ Moreover, their poor selectivity between cancer cells and normal cells limits their clinical potential.¹⁴⁾ It is therefore of great importance to develop new forms of Se with greater clinical potential.

Compared with inorganic Se, organoselenium compounds show several advantages, such as better absorption, stronger anticancer activity and lower cytotoxicity against normal cells.^{12,13)} Therefore, in recent decades, a number of potent organoselenium compounds have been designed in pursuit of greater chemopreventive efficacy and lower side effects. Examples include ebselen,¹⁵⁾ selenocyanate,¹⁶⁾ selenobetaine¹⁷⁾ and Se analogues of amino acids.¹⁸⁾ For instance, 1,2,5-selenadiazolo-[3,4-*d*]pyrimidine-5,7-(4*H*,6*H*)-dione was identified as a potent antiproliferative agent when tested in several human cell lines, including MCF-7 breast carcinoma, HepG2 hepatoma and A375 melanoma cells.¹⁸⁾ In addition, 1,4-diselephenone-1,4-diketone was shown to induce caspase-dependent mitochondrial apoptosis in A375 human melanoma cells,¹⁹⁾

and 1,2-(5,5'-dimethoxybis(1,2-benziselenazol-3(2*H*)-one))-ethane was shown to inactivate thioredoxin reductase (TrxR) in several cancer cell lines.²⁰⁾ However, the selectivity of these compounds is poor, and their mechanisms of action have not been well clarified.

In this paper, we show that an organoselenium compound, 4-(benzo[*c*][1,2,5]selenadiazol-6-yl) benzene-1,2-diamine (BSBD), induced death in several cancer cell lines, with minimal effects on normal cells. Further mechanistic investigation showed that BSBD induced the dephosphorylation of AKT and DNA damage-mediated activation of p53, leading to extensive mitochondrial apoptosis in SWO-38 glioma cells.

Results and Discussion

We synthesised BSBD (Fig. 1A), a novel selenium compound that we expected to exhibit selective anticancer activity. The inhibitory effect of BSBD on the growth of several cell lines was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay; IC₅₀ values are shown in Table 1. Compared with selenite and selenate, BSBD exhibited excellent, selective anticancer activity

Table 1. IC₅₀ Values (μM) of BSBD on Various Human Cancer and Normal Cells

	BSBD	Selenite	Selenate
A375	80.5±5.5	16.8±6.1	22.7±6.7
HepG2	57.8±11.7	19.3±13.2	21.3±9.7
MCF-7	38.9±6.2	15.7±9.5	29.4±10.2
Neuro-2a	32.0±7.9	24.5±10.1	22.1±9.5
SWO-38	25.5±5.2	31.2±5.8	36.8±6.1
PC-12	35.4±12.4	27.3±14.6	33.5±12.7
HK-2	>600	11.9±3.5	20.1±4.8
HOPC	>600	20.4±4.3	26.5±3.9
MCF-10A	>600	19.5±3.9	18.6±3.7
LO-2	>600	14.6±3.4	25.8±3.6

Cells were treated with various concentrations of BSBD for 48h. Cell viability was determined by MTT assay and IC₅₀ values were calculated as described in Experimental. Each value represents the mean±S.D. of three independent experiments.

The authors declare no conflict of interest.

[#]These authors contributed equally to this work.

* To whom correspondence should be addressed. e-mail: tzhwj@jnu.edu.cn; tzxy@jnu.edu.cn

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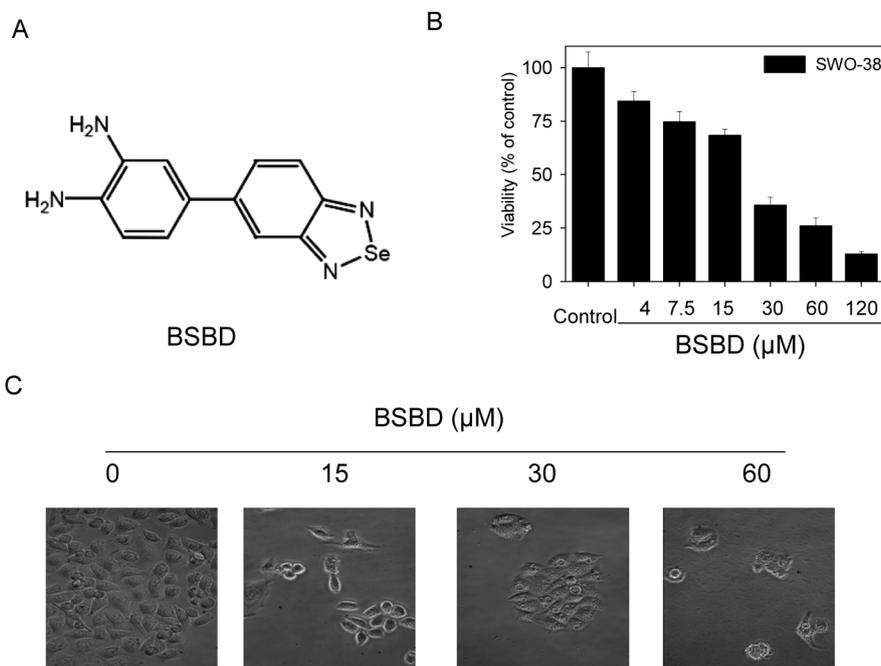


Fig. 1. Growth-Inhibition Effect of BSBD in SWO-38 Cells

(A) Chemical structure of BSBD. (B) Growth-inhibit effect of BSBD on SWO-38 cells measured by MTT assay. (C) Morphology of SWO-38 cells after treated with different concentrations of BSBD. Cells were exposed to different concentrations of BSBD for 48h.

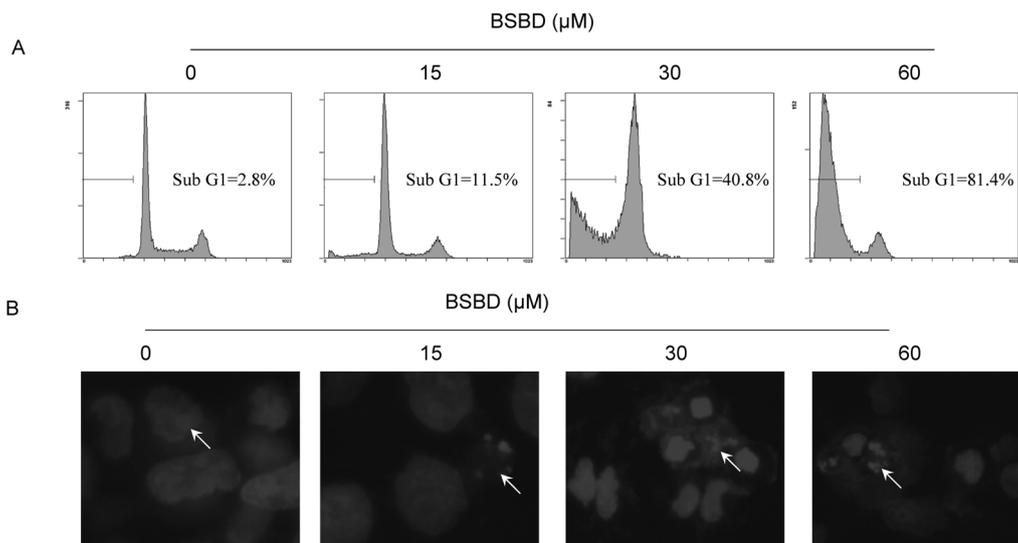


Fig. 2. BSBD Induces Apoptosis in SWO-38 Cells

(A) Cell cycle distribution of SWO-38 cells after treated with BSBD for 48h was detected by flow cytometry. (B) BSBD-induced nuclear condensation evaluated by DAPI assay.

towards A375, HepG2, MCF-7, Neuro-2a, SWO-38 and PC-12 cancer cells; this activity was particularly strong against SWO-38 glioma cells. Moreover, BSBD exhibited minimal cytotoxicity towards the normal cell lines HK-2, LO-2, MCF-10A and HOPC-5, which represents a significant improvement in selectivity compared with previous reports.^{18,19} As shown in Fig. 1B, BSBD treatment led to a dose-dependent inhibition of cell growth: 12–88% inhibition after 48h of treatment with BSBD (4–120 μM). After exposure to BSBD, vacuolation was observed in SWO-38 cells (Fig. 1C). These results suggest that BSBD kills glioma cells selectively, with a wide margin between effective and cytotoxic dosages.

We next explored the mechanism of BSBD-induced cell death. The inhibitory effect of BSBD on SWO-38 cells may result from the induction of apoptosis, cell cycle arrest or a combination of the two. First, we determined whether apoptosis and/or cycle arrest were involved in BSBD-induced cell death using flow cytometry, an *in vitro* method for the detection of apoptosis. Treatment of SWO-38 cells with BSBD resulted in a dose-dependent increase in the percentage of apoptotic cells (Fig. 2A), as reflected in the sub-G1 populations (11.5–81.4%). These results suggest that BSBD, like many other selenium compounds,^{7,18,19} induces apoptosis in SWO-38 cells. These findings were confirmed by 4',6-diami-

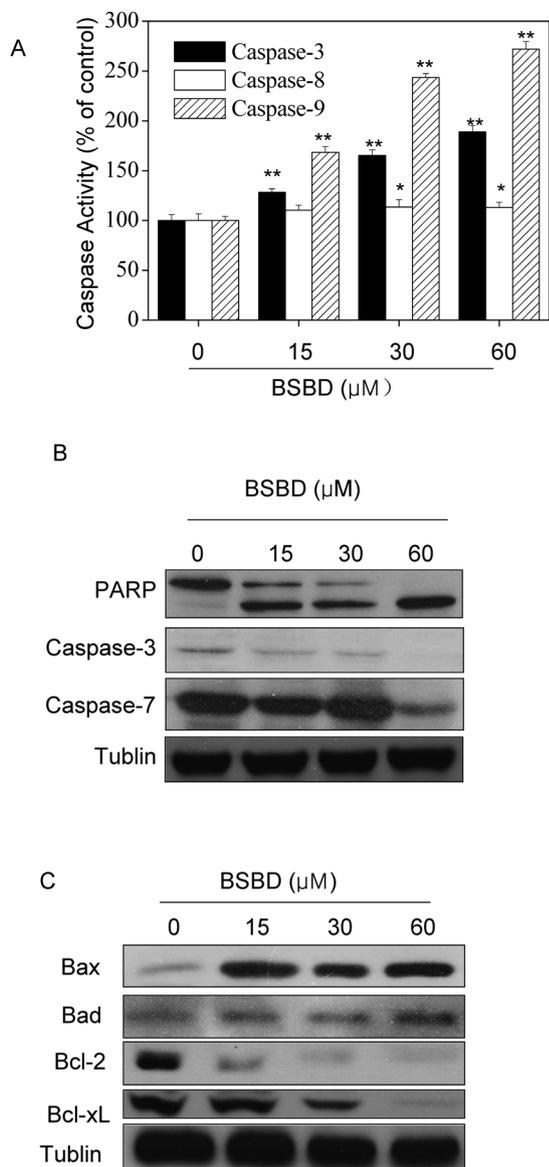


Fig. 3. BSBD Induced Mitochondrial Apoptosis in SWO-38 Cells

(A) Analysis of caspase activation in BSBD induced apoptosis in SWO-38 cells. Caspase activities were measured using synthetic fluorescent substrates for caspase-3, -8, and -9. (B) The cleavage of PARP, caspase-3 and -7 in cells that were treated with BSBD for 48h. (C) Expression of Bax, Bad, Bcl-2 and Bcl-xL in SWO-38 cells treated by BSBD. Results were obtained from three independent experiments. Significant difference between treatment and control groups is indicated at * $p < 0.05$ level and ** $p < 0.01$ level.

dino-2-phenylindole (DAPI) staining. As shown in Fig. 2B, after exposure to different concentrations of BSBD, a dose-dependent increase in nuclear condensation was apparent in SWO-38 cells. These results reflect BSBD-induced apoptosis in SWO-38 human glioma cells.

There are two well-characterised caspase activation pathways mediating apoptosis. The mitochondrial (intrinsic) pathway involves mitochondrial membrane permeabilisation and the activation of caspase-9, whereas the death receptor pathway involves the death receptor, the adaptor protein FADD, caspase-8 and -10.²¹⁾ In this study, treatment of SWO-38 cells with BSBD resulted in the cleavage of caspase-7, -3 and poly(ADP-ribose) polymerase (PARP) (Fig. 3B), as well as the activation of caspase-3 and -9 (Fig. 3A). We interpreted these results as a biochemical indicator of mitochondrial apoptosis.

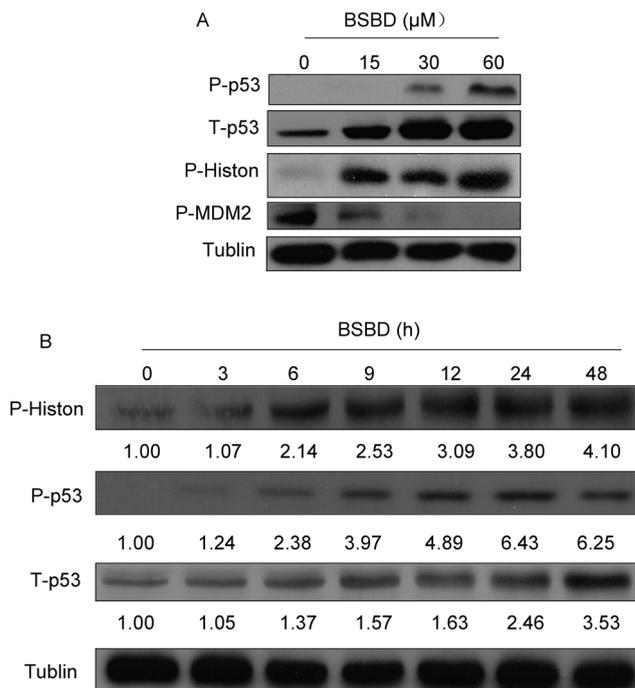


Fig. 4. BSBD Triggers DNA Damage Mediated p53 Activation

(A) Western blotting analysis of the phosphorylation level of p53, Histone H2A, MDM2 and the expression level of total p53 in SWO-38 cells exposed to different concentrations of BSBD for 48h. (B) The level of phospho-H2A.X, phospho-p53 and total p53 after treated by BSBD (10 $\mu\text{g}/\text{mL}$) for 3–48h were detected by Western blot. All experiments were carried out at least in triplicates.

Furthermore, clinical data show that antiapoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-XL, are associated with drug resistance and tumour recurrence in multiple clinical scenarios.²²⁾ In this study, down-regulation of Bcl-2 and Bcl-XL and up-regulation of Bax and Bad were detected in the presence of BSBD (Fig. 3C). These results indicate that BSBD induces mitochondrial apoptosis, suggesting that BSBD may have potential in overcoming drug resistance and cancer recurrence.

Accumulating evidence suggests that p53 is a key factor through which selenium compounds exhibit anticancer activity. The function and activity of p53 is regulated by transcription, translation, protein–protein interactions and post-translational modifications such as phosphorylation or acetylation on specific amino acid.²³⁾ Moreover, the pro-apoptotic function of p53 is believed to influence the levels of Bcl-2 family members.²⁴⁾ Our results show that BSBD up-regulated the expression of Bax and Bad (Fig. 3C), which can be regulated by p53. This suggests the potential involvement of DNA damage and p53 activation. Consistent with this, we detected phosphorylation of p53. As shown in Fig. 4A, treatment with BSBD for 48h resulted in the phosphorylation of p53 at Ser15, followed by an accumulation of p53 protein. BSBD also induced the phosphorylation of H2A.X, a DNA damage-related signalling molecule, which further suggests the involvement of DNA damage-mediated activation of the p53 pathway. A p53 heterodimer that induces degradation of p53, phospho-MDM2, was also decreased after treatment with BSBD. To confirm the role of the p53 pathway in apoptosis, the levels of phospho-H2A.X, phospho-p53 and total p53 were evaluated by Western blotting after treatment with BSBD for 3–48h (Fig. 4B). BSBD increased the phosphorylation of p53 and H2A.X by a factor

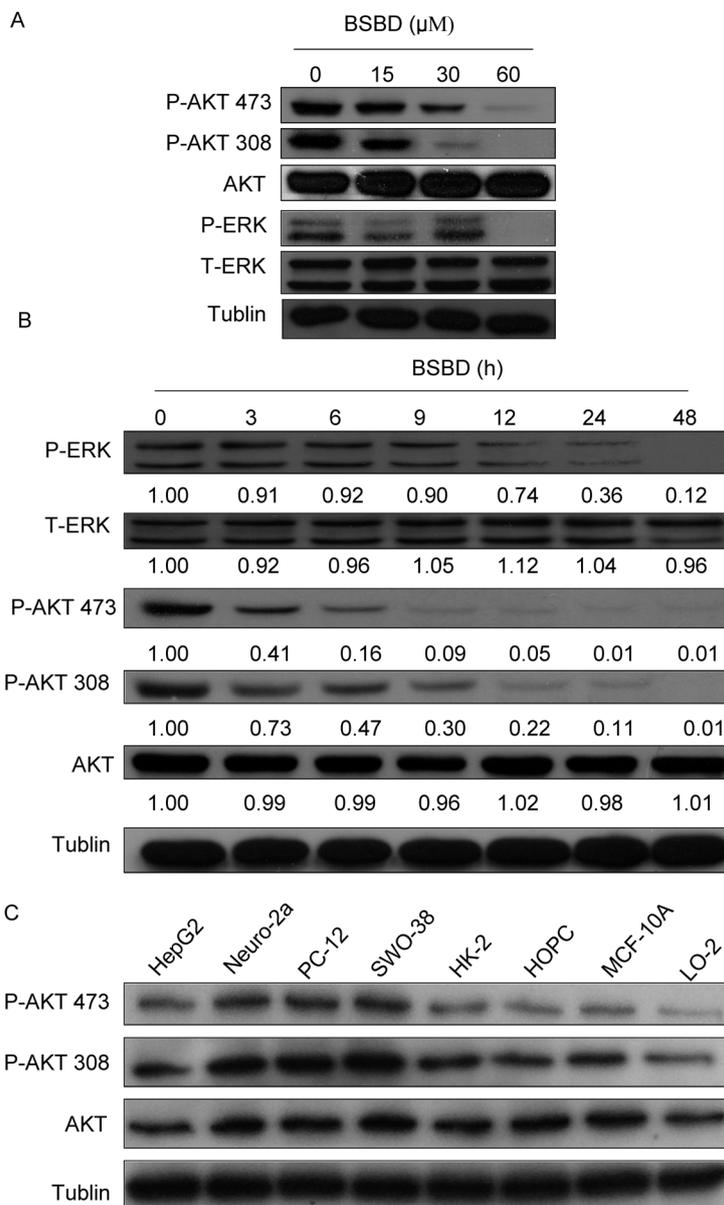


Fig. 5. BSBD Induces Apoptosis in SWO-38 Cell by Triggering AKT Inactivation

(A) The expression and phosphorylation levels of AKT and ERK in SWO-38 cells exposed to different concentration of BSBD for 48 h were evaluated by Western blot. (B) Expression and phosphorylation levels of total ERK and AKT in SWO-38 cells exposed to BSBD (10 μ g/mL) for 3–48 h detected by Western blot assay. (C) The activation status and expression level of AKT in different cancer and normal cell lines detected by Western blot assay. All results were carried out at least in triplicates.

of two after 9-h exposure to BSBD. Total p53 protein also doubled after treatment with BSBD for 24 h. Together, these results indicate that DNA damage-mediated activation of p53 plays a vital role in the induction of apoptosis by BSBD.

Several protein kinases, including AKT and extracellular-signal-regulated kinase (ERK), are crucial in survival signalling, drug resistance and regulating the activation of p53 in many cancer cell lines.²⁵⁾ Activation of the p53 pathway (Fig. 4) and Bad (Fig. 3C) also suggest the possible involvement of AKT or/and ERK.^{26,27)} We therefore investigated the role of AKT and ERK in the induction of apoptosis by BSBD. The results showed down-regulation of AKT (Thr308 and Ser473) and ERK phosphorylation after treatment with BSBD (Fig. 5A). To investigate the roles of AKT and ERK in the induction of apoptosis by BSBD, the AKT and ERK phosphorylation levels were monitored by Western blotting

following BSBD treatment. The phosphorylation of AKT at Ser473 decreased to 40% after 3-h exposure to BSBD, and this level further dropped to 10% at 6 h post-exposure. The level of phospho-AKT (Thr308) also decreased to 50% of the control after treatment with BSBD for 6 h. However, ERK remained activated after 12-h exposure to BSBD; thus, ERK may not regulate the p53 pathway in BSBD-induced apoptosis of SWO-38 cells (Fig. 5B). These results also suggest that AKT inactivation may be the initial event in the induction of apoptosis by BSBD in SWO-38 cells. To further clarify the role of AKT in the selective induction of apoptosis by BSBD, we measured the activation status (phospho-AKT at both S473 and T308) and expression level of AKT in HepG2, Neuro-2a, PC-12 and SWO-38 cancer cells and HK-2, HOPC, MCF-10A and LO-2 normal cells. AKT activation was significantly higher in cancer cells compared with normal cells, which is in line

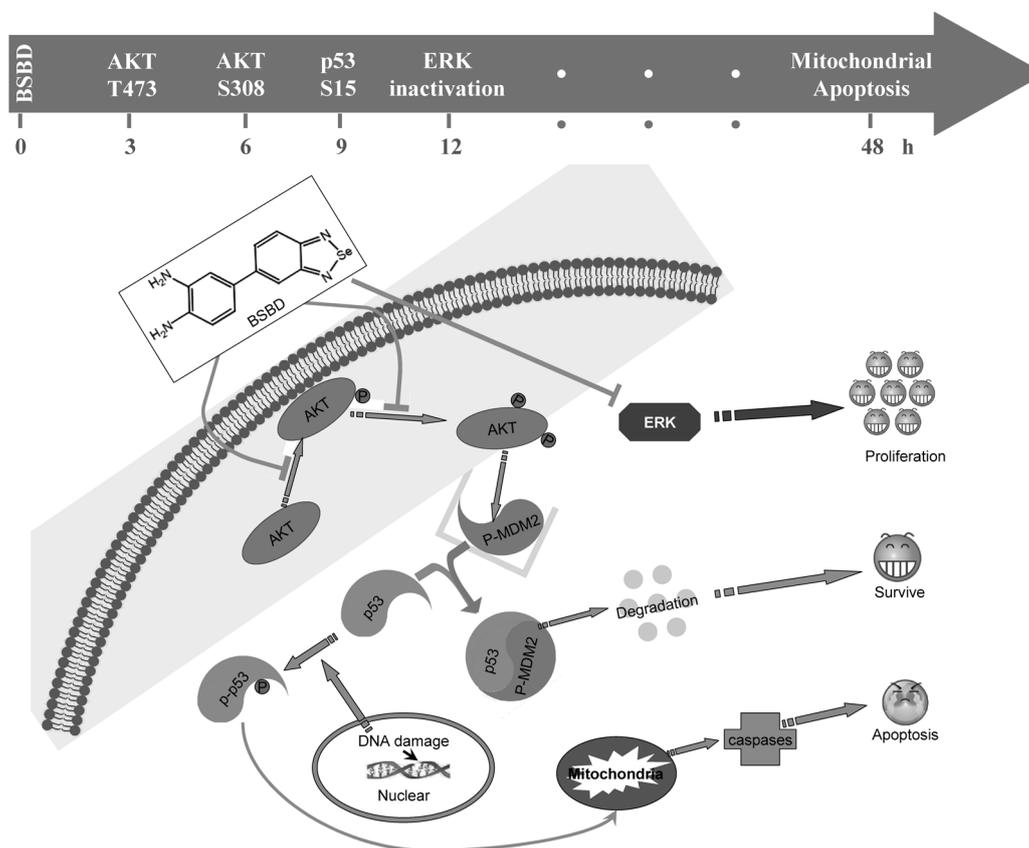


Fig. 6. The Potential Signaling Transduction of BSBD-Induced Apoptosis in SWO-38 Cells

with the concept that constitutive phosphorylation of AKT is required for the survival of cancer cells but not normal cells. These results suggest that activation of AKT may account for the selectivity of BSBD. Collectively, our results suggest that BSBD selectively induces apoptosis in SWO-38 cells *via* the dephosphorylation of AKT.

In summary, our results suggest that BSBD induces dephosphorylation of AKT and DNA damage-mediated activation of p53, leading to extensive mitochondrial apoptosis in SWO-38 glioma cells (Fig. 6). Based on these results, we suggest that BSBD may represent a potential therapeutic agent against human glioma.

Experimental

Materials 3,3',4,4'-Biphenyltetramine and SeO_2 (AR, Aldrich), thiazolyl blue tetrazolium bromide (MTT), DAPI and propidium iodide (PI) were obtained from Sigma. Substrates for caspase-3 (Ac-DEVD-AFC), -8 (Ac-IETD-AFC), and -9 (Ac-LEHD-AFC) were purchased from Calbiochem. Antibodies were purchased from Cell Signaling Technology.

Synthesis and Characterisation BSBD was synthesised as described previously.²⁸ 3,3',4,4'-Biphenyltetramine (1 mM) was dissolved in HCl solution, and then 1 mM SeO_2 -water was added slowly at room temperature and stirred for 2 h. The pH of the mixture was then adjusted with NaOH to 7.2, and the precipitate was collected and dried (60%). $^1\text{H-NMR}$ (300 MHz, dimethyl sulfoxide (DMSO)) δ : 7.79–7.82 (m, 3H, Ar-H), 6.95–7.06 (m, 2H, Ar-H), 6.63 (d, $J=1.2\text{ Hz}$, 1H), 4.87 (s, 2H), 4.65 (s, 2H); IR (KBr) ν : 3426, 1620, 1512, 671, 455 cm^{-1} ; electrospray ionization (ESI)-MS m/z : 291.1 (M+H)⁺.

Cell Culture The A375, HepG2, MCF-7, Neuro-2a,

PC-12, LO-2, HK-2 and MCF-10A cell lines were obtained from ATCC, and HOPC cells were obtained from ScienCell. The SWO-38 cell line was generated in our own laboratory and maintained in RPMI 1640 medium or Dulbecco's modified Eagle's medium (DMEM) supplemented with foetal bovine serum (10%), penicillin (100 units/mL) and streptomycin (50 unit/mL) at 37°C in a humidified incubator under a 5% CO_2 atmosphere.¹⁸

Cell Viability and Flow Cytometry After treatment, cell viability was determined by measuring the transformation of MTT to a purple formazan dye. Cell cycle distribution was analysed by flow cytometry as previously described.¹⁸

DAPI Staining Treated cells were fixed with 3.7% formaldehyde for 15 min and incubated with 1 $\mu\text{g/mL}$ DAPI for 20 min at 37°C. Cells were then washed three times with phosphate buffered saline (PBS) and examined under a fluorescence microscope.¹⁸

Caspase Activity Treated cells were harvested and incubated with lysis buffer on ice for 2 h. Total protein (80 μg /well) was incubated with specific caspase substrates (Ac-DEVD-AMC for caspase-3, Ac-IETD-AMC for caspase-8 and Ac-LEHD-AMC for caspase-9) at 37°C for 2 h in darkness. Caspase activity was determined by fluorescence intensity with excitation and emission wavelengths of 380 and 440 nm, respectively.¹⁸

Western Blot Analysis Total cellular proteins were extracted by incubating cells in lysis buffer (Cell Signaling Technology). The protein concentration in cell lysates was determined using the bicinchoninic acid assay (Sigma) according to the manufacturer's instructions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was

performed on 12% gels, after loading equal amounts of protein per lane. After electrophoresis, separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes and then blocked with 5% non-fat milk in TBST buffer for 1 h. Next, the membranes were incubated with primary antibodies (Cell Signaling Technology) at a 1:1000 dilution in 5% bovine serum albumin (BSA) overnight at 4°C, followed by incubation with secondary antibodies conjugated to horseradish peroxidase (Cell Signaling Technology) at a 1:3000 dilution for 1 h at room temperature. Protein bands were visualised on X-ray film using an enhanced chemiluminescence system (Kodak). To compare the amount of protein in each lane, the membranes were stripped to detect α -tubulin.

Statistical Analysis Experiments were carried out at least in triplicate, and results are expressed as the mean \pm S.D. Statistical analysis was performed using the SPSS statistical package (SPSS 13.0 for Windows; SPSS, Inc., Chicago, IL, U.S.A.). Differences between two groups were analysed using the two-tailed Student's *t* test, whereas those between three or more groups were analysed using one-way ANOVA comparisons. Differences with $p < 0.05$ (*) or $p < 0.01$ (**) were considered statistically significant.

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