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Synthesis and radioprotective effects of novel benzyl naphthyl sulfoxide (sulfone) derivatives transformed from Ex-RAD†

Lin Tang, Tao Peng, Gang Wang, Xiaoxue Wen, Yunbo Sun, Shouguo Zhang,* Shuchen Liu* and Lin Wang *

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In this work, a series of novel benzyl naphthyl sulfoxides (sulfones) derived from Ex-RAD were designed and synthesized as potential radioprotective agents. Some of the compounds considerably protected HUVECs against ^{60}Co γ-irradiation, accompanied by the absence of cytotoxicity. Compared to Ex-RAD, compound **8n** not only exhibited a significant protective effect on cell survival and radiation-induced DNA damage, but also remarkably enhanced the survival (100%) of mice in 30 days after being exposed to irradiation. The results suggested that some target compounds are valuable for further research as promising radioprotectors.

1. Introduction

Providing protection from ionizing radiation injuries is not only a matter of concern in therapeutic radiology, but also has become an important issue due to the ever increasing threats associated with the proliferation of nuclear materials, terrorism and occupational risks associated with space exploration.[1,2] At present, amifostine (WR2721) is the only radioprotector that has been clinically approved by the Food and Drug Administration for reducing side effects in patients undergoing radiotherapy.[3] This drug has a good effect on radiation injuries, but causes serious side effects, such as nausea, vomiting and hypotension.[4] Therefore, it is essential to develop easily self-administered, less toxic, and more effective radioprotectors.[4]

Ex-RAD (**I** in Fig. 1), also known as ON 01210.Na (4-carboxystyryl-4-chlorobenzyl sulfone, sodium salt), is a chlorobenzyl sulfone derivative developed by Onconova Therapeutics (Newtown, PA, USA) as a radioprotector and mitigator.[5] Unlike most radioprotectors, Ex-RAD is not a free-radical scavenger or responsible for cell cycle arrest. Available data suggest that Ex-RAD has a novel mechanism for radiation protection involving DNA repair pathways.[6] It is a water soluble, non-toxic, synthetic molecule with potent radioprotective properties both *in vitro* and *in vivo*.[5,7–9] In *in vitro* studies, Ex-RAD has shown potent radioprotective efficacy in several human cell lines, including human umbilical vein endothelial cells (HUVECs), lung fibroblast cells (HFL-1) and skin fibroblast cells (AG1522).[5] In *in vivo* studies, Ex-RAD has also demonstrated significantly greater protection against ^{60}Co γ-irradiation than the vehicle when administered to mice before radiation exposure (subcutaneous injection (s.c.) or intragastric administration (i.g.)).[5–8] Moreover, pre-clinical pharmacokinetic studies in rats, dogs, rabbits and monkeys demonstrate that Ex-RAD is well-absorbed following extravascular administration, resulting in significant plasma exposure.[10,11] Mechanistically, the radioprotective effects of Ex-RAD may involve the prevention of p53-dependent apoptosis.[8] Attenuation of ataxia telangiectasia-mutated gene-p53-mediated (ATM-p53-mediated) DNA damage response (DDR) by Ex-RAD contributes to the mitigation of radiation-induced hematopoietic toxicity.[6] Further, Ex-RAD manifests its protective effects through the up-regulation of PI3-kinase/AKT pathways in cells exposed to radiation.[9] Onconova Therapeutics has completed four Phase I trials with Ex-RAD, three trials with subcutaneous Ex-RAD in more than 50 healthy adults and one trial with oral Ex-RAD in nine healthy adults; none of these trials reported evidence of systemic side effects.[12]

The structure of Ex-RAD, associated with three types of chemical structure, has been protected by a patent. The three chemical structures (Fig. 1) are: styryl benzyl sulfoxides (sulfones) (**II**), diaryl acrylketone sulfoxides (sulfones) (**III**) and diaryl vinyl sulfoxides (sulfones) (**IV**).

The structure–activity relationship (SAR) analysis of the above aryl sulfoxides (sulfones) indicated the following rules: (1) in the part of the styryl segment, the bioactivity was higher when 2-chloro, 4-chloro, 4-fluoro, 2-methoxy, 2,4-dimethoxy or 4-carboxyl was introduced to the styrene side; alternatively, the benzene ring was replaced by a 3-furan

Beijing Institute of Radiation Medicine, Beijing 100850, P. R. China.
E-mail: wanglin@bmi.ac.cn, wanglin07@sina.com; Tel: +86 010 66932239

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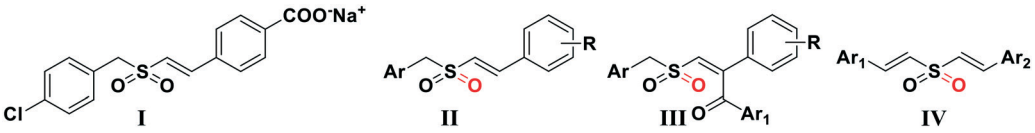


Fig. 1 Structure of Ex-RAD (I). Structures of aryl sulfoxides (sulfones) protected by a patent (II, III and IV).

ring. (2) In the part of the benzyl segment, introduction of 4-cyano, 4-chloro or 4-hydrogen could enhance the bioactivity. However, a decrease in activity was observed when the *para*-position of the benzene ring was substituted with methoxy or nitro. (3) The bioactivity of sulfoxides was similar to the corresponding sulfones. (4) The *trans*-forms could exist more stably and the synthetic yield was higher. But a similar bioactivity was also retained in *cis*-forms.

Based on the results of SAR analysis, the *ortho*-position of the styrene side could be chemically processed to get various derivatives. In this article, to connect the *ortho*-position of the styrene side with the 2-position of vinyl according to the principle of isotones, a series of novel 2-naphthyl benzyl sulfoxide (sulfone) derivatives (Fig. 2) were designed rationally. Subsequently, thirty-seven target compounds were prepared. Their stereochemical and electronic structures were similar to Ex-RAD while avoiding the extent of patent protection. Furthermore, looking forward to obtaining more effective anti-radiation agents, the radioprotective effects of the target compounds were evaluated *in vitro* and *in vivo*.

2. Results and discussion

2.1. Chemistry

We first synthesized compound **5b** by using 2-naphthol as a starting material, as described in the literature.[13–15] Compounds **7a–7t** were synthesized from the reaction of **5a–5b** with **6a–6j** in the presence of NaOH.[16] Finally, the sulfides **7a–7t** were oxidized to obtain the corresponding sulfoxides (**8a–8r**) and sulfones (**9a–9t**) with H_2O_2 in acetic acid.[17] All the synthesized compounds were purified by recrystallization or silica gel column chromatography, and the structures of some target compounds with better biological activity were characterized by ^{1}H NMR, ^{13}C NMR and HR-MS spectra analyses.

2.2. Biological activity

2.2.1. Evaluation of the radioprotective activity and cytotoxicity of target compounds. The *in vitro* radioprotective activity of the target compounds was first evaluated by a radiation assay using HUVECs as a model of normal cells.[5] Cell survival after irradiation was measured by the MTS tetrazolium assay. Cells were pretreated with the target compounds 24 h before irradiation (8.0 Gy), and Ex-RAD was used as a positive control. Ex-RAD was synthesized by our lab. Unfortunately, most of the compounds did not show significant radioprotective activity for the first screening at a concentration of 40 μM (data are provided in the ESI†). However, 9 compounds (**8d**, **8g**, **8j**, **8l**, **9l**, **8n**, **9o**, **8p** and **9t**) exhibited obvious radioprotective effects at concentrations of 40 μM and 20 μM (second screening). In particular, the survival of **8n**-treated cells was significantly ($p < 0.05$) higher than that of Ex-RAD-treated cells. Results from cell survival after irradiation indicated that **8n** exhibited good radioprotective activity. The survival rates of cells treated with the 9 compounds (20 μM) are summarized in Table 1. The cytotoxic activity of the 9 compounds (100 μM) in HUVECs was also determined by the MTS tetrazolium assay. The results demonstrated that they were not toxic (data not shown).

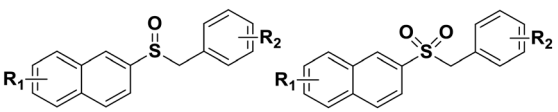
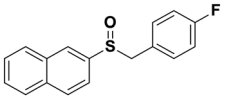
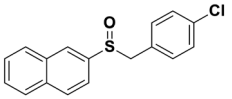
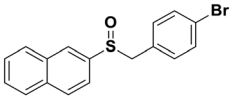
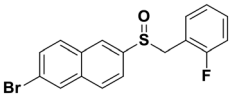
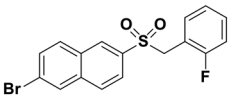
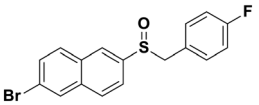
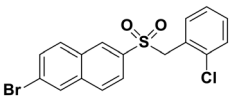
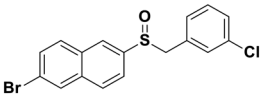
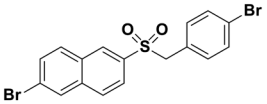


Fig. 2 The structures of 2-naphthyl benzyl sulfoxide (sulfone) derivatives.

2.2.2. Evaluation of the protective effect of 8n on radiation-induced DNA damage. The alkaline comet assay was employed to monitor the efficacy of **8n** in protecting HUVECs from radiation-induced DNA damage.[5] The comet assay is a common technique for measurement of DNA damage in individual cells. Under an electrophoretic field, damaged cellular DNA (containing fragments and strand breaks) is separated from intact DNA and migrates further from the intact DNA, yielding a classic "comet tail" shape under the microscope. The extent of DNA damage is usually visually estimated by comet tail measurement. Radiation-induced DNA damage results in increased numbers of single-strand breaks (SSBs), double-strand breaks (DSBs), and alkali-labile lesions. The degree of DNA damage was measured by calculating the tail lengths with Comet Assay Software Project Lab (CASP1.2.3 beta 2) after irradiation with 6.0 Gy. Fifty randomly selected comets were captured by fluorescence microscopy after staining with vista green DNA dye. In control cells, there was a significant increase in tail lengths. As shown in Fig. 3, **8n** treated HUVECs had significantly ($p < 0.05$) shorter tail lengths compared to the vehicle control and Ex-RAD treated cells, which indicated that **8n** played an important role in the repair of DNA damage after exposure to ionizing radiation.

2.2.3. Radioprotective efficacy of 8n in mice. In *in vitro* studies, compound **8n** exhibited a significant radioprotective effect. In order to complete the study of this compound, we tested the radioprotective effectiveness of **8n** *in vivo* on C57/BL male mice. The survival was monitored for 30 days post-irradiation.[5,8] Ex-RAD and nylestriol were used as positive controls. As shown in Fig. 4, **8n** was given *via* intraperitoneal injection (i.p.) at a dose of 300 mg kg^{-1} 24 h and 15 min (two doses) before irradiation at 8.0 Gy, resulting in a net survival of 100% ($p < 0.05$, Fisher's extract test). The survival in the group given nylestriol i.g. 24 h (one dose) before irradiation

Table 1 The survival rates in some compound-pretreated cells after irradiation

Compound (20 μM)	Structure	Survival rate (%)
Vehicle control	—	43.7 ± 3.4
8d		52.0 ± 1.9
8g		57.2 ± 0.9
8j		59.3 ± 2.0
8l		51.3 ± 3.5
9l		52.4 ± 1.2
8n		77.2 ± 2.6
9o		56.7 ± 2.4
8p		55.1 ± 1.6
9t		53.1 ± 4.6
Ex-RAD	—	57.5 ± 4.9

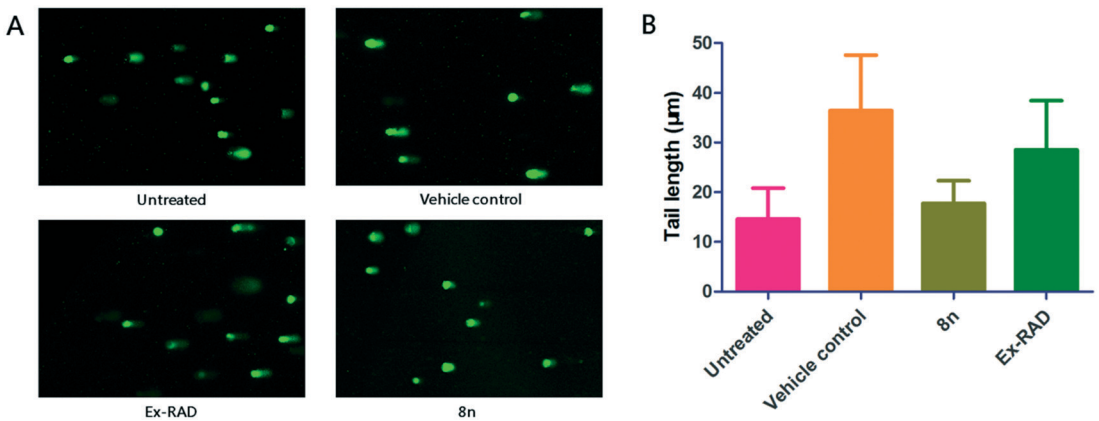


Fig. 3 DNA damage as determined by the alkaline comet assay in HUVECs. The untreated group was composed of normal cultured cells without irradiation. A. The fluorescence microscopy images of HUVECs 24 h post-irradiation. B. DNA damage was measured by calculating the tail lengths 24 h post-irradiation (6.0 Gy). **8n**-treated HUVECs had significantly ($p < 0.05$) shorter tail lengths compared to the vehicle control and Ex-RAD treated cells.

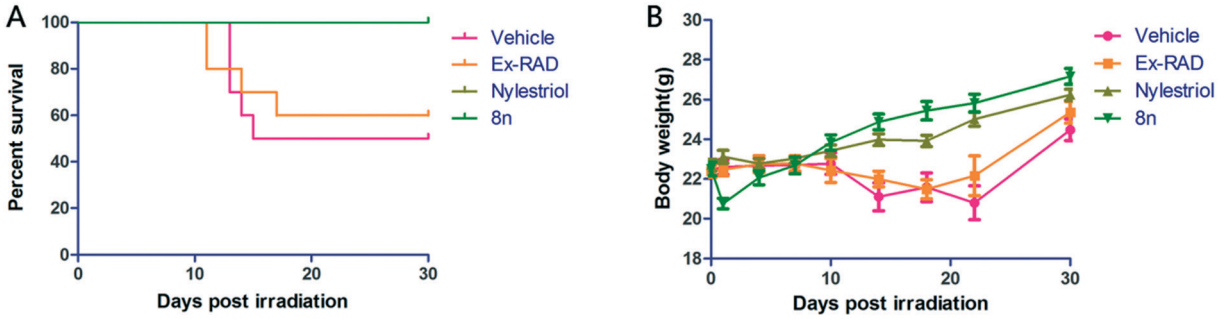


Fig. 4 A. Kaplan–Meier 30 day survival rates were observed in male C57/BL mice (n = 10 per group). Mice that received **8n** exhibited a significant increase in survival (100%) as compared with the vehicle control group and the Ex-RAD group. Note: There was a significant difference among the survival curves (p = 0.0104, df = 3, chi squared value = 11.25, log-rank test). B. The body weights of male C57/BL mice (n = 10 per group) were measured 30 days post-irradiation. The body weights of mice in group **8n** were statistically significant ($p < 0.05$) and heavier than those of the control and Ex-RAD groups between 10 d and 30 d post-irradiation.

at 8.0 Gy also showed a net survival of 100%. However, the survival rate in the group that was given Ex-RAD s.c. (300 mg kg^{-1}) 24 h and 15 min (two doses) before irradiation was only 60%. The survival in the vehicle control group was 50%. Moreover, the body weights of mice in group **8n** (Fig. 4B) were statistically significant ($p < 0.05$) and heavier than those of the control and Ex-RAD groups between 10 d and 30 d post-irradiation. The results showed that **8n** could prolong the survival of mice after exposure to ionizing radiation.

3. Conclusions

The structure–activity relationship (SAR) study revealed that most sulfoxides exhibited better radioprotective activity than the corresponding sulfones. The introduction of halogen groups at the 4-position of the benzene ring enhanced the biological activity to a much greater extent.

In summary, we report on the design and synthesis of some 2-naphthyl benzyl sulfoxide (sulfone) derivatives for use as radioprotective drugs. Evaluations of their radioprotective activities *in vitro* indicated that some derivatives such as **8d**, **8g**, **8j**, **8l**, **9l**, **8n**, **9o**, **8p** and **9t** have a high radioprotective activity and no cytotoxicity for HUVECs. Among them, **8n** exhibited a significant radioprotective effect on cell survival and DNA damage. In addition, **8n** also showed remarkable biological activity by significantly increasing the survival rate of mice after being exposed to irradiation compared to Ex-RAD. The findings strongly suggest the great potential of this class of compounds as radioprotective drugs for radiation therapy and other purposes. Further detailed research will be conducted to evaluate the molecular mechanism underlying the radioprotective activity of these compounds.

4. Experimental section

4.1. Chemistry

All chemicals and reagents were purchased from commercial suppliers, of reagent grade and used without further purification. Melting points were recorded in an open capillary tube and uncorrected. Reactions were monitored using TLC and performed on silica gel glass plates containing 60 GF-254. Visualization was achieved using UV light (λ_{max} = 254 or 365 nm). The purification of compounds was performed using silica gel (200–300 mesh) column chromatography. ^{1}H-NMR and ^{13}C-NMR spectra were recorded using Bruker (Palo Alto, CA, USA) AV-400 spectrometers in DMSO-d_6 with TMS as the internal standard. Mass spectral data were obtained using electron spray ionization on a Micromass ZabSpec high-resolution mass spectrometer (Karlsruhe, Germany).

4.1.1. General procedure for the synthesis of compounds (8d, 8g, 8j, 8l, 8n and 8p). To an ice cold solution of 7 (1.0 mmol) in 50 mL acetic acid was added 2.0 mmol 30% H_2O_2, then the mixture was stirred at room temperature for about 3–5 h. After the completion of the reaction (monitored by TLC), the mixture was poured into ice water, and the formed white precipitate was filtered, washed with water and dried under vacuum to get the target compounds (8).

2-((4-Fluorobenzyl)sulfinyl)naphthalene (8d). Obtained in 87.5% yield, white solid, m.p. 206–208 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 4.16(d, 1H, J = 12.9 Hz), 4.39(d, 1H, J = 12.9 Hz), 7.05–7.13(m, 4H), 7.60–7.66(m, 3H), 7.99–8.09(m, 4H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ (ppm): 163.13, 160.71, 140.53, 138.86, 132.42, 132.34, 132.23, 128.85, 128.39, 128.00, 127.74, 127.25, 126.58, 126.56, 124.72, 120.64, 115.07, 114.86, 59.92; HRMS-ESI (m/z) calcd. for $C_{17}H_{14}FOS$ $[M + H]^+$: 285.0749, found: 285.0744.

2-((4-Chlorobenzyl)sulfinyl)naphthalene (8g). Obtained in 93.0% yield, white solid, m.p. 214–216 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 4.18(d, 1H, J = 12.6 Hz), 4.42(d, 1H, J = 12.9 Hz), 7.08(d, 2H, J = 8.4 Hz), 7.30(d, 2H, J = 8.4 Hz), 7.60–7.67(m, 3H), 7.99–8.02(m, 4H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ (ppm): 140.45, 138.83, 132.63, 132.19, 132.12, 129.32, 128.82, 128.34, 128.02, 127.95, 127.71, 127.21, 124.69, 120.56, 59.95; HRMS-ESI (m/z) calcd. for $C_{17}H_{14}ClOS$ $[M + H]^+$: 301.0454, found: 301.0448.

2-((4-Bromobenzyl)sulfinyl)naphthalene (8j). Obtained in 93.3% yield, white solid, m.p. 219–220 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 4.16(d, 1H, J = 12.8 Hz), 4.41(d, 1H, J = 12.8 Hz), 7.03(d, 2H, J = 8.5 Hz), 7.44(d, 2H, J = 8.5 Hz), 7.60–7.67(m, 3H), 8.00–8.03(m, 3H), 8.09(d, 1H, J = 8.5 Hz);

^{13}C-NMR (100 MHz, DMSO-d_6) δ (ppm): 140.46, 132.45, 132.19, 131.23, 130.95, 129.74, 128.83, 128.34, 127.95, 127.71, 127.21, 124.69, 121.25, 120.56, 60.03; HRMS-ESI (*m*/*z*) calcd. for $C_{17}H_{14}BrOS$ $[M + H]^+$: 346.9928, found: 346.9923.

2-Bromo-6-((2-fluorobenzyl)sulfinyl)naphthalene ***(8l).*** Obtained in 70.0% yield, white solid, m.p. 235–237 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 4.27(d, 1H, J = 13.2 Hz), 4.41(d, 1H, J = 13.2 Hz), 7.03–7.33(m, 4H), 7.68–7.75(m, 2H), 7.95–8.07(m, 3H), 8.33(s, 1H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ (ppm): 161.98, 159.53, 141.28, 135.10, 132.85, 132.82, 130.74, 130.59, 130.32, 130.24, 129.95, 129.36, 128.99, 128.09, 124.79, 124.12, 124.09, 121.75, 121.14, 117.20, 117.05, 115.20, 114.99, 54.37; HRMS-ESI (*m*/*z*) calcd. for $C_{17}H_{13}BrFOS$ $[M + H]^+$: 362.9855, found: 362.9849.

2-Bromo-6-((4-fluorobenzyl)sulfinyl)naphthalene ***(8n).*** Obtained in 89.1% yield, white solid, m.p. 195–197 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 4.17(d, 1H, J = 12.9 Hz), 4.41(d, 1H, J = 12.9 Hz), 7.06–7.08(m, 4H), 7.68–7.75(m, 2H), 7.97–8.08(m, 3H), 8.33(s, 1H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ (ppm): 163.10, 160.67, 141.26, 134.93, 132.35, 132.27, 130.74, 130.50, 130.18, 129.89, 127.98, 126.33, 126.30, 124.76, 121.84, 121.00, 115.00, 114.78, 59.74; HRMS-ESI (*m*/*z*) calcd. for $C_{17}H_{13}BrFOS$ $[M + H]^+$: 362.9855, found: 362.9849.

2-Bromo-6-((3-chlorobenzyl)sulfinyl)naphthalene ***(8p).*** Obtained in 86.4% yield, white solid, m.p. 149–150 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 4.18(d, 1H, J = 12.9 Hz), 4.43(d, 1H, J = 12.6 Hz), 6.99(d, 1H, J = 7.6 Hz), 7.14(s, 1H), 7.25(t, 1H, J = 7.6 Hz), 7.33(d, 1H, J = 7.3 Hz), 7.70–7.76(m, 2H), 7.97–8.09(m, 3H), 8.34(s, 1H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ (ppm): 141.15, 134.97, 132.59, 132.55, 130.74, 130.52, 130.21, 130.10, 129.88, 129.79, 128.98, 128.03, 127.70, 124.78, 121.79, 121.04, 59.96; HRMS-ESI (*m*/*z*) calcd. for $C_{17}H_{13}BrFOS$ $[M + H]^+$: 380.9539, found: 380.9527.

4.1.2. General procedure for the synthesis of compounds (9l, 9o and 9t). To an ice cold solution of 7 (1.0 mmol) in 50 mL acetic acid was added 6.0 mmol 30% H_2O_2, then the mixture was heated to 50 °C and stirred for about 2–5 h. After the completion of the reaction (monitored by TLC), the mixture was poured into ice water, and the formed white precipitate was filtered, washed with water and dried under vacuum to get the target compounds (**9**).

2-Bromo-6-((2-fluorobenzyl)sulfonyl)naphthalene ***(9l).*** Obtained in 58.8% yield, white solid, m.p. 130–132 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 4.79(s, 2H), 7.08–7.39(m, 4H), 7.81(t, 2H, J = 8.7 Hz), 8.11–8.14(m, 2H), 8.42(d, 2H, J = 6.7 Hz); ^{13}C-NMR (100 MHz, DMSO-d_6) δ (ppm): 162.07, 159.60, 135.91, 133.30, 131.63, 131.12, 131.04, 130.85, 130.18, 129.97, 129.87, 128.50, 124.41, 124.20, 123.11, 115.92, 115.77, 115.51, 115.30, 54.59; HRMS-ESI (*m*/*z*) calcd. for $C_{17}H_{12}BrFKO_2S$ $[M + K]^+$: 418.9342, found: 418.9339.

2-Bromo-6-((2-chlorobenzyl)sulfonyl)naphthalene ***(9o).*** Obtained in 70.4% yield, white solid, m.p. 136–138 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 4.88(s, 2H), 7.31–7.37(m, 4H), 7.74–7.83(m, 2H), 8.12(t, 2H, J = 8.7 Hz), 8.41(s, 2H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ (ppm): 136.00, 135.96, 134.52, 133.48, 131.65, 130.85, 130.59, 130.24, 130.08, 129.97, 129.48, 128.54, 127.23, 126.56, 124.27, 123.15, 58.16; HRMS-ESI (*m*/*z*) calcd. for $C_{17}H_{12}BrClKO_2S$ $[M + K]^+$: 434.9047, found: 434.9041.

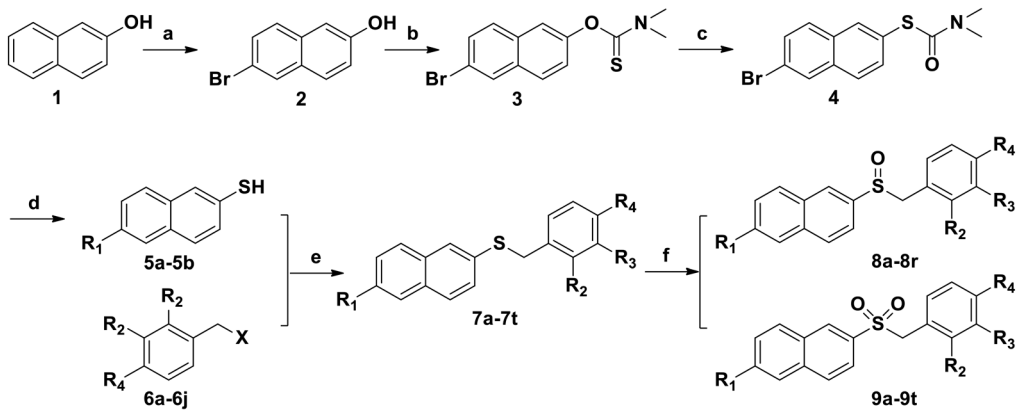
2-Bromo-6-((4-bromobenzyl)sulfonyl)naphthalene ***(9t).*** Obtained in 100% yield, white solid, m.p. 203–205 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 4.80(s, 2H), 7.10(d, 2H, J = 8.5 Hz), 7.49(d, 2H, J = 8.3 Hz), 7.79–7.84(m, 2H), 8.12–8.15(m, 2H), 8.42(m, 2H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ (ppm): 135.43, 135.40, 132.59, 131.12, 130.85, 130.37, 129.69, 129.49, 129.27, 127.99, 127.62, 123.74, 122.56, 121.55, 59.34; HRMS-ESI (*m*/*z*) calcd. for $C_{17}H_{12}Br_2KO_2S$ $[M + K]^+$: 478.8541, found: 478.8551.

Note: Except for some target compounds with better biological activity, the synthesis of all other compounds mentioned in Scheme 1 is described in the ESI.†

4.2. Biological evaluation

4.2.1. Cell culture and irradiation. Human umbilical vein endothelial cells (HUVECs) were used for *in vitro* studies. HUVECs were cultured aseptically in Roswell Park Memorial Institute-1640 (RPMI-1640) supplemented with 10% (v/v) fetal bovine serum (FBS) and penicillin (100 units per mL)/streptomycin (100 μg mL^{-1}) at pH 7.2 and a 5% CO_2 humidified atmosphere at 37 °C. After attaining 80% confluence, the cells were trypsinized with 0.25% trypsin–EDTA and diluted with media to a fixed number of cells. The cell proliferation capacity was determined by the MTS tetrazolium assay in the presence of target compounds, according to the protocol of the manufacturer (Promega, USA). Monolayer cells were incubated with the target compounds for 24 h in 96-well plates before irradiation. The plates were placed under separate Plexiglas covers and irradiated with the needed dose (8.0 Gy for cell survival and 6.0 Gy for the comet assay) at a dose rate of 0.93 Gy min^{-1}. Cell irradiation was done at the ^{60}Co γ-radiation facility of the Beijing Institute of Radiation Medicine, Beijing, China.

4.2.2. Cell survival. The cell survival after irradiation was measured by the standard MTS tetrazolium assay. The cells were seeded into 96-well plates containing the medium at a density of 4000 cells per mL (100 μL per well). The compounds were dissolved in DMSO to a concentration of 100 mM, and diluted in culture medium to the concentrations needed. After 24 h, the cultured cells were treated with the synthesized compounds (40 μM or 20 μM) for 24 h. After 24 h of incubation, the cells were exposed to 8.0 Gy of ^{60}Co γ-irradiation. The cells were continued to be incubated for 4 days after radiation and the supernatant was replaced by fresh medium (100 μL per well) every two days. After 4 days of incubation, the supernatant was replaced by fresh medium (100 μL per well) once more and 10 μL MTS reagent ([3-(4,5-dimethyl thiazol-2-yl)-5-(3-carboxy methoxy phenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt]) was added to each well. The plate was further incubated for 3 h at 37 °C in 5% CO_2. The optical absorbance in individual wells was determined at 492 nm using a Microplate Reader. The inhibition rates were calculated using the following formula. The final



5a:R_1=H	6a:R_2=H, R_3=H, R_4=H	7a, 8a, 9a: R_1=H, R_2= H R_3=H, R_4=H	7k, 8k, 9k: R_1=Br, R_2= H R_3=H, R_4=H
5b:R_1=Br	6b: R_2=F, R_3=H, R_4=H	7b, 8b, 9b: R_1=H, R_2= F, R_3=H, R_4=H	7l, 8l, 9l: R_1=Br, R_2= F, R_3=H, R_4=H
X: F,Cl,Br	6c: R_2=H, R_3=F, R_4=H	7c, 8c, 9c: R_1=H, R_2=H, R_3=F, R_4=H	7m, 8m, 9m: R_1=Br, R_2=H, R_3=F, R_4=H
	6d: R_2=H, R_3=H, R_4=F	7d, 8d, 9d: R_1=H, R_2=H, R_3=H, R_4=F	7n, 8n, 9n: R_1=Br, R_2=H, R_3=H, R_4=F
	6e: R_2=Cl, R_3=H, R_4=H	7e, 8e, 9d: R_1=H, R_2=Cl, R_3=H, R_4=H	7o, 8o, 9o: R_1=Br, R_2=Cl, R_3=H, R_4=H
	6f: R_2=H, R_3=Cl, R_4=H	7f, 8f, 9f: R_1=H, R_2=H, R_3=Cl, R_4=H	7p, 8p, 9p: R_1=Br, R_2=H, R_3=Cl, R_4=H
	6g: R_2=H, R_3=H, R_4=Cl	7g, 8g, 9g: R_1=H, R_2=H, R_3=H, R_4=Cl	7q, 8q, 9q: R_1=Br, R_2=H, R_3=H, R_4=Cl
	6h: R_2=Br, R_3=H, R_4=H	7h, 8h, 9h: R_1=H, R_2=Br, R_3=H, R_4=H	7r, 8r, 9r: R_1=Br, R_2=Br, R_3=H, R_4=H
	6i: R_2=H, R_3=Br, R_4=H	7i, 8i, 9i: R_1=H, R_2=H, R_3=Br, R_4=H	7t, 9t: R_1=Br, R_2=H, R_3=H, R_4=Br
	6j: R_2=H, R_3=H, R_4=Br	7j, 8j, 9j: R_1=H, R_2=H, R_3=H, R_4=Br	

Scheme 1 Reagents and conditions: (a) Br_2, acetic acid, reflux, 3 h; (b) (i) NaH, DMF, 0 °C, 15 min, (ii) dimethyl carbamoyl chloride, 80 °C (2 h), r.t. (15 h); (c) 220 °C, 6 h; (d) KOH, MeOH, 80 °C, 2.5 h; (e) NaOH, EtOH, reflux, 3 h; (f) H_2O_2, acetic acid, r.t. or 40 °C, 4–5 h.

survival rates reported here represent the average of three independent experiments. The survival rate calculation was calculated using GraphPad Prism Software (GraphPad Prism 5, Version 5.01).

Survival rate (%) = $(OD_{sample} - OD_{blank})(OD_{negative\ control*} - OD_{blank}) \times 100\%$.

*The negative control group was composed of normal cultured cells without irradiation.

4.2.3. Single-cell electrophoresis (comet assay). The alkaline comet assay was performed to determine the radioprotective effect of Ex-RAD on DNA damage. The assay was performed according to instructions provided by the manufacturer (OxiSelect™ Comet Assay Kit, Cell Biolabs). Briefly, 24 h after irradiation (6.0 Gy), the cells (control or treated) were seeded at a concentration of 1×10^5 mL^{-1}, combined with comet agarose at a ratio of 1 : 10 (v/v), titrated to mix, and immediately 75 μL per well was onto the 3-well comet slides. The slides were maintained at 4 °C for 15 min in the dark for gel solidification. The slides were then submerged horizontally in the precooled provided lysis buffer in the dark at 4 °C for 30 min. Finally, the slides were submerged in an alkaline buffer (300 mM NaOH and 1 mM EDTA, pH > 13) at 4 °C for 30 min. Electrophoresis was conducted in a fresh chilled alkaline electrophoresis buffer (300 mM NaOH and 1 mM EDTA, pH > 13). The slides were placed side by side in a horizontal electrophoresis apparatus at 1.0 V cm^{-1} at a current of 300 mA for 20 min. After electrophoresis, the slides were dried in 70% ethanol for 5 min, air-dried (1–2 h), and stained for 5 min with vista green DNA dye (1/10 000 dilution of stock supplied by Cell Biolabs). The slides were observed at 400× magnification under an epifluorescence microscope equipped with an excitation filter of 494–521 nm. A total of 50 randomly captured comets from each slide were examined. To quantify the DNA damage, the tail lengths were evaluated. The tail length (length of DNA migration) is related directly to the DNA fragment size and is measured in micrometers. Lengths were calculated and analyzed with Comet Assay Software Project Lab (CASP1.2.3 beta 2). The experiment was repeated three times.

4.2.4. Mice and irradiation. 20–24 g male C57/BL mice were purchased from SPF (Beijing) Biotechnology (China) and were housed five per cage in an air-conditioned facility at the Laboratory Animal Center of Academy of Military Medical Sciences (Beijing, China). The holding rooms for the mice were maintained at a temperature of 25°C with 10–15 hourly cycles of fresh air and a relative humidity of 50% ± 10%. All mice were kept in rooms with a 12 h light/dark cycle with lights on from 08:00 to 20:00. Before starting the experiments, all mice were allowed one week of acclimatization in the facility. Mice were irradiated in well-ventilated Lucite

boxes at a dose rate of 0.62 Gy min^{-1}. After irradiation, the mice were returned to their cages and monitored for 30 day survival. Mice irradiation was done at the ^{60}Co γ-radiation facility of the Beijing Institute of Radiation Medicine, Beijing, China.

Ethical statement: All animal procedures were performed according to a protocol approved by the Committee on the Ethics of Animal Experiments of the Animal Center at the Beijing Institute of Radiation Medicine (IACUC of AMMS 2013022).

4.2.5. Survival study in mice. Mice were divided into four groups: irradiated vehicle-treated (**Vehicle**), irradiated Ex-RAD-treated (**Ex-RAD**), irradiated nylestriol-treated (**Nylestriol**) and irradiated **8n**-treated (**8n**). Each group had 10 mice. Each mouse in group **8n** and **Vehicle** received 0.25 mL of either **8n** (300 mg kg^{-1}) or a vehicle i.p. 24 h and 15 min (two doses) before irradiation. Each mouse in group **Ex-RAD** received 0.25 mL of Ex-RAD (300 mg kg^{-1}) s.c. 24 h and 15 min (two doses) before irradiation. Each mouse in group **Nylestriol** received 0.25 mL of nylestriol (5 mg kg^{-1}) i.g. 24 h (one dose) before irradiation. Compound **8n** and Ex-RAD were suspended in a vehicle consisting of 20% HPCD in normal saline. Nylestriol was suspended in 0.5% CMCNa. All mice were whole-body irradiated with 8.0 Gy. The survival was monitored for 30 days post-radiation.

4.3. Statistical analysis

For data of cell survival, comet assay and mice weight, statistical analysis to find the significance between two groups was performed using the two tailed paired Student's *t*-test, and $p < 0.05$ was taken as statistically significant. Error bars represent ± standard error of mean (SEM). A Kaplan–Meier survival plot was drawn using GraphPad Prism Software. For mice survival data, the Fisher's exact test was used to compare the survival at 30 days and a log-rank test was used to compare survival curves, and $p < 0.05$ was taken as statistically significant. Statistical software (GraphPad Prism 5, Version 5.01) was used for statistical analyses.

Conflicts of interest

The authors declare no conflict of interest.

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References

1 G. I. Reeves, *Crit. Care Clin.*, 1999, **15**, 457–473.
2 T. M. Fliedner, W. Nothdurft and H. Heit, Biological factors affecting the occurrence of radiation syndromes, in *Response of Different Species to Total Body Irradiation*, ed. J. J. Broerse and T. J. MacVittie, Martinas Nijhoff Publishers, Leiden, 1984, pp. 209–219.
3 G. Cakmak, L. M. Miller and F. Zorlu, *et al.*, *Arch. Biochem. Biophys.*, 2012, **520**, 67–73.
4 M. I. Koukourakis, G. Kyrias and S. Kakolyris, *et al.*, *J. Clin. Oncol.*, 2000, **18**, 2226–2233.
5 S. P. Ghosh, M. W. Perkins and K. Hieber, *et al.*, *Radiat. Res.*, 2009, **171**, 173–179.
6 S. Suman, M. Maniar and A. J. Fornace Jr, *et al.*, *Radiat. Oncol.*, 2012, **7**, 6.
7 S. Suman, K. Datta and K. Doiron, *et al.*, *Radiat. Res.*, 2012, **53**, 368–376.
8 S. P. Ghosh, S. Kulkarni and M. W. Perkins, *et al.*, *Radiat. Res.*, 2012, **53**, 526–536.
9 A. D. Kang, S. C. Coscenza and M. Bonagura, *et al.*, *PLoS One*, 2013, **8**, e58355.
10 R. Kumar, Radioprotection and radiomitigation properties of Ex-Rad upon oral administration, *56th Annual Meeting of the Radiation Research Society*, Maui, Hawaii, 2010.
11 A. W. Chun, R. E. Freshwater and D. R. Taft, *et al.*, *Biopharm. Drug Dispos.*, 2011, **32**, 99–111.
12 V. K. Singh, V. L. Newman and P. L. Romaine, *et al.*, *Expert Opin. Ther. Pat.*, 2014, **24**, 1229–1255.
13 K. S. Jagadish, S. Prashant and F. L. Wong, *et al.*, *Phys. Chem. Chem. Phys.*, 2014, **16**, 23320–23328.
14 V. H. Marcelo, M. S. Juan and R. F. Marco, *et al.*, *Bioorg. Med. Chem.*, 2009, **17**, 2452–2460.
15 G. Sergio, H. Roman and W. Songmei, *et al.*, *Eur. J. Org. Chem.*, 2010, 833–845.
16 P. B. Konstantin and P. T. Evgenii, *Eur. J. Org. Chem.*, 2011, 4693–4698.
17 T. Peng, H.-Y. Yan and X.-X. Wen, *et al.*, *Chin. J. New. Drugs.*, 2014, **23**, 1689–1691.