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AS-2, a novel inhibitor of p53-dependent apoptosis, prevents apoptotic mitochondrial dysfunction in a transcription-independent manner and protects mice from a lethal dose of ionizing radiation

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ABSTRACT

In a previous study, we reported that some tetradentate zinc(II) chelators inhibit p53 through the denaturation of its zinc-requiring structure but a chelator, Bispicen, a potent inhibitor of *in vitro* apoptosis, failed to show any efficient radioprotective effect against irradiated mice because the toxicity of the chelator to mice. The unsuitability of using tetradentate chelators as radioprotectors prompted us to undertake a more extensive search for p53-inhibiting agents that are weaker zinc(II) chelators and therefore less toxic. Here, we show that an 8-hydroxyquinoline (8HQ) derivative, AS-2, suppresses p53-dependent apoptosis through a transcription-independent mechanism. A mechanistic study using cells with different p53 characteristics revealed that the suppressive effect of AS-2 on apoptosis is specifically mediated through p53. In addition, AS-2 was less effective in preventing p53-mediated transcription-dependent events than pifithrin-μ (PFTμ), an inhibitor of transcription-independent apoptosis by p53. Fluorescence visualization of the extranuclear distribution of AS-2 also supports that it is ineffective on the transcription-dependent pathway. Further investigations revealed that AS-2 suppressed mitochondrial apoptotic events, such as the mitochondrial release of intermembrane proteins and the loss of mitochondrial membrane potential, although AS-2 resulted in an increase in the mitochondrial translocation of p53 as opposed to the decrease of cytosolic p53, and did not affect the apoptotic interaction of p53 with Bcl-2. AS-2 also protected mice that had been exposed to a lethal dose of ionizing radiation. Our findings indicate that some types of bidentate 8HQ chelators could serve as radioprotectors with no substantial toxicity *in vivo*.

1. Introduction

p53, a multifunctional protein that has a role in DNA damage response associated with the cell's decision between apoptosis and other fates, has been extensively studied [1,2]. However, the acute response by p53 clearly induces "unnecessary apoptosis" that does not contribute to tumor suppression [3]. Thus, p53 is considered to be a target for therapeutic and mitigative radioprotection for suppressing the excess apoptosis that is observed in highly radiosensitive organs, and chemical p53 inhibitors would be expected to selectively prevent the damage of normal tissues during radio (chemo) therapy for p53-deficient tumors [4].

Three radioprotective p53 inhibitors have been reported, namely, pifithrin-α (PFTα), pifithrin-μ (PFTμ), and sodium orthovanadate (vanadate) [5–11]. Among the three radioprotective p53 inhibitors, the highest radioprotective efficacy in total-body-irradiated mice was produced by vanadate, which has a unique activity in inducing the denaturation of p53 relative to PFTα and PFTμ. We therefore postulated that the activity of vanadate is what should be responsible for its potent radioprotective activity. On the other hand, the p53 protein is a zinc(II)-requiring protein that can be denatured and inactivated by removing zinc(II) ions from p53 using zinc(II) chelators [12–17]. Thus, in a previous study, we evaluated several multidentate zinc(II) chelators

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(>tridentate), and two tetradentate zinc(II) chelators were then found to suppress p53-dependent apoptosis in irradiated MOLT-4 cells [11]. Bispicen (*N,N′*-Bis(2-pyridylmethyl)-1,2-ethanediamine) was found to be highly efficacious in terms of inhibiting apoptosis, showing activity for p53 denaturation as well as on the inhibition of both the transcription-dependent and -independent apoptotic pathways [11,18–21].

This is the first study to demonstrate that certain types of zinc(II) chelators can be used to inhibit p53-mediated apoptosis in irradiated cultured cells. However, because the highest dose of Bispicen tolerable in mice was substantially lower than the dose required to suppress p53-dependent apoptosis in cultured cells, it cannot be considered to be unsuitable for rescuing mice that had been exposed to a lethal dose of ionizing radiation [11]. In order to reduce the cytotoxic effects, we conducted a more extensive search for weaker zinc(II) chelators that are less toxic. As candidates for p53-inhibiting zinc(II) chelators, we synthesized a series of 8-hydroxyquinoline (8HQ) derivatives that function as bidentate ligands [22–24], and evaluated their anti-apoptotic activities in irradiated MOLT-4 cells. As a result, some 8HQ derivatives showed anti-apoptotic activity and had a low cytotoxicity [22].

The findings reported in this study show that a bidentate 8HQ derivative, AS-2 (5,7-bis(*N*-methylaminosulfonyl)-8-hydroxyquinaldine), suppressed p53-dependent apoptosis through a p53-dependent, transcription-independent mechanism with its characteristic extranuclear localization, and protected mice from a dose of radiation sufficient to cause the lethal hematopoietic syndrome. The transcription-independent mechanism exerts some mitoprotective effects in spite of the increase in mitochondrial p53 levels. These results suggest that AS-2 disables certain extranuclear functions of p53.

2. Materials and methods

2.1. Cell culture and treatment

MOLT-4 cells and their derivative transformed cell lines (MOLT/Nega, MOLT/p53KD-1, and MOLT/p53KD-1/R-p53-1), IM-9, KU812, CCRF-CEM, and U937 cells were cultured in RPMI 1640 medium (Wako) supplemented with 10% fetal bovine serum (FBS; Sigma) and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin (Nacalai Tesque)) [8,11]. The medium was also supplemented with 0.5 mg/ml G418 (Enzo Life Sciences) and/or 0.25 mg/ml Hygromycin B (Wako) in order to maintain stable transformants. The human cervical carcinoma cell line HeLa cells were cultured in DMEM/F12 medium (Wako) supplemented with 10% FBS and antibiotics. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2, and irradiated at room temperature with an X-ray generator (MBR-1520R-3, Hitachi) operating at 150 kV–20 mA with a filter of 0.3 mm Cu and 0.5 mm Al at a dose rate of 1.6 Gy/min, or were treated with etoposide (Wako). AS-2 was synthesized and purified as described in a previous report [22]. AS-2 or PFTμ (StressMarq) was added to the culture medium 1 h before irradiation (IR) or the etoposide treatment. The protein concentrations of all the protein samples were determined using the BCA Protein Assay Reagent (Thermo Fisher Scientific) and equalized.

2.2. Apoptosis assay

Cell viability was determined by the WST-8 reduction assay (Cell counting kit-8; Dojindo) or the erythrosin B dye-exclusion test. The percentage of cells losing mitochondrial membrane potential ($\Delta\psi m$) was measured by MitoTracker Red CMXRos (Molecular Probes) staining with a flow-cytometer (FACS Calibur, Becton Dickinson) as described previously [8]. Statistical significance was determined by one-way analysis of variance (*F*-test) followed by individual two-tailed *t*-test by use of the statistics module of Microsoft Excel 2011 for Mac software (Microsoft).

2.3. Immunoblotting analysis

Immunoblotting was performed essentially as described in a previous report [25]. We used the following antibodies as primary antibodies: caspase-3 (ab90437, abcam), caspase-7 (clone 4G2, MBL), β-actin (clone AC-15, Sigma), p53 (clone DO-1, sc-126 HRP, Santa Cruz Biotechnology), PUMA (Ab-1, Calbiochem), p21 (clone EA10, Calbiochem), Bcl-2 (clone Bcl-2/100, sc-509 HRP, Santa Cruz Biotechnology), COX IV (4850, Cell Signaling), Apaf-1 (clone 24, BD Transduction Laboratories), cytochrome *c* (Cyt. *c*; clone 6H2.B4, BD Pharmingen), or Smac/DIABLO (Smac; PM004, MBL).

2.4. Analysis of nuclear p53 response

Quantitative real-time reverse transcription-PCR analyses were performed on an Applied Biosystems 7500 real-time PCR system as described previously [11]. Chromatin immunoprecipitation (ChIP) assay was performed as described previously using a ChIP assay kit (Millipore) [8].

2.5. Fluorescence microscopy

The fluorescence properties of AS-2 were analyzed using a spectrofluorometer (FP-6500, JASCO). HeLa cells were plated and cultured overnight in an 8-well chamber slide (SCS-008, Matsunami). The cells were then treated with 300 μM AS-2 for 7 h, washed once with PBS, sealed with a glass coverslip, and observed by fluorescence microscopy (BZ-9000, Keyence).

2.6. Subcellular fractionation

MOLT-4 cells were homogenized and fractionated into the cytosolic fraction and the heavy membrane fraction, as described previously, using a cell fractionation kit involving syringe homogenization and differential centrifugation (WSE-7422, EzSubcell Fraction, ATTO) [26]. Cytosolic extraction to measure the release of Cyt.*c* and Smac was performed using a detergent-based cell fractionation kit (ab109719, Cell fractionation kit standard, abcam).

2.7. Immunoprecipitation

Immunoprecipitation was performed with an anti-Bcl-2 antibody (clone 7/Bcl-2, BD Transduction Laboratories) as described previously [8]. In this immunoprecipitation method, control normal mouse IgG does not precipitate any detectable levels of Bcl-2 or p53 [9].

2.8. Total-body irradiation (TBI)

Imprinting control region (ICR) female mice (SLC, Inc.), aged 8 weeks, were irradiated with an X-ray generator (Pantak-320S, Shimadzu) operated at 200 kV–20 mA at a dose rate of 0.66 Gy/min. Mice were i.p.-injected with vehicle (20% DMSO in olive oil) or 80 mg/kg AS-2 30 min before TBI. All experimental protocols involving mice were reviewed and approved by the Animal Care and Use Committee of the National Institute of Radiological Sciences (NIRS), and were performed in strict accordance with the NIRS Guidelines for the Care and Use of Laboratory Animals.

3. Results

3.1. AS-2 exerted anti-apoptotic activity in a p53-dependent manner

Fig. 1A shows the chemical structure of AS-2. Basically, 8HQ derivatives bind various metal ions including zinc(II) ions through coordination bonds from the nitrogen atom at 1-position and the phenolate oxygen of the quinoline ring. We synthesized several 8HQ derivatives and found that 8HQ analogues, which contained two methylaminosulfonyl groups at the 5- and 7-positions of the quinoline ring, had the optimal combination of antagonistic activity against radiation-induced MOLT-4 apoptosis and a low cytotoxicity. Among the 8HQ derivatives, AS-2 was found to have a relatively high potency for inhibiting apoptosis at high doses [22] (80–320 μM; Fig. 1B). The high-dose requirement can be attributed to the low permeability of AS-2. As a matter of fact, AS-2 has three (week) acidic protons, whose pK_{a1}, pK_{a2}, and pK_{a3} values were determined to be 6.1, 10.8, and >12, respectively, by potentiometric pH titration (see Supplementary Fig. S1). The pK_a value for the 8-OH group is almost same as that of the similar analog of AS-2 [27]. These three pK_a values imply that over 90% of AS-2 exists as monoanionic ($(AS\text{-}2)^-$) or dianionic ($(AS\text{-}2)^{2-}$) form at neutral pH, which should go across the cell membrane less efficiently than its neutral form. The anti-apoptotic activity of AS-2 was also demonstrated by its inhibitory effects on caspase activation (Fig. 1C).

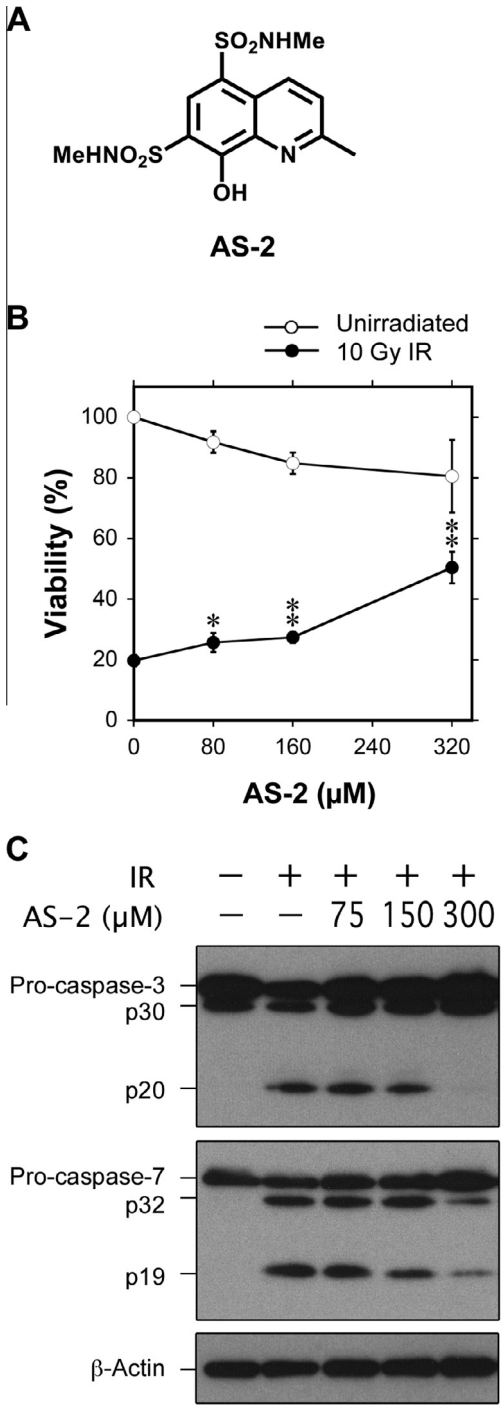

Fig. 1. AS-2 partially suppresses apoptosis and caspase activation in irradiated MOLT-4 cells. (A) Chemical structure of AS-2. (B) Dose response of AS-2 on apoptosis. Cell viability was determined by a WST-8 assay 20 h after 10 Gy-IR. Data shown are the means ± SD from at least three independent experiments. Asterisks denote statistically significant increases in viabilities of irradiated cells relative to those in irradiated control without AS-2: $^{**}P < 0.01$; $^{*}P < 0.05$. (C) Caspase activations were detected by immunoblotting. β-Actin was used as an internal control. Cells were harvested 10 h after 10 Gy-IR.

We next confirmed and characterized the effects of AS-2 on p53-dependent and -independent apoptosis using several cell systems. Fig. 2A shows the effect of AS-2 on etoposide-induced apoptosis in cultured leukemia or lymphoma cell lines, each with a different p53 status, IM-9 (wt-p53), KU812 (K132R), CCRF-CEM (R175H/R248Q), and U937 (a 46-base deletion at the 3′ end of exon 5 of TP53) [28–30]. Significant suppression of apoptosis by AS-2 was observed only in wt-p53-bearing IM-9 cells (approximately a 20% increase at 300 μM relative to etoposide-treated controls without AS-2, $P < 0.05$), while no protective effect was observed in any of the p53-impaired cell lines. Furthermore, in an analysis of the various MOLT-4 shRNA transformants [8,11], the anti-apoptotic activity of AS-2 was limited to cells expressing p53 or shRNA-resistant p53, but not the p53-knockdown KD-1 cells that showed partial death after IR (Fig. 2B). These data strongly suggest that the suppression of DNA-damage-induced apoptosis by AS-2 is specifically mediated via p53.

3.2. AS-2 does not significantly affect the nuclear p53 response

To further investigate the p53-dependent anti-apoptotic mechanism by AS-2, we evaluated the effects of AS-2 on p53-mediated nuclear events in irradiated MOLT-4 cells. We initially examined the effects of AS-2 on p53 transactivation after IR. p53 transactivation was assessed by the mRNA and protein expression of p53 target gene products, PUMA and p21. As presented in Fig. 3A, although p21 mRNA expression tended to be decreased by AS-2, AS-2 did not significantly suppress the upregulation of mRNA ($P > 0.05$). On the other hand, PFTμ, an inhibitor of the p53-mediated transcription-independent pathway, significantly suppressed p21 mRNA expression at a concentration of 10 μM ($P < 0.05$). The optimal concentration of PFTμ for irradiated MOLT-4 cells was 10 μM, as assessed by a cell viability measurement (Fig. 3B). AS-2 also did not show any suppressive effect against the protein expression of p53 or its target gene products (Fig. 3C). Moreover, a ChIP assay revealed that AS-2 had no effect on the intranuclear DNA-binding of p53 to the p53 target gene promoters, while PFTμ partially suppressed this binding, in addition to the binding of RNA polymerase II (RNA pol II) to the gapdh promoter, which appears to be a side effect of PFTμ (Fig. 3D). These data clearly indicate that AS-2 is a less effective compound in terms of inhibiting the transcription-dependent pathway than PFTμ, and that it is not so effective in suppressing the gene expression of p53 and p53 target genes.

To support this conclusion, we next investigated the intracellular localization of AS-2 by fluorescence microscopy using HeLa cells as a representative cultured adherent cells in order to visualize the intracellular structure more clearly, because AS-2 is a fluorescence-emitting compound when excited by UV light (see Supplementary

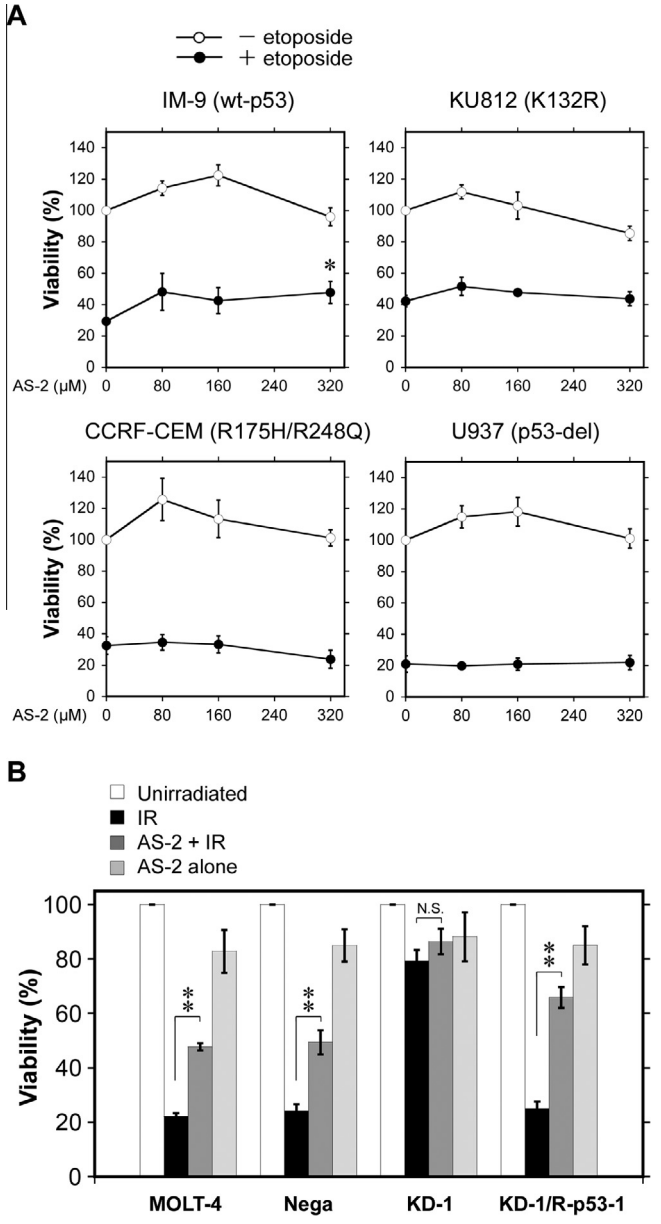

Fig. 2. p53 is required for the suppression of DNA damage-induced apoptosis by AS-2. Data shown are the means ± SD from at least three independent experiments. Asterisks denote statistically significant increases in viabilities of apoptosis-stimulated cells: $^{**}P < 0.01$; $^{*}P < 0.05$; N.S., Not significant. (A) Effect of AS-2 on etoposide-induced apoptosis in cultured leukemia or lymphoma cell lines with different p53 status. IM-9, KU812, CCRF-CEM, and U937 cells were treated with 1 μM, 50 μM, 2.5 μM, and 5 μM, respectively. Cell viability was determined by a WST-8 assay 20 h after the addition of etoposide. (B) The effect of 300 μM AS-2 on parental MOLT-4 cells and the various MOLT-4 transformants after IR. Nega, negative shRNA-transfected control cells; KD-1, p53-knockdown cells; KD-1/R-p53-1, p53 shRNA-resistant, silent mutated FLAG-p53-transfected KD-1 cells. Cell viability was determined by a WST-8 assay 20 h after 10 Gy-IR.

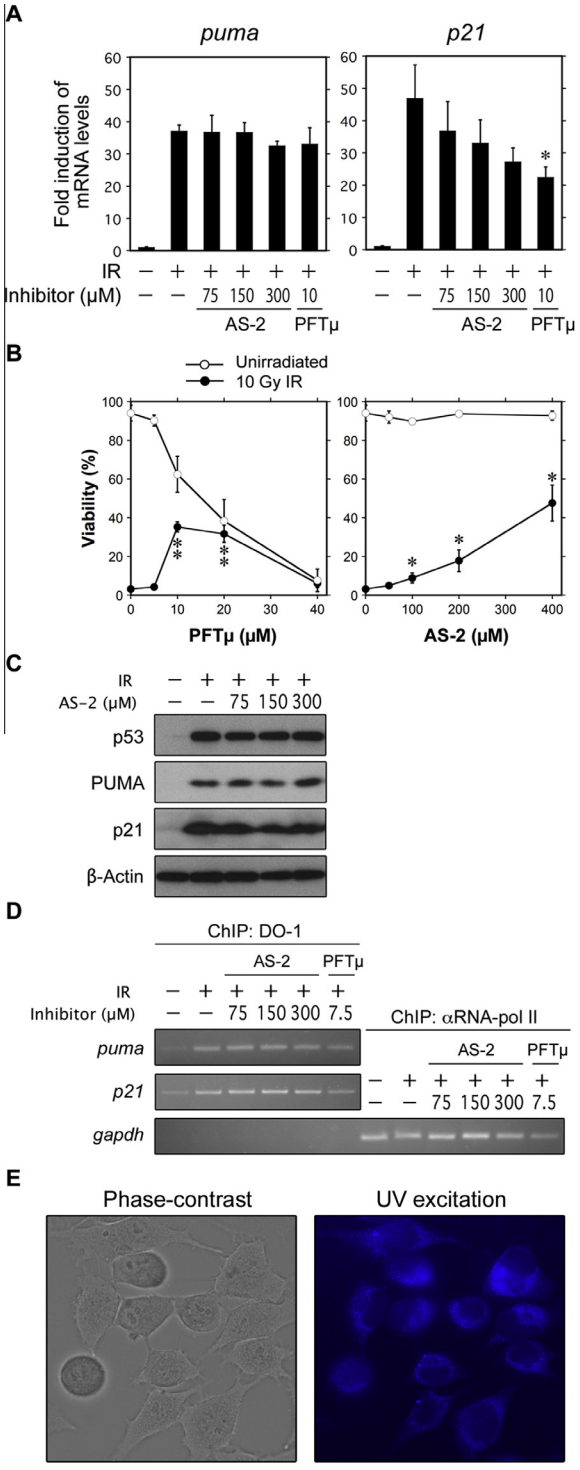

Fig. 3. AS-2 has a negligible effect on p53-mediated nuclear events in irradiated MOLT-4 cells. (A and B) Data shown are the means ± SD from at least three independent experiments. Asterisks denote statistically significant differences in irradiated cell samples relative to irradiated control without inhibitor: $^{**}P < 0.01$; $^{*}P < 0.05$. (A) Real time-PCR analysis of transcription of *puma* and *p21* in the absence or presence of indicated concentrations of AS-2 or PFTμ. Cells were harvested 6 h after 10 Gy-IR. (B) Dose response of PFTμ or AS-2 on the apoptosis quantified by a dye-exclusion test (20 h after 10 Gy-IR). (C) Dose–response of AS-2 on the accumulation of p53 and the induction of p53 target gene products, PUMA and p21. Cells were harvested 6 h after 10 Gy-IR, and the proteins were detected by immunoblotting. (D) Effect of AS-2 or PFTμ on the DNA-binding of nuclear p53 to the *p21*, *puma*, and *gapdh* (as a negative control) promoters was analyzed by ChIP assays. DNA-binding of RNA pol II to the *gapdh* promoter was used as an internal control. (E) Fluorescence visualization of AS-2 in HeLa cells under UV excitation.

Fig. S2). AS-2 was clearly located in the extranuclear space, as evidenced by its UV-excited blue emission, strongly supporting the conclusion that it has no effect on p53-mediated nuclear events (Fig. 3E).

3.3. Mitoprotective and in vivo radioprotective effects of AS-2

Finally, we investigated the effect of AS-2 on the mitochondrial apoptotic events in irradiated MOLT-4 cells. We first analyzed the

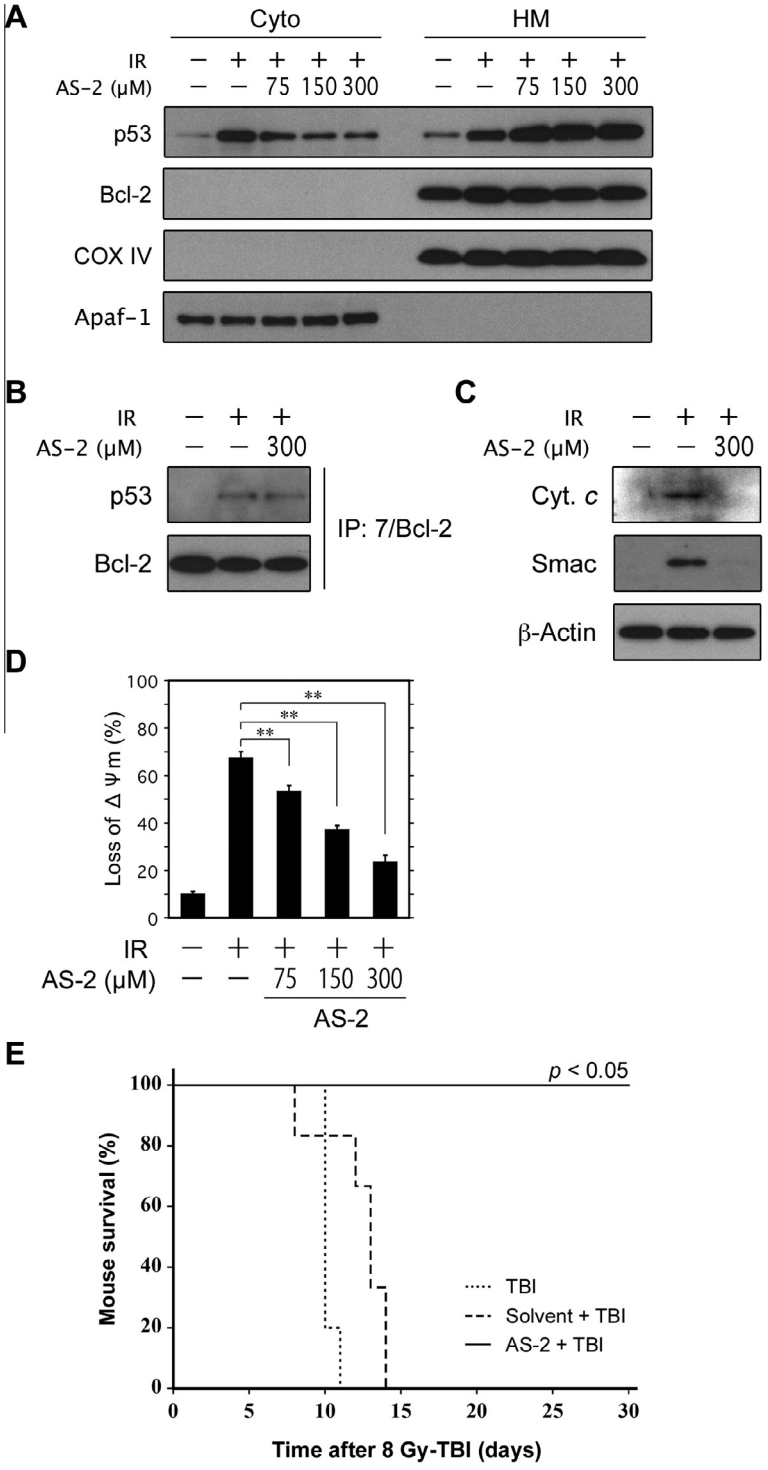

Fig. 4. AS-2 prevents apoptotic mitochondrial dysfunction and protects mice from a lethal dose of ionizing radiation. (A) MOLT-4 cells were homogenized and fractionated into cytosolic (Cyto) and heavy membrane (HM) fractions by differential centrifugation 6 h after 10 Gy-IR, and then subjected to immunoblotting analysis of p53. Bcl-2 and COX IV were used as mitochondrial markers. Apaf-1 was used as a cytosolic marker. (B) Immunoprecipitation (IP) of Bcl-2 and immunocoprecipitation of p53 in irradiated MOLT-4 cells (6 h after 10 Gy-IR). (C) Effect of AS-2 on the release of mitochondrial proapoptotic molecules into the cytosol in irradiated MOLT-4 cells (7 h after 10 Gy-IR). Cytosolic proteins were detected by immunoblotting. (D) The loss of $\Delta\psi m$ was measured by flow cytometry after MitoTracker staining. MOLT-4 cells were harvested 12 h after 10 Gy-IR. Asterisks denote statistically significant differences: $^{**}P < 0.01$. (E) Thirty-day survival tests of ICR mice subgroups of 8 Gy-TBI alone (five mice), vehicle plus 8 Gy-TBI (six mice), and AS-2 plus 8 Gy-TBI (three mice). ICR mice were i.p.-injected with vehicle or 80 mg/kg AS-2 30 min before TBI. *P*, comparison between the AS-2-treated and untreated subgroups (Chi-square test).

effect of AS-2 on the translocation of p53 to mitochondria, a key initial event in this pathway [18–21]. Surprisingly, AS-2 did not block the translocation, but caused a dose-dependent increase, as assessed by the amount of p53 in the heavy membrane fraction that mainly contained mitochondrial components [26], whereas the level of cytosolic p53 was markedly decreased by AS-2 (Fig. 4A). Despite the increase in mitochondrial p53 levels, AS-2 did not suppress the interaction of p53 with Bcl-2, which is

thought to be required for the direct initiation of transcription-independent apoptosis [18,19] (Fig. 4B), suggesting that AS-2 does not inhibit the typical events of the transcription-independent pathway. However, AS-2 was highly effective in inhibiting mitochondrial apoptotic dysfunction, as evidenced by the release of mitochondrial proapoptotic molecules, Cyt. c and Smac, into the cytosol (Fig. 4C), and the loss of $\Delta\psi m$ (Fig. 4D). The mitoprotective effects appear to be responsible for the anti-apoptotic activity of AS-2.

As a trial to evaluate the in vivo radioprotective efficacy of AS-2, we performed 30-day survival tests using ICR mice after 8 Gy-TBI, which induces the hematopoietic syndrome in these mice [9]. The dosage of AS-2 (80 mg/kg with vehicle), which was delivered i.p. into ICR mice, produced no apparent harmful effects, as assessed in our pilot study. In the radioprotection assays, all of the AS-2-treated mice survived, while all of the control mice that had been treated with 8 Gy alone or vehicle plus 8 Gy died within 2 weeks (Fig. 4E). The only observable change in the surviving mice is a slight decrease without statistical significance in body weight gain compared to that of the untreated control. Considering the additional information that AS-2 did not protect mice from a superlethal dose of 9 Gy-TBI (data not shown), the radioprotective efficacy of AS-2 is limited to approximately ~8 Gy-TBI-induced acute radiation syndrome.

4. Discussion

The findings reported here show that a bidentate 8HQ derivative, AS-2, which is characteristically localized in the extranuclear space, prevents apoptotic mitochondrial dysfunction in a transcription-independent manner, and protects mice from a dose of radiation sufficient to cause the lethal hematopoietic syndrome. The transcription-independent radioprotective mechanism can be attributed to its mitoprotective effects that override the interaction of p53 with Bcl-2 and the increase in mitochondrial p53 levels, which are thought to be essential for the initiation of the typical transcription-independent pathway [18–21].

The disablement of the initiation processes by p53 suggests that AS-2 neutralizes certain extranuclear apoptotic functions of p53 [31]. However, our analysis of circular dichroism (CD) spectroscopic data, which is capable of detecting the conformational denaturation of p53, and an electrophoretic mobility shift assay (EMSA), which measures the DNA-binding activity of p53 [11] revealed that AS-2 has no direct effect on either the conformation or the DNA-binding properties of p53 [22]. These results indicate that AS-2 does not directly affect the conformation of p53. Possibly the appropriate coordination-property of 8HQ derivatives without eliminating zinc(II) ions [23,24] may be responsible for the ineffectiveness of AS-2 on conformational changes. However, this feature of AS-2 might be sufficiently effective so as to interfere some type of extranuclear p53 interaction that is involved in initiation processes of the mitochondrial transcription-independent pathway.

Alternatively, the disablement can be explained by the simple blocking of the mitochondrial release of intermembrane proteins by AS-2-protected mitochondria. In this case, AS-2 would be predicted to function as an inhibitor of mitochondrial dysfunction rather than an inhibitor of mitochondrial p53. However, this mitochondrial protection is probably not due to the direct regulation of mitochondrial redox, because AS-2 has no obvious radical scavenging activity [22]. In this respect, the effect of AS-2 on decreasing cytosolic p53 levels is probably linked to its extranuclear localization, and might be involved in the protection of mitochondria (Fig. 4A). Since cytosolic p53 inhibits autophagy [32], a decrease in cytosolic p53 levels would likely shift the balance away from apoptosis towards autophagy, which has pro-survival effects [33]. However, this model cannot explain the ineffectiveness of AS-2 on the apoptosis observed in the case of DNA-binding domain (DBD)-mutant cell lines (Fig. 2B; KU812 and CCRF-CEM), because DBD is not responsible for the inhibition of autophagy by p53 [34]. These two cell lines expressed substantial amounts of DBD-mutant p53 [9]. It is reasonable to assume that the autophagy-facilitating model is not an adequate explanation for the anti-apoptotic effects of AS-2. The cytosolic p53 might have an unknown function that directly induces cell death. Further studies will clearly be needed in order to completely elucidate the p53-dependent mechanisms for AS-2 that affects extranuclear p53, and inhibits mitochondria-mediated apoptosis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.07.037.

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