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A chemical modulator of p53 transactivation that acts as a radioprotective agonist

Akinori Morita[1,2,*], Ippei Takahashi[1,3,*], Megumi Sasatani[1], Shin Aoki[4,5], Bing Wang[6], Shinya Ariyasu[5], Kaoru Tanaka[6], Tetsuji Yamaguchi[1], Akiko Sawa[4], Yurie Nishi[4], Tatsuro Teraoka[4], Shohei Ujita[2], Yosuke Kawate[2], Chihiro Yanagawa[2], Keiji Tanimoto[1], Atsushi Enomoto[7], Mitsuru Nenoi[6], Kenji Kamiya[1], Yasushi Nagata[3], Yoshio Hosoi[1,8], Toshiya Inaba[1]

[1]Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima 734-8553, Japan.
[2]Department of Biomedical Science and Technology, Graduate School of Biomedical Sciences, Tokushima University, Tokushima 770-8503, Japan.
[3]Department of Radiation Oncology, Hiroshima University, Hiroshima 734-8553, Japan.
[4]Department of Medicinal and Life Science, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Chiba 278-8510, Japan.
[5]Center for Technologies against Cancer, Tokyo University of Science, Chiba 278-8510, Japan.
[6]National Institute of Radiological Sciences, National Institutes for Quantum and Radiological Science and Technology, Chiba 263-8555, Japan.
[7]Laboratory of Molecular Radiology, Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo 113-0033, Japan
[8]Department of Radiation Biology, Graduate School of Medicine, Tohoku University, Sendai 980-8575, Japan.

*These authors contributed equally to this work.

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Corresponding Author:
Akinori Morita, Department of Biomedical Science and Technology, Graduate School of Biomedical Sciences, Tokushima University, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan. Phone & Fax: 81-88-633-9052; E-mail: morita@tokushima-u.ac.jp.

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Abstract:

Inhibiting p53-dependent apoptosis by inhibitors of p53 is an effective strategy for preventing radiation-induced damage in hematopoietic lineages, while p53 and p21 also play radioprotective roles in the gastrointestinal epithelium. We previously identified some zinc(II) chelators, including 8-quinolinol derivatives that suppress apoptosis in attempts to discover compounds that target the zinc-binding site in p53. We found that 5-chloro-8-quinolinol (5CHQ) has a unique p53-modulating activity that shifts its transactivation from proapoptotic to protective responses including enhancing p21 induction and suppressing PUMA induction. This p53-modulating activity also influenced p53 and p53-target gene expression in unirradiated cells without inducing DNA damage. The specificity of 5CHQ for p53 and p21 was demonstrated by silencing the expression of each protein. These effects seems to be attributable to the sequence-specific alteration of p53 DNA-binding, as evaluated by chromatin immunoprecipitation and electrophoretic mobility shift assays. In addition, 5-chloro-8-methoxyquinoline itself had no antiapoptotic activity, indicating that the hydroxyl group at the 8-position is required for its antiapoptotic activity. We applied this remarkable agonistic activity to protecting the hematopoietic and gastrointestinal system in mouse irradiation models. The dose-reduction factors of 5CHQ in total-body and abdominally irradiated mice were about 1.2 and 1.3, respectively. 5CHQ effectively protected mouse epithelial stem cells from a lethal dose of abdominal irradiation. Furthermore, the specificity of 5CHQ for p53 in reducing the lethality induced by abdominal irradiation was revealed in *Trp53*-KO mice. These results indicate that the pharmacological upregulation of radioprotective p53-target genes is an effective strategy for addressing the gastrointestinal syndrome.

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Introduction

Apoptosis-regulating compounds have been developed for use as radioprotectors (1). These drugs are expected to prevent damage to normal tissues during radio (chemo) therapy as well as that caused by a nuclear disaster. The vital radiosensitive tissues are bone marrow (BM) and the gastrointestinal (GI) tract, albeit the sensitivities are different. Death caused by a total-body exposure in excess of 10 Gy is generally referred to as the GI syndrome or GI death. The death occurs within approximately 2 weeks after exposure and is associated with extensive bloody diarrhea and the destruction of the GI mucosa. At lower doses of ionizing radiation, death occurs within several weeks (rodents) to 2 months (humans) after exposure and is caused by radiation injury of the hematopoietic (HP) system; this mode of death is called BM death or the HP syndrome (2), whereas the HP syndrome, at least in part, can be cured by appropriate treatment such as antibiotic therapy or by shielding part of the BM (2-6).

In addition, death by around 12 Gy is probably due to a combination of BM and GI death, because BM transplantation has been shown to be effective at these doses (6, 7). The radiosensitivity of BM stem cells is associated with their sensitivity to apoptosis, because BM-specific genetically engineered mice that lack both of the proapoptotic genes, *Bax* and *Bak1* in hemangioblast-derived tissues, are resistant to 12.5 Gy γ-irradiation (IR); however, these mice failed to show any resistance to more severe abdominal radiation injuries caused by shielding the BM of the front legs for sub-total-body IR (SBI) (3). These results imply that GI death proceeds via two modes: BM injury-associated or not. Other important demonstrations in SBI models are that p53 and p21 play radioprotective roles in the GI epithelium (3). Based on these observations, the efficacy of antiapoptotic radioprotectors is thought to be limited to apoptosis-prone HP lineages. Indeed, several potent antiapoptotic radioprotectors such as some innate immune agonists and sodium orthovanadate, have been reported to be effective for the lower dose GI syndrome of around 12 Gy, but not at higher doses (8-10), suggesting limited efficacy against the GI syndrome, although these innate immune agonists inherently possess some protective effects on GI epithelium in addition to their antiapoptotic effects on HP lineages (8, 9). Thus, strengthening the anti-cell death function of p53 that enables GI epithelial cells to survive (11) would be an ideal pharmacological

approach to radioprotection against the GI syndrome, which could be readily applicable for cancer therapy without any concern for carcinogenic risk if a p53 inhibitor were to be used, although the risk is thought to be low (12).

In a recent study, we reported that some zinc(II) chelators, including 8-quinolinol (8-HQ) derivatives, suppress radiation-induced p53-dependent apoptosis (13-16). These findings were the result of our attempts to discover compounds that target the zinc-binding site (ZBS) in p53 (17-20). These 8-HQ derivatives function as bidentate ligands for zinc(II) ions, and some of them were found to be less toxic than zinc(II)-removing chelators such as Bispicen and TPEN (13, 14). The bioavailability of 8-HQ derivatives is demonstrated by a derivative, AS-2, which suppress p53-dependent apoptosis and protects mice from radiation-induced BM death (15). Among these 8-HQ derivatives, 5-chloro-8-quinolinol (5CHQ; Fig. 1A), compound 44 in Ref. 14, was chosen in this study due to its potent radioprotecting activity at low doses against p53 transactivation, although cytotoxicity was observed at high doses.

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Materials and Methods

Cell culture and treatment

Wild-type (wt)-p53-bearing human T-cell leukemia MOLT-4 cells (21-25) and their derivative transformed cell lines (Nega, KD-1, KD-1/ Hygro, and KD-1/R-p53-1) (26, 13), and p21KD-1 cells (a stable p21-knockdown MOLT-4 transformant established as described in Supplementary Methods) and control shRNA-transformant were cultured in RPMI 1640 medium (Wako, Japan) supplemented with 10% fetal bovine serum (FBS; Sigma) and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin (Nacalai Tesque, Japan)). Wt-p53-bearing murine FM3A cells (subclone F28-7), originally established from a spontaneous mammary carcinoma in a C3H/He mouse (27, 28) were a gift from Dr. Hideki Koyama (Yokohama City University), and confirmed to be mycoplasma-free (JCRB). FM3A cells were cultured in E-MEM medium (Wako) supplemented with 10% FBS and antibiotics. MOLT-4 cells were a gift from Dr. Jun Minowada (Roswell Park Memorial Institute), confirmed to be mycoplasma-free (JCRB), and authenticated using STR profiling service (BEX, Japan). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and irradiated at room temperature with a ^{137}Cs γ-ray source (Gammacell 40 Exactor, Nordion International, Canada) at a dose rate of 0.90 Gy/min or an X-ray generator (MBR-1520R-3, Hitachi, Japan) operating at 150 kV–20 mA with a 0.3 mm Cu and 0.5 mm Al filter at a dose rate of 1.6 Gy/min, or treated with etoposide (Wako). 5CHQ and chloroquine diphosphate (chloroquine) were purchased from Wako, and recrystallized from ethanol before the biological assay. This recrystallization is important to reduce the toxicity of the commercial product of 5CHQ. The melting point of the recrystallized 5CHQ was 127-128 °C, which is identical to its reported value of 130 °C (29). 5-Chloro-8-methoxyquinoline (5CMQ) was synthesized and purified as described in Supplementary Methods. Each compound was added to the culture medium 1 h before irradiation (IR) or the etoposide treatment.

cDNA sequencing of the *TP53* gene in MOLT-4 cells

MOLT-4 cells were confirmed to express wt-p53 by cDNA sequencing. For more details, see Supplementary Methods.

Apoptosis assay

For more details, see Supplementary Methods.

Statistical analysis

Statistical significance was determined by the two-tailed *t*-test using the statistics module of Microsoft Excel:mac 2011 (Microsoft) assuming homoscedasticity, except for the mouse survival tests in the radioprotection assays, the statistical significance of which was determined by the Chi-square test.

Immunoblotting analysis

Immunoblotting was performed essentially as described in a previous report (30). For more details, see Supplementary Methods.

Quantitative PCR (Q-PCR) analysis and Chromatin immunoprecipitation (ChIP) assays

For more details, see Supplementary Methods.

Electrophoretic mobility shift assay (EMSA)

For more details, see Supplementary Methods.

Circular dichroism (CD) spectroscopy

CD spectra were obtained, as described previously (13, 14).

DPPH free radical scavenging assay

The DPPH free radical assay was performed, as described previously (14).

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Cell cycle analysis

For more details, see Supplementary Methods.

Maximum tolerated dose (MTD) study and Total-body irradiation (TBI)

Mice were intraperitoneally (i.p.) injected with vehicle (20% DMSO in olive oil) or the indicated concentrations of 5CHQ, for MTD study or 30 min before TBI. The Imprinting control region (ICR) female mice (SLC, Inc.), at 8 weeks of age, were irradiated with an X-ray generator (Pantak-320S, Shimadzu) operating at 200 kV‐20 mA with a filter of 0.5 mm Cu and 0.5 mm Al at a dose rate of 0.66 Gy/min. All TBI protocols 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. For more details, see Supplementary Methods.

Subtotal-body irradiation (SBI) and histologic evaluation of epithelial survival

SBI was performed essentially as described by Kirsch *et al.* (3). ICR female mice were purchased from CLEA JAPAN, Inc.. To demonstrate the generality of the radioprotective effect of 5CHQ and to compare wild-type mice with *Trp53*-deficient mice in the same genetic background, C57BL/6 mice (Charles River Laboratories, Japan) were also tested. *Trp53*-deficient mice (31) were generated by crossing C53BL/6-*Trp53*$^{+/-}$ mice (Accession No. CDB 0001K), provided by RIKEN BRC. For more details, see Supplementary Methods.

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Results

5CHQ suppresses DNA damage-induced apoptosis in a p53-dependent manner.

We initially investigated the radioprotective property of 5CHQ in the radiation-induced p53-dependent apoptosis of human T-cell leukemia MOLT-4 cells. 5CHQ dose-dependently decreased apoptosis at 5-40 μM in Annexin V-FITC staining (Fig. 1B) and measuring loss of mitochondrial membrane potential (Δψm; Fig. 1C). The optimal dose for suppressing the apoptosis was 20-40 μM. On the other hand, there was no significant difference for the efficacy of 5CHQ at 10-40 μM in decreasing a conformational change of Bax (Fig. 1D) and effector caspase activation (Fig. 1E). The conformational change of Bax is thought to be related to mitochondrial outer membrane permeabilization as an upstream event of caspase activation (32). 5CHQ also showed some cytotoxic effects in unirradiated MOLT-4 cells, but our initial focus was on the antiapoptotic mechanism of 5CHQ, because we also confirmed that 5CHQ can be administered to mice at high doses (up to 80 mg/kg) with no overt signs of toxicity (see the results of experiments using mouse models). To study the antiapoptotic mechanism of 5CHQ, we next investigated the effects of 5CHQ regarding its p53-dependency. In an analysis of the MOLT-4 transformants expressing p53-targeting shRNA (KD-1 and KD-1/Hygro) (26, 13), the antiapoptotic activity of 5CHQ was limited to cells expressing p53 (MOLT-4 and Nega) or shRNA-resistant p53 (KD-1/R-p53-1) (13), while p53-knockdown KD-1 and KD-1/Hygro cells were more sensitive to 5CHQ plus IR than IR alone (Fig. 2A). Similar results were also observed when etoposide was administered to induce severe DNA damage to these cell-lines (Fig. S1). These results indicate that 5CHQ suppressed the apoptosis in a p53-dependent manner. Consequently, it can be assumed that 5CHQ suppresses a canonical caspase-dependent apoptosis at low doses and other apoptotic pathway at high doses, respectively, in some p53-dependent processes of cell death.

5CHQ shifts p53-mediated transactivation from proapoptotic to protective responses resulting in the enhancement of p21 induction and the suppression of PUMA induction.

To further investigate the mechanism responsible for the p53-dependent antiapoptotic effects caused by 5CHQ, we examined its effects on p53-mediated transactivation after IR. 5CHQ suppressed the induction of PUMA and its coding mRNA (*BBC3*), but surprisingly, it enhanced the induction of p21 and its coding mRNA (*CDKN1A*). Compared to the IR alone cells, 5CHQ enhanced p21-induction by 2-fold at a concentration of 40 μM, and suppressed PUMA-induction by approximately 50% at 40 μM (Fig. 2 B). Likewise, a Q-PCR analysis revealed that 5CHQ enhanced and suppressed the transactivation of p53-target genes, *CDKN1A* mRNA (~4-fold increase) and *BBC3* mRNA (~60% decrease), respectively (Fig. 2C). Chromatin immunoprecipitation (ChIP) assays revealed that 5CHQ enhanced and suppressed the intracellular DNA-binding of p53 to *CDKN1A* (~2-fold increase) and *BBC3* promoters (~85% decrease), respectively (Fig. 2D).

Biochemical characterization of 5CHQ.

We also reconstituted the upregulation of the DNA-binding of p53 to consensus oligonucleotides (33) (Fig. 3A) and the *CDKN1A* promoter oligonucleotides (Fig. S2A) by 5CHQ in an electrophoretic mobility shift assay (EMSA). In contrast to that both binding ratios reached more than 90% by 5CHQ, the findings showed that the binding to *BBC3* promoter oligonucleotides (Fig. S2B) was less affected, suggesting a sequence-specific altering activity of 5CHQ on p53. In this respect, using circular dichroism (CD) spectroscopy, we investigated the conformational changes of p53 that had been treated with various p53-regulating compounds (Fig. 3B). Negative Cotton effects around 210-230 nm, which can be assigned to α-helix and β-sheet structures in p53 (34), were clearly reduced by the addition of Bispicen (13), indicating that Bispicen removed the zinc(II) ion from the ZBS in p53, thus resulting in denaturation. A small chemical shift was observed in the 210-215 nm wavelength region in the case of 5CHQ-treated p53, but a negligible effect was observed in the 215-230 nm region, thus indicating that 5CHQ has little, if any, effect on the conformation of p53. AS-2 (15) also had a negligible effect on the conformation of p53. We also examined the radical scavenging activity of 5CHQ by using DPPH free radicals. 5CHQ showed negligible radical scavenging activity, while a positive control (the antioxidant vitamin E) rapidly

scavenged DPPH free radicals (Fig. 3C). To investigate the role of the hydroxyl group at the 8-position of the quinoline ring of 5CHQ in this process, we tested 5-chloro-8-methoxyquinoline (5CMQ), in which the 8-hydroxyl group is methylated, and chloroquine that contains a chloro moiety but no hydroxyl group and acts as a resistant agent for genotoxic stress by activating the ATM signaling pathway (35, 36). As shown in Figure 3D, these quinoline derivatives had no protective effects on irradiated MOLT-4 cell death, indicating that the 8-hydroxyl group of 5CHQ is required for its activity.

5CHQ has p21-dependent antiapoptotic activity, and some inherent p53-activating activity.

Considering the fact that the reduction of p53-target genes by an RNA synthesis inhibitor is less affected with the p53-dependent apoptosis of irradiated MOLT-4 cells (30), it is reasonable to speculate that some radioprotective p53-target genes are upregulated in the 5CHQ-mediated apoptosis suppression. Thus, we established a p21-knockdown MOLT-4 transformant expressing p21-targeting shRNA (p21KD-1). Although the knockdown was partial (Fig. 4A), the antiapoptotic effect of 5CHQ was decreased as the result of the knockdown of p21 (Fig. 4B), despite the fact that the suppression of PUMA by 5CHQ was not affected (Fig. 4A), suggesting that the enhancement of p21 contributes to the antiapoptotic effect of 5CHQ.

In further studies, we also found that 5CHQ alone has a unique activity that stabilizes p53 and induces p21 without inducing PUMA and a DNA damage response (DDR; 37) including p53 and H2AX phosphorylation (pS15-p53 and γ-H2AX) (Fig. 4 A, C, and D). These stabilizing and activating activities appear to be correlated with the antiapoptotic effect of 5CHQ, since they were observed at the optimal antiapoptotic dose of 5CHQ ranging 20-40 μM (Fig. 4C and Fig. S3 and Fig. 1 B and 1C). These results indicate that 5CHQ has some inherent agonistic activity for p53, in addition to its p53 transactivation-modulating activity. The upregulation of the transactivation of p53 target genes by 5CHQ alone is p53-dependent, because, in p53-knockdown KD-1 cells, the transactivation levels of mRNA and protein were suppressed (Fig. S3 and Fig. 4C). We also examined this issue of whether 5CHQ alters cell cycle distribution. Irradiated MOLT-4 cells showed a decrease in G_1 population and an increase in apoptotic

Sub-G_1 cells, and 5CHQ suppressed this change, but no obvious cell cycle arrest was observed in 5CHQ-treated irradiated MOLT-4 cells (Fig. S4). In addition, 5CHQ alone slightly increased the G_1 population, presumably due to a slight induction of p21 (Fig. S4 and Fig. 4C).

Radioprotective application to protecting lethally irradiated mouse models.

Before mouse radioprotection assays, we examined a maximum tolerated dose (MTD) study (Fig. S5). MTD was defined as the dose at which no animals died as the result of treatment and at which no abnormal behavior and no adverse effects were observed. In this study, in the mice treated with 120 mg/kg of 5CHQ, only one mouse died 10 days after treatment, and the data obtained from the surviving 9 mice showed a significant increase of WBC value. All mice treated with less than 80 mg/kg of 5CHQ did not show any overt signs of toxicity. Therefore, we concluded that MTD of 5CHQ is below 120 mg/kg, and less than 80 mg/kg is obviously safe.

In a 30-day survival test after total-body irradiation (TBI), 5CHQ was found to protect 50% of the mice from a lethal dose of 7.5 Gy TBI (Fig. 5A). Since the dose lethal for 50% within 30 days ($LD_{50/30}$) of control ICR mice in our previous study was determined to be 6.1 Gy (38), the dose reduction factor (DRF; the fold change in IR dose to produce a given level of lethality) for 5CHQ was estimated to be approximately 1.2. We also found that 5CHQ markedly protected BM cells from TBI injury (Fig. 5B). The number of white blood cells, platelets, BM stem cells, and BM progenitor cells between the 5CHQ-treated and vehicle DMSO-treated irradiated mice also indicated a marked radioprotective action by 5CHQ (Fig. 5B and 5C).

Furthermore, the remarkable activity of 5CHQ as an enhancer of p21 induction appeared to be useful for protecting the GI system, because p53 and p21 have been reported to be resistant factors in the radiation-induced GI syndrome in mice (3). Thus, we investigated the effect of 5CHQ on abdominal radiation injury using the SBI technique to avoid BM aplasia (Fig. S6). Before the SBI experiments, we also performed two additional, independent TBI experiments, a 30-day survival test (Fig. S7A and B) and an 8-day hematological test (Fig. S7C) for comparison between 35 and 60 mg/kg of 5CHQ-treated mice.

Although 8-day hematological test did not show any significant difference, the second 30-day survival test showed a superiority of 60 mg/kg of 5CHQ in radioprotective assay after TBI. Furthermore, a pilot test after SBI using C57BL/6 mice showed that the protective effect of 5CHQ at 60 mg/kg was greater than that at 35 mg/kg (Fig. S8). Thus, we tested 60 mg/kg of 5CHQ to the SBI model, and 5CHQ protected mice from a lethal dose of 18 or 24 Gy-SBI (Fig. 6A). The DRF for a 60% lethality was approximately 1.3, which is a better value than that for BM death induced by TBI. The transactivation-modulating activity of 5CHQ was confirmed in mouse GI epithelia treated with 21 Gy-SBI. Radioprotective *Cdkn1a* mRNA was upregulated, and proapoptotic *Bbc3* and *Pmaip1* mRNAs were downregulated by 5CHQ (Fig. 6B). We also obtained similar results in immunoblotting detection for the transactivation-modulating activity of 5CHQ (Fig. S9). The upregulation of *Cdkn1a* gene expression by 5CHQ was also observed in wild-type p53-bearing mouse mammary carcinoma cell line FM3A cells, although there was a discrepancy between the mRNA and the protein abundance at 20 μM in the *Bbc3* gene expression, suggesting some posttranscriptional mechanisms (Fig. S10). We also performed some pathological studies on small intestinal tissue, and compared the effect of 21 Gy-SBI on the epithelial proliferation in 5CHQ-treated and vehicle DMSO-treated mice using a hematoxylin and eosin (H&E) staining and a bromodeoxyuridine (BrdU) incorporation assay. 5CHQ efficiently relieved epithelial damage, as indicated by vigorous regeneration in crypts and protection from the disappearance of crypts and shortening of the villi (Fig. 6C). The increase in the survival of GI epithelial stem cells by 5CHQ was also confirmed by crypt survival assays (60% increase compared to DMSO-treated GI epithelium 3.5 days after SBI; Fig. 6D) and Q-PCR analysis of the stem cell marker *Lgr5* expression (39) (1.6-fold expression compared to DMSO-treated GI epithelium 48 h after SBI; Fig. S11). Furthermore, to demonstrate the specificity of 5CHQ for p53 in reducing the lethality after SBI, we performed a 60-day survival test after SBI using *Trp53*-knockout mice and wild-type C57BL/6 mice, and, in contrast to wild-type mice, the *Trp53*-knockout mice showed the ineffectiveness of 5CHQ (Fig. 6E).

Discussion

In radiotherapy, the radiation damages both tumor and normal tissue. This lack of specificity is a major limitation of radiation therapy. To define therapeutic ratios in clinical settings, the balance between the tumor control probability (TCP) and the normal tissue complication probability (NTCP) must be taken into account (2). In order to increase TCP and to reduce NTCP, technical improvements, such as intensity-modulated radiation therapy (IMRT), image-guided radiation therapy (IGRT), or particle therapy, are used to maximize the therapeutic effect and to minimize toxicity. However, these advanced methods cannot completely overcome the problems that the organ at risk (OAR) and target volume (TV) are overlapping or close to each other, or are moving. In fact, radiotherapy for the abdominal and pelvic region is especially challenging, because the GI tract has an empirically low dose tolerance and is constantly moving. Therefore, pharmacological approaches to the selective radioprotection of normal tissues must be developed (1, 40-42) in order to allow clinicians to increase radiotherapy doses to lead to better tumor control rates. 5CHQ and 5CHQ-like transcriptional modulators for p53 may serve as selective protectors of normal tissues in abdominal or pelvic p53-deficient cancer therapy.

Some transcriptional modulators such as tamoxifen and retinoids are already being used for cancer therapy (43). For example, the anticancer activities of tamoxifen and their derivatives are not solely due to their antagonistic activity against estrogen receptors. They function as selective estrogen receptor modulators (SERMs) that exert complex anti-estrogenic and pro-estrogenic effects, and modulate the transcriptional activity of these receptors in a ligand structure-dependent manner. Thus, SERMs such as tamoxifen, raloxifene, and all compounds that bind to estrogen receptors induce the formation of distinctly different receptor conformations, and the resulting conformations transcribe their target genes in different ways (44). The findings reported herein demonstrate that 5CHQ increases the DNA-binding of p53 to specific oligonucleotides in a sequence-specific manner (Fig. 3A and Fig. S2), suggesting that 5CHQ alters the conformation of p53 and its transcriptional activity. Thus, we investigated the conformational changes of p53 that had been treated with various p53-regulating compounds including 5CHQ by CD spectroscopy. The resulting data indicated that 5CHQ has little, if any, effect on the

conformation of p53 (Fig. 3B). Considering the present data and our previously reported CD data (14) showing that 8-HQ-derived p53-regulating compounds have little effect on the conformation of p53, these compounds are not likely to eliminate the zinc(II) ion from the ZBS in p53, as is in the case of zinc(II) peptidase reported in our previous structural analysis (45). The ZBS is a cysteine-rich zinc-finger-like motif that is located in the DNA binding domain (17, 20). It is reasonable to assume that 5CHQ alters the affinity of p53 for DNA by coordinating between the ZBS and DNA. To support this hypothesis, the ineffectiveness of OH-protected and non-hydroxylated quinoline derivatives indicates that the hydroxyl group of 5CHQ is required for its metal-chelating activity in suppressing apoptosis (Fig. 3D). This requirement suggests that 5CHQ exerts its apoptosis-suppressing activity by specific coordination to the p53-DNA complex. The distance between DNA and an important basic amino acid (R248 in p53) has been estimated as a long-range interaction within 4.0 Å (17). The long-range niche might provide sufficient space to permit 5CHQ to be accommodated. The tertiary structure of the tripartite complex of p53-5CHQ-DNA needs to be addressed in future research.

Regarding to the cytotoxic effect of 5CHQ observed in unirradiated MOLT-4 cells, we conclude that this toxicity is p53-independent and that it is not associated with the stabilizing and activating activities of 5CHQ on p53 (Figs. 4, S3, and S9), because p53-knockdown KD-1 cells showed the same sensitivity to 5CHQ alone as the parental MOLT-4 cells (Fig. 2A). It is possible that p53-independent cytotoxicity could be reduced by chemical modification; however, the fact that 5CHQ can be administered to mice at high doses (up to 80 mg/kg) with no overt signs of toxicity (Fig. S5) provides an optimistic view that the toxicity can be ignored in *in vivo* experiments and that 5CHQ may serve as a seed compound for a p53 agonist. The difference in sensitivity observed between *ex vivo* and *in vivo* experiments should be considered in future studies.

Interestingly, a recent study has shown that some ZBS-targeting chelators can act as p53 activators (46, 47), the properties of which might have some relation to the p53-agonistic activity of 5CHQ. The p53-agonistic activity of 5CHQ also includes some inherent activity that stimulates p53 in unirradiated cells without DDR (Fig. 4). Remarkably, the PUMA protein was hardly observed in 5CHQ alone-treated

MOLT-4 cells (Fig. 4C) in spite of the upregulation of *BBC3* mRNA (Fig. S3), suggesting that the cells have a defense mechanism that permits the expression of the PUMA protein to be suppressed under no DDR environments. These findings further raise the possibility that 5CHQ may differentially regulate cell cycle and apoptosis mediators of p53 through some regulators. It is known that some posttranslational mechanisms can control p53-mediated decision on either cell survival or induction of apoptosis (48).

In this study, we demonstrate that a chemical modulator that elicits radioprotective ability for p53 can efficiently protect mice from severe radiation injury caused by abdominal irradiation. It is particularly noteworthy that p21 has an important role in suppressing p53-mediated cell death as supported by previous studies showing that p21 has a radioprotective role in irradiated mice (3, 49, 50), although the precise and detailed mechanism by which p21 suppresses cell death remains unknown. The radioprotective action by 5CHQ or its derivatives may also be useful as a radioprotector for carcinogenesis by providing radiation damaged cells with some additional recovery time through the upregulation of p21. Multiple applications are being considered and will be pursued in future studies.

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References

1. Gudkov AV, Komarova EA. Radioprotection: smart games with death. J Clin Invest 2010;120:2270-3.
2. Hall EJ, Giaccia AJ. Radiobiology for the radiologist. 7th ed. Philadelphia: Lippincott Williams & Wilkins; 2012.
3. Kirsch DG, Santiago PM, di Tomaso E, Sullivan JM, Hou WS, Dayton T, et al. p53 controls radiation-induced gastrointestinal syndrome in mice independent of apoptosis. Science 2010;327:593-6.
4. van Bekkum DW, Schotman E. Protection from haemopoietic death by shielding versus grafting of bone-marrow. Int J Radiat Biol Relat Stud Phys Chem Med 1974;25:361-72.
5. Mason KA, Withers HR, McBride WH, Davis CA, Smathers JB. Comparison of the gastrointestinal syndrome after total-body or total-abdominal irradiation. Radiat Res 1989;117:480-8.
6. Terry NHA, Travis EL. The influence of bone marrow depletion on intestinal radiation damage. Int J Radiat Oncol Biol Phys 1989;17:569-73.
7. Paris F, Fuks Z, Kang A, Capodieci P, Juan G, Ehleiter D, et al. Endothelial apoptosis as the primary lesion initiating intestinal radiation damage in mice. Science 2001;293:293-7.
8. Burdelya LG, Krivokrysenko VI, Tallant TC, Strom E, Gleiberman AS, Gupta D, et al. An agonist of toll-like receptor 5 has radioprotective activity in mouse and primate models. Science 2008;320:226-30.
9. Saha S, Bhanja P, Liu L, Alfieri AA, Yu D, Kandimalla ER, et al. TLR9 agonist protects mice from radiation-induced gastrointestinal syndrome. PLoS One 2012;7:e29357.
10. Morita A, Yamamoto S, Wang B, Tanaka K, Suzuki N, Aoki S, et al. Sodium orthovanadate inhibits p53-mediated apoptosis. Cancer Res 2010;70:257-65.
11. Potten CS, Owen G, Roberts SA. The temporal and spatial changes in cell proliferation within the irradiated crypts of the murine small intestine. Int J Radiat Biol 1990;57:185-99.

12. Christophorou MA, Ringshausen I, Finch AJ, Swigart LB, Evan GI. The pathological response to DNA damage does not contribute to p53-mediated tumour suppression. Nature 2006;443:214-7.

13. Morita A, Ariyasu S, Ohya S, Takahashi I, Wang B, Tanaka K, et al. Evaluation of Zinc (II) chelators for inhibiting p53-mediated apoptosis. Oncotarget 2013;4:2439-50.

14. Ariyasu S, Sawa A, Morita A, Hanaya K, Hoshi M, Takahashi I, et al. Design and synthesis of 8-hydroxyquinoline-based radioprotective agents. Bioorg Med Chem 2014;22:3891-905.

15. Morita A, Ariyasu S, Wang B, Asanuma T, Onoda T, Sawa A, et al. 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. Biochem Biophys Res Commun 2014;450:1498-504.

16. Matsumoto A, Aoki S, Ohwada H. Comparison of random forest and SVM for raw data in drug discovery: prediction of radiation protection and toxicity case study. IJMLC 2016;6:145-8.

17. Cho Y, Gorina S, Jeffrey PD, Pavietich NP. Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. Science 1994;265:346-55.

18. Butler JS, Loh SN. Structure, function, and aggregation of the zinc-free form of the p53 DNA binding domain. Biochemistry 2003;42:2396-403.

19. Butler JS, Loh SN. Zn(2+)-dependent misfolding of the p53 DNA binding domain. Biochemistry 2007;46:2630-9.

20. Joerger AC, Fersht AR. Structure–function–rescue: the diverse nature of common p53 cancer mutants. Oncogene 2007;26:2226-42.

21. Cheng J, Haas M. Frequent mutations in the p53 tumor suppressor gene in human leukemia T-cell lines. Mol Cell Biol 1990;10:5502-9.

22. Oconnor PM, Jackman J, Bae I, Myers TG, Fan S, Mutoh M, et al. Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency of 123 anticancer agents. Cancer Res 1997;57:4285-300.

23. Gong BD, Chen Q, Endlich B, Mazumder S, Almasan A. Ionizing radiation-induced, Bax-mediated cell death is dependent on activation of cysteine and serine proteases. Cell Growth Differ 1999;10:491-502.

24. Jia LQ, Osada M, Ishioka C, Gamo M, Ikawa S, Suzuki T, et al. Screening the p53 status of human cell lines using a yeast functional assay. Mol Carcinog 1997;19:243-53.

25. Nakano H, Kohara M, Shinohara K. Evaluation of the relative contribution of p53-mediated pathway in X-ray-induced apoptosis in human leukemic MOLT-4 cells by transfection with a mutant p53 gene at different expression levels. Cell Tissue Res 2001;306:101–6.

26. Morita A, Zhu J, Suzuki N, Enomoto A, Matsumoto Y, Tomita M, et al. Sodium orthovanadate suppresses DNA damage-induced caspase activation and apoptosis by inactivating p53. Cell Death Differ 2006;13:499-511.

27. Ayusawa D, Koyama H, lwata K, Seno T. Selection of mammalian thymidine auxotrophic cell mutants defective in thymidylate synthase by their reduced sensitivity to methotrexate. Somatic Cell Genet 1981;7:523-34.

28. Saito Y, Mitsuhashi N, Sakurai H, Ishikawa H, Hasegawa M, Akimoto T, et al. Apoptosis and appearance of *Trp53*-positive micronuclei in murine tumors with different radioresponses *in vivo*. Radiat Res 1999;152:462–7.

29. O'Neil, MJ, Ed. The Merck Index. 15 th ed.: An encyclopedia of chemicals, drugs, and biologicals: Royal Society of Chemistry;2013.

30. Ito A, Morita A, Ohya S, Yamamoto S, Enomoto A, Ikekita M. Cycloheximide suppresses radiation-induced apoptosis in MOLT-4 cells with Arg72 variant of p53 through translational inhibition of p53 accumulation. J Radiat Res 2011;52:342-50.

31. Tsukada T, Tomooka Y, Takai S, Ueda Y, Nishikawa S, Yagi T, et al. Enhanced proliferative potential in culture of cells from p53-deficient mice. Oncogene 1993;8:3313-22.

32. Roucou X, Martinou JC. Conformational change of Bax: a question of life or death. Cell Death Differ 2001;8:875-7.

33. Funk WD, Pak DT, Karas RH, Wright WE, Shay JW. A transcriptionally active DNA-binding site for human p53 protein complexes. Mol Cell Biol 1992;12:2866-71.

34. Nichols NM, Matthews KS. Protein-DNA binding correlates with structural thermostability for the full-length human p53 protein. Biochemistry 2001;40:3847-58.

35. Lim Y, Hedayati M, Merchant AA, Zhang Y, Yu HH, Kastan MB, et al. Chloroquine improves survival and hematopoietic recovery after lethal low-dose-rate radiation. Int J Radiat Oncol Biol Phys 2012;84:800-6.

36. Loehberg CR, Thompson T, Kastan MB, Maclean KH, Edwards DG, Kittrell FS, et al. Ataxia telangiectasia-mutated and p53 are potential mediators of chloroquine-induced resistance to mammary carcinogenesis. Cancer Res 2007;67:12026-33.

37. Bakkenist CJ, Kastan MB. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature 2003;421:499-506.

38. Wang B, Tanaka K, Morita A, Ninomiya Y, Maruyama K, Fujita K, et al. Sodium orthovanadate (vanadate), a potent mitigator of radiation-induced damage to the hematopoietic system in mice. J Radiat Res 2013;54:620-9.

39. Metcalfe C, Kljavin NM, Ybarra R, de Sauvage FJ. Lgr5(+) stem cells are indispensable for radiation-induced intestinal regeneration. Cell Stem Cell 2014;14:149-59.

40. Liauw SL, Connell PP, Weichselbaum RR. New paradigms and future challenges in radiation oncology: an update of biological targets and technology. Sci Transl Med 2013;5:173sr2.

41. Emami B, Lyman J, Brown A, Coia L, Goitein M, Munzenrider JE, et al. Tolerance of normal tissue to therapeutic irradiation. Int J Radiat Oncol Biol Phys 1991;21:109-22.

42. Marks LB, Yorke ED, Jackson A, Ten Haken RK, Constine LS, Eisbruch A, et al. Use of normal tissue complication probability models in the clinic. Int J Radiat Oncol Biol Phys 2010;76(3 Suppl):S10-19.

43. Sporn MB, Suh N. Chemoprevention: an essential approach to controlling cancer. Nat Rev Cancer 2002;2:537-43.

44. Katzenellenbogen BS, Katzenellenbogen JA. Defining the 'S' in SERMs. Science 2002;295:2380-1.

45. Hanaya K, Suetsugu M, Saijo S, Yamato I, Aoki S. Potent inhibition of dinuclear zinc(II) peptidase, an aminopeptidase from Aeromonas proteolytica, by 8-quinolinol derivatives: inhibitor design based on Zn^{2+} fluorophores, kinetic, and X-ray crystallographic study. J Bio Inorg Chem 2012;17:517-29.

46. Yu X, Vazquez A, Levine AJ, Carpizo DR. Allele-specific p53 mutant reactivation. Cancer Cell 2012;21:614-25.

47. Yu X, Narayanan S, Vazquez A, Carpizo DR. Small molecule compounds targeting the p53 pathway: are we finally making progress? Apoptosis 2014;19:1055-68.

48. Charvet C1, Wissler M, Brauns-Schubert P, Wang SJ, Tang Y, Sigloch FC, et al. Phosphorylation of Tip60 by GSK-3 determines the induction of PUMA and apoptosis by p53. Mol Cell 2011;42:584-96.

49. Komarova EA, Kondratov RV, Wang K, Christov K, Golovkina TV, Goldblum JR, et al. Dual effect of p53 on radiation sensitivity *in vivo*: p53 promotes hematopoietic injury, but protects from gastro-intestinal syndrome in mice. Oncogene 2004;23:3265-71.

50. Sullivan JM, Jeffords LB, Lee CL, Rodrigues R, Ma Y, Kirsch DG. p21 protects "Super p53" mice from the radiation-induced gastrointestinal syndrome. Radiat Res 2012;177:307-10.

Figure legends:

Figure 1. Dose response of 5CHQ on radiation-induced apoptosis in MOLT-4 cells.

A, Chemical structure of 5CHQ. B-E, Dose response of 5CHQ in 10 Gy-irradiated MOLT-4 cells. B-D, Means and standard deviations from three independent experiments are shown. B, Annexin V-FITC staining 17 h after IR. 5CHQ decreased apoptosis at 5-80 μM ($p < 0.002$). C, Flow cytometric analysis of cells losing mitochondrial membrane potential ($\Delta\psi m$) using MitoTracker Red (15 h after IR). 5CHQ decreased the apoptosis at 10-80 μM ($p < 0.02$). D, Flow cytometric analysis of the conformational change of Bax measured by anti-Bax immunoreactivity (6 h after IR). 5CHQ decreased the conformational change at 10-40 μM ($p < 0.01$). E, Caspase activation is inhibited by 5CHQ, as evidenced by immunoblotting (10 h after IR).

Figure 2. 5CHQ suppresses apoptosis in a p53-dependent manner and functions as a chemical modulator of p53 transcription.

A, The effect of 40 μM 5CHQ on parental MOLT-4 cells and various MOLT-4 transfectants 17 h after 10 Gy-IR. Means and standard deviations from 3-5 independent experiments are shown and asterisks denote statistical significance: **, $P < 0.01$; *, $P < 0.05$. B-D, MOLT-4 cells were harvested 6 h after a 10 Gy-IR treatment. B, Immunoblotting detection of p53 and p53 target gene products p21 and PUMA. β-actin was used as an internal control. Band intensities were quantified by densitometry. C, Q-PCR analysis of the transcription of *CDKN1A* and *BBC3*. D, ChIP assay quantified by Q-PCR for the promoters of *CDKN1A*, *BBC3,* and *GAPDH* (control).

Figure 3. *In vitro* potentiation of the DNA-binding activity of p53 by 5CHQ, and the chemical importance of the hydroxyl group at the 8-position of the quinoline ring of 5CHQ.

A, EMSA showing that 5CHQ enhances the DNA-binding activity of recombinant p53. FITC-labeled double stranded consensus oligonucleotides (33) were used. B, The conformational changes of p53

treated with various p53-regulating compounds were examined by CD spectroscopy (14). CD spectra of recombinant FLAG-p53 (20 nM) in PBS buffer (pH 7.4) in the absence and presence of zinc(II) chelators (2 μM each) at 25 °C. C, DPPH free radical scavenging assay was performed in methanol followed by UV–vis spectroscopy. The initial DPPH and radioprotectors concentrations were 50 μM and 200 μM, respectively. The changes in absorbance at 516 nm by the addition of vitamin E and 5CHQ are shown as dashed and solid curves, respectively. D, The ineffectiveness of non-hydroxylated quinoline derivatives on the apoptosis. MOLT-4 cells were harvested at 17 h after 10 Gy-IR. Annexin V-positive cells were measured by flow cytometry. Means and standard deviations from three independent experiments are shown.

Figure 4. 5CHQ exerts a p21-dependent antiapoptotic activity, and has an intrinsic activity to stimulate p53.

A, C, and D, Cells were harvested 7 h after compound treatment or 6 h after the 10 Gy-IR treatment. A, Parental MOLT-4 cells, control shRNA-transformant (puromycin-resistant), p21-knockdown transformant (p21KD-1) were subjected to immunoblotting. B, The effect of 40 μM 5CHQ on three cell lines 17 h after 10 Gy-IR. 5CHQ was less effective in p21KD-1 cells than in the other two cell lines (**, $P < 0.01$). C and D, 5CHQ alone dose-dependently induces p53 accumulation and p21 without inducing PUMA and DDR. C, KD-1 cells were used as a negative control for p53 expression.

Figure 5. 5CHQ protects mice from BM death caused by TBI.

ICR mice were i.p. injected with 5CHQ 30 min before 7.5 Gy-TBI. Numbers in parenthesis denote the number of mice. A, Thirty-day survival tests of mouse subgroups of TBI alone, 35 mg/kg or 60 mg/kg 5CHQ plus TBI. B and C, Change in HP parameters and body weight at 8 days after 7.5Gy-TBI-treated mice subgroup of DMSO alone or 35 mg/kg 5CHQ. There was no significant influence on any of the tested HP parameters between 5CHQ-treated and vehicle DMSO-treated unirradiated mice. B, BM cells were obtained from two femurs and two tibias, which are correspond to “BM tissues” in this figure. The

assay showed that 5CHQ markedly suppressed the decrease in the number of BM cells, WBC, and PLT by TBI (**, $P < 0.01$). C, Flow cytometric profiles of BM stem (LSK) and progenitor cells. The average number of each kind of cells in 5CHQ-treated irradiated mice was higher than that in vehicle DMSO-treated irradiated mice (**, $P < 0.01$).

Figure 6. 5CHQ protects mice from GI death caused by abdominal IR.

Mice were i.p. injected with 5CHQ 30 min before SBI (A-D: ICR mice, E: C57BL/6 mice). Numbers in parenthesis denote the number of mice used. A, Sixty-day survival tests after 18 or 24 Gy-SBI of ICR mouse subgroups of vehicle alone or 60 mg/kg 5CHQ. *P* values are the comparison between the 5CHQ-treated and DMSO-treated subgroups. B, Q-PCR analysis revealed that 5CHQ shifted p53-mediated transactivation after 21 Gy-SBI from proapoptotic to protective responses in GI epithelium. Means and standard deviations from 4 mice are shown, and *P* values in each time point compared between 5CHQ-treated and vehicle-treated mice are all less than 0.01 (**). C and D, All histological samples were collected 3.5 days after the treatments. C, The panels of the upper two rows show a series of microphotographs of H&E-stained intestines at different magnifications. The panels of the lower two rows show a series of microphotographs of BrdU-incorporated intestines at different magnifications. D, Intestinal crypt microcolony survival per crypt section was estimated quantitatively by light microscopy. The assay showed that 5CHQ increased crypt survival by 60%, compared to DMSO-treated GI epithelium. E, Sixty-day survival tests after 20 Gy-SBI of subgroups of *Trp53*-KO mice or wild-type C57BL/6 mice. *P* values are the comparison between the 5CHQ-treated and DMSO-treated subgroups.

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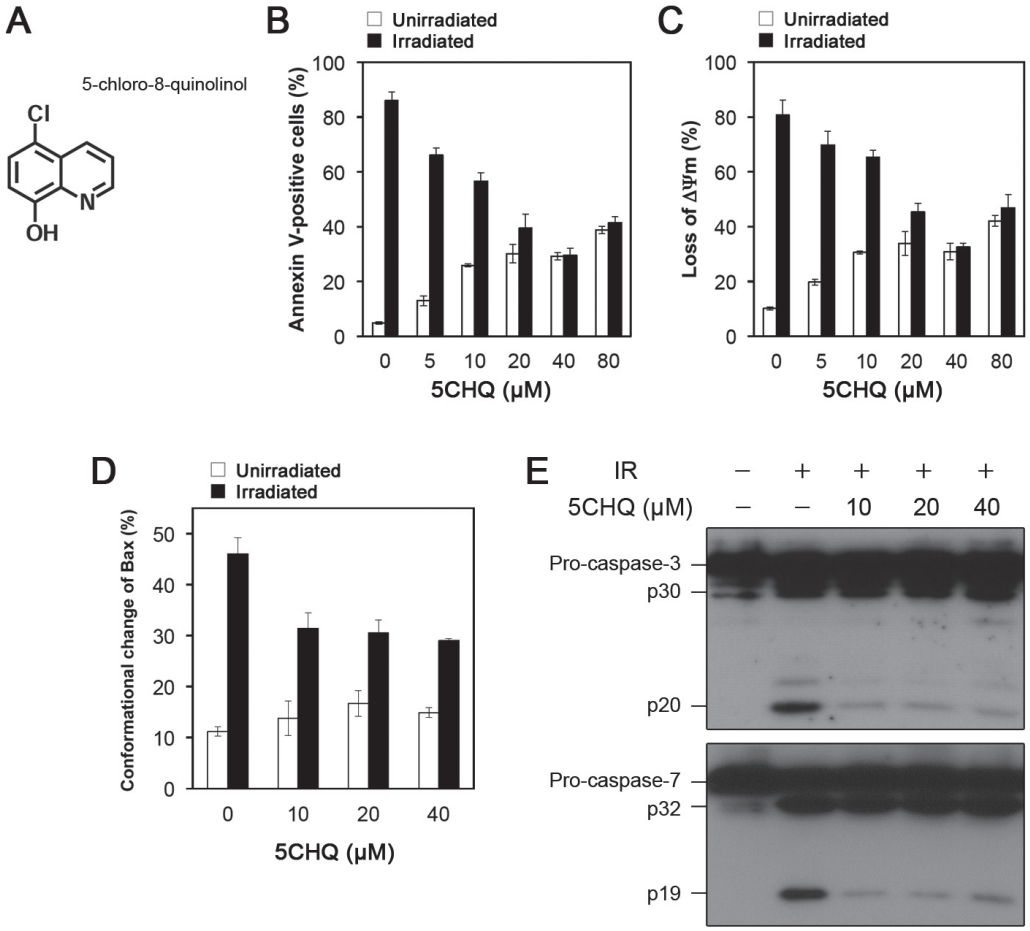
A. Morita et al., Figure 1.



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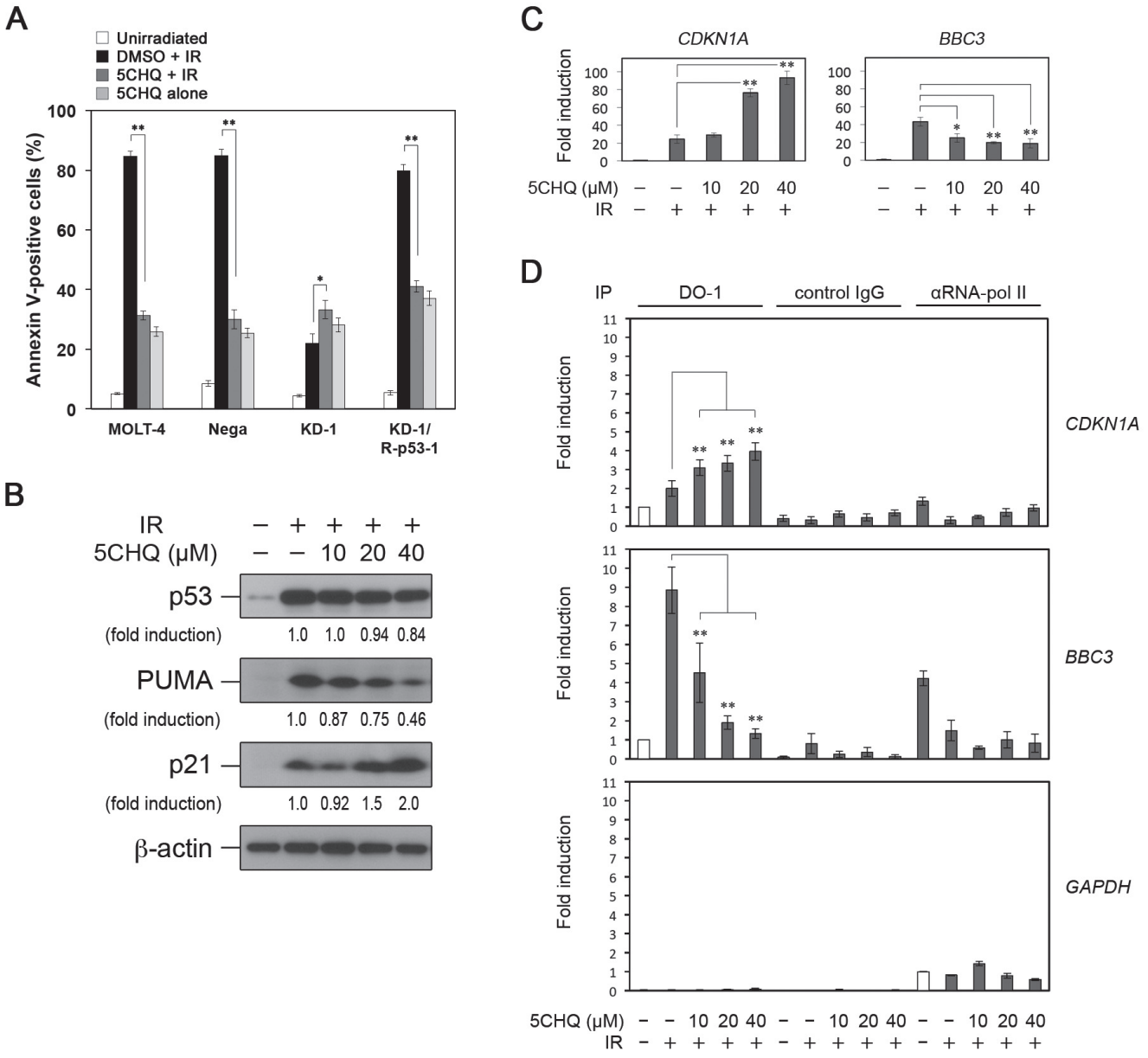

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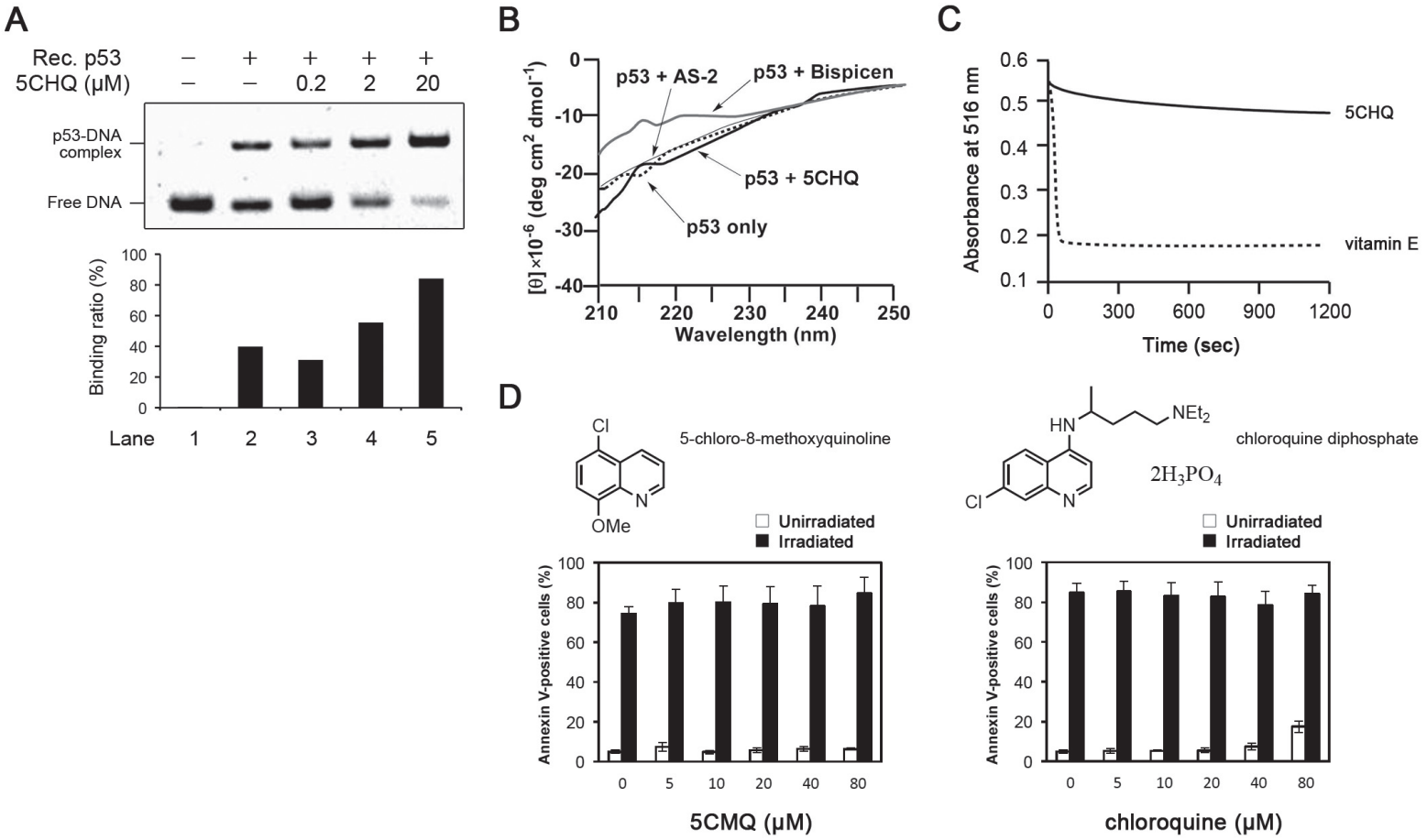

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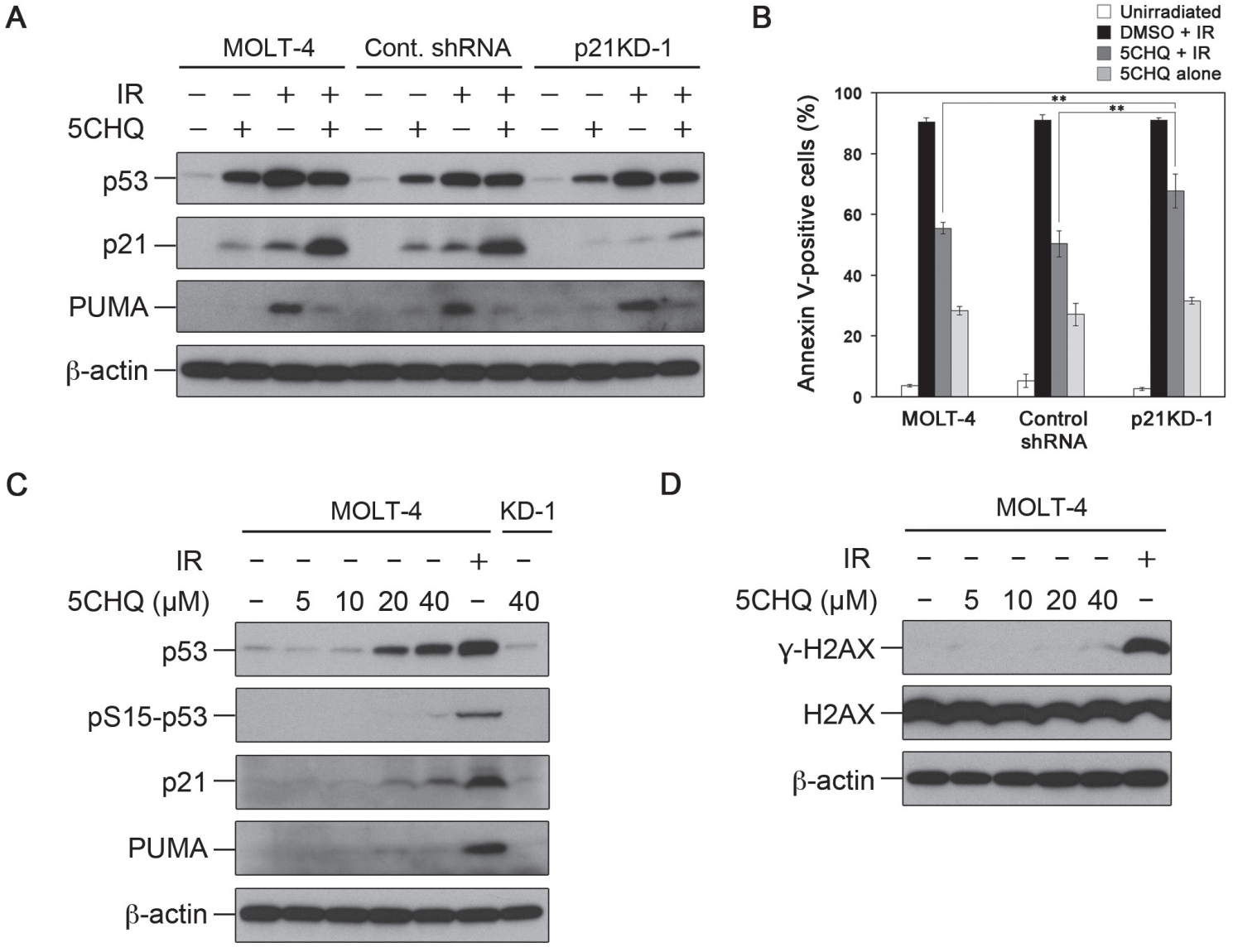

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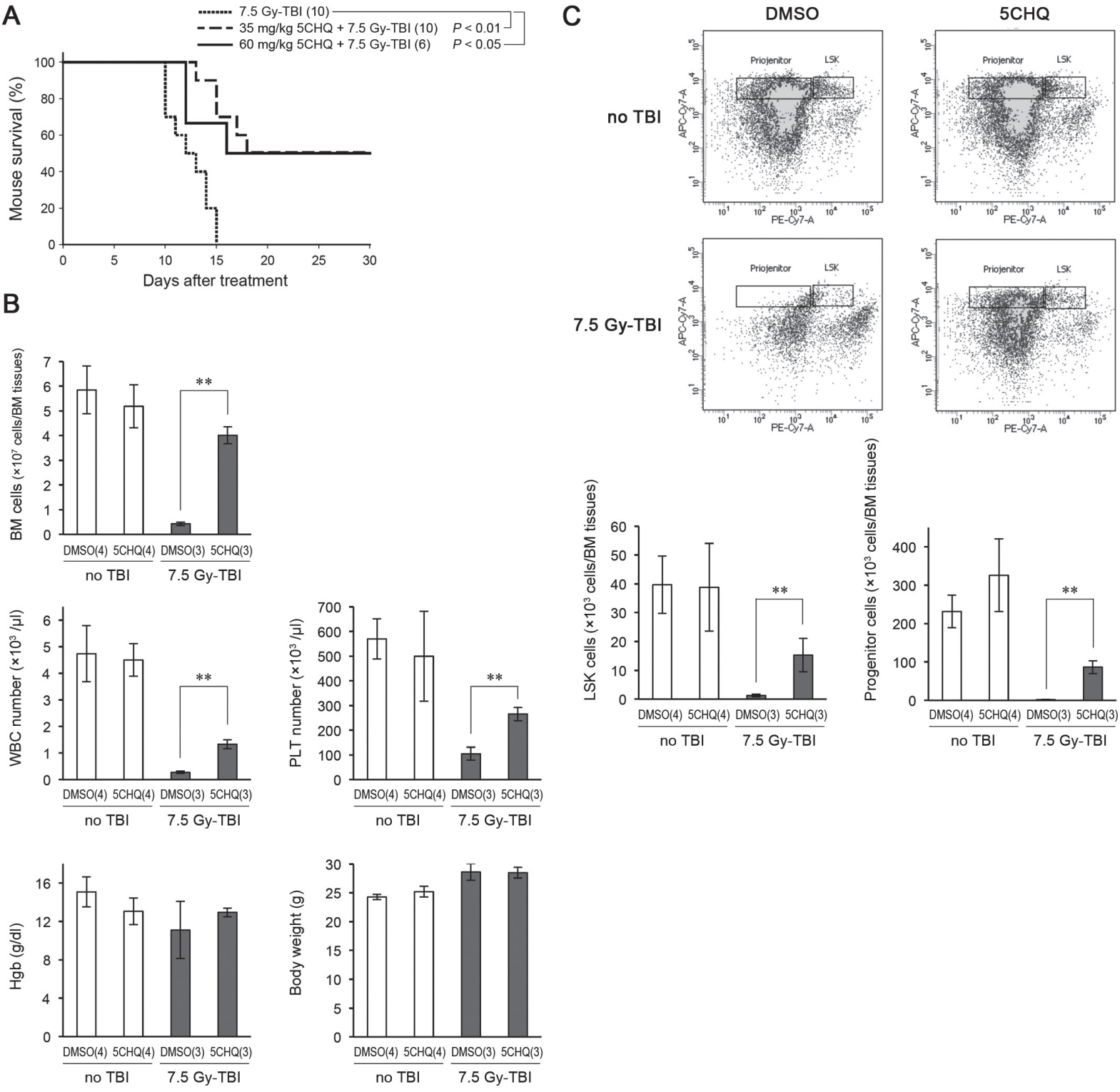

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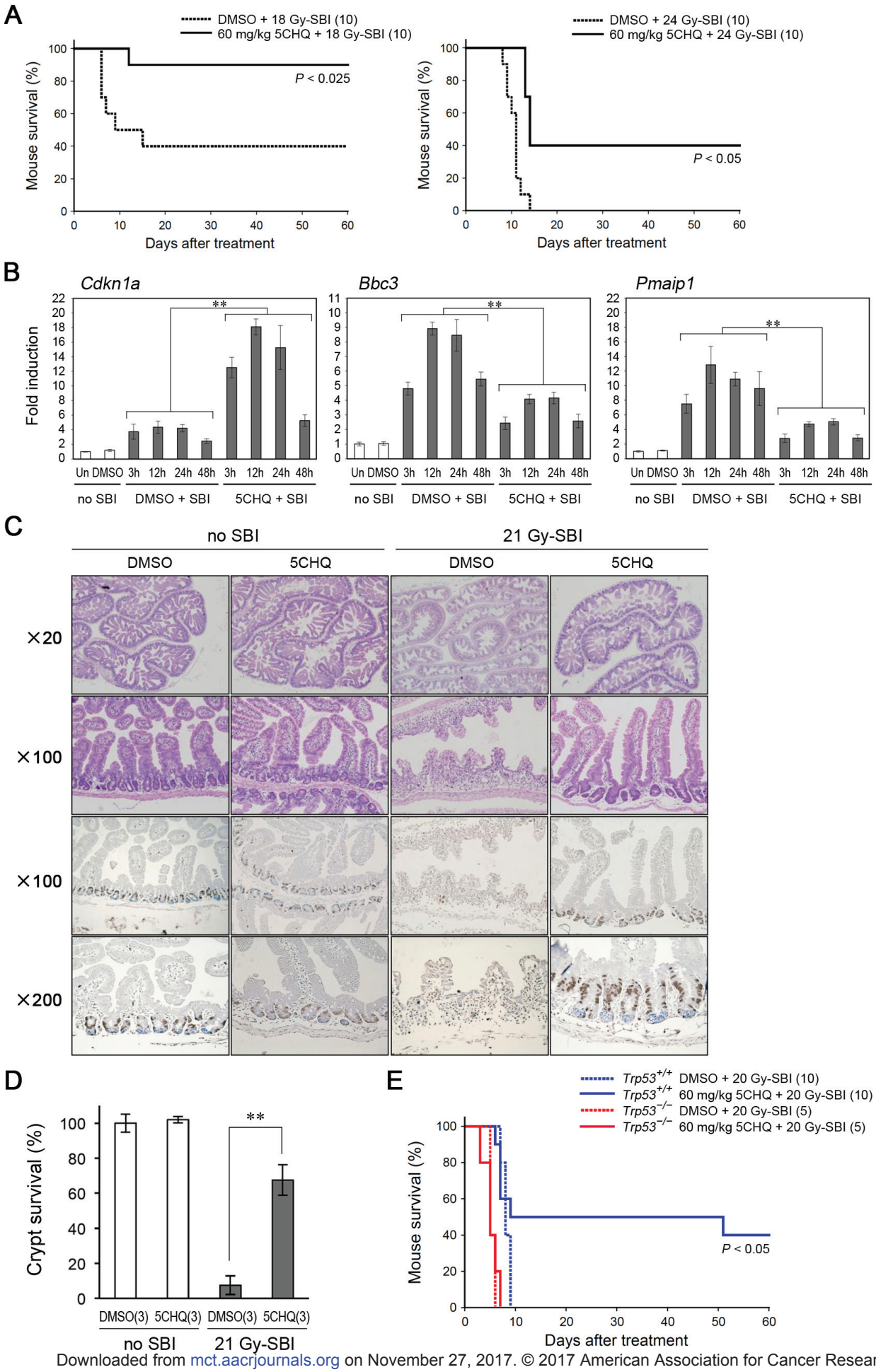

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Molecular Cancer Therapeutics

A chemical modulator of p53 transactivation that acts as a radioprotective agonist

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