Sodium Orthovanadate Is a Bifunctional Inhibitor of Transcription-Dependent and -Independent p53-Mediated Apoptosis

Supplemental Data

Akinori Morita,1 Shinichi Yamamoto,1 Bing Wang,3 Kaoru Tanaka,3 Norio Suzuki,4 Shin Aoki,5 Azusa Ito,1 Tomohisa Nanao,1 Soichiro Ohya,1 Minako Yoshino,1 Jin Zhu,6 Atsushi Enomoto,6 Yoshihisa Matsumoto,6 Osamu Funatsu,2 Yoshiho Hosoi,6 and Masahiko Ikekita1,2

1Department of Applied Biological Science, Faculty of Science and Technology, and 2Genome and Drug Research Center, Tokyo University of Science, Noda, Japan; 3Research Center for Radiation Protection, National Institute of Radiological Sciences, Chiba, Japan; 4Emeritus Professor, University of Tokyo, 5Department of Radiological Health, Graduate School of Medicine, and 6Laboratory of Molecular Radiology, Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan
Supplemental Materials and Methods

Cell culture and treatment.

The MOLT-4 stable transfectants expressing the short hairpin (sh)-type of p53 siRNA (MOLT/p53KD-1, -2) (1), the negative control shRNA (MOLT/Nega) (1), human papillomavirus type 18 (HPV18)-E6 (MOLT/E6-1, -2, and –3), and a Myc-His-tagged, myristoylated active Akt1 (MOLT/Akt-1) were cultured in RPMI 1640 medium supplemented with 10% FBS. To generate MOLT-4 stable transfectants expressing HPV18-E6, MOLT-4 cells were transfected by electroporation (Gene Pulsar II, Bio-Rad; 0.25 kV, 950 microfarads) with \( Bgl \ II \)-linearized HPV18-E6 vector or mock vector (pUSEamp(+), Upstate) (MOLT/Mock-1), and selected on 0.16% soft agar culture containing 0.4 mg/ml G418 for 3 weeks. To generate the MOLT/Akt-1 cells, MOLT-4 cells were transfected with a pUSEamp-myr-Akt1 plasmid (Upstate) by electroporation, and selected like the MOLT/E6 cells. The human embryonic kidney cell line HEK293T was cultured in DMEM/F12 medium supplemented with 10% FBS. HEK293T cells were plated in 6-well plates, and transiently transfected with 1 µg of pUSEamp(+) or pUSEamp-Bax plasmid (Upstate) using the FuGENE 6 transfection reagent (Roche), and the percentage of dead cells was determined by the 0.2% erythrosin B dye exclusion test. Cell density was determined with a cell counter (Z1 Cell and particle counter, Beckman Coulter). CdCl₂ (Wako) and sodium salicylate (Wako) were dissolved in water at 10 mM and 3 M, respectively. Etoposide (Wako), Dephostatin (3,4-Dephostatin, Calbiochem), Cypermethrin (Calbiochem), Okadaic acid (Calbiochem), LY294002 (Promega), and Anisomycin (Calbiochem) were dissolved in Me₂SO at concentrations of 10 mM, 10 mM, 25 mM, 250 µM, 10 mM, and 2 mg/ml, respectively. These
reagents, except for Etoposide and Anisomycin, were added to the culture medium immediately after IR.

Vector construction. To generate the HPV18-E6 expression vector, a HPV18-E6 coding insert was obtained by PCR using DNA extracted from HeLa cells (HPV18-positive) as a template (2), and the product was ligated into the Hind III/Eco R I site of the pUSEamp(+) vector. The primer sequences for E6 cloning were as follows: (forward) 5’-GCATAAGCTTGCCGCCACCATGGCGCGCTTTGAGGATCCGACCGCGA-3’, (reverse) 5’-CGCTGAATTCCTCGTGACATAAGCTGTCGTCGAACGGA-3’. All of the constructs were verified by sequencing their full length.

Semi-quantitative RT-PCR analysis
The primer sequences for E6 were as follows:

(forward) 5’-CTGTGTATGGAGACACATTGGAA-3’,
(reverse) 5’-ATACTTGTGTTTCTCTGCGTCGT-3’.

The primer sequences in mouse study were as follows:

noxα, (forward) 5’-GGCAGAGCTACCACCTGAGTTC-3’,
(reverse) 5’-TCTGGAGACCTGAGTTC-3’;

puma, (forward) 5’-AGCCCAGCAGCAGCTTAGTC-3’,
(reverse) 5’-GAGGAGTCCCATGAGGAGATG-3’;

β-actin, (forward) 5’-TCTGGACCTGGCTGGCCGACCTGA-3’,
(reverse) 5’-TACTCCTGCTTGGCAGATCCACAT-3’.
**Immunoblotting analysis.**

To detect Akt, activated myr-Akt1, and phospho-Akt, we used antibodies against Akt1/2/3 (H-136, Santa Cruz Biotechnology), Myc-tag (MBL), and phospho-Akt1/2/3 (p-Akt1/2/3 (Ser 473)-R, Santa Cruz Biotechnology).

**Preparation of thymocytes.**

All experimental protocols involving mice in this experimental section were reviewed and approved by the Animal Care and Use Committee of the Tokyo University of Science (TUS), and were performed in strict accordance with the TUS Guidelines for the Care and Use of Laboratory Animals. C57BL/6J male mice and B6.129-Trp53tm1Brd N12 male mice aged 5 weeks were purchased from CLEA Japan, Inc. and Taconic, respectively, and were used as a source for thymus. Thymocytes were prepared mechanically by sieving minced thymus through stainless steel mesh into PBS supplemented with 0.6% glucose. Cells were spun at 200 × g for 5 min, and the cell pellet was suspended with RPMI-1640 medium supplemented with 10% FBS (heat-inactivated at 56 °C for 30 min), 1 mM non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μM β-ME. The cell suspensions (2.5 × 10^5 cells/ml) were plated in 12-well plates, treated with IR and/or vanadate immediately after plating, and maintained at 37 °C in a humidified atmosphere containing 5% CO₂.

**Immunofluorescence microscopy.**

Cells were plated and cultured in 6-well plates. They were then washed once with 2% FBS/PBS (PBS-F), fixed with 4% paraformaldehyde/PBS for 15 min, washed once with
PBS-F, permeabilized with 100 µg/ml digitonin/PBS for 10 min, washed twice with PBS-F, blocked with normal goat serum (Dako) for 10 min, and washed once with PBS. The DO-1 antibody (Calbiochem) and anti-VDAC1 rabbit polyclonal antibody (ab15895, Abcam) were diluted 1:500 in 3% bovine serum albumin (BSA)/PBS and incubated with the cells at room temperature for 2 h. The cells were then washed three times with PBS, incubated with a 1:1000 dilution of an Alexa 488-labeled goat anti-mouse immunoglobulin antibody and Alexa 546-labeled goat anti-rabbit immunoglobulin antibody (Molecular Probes) with 3% BSA/PBS at room temperature for 1 h, washed three times with PBS, mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories), and then observed by fluorescence microscopy (Olympus). Each captured fluorescence image was merged using Adobe Photoshop CS software (v. 8.0.1).
Supplemental References


Supplemental Figure legends

Figure S1. Effects of p53 inhibitors on the transcriptional activity and accumulation of p53, transactivation of p53 target genes, and p53-dependent apoptosis in irradiated MOLT/p53-Luc1 cells.

The effects of salicylate and cadmium were analyzed as described in Figure 2.

Figure S2. Vanadate suppresses p53-dependent DNA-damage-induced apoptosis.

Apoptotic cells were quantified by Annexin V-FITC staining and flow cytometry. The loss of Δψm after treatment was measured by flow cytometry after MitoTracker staining.

A, B, Apoptotic cells were quantified 18 h after treatment with 10 Gy IR and/or 800 µM vanadate. Columns, mean (n ≥ 3); bars, SD. Lower panel, Cells were harvested 6 h after 10 Gy IR. Proteins were detected by immunoblotting. U, unirradiated control; I, 10 Gy IR; IV, 10 Gy IR plus 800 µM vanadate. Nega, MOLT/Nega; p53KD-1 and p53KD-2, MOLT/p53KD-1 and -2; Mock-1, MOLT/Mock-1; E6-1, E6-2, and E6-3, MOLT/E6-1, -2, and -3. Two mock-transfectants (MOLT/Nega and MOLT/Mock-1) showed similar p53 accumulation and apoptosis sensitivity after treatment as the parental cells.

A, Even after IR, p53 was almost undetectable in the MOLT/p53KD-1, -2 cells, which were resistant to radiation-induced apoptosis. Vanadate did not suppress the modest increases in apoptosis that followed IR in these cell lines. In fact, these knockdown transfectants were even more susceptible to IR when treated with vanadate.

B, In the HPV18-E6-expressing transfectants, although the cells showed resistance to IR, p53 expression was not fully inhibited, and the suppressive effects of vanadate were greatly
reduced. The effects that were seen may have been due to vanadate’s inhibition of the already-reduced p53 expression, the mechanism of which is unknown. Upper panel, RT-PCR analysis of HPV18-E6 expression.

C, Thymocytes from wild-type C57BL/6J mice or Trp53-/− mice were irradiated and treated with or without vanadate. Upper and Middle panels, Open columns, unirradiated samples 6 h after treatment; closed columns, 10 Gy-irradiated samples 6 h after treatment. Columns, mean (n =3); bars, SD. Lower panel, Open circles, unirradiated Trp53-/− samples 12 h after treatment; closed diamonds, 10 Gy-irradiated Trp53-/− samples 12 h after treatment; closed circles, 20 Gy-irradiated Trp53-/− samples 12 h after treatment. Points, mean (n = 3); bars, SD. Vanadate suppressed the radiation-induced apoptosis in thymocytes from wild-type mice at 100 and 150 µM, but not in those of Trp53-/− mice, at any dose of vanadate and IR tested, and at both 6 h and 12 h after IR. We also investigated the loss of mitochondrial membrane potential (Δψm) after the treatments. The percentage of Δψm lost in the wild-type p53 thymocytes roughly correlated with the amount of apoptosis measured by Annexin V-FITC staining, and the p53-null thymocytes showed no loss of Δψm after IR, suggesting that the loss of Δψm was tightly coupled to the loss of p53 function.

D, Effect of vanadate on etoposide-induced apoptosis in p53-mutated leukemia cell lines, KU812 (K132R), CCRF-CEM (R175H/R248Q), and Ball-1 (D281G). MOLT-4, KU812, CCRF-CEM, and Ball-1 cells were treated with 1 µM, 50 µM, 2.5 µM, and 1 µM etoposide, respectively. Etoposide was added to the culture medium immediately after vanadate. Closed circles, etoposide-treated samples with various concentrations of vanadate 18 h after treatment; open circles, etoposide-untreated samples with various concentrations of vanadate 18 h after treatment. Points, mean (n ≥ 3); bars, SD. Lower panel, Cells were harvested 8 h
after treatment. Proteins were detected by immunoblotting, \(U\), untreated control; \(E\), etoposide.

Vanadate had no protective effect on KU812 treated with etoposide, and little effect on CCRF-CEM and Ball-1 cells. By contrast, etoposide treatment induced p53 accumulation in MOLT-4 cells, and vanadate largely blocked their apoptosis. Interestingly, like the knockdown MOLT-4 transfectants, p53-null thymocytes and p53-mutated cell lines tended to be more sensitive to vanadate than the corresponding wild-type p53 controls.

Figure S3. Investigations of possible mechanisms for vanadate’s effect.

\(A\), Effect of several known phosphatase inhibitors, 3,4-Dephostatin (protein tyrosine phosphatase inhibitor) (3), Cypermethrin (protein phosphatase 2B inhibitor) (4), and Okadaic acid (protein phosphatase 2A inhibitor) (5) on the radiation-induced apoptosis of MOLT-4. Closed circles, 10 Gy-irradiated samples 18 h after treatment; open circles, unirradiated samples 18 h after treatment. Points, mean \((n \geq 3)\); bars, SD.

\(B\), Effects of the PI3K inhibitor LY294002 on the suppressive effect of vanadate. MOLT-4 cells were harvested 18 h after treatment. Closed circles, 10 Gy IR; closed diamonds, 10 Gy IR plus 800 µM vanadate; open circles, 800 µM vanadate. Points, mean \((n \geq 3)\); bars, SD. Lower panels, MOLT-4 cells were harvested 6 h after treatment. Phospho-Akt (Ser 473) and the total Akt proteins were detected by immunoblotting.

\(C\), Active myr-Akt1 negatively regulates the suppressive effect of vanadate. \(Mock-1\), MOLT/Mock-1; \(Akt-1\), MOLT/Akt-1. Upper panel, Immunoblotting analysis of the myr-Akt1 expression. Lower panel, Apoptotic cells were quantified 18 h after treatment with 10 Gy IR and/or 800 µM vanadate. Columns, mean \((n \geq 3)\); bars, SD.

\(D\), Neither vanadate nor PFT\(\alpha\) suppressed p53-independent apoptosis, or was an inhibitor of
mitochondria. Vanadate (V), PFTα (α), and anisomycin (A) were used at final concentrations of 800 µM, 50 µM, and 1 µg/µl, respectively. Columns, mean (n ≥ 3); bars, SD; C, Control.

Left panel. Loss of Δψm after anisomycin treatment measured by flow cytometry after MitoTracker staining. MOLT-4 cells were harvested 3 h after treatment. Right panel, Dead cells from the apoptosis induced by the expression of exogenous Bax were measured by the dye-exclusion test. Open columns, mock-transfected samples; closed columns, Bax-expressing samples. HEK293T cells were transiently transfected with each plasmid and treated with each inhibitor 12 h after transfection. The percentage of dead cells was then measured 12 h after the inhibitor treatment.

Figure S4. Loss of Δψm and the conformational change of Bax and Bak after IR were induced in a p53-dependent manner.

Nega, MOLT/Nega; p53KD-1 and p53KD-2, MOLT/p53KD-1 and -2. Open columns indicate unirradiated samples, and closed columns indicate 10 Gy-irradiated samples. Columns, mean (n ≥ 3); bars, SD.

A, The loss of Δψm after IR measured with a flow cytometer after MitoTracker staining. Cells were harvested 12 h after IR.

B, C, The conformational change of Bax and Bak after IR, measured with a flow cytometer after immunofluorescence staining with the anti-Bax or -Bak antibody. Cells were harvested 6 h after IR. Lower panels show immunoblots of Bax and Bak proteins with the same antibody used in the flow cytometric analysis.

Figure S5. Immunofluorescent staining of ts p53 in SaOS-2 stable transfectants.
SaOS-2 stable transfectants were incubated at 39 °C or 30 °C for 24 h. ts p53, mitochondria, and nuclei were visualized by anti-p53, anti-VDAC1 antibody, and DAPI, respectively. Lp53 was mainly localized to mitochondria. After the temperature shift, the mitochondria appeared to aggregate, a known occurrence during apoptosis (6). In the case of Np53, since we captured the temperature-shifted images with the same exposure time as the nonpermissive temperature control, the fluorescence intensity of the nuclear Np53 after the temperature shift was too high for us to detect Np53 at mitochondria. However, Np53’s interaction with Bcl-2 clearly indicated that residual Np53 indeed was localized to mitochondria (Fig. 5D).

Figure S6.

A. Neither vanadate alone nor cPFTα alone (20 mg/kg each) affected the survival of mice. These unirradiated controls were treated as described in Figure 6A except no IR was performed, and no mice died in these control groups. Ten mice were used in each experimental subgroup.

B. Vanadate did not protect mice treated with 13 Gy-TBI. ICR mice were injected i.p. with 20 mg/kg vanadate 30 min before TBI as described for Figure 6A. Ten mice were used in the vanadate-treated subgroup. The data from the group treated with 12 Gy TBI alone were identical to those shown in Figure 6A.

C. RT-PCR analysis of the p53-target genes in bone marrows and small intestines (jejuni). ICR mice were treated as described in Figure 6A, and the tissues were surgically isolated 5 h after TBI. Since noxa and puma well responded to TBI in bone marrow and small intestine, respectively (Left panel), each of them was chosen as an indicator of the transcriptional suppression of p53 by both inhibitors in the respective tissues (Right panel). BM, bone
marrow; SI, small intestine; V, vanadate; α, cPFTα.