Trimidox-Induced Apoptosis Is Mediated Through Induction of p53 in NALM-6 Cells

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Abstract. We examined the effect of trimidox-induced apoptosis involvement of p53 in the NALM-6 cell line of acute lymphoblastic leukemia. Trimidox has been shown to increase the induction of p53. Phosphorylation of p53 protein at Ser-15 and Ser-20 residues was activated earlier than the obvious increase in p53 expression. Pifithrin-α, a p53 inhibitor, significantly prevented trimidox-induced apoptotic characteristics, as detected by nuclear morphological observation and DNA fragmentation. Trimidox-induced apoptosis was enhanced or attenuated by transfection with wild-type or dominant-negative p53 containing expression vectors, respectively. These results indicate that one of the induction mechanisms of apoptosis by trimidox is the mediated augmentation of p53.

Keywords: trimidox, apoptosis, p53

The p53 protein is traditionally believed to be a tumor suppressor. In this regard, various chemotherapeutic drugs have been reported to exert p53-dependent anti-tumor effects by the induction of cancer cell apoptosis, and this therapeutic strategy has been aggressively explored by many researchers, with the modest success explained by the challenge of the effective delivery of p53-expressing vectors into tumor cells (1, 2).

Ribonucleotide reductase (RR) is a rate-limiting enzyme for the de novo formation of deoxyribonucleotides and plays an important role in DNA synthesis. Inhibition of RR is considered a specific target for cancer chemotherapy because the activity of this enzyme is greatly increased in neoplastic cells (3). A novel group of RR inhibitors are polyhydroxy-substituted benzoic acid derivatives (4). Trimidox (3,4,5-trihydroxybenzamidoxime) was shown to be one of the most potent in this series of compounds (5). Our lab has previously reported that trimidox induced apoptosis in a number of typical human leukemia cell lines, and the most susceptible cell was NALM-6, a B-cell line of acute lymphoblastic leukemia, which expresses wild-type p53 (6). The aim of this study was to determine the contribution of p53 to trimidox-induced apoptosis. To address this question we used a reversible p53 inhibitor, pifithrin-α, which has been shown to have anti-apoptotic effects in a number of systems (7) by preventing p53 transactivation (8). More recently, pifithrin-α has been demonstrated to inhibit p53 phosphorylation and subsequent apoptosis (9). Furthermore, we have transfected cells with wild-type p53 or the dominant-negative form of mutant p53-containing expression vectors and examined the effect of trimidox-induced apoptosis.

Trimidox was synthesized as described by van’t Riet et al. (4). Pifithrin-α and other reagents were supplied by either Sigma (St. Louis, MO, USA) or Nacalai Tesque (Kyoto) and were of the highest grade available. All cell culture reagents were obtained from Invitrogen Corp. (Carlsbad, CA, USA).

NALM-6 cells were supplied by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai). Cells were routinely kept in RPMI 1640 medium supplemented with 10% fetal bovine serum.

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and penicillin G (100 U/ml) / streptomycin (100 µg/ml) at 37°C in a humidified 5% CO₂ – 95% air incubator under standard conditions (10).

The expressions of p53, phosphor-p53 (Ser 15 and 20), and β-actin proteins were detected by Western blotting, modified by our previous method (4). Briefly, cells were harvested and extracted by lysis buffer. Samples of each protein (30 µg) were loaded onto a 10% SDS-polyacrylamide gel. After electrophoresis, the protein was transferred to a PVDF membrane, blocked with blocking solution for 4 h and reacted with antibody overnight at 4°C, and then incubated with horseradish peroxidase-linked secondary antibody for 1 h. After another wash, the levels of protein were analyzed by enhanced chemiluminescence with an ECL plus Western blotting detection system (Amersham, Arlington Heights, IL, USA).

Apoptotic cells were estimated by nuclear morphological observation and DNA fragmentation analysis using our previous methods (6, 10).

Briefly, to analyze changes in nuclear morphology, cells were stained in 5 µM bisbenzimide H 33342 fluorochrome trihydrochloride and observed under a fluorescence microscope. Apoptosis was characterized by chromatin condensation followed by partitioning into multiple bodies, and the percentage of apoptotic cells was calculated.

For DNA fragmentation analysis, the qualitative damage to genomic DNA was estimated by agarose gel electrophoresis. The cells were lysed by adding lysis buffer and centrifuged to obtain supernatants of DNA fragmented fractions. They were then incubated for 1 h with RNase A (10 µg/ml) at 37°C, before being digested for 30 min with proteinase K (100 µg/ml) at 56°C to obtain the cell lysate. DNA was precipitated with isopropyl alcohol and 0.5 M NaCl. DNA samples were electrophoresed on a 1.4% agarose gel containing ethidium bromide, visualized under UV light, and photographed.

For transfection, we used the p53 dominant-negative vector set (Clontech Takara-bio, Tokyo), containing p-CMV vectors, wild-type p53 (wtp53), and dominant-negative mutation of p53 (p53mt135); and it was transfected into NALM-6 cells using DMRIE-C transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the instructions provided by the manufacturer.

The results were statistically analyzed by Student’s t-test for two-group comparisons and one-way analysis of variance followed by Dunnett’s test. A P-value of less than 0.05 was considered significant.

We used a trimidox at 250 µM in this experiment as this concentration was shown to cause a time-dependent increase in DNA damage after incubation for 4 h in our previous study (6). First, we examined the effect of trimidox-induced increases in the expression of p53 by Western blotting (Fig. 1a). The expression of p53 slightly increased in early incubation after 30 min and gradually increased with the incubation period. The higher expression of p53 observed with incubation from 2 – 8 h subsequently decreased. The p53 signaling pathway might be down-regulated after incubation with trimidox for 12 h followed by the progression of apoptosis. All lanes exhibited equivalent intensities of β-actin, assayed by Western blotting as the loading control. Phosphorylation plays an important role in regulating the biological activities of p53 (11). Once phosphorylated, the stability of p53 protein is increased, allowing it to act as a transcriptional factor to enhance and repress genes involved in the apoptotic process (12). The important function of activated p53 is critically dependent on the phosphorylation of p53 protein in Ser-15 and Ser-20 residues (13). We therefore assessed the effect of trimidox on the level of phosphorylated p53 at Ser-15 and Ser-20 (Fig. 1b). Following trimidox incubation for 30 min and 1 h, these phosphorylated proteins were significantly increased, but decreased after incubation for 2 h. This finding indicates that trimidox evokes the earlier and transient increase of p53 phosphorylation. Phosphorylation in these residues did not cause a sustained increase in p53 expression after incubation for 2 h. This result suggests that these p53 phosphorylation events were not involved in the long-term stabilization of p53 and were activated earlier, before the significant
increase in p53 expression. Hydroxyurea is one of the RR inhibitors in the same group as trimidox, in which it has been reported that RR inhibitory and cytotoxic effects relate to p53R2, a p53-inducible form of the small subunit of RR (14). Trimidox-induced p53-dependent apoptosis could also be implicated in p53R2. The relation between the induction mechanism of apoptosis by trimidox and the effect on the p53 pathway remain to be elucidated.

To determine if p53 is involved in trimidox-induced apoptosis, cells were treated with a p53 inhibitor, pifithrin-α (20 µM), prior to exposure to trimidox (250 µM) for 1 h (Fig. 2: a–c). Other studies have already shown that pifithrin-α prevents transactivation and phosphorylation of p53 (7–9). Pifithrin-α could attenuate trimidox-induced phosphorylation of p53 protein in Ser-15 and Ser-20 residues in this study (data not shown). There was no observation of apoptotic cells and no detection of DNA fragmentation by incubation with pifithrin-α alone in this experiment (Fig. 2: a and c).

Fig. 2. Effect of pifithrin-α, a p53 inhibitor, on trimidox-induced apoptosis. Cells were pretreated with 20 µM pifithrin-α for 1 h, followed by the addition of trimidox at 250 µM. a: Nuclear morphological observation (×400) and quantitative detection of apoptotic cells after incubation for 12 h. A, Control; B, 20 µM pifithrin-α alone; C, 250 µM trimidox alone; D, pifithrin-α + trimidox. Each value represents the mean ± S.E.M. of three different experiments performed in triplicate. **P<0.01 vs trimidox alone incubation groups. b: Time-dependent increase in DNA fragmentation by 250 µM trimidox. c: Effect of pifithrin-α on trimidox-induced DNA fragmentation after incubation for 12 h. Triplicate experiments gave similar results.

![Fig. 2](image1)

Fig. 3. Effect of transfected wild-type p53 (wtP53) or dominant-negative form of mutant p53 (p53mt135) on trimidox-induced apoptosis. Cells were transfected with wtP53 or p53mt135 for 24 h, followed by the incubation with trimidox at 250 µM for 12 h. a: After transfection with each expression vectors for 24 h, p53 protein was detected by Western blotting and quantitative detection apoptotic cells after incubation with trimidox for 12 h. Each value represents the mean ± S.E.M. of three different experiments performed in triplicate. *P<0.05 or **P<0.01 vs trimidox alone incubation groups in the groups transfected with p-CMV empty vector (mock). b: Effect of transfection with wtP53 or p53mt135 on trimidox-induced DNA fragmentation after incubation for 12 h. Triplicate experiments gave similar results.
As shown in Fig. 2a, incubation for 12 h with trimidox induced apoptosis (52.1 ± 6.3%, n = 3), estimated by nuclear morphological observation, which was significantly prevented by pifithrin-α (24.2 ± 3.6%, n = 3, P < 0.01). The detection of DNA fragmentation using agarose gel electrophoresis showed the time-dependent elevation of DNA fragmentation by incubation with trimidox, which was strongly detected with incubation for 12 h (Fig. 2b). Pifithrin-α also attenuated trimidox-induced DNA fragmentation at that time (Fig. 2c). To confirm the p53-mediated pathway, we have transfected p-CMV vectors containing wtp53 or a dominant-negative form p53mt135, which because of a conformational change, blocks normal p53 function, and examined the effect of trimidox-induced apoptosis. As shown in Fig. 3, there were no inductions of apoptosis by only transfection with each vector. Trimidox-induced apoptosis (53.6 ± 7.3%, n = 3) was enhanced by transfection of wtp53 (75.6 ± 7.8%, n = 3, P < 0.05) and prevented by p53mt135 (18.2 ± 5.5%, n = 3, P < 0.01). Trimidox-induced DNA fragmentation has also enhanced by wtp53 and prevented by p53mt135 (Fig. 3b). These results indicate that trimidox-induced apoptosis is mediated by p53 induction. What is the p53-mediated pathway in trimidox-induced apoptosis? Following the increase of p53 in the induction of apoptosis and cytochrome c translocation to the cytosol, subsequent activation of the executioner cysteine protease, caspase-3, induced apoptotic features such as chromatin condensation and DNA fragmentation (15). We have previously shown that cytochrome c release and caspase-3 activation are central to the apoptotic cascade triggered by trimidox in NALM-6 cells (6). The increase in p53 induction and phosphorylation by trimidox (Fig. 1) occurred prior to the elevation of DNA fragmentation after incubation for 4 h (Fig. 2) in the present study; thus, p53 exists upstream on the apoptosis signaling cascade. These results support that pifithrin-α or p53mt135 inhibits the subsequent apoptosis pathway, which in turn leads to blocking the induction of apoptosis by trimidox (Fig. 3); however, the induction of apoptosis by trimidox is not completely blocked. This result suggests that trimidox can still induce apoptosis via other mechanistic pathways.

In conclusion, the induction of apoptosis by trimidox was dependent on increased expression of p53 and phosphorylation in NALM-6 cells. These results imply that cancer chemotherapy using trimidox may be potently affected by the p53 signal transduction pathway.

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