Triptolide-induced apoptosis in p53 mutant hepatoma cells involves ROS-mutant p53-dependent inhibition effect on p21 expression

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Abstract

Chemotherapeutic drugs had poor cytotoxic effects on cancer cells with mutation in the p53 gene. However, triptolide induced apoptosis in tumor cells with mutant (mt) p53. Here we study the potential therapeutic effects and underlying mechanisms of triptolide against human liver cancer cells bearing mt p53. One hepatic cancer cell line with mt p53 (Huh7) and another p53-deficient hepatic cancer cell line (Hep3B) were evaluated for cell proliferation inhibition and apoptosis induction following treatment with triptolide. Triptolide inhibited cell proliferation and induced apoptosis in Huh7 but not Hep3B cells at lower concentrations. In triptolide-treated Huh7 cells, mt p53 and p21 expressions were induced but significant increase of p21 was later than that of mt p53. Increases in the mt p53 and p21 proteins were mediated by enhanced transcriptions resulting from overproduction of ROS. Triptolide rendered Huh7 cells accumulate in S phase. Additionally, triptolide induced p21 and caused cell cycle to be arrested in G1 phase in Hep3B cells. The expression of exogenous mt p53 prevented increase in p21 induced by triptolide and increased apoptosis in Hep3B cells. The above findings suggest that triptolide-induced mt p53, by repressing p21 elevation, accelerates apoptosis in hepatoma cells.

Introduction

Studies demonstrated that some conventional chemotherapy drugs were inefficient in killing p53 gene mutated tumor cells [18, 19]. However, triptolide, a purified compound extracted from Chinese herb *Tripterygium Wilfordii* hook F, was able to trigger apoptosis in cancer cells whose p53 activity was lost or p53 gene was mutated. For example, triptolide was proven to be capable of inducing apoptosis in HeLa and PANC-1 cells, both of which contained little p53 activity [23]. This was because the p53 proteins in HeLa cells were quickly degraded by the E6 proteins expressed by Papillomavirus [15, 25] and PANC-1 cells had a point mutation in the p53 gene which made the p53 protein inactive [20, 21]. Kuvigarju et al. showed that higher concentration of triptolide induced apoptosis in the primary cultures of human prostatic epithelial cells by increasing p53 but reducing p21 level although function of the wild-type p53 protein was impaired in the prostatic epithelial cells [10]. In addition, Chang et al. found that triptolide enhanced chemotherapy-induced apoptosis by increasing p53 expression but blocking p21 mRNA transcription in HT1080 cells with wild-type p53 [4]. Thus, the above studies indicated that triptolide induced apoptosis in cancer cells independently of the basal function of p53 and was able to activate the expression of p53, but induced apoptosis by inhibiting the transactivation function of p53 and blocking p21-mediated growth arrest in cancer cells. Inconsistent with such mechanism, Jiang et al. showed that triptolide-mediated activation of p53 induced p21 expression in gastric cancer cells [9]. Thus, whether transactivation or transcriptional repression of p53 played a role in the apoptosis induction might vary for cell types and/or stress intensities.

Presently, the mechanism by which triptolide suppressed the transcription of p21 is unclear. Chang et al. reported that triptolide repressed transcription of p21 in HT1080 cells but had no effect on p21 expression in HT-29 colon cancer cell line with mutant (mt) p53. In addition, they observed that triptolide induced phosphorylation of p53 in HT1080 cells although the site of p53 phosphorylated by triptolide

was not revealed [4]. These results implied that p53 might be involved in inhibiting p21 expression in triptolide-treated tumor cells.

In this study, we investigate the mechanism of triptolide resisting human liver cancer cells containing mt p53. We show that triptolide carries out its anti-mt p53 liver cancer cells effect by inducing apoptosis. Triptolide increased the transcriptions of mt p53 and p21 by inducing ROS in hepatoma cells but the expression of p21 protein appeared to be suppressed by the mt p53 protein. In addition, triptolide induced p21 expression and failed to bring out apoptosis in p53-null hep3B cells. But the expression of exogenous mt p53 inhibited an increase in p21 caused by treatment with triptolide and enhanced apoptosis in Hep3B cells. Our results suggest that triptolide induces apoptosis in hepatoma cells with mt p53 possibly in part by mt p53-mediated blocking of p21 expression which is induced by triptolide itself.

Materials And Methods

Cell lines and chemicals

Two human hepatocellular carcinoma-derived cell lines were used: (a) Huh7 cells expressing mutant p53 (tyrosine → cysteine at codon 220, Y220C) [8]; (b) Hep3B cells lacking p53 [1]. Huh7 and Hep3B cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Triptolide (purity, ≥ 97%; molecular weight, 360.40096) was purchased from Shanghai Tongtian Biotechnology Co., Ltd. (Shanghai, China). Tempol was bought from MedChemExpress (MCE) (Shanghai, China).

Cell culture and transfection of plasmids

HuH7 and Hep3B cells were maintained in High Glucose Dulbecco's modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin and 100 ug/ml streptomycin (Gibco).

The mt p53 (Y220C) ORF clone of Homo sapiens tumor protein p53 which was used as a p53 mutant (Y220C) expression plasmid and the vector control were purchased from OriGene (Rockville, MD, USA). Lipofectamine 3000 kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was used for transfection of plasmids into Hep3B cells. Based on the manufacture's instruction, Hep3B cells at approximately 80% confluence were transfected with mt p53 (Y220C) ORF clone expression plasmid or vector control in duplicate in a 6-well plate. After incubation in the atmosphere contained 5% CO_2 for 8 h at 37 ℃ in a humidified incubator, the medium of a copy of transfected cells was replaced with fresh medium in which triptolide was added. After 40 h of culture, cells from each well were harvested for protein/mRNA expression or apoptosis induction analysis.

CCK-8 assay

Approximately 2 000 cells per well were seeded in 96-well cell culture plates and incubated overnight in 100 microliter cell culture medium. Cells were subsequently treated with four different concentrations of

triptolide for 48 h. Then, 10 microliter cell counting kit 8 (CCK-8) (MCE, Shanghai, China) was added into each well and the cells were incubated for another 3 h in the cell incubator. A microplate reader (BioRad, CA, USA) was used for measuring the absorbance at wavelength of 450 nm.

Annexin V-FITC/PI analysis

To determine the apoptosis and necrosis, cells were firstly incubated with various concentrations of triptolide for approximately 32 h and then were processed according to the instruction manual of FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA, USA). Finally, the stained cells were immediately analyzed by a flow cytometer LSR Fortessa (BD Biosciences, San Jose, CA, USA) using the excitation wavelengths of 488 nm and 535 nm. At least 10 000 cells were used for analysis in each group.

Quantitative real-time PCR

The total RNA of cells was extracted with RNAiso Plus (TaKaRa, Otsu, Japan). The concentration and purity of RNA was measured by Nanodrop 2000c (Thermo Fisher Scientific, USA). cDNA was prepared using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa). The primers for Real-time PCR analysis were listed in Table 1. The PCR amplification was completed on ABI PRISM 7900HT/FAST using TB Green Premix Ex Taq (Tli RNaseH Plus) (TaKaRa) and 40 cycles of 95 ℃ for 5 s and 60 ℃ for 30 s. GAPDH was used as an internal parameter. Fold change for the mRNA level of molecule in the treated groups versus that in the control group was calculated using the $2^{-\Delta\Delta Ct}$ method [13, 27].

Cell cycle determination

Huh7 and Hep3B cells (1×10^6) were treated with triptolide (20 ng/ml) for 24 h. Cells were harvested and washed with ice-cold PBS. Cells were then gently resuspended in 5 ml of ice-cold ethanol (70–80%) and stored at 4 ℃ for at least 18 h. After washing with PBS and staining buffer (BD Pharmingen) successively, the cells were incubated with PI/RNase staining buffer (BD Pharmingen) at room temperature for 15 min. Subsequently, the samples were determined by flow cytometry and cell cycle was analyzed with ModFit LT software (version 3.1) (Verity Software House, Topsham, ME, USA).

Western blotting

BCA Protein Assay kit (Pierce Chemical Co., Rockford, IL, USA) was used for evaluating the concentration of total protein. Forty microgram proteins of each group were separated by 4–20% SurePAGE, Bis-Tris gels (GenScript, Nanjing, China); and transferred to polyvinylidene difluoride membrane (Merck Millipore, Billerica, USA); and then were probed with the specific primary antibodies and horseradish peroxidase-conjugated anti-IgG antibodies. Target proteins were displayed by enhanced chemiluminescence (ECL) kit (BeyoECL Plus) (Beyotime, Shanghai, China) and the signals were captured by chemiluminescence detection system (Clinx Science Instruments, Shanghai, China). Rabbit monoclonal antibody against caspase-8, caspase-9, caspase-3, PARP, p21, p53, Phospho-p53 (Serine20) or c-myc as well as peroxidase-

conjugated anti-rabbit IgG were purchased from Cell Signaling Technology (Danvers, MA, USA), Rabbit multiclonal antibody against GAPDH was purchased from Bioker Biotechnology (Hangzhou, China). Densitometric calculations of protein bands were performed by using ImageJ software (NIH, Rockville Pike, Bethesda, Maryland, USA)

Statistical analysis

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was employed for the statistical analysis. Values were presented as mean ± S. D. of at least three experiments. Statistical comparisons were conducted using one-way analysis of variance (ANOVA). The level of significance was set at $P < 0.05$ or $P < 0.01$.

Results

Effects of triptolide on the proliferation of hepatocellular carcinoma cells

We used CCK-8 method to analyze the inhibition effects of triptolide on Huh7 and Hep3B cells proliferation. Figure 1 shows that triptolide inhibited the proliferation of Huh7 cells (Fig. 1A) but not Hep3B (Fig. 1B) cells in a dose-dependent manner when used for treatment of cells at concentrations ranging from 10 to 30 ng/ml. After 48 h of treatment with 30 ng/ml of triptolide, approximately 60% Huh7 cells survived.

Triptolide induced apoptosis in Huh7 cells

Annexin V staining analyzed by flow cytometry shows that treatment with triptolide significantly increased Huh7 cells (Fig. 2A) but not Hep3B cells (Fig. 2B) stained positive for both Annexin V and PI dose-dependently. It was reported that caspases including caspase-8, caspase-9 and caspase-3 participated in apoptosis induced by triptolide [11, 23]. To further verify triptolide induced apoptosis in Huh7 cells, we determined activation of the above caspases. In addition, we also examined poly (ADP-ribose) polymerase (PARP) cleavage by western blot. Figure 2C demonstrates that the procaspase 3/8/9 was cleaved into subunits after 22 h of treatment with triptolide. Meanwhile, an 89 kDa fragment cleaved from 116 kDa PARP was detected in the extracts from Huh7 cells treated with triptolide. These results indicate that triptolide stimulates apoptosis in Huh7 cells. In contrast, no significant procaspase or PARP cleavage was shown in triptolide (20 ng/ml)-treated Hep3B cells by Western blotting (data not shown).

Triptolide induced mt p53 and p21 expression

There were studies indicating that triptolide induced apoptosis in tumor cells with wild-type or function-impaired p53 by increasing p53 and blocking p21 expression [4, 10]. Therefore, time-course tests were conducted to examine the protein levels of mt p53 and p21 after treatment with triptolide. Figure 3A shows that there was little basal mt p53 and p21 level in untreated Huh7 cells, but significant increase of the mt p53 protein in triptolide (20 ng/ml)-treated Huh7 cells began from 12 h of treatment and reached a maximal level after treatment for 36 h. In contrast, the protein level of p21 started to rise after 36 h of

treatment and achieved the highest level after 48 h within the time frame of this test. We also treated Huh7 cells with triptolide at a higher concentration (30 ng/ml) and determined the protein levels of mt p53 and p21. As shown in Fig. 3A, mt p53 protein level was enhanced to the maximal level after 24 h and p21 level was significantly increased after 36 h. Therefore, the above results show that treatment with triptolide increases the accumulations of mt p53 and p21 but induce the greatest increase of p21 always 12 h later than induce that of mt p53, which gives a hint that mt p53 may repress p21 expression. The time-course experiments were also carried out to determine the protein levels of p21 in p53-null Hep3B cells after treatment with triptolide. In Hep3B cells, p21 levels were increased by treatment with triptolide (20 ng/ml) time-dependently and reached the maximal level after 48 h which was shorten to 24 h by treatment with 30 ng/ml triptolide (Fig. 3B). This result indicates that triptolide increases p21 in hepatoma cells in a p53-independent manner and in Huh7 cells, the delayed expression of p21 under treatment with the same dose of triptolide possibly results from the suppression effect produced by triptolide-induced mt p53.

Triptolide increased mt p53 and p21 mRNA expression by inducing ROS

We next checked whether increases in the mt p53 and p21 protein levels attributed to increase in the mt p53 and p21 mRNA level. Our result showed that the mRNA level of mt p53 and p21was enhanced by triptolide in a dose-dependent manner (Fig. 4A and Fig. 4B). Previous studies demonstrated that triptolide treatment forced generation of a large amount of ROS in tissues or cells [2, 26] and ROS could induce p53 or p21 expression at the transcriptional level [7, 24]. As expected, in Huh7 cells, elevation of the mt p53 and p21 mRNA level promoted by triptolide were dose-dependently inhibited by Tempol, a general SOD-mimetic drug that neutralized ROS efficiently (Fig. 4C and Fig. 4D). And elevation of the mt p53 protein level was also dose-dependently repressed by Tempol (Fig. 4E). Interestingly, the p21 protein level appeared to increase firstly and then decrease with the increasing dose of Tempol (Fig. 4F), which also implies that the possible inhibiting effect of mt p53 on p21 protein expression.

Triptolide mediated S arrest in Huh7 cells

It was documented that triptolide caused accumulation of cells in S phase and inhibited growth arrest by blocking p21 expression [4, 10]. We thus examined the effect of triptolide treatment on the cell cycle progression in Huh7 cells. Figure 5A and Fig. 5B show that Huh7 cells were arrested in the S phase after 24 h of 20 g/ml triptolide treatment (to 50.83 ± 0.70% from 33.74 ± 0.22% in untreated cells). However, triptolide at 20 ng/ml for 24 h enhanced the proportion of p53-null Hep3B cells in G1 phase from 54.59 ± 0.00% in control to 63.30 ± 0.95% in treated cells (Fig. 5C and Fig. 5D). This result probably correlates with triptolide-mediated increase of p21 in Hep3B cells for p21 had greatest affinity to G1 cyclin-CDK complexes [3, 5, 22]. Thus, our results provide a clue that in Huh7 cells, triptolide induces cell cycle arrest in S phase possibly by inhibiting the increase in p21.

Exogenous mt p53 expression inhibited the expression of p21 induced by triptolide and enhanced apoptosis in Hep3B cells

To confirm that accumulation of the mt p53 produced by the effect of triptolide on Huh7 cells was conversely involved in inhibiting the expression of p21 which was also induced by treatment with triptolide, we expressed mt p53 in Hep3B cells using a mt p53 vector. Transfection with Lipofectamine 3000 reagent strongly induced exogenous mt p53 expression that was reduced by triptolide (20 ng/ml) treatment (Fig. 6A). The p21 protein expression induced by triptolide increased to 2.63-fold of control (p21 level in untreated cells) in cells transfected with vector control but increased to 1.81-fold of control in cells transfected with mt p53 expression plasmid (Fig. 6A and Fig. 6B). Subsequently, we quantified p21 mRNA level to investigate whether the expression of exogenous mt p53 downregulated p21 mRNA at the transcriptional level. Our result demonstrated that p21 mRNA level enhanced by triptolide treatment in Hep3B cells was downregulated by the exogenous mt p53 (data not shown). These results manifest that the expression of exogenous mt p53 suppresses the increase in p21 protein, at least in part, by repressing the transcription of p21 mRNA.

To assess whether apoptosis was enhanced by the exogenous mt p53 in triptolide-treated Hep3B cells, we analyzed the Annexin V and PI staining results of cells using flow cytometry. Figure 6C shows that exogenous mt p53 expression enhanced cytotoxic synergy between triptolide and transfection reagent in Hep3B cells.

Exogenous mt p53 enhanced c-myc expression and was phosphorylated

As c-myc could suppress the transcription of p21 by binding with the promoter [12], we investigated if the expression of mt p53 increased c-myc, by which it suppressed p21 expression. In Hep3B cells, the endogenous c-myc protein levels were significantly reduced by the lipoplex transfection or treatment with triptolide (0.60-fold of control) but increased by the expression of exogenous mt p53 (1.21-fold of control); the exogenous mt p53 expression also resisted the decline in the c-myc level effected by treatment with triptolide (0.90-fold of control) (Fig. 7B). These results suggest that the exogenous mt p53 inhibits the expression of p21 induced by triptolide possibly by activating c-myc-mediated transcriptional repression.

Many studies demonstrated that the transcriptional activity of p53 was regulated by phosphorylation modification [14, 16, 17]. Thus, we examined if the in vitro expression of mt p53 undergone phosphorylation. Figure 7A shows that Liposome transfection-mediated the expression of mt p53 in Hep3B cells was phosphorylated at Serine (Ser)20.

Discussion

Triptolide induced apoptosis in several p53 mutant tumor cells or tumor cells that were loss of p53 activity [10, 11, 23]. However, some anticancer drugs could not induce significant apoptosis in tumor cells with mutated p53 [18, 19]. This suggested that triptolide might have potential of anti-chemoresistance. In the present study, we investigate whether triptolide can induce apoptosis in p53 mutant or p53 missing human liver cancer cells. Our results show that triptolide induced apoptosis in p53 mutant hepatocarcinoma cell line Huh7 but did not cause significant apoptosis in p53 deficient Hep3B.

Chang et al. evidenced that triptolide induced apoptosis in the wild-type p53 tumor cells by increasing the translation of p53 protein but reducing the transcription of basal p21 or p21 induced by doxorubicin [4]. In contrast, our findings display that triptolide induced mt p53 accumulation in Huh7 cells by increasing the transcription of mt p53 mRNA. We further elucidated that ROS produced by Huh7 cells under treatment with triptolide promoted the transcription of mt p53 mRNA. In addition, our results reveal that the p21 mRNA transcription was also enhanced by ROS accumulation. However, the kinetics of p21 protein did not synchronize with that of the mt p53 and the marked increase of p21 occurred at a timepoint which the protein level of mt p53 was reduced after the prior increase, which presents a possibility that p21 expression is not directly induced but suppressed by the mt p53. We demonstrated that Huh7 cells were accumulated in S phase after treatment with triptolide. Interestingly, triptolide did induce cell cycle arrest in S phase when it triggered apoptosis by increasing p53 but inhibiting p21 [4, 10]. Subsequently, we illustrated that the expression of exogenous mt p53 was involved in inhibiting the expression of basal or triptolide-induced p21 and increasing apoptosis in Hep3B cells treated with triptolide, which confirms that mt p53 accumulation repressed the expression of p21.

Li et al. found that Trichostatin A, the histone deacetylase inhibitor, activated p21 expression independently of p53 through downregulation of c-myc [12]. Coincidentally, our results show that treatment with triptolide increased p21 expression and reduced the protein level of c-myc in p53-null Hep3B cells. Thus, it is possible that triptolide increases the expression of p21 by downregulating c-myc in Hep3B cells. Basing on this, we hypothesized that mt p53 suppressed the expression of p21 by increasing c-myc. As expected, we observed that the expression of exogenous mt p53 enhanced the endogenous c-myc levels in Hep3B cells. This result is consistent with a study which proved that tumor-derived p53 mutants were potent activators of the *c-myc* promoter and could upregulate the expression of endogenous *c-myc* gene in a N- and C-terminal- domain-dependent manner [6]. Taken together, our study suggests that mt p53 inhibits the expression of basal or triptolide-induced p21, possibly by activating *c-myc* gene.

It was proposed that triptolide-induced apoptosis which probably resulted from p21 expression blocked involved p53 phosphorylation [4]. In the present study, mt p53 was significantly phosphorylated at Ser20 when expressed in Hep3B cells in the presence or absence of triptolide, which implies that cytotoxicity brought out by the transfection regent results in mt p53 phosphorylation at Ser20. However, currently, we cannot ensure that phosphorylation of Ser20 in mt p53 is required for mt p53-mediated inhibition of p21 expression.

In all, our results indicate that mt p53-mediated inhibition of p21 expression which likely derived from the increase in the transcription of c-myc is involved in triptolide-induced apoptosis in hepatocellular carcinoma cells. In future, we intend to firstly investigate the activation effect of mt p53 phosphorylation at Ser20 on *c-myc* transcription to disclose the mechanism by which triptolide restrains the expression of p21.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Weixia Liu: Methodology, Data analysis, Writing-Original draft preparation. **Haihong Zhu:** Software, Investigation. **Min Zheng:** Conceptualization, Methodology. **Feng Chen:** Software, Editing. **Zhi Chen:** Supervision.

Data Availability

The datasets generated during the current study are not publicly available due to the study has not been published but are available from the corresponding author on reasonable request.

Ethics approval

This is an in vitro study without involving human or animal subjects. Research Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University has confirmed that no ethical approval is required.

Consent to participate

The manuscript does not contain clinical studies or patient data. Therefore, a statement on informed consent is not applicable.

Consent to publish

The manuscript does not contain clinical studies or patient data. Thus, a statement confirming that consent to publish is not appliable.

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Tables

Table 1 The primer sequences for quantitative RT-PCR analysis

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
p53	AGCTTTGAGGTGCGTGTTTG	TCAGCTCTCGGAACATCTCG
p21	TGATTAGCAGCGGAACAAG	AAACAGTCCAGGCCAGTATG
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

Figures

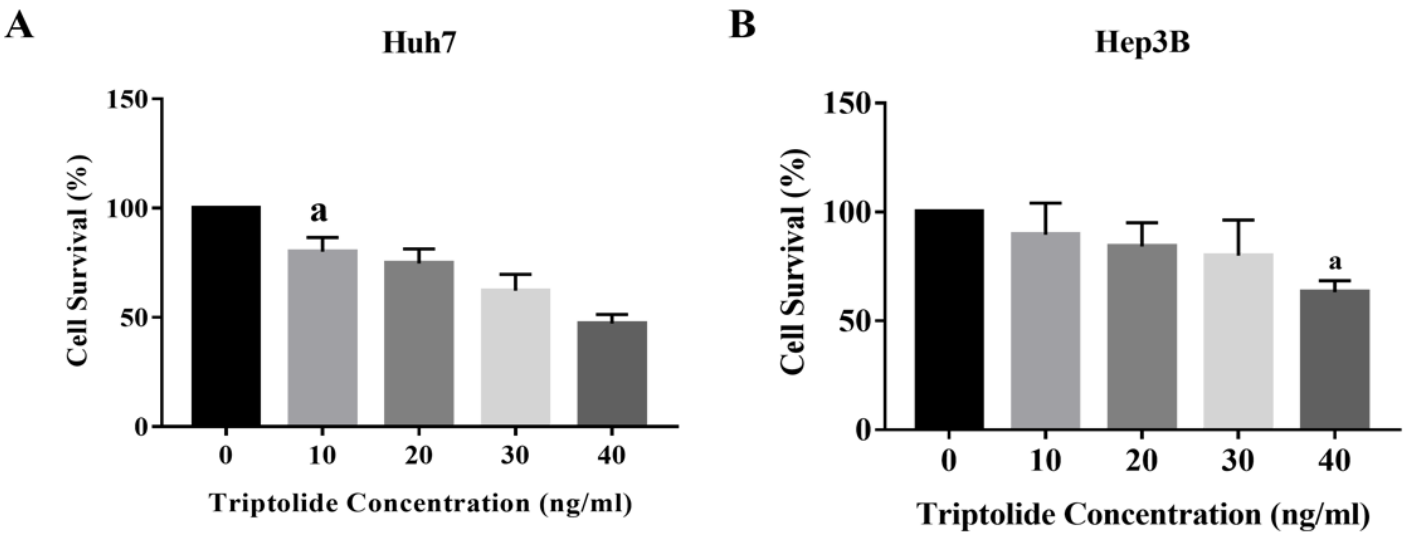

Figure 1

Effects of triptolide on cell proliferations in Huh7 and Hep3B cells. Cell growth inhibition responses in Huh7 (A) and Hep3B cells (B) to various concentrations of triptolide assayed by CCK-8. The percentages of cells survived are expressed as mean ± S. D. from three independent experiment. a: $P < 0.05$, compared with 0 ng/ml triptolide (A) or 30 ng/ml triptolide (B).

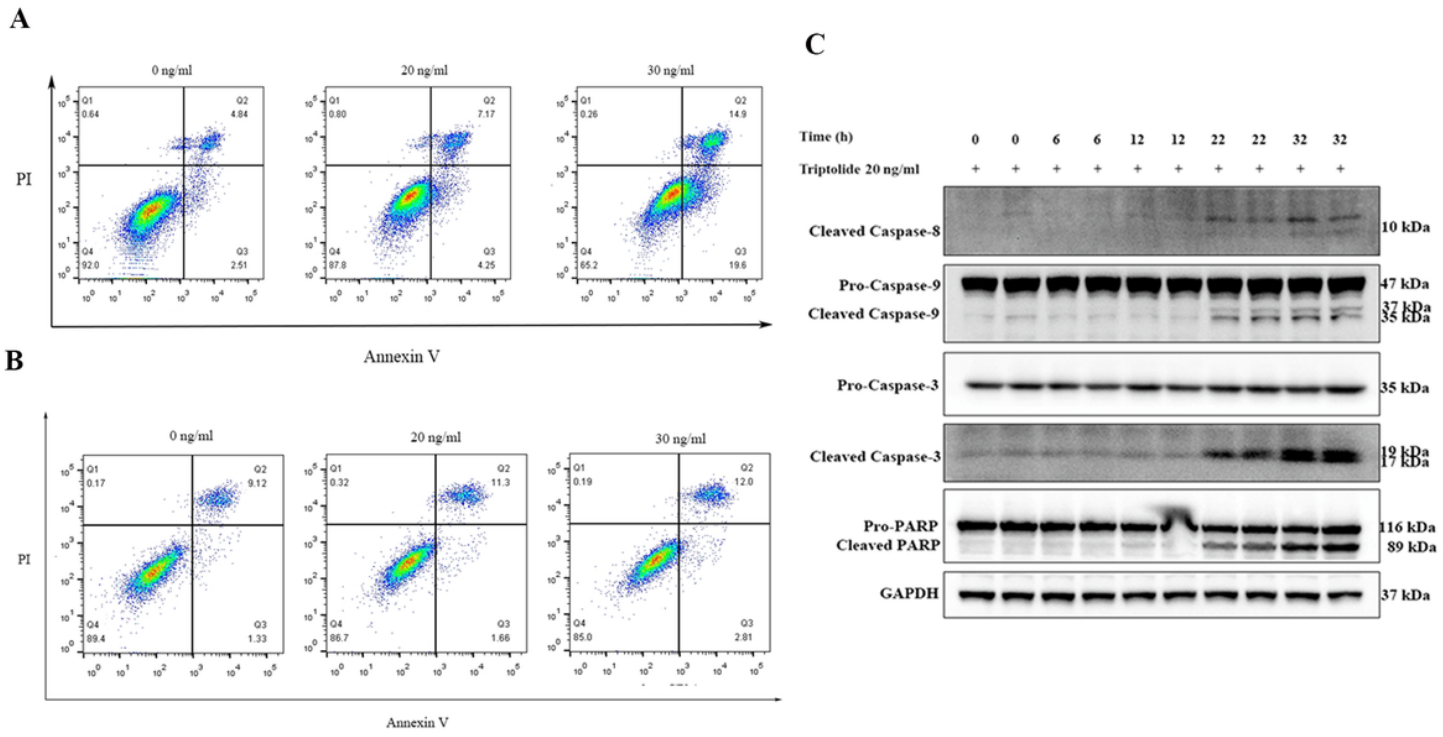

Figure 2

Triptolide induced apoptosis by producing externalization of phosphatidylserine and cleavage of procaspases/PARP in Huh7 cells. Huh7 cells and Hep3B cells were treated with 20, 30 ng/ml triptolide for 24 h, respectively. Cells stained with annexin V and PI were examined by flow cytometry. Ratios of early apoptotic cells and late/necrotic apoptotic cells are shown in scattergrams of control and triptolide-treated Huh7 cells (A) or Hep3B cells (B). (C) Analysis of caspase-8, caspase-9, caspase-3 and PARP expression in Huh7 cells treated with triptolide. Cells were treated with 20 ng/ml triptolide for 0, 6,12, 22 and 32 h. After that, the cleavages of caspase-8, caspase-9, caspase-3 and PARP were assayed by Western blotting. All these experiments were repeated at least three times.

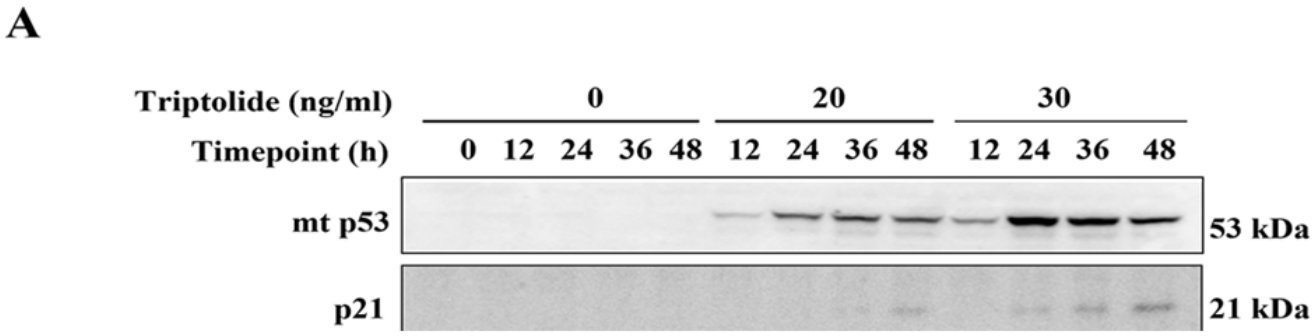

Figure 3

The expression of mt p53 and p21 protein regulated by treatment with triptolide. Triptolide (20, 30 ng/ml) was used to treat the cell line Huh7 and Hep3B for various time intervals (12, 24, 36, 48 h). The protein levels were determined by Western blots. (A) Huh7 cells; (B) Hep3B cells. The experiment was repeated three times.

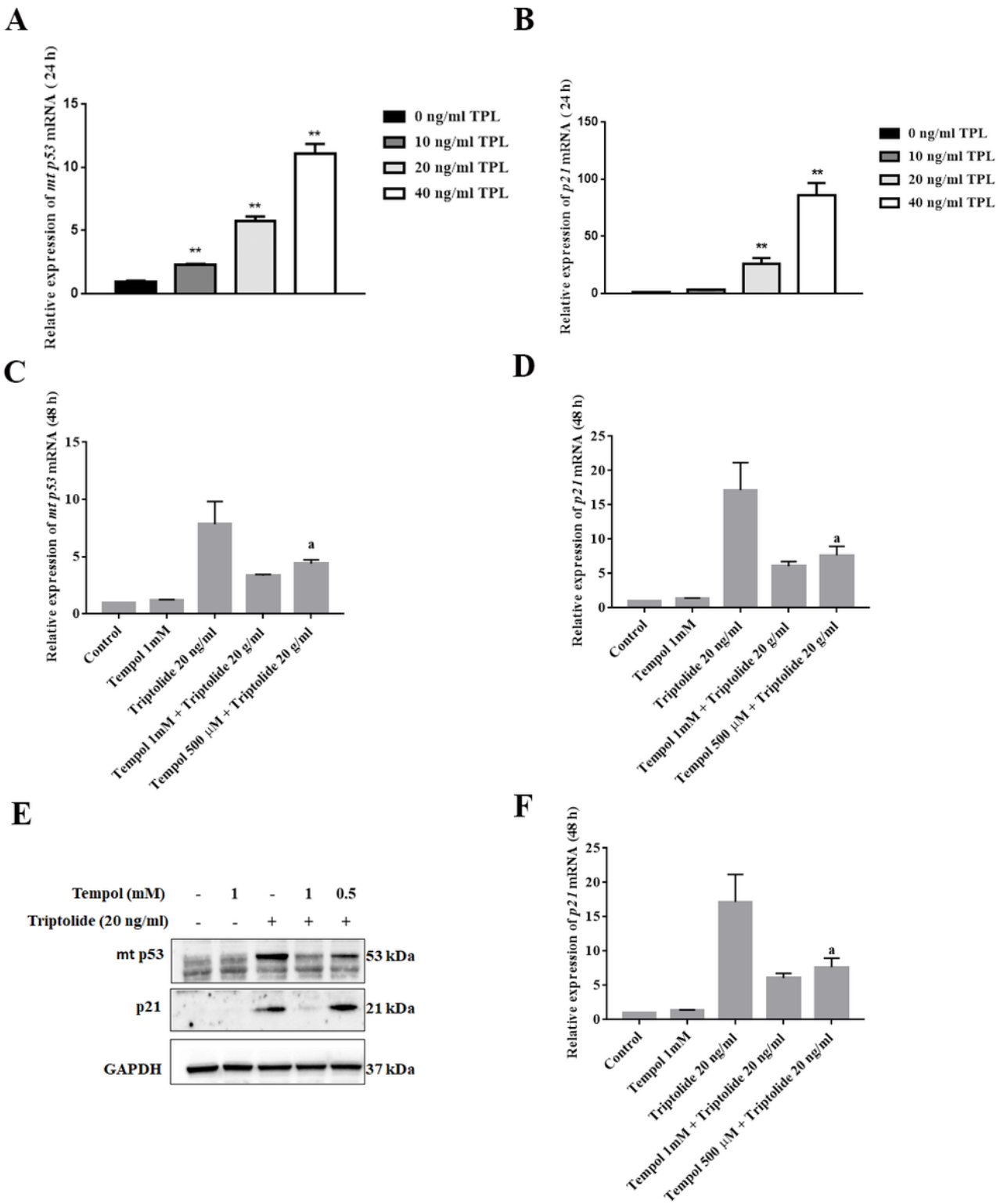

Figure 4

Triptolide promoted mt p53 and p21 mRNA expression by inducing ROS. (A and B) Quantitative RT-PCR analysis of *mt p53* and *p21* mRNA level was conducted after treatment with triptolide (TPL) (10, 20, 40 ng/ml) in Huh7 cells for 24 h. mRNA expression is presented relative to *GAPDH* and expressed as mean ± S.D. from three individual experiments. **: Comparison analysis of mRNA level in Huh7 cells treated with various concentrations of TPL ($P < 0.01$). (C and D) Tempol (500 μM, 1 mM) was added or not for 2 h to

Huh7 cells followed by the addition of 20 ng/ml triptolide for 46 h. Then *mt p53* and *p21* mRNA expression was analyzed and is shown as described above. a: $P < 0.01$, compared with 20 ng/ml triptolide. (E) Cells were harvested for Western blot assay with anti-p53, anti-p21, anti-PARP or anti-GAPDH antibody. GAPDH was used as a control for loading. (F) The relative intensities of mt p53 and p21 protein expression in Huh7 cells were quantified by ImageJ software and are expressed relative to GAPDH.

Figure 5

Cell cycle examination. Huh7 and Hep3B cells were treated with 20 ng/ml triptolide for 24 h. Then their DNA contents were determined by flow cytometry based on PI-mediated DNA staining. Data analyses were performed with ModFit LT 3.1 program. The percentage of cells in each phase of the cell cycle represents mean of triplicates from two experiments ± S. D. (A) Huh7 control; (B) HuH7 treated with triptolide; (C) Hep3B control; (D) Hep3B treated with triptolide.

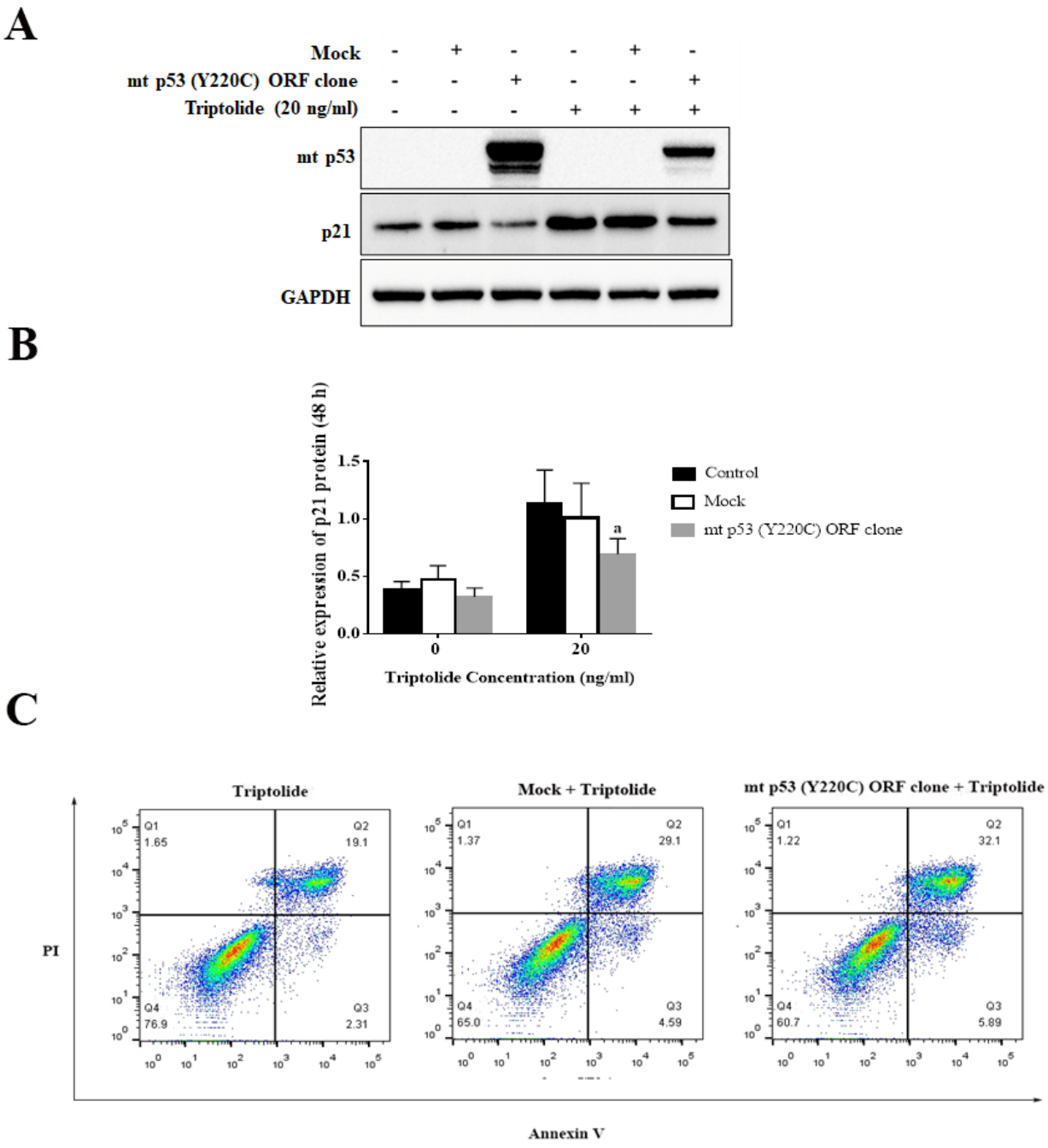

Figure 6

Exogenous mt p53 expression inhibited the expression of endogenous p21 protein and increased apoptosis upon triptolide treatment. (A) Either a mt p53 (Y220C) ORF clone in which the expression of mt p53 gene is controlled by the CMV promoter, or the corresponding control plasmid lacking the mt p53 insert (*mock*) was transiently transfected into $p53^{-/-}$ Hep3B cells in duplicate. After 8 h of the transit transfection, 20 ng/ml triptolide was added into a half of the cells and incubating cells for 40 h. Cells

were then harvested for immunoblot analysis with anti-mt p53 and anti-p21, respectively. GAPDH levels were used for normalization of the protein level of p21. The results are representative of three separate experiments. (B) The p21 protein levels detected by Western blotting in Hep3B cells after treatments described above. a: Comparison analysis of the relative p21 protein expressions amongst Hep3B cells treated as depicted previously ($P < 0.05$, compared with transfection with vector control supplemented with triptolide or not). (C) Cells identically treated were collected to analyze apoptosis by examining Annexin-V and PI staining results of cells.

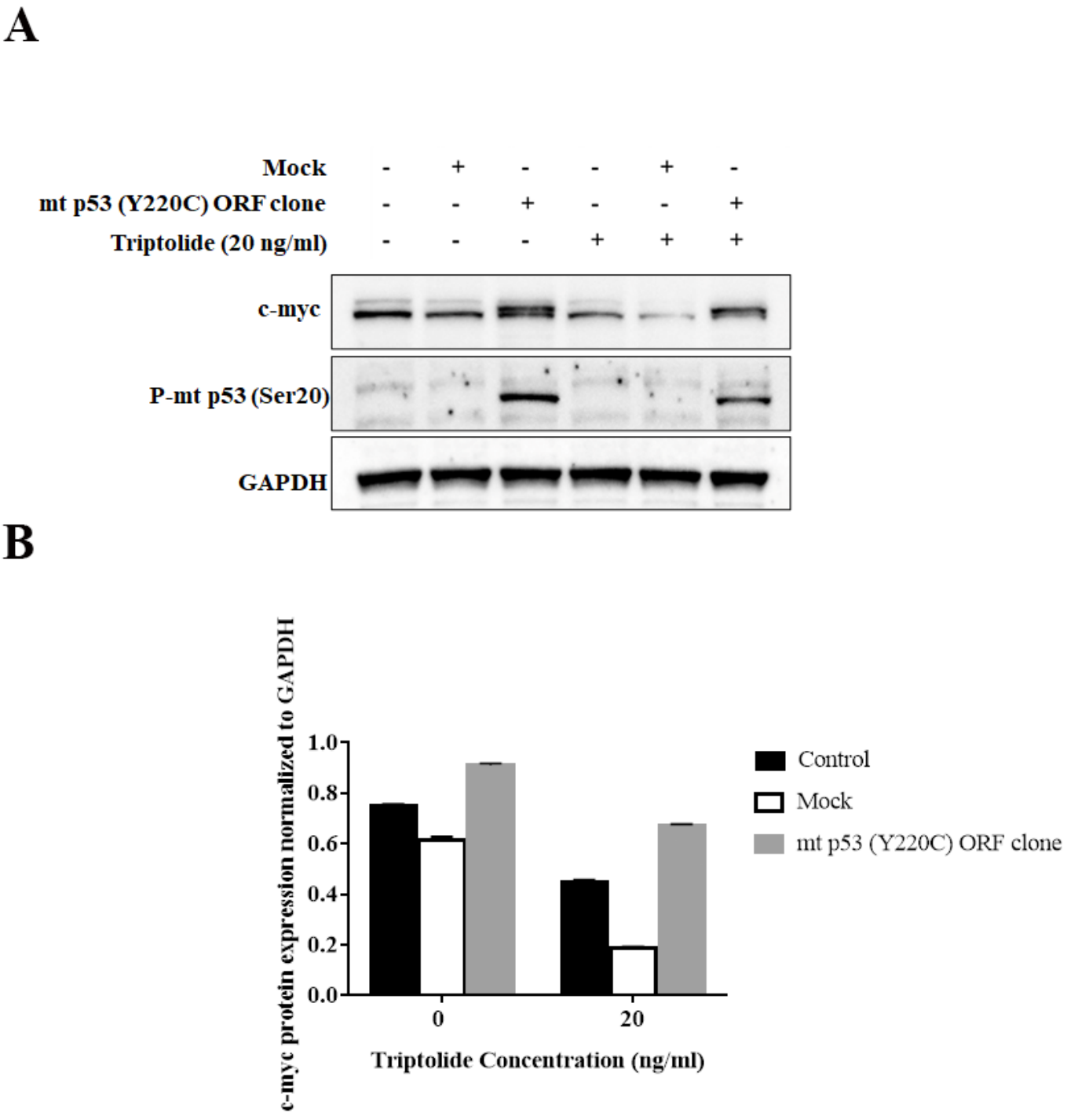

Figure 7

Expression of the exogenous mt p53 induced expression of the endogenous c-myc and was phosphorylated at Ser20. Cells were treated as described in the figure legend of figure 6A. (A) c-myc and P-mt p53 (Ser20) levels were mesured by Western blots. (B) The relative intensity of c-myc and GAPDH expression was determined using ImageJ program. c-myc expression is expressed relative to GAPDH.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- OriginaldataCtvaluesofqPCR.pdf
- OriginaldataFullimagesofWesternblot.pdf