Triptolide inhibits viability and induces apoptosis in liver cancer cells through activation of the tumor suppressor gene p53

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Abstract. The present study investigated the effect of triptolide on viability and apoptosis along with underlying mechanism in liver cancer cells. CCK-8 assay showed that triptolide treatment for 48 h significantly reduced the viability of HepG2 and QSG7701 cells at 50 μM concentration. Annexin V-FITC and propidium iodide staining showed that triptolide treatment of HepG2 cells at 50 μM concentrations induced apoptosis in 56.45% cells compared to only 2.36% cells in the control cultures. Western blot assay showed that treatment of HepG2 cells with 50 μM concentration of triptolide significantly induced phosphorylation of p53 in a 2 h-treatment. Phosphorylation of histone H2A.X indicator of DNA damage was induced by triptolide treatment after 12 h in HepG2 cells. The level of nuclear p53 in a 6 h-treatment with 0, 10, 20, 30, 40 and 50 μM concentration of triptolide was found to be 15.3, 19.6, 28.5, 43.7, 63.8 and 91.5%, respectively. Treatment of HepG2 cells with triptolide at 50 μM concentration caused a significant increase in the binding potential of p53 to DNA. Triptolide treatment of HepG2 cells caused a significant increase in the expression of p21, Bax and DR5 genes in HepG2 cells. It also increased the expression of miR-34b and miR-34c in HepG2 cells markedly. Treatment of HepG2 cells with p53 inhibitor, pifithrin-α prior to incubation with triptolide significantly prevented induction of cell apoptosis. Suppression of p53 expression by siRNA inhibited the expression of p53 as well as its target genes along with the prevention of apoptosis induction. In conclusion, triptolide inhibits viability and induces apoptosis in liver cancer cells through activation of the tumor suppressor gene p53. Thus, triptolide can be used for the treatment of liver cancer.

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Introduction

Triptolide, one of the members from diterpenoid family is obtained during the phytochemical investigation of *Tripterygium wilfordii Hook F* (1). It has a long history of use in traditional Chinese medicine for various diseases. Biological screening of triptolide has revealed its potential as anticancer agent, an immunosuppressor and as contraceptive (1-3). Triptolide treatment induces apoptosis in cancer cells through cytochrome *c* transfer into the cytosol and inhibition of expression of anti-apoptotic proteins (4). The apoptotic proteins including caspases play a vital role in the induction of apoptosis in cancer cells leading to alteration in cell morphology and biochemical changes (5-8).

Hepatocellular carcinoma is a commonly detected cancer and the third leading cause of cancer related deaths throughout the world (9,10). Studies have revealed that every year more than 600,000 new cases of primary liver cancer are detected (9,10). Despite the improvement in the 5-year survival rate of liver cancer patients, the rate of prognosis of liver cancer patients is very low (11). Liver cancer because of resistance to currently available chemotherapeutics and radiotherapy easily shows metastasis in other tissues such as lungs, lymph nodes, bones and adrenal glands (12). Therefore, development of novel and effective treatment strategies for liver cancer is required.

Tumor suppressor gene (p53) controls the function of several other genes involved in various cellular processes such as apoptosis induction, arrest of cell cycle and repair of DNA damage (13-16). Thus, p53 is involved in the formation and progress of various types of cancer. Activation of p53 gene takes place during cell stress through phosphorylation. Among various activation sites available on p53 the most important site of its activation is the phosphorylation at serine-15 (17,18). Studies have shown that during stress by oxidants and radiations p53 is phosphorylated which then induces cell apoptosis (19-21). On activation p53 induces the expression of various other genes such as p21 and Bax which are well known for their role in cellular apoptosis (22-25). Thus, activation of p53 in cancer cells can play an important role in the arrest of cancer development and progress. In the present study, effect of triptolide on the viability and apoptosis of HepG2 and QSG7701 liver cancer cells as well the underlying mechanism was investigated. The present study demonstrated that trip-

tolide induced inhibition of liver cancer cell viability involving activation of the tumor suppressor gene p53.

Materials and methods

Cell culture and reagents. HepG2 and QSG7701 liver cancer cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Cell culture was carried out in a standard cell culture medium containing L-glutamine (2 mM), streptomycin (100 μg/ml) and penicillin (100 U/ml) in an incubator with humidified 5% CO_2 and 95% air atmosphere. Triptolide was obtained from Sigma-Aldrich (St. Louis, MO, USA) and its stock solution in dimethyl sulfoxide (DMSO) was stored at -10°C. Pifithrin-α was obtained from Calbiochem (La Jolla, CA, USA).

Cell viability assay. Viability of HepG2 and QSG7701 cells was determined using a CCK-8 kit (Dojindo Molecular Technologies Inc., Rockville, MD, USA). HepG2 and QSG7701 cells were seeded at a density of 2×10^6 cells/well into 96-well plates and treated with 10-100 μM concentrations of triptolide for 48 h. After incubation, the CCK-8 solution (10 μl) was added to each well of the plate and the plates were incubated in incubator at 37°C for 3 h. Absorbance was recorded three times independently using a microplate reader (Bio-Rad Laboratories, Richmond, CA, USA) at 450 nm.

Apoptosis analysis using fluorescent microscopy and flow cytometry. HepG2 cells put into the tissue culture slides were allowed to undergo attachment for 48 h. The cells were then treated with various concentrations of triptolide for 48 h, washed with phosphate-buffered saline (PBS) and subsequently put into the 1X binding buffer (500 μl). A total of 5 μl each of Annexin V-FITC and propidium iodide were added into the plates and the cells were incubated under dark conditions for 5 min. The plates were analyzed using flow cytometry (BD FACSCalibur; BD Biosciences, San Jose, CA, USA) to determine the apoptotic cells.

Western blot analysis. HepG2 cells after incubation with various concentrations of triptolide were harvested and then were washed with ice-cold PBS. The cells were treated with RIPA lysis buffer (Roche Diagnostics, Shanghai, China) under ice-cold conditions for 45 min. The lysates were centrifuged at 12,000 rpm for 15 min at 4°C to collect the supernatant. Bradford method was used for the determination of the protein concentration in lysate. The proteins were separated by electrophoresis using 8% SDS-polyacrylamide gel and subsequently were transferred to NC membrane (Millipore, Billerica, MA, USA). The membrane incubation was performed with 10% dry milk in TBST to block non-specific binding sites. The membrane incubation with primary antibodies against Bax, DR5, p53, p53, histone-H2A.X and p-H2A.X (Cell Signaling Technology, Danvers, MA, USA) was performed overnight. The membranes were then washed with PBS and were incubated with secondary antibodies for 1 h. Expression of the desired proteins was examined using an ECL kit (Millipore) according to the instructions from the manufacturer. The

bands were visualized using autoradiography with Hyperfilm (Millipore).

Isolation of nuclei. Into the culture plates (100 mm diameter) HepG2 cells were seeded at a density of 2×10^6/plate and grown to confluence. The cells were then treated with triptolide for different durations. The cells were collected, PBS washed and subjected to fractionation into separate nuclear and cytosolic fractions using kit from Qiagen (Valencia, CA, USA). The sample purity of the nucleus and cytosol was checked using Lamin B and GAPDH as markers, respectively.

Analysis of DNA binding potential. HepG2 cells seeded into the plates were treated with triptolide for analysis of DNA binding potential of p53. The nuclear fractions obtained as mentioned above were examined using the TransAM p53 kit according to the protocol provided by the manufacturer (Active Motif, Carlsbad, CA, USA).

RT-PCR assay. Following treatment with triptolide for different durations HepG2 cells were collected to isolate total cell RNA using Isogen (Nippon Gene, Co., Ltd., Tokyo, Japan). Incubation of the RNA with DNase I was carried out to digest DNA that was contaminated. The RNA samples were purified by extraction using phenol and precipitation in ethyl alcohol. Ready-To-Go RT-PCR Beads (Amersham Pharmacia Biotech, Uppsala, Sweden) were used for the preparation of cDNA. AmpliTaq Gold® DNA Polymerase, LD (Applied Biosystems, Foster City, CA, USA) was employed for CR of cDNA using the primers: p21, 5'-TTAGCAGCGGAACAAGGAGT-3' and 5'-ATTCAGCATTGTGGGAGGAG-3' upstream and downstream, respectively); Bax, 5'-ATCCAGGATCGAGCAG GGCG-3' and 5'-GGTTCTGATCAGTTCCGGCA-3'; DR5, 5'-AAGACCCTTGTGCTCGTTG-3' and 5'-TCACCTGAA TCACACCTGG-3'; GAPDH, 5'-TGAAGGTCGGAGTCAA CGGATTTGGGT-3' and 5'-CATGTGGGCCATGAGGTCC ACCAC-3'. Proteins were treated at 90°C for 15 min followed by amplification. Amplification of the cDNA was performed by the sequence of 94°C for 50 sec, 55°C for 50 sec and 72°C for 50 sec. Quenching of the reaction was done using elongation step at 75°C for 5 min. The 2% agarose gel and ethidium stain was used for the analysis of PCR products.

ELISA assay for apoptosis analysis. HepG2 cells were seeded at a density of 2×10^6 into 60-mm cultural dishes (Falcon) and allowed to attain confluence. The cells were then treated with triptolide for 48 h. Following incubation, the cells were collected, washed with PBS and lysed. The Cell Death Detection ELISA Plus kit (Roche Diagnostics, Indianapolis, IN, USA) was used for the measurement of DNA degradation into the nucleosomal fragments.

p53 silencing using RNA. HepG2 cells seeded at a density of 2×10^6 into 60-mm cultural dishes were subjected to incubation with RNA for silencing p53 in transfection reagent (Mirus Bio, Madison, WI, USA). The plates were incubated for 48 h and then treated with triptolide for 48 h. After incubation, apoptosis induction in HepG2 cell cultures was analyzed using ELISA assay. Western blot assay was used to examine the expression of various proteins.
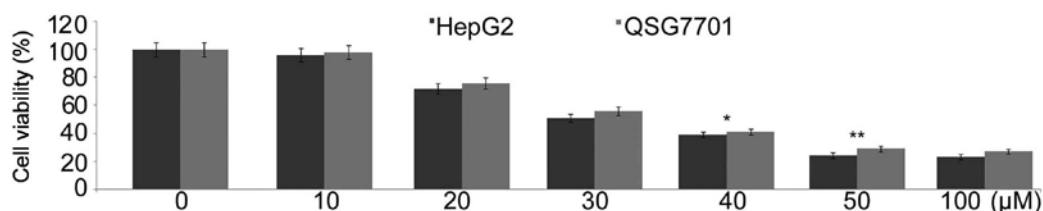
Figure 1. Triptolide treatment reduces viability of HepG2 and QSG7701 cells. The cells treated with various concentrations of triptolide for 48 h were subjected to MTT assay for analysis of cell viability. A concentration based reduction in viability was observed in HepG2 and QSG7701 cells at 48 h of treatment with triptolide. The expressed data are mean of three independently performed experiments. *P<0.05; **P<0.01.
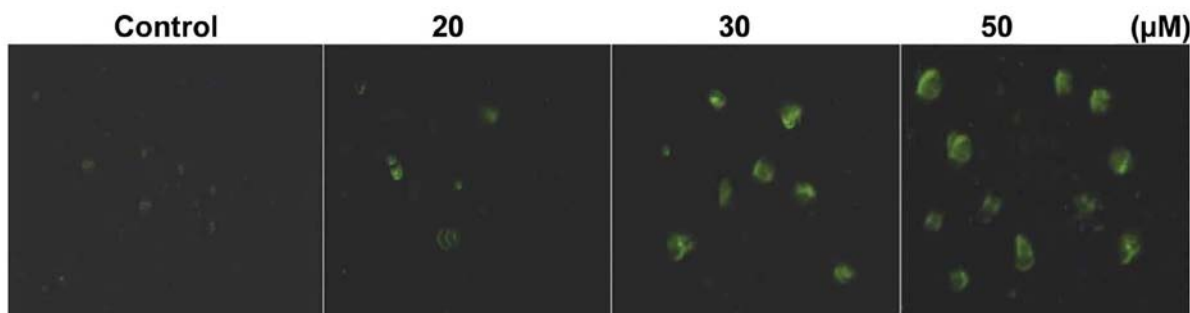

Figure 2. Triptolide treatment induces apoptosis in HepG2 cells. The cells treated with triptolide for 48 h were analyzed for apoptosis induction using Annexin V-FITC staining. The data presented are the mean of three experiments performed independently.
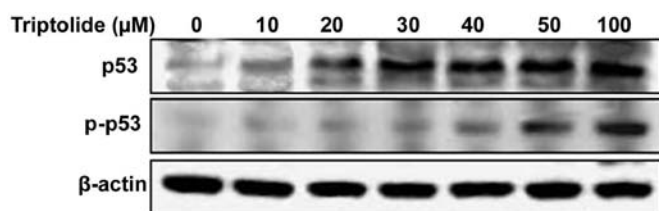

Figure 3. Triptolide treatment increases p53 phosphorylation in HepG2 cells. The cells seeded at a density of $2x10^6$/plate were incubated with various concentrations of triptolide for 48 h. Following treatment, western blot assay was used for the analysis of p53 phosphorylation in HepG2 cells. The internal loading control was β-actin.
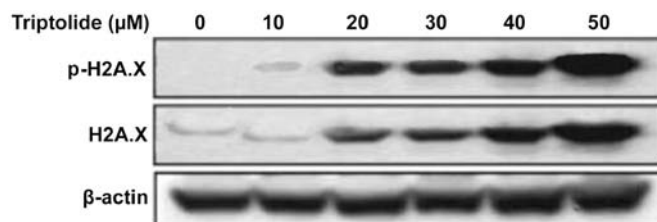
Figure 4. Effect of triptolide treatment on histone H2A.X phosphorylation HepG2 cells were treated with triptolide treatment for 48 h and the phosphorylation of histone H2A.X was analyzed using western blot assay.

Statistical analysis. The data presented are the means ± standard error or division of the mean (SEM or SD). Student's t-tests and two-way ANOVA were used for the analysis of the data using the statistical program in GraphPad Prism Version 4.0 (GraphPad Software, Inc., San Diego, CA, USA). All experiments were performed in triplicate. A P<0.05 was taken to indicate statistically significant difference.

Results

Triptolide treatment inhibits HepG2 and QSG7701 cell growth and proliferation. In HepG2 and QSG7701 cells treatment with various doses of triptolide for 48 h caused a concentration-dependent proliferation reduction. Treatment with range of triptolide concentrations from 10 to 100 μM led to a significant viability reduction at 50 μM of 48 h treatment in both HepG2 and QSG7701 cell lines (Fig. 1).

Triptolide treatment of HepG2 cells induces apoptosis. Annexin V-FITC staining showed that triptolide treatment of HepG2 cells for 48 h induced apoptosis. HepG2 cells were treated with 10-50 μM concentrations of triptolide for 48 h and then were analyzed by fluorescent microscopy. Triptolide treatment of HepG2 cells at 50 μM concentrations induced apoptosis in 56.45% cells compared to only 2.36% cells in the control cultures (Fig. 2).

Triptolide increases tumor suppressor p53 phosphorylation. Treatment of HepG2 cells with triptolide at various concentrations induced phosphorylation of p53 at serine-15 residue. The phosphorylation of p53 was significant at 2 h of treatment with 50 μM concentration of triptolide in HepG2 cells (Fig. 3).

Phosphorylation at serine-15 of p53 in HepG2 cells by triptolide is independent of DNA damage signals. Analysis of the phosphorylation of histone H2A.X at serine 139, indicator of DNA damage showed a significant increase by triptolide treatment from 12 h in HepG2 cells. However, no significant increase in the phosphorylation of histone H2A.X was observed at 1 h of triptolide treatment (Fig. 4).
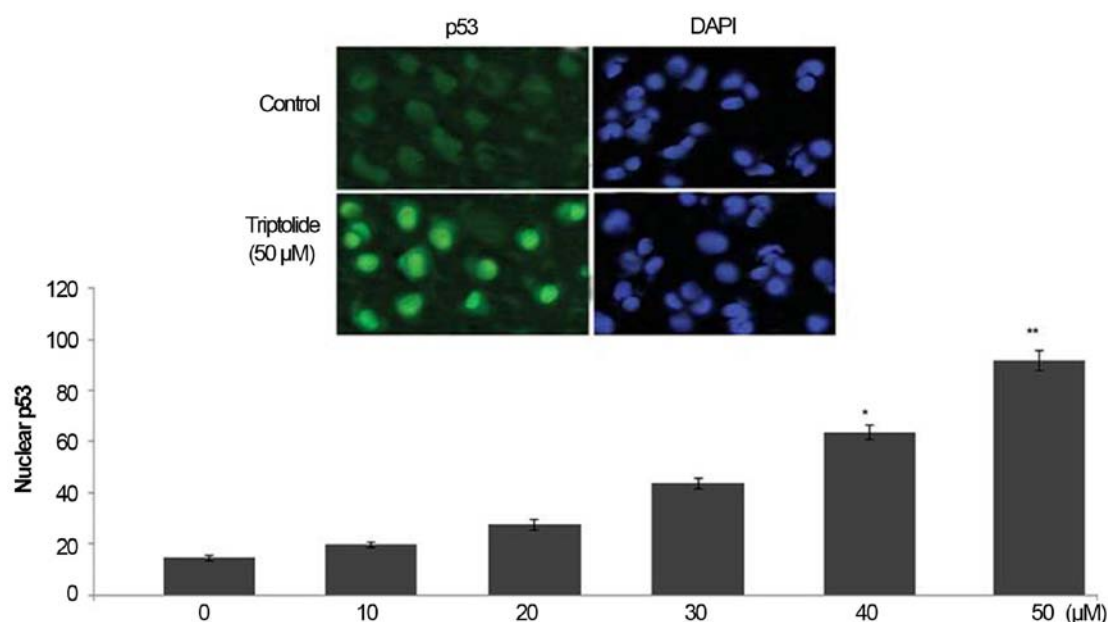
Figure 5. Effect of various concentrations of triptolide on expression levels of nuclear p53. HepG2 cell were treated with range of triptolide concentrations and stained with DAPI for examination of p53 expression. *P<0.05; **P<0.01.
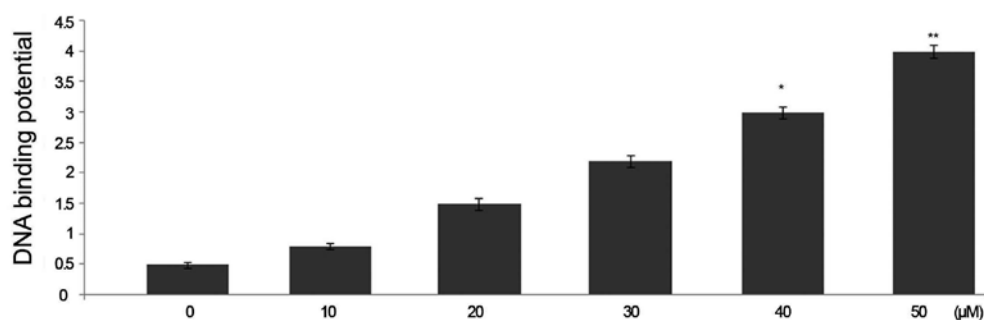

Figure 6. Triptolide treatment increases binding potential of p53 with DNA. HepG2 cells were treated with 50 μM concentration of triptolide and then binding potential of p53 with DNA was examined using the TransAM-p53 kit. The cells after treatment with triptolide were fractioned into nuclear and cytosolic components. The nuclear protein (4 μg) was used for measurement of binding of nuclear p53 with the consensus DNA sequence using the TransAM-p53 kit according to the manufacturer's instructions. *P<0.05; **P<0.01.

Triptolide treatment increases p53 level in HepG2 cell nuclei. Treatment of HepG2 cells with triptolide caused a significant increase in the level of p53 in HepG2 cell nuclei (Fig. 5). In 6 h-treatment with 0, 10, 20, 30, 40 and 50 μM concentration of triptolide the level of nuclear p53 was 15.3, 19.6, 28.5, 43.7, 63.8 and 91.5%, respectively (Fig. 5).

Triptolide treatment increases nuclear p53 DNA-binding activity in HepG2 cells. Treatment of HepG2 cells with triptolide at 50 μM concentration caused a significant increase in the binding potential of p53 to DNA. The binding potential of p53 to DNA was significantly higher in 6 h triptolide treatment (Fig. 6).

Treatment with triptolide induces rapid transcription of p53 target genes in liver cancer cells. Triptolide treatment of HepG2 cells caused a significant increase in the expression of various genes such as p21, Bax and DR5 at 6 h. Compared to control HepG2 cells the expression of p21, Bax and DR5 genes

was markedly higher on treatment with 50 μM concentration of triptolide (Fig. 7). In addition, triptolide treatment also increased the expression of miR-34b and miR-34c in HepG2 cells markedly (Fig. 7). However, no increase was observed in the expression levels of miR-34a in HepG2 cells on triptolide treatment (Fig. 7).

Inhibition of p53 prevents triptolide induced HepG2 cell apoptosis. Treatment of HepG2 cells with p53 inhibitor, pifithrin-α prior to incubation with triptolide significantly prevented induction of cell apoptosis (Fig. 8). Suppression of p53 expression by siRNA inhibited the expression of p53 as well as its target genes along with the prevention of apoptosis induction (Fig. 8).

Discussion

In the present study, triptolide treatment reduced viability and induced apoptosis in HepG2 liver cancer cells. The mecha-
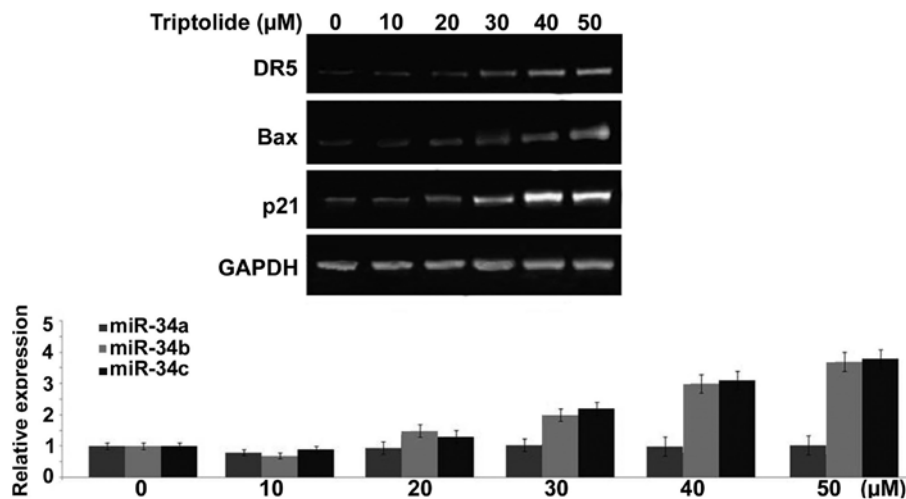
Figure 7. Effect of triptolide on p53 expression in HepG2 cells. The cells were treated triptolide for various time periods and then analyzed for the expression of p21, Bax and DR5 genes using RT-PCR assay. The cells after treatment with triptolide were analyzed for the expression of miR-34b, miR-34c and miR-34a genes.
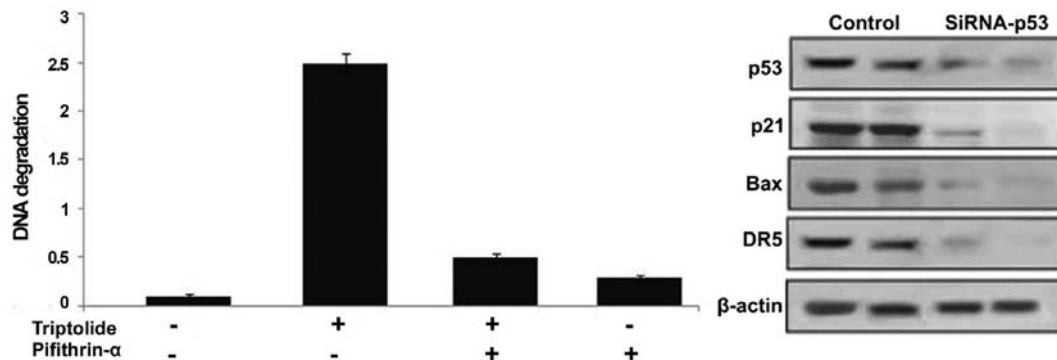

Figure 8. Suppression of p53 by pifithrin-α or siRNA inhibits triptolide-induced apoptosis. HepG2 cells were incubated for 24 h with 20 μM concentration of pifithrin-α and then with 50 μM concentration of triptolide for 48 h. Following incubation ELISA was used for the analysis of induction of apoptosis in HepG2 cells. HepG2 cells were incubated for 48 h with p53-siRNA followed by treatment with triptolide for 48 h. Western blot assay was used for analysis of expression of various proteins. Control, non-targeting siRNA.

nistic study revealed that triptolide exhibited its effect through activation of p53 tumor suppressor gene. Activation of p53 inhibits its interaction and subsequent degradation by MDM2 as well as increases the expression of nuclear p53 (26,27). Various kinases have been found to activate p53 by phosphorylating it at serine-15 during stress (28,29). Our results revealed that triptolide treatment of HepG2 cells at various concentrations induced phosphorylation of p53 at serine-15 residue. It is reported that activation of p53 can occur because of DNA damage signals as well during absence of these signals (30,31). In the present study, analysis of the activation of histone H2A.X indicator of DNA damage revealed its phosphorylation in 6 h-treatment. However, phosphorylation of p53 was induced only after 2 h of treatment with triptolide in HepG2 cells. These findings revealed that activation of p53 in HepG2 cells by triptolide treatment is independent of the signals produced by DNA damage. Our results showed that triptolide treatment of HepG2 cells increased expression level of p53 by its transcription. The expression of activated p53 was found to be more in nuclear region of triptolide treated HepG2 cells. Following activation p53 binds with various gene promoters

and induces their expression in the cells. Our results showed that triptolide treatment of HepG2 cells induced expression levels of p21, Bax and DR5 mRNAs. The expression level of miR-34b, miR-34c, and microRNAs in HepG2 cells was also increased on treatment with triptolide. In various types of cancers it has been found that miR-34a gene is deleted from its location in the chromosome (32). Our results showed that triptolide treatment of HepG2 cells induced no alteration in its expression. Different types of stresses are able to induce cancer cell apoptosis either in p53-dependent or -independent manner. In the present study, the role of p53 in induction of apoptosis by triptolide treatment was examined. The results from p53 suppression experiments revealed that p53 plays an important role in HepG2 cancer cell apoptosis induction on treatment with triptolide. The suppression of p53 by siRNA prevented induction of apoptosis in HepG2 cell cultures by triptolide treatment.

In conclusion, the present study demonstrated that triptolide inhibits viability and induces apoptosis in liver cancer cells through activation of tumor suppressor gene p53. Thus, triptolide can be used for the treatment of liver cancer.

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