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Triptolide inhibits MDM2 and induces apoptosis in acute lymphoblastic leukemia cells through a p53-independent pathway

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Abstract

Triptolide, a natural product derived from the Chinese plant Tripterygium wilfordii, is reported to exhibit antitumor effects in a broad range of cancers. The antitumor activity of triptolide is associated with its biological activities, as it inhibits various pro-proliferative or anti-apoptotic factors that are dominantly expressed in given types of cancer cells. Herein, we demonstrate that triptolide induced apoptosis in a subgroup of acute lymphoblastic leukemia (ALL) cells overexpressing the MDM2 oncoprotein, by inhibiting MDM2 expression. More specifically, we found that triptolide inhibited MDM2 at the transcriptional level by suppressing its mRNA synthesis. This MDM2 inhibition led in turn to increased levels of p53 protein; however, p53 functionality was not activated, due to the fact that triptolide-treated cells lacked induction of p21 and PUMA as well as in G1 cell-cycle arrest. Triptolide-mediated downregulation of MDM2 increased inhibition of XIAP, its translational target, in a manner distinct from reactions to cellular stress and DNA-damaging agent ionizing radiation (IR) that induce XIAP due to p53-activated MDM2. These results suggest that increased inhibition of XIAP due to downregulation of MDM2 may play a critical role in triptolide-induced apoptosis in MDM2-overexpressing cancers.

Introduction

Triptolide is a natural product isolated from the native Chinese plant Tripterygium wilfordii Hook.f. For centuries, Tripterygium wilfordii Hook.f has been used in traditional Chinese medicine to treat autoimmune diseases such as rheumatoid arthritis, nephritis and systemic lupus erythematosus [1–3]. Triptolide is reported to exhibit anticancer properties in a broad range of human cancers [4–8], so it has been recently entered into clinical trials [9,10]. So far, there is a large body of work that describes the anticancer effects of triptolide as occurring through multiple mechanisms, by suppression of various signaling pathways, pro-proliferative and anti-apoptotic factors in a given cell type and under specific conditions. For example, Triptolide inhibits cell growth and induces apoptosis in cancers that usually express constitutively high levels of NF-kB activation, such as multiple myeloma and thyroid carcinoma, through repression of their NF-kB signaling pathway [11,12]. Triptolide induces apoptosis in chronic myelogenous leukemia (CML) via downregulation of bcr-abl, which is often expressed at a high level due to the Philadelphia chromosome t(9;22) [13,14]. Pancreatic cancer cells commonly express high levels of heat shock proteins (HSP); triptolide kills them by inhibiting HSP expression [15,16]. Recent studies show triptolide
inhibits RNA polymerase-mediated transcription by targeting a subunit of the transcription factor TFIIH named XPB, which leads to downregulation of certain short-lived mRNA [17, 18]. This suggests that triptolide-repressed expression of a variety of anti-apoptotic or survival factors may likely occur through inhibition of their mRNA synthesis. Once proliferation or survival of a given type of cancer that is driven by predominant expression of a specific growth or survival factor is altered by inhibition of this factor, suppression of cell growth and induction of cancer cell death ensue.

MDM2, an oncoprotein, is also considered to be an anti-apoptotic or survival factor that can protect cancer cells from apoptosis. MDM2 gained considerable attention following its identification as the protein that negatively regulates the tumor suppressor p53. The N-terminus of MDM2 protein binds to p53, restraining p53-mediated transcription [19], while its C-terminus acts as an E3 ubiquitin ligase, mediating p53 degradation [20]. MDM2 also plays p53-independent roles in oncogenesis. In addition to interacting with and regulating p53, MDM2 interacts with other molecules that include specific proteins, DNA and RNA. These interactions likely contribute to the p53-independent role of MDM2 in oncogenesis.

For example, MDM2 interacts with Rb and E2F1, which promote cell cycle progression [21,22]. Also, MDM2 induces NF-kB/p65 expression transcriptionally, through Sp1-binding sites, to induce resistance to apoptosis [23]. In addition, the C-terminal RING finger domain of MDM2 exhibits specific RNA binding ability [24]. We recently reported that binding of the C-terminal RING domain of the MDM2 protein to XIAP mRNA regulates translation of this apoptosis regulator, which allows for development of resistance to anticancer treatment [25].

Overexpression of MDM2 due to genomic amplification occurs in a variety of human solid cancers, particularly in soft tissue tumors [26, 27]. Overexpression of MDM2 is even detected in leukemia and other malignancies that lack MDM2 gene amplification [28]. Regardless of the cellular pathway involved, MDM2 overexpression is associated with promotion of cancer and poor treatment outcome. For example, a single nucleotide polymorphism in the MDM2 gene promoter, which enhances MDM2 transcription, serves as a marker for an increased predisposition to develop tumors, as well as a marker for neuroblastoma disease aggressiveness [29]. Although the mechanism for MDM2 overexpression in leukemia is not known, overexpression of MDM2 is observed in 20% to 30% of pediatric patients with ALL [30–32] and is found to be associated with chemoresistance and a poor prognosis [32–36].

In the present study, we examined the effects of triptolide on MDM2 expression and induction of apoptosis in ALL. We found that triptolide strongly inhibited MDM2 expression and induced potent apoptosis of MDM2-overexpressing ALL cells. Herein, we delineate the mechanism by which triptolide represses MDM2 expression and the mechanistic steps involved in MDM2 downregulation that induce ALL cell apoptosis.

**Materials and methods**

**Cells and reagents**

This study used nine established cell lines derived from children with ALL. Three of these cell lines (EU-1, EU-3 and EU-4) were established at Emory University, and five (SUP-B13, SUP-B15, UOC-B1, UOC-B3 and UOC-B4) were obtained from Stephen D. Smith (Department of Pediatrics, University of Illinois College of Medicine at Peoria, Peoria, IL, USA). The Reh ALL cell line was obtained from C. Rosenfeld (INSERM, Villejuif, France). The EU-4/MDM2 cell line was generated previously [23] by stable MDM2 gene transfection into EU-4 cells that are p53/MDM2 negative. Authentication of all cell lines was performed by testing their immune-phenotypes and cytogenetic profiles in prior
publications [23,30,37–40] and are summarized in Table I. All cell lines were grown in standard culture medium (RPMI 1640 containing 10% FBS, 2 mmol/L L-glutamine, 50 U penicillin, and 50 μg/ml streptomycin), at 37°C in 5% CO₂.

Patient isolates included in this study came from patients treated for ALL (either diagnosis or first relapse) at Emory. Following informed consent, pre-treatment bone marrow specimens were obtained from 8 patients with ALL. Of these 8 patients, 6 had B-cell precursor (BCP) ALL and 2 had T-ALL, as diagnosed by standard immunologic, morphologic and cytochemical criteria. Mononuclear cells were separated by centrifugation on Ficoll-Hypaque (1.077g/ml), washed twice in phosphate-buffered saline (PBS), and then resuspended at a density of 10⁶ cells/ml in RPMI 1640 containing 10% fetal bovine serum (FBS). These cells were incubated on plastic petri dishes for one h at 37°C to remove the monocytes, after which nonadherent cells were recovered by gently washing the dishes with PBS. All specimens collected for these studies contained more than 90% blasts, following purification.

The triptolide, purchased from Sigma-Aldrich (Cat. No: T3652), was dissolved in DMSO to create a 10 mM stock solution, which was stored in small aliquots at −120°C. To treat cells, they were exposed to 10–100 nM of triptolide for the time period indicated, with the final DMSO concentration kept constant in each experiment. MDM2 antibody (SMP14) was purchased from Sigma; p53 (DO-1) and PUMA (H-136) antibodies were purchased from Santa Cruz; p21 (12D1), caspase 3 (3G2), caspase 9 (C9) and PARP (7D3-6) were purchased from cell Signaling; XIAP (2F1) was purchased from Abcam. The concentrations of all antibodies were used according to the manufacturers’ instruction.

Western blot analysis

Cells were lysed for 30 min at 4°C in a lysis buffer composed of 150 mM NaCl, 50 mM Tris (pH 8.0), 5mM EDTA, 1% (v/v) Nonidet p-40, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin and 25 μg/ml leupeptin. Equal amounts of protein extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following transfer to a nitrocellulose filter, it was blocked for 1 h at room temperature with buffer containing 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 5% non-fat milk; incubated with specific antibodies for 3 h at room temperature; washed; and incubated with a HRP-labeled secondary antibody for 1 h. Finally, the blots were developed using a chemiluminescent detection system (ECL, Amersham Life Science, Buckinghamshire, England).

Quantitative RT-PCR

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen). First-strand cDNA synthesis was performed with a mixture of random monomers and oligo-dT as primers. Amplification was performed with a 7500 Real-Time PCR System (Applied Biosystems), using the QuantiFast SYBR Green RT-PCR kit (Qiagen), according to the manufacturer’s instructions. All specific primers for amplification of specific genes, as well as the housekeeper gene GAPDH, were purchased from Qiagen.

Pulse-chase assay

The degradation rate of mRNA was examined by performing a standard actinomycin D analysis. At different times after addition of 5 μg/ml actinomycin D, in the presence or absence of triptolide, cells were harvested and their total RNA isolated. The MDM2 mRNA was detected by quantitative RT-PCR, as described above. Protein turnover was assessed by a standard protein-synthesis inhibitor cycloheximide (CHX) assay. Briefly, cells were treated with 50μg/ml CHX for different times before lysis, in the presence or absence of

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triptolide, then western blot analysis revealed concurrent expression levels of MDM2, p53 and XIAP.

**Polysome preparation and analysis**

Cells were incubated with 100 μg/ml CHX for 15 min, to arrest polyribosome migration, and then they were lysed in order to isolate cytoplasmic extracts in a buffer containing 20 mM Tris-HCl at pH 8.0, 100 mM NaCl, 5 mM MgCl₂, 0.5% Triton X-100, 500 U/ml RNAsin, and a cocktail of protease inhibitors. Fractionation was performed on a 15–45% (w/v) sucrose gradient, centrifuged in a SW41Ti rotor at 39,000 rpm for 1 hr. Fractions were collected from each gradient tube by upward replacement and absorption monitored at OD_{254}, using a fractionator (Brandel, Inc.). RNA from each fraction was extracted and subjected to quantitative PCR, as described below.

**Plasmids and transfection**

The MDM2 gene promoter 1 and promoter 2 were generated by PCR and cloned into the pGL3 basic vector to produce the MDM2 p1-Luciferase and MDM2 p2-Luciferase plasmids. EU-4 cells were transiently transfected with these MDM2 promoter luciferase plasmids by electroporation. As an internal control, the pRL (Renilla luciferase)-CMV vector was cotransfected. After a 24-h transfection, cells were treated with different concentrations of triptolide for another 4 h. Their cell extracts were then prepared for testing FL and RL activities using Assay Reagent II (Promega) and a Microplate Luminometer.

**WST assay**

The cytotoxic effect of triptolide on ALL cells was determined using the water-soluble tetrazolium salt (WST) assay. Briefly, cells cultured in 96-well microtiters plates were given different concentrations of triptolide, for a 20-h period. Following this, WST (25 μg/well) was added and incubation continued for an additional 4 h before the optical density (OD) of the wells was read with a microplate reader (set at a test wavelength of 450 nm and a reference wavelength of 620 nm). Appropriate controls lacking cells were included, to determine background absorbance.

**Flow cytometry**

Flow cytometry was performed to analyze the cell cycle position and degree of apoptosis induced by triptolide. For the cell-cycle analysis, cells were collected, rinsed twice with PBS, fixed in 70% ethanol for 1 h at 4°C, washed twice in PBS and re-suspended in 0.5 ml PBS containing 20 μg/ml of propidium iodide (PI) and 20 μg/ml of RNase A. Following incubation at 4°C for at least 30 min, samples were analyzed using a FACScan (Becton Dickinson) with WinList software (Verity Software House, Inc).

For the quantitative detection of apoptotic cells, flow cytometry of annexin-V stained cells was performed. Briefly, cells with or without triptolide treatment were washed once with PBS and stained with PE-annexin-V, according to the manufacturer’s instructions, prior to flow analysis.

**Results**

**Triptolide inhibits MDM2 expression in ALL**

To investigate whether triptolide can inhibit MDM2, we first treated a group of ALL cell lines that overexpress MDM2 with triptolide. We found that triptolide potently downregulated MDM2 in all 8 MDM2-overexpressing ALL cell lines studied (Fig. 1A). Triptolide inhibited MDM2 expression in a dose-dependent manner, even at low
concentrations spanning 20–100 nM (Fig. 1B). Inhibition of MDM2 by triptolide occurred at about 2 h after treatment and was followed by steady-state downregulation (Fig. 1C), in a manner distinct from a stress challenge with IR, which instead induced a rapid and transient (1–2 h) reduction of MDM2, followed by a remarkable upregulation of this protein (Fig. 1D). As a control for specificity of action, another component extracted from *Tripterygium wilfordii*, Celastrol (tripterine) did not inhibit MDM2 (Fig. 1E). Fig. 1F shows the chemical structure of triptolide.

### Inhibition of MDM2 expression by triptolide is likely through repression of mRNA synthesis

We evaluated the mechanism by which triptolide inhibits MDM2. First, we performed RTPCR and found that triptolide strongly suppressed MDM2 mRNA expression (Fig. 2A). Next, we investigated whether the inhibition of MDM2 mRNA in triptolide-treated cells is due to repression of promoter activity on MDM2 genes by performing gene transfections and a reporter assay in an ALL cell line EU-4. Our results showed that triptolide did not regulate MDM2 promoter activity (Fig. 2B). Pulse-chase and quantitative RT-PCR indicated that MDM2 mRNA stability was not affected by triptolide treatment (Fig. 2C). Results from polysome profiling demonstrated that triptolide does not regulate MDM2 translation, as no effect of triptolide on the polyribosome profile of MDM2 was detected (Fig. 2D). In addition, we found that triptolide did not regulate MDM2 protein stabilization, as detected by a standard CHX pulse-chase assay, as there was no difference in the protein’s half-life in cells, whether triptolide-treated or untreated (Fig. 2E). These results imply that most likely, triptolide inhibits MDM2 through suppression of mRNA synthesis.

### Enhanced p53 expression by triptolide is a result of triptolide-mediated inhibition of MDM2

Because MDM2 is an E3 ligase that strongly ubiquitinates and degrades the tumor suppressor p53, we tested to see the consequences of triptolide-mediated downregulation of MDM2 on p53 expression. Before this, we had first sought for a possible direct effect of triptolide on p53 mRNA expression, comparing it with the known role of IR stress on wt-p53/MDM2-overexpressing ALL cells, namely, EU-1. It is well known that IR activates p53 through multiple mechanisms involving regulation of its transcription, translation and posttranslational modification. As is seen in Fig. 3A, while IR induced p53 transcription in these cells, triptolide did not show any directly inductive or inhibitory effects on p53 mRNA expression. We also evaluated whether there was a role for triptolide in regulation of p53 translation. We treated EU-1 cells with either IR or triptolide, in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX). In the absence of CHX, IR did induce p53, whereas p53 was not induced by the same IR exposure combined with the presence of CHX (Fig. 3B, upper panel). In contrast, triptolide treatment increased p53 expression in either the absence or presence of CHX (Fig. 3B, low panel). This suggests that unlike IR that induces p53 translation, triptolide does not affect p53 translation. When we measured the turnover of p53 protein after triptolide treatment by pulse-chase assay, the half-life of p53 in untreated cells was found to be less than 30 min, while it was remarkably increased by triptolide (Fig. 3C), suggesting that the observed triptolide-upregulated p53 expression in MDM2-overexpressing cells occurs only due to activity at the post-translational level. Triptolide-induced accumulation of p53 was most likely due to release of MDM2-mediated degradation. To prove this, we examined p53 levels after triptolide treatment in both MDM2-expressing and MDM2-negative ALL cells. Because cancer cell lines with wt-p53/MDM2-negative phenotype were unavailable, we sought to use primary ALL cells derived from patients. Triptolide increased expression of p53 in MDM2-expressing cells following downregulation of MDM2, but did not do so in MDM2-negative cells that already expressed a relatively high level of p53 (Fig. 3D).
The p53 cell-cycle arrest function is not activated in triptolide-treated ALL cells

The major anti-cancer function of p53 is to arrest cell-cycle in G1 phase, then induce apoptosis through induction of p21 and PUMA, its transcriptional targets, as can be observed in IR-treated cells. Here, we tested for the expression of p21 and PUMA in our wt-p53/MDM2 overexpressing ALL cells, after triptolide treatment, as compared with IR. We performed quantitative RT-PCR and found that while IR induced remarkable increases in p53-targets p21 and PUMA, as well as MDM2 mRNA expression, triptolide failed to increase and even decreased expression of p21, PUMA and MDM2 mRNA (Fig. 4A); however triptolide treatment did increase the p53 protein to a level comparable to that of IR-treated cells (Fig. 4B). Western blots to investigate protein expression of p21 and PUMA found changes corresponding to those in mRNA, i.e. IR induced p21 and PUMA protein expression, whereas triptolide reduced their expression (Fig. 4B). In addition, triptolide did not induce phosphorylation of p53 on serine 15 (S15), an activated form of p53, in contrast to IR that increased p53 (S15) expression [41]. To confirm that p21 was not functional in triptolide-treated cells, we performed cell-cycle analysis in EU-1 cells. In contrast to IR, which induced cell-cycle arrest in G1 phase, triptolide treatment did not induce G1 arrest. As shown in Fig. 4C, while there were rapid depletions of S and G2/M, and increasing numbers of G1 cells in IR-treated cells, the triptolide-treated cells exhibited proportional reductions in the numbers of cells in G1, S and G2/M.

Triptolide-treated MDM2-overexpressing ALL cells have increased XIAP inhibition

Previous studies already demonstrated that triptolide inhibits XIAP expression at the mRNA level [42]. Because XIAP is a translational target of MDM2, inhibition of MDM2 by siRNA resulted in reduced translation and expression of XIAP [25]. When we tested for XIAP inhibition by triptolide, we found it was enhanced in MDM2-expressing cells due to additional translation repression occurring through triptolide-mediated downregulation of MDM2. In fact, we found that MDM2 and XIAP expression was remarkably reduced in the MDM2-overexpressing EU-1 cells following triptolide treatment. Instead, both were increased in the same cells treated with IR (Fig. 5A). To see if MDM2 plays a role in the reduction of XIAP in triptolide-treated cells, we treated a stably-transfected (with MDM2) ALL cell line, EU-4, with triptolide. A far greater reduction of XIAP expression was detected in triptolide-treated MDM2-transfected EU-4 cells than in triptolide-treated control plasmid-transfected EU-4 (Fig. 5B). In addition, when we performed linear sucrose gradient fractionation to assess the polyribosome association of the XIAP mRNA in EU-1 cells subjected to triptolide and the mock-treatment, we found XIAP mRNA clearly shifted away from fractions enriched with translating polyribosomes (Fig. 5C, bottom, fractions 5–11) to fractions containing translation-dormant complexes (Fig. 5C, bottom, fractions 1–4). These results are indicative of reduced translation as a consequence of triptolide treatment. Triptolide treatment had no effect on the polyribosome profile of Actin mRNA, a control (Fig. 5C, top). Furthermore, no appreciable difference was found in XIAP protein stability between EU-1 cells treated with triptolide and those with no triptolide treatment (Fig. 5D). Taken together, these results demonstrate that triptolide strongly inhibits XIAP expression in MDM2-overexpressing ALL cells at both the transcriptional and translational levels.

Triptolide induces potent apoptosis of MDM2-overexpressing/IR-resistant ALL cells

We examined the effect of triptolide on cell viability using the WST cytotoxic assay in the 10 ALL lines including EU-4 and EU-4 transfected with MDM2 (EU-4/MDM2). Triptolide exhibited strongly cytotoxic activity in all 8 cell lines having native MDM2 overexpression, with IC50 values ranged from 47 to 73 nM (Table 1). Triptolide exhibited much less cytotoxic effect on EU-4 cells that express very low level of MDM2, while it effectively kill these cells when MDM2 was stably transfected (IC50 values: 725 nM vs. 88 nM). To clarify whether the observed cell death induced by triptolide was associated with induction of
apoptosis, we stained the cells with Annexin V and quantitated them by flow cytometry. The outcome was consistent with the WST results: triptolide induced apoptosis in the 9 MDM2-overexpressing ALL cells including EU-4 transfected with MDM2 (Table 1). Triptolide-induced apoptosis, which occurred in a caspase-dependent manner, was further confirmed by a western blot assay to evaluate the activation of caspases -3 and -9, as well as cleavage of the death substrate PARP. As shown in Fig. 6A, cleavage of caspases -3, -9, and PARP can be detected 8 h after triptolide treatment in EU-1 cells. Simultaneously, we treated EU-1 cells with a therapeutic dose (10 Gy) of IR that induced p53, p21, and PUMA; and provided cell-cycle arrest (Fig. 4). Cleavage of caspases -3, -9 and PARP were not detected until 24 h after IR treatment. To confirm that the increased p53 protein does not confer apoptosis in triptolide-treated cells, we transfected p53 siRNA and control siRNA into the cells treated by triptolide. Apoptosis was not significantly reduced in cells transfected with p53 siRNA as compared to cells without p53 siRNA transfection or transfected with control siRNA (p>0.5) (Fig. 6B).

We also tested for the apoptotic effects of triptolide on six patient’s primary ALL cells, including three with high level expression of MDM2 and three without MDM2 expression (data not shown). Interestingly, all these fresh ALL cells were sensitive to triptolide (Fig. 6C), suggesting that additional mechanism other than inhibition of MDM2 exists for triptolide to kill ALL cells. As we reported previously [37], three ALL cell lines (EU-1, UOC-B1 and Reh) are resistant to IR treatment, as demonstrated by testing that revealed no DNA fragmentation following 10 Gy IR for 5 h. All 3 ALL cell lines were equally sensitive to triptolide (Table 1). We tested to see whether triptolide provided a possible synergistic effect in IR-induced apoptosis by treating these 3 cell lines with both. When given alone, a 24-h treatment induced 10-30% apoptosis; when the same doses were given to cells in combination, we found that a significant number (over 65%, p≤0.01) of cells underwent apoptosis after 24 h (Fig. 6D), which suggested that triptolide may provide synergy, or at least an IR-resistant-reversing effect, to cancer cells with MDM2-overexpressing phenotypes.

Discussion

Triptolide has been widely investigated for its anticancer activity. Mechanistic studies demonstrate that triptolide inhibits cell growth and induces apoptosis in various cancers, mainly through repression of oncoproteins or cell growth/survival factors. In the present study, we evaluated the potential effects of triptolide on MDM2 expression in ALL. We found that triptolide strongly inhibited MDM2 at the transcriptional level, which resulted in potent apoptosis of MDM2-overexpressing ALL cells that constitute a high-risk subgroup of ALL. Investigating further into the mechanism of action, we found that inhibition of MDM2 by triptolide led to increased p53 accumulation. However, the primary function of p53 to induce p21 and PUMA for cell-cycle arrest and apoptosis was not activated. Triptolide strongly inhibited XIAP expression, which was partially regulated by the triptolide-mediated downregulation of MDM2. Based on our results, we now believe that the potent apoptosis induced by triptolide in MDM2-overexpressing cancer cells is predominantly attributable to downregulation of XIAP.

XIAP is an important member of the “inhibitor of apoptosis protein” (IAP) family. The XIAP protein has been shown to bind specifically to and inhibit the activated forms of caspase-3, -7 and -9, which are the enzymes that induce the intrinsic (mitochondrial) pathway of apoptosis [43]. Previous studies in AML have shown that triptolide induces caspase-dependent cell death via the mitochondrial pathway by inhibition of XIAP [42,44]. It was found that triptolide inhibits XIAP at both the mRNA and protein levels, as detected by RT-PCR and Western blot [42]. Because we found in our previous work that the
expression of XIAP is regulated by MDM2 at the translational level [25], we evaluated whether triptolide-mediated downregulation of MDM2 results in inhibition of XIAP translation. More inhibition of XIAP by triptolide was detected in MDM2-overexpressing cells than in non-MDM2-overexpressing cells. Polysome profile results definitively demonstrated that triptolide inhibited XIAP translation in MDM2-overexpressing ALL cells. Consistent with our previous MDM2 siRNA treatment results [25], we found that in the same MDM2-overexpressing ALL cells, downregulation of MDM2 by triptolide led to potent cell death.

Our finding that cancer cell death is mainly caused by triptolide-mediated downregulation of XIAP in wt-p53/MDM2-overexpressing ALL was further confirmed by finding that there was no induction of p53 function in these cells. We showed that although downregulation of MDM2 by triptolide remarkably increased p53 expression in these wt-p53/MDM2-overexpressing ALL cells, there was no corresponding increase in expression of p21 or cell-cycle arrest in G1 phase. In contrast, we found that p21 expression was inhibited in the triptolide-treated ALL cells, which was consistent with previously-reported results in A549 lung cancer and HT1080 fibrosarcoma cells [45]. Similarly, another important p53 transcriptional target for induction of apoptosis, PUMA, was also not induced and even inhibited in the same triptolide-treated ALL cells. Although the present study did not test for the reason why p21 and PUMA were not induced by the triptolide-increased p53, we hypothesize that, like MDM2, the synthesis of p21 and PUMA mRNA was most likely repressed by triptolide, even though their promoters could potentially become activated by the increase in p53. The molecular dynamics between these molecules remains unknown.

The most important observations of the present study were that IR-resistant ALL cells were sensitive to triptolide and that triptolide showed an IR-resistant-reversing effect in ALL cells having the MDM2-overexpressing phenotype. It is well known that IR and many chemotherapeutic drugs kill cancer cells through cellular stress and DNA damage that induce an immediate accumulation and activation of p53 through multiple mechanisms. The activated p53, in turn, induces its transcriptional targets p21 and PUMA to bring about cell-cycle arrest and apoptosis. The activated p53 also induces expression of its inhibitor MDM2. This p53-mediated increase in MDM2 expression begins to degrade p53 and repress its function. In addition, cellular stress and DNA damage also regulate translocation of MDM2 (if it originally existed in the cells) from the nucleus to the cytoplasm [46,47], where it plays many p53-independent roles, such as the induction of XIAP [25]. Later events such as p53 inhibition and XIAP induction are likely combined to be part of an important mechanism for the development of IR- or chemo-resistance in wt-p53/MDM2 overexpressing ALL cells. In contrast, it appears that triptolide is not a cellular stress and DNA-damaging agent, even though it induces cell death. It primarily inhibits MDM2 that either already existed or was p53-induced, resulting in eventual inhibition of XIAP and potent cell death, even when these cancer cells are resistant to IR or chemotherapy. Thus, we believe triptolide should be an interesting candidate drug in the development of therapies against high risk, refractory ALL in patients whose leukemic cells overexpress MDM2. Improving the chances for a good outcome in these patients would be the future goal of developing this naturally-derived drug.

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References


Fig. 1.
The inhibitory effect of triptolide on MDM2 expression in ALL. A, western blot assay for MDM2 expression in ALL cell lines treated for 24 h with 100 nM triptolide. B and C, western blot assays showing the dose-response (B) and time-course (C) of MDM2 inhibition by triptolide. D, regulation of MDM2 expression in EU-1 cells treated with 10 Gy IR for the times indicated. E, comparison of the effects of triptolide and celastrol on MDM2 inhibition in EU-1 cells treated for 24 h. F, Chemical structure of triptolide.
Fig. 2. Evaluation of the mechanism by which triptolide inhibits MDM2. 

A, RT-PCR for MDM2 mRNA expression in EU-1 cells treated with 100 nM triptolide for different times as indicated. 

B, EU-4 cells were transfected with either 5 μg MDM2 promoter 1 luciferase (MDM2 p1-Luc) or MDM2 p2-Luc. Transfected cells were treated with or without increasing amounts (25, 50 and 100 nM) of triptolide for 4 h before harvesting. Cell extracts were analyzed by luciferase activity assay. 

C, EU-1 cells were treated with or without 100 nM triptolide for 4 h, followed by addition of 5mg/ml actinomycin D. Cells were harvested at the indicated time points and the amount of MDM2 mRNA was determined by quantitative RT-PCR. 

D, EU-1 cells were treated with or without 100 nM triptolide for 4 h and their cytoplasmic lysates were fractionated on a sucrose gradient. RNA was extracted from each of the fractions and subjected to quantitative RT-PCR for analysis of the distribution of MDM2 and Actin mRNAs. Data represent the percentage of the total amount of corresponding mRNA in each fraction. 

E, CHX pulse-chase assay for detection of MDM2 turnover in EU-1 cells treated with or without 100 nM triptolide for 4 h. Numerical labels under each protein band represent the expression levels after normalization for GAPDH, compared with untreated (0) samples (defined as 1 unit).
Fig. 3.
The effect of triptolide-mediated downregulation of MDM2 on p53 expression. A, EU-1 cells were either treated with increasing amounts (25, 50 and 100 nM) of triptolide or exposed to increasing amounts (2.5, 5 and 10 Gy) of IR for 4 h, then p53 mRNA was detected by quantitative RT-PCR. B, EU-1 cells were treated with and without 10 μg/ml CHX for 10 min, followed by treatment with either 10 Gy IR or 100 nM triptolide. Cell extracts from untreated (0) cells or harvested 1 or 2 h after treatment with IR or triptolide were assessed for p53 expression by Western blot assay. C, pulse-chase assay for p53 turnover in EU-1 cells treated with triptolide. D, primary ALL cells from patients (UPN, unique patient number) treated with 100 nM triptolide for the times indicated were assessed for MDM2 and p53 expression by Western blot.
Fig. 4.
The effect of triptolide-mediated downregulation of MDM2 on the p53 targets p21, PUMA and on its repressor MDM2, as compared with IR treatment. 

A, EU-1 cells were treated with 10 Gy IR or 100 nM triptolide for 5h and then assayed by quantitative RT-PCR for expression of mRNA as indicated. Data represent the fold induction of treatment compared to no treatment.

B, EU-1 cells were treated with either 10 Gy IR or 100 nM triptolide for different times, and then expression levels of proteins as indicated were detected by western blot.

C, cell-cycle analysis in EU-1 cells performed 4 h post-treatment with 10 Gy IR or 100 nM triptolide.
Fig. 5. The effects of IR-upregulated and triptolide-downregulated MDM2 on XIAP expression. **A**, EU-1 cells were treated with either 10 Gy IR or 100 nM triptolide for the times indicated. MDM2 and XIAP protein expression was detected by western blot. **B**, EU-4 cells transfected with either MDM2 or vehicle controls were treated with 100 nM triptolide for the times indicated. Expression of transfected MDM2 and endogenous XIAP were detected by western blot. **C**, the effect of triptolide-mediated downregulation of MDM2 on XIAP translation, as detected by polyribosome profiling described in Fig. 2D. **D**, effect of triptolide on XIAP protein stability, as detected by pulse-chase assay described in Fig. 2E.
Fig. 6.
The apoptotic effect of triptolide on ALL. A, EU-1 cells were treated with 100 nM triptolide or 10 Gy IR for the times indicated, then expression of the indicated proteins was detected by western blot. B, EU-1 cells were treated with different doses of triptolide for 24 h in the absence and presence of 200 nM sip53 or siRNA control. Cell apoptosis was detected by Annexin-V staining and quantitated by flow cytometry. Data represent the mean percentage ± SD of apoptosis from three independent experiments. *p>0.5. C, primary ALL cells from patient BM were treated with different concentrations of triptolide or without treatment (control) for 24 h, and apoptosis was detected as in (B). D, EU-1, UOC-B1 and Reh cells were treated with triptolide (50 nM) or IR (10 Gy), alone or in combination, for 24 h. Apoptosis was detected as in (B).
Table 1

Apoptotic effect of triptolide on ALL cell lines with wt-p53 and MDM2 overexpression

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Type</th>
<th>p53 status</th>
<th>MDM2 mRNA levels*</th>
<th>XIAP expression</th>
<th>IC50 (nM)**</th>
<th>Apoptosis (%)***</th>
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<tbody>
<tr>
<td>EU-1</td>
<td>BCP</td>
<td>wt</td>
<td>39</td>
<td>+</td>
<td>49</td>
<td>68</td>
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<tr>
<td>EU-3</td>
<td>BCP</td>
<td>wt</td>
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<td>+</td>
<td>73</td>
<td>77</td>
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<tr>
<td>Sup-B13</td>
<td>BCP</td>
<td>wt</td>
<td>35</td>
<td>+</td>
<td>53</td>
<td>69</td>
</tr>
<tr>
<td>Sup-B15</td>
<td>BCP</td>
<td>wt</td>
<td>32</td>
<td>+</td>
<td>50</td>
<td>62</td>
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<tr>
<td>UOC-B1</td>
<td>BCP</td>
<td>wt</td>
<td>41</td>
<td>+</td>
<td>47</td>
<td>60</td>
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<tr>
<td>UOC-B3</td>
<td>BCP</td>
<td>wt</td>
<td>47</td>
<td>+</td>
<td>61</td>
<td>81</td>
</tr>
<tr>
<td>UOC-B4</td>
<td>BCP</td>
<td>wt</td>
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<tr>
<td>Ren</td>
<td>BCP</td>
<td>wt</td>
<td>28</td>
<td>+</td>
<td>55</td>
<td>59</td>
</tr>
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<td>17</td>
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<td>EU-4/MDM2</td>
<td>BCP</td>
<td></td>
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<td>+</td>
<td>88</td>
<td>58</td>
</tr>
</tbody>
</table>

Abbreviations: BCP, B-cell precursor; wt, wild-type;

* Relative amount of mRNA compared with that in normal peripheral blood lymphocytes (fold),

** Tested by WST assay,

*** Percent of apoptotic cells induced by 100 nM triptolide after 48 h.