Triptolide Inhibits Bcr-Abl Transcription and Induces Apoptosis in STI571-resistant Chronic Myelogenous Leukemia Cells Harboring T315I Mutation

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Abstract

Purpose: Resistance to STI571 is an emerging problem for patients with chronic myelogenous leukemia (CML). Mutation in the kinase domain of Bcr-Abl is the predominant mechanism of the acquired resistance to STI571. In the present study, we investigated the effect of triptolide on cell survival or apoptosis in CML cells bearing Bcr-Abl-T315I or wild-type Bcr-Abl. Experimental Design: CML cell lines (KBM5 versus KBM5-T315I, BaF3-Bcr-Abl versus BaF3-Bcr-Abl-T315I) and primary cells from CML patients with clinical resistance to STI571 were treated with triptolide, and analyzed in terms of growth, apoptosis, and signal transduction. Nude mouse xenograft model was also used to evaluate the antitumor activity. Results: Triptolide potently down-regulated the mRNA and protein levels of Bcr-Abl independently of the caspase or proteosome activation in CML cells. It induced mitochondrial-dependent apoptosis in Bcr-Abl-T315I CML cells and primary cells from CML patients with clinical resistance to STI571. Additionally, triptolide inhibited the growth of STI571-sensitive KBM5 and STI571-resistant KBM5-T315I CML cells in nude mouse xenografts. Triptolide also down-regulated the expression of survivin, Mcl-1, and Akt in CML cells, which suggests that it may have multiple targets. Conclusions: These findings suggest that triptolide is a promising agent to overcome STI571-resistant CML cells, and warrant a clinical trial of triptolide derivatives for CML with Bcr-Abl-T315I mutation.

Bcr-Abl, the product of a fusion gene formed by a reciprocal chromosomal translocation t(9;22)(q34;q11), is found in ~95% patients with chronic myelogenous leukemia (CML) and 30% of adult patients with acute lymphoblastic leukemia (1–3). The aberrant Ab1 tyrosine kinase activity of this chimeric protein is responsible for inducing malignant transformation (4). Bcr-Abl has been an important target for leukemia therapeutics (5). STI571 (imatinib mesylate; Gleevec; Novartis) effectively inhibits tyrosine kinase activity by occupying the ATP-binding pocket of Bcr-Abl, thus abrogating subsequent signal transduction pathways (6). Of newly diagnosed patients with chronic-phase CML, 82% showed complete cytogenetic response on treatment with STI571 over a median follow-up of 54 months (7). However, resistance to STI571 develops over time and is an emerging problem for CML patients. Multiple mechanisms have been proposed to explain the resistance, including Bcr-Abl–independent Lyn kinase activation (8), binding of imatinib to serum α-1 acid glycoprotein (9), increased drug efflux through the multidrug resistance gene (10), Bcr-Abl gene amplification (11), and Bcr-Abl gene mutation (11, 12). Among them, mutation in the kinase domain of Bcr-Abl is believed to be the predominant mechanism underlying the resistance. To overcome the acquired resistance, new tyrosine kinase inhibitors such as AMN107 (nilotinib), dasatinib, and INNO-406 have been developed and shown to be effective, to some extent, against all
Triptolide Kills STI571-Resistant CML Cells

Translational Relevance

STI571 (Gleevec) has had major effect on chronic myelogenous leukemia (CML) therapy, but resistance to STI571 is now an emerging problem for CML patients. Mutations in the kinase domain of Bcr-Abl have been identified as the predominant mechanism of acquired drug resistance to STI571. Novel tyrosine kinase inhibitors such as AMN107 and dasatinib are effective against all but the T315I mutation in Bcr-Abl. The prognosis for patients who fail STI571, AMN107, and dasatinib therapy is very poor, and treatment options are extremely limited. In this article, we report our findings of triptolide, a biologically active component isolated from the traditional Chinese herbal medicine Tripterygium wilfordii Hook.f., against CML cells bearing Bcr-Abl-T315I or wild-type Bcr-Abl. We found triptolide exerts a striking inhibitory effect on the growth of CML cells. We confirmed this potent activity in vitro and in xenograft model. Our findings warrant a clinical trial that would benefit patients with T315I Bcr-Abl.

Materials and Methods

Chemicals. Triptolide was purchased from Sigma-Aldrich and prepared as a 20 mmol/L stock solution in DMSO. The stock solution was stored in aliquots at -20°C. STI571 was a product of Novartis Pharmaceuticals (34). Caspase inhibitors z-DEVD-fmk and z-VAD-fmk were obtained from BD Biosciences. MG132 and N-acetyl-l-cysteine (NAC) were purchased from EMD Biosciences.

Cell lines. The culture of KBM5 and KBM5-T315I cells was described previously (34). In brief, KBM5 cells expressing 210 kDa wild-type Bcr-Abl were cultured in Iscove’s modified Dulbecco’s medium (Invitrogen) supplemented with 10% heat-inactivated FCS. KBM5-T315I cells were routinely maintained in the same medium but with 1 μmol/L STI571, which was removed before experiments with a wash-out period of 2 to 3 d. KBM5 cells expressing the 210 kDa wild-type Bcr-Abl was derived from a female CML patient. The KBM5-T315I subline was originally established by exposure to increasing concentrations of STI571. KBM5-T315I cells, harboring a threonine-to-isoleucine substitution at position 315 of Abl, are resistant to STI571, and they only have a marginal increase Bcr-Abl gene copies and expression (35–37). BaF3-Bcr-Abl cells and BaF3-Bcr-Abl-T315I cells were established from murine BaF3 cells by stable transfection with plasmids expressing wild-type or T315I (34). BaF3-Bcr-Abl, BaF3-Bcr-Abl-T315I, K562, Molm13, MV4-11, and HEL cells were cultured in RPMI 1640 supplemented with 10% FCS.

Primary cells from CML patients. Peripheral blood samples were obtained from seven CML patients with imatinib resistance in The First Affiliated Hospital of Sun Yat-sen University after informed consent according to the institutional guidelines and the Declaration of Helsinki principles. The information of the seven patients is summarized in Supplementary Table 1. Mononuclear cells were isolated by Histopaque gradient centrifugation (density 1.077; Sigma-Aldrich). Contaminating red cells were lysed in 0.8% ammonium chloride solution for 10 min. After a washing, cells were suspended in RPMI 1640 supplemented with 10% FCS. All drug treatments started after the cells were preincubated in fresh medium for 24 h.

Cell viability assay. Cell viability was evaluated by MTS assay (CellTitre 96 Aqueous One Solution reagent, Promega) as described previously (38). The drug concentration resulting in 50% inhibition of cell growth (IC50) was determined by curve fitting of the dose-response curve.

Clonogenicity assay. KBM5 and KBM5-T315I cells (2 × 103/mL) were treated with increasing concentrations of triptolide or diluent (DMSO, control) for 24 h, then washed with PBS and seeded in 6-well plates in Iscove’s medium containing 0.3% agar and 20% FCS in the absence of drug treatment. After incubation for 10 to 14 d at 37°C, colonies composed of >50 cells were counted by use of an inverted phase-contrast microscope.

Western blot analysis. Western blot analysis, except for cytochrome c release detection, was with whole cell lysates prepared in radio-immunoprecipitation assay buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with freshly added 10 mmol/L β-glycerophosphate, 1 mmol/L sodium orthovanadate, 10 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, and 1× Roche Complete Mini Protease Inhibitor Cocktail (Roche; ref. 39). Antibodies against c-Abl (C-19), Mcl-1 (S1-19), caspase-3, and Bax were from Santa Cruz Biotechnology. Antibodies against poly(ADP-ribose) polymerase (PARP, clone 4C10-5) were from BD Biosciences. Antibodies against phospho-c-Abl at Y245, phospho-Erk1/2 (T202/ T204), Erk1/2, phospho-CrkL at Y207, Akt, and XIAP were from Cell Signaling Technology. Antibodies against phospho-STAT5A/B (Y694/Y699; clone 8-5-2), STAT5A, phospho–signal transducers and activators of transcription 3 (STAT3) at Y705 (clone 9E12), STAT3, and Bcl-2 were from Upstate Technology; Mouse monoclonal antibody against actin was from Sigma-Aldrich. Anti-mouse IgG and anti-rabbit IgG horseradish peroxidase–conjugated antibodies were from Pierce Biotechnology.

To detect the level of cytochrome c in the cytosol, the cytosolic fraction was prepared with digitonin extraction buffer as described previously (40). Mouse anti-cytochrome c (clone 6H2.B4) was from BD Biosciences.
Semiquantitative reverse transcription-PCR. Total RNA was extracted from KBM5 and KBM5-T315I cells treated with or without triptolide by using Trizol reagent (Invitrogen). After quantification by spectrophotometry, the first-strand cDNA was synthesized from 1 μL of total RNA with the use of the RNA PCR kit (AMV) Ver. 3.0 (TaKaRa) and random primers. Bcr-Abl gene amplification involved the following primers (35): forward 5'-TTCAGAAGCTTCTCCCTGACAT'-3' (exon 13 of Bcr gene), reverse 5'-CTTCGTCTGAGATACTGGATTCCT'-3' (exon 9 of Abl gene).

Fig. 1. Triptolide inhibits growth of CML cells expressing wild-type Bcr-Abl and T315I-mutant Bcr-Abl at nanomolar concentrations. Cell viability (percent relative to control) was determined by MTS assay. Triptolide dose response curves are shown. Triptolide (72-h treatment) potently inhibited the cell viability in STI571-sensitive KBM5 and STI571-resistant KBM5-T315I cells, BaF3 cells transfected with wild-type p210 Bcr-Abl or T315I Bcr-Abl, K562 expressing wild-type p210 Bcr-Abl (A), and primary mononuclear cells from peripheral blood of patients with clinically STI571-resistant CML (B). Colony formation (C) and cell cycling (D) of CML cells as analyzed by fluorescence-activated cell sorting. KBM5 cells were exposed to triptolide for 24 h. Histograms show data from a representative experiment.
were measured every other day with use of calipers. Tumor volumes in inoculated s.c. on the flanks of 6- to 8-wk-old male nude mice. Tumors were counterstained with hematoxylin. A negative control for c-Abl was included for each xenograft specimen by substituting the primary antibody with preimmune goat serum.

Statistical analysis. All experiments were performed at least thrice, and results are expressed as mean ± SE unless otherwise stated. GraphPad Prism 4.0 software (GraphPad Software) was used for statistical analysis. Comparisons between variables involved Student’s t test. P values of <0.05 was considered statistically significant.

Results

Triptolide inhibits growth of STI571-sensitive and STI571-resistant CML cells at low nanomolar concentration. We recently reported a CML cell line harboring wild-type Bcr-Abl (KBM5) and a variant line harboring T315I Bcr-Abl (KBM5-T315I) with different sensitivities to STI571; IC50 values were 0.28 and 5.4 μmol/L, respectively (34). To investigate the effect of triptolide on growth of CML cells bearing T315I Bcr-Abl, KBM5 and KBM5-T315I cells were incubated for 72 hours with increasing concentrations of triptolide. Cell viability (measured by MTS assay) of both cell types was inhibited, with IC50 values of 6.1 and 5.5 nmol/L, respectively (Fig. 1A, left), which suggests similar sensitivities of these two CML lines to triptolide. To further validate this finding, we treated a pair of BaF3 murine cell lines stably expressing wild-type or T315I-mutant Bcr-Abl with triptolide and found their growth significantly inhibited, with IC50 values of 6.5 and 5.4 nmol/L, respectively (Fig. 1A, middle). Even in the presence of IL-3, the IC50 values were 7.4 and 9.0 nmol/L, respectively (data not shown). So both cell line types were equally sensitive to triptolide. K562 cells bearing wild-type Bcr-Abl were also sensitive to triptolide (IC50, 5.7 nmol/L; Fig. 1A, right). Additionally, triptolide inhibited the cell viability of leukemia cell lines MolM13 and MV-4-11, which carry FLT-3-ITD mutation (IC50, 2.9 and 3.8 nmol/L, respectively), and HEL, which carries Jak2 V617F mutation (IC50 value of 42.3 nmol/L; data not shown). These results suggest that triptolide may be effective against cells expressing activated protein tyrosine kinases Bcr-Abl, FLT-3, and Jak2.

We next examined the effect of triptolide on STI571-resistant primary CML cells. Mononuclear cells in peripheral blood from CML patients who were clinically resistant to STI571 were exposed to various concentrations of triptolide for 72 hours. The primary CML cells were sensitive to triptolide as well, with IC50 of 8.17 ± 2.61 (range, 4.8 to 13.3 nmol/L; n = 3; Fig. 1B). Because clonogenicity is believed to better reflect malignant behavior of tumor cells, we determined the effect of triptolide on clonogenicity in CML cells. KBM5 or KBM5-T315I cells were exposed to increasing concentrations of triptolide for 24 hours, and then were assayed for colony formation in the absence of drugs. Triptolide potently inhibited the number of surviving clonogenic KBM5 and KBM5-T315I cells in a dose-dependent manner, with IC50 values of 1.7 and 1.6 nmol/L, respectively (Fig. 1C).

We also assessed whether triptolide disturbed cell cycle distribution. The results revealed that it did not lead to significant alteration in cell cycle (Fig. 1D), which is consistent with the previous report (26).

Taken together, triptolide potently inhibits cell growth in CML cells in a dose-dependent manner.

Triptolide down-regulates Bcr-Abl transcription. To address the mechanism of the triptolide-mediated growth inhibition in CML cells, we analyzed the expression of Bcr-Abl protein in CML cells treated with or without triptolide by immunoblotting. Twenty-four-hour exposure of KBM5 and KBM5-T315I cells to nanomolar concentrations of triptolide dose-dependently decreased Bcr-Abl level in both cell types (Fig. 2A). Exposing KBM5 or KBM5-T315I cells to a fixed concentration (50 nmol/L) of triptolide revealed a time-dependent decrease of Bcr-Abl protein (Fig. 2B). Notably, the decreased Bcr-Abl
Fig. 2. Triptolide decreases Bcr-Abl mRNA level. A, immunoblots of Bcr-Abl, PARP, and actin are shown. Twenty-four-hour treatment of triptolide reduced Bcr-Abl protein level in a dose-dependent manner in KBM5 (lanes 1-5) and KBM5-T315I cells (lanes 8-12). Inhibition of proteasome by use of MG132 (0.5 μmol/L) did not reverse the inhibitory action of triptolide in KBM5 (lanes 6-7) and KBM5-T315I cells (lanes 13-14). B, time-dependent down-regulation of Bcr-Abl protein by triptolide. Immunoblots of Bcr-Abl, phospho-Bcr-Abl, PARP, and actin are shown. Triptolide (50 nmol/L) decreased Bcr-Abl and phospho-Bcr-Abl levels in a time-dependent manner in KBM5 (lanes 1-6) and KBM5-T315I (lanes 7-12) cells. The onset of specific cleavage of PARP with the down-regulation of Bcr-Abl and phospho-Bcr-Abl. C, triptolide decreases Bcr-Abl and c-Abl mRNA levels in a dose- and time-dependent manner. Ethidium bromide-stained reverse transcription-PCR products of Bcr-Abl, c-Abl, and glyceraldehyde-3-phosphate dehydrogenase mRNAs from KBM5 and KBM5-T315I cells treated with various concentrations of triptolide for 15 h (top) or a fixed concentration (50 nmol/L) for various durations (bottom) are shown. c, DMSO control. D, the effect of triptolide on the downstream targets of Bcr-Abl in CML cells. CML cell lines were exposed to triptolide at the indicated concentrations for 24 h. Cell lysates were analyzed by Western blotting with the specific antibodies against the proteins as indicated.
protein level occurred concomitantly with apoptosis, as indicated by specific PARP cleavage after triptolide treatment (Fig. 2A and B). To investigate whether the proteosome pathway was involved in the triptolide-mediated down-regulation of Bcr-Abl, we pretreated KBM5 cells with a subcytotoxic concentration (0.5 μmol/L) of the proteosome inhibitor MG132 but found no effect on decrease in Bcr-Abl protein level (lane 1 versus lanes 5 and 6, lane 8 versus lanes 11-13; Fig. 2A) in KBM5 and KBM5-T315I cells. An increased concentration (1.0 μmol/L) of MG132 also failed to prevent the degradation of the wild-type or T315I mutant Bcr-Abl (data not shown). However, the MG-132 exhibited an activity to reverse the degradation of Mcl-1 mediated by another small molecule compound EXEL-0862 as described previously (39).

C-Abl is a substrate of caspase-3 (34), an effector of apoptosis, so we tested whether the triptolide-induced reduction in Bcr-Abl level was a result of apoptosis. We treated KBM5 cells with 50 nmol/L triptolide for 24 hours in the absence or...
The presence of 10 μmol/L z-DEVD-fmk (caspase-3 inhibitor) and then detected Bcr-Abl level. Inhibition of caspase-3 did not abrogate the triptolide-mediated decrease in Bcr-Abl level (Supplementary Fig. S1), which suggests that triptolide decreases Bcr-Abl independently of caspase-3 activation. Similar observations were obtained with 20 μmol/L z-VAD-fmk (a pan-caspase inhibitor; data not shown), which further confirmed that the Bcr-Abl decrease induced by triptolide did not result from caspase activation.

Because triptolide can suppress the transcription of proteins such as HIF1 and nuclear factor-κB (24, 41), we hypothesized that triptolide might inhibit Bcr-Abl at the transcriptional level. We treated KBM5 and KBM5-T315I cells with increasing concentrations of triptolide for 24 hours; semiquantitative reverse transcription-PCR revealed the mRNA level of both Bcr-Abl and c-Abl diminished even with treatment of low concentrations (5 or 10 nmol/L) of triptolide (Fig. 2C). The kinetics of mRNA decrease is consistent with the decrease in protein level (Fig. 2C, bottom, versus Fig. 2B).

Triptolide inhibits the activation of Bcr-Abl kinase and its downstream targets. Bcr-Abl is a constitutively active tyrosine kinase that phosphorylates several substrates and activates multiple signal transduction pathways such as mitogen-activated protein kinase/extracellular signal-related protein kinase, CrkL, STAT5, STAT3, and PI3K/Akt all of which can stimulate cell proliferation and resistance to apoptosis (4). We reasoned that the loss of total Bcr-Abl protein would lead to a decrease in its kinase activity. We measured the kinase activity by immunoblotting, using the phospho-specific antibody against the phosphorylated Y245 c-Abl as described previously (16). Baseline phosphorylation of Bcr-Abl was detectable in both KBM5 and KBM5-T315I cells (Fig. 2B, lanes 1 and 7). As expected, triptolide potently decreased the phosphorylation of Bcr-Abl, which agreed with alteration in expression of total Bcr-Abl protein (Fig. 2B). Accordingly, the phosphorylation of STAT5, STAT3, and CrkL Erk1/2 was also inhibited by triptolide treatment (Fig. 2D, top). Additionally, phosphorylation of STAT3 and CrkL was potently inhibited by triptolide in BaF3 cells.

Fig. 4. Triptolide leads to changes in expression of apoptosis-related proteins and mitochondrial damage. A, triptolide decreased expression of Mcl-1 and survivin. KBM5 and KBM5-T315I cells were treated with increasing concentrations of triptolide for 24 h. Expression of apoptosis-related proteins was detected by Western blots with the specific antibodies indicated. B and C, mitochondrial membrane depolarization was induced by triptolide in CML cells. KBM5 and KBM5-T315I exposed to triptolide were stained with chloromethyl-X-rosamine and MTGreen, and analyzed by flow cytometry. R2, (B) the population of cells with intact mitochondria; R3, the population with partial loss of mitochondrial potential; and R4, the population with complete loss of mitochondrial potential. C, representative fluorescent histograms from three independent experiments performed in triplicate. Vertical axis, the sum of R3 and R4. Columns, mean; bars, SE. D, triptolide induced mitochondrial release of cytochrome c. KBM5 cells were treated with triptolide for 12 h, and cytochrome c (cyto c) in cytosolic fractions was measured by Western blot analysis.
cells stably expressing wild-type or T315I Bcr-Abl (Fig. 2D, bottom). Because the total level of Akt was decreased in triptolide-treated CML cells, triptolide may have targets other than Bcr-Abl (Fig. 2D, top). However, c-Jun-NH2-kinase level was not significantly reduced in treated CML cells. Thus, triptolide abolishes the kinase activity of Bcr-Abl and activation of its downstream targets.

**Triptolide induces apoptosis in STI571-sensitive and STI571-resistant CML cells.** Given that triptolide elicits a dose- and time-dependent specific cleavage of PARP (Fig. 2A and B), a hallmark of apoptosis, and substantial sub-G1 proportions (Fig. 1D), we further explored the ability of triptolide to induce cell death in CML cell lines by flow cytometry after Annexin V/propidium iodide staining. Exposure of KBM5 and KBM5-T315I cells to increasing concentrations (range, 25-150 nmol/L) of triptolide resulted in apoptotic cell death in a large percentage of cells (Fig. 3A). Similarly, a nanomolar concentration induced a significant increase in proportion of Annexin V-positive cells in BaF3-Bcr-Abl and BaF3-Bcr-Abl-T315I cells, although these two lines were less sensitive than KBM5 and KBM5-T315I lines (Fig. 3B). Triptolide induced a dose- and time-dependent decrease of the precursor form of caspase-3 (Fig. 3C and D), which reflected the activation of caspase-3. Of note, the kinetics of caspase-3 activation by dynamic monitoring (Fig. 3D) paralleled the PARP cleavage results (Fig. 2B) and was inversely related to the alteration of Bcr-Abl level. Therefore, triptolide induced apoptosis in STI571-sensitive and STI571-resistant CML cells.

**Triptolide decreases Mcl-1 and survivin and leads to mitochondrial damage.** To elucidate the mechanism of triptolide-induced apoptosis, we evaluated the expression of apoptosis-related proteins. KBM5 and KBM5-T315I cells were treated with triptolide doses (25-100 nmol/L) for 24 hours. Western blot analysis revealed no change in expression of

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**Fig. 5.** Triptolide induces apoptosis in primary CML cells. **A** and **B**, peripheral blood mononuclear cells from CML patients with STI571 resistance were incubated with DMSO containing medium (control) or 100 or 1,000 nmol/L triptolide for 20 h. Cell death was measured by flow cytometry with Annexin V/propidium iodide double staining. Representative results in two patients are shown in **A**. Statistical analysis of apoptosis induced by triptolide in patients with STI571-resistant CML (n = 5; B). *, P < 0.0001, Student’s t test. **C**, PARP cleavage was observed by Western blot analysis after 20 h of treatment of peripheral blood mononuclear cells with triptolide.
antiapoptotic protein Bcl-X<sub>L</sub>, Bcl-2, and proapoptotic protein Bax but a substantial decrease in survivin and Mcl-1 level and a slight decrease in XIAP level (Fig. 4A). Because survivin, Mcl-1, and XIAP are predominantly localized to mitochondria, we evaluated whether triptolide damaged mitochondria. KBM5 and KBM5-T315I cells were exposed to increasing triptolide concentrations for 24 hours, and the uptake of chloromethyl-X-rhodamine and MTFgreen double staining was measured by flow cytometry (Fig. 4B). The proportion of CML cells with loss of mitochondrial potential substantially increased with triptolide treatment (Fig. 4C). Notably, the pattern of mitochondrial transmembrane potential change was in line with results of other apoptotic indices, including caspase-3 activation (Fig. 3C) and PARP cleavage (Fig. 2A). Furthermore, the level of cytochrome <i>c</i> in the cytosolic fraction detected by Western blot analysis was elevated even after 12-hours treatment with 50 nmol/L triptolide (Fig. 4D). Thus, triptolide triggered the mitochondrial pathway of apoptosis.

Because triptolide has been reported to induce ROS in macrophages (42), we investigated whether ROS is involved in the mechanism of triptolide-mediated apoptosis in CML cells. KBM5 cells were exposed to 25 nmol/L triptolide for various durations, and intracellular ROS was monitored by CM-H2DCF-DA fluorescent dye. Twelve- and 24-hour triptolide treatment increased median ROS content by 143% and 165%, respectively (Supplementary Fig. S2A). The kinetics of this slight increase of ROS did not precede apoptosis. To further characterize the role of ROS, we examined the reducing agent NAC. We have reported that NAC at 2 mmol/L could effectively quench ROS by increasing intracellular glutathione in KBM5 cells (34). We therefore pretreated KBM5 cells with 2 mmol/L NAC for 1 hour and then exposed cells to 25 or 50 nmol/L triptolide for 24 hours. As shown in supplementary Fig. 2B, NAC was unable to prevent the triptolide-mediated decrease of Bcr-Abl expression and apoptosis (cleavage of PARP). Therefore, the ROS increase was not a mediating factor in triptolide-induced Bcr-Abl inhibition and apoptosis. Considering that mitochondrial damage may elevate the level of ROS, triptolide might damage mitochondria through a not-yet completely known mechanism, leading to subsequent increase in ROS generation.

**Triptolide induces apoptosis in primary CML cells.** Our <i>in vitro</i> findings prompted us to assess the efficacy of triptolide in primary tumor cells from CML patients. Peripheral blood mononuclear cells isolated from 5 patients with imatinib-resistant CML were exposed to DMSO (3.01% v/v) containing medium (control), or 100 or 1,000 nmol/L triptolide for 20 hours, and cell death was measured by flow cytometry with Annexin V/propidium iodide double staining. Triptolide at 100 nmol/L was sufficient to induce substantial apoptosis in these primary CML cells (<i>P</i> < 0.0001; Fig. 5A and B). Western blot analysis of PARP cleavage after 20-hour triptolide treatment of peripheral blood mononuclear cells suggested apoptosis, with no appreciable change in Bcl-X<sub>L</sub> protein level (Fig. 5C). The <i>ex vivo</i> results, like the <i>in vitro</i> results, suggest that triptolide induces apoptosis.

**Triptolide abrogates the growth of xenografted CML cells in nude mice.** We further examined the <i>in vitro</i> effect of triptolide on STI571-resistant CML cells using the nude mouse xenograft model. Twenty <i>nu/nu</i> BALB/c mice were injected with KBM5 and another 20 mice with KBM5-T315I cells. Five days after inoculation of tumor cells, when the size of tumor reached ~50 mm<sup>3</sup>, mice inoculated with each type of cells were randomized to receive treatment with DMSO (0.1% v/v) containing medium (control) or triptolide, 0.15 mg/kg/d, for 21 days (10 animals each group). We and others have found this triptolide dosage to be well-tolerated (27). The growth curves (the estimated tumor size calculated from the tumor dimension versus time) are shown in Fig. 6A. Triptolide potently inhibited the growth of both KBM5 and KBM5-T315I tumors. The sizes of dissected and weighed tumors were remarkably lower in the treated group than in the control group (Fig. 6B, <i>top</i>). Triptolide-treated tumors were significantly lower in weight than control tumors for both cell tumor types (<i>P</i> < 0.0001; Fig. 6B, <i>bottom</i>). Immunohistochemical analysis with an anti–c-Abl antibody (to detect both c-Abl and Bcr-Abl) revealed c-Abl immunoreactivity greatly decreased by triptolide treatment (Fig. 6C). These data again reflected the potent antitumor activity of triptolide. The body weight of the mice remained stable, with no significant differences between treated and control mice (data not shown). Motor activity and feeding behavior of the mice were all normal. Whole blood cell counts did not reveal any significant myelosuppression in the treated mice (data not shown), nor were aspartate and alanine aminotransferase activities increased in the treatment group. No mice died. Overall, surveillance of morbidity and mortality did not reveal any significant toxicity of the triptolide at the dosage used.

**Discussion**

The prognosis of CML resistant to STI571 is poor (13). Point mutations in Bcr-Abl, which prevent STI571 binding, are responsible for most of the cases of acquired clinical resistance to STI571. We aimed to identify effective chemotherapy against leukemic cells carrying the notorious Bcr-Abl-T315I mutation that confers resistance to STI571 as well as the second generation of tyrosine kinase inhibitors. Our prime candidate was triptolide, the major active component in the traditional Chinese medicine herb <i>T. wilfordii</i> Hook. L., because it has antitumor activities in a broad range of human cancer cells (25–28) and can down-regulate Bcr-Abl expression in K562 cells via an unknown mechanism (33). Triptolide potently down-regulated Bcr-Abl at the level of transcription and inhibited the growth and induced apoptosis in CML cells harboring wild-type Bcr-Abl or Bcr-Abl-T315I mutation. We confirmed this potent activity with two pairs of CML cell lines (KBM5 versus KBM5-T315I, BaF3-Bcr-Abl versus BaF3-Bcr-Abl-T315I) and primary cells from CML patients with clinical resistance to STI571. Additionally, triptolide inhibited the growth of STI571-resistant Bcr-Abl-T315I as well as STI571-sensitive CML cells in nude mouse xenografts; the protein level of Bcr-Abl in STI571-sensitive (KBM5) or STI571-resistant (KBM5-T315I) CML cells was decreased after triptolide treatment. To our knowledge, this is the first report to show that triptolide is effective <i>in vitro</i> and <i>in vivo</i> against CML cells, including those with the T315I mutation.

Extensive efforts have been made to overcome STI571 resistance. Several second-generation Abl kinase inhibitors, such as AMN107, dasatinib (15) and INNO-406, SKI-606, and PD166326, have been developed (4). They have stronger affinities for the ABL-binding site than...
does STI571, to some extent, and thus are effective for STI571-resistant patients. These novel inhibitors can effectively inhibit the phosphorylation of the mutated Bcr-Abl (E255K, M351T) but not Bcr-Abl-T315I. An ATP-noncompetitive inhibitor (ONO12380; ref. 43), aurora kinase inhibitor (VX-680; ref. 44), p38 MAP kinase inhibitor (BIRB-796; ref. 45), and ROS-promoting agents (PEITC and adaphostin; refs. 34, 46) have been evaluated to overcome the problem of the Bcr-Abl-T315I mutation. VX-680 may be effective against Bcr-Abl-T315I in vitro (44, 47, 48), but its efficiency and safety in vivo remain to be explored. However, an effective strategy may be minimizing the activity of Bcr-Abl by inhibiting the expression of Bcr-Abl at the protein or mRNA level (16, 17, 49).

Triptolide-mediated down-regulation of Bcr-Abl was independent of caspase or proteosome activation. Instead, triptolide, at low nanomolar concentrations, potently inhibited the transcription of Bcr-Abl. At the present time, the underlying mechanism is not clear. During preparation of this article, Leuenroth et al. (50) reported that triptolide induces distinct rounding of nuclear speckles and global transcriptional arrest.

Fig. 6. Triptolide abrogates the growth of tumors transplanted in nude mice with down-regulation of c-Abl. A, the growth curves of subcutaneous xenografts of KBM5 or KBM5-T315I cells are shown. The estimated tumor sizes are plotted against the number of days since triptolide treatment began (day 5 after inoculation). Columns, mean; bars, SE. B, on day 21, xenografts were dissected, photographed, and weighed. Representative tumors removed from four mice of each group are shown (B, top). The bar chart (B, bottom) shows the weight of the tumors from each group (n = 10). Columns, mean; bars, SE. *, P < 0.0001 by Student’s t test. C, the expression of c-Abl was greatly inhibited by triptolide. Immunohistochemical analysis with anti–c-Abl in xenograft tissues from mice on day 21 after triptolide treatment and H&E staining.
by inhibiting RNA polymerase I and II. It will be interesting to further explore the decline of Bcr-Abl induced by triptolide is associated with these changes.

Our findings suggest that inhibition of Bcr-Abl is essential for the inhibitory activity of triptolide in CML cells. First, Bcr-Abl plays a pivotal role in the pathogenesis of CML. Second, overexpression of the Bcr-Abl oncoprotein inhibits apoptosis induced by multiple stimuli (51). Third, decreasing Bcr-Abl expression by compounds (16, 17) or specific antisense oligonucleotides induces apoptosis (52). Fourth, our time course experiments showed that the levels of total and phosphorylated Bcr-Abl declined along with apoptosis after triptolide treatment (Fig. 2B). Finally, the phosphorylation of downstream targets of Bcr-Abl (i.e., CrkL, STAT3, STAT3, Erk1/2) was inhibited by triptolide (Fig. 2D).

Additional mechanisms may enhance the inhibitory effect of triptolide. Our findings indicated that the level of survivin, Mcl-1, and Akt was decreased after treatment with triptolide, so triptolide may have multiple targets. Triptolide is a potent inhibitor of nuclear factor-κB and NF-AT–mediated transcription (24) and can abolish Hsp70 expression in pancreatic cancer cells (28).

Our results of loss of mitochondrial transmembrane potential and release of cytochrome c into the cytoplasm in triptolide-treated CML cells (Fig. 4) are consistent with the report by Carter et al. (26), showing that triptolide triggers a mitochondrial-dependent pathway. The precise mechanism of triptolide-mediated mitochondrial damage is not yet known. It may be related to the pronounced decline in Mcl-1 and survivin level. Mcl-1 and survivin are frequently overexpressed in leukemias and play a crucial role in cell proliferation and survival or death, their inhibition by triptolide likely contributes to the antineoplastic activity of triptolide.

Bao et al. (42) reported that ROS plays a role in triptolide-induced apoptosis in macrophage cells. However, our data do not support a critical role of ROS in triptolide-induced apoptosis in CML cells. First, the scale of ROS increase is mild. Second, the ROS increase did not precede apoptosis. Third, NAC, a potent inhibitor of ROS generation, had no significant effect on triptolide-induced inhibition of Bcr-Abl expression and apoptosis. The alteration of ROS level is more likely a secondary change induced by triptolide that had little or no contribution to apoptosis.

In summary, our in vitro and in vivo results show that triptolide has potent activity against cells bearing wild-type or Bcr-Abl/T315I mutation. Our in vitro study (triptolide at 0.15 mg/kg) and another study by others (PG490-88 at ~0.75 mg/kg) show that triptolide and its derivatives have significant antineoplastic activity without apparent toxicity in nude mouse xenograft models (23). A phase I clinical trial of a water-soluble derivative of triptolide on solid tumors is ongoing in Europe (26). Compared with primary leukemia cells, CD34-positive cells from normal human bone marrow cells are less sensitive to triptolide (26). A clinical trial of triptolide derivatives such as PG490-88 for patients with imatinib-resistant CML may be warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Sai-Ching J. Yeung (The University of Texas M. D. Anderson Cancer Center, Houston, TX, USA) for a critical reading of the manuscript.

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