Triptolide is an inhibitor of RNA polymerase I and II–dependent transcription leading predominantly to down-regulation of short-lived mRNA

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Abstract

Triptolide, a natural product extracted from the Chinese plant *Tripterygium wilfordii*, possesses antitumor properties. Despite numerous reports showing the proapoptotic capacity and the inhibition of NF-κB–mediated transcription by triptolide, the identity of its cellular target is still unknown. To clarify its mechanism of action, we further investigated the effect of triptolide on RNA synthesis in the human non–small cell lung cancer cell line A549. Triptolide inhibited both total RNA and mRNA *de novo* synthesis, with the primary action being on the latter pool. We used 44K human pan-genomic DNA microarrays and identified the genes primarily affected by a short treatment with triptolide. Among the modulated genes, up to 98% are down-regulated, encompassing a large array of oncogenes including transcription factors and cell cycle regulators. We next observed that triptolide induced a rapid depletion of RPB1, the RNA polymerase II main subunit that is considered a hallmark of a transcription elongation blockage. However, we also show that triptolide does not directly interact with the RNA polymerase II complex nor does it damage DNA. We thus conclude that triptolide is an original pharmacologic inhibitor of RNA polymerase activity, affecting indirectly the transcription machinery, leading to a rapid depletion of short-lived mRNA, including transcription factors, cell cycle regulators such as CDC25A, and the oncogenes MYC and Src. Overall, the data shed light on the effect of triptolide on transcription, along with its novel potential applications in cancers, including acute myeloid leukemia, which is in part driven by the aforementioned oncogenic factors. [Mol Cancer Ther 2009;8(10):2780–90]

Introduction

Triptolide is a natural product extracted from the Chinese plant *Tripterygium wilfordii*, used in Chinese traditional medicine to treat immune inflammatory diseases (1, 2). Its potent antiproliferative activity *in vitro* has prompted its evaluation on human tumors xenografted in mice, where it clearly showed antitumor activities (3–5). Interestingly, triptolide elicited clinical responses when tested in a phase I clinical trial in patients with leukemia (6). Numerous reports show that triptolide inhibits NF-κB–mediated transcription, which was presented as the basis for its anti-inflammatory but also for its antiproliferative and proapoptotic properties (7–9). Recent data show that triptolide inhibits transcription mediated by the heat shock factor 1 (HSF1) and activator protein-1 factors as well (10, 11). McCallum et al. showed that triptolide inhibits *de novo* total RNA transcription in A549 and THP-1 tumor cells (10). Because rRNA represents >80% of total RNA (12), this latter study suggests that triptolide inhibits the production of this RNA pool. However, the potential activity of this molecule on the pool of mRNA was not extensively investigated. More recently, Leuenroth and Crews published data, indicating that triptolide induces nuclear morphologic changes, that is, disruption of nucleolar integrity and nuclear speckle rounding, which were proposed to reflect a global effect of triptolide on transcription (13). In the same study, relocalization of nucleolin and SC35, respectively, a ribosomal protein and a RNA splicing factor, was evidenced 16 h after compound addition. Furthermore, triptolide was shown to induce an inhibition of RNA polymerase II Ser^2 phosphorylation, which could account for an inhibition of mRNA transcription. Actually, a gene expression analysis of human cancer Jurkat cells revealed the underexpression of 117 genes on triptolide treatment (14). But again, the precise effect of triptolide on rRNA versus mRNA, along with its molecular target(s), remains to be elucidated. All these data strongly suggest that triptolide is a general transcription inhibitor that would modulate a larger set of genes than those controlled

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by NF-κB, HSF1, and activator protein-1 as originally proposed. Because triptolide was shown to act downstream of NF-κB and HSF1 binding to DNA, one can hypothesize that it potentially interacts with the rRNA and mRNA transcription machinery (7, 8, 11). Several targets could account for the transcription inhibition properties of triptolide. First, DNA topoisomerase I, through its decatenating property, is required for proper gene transcription; indeed camptothecin, a specific topoisomerase I inhibitor, efficiently impedes RNA synthesis (15, 16). Second, DNA-interacting molecules, such as actinomycin D, and DNA-damaging agents, including UV radiation, have also been reported to block transcription (17–19). Finally, α-amanitin, a specific RNA polymerase II inhibitor, acts through direct interaction with the RPB1 catalytic subunit of this enzyme (20–23). With the aim to clarify the mechanism of action of triptolide, we investigated the ability of this natural product to interfere with transcription and to interact with the abovementioned molecular targets. Therefore, the activity of triptolide on intracellular RNA polymerase I and II–mediated transcription was investigated. To complete this study, a direct interaction between triptolide and the purified functional RNA polymerase II transcription machinery was evaluated.

Materials and Methods

Cell Lines and Reagents

All cell lines were obtained from the American Type Culture Collection, except Miapaca2, MX1, G361, WM115, and WM266, which were obtained from the European Collection of Cell Cultures. These cells were cultivated in either DMEM (Life Technologies) for COS cells or RPMI 1640 (Life Technologies) for the others, both supplemented with 10% heat-inactivated FCS (Sigma), penicillin (65 μg/mL), and streptomycin (100 μg/mL). Human RNA polymerase II with a FLAG-tagged Rpb3 subunit was purified from HEK293 cells as described previously (24). In each experiment, 4.3 pmol polymerase was used. Triptolide was obtained from Pharmagenesis. An extensive chemical characterization of the molecule was done including Fourier transform infrared spectrum, ^{1}H and ^{13}C nuclear magnetic resonance, elemental analysis (C and H), high-performance liquid chromatography, TLC, and melting point measure (232°C). The purity of triptolide was 99.5%. Unless specified, all other drugs and reagents were from Sigma, except α-amanitin, which was purchased from Fluka.

Proliferation Assay

The antiproliferative activity of test compounds was measured using the ATPlite assay (Perkin-Elmer) on a comprehensive panel of 16 cell lines. Adherent cells were seeded in 96 well plates (1×10^4-2×10^4/mL) at day 0 and then incubated for 24 h before treatment. Nonadherent cells (Namalwa, COST, PIO, FEPD, and Karpas-299) were seeded in 96-well plates (3×10^5/mL) at day 1; these seeding densities were set to ensure that cells remained in logarithmic cell growth phase throughout the 72 h drug treatment period. All the cells were treated at day 1; on day 4, cell viability was evaluated by measuring the ATP released by viable cells. EC_{50} values were determined with curve fitting analysis (nonlinear regression model with a sigmoidal dose response, variable Hill slope coefficient), done with the algorithm provided by GraphPad Prism application (GraphPad Software). The GI_{50} values for triptolide (National Cancer Institute accession no. NSC163062) obtained on the COMPARE NCI-60 panel of cancer cell lines were retrieved from the following link: http://dtp.nci.nih.gov/compare-web-public_compare/SearchAndDisplay (25). The comparison of triptolide growth inhibition profile with those from anticancer compounds was done with the three-dimensional MIND tool.[6]

RNA Synthesis Measurements

A549 cells were grown to 70% confluence in 75 cm^2 flasks for 48 h and preincubated for 30 min in the presence of various concentrations of the test drug followed by a 90 min RNA labeling in the presence of 6 μCi [H^3]uridine (GE Healthcare). Twenty-five percent of the cells were then lysed in water for total RNA quantification, and the remaining 75% were used to extract mRNA using the MicroPoly(A) Purist kit (Ambion). The radioactivity of the samples was quantified on a 1450 MicroBeta Trilux microplate scintillation counter (Perkin-Elmer). To assess the effect of triptolide on both 28S and 18S rRNA, exponentially growing 2×10^6 A549 cells were exposed to the indicated triptolide concentrations for 8 h and lysed in 350 μL lysis solution and total RNA was extracted with the RNeasy Plus Mini Kit (Qiagen) followed by agarose electrophoresis. RNA was then visualized with ethidium bromide.

DNA Microarrays

A549 cells were seeded in 75 cm^2 plates up to 70% confluence and exposed to various concentrations of triptolide for 1, 2, and 4 h before RNA was extracted using the TRizol reagent (Invitrogen). Their quality was assessed using the BioAnalyzer 2100 (Agilent Technologies) before the One-Color Microarray–based expression analysis was done on 36 arrays [3 incubation times × 4 concentrations including control × 3 replicates]. Each sample was labeled (Cy3, One Color Spike Mix, Agilent Technologies) and hybridized on whole human genome 4 × 44K oligonucleotides microarrays (45,015 probes) according to the manufacturer's recommendations (Agilent Technologies). Fluorescent signals were scanned with default parameters (Agilent Technologies), and data were extracted with the Feature Extraction 9.1 software (Agilent Technologies). Data cleaning consisted in background subtract. After selection according to the "well above background" criterion, 30,499 probes remained for the analysis. To identify probe expression changes under different concentrations and time conditions, the median signal was analyzed by a two-way ANOVA (SAS System R9.1) involving concentration and time; false discovery rate was used to adjust for multiple comparisons, which generated a list of 4,436 genes (5,133 probes) deregulated by triptolide. Microarray data management was conducted using Arraytrack, a FDA microarray software, Pipeline Pilot version 7.1 from Accelrys and SAS R9.1.

[6] http://spheroid.ncifcrf.gov/spheroid/

These genes were used to identify functional networks with the Ingenuity Pathway Analysis knowledge base (Ingenuity Systems).[7] Green indicates underexpressed and red overexpressed transcripts by a factor of at least 2 compared with untreated cells. The microarray data have been submitted to the Gene Expression Omnibus database.[8]

Quantitative PCR

A549 were grown in 75 cm^2 flasks up to 70% confluence before total RNA was isolated using RNeasy Plus kit (Qiagen). Total RNA (1 μg) was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad), and the resulting first-strand cDNA was diluted and used as template in the real-time quantitative PCR analysis. All measurements were done in duplicate. Glyceraldehyde-3-phosphate dehydrogenase and P0 mRNA served as internal controls and were used to normalize for the input cDNA. Primers for each gene (real-time quantitative PCR Primer Assays SYBR Green) were provided by SABiosciences. Detection of gene expression was done with iQ SYBR Green Supermix (Bio-Rad) and iCycler iQ (Bio-Rad) using the relative standard curve method.

Antibodies and Western Blots

Control and treated A549 cells were lysed in a buffer containing 20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L DTT, 5 mmol/L $MgCl_2$, 5 mmol/L EDTA, 5 mmol/L NaF, 1 mmol/L Na_2VO_4, 60 mmol/L β-glycerophosphate, 0.1% NP-40, and 0.1% Triton X-114, to which 100 μg/mL DNase I and proteases inhibitors (Complete; Boehringer) were added before use. Total proteins (40 or 80 μg) were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (GE Healthcare; ref. 26). The membrane was blocked in TBS-T buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, and 0.1% Tween 20] containing 3% skimmed milk and then incubated with either anti-c-Myc (1:1,000; Cell Signaling Technology), anti-p53 (1:200; Santa Cruz Biotechnology), anti-RPB1 (1:200; Santa Cruz Biotechnology), or anti-α-actin (1:500; Santa Cruz Biotechnology). Bands were revealed by electrochemiluminescence using Immobilon (Millipore) Western and their intensity (intensity × mm^2) was measured with a Fluor-S CCD camera (Bio-Rad).

Cell Transfection and Luciferase Reporter Assay

Cos-7 cells at 70% confluence in 100 mm dishes were transfected with Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions using plasmids from the Pathdetect *trans*-Reporting Systems for Elk1 and CREB (Stratagene Europe). For Elk1 reporting, 6 μg pFR-Luc reporter gene plasmid expressing firefly luciferase was cotransfected with 2 μg pFC-MEK1 (S218E/S222E and 32-51 constitutively activating mutant) and 2 μg pFA2-Elk1 transactivator plasmid. For CREB reporting, 6 μg pFR-Luc reporter gene plasmid was cotransfected with 2 μg pFC-PKA (catalytic subunit) and 2 μg pFA2-CREB transactivator plasmid. Transfected cells were replated 24 h post-transfection in 96-well plates and 24 h later changed to Opti-MEM I (Invitrogen) 2 h before treatments. Cells were challenged with triptolide or camptothecin for 5 h before addition of an equal volume of Brite-Glo assay buffer (Promega). Each point was done in quadruplicate. Luminescence was detected in a Topcount luminometer (Packard) and arbitrary luminescent units were expressed as percentage of counts from unchallenged cells. Concentration response curves of triptolide- or camptothecin-inhibited luciferase activity were analyzed by nonlinear regression using PRISM (GraphPad Software) to yield IC_{50} values.

In vitro RNA Polymerase II–Mediated Transcription Assay

RNA polymerase II ternary complexes were assembled as described (27) using synthetic RNA oligonucleotides purchased from Dharmacon and DNA oligonucleotides from DNA Technology. Transcribed strand (2.5 pmol) was used in each reaction, to which 2.5 pmol of a complementary ^{32}P end-labeled 9mer RNA oligonucleotide were annealed. RNAPII was subsequently added into the reaction followed by 5.0 pmol of a nontranscribed oligonucleotide, completing the transcription bubble. Ternary complexes were bound to streptavidin magnetic beads via biotin on the transcribed strand and washed in a buffer containing 20 mmol/L Tris (pH 8.0), 40 mmol/L KCl, 5 mmol/L $MgCl_2$, and 1 mg/mL bovine serum albumin. When required, inhibitors were diluted in DMSO and titrated into the assay to give a range of final concentrations between 10^{-4} and 10^{-8} mol/L in a total volume of 50 μL. Inhibitors were either preincubated with the ternary complexes or added simultaneously with nucleotide triphosphates. Transcription was initiated by the addition of Transcription Buffer [20 mmol/L Tris (pH 8.0), 40 mmol/L KCl, 5 mmol/L $MgCl_2$] and 500 μmol/L ATP, CTP, and GTP, 25 μmol/L UTP, and [$\alpha^{32}P$]UTP, which proceeded for 2 min at 29°C. Transcription was terminated by the addition of 20 mmol/L EDTA, 0.5% SDS, and 0.8 mg/mL proteinase K. Transcription products were subsequently purified via phenol-chloroform extraction and ethanol precipitation. Samples were analyzed on a 12% denaturing PAGE.

Results

Triptolide Has a Unique Antiproliferative Profile on a Panel of Tumor Cells

In a first attempt to identify cells either resistant or sensitive to triptolide, its antiproliferative properties have been evaluated on a panel of 16 tumor cell lines. For each cell line, derived from leukemia and solid tumors, EC_{50} values were determined after exposure to graded concentrations of triptolide for 72 h. All EC_{50} values proved to be in the nanomolar range, with the most sensitive cell line being the prostate carcinoma DU145 and the least sensitive being the colon cancer cell line Lovo with EC_{50} values of 2.3 and 77 nmol/L, respectively (Table 1). All cell lines tested appeared to be sensitive to triptolide. Similarly, triptolide has been evaluated on the NCI-60 cell lines panel and proved to be a potent growth inhibitor with GI_{50} values ranging from 2.6 to 103 nmol/L, similar to our EC_{50} values (Table 1).[9] Additionally, the GI_{50} profiles, determined at the National Cancer

[7] http://ingenuity.com
[8] http://www.ncbi.nlm.nih.gov/geo/
[9] http://dtp.nci.nih.gov/compare-web-public_compare/login.do

Table 1. Antiproliferative activity of triptolide (EC_{50}, nmol/L)

Cell line	EC_{50}	Cell line	EC_{50}
A549	20	NCI-H929	4.8
BxPC3	20	SKOV3	12
Lovo	77	A375	8
Miapaca2	5.2	G361	10
MCF7	10	WM115	40
MX1	3.2	WM266	20
Namalwa	10	Ls174T	3.5
U937	6.5	DU145	2.3

NOTE: EC_{50} values were calculated after 72 h of incubation of the cells with graded concentrations of triptolide.

Institute, allow the compounds to be grouped according to their common mechanism of action (25). Hence, the already available GI_{50} values for triptolide and anticancer compounds were submitted to the COMPARE algorithm to identify the closest reference compounds related in terms of mode of action. This analysis shows that triptolide falls into a remote area and did not significantly correlate with any of the reference compounds present in this study (Fig. 1). According to these results, triptolide seems to display a unique pharmacologic mode of action, different from the 118 reference compounds found in the NCI-60 internal database. These data suggest that triptolide interacts with a cellular target present in all cell lines tested but with a mechanism of action potentially distinct from most of the conventional modes of action for anticancer agents such as tubulin polymerization inhibitors/stabilizers, DNA binders/alkylators, antimetabolites, or topoisomerase I/II poisons. This conclusion is supported by additional (data not shown) experiments revealing that triptolide did not significantly inhibit these enzymes, nor does it interact with DNA or inhibit DNA synthesis using *in vitro* assays. Triptolide is thus likely to possess an original, as yet unknown, mechanism of action, distinct from that of conventional anticancer drugs.

Triptolide Inhibits Both Total RNA and mRNA Neosynthesis

In light of the triptolide effect on nucleolar structure, recently revealed by Leuenroth and Crews (13), we decided to explore its activity on both total RNA and mRNA synthesis using radiolabeled uridine. The first set of data shows that triptolide inhibits total RNA production, with a maximum of 60% inhibition for 1 μmol/L, after only 90 min incubation (Fig. 2A). Actinomycin D was used as a reference and exhibits a stronger potency than triptolide (Fig. 2A). For example,

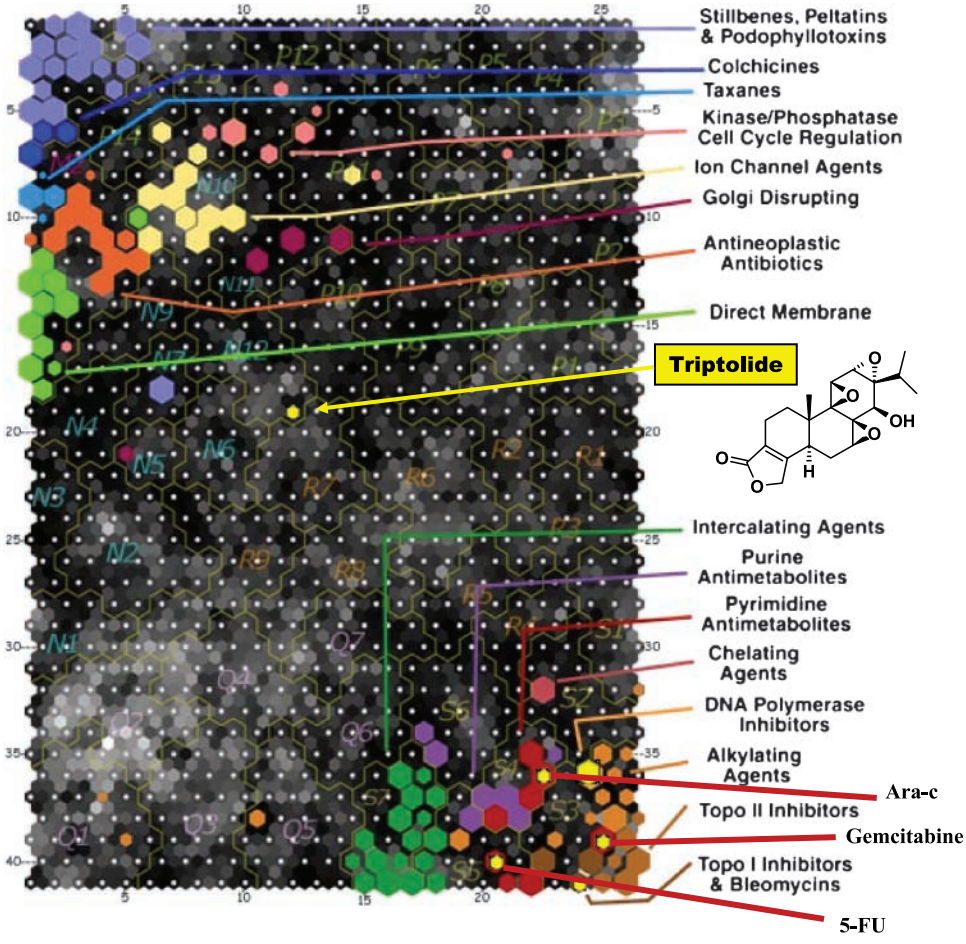

Figure 1. Triptolide has a unique antiproliferative profile in the NCI-60 panel of tumor cell lines. The GI_{50} profile for triptolide was compared with one of the reference inhibitors as described in Materials and Methods. The structure of triptolide is indicated.

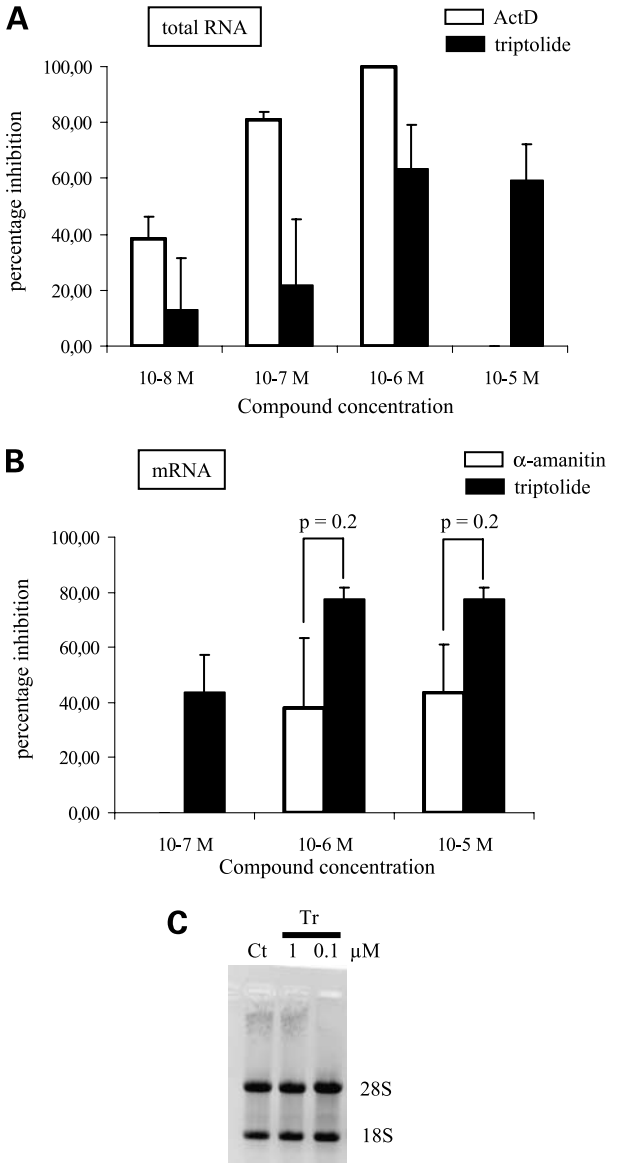

Figure 2. Triptolide inhibits synthesis of both rRNA and mRNA. **A** and **B,** A549 cells were preincubated for 30 min with the indicated concentrations of triptolide before [^{3}H]uridine was added for a further 90 min incubation. Total RNA (**A**) and mRNA (**C**) were extracted and quantified as described in Materials and Methods. Mean ± SD of a minimum of three independent experiments in which each condition was done in duplicate. **C,** A549 cells were treated for 8 h with either 0.1 or 1 μmol/L triptolide before total RNA extraction and separation on agarose gel, and DNA was visualized with ethidium bromide.

100 nmol/L triptolide gives a 25% inhibition, whereas actinomycin D exhibits an 80% inhibition of total RNA *de novo* synthesis (Fig. 2A). Because rRNAs represent the vast majority (80% to >90%) of total RNA synthesized in a cell (12), we thus conclude that triptolide inhibits, at least partially, *de novo* rRNA production. However, triptolide does not significantly affect the endogenous level of rRNA over a period of 8 h of incubation for up to 1 μmol/L of the drug (Fig. 2C), which could be explained by the long half-life of rRNA (28). We next purified the mRNA pool from total RNA, and the radioactivity counting clearly shows that triptolide inhibits mRNA *de novo* synthesis as well, with a potency equivalent (P = 0.2, Wilcoxon test) to that of the RNA polymerase II reference inhibitor α-amanitin (Fig. 2B). A similar statistical analysis indicates no significant differences in the potency of triptolide to inhibit total RNA versus mRNA ($P > 0.05$). We also observed a plateau between 40% and 80% of inhibition for both triptolide and α-amanitin (Fig. 2A and B). Two hypothesis could explain this phenomenon: (*a*) triptolide interacts with a transcription factor not shared by all transcription complexes, resulting in a low background transcription activity, or (*b*) a competition between triptolide and another molecule (e.g., transcription factor and nucleotide) allows transcription to occur at a lower rate. Actually, this last explanation would also explain the plateau observed for α-amanitin, which has been shown to slow down the RNA polymerase II activity (29). Finally, we observed through transmission electron microscopy that triptolide induces, in A549 cells, dramatic changes in the nuclear structures (Supplementary Fig. S3).

Effect of Triptolide on Gene Expression

To better define the genes primarily affected by triptolide, we employed pan-genomic DNA microarrays using total RNA extracted from A549 cells treated for 1, 2, and 4 h by three concentrations (0.05, 0.15, and 0.45 μmol/L) of triptolide corresponding to 2.5-, 7.5-, and 22.5-fold the EC_{50} value (20 nmol/L) of the compound determined with the antiproliferative assay (Table 1). The hybridization results were then analyzed to identify the genes statistically modulated as a function of both the incubation time and the concentration of triptolide. This generated a list of 4,436 genes from which we extracted those modulated by at least 2-fold compared with untreated cells (Supplementary Material S1). We first scored the number of genes that were either up-regulated or down-regulated (Fig. 3A and B). The 1 h condition revealed a very low (<10) number of genes modulated >2-fold. However, for the 2 h incubation time, 100% and 98% of the modulated genes were underexpressed at 0.15 and 0.45 μmol/L, respectively (Fig. 3A). For the latter condition, we scored 9 overexpressed versus 508 underexpressed genes (Fig. 3A). The 4 h treatment with 0.15 μmol/L triptolide revealed a more balanced effect with 94 overexpressed versus 107 underexpressed genes (Fig. 3B). However, for the highest concentration of triptolide, that is, 0.45 μmol/L, 160 genes were overexpressed versus 1,511 genes that were underexpressed (Fig. 3B). Because of these large numbers of deregulated genes, a manual gene-to-gene analysis was not possible. To identify potential clusters of genes, we submitted the list of 4,436 genes, and their respective fold changes, to the Ingenuity Knowledge base. The number of affected genes being high, this analysis generated a large number of networks, which precludes their exhaustive presentation in this article. The analysis of the most documented networks reveals that numerous genes regulated by NF-κB are down-regulated by triptolide, which confirms previous reports, thus reinforcing the validity of our experiment (30). For instance, CXCL2, a chemokine whose expression is regulated by NF-κB, is

repressed by triptolide by a factor of 2.6 and 4.8 at the highest concentration for 2 and 4 h incubation time, respectively (Fig. 3C; Supplementary Material S1). Another striking observation is that transcription regulators, both general such as TAFs, TBP, CEBPA-CEBPB, MYC, FOS, SP1, and JunB and more specific such as ZNFs, NRF1, NFYA, and HSF2, are largely represented among the triptolide down-regulated genes (Fig. 3D; Supplementary Material S1). The cell cycle regulators CDC25A, but not CDC25B or CDC25C, as well as Polo-like kinases Plk2, Plk3, and Plk4 were significantly down-regulated by triptolide (Fig. 3D; Supplementary Material S1). In addition, we selected 7 genes to confirm, by quantitative PCR, their deregulation (Table 2). CXCL2 was chosen as a reporter of NF-κB activity along with a series of transcription factors not previously described to be down-regulated by triptolide. Our results confirm that ELF2,

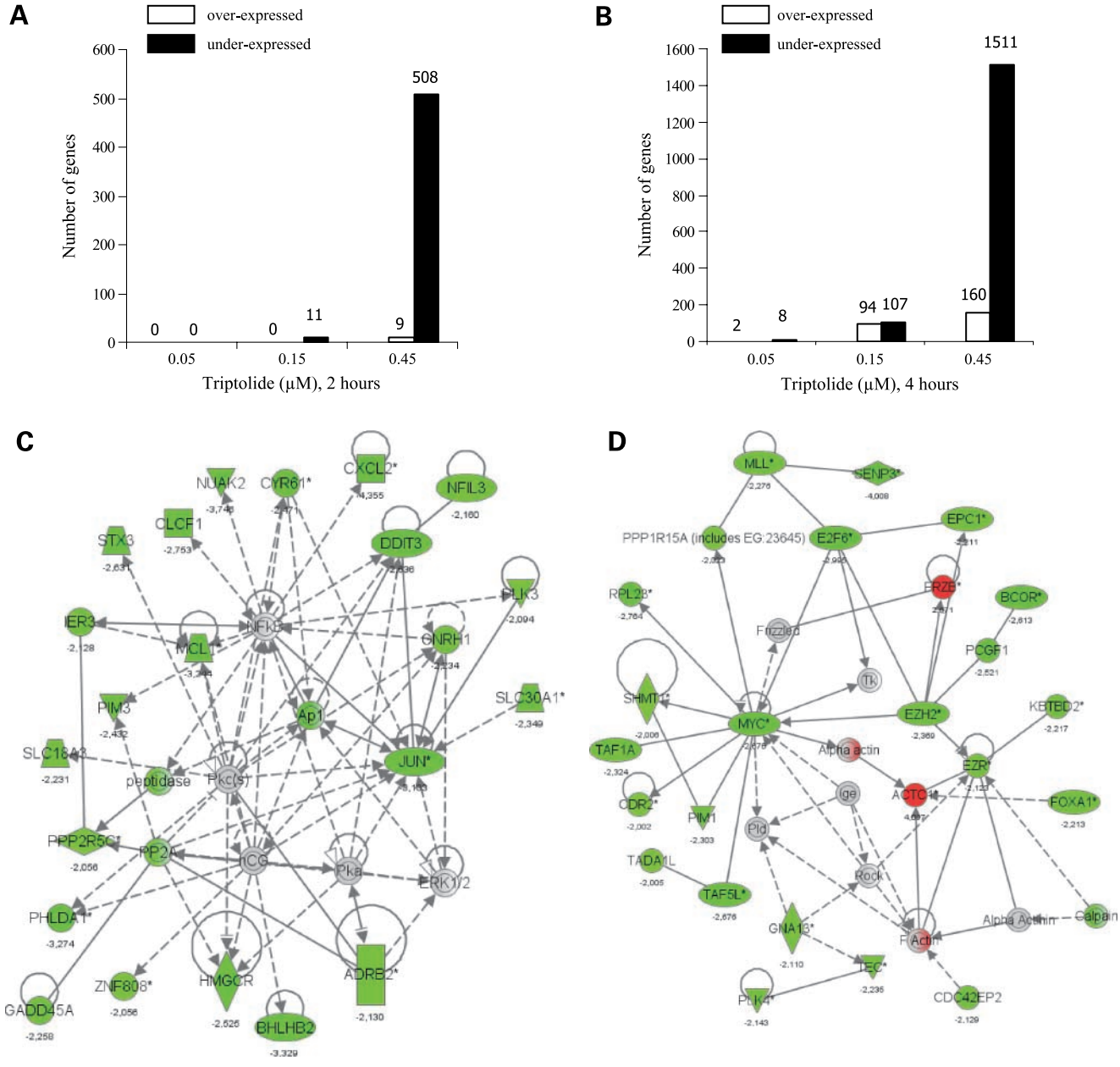

Figure 3. Triptolide inhibits a large variety of mRNA. A549 cells were treated with the indicated concentrations of triptolide 1, 2, or 4 h before RNA was extracted; the corresponding cDNA were synthesized and hybridized with pan-genomic DNA microarrays. **A** and **B,** number of both underexpressed and overexpressed genes by at least 2-fold for each condition. For each condition, the number of both overexpressed and underexpressed genes is indicated on top of each column. **C** and **D,** two examples of networks generated by the Ingenuity Pathway Analysis database with the genes modulated by triptolide (0.45 μmol/L, 2 h). In this analysis, the genes modulated by at least 2-fold are colored in either green or red for down-regulated or up-regulated genes, respectively.

Table 2. Comparison of gene expression ratios between the microarray analysis and the real-time quantitative PCR analysis for a selection of genes modulated by a 4 h treatment with 0.45 μmol/L triptolide

Gene name	Microarray* expression ratios	Real-time quantitative PCR† ΔΔCt
ELF2	0.12	0.23 ± 0.06
TAF15	0.36	0.79 ± 0.21
MYC	0.21	0.10 ± 0.07
JUN	0.24	0.10 ± 0.04
CXCL2	0.32	0.04 ± 0.03
ACTA1	14	0.92 ± 0.15
FOSB	2.3	4.61 ± 4.26

*Expression ratios relative to the untreated cells.
†Mean ± SD expression ratios, relative to the untreated cells, from three independent experiments.

MYC, JUN, and CXCL2, but not TAF15, are down-regulated >2-fold by a 4 h treatment by 0.45 μmol/L triptolide in accordance with the microarray analysis (Table 2). Moreover, the extent of modulation was significantly stronger using quantitative PCR for the genes CXCL2, MYC, and JUN, which were down-regulated 25-, 10-, and 10-fold, respectively, compared with 3-, 5-, and 4-fold in the microarray analysis. We also selected two overexpressed genes, that is, FOSB, and ACTA1, and confirmed the overexpression of the former but not of the latter (Table 2).

Triptolide Induces a Rapid RPB1 and MYC Depletion Associated with p53 Induction

Because the microarray approach revealed a rapid underexpression of the MYC transcript after triptolide treatment, we analyzed its consequence at the protein level in A549 cells. The results reveal that the Myc protein is underexpressed 2 h following triptolide addition to the cell culture, an effect that lasts for at least 16 h, our latest time point (Fig. 4A). Due to its central role in transcribing mRNA, we also decided to look at RPB1, the catalytic and largest RNA polymerase II main subunit. Our results show that underexpression of RPB1 starts 2 h after triptolide addition and is further amplified with longer incubation times up to 16 h (Fig. 4B). Shorter incubations show that RPB1 levels remain similar to the control up to 1 h (Supplementary Fig. S5). This effect on RPB1 is paralleled by a strong and persistent p53 accumulation (Fig. 4B). This triptolide-induced RPB1 depletion was also observed in the KG1 leukemia and DLD1 colon cancer cell lines (Fig. 4C). It has been reported previously that a stalled RNA polymerase II complex is a signal for its proteasome-mediated degradation (24). We thus measured the triptolide-induced RPB1 down-regulation in the presence of two different proteasome inhibitors, that is, bortezomib and epoxomycin. Both molecules were able to quantitatively prevent this down-regulation, showing that triptolide acts by stimulating RPB1 degradation (Fig. 4D). Furthermore, we similarly analyzed the RPB1 depletion in A549 cells that have

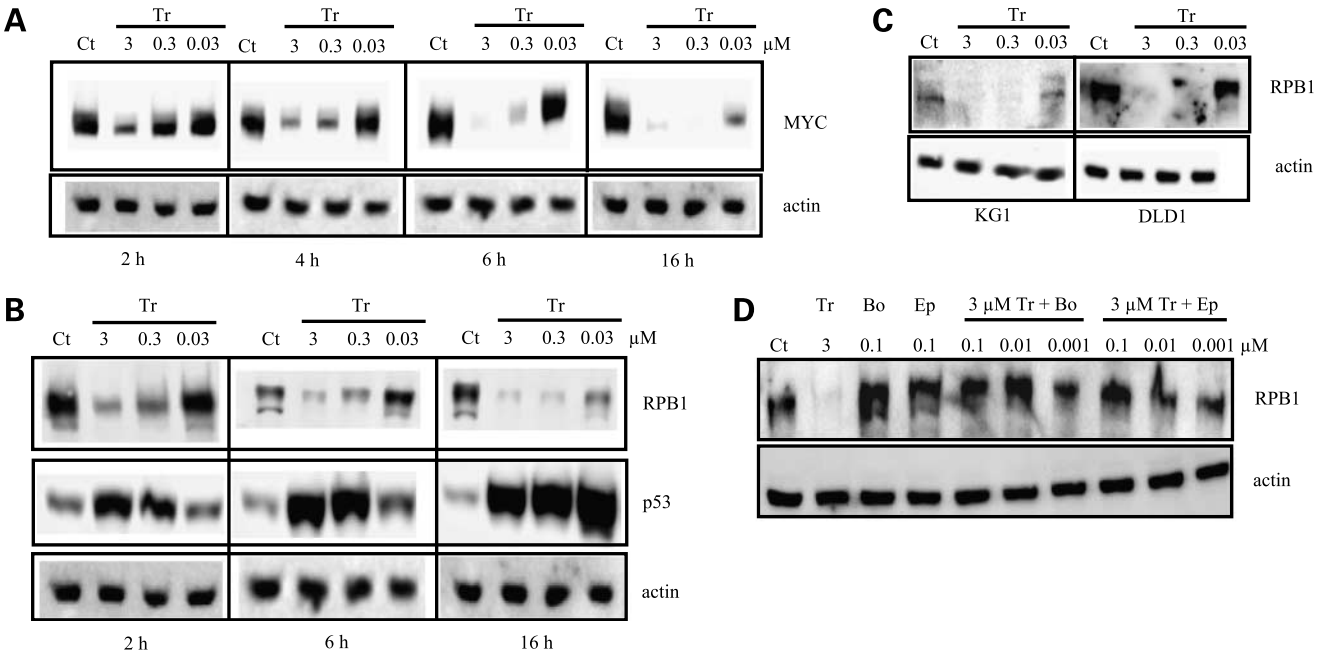

Figure 4. Triptolide induces MYC and RPB1 depletion associated with p53 accumulation in A549 cells. A549 cells were treated for 2, 4, 6, and 16 h with the indicated concentrations of triptolide before total proteins were extracted and analyzed by Western blotting. **A,** MYC expression in response to 3, 0.3, and 0.03 μmol/L triptolide. **B,** RPB1 and p53 expression in response to 3, 0.3, and 0.03 μmol/L triptolide. **C,** RBP1 expression in response to 3, 0.3, and 0.03 μmol/L triptolide in KG1 and DLD1 cells. **D,** RPB1 expression in the presence of 3 μmol/L triptolide (*Tr*), 0.1 μmol/L bortezomib (*Bo*), or 0.1 μmol/L epoxomicin (*Ep*) or in cells pretreated 4 h with 0.1, 0.01, or 0.001 μmol/L of either bortezomib or epoxomicin followed by a further 4 h incubation with 3 μmol/L triptolide. In all cases, actin was used as a loading control.

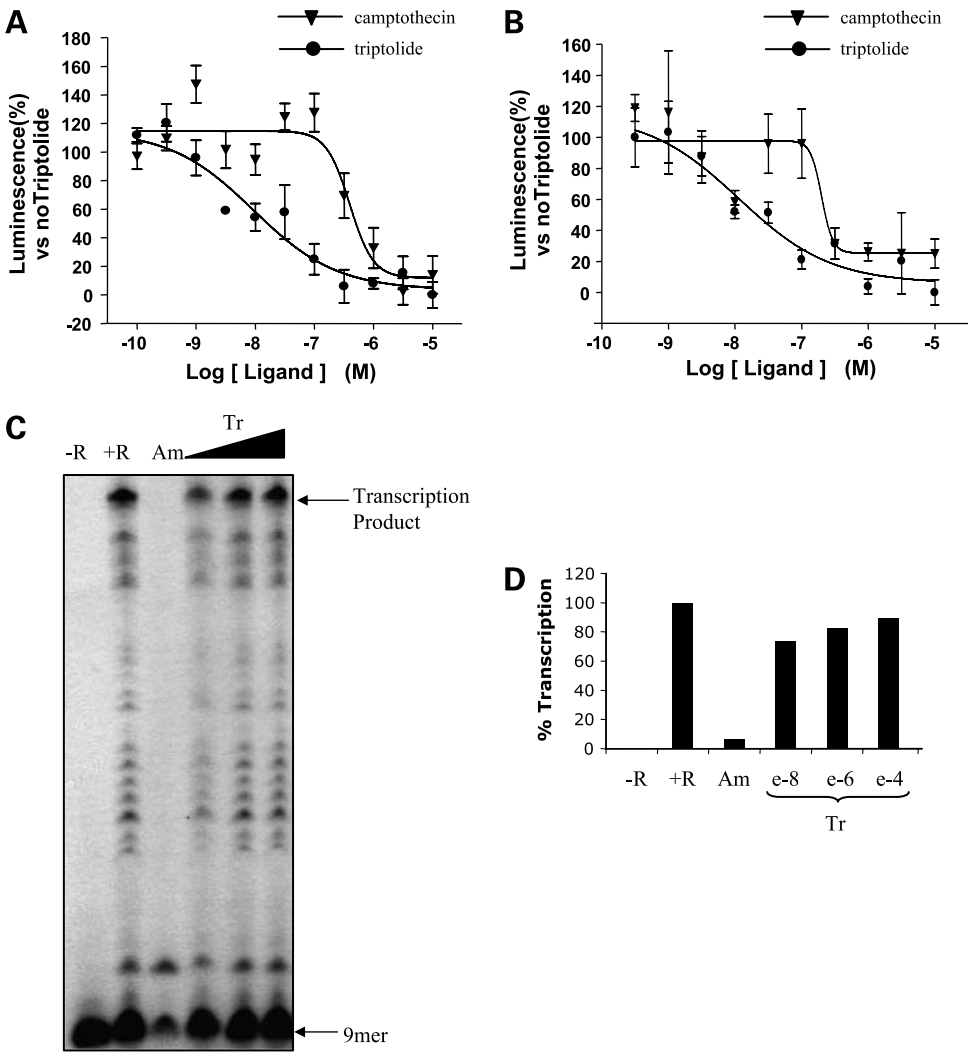

Figure 5. Triptolide inhibits transcription stimulated by either Elk1 or CREB transcription factors without inhibiting directly the purified RNA polymerase II active complex. **A** and **B,** inhibition of luciferase expression in Cos-7 cells (*filled triangles,* camptothecin; *filled circles,* triptolide). Mean ± SD. **A,** triptolide and camptothecin concentration-dependently inhibit MEK1 + Elk1–stimulated luciferase activity. Representative experiment ($n = 7$). **B,** triptolide and camptothecin concentration-dependently inhibit protein kinase A + CREB–stimulated luciferase activity. Representative experiment ($n = 2$). **C,** *in vitro* RNA polymerase II transcription assay on a short DNA duplex in the presence of either 100 μmol/L α-amanitin (*Am*) used as an inhibitor or a concentration range of 100 μmol/L (*e-4*), 1 μmol/L (*e-6*), or 0.01 μmol/L (*e-8*) triptolide. **D,** transcription products were quantified and normalized to the control condition, +R positive control in the presence of nucleotide triphosphates, and −R negative control in the absence of nucleotide triphosphates. Representative of one of two experiments.

been derived to resist to the cytotoxic activity of triptolide, named A549TRP4. These cells require a 10-fold higher drug concentration to achieve a similar RPB1 depletion (Supplementary Fig. S6). Finally, and unlike the RNA polymerase II, the RPA135 and RPA194 subunits of the RNA polymerase I are not degraded in response to triptolide exposure up to 16 h (Supplementary Fig. S4).

Triptolide Inhibits Transcription from Two Different Promoters without Directly Inhibiting the RNA Polymerase II Transcription Complex

To further narrow down potential targets of triptolide at the level of the transcription machinery, we performed two independent assays using the luciferase reporter gene. Both assays are based on the constitutive activation of a specific transactivator transcription factor, Elk1 or CREB, by their respective overexpressed upstream activators, that is, MEK1 or protein kinase A, respectively. Triptolide inhibits both Elk1- and CREB-dependent luciferase activity with similar IC_{50} values (10^{-8} and 1.3×10^{-8} mol/L, respectively) and is significantly more potent than camptothecin (IC_{50} values of 4×10^{-7} and 2×10^{-7} mol/L, respectively) used as a reference transcription inhibitor in these assays (Fig. 5A and B). The fact that triptolide potently and rapidly inhibits the activity of a reporter gene stimulated by two unrelated signaling pathways points to a general mechanism of action at the transcriptional level, potentially involving a common transcription factor. At this stage, we hypothesized that this factor could be the RNA polymerase II complex itself, for which the main RBP1 subunit is the target for α-amanitin. A reconstituted *in vitro* RNA polymerase II–dependent transcription assay was thus used to test this hypothesis. Triptolide was preincubated with the reconstituted ternary complexes for 2 min at 29°C before transcription was initiated. We would expect any inhibition to be pronounced during preincubation, if inhibition is in fact occurring. The results of this experiment indicate that, whatever the conditions, triptolide did not cause any significant inhibition of transcript elongation, whereas α-amanitin (at 100 μmol/L) induced a complete inhibition (Fig. 5C and D).

Discussion

Triptolide is a natural product that possesses a large spectrum of clinical and traditional applications, including inflammatory diseases such as rheumatoid arthritis and cancer (2, 6). It is a potent apoptosis inducer in cancer cells and an inhibitor of NF-κB–mediated transcription (7, 8, 31–33). However, the precise genes affected by triptolide and its molecular targets remain to be identified. The main goal of this study was to dissect the molecular events primarily triggered by the exposure of human A549 cancer cells to triptolide. Our first set of proliferation data indicates that triptolide interacts with a target shared by all tumor cell lines tested because they all appeared to be sensitive to the molecule. However, triptolide is not a general poison because the EC_{50} values measured in the *in vitro* proliferation assay vary by 33-fold between the tested cell lines. In addition, its unique growth inhibition profile on the NCI-60 panel strongly suggests that it acts through an original mechanism of action. In accordance with a previous published work from McCallum et al., our data confirm that triptolide inhibits total RNA *de novo* synthesis (10). More interestingly for the same concentrations and incubation timeframe, we also observed an inhibition of mRNA neosynthesis. This is the first observation that triptolide is a potent general inhibitor of mRNA *de novo* synthesis.

Because our study confirms the stability of the rRNA pool, we decided to further explore the effect of triptolide on the mRNA pool (28). In addition to NF-κB–dependent genes, such as CXCL2, the microarray analysis of triptolide-treated A549 cells revealed a much larger set of genes whose transcription is inhibited including cell cycle regulators and cell survival factors. Moreover, the effect of triptolide on a large set of transcription factors would explain its double-blade weapon action by (*a*) depleting the oncogene-driven cancer cells of proteins required for their survival and (*b*) ensuring a sustained down-regulation of these essential genes through the depletion of the transcription factors required to allow for the recovery of their respective mRNA. The genome is considered to code for ~23,000 genes.[10] Thus, the 1,511 genes down-regulated by at least 2-fold by 0.45 μmol/L triptolide after 4 h would then represent 7% of the human genome. We should, however, keep in mind that these 7% probably correspond to the short-lived mRNA pool only. It is thus highly probable that triptolide inhibits the transcription of a higher proportion of genes possessing longer half-lives. This is actually supported by our findings that triptolide can inhibit up to 80% of mRNA *de novo* synthesis. At this stage, one can thus conclude that triptolide is a general transcription inhibitor, primarily affecting short-lived mRNA comprising many cell cycle, survival, and transcription factors. This hypothesis is strengthened by the identification, in murine and human cells, that transcription factors are particularly enriched in fast-decaying mRNAs with half-lives <2 h (34, 35). Indeed, ~50% of these unstable mRNA are found in our list of genes down-regulated by triptolide (Supplementary Fig. S2). Interestingly, the lower concentration of 0.15 μmol/L induces similar numbers of overexpressed and underexpressed genes. We suggest that this reflects a partial inhibition of mRNA transcription, which induces cells to overexpress specific genes to compensate for the gene expression loss.

Koumenis and Giaccia showed that exposure of cancer cells to either α-amanitin or the nucleoside analogue DRB induces apoptosis as a result of down-regulation of survival genes, including the oncogenic transcription factor c-Myc, which appears to be one of the numerous short-lived mRNA and protein potently down-regulated by triptolide (36, 37). One should, however, not ignore that this first effect on mRNA is probably relayed by a potential inhibitory effect of triptolide on both rRNA and tRNA, eventually preventing protein synthesis (38).

We then looked for potential molecular targets responsible for this transcriptional effect. Unlike actinomycin D and camptothecin, triptolide is not a DNA binder nor a DNA topoisomerases inhibitor. At this stage, we could propose that triptolide stimulates the proteasomal degradation of proteins, including RBP1, the main RNA polymerase II subunit, ultimately leading to transcription inhibition. Nevertheless, our data clearly show that transcription inhibition occurs before RPB1 level decreases. Furthermore, α-amanitin, a RPB1 binder, has been shown to induce RPB1 degradation through the proteasome as a response to a stalling of the elongating transcription machinery (24). This triptolide-mediated RPB1 depletion is likely to represent an antiproliferative marker because cells resistant to triptolide requires 10 times higher drug concentration to achieve a similar RPB1 depletion. Combined with our observation that triptolide inhibits with the same potency the transcription from epigenetic constructions containing two different promoters, we proposed that triptolide could bind directly to the RNA polymerase II core enzyme. However, an *in vitro* transcription assay showed that triptolide does not have the ability to directly interact and inhibit the elongating RNA polymerase II complex under the experimental conditions. Actually, a 90 kDa nuclear protein has recently been identified to bind triptolide (39). We thus suspect that this protein could be a general transcription factor not present in our reconstituted RNA polymerase II–mediated transcription assay. One can also propose that triptolide either interacts with a higher affinity with the phosphorylated COOH terminus domain of RPB1 or interferes with its phosphorylation, as proposed by Leuenroth and Crews (13). Because triptolide exhibits a mechanism of action different from that of previously characterized transcription inhibitors, we can thus anticipate that triptolide will not expose the patients to the side effects associated to these molecules. Actually, this is supported by the long-established use of *T. wilfordii* in Chinese traditional medicine for inflammatory diseases (1, 2). In addition, triptolide has also been shown to possess male contraceptive properties (40). In view of our data, we suggest that this action is at least partly mediated by the general transcription inhibition action of triptolide in a tissue previously

[10] http://www.ornl.gov/sci/techresources/Human_Genome/faq/genenumber.shtml

described to possess a highly active transcription machinery, required for the gametes formation (41).

Actinomycin D has shown antitumor properties in clinical trials including patients harboring various tumors such as kidney cancers and rhabdomyosarcomas (42, 43). This strongly suggests that targeting transcription is a valuable anticancer approach. Moreover, additive combinations of actinomycin D with *Vinca* alkaloids were reported regarding their respective antiproliferative properties, suggesting the potential therapeutic benefit of combining this class of agents with triptolide (44). Furthermore, a recent publication showed that cytosine arabinoside sensitizes acute myeloid leukemia cells to low concentrations of triptolide (45). We can now propose a molecular explanation for such a combination where these two molecules cooperate through two oncogenic survival processes, that is, DNA replication and mRNA transcription, both essential for tumor cell survival. In addition, the survival and proliferation genes CDC25A, Myc, and Src, which we identified as indirect downstream targets for triptolide, have previously been identified as important oncogenic regulators of acute myeloid leukemia (46–48). Myc has also been described to be directly involved in the tumorigenicity of ovarian, colon, and esophageal cancers (48–51). Altogether, these results represent a breakthrough toward the potential therapeutic benefits from a triptolide-based therapy in cancer.

Disclosure of Potential Conflicts of Interest

Stéphane Vispé, Luc DeVries, Laurent Créancier, Jérome Besse, Sophie Bréand, Jean-Philippe Annereau, Didier Cussac, Nicolas Guilbaud, Jean-Marc Barret, and Christian Bailly: Employment, Pierre Fabre Laboratories. The other authors disclosed no potential conflicts of interest.

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