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XPB, a Subunit of TFIIH, Is a Target of the Natural Product Triptolide

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Abstract

Triptolide (**1**) is a structurally unique diterpene triepoxide isolated from a traditional Chinese medicinal plant with anti-inflammatory, immunosuppressive, contraceptive and antitumor activities. Its molecular mechanism of action, however, has remained largely elusive to date. We report that triptolide covalently binds to human XPB/ERCC3, a subunit of the transcription factor TFIIH, and inhibits its DNA-dependent ATPase activity, which leads to the inhibition of RNA Polymerase II mediated transcription and likely nucleotide excision repair. The identification of XPB as the target of triptolide accounts for the majority of the known biological activities of triptolide. These findings also suggest that triptolide can serve as a novel molecular probe for studying transcription and, potentially, as a new type of anticancer agents through inhibition of the ATPase activity of XPB.

Natural products have played an important role in the discovery and development of drugs[1]. In recent years, they have also become important molecular probes for studying different cellular processes by virtue of their ability to bind to specific protein targets and interfere with their cellular functions. For example, the identification of calcineurin as the target of the immunosuppressive drugs cyclosporine A and FK506[2] and of TOR as the target of rapamycin[3] opened the gateways to the subsequent studies of calcium-calcineurin and TOR signaling pathways, respectively. More recently, we and others identified the type 2 methionine aminopeptidase as the target for the potent angiogenesis inhibitors fumagillin and ovalicin[4] and the eukaryotic translation initiation factor 4A as the target for the marine sponge-derived antitumor natural product pateamine A[5]. These studies have led to the exploitation of MetAP2 and eIF4A1 as new molecular targets for discovering and

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developing new anti-angiogenic and anticancer drugs, respectively. Thus, natural products can serve as bridges between chemistry, biology and medicine, constituting a major tool set for chemical biologists today. Elucidation of the mechanisms of action of natural products not only offers new insights into the cellular functions of their protein targets but also facilitates the ensuing use of natural products as leads in drug development.

Triptolide[6] is a diterpene triepoxide purified from *Tripterygium wilfordii* Hook F, commonly known as Lei Gong Teng or Thunder God Vine, a medicinal plant whose extracts have been used in traditional Chinese medicine for treating a wide variety of diseases from inflammation to arthritis for centuries[7]. It is structurally distinct in that it contains three epoxide groups next to each other (Fig. 1a). It also possesses a unique profile of biological activities. Triptolide has been shown to exhibit potent antiproliferative and immunosuppressive activities. Preclinical studies have revealed that triptolide is effective against cancer, collagen-induced arthritis, skin allograft rejection and bone marrow transplantation in animal models[8–10]. Triptolide and derivatives have entered human clinical trials for cancer among other diseases[11].

Extensive scrutiny of its mechanism of action in the past few decades has yielded important insights. At the cellular level, triptolide exhibits strong anti-proliferative activity, inhibiting the proliferation of all 60 NCI cancer cell lines with IC_{50} values in the low nanomolar range (average IC_{50} = 12 nM). It also induces apoptosis in a number of cancer cell lines. At the molecular level, triptolide was shown to interfere with a number of transcription factors including NF-κB, p53, NF-AT and HSF-1[12–14]. An interesting common feature of the effects of triptolide on all those transcription factors is that it seems to block their transactivation activity without affecting DNA binding. More recently, it was shown that triptolide inhibits *de novo* RNA synthesis which was suggested to be due to indirect inhibition of transcription mediated by RNA polymerases I and II[15–18]. Attempts to isolate the molecular target of triptolide have led to the identification of a calcium channel polycystin-2 and detection of a 90-kDa nuclear protein as potential molecular targets of triptolide[15,19]. However, polycystin-2 cannot account for most, if not all, aforementioned biological activities of triptolide while the identity of the 90-kDa putative nuclear triptolide-binding protein has remained unknown.

In this study, we took a “top-down” approach to identifying the molecular target of triptolide. Taking advantage of the extensive prior knowledge on eukaryotic transcription initiation, we systematically examined the effects of triptolide on different steps and players involved and eventually identified the molecular target of triptolide as the XPB/ERCC3 subunit of the general transcription factor TFIIH. Inhibition of XPB by triptolide offers a unified molecular mechanism for the diverse biological activities of triptolide. Our preliminary evidence also revealed a previously unknown activity of triptolide—inhibition of nucleotide excision repair, which has important implications in the application of triptolide for the treatment of cancer.

RESULTS

Triptolide inhibits RNAPII-dependent transcription

To determine whether triptolide has a specific effect on transcription, we examined its effects on global protein, RNA and DNA synthesis using incorporation of [^{35}C]-methionine, [^{3}H]-uridine and [^{3}H]-thymidine as readouts, respectively. Treatment of HeLa cells with triptolide for 1 h led to a dose-dependent inhibition of [^{3}H]-uridine incorporation with an IC_{50} of 109 nM, which is in agreement with previous results in A549 cells[16] (Fig. 1b). In contrast, protein and DNA synthesis were not affected under these conditions (Fig. 1b). Inhibition of protein and DNA synthesis required longer incubation with triptolide.

Interestingly, the IC_{50} value for the inhibition of HeLa cell proliferation by triptolide is significantly lower than 109 nM (IC_{50} = 5 nM, Supplementary Results, Supplementary Fig. 1). It turned out that the effects of triptolide on RNA synthesis and cell proliferation were time-dependent and the apparent difference in IC_{50} values was due to different drug treatment times for these assays. An increase in triptolide treatment time from 1 hour to 3 hours led to a 50% decrease in IC_{50} for [^{3}H]-uridine incorporation (from 109 nM to 62 nM, Fig. 1b and Supplementary Fig. 2 and 3). Similarly, an increase in treatment from 1 hour to 3 hours to 24 hours led to a progressive decrease in IC_{50} for [^{3}H]-thymidine incorporation (1 h, no effect; 3 h, 41 nM; 24 h, 12 nM. see Supplementary Fig. 4). Thus, temporally, triptolide exhibited earlier inhibition of RNA synthesis. Nevertheless, the IC_{50} values for inhibition of RNA synthesis (62 nM) and DNA synthesis as a measure of cell proliferation (41 nM) are similar after 3-h treatment (Supplementary Fig. 3 and 4).

We next sought to compare the effects of triptolide with those of known inhibitors of RNA synthesis. In comparison with three other transcription inhibitors, α-amanitin (RNAPII inhibitor)[20], flavopiridol (a CDK9 inhibitor)[21] and actinomycin D (a DNA binding ligand)[22], the dose-response curve for triptolide is similar to that of flavopiridol in that it levels off at the higher concentration range with the maximal inhibition staying at around 80% (90% for flavopiridol), suggesting that, unlike actinomycin D, it does not inhibit the synthesis of all RNA species in cells (Supplemental Fig. 2 and 3). As expected, methionine incorporation was not significantly inhibited by any of those compounds (Supplemental Fig. 5 and 6). Interestingly, α-amanitin was unable to inhibit RNA synthesis until its concentration reached 50 μM, which is unexpected considering that it has subnanomolar K_d for RNAPII and its lack of cellular activity is likely due to previously reported low cell permeability[23]. Next, we determined and compared the effects of triptolide on phosphorylation and degradation of the largest subunit of RNA Polymerase II with those of other known transcription inhibitors including 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (another CDK9 inhibitor)[24], actinomycin D and α-amanitin. Unlike DRB, triptolide had no effect on the phosphorylation of RNAPII, suggesting that it is not an inhibitor of CDK9 (Fig. 1c). As previously reported[17], after prolonged treatment (4 h), triptolide induced degradation of the catalytic subunit of RNAPII, similar to DRB and α-amanitin. Prolonged treatment with triptolide also led to faster disappearance of transcribing/phosphorylated form of RNAPII than nontranscribing/unphosphorylated form, which suggested that it might inhibit transcription initiation or promoted transcription termination. This last observation was previously reported as cdk9 inhibition, which is not the case based on the kinetics and magnitude of this effect compared to DRB[25]. In contrast, actinomycin D had no obvious effect on the stability of RNAPII and seemed to shift the equilibrium towards the phosphorylated form compared to control (Fig. 1c). Together, these results suggested that the effect of triptolide on RNAPII is distinct from those of Cdk9 inhibitors (DRB and flavopiridol) and actinomycin D, but similar to that of α-amanitin.

We next determined the effect of triptolide on transcription mediated by all three RNA polymerases *in vitro*. Triptolide inhibited RNAPII-mediated transcription in a dose-dependent manner with an IC_{50} of ca. 200 nM (Fig. 1d). Using the same *in vitro* transcription assay with templates under the control of RNAPI and RNAPIII specific promoters, we found that triptolide had no effect on RNAPI- or RNAPIII-dependent transcription at concentrations up to 10 μM, suggesting that triptolide is a selective inhibitor of the RNA polymerase II transcription machinery (Fig. 1e). To determine whether triptolide works by directly binding to and inhibiting RNAPII catalytic activity like α-amanitin, we employed purified calf RNAPII to direct the RNA synthesis from a DNA template with a poly(dC) overhang in the absence of general transcription factors[26]. As previously reported[17], triptolide had no effect on the catalytic activity of purified RNAPII (Fig. 1f),

ruling out the possibility that triptolide shares the same molecular target as α-amanitin and suggesting that triptolide targets one of the general transcription factors associated with RNAPII.

Triptolide inhibits TFIIH-dependent transcription and repair

To identify which general transcription factor is targeted by triptolide, we began by determining the effect of triptolide in a reconstituted *in vitro* transcription assay with purified or recombinant general transcription factors and RNAPII[27]. Triptolide inhibited transcription from a linear template with the Adenovirus major late promoter (−53 to +10) fused to a 380-bp G-less cassette in the presence of TFIIB, TBP, TFIIH, TFIIE, TFIIF and RNAPII[28] (Fig. 2a, Lanes 2 vs. 1 and 4 vs. 3). Surprisingly, transcription from a negatively supercoiled template was refractory to inhibition by triptolide (Fig. 2a, Lanes 8 vs. 7). It is known that transcription from supercoiled templates can bypass the need for TFIIH and TFIIE, suggesting that triptolide might inhibit the function of TFIIH or TFIIE[27]. In addition to negatively supercoiled template, it has been previously shown that a linear template containing a preexisting bubble at the transcription start site with mismatched sequence from −9 to +3 of the AdMLP can also undergo transcription initiation independent of TFIIH and TFIIE[29]. Indeed, transcription from this bubble-containing template is also insensitive to triptolide as well as to the addition of TFIIH and TFIIE (Fig. 2b). To further dissect the site of action of triptolide on transcription, we exploited the ability of transcription initiation complexes to synthesize multiple copies of 3mers in a process known as abortive initiation[30]. Abortive initiation can occur in the absence of TFIIH and TFIIE, but is strongly stimulated by them. While triptolide had no effect on basal abortive initiation in the absence of TFIIH and TFIIE, it inhibited the enhancement of abortive initiation by TFIIH and TFIIE (Fig. 2c). Together, these results narrowed down the target for triptolide to either TFIIH or TFIIE.

Although TFIIH and TFIIE work in concert to enable RNAPII-mediated transcription, TFIIH, unlike TFIIE, possesses a unique function—it is also required for Nucleotide Excision Repair (NER)[31]. We thus determined whether triptolide affected NER. We took advantage of the ability of HeLa whole cell extract to support NER *in vitro*. An oligonucleotide containing one cisplatin-damaged site with a ^{32}P label 5′ to the site was incubated with HeLa cell extract, leading to accumulation of 22 to 25-nt long excised fragments as a result of the NER activity (Fig. 2d and Supplementary Fig. 7). Triptolide inhibited the NER activity at concentrations similar to those required for *in vitro* transcription. Given the dependence of NER on TFIIH, this result suggested that TFIIH, but not TFIIE, is likely the target of triptolide.

Triptolide binds to and inhibits ATPase activity of XPB

TFIIH contains a total of ten subunits, four of which exhibit detectable enzymatic activities. Cdk7 possesses RNAPII kinase activity, both XPB and XPD contain DNA helicase and ATPase activities and p44 has ubiquitin ligase activity[32]. As triptolide had no effect on the phosphorylation of the catalytic subunit of RNAPII (Fig. 1c), Cdk7 was ruled out as a target. Ubiquitin ligase activity of p44 was ruled out since it was shown to not be important for either *in vitro* transcription or NER[32]. We then determined whether triptolide affected the DNA helicase and ATPase activities of XPB and XPD. Using immunoaffinity purified TFIIH holocomplex containing all ten subunits (Supplementary Fig. 8), we were able to observe both the 3′-5′ DNA helicase activity associated with XPB and the 5′-3′ helicase activity associated with XPD. Neither helicase activity of TFIIH was affected by triptolide (Fig. 3a). In contrast, triptolide inhibited the DNA-dependent ATPase activity of the TFIIH holocomplex at concentrations similar to those required for *in vivo* [^{3}H]-uridine incorporation, *in vitro* transcription and *in vitro* NER assays (Fig. 3b and Supplementary

Fig. 9). To further determine whether triptolide inhibited the ATPase arising from XPB or XPD subunit of TFIIH, we purified the TFIIH core complex that is devoid of the XPD subunit and used it in the ATPase assay in the absence and presence of triptolide[33] (Supplementary Fig. 5). Similar to the holocomplex, the DNA-dependent ATPase activity of the core complex of TFIIH is also inhibited by triptolide at similar concentrations (Fig. 3c and Supplementary Fig. 10), suggesting that XPB is the molecular target for triptolide. It is noteworthy that triptolide selectively inhibited the ATPase activity of both the core and holo-TFIIH complexes without affecting the DNA helicase activity.

It was previously reported that triptolide binds covalently to a 90-kDa protein, and coincidently, this molecular weight is closest to that of XPB among all the subunits of TFIIH[15]. We thus determined whether triptolide binds to XPB. HeLa cell nuclear extract was incubated with [^{3}H]-triptolide, followed by immunoprecipitation with an anti-XPB antibody. The protein covalently labeled by [^{3}H]-triptolide is quantitatively immunoprecipitated by the anti-XPB antibody with no labeled protein remaining in the supernatant (Fig. 4a, Lane 3 vs. 1). The binding of [^{3}H]-triptolide to the protein was sensitive to competition by excess unlabeled triptolide (Fig. 4a, Lane 3 vs. 4). Western blot analysis revealed that under those conditions XPB was nearly quantitatively pulled down from the cell lysate by the anti-XPB antibody (Supplementary Fig. 11), suggesting that XPB is the triptolide-binding protein. To further confirm that XPB is the 90-kDa triptolide-binding protein, we immunoprecipitated TFIIH complex after labeling nuclear extract with [^{3}H]-triptolide, followed by 5% SDS-PAGE in duplicate. One gel was stained with silver to visualize the XPB, XPD and p62 subunits of TFIIH while the second identical gel was subjected to fluorography. As shown in Fig. 4b, the 90-kDa band labeled by [^{3}H]-triptolide had the same gel mobility as the XPB subunit. Lastly, we overexpressed and purified recombinant XPB from baculovirus-driven insect cells (Supplementary Fig. 12) and determined whether it is capable of binding to triptolide and whether its DNA-dependent ATPase activity is inhibited by triptolide. The recombinant XPB also formed a covalent complex with [^{3}H]-triptolide and the binding was sensitive to competition by unlabeled triptolide (Fig. 4c), clearly demonstrating that XPB is the direct target of triptolide. Moreover, when we performed the ATPase assay, although the intrinsic DNA-dependent ATPase activity of purified XPB is much lower than that of the TFIIH complex, the residual ATPase activity was inhibited by triptolide in a dose-dependent manner (Fig. 4d and Supplementary Fig. 13). It took a much higher concentration of triptolide to inhibit the ATPase activity of purified XPB than that in the TFIIH complex, which suggests that other subunits of TFIIH may be involved in the binding of triptolide to XPB.

Correlating XPB ATPase and cell proliferation inhibition

To further confirm that the antiproliferative effect of triptolide is indeed mediated by inhibition of XPB ATPase activity, we synthesized twelve analogs of triptolide with reported IC_{50} for inhibition of cell proliferation spanning 3 orders of magnitude[34–37]. We then determined their activities in the ATPase assay and HeLa cell proliferation assay, respectively (Table 1). There is a significant correlation ($r = 0.98$) between IC_{50} values of the analogs for inhibition of TFIIH ATPase activity and cell proliferation, offering further support that triptolide inhibits cell proliferation by blocking XPB DNA-dependent ATPase activity (Fig. 4e).

DISCUSSION

Since triptolide was first identified in 1972[6], its mechanism of action has remained a mystery. In this study, we identified the XPB subunit of TFIIH as a key target of triptolide. The covalent binding of triptolide to XPB and the consequent inhibition of the ATPase activity of XPB offers a unified and coherent mechanism that can account for the majority of

the known cellular and physiological activities of triptolide reported to date. Thus, the effect of triptolide on the activity of a number of transcription factors from NF-κB to p53 and from AP-1 to HSF-1 at the transactivation step can be explained by the inhibition of XPB and TFIIH activity; inhibition of XPB and TFIIH blocks RNAPII-mediated transcription initiation, hence the transactivation by all those transcription factors. This general inhibition of RNAPII-mediated transcription may underlie the inhibition of T cell activation and proliferation of nearly all cancer cell lines, thus explaining the anti-inflammatory, immunosuppressive and antiproliferative activity of triptolide. Given the essential role of RNAPII-mediated transcription in cell growth and survival, it is possible that inhibition of XPB ATPase activity by triptolide may also be the underlying cause of its toxicity as well.

The effects of triptolide on the ATPase activity of XPB and PolII-mediated transcription are consistent with previous findings on the inhibitory activity of the drug on the transactivation of several transcription factors *in vitro*[16]. More recently, effects of triptolide on RNA synthesis were further investigated using multiple approaches[17]. Triptolide was found to have an inhibitory effect on the majority of mRNA species, in agreement with an effect on PolII-mediated transcription. Using microarray, expression of over 4,400 genes was found to be affected by triptolide, the majority of which were downregulated. It was concluded that triptolide predominantly downregulated the transcription of short-lived mRNA[17]. As microarray only detects the steady levels of RNA, the observed perturbation by triptolide resulted from a combination of effects on both *de novo* transcription and mRNA degradation. As such, inhibition of XPB and PolII-mediated transcriptional initiation can explain the inhibitory effect of triptolide on the expression levels of short-lived mRNA. Additional experiments to examine the effect of triptolide on the association of PolII and TFIIH with the different loci throughout the genome will be needed to further verify the relevance of its interaction with XPB to its cellular activity.

Two putative triptolide targets have been previously reported. One is a 90-kDa unknown nuclear protein that forms a covalent complex with [^{3}H]-triptolide[15] and the other is the calcium channel polycystin-2[19]. Our results are in agreement with the former and the 90-kDa putative triptolide-binding protein is XPB. The possibility cannot be ruled out that triptolide also directly binds to polycystin-2, regulating its channel activity[19]. If that proves to be true, however, it would be extraordinary that the relatively small triptolide is capable of binding to two structurally unrelated proteins, a DNA-dependent ATPase and a transmembrane ion channel. While inhibition of XPB can account for most of the known biological activities of triptolide, the effect of triptolide on polycystin-2 seems to be unrelated to those activities. It will be interesting to determine whether the effect of triptolide on polycystin-2 is indirect and secondary to the inhibition of transcription, and hence the expression level of the calcium channel and its mutants.

We were surprised to observe that triptolide inhibited more then 80% of uridine incorporation in cells without inhibiting RNAPI activity *in vitro*, as it is known that more than 90% of RNA inside the cell is rRNA. Furthermore, two other RNAPII-specific inhibitors, flavopiridol and α-amanitin (in permeabilized cells), have the same effect on uridine incorporation[23]. The existence of three different inhibitors of RNAPII machinery that inhibited the majority of uridine incorporation suggests that either RNAPII is responsible for the synthesis of the majority of RNA inside the cell or RNAPI-mediated rRNA synthesis is also dependent on RNAPII activity, potentially through the regulated expression of some key component of RNAPI-mediated transcription.

As a key component of the multi-protein TFIIH complex, XBP has been shown to possess at least two distinct enzymatic activities *in vitro*: ATP-dependent DNA helicase activity and DNA-dependent ATPase activity. To our surprise, we found that triptolide only inhibited the

ATPase activity of XPB in the core- and holocomplexes of TFIIH without affecting its conventional DNA helicase activity. Although the helicase activity of XPB has been thought to play a key role in opening double-stranded DNA to facilitate transcription initiation by RNAPII, this notion was called into question when it was observed that TFIIH appeared to work as an ATP-dependent DNA unwinding machine rather than a conventional DNA helicase to promote promoter melting during transcription initiation[38]. The selective inhibition of the ATPase activity of XPB by triptolide offers new evidence in support of the latter model.

Triptolide is structurally distinct in that it contains multiple reactive functional groups, an α, β-unsaturated lactone, which can serve as a Michael acceptor, and three consecutive epoxide groups. It is thus not surprising that it forms a covalent complex with XPB that can survive the denaturing and reducing conditions of gel electrophoresis. This is reminiscent of the natural products fumagillin and ovalicin, which specifically interact with MetAP2 despite the presence of two potentially reactive epoxide groups (Griffith et al., 1997; Griffith et al., 1998 PNAS). It seems that Nature has elaborated natural products containing reactive chemical groups in such a way that their presence does not compromise the binding specificity of natural products. In a model reaction with propanethiol *in vitro*, it was previously shown that the 9,11-epoxide was attacked by the thiol, assisted by the neighboring 14β-hydroxyl group, to form a covalent adduct[39]. It remains to be seen whether a cysteine residue in XPB is involved in the formation of the triptolide-XPB covalent complex. The presence of multiple epoxides in triptolide also raised the possibility that it may have non-specific interactions with cysteine-containing proteins in general. However, only a single covalent complex was detected when the nuclear lysate was exposed to [^{3}H]-triptolide, suggesting that triptolide is highly specific for XPB. In support of XPB as a physiologically relevant target of triptolide, we found a significant correlation between the antiproliferative activities and the potencies for inhibition of XPB ATPase activity by a number of triptolide analogs with a wide range of activities. Moreover, we found that overexpresion of XPB, but not XPD, conferred a relatively small but significant resistance to 293T cells towards triptolide (Supplementary Fig. 14). Despite these lines of supportive evidence, however, we cannot completely rule out the possibility that triptolide may have additional targets with lower abundance but higher affinity that might have eluded detection under the current experimental conditions.

The potent inhibition of XPB and the accompanying RNAPII-mediated transcription makes triptolide a unique and useful molecular tool among the existing inhibitors of transcription due in part to its higher potency and greater cell permeability than α-amanitin and its greater specificity than either actinomycin D or DRB. A deeper understanding of the interaction between triptolide and XPB will facilitate the design of novel inhibitors of XPB and other homologous DNA helicases as anticancer and antiproliferative drug leads. Moreover, our preliminary evidence also suggested a previously unknown activity of triptolide, i.e., inhibition of nucleotide excision repair. Although an effect of triptolide on nucleotide excision repair remains to be demonstrated *in vivo*, it is expected as the ATPase activity has been shown to be crucial for that activity of TFIIH. The ability of triptolide to block DNA repair has important implications as its impairment has been shown to be responsible for resistance of cancer cells to certain classes of anticancer drugs. For example, it has been shown that triptolide can enhance the anticancer activity of such DNA damaging drugs as cisplatin and topoisomerase I inhibitors[40,41]. It is tempting to speculate that this unique inhibitory effect of triptolide on nucleotide excision repair in addition to transcription may be further exploited for the development of new anticancer modalities.

METHODS

In vitro transcription assay

The *in vitro* transcription assay was conducted as previously described[42]. HeLa cells nuclear extract was prepared as previously described[43]. Cytomegalovirus (CMV) promoter from Positive Control DNA (Promega), human rDNA promoter from pETS-RB digested with XhoI[44] and adenovirus VAI promoter from pVATK digested with SacI[45] were used for RNA polymerase II, RNA polymerase I and RNA polymerase III directed transcription, respectively. pETS-RB and pVATK were gifts from Dr. Barbara Sollner-Webb (Johns Hopkins University).

RNA polymerase II activity on oligo(dC) tailed template

The tailed template assay for RNA polymerase II catalytic activity was performed as previously described[26]. Purified bovine RNA polymerase II was a gift from Dr. Averell Gnatt (University of Maryland).

In vitro transcription assay with purified or recombinant transcription factors

Recombinant (TBP, TFIIA, TFIIB, TFIIE, and TFIIF) and native (TFIID, TFIIH and Pol II) human transcription factors were prepared as previously described[46]. *In vitro* transcription on the linear and negatively supercoiled templates containing the Adenovirus major late promoter (−53 to +10) fused to a 380 basepair G-less cassette was performed as previously described[28,46]. The heteroduplex template consisted of the Adenovirus major late promoter with a mismatched region from −9 to +3[29].

Abortive initiation assay

Abortive initiation was performed as previously described[30].

In vitro Nucleotide Excision Repair assay

A site specific 1,3 GTG Pt lesion was placed in the center of a 150mer substrate as previously described[47]. The preparation of HeLa whole cell extracts and the NER excision assay were carried out as previously outlined in detail[48].

ATPase assay

The DNA-dependent ATPase assay was performed as previously described[49]. Briefly, a 10-μl reaction mixture contained 20 mM Tris (pH 7.9), 4 mM $MgCl_2$, 1 μM ATP, 1 μCi ATP (3000 Ci/mmol), 100 μg/ml BSA, 50 ng RNA polymerase II promoter Positive Control DNA (Promega), 1–2 nM TFIIH or 100 nM his-XPB and indicated concentration of drugs. The reactions were started by either addition of TFIIH/his-XPB or ATP and incubated at 37 °C for 2 h. The reactions were stopped by addition of 2 μl 0.5 M EDTA and dilution in up to 100 μl with TE buffer. An aliquot of 1 μl reaction mixture was spotted on PEI-cellulose and the chromatogram was developed with 0.5 M LiCl and 1 M HCOOH. The percent of ATP hydrolysis was quantified using a PhosphorImager.

TFIIH helicase assay

The helicase assay was performed as previously described[50]. Briefly, a 10-μl reaction mixture contained 20 mM Tris (pH 7.9), 4 mM $MgCl_2$, 4 mM ATP, 100 μg/ml BSA, 0.12 nM of M13mp18-based bidirectional helicase substrate, and 0.4 nM TFIIH. The reactions were started by either addition of TFIIH/his-XPB or ATP and incubated at 37 °C for 2 h. The reactions were stopped by addition of 5 μl of a quenching buffer (60 mM EDTA, 50% glycerol, 0.75% SDS). An aliquot of 10 μl of the mixture was loaded on a 10% non-

denaturing polyacrylamide gel containing 0.1% SDS and run at 200 V in 0.5x TBE buffer with 0.1% SDS. The gel was dried and subjected to quantification using a PhosphorImager.

[^{3}H]-triptolide binding to XPB in HeLa nuclear extract

HeLa nuclear extract (containing 400 μg of total proteins) was incubated with 1 μM [^{3}H]-triptolide with or without 50 μM unlabeled triptolide in 50 μl of 10 mM HEPES (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 0.1 mM EDTA, 0.5 mM DTT and 50 μM PMSF for 1 h at 30 °C. Three micrograms of affinity purified rabbit anti-XPB antibody (A301–337A, Bethyl Laboratories) was added and the mixture was incubated for an additional 30 min at 25 °C. The mixture was added to 50 μl of Dynabeads Protein A (100.01D, Invitrogen, storage solution removed) and further mixed by rotation for 10 min at 25 °C. The supernatant was aspirated and the beads were washed 3 times with PBS. The beads were resuspended in 40 μl of sample buffer, boiled for 5 min and subjected to SDS-PAGE. After electrophoresis, the gel was soaked in En3hance solution according to the manufacturer's instructions and exposed to pre-flashed x-ray film for 2 weeks prior to autoradiography.

[^{3}H]-triptolide binding to recombinant his-XPB

His-XPB (300 ng) was incubated with 1 μM [^{3}H]-triptolide with or without 50 μM cold triptolide in 40 μl binding buffer [20 mM Tris (pH 7.9), 4 mM $MgCl_2$, 1 μM ATP, 100 μg/ml BSA], and 500 ng RNA polymerase II promoter Positive Control DNA (Promega) for 1 h at 30 °C. Samples were boiled in sample buffer and subjected to 12% SDS-PAGE. After electrophoresis, the gel was soaked in En3hance solution (Perkin Elmer) according to manufacturer instructions and exposed to pre-flashed x-ray film for 2 weeks prior to development.

Other methods

For the remaining experimental procedures and a more detailed description of the above procedures, see Supplementary Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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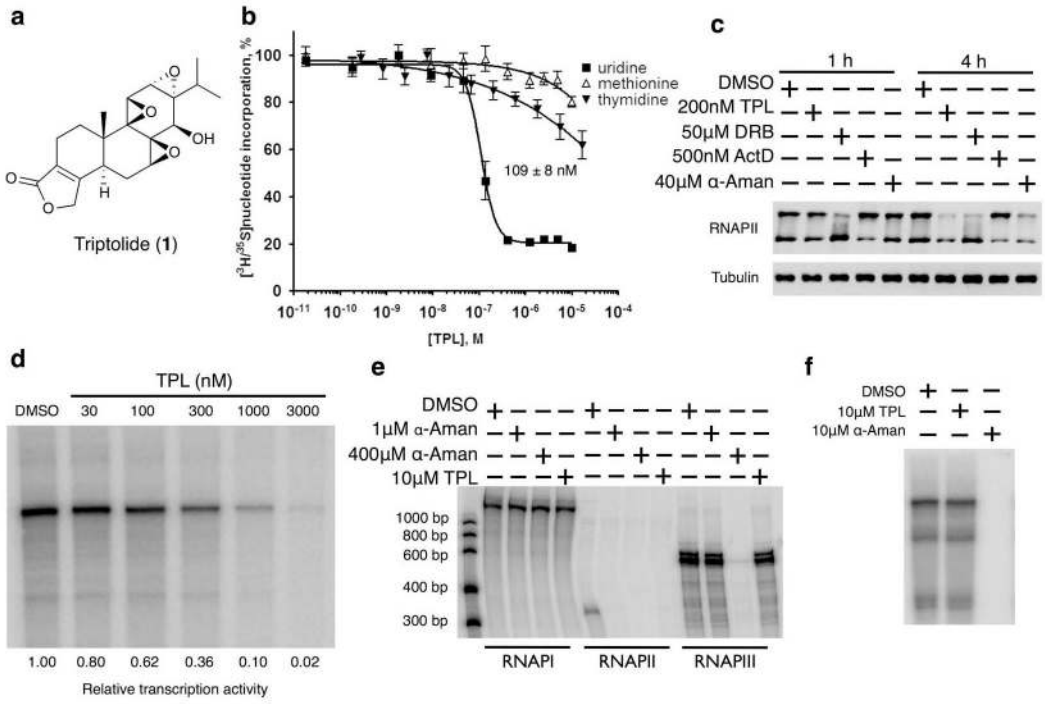


Figure 1. Triptolide is a novel type of inhibitor of RNAPII-mediated transcription

(a) Chemical structure of triptolide. **(b)** Inhibition of protein, RNA and DNA synthesis after 1-h treatment with triptolide followed by 1-hour pulse with [^{32}S]methionine, [^{3}H]uridine or [^{3}H]thymidine. Mean values ± SEM (error bars) from 3 independent experiments are shown. IC_{50} values are listed next to corresponding curves ± SE. **(c)** Western blot analysis of the degradation and dephosphorylation of RNA polymerase II largest subunit after treatment of HeLa cells with different transcription inhibitors. Upper band is phosphorylated RNAPII. **(d** and **e)** *In vitro* transcription assays using HeLa nuclear extracts showing that triptolide selectively inhibits RNA synthesis driven by RNA polymerase II promoter. Relative transcription activity was quantified with a PhosphorImager. **(f)** Effects of triptolide and α-amanitin on transcription of a tailed template with purified calf RNA polymerase II. Abbreviations: TPL, triptolide; α-aman, α-amanitin; ActD, actinomycin D.

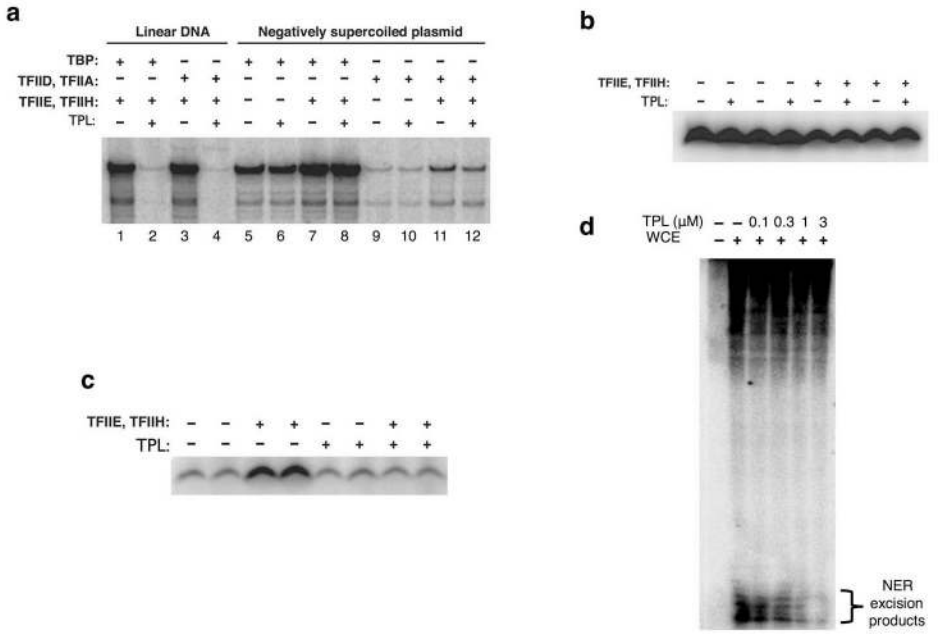


Figure 2. Triptolide inhibits TFIIH-dependent basal transcription and nucleotide excision repair

(a) *In vitro* transcription assays with purified or recombinant basal transcription factors on linear and supercoiled templates in the absence and presence of 10 μM triptolide. **(b)** Effect of triptolide (3 μM) on *in vitro* transcription from a bubbled template. **(c)** Inhibition of TFIIH/TFIIE-dependent enhancement of abortive initiation by triptolide (1 μM). **(d)** Inhibition of nucleotide excision repair by triptolide. Shown are a representative sequencing gel and the corresponding quantitation graph of the inhibition of nucleotide excision repair by triptolide.

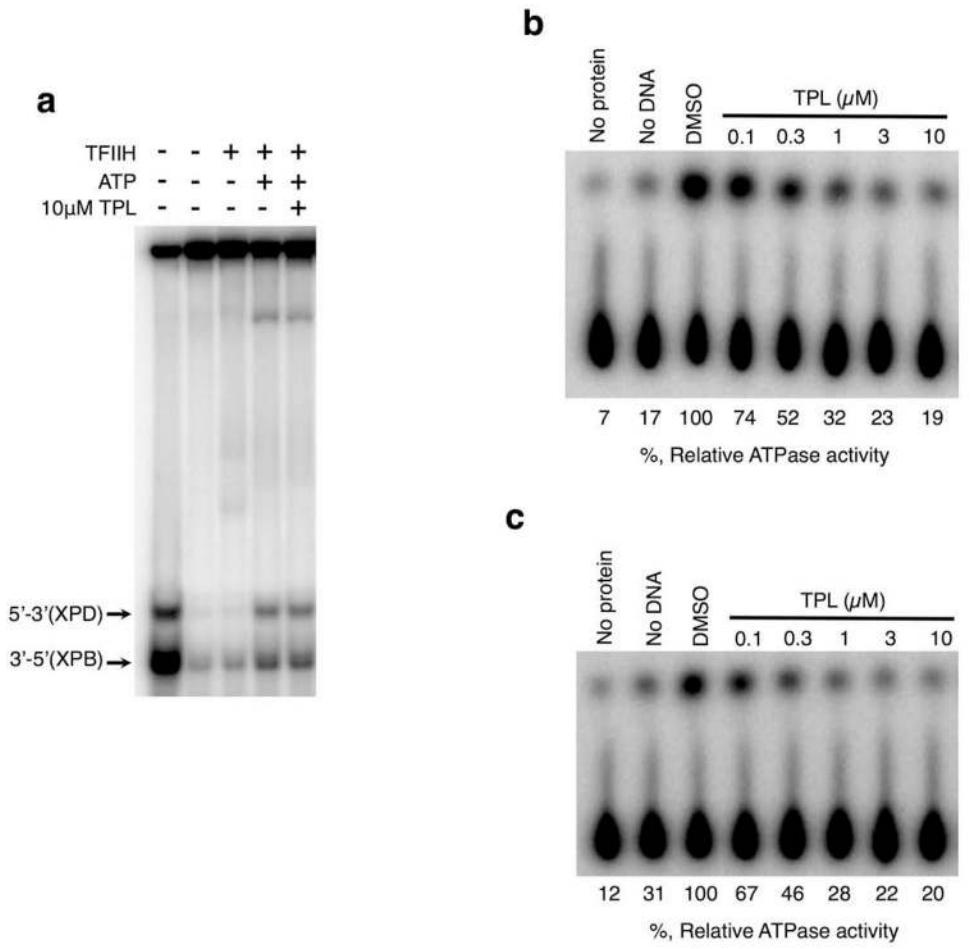


Figure 3. Triptolide inhibits the DNA-dependent ATPase activity of TFIIH without affecting its DNA helicase activity

(a) Effect of triptolide on the two helicase activities of TFIIH in a bidirectional helicase assay with the holoTFIIH complex. **(b** and **c)** Inhibition of DNA-dependent ATPase of the holo (B) and core (C) complex of TFIIH (1 nM) by triptolide. 100% activity corresponds to hydrolysis of 14.3% of ATP for core TFIIH and 27.8% for holo TFIIH. Relative ATPase activity was quantified with a PhosphorImager.

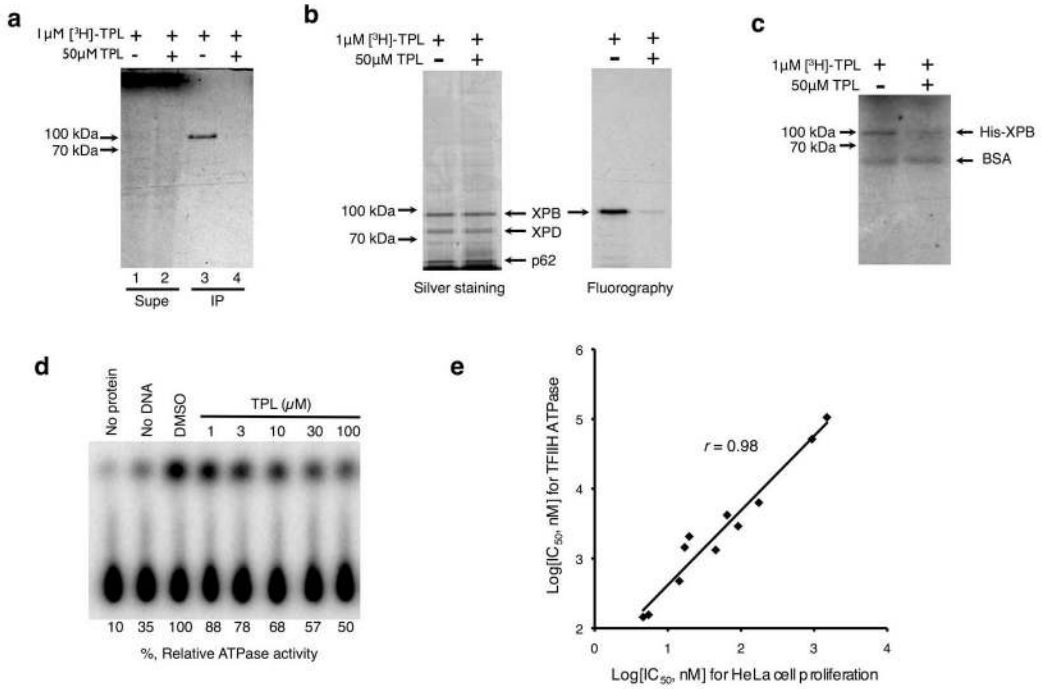
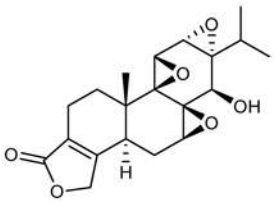
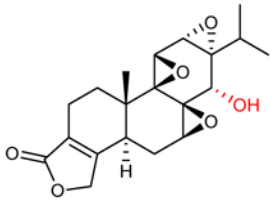
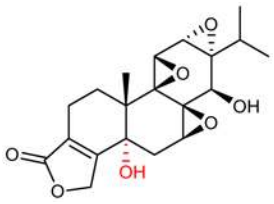
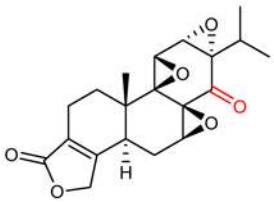
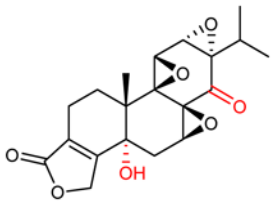
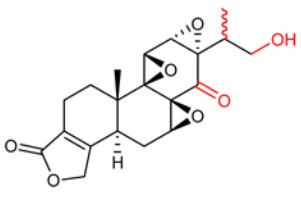


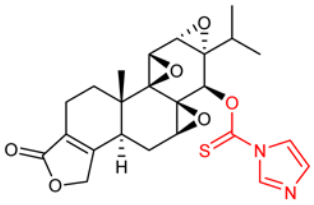
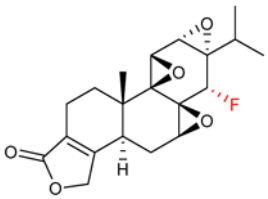
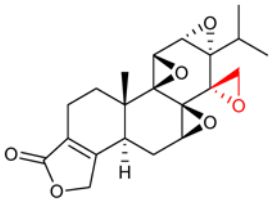
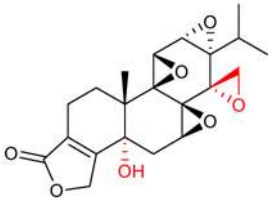
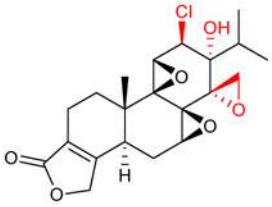
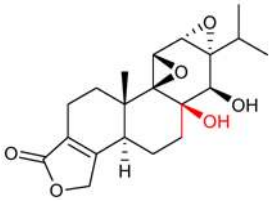
Figure 4. Triptolide binds covalently to XPB subunit of TFIIH and correlation between inhibition of TFIIH ATPase activity and inhibition of cell proliferation by triptolide analogs

(**a**) [^{3}H]-triptolide binds covalently and selectively to a 90-kDa protein in HeLa nuclear extract that can be immunoprecipitated by anti-XPB antibody. (**b**) The 90-kDa protein labeled by [^{3}H]-triptolide comigrates with XPB on SDS-PAGE gels. (**c**) [^{3}H]-triptolide binds covalently to recombinant his-XPB which can be competed away with excess unlabeled triptolide. (**d**) Triptolide inhibits DNA-dependent ATPase activity of recombinant his-XPB (100 nM). 100% activity corresponds to hydrolysis of 23% of ATP. Relative ATPase activity was quantified with a PhosphorImager. (**e**) Correlation between inhibition of TFIIH ATPase activity and inhibition of cell proliferation by analogs of triptolide. Data points were fitted to the linear equation y = 1.0691x + 1.55 with R^2 = 0.95184. Correlation coefficient (*r*) is calculated using CORREL function in Excel.

Table 1

Bioactivities of triptolide (**1**) and its analogs

Entry	Structure	IC ± SE (M)	
		ATPase activity	HeLa Proliferation
1		**0.145** (± 0.01)	**0.00459** (± 0.001)
2		**1.32** (± 0.03)	**0.0451** (± 0.01)
3		**2.9** (± 0.11)	**0.0913** (± 0.021)
4		**0.156** (± 0.024)	**0.00545** (± 0.001)
5		**6.32** (± 0.23)	**0.175** (± 0.024)
6		**106** (± 8)	**1.50** (± 0.29)

Entry	Structure	IC ± SE (M)	
		ATPase activity	HeLa Proliferation
7		**1.44**(± 0.08)	**0.017**(± 0.007)
8		**0.475** (±0.019)	**0.0143** (± 0.004)
9		**2.07** (± 0.2)	**0.0196** (± 0.0041)
10		**51.4** (± 3.0)	**0.935** (± 0.406
11		**4.19**(± 0.2)	**0.0646** (± 0.0094
12		**>500**	**>10**