Antitumor Agents Hot Paper

International Edition: DOI: 10.1002/anie.201606121
German Edition: DOI: 10.1002/ange.201606121

Targeted Delivery and Sustained Antitumor Activity of Triptolide through Glucose Conjugation

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Abstract: *Triptolide, a key ingredient from the traditional Chinese medicinal plant thunder god vine, which has been used to treat inflammation and autoimmune diseases for centuries, has been shown to be an irreversible inhibitor of the XPB subunit of the transcription factor TFIIH and initiation of RNA polymerase II mediated transcription. The clinical development of triptolide over the past two decades has been limited by its toxicity and low water solubility. Herein, we report the development of a glucose conjugate of triptolide, named glutriptolide, which was intended to target tumor cells overexpressing glucose transporters selectively. Glutriptolide did not inhibit XPB activity in vitro but demonstrated significantly higher cytotoxicity against tumor cells over normal cells with greater water solubility than triptolide. Furthermore, it exhibited remarkable tumor control in vivo, which is likely due to sustained stepwise release of active triptolide within cancer cells. These findings indicate that glutriptolide may serve as a promising lead for developing a new mechanistic class of anticancer drugs.*

Triptolide is a structurally unique diterpenoid isolated from the traditional Chinese medicinal plant thunder god vine, the extract of which has been used as an immunosuppressive and anti-inflammatory medicine for centuries (**1**; Figure 1 a).[1] Aside from its immunosuppressive activity, triptolide has been shown to be a potent inhibitor of cancer cell growth in vitro and to exhibit strong antitumor activity in vivo.[2] We recently identified XPB (ERCC3), a subunit of the general transcription factor TFIIH, as the major target mediating the anticancer activity of triptolide.[3] In ensuing studies, we and others gathered further supporting evidence that XPB is the physiologically relevant target of triptolide.[4] Those observations suggested that triptolide is a novel and highly specific inhibitor of the general transcription factor TFIIH, which is

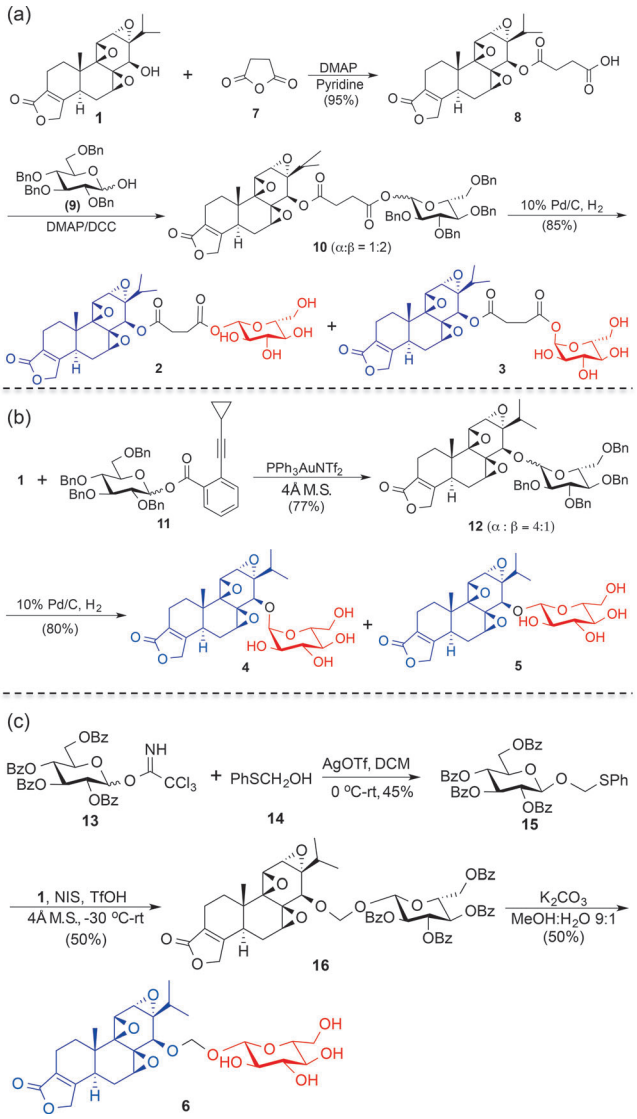

Figure 1. a) Synthesis of glutriptolides **2** and **3**. b) Synthesis of glutriptolides **4** and **5**. c) Synthesis of glutriptolide **6**. Triptolide **1**: blue; glucose: red. Bn = benzyl, Bz = benzoyl, DCC = dicyclohexylcarbodiimide, DCM = dichloromethane, DMAP = 4-dimethylaminopyridine, M.S. = molecular sieves, NIS = *N*-iodosuccinimide, Tf = trifluoromethanesulfonyl.

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Supporting information for this article can be found under: http://dx.doi.org/10.1002/anie.201606121.

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responsible for transcriptional initiation by RNA polymerase II (RNAPII) as well as nucleoside excision repair.

Despite its great promise as an anticancer drug lead, attempts to develop triptolide and its synthetic derivatives over the past few decades have not succeeded.[5] Several generations of clinical candidates have failed except for Minnelide™, the latest triptolide derivative that has entered a phase I human clinical trial.[6] The major obstacles for triptolide to becoming a clinically useful drug include its general toxicity and lack of water solubility.[7]

One strategy to reduce the toxicity of triptolide is to deliver it selectively to tumor cells over their normal counterparts. A unique characteristic of tumor cells is that they have a much higher demand for glucose than normal cells partially owing to the Warburg effect.[8] Consequently, most cancer cells overexpress one or more isoforms of glucose transporters (GLUTs), particularly GLUT1 and GLUT3, to sustain their growth and survival.[9] The overexpression of GLUTs in tumor cells has been exploited to target tumor cells selectively by conjugating cytotoxic drugs to glucose.[10] Glucose conjugates have been made for a number of cytotoxic drugs such as ifosfamide and taxol[11] and have been shown to have lower toxicity and higher tumor cell selectivity in vitro. We envisioned that conjugating triptolide with glucose could also increase its selectivity for tumor cells, thereby decreasing its toxicity. The resultant glucose–triptolide conjugates (glutriptolides) would enter cancer cells through GLUTs whereby the linker would undergo cleavage to release triptolide, allowing it to bind to XPB and block cell proliferation or induce apoptosis.

We designed and synthesized five glutriptolide derivatives, compounds **2**–**6** (Figure 1). We chose the C14 hydroxy group as the site of linkage to glucose as it is the most reactive functional group in triptolide and can undergo facile chemical modification. Whereas there are multiple sites in glucose that can be used to connect to triptolide, the C1 hydroxy group in glucose has been successfully used to form active conjugates with a number of known drugs.[10] The glutriptolides contain one of three distinct linkages, namely a succinate (**2**, **3**) or an acetal (**6**) linker or a direct linkage through an ether bond (**4**, **5**). Whereas the succinate linker is susceptible to hydrolysis by cellular esterases, the acetal linker is more stable, and the ether linkage likely remains intact inside cells.

The syntheses of glutriptolides **2** and **3** (Figure 1a) commenced with acylation of the C14 hydroxy group of triptolide with succinic anhydride (**7**) in the presence of DMAP and pyridine to give intermediate **8**.[12] Condensation of **8** with protected glucose **9** in the presence of DMAP and DCC yielded **10** as an anomeric mixture with a 1:2 ratio for the α and β anomers. The benzyl protecting groups on the glucose moiety were removed by hydrogenation in the presence of palladium on charcoal, giving rise to glutriptolides **2** and **3**, with **2** as the predominant product. Glutriptolides **4** and **5** were synthesized by a two-step sequence including condensation of triptolide with compound **11** in the presence of PPh_3AuNTf_2 and 4 Å molecular sieves to give **12**, followed by removal of the benzyl protecting groups by hydrogenolysis in the presence of palladium on charcoal (Figure 1b). To prepare glutriptolide **6**, the trichloroacetimidate donor **13** was first converted into intermediate **15** upon reaction with alcohol **14** in the presence of AgOTf, followed by condensation with triptolide under the promotion of NIS and TfOH to yield conjugate **16** (Figure 1c). Upon removal of the benzoyl protecting groups with potassium carbonate in aqueous methanol, **6** was obtained in 50% yield. The structures of key reaction intermediates and all glutriptolides (**2**–**6**) were confirmed by ^1H and ^{13}C NMR spectroscopy and high-resolution mass spectrometry (see the Supporting Information).

We determined the effects of the glutriptolides on the DNA-dependent ATPase activity of TFIIH isolated from HeLa cell nuclear extract as previously described.[3] Surprisingly, none of the glutriptolide analogues showed appreciable inhibitory activity (Figure 2a; see also the Supporting Information, Figure S1). Based on the solution structure of **2** determined by 2D NMR spectroscopy, the lack of activity of **2** may be attributable to intramolecular interactions between the succinate linker and the A and B rings of triptolide (Figures S2 and S3), preventing the triptolide moiety from interacting with XPB. The inability of **2** and **3** to inhibit XPB renders them ideal prodrugs devoid of undesirable activity prior to entry into cells where the linker is cleaved by cellular esterases.

Next, we determined the effects of the glutriptolides on the proliferation of HEK293T cells. Glutriptolides **2** and **3** retained considerable antiproliferative activity with IC_{50} values of 268 and 615 nM, respectively (Figure 2b). In contrast, glutriptolides **4**–**6** had little effect on cell proliferation even at the highest concentrations. Those results are likely due to the inability of glutriptolides **4**–**6** to undergo linker cleavage inside cells, which is consistent with their lack of activity towards XPB/TFIIH in vitro. Given that **2** had a slightly higher antiproliferative activity than **3** (Figure 2b) and was the major product of the synthesis (Figure 1a), we decided to focus on **2** in ensuing studies.

To confirm that the antiproliferative activity of **2** was due to inhibition of endogenous XPB, we determined its effect on the stability of the catalytic subunit of RNAPII, which has been shown to undergo degradation upon binding of triptolide to XPB.[3] Glutriptolide **2** induced degradation of the RPB subunit of RNAPII, although at higher concentrations in comparison to triptolide (Figure 2c). Upon longer incubation (72 h), the degradation of RPB became more pronounced (Figure S4). In contrast, neither taxol nor doxorubicin affected the stability of RPB (Figure S5), suggesting that the induction of RPB degradation is specific for triptolide and **2**. To further assess whether the antiproliferative activity of **2** was mediated through inhibition of XPB, we determined the sensitivity of an engineered XPB-C342T mutant cell line that is resistant to triptolide as a result of a mutation of the active-site cysteine that is covalently modified by triptolide.[4a] Glutriptolide **2**, like triptolide, had no inhibitory effect whereas both taxol and doxorubicin retained their inhibitory effects on the XPB-C342T cell line (Figure S6), indicating that the antiproliferative activity of glutriptolide **2** is mediated through inhibition of XPB.

To determine whether **2** depended on GLUT to enter cells, we tested **2** in combination with a known inhibitor of

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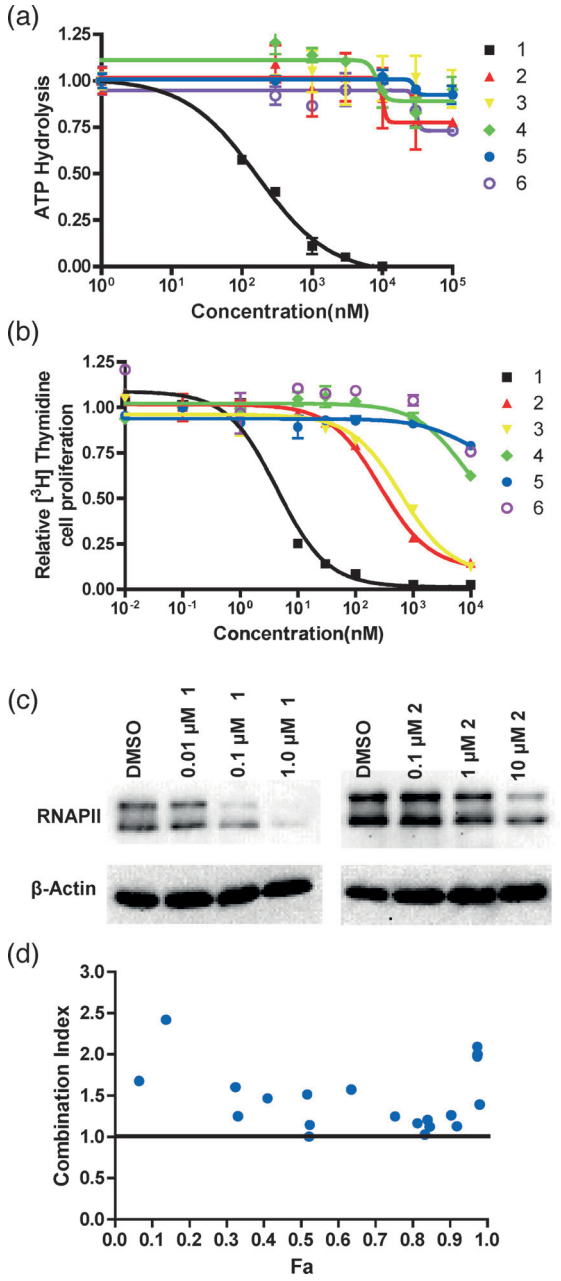

Figure 2. Characterization of the antiproliferative activity and mechanism of glutriptolides in vitro. a) Effects of triptolide and glutriptolides on the DNA-dependent ATPase activity of TFIIH. b) Antiproliferative effects of triptolide and glutriptolides on the proliferation of HEK293T cells. c) Degradation of the RPB subunit of RNPII induced by triptolide (**1**) and glutriptolide (**2**) as judged by western blot analysis. d) Mutual antagonism between GLUT inhibitor WZB117 and glutriptolide **2**. CI = combination index, Fa = fraction affected.

GLUT1, WZB117,[13] which alone also inhibits the proliferation of HEK293T cells. We found that over the entire concentration range, **2** and WZB117 were mutually antagonistic as judged by their combination indexes (CI = 1: additive; CI > 1: antagonistic; Figure 2d), suggesting that glutriptolide **2** is dependent on GLUT1 to exert its antiproliferative effect. We also took a complementary approach to assess the involvement of GLUT in the antiproliferative activity of **2** by comparing the sensitivity of two isogenic cancer cells lines, DLD1-WT and DLD1-Mut, that have been shown to overexpress GLUT1[14] (Figure S7). The mutant cell line proved more sensitive to glutriptolide **2** (Figure S8). Similar to glucose, glutriptolide **2** also showed dose-dependent inhibition of cellular [^{3}H]glucose uptake (Figure S9). Together, these results suggest that GLUT1 is capable of facilitating the uptake of **2** into cells, which contributes to its selective targeting of tumor cells overexpressing GLUT1 and other isoforms of GLUT.

We determined the partition coefficients of triptolide and **2** to assess their water solubility. With a log P value of −0.75, glutriptolide **2** has a significantly higher water solubility (> 25 fold) than triptolide itself (log P = 0.69), indicating that conjugation of triptolide to glucose effectively resolved its low solubility issue.

We used a panel of primary and transformed cancer cell lines to obtain a profile of the general cytotoxicity of glutriptolide **2**, reasoning that **2** should be more toxic to tumor cells than to normal cells. For the four types of primary cells tested, namely airway epithelial cells, astrocytes, human umbilical vein endothelial cells (HUVEC), and human fibroblasts, the average IC_{50} value was 1.6 μM. In comparison, the average IC_{50} value for the four cancer cell lines PC3, SK-RC-52, H460, and H1975 was about 0.2 μM (Table 1 and Figure S10). In contrast, the IC_{50} values for triptolide are similar for the primary and the transformed cancer cell lines (Table 1 and Figure S11), indicating that glutriptolide **2** was more selective for tumor cells than primary, non-malignant cells.

The in vivo antitumor activity of glutriptolide **2** was determined by using a metastatic prostate cancer mouse model that has been established in one of our laboratories.[15] In pilot experiments, we found the tolerable dose of triptolide to be 0.2 mg kg^{-1} and that of **2** to be 1 mg kg^{-1}. Accordingly, three weeks after tumor cell injection, each compound was administered once daily by intraperitoneal injection at those doses for a total of four weeks. We continually monitored survival of the animals upon cessation of administration of triptolide or **2**. As shown in Figure 3a, mice treated with **2** had lower tumor burdens during weeks 1 and 2 than those treated with triptolide. At the end of week 4, both treatment groups showed no detectable tumor cells whereas all animals in the untreated groups had died. Upon termination of treatment, tumors returned immediately in animals treated with triptolide. In contrast, no tumor cells were detectable in those treated with **2** until three weeks after cessation of treatment. In another test with varying doses of triptolide and **2**, both triptolide and **2** prolonged the survival of animals in a dose-dependent manner. In comparison to triptolide, however, **2** at both 0.5 and 1 mg kg^{-1} led to significantly longer survival than triptolide at 0.2 mg kg^{-1} (79 and 89 days vs. 46 days; Figure 3b).

To determine the possible path of activation of **2** in cancer cells, we assessed the conversion of **2** into triptolide and degradation intermediate **8** in HEK293T cell lysates. There was a time-dependent conversion of **2** into **8** and a much slower accumulation of triptolide itself (Figure S12). As intermediate **8** retained a significant amount of activity

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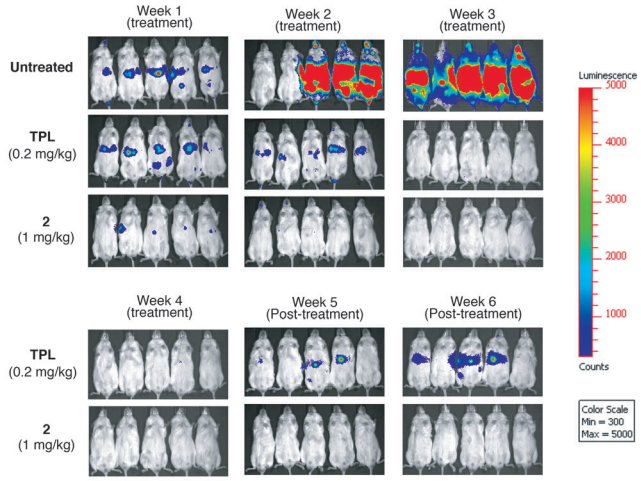
Table 1: IC_{50} values of triptolide **1** and glutriptolide **2** in different cell lines.

Primary cells[a]	**1** [nM]	**2** [μM]	Cancer cell line[a]	**1** [nM]	**2** [μM]
AEC	7.64 ± 0.02	0.64 ± 0.01	PC3	10.9 ± 0.05	0.14 ± 0.03
astrocytes	23.5 ± 0.02	3.09 ± 0.01	SK	14.0 ± 0.03	0.37 ± 0.05
HUVEC	6.40 ± 0.01	1.69 ± 0.01	H460	2.75 ± 0.05	0.17 ± 0.07
fibroblasts	4.20 ± 0.03	1.16 ± 0.13	H1975	13.4 ± 0.05	0.08 ± 0.03

[a] AEC = airway epithelial cells, HUVEC = human umbilical vein endothelial cells, SK = SK-RC-52.

(a)



(b)

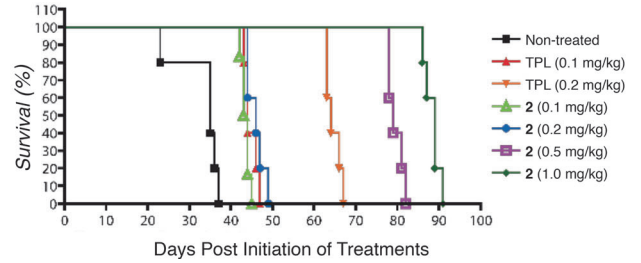


Figure 3. Antitumor activity of glutriptolide **2** in comparison to triptolide in vivo. a) Antitumor activity of triptolide and glutriptolide **2** as assessed by bioluminescence imaging in live animals. b) Effects of triptolide and glutriptolide **2** on the survival of mice. Kaplan–Meier curves showing the survival time (days after initiation of the treatments). Median survival times: non-treated: 35 d; TPL (0.1 mg kg^{-1}): 44 d; TPL (0.2 mg kg^{-1}): 64 d; **2** (0.1 mg kg^{-1}): 43.5 d; **2** (0.2 mg kg^{-1}): 46 d; **2** (0.5 mg kg^{-1}): 79 d; **2** (1 mg kg^{-1}): 89 d ($n = 5$). TPL = triptolide.

compared to triptolide (Figure S13), it is likely that it was the active species responsible for the antiproliferative activity of glutriptolide **2**. It is possible that different types of cancer cells may have distinct esterases, leading to different profiles of metabolism of glutriptolide **2**.

In conclusion, glutriptolide **2** has shown unexpectedly sustained antitumor activity, keeping tumor cells under control weeks after cessation of administration, though the underlying mechanism remains to be elucidated. It was further shown to be among the most efficacious of all agents tested to date in this model. Should that hold true in humans, treatment with glutriptolide **2** could be readily integrated into standard anticancer therapy, wherein patients are often afforded structured treatment interruptions. Glutriptolide **2** represents a promising new lead that allows for the exploitation of the potent and mechanistically novel antitumor activity of triptolide through a combination of tumor targeting with the glucose transporter and specific inhibition of RNAPII-mediated transcription.

Acknowledgements

This work has been supported by a Synergy Award from Johns Hopkins School of Medicine (J.O.L. and M.G.P.), FAMRI (J.O.L.), the Liu laboratory discretionary fund, and the Johns Hopkins Institute for Clinical and Translational Research (ICTR), which is funded in part by Grant Number UL1 TR 001079. We thank Dr. Bert Vogelstein for generous provision of the isogenic DLD1 cell lines. We are grateful to Dr. Hanjing Peng and Brandon Peiffer for assistance and members of the Liu laboratory for helpful discussions.

Keywords: antitumor agents · cytotoxicity · glucose transporters · prostate cancer · triptolide

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Received: June 23, 2016
Published online: ■■ ■■, ■■■■

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Communications

Antitumor Agents

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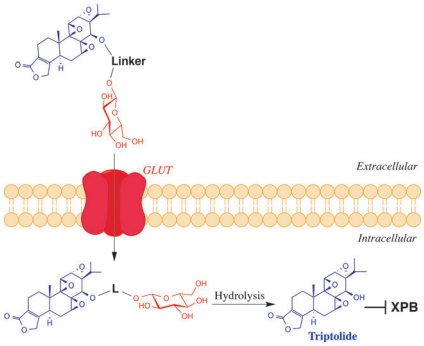
Targeted Delivery and Sustained Antitumor Activity of Triptolide through Glucose Conjugation



A glucose conjugate of the anti-inflammatory natural product triptolide, glutriptolide, was developed that selectively targets tumor cells overexpressing glucose transporters. Glutriptolide demonstrated significantly higher cytotoxicity against tumor cells than against normal cells and also benefitted from improved water solubility compared with triptolide.

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