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Podophyllotoxin analogues active versus *Trypanosoma brucei*

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ABSTRACT

In an effort to discover novel anti-trypanosomal compounds, a series of podophyllotoxin analogues coupled to non-steroidal anti-inflammatory drugs (NSAIDs) has been synthesized and evaluated for activity versus *Trypanosoma brucei* and a panel of human cell lines, revealing compounds with low nano-molar potencies. It was discovered that coupling of NSAIDs to podophyllotoxin increased the potencies of both compounds over 1300-fold. The compounds were shown to be cytostatic in nature and seem to act via de-polymerization of tubulin in a manner consistent with the known activities of podophyllotoxin. The potencies against *T. brucei* correlated directly with Log *P* values of the compounds, suggesting that the conjugates are acting as hydrophobic tags allowing podophyllotoxin to enter the cell.

The eukaryotic parasite *Trypanasoma brucei* is the causative agent of Human African Trypanosomiasis, a major health concern in much of sub-Saharan Africa.[1] Current treatments for this disease are highly toxic or suffer from poor pharmacokinetic properties which necessitate lengthy dosing regimens.[2] The need for novel therapeutics is further exacerbated by developing resistance to clinically used drugs.[3]

Previous work in developing anti-trypanosomal compounds targeted at the related parasite *Trypanosoma cruzi* showed that indomethacin amides were potent inhibitors of *T. cruzi* sterol 14α-demethylase (TCCYP51), an important enzyme in the sterol biosynthetic pathway.[4–6] These compounds were also active in in vitro whole cell assays. Since *T. brucei* shares this pathway and also possesses an active sterol 14α-demethylase, it was decided to test this compound series for anti-trypanosomal activity in this parasite as well.[7]

While most of the analogues were relatively inactive (IC_{50} > 10 μM) versus *T. brucei*, a small set of compounds (**1–3**) linked to podophyllotoxin showed very good potencies (IC_{50} values from 51 ± 6 nM to 5.3 ± 0.8 μM) as well as a preliminary structure–activity relationship.

The observed activities are unlikely to be a result of CYP51 inhibition, since the *T. cruzi* and *T. brucei* enzymes are highly homologous, and the observed SAR for these compounds is significantly different from that observed for trypanosomal CYP51 inhibitors.[4]

Following this discovery, the effects of podophyllotoxin (Fig. 1**B**) and indomethacin (**C**) as both single agents and in various fixed ratio combinations (1:1, 1:10, and 1:100 in both directions) were tested. The compounds were inactive (IC_{50} > 50 μM) under all tested conditions, indicating that this was not a simple case of synergy. While podophyllotoxin was inactive in *T. brucei*, it was active in transformed mammalian cell lines, with similar potencies to compound **1**, but not in primary human cell lines. (Table 1)

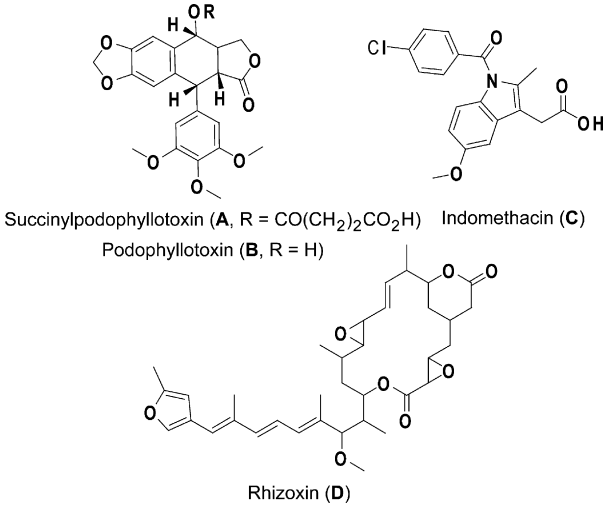

Figure 1. Strucutres of compounds **A**–**D**.

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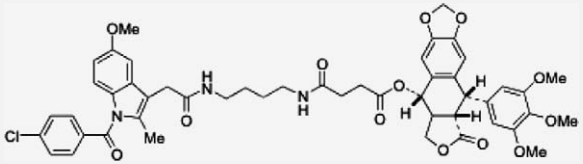
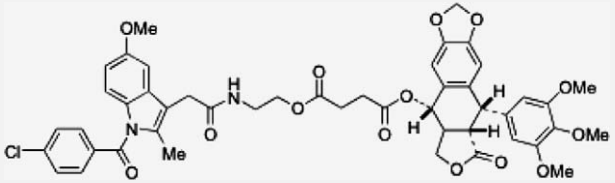
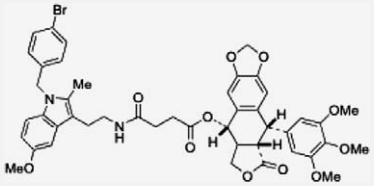
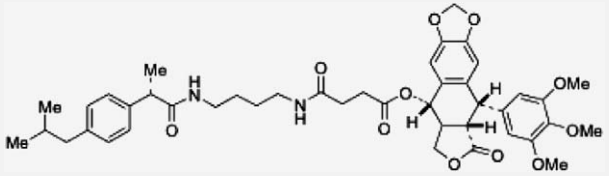
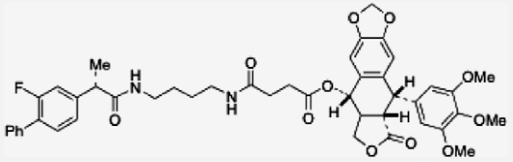
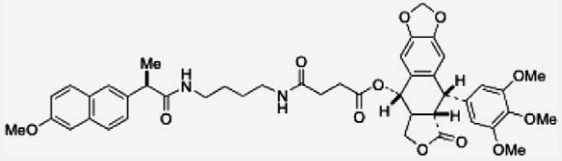
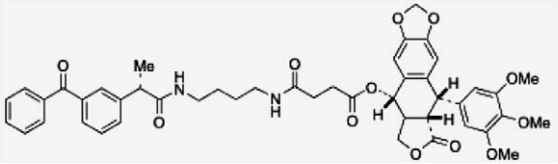
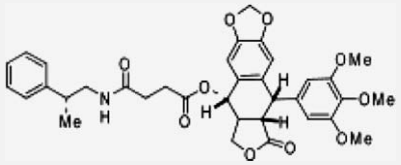
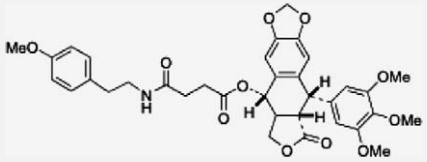
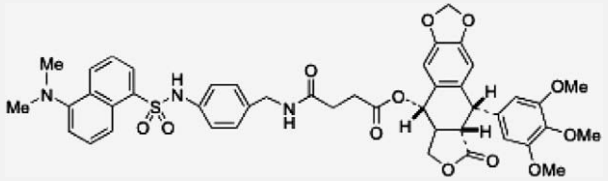
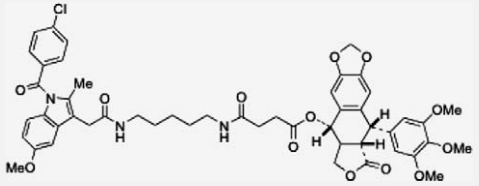
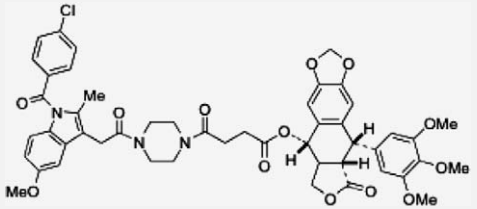
Table 1
Primary hits (**1**–**3**) NSAID COX inhibitor analogues (**4**–**7**), non-NSAID based analogues (**8**–**10**), and linker modifications (**11**,**12**) tested for anti-trypanosomal activities

No.	Compounds	*T. brucei* IC_{50}[a] (μM)	HEPG2 IC_{50}[a] (μM)	BJ IC_{50}[a] (μM)	% Tubulin inhibition[b]	COX-1 IC_{50}[c] (μM)	COX-2 IC_{50}[c] (μM)	$T_{1/2}$ plasma[d] (h)	*A* Log *P*
1		0.051 (0.006)	0.20 (0.06)	>50	60 (8)	>4	0.295	2.7 (0.1)	6.2
2		3.4 (0.2)	1.0 (0.2)	>50	NT	>4	0.254	NT	6.2
3		5.3 (0.8)	0.32 (0.09)	>50	NT	>4	>4	NT	6.8
4		0.46 (0.08)	0.22 (0.07)	>50	64 (8)	>4	>4	4.1 (0.3)	5.6
5		0.17 (0.01)	0.27 (0.1)	>50	NT	>4	>4	9.7 (0.6)	5.6
6		4.5 (0.4)	0.54 (0.36)	>50	NT	>4	>4	NT	4.8
7		>50	>50	>50	89 (9)	>4	>4	6.2 (0.4)	5.3

8		32 (12.4)	0.44 (0.22)	>50	74 (6)	>4	>4	NT	4.3
9		31 (12.7)	0.090 (0.061)	>50	NT	>4	>4	NT	3.8
10		27 (15)	0.028 (0.015)	>50	58 (5)	>4	>4	2.6 (0.1)	5.1
11		0.22 (0.02)	0.50 (0.23)	>50	NT	>4	0.147	2.9 (0.1)	6.6
12		0.99 (0.08)	0.71 (0.33)	>50	NT	>4	0.602	2.9 (0.2)	5.8
A	Succinylpodophyllotoxin	>50	0.17 (0.019)	>50	NT	>4	>4	NT	2.3
B	Podophyllotoxin	>50	0.024 (0.005)	>50	103 (0.8)	>4	>4	9.8 (0.4)	2.1
C	Indomethacin	>50	>50	>50	NT	0.05	0.75	NT	4.2
D	Rhizoxin	0.15 (0.2)	0.40 (0.19)	>50	102 (2)	NT	NT	NT	5.5

(NT = not tested).

[a] Values are expressed as the mean of three experiments with standard deviation given in parentheses. HEPG2 is a liver carcinoma cell line while BJ are normal human foreskin fibroblasts. The IC_{50} values for pentamidine and eflornithine versus *T. brucei* under the tested conditions were 25 ± 10 nM and 243 ± 32 μM, respectively.

[b] Values are expressed as the mean of four experiments with standard deviation given in parentheses. Compounds were tested at a single concentration of 10 μM for their ability to inhibit tubulin polymerization.

[c] IC_{50} values were determined by incubating several concentrations of inhibitors in DMSO with purified murine COX-2 (63 nM) and ovine COX-1 (22.5 nM) for 20 min followed by treatment with 1-^{14}C-AA (50 μM) at 37 °C for 30 s. Assays were run in duplicates and IC_{50} values are reported as the mean of both experiments. IC_{50} values for control COX inhibitors are as follows: Celecoxib (COX-1 IC_{50} = >4 μM, COX-2 IC_{50} = 0.21 μM) Rofecoxib (COX-1 IC_{50} > 4 μM, COX-2 IC_{50} = 0.15 μM).

[d] Values for half-life in mouse plasma are expressed as the mean of three experiments with standard deviation given in parentheses.

Indomethacin did not show toxicity in any of the cell lines tested. Following these observations, further experiments aimed at deconvoluting the mechanism of action of compound **1** were performed.

Compound **1** was shown to be reversibly cytostatic in *T. brucei* and mammalian cell lines with similar potencies in both. (Fig 2, Table 1) However, when tested against primary human mammary epithelial or foreskin fibroblast cell lines, **1** appeared completely non-toxic. Since podophyllotoxin, a tubulin destabilizing agent, is known to be a reversible cytostatic compound the effects of **1** on *T. brucei* tubulin was evaluated. Staining of *T. brucei* in the presence of 1 μM compound **1** with Tubulin Tracker (Molecular Probes, Eugene, OR), a fluorescently labeled taxol analogue which binds to polymerized tubulin, showed that the test compound caused a decrease in the amount of polymerized tubulin present in the cell. Rhizoxin (**D**), a microtubule toxin known to be active in *T. brucei* displayed similar potencies (IC_{50} = 150 ± 20 nM) and phenotypes in these assays. After 24 h of exposure to either **1** or **D**, the cells became rounded and began displaying aberrant nuclei counts. (Fig 3) This phenotype has been reported for 100 nM rhizoxin and has been interpreted as an indication of inhibition of microtubule dependent cellular processes.[8] These results suggest that the compound is acting via the classical podophyllotoxin mechanism in which polymerization of the microtubules is inhibited.[9]

This caused us to hypothesize that indomethacin was increasing the import of podophyllotoxin into the cell. Indomethacin is a non-steroidal anti-inflammatory drug (NSAID) which works by inhibiting the cyclooxygenase enzymes (COX-1 and COX-2), which are not present in *T. brucei*.[10,11] Therefore, it is unlikely that the NSAID was acting in COX dependent manner. A second possible mechanism for the increased uptake is the sterol pathway. *T. brucei* is known to possess high affinity transporters for low density lipoprotein (LDL) and it is possible that the linked NSAID could be acting as a cholesterol mimetic and entering the cells via this pathway.[12]

To determine if the observed increase in potencies was NSAID dependent, a set of podophyllotoxin analogues conjugated to various NSAIDs as well as non-NSAID groups were synthesized. The effects of varying the linker were also evaluated in this effort.

Synthesis of the test library of podophyllotoxin conjugates was performed using liquid phase parallel synthetic methodologies, where succinyl-podophyllotoxin was reacted with tethered NSAIDs or other moieties containing terminal amino-groups. The succinylpodophyllotoxin (**A**) was synthesized from a reaction of succinic anhydride and podophyllotoxin. Tethered NSAIDs containing a terminal amino-group were synthesized from the reaction of carboxylic acid containing NSAIDs with BOC-protected alkyldimine or pyperzinyl, or aminomethylaniline in the presence of ethyl-1-{3-(dimethylamino)propyl}-3-ethylcarbodiamide (EDCI) followed by treatment with HCl gas. Compounds were purified via LC/MS and Prep-HPLC. Structures were assigned by NMR and mass spec. analysis. All compounds were tested in cytotoxicity assays versus *T. brucei*, HEPG2, HEK293, BJ, and Raji cell lines. In addition, compounds were tested for their ability to inhibit COX-I and COX-II enzymes as well as tubulin polymerization.

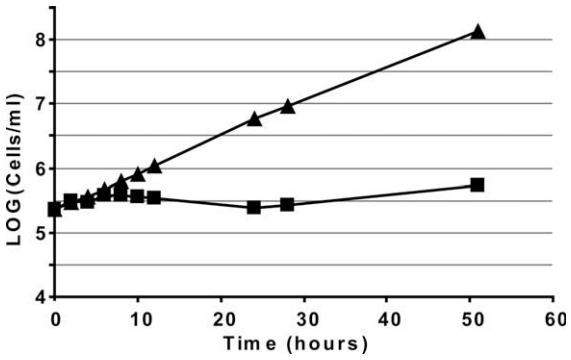


Figure 2. *T. brucei* growth curve of 1 μM **1** (■) and DMSO control (▲). Cells were counted manually by haemocytometer. Values are a mean of three experiments.

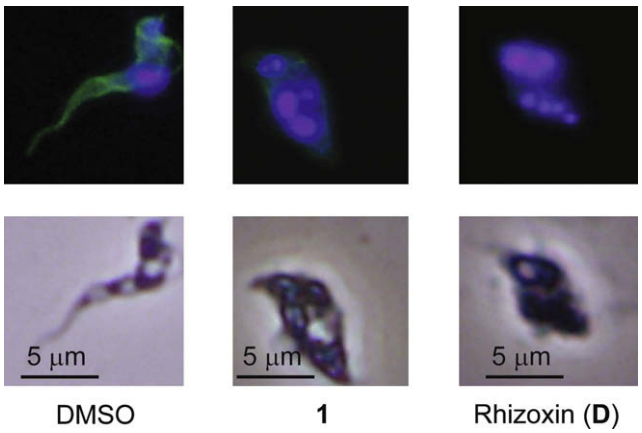


Figure 3. Phase contrast and fluorescence images of *T. brucei* showing the effect of **1** (1 μM, 24 h exposure) and **D** (100 nM, 24 h exposure) on tubulin polymerization and DNA content. Green fluorescence indicates polymerized tubulin as determined by Tubulin Tracker™ while blue is Hoescht stained DNA.

After testing, it was clear that compounds containing NSAID groups (**4**–**7**) were more potent against *T. brucei* than those without NSAIDs (**8**–**10**). However, with the exception of some indomethacin containing compounds (**1**, **2**, **11**, **12**), most of the compounds were unable to directly inhibit purified human COX enzymes. This suggests that if a NSAID binding target in *T. brucei* is responsible for the observed activities, the pharmacophore necessary for activity is substantially different than that for mammalian COX enzymes. All of the tested compounds displayed significant selectivity (>100-fold) for *T. brucei* and transformed human cell lines versus primary fibroblast and epithelial human cells.

The modest set of linker modifications explored (**1**, **2**, **11**, and **12**) show that some modifications are tolerated. The lengthening by a single methylene group (**11**), only lowers potency fourfold against *T. brucei* while the piperizine linker (**12**) lowers potency 20-fold. Shortening the linker and substituting an ester linkage for the amide (**2**), lowers potency by 60-fold. Compounds **4** and **8**, also follow this pattern.

Since the linkers used in this study contained ester and amide moieties, a subset of the compounds were tested for stability in simulated gastric fluid (SGF, pH 1) as well as stability in mouse plasma. All tested compounds displayed $T_{1/2} > 24$ h in SGF. Half-lives in plasma ranged from 2.6 to 9.8 h. Plasma $T_{1/2}$ was affected more by the NSAID moiety than linker type, indicating that stable compounds based on this scaffold may be achievable. (see compounds **1**, **5**, **10**–**12**).

The ability of the compounds to inhibit tubulin polymerization did not correlate with anti-trypanosomal potencies, suggesting that cellular uptake was driving the observed differential activities. In support of this, it was observed that the potencies against *T. brucei* directly correlated with *A*Log *P* values. ($P < 0.001$) *T. brucei* possesses an extremely high rate of membrane turnover, recycling the cellular surface once every 12 min.[13] If the hydrophobic conjugates are interacting directly with the cellular membrane, this could result in the rapid uptake and concentration of the compounds.

In summary, we have reported the ability of NSAID motifs conjugated to podophyllotoxin to increase the potency of both compounds against *T. brucei* by over 1300-fold. This phenomenon is not a result of synergy of the two compounds and is hypothesized

to be the result of increased cellular uptake, either via passive diffusion or via active uptake through the LDL import pathway. However, it remains a possibility that these compounds are acting through other mechanisms, such as inhibition of topoisomerase II, a known target of other podophyllotoxin analogues. Though the use of drug conjugates has been previously reported in other cases, it is extremely rare for the combination of two inactive compounds to result in such dramatic increases in potency. Efforts to further characterize this phenomenon are currently underway in our laboratory.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.01.009.

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