In Vitro Evaluation of *s*-Triazine Derivatives for African Trypanosomiasis

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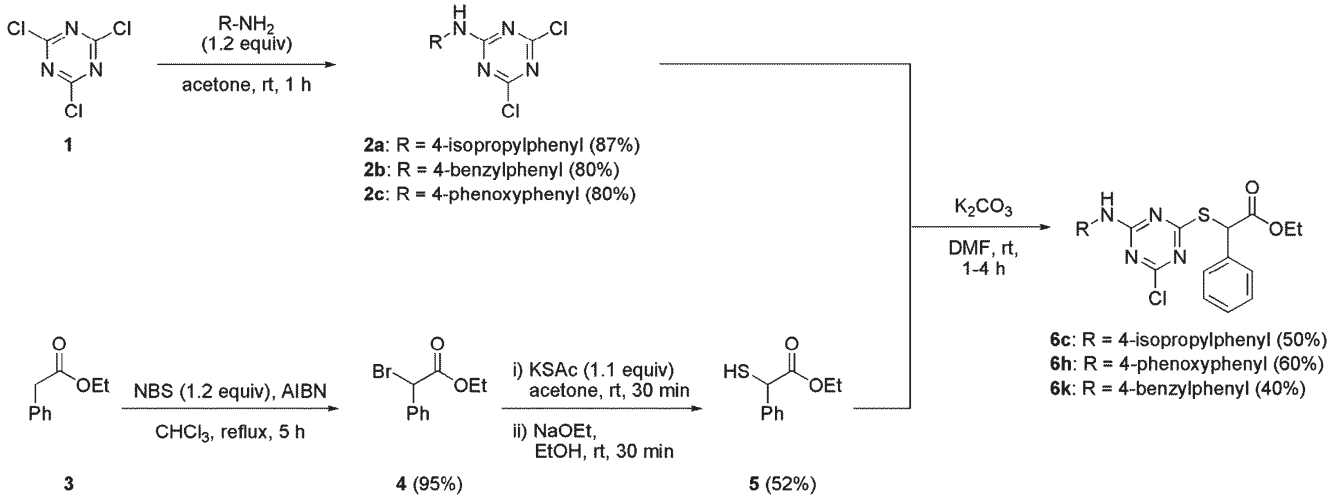
African trypanosomiasis (sleeping sickness) is one of the neglected tropical diseases in vertebrates caused by protozoa of the species *Trypanosoma brucei*.[1] Many countries of sub-Saharan Africa suffer from human African trypanosomiasis, which is caused by *Trypanosoma brucei gambiense* (*T. b. gambiense*) and *Trypanosoma brucei rhodesiense* (*T. b. rhodesiense*).[1–3] The other subspecies *Trypanosoma brucei brucei* (*T. b. brucei*) causes animal African trypanosomiasis along with several other species of trypanosome and is not human infective due to its susceptibility to lysis by trypanosome lytic factor-1 (TLF-1).[4] As *T. b. brucei* is closely related to and shares fundamental features with the human infective subspecies,[5] it is used as a model for human infections in laboratory and animal studies. Transmission of *T. brucei* between mammal hosts is usually by an insect vector, namely the tsetse fly (*Glossina* genus). More importantly, *T. brucei* is one of the very few pathogens that can cross the blood–brain barrier.[6] There is an urgent need for the development of new drug therapies, as the drugs currently available for clinical use have limitations such as drug resistance and toxicity.[7,8]

Several derivatives of *s*-triazine show antimicrobial,[9] antibacterial,[10] antitumor,[11] anti-HIV,[12] and herbicidal activities.[13] We have previously reported the potent PGE_2 production inhibition of our *s*-triazine derivatives as potential inhibitors of microsomal prostaglandin E synthase-1 (mPGES-1).[14] Based on the various biological activities of *s*-triazine nucleus,[12,15] we became interested in testing our *s*-triazine compounds against *T. b. brucei*. Here, we present the results of the antiprotozoal activity of our in-house prepared *s*-triazine compounds as well as the additional synthesis of new *s*-triazine derivatives.

Three new compounds (**6c**, **6h**, and **6k**) included in Table 1 were synthesized according to a reported procedure by our group, as shown in Scheme 1.[14]

Initially, cell viability was assessed with MTT assay based on the experimental procedurbes described previously.[17] Three new synthetic compounds (**6c**, **6h**, and **6k**) did not exhibit any cytotoxic effects against RAW 264.7 cells at concentrations above 10 μM in the presence or absence of LPS (data not shown), indicating that their suppressive effects on PGE_2 production could not be attributed to nonspecific



Scheme 1. Synthesis of new *s*-triazine derivatives.

Correction added on 01 October 2015, after first online publication: ISSN (Print) has been corrected.

cytotoxicity. Therefore, they were screened for their ability to inhibit PGE_2 production in LPS-induced RAW 264.7 cells at 0.1 μM concentration using NS398 as a positive control. PGE_2 concentration in the medium was measured using an enzyme immunoassay (EIA) kit for PGE_2 according to the manufacturer's recommendation.[18] All experiments were carried out at least twice, and these new compounds (**6c**, **6h**, and **6k**) exhibited weak or moderate inhibitory activity of PGE_2 production (15.6%–30.5% inhibition at 0.1 μM concentration) compared to the previous compounds at the given concentrations (Table 1).

Next, all synthetic compounds were tested *in vitro* against *T. b. brucei* strain 427 using the resazurin assay,[19] which demonstrates cell viability. Pentamidine was included in the assays as a positive control for *T. b. brucei*. In this evaluation, the activity was expressed as EC_{50} (half maximum effective concentration on parasite growth). *In vitro* cytotoxicity was also evaluated toward both a human embryonic kidney cell line (HEK293T) and a human liver carcinoma cell line (HepG2) using chlorpromazine as a positive control. Antiprotozoal activity against *T. b. brucei* and cytotoxicity data of all tested compounds are given in Table 1.

In general, all *s*-triazine compounds showed acceptable antiprotozoal effects on *T. b. brucei* strains 427 (EC_{50} = 5.51–13.60 μM) independent of their inhibitory activities of PGE_2 production, but their cytotoxicity toward two human cell lines exhibited a linear relationship with their PGE_2-related activities, in accordance with the inextricable link between inflammation and cancer.[20] This overall result suggests that the PGE_2-related activity was not predictive of *in vitro* antiprotozoal activity. Of the compounds, **6e** bearing 4-cyclohexylpheyl and 1-naphthyl groups on R^1 and R^3 positions, respectively, was found to be the most active compound (EC_{50} = 5.51 μM) and also the least toxic one of the series (CC_{50} = >200 μM), with T.I. (therapeutic index) value of ~36.

Three compounds showing high cytotoxicity (**6c**, **6h**, and **6k**), as well as the active compound **6e,** were further screened on *Leishmania donovani*-infected THP-1 macrophage cells (a human acute monocytic leukemia cell line) and *T. cruzi*-infected U2OS cells (a human osteosarcoma cell line) according to the reported procedure by our research team.[21] Amphotericin B and benznidazole were used as positive controls, respectively, for activity against each intracellular parasites (Table 2). Cytotoxicity was measured by counting host cell number in an image-based assay. Most of compounds displayed weak or no parasite inhibition against *L. donovani*-infected THP-1 cells, with less cytotoxicity in host THP-1 macrophage cells compared to amphotericin B. Compound **6h** was only compound that showed *L. donovani* clearance in host macrophage cells without any cytotoxicity (Figure 1). In the case of *T. cruzi*-infected U2OS cells, on the other hand, three compounds except **6e** showed strong parasite inhibition with significant cytotoxicity in host U2OS cells compared to benznidazole, indicating that their antiparasite activities could be attributed to nonspecific cytotoxicity on the host cells. Interestingly, compound **6e** was found to be inactive against two parasites and also not cytotoxic up to 100μM in two host cells when compared with its data against *T. b. brucei*. Taken together, these data showed that not all trypanosomatids are sensitive to *s*-triazine derivatives, and not all *s*-triazine derivatives are equally active on sensitive parasites. Overall, compound **6e** was found to be specifically active *in vitro* against *T. b. brucei* without serious cytotoxicity on human cell lines and thus can be considered a hit compound for African trypanosomiasis.

In summary, a set of *s*-triazine derivatives as potential mPGES-1 inhibitors were evaluated for their *in vitro* antiparasite activities against three parasites, namely *T. b. brucei*, *T. cruzi,* and *L. donovani*. Compound **6e** exhibited an EC_{50} of 5.51μM against only *T. b. brucei* and lower toxic effect on two human cell lines (CC_{50} = >200 μM). Therefore, *s*-triazine ring could be a useful scaffold for the discovery of novel trypanocidal drug candidates without serious toxic effects. Accordingly, future work will address the intensive structure–activity relationship (SAR) of *s*-triazine derivatives with a goal to increase the efficacy. Studies on the mechanism of action of the *s*-triazine derivatives against *T. b. brucei* are under way and will be reported in the future.

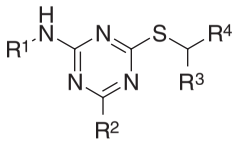
Experimental

The Inhibitory Activity Assay of COX-2-catalyzed PGE_2. The RAW 264.7 macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). Cells were grown at 37 °C in DMEM supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin sulfate (100 μg/mL) in a humidified 5% CO_2 atmosphere. Cells were incubated with the tested samples at increasing concentrations or positive control chemical (NS398) and then stimulated with LPS 1 μg/mL for the indicated time. PGE_2 concentration in the medium was quantified using EIA kits (R&D Systems, Minneapolis, MN, USA).

***T. brucei brucei* Activity Assays.** *T. b. brucei* strain 427 bloodstream forms were cultivated at 37 °C with 5% CO_2 in HMI-9 medium supplemented with 10% FBS. Assay was performed in 384 multiwell plates with 2500 cultivated parasites in a well with pre-prepared compounds. Compounds were exposed for 72 h and 120 μM of resazurin sodium salt (Sigma-Aldrich, St. Louis, MO, USA) and were treated for additional 5 h. After incubation, the parasites were fixed with 4% paraformaldehyde (Fisher Scientific Co., Fair Lawn, NJ, USA) and the assay plates were read by a Victor 3 plate reader (PerkinElmer, Inc., Waltham, MA, USA) at 530 nm_{Ex}/590 nm_{Em}. Pentamidine was used as a drug-positive control, and 0.5% of DMSO was used as a drug-negative control.

Cytotoxicity Assays. HEK293T and HepG2 cells lines were used to evaluate cytotoxicity. Both cell lines were cultured at 37 °C with 5% CO_2 in Dulbecco's Modified Eagle Medium containing 10% FBS. Four thousand HEK239T and 2000 HepG2 cells were seeded individually in 384 multiwell plates and incubated for 72 h with compounds at twofold dilution from 100 μM in 10-point concentration. After incubation, cells were exposed to 40 μM of resazurin sodium salt

Table 1. *In vitro* activity of *s*-triazine derivatives against PGE_2 production and *T. b. brucei* strain 427, and their cytotoxicity.



Entry	R^1	R^2	R^3	R^4	PGE_2 %inhibition @ 10 μM[a]	*T. b. brucei* EC_{50} (μM)[b]	HEK293T[c] CC_{50} (μM)[e]	HEK293T[c] TI[f]	HepG2[d] CC_{50} (μM)	HepG2[d] TI
6a	4-Isopropylphenyl	Me	Ph	CO_2Me	85.65	8.12	25.78	3.17	49.32	6.07
6b	4-Isopropylphenyl	Cl	Ph	CO_2Me	91.81	13.60	10.26	0.75	19.15	1.41
6c	4-Isopropylphenyl	Cl	Ph	CO_2Et	30.46 (0.1)	9.96	4.01	0.40	9.89	0.99
6d	4-Isopropylphenyl	Cl	1-naphthyl	CO_2Me	88.69	8.43	15.47	1.84	21.02	2.49
6e	4-Cyclohexylphenyl	Cl	1-naphthyl	CO_2Et	43.68	5.51	>200.00	>36.3	>200.00	>36.3
6f	4-Biphenylyl	Cl	1-naphthyl	CO_2Et	78.56	6.26	73.24	11.7	112.66	18
6g	4-Phenoxyphenyl	Cl	Ph	CO_2Me	85.77	8.41	14.04	1.67	22.49	2.67
6h	4-Phenoxyphenyl	Cl	Ph	CO_2Et	23.47 (0.1)	7.10	4.28	0.60	14.20	2.00
6i	4-Phenoxyphenyl	Cl	1-naphthyl	CO_2Me	72.46	9.37	24.38	2.60	39.40	4.20
6j	4-Benzylphenyl	Cl	Ph	CO_2Me	90.81	8.72	13.97	1.60	27.75	3.18
6k	4-Benzylphenyl	Cl	Ph	CO_2Et	15.61 (0.1)	7.68	4.56	0.59	14.60	1.90
6l	4-Benzylphenyl	Cl	1-naphthyl	CO_2Et	72.60	7.11	43.86	6.17	104.05	14.63
6m	4-Isopropylphenyl	Me	Ph	$CONH_2$	26.50 (1)	7.81	23.12	2.96	26.98	3.45
NS398[g]					99.42 (3)					
Pentamidine[h]						0.004 (6.43)[i]			(159.71)[i]	(24.84)[i]
Chlorpromazine[j]							13.57		9.88	

[a] %PGE_2 production inhibition in LPS-induced RAW 264.7 macrophage cells at given concentration.
[b] EC_{50}: half maximal effective concentration on bloodstream form of *T. b. brucei*.
[c] HEK293T: a human embryonic kidney cell line.
[d] HepG2: a human liver carcinoma cell line.
[e] CC_{50}: half maximal cytotoxic concentration.
[f] Therapeutic Index (TI) = CC_{50}/EC_{50}.
[g] Positive control for PGE_2 production inhibition.
[h] Positive control for *T. b. brucei* and its EC_{100} = 120 nM.
[i] Reported data: Ref. [16].
[j] Positive control for cytotoxicity and its CC_{100} = 83.0 μM.

Table 2. *In vitro* activity of *s*-triazine derivatives against amastigote forms of *L. donovani* and *T. cruzi*.

	L. donovani			*T. cruzi*		
Compound	EC_{50} (μM)[a]	CC_{50} (μM) of THP-1[b]	TI[c]	EC_{50} (μM)[d]	CC_{50} (μM) of U2OS[e]	TI
6c	86.1	87.1	1	4.64	3.20	0.7
6e	>100	>100	N/A[f]	>100	>100	N/A
6h	45.2	>100	>2.2	7.89	3.65	0.5
6k	>100	>100	N/A	2.18	2.69	1.2
Amphotericin B[g]	0.31					
Benznidazole[h]				73.2		

[a] EC_{50}: Half maximal inhibitory concentration on intracellular *L. donovani* in THP-1 cells (a human acute monocytic leukemia cell line).
[b] CC_{50}: Half maximal cytotoxic concentration against host THP-1 cells.
[c] Therapeutic Index (TI) = CC_{50}/EC_{50}.
[d] EC_{50}: Half maximal inhibitory concentration on intracellular *T. cruzi* in U2OS cells (a human osteosarcoma cell line).
[e] CC_{50}: Half maximal cytotoxic concentration against host U2OS cells.
[f] N/A: not available.
[g] Positive control for *L. donovani*.
[h] Positive control for *T. cruzi*.

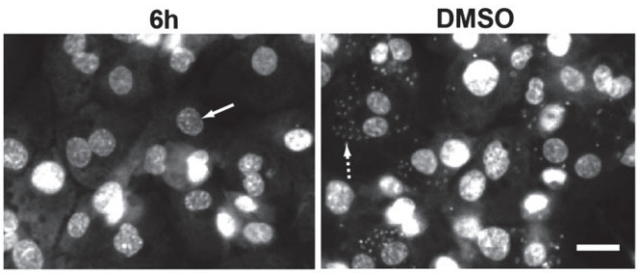


Figure 1. Confocal microscopy images of intracellular *L. donovani* treated with compound **6h**: Treatment with 50 μM of compound **6h** (left) showed complete parasite clearance from the host cells (arrow), while 1% DMSO (right) showed high number of parasites (dotted arrow) inside. The fluorescent DNA dye DRAQ5 was used to visualize host cells and parasites. The solid arrow indicates nucleus of THP-1 cells and the dotted arrow indicates *L. donovani* in cytosol. Scale bar, 50 μm.

(Sigma-Aldrich) for 5 h to allow resazurin reduction by aerobic respiration. Then, 4% paraformaldehyde was added to fix the cells, and the assay plates were analyzed in a Victor 3 plate reader at 530 nm_{Ex}/590 nm_{Em}. Chlorpromazine was used as a drug-positive control and 0.5% of DMSO was used as a drug-negative control.

Statistical Analysis. Dose–response curves were fitted by sigmoidal dose–response with variable slope. The equation described as $Y = \text{bottom} + (\text{top} - \text{bottom})/(1 + 10^{(\text{LogEC50}-X)\times\text{HillSlope}})$ using GraphPad Prism 6 Software (GraphPad Software, San Diego, CA, USA).

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