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Design, Synthesis, and Testing of Antiprotozoal Activity of Primin and Analogues

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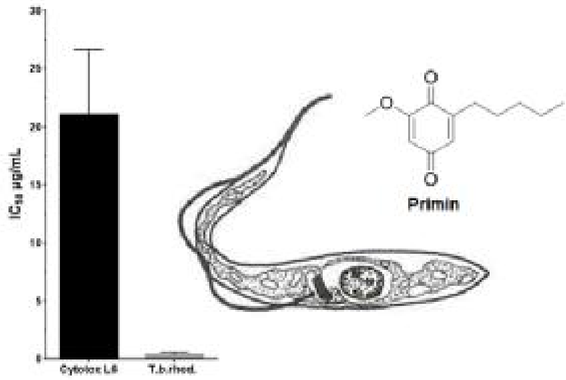
ABSTRACT

Conformationally restricted analogues of the natural product primin were synthesized as potential antiprotozoal agents. The synthesis utilizes quinone C-H functionalization methods to enable an efficient and easy access to primin analogues. The antiprotozoal activities of this series were evaluated in a panel of parasites and compared to the natural product primin. For all the synthesized primin analogues a potent in vitro activity was found against the pathogen *Trypanosoma brucei rhodesiense* (IC_{50}<0.05 µg/mL). The observed antiprotozoal activity is not related to the production of reactive oxygen species (ROS). Initial results of the in vivo experiments with a *T. b. rhodesiense* rodent animal model of the human disease were also reported. Intraperitoneal injection administration of compound **7** resulted in complete clearance of *T. b. rhodesiense* in the tested rodent animals 24 hours after the last treatment. Our results show that the primin scaffold represents a new scaffold for further development of potent inhibitors of *Trypanosoma brucei rhodesiense*.

GRAPHICAL ABSTRACT



1. Introduction

Primin (**1**) which is a natural product is, first of all, isolated from surface extracts of Primula obconica[1]. This natural product and related methoxy-1,4-benzoquinones, verapliquinone A (**2**)[2] griffithane D (**3**)[3], SAN5201 (**4**)[4] and betulinan A (**5**)[5] (**Fig.1**) have been reported to demonstrate a wide range of biologically interesting properties. Primin (**1**) in particular reveals significant antibacterial and antitumor activities[6].In addition, primin has been reported to exhibitantiprotozoal activity[7] .

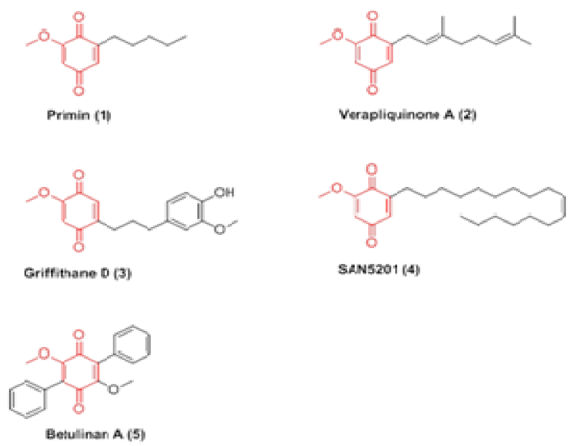


Fig.1. Structure of exemplary methoxy 1,4-benzoquinone containing natural products; primin (**1**)[1], verapliquinone (**2**)[2], griffithane D (**3**)[3],

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SAN5201 (**4**)[4] and betulinan A (**5**)[5]. Red color denotes the methoxy benzoquinone scaffold.

According to the World Health Organization, infectious diseases caused by protozoan parasites such as human African trypanosomiasis, Chagas' disease, Leishmaniosis and malaria remain a major problem in tropical and subtropical countries. There is a pressing need for new chemical entities, which can be further developed in antiprotozoal agents due to the toxicity and inefficacy of currently used treatments as well as the development of resistance. In this regard, natural products and compounds derived from natural products represent a promising starting point for medicinal chemistry[8,9] and have played a pivotal role in the development of antiprotozoal agents[10]. The scope of this letter is to use primin as a starting point for the design and synthesis of a primin-derived set of compounds and test their antiprotozoal activity.

Due to thesynthetic challenges of primin, only limited studies have been reported on the structure-activity relationship (SAR) of this natural product[11]. The reported modifications were only limited to the length of the pentyl- side chain[12,13]. Recently, we developed a one-step synthesis of primin (**1**) and iso-primin (**6**) utilizing a C-H functionalization methodology[14]. This highly concise synthetic route allows the quick and efficient access to primin and its analogues for biological evaluation. Following this strategy, a focused set of conformationally restricted primin derived compounds (**7**-**10**) was designed, synthesized and characterized by NMR spectroscopy (**Fig.2**). All compounds were tested against several parasite panels. All tested compounds showed activities in the low µg/ml range. Compound **9** was the most active compound, with comparable activity to the positive control melarsoprol.

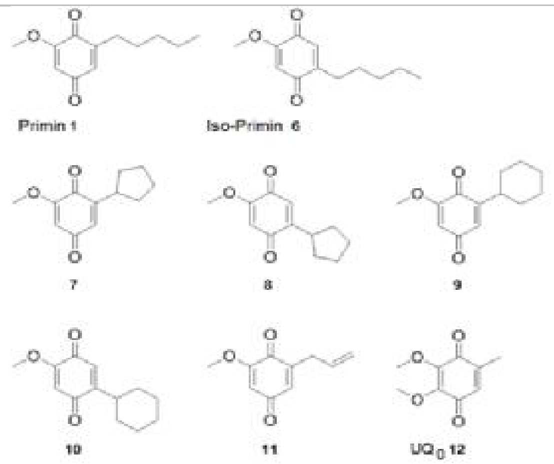
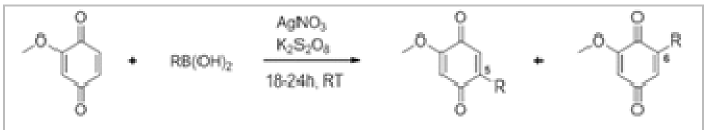


Fig. 2. Structures of the benzoquinones and primin analogues (7-10) used in this study

Primin analogues (**7**-**10**) were synthesized following the reaction route presented in scheme 1. 2-methoxy-1,4-benzoquinone was directly functionalized with cycloalkyl groups. The cyclopentyl- and cyclohexyl derivatives were designed as conformationally restricted side chain analogues of primin.



Scheme1. Synthetic route of primin analogues.

The functionalization occurs at C-5 or C-6 position of the precursor 2-methoxy-1,4-benzoquinone, producing a mixture of both isomers. Previously, 1,2 ADEQUATE NMR was applied to unambiguously assign the isomers[14]. In this study, we demonstrate that ^{1}H 1-D-NMR experiments are suitable to confirm the constitutions of the synthesized analogues and distinguish between C-5 and C-6 isomers. This assignment is demonstrated for compounds **7** and **8** in Figure 3.

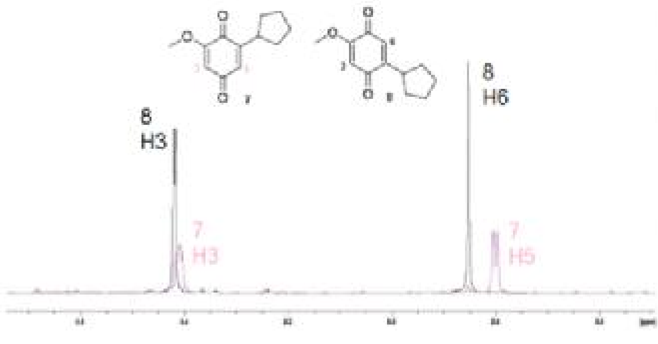


Fig. 3. ^{1}H-NMR Spectra of C6 (compound 7) and the C5 (compound 8) isomers.

While the proton H6 in compound **8** reveals a singlet around 5.85 ppm, the proton H5 of compound **7** has a doublet at 5.8 ppm. Additionally, the proton H3 in **8** has roughly the same chemical shift as theH3 proton in compound **7** (~6.4 ppm) but differs in its multiplicity. Vitamin UQ_0 and compound **11** were also included in this study. Compound **11** was synthesized according to a literature procedure by Saa et al[15].

Primin, isoprimin and compounds **7-12**were screened in vitro against the protozoa *T. brucei rhodesiense* (Tbr), *T. cruzi* (Tc), *L. donovani* (Ld), and *P. falciparum* (Pf) and for cytotoxicity towards the rat skeletal myoblast cell line L6. As summarized in Table 1, all the tested compounds show potent activity against *T. brucei rhodesiense*. Iso-primin and the analogues **7-12**synthesized in this workrevealed higher growth inhibition compared to the actual natural product primin.Compounds **7-10** have a much higher selectivity than primin (selectivity index, SI 270, 843, 246, 4433). Isoprimin, **7**, **9**also exhibited selective activity against *L. donovani*. The tested compounds were not selective active against *T. cruzi* and *P.falciparum*.

Table 1. In vitro antiprotozoal and cytotoxic activity of primin, isoprimin and compounds **7**-**12**. $^{a}IC_{50}$ values reported are the average of two independent assays, individual values varying less than ±50%; ^{b}SI selectivity index (IC_{50} L6/IC_{50} parasite); Positive controls: melarsoprol (Tbr), benznidazole (Tc), miltefosine (Ld), chloroquine (Pf), podophyllotoxin (Cytotox.L6).

	$^{a}IC_{50}$ [µg/ml] (SI)^{b}				
	Tbr	Tc	Ld	Pf	L6
PRIMIN	0.372 (57)	40.3	2.33	6.04	21.1
ISO-PRIMIN	0.064 (86)	12.8	0.496 (11)	1.71	5.53
7	0.006 (843)	10.5	0.168 (30)	0.91	5.06
8	0.019 (270)	12.6	1.27	1.63	5.12
9	0.003 (4433)	11.8	0.205 (64)	1.95	13.3
10	0.019 (246)	12.6	1.07	1.02	4.67
11	0.022 (80)	4.33	0.198	0.849	1.76
12	0.05 (58)	4.88	0.923	0.581	2.88
pos. Ctrl	0.003	0.664	0.061	0.003	0.006

Compounds**7** and **8** were selected for further studies. Both compounds have good physicochemical properties with low molecular weight (206 g/mol) and low polar surface area (PSA = 43 Å^2) and good predicted delivery with good brain-blood barrier penetration[16]. Both compounds were selected for in vivo evaluation in *T.brucei rhodesiense* infected mice. Both compounds did not cure infected mice at an ip dose of 50 mg/kg administered on 4 consecutive days. Compound **7** was able to reduce the parasitaemia below detection limit 24 hours after the last treatment in two out of 4 infected mice. The mice relapsed two days later. The mice treated with compounds **8** showed similar parasitaemia as the untreated control24 hours after the last treatment.

As described in the literature, the antiprotozoal activity of several identified natural products is associated with the generation of reactive oxygen species (ROS)[17,18]. Inhibitors with a ROS-based mechanism of action, however, are deemed to be unspecific compounds (also called PAINS; Pan assay interference compounds). The negative issue with PAINS has been raised by several groups and there is a general awareness to avoid these types of inhibitors[19,20]. The fact that the tested compoundsin this studyshow a parasite specific selectivity and low toxicity speaks against an unspecific PAIN-type inhibition. Nevertheless, we set-up an in vitro assay to measure the ability of tested compounds to produce hydrogen peroxide via an ROS based mechanism.
The assay monitors the formation of hydrogen peroxide by the methoxy-benzoquinones upon reduction with dithiothreitol (DTT)[21]. The amount of the produced hydrogen peroxide is coupled to the oxidation of phenol red via horseradish peroxidase enzyme and quantified by the detection of light absorbance at 610nm (Fig.4). Menadione, a known ROS generating compound, was used as a small molecule control (SC).

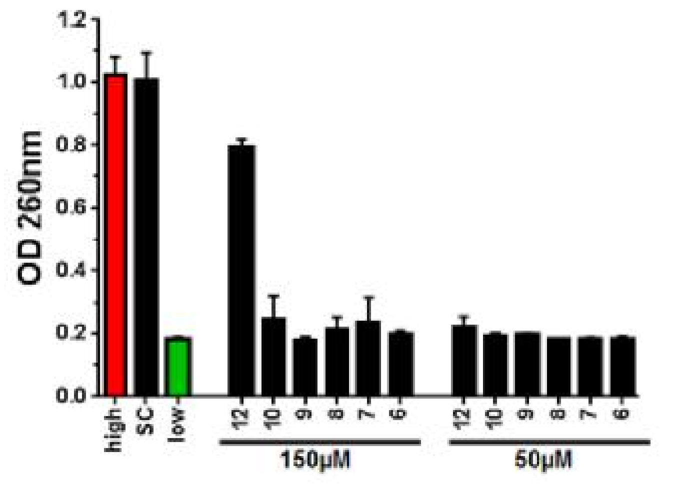


Fig. 4. Testing of primin analogues 6-12for their ability to produce ROS by using an in vitro colorimetric assay. High control: 100μM hydrogen peroxide; low control: DMSO and small molecule control (SC): 40μM menadione. Compounds were tested at two different conventions (50μM and 150μM) in replicates

None of the primin analogues with desired antiprotozoal activity generated hydrogen peroxide at a concentration of 50μM and therefore a ROS based mechanism can be excluded.

In summary, for a better understanding of theSAR of primin, novel analogues with substitutions at the C-5 and C-6 position were synthesized following a concise synthesis route. Biological activities were evaluated by being tested against a panel of parasites. The parasite *Trypanosoma brucei* is the cause of human African trypanosomiasis and transmitted by the tsetse fly. The disease causes the infected person to fall asleep and lethal. Clinical application of existing drugs is limited due to severe side effects, low efficacy and high cost. We have used primin as a starting point in the search for novel chemical matter.

The experimental details for the synthesis of primin analogues and the biological assays can be found in reference and notes[22].

Acknowledgments

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22. *Caution*: Primin (**1**) and its analogues are skin sensitizers. The contact with skin should be avoided. 2-Methoxy-1,4-benzoquinoe and quinone (12) were commercially available. *General synthesis procedure*: This C-H direct functionalization of quinones was first described by Baran as a scalabe reaction, which procceds at room temperature in an open flask.[23] 0.5g (3.6mmol) 2-methoxy-1,4-benzoquinone and the corresponding boronic acids (1eq. 3.6mmol) were dissolved in dichloromethane (DCM). To this reaction mixture 0.12g (0.72mmol, 0.2 eq) Silver nitrate in 18mL water and 1.9g (7.2 mmol, 2eq) potassium persulfate in 11mL DCM was added. The reaction mixture was stirred at room temperature for 18h. For the workup, 20mL DCM was added and the organic phase washed with 5% hydrogen carbonate solution. The product was purified on silica chromatography using hexane:ethylacetate as eluent. **Compound 7: ^{1}H – NMR:** (400 MHz, $CDCl_3$): δ [ppm] = 6.42 (s, 1H, C[3]), 5.85 (s, 1H, C[5]), 3.71 (s, 3H, C[7]), 3.02 (q, 1H, C[1‘]), 1.90 (m, 4H, C[2‘], C[5‘]), 1.65 (m, 2H, C[3‘]), 1.36 (m, 2H, C[4‘]) **^{13}C – NMR:** (400 MHz, $CDCl_3$): δ [ppm] = 187.6 [C], 182.8 [C], 158.5 [C], 154.3 [C], 128.3 [CH], 108.1 [CH], 56.2 [CH_3], 38.7 [CH], 32.3 [2x CH_2], 25.3 [2x CH_2] **Compound 8: ^{1}H – NMR:** (400 MHz, $CDCl_3$): δ [ppm] = 6.40 (s, 1H, C[3]), 5.79 (s, 1H, C[6]), 3.75 (s, 3H, C[7]), 3.01 (q, ^{3}J = 7.52 Hz, 1H, C[1‘]), 1.90 (m, 4H, C[2‘], C[5‘]), 1.65 (m, 2H, C[3‘]), 1.36 (m, 2H, C[4‘]) **^{13}C – NMR:** (400 MHz, $CDCl_3$): δ [ppm] = 188.1 [C], 182.3 [C], 159.3 [C], 151.3 [C], 130.7 [CH], 107.0 [CH], 56.4 [CH_3], 38.7 [CH], 32.1 [2x CH_2], 25.1 [2x CH_2] **Compound 9: ^{1}H-NMR:** (500 MHz, CDCl3): δ [ppm] = 6.36 (s, 1H, C[3]), 5.79 (s, 1H, C[5]), 3.74 (s, 3H, C[7]), 2.65 (t, ^{3}J = 11.89, 1H, C[1']), 1.72 (m, 4H, C[2'], C[6']), 1.35 (m, 4H, C[3'], C[5']), 0.77 (m, 2H, C[4']) **^{13}C-NMR:** (500 MHz, $CDCl_3$): δ [ppm] = 188.06 [C], 181.80 [C], 158.80 [C], 151.96 [CH], 131.29 [C], 106.84 [CH], 58.18 [CH_3], 36.09 [CH], 32.02 [2x CH_2], 29.84 [2x CH_2], 26.34 [CH_2] **Compound 10: ^{1}H-NMR:** (500 MHz, CDCl3): δ [ppm] = 6.36 (s, 1H, C[3]), 5.85 (s, 1H, C[6]), 3.74 (s, 3H, C[7]), 2.64 (t, ^{3}J = 12.16, 1H, C[1']), 1.72 (m, 4H, C[2'], C[6']), 1.35 (m, 4H, C[3'], C[5']), 1.09 (m, 2H, C[4']) **^{13}C-NMR:** (500 MHz, $CDCl_3$): δ [ppm] = 187.18 [C], 182.67 [C], 158.22 [C], 154.72 [CH], 128.67 [C], 107.86 [CH], 55.89 [CH_3], 36.10 [CH], 32.45 [2x CH_2], 27.07 [2x CH_2], 26.20 [CH_2]
23. *Biological assays* In vitro activity. The in vitro activity of the compounds were determinded against *T.b.rhodesiense* (bloodstream trypomastigotes, STIB 900 strain), *T.cruzi* (amastigotes, Tulahuen C4 strain), *L.donovani* (axenic grown amastigotes, MHOM-ET-67/L82 strain), *P. falciparum* (intra-erythrocytic forms, NF54 strain), and cytotoxicity against mammalian cells (L6 cells, rat-skeletal myoblasts) as previously described.[24] In vivo efficacy studies: In vivo experiments were performed in *T.b. rhodesiense* (STIB900) infected mice as previously described.[25] In vivo efficacy studies in mice were conducted according to the rules and regulations for the protection of animal rights ("Tierschutzverordnung") of the Swiss "Bundesamt für Veterinärwesen". They were approved by the veterinary office of Canton Basel-Stadt, Switzerland.
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