EVALUATION OF IN VITRO AND IN VIVO ACTIVITY OF NAPHTHINDAZOLE-4,9-QUINONES AGAINST *CRYPTOSPORIDIUM PARVUM*

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ABSTRACT

A series of naphthindazol-4,9-quinones was tested for growth-inhibitory effects on *Cryptosporidium parvum* in vitro and in vivo. Most compounds showed considerable activity at concentrations from 25 to 100 µM. Seven of the 23 compounds tested caused ≥90 % growth inhibition at 50 µM. Examples for highly active derivatives are 5-Hydroxy-8-chloro-N^1-methylbenz[*f*]indazol-4,9-quinone and 5-Chloro-N^2-methylbenz[*f*]indazol-4,9-quinone which, at 25 µM, inhibited growth of *C. parvum* in vitro by 78, resp. 100 %. Their anti-cryptosporidial activity was confirmed in vivo in TCR-alpha-deficient mice infected with *C. parvum* oocysts, as a model for human AIDS patients. These compounds reduced the infectivity score in the *caecum* to 0.63 resp. 0.20 compared to 0.81 in sham-treated mice. In the *ileum*, the infectivity score was 1.12, resp. 0.20 compared to 1.25. No acute or chronic toxicity was observed for any compound tested in vivo,.

KEYWORDS

Naphthoquinones, naphthindazol-4,9-quinones, *Cryptosporidium*, in vitro, in vivo, antiprotozoal, cytotoxicity, drug testing

INTRODUCTION

Cryptosporidium parvum is a protozoan parasite which may cause self-limited disease (cryptosporidiosis) in immunocompetent hosts with watery diarrhea, cramps, nausea and anorexia. Cryptosporidiosis can be life threatening to immunocompromised individuals including cancer and organ transplant patients undergoing immunosuppressive therapy, and AIDS patients. Here, disease is prolonged and may even be life-threatening with diarrhea persisting for months to years. As yet there is still no specific treatment for prevention or therapy of cryptosporidiosis available (1-4).

A large number of drugs has been tested in the past in vitro and in vivo for the treatment of cryptosporidiosis (5) but none proved to be sufficiently effective to warrant extended human trials e.g. with AIDS patients. Drugs presently in clinical use include paromomycin (6), nitazoxanide (7), azithromycin + paromomycin (8), roxithromycin (9), and the combination of protease inhibitors used in 'highly active antiretroviral therapy' (HAAT). Again, none is sufficiently efficacious to be recommended as standard therapy. Also, most of these drugs have serious side effects or treated patients experiences repeated relapse, or both. (10). Therefore, there is an urgent need both for innovative new, and for pharmaceutically improved drugs.

A series of naphthindazol-4,9-quinones a drug lead initially developed for visceral leishmaniasis was tested for their growth inhibitory activity against *C. parvum* in vitro and in vivo. According to their parent structure, naphthinadzol-4,9-quinones belong to the chemical class of naphthoquinones with an additional imidazol ring (11). Naphthoquinones and other related quinoid compounds are among the major chemical classes with significant inhibitory effects on the growth of parasites like *Leishmania*, *Trypansoma* and *Plasmodium* (12). Many have been isolated from plant or microbiological sources, but in most cases their potential usefulness is limited by toxic side effects and low bioavailability. In contrast to these findings the naphthindazol-4,9-quinones tested in this study and chosen for in vivo studies with *C. parvum* showed no or only moderate cytotoxicity.

MATERIALS AND METHODS

Compounds

Naphthinadzol-4,9-quinones were synthesised by one of us (Laatsch). Structure elucidation and purity (> 95%) of the compounds were determined by nuclear magnetic resonance spectroscopy (H/C-NMR) and high-performance liquid chromatography (HPLC) (11, 13). All compounds were first dissolved in dimethylsulphoxide (DMSO), aliquoted and stored frozen until use when they were dilution with phosphate-buffered saline (PBS) to the desired concentration (25 - 100 μM).

In vitro testing for anticryptosporidial activity

A well-established *in vitro* assay was used to test the efficacy of inhibitors against *C. parvum* (14). In short, human ileocecal epithelial cells (HCT-8; ATCC, CCL 244) were cultured in 75 cm^2 tissue culture flasks in a maintenance medium consisting of RPMI 1640 supplemented with 10 % Opti-MEM (GIBCO-BRL), 2 % fetal bovine serum (FBS) and 2 mM L-glutamine at 37 °C in a humidified, CO_2-enriched (5 %) atmosphere (15-17). 96-well flat-bottom microtiter plates were seeded with 5.0×10^4 HCT-8 cells/ well and incubated for 14-24 h. For infection, the maintenance medium was replaced by 100:1 parasite growth medium containing 3.0×10^4 sterilized oocysts. Negative controls consisted of the same number of non-viable oocysts that had been frozen and thawed between liquid nitrogen and a 37°C water bath. Parasites were allowed to invade host cells for 90 min at 37°C, when free parasites were then removed by rinsing once with warm saline. After rinsing, 150 μL fresh growth medium containing drugs at appropriate concentrations was added into each well. Negative controls contained drug diluent and medium only. Four to 8 replicate wells were used for each experimental condition.

Infected HCT-8 monolayers were incubated for 48 h, then fixed with 8 % formalin in PBS (pH 7.3) for 2 h at room temperature. After fixation, plates were blocked for 1 h with 1 % bovine serum albumin (BSA) with 0.002 % Tween-20 in PBS and then labeled for 30 min with polyclonal rat antibodies directed against *C. parvum* membrane proteins. A goat-anti-rat polyvalent antiserum conjugated with horseradish peroxidase in combination with a TMB substrate kit was used for detection and color development read at $\lambda_{abs\,630}$ using a BioTek EL311s ELISA plate reader. Each plate also contained 4 or more positive controls using 200 μg paromomycin/ mL of, which at this concentration consistently showed 60-70 % inhibition of parasite growth in the ELISA assay. Inconsistent infection data were excluded from calculations if their optimal densities deviated over 3 x SD from the mean of the group Cytotoxicity for host cells was evaluated by cell death and/ or detachment. Independent cytotoxicity assays using XTT metabolization as readout were also used.

In vivo testing for anticryptosporidial activity

Experimental design of *in vivo* studies TCR-α-deficient mice are incapable of spontaneous clearance of *C. parvum* (18); thus, they are useful for screening compounds for potential use in cryptosporidiosis. In this study, neonatal TCR-α^{neg} mice were first infected *per os* with *C. parvum* at 7 days of age, then treated either PBS with (controls) or with test compounds beginning at 10 days of age. The drugs were administered p.o. by gavage using a 24 G animal feeding needle twice daily for 6 or 7 days. Mice were euthanized at 21 days of age and intestinal sections obtained and examined for *C. parvum* and for histopathological changes.

***C. parvum* inoculum** Purified oocysts were isolated from feces collected from calves experimentally inoculated with *C. parvum* oocysts by a method described previously (19).

Oral challenge of mice consisted of 10^3 oocysts in 100 µl of 0.15 M phosphate-buffered saline (PBS). Mice were infected with *C. parvum* oocysts at 1 week of age by gavage using a 24-gauge animal feeding needle.

Assessment of *C. parvum* infection Intestinal sections from the distal ileum and cecum were fixed in 10% formalin and embedded in paraffin. Histologic sections (4 µm) were cut, stained with hematoxylin and eosin, and examined microscopically for *C. parvum* and intestinal lesions. Infectivity scores were determined as described previously (20). Briefly, a score of 0 represents no *C. parvum* detected; 1 represents few *C. parvum* detected; and 2 represents many *C. parvum* detected. Scores were determined upon examination of individual tissue sections, means calculated for each treatment group, and data presented as group means ± SEM.

Assay for cytotoxic activity against host cells For toxicity assays, uninfected HCT-8 monolayers were incubated in various concentrations of test compound for 48 h, then developed for 1 hr at 37 °C after adding 50 µl of medium containing 0.8 mg/ mL sodium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate (XTT; Sigma X-4251) and 100 µM phenazine methosulfate (Sigma P-9625) (21). The absorbance was read at 450 nm using a BioTek EL311s ELISA plate reader.

Statistics

Data were analyzed by 1-way analysis of variance followed by Tukey-Kramer multiple comparisons tests (mean infectivity scores) or 2 x 2 contingency tables were formulated and data analyzed by Fisher's Exact test (percent infected). Data were considered significant if P values <0.05 were obtained.

RESULTS

In vitro and in vivo activity of naphthindazole-4,9-quinones (Fig. 1) against *Cryptosporidium parvum* was evaluated (Tab. 1 and 2). When tested in vitro at concentrations of 10, 25, 50, and 100 µM, 13 out of 23 compounds showed significant inhibition of the pathogen (> 40% at 50 µM). Anticryptosporidial activity was associated with moderate or no toxicity for HCT-8 host cells (data not shown).

The most efficacious compounds were N^1-methyl-naphthinadzoles-4,9-quinones with significant anticryptosporidial activity below effective concentrations of 50 µM. When compounds showed more than 90 % inhibition 25 - 100 µM such as compounds **2**, **5**, **6**, **10**, **13** and **19**, they were considered highly active. Again, none of these were toxic to host cells. Among this group, compounds **2, 6, 10 and 13** were the most active showing 100 % inhibition already at 25 µM. Naphthindazoles like **3-5, 8**, **17**, and **19** also showed significant activity at 100 µM, but at lower concentrations these compounds inhibited growth of *C. parvum* by only 90 % or below. All moderately active compounds exhibited no detectable cytotoxicity. From the series of compounds tested only 7 naphthindazoles-4,9-quinones (**1**, 7, **14** - **16**, **22**, **23**) were poorly or non-effective with less than 30 % inhibition at any concentration. Anticryptosporidial activity was associated with toxicity only for compounds **8** and **13** at 100 µM.

In order to determine whether one of the active and non-toxic agents were also active in vivo, compounds **12** (low in vitro activity), **13** (high) and **19** (intermediate) were administered orally in neonate TCR-alpha-deficient mice that had been infected p.o. with *C. parvum* 3 days earlier. For all three compounds anticryptosporidial activity was confirmed in vivo (Tab 2), interestingly in the same order of effectiveness as shown in vitro. These naphthinadzoles-4,9-quinones significantly reduced the number of *Cryptosporidium* meronts within enterocytes of the caecum with infectivity scores of = 0.42, 0.20, 0.63, respectively compared to 0.81 in PBS-treated mice.

DISCUSSION

Regarding anticryptosporidial activity, these in vitro and in vivo studies show that naphthindazol-4,9-quinone derivatives have interesting potentials. Inhibition of *C. parvum* growth was intensively studied in vitro and analysis of the inhibitory concentration indicated pronounced activity for N^1-methylnaphthindazole-4,9-quinones represented by compounds **2** - **6**, **10**, and **17**. In comparison to moderately or non-effective analogs, antiparasitic activity was basically associated with the parent structure allowing minor changes in the substitution pattern of the aromatic ring. Introduction of oxygen groups (compounds **6**, **10**), halogenation (compounds **5**, **13**) or methylation (compound **8**) increased activity against *C. parvum*. When comparing N^1-methylnaphthindazole-4,9-quinones with N^2-methylated analogs reduction of activity is leading to less active agents in the lower concentration range. This can be demonstrated for compounds **6** (N^1) and **20** (N^2) both having a hydroxy group at C-5. Regarding these compounds different N-methylation is reflected by different anticryptosporidial activity raging from 100 % to below 10 % at 25 μM. Due to the limited number of compounds tested, the importance of the N-methylation in the imidazol ring is still unclear. Depending on the substitution pattern, non-alkylated compounds were either highly active like compound **2** (100 % at 25 μM) or inactive like the parent compound **1** (< 10%). Interestingly, even minor modification leading to ethyl substition at N^1 as documented for compounds **14** - **16** reduced the anticryptosporidal activity significantly (<10 %). Analysis of the inhibition rates of tested naphthinadzole-4,9-quinones also showed that introduction of an aromatic (compound **22**) or cyclohexan ring (compound **23**) at the parent structure will reduce the anticryptosporidal activity drastically (<10 % at 50.0 µM). Taking a closer look at the substitution pattern of the aromatic ring reveals that two positions are favoured. Hydroxy groups or halogenation at position C-5 and C-8 seem give dominant contributions to the anticryptosporidal activiy of certain naphthindazol-4,9-quinones. It seems that, *para*-substitution in the aromatic ring is important as a distinct structural feature. For all the potentially active compounds *meta*-substition of a methyl and a hydroxy group lead to a marked loss in activity as displayed by compounds **7**, **11**, **12** (IS = <10 %, 45 %, 43 %, respectively at 50.0 µM).

Further testing of selected naphthindazol-4,9-chinones in a sophisticated in vivo model using α-TCR-deficient mice mimicking the situation of AIDS in man so far confirmed their the respective in vitro activites. Compounds **12** (low in vitro activity), **13** (high), and **19** (intermediate) showed significant reduction of the parasite load in the *cecum* of TCR-α-deficient mice with Infectivity Scores of 0.20, 0.42, and 0.63, respectively compared to 0.63 of PBS-treated controls. We should emphasize, that therapeutic treatment with compound **12** produced nearly complete cure of the experimental *C. parvum* oocyst infection. At the administered dose none of the compounds showed any significant side effects. Oral application even of 169 µg/ kg b.w. was well tollerated.

The in vitro studies demonstrated that marked improvement but also substantial loss in anticryptosporidal activity could be brought about by only minor changes in the functional groups of the aromatic region and the alkylation of the nitrogen in the imidazole ring. A plausible explanation for the strong activities against *Cryptosporidium parvum* of distinct naphthindazole-4,9-quinones, combined with only weak general cytotoxicity, may lie in the presence of a redox-groups as known for simple naphthoquinones. Similar observations were recorded in the studies on antileishmanial and antiplasmodial effects of tested naphthoquinones from plant origin (12, 22). By virtue of structural analogy to naphthoquinones and their mode of action, such quinones are expected to inhibit parasite growth by causing disruption in their mitochondrial electron transport chain (23) as they are mainly involved in the electron transfer system. For instance, Molina Portela et al. (24) demonstrated by very thorough analysis how trypanosomatids can generate radicals from redox cycling of *ortho*-naphthoquinones. The antiparasitic effects based on the oxygen consumption of *Leishmania* species was referred by Croft et al. (25). A further explanation for the strong anticryptosporidial activity of certain naphthindazole-4,9-quinones associated with no or only moderate cytotoxicity can not be given at the moment. In contrast to the widely tested naphthoquinones the introduction of the imidazole ring seems to be important for the reduction of toxicity. Following this requirement for a safe and effective lead compound a larger number of modified compounds must be synthesized and the molecular target must identified and finally molecular modeling performed in order to get an idea on the mechanism of action of this pharmacophore.

In conclusion, our study shows that naphthindazole-4,9-quinones exhibit interesting anticryptosporidial properties with low toxicity for mammalian host cells. These results possibly bear implications for other intracellular pathogens like *Leishmania*, *Plasmodium* and and *Trypanosoma*. Also, other pharmaceutical formulations and application protocols may further improve the already appreciable antiparasitic activities in vivo. The anticryptosporidial potential of certain naphthindazole-4,9-quinones described here could represent an exciting advance in the search for a novel and selective remedy for cryptosporidiosis especially in view that fact that a safe and efficacious treatment of this HIV associated opportunistic disease is still not available.

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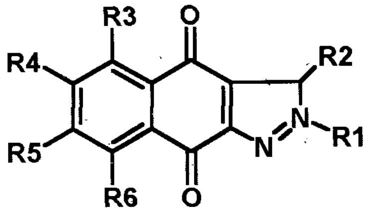
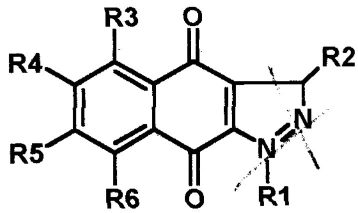
Fig. 1 **Chemical structures of the naphthindazol-4,9-quinones used in this study**



(1 - 17)



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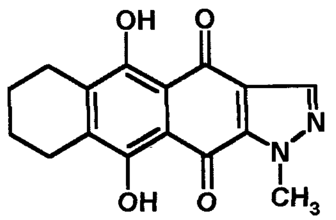

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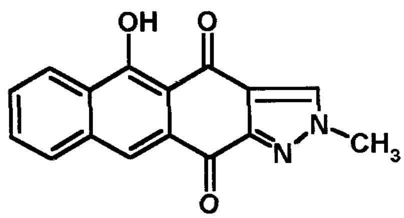

(23)

No	Name	R_1	R_2	R_3	R_4	R_5	R_6
(1)	Benz[*f*]indazol-4,9-quinone	H	H	H	H	H	H
(2)	5,8-Dihydroxy-benz[*f*]indazol-4,9-quinone	H	H	OH	H	H	OH
(3)	3-Methyl-5,8-dihydroxy-benz[*f*]indazol-4,9-quinone	H	CH_3	OH	H	H	OH
(4)	*N*[1]-Methylbenz[*f*]indazol-4,9- quinone	CH_3	H	H	H	H	H
(5)	5-Bromo-*N*[1]-methylbenz[*f*]indazol-4,9-quinone	CH_3	H	Br	H	H	H
(6)	5-Hydroxy-*N*[1]-methylbenz[*f*]indazol-4,9-quinone	CH_3	H	OH	H	H	H
(7)	5-Methyl-*N*[1]-methylbenz[*f*]indazol-4,9-quinone	CH_3	H	CH_3	H	H	H
(8)	7-Methyl-*N*[1]-methylbenz[*f*]indazol-4,9-quinone	CH_3	H	H	H	CH_3	H
(9)	5-Methoxy- *N*[1]-methylbenz[*f*]indazol-4,9-quinone	CH_3	H	OCH_3	H	H	H
(10)	8-Acetoxy-*N*[1]-methylbenz[*f*]indazol-4,9-	CH_3	H	H	H	H	OAc

quinone 953						
(11) 7-Methyl-5-hydroxy-*N*[1]-methylbenz[*f*]-indazol-4,9- quinone	CH_3	H	OH	H	CH_3	H
(12) 7-Methyl-5-methoxy-*N*[1]-methylbenz[*f*]-indazol-4,9-quinone	CH_3	H	OCH_3	H	CH_3	H
(13) 5-Hydroxy-8-chloro-*N*[1]-methylbenz[*f*]-indazol-4,9- quinone	CH_3	H	OH	H	H	Cl
(14) 5,8-Dihydroxy- *N*[1]-ethylbenz[*f*]indazol-4,9-quinone	CH_2CH_3	H	OH	H	H	OH
(15) 3-Benzoyl-6,7-dimethyl-5,8-diacetoxy- *N*[1]-ethylbenz[*f*]indazol-4,9-quinone	CH_2CH_3	Bz	OAc	CH_3	CH_3	OAc
(16) 3-Methyl- *N*[1]-ethylbenz[*f*]indazol-4,9-quinone	CH_2CH_3	CH_3	H	H	H	H
(17) 10-Acetoxy-*N*[1]-methyl-naphth[*f*]indazol-4,9-quinone	-	-	-	-	-	-
(18) *N*[2]-Methylbenz[*f*]indazol-4,9- quinone 941	H	H	H	H	H	H
(19) 5-Chloro-*N*[2]-methylbenz[*f*]indazol-4,9-quinone	H	H	Cl	H	H	H
(20) 5-Hydroxy-*N*[2]-methylbenz[*f*]indazol-4,9-quinone	H	H	OH	H	H	H
(21) 5-Chloro-6-methyl-8hydroxy-*N*[2]-methylbenz[*f*]indazol-4,9- quinone	H	H	Cl	CH_3	H	OH

1 Ac = acetate, Bz = benzoyl

Tab. 1: **In vitro anticryptosporidal activity of naphthindazol-4,9-quinones**[1)]

No.	**Concentration [µM]**			
	10	**25**	**50**	**100**
(1)	<10	<10	<10	<10
(2)	20	100	100	100
(3)	<10	<10	79	90
(4)	<10	<10	85	90
(5)	<10	42	95	99
(6)	32	100	100	100
(7)	<10	<10	<10	<10
(8)	10	34	77	Tx
(9)	<10	<10	<10	<10
(10)	38	100	100	100
(11)	<10	11	45	42
(12)	<10	<10	43	71
(13)	79	100	99	Tx
(14)	<10	<10	<10	<10
(15)	<10	<10	<10	<10
(16)	<10	<10	<10	<10
(17)	64	86	90	93
(18)	<10	<10	34	100
(19)	17	78	99	100
(20)	<10	<10	44	100
(21)	<10	<10	<10	<10
(22)	<10	<10	<10	<10
(23)	<10	<10	<10	<10

[1)] Values indicate percent growth inhibition of *C. parvum* related to untreated controls; n.d., not determined; Tx, nonspecific cytotoxicity as indicated by cytopathic effects also on feeder cell

Tab. 2: **In vivo anticryptosporidal activity of selected naphthindazol-4,9-quinones[1)]**

No.[2)]	**Mice** infected/ treated	**Infectivity Score**	
		Ileum	**Caecum**
(12)	6 / 7	1.29 (±0.29)	0.42 (±0.30)
(13)	4 / 5	0.20 (±0.20)	0.20 (±0.25)
(19)	7 / 8	1.12 (±0.23)	0.63 (±0.18)
Paro			
PBS	24 / 27	1.25 (±0.12)	0.81 (±0.15)

[1)] Values indicate an infectivity score (IS) as described in Methods; Paro, Paromomycin

[2)] Naphthindazol-4,9-chinones as listed in Table 1; Paro, paromomycin; PBS, phosphate-buffered saline