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Evaluation of *in vitro* and *in vivo* activity of benzindazole-4,9-quinones against *Cryptosporidium parvum*

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A series of benzindazole-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. For instance, at 25 µM the derivatives 5-hydroxy-8-chloro-*N*[1]-methylbenz[*f*]-indazole-4,9-quinone and 5-chloro-*N*[2]-methylbenz[*f*]indazole-4,9-quinone inhibited growth of *C. parvum* 78–100%, and at 50 µM seven of the 23 derivatives inhibited growth ≥90%. The activity of the former two compounds was confirmed in a T-cell receptor α (TCR-α)-deficient mouse model of chronic cryptosporidiosis. In these mice, the mean infectivity scores (IS) in the caecum were 0.63–0.20, whereas in sham-treated mice the score was 1.44 ($P < 0.05$). There were similar differences in IS in the ileum, where the score for treated mice was 1.12–0.20 and that for mice receiving no drug was 1.32. There was no acute or chronic toxicity for any compound tested *in vivo*.

Keywords: naphthoquinones, benzindazole-4,9-quinones, *Cryptosporidium*, *in vitro*, *in vivo*, antiprotozoal, cytotoxicity, drug testing

Introduction

Cryptosporidium parvum is an intracellular parasite of human enterocytes that may cause watery diarrhoea, cramps, nausea and anorexia, which is generally self-limiting in immunocompetent hosts. However, cryptosporidiosis can be life-threatening to immunocompromised individuals, including patients undergoing immunosuppressive therapy or organ transplantation or who are infected with the human immunodeficiency virus (HIV). Here, the disease is prolonged, and diarrhoea may persist for months to years. As yet, there is no specific prevention or therapy available for cryptosporidiosis.[1–4]

Although hundreds of drugs have been tested against cryptosporidiosis,[5] none have proved effective enough to warrant extended clinical trials in AIDS patients. Drugs now used clinically include paromomycin,[6] nitazoxanide,[7] azithromycin plus paromomycin,[8] roxithromycin[9] and 'highly active antiretroviral therapy' (HAART). Again, none is sufficiently efficacious to be recommended universally, and most of them either have significant side effects or do not prevent relapse.[10] Therefore, there is an urgent need for both innovative and pharmaceutically improved drugs to treat this AIDS-associated disease.

A series of benzindazole-4,9-quinones (BIQs) initially developed to treat visceral leishmaniasis have recently been tested against *C. parvum in vitro* and *in vivo*. Structurally,

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BIQs are naphthoquinones that possess an additional imidazole ring.[11] Naphthoquinones and other related quinoids are one of the major drug classes with significant activity against parasitic protozoa like *Leishmania*, *Trypanosoma* and *Plasmodium*.[12] Many naphthoquinones have been isolated from plant or microbial sources, but in most cases their potential usefulness has been limited by cytotoxicity and low bioavailability. In contrast, the BIQs tested here showed no, or only moderate, cytotoxicity. For this reason, BIQs have been tested as lead compounds in a murine model of chronic cryptosporidiosis.

Materials and methods

Compounds

BIQs were synthesized by one of us (H. L.). Structures and >95% purity were determined by both 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 then stored frozen until used, when they were diluted 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 these inhibitors against *C. parvum*.[14] Briefly, human ileocaecal 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, 5% CO_2-enriched atmosphere.[15–17] Ninety-six-well flat-bottomed microtitre 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 μL/well parasite growth medium[17] containing 3.0×10^4 sterilized oocysts. Non-viable, negative controls consisted of the same number of oocysts that had been frozen and thawed using liquid nitrogen and a 37°C water bath. Parasites were allowed to invade host cells for 90 min at 37°C, and any non-invading parasites were removed by rinsing once with warm PBS. After rinsing, 150 μL of fresh growth medium containing drugs at appropriate concentrations was added to each well. Negative controls contained drug diluent and growth medium only. Four to eight replicate wells were used for each experimental condition. All compounds were tested at least twice in separate experiments.

Infected HTC-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) containing 0.002% Tween-20 in PBS, and they were then labelled for 30 min with rat polyclonal antibodies directed against *C. parvum* membrane proteins. A goat-anti-rat polyvalent antiserum conjugated with horseradish peroxidase was used in combination with a 3,3′,5,5′,-tetra-methyl-benzidine (TMB) substrate kit to check for colour development at $\lambda_{abs\ 630}$ using a BioTek EL311s ELISA plate reader. Each plate contained four or more positive controls including paromomycin 200 μg/mL, which consistently inhibits parasite growth 60–70% in this assay. Inconclusive data, i.e. those in which the optimal parasite densities deviated more than ±3 standard deviations from the mean, were excluded from the calculations. Cytotoxicity for host cells was evaluated by cell death and/or detachment from the tissue culture plastic, as well as by an MTT cytotoxicity assay as described previously.[14]

In vivo testing for anticryptosporidial activity

Experimental design. Mice deficient in T-cell receptor α (TCR-α) are incapable of clearing *C. parvum* infections.[18] Therefore, they are useful for screening lead compounds against cryptosporidiosis. Oocysts were isolated and purified from the faeces of calves experimentally inoculated with *C. parvum* as described previously.[19] Neonatal TCR-α-deficient mice were infected by gavage with 1×10^3 *C. parvum* oocysts in 100 μL of 0.15 M PBS at 7 days of age. Mice were then treated either with PBS (controls) or test compounds beginning at 10 days of age. The drugs were administered orally by gavage twice daily for 6 or 7 days using a 24G animal feeding needle at a dose of 0.67 mg/kg body weight. Mice were euthanized at 21 days of age when intestinal biopsies were performed, and sections from them were examined both for the presence of *C. parvum* and for histopathological changes due to the infection.

Assessment of infection. Intestinal sections from the distal ileum and caecum were fixed in 10% formalin and embedded in paraffin. Histological sections (4 μm) were cut, stained with haematoxylin–eosin and examined microscopically both for intracellular stages of *C. parvum* and for pathological lesions due to the infection. Infectivity scores (IS) were determined as described previously.[20,21] Briefly, a score of 0 means that no intracellular stages of *C. parvum* were detected; 1 means that a few enterocytes containing parasites were observed; and 2 means that many intestinal cells contained asexual, intracellular stages of *C. parvum*. Scores were determined by examining at least 100 fields in individual tissue sections. Means were calculated for each treatment group, and the data were expressed as group mean ± S.E.M.

Assay for cytopathic activity against host cells. Briefly, uninfected HCT-8 monolayers were incubated in several concentrations of experimental drugs for 48 h and were then exposed to 50 μL of medium containing 0.8 mg/mL sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis-

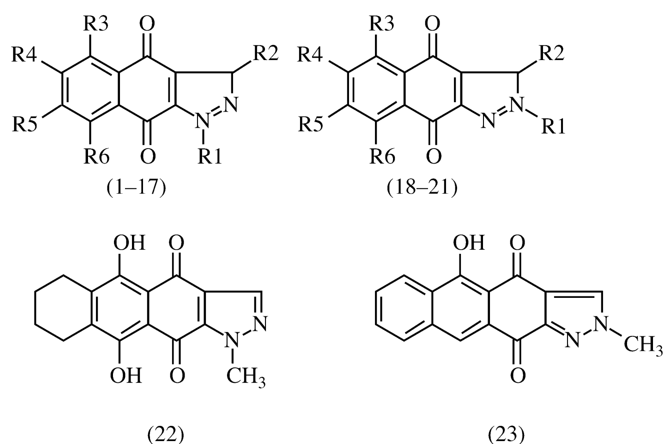

Figure 1. Chemical structures of the benzindazole- and naphthindazole-4,9-quinones used in this study. See Table 1 for details.

(4-methoxy-6-nitro)benzene-sulphonic acid hydrate (XTT; Sigma) and 100 µM phenazine methosulphate (Sigma) for 1 h at 37°C, so that a change in colour could develop as described previously.[21] The absorbance was read at λ_{450} using a BioTek EL311s ELISA plate reader.

Statistical significance

Mean IS were analysed by one-way analysis of variance followed by Tukey–Kramer multiple comparison tests, or 2 × 2 contingency tables were formulated and the percentage infected analysed by Fisher's Exact Test. Data were considered significant when $P < 0.05$.

Results

The structure and *in vitro* and *in vivo* activities of BIQs against *C. parvum* are shown in Figure 1 and Tables 1–3. When BIQs were tested *in vitro* at 10, 25, 50 and 100 µM concentrations, 13 out of 23 significantly inhibited growth of the pathogen (>40% at 50 µM), and only moderate or no toxicity for HCT-8 host cells was observed (data not shown).

The most efficacious BIQs were N^1-methylbenzindazole-4,9-quinones, which were effective at concentrations of <50 µM. BIQs were considered highly active if growth was inhibited 90% at 25–100 µM, e.g. BIQs 2, 5, 6, 10, 13 and 19. Among this group, BIQs 2, 6, 10 and 13 were the most active, showing 100% inhibition at 25 µM. Although BIQs 3–5, 8, 17 and 19 were significantly active at 100 µM, growth inhibition was incomplete at lower concentrations. Nine indazole-quinones (1, 7, 9, 14–16 and 21–23) showed <30% inhibition at any concentration and were therefore considered inactive. Among the most active BIQs, only 8 and 13 were cytotoxic, and then only at 100 µM, the highest concentration tested. All moderately active or inactive BIQs were non-toxic to host cells.

Table 1. Benzindazole-4,9-quinones and naphthindazole-4-9-quinones (see Figure 1 for chemical structures)

No.	Name	R_1	R_2	R_3	R_4	R_5	R_6
1	Benz[*f*]indazole-4,9-quinone	H	H	H	H	H	H
2	5,8-Dihydroxy-benz[*f*]indazole-4,9-quinone	H	H	OH	H	H	OH
3	3-Methyl-5,8-dihydroxy-benz[*f*]indazole-4,9-quinone	H	CH_3	OH	H	H	OH
4	N^1-Methylbenz[*f*]indazole-4,9-quinone	CH_3	H	H	H	H	H
5	5-Bromo-N^1-methylbenz[*f*]indazole-4,9-quinone	CH_3	H	Br	H	H	H
6	5-Hydroxy-N^1-methylbenz[*f*]indazole-4,9-quinone	CH_3	H	OH	H	H	H
7	5-Methyl-N^1-methylbenz[*f*]indazole-4,9-quinone	CH_3	H	CH_3	H	H	H
8	7-Methyl-N^1-methylbenz[*f*]indazole-4,9-quinone	CH_3	H	H	H	CH_3	H
9	5-Methoxy-N^1-methylbenz[*f*]indazole-4,9-quinone	CH_3	H	OCH_3	H	H	H
10	8-Acetoxy-N^1-methylbenz[*f*]indazole-4,9- quinone	CH_3	H	H	H	H	OAc
11	5-Hydroxy-7-methyl-N^1-methylbenz[*f*]-indazole-4,9-quinone	CH_3	H	OH	H	CH_3	H
12	7-Methyl-5-methoxy-N^1-methylbenz[*f*]-indazole-4,9-quinone	CH_3	H	OCH_3	H	CH_3	H
13	5-Hydroxy-8-chloro-N^1-methylbenz[*f*]-indazole-4,9-quinone	CH_3	H	OH	H	H	Cl
14	5,8-Dihydroxy-N^1-ethylbenz[*f*]indazole-4,9-quinone	CH_2CH_3	H	OH	H	H	OH
15	3-Benzoyl-6,7-dimethyl-5,8-diacetoxy-N^1-ethylbenz[*f*]indazole-4,9-quinone	CH_2CH_3	Bz	OAc	CH_3	CH_3	OAc
16	3-Methyl-N^1-ethylbenz[*f*]indazole-4,9-quinone	CH_2CH_3	CH_3	H	H	H	H
17	10-Acetoxy-N^1-methyl-naphth[*f*]indazole-4,9-quinone	—	—	—	—	—	—
18	N^2-Methylbenz[*f*]indazole-4,9-quinone	H	H	H	H	H	H
19	5-Chloro-N^2-methylbenz[*f*]indazole-4,9-quinone	H	H	Cl	H	H	H
20	5-Hydroxy-N^2-methylbenz[*f*]indazole-4,9-quinone	H	H	OH	H	H	H
21	5-Chloro-6-methyl-8hydroxy-N^2-methylbenz[*f*]indazole-4,9-quinone	H	H	Cl	CH_3	H	OH

Ac, acetate; Bz, benzoyl.

Table 2. *In vitro* anticryptosporidial activity of benzindazole-4,9-quinones and naphthindazole-4-9-quinones[a]

No.[b]	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
Paro	<10	<10	19	40

[a]Values indicate percentage growth inhibition of *C. parvum* related to untreated controls; Tx, non-specific cytotoxicity as indicated by cytopathic effects on host cells.
[b]Benzindazole-4,9-quinones as listed in Table 1; structures shown in Figure 1; Paro, paromomycin.

Table 3. *In vivo* anticryptosporidial activity of selected benzindazole-4,9-quinones[a]

No.[b]	Mice (infected/treated)	Infectivity score ileum	caecum
12	6/7	1.29 (±0.29)	0.42 (±0.30)
13	2/5	0.20 (±0.20)	0.20 (±0.25)
19	7/8	1.12 (±0.23)	0.63 (±0.18)
Paro	7/8	0.75 (±0.25)	0.62 (±0.19)
PBS	18/21	1.32 (±0.12)	1.44 (±0.16)

[a]Values indicate an infectivity score as described in Materials and methods; differences compared with PBS-treated mice are significant at $P < 0.05$.
[b]Benzindazole-4,9-quinones as listed in Table 1 and illustrated in Figure 1; Paro, paromomycin; PBS, phosphate-buffered saline.

In order to determine whether any of the active, non-toxic BIQs were also effective *in vivo*, one compound each with low, high and intermediate *in vitro* activity (12, 13 and 19, respectively) was administered orally to neonatal TCR-α-deficient mice that had been infected with *C. parvum* 3 days earlier. Anticryptosporidial activity for all three BIQs tested *in vivo* (Table 3) was comparable to that observed *in vitro*, i.e. BIQ 13 > 19 > 12. These BIQs significantly ($P < 0.05$) reduced the number of intracellular, asexual stages of *C. parvum* within epithelial cells of the caecum as measured by IS of 0.42, 0.20 and 0.63, respectively, whereas the IS of mock-treated mice was 1.44.

Discussion

Perhaps not unexpectedly, these data indicate that BIQs have potential activity against *C. parvum* both *in vitro* and in a murine model of chronic cryptosporidiosis that mimics the disease in AIDS patients. An extensive analysis of the *in vitro* inhibition of *C. parvum* growth indicated that N^1-methylbenzindazole-4,9-quinones 2–6, 10 and 17 are efficacious and show promise as lead BIQs. When compared with moderately active or inactive analogues, antiparasitic activity appeared to be associated with the parent structure, and only minor substitutions on the aromatic ring are permitted if activity is to be sustained. The introduction of oxygen groups (BIQs 6 and 10), halogenation (BIQs 5 and 13) or methylation (BIQ 8) increased activity against *C. parvum*. Activity was reduced when N^1-methylbenzindazole-4,9-quinones were substituted by N^2-methylated analogues, especially at lower drug doses. This is noted for compounds 6 (N^1) and 20 (N^2), both having a hydroxy group at C-5. Specifically, at 25 μM concentrations the change from N^1- to N^2-methylation resulted in a drop from 100% anticryptosporidial activity to <10%. Although the limited number of BIQs tested prevented an exact evaluation of why N^1-methylation of the imidazole ring is important, the trend for reduced activity by N^2 analogues is quite clear.

Depending upon the substitution pattern, non-alkylated compounds were either highly active (BIQ 2, 100%) or inactive (BIQ 1, <10%) at 25 μM. Interestingly, even minor modifications like an ethyl substitution at N^1 (BIQs 14–16) reduced the anticryptosporidial activity to <10%. Analysis of the BIQ rates of inhibition at 50 μM also showed that introducing an aromatic (BIQ 22) or cyclohexane (BIQ 23) ring on the parent structure drastically reduced activity to <10%. Taking a closer look at the substitution pattern on the aromatic ring revealed that hydroxy groups or halogenation at positions C-5 and C-8 seemed to contribute significantly to activity against *C. parvum*. Both *peri*-substitution on the aromatic ring and *meta*-substition of a methyl or hydroxy group lead to a marked loss in activity, e.g. at 50 μM the inhibition of

growth by BIQs 7, 11 and 12 was <10%, 45% and 43%, respectively.

As mentioned previously, additional testing of selected BIQs in a TCR-α-deficient murine model of chronic cryptosporidiosis that mimics the disease in AIDS patients confirmed the efficacy observed *in vitro*. Remarkably, treatment with BIQ 13 resulted in a nearly complete cure of experimental mouse infections. There were no significant side effects at any dose tested, even including oral dosing at 169 μg/kg body weight.

As with simple naphthoquinones, the presence of redox groups may contribute to the considerable activity and low toxicity of BIQs against *C. parvum* and plant naphthoquinones against species of *Leishmania* and *Plasmodium*.[12,22] By virtue of structural analogy to naphthoquinones and their mode of action, BIQs are postulated to inhibit parasite growth by causing disruption of mitochondrial electron transport,[23] especially at sites III (bc_1 complex) and IV (cytochrome *c* oxidase) where quinones are known to play a role.[24,25] For instance, it is well documented that trypanosomatids can generate radicals from redox cycling of *ortho*-naphthoquinones[26] and that naphthoquinones inhibit the consumption of oxygen by *Leishmania* species.[27] Although the remarkable efficacy of BIQs for *C. parvum* both *in vitro* and *in vivo* was unknown, using the mitochondria-specific vital fluorescent dyes $DIOC_6$, MitoTracker Green FM, Rhodamine B and Rhodamine 123 (Molecular Probes), we have recently shown that the relic mitochondrion of *C. parvum*[28] possesses a membrane potential ($\Delta\Psi_{mt}$) that can be disrupted by 1 nM KCN or 100 μM oligomycin (J. S. Keithly, unpublished results). Therefore, as in trypanosomatids and other apicomplexans,[29,30] it is likely that some part of BIQ efficacy against *C. parvum* is related to mitochondrial function.

In contrast to the widely tested naphthoquinones, the introduction of an imidazole ring in BIQs seemed to be important for reducing toxicity. Therefore, in order to develop a safe and effective lead compound, the precise target and mechanism of action of this pharmacophore needs to be determined. We are currently testing lead BIQs for their ability to inhibit O_2 uptake by sporozoites of *C. parvum* and to disrupt $\Delta\Psi_{mt}$ as measured by the dissipation of fluorescence of mitochondria-specific vital dyes. Similar methods have been used successfully both *in vitro* and *in vivo* to determine the mode of action of mitochondrial inhibitors against the apicomplexans *Plasmodium yoelii* and *Toxoplasma gondii*,[29,30] as well as trypanosomatids.[24] In the future, molecular modelling might also be useful for synthesizing more efficacious BIQs.

In conclusion, our study shows that BIQs exhibit interesting anticryptosporidial properties with low toxicity for mammalian host cells. These results may have implications for other intracellular apicomplexans like *Plasmodium* and *Toxoplasma*, as well as the kinetoplastids *Leishmania* and *Trypanosoma*. *In vivo* experiments in a murine model confirmed the *in vitro* results. Additional drug formulations must now be tested and experimental procedures varied to substantiate further and possibly improve the already appreciable antiparasitic activities of BIQs *in vivo*. The anticryptosporidial potential of the BIQs described here may well contribute to the search for new and selective therapies against cryptosporidiosis. This is especially important, because a safe and efficacious treatment for this AIDS-associated opportunistic disease is still not available.

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