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Bobel-24 Activity against *Cryptosporidium parvum* in Cell Culture and in a SCID Mouse Model[▽]

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The anticryptosporidial activity of Bobel-24 (2,4,6-triiodophenol) was studied for the first time, resulting in a reduction of the in vitro growth of *Cryptosporidium* of up to 99.6%. In a SCID mouse model of chronic cryptosporidiosis, significant differences ($P < 0.05$) in oocyst shedding were observed in animals treated with 125 mg/kg/day. These results merit further investigation of Bobel-24 as a chemotherapeutic option for cryptosporidiosis.

Cryptosporidium parvum is considered one of the top four causes of self-limiting diarrhea in humans and in several animal species (15). In immunocompromised individuals, cryptosporidial infection may become chronic and life threatening because no completely effective treatment is available (7).

The molecular mechanism involved in the initial interaction between *C. parvum* sporozoites and epithelial cells is still unclear. One of the mechanisms of *C. parvum* attachment is the interaction between galactose-*N*-acetylgalactosamine (Gal/GalNAc) epitopes on the epithelial apical membrane and Gal/GalNAc-specific sporozoite surface lectins (3). Invasion by and intracellular development of the parasite lead to the destruction of epithelial cells, resulting in blunting of intestinal villi, crypt hyperplasia, and cytoskeletal remodeling. In addition, there are decreased sodium absorption, epithelial chemokine production, and increased prostaglandin production (16), which are directly related to the notable inflammatory response that follows *Cryptosporidium* infection and characterizes its pathology (9).

To further the improvement of treatment against cryptosporidiosis, we studied the anticryptosporidial activity of Bobel-24 (2,4,6-triiodophenol). This compound is able to inhibit lectin expression (11). In addition, Bobel-24 is considered a dual inhibitor of lipoxygenase and cyclooxygenase, which are involved in the resolution of inflammatory diseases (10, 13).

The *C. parvum* IOWA bovine isolate used for this study was kindly provided by M. J. Arrowood (Centers for Disease Control and Prevention, Atlanta, GA). Bobel-24 is a nonsteroidal antiinflammatory compound (Fig. 1) (6, 10, 11). It is under clinical development as a potent leukotriene B_4 synthesis inhibitor (17). For in vitro studies, Bobel-24, used as a pure compound (purity of 99.6%, obtained from Chemical Iberica SL, Salamanca, Spain), was first dissolved in dimethyl sulfoxide (DMSO) and then diluted with phosphate-buffered saline. In an MTT cytotoxicity assay (5), viability percentages (>95%) of HCT-8 cells with Bobel-24 concentrations lower than 100 μM were observed. To study in vitro activity of Bobel-24, 8×10^5 excysted oocysts/ml (1) were inoculated in confluent HCT-8 cell monolayers as previously described (2). To assess the effect on sporozoite attachment to HCT-8 cells, we incubated sporozoite suspensions with Bobel-24 (90 μM) before use, we incubated HCT-8 cells with Bobel-24 (90 μM) before infection, and to evaluate the effect of Bobel-24 on *C. parvum* development in HCT-8, we incubated infected cells afterwards with 90 μM Bobel-24 for 48 h. Paromomycin (PRM) (2 mg/ml) and mucin (0.2 mg/ml) were used as treatment controls. Infected HCT-8 cells were also incubated with DMSO and with medium as controls. To study the effect of the dose on the response to Bobel-24 treatment, HCT-8 cells were inoculated with 8×10^5 oocysts/ml and then incubated with 90, 45, or 22 μM Bobel-24.

Fixed cultures were incubated with rhodamine-labeled C3C3 monoclonal antibody to enumerate meronts and gamonts (>3 μm) (1). Developing stages were quantified as the number per field contained in 30 microscopic fields per well. Results are expressed as the number of developmental stages (DE) per field (mean number of DE in four replicate wells) and percent inhibition. Inhibition scores (IS) were assigned to all results as previously described (15). The IS assigned were 0, 1, 2, 3, and 4 for inhibition levels of 0 to 30%, 31 to 55%, 56 to 70%, 71 to 90%, and 91 to 100%, respectively.

SCID mice are useful in evaluating new drugs against cryptosporidiosis (12). Thirty female C.B-17 scid/scid (SCID) mice, aged 7 weeks, were orally infected with 1×10^7 oocysts in 100 μl phosphate-buffered saline. Animals in individualized cages were allowed to develop chronic cryptosporidiosis for 25 days. For in vivo studies, Bobel-24 suspension with polyvinylpyrrolidone (PVP) was performed. Infected mice were paired and divided as follows. Treatment A mice ($n = 10$) received 250 mg/kg/day of Bobel-24, treatment B mice ($n = 10$) received 125 mg/kg/day of Bobel-24, control PVP mice ($n = 4$) received PVP diluted in water, and infection control group mice ($n = 6$) received no treatment. All animals were treated orally for 2 weeks and euthanized 3 to 7 days after treatment ceased. Oocyst shedding was determined every 3 days by IIF from each group of paired infected animals. Concentrated fecal samples

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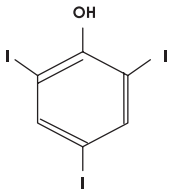

FIG. 1. Chemical structure of Bobel-24.

were examined by IIF (4). Results are shown as the number of oocysts per 50-μl concentrated fecal sample.

Data were analyzed by analyses of variance and least significant differences (14).

A significant ($P < 0.05$) inhibitory activity of Bobel-24 on *C. parvum* development was first observed when the infected HCT-8 cells were treated with 90 μM Bobel-24, with rates of growth inhibition reaching 99.6% (Table 1). No significant differences were found between Bobel-24 and PRM activities. However, treatment of sporozoite suspensions with Bobel-24 before using them to infect cells and previous treatment of noninfected cells showed IS of 0 and 1, respectively, suggesting that the effect of the compound on the *C. parvum* attachment and invasion processes was weaker. A dose-dependent inhibitory effect of Bobel-24 on *C. parvum* development was also observed (Table 2), and an IS of 4 was noted at concentrations between 45 and 90 μM, with growth inhibition rates of 99.5 to 96.5%.

Results for the in vivo activity of Bobel-24 are shown in Fig. 2. Bobel-24 was not able to completely eliminate *C. parvum* development in treated SCID mice. However, statistical analysis of the number of oocysts excreted every 3 days during treatment showed significant differences when animals were treated with 125 mg/kg/day of Bobel-24 ($P < 0.05$).

Testing drugs for efficacy against *C. parvum* continues to be of clinical and veterinary interest because a drug that is completely effective against cryptosporidiosis is not available (7, 16).

TABLE 1. Inhibition of *C. parvum* forms in cell culture by prophylactic and therapeutic treatments with Bobel-24

Treatment	Concn	Parasite count[a]	Growth inhibition	
			%	Score
Bobel-24				
Prophylactic treatment against sporozoites[b]	90 μM	14.11 ± 2.28	27.8	0
Prophylactic treatment against cells[c]	90 μM	12.50 ± 2.18	36	1
Therapeutic treatment against DE[d]	90 μM	0.43 ± 0.02	99.6	4
PRM	2 mg/ml	0.89 ± 0.02	95.4	4
Mucin	0.2 mg/ml	7.75 ± 0.39	60.4	2
Medium + DMSO	0.02%	15.31 ± 2.11	21.7	0
Medium		19.56 ± 1.78	0	0

[a] Shown is the mean number of DE per field ± the standard deviation. Values were determined by counting meronts and gamonts (<3 μm) to avoid counting nonviable but adherent sporozoites and merozoites.
[b] Sporozoite suspension incubated with Bobel-24 at 37°C for 30 min before use.
[c] HCT-8 cells incubated with Bobel-24 at 37°C for 30 min before infection.
[d] Treatment of infected HCT-8 cells with Bobel-24 at 37°C for 24 h in 5% CO_2.

TABLE 2. Dose-dependent inhibitory effect of Bobel-24 on *C. parvum* development

Treatment	Concn	Parasite count[a]	Growth inhibition	
			%	Score
Bobel-24	90 μM	2.6 ± 0.2	96.5	4
Bobel-24	45 μM	5.6 ± 0.2	92.5	4
Bobel-24	22 μM	37.5 ± 1.4	50.1	1
PRM	2 mg/ml	3.4 ± 0.3	94.8	4
Medium + DMSO	0.02%	68.3 ± 4.9	18.2	0
Medium		75.2 ± 2.9		

[a] Shown is the mean number of DE per field ± the standard deviation. Values were determined by counting.

Bobel-24 is a compound with an antiinflammatory activity similar to those of other nonsteroidal antiinflammatory drugs (10). It is a dual inhibitor of lipoxygenase and cyclooxygenase, which play an important role in inflammatory response during diarrheic disease. For this reason, it would be reasonable to suppose that inhibition of those enzymes might result in an increase in fluid absorption, an important part of treatment for cryptosporidial infection. Moreover, Bobel-24 is considered an inhibitor of L-selectin, a molecule with the same origin as the sporozoite surface lectin (3). This lectin is considered a mediator of the initial interaction process between the parasite and the host cell, confirmed because exposure of *C. parvum* sporozoites to Gal/GalNAc and to bovine mucin reduced *C. parvum* attachment to biliary and intestinal epithelia up to 70% (3). Even though no antiprotozoal activity has been described for this compound to date, due to its action mechanisms described above, it may constitute a novel approach for treating cryptosporidiosis. To date, Bobel-24 is under clinical development as a potent leukotriene B_4 synthesis inhibitor (17). Furthermore, Parreño et al. have synthesized three derivates of Bobel-24 (Bobel-4, Bobel-16, and Bobel-30) and have tested their activities as putative antileukemic agents (13). These authors found that Bobel-24 and Bobel-16 were dual inhibitors of cyclooxygenase and 5-lipoxygenase, whereas Bobel-4 and Bobel-30 were selective against 5-lipoxygenase.

In the present study, Bobel-24 showed notable and significant therapeutic activity in vitro, reaching 99.6% growth inhibition at a concentration of 90 μM. A dose-dependent inhibitory activity was also observed. There was no significant difference from the activity of PRM in vitro. This is outstanding, because PRM is a well-recognized drug with anticryptosporidial activity that is currently used to compare the activities of new candidates for cryptosporidiosis treatment. However, the in vitro prophylactic activity of Bobel-24 did not exceed an IS of 1. In contrast, the in vitro prophylactic activity of mucin was higher than when it was used for prophylactic treatment (IS of 2) but significantly lower ($P < 0.05$) than that of therapeutic treatment with 90 μM Bobel-24.

When the anticryptosporidial activity of Bobel-24 was studied in an animal model, a significant reduction in the number of oocysts excreted in the feces of infected SCID mice was only observed in animals treated with 125 mg/kg/day. However, Bobel-24 was not able to eradicate the parasite. Since lower doses of Bobel-24 showed higher anticryptosporidial activity, it is feasible that a problem in compound bioavailability may

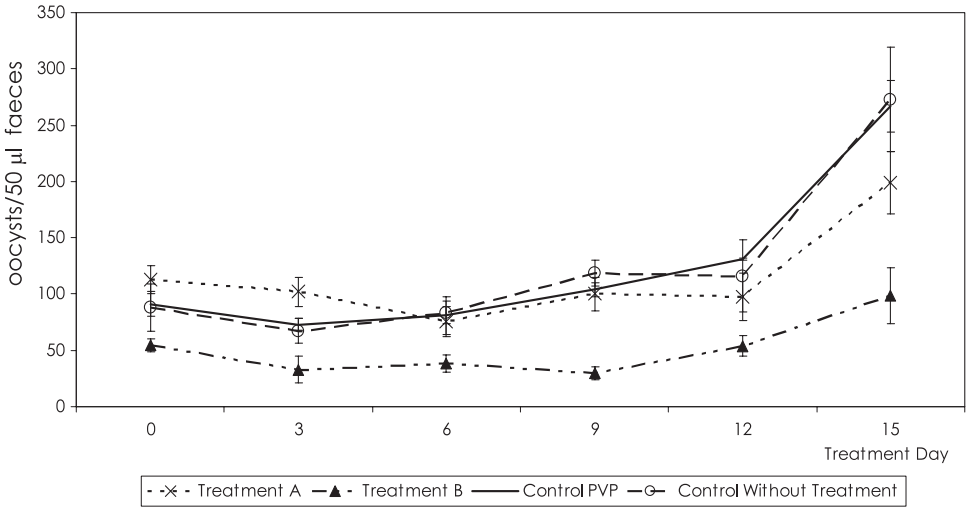

FIG. 2. *C. parvum* oocyst shedding during 2 weeks of treatment with Bobel-24.

have occurred, as Bobel-24 is a poorly water-soluble agent that needs to be formulated with a polymer.

It must be pointed out that the doses used in this study are expected to be well tolerated on the basis of the general information provided by the manufacturers, who have studied the mutagenic potential of the drug in vitro and have conducted in vivo safety studies with rodents and beagle dogs (product profile; Farma-Cros Ibérica, S.A., Madrid, Spain). Furthermore, a preliminary phase I clinical trial has been conducted with humans (17) with the only finding a slight, transient, and not clinically relevant hypothyroidism that agrees with a previous report of T3 receptor blockade at high concentrations in *Xenopus laevis* (8).

It is known that demonstration of the activity of a drug in vitro does not guarantee its function in in vivo models. However, it is generally accepted that drugs are unlikely to inhibit *C. parvum* in vivo without inhibiting it in vitro. Moreover, nitazoxanide, considered to date a very promising drug for human cryptosporidiosis treatment, was highly effective in cell culture, partially effective in the piglet diarrhea model, and ineffective in the anti-gamma interferon-conditioned SCID mouse model (15). Thus, Bobel-24 merits consideration and further investigation as a therapeutic option in the treatment of cryptosporidiosis.

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