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APPROVAL PACKAGE FOR:

APPLICATION NUMBER

21-498

Microbiology Review(s)

MICROBIOLOGY REVIEW
DIVISION OF SPECIAL PATHOGEN AND IMMUNOLOGIC DRUG PRODUCTS (HFD-590)

NDA #: 21-498	**REVIEWER** : Kalavati Suvarna
	CORRESPONDENCE DATE : 05-29-02; 07-22-02; 08-30-02
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	REVIEW COMPLETE DATE : 11-06-02

SPONSOR: Romark Laboratories Inc.
6200 Courtney Campbell Causeway
Suite 880
Tampa, FL 33607

SUBMISSION REVIEWED: N-000, BZ, BI

DRUG CATEGORY: Anti-parasitic

INDICATION: Treatment of diarrhea caused by *Cryptosporidium parvum* and *Giardia lamblia*

DOSAGE FORM: oral suspension

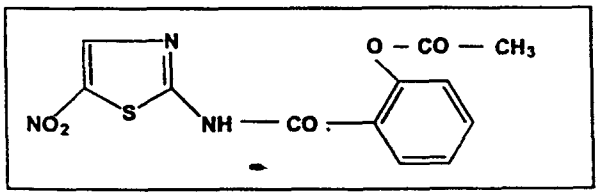
PRODUCT NAMES:

a. **PROPRIETARY:** None

b. **NONPROPRIETARY:** Nitazoxanide
CAS: 55981-09-4

c. **CHEMICAL:** 2-(acetolyloxy)-N-(5-nitro-2-thiazolyl) benzamide

STRUCTURAL FORMULA:



Molecular weight: 307.2
Empirical formula: $C_{12}H_9N_3O_5S$

SUPPORTING DOCUMENTS: NDA # 20-871; IND # [redacted], and [redacted]
Type II DMF # [redacted], DMF [redacted] DMF [redacted] DMF [redacted]

TABLE OF CONTENTS:

1. INTRODUCTION AND BACKGROUND:

The subject of this NDA is Nitazoxanide for (a) the treatment of diarrhea due to *Cryptosporidium parvum* and and (b) the treatment of diarrhea due to *Giardia lamblia*
The sponsor has proposed the following doses of nitazoxanide for the treatment of cryptosporidial diarrhea and giardiasis: 200 mg oral suspension b.i.d for 3 days in children ages 4 - 11 years, and 100 mg oral suspension b.i.d for 3 days in children ages 1 - 3 years.

Nitazoxanide is registered in Latin America for the treatment of a wide range of parasitic infections including *C. parvum* and *G. lamblia.* It is approved for veterinary use (for the treatment of helminthic infections in cats and dogs) in Switzerland and France. In the United States, the drug was the subject of NDA 20-871 (submitted in 1998) for the treatment of cryptosporidial diarrhea in AIDS patients but was not approved.

There is no approved therapy for the treatment of cryptosporidiosis in the United States. Furazolidone and Quinacrine have been approved for treatment of giardiasis in the United States. In addition to these drugs, several drugs such as metronidazole, albendazole, and paromomycin, although not approved are available in the United States for the treatment of giardiasis.

Nitazoxanide is a nitro-thiazolyl with a salicylic acid amide moiety. It is soluble in DMSO and in aqueous media at alkaline pH. The drug is highly unstable and is rapidly metabolized to various metabolites (2 major and 5 minor metabolites have been identified). The two major metabolites are tizoxanide and tizoxanide glucuronide. Following oral administration, nitazoxanide is hydrolyzed to tizoxanide (desacetyl nitazoxanide) that undergoes glucuronidation to form tizoxanide glucuronide. The time to maximum concentration (T_{max}) for both metabolites (tizoxanide and tizoxanide glucuronide) was ≤ 4.5 hours after administration of a single oral dose of nitazoxanide [500 mg to healthy adults (≥ 12 years), 100 mg to children (≤ 3 years) or 200 mg to children (4 - 11 years)]. The maximum plasma concentration (C_{max}) and the area under the concentration versus time curve (AUC) for both metabolites were about 3-fold higher in adults than children (Table 1). Both nitazoxanide and tizoxanide were shown to exhibit high protein binding (> 99%). The protein binding property of tizoxanide glucuronide was not examined.

Table 1: Pharmacokinetic parameters of tizoxanide and tizoxanide glucuronide.

Population	Dose (mg)	Tizoxanide			Tizoxanide glucuronide		
		C_{max} (µg/ml)	T_{max} (hours)	AUC (µg.h/ml)	C_{max} (µg/ml)	T_{max} (hours)	AUC (µg.h/ml)
Adults	500	10.4	3.0	41.8	10.4	4.5	64.7
12-17 years	500	91.2	4.0	39.5	7.27	4.0	46.5
4-11 years	200	3.00	2.0	13.5	2.84	4.0	16.9
12-47 months	100	3.11	3.5	11.7	3.64	4.0	19.0

The sponsor has examined the mechanism of action of nitazoxanide and its activity *in vitro* and/or in animal models against protozoa (*Cryptosporidium, Giardia,* and others), helminths (nematodes, cestodes, and trematodes), and bacteria (anaerobic and aerobic gram positive and negative bacteria). However, in this review only studies examining the activity against *Cryptosporidium* and *Giardia* (the infective agents for the indication under consideration) are discussed in detail. The activity against other parasites and bacteria are summarized briefly.

A. ***Cryptosporidium parvum*:**

1.1. Biology of *Cryptosporidium parvum*:

Cryptosporidium is an intracellular parasite present in the gastrointestinal and respiratory tract. The infection is caused by ingestion of oocysts. The oocyst contains four sporozoites within a membrane. Upon ingestion, the sporozoites excyst from the oocyst and invade the epithelial cells and become enveloped in a parasitophorous vacuole. Sporozoites undergo maturation into type I meronts, which release merozoites. The merozoite stage can undergo asexual replication and reinvade the host cells or form type II meronts by sexual replication. The Type II meronts release the macrogametocytes or microgametocytes that fertilize to give rise to a zygote. The zygote can develop into oocyst that may either rupture releasing sporozoites *in vivo* or is shed via the feces. Therefore, the presence of oocyst(s) in the stool samples may be intermittent.

The mechanism by which oocysts rupture allowing sporozoites to invade the mammalian cells is not known. Studies conducted *in vitro* show that oocysts can excyst spontaneously. However, the excystation of oocysts can be enhanced by exposure to acids, bile salts or enzymes. Thus, the exposure of oocysts to acids or enzymes in the gastrointestinal tract may play a role in the rupture of the oocyst cell wall.

1.2. Pathogenesis of cryptosporidial infection:

The major clinical manifestation observed with cryptosporidial infection is diarrhea. However, the mechanism by which *Cryptosporidium* causes diarrhea is not known. The severity of the infection and ultimate pathology is influenced by the immune status of the host. Cryptosporidial diarrhea is self-limiting in immunocompetent individuals but may be life threatening in AIDS or immunocompromised patients.

2. MECHANISM OF ACTION:

The survival of protozoa, that lack mitochondria, under anaerobic conditions depend on the presence of the enzyme pyruvate:ferredoxin oxidoreductase (PFOR). The enzyme PFOR is involved in carbon metabolism and oxidizes pyruvate to acetylCoA using ferredoxin as an electron acceptor *in vivo*. Nitazoxanide can act as an alternate electron acceptor for this enzyme and be activated. The activated product has not been identified, however, it is thought to play a role in the mechanism by which nitazoxanide exhibits activity against protozoa by generation of a toxic radical.

Genome analyses of *C. parvum* revealed the presence of a gene that encodes a PFOR like protein. In report RM01-0401[1], the *C. parvum* DNA derived PFOR peptide sequence was compared to peptide sequences from other organisms. The percent similarity between the peptide sequence of *C. parvum* PFOR and that of *Giardia lamblia, Entameoba histolytica, Trichomonas vaginalis* and *Clostridium pasteuranium* was 31%, 49%, 43%, and 51%, respectively. However, the effect of nitazoxanide on the activity of *C. parvum* PFOR enzyme was not measured.

3. ACTIVITY *IN VITRO*:

The *in vitro* activity of nitazoxanide against *C. parvum* was measured using different cell lines such as Madin-Darby bovine kidney (MDBK-F5D2), human adenocarcinoma ileocecal (HCT-8) or human lung carcinoma (A-549) cells infected with oocysts or sporozoites. The sponsor has submitted 3 published and 2 unpublished studies in support of the activity of nitazoxanide and its metabolites against *C. parvum in vitro*.

3.1. Activity of nitazoxanide and its metabolite against a human strain:

In study ROM-022[2], five experiments were conducted (all in the same laboratory) to determine the *in vitro* activity of nitazoxanide and/or its metabolite against the GCH1 strain of *C. parvum* [these reports were reviewed previously NDA# 20-871 (N-000), microbiology review dated 06-01-98]. All of these experiments were conducted using MDBK-F5D2 cells as the feeder layer and GCH1 oocysts at a concentration of 5×10^4 per well in DMEM with 5% fetal bovine serum. The cultures were incubated at 37°C for 24 hours and/or 48 hours with different concentrations of nitazoxanide (dissolved in DMSO; final concentration 0.025-0.5%) or paromomycin (dissolved in water/medium). The drugs were added at the time of initiation of infection in culture. The anti-cryptosporidial effect of nitazoxanide was measured by immunofluorescence using anti-*C. parvum* sporozoite rabbit serum. The anti-sporozoite antiserum raised in rabbits reacted with all developmental forms except the oocyst wall. The toxic effects of the drug on the uninfected mammalian (MDBK) cells was determined by measuring the absorbance of the supernatant at 490 nm after incubation of the cells in the presence of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) for 2 hours in the dark. The results of the five experiments are shown below:

Table 2A: *C. parvum* Oocysts Assay (24 hours) - Experiment #17 (final DMSO concentration = 0.5%)

Trial 1 - 24hr

Compound	Conc.	Mean (± SD)*	Percent Toxicity	Percent Inhibition
Infected Media	0	983.5 (±128.2)	0	0
Paromomycin	2mg/ml	482 (±47.1)	23.8	51
NTZ	100µg/ml	Lost	88.1	NA**
	10µg/ml	55.5 (±13.5)	65.1	94.4
	1µg/ml	224.5 (±28.5)	8.3	77.2
	0.1µg/ml	474.5 (±29.3)	19.3	51.8

* Parasite Count/10 fields
** Not available due to toxicity

Table 2B: *C. parvum* Oocysts Assay (48 hours) - Experiment #17

Trial 2 - 48hr

Compound	Conc.	Mean (± SD)*	Percent Toxicity	Percent Inhibition
Infected Media	0	2231.25 (+90.03)	0	0
Paromomycin	2mg/ml	580 (+33.42)	40.8	74.01
NTZ	20µg/ml	68.75 (+13.77)	92.87	96.92
	2µg/ml	113.75 (+21.36)	24.93	94.90
	0.2µg/ml	1020 (+158.48)	16.56	54.29
	0.02µg/ml	1041 (+191.46)	21.23	53.33

* Parasite Count/10 fields

Table 3: *C. parvum* Oocysts Assay (24 hours)- Experiment #19

Combined Drugs vs. *C. parvum* Oocysts

Paromomycin (µg/ml) (water base)	NTZ (µg/ml) (DMSO base)	Par/10	± SD	Tox/OD	± SD	%Inhib	%Tox	Score
0	0	928.50	±79.32	1.187	±.023	0	0	0
2	0	270.00	±12.65	1.023	±.006	70.92	13.82	1
1	0	373.00	±83.66	1.118	±.066	59.83	5.82	1
0.5	0	490.50	±98.36	NA*	NA	47.17	NA	NA
0.25	0	599.00	±74.13	NA	NA	35.49	NA	NA
0	0	779.50	±63.08	1.049	±.066	0	0	0
0	20	88.50	±15.86	0.329	±.074	88.65	68.62	3
0	10	110.0	±16.57	0.633	±.093	85.89	39.68	2
0	5	72.50	±22.23	1.071	±.052	90.70	0	0
0	2.5	168.50	±16.60	1.131	±.294	78.38	0	0
2	20	52.00	±18.11	0.532	±.101	93.33	49.26	2
2	10	84.50	±12.37	0.610	±.066	89.16	41.87	2
2	5	91.00	±25.32	0.901	±.152	88.33	14.07	1
2	2.5	87.50	±2.52	1.011	±.156	88.77	3.58	0
1	20	84.50	±36.93	0.601	±.041	89.16	42.68	2
1	10	75.00	±15.56	0.645	±.049	90.38	38.48	2
1	5	88.50	±25.16	0.811	±.045	88.65	22.70	1
1	2.5	135.50	±19.49	1.030	±.021	82.62	1.76	0
0.5	20	137.00	±27.25	0.350	±.034	82.42	66.62	3
0.5	10	83.33	±14.05	0.611	±.008	89.31	41.73	2
0.51	5	95.50	±22.71	0.912	±.104	87.75	13.07	1
0.5	2.5	116.50	±25.68	1.021	±.052	85.05	2.58	0
0.25	20	70.67	±12.86	0.349	±.073	90.93	66.71	3
0.25	10	65.50	±37.07	0.647	±.062	91.60	38.29	2
0.25	5	102.00	±37.63	0.896	±.007	86.91	14.56	1
0.25	2.5	126.00	±19.66	1.082	±.075	83.84	0	0

Par/10 = Parasite counts per 10 high power fields
%Inhib = Percent Inhibition of parasite infection
%Tox = Percent toxicity to cells by the drug
*NA - Information not available

Table 4A: *C. parvum* Oocysts Assay (48 hours) Experiment #28- submitted as part of Experiment #29A

Drugs	Conc.	Parasite	± SD	Tox/OD	± SD	%Inhib	%Tox	Score
Aq. Media	0	1218.4	±210.22	1.013	±.024	0	0	0
Paromomycin	2mg/ml	219.08	±70.69	.873	±.016	82.02	13.82	1
	1	279.17	±100.80	1.061	±.061	77.09	≤0	0
	0.5	309.83	±77.92	.874	±.158	74.57	13.72	1
	0.25	485.67	±94.33	.697	±.006	60.14	31.19	2
0.25% DMSO Media	0	824.92	±173.73	.928	±.071	0	0	0
Nitazoxanide	100mg/kg	LOST	NA*	.515	±.107	NA	NA	4
	10	43.42	±14.69	.201	±.023	94.74	78.34	4
	1	120.00	±40.25	.922	±.017	85.45	.65	0
	0.1	782.75	±251.45	.824	±.086	5.11	11.21	1

Table 4B: *C. parvum* Oocysts Assay (48 hours)-Experiment #29A

Drugs	Conc.	Parasite	± SD	Tox/OD	± SD	% Inhib	%Tox	Score
Aqueous Media	0	895.13	±248.28	1.753	±.068	0	0	0
Fresh Paromomycin	*2000*	*265.00*	*±63.44*	*1.527*	*±.250*	*70.40*	*12.92*	*1*
0.25% DMSO Media	0	678.50	±114.69	1.741	±.194	0	0	0
Fresh Nitazoxanide	100	LOST	NA	.243	±.037	NA	86.04	4
	10	52.50	±15.88	.246	±.012	92.26	85.87	4
	1	479.67	±94.98	1.718	±.261	29.30	1.32	0
	0.1	549.00	±145.22	1.834	±.274	19.09	≤0	0

Table 4C: *C. parvum* Oocysts Assay (48 hours)-Experiment #29A

Drugs	Conc.	Parasite	± SD	Tox/OD	± SD	% Inhib	%Tox	Score
Aqueous Media	0	709.89	±343.85	1.544	±.066	0	0	0
Fresh Paromomycin	2000	174.50	±58.49	1.188	±.030	75.42	23.03	1
0.25% DMSO Med	0	535.58	±242.96	1.479	±.041	0	0	0
11 Day Old Nitazoxanide	100	LOST	NA	.479	±.001	NA	67.60	3
	10	46.78	±21.66	.230	±.016	91.27	84.41	4
	1	118.17	±63.16	1.420	±.013	77.94	3.99	0
	0.1	405.33	±142.79	1.515	±.086	24.32	≤0	0

Table 4D: *C. parvum* Oocysts Assay (48 hours)-Experiment #29A

Drugs	Conc.	Parasite	± SD	Tox/OD	± SD	% Inhib	%Tox	Score
Aqueous Media	0	406.33	±115.38	1.698	±.248	0	0	0
Fresh Paromomycin	2000	146.83	±50.71	1.455	±.130	63.86	14.32	1
0.25% DMSO Med	0	370.91	±118.02	1.474	±.064	0	0	0
17 Day Old Nitazoxanide	100	LOST	NA	.749	±.008	NA	49.20	2
	10	32.44	±16.84	.324	±.008	91.25	78.05	4
	1	56.00	±11.97	1.693	±.056	84.90	≤0	0
	0.1	344.67	±43.87	1.389	±.126	7.07	5.73	0

Table 4E: *C. parvum* Oocysts Assay (48 hours)-Experiment #29A

Drugs	Conc.	Parasite	± SD	Tox/OD	± SD	% Inhib	%Tox	Score
Aqueous Media	0	1218.42	±210.22	1.013	±.024	0	0	0
Fresh Paromomycin	2000	219.08	±70.69	.873	±.016	82.02	13.82	1
0.25% DMSO Media	0	824.92	±173.73	.928	±.071	0	0	0
Fresh Nitazoxanide	100	LOST	NA	.515	±.107	NA	44.56	2
	10	43.42	±14.69	.201	±.023	94.74	78.34	4
	1	120.00	±40.25	.922	±.017	85.45	0.65	0
	0.1	782.75	±251.45	.824	±.086	5.11	11.21	1

Note: It appeared that the data shown in Table #4A and #4E are the same. However, the sponsor stated that "while the results appear to be similar, they are not all the same". Given that all of the values reported in both tables were identical out to two or three decimal points it is unclear what data were new or different. Also, the results in Table 4B were stated to be anomalous.

Table 5: *C. parvum* Oocysts Assay (48 hours)-Experiment #30

***C. parvum* Oocysts Assay (48 hr.)**

Drugs	Conc.	Parasite	± SD	Tox/OD	± SD	%Inhib	%Tox	Score
Aqueous Media	0	681.58	±271.02	2.024	±.018	0	0	0
Paromomycin	2000	115.75	±44.65	1.219	±.009	83.02	39.79	2
0.025% DMSO Media	0	628.50	±171.94	1.799	±.145	0	0	0
NTZ	10	11.75	±7.33	.413	±.013	98.13	77.07	4
	1	39.67	±13.13	1.618	±.326	93.69	10.09	1
	0.1	643.42	±229.73	1.878	±.154	≤0	≤0	0
	0.01	714.33	±194.79	1.617	±.072	≤0	10.12	1
New NTZdes	10	13.75	±6.66	.337	±.005	97.81	81.27	4
	1	39.92	±13.49	1.710	±.033	93.65	4.97	0
	0.1	649.86	±152.19	1.506	±.119	≤0	16.29	1
	0.01	749.33	±139.49	1.721	±.144	≤0	4.36	0

Conc. - µg/ml
Parasite - Mean parasite count/field (12 fields analyzed)
%Inhib - Percent Inhibition of parasite infection
%Tox - Percent toxicity to cells by the drug

Table 6: *C. parvum* Oocysts Assay (48 hours)-Experiment #31

C. parvum **Oocysts Assay (48 hr.)**

Drugs 1	Drug 2	Parasite	± SD	Tox/OD	± SD	%Inhib	%Tox	Score
Aqueous Media	--------	611.58	±160.21	1.616	±.019	0	0	0
Paromomycin 1mg/ml	--------	180.50	±64.27	1.324	±.073	70.49	18.13	1
0.01% DMSO Media	--------	694.92	±163.42	1.612	±.113	0	0	0
Nitazoxanide (des) 0.75µg/ml	--------	237.50	±134.48	1.356	±.123	65.82	15.89	1
HL 2945 10µM	--------	134.08	±49.56	1.240	±.122	80.71	23.05	1
HL 2945 5µM	--------	692.67	±256.21	1.232	±.141	0.32	23.55	1
HL 2945 10µM	Paromomycin 1mg/ml	233.89	±176.79	1.192	±.147	66.34	26.03	2
HL 2945 5µM	Paromomycin 1mg/ml	393.60	±214.53	1.217	±.163	43.36	24.48	1
HL 2945 10µM	NTZdes 0.75µg/ml	126.42	±56.37	1.218	±.179	81.81	24.42	1
HL 2945 5µM	NTZdes 0.75µg/ml	139.11	±50.61	1.224	±.101	79.98	24.01	1

Parasite - Mean parasite count/field (12 fields analyzed)
%Inhib - Percent Inhibition of parasite infection
%Tox - Percent toxicity to cells by the drug

The results of the *in vitro* studies (shown in Tables 2 to 6 and summarized in Table 7) indicate that at 24 hours, nitazoxanide at concentrations between 1 and 5 µg/ml inhibited parasite count by 77 to 91% with ≤ 8.35% toxicity to MDBK cells. These data are based on a single experiment. At 48 hours of incubation, the data collected from 5 different experiments (conducted in the same laboratory) show that nitazoxanide at a concentration of 1 µg/ml inhibited parasite number from 29 to 85% with minimal toxicity to uninfected feeder cells. Higher concentrations (≥ 10 µg/ml) of the drug were highly cytotoxic (≥ 40%).

The results in Table 4B (which show comparatively lower inhibition of the parasite i.e. 29%) were stated to be anomalous but the reasons for that are unclear. The parasite count in the untreated cultures was within the range observed in other experiments. Also, paromomycin, which was used for comparison of drug activity, showed results comparable to that observed in other experiments.

Table 7: Summary of results shown in Tables 2 to 6.

Experiment #	Parasite count in untreated control	Drug concentration µg/ml	% inhibition	% toxicity
After 24 hours incubation with NTZ				
# 17 and # 19	984 ± 128; 780 ± 63	0	0	0
		100	NA	88.1
		20	89	69
		10	94.4	65.1
		10	86	40
		5	91	0
		2.5	78	0
		1	77.2	8.3
		0.1	51.8	19.3
After 48 hours incubation with NTZ				
# 17, # 29A, and # 30	2231 ± 90; 825 ± 174; 679 ± 115; 825 ± 174; 536 ± 243; 371 ± 118; 629 ± 172	0	0	0
		100	NA NA[a] NA[a] NA[b] NA[c]	NA 86[a] 45[a] 68[b] 49[c]
		20	97	93
		10	95 93[a] 95[a] 91[b] 91[c]	78 86[a] 78[a] 84[b] 78[c]
		10	98	77
		2	95	25
		1	85 29[a] 85[a] 78[b] 85[c]	0.7 1[a] 0.7[a] 4[b] 0[c]
		1	94	10
		0.2	54	17
		0.1	5 19[a] 5[a] 24[b] 7[c]	11 0[a] 11[a] 0[b] 6[c]
		0.1	0	0
		0.02	53	21
		0.01	0	4
After 48 hours incubation with NTZdes				
# 30 and # 31	629 ± 172; 694 ± 92	0	0	0
		10	98	81
		1	94	5
		0.75	66	16
		0.1	0	16
		0.01	0	4

NTZ = nitazoxanide, NTZdes = Nitazoxanide desacetyl
[a] fresh NTZ (nitazoxanide)
[b] 11 day old NTZ
[c] 17 day old NTZ
Results of different experiments are represented as without, single, double or thick underline.
Bold numbers appeared to be same except for toxicity result with 100µg/ml NTZ; sponsor has stated that they are from different experiments.

In another study by the same group of investigators[3], the *in vitro* activity of nitazoxanide against the same strain of *C. parvum* was examined. The experimental design was the same as that described above. The cultures were incubated for 48 hours in the presence or absence of drug and the cytotoxic effect of the drug on uninfected MDBK cells was determined using the CellTiter 96 aqueous cell proliferation assay kit containing the same reagents (MTS and PMS) as in the previous study (see page 6). However, the incubation period with the reagents was longer (4 hours) than in the previous study (2 hours). The activity of nitazoxanide (1 µg/ml and 10 µg/ml) and paromomycin (2 mg/ml) was within the range of activity of the two drugs observed in the previous study (Tables 7 and 8). In this single experiment, minimal cytotoxicity (11%) was observed at 1 µg/ml nitazoxanide and no toxicity was observed at 10 µg/ml of nitazoxanide or 2 mg/ml of paramomycin. The reason(s) for this discrepancy are unclear.

Table 8: Dose responses for inhibition by paromomycin (PRM) and nitazoxanide (NTZ) of *C. parvum* forms in cell cultures.

Medium or drug(s)	Concn	Parasite count[b]	Growth inhibition		Toxicity[c]	
			%	Score	%	Score
Medium		1,416.4 ± 302	NA[d]	NA	0.0	0
Medium + DMSO		1,231.7 ± 281	NA	NA	1.06	0
PRM	3.2 mM (2 mg/ml)	256.4 ± 64.8	81.9	3	−1.7	0
	1.6 mM (1 mg/ml)	293.6 ± 96.7	79.3	3	7.7	1
	0.8 mM (0.5 mg/ml)	398.3 ± 87.1	71.9	3	2.3	0
	0.4 mM (0.25 mg/ml)	453.9 ± 75	68	2	−7.5	0
NTZ	325 µM (100 µg/ml)	ND[e]	ND	ND	74.1	3
	32.5 µM (10 µg/ml)	87.3 ± 20.1	93	4	−25	0
	3.25 µM (1 µg/ml)	695 ± 173	44	1	11.3	1
	0.325 µM (0.1 µg/ml)	1,105 ± 127	10.3	0	18	1
PRM-NTZ[f]	0.4 mM/3.25 µM	422.4 ± 65	70	2	16	1
	0.2 mM/3.25 µM	619 ± 158	47	1	12.2	1
Medium[g]		ND	NA	NA	20	1
Lysate[h]		ND	NA	NA	17	1

[a] The dose responses were evaluated after all parasites and treatments were applied to the MDBK cells and incubated for 48 h.
[b] Mean number of parasites per field ± standard deviation. Values were determined by counting parasites in 16 fields per well for a total of 4 wells per treatment.
[c] Toxicity values were determined with uninfected cells.
[d] NA, not applicable.
[e] ND, not determined.
[f] Combined treatment with PRM and NTZ.
[g] Toxicity values were determined for cells infected with 3×10^6 oocysts/well.
[h] Toxicity values were determined for cells exposed to 3×10^6 oocyst equivalents per well.

It should be noted that in all of these studies, parasite count was determined either at 24 or 48 hours of incubation. Later time points were not tested. The enumeration of parasites does not include oocyst(s) present in the culture since the anti-sporozoite polyclonal serum used for performing parasite count does not detect the oocyst wall. The medium was not supplemented with bile salts, or any other factors, nor were the oocysts pretreated with any agent that could maximize the rupture of oocyst wall and release the sporozoites for initiating infection of mammalian cells. Some investigators have reported a reduction or disappearance of oocyst(s) within 24 hours of infection *in vitro* suggesting that the majority of the oocysts were ruptured. However, such an excystation of the oocyst could be influenced by the culture conditions (e.g., the cell lines used as a feeder layer, components of the media, age/viability/infectivity of the oocyst, pretreatment of the oocyst to enhance the excystation of the oocyst, etc.). Also, while the polyclonal serum used for identifying parasites in the cultures was stated to react with all other developmental stages (except oocyst) of the *Cryptosporidium* parasite including trophozoite and

merozoite forms, there is no information available to show which developmental forms were actually present in cultures within the time frame tested. Based on literature reports using other cell lines such as Caco-2 or colonic epithelial cells, studies have shown that asexual forms develop between 24 to 48 hours but the development of macrogametes occurs after extended periods of incubation (3 to 5 days).

3.2. Activity of nitazoxanide against clinical isolates:

The activity of nitazoxanide was measured *in vitro* against clinical isolates, collected at different timepoints from a single patient with AIDS (CD4 count = 55/mm^3; and viral load = 104,472 copies/ml plasma)[4]. The patient had failed treatment with paromomycin (500 mg bid for 25 days), azithromycin (1200 mg bid for 27 days) and nitazoxanide (1000 mg bid for 28 days). The isolates Cp 98-1, Cp 98-3, Cp 98-7, Cp 98-8 and Cp 98-10 were collected after treatment of the patient with paromomycin or azithromycin while isolates Cp 99-2 and Cp 99-4 were collected after the patient was treated with nitazoxanide (Table 9). The oocysts from stool samples (stored in 2.5% potassium dichromate for 6 months) were sterilized with sodium hypochlorite, washed and resuspended in Dulbecco's modified eagles medium (DMEM). Excystation was achieved by incubating the oocysts in 0.25% trypsin and 0.75% sodium taurocholate for 60 minutes at 37°C and the sporozoites were collected by centrifugation at 200 x g for 20 minutes. Sporozoites (10^5) were then added to a monolayer of A-549 feeder cells in DMEM with 10% fetal calf serum and incubated at 37°C for 4 hours in 5% CO_2. Non-invasive sporozoites and residual oocysts were removed by washing with fresh medium. Different concentrations of the drugs (nitazoxanide, paromomycin and azithromycin) were added to the cultures and incubation continued for an additional 48 hours. Meront and microgamont stages of the parasite were observed within 48 hours of infecting A-549 cells with sporozoites. The number of meronts and gamonts per 50 oil immersion fields was determined. The toxic effect of the drug on the uninfected A-549 cells was examined by the trypan blue dye-exclusion assay. The activity of nitazoxanide against the different isolates obtained before and after treatment of the patient with nitazoxanide was similar (Table 9). Nitazoxanide at 1 µg/ml (low concentration) and 10 µg/ml (intermediate concentration) reduced meronts and gamonts by about 20% and 45%, respectively. The reduction in meronts and gamonts was about 65% at 100 µg/ml nitazoxanide, however, at this concentration the drug was stated to be cytotoxic to the cell line. The raw data on drug toxicity to the cell line were not included. It is of note, however, that studies by another group of investigators[2] showed nitazoxanide at 10 µg/ml to cause about 40% cytotoxicity to MDBK-F5D2 cells (for details see pages 6-11). The activity of nitazoxanide against the isolates was similar to paromomycin. Azithromycin was less effective. No correlation was observed between the clinical outcome and the *in vitro* activity of nitazoxanide against the small number of isolates tested from a single patient.

Table 9: Inhibitory effect of low, intermediate, and high concentrations of different drugs on *Cryptosporidium parvum*, expressed as percent reduction in the number of parasites.

Antimicrobial agent	Strain	Percent reduction in *C. parvum*		
		Low concentration[a]	Intermediate concentration[a]	High concentration[a]
Paromomycin	Cp 98-1	18.8	42.8	63.4
	Cp 98-3	17.9	40.9	64.0
	Cp 98-7	19.0	41.0	58.3
	Cp 98-8	16.9	45.6	61.7
	Cp 98-10	17.3	39.7	56.9
	Cp 99-2	18.4	42.6	60.8
	Cp 99-4	17.6	40.7	61.1
Azithromycin	Cp 98-1	5.7	15.3	26.5
	Cp 98-3	6.0	16.5	27.8
	Cp 98-7	4.8	17.3	29.5
	Cp 98-8	4.6	14.8	24.8
	Cp 98-10	5.1	13.5	26.0
	Cp 99-2	5.3	15.6	25.3
	Cp 99-4	5.8	14.5	30.1
Nitazoxanide	Cp 98-1	19.0	44.3	67.2
	Cp 98-3	20.1	41.5	65.0
	Cp 98-7	18.7	50.2	68.4
	Cp 98-8	20.9	43.6	60.1
	Cp 98-10	19.4	47.2	66.3
	Cp 99-2	17.3	44.0	64.8
	Cp 99-4	22.6	42.8	65.0

[a] Low, intermediate, and high concentrations were defined as 0.05 mg/L, 0.5 mg/L, and 1 mg/l for paromomycin; 1 mg/L, 4 mg/L, and 8 mg/l for azithromycin; 1 mg/L, 10 mg/l, and 100 mg/l for nitazoxanide

In another study[5], the *in vitro* activity of nitazoxanide alone or in combination with azithromycin and rifabutin against *C. parvum* isolates (obtained from 4 AIDS patients) was examined using A-549 feeder cells. Drugs were dissolved in methanol/acetone. The experimental conditions, the method used for excystation of oocysts, and the method for determination of toxicity were same as that described in the previous study. However, the inoculum size was 10 fold lower (10^4 sporozoites). The number of meronts and gamonts per 50 oil immersion field was determined. The results in Table 10 show that nitazoxanide (8 µg/ml) decreased the parasite counts by 50%, rifabutin (8 µg/ml) and azithromycin (8 µg/ml) by 23% and 25%, respectively. The toxicity to A-549 cells ranged from -8.9 to 11.2% at the different concentrations (≤ 8µg/ml) of the 3 drugs. The activity of nitazoxanide in combination with azithromycin or rifabutin was better than the activity of either drug alone, suggesting an additive effect. The toxicity to A-549 cells was ≤ 8.4% when the drugs were used in combination. The raw data showing the anti-parasitic activity and the toxicity to A-549 cells in the presence of the different drugs alone or in combination were not included.

Table 10: Inhibitory effects of nitazoxanide in combination with azithromycin and rifabutin on *C. parvum* in A-549 cells: parasite count and percentage reduction versus control plates without antimicrobials.

	Parasite count[a] (% reduction) with nitazoxanide (mg/L)			
Drug (mg.L)	0	0.5	2	8
Azithromycin				
control	40.8 (0.0)	36.0 (11.8)	24.1 (41.0)	18.2 (55.4)
0.5	39.1 (4.2)	34.2 (16.2)	22.7 (44.4)	16.4 (59.9)
2	35.3 (13.5)	30.3 (25.8)	18.3 (55.2)	12.5 (69.4)
8	30.4 (25.5)	26.4 (35.3)	13.6 (66.7)	6.6 (83.9)
Rifabutin				
control	42.9 (0.0)	38.3 (10.8)	25.1 (41.5)	19.3 (55.1)
0.5	40.3 (6.1)	36.0 (16.1)	22.5 (47.6)	17.6 (59.0)
2	38.2 (11.0)	33.1 (22.9)	21.0 (51.1)	15.2 (64.6)
8	33.1 (22.9)	29.5 (31.3)	15.4 (64.2)	8.7 (79.8)

[a]Each value is based on the mean count of three experiments from each isolate.

Number of parasites in the control group ranged from 29.4 to 52.8 (mean 41.1 per 50 oil immersion fields.

3.3. Activity of nitazoxanide and its metabolites against a bovine isolate:

The activity of nitazoxanide and its metabolites was measured against the development of *C. parvum* sporozoites using HCT-8 cells as feeder layer in Eagle's modified Dulbecco's medium (BHK21) containing 20% fetal calf serum[6]. Calf oocysts were purified from fecal samples (stored for less than 3 months in potassium dichromate solution) by layering over discontinuous sucrose gradient. The oocysts were washed, treated with sodium hypochlorite for cell surface sterilization, and washed again prior to incubation with 1.5% taurocholic acid at 37°C for 90 minutes for excystation of oocysts. The sporozoites released were separated from non-excysted oocysts and empty shells by filtration using a filter. Sporozoites (1.5 to 2 x 10^5) suspended in BHK21 medium were added to HCT-8 cell monolayers within 15 minutes of isolation and the cultures incubated for 2 hours at 37°C. The cultures were then supplemented with para-aminobenzoic acid, ascorbic acid, fetal calf serum, etc. Nitazoxanide and its metabolites (tizoxanide and tizoxanide glucuronide) were added at different time intervals after inoculation of sporozoites to the cell culture (0, 2, and 18 hours, see Table 11). The cultures were incubated for up to 46 hours in the presence or absence of drug. The number of parasites was measured in 20 microscopic fields ( ) by immunofluorescence assay using hyperimmune sera (raised in rats by immunization with the sporozoites in complete/incomplete Freund's adjuvant). It was stated that the antiserum cross-reacts with all stages of *C. parvum* including oocysts.

Table 11: Time of adding the agents and contact duration in culture for the enzyme immunoassay.

Time of adding agents (hrs from time of adding sporozoites)	Contact duration in culture (hrs)	Corresponding parasite stage(s)
0	2	sporozoite
+2	4	meront with 8 nuclei (asexual stage)
+18	4	meront with 4 nucleui and other sexual stages
+2	46	complete parasite development (asexual and sexual stages)

The sponsor has enumerated the number of trophozoites, meronts (4 or 8 nuclei) and other sexual stages of *C. parvum* in the presence or absence of nitazoxanide and its metabolites (Tables 12 to 14). It would have been useful to confirm the different stages by morphological identification. It appears that the different stages of the parasite enumerated were based on the times indicated in Table 11. The sponsor has stated that photographs depicting the morphology of the different stages after drug treatment were not taken. The number of parasites referred to by the sponsor as sexual meronts (4 nuclei) observed in 18 to 22 hour old control cultures (i.e., drug added at 18 hours and cultures incubated for 4 hours) was relatively small (Tables 12 to 14). In 2-hour and 18-hour old cultures exposed to nitazoxanide (≥ 10 µg/ml) for 4 hours, a reduction in the number of parasites was observed (Table 12). The reduction in parasite counts was 88%, when the 2-hour old cultures were exposed to 10 µg/ml nitazoxanide for 46 hours (Table 12). The raw data for the drug toxicity at the different drug concentrations were not included. The sponsor has stated that at concentrations of 10 to 50 µg/ml nitazoxanide, the toxicity to HCT-8 cells ranged from 14% to 39% using the trypan blue exclusion and nitroblue tetrazolium chloride monohydrate reduction assays.

The activity of tizoxanide was similar to nitazoxanide (Table 13), while tizoxanide glucuronide was less effective (Table 14). However, fragilation and/or peeling of HCT-8 cells were observed at tizoxanide and tizoxanide glucuronide concentrations of 10 and 50 µg/ml. No other drugs were used for comparison.

Table 12: Immunofluorescent evaluation of stage dependent anticryptosporidial activity of nitazoxanide on *Cryptosporidium parvum* development on HCT-8 cells[1].

Hours of incubation with NTZ / NTZ concentration (µg/ml)	Number (mean ± S.D.) of parasites/20 microscopic fields				
	0 - 2 hours* (2 hours)	2 - 6 hours* (4 hours)	18 - 22 hours* (4 hours)		2 - 48 hours* (46 hours)
	Trophozoites	Meronts 8N	Meronts 4N	Other sexual stages (gametocytes, gametes and oocysts)	All parasite stages
0	82.0 ± 17.0	60.0 ± 8.5	14.0 ± 2.8	106.0 ± 14.1	262.0 ± 42.4
10	22.0 ± 2.3	2.3 ± 1.7	0.5 ± 1.0	8.0 ± 6.7	32.0 ± 10.3
30	28.0 ± 0.0	4.0 ± 5.7	0.0 ± 0.0	13.0 ± 7.1	45.0 ± 12.7
50	7.0 ± 9.9	0.0 ± 0.0	0.0 ± 0.0	7.0 ± 9.9	14.0 ± 19.8

[1] Pooled results of 2 experiments (with each experiment conducted in duplicate for the 10 µg/ml concentration).
* The time of addition of the drug (duration of drug exposure).
NTZ = nitazoxanide.

Table 13: Immunofluorescent evaluation of stage dependent anticryptosporidial activity of tizoxanide on *Cryptosporidium parvum* development on HCT-8 cells[2].

Hours of incubation with TZ / TZ concentration (µg/ml)	Number (mean ± S.D.) of parasites/20 microscopic fields				
	0 - 2 hours* (2 hours)	2 - 6 hours* (4 hours)	18 - 22 hours* (4 hours)		2 - 48 hours* (46 hours)
	Trophozoites	Meronts 8N	Meronts 4N	Other sexual stages (gametocytes, gametes and oocysts)	All parasite stages
0	124.7 ± 20.4	14.7 ± 13.6	2.0 ± 2.0	120.0 ± 13.1	261.3 ± 28.0
10	67.58 ± 30.8	5.5 ± 6.4	0.5 ± 1.0	36.0 ± 15.7	109.5 ± 25.9
20	20.7 ± 23.7	3.3 ± 3.1	0.0 ± 0.0	5.3 ± 9.2	29.3 ± 22.5
30	8.0 ± 5.7	7.0 ± 4.2	0.0 ± 0.0	0.0 ± 0.0	15.0 ± 1.4
50	12.0 ± 8.7	3.3 ± 3.1	0.0 ± 0.0	0.0 ± 0.0	15.3 ± 11.5

[2] Pooled results of 3 experiments (with one experiment conducted in duplicate for the 10 µg/ml concentration in lieu of the 30 µg/ml concentration).
* The time of addition of the drug (duration of drug exposure).
TZ = tizoxanide.

Table 14: Immunofluorescent evaluation of stage dependent anticryptosporidial activity of tizoxanide glucuronide on *Cryptosporidium parvum* development on HCT-8 cells[3].

Hours of incubation with TZg / TZg concentration (µg/ml)	Number (mean ± S.D.) of parasites/20 microscopic fields				
	0 - 2 hours* (2 hours)	2 - 6 hours* (4 hours)	18 - 22 hours* (4 hours)		2 - 48 hours* (46 hours)
	Trophozoites	Meronts 8N	Meronts 4N	Other sexual stages (gametocytes, gametes and oocysts)	All parasite stages
0	197.5 ± 34.5	27.0 ± 17.1	11.0 ± 13.1	291.0 ± 44.3	526.5 ± 88.4
10	223.0 ± 4.2	34.0 ± 0.0	9.0 ± 7.1	301.0 ± 77.8	567.0 ± 66.5
20	114.0	8.0	4.0	232.0	358.0
30	72.0	4.0	0.0	144.0	220.0
50	44.0 ± 14.0	10.0 ± 5.7	4.0 ± 0.0	76.0 ± 17.0	134.0 ± 36.8

[3] Pooled results of 4 experiments (with the 10 µg/ml and 50 µg/ml concentrations tested in only two of the experiments, and the 20 µg/ml and 30 µg/ml concentrations tested in only one experiment).
* The time of addition of the drug (duration of drug exposure).
TZg = tizoxanide glucuronide.

In summary, the results show that at 46 hours, nitazoxanide (10 µg/ml) reduced parasite counts in 2-hour old cultures by 88% with 14% toxicity to HCT-8 cells. Exposure of 2-hour old and 18-hour old cultures to nitazoxanide (10 µg/ml) for 4 hours also resulted in a similar reduction in parasite counts. Concentrations of nitazoxanide above 10 µg/ml were cytotoxic (36% - 39%). The concentration of tizoxanide and tizoxanide glucuronide (10 to 50 µg/ml) that reduced parasite counts in 2-hour and 18-hour old cultures also caused fragilation and peeling of HCT-8

cells. In the absence of complete information, these results show a decrease in parasite counts when nitazoxanide (10 µg/ml) was added to 2-hour and 18-hour old cultures for 4 or 46 hours. However, the effect of the drugs on the different stages is unclear.

In another experiment, the effect of nitazoxanide and its metabolites on cryptosporidial growth was measured using an enzyme immunoassay (EIA)[6,7]. The experimental conditions and the antiserum used for detection of the parasite were same as in the immunofluoresence assay. The percentage inhibition (I) was calculated as follows:

$$I = \frac{\text{(optical density in infected wells containing drug) - (optical density in uninfected wells containing drug)}}{\text{(optical density in infected wells without drug) - (optical density in uninfected wells without drug)}} \times 100$$

The results in Figures 1 to 4 show inhibition of *C. parvum* at different time points in the presence of different concentrations (10 - 50 µg/ml) of nitazoxanide or its metabolites. Please note that at these concentrations, toxicity to HCT-8 cells ranged between 14 - 39% with nitazoxanide and fragilation/peeling of HCT-8 cells was observed with tizoxanide and tizoxanide glucuronide. The results in Figure 1 show 70% inhibition of parasites (sporozoite stage) after exposure of cultures to nitazoxanide (10 µg/ml) for 2 hours. The metabolites, tizoxanide and tizoxanide glucuronide, were less effective against the sporozoites (about 50% and 10% inhibition at a concentration of 10 µg/ml, respectively). Parasite inhibition was lower (40 - 60%) when 2-hour old cultures were exposed to tizoxanide glucuronide and nitazoxanide (10 µg/ml) for 4 hours (Figure 2). The sponsor has stated that asexual meront stages were observed at this time point. Under similar conditions, tizoxanide was less active (5% parasite inhibition; Figure 2). The activity of nitazoxanide and its metabolites seems to be better when the cultures (2-hour old) were exposed to the drug for 46 hours (Figure 4) compared to 4 hours (Figure 2). Exposure of 18-hour old cultures of *C. parvum* (sponsor has stated that the sexual stages are observed at this time point; Figure 3) to nitazoxanide (10 µg/ml) for 4 hours inhibited parasites by about 30% compared to tizoxanide (50%) and tizoxanide glucuronide (98%). Please note that in the previous study examining activity of nitazoxanide in sporozoite infected A-549 cells, the meront and gamont stages were observed at 48 hours post-infection. Although, the sponsor has stated that nitazoxanide is active against the different stages (asexual meronts and sexual stages), it is unclear if the drug has an effect on these different stages as the morphology of the stages of the parasite at the different time points was not described nor were the photographs taken.

Figure 1: Effects of nitazoxanide, tizoxanide and tizoxanide glucuronide on *Cryptosporidium parvum* sporozoites in HCT-8 cells. Agents were added in cultures at the time of incubation of 1.5 to 2 x 10^5 sporozoites per well, and left in culture for 2 hours. Results of EIA detection, performed after a further 46 hour incubation, are expressed as mean (± 1 SD) inhibition percentages. Pooled data from 5 independent experiments.

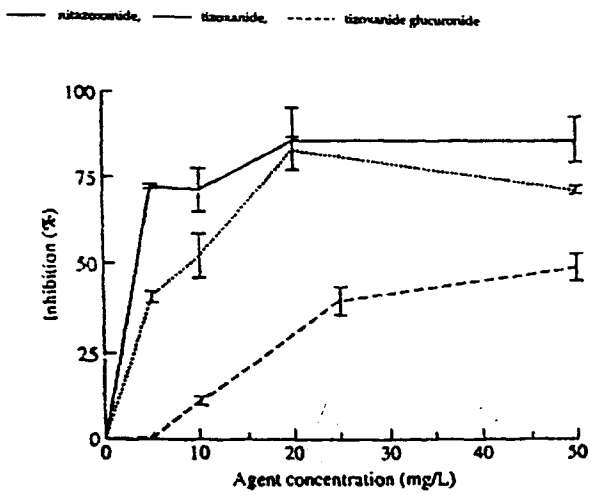


Figure 2: Effects of nitazoxanide, tizoxanide and tizoxanide glucuronide on the asexual development of *Cryptosporidium parvum* in HCT-8 cells. Agents were added in cultures 2 hours after addition of 1.5 to 2 x 10^5 sporozoites per well, and left in culture for 4 hours. Results of EIA detection, performed after a further 46 hour incubation, are expressed as mean (± 1 SD) inhibition percentages. Pooled data from 3 independent experiments.

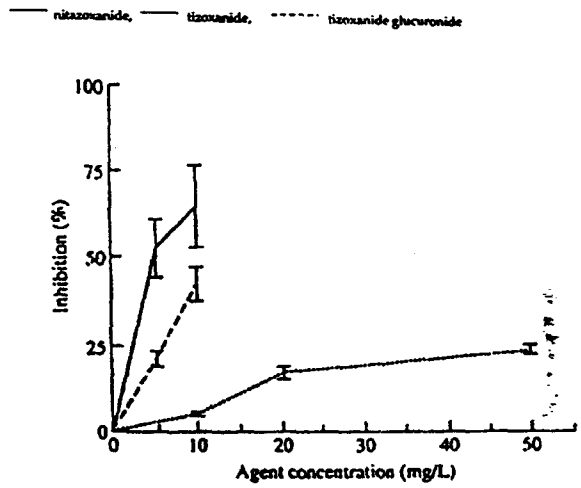


Figure 3: Effects of nitazoxanide, tizoxanide and tizoxanide glucuronide on the sexual development of *Cryptosporidium parvum* in HCT-8 cells. Agents were added in cultures 18 hours after addition of 1.5 to 2 x 10^5 sporozoites per well, and left in culture for 4 hours. Results of EIA detection, performed after a further 46 hour incubation, are expressed as mean (± 1 SD) inhibition percentages. Pooled data from 5 independent experiments.

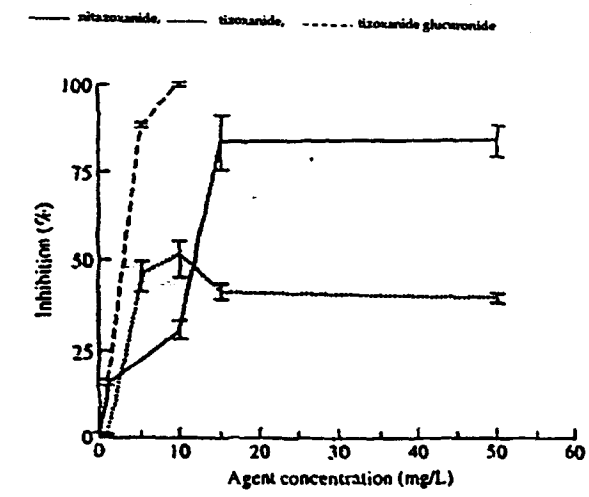
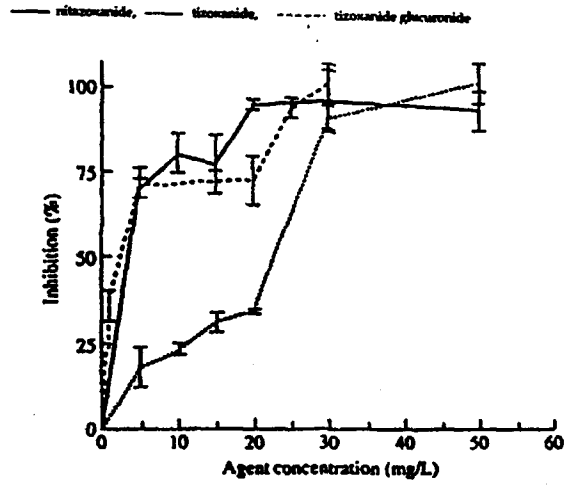


Figure 4: Effects of nitazoxanide, tizoxanide and tizoxanide glucuronide on asexual and sexual stage development of *Cryptosporidium parvum* in HCT-8 cells. Agents were added in cultures 2 hours after addition of 1.5 to 2 x 10^5 sporozoites per well, and left in culture for 46 hours. Results of EIA detection are expressed as mean (± 1 SD) inhibition percentages. Pooled data from 20 independent experiments.



Based on the above results, the concentration of the drug required to inhibit the different stages of the parasite by 50% (IC_{50s}) was determined. The nitazoxanide IC_{50} against asexual and sexual stages of the parasite was <12 µg/ml (Table 15). The IC_{50} values for tizoxanide was comparable to nitazoxanide against the sporozoite and sexual stages and higher (> 10 fold) against the asexual meront stages (Table 15). Tizoxanide glucuronide was the most active agent against the sexual stages but least effective against the sporozoite stage (Table 15).

Table 15: EIA evaluation of IC_{50} (mg/L) of nitazoxanide, tizoxanide, and tizoxanide glucuronide on sporozoite, asexual and sexual stages and complete development of *Cryptosporidium parvum* in HCT-8 cells. Mean values from 6 wells carried out in triplicate.

	IC_{50} (mg/L)			
Agent	Sporozoite stage	Asexual stages	Sexual stages	Complete development
Nitazoxanide	5.8	4.7	11.8	1.2
Tizoxanide	8.6	>50	9	22.6
Tizoxanide-glucuronide	45.2	11.7	2.8	2.2

The inhibitory effect of nitazoxanide (10 µg/ml) measured by the two methods i.e., immunofluorescence and EIA were similar (88% by the immunofluorescence assay and 75-85% by EIA; Table 12 and Figure 4). However, a difference in the inhibitory effects of tizoxanide and tizoxanide glucuronide were observed using the two methods. Tizoxanide at a concentration of 10 µg/ml showed 58% inhibition of parasite by immunofluorescence (Table 13) and about 25% inhibition by EIA (Figure 4). In the case of tizoxanide glucuronide, no effect was observed at 10 µg/ml by immunofluorescence (Table 14) and about 70% parasite inhibtion was observed by EIA (Figure 4). The sponsor has stated that this difference was possibly due to interference of the color in absorbance measurements at 405 nm by tizoxanide and tizoxanide glucuronide (yellow) or due to effect of different mass of the various stages on the optical density values obtained by EIA. Based on literature review, Nomarski interference contrast microscopy and electron microscopy methods are used for morphological identification of the different stages of *C. parvum* in cell culture. It would have been worthwhile to measure the activity of the drug against different stages of the parasite by these methods.

In another experiment, the effect of nitazoxanide against the different stages of *C. parvum* was examined using electron microscopy. For this, HCT-8 cells grown on tissue culture inserts (— µm pore size) were infected with sporozoites (inoculum same as in previous experiments). Two hours after infection, nitazoxanide (10 µg/ml) was added and the incubation continued for 46 additional hours. Thin sections from the inserts were processed for electron microscopic examination. The results in Table 16 show the number of parasites per 45-46 mm of tissue culture insert. In this single experiment, the number of zygotes in cultures in the presence of nitazoxanide was reduced compared to control cultures without drug (Table 16). However, 2 macrogametes were observed in the nitazoxanide treated cultures but not in control cultures. The number of parasites counted in the cultures (in the presence and absence of drug) is too small to conclusively state the effect of nitazoxanide on the different stages of the parasite. At 10 µg/ml nitazoxanide, the toxicity to HCT-8 cells was 14%. The metabolites and other comparator drugs were not used in this experiment.

Table 16: Transmission electron microscopy counting of *Cryptosporidium parvum* stages in cultures in the presence of nitazoxanide (10 mg/L) for 46 hours.

	Cryptosporidium parvum stages (number of parasites/tissue culture insert length)				
Culture condition	zygote	macrogamete	microgamete	meront 8N	meront 4N
Cryptosporidium parvum infected culture (control)	5	0	1	1	1
Cryptosporidium parvum infected culture with nitazoxanide (10mg/L)	0	2	0	1	0

3.4. Effect of protein binding on the activity of nitazoxanide:

Nitazoxanide and tizoxanide were both shown to exhibit high protein binding (> 99%). All the *in vitro* experiments were conducted in the presence of 5% to 20% fetal calf or bovine serum. The specific effect of protein binding on *in vitro* activity was not examined.

4. ACTIVITY *IN VIVO*:

The activity of nitazoxanide against *C. parvum* was examined in several animal models such as the (a) suckling mice, (b) scid mice, (c) immunosuppressed rat, and (d) gnotobiotic piglets.

4.1. Suckling mice:

Two studies examined the activity of nitazoxanide against *C. parvum* in suckling mice. In one study[8], 2-day old normal (immunocompetent) suckling mice were infected with *C. parvum* oocysts (10^5: obtained from infected calves) by the oral route [this study is the same as reviewed previously, NDA# 20-871 (N-000), microbiology review dated 06-01-98]. Oocysts were observed in the rectal swabs obtained on day 2 of infection. Treatment with nitazoxanide (1.3 mg b.i.d. for 7 days) by the oral route was initiated 3 days post-infection. Oocyst count in rectal swabs was evaluated daily for up to 7 days after discontinuation of treatment. The results (expressed as the number of oocyst per 100 oil immersion fields) show that treatment of infected mice with nitazoxanide decreased the oocyct count compared to the untreated control animals (Table 17). No vehicle treated mice were used for comparison of drug activity. Also, no attempts were made to measure the activity of the drug in tissues obtained from different parts of the intestine.

Table 17: Efficacy of nitazoxanide against *Cryptosporidium* infection in experimentally infected mice.

Mice No.	No. of oocyst detected per oil immersion field							
	At 3rd day of treatment		At last day of treatment		At 3rd day post-treatment		At 7th day post-treatment	
	Control group	Treated group	Control group	Treated group	Control group	Treated group	Control group	Treated group
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
Total	35	8.0	4.2	0.0	30	0.0	10	0.0
Mean	3.5	0.8	4.2	0.0	3.0	0.0	1.0	0.0
Efficacy		60%		100%		100%		100%

Note: Treatment started 2 days after the beginning of oocyst shedding.
Microscopic examination of oocysts after Ziehl-Neelsen staining.

In another study in suckling mice[9], the activity of 2 different formulations of nitazoxanide was examined against a bovine strain of *C. parvum* (strain AUCp1). Mice (16-24 per group) were infected with 1 to 2 x 10^5 oocysts by oral gavage. Treatment with 2 different formulations (oral and injectible) of nitazoxanide (100 or 150 mg) by the oral route was initiated 4 hours after infection and continued for 5 additional days. Paromomycin was used as the comparator. Vehicle (water or 1% DMSO) treated animals were used as controls. The number of oocysts in the tissue (pylorus to rectum) was determined at the end of treatment i.e., day 6. The results in Table 18 show the calculated mucosal oocyst levels in treated animals (expressed as mean percentage oocyst counts ± SE compared to controls). The oocysts counts for each of the treatment groups were not included. A 50 to 74% reduction in the mucosal oocyst counts was observed in animals treated with 100 mg/kg of the two nitazoxanide formulations (Table 18). The authors have stated that the powder formulation was obtained from Romark laboratories and contained 70.8% active nitazoxanide while the injectible formulation was obtained from Blue Ridge Pharmaceuticals and contained 20% active nitazoxanide. Additional details on the two formulations were not provided. Although, the powder formulation contained a higher concentration of active nitazoxanide, it was not as effective as the injectible formulation in reducing oocyst counts in the intestinal tissues when administered orally. This could be due to poor bioavailability of the powder formulation. At a higher dose of nitazoxanide (150 mg/kg), the reduction in mucosal oocysts counts was about 95%. The sponsor has stated in the footnote of Table 18 that this dose was moderately toxic and only 14 out of 25 mice survived treatment. Paromomycin (50 mg/kg) was more effective in reducing oocysts counts than nitazoxanide. The shedding of oocysts in the stool was not examined.

Table 18: Efficacy of NTZ and paromomycin against *C. parvum* in the neonatal mouse model.

Compound	Dose (mg/kg of body weight)[a]	Formulation	Oocyst level (% of con trol)[b] (mean ± SE)
NTZ	100	Powder	42.3 ± 4.6[c]
	100	Injectible	26.0 ± 4.5[c]
	150[d]	Injectible	4.3 ± 1.0[c]
Paromomycin	50	Powder	1.2 ± 0.6[c]

[a]Mice were treated at a constant dose rate daily for 6 days.
[b]Mean numbers of oocysts in treated mice are expressed as percentages of the mean number of oocysts recovered from control mice (taken as 100%).
[c]Treated mice and control mice were significantly different ($p \leq 0.05$).
[d]This dosage appeared moderately toxic; 14 of 25 mice survived the treatment period.

4.2. Scid mice:

The activity of nitazoxanide and desacetyl nitazoaxanide alone or in combination with paromomycin against *C. parvum* was determined using a **scid mouse model** [this study is the same as reviewed previously, NDA# 20-871 (N-000), microbiology review dated 06-01-98]. Mice were injected intraperitoneally with antibodies to interferon gamma and 2 hours later infected with *C. parvum* oocysts (10^7) by the oral route. Six days post-infection, different doses (50, 100 or 200 mg/kg) of nitazoxanide (lot# 12049) were administered twice daily for 10 days by oral gavage. Paromomycin was used as a positive control. Body weight of the mice was not altered by infection or treatment. Mice were examined for the presence of oocyst in the fecal samples at multiple time points during the course of the study. The presence of parasites in the tissues, including the pyloric region of the stomach, mid section of the small intestine, ileum, cecum, proximal colon, and liver/gall bladder was determined on day 20 of challenge (i.e., 5 days after discontinuation of treatment). The results in **Figure 5** indicate that in animals treated with nitazoxanide at 100 mg/kg from days 6 to 16 of infection, oocyst shedding decreased in comparison to the vehicle treated mice ($p < 0.001$). The extent of mucosal infection (**Figure 6**) was also low in mice treated with 100 mg/kg nitazoxanide ($p = 0.0002$). In this study the anticryptosporidial activity of nitazoxanide was comparable to paromomycin.

Figure 5: Oocyst shedding of 6 groups (7 mice each) of weaned male C.B-17 SCID mice infected with 10^7 oocysts of the GCH1 isolate. The experimental drug, Phavic-1 (nitazoxanide) was dissolved in DMSO and treatments were administered as follows:
Group 1 = 200 mg/kg/day Phavic-1,
Group 2 = 100 mg/kg/day Phavic-1,
Group 3 = 50 mg/kg/day Phavic-1,
Group 4 = 200 mg/kg/day Phavic-1 (uninfected control to determine toxicity of drug),
Group 5 = 2000 mg/kg/day Paromomycin (positive control),
Group 6 = DMSO (vehicle control).
All treatments were administered orally in two divided doses/day for 10 days.
The mice were maintained for an additional 5 days following the cessation of treatment.

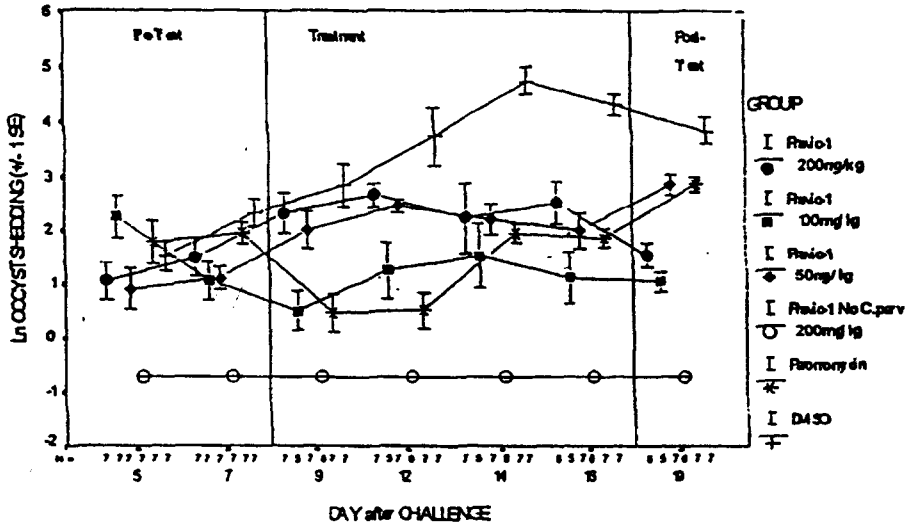
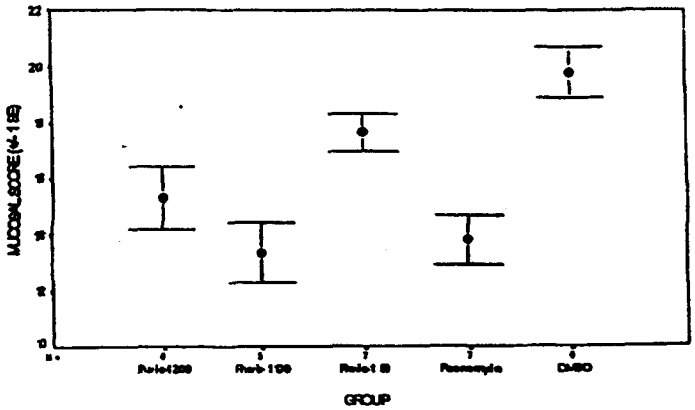


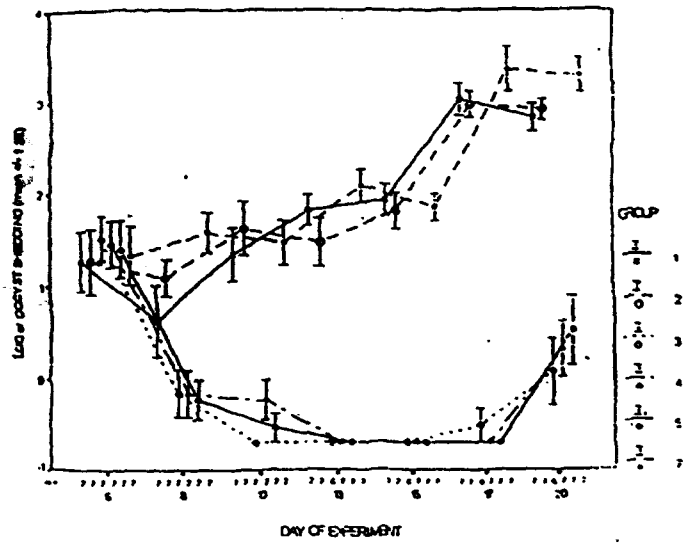
Figure 6. Extent of mucosal infection on day 20 of infection, expressed as a mean total score of 5 intestinal sites (pyloric region of the stomach, mid-small intestine, ileum, cecum and proximal colon) for the different treatment groups described in Figure 5.



The anti-cryptosporidial activity of nitazoxanide in the groups of animals treated with either 200 mg/kg or 50 mg/kg was statistically similar. The sponsor stated that this could be due to the fact that 50 mg/kg was an insufficient dose and 200 mg/kg could cause subclinical toxicity by reducing or eliminating normal gut flora.

Another experiment conducted in scid mice used the same design as the first one but a different lot of nitazoxanide (lot # 001). The study was also **published by Theodas *et al.*,** (1998)[3] and has been reviewed earlier [NDA# 20-871 (N-000), microbiology review dated 06-01-98]. Paromomycin alone or in combination with nitazoxanide was effective in reducing the parasite load during the period of drug administration (Figures 7 and 8). However, oocyst count increased in all the groups after discontinuation of treatment (Figure 7). Unlike the first experiment, nitazoxanide was not effective in reducing the shedding of oocyst in the stool (Figure 7) nor in decreasing the extent of mucosal infection (Figure 8) at the doses tested i.e., 200 and 100 mg/kg. The reason for this variability in activity between the two different experiments is unclear. Although differences in the particle size were observed between the 2 lots, the specifications of the lot #001 were within the range of those observed among the various lots used in the clinical trials (for details see chemistry review to the original NDA# 20-871)

Figure 7: Oocyst shedding of 7 groups (7 mice each) of weaned male C.B-17 SCID mice infected with 10^7 oocysts of the GCH1 isolate. The experimental drug, Phavic-1 (nitazoxanide) was dissolved in 100% DMSO and administered orally in two divided doses of 30 µl each per day. Paromomycin was dissolved in drinking water to a concentration of 10 mg/ml. Treatments were administered as follows:
Group 1 = 200 mg/kg/day Phavic-1,
Group 2 = 100 mg/kg/day Phavic-1,
Group 3 = 200 mg/kg/day Phavic-1 + 2500 mg/kg/day paromomycin,
Group 4 = 100 mg/kg/day Phavic-1 + 2500 mg/kg/day paromomycin,
Group 5 = 2500 mg/kg/day Paromomycin (positive control),
Group 6 = 200 mg/kg/day Phavic-1 + 2500 mg/kg/day paromomycin (uninfected control for determining toxicicty)
Group 7 = 30 µl DMSO orally twice a day (vehicle control).
The mice were maintained for an additional 5 days following the cessation of treatment



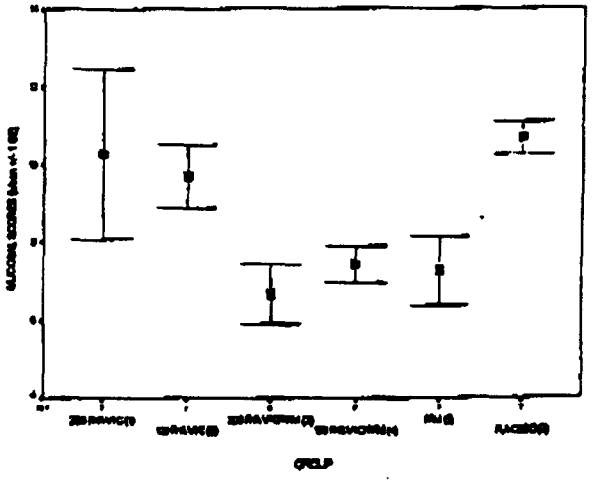


Figure 8: Extent of mucosal infection on day 20 of infection, expressed as a mean score of 5 intestinal sites (pyloric region of the stomach, mid-small intestine, ileum, cecum and proximal colon) for the different treatment groups described in Figure 7.

In another experiment, the activity of desacetyl nitazoxanide alone or in combination with paromomycin against *C. parvum* was examined. The experimental design was similar to that described above except that nitazoxanide (the parent compound) was not used for comparison. The results in Figures 9 and 10 show that desacetyl nitazoxanide does not exhibit any activity against *C. parvum* as measured by presence of oocysts in the stool as well as the mucosal scores. Paromomycin, however, significantly decreased the shedding of oocyst in the stool as well as the mucosal scores in the gastro-intestinal tract. The activity of desacetyl nitazoxanide in combination with paromomycin against *C. parvum* was similar to paromomycin alone.

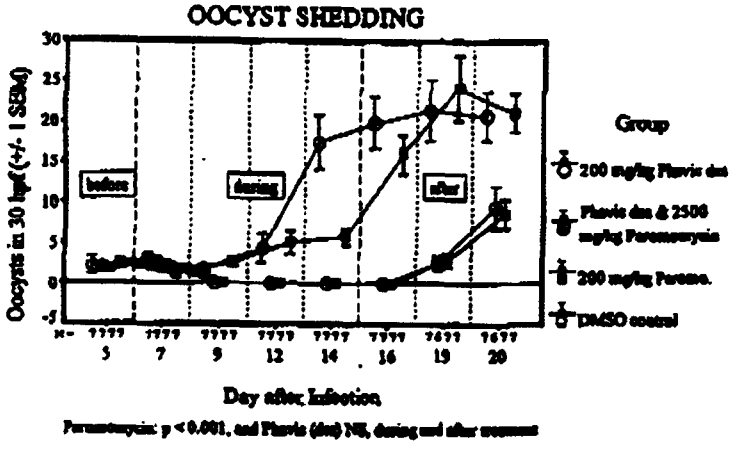


Figure 9: Oocyst shedding of 4 groups (7 mice each) of weaned male C.B-17 SCID mice infected with 10^7 oocysts of the GCH1 isolate. Treatments were administered as follows:

Group 1 = 200 mg/kg/day Nitazoxanide (des),

Group 2 = 200 mg/kg/day Nitazoxanide (des) + 2500 mg/kg/day paromomycin,

Group 3 = 2500 mg/kg/day paromomycin dissolved in drinking water,

Group 4 = 30 µl DMSO orally twice a day (vehicle control).

The mice were maintained for an additional 5 days following the cessation of treatment

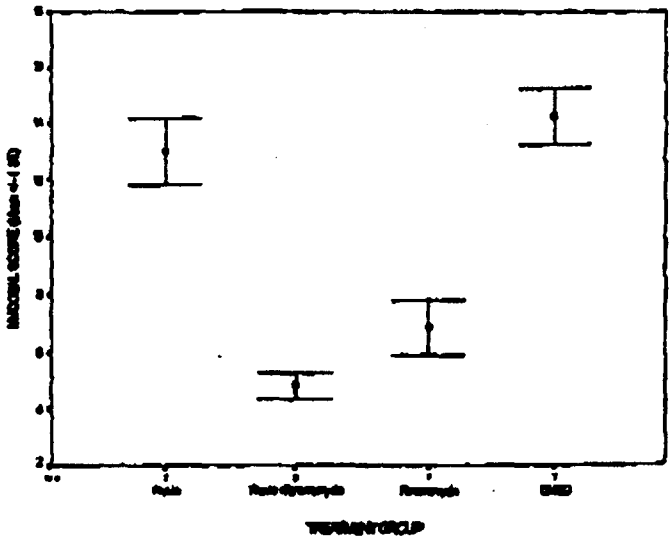


Figure 10: Extent of mucosal infection on day 20 of infection, expressed as a mean score of 5 intestinal sites (pyloric region of the stomach, mid-small intestine, ileum, cecum and proximal colon) for the different treatment groups described in Figure 9.

4.3. Immunosuppreased rats:

In immunosuppressed rats, the activity of nitazoxanide against *C. parvum* was compared to paromomycin and sinefungin[11]. The rats were immunosuppressed by subcutaneous administration of 25 mg hydrocortisone acetate twice a week for 5 weeks before infection and immunosuppression maintained for 3 additional weeks. Rats were infected with 10^5 oocysts (obtained from infected calves) by oral gavage. On day 7 post-infection, the drugs (dissolved in 5% DMSO) were administered orally three times daily for 8 days. Vehicle-treated animals were used as controls. The fecal samples collected at different time points for up to 21 days post-infection were examined using a phase contrast microscope (after carbolfuchsine staining) and the oocyst counted per 10 microscopic fields ( ). The percentage inhibition of oocyst in drug treated animals was compared to the control groups. The results in Table 19 show that the oocyst counts in the control animals decreased with time, suggesting that the infection was cured spontaneously despite immunosuppression. In rats treated with nitazoxanide (100 mg and 200 mg), a 2-fold reduction in oocyst counts was observed compared to vehicle-treated control animals. The activity of paromomycin was similar to nitazoxanide while sinefungin was 2-fold more active. The oocyst counts increased in the sinefungin and paromomycin treated animals after discontinuation of treatment while no increase was observed in the nitazoxanide treated group. The mean oocyst counts for all treatment groups on days 16 to 21 (i.e., 1 to 6 days after discontinuation of drug treatment) appear to be similar to the control group (Table 19). However, the sponsor has stated that the oocyst counts in nitazoxanide treated animals and controls were significantly different.

Table 19: Mean number of oocysts shed per 10 microscopic fields (x 400 magnification) by treatment group.

Drug (mg/kg)	Period D7	D8-9	D10-11	D12-13	D14-15	D16-17	D18-19	D20-21	D8-15 (during Rx)	D16-21 (After Rx)
NTZ 50										
Mean	83.55	38	33.33	26.6	12.45	9.1	5.45	4.2	27.6	6.19
SD	(52.9)	(19.3)	(19.9)	(22.4)	(12.5)	(7.9)	(5.46)	(4)	(20.9)	(6.4)
% inhibition	0%	31.7%	0%	14.4%	44.1%	16.5%	48%	50%	14.2%	37.9%
P*	.1805	.2338	.6228	.1641	.0351	.8148	.0184	.0419	.0444	.0110
NTZ 100										
Mean	87.45	28.2	15.2	14.45	10.65	6.65	3.3	2.75	17.12	4.23
SD	(56.1)	(27.1)	(12.5)	(11.9)	(11)	(8)	(2.9)	(1.86)	(18.0)	(5.3)
% inhibition	0%	49.3%	50.6%	58.9%	52.2%	39%	68.5%	67.2%	53.0%	57.6%
P*	.1595	.0053	.0038	.0020	.0158	.2577	.0006	.0050	<.0001	<.0001
NTZ 200										
Mean	81.85	28	19.45	10.3	5.15	7.75	3.9	2	15.73	4.55
SD	(42.6)	(18.4)	(15.4)	(7.6)	(4.4)	(7.18)	(3)	(11.4)	(15.3)	(5.1)
% inhibition	0%	49.7%	36.8%	70.7%	76.9%	28.9%	2.8%	76.2%	56.8%	54.4%
P*	.1016	.0186	.0523	.0005	.0002	.5187	.0044	.0009	<.0001	.0002
Paromomycin 100										
Mean	81.25	61	20	1.76	3.9	5.87	6.72	9.35	21.67	8.15
SD	(32.5)	(49.7)	(22.3)	(2)	(5.5)	(10)	(10.9)	(11.5)	(34.7)	(9.7)
% inhibition	0%	0%	35%	95%	82.5%	46.1%	36%	0%	40.5%	18.3%
P*	.2298	.6701	.2904	.0093	.0153	.1861	.7014	.8313	.0035	.2310
Sinefungin 10										
Mean	79.25	15.75	13.75	1.77	1.05	1.7	3.1	9.65	8.08	4.82
SD	(91.6)	(19.1)	(17.6)	(3.1)	(1.96)	(3.2)	(5.9)	(18.9)	(13.7)	(11.1)
% inhibition	0%	71.7%	55.3%	94.9%	95.3%	84.4%	70.4%	0%	77.8%	51.7%
P*	.7862	.0364	.1042	.0051	.0049	.0150	.0331	.1865	<.0001	.0006
Controls										
Mean	60.9	55.7	30.78	35.2	22.3	10.9	10.5	8.4	36.4	9.97
SD	(38.3)	(41)	(16.9)	(21.4)	(16.3)	(9.7)	(7)	(6.9)	(28.7)	(8.0)

* Mean oocysts shed for the treatment group compared to mean oocysts shed for controls, non-parametric Wilcoxon rank sum test

The ileum tissue from nitazoxanide (different doses) treated animals only was examined for the presence of parasite after hematoxylin-eosin staining. A 2 to 4 fold decrease in mucosal infection was observed in nitazoxanide treated animals compared to controls (Table 20). The variability in the number of parasites among the different rats within a group was not shown. Paromomycin and sinefungin treated animals were not used for comparison of drug activity.

Table 20: Extent of mucosal infection in sample of rats from the nitazoxanide-treated and untreated control groups presented in comparison with oocysts shedding on days 20-21.

Dose (mg/kg/day)	Mean no. of oocysts/10 fields days 20-21	Inhibition %	No. of parasites in 10 villi of the ileum	Inhibition %
NTZ 50	4.20	50%	22	46%
NTZ 100	2.75	67%	17	59%
NTZ 200	2.00	76%	10	76%
Untreated control	8.4	-	41	-

4.4. Gnotobiotic piglets:

The effect of nitazoxanide treatment was examined in gnotobiotic piglets (24 hour old, derived from 2 litters) infected with the GCH1 strain of *C. parvum* [this study was reviewed previously NDA# 20-871 (N-000), microbiology review dated 06-01-98][17]. The piglets were inoculated orally with 2 x 10^7 oocysts. At 56 hours post-infection, the animals were treated with nitazoxanide (125 mg/kg b.i.d. for 11 days) by the oral route (mixed with milk). Activity of nitazoxanide was compared with paromomycin (250 mg/kg bid for 11 days) or vehicle administered by the same route. Fecal oocyst counts and extent of mucosal infection in 12 intestinal sections (pylori of stomach, duodenum, 7 other small intestinal sections, terminal ileum, cecum, and colon) were determined. Body weights were not significantly altered in animals from either of the groups, however, results in Table 21 show paromomycin to be more effective than nitazoxanide with respect to reduction in oocyst or mucosal scores. Nitazoxanide decreased the mucosal scores by about 50% (Figure 13). However, due to the high variability in the oocyst score there appears to be no significant difference in nitazoxanide treated and vehicle treated groups at these time points (Figure 14). While the sponsor has analyzed the cumulative oocyst shedding over a period of 13 days and has shown a decrease in the number of oocysts in the group treated with nitazoxanide compared to infected controls ($p = 0.0039$; Figures 15 and 16), the statistical significance of the difference between the nitazoxanide treated vs vehicle treated animals at different time points was not determined. The incidence of diarrhea also appears to be same in the 2 groups. The sponsor has stated that all the infected animals irrespective of treatment, exhibited watery diarrhea which was white gray in color. Diarrhea was also observed in uninfected piglets treated with nitazoxanide although the appearance of the diarrhea (pasty and yellow in color) was different. The diarrhea resolved in piglets administered paromomycin.

Table 21: Oocyst shedding and extent of mucosal infection in *C. parvum* infected gnotobiotic piglets.

	Days after challenge												Mean Mucosal
Pig #	2@	3	4	5	6	7	8	9	10	11	12	13	Scores (SD)
1. Plach.	0/	0/d	1/d	3/d	4/d	3/d	2/d	2/d	2/d	2/d	2/d	3/d	16
2. Plach.	0/d	0/d	4/d	4/d	3/d	3/d	3/d	4/d	3/d	2/d	2/d	3/d	15
3. Plach.	1/d	2/d	5/d	3/d	2/d	2/d	4/d	3/d	5/d	4/d	2/d	3/d	10
4. Plach.	1/	2/d	2/d	3/d	2/d	3/d	5/d	3/d	4/d	3/d	3/d	3/d	6
5. Plach.	0/d	1/d	2/d	1/d	3/d	2/d	2/d						N/A
													11(±4.6)
6. NTZ	0/d	1/d	1/d	4/d	3/d	2/d	2/d	3/d	3/d	3/d	2/d	2/d	7
7. NTZ	0/	2/d	2/d	4/d	3/d	2/d	3/d	2/d	1/d	2/d	1/d	1/d	3
8. NTZ	0/	1/d	2/d	3/d	2/d	1/d	3/d	1/d	1/d	1/d	1/d	1/d	5
9. NTZ	1/d	3/d	2/d	2/d	2/d	2/d	3/d	2/d#					N/A
10. NTZ	0/d	0/d	2/d	1/d	2/d	2/d	3/d#						N/A
													5(±2)
11. PRM	0/d	1/d	2/d	2/d	0/d	1/d	1/d	0/1	0/1	0/1	0/1	0/1	0
12. PRM	1/d	2/d	2/d	2/d	1/d	0/d	0/1	1/1	0/1	0/1	0/1	0/1	1
13. PRM	0/	1/d	2/d	1/d	0/d	0/d	0/d						N/A
													0.5(±.2)
14. Cont.	0/	0/	0/	0/d*	0/d*	0/d*	0/d*	0/d*	0/d*	0/d*	0/d*	0/d*	N/A
15. Cont.	0/	0/	0/	0/d*	0/d*	0/d*	0/d*	0/d*	0/d*	0/d*	0/d*	0/d*	N/A
16. Cont.	0/	0/	0/	0/d*	0/d*	0/d*	0/d*						N/A

Placebo = milk
NTZ = nitazoxanide
PRM = paromomycin
Control = uninfected control treated with 250 mg/kg NTZ

@ Onset of treatment 56 hours after challenge coinciding with the onset of diarrhea in more than 50% of piglets.
Oocyst shedding: 0 = no oocysts detected in fecal smear; 1 = ≤10 detected in the entire smear; 2 = ≤ 25; 3 = ≤ 50; 4 = ≤ 100; and 5 = ≥ 100.
d = watery white-grey diarrhea (representing maldigestion and malabroption); d* drug-related yellow diarrhea (normal digestion); l = loose feces (higher water content than normal).
# piglet euthanized because of poor health associated with diarrhea.

Figure 13: Individual mucosal scores of piglets in varying treatment groups. Despite the small numbers, mucosal scores were significantly related to group (p = 0.0355).

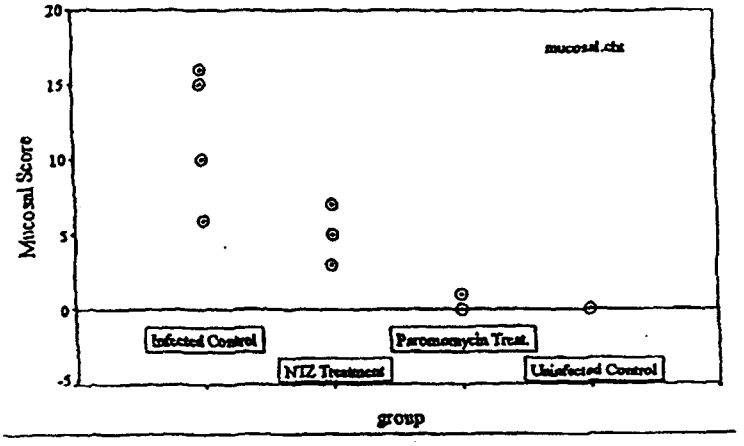
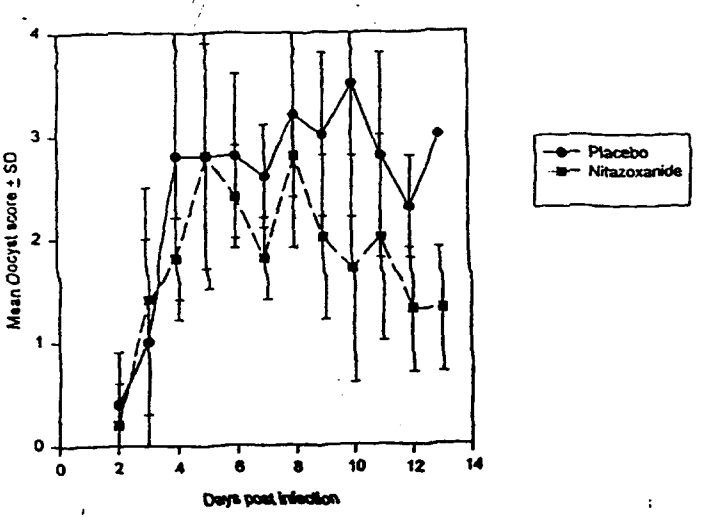


Figure 14: Effect of nitazoxanide on oocyst score in gnotobiotic piglets.



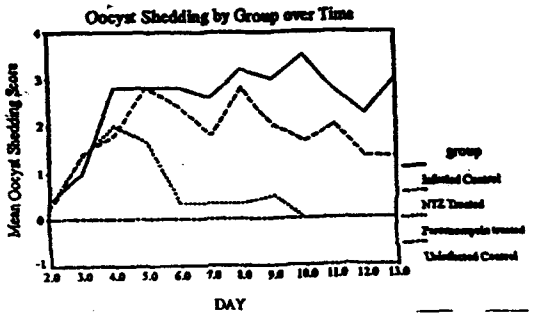
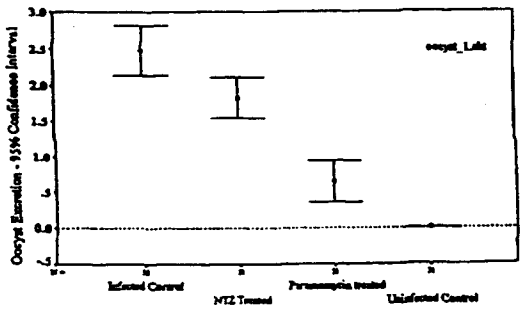


Figure 15: The mean daily oocyst excretion score by group.

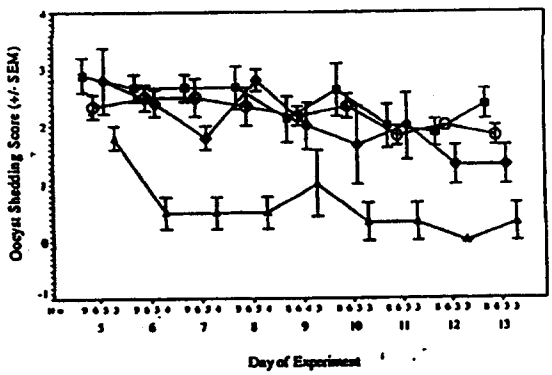
Figure 16: Overall mean oocyst excretion score in each group. Each group is significantly different from each of the others. These differences are detectable despite inclusion of the score from the first few days of treatment, when little effect of treatment would have been expected.



groups	Mann-Whitney U- Wilcoxon Rank Sum test	
control uninfected and paromomycin treated	p <	0.0001
paromomycin vs. nitazoxanide treated	<	0.0001
nitazoxanide treament vs. infected control	=	0.0039
paromomycin vs. infected control	<	0.0001

In another study in gnotobiotic piglets[3], the effect of nitazoxanide on oocyst shedding was examined using the same experimental design as the previous study except that two different doses of nitazoxanide (62.5 or 125 mg b.i.d for 11 days) were tested. The results show that the oocyst scores in animals treated with nitazoxanide (125 or 250 mg) were similar to that of untreated animals (Figure 17). The activity of paromomycin against *C. parvum* was same as that observed in the previous study. It was stated that nitazoxanide induced diarrhea in these animals whereas paromomycin was effective in resolving diarrhea.

Figure 17: Fecal oocyst excretion scores of infected piglets treated with nitazoxanide at either 125 (o) or 250 ( ) mg/kg/day, placebo (■) or paromomycin at 500 mg/kg/day (▲). In multiple regression analysis, the oocyst excretion score was found to be significantly related to treatment group, with highest scores being observed in the placebo group, followed by the lower-dose nitazoxanide group and then the higher dose nitazoxanide group, and the lowest scores being observed in the paromomycin group (F = 42.507; p <0.001). Further comparison revealed that during days 7 through 14, the scores for the piglets treated with nitazoxanide 250 mg/kg/day were significantly lower than those for the placebo treated piglets (Z = -3.258; p = 0.001, two tailed Wilcoson signed rank test). In contrast, subgroup comparison of the infected placebo-treated control piglets and piglets treated with nitazoxanide at 125 mg/kg/day did not reveal any significant difference. Values are means ± standard errors of the means (SEM).



5. CLINICAL MICROBIOLOGY:

5.1. Diagnosis of *Cryptosporidium parvum* infection:

Diagnosis of *Cryptosporidium* is based on identification of oocyst(s) in stool samples. There is no consensus on the number of stool samples that should be tested to confirm absence of the parasite since shedding of oocysts in the fecal samples is intermittent. However, examination of three or more stool samples within 5 days of initiation of therapy is generally recommended for drug efficacy testing[13,14]. In cases where the oocyst counts are low, concentration of stool samples was shown to improve the detection of oocysts. Several staining methods are available for detection of oocysts in stool samples such as iodine staining, acid fast staining, auramine-rhodamine staining, auramine-phenol staining, Giemsa's staining, and acridine orange staining. However, acid fast staining is the most sensitive of the staining methods and is routinely used for diagnosis. Although acid fast staining of stool sample is easy to perform and cost effective, variation in staining with age of oocysts has been observed and identification is dependent on the skills of the laboratory technician. The acid fast staining technique does not detect ghost oocysts (oocysts that have shed the sporozoites), although patients with ghost oocysts continue to have the infection.

Besides staining, other assays such as immunofluorescence and enzyme linked immunoassays are available for the detection of oocysts. These methods are more sensitive than the staining methods for diagnosis of *C. parvum* infection, however, they require specialized equipment. The advantage of the immunofluoresence method is that it can detect ghost oocysts.

Concentration of stool samples in combination with more sensitive identification techniques and quantitative expression of results using multiple stool samples may be useful in enumeration of oocyst(s). Examination of stool samples detects the oocyst stage of the parasite. The other stages of the parasite are intracellular and would require examination of tissue biopsy samples.

5.2. Clinical studies:

Three phase III randomized, double blind, placebo controlled studies (**RM-NTZ-98-002, RM02-3007, and RM02-3008**) were conducted to examine the safety and efficacy of nitazoxanide (100 mg, 200 mg or 500 mg, administered orally twice daily for 3 days with food) in the treatment of crpytosporidial diarrhea in adults and children. Subjects with diarrhea (defined as ≥ 3 bowels/day) with or without other symptoms (i.e., tenesmus, loose stools, bloody stools, rectal bleeding or enlarged colon) and a positive diagnosis for *C. parvum* oocysts in stool samples collected 1 to 7 days prior to initiation of therapy were enrolled. HIV positive subjects were excluded. In addition, patients receiving other antiparasitic/antihelminthic therapies and/or those with hypersensitivities to nitazoxanide or nitroimidazoles were excluded.

In study RM-NTZ-98002, 50 adults (≥ 12 years) and 50 children (<12 years) from Egypt with crypotosporidial diarrhea and associated protozoan and helminthic infections were enrolled. The diagnosis of *C. parvum* was based on microscopic examination of a drop of fecal sample after staining with the Ziehl-Neelson acid-fast stain (ZNN; 1000X magnification). Concentrated stool samples were not examined. In some cases the direct immunofluorescence assay (IFA) using the

[redacted] for diagnosis of *C. parvum*) was used in addition to ZNN. Although the protocol stated that patients should have a positive *C. parvum* diagnosis 7 days prior to start of therapy, in 20 patients diagnosis was made 8 to 19 days prior to enrollment. The protocol also called for detection of *C. parvum* in stool samples by PCR, however, the sponsor has stated that the assay was not performed because it could not be standardized. The diagnostic methods used for identification of other protozoa/helminths/bacteria are shown in Table 22. HIV infections were diagnosed using the [redacted] Rapid Test.

Table 22: Diagnostic methods for detection of protozoa/helminths/bacteria

Protozoa/Helminth/Bacteria	Diagnostic method
Entamoeba coli	Microscopic examination of stool
Entamoeba histolytica	Microscopic examination of stool, PCR-SHELA technique
Giardia lamblia	Microscopic examination of stool
Endolimax nana	Microscopic examination of stool
Iodamoeba butschii	Microscopic examination of stool
Dientamoeba fragilis	Microscopic examination of stool
Isospora belli	Microscopic examination of stool
Blastocystis hominis	Microscopic examination of stool
Cyclospora cayaetanensis	Microscopic examination of stool
Balantidium coli	Microscopic examination of stool
Enterobius vermicularis	Microscopic examination of stool, Graham scotch test
Ascaris lumbricoides	Microscopic examination of stool, Egg count by Kato-Katz kit
Necator americanus	Microscopic examination of stool, Egg count by Kato-Katz kit
Ancylostoma duodenale	Microscopic examination of stool, Egg count by Kato-Katz kit, coproculture using Harada/Mori technique
Trichuris trichura	Microscopic examination of stool Egg count by Kato-Katz kit
Strongyloides stercoralis	Microscopic examination of stool Baermann concentration tests
Taenia saginata	Microscopic examination of stool
Taenia solium	Microscopic examination of stool
Hymenolepis nana	Microscopic examination of stool, Egg count by Kato-Katz kit
Fasciola hepatica	Microscopic examination of stool
Heterophyes heterophyes	Microscopic examination of stool
Salmonella	Culture
Shigella	Culture

The primary endpoints of the study were (a) resolution of clinical symptoms, and (b) absence of *C. parvum* oocysts in two stool samples collected 24 hours apart between days 5 and 12 of initiation of treatment. Please note that although the protocol defined endpoint was parasitological cure between days 5 and 12, the parasitological evaluations were performed between days 6 and 23. The clinical response was defined as "well" if at the time of clinical evaluation (day 7 ± 2 after initiation of treatment) the patient had (a) no symptoms, no watery stools or ≤ 2 soft stools and no hematochezia within the past 24 hours, or (b) no symptoms and no unformed stools within the past 48 hours. The secondary endpoint was absence of cyst/trophozoites/eggs/larvae of other protozoa or helminths in two stool samples obtained between days 5 and 12 of initiation of treatment. For efficacy analysis, patients with mixed infections (*C. parvum* + other protozoa/helminths) at baseline were excluded.

The results of the parasitological and clinical efficacy of nitazoxanide and placebo in the treatment of different patient populations which includes children (ages 1-3 and 4-11 years) and adults (≥ 12 years) with cryptosporidial diarrhea are shown in Tables 23 to 26 and summarized in Table 27. The detection of oocysts by the IFA test appears to be better than that of ZNN staining based on a small number of patients (2 each in the placebo and NTZ arm; Table 23). The use of two different methods appears to improve the detection of oocyst.

Subjects positive for oocysts in one post-treatment stool sample and negative in another stool sample were observed in both placebo (8/50; 16%) and nitazoxanide treatment (7/50; 14%) groups (Tables 23 to 26) thereby suggesting that examination of two or more post-treatment stool samples is helpful for evaluation of drug efficacy.

Of the 7 children under the age of 3 with cryptosporidial diarrhea, 4 (57%) children showed absence of oocysts in stool samples within 6 to 16 days of initiation of therapy with nitazoxanide (100 mg b.i.d. for 3 days, see Tables 23 and 27) and diarrhea was resolved in 3 of the 4 patients. The remaining 3 (43%) children were clinically well but continued to shed oocysts. Placebo was less effective than nitazoxanide and eradicated oocysts in 2/9 (22%) children and one of the 2 patients resolved diarrhea (Tables 23 and 27). In addition, one patient in the placebo group was clinically well but continued to shed oocysts. The remaining 6 patients continued to have diarrhea and shed oocysts. The time of evaluation of clinical response in these patients was not specified. Based on the information on the small number of patients there appears to be a lack of correlation between parasitological and clinical responses.

In older children (ages of 4-11 years) with cryptosporidial diarrhea, nitazoxanide (200 mg b.i.d for 3 days) was effective in eradicating oocysts within 6 to 16 days of initiation of therapy in 7/8 (88%) patients (Tables 24 and 27). All of the 7 children were clinically well. One patient failed therapy both clinically and parasitologically. In children treated with placebo, however, 2/12 (17%) showed absence of oocysts in the post-treatment stool samples and both of them continued to have diarrhea (Tables 24 and 27). Four children (36C, 39C, 31C, and 19C) in the placebo arm although clinically well continued to shed oocysts in the stool. The remaining 6 subjects continued to have diarrhea and shed oocysts in stool. The time of evaluation of clinical response was not specified. There appears to be a correlation between parasitological and clinical outcomes in a small number of children treated with nitazoxanide.

In adults (≥ 12 years) treated with nitazoxanide (500 mg b.i.d. for 3 days), eradication of oocysts was observed in stool samples in 12/21 (57%) patients and resolution of diarrhea in 7 of the 12 patients (Tables 26 and 27). One patient failed therapy both clinically and parasitologically. Placebo treatment eradicated oocysts in 6/21 (28%) patients, and 3 of the 6 patients resolved diarrhea (Tables 25 and 27). Nine patients continued to have diarrhea and shed oocysts. Six out of 21 patients in the placebo arm and 8/21 in the nitazoxanide arm resolved diarrhea but continued to shed oocysts. The time of evaluation of clinical response for these patients was not specified. There appears to be a lack of correlation between parasitological and clinical responses in adults treated with nitazoxanide.

Table 23: Parasitological and clinical responses in children under the age of 3 years with cryptosporidial diarrhea after treatment with nitazoxanide or placebo for 3 days.

Age in Years (Treatment Group)	Patient ID	Oocysts at baseline stool sample ZNN (IFA)	Oocysts in stool samples on different days after initiation of therapy ZNN (IFA)										Parasitologic response	Clinical response
			6	7	8	9	10	11	12	13	14	16		
1-3 years (Placebo b.i.d 3 days)	3C[bd]	0-1 (+)	- (-)							- (-)			Eradicated	Well
	4C[b]	0-2 (+)	- (-)				- (-)						Eradicated	CI
	5C[i]	+ (30-40)	0-1 (3-10)									- (0-1)	Failed	CI
	6C	+ (30-40)	0-2 (5-15)		- (-)								Failed	CI
	26C	1-2 (ND)				- (ND)					3-4 (ND)		Failed	CI
	17C[*]	1-3 (ND)			0-2 (ND)								Failed	Well
	22C	1-3 (ND)		1-4 (ND)		1-3 (ND)							Failed	CI
	27C	2-4 (ND)					1-3 (ND)	8-10 (ND)					Failed	CI
	41C[d]	2-4 (ND)				1-3 (ND)			3-5 (ND)				Failed	CI
	50C	3-5 (ND)	4-7 (ND)			8-12 (ND)							Failed	Well
	1C[*]	3-5 (ND)		- (ND)			(ND)						Eradicated	Well
	2C[*]		ND			(ND)								Well
1-3 years (NTZ 100 mg b.i.d 3 days)	2C[k]	0-3 (+)	0-1 (5-25)							- (-)			Failed	Well
	9C[b]	0-3 (0-3)	- (-)	- (-)									Eradicated	Well
	44C[b]	0-3 (ND)	- (ND)		0-1 (ND)								Failed	Well
	30C[b]	1-3 (ND)		- (ND)					- (ND)				Eradicated	Well
	43C	1-3 (ND)		- (ND)	- (ND)								Eradicated	Well
	7C[b]	3-5 (10-20)	+ (0-1)						- (-)				Failed	Well
	28C[*]	1-3 (ND)		(ND)			(ND)						Eradicated	Well
	42C	4-6 (ND)				- (ND)						- (ND)	Eradicated	CI
	34C[*]	4-6 (ND)												Well
	21C[**]	6-8 (ND)												NA
	6C[*]	2-4 (+)		(-)		(-)							Cured	Well

* excluded (shaded cells)
** did not take medication and excluded
NTZ = nitazoxanide
ZNN = Ziehl-Neelson acid fast staining
IFA = immunofluorescence assay
CI = Continuing illness
+ = positive result

[a] *Entameoba histolytica* cyst/trophs at baseline
[b] *Entameoba histolytica* cyst/trophs at post-treatment
[c] *Blastocystis hominis* at baseline
[d] *Blastocystis hominis* at post-treatment
[e] *Giardia lamblia* at baseline
[f] *Giardia lamblia* at post-treatment
[g] *Enterobius vermicularis* at baseline
[h] *Enterobius vermicularis* at post-treatment
[i] *Hymenolepis nana* at baseline
[j] *Hymenolepis nana* at post-treatment
[k] *Taenia* spp
NA = Not available
- = negative result
ND = Not done

Table 24: Parasitological and clinical responses in children (4-11 years) with cryptosporidial diarrhea after treatment with nitazoxanide or placebo for 3 days.

Age in Years (Treatment Group)	Patient ID	Oocysts at baseline stool sample ZNN (IFA)	Oocysts in stool samples on different days after initiation of therapy ZNN (IFA)										Parasitologic response	Clinical response
			6	7	8	9	10	12	13	14	15	16		
4-11 years (Placebo b.i.d 3 days)	14C[e]	1-3 (ND)	1-3 (ND)						0-3 (ND)				Failed	CI
	32C	1-3 (ND)									1-3 (ND)	1-3 (ND)	Failed	CI
	15C[c]	+ (ND)	+ (ND)				0-2 (ND)						well	well
	16C	3-5 (ND)		0-2 (ND)				- (ND)					Failed	CI
	20C[e]	3-5 (ND)		- (ND)			- (ND)						Eradicated	CI
	36C	3-5 (ND)			3-5 (ND)			4-12 (ND)					Failed	well
	23C[f]	4-6 (ND)	1-3 (ND)						3-5 (ND)				Failed	CI
	39C[d]	4-7 (ND)	2-4 (ND)				2-4 (ND)						Failed	well
	31C	5-7 (ND)		3-5 (ND)						3-5 (ND)			Failed	well
	46C[e]	5-7 (ND)	- (ND)		- (ND)								Eradicated	CI
	33C[c]	+ (ND)				2-5 (ND)	8-12 (ND)						Failed	CI
	19C	5-11 (ND)		1-3 (ND)				0-2 (ND)					Failed	well
	48C	7-10 (ND)	1-3 (ND)		6-9 (ND)								Failed	CI
	40C	8-10 (ND)				2-4 (ND)	2-3 (ND)						Failed	CI
4-11 years (NTZ 200 mg b.i.d 3 days)	8C	0-1 (-)	- (-)				- (-)						Eradicated	well
	10C	1-2 (ND)		- (ND)		- (ND)							Eradicated	well
	37C[b]	1-2(ND)			- (ND)			- (ND)					Eradicated	well
	38C*[b]	1-2 (ND)	+ (ND)					+ (ND)					Failed	well
	45C*	+ (ND)	+ (ND)		+ (ND)								Failed	CI
	12C*	+ (ND)	+ (ND)			+ (ND)							Eradicated	well
	29C	2-4 (ND)	- (ND)									- (ND)	Eradicated	well
	18C	3-5 (ND)	- (ND)			- (ND)							Eradicated	well
	24C*	+ (ND)	+ (ND)										Failed	well
	13C	4-6 (ND)	0-1 (ND)			2-4 (ND)							Failed	CI
	49C	4-6 (ND)	- (ND)									- (ND)	Eradicated	well
	47G*	+ (ND)	+ (ND)		+ (ND)								Failed	well
	35C	5-7 (ND)							- (ND)	- (ND)			Eradicated	well

* excluded (shaded cells)
NTZ = nitazoxanide
ZNN = Ziehl-Neelson acid fast staining
IFA = immunofluorescence assay
+ = positive result

[a] *Entameoba histolytica* cyst/trophs at baseline
[b] *Blastocystis hominis* at baseline
[c] *Giardia lamblia* at baseline
CI = Continuing illness
- = negative result

[d] *Blastocystis hominis* at post-treatment
[e] *Giardia lamblia* at post-treatment
[f] *Enterobius vermicularis* at post-treatment
ND = Not done

Table 25: Parasitological and clinical responses in adults (≥ 12 years) with cryptosporidial diarrhea after treatment with placebo for 3 days.

Patient ID	Oocysts at baseline stool sample ZNN (IFA)	Oocysts in stool samples on different days after initiation of therapy ZNN (IFA)											Parasitologic response	Clinical response
		6	7	8	9	10	11	12	13	14	17	23		
31A	0-2 (ND)		1-3 (ND)			2-5 (ND)							Failed	well
16A	0-2 (ND)	- (ND)			3-5 (ND)								Failed	CI
[redacted]	[redacted]	[redacted]											[redacted]	[redacted]
28A	0-3 (ND)	0-2 (ND)			0-2 (ND)								Failed	well
7A	1-2 (ND)	- (ND)						- (ND)					Eradicated	CI
17A	1-2 (ND)	- (ND)			- (ND)								Eradicated	CI
39A	1-2 (ND)		4-6 (ND)			7-9 (ND)							Failed	CI
43A	1-2 (ND)		1-3 (ND)									15-18 (ND)	Failed	CI
48A	1-2 (ND)			3-5 (ND)			3-7 (ND)						Failed	CI
5A	1-3 (ND)									- (ND)	- (ND)		Eradicated	CI
25A	1-3 (ND)	- (ND)					1-2 (ND)						Failed	well
27A	1-3 (ND)			4-6 (ND)	1-3 (ND)								Failed	CI
40A	1-3 (ND)	4-7 (ND)				5-7 (ND)							Failed	well
[redacted]	[redacted]	[redacted]											[redacted]	[redacted]
44A[b]	1-3 (ND)			2-4 (ND)	12-17 (ND)								Failed	well
[redacted]	[redacted]	[redacted]											[redacted]	[redacted]
[redacted]	[redacted]	[redacted]											[redacted]	[redacted]
33A[d]	2-4 (ND)				0-2 (ND)				- (ND)				Failed	well
11A	3-5 (ND)		- (ND)					- (ND)					Eradicated	well
15A	3-5 (ND)		- (ND)	- (ND)									Eradicated	well
4A	3-6 (ND)	4-6 (ND)		4-6 (ND)									Failed	CI
29A[b]	4-6 (ND)	1-2 (ND)			0-2 (ND)								Failed	CI
38A	8-12 (ND)		- (ND)			1-3 (ND)							Failed	CI
21A	10-15 (ND)	- (ND)							0-2 (ND)				Failed	CI
36A[e]	10-15 (ND)					- (ND)		- (ND)					Eradicated	well

* excluded (shaded cells)
NTZ = nitazoxanide
ZNN = Ziehl-Neelson acid fast staining
CI = Continuing illness
+ = positive result

[a] *Entamoeba histolytica* cyst/trophs at baseline
[c] *Giardia lamblia* at baseline
IFA = immunofluorescence assay
ND = Not done
- = negative result

[b] *Entamoeba histolytica* cyst/trophs at post-treatment
[d] *Giardia lamblia* at post-treatment
[e] *Hymenolepis nana* at post-treatment

Table 26: Parasitological and clinical responses in adults (≥ 12 years) with cryptosporidial diarrhea after treatment with nitazoxanide (500 mg b.i.d) for 3 days.

Patient ID	Oocysts at baseline stool sample	Oocysts in stool samples on different days after initiation of therapy ZNN (IFA)										Parasitologic response	Clinical response
	ZNN (IFA)	6	7	8	9	10	11	12	13	15	16		
1A	0-1 (ND)	- (-)		- (-)								Eradicated	well
2A*	- (0-4)			(-)									
19A	0-1 (ND)	0-2 (ND)		0-2 (ND)								Failed	well
32A[d]	0-2 (ND)	- (ND)			- (ND)							Eradicated	well
26A	0-2 (ND)	- (ND)			- (ND)							Eradicated	CI
47A[d]	0-2 (ND)	- (ND)			- (ND)							Eradicated	CI
50A	0-2 (ND)		- (ND)				- (ND)					Eradicated	CI
14A*[b]	1-3 (ND)	(ND)			(ND)								
18A	1-3 (ND)	3-5 (ND)			2-4 (ND)							Failed	CI
22A	1-3 (ND)	2-4 (ND)							1-3 (ND)			Failed	well
34A	1-3 (ND)	- (ND)		2-4 (ND)								Failed	well
49A	1-3 (ND)			- (ND)			- (ND)					Eradicated	CI
6A	2-4 (ND)	- (ND)		- (ND)								Eradicated	well
10A	2-4 (ND)				- (ND)	- (ND)						Eradicated	CI
20A	2-4 (ND)	1-2 (ND)							4-6 (ND)			Failed	well
41A	2-4 (ND)		- (ND)		- (ND)							Eradicated	well
46A	2-4 (ND)							- (ND)	- (ND)			Eradicated	well
8A*[b]	3-5 (ND)	0-2 (ND)			1-2 (ND)								
9A	3-5 (ND)		- (ND)				- (ND)					Eradicated	well
24A	3-5 (ND)	- (ND)				2-5 (ND)						Failed	well
10A*	5-7 (ND)	(ND)					(ND)						CI
37A	5-7 (ND)		- (ND)		- (ND)							Eradicated	well
13A	6-8 (ND)	- (ND)			2-4 (ND							Failed	well
3A	8-15 (ND)						1-2 (ND)			- (ND)		Failed	well
30A	10-12 (ND)	3-5 (ND)							8-12 (ND)			Failed	well

* excluded (shaded cells)
NTZ = nitazoxanide
ZNN = Ziehl-Neelson acid fast staining
IFA = immunofluorescence assay
+ = positive result
ND = Not done

[a] *Entameoba histolytica* cyst/trophs at baseline
[b] *Entameoba histolytica* cyst/trophs at post-treatment
[c] *Giardia lamblia* at baseline
[d] *Giardia lamblia* at post-treatment
[e] *Hymenolepis nana* at post-treatment
CI = Continuing illness
- = negative result

Table 27: Summary of the efficacy of nitazoxanide in immunocompetent patients with *C. parvum* infection (RMNTZ 98-002).

Treatment group	Age in years	baseline parasite counts using ZNN (no of subjects)	No. of patient		Parasitologic and clinical responses		patients with eradication of oocysts N (%)	Clinical well response N (%)	patients clinical well and eradicated oocysts N (%)
			total	excluded	Eradicated (CR)	Persisted (CR)			
Placebo b.i.d 3 days	1-3	1-5	11	2	1(well); 1 (CI)	1(well); 6 (CI)	2 (22)	2 (22)	1 (11)
	4-11	≤3 (2) >3 <10 (11) >10 (1)	14	2	2 (CI)	4 (well); 6 (CI)	2 (17)	4 (33)	0 (0)
	≥ 12	≤3 (16) >3 <10 (6) >10 (3)	25	4	3 (well); 3 (CI)	6 (well); 9 (CI)	6 (28)	9 (43)	3 (14)
NTZ 100 mg b.i.d 3 days	1-3	≤3 (6) 12 >3 <10 (6)		5	3 (well); 1 (CI)	3 (well)	4 (57)	6 (86)	3 (43)
NTZ 200 mg b.i.d 3 days	4 -11	≤3 (5) 13 >3 <10 (8)		5	7 (well)	1 (CI)	7 (88)	7 (88)	7 (88)
NTZ 500 mg b.i.d 3 days	≥ 12	≤3 (12) >3 <10 (11) >10 (2)	25	4	7 (well); 5 (CI)	8 (well); 1 (CI)	12 (57)	15 (71)	7 (33)

NTZ = Nitazoxanide;
CI = continuing illness;
CR = clinical response;
N = number of subjects.

In summary, examination of ≥ 2 stool samples is helpful for determination of parasitological efficacy of the drug. Evaluation of stool samples by two different methods improved the detection of oocysts in stool samples. A total of 23 out of 36 (64%) patients eradicated the oocysts within 6 to 23 days after initiation of therapy with nitazoxanide (100 mg, 200 mg, or 500 mg) compared to 10/42 (24%) patients in the placebo arm. There appears to be a lack of correlation between the parasitological and clinical outcomes in patients with cryptosporidial diarrhea treated with nitazoxanide except in children 4-11 years of age (Table 27).

In study RM02-3001, 50 HIV negative children (≤ 3 years old) with cryptosporidial diarrhea from Zambia were enrolled. The inclusion/exclusion criteria were same as study RM-NTZ-98-002 except that only subjects with *C. parvum* oocysts in 2 stool samples collected at the time of screening (7-10 days prior to enrollment) and at baseline were enrolled. Exclusion of subjects with ***Entamoeba histolytica*** and *Giardia lamblia* infections was based on a positive test using a FDA approved enzyme immuno-assay kit. The HIV infections were

diagnosed using the [redacted] ; Rapid test. Only subjects who were HIV negative were enrolled. Subjects were treated with nitazoxanide (100 mg b.i.d) for 3 days.

The primary endpoint of this study was resolution of clinical symptoms. The secondary endpoints were (a) absence of *C. parvum* oocysts in two stool samples collected 24 hours apart between days 7 and 15 after initiation of therapy, and (b) time to passage of last unformed stool. Microscopic examination of unconcentrated and/or concentrated stool samples stained with auramine-phenol was used to identify *C. parvum* oocysts. The sponsor has stated that concentrated stool samples were processed for the presence of oocyts only if the unconcentrated stool samples were negative for oocysts. However, it appears that this criterion was not used uniformly. No other method was used for detection of oocysts. In 3 patients, the diagnosis of *C. parvum* infection at screening was made using the enzyme immunoassay kit ([redacted] only. It should be noted that the [redacted] test consists of antibodies to the *G. lamblia* alpha-1-giardin, *E. histolytica* 29 kDa surface protein and *C. parvum* protein disulfide isomerase and has been approved for diagnosis of *C. parvum*, *G. lamblia* and *E. histolytica* infections. The test can be performed only using fresh stool sample within 72 hours of collection.

One of the patients (#10) had infection due to *G. lamblia* and was excluded from the efficacy analysis (Table 28).

Discordance in the test results of the 2 stool samples collected at post-treatment was observed in some of the patients (Table 28). In the placebo and nitazoxanide treatment groups, 35% (6/17) and 8% (2/24) patients, respectively, were positive for oocysts in one stool sample and negative in the other stool sample. This could be due to intermittent shedding of oocysts. Examination of two or more post-treatment samples appears to be necessary for evaluation of drug efficacy.

Eradication of oocysts was observed in the stool samples from 3/25 (12%) patients collected between days 7 and 11 in the placebo group. Of the 3 patients, one was clinically well (Tables 28 and 29). Three patients (# 32, 34 and 46) in the placebo arm were positive for *C. parvum* oocysts at screening but were negative on the day of initiation of treatment. The clinical outcome of these patients was not described. The sponsor has excluded these patients from efficacy analysis. However, for the purpose of this review these patients were included in the analysis so that comparison can be made across different studies using the same inclusion criteria i.e., presence of oocysts 7 to 10 days prior to enrollment. Diarrhea and shedding of oocysts was observed in 10/25 (40%) patients. Five patients were lost to follow-up. Oocysts were absent in post-treatment stool samples (collected between days 7 and 15) from 12/24 (50%) patients treated with nitazoxanide (Tables 28 and 29). Of these, 7 patients were clinically well. Six patients failed therapy both clinically and parasitologically. Four patients in the placebo group and six in the nitazoxanide group were clinically well but continued to shed oocysts. The time of evaluation of the clinical response was not specified. There appears to be no correlation between the clinical and parasitological outcomes in these children.

Table 28: Parasitological and clinical efficacy of Zambian children with *C. parvum* infection in the placebo and nitazoxanide treatment groups (treatment administered for 3 days).

Group	Patient ID	Oocysts at baseline stool sample UC (C)	Oocysts in stool samples on different days after initiation of therapy UC (C)							Parasitologic response	Clinical response
			7	8	9	10	11	13	15		
Placebo b.i.d 3 days n = 25	2	+ (ND)	+ (ND)			- (0)					well
	4	+ (ND)		- (0)	- (0)					Eradicated	well
	6	+ (ND)			+ (ND)						CI
	9	+ (ND)	Lost to follow up								
	16	+ (ND)	Lost to follow up								
	26	+ (ND)	Lost to follow up								
	7	+ (1+)		+ (ND)			- (0)				CI
	12	+ (1+)		- (0)			+ (ND)				well
	24	+ (1+)		- (0)	- (ND)					Eradicated	CI
	27	+ (1+)		- (0)	- (ND)					Eradicated	CI
	37	+ (1+)	Lost to follow up								
	39	+ (1+)		+ (1+)	+ (1+)						CI
	47	+ (1+)		+ (2+)	+ (2+)						CI
	49	+ (1+)		+ (1+)	- (0)						well
	14	+ (2+)		+ (3+)	+ (1+)						CI
	17	+ (2+)		+ (ND)		- (0)					CI
	29	+ (2+)		+ (1+)	+ (1+)						CI
	42	+ (2+)		+ (1+)	- (0)						CI
	44	+ (2+)	Lost to follow up								
	22	+ (3+)		+ (2+)	+ (3+)						CI
	36	+ (3+)		+ (3+)	+ (2+)						CI
	19	+ (4+)		+ (1+)	- (0)						well
	32**	-								Eradicated	NA
	34**	-								Eradicated	NA
	46**	-								Eradicated	NA
NTZ 100 mg b.i.d 3 days n = 24	1	+ (ND)		- (0)				- (0)		Eradicated	CI
	3	+ (ND)		- (0)		- (0)				Eradicated	well
	5	+ (ND)		+ (ND)							CI
	8	+ (ND)		- (0)					- (0)	Eradicated	CI
	15	+ (ND)		- (0)	- (0)					Eradicated	well
	11	+ (1+)		+ (1+)	+ (1+)						CI
	13	+ (1+)		+ (1+)	+ (1+)						CI
	23	+ (1+)		- (0)	- (0)					Eradicated	CI
	25	+ (1+)	+ (+1)	+ (+1)							well
	28	+ (1+)		- (0)	- (0)					Eradicated	well
	31	+ (1+)	- (0)	- (0)						Eradicated	CI
	38	+ (1+)		+ (1+)	- (0)						well
	41	+ (1+)		- (0)	- (0)					Eradicated	CI
	45	+ (1+)		- (0)	- (0)					Eradicated	well
	48	+ (1+)		+ (1+)	+ (1+)						CI
	50	+ (1+)			- (0)	+ (1+)					well
	21	+ (2+)	+ (+1)		+ (1+)						well
	35	+ (2+)		- (0)	- (0)					Eradicated	well
	40	+ (2+)			- (0)		- (0)			Eradicated	well
	43	+ (2+)	- (0)	- (0)						Eradicated	well
	18	+ (3+)		+ (1+)	+ (2+)						well
	30	+ (3+)		+ (1+)	+ (1+)						CI
	33	+ (3+)		+ (1+)	+ (1+)						CI
	20	+ (4+)		+ (3+)	+ (4+)						well
	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]

NTZ = Nitazoxanide; UC = unconcentrated stool; C = concentrated stool; + = positive result;
- = negative result; ND = Not done; CI = continuing illness; NA = not available

*excluded due to mixed infection with *Giardia* (shaded cell)

** positive for oocysts at screening (7-10 days prior to start of therapy) and negative at baseline;

0 = no oocyst entire smear; 1+ = <5 oocyst per microscopic field (100X magnification);
2+ = <5 oocyst per microscopic field (100X magnification); 3+ = 5-10 oocyst per microscopic field (100X magnification);
4+ = 11-20 oocyst per microscopic field (100X magnification); 5+ = >20 oocyst per microscopic field (100X magnification).

Table 29: Summary of parasitological and clinical responses of immunocompetent children with *C. parvum* infection (RM02-3007)

Treatment group	Age in years	Baseline parasite score (no of subjects)	No. of patient		Parasitologic and clinical response		Patients with eradication of oocysts N (%)	Patients with Clinical well response N (%)	Patients clinical well and eradicated oocysts N (%)
			total	excluded	Eradicated (CR)	Persisted (CR)			
Placebo* b.i.d 3 days	1-3	≤ 2+ (22) ≤ 4+ (3)	25	0	1 (well); 2 (CI); 3 (NA)	4 (well); 10 (CI)	6 (24)	5 (20)	1 (4)
100 mg NTZ b.i.d 3 days	1-3	≤ 2+ (21) ≤ 4+ (4)	25	1	7 (well); 5 (CI)	6 (well); 6 (CI)	12 (50)	13 (54)	7 (29)

NTZ = Nitazoxanide
NA = clinical response not available
* 5 patients lost to follow-up
CR = clinical response;
N = number of subjects

Parasite score: 0 = no oocyst entire smear;
2+ = <5 per microscopic field (100X magnification)
3+ = 5-10 per microscopic field (100X magnification)
4+ = 11-20 per microscopic field (100X magnification)

Some of the patients who continued to shed oocysts or were clinically ill after treatment with the placebo or nitazoxanide were treated with nitazoxanide (100 mg bid for 3 days) in an open-label protocol (Table 30). Two patients previously treated with placebo (#2 and #27) and one patient (#1) previously treated with nitzoxanide had parasitological evaluations based on one stool sample and were excluded from analysis. The results in Table 30 show that eradication of oocyst and resolution of diarrhea was observed in 6 patients (1/7 patients with prior placebo treatment and 5/12 patients with prior nitazoxanide treatment). In addition, the results also support the fact that detection of oocysts in < 2 samples may be insufficient for measuring parasitological outcome after treatment with an anti-cryptosporidial drug

In summary, 7/24 (29%) children (≤ 3 years) showed a parasitological and clinical cure after treatment with nitazoxanide (100 mg b.i.d for 3 days) compared to 1/25 (4%) in the placebo arm.