before shaking to detach the parasites. The parasites were separated from the tissue by filtration and counted using a hemocytometer. The results in Table 36 show the trophozoite counts in the control group to vary from 14 to 330. Based on these results the % trophozoite counts in drug treated animals compared to control animals was calculated and used to determine the 50% effective dose (ED_{50}) by regression analysis (Table 37). The ED_{50} values for nitazoxanide, metronidazole and albendazole were 31.2 mg/kg, 125.8 mg/kg and 44.2 mg/kg, respectively. The sponsor has stated that the 100 mg/kg and 200 mg/kg dose of nitazoxanide was effective in the treatment of infected animals. However, this effect may not be significant due to the variation in the trophozoite counts observed in the control groups.

Table 36: Number of trophozoites per ml in untreated control (C) and treated (T) newborn mice.

Mice	Metronidazole								Nitazoxanide								Albendazole							
	10		50		100		200		10		50		100		200		10		50		100		200	
	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T
1																								
2																								
3																								
4																								
5																								
6																								
7																								
8																								
m ± sd	33.7 ± 2.6	37.8 ± 3	30.2 ± 3.9	22.7 ± 3.1	46.1 ± 3.9	28.7 ± 2.7	143 ± 8	25 ± 2.6	48 ± 3.7	28.6 ± 4.3	17.2 ± 1.8	7.7 ± 1.8	100 ± 8.6	25.5 ± 1.7	30.2 ± 3.2	3.6 ± 0.8	227 ± 40.7	158 ± 17.4	106 ± 7.8	43.7 ± 6	66.3 ± 13	17.4 ± 7.1	49.5 ± 12.6	0.4 ± 0.4

Table 37: Effects of drugs on new born mice infection (% trophozoite count compared to control animals).

Dose (mg/kg)	Metronidazole	Nitazoxanide	Albendazole
10	112.6 ± 8.9	59.6 ± 8.9	69.6 ± 7.6
50	75.1 ± 10.2	44.9 ± 10.4	41.2 ± 5.7
100	62.2 ± 5.8	25.3 ± 1.7	26.2 ± 10.7
200	17.4 ± 1.8	11.8 ± 2.5	0.8 ± 0.8

No other studies were done to measure the activity of nitazoxanide *in vivo*.

5. CLINICAL MICROBIOLOGY:

5.1. Diagnosis of Giardiasis:

Diagnosis of Giardiasis is based on detection of trophozoites or cysts by microscopic examination of unconcentrated and concentrated stool samples. Since the trophozoites attach to the duodenal walls and the cysts are shed intermittently in stool, the possibility of missing the parasites exists. Staining of fecal smears is recommended as it improves the sensitivity of the microscopic examination. Beside microscopic examination, immunofluorescence and enzyme linked immunosorbent assays are available for the detection of *Giardia* cysts and have been shown to be more sensitive than microscopic examinations. Although these tests need specialized equipment, they are easy to perform and less prone to error. In cases where stool samples are negative, testing of duodenal aspirates or duodenal contents using the Entero-test capsule (the capsule contains a string that can be used to retrieve duodenal contents) may be useful to rule out infection.

Methods detecting anti-Giardia IgG antibodies have been used more for epidemiological purposes than diagnosis due to the prolonged persistence of the antibodies in circulation. PCR methods have been developed for detection of *Giardia* cysts, however, the usefulness of PCR for diagnosis and evaluation of drug efficacy is not known.

5.2. Clinical studies:

Two randomized studies (RM-NTZ-98-001 and RM-NTZ-99-010) were conducted in Egypt and Peru to examine the safety and efficacy of nitazoxanide in the treatment of giardiasis.

In study RM-NTZ-98-001, about 100 adults (12 - 65 years) from Egypt, with diarrhea (> 4 bowel movements/day with or without other symptoms such as bloody stools, rectal bleeding or enlarged colon) and positive for cysts or trophozoites of *Giardia lamblia* and/or *Entamoeba histolytica/dispar* in stool samples within 7 days prior to enrollment were included. This was a randomized, placebo controlled, double-blind study. Subjects receiving other antiparasitic or antihelminthic drugs, those with hypersensitivity to nitazoxanide or nitroimidazole, and those who tested HIV positive , were excluded. Subjects were treated with nitazoxanide (500 mg, b.i.d) or placebo for 3 days. The primary endpoints of the study were (a) resolution of clinical symptoms, and (b) absence of cysts or trophozoites in two stool samples (unconcentrated and/or concentrated) collected atleast 24 hours apart between days 6 and 13 after initiation of treatment. The secondary endpoint was time to passage of last unformed stool.

The diagnosis of *G. lamblia* and *E. histolytica* was made using the test (FDA approved), an enzyme immuno assay for detection of cysts of *G. lamblia* and *E. histolytica* and oocysts of *C. parvum*. These results were confirmed by microscopic examination of unconcentrated and/or concentrated stool samples (400x magnification over the entire cover slip area). In addition to these tests, the immunofluorescence assay (FDA approved for diagnosis of *G. lamblia* cysts in stool samples) was used in some cases. All these methods except the test were used for parasitological evaluations of post-treatment samples. The quantity of stool for direct examination was not specified, however, 5 gram of formed stool or 0.5 ml of watery stool was used for concentration by the formalin-ether method. The methods used for identification of other protozoans/helminths are shown in Table 22 (page 33).

The efficacy analysis for patients with infections due to *G. lamblia* alone, *G. lamblia* and *E. histolytica*, and *E. histolytica* alone at baseline, was performed separately. A total of 22 patients (11 per treatment arm) had infection due to *G. lamblia* alone at baseline. Few patients had mixed infection with *B. hominis* and were excluded from analysis. Patients who did not return for follow-up visit and those who discontinued from the study were considered as failures.

The results in Table 38a suggest the concentration and immunofluorescence methods were more sensitive in the detection of *Giardia* cysts than examination of unconcentrated stool when the cysts were present in low numbers. In addition, examination of two or more stool samples is helpful for evaluation of drug efficacy. For example, only one of the two stool samples were positive for cysts in 5/10 (50%) patients in the placebo arm (Table 38a) and 1/11 (9%) patients in

the nitazoxanide arm (Table 38b). It is of note that trophozoites were detected in one patient (#42) in the nitazoxanide arm at the post-treatment visit (Table 38b).

The parasitological and clinical responses of patients with giardiasis to nitazoxanide treatment are summarized in Table 39. In patients with infection due to *Giardia* alone (n = 8), 6 patients were clinically well and did not show presence of cysts after treatment with nitazoxanide (500 mg b.i.d for 3 days; Tables 38b and 39). Two patients were clinically well but continued to shed cysts. In the placebo arm, none of the 10 patients were cured of the parasite although 3 were clinically well (Tables 38a and 39). The time of evaluation of the clinical response was not specified. Based on a small number of patients, nitazoxanide (500 mg, b.i.d for 3 days) appears to be effective in the treatment of giardiasis in adults.

Table 38a: Parasitological and clinical responses of adults with *G. lamblia* infection in the placebo arm (study RM-NTZ-98-001).

Patient ID	Cysts in baseline stool sample UC (C) [IFA]	Cysts in stool samples on different days after initiation of therapy UC (C) [IFA]							Parasitologic response	Clinical response
		5	6	7	8	9	11	13		
27	0-2 (0-4) [6-8]								Failed	CI
37	- (-) [0-2]		- (6-10) [+]					- (-) [-]	Failed	CI
38	- (2-3) [0-3]								Failed	CI
41	0-2 (1-3) [-]		- (-) [0-3]		- (-) [-]				Failed	CI
45	2-6 (6-12) [+]			0-3 (3-6) [5-10]				- (-) [-]	Failed	CI
47	- (-) 5-8		- (-) [-]			0-1 (0-1) [5-8]			Failed	CI
56	2-6 (6-10) [5-10]			1-3 (2-3) [ND]			+ (+) [ND]		Failed	Well
68	0-2 (2-4) [ND]		- (-) [ND]			- (3-5) [ND]			Failed	Well
74*	1-2 (3-5) [ND]		0-1 (3-5) [ND]						Failed	Well
75	1-3 (3-9) [ND]		1-3 (3-5) [ND]						Failed	CI
88	1-3 (3-6) [ND]	- (1-3) [ND]							Failed	Well

* excluded due to *B. hominis* infection
IFA = merifluor immunofluorescence;
ND = Not done
UC = unconcentrated stool;
- = negative result
CI = ccontinuous illness
C = concentrated stool;
+ = positive result

Table 38b: Parasitological and clinical responses of adults with *G. lamblia* infection in the nitazoxanide arm (study RM-NTZ-98-001).

Patient ID	Cysts in baseline stool sample UC (C) [IFA]	Cysts in stool samples on different days after initiation of therapy UC (C) [IFA]						Parasitologic response	Clinical response
		6	7	8	11	12	13		
8	6-10 (8-15) [30]	- (-) [-]			- (-) [-]			Eradicated	Well
9	15-20 (15-20) [30]	- (-) [-]			- (0-1) [-]			Failed	Well
32*	- (-) [0-1]	- (-) [-]		- (-) [-]				Eradicated	Well
33	- (-) [0-1]	- (-) [-]		- (-) [-]				Eradicated	Well
42**	2-4 (2-5) [5-15]	- (-) [1-3]		0-3 (3-10) [15-20]				Failed	Well
46	- (0-1) [-]	- (-) [-]					- (-) [-]	Eradicated	Well
50	- (-) [1-3]				- (-) [ND]	- (-) [ND]		Eradicated	Well
55	- (-) [0-2]	- (-) [ND]		- (-) [ND]				Eradicated	Well
71	0-2 (3-5) [ND]		- (-) [ND]	- (-) [ND]				Eradicated	Well
78*	- (3-5) [ND]	- (-) [ND]				- (-) [ND]		Eradicated	Well
80*	- (3-5) [ND]	- (-) [ND]		- (-) [ND]				Eradicated	CI

* excluded due to *B. hominis* infection
** 0-1 trophozoites observed in one unconcentrated stool sample;
UC = unconcentrated stool; C = concentrated stool; IFA = [redacted] immunofluorescence;
- = negative result
+ = positive result
ND = Not done
CI = ccontinuous illness

Table 39: Summary of parasitological and clinical responses of adults with *G. lamblia* infection at days 5 - 13 after treatment with nitazoxanide (for 3 days).

Treatment group	No. of patient		Parasitological and clinical responses		Patients with eradication of cysts N (%)	Patients with clinical well response N (%)	Patients clinical well and showing eradication of cysts N (%)
	total	excluded	Cysts Eradicated (CR)	Cysts Persisted (CR)			
Placebo b.i.d 3 days	11	1	0	3 (well); 7 (CI)	0 (0)	3 (30)	0 (0)
500 mg NTZ b.i.d 3 days	11	3	6 (well)	2 (well)	6 (75)	8 (100)	6 (75)

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

Of the 14 patients with infection due to *G.lamblia* and *E. histolytica* at enrollment, 8 were treated with placebo and 6 with nitazoxanide (500 mg b.i.d for 3 days). Parasitological and clinical cure

for both parasites was observed in one patient (Table 40, shown in bold). Three patients showed eradication of *Giardia* cysts but only one was clinically well. Although one patient treated with placebo resolved diarrhea, parasitological cure was not observed (Table 40).

Table 40: Summary of parasitological and clinical responses of adults with mixed *G. lamblia* and *E. histolytica* infection at days 5 -13 after treatment with nitazoxanide (for 3 days).

Treatment group	No. of patient		Parasitological and clinical responses		Patients with eradication of *Giardia* cysts N (%)	Patients clinical well response and eradication of *Giardia* cysts N (%)
	total	excluded	Cysts Eradicated (CR)	Cysts Persisted (CR)		
Placebo b.i.d 3 days	8	3	2[a] (CI)	1[f] (well); 2[f] (CI), 2[e] (CI)	0 (0)	0 (0)
500 mg NTZ b.i.d 3 days	6	0	1[c] (well)*, 1[a] (well); 1[c] (CI), 1[b] (CI)	2[f] (well), 1[e] (well); 1[d] (CI)	3 (50)	1 (17)

NTZ = Nitazoxanide;
[a] eradicated Amoeba cysts;
[b] eradicated *Giardia* cysts;
[c] eradicated Amoeba and *Giardia* cysts
*eradicated Amoeba and *Giardia* cysts and clinically well;
N = number of subjects
CI = continuing illness
[d] persisting amoebiasis
[e] persisting giardiasis
[f] persisting ameobiasis and giardiasis
CR = clinical response

A total of 55 patients with amoebiasis were enrolled. The results in Table 41 show that treatment with nitazoxanide (500 mg b.i.d for 3 days) eradicated the *E. histolytica* cysts in 19/25 (76%) patients and resolved diarrhea in 17 of the 19 patients. However, placebo was also effective in eradicating cysts in 9/20 (45%) patients and resolving diarrhea in 8 of these 9 patients. Although, 4 patients in the nitazoxanide arm and one in the placebo arm were clinically well, they continued to shed *E. histolytica* cysts. There appears to be a correlation between the parasitological and clinical outcomes in both groups.

Table 41: Summary of parasitological and clinical responses of adults with *E. histolytica* infection at days 5 -13 after treatment with nitazoxanide (for 3 days).

Treatment group	No. of patient		Parasitological and clinical responses		Patients with eradication of cysts N (%)	Patients with clinical well response N (%)	Patients clinical well and eradicated cysts N (%)
	total	excluded	Cysts Eradicated (CR)	Cysts Persisted (CR)			
Placebo b.i.d 3 days	24	4	8 (well); 1 (CI)	1 (well); 10 (CI)	9 (45)	9 (45)	8 (40)
500 mg NTZ b.i.d 3 days	31	6	17 (well); 2 (CI)	4 (well); 2 (CI)	19 (76)	21 (84)	17 (68)

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

Overall, the concentration and immunofluorescence methods appear to be more sensitive in the detection of *Giardia* cysts than examination of unconcentrated stool when the cysts were present in low numbers. Examination of 2 or more samples is necessary for evaluation of drug efficacy,

as the shedding of cysts in the stool can be intermittent. Nitazoxanide was effective in the treatment of 6/8 adults with giardiasis compared to placebo (0/10 adults). Of the 6 patients with mixed *Giardia* and *Entameoba* infections, eradication of both parasites and resolution of diarrhea was observed in one patient after treatment with nitazoxanide. Eradication of *E. histolytica* cysts and resolution of diarrhea was observed in 68% (17/25) amoebiasis patients treated with nitazoxanide compared to 40% (8/20) patients in the placebo arm. Nitazoxanide appears to be more effective than placebo in the treatment of amoebiasis. However, the number of patients with giardiasis or amoebiasis enrolled in the study was too small.

In study RM-NTZ-99010, 110 children (2-11 years old) from Peru with acute diarrhea (> 3 unformed stools/day) or chronic diarrhea (unformed stools with or without increased stool frequency for more than 4 weeks) and positive for *Giardia lamblia* in stool samples by the — enzyme immunoassay were included. The initial diagnosis for *Giardia* by the — test at screening was confirmed by microscopic examination of unconcentrated and/or concentrated stool samples at baseline. The exclusion criteria were same as study RM-NTZ-98-001 except patients with *E. histolytica* and *C. parvum* infections were also excluded. The post-treatment diagnosis was made by microscopic examination of unconcentrated and concentrated stool samples, however, the cysts/trophozoites were not quantified. The primary endpoint was resolution of clinical symptoms. The secondary endpoints were (a) absence of cysts or trophozoites in two stool samples (unconcentrated and/or concentrated) collected atleast 24 hours apart between days 7 and 16 after initiation of treatment, (b) time to passage of last unformed stool, and (c) therapeutic cure (clinically well and absence of parasite).

For the efficacy analysis, patients with mixed infections and those who did not take all the doses of the medication were excluded. The results in Table 42 show that nitazoxanide was as effective as metronidazole in the treatment of *Giardia lamblia* infection in children. In children with giardiasis, there appears to be a correlation between the parasitological and clinical outcomes after treatment with nitazoxanide or metronidazole.

Table 42: Parasitological and clinical responses of children with *G. lamblia* infections to nitazoxanide or metronidazole treatment.

Treatment group	Age in years	No. of patient		Parasitological and clinical responses		Patients with eradication of cysts N (%)	Patients with clinical well response N (%)	Patients clinical well and eradicated cysts N (%)
		total	excluded	Eradicated (CR)	Persisted (CR)			
Metronidazole (125 mg) b.i.d 3 days	1-3	11	4	6 (well)	1 (CI)	**6 (86)**	**6 (86)**	**6 (86)**
100 mg NTZ b.i.d 3 days	1-3	14	2	9 (well)	2 (well); 1 (CI)	**9 (75)**	**11 (92)**	**9 (75)**
Metronidazole (250 mg) b.i.d 3 days	4-11	44	11	21 (well); 4 (CI)	5 (well); 3 (CI)	**25 (76)**	**26 (79)**	**21 (64)**
200 mg NTZ b.i.d 3 days	4-11	41	15	20 (well); 3 (CI)	2 (well); 1 (CI)	**23 (89)**	**22 (85)**	**20 (77)**

NTZ = Nitazoxanide; N = numberof subjects; CR = clinical response; CI = continuing illness;

In summary, parasitological evaluations in the clinical trials conducted to determine efficacy of nitazoxanide in the treatment of giardiasis was based on microscopic examination of unconcentrated and/or concentrated wet stool samples. Fixed fecal smears after staining (known to improve sensitivity of parasite detection) were not examined. In one study (RM-NTZ-98-001), the immunofluorescence assay was used to confirm the diagnosis. No other assay was used for confirmation in the other study (RM-NTZ-99-010). Concentration and immunofluorescence detection methods were more sensitive when the number of cysts in the stool was low. Additionally, examination of 2 or more stool samples was useful for evaluation of parasitological efficacy. Nitazoxanide was as effective as metronidazole in the treatment of children with giardiasis in Peru. Nitazoxanide was also effective in adults with *G. lamblia* infection in Egypt compared to placebo.

C. Protozoa (other than *Cryptosporidium* and *Giardia*), Helminths or Bacteria:

2. MECHANISM OF ACTION:

2.1. Protozoa:

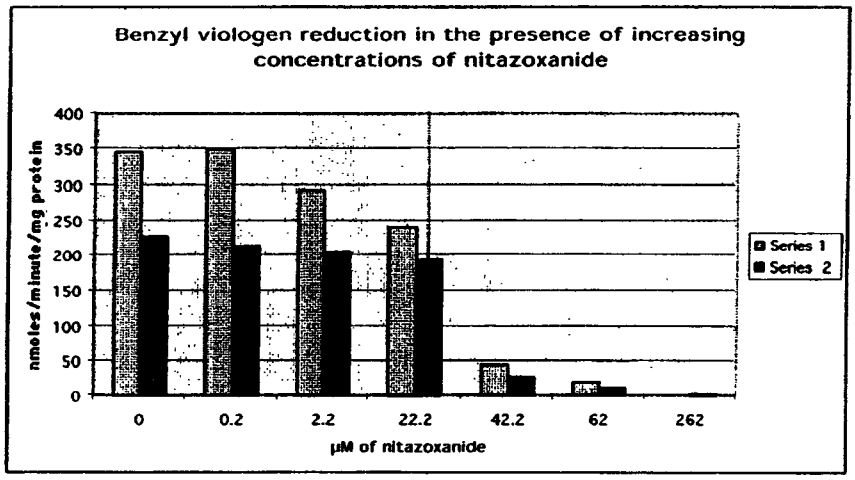
The ability of nitazoxanide to act as an electron acceptor for the PFOR enzyme of *Entamoeba histolytica* and *Trichomonas vaginalis* was examined under aerobic conditions[1]. For this, the parasites were grown to log phase in Diamond's TYI-S-33 medium and then harvested, disrupted by sonication or by using a homogenizer and fractions obtained by centrifugation. The soluble fraction of the *E. histolytica* cell extract (supernatant obtained after centrifugation at 14,000 x g) was used to measure enzyme activity. In the case of *T. vaginalis*, the fraction containing hydrogenosomes was used. The fractions were incubated in the presence of the pyruvate (substrate) and nitazoxanide and the absorbance was measured at 412 nm. Other electron acceptors (such as benzyl viologen and nitroblue tetrazolium were used as comparators. The absorbance of products of benzyl viologen and nitroblue tetrazolium reduction was measured at 546 nM. The results in Table 43 show that the specific activity of the *E. histolytica* PFOR enzyme in the presence of nitazoxanide was about 2-fold less than that observed in the presence of benzyl viologen and 17-fold more than that observed in the presence of nitroblue tetrazolium. The results suggest that nitazoxanide acts as an electron acceptor for the *E. histolytica* PFOR enzyme. Nitazoxanide was a better electron acceptor than nitroblue tetrazolium. However, benzyl violgen was the best electron acceptor for the enzyme.

Table 43: PFOR activity in *Entamoeba histolytica* with different electron acceptor.

Electron acceptor	Specific activity (nmol/min/mg protein)
benzyl viologen	1,485
nitroblue tetrazolium	37
nitazoxanide	645

The ability of nitazoxanide to act as an electron acceptor was also examined under anaerobic conditions using benzyl viologen (5 mM). For this, the soluble fraction was incubated in the presence of pyruvate, benzyl viologen and different concentrations of nitazoxanide and activity of PFOR measured spectrophotometrically at 546 nm. Anaerobic condition in the cuvettes was achieved by bubbling H_2 gas or by addition of sodium dithionite. The results in Figure 18 show that nitazoxanide (≥ 42 µM i.e., 13 µg/ml) decreased the ability of *E. histolytica* PFOR enzyme to reduce benzyl viologen, suggesting that nitazoxanide can compete with benzyl viologen as the electron acceptor.

Figure 18: Nitazoxanide inhibition of benzyl viologen reduction in *E. histolytica*.



Series 1: H_2 bubbled to make cuvette anaerobic
Series 2: Sodium hydrosulfite (dithionite) used instead of hydrogen to render cuvette anaerobic

Using the same assay, nitazoxanide, at a concentration of 20 µM (i.e. 6 µg/ml), was shown to compete with benzyl viologen for electrons and act as an electron acceptor for the PFOR enzyme from 2 strains of *T. vaginalis* (Table 44).

Table 44: Effect of nitazoxanide on inhibition of PFOR activity in *T. vaginalis*.

	Benzyl viologen reduction (nmol/min/mg protein)		
Strain	0 µM nitazoxanide	20 µM nitazoxanide	Percent inhibition
014 (metronidazole-sensitive)	105	5.12	95%
043 (metronidazole-resistant)	113	20.5	82%

2.2. Anaerobic and microaerophilic bacteria:

The ability of nitazoxanide to act as an electron acceptor for the PFOR enzyme of *Clostridium difficile and Clostridium perfringes* was examined[1]. For this, one strain of each of the 2 species was grown in Wilkins-Chalgreen broth under anaerobic conditions for 24 hours. Cell-free extracts were prepared and used to test for PFOR activity by the same assay as described in the previous study. The results in Table 45 show 3 to 4 fold increase in specific activity of PFOR in

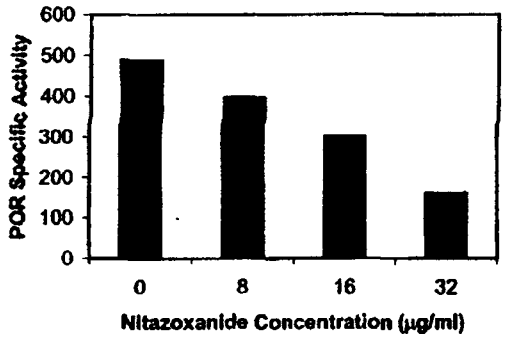
the presence of nitazoxanide compared to benzyl viologen suggesting that nitazoxanide can serve as an electron acceptor for the PFOR enzyme of *Clostridium.*

Table 45: PFOR activity for *Clostridium difficile* and *Clostridium perfringes.*

	Specific Activity (nmol/min/mg P)	
	Benzyl viologen	Nitazoxanide
Clostridium difficile	211	638
Clostridium perfringens	450	1,920

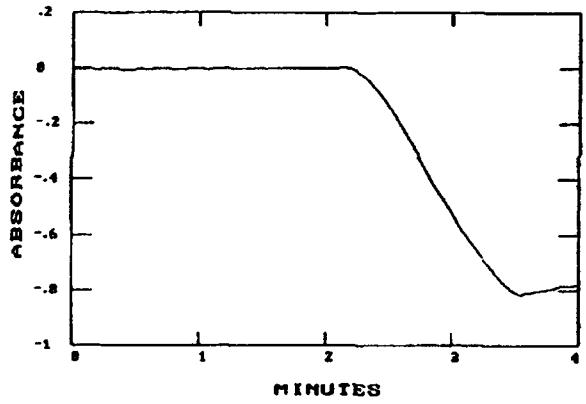
The mechanism by which nitazoxanide exhibits activity against *Helicobacter pylori* was examined under anaerobic conditions[19,20]. Cell extract prepared from late log phase cultures of *H. pylori* grown in Brucella broth was used to determine PFOR activity. The PFOR activity was measured spectrophotometrically at 546 nm using benzyl viologen as described in the previous studies. However, the reaction mixture also contained coenzyme A and thiamine pyrophosphate (additional co-factors required for enzyme activity). Anaerobic condition was achieved by adding few grains of sodium hydrosulfite to the reaction mixture. The reduction of benzyl viologen by PFOR in the presence of nitazoxanide (0-32 μg/ml) was determined. The results in Figure 18 show that nitazoxanide inhibits the reduction of benzyl viologen by PFOR, suggesting that it can act as an alternative electron acceptor for the PFOR enzyme of *H. pylori.*

Figure 18: Competitive inhibition of PFOR activity (benzyl viologen reduction) in *H. pylori* extracts as a function of NTZ concentration. POR = PFOR= pyruvate oxidoreductase.



In another experiment, the ability of nitazoxanide to act as an electron acceptor for PFOR enzyme was measured spectrophotometrically at 412 nm. The results in Figure 19 show a decrease in absorbance at 412 nm suggesting that nitazoxanide is reduced. This confirms the observation made in the previous experiment that nitazoxanide can act as an electron acceptor for the PFOR enzyme of *H. pylori.*

Figure 19: PFOR assay by monitoring reduction of nitazoxanide at 412 nm.



The role of other enzymes such as nitroreductases (e.g., RdxA and FrxA) in the reduction/activation of nitazoxanide was investigated in *H. pylori*. In one study[19], the minimum inhibitory concentration (MIC) required to completely inhibit growth of *H. pylori* in the presence of the *rdxA* gene was 8-fold lower than in its absence. Such testing was done by the standard agar dilution method using Brucella agar medium supplemented with 7% fetal bovine serum under microaerobic conditions at 37°C (Table 46). The results suggest that bacteria with *rdxA* gene were susceptible to nitazoxanide and that this gene may be involved in the activation of the drug.

Table 46: Role of RdxA in susceptibility to nitazoxanide.

Strain	MIC (µg/ml)	
	Metronidazole	**Nitazoxanide**
1061 (*rdxA* -ve mutant)	64	32
1061 + pRdxA*	1	4

*Strain 1061 with plasmid carrying wild-type RdxA.

In another study[20], the ability of two nitroreductases (encoded by the *rdxA* and *frxA* gene) of *H. pylori* to reduce nitazoxanide was examined. For this, the *H. pylori rdxA* and *frxA* genes were expressed in *E.coli* by cloning into an overexpression vector pET29. The enzymes were purified using a nickel column and the substrate specificity for nitazoxanide and other compounds was determined spectrophotometrically in the presence of NADPH. The wavelength at which the reduction of each compound was measured is shown in Table 47. The results in Table 47 show that the nitroreductases encoded by the *rdxA* and *frxA* gene of *H. pylori* were capable of reducing nitazoxanide.

Table 47: Substrate specificity of RdxA and FrxA nitroreductases of *H. pylori*.

Substrate	Wavelength	Specific activity (μmol/min/mg protein)[a]	
		RdxA nitroreductase	FrxA nitroreductase
Metronidazole	320nm	5.13	<0.0002
NADPH (metronidazole)[b]	340nm	9.01	<0.0002
Nitazoxanide	412nm	13.4	22.2
Nitrofurazone	400nm	<0.0002	0.50
Furazolidone	400nm	<0.0002	1.50
Nitrofurantoin	420nm	<0.0002	2.01

[a] Specific activities are calculated as the means for five assays of each of two independent batches of enzyme for FrxA and RdxA. The error in these assays is <15%.
[b] NADPH oxidation was followed at 340 nm with metronidazole as the electron acceptor.

Overall, the results show that nitazoxanide can be reduced or activated by PFOR and the nitroreductases (RdxA and FrxA) of *H. pylori*.

In another study[21], the mechanism of action of nitazoxanide was compared to that of metronidazole (another nitroimidazole) in *H. pylori*. Activated products of metronidazole are known to cause base substitutions (transversions and transition) in DNA resulting in an increase in papillation frequency. The ability of nitazoxanide to cause mutation was determined by a papillation assay using *Escherichia coli* tester strains carrying the *H. pylori rdxA* gene on plasmid pGS590. The strains carrying the *rdxA* gene express nitroreductase that confers suceptibility to metronidazole. Strains carrying pBluescript (vector without the *rdxA* gene) were used as controls. Dilution of overnight cultures of the different strains were plated on minimal glucose agar medium supplemented with phenyl-β-D-galactoside, ampicillin (100 μg/ml), 5-bromo-4-chloro-3-indolyl -β-D-galactoside (X-gal) and nitazoxanide or metronidazole (5 or 10 μg/ml) and incubated for 24 to 36 hours. The number of blue (β-galactosidase positive) papillae per total viable count was used to determine the papillation frequency. The results in Table 48 show that in the presence of nitazoxanide, the papillation frequency did not increase significantly in strains carrying the *rdxA* gene. However, metronidazole was shown to increase the papillation frequency in these strains (Table 49). The results suggest that although metronidazole and nitazoxanide are activated by RdxA, the mechanism of action of nitazoxanide is different from that of metronidazole. The study shows that nitazoxanide is not mutagenic in *E.coli* expressing the *H. pylori* RdxA nitroreductase. However, weak mutagenic effects were observed against the *Salmonella typhimurium* strain TA 100 using the Ames test [see NDA #20871 (N-000) pharmacologist review dated 24-4-96].

Table 48: Papillation frequency with Nitazoxanide.

Strain	Base Substitution In lacZ	Mutation Type	pBluescript Ntz (µg/ml) 0	5	10	pGS950 Ntz (µg/ml) 0	5	10
WT	GGG AAT GAG TCA GGC Glu							
CC101	GGG AAT TAG TCA GGC G	TV	32	29.8	30.1	53	72.6	115
CC103	GGG AAT CAG TCA GGC G	TV	0.39	3.39	2.86	1.12	1.28	2.55
CC104	GGG AAT GCG TCA GGC A	TV	35	19	11.5	100	90	51.4
CC105	GGG AAT GTG TCA GGC A	TV	13.3	12.9	8.56	0.39	0.42	3.71
CC106	GGG AAT AAG TCA GGC G	TS	0.86	0.77	6.48	124	50.9	76

TV = transversions
TS = Transitions.

Table 49: Papillation frequency with Metronidazole.

Strain	Base Sub. lacZ	Metronidazole (µg/ml) pBluescript 0	5	10	15	pGS950 0	5	10	15
WT	GGG AAT GAG TCA GGC Glu								
CC101	GGG AAT TAG TCA GGC G	25	24	34	25	59	217	793	697
CC103	GGG AAT CAG TCA GGC G	3.16	5.5	2.78	6.1	5.4	48	724	46,053
CC104	GGG AAT GCG TCA GGC A	75	12	69	91	69	220	4,404	39,310
CC105	GGG AAT GTG TCA GGC A	7	8.4	7	3.65	0.24	17.7	349	3,614
CC106	GGG AAT AAG TCA GGC G	1.16	2.4	2.0	2.8	18.9	134	617	896

In another experiment[20, 21], the ability of nitazoxanide and metronidazole to cause DNA damage/ fragmentation were compared. For this, *E. coli* tester strain CC104 harboring the vector, pBluecript (pBSK) or pGS950 (carrying the *rdxA* gene) was incubated with different concentrations of nitazoxanide or metronidazole in minimal glucose medium at 37°C for 5 hours. Cells treated with hydrogen peroxide (20 mM) for 15 minutes were used as positive control. DNA extracted from the drug or hydrogen peroxide treated cells was subjected to alkaline gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light. The results in Figure 20 show that no smearing of DNA (indicating DNA damage) was observed in nitazoxanide treated *E.coli* cells carrying the *rdxA* gene (pGS950) or vector (pBSK). However,

DNA damage is observed in metronidazole treated *E.coli* cells carrying the *rdxA* gene on the plasmid pGS950 (Figure 21).

Figure 20: DNA fragmentation of *E.coli* strains challenged with nitazoxanide.

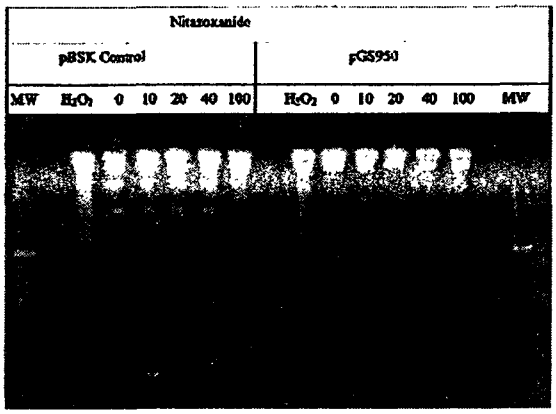
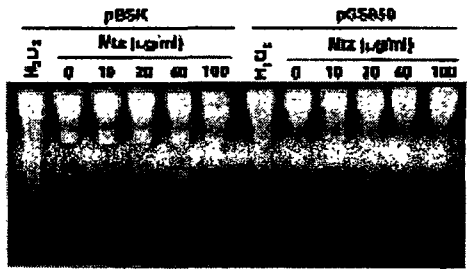


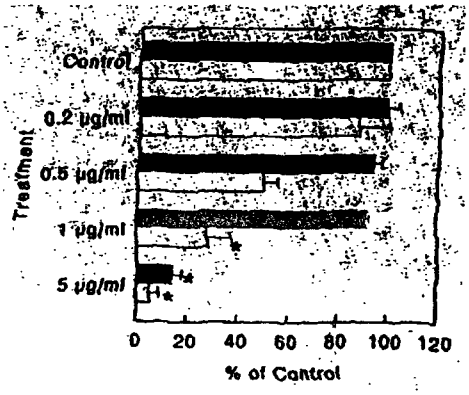
Figure 21: DNA fragmentation of *E.coli* strains challenged with metronidazole.



The results of the DNA fragmentation assay suggest that unlike metronidazole, nitazoxanide does not damage DNA in the presence of the *H. pylori rdxA* gene and the mechanism of action of the two drugs in *H. pylori* is different.

In addition to the effect of nitazoxanide on DNA damage, its ability to inhibit vacuolating toxin produced by *H. pylori* (possible cause of mucosal damage in infected individuals) was investigated[22]. For this, the bacterial cells were cultured in Brucella broth supplemented with 10% fetal calf serum with or without nitazoxanide at 37°C for 2 days. The culture supernatant was collected by centrifugation and concentrated 14-fold by ultrafiltration technique. Two-fold dilutions of the supernatants were added to a monolayer of HeLa cells in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum and 10 mM ammonium chloride. The intracellular vacuole development was observed under the microscope (magnification x 250) for a period of 24 hours. The vacuolating toxin activity was expressed as the reciprocal dilution of the culture supernatant showing cytotoxic effect (≥ 50% cells with vacuoles). The results for vacuolating toxin production (% of control) and bacterial growth in the presence of nitazoxanide are shown in Figure 22. The addition of nitazoxanide (1 µg/ml) reduced the bacterial growth by about 5% and the vacuolating toxin activity by 60%. At a higher concentration of nitazoxanide (5 µg/ml), both bacterial growth and vacuolating toxin activity were inhibited by > 80%.

Figure 22: Inhibition of vacuolating toxin production in *H. pylori* cultures by nitazoxanide. The bacterial growth was measured spectrophotometrically. The optical density of the control group at the end of culture was 0.502 ± 0.012. The vacuolating activity of control cultures was 128 ± 32 (reciprocal dilution titer of culture supernatant which showed vacuolating effect). Each bar represents the mean ± standard error for 3 experiments. * $p < 0.05$ compared with control group. ■ = bacterial growth; = vacuolating activity.



3. ACTIVITY *IN VITRO* AND *IN VIVO*:

The activity of Nitazoxanide was measured against selected protozoans (other than *Cryptosporidium* and *Giardia*), helminths, anaerobic and microaerophilic bacteria. These studies were conducted *in vitro* or in animals. A brief summary of the studies is as follows:

3.1. *Trichomonas vaginalis*:

3.1.1. *In vitro:*

The *in vitro* activity of nitazoxanide and tizoxanide was measured against 16 clinical isolates of *T. vaginalis* collected from vaginal secretions from women and prostatic/urine samples from men within the US[23]. The trophozoites (10, 000/well) were incubated in the presence or absence of drug in modified Diamond's medium supplemented with 10% fetal calf serum, pH 5.9 under aerobic or anaerobic conditions for 46-50 hours at 37°C. The minimum lethal concentration (MLC) was the first dilution at which no motile trichomonads were observed.

The results in Table 50 show that all the isolates exhibited higher metronidazole MLC values under aerobic conditions compared to anaerobic conditions. It is also of note that the MLC values for nitazoxanide and tizoxanide were not altered by aerobic vs anaerobic conditions and were ≥ 8-fold lower than metronidazole (Table 50). The actual parasite count was not included.

Table 50: Activity of nitazoxanide and tizoxanide against *T. vaginalis*.

	MLC_{90} (ug/ml)*		MLC_{50} (ug/ml)*	
	Anaerobic	Aerobic	Anaerobic	Aerobic
Metronidazole	25	>400	12.5	100
Nitazoxanide	3.2	6.4	1.6	1.6
Tizoxanide	0.8/1.6	1.6	0.4	0.8

* the results are based on the testing of 16 isolates.

In another study[24], the *in vitro* activity of nitazoxanide against clinical isolates of *T. vaginalis* was measured using Magara medium containing 5-10% human plasma (under anaerobic conditions). A 3-day old culture was used to determine the activity of nitazoxanide and metronidazole. The results were expressed as MLC or MIC (i.e., concentration required to completely inhibit the growth of the trophozoites). The concentration of the inoculum prepared for determining MLCs and MICs was different (MLCs: 150,000 to 600, 000 and MICs 100,000). However, the trophozoite count used per well for determining MLCs and MICs was not specified. The cultures were incubated at 37°C for 48 hours. The results in Table 51 show that the nitazoxanide and metronidazole MIC values to be comparable. However, the MLCs were about 4-fold higher for metronidazole compared to nitazoxanide. The number of isolates tested was not specified. The actual parasite count in the absence or presence of different concentrations of drug was not included.

Table 51: *In vitro* minimum lethal concentrations and minimum inhibitory concentrations.

Test Drug	Minimum Inhibitory Concentrations	Minimum Lethal Concentrations
Nitazoxanide	0.50-1.25 µg/ml	6 µg/ml
Metronidazole	0.25-1.00 µg/ml	25 µg/ml

3.1.2. *In vivo*:

The *in vivo* activity of nitazoxanide against *T. vaginalis* was measured in 4-week old Sprague Dawley female rats[24]. The rats were castrated and permanent estrus maintained by subcutaneous administration of estradiol cyclopentenylpropionate. The sponsor has stated that *T. vaginalis* trophozoites were inoculated, however, the route of inoculation was not specified. Nevertheless, vaginal fluids were examined microscopically to confirm Trichomonas infection. Rats were treated for 6 days with either nitazoxanide or metronidazole by gavage or locally using vaginal suppositories (prepared in the lab) and the rats were followed for the presence of trophozoites in vaginal secretion.

The results were expressed as

+ numerous mobile trophozoites: treatment failures
± very few trophozoites with no or low mobility: treatment partially effective
- no trophozoites: effective treatment.

The results in Table 52 show that metronidazole was more effective than nitazoxanide when administered by gavage. However, the activity of nitazoxanide was similar to metronidazole when administered as an intravaginal suppository.

Table 52: Activity of nitazoxanide (a) oral solutuions and (b) vaginal suppository against *T. vaginalis* in rats.

(a) oral solutions

Compound	Dose (mg/kg)	No. of Animals	1st Control			2nd Control			3rd Control		
			+	±	-	+	±	-	+	±	-
Nitazoxanide	200	10	6	2	2	2	0	8	1	0	9
Metronidazole	20	9	0	3	6	3	2	4	2	0	7
Metronidazole	50	10	0	2	8	0	0	10	0	0	10
Untreated Controls	-	11	8	3	0	9	2	0	11	0	0

(b) vaginal suppositories

Compound	Dose (mg)	No. of Animals	1st Control			2nd Control			3rd Control		
			+	±	-	+	±	-	+	±	-
Nitazoxanide	5	10	0	0	10	0	0	10	0	0	10
Metronidazole	5	10	0	0	10	0	0	10	0	0	10
Metronidazole	1	9	4	0	5	4	0	5	4	0	5
Untreated Controls	-	11	11	0	0	11	0	0	11	0	0

3.2. *Entameoba histolytica*:

3.2.1. *In vitro*:

The activity of nitazoxanide and its metabolites against 5 strains of *Entamoeba histolytica* was examined *in vitro*[15]. Metronidazole was used as a positive control. Log phase culture of *E. histolytica* containing trophozoites (10^4) were incubated with two fold dilutions of the drugs in Diamond's TYI-S-33 complete medium supplemented with 10% inactivated bovine serum and 3% vitamin-tween 80 mixture at 35°C in 4% CO_2. The activity was measured by incorporation of ^{3}H-thymidine and the cultures incubated for 24 additional hours. The drug concentration required for 50% inhibition of parasites (IC_{50}) was determined by regression analysis.

The results in Table 53 show that nitazoxanide and its metabolites, tizoxanide (TIZ) and tizoxanide glucuronide (TIZg) were active against 5 strains of *E. histolytica*. The activity of NTZ was similar to metronidazole (MTZ). The mean IC_{50s} for NTZ, TIZ, TIZg, and MTZ were 9.9 ± 9.4 μM (32.3 ± 30.6 μg/ml), 9.3 ± 9.3 μM (35.1 ± 35.1 μg/ml), 13.8 ± 9.5 μM (31.3 ± 21.5 μg/ml) and 14.6 ± 30.4 μM (85.3 ± 68.9 μg/ml), respectively.

Table 53: *In vitro* activity of nitazoxanide and its metabolites against *Entameoba histolytica.*

Mean [SD] IC_{50} in µM

Strain	MTZ	NTZ	TIZ	TIZ_g
HM1:IMSS	11.21 [4.2]	8.16 [0.6]	9.01[1.2]	25.86 [4.8]
NIH 200	9.52 [1.2]	4.85 [0.6]	7.39[1.1]	7.31 [2.3]
H303	14.14 [6.4]	6.5 [1.3]	12.22 [2.2]	4.83 [0.5]
IULA:0593:2	28.49 [9.76]	15.55 [4.1]	7.01 [1.66]	13.3 [3.0]
IULA:1092:1	9.69 [0.5]	14.57 [0.7]	10.97 [2.0]	17.86[7.7]
MEAN	14.61 [30.4]	9.93 [9.4]	9.32 [9.3]	13.83 [9.5]
t-test vs MTZ	-	NS	NS	NS

MTZ= metronidazole
NTZ = nitazoxanide
TIZ = tizoxanide
TIZg = tizoxanide glucuronide
NS = not studied

In another study[25], the *in vitro* activity of nitazoxanide against *Entamoeba histolytica* was measured by adding drug to either fresh or 24 hours old cultures of the parasites. The study has been reviewed previously [NDA# 20-871 (N-000), microbiology review dated 06-01-98]. The raw data were not provided in the report. It has been stated that nitazoxanide at concentrations of 0.5-1 µg/ml and 1-1.25 µg/ml inhibited fresh *Entamoeba histolytica.* However, inhibition of 24 hour old cultures required much higher concentrations of the drug (6 and 12.5 µg/ml, respectively). It was stated that this activity was similar to that observed with metronidazole, however, complete details of the experimental design and results were not provided for an independent evaluation.

3.2.2. *In vivo*:

No animal studies were conducted to examine the *in vivo* activity of nitazoxanide against *E. histolytica.* However, nitazoxanide (500 mg b.i.d for 3 days) was shown to be effective in patients with *E. histolytica* infections (for details see page 52)

3.3. *Microsporidium*:

3.3.1. *In vitro*:

Activity against *Encephalitozoon (Septata) intestinalis* was measured *in vitro* by infecting rabbit kidney cells (RK-13) with tissue culture derived microsporidia[26]. This study is same as reviewed earlier [NDA# 20-871 (N-000), microbiology review dated 06-01-98]. The results show that nitazoxanide and desacetyl nitazoxanide at a concentration of 1 µg/ml resulted in about 70% parasite inhibition with minimal toxicity. Higher concentrations of the drug were toxic to the mammalian cells. Similar results were observed against *Vittaforma corneae.*

3.3.2. *In vivo:*

Studies examining the *in vivo* actvity of nitazoxanide against *Microsporidium* were not conducted.

3.4. Trematodes (*Fasciola hepatica* and *Fasciola gigantica*):

3.4.1. *In vitro*:

The mature flukes (obtained from infected calves) in RPMI-Rabbit serum with 2% rabbit blood plus 100 ppm penicillin and 100 ppm streptomycin were exposed *in vitro* to different concentrations of nitazoxanide or its metabolite[27]. Presence of the nitazoxanide at a concentration of ≥ 10 µg/ml was shown to decrease motility and to cause more death of the worms as compared to control cultures. A higher concentration of deacetyl-nitazoxanide (≥ 25 µg/ml) was required for similar activity.

3.4.2. *In vivo*:

The activity of nitazoxanide against *Fasicola gigantica* was tested in rabbits[28]. In one experiment, rabbits were infected orally with 35-40 encysted metacercariae (placed in a lettuce leaf). It was stated that immature fluke stages were observed in rabbits at 4 weeks post-infection. Four weeks after infection, nitazoxanide (35 mg/kg or 75 mg/kg) was administered by the oral route for 7 days. Untreated animals were used as controls. At the end of treatment, the number of flukes in the liver tissue and washings of the abdominal cavity and visceral surface was determined. The raw data were not included in the submission. The sponsor has stated that 40-60% reduction in immature flukes was observed in nitazoxanide treated animals.

In another experiment, metacercariae (10-15) were used to infect rabbits and the treatment was initiated 10 weeks post-infection. Treatment with nitazoxanide was at the same dose as described above. It was stated that mature fluke stages were observed at 10 weeks post-infection. Here again, the raw data were not included. The sponsor has stated that no flukes were observed in nitazoxanide treated animals compared to untreated controls. Based on these results the sponsor has concluded that nitazoxanide at 35 mg/kg or 75 mg/kg dose for 7 days was effective against mature flukes.

3.5. Nematodes and Cestodes:

3.5.1. *In vitro*:

No studies examining the *in vitro* activity of nitazoxanide against nematodes and cestodes were included in the submission.

3.5.2. *In vivo*:

The activity of nitazoxanide against helminthic infections was examined in dogs[29,30]. This study has been reviewed earlier [NDA# 20-871 (N-000), microbiology review dated 06-01-98]. Studies were conducted in 15 dogs experimentally infected with *Taenia pisiformis* and 13 farm dogs naturally infected with nematodes (round worms, hookworms and whipworms) or a cestode (*Dipyllidium caninum*). For the experimental infection model, dogs were administered 14 to 20 viable cysts of *Taenia pisiformis* by the oral route and treatment with nitazoxanide (75 mg/kg) was administered as a single dose at 6 (group 1) or 8 weeks (group 2) post-infection (the route of

drug administration was not specified). At these times, immature and mature worms, respectively, would be present in the dogs. The fecal samples were examined at different time intervals (1, 8, 12, 24, and 48 hours) after drug administration for the presence of strobila with the naked eye and also concentrated by filtering through a sieve. No untreated or vehicle treated group was used for comparison. At 19 and 4 days post-administration of a single dose of nitazoxanide for the first and second group respectively, the dogs were necropsied and the gastro-intestinal tract was examined for the presence of *Taenia* species. The results in Table 54 show the continued presence of parasite in fecal samples up to a period of 24 hours post-therapy in both groups. Although the sponsor has stated that the dogs were examined for a period of up to 48 hours post treatment, only results obtained between 0-9 and 10-24 hours were shown. At necropsy on day 4 or 19, however, no parasites were observed in the intestine. Nevertheless, in the absence of untreated controls it is difficult to evaluate the activity of the drug because infections with the parasite can be cured up to certain point in immunocompetent hosts. Also, no baseline measurement(s), before initiating treatment, were performed.

Group No	Dog No	Sex	Hours Post-treatment 0-9	10-24	Number of Worms Recovered at Necropsy	Day of Sacrifice
1	N 01	F	2	0	0	DAY 19
	N 04	M	3	1	0	
	N 05	F	0	0	0	
	N 06	M	4	0	0	
	N 10	F	0	0	0	
	N 14	M	1	3	0	
	N 15	M	0	2	0	
2	N 03	F	2	1	0	DAY 4
	N 02	F	0	0	0	
	N 05	F	3	0	0	
	N 07	F	2	2	0	
	N 09	M	3	1	0	
	N 18	M	3	0	0	
	M 09	M	0	0	0	
	M 16	M	3	1	0	

Table 54: Activity of nitazoxanide against *Taenia pisiformis*

In 13 naturally infected dogs, 3 different dosage regimens of nitazoxanide were administered by oral route (mixed with meat) as shown in Table 55.

Table 55: Activity of nitazoxanide against helminths.

Group (Number)	Drug administration Dose (mg/kg)	Days	Activity against parasites examined* *Dc*	*Tv*	*Us*	*Tc*
3 (3)	100, 100 & 200	1, 2 & 8	+	±	±	-
4 (4)	100, 100 & 250	1, 2 & 8	+	±	-	-
5 (3)	150 & 150	1 & 2	+	+	+/-	-
6 (3)	200 & 200	1 & 2	+	-	-	-

Dc = *Dipyllidium caninum*
Tv = *Trichuris vulpis*
Us = *Uncinaria stenocephala*
Tc = *Toxocara canis*

The animals were followed for toxicity and the presence of parasites in the fecal samples at different intervals. The fecal samples were examined with the naked eye for the presence of strobila and also by passing through a sieve. The dogs in groups 5 and 6 were necropsied 24 hours after discontinuation of treatment and 48 hours later for those in groups 3 and 4 and the intestine was processed to determine if parasites were present. The sponsor has identified the parasites recovered and their number at different time intervals and has concluded that nitazoxanide at a dose of ≥ 100 mg/kg was effective in eliminating the cestode *Dipyllidium caninum* since no parasites were observed beyond 24 hours in fecal samples nor in the small intestine at necropsy. The sponsor has also stated that in a previous study administration of 75 mg/kg of nitazoxanide was not effective against this infection. However, the details of these results were not included for review.

Drug activity against 3 species of nematodes, based on examination of the intestine at necropsy, is summarized in Table 55 above. Baseline measurements (that is the number of parasites present in the fecal samples before initiation of treatment) were not performed. Unfortunately, due to the absence of untreated controls in the experiment it is difficult to interpret these data.

In another study[28], cats naturally infected with *Taenia taeniaeformis* were used to examine the activity of nitazoxanide. Nitazoxanide was administered as a single oral dose of 100 mg/kg. The sponsor has stated that the cestodes were eliminated between 8 hours and 3 days post-treatment. The raw data were not included for an independent evaluation.

In another experiment, studies were conducted using sheep naturally infected with *Monieza expansa*, *Thysanieza ovilla*, *Stilesia globipunctata* and *Avitellina centripunctata*. The sheep were administered nitazoxanide (200 mg/kg or 400 mg/kg) as a single dose by the oral route. The sponsor has stated that no tapeworms were observed in the animals at necropsy 4-5 days after treatment. Here again, the raw data were not included for an independent evaluation.

In another study[25], the activity of nitazoxanide was measured against *Syphacia obvelata* (a nematode), and *Hymenolepis nana* (a cestode) *in vivo*. This study has been reviewed previously [NDA# 20-871 (N-000), microbiology review dated 06-01-98]. Mice were infected with *Syphacia obvelata* by placing uninfected mice with infected mice in the same cage for 5 days. Treatment with nitazoxanide (200 mg/kg) or the vehicle was initiated on day 8 for 4 days by the oral route. Two days after discontinuation of treatment, the mice were necropsied and the cecum was examined for the presence of adult worms (≥ 0.5 mm in length). The sponsor has stated that all the mice treated with the vehicle were parasitized, whereas no parasites were observed in 60% of the treated mice. The remaining 40% of the mice treated with nitazoxanide were partly free of worms. The raw data demonstrating the number of parasites recovered were not provided for an independent evaluation and the effect on other stages of the parasite was not evaluated.

In order to measure the activity of nitazoxanide against *Hymenolepis nana*, mice were infected with the egg stage of the parasite and the establishment of infection confirmed by the excretion of eggs in the feces on days 20 to 25 after infection. Mice were then treated with either nitazoxanide (200 mg/kg) or the vehicle for 4 days by the oral route and examined for the presence of worms in the small intestine. The sponsor has stated that mice treated with nitazoxanide were completely deparasitized, whereas untreated mice were completely

parasitized. The raw data were not provided for an independent evaluation. In another experiment conducted as described above, different doses of the drug were administered and the activity of the drug compared to that of niclosamide. The results show that a dose of ≥ 50 mg/kg for 4 days was effective in clearing the worms from the small intestine.

3.6. Bacteria:

3.6.1. *In vitro*:

The activity of nitazoxanide against 241 anaerobic bacteria and 9 aerobic bacteria using clinical isolates and 4 reference ATCC control strains (aerobic and anaerobic) was examined by the NCCLS method[31,32]. This study has been reviewed previously [NDA# 20-871 (N-000), microbiology review dated 06-01-98]. The results show that the anaerobic strains that are susceptible to metronidazole were also susceptible to nitazoxanide. Activity of the metabolite of nitazoxanide was one fold less than that of the parent compound. Aerobic organisms were not susceptible to nitazoxanide under aerobic conditions. *Staphylococccus aureus* (34 clinical isolates) showed about 8 fold lower MIC values under anaerobic conditions as compared to aerobic conditions. Also, about 8 fold lower MIC values were observed against 2 *Staphylococcus epidermidis* strains under anaerobic compared to aerobic conditions. Break-points for susceptibililty were not established.

Activity of nitazoxanide was measured against 103 strains of *Helicobacter pylori* isolated from patients with duodenal ulcer or non-ulcer dyspepsia in France[33]. The MICs were determined by the agar dilution method using Wilkins Chalgreen agar supplemented with 10% sheep blood and polyvitex at pH 6.5. The MIC values indicate that the activity of nitazoxanide and tizoxanide may be similar to that of metronidazole.

The activity of nitazoxanide against *Clostridium difficile* was examined *in vitro*[34,35]. The *in vitro* susceptibility of 15 toxigenic strains of *C. difficile* to nitazoxanide was measured using Wilkins-Chalgreen broth by the microbroth dilution method. Vancomycin and metronidazole were used as comparators. Nitazoxanide was solubilized in DMSO while vancomycin and metronidazole was solubilized in saline or medium. All drugs were then diluted in medium. The cultures were incubated in the presence of different dilution of the drugs at 37°C for 48 hours under anaerobic conditions. The minimum inhibitory concentration (MIC) defined as the lowest concentration of the drug that completely inhibited bacterial growth was determined. The mimimum bactericidal concentration (MBC) defined as the concentration that killed 99.9% of the bacteria was also determined by subculturing aliquots of cultures onto Wilkins-Chalgreen agar at the end of the 48 hour incubation. Additonally, the stability of nitazoxanide in the presence of 5% hamster cecal content (— , was determined. The results in Table 56 show that the activity of nitazoxanide was similar to metronidazole and vancomycin. However, the MICs required to inhibit 90% of the strains (MIC_{90}) was 64 fold higher in the presence of 5% hamster cecal content than in its absence. The activity of metronidazole and vancomycin were not significantly altered in the presence of 5% hamster cecal content. The mean MBCs ± standard deviation for nitazoxanide in the absence and presence of 5% hamster cecal content against *C. difficile* were 0.48 ± 0.47 μg/ml and 13.6 ± 12.7 μg/ml, respectively. The break-points for susceptibility have not been established.

Table 56: MIC of antimicrobial agents for 15 toxigenic *C. difficile* strains.

Antimicrobial agent	MIC (µg/ml)		
	MIC_{50}	MIC_{90}	Range
NTZ	0.25	0.50	0.13–1
Metronidazole	0.25	0.50	0.13–0.50
Vancomycin	0.50	0.50	0.13–1.0
NTZ in 5% cecal contents	4	32	0.50–32
Metronidazole in 5% cecal contents	0.25	0.50	0.13–1
Vancomycin in 5% cecal contents	1	1	0.50–1

MIC_{50} = MIC for inhibition of 50% strains
MIC_{90} = MIC for inhibition of 90% strains.

3.6.2. *In vivo*:

The ability of nitazoxanide to prevent clindamycin-induced ileocecitis was examined in hamsters[34,35]. For this hamsters were administered clindamycin (3 mg/100 gram body weight) orally and inoculated with 10^5 cfu of a *C. difficile* strain 24 hours later. One day post infection, nitazoxanide (3, 7.5 or 15 mg/100 gram body weight) was administered orally once a day for 6 days. The sponsor has stated that the control animals died within 72 hours of infection. All hamsters treated with nitazoxanide died within two week of discontinuation of treatment.

D. Effect of nitazoxanide on the inflammatory responses:

Nitazoxanide contains a salicylic acid moiety that is also present in the structures of non-steroidal anti-inflammatory compounds such as aspirin. Therefore, the drug may have the potential to exhibit anti-inflammatory activity. The anti-inflammatory effect of nonsteroidal compounds has been shown to be due to inhibition of cytokines, chemokines, superoxide radicals, nitric oxide and other mediators produced by neutrophils and macrophages. These activities are important for protection against infection by microorganisms. Thus, modulation of these activities may impact the progression of diarrhea due to *Cryptosporidium* or *Giardia*.

The effect of nitazoxanide on inflammatory response was examined using unstimulated and cytokine stimulated human intestinal epithelial (HCT-8) cells[36]. For this, the monolayers of HCT-8 cells in DMEM supplemented with 10% heat inactivated fetal calf serum (FCS), 1% non-essential amino acids, antibiotics, and 2 mM L-glutamine were stimulated by incubating in the presence of interferon gamma (IFN-γ,1 ng/ml), transforming growth factor-alpha (TNF-α; 20 ng/ml), and interleukin-1β (IL-1β; 10 ng/ml) for 2 or 6 hours at 37°C in 5% CO_2. Nitazoxanide (5 ng/ml) was added and the incubation continued for additional 24 hours. RNA was obtained from the cells at different time points (3, 6 and 24 hours). The mRNA level of interferon inducible protein-10 (IP-10), interleukin-8 (IL-8) and inducible nitric oxide synthase (iNOS) was measured by reverse transcription-polymerase chain reaction (RT-PCR). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as a control. The primers used for the PCR reaction are shown in Table 57. The PCR products were quantified using a — after autoradiography.

Table 57: Oligonucleotide primers for detection of IP-10, IL-8, iNOS and GAPDH.

Gene	Oligonucleotide primer sequences 5'-3'(up/down)	Product size (bp)
IP-10	AGTGGCATTCAAGGAGTACC/ ATCCTTGGAAGCACTGCATC	288
IL-8	TCTGATGGAAGAGAGCTCTG/ GATATTCTCTTGGCCCTTGG	413
iNOS	TTCAGGTACGCTGTGTTTGG/ GGGGATCTGAATGTGCTGTT	231
GAPDH	AATCCCATCACCATCTTCCA/ TGTGGTCATGAGTCCTTCCA	318

The results show that nitazoxanide (5 ng/ml) did not alter the levels of IP-10, IL-8 and iNOS mRNA in HCT-8 cells stimulated by cytokines for 2 hours (Figure 23) or 6 hours (Figure 24). Exposure of unstimulated HCT-8 cells to nitazoxanide increased the IL-8 mRNA levels by 2 fold (Figure 24)

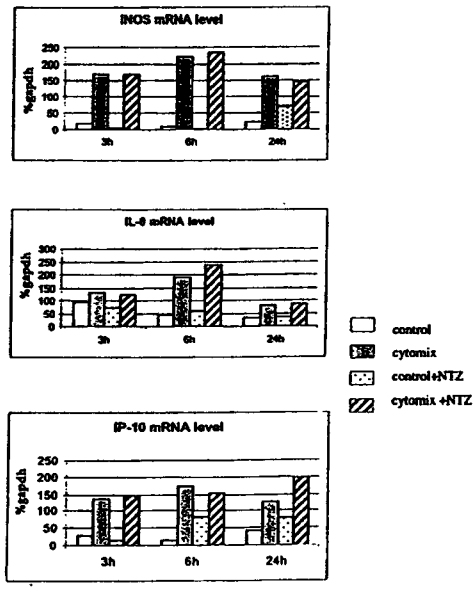


Figure 23: Kinetics of iNOS, IL-8 and IP-10 mRNA response in HCT-8 cells exposed to cytokines in the absence or presence of nitazoxanide. The results (% gapdh) are expressed as ratio of level of target gene product to control gene (gapdh) product (one experiment).

iNOS = inducible nitric oxide synthase.
IL-8 = interleukin-8
IP-10 = interferon inducible protein 10

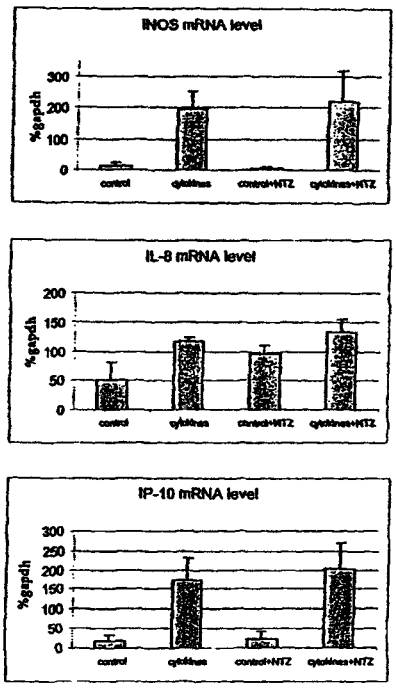


Figure 24: Level of iNOS, IL-8 and IP-10 mRNA in HCT-8 cells exposed to cytokines in the absence or presence of nitazoxanide. The results (% gapdh) are expressed as mean ratio of level of target gene product to control gene (gapdh) product (three experiments). Bars represent one SD from the mean

iNOS = inducible nitric oxide synthase.
IL-8 = interleukin-8
IP-10 = interferon inducible protein 10

In another experiment, the effect of nitazoxanide on the amount of nitrites and nitrates (NO) formed by the metabolism of nitric oxide in unstimulated and cytokine stimulated HCT-8 cells was measured. The HCT-8 cells were stimulated with cytokines as described above for 6 hours. The cells were then exposed to nitazoxanide (5 ng/ml) for 4 hours. The concentrations of nitrites and nitrates in culture supernatants were determined spectrophotometrically using nitrate reductase and Griess reagent [redacted]. The results in Figure 25 show a 3-fold reduction in the amount of nitrites and nitrates produced by HCT-8 cells stimulated with cytokines and exposed to nitazoxanide.

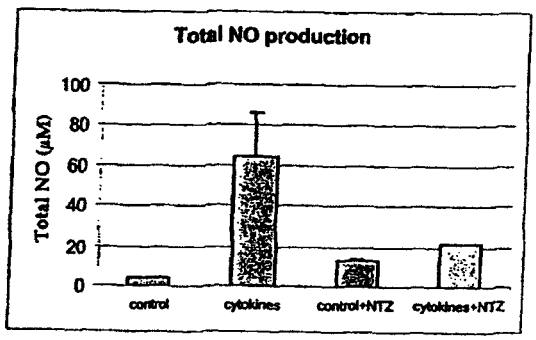


Figure 25: Effect of 6 hour cytokine and/or 4 hour nitazoxanide exposure on total nitrite and nitrate (NO) production. Values are means ± SD of three experiments.

In another experiment, the concentration of IL-8 in the culture supernatants of unstimulated and cytokine stimulated HCT-8 cells in the presence or absence of nitazoxanide was examined. The experimental design was same as described above. The results in Figure 26 show that

nitazoxanide at a concentration of 5 ng/ml did not alter the concentration of IL-8 in unstimulated or stimulated HCT-8 cells

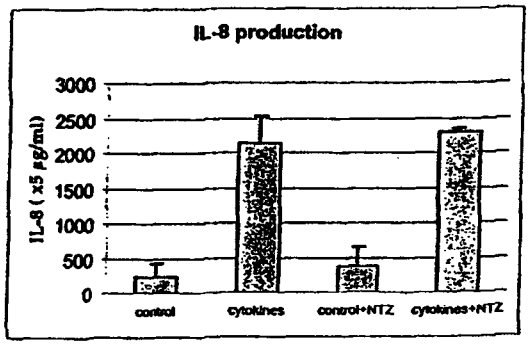


Figure 26: Effect of 6 hour cytokine and/or 4 hour nitazoxanide exposure on IL-8 production. Values are means ± SD of three experiments. The IL-8 levels were determined using IL-8 specific monoclonal antibody by an enzyme-linked immunosorbent assay (ELISA).

Overall, the results show that nitazoxanide at a concentration of 5 ng/ml did not alter the mRNA levels of iNOS, IL-8 and IP-10 in cytokine (IL-1β, INF-γ, and TNF-α) stimulated HCT-8 cells *in vitro*. A 2-fold increase in the mRNA level of IL-8 was observed in unstimulated HCT-8 cells exposed to nitazoxanide. However, nitazoxanide did not alter the production of IL-8 in unstimulated or cytokine stimulated HCT-8 cells. Nitazoxanide was also shown to decrease the production of nitric oxide in cytokine stimulated HCT-8 cells. Higher concentrations of nitazoxanide were not tested in these experiments. Additionally, *C. parvum* infected HCT-8 cells were not used. It is unclear if nitazoxanide will have similar effects on infected versus uninfected cells *in vivo*.

The target enzymes for nonsteroidal anti-inflammatory compounds are cyclooxygenases that are involved in the production of prostaglandins (PGE_2 and PGH). The effect of nitazoxanide on cyclooxygenases, PGE_2 secretion, and other inflammatory responses in the rat carrageenan-induced paw edema model was examined (for details see pharmocologist's review). Nitazoxanide and tizoxanide were shown to inhibit cyclooxygenase (Cox-1 and Cox-2) and the production of PGE_2. Using the rat carrageenan-induced paw edema model, nitazoxanide (500 mg/kg) was shown to be less effective (29%) than the nonsteroidal anti-inflammatory agent, indomethacin (46%), in reducing the edema.

The above studies suggest that nitazoxanide has some anti-inflammatory activity and may modulate the host immune response. However, in studies examining the efficacy of nitazoxanide in the treatment of diarrhea due to *Cryptosporidium* or *Giardia,* a correlation of parasitological and/or clinical outcome with immune/inflammatory responses was not examined.

E. Drug Resistance:

A potential for development of resistance by *C. parvum* and *G. lamblia* to nitazoxanide was not examined.

F. CONCLUSIONS:

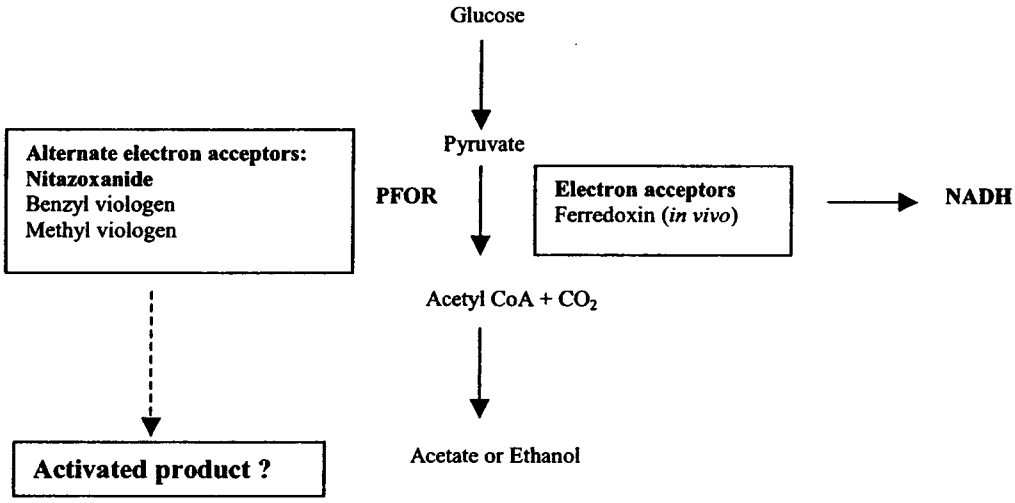
The sponsor is seeking an approval for use of nitazoxanide in the treatment of diarrhea due to *C. parvum* and *G. lamblia*

Mechanism of Action:

The survival of protozoa that lack mitochondria and microaerophilic bacteria under anaerobic conditions depend on the presence of the enzyme pyruvate:ferredoxin oxidoreductase (PFOR) which plays an important role in glucose metabolism (Figure 27). PFOR catalyzes the conversion of pyruvate to acetylCoA and CO_2 using ferredoxin as an electron acceptor *in vivo*. Benzyl viologen and methyl viologen have been shown to act as alternate electron acceptors *in vitro*.

Studies show that nitazoxanide can also act as an electron acceptor and is reduced by the PFOR enzyme from protozoa (*G. lamblia*, *T. vaginalis*, and *E. histolytica*), and anaerobic bacteria (*C. difficile*, *C. perfringes*, and *H. pylori*). It is possible that the reduction of nitazoxanide by PFOR can lead to generation of a toxic radical. Although, the *C. parvum* genome analysis revealed the presence of a PFOR like enzyme with 31-51% sequence similarity to PFOR sequences from other protozoa and bacteria, the ability of this enzyme to reduce/activate nitazoxanide was not investigated.

Figure 27: Activation of nitazoxanide by pyruvate:ferredoxin oxidoreductase (PFOR) enzyme from protozoa.



It is possible that nitazoxanide may exhibit its effects through other pathways. For example, two other enzymes, the RdxA and FrxA nitroreductases of *H. pylori* were shown to activate the drug. Nitazoxanide did not induce mutations and damage DNA of *Escherichia coli* expressing the *H. pylori rdxA* gene product.

Overall, the studies show that nitazoxanide acts as an electron acceptor (Table 57). The ability of nitazoxanide to inhibit the PFOR enzyme was not measured. The precise mechanism by which nitazoxanide exhibits activity against *C. parvum* or *G. lamblia* is not known.

Table 57: Summary of the studies examining mechanism of action of nitazoxanide in protozoa and bacteria.

Organism	Activity of enzyme PFOR	Nitazoxanide as electron acceptor for PFOR	Mutation frequency	DNA damage	Other nitroreductase activating nitazoxanide	Anti-vacuolating activity of nitazoxanide	Other	Genome Sequence similarity to the *C. parvum* PFOR sequence (%)
C. parvum	ND	ND	ND	ND	ND	ND	Genome analysis	
G. lamblia	+	yes	ND	ND	ND	ND	Genome analysis	31%
E. histolytica	+	yes	ND	ND	ND	ND	Genome analysis	49%
T. vaginalis	+	yes	ND	ND	ND	ND	Genome analysis	43%
Cl.pasteuranium	ND	ND	ND	ND	ND	ND	Genome analysis	51%
Cl. perfringes	+	yes	ND	ND	ND	ND	ND	
Cl. difficile	+	yes	ND	ND	ND	ND	ND	
H. pylori	+	yes	ND	ND	RdxAFrxA	+	ND	
H. pylori rdxA⁻	ND	ND	ND	ND	ND	ND	ND	
E. coli + *H. pylori rdxA*	ND	ND	No mutation	absent	ND	ND	Susceptibility to nitazoxanide in the presence of RdxA nitroreductase	

ND = not done
+ = present

Activity *in vitro* and *in vivo*:

C. parvum:

The activity of nitazoxanide against *C. parvum* was measured *in vitro* and *in vivo*. *In vitro* activity was determined using different cell lines such as MDBK-F5D2, HCT-8 or A-549 cells infected with oocysts or sporozoites. However, due to variation in methodology (such as time of initial exposure to drug, incubations time, methods used for quantitative analysis) comparison of results is difficult.

MDBK cells infected with the oocyst stage and incubated with nitazoxanide (1 μg/ml) for 48 hours decreased the number of parasites by 29 to 94% with minimal toxicity (≤ 8 %) to the mammalian cells. The activity of the metabolite (tizoxanide) appears to be similar to nitazoxanide when tested under similar experimental conditions. The activity of the other metabolite, tizoxanide glucuronide, was not measured.

In another study, using A-549 cells infected with sporozoites, a higher concentration of nitazoxanide (8 μg/ml) was required to inhibit parasite growth by 50% at 48 hours. The toxicity to A-549 cells was ≤ 11% at this concentration. In a single experiment, a combination of nitazoxanide with azithromycin or rifabutin was shown to be more active against *C. parvum* than nitazoxanide alone.

In another study, the activity of nitazoxanide and its metabolites was examined using HCT-8 cells infected with sporozoites using immunofluorescence and enzyme immuno assays. Exposure of 2-hour old cultures to nitazoxanide (10 μg/ml) for 4 or 46 hours reduced the parasite counts by ≥ 88% using the immunofluorescence assay with 14% toxicity to the HCT-8 feeder cells. Similar observation was made when older cultures (18-hour old) were exposed to the drug for 4 hours. The inhibition of parasite in older cultures (18-hour old) exposed to nitazoxanide

was lower (30%) using enzyme immunoassay compared to the immunofluorescence assay. However, the inhibition of parasite in younger cultures (2-hour old) exposed to nitazoxanide was comparable using the two methods. At a concentration of 10 μg/ml, the metabolites (tizoxanide and tizoxanide glucuronide) caused fragilation and peeling of the HCT-8 cell. The sponsor has stated that asexual stages and sexual stages were observed in cultures at 2-6 hours, and 18-22 hours, respectively. The antiserum used in the above assays cross-reacted with all stages of the parasite including oocysts. However, photographs depicting the different stages of the parasite at 2 hours and 18 hours were not taken. In another study using a different cell line (A-549), meront and gamont stages were observed 48 hours after infection of cells with *C. parvum* sporozoites. In the absence of complete information, the effect of the drug on the different stages in unclear. In a single experiment, nitazoxanide (10 μg/ml) was shown to reduce the number of zygotes in *C. parvum* infected HCT-8 cells in electron microscopic sections. However, macrogametes were observed in nitazoxanide treated cultures but not in controls. The number of parasites reported is too small to draw conclusions on the effect of the drug on the different stages. Also, no comparator was used in these studies.

The *in vivo* studies examining activity of nitazoxanide were conducted in 4 different animal models (rodents and gnotobiotic piglets). It is of note that rodents infected with *C. parvum* do not exhibit diarrhea whereas gnotobiotic piglets do. Studies in **suckling mice** show that nitazoxanide (1.3 mg b.i.d. for 7 days) was effective in reducing the oocycst count detected by the rectal swabs compared to untreated control animals. Such an effect was observed up to 7 days after discontinuation of treatment. Residual parasitic load was not examined in the tissues and paromomycin was not used for comparison of activity. In another study using a powder and an injectible formulation of nitazoxanide (100 mg/day), the powder formulation showed about 50% reduction in mucosal oocysts counts while the injectible formulation showed a 75% reduction in mucosal oocyst counts at the end of 5 days of treatment. This may be due to poor bioavailability of the powder formulation. Paromomycin (50 mg/day for 5 days) was more effective than nitazoxanide in reducing mucosal oocyst counts. The effect of nitazoxanide on shedding of oocysts in stool was not examined in this study.

In the **scid mice** model, two experiments were conducted. In one experiment treatment with nitazoxanide (100 mg/kg) decreased oocyst shedding in the stool samples compared to vehicle treated mice. Parasitic load in the mucosa was also decreased when measured 5 days after discontinuation of treatment. In the second experiment, however, nitazoxanide did not show any activity. Paromomycin was active in both experiments. Reasons for the observed difference in the activity of nitazoxanide in the 2 experiments are unknown. In another experiment performed in scid mice, desacetyl nitazoxanide (tizoxanide) failed to show any activity. While nitazoxanide was not used for comparison in this study, paromomycin was shown to be active.

Studies in **immunosuppressed rats** show that the oocysts counts decreased in control animals over a period of 21 days post-infection. A 2-fold decrease in oocyst counts was observed during treatment with 100 or 200 mg nitazoxanide. The activity of paromomycin was similar to nitazoxanide while sinefungin was more effective than nitazoxanide during treatment. However, no difference in the oocyst counts was observed in the drug treated and control animals after discontinuation of treatment. A 2 to 4-fold decrease in mucosal infection was observed in

nitazoxanide treated animals compared to untreated control animals. Paromomycin and sinefingin were not used as comparators in this study.

In a study in **gnotobiotic piglets** a trend towards a decrease in oocyst scores from days 9 to 13 in nitazoxanide-treated animals was observed. Due to a small number of animals studied and the variability in the oocyst score, it is not clear whether the differences observed between the treated and untreated groups were significant. The incidence of diarrhea was same in all the untreated and nitazoxanide treated animals. In these studies paromomycin was more effective than nitazoxanide in reducing oocyst numbers, mucosal infection scores and in resolution of diarrhea. In another study, no reduction in oocyst counts was observed in animals treated with nitazoxanide. Similar experimental designs and drug doses were used in the two studies. The reason(s) for this discrepancy are not known.

The development of resistance by *C. parvum* to nitazoxanide was not examined.

Immunocompetent patients (children and adults) with cryptosporidial diarrhea, 40% (24/60) of patients treated with nitazoxanide showed absence of oocysts in stool samples at 6-23 days after initiation of therapy compared to 7% (5/67) patients in the placebo group. The parasitological evaluations in these studies were limited to microscopic examination of small amount of stool after acid fast or auramine-phenol staining. Concentrated stool samples for the detection of parasites were not used consistently across studies and the testing of stool samples using more sensitive techniques such as immunofluorescence was performed only in 9 patients. Examination of 2 or more stool samples by 2 different methods (ZNN and IFA) appears to provide a better measure of oocyst levels in the stool. There appears to be no correlation between clinical and parasitological response except in children 4-11 years of age. In HIV positive children, nitazoxanide at the dose of 100 mg b.i.d. for 3 days was ineffective in the treatment of cryptosporidial diarrhea.

G. lamblia:

The studies examining the *in vitro* and *in vivo* activity of nitazoxanide against *G. lamblia* were limited. One study examined the *in vitro* activity of nitazoxanide and its metabolites against 6 strains of *G. lamblia* by incorporation of radiolabeled thymidine. The nitazoxanide IC_{50} values against the 6 strains ranged between 3.5 and 25.4 μg/ml and were 2 to 5 fold lower than the metronidazole IC_{50} values. The activity of tizoxanide was similar to nitazoxanide, however, tizoxanide glucuronide was less active (IC_{50} values 2 to 30-fold higher) than nitazoxanide. Although, the test measures multiplication of trophozoites, it is possible that the trophozoites are still viable. Motility or other viability tests were not performed. In another study, using a different adherence assay and Caco-2 cells as feeder layer, the IC_{50} values of nitazoxanide against 3 clinical isolates were between 61 and 74 μg/ml. The activity of tizoxanide was similar to nitazoxanide (IC_{50} values 50 to 82 μg/ml). The toxicity of nitazoxanide or tizoxanide to Caco-2 cells was not examined. The IC_{50} values based on the adherence assay were 3 to 18-fold higher than that obtained using incorporation of ^{3}H-thymidine. There are no established methods for susceptibility testing of *Giardia*. In the absence of toxicity data and standardized methods, the usefulness of the IC_{50} values in predicting *in vivo* activity is not known.

In **neonatal mice** infected with the [redacted] strain of *G. lamblia*, nitazoxanide (100 and 200 mg/kg) showed a decrease in trophozoite counts in the intestinal tissue at 48 hours post-treatment. The variability in the trophozoite counts in the untreated control animals was very large (14 - 330). The *in vivo* activity of nitazoxanide against other strains of *G. lamblia* was not tested.

Nitazoxanide was shown to be effective in the treatment of 6/8 immunocompetent adults with giardiasis compared to the placebo (0/10). In immunocompetent children, nitazoxanide was shown to be as effective as metronidazole in the treatment of giardiasis. Examination of 2 or more post-treatment samples was useful in the detection of cysts after drug treatment. The concentration and IFA methods appear to be more sensitive in detection of cysts in stool samples when the numbers were low.

Studies examining the development of resistance by *G. lamblia* to nitazoxanide were not performed.

Overall, nitazoxanide and its metabolite, tizoxanide were effective in inhibiting of the growth of sporozoites and oocysts of *C. parvum* and trophozoites of *G. lamblia* (Table 59). The metabolite, tizoxanide glucuronide, was less effective in inhibiting the growth of sporozoites of *C. parvum*, however, its activity against the oocyst stage was not tested. Tizoxanide glucuronide was also less effective than nitazoxanide or tizoxanide against the trophozoites of *G. lamblia*.

Table 59: Effect of nitazoxanide and its metabolites on the growth of *C. parvum* and *G. lamblia*.

Drug	*C. parvum*		*G. lamblia*
	Sporozoite	**Oocyst**	**Trophozoite**
Nitazoxanide (NTZ)	Inhibition	Inhibition	Inhibition
Tizoxanide (TZ)	Inhibition but cytotoxic	Inhibition	Inhibition
Tizoxanide glucuronide (TZg)	Inhibition but cytotoxic	Not tested	Inhibition (2-30 fold lower than NTZ and TZ)

Other microorganisms:

The activity of nitazoxanide against protozoans (other than *Cryptosporidium* and *Giardia*), helminths and bacteria was measured. Nitazoxanide was shown to be active againt the trophozoites of *E. histolytica in vitro* and effective in the treatment of patients with ameobiasis. The information provided in the study reports on other protozoa, helminths and bacteria is very limited. Since the broad spectrum anti-bacterial and anti-parasitic indications are not under consideration at the present time no attempts were made to obtain additional information from the sponsor about these studies.

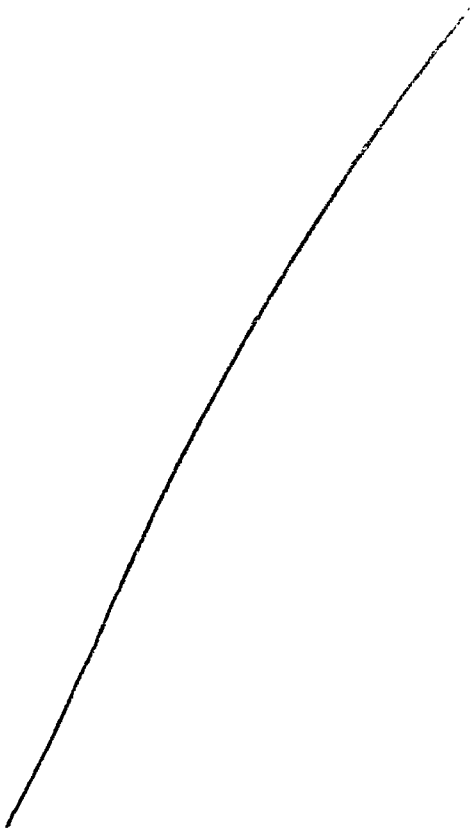
Effect of nitazoxanide on inflammatory and immune response:

In vitro, nitazoxanide at a concentration of 5 ng/ml did not have an effect on the mRNA levels of inducible nitric oxide synthase, interleukin-8 (IL-8) and interferon inducible protein 10 in cytokine stimulated human intestinal epithelial (HCT-8) cells. However, the drug inhibited the

production of nitric oxide in these cells. A 2-fold increase in IL-8 mRNA levels was observed in unstimulated HCT-8 cells exposed to nitazoxanide. However, the production of IL-8 was not altered in unstimulated and stimulated HCT-8 cells in the presence of nitazoxanide. It is unclear whether nitazoxanide will have a similar effect on these immune modulators *in vivo*. Like other anti-inflammatory compounds, nitazoxanide inhibited the activity of cyclooxygenases and production of prostaglandins *in vitro*.

In vivo, nitazoxanide reduced carrageenan induced paw edema in rats by 29% compared to 46% in animals treated with indomethacin. The effect of nitazoxanide on immune function in patients with cryptosporidial diarrhea or giardiasis has not been examined.

G. THE LABEL:



4 Draft Labeling Page(s) Withheld

Susceptibility Tests:

INDICATIONS AND USAGE (for Oral Suspension):

H. RECOMMENDATIONS:

This NDA is approvable with respect to Microbiology pending an accepted version of the label. The following recommendation should be considered for future drug development.

1. For future clinical studies, please consider performing parasitological evaluations using multiple stool samples at different time points such as, at baseline, end of therapy and 3-4 weeks post therapy. Concentration techniques for stool samples in combination with more sensitive immunofluorescence or enzymeimmunoassays should be used for detection of the parasite. Attempts should be made to correlate parasitologic outcome with clinical outcome.

/S/

Kalavati Suvarna
Microbiologist, HFD-590

CONCURRENCES:

HFD-590/Deputy Dir. _____Signature _____Date
HFD-590/Micro TL _____Signature _____Date
CC:
HFD-590/Original IND
HFD-590/Division File
HFD-590/MO
HFD-590/Pharm
HFD-590/Chem
HFD-590/Review Micro
HFD-590/CSO/MillerK

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