

# W Effect of nitazoxanide for treatment of severe rotavirus diarrhoea: randomised double-blind placebo-controlled trial

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## Summary

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**Background** Rotavirus is a leading cause of morbidity and mortality in children younger than 5 years, but there is no effective treatment. We assessed the activity of nitazoxanide, a broad-spectrum anti-infective drug, against rotavirus in cell culture and in a clinical trial in paediatric patients hospitalised with severe rotavirus diarrhoea.

**Methods** We did a randomised double-blind placebo-controlled trial in 50 children admitted to the Cairo University Children's Hospital between June 15 and Aug 23, 2005, with severe rotavirus diarrhoea. 38 children aged 5 months to 7 years (median age 11 months) with rotavirus as the sole identified cause of gastroenteritis were enrolled in the clinical study. Patients were randomly assigned either 7·5 mg/kg nitazoxanide as an oral suspension or placebo twice a day for 3 days, and all remained in hospital for 7 days after start of treatment. The primary endpoint was time from first dose to resolution of illness, and analysis was by modified intention-to-treat. This study is registered with ClinicalTrials.gov, number NCT00302640.

**Findings** Survival analysis showed that the median time to resolution of illness was 31 h (IQR 22–73) for the nitazoxanide-treated group compared with 75 h (51–124) for the placebo group ( $p=0\cdot0137$ ). No significant adverse events were reported.

**Interpretation** A 3-day course of nitazoxanide significantly reduced the duration of rotavirus disease in hospitalised paediatric patients. These results are encouraging, and might lead us to think about new approaches to managing rotavirus disease in children.

## Introduction

Rotavirus is the most common cause of severe dehydrating diarrhoea and gastroenteritis in young children worldwide, and a major cause of mortality. The disease causes an estimated 500 000 deaths every year, most of which occur in poor countries. In developed nations, around 1 in 40 children younger than 5 years are hospitalised every year because of rotavirus diarrhoea. The medical and societal costs of rotavirus disease in the USA have been estimated at more than US\$1 billion annually.<sup>1–3</sup>

Oral rehydration and maintenance of proper fluid and electrolyte balance remain the primary treatment for children with rotavirus gastroenteritis.<sup>4,5</sup> There is currently no drug for treating rotavirus infection. Racecadotril, an enkephalinase inhibitor, inhibits intestinal hypersecretion and has been shown to reduce stool output and duration of rotavirus diarrhoea.<sup>6</sup> Some studies have shown that use of probiotics early in the course of diarrhoea can reduce duration of illness and rotavirus shedding.<sup>7–9</sup> Hyperimmune bovine colostrum from cows immunised with human rotavirus has been shown to reduce the duration and severity of childhood diarrhoea due to rotavirus.<sup>10</sup> A rotavirus vaccine was first licensed in 1998, but was withdrawn after less than 1 year of use because of risk of intussusception. Two new vaccines have been or will soon be licensed and could be available in the next 2–3 years.<sup>1</sup> However, there remains a need for an effective treatment for rotavirus infection when it does occur.

Nitazoxanide is a thiazolide anti-infective licensed in the USA for treating diarrhoea caused by *Cryptosporidium*

*parvum* and *Giardia lamblia* in children and adults.<sup>11–13</sup>

The drug is also effective for treatment of diarrhoea associated with *Entamoeba histolytica*, *Blastocystis hominis*, and *Clostridium difficile*.<sup>13–15</sup> One study showed that a 3-day course of nitazoxanide suspension reduced mortality in severely malnourished paediatric patients who were not infected with HIV and were hospitalised with diarrhoea caused by *Cryptosporidium* spp.<sup>16</sup> In-vitro studies have shown that nitazoxanide inhibits replication of a broad range of viruses.<sup>17,18</sup> These studies have led to clinical development of nitazoxanide for treating chronic hepatitis B and hepatitis C.

We tested nitazoxanide (the active circulating metabolite of nitazoxanide) in cell culture for activity against rotavirus because of its use in treating diarrhoea caused by a broad range of enteric pathogens. Based on our findings, we then did a preliminary randomised double-blind placebo-controlled clinical trial in hospitalised paediatric patients with severe rotavirus gastroenteritis to assess the use of nitazoxanide in reducing the duration of this disease.

## Methods

### In vitro studies

Monkey kidney MA104 cells were grown at 37°C in medium 199 (EuroClone Ltd, Paignton, UK), supplemented with 10% inactivated fetal calf serum, 2 mmol glutamine, and antibiotics. Nitazoxanide dissolved in dimethyl sulphoxide stock solution (50 mg/mL) was diluted to the appropriate concentration in culture medium and added to the infected cells immediately

after the adsorption period. Controls received equal amounts of dimethyl sulphoxide diluent. Every concentration of the drug was tested in duplicate and each experiment was repeated three times.

We used simian rotavirus SA-11 (SA-11). Confluent MA104 monolayers were inoculated with preactivated SA-11 rotavirus for 1.5 h at 37°C at a multiplicity of infection of 0.1, 1, 5, or 10 plaque-forming units per cell. After the adsorption period, the viral inoculum was removed and cell monolayers were washed three times with phosphate-buffered saline. Cells were maintained at 37°C in medium 199 in the absence of serum. Tizoxanide was added immediately after the adsorption period and kept in the culture medium throughout the experiment. Virus yield was determined 24 h post-infection after three cycles of freeze-thawing of infected cultures by haemagglutinin titration according to standard procedures.<sup>19,20</sup>

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to MTT formazan conversion assay (Sigma-Aldrich, St Louis, MO, USA). For MTT assay, reduced MTT (formazan) was extracted from cells by adding 100 µl of acidic isopropanol containing 10% Triton X-100, and formazan absorbance was measured in an ELISA microplate reader at two different wavelengths (540 and 690 nm).<sup>21</sup> Microscopic examination of mock-infected or virus-infected cells was done to detect the cytopathic effect induced by rotavirus infection, and possible morphological changes or cytoprotection induced by the drug. Microscopy studies were done with a Leica DM-IL microscope and images were captured on a Leica DC 300 camera with Leica Image-Manager500 software. The expression of results is shown in the panel.

### Study design

We did a randomised, double-blind, placebo-controlled study to assess the effectiveness of nitazoxanide oral suspension compared with a placebo suspension in paediatric patients hospitalised with severe rotavirus disease. The primary endpoint of the study was time from first dose to resolution of illness. Resolution of illness was defined as resolution of all gastrointestinal symptoms associated with rotavirus disease at enrolment with the patient not needing anti-motility or other palliative treatment. Symptom resolution needed to have been maintained for at least 72 h to be valid. The study was designed to enrol 50 patients. The sample size was established with a log-rank test of survival in two groups followed up for a fixed time with the hazard ratio constant over time (nQuery Advisor software, version 5.0). With a sample size of 19 patients per group, a 0.05 level two-sided log-rank test for equality of survival curves has an 80% power to detect a difference between an 85% response rate for one group and a 40% response rate for a second group at a given time. A

sample size of 25 patients per group was selected to allow for exclusion of up to 20% of patients for other identified causes of diarrhoea. Patients were followed up for 14 days, since most patients would be expected to resolve illness during that time. The study was done in compliance with the guidelines of the US Department of Health and Human Services. The protocol and informed consent forms were approved by the ethics committee of the participating institution.

### Patients

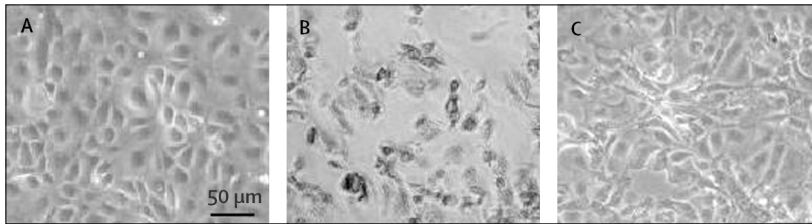
Patients younger than 12 years presenting with watery diarrhoea at the emergency room of the Cairo University Children's Hospital and subsequently hospitalised in the paediatric gastroenterology department because of the severity of their illness were considered for enrolment. Before screening, written informed consent was obtained from the parent or guardian of each patient. Patients with diarrhoea (at least 3 liquid stools per day) and stools positive for rotavirus at screening were eligible for enrolment. Patients with other known or suspected causes of diarrhoea and those with serious diseases or disorders incompatible with the study were excluded.

### Procedures

Children hospitalised with severe watery diarrhoea submitted stool samples, which were tested for diarrhoea-causing pathogens. Patients with stools positive for rotavirus were enrolled in the study and underwent a complete physical examination including determination of bodyweight and temperature before starting medication. The blinded study medication was administered by the nursing staff as 10 mL (200 mg nitazoxanide) twice a day for 3 days in patients 4–11 years of age, 5 mL (100 mg nitazoxanide) twice a day in patients 12–47 months of age, and 0.375 mL/kg of bodyweight (7.5 mg/kg nitazoxanide) twice a day in patients younger than 12 months. The placebo suspension was identical to the nitazoxanide suspension except that it did not contain the active ingredient. In addition to the study medication, all patients received routine care including fluid replacement therapy and nutritional and metabolic management of diarrhoea. The nursing staff recorded the number and consistency of stools and other symptoms or adverse events for every patient throughout the first 7 days of the study. Well patients were released from the hospital on day 7.

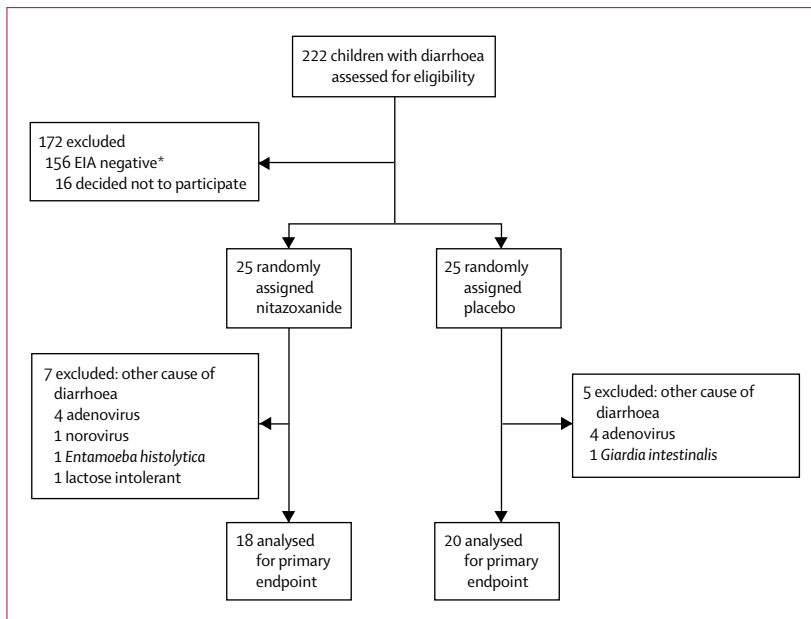
#### Panel: Expression of results

$EC_{50}$  = drug concentration at which a 2-fold reduction of viral yield (relative to the average rates in untreated cultures) was seen.  $CC_{50}$  = drug concentration at which a 2-fold reduction of reduced MTT was seen relative to average levels in untreated cultures. SI (selectivity index) =  $CC_{50}/EC_{50}$ .



**Figure 1: Cytoprotective effect of tizoxanide in rotavirus-infected cells**

Confluent monolayers of MA104 cells were mock-infected (A) or infected with rotavirus SA-11 (1 plaque-forming unit per cell; B, C) and treated with tizoxanide (10 µg/mL; C) soon after the adsorption period. Phase contrast microscopy images from a representative experiment are shown. SA-11 virus infection is highly cytopathic, causing cell shrinkage and loss of adhesion at 24 h post-infection (B). Tizoxanide has a profound cytoprotective effect in rotavirus-infected cells for at least 24 h after infection (C).



**Figure 2: Trial profile**

\*EIA negative=enzyme immunoassay negative for rotavirus.

Patients returned to the hospital for a final assessment at day 14. The patients, their parents or guardians, or both were questioned on day 14 to establish if there had been any adverse events.

All stool samples obtained at screening were tested by: (1) enzyme immunoassay (EIA) tests for detecting enteric viruses (RIDASCREEN for rotavirus and adenovirus, R-Biopharm AG, Darmstadt, Germany; IDEIA for norovirus, DakoCytomation Denmark A/S, Glostrup, Denmark); (2) microscopic examination for ova and parasites including examination after stool concentration, a Ziehl-Neelsen stain and immunofluorescence assay (MeriFluor; Meridian Diagnostics, Cincinnati, OH, USA); and (3) a stool culture to identify bacterial causes of diarrhoea including adherent or toxigenic *Escherichia coli*.

At enrolment, the investigator sequentially assigned each patient a number corresponding to the number on his or her bottle of study medication. The computer-

generated randomisation list and the packaging of study medication were prepared by the study sponsor, Romark Laboratories (Tampa, FL, USA). The randomisation list was sealed and maintained by the study sponsor until the study was completed. The patients, principal investigators, and their staff and laboratory personnel were masked to the treatment assignment.

### Statistical analysis

The statistical analyses were done with JMP software version 5.1.1 (SAS Institute Inc, Cary, NC, USA). The population used for efficacy analyses was defined prospectively as all patients randomised to the study excluding patients with other identified causes of diarrhoea (for example, *Giardia lamblia*, *Cryptosporidium* spp, *Entamoeba histolytica*, bacterial pathogens). Time from first dose to resolution of illness was compared by treatment group with a survival analysis and a Prentice modified Wilcoxon test with an alpha of 0.05. The Wilcoxon test has been used for other studies of interventions for acute diarrhoeal illness and was selected because the ratio of hazards was expected to be greater at earlier survival times than at later ones.<sup>22</sup>

### Role of the funding source

The authors designed the protocol for the clinical study in collaboration with the study sponsor, Romark Laboratories. The clinical investigators enrolled the patients, did the clinical trial, and recorded the data independently of any involvement of the sponsor. The sponsor provided monitors to ensure quality of the conduct of the study and that data collected by the investigators on case report forms were consistent with the records in the hospital files. Data entry and analysis was done by the sponsor under the supervision of the authors. All authors had free access to the data at all times. The sponsor was allowed to review the manuscript and provide comments before submission.

### Results

In cell culture infected with 1 plaque-forming unit per cell, tizoxanide inhibited replication of rotavirus with an  $EC_{50}$  of 0.5 µg/mL and a selectivity index higher than 100. The antiviral effect was consistently seen for different levels of infection. At concentrations of 1, 5, or 10 plaque-forming units per cell, tizoxanide (10 µg/mL) reduced virus yield by 94%, 93%, and 88% of control, respectively. No virus was detected in treated cells infected at low (0.1 plaque-forming units per cell) multiplicity of infection. Microscopic examination showed a cytoprotective effect of the drug (figure 1). Further studies of the mechanism of action of tizoxanide against rotavirus are being done in our laboratories. Preliminary findings suggest a selective effect on synthesis of rotavirus structural proteins.

222 children with diarrhoea were assessed between June and August, 2005, for eligibility to participate in the trial

(figure 2). 50 children were enrolled and all completed the study. 12 children were excluded from the efficacy analysis because of other identified causes of diarrhoea: eight with adenovirus, one with norovirus, one with *Giardia lamblia*, one with *Entamoeba histolytica*, and one with lactose intolerance. No bacterial causes of diarrhoea were identified in this population. Demographic and disease-related characteristics of the 38 patients included in the efficacy analysis are summarised in the table. Survival analysis showed that the times from first dose to resolution of illness were significantly less for nitazoxanide-treated patients than for patients in the placebo group ( $p=0.0137$ , Wilcoxon test; figure 3). The median time from first dose to resolution of illness was 31 h (IQR 22–73) for patients treated with nitazoxanide compared with 75 h (51–124) for patients randomised to the placebo group.

Two of the children were roughly 5 and 7 years of age, whereas the other 36 patients were all younger than 24 months. To exclude any potential bias due to inclusion of the two older children, a separate efficacy analysis was done for the subset of patients younger than 24 months of age. The results were similar within this subset of patients ( $p=0.0251$ , Wilcoxon test).

Linear regression analyses were done to identify potential relations between specific demographic or disease-related factors listed in the table, and the time from first dose to resolution of illness for each of the treatment groups. For patients enrolled in the placebo group, longer times from first dose to resolution of illness were associated with lower weight-for-age Z scores (WAZ;  $p=0.12$ ), shorter durations of hospitalisation at time of first dose ( $p=0.08$ ), and longer durations of diarrhoea at baseline ( $p=0.09$ ). These relations were not apparent for patients in the nitazoxanide-treated group, presumably because of the effect of treatment.

There were no significant adverse events. Only two adverse events were reported during the course of the study: one case of mild otitis media and one case of mild bronchitis. Both these patients were in the placebo treatment group.

## Discussion

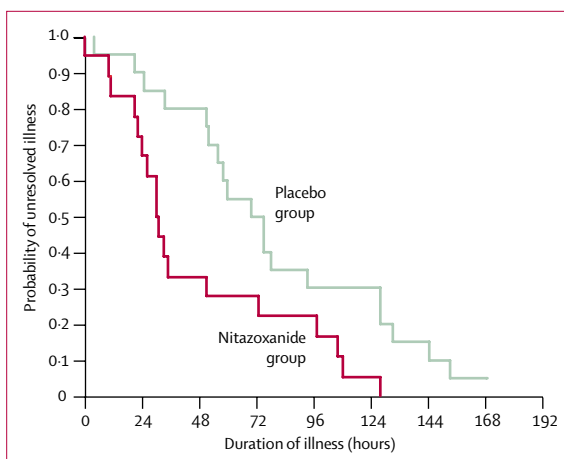
Our studies show the antiviral effect of the active nitazoxanide metabolite, tizoxanide, against rotavirus in cell culture. This activity was confirmed in vivo by a double-blind, placebo-controlled clinical trial in paediatric patients hospitalised with severe rotavirus disease. Treatment with nitazoxanide was associated with a significant reduction in the duration of illness.

As expected, most of our patients were malnourished young children with reduced immunity. The duration of diarrhoea at enrolment (median 6.5 days, range 3–16 days) indicated the severity of illness in this population. Other investigators have reported rotavirus as an important cause of protracted diarrhoeal illness,<sup>23–27</sup> which is not uncommon in children hospitalised at Cairo University Children's Hospital.

	All patients	Active suspension	Placebo suspension	p*
<b>Race</b>				
White	38	18	20	1.0
<b>Sex</b>				
Male	21	9	12	0.75
Female	17	9	8	
<b>Age (months)</b>				
Mean (SD)	15.1 (15.5)	16.9 (22.1)	13.4 (4.9)	0.52
Median (range)	11.0 (5–92)	9.0 (5–92)	12.0 (5–24)	
<b>Weight (kg)</b>				
Mean (SD)	8.2 (2.6)	8.2 (3.6)	8.2 (1.5)	0.93
Median (range)	7.8 (5.0–20.0)	7.2 (5.0–20.0)	8.3 (6.1–11.4)	
<b>Stool frequency</b>				
3–4/day	5	4	1	0.27
5–10/day	26	11	15	
>10/day	7	3	4	
<b>Stool consistency</b>				
Liquid	38	18	20	1.0
<b>Vomiting</b>				
Yes	30	13	17	0.44
No	8	5	3	
<b>Malnutrition status†</b>				
Severely underweight	11	5	6	0.95
Moderately underweight	11	6	5	
Mildly underweight	9	4	5	
Not underweight	7	3	4	
<b>Duration of diarrhoea (days) at time of enrolment</b>				
Mean (SD)	7.0 (3.5)	7.4 (3.4)	6.7 (3.6)	0.49
Median (range)	6.5 (3–16)	7.0 (4–16)	6.0 (3–16)	
<b>Duration of hospitalisation at time of first dose</b>				
Mean (SD)	46 (22)	47 (20)	46 (24)	0.87
Median (range)	47 (0–119)	47 (0–71)	47 (23–119)	

\*Fisher's exact test or  $\chi^2$  test used for comparing proportions, t test for means. †Based on weight-for-age Z scores:  $Z < -3.0$ =severely underweight,  $Z < -2.0$ =moderately underweight,  $Z < -1.0$ =mildly underweight.

**Table: Demographic and disease-related characteristics**



**Figure 3: Effect of nitazoxanide on duration of rotavirus illness**  
Survival analysis of time from first dose to resolution of illness ( $p=0.0137$ , Wilcoxon test).

The main limitation of our clinical trial was the small number of patients enrolled in each group. In view of this limitation, we examined the data for possible biases that might provide alternate explanations of our results. There were no significant differences in the demographic characteristics of the patients enrolled in the two groups, their symptoms (severity of illness), nutritional status, duration of diarrhoea at the time of enrolment, or duration of hospital care received before the first dose of blinded study medication. To exclude the possibility of bias due to inclusion of two patients with outlying ages, we did a separate analysis of the 36 patients younger than 24 months of age, and found the same results for this subset of patients.

As noted from our exploratory analyses, the three factors most predictive of an early response in the placebo group were higher WAZ scores (improved nutritional status), longer durations of hospitalisation at the time of first dose, and longer durations of diarrhoea at baseline. Although improved nutritional status and hospital care might be predicted to lead to early response, the association of longer durations of diarrhoea at baseline with longer times to response might not have been predicted. This association, nevertheless, draws attention to the difficulty that can be encountered in managing young children with severe and protracted rotavirus disease.

All of the factors identified as being associated with early response times (high WAZ scores, longer hospitalisation at time of first dose, and longer duration of diarrhoea at baseline) were quite similar for the two treatment groups. Although duration of diarrhoea at baseline was slightly longer for the nitazoxanide-treated patients, this fact should have biased the outcome in favour of the placebo group.

We cannot rule out the possibility of hidden intestinal infections or comorbidities that could have affected the outcome of our clinical trial. As noted earlier, the patients enrolled in our study were young children with severe rotavirus diarrhoea and without other significant comorbidities. We thoroughly searched for other causes of diarrhoea, and we found no evidence of co-infection with other enteropathogens. Co-infection with rotavirus and bacterial enteropathogens have been shown to be rare.<sup>28–30</sup>

We did not gather data on fecal rotavirus excretion during our study. The effect of nitazoxanide treatment seen in these patients could theoretically be attributed to another pharmacodynamic effect of the drug. Ideally, stool samples should have been collected during the study to assess the relation between fecal rotavirus excretion and clinical response to treatment. This issue will be addressed in future studies.

Future studies might also more rigorously assess the effect of nitazoxanide on extended excretion of rotavirus. Other investigators have suggested that extended excretion of rotavirus after severe rotavirus diarrhoea

could be responsible for postgastroenteritis syndrome.<sup>25,26</sup> Other parameters that should be monitored as endpoints in future studies include stool weight, need for oral or intravenous rehydration, and the duration of hospitalisation needed. Although we did not serotype the strains of rotavirus in our study, most strains currently detected in our hospital are G1, with G2 and G4 also common. Future studies will assess the serotypes and provide analysis of results by serotype.

We know of no other clinical trials of nitazoxanide in paediatric patients younger than 12 months of age. Earlier studies of nitazoxanide in paediatric patients have been limited to patients at least 1 year of age. Based on previous experience with nitazoxanide in treating children 12–36 months of age with diarrhoeal diseases and the frequency of rotavirus diarrhoea in those younger than 12 months of age, we enrolled patients down to 5 months of age in our study. Although the number of patients enrolled was small, we found no adverse experiences associated with the use of nitazoxanide.

The availability of an effective drug for treating rotavirus infection in conjunction with oral rehydration and other supportive therapy could play an important role in managing rotavirus disease. New rotavirus vaccines are finding their way to the marketplace with the hope of reducing the burden associated with this disease,<sup>1</sup> but rotavirus will probably remain an important problem in children without access to the vaccine, those who elect not to be vaccinated, or those for whom the vaccine is not effective. An effective treatment could be used to reduce the duration of hospitalisation, perhaps preventing hospitalisations in some cases, and reducing medical and societal costs associated with the disease.

In developing countries, where morbidity and mortality associated with paediatric diarrhoea are highest, the availability of a treatment for rotavirus disease might be especially important. Nitazoxanide has been available in Latin America for 10 years, and has more recently become available in India where it is used as a 3-day course of treatment for a broad spectrum of intestinal protozoan and helminthic infections. Activity treating rotavirus disease would enhance the value of a product that is already used in these countries. Rapid ELISA tests to diagnose rotavirus in stool specimens are available in developing countries, and especially in hospital settings. In the absence of laboratory diagnosis, however, empirical treatment with nitazoxanide could be considered in cases of severe illness where rotavirus or perhaps *Cryptosporidium* is suspected. Symptoms of rotavirus and cryptosporidiosis can be similar, and both diseases are known to affect young children because of lack of immunity.

Additional clinical trials are being done in larger numbers of patients to confirm our results. In the interim, the results reported here are encouraging and might lead us to think about new approaches to managing rotavirus disease in children.

**Contributors**

M G Santoro conducted the studies of tizoxanide in cell culture. J-F Rossignol and M Abu-Zekry designed the clinical trial. M Abu-Zekry was the principal investigator for the clinical trial, assisted by A Hussein, and was responsible for data collection. J-F Rossignol and M Abu-Zekry did the analysis of the data from the clinical trial. All authors contributed to the interpretation of the data and writing of the manuscript.

**Conflict of interest statement**

J-F Rossignol is an employee and stockholder of Romark Laboratories, LC, the company that owns the intellectual property rights for nitazoxanide. M G Santoro has served as a consultant to Romark Laboratories, LC.

**Acknowledgments**

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