Identification of a potent benzoxaborole drug candidate for treating cryptosporidiosis

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Cryptosporidiosis is a leading cause of life-threatening diarrhea in young children and causes chronic diarrhea in AIDS patients, but the only approved treatment is ineffective in malnourished children and immunocompromised people. We here use a drug repositioning strategy and identify a promising anticryptosporidial drug candidate. Screening a library of benzoxaboroles comprised of analogs to four antiprotozoal chemical scaffolds under preclinical development for neglected tropical diseases for Cryptosporidium growth inhibitors identifies the 6-carboxamide benzoxaborole AN7973. AN7973 blocks intracellular parasite development, appears to be parasiticidal, and potently inhibits the two Cryptosporidium species most relevant to human health, C. parvum and C. hominis. It is efficacious in murine models of both acute and established infection, and in a neonatal dairy calf model of cryptosporidiosis. AN7973 also possesses favorable safety, stability, and PK parameters, and therefore, is an exciting drug candidate for treating cryptosporidiosis.
Despite efforts to improve hygiene, infectious diarrhea is responsible for \(~11\%) of early childhood deaths\(^1\). A recent multicenter study of life threatening diarrhea conducted in the Indian subcontinent and Sub-Saharan Africa found that the little-studied apicomplexan parasite *Cryptosporidium*, predominantly either *Cryptosporidium parvum* or *Cryptosporidium hominis*, is amongst the two or three most important causes of childhood diarrhea\(^2,3\). *Cryptosporidium* is also strongly associated with child malnutrition and developmental stunting\(^4,5\). *Cryptosporidium* parasites were previously best known as a frequent cause of chronic diarrhea in immunocompromised people, such as those with AIDS or following organ transplantation, and in the United States they account for more than 85% of waterborne diarrheal disease for which a pathogen is known\(^6,7\). *Cryptosporidiosis* is also an important economic concern for beef and milk producers, and infection of cattle may contribute to contamination of water supplies and human outbreaks\(^8,9\). In a nationwide survey of farms in the United States, *C. parvum* was present on more than 50% of dairy farms, and 48% of all calves aged 1–3 weeks were infected\(^9\). Despite the public health and economic impacts of *Cryptosporidium*, there is no preventive vaccine and treatment options for both humans and cattle are limited. The only approved treatment, nitazoxanide, reduces the duration of diarrhea in immunocompetent adults, but is only moderately effective in children and is equivalent to a placebo in immunocompromised patients\(^11,13\). Its activity in dairy calves is controversial and at best marginal\(^14,15\). New anti-*Cryptosporidium* drugs are urgently needed, and several promising drug leads have been reported recently\(^16-19\).

Benzoxaboroles are easily synthesized boron-heterocyclic compounds that have garnered interest from the pharmaceutical industry in the last decade\(^20\). They typically engage with proteins through interaction of an electrophilic boron atom with nucleophilic partner residues (e.g., serine, threonine or tyrosine) or with metal cations (e.g., Zn\(^{2+}\), Mg\(^{2+}\)) present in the active site of enzymes, acting as a phosphate mimetic. Potent benzoxaborole inhibitors of bacterial\(^21,22\), fungal\(^23\), and protozoan\(^24\) pathogens have been identified that work by inhibiting various essential microbial enzymes\(^22,23,25-28\). *Tavaborole*, a leucyl-tRNA synthetase inhibitor, is approved for treatment of onychomycosis, and *crisaborole*, which targets human phosphodiesterase 4, was recently approved for treatment of atopic dermatitis\(^21,29\).

*Plasmodium* parasites, which cause malaria, are members of the phylum Apicomplexa, and are therefore genetically related to *Cryptosporidium* parasites. Given this and the recent identification of benzoxaboroles with potent antimalarial activity\(^28,30,31\), we screened a library of benzoxaborole compounds for anticytotoxic activity. Here, we report identification of a novel drug candidate, AN7973, for treatment of *cryptosporidiosis*. AN7973 potently inhibits multiple *C. parvum* isolates and the *C. hominis* TU502 isolate in vitro, appears to be parasitcidal, has outstanding activity in rodent models of both acute and established infection, and was efficacious against *C. parvum* in a dairy calf clinical model. Its initial pharmacokinetic (PK), stability, and safety profiles are highly favorable, and indicate its potential as a new drug for treating *cryptosporidiosis* in all target populations.

## Results

### Drug repositioning strategy and compound screen

To meet the need for new anticytotoxic drug candidates, we adopted a drug repositioning strategy to capitalize on existing benzoxaborole scaffolds and knowledge from advanced programs within Anacor’s Neglected Tropical Disease portfolio, which included antikinetoplastid programs with the Drugs for Neglected Diseases initiative (DNDi) and the Global Alliance for Veterinary Medicine (GALVmed), an antimalarial program with the Medicines for Malaria Venture (MMV), and an internal antibacterial leucyl-tRNA synthetase (LeuRS) inhibitor program. A library of 7802 compounds, including analogs similar to four chemical scaffolds with antiprotozoal activity, was screened in duplicate for inhibition of *C. parvum* growth within MDCK cells using high content microscopy and adaptation of a previously described screening assay (Fig. 1)\(^32\). This screen yielded 403 hits with \(>70\%) inhibition at a concentration of 1 \(\mu\)M. Confirmatory dose-response assays were conducted in triplicate on 400 compounds available for follow-up, and an additional 222 compounds were tested to complete structure activity relationship (SAR) analyses for each scaffold. Further prioritization was then done by conducting a preliminary mouse efficacy study with one compound representative of each chemical scaffold that was selected based on availability of chemical stocks, existing potency and mouse PK data, and the potential to leverage other programs within Anacor’s Neglected Tropical Disease portfolio. AN7973 was selected

![Fig. 1 Screening results and prioritization considerations. Confirmed anticytotoxic screening hits, lowest EC\(_{50}\), and prioritization considerations for four benzoxaborole chemical scaffolds are shown. The 6-carboxamide antitrypanosomal chemical scaffold was prioritized for further study based on a large number of available analogs, existing safety data from a human African trypanosomiasis (HAT) drug development program, and the results of a preliminary murine efficacy study](https://doi.org/10.1038/s41467-019-10687-y)
for further development based on these considerations, and the remaining scaffolds were retained as alternates worthy of revisiting if necessary.

AN7973 (Fig. 2a) is a benzoxaborole 6-carboxamide representative of a class of compounds active against *Trypanosoma brucei*, the cause of human African trypanosomiasis (HAT). This class of compounds is currently in human trials. The 6-carboxamide SCYX-7158 (AN5568/acoziborole) exhibits favorable PK properties in animals and humans, e.g., prolonged exposure and half-life, and has the potential to provide a single-dose cure for HAT. Related benzoxaboroles from the 6-carboxamide class have also shown good in vitro activity against human African trypanosomiasis (HAT) due to *T. brucei*, *T. br. gambiense*, and *T. br. rhodesiense*, and African animal trypanosomiasis (AAT) due to *T. congolense* and *T. vivax*. Members of this class have also shown favorable safety profiles, which enhanced the status of AN7973 for the repositioning strategy of this program.

AN7973 selectively inhibits *Cryptosporidium* development. The structure and purity of AN7973 were determined by 1H NMR, 13C NMR, high resolution mass spectral analysis (HRMS), and high performance liquid chromatography (HPLC) (Supplementary Figs. 1 and 2).

In vitro inhibition of the Bunch Grass Farm (BGF) *C. parvum* Iowa isolate by 48 h of exposure to AN7973 in HCT-8 cells was confirmed repeatedly, with the measured EC50 ranging from 0.13 µM to 0.43 µM. Comparable activity was observed for inhibition of field *C. parvum* isolates, and the BGF *C. parvum* Iowa isolate sourced from the University of Arizona Sterling Laboratory (this isolate has been propagated independently of the BGF isolate for ~20 years). AN7973 also inhibited the *C. hominis* TU502 isolate, but with 3-fold to 4-fold higher EC50 (Fig. 2b). Cytotoxicity as assessed in three cell lines was not observed at concentrations up to 25 µM (i.e., the highest concentration tested (Supplementary Table 1)), yielding a selectivity index for inhibition of *Cryptosporidium* of greater than 50 in all cases.

The anticytospordial activity of AN7973 was further examined using a panel of in vitro assays to determine its mode of action. AN7973 had no activity in an assay for *C. parvum* invasion of HCT-8 host cells (Supplementary Fig. 3). It predominantly affected intracellular *Cryptosporidium* development, since it arrested new DNA synthesis as reflected by inhibition of incorporation of the thymidine analog 5-ethynyl-2′-deoxyuridine (EdU) into newly synthesized DNA (incorporated EdU) shown in green. Arrows indicate EdU positive parasites, which are seen only in the absence of AN7973. Representative images from 2 independent experiments are shown. Scale bars are 5 µm. Potential of AN7973 against asexual growth (0–48 h (black line and circles)) and asexual-to-sexual conversion (48–72 h (cyan line and squares)) of *C. parvum* (Bunch Grass Farms Iowa isolate). Data are the means and SD for each concentration with data combined from 2 independent experiments (n = 4 per experiment).
Fig. 3 AN7973 acts rapidly to eliminate Cryptosporidium in vitro. Time-kill curves showing in vitro effects of nitazoxanide (NTZ) or AN7973 on C. parvum (Bunch Grass Farms Iowa isolate) following compound exposure. NTZ or AN7973 were added at varying concentrations ~24 h after infecting HCT-8 cell monolayers, and then parasites were stained and enumerated by high-content microscopy at the indicated time points. Different compound concentrations are indicated in all graphs as follows: open blue squares and blue lines (DMSO control); solid black circles and black lines (EC50); solid black squares and black lines (EC90); open gray triangles and gray lines (3 × EC90); solid black circles and black lines (EC50); solid black squares and black lines (EC90); open gray triangles and gray lines (3 × EC90); solid red triangles and red lines (6 × EC90); solid black circles and black lines (EC50); solid black squares and black lines (EC90); open cyan circles and lines (12 × EC90).

(a) NTZ time-kill curves
(b) NTZ rate of parasite elimination
(c) AN7973 time-kill curves
(d) AN7973 rate of parasite elimination

| Compound | EC50 | EC90 | 3 × EC90 | 6 × EC90 | 12 × EC90 |
|----------|------|------|----------|----------|----------|
| NTZ      |      |      |          |          |          |
| AN7973   |      |      |          |          |          |

Pharmacokinetic properties of AN7973. In anticipation of progression to mouse and calf models of cryptosporidiosis, we evaluated the pharmacokinetic properties of AN7973 in these species. When administered intravenously to CD-1 mice at a dose of 2 mg per kg as a solution in a polyethylene glycol/propylene glycol/water vehicle, AN7973 exhibited a high Cmax (6.82 µg per mL), very low clearance (CL = 40 mL·h⁻¹·kg⁻¹), high exposure (AUC₀–inf = 49.7 h·µg⁻¹·mL⁻¹), and long half-life (t₁/₂ = 9.2 h). When administered orally to CD-1 mice at a dose of 10 mg per kg as a solution in 1% CMC/0.1% Tween-80 aqueous vehicle, AN7973 showed very high Cmax (8.63 µg per mL) and exposure (AUC₀–inf = 92.7 h·µg⁻¹·mL⁻¹), a long half-life (t₁/₂ = 6.6 h) and modest bioavailability (F = 37%) (Supplementary Table 1 and Supplementary Fig. 4a). When dosed orally to calves at a dose of 2 mg per kg as a solution in a polyethylene glycol/propylene glycol/water vehicle, AN7973 exhibited high Cmax (3.57 µg per mL), very high exposure (AUC₀–inf = 190 h·µg⁻¹·mL⁻¹), and a half-life about five times longer than in CD-1 mice.
paromomycin reduced fecal parasite shedding by ~90% after 4 days of treatment. On the other hand, after 4 days of treatment, AN7973 reduced parasite shedding by >99% at a dose of 25 mg per kg and by >90% at a dose of 10 mg per kg administered once daily by oral gavage (Fig. 4a). NSG mice treated with AN7973 gained weight normally, and no adverse effects were observed.

The interferon gamma knockout (IFN-γ KO) mouse is another widely used Cryptosporidium animal model. Unlike lethal infection reported by some investigators, C. parvum infection of IFN-γ KO mice produces a self-resolving acute infection in our hands. Various doses of AN7973 were tested using this model and the C. parvum University of Arizona Iowa isolate. Clofazimine, which is parasitistatic and lacks efficacy against Cryptosporidium in the NSG mouse, was used as a positive control. AN7973 demonstrated dose-dependent efficacy, including efficacy at a dose of 10 mg per kg once daily. Maximal efficacy was similar to that seen with clofazimine (Fig. 4b). As was the case for NSG mice, AN7973 caused no adverse effects in IFN-γ KO mice.

AN7973 efficacy in neonatal calves. Mice infected with C. parvum do not develop diarrhea, but newborn calf calves infected with C. parvum develop a self-limited illness much like that seen in infants and characterized by severe watery diarrhea, dehydration, and fecal oocyst shedding. We therefore used a neonatal calf model to test the effect of AN7973 treatment on diarrhea and dehydration, in addition to its effect on parasite shedding. For this, an initial small efficacy study was conducted during which the effect of infection on PK was also assessed. These data were then used to guide the design of additional dosing regimens (Fig. 5a). One-day-old to two-day-old bull calves were infected by oral administration of ~5 x 10⁵ C. parvum Bunch Grass Farms Iowa isolate oocysts. Fecal parasite shedding was measured daily using qPCR, and the animals were assessed clinically at least twice daily for diarrhea, hydration status, appetite, and overall health status. Clinical observations were quantified using previously described scales ranging from 1 (normal) to 3 (severely abnormal) for fecal consistency, overall health status, hydration status, and appetite (see Supplementary Table 2). Finally, the areas under each time course curve (AUC) were calculated to provide an overall measure of the impact of AN7973 on each parameter and facilitate statistical comparison.

**Efficacy in acute and established murine cryptosporidiosis.** AN7973 efficacy was first tested in vivo using a NOD scid IL2γnull (NOD scid gamma (NSG)) mouse model, which is a model of established C. parvum infection. Treatment was begun 7 days after infecting mice by oral gavage with the C. parvum Bunch Grass Farms Iowa isolate. The positive control compound paromomycin reduced fecal parasite shedding by ~90% after 4 days of treatment. The other hand, after 4 days of treatment, AN7973 reduced parasite shedding by >99% at a dose of 25 mg per kg and by >90% at a dose of 10 mg per kg administered once daily by oral gavage (Fig. 4a). NSG mice treated with AN7973 gained weight normally, and no adverse effects were observed.

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In both the mouse and calf PK studies, we also quantified AN7973 in feces, as we expected that at least some of the antiparasitic activity could be related to direct exposure of parasites to the drug in this matrix. In the mouse study, concentrations of AN7973 in feces generally increased over the 16 h period following oral dosing but were highly variable between animals, and concentrations of AN7973 remained high through the entire 168 h observation period (Supplementary Fig. 4c).

**Fig. 4** AN7973 efficacy in established and acute murine C. parvum infections. **a** Effect of AN7973 on established cryptosporidiosis in NOD scid gamma mice. C. parvum (Bunch Grass Farms Iowa isolate) infection was established by oral gavage of ~10⁵ oocysts and allowed to progress for 7 days prior to once daily oral administration of paromomycin (positive control), or AN7973 at the indicated dosages. Data are the mean and SEM (n = 4 mice per experimental group) of parasite fecal shedding per mg of feces measured by qPCR. Note that no oocyst shedding was detected on day 5 for 3 of 4 mice treated with 25 mg per kg AN7973, and data points are shown at the assay’s limit of detection (0.1 oocysts per mg feces). Asterisk (*) indicates p < 0.02 by one-way ANOVA test with Dunnett’s Method for multiple comparisons. **b** Effect of AN7973 in an acute, self-resolving IFN-γ knockout mouse model of cryptosporidiosis. C. parvum infection was established by oral gavage of ~10⁵ C. parvum (University of Arizona Iowa isolate) oocysts followed by daily oral treatment beginning on day 4 post-infection using the indicated dose of AN7973 or clofazimine (positive control). Lines and dosing regimen are color coded as follows: black line (vehicle); gray line (clofazimine 25 mg per kg); purple line (AN7973 5 mg per kg); pink line (AN7973 10 mg per kg); and orange line (AN7973 25 mg per kg). Data are the mean and SEM (n = 4 mice per experimental group) of fecal oocyst shedding as measured by immunostaining and flow cytometry.
All calves tolerated AN7973 well, based on at least twice daily assessment of alertness, interaction with caregivers, and appetite (see Supplementary Table 2 for clinical scoring rubric). The effects of oral 5 mg per kg twice daily AN7973, 10 mg per kg once daily AN7973, or vehicle alone were first compared, each begun 2 days after infection and continued for 7 days. Plasma and fecal exposures during infection were in-line with predictions based on results in uninfected calves, although fecal levels varied widely on the first day of dosing (Supplementary Fig. 5). Twice daily 5 mg per kg AN7973 curtailed parasite shedding and completely eliminated diarrhea.
Fig. 5. Efficacy of AN7973 in a neonatal calf model of cryptosporidiosis. a Summary of calf model and AN7973 microbiologic and clinical efficacy studies conducted. Bull Holstein calves were challenged within 48 h of birth by oral administration of $5 \times 10^7$ C. parvum (Bunch Grass Farms Iowa isolate) oocysts. Fecal oocyst shedding was quantified daily using qPCR, and clinical assessments were conducted twice daily and quantified on a scale of 1 to 3 (1 = normal, 2 = severely abnormal (e.g., severe diarrhea, or severe dehydration)). Treatments were given at the times highlighted in color in a to c, using the following dosing regimens: no color (vehicle control given on days 2–8); tan (AN7973 5 mg per kg every 12 h on days 2–8); green (AN7973 10 mg per kg every 12 h on days 4–10); and blue (AN7973 6.67 mg per kg every 8 h on days 2–4). b Initial AN7973 calf study. Each graph shows the mean and SEM of fecal oocyst shedding (red) or diarrhea score (blue) vs. time for the indicated treatment regimen ($n = 3$ calves per experimental group, except for $n = 2$ for the 5 mg per kg group). Dotted horizontal lines indicate the approximate limit of detection for the qPCR assay. c Follow-up AN7973 calf study, demonstrating clinical and microbiologic efficacy with delayed administration. A 3-day course was effective for diarrhea, but was followed by a relapse of shedding. Each graph shows the mean and SEM of fecal oocyst shedding (red) or diarrhea score (blue) vs. time ($n = 3$ calves per experimental group). Dotted horizontal lines indicate the approximate limit of detection for the qPCR assay. d Scatter plot showing the area under the curve (AUC) for Log_{10} transformed fecal oocyst shedding for individual animals. The lines indicate means and SD. Asterisk (*) indicates $p < 0.02$ compared with the corresponding vehicle control by one-way ANOVA with Dunnett’s Correction for multiple comparisons. e and f Scatter plots showing the AUC for the diarrhea and dehydration scores for individual animals receiving each treatment regimen. The lines indicate means and SD. Asterisk (*) indicates $p < 0.02$ compared with the corresponding vehicle control by one-way ANOVA with Dunnett’s Correction for multiple comparisons.

(Fig. 5b, d and e). Once daily 10 mg per kg AN7973 also reduced peak parasite shedding and diarrhea. However, despite equal or better mean plasma exposures in animals treated with once daily 10 mg per kg vs. twice daily 5 mg per kg AN7973, fecal parasite shedding resumed and persisted in the once daily treated animals (Fig. 5b). Based on these data, it was unlikely that plasma exposures alone were driving efficacy, suggesting that gastrointestinal exposure to AN7973 was important.

Major questions addressed in a second calf study included whether the dosing duration could be shortened, and whether or not fully sick animals would respond to treatment. These questions were addressed, respectively, by testing a 3-day regimen of 6.67 mg per kg three times daily, and testing the efficacy of 10 mg per kg twice daily for 7 days when treatment was delayed until the peak of diarrhea. Even when administered beginning at the peak of symptoms on day 4 after infection, twice daily 10 mg per kg AN7973 cured diarrhea 3 days faster than in controls, and parasite shedding was abolished by day 14 (Fig. 5c). Total parasite shedding was reduced by greater than 1 log (Fig. 5d). Treatment with 6.67 mg per kg three times per day for 3 days eliminated diarrhea, and reduced the rate of parasite shedding by ~3 log during treatment. However, a parasitological relapse followed (Fig. 5c), suggesting that >3 days and ≥7 days of treatment will be required. Finally, several of the AN7973 treatment regimens significantly reduced the overall severity of dehydration (Fig. 5f), a surprising result given that all of the animals received aggressive treatment to minimize dehydration.

Safety profiling. AN7973 possessed favorable stability, PK (e.g., mouse $t_{1/2} = 6$ h), and safety characteristics (Supplementary Table 1 and Supplementary Table 3). It was negative in the Ames test for mutagenic potential, was negative in an in vitro micro-nucleus genotoxicity assay, had no significant findings in Ricerca Bioscience’s Lead Profiling and Adverse Reaction Enzyme off-target panel (total # of assays: 109), and was negative for inhibition of the hERG K$^+$ ion channel, which is associated with cardiac tachyarrhythmias.

Administration of AN7973 by daily oral gavage to male rats at 80 mg per kg per day for seven days was well tolerated, and there were no clinical signs (e.g., changes in activity or grooming (see methods)), weight loss, changes in food consumption, or pathology observed. Slight changes to red blood cell parameters, serum alkaline phosphatase, and serum triglycerides occurred, which were not considered clinically significant. No compound accumulation was observed in a toxicokinetic analysis done in parallel (Supplementary Table 3). Based on these data, the no observed adverse effect level (NOAEL) of AN7973 was concluded to be greater than 80 mg per kg per day in rats. Additional toxicology studies, including higher doses, will be needed to ascertain the complete pre-clinical picture for AN7973.

Discussion

These studies collectively identify AN7973 as a promising candidate for a new cryptosporidiosis treatment. AN7973 is active in vitro against both C. hominis and C. parvum, and unlike compounds such as nitazoxanide that lack efficacy in severely immunocompromised mice and people, AN7973 rapidly eliminates C. parvum in vitro and is efficacious in immunocompromised mice. Thus, it may be useful for treatment of all patient populations affected by cryptosporidiosis, including AIDS patients, transplant recipients, and malnourished children. In addition to efficacy in mouse models of both acute and established infection, AN7973 reduced C. parvum fecal shedding, diarrhea, and dehydration in a neonatal calf model in which disease closely mimics that seen in infants. AN7973 also meets many of the stability and safety requirements for a novel anticytospordial44, although additional toxicology and PK studies of AN7973 are needed. Note that AN7973 was 3-fold to 4-fold less potent in vitro against the C. hominis TU502 isolate, which highlights the need to test in vivo efficacy of AN7973 against C. hominis.

The anticytospordial mechanism of action of AN7973 remains to be experimentally determined. The EdU incorporation assay (Fig. 2c) results demonstrate only that AN7973 arrests intracellular Cryptosporidium development prior to DNA synthesis, and do not imply that the compound directly affects DNA synthesis. Many compounds that do not directly target DNA synthesis (e.g., several tRNA-synthetase inhibitors presumed to inhibit protein synthesis) look similar to AN7973 in this assay38. Recent studies of AN7973 demonstrated that its trypanocidal activity results from inhibition of trypanosome mRNA processing45. Furthermore, related HAT and AAT lead compounds, also 6-amides like AN7973, target Cleavage and Polyadenylation Specific Factor 3 (CPSF3), an endonuclease that functions in mRNA processing46. It is known that other oxaboroles inhibit CPSF3 in Plasmodium falciparum50, and Cryptosporidium CPSF3 is greater than 40% identical to that of P. falciparum at the amino acid level. It therefore seems possible that AN7973 inhibits Cryptosporidium CPSF3 as well.

Given the modest efficacy of nitazoxanide, improved drugs to treat cryptosporidiosis in the most vulnerable populations (i.e., malnourished children and immunocompromised people) are eagerly awaited by clinicians47. Multiple lead compound series that affect a variety of targets may be needed, given the high attrition rate that is typical during drug development. This may also help to address the inevitable issue of drug resistance, either
simply by providing alternative drugs or by facilitating development of drug combinations to reduce the likelihood that resistance will evolve. Towards this goal, phenotypic screening assays have been developed and a growing number of lead compounds have been reported\(^{17-19,32,48-52}\). The phosphatidyl-inositol 4 kinase (PI4K) inhibitor KDU731, which was repurposed from a malaria drug development effort, is currently the most advanced compound in the Cryptosporidium pipeline\(^{18}\).

AN7973 reduced the fecal C. parvum shedding rate by nearly five logs in the first three days of treatment in the neonatal calf model, which we believe is the most rapid effect yet reported in this model\(^{16,18,43}\). Furthermore, AN7973 treatment reduced the total burden of fecal C. parvum shedding (i.e., the AUC) by over 90%. Based on its efficacy, stability, and preliminary in vitro and rat safety characteristics, AN7973 is amongst the most advanced compounds in the current pipeline, and is poised for pre-clinical toxicology studies in hopes of advancement to human clinical trials.

### Methods

#### PCR primers

Where noted, the following primers were used for quantitative PCR amplification of C. parvum 18s rRNA: CqP18s forward: TTAGAGTAGGAGGT GTTCC, and CqP18s reverse:CCACCAACTAAGAACGGCC\(^{39}\).

#### Compound characterization

The chemical identity of AN7973 was established through \(^1\)H NMR, \(^{13}\)C NMR, and high-resolution mass spectral (HRMS) analysis. Compound purity was assessed by high performance liquid chromatography (HPLC) (Instrument and column: HPLC-01 Venus M2 C18 5 µm 4.6 x 50 mm).

### In vitro activity of compounds against Cryptosporidium

A high-content microscopy assay was used to measure activity of compounds against C. parvum\(^{32,44}\). Screening was performed at the University of California San Francisco using Madin-Darby canine kidney (MDCK) type 2 (ATCC CRL-2936) cells grown to confluence in clear-bottomed 384-well plates in 50 µL per well of Dulbecco’s minimum essential medium (DMEM; Life Technologies) with 5% heat inactivated fetal bovine serum (FBS). Activity was reduced to 100% on the addition of parasites. C. parvum Iowa isolate oocysts used for screening were purchased from the Sterling Laboratory at the University of Arizona and stored at 4 °C until use. Oocysts were induced to excyst by treatment for 10 min at 37 °C with 10 mM HCl and then 10 min at 15 °C in 2 mM sodium taurocholate, washed in the above medium with 1% FBS, and then added to cell monolayers in the presence of compounds. The number of oocysts added to each well was dependent on the viability of the parasite stock, and varied between experiments. Compounds were screened at a concentration of 1 µM. After incubation at 37 °C under 5% CO\(_2\) for 48 h, the cells were fixed for at least 24 h by adding an equal volume of 4% formaldehyde in PBS. After fixation, the cells were blocked with 0.1% Triton X-100, 0.25% bovine serum albumin (BSA) in saline, which was also used for all subsequent steps. Cells were incubated with 0.5 µg per mL of biotinylated Vicia villosa lectin (VVL, Vector Laboratories, catalog# FL-1231) for 1 h at room temperature, washed three times, and stained in the dark for 1 h at room temperature in 0.5 µg per mL V. villosa lectin and DAPI (4,6-diamidino-2-phenylindole; Sigma) conjugated with fluorescein isothiocyanate (FITC, Molecular Probes, catalog# F-63341) according to the manufacturer’s protocol, and then HCT-8 cells were grown in RPMI media supplemented with 120 U per mL penicillin and 120 µg per mL streptomycin and 10% heat inactivated fetal bovine serum (complete media). C. parvum Iowa isolate oocysts from Bunker, DE were induced to excystation and used for infection. When cells reached a confluence of >99%, media was removed and replaced with 25 µL per well of fresh media containing twice the indicated concentrations of compounds and incubated for 1 h at 37 °C in a humidified CO\(_2\) incubator. In the meantime, oocysts were induced for excystation by first treating with 10 mM hydrochloric acid (diluted with distilled water) for 10 min at 37 °C followed by 2 mM sodium taurocholate (in phosphate buffered saline (PBS) with calcium and magnesium) for 10 min at 16 °C. Before and after each treatment, oocysts were spun down at 14,000 x g for 4.5 min and supernatant was removed. Oocysts were then diluted in complete media and added to cells at 5 x 10\(^4\) oocysts in 25 µL per well such that the final concentration of compounds was the same as the indicated controls. Oocysts were added to the excyst and sporozoites allowed to invade in presence of indicated compound concentrations for 3 h after which the cells were washed three times with PBS containing 111 mM D-galactose, fixed with 4% formaldehyde for 15 min at room temperature, followed by permeabilization with 0.25% triton X-100 for 10 min at 37 °C. Cells were then washed with PBS three times with PBS containing 0.1% tween 20, 2% 4% bovine serum albumin (BSA) in PBS overnight at 4 °C. Invaded parasites were stained with 1.33 µg per mL of fluorescein-labeled Vicia villosa lectin (Vector Laboratories, catalog# FL-1231) for 1 h at 37 °C followed by nuclei staining with 0.99 mM Hoechst 33258 (AnaSpec, catalog# AS-83219) for 15 min at 37 °C and then washed with PBS containing 0.1% tween 20. Each of the negative controls were treated the same, except for one of the following additional pre-steps. Heat killed oocysts were first heat killed at 70 °C for 30 min before being induced for excystation. For fixed host cells, HCT-7 cells seeded in 384 well plates at >99% confluence were fixed with 4% formaldehyde for 15 min at room temperature, followed by 3 washes with complete media before the experiment. Measurement of DNA replication was used as a surrogate marker of Cryptosporidium development within HCT-8 cells\(^{38}\). For this, glass bottom 96-well plates were coated with 50 µg per mL fibronectin (BD Pharmingen, catalog# 354008) according to the manufacturer’s protocol, and then HCT-7 cells were grown to >90% confluence in the wells. All mode of action and time-kill curve assays were performed in 10% FBS. C. parvum Iowa isolate oocysts (Bunch Grass Farms, Deary, ID) were triggered for excystation and added at a density of 5.5 x 10\(^4\) per well. After a 3-h delay, AN7973 was added at ~2x EC\(_{50}\) (0.42 µM) followed by incubation for 6 h and then addition of 10 nM 5-ethyl-2'-deoxyuridine (EdU). After incubation with EdU for 2 more hours, the cells were washed and fixed with 4% formaldehyde in saline. Cells were then permeabilized and stained for incorporation of EdU using the Click-IT assay kit (Thermo Fisher Scientific, catalog# 10340). Images were acquired by focusing on the parasite focal plane, which is on top of the host cell monolayer, using a x40 objective (0.7 NA) and EdU and nuclei numbers were quantified using ImageJ software.

The effect of AN7973 on asexual vs. sexual stage Cryptosporidium was assessed by comparing its potency for reducing the percent of host cells infected during the first 48 h of development within HCT-8 cells, which reflects asexual parasite development, to its effect on expression of the meiotic recombination protein DMC1, which is a previously identified sexual stage-specific marker\(^8\). For this, AN7973 was added after 48 h of culture (the approximate timing of sexual differentiation) and its effect on appearance of DMC1 positive parasites was measured using high-content microscopy after, staining with FITC-conjugated Vicia villosa lectin and DAPI as above, and with an anti-C. parvum DMC1 mouse monoclonal antibody (clone IH10/97G, IgG2b, kappa isotype used as a cultivated culture supernatant) with a secondary Alexa Fluor 568 goat anti-mouse IgG antibody (Invitrogen, catalog A-11004) at 1:300 dilution (4 µg per mL). Amphotericin B (Sigma, catalog# A2942) was added to the culture media at 0.1 to 0.5 µg per mL for the DMC1 dose response assays.

Time-kill curve assays were used to assess if AN7973 is parasiticidal or parasiticistatic for C. parvum\(^19\). C. parvum Iowa isolate oocysts (Bunch Grass Farms, Deary, ID) were treated to induce excystation and added to >90% confluent HCT-8 cells in 384-well plates. AN7973 was added at the EC\(_{50}\) (0.13 µM) or multiples of the EC\(_{50}\) (0.42 µM) ~24 h after excystation. The monolayers were then fixed and stained with Vicia villosa lectin and DAPI at the indicated time points, and imaged as for the C. parvum growth assay. Separate 384-well plates were used for each time point. Parasite numbers were normalized to host cell nucleus numbers and expressed as the percentage of host cells infected. Drug-induced parasite decay was isolated from the reduction in parasites over time that occurs spontaneously in culture by expressing the number of parasites as the percentage of the number of parasites present at each time point under control (DMSO-treated) conditions.
Pharmacokinetic studies. Single-dose oral and intravenous murine PK studies were performed by WuXi Biologics (Wuxi, China) in female CD-1 mice in compliance with the institutional animal care guidelines. AN7973 was formulated at 1 mg per mL in 50/50 20/30 PEG300/propanolol glycol (PG)/water for IV administration, and at 2 mg per mL in 1% carboxymethylcellulose in water with 0.1% Tween 80 for oral administration. Single-dose oral PK studies were performed at the University of Vermont in compliance with animal care guidelines and with approval from the University of Vermont Institutional Animal Care and Use Committee. Holstein bull calves were acclimatized for 3 days prior to infection by oral gavage with C. parvum. Single-dose oral and separate toxicokinetics (TK) study were performed at the University of Vermont in compliance with animal care guidelines and with approval from the University of Vermont Institutional Animal Care and Use Committee. Holstein bull calves were acclimatized for 3 days prior to infection by oral gavage with C. parvum. Single-dose oral and separate toxicokinetics (TK) study were performed at the University of Vermont in compliance with animal care guidelines and with approval from the University of Vermont Institutional Animal Care and Use Committee. Holstein bull calves were acclimatized for 3 days prior to infection by oral gavage with C. parvum.
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Author contributions

C.S.L. contributed to experimental design and writing the manuscript, and had overall responsibility for managing the project at Anacor. E.E.S. contributed to design of the calf efficacy studies and conducted the studies. R.S.I. contributed to in vitro characterization of AN7973 and other screening hits. M.M.H. contributed to in vitro characterization of AN7973. P.M. conducted the NSG mouse efficacy study. J.B. aided with the design and conduct of the calf study. Y.R.F. helped with experimental design and writing the manuscript. P.B. conducted pharmacokinetics studies and modeling, and aided with writing the manuscript. R.S. helped with experimental design and writing the manuscript. J.G. was in charge of library screening. P.J.R. aided with design of library screening, and with writing the manuscript. M.S.L. conducted testing with *C. parvum* strains and C. hominis, and was responsible for the IFN-gamma knockout mouse studies. C.W.M. led efforts at Calibre, and aided with experimental design, and writing the manuscript. E.E. had oversight responsibility of the Neglected Diseases program at Anacor and provided overall guidance on the project. J.J.P. was the head of research at Anacor, and had intellectual contributions to the project. R.T.J. provided medicinal chemistry oversight at Anacor, helped to write the manuscript. C.D.H. was primarily responsible for all in vitro and in vivo compound characterization at UVM, and wrote the manuscript.

Additional information

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