Paper

Development of a novel in vitro method for drug development for fish; application to test efficacy of antimicrosporidian compounds

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Few drugs are approved for treating diseases caused by parasites in minor species such as fish. This is due, in part, to the expense of drug development and to the comparatively small market. In vivo effectiveness trials for antiparasitic drugs are costly, time consuming and require ethics approval, therefore an in vitro screening approach is a cost-effective alternative to finding promising drug candidates. We developed an in vitro testing system to test antimicrosporidial compounds against a microsporidian pathogen *Heterosporis saurida*. Five antiparasitic compounds, albendazole, fumagillin, TNP-70, nitazoxanide and lufenuron, were assayed for antimicrosporidial activity. All compounds reduced the number of *H saurida* spores in infected cells when applied at a concentration that did not appear to be toxic to the host cells. Albendazole inhibited replication of *H saurida* by >60 per cent, fumagillin and its analogue TNP-470 inhibited *H saurida* >80 per cent, nitazoxanide and lufenuron inhibited growth >70 per cent. The data suggest that both fumagillin and its analogous TNP-70 hold the best promise as therapeutic agents against *H saurida*. The ability to use fish cell cultures to assess drugs against *H saurida* demonstrates an approach that may be helpful to evaluate other drugs on different microsporidia and host cells.

Introduction

Microsporidia are obligate intracellular parasites of most animal groups, including fish (Schmahl & Benini 1998). More than 1000 species cause disease in invertebrates and vertebrates (Weiss and others 1999, Weber and others 2000, Didier and others 2004). Microsporidian infections in fish are always deleterious and endanger the lives of their hosts. Under suitable conditions, especially in hatcheries, disease outbreaks can reach epizootic levels (Lom and Dykova 1992).

Current therapies for microsporidiosis vary in their effectiveness. The main drugs studied for their abilities to inhibit microsporidian growth are albendazole and fumagillin, though recently several others have been explored to a limited degree (Monaghan 2011). The ability of albendazole, fumagillin, the fumagillin analogous TNP-470, nitazoxanide and lufenouron to inhibit the growth of microsporidia has been reported (Didier and others 1998).

Albendazole has been reported to affect the intracellular development of the microsporid *Glugea anomala* by extensively damaging its merogonic, sporogonic and prespore stages, as well as the mature spores in the connective tissue of sticklebacks (*Gasterosteus aculeatus*) (Schmahl and Benini 1998). *Enterocytozoon cuniculi* replication was inhibited in vitro, and malformation of giant meronts and sporonts containing bundles of 35 nm tubules were developed in response to albendazole (Colbourn and others 1994). Blanshard and others (1993) reported that all stages of *Enterocytozoon bieneusi* were abnormal, particularly the proliferative stages, which contained ballooned plasmalemmas and irregularly shaped nuclei after albendazole treatment. These observations suggested that albendazole performs by inhibiting karyokinesis and cytokinesis of the microsporidia by virtue of inhibiting microtubule polymerisation (Didier 1997).

Fumagillin has long been reported as a candidate for the treatment of many fish parasitic diseases, including proliferative kidney disease (Hedrick and others 1988) and whirling disease (El-Matbouli and Hoffmann 1991). The antimicrosporidial activity of fumagillin has been demonstrated in vitro against *E cuniculi* in primary mammalian cell cultures (Shadduck 1980). Beauvais and others (1994), Didier and others (1998) and Franssen and others (1995) demonstrated that fumagillin was among the most promising drugs assayed against *E cuniculi* in vitro. Fumagillin has been extensively used as an oral treatment for fish microsporidiosis, usually with good success (Shaw and Kent 1999). This drug reduced the disease severity in ayu (*Plecoglossus altivelis)* infected with *Glugea plecoglossi* (Takahashi and Egusa 1976). Fumagillin was also effective against the microsporidium *Heterosporis anguillarum* in eels (*Anguilla japonica*) (Kano and others 1982) and against *E salmonis*, *Loma salmonae* and *Nucleospora salmonis* in salmon (Hedrick and others 1991, Kent and Dawe 1994, Speare and others 1999). Cultures of the zebra fish embryo cell line, ZEB2J, which had

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been infected with *Anncaliia algerae* spores were used as an in vitro test system and showed that fumagillin has potential as a treatment against microsporidia (Monaghan 2011). The sensitivity of the fish cell line, ZEB2J, infected with *A algerae* to fumagillin in vitro was comparable with that reported for mammalian cells.

TNP-470 showed a broad-spectrum efficacy against the microsporidia by inhibition of replication of both *Encephalitozoon intestinalis* and *Vittaforma corneae* in vitro (Didier 1997). It has also been demonstrated that TNP-470 is highly active against *Encephalitozoon cuniculi* and *Encephalitozoon hellem*, both in vitro and in vivo (Coyle and others 1998). Additionally, TNP-470 was found to inhibit the replication of *N salmonis* and *L salmonae* in salmonid fish (Higgins and others 1998).

Nitazoxanide has been used for the treatment of cryptosporidiosis and demonstrated therapeutic potential on *E intestinalis* and *V cornea* in cell cultures (Bicart-Sée and others 2000, Costa & Weiss 2000, Groß 2003). It has been effective also for the treatment of *E bieneusi* infections in clinical cases (Bicart-Sée and others 2000).

Rabbit kidney cells, RK-13 which had been infected with *E intestinalis*, was used to screen the effectivity of lufenuron that was used at 10 µg/ml which was the highest dose tested and was not toxic to the host cells (Didier and others 1998).

New and efficient methods to screen drugs while reducing animal use are needed to counter increased drug resistance. To date, the in vitro approach for testing potential compounds has been limited to a few combinations of cell lines and microsporidia. Insect cell lines have been used: from the moth *Heliothis zea*, infected with either *Glugea disstriae* or *Nosema* species (Kurtti and Brooks 1977); fall armyworm, *Spodoptera frugiperda* S9 with *Nosema bombycis* (Haque and others 1993, Sichtova and others 1993). Treatments for eliminating *Encephalitozoon cuniculi* have been studied in primary mamalian cell cultures from rabbit choroid plexus and kidney and from canine embryos (Shadduck 1980) and in cultures of three cell lines, Madin-Darby kidney (MDCK) (Beauvais and others 1994), rabbit kidney RK13 (Franssen and others 1995) and monkey kidney Vero (E6) (Ditrich and others 1994, Sobottka and others 2002). Inhibition of *V corneae* growth using albendazole was tested in cultures of MDCK (Silveira and Canning 1995). Effects of several compounds on the inhibition of *V corneae* and *E intestinalis* growth in RK13 (Didier 1997), and the sensitivity of *E cuniculi* and *E hellem* to albendazole in Vero cell lines were examined (Ditrich and others 1994). Similarly, mouse myoblast cell lines C2 and C12 were used to study inhibition of *Trachipleistophora hominis* by albendazole (Lafranchi-Tristem and others 2001).

The microsporidian *Heterosporis saurida* infects lizard fish and has been recently propagated in the mammalian cell line, rabbit kidney RK-13 (Kumar and others 2014), as well as in the fish cell line eel kidney EK-1 (Saleh and others 2014). Demonstration that the EK-1 cell line supports *H saurida* propagation and opens up the possibility of testing the efficacy of potential chemotherapeutic drugs on experimentally infected fish cells. Neither *H saurida* nor the fish cells have been objects for previous in vitro investigations of antimicrosporidial compounds. The aim of our present study was to evaluate the therapeutic effects of albendazole, fumagillin, the fumagillin analogous TNP-470, nitazoxanide and lufenouron on the replication of the fish microsopridium *H saurida* in vitro.

Materials and methods

H saurida growth

Microsporidia were grown in EK-1 cells using procedures previously described (Saleh and others 2014). Briefly, the spores of *H saurida* were collected from lizard fish, *Saurida undosquamis* (Al-Quraishy and others 2012). EK-1 cells were subcultured and seeded in 24-well plates in triplicate. Leibovitz, L-15 medium supplemented with 10 per cent fetal bovine serum (FBS) was added to the cells, and 24 hours after seeding, 100 µl of the spore suspensions were added to each cell line monolayer. After 24 hours postinfection (p.i.), the medium was removed from each well and the well was rinsed three times with fresh medium. The medium was replaced twice a week.

Compounds used

Albendazole, fumagillin, the fumagillin analogous TNP-470, nitazoxanide and lufenouron were tested. All compounds were purchased from Sigma-Aldrich GmbH (Schnelldorf, Germany). Stock solutions were prepared in dimethyl sulfoxide (DMSO) at a final concentration of 10 mg/ml and diluted in cell culture medium for use in the assays.

Measuring of antimicrosporidial activities

EK-1 cells were plated onto 24-well culture plates at a concentration of 1.5×10^5 cells/ml in L-15 (Leibovitz) medium containing L-glutamine (Sigma-Aldrich), 10 per cent FBS (Invitrogen) and 100 IU/ml penicillin/streptomycin (Sigma-Aldrich). The plates were incubated overnight at 26°C to allow the cells to reach confluency. *H saurida* spores were added in 1 ml volumes in medium at a concentration of 10^6–10^7 spores/ml to achieve a final ratio of 3:1 spores/cell. After 24 hours, non-internalised or non-adherent spores were washed off, and fresh medium with or without drugs was added and incubated for seven days. Control cultures, which were not given chemotherapy, were supplied with medium containing equal amounts of DMSO used in preparing drug stock solutions. For cultures without chemotherapy, the spores were suspended in medium (L-15/FBS) with 0.5 per cent DMSO, and media replaced every three days with fresh medium and incubated for seven days. The monolayers were examined daily with an inverted microscope and 10 cells in 10 fields were viewed per well (6 wells each treatment and control), and each assay was repeated three times.

Treatments were assayed in triplicate, and the per cent inhibition of *H saurida* replication was calculated: per cent inhibition= 100–[(mean number of parasites counted in treated cultures/mean number of parasites counted in non-treated cultures)×100]. The differences between drug-treated and non-treated spores were analysed using *t* tests with Bonferroni α-correction. For all statistical tests, a P value<0.05 was regarded as significant. Statistical analyses were conducted with SPSS V.20 software.

Visual observations and MTT assay of cell viability for measurement of drug toxicity

EK-1 cells were plated in 96-well plates at approximately 1.5×10^4 cells per well. After 24 hours, the medium was removed and replaced with fresh medium with equal amounts of DMSO with different concentrations of the antiparasitic compounds. Cellular viability of EK-1 cultures after exposure to the compounds was monitored. EK-1 cells incubated with the test compounds without microsporidia were examined by light microscopy using an inverted microscope. Drug toxicity was noted if the cells became subconfluent or changed in morphology when compared with non-treated cells. Plates were held at 26°C for seven days. At this point, the media were removed and the viability of cultures was evaluated by incubating replicate wells of treated and non-treated control EK-1 cells with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), Sigma-Aldrich. Briefly, 20 µl of the MTT (5 mg/ml dissolved in balanced salt solution) was added to each well of 96-well plates and incubated for two hours. After the incubation period, the medium of each well was discarded and 200 µl of MTT solubilisation solution (Sigma-Aldrich) was added into each well to solubilise formazan crystals and then mixed to ensure complete solubilisation using an orbital shaker. The absorbance values were read on a spectrophotometer at a test wavelength of 570 nm and a reference wavelength of 690 nm by a microplate reader (EnSpire 23000 Multilabel Reader Perkin Elmer). The percentage of cell viability was calculated as the optical density values of treated cells/the mean optical density of non-treated cells×100. Three replicates were used for each group of treatments.

Reinfection of EK-1 cells with *H saurida* recovered after treatments

To determine if microsporidia remained infectious after treatment, *H saurida* was recovered from culture wells on day 7, by addition of 100 µl of 10 per cent (w/v) sodium dodecyl sulfate to each well, to release spores from host cells. Spores were centrifuged and washed three times at 400 g for 15 minutes with Tris-buffered saline (TBS) containing 0.3 per cent Tween 20 (TBS-Tween). Spore pellets were resuspended in L-15 medium and used to infect new cultures of EK-1 cells, maintained as described previously. The total numbers of drug-treated and non-treated spores were counted using a haemacytometer, and 1 ml volumes of spores adjusted to 1×10^4 spores/ml were added to each well.

Fresh media without drugs was added on day 3. Using an inverted microscope, 10 fields were viewed per well and counts repeated three times.

Results

Five antiparasitic compounds were assayed for antimicrosporidial activity. Effects of these antimicrospordial agents on cell viability were measured using MTT assay after treatment for seven days with different concentrations for each compound.

The therapeutic compounds tested reduced highly significantly ($P<0.0001$) the number of the microsporidium *H saurida* in infected cells. Cellular and nuclear shapes were unchanged when cultures were examined for changes in appearance. Effects of each drug are shown in Table 1. In summary, albendazole inhibited replication of *H saurida* by more than 60 per cent at doses that did not appear toxic to the EK-1 cells (Fig 1a), but at 1 µg/ml, it caused a loss of EK-1 cell viability. Cultures with 0.1 µg/ml fumagillin had notably fewer *H saurida* than control cultures (Fig 1b). Both fumagillin and its analogue TNP-470 inhibited *H saurida* by more than 80 per cent at a concentration of 0.1 µg/ml (Fig 1c). The growth of the microsporidium was inhibited by more than 70 per cent when nitazoxanide was used by a concentration of 1 µg/ml (Fig 1d). After lufenuron treatment at concentration of 10 µg/ml, more than 70 per cent inhibition of the microsporidium was observed (Fig 1e).

H saurida was observed in all cultures after treatment with albendazole, fumagillin, TNP-470, nitazoxanide or lufenuron. Since it was not possible to determine if these spores were mature or infectious by microscopic examination, EK-1 cells were reinfected with recovered *H saurida* after treatment. Fig 2 shows that significantly fewer infectious microsporidia were recovered after treatments with albendazole, fumagillin, TNP-470, nitazoxanide or lufenuron, compared with non-treated cultures.

Discussion

Five antimicrosporidial compounds were evaluated for their potential to prevent *H saurida* growth without affecting the host cells, the fish cell line EK-1. Nitazoxanide and lufenuron had moderate impact on the development of *H saurida*. Albendaozle impeded the growth of *H saurida*, but at concentrations that caused sublethal damage to EK-1 cells. Contrastingly, fumagillin and its analogous TNP-70 inhibited *H saurida* growth at concentrations that had negligible impact on EK-1 cells.

TABLE 1: Effects of albendazole, fumagillin, TNP-70, nitazoxanide and lufenuron on *Heterosporis saurida* growth in EK-1 cells

Compound	Treatment concentration	Average *H saurida* spore count with SD	Average % inhibition on day 7	Cell viability % (MTT assay)
Albendazole	0.01 µg/ml	306±15.92	37.6	90.32
	0.1 µg/ml	149±6.71	69.6	61.93
	1 µg/ml	102±6.88	79.2	30.96
	Non-treated	490±15.16	–	–
Fumagillin	0.001 µg/ml	116±11.83	66.7	96.80
	0.01 µg/ml	85±6.53	75.6	91.37
	0.1 µg/ml	62±5.60	82.2	83.38
	Non-treated	348±14.88	–	–
TNP-70	0.001 µg/ml	132±6.62	66.6	81.10
	0.01 µg/ml	66±6.03	83.3	77.25
	0.1 µg/ml	60±4.56	84.9	58.41
	Non-treated	395±11.40	–	–
Nitazoxanide	0.01 µg/ml	305±5.84	12.1	85.46
	0.1 µg/ml	139±5.11	59.9	71.39
	1 µg/ml	80±6.83	76.9	54.88
	Non-treated	347±10.30	–	–
Lufenuron	0.1 µg/ml	164±8.14	60.2	64.85
	1 µg/ml	125±6.89	69.7	61.43
	10 µg/ml	91±4.36	77.9	55.68
	Non-treated	412±8.12	–	–

Average (±SD) numbers of spores are collected from six wells of 10 infected EK-1 cells in 10 fields. Drug toxicity effect of test compounds on cellular activity was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay

In the study presented here, addition of albendazole to EK-1 cells did not prevent *H saurida* from infecting EK-1 cells, but impeded further growth of the microsporidian. Albendazole treatment for seven days in vitro notably reduced replication of *H saurida*. Few infectious spores were recovered after treatment, which suggested that albendazole is antimicrosporidial in vitro. Seven days after simultaneous infection with *H saurida* spores and treatment with albendazole, EK-1 cells contained sporonts larger than those in untreated cells. Individual *H saurida* spores were abnormal, with distended plasmalemmas and irregularly shaped nuclei following albendazole treatment. These observations are consistent with previously reported effects of albendazole on other microsopridia (Blanshard and others 1993, Colbourn and others 1994, Didier and others 1997, Monaghan 2011). Albendazole at concentrations above 1 µg/ml caused a loss of EK-1cell viability. Albendazole is a broad spectrum antiprotozoal benzimidazole that binds to the colchicines binding site of b-tubulin and thus inhibits microtubule assembly (Lacey 1985) and has been reported as being cytotoxic in mammalian cell lines at lower concentrations (Whittaker and Faustman 1991, Baliharová and others 2003). Fish and mammalian cells might respond differently to albendazole due to differences in metabolism or tubulins in mammalian cells which may be affected at lower concentrations. Despite potential toxicity, albendazole has been used effectively to reduce the severity of microsporidia infections in vivo. These include infections of *Encephalitozoon intestinalis* and *E cuniculi* in human beings (Gross 2003) and of *G anomala* and *L salmonae* in fish (Schmahl and Benini 1998, Speare and others 1999). Although addition of albendazole to ZEB2J cultures impeded the growth of *A algerae* (Monaghan 2011), Coyle and others (2004) reported that albendazole was able to cure *A algerae* in clinical infections, including a deep tissue infection and infection of the vocal folds (Cali and others 2010).

In our study, when fumagillin was added to cultures, it did not prevent *H saurida* from infecting EK-1cells, but impeded the growth of *H saurida*. Fumagillin treatment hampered the subsequent development of *H saurida* spores in EK-1 cells. *H saurida* was inhibited by more than 80 per cent at a concentration of 0.1 µg/ml which was not toxic for the host cells. Few infectious spores could be recovered after seven days treatment with fumagillin. We obtained similar results with the fumagillin analogue TNP-70. Fumagillin inhibits methionine aminopeptidases, MetAP-2 but not MetAP-1 (Sin and others 1997). Microsporidia are known to have only MetAP2, while mammalian cells have both MetAP-1 and MetAP-2, hence the latter can survive inhibition of MetAP-1 by fumagillin (Weiss and others 2003, Zhang and others 2005). However, though fumagillin and analogous drugs are currently used for treatment of microsporidiosis (Alvarado and others 2009), their inhibitory effect on MetAP-2 has a toxic effect on human beings and other vertebrates, as the enzyme is involved in protein maturation and post-translation processes (Lefkove and others 2007, Huang and others 2013). The fumagillin analogue TNP-470 was found to be less toxic in laboratory animals when used in vivo (Ingber and others 1990).

Nitazoxanide inhibits the microsporidian replication through interacting with enzymes of the electron transport systems, such as pyruvate ferrodoxine oxydoreductase, which are considered as potential target structures (Groß 2003). In this study, nitazoxanide was able to inhibit the growth of *H saurida* by more than 70 per cent in EK-1 cultures at concentrations that were not toxic to the EK-1 cells. Hence, this drug could be useful in the treatment of microsporidiosis of fish.

In the present study, lufenuron inhibited *H saurida* growth by more than 70 per cent assumably in a similar mechanism as reported previously. Lufenuron is a benzoylphenylurea drug that inhibits the production of chitin, which is an important component of the cell wall of microsporidia (Erickson & Blanquet 1969, Vavra and others 1993, Bigliardi and others 1996). Didier and others (1998) screened a range of chitin synthesis/assembly inhibitors in vitro, but found they were toxic for the host cells (RK-13) with the exception of lufenuron, which inhibited microsporidian species tested by more than 50 per cent at concentrations that were not toxic to the host cells.

In summary, while all tested compounds inhibited growth of the microsporidian *H saurida*, both fumagillin and its analogue TNP-70

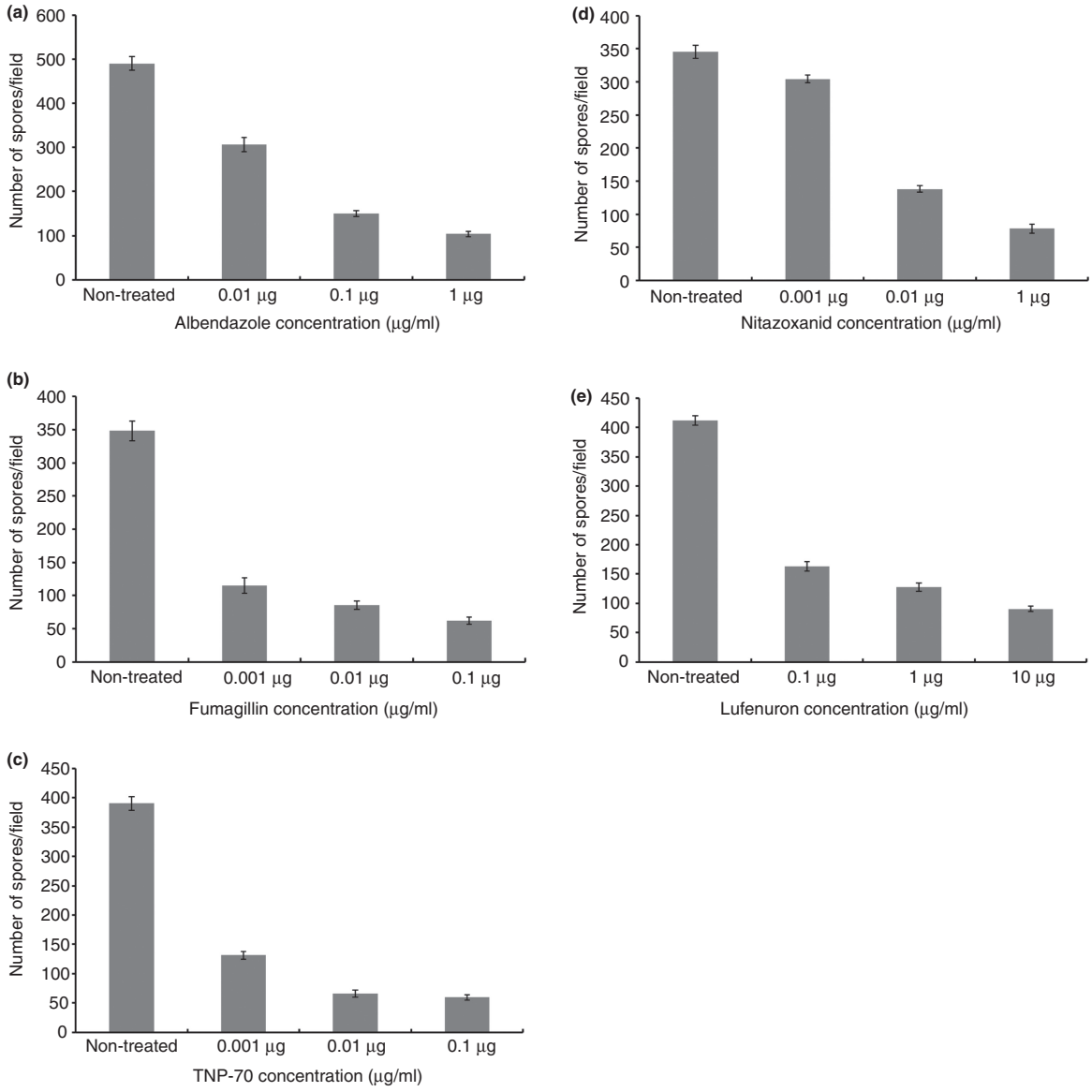

FIG 1: Effects of used compounds on the growth of *Heterosporis saurida* in EK-1 cells. (a) Albendazole, (b) fumagillin, (c) TNP-70, (d) nitazoxanide, (e) lufenuron. Data are average (±SD) numbers of spores in six wells of infected EK-1 cells.

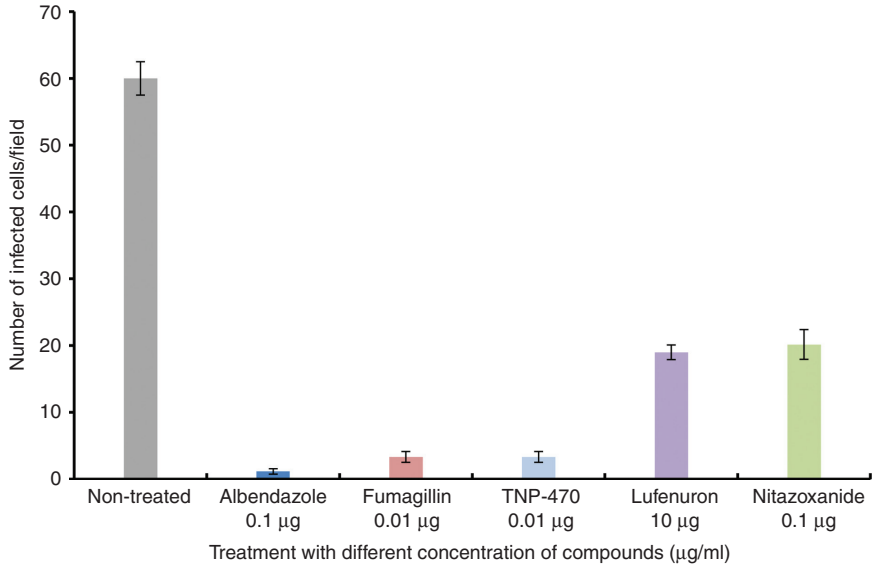

FIG 2: Viability of treated *Heterosporis saurida* spores to infect EK-1 cells. Comparisons were made with *H saurida* between treated and non-treated cell cultures and assayed for infectivity. Data are average (±SD) numbers of spores in six wells of infected EK-1 cells. Details of *H saurida* spores count are presented in Table 1.

were most effective and thus hold promise as potential therapeutic compounds against *H saurida*. We demonstrated that fish cell cultures can be used effectively to assess drug treatment against *H saurida* and we suggest that establishment of other host-parasite systems in vitro would be helpful in evaluating drugs on different microsporidia and host cells.

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