Title

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Identification of Cysteine Protease Inhibitors as New Drug Leads against *Naegleria fowleri*

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Abstract

Primary amebic meningoencephalitis (PAM) is a rapidly fatal infection caused by the free-living ameba *Naegleria fowleri*. PAM occurs principally in healthy children of less than 13 years old with a history of recent exposure to warm fresh water. While as yet not a reportable disease, the Centers for Disease Control and Prevention (CDC) documents a total of 143 cases in the United States. Only four patients have survived. Infection results from water containing *N. fowleri* entering the nose, followed by migration of the amebae to the brain. Within the brain, *N. fowleri* infection results in extensive necrosis, leading to death in 3 to 7 days. Mortality among patients with PAM is greater than 95%. The drugs of choice in treating PAM are the antifungal amphotericin B, and the antileishmanial, miltefosine. However neither drug is FDA-approved for this indication and the use of amphotericin B is associated with severe adverse effects. Moreover, very few patients treated with amphotericin B have survived PAM. Therefore, development of new, safe and effective drugs is a critical unmet need to avert future deaths of children. The molecular mechanisms underlying the pathogenesis of PAM are poorly understood but it is known that cysteine proteases of *N. fowleri* play a role in the progression of PAM. We therefore assessed the *in vitro* activity of the synthetic vinyl sulfone cysteine protease inhibitor, K11777, and 33 analogs with valine, phenylalanine or pyridylalanine at P2 position, against cysteine protease activity in the lysate of *N. fowleri*. Inhibitors with phenylalanine or pyridylalanine at P2 position were particularly effective in inhibiting the cysteine protease activity of *N. fowleri* cell lysate with IC_{50} ranging between 3 nM to 6.6 μM. Three of the 34 inhibitors also showed inhibitory activity against *N. fowleri* in a cell viability assay and were 1.6- to 2.5-fold more potent than the standard of care drug miltefosine. Our study provides the first evidence of the activity of synthetic, small molecule cysteine protease inhibitors against *N. fowleri*.

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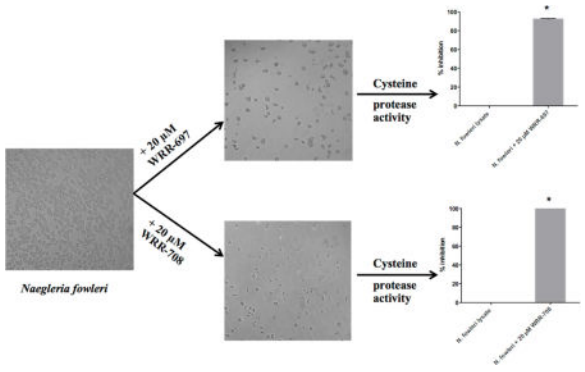
Graphical abstract



Keywords

Naegleria fowleri; primary amebic meningoencephalitis; cysteine protease; inhibitors; drug discovery; drug leads

1. Introduction

Naegleria fowleri is a free-living ameba found in warm fresh water lakes, ponds and other warm-bodied waters. It is the cause of primary amebic meningoencephalitis (PAM) in more than 16 countries (1). As of 2012, 310 cases had been reported globally with a fatality rate of more than 95% (2). *N. fowleri* infection is not a notifiable disease in the US and therefore is likely significantly underreported. Based on the free-living ameba registry maintained by the CDC and other data sources, only 143 cases of PAM were reported in the US from 1962-2016 (3). Infections occur mostly in warmer, southern-tier states. One reason why the infection is underreported is that states differ significantly in their capacity to identify, investigate or report cases (4). Even among the 143 known cases, 139 were fatal.

In the USA, especially in the southern warm-weather states, most of the PAM infections occurred after swimming in fresh-water bodies naturally heated by the sun. *N. fowleri* thrives in warm water and can grow in temperatures of up to 45°C. A few infections occurred after swimming in geothermal waters or by using tap water originating from warm groundwater (5). In the US, during 2003–2012, three of 31 persons infected with *N. fowleri* became infected after performing nasal rinsing with contaminated tap water. Two of the three patients performed nasal rinsing using a neti pot or similar device (6). Recently, ablution has been associated with *N. fowleri* cases globally. In 2008-2009, 13 cases were reported in Karachi, Pakistan and the infection likely occurred through ablution with tap water (7). In 2012, a first documented U.S. case of PAM potentially associated with ablution was also reported (8).

N. fowleri enters the host through the nasal cavity and travels via the olfactory nerve into the brain where it causes PAM (1). The ameba most commonly infects healthy young adults and children and is able to evade the immune system but also elicits extensive damaging inflammation, tissue necrosis and hemorrhage of the brain and meninges (1). The time from

initial exposure to onset of illness is usually 5-7 days but may be as early as 24 h. Initial symptoms include sudden onset of bifrontal or bitemporal headaches, high fever, nuchal rigidity, anorexia, vomiting, irritability and restlessness. Other symptoms such as photophobia, neurological abnormalities, including altered mental status, lethargy, dizziness, ataxia, cranial nerve palsy, hallucinations, delirium, coma may occur late in the clinical course, leading to death in 3 to 7 days (9). Current treatments are inadequate and provide no guarantee for survival. The drug of choice to treat *N. fowleri* is the antifungal amphotericin B but is not FDA-approved for this indication. Treatment with amphotericin B requires high dosage (1.5 mg/kg/day intravenous in 2 doses × 3 days, then 1 mg/kg/day × 6 days plus 1.5 mg/day intrathecally × 2 days, then 1 mg/day every other day × 8 days) and its use is frequently associated with renal toxicity, manifested as azotemia and hypokalemia (10). Amphotericin B can also cause anemia, and many patients experience chills, fever, nausea, vomiting, and headache. Moreover, no more than a dozen persons with PAM have been treated successfully worldwide with amphotericin B alone or in combination with other drugs (10). Recently, an investigational breast cancer and anti-*Leishmania* drug, miltefosine, has shown some promise in combination with other drugs and a patient was successfully treated with miltefosine and hypothermia (11). But another patient, though treated with miltefosine, suffered permanent brain damage. Therefore, it is imperative to find safe and effective drugs that can treat PAM and increase host survival. Identification of new leads that can be used as a basis to develop drugs to treat *Naegleria* infection is a critical unmet need to prevent future deaths of children and young adults.

In this study, we first show that the cysteine protease inhibitors inhibited *N. fowleri* cysteine protease activity present in cell lysates. Because of the inhibitory activity against parasite cysteine proteases, we also tested the effect of the inhibitors on the viability of *N. fowleri*.

2. Materials and Methods

2.1. Maintenance of *N. fowleri*

Trophozoites of *N. fowleri* KUL strain (ATCC 30808) were cultured in Nelson's media supplemented with 10% FBS at 37°C (12). Cells were counted using a hemocytometer. All experiments were performed using trophozoites harvested during logarithmic phase of growth.

2.2. Cysteine protease inhibitors

Vinyl sulfone cysteine protease inhibitor K11777 (*N*-methylpiperazine-urea-phenylalanyl-homophenylalanyl-vinylsulfone-benzene) and 33 analogs (WRR-636, WRR-673, WRR-679, WRR-680, WRR-682, WRR-683, WRR-689, WRR-692, WRR-693, WRR-694, WRR-695, WRR-696, WRR-697, WRR-698, WRR-699, WRR-700, WRR-701, WRR-703, WRR-704, WRR-705, WRR-706, WRR-707, WRR-708, WRR-709, WRR-710, WRR-711, WRR-712, WRR-713, WRR-714, WRR-716, WRR-717, WRR-718, WRR-719) with valine, phenylalanine or pyridylalanine at P2 position (Table 1) were dissolved in DMSO. K11777 analogs were synthesized from appropriate amino acids by adapting previously published experimental procedures (13–15). A broad-spectrum irreversible cysteine protease inhibitor,

E-64 (*N*-[*N*-{L-3-trans-carboxirane-2-carbonyl}-L-leucyl]-agmatine) was included as a positive control.

2.3. Effect of cysteine protease inhibitors on protease activity present in *N. fowleri* lysate

N. fowleri trophozoites (10×10^6) were collected by centrifugation at 600 × g for 5 min at 4°C and the cells were washed with PBS (pH 7.2). Cells were subsequently lysed using a 27 gauge needle and spun at top speed for 5 min at 4°C. The supernatant was collected and protein concentration was quantified by the method of Bradford. One microgram of lysate was used to determine the IC_{50} of vinyl sulfone cysteine protease inhibitors (K11777 and WRR compounds) by incubating the lysate with different concentrations of inhibitors for 20 min in a reactivation buffer (pH 7.4; 10 mM Tris, 5 mM EDTA, 50 mM NaCl, 10 mM DTT). E-64, the prototypical cysteine protease inhibitor was also incubated with the lysate to validate the presence of cysteine protease in the lysate. The cysteine protease activity present in cell lysate was measured at room temperature by detection of the fluorescent leaving group, 7-amino-4-methyl coumarin (AMC), from the peptide substrate Z–Phe–Arg–AMC (40 μM) (where Z is benzyloxycarbonyl, R&D Systems). At the pH tested (7.4) and in the presence of 10 mM DTT, this derivatized peptide is a known substrate of Clan CA cysteine proteases (16). Relative fluorescent units (RFU) were measured in an automated microtiter plate spectrofluorometer (EnVision, PerkinElmer) with excitation wavelength at 355 nm and emission wavelength at 460 nm. The rate of substrate hydrolysis at ambient temperature was determined from the rate of increase of fluorescence, monitored on a continuously recording spectrofluorometer and measured as RFU/min/μg protein. The IC_{50} determination and statistical analysis of the data were performed using GraphPad Prism 5.0 software.

2.4. *In vitro* activity of K11777 and WRR inhibitors against *N. fowleri*

Primary screens of K11777 and the WRR inhibitors were performed after transferring 0.5 μL of a 10 mM solution of compound in DMSO to a 96-well microtiter plate to achieve final concentrations of test compound and DMSO of 50 μM and 0.5%, respectively. Finally, 99.5 μL of Nelson medium with 10,000 *N. fowleri* trophozoites was added in each well of 96-well plate and incubated for 48 h at 37°C. Negative controls in the screen plates contained 0.5% DMSO and positive controls contained 50 μM amphotericin B (Sigma-Aldrich). At the end of incubation, the assay plates were equilibrated to room temperature for 30 min, followed by addition of 50 μL of CellTiter-Glo Luminescent Cell Viability Assay (Promega) in each well of the 96-well plates. Cell lysis was induced by placing the plates on an orbital shaker for 10 min. The resulting ATP-bioluminescence of the trophozoites was measured at room temperature using an EnVision Multilabel Plate Reader from PerkinElmer (12). Compounds showing more than 50% inhibition in the primary screen were followed up in a secondary screen.

For 8-point EC_{50} determination experiments, 2.5 μL of 20 mM stock compounds was serially diluted with equal volume of DMSO to yield a concentration range of 156 μM-20 mM. From this dilution plate, 0.5 μL of each compound was transferred in triplicate into the 96-well screen plates followed by addition of 99.5 μL of trophozoites (10,000 amebae) to yield a final 8-point concentration range spanning 0.78-100 μM in 0.5% DMSO. The assays were performed using the CellTiter-Glo Luminescent Cell Viability Assay.

2.5. Effect of WRR-697 and WRR-708 on *N. fowleri* growth and morphology

N. fowleri trophozoites (1×10^4) were treated with 20 μM of WRR-697 and WRR-708 in a 96-well plate for 48 h. Cells treated with 0.5% DMSO were used as control. After 48 hours, cells were visualized with Axiovert 40 CFL phase contrast microscope (Carl Zeiss).

2.6. Determination of cysteine protease activity present in cell lysate after treating the trophozoites with WRR-697 and WRR-708 for 24 hours and 48 hours

N. fowleri trophozoites (1×10^6) were incubated in 12-well plates with 20 μM, 10 μM and 5 μM of WRR-697 and WRR-708 for 24 hours and 48 hours. 0.5% DMSO-treated cells were used as a control. The lysate was collected after 24 hours and 48 hours and one microgram of lysate protein was used to determine the cysteine protease activity based on detection of the fluorescence following the cleavage of the substrate Z-Phe-Arg-AMC in an EnVision® spectrofluorometer at 355 nm excitation wavelength and 460 nm emission wavelength.

3. Results

3.1. Effect of cysteine protease inhibitors on protease activity present in *N. fowleri* lysate

A vinyl sulfone cysteine protease inhibitor K11777 and its 33 analogs, derived from a prior effort to develop inhibitors of the cathepsin B-like *Entamoeba histolytica* cysteine protease EhCP4 (17), were screened against *N. fowleri* lystate. The majority of the EhCP4 inhibitors that were made had valine at the P2 position, however the *N. fowleri* screening consistently demonstrated that inhibitors with phenylalanine (WRR-693, WRR-695, WRR-696, WRR-697, WRR-705, WRR-707, WRR-708, WRR-709, WRR-718, WRR-719, K11777) or pyridylalanine (WRR-698, WRR-699, WRR-700, WRR-701, WRR-703, WRR-704, WRR-706, WRR-710, WRR-711, WRR-712, WRR-713, WRR-714, WRR-716, WRR-717) at this position had superior activity in biochemical assays against the *N. fowleri* cell lysate with IC_{50} ranging between 3 nM to 6.6 μM (Table 2).

3.2. *In vitro* activity of K11777 and WRR compounds against *N. fowleri*

We tested the activity of K11777 and 33 of its analogs against *N. fowleri* trophozoites using ATP-bioluminescent cell viability assay (12). Three inhibitors (WRR-697, WRR-708, WRR-712) of the 34 inhibitors tested were active against *N. fowleri* in a primary screen at 50 μM concentration. WRR-697 and WRR-708 had EC_{50} of 22.7 μM and 20.8 μM, respectively. WRR-712 exhibited an EC_{50} of 33.6 μM (Fig. 1). All three inhibitors were found to be 1.6- to 2.5-fold more potent than the CDC-recommended current anti-PAM drug miltefosine (EC_{50} = 54.5 μM) (18).

3.3. Effect of WRR-697 and WRR-708 on *N. fowleri* growth and morphology

Since WRR-697 and WRR-708 were more potent than WRR-712, these two inhibitors were analyzed microscopically for their effect on *N. fowleri* morphology. *N. fowleri* trophozoites were incubated with 20 μM of WRR-697 and WRR-708 for 48 h. 0.5% DMSO-treated control cells appeared elongated and/or irregularly shaped with visible cytoplasm while protease inhibitors led to dead or non-dividing cells (Fig. 2).

3.4. Determination of cysteine protease activity present in cell lysate after treating the trophozoites with WRR-697 and WRR-708 for 24 hours and 48 hours

N. fowleri trophozoites were treated with 5 μM, 10 μM and 20 μM of WRR-697 and WRR-708 for 24 h and 48 h and cysteine protease activity present in the 0.5% DMSO-treated control lysate and inhibitor-treated cell lysate was measured. Both the inhibitors significantly reduced the cysteine protease activity present in the cell lysate (Fig. 3, 4), confirming that these inhibitors are cell penetrant.

4. Discussion

The role of cysteine proteases in *N. fowleri* is not clearly defined. Several studies suggest that cysteine protease may play a role in *Naegleria* pathogenesis. A 30 kDa secreted *N. fowleri* cysteine protease was found to have a cytopathic effect on BHK cells (19). A 37 kDa cysteine protease with mucinolytic activity was hypothesized to have a role in mucin degradation and host immune response evasion (20). Treatment of *N. fowleri* with the canonical cysteine protease inhibitor, E64, decreased their migration through an extracellular matrix construct, suggesting a role for cysteine protease activity in invasion of the central nervous system (21). In the absence of a complete *N. fowleri* genome, recent studies reported that *N. fowleri* lysate and excretory-secretory proteins contain cathepsin B and cathepsin B-like cysteine proteases that are likely virulence factors of *N. fowleri* and involved in pathogenesis, as they are in *E. histolytica* infections. The *N. fowleri* cathepsin B gene was found differentially expressed only in the pathogenic trophozoite stage but not in the cyst stage, compatible with a role in pathogenesis (22). Biochemical characterization of *N. fowleri* protease activity in total crude extract and in conditioned medium was performed and the effect of protease inhibitors, such as E-64 and NEM, on proteolytic activity was also quantified (23).

Cysteine protease inhibitors representing several chemical scaffold types are effective in halting parasite replication without toxicity to the host (24). A vinyl sulfone cysteine protease inhibitor, K11777, is completing final Good Laboratory Practice (GLP) preclinical tests as a prelude to Phase I clinical trials against Chagas Disease. Several of the inhibitors we tested are direct analogs of the vinyl sulfone K11777, with a variety of substituents in the P1′, P1 and P3 positions. Our studies identified K11777 and related vinyl sulfone inhibitors that are effective inhibitors of the cysteine protease activity of *N. fowleri*. Three of the inhibitors tested *in vitro* were also cidal versus *N. fowleri*. Vinyl sulfone inhibitors can serve as the starting points to optimize compounds that inhibit *N. fowleri* as safe and effective drug candidates.

Acknowledgments

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Highlights

- Cysteine protease may play a role in the pathogenesis of *Naegleria fowleri*
- Vinyl sulfone protease inhibitors inhibit cysteine protease activity of *N. fowleri*
- Inhibitors with phenylalanine or pyridylalanine at P2 position were more effective
- Three inhibitors were cidal against *N. fowleri in vitro*

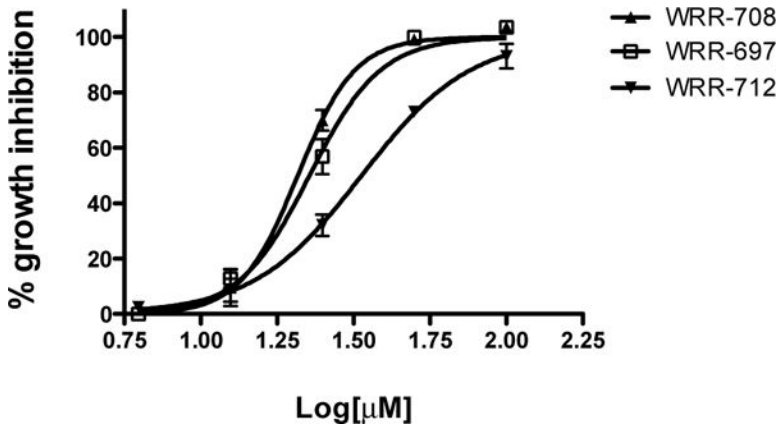

Fig. 1.

Percent inhibition of *N. fowleri* growth by WRR-697, WRR-708 and WRR-712. The EC_{50} dose response curves for WRR-697, WRR-708 and WRR-712 against *N. fowleri*.

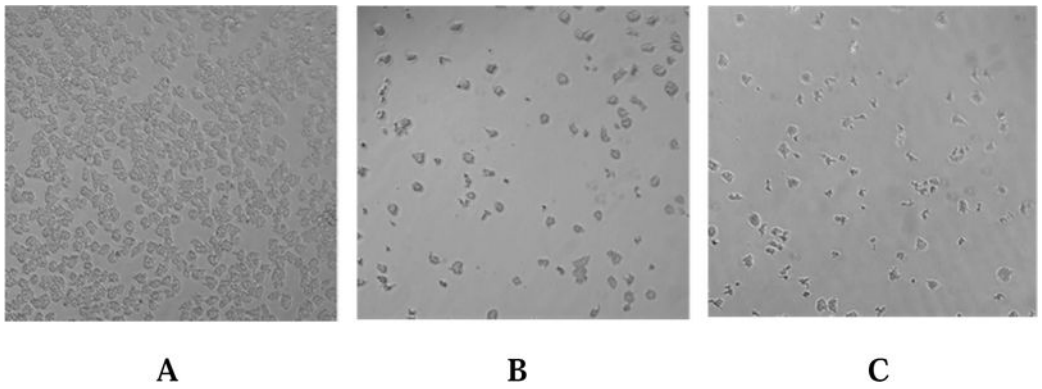

Fig. 2.

Effect of WRR-697 and WRR-708 on *N. fowleri* growth and morphology. (A) *N. fowleri* treated with 0.5% DMSO for 48 h. (B) *N. fowleri* treated with 20 μM WRR-697 for 48 h. (C) *N. fowleri* treated with 20 μM WRR-708 for 48 h. Magnification, ×20.

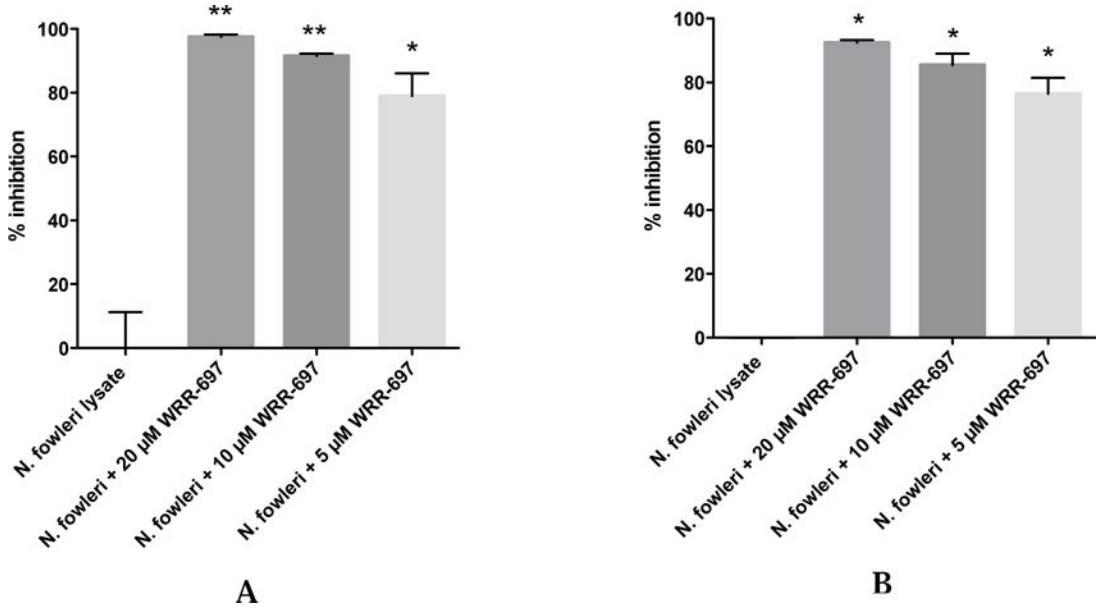

Fig. 3.

Inhibition (%) of protease activity present in *N. fowleri* lysate after treating the cells with WRR-697 (20 μM, 10 μM and 5 μM) for 24 hr (A) and 48 hr (B). One microgram of lysate protein was used in the cysteine protease assay. Cysteine protease activity was measured as RFU/min/μg protein. The data represent the mean and standard error of mean of three independent experiments. *P < 0.05 by Student's t test compared to DMSO-treated *N. fowleri* lysate.

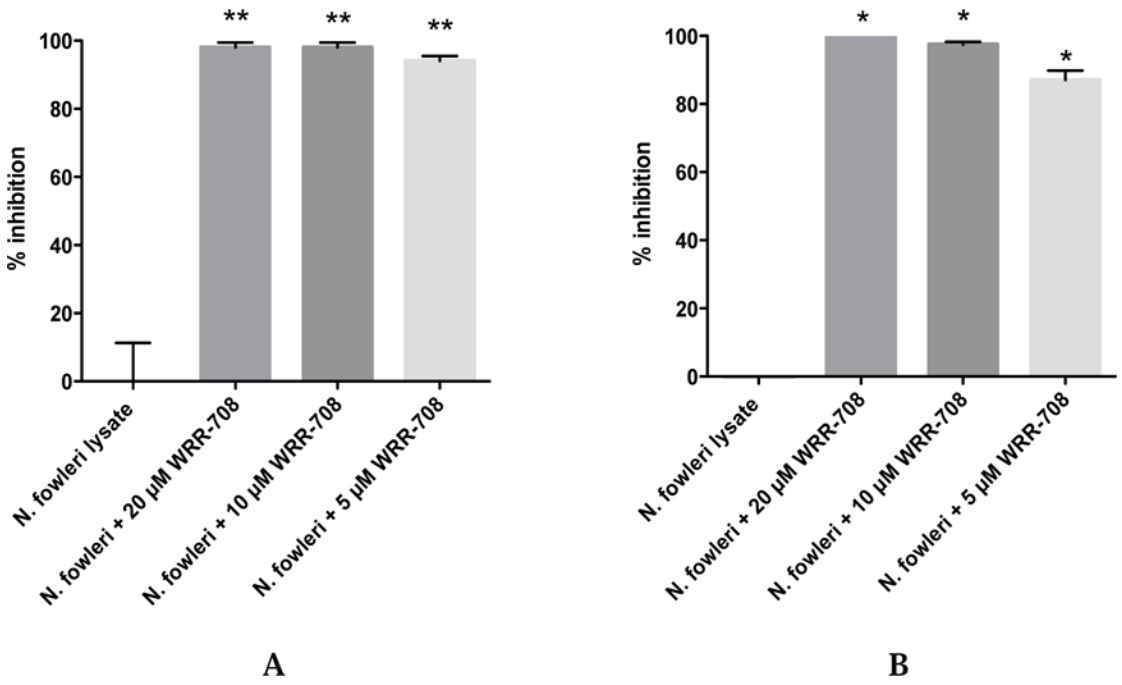

Fig. 4.

Inhibition (%) of protease activity present in *N. fowleri* crude extract after treating the cells with WRR-708 (20 μM, 10 μM and 5 μM) for 24 hr (A) and 48 hr (B). One microgram of lysate protein was used in the cysteine protease assay. Cysteine protease activity was measured as RFU/min/μg protein. The data represent the mean and standard error of mean of three independent experiments. *P < 0.05 by Student's t test compared to DMSO-treated *N. fowleri* lysate.

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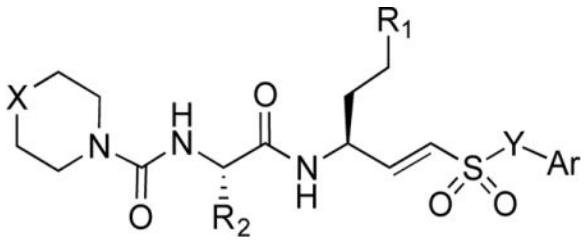
Table 1

Structures of vinyl sulfone inhibitors.



Compound ID	R_1	R_2	X	Y	Ar
K11777	phenyl	PhenylCH_2-	Me-N	–	phenyl
WRR-636	phenyl	i-Pr-	O	–	phenyl
WRR-673	phenyl	i-Pr-	Me-N	NH	(2-tetrahydrofuryl)methyl
WRR-679	phenyl	i-Pr-	Me-N	NH	2-fluorophenyl
WRR-680	phenyl	i-Pr-	Me-N	NH	2,6-difluorophenyl
WRR-682	phenyl	i-Pr-	Me-N	NH	2-pyrimidyl
WRR-683	3-pyridyl	i-Pr-	Me-N	–	phenyl
WRR-689	4-fluorophenyl	i-Pr-	Me-N	–	phenyl
WRR-692	phenyl	i-Pr-	Me-N	N(Me)	2-pyridyl
WRR-693	phenyl	PhenylCH_2-	Me-N	–	2-pyridyl
WRR-694	phenyl	i-Pr-	Me-N	–	2-pyrimidyl
WRR-695	phenyl	PhenylCH_2-	Me-N	N(Me)	2-pyridyl
WRR-696	phenyl	PhenylCH_2-	Me-N	–	2-pyrimidyl
WRR-697	phenyl	PhenylCH_2-	Me-N	–	4-pyridyl
WRR-698	phenyl	(2-pyridyl)-CH_2-	Me-N	–	2-pyridyl
WRR-699	phenyl	(3-pyridyl)-CH_2-	Me-N	–	4-pyridyl
WRR-700	phenyl	(4-pyridyl)-CH_2-	Me-N	–	2-pyridyl
WRR-701	phenyl	(4-pyridyl)-CH_2-	Me-N	–	2-pyrimidyl
WRR-703	phenyl	(4-pyridyl)-CH_2-	Me-N	–	4-pyrimidyl
WRR-704	phenyl	(3-pyridyl)-CH_2-	Me-N	–	2-pyridyl

Compound ID	R_1	R_2	X	Y	Ar
WRR-705	phenyl	PhenylCH_2-	(BocNHCH_2)C	–	phenyl
WRR-706	phenyl	(2-pyridyl)-CH_2-	Me-N	–	phenyl
WRR-707	phenyl	PhenylCH_2-	(AcNHCH_2)C	–	phenyl
WRR-708	phenyl	PhenylCH_2-	(H_2NCH_2)C	–	phenyl
WRR-709	phenyl	PhenylCH_2-	($MeSO_2$NHCH_2)C	–	phenyl
WRR-710	phenyl	(4-pyridyl)-CH_2-	(BocNHCH_2)C	–	phenyl
WRR-711	phenyl	(4-pyridyl)-CH_2-	(MeO_2C)C	–	phenyl
WRR-712	phenyl	(4-pyridyl)-CH_2-	(H_2NCH_2)C	–	phenyl
WRR-713	phenyl	(4-pyridyl)-CH_2-	(AcNHCH_2)C	–	phenyl
WRR-714	phenyl	(4-pyridyl)-CH_2-	($MeSO_2$NHCH_2)C	–	phenyl
WRR-716	phenyl	(3-pyridyl)-CH_2-	Me-N	–	phenyl
WRR-717	phenyl	(4-pyridyl)-CH_2-	Me-N	–	phenyl
WRR-718	4-fluorophenyl	PhenylCH_2-	Me-N	–	phenyl
WRR-719	4-fluorophenyl	PhenylCH_2-	Me-N	–	2-pyridyl

Table 2

IC_{50} values of vinyl sulfone inhibitors against *N. fowleri* cysteine protease present in the lysate

Inhibitor	IC_{50}±SE (μM)
WRR-636	0.5±0.05
WRR-673	0.2±0.1
WRR-679	0.07±0.08
WRR-680	0.2±0.04
WRR-682	11.9±0.1
WRR-683	0.3±0.05
WRR-689	0.7±0.05
WRR-692	0.09±0.05
WRR-693	0.6±0.08
WRR-694	11.9±0.1
WRR-695	0.009±0.1
WRR-696	1±0.07
WRR-697	2±0.06
WRR-698	0.5±0.04
WRR-699	5.9±0.05
WRR-700	3.6±0.07
WRR-701	0.7±0.2
WRR-703	6.6±0.1
WRR-704	1.1±0.05
WRR-705	0.003±0.06
WRR-706	0.9±0.08
WRR-707	1.0±0.06
WRR-708	0.4±0.07
WRR-709	0.01±0.05
WRR-710	0.2±0.06
WRR-711	0.2±0.08
WRR-712	0.2±0.04
WRR-713	0.03±0.2
WRR-714	0.1±0.07
WRR-716	0.03±0.04
WRR-717	0.2±0.03
WRR-718	0.07±0.04
WRR-719	0.19±0.05
K11777	0.006±0.04
E-64	0.002±0.04