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Novel *in vitro* approaches for screening anti-parasitic drugs against the brain-eating amoeba *Naegleria fowleri*

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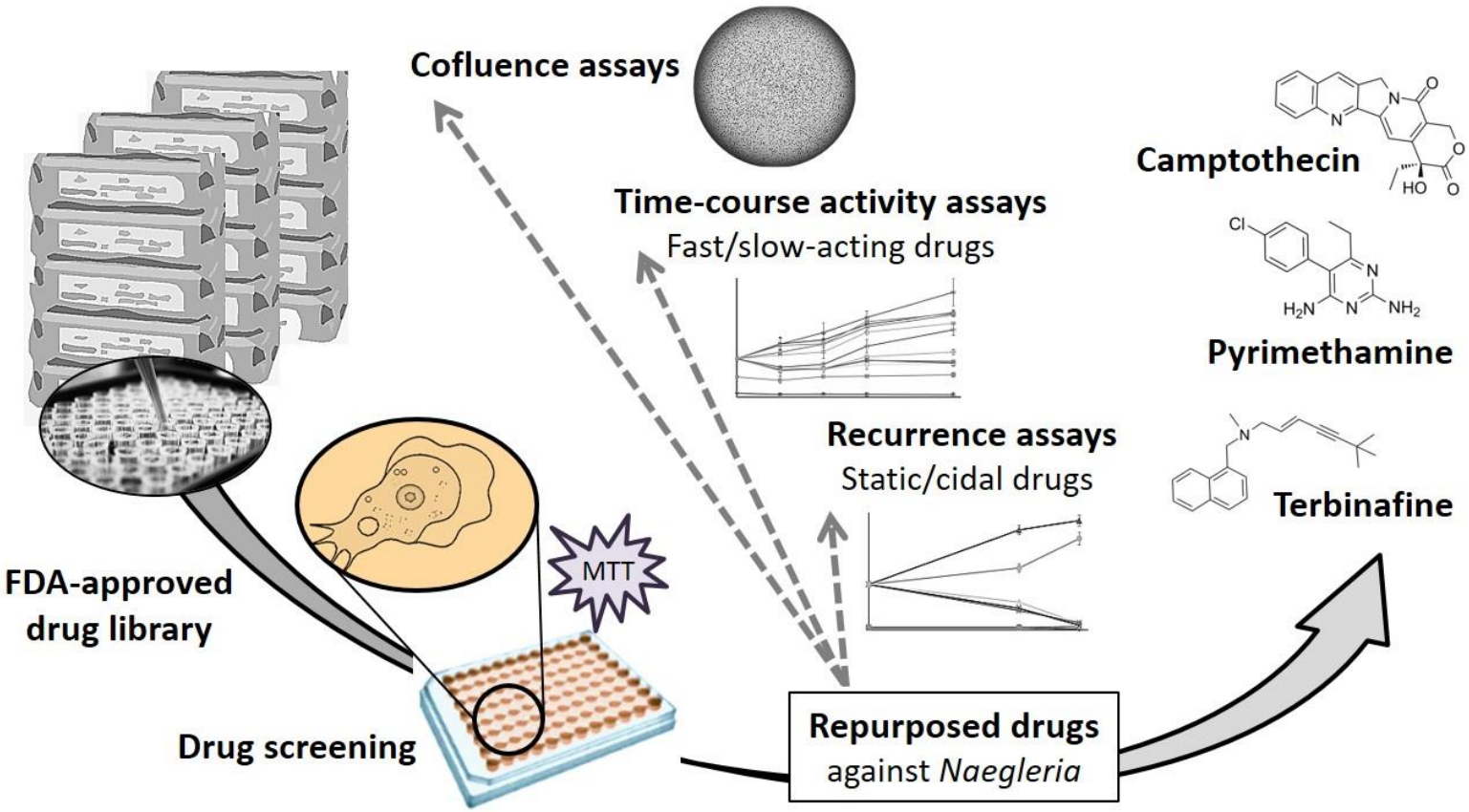
Novel *in vitro* approaches for screening anti-parasitic drugs against the brain-eating amoeba *Naegleria fowleri*



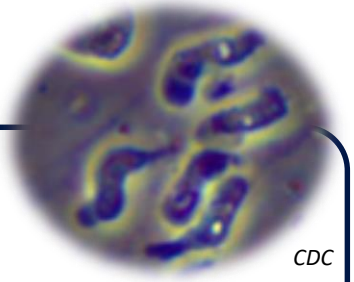
Abstract:

Naegleria fowleri is both a pathogenic and free-living microbial eukaryote, responsible for the development of primary amoebic meningoencephalitis (PAM) in humans. PAM is a rapid, severe and fatal underestimated infectious disease which has been reported worldwide. The major drawback with PAM is the lack of effective therapies. The current frontline treatment presents a low rate of recovery (5%) and severe adverse effects. For example, many drug candidates lack efficacy, because they do not effectively cross the blood-brain-barrier. Consequently, more effective drugs are urgently needed. Herein, we report a new in vitro method suitable for medium- and high-throughput drug discovery assays, using the closely related *Naegleria gruberi* as a model. We have subsequently used this method to screen a library of 1,175 Food and Drug Administration-approved drugs. As a result, we present three new drugs (Camptothecin, Pyrimethamine, and Terbinafine) that are anticipated to readily cross the blood-brain-barrier with activity against *Naegleria* species in therapeutically achievable concentrations. Successively, we integrated several in vitro assays that resulted in identifying fast-acting and high amoebicidal drugs. In conclusion, we present a new approach for the identification of anti-*Naegleria* drugs along with three novel drug candidates for further development for the treatment of PAM.

Keywords: Brain-eating amoeba; Drug screening; Drug repurposing; *Naegleria*; PAM

Introduction

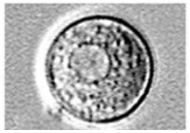
Naegleria fowleri & Brain-eating amoeba



CDC

- Eukaryotic microbe commonly found in warm freshwater and soil around the world.
- Responsible for the development of **primary amoebic meningoencephalitis (PAM)**.
  - Sudden, severe and fatal disease reported in both developed and developing countries worldwide.

- *N. fowleri* can thrive in a wide range of osmotic and oxygenic conditions, where it can exist as one of **three forms**:

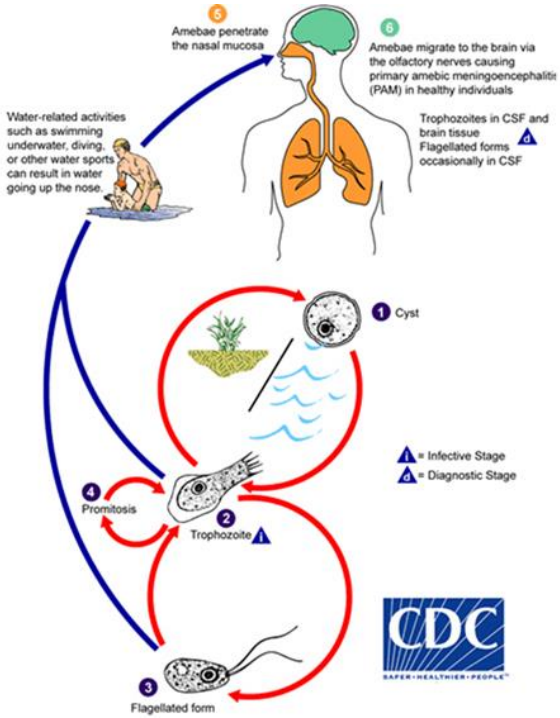


Encyst
(protective dormant stage)

Amoebic form
(active feeding trophozoite)

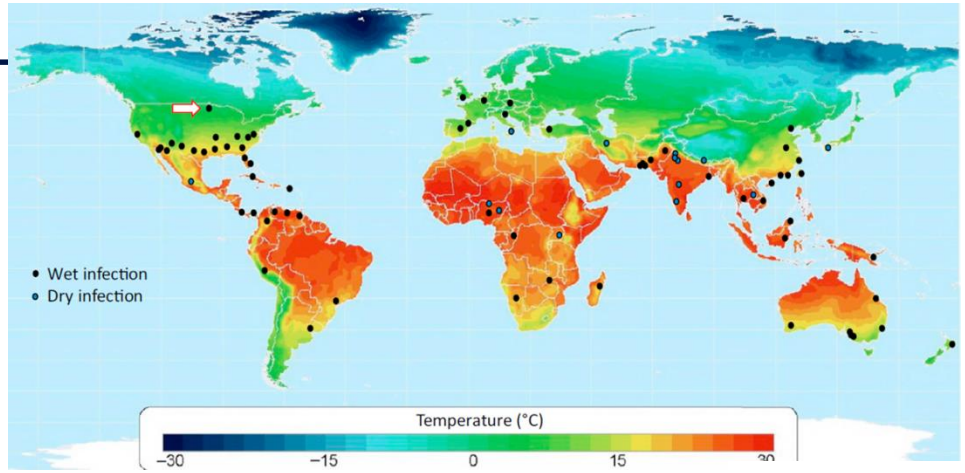
Flagellate
(motile swimming stage)

Once trophozoites reach the brain, they cause hemorrhagic meningoencephalitis.
97% of untreated cases leading to patient death within two weeks.



Introduction

Naegleria fowleri & Epidemiology



Maciver et al., 2019

- Reported in **developed and developing countries**, especially in areas that lack of control procedures against *N. fowleri.*
- **The number of infection reports is unclear**, and only a few epidemiological studies have been published:
  - Either 235 (*Jonckheere, 2011*), or 300 (*Trabelsi et al., 2012*) or even 440 (*Azlan et al., 2017*) cases worldwide.
  - However, the number of PAM reported cases is likely to be significantly **underestimated** due to common misdiagnosis as viral or bacterial meningitis.
  - Moreover, **PAM cases appear to have been increasing over recent years.**

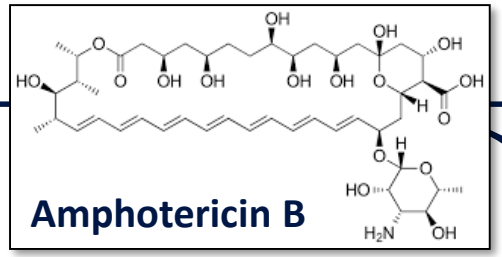
Despite being fatal and a potential increase in PAM cases, *Naegleria fowleri* receives little public health attention.

Introduction

Naegleria fowleri & Current treatments

PAM is currently a disease with no efficient treatment

- **key to the few cases of survival** is early diagnosis and a treatment regimen that includes intravenous amphotericin B.



However

- PAM is **not commonly confirmed during the early infection stages**, and most infected individuals decease.

In any case

- Amphotericin B presents:
  - **A low rate of recovery** (5%) after treatment.
  - Multiple and severe **adverse effects.**

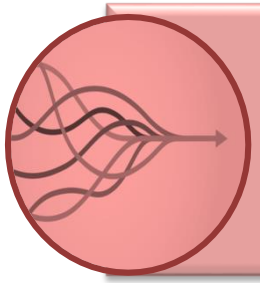
The efficacy of many other suggested drugs is limited by the blood-brain barrier (BBB) after intravenous administration

Therefore

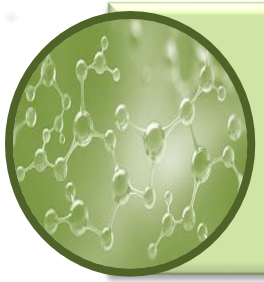
More effective drugs are urgently needed

Introduction

Objectives



Develop a **novel *in vitro* approach for screening anti-parasitic drugs** against *Naegleria* spp.



Identify **fast-acting and efficient drugs** to combat PAM.

For this purpose

- ✓ **A library of 1,175 Food and Drug Administration (FDA)-approved drugs and *N. gruberi* – a close non-pathogenic relative of *N. fowleri* – were used.**
- ✓ **Drug candidates were screened using a combination of new methodologies – MTT viability and confluence assays.**

Results and discussion

Screening strategy & Activity assays

First-round HTS

1175 drugs → 103 drugs

Single concentration → 10 µM

Selection criterion → Inhibition ≥ 80 %

Second-round HTS

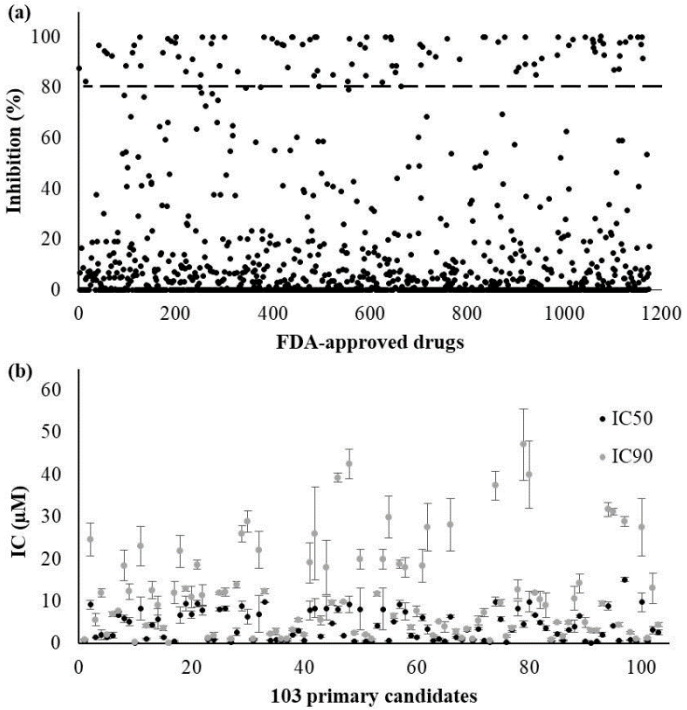
103 drugs → 9 drugs (8 drugs + Amphotericin B)

Concentrations from 40 to 0.02 µM

Selection criteria:
- IC50 and/or IC90 values lower than Amphotericin B
- Able to cross the BBB: Yes, n/a, and limited (as Amphotericin B)

6 x 10^4 *Naegleria gruberi* cells·mL^{-1} — 120 h of treatment

Endpoint: MTT activity, Confluence (JS)



High-Throughput Screening (HTS) strategy (rounds, doses, time of treatment, and selection criteria) using Food and Drug Administration (FDA)-approved drug library against *Naegleria gruberi*. BBB, blood-brain barrier.

Results and discussion

Screening strategy & Activity assays

22 drugs (IC50 values < 1 µM) selected as potential drugs for the treatment of PAM

- ✓ Examined for two aspects of major importance for **potential clinical efficacy**:
  - Therapeutic plasma concentration
  - Brain uptake ability

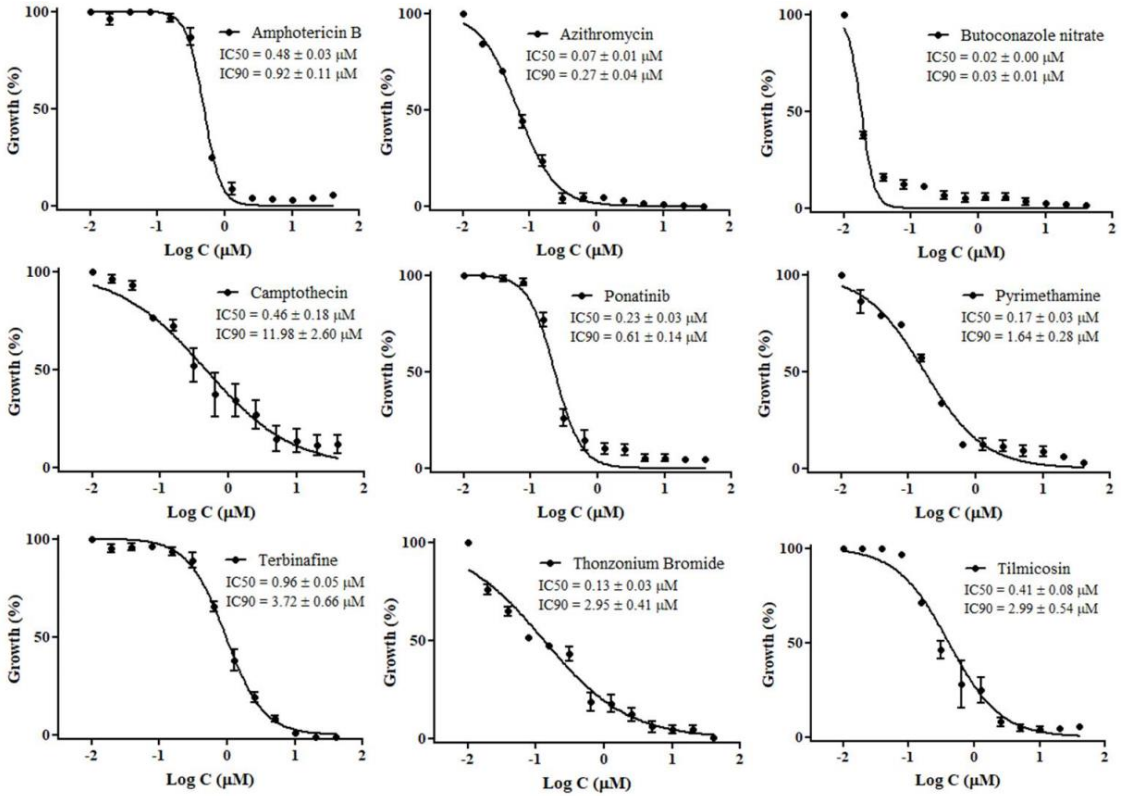
Camptothecin, Pyrimethamine, and Terbinafine are shown as potential drugs to treat PAM infections

Drug	Therapeutic plasma concentration / C_{max} (µg/mL)	IC_{50} (µg/mL)	IC_{90} (µg/mL)	Brain uptake
Amphotericin B (Abelcet)	0.23-0.36 / 2.90	0.44	0.85	Poor
Azithromycin (Zithromax)	0.21-0.54	0.05	0.20	Poor
Camptothecin	12.00-20.00	0.16	4.17	Yes
Clarithromycin (Biaxin. Klacid)	0.78-2.12	0.65	0.89	Poor
Clotrimazole (Canesten)	0.20-.035 / 1.29	0.26	0.38	Unknown. Predicted (+, 0.98)
Dirithromycin	0.10-0.60 / 1.70	0.58	0.93	Unknown. Predicted (-, 0.97)
Econazole nitrate (Spectazole)	1.00-13.00	0.30	0.84	Poor
Emetine	0.05-0.08	0.37	1.32	Unknown. Predicted (no data)
Entecavir hydrate	8.20×10^{-3}	0.20	0.31	Unknown. Predicted (+, 0.87)
Erythromycin (E-Mycin)	1.40	0.43	0.82	Poor
Ibandronate sodium	4.10×10^{-3}-0.13	0.17	0.84	Unknown. Predicted (-, 0.52)
Itraconazole (Sporanox)	0.30-1.13	0.34	0.70	Poor
Miconazole (Monistat)	0.04-1.00	0.25	0.77	Poor
Niclosamide (Niclocide)	0.25-6.00	0.29	0.42	Unknown. Predicted (+, 0.73)
Pemetrexed	72.2	0.26	0.42	Poor
Pimozide	0.01-0.02	0.30	3.37	Yes
Ponatinib (AP24534)	0.02-0.07	0.12	0.32	Unknown. Predicted (+, 0.94)
Pyrimethamine	1.00-3.00	0.04	0.41	Yes
Terbinafine (Lamisil. Terbinex)	1.00	0.28	1.08	Yes
Thioridazine HCl	0.1-2.0	0.20	4.05	Yes
Trifluprom azine HCl	Erratic absorption	0.22	1.73	Yes
Voriconazole	4.40	0.30	6.57	Yes

Results and discussion

MTT validation

Dose-response curves using MTT



8 drugs + the reference drug amphotericin B were selected to develop the novel screening approach because their higher activities

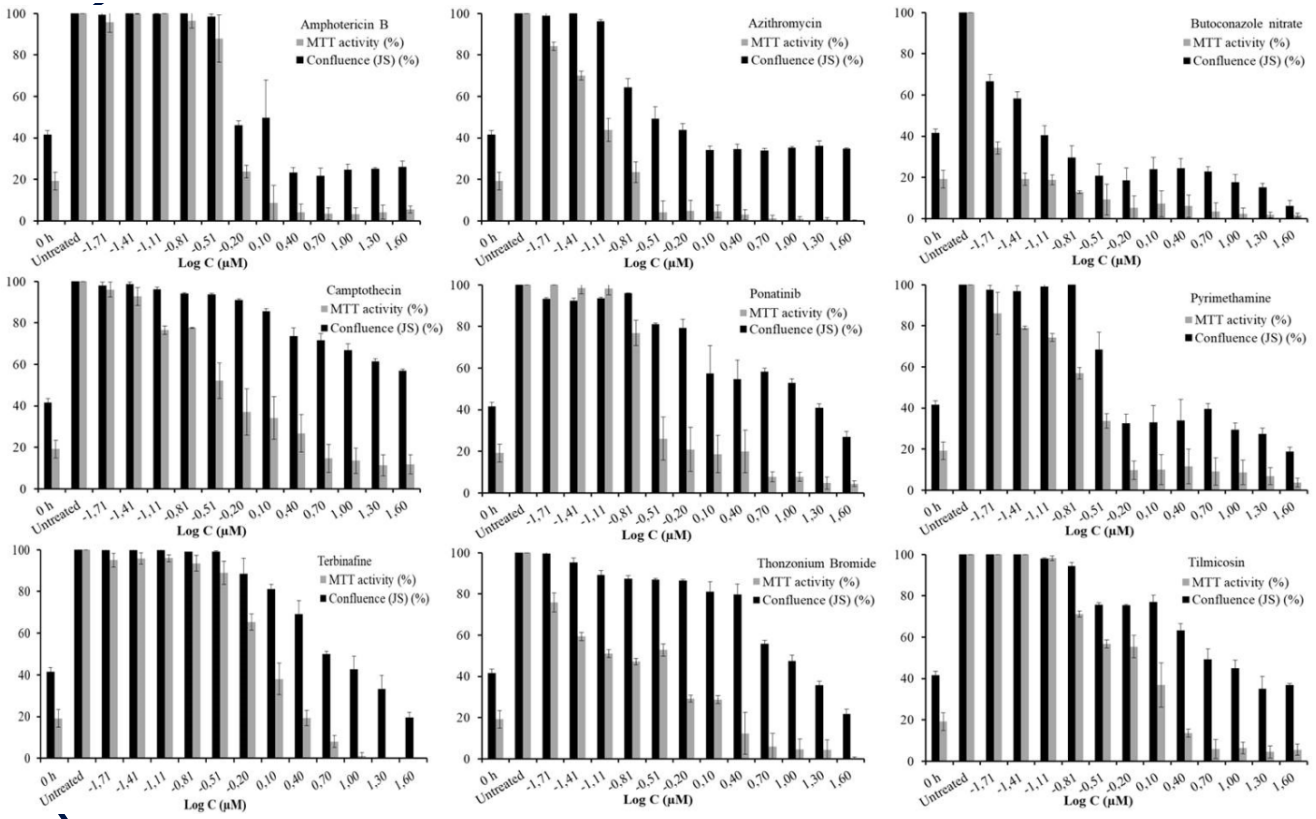
- ✓ Azithromycin
- ✓ Butoconazole nitrate
- ✓ Camptothecin
- ✓ Ponatinib
- ✓ Pyrimethamine
- ✓ Terbinafine
- ✓ Thonzonium bromide
- ✓ Tilmicosin

Results obtained using these new methods for drug discovery against PAM have been compared to those from the literature.

Results and discussion

MTT & Confluency (Juli Stage system)

MTT vs Confluency

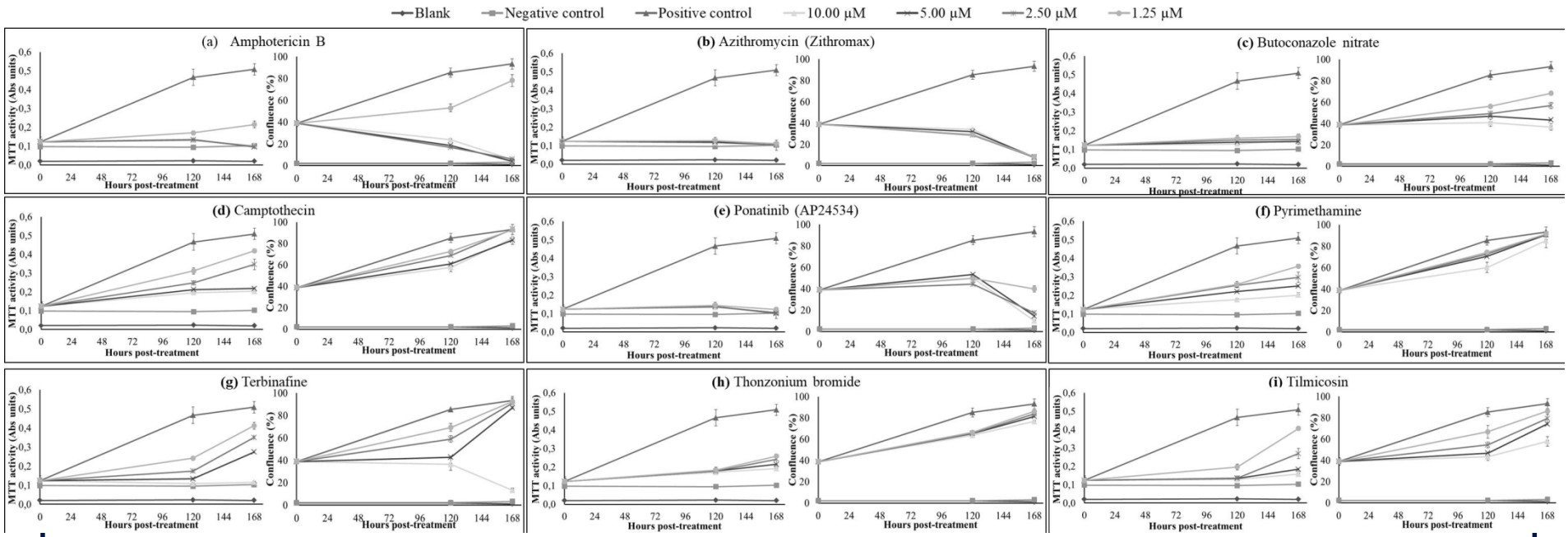


Differences observed between the MTT viability and the confluency can be linked to the different mechanisms of action (MoA) of each drug candidate.

These differences explain the inherent lack of potency of most of the current drugs identified with traditional methods and used to treat PAM infections.

Results and discussion

Recurrence assays: static/cidal drugs



Dose–response assessment for each drug against *Naegleria gruberi* after 120-h treatment and 48-h incubation without drugs by MTT assay and cell analysis software (Juli Stage system). Values constitute means of three separate determinations ± standard deviation.

Results and discussion

Recurrence assays: static/cidal drugs

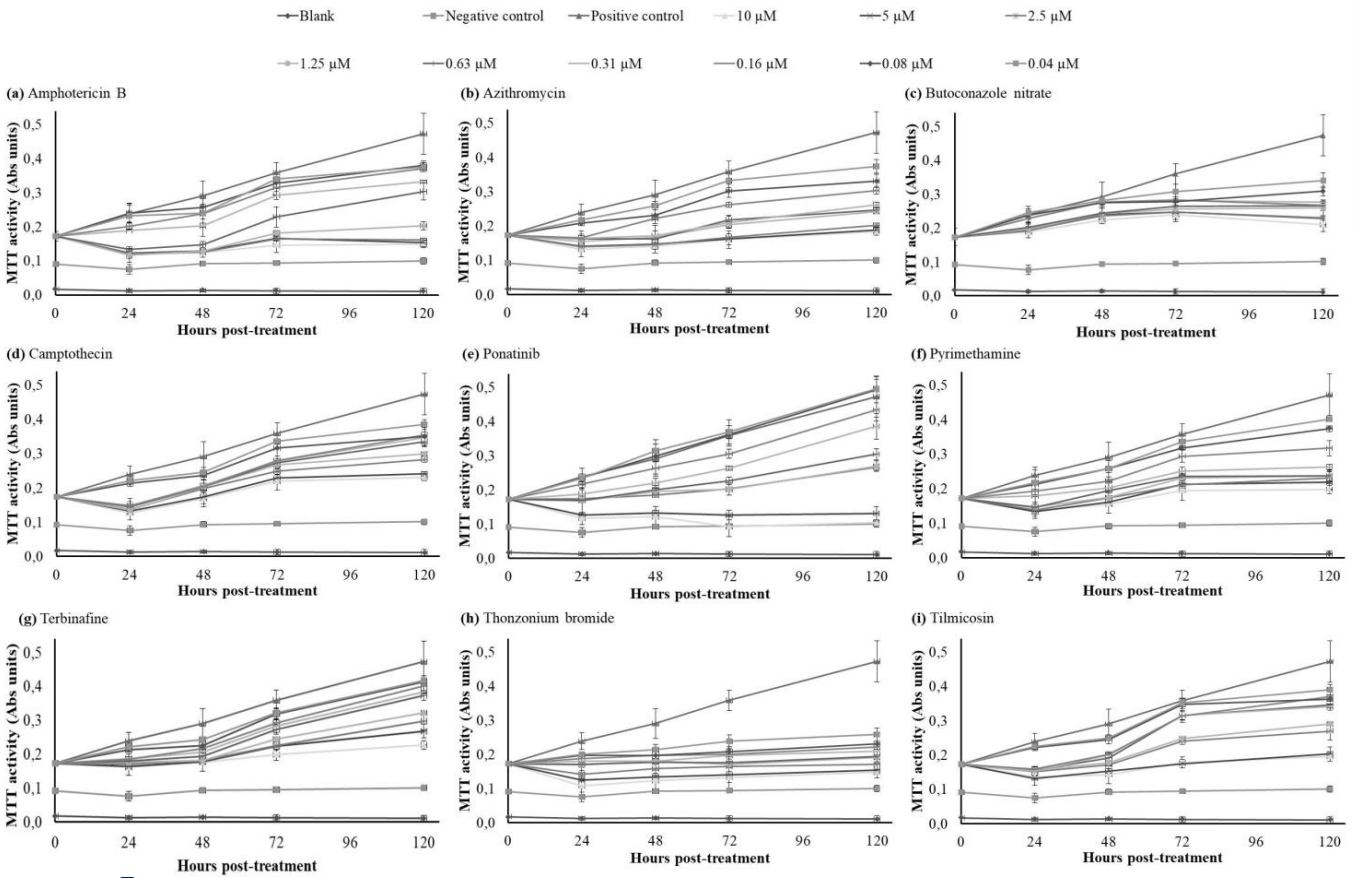
Drug	MIC (µM)
Amphotericin B (Abelcet)	> 1.25, < 2.5
Azithromycin (Zithromax)	< 1.25
Butoconazole nitrate	< 1.25
Camptothecin	> 10
Ponatinib (AP24534)	< 1.25
Pyrimethamine	> 10
Terbinafine (Lamisil, Terbinex)	> 5, < 10
Thonzonium bromide	> 10
Tilmicosin	> 10

MIC, minimum inhibitory concentration.

Minimum inhibitory concentration (MIC) values were determined to identify the lowest concentrations that effectively kill *N. gruberi* trophozoites

Results and discussion

Time-course assays: fast/slow-acting drugs



These drugs produced a decrease in cell viability in a time-dependent manner; even showing activity within the first 24-h treatment

Given the rapid and fatal development of PAM, it is required to focus drug discovery efforts on amoebicidal agents with fast-acting activity and to prioritize them over other drugs for lead optimization.

Results and discussion

Most previous *in vitro* assays for the assessment of amoebicidal drug screening are not suitable to medium- or high-throughput screening.

Endpoints for growth often include:

- Morphology and visual counting of amoebae.
- Viability assessment that requires weeks.
- Large volumes of culture media.
- Release of lactate dehydrogenase.

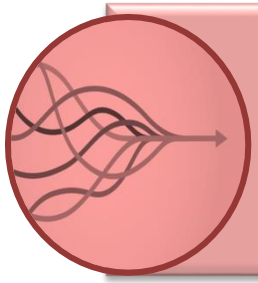
which are simply too time-consuming to support modern drug discovery requisites.

Herein, we developed new methods for HTS in 96-well microtiter plates and used them for the first time with *Naegleria*.

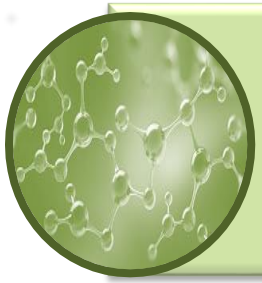
Multiple advantages:

- Reproducible
- Qantitative endpoints
- Enabling the detection of drugs with a more rapid onset of action

Conclusions



A **new method for quantitative dose-response** has been established to identify new drugs for PAM.



Three drugs have been identified as new potential candidates for the treatment of PAM, with higher amoebicidal activity, improved ADMET profile and bioavailability than the reference drug amphotericin B.

Acknowledgments

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