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

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ABSTRACT

*Naegleria fowleri* is both a pathogenic and a 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 in countries with warmer climates. The major drawbacks with PAM are the lack of effective therapies and delay in diagnosis. The current frontline treatment presents a low rate of recovery (5%) and severe adverse effects. For example, many drug candidates lack efficacy, since 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 1175 Food and Drug Administration-approved drugs. As a result, we present three drugs (camptothecin, pyrimethamine, and terbinafine) that can be repurposed, and 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 potential drug candidates for further development for the treatment of PAM.

## 1. Introduction

*Naegleria fowleri*, the so-called "brain-eating amoeba", is a free-living microbial eukaryote and the only pathogenic species of the group (Schoch et al., 2019). *N. fowleri* is responsible for the development of primary amoebic meningoencephalitis (PAM), which is a sudden, severe and fatal disease reported in both developed and developing countries with warmer climates (Fowler and Carter, 1965).

Typically found in soils and freshwater worldwide, *N. fowleri* can thrive in a wide range of osmotic and oxygenic conditions (De Jonckheere, 1979; Jonckheere, 2014; Tyml et al., 2016), where it can exist as one of three forms: it appears primarily as an amoebic form (active feeding trophozoite), but upon environmental stimuli can transform into a flagellate (motile swimming stage) or encyst (protective dormant stage) (De Jonckheere et al., 2001). Trophozoites are thought to be the only infective stages. Infection occurs when they pass through the nasal cavity and penetrate the olfactory neuroepithelium, where they migrate through the olfactory nerves across the cribriform plate until they reach the frontal cerebral cortex (Jarillo-luna et al., 2004; Visvesvara, 2014). Once trophozoites reach the brain, they can proliferate and subsequently cause hemorrhagic meningoencephalitis with the classical PAM symptoms: 97% of the untreated cases lead to patient death within two weeks (Cervantes-sandoval et al., 2008; Jarolim et al., 2000; Visvesvara et al., 2007).

PAM incidents have been frequently reported in healthy children and young adults who have participated in swimming activities in contaminated water. Such incidents are reported in both developed and developing countries, especially in areas that lack of control procedures against *N. fowleri* (Marciano-cabral, 1988; Siddiqui and Khan, 2014). The number of infection reports is unclear, and only a few epidemiological studies have been published, reporting inconsistent case numbers, which could also be rising: either 235 (Jonckheere, 2011), or

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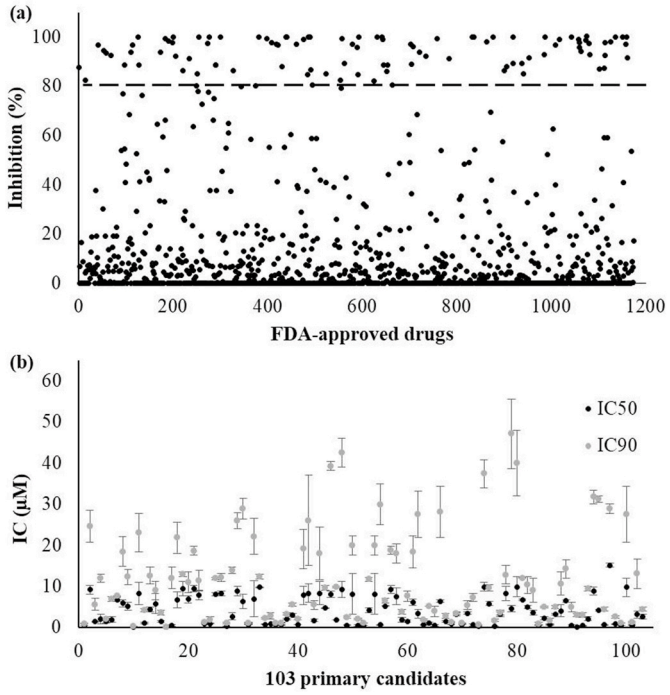


**Fig. 1.** High-Throughput Screening (HTS) of the Food and Drug Administration (FDA)-approved drugs against *Naegleria gruberi*. (a) First-round HTS. Inhibition values (%) of the 1175 FDA-approved drugs at 10 μM. The dashed line shows 80% inhibition. (b) Second-round HTS. Inhibitory concentrations (IC) of the 103 primary candidates. Points constitute means of three separate determinations ± standard deviation.

300 (Trabelsi et al., 2012) or even 440 (Abdul Majid et al., 2017; Coupat-goutaland et al., 2016) cases worldwide. The scarcity of cases seems to indicate a very rate type of infection. However, the number of PAM reported cases is likely to be significantly underestimated due to common misdiagnosis as viral or bacterial meningitis (Heggie, 2010). Moreover, PAM cases appear to have been increasing over recent years (Cope et al., 2015, 2016; Linam et al., 2015; Stowe et al., 2017). Despite being fatal and a potential increase in PAM cases, *N. fowleri* receives little public health attention (Bellini et al., 2018).

PAM caused by *Naegleria fowleri* is currently a disease with no efficient treatment, and key to the few cases of survival is early diagnosis and a treatment regimen that includes intravenous amphotericin B alone or in combination with other drugs (Kim et al., 2008a). However, PAM is not commonly confirmed during the early infection stages, and most infected individuals die. In any case, amphotericin B presents a low rate of recovery (5%) after treatment, in addition to multiple and severe adverse effects, mainly nephrotoxicity (Martínez-Castillo et al., 2016; Schuster and Visvesvara, 2004; Stevens et al., 1981). The efficacy of many other suggested drugs is limited by the blood-brain barrier (BBB) after intravenous administration. Therefore, high drug doses are needed to achieve the minimum inhibitory concentration (MIC) at the target site, which is often prevented by dose-limiting toxic side effects. Consequently, more effective drugs are urgently needed.

Remarkably, most previous *in vitro* assays for the assessment of amoebicidal drug screening are not suitable to medium- or high-throughput screening (MTS or HTS respectively). Endpoints for growth often include morphology and visual counting of amoebae, viability assessment that requires weeks, large volumes of culture media, or release of lactate dehydrogenase (Iturbe and Herna, 2006; Kim et al., 2008a, 2008b), which are simply too time-consuming to support modern drug discovery requisites. Herein, we developed new methods for either MTS or HTS in 96-well microtiter plates and used them for the first time with *Naegleria*. These methods have multiple advantages, including reproducible, quantitative endpoints, and enabling the detection of drugs with a more rapid onset of action, like alamarBlue (Rice et al., 2015). 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.

Currently, a widely used process called drug repositioning is performed as an effective strategy to accelerate drug discovery (Nielsch, 2016; Wohlleben et al., 2016; Xue et al., 2018). Drug repositioning has several strategic advantages compared to classical drug discovery including faster, safer and cheaper preclinical and clinical validation steps (Xue et al., 2018). Therefore, the focus of this work was to develop a different *in vitro* approach for screening anti-parasitic drugs against *Naegleria* spp. For this purpose, we commissioned a library of 1175 Food and Drug Administration (FDA)-approved drugs and *N. gruberi* – a close non-pathogenic relative of *N. fowleri* (Schoch et al., 2019). While drugs affective against *N. gruberi* are not necessarily successful against *N. fowleri*, the free-living relative has been proven to be a good model for drug screening expeditions (Debnath et al., 2012, 2018). Drug candidates were screened using a combination of new methodologies – MTT viability and confluence assays – for their effects against *Naegleria*. As a result, three FDA-approved drugs were identified, that have not previously been reported to have activity against *Naegleria,* showing higher amoebicidal activity, improved ADMET profile and bioavailability than the reference drug amphotericin B.

## 2. Materials and methods

### 2.1. FDA-approved drug library

FDA-approved drug library was purchased from Stratech. A total of 1175 drugs were used to perform a throughput screening (Supplementary Material Fig. S1). Drugs were dissolved at 10 mM stock solutions in Dimethyl sulfoxide (DMSO) in 96 well-plate format and were kept at −80 °C.

### 2.2. N. gruberi cell culturing

*N. gruberi* trophozoites (NEG-M strain) were axenically cultured at 27 °C in M7 Medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Thermofisher) (Fulton, 1974; Fulton et al., 1984) and passaged every three to five days depending on their density (Fulton, 1974). Trophozoites were harvested during the logarithmic growth phase to perform the experiments.

### 2.3. Screening using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent: endpoint assay

1175 FDA-approved drugs were tested against *N. gruberi* in 96-well microtiter plates after seeding trophozoites at $6 \times 10^4$ mL$^{-1}$ in M7 Medium supplemented with 10% (v/v) FBS and incubated them overnight at 27 °C. The screening strategy was carried out in two different rounds (Supplementary Material Scheme 1).

The first-round of screening was performed by adding the 1175 drugs at a single concentration of 10 μM in 100 μL·well$^{-1}$ volumes at 27 °C for 120 h (endpoint). Blanks, negative and positive (untreated growth trophozoites) controls were also included. Thereafter, 25 μL of MTT reagent (2 g L$^{-1}$) (Universal biologicals; 20395.02) was added into each well to be incubated for further 4 h. Finally, 25 μL of 20% w/v sodium dodecyl sulphate (SDS) was added, and after overnight incubation, the cell viability (%) was assessed by absorbance measurements (in a BMG SpectroStar plate reader) at 570/690 nm. Each drug candidate was tested in triplicate in three separate determinations.

The second-round of screening was performed by adding the primary candidates identified using the 80% inhibition endpoint criterion. 103 FDA-approved drugs were rescreened from 40 to 0.02 μM via 12 serial

**Table 1**
Therapeutic plasma concentration, IC values and brain uptake ability of potential compounds ($IC_{50} < 1$ μM, excluding topical and/or veterinary use) against *Naegleria* species.

| 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 | $8.20\times10^{-3}$ | 0.20 | 0.31 | Unknown. Predicted (+, 0.87) |
| Erythromycin (E-Mycin) | 1.40 | 0.43 | 0.82 | Poor |
| Ibandronate sodium | $4.10\times10^{-3}$-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 |

Green/yellow/red code with regard to the $IC_{50}$/$IC_{90}$ concentrations in the context of the plasma concentrations. Green/yellow/red code for the brain uptake (yes/unknown/poor).

dilutions in 100 μL·well$^{-1}$ volumes at 27 °C. After 120-h incubation (endpoint), the same procedure as described to perform the first-round screening was followed. The viability was determined using GraphPad Prism 5 software and expressed as the $IC_{50}$ and the $IC_{90}$, i.e., the concentrations required to result in 50% and 90% inhibition, respectively. Each drug concentration was tested in triplicates in three separate determinations.

### 2.4. Images taken using the JuLi™ stage system

Nine candidate drugs, the reference drug amphotericin B among them, were the selected FDA-approved drugs after the second-round screening (Supplementary Material Scheme 1) to develop this screening approach. *N. gruberi* trophozoites were seeded in 96-well microtiter plates at $6 \times 10^4$ mL$^{-1}$ in M7 Medium supplemented with 10% (v/v) (FBS) at 27 °C. After overnight incubation, the nine drugs were tested at a single concentration of 10 μM in 100 μL·well$^{-1}$ volumes. Finally, the plates were recorded by taking frames every minute during 24 h (1440 frames) using the real-time cell history recorder software (JS system).

### 2.5. Confluence assay using the JuLi™ stage system: endpoint assay

The nine candidate drugs were counter-screened from 40 to 0.02 μM

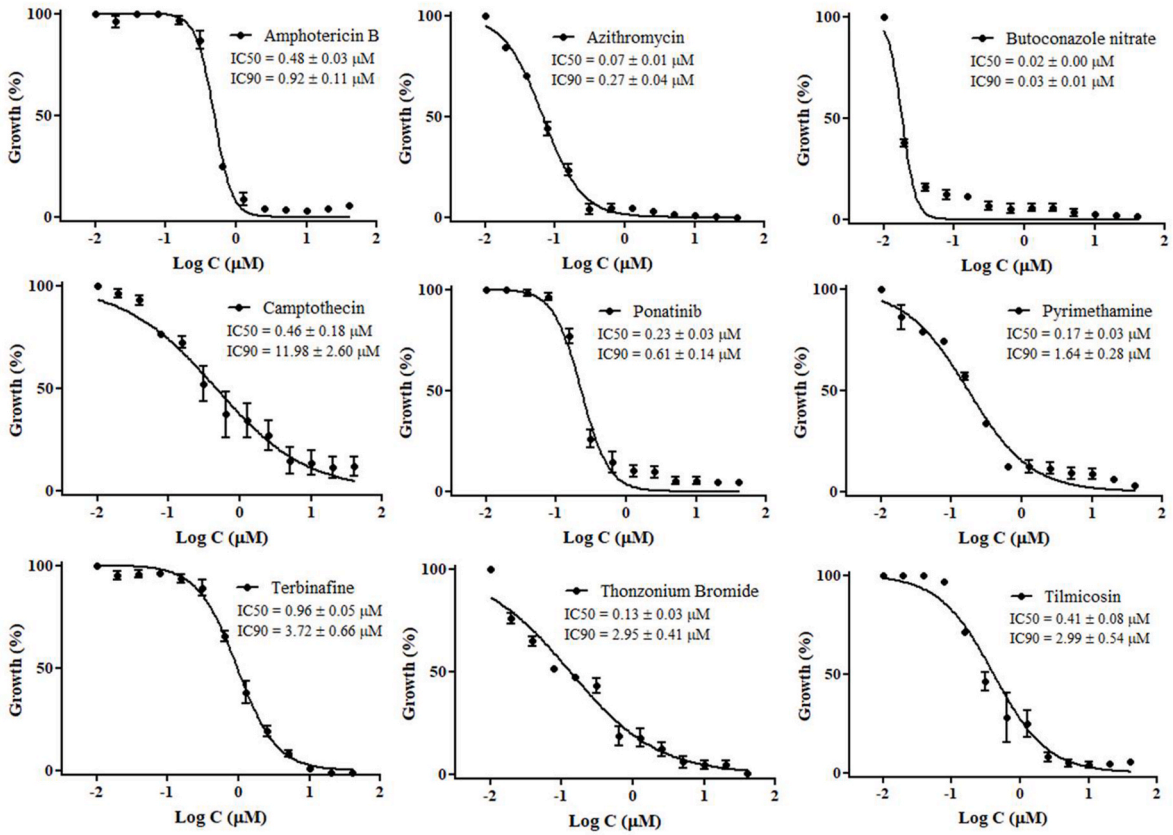


**Fig. 2.** Dose-response curves to determine the inhibitory concentrations (IC) 50 and 90 for each drug against *Naegleria gruberi* using GraphPad Prism 5 software. Values constitute means of three separate determinations ± standard deviation.

via 12 serial dilutions in 96-well microtiter plates (100 μL·well$^{-1}$ volumes) after seeding trophozoites at $6 \times 10^4$ mL$^{-1}$ and incubating them overnight at 27 °C. The same procedure as described to perform the second-round screening was followed until 120-h incubation (endpoint). Finally, the confluence (%) was measured using the cell analysis software (JuLi™ Stage system) (Supplementary Material Scheme 1). Each drug concentration was tested in triplicate in three separate determinations.

### 2.6. Recurrence assay: static/cidal drugs

The nine selected drugs were examined from 10 to 1.25 μM via four serial dilutions in 96-microtiter plates (100 μL·well$^{-1}$ volumes) after seeding trophozoites at $6 \times 10^4$ mL$^{-1}$ and incubating them overnight at 27 °C. After 120-h incubation, plates were carefully washed three times with 200 μL·well$^{-1}$ volumes pre-warmed phosphate buffered saline (PBS), followed by 48 h without drug treatments prior to endpoint. Finally, the cell viability (%) by absorbance measurements and the confluence (%) using the cell analysis software (JuLi™ Stage system) were determined as described above (Supplementary Material Scheme 2). Each drug concentration was tested in triplicate in three separate determinations.

### 2.7. Time-course activity assay: fast/slow-acting drugs

The nine candidate drugs were tested from 10 to 0.04 μM via nine serial dilutions in 96-microtiter plates (100 μL·well$^{-1}$ volumes) after seeding trophozoites at $5 \times 10^5$ mL$^{-1}$ and incubating them overnight at 27 °C. Following this incubation, the cell viability (Absorbance units) was tested using MTT reagent at each timepoint (0 h, 24 h, 48 h, 72 h, and 120 h) (Supplementary Material Scheme 3), as described above. Each drug concentration was tested in triplicates in three separate determinations.

## 3. Results and discussion

### 3.1. Drug screening

Drug discovery is a time-consuming, high-risk, and high-investment process in traditional drug development used to identify potential new medicines for different disciplines, including biology, chemistry and pharmacology (Nielsch, 2016; Xue et al., 2018). In 1969, Rodney F. Carter employed this process (microscopic examination and visual counting every 24 h) against *Naegleria* and found that only amphotericin B showed amoebicidal activity (Carter, 1969). This outcome was then further corroborated later on by Schuster & Rechthand (Schuster and Rechthand, 1975). Since then, amphotericin B is the reference drug against PAM, even though it does not target anything specific (e.g. pathway) against these parasites; the drug is typically involved in cell membrane lysis through interactions with sterols in the membrane in both parasites and fungi (Brajtburg and Bolard, 1996; Martínez-Castillo et al., 2016). For this reason, the profile of the reference drug amphotericin B presents multiple side effects, in addition to a low rate of recovery (5%) after treatment (Grace et al., 2015; Martínez-Castillo et al., 2016; Schuster and Visvesvara, 2004). In addition to slow-infusion parenteral administration and high nephrotoxicity, amphotericin B side effects include shaking chills, fever, headache, nausea, anorexia, and dyspnea (Fosu-Mensah et al., 2016; McCurdy et al., 1968). Lastly, many of the issues with this drug (e.g Amphotericin B is also available as a liposomal formulation called AmBisome) can be linked to its low solubility, which affects dissolution, absorption, bioavailability, and clearance (Grace et al., 2015). Therefore, fast-acting and efficient drugs are urgently needed for the treatment of PAM.

Herein, we aimed to establish a detection platform to identify,

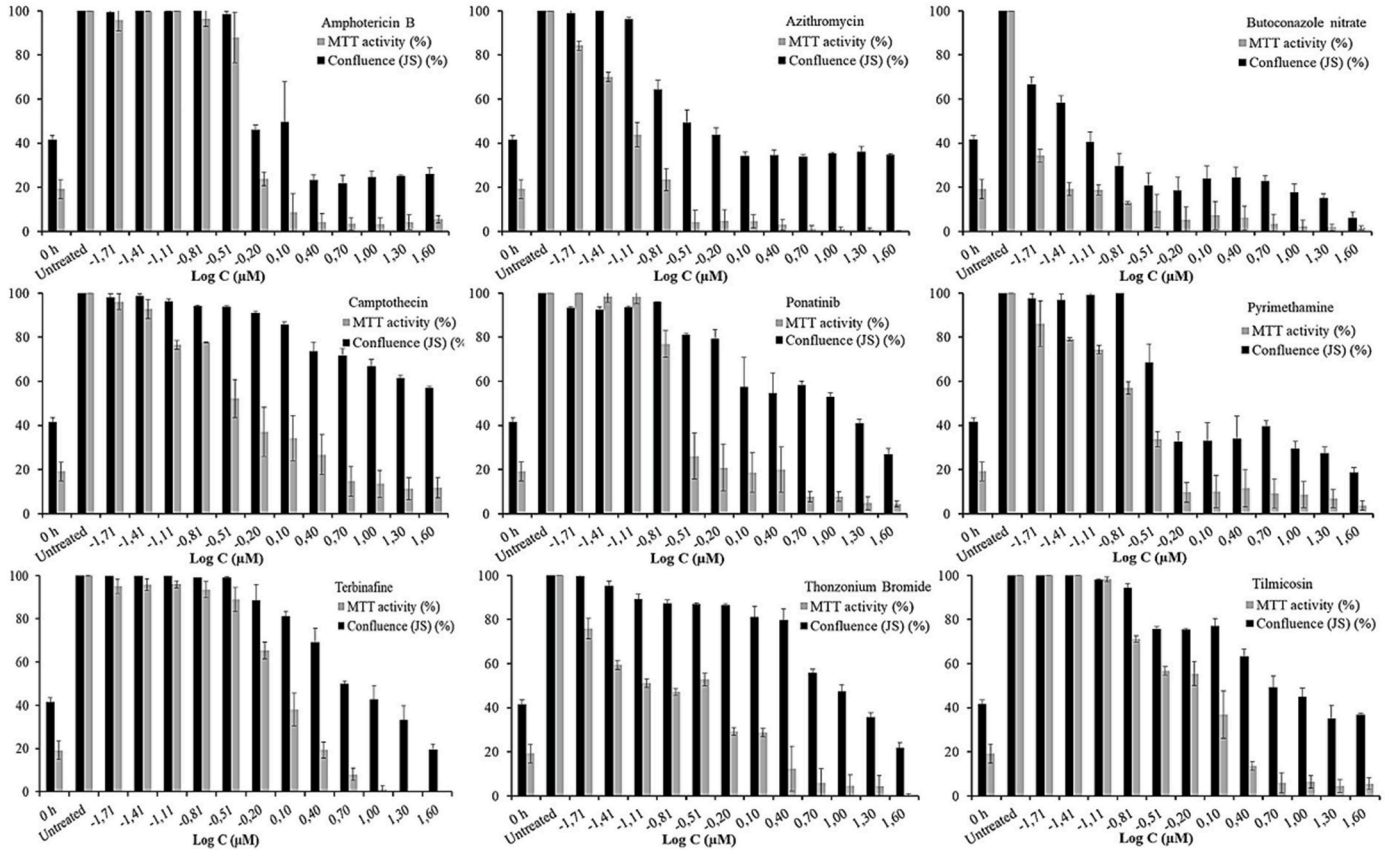


**Fig. 3.** Dose–response assessment for each drug against *Naegleria gruberi* by MTT assay (viability in percentage) and cell analysis software (confluence in percentage). Values constitute means of three separate determinations ± standard deviation.

evaluate and optimize new clinical therapy candidates for the treatment of PAM. We thus utilized the non-pathogenic *N. gruberi* as a model and we subsequently established a formazan-based MTT assay by also determining the organism's cell viability versus a previous well-established assay (Supplementary Material Fig. S2). Successively, a library of 1175 FDA-approved drugs was screened against *N. gruberi* using the same MTT assay in two series. Initially, all compounds were identified that caused a ≥80% inhibition of *N. gruberi* at a concentration of 10 μM after 120-h incubation. It's worth mentioning that the cell density was adjusted for an incubation time of 120-h days, for two main reasons: (1) this was the time required for the cultures to reach and maintain confluence in the control; (2) to investigate whether there was any recovery of the cultures post treatment (e.g. potential resistance to the drugs). *Naegleria*'s ability to transform to a cyst (which is a metabolically inactive stage), could have resulted in some false-positive/negative results if shorter incubations. Then, drugs were classified based on having lower $IC_{50}$ and/or $IC_{90}$ values than amphotericin B, and an anticipated higher ability to cross the BBB than amphotericin B (Supplementary Material Scheme 1). The ability of crossing the BBB was taken as a selection criterion, since drug delivery is a key issue for the treatment of the amoebic infections. This approach aimed to find molecules able to successfully reach the brain parenchyma and with high amoebicidal activity to improve the therapeutic arsenal against PAM (Bellini et al., 2018; Schuster et al., 2006). The inhibition values (%) of the 1175 drugs in the first-round screening are shown in Fig. 1a and in Supplementary Material Table S1. Subsequently, 103 primary candidates (Supplementary Material Fig. S3a) were selected. Thenceforth, the activity of the 103 primary candidates, expressed as the IC values ($IC_{50}$ and $IC_{90}$), are shown in Fig. 1b and in Supplementary Material Table S2.

As a result, 22 drugs (Table 1) with $IC_{50}$ values lower than 1 μM were selected as potential drugs for the treatment of PAM. These drugs were examined for two aspects of foremost importance for potential clinical efficacy: therapeutic plasma concentration and brain uptake capability. We thus narrowed our selection to eight drugs [azithromycin, butoconazole nitrate, camptothecin, ponatinib (AP24534), pyrimethamine, terbinafine, thonzonium bromide, and tilmicosin] to be further studied.

These eight drug candidates, together with the reference drug amphotericin B (Supplementary Material Fig. S3b), were selected to develop the screening approach because their higher *in vitro* activities. The dose-response curves of the eight drugs and amphotericin B are shown in Fig. 2. The reference drug amphotericin B showed $IC_{50}$ and $IC_{90}$ lower than 1 μM after five days of treatment. From the eight drugs, it is noteworthy that the activity of azithromycin, butoconazole nitrate and ponatinib showed $IC_{50}$ and $IC_{90}$ lower than amphotericin B. Azithromycin has already been used in clinical trials for PAM (Cope et al., 2016; Linam et al., 2015), but it is considered a drug with low ability to cross the BBB ("Drugbank," 2021a), similarly to amphotericin B ("Drugbank," 2021b). To further investigate the potential of these candidate drugs, *N. gruberi* trophozoites were monitored for 24 h at a high concentration (10 μM) of the eight drugs and amphotericin B. The video stills were performed by taking frames every minute for the first 24 h in order to follow the effect of the drugs on *Naegleria*'s trophozoites (Supplementary material Table S3). Three candidate drugs (camptothecin, pyrimethamine, and terbinafine) are shown as potential drugs, upon further optimization, to treat PAM infections.

It should be noted that the results obtained using these new methods for drug discovery against PAM have been compared to those from the previous reports (Supplementary Material Table S4). As such, we obtained similar outcomes, while there were some minor disparities that could be due to several factors, such as the *Naegleria* strain and species (*N. gruberi* vs *N. fowleri*) used, the initial number of cells and the time of exposure to the drugs. It is notwithstanding that we have now validated

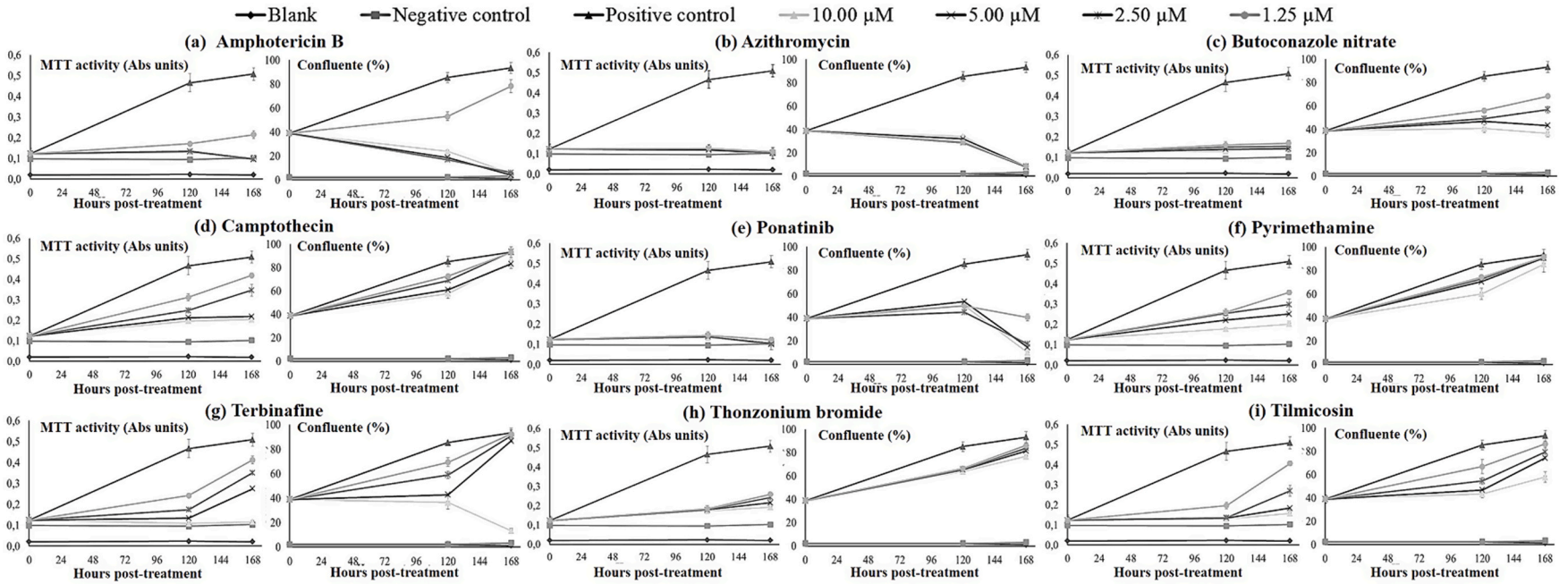


**Fig. 4.** Dose–response assessment for each drug against *Naegleria gruberi* after 120-h treatment and 48-h incubation without drugs by MTT assay (viability in percentage) and cell analysis software (confluence in percentage). Values constitute means of three separate determinations ± standard deviation.

**Table 2**
Activity for drugs used to develop the novel screening approach on *Naegleria gruberi*.

| Drug | $IC_{50}$ (μM) | $IC_{90}$ (μM) | MIC (μM) |
|---|---|---|---|
| Amphotericin B (Abelcet) | 0.48 ± 0.03 | 0.92 ± 0.11 | >1.25, <2.5 |
| Azithromycin (Zithromax) | 0.07 ± 0.01 | 0.27 ± 0.04 | <1.25 |
| Butoconazole nitrate | 0.02 ± 0.00 | 0.03 ± 0.01 | <1.25 |
| Camptothecin | 0.46 ± 0.18 | 11.98 ± 2.60 | >10 |
| Ponatinib (AP24534) | 0.23 ± 0.03 | 0.61 ± 0.14 | <1.25 |
| Pyrimethamine | 0.17 ± 0.03 | 1.64 ± 0.28 | >10 |
| Terbinafine (Lamisil, Terbinex) | 0.96 ± 0.05 | 4.59 ± 0.62 | >5, <10 |
| Thonzonium bromide | 0.13 ± 0.03 | 2.95 ± 0.41 | >10 |
| Tilmicosin | 0.41 ± 0.08 | 2.99 ± 0.54 | >10 |

IC, inhibitory concentration; MIC, minimum inhibitory concentration.

and reproduced these new *in vitro* assays that could be further employed for quantitative dose-response and medium- or high-throughput drug discovery assays against the brain eating amoeba.

### 3.2. MTT viability assay versus confluency (using the JuLi™ stage system)

The effects of amphotericin B and the eight identified drug candidates on *N. gruberi* cultures were additionally determined in a confluence assay to confirm the MTT viability assay results (Fig. 3). As shown in Supplementary Material Fig. S4, combining both assays showed that azithromycin and butoconazole nitrate exhibited higher activity against *N. gruberi* than amphotericin B.

The MTT viability assay is a colorimetric assay used to assess the cell metabolic activity. This assay is based on the reduction of the tetrazolium dye MTT to its insoluble formazan by NAD(P)H-dependent cellular oxidoreductase enzymes of viable cells. Hence, it is a quantitative assay that reflects the number of viable cells present under defined conditions.

It is noteworthy that the differences observed between the MTT viability and the confluency can be linked to the different mechanisms of action (MoA) of each drug candidate. For instance, amphotericin B and butoconazole nitrate show the slightest differences between MTT viability assay versus confluency (Fig. 3), since they both have similar mechanisms of action. It has been reported that amphotericin B induces morphological changes and pore formation by binding to ergosterol in membranes, altering the membrane permeability and producing apoptosis-like programmed cell death (PCD) (Cardenas-Zúñiga et al., 2017; Pugh and Levy, 2016; Schuster and Rechthand, 1975). In contrast, butoconazole nitrate inhibits the steroid synthesis by inhibiting the cytochrome P450 14α-demethylase, altering the cell membrane permeability and producing osmotic disruption ("Drugbank," 2021c; Jeffreys et al., 2019). Nevertheless, azithromycin, ponatinib or tilmicosin show higher differences between both methods, inhibiting different metabolic pathways: azithromycin and tilmicosin inhibit RNA-dependent protein synthesis by binding to ribosomal subunits and blocking transpeptidation/translocation step of protein synthesis (Je and Levy, 2016), and ponatinib is a multi-target kinase inhibitor ("Drugbank," 2021d). Conclusively, these differences between the two methodologies explain the inherent lack of potency of most of the candidate drugs identified with *in vitro* methods versus the ones used to treat PAM infections.

### 3.3. Recurrence results: static/cidal drugs

To investigate whether drugs primarily interfere with cell proliferation or also induce cytotoxic effects that kill the trophozoites, we implemented recurrence assays. The recurrence of *N. gruberi* trophozoites after 120-h treatment with amphotericin B along with the eight candidate drugs followed by a 48-h incubation without drugs was further assessed using both the MTT viability assay and the cell analysis software (JuLi™ Stage system) (Fig. 4). As a result, minimum inhibitory concentration (MIC) values were determined to identify the lowest

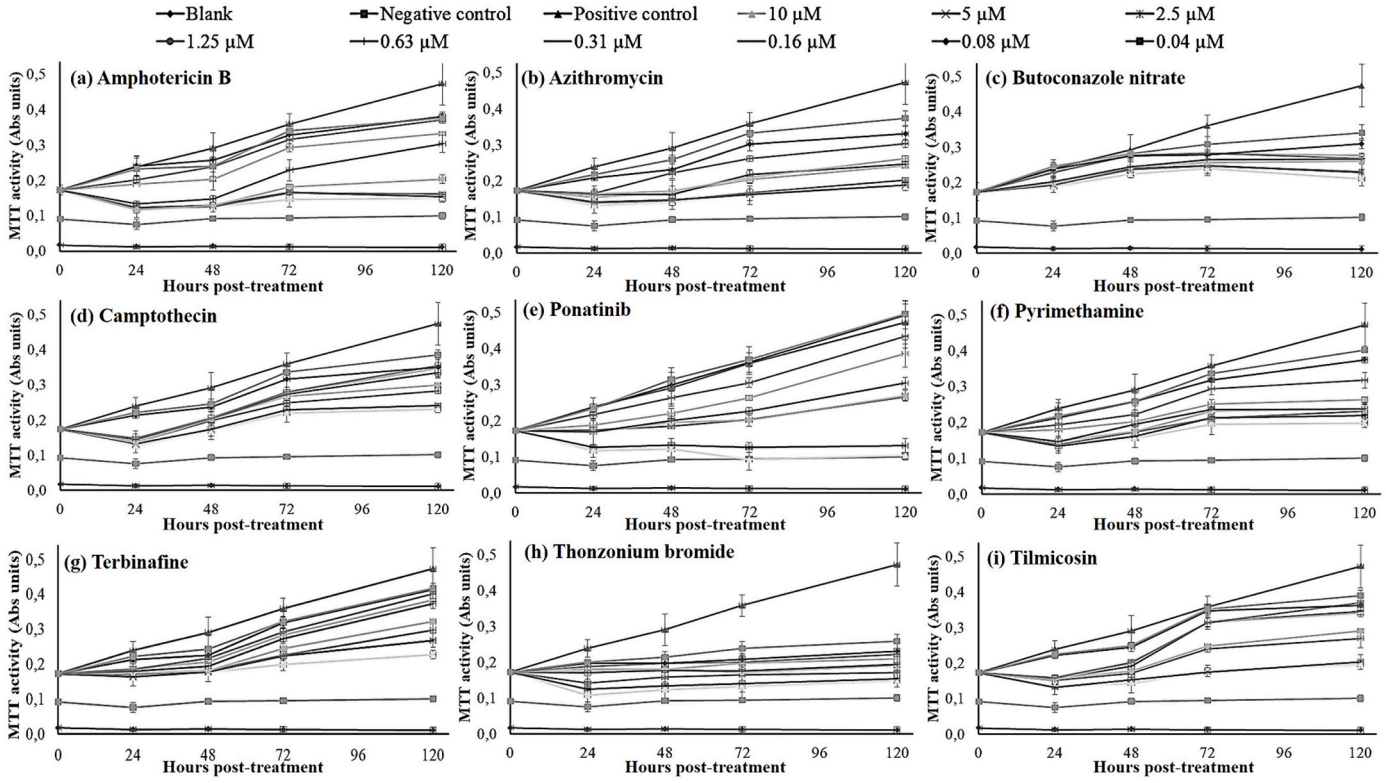


**Fig. 5.** Dose-response curves for each drug against *Naegleria gruberi* during the first 120 h using MTT assay (viability in percentage). Values constitute means of three separate determinations ± standard deviation.

concentrations that effectively kill *N. gruberi* trophozoites (Rajendran et al., 2017). Likewise, azithromycin, butoconazole nitrate and ponatinib showed higher amoebicidal activity than amphotericin B (Table 2). These three drugs showed MIC values lower than 1.25 μM for both methods, thus demonstrating the lowest concentration tested for this assay.

### 3.4. Time-course results: fast/slow-acting drugs

Finally, the eight selected FDA-approved drugs were assayed from 10 to 0.04 μM against *N. gruberi* trophozoites every 24 h from the initiation of the treatment to determine whether the eight candidate drugs are fast or slow-acting drugs, and to compare with amphotericin B (Fig. 5). All these drugs produced a reduction in cell viability in a time-dependent manner, and they even showed activity within the first 24-h treatment (images of *N. gruberi* trophozoites, using the JS system, are shown in Supplementary Material Fig. S5). As stated above, fast-acting and efficient drugs are urgently needed for the treatment of this disease, and all these drugs can be considered fast-acting drugs since they show activity within the first 24 h-treatment (video stills available in Supplementary Material Table S3).

## 4. Conclusions

In this study, we have applied the tenets of modern drug discovery by using a phenotypic screening against *Naegleria*. To accomplish this goal, we have used a new method for quantitative dose-response and screening of FDA drugs for PAM. We conclude that this screening is a promising strategy for drug discovery, allowing us to propose repurposed drugs for clinical trials. After evaluating the amoebicidal activity of 1175 FDA-approved drugs in two rounds of screening, eight drugs and the reference drug amphotericin B were further investigated. Amongst those, three drugs (camptothecin, pyrimethamine, and terbinafine) have shown some potential and would need to be investigated even further. Campothecin is an anticancer drug that inhibits the topoisomerase I, causing DNA damage which results in apoptosis (https://go.drugbank.com/drugs/DB04690). Pyrimethamine is an antiparasitic drug used in the prevention and treatment of toxoplasmosis and malaria that inhibits the dihydrofolate reductase and thereby blocks the biosynthesis of purines and pirymidines (https://go.drugbank.com/drugs/DB00205). Terbinafine is an antifungal drug used to treat skin infections that inhibits ergosterol synthesis by inhibiting the fungal squalene monooxygenase (https://go.drugbank.com/drugs/DB00857). While these are promising candidates for the treatment of PAM (higher amoebicidal activity, improved ADMET profile and bioavailability than the reference drug amphotericin B), further multiphasic investigations combining both 'omics (Herman et al., 2021) and this newly established methods, are required to identify suitable fast-active drugs against the brain-eating amoeba.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2021.10.003.

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