

1 **Screening the MMV Open Access Pathogen box unveils novel and potent**
2 **inhibitors of Amoebiasis agent: *Entamoeba histolytica***

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20 **Abstract**

21 Amoebiasis caused by the protozoan parasite *Entamoeba histolytica* remains a major public health
22 hazard, as being the second cause of death among parasitic infections. Although currently
23 prescribed drugs have shown to be effective in the treatment of amoebiasis, side effects and
24 emergence of parasites resistance prompted the search for novel drug to control this disease. In
25 this regard, the Medicines for Malaria Venture (MMV) Pathogen Box library of selected
26 compounds was screened to identify anti-*Entamoeba histolytica* agents using the resazurin based
27 fluorescence assay. Overall, the results revealed three novel anti-*Entamoeba histolytica* scaffolds
28 with low micromolar activity including MMV675968 ($IC_{50} = 2.10 \mu\text{M}$), MMV688179 ($IC_{50} = 2.38$
29 μM) and MMV688844 ($IC_{50} = 5.63 \mu\text{M}$). Structure-Activity-Relationship (SAR) studies led to
30 identification of two analogs ~100 fold more potent and selective than the original hit compound
31 **1** (MMV675968): **1k** ($IC_{50} = 0.043 \mu\text{M}$) and **1l** ($IC_{50} = 0.055 \mu\text{M}$). Predictive analysis using
32 Maestro 11.6 suggested that these hit compounds possess acceptable physicochemical and
33 metabolism properties. These lead compounds are therefore good starting points for lead
34 optimization studies towards identification of drug candidate against amoebiasis.

35 **Key words:** MMV Pathogen Box, Anti-*Entamoeba histolytica*, Novel scaffolds, Lead compounds

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41 **Author Summary**

42 Diarrhoea is a leading cause of death for millions of children worldwide. One of the top 15 causes
43 of severe diarrhoea is *Entamoeba histolytica*, causing amoebiasis. What makes *E. histolytica*
44 dangerous is its ability to disseminate easily through a given population via contaminated food and
45 water supplies. Moreover, *E. histolytica* is quite comfortable in the environment, difficult to kill
46 with chlorine and infect people at a very low dose, making it a priority pathogen to eradicate. Many
47 drugs have been developed so far to cure this infection. However, they are not efficient enough to
48 control the disease due to pathogen resistance that is becoming a big issue. In addition to that,
49 almost all the drugs in use are highly toxic to human causing several side effects upon medications.
50 Therefore, new, more efficient and less toxic drugs are urgently needed for the better management
51 of amoebiasis. Since the development of a new drug takes years, repurposing existing drugs has
52 been shown to shortcut the process and boost the discovery rate of new medicines. Using this same
53 approach, we have identified two compounds that potently inhibit *E. histolytica* and are nontoxic
54 that can enter the drug discovery pipeline for new amoebicidal drug development. Moreover, these
55 new inhibitors could also serve as starting points for the synthesis of a library of amoebicidal
56 compounds.

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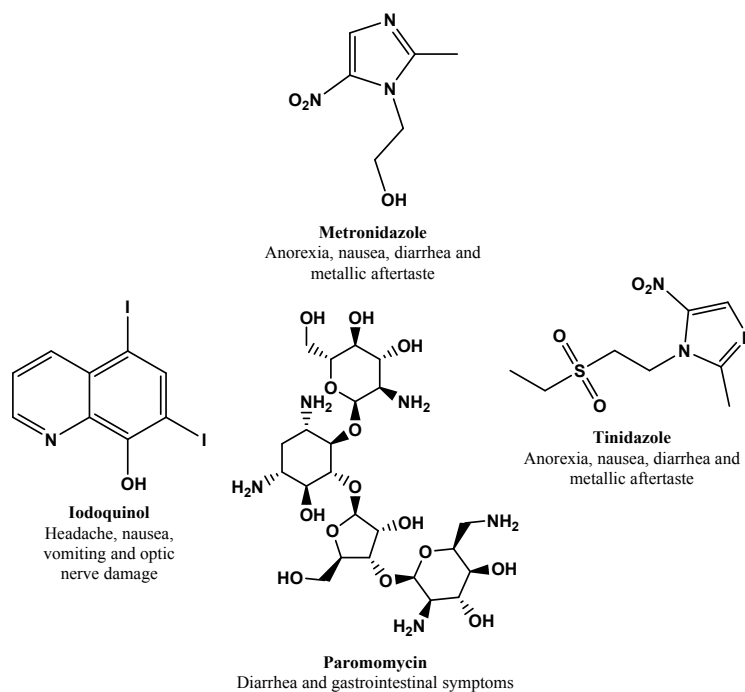
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62 **Introduction**

63 Diarrhoea that is credited to have caused approximately 8% of all deaths among children under
64 age 5 worldwide in 2016 is considered as a leading executioner of children. Basically, the number
65 of yearly deaths among children is about 480,000, meaning over 1,300 children dying each day
66 [1]. Amoebiasis caused by the protozoan parasite *Entamoeba histolytica* is listed among the top
67 15 causes of severe diarrhoea in the first 2 years of life in children living in the developing world
68 [2]. Moreover, approximately 4 to 10% of the carriers of this amoeba infection develop clinical
69 symptoms within a year and amoebic dysentery is considered as the third leading cause of death
70 from parasitic disease worldwide after malaria and schistosomiasis [3-4]. Acute amoebiasis
71 presents symptoms such as diarrhea with frequent and often bloody stools, whereas chronic
72 amoebiasis can present gastrointestinal symptoms plus fatigue, weight loss and occasional fever.
73 Extra-intestinal amoebiasis can occur if the parasite spreads to other organs, most commonly the
74 liver, where it causes amoebic liver abscesses with fever and right upper quadrant abdominal pain
75 [5-6].

76 Estimates indicate that *E. histolytica* infects approximately 500 million people worldwide,
77 resulting in 50 million cases of invasive disease and about 70,000 deaths annually [7-8]. Because
78 of its easy dissemination through contaminated food and water supplies, in addition to its low
79 infectious dose, chlorine resistance, and environmental stability, *E. histolytica* is classified as a
80 category B priority biodefense pathogen by the National Institute of Allergy and Infectious
81 Diseases [9]. For the control of this infection, the treatment relies on structurally diverse drugs (fig
82 1) and the administration of the treatment depends on the diagnosis and severity of the illness.
83 Usually, the drugs are effective for the treatment of invasive amoebiasis, but are less effective in
84 eliminating parasites located in the intestinal lumen. Overall, in symptomatic patients and in

85 invasive disease, the most widely used drugs against *E. histolytica* are the nitroimidazoles
86 (metronidazole and tinidazole) [10-12]. However, several side effects are reported, ranging from
87 vomiting and diarrhea, hallucinations [13], encephalopathy [14] to cancer [15] besides emergence
88 of resistant strains that have been reported against these drugs [16]. Therefore, to face these
89 shortcomings, new and better drugs are required to control amoebiasis.



90

91 **Fig 1.** Structure of available drugs against amoebiasis and their respective side effects.

92 Prompt treatment accounts among the global strategy to control infectious diseases including
93 amoebiasis. However, the efficiency of this intervention highly relies on drugs which should not
94 only be safe and efficacious, but also affordable, and available to the population in need. Ideally,
95 any new anti-amoeba agent should bear all the above characteristics. In the quest for such new
96 treatments against amoebiasis, many strategies have been adopted, including rational drugs design
97 [17] and screening of synthetic and natural products libraries [8, 18]. Several synthesized and
98 naturally occurring compounds have been tested, yet there is no molecule considered to be ideal

99 for the treatment of amoebiasis, particularly for the treatment of severe infections [19]. However,
100 Chacín-Bonilla et al. [20] reported the potential of nitazoxanide prescribed as medication against
101 *Cryptosporidium parvum* and *Giardia lamblia* infection as a key compound for the therapy against
102 both luminal and invasive *E. histolytica* forms, although no further study has been reported since.
103 On another hand, repurposing previously developed drugs or those under development stages
104 against other parasitic or non-parasitic diseases represents a shortest and cheapest way to accelerate
105 the discovery of drugs for neglected tropical diseases, including amoebiasis.

106 Within this framework, the Medicines for Malaria Venture (MMV) has made available open access
107 libraries of hits generated through screening corporates and academic libraries [21]. The success
108 of this approach went beyond malaria parasites [22-24], where inhibitors of several pathogens of
109 other diseases including tuberculosis [25-26], schistosomiasis [27-29], those caused by
110 kinetoplastids [30], cryptosporidiosis [31], toxoplasmosis [32] and cancers [33] were identified.
111 Based on the same concept, we investigated the MMV Malaria Box which led to identification of
112 compounds exhibiting moderate inhibition against *Entamoeba histolytica* [32]. Following these
113 previous footsteps, we hypothesized that the investigation of the MMV Pathogen Box (MMVPB),
114 consisting in 400 drug-like compounds with low toxicity for mammalian cells and activity against
115 specific microbial pathogens could lead to identification of hit scaffolds different from those of
116 the mainstay treatment to feed and accelerate the discovery and development of new anti-
117 amoebiasis drugs with different modes of action.

118 **Methods**

119 **Parasites culture and maintenance**

120 The *E. histolytica* strain NR-176 provided by BEI Resources (www.beiresources.org) was
121 maintained in culture in Eagle's minimum essential medium (EMEM) (Sigma, Germany)
122 supplemented with 10% albumin bovine serum (ABS) and 1% penicillin-streptomycin solution.
123 Parasites were sub-cultured twice weekly. For the assays, cells were harvested by chilling the tube
124 on ice for 15 min to detach the parasites, and then centrifuged at 300×g for 5 min. The supernatant
125 was decanted, and cells pellets were resuspended in fresh medium. The number of viable cells was
126 calculated using a haemocytometer and 0.4% (w/v) trypan blue dye. The criteria for viability were
127 motility and dye exclusion.

128 **The MMVPB compounds library**

129 The Pathogen Box was kindly provided by Medicines for Malaria Venture (MMV, Switzerland)
130 and consisted in 400 drug-like compounds. Compounds were supplied in 96-well microtiter plate
131 format containing 20µL/well of 10 mM solution dissolved in 100% DMSO. Intermediary solution
132 for each compound was prepared in 96-well plate at 100µM by diluting compounds in incomplete
133 culture medium. All plates were stored at -20°C.

134 **Assessment of amoebicidal activity of compounds via resazurin reduction assay**

135 **Determination of *E. histolytica* inoculum size using fluorescence intensity**

136 *E. histolytica* trophozoites at 9.69×10^3 cell/mL, 1.94×10^4 cell/mL, 3.88×10^4 cell/mL,
137 7.75×10^4 cell/mL, and 1.55×10^5 cell/mL in 100µL were added in triplicate in the 96-wells flat-
138 bottomed plates and incubated for 48 h at 37°C in an atmosphere of 5% CO₂. Upon 48 h of
139 incubation, 10 µL of resazurin (0.15mg/mL) were added and mixed gently and incubated in the
140 dark at 37°C for 30 min. Fluorescence was subsequently measured using Infinite M200 plate reader
141 (Tecan) with excitation and emission at 530 and 590 nm respectively.

142 Assay validation through Z-factor determination

143 To validate our resazurin reduction assay, the statistical effect size (Z-factor) was calculated. In a
144 96 wells plate format, *E. histolytica* (2×10^4 cells/mL) was cultured in Eagle's minimum essential
145 medium (EMEM) (Sigma, Germany) supplemented with 10% albumin bovine serum (ABS) and
146 1% penicillin-streptomycin solution for 48 h at 37°C, 5% CO₂. After 48h incubation, 10 µL
147 resazurin at 0.01% (wt/vol) were added in each well and incubated in the dark for 30 min at 37°C.
148 Plates were scanned using a Tecan Infinite M200 fluorescence multi-well plate reader (Männedorf,
149 Switzerland) with excitation and emission at 530 and 590 nm respectively. More than 30 replicate
150 of the negative control (Parasite-free inhibitor), positive control (1mg/mL metronidazole in 100%
151 DMSO) and the blank (medium-free parasite) were prepared. The assay was performed twice and
152 the data were used to calculate the Z-factor using the formula:

$$Z - factor = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

153 Where σ_p and σ_n are the standard deviations of the positive and negative controls respectively, and
154 μ_p and μ_n are the corresponding mean values. A Z-factor between 0.5 and 1.0 indicates an excellent
155 assay and statistically reliable separation between the positive and negative controls.

156 Determination of Single Point Growth Inhibition of the MMVPB compounds

157 Screening of the 400 compounds was conducted in 96-well sterile polystyrene flat-bottom
158 microtiter plates (Corning). *E. histolytica* culture at 2×10^4 cells/mL was exposed to 10 µM drug in
159 a final volume of 100 µL of EMEM per well. Plates were incubated at 37°C under an atmosphere
160 of 5% CO₂ for 48h. Metronidazole at 1 mg/mL and 0.4% DMSO were used respectively as positive
161 and negative controls. After 48 h of incubation, 10µL of resazurin (0.15 mg/mL) (Sigma, USA)
162 were added to each well, mixed gently, and incubated in the dark at 37°C for 30 min. Subsequently

163 fluorescence was measured using a Tecan Infinite M200 fluorescence multi-well plate reader
164 (Austria) with excitation and emission at 530 and 590 nm, respectively. Tests were performed in
165 three independent experiments. Compounds with inhibition percentage $\geq 70\%$ were selected for
166 dose-response assay.

167 **Dose-Response Growth Inhibition assay of the selected MMVPB Compounds**

168 Median inhibitory concentrations (IC_{50}) of the selected MMVPB compounds were determined as
169 described above, with little modifications consisting in tested concentrations ranging from 0.02-
170 25 μ M for compounds and 1.11-584.24 μ M for the reference drug (metronidazole). Experiments
171 were performed in triplicate and repeated twice. Dose-response curves were constructed by
172 plotting mean percent inhibition calculated from the fluorescence counts versus the drug
173 concentrations, and activity was expressed as 50% inhibitory concentration (IC_{50}) using the IC
174 Estimator-version 1.2 software.

175 **Cytotoxicity study of potent anti- *Entamoeba histolytica* compounds**

176 The cytotoxicity of anti-amoebic compounds was assessed using the MTT assay [34], targeting
177 Vero cells line (ATCC[®] CCL-81[™]) cultured in complete medium containing 13.5 g/L EMEM
178 (Gibco, Waltham, MA USA), 10% foetal bovine serum (Gibco, Waltham, MA USA), 0.21%
179 sodium bicarbonate (Sigma-Aldrich, Waltham, MA USA) and 50 μ g/mL gentamicin (Gibco,
180 Waltham, MA USA). Essentially, Vero cells at 10^4 cells/200 μ L/well were seeded into 96-well flat-
181 bottomed tissue culture plates (Corning, USA) in complete medium. Fifty μ L of serially diluted
182 compounds solutions (concentrations $\leq 50\mu$ M) were added after 24 h of seeding then incubated
183 for 48 h in a humidified atmosphere at 37°C and 5% CO₂. DMSO was added as negative inhibitor
184 at 0.4% (v/v). Twenty μ L of a stock solution of MTT (5mg/mL in 1X phosphate buffered saline)

185 were added to each well, gently mixed, and incubated for an additional 4 h. After spinning the
186 plate at 1,500 rpm for 5 min, the supernatant was carefully removed and 100 μ L of 100% DMSO
187 (v/v) were added. Formazan formation was measured on a Magelan Infinite M200 fluorescence
188 multi-well plate reader (Tecan) at 570 nm. The 50% cytotoxic concentrations (CC_{50}) of compounds
189 were determined by analysis of dose – response curves using GraphPad Prism 7.0. The selectivity
190 Indices (CC_{50} Mammalian cell/ IC_{50} *E. histolytica*) were calculated for each compound.

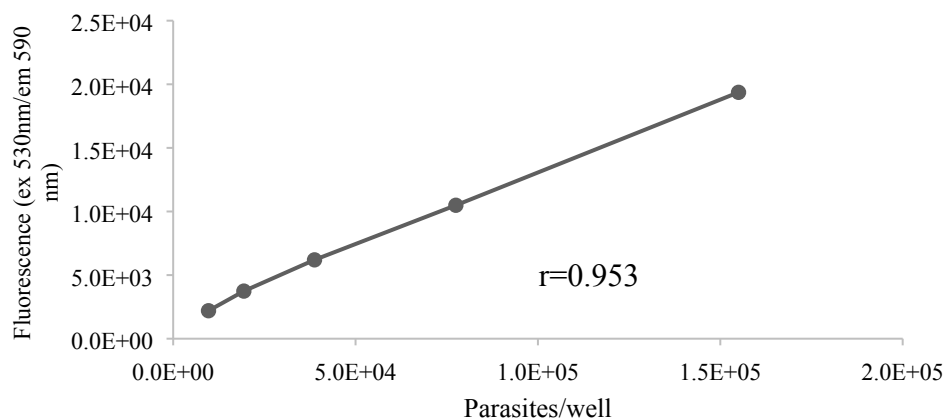
191 **Statistical analysis**

192 The data was analysed in Microsoft Excel and Prism 7.0 software (GraphPad Software, San Diego,
193 CA). A nonlinear regression sigmoidal dose-response curve fit was applied to dose-response data
194 for both 50% inhibitory concentration and 50% cytotoxic concentration.

195 **Results**

196 **Choice of *E. histolytica* inoculum size for the assays**

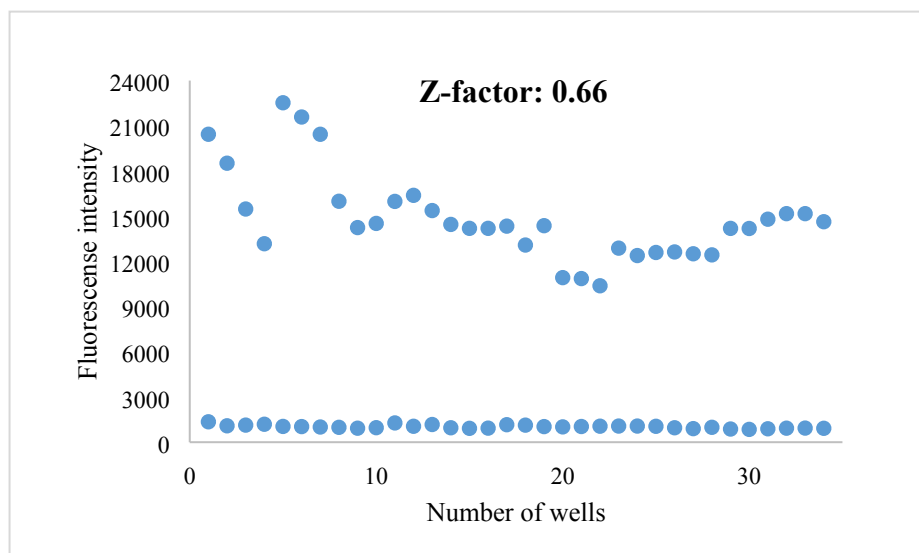
197 The correlation coefficient of the line was 0.953 (fig 2), indicating a linear response between
198 cell/parasite number and fluorescence values at 530 nm (excitation) and 590 nm (emission).



199 Fig 2. Correlation of *E. histolytica* inoculum size with fluorescence values at 530 nm (excitation)
200 and 590 nm (emission) measured using the Resazurin Assay. Different inoculum sizes of *Entamoeba*
201 *histolytica* parasites were added in triplicate to the wells of a 96-well plate in EMEM supplemented with 10% ABS,
202 and 1% penicillin-streptomycin. The medium was allowed to equilibrate for 48 h; then 10 μ l/well of Resazurin Reagent
203 was added. After 30 min at 37°C in a humidified, 5% CO₂ atmosphere, the fluorescence at 530 nm (excitation) and
204 590 nm (emission) was recorded using an Infinite M200 plate reader (Tecan). Each point represents the mean \pm SD
205 of 3 replicates.

206 **Z-factor value for assay validation**

207 The quality of the screen was evaluated using the Z-factor based on the percent inhibition against
208 *E. histolytica* between the 1.0% DMSO and 1mg/mL metronidazole-treated parasites taken as the
209 negative and positive controls respectively. Fig 3 shows the scatter-plot distribution of the percent
210 inhibition for 1.0% DMSO and 1mg/mL metronidazole. The average Z-factor between the 0.4%
211 DMSO and 1mg/mL metronidazole in the 96-well test plates was 0.66 (fig 3) indicating that the
212 assay could reliably separate positive and negative controls. These findings supported the
213 feasibility of our drug screening assay for use in *E. histolytica* screening.



214 **Fig 3. Screening validation.** The Z-factor of 0.66 demonstrates an excellent assay respectfully to the guidance defined by
215 Zhang et al. (1999) indicating that for $0.5 \leq Z < 1$, there is a good separation of the distributions between the signal of negative and
216 positive controls, indicating an excellent assay.

217

218 **Screening of the Pathogen Box against *E. histolytica* identifies MMV675968, MMV688179**
219 **and MMV688844 as potent hits**

220 The preliminary screening of the MMVPB led to the identification of six compounds exhibiting
221 percent inhibition ranging 96-100% against *E. histolytica* in culture including, MMV688978
222 (Auranofin), MMV688775 (Rifampicin), MMV687798 (Levofloxacin (-)-ofloxacin),
223 MMV675968 (compound 1), MMV688179 (compound 2) and MMV688844 (compound 3).
224 Besides, three other compounds including Linezolid (MMV687803), MMV272144 (compound 4)
225 and MMV393995 (compound 5) weakly inhibited the growth of the parasites at the same
226 concentration. The 9 compounds were selected and submitted to dose-response studies as
227 described above. The results achieved indicated, with the exception of compounds 4 and 5, that 7
228 compounds could inhibit the growth of *E. histolytica* with IC_{50} values ranging below 10 μ M (Table
229 1).

230 Table 1: Anti-*E. histolytica* activity profile of nine selected MMVPB compounds

| *MMV ID | Common name or manuscript ID | IC₅₀ (μM) | #CC₅₀ (μM) | SI (CC₅₀/IC₅₀) | Molecular weight (g/mol) | *cLogP | ** Known biological activity |
|----------------|-------------------------------------|-----------------------------|------------------------------|---|---------------------------------|---------------|-------------------------------------|
| MMV688978 | Auranofin | 0.07±0.01 | >50 | >714.29 | 678.48 | 1.40 | Rheumatoid arthritis/Amoebiasis |
| MMV688775 | Rifampicin | 0.05±0.00 | >50 | >1000 | 822.94 | 1.29 | Tuberculosis/ Buruli ulcer |
| MMV687798 | Levofloxacin(-)-ofloxacin | 0.045±0.00 | >50 | >1111.11 | 361.37 | -0.34 | Broad-spectrum antibiotic |
| MMV675968 | 1 | 2.10±0.37 | >50 | >23.80 | 359.81 | 2.31 | Cryptosporidiosis |
| MMV688179 | 2 | 2.38±0.64 | 35.43±1.17 | 14.88 | 476.19 | 2.87 | Kinetoplastids |
| MMV688844 | 3 | 5.63±0.6 | >50 | >8.88 | 424.92 | 3.83 | Tuberculosis |
| MMV687803 | Linezolid | 9.17±1.91 | >50 | >5.45 | 337.35 | 0.52 | Tuberculosis |

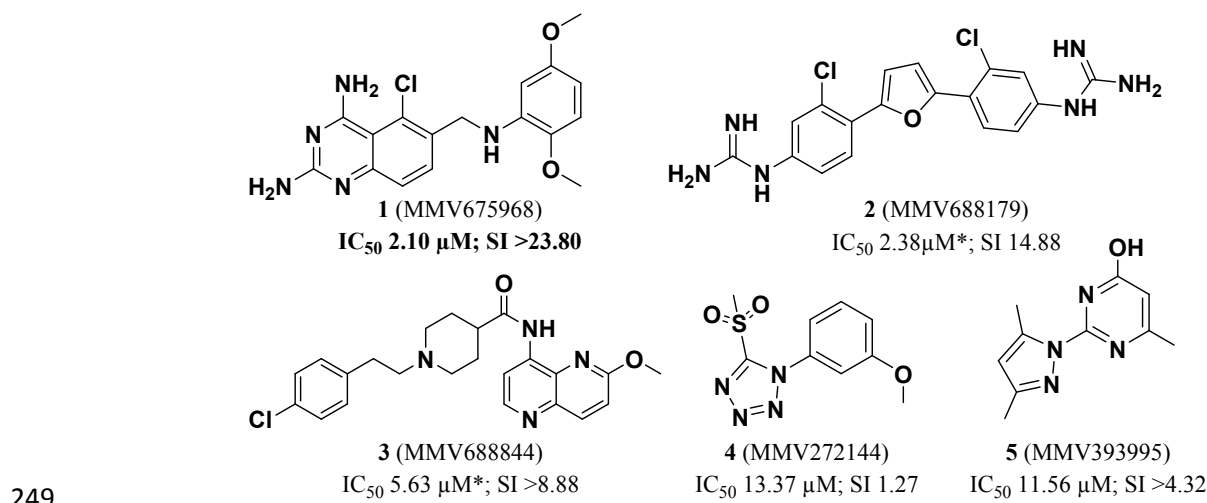
| | | | | | | | |
|---------------|---|------------|------------|-------|--------|-------|--------------|
| MMV272144 | 4 | 13.37±0.68 | 16.98±1.23 | 1.27 | 254.27 | -0.27 | Tuberculosis |
| MMV393995 | 5 | 11.56±0.07 | >50 | >4.32 | 204.23 | 1.10 | Tuberculosis |
| Metronidazole | - | 9.34±0.21 | - | - | 171 | - | Amoebiasis |

231 *cLogP= $\log(C_{\text{octanol}}/C_{\text{water}})$, is the measure of the compound's hydrophilicity; **Known activity of compounds against other diseases. IC₅₀ obtained

232 from two independent experiments performed in triplicate each; #CC₅₀ values of compounds against Vero cell line.

233 Interestingly, among the six compounds that significantly inhibited *E. histolytica* with IC₅₀ values
234 ranging 0.045-5.63 μM, three are approved drugs. In fact in this study, Auranofin (MMV688978)
235 completely prevented *E. histolytica* proliferation (100% inhibition) over the incubation time frame.
236 This finding was not surprising given that Auranofin which is used for the management of
237 rheumatoid arthritis has recently been repurposed as drug for the treatment of amoebiasis [18]. As
238 a result, the activity of Auranofin against *E. histolytica* growth further validated our screening
239 approach. Rifampicin (MMV688775) is an important antibiotic drug used for the treatment of
240 buruli ulcer and tuberculosis, and Levofloxacin (-)-ofloxacin (MMV687798), a broad-spectrum
241 antibiotic of the fluoroquinolone class known to exhibit bactericidal activity.

242 Of particular interest, this study has allowed to identify three hits showing selectivity indexes
243 >8.88 against Vero cell lines and low micromolar activity against *E. histolytica*: IC₅₀= 2.10 μM,
244 2.38 μM, and 5.63 μM for compounds 1, 2 and 3 respectively. These compounds are being reported
245 here for the first time as having amoebicidal activity (Fig 4; Table 1). Given their lipophilicity in
246 an acceptable range (cLogP 2.31-3.83) and small molecular weight (MW 359-476), they might
247 represent novel and attractive anti- *E. histolytica* chemical starting points for medicinal chemistry
248 efforts and therefore deserve further attention.



250 **Fig 4.** Structures and activity of novel amoebicidal compounds identified from the screening of
251 the Pathogen Box (*activity not reconfirmed on resynthesized hits)

252 **Screening of resynthesized hits and their analogs identifies two highly potent *E. histolytica***
253 **inhibitors: MMV1578523 (1k) and MMV1578540 (1l)**

254 In an attempt to confirm their potency upon retesting, and identify more potent and selective anti-
255 amoebic hits, compounds **1**, **2** and **3** were resynthesized and fresh DMSO solutions were prepared
256 from solid samples before testing against *E. histolytica* as described above.

257 Surprisingly upon testing, the activity was not confirmed for compounds **2** and **3** since a complete
258 loss of inhibitory effect against *E. histolytica* was observed at concentrations below 25 μM. Further
259 investigation through available structural analogs of these 2 hits did not show any inhibition
260 against *E. histolytica* while their cytotoxicity values were significant (see Supporting Information).

261 These results suggest that primary activity observed for compounds **2** and **3** might be due to their
262 cytotoxicity or to degradation of the compound under the storage condition of the Pathogen Box.

263 Therefore, they may not be suitable inhibitors against the target parasite. The loss of potency of
264 resynthesized hits is one of the main challenges encountered during hit identification and lead

265 discovery phase of the drug discovery process. This observation led Hughes et al. [35] to define a
266 true ‘hit’ molecule as a compound which has the desired activity in a compound screen and whose
267 activity is confirmed upon retesting. This is as true as the success to identify candidate molecules
268 for clinical development depends upon the stability of the potency over time.

269 Upon retest, compound **1** showed a 3.6-fold decrease in potency (IC_{50} from 2.100 to 7.495 μ M) as
270 well as the selectivity against Vero cell line (SI 5.5). Although slightly disappointed by this loss
271 of potency, this activity level confirmed to be below 10 μ M prompted us to test structural analogs
272 of compound **1**. This has allowed to establish rudimentary structure-activity-relationship (SAR)
273 and to identify two analogs ~100 fold more potent than the parent hit compound.

274 **Structure activity relationship**

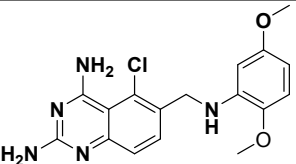
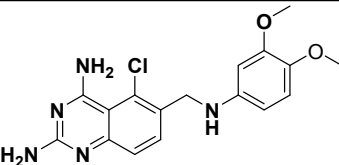
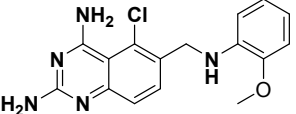
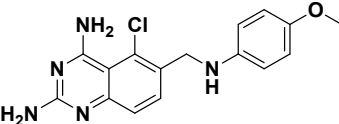
275 Twenty-three analogs of compound **1** were available as solids from MMV as part of the Pathogen
276 Box initiative and tested against *E. histolytica* as an opportunistic approach (see Supporting
277 Information for data on all compounds). The key results allowing to establish very preliminary
278 SAR around compound **1** are summarized in Table 2 below.

279 Compounds **1a-1d** show different substitution pattern around the phenyl moiety of the hit
280 compound **1** while keeping unchanged the 5-chloro-2,4-diaminoquinazoline core. Among these
281 structural analogs, **1b** was found to be the most potent (IC_{50} = 0.123 μ M). This 61-fold increase in
282 potency vs. the hit compound **1** results from incorporation of an *ortho*-methoxy as the only
283 substituent of the phenyl moiety. Replacement of this methoxy with a methyl group did not result
284 in significant activity improvement (compound **1d**).

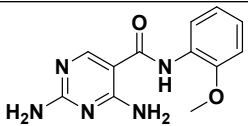
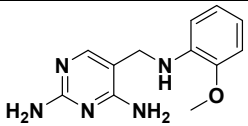
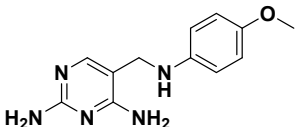
285 The additional analogs depicted in Table 2 deal with modifications of the core moiety. Removal
286 of the chlorine atom from compounds **1a** and **1c** led to complete loss of activity in compounds **1e**

287 and **1g** at concentrations below 25 μ M. Moreover, removal of the chlorine atom from the most
288 potent analogue **1b** led to compound **1f**, showing about 22-fold decrease in activity. Overall, these
289 activity changes indicate that the chlorine atom is critical for activity maintenance.

290 **Table 2: Amoebicidal activity, selectivity and predictive ADME parameters of compound 1 (MMV675968) and structural**
 291 **analogs**

| Compounds | Chemical Structure | IC ₅₀ (μ M) | CC ₅₀ (μ M) | QPlogS | QPlogHERG | QPPCaco | QPlogBB | QPPMDCK | cLogP | QPlogKhsa | HOA |
|-----------|---|--------------------------------|--------------------------------|--------|-----------|---------|---------|---------|-------|-----------|-----|
| 1 |  | 7.495* | 41.27 | -4.061 | -5.464 | 343.441 | -1.282 | 267.239 | 2.41 | -0.106 | 3 |
| 1a |  | 0.357 | 24.77 | -4.06 | -5.471 | 316.921 | -1.325 | 245.526 | 2.68 | -0.117 | 3 |
| 1b |  | 0.123 | >50 | -3.788 | -5.587 | 338.88 | -1.197 | 260.693 | 2.8 | -0.148 | 3 |
| 1c |  | 5.104 | >50 | -3.138 | -5.503 | 96.13 | -1.719 | 67.708 | 2.8 | -0.383 | 2 |

| | | | | | | | | | | | |
|----|--|-------|------|--------|--------|---------|--------|---------|------|--------|---|
| 1d | | 4.567 | >50 | -4.065 | -5.692 | 371.154 | -1.099 | 310.498 | 3.42 | -0.063 | 3 |
| 1e | | >25 | 4.66 | - | - | - | - | - | - | - | - |
| 1f | | 2.716 | 2.04 | -3.459 | -5.727 | 273.028 | -1.41 | 121.606 | 2.24 | -0.223 | 3 |
| 1g | | >25 | 3.00 | - | - | - | - | - | - | - | - |
| 1h | | 7.670 | 1.59 | -3.524 | -5.678 | 299.034 | -1.291 | 134.173 | 2.86 | -0.158 | 3 |
| 1i | | >25 | >50 | - | - | - | - | - | - | - | - |

| | | | | | | | | | | | |
|----|---|--------------|-------|--------|--------|---------|--------|---------|------|--------|---|
| 1j |  | >25 | >50 | - | - | - | - | - | - | - | |
| 1k |  | 0.043 | 32.01 | -2.351 | -4.979 | 273.26 | -1.314 | 121.718 | 0.82 | -0.514 | 3 |
| 1l |  | 0.055 | >50 | -2.334 | -4.957 | 250.144 | -1.352 | 110.628 | 0.82 | -0.522 | 3 |

292 IC_{50} obtained from three independent experiments performed in triplicate each; # CC_{50} values of compounds against Vero cell line; *Hit compound from the screening of the
293 Pathogen Box (IC_{50} 2.10 μ M from the DMSO solution provided by MMV); MW: Molecular weight of the compounds; QPlogS: Predicted aqueous solubility, log S. S in mol
294 dm^{-3} is the concentration of the solute in a saturated solution that is in equilibrium with the crystalline solid (recommended values: -6.5 – 0.5); QPlogHERG: Predicted IC_{50}
295 value for blockage of hERG K^+ channels (recommended values: concern below -5); QPPCaco: Predicted apparent Caco-2 cell permeability in nm/sec. Caco2 cells are a model
296 for the gut-blood barrier (Range: <25 poor and >500 great); QPlogBB: Predicted brain/blood partition coefficient (recommended values: -3.0 – 1.2); QPPMDCK: Predicted
297 apparent MDCK cell permeability in nm/sec. MDCK cells are considered to be a good mimic for the blood brain barrier (Range: <25 poor and >500 great); QPlogKhsa:
298 Prediction of binding to human serum albumin (range: -1.5 – 1.5); HOA: Human Oral Absorption Predicted qualitative human oral absorption: 1, 2, or 3 for low, medium, or
299 high respectively. LogP: lipophilicity (recommended values: LogP <5).

300 Interestingly, 2,4-diaminopyrimidine as core moiety led to a dramatic improvement in potency
301 with compounds **1k** and **1l** showing 3- and 93-fold activity increase respectively when compared
302 to their 5-chloro-2,4-diaminoquinazoline analogs **1b** and **1c**. Overall, screening of the available
303 analogs of the hit compound **1** has allowed to improve the *in vitro* potency against *E. histolytica*
304 by 147-fold.

305 **1l** and **1k** constitute promising starting points. However, it would be key to assess the scope for
306 modifications around the phenyl moiety and to determine which functionalities could be tolerated.
307 Removal of the benzylic position in compounds **1k** and **1l** by incorporation of an amide
308 functionality resulted in complete loss of activity for **1i** and **1j**. The length and rigidity of the spacer
309 connecting the diaminopyrimidine core to the methoxyphenyl group deserves also further
310 investigation.

311 **Discussion**

312 In the quest for new drugs against infectious diseases, repurposing existing active principles is
313 among the novel and fast-track approaches in drug discovery research. This approach significantly
314 reduces the time frame, cost, effort and clinical failure risk associated with conventional drug
315 discovery approaches [36]. In fact, repurposing unsuccessful drug candidates and existing drugs
316 has successfully discover new active approved drugs for other indications [37].

317 In the present study, some MMVPB compounds have shown highly potent activity against *E.*
318 *histolytica*, the causative agent of amoebiasis. Auranofin (MMV688978) was identified to exhibit
319 very good amoebicidal activity (IC_{50} = 0.07 μ M). This result corroborates a recent report of potent
320 activity of Auranofin against *E. histolytica* with IC_{50} value 10-fold better *in vitro* than
321 metronidazole (0.5 vs 5 μ M) [18], the best treatment for amoebiasis, together with tinidazole.

322 Auranofin exerts its action through the inhibition of reduction/oxidation (redox) enzymes that are
323 essential for maintaining intracellular levels of reactive oxygen species. This inhibition leads to
324 cellular oxidative stress and intrinsic apoptosis [38-41]. Through this mechanism of action,
325 Auranofin has been reported as potential drug lead against several diseases including cancer,
326 neurodegenerative disorders, HIV/AIDS, parasitic and bacterial infections [41].

327 Also, two of the hits that were identified in this study, MMV688775 (Rifampicin) and
328 MMV687798 (Levofloxacin (-)-ofloxacin) exhibited potent amoebicidal activities (IC_{50} of 0.05
329 μ M and 0.045 μ M respectively). They are well known for their broad-spectrum antibiotic activity
330 against a variety of bacterial pathogens. However, their amoebicidal properties have not been
331 reported before. Three other MMVPB compounds exerted significant activity against *E.*
332 *histolytica*. Compound **2** (IC_{50} of 2.38 μ M) is thought to be a DNA groove binder as likely
333 mechanism of action, and is active against several bacterial and fungal pathogens [42]. Besides,
334 this compound was among the seven MMV Pathogen Box compounds that exhibited bacteriostatic
335 or bactericidal activity against *Burkholderia pseudomallei*, the causative agent of melioidosis, a
336 disease that requires long-term treatment regimens with no assurance of bacterial clearance [43].
337 To a lesser extent, compound **3** also exhibited promising activity against *E. histolytica* (IC_{50}
338 5.63 μ M). It was originally identified as non-cytotoxic *Mycobacterium tuberculosis* hit in
339 GlaxoSmithKline (GSK) whole cell screens and was predicted, based on *in silico* analyses, to
340 target ABC transporters (Rv0194) in *M. tuberculosis* [26, 44] also involved in the resistance
341 mechanisms of several parasitic protozoa including *E. histolytica* [45]. This compound also
342 showed potency against non-tuberculous mycobacteria (*M. abscessus* and *M. avium*) [46-47] and
343 other intestinal protozoan parasites such *Giardia lamblia* and *Cryptosporidium parvum* [48].

344 Compound **1** (MMV675968) has previously been reported to have anti-cryptosporidiosis activity
345 and to target the dihydrofolate reductase (DHFR) in *Pneumocystis carinii* and *Toxoplasma gondii*
346 [49-50]. Moreover, the chemical structure of compound **1** show some similarity to piritrexim and
347 trimetrexate, two nonclassical folic acid inhibitors approved for the treatment of *Pneumocystis*
348 *carinii* infection in AIDS patients [51]. A recent interrogation of the MMV Pathogen Box has
349 identified **1** as the most active of the box compounds against *Toxoplasma gondii* with IC₅₀ of
350 0.02μM and a selectivity index of 275 [52]. This compound was also identified as a dual hit of
351 *Cryptosporidium parvum* and *Giardia lamblia* with 88% and 90% inhibition respectively in the
352 initial screen of the box compounds by Hennessey et al. [48]. Another study by Lim et al. [53] has
353 found compound **1** among the 13 most potent MMV Pathogen Box compounds against *Madurella*
354 *mycetomatis*, a fungus primarily reported in Central Africa as the causative agent of mycetoma in
355 humans, which is a chronic infectious and inflammatory disease. Beyond the numerous activities
356 reported of compound MMV675968, it was also showed in this study to be active against *E.*
357 *histolytica* with IC₅₀ of 2.10μM, rationally inhibiting the amoebal DHFR enzyme as suggested by
358 the findings of Lau et al. [50]. In fact, competitive inhibitors of DHFR are used in the
359 chemotherapy or prophylaxis of many protozoan pathogens, including the eukaryotic parasites
360 *Plasmodium falciparum*, *Entamoeba histolytica* and *Toxoplasma gondii* [50].

361 It is likely that lead compounds identified in this study inhibits *Entamoeba histolytica* through
362 inhibition of the enzyme dihydrofolate reductase. In fact, the enzyme dihydrofolate reductase
363 (DHFR) catalyzes the reduction of folate to dihydrofolate(DHF) and DHF to tetrahydrofolate
364 (THF) by use of the cofactor NADPH. The methylenated form of THF serves as a carbon donor
365 for the synthesis of thymidylic acid in a reaction catalyzed by thymidylate synthase (TS). Knowing
366 that thymidylic acid is essential for DNA synthesis, blocking of either DHFR or TS activity will

367 lead to cell death [54]. Indeed, success of DHFR inhibitors in treating various infectious diseases
368 can be attributed to the divergence in the DHFR sequence, which imparts a high degree of
369 selectivity for certain antifolates for one organism versus others. This could be particularly
370 important for parasitic protozoa, which, unlike humans, express DHFR as part of a bifunctional
371 enzyme containing both DHFR and TS activity in two domains of the same polypeptide joined by
372 a linker [55]. This structural and mechanistic distinction of protozoan DHFRs offers a unique
373 opportunity to develop new drug with greater selectivity [51].

374 Considered as a promising starting point for drug discovery against *E. histolytica*, a preliminary
375 hit expansion was performed in this study through the screening of twenty-three structural analogs
376 of compound **1** to establish rudimentary structural activity relationship. Four analogs displayed
377 good potency, including compounds **1k** and **1l** that were >100-fold more potent than the original
378 hit. DMPK profiling, additional modifications to understand the SAR and to deal with possible
379 metabolic hotspots (e.g. methoxy group, benzylic position) would be required as next steps to
380 assess further the potential of this chemotype.

381 Overall, all the novel anti-*E. histolytica* hits identified from this study are structurally different
382 from currently available drugs. Furthermore, they are selective against Vero cell lines and have
383 favorable physicochemical properties. Very preliminary structure-activity-relationship has
384 allowed to identify double-digit nanomolar inhibitors against *E. histolytica* in cellular assays. This
385 suggests that medicinal chemistry efforts focusing on lead optimization could result in successful
386 selection of a drug candidate for amoebiasis drug development.

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397

398 **Competing Interests**

399 The authors declared there is no competing interests

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568

569 **Supporting Information Legends**

570 **Table:** Amoebicidal potency of the hit compounds and their structural analogues.

571 IC_{50} = Median inhibitory concentration of active compounds against *E. histolytica*; CC_{50} = Median cytotoxic
572 concentration of active compounds against Vero cells; $SI (CC_{50}/IC_{50})$ = Selectivity index

573