Screening The Pathogen Box for Identification of New Chemicals Agents With Anti *Fasciola hepatica* Activity

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22

23 **Abstract**

24 Fascioliasis is an infectious parasitic disease distributed globally and caused by the liver
25 flukes *Fasciola hepatica* or *F. gigantica*. This neglected tropical disease affects both
26 animals and humans and it represents a latent public health problem due to the significant
27 economic losses related to animal husbandry. For decades, Triclabendazole has been the
28 unique anti-Fasciola drug that can effectively treat this disease. However, triclabendazole
29 resistance in Fascioliasis has been more recently reported around the world, and thus the
30 discovery of novel drugs is an urgent need. The aim of this study was to investigate the
31 fasciocidal properties of 400 compounds contained in the Pathogen Box. The first stage of
32 the screening was carried out by measuring the fasciocidal activity on metacercariae at a
33 concentration of 33 µM of each compound (standard dose). Subsequently the 50%
34 inhibitory concentration (IC_{50}) values of the most active compounds (n=33) were assayed
35 on metacercariae and resulted in 13 compounds with $IC_{50} \leq 10$ µM. The second stage
36 queried these compounds at 33 µM on adult flukes where seven showed high mortality
37 rates > 50%. Four hit compounds were selected based on predicted nontoxic properties and
38 IC_{50} values obtained on adult worms resulted < 10 µM thus representing the best
39 fasciocidal compounds tested here. Cytotoxicity assay on four types of cell lines
40 demonstrated that three compounds are nontoxic at its most active concentration. In
41 conclusion, three hit compounds identified in this proof-of-concept study are potential
42 candidates in the discovery of new fasciocidal drugs. Further studies are warranted.

INTRODUCTION

Fasciola hepatica is the etiological agent of fascioliasis, the most widespread trematodiasis that affects both humans and herbivorous mammals such as sheep, cattle, goats and other species (1, 2). In humans, fascioliasis can be acquired by the consumption of contaminated vegetables. Up to 17 million people in 51 countries are estimated to be infected with *F. hepatica* worldwide and more than 91 million are at risk of infection by this parasite (3, 4). Among all continents, the Andean Region of South America is the most affected by *Fasciola* where prevalence rates above 10% have been documented (5-8) and national treatment programs are being scaled up.

Triclabendazole (TCBZ) is the most single effective fasciocidal drug, with activity against both the infective larvae (Metacercaria) and adult worms, and an efficacy that exceeds 90% in humans after a single oral dose (9, 10). Nonetheless, after decades of successful efficacy, TCBZ resistance has developed in both animals and humans (11). Cases of TCBZ-resistant *Fasciola* in both animals and humans have been reported in Australia, Europe and Latin America (12-18). The development of TCBZ resistance represents an important public health concern throughout the world that mainly affects animal husbandry and leads to enormous economic losses (19). As a consequence, the discovery of novel effective drugs and vaccines against *Fasciola* is an urgent need for the global control of fascioliasis. Repurposing of praziquantel (PZQ) as anti-Fasciola drug failed whereas oxfebendazole showed to be an effective drug in animals (20, 21). Currently, there is no other fasciocidal drug in clinical practice for humans, and thus TCBZ remains the unique treatment against this infectious disease.

Open-access drug discovery provides a substantial resource in the research of those diseases that affect primarily people living in low-resources locations. The Medicines for Malaria Venture (MMV) foundation assembled a set of compounds, called "Malaria Box", which has been tested against various infectious agents including *Cryptosporidium parvum* (22), *Plasmodium falciparum* (23, 24) *Schistosoma mansoni* (25, 26), *Toxoplasma gondii* (27), and mycobacteria (28, 29). Later, a new set of chemical entities was assembled and named the Pathogen Box collection. It contains 400 drug-like compounds that have showed inhibitory activity on various infectious diseases such as haemonchosis, toxoplasmosis, tuberculosis, neosporosis, malaria, sleeping sickness, Chagas, leishmaniasis and trypanosomiasis (30-36). The Pathogen Box has been tested also in fungal diseases caused by *Cryptococcus neoformans* and *Candida albicans* (37-39). The aim of this study was to identify the fasciocidal activity of 400 compounds contained in the Pathogen Box by *in vitro* testing.

MATERIALS AND METHODS

Study design. The study was conducted in three stages: (i) bioassays on metacercariae; (ii) bioassays on adult worms; and (iii) cytotoxicity on cell (Figure 1). The best ffasciocidal compounds were selected in each stage to be tested in the next phase based on *in vitro* biological activity. To complement our knowledge on the active compounds obtained by the experimental assays, computational resources were consulted to describe the chemical properties as well as the *in silico* toxicology features and biological targets from these active compounds.

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88 Drugs and Media

89 The Pathogen Box was provided by the MMV agency (Geneva, Switzerland) and
90 manufactured by Evotec (USA). The 400 drug-like molecules were supplied in 96-well
91 plates as stock solutions of 10 mM dissolved in dimethyl sulfoxide (DMSO). Full data of
92 The Pathogen Box compounds is available at https://www.pathogenbox.org (40). TCBZ
93 was purchased from Sigma-Aldrich (Buchs, Switzerland). All of the compounds of The
94 Pathogen Box were dissolved in DMSO (Sigma-Aldrich, Irvine, UK) for drug stock
95 solutions of 200 µM. Additional vials of MMV063404, MMV003270, MMV085210,
96 MMV676380, MMV687730, MMV687251, MMV1030799, MMV690102, MMV1029203,
97 MMV676053, MMV688179, MMV023969 and MMV688921 were manufactured by
98 Evotec (France). RPMI 1640 culture medium (Sigma- Aldrich, St. Louis, US) was used for
99 both stages, metacercariae and adult worm; supplemented with penicillin (100 U/ml) and
100 streptomycin (100 µg/ml) (Sigma-Aldrich, St. Louis, US).

101 Parasites

102 Metacercariae of *F. hepatica* were obtained following the protocol described by Ortiz et al.
103 (16), at the Immunology and Research Laboratory of the Faculty of Veterinary Sciences of
104 the *Universidad Nacional de Cajamarca* in Peru. Eggs of *F. hepatica* were collected
105 directly from the gallbladder of sheep slaughtered in a popular abattoir in the city of
106 Cajamarca (TCBZ-resistant endemic area for fascioliasis). Miracidia were from Fasciola
107 eggs after incubation for 15 days at 25°C. Afterwards, they were used to infect *Lymnaea sp.*
108 snails (5-6 mm) in a proportion of two miracidia per snail. The infected snails were kept in

plastic containers for 45 to 60 days at room temperature. After this time, the snails were stimulated by direct solar exposure and water at 4-8°C to produce metacercaria. Approximately 20,000 metacercariae were obtained for this study and stored in cryovials on distilled water at 4-8°C. Adult worms were collected from bile ducts of infected cattle from a slaughterhouse in Lima, Peru; and maintained at 37°C until usage (within 2h). Before its incubation, three washes with phosphate-buffered saline (PBS) (HiMedia, India) and one additional with supplemented RPMI were performed to remove host debris. All the incubations, for both metacercariae and adults, were carried out at 37°C with 5% CO_2.

In vitro screening on metacercariae

The 400 compounds were initially tested at 33 µM on *F. hepatica* metacercariae. Drug stock solutions were diluted in 96-well plates (BD Falcon, US) with RPMI 1640 supplemented with antibiotic up to a final volume of 180 µL. In all *in vitro* assays, positive and negative controls were run in parallel for each assay batch. A range between seven and ten metacercariae were added to each well, previously analyzed microscopically to confirm its viability (microscopic features intact). The metacercariae viability considered some physical properties of the parasite determined by microscopy as described previously (41, 42). The MCs viability was surveyed as a function of both damage in membrane and fluke colour (translucence). Therefore, a low viability corresponded to big damage and high translucency. The viability scale was scored as follows: +++, total damage (dead parasite, shattered membrane and mostly translucent); ++, partial damage (partial membrane damage and highly translucent); +, mild damage (partial membrane damage, poorly translucent) and no damage (intact membrane, dark metacercariae, lack of translucency).

Positive-control wells contained TCBZ 10 µM whereas *F. hepatica* metacercariae incubated in the presence of the highest concentration of DMSO served as negative control. Each test was performed in triplicate. Culture plates were incubated at 37°C in a humidified 5% CO_2 atmosphere for 72 h. First, metacercariae were evaluated by inverted microscopy (PhotoZoom, Cambridge Instruments) at magnification 10X and 20X at 24, 48 and 72 h post drug exposure to determine its viability. Only the compounds that caused, on average, at least 25% of metacercariae mortality at 72 hours were considered for IC_{50} (50% inhibitory concentration) determination. Experiments were run in sets of triplicates. The mean mortality percent of the study compounds were compared to that of DMSO. A standard deviation (SD) was also estimated.

In the second part, we determined the IC_{50} of the selected compounds chosen in the previous bioassay. Drugs were tested at concentrations of 2.1, 4.2, 8.4 and 33 µM using supplemented culture medium. The incubation was done under the conditions described above, by triplicate and considering TCBZ and DMSO as controls. Anti-parasite activity was evaluated at 24, 48 and 72 h post exposure, using the above-mentioned metacercariae viability scale. Viability (mean % of viable parasites) at 72h was considered for the estimation of IC_{50}. IC_{50} values of test compounds were determined by linear regression analysis using CompuSyn software (Version 3.0.1, 2007; ComboSyn Inc., USA). The linear correlation coefficient (r) was obtained.

Assessment of anti-*Fasciola* activity *in vitro* on adult worms

Those compounds that showed activity $IC_{50} \leq 10$ µM on metacercaria were subsequently tested in adult stage of *F. hepatica*. In all *in vitro* assays, positive and negative controls were run in parallel for each assay batch. First, the selected compounds were tested at 33

154 µM by triplicate, using drug stock solutions diluted in supplemented RPMI on 6-well plate
155 up to a final volume of 4 ml. Adult worms were thoroughly washed with PBS to remove
156 host debris and then three worms were placed in each well. The incubation was done under
157 the same conditions as those applied in bioassays with metacercariae. Positive control
158 consisted of 50 µM TCBZ and the negative control was DMSO at the highest
159 concentration. The viability of adult flukes was scored after 24 and 48 h using a motility
160 criterion previously described (43) and also color and rigidity criteria previously applied by
161 our team (data not published). Motility was assessed only in adults and not in MC because
162 this latter has no movements. Rigidity was a parameter used to confirm the damage caused
163 by the drug once the incubation time finished. In general, low motility level corresponded
164 to transparent and rigid worms. Those changes were attributed to the damage caused by a
165 drug. The viability scale was determined as follows: (i) worm motility: 3, normal
166 movements; 2, reduced movements; 1, very weak movements and 0, absence of movements
167 (i.e. death of worm); (ii) worm color: +++, dark red; ++, pink; +, slightly transparent and -,
168 totally transparent; and (iii) worm rigidity: -, no rigidity; +, rigidity and ++, cell break when
169 touched. Assessments at 72h post drug exposure were not done because death of worms
170 always occurred $\leq$ 48h. Experiments were run in sets of triplicates. The mean mortality
171 percent and SD of the study compounds were estimated. The selected compounds were
172 those that caused an average mortality > 50% in adult parasites. Then IC_{50} assays were
173 conducted by testing the selected compounds at five different concentrations 0.31, 0.93,
174 2.78, 8.33 and 25.0 µg/ml. DMSO and TBZ were used as negative and positive controls,
175 respectively. Parasite viability at 24 h were estimated based on survival in DMSO. The
176 IC50s and 95% CI were estimated using GraphPad Prism 7.0 software using a variable
177 slope of the sigmoidal curve from normalized percent activity values and log10-

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transformed concentrations. Top and bottom values were constrained to 100 and 0, respectively. The fasciocidal activity was determined by considering the adult viability scale described before.

Computational analysis.

Evaluation of biological targets of small compounds. To learn about biological targets, those compounds that showed promising anti-fasciola activity in the adult stage as well as TCBZ were entered in the ChEMBL database (https://www.ebi.ac.uk/chembl/) (44). First, the SMILES (Simplified Molecular-Input Line-Entry System) of each selected compound were obtained from the supplementary material provided by the MMV (also available at www.mmv.org). Then the SMILES were entered in ChEMBL and known targets of each compound were retrieved. ChEMBL compares the query compound to a large database of compounds and their targets available from multiple sources including the projects funded by MMV (45). The target name, organism and protein target classification were collected.

In silico cytotoxicity prediction. Lazar (lazy structure–activity relationships), a modular framework for predictive toxicology, was consulted to predict the toxic effects of the selected compounds that showed activity on metacercariae (46-48). Lazar was accessed through https://lazar.in-silico.de/predict and SMILES of each compound were entered. Relevant data including carcinogenicity in rodents, mutagenicity in *Salmonella typhi* and acute toxicity on *Fathead minnow,* Blood Brain Barrier Penetration and the maximum recommended daily dose in humans were predicted.

Cell Growth Inhibition Bioassay.

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Cytotoxicity of the compounds was evaluated in tumor and non-tumor cell lines using the sulforhodamine B (SRB) assay method (49, 50). Cell lines tested include BALB/3T3 (Non-tumorogenic, BALB/c mouse embryo cells), H460 (human lung large cell carcinoma), DU145 (human prostate carcinoma) and HT-29 (human colon adenocarcinoma).

To determine the cytotoxicity of the compounds, cells were plated into 96-well tissue culture plates and in their corresponding growth medium Dulbecco's Modified Eagle Medium (DMEM) at approximately 10% confluency (BALB/3T3 at 3,500 cells/well, H460 at 1,500 cells/well, DU145 at 3,500 cells/well and HT-29 at 3,000 cells/well) and incubated at 37˚C in a 5% CO_2 and 95% air humidified atmosphere for 24 h to allow cells to attach. A plate containing each of these cells was fixed *in situ* with trichloroacetic acid (TCA) in order to obtain the cell values at zero time before adding the compounds. The rest of the plates containing the different cell lines received serial dilutions of the compound to be tested at the following final concentrations: 4, 1, 0.25 and 0.0625 µg/mL. The plates were then incubated at 37˚C in a 5% CO_2 and 95 % air humidified atmosphere for 48 h. The assay was terminated by the addition of cold TCA. TCA treated plates were incubated at 4˚C for 1 hour and then washed five times with tap water to remove TCA and air dried. Background optical densities were measured in wells incubated with growth medium without cells. TCA-fixed cells were stained for 20 minutes with 0.4% (w/v) SRB dissolved in 1% acetic acid. At the end of the staining period unbound dye was removed by washing four times with 1% acetic acid. After air drying the plates, bound dye was solubilized with 10 mM Tris base (pH 10.5) and the absorbance read on an automated plate reader at a wavelength of 550 nm. The GI_{50} value was defined as the concentration of test sample resulting in a 50% reduction of absorbance as compared with untreated controls that

received a serial dilution of the solvent in which the test samples were dissolved and was determined by linear regression analysis. The optical density values obtained were used to determine the cell growth and cytotoxicity from each compound.

Ethics. This study was approved by the Animal Ethics Committee of the Universidad Peruana Cayetano Heredia (Approval ID Code 41-07-16).

RESULTS

In vitro activity of The Pathogen Box determined on *F. hepatica* metacercariae

In the first stage of the study, the 400 compounds contained in the Pathogen Box were *in vitro* screened against *F. hepatica* metacercaria. A total of 33 compounds showed mean mortality rates above 25% at 33 µM but all these resulted being less active than TCBZ (mortality rate of 90%) as shown on Table 1. Fasciocidal activity of these 33 compounds was then assessed by determining the IC_{50} values (Table 1). As a result, 13 compounds showed potent inhibitory activities with IC_{50} values between 0.31 µM and 8.23 µM and were then assayed in adult worms although its low r values (Table 1).

In vitro activity of selected compounds on *F. hepatica* adult worms and i*n silico* toxicology prediction.

The 13 selected compounds listed on Table 2 were assayed at 33 µM in adult worms. Seven compounds produced moderate or high mean mortality rates (> 50%) (Table 2). These were MMV003270, MMV676380, MMV690102, MMV1029203, MMV063404, MMV1030799, and MMV688921. Six compounds showed low mortality rates (<50%) and for that reason these were not considered in the next assays. Before to proceed with the IC_{50} assay, *in silico* safety profiles of the seven selected compounds were predicted by lazar

program (Table 1). Whereas MMV003270 and MMV676380 were predicted non-carcinogenic and non-tumorigenic compounds, MMV690102 was deemed non-carcinogenic and tumorigenic (Table 1). MMV1029203, MMV063404, MMV1030799, and MMV688921 were predicted carcinogenic and tumorigenic substances. Thus, the three deemed non-carcinogenic compounds as well as MMV1029203, a predicted carcinogenic substance that had the highest mean mortality rate (78%), were tested in adult worms. Such four compounds constitute our hit compounds.

To determine which of the four hit compounds were most potent at inhibiting the growth of *F. hepatica* adult worms, the IC_{50} values were determined. The hit compounds had IC_{50} values < 10 µM in adult worms (Table 3, Fig. S1, Table S1). These four hit compounds were tested in the cytotoxicity study on cell cultures.

In vitro cytotoxicity on cell lines.

Cytotoxicity of the four hit compounds against cell lines was evaluated in culture (Table 3). The GI_{50} values ranged from 0.95 and >23.73 µM across the four types of cell lines assayed (Table 3). MMV003270, MMV676380, MMV1029203 and TCBZ presented GI_{50} values above its IC_{50} values thus meaning that these compounds are not toxic at their active concentrations. In one of the four cell lines, MMV690102 had a GI_{50} value below its IC_{50} value thus suggesting that it may cause a level of toxicity in certain cell types at its active concentration (Table 3).

Computational recognition of targets.

As a result of the search in the ChEMBL database, a total of 27 targets were recognized for TCBZ whereas MMV003270 resulted to have 19 known target, most of them in humans

(Table 4). MMV003270 and TCBZ have common human targets that comprise Nuclear factor erythroid 2-related factor 2, Microtubule-associated protein tau and TAR DNA-binding protein 43. According to the data deposited in ChEMBL, MMV003270 targets a number of cytochrome p450 of family 1, 2 and 3. MMV676380 and MMV023969 have identical cell targets that include human glucose transporter and hexose transporter of *Plasmodium falciparum* and *Leishmania mexicana* (Table 4). Targets for MMV1029203 and MMV676053 also resulted to have known targets including human ferrochelatase and Inosine-5'-monophosphate dehydrogenase of *Cryptosporidium parvum,* respectively. The remaining eight compounds had no known targets according to the ChEMBL database (Table 4).

DISCUSSION

In the present study, the Pathogen Box was queried to identify compounds with *in vitro* anti-Fasciola activity against both metacercariae and adult worms (Figure 1). We found 13 compounds with potent inhibitory activity on metacercariae (IC_{50} < 10 μM), meaning that 3% of the substances within the Pathogen Box are effective against the infective form of *F. hepatica*. Two out of the 13 compounds (MMV687730 and MMV687251) had the most potent activity against metacercariae with IC_{50} values below 1 μM but showed mild effects on adult worms (Tables 1 and 2). Since we were interested in identifying hit compounds that were active on larvae and adult stages, these two compounds were not further studied (Table 2). When assayed on adult worms, seven promising compounds showed mortality rates above 50% (Table 2). As a criterion for hit prioritization during the screening on adult worms, we prepared a list of hit compounds that mostly excluded the predicted

carcinogenic/tumorigenic compounds (Table 3). Thus, three (MMV676380, MMV003270 and MMV690102) of the seven most promising candidates were included in the list of hit compounds since they were predicted noncarcinogenic agents (Table 1). One additional compound (MMV1029203) that was predicted as a carcinogenic compound was also included due to its very high effect on adult worms. According to our results, the four hit compounds resulted potent inhibitory molecules both on MC and adult stages (Table 3). The cytotoxicity assay revealed that three hit compounds (MMV676380, MMV003270 and MMV1029203) were non-toxic agents at its most active concentrations when assayed on cell lines (Table 3). In contrast, MMV690102 may cause cell cytotoxicity at its most active concentration meaning that it is not a primary candidate for drug development (Table 3). Our results are consistent with previous cytotoxicity assays on HepG2, HL60 and MRC5 as shown on Table 3 (data provided by the MMV as part of the supporting information for the Open Access Malaria Box).

Repurposing of hits, using the Pathogen Box, against *F. hepatica* is highly relevant since TCBZ is the only existing effective drug for which resistance is known (51-53). Previous works tried to repurpose albendazole, nitroxynil and closantel as candidate fasciocidal drugs but treatment failed (54, 55). In the present study, four out of 400 compounds contained in The Pathogen Box showed potent inhibitory activity against the infective form of *F. hepatica* as well as its adult form (Table 3). Such finding represents a relevant contribution in the identification of dual drug candidates that are able to act against the initial stages of the infective larvae (metacercaria) and adult forms of liver flukes, similar to TCBZ. Additionally, other 13- compounds showed biological activity at < 20 μM against metacercaria (Table 1). Since MC represents the initial infective form of parasites, it should

be primarily controlled through potent compounds such as those identified here (Table 1). Future exploration of The Pathogen Box in newly juvenile metacercaria is desirable given that some compounds may have not penetrated the cyst wall of larvae. By testing compounds on juvenile worms, some additional molecules might be recognized that are active in adult worms.

The four hit compounds identified in this study have been previously characterized against *Plasmodium falciparum, Ancylostoma ceylanicum, Trypanosoma cruzi* and *Leishmania donovani* (data provided by the MMV as part of the supporting information for the Open Access Malaria Box). Therefore, a common mechanism of action or target is plausible among the hit compounds across such pathogens. For instance, MMV676380 has previously shown to have a lethal effect on *P. falciparum* and here was found to be a potent inhibitory compound against *F. hepatica* (36, 56). Known targets of MMV676380 are the glucose and hexoses transporters suggesting that such mechanism may be affected in both parasites in presence of such compound (Table 4). In the other hand, MMV003270 (Zoxazolamine), that is also active against *A. ceylanicum,* resulted to have 19 targets including three human proteins that are also targeted by TCBZ (Table 4). Two of these proteins are transcription regulators (Nuclear factor erythroid 2-related factor 2 and TAR DNA-binding protein 43) whose disruption may affect the gene expression. Such finding is in accordance with a hypothetical mechanism of action of TCBZ that involves a direct effect of the drug on protein synthesis (11, 57). Similarly, the microtubule-associated protein tau is a known target both of TCBZ and MMV003270. TCBZ is a benzimidazole-derivative that disrupts the assembly of microtubules in helminths by binding to tubulin

335 molecules (58). Our results suggest that MMV003270 also affects the microtubules
336 formation mechanism. Common targets of TCBZ and MMV003270 may be partially
337 explained by the similar scaffold structures. MMV1029203, one of the four hit compounds,
338 targets a human ferrochelatase that is a mitochondrial factor involved in protoheme
339 biosynthesis. This latter is a vital process that exists also in *F. hepatica* and whose
340 disruption may be lethal. Some known targets of the hit compounds here identified
341 correspond to human proteins which suggests that a level of toxicity may exist in humans.
342 However according to our results with cell lines, the compound concentrations needed to
343 kill *F. hepatica* (IC_{50}) are considerably less than that to cause cell death (GI_{50}) which means
344 that these are nontoxic (Table 3), except for MMV690102. Although no *F. hepatica* target
345 is recognized for our hit compounds, the demonstration of inhibitory activity of such
346 chemical agents both in metacercariae and adult forms suggests that common targets may
347 exist in both liver fluke stages. The identification of drug targets becomes an important step
348 that drive the discovery of novel antiparasitic agents administered by various ways (34).
349 For that reason, further studies to identify potential *F. hepatica* targets of hit compounds are
350 desirable. Such a study should consider the recognition of human homologs in *F. hepatica*
351 according to our results (Table 4).

352 Our study has some limitations. First, TCBZ metabolites (TCBZ-sulfoxide and TCBZ-
353 sulfone) that are quickly released *in vivo* were not included in this pilot study. However
354 given that TCBZ has a moderate *in vivo* and *in vitro* fasciocidal effects it is suitable as
355 positive control in bioassays (59, 60). A second limitation is that alive *F. hepatica* worms
356 were collected from a local abattoir where some animals may have been infected by various
357 other pathogens or may have been treated with TCBZ. To guarantee the best quality of

adult worms for bioassays, we performed a quality control on adult fasciolas before using these in the experiments. Thus, only worms that presented intense brown or red color and that have active motility were selected. All the remaining were discarded. A third limitation is the low number of parasites used for the assays, that did not allow performing formal statistical comparisons of activity between TCBZ and test drugs. Obtaining MC and adults was a challenging task since both MC and adult worms were collected from natural reservoirs. Therefore we had limited access to parasites for bioassays. However, our exploratory study aimed to identify fasciocidal compounds, we found that negative controls were enough for such purposes.

In conclusion, we identified three promising non-cytotoxic drug-like compounds, MMV003270, MMV676380 and MMV1029203, that showed a potent biological activity against *F. hepatica* metacercaria and adult worms. Such compounds represent new lead candidates to potentially become future anti-*F. hepatica* drugs. By acting both on infective form and adult worms, such agents may provide an appropriate treatment against fascioliasis.

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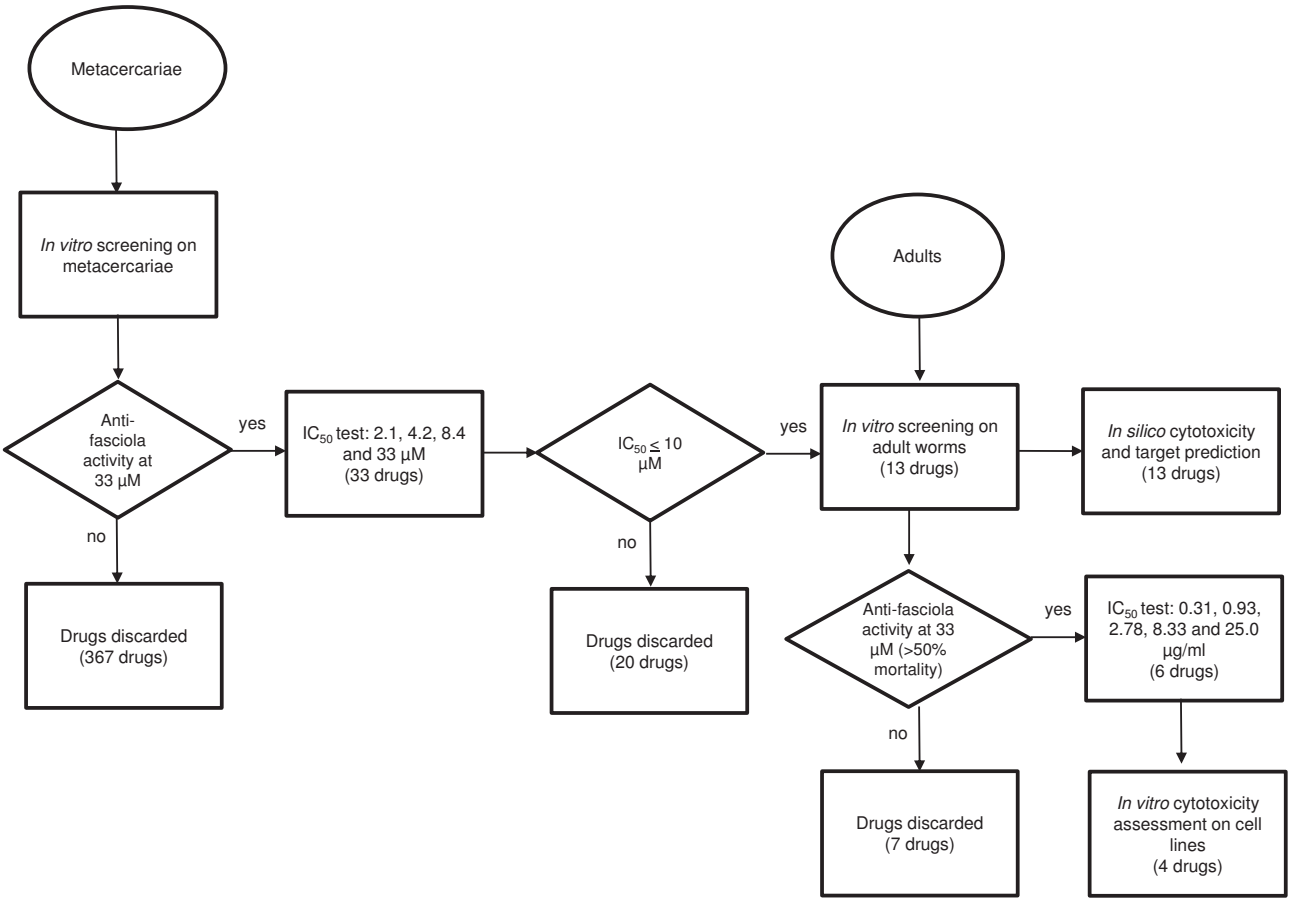


Figure 1. Flowchart of the tests carried out during the study

Table 1. Summary of the chemical compounds that showed the best biological activity against metacercariae.

Compound plate code [1]	MMV ID [2]	Molecular Formula	Molecular weight (g/mol)	Mean % mortality on MC [3]	SD(%)	IC50 μM[4]	R	Other infectious microorganisms [5]	*In silico* toxicity features [6]				
									Acute cytotoxicity (Fathead minnow)	Blood Brain Barrier Penetration (Human)	Carcinogenicity (Rodent)	Mutagenicity (*Salmonella typhimurium*)	Maximum Recommended Daily Dose (Human)
TCBZ	N.A.	C14H9Cl3N2OS	359.7	100 †	0 †	15*	N.A.	Schistosoma	4.57 (mg/L)	penetrating	non-carcinogenic	non-mutagenic	N.A.
PAA2	MMV010764	C14H16N4OS2	320.4	22	38.5	24.1	-0.3	Plasmodium	N.A.	N.A.	non-carcinogenic	non-mutagenic	N.A.
PAF4	MMV676388	C15H14N4O3S	330.4	29	24.7	16.9	0.8	Mycobacterium	254.0 (mg/L)	penetrating	carcinogenic	mutagenic	2.44 (mg/kg_bw/day)
PAF5	MMV202553	C15H15N3O2	269.3	29	24.7	14.9	0.9	Kinetoplastids	7.58 (mg/L)	penetrating	non-carcinogenic	mutagenic	0.993 (mg/kg_bw/day)
PAG6	MMV063404	C19H24N3OCl	345.9	54	7.2	5.3	1.0	Mycobacterium	N.A.	penetrating	carcinogenic	mutagenic	N.A.
PAH6	MMV676539	C20H16N2O3	332.4	17	28.9	24.7	-1.0	Mycobacterium	25.9 (mg/L)	penetrating	carcinogenic	mutagenic	4.05 (mg/kg_bw/day)
PBD3	MMV637953	C51H40N6O23S6	1435.3	25	9.9	21.8	-0.6	Trypanosoma and Onchocerca	N.A.	penetrating	non-carcinogenic	non-mutagenic	N.A.
PBD7	MMV019838	C18H10N4OF6	412.3	26	11.6	12.4	0.0	Plasmodium	N.A.	penetrating	non-carcinogenic	mutagenic	N.A.
PBF4	MMV003270	C7H5N2OCl	168.6	26	25.1	8.2	-0.7	Ancylostoma	6.75 (mg/L)	penetrating	non-carcinogenic	non-mutagenic	15.5 (mg/kg_bw/day)
PBF6	MMV688853	C19H23NSO2	389.9	25	22.5	31.9	-0.8	Cryptosporidium	N.A.	non-penetrating	non-carcinogenic	mutagenic	N.A.
PBF11	MMV085210	C22H24N3O3ClS	446.0	40	15.3	2.4	0.8	Plasmodium	N.A.	penetrating	non-carcinogenic	non-mutagenic	1.64 (mg/kg_bw/day)
PBH10	MMV676380	C18H15N4O3Cl	370.8	33	33.3	1.3	0.1	Plasmodium	132.0 (mg/L)	penetrating	non-carcinogenic	non-mutagenic	101.0 (mg/kg_bw/day)
PCA2	MMV675997	C24H29N4O2F	424.5	22	38.4	18.1	-0.2	Kinetoplastids	N.A.	penetrating	non-carcinogenic	mutagenic	1.51 (mg/kg_bw/day)
PCA6	MMV688852	C16H17N5ClF	333.8	29	37.4	17.2	-0.7	Toxoplasma	N.A.	penetrating	non-carcinogenic	mutagenic	N.A.
PCC2	MMV688508	C19H19N2O4F	358.4	26	3.7	16.9	-0.5	Mycobacterium	N.A.	penetrating	non-carcinogenic	mutagenic	N.A.
PCC5	MMV687730	C22H32N4O2	384.5	28	13.4	0.4	-0.5	Mycobacterium	N.A.	penetrating	carcinogenic	non-mutagenic	N.A.
PCC6	MMV687251	C8H9N3O4S2	275.3	30	12.0	0.3	-0.5	Mycobacterium	N.A.	penetrating	non-carcinogenic	non-mutagenic	13.3 (mg/kg_bw/day)
PCC9	MMV688361	C21H19N5O	357.4	32	11.5	17.2	-0.7	Kinetoplastids	N.A.	penetrating	carcinogenic	mutagenic	N.A.
PCC10	MMV689029	C26H26N4O4S	490.6	33	19.1	10.5	0.8	Kinetoplastids	N.A.	penetrating	carcinogenic	mutagenic	11.9 (mg/kg_bw/day)
PCD11	MMV1030799	C20H18N4O	330.4	28	11.7	1.5	-0.3	Plasmodium	6.62 (mg/L)	non-penetrating	carcinogenic	mutagenic	N.A.
PCE5	MMV687146	C19H26N2O	298.4	21	25.8	15.6	0.6	Mycobacterium	N.A.	penetrating	non-carcinogenic	mutagenic	N.A.
PCE6	MMV687696	C29H28N4O2ClF3	557.0	26	20.6	18.2	-0.7	Mycobacterium	N.A.	non-penetrating	carcinogenic	mutagenic	N.A.
PCE7	MMV687170	C17H13N4O2Cl	340.8	34	25.3	13.1	0.0	Mycobacterium	N.A.	penetrating	carcinogenic	mutagenic	N.A.
PCE8	MMV690102	C22H23N7O2	417.5	38	15.6	2.1	0.7	Kinetoplastids	N.A.	penetrating	non-carcinogenic	mutagenic	3.27 (mg/kg_bw/day)
PCE11	MMV1029203	C20H17N5OS	375.5	33	29.7	7.1	-0.4	Plasmodium	100.0 (mg/L)	penetrating	carcinogenic	mutagenic	N.A.
PCF2	MMV676053	C18H16N3O3Cl	357.8	38	12.5	1.9	0.6	Cryptosporidium	194.0 (mg/L)	penetrating	non-carcinogenic	mutagenic	0.991 (mg/kg_bw/day)
PCF3	MMV688179	C18H16N6OCl2	476.2	35	32.0	3.1	-0.1	Kinetoplastids	4.62 (mg/L)	penetrating	carcinogenic	mutagenic	1.41 (mg/kg_bw/day)
PCF4	MMV023969	C24H24N4OS	453.0	48	21.8	1.5	0.3	Mycobacterium	N.A.	N.A.	carcinogenic	mutagenic	N.A.
PCF5	MMV687138	C19H17NO3S	339.4	26	11.6	14.7	-0.2	Mycobacterium	524.0 (mg/L)	penetrating	non-carcinogenic	mutagenic	89.7 (mg/kg_bw/day)
PCF11	MMV688921	C23H18N3O5Cl	451.9	31	43.0	2.4	-0.4	Aedes aegypti - chikungunya	N.A.	penetrating	carcinogenic	mutagenic	N.A.
PCG9	MMV688891	C18H11NO4BrF3	442.2	25	10.9	25.7	-0.5	Mycobacterium	N.A.	penetrating	carcinogenic	mutagenic	1.25 (mg/kg_bw/day)
PDH11	MMV688980	C16H18N3O2FS	335.4	33	38.2	21.2	0.2	Plasmodium	N.A.	penetrating	carcinogenic	mutagenic	N.A.
PEC8	MMV687765	C25H26N6O	463	28	25.5	20.6	-0.8	Mycobacterium	N.A.	penetrating	non-carcinogenic	mutagenic	N.A.
PEG9	MMV084864	C17H12N6O	316.3	40	18.7	17.3	0.8	Plasmodium	14.2 (mg/L)	penetrating	non-carcinogenic	mutagenic	N.A.

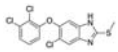
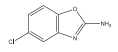
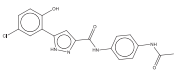
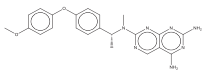
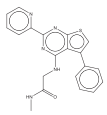
[1] Coordinates used to identify compounds in each plate. TCBZ is Triclabendazole.
[2] ID codes assigned by the Medicines for Malaria Venture (MMV) agency. N.A. is not applicable
[3] Measured at 72-hr post drug exposure on metacercariae (MC) stage. Results are the mean and standard deviation of triplicate experiments at a concentration of 33 μM.
[4] Compounds were serially diluted and tested in culture. Results are means from triplicate experiments. Fasciolicidal activity values determined by CompuSyn. R is the correlation coefficient
[5] Activity shown in agents causing others infectious diseases as obtained from www.mmv.org.
[6] Predictions using lazar program (https://lazar.in-silico.de/predict). N.A. is not available
SD is standard deviation
† Mean and standard deviation of 10 individual experiments performed in 5 plates
* Data obtained from https://drugs.ncats.io
Molecular formula and weights were obtained from www.mmv.org. For TCBZ these values were obtained from ChEMBL (https://www.ebi.ac.uk/chembl/).

Table 2. Biological activity of the compounds screened on adult worms.

Compound plate code [1]	MMV ID [2]	Mean % Mortality on adults [3]	SD (%)
TCBZ	N.A.	100 †	0
PAG6	MMV063404	67	33.3
PBF4	*MMV003270*	67	0
PBF11	MMV085210	0	0
PBH10	*MMV676380*	78	19.2
PCC5	MMV687730	11	19.2
PCC6	MMV687251	33	33.3
PCD11	MMV1030799	67	33.3
PCE8	*MMV690102*	56	19.2
PCE11	*MMV1029203*	78	19.2
PCF2	MMV676053	0	0
PCF3	MMV688179	22	19.2
PCF4	MMV023969	33	33.3
PCF11	MMV688921	67	33.3

[1] Coordinates used to identify compounds in each plate. TCBZ is Triclabendazole.
[2] ID codes assigned by the MMV agency.
[3] Measured at 48-hr post drug exposure on adult worms. Results are means and standard deviation from triplicate experiments at a concentration of 33 µM.
† Mean and standard deviation of 6 individual experiments performed in 3 plates
Compounds in italics were selected for IC50 on adult worms and cytotoxicity assay on cell lines.
N.A. is not applicable

Table 3. Hits compounds selected for their fasciocidal activity as a new effective drugs against *F. hepatica*.

Structure/Smiles[1]	Compound ID/Drug name[1]	Drug name	Molecular weight	AlogP[2]	*In vitro* fasciocidal assesment[3]		*In vitro* cytotoxicity[4]				Cytotoxicity data from other studies[5]		
					Adult IC_{50} (µM)	CI 95%	3T3 GI50 (µM)	H460 GI50 (µM)	DU145 GI50 (µM)	HT29 GI50 (µM)	HepG2 CC20 (µM)	HL60 CC50 (µM)	MRC5 CC50 (µM)
 CSC1=NC2=C(N1)C=C(Cl)C(OC1=CC=CC(Cl)=C1Cl)=C2	Triclabendazole	6-Chloro-5-{2,3-dichlorophenoxy}-2-{methylthio}-1H-benzo[d]imidazole	359.66	6		15*	22.80	32.62	35.22	37.80	N.A.	N.A.	N.A.
 ClC1=CC(N=C(N)O2)=C2C=C1	MMV003270/Zoxazolamine	2-Amino-5-chlorobenzoxazole	168.58	2.1	9.37	1.45 to 53.88	> 23.73	> 23.73	> 23.73	> 23.73	> 80	N.A.	N.A.
 CC(=O)Nc1ccc(NC(=O)c2cc([nH]n2)c3cc(Cl)ccc3O)cc1	MMV676380	N-(4-Acetamidophenyl)-3-(5-chloro-2-hydroxyphenyl)-1H-pyrazole-5-carboxamide	370.79	3.7	6.68	4.39 to 10.06	> 10.79	> 10.79	> 10.79	> 10.79	> 80	> 50	N.A.
 COc1ccc(Oc2ccc(cc2)[C@@H](C)N(C)c3ncc4c(N)nc(N)nc4n3)cc1	MMV690102	2-N-[1-[4-(4-methoxyphenoxy)phenyl]ethyl]-2-N-methylpyrimido[4,5-d]pyrimidine-2,5,7-triamine	417.46	3.6	2.14	1.16 to 4.82	4.86	0.95	9.58	11.00	2.87	N.A.	5.44
 CNC(=O)CNc1nc(nc2scc(c3ccccc3)c12)c4ccccn4	MMV1029203	N-methyl-2-[[5-phenyl-2-(2-pyridyl)thieno[3,2-e]pyrimidin-4-yl]amino]acetamide	375.45	3.58	4.32	2.82 to 6.60	> 10.65	> 10.65	> 10.65	> 10.65	22	N.A.	N.A.

[1] ID codes assigned by the MMV agency.
[2] Data obtained from the MMV agency website 'Biological activity'.
[3] Compounds were serially diluted and tested in culture. Results are mean from triplicate assays.
[4] Cytotoxicity assays on cancer cell lines 3T3, H460, DU145 AND HT29. Results are mean from duplicate tests.
[5] As obtained from www.mmv.org. HepG2 is hepatocellular carcinoma, HL60 is Human promyelocytic leukemia cells, MRC5 is fibroblasts derived from lung.
* Data obtained from https://drugs.ncats.io

Table 4. Potential targets of the 13 hits and TCBZ tested in adult worms assays.

Compound plate code [1]	MMV code [2]	Target [3]				
		Targets predicted	CHEMBL ID	Preferred name	Organism	Protein target classification
PBH10	MMV676380	3	CHEMBL2535	Glucose transporter	*Homo sapiens*	transporter > electrochemical transporter > slc superfamily of solute carriers > slc02 family of hexose and sugar alcohol transporters
			CHEMBL4697	Hexose transporter 1	*Plasmodium falciparum*	
			CHEMBL3431938	Glucose transporter	*Leishmania mexicana*	transporter
PCE11	MMV1029203	1	CHEMBL3879831	Ferrochelatase	*Homo sapiens*	unclassified protein
PCF2	MMV676053	1	CHEMBL6145	Inosine-5'-monophosphate dehydrogenase, probable	*Cryptosporidium parvum*	enzyme
PCF4	MMV023969	3	CHEMBL2535	Glucose transporter	*Homo sapiens*	transporter > electrochemical transporter > slc superfamily of solute carriers > slc02 family of hexose and sugar alcohol transporters
			CHEMBL4697	Hexose transporter 1	*Plasmodium falciparum*	
			CHEMBL3431938	Glucose transporter	*Leishmania mexicana*	unclassified protein
PBF4	MMV003270	19	CHEMBL340	Cytochrome P450 3A4	*Homo sapiens*	enzyme > cytochrome p450 > cytochrome p450 family 3 > cytochrome p450 family 3a > cytochrome p450 3a4
			CHEMBL289	Cytochrome P450 2D6	*Homo sapiens*	enzyme > cytochrome p450 > cytochrome p450 family 2 > cytochrome p450 family 2d > cytochrome p450 2d6
			CHEMBL3397	Cytochrome P450 2C9	*Homo sapiens*	enzyme > cytochrome p450 > cytochrome p450 family 2 > cytochrome p450 family 2c > cytochrome p450 2c9
			CHEMBL3622	Cytochrome P450 2C19	*Homo sapiens*	enzyme > cytochrome p450 > cytochrome p450 family 2 > cytochrome p450 family 2c > cytochrome p450 2c19
			CHEMBL3356	Cytochrome P450 1A2	*Homo sapiens*	enzyme > cytochrome p450 > cytochrome p450 family 1 > cytochrome p450 family 1a > cytochrome p450 1a2
			CHEMBL4040	MAP kinase ERK2	*Homo sapiens*	enzyme > kinase > protein kinase > cmgc protein kinase group > cmgc protein kinase mapk family > cmgc protein kinase erk subfamily

			CHEMBL2903	Arachidonate 15-lipoxygenase	*Homo sapiens*	enzyme
			CHEMBL2756	Monoamine oxidase B	*Bos taurus*	enzyme
			CHEMBL3254	Monoamine oxidase A	*Bos taurus*	enzyme
			CHEMBL1075094	Nuclear factor erythroid 2-related factor 2	*Homo sapiens*	unclassified protein
			CHEMBL1293224	Microtubule-associated protein tau	*Homo sapiens*	unclassified protein
			CHEMBL2362981	TAR DNA-binding protein 43	*Homo sapiens*	unclassified protein
			CHEMBL1293235	Prelamin-A/C	*Homo sapiens*	unclassified protein
			CHEMBL1781865	78 kDa glucose-regulated protein	*Homo sapiens*	unclassified protein
			CHEMBL1977	Vitamin D receptor	*Homo sapiens*	transcription factor > nuclear receptor > nuclear hormone receptor subfamily 1 > nuclear hormone receptor subfamily 1 group i > nuclear hormone receptor subfamily 1 group i member 1
			CHEMBL1947	Thyroid hormone receptor beta-1	*Homo sapiens*	ranscription factor > nuclear receptor > nuclear hormone receptor subfamily 1 > nuclear hormone receptor subfamily 1 group a > nuclear hormone receptor subfamily 1 group a member 2
			CHEMBL1697668	Solute carrier organic anion transporter family member 1B1	*Homo sapiens*	transporter > electrochemical transporter > slc superfamily of solute carriers > slc21/slco family of organic anion transporting polypeptides
			CHEMBL1743121	Solute carrier organic anion transporter family member 1B3	*Homo sapiens*	
			CHEMBL1741193	Chromobox protein homolog 1	*Homo sapiens*	epigenetic regulator > reader > methyl-lysine/arginine binding protein > chromodomain
TCBZ	N.A.	27	CHEMBL1293278	Geminin	*Homo sapiens*	unclassified protein
			CHEMBL1075094	Nuclear factor erythroid 2-related factor 2	*Homo sapiens*	unclassified protein
			CHEMBL1293224	Microtubule-associated protein tau	*Homo sapiens*	unclassified protein
			CHEMBL1293258	Mothers against decapentaplegic homolog 3	*Homo sapiens*	unclassified protein
			CHEMBL2362981	TAR DNA-binding protein 43	*Homo sapiens*	unclassified protein

CHEMBL2146310	Aberrant vpr protein	*Human immunodeficiency virus 1*	unclassified protein
CHEMBL2029198	Rap guanine nucleotide exchange factor 4	*Homo sapiens*	unclassified protein
CHEMBL6152	Alpha-synuclein	*Homo sapiens*	unclassified protein
CHEMBL1293191	Transcriptional regulator ERG	*Homo sapiens*	unclassified protein
CHEMBL2007624	Peripheral myelin protein 22	*Rattus norvegicus*	unclassified protein
CHEMBL1795086	HSP90	*Plasmodium falciparum 3D7*	unclassified protein
CHEMBL5567	Luciferin 4-monooxygenase	*Photinus pyralis*	enzyme
CHEMBL2007625	Isocitrate dehydrogenase [NADP] cytoplasmic	*Homo sapiens*	enzyme
CHEMBL3563	Cruzipain	*Trypanosoma cruzi*	enzyme > protease > cysteine protease > cysteine protease ca clan > cysteine protease c1a family
CHEMBL1293248	4'-phosphopantetheinyl transferase ffp	*Bacillus subtilis*	enzyme
CHEMBL1795087	Ubiquitin carboxyl-terminal hydrolase 1	*Homo sapiens*	enzyme
CHEMBL1293234	Putative fructose-1,6-bisphosphate aldolase	*Giardia intestinalis*	enzyme
CHEMBL1293228	Streptokinase A	*Streptococcus pyogenes serotype M1*	enzyme > kinase
CHEMBL2524	Alpha-galactosidase A	*Homo sapiens*	enzyme
CHEMBL1784	Glucagon-like peptide 1 receptor	*Homo sapiens*	membrane receptor > family b g protein-coupled receptor > peptide receptor (family b gpcr) > glucagon-like receptor > glucagon-like peptide receptor
CHEMBL1793	Parathyroid hormone receptor	*Homo sapiens*	membrane receptor > family b g protein-coupled receptor > peptide receptor (family b gpcr) > parathyroid hormone receptor > parathyroid hormone receptor
CHEMBL5162	Neuropeptide S receptor	*Homo sapiens*	membrane receptor > family a g protein-coupled receptor > peptide receptor (family a gpcr) > short peptide receptor (family a gpcr) > neuropeptide receptor

			CHEMBL1293231	Nuclear receptor ROR-gamma	*Mus musculus*	transcription factor > nuclear receptor > nuclear hormone receptor subfamily 1 > nuclear hormone receptor subfamily 1 group f > nuclear hormone receptor subfamily 1 group f member 3
			CHEMBL1871	Androgen Receptor	*Homo sapiens*	transcription factor > nuclear receptor > nuclear hormone receptor subfamily 3 > nuclear hormone receptor subfamily 3 group c > nuclear hormone receptor subfamily 3 group c member 4
			CHEMBL3880	Heat shock protein HSP 90-alpha	*Homo sapiens*	other cytosolic protein
			CHEMBL6032	Histone-lysine N-methyltransferase, H3 lysine-9 specific 3	*Homo sapiens*	epigenetic regulator > writer > protein methyltransferase
			CHEMBL4377	Guanine nucleotide-binding protein G(s), subunit alpha	*Homo sapiens*	other membrane protein
PAG6	MMV063404	No target	N.A.	N.A.	N.A.	N.A.
PCC5	MMV687730	No target	N.A.	N.A.	N.A.	N.A.
PCC6	MMV687251	No target	N.A.	N.A.	N.A.	N.A.
PCD11	MMV1030799	No target	N.A.	N.A.	N.A.	N.A.
PCE8	MMV690102	No target	N.A.	N.A.	N.A.	N.A.
PBF11	MMV085210	No target	N.A.	N.A.	N.A.	N.A.
PCF3	MMV688179	No target	N.A.	N.A.	N.A.	N.A.
PCF11	MMV688921	No target	N.A.	N.A.	N.A.	N.A.

[1] Coordinates used to identify compounds in each plate

[2] ID codes assigned by the MMV agency.

[3] By consulting ChEMBL.

N.A is not available