**Repurposing Infectious Disease Hits as Anti-*Cryptosporidium* Leads**

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**ABSTRACT**

New drugs are critically-needed to treat *Cryptosporidium* infections, particularly for malnourished children under 2 years old in the developing world and persons with immunodeficiencies. Bioactive compounds from the Tres-Cantos GSK library that have activity against other pathogens were screened for possible repurposing against *Cryptosporidium parvum* growth. Nineteen compounds grouped into 9 structural clusters were identified using an iterative process to remove excessively toxic compounds and screen related compounds from the Tres-Cantos GSK library. Representatives of four different clusters were advanced to a mouse model of *C. parvum* infection, but only one compound, an imidazole-pyrimidine, led to significant clearance of infection. This imidazole-pyrimidine compound had a number of favorable safety and pharmacokinetic properties and was maximally active in the mouse model down to 30 mg/kg given daily. Though the mechanism of action against *C. parvum* was not definitively established, this imidazole-pyrimidine compound inhibits the known *C. parvum* drug target, calcium-dependent protein kinase 1, with a 50% inhibitory concentration of 2 nM. This compound, and related imidazole-pyrimidine molecules, should be further examined as potential leads for *Cryptosporidium* therapeutics.

**Keywords:** *Cryptosporidium*, cryptosporidiosis,N-carbamoylazole, imidazole-pyrimidine, calcium dependent protein kinase 1, cyclic GMP-dependent protein kinase *Cryptosporidium* is one of the leading causes of diarrhea in children ages 6 to 18 months in Africa and Asia 1. Cryptosporidiosis leads to high mortality and prolonged developmental delays and malnourishment among this target age group 1-2. Healthy adults normally self-cure after a prolonged period of infection and watery diarrhea. However, patients with compromised immune systems, such as HIV positive individuals, the disease becomes chronic, infects the biliary tract, probably leading to relapses after therapy, and debilitating diarrhea often results in eventual death 3. The only approved drug for *Cryptosporidium*, Nitazoxanide, has poor efficacy for malnourished children and immunocompromised individuals and is not approved for use in children less than one year-old 4. Oocysts that are spread into the environment through fecal shedding are resistant to chlorine treatment, making them a leading cause of diarrheal infection even in the developed world through contaminated drinking water or recreational water use 5. New treatments are still needed, and progress in drug development research has been recently made due to advances in in vitro screening procedures and genetic manipulations of the parasite 6-9.

A common and often utilized tactic for discovering new therapies is repurposing of already approved drugs. This tactic provides a potential path to rapidly find new therapies that have already been tested for safety in humans. Many active compounds have been found to be effective against infectious agents not related to their original treatment purpose. Examples are: the chemotherapy drug Tipifarnib 10 and the antifungal Posaconazole 11, both of which are also potent inhibitors of *Trypanosoma cruzi* by means of sterol synthesis inhibition; the arthritis drug Auranofin, which is potent against *Entamoeba histolytica, Giardia,* and *Cryptosporidium* 12-13; the leprosy drug Clofazimine, which is potent against both breast cancer cells 14 and *Cryptosporidium* 7, 13; and Disulfiram, used to treat alcohol dependence, but is also potent against non-tuberculosis mycobacteria 15. Criticisms of the repurposing approach include the rarity with which repurposing is successful, perhaps because activity against an organism found by in vitro screening doesn’t necessarily lead to activity in vivo16. Indeed, Posaconazole for therapy of *T. cruzi* and Clofazimine for cryptosporidiosis both recently failed in clinical trials 17-18.

The repurposing method may prove useful to find leads from compounds that are biologically active “hits” against other microorganisms. Repurposing infectious diseases hit compounds may be even more useful when applied among related organisms, such as apicomplexan parasites. Apicomplexans include *Toxoplasma*, *Babesia*, *Plasmodium*, and *Cryptosporidium* and are among the most prevalent parasites to infect both humans and livestock. Due to their close phylogenetic relationship, drug targets are likely preserved to the extent that the same drug or drug class could be safe and effective for therapy against multiple apicomplexa diseases 19. An additional advantage is that a hit or a lead that was abandoned for a pharmacological reason, such as inadequate systemic exposure for therapy of *Toxoplasma* or *Plasmodium*, might be useful for an infection with different pharmacological requirements, such as *Cryptosporidium* or *Giardia*, which are isolated to the gastrointestinal tract and would not theoretically require large systemic exposures 6, 8, 20. If the hit compound properties are a fit for a new indication, then otherwise unused research results can be repurposed for a different disease.

To examine and identify possible hits and leads for candidates for repurposing for the therapy of cryptosporidiosis, a subset of the GlaxoSmithKline (GSK) Bioactives library known to inhibit other microorganisms, including *Plasmodium falciparum (Pf)*, *Trypanosoma brucei (Tb)*, *Mycobacterium tuberculosis (Mtb)*, *Leishmania donovani*, and *Trypanosoma cruzi*, was screened against *C. parvum* (*Cp*) in vitro and *a* *Cp*-infected mouse model. Several compounds were identified that inhibited multiple organisms and were potential treatment candidates for *Cryptosporidium*, with one, compound **1**, emerging as a very promising cryptosporidiosis candidate.

**RESULTS**

**Screening of Bioactives Library against *Cryptosporidium parvum***. To search for new *Cryptosporidium* treatment leads, assess possible mechanisms of action using compound hits with known targets for other apicomplexan parasites, and search for possible multi-parasite treatments, a subset of the GSK Library with known bioactive activity was screened for inhibition of Nanoluciferase (Nluc)-expressing *Cp* growth in HCT-8 cells (Figure 1).

**Figure 1:** **High level analysis of the screening cascade employed with the Tres-Cantos Bioactive Library against *Cryptosporidium parvum.*** Compounds were initially screened at a single concentration of 10 µM. Cmpds = compounds, Inh = inhibition, #Ar = number of fused aryl rings.

Compounds were selected based on availability, quality control compliance, previous screening and activity against *Pf*, *Tb*, *Mtb*, *L. donovani*, and *T. cruzi*, and structural diversity. A total of 2199 compounds were screened in duplicate at an initial single concentration of 10 µM. Each well was observed microscopically, 66 compounds displayed microscopically-observable toxicity to the HCT-8 cell monolayer, and these 66 compounds were removed from further consideration. Two hundred and forty-six compounds with > 80% *Cp* growth inhibition in both replicates, were observed, a hit rate of 11.1%. Compounds displaying a >80 % inhibition were moved to in vitro dose response to determine the effective concentration to inhibit *Cp* growth by 50% (EC50). Also, an additional 76 compounds from the original screen were retested in dose response after showing > 80% inhibition in at least 1 replicate. These hits were analyzed for structure-activity relationships (SAR) and an additional 260 compounds with structural-similarity were screened for hit expansion from available analogs within the GSK library. Thus, a total of 582 compounds were tested in dose response and the most potent compounds were also tested for 50 % cytotoxic concentration (CC50) against to growing human liver derived HepG2 cells using a luminescence based assay to measure acute cytotoxicity. The basis of this assay is to quantitate intracellular ATP concentrations after exposure to compounds; mitochondrial function and cell redox state are both markers for cell health, and impairment of either results in a decrease in intracellular ATP. The relatively short treatment time of cells with compound (24-48 hours) allows acutely toxic compounds to be identified. Results showed 19 compounds with an EC50 value of < 1 µM, number of fused aryl rings <3, and a ratio of *Cp* EC50 to HepG2 CC50 of ≥ 10. These 19 compounds were divided into 9 clusters, based on structural similarities, with varied absorption, distribution, metabolism, and excretion (ADME) profiles. One or more representative compounds were chosen from each cluster for possible further evaluation (Figure 2), based on inhibition of *Cp*, inhibition of at least one other pathogen screened with known targets for that pathogen, and cytotoxicity (Figure 2).

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**Figure 2: Hits in 9 different clusters identified from the Tres-Cantos Bioactives library.** 5 compounds were available to be tested in vivo. EC50 (μM) represents the compound concentration to give a 50% reduction in growth for Cp (*Cryptosporidium parvum)*. HepG2 CC50 (μM) represents the compound concentration to give 50% growth inhibition of the mammalian cell line, HepG2.

An additional screen for *Cp* EC50 was performed on compounds from 5 of these clusters using high-content imaging on 3 different strains of *Cp* and one strain of *C. hominis* (Table 1). Some variation was observed between the strains, but acceptable potency was seen for all 5 of these compounds on all *Cryptosporidium* strains tested. Such variation is commonly observed between different strains of *Cryptosporidium*  21.

**Table 1. 50% effective dose against multiple *Cryptosporidium* species as determined by high-content imaging.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **EC50 (μM)** | | | |
| Compound | Sterling Lab *C. parvum* | Bunch Grass Farm *C. parvum* | Wild *C. parvum* | *C. hominis* |
| **1** | 0.29 | 1.3 | 0.0031 | 1.4 |
| **7** | 0.0028 | 0.0017 | 0.0028 | 0.021 |
| **6** | 0.0010 | 0.0005 | 0.0004 | 0.0030 |
| **10** | 0.066 | 0.022 | 0.0044 | 0.049 |
| **9** | 0.39 | 0.91 | 0.44 | 0.78 |

**SAR for inhibition of *Plasmodium* or *Cryptosporidium* – case study cluster 2**. Cluster 2, which is based on a N-carbamoylazole core (compounds **2** and **3**), is of note because its consistent SAR against the apicomplexa *Pf* make of this chemical series an attractive starting point for a parasite-hopping strategy 22. In addition, this core was interesting due to their efficacy on increasing glucose tolerance by inhibiting Dipetptidyl peptidase IV (DPP-IV) which is mainly located on the brush border epithelium of the small intestine 23. It is an open debate in the crypto community if systemic drug exposure is needed to control the infection, therefore, a highly-metabolizable scaffold in blood, like the N-carbamoylazole series, but with a good residence time in the intestine, could be an attractive chemical series to treat cryptosporidiosis, as potential side effects due to distribution in the organisms would be mitigated.21 For this purpose 40 N-carbamoylazole compounds from the GSK collection with varying *Pf* EC50s between 100 µM and 10 nM 22 were compared against their *Cp* EC50s (Figure 3). Of these 40 compounds, 24 had *Cp* EC50s of ≤ 10 µM. SAR differs between the *Pf* and *Cp* and no correlation was found between the potencies of the two apicomplexa species. *In vivo* activity is discussed in the next section.

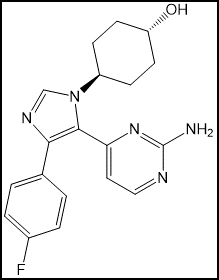
**Figure 3: N-carbamoylazole compounds’ structure-activity relationship (SAR) comparing activity (pEC50) against *P. falciparum* (Y-axis)and *C. parvum (X-axis).***Forty N-Carbamoylazole compounds were tested and 24 inhibited *Cp* growth at <10 μM (pEC50 >5.0). There was no clear *Pf-Cp* SAR correlation. Two compounds were progressed to in vivo *Cp* testing (Compound 2 and 3).

**Efficacy in *C. parvum* infected mice.** Five representative compounds were chosen to be tested for efficacy in interferon-γ knock out (IFN-γ KO) mice infected with Nluc expressing *Cp*. Compounds were chosen based on availability from 4 of the 9 clusters. Compounds **2** and **3** were chosen from the aforementioned N-carbamoylazoles. Compound **3**is a known protease inhibitor and was used as lead optimization candidate against Malaria 22. However, this compound showed low stability in mouse blood without protease inhibitors 22 which severely limits it as a potential malaria treatment. Its analog, compound **2** 22, was also potent against *Pf* and had improved metabolic stability over **3**. Compound **3** had a *Cp* EC50 of <100 nM and **2** had a *Cp* EC50 of 400 nM. Since plasma exposure and stability are not likely to effect the efficacy of a gastrointestinal infection like *Cryptosporidium*, **3** was dosed at 100 mg/kg and **2** was dosed at 60 mg/kg, both once daily by oral gavage (Figure 4A). Three additional compounds, including *Pf* and *Tb* inhibitor **1**, *Pf* inhibitor **6** and *Pf* and *Tb* inhibitor **7**, were also orally dosed at 60 mg/kg once daily. All mice were dosed on days 6 through 10 post infection (PI) to allow the infection to become fully established 6.

The compounds showed varied levels of efficacy (Figure 4A). Vehicle only treated control mice showed a 2 log rise in infection from day 6 to 15 PI and gradually lost weight until they needed to be euthanized for 20% weight loss in accordance with institutional animal use guidelines. Gradual weight loss resulting from infection and dehydration, with associated declines in overall health are the typical observations for ineffective treatments in this assay 6, 24. BKI-1369 was administered as a positive control for efficacy 21.

**Figure 4. Compound efficacy in adult female interferon-γ knockout mice (n=3) infected with nanoluciferase expressing *C. parvum*. A**. Initial efficacy testing of Compounds **1**, **2**, **3**, **6**, and **7** at a single concentration for each. **B.** Dose response efficacy of compound **1**. **C.** Plasma concentrations of compound **1** taken before and after 4 doses on day 9 post infection.

N-carbamoylazoles **2** and **3** not only showed no signs of efficacy, the infection levels rose more rapidly and the mice lost weight and showed deteriorating signs of health when compared to the control group and had to be euthanized at 20% weight loss on days 8 and 9 PI, respectively. Infection levels in mice dosed with **6** stayed approximately the same as the control group, but these mice also lost weight slightly faster than controls and had to be euthanized for 20% weight loss on day 10 PI. **7** held infection levels steady through the dosing period with a slow decline in infections through day 20 PI, but again, inferior to BKI-1369. **1**, an imidazole-pyrimidine compound, which was also a potent in vitro inhibitor with an EC50 of 49 ± 9 nM (Table 2), proved to be the most efficacious compound, matching BKI-1369 with a rapid decline in infection that remained low with a 4.1 log reduction over untreated controls by day 20 PI.

**Table 2. In vitro properties of compound 1.**

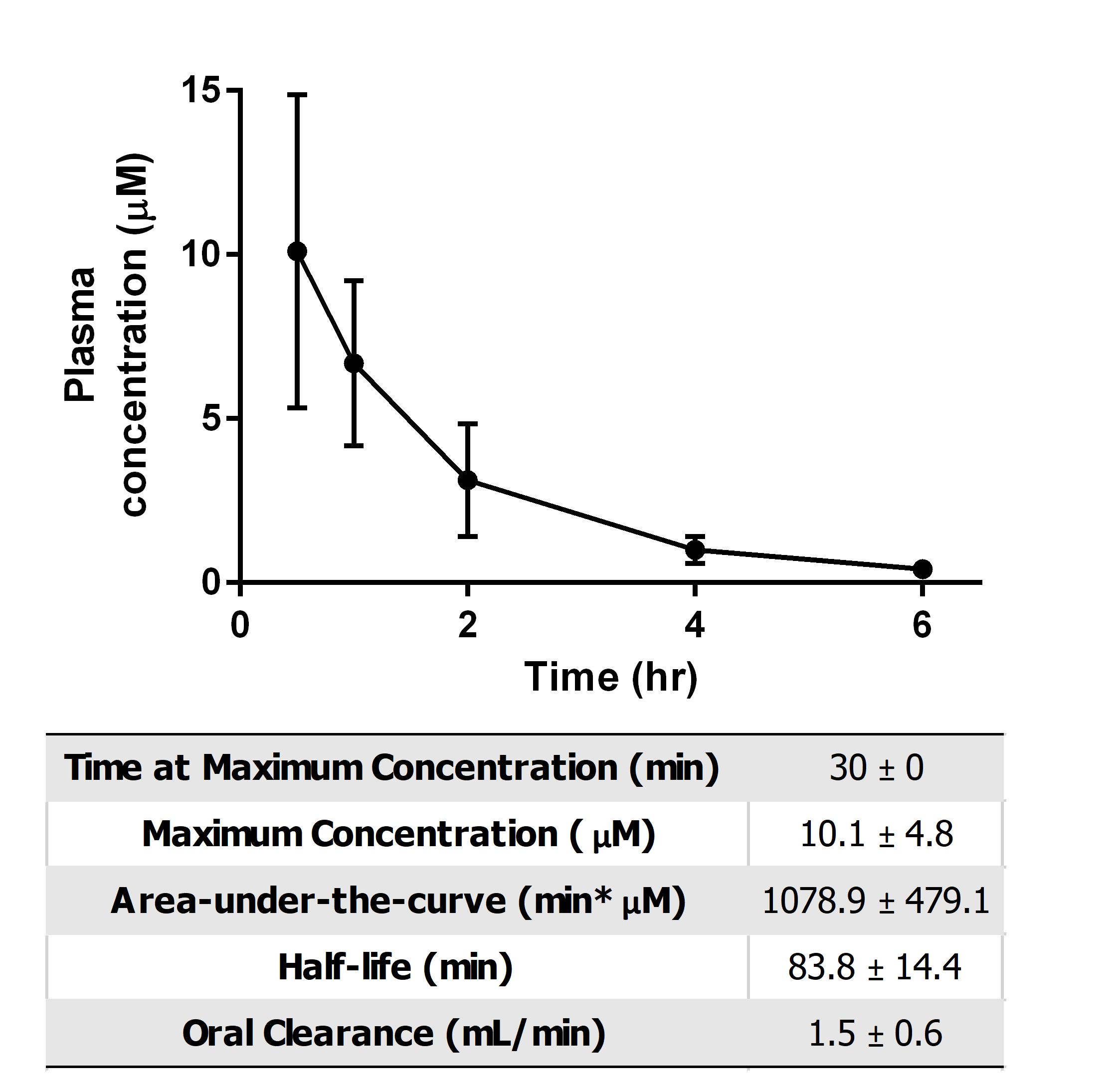
|  |  |
| --- | --- |
| **Cellular Inhibition** | **EC50 (μM)** |
| Nluc expressing *Cp* | 0.049 |
| **Enzyme Inhibition** | **IC50 (μM)** |
| *Pf* PKG WT25 | 0.08 |
| *Pf* PKG T-Q mutant25 | >100 |
| *Pf* CDPK1 | 0.07 |
| Human PKG25 | >100 |
| *Cp* CDPK1 WT | 0.002 |
| *Cp* CDPK1 G-M mutant | 6.2 |
| **Cardiotoxicity** | **IC50 (μM)** |
| hERG | >50 |
| NaV1.5 | >100 |
| **Kinase antagonism** | **IC50 (μM)** |
| AuroraB25 | 2.5 |
| PI3Kγ25 | 31.6 |
| LCK25 | 1.25 |
| **Cytotoxicity** | **CC50 (μM)** |
| HepG2 | 19 |
| HEK293T | >40 |
| HCT-8 | >25 |
| **Solubility** | (**μM)** |
| FeSSIF pH 5 | 116 |
| FaSSIF pH 6.5 | 56 |
| PBS pH 7.4 | 45 |
| **Liver Microsome Stability** | **Clearance (mL/min/g)** |
| Rat | 0.4894 |
| Human | 0.397 |
| Mouse | 0.7565 |

**Compound 1 inhibits kinases in Malaria and *Cryptosporidium***. Compound **1** was examined for inhibition of *Pf* kinases including calcium dependent protein kinase 1 (CDPK1) and cyclic GMP-dependent protein kinase (PKG). **1** had had an IC50 of 0.07 µM against *Pf* CDPK1 (Table 2) and 0.12 µM against *Pf* PKG 25. Regarding the latter, the binding mode was explored by testing the compound against a mutant enzyme at the gatekeeper. A shift from EC50 0.07 µM to > 100 µM was detected, demonstrating a clear interaction with the gatekeeper pocket 25.

Assuming these kinase targets retain high amino acid sequence homology from malaria to *Cryptosporidium*, enzyme assays were performed on wild-type and mutant strains of *Cp* CDPK1. **1** showed an IC50 of 0.002 μM for the wild-type *Cp* CDPK1 enzyme and 6.2 μM for the *Cp* CDPK1 enzyme with a Glycine to Methionine gatekeeper mutation (Table 2) 26-28. The generation of a *Cp* strain of parasites with a mutation to the CDPK1 gatekeeper residue has been unsuccessful, presumably because mutated G->M gatekeeper residue strains are non-viable, leading to the assumption that the wild-type CDPK1 is essential to parasite survival (unpublished data). Nonetheless, the large shift in IC50 between the wild type and mutant enzymes suggests that *Cp* CDPK1 enzyme is targeted at or near the gatekeeper by this compound. Unfortunately, despite many efforts, an active *Cp* recombinant PKG enzyme could not be produced, so no IC50 assays could be performed.

**Further characterization of compound 1**. A dose-response efficacy of compound **1** was performed in *Cp* infected IFN-γ KO mice to repeat the 60 mg/kg QD dose and establish how low of a dose could be used for successful treatment (Figure 4B). Both the 60 mg/kg and 30 mg/kg QD doses were highly efficacious, reducing the infection levels by the end of the dosing period (day 10 PI). The 15 and 5 mg/kg QD doses were slower to show an effect, but both resulted in substantial decreases in infection, with 15 mg/kg giving a 2.3 log reduction and 5 mg/kg a 1.5 log reduction, compared to the untreated control group, by day 20 PI. Plasma was sampled for all groups around the 4th dose on day 9 PI and compound concentration was measured by analysis on LC-MS/MS (Figure 4C).

Three female BALBc mice were dosed with a single oral dose at 25 mg/kg and plasma was sampled at various time points for 24 hours for pharmacokinetic (PK) analysis (Figure 5). The resulting average maximum concentration (Cmax) of 10.1 μM was over 20 times the compound’s EC50. Although the free concentration of compound **1** is likely to be substantially lower, average total plasma concentrations remained at or above 8 times the EC50 for at least 6 hours post dose.

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**Figure 5. Single oral dose pharmacokinetics of compound 1 at 25 mg/kg in uninfected adult female Balb/C mice (n=3).**

A number of in vitro assays were also performed to check for possible toxicity liabilities and ADME properties of **1** (Table 2). No cardiotoxicity was indicated from the 2 assays performed on hERG or NaV1.5. All 3 cell lines tested resulted in CC50s above 25 μM. Finally, LCK (tyrosine kinase), Phosphoinositide-3-kinase gamma-(PI3Kγ) (lipid kinase) and Aurora B (serine/threonine kinase) were chosen to represent the phylogenetic diversity of kinases. As there is wide sequence overlap between the conserved ATP binding site of kinases and hence crossover of inhibitory activity to other kinases, the behavior of **1** against these set can offer a prediction of the promisquity of the scaffold. It was observed that none were sub-micromolar. Compound **1** showed good solubility at 3 different biorelevant media modelling fasting state (FaSSIF), fed state (FeSSIF) and physiological (PBS) pHs and pooled liver microsome metabolism clearance rates were determined for mice, rats, and humans to be low.

**DISCUSSION**

By repurposing actives for other infectious indications, 246 compounds were identified that were initial hits against *Cp* growth in vitro, without noticeable toxicity to the mammalian cells in which the *Cp* is grown. The GSK libraries allowed for the selection of structurally related compounds, to obtain SAR, and thus 582 compounds were tested in dose response. Nineteen compounds were identified, representing 9 structurally related clusters, with an EC50 of < 1 µM on *Cp* proliferation and a selectivity index (SI) of >10 for mammalian liver cell line proliferation.

The largest cluster of anti-*Cp* hits in GSK libraries was composed of N-carbamoylazole compounds. Twenty-four of 42 of the N-carbamoylazoles were active with an EC50 of <10 μM on *Cp* proliferation. These compounds had previously been explored for malaria therapy. However, the SAR for inhibition of *Pf* appears distinct from *Cp,* even though both are related apicomplexa parasites. This difference in SAR could be due to distinct target engagement, distinct difference on penetration into the different host cells (erythrocytes vs. intestinal epithelial cells), or even differences in pumps and transporters between the two parasites. Two N-carbamoylazole compounds, **2** and **3**, were tested in the mouse model of *Cp* infection, but both failed to clear parasites substantially and even appeared to accelerate weight loss of *Cp*-infected animals. Some N-carbamoylazoles have been shown to be potent dipeptidyl peptidase (DPP)-IV inhibitors, with U20036 showing a DPP-IV IC50 of 9 nM. DDP-IV inhibitors have been shown to limit glucose uptake and cause weight loss in rodents 29. This may explain the accelerated decline in health among mice treated with these compounds in the nLuc-*Cp* mouse efficacy model, which already displays weight loss from infection as one of the primary indications of health decline in control and unsuccessfully-treated mice 6, 24.

Despite six compounds from four different scaffolds tested in the mouse model of *Cp*, only **1**, an imidazole-pyrimidine compound, led to a significant in vivo activity to reduce *Cp* excretion. Maximal *Cp* activity could be seen even down to 30 mg/kg administered daily. **1** had a number of favorable safety attributes.

*Cryptosporidium* infection is typically localized to the gut and systemic exposures have been previously shown to lack correlation to efficacy 6, 8, 20, 24. However, the PK parameters demonstrating systemic exposure after oral dosing are helpful in determining some of the compound’s ADME properties as well as being helpful in establishing therapeutic windows between exposure and potential toxicities. For example, the ample systemic exposure in mice combined with the clearance rates in human liver microsomes being almost half that of mouse liver microsomes (Table 2), indicates that the compound **1** would likely remain stable enough in humans to provide prolonged exposure, which may be beneficial for efficacy against *Cp* in vivo 21.

Solubility is likely not a concern with **1** as it was high enough to not affect the efficacy results, as has been seen with other compound classes with lower solubility 24. All of the preliminary assays testing for toxicity indicators, including the tested mammalian cell lines for cytotoxicity, and assays to evaluate cardiotoxicity channels, are well above the highest total plasma concentration of 9.98 μM from the efficacious dose of 30 mg/kg, leaving the compound a reasonable range between toxicity and exposure, especially since the free plasma levels of compound 1 are likely to be substantially lower. The low number of kinases that showed activity and the fact that none were sub-micromolar is also a good indication that treatment with **1** can avoid toxic side effects during treatment.

Although the mechanism of action of **1** on *Cp* is unclear, it is notable that it has an IC50 of 2 nM against *Cp* CDPK1, suggesting this known drug target for *Cp* could be the target for **1***.*  Nonetheless, recombinant *Cp* PKG could not be obtained and so the relative potency of **1** on that potential target was not determined. Thus, it remains possible that **1** inhibits multiple protein kinase targets in *Cp.*

There was strong inhibition of *Pf* CDPK1 in vitro by **1**, but no CDPK1 mutations were observed in the **1**-resistant *Pf* strain, only mutations in *Pf* PKG. Though *Pf* CDPK1 has been shown to be important for asexual growth, *Pf* parasites grown in human erythrocytes with mutated CDPK1 have shown that certain PKG signaling pathways can compensate for abnormal CDPK1 expression 31-32. This would result in reduced pressure on *Pf* CDPK1 to mutate in the erythrocyte culture, even in the presence of a CDPK1 inhibitor, as the PKG signal pathways would be compensating and allowing for continued growth. However, in this case *Pf* PKG is also targeted, and an EC50 shift results after the *Pf* PKG became mutated. This result demonstrates how target identification may fail to identify certain targets, especially with compounds that act on multiple targets or targets that are only essential during certain stages of a life cycle.

The potential targeting of **1** against multiple protein kinases could strengthen **1** as a potential therapeutic, from the standpoint of reduced chances of generating resistance. A compound that is active against multiple targets should be less likely to have resistant parasites arise from its use, as mutations would presumably have to develop in both targets simultaneously. Also, with regards to *Cryptosporidium*, **1** is active on *Cp* CDPK1 and all attempts to make a *Cp* strain with mutations to CDPK1 have led to non-viable parasites, potentially lowering the likelihood that a resistant population with a CDPK1 mutation could arise.

**METHODS**

Methods have been previously reported for determining *C. parvum* inhibition and EC50s for both Nluc expressing *Cp* in HCT-8 cells and *Cp* high content imaging in HCT-8 cells 6, 9, efficacy in *Cp* infected IFN-γ KO mice 6, enzyme IC50s 27, mouse PK 33, liver microsomal stability 33, solubility 33, cytotoxicity 33-34, hERG 35-36, kinases 37-39, and screening with high-content imaging 7.

**Animal Ethics**. All studies were conducted in accordance with the UW or GSK Policy on the Care, Welfare and Treatment of Laboratory Animals and were reviewed the Institutional Animal Care and Use Committee either at UW, GSK, or by the ethical review process at the institution where the work was performed. The human biological samples were sourced ethically, and their research use was in accord with the terms of the informed consents under an IRB/EC approved protocol

**ASSOCIATED CONTENT**

Supporting Information on compound characterization. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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**Supporting Information**

**Repurposing Infectious Disease Hits as Anti-*Cryptosporidium* Leads**

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LC-MS analyses for purity were performed using a diode-array detector and an ESI ion source. Signal separation was carried out by use of Acquity UPLC BEH C18 column (1.7 μm, 3.0 mm × 50 mm) or Luna C18 (5 μm, 4.6 mm × 50 mm), eluent: 25 mM NH4OAc + 10% MeCN at pH 6.6/MeCN (gradient run 100:0 → 10:90 for Acquity and 100:0 → 0:100 for Luna), and flow = 0.8 mL/ min. Purity of the all tested compounds was 95% or higher.

**Characterization of compounds from GSK corporate compound collection tested *in vivo***

**Compound 1**: 1H NMR (400 MHz, DMSO-*d*6) δ ppm = 8.18 - 8.25 (m, 1 H) 7.94 - 8.02 (m, 1 H) 7.38 - 7.50 (m, 2 H) 7.07 - 7.18 (m, 2 H) 6.76 - 6.87 (m, 2 H) 6.39 - 6.46 (m, 1 H) 4.59 - 4.71 (m, 1 H) 4.20 - 4.35 (m, 1 H) 3.40 - 3.57 (m, 1 H) 1.65 - 2.00 (m, 6 H) 1.12 - 1.34 (m, 2 H). 13C NMR (DMSO-d6, 101 MHz) δ ppm = 164.3, 162.8, 160.4, 159.3, 158.8, 139.1, 136.4, 131.5, 129.5, 125.9, 115.5, 111.5, 68.3, 54.7, 34.8, 31.9. [M+H]+ : 354

**Compound 2**: 1H NMR (400 MHz, CHLOROFORM-d) δ ppm = 8.84 (brs, 1H), 6.86-7.43 (m, 1H), 6.44 (brs, 2H), 4.52-4.99 (m, 2H), 3.80 (s, 6H), 3.50-3.97 (m, 1H), 2.85-3.38 (m, 3H), 2.75 (brs, 1H), 2.41 (brs, 1H), 2.18-2.35 (m, 1H), 1.30-1.96 (m, 7H). [M+H]+ : 435

**Compound 3:** 1H NMR (400 MHz, CHLOROFORM-d) δ ppm = 8.83 (s, 1H), 4.57 (brs, 1H), 3.68-3.84 (m, 1H), 3.06 (brs, 3H), 2.76 (brs, 1H), 2.43 (brs, 1H), 2.21-2.37 (m, 1H), 1.87 (d, J = 6.8 Hz, 2H), 1.56-1.76 (m, 1H), 1.38-1.54 (m, 4H), 1.28 (d, J = 6.6 Hz, 6H). [M+H]+ : 327

**Compound 6**: 1H NMR (DMSO-d6, 400 MHz) δ 12.62 (s, 1H), 9.74 (s, 1H), 9.36-9.38 (m, 1H), 8.4 (s, 1H), 8.30-8.32 (m, 2H), 8.01 (m, 1H), 7.65-7.67 (m, 2H), 7.56-7.59 (m, 1H), 7.35-7.38 (m, 2H), 4.4 (s, 2H), 2.9 (s, 3H). [M+H]+ : 433

**Compound 7** : 1 H NMR (400 MHz, METHANOL-d4) δ ppm 8.82 - 8.90 (m, 1 H), 8.19 - 8.35 (m, 1 H), 7.73 - 7.82 (m, 1 H), 7.61 - 7.70 (m, 1 H), 7.38 - 7.48 (m, 1 H), 7.20 - 7.26 (m, 1 H), 7.14 - 7.19 (m, 1 H), 7.07 - 7.14 (m, 1 H), 6.95 - 7.01 (m, 1 H), 4.00 (s, 3 H), 3.84 (s, 3H), 2.97 (s, 3H). [M+H]+ : 464

**Compounds not tested in vivo were characterized by LC-MS:**

|  |  |
| --- | --- |
| **Compound** | **[M+H]+** |
| **4** | 693 |
| **5** | 333 |
| **8** | 403 |
| **9** | 343 |
| **10** | 330 |
| **11** | 269 |