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1 **From drug screening to target deconvolution: A target-based drug discovery**
2 **pipeline using *Leishmania casein kinase 1 isoform 2* to identify compounds with**
3 ***anti-leishmanial activity*.**

4
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21

22 Running Title: *Leishmania CK1.2 drug screening*

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27 **Keyword:** Casein kinase I, Leishmaniasis, Therapeutic target, drug screening

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32

33 **ABSTRACT**

34 Existing therapies for leishmaniasis present significant limitations, such as toxic side
35 effects, and are rendered inefficient by parasite resistance. It is of utmost importance to
36 develop novel drugs targeting *Leishmania* that take these two limitations into
37 consideration. We thus chose a target-based approach using an exo-protein kinase,
38 *Leishmania* casein kinase 1.2 (LmCK1.2) that was recently shown to be essential for
39 intracellular parasite survival and infectivity. We developed a four-step pipeline to
40 identify novel selective anti-leishmanial compounds. In step 1, we screened 5018
41 compounds from kinase-biased libraries with *Leishmania* and mammalian casein kinase 1
42 in order to identify hit compounds and assess their specificity. For step 2, we selected 88
43 compounds among those with the lowest IC₅₀ to test their biological activity on host-free
44 parasites using a resazurin reduction assay and on intramacrophagic amastigotes using a
45 high content phenotypic assay. Only seventy-five compounds showed anti-leishmanial

46 activity and were retained for step 3 to evaluate their toxicity against mouse macrophages
47 and human cell lines. The four compounds that displayed a selectivity index above 10
48 were then assessed for their affinity to LmCK1.2 using a target deconvolution strategy in
49 step 4. Finally, we retained two compounds, PP2 and compound 42, for which LmCK1.2
50 seems to be the primary target. Using this four-step pipeline, we identify from several
51 thousand molecules, two lead compounds with a selective anti-leishmanial activity.

52

53 INTRODUCTION

54 The protozoan parasite *Leishmania* is the causative agent of leishmaniasis, a potentially
55 fatal disease with worldwide distribution. Depending on the species, three clinical forms
56 of the disease can be distinguished, cutaneous (CL, e.g. *L. major*), mucocutaneous (MCL,
57 e.g. *L. braziliensis*) and fatal visceral leishmaniasis (VL, e.g. *L. donovani*) (1). Several
58 treatment options are available, which either show important side effects or are
59 unaffordable. In all endemic regions, the first line of treatment is pentavalent antimonials,
60 despite their important side effects and the appearance of parasite resistance. Although
61 their target has not been identified, one member, sodium stibogluconate was shown to
62 inhibit the energy metabolism and macromolecule biosynthesis (2) (3). The second line of
63 treatment is Amphotericin B, a very potent but highly toxic antifungal drug. This
64 compound creates pores targeting ergosterol only present in the plasma membrane of
65 parasites but not in that of mammalian cells. Its less toxic lipid formulation is extremely
66 expensive and thus incompatible with treatment in developing countries (3, 4). Among
67 the other drugs that have been recently developed, miltefosine was a major breakthrough
68 in leishmaniasis therapy as this anticancer drug is the first oral treatment against VL.

69 Unfortunately, its teratogenicity excludes the treatment of pregnant women and its slow
70 turnover could promote the emergence of clinical parasite resistance (4). Miltefosine
71 plays a role in the perturbation of the lipid metabolism, the induction of apoptosis-like
72 cell death and has immuno-stimulatory effects; however its mode of action has not been
73 precisely identified (5). The activity of miltefosine is due to its accumulation inside the
74 parasite (6), which is prevented in resistant lines generated *in vitro* by the overexpression
75 of members of the ABC (ATP-binding cassette) transporter family and/or mutation of the
76 flippase LdMT (6) (7). Three more drugs complete the list of available treatments for
77 Leishmaniasis: (i) pentamidine, which has been used for VL, CL and MCL treatment,
78 induces the inhibition of polyamine biosynthesis and a decrease of the mitochondrial
79 inner membrane potential, (ii) the aminoglycosidic antibiotic paromomycin, which is
80 restricted to endemic areas, cures both VL and CL efficiently by targeting mitochondria,
81 and (iii) sitamaquine, an 8-aminoquinoline, which intercalates within biological
82 membranes to accumulate in *Leishmania* cytosolic acidic compartments (4).

83 Despite the various drugs available, none of these treatments are ideal because of two
84 main aspects: (i) their side effects, due mainly to off-target effects that cannot be
85 eliminated by drug optimization as the target responsible for the anti-leishmanial effect is
86 unknown, and (ii) the emergence of parasite resistance, due to the plasticity of the
87 parasite. Therefore there is an urgent need to discover new molecules and to develop new
88 drug discovery pipelines that take these two aspects into consideration. First, the use of
89 known validated targets for drug screening represents a major advantage as the
90 compound could be optimized to fit exclusively the target, avoiding off-target effects
91 mainly responsible for side effects. Protein kinases are among the best candidates as drug

92 targets for leishmaniasis because: (i) kinase inhibitors are one of the most important
93 group of FDA-approved drugs for the treatment of diseases such as cancer or Alzheimer
94 disease, (ii) they are considered as valid targets for diseases caused by unicellular
95 parasites, such as malaria, and (iii) kinases regulate many key processes, such as cell
96 cycle or signal transduction and thus the inhibition of their activity decreases cell viability
97 (8) (9-14). Second, targeting proteins secreted by the parasite could delay the emergence
98 of drug resistance. Indeed it has been recently shown that parasitic proteins could be
99 exported, via exosomes, into the host cell to modify its biology or its innate immune
100 response (15) (16). To perform their function in the host, these proteins need to interact
101 with host proteins and thus any mutations could abrogate their functions in the host cell,
102 which could be detrimental for the intracellular parasite survival.

103

104 Among the proteins identified in the recent proteomic analysis of *Leishmania* exosomes,
105 13 could qualify as good drug targets as defined above because they are excreted kinases.
106 Most of these kinases are involved in purine or glucose metabolism, and only one is
107 involved in signal transduction, casein kinase 1. Member of a highly conserved Ser/Thr
108 protein kinase family (17), casein kinase I contains six isoforms in *Leishmania* (15, 18-
109 20). LmjF35.1010 (LmCK1.2), the major isoform, has been validated pharmacologically
110 as a drug target based on the findings that the inhibition of CK1 activity by the specific
111 inhibitor D4476 strongly compromises axenic amastigote viability and decreases the
112 percentage of infected macrophages (21). We hypothesize that the capacity of LmCK1.2
113 to recognize and phosphorylate host proteins could allow the parasite to regulate essential
114 host cell processes (22) and therefore to survive. This hypothesis is based on our previous

115 findings showing that (i) the protein sequence of LmCK1.2 kinase domain is 100%
116 identical in all sequenced *Leishmania* species (except the lizard-isolated *L. tarentolae* and
117 unclassified *L. sp. MAR LEM2494*), suggesting that there is a selection pressure to
118 maintain the integrity of the protein sequence, and (ii) LmCK1.2 is the most closely
119 related kinase to its human orthologs in *Leishmania*. These two elements suggest that
120 LmCK1.2 cannot be mutated without compromising the survival of the intracellular
121 parasite, which would render the emergence of drug resistant parasites expressing
122 mutated LmCK1.2 unlikely (21).

123 Here we present a four-step pipeline that allows the discovery of novel lead compounds.
124 First, we generated an active recombinant LmCK1.2 and purified mammalian CK1 from
125 porcine brain (SsCK1, (23)). We developed an enzymatic assay to screen 4030
126 compounds from kinase-biased and focused libraries as well as 988 analogs with both
127 *Leishmania* and mammalian kinases in order to identify hit compounds and assess their
128 specificity. We selected 88 compounds with an IC_{50} below 10 μ M. Second, we tested the
129 anti-leishmanial effect of these compounds on cultured parasites using a rezasurin-based
130 assay as well as on intracellular parasites using a high content phenotypic screen. We
131 retained seventy-five compounds with an anti-leishmanial activity. Third, after evaluation
132 of the toxicity of the selected anti-leishmanial compounds against mouse macrophages
133 and human cell lines, only four compounds had a selectivity index (SI) above 10. Fourth,
134 the affinity for LmCK1.2 for these compounds was tested using a target deconvolution
135 approach. Two compounds for which LmCK1.2 seems to be the primary target were
136 eventually selected. The identification of these two lead compounds validates our

137 pipeline, which will be used to screen diversified libraries to identify more lead
138 compounds.

139

140 **MATERIAL AND METHODS**

141 ***L. donovani* culture and axenic amastigote differentiation.**

142 *L. donovani* 1S2D (MHOM/SD/62/1S-CL2D), clone LdB, was obtained from Steve
143 Beverley, Washington University School of Medicine, St. Louis, USA and cultured as
144 described (24-26).

145

146 **Parasite growth inhibition assay.**

147 *L. donovani* promastigotes and axenic amastigotes (2×10^6 cells/ml) in their respective
148 media were distributed in 96 well plates (125 μ l/well). An equal volume of medium
149 containing inhibitor at the indicated concentrations (in 1% of dimethyl sulfoxide final
150 concentration, DMSO) was added. After 24h incubation in the dark at 26°C
151 (promastigotes) or 37°C (amastigotes), 25 μ l of resazurin solution at 0.001% was added
152 and the plates were incubated for an additional 24h in the dark at appropriate
153 temperatures. The plates were read (λ_{ex} : 544 nm; λ_{em} : 590 nm) using a fluorescent
154 microplate reader (Safas xenius XML) (27).

155

156 **Human cell lines MTT.**

157 HFF1, SH-SY5Y and U-2 OS cells were cultured in Dulbecco's modified Eagle's medium
158 (DMEM), hTERT RPE-1 were cultured in DMEM:F12 medium. All media were
159 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 IU penicillin and

160 streptomycin. Cell viability was assayed using the ‘CellTiter96 AQueous non-radioactive
161 cell proliferation assay’ test (MTS) from PROMEGA according to manufacturer’s
162 instructions.

163

164 **Macrophage infection and assessment of intracellular parasite survival.**

165 A high content, biologically relevant cell-based assay was used to determine the anti-
166 leishmanial activity of D4476 as previously described (21, 28). Briefly, the assay
167 combines (1) the use of primary bone marrow-derived mouse macrophages as natural
168 host cells and *DsRed2*-expressing amastigotes of *L. amazonensis*
169 (MPRO/BR/1972/M1841), the clinically relevant parasite stage of *Leishmania* with (2)
170 the detection of fluorescent markers as reporter molecules. 10,000 macrophages were
171 counted per well depending on the number of replicates per tested compound.

172

173 **Ethics statement.**

174 All animals were housed in our A3 animal facilities in compliance with the guidelines of
175 the A3 animal facilities at the Institut Pasteur, which is a member of Committee 1 of the
176 “Comité d’Ethique pour l’Expérimentation Animale” (CEEAA) - Ile de France - Animal
177 housing conditions and the protocols used in the work described herein were approved by
178 the “Direction des Transports et de la Protection du Public, Sous-Direction de la
179 Protection Sanitaire et de l’Environnement, Police Sanitaire des Animaux under number
180 B75-15-27 and B75-15-28 in accordance with the Ethics Charter of animal
181 experimentation that includes appropriate procedures to minimize pain and animal
182 suffering. GS and EP are authorized to perform experiment on vertebrate animals (licence

183 B75-1159 and 75-1265, respectively) issued by the “Direction Départementale de la
184 Protection des Populations de Paris” and is responsible for all the experiments conducted
185 personally or under his supervision as governed by the laws and regulations relating to
186 the protection of animals.

187

188 **Automated microtiter plate CK-S kinase assay.**

189 A mixture of native CK1 isoforms (essentially CK1 δ and CK1 ϵ) was extracted from
190 porcine brain (SsCK1) and purified by affinity chromatography on immobilised axin
191 (23). LmCK1.2 was produced and purified as previously described (21). Both SsCK1 and
192 recombinant LmCK1.2 were assayed, with 27 μ M CK-specific peptide substrate CK-S
193 (RRKHAAIGpSAYSITA) synthesized by Proteogenix (Oberhausbergen, France), in
194 buffer C pH 7 (60 mM β -glycerophosphate, 30 mM p-nitrophenyl phosphate, 25 mM
195 MOPS, 5 mM EGTA, 15 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM sodium vanadate),
196 with 15 μ M [γ -³³P] ATP in a final volume of 30 μ l. After 30 min incubation at 30°C, 30
197 μ L aliquots were filtered onto whatman P81 phosphocellulose paper. The filters were
198 washed with a solution of 1% phosphoric acid then counted in the presence of 20 μ l
199 scintillation fluid per well. Blank values were subtracted and activities calculated as
200 pmoles of phosphate incorporated during the 30 min incubation. The activities were
201 expressed in percent of maximal activity, i.e. in the absence of inhibitors. Controls were
202 performed with appropriate dilutions of DMSO.

203

204 **ATP depletion and competition**

205 Seven mg of axenic amastigote total protein extract were dialyzed overnight at 4°C in
206 dialysis solution (1X PBS, 1 mM EDTA, 1 mM DTT) using a Slide-A-Lyzer 10kD
207 dialysis cassette (Pierce) to eliminate free ATP. One mg of dialyzed extract per condition
208 was mixed with the binding solution (1X Binding solution, 1mM DTT, 1X Protease
209 inhibitor) of the ATP affinity test kit (Jena Bioscience) and 500 µM of inhibitor. The
210 samples were incubated 30 min at 4°C and added to a mixture of 12.5 µl of each ATP
211 agarose (ATP affinity test kit from Jena Bioscience). ATP binding proteins from the
212 assay and the competition samples were enriched according to the manufacturer's
213 instructions. Finally, eluted samples were concentrated using Amicon Ultra 10K
214 centrifugal filters (Millipore) to a final volume of approximately 100 µl.

215 12.5 µl of the flow through and 30 µl of the eluate were separated on Novex NuPAGE 4-
216 12% bis tris gel (Life technologies) from both the assay and the competition. The gel was
217 stained with SYPRO Ruby (Life technologies) according to the manufacturer's
218 instructions and revealed using a Typhoon scanner. Alternatively, proteins separated by
219 SDS-PAGE were transferred onto a PVDF membrane and probed with SY3535 antibody
220 (21). Signals were revealed by Super Signal ECL (Pierce).

221

222 **Compound libraries**

223 We screened 5018 compounds from the Roscoff library including 588 purine derivatives
224 (29) and 400 indirubin derivatives (30) (31) (32).

225

226 **Homology modeling and structural alignment**

227 The amino acid sequence of *L. major* CK1.2 was retrieved from NCBI database
228 (Accession number: XP_003722496) in FASTA format. The homology modeling of the
229 sequence was performed by SWISS-MODEL program (33) and the protein with PDB
230 code 3SV0 was selected as a template. PyMOL program (34) was used for the structural
231 alignment of *Schizosaccharomyces pombe* casein kinase 1 in complex with the specific
232 CK1 inhibitor, IC261 (PDB code: 1EH4) and *Homo sapiens* casein kinase 1 δ (PDB code:
233 4KB8) to the generated *L. major* CK1.2 homology model. The figures were also prepared
234 using PyMOL (34).

235

236 **RESULTS**

237 We present below, a comprehensive drug discovery pipeline encompassing four steps.

238 **STEP 1: Identification of specific LmCK1.2 inhibitors.**

239 Primary screening comparing *SsCK1* and *LmCK1.2*

240 We purified recombinant LmCK1.2-V5-His₆ from *E. coli* and a mixture of *Sus scrofa*
241 CK1 δ and CK1 ϵ (*SsCK1*) from porcine brain (21) (23). The conditions used previously
242 for the manual kinase assay were adapted to an automated 96-well plate format (23) (21).
243 We used CK-S as substrate for both kinases, and 15 μ M [γ -³³P] ATP (23) (21). We
244 screened 4030 compounds at 10 μ M from a kinase-biased library that has been previously
245 tested on mammalian kinases, such as Cyclin-Dependent Kinases (CDK) (29). For each
246 compound, the percentage of inhibition of LmCK1.2 versus that of *SsCK1* is presented in
247 Figure 1A. We classified as hit compounds those that decreased the kinase activity by
248 more than 40% (Figure 1A a and b). We obtained twice as much hit compounds for the
249 mammalian CK1 than for LmCK1.2 (Figure 1B). Indeed, we identified 245 hit

250 compounds against SsCK1 (6.1% Hit rate) and 128 against LmCK1.2 (3.2% Hit rate)
251 with only 37 compounds with a similar potency against both kinases. This finding, which
252 is surprising considering the high similarity between the protein sequences of LmCK1.2
253 and the mammalian CK1s (about 70% (21)), suggests that the ATP binding pocket of the
254 two kinases is sufficiently divergent to accommodate different inhibitors. We next
255 classified the compounds according to their potency and specificity (Class 1 corresponds
256 to compounds that inhibit the kinase activity between 80-100%, Class 2 between 60-80%,
257 and Class 3 between 40-60%, Figure 1C). We obtained a similar number and distribution
258 of hit compounds active on SsCK1 (30 compounds) and on LmCK1.2 (31 compounds) in
259 Class 1. In contrast, we observed an increased number of compounds inhibiting
260 specifically SsCK1 in Class 2 and 3 (Figure 1C). This could suggest that the ATP binding
261 pocket of SsCK1 could be more permissive than that of LmCK1.2. We selected 45
262 compounds either belonging to Class 1 that inhibited LmCK1.2 activity by more than
263 90% or that belong to Class 2 and were specific to LmCK1.2 to determine their IC_{50} . As
264 shown in Figure 2, all the compounds with an IC_{50} below 1.3 μ M (most potent) were not
265 specific, whereas all the specific compounds had an IC_{50} above 1.3 μ M.

266 Among the 45 compounds that were potent against LmCK1.2, we identified several
267 inhibitors described to have anti-leishmanial activity, including known CK1 inhibitors,
268 such as anthraquinone (35-37), or compounds for which we revealed CK1 as a new
269 target, such as Gossypol, purpurogallin and some flavonoids (38-41) (42) (43). These
270 compounds, identified from several libraries, were found at least twice with similar IC_{50} ,
271 indicating that our assay is reproducible (data not shown). Altogether, these data
272 demonstrate the efficiency of using CK1 as a target to identify compounds with anti-

273 leishmanial activities and confirm LmCK1.2 as a valid drug target. However we did not
274 retain these compounds for subsequent characterization as they have been already
275 extensively studied.

276 Secondary screening of purine and indirubin libraries

277 Out of the 45 compounds for which we determined the IC₅₀, we selected 2 compounds
278 with low IC₅₀ but only moderate specificity towards LmCK1.2, purvalanol B and
279 indirubin-3'-monoxime, and tested analog libraries to perform SAR analysis in order to
280 identify more specific compounds. We chose purvalanol B because we showed
281 previously that *Leishmania* CK1.2 binds to purvalanol B better than its mammalian
282 counterpart, suggesting that the sensitivity to purvalanol B could be higher for LmCK1.2
283 than for SsCK1 (44). Confirming this finding, the IC₅₀ of purvalanol B towards LmCK1.2
284 (2 +/- 0.3 μM) is slightly lower than that towards SsCK1 (2.9 +/- 1.2 μM). We tested the
285 potency of 588 purine analogs (Figure 3A) at 10 μM against LmCK1.2 and SsCK1 to
286 identify compounds with better potency and/or specificity. As shown in Figure 3B and
287 3C, most compounds were more potent against mammalian CK1 than against LmCK1.2
288 (below the black line, Figure 3B and dark grey in Figure 3C). Next, we determined the
289 IC₅₀ of the 21 most potent purine derivatives against LmCK1.2 (Figure 3D). For all the
290 compounds, except one, the IC₅₀ was systematically higher against LmCK1.2 (0.44 to 2.4
291 μM) than against mammalian CK1 (0.081 to 1.6 μM). Only compound **26** had a lower
292 IC₅₀ against LmCK1.2 (1 +/- 0.4 μM) than against SsCK1 (3.7 +/- 1.2 μM), which could
293 be due to the presence of a long carbon chain, a unique feature compared to the other
294 derivatives. Thus although the purines were very potent towards LmCK1.2, they present a
295 higher affinity for SsCK1.

296 The second family of compounds we screened are the indirubins (Figure 4A). We showed
297 that the IC₅₀ of indirubin-3'-monoxime is lower against LmCK1.2 (0.13 +/- 0.03; Table
298 S1) than against mammalian CK1 (0.39 +/- 0.08 μM; Table S1). To identify better
299 compounds with higher selectivity, we tested 400 indirubin derivatives (Figure 4B).
300 Contrary to the purine analogs, the indirubins were globally more potent against
301 LmCK1.2 than SsCK1 (Figure 4B). For instance, in Class 1, forty-six compounds were
302 more specific toward LmCK1.2 than SsCK1 versus only 9 were more specific towards
303 SsCK1 than LmCK1.2 (Figure 4C). Indeed, the most active compounds were the most
304 selective toward LmCK1.2, whereas compounds with lower potency (Class 3) were more
305 specific toward SsCK1 (Figure 4C). We selected the 55 most potent compounds to
306 measure their IC₅₀. They were comprised between 0.08 and 10 μM, and for almost all the
307 compounds, the IC₅₀ against LmCK1.2 was systematically lower than that against
308 mammalian CK1 (Figure 4D), suggesting that the indirubin compounds have more
309 affinity towards LmCK1.2 than SsCK1.

310 The differences in specificity observed with the purine and indirubin compound families
311 confirm that important differences exist between the ATP binding pocket of both kinases
312 (21). It also suggests that due to the strong affinity of the purine for the mammalian CK1,
313 it is likely that these compounds will be toxic for the host cell.

314 Among all the compounds identified in the primary and secondary screenings, we
315 eliminated all the compounds for which the chemical optimization was unfeasible and
316 selected 12 compounds from the main library, 21 compounds from the purine library and
317 55 compounds from the indirubin library to assess their anti-leishmanial activity.

318

319 **STEP 2: Evaluation of the anti-leishmanial activity of selected compounds.**

320 We evaluated the anti-leishmanial activity of the 88 compounds selected in STEP 1 on
321 cultured *L. donovani* promastigotes and axenic amastigotes by measuring the percentage
322 of metabolically active parasites in liquid culture using the rezasurin-based assay (27)
323 (Table S1). As a positive control, we treated the parasites with 1 μ M of the anti-
324 leishmanial reference drug, AMB and obtained a growth inhibition of 90.4 ± 1.5 % and
325 79.9 ± 1.3 % for promastigotes and amastigotes, respectively, with excellent
326 reproducibility as reported by the small standard deviation values.

327 We then tested the compounds against intracellular *L. amazonensis* using a visual high
328 content phenotypic assay (21, 28). We measured three parameters: (i) the percentage of
329 cells remaining after treatment compared to the vehicle control (DMSO) to evaluate cell
330 detachment (Total Macrophages, TM), (ii) the percentage of healthy cells compared to
331 the total number of cells remaining after treatment to evaluate cell mortality (Viability
332 Index, VI), and (iii) the percentage of parasitophorous vacuoles per healthy cell to
333 evaluate the parasite burden (PB). We used AMB at 0.5 μ M and cycloheximide (CHX) at
334 150 μ M as anti-leishmanial and cytotoxic control compounds, respectively. Figure S1
335 presents the data of all the controls performed during the screening campaign. As
336 expected, AMB is reducing parasite burden without affecting the number of macrophages
337 or their viability, whereas CHX does not affect significantly the number of macrophages
338 but decreases dramatically the viability, as it is extremely toxic to macrophages. The data
339 were reproducible as judged by the small standard deviation values, (Figure S1).

340 For each of the 88 compounds, we plotted the percentage of metabolically active
341 promastigotes or amastigotes at 10 μ M versus the percentage of parasite burden at 10 μ M

342 (Figure 5). We considered as efficient, those compounds that decreased the percentage of
343 metabolically active parasites or the percentage of parasite burden by 40%. We
344 eliminated compounds that had no effect on the percentage of intracellular parasites;
345 remarkably those compounds were also mainly inefficient on cultured parasites (Figure 5,
346 b and d). Among the 65 compounds that were potent towards the intracellular parasites,
347 we identified two categories: compounds equally efficient on cultured and intracellular
348 parasites (Figure 5, c) and compounds that were only efficient on intracellular parasites,
349 which represent the majority of the compounds (43 out of 65 for promastigotes and 57
350 out of 65 for amastigotes, Figure 5, a). This finding suggests that the exclusion of
351 compounds based on their lack of efficacy on cultured parasites could lead to the
352 elimination of compounds very efficient on intracellular parasites, among which,
353 inhibitors that kill indirectly parasites by targeting host cell proteins.

354

355 Main library

356 We selected seven out of twelve compounds from the main library including i) Rottlerin,
357 NSC 146771, gefitinib and sunitinib, all potent against intracellular parasites at 10 μ M,
358 and ii) 5'ITu, PP2 (1-tert-butyl-3-(4-chlorophenyl)-1h-pyrazolo[3,4-d]pyrimidin-4-
359 amine,(45)) and NSC 699479 that were efficient against intracellular parasites at 10 and 1
360 μ M as well as against cultured parasites (Table S1). 5'ITu, which is described as a
361 general kinase inhibitor (46), is potent against promastigotes and axenic amastigotes, with
362 an EC_{50} of 0.4 ± 0.1 and 5.4 ± 1.8 μ M respectively (Table S1). It has also a strong effect
363 at 10 and 1 μ M on intracellular parasites with only $10 \pm 1\%$ and $13 \pm 4.5\%$ remaining PB,
364 respectively, which is similar to 0.5 μ M AMB. PP2 is potent against intracellular

365 amastigotes at 1 μM ($54 \pm 3\%$ PB; Table S1). NSC 699479 (4-[(E)-[2-(4-chlorophenyl)-
366 1-methylpyrazolo[1,5-a]indol-1-ium-4-ylidene]methyl]-N,N-dimethylaniline;tri-fluorome-
367 thane-sulfonate) is known for anti-cancer activity and has been shown to target a wide
368 range of proteins including the DNA polymerase iota (47). It is extremely potent against
369 promastigotes and axenic amastigotes, with an EC_{50} below 1 μM as well as intracellular
370 parasites at 10 μM ($3.6 \pm 1.9\%$ PB), and 1 μM ($11 \pm 1.4\%$ PB; Table S1), activity which
371 is comparable to AMB.

372

373 Purine library

374 We selected 13 out of 21 purine compounds that were able to kill efficiently intracellular
375 parasites at either 1 μM or 10 μM (compound 21 and 30, Table S1). Consistent with their
376 high potency against recombinant LmCK1.2, most compounds were active against
377 intracellular parasites. Surprisingly the purine derivatives were not very potent against
378 promastigotes and axenic amastigotes. With the exception of compound **22** and **30**, which
379 present an EC_{50} of $0.72 \pm 0.03 \mu\text{M}$ and $6.2 \pm 0.8 \mu\text{M}$ against promastigotes respectively,
380 most of the compounds were weakly active against promastigotes and inactive against
381 axenic amastigotes at 50 μM (Table S1). This lack of potency against cultured parasites
382 cannot be explained by cell permeability as these compounds efficiently decrease the
383 parasite burden of infected macrophages.

384

385 Indirubin library

386 Fifty-five indirubins were tested against promastigotes and axenic amastigote. Twenty-
387 one compounds showed an EC_{50} below 10 μM against promastigotes (ranging from 0.4 to

388 2 μM) whereas only 6 showed an EC_{50} below 10 μM against amastigotes (ranging from
389 3.5 to 8 μM). These compounds were all members of a subfamily of indirubins,
390 containing a diethanolamine substitution in position-3', suggesting that the presence of
391 this substitution could be important for their anti-leishmanial activity against cultured
392 parasites. It is remarkable that the EC_{50} against promastigotes was systematically lower
393 than that against axenic amastigotes (21). We next tested all the indirubin derivatives
394 against intracellular parasites. In contrast to purine derivatives, all indirubin compounds
395 were efficient against intracellular parasites at 10 μM , with 9 also efficient at 1 μM
396 (Table S1). The most efficient indirubin is compound **42** with a remaining $22 \pm 5\%$ PB,
397 corresponding to a decrease of 78% compared to the DMSO-treated controls. Altogether,
398 these data confirm what we observed with recombinant LmCK1.2 (Figure 4C and D) that
399 the indirubin compound family, which has a stronger affinity for LmCK1.2 is also
400 showing a higher anti-leishmanial activity.

401

402 **STEP 3: Evaluation of the toxicity of the compounds.**

403 *Cytotoxicity against mouse Bone Marrow Derived Macrophages*

404 We first assessed the toxicity towards mouse bone marrow-derived macrophages of the
405 75 compounds that displayed anti-leishmanial activity against intracellular parasites
406 (Table S1, column VI%). We plotted the percentage of parasite burden versus the
407 percentage of viable macrophages for each of the three libraries (Figure 6). As shown in
408 Figure 6A, 3 compounds out of the 7 selected from the main library were toxic towards
409 macrophages at 10 μM (Figure 6A, c, black dots) but none were toxic at 1 μM (Figure
410 6A, b and d, grey dots). However, decreasing their concentration from 10 μM to 1 μM to

411 prevent cytotoxicity led, in some cases such as with sunitinib, to a decrease in potency
412 against intracellular parasites. Nevertheless, we identified compounds that were not toxic
413 and able to efficiently decrease PB (Figure 6A d). We obtained a similar result with the
414 indirubin derivatives (Figure 6B, b and d) as we identified compounds with anti-
415 leishmanial activity and no toxicity against macrophages: 35 compounds at 10 μ M and 9
416 compounds at 1 μ M (Figure 6B d). These results are in contrast to the results obtained for
417 the purine library, as most of the 21 purine derivatives that we tested led to cell death,
418 preventing proper analysis of their effect on intracellular parasites. Indeed, we did not
419 identify any compound that decreased the percentage of parasite burden without
420 cytotoxicity (Figure 6C, d). To investigate whether these derivatives could be efficient
421 toward intracellular parasites at lower concentration, we tested compound **16**, **22** and **30**
422 at 0.01 μ M and 0.1 μ M (data not shown). These compounds were no longer toxic to host
423 cells at 0.1 μ M, 0.1 μ M and 0.01 μ M respectively, and no longer active against
424 intracellular parasites. It seems that the efficient concentration to kill intracellular
425 parasites could be similar to the cytotoxic concentration. This finding could be explained
426 by the higher affinity of this compound family for the mammalian CK1 compared to
427 *Leishmania* CK1.2 (Figure 3B and C).

428 Based on these results, we eliminated the sunitinib from the main library, all the
429 remaining compounds from the purine library and 11 compounds from the indirubin
430 library because of their toxicity against macrophages. We thus retained 6 compounds
431 from the main library and 7 indirubins from those with anti-leishmanial activity at 1 μ M
432 and without cytotoxicity against BMDM.

433 *Cytotoxicity against human cell lines*

434 To establish the selectivity index (SI, this unit corresponds to the ratio between the EC₅₀
435 against intracellular parasites and the EC₅₀ against mammalian cells), we determined the
436 EC₅₀ against intracellular parasites, macrophages as well as human cell lines (RPE-1,
437 SHSY-5Y, HFF-1 and U2OS, Table S1) for the remaining 13 compounds (Table S1). The
438 SI ranged from 0.15 to 50, which is consistent with small molecules being able to
439 discriminate between *Leishmania* CK1.2 and mammalian CK1, as we identified
440 compounds that show leishmanicidal activity without cytotoxicity (Table S1). We
441 eliminated all the compounds with a SI below 10, as they were likely to lead to side
442 effects, retaining only five compounds: 5'ITu, PP2, NSC 699479 from the main library
443 and compound **38** and **42** from the indirubin library. The EC₅₀ of 5'ITu against
444 intracellular parasites is in the nanomolar range ($0.06 \pm 0.01 \mu\text{M}$) while that against
445 mouse macrophages is in the micromolar range ($3.5 \mu\text{M} \pm 0$), which represents a 60-fold
446 difference between the cytotoxic and the anti-leishmanial concentrations (Table S1). The
447 toxicity of this compound towards the human cell lines seems to be cell-dependent;
448 indeed the EC₅₀ against RPE-1 and U2OS is $1.2 \mu\text{M}$, whereas it is above $25 \mu\text{M}$ against
449 SHSY-5Y and HFF-1 (Table S1). Taking in account both cell lines and macrophages the
450 minimum SI is thus 20, indicating that the leishmanicidal concentration is 20 fold lower
451 than the toxic concentration. NSC699479 has also an EC₅₀ against intracellular parasites
452 in the nanomolar range ($0.33 \pm 0.05 \mu\text{M}$) but a SI of only 10, due to the low EC₅₀ towards
453 macrophages and U2OS, respectively at $3.5 \pm 0.24 \mu\text{M}$ and $3 \pm 0.5 \mu\text{M}$. Indirubin **38** has
454 an EC₅₀ of 0.6 ± 0.1 with a SI above 17, whereas compound **42**, more potent, has an EC₅₀
455 of $0.06 \pm 0.005 \mu\text{M}$, with a high SI of 50 (Table S1). For PP2, it was impossible to
456 determine the exact EC₅₀, because the parasite burden at $10 \mu\text{M}$ of PP2 was $98 \pm 8.5\%$,

457 whereas it was only $54 \pm 3\%$ at $1 \mu\text{M}$. This result could be explained by the detachment
458 of non-infected macrophages. Indeed, we showed that treatment of infected BMDM with
459 $10 \mu\text{M}$ of PP2 led to cell detachment as judged by the percentage of total remaining cells
460 in the well ($60 \pm 1\%$, Table S1). We cannot completely exclude that cell detachment
461 could be the consequence of cell mortality, but it seems unlikely, as the cells that
462 remained attached were viable in the presence of the drug (VI of $83 \pm 4\%$). This is
463 consistent with what has been previously observed for other cell types, as PP2 is known
464 to directly interfere with cell attachment (48). We estimated the EC_{50} of PP2 around 1
465 μM as the PB is $54.3 \pm 3.2\%$ at $1 \mu\text{M}$ (Table S1), with a SI above 10. For STEP4, we
466 only selected 5'ITu, NSC699479, PP2 and compound **42** (Table 1).

467

468 **STEP 4: Target deconvolution.**

469 To confirm that 5'ITu, NSC699479, PP2 and compound **42** (Table 1) target *Leishmania*
470 CK1.2 in the parasite and to estimate their affinity for this kinase, we investigated
471 whether they could prevent the binding of CK1.2 to ATP-agarose (21). We treated
472 amastigote lysates with PP2, 5'ITu, compound **42**, NSC699479 or D4476 (positive
473 control) before performing an affinity chromatography; we used an untreated sample as
474 negative control. The proteins eluted from the ATP-agarose were separated by SDS-
475 PAGE and either stained with SYPRO-Ruby or analyzed by Western blotting using an
476 anti-LmCK1.2 antibody (21). By comparing the protein elution profiles obtained with the
477 untreated sample (Ama) to that obtained with the treated samples (D4476, PP2, Iodo, **73**
478 and **42**) we were able to assess compound selectivity (Figure 7A). Most of the treated
479 samples showed a profile similar to that of the untreated sample, except for the 5'ITu

480 (Figure 7A). As judged by the disappearance of several bands, 5'ITu could be targeting
481 many ATP-binding proteins aside CK1.2. Because this absence of selectivity prevents
482 any possibility for compound optimization, which could in turn lead to side effects, we
483 eliminated 5'ITu. Based on the Western blot analysis, we also discarded NSC699479, as
484 LmCK1.2 could still bind to the ATP beads in presence of this compound, suggesting that
485 this kinase is not the primary target of NSC699479 (Figure 7B). This is consistent with
486 the fact that the IC₅₀ of NSC699479 against LmCK1.2 is 8 μM, which is higher than that
487 of the other compounds (Table S1). However because of its strong anti-leishmanial
488 activity, it would be interesting to identify the primary target of NSC699479. As shown
489 in Figure 7B, similarly to D4476, only PP2 and compound **42** prevent the binding of
490 LmCK1.2 to the ATP beads without affecting the elution profile. This result suggests that
491 LmCK1.2 could be one of the primary targets of these two compounds.

492

493 **DISCUSSION**

494 The anti-leishmanial drugs currently available are compromised mostly because they lead
495 to parasite resistance and have important side effects. Considering these parameters early
496 in the process of drug development is therefore crucial to discover more efficient drugs.
497 We established a pipeline consisting of 4 steps from target-based screening to target
498 deconvolution. In order to integrate these parameters, we used LmCK1.2 (a *Leishmania*
499 *exo*-kinase) as a target to address parasite resistance, and excluded compounds based on
500 their absence of anti-leishmanial activity, on their lack of specificity or on their poor
501 affinity for LmCK1.2 to address side effects. Our pipeline introduces two improvements
502 compared to previous screening campaigns. First, as *Leishmania* and mammalian CK1s

503 are closely related and to limit the possibility of cell toxicity, which leads to side effects,
504 we screened, in parallel, the leishmanial and mammalian CK1 to assess specificity. Using
505 this approach we could discriminate between compounds with low and high specificity.
506 For instance, we showed that the purine derivatives displayed a better potency against
507 mammalian CK1 than against LmCK1.2, which led to toxicity against mammalian host
508 cells. Reversely, compounds of the indirubin family displayed a better potency against
509 LmCK1.2 than mammalian CK1, which was subsequently confirmed by their absence of
510 toxicity toward the mammalian host cell at 1 μ M. This finding suggests that a strong
511 correlation between the specificity toward the target and the subsequent effect on
512 intracellular parasite survival. Moreover, our results also confirm that LmCK1.2 has an
513 ATP binding pocket sufficiently divergent from that of mammalian CK1 to identify
514 discriminating compounds (21). Indeed, more than 70% of the small molecules that we
515 tested showed a differential potency against both kinases. We modelled the structure of
516 *Leishmania* CK1.2 based on existing crystal structures of CK1s found in Protein Data
517 Bank and noticed a few differences between the LmCK1.2 model (green, Figure S2) and
518 the crystal structure of human CK1 δ or of *Schizosaccharomyces pombe* CK1 (magenta or
519 cyan (SpCK1) respectively, Figure S2) that could account for the specificity of LmCK1.2
520 towards certain compounds. Indeed, residues in the active site of LmCK1.2 such as F22
521 and K40 could be positioned differently, which could overall change the shape of the
522 active site. Moreover, the structural alignment of LmCK1.2, human CK1 δ and SpCK1
523 (49), which is in complex with IC261 (specific CK1 inhibitor) shows that the position of
524 K40 in the active site of LmCK1.2 compared to that of K41 or K38 in the active site of
525 CK1 δ and SpCK1 respectively, may account for the differential response to compound

526 inhibition. Indeed, contrary to K38 and K41, K40 could lead to conformational clash with
527 IC261. This finding supports our previous results showing that the IC₅₀ of IC261 towards
528 LmCK1.2 is above 10 μM whereas it is 0.47 μM towards mammalian CK1 (21). Our
529 results demonstrate that *Leishmania* CK1.2, which would have been rejected based on the
530 strong identity to its mammalian orthologs, is a good drug target.

531 A second improvement was to assess whether compounds have multiple targets or low
532 affinity for LmCK1.2. To address this issue, we performed target deconvolution. This
533 strategy, which depends on affinity purification and competition, allowed the elimination
534 of compounds based on their lack of specificity or their lack of affinity towards
535 LmCK1.2. Indeed, we excluded 5'ITu that could be targeting many proteins as revealed
536 by their depletion following competition assay and ATP-affinity chromatography. This
537 finding is consistent with recent publications showing that 5'ITu is a general kinase
538 inhibitor due to its broad inhibitory activity (46). Although this compound could be a
539 good lead compound purely based on its SI, its optimisation could be difficult as the
540 target responsible for the leishmanicidal activity is unknown. We also excluded
541 NSC699479 because of its weak affinity towards LmCK1.2, suggesting that this kinase
542 might not be its primary target. Based on previous results in mammalian cells, the
543 primary target of NSC699479 could be topoisomerases in *Leishmania*, enzymes already
544 known as good drug targets (50) (51) (52).

545

546 Using this pipeline, we screened 5018 compounds in total and identified two lead
547 compounds, PP2 and compound **42**. PP2 is an inhibitor for which no anti-leishmanial
548 activity against intracellular parasites in THP-1 cells *in vitro* has been previously

549 described but appears to be efficient in animal model (53). It has an EC₅₀ on intracellular
550 parasites around 1 μM, but a selectivity index above 10 for murine macrophages and
551 above 25 for human cell lines. The known targets of PP2 are tyrosine kinases, Src, Lck,
552 Csk, Rip2 and Gak, for which there are no orthologs identified in *Leishmania* (54, 55).
553 The two other kinases that are targeted by PP2 are p38 (52% of inhibition at 1 μM (54))
554 and CK1δ (93% of inhibition at 1 μM (54)), suggesting that the anti-leishmanial activity
555 of PP2 is more likely mediated by the inhibition of CK1.2 in *Leishmania*. This finding is
556 consistent with our experimental data showing that *Leishmania* CK1.2 is one of the
557 primary targets of PP2. However, treatment with high concentrations of PP2 leads to
558 macrophage detachment (40% at 10 μM) similarly to what has been shown previously for
559 other cell types (48). Our results seem to indicate that most of the macrophages that
560 detach from the slides are those non-infected by *Leishmania*. This hypothesis is supported
561 by Tejle *et al*, which showed that the presence of *L. donovani* affect the detachment of
562 monocyte-derived dendritic cells, suggesting that the presence of the parasite could
563 promote cell adhesion (56).

564 Indirubins are particularly potent against cultured as well as intracellular parasites and
565 among the 55 indirubin derivatives showing leishmanicidal activity at 10 μM, only 37%
566 were cytotoxic against macrophages. Compound **42** is our best lead compound, with an
567 EC₅₀ on intracellular parasites of 60 ± 5 nM and a SI of 50. Although several authors
568 have already described the anti-leishmanial effect of indirubins (57-59), this particular
569 derivative has not been previously tested on intracellular parasites. From previous
570 published work, we already know some of the targets of the indirubins, such as
571 *Leishmania* CRK3 or GSK3 (57, 58). In our study, we reveal for the first time CK1.2 as a

572 novel target for this family of compounds. This is particularly striking as in higher
573 eukaryotes GSK3 and CK1 are often involved in similar signalling pathways such as the
574 Wnt/ β -catenin or the Hedgehog pathways, where they act as priming kinases for one
575 another (60, 61) (62) (63). Using affinity purification, we found that GSK-3 is also a
576 target of compound **42** (data not shown). We will determine precisely, using biochemical
577 approaches, whether this compound targets other kinases and which one causes the anti-
578 leishmanial effect.

579

580 In conclusion, we have established a comprehensive pipeline that identify and select
581 LmCK1.2 inhibitors based on their specificity, anti-leishmanial activity, absence of
582 cytotoxicity and selectivity. As a proof of principle, we identified two lead compounds,
583 PP2 and compound **42** that will be studied further to understand their mode of action and
584 could also be used as pharmacological tools to study parasite-specific signal transduction.
585 We will use this pipeline to screen diversified libraries that have not yet been screened
586 against *Leishmania* kinases, in order to identify lead compounds.

587

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604

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796

797 **FIGURE LEGENDS**

798 **FIGURE 1. Differential target-based screen of 4030 compounds from various**
799 **libraries. A.** Representation of the percentage of inhibition towards LmCK1.2 activity
800 versus the percentage of inhibition toward SsCK1 activity. Compounds in a and b are
801 potent toward LmCK1.2 as they show more than 40% inhibition, whereas compounds in
802 b and d are potent toward SsCK1. **B.** 336 hit compounds were identified in the screen
803 from which 245 inhibit SsCK1 (6.1 % hit rate) and 128 inhibit LmCK1.2 (3.2% hit rate).
804 Only 37 compounds showed equal potency against both CK1s. **C.** Compounds were
805 classified according to their specificity: compounds only potent against SsCK1 (only
806 SsCK1), more potent against SsCK1 than LmCK1.2 (SsCK1 > LmaCK1.2), equally
807 potent on both kinases (SsCK1 = LmCK1.2), more potent against LmCK1.2 than SsCK1
808 (SsCK1 < LmaCK1.2) and only potent on LmCK1.2 (only LmCK1.2). Compounds were
809 also classified according to their % of inhibition: Class 1 corresponds to compounds that
810 inhibit the kinases activity between 80-100%, Class 2 between 60-80% and class 3
811 between 40-60%. Twenty-three compounds are more potent toward LmCK1.2 than
812 SsCK1 and 68 compounds are specific to LmCK1.2 (the numbers in the histograms
813 indicate the percentage of compounds in each category).

814

815 **FIGURE 2. Determination of the IC₅₀ of the 45 compounds belonging to Class 1 that**
816 **have a percentage of inhibition above 90%.** Each point represents the IC₅₀ of a
817 particular compound towards LmCK1.2. Non-specific compounds have a potency below
818 10 μM towards both kinases, whereas specific compounds have a potency below 10 μM
819 only towards LmCK1.2.

820

821 **FIGURE 3. Screening of the purine derivative library. A.** Structure of the purine
822 backbone. R1, R2 and R3 represent different substitutions of the purines. **B.** We
823 performed a target-based screening of 588 derivatives. Each point represents the
824 percentage of inhibition towards LmCK1.2 activity versus the percentage of inhibition
825 towards SsCK1 activity of each compound. The compounds in the top left are more
826 potent toward LmCK1.2 whereas the compounds in the bottom right are more potent
827 towards SsCK1. **C.** Compounds were classified according to their specificity: only potent
828 on SsCK1, more potent on SsCK1 than LmCK1.2 (SsCK1 > LmCK1.2), equally on both
829 kinases (SsCK1 = LmCK1.2), more potent on LmCK1.2 than SsCK1 (SsCK1 <
830 LmCK1.2) and only potent on LmCK1.2. Compounds were also classified according to
831 their % of inhibition: Class 1 corresponds to compounds that inhibit the kinases between
832 80-100%, Class 2 between 60-80% and class 3 between 40-60%. Only 4 percent of the
833 compounds are more potent toward LmCK1.2 than SsCK1 or specific to LmCK1.2. **D.**
834 We determined the IC₅₀ of the 21 compounds belonging to class 1 that have a percentage
835 of inhibition above 90%. Each point represents the IC₅₀ of a particular compound towards
836 LmCK1.2 versus SsCK1. The IC₅₀ are lower towards SsCK1 than LmCK1.2.

837

838 **FIGURE 4. Screening of the indirubin derivative library.** **A.** Structure of the indirubin
839 backbone. **B.** Target-based screening of 400 derivatives. Each point represents the
840 percentage of inhibition towards LmCK1.2 activity versus the percentage of inhibition
841 toward SsCK1 activity for each compound. The compounds in the top left are more
842 potent towards LmCK1.2 whereas the compounds in the bottom right are more potent
843 towards SsCK1. **C.** Compounds were classified according to their specificity: only potent
844 on SsCK1, more potent on SsCK1 than LmCK1.2 ($SsCK1 > LmCK1.2$), equally on both
845 kinases ($SsCK1 = LmCK1.2$), more potent on LmCK1.2 than SsCK1 ($SsCK1 <$
846 $LmCK1.2$) and only potent on LmCK1.2. Compounds were also classified according to
847 their % of inhibition: Class 1 corresponds to compounds that inhibit the kinases between
848 80-100%, Class 2 between 60-80% and class 3 between 40-60%. Fifty-seven percent of
849 the compounds are more potent towards LmCK1.2 than SsCK1 and 46% are specific to
850 LmCK1.2. **D.** IC_{50} of the 55 compounds that are specific to LmCK1.2 or that belong to
851 class 1 with a percentage of inhibition above 90%. Each point represents the IC_{50} of a
852 particular compound towards LmCK1.2 versus SsCK1. The IC_{50} are lower against
853 LmCK1.2 than SsCK1.

854

855 **FIGURE 5. Comparison of the anti-leishmanial activity of compounds on cultured**
856 **and intracellular parasites.** We performed a screening of 88 compounds from the main,
857 the purine and the indirubine libraries on cultures promastigotes, axenic amastigotes and
858 intracellular parasites. Each point represents the percentage of metabolically active
859 promastigotes or amastigotes at 10 μ M versus the percentage of parasite burden at 10 μ M

860 for each compound. Black squares correspond to the percentage of metabolically active
861 promastigotes at 10 μ M versus the percentage of parasite burden at 10 μ M and grey dots
862 correspond to the percentage of metabolically active amastigotes at 10 μ M versus the
863 percentage of parasite burden at 10 μ M. a: compounds that are potent against intracellular
864 parasites but not against cultured parasites, b: compounds that are not potent against
865 intracellular and cultured parasites, c: compounds that are potent against intracellular and
866 cultured parasites and d: compounds that are not potent against intracellular but potent
867 against cultured parasites.

868

869 **FIGURE 6. Parasite burden versus macrophage viability.** Using a visual high content
870 phenotypic assay, we calculated the percentage of viable macrophages and the percentage
871 of infected cells. We analysed the anti-leishmanial effect of the selected compounds from
872 the main library (A), the indirubin library (B) and the purine library (C) versus their
873 toxicity against macrophages. a: compounds that are not potent against intracellular
874 parasites but cytotoxic, b: compounds that are not potent against intracellular parasites
875 and not cytotoxic, c: compounds that are potent against intracellular but cytotoxic and d:
876 compounds that are potent against intracellular and not cytotoxic.

877

878 **FIGURE 7. PP2 and compound 42 are the most specific compounds towards CK1.2.**
879 Competitive ATP affinity chromatography assays were performed on amastigote cell
880 lysates in presence or not of D4476, PP2, 5'ITu (Iodo), NSC699479 (73) and 42. ATP-
881 binding proteins (Elution) were eluted with an excess of ATP, resolved by SDS-PAGE

882 electrophoresis and stained by SYPRO Ruby (**A**). CK1.2 was revealed by Western blot
883 using an anti-LmCK1.2 antibody (SY3535, **B**).

884

885 **FIGURE S1. Controls for the phenotypic screen.** We analysed the effect of DMSO,
886 AMB and CHX on intracellular parasites using a visual high content phenotypic assay.
887 We assessed several parameters including the percentage of total cells compared to
888 control cells with DMSO (TM%), the percentage of viable cells (VI%) and the
889 percentage of viable cells with parasitophorous vacuoles that is considered as an
890 indication of parasite burden (PB%).

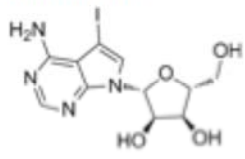
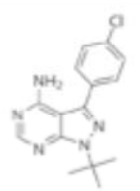
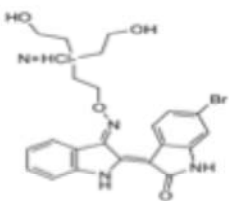
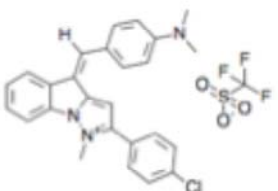
891

892 **FIGURE S2. Model of the ATP binding pocket of LmCK1.2 compared to that of**
893 **human and yeast CK1s.** Comparison of yeast (cyan) and human (magenta) CK1 crystal
894 structures with a structural model of LmCK1.2 (green). IC261 is represented in the ATP-
895 binding pocket.

896

897

Table 1: Selected compounds against intracellular parasites tested for target deconvolution.

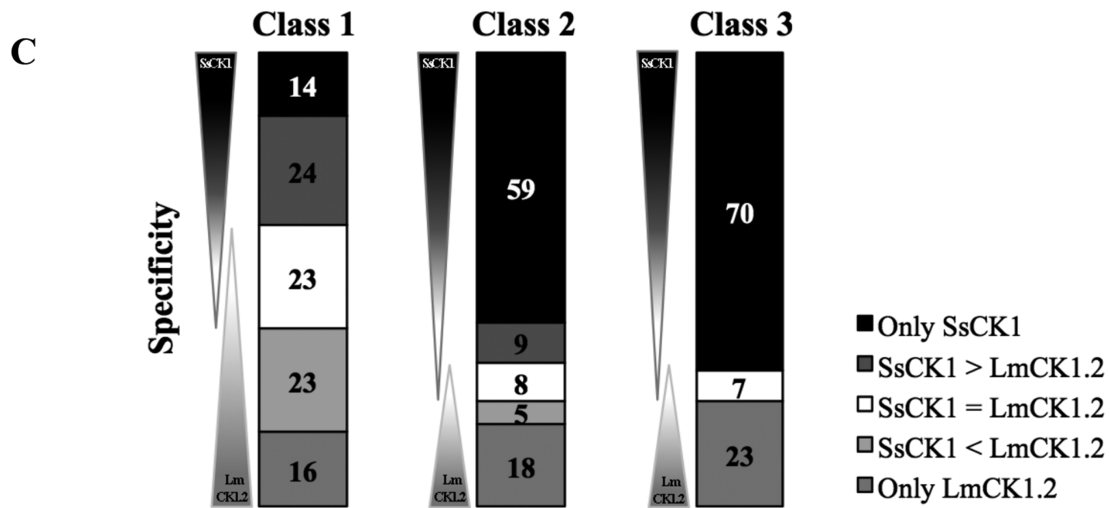
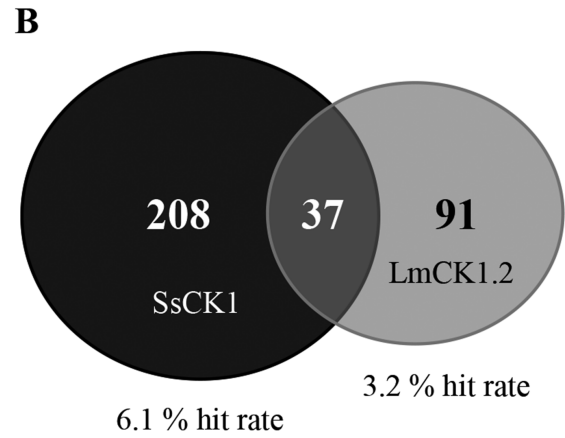
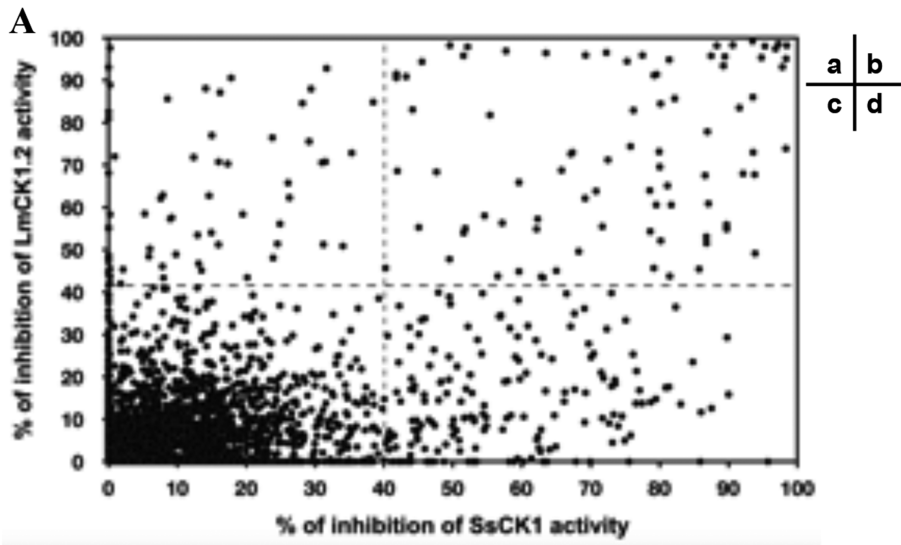
Compound name and structure	LmCK1.2 IC ₅₀ in μM	EC ₅₀ on cultured Promastigote in μM	EC ₅₀ on cultured Amastigote in μM	EC ₅₀ on intracellular parasites in μM	SI
5' Iodotubercidin 	0.18 ± 0.04	0.40 ± 0.10	5.40 ± 1.80	0.06 ± 0.01	20
PP2 	1.60 ± 0.30	> 10	> 50	1*	> 10
42 	0.93 ± 0.20	2.00 ± 0.00	> 10	0.06 ± 0.00	50
NSC699479 	8.00 ± 0.30	<1	<1	0.33 ± 0.05	10

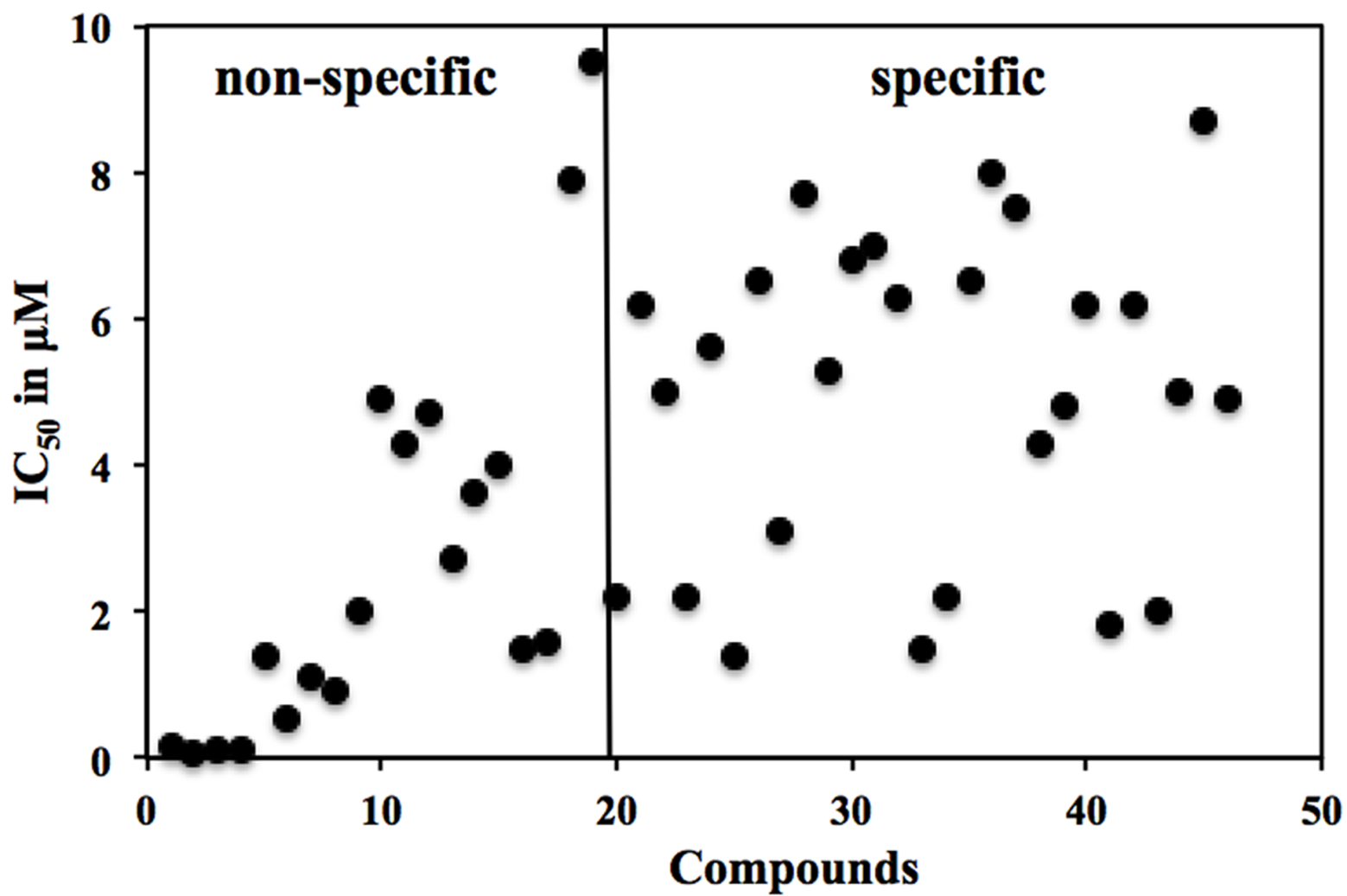
898

*: Estimation of the EC₅₀.

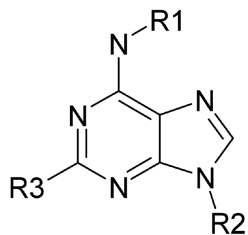
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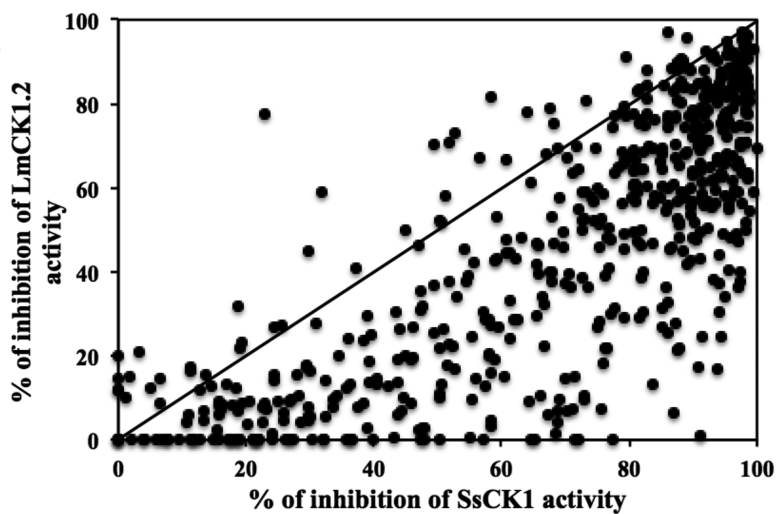




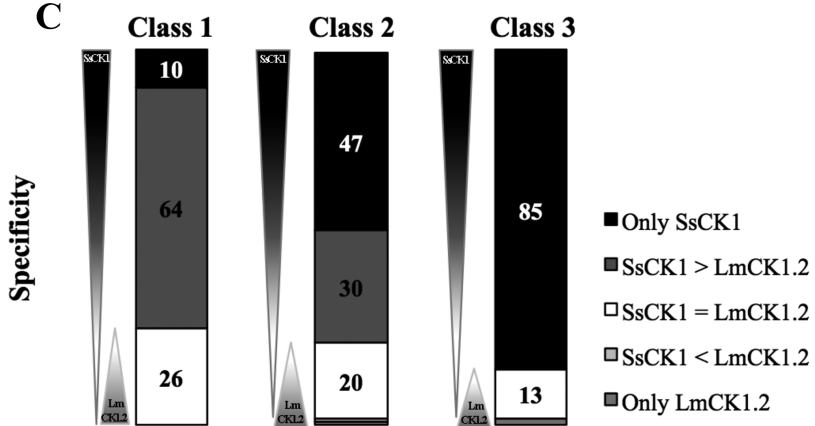
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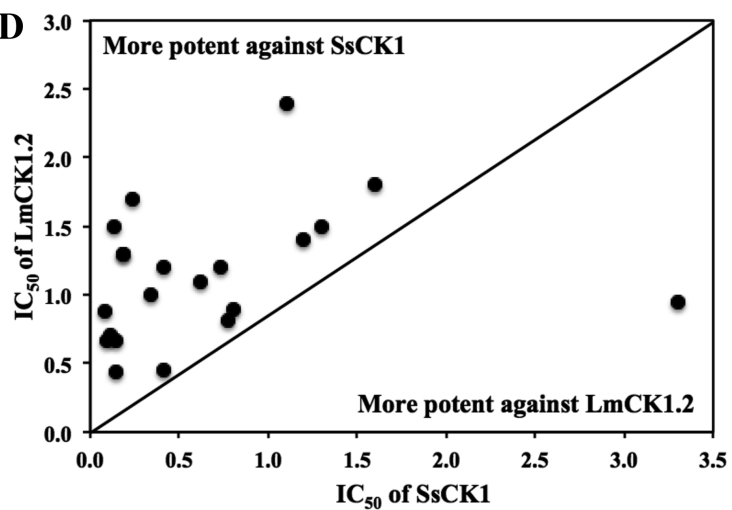
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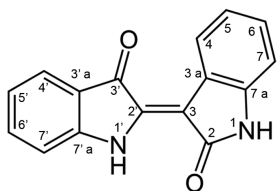
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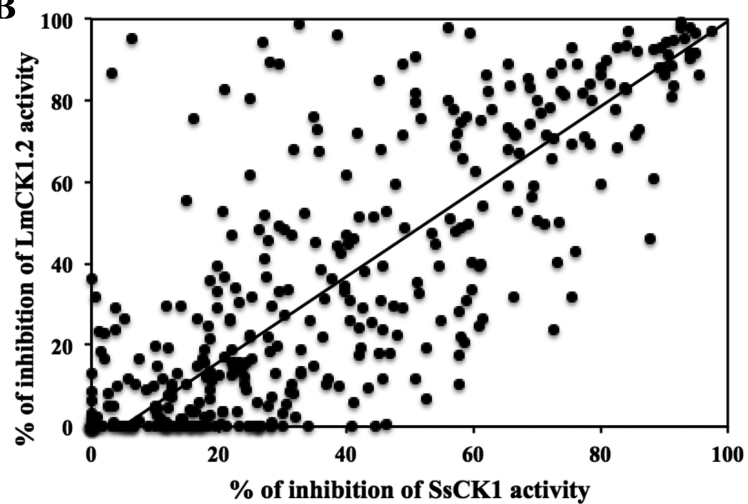
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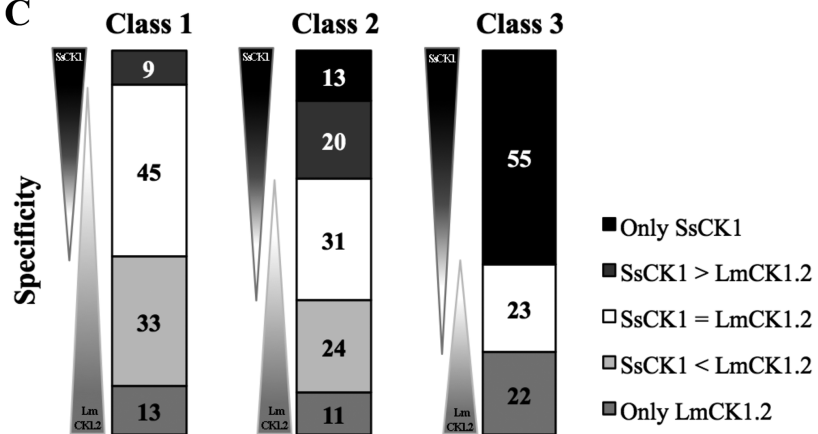
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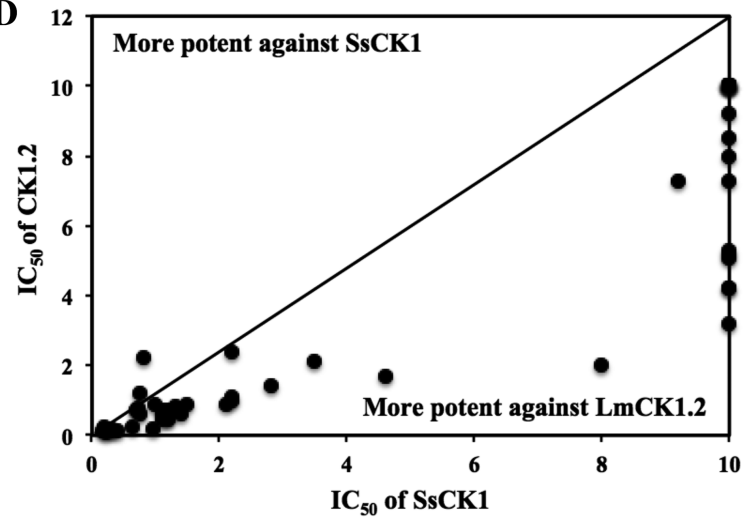
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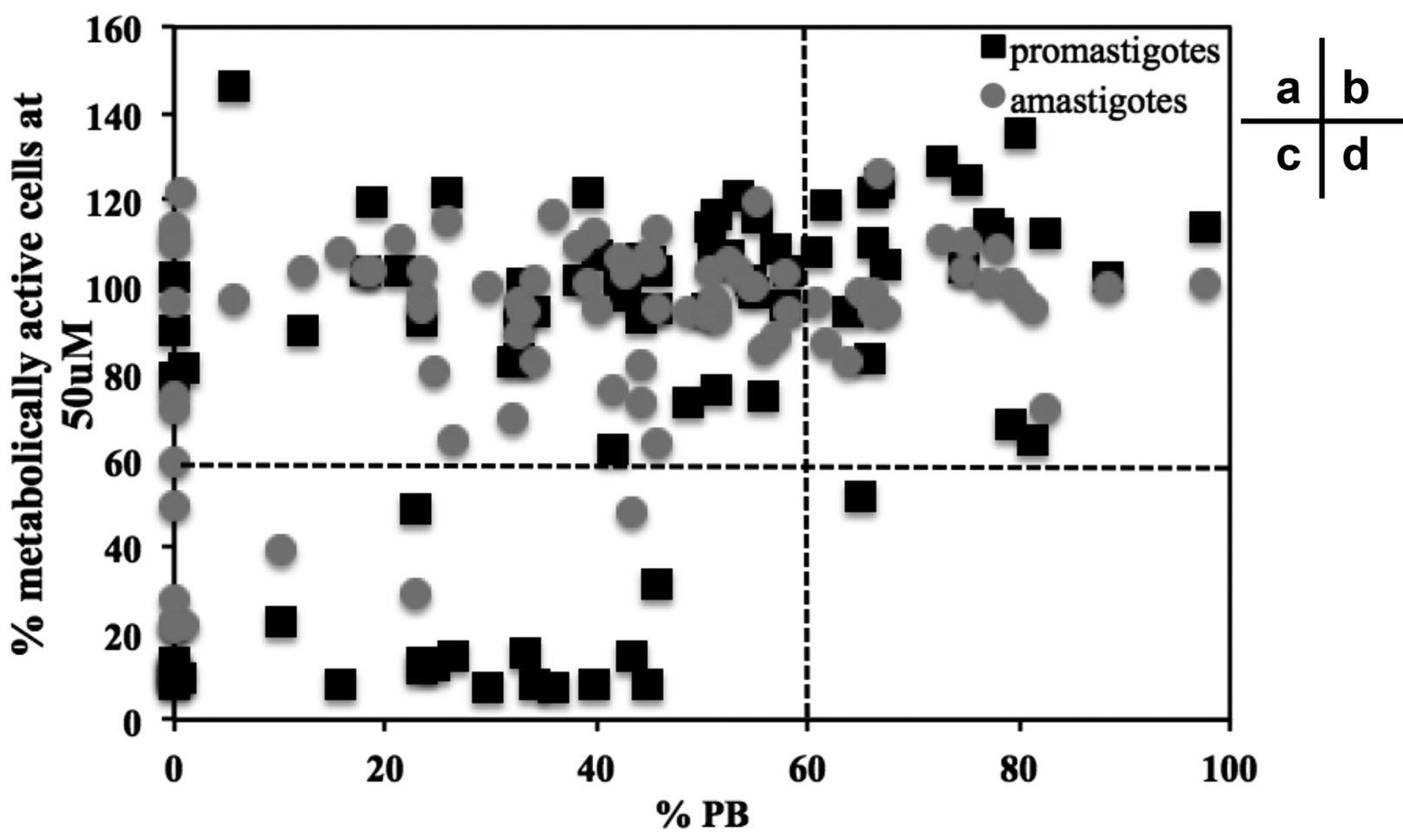


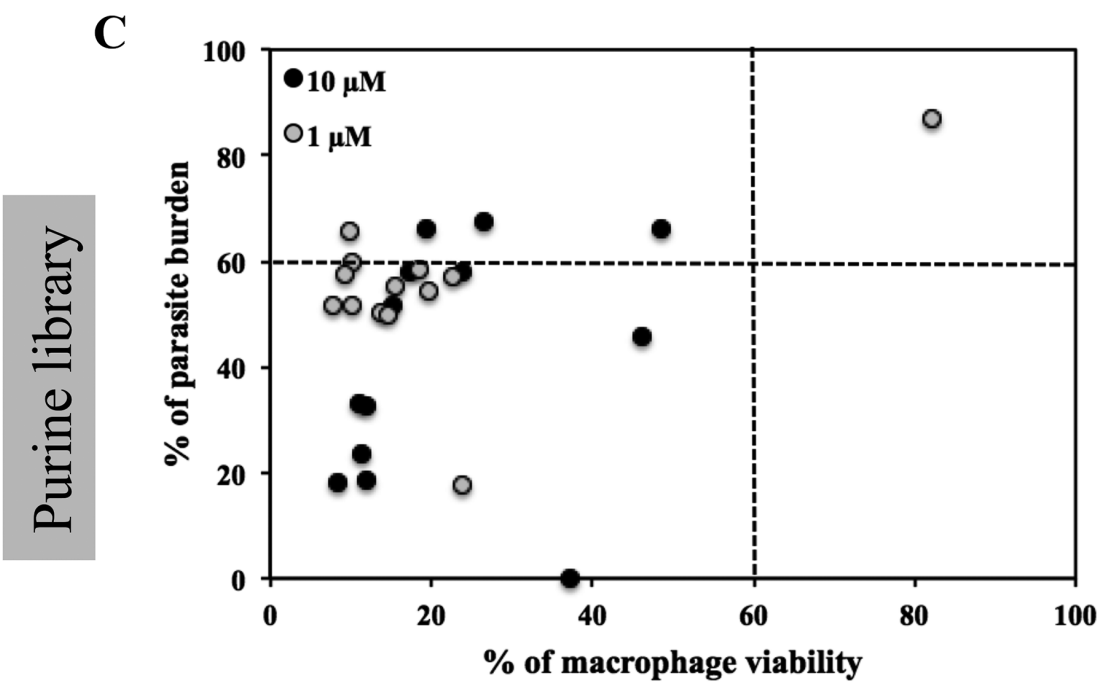
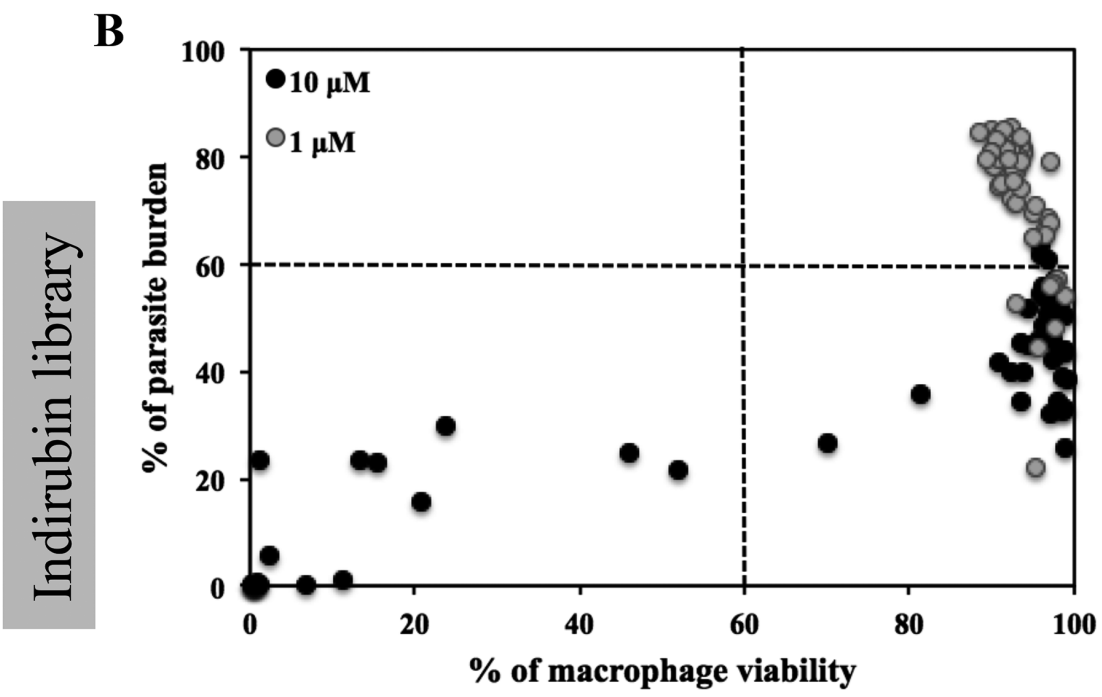
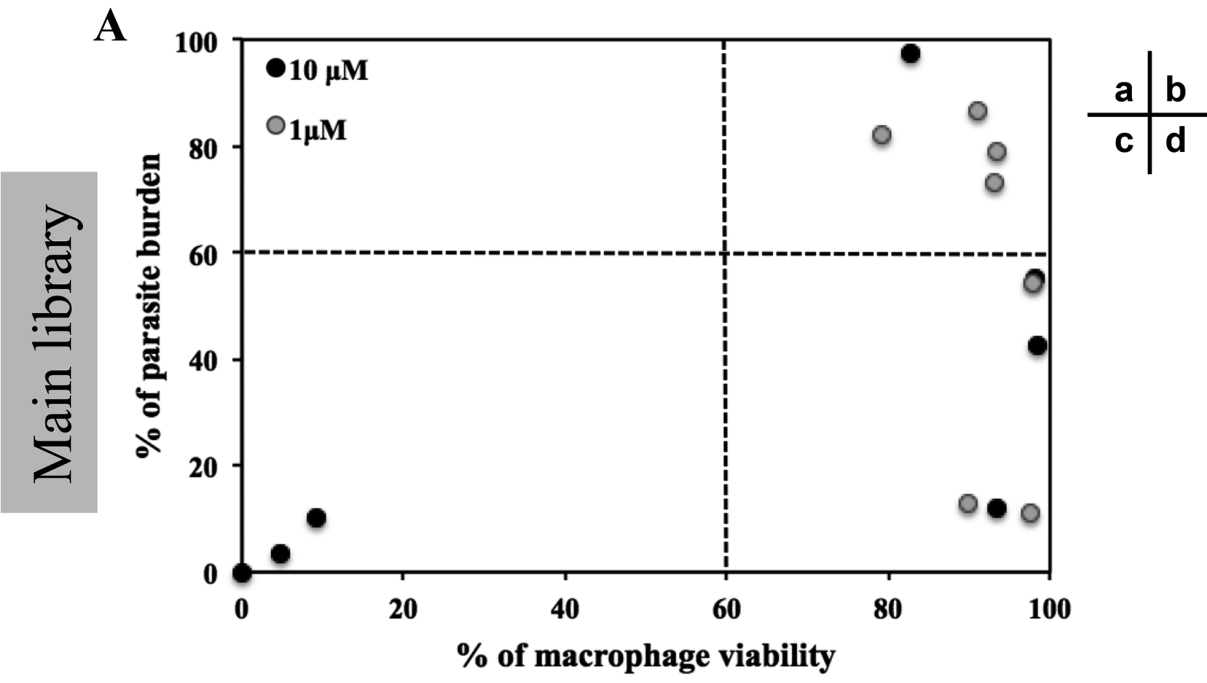
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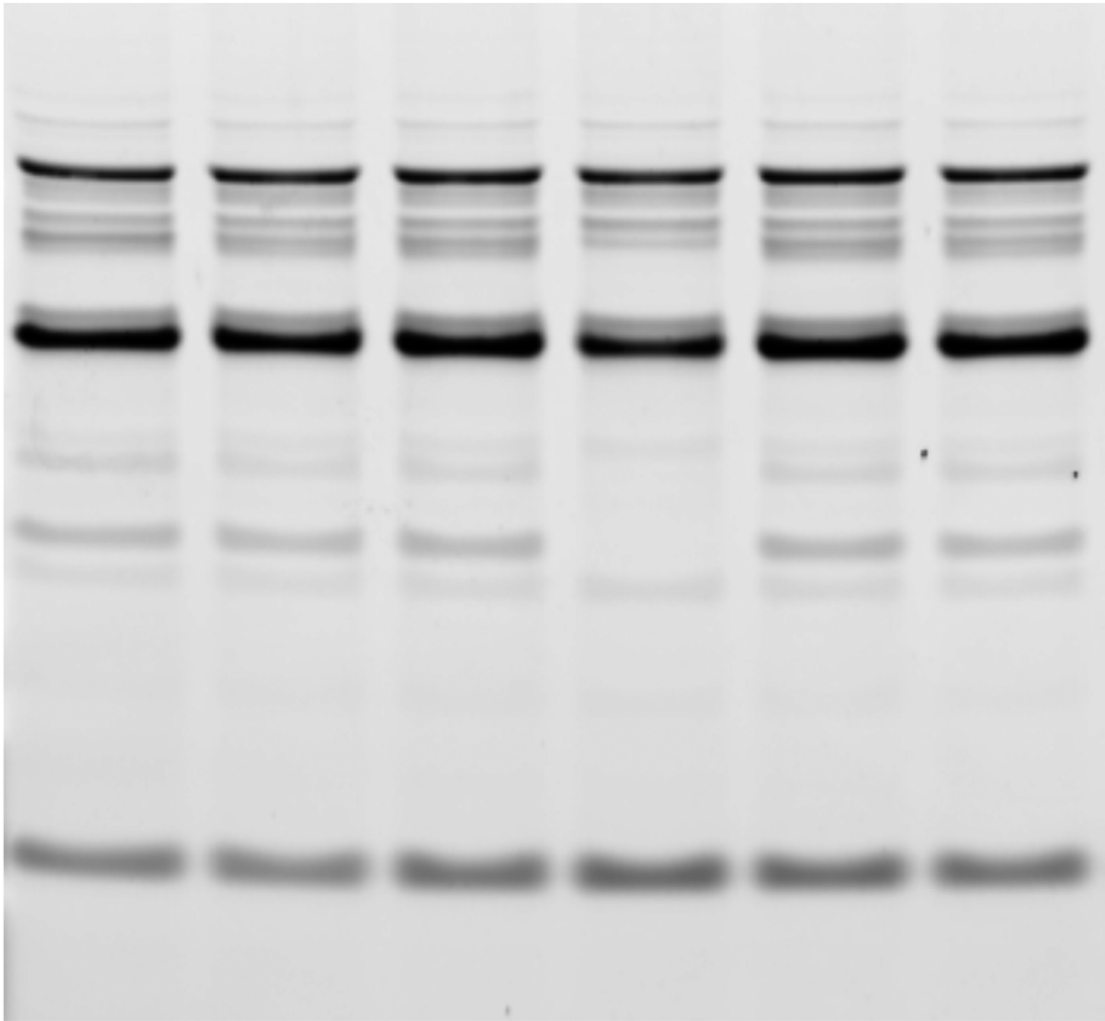


Elution

Ama D44 PP2 Iodo 73 42

A

140 -
115 -
80 -
70 -
50 -
40 -
30 -
25 -
15 -



B

40 -



α CK1.2

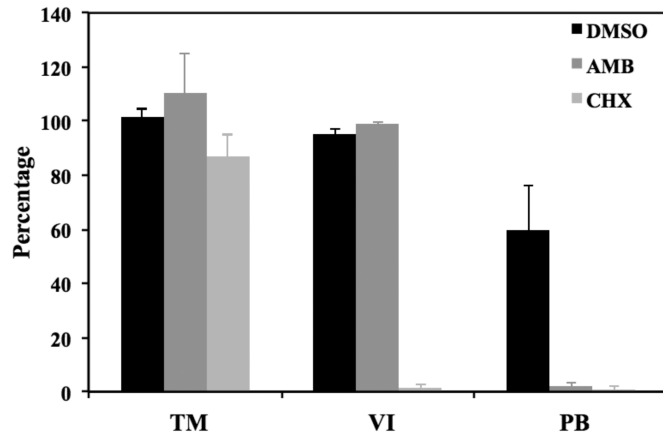


FIGURE S1. Controls for the phenotypic screen. We analysed the effect of DMSO, AMB and CHX on intracellular parasites using a visual high content phenotypic assay. We assessed several parameters including the percentage of total cells compared to control cells with DMSO (TM%), the percentage of viable cells (VI%) and the percentage of viable cells with parasitophorous vacuoles that is considered as an indication of parasite burden (PB%).

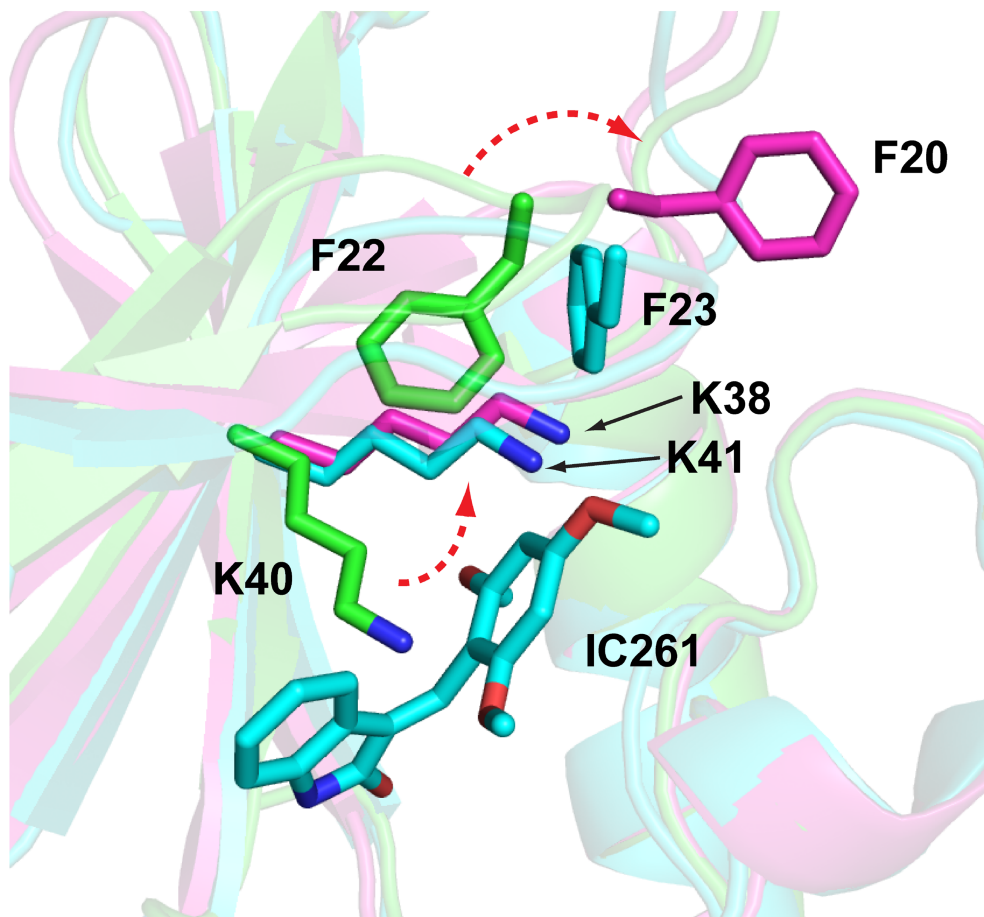


FIGURE S2. Model of the ATP binding pocket of LmCK1.2 compared to that of human and yeast CK1s. Comparison of yeast (cyan) and human (magenta) CK1 crystal structures with a structural model of LmCK1.2 (green). IC261 is represented in the ATP-binding pocket.

