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1 **Proteasome inhibition for treatment of leishmaniasis, Chagas disease and**
2 **sleeping sickness**

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10 **Chagas disease, leishmaniasis, and sleeping sickness affect 20 million people worldwide and lead to**
11 **more than 50,000 deaths annually¹. The diseases are caused by infection with the kinetoplastid**
12 **parasites *Trypanosoma cruzi*, *Leishmania* spp. and *Trypanosoma brucei* spp., respectively. These**
13 **parasites have similar biology and genomic sequence, suggesting that all three diseases could be**
14 **cured with drug(s) modulating the activity of a conserved parasite target². However, no such**
15 **molecular targets or broad spectrum drugs have been identified to date. Here we describe a**
16 **selective inhibitor of the kinetoplastid proteasome (GNF6702) with unprecedented *in vivo* efficacy,**
17 **which cleared parasites from mice in all three models of infection. GNF6702 inhibits the**
18 **kinetoplastid proteasome through a non-competitive mechanism, does not inhibit the mammalian**
19 **proteasome or growth of mammalian cells, and is well-tolerated in mice. Our data provide genetic**
20 **and chemical validation of the parasite proteasome as a promising therapeutic target for treatment**
21 **of kinetoplastid infections, and underscore the possibility of developing a single class of drugs for**
22 **these neglected diseases.**

23 Kinetoplastid infections affect predominantly poor communities in Latin America, Asia and Africa.
24 Available therapies suffer from multiple shortcomings, and new drug discovery for these diseases is
25 limited by insufficient investment³. We sought low molecular weight compounds with a growth
26 inhibitory effect on *Leishmania donovani* (*L. donovani*)^{4,5}, *Trypanosoma cruzi* (*T. cruzi*)^{6,7} and
27 *Trypanosoma brucei* (*T. brucei*)^{5,8}. Our approach was to test 3 million compounds in proliferation assays
28 on all three parasites (Supplementary Information Tables 1-3), followed by triaging of active compounds
29 (half-maximum inhibitory concentration value $EC_{50} < 10 \mu\text{M}$) to select those with a clear window of
30 selectivity (>5-fold) with respect to growth inhibition of mammalian cells. An azabenzoxazole,
31 GNF5343, was identified as a hit in the *L. donovani* and *T. brucei* screens. Although GNF5343 was not
32 identified in the *T. cruzi* screen, we noted potent anti-*T. cruzi* activity of this compound in secondary
33 assays.

34 Optimization of GNF5343 involved the design and synthesis of ~3,000 compounds, and focused on
35 improving bioavailability and potency on inhibition of *L. donovani* growth within macrophages (Fig. 1).
36 A critical modification involved replacement of the azabenzoxazole center with C6-substituted imidazo-
37 and triazolopyrimidine cores, which yielded compounds up to 20-fold more potent on intra-macrophage
38 *L. donovani* (e.g. GNF2636). Replacement of the furan group with a dimethyloxazole ring reduced the
39 risk of toxicity associated with the furan moiety, and replacement of the chlorophenyl group with a
40 fluorophenyl improved selectivity over mammalian cell growth inhibition (e.g. GNF3849). These
41 changes also resulted in low clearance and acceptable bioavailability. Further substitutions at the core C6
42 position led to GNF6702 and a 400-fold increase in intra-macrophage *L. donovani* potency compared to
43 GNF5343.

44 *L. donovani* parasites cause a majority of visceral leishmaniasis (VL) cases in East Africa and India⁹. In
45 mice infected with *L. donovani*¹⁰, oral dosing with GNF6702 effected a more pronounced reduction in

46 liver parasite burden than miltefosine, the only oral anti-leishmanial drug available in clinical practice⁵
47 (Fig. 2a). The miltefosine regimen for VL efficacy studies was chosen to approximate the drug plasma
48 concentration of the clinical regimen¹¹. We noted a greater than three log reduction in parasite load after
49 eight day treatment with 10 mg/kg of GNF6702 twice-daily with the free concentration of GNF6702
50 (fraction unbound in plasma=0.063) staying above the *L. donovani* EC₉₉ value (the concentration
51 inhibiting 99% of intra-macrophage parasite growth *in vitro*) for the duration of the dosing period
52 (Extended Data Fig. 1a). Characterization of efficacy of ten analogues in the series at various doses
53 revealed a significant correlation ($r^2=0.89$, $p<0.01$) between i) the ratio of mean free plasma compound
54 concentration to the *L. donovani* EC₉₀ value and ii) reduction of the liver parasite burden. We found that
55 90% parasite burden reduction in the mouse model was achieved when the mean free compound plasma
56 concentration during treatment equaled a 0.94-fold multiple of the *L. donovani* EC₉₀ value (Fig. 2b).
57 Cutaneous leishmaniasis (CL) affects about a million people per year, causing skin lesions that can
58 resolve into scar tissue¹². In parts of the Middle East, CL has reached epidemic proportions¹³. After
59 footpad infection of BALB/c mice with the dermatotropic *L. major* strain^{14,15}, treatment with GNF6702 at
60 10 mg/kg twice-daily caused a 5-fold decrease in footpad parasite burden and a reduction in footpad
61 swelling (Fig. 2c). Both 3 mg/kg and 10 mg/kg twice-daily regimens of GNF6702 were superior to 30
62 mg/kg once-daily miltefosine regimen ($p<0.01$), which translates into ~2-fold higher miltefosine plasma
63 concentration in mice than observed in clinical dosing¹¹.
64 We further tested if GNF6702 can cure additional kinetoplastid parasite infections. An estimated 25% of
65 the 8 million people infected with *T. cruzi* will develop chronic Chagas disease, manifesting as cardiac or
66 intestinal dysfunction^{16,17}. Benznidazole is broadly used for treatment of acute and indeterminate stages
67 of Chagas disease in Latin America^{18,19}. However, benznidazole has side-effects that frequently lead to
68 treatment interruption^{18,20-22} and a better tolerated drug is needed. To model treatment in the

69 indeterminate disease stage, we infected mice with *T. cruzi* parasites and began treatment 35 days after
70 infection, when the immune system of the mice had controlled parasite burden²³. We increased the
71 parasite detection sensitivity by immunosuppressing the mice after 20 days of treatment^{23,24}. In this
72 model, GNF6702 dosed twice-daily at 10 mg/kg matched the efficacy of benznidazole at 100 mg/kg
73 once-daily; all but one treated mice had no detectable parasites in blood, colon or heart tissue, even after
74 4 weeks of immunosuppression (Fig. 2d).

75 Finally, we tested GNF6702 in a mouse model of stage II sleeping sickness (human African
76 trypanosomiasis - HAT)²⁵. Mortality of stage II HAT is caused by infection of the CNS and, in this
77 mouse model, luciferase-expressing *T. brucei* parasites establish a CNS infection by day 21 post-
78 infection. GNF6702 was administered at 100 mg/kg once-daily to account for low exposure in the brain
79 relative to the plasma (~10%, Extended Data Fig. 1b). Diminazene aceturate, a stage I drug that poorly
80 crosses the blood-brain barrier, effected apparent clearance of parasites from the blood after a single dose,
81 but did not prevent parasite recrudescence 21 days later. By contrast, treatment with GNF6702 for seven
82 days caused a sustained clearance of parasites (days 42 and 92 post-infection in Fig. 2e, Extended Data
83 Fig. 2a, Supplementary Information Tables 4 and 5). Significantly, mice treated with GNF6702 had no
84 detectable parasites in the brain at termination of the experiment, though parasites were clearly detected
85 in the brains of mice treated with diminazene aceturate (Extended Data Fig. 2b, Supplementary
86 Information Table 6).

87 As GNF6702 showed compelling efficacy in four mouse models of kinetoplastid infections: VL, CL,
88 Chagas disease and stage II HAT, we reasoned that mechanistic studies of GNF6702 might identify a
89 pan-kinetoplastid drug target that could inform target-based drug discovery efforts. We attempted to
90 evolve *L. donovani* strains resistant to GNF3943 and GNF8000 (early analogues from the series,
91 Extended Data Fig. 3) through 12 months of parasite culture under drug pressure without success.

92 However, we were able to select two drug-resistant *T. cruzi* epimastigote isolates, one resistant to
93 GNF3943, and another to GNF8000. Both *T. cruzi* lines exhibited at least 40-fold lower susceptibility to
94 GNF6702 than wild type *T. cruzi* (Extended Data Fig. 4a and 4b). Using whole genome sequencing, we
95 found that the GNF3943-resistant line had a homozygous mutation encoding a substitution of isoleucine
96 for methionine at amino acid 29 in the proteasome beta 4 subunit ($PSMB4^{I29M/I29M}$) and a heterozygous
97 mutation P82L in dynein heavy chain gene. The GNF8000-resistant line had a heterozygous F24L
98 mutation in *PSMB4*, and four other heterozygous mutations (Extended Data Table 1). We focused our
99 attention on the proteasome as a likely target for the compound series because we found two independent
100 mutations in the *PSMB4* gene, and because the proteasome is an essential enzyme in eukaryotic cells. We
101 also note that the *Plasmodium falciparum* proteasome has recently been the target of promising drug
102 discovery efforts for malaria²⁶.

103 We first asked whether two prototypic inhibitors of mammalian proteasome, bortezomib and MG132,
104 could also block *T. cruzi* growth. Indeed, both compounds inhibited *T. cruzi* epimastigote proliferation
105 with sub-micromolar potency. However, in contrast to GNF6702, bortezomib and MG132 inhibited
106 proliferation of the two resistant lines ($PSMB4^{I29M/I29M}$, $PSMB4^{wt/F24L}$) with comparable potency to the
107 wild type parasites. Additionally, the *PSMB4* mutant lines were not resistant to nifurtimox, an anti-
108 kinetoplastid drug with an unrelated mechanism of action (Extended Data Fig. 4a and 4b). To determine
109 whether the F24L mutation was sufficient to confer resistance to GNF6702, we engineered *T. cruzi*
110 epimastigote lines that ectopically expressed either wild type or F24L-mutated *PSMB4*. Overexpression
111 of $PSMB4^{WT}$ had little effect on the EC_{50} value for GNF6702, whereas overexpression of $PSMB4^{F24L}$
112 caused a greater than 10-fold reduction in GNF6702 potency, but not in that of bortezomib (Fig. 3a,
113 Extended Data Fig. 4c). Previously, bortezomib was also shown to inhibit the growth of *T. brucei*,
114 suggesting that proteasome activity is essential for growth in this parasite as well²⁷. To test whether

115 PSMB4^{F24L} can rescue growth inhibition by GNF6702 in *T. brucei*, we engineered two parasite strains
116 that ectopically expressed wild type and F24L-mutated PSMB4, respectively. Similar to *T. cruzi*,
117 overexpression of PSMB4^{F24L} in *T. brucei* conferred a high level of resistance to GNF6702 (~70-fold
118 shift in EC₅₀ value), while having no effect on parasite susceptibility to bortezomib (Fig. 3b, Extended
119 Data Fig. 4c).

120 We next asked whether GNF6702 could inhibit any of three *T. cruzi* proteasome proteolytic activities in
121 biochemical assays. As predicted from the *T. cruzi* genome²⁸, mass spectrometry analysis of purified *T.*
122 *cruzi* proteasome identified seven alpha and seven beta proteasome subunits, including PSMB4
123 (Supplementary Tables 7 and 8). Using substrates that are specific for each of the chymotrypsin-like,
124 trypsin-like and caspase-like proteolytic activities, we found that only the chymotrypsin-like activity of
125 the *T. cruzi* proteasome was inhibited by GNF6702 (IC₅₀=35 nM), while the other two activities were not
126 affected (IC₅₀>10 μM). In contrast, bortezomib inhibited the chymotrypsin-like (IC₅₀=91 nM), the
127 caspase-like (IC₅₀=370 nM) and the trypsin-like (IC₅₀=1.7 μM) activities. We further found that the
128 chymotrypsin-like activity of the PSMB4^{I29M} *T. cruzi* proteasome was at least 300-fold less susceptible to
129 GNF6702 (IC₅₀>10 μM) and ~3-fold less susceptible to bortezomib (IC₅₀=0.26 μM), while susceptibility
130 of the other two mutant proteasome proteolytic activities to the two inhibitors were not affected (Fig. 4a,
131 Extended Data Table 2).

132 We reasoned that if the primary mechanism of parasite growth inhibition by the compound series was
133 through inhibition of the proteasome chymotrypsin-like activity, then the IC₅₀ values for this proteolytic
134 activity should correlate with EC₅₀ values for parasite proliferation. Indeed, a tight correlation between
135 the two parameters was observed for *L. donovani* axenic amastigotes and *T. brucei* bloodstream form
136 trypomastigotes (r²=0.78 and r²=0.67, respectively) over a 2,000-fold potency range for 317 analogues,
137 thus indicating that inhibition of parasite proteasome activity was driving the anti-parasitic activity of

138 these compounds. We observed a weaker correlation between IC₅₀ and EC₅₀ values for intracellular
139 *T. cruzi* ($r^2=0.36$, $p<0.01$), perhaps reflecting more complex cellular pharmacokinetics resulting from
140 compounds having to access *T. cruzi* parasites within the cytosol of mammalian cells (Fig. 4b, Extended
141 Data Fig. 5).

142 Both resistant *T. cruzi* lines retained sensitivity to bortezomib, which is a substrate-competitive inhibitor,
143 suggesting that GNF6702 might have an alternative mode of inhibition. A Lineweaver-Burk plot of
144 chymotrypsin-like activity at increasing concentrations of peptide substrate showed that GNF6702 has a
145 non-competitive mode of inhibition clearly distinct from the competitive mechanism described for
146 MG132 and bortezomib^{29,30}. We were also able to extend these observations to proteasome from *L.*
147 *donovani* (Fig. 4c, Extended Data Table 3). We further note that GNF6702 had no measurable activity on
148 the human proteasome (Fig. 4d, Extended Data Table 2). Interestingly, human proteasome beta 4 subunit
149 has a methionine at the 29th amino acid position, mirroring the I29M mutation in the GNF3943-resistant
150 *T. cruzi* line (Extended Data Fig. 6a).

151 In summary, GNF6702 blocks the chymotrypsin-like activity harbored by the beta 5 subunit without
152 competing with substrate binding, and mutations in the beta 4 subunit, which is in direct physical contact
153 with the beta 5 subunit, confer resistance to this inhibition. Next we used homology modeling of the *T.*
154 *cruzi* proteasome to look for evidence of an allosteric inhibitor binding site. In the *T. cruzi* proteasome
155 model, the F24 and I29 beta 4 residues are positioned at the interface between the beta 4 and beta 5
156 subunits, on the outer limit of the beta 5 active site. Adjacent to these two beta 4 residues and the beta 5
157 active site is a plausible binding pocket for GNF6702 (Extended Data Fig. 6b and 6c).

158 Finally, we tested whether GNF6702 can inhibit proteasome activity in intact *T. cruzi* cells. Cellular
159 proteins entering the proteasome degradation pathway are first tagged with ubiquitin, and proteasome
160 inhibition results in intracellular accumulation of ubiquitylated proteins. Treatment of *T. cruzi*

161 epimastigotes with GNF6702 led to significant buildup of ubiquitylated proteins (Extended Data Fig. 7a)
162 with the half-maximal effect (EC_{50}) achieved at 130 nM compound concentration (Extended Data Fig.
163 7c). This EC_{50} value correlated well with the half-maximal growth inhibitory concentration of GNF6702
164 on *T. cruzi* epimastigotes (EC_{50} =150 nM; Extended Data Fig. 4b). For comparison, similar experiments
165 with bortezomib yielded comparable inhibitor potencies in the two *T. cruzi* assays (ubiquitylation
166 EC_{50} =62 nM vs growth inhibition EC_{50} =160 nM; Extended Data Fig. 4b and 7c). We did not observe any
167 detectable accumulation of ubiquitylated proteins in mammalian 3T3 cells treated with GNF6702
168 (Extended Data Fig. 7b and 7c), further confirming high selectivity of this compound.

169 Validation of the parasite proteasome as the target of GNF6702 is supported through several lines of
170 evidence: i) point mutations in the *PSMB4* gene are sufficient to confer resistance to biochemical
171 proteasome inhibition and cellular *T. cruzi* growth inhibition; ii) GNF6702 is a selective inhibitor of
172 parasite proteasome activity and does not inhibit the human proteasome, mirroring the selective
173 inhibition of parasite growth over mammalian cell growth; and iii) potency of GNF6702 and analogues in
174 parasite proteasome assays predict potency in parasite growth inhibition assays.

175 In this work we show that in mouse disease models, GNF6702 was able to eradicate parasites from
176 diverse niches that included the cytosol (*T. cruzi*), phagolysosome (*L. donovani*, *L. major*) of infected
177 host cells, and brain (*T. brucei*). GNF6702 has also good pharmacokinetic properties, and the compound
178 did not show activity in panels of human receptor, enzyme and ion channel assays (Supplementary
179 Tables 9-11). Going forward, GNF6702, or analogues thereof, has potential to yield a new treatment for
180 several kinetoplastid infections and it is currently being evaluated in preclinical toxicity studies. It is
181 unclear if the clinical utility of GNF6702 could extend to the treatment of stage II HAT as GNF6702 was
182 tested in the HAT mouse model only at one high dose (100 mg/kg once-daily). We also note that
183 identification of a broadly active pan-kinetoplastid drug might not be feasible (or desirable) as such a

184 drug would need to reach high concentrations in varied tissues/subcellular compartments, and might
185 carry increased toxicity risk. Instead, alternative analogues from this series with different
186 pharmacological profiles might be needed for treatment of different kinetoplastid infections. Nevertheless,
187 there are only scarce resources for drug development in these diseases, and identification of a common
188 target and chemical scaffold with potential across multiple indications provides new hope for improved
189 treatment options for some of the world's poorest people.

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300 **FIGURE LEGENDS**

301 **Figure 1: Chemical evolution of GNF6702 from the phenotypic hit GNF5343.** *L. donovani*:
302 amastigotes proliferating within primary mouse macrophages; *T. brucei*: the bloodstream form
303 trypomastigotes; *T. cruzi*: amastigotes proliferating in 3T3 fibroblast cells; macrophage: mouse primary
304 peritoneal macrophages; EC₅₀ and CC₅₀: half-maximum growth inhibition concentration; F: oral
305 bioavailability in mouse after administering single compound dose (20 mg/kg) as a suspension; CL:
306 plasma clearance in mouse after single iv bolus dose (5 mg/kg); N.D.: not determined; all EC₅₀ and CC₅₀
307 values correspond to means ± s.e.m. (n=4 technical replicates).

308 **Figure 2: GNF6702 clears parasites in mouse models of kinetoplastid infections.** **a**, Post-treatment *L.*
309 *donovani* liver burdens in mouse model of VL as assessed by qPCR (n=5 mice). **b**, PK/PD relationship
310 for ten GNF6702 analogues, each administered at several doses; circles: mean liver burdens associated
311 with individual compound regimens (30 regimens in total; n=5 mice per regimen) relative to vehicle;
312 horizontal dotted line: 90% reduction in the liver *L. donovani* burden; vertical dotted line: 0.94-fold
313 multiple of the mean free compound plasma concentration/ the *L. donovani* EC₉₀ value ratio. **c**, Post-
314 treatment *L. major* footpad burdens in the BALB/c mouse model of CL as assessed by qPCR (n= 6 mice);

315 the p values (two-tailed distribution) relate parasite burdens in compound-treated mice with those from
316 vehicle-treated mice; left inset picture: a representative mouse footpad after treatment with vehicle; right
317 inset picture: a representative mouse footpad after treatment with GNF6702 10 mg/kg twice-daily
318 regimen. **d**, *T. cruzi* burden in mouse blood (circles), colon (triangles) and heart (diamonds) as assessed
319 by qPCR after 20 days of treatment and four weeks of immunosuppression (n=8 mice). **e**, Whole body *in*
320 *vivo* imaging of bioluminescent *T. brucei* before and after treatment; *Trypanosoma brucei*-infected mice
321 were treated by a single intraperitoneal injection of diminazene aceturate (n=3 mice) or by oral
322 administration of GNF6702 once-daily for 7 days (n=6 mice); filled symbols show whole body
323 bioluminescence values for individual mice; several mice from the untreated and diminazene aceturate-
324 treated groups were euthanized between days 28 and 56 due to CNS infection symptoms; background
325 bioluminescence values shown for uninfected mice (grey-filled squares; n=4) were collected
326 independently from mice aged-matched for day 0 using the same acquisition settings. Red dotted lines in
327 **a**, **c** and **d** plots show limit of parasite detection by qPCR; plot symbols below the red dotted line: mice
328 with no detectable parasites; data points below the limit of detection are 'jittered' to show number of
329 animals in a group; thick horizontal lines: means of the treatment groups; RU: relative units (parasite
330 burden relative to the mean burden of the vehicle-treated group).

331 **Figure 3: F24L mutation in proteasome beta 4 subunit confers selective resistance to GNF6702. a**,
332 growth inhibition of *T. cruzi* epimastigote strains ectopically expressing PSMB4^{WT} or PSMB4^{F24L} protein
333 by GNF6702 and bortezomib; non-induced/induced: culture medium without/with tetracycline to
334 modulate expression of tetracycline-inducible *PSMB4* genes. **b**, growth inhibition of *T. brucei*
335 bloodstream form trypomastigotes constitutively overexpressing PSMB4^{WT} or PSMB4^{F24L} protein by
336 GNF6702 and bortezomib. EC₅₀ values for each strain/compound pair are listed inside **a** and **b** plot
337 panels next to corresponding strain/compound symbol (defined in plot legends); means from n=3

338 technical replicates are shown; error bars represent s.e.m. values; for data points lacking error bars, s.e.m.
339 values are smaller than circles representing means; due to limited aqueous solubility, the highest tested
340 GNF6702 concentration was 10 μ M. RU (relative units) in **a** and **b** corresponds to parasite growth
341 relative to the DMSO control (%).

342 **Figure 4: Compounds from GNF6702 series inhibit growth of kinetoplastid parasites by inhibiting**
343 **parasite proteasome chymotrypsin-like activity. a**, Inhibition of three proteolytic activities of purified
344 wild type (PSMB4^{WT}) and PSMB4^{I29M} *T. cruzi* proteasomes by GNF6702 and bortezomib; IC₅₀ values
345 for proteasome proteolytic activities are listed inside plots. **b**, Correlation between inhibition of
346 chymotrypsin-like activity of purified *L. donovani* proteasome (IC₅₀) and *L. donovani* axenic amastigote
347 growth inhibition (EC₅₀; data points correspond to means of 2 technical replicates); red circles: IC₅₀>20
348 μ M; blue circles: EC₅₀>25 μ M; yellow circles: IC₅₀>20 μ M and EC₅₀>25 μ M; data for 317 analogues are
349 shown. **c**, Lineweaver-Burk plot of inhibition of *T. cruzi* proteasome chymotrypsin-like activity by
350 GNF6702 at increasing concentrations of a peptide substrate. **d**, Effect of GNF6702 and bortezomib on
351 three proteolytic activities of human constitutive proteasome; IC₅₀ values for proteasome proteolytic
352 activities are listed inside plots. Data shown in **a**, **c** and **d** represent means \pm s.e.m. (n=3 technical
353 replicates; for data points lacking error bars, s.e.m. values are smaller than circles representing means).
354 Due to limited aqueous solubility, the highest tested GNF6702 concentration in experiments shown in **a**
355 and **d** was 10 μ M.

356 **METHODS**

357 **Ethics statement for animal models.** All procedures involving mice were performed in accordance with
358 AAALAC standards or under UK Home Office regulations, and were reviewed and approved in
359 accordance with the Novartis Animal Welfare Policy. Sample size was determined on the basis of the

360 minimum number of animals required for good data distribution and statistics. Blinding was not possible
361 in these experiments but animals were selected randomly for each group.

362 **Determination of IC₅₀, EC₅₀ and CC₅₀ values, and data correlation.** Reported IC₅₀/ EC₅₀/ CC₅₀ values
363 were calculated by averaging IC₅₀/ EC₅₀/ CC₅₀ values obtained from individual technical replicate
364 experiments (n; specified in relevant Figure captions and Methods sub-sections). Each technical replicate
365 experiment was performed on a different day with freshly prepared reagents. Reported standard errors of
366 mean (s.e.m.) were calculated using IC₅₀/ EC₅₀/ CC₅₀ values determined in individual technical replicate
367 experiments. To calculate IC₅₀/ EC₅₀/ CC₅₀ values, measured dose response values were fitted with 4-
368 parameter logistic function $y=A+(B-A)/(1+(x/C)^D)$ (model 201, XLfit, IDBS), where x refers to
369 compound concentration and y corresponds to an assay readout value.

370 VL efficacy data for ten GNF6702 analogues (Fig. 2a) were fitted with 4-parameter logistic function
371 $y=A+(B-A)/(1+(x/C)^D)$ (model 201, XLfit, IDBS), where x values correspond to free mean compound
372 plasma concentrations and y values correspond to $\log_{10}(L. donovani$ liver burden).

373 To correlate parasite proteasome inhibition with parasite growth inhibition (Fig. 4b and Extended data
374 Fig. 5), we fitted data with $y=a*x+b$ function using the least square method (x corresponds to $\log_{10}(IC_{50})$;
375 y corresponds to $\log_{10}(EC_{50})$).

376 ***Leishmania donovani* axenic amastigote growth inhibition assay.** RPMI 1640 medium (HyClone) was
377 supplemented with 20% heat-inactivated fetal bovine serum (Omega Scientific), 23 μ M folic acid
378 (Sigma-Aldrich), 100 μ M adenosine (Sigma-Aldrich), 22 mM D-glucose (Sigma-Aldrich), 4 mM L-
379 glutamine (Hyclone), 25 mM 2-(4-morpholino) ethanesulfonic acid (Sigma-Aldrich) and 100 IU
380 penicillin/ 100 μ g/mL streptomycin (HyClone), and adjusted to pH= 5.5 with 6 M hydrochloric acid
381 (Fisher Scientific) at 37 °C. *Leishmania donovani* MHOM/SD/62/1S-CL2D axenic amastigotes were

382 cultured in 10 mL of this medium (Axenic Amastigote Medium) in T75 CELL-STAR flasks (Greiner
383 Bio-One) at 37 °C/ 5% CO₂ and passaged once a week.

384 To determine compound growth inhibitory potency on *L. donovani* axenic amastigotes, 100 nL of serially
385 diluted compounds in DMSO were transferred to the wells of white, solid bottom 384-well plates
386 (Greiner Bio-One) by Echo 555 acoustic liquid handling system (Labcyte). Then, 1 x 10³ of *L. donovani*
387 axenic amastigotes in 40 µL of Axenic Amastigote Medium were added to each well, and plates were
388 incubated for 48 hours at 37 °C/ 5% CO₂. Parasite numbers in individual plate wells were determined
389 through quantification of intracellular ATP. The CellTiter-Glo luminescent cell viability reagent
390 (Promega) was added to plate wells, and ATP-dependent luminescence signal was measured on an
391 EnVision MultiLabel Plate Reader (Perkin Elmer). Luminescence values in wells with compounds were
392 divided by the average luminescence value of the plate DMSO controls, and used for calculation of
393 compound EC₅₀ values as described above.

394 Axenic amastigote EC₅₀ values shown in Fig. 4b correspond to means of 2 technical replicates.

395 **Isolation and maintenance of *Leishmania donovani* splenic amastigotes.** Female BALB/cJ mice
396 (Envigo) infected with *L. donovani* MHOM/ET/67/HU3 (ATCC) for 50-80 days were euthanized, and
397 infected spleens were removed and weighed. The weight of an infected spleen ranged from 300 to 600
398 mg. For comparison, spleens from non-infected age-matched BALB/cJ mice weighed ~100 mg. Infected
399 spleens were washed in Axenic Amastigote Medium (composition described above) and placed into
400 Falcon 50 mL conical centrifuge tubes (Fisher Scientific) containing ice-cold Axenic Amastigote
401 Medium (15 mL per infected spleen). Spleens were homogenized on ice in a Dounce homogenizer and
402 centrifuged at 200 x g for 15 minutes at 4 °C to remove tissue debris. *Leishmania donovani* amastigotes
403 present in the supernatant were pelleted by centrifugation at 1,750 x g for 15 min at 4 °C and re-
404 suspended either in Axenic Amastigote Medium (when used for *in vitro* macrophage infections) or in

405 Hanks' Balanced Salt Solution (when used for mouse infections; Hyclone). Suspensions of splenic
406 amastigotes were kept on ice and used for *in vitro* or *in vivo* infections within 2-3 hours. To propagate *L.*
407 *donovani* amastigotes *in vivo*, 6 to 7 weeks old female BALB/cJ mice were infected with 8×10^7 purified
408 splenic amastigotes in 200 μ L of Hanks' Balanced Salt Solution by tail vein injection.

409 ***Leishmania donovani* intra-macrophage amastigote growth inhibition assay.** *In vitro* compound
410 potencies on intra-macrophage *L. donovani* MHOM/ET/67/HU3 were determined using primary murine
411 peritoneal macrophages infected with *L. donovani* splenic amastigotes. Primary macrophages were
412 elicited in female BALB/c mice for 72 hours following the injection of 500 μ L of sterile aqueous 2%
413 starch (J. T. Baker) solution into the mouse peritoneal cavity. The protocol used for isolation of
414 peritoneal macrophages was described in detail previously³¹. The isolated macrophages were re-
415 suspended in Macrophage Infection Medium (RPMI-1640 medium supplemented with 2 mM L-
416 glutamine, 10% heat-inactivated fetal bovine serum, 10 mM sodium pyruvate (Hyclone), and 100 IU
417 penicillin/ 100 μ g/mL streptomycin), and 50 μ L of macrophage suspension (8×10^5 macrophages/mL)
418 were added to microscopy-grade, clear-bottom, black 384-well plates (Greiner Bio-One). Following
419 overnight incubation at 37 °C/ 5% CO₂, plate wells were washed with Macrophage Infection Medium to
420 remove non-adherent cells using ELx405 Select microplate washer (BioTek), and then filled with 40 μ L
421 of Macrophage Infection Medium. *Leishmania donovani* HU3 splenic amastigotes isolated from infected
422 spleens were re-suspended in Macrophage Infection Medium at a concentration of 6×10^7 cells/mL, and
423 10 μ L of the suspension were added to assay plate wells containing adherent macrophages. After a 24-
424 hour infection period at 37 °C/ 5% CO₂, plate wells were washed with Macrophage Infection Medium to
425 remove residual extracellular parasites and re-filled with 50 μ L of the medium. *Leishmania donovani*-
426 infected macrophages were subsequently treated with DMSO-dissolved compounds (0.5% final DMSO
427 concentration in the assay medium) in dose response for 120 hours at 37 °C/ 5% CO₂. Next, treated

428 macrophages were washed with the phosphate-buffered saline buffer (PBS; Sigma-Aldrich)
429 supplemented with 0.5 mM magnesium chloride (Sigma-Aldrich) and 0.5 mM calcium chloride (Sigma-
430 Aldrich), fixed with 0.4% paraformaldehyde (Sigma-Aldrich) in PBS, permeabilized with 0.1% Triton X-
431 100 (Sigma-Aldrich) in PBS, and stained with SYBR Green I nucleic acid stain (Invitrogen, 1:100,000
432 dilution in PBS) overnight at 4 °C. Image collection and enumeration of macrophage cells and
433 intracellular *L. donovani* amastigotes was performed using the OPERA QEHS automated confocal
434 microscope system equipped with 20x water immersion objective (Evotec Technologies) and the OPERA
435 Acapella software (Evotec Technologies) as described previously³².

436 All reported intra-macrophage *L. donovani* EC₅₀ values were calculated from at least 3 technical
437 replicates (n= 3 or n= 4; specified in relevant Figure captions).

438 ***Trypanosoma brucei* growth inhibition assay.** Bloodstream form *Trypanosoma brucei* Lister 427
439 parasites were continuously passaged in HMI-9 medium formulated from IMDM medium (Invitrogen),
440 10% heat-inactivated fetal bovine serum, 10% Serum Plus medium supplement (SAFC Biosciences), 1
441 mM hypoxanthine (Sigma-Aldrich), 50 μM bathocuproine disulfonic acid (Sigma-Aldrich), 1.5 mM
442 cysteine (Sigma-Aldrich), 1 mM pyruvic acid (Sigma-Aldrich), 39 μg/mL thymidine (Sigma-Aldrich),
443 and 14 μL/L beta-mercapthoethanol (Sigma-Aldrich); all concentrations of added components refer to
444 those in complete HMI-9 medium. The parasites were cultured in 10 mL of HMI-9 medium in T75
445 CELL-STAR tissue culture flasks at 37 °C/ 5% CO₂.

446 To determine compound growth inhibitory potency on *T. brucei* bloodstream form parasites, 100 nL of
447 serially diluted compounds in DMSO were transferred to the wells of white, solid bottom 384-well plates
448 (Greiner Bio-One) by Echo 555 acoustic liquid handling system. Then, 5 x 10³ of *T. brucei* parasites in
449 40 μL of HMI-9 medium were added to each well, and the plates were incubated for 48 hours at 37 °C/ 5%
450 CO₂. Parasite numbers in individual plate wells were determined through quantification of intracellular

451 ATP amount. The CellTiter-Glo luminescent cell viability reagent was added to plate wells, and ATP-
452 dependent luminescence signal was measured on an EnVision MultiLabel Plate Reader. Luminescence
453 values in wells with compounds were divided by the average luminescence value of the plate DMSO
454 controls, and used for calculation of compound EC₅₀ values as described above.

455 *Trypanosoma brucei* EC₅₀ values shown in Fig. 1 and Extended Data Fig. 3 correspond to means of 4
456 technical replicates.

457 ***Trypanosoma cruzi* amastigote growth inhibition assay.** NIH 3T3 fibroblast cells (ATCC) were
458 maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% heat-inactivated fetal
459 bovine serum and 100 IU penicillin/ 100 µg/mL streptomycin at 37 °C/ 5% CO₂. *Trypanosoma cruzi*
460 Tulahuen parasites constitutively expressing *Escherichia coli* beta-galactosidase³³ were maintained in
461 tissue culture as an infection in NIH 3T3 fibroblast cells. Briefly, 2 x 10⁷ *T. cruzi* trypomastigotes were
462 used to infect 6 x 10⁵ NIH 3T3 cells growing in T75 CELL-STAR tissue culture flasks and cultured at
463 37 °C/ 5% CO₂ until proliferating intracellular parasites lysed host 3T3 cells and were released into the
464 culture medium (typically 6-7 days). During the infection, the tissue culture medium was changed every
465 two days. Number of *T. cruzi* trypomastigotes present in one mL of medium was determined using a
466 hemocytometer.

467 To determine compound potency on intracellular *T. cruzi* amastigotes, NIH 3T3 cells were re-suspended
468 in phenol red-free RPMI 1640 medium containing 3% heat-inactivated fetal bovine serum and 100 IU
469 penicillin/ 100 µg/mL streptomycin, seeded at 1,000 cells/ well (40 µL) in white, clear bottom 384-well
470 plates (Greiner Bio-One), and incubated overnight at 37 °C/ 5% CO₂. The following day, 100 nL of each
471 compound in DMSO were transferred to individual plate wells by Echo 555 acoustic liquid handling
472 system. After one hour incubation, 1 x 10⁶ of tissue culture-derived *T. cruzi* trypomastigotes, in 10 µL of
473 phenol red-free RPMI 1640 medium supplemented with 3% heat-inactivated fetal bovine serum and 100

474 IU penicillin/ 100 µg/mL streptomycin were added to each well. Plates were then incubated for 6 days at
475 37 °C/ 5% CO₂. Intracellular *T. cruzi* parasites were quantified by measuring the activity of parasite-
476 expressed beta-galactosidase. Ten microliters of a chromogenic beta-galactosidase substrate solution (0.6
477 mM chlorophenol red-β-D-galactopyranoside/ 0.6% NP-40 in PBS; both reagents from Calbiochem)
478 were added to each well and incubated for 2 hours at room temperature. After incubation, absorption was
479 measured at 570 nM on SpectraMax M2 plate reader (Molecular Devices). Measured absorbance values
480 in wells with compounds were divided by the average absorbance value of the plate DMSO controls, and
481 used for calculation of compound EC₅₀ values as described above.

482 *Trypanosoma cruzi* amastigote EC₅₀ values shown in Fig. 1 and Extended Data Fig. 3 correspond to
483 means of 4 technical replicates.

484 ***Trypanosoma cruzi* epimastigote proliferation assay.** *Trypanosoma cruzi* CL epimastigotes were
485 continuously passaged in LIT medium containing 9 g/L liver infusion broth (Difco), 5 g/L bacto-tryptose
486 (Difco), 1 g/L sodium chloride, 8 g/L dibasic sodium phosphate (Sigma-Aldrich), 0.4 g/L potassium
487 chloride (Sigma-Aldrich), 1 g/L D-glucose, 10 % heat-inactivated fetal bovine serum and 10 ng/mL of
488 hemin (Sigma-Aldrich). The medium was adjusted to pH= 7.2 with 6 M hydrochloric acid. The parasites
489 were cultured in 10 mL of LIT medium in T75 CELL-STAR tissue culture flasks at 27 °C.

490 To determine compound growth inhibitory potency on *T. cruzi* epimastigotes, 100 nL of serially diluted
491 compounds in DMSO were transferred to the wells of white, solid bottom 384-well plates (Greiner Bio-
492 One) by an Echo 555 acoustic liquid handling system. Then, 5 x 10³ of *T. cruzi* epimastigotes in 40 µL
493 of LIT medium were added to each well, and the plates were incubated for 7 days at 27 °C. Parasite
494 numbers in individual plate wells were determined through quantification of intracellular ATP amount.
495 The CellTiter-Glo luminescent cell viability reagent was added to plate wells, and ATP-dependent
496 luminescence signal was measured on an EnVision MultiLabel Plate Reader. Luminescence values in

497 wells with compounds were divided by the average luminescence value of the plate DMSO controls, and
498 used for calculation of compound EC₅₀ values as described above.

499 *Trypanosoma cruzi* epimastigote EC₅₀ values shown in Extended Data Fig. 4 correspond to means of 3
500 technical replicates.

501 **Mouse fibroblast NIH 3T3 growth inhibition assay.** NIH 3T3 fibroblast cells were maintained in
502 RPMI medium 1640 with glutamine (Life Technologies) supplemented with 5% heat-inactivated fetal
503 bovine serum and 100 IU penicillin/ 100 µg/mL streptomycin (3T3 Medium) at 37 °C/ 5% CO₂. NIH 3T3
504 fibroblast cells were purchased from ATCC. We did not perform cell line authentication and did not test
505 the cells for mycoplasma contamination. This cell line is not listed in the database of commonly
506 misidentified cell lines maintained by ICLAC and NCBI Biosample.

507 To determine compound potency, NIH 3T3 cells re-suspended in 3T3 medium were seeded at 1,000
508 cells/ well (50 µL) in white 384-well plates (Greiner Bio-One) and incubated overnight at 37 °C/ 5% CO₂.

509 The following day, 100 nL of each compound in DMSO were transferred to individual plate wells by
510 Echo 555 acoustic liquid handling system and plates were incubated for five days at 37 °C/ 5% CO₂. Cell
511 numbers in individual plate wells were determined through quantification of intracellular ATP amount.

512 The CellTiter-Glo luminescent cell viability reagent was added to plate wells, and ATP-dependent
513 luminescence signal was measured on an EnVision MultiLabel Plate Reader. Luminescence values in
514 wells with compounds were divided by the average luminescence value of the plate DMSO controls, and
515 used for calculation of compound CC₅₀ values as described above.

516 NIH 3T3 CC₅₀ values shown in Fig. 1 and Extended Data Fig. 3 correspond to means of 4 technical
517 replicates.

518 **Primary macrophage cytotoxicity assay.** Primary macrophage cell viability was determined on mouse
519 peritoneal macrophages infected with *L. donovani* and was expressed as the ratio of the number of

520 macrophage cells in wells treated with a compound to those in wells treated with DMSO. The number of
521 macrophage cells in wells was determined by high content microscopy as described previously³².
522 All reported macrophage CC₅₀ values were calculated from 4 technical replicates (n= 4; also specified in
523 Figure 1 and Extended Data Figure 3 captions).

524 **Selection of GNF3934- and GNF8000-resistant *T. cruzi* mutants.** *T. cruzi* epimastigotes cultures
525 resistant to GNF3943 and GNF8000 were generated using a methodology described previously³². Briefly,
526 epimastigotes were initially cultured in the presence of compound concentration equivalent to its
527 EC₂₀ value (GNF3943 EC₂₀= 1.5 μM and GNF8000 EC₂₀= 0.2 μM in 0.2% DMSO) or 0.2% DMSO
528 (control). Once a week, parasites were counted and growth rates were determined. If the parasite cultures
529 exhibited a reduced growth rate compared to 0.2% DMSO-treated parasites, epimastigotes were cultured
530 at the same compound concentration. Once the growth rates matched that of the control epimastigote
531 culture (0.2% DMSO), parasites were transferred into medium containing two-fold higher compound
532 concentration. The process was repeated until significant resistance was achieved (~10- to 20-fold
533 increase in corresponding EC₅₀ value). The time required for generation of cultures with such a level of
534 resistance was approximately five months. Resistant clones were isolated via cloning by limiting dilution,
535 and two independent clones were analyzed by whole genome sequencing.

536 ***T. cruzi* whole genome sequencing.** Chromosomal DNA isolation from GNF3943- and GNF8000-
537 resistant *T. cruzi* clones, whole genome sequencing and sequence analysis were performed as described
538 previously³². Sequencing reads were aligned to the *T. cruzi* CL Brenner genome³⁴.

539 **Generation of *T. cruzi* strains ectopically expressing proteasome beta 4 subunit variants.** *PSMB4*
540 TcCLB503891.100 was amplified from *T. cruzi* CL Brenner genomic DNA using KOD Hot Start DNA
541 Polymerase (EMD Millipore), and sense (5'-AAAGCGGCCGCATGTTCGGAGACAACCATTG-3) and
542 antisense (5'-CCATGATCTTGATGTAATATAAGGCATTCAGCCCTGCTG-3) primers. The

543 *PSMB4*^{F24L} gene was generated from the wild type *PSMB4* construct by site-directed mutagenesis using
544 mutagenic sense (5-CAGCAGGGCTGAATGCCTTATATTACATCAAGATCATGG-3') and antisense
545 (5'-CCATGATCTTGATGTAATATAAGGCATTCAGCCCTGCTG-3') primers and QuikChange II
546 Site-Directed Mutagenesis Kit (Stratagene). The sequences of the wild type and mutant *PSMB4* genes
547 were verified by sequencing and both gene versions were subcloned into the *T. cruzi* expression vector
548 pTcIndex1 under control of a T7 promoter³⁵. *Trypanosoma cruzi* CL Brenner epimastigotes were first
549 transfected as described previously³⁶ with the pLEW13 plasmid³⁷ harboring a tetracycline-inducible T7
550 RNA polymerase gene. Transfected epimastigotes were selected in medium supplemented with neomycin
551 (G418) at 500 µg/ml, and then transfected a second time with either pTcIndex1-*PSMB4*^{wt} or pTcIndex1-
552 *PSMB4*^{F24L} plasmid. Double transfected epimastigotes were selected in the presence of 500 µg/mL of
553 G418 (Sigma-Aldrich) and 500 µg/mL of hygromycin (Sigma-Aldrich). Susceptibility of double
554 transfected epimastigote cell lines to compounds was assessed using induced (+5 mg/mL of tetracycline)
555 and non-induced parasite cultures after five days of compound treatment. Parasite viability was
556 determined with AlamarBlue (ThermoFisher Scientific).

557 Reported EC₅₀ values for *T. cruzi* epimastigotes ectopically expressing PSMB4 proteins were calculated
558 from 3 technical replicates (n= 3; also specified in the Figure 3a caption).

559 **Generation of *T. brucei* strains ectopically expressing proteasome beta 4 subunit variants.** *PSMB4*
560 (Tb927.10.4710) was amplified from *T. brucei* Lister 427 genomic DNA using PCR SuperMix High
561 Fidelity (Invitrogen), sense (5'-GCAAGCTTATGGCAGAGACGACTATCGG-3) and antisense (5'-
562 GCGGATCCCTAGCTTACAGATTGCACTC-3') primers. The *PSMB4*^{F24L} gene was generated from the
563 wild type PSMB4 construct by site-directed mutagenesis using mutagenic sense (5'-
564 gctgcgggggttaaagcgttataactacattaagataacgg-3'), antisense (5'-ccgttatcttaagttagtataacgcatttaaccccgagc-3')
565 primers and QuikChange II Site-Directed Mutagenesis Kit (Stratagene). The sequences of the wild type

566 and mutant *PSMB4* genes were verified by sequencing and both gene versions were cloned into the *T.*
567 *brucei* expression vector pHD1034 under control of a ribosomal RNA promoter. Transfected *T. brucei*
568 Lister 427 cells were selected in medium supplemented with puromycin at 1 µg/ml. Susceptibility of
569 transfected *T. brucei* cell lines to compounds were assessed after 2 days of compound treatment. Parasite
570 viability was determined with CellTiter-Glo.

571 Reported EC₅₀ values for *T. brucei* parasites ectopically expressing PSMB4 proteins were calculated
572 from 3 technical replicates (n= 3; also specified in the Figure 3b caption).

573 **Purification of parasite 20S proteasomes.** *T. cruzi* CL epimastigotes, *L. donovani* MHOM/SD/62/1S-
574 CL2D axenic amastigotes and *T. brucei* Lister 27 bloodstream form trypomastigotes were grown to log
575 phase and harvested by centrifugation. The corresponding cell pellets were stored at -80 °C until further
576 use. Prior to purification, 10 g of cell pellets were thawed, re-suspended in lysis buffer (50 mM Tris-HCl
577 pH = 7.5, 1 mM TCEP, 5 mM EDTA, and 10 µM E-64), and lysed by passing cell suspension three times
578 through a needle (22 gauge) and by subsequent three freeze/ thaw cycles. The lysate was first cleared of
579 cellular debris by two centrifugation steps (15,000 x g at 4 °C for 15 minutes followed by 40,000 x g at
580 4 °C for 60 minutes) and then fractionated through ammonium sulfate precipitation. The protein fraction
581 precipitated between 45% and 65% of ammonium sulfate saturation was re-suspended in 25 mM Tris-
582 HCl pH = 7.5, 1 mM TCEP buffer, and dialyzed overnight at 4 °C against the same buffer. Proteasomes
583 were further purified by anion exchange chromatography (Resource Q column, GE Healthcare Life
584 Sciences) and size exclusion chromatography (Superose 6 column, GE Healthcare Life Sciences) as
585 described elsewhere³⁸. Active fractions from the latter purification step were pooled and used in
586 proteasome biochemical assays.

587 **Subunit composition analysis of purified *T. cruzi* 20S proteasome by LC/MS/MS.** Purified *T. cruzi*
588 proteasome sample was buffer-exchanged and concentrated into 100 mM trimethylamine bicarbonate-

589 HCl pH= 8.0, 150 mM NaCl buffer using a 10 kDa molecular weight cut-off micro-concentrator
590 (Milipore Amicon Ultra). The resulting proteasome sample (200 µl, 1 mg/ml) was mixed with 5 µl of a
591 TMTsixplex reagent (Pierce). After 60 second incubation to label primary amines, the reaction was
592 stopped by adding 25 µl of 5% hydroxylamine. The labeled sample was run on 4-20% Bis-Tris PAGE gel
593 (Invitrogen) to separate polypeptides. The gel was stained with eStain 2.0 (GenScript). Stained protein
594 bands were cut out and in-gel digested separately with elastase (Promega) and asparaginase (Roche).
595 Peptides generated by the digestions were resolved by HPLC using a vented column setup with a 2 cm
596 Poros 10 R2 (Life Technologies, Carlsbad, CA) self-packed pre-column, and a PepMap Easy-Spray C18
597 analytical column (15 cm x 75 µm ID, Thermo Scientific). Resin-bound proteolytic fragments were
598 eluted with 2 to 40% acetonitrile / 0.1% formic acid operated at 300 nL/min for 120 min. Spectra of
599 eluted peptide species were determined by a column-coupled Q Exactive hybrid quadrupole orbitrap
600 mass spectrometer (Thermo Scientific). Proteome Discoverer v1.4 software (Thermo Scientific) was used
601 to search the *T.cruzi* genome²⁸ with identified spectra for presence of 20S proteasome subunits
602 (Supplementary Table 7). Search parameters included fixed carbamidomethyl modification of cysteine,
603 and variable oxidation of methionine, deamidation of asparagine, pyro-glu of N-terminal glutamine, and
604 TMT(6-plex) modification of lysine residues.

605 **Measuring proteasome proteolytic activities.** The activity of purified parasite and human 20S
606 proteasomes was monitored by measuring cleavage of various rhodamine-labelled fluorogenic substrates.
607 Purified 20S proteasomes were diluted in proteasome assay buffer (25 mM Tris-HCl pH 7.5, 1 mM
608 dithiothreitol (Sigma-Aldrich), 10 mM sodium chloride, 25 mM potassium chloride, 1 mM magnesium
609 chloride, 0.05% (w/v) CHAPS (Sigma-Aldrich) and 0.9% DMSO) at a final concentration of 162 nM
610 (parasite proteasomes) or 25 nM (human proteasome), and pre-incubated with compound (40 nL; 0.2%
611 final DMSO concentration) for 1 hour. Next, the following substrates (Biosynthan GmbH) were added at

612 3 μ M final concentration to monitor specific proteolytic activities (Suc-LLVY-Rh110-dPro:
613 chymotrypsin-like activity; Ac-RLR-Rh110-dPro: trypsin-like activity; Ac-GLPD-Rh110-dPro: caspase-
614 like activity). The reaction was allowed to proceed for two hours at room temperature and fluorescence as
615 a measure of purified 20S proteasome activity was monitored using the EnVision® plate reader
616 (excitation at 485 nm/ emission at 535 nm). Km and Ki values were calculated using GraphPad Prism
617 (GraphPad Software) 'Non-competitive enzyme inhibition' function.

618 Data shown in Fig. 4a, 4c, 4d and Extended Data Table 3 represent means of 3 technical replicates (n= 3).
619 Data shown in Fig. 4b and Extended Data Fig. 5 represent means of 2 technical replicates (n= 2).

620 **Monitoring accumulation of ubiquitylated proteins in intact cells.** Growing *T. cruzi* epimastigotes
621 were seeded into 24-well tissue culture plate (1×10^7 cells/per well) in LIT medium and treated for 2-12
622 hours with DMSO (0.2%) or various concentrations of bortezomib and GNF6702 at 27 °C. Following the
623 treatment, parasites were collected by centrifugation (3,500 g for 6 minutes) and washed twice with
624 phosphate-buffered saline (PBS). Epimastigotes were lysed by resuspending washed cells in a buffer
625 containing 50 mM Tris-HCl pH= 7.4, 150 mM sodium chloride, 1% CHAPS, 20 μ M E-64 (Sigma-
626 Aldrich), 10 mM EDTA(Sigma-Aldrich), 5 mM N-ethylmaleimide(Sigma-Aldrich), 1 mM
627 phenylmethylsulfonyl fluoride (Sigma-Aldrich), 10 μ g/mL leupeptin (Sigma-Aldrich), 10 μ g/mL
628 aprotinin (Sigma-Aldrich), and incubating the suspension on ice for 20 minutes. Cell lysates were cleared
629 by centrifugation at 21,000 g for 30 min at 4 °C.

630 For 3T3 cells, 2×10^5 cells/ well were seeded into 24-well tissue culture plates in RPMI medium 1640
631 supplemented with 10% heat-inactivated fetal bovine serum, and incubated overnight at 37 °C to allow
632 cells to attach. Attached cells were treated for 2 hours with DMSO (0.25%) or various concentrations of
633 bortezomib and GNF6702. Treated cells were washed twice with PBS and then lysed by incubating cells
634 in modified RIPA buffer (50 mM Tris-HCl pH= 7.4, 1% Triton X-100, 0.2% sodium dodecylsulfate, 1

635 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 µg/mL aprotinin, 5 µg/mL leupeptin) for 30 min at
636 4 °C. Cell lysates were cleared by centrifugation at 21,000 g for 30 min at 4 °C.

637 Protein concentration in cell extracts was determined with BCA assay (ThermoFisher), and 10 µg of cell
638 extracts were loaded on NuPAGE Novex 4-12% Bis-Tris gel (Invitrogen). After electrophoresis, resolved
639 proteins were transferred to nitrocellulose membrane. Ubiquitylated proteins were detected with
640 polyclonal anti-ubiquitin primary antibody (Proteintech, catalogue number 10201-2-AP) and rabbit anti-
641 mouse IgG-peroxidase antibody (Sigma-Aldrich, catalogue number A0545), and then imaged using ECL
642 Prime Western Blotting Detection Reagent (Amersham) on Chemidoc XR+ imaging system (BioRad).

643 Collected western blot images were quantified using Image Lab software (BioRad). Briefly, rectangles of
644 identical size and shape were drawn around each blot lane to include inside the shape all ubiquitylated
645 protein bands within 17 - 198 kDa molecular mass range. Next, integrated signal intensities within the
646 rectangles (reported by the Image Lab software) were used for calculation of EC₅₀ values. Three
647 technical replicate experiments (n= 3) for each different dose response experiment (GNF6702 on *T. cruzi*
648 epimastigotes; GNF6702 on 3T3 cells; bortezomib on *T. cruzi* epimastigotes; bortezomib on 3T3 cells)
649 were performed.

650 ***Trypanosoma cruzi* proteasome modeling studies.** The homology model of *T. cruzi* 20S proteasome
651 was built using ‘Prime’ protein structure prediction program (Schrödinger) and X-ray structure of bovine
652 20S proteasome (pdb accession code 1IRU)³⁹ as the template. The model was subjected to restrained
653 minimization to relieve inter-chain clashes. ‘SiteMap’ program (Schrödinger) was used to identify
654 pockets on a protein surface suitable for small molecule binding. Flexible ligand docking was performed
655 using ‘Glide 5.8’ (Schrödinger). The grid box was centered in a middle of the identified pocket and
656 extended by 10 Å, with outer box extending additional 20Å. The ligand was docked using the standard

657 precision (SP) algorithm and scored using 'GlideScore' (Schrödinger). The GNF6702 GlideScore is
658 equal to -8.5.

659 **Receptor, enzyme and ion channel assays.** GNF6702 profiling was performed at 10 μ M concentration
660 in a selectivity panel at Eurofins (www.eurofinspanlabs.com/Catalog/AssayCatalog/AssayCatalog.aspx).
661 Listed values % change in the assay readout relative to the DMSO control. To determine inhibition of a
662 subset of human tyrosine kinases by GNF6702, the inhibitor was profiled on a panel of Ba/F3 cell lines
663 expressing individual Tel-activated kinases as described previously⁴⁰. All assays were performed as
664 single technical repeats.

665 **Determination of GNF6702 thermodynamic solubility.** The solubility of GNF6702 was assessed in a
666 high throughput thermodynamic solubility assay as described previously⁴¹. First, 25 μ L of GNF6702
667 DMSO solutions were transferred to individual wells of a 96-well plate. DMSO was evaporated and 250
668 μ L of 67 mM potassium phosphate buffer pH 6.8 were added to yield projected final compound
669 concentrations from 1 μ M to 100 μ M. The plate was sealed to prevent solvent loss and shaken for 24
670 hours at room temperature. The plate was then filtered to remove non-dissolved material. Concentration
671 of GNF6702 in individual plate wells was determined by measuring solution UV absorbance with
672 reference to a GNF6702 calibration curve.

673 **Determination of GNF6702 permeability in Caco-2 assay.** A 96-Multiwell Insert System (BD
674 Biosciences) was used for the Caco-2 cell culture and permeability assay as described previously⁴². Caco-
675 2 cells were seeded onto insert wells at a density of 1.48×10^5 cells per ml and allowed to grow for 19-23
676 days before assays. To measure both absorptive (apical to basolateral [A-B]) and secretory (basolateral to
677 apical [B-A]) compound transport, a solution of GNF6702 at 10 μ M concentration in 0.5% DMSO were
678 added to donor wells. The plate was incubated at 37°C for 2 hours, with samples taken at the beginning

679 and end of the incubation from both donor and acceptor wells. The concentration of GNF6702 was
680 determined by LC-MS/MS.

681 Apparent drug permeability (P_{app}) was calculated using the following equation:

$$682 \quad P_{app} = \frac{dQ}{dt} * \frac{1}{(A * C_{in})}$$

683 where dQ/dt is the total amount of a test compound transported to the acceptor chamber per unit of time
684 (nmol/s), A is the surface area of the transport membrane (0.0804 cm^2), C_{in} is the initial compound
685 concentration in the donor chamber ($10 \text{ }\mu\text{M}$), and P_{app} is expressed as cm/s .

686 **Determination of human CYP450 inhibition by GNF6702.** Extent of inhibition of major human
687 CYP450 isoforms 2C9, 2D6 and 3A4 by GNF6702 was determined using pooled human liver
688 microsomes and the known specific substrates of various CYP450 isoforms: diclofenac ($5 \text{ }\mu\text{M}$), bufuralol
689 ($5 \text{ }\mu\text{M}$), midazolam ($5 \text{ }\mu\text{M}$), and testosterone ($50 \text{ }\mu\text{M}$). Probe substrate concentrations were used at
690 concentrations equal to their reported K_m values. The CYP450 inhibition assays with probe substrates
691 diclofenac (2C9) or midazolam (3A4) were incubated at $37 \text{ }^\circ\text{C}$ for 5 to 10 minutes using a microsomal
692 protein concentration of 0.05 mg/mL . Probe substrates bufuralol (2D6) and testosterone (3A4) were
693 incubated at $37 \text{ }^\circ\text{C}$ for 20 minutes using microsomal concentration 0.5 mg/mL . The test concentrations of
694 GNF6702 ranged from 0.5 to $25 \text{ }\mu\text{M}$ in the presence of 1% DMSO. The reactions were initiated by
695 adding NADPH (1 mM final concentration; Sigma-Aldrich) after a 5-min pre-incubation. Incubations
696 were terminated by the addition of $300 \text{ }\mu\text{L}$ of acetonitrile to $100 \text{ }\mu\text{L}$ of a sample. No significant
697 cytochrome P450 inhibition was observed. Extent of CYP450 isoform inhibition was determined by
698 quantifying residual concentrations of individual CYP450 substrate probes at the end of reactions by
699 LC/MS/MS.

700 **Determination of GNF6702 *in vitro* metabolic stability.** The intrinsic metabolic stability of GNF6702
701 was determined in mouse and human liver microsomes using the compound depletion approach and

702 LC/MS/MS quantification. The assay measured the rate and extent of metabolism of GNF6702 by
703 measuring the disappearance of the compound. The assay determined GNF6702 *in vitro* half-life ($T^{1/2}$)
704 and hepatic extraction ratios (ER) as described previously⁴³. GNF6702 was incubated for 30 minutes at
705 1.0 μ M concentration in a buffer containing 1.0 mg/ mL liver microsomes. Samples (50 μ L) were
706 collected at 0, 5, 15 and 30 minutes and immediately quenched by addition of 150 μ L of ice-cold
707 acetonitrile/ methanol/water mixture (8/1/1). Quantification of GNF6702 in samples was performed by
708 LC/MS/MS, and the *in vitro* intrinsic clearance was determined using the substrate depletion method.

709 The intrinsic clearance, CL_{int} was calculated using the following equation:

$$710 \text{CL}_{\text{int}} = (0.693/ T^{1/2}) * (V/ M) ,$$

711 where $T^{1/2}$ is the *in vitro* half-life, V (μ L) is the reaction volume, and M (mg) is the microsomal protein
712 amount. Finally the hepatic extraction ratio is calculated as:

$$713 \text{ER} = \text{CL}_h / \text{Q}_h ,$$

714 where CL_h = hepatic clearance, Q_h = hepatic blood flow.

715 CL_h was calculated using the following equation:

$$716 \text{CL}_h = (\text{Q}_h * f_u * \text{CL}_{\text{int}}) / (\text{Q}_h + f_u * \text{CL}_{\text{int}}) ,$$

717 where f_u = fraction unbound to protein (assumed to be 1).

718 **Pharmacokinetic studies.** An outline of various *in vitro* and *in vivo* DMPK assays used in this study for
719 compound profiling was summarized previously⁴⁴. The pharmacokinetic properties of GNF compounds
720 and calculation of pharmacokinetic parameters was performed as described previously²³. Mean
721 compound plasma concentrations were calculated from fitted functions approximating compound plasma
722 profile throughout 8 days of dosing. Blinding was not possible in these experiments.

723 **Bioanalysis of GNF6702 in plasma.** Plasma concentration of GNF6702 was quantified using a
724 LC/MS/MS assay. Solution of 20 ng/mL of verapamil hydrochloride (Sigma-Aldrich) in

725 acetonitrile/methanol mixture (3/1 by volume), was used as an internal standard. Twenty microliters of
726 plasma samples were mixed with 200 μ l of internal standard solution. The samples were vortexed and
727 then centrifuged in an Eppendorf Centrifuge 5810R (Eppendorf) at 4,000 rpm for 5 minutes at 4 $^{\circ}$ C to
728 remove precipitated plasma proteins. The supernatants (150 μ l) were transferred to a 96-well plate and
729 mixed with 150 μ l H₂O. The samples (10 μ l) were then injected onto a Zorbax SB-C8 analytical column
730 (2.1 x 30 mm, 3.5 μ m; Agilent Technologies) and separated using a three step gradient (1st step: 1.5 mL
731 of 0.05% formic acid in 10% acetonitrile; 2nd step: 0.5 mL of 0.05% formic acid in 100% acetonitrile; 3rd
732 step: 0.5 mL of 0.05% formic acid in 10% acetonitrile) at flow rate of 700 μ l/min. GNF6702 and
733 verapamil were eluted at retention time 1.19 and 1.17 minutes, respectively. The HPLC system,
734 consisting of Agilent 1260 series binary pump (Agilent Technologies), Agilent 1260 series micro
735 vacuum degasser (Agilent Technologies) and CTC PAL-HTC-xt analytics autosampler (LEAP
736 Technologies) was interfaced to a SCIEX API 4000 triple quadrupole mass spectrometer (Sciex). Mass
737 spectrometry analysis was carried out using atmospheric pressure chemical ionization (APCI) in the
738 positive ion mode. GNF6702 (430.07 > 333.20) and verapamil (455.16 > 164.90) peak integrations were
739 performed using AnalystTM 1.5 software (Sciex). The lower limit of quantification (LLOQ) in plasma
740 was 1.0 ng/mL. Samples were quantified using seven calibration standards (dynamic range 1 – 5,000
741 ng/mL) prepared in plasma and processed as described above.

742 **Formulation of study drugs for *in vivo* efficacy experiments.** All compounds administered to mice
743 during efficacy experiments were formulated as suspensions in distilled water containing 0.5%
744 methylcellulose (Sigma-Aldrich) and 0.5% Tween 80 (Sigma-Aldrich). During a treatment course, each
745 mouse received 0.2 ml of drug suspension per dose by oral gavage.

746 **Mouse model of visceral leishmaniasis.** Female BALB/c mice (Envigo; 6-8 weeks old) were infected by
747 tail vein injection with 4×10^7 *L. donovani* MHOM/ET/67/HU3 splenic amastigotes (protocol number

748 P11-319). Seven days after infection, animals were orally dosed for eight days with vehicle (0.5%
749 methylcellulose/ 0.5% Tween 80, miltefosine (12 mg/kg once-daily; Sigma-Aldrich), or a GNF
750 compound (twice-daily). On the first day of dosing, three mice were used for collection of blood for PK
751 determination and euthanized afterwards. On the last day of dosing, PK samples were collected from
752 remaining five mice, which were also used for determination of compound efficacy (n= 5 mice per
753 group). Liver samples were collected from these five mice and *L. donovani* parasite burdens were
754 quantified by qPCR as follows. Total DNA was extracted from drug-treated mice livers using the
755 DNeasy Blood and Tissue Kit (Qiagen). Two types of DNA were quantified in parallel using the
756 TaqMan assay: *L. donovani* major surface glycoprotein gp63 (Ldon_GP63) and mouse GAPDH. *L.*
757 *donovani* GP63 DNA was quantified with the following set of primers:
758 TGCGGTTTATCCTCTAGCGATAT (forward), AGTCCATGAAGGCGGAGATG (reverse), and
759 TGGCAGTACTTCACGGAC (TaqMan MGB probe, 5'-FAM-labeled reporter dye, non-fluorescent
760 quencher). Mouse GAPDH DNA was quantified with the following set of primers:
761 GCCGCCATGTTGCAAAC (forward primer), CGAGAGGAATGAGGTTAGTCACAA (reverse
762 primer), and ATGAATGAACCGCCGTTAT (TaqMan MGB probe, 5'-FAM-labeled reporter dye, non-
763 fluorescent quencher). Each qPCR reaction (10 μ L) included 5 μ l of TaqMan Gene Expression Master
764 Mix (Life Technologies), 0.5 μ L of a 20X primer/probe mix (Life Technologies), and 4.5 μ L (50 ng) of
765 total DNA from liver samples. DNA amount was quantified using the Applied Biosystems 7900HT
766 instrument. *L. donovani* parasite burden (RU: relative units) was expressed as the abundance of *L.*
767 *donovani* GP63 DNA relative to the abundance of mouse GAPDH DNA.

768 **Mouse footpad model of cutaneous leishmaniasis.** *L. major* MHOM/SA/85/JISH118 metacyclic
769 promastigotes were generated and purified by the peanut agglutinin method as described elsewhere⁴⁵. To
770 establish the *L. major* footpad infection, female BALB/c mice (Envigo; 6-8 weeks old; protocol number

771 P11-319) were injected with suspension of *L. major* metacyclic promastigotes (1×10^6 parasites in 50
772 μL) into each hind footpad. After eight days of infection, animals were dosed with vehicle, miltefosine
773 (30 mg/kg once-daily), or indicated regimens of GNF6702 for seven days ($n=6$ mice per group). The
774 progress of infection was monitored by measuring the size (length and thickness) of hind footpad
775 swelling using digital calipers. At the end of the study, the mice were euthanized, and the footpad tissues
776 were extracted and used for genomic DNA isolation with the DNeasy Blood and Tissue kit (Qiagen). The
777 *L. major* footpad burden was determined by qPCR quantification of kinetoplastid minicircle DNA
778 (forward primer: 5'-TTTTACACCTCCCCCAGTTT-3'; reverse primer: 5'-
779 CCCGTTTCATAATTTCCCGAAA-3'; Taqman MGB probe: 5'-AGGCCAAAAATGG-3', 5'-FAM [6-
780 carboxyfluorescein]-labeled reporter dye, non-fluorescent quencher). The amounts of mouse
781 chromosomal DNA in extracted samples were quantified in parallel qPCR using a glyceraldehyde-3-
782 phosphate dehydrogenase (GAPDH) TaqMan assay as described for mouse VL model above. *L. major*
783 burden in footpad was expressed as the ratio of kinetoplast minicircle DNA to mouse GAPDH. P values
784 for the between-groups differences in efficacies were calculated with a Student's paired t test with a two-
785 tailed distribution.

786 **Mouse model of Chagas disease.** Compound efficacy in mouse model of Chagas disease was
787 determined as described previously²³. Female C57BL/6 mice (Envigo; 6-8 weeks old; protocol number
788 P11-316) were infected by intraperitoneal injection with 10^3 tissue culture-derived *T. cruzi* CL
789 trypomastigotes. Starting at 35 days after infection, the animals were dosed orally once-daily with 100
790 mg/kg benznidazole (Sigma-Aldrich) and indicated doses of GNF6702 (1, 3, and 10 mg/kg twice-daily,
791 $n=8$ per group) for 20 days. Ten days following the end of drug treatment, the mice underwent four
792 cycles of cyclophosphamide immunosuppression, each cycle lasting one week. During each
793 immunosuppression cycle, mice were dosed by oral gavage once-daily with 200 mg/kg

794 cyclophosphamide (suspension in 0.5% methylcellulose/ 0.5% Tween80 aqueous solution) on day 1 and
795 day 4 of the cycle. After the fourth immunosuppression cycle, blood samples were collected from the
796 orbital venous sinus of each mouse, mice were euthanized and heart and colon samples were collected.
797 Samples from treated mice were used for extraction of total DNA using the High Pure PCR template
798 preparation kit (Roche). The amounts of *T. cruzi* satellite DNA (195-bp fragment) in extracted DNA
799 samples were quantified by real-time qPCR TaqMan assay (Life Technologies) with the following set of
800 primers: AATTATGAATGGCGGGAGTCA (forward primer), CCAGTGTGTGAACACGCAAAC
801 (reverse primer), and AGACACTCTCTTTCAATGTA (TaqMan MGB probe, 5'-FAM [6-
802 carboxyfluorescein]-labeled reporter dye, non-fluorescent quencher). The amounts of mouse
803 chromosomal DNA in extracted samples were quantified in parallel qPCR reactions using a GAPDH
804 (glyceraldehyde-3-phosphate dehydrogenase) TaqMan assay as described for mouse VL model above.
805 Each qPCR mixture (10 µl) included 5 µl of TaqMan Gene Expression master mix (Life Technologies),
806 0.5 µl of a 20x primer/ probe mix (Life Technologies), and 4.5 µl (50 ng) of total DNA extracted from
807 blood samples. PCRs were run on the Applied Biosystems 7900HT instrument. *T. cruzi* parasitemia was
808 expressed as the abundance of *T. cruzi* microsatellite DNA relative to the abundance of mouse GAPDH
809 DNA.

810 **Mouse model of stage II HAT.** Female CD1 (Charles River UK; ~8 weeks old; protocol number PPL
811 60/4442) mice were infected by injection into the peritoneum with 3×10^4 *T. brucei* (GVR35-VSL2)
812 bloodstream form parasites⁴⁶. Starting on day 21, mice were dosed by oral gavage once-daily with
813 GNF6702 (n= 6) at 100 mg/kg for 7 days or a single dose of diminazene aceturate (Sigma-Aldrich) at 40
814 mg/kg in sterile water was administered by ip injection (n= 3). A group of untreated mice (n= 3) was
815 included as controls.

816 Mice were monitored weekly for parasitemia from day 21 post-infection. *T. brucei* was quantified in
817 blood samples from the tail vein by microscopy, and *in vivo* bioluminescence imaging of infected mice
818 was performed before treatment on day 21 post-infection and in weeks following the treatment (day 28,
819 35, 42, 56, 63, 72, 84, 92 post-infection). Imaging on groups of three mice was performed 10 min after ip
820 injection of 150 mg D-luciferin (Promega)/kg body weight (in PBS) using an IVIS Spectrum
821 (PerkinElmer) as described previously²⁵. A group of uninfected mice (aged-matched for day 0 time point;
822 n= 4) were imaged using the same acquisition settings to show the background bioluminescence (Fig. 2e,
823 grey-filled squares) in the absence of luciferase-expressing *T. brucei* after day 92 of the experiment.
824 Untreated and diminazene-treated mice were euthanized on days 32 and 35, and day 42, respectively, due
825 to high parasitemia or the development of symptoms related to CNS infection. GNF6702-treated mice
826 were euthanized on day 92. No parasitemia or clinical symptoms were observed at this point. At the
827 specified endpoints mice were sacrificed by cervical dislocation, after which whole brains were removed
828 and imaged *ex vivo* within 10 minutes after administration of 100 μ L of D-luciferin onto the brain
829 surface. Data analysis for bioluminescence imaging was performed using Living Image Software. The
830 same rectangular region of interest (ROI) covering the mouse body was used for each whole body image
831 to show the bioluminescence in total flux (photons per second) within that region. Image panels of whole
832 mouse bodies are composites of the original images with areas outside the ROI cropped out to save
833 space. For *ex vivo* brain images the same oval shaped ROI was used to display the bioluminescence
834 detected for each mouse brain at the respective endpoints.

835 **Chemical synthesis.** The detailed procedures for chemical synthesis are presented in Supplementary
836 Information.

837 **END NOTES**

838 **Supplementary Information** can be found at the end of this manuscript.

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848 **Contributions**

849 A.B., F.L., C.J.N.M., P.K.M., A.S.N., J.L.T. and V.Y. designed chemical analogues, and performed
850 chemical synthesis and purification of synthesized analogues. F.S.B., J.B., J.R.G., S.K., H.X.Y.K.,
851 Y.H.L., S.P.S.R., F.S., and X.L. conducted and analyzed data from *in vitro* growth inhibition assays.
852 L.C.D., X.L., J.C.M., E.M., I.C.R., S.P.S.R., M.S., F.S., and B.G.W. conducted and analyzed data from
853 *in vivo* efficacy assays. J.B., M.-Y.G., S.K., and F.S. conducted proteasome purification, proteasome
854 inhibition assays and biochemical data analysis. S.W.B., G.F., S.K., F.S., and J.R.W. designed, conducted
855 and analyzed experiments resulting in identification of proteasome resistance mutations. G.S. and B.B.
856 built the homology model of *T. cruzi* proteasome structure and performed GNF6702 docking. A.B. and
857 J.D.V. analyzed *T. cruzi* proteasome by mass spectrometry. A.N., T.G., M.S., F.S., and T.T. designed,
858 conducted, and analyzed PK data. A.N. and V.M. led the chemistry team. F.S. led the biology team.
859 R.J.G. and F.S. supervised and led the overall project, and led the writing of the manuscript. All authors
860 contributed to writing of the manuscript.

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869 *These authors contributed equally to this work.

870 **Competing financial interests**

871 Patents related to this work has been filed (WO 2015/095477 A1, WO 2014/151784 A1, WO
872 2014/151729). Several authors own shares of Novartis.

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875

876 **EXTENDED DATA LEGENDS**

877 **Extended Data Figure 1: Pharmacokinetic profile of GNF6702 in mouse. a,** Time profiles of mean
878 free plasma concentration of GNF6702 in mouse model of visceral leishmaniasis; free GNF6702
879 concentration values were predicted from measured total plasma concentration values collected on day 1
880 and day 8 of treatment. Dashed blue lines correspond to intra-macrophage *L. donovani* EC₅₀ of 18 ± 1.8
881 nM and EC₉₉ of 42 ± 5.6 nM. Circles: means ± s.d.; n=3 mice for treatment day 1; n=5 mice for treatment
882 day 8; fraction unbound in mouse plasma=0.063. For data points lacking error bars, standard deviations
883 are smaller than circles representing means. **b,** Time course of total GNF6702 concentration in mouse
884 plasma and brain after single oral dose (20 mg/kg); n=2 mice per time point; circles: measured values;
885 rectangles: means.

886 **Extended Data Figure 2: GNF6702 clears parasites from mice infected with *T. brucei*.** **a**, *In vivo*
887 quantification of bioluminescent *T. brucei* in infected mice before and after treatment. ip: intraperitoneal;
888 day 21: start of treatment; day 28: 24 hours after last GNF6702 dose; day 42: evaluation of early parasite
889 recrudescence in mice treated with diminazene aceturate (n=3); day 42 and 92: absence of parasite
890 recrudescence in mice treated with GNF6702 (n=6). Images from uninfected mice (3 mice of 4 are shown)
891 aged-matched for day 0 were collected independently using the same acquisition settings. Parasitemia
892 (blue font) and whole mouse total flux (black font) values of each animal are shown above the image;
893 N.D.: not detectable. Within each group the mouse numbers in yellow (top left in each image) refer to the
894 same mouse imaged throughout. Complete sets of parasitemia and whole mouse total flux values
895 collected on individual mice throughout the experiment are listed in Supplementary Tables 4 and 5. **b**,
896 Brains from mice shown in panel a were soaked in luciferin and imaged for presence of bioluminescent *T.*
897 *brucei* at the indicated time points. For three diminazene-treated mice, two images of each brain are
898 shown, one at a lower sensitivity (left) and the other at a high signal intensity scale.

899 **Extended Data Figure 3: Structures and profiles of GNF3943 and GNF8000 used for selection of**
900 **resistant *T. cruzi* lines.** *L. donovani*: amastigotes proliferating within primary mouse macrophages; *T.*
901 *brucei*: the bloodstream form trypomastigotes; *T. cruzi*: amastigotes proliferating in 3T3 fibroblast cells;
902 macrophage: mouse primary peritoneal macrophages; EC₅₀ and CC₅₀: half-maximum growth inhibition
903 concentration; F: oral bioavailability in mouse after administering single compound dose (20 mg/kg) as a
904 suspension; CL: plasma clearance in mouse after single iv bolus dose (5 mg/kg); all EC₅₀ and CC₅₀
905 values correspond to means ± s.e.m. (n=4 technical replicates).

906 **Extended Data Figure 4: Mutations in proteasome beta 4 subunit confer resistance to GNF6702 in**
907 ***T. cruzi* and *T. brucei*.** **a**, growth curves of wild type, GNF3943-resistant and GNF8000-resistant *T.*
908 *cruzi* epimastigote strains in the presence of increasing concentrations of GNF6702, nifurtimox,

909 bortezomib and MG132; RU (relative units) corresponds to parasite growth relative to the DMSO
910 control (%); for data points lacking error bars, standard errors are smaller than circles representing means;
911 due to limited aqueous solubility, the highest tested GNF6702 concentration was 10 μ M. **b**, growth
912 inhibition EC₅₀ values of GNF6702, bortezomib, MG132 and nifurtimox on indicated *T. cruzi* strains. **c**,
913 growth inhibition EC₅₀ values of GNF6702 and bortezomib on *T. cruzi* epimastigotes and *T. brucei*
914 bloodstream form trypomastigotes overexpressing PSMB4^{WT} or PSMB4^{F24L}. Data shown in panels **a**, **b**
915 and **c** correspond to means \pm s.e.m. (n=3 technical replicates).

916 **Extended Data Figure 5: Correlation between inhibition of parasite proteasome chymotrypsin-like**
917 **activity and parasite growth inhibition by the GNF6702 compound series.** IC₅₀: half-maximum
918 inhibition of indicated parasite proteasome; *T. brucei* EC₅₀: half-maximum growth inhibition on *T. brucei*
919 bloodstream form trypomastigotes; *T. cruzi* EC₅₀: half-maximum growth inhibition on *T. cruzi*
920 amastigotes proliferating inside 3T3 cells; data points correspond to means of 2 technical replicates; red
921 circles: IC₅₀>20 μ M; yellow circles: IC₅₀>20 μ M and EC₅₀>25 μ M; data for 317 analogues are shown.

922 **Extended Data Figure 6: Hypothetical model of GNF6702 binding to *T. cruzi* proteasome beta 4**
923 **subunit. a**, Alignment of amino acid sequences of proteasome beta 4 subunits (PSMB4) from *L.*
924 *donovani*, *T. cruzi*, *T. brucei* and *H. sapiens*. Green: amino acid residues conserved between human and
925 kinetoplastid PSMB4 proteins; blue: amino acid residues conserved only among kinetoplastid PSMB4
926 proteins; black: amino acids mutated in *T. cruzi* mutants resistant to analogues from the GNF6702 series.
927 **b**, Surface representation of the modeled *T. cruzi* 20S proteasome structure showing relative positions of
928 the beta 5 and beta 4 subunits. Beta 4 amino acid residues F24 and I29 (colored yellow) are located at the
929 interface of the two beta subunits. GNF6702 is depicted in a sphere representation bound into a predicted
930 pocket on the beta 4 subunit surface with carbon, nitrogen, oxygen and hydrogen atoms colored magenta,
931 blue, red and grey, respectively. The other *T. cruzi* 20S proteasome subunits are colored gray. **c**, Close-up

932 of the beta 5 and beta 4 subunits. The beta 5 subunit active site (pocket 1, chymotrypsin-like activity) is
933 colored pale green. The predicted beta 4 pocket (pocket 2) with bound GNF6702 is colored blue. The
934 inhibitor is shown in a stick representation with atoms colored as described in caption for the b panel.
935 Beta 4 residues F24 and I29 are colored yellow. The proteasome model shown in panels b and c was
936 produced by The PyMol Molecule Graphics System, Version 1.8, Schrodinger, LLC.

937 **Extended Data Figure 7: Effect of GNF6702 on accumulation of ubiquitylated proteins by *T. cruzi***
938 **epimastigotes and 3T3 cells. a,** Western blot analysis of *T. cruzi* whole cell extracts with anti-ubiquitin
939 antibody after treatment with GNF6702 and bortezomib. **b,** Western blot analysis of 3T3 whole cell
940 extracts with anti-ubiquitin antibody after treatment with GNF6702 and borteomib. **c,** Concentrations of
941 GNF6702 and bortezomib effecting half-maximum accumulation of ubiquitylated proteins in *T. cruzi* and
942 3T3 cells (means \pm s.e.m.; n=3 technical replicates); total ubiquitin signal values in individual blot lanes
943 shown in panels **a** and **b** were quantified and used for calculation of the listed EC₅₀ values. In **a** and **b**,
944 numbers above the blot lanes indicate compound concentrations and D indicates control, DMSO-treated
945 cells. For western blot source data, see Supplementary Figure 1.

946 **Extended Data Table 1: Point mutations identified by whole genome sequencing in GNF3943- and**
947 **GNF8000-resistant *T. cruzi* epimastigotes.**

948 **Extended Data Table 2: Enzyme inhibition IC₅₀ values of bortezomib and GNF6702 on three**
949 **proteolytic activities of wild type *T. cruzi*, PSMB4^{I29M} *T. cruzi*, and *H. sapiens* proteasomes.**

950 **Extended Data Table 3: Inhibition kinetics parameters of GNF6702 on *L. donovani* and *T. cruzi***
951 **proteasomes.**

952 SUPPLEMENTARY METHODS

953 1. Genomics Institute of the Novartis Research Foundation (GNF) chemical library

954 The GNF chemical library consists of ~3 million low molecular weight compounds.

955 **2. High throughput screening (HTS) campaigns and hit identification**

956 The high throughput screens were performed using 1,536 well polystyrene solid bottom white
957 microplates (Greiner Bio-One). The GNF chemical library was tested against *L. donovani*, *T. brucei* and
958 *T. cruzi* in whole-cell growth inhibition screens at single compound concentrations specified in sections
959 below describing individual parasite screens. Parasite proliferation protocols described in the Methods
960 section were optimized for 1,536 well plate assay format to provide optimal assay window and Z-factor.
961 Primary hits included compounds that reduced growth of parasites by more than 50% relative to the
962 relevant DMSO controls.

963 **2.1. *Leishmania donovani* HTS**

964 *Leishmania donovani* MHOM/SD/62/1S-CL2D axenic amastigotes in cell suspension were dispensed
965 into 1,536-well assay plates (2,000 parasite cells in 5 μ L of medium) and library compounds dissolved in
966 DMSO were added to 4 μ M final concentration (0.4% final DMSO concentration). After 48 hour
967 incubation at 37 °C, parasite viability was assessed using the CellTiter-Glo Luminescent Cell Viability
968 Assay (Promega) as described previously³². Compounds causing more than 50% reduction in parasite
969 viability were considered hits. Identified hits were subsequently evaluated in the screening assay in
970 triplicates at 4 μ M compound concentration. Compounds that inhibited *L. donovani* growth in at least
971 two replicates were considered confirmed hits.

972 **2.2. *Trypanosoma brucei* HTS**

973 *Trypanosoma brucei* Lister 427 bloodstream trypomastigotes in cell suspension were dispensed into
974 1,536-well assay plates (900 parasite cells in 7 μ L of medium) and library compounds dissolved in
975 DMSO were added to 7 μ M final concentration (0.7% final DMSO concentration). After 48 hour
976 incubation at 37 °C, parasite viability was assessed using the CellTiter-Glo Luminescent Cell Viability
977 Assay (Promega) as described previously³². Compounds causing more than 50% reduction in parasite

978 viability were considered hits. Identified hits were subsequently evaluated in the screening assay in
979 triplicates at 7 μ M compound concentration. Compounds that inhibited *T. brucei* growth in at least two
980 replicates were considered confirmed hits.

981 **2.3. *Trypanosoma cruzi* HTS**

982 A suspension of mouse fibroblast 3T3 cells was dispensed into 1,536-well assay plates (750 cells in 5 μ L
983 of medium). After overnight incubation at 37 $^{\circ}$ C, adhered 3T3 cells were infected with *T. cruzi*
984 trypomastigotes (2,500 trypomastigotes per well in 3 μ L of medium) and library compounds dissolved in
985 DMSO were added to 6.3 μ M final concentration (0.63% final DMSO concentration). After an additional
986 96 hour incubation at 37 $^{\circ}$ C, parasite viability was assessed using the BetaGlo Luminiscent Assay
987 (Promega) as described previously³². Compounds causing more than 50% reduction in parasite viability
988 were considered hits. Because of a large number of screen hits, we further followed upon only on a small
989 subset of hits that were also identified as confirmed hits in *L. donovani* and *T. brucei* high throughput
990 screens. Out of 93 such hits, 77 compounds were confirmed to be selective pan-kinetoplastid inhibitors (*L.*
991 *donovani*, *T. brucei*, *T. cruzi* EC₅₀ values < 10 μ M, selectivity index relative to 3T3 CC₅₀> 5).

992 **3. Chemical synthesis**

993 Unless otherwise noted, materials were obtained from commercial suppliers and were used without
994 purification. Removal of solvent under reduced pressure refers to distillation using Büchi rotary
995 evaporator attached to a vacuum pump (~3 mm Hg). Products obtained as solids or high boiling oils were
996 dried under vacuum (~1 mm Hg). Purification of compounds by high pressure liquid chromatography
997 was achieved using a Waters 2487 series with Ultra 120 5 μ m C18Q column with a linear gradient from
998 10% solvent A (acetonitrile with 0.035% trifluoroacetic acid) in solvent B (water with 0.05%
999 trifluoroacetic acid) to 90% A in four minutes, followed by two and half minute elution with 90% A.

1000 ¹H NMR spectra were recorded on Bruker XWIN-NMR (400 MHz or 600 MHz). Proton resonances are
1001 reported in parts per million (ppm) downfield from tetramethylsilane (TMS). ¹H NMR data are reported
1002 as multiplicity (s - singlet, d - doublet, t - triplet, q - quartet, quint - quintet, sept - septet, dd - doublet of
1003 doublets, dt - doublet of triplets, bs - broad singlet), number of protons and coupling constant in
1004 Hertz. For spectra obtained in CDCl₃, DMSO-*d*₆, CD₃OD, the residual protons (7.27, 2.50 and 3.31 ppm
1005 respectively) were used as the reference.

1006 Analytical thin-layer chromatography (TLC) was performed on commercial silica plates (Merck 60-F
1007 254, 0.25 mm thickness); compounds were visualized by UV light (254 nm). Flash chromatography was
1008 performed either by CombiFlash® (Separation system Sg. 100c, ISCO) or using silica gel (Merck
1009 Kieselgel 60, 230-400 mesh). Agilent 1100 series liquid chromatograph/ mass selective detector (LC/
1010 MSD) was used to monitor the progress of reactions and check the purity of products using 254 nm and
1011 220 nm wavelengths, and electrospray ionization (ESI) positive mode. Mass spectra were obtained in ESI
1012 positive mode. Elemental analyses were carried out by Midwest microlabs LLC, Indianapolis.

1013 **3.1. Synthesis of GNF5343**

1014 GNF5343 is a commercially available compound and was purchased from Chembridge laboratories
1015 (catalogue # 5840200).

1016 **3.2. Synthesis of GNF6702; N-(4-fluoro-3-(6-(pyridin-2-yl)-[1,2,4]triazolo[1,5-a]pyrimidin-2- 1017 yl)phenyl)-2,4-dimethyloxazole-5-carboxamide**

1018 **3.2.1. Synthesis of 2-fluoro-5-nitrobenzoyl chloride (1)**

1019 A solution of 2-fluoro-5-nitrobenzoic acid (50 g, 270 mmol) in thionyl chloride (100 mL) was heated to
1020 80 °C and stirred for 4 hours. The mixture was allowed to cool down to room temperature and the solvent
1021 was removed to give compound **1** (54 g, 98% yield).

1022 **3.2.2. Synthesis of 2-(2-fluoro-5-nitrobenzoyl)hydrazine-1-carboximidamide (2)**

1023 To a solution of aminoguanidine carbonate (36.2 g, 266 mmol) in dry toluene (300 mL) at 0 °C, was
1024 added compound **1** (54 g, 0.266 mol) over 30 minutes. The mixture was stirred at room temperature for
1025 12 hours. The formed precipitate was removed by filtration, and the residue was treated with H₂O (400
1026 mL) and made alkaline with sodium carbonate. The solid was collected and recrystallized from water to
1027 obtain compound **2** (62 g, 97% yield). M/Z 241.1 (M+1).

1028 **3.2.3. Synthesis of 5-(2-fluoro-5-nitrophenyl)-4H-1,2,4-triazol-3-amine (3)**

1029 A solution of compound **2** (62 g, 0.257 mol) in H₂O (800 mL) was stirred for 8 hours at 100 °C. After
1030 cooling, the obtained solid was filtered, and the cake was washed with H₂O (100 mL), tetrahydrofuran
1031 (100 mL), and dried to give compound **3** (34 g, 51% yield). ¹H NMR (400 MHz, DMSO) 12.42 (s, 1H),
1032 8.74 (dd, *J* = 6.27, 3.01, 1H), 8.26 (dt, *J* = 8.97, 3.42, 1H), 7.57 (t, *J* = 9.54, 1H), 6.29 (s, 2H).

1033 **3.2.4. Synthesis of 2-(2-fluoro-5-nitrophenyl)-6-(pyridin-2-yl)-[1,2,4]triazolo[1,5-a] pyrimidine (4)**

1034 To a solution of compound **3** (1 g, 4.48 mmol) in acetic acid (20 mL) 2-(pyridin-2-yl)malonaldehyde (0.8
1035 g, 5.376 mmol) was added at room temperature. The mixture was heated to 100 °C and stirred for 4 hours.
1036 The mixture was allowed to cool to room temperature before adding water (50 mL), filtered, and the filter
1037 cake was washed with saturate sodium bicarbonate solution (100 mL), H₂O (100 mL), and
1038 tetrahydrofuran (100 mL) and dried under vacuum to give compound **4** (0.9 g, 60% yield). ¹H NMR (400
1039 MHz, DMSO) 10.13 (d, *J* = 2.01, 1H), 9.68 (d, *J* = 2.01, 1H), 9.09- 9.02 (m, 1H), 8.77 (d, *J* = 4.27, 1H),
1040 8.28-8.19 (m, 1H), 8.15-7.96 (m, 2 H), 7.77 (t, *J* = 9.54, 1H), 7.56-7.43 (m, 1H).

1041 **3.2.5. Synthesis of 4-fluoro-3-(6-(pyridin-2-yl)-[1,2,4]triazolo[1,5-a]pyrimidin-2-yl)aniline (5)**

1042 To a solution of compound **4** (0.15 g, 0.443 mmol) in tetrahydrofuran (5 mL) was added Raney Nickel
1043 (0.2 g) and ZnI₂ (71 mg) at room temperature. The mixture was stirred under H₂ (50 psi) at 25 °C for 2.5
1044 hours. The mixture was diluted with methanol (10 mL) and filtered. The solvent was removed and the
1045 crude product was washed with methanol (5 mL x 2) and dried under vacuum to give compound **5** (90

1046 mg, 66% yield). ¹H NMR (400 MHz, DMSO) 10.01-10.06 (m, 1H), 9.62-9.58 (m, 1H), 8.73-8.78 (m,
1047 1H), 8.24-8.20 (m, 1H), 8.02-7.96 (m, 1H), 7.57-7.47 (m, 2H), 7.08-7.05 (m, 1H), 6.76-6.70 (m, 1H),
1048 5.24 (s, 2H) M/Z 307.01 (M+1).

1049 **3.2.6. Synthesis of N-(4-fluoro-3-(6-(pyridin-2-yl)-[1,2,4]triazolo[1,5-a]pyrimidin-2-yl)phenyl)-2,4-**
1050 **dimethyloxazole-5-carboxamide (GNF6702; 6)**

1051 To a solution of 2,4-dimethyloxazole-5-carboxylic acid (40.6 mg, 0.28 mmol) in dimethylformamide (5
1052 mL) was added HATU (118.6 mg, 0.31 mmol) and DIEA (72.4 mg, 0.56 mmol) at room temperature.
1053 The mixture was stirred for 30 min, the intermediate **5** (80 mg, 0.26 mmol) was added at room
1054 temperature. The mixture was stirred for 3 hours, water (10 mL) was added, the mixture was filtered, and
1055 the filter cake was washed with H₂O (5 mL x 2), tetrahydrofuran (5 mL x 2) and purified by HPLC to
1056 give product **6** (33 mg, 31% yield). ¹H NMR (400 M, MeOD) 9.84 (d, *J* = 2.4, 1H), 9.61 (d, *J* = 2.3, 1H),
1057 8.76 (dt, *J* = 4.8, 1.4, 1H), 8.54 (dd, *J* = 6.4, 2.7, 1H), 8.12 (dt, *J* = 8.0, 1.1, 1H), 8.00 (td, *J* = 7.8, 1.8,
1058 1H), 7.93 (ddd, *J* = 8.9, 4.1, 2.7, 1H), 7.49 (ddd, *J* = 7.5, 4.9, 1.0, 1H), 7.34 (dd, *J* = 10.4, 9.0, 1H), 2.57
1059 (s, 3H), 2.48 (s, 3H). M/Z= 430.13 (M+1).

1060 **3.3. Synthesis of GNF3943; Isopropyl (2-(2-chloro-5-(furan-2-carboxamido)phenyl)-1H-**
1061 **imidazo[4,5-b]pyridin-6-yl)carbamate**

1062 **3.3.1. Synthesis of 2-chloro-5-(furan-2-carboxamido)benzoic acid (7)**

1063 To a suspension of 5-amino-2-chlorobenzoic acid (13.7 g, 79.85 mmol, 1.00 equiv) in tetrahydrofuran
1064 (100 mL) was added furan-2-carbonyl chloride (11.5 g, 88.10 mmol, 1.10 equiv) at 0 °C. The ice bath
1065 was then removed and the reaction was stirred overnight at room temperature. The resulting mixture was
1066 concentrated under vacuum and diluted with DCM. The solid was collected by filtration to give 17 g
1067 (80%) of 2-chloro-5-(furan-2-amido)benzoic acid (**7**) as a gray solid.

1068 **3.3.2. Synthesis of *N*-(4-chloro-3-[6-nitro-1*H*-imidazo[4,5-*b*]pyridin-2-yl]phenyl)furan-2-**
1069 **carboxamide (8)**

1070 A mixture of 5-nitropyridine-2,3-diamine (6 g, 38.93 mmol, 1.00 equiv) and 2-chloro-5-(furan-2-
1071 amido)benzoic acid (7) (10.4 g, 39.15 mmol, 1.00 equiv) in polyphosphoric acid (PPA) (100 mL) was
1072 stirred overnight at 130 °C. The reaction was then poured into water/ice and the pH value of the mixture
1073 was adjusted to 9 with sodium carbonate. The solids were collected by filtration and applied onto a silica
1074 gel column with ethyl acetate/petroleum ether (3/1) to give 3.9 g (26%) of *N*-(4-chloro-3-[6-nitro-1*H*-
1075 imidazo[4,5-*b*]pyridin-2-yl]phenyl)furan-2-carboxamide (8) as a light yellow solid. ¹H NMR (400 MHz,
1076 DMSO) δ 10.50 (s, 1H), 9.19 (d, *J* = 2.6 Hz, 1H), 8.73 (s, 1H), 8.43 (d, *J* = 2.6 Hz, 1H), 8.03 – 7.90 (m,
1077 4H), 7.61 (d, *J* = 8.9 Hz, 1H), 7.41 (d, *J* = 3.6 Hz, 1H), 6.79 – 6.67 (m, 1H). MS *m/z* 383.9 (M+H)⁺.

1078 **3.3.3. Synthesis *N*-(3-[6-amino-1*H*-imidazo[4,5-*b*]pyridin-2-yl]-4-chlorophenyl)furan-2-**
1079 **carboxamide (9)**

1080 To a suspension of *N*-(4-chloro-3-[6-nitro-1*H*-imidazo[4,5-*b*]pyridin-2-yl]phenyl)furan-2-carboxamide
1081 (3.9 g, 10.16 mmol, 1.00 equiv) in ethanol (50 mL) was added SnCl₂·2H₂O (3.4 g, 15.04 mmol, 1.48
1082 equiv) and the resulting mixture was heated to reflux overnight. The reaction mixture was concentrated
1083 under vacuum and diluted with H₂O. The pH value of the mixture was adjusted to 9 with saturated
1084 sodium carbonate. The solids were collected by filtration and applied onto a silica gel column with ethyl
1085 acetate/PE (3/1) to give 1.95 g (54%) of *N*-(3-[6-amino-1*H*-imidazo[4,5-*b*]pyridin-2-yl]-4-
1086 chlorophenyl)furan-2-carboxamide (9) as a yellow solid. ¹H-NMR: (CD₃OD, 400 MHz): 8.16 (d, *J* = 2.4
1087 Hz, 1H), 7.97-8.10 (m, 2H), 7.78 (d, *J* = 0.8 Hz, 1H), 7.65 (d, *J* = 20.0 Hz, 1H), 7.31-7.41 (m, 2H), 6.68
1088 (dd, *J* = 3.6, 2.0 Hz, 1H). MS (M+H)⁺=354.

1089 **3.3.4. Synthesis of Isopropyl (2-(2-chloro-5-(furan-2-carboxamido)phenyl)-1*H*-imidazo[4,5-**
1090 ***b*]pyridin-6-yl)carbamate (GNF3943) (10)**

1091 To a 20 mL vial was transferred N-(3-(6-amino-1H-imidazo[4,5-b]pyridin-2-yl)-4-chlorophenyl)furan-2-
1092 carboxamide **9** (80 mg, 0.225 mmol) in dimethylformamide (4 mL) followed by addition of pyridine (2
1093 drops), and the reaction mixture was stirred at 0 °C for 10 minutes. At this point was added isopropyl
1094 carbonochloridate (1 M solution in toluene, 1.45 mmols, 6.4 eq). The reaction mixture was stirred
1095 overnight while slowly warming up to room temperature. The presence of desired peak (M+H (440))
1096 was confirmed by LC/MS. The reaction mixture was then quenched with saturated sodium carbonate
1097 solution to neutralize the extra acid chloride and to make the solution basic (pH 8-9). The reaction was
1098 extracted with ethyl acetate (3x10 mL), and the resulting organics were dried over sodium sulfate,
1099 filtered, and dried under vacuum. The resulting residue was purified via ISCO column chromatography
1100 using (0-100% ethyl acetate/hexane) to provide 53 mg, 0.119 mmol, 53% of the desired compound. ¹H
1101 NMR (400 MHz, MeOD) δ 8.28 (d, *J* = 22.1, 2H), 8.10 (s, 1H), 7.88 (s, 1H), 7.67 (d, *J* = 1.0, 1H), 7.51
1102 (d, *J* = 8.8, 1H), 7.24 – 7.16 (m, 1H), 6.56 (dd, *J* = 1.7, 3.5, 1H), 4.91 (dt, *J* = 6.2, 12.5, 1H), 1.24 (d, *J* =
1103 6.2, 6H). M/Z=440.1(M+1)

1104 **3.4. Synthesis of GNF8000; isopropyl (2-(2-fluoro-5-(furan-2-carboxamido)phenyl) imidazo[1,2-** 1105 **a]pyrimidin-6-yl)carbamate**

1106 **3.4.1. Synthesis of 1-(2-fluoro-5-nitrophenyl)ethan-1-one (11)**

1107 A 3,000 mL three necked flask equipped with a mechanic stirrer was charged with concentrated H₂SO₄
1108 (720 mL) and cooled to -40 °C. 1-(2-fluorophenyl)ethanone (180 g, 1.3 mol) was added, followed by
1109 addition of a mixture of fuming HNO₃ (106.2 mL) in concentrated H₂SO₄ (260 mL) dropwise over 45
1110 minutes. This mixture was stirred at this temperature for 15 minutes, poured into ice (8 kg), and extracted
1111 with ethyl acetate (2000 mL x 2). The combined ethyl acetate layer was washed with saturated NaHCO₃
1112 solution (800 mL x 3), brine (800 mL), dried with anhydrous sodium sulfate, and concentrated under
1113 vacuum. The residue was crystallized with petroleum ether to give compound **11** (200 g, yield: 84%) as a

1114 yellow solid. ¹H NMR (400 MHz, CDCl₃) δ 7.34 (t, *J* = 9.29 Hz, 1H), 8.33-8.48 (m, 1H), 8.78 (dd, *J* =
1115 6.15, 2.89 Hz, 1H).

1116 **3.4.2. Synthesis of 2-bromo-1-(2-fluoro-5-nitrophenyl)ethan-1-one (12)**

1117 To a solution of compound **11** (126 g, 0.688 mol) in acetic acid (860 mL) and 40% HBr solution (825.6
1118 mL) at 0 °C, was added a solution of Br₂ (110 g, 0.688 mol) in acetic acid (344 mL) in one portion. This
1119 mixture was stirred at room temperature overnight, diluted with water (3000 mL), and extracted with 50%
1120 ethyl acetate/petroleum ether (1500 mL x 2). The combined organic layer was washed with a saturated
1121 NaHCO₃ solution (1000 mL x 2), brine (1000 mL), dried with anhydrous sodium sulfate and
1122 concentrated. The residue was purified by column chromatography on silica gel (20% EA/PE) to give the
1123 compound **12** (150 g, yield: 83%) as a white solid. ¹H NMR (400 MHz, CDCl₃) δ 8.85 (dd, *J* = 5.90, 2.89
1124 Hz, 1H), 8.42-8.58 (m, 1H), 7.42 (t, *J* = 9.29 Hz, 1H), 4.52 (d, *J* = 2.01 Hz, 2H).

1125 **3.4.3. Synthesis of Isopropyl (2-aminopyrimidin-5-yl)carbamate (13)**

1126 A suspension of 5-nitropyrimidine-2-amine (1 eq.) and Pd/C (0.05 eq.) in ethanol (0.1 mM) was stirred
1127 under hydrogen atmosphere overnight at room temperature to give of 2,5-diaminopyrimidine. The
1128 mixture was then filtered and concentrated under vacuum. The residue (1 eq.) was subjected to coupling
1129 with isopropylcarbonochloridate (1.5 eq.) in anhydrous pyridine (0.3 mM) overnight at room temperature.
1130 The mixture was concentrated under vacuum, and the residue was extracted with ethyl acetate, washed
1131 with brine, dried over anhydrous MgSO₄ (s), filtered and concentrated under vacuum to give **13** as a
1132 yellow solid. *m/z* (ESI): 196 (M + H⁺).

1133 **3.4.4. Synthesis of isopropyl (2-(2-fluoro-5-nitrophenyl)imidazo[1,2-a]pyrimidin-6-yl)carbamate** 1134 **(14)**

1135 Into a 500 mL round-bottom flask, was placed 2-bromo-1-(2-fluoro-5-nitrophenyl)ethan-1-one **12** (30 g,
1136 114.49 mmol, 1 eq.), propan-2-yl N-(2-aminopyrimidin-5-yl)carbamate (11.2 g, 57.08 mmol, 0.5 eq.) and

1137 acetone (200 mL). The resulting solution was stirred overnight at 70 °C. The reaction mixture was cooled
1138 down and the solids were collected by filtration resulting in 15 g (36%) of propan-2-yl N-[2-(2-fluoro-5-
1139 nitrophenyl)imidazo[1,2-a]pyrimidin-6-yl]carbamate (**14**) as a brown solid.

1140 **3.4.5. Synthesis of isopropyl (2-(5-amino-2-fluorophenyl)imidazo[1,2-a]pyrimidin-6-yl)carbamate** 1141 **(15)**

1142 Into a 1 L round-bottom flask was placed tetrahydrofuran (500 mL), Raney Ni (15 g) and propan-2-yl N-
1143 [2-(2-fluoro-5-nitrophenyl)imidazo[1,2-a]pyrimidin-6-yl]carbamate **14** (8 g, 22.26 mmol, 1 eq.). The
1144 resulting solution was stirred overnight at room temperature under an atmosphere of hydrogen. The solids
1145 were filtered out, and washed with methanol (200 mL x 4). The resulting mixture was concentrated under
1146 vacuum to give 7 g (95%) of propan-2-yl N-[2-(5-amino-2-fluorophenyl)imidazo[1,2-a]pyrimidin-6-
1147 yl]carbamate (**15**) as a brown solid. ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.94 (s, 1H), 9.24 (s, 1H), 8.46-
1148 8.47 (m, 1H), 8.26-8.28 (m, 1H), 7.51-7.53 (m, 1H), 6.96-7.02 (m, 1H), 6.55-6.59 (m, 1H), 4.89-4.98 (m,
1149 1H), 3.17 (s, 2H), 1.07-1.30(m, 6H). MS *m/z*= 330 (M+1).

1150 **3.4.6. Synthesis of isopropyl (2-(2-fluoro-5-(furan-2-carboxamido)phenyl)imidazo[1,2-a]pyrimidin-** 1151 **6-yl)carbamate (GNF8000) (16)**

1152 In a 40 mL vial, pyridine (10 mL) was added to intermediate **15** (0.5 g, 1.518 mmol) to give a yellow
1153 solution. To this solution was added furan-2-carbonyl chloride (0.198 g, 1.518 mmol) at 0 °C and the
1154 resulting mixture was stirred for 1 hour. The reaction mixture was quenched with 60 mL of water and
1155 extracted with ethyl acetate. The same step was repeated once more time to remove any extra pyridine.
1156 All organic phases were combined, dried over sodium sulfate and purified by flash chromatography to
1157 give product **16** (ethyl acetate/methanol= 0-10%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.43 (s, 1H), 10.06
1158 (s, 1H), 9.36 (s, 1H), 8.69 (dd, *J* = 2.8, 6.9 Hz, 1H), 8.56 (d, *J* = 2.7 Hz, 1H), 8.45 (d, *J* = 4.2 Hz, 1H),
1159 8.02 (d, *J* = 1.0 Hz, 1H), 7.95-7.85 (m, 1H), 7.46 (d, *J* = 3.4 Hz, 1H), 7.37 (dd, *J* = 9.0, 10.9 Hz, 1H),

1160 6.78 (dd, $J = 1.7, 3.5$ Hz, 1H), 5.00 (dt, $J = 6.3, 12.5$ Hz, 1H), 1.35 (d, $J = 6.2$ Hz, 6H). MS $m/z = 424$
1161 (M+1).

1162 **3.5. Synthesis of GNF3849; N-(4-fluoro-3-(6-phenyl-[1,2,4]triazolo[1,5-a]pyrimidin-2-yl)phenyl)-**
1163 **2,4-dimethyloxazole-5-carboxamide**

1164 **3.5.1. Synthesis of 2-(2-fluoro-5-nitrophenyl)-6-phenyl-[1,2,4]triazolo[1,5-a]pyrimidine (17)**

1165 To a solution of compound **3** (0.5 g, 2.24 mmol) in AcOH (5 mL) was added 2-phenylmalonaldehyde
1166 (0.39 g, 2.7 mmol). The mixture was then heated to 100 °C and stirred for 4 hours. The mixture was
1167 allowed to cool to room temperature, water (10 mL) was added, the solids filtered, and the filter cake was
1168 washed with tetrahydrofuran, and dried under vacuum to give compound **17** (0.36 g, 48% yield). ^1H
1169 NMR (400 MHz, DMSO) 9.93 (d, $J = 2.4$, 1H), 9.38 (d, $J = 2.8$, 1H), 8.90 (s, 1H), 7.93 (d, $J = 7.78$, 2H),
1170 7.69 (d, $J = 8.53$, 1H), 7.61-7.50 (m, 2H), 7.31 (t, $J = 7.40$, 1H), 6.88 (s, 1H).

1171 **3.5.2. Synthesis of 4-fluoro-3-(6-phenyl-[1,2,4]triazolo[1,5-a]pyrimidin-2-yl)aniline (18)**

1172 To a solution of compound **17** (2.5 g, 7.4 mmol) in tetrahydrofuran (200 mL) was added ZnI_2 (1.2 g, 3.7
1173 mmol) and Raney Nickel (3.5 g). This mixture was stirred at room temperature for 4 hour under H_2 at 50
1174 psi, then the mixture was filtrated and washed with methanol (20 mL) to give compound **18** (2.0 g, 87%
1175 yield). ^1H NMR (400 MHz, DMSO) 9.81 (d, $J = 2.4$, 1H), 9.27 (d, $J = 2.8$, 1H, 7.90 (d, $J = 7.6$, 2H),
1176 7.58-7.53 (m, 2H), 7.45-7.50 (m, 2H), 7.09-7.05 (m, 1H), 6.74-6.70 (m, 1H), 5.22 (s, 2H). M/Z 306.1
1177 (M+H⁺).

1178 **3.5.3. Synthesis of N-(4-fluoro-3-(6-phenyl-[1,2,4]triazolo[1,5-a]pyrimidin-2-yl)phenyl)-2,4-**
1179 **dimethyloxazole-5-carboxamide (GNF3849) (19)**

1180 To a solution of 2,4-dimethyloxazole-5-carboxylic acid (0.56 g, 3.9 mmol) in dimethylformamide (30 mL)
1181 was added DIEA (0.85 g, 6.66 mmol) and HATU (1.5 g, 3.9 mmol). This mixture was stirred at room
1182 temperature for 30 minutes, then compound **18** (1.0 g, 3.28 mmol) was added. The mixture was then

1183 stirred at room temperature for 4 hours, diluted with water (50 mL) and extracted with tetrahydrofuran/
1184 ethyl acetate (100 mL /50 mL), the organic layer was dried over sodium sulfate and concentrated to give
1185 the crude product. It was purified by HPLC to give product **19** (0.91 g, yield, 65%) as a white solid. ¹H
1186 NMR (400 MHz, MeOD) 9.49 (d, *J* = 2.4, 1H), 9.22 (d, *J* = 2.4, 1H), 8.51 (dd, *J* = 6.4, 2.8, 1H), 7.90
1187 (ddd, *J* = 8.9, 4.2, 2.8, 1H), 7.86-7.76 (m, 2H), 7.63-7.55 (m, 2H), 7.54-7.45 (m, 1H), 7.32 (dd, *J* = 10.4,
1188 9.0, 1H), 2.56 (s, 3H), 2.47 (s, 3H). *M/Z*= 429.2 (M+H⁺).

1189 **3.6. Synthesis of GNF2636; isopropyl (2-(2-chloro-5-(furan-2-carboxamido)phenyl)imidazo[1,2-**
1190 **a]pyrimidin-6-yl)carbamate**

1191 **3.6.1. Synthesis of isopropyl (2-(2-chloro-5-nitrophenyl)imidazo[1,2-a]pyrimidin-6-yl)carbamate**
1192 **(20)**

1193 Into a 500-mL round-bottom flask, was placed **13** (1.75 g, 6.3 mmol, 1.2 equiv), acetone (400 mL) and 2-
1194 bromo-1-(2-chloro-5-nitrophenyl)ethan-1-one (1.0 g, 5.3 mmol). The resulting solution was stirred
1195 overnight at 70 °C. The reaction mixture was cooled, the solvent evaporated, the resulting material
1196 suspended in methanol, and then solids collected by filtration resulting in product **20** (0.75 g, 38% yield).
1197 ¹H NMR (400 MHz, DMSO-D₆) δ 10.08 (s, 1H), 9.34 (s, 1H), 9.08 (s, 1H), 8.86 (s, 1H), 8.56 (s, 1H),
1198 8.19 (d, *J* = 8.7, 1H), 7.88 (d, *J* = 8.8, 1H), 4.95 (m, 1H), 1.30 (m, 6H). MS *m/z* (ESI) = 377 (M +).

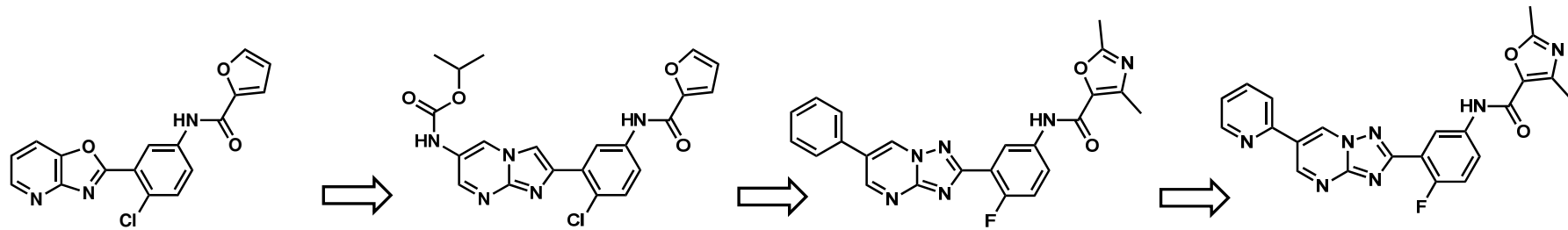
1199 **3.6.2. Synthesis of isopropyl (2-(5-amino-2-chlorophenyl)imidazo[1,2-a]pyrimidin-6-yl)carbamate**
1200 **(21)**

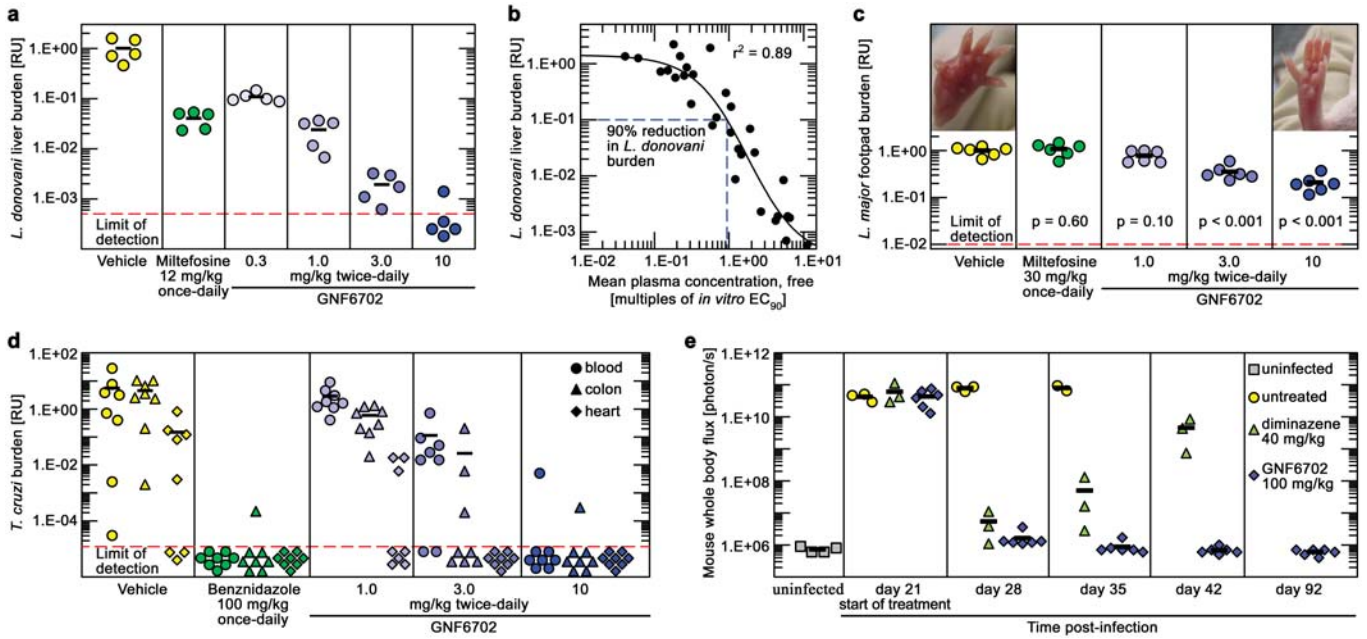
1201 In a round-bottom flask, **20** (300 mg, 0.77 mmol) was taken up in methanol (20 mL) and SnCl₂ (3
1202 equivalents) was added. The resulting mixture was stirred for 2 hours at reflux. The reaction mixture was
1203 concentrated under vacuum and the crude material was purified by flash column chromatography
1204 (hexane/ ethyl acetate solvent system followed by DCM/methanol solvent system) resulting in **21** (265
1205 mg, 96%) as a yellow solid. ¹H-NMR: (300 MHz, MeOD): 9.30 (s, 1H), 8.51 (d, *J* = 2.1 Hz, 1H), 8.37 (s,

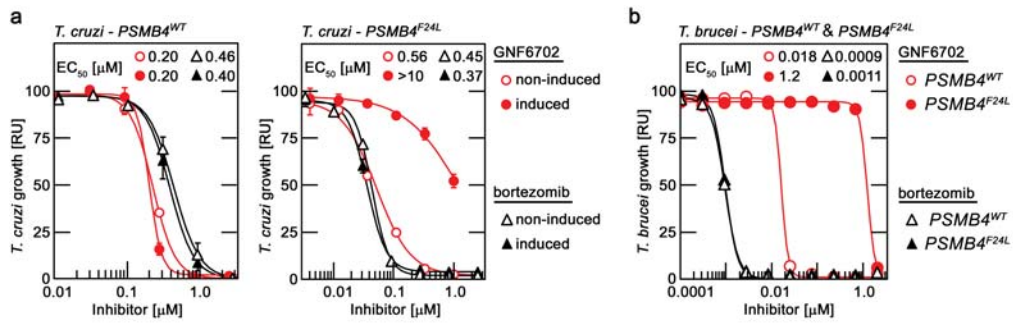
1206 1H), 7.37 (d, $J = 1.8$ Hz, 1H), 7.23 (d, $J = 6.6$ Hz, 1H), 6.72-6.74 (m, 1H), 5.01-5.07 (m, 1H), 1.24-1.36
1207 (m, 6H). MS $m/z = 346$ (M+H+).

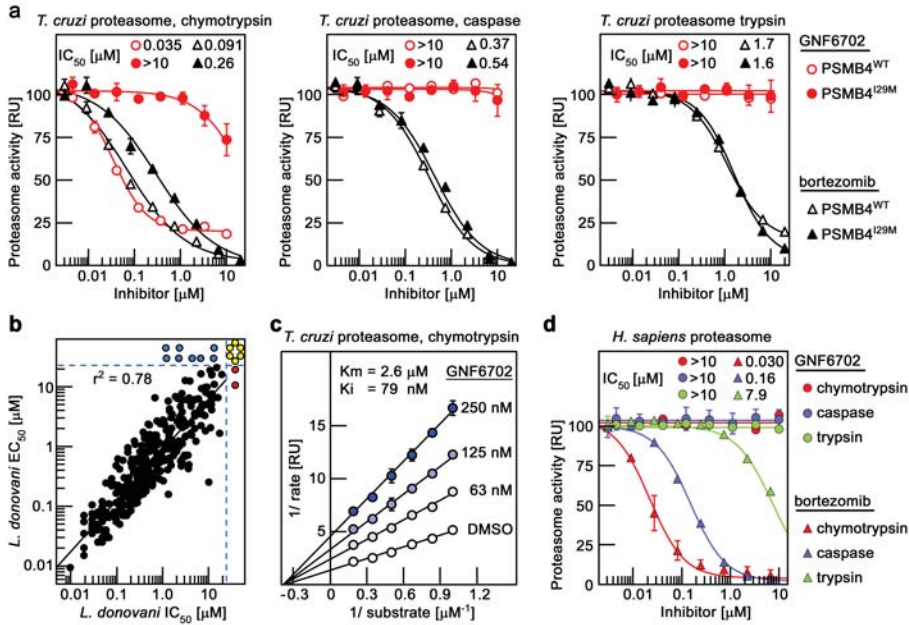
1208 **3.6.3. Synthesis of isopropyl (2-(2-chloro-5-(furan-2-carboxamido)phenyl)imidazo[1,2-a]**
1209 **pyrimidine-6-yl)carbamate (GNF2636) (22)**

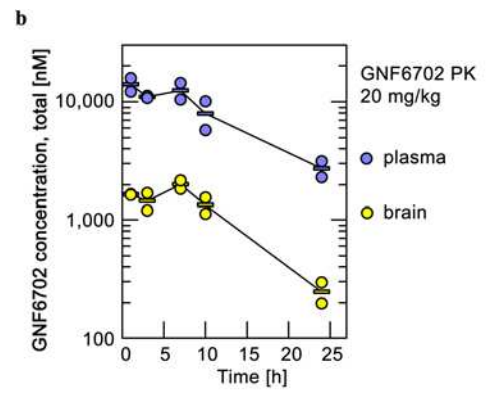
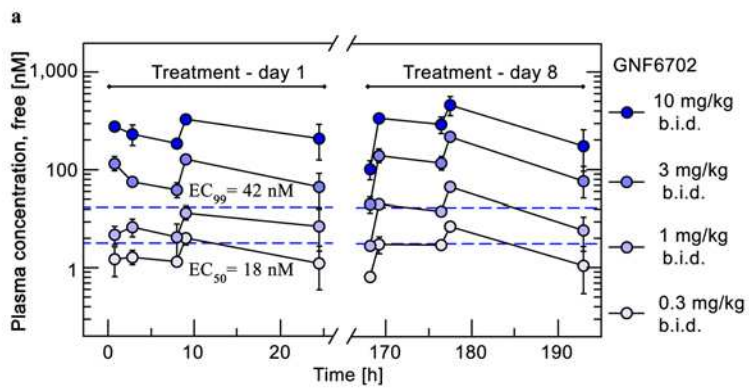
1210 To a suspension of compound **21** (20 mg, 0.06 mmol) in pyridine (2 mL) in a vial was added 2-furoyl
1211 chloride (1.5 equivalents) at room temperature. After stirring overnight, the reaction was concentrated
1212 and the resulting residue was purified by prep HPLC to afford the product **22** (5 mg, 19% yield). ¹H
1213 NMR (400 MHz, methanol-*d*₄) δ 9.57 (s, 1H), 8.76 (d, $J = 2.6$ Hz, 1H), 8.52 (s, 1H), 8.31 (d, $J = 2.6$ Hz,
1214 1H), 7.89 – 7.69 (m, 2H), 7.62 (d, $J = 8.8$ Hz, 1H), 7.32 (d, $J = 3.5$ Hz, 1H), 6.68 (dd, $J = 3.5, 1.7$ Hz,
1215 1H), 5.14 – 4.97 (m, 1H), 1.35 (d, $J = 6.3$ Hz, 6H). MS $m/z = 440.2$ (M+H).

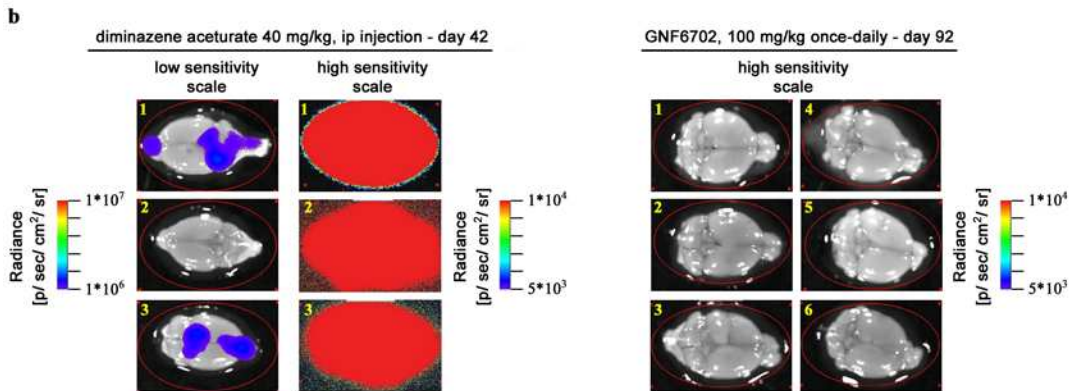
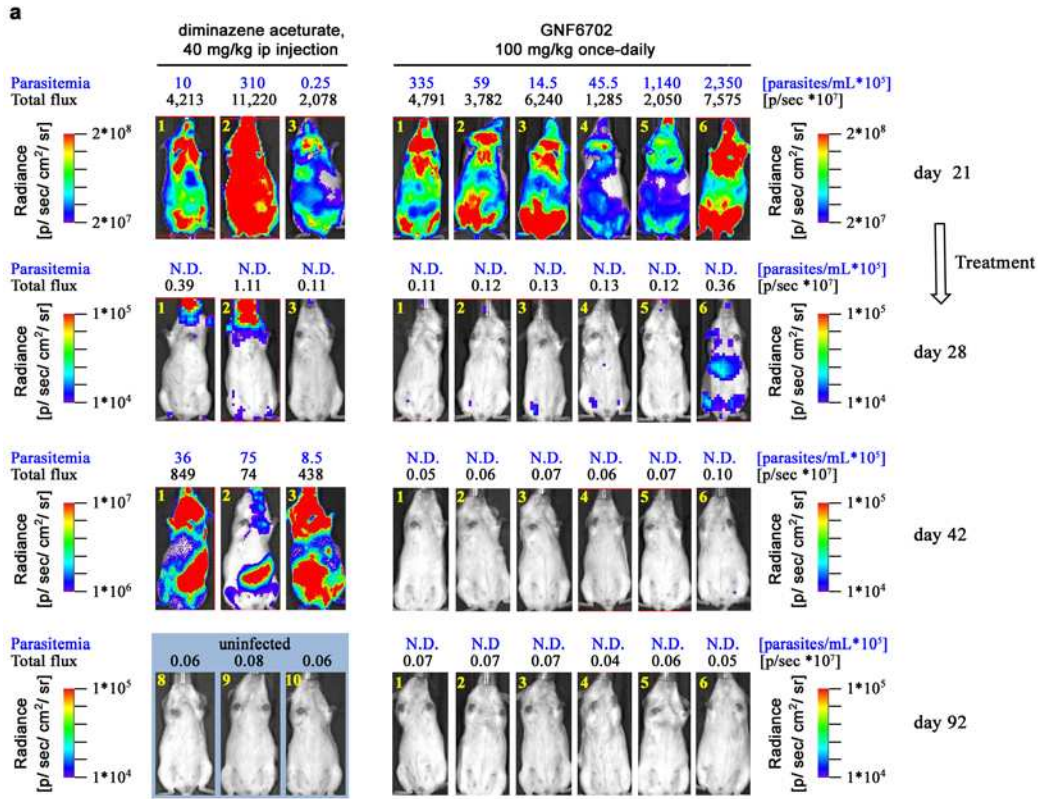


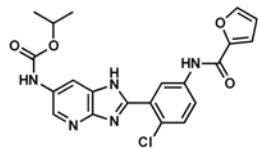






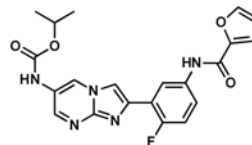






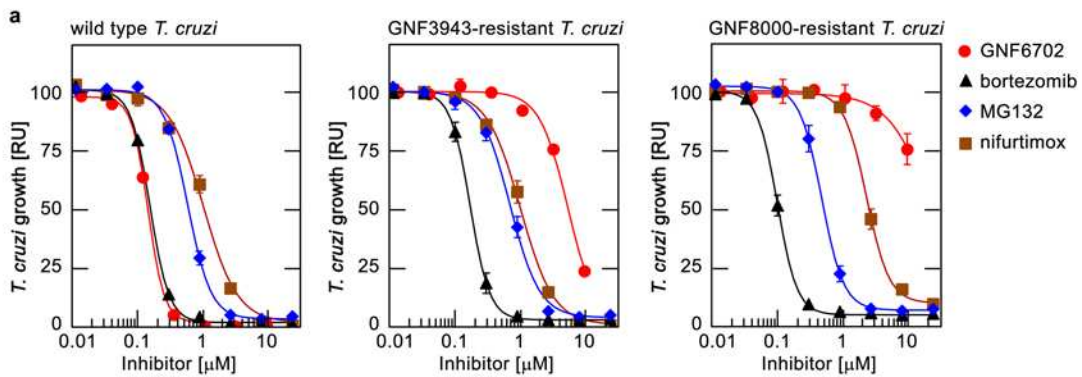
GNF3943

L. donovani EC₅₀ = 380 ± 23 nM
T. brucei EC₅₀ = 33 ± 9.4 nM
T. cruzi EC₅₀ = 120 ± 12 nM
 3T3 CC₅₀ = 4.5 ± 0.9 μM
 Macrophage CC₅₀ = 9.8 ± 2.4 μM
 F = 75 %
 CL = 17.7 mL*min⁻¹*kg⁻¹



GNF8000

L. donovani EC₅₀ = 320 ± 7.1 nM
T. brucei EC₅₀ = 73 ± 2.9 nM
T. cruzi EC₅₀ = 154 ± 12 nM
 3T3 CC₅₀ > 20 μM
 Macrophage CC₅₀ = 18 ± 2.1 μM
 F = 10 %
 CL = 8.8 mL*min⁻¹*kg⁻¹

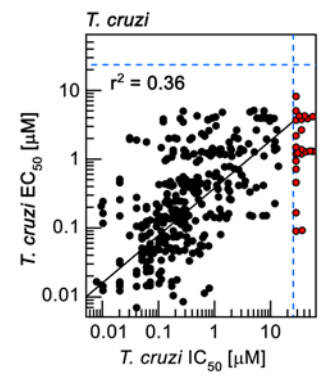
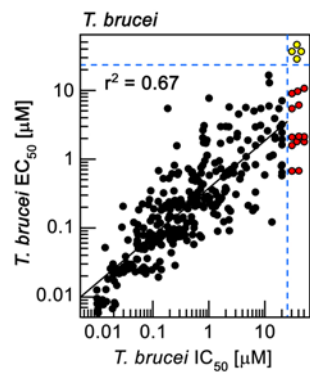


b

	wt <i>T. cruzi</i>	GNF3943 ^R <i>T. cruzi</i>	GNF8000 ^R <i>T. cruzi</i>
GNF6702 EC ₅₀ [μM]	0.15 ± 0.002	5.5 ± 0.016	> 10
bortezomib EC ₅₀ [μM]	0.16 ± 0.006	0.12 ± 0.020	0.10 ± 0.007
MG132 EC ₅₀ [μM]	0.61 ± 0.015	0.76 ± 0.071	0.48 ± 0.052
nifurtimox EC ₅₀ [μM]	1.0 ± 0.09	1.0 ± 0.11	2.4 ± 0.15

c

	<i>T. cruzi</i> ectopic PSMB4 ^{WT}		<i>T. cruzi</i> ectopic PSMB4 ^{F24L}		<i>T. brucei</i> ectopic PSMB4 ^{WT}		<i>T. brucei</i> ectopic PSMB4 ^{F24L}
	non-induced	induced	non-induced	induced	constitutive	constitutive	constitutive
GNF6702 [μM]	0.20 ± 0.007	0.20 ± 0.023	0.56 ± 0.029	> 10	0.018 ± 0.0018	1.2 ± 0.013	
bortezomib [μM]	0.46 ± 0.059	0.40 ± 0.057	0.45 ± 0.008	0.37 ± 0.015	0.00094 ± 0.00005	0.0011 ± 0.00026	



a

```
L. donovani 1 MAETA I A FRCQDYVMVAAAGLNAFYIYIKITDAEDKI TQLDTHQLI ACTGE
T. cruzi 1 MSETT I A FRCNSFVLVAAAGLNAFYIYIKIMDTEKVTQLDSHKVVACAGE
T. brucei 1 MAETT I GFRCDFFVLVAAAGLNAFYIYIKITDTEK I TELDSHKVVACAGE
H.sapiens 1 MEYLI G IQGPDYVLVASDRVAASNIVQMKDDHDKMFKMSEK I LLL CVGE

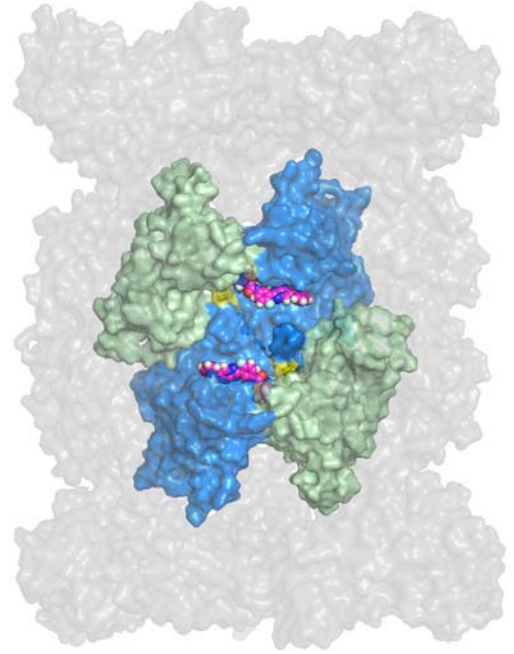
L. donovani 51 NGPRVNF TEYI KCNLM LNMRQHGRHSSCDSTANFMRNCLASA I RSREGA
T. cruzi 51 NGPRVNF VEYI KCNML KMRREHGRV I RTSAAA SFMRNALA GALRSRDGA
T. brucei 51 NGPRTHF VEYV KCNML KMRREHGRMI STHATAS FMRNT LAGALRSRDGL
H.sapiens 50 AGDTVQF AEYI QKNVQL YKMR - NGYELSP TAAANFT RRNLADCLRSRT - P

L. donovani 101 YQVNCL F AGYDMPVSEDDGAVGQPLFYLDYLGTLQAVPYGCHGYGACFV
T. cruzi 101 YL VNCL L AGYDVAASDDDI ATGPHLYYMDYLGTMQEVY GCHGYGASFV
T. brucei 101 YPVNCL L AGFDVPASAEDDVATGAHLYYLDYLGTMQEVY GCHGYGAPFV
H.sapiens 98 YHVNLL L AGYDE - - - - - HEGP ALYYMDYLAALAKAPFAAHGYGAFLT

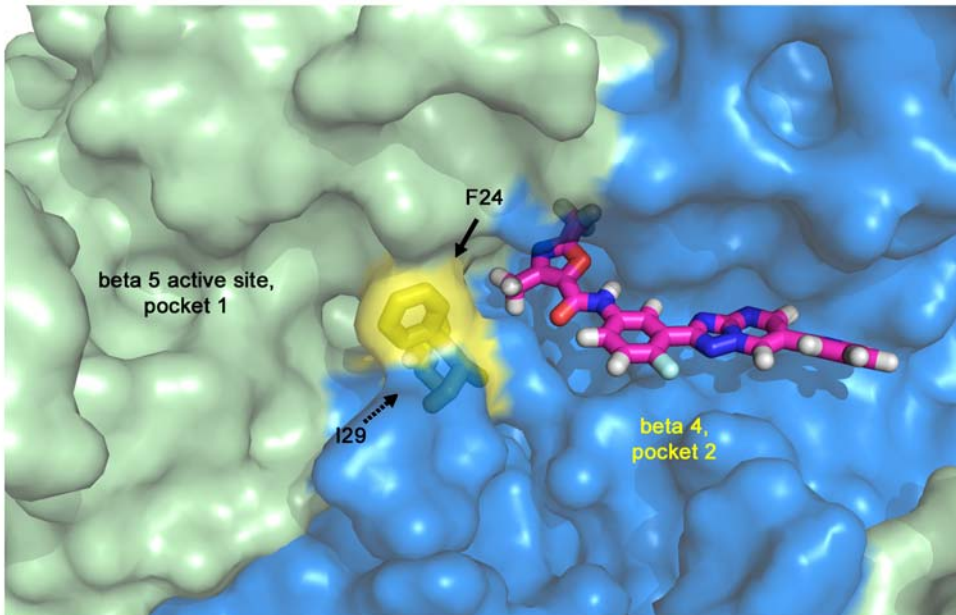
L. donovani 151 T ALLDCL WRPDL TQEGLEL MQKCCDEVKRRV I SNSYF FVKAVTKNGVE
T. cruzi 151 I AMLDRL WRPDL TAQEAVDL MQKCCDEVKRRV I SNDKF I CKAVTENGVE
T. brucei 151 TAMLDRMWRPNL TAQEGVDL MQKCCDEVKRRVVSNTF I CKAVTKDGVE
H.sapiens 140 L S I LDRY YTP T I SRERAVEL L RKCLEELQKRF I LNLPTFSVRI I DKNGIH

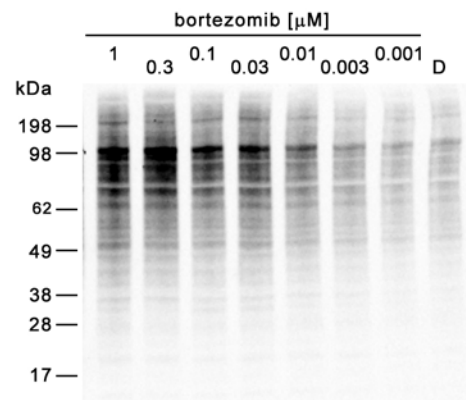
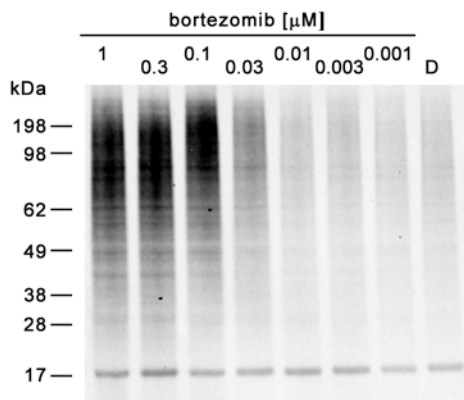
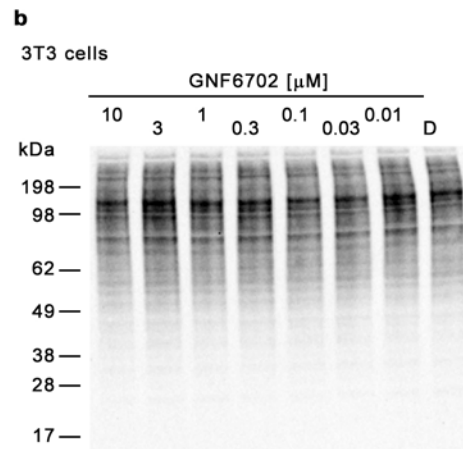
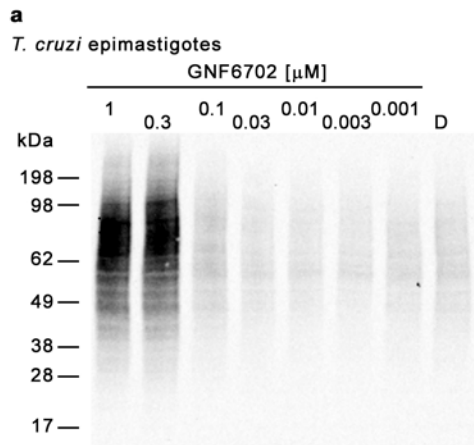
L. donovani 201 VITAVH
T. cruzi 201 LVNTVS
T. brucei 201 LVNTVS
H.sapiens 190 DLDNIS FPKQGS
```

b



c



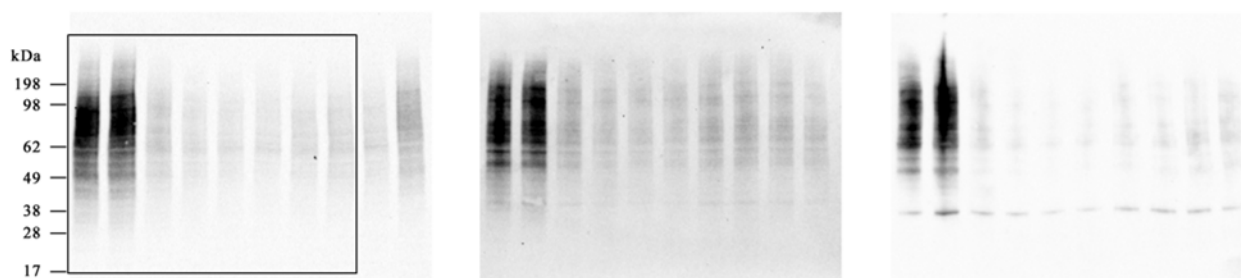


c

	<i>T. cruzi</i>	3T3
GNF6702 EC ₅₀ [μM]	0.13 ± 0.010	> 10
bortezomib EC ₅₀ [μM]	0.062 ± 0.001	0.040 ± 0.008

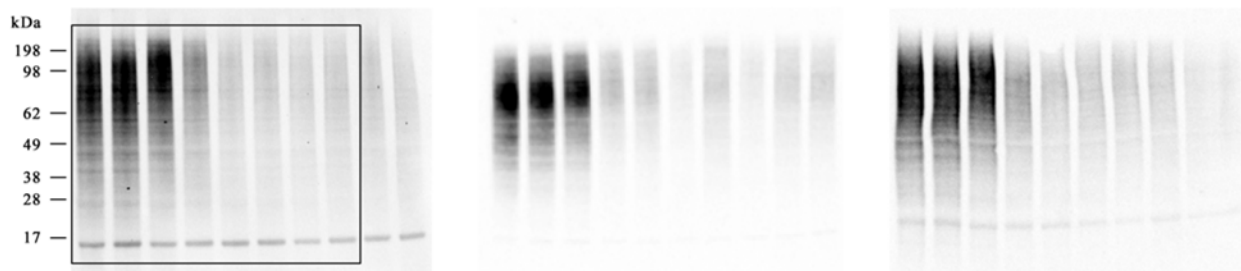
Supplementary Figure 1

a Western blots of three sets of cell lysates from GNF6702-treated *T. cruzi* epimastigotes



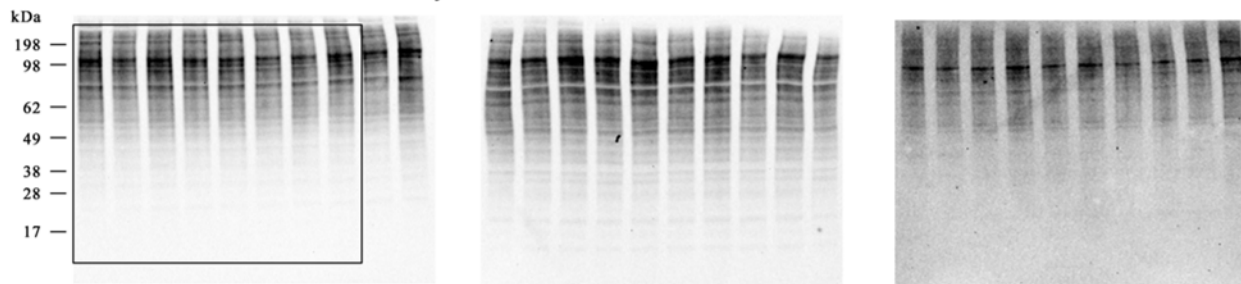
Extended Data Fig. 7a, upper panel

b Western blots of three sets of cell lysates from bortezomib-treated *T. cruzi* epimastigotes



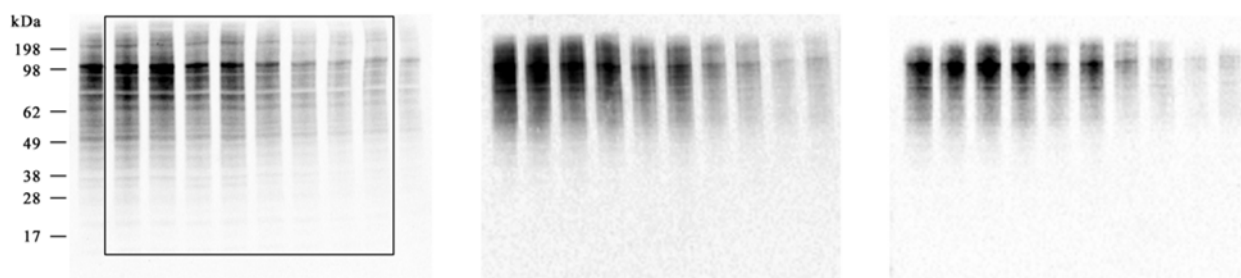
Extended Data Fig. 7a, lower panel

c Western blots of three sets of cell lysates from GNF6702-treated 3T3 cells



Extended Data Fig. 7b, upper panel

d Western blots of three sets of cell lysates from bortezomib-treated 3T3 cells



Extended Data Fig. 7b, lower panel

Excel files

1. [Supplementary Table 1](#)
Small molecule screening data from *Leishmania donovani* axenic amastigote growth inhibition HTS.
2. [Supplementary Table 2](#)
Small molecule screening data from *Trypanosoma brucei* bloodstream form trypomastigote growth inhibition HTS.
3. [Supplementary Table 3](#)
Small molecule screening data from *Trypanosoma cruzi* intracellular trypomastigote growth inhibition HTS.
4. [Supplementary Table 4](#)
Time course of parasitemia in mice infected with bioluminescent *T. brucei* during and after treatment with diminazene aceturate and GNF6702
5. [Supplementary Table 5](#)
Time course of whole body bioluminescence in mice infected with bioluminescent *T. brucei* during and after treatment with diminazene aceturate and GNF6702
6. [Supplementary Table 6](#)
Trypanosoma brucei bioluminescence of *ex vivo* brains obtained from parasite-infected mice after treatment with diminazene aceturate and GNF6702.
7. [Supplementary Table 7](#)
20S proteasome subunits identified in purified *T. cruzi* proteasome.
8. [Supplementary Table 8](#)
Amino acid sequences of predicted *Trypanosoma cruzi* 20S proteasome alpha and beta subunits.
9. [Supplementary Table 9](#)
GNF6702 profile in a panel of mammalian receptors, enzymes and ion channels.
10. [Supplementary Table 10](#)
GNF6702 inhibition profile in a cell-based Ba/F3 panel of Tel-activated human tyrosine kinases.
11. [Supplementary Table 11](#)
GNF6702 ADME profile.