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2 **Targeting *Echinococcus multilocularis* PIM kinase for improving**
3 **anti-parasitic chemotherapy**

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16 **Short title:** *Echinococcus multilocularis* PIM kinase

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20 **Abstract**

21 **Background:** The potentially lethal zoonosis alveolar echinococcosis (AE) is caused
22 by the metacestode larval stage of the tapeworm *Echinococcus multilocularis*. Current
23 AE treatment options are limited and rely on surgery as well as on chemotherapy
24 involving benzimidazoles (BZ). BZ treatment, however, is parasitostatic only, must be
25 given for prolonged time periods, and is associated with adverse side effects. Novel
26 treatment options are thus urgently needed.

27 **Methodology/Principal findings:** By applying a broad range of kinase inhibitors to *E.*
28 *multilocularis* stem cell cultures we identified the proto-oncogene PIM kinase as a
29 promising target for anti-AE chemotherapy. The gene encoding the respective *E.*
30 *multilocularis* ortholog, EmPIM, was characterized and *in situ* hybridization assays
31 indicated its expression in parasite stem cells. By yeast two-hybrid assays we
32 demonstrate interaction of EmPIM with *E. multilocularis* CDC25, indicating an
33 involvement of EmPIM in parasite cell cycle regulation. Small molecule compounds
34 SGI-1776 and CX-6258, originally found to effectively inhibit human PIM kinases,
35 exhibited detrimental effects on *in vitro* cultured parasite metacestode vesicles and
36 prevented the formation of mature vesicles from parasite stem cell cultures. To improve
37 compound specificity for EmPIM, we applied a high throughput *in silico* modelling
38 approach, leading to the identification of compound Z196138710. When applied to *in*
39 *vitro* cultured metacestode vesicles and parasite cell cultures, Z196138710 proved
40 equally detrimental as SGI-1776 and CX-6258, but displayed significantly reduced
41 toxicity towards human HEK293T and HepG2 cells.

42 **Conclusions/Significance:**

43 Repurposing of kinase inhibitors initially designed to affect mammalian kinases for
44 helminth disease treatment is often hampered by adverse side effects of respective
45 compounds on human cells. Here we demonstrate the utility of high throughput *in silico*
46 approaches to design small molecule compounds of higher specificity for parasite cells.
47 We propose EmPIM as a promising target for respective approaches towards AE
48 treatment.

49 **Author summary**

50 The larva of the tapeworm *E. multilocularis* grows tumor-like within the host liver, thus
51 causing the lethal disease alveolar echinococcosis (AE). Anti-parasitic treatment relies
52 on chemotherapy with benzimidazoles, which do not kill the parasite and must be
53 applied for years. As druggable enzymes with key functions in growth control, protein
54 kinases are promising drug targets and many kinase inhibitors have been identified
55 during cancer research. Optimized for binding to human kinases, however,
56 repurposing of such drugs for parasitic disease treatment is associated with adverse
57 side effects. Herein, the authors applied an *in silico* approach to identify small molecule
58 compounds that show higher specificity for a parasite kinase, EmPIM, over its
59 mammalian homologs. The authors demonstrate expression of EmPIM in
60 *Echinococcus* stem cells, which are the drivers of parasite growth, and show that
61 mammalian PIM kinase inhibitors SGI-1776 and CX-6258 also affect parasite
62 development *in vitro*. Finally, they show that one of the *in silico* screened compounds
63 is equally effective against the parasite as SGI-1776 and CX-6258, but significantly
64 less toxic to human cells. These results demonstrate the utility of *in silico* approaches
65 to identify parasite-specific kinase inhibitors.

66 Introduction

67 The metacestode (MC) larval stage of the cestode *E. multilocularis* is the causative
68 agent of alveolar echinococcosis (AE), a potentially lethal zoonosis prevalent in the
69 Northern Hemisphere [1]. Intermediate hosts (rodents, humans) are infected by oral
70 uptake of infectious eggs, which contain an embryonic larval stage, called the
71 oncosphere. Within the intestine of the intermediate host, the oncosphere hatches from
72 the egg, penetrates the intestinal barrier, and gains access to the inner organs. Usually
73 within the host liver, the oncosphere then undergoes a metamorphosis-like transition
74 towards the MC stage [2]. The *E. multilocularis* MC consists of numerous vesicles,
75 which grow infiltratively, like a malignant tumour, into the surrounding liver tissue,
76 eventually resulting in organ failure if no adequate treatment is applied [3]. As we have
77 previously shown, MC growth and proliferation strictly depend on a population of
78 pluripotent stem cells (called 'germinative cells' (GC) in the case of *Echinococcus*),
79 which, as typical for flatworms, are the only mitotically active cells of the MC and give
80 rise to all differentiated cells [4]. Currently, the only option to cure AE is surgical
81 removal of the invading MC tissue, supported by chemotherapy using benzimidazoles
82 (BZ; albendazole, mebendazole), which target parasite β -tubulin [5]. Surgical removal
83 of parasite tissue is, however, only possible in around 20% of cases, leaving BZ
84 chemotherapy as the only remaining treatment option for non-operable patients [3].
85 Although the prognosis of such patients has significantly improved after the
86 introduction of BZ chemotherapy around 40 years ago, adverse side effects are
87 frequently observed [6]. Furthermore, BZ are parasitostatic only and must be applied
88 for years to decades, sometimes even life-long [7]. Since GC are the only mitotically
89 active cells of the MC [4], it has already been proposed that the high recurrence rates
90 after anti-AE chemotherapy are due to limited activity of BZ against the parasite's stem

91 cell department [8]. Hence, alternative drugs are urgently needed, which are also
92 active against the *Echinococcus* GC [5].

93 Due to their catalytical process in transferring phosphate onto protein targets, protein
94 kinases are particularly druggable enzymes [9] and most small molecule compounds
95 that interfere with kinase activity bind to the ATP binding pocket [10]. Furthermore,
96 protein kinases are crucially involved in regulating proliferation and differentiation of
97 eukaryotic cells, making them highly attractive targets for strategies to chemically
98 interfere with aberrant cell proliferation, e.g. in the context of malignant transformation
99 [11]. One of these protein kinases, the PIM (proviral integration site for murine
100 leukemia virus) kinase, has recently drawn much attention as a potential target for
101 treating multiple forms of cancer [12–19]. Mammals typically express three PIM kinase
102 isoforms, Pim-1, Pim-2, and Pim-3, which are constitutively active serine/threonine
103 kinases [20] that phosphorylate numerous protein substrates and are downstream
104 effectors of a variety of cytokine signalling pathways [21]. Via activation of CDC25
105 phosphatase, mammalian Pim-1 is involved in the regulation of the cell cycle [22,23]
106 and aberrant expression of PIM kinases has been associated with numerous forms of
107 malignant transformation [21]. A hallmark of PIM kinases is their unusual hinge region,
108 which facilitates the development of specific kinase inhibitors and, during recent years,
109 several small molecule compounds with activities against PIM kinases have been
110 identified. Of these, the imidazole pyridazine compound SGI-1776 proved to be an
111 effective pan-PIM inhibitor with IC_{50} values of 7, 363, and 69 nM against human Pim-
112 1, Pim-2, and Pim-3, respectively, but also inhibited the kinases FLT3 (44 nM) and
113 haspin (34 nM) [15]. Among second generation inhibitors, the oxindole-based
114 compound CX-6258 displayed even higher specificity for PIM kinases than SGI-1776
115 (IC_{50} Pim-1, 5 nM; Pim-2, 25 nM, Pim-3, 16 nM) [24] and less activity against FLT3

116 (IC₅₀: 134 nM) [25]. At least in melanoma cell lines, CX-6258 also showed activities
117 against haspin kinase, although also at much higher IC₅₀ values than against Pim-1
118 and Pim-3 [26]. Although SGI-1776 has proceeded to clinical phase I trials against
119 non-Hodgkin lymphoma and prostate cancer, the respective studies have been
120 terminated due to toxicity in cardiac electric cycle prolongation [27], probably due to
121 their activities against PIM kinases in non-cancer cells.

122 In the present work, we demonstrate that SGI-1776 and CX-6258 also exert
123 detrimental effects on *in vitro* cultivated stem cells and larval stages of *E. multilocularis*.
124 We characterized the *Echinococcus* PIM kinase ortholog, show that it contains the
125 majority of amino acid residues that mediate the binding of PIM inhibitors to the ATP
126 binding pocket, and demonstrate that, like the mammalian counterpart Pim-1, the
127 *Echinococcus* enzyme interacts with CDC25 phosphatase. Using an *in silico* modelling
128 approach to discriminate between mammalian and parasite PIM sequences, we then
129 identified compound Z196138710, which displays equal toxicity against parasite larvae
130 as SGI-1776 or CX-6258, but which is much less toxic for mammalian cells. The impact
131 of these findings on drug design strategies against AE are discussed.

132

133 **Methods**

134 **Ethics statement**

135 *In vivo* propagation of parasite material was performed in mongolian jirds (*Meriones*
136 *unguiculatus*), which were raised and housed at the local animal facility of the Institute
137 of Hygiene and Microbiology, University of Würzburg. This study was performed in
138 strict accordance with German (*Deutsches Tierschutzgesetz, TierSchG*, version from
139 Dec-9-2010) and European (European directive 2010/63/EU) regulations on the

140 protection of animals. The protocol was approved by the Ethics Committee of the
141 Government of Lower Franconia (Regierung von Unterfranken) under permit numbers
142 55.2–2531.01-61/13 and 55.2.2-2532-2-1479-8.

143

144 **Organisms and culture methods**

145 All experiments were performed with the *E. multilocularis* isolates H95 and GH09 [28]
146 which either derive from a naturally infected fox of the region of the Swabian
147 Mountains, Germany (H95) [29] or from Old World Monkey species (*Macaca*
148 *fascicularis*) that had been naturally infected in a breeding enclosure (GH09) [30] The
149 isolates were continuously passaged in mongolian jirds (*Meriones unguiculatus*)
150 essentially as previously described [31,32]. *In vitro* culture of parasite metacystode
151 vesicles (MV) under axenic conditions was performed as previously described [32] and
152 the isolation and maintenance of *Echinococcus* primary cell cultures (PC) was carried
153 out essentially as established by Spiliotis et al. [33]. In all cases, media were changed
154 every three to four days (d). For inhibitor studies, specific concentrations of
155 compounds, dissolved as 10-50 mM stock solutions and stored at -80°C, were added
156 to parasite cultures as indicated and as negative control DMSO (0.1 %) was used. SGI-
157 1776 was purchased from Selleckchem (Houston, Texas) and CX-6258 was from
158 Cayman chemical (Ann Arbor, Michigan). Z196138710 was purchased from SIA
159 Enamine (Riga, Latvia). Providers of other kinase inhibitors are listed in S1 Table. A6
160 medium was prepared by seeding 1.0×10^6 rat Reuber hepatoma cells [32] in 175 cm²
161 culture flasks with 50mL DMEM (Dulbecco's Modified Eagle Medium) + GlutaMAX-I
162 (life technologies) including 10% Fetal Bovine Serum Superior (life technologies) and
163 incubated for 6 days under aerobic condition. The supernatant was sterile filtrated to
164 remove hepatocytes. Similarly, B4 medium was prepared by seeding 1.0×10^7 rat

165 Reuber hepatoma cells in 175 cm² culture flasks with 50mL DMEM+GlutaMAX-I
166 including 10% FBS and incubated for 4 days under aerobic conditions before sterile
167 filtration.

168

169 **Anti-parasitic inhibitor assays**

170 In cell viability assays for initial screening of kinase inhibitors, PC were isolated from
171 mature MV using a previously established protocol [33] and PC density was measured
172 by densitometry. 1 Unit (U) of PC is defined as the amount which increases OD₆₀₀ by
173 0.01. 15 U of isolated PC ($\sim 2.25 \times 10^3$ cells/well) were seeded into 384 well plates
174 with 100 μ l of conditioned medium (50% A6 medium + 50% B4 medium) including
175 defined concentration of inhibitors as indicated. Plates were incubated at 37 °C under
176 nitrogen atmosphere. After 3 d, cell viability was measured using the Cell Titer Glo 2.0
177 cell viability assay (Promega), according to the manufacturer's instructions.
178 Luminescence was measured using a Spectramax iD3 Multi-mode Microplate reader
179 (Molecular Devices; San Josè, CA, USA). Measured luminescence units were
180 normalized to those of the DMSO control and visualized as heatmaps with GraphPad
181 Prism version 9.3.1 (Graphpad software). All kinase inhibitors were tested
182 independently in three technical replicates.

183 In mature MV assays, 10 individual MV each were cultured in 2 ml of conditioned
184 medium (100% A6 medium) in the presence of inhibitors in 12 well plates under axenic
185 conditions for 28 d as described in [34]. Structural integrity of MV was assessed using
186 an optical microscope (Nikon eclipse Ts2-FL). Criteria for intact or damaged vesicles
187 were essentially as previously described [35,36]. All experiments were performed
188 using 3 biological replicates. Percentages of structurally intact vesicles were

189 statistically analyzed with one-way ANOVA with Dunnet's multiple comparison tests in
190 Graphpad prism 9.3.1(Graphpad software).

191 In vesicle formation assays, PC were isolated as described above and 100 U of
192 isolated PC ($\sim 1.5 \times 10^4$ cells/well) were seeded in 96 well plates with 200 μ l of
193 conditioned medium (50% A6 medium + 50% B4 medium) for 21 d under nitrogen
194 atmosphere. The number of newly formed vesicles was counted using an optical
195 microscope (Nikon eclipse Ts2-FL) essentially as previously described [37] Kruskal-
196 Wallis test followed by Dunn's multiple comparisons test was used in GraphPad Prism
197 version 9.3.1(Graphpad software) to analyze the difference of vesicle numbers in
198 control and treatment groups. In this analysis, all concentrations were compared with
199 the negative control DMSO. Experiments with SGI-1776 and CX-6258 were performed
200 in three biological and technical replicates. Experiments with SGI-1776 and
201 Z196138710 were performed in three technical replicates.

202

203 **Mammalian cell cultivation and drug treatment**

204 The toxicity of inhibitors against mammalian cells was evaluated by treatment of the
205 commonly used cell lines HEK293T [38] and HepG2 [39], which were cultivated and
206 passaged as-described in [40,41]. Semi-confluent cultured cells up to ten passages
207 after vial thawing were trypsinized and 1.0×10^3 cells were seeded in 384 well plates
208 with 50 μ l of DMEM (Dulbecco's Modified Eagle Medium) + GlutaMAX-I (life
209 technologies) including 10% Fetal Bovine Serum Superior (life technologies). 24 h after
210 seeding, 50 μ l of DMEM+GlutaMAX-I including FBS and inhibitors were added. Final
211 concentrations of inhibitors were adjusted to 0-30 μ M as indicated, DMSO alone was
212 used as a control. Plates were incubated for 3 d under aerobic conditions and cell
213 viability was measured using the Cell Titer Glo 2.0 system (Promega) according to the

214 manufacturer's instructions. Luminescence was measured by a Spectramax iD3 Multi-
215 mode Microplate reader (Molecular Devices). Three independent experiments with
216 three technical replicates were carried out for both cell lines. Luminescence units were
217 normalized to the DMSO control of each independent experiment and expressed as
218 percentage of luminescence unit. One-Way-ANOVA test followed by Tukey's multiple
219 comparisons test was used in GraphPad Prism version 9.3.1 (Graphpad software) for
220 statistical analysis.

221

222 **Nucleic acid isolation, cloning and sequencing**

223 RNA isolation from *in vitro* cultivated axenic metacystode vesicles and primary cells
224 was performed using a Trizol (5Prime, Hamburg, Germany)-based method as
225 previously described [42]. For reverse transcription, 2 µg total RNA was used for cDNA
226 synthesis using oligonucleotide CD3-RT (5'-ATC TCT TGA AAG GAT CCT GCA
227 GGT₂₆ V-3'). PCR products were cloned using the PCR cloning Kit (QIAGEN, Hilden,
228 Germany) or the TOPO XL cloning Kit (Invitrogen). The complete list of primer
229 sequences used for *empim* and *emcdc25* cDNA amplification and characterization is
230 given in S2 Table. Upon cloning, PCR products were directly sequenced using primers
231 binding to vector sequences adjacent to the multiple cloning site by Sanger
232 Sequencing (Microsynth Seqlab, Göttingen, Germany). The sequences of all genes
233 newly characterized in this work have been submitted to the GenBank, EMBL, and
234 DDJB databases under accession numbers listed in S3 Table.

235

236 ***In situ* hybridization and 5-ethynyl-2'-deoxyuridine (EdU) labeling**

237 Digoxygenin (DIG)-labeled probes were synthesized by *in vitro* transcription with T7
238 and SP6 polymerase (New England Biolabs), using the DIG RNA labelling kit (Roche)
239 according to the manufacturer's instructions from *empim*-cDNA fragments cloned into
240 vector pJET1.2 (Thermo Fisher Scientific). Primers for probe production are listed in
241 S2 Table. Probes were subsequently purified using the RNEasy Mini Kit (Qiagen),
242 analysed by electrophoresis, and quantified by dot blot serial dilutions with DIG-labeled
243 control RNA (Roche). Whole-mount *in situ* hybridization (WISH) was subsequently
244 carried out on *in vitro* cultivated metacestode vesicles as previously described [4],
245 using vesicles (isolate H95) of at least 1 cm in diameter to avoid losing material during
246 washing steps. Fluorescent specimens were imaged using a Nikon Eclipse Ti2E
247 confocal microscope and maximum projections created using ImageJ as previously
248 described [43]. In all cases, negative control sense probes yielded no staining results.
249 *In vitro* labelling with 50 μ M EdU was done for 5, 8, or 16 hours (h) and fluorescent
250 detection with Alexa Fluor 555 azide was performed after WISH essentially as
251 previously described [4]. Series of pictures were taken at randomly chosen sections of
252 the germinal layer of 5 MC vesicles with 40 \times objective lens as Z-stack. Among the
253 picture of each Z-stack, the layer of strongest signal was selected by the function of Z
254 project in Fiji/ImageJ and processed [44]. EdU positive cells, WISH positive cells and
255 double positive cells were counted manually and independently. The number of cells
256 with each signal were calculated to cell number per mm² on the germinal layer.

257

258 **Yeast Two hybrid (Y2H) analyses**

259 The Gal4-based Matchmaker System (Takara Bio, USA) was used as described by
260 Zavala-Góngora et al. [45,46] and Stoll et al [43]. Full-length cDNAs encoding EmPim

261 kinase and EmCdc25 phosphatase were PCR amplified from parasite cDNA using
262 primers as listed in S2 Table and cloned into pGADT7 or pGBKT7 (Takara/Clontech).
263 The *Saccharomyces cerevisiae* Gold strain (Clontech) was transformed with these
264 plasmids by a one-step protocol described in Tripp et al. [47] and inoculated on Leu⁻
265 /Trp⁻ double dropout agarose plates. After incubation at 30 °C for 2 d, three colonies
266 were picked from each transformation and inoculated independently in 2 ml of liquid
267 Leu⁻/Trp⁻ medium and incubated at 30 °C and 200 rpm until above OD₆₆₀=1.0. Yeast
268 cultures were then diluted to equalized densities of OD₆₆₀=1.0, 0.1 and 0.01. Diluted
269 yeast cultures were then dropped (5 µl each) onto Leu⁻/Trp⁻/His⁻ triple dropout plates
270 and Leu⁻/Trp⁻/Ade⁻/His⁻ quadruple dropout plates. After 48 - 72 h incubation at 30 °C,
271 pictures of plates were taken with ProtoCOL SR colony counter (Synbiosis). The
272 pictures were then converted into gray scale and processed using Fiji/ImageJ [44]
273 according to the protocol described in [48]. The level of growth on quadruple dropout
274 plates with the inoculation density OD₆₆₀=1.0 was quantified as gray value. The
275 quantified level of growth was statistically analysed with one-way ANOVA with Tukey's
276 multiple comparison tests in Graphpad prism 9.3.1 (Graphpad software).

277

278 **Bioinformatic procedures**

279 Amino acid sequences of human Pim-1, Pim-2, Pim-3, Cdc25A, Cdc25B, and Cdc25C
280 were used as queries in BLASTP searches against the *E. multilocularis* genome on
281 Wormbase ParaSite [28,49,50]. Reciprocal BLASTP searches were performed again
282 the SWISSPROT database as available under the KEGG database at Genomenet [51].
283 Dmain structure was analyzed with SMART8.0 [52–54]. Percent identity/percent
284 similarity values of amino acid sequences were calculated through Sequence
285 Manipulation Suite [55]. Multiple sequence alignments were performed using Clustal

286 omega [56] or CLUSTALW2.1 [57] in MEGA11 [58] under the following settings: Gap
287 Opening Penalty = 10.00, Gap Extension Penalty = 0.20, Delay Divergent Cutoff =
288 30%. Aligned sequences were visualized by SnapGene Viewer (Snappene software).
289 Based on these alignments, phylogenic trees were generated by MEGA11. The
290 statistical method for tree building was maximum likelihood, substitution model was
291 Jones-Taylor-Thompson model, ML Heuristic method was Nearest-Neighbor
292 Interchange. All transcripts of *Echinococcus* genes were analysed using Integrative
293 Genomics Viewer [59,60] and previously published transcriptome data [28] to check
294 for correct prediction of the sequences available through UniProt.

295 For virtual compound identification procedures, the target of interest (EmPim) was
296 screened against Fluency, a proprietary deep learning-based platform [61]. The
297 EmPim sequence tested was retrieved from Uniprot, functional domains were retrieved
298 from the Pfam database. The target of interest was screened against 2 small molecule
299 libraries: the Enamine Hinge Binders library [62] (n=24,000), and Enamine Diverse
300 REAL drug-like library [63] version 2021q1-2, further filtered for drug-like properties
301 based on Lipinski's rule of 5 [64] (n=21.4M). We applied predictions from 2 versions of
302 the Fluency model, termed model 1 and model 2, which were trained on varying data
303 sets and settings. Every combination of protein, compound library, and model was
304 predicted by Fluency, resulting in 4 sets of predictions, ranking molecules from
305 strongest predicted binder to weakest. Out of the 200 top-ranked *in silico* hits from the
306 Fluency screen, 20 compounds were selected for purchase and profiling based on their
307 Fluency Screen score, diversity of structures representing best the chemical space of
308 the 200 hits and molecular modeling in hPIM (6VRU) employing seeSAR modeling
309 software from BioSolveIT (version 11.2.). The generated poses were assessed for a

310 meaningful binding mode into the ATP-pocket, absence of intra- and intermolecular
311 clashes and torsion quality.

312 Modeling of SGI-1776 and Z196138710 against human Pim-1 was performed using
313 modeling software seeSAR Version 12.0.1 (BioSolveIT). To create broad diversity and
314 to continue further, 200 poses were demanded
315 within SeeSAR [65] per structure; the integrated Analyzer Mode was used
316 to select those poses that were clash-free and only exhibited green torsions, i.e.,
317 torsions that are statistically prevalent in small-molecule crystal structures [66].

318

319 **Results and Discussion**

320 **Initial screen of broad-range kinase inhibitors against *E. multilocularis* cell** 321 **cultures**

322 Based on sequence similarities between the catalytic domains and the presence of
323 accessory domains, conventional protein kinases are basically divided into seven sub-
324 groups, against which specific kinase inhibitors are available [67]. Homologs belonging
325 to all these sub-groups are also expressed by *E. multilocularis* [28]. To identify kinase
326 sub-groups that are important for *Echinococcus* stem cell function, we carried out an
327 initial screen of 14 available kinase inhibitors covering all sub-groups, against *E.*
328 *multilocularis* PC, which are strongly enriched in germinative (stem) cells [4] (Fig 1).
329 To assess for direct cytotoxic effects, we performed cell viability assays with all
330 inhibitors at a concentration of 10 μ M and measured cell viability after 3 d of drug
331 exposure. As shown in Fig 1, several inhibitors against the AGC and the CAMK groups
332 showed clear effects against *Echinococcus* PC, whereas Dasatinib, directed against
333 the BCR-Abl subfamily of tyrosine kinases, even stimulated parasite cell proliferation

334 under these conditions. The strongest anti-parasitic effect was achieved using the PIM-
335 specific inhibitor SGI-1776, with more than 50% growth inhibition after 3 d. In all further
336 experiments we therefore decided to concentrate on the PIM kinase family and their
337 role in *Echinococcus* stem cell biology.

338

339 **Fig 1. Activities of selected kinase inhibitors against *E. multilocularis* PC.** (A)

340 Phylogenetic tree of human protein kinases, based on homologies within the kinase
341 domain. Seven groups according to current nomenclature [68] are indicated. (B)
342 Heatmap showing the effects of different kinase inhibitors, covering all 7 groups, on *E.*
343 *multilocularis* PC. Colour-code below indicates levels of luminescence after 3 d of
344 incubation with 10 μ M of inhibitor. Inhibitor names, human target proteins, and kinase
345 sub-families are indicated in the table to the left.

346

347 **Characterization of an *E. multilocularis* PIM kinase**

348 Since SGI-1776 was originally designed to inhibit human PIM kinases and showed
349 strong effects against *Echinococcus* PC, we were interested in characterizing parasite
350 PIM kinase orthologs. To this end, we performed BLASTP analyses using all three
351 human PIM isoforms as queries against the published *E. multilocularis* genome
352 sequence [28]. In all three cases we identified one single locus (EmuJ_000197100),
353 which encoded a protein with significant homologies. Reciprocal BLASTP analyses
354 against the SWISSPROT database using the EmuJ_000197100 gene product as a
355 query revealed high homologies to human Pim-1, particularly within the kinase domain
356 (47% identical, 65% similar residues) (Fig 2). Significant homologies were also
357 detected between the kinase domains of the EmuJ_000197100 gene product and

358 PRK2 (38%, 58%) and PSK2 (30%, 50%), which are PIM kinase orthologs of
359 *Caenorhabditis elegans* and yeast, respectively. We thus named the *Echinococcus*
360 gene *empim*, encoding the serine/threonine kinase EmPim (657 amino acids; 74 kDa
361 theoretical MW). Since BLASTP searches against the *E. multilocularis* genome using
362 the amino acid sequences of EmPim or all three human PIM kinase isoforms did not
363 reveal any other gene with significant homologies, we concluded that *empim* is a single
364 copy gene and that, in contrast to mammals, *E. multilocularis* only encodes one single
365 PIM kinase isoform.

366 We then conducted similar analyses for the genome of the related trematode parasite
367 *Schistosoma mansoni*, and, likewise, found one single locus (Smp_090890) encoding
368 a PIM ortholog with significant homologies to EmPim, which we named SmPim (S1
369 Figure). The presence in the genome of just one gene encoding a PIM kinase ortholog
370 appears to be a specific trait for parasitic flatworms since, as already mentioned,
371 mammals express three PIM kinase isoforms [69,70]. Furthermore, three isoforms
372 have already been described to be expressed by the related, but free-living, planarians,
373 in which they belong to the group of 'immediate early genes', the expression of which
374 is drastically upregulated during wound-induced responses [71]. Most interestingly,
375 both EmPim and SmPim not only harbour the typical serine/threonine kinase domain
376 but also a large C-terminal extension (S1 Figure), which is missing in human and
377 planarian PIM kinase isoforms. Due to the absence of a regulatory domain, the human
378 PIM kinase isoforms are considered constitutively active kinases, the activity of which
379 is largely regulated at transcriptional, translation, and proteosomal degradation level
380 [20,72]. Hence, in contrast to these enzymes, the PIM kinases of parasitic flatworms
381 are likely subject to more elaborate regulatory mechanisms, although we could not

382 identify consensus regulatory regions, such as conserved phosphorylation sites, within
383 the C-terminal extension.

384

385 **Fig 2. Homologies and structural features of EmPim.** (A) Amino acid sequence
386 alignment of the kinase domains of human Pim-1 (HsPIM1), *E. multilocularis* Pim
387 (EmPim), human FLT3 kinase (HsFLT3), human haspin kinase (HsHASPIN) and an
388 *E. multilocularis* haspin kinase ortholog (EmHASPIN1). Residues identical to human
389 Pim-1 are shown in black on grey. Kinase DFG motifs and the hinge regions are
390 marked in red. Black triangles indicate residues known to be involved in the interaction
391 between human Pim-1 and compound CX-6258 (numbered according to human Pim-
392 1). (B) Presence of amino acid residues important for the interaction between human
393 Pim-1 and CX-6258 in different kinases. For each of the 14 known residues of human
394 Pim-1 (HsPIM1), the corresponding residue and position in *E. multilocularis* Pim
395 (EmPIM), human FLT3 kinase (HsFLT3), human haspin kinase (HsHASPIN), and the
396 *E. multilocularis* haspin kinase isoform (EmHASPIN1) are shown. Residues identical
397 to those of human Pim-1 are marked in yellow, residues with similar biochemical
398 properties are marked in green. The numbers of identical/similar residues compared
399 to human Pim-1 are listed to the right as well as IC₅₀ values of compounds CX-6258
400 and SGI-1776 to human enzymes.

401

402 Although the precise mode of interaction between SGI-1776 and PIM kinases is not
403 known, crystallographic studies have already been conducted for the binding mode of
404 CX-6258 to human Pim-1 [25]. These studies identified 14 amino acid residues of
405 particular importance for the inhibitor-kinase interaction (indicated in Fig 2).

406 Interestingly, of these 14 residues, 10 are invariantly present in EmPim and two further
407 residues represent exchanges with conserved biochemical properties (Fig 2). In the
408 case of human FLT3 kinase, which is also inhibited to a certain extent by CX-6258,
409 only 7 of these residues are conserved (Fig 2). We thus propose that CX-6258 (and
410 most probably also SGI-1776) binds to EmPim with intermediate affinities when
411 compared to Pim-1 and FLT3. Notably, an FLT3 ortholog is apparently not expressed
412 by *E. multilocularis* since BLASTP genome mining using mammalian FLT3 as a query
413 did not reveal clear orthologs. This is supported by phylogenetic studies indicating that
414 FLT3 kinases have evolved before the chordate/urochordate split, but after the
415 divergence of protostomes and deuterostomes [73].

416 Apart from the tyrosine kinase FLT3, both SGI-17776 [15] and CX-6258 [26] have been
417 demonstrated to inhibit, although with lower affinities, the kinase *haspin* (haploid germ
418 cell-specific nuclear protein kinase), which mediates histone modification in mammals
419 [74]. Interestingly, a *haspin* ortholog is also expressed by the *E. multilocularis* genome
420 (EmuJ_000667600). Within the kinase domain, both the *Echinococcus* and the human
421 *haspin* kinases share 6 or 7, respectively, of the 14 residues involved in the CX-6258-
422 kinase interaction (Fig 2). It thus cannot be excluded that the parasite *Haspin* kinase
423 might also be targeted by CX-6258, at least to a certain extent.

424 Taken together, our analyses indicated that *E. multilocularis* expresses a single PIM
425 ortholog with substantial homologies to mammalian PIM kinases within the kinase
426 domain. Unlike PIM kinases of mammals or planarians, the PIM kinase enzymes of
427 parasitic flatworms contain a large C-terminal extension, indicating a more complex
428 mode of regulation than in the case of conventional (mammalian) PIM kinases. Based
429 on the conservation of the majority of amino acid residues that mediate binding of CX-
430 6258 to PIM kinases, we also concluded that available PIM kinase inhibitors should

431 primarily target EmPim in *Echinococcus* cells. We cannot rule out, however, that part
432 of the effects of CX-6258 (and of SGI-1776) described below might be due to inhibition
433 of the *Echinococcus haspin* ortholog.

434

435 **Expression of *empim* in *Echinococcus* stem cells**

436 In mammalian cells, PIM kinases are involved in cell proliferation and cell cycle
437 regulation [75]. Since the germinative (stem) cell population is the only mitotically
438 active *Echinococcus* cell type [4], we would expect expression of *empim* in germinative
439 cells if EmPim has equivalent functions as mammalian PIM kinases. According to
440 transcriptome analyses that had been produced during the *E. multilocularis* whole
441 genome project [28], *empim* displayed higher expression in primary cell cultures after
442 2 d of incubation when compared to metacestode vesicles (S2 Figure). Since these
443 cultures are highly enriched in germinative cells [4], we assumed that *empim* might
444 show a dominant expression in this cell type. To clarify the situation, we carried out
445 WISH analyses on MC vesicles that had been incubated with EdU, thus identifying the
446 proliferating stem cell compartment. As shown in Fig 3, in the germinative layer of *in*
447 *vitro* cultivated MV we detected *empim* signals in both EdU⁺ and EdU⁻ cells. After an
448 8 h EdU pulse, around 25% of *empim*⁺ cells were also EdU⁺. For the majority of
449 *empim*⁺ cells, however, we could not detect co-staining with EdU, indicating that they
450 either represent post-mitotic, differentiated cells, or stem cells which were not in S-
451 phase during the EdU pulse.

452

453 **Fig 3. Expression of *empim* in *Echinococcus* MV.** (A) WISH on *E. multilocularis* MV
454 directed against *empim*. Channels shown are DAPI (blue, nuclear staining), WISH

455 (green, *empim+*), EdU (red, S-phase stem cells), and merge as indicated. Green arrow
456 indicates example of *empim+*/EdU- cell, yellow arrow indicates example of
457 *empim+*/EdU+ cell. Size bar represents 25 μm . (B) Statistical analysis of
458 EdU+/*empim+* staining patterns. Displayed are counted cell number per mm^2 of
459 germinal layer in Z-stack analyses.

460

461 Previous studies on human chronic myelogenous leukemia cells indicated that Pim-1 is
462 cell cycle-regulated with highest expression levels at G1-S and G2-M transitions,
463 whereas a significant drop in Pim-1 expression occurs during S-phase [76]. Since EdU
464 exclusively stains cells that have been in S-phase during the 8 h pulse, it is thus
465 possible that the fraction of germinative cells which express *empim* is significantly
466 higher than 25%, provided that the PIM kinase gene is also cell cycle-regulated in
467 *Echinococcus*. In any case, our WISH/EdU experiments clearly indicate that a certain
468 fraction of parasite stem cells expresses *empim*.

469

470 **Interaction between EmPim and CDC25C phosphatase**

471 Modulation of mammalian cell cycle progression through Pim-1 is mainly mediated by
472 phosphorylation, and thereby activation, of dual specific phosphatases of the CDC25
473 family [22,23]. CDC25 phosphatases are highly conserved from yeast to mammals,
474 are expressed in differing numbers of isoforms (e.g. 3 in humans, 1 in yeast, 2 in
475 *Drosophila*, 4 in *C. elegans*), and induce the M-phase of the cell cycle by removing
476 inhibitory phosphates from cyclin-dependent kinases [22,77,78]. Provided that EmPim,
477 despite its unusual C-terminal extension, also mediates cell cycle progression in
478 *Echinococcus*, we would expect that it physically interacts with CDC25 isoforms in this

479 parasite. To investigate these aspects, we first mined the available *E. multilocularis*
480 genome sequence for the presence of CDC25 encoding genes. Using either of the
481 three human CDC25 isoforms (CDC25A-C) as a query against the *E. multilocularis*
482 genome in BLASTP analyses, we constantly identified one single locus
483 (EmuJ_001174300) encoding a 762 amino acid (theoretical MW = 85,3 kDa) protein
484 tyrosine phosphatase. The EmuJ_001174300 product displayed relatively weak
485 overall homologies to CDC25 family members of humans, insects, or nematodes, but
486 contained a Rhodanese domain, which is a hallmark of CDC25 family M-phase
487 inducers [79] (Fig 4). Furthermore, amino acid residues within the Rhodanese domain
488 that are critical for enzymatic function were highly conserved between the
489 EmuJ_001174300 product and human CDC25 orthologs (Fig 4). Furthermore, in
490 reciprocal BLASTP analyses against the SWISSPROT database using the
491 EmuJ_001174300 gene product as a query, we detected highest homologies with
492 CDC25 orthologs of mammals and invertebrate model organisms. We thus concluded
493 that *E. multilocularis* genome contains a single locus encoding a CDC25 family
494 phosphatase and named the respective gene *emcdc25* (encoding the protein
495 EmCDC25).

496

497 **Fig 4. Domain structure and homologies of EmCDC25.** (A) Amino acid sequence
498 alignment of the Rhodanese homology domains of EmCDC25 (EmCdc25), two *S.*
499 *mansoni* CDC25 orthologs (SmCDC25A, SmCDC25B), and three human CDC25
500 orthologs (HsCDC25A-C). Conserved Rhodanese domain DCR motifs and the active
501 site are indicated. Residues identical to EmCDC25 are shown in black on grey. (B)
502 Phylogenetic tree based on Rhodanese domains of different CDC25-like
503 phosphatases. Sequences derived from *E. multilocularis* (EmCDC25), *S. mansoni*

504 (SmCDC25A/B), *H. sapiens* (HsCDC25A-C), *C. elegans* (CeCDC25 1-4), *D.*
505 *melanogaster* (TEW, STG), and *Saccharomyces cerevisiae* (MIH1). Statistical method
506 for the tree was maximum likelihood (ML), substitution model was Jones-Taylor-
507 Thompson, ML heuristic method was Nearest Neighbour Interchange. (C) Domain
508 structures of EmCDC25, two different CDC25 orthologs of *S. mansoni* (SmCDC25A/B),
509 and three human CDC25 isoforms (HsCDC25A-C). Shown are Rhodanese domains
510 and M-phase inducer phosphatase domains, which are typical for mammalian
511 isoforms.

512

513 To investigate possible interactions between EmPim and EmCDC25 we employed the
514 yeast two-hybrid (Y2H) system which we had previously used to study protein-protein
515 interactions between *Echinococcus* factors [43,45,46,80]. To this end, we cloned the
516 full-length cDNAs for EmPim and EmCDC25 into vectors pGBKT7 and pGADT7,
517 respectively, and assessed colony growth under medium (triple dropout plates) and
518 high (quadruple dropout plates) stringency conditions. As shown in Fig 5, under
519 medium stringency conditions we obtained growth for the combination EmPim-
520 pGBKT7 x EmCDC25-pGADT7 but we also observed some growth capacity for
521 EmPim-pGBKT7 with the empty vector control. Under high stringency conditions, on
522 the other hand, only EmPim-pGBKT7 x EmCDC25-pGADT7 yielded positive results,
523 indicating specific interaction between these proteins. Statistically significant
524 differences between EmPim-pGBKT7 x EmCDC25-pGADT7 and empty vector
525 controls were also observed in quantitative assays measuring yeast growth on
526 quadruple dropout plates (OD₆₆₀=1.0) (Fig 5). We thus concluded that, like in
527 mammalian systems, the *Echinococcus* Pim kinase acts upstream of a CDC25 family
528 phosphatase. Whether this interaction is involved in *Echinococcus* M-phase entry

529 control remains to be established. Due to the expression of EmPim in *Echinococcus*
530 stem cells and the high conservation of Pim/CDC25-dependent M-phase entry control
531 from yeast to mammals [22,23], such a role is, however, highly likely.

532

533 **Fig 5. Interaction between EmPim and EmCDC25.** (A) Representative pictures of
534 yeast transformant growth on plates selecting for plasmids (-Leu, -Trp) as well as triple
535 dropout (-Leu, -Trp, -His) and quadruple dropout (-Leu, -Trp, - His, -Ade) plates for
536 interaction under medium and high stringency conditions, respectively. Plasmid
537 combinations are indicated to the right, OD₆₀₀ values for dropout density above. (B)
538 Quantitative assay measuring growth densities of yeast transformants. Plasmid
539 combinations are indicated below the graph. **** indicates $p \leq 0.0001$

540

541 We cannot yet tell whether the EmPim-EmCDC25 interaction indeed involves
542 phosphorylation of the M-phase regulator by the PIM kinase, although this is clearly
543 the case for human Pim-1 and CDC25A [23]. Currently available PIM kinase activity
544 assays rely on small peptide substrates basing on known target consensus sequences
545 (K/R-K/R-R-K/R-L-S/T-a; a = small amino acid residue) for human PIM kinases [81].
546 Unfortunately, we could not identify a sequence motif in EmCDC25 that exactly
547 matches the consensus of human PIM kinases, thus making it very difficult to establish
548 a functional EmPim kinase assay at present. Hence, further investigations are
549 necessary to clearly define phosphorylation sites for EmPim to facilitate kinase assays
550 e.g. for high throughput screening.

551

552 **Effects of SGI-1776 and CX6258 on *Echinococcus* larvae and stem cells**

553 Thus far, we had only measured effects of SGI-1776 on *Echinococcus* primary cells in
554 a cell viability assay. However, the actual target for anti-AE therapy are MC vesicles.
555 Furthermore, for effective elimination of parasite tissue, the capacity of stem cells to
556 differentiate into MV vesicles must be eliminated [5]. We thus employed in further
557 experiments previously established *in vitro* cultivation systems for mature MV and for
558 the production of MV from stem cells. Furthermore, since EmPim contained the
559 majority of residues that mediate the interaction between Pim kinases and CX-6258,
560 we also included this inhibitor in our analyses. As shown in Fig 6, both SGI-1776 and
561 CX-6258 had a detrimental and dose-dependent impact on the structural integrity of
562 mature MV. Although incubation of MV with 3 μ M of both inhibitors for 28 d did not lead
563 to statistically significant effects, a concentration of 10 μ M of these inhibitors led to a
564 drastic loss of structural integrity of all (CX-6258) or almost all (SGI-1776) vesicles (Fig
565 6). In the case of 3 μ M of these inhibitors, many vesicles lost structural integrity but still
566 had the germinative layer attached to the parasite surface laminated layer (Fig 6). In
567 the case of 10 μ M of both inhibitors, however, complete detachment of the parasite
568 tissue from the laminated layer was observed (Fig 6).

569

570 **Fig 6. Effects of SGI-1776 and CX-6258 on MV and PC.** (A) Inhibitor effects on
571 mature MV. *E. multilocularis* MV were incubated for 28 d in the presence of different
572 inhibitor concentrations as indicated below, and the number of structurally intact MV
573 was inspected microscopically. ** indicates $p \leq 0.0021$. (B) Representative examples
574 of MV incubated with different concentrations of inhibitors as indicated to the left. (C)
575 Inhibitor effects on the formation of MV from PC. Parasite stem cell cultures were

576 incubated for 21 d in the presence of different inhibitor concentrations as indicated
577 below. Numbers of fully mature MV were subsequently counted. * represents $p \leq$
578 0.0332.

579

580 Since even after loss of structural integrity, parasite vesicles can in theory still harbor
581 living stem cells, we then tested both inhibitors for their capacity to affect the formation
582 of MV from cultivated stem cells. As shown in Fig 6, both inhibitors affected MV
583 formation from stem cells in a dose-dependent manner. In the case of SGI-1776,
584 vesicle formation, which in this system is usually achieved after 21 d [32], was
585 completely prevented in the presence of 30 μM SGI-1776, and almost completely in
586 the presence of 10 μM . At 3 μM concentration, SGI-1776 did not lead to statistically
587 significant effects. CX-6258, on the other hand, already drastically affected MV
588 formation at 3 μM and completely inhibited MV development at higher concentrations
589 (Fig 6).

590 Taken together, these analyses demonstrated clear detrimental effects of both PIM
591 kinase inhibitors on *Echinococcus* larvae and stem cells. Based on the homologies of
592 EmPim to human PIM kinases in regions that are important for inhibitor-kinase
593 interaction (Fig 2), we concluded that most of these effects should be due to an
594 inhibition of EmPim, although we cannot fully exclude that a certain degree of inhibition
595 of the *Echinococcus haspin* kinase might have contributed.

596

597 ***In silico* screening of EmPim inhibitors and effects against *Echinococcus***

598 **larvae**

599 Due to their effects on human kinases, the utilization of currently available PIM
600 inhibitors for chemotherapeutic approaches is associated with severe adverse effects
601 [27,82–84]. At least in the case of SGI-1776, clinical trials against different forms of
602 cancer had to be terminated since adverse effects on the cardiac electric cycle of
603 patients were observed (NCT0084860, NCT01239108). We thus aimed at the
604 identification of small molecule compounds that more specifically interact with the
605 parasite enzyme isoforms when compared to human PIM kinases.

606 To this end, we first employed a very recently established *in silico* approach, the
607 *Fluency* computational platform [60], which predicts quantitative binding affinities of
608 compounds to target enzymes exclusively from amino acid sequences. Briefly, *Fluency*
609 input consists of a protein amino acid sequence with domains optionally defined, and
610 a small molecule structure in the form of SMILES. For each input-pair, *Fluency* predicts
611 the protein-molecule binding affinity. Therefore, a natural application of *Fluency* is
612 virtual screening of large molecular libraries against a target of interest to prioritize a
613 top list of tractable size for downstream analysis such as medicinal chemistry analysis,
614 docking, and experimental validation.

615 In a first *Fluency* screen of roughly 24 million compounds, using the EmPim amino acid
616 sequence as a query, we obtained a list of 19,000 potential binders with predicted
617 affinities between 10 nM and 1 μ M for the parasite protein. Out of the 200 top-ranked
618 *in silico* hits (S4 Table), 20 compounds were then selected for profiling based on (i) the
619 *Fluency* screen score; (ii) diversity of chemical structures; and (iii) molecular modelling
620 using the seeSAR software, thus assessing the ATP pocket binding mode as well as
621 the absence of intra- and intermolecular clashes (S5 Table).

622 We then tested the 20 selected compounds against *E. multilocularis* MV and stem
623 cells. First, we again employed the MV assay and found 4 of the compounds
624 (Z30898879, Z196138710, Z65225039, Z354576500) being highly effective in
625 inducing structural vesicle damage at concentrations of 3 and 10 μ M (Fig 7). These 4
626 compounds were then employed in the PC vesicle formation assay, leading to the
627 identification of compound Z196138710 which, at a concentration of 10 μ M, completely
628 prevented MV formation (Fig 7). Finally, we focussed on the thienopyrimidine
629 Z196138710 (*N*-(4-(difluoromethoxy)-3-methoxybenzyl)-thieno-[3,2-*d*]-pyrimidin-4-
630 amine) and tested it in comparison to SGI-1776 on mature MV and the PC cultivation
631 system for MV development. As shown in Fig 7, a concentration of 10 μ M Z196138710
632 led to structural disintegration of 100% of MV after 28 d, which was even more effective
633 than SGI-1776. In the case of MV development from primary cells, Z196138710
634 showed effects similar to those of SGI-1776 (Fig 7).

635
636 **Fig 7. Effects of Pim inhibitors on *Echinococcus* larvae and human cells.** (A) Heat
637 map showing the effects of 20 *in silico* screen compounds on MV. Different
638 concentrations (indicated below) of each compound (indicated to the left) were
639 incubated *in vitro* with MV for 28 d and structural integrity was assessed. Colour-code
640 indicating percentages of surviving vesicles is indicated below. (B) Effects of four *in*
641 *silico* screen compounds on MV production from PC. 10 μ M of each compound
642 (indicated below) were incubated for 21 d with PC *in vitro* and the production of MV
643 was assessed. For comparison, SGI-1776 was tested at 10 μ M. (C) Effects of
644 Z196138710 and SGI-1776 on MV. Both compounds were tested at different
645 concentrations (shown below) on MV *in vitro*. Structural integrity was measured after
646 28 d. P values less than 0.0001 are summarized with **** and p values less than 0.0332

647 are summarized with *. (D) Effects of Z196138710 and SGI-1776 on the *in vitro*
648 formation of MV from PC. Both inhibitors were incubated at different concentrations
649 (indicated below) for 21 d with PC and the formation of mature MV was measured. (E)
650 Effects of Z196138710, SGI-1776, and CX-6258 on human HEK293T cells. HEK293T
651 cells were incubated with different concentrations of inhibitors as indicated below. Cell
652 viability was measured after 3 d. (F) Effects of inhibitors on human HepG2 cells. For
653 experimental procedure, see (E). P values less than 0.0001 are summarized with ****
654 and p values less than 0.0021 are summarized with **.

655

656 **Effects of Pim inhibitors on human cell lines.**

657 Having shown that Z196138710 shows similar (PC) or even higher (MV) toxicity
658 towards *E. multilocularis* than SGI-1776, we were, in a final set of experiments,
659 interested in possible toxicities of the thienopyrimidine compound on human cells. To
660 this end, we employed the cell lines HEK293T and HepG2, which, according to
661 previous studies, strongly depend on functional PIM kinases for cell viability [85-87].
662 As shown in Fig 7, at concentrations of 30 and 10 μM , both SGI-1776 and CX-6258
663 fully eliminated HEK293T and HepG2 cells within 3 d, whereas at the same
664 concentrations, Z196138710 only inhibited both cell lines to 40-60% (Fig 7). At a
665 concentration of 3 μM , the effects were even more drastic since SGI-1776 and CX-
666 6258 still almost completely inhibited both cell lines whereas Z196138710 had no
667 statistically significant effects (Fig 7). To assess whether the low toxicity of
668 Z196138710 towards human cell lines was due to reduced binding of the
669 thienopyrimidine compound to human PIM kinases, we finally performed *in silico*
670 modelling assays of Z196138710 and SGI-1776 on the structure of Pim-1. As shown
671 in S3 Figure, these analyses revealed a binding affinity of SGI-1776 in the nanomolar

672 range, which is in line with the results of previous biochemical assays [15]. For
673 Z196138710, on the other hand, binding affinities in the micromolar range were
674 obtained (S3 Figure), indicating that the low toxicity of the thienopyrimidine compound
675 towards human cell lines is due to low binding to human PIM kinases. Although
676 biochemical assays for measuring EmPim activity in the presence of kinase inhibitors
677 will have to be established to verify these *in silico* analyses, our data at least point to
678 Z196138710 as a promising candidate of an anti-*Echinococcus* compound with low
679 adverse side effects.

680 In summary, we herein characterized an *E. multilocularis* single copy gene, which is
681 expressed in the parasite's stem cell department, and which encodes a PIM kinase
682 family member that interacts with an *Echinococcus* CDC25 ortholog in Y2H assays.
683 These data at least point to a role of EmPim in *Echinococcus* cell cycle regulation,
684 which appears to be one of the conserved functions of PIM kinases in vertebrate and
685 invertebrate organisms [22,23,88]. An important role of EmPim in *Echinococcus* stem
686 cell function is further supported by our data on the detrimental effects of known PIM
687 kinase inhibitors, SGI-1776 and CX-6258, on *in vitro* cultivated MV and, particularly,
688 PC, which are highly enriched in stem cells [4]. Since EmPim shares the majority of
689 amino acid residues that are critical for inhibitor binding to mammalian Pim-1, it is
690 highly likely that these effects are primarily due to the inhibition of EmPim, although a
691 certain level of off-target effects, which might involve a parasite *haspin* ortholog, cannot
692 be fully excluded. Since the germinative (stem) cells are the crucial cell type for
693 parasite growth within the host [4], molecules that regulate their proliferative capacity
694 are, *per se*, attractive targets for anti-parasitic chemotherapy, provided that small
695 molecule compounds can be identified which discriminate between these factors and
696 their (usually) highly conserved mammalian orthologs. In the case of *Echinococcus*,

697 high-throughput screening approaches towards the identification of specific inhibitory
698 compounds from extensive small-molecule libraries are hampered by the fact that the
699 complex conditions of parasite cultivation, particularly those for stem cell cultures, only
700 allow parallel screening of dozens to maybe a few hundreds of molecules, and usually
701 must be carried out over several weeks. Even though elegant approaches such as the
702 PGI-assay for measuring MV integrity [89] or PC-based cell activity assays [90] allow
703 compound screening against *E. multilocularis* in shorter time, a pre-selection of
704 molecules from complex compound libraries is still necessary to narrow down
705 screening procedures to manageable sizes. We herein combined a novel, target-based
706 computational approach and *in silico* modeling techniques to select 20 compounds
707 from complex libraries of roughly 24 million molecules. Of these 20 compounds, 4
708 displayed detrimental effects on *in vitro* cultivated parasite larvae and stem cells, and
709 one of these, Z196138710, even out-matched known inhibitors against the target
710 kinase family concerning side effects on human cells. Although the true capacity of
711 Z196138710 in echinococcosis therapy still has to be established in future studies,
712 particularly involving biochemical assays against Pim kinases and, of course, *in vivo*
713 testing in murine models for echinococcosis [90], we propose the strategy employed
714 in this study as highly effective in identifying promising candidates for downstream
715 analyses towards this aim.

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984 **Supporting information Captions**

985 **S1 Table.** Providers of small molecule compounds and inhibitors used in this study.

986 **S2 Table.** Sequences of primers used in this study.

987 **S3 Table.** Accession numbers of genes and proteins analysed in this study.

988 **S4 Table.** Top 200 list of compounds after Fluency *in silico* screening against EmPim.

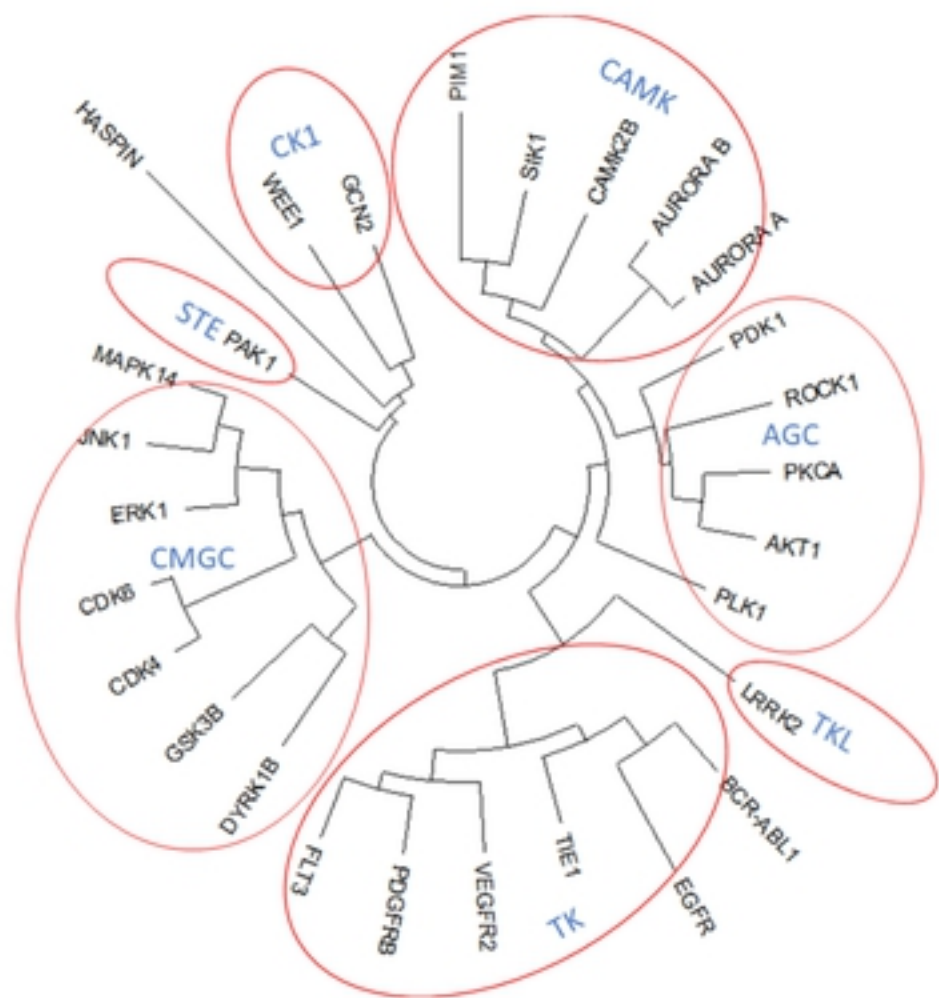
989 **S5 Table.** Structures and features of 20 compounds selected after Fluency *in silico*
990 screening.

991 **S1 Figure.** Structural features and homologies of EmPim and SmPim.

992 **S2 Figure.** Expression of *empim* in PC and MV.

993 **S3 Figure.** SeeSAR analysis of SGI-1776 and Z196138710 binding to human Pim-1.

A



B

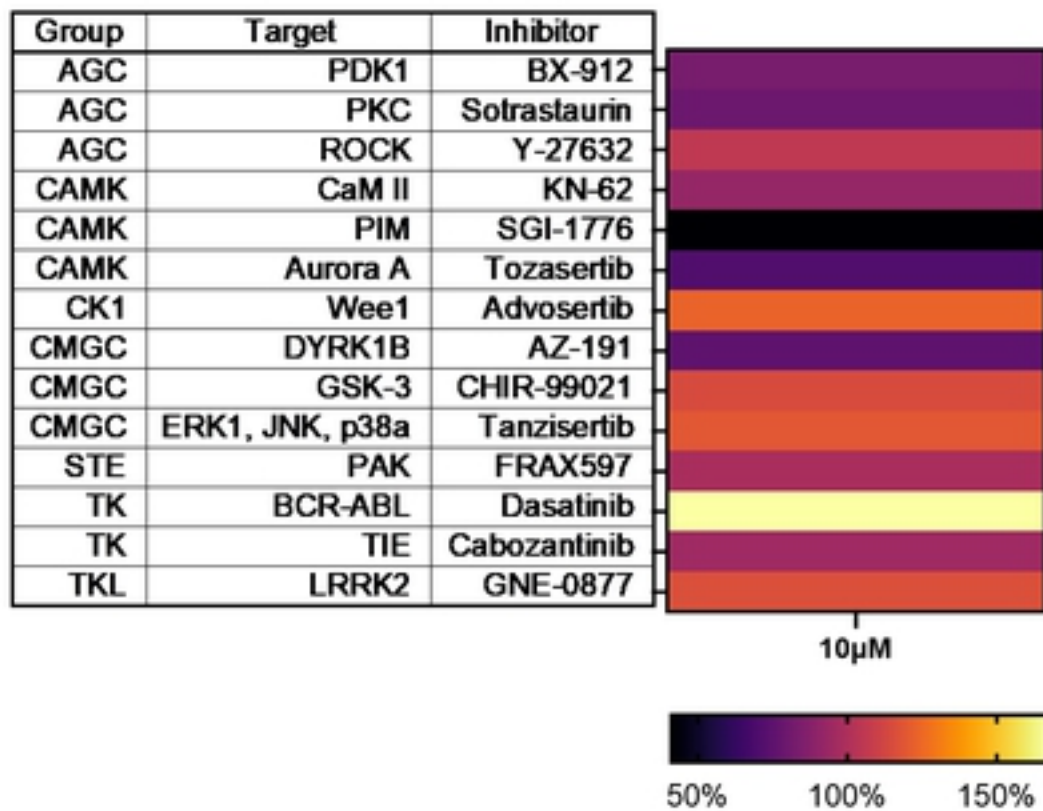
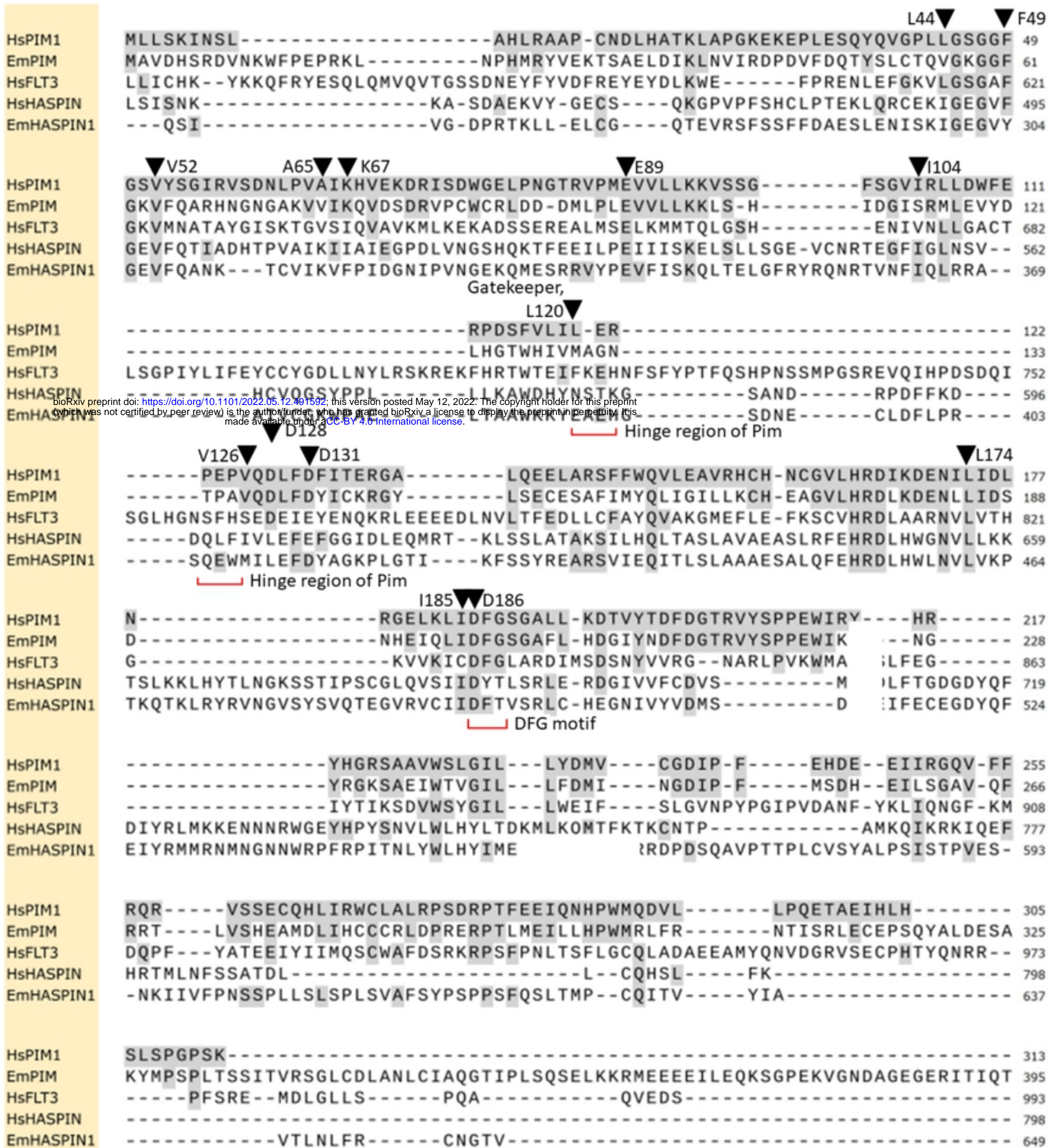


Fig 1

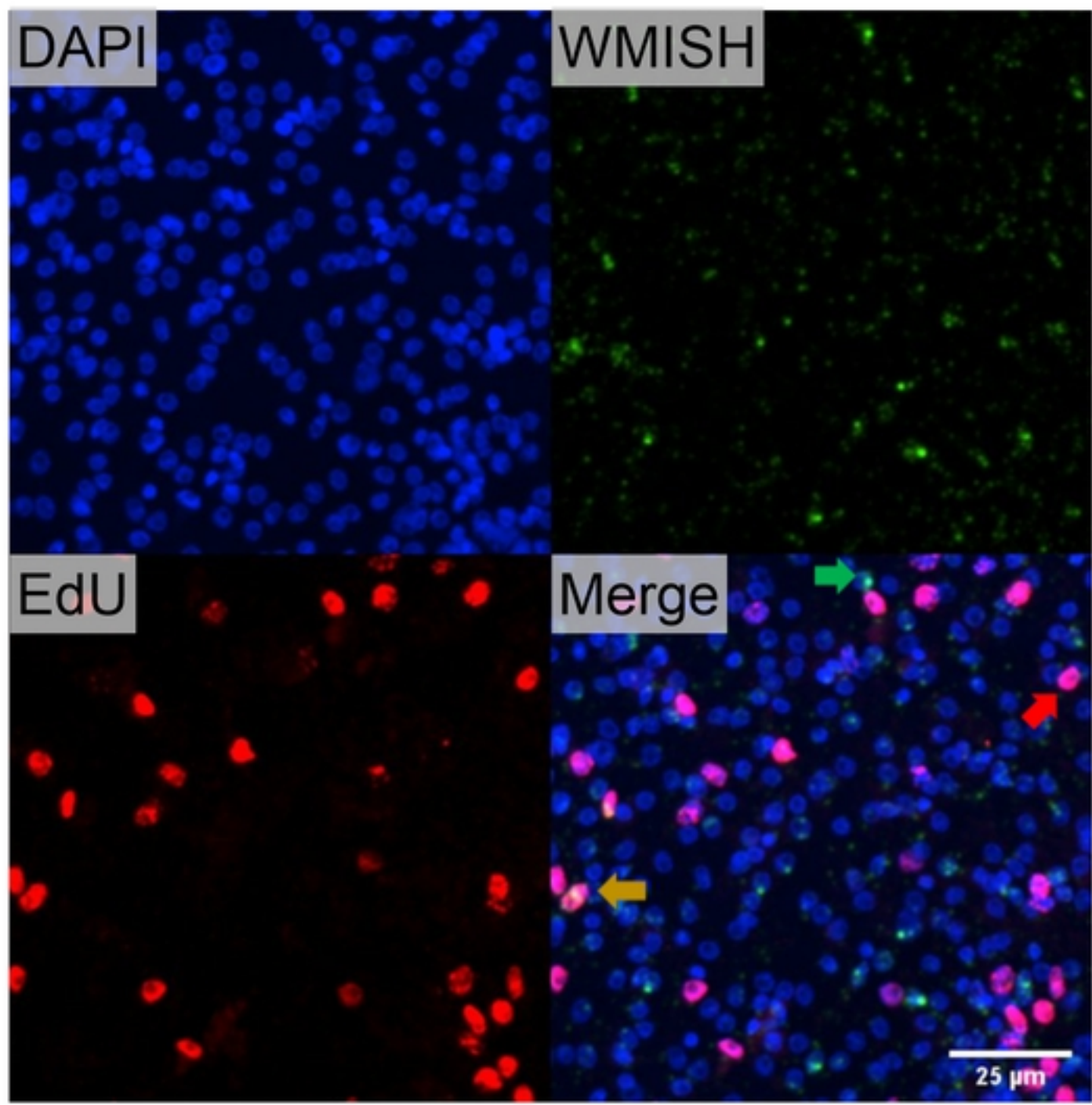
A



B

		identical residues	similar residues	IC ₅₀ of CX-6258(nM)	IC ₅₀ of SGI-1776(nM)
HsPIM1	L44 F49 V52 A65 K67 E89 I104 L120 V126 D128 D131 L174 I185 D186	14	0	5	7
EmPIM	V56 F61 V64 V77 K79 E101 S114 M130 V137 D139 D144 L185 I196 D197	10	2		
HsFLT3	L616 F621 V624 V637 I639 E661 V675 F718 S762 D764 E769 L818 C828 D829	7	3	134	44
HsHASPIN	I490 F495 V498 K511 F513 E535 I557 N581 I601 L603 E608 L656 I686 D687	7	3		34
EmHASPIN1	I299 Y304 V307 K317 F319 E341 I364 E388 M408 L410 D415 L461 I491 D492	7	1		

Fig 2

A

← WMISH positive
← EdU positive

← Double positive

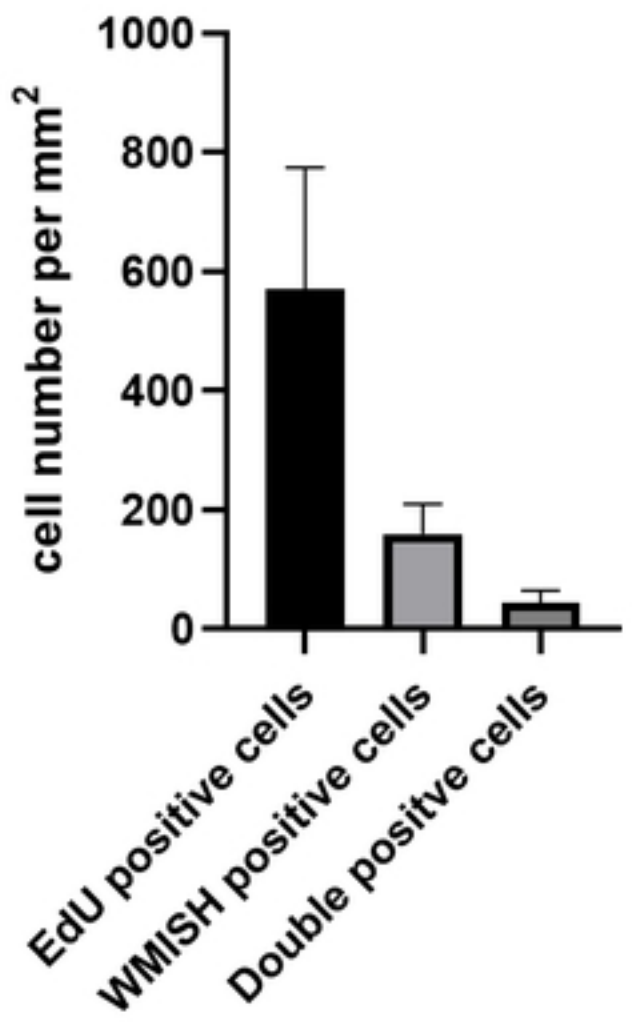
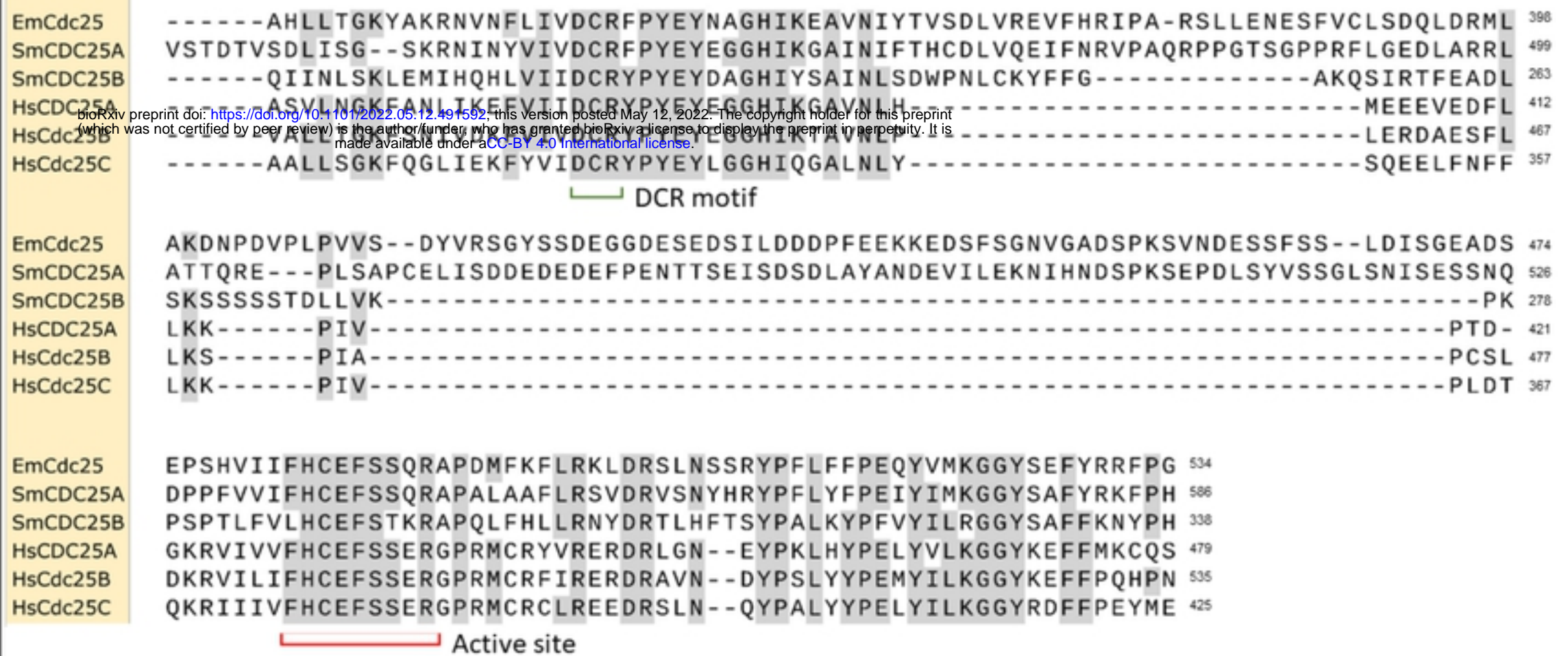
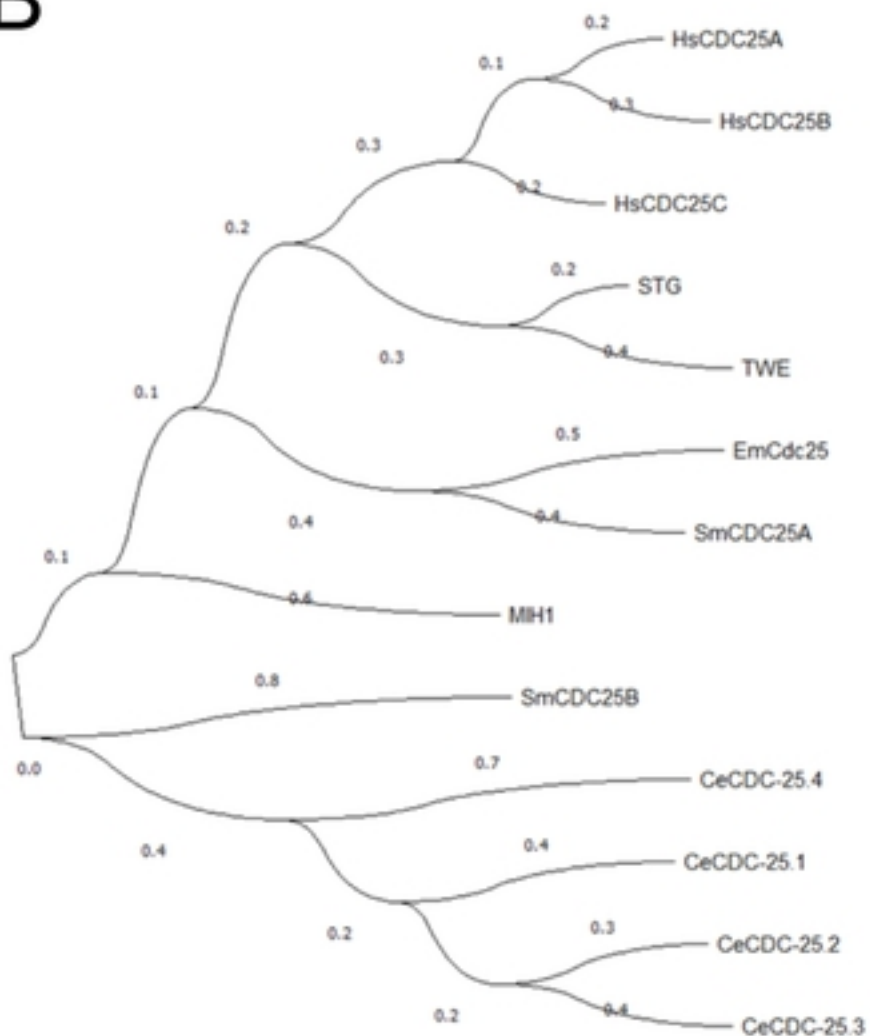
B

Fig 3

A



B



C

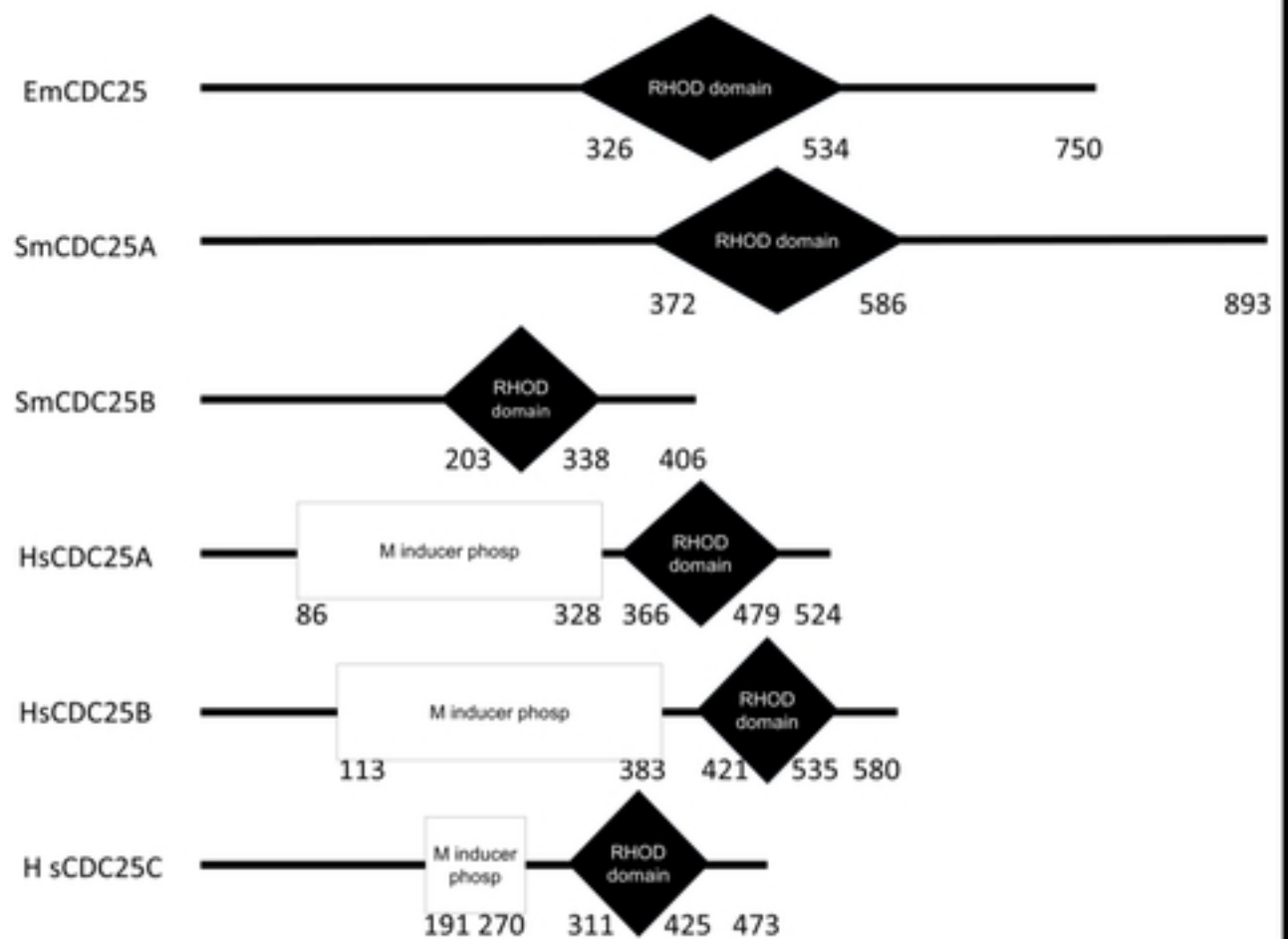


Fig 4

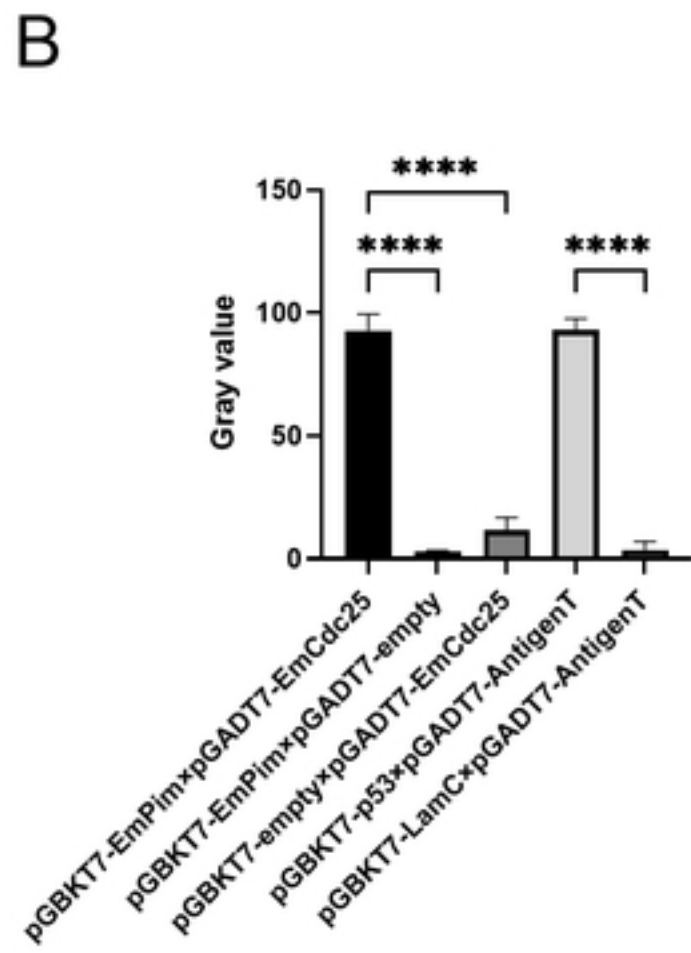
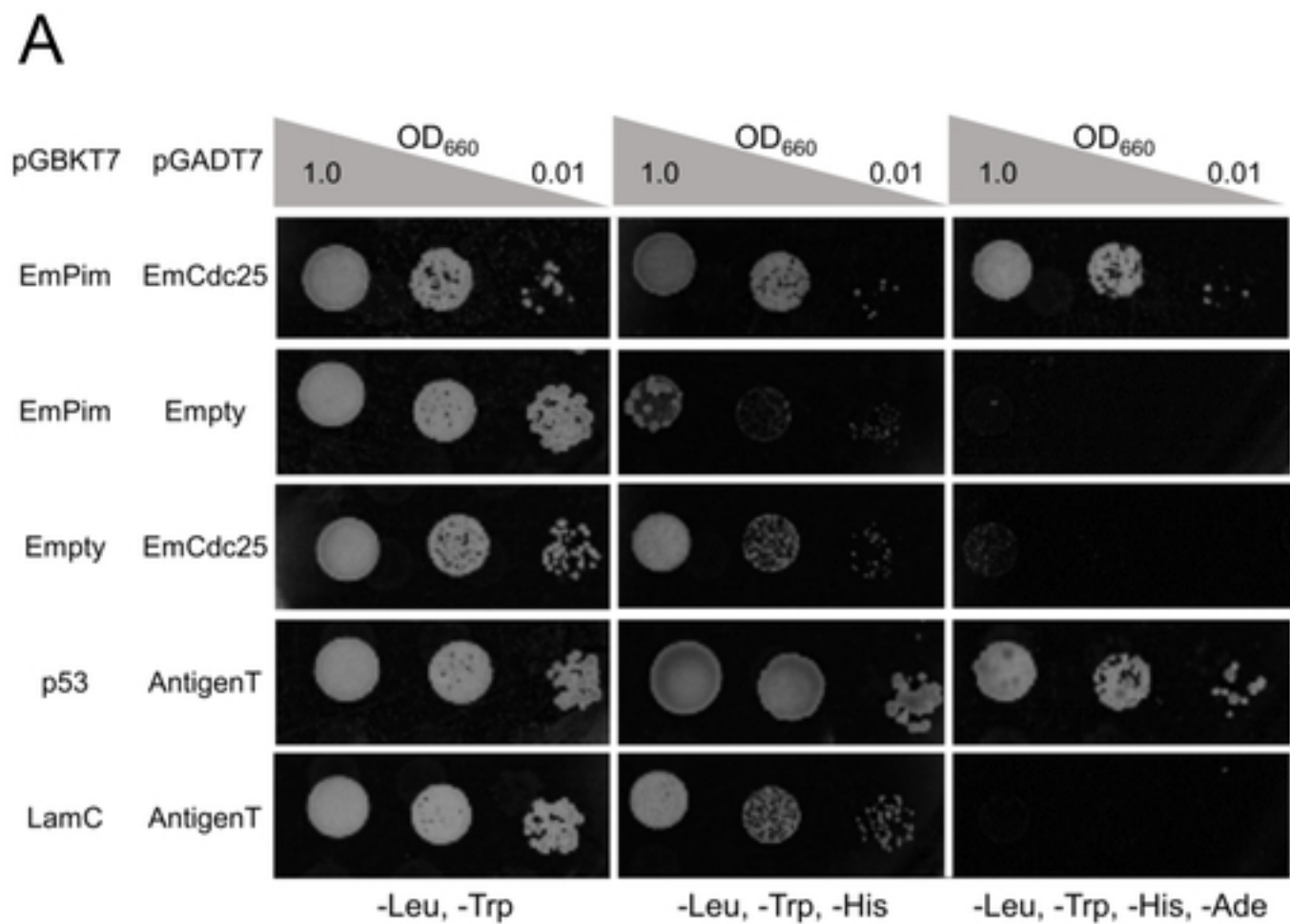
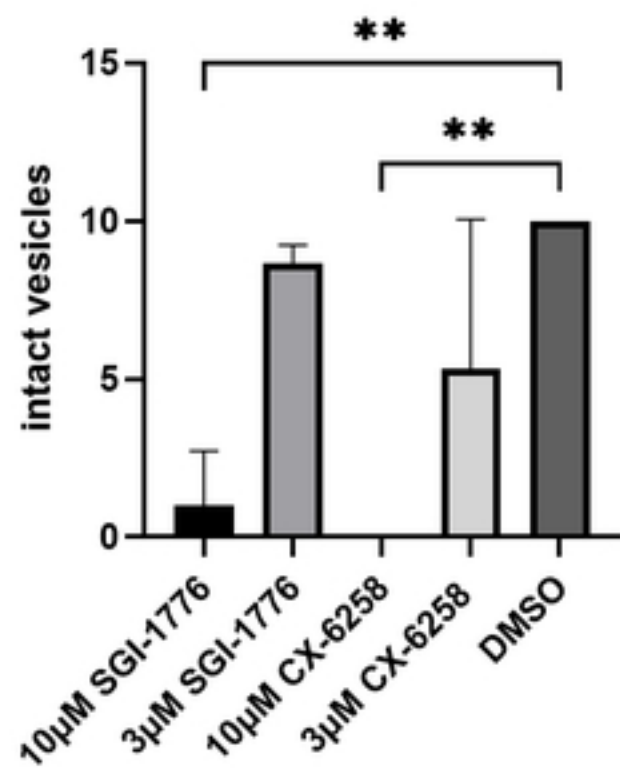
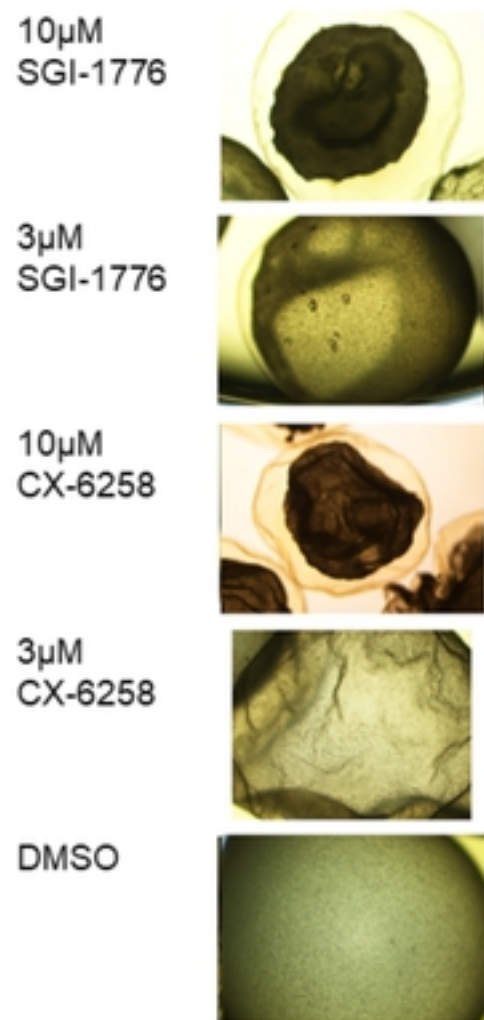


Fig 5

A



B



C

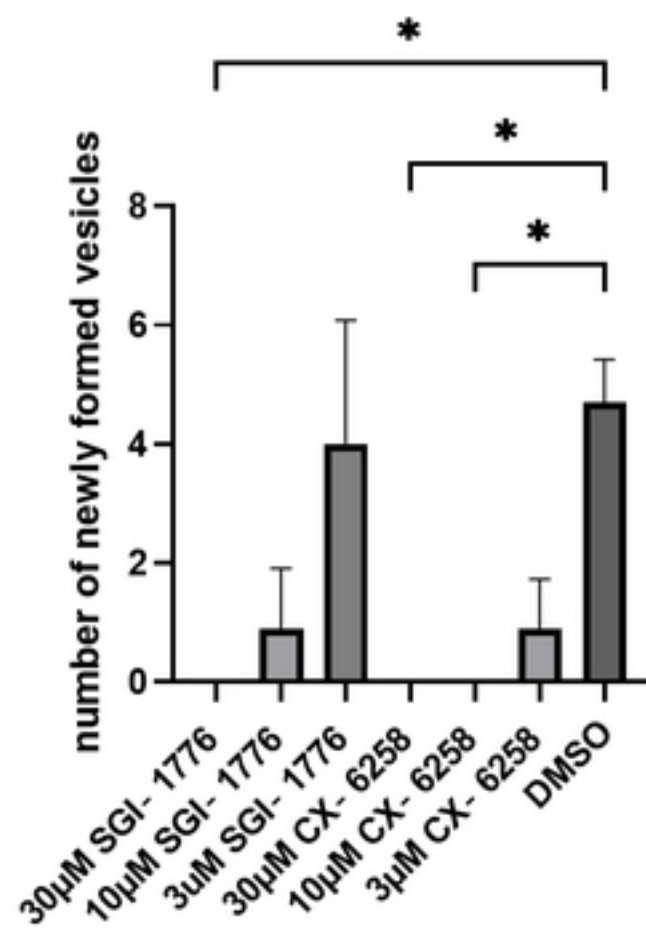
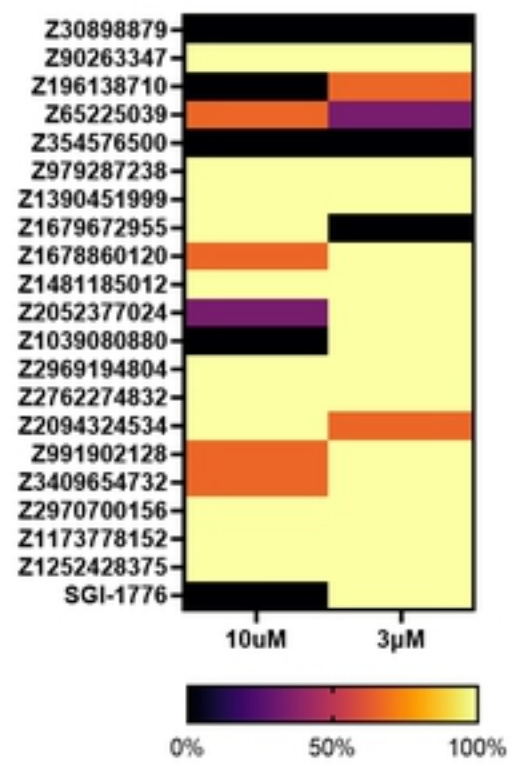
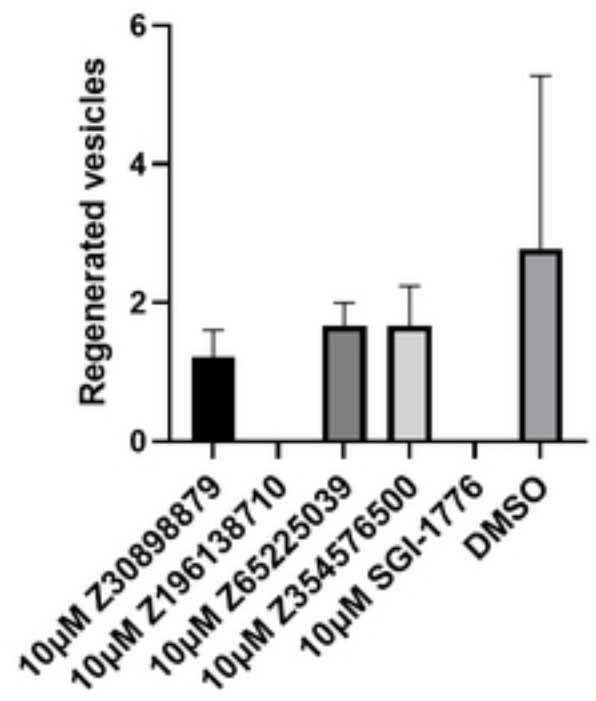


Fig 6

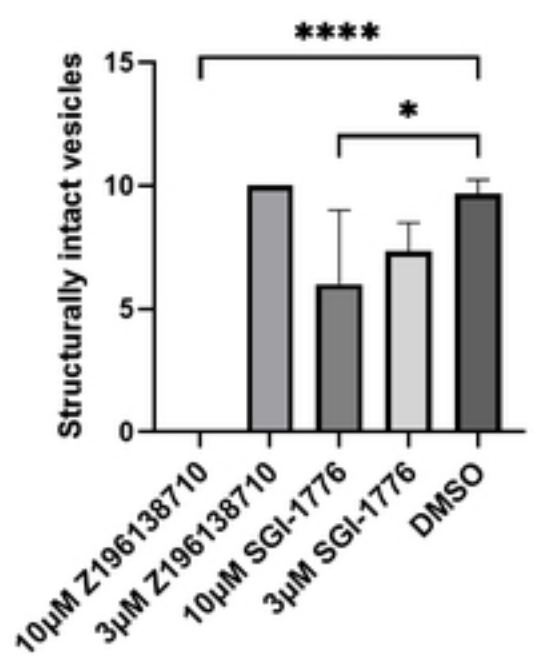
A



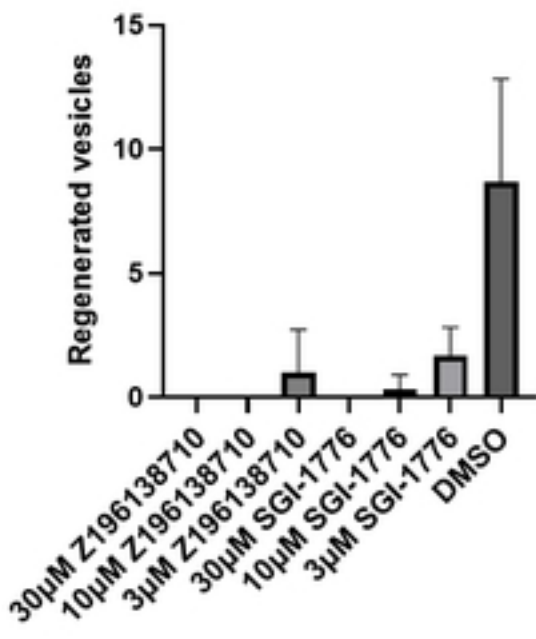
B



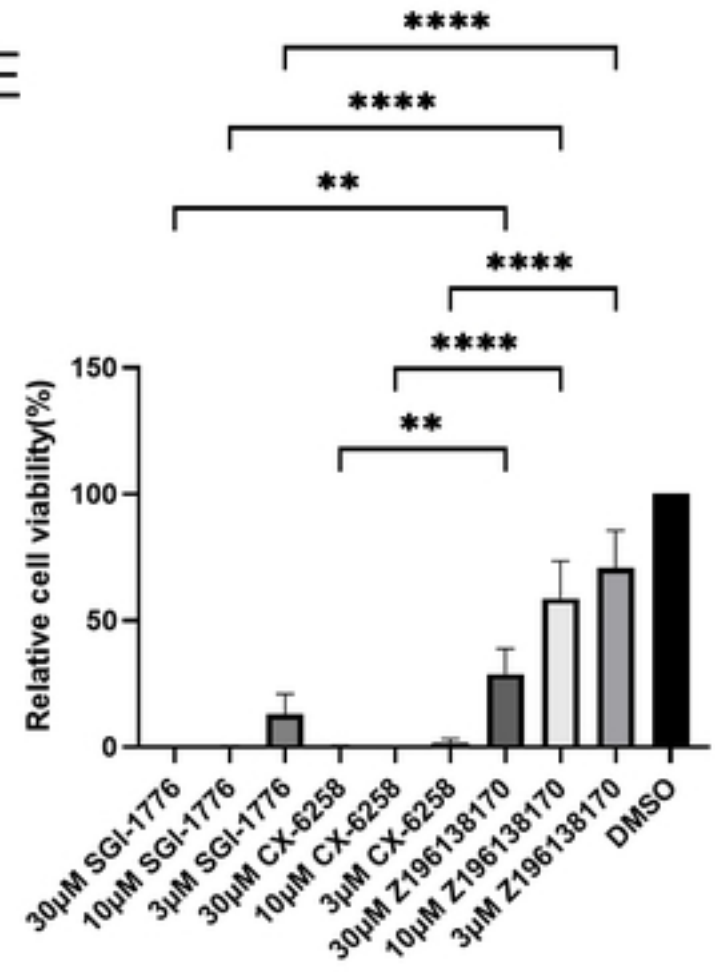
C



D



E



F

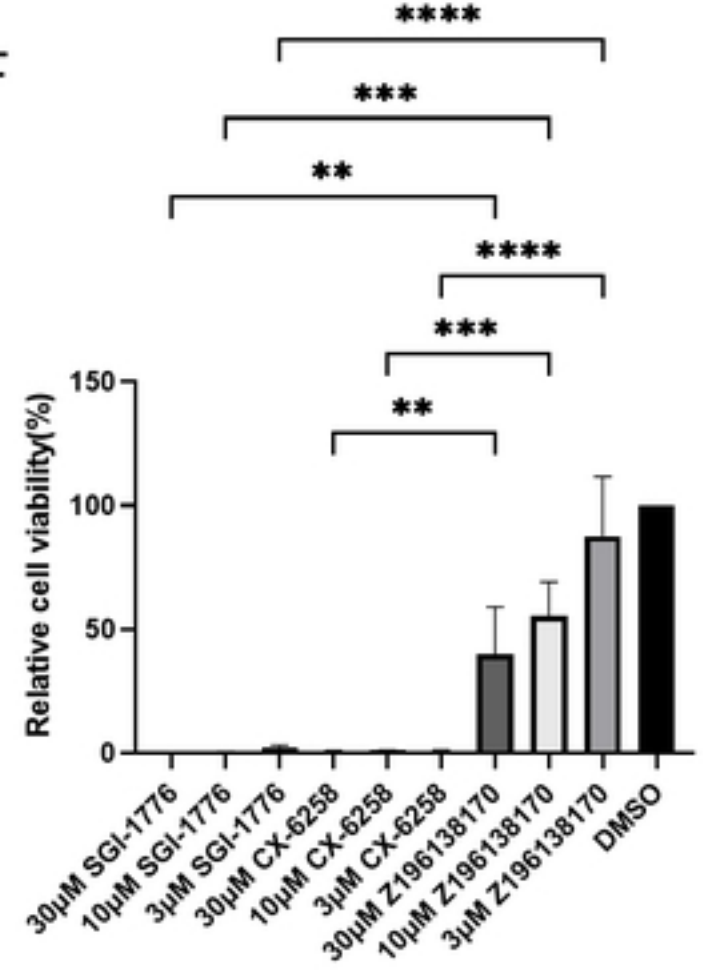


Fig 7