

1 *Naegleria fowleri*: protein structures to facilitate drug discovery for the
2 deadly, pathogenic free-living amoeba

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33

34 **ABSTRACT:** *Naegleria fowleri* is a pathogenic, thermophilic, free-living amoeba which causes
35 primary amebic meningoencephalitis (PAM). Penetrating the olfactory mucosa, the brain-eating
36 amoeba travels along the olfactory nerves, burrowing through the cribriform plate to its
37 destination: the brain's frontal lobes. The amoeba thrives in warm, freshwater environments,
38 with peak infection rates in the summer months and has a mortality rate of approximately 97%.
39 A major contributor to the pathogen's high mortality is the lack of sensitivity of *N. fowleri* to
40 current drug therapies, even in the face of combination-drug therapy. To enable rational drug
41 discovery and design efforts we have pursued protein production and crystallography-based
42 structure determination efforts for likely drug targets from *N. fowleri*. *N. fowleri* genes were
43 selected if they had homology to drug targets listed in Drug Bank or were nominated by primary
44 investigators engaged in *N. fowleri* research. In 2017, 178 *N. fowleri* protein targets were queued
45 to the Seattle Structural Genomics Center of Infectious Disease (SSGCID) pipeline, and to date
46 89 soluble recombinant proteins and 19 unique target structures have been produced. Many of
47 the new protein structures are potential drug targets and contain structural differences compared
48 to their human homologs, which could allow for the development of pathogen-specific inhibitors.
49 Five of the structures were analyzed in more detail, and four of five show promise that selective
50 inhibitors of the active site could be found. The 19 solved crystal structures build a foundation
51 for future work in combating this devastating disease by encouraging further investigation to
52 stimulate drug discovery for this neglected pathogen.

53 1. INTRODUCTION

54 In Australia in 1965, Fowler and Carter reported the first case of *Naegleria fowleri* infection,
55 commonly referred to as the “brain-eating amoeba”, which is the only known species of the
56 *Naegleria* genus associated with human disease (1). The free-living amoeba is found in soil and
57 freshwater on all seven continents, but is mainly found in warmer regions flourishing in
58 freshwater and soils with higher temperatures up to 115°F (46°C) (2).

59
60 Infection rates of the amoeba increase during summer months causing the disease, primary
61 amebic meningoencephalitis (PAM) (2,3). PAM is a fatal CNS disease that displays severe
62 meningitis and cranial pressure caused by inflammation of the brain (3,4). The National Institute
63 of Allergy and Infectious Diseases (NIAID) has classified *N. fowleri* as a category B priority
64 pathogen, the second highest class of priority organisms/biological agents. Category B pathogens
65 typically have high mortality rates, are easily disseminated, may cause public panic and social
66 disruption, and require special action for public health preparedness (2,5).

67
68 Between the years 1962 and 2016, 145 cases were reported by the CDC within the USA, and
69 only 4 patients (3%) survived *N. fowleri* infection and subsequent PAM (6). This 54-year
70 reporting tally might suggest only several PAM cases occur in the USA a year. However, a
71 recent review found that hundreds of undiagnosed cases of fatal “meningitis and encephalitis”
72 were reported in the summers between the years 1999 and 2010 in persons aged 2-22 years in the
73 Southeast USA (7). Cases lacking diagnostic brain biopsy or spinal fluid analysis of PAM may
74 account for a portion of the undiagnosed, inconclusive cases (7). Thus, the incidence of *N.*
75 *fowleri* infections in the USA is probably much higher than several a year arguing, that *N.*

76 *fowleri* poses a much higher public health threat than the documented cases would suggest (7).
77 Rising global temperatures has also led to claims of increased risk of infections due to spread of
78 suitable water conditions for *N. fowleri* (3,8).

79
80 The global burden of *N. fowleri* PAM cases is likely greater than anticipated, yet undefined.
81 Aga Khan University in Karachi, Pakistan has reported a rise in the number of PAM cases. Aga
82 Khan Hospital serves only a small fraction of the Karachi population (~23 million total) and
83 reports around 20 cases/year of PAM. Other hospitals in Karachi did not report a single case
84 during the same time, likely due to the lack of awareness, autopsies, and microscopy (9). Only
85 about 10 cases of *N. fowleri* PAM have been reported in Africa, though the numbers are likely
86 impacted by a reporting bias, as the resources for diagnostic brain biopsy are rarely present, and
87 autopsies are almost never performed (10).

88
89 The combination of a devastating mortality rate, a warming climate, and a rapid-onset
90 infection emphasize why *N. fowleri* should not stay a neglected organism. Treatment is
91 extremely limited and not well defined, using a combination of known antifungals, antibiotics
92 and microbicides (11). Amphotericin B is the current drug of choice, in high dosages, in
93 combination with other repurposed drugs such as rifampin, miltefosine, and fluconazole (9,12).
94 Recent studies suggest that posaconazole is more efficacious than fluconazole in vitro and in
95 animal models of PAM (12). Miltefosine was used in combination to successfully treat two
96 patients, one reported and one unreported in the literature. Miltefosine in combination is not
97 always helpful, in that a patient was treated with miltefosine and suffered permanent brain
98 damage and another had a fatal outcome (11,13). The multi-drug therapy is associated with

99 severe adverse effects and requires higher than normal dosages to penetrate the blood-brain
100 barrier and to reach the CNS (11,14). New development of rapid-onset, brain permeable,
101 efficient, and safe drugs is urgently needed.

102
103 Given the lack of understanding about causes of drug resistance of *N. fowleri* and the urgent
104 need for new drugs, we investigated the proteome of *N. fowleri* for likely drug targets attempting
105 to enable further drug discovery efforts by producing material for characterization of the
106 proteins. This work is a first step towards the discovery of drugs specifically designed against *N.*
107 *fowleri*.

108

109 **2. RESULTS**

110 **2.1 The *N. fowleri* proteome contains hundreds of potential drug targets.**

111 Potential drug targets were selected by sequence homology to DrugBank protein targets
112 (15). Additional targets were requested by the amoeba research community, leading to a total of
113 178 *N. fowleri* targets entering the Seattle Structural Genomics for Infectious Disease (SSGCID)
114 structure determination pipeline. The SSGCID is a National Institutes for Allergy and Infectious
115 Disease (NIAID) supported preclinical service for external investigators (www.SSGCID.org)
116 (16). All targets were filtered according to the standard SSGCID target selection protocol and
117 criteria (13): eliminating proteins with over 750 amino acids, 10 or more cysteines, or 95%
118 sequence identity with 70% coverage to proteins already in the PDB, targets claimed or worked
119 on by other scientific groups, and targets with transmembrane domains (except where a soluble
120 domain could be expressed separately) (18). Target criteria resulted in selection of 178 proteins
121 which entered the SSGCID production pipeline. These proteins, homologous to other known

122 drug targets, consisted of metabolic enzymes, protein synthetases, kinases, and others. Real-time
 123 updates to target status progress can be viewed at the SSGCID website. **Figure 1** shows a view
 124 of current status. Additionally, a detailed table of the *Naegleria* protein crystallography statistics
 125 is available in Supplemental Information.
 126

SSGCID ID [Center ID]	Family ID	Annotation	Target Approved	Selected	Cloned	Expressed	Soluble	Purified	Crystal- lized	Diff- raction Data	HSQC	NMR Assigned	In PDB	Work Stopped	Expression Clones* Available	Protein Available	Most Recent Activity
NafoA.20238.a	20238	deoxyhypusine hydroxylase													0	0	9/24/2020
NafoA.18219.a	18219	transcript1: glycolipide n-tetradecanoyltransferase 1; n-myristoyltransferase (nmt1)													0	0	8/27/2020
NafoA.20414.a	20414	phosphatidylinositol 3-kinase 2, mRNA1 (PI3K)													0	0	7/10/2020
NafoA.20399.a	20399	Phosphatidylinositol 3-kinase 2 (PI3K)													0	0	7/10/2020
NafoA.18272.b	18272	peptidyl-prolyl cis-trans isomerase fxbp1a													0	0	7/10/2020
NafoA.01088.a	01088	heterotrimeric G-protein alpha subunit													0	0	6/4/2020
NafoA.01541.a	01541	deoxyhypusine synthase													1	0	5/25/2020
NafoA.01340.a	01340	dihydrolipoyl dehydrogenase													1	0	5/25/2020
NafoA.19531.a	19531	regulator of heterotrimeric G proteins RGS4													1	0	5/4/2020
NafoA.01011.a	01011	calcium calmodulin-dependent protein phosphatase													2	1	4/1/2020
NafoA.19390.a	19390	phosphatidylinositol 3-kinase 2, mRNA2 (PI3K)													0	0	2/28/2020
NafoA.01391.a	01391	casein kinase													1	0	2/24/2020
NafoA.20395.a	20395	hsp90 co-chaperone													0	0	2/13/2020
NafoA.18664.a	18664	calcium calmodulin-dependent protein kinase c													0	0	2/13/2020
NafoA.01515.a	01515	cyclin-dependent kinase 1-like, cell division control protein 2													2	1	2/2/2020
NafoA.20237.a	20237	translation initiation factor eif-5a family protein													0	0	1/2/2020
NafoA.19531.b	19531	regulator of heterotrimeric G proteins RGS2													1	1	12/28/2019
NafoA.01088.d	01088	heterotrimeric G-protein alpha subunit Galpha2													2	1	12/28/2019
NafoA.01088.e	01088	heterotrimeric G-protein alpha subunit Galpha7													1	0	12/28/2019
NafoA.00032.a	00032	s-adenosyl-L-homocysteine hydrolase													1	2	12/28/2019

127 **Figure 1. Status of *Naegleria* targets in the SSGCID pipeline as of 10/1/2020.** This view is publicly
 128 available at www.ssgcid.org through the Targets tab, sorting by genus. Targets are displayed by SSGCID identifier
 129 and annotation with current status shown by green bars, sorted here by most recent activity reported to the database.
 130 The number of expression clones is shown and indicates different forms of the target that are available to external
 131 users and can be ordered. Protein availability also indicates samples of protein available to the external community
 132 and represents at least one vial of concentrated protein of approximately 100 microliters ranging from 10-50 mg/mL
 133 of >95% purity material.

134

135 2.2 One third of targets attempted produced soluble protein.

136 The open reading frames of each target were obtained from AmoebaDB.org. Progression of
 137 the targets through the SSGCID protein production pipeline is shown in **Table 1**. Of the 178
 138 NIAID approved targets, 177 were selected for cloning. One protein target was eliminated due to
 139 redundancy given its 100% identity match and 84% coverage to another protein target already in
 140 the SSGCID pipeline. Of the 177 targets attempted in PCR amplification using *N. fowleri* cDNA,

141 133 were successfully amplified and cloned into SSGCID expression vectors (75%) (17). In
142 small-scale expression screening, 82 of the 133 successfully cloned targets (61.6%)
143 demonstrated soluble expression with a N-terminal His6-tag vector (17). Of these 82 soluble
144 proteins, 64 proteins were purified to >95% purity with yields ranging from 1.1 mg to 348 mg.
145 These proteins are available, under request, at SSGCID.org.

146

147 **Table 1. Target status of *N. fowleri* proteins within SSGCID pipeline.**

Target approved	178
Selected	177
Cloned	133
Expressed	98
Soluble	82
Purified	64
Crystallized	29
Diffraction	27
Native diffraction data	19
In PDB (apo)	19

148

149 **2.3 *N. fowleri* proteins resulted in a 11% structure determination success rate.**

150 Following purification of high-purity preparations of protein, the targets were submitted for
151 structure determination by X-ray crystallography. Of the 64 proteins produced, 26 crystallized
152 (29%), and 20 diffracted, 19 of which met the SSGCID resolution quality criteria and were
153 submitted to the Protein Data Bank (PDB). **Table 2** lists the 19 targets deposited in PDB that will
154 be reported in this paper, including five structures with a unique ligand bound, for a total of 23
155 PDB deposits. Overall, this resulted in a structure determination success rate of 11%, which is
156 comparatively higher than usual structural genomic pipeline rates reported by us or other
157 structural genomic groups (19).

158

159 **Table 2. Listing of *Naegleria fowleri* structures deposited by SSGCID in the PDB.**

SSGCID ID	Annotation	AmoebaDB	PDB ID	Metabolic pathway
NafoA.00005.a	Malate dehydrogenase 2 (mitochondrial)	BF0021050	6UM4	TCA cycle
NafoA.01242.a	dUTP pyrophosphatase	NF0068730	5VJY, 6MJK	DNA replication
NafoA.00085.a	GDP-L-fucose synthetase	NF0109030	6AQY, 6AQZ	Glycolysis
NafoA.00085.b	GDP-mannose dehydratase	NF0016040	5UZH	Glycolysis
NafoA.00855.a	Glyceraldehyde-3-phosphate dehydrogenase	NF0055660	6NLX	Glycolysis
NafoA.19900.a	Glucokinase	NF0035880	6DA0	Glycolysis
NafoA.01088.e	Heterotrimeric G-protein alpha subunit Galpha7	NF0037180	6NE6	GPCR signaling
NafoA.00438.a	Nucleoside diphosphate kinase	NF0036070	5U2I	Purine metabolism
NafoA.18272.a	Peptidylprolyl isomerase	NF0084240_2	6B4P, 6MKE	Post-translational protein modification
NafoA.01013.a	Phosphoglycerate mutase	NF0048460	5VVE	Glycolysis
NafoA.18681.a	Prolyl-tRNA synthetase	NF0061610	6NAB, 6UYH	Amino acid response
NafoA.01523.a	Protein arginine n-methyltransferase	NF0022020	6CU3, 6CU5	Post-translational protein modification
NafoA.19251.a	Polyubiquitin with 3 ub domains	NF0029300_2	5VIX	Ubiquitination
NafoA.01385.a	Rab GDP dissociation inhibitor alpha	NF0049320	6C87	Signaling pathways
NafoA.00927.a	Ras-related c3 botulinum toxin substrate 1 isoform x2	NF0029600_1	5VCU	Signaling pathways
NafoA.00032.a	S-adenosyl-L-homocysteine hydrolase	NF0035640	5V96	Methylation metabolism
NafoA.01238.a	Serine-tRNA synthetase	NF0119870	6BLJ	Protein synthesis
NafoA.00964.a	Trafficking protein particle complex subunit 3	NF0066220	6AQ3	Protein trafficking
NafoA.00601.c	Ubiquitin-conjugating enzyme e2	NF0031160_1	5V0R, 6MJ9	Ubiquitination

160

161 **2.4 Comparative analysis of *N. fowleri* and *Homo sapiens* enzyme active sites suggest**

162 **some targets have promise for selective inhibition.**

163 We have already published that glucokinase inhibitors can be obtained that are selective for

164 *N. fowleri* vs. the human homolog, supporting glucokinase as a target for *N. fowleri* therapeutics

165 (20). We analyzed the other structures determined, comparing the *N. fowleri* structure to human

166 homolog structures, in order to determine opportunities for selective design of chemical

167 inhibitors. Comparison of the *N. fowleri* determined structure to human structures available in

168 the PDB was done by superimposition of the coordinate files (**Table 3**). With the exception of a

169 pair of 96% identical *N. fowleri* and human ubiquitin-conjugating enzymes e2, all of the *N.*

170 *fowleri* enzymes differed from human homologs by more than 38% (**Table 3**). We wanted to

171 focus on the known ligand binding sites, to search for potential differences for inhibitors. A

172 PDB search revealed that five of the 19 *N. fowleri* structures determined also had human

173 homolog structures determined which contained a known inhibitor of the human protein (**Table**
 174 **3**). We then manually inspected and compared the binding sites of these five proteins, described
 175 below for each protein. Despite sequence similarities of the active sites, there were four cases
 176 where a case for active site specificity could be made, supporting these proteins as targets for
 177 therapeutics for *N. fowleri*.

178

179 **Table 3. Comparative analysis of *N. fowleri* and human structures deposited the PDB.**

Annotation	PDB ID	# Amino acids	Human PDB ID	Coverage (#aa)	% Coverage	% Identity of coverage	Inhibitor or ligand in human structure
malate dehydrogenase (mitochondrial) (MDH2)	6UM4	436	4WLF	221	51%	26%	-
dUTP pyrophosphatase	6MJK, 5VJY	147	2HQU_A	136	93%	63%	-
GDP-L-fucose synthetase	6AQY, 6AQZ	333	4E5Y_A	311	93%	52%	-
GDP-mannose-dehydratase	5UZH	380	6GPJ_A	343	90%	68%	-
glyceraldehyde-3-phosphate dehydrogenase	6NLX	333	6YNF_A	324	97%	65%	-
heterotrimeric G-protein alpha subunit Galpha7	6NE6	321	6K41_A	312	97%	41%	-
nucleoside diphosphate kinase	5U2I	151	1JXV_A	151	100%	63%	-
peptidylprolyl isomerase	6MKE, 6B4P	119	4DRO_A	113	95%	53%	FK506-AN
phosphoglycerate mutase	5VVE	250	5Y65_C	250	100%	61%	KH2
prolyl-tRNA synthetase	6NAB, 6UYH	535	5VAD_A	526	98%	54%	91Y
protein arginine n-methyltransferase	6CU3, 6CU5	328	6NT2_A	310	95%	53%	GSK3368715
polyubiquitin with 3 ub domains	5VIX	230	5H07_A	227	99%	96%	-
rab GDP dissociation inhibitor alpha	6C87	444	n/a				-
ras-related c3 botulinum toxin substrate 1 isoform x2	5VCU	200	1I4D_D	191	96%	55%	-
S-adenosyl-L-homocysteine hydrolase	5V96	472	1LI4_A	427	90%	57%	Neplanocin
Serine-tRNA ligase	6BLJ	477	4L87_A	458	96%	52%	-
trafficking protein particle complex subunit 3	6AQ3	187	2CFH_A	176	94%	47%	-
ubiquitin-conjugating enzyme e2	6MJ9, 5V0R	161	4ONM_A	126	78%	48%	-

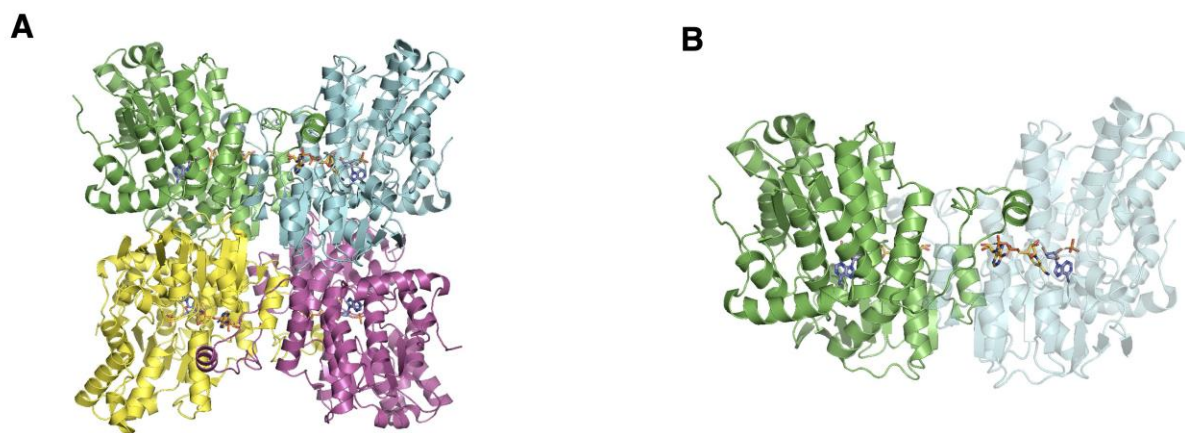
180

181 *N. fowleri* S-adenosyl-L-homocysteine hydrolase (*NfSAHH*) catalyzes the breakdown of

182 S-adenosyl-homocysteine (SAH) into adenosine and homocysteine. SAH is a byproduct of S-

183 adenosyl-L-methionine as a methyltransferase; the transfer of a methyl group to its respective
184 cellular substrates such as DNA or rRNA, produces SAH (21). SAH hydrolases play a central
185 role in methylation reactions required for growth and gene regulation, and inhibitors of SAH
186 hydrolase are expected to be antimicrobial drugs, especially for eukaryotic parasites (21).
187 Ribavirin is structurally similar to adenosine and has been proved to produce a time-dependent
188 inactivation of human (*Hs*) SAHH and *Trypanosoma cruzi* (*Tc*) SAHH (22).

189
190 The *Nf*SAHH asymmetric unit contains a homo-tetramer (**Figure 2**). Although each chain
191 contains an active site, structural analysis indicates that two chains must be present for the
192 hydrolysis reaction to occur successfully. Each chain consists of three domains: a substrate-
193 binding, a cofactor-binding, and a C-terminus domain (23). When substrates are not bound, the
194 substrate-binding domain is located on the exterior, far from the meeting point of all four
195 subunits of the asymmetric unit (24). The C-terminus domain is involved in both cofactor
196 binding and protein oligomerization (23). In addition to the three main constituents, the structure
197 contains two hinge regions that connect the substrate-binding and cofactor-binding domains.
198 When substrates bind, the hinge region changes conformation, closing the cleft between the
199 substrate-binding domain and the cofactor-binding domain of the respective chain (24). In the
200 structure of *Nf*SAHH, all subunits exhibit a closed conformation.



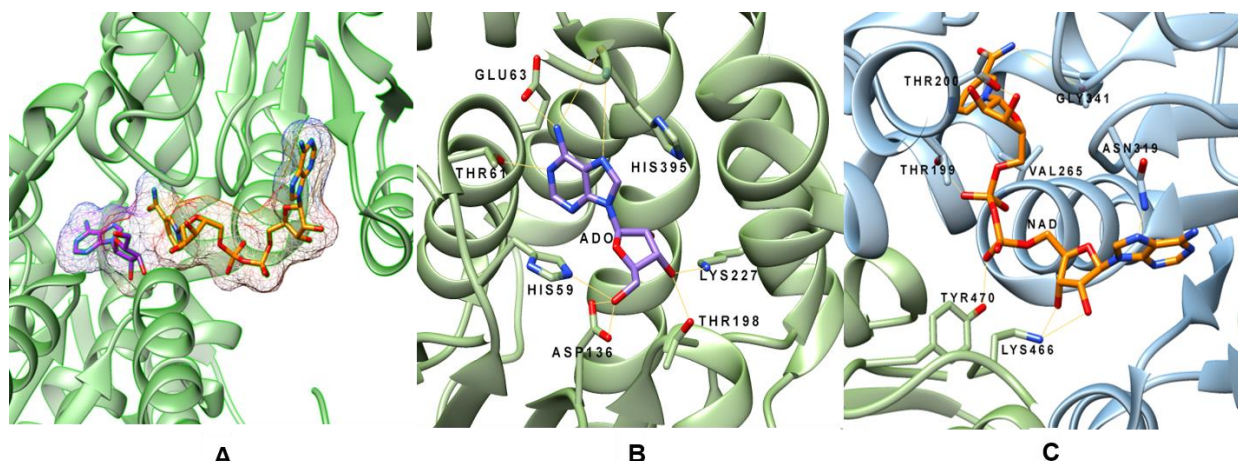
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202 **Figure 2. Crystal structure of *NfSAHH* (PDB: 5V96) solved at a 2 Å resolution. (A)** The asymmetric unit
203 of SAHH. Individual polypeptide chains are colored green (chain A), pink (chain B), yellow (chain C), and cyan
204 (chain D). **(B)** The biological unit is a homotetramer with a 2-fold axis of symmetry. Each of the four chains has its
205 own active site containing one NAD⁺ molecule (yellow), one adenosine molecule (purple), and a phosphate
206 (orange).

207

208 SAHH is one of the most highly conserved proteins among species, with many of the same
209 amino acids binding the same substrates across homologs. *NfSAHH* is 62% identical to the
210 human homolog. In the NAD binding region, conserved Lys and Tyr bind via hydrogen bonds to
211 oxygens of NAD in both *NfSAHH* and *HsSAHH* (24) (**Figure 3**). Residues involved with
212 binding adenosine (ADO) are also highly conserved, with Gly341-His342-Phe333 being
213 completely conserved (24). To regulate the entrance of substrates into/out of the active site, there
214 is a highly conserved His-Phe sequence within the cofactor-binding domain. This works as a
215 molecular gate that, when the protein is in open conformation, allows access to the substrate-
216 pocket (23).

217



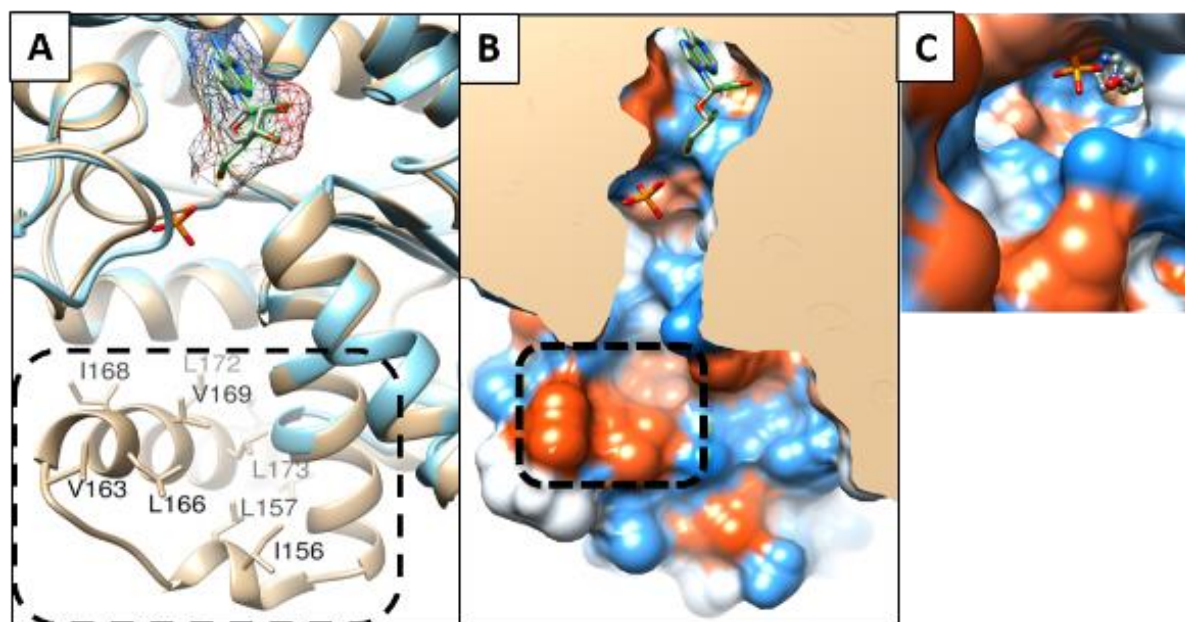
218 **Figure 3. Overview of *NfSAHH* active site binding.** (A) Nicotinamide adenine dinucleotide (NAD/orange
219 sticks) and adenosine (ADO/purple sticks) both fit well within the structure of SAHH. (B) Hydrogen-bond network
220 denoted by yellow dashed lines around ADO involving chain A. (C) Hydrogen-bond network involving NAD with
221 chain A (blue) and chain B (green).

222

223 A search of the PDB for *HsSAHH* structures found multiple inhibitor bound human
224 structures including 1LI4 (neplanocin) and 5W49 (oxadiazole compound). A comparison of the
225 *NfSAHH* structure to the neplanocin bound human structure revealed a highly conserved
226 conformation of the protein. In the neplanocin bound structure, the two domains of the monomer
227 are similar to the *NfSAHH* structure. However, in the oxadiazole bound structure, two domains
228 of the monomer are in a more open conformation, where the C-terminal and N-terminal domains
229 have opened up relative to each other in a hinge-opening motion. The oxadiazole compound
230 stretches across the interface and is surrounded by 11 residues within 4 Å. Of the 11 residues
231 coordinating the inhibitor oxadiazole compound of the 5W49 structure, 10 are identical between
232 the *Naegleria* and human SAHH. Only one change of M351T relative to the human enzyme is
233 present, suggesting a highly conserved inhibitor binding site.

234

235 The crystal structure of *Nf*SAHH (**Figure 4**) contains the adenosine substrate and NAD
236 cofactors bound to the active site to guide structure-activity relationships that could help to
237 optimize adenosine analog compounds. The sequence differences that line the access channel at
238 the dimer interface allow a rational approach to selectively inhibit the otherwise highly
239 conserved active site (25). Amoeba SAHs have an additional helix insertion that in *Nf*SAHH
240 forms a hydrophobic groove accessible from the adenosine binding site (**Figure 4 B, C**).
241 Specificity could be achieved by designing compounds that simultaneously target this
242 hydrophobic pocket and the active site (**Figure 4**). Thus, we feel a reasonable case can be made
243 that structural differences, close to the active site, would allow development of specific
244 *Ng*SAHH inhibitors supporting development of a therapeutic.
245



246
247 **Figure 4. SAHH active site analysis.** (A) Adenosine-bound *Nf*SAHH (PDB: 5V96, tan) vs. *Hs*SAHH bound
248 to adenosine analogue (PDB: 1A7A, blue); box highlights *Nf*-specific insertion with labelled hydrophobic residues.
249 (B) Slice through *Nf*SAHH, with surface colored on Kite-Doolittle (blue-red) hydrophobicity scale; dashed box

250 indicates hydrophobic groove formed by Val163, Leu166, Val169 and Leu173 at opening of deep-seated adenosine
251 pocket. (C) Same view as (B) tilted 90° shows opening from hydrophobic groove to adenosine pocket.

252

253 ***N. fowleri* phosphoglycerate mutase (NfPGM)**, a glycolysis enzyme, catalyzes the

254 isomerization of 3-phosphoglycerate and 2-phosphoglycerate during glycolysis and

255 gluconeogenesis and is regarded as a key enzyme in most organism's central metabolism (26).

256 There are two distinct forms of PGMs, differentiated by their need of 2, 3-bisphosphoglycerate

257 as a cofactor. PGM in mammals require the cofactor whereas PGM present in nematodes and

258 bacteria do not (27). The NfPGM is likely the cofactor-dependent PGM type. The crystal

259 structure of NfPGM (PDB: 5VVE) was solved at a resolution of 1.7 Å and consists of 250 amino

260 acid residues (~30 kDa).

261 The HsPGM and NfPGM structures share 61% identity. Residues surrounding the binding

262 pocket for citrate acid are all conserved, with the exception of a conservative change from a

263 Thr30 (*Nf*) to Ser30 (*Hs*). A comparison of the NfPGM structure to the homologous human

264 enzyme HsPGM (PDB: 5Y65) shows a conformational opening of the substrate binding site to

265 accommodate the KH2 ligand. However, the residues surrounding the inhibitor molecule and

266 supporting the movement of the peptide are identical between the two enzymes. It is likely that

267 with this high homologous identity that NfPGM is not a strong candidate for selective active site

268 inhibitor design.

269

270 ***N. fowleri* protein arginine N-methyltransferase (NfPRMT1)** methylates the nitrogen

271 atoms found on guanidinium side chains of arginine residues within proteins. The methylation of

272 nucleotide bases is a well-known mechanism of importance that influences DNA, nucleosomes,

273 and transcription functionalities (28). The enzyme is highly conserved across eukaryotes. Faulty

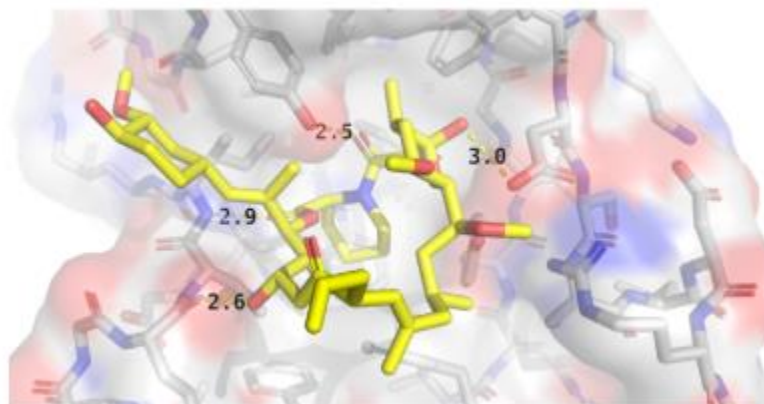
274 regulation or deviating expression of PRMTs is associated with various diseases including
275 inflammatory, virus-related, pulmonary, and carcinogenesis (29). Overexpression of PRMTs has
276 been observed in multiple forms and types of cancer, including PRMT1v1 overexpression in
277 colon cancer (30) and large increases of PRMT1v2 in breast cancer (31). Inhibitor discovery and
278 testing using PRMTs in cancer has been frequently employed (29). *Nf*PRMT1 was compared to
279 the drug bound structure of *Hs*PRMT1 (PDB: 6NT2). The protein binds ligands at a dimer
280 interface closing around two inhibitor molecules, one on each monomer. A large ligand binding
281 loop is disordered in the *Nf*PRMT1 structure, presumably becoming ordered and visible in the
282 crystal structure in the presence of inhibitor in the human structure. Due to the large binding
283 surface for peptide substrates, PRMTs typically are promiscuous in nature with a wide range of
284 binding substrates (29). Comparison of over 40 PRMT-inhibitor complexes revealed 5 distinct
285 binding mechanisms at multiple sites including active site and allosteric binding pockets (32).
286 Isozyme specific peptide mimics have been identified which preferentially bind *Hs*PRMT1 vs.
287 *Hs*PRMT5 enzyme. A similar approach could be considered for selective *Nf*PRMT inhibitor
288 development (33,34). There is still a need to improve both the affinity and selectivity of these
289 micromolar, sub-micromolar potent PRMT inhibitors as well as to better understand the
290 enzyme's biological and disease processes in greater scope (35).

291
292 Despite high sequence identity in the ligand binding pocket, there are distinct differences in
293 side chain orientation between the structures. These residues may change conformation upon
294 binding inhibitor. A number of distinct features of *Nf*PRMT1 exist which can be exploited for
295 potential structure-based approach to developing selective allosteric inhibitors against the *Nf*
296 enzyme. A methionine is present in the *Nf*PRMT1 structure adjacent to the adenine moiety of the

297 S-adenosyl homocysteine (SAH) which differs significantly from all nine-known human
298 PRMTs. The substrate binding region is lined by residues variant between *Nf* and all nine-known
299 human PRMTs; for example, though the *Nf*PRMT1 pocket is similar to the allosteric inhibition
300 pocket of *Hs*PRMT3, there are two tyrosine substitutions lining the pocket (36). Additionally, N-
301 terminal residues which interact with inhibitors of *Hs*PRMT1 are largely not present or have
302 limited interactions in *Nf*PRMT1 (37). Thus, inhibitors that selectively target *Nf*PRMT1 vs. the 9
303 *Hs*PRMTs are envisioned due to structural differences near the ligand binding sites.

304
305 ***N. fowleri* peptidylprolyl isomerase (*Nf*PPI)** is a member of a superfamily of proteins
306 comprised of three structurally distinct main families: cyclophilins, FK506 binding proteins
307 (FKBPs), and parvulins. Based on structural and sequence alignment, the *N. fowleri* structure
308 falls in the FKBP family, a group of enzymes inhibited by compounds such as FK506 and
309 rapamycin (38). PPIs assist protein folding and influence protein denaturation kinetics by
310 catalyzing the cis/trans isomerization of peptide bonds preceding prolyl residues (39). The
311 enzymes participate in a diverse array of processes ranging from signal transduction to gene
312 regulation and have been found to have close interaction with heat shock 90 proteins (40). PPI
313 inhibitors are an emerging class of drugs for many therapeutic areas including infectious diseases
314 and many potent small molecule inhibitors have been derived for each of the members of the
315 superfamily. However, selective inhibitor design has been difficult due to the shallow, broad,
316 solvent-exposed active sites and their conservation between homologs and protein families (41).

317



318 **Figure 5. Interactions between *NfPPI* and FK506.** FK506 (yellow-carbon stick model) sits inside a mostly
319 hydrophobic binding pocket of *NfPPI* (white-carbon stick and surface model) consisting of Tyr38, Phe58, Trp71,
320 and Phe111 on the distal side of the substrate. Hydrogen-bonding interactions exist between FK506 and the side
321 chains of Asp49 and Tyr94, and the backbones of Glu66 and Ile68.

322

323 The interior of the binding pocket of *NfPPI* is mostly hydrophobic (**Figure 5**). Only four
324 putative hydrogen-bonding interactions are observed between the enzyme and substrate. All
325 residues involved in polar interactions in the *NfPPI* are also present in the human homolog
326 *HsFKBP51* (PDB: 1KT0), but the regions occupied by two hydrophilic residues in
327 *HsFKBP51*(Ser118 and Lys121) are instead occupied by hydrophobic residues, (Ile and Leu,
328 respectively) (40). Another difference found in the conformation of this loop region is the
329 insertion of an additional residue after Gly95 of *NfPPI*. These changes in structure and sequence
330 may lead to selective inhibition and thus establish PPIs as a selective drug target for *Naegleria*.

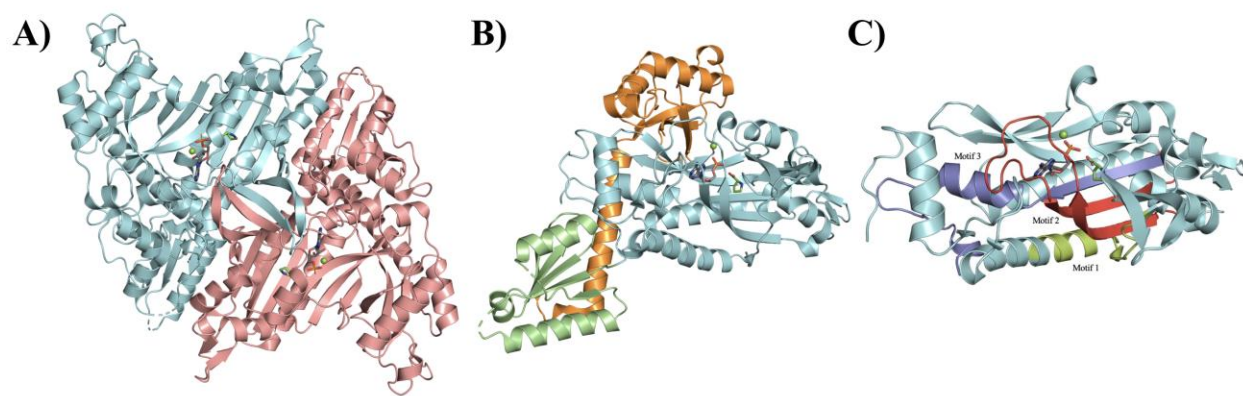
331

332 ***N. fowleri* Prolyl-tRNA synthetase (*NfProRS*).** Aminoacyl-tRNA synthetases (ARSs) are
333 globally essential enzymes among all living species. Their roles in protein translation and
334 biosynthesis have been heavily researched and understood as attractive therapeutic targets.
335 Recently, evidence of their propensity for adding new sequences or domains during ARS

336 evolution hints at broader functions and complexity outside of translation (42). Protein
337 translation as a drug target has been validated for anti-infective compounds for a wide array of
338 microbes (43). The natural product known as febrifugine, a quinazolinone alkaloid, and its
339 analogues have shown antiparasitic activity in targeting ProRS. Halofuginone, a halogenated
340 derivative of febrifugine, has shown promising potency though a lack of specificity, in that it
341 inhibits both the parasite and human ProRS (43).

342

343 The structure of *Nf*ProRS folds into a $\alpha 2$ homodimer (**Figure 6A**) with each subunit
344 containing three domains characteristic of Class II ARSs: the catalytic domain, the anticodon
345 binding domain, and the editing domain (**Figure 6B**). The *Nf*ProRS catalytic domain features the
346 three motifs which are exclusively conserved between class II ARSs for both sequence and
347 structure-function (**Figure 6C**). Motif 1 is located at the interface of the dimer and is
348 hypothesized to facilitate communication between the active sites of the two subunits (44). Motif
349 2 consists of β -strands connected by a variable loop which makes critical contacts with the
350 acceptor stem of tRNA^{Pro} and thus plays an important role in proper tRNA recognition (45).
351 Motif 3 is made up of entirely hydrophobic residues and comprises an integral part of the
352 aminoacylation active site.

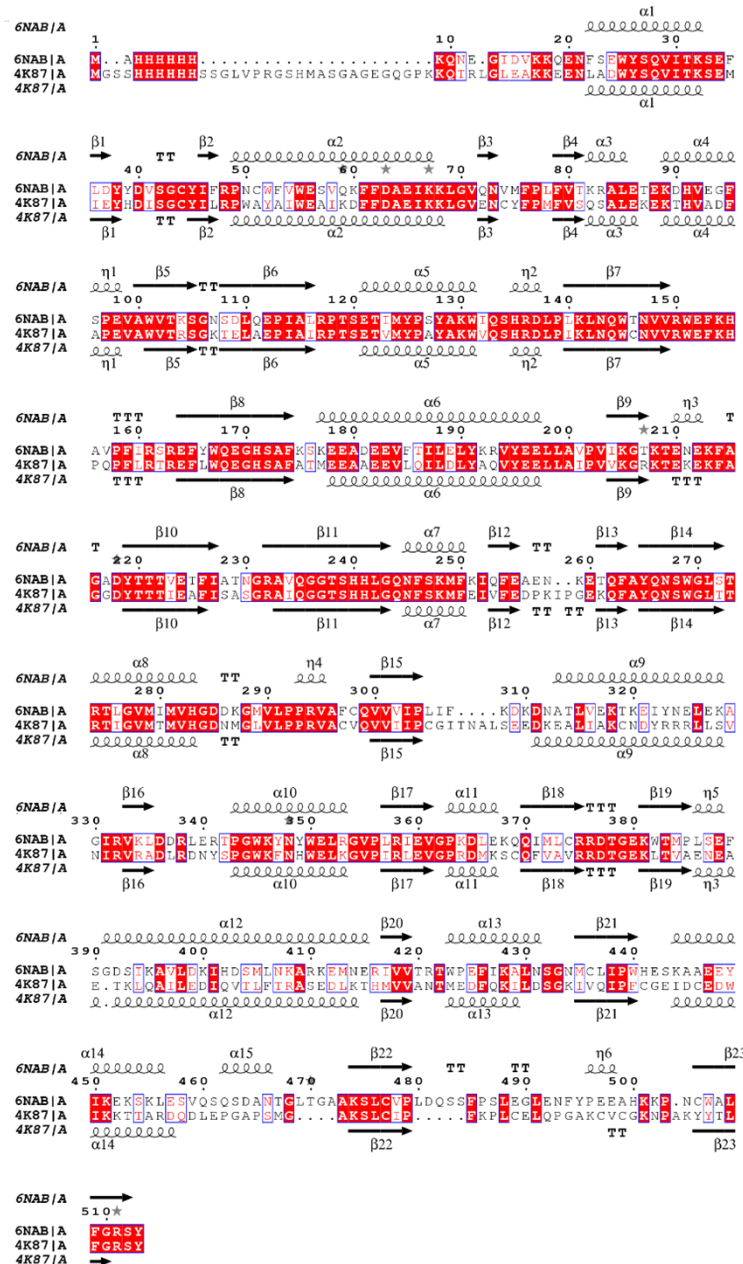


353

354 **Figure 6. Structure of the *Nf*ProRS.** (A) Both the biological and asymmetric unit of the structure are
355 homodimeric. Individual polypeptide chains are shown in cyan and salmon. AMP, proline, and magnesium ligand
356 molecules are also shown in yellow, purple, and green; respectively. (B) The three structure-function domains of
357 *Nf*ProRS. The catalytic, anticodon binding, and editing domains are colored cyan, green, and orange; respectively.
358 (C) The three highly conserved sequence motifs that characterize class II ARSs. Motif 1, colored lime, comprises
359 the dimer interface. Motif 2, colored red, forms part of the acceptor stem. Motif 3, colored blue, is involved in
360 forming the activated prolyl-adenylate.

361
362 Alignment of *Nf*ProRS bound to AMP and proline ligands (PDB: 6NAB) with apo *Hs*ProRS
363 (PDB: 4K87) exhibits no significant structural changes between the apo and ligand forms of the
364 ARS. The eukaryotic and archaeal origins of these ProRS make them suitable comparisons for
365 the reason mentioned earlier: their strict conservation in all three structural domains. Both the
366 proline and AMP bound *Nf*ProRS (PDB: 6NAB) and the halofuginone and AMP-PNP bound
367 *Nf*ProRS (PDB: 6UYH) structures have been solved. The proline and AMP *Nf*ProRS (6NAB)
368 shares structural homology with the halofuginone liganded ProRS (6UYH) and halofuginone
369 binding induces a conformational change of residues 80-87 of the *N. fowleri* enzyme. In the
370 proline bound 6NAB structure, residues 80-88 form a two-turn alpha helix ($\alpha 4$ in **Figure 7**).
371 However, the halofuginone compound displaces Phe87 and disrupts the short helical structure.
372 Residues making up this helix (EKDHVEGFS) are disordered in the 6UYH coordinate set. The
373 equivalent region of the human ProRS (PDB: 4K87) is structurally homologous to the proline
374 bound *N. fowleri* in the absence of halofuginone binding. The equivalent helix in 4K87, residues
375 90-98 (EKTHVADFA), includes non-conservative amino acid substitution adjacent to the crucial
376 phenylalanine which must be displaced for halofuginone to bind the human enzyme, including
377 E85-G86 which are A-D in the human sequence. Exploiting differences in the mobility of this
378 non-conserved loop adjacent to the active site of *Nf*ProRS and *Hs*ProRS could enable selective

379 targeting. In addition, allosteric inhibitors that take advantage of sequence differences
 380 throughout the *Nf*ProRS might be found by screening, as was the case for *P. falciparum* ProRS
 381 (*Pf*ProRS) (46). Thus, ProRS may be a reasonable drug target for *N. fowleri* drug development.
 382



383
 384 **Figure 7. Alignment of *Nf*ProRS against its human homolog.** Red background denotes residue conservation
 385 between the two structures, black text with white background signifies differences and red text encompassed in a

386 blue box demonstrates differences in residues that are not categorized as significant and the residues belong to the
387 same grouping. Secondary structure annotations signaling helices and sheet are reflective of both structures
388 respectively. Other structures used in alignment: *H. sapiens* (PDB: 4K87_A).

389

390 **3. CONCLUSION**

391 This manuscript reports 19 new protein structures from *N. fowleri* that are potential targets
392 for structure-based drug discovery. Eighteen of 19 possess a >38% difference in AA alignment
393 in comparison to the human homologs, suggesting selective inhibitors may be found by
394 screening campaigns. In this paper we analyzed five of the *N. fowleri* enzymes that have ligands
395 that define the active sites and compared them to human homologs. Though all are somewhat
396 homologous at the active site, differences in four of the five *N. fowleri* enzymes analyzed support
397 the hypothesis that selective active site inhibitors could be developed as therapeutics.

398

399 There are therapeutic opportunities, as well for some of the other 14 unexamined proteins as
400 well. For example, the *Nf* serine tRNA synthetase (*Nf*SerRS) structure (PDB: 6BLJ). SerRS is
401 required for charging tRNAs with serine critical for protein synthesis and thus is an essential
402 gene. An insertion of four residues (391-395) adjacent to the substrate and tRNA binding sites
403 creates a pocket with differential sequence identity to *Hs*SerRS and provides a foothold for the
404 design of selective inhibitors blocking tRNA charging.

405

406 Even if selective active site inhibitors cannot be identified, high-throughput screening of
407 compound libraries can still reveal selective inhibitors, as was found for *Plasmodium falciparum*
408 ProRS compared with human *Hs*ProRS (46). In this case, two allosteric inhibitors were found to
409 bury themselves into a lobe of the *Pf*ProRS enzyme, distant from the active site, and inhibit the

410 activity of the *Pf*ProRS enzyme, but not *Hs*ProRS. Selective high throughput screening of a
411 eukaryotic enzyme including counter screening against the homologous human enzyme, can also
412 identify selective inhibitors as has been shown by us in the case of *Plasmodium* N-
413 myristoyltransferase (32). Focusing on essential genes and drug targets of other eukaryotes and
414 producing a pool of potential drug target structures, SSGCID has created a foundation on which
415 to build structure-based drug discovery. The relatively quick successful progress through the
416 pipeline has catalyzed a consortium of investigators interested in addressing *N. fowleri* drug
417 discovery.

418

419 **4. MATERIALS AND METHODS**

420 **4.1 Bioinformatics**

421 The complete genome and transcriptome is available on the EupathDB BRC website
422 (www.amoebadb.org) (2). The complete ORFs and annotated predicted proteome from
423 *Naegleria fowleri* strain ATCC30863 was downloaded from AmoebaDB release 24. Analysis of
424 the ORFs indicated that 39% were missing a start codon and 12% were missing a stop codon.
425 The sequence authors, the Wittwer group at the Spiez Laboratory, confirmed that the 40% of
426 transcripts without an AUG start codon were most likely due to the ORF finder they used, which
427 searches for the longest ORFs in the RNA assembly, but has no start codon finding function. To
428 address this issue, we applied a conservative strategy to select high quality sequences from the
429 draft genome. A sequence homology search using BlastP against DrugBank v.4.3 targets (4,212
430 sequences) (15) and potential drug targets in the SSGCID pipeline (9,783 sequences) was
431 performed. Sequences with at least 50% amino acid sequence identity over 70% coverage were
432 selected for further filtering. Manual inspection indicated that half the potential targets without a

433 start codon appeared to be significantly truncated when compared to the *Naegleria gruberi* and
434 other closely related Eukaryota orthologues. Therefore, additional filters were applied to remove
435 likely truncated sequences: (1) targets without a start or stop codon were discarded, (2)
436 remaining candidates were blasted against the *Naegleria gruberi* proteome and sequences with
437 over 10% length difference to their *Naegleria gruberi* orthologues were discarded, and (3)
438 shorter variants with 100% match to a longer ORF transcript were discarded. In the end, 178
439 ORFs with a start and stop codon were identified, nominated, and approved by the SSGCID
440 target selection board and NIAID to attempt structure determination.

441

442 **4.2 High-throughput Protein Expression and Purification**

443 All proteins discussed were PCR-amplified using cDNA as a template. RNA template of
444 *Naegleria fowleri* ATCC30215 was provided by Dr. Christopher Rice (University of Georgia,
445 Athens) through RNA extraction and cDNA synthesis using previously published methodology
446 in *Acanthamoeba* (47). PCR, cloning, screening, sequencing, expression screening, large-scale
447 expression and purification of proteins were performed as described in previous SSGCID
448 publications (17,48). All described constructs were cloned into a ligation-independent cloning
449 (LIC) pET-14b derived, N-terminal His tag expression vector, pBG1861. Targets were expressed
450 using chemically competent *E.coli* BL21(DE3)R3 Rosetta cells and grown in large-scale
451 quantities in an auto-induction media (49). All purifications were performed on an
452 ÄKTAexplorer (GE) using automated IMAC and SEC programs in adherence to prior
453 established procedures (17).

454

455 **4.3 Crystallization and Structure Determination**

456 Crystal trials, diffraction, and structure solution were performed as previously published
457 (16). Protein was diluted to 20 mg/mL and single crystals were obtained through vapor diffusion
458 in sitting drops directly. The screens and conditions that yielded the crystals are listed in
459 **Supplementary Table 1**. The screens that were used to find the crystallization conditions were
460 typically JCSG+ (Rigaku Reagents), MCSG1 (Microlytic/Anatrace), Morpheus (Molecular
461 Dimensions), in some cases supplemented by ProPlex (Molecular Dimensions) and JCSG Top96
462 (Rigaku Reagents). All data was integrated and scaled with *XDS* and *XSCALE* (50). Structures
463 were solved by molecular replacement with *MOLREP* (51-53), as implemented in *MoRDa*. The
464 structures were refined in iterative cycles of reciprocal space refinement in *Phenix* and real space
465 refinement in *Coot* (54,55). The quality of all structures was continuously checked using
466 *MolProbity* (56) as implemented in *Phenix*. Structural comparisons for analysis among
467 homologues was done using DALI Protein Structure Comparison Server.

468

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481 paper.
482

483 **6. REFERENCES**

- 484 1. Marciano-Cabral F, Cabral GA. The immune response to *Naegleria fowleri* amebae and
485 pathogenesis of infection. *FEMS Immunol Med Microbiol*. 2007 Nov;51(2):243–59.
- 486 2. Grace E, Asbill S, Virga K. *Naegleria fowleri*: pathogenesis, diagnosis, and treatment
487 options. *Antimicrob Agents Chemother*. 2015 Nov;59(11):6677–81.
- 488 3. Yoder JS, Eddy BA, Visvesvara GS, Capewell L, Beach MJ. The epidemiology of primary
489 amoebic meningoencephalitis in the USA, 1962–2008. *Epidemiol Infect*. 2010
490 Jul;138(07):968–75.
- 491 4. Visvesvara GS, Moura H, Schuster FL. Pathogenic and opportunistic free-living amoebae:
492 *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri* and *Sappinia diploidea*.
493 *FEMS Immunol Med Microbiol*. 2007 Jun;50(1):1–26.
- 494 5. NIAID Emerging Infectious Diseases/ Pathogens | NIH: National Institute of Allergy and
495 Infectious Diseases [Internet]. [cited 2020 Oct 12]. Available from:
496 <http://www.niaid.nih.gov/research/emerging-infectious-diseases-pathogens>
- 497 6. Vugia DJ. Notes from the Field: Fatal *Naegleria fowleri* Meningoencephalitis After
498 Swimming in Hot Spring Water — California, 2018. *MMWR Morb Mortal Wkly Rep*.
499 2019;68.
- 500 7. Matanock A, Mehal JM, Liu L, Blau DM, Cope JR. Estimation of undiagnosed *Naegleria*
501 *fowleri* primary amoebic meningoencephalitis, United States1. *Emerg Infect Dis*. 2018
502 Jan;24(1):162–4.
- 503 8. El-Sayed A, Kamel M. Climatic changes and their role in emergence and re-emergence of
504 diseases. *Environ Sci Pollut Res*. 2020 Jun;27(18):22336–52.
- 505 9. Siddiqui R, Khan NA. Primary amoebic meningoencephalitis caused by *Naegleria fowleri*:
506 an old enemy presenting new challenges. *PLoS Negl Trop Dis*. 2014 Aug;8(8):e3017.
- 507 10. Chomba M, Mucheleng'anga LA, Fwoloshi S, Ngulube J, Mutengo MM. A case report:
508 primary amoebic meningoencephalitis in a young Zambian adult. *BMC Infect Dis*. 2017
509 Dec;17(1).
- 510 11. Debnath A, Nelson AT, Silva-Olivares A, Shibayama M, Siegel D, McKerrow JH. In Vitro
511 Efficacy of Ebselen and BAY 11-7082 Against *Naegleria fowleri*. *Front Microbiol*.
512 2018;9:414.
- 513 12. Colon BL, Rice CA, Guy RK, Kyle DE. Phenotypic screens reveal posaconazole as a
514 rapidly acting amoebicidal combination partner for treatment of primary amoebic
515 meningoencephalitis. *J Infect Dis*. 2019 Mar 15;219(7):1095–103.
- 516 13. Cope JR, Conrad DA, Cohen N, Cotilla M, DaSilva A, Jackson J, et al. Use of the novel
517 therapeutic agent miltefosine for the treatment of primary amoebic meningoencephalitis:
518 report of one fatal and one surviving case. *Clin Infect Dis Off Publ Infect Dis Soc Am*. 2016
519 Mar 15;62(6):774–6.

- 520 14. Bellini NK, Santos TM, da Silva MTA, Thiemann OH. The therapeutic strategies against
521 Naegleria fowleri. *Exp Parasitol.* 2018 Apr;187:1–11.
- 522 15. Law V, Knox C, Djoumbou Y, Jewison T, Guo AC, Liu Y, et al. DrugBank 4.0: shedding
523 new light on drug metabolism. *Nucleic Acids Res.* 2014 Jan;42:D1091-1097.
- 524 16. Myler PJ, Stacy R, Stewart L, Staker BL, Van Voorhis WC, Varani G, et al. The Seattle
525 Structural Genomics Center for Infectious Disease (SSGCID). *Infect Disord Drug Targets.*
526 2009 Nov;9(5):493–506.
- 527 17. Bryan CM, Bhandari J, Napuli AJ, Leibly DJ, Choi R, Kelley A, et al. High-throughput
528 protein production and purification at the Seattle Structural Genomics Center for Infectious
529 Disease. *Acta Crystallograph Sect F Struct Biol Cryst Commun.* 2011 Sep 1;67(Pt
530 9):1010–4.
- 531 18. Baugh L, Gallagher LA, Patrapuvich R, Clifton MC, Gardberg AS, Edwards TE, et al.
532 Combining functional and structural genomics to sample the essential Burkholderia
533 structome. *PloS One.* 2013;8(1):e53851.
- 534 19. Stacy R, Begley DW, Phan I, Staker BL, Van Voorhis WC, Varani G, et al. Structural
535 genomics of infectious disease drug targets: the SSGCID. *Acta Crystallograph Sect F*
536 *Struct Biol Cryst Commun.* 2011 Sep 1;67(Pt 9):979–84.
- 537 20. Milanes JE, Suryadi J, Abendroth J, Van Voorhis WC, Barrett KF, Dranow DM, et al.
538 Enzymatic and structural characterization of the Naegleria fowleri glucokinase. *Antimicrob*
539 *Agents Chemother.* 2019 Apr 25;63(5).
- 540 21. Tehlivets O, Malanovic N, Visram M, Pavkov-Keller T, Keller W. S-adenosyl-L-
541 homocysteine hydrolase and methylation disorders: yeast as a model system. *Biochim*
542 *Biophys Acta.* 2013 Jan;1832(1):204–15.
- 543 22. Cai S, Li Q-S, Borchardt RT, Kuczera K, Schowen RL. The antiviral drug ribavirin is a
544 selective inhibitor of S-adenosyl-L-homocysteine hydrolase from *Trypanosoma cruzi*.
545 *Bioorg Med Chem.* 2007 Dec 1;15(23):7281–7.
- 546 23. Czyrko J, Sliwiak J, Imiolczyk B, Gdaniec Z, Jaskolski M, Brzezinski K. Metal-cation
547 regulation of enzyme dynamics is a key factor influencing the activity of S-adenosyl-L-
548 homocysteine hydrolase from *Pseudomonas aeruginosa*. *Sci Rep.* 2018 Jul 27;8(1):11334.
- 549 24. Kusakabe Y, Ishihara M, Umeda T, Kuroda D, Nakanishi M, Kitade Y, et al. Structural
550 insights into the reaction mechanism of S-adenosyl-L-homocysteine hydrolase. *Sci Rep.*
551 2015 Dec;5(1).
- 552 25. Reddy MCM, Kuppan G, Shetty ND, Owen JL, Ioerger TR, Sacchettini JC. Crystal
553 structures of *Mycobacterium tuberculosis* S-adenosyl-L-homocysteine hydrolase in ternary
554 complex with substrate and inhibitors. *Protein Sci Publ Protein Soc.* 2008
555 Dec;17(12):2134–44.
- 556 26. Oost J, Huynen MA, Verhees CH. Molecular characterization of phosphoglycerate mutase
557 in archaea. *FEMS Microbiol Lett.* 2002 Jun;212(1):111–20.

- 558 27. Sainas S, Dosio F, Boschi D, Lolli ML. Targeting human onchocerciasis: recent advances
559 beyond ivermectin. *Annual Reports in Medicinal Chemistry*. Elsevier. 2018.1–38.
- 560 28. Krause CD, Yang Z-H, Kim Y-S, Lee J-H, Cook JR, Pestka S. Protein arginine
561 methyltransferases: Evolution and assessment of their pharmacological and therapeutic
562 potential. *Pharmacol Ther*. 2007 Jan;113(1):50–87.
- 563 29. Hu H, Qian K, Ho M-C, Zheng YG. Small molecule inhibitors of protein arginine
564 methyltransferases. *Expert Opin Investig Drugs*. 2016;25(3):335–58.
- 565 30. Mathioudaki K, Papadokostopoulou A, Scorilas A, Xynopoulos D, Agnanti N, Talieri M. The
566 PRMT1 gene expression pattern in colon cancer. *Br J Cancer*. 2008 Dec 9;99(12):2094–9.
- 567 31. Baldwin RM, Morettin A, Paris G, Goulet I, Côté J. Alternatively spliced protein arginine
568 methyltransferase 1 isoform PRMT1v2 promotes the survival and invasiveness of breast
569 cancer cells. *Cell Cycle*. 2012 Dec 15;11(24):4597–612.
- 570 32. Tewary SK, Zheng YG, Ho M-C. Protein arginine methyltransferases: insights into the
571 enzyme structure and mechanism at the atomic level. *Cell Mol Life Sci CMLS*. 2019
572 Aug;76(15):2917–32.
- 573 33. Mann SA, DeMart MK, May B, Causey CP, Knuckley B. Histone H4-based peptoids are
574 inhibitors of protein arginine methyltransferase 1 (PRMT1). *Biochem J*. 2020 Aug
575 28;477(16):2971–80.
- 576 34. Mann SA, Salsburg A, Causey CP, Knuckley B. The development and characterization of a
577 chemical probe targeting PRMT1 over PRMT5. *Bioorg Med Chem*. 2019 01;27(1):224–9.
- 578 35. Fulton MD, Brown T, Zheng YG. Mechanisms and Inhibitors of Histone Arginine
579 Methylation. *Chem Rec*. 2018 Dec;18(12):1792–807.
- 580 36. Kaniskan HÜ, Eram MS, Zhao K, Szewczyk MM, Yang X, Schmidt K, et al. Discovery of
581 potent and selective allosteric inhibitors of protein arginine methyltransferase 3 (PRMT3). *J*
582 *Med Chem*. 2018 Feb 8;61(3):1204–17.
- 583 37. Fedoriw A, Rajapurkar SR, O'Brien S, Gerhart SV, Mitchell LH, Adams ND, et al. Anti-
584 tumor Activity of the Type I PRMT Inhibitor, GSK3368715, Synergizes with PRMT5
585 Inhibition through MTAP Loss. *Cancer Cell*. 2019 08;36(1):100-114.e25.
- 586 38. Stocki P, Sawicki M, Mays CE, Hong SJ, Chapman DC, Westaway D, et al. Inhibition of
587 the FKBP family of peptidyl prolyl isomerases induces abortive translocation and
588 degradation of the cellular prion protein. *Mol Biol Cell*. 2016 Mar 1;27(5):757–67.
- 589 39. Ünal CM, Steinert M. Microbial peptidyl-prolyl cis/trans isomerases (PPIases): virulence
590 factors and potential alternative drug targets. *Microbiol Mol Biol Rev MMBR*. 2014
591 Sep;78(3):544–71.
- 592 40. Sinars CR, Cheung-Flynn J, Rimerman RA, Scammell JG, Smith DF, Clardy J. Structure of
593 the large FK506-binding protein FKBP51, an Hsp90-binding protein and a component of
594 steroid receptor complexes. *Proc Natl Acad Sci*. 2003 Feb 4;100(3):868–73.

- 595 41. Dnyak BM, Gestwicki JE. Peptidyl-Proline Isomerases (PPlases): Targets for natural
596 products and natural product-inspired compounds. *J Med Chem.* 2016 10;59(21):9622–44.
- 597 42. Lee E-Y, Kim S, Kim MH. Aminoacyl-tRNA synthetases, therapeutic targets for infectious
598 diseases. *Biochem Pharmacol.* 2018 Aug;154:424–34.
- 599 43. Pham JS, Dawson KL, Jackson KE, Lim EE, Pasaje CFA, Turner KEC, et al. Aminoacyl-
600 tRNA synthetases as drug targets in eukaryotic parasites. *Int J Parasitol Drugs Drug*
601 *Resist.* 2014 Apr;4(1):1–13.
- 602 44. Pang YLJ, Poruri K, Martinis SA. tRNA synthetase: tRNA aminoacylation and beyond.
603 *Wiley Interdiscip Rev RNA.* 2014 Aug;5(4):461–80.
- 604 45. Burke B, Yang F, Chen F, Stehlin C, Chan B, Musier-Forsyth K. Evolutionary coadaptation
605 of the motif 2--acceptor stem interaction in the class II prolyl-tRNA synthetase system.
606 *Biochemistry.* 2000 Dec 19;39(50):15540–7.
- 607 46. Hewitt SN, Dranow DM, Horst BG, Abendroth JA, Forte B, Hallyburton I, et al. Biochemical
608 and structural characterization of selective allosteric inhibitors of the *Plasmodium*
609 *falciparum* drug target, prolyl-tRNA-synthetase. *ACS Infect Dis.* 2017 Jan 13;3(1):34–44.
- 610 47. Rice CA, Campbell SJ, Bisson C, Owen HJ, Sedelnikova SE, Baker PJ, et al. Structural
611 and functional studies of histidine biosynthesis in *Acanthamoeba* spp. demonstrates a
612 novel molecular arrangement and target for antimicrobials. *PloS One.*
613 2018;13(7):e0198827.
- 614 48. Choi R, Kelley A, Leibly D, Nakazawa Hewitt S, Napuli A, Van Voorhis W. Immobilized
615 metal-affinity chromatography protein-recovery screening is predictive of crystallographic
616 structure success. *Acta Crystallogr Sect F Struct Biol Cryst Commun.* 2011 Sep
617 1;67(9):998–1005.
- 618 49. Studier FW. Protein production by auto-induction in high-density shaking cultures. *Protein*
619 *Expr Purif.* 2005 May;41(1):207–34.
- 620 50. Kabsch W. XDS. *Acta Crystallogr D Biol Crystallogr.* 2010 Feb;66(Pt 2):125–32.
- 621 51. Vagin A, Teplyakov A. Molecular replacement with MOLREP. *Acta Crystallogr D Biol*
622 *Crystallogr.* 2010 Jan;66(Pt 1):22–5.
- 623 52. Lebedev AA, Vagin AA, Murshudov GN. Model preparation in *MOLREP* and examples of
624 model improvement using X-ray data. *Acta Crystallogr D Biol Crystallogr.* 2008 Jan
625 1;64(1):33–9.
- 626 53. Murshudov GN, Skubák P, Lebedev AA, Pannu NS, Steiner RA, Nicholls RA, et al.
627 *REFMAC 5* for the refinement of macromolecular crystal structures. *Acta Crystallogr D Biol*
628 *Crystallogr.* 2011 Apr 1;67(4):355–67.
- 629 54. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. *Acta Crystallogr D*
630 *Biol Crystallogr.* 2004 Dec;60(Pt 12 Pt 1):2126–32.

- 631 55. Emsley P, Lohkamp B, Scott WG, Cowtan K. Features and development of Coot. *Acta*
632 *Crystallogr D Biol Crystallogr*. 2010 Apr;66(Pt 4):486–501.
- 633 56. Chen VB, Arendall WB, Headd JJ, Keedy DA, Immormino RM, Kapral GJ, et al.
634 MolProbity: all-atom structure validation for macromolecular crystallography. *Acta*
635 *Crystallogr D Biol Crystallogr*. 2010 Jan;66(Pt 1):12–21.
- 636