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## **A comprehensive model of purine uptake by the malaria parasite *Plasmodium falciparum*: identification of four purine transport activities in intraerythrocytic parasites**

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1 **A comprehensive model of purine uptake by the malaria**  
2 **parasite *Plasmodium falciparum*: identification of four**  
3 **purine transport activities in intraerythrocytic parasites.**

4

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7

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13

14 Running head left: QUASHIE et al

15 Running head right: PURINE TRANSPORT IN *PLASMODIUM*

16 Designation: RED CELLS

17

18 Running title: Purine transport in intraerythrocytic *Plasmodium*

19 **Synopsis**

20

21 *Plasmodium falciparum* is incapable of *de novo* purine biosynthesis, and is absolutely  
22 dependent on transporters to salvage purines from the environment. Only one low-  
23 affinity adenosine transporter has been characterised to date. Here we report a  
24 comprehensive study of purine nucleobase and nucleoside transport by  
25 intraerythrocytic *P. falciparum* parasites. Isolated trophozoites expressed (a) a high  
26 affinity hypoxanthine transporter with a secondary capacity for purine nucleosides, (b)  
27 a separate high affinity transporter for adenine (c) a low affinity adenosine transporter  
28 and (d) a low affinity/high capacity adenine carrier. Hypoxanthine was taken up with  
29 12-fold higher efficiency than adenosine. Using a parasite clone with a disrupted  
30 *PfNT1* gene we found that the high affinity hypoxanthine/nucleoside transport activity  
31 was completely abolished whereas the low affinity adenosine transport activity was  
32 unchanged. Adenine transport was increased, presumably to partly compensate for the  
33 loss of the high affinity hypoxanthine transporter. We thus propose a model for purine  
34 salvage in *P. falciparum*, based on the highly efficient uptake of hypoxanthine by  
35 PfNT1 and a high capacity for purine nucleoside uptake by a lower affinity carrier.

36

37 **Keywords:** Purine salvage; *Plasmodium falciparum*; nucleobase transporter;  
38 Equilibrative Nucleoside Transporter family; drug target; PfNT1.

39

40 Short title: purine transporters of *Plasmodium falciparum*.

41

42 **Abbreviations used:**

43 ENT, Equilibrative Nucleoside Transporter; hFNT1, human Facilitative Nucleoside  
44 Transporter; NPP, New Permeation Pathways; PfADET, *Plasmodium falciparum*  
45 Adenine Transporter; PfLAAT, *Plasmodium falciparum* Low Affinity Adenosine  
46 Transporter; PfNT1, *Plasmodium falciparum* Nucleoside Transporter 1; PV

46

Stage 2(a)

## 47 Introduction

48

49 *Plasmodium* spp. are auxotrophic for purines, as are all other parasitic protozoa  
50 studied to date, and require an efficient salvage system for these essential nutrients  
51 [1]. Purine salvage may be especially important during the fast dividing intra-  
52 erythrocytic stages, and it has long been known that *Plasmodium* trophozoites  
53 accumulate some purines very rapidly [1,2]. Tracy and Sherman [2] found that  
54 hypoxanthine, adenosine and inosine but not adenine or nucleotides were efficiently  
55 taken up by the avian malaria parasite *P. lophurae*. Similarly, the rodent malaria *P.*  
56 *berghei* was reported to have two separate uptake systems for purines: one for adenine  
57 and one for adenosine, inosine and hypoxanthine [3]. The study of purine metabolism  
58 in the human malaria species *P. falciparum*, however, showed clearly that  
59 hypoxanthine was by far the preferred purine source for the parasite, with very high  
60 activities of hypoxanthine-guanine phosphoribosyltransferase and inosine  
61 phosphorylase but not of adenine phosphoribosyltransferase or adenosine kinase [4].  
62 Adenosine salvage appeared to proceed through rapid deamination to inosine and  
63 thence to hypoxanthine [4], and this process mostly occurs extracellularly [5]. This  
64 explains the observation of Hansen et al. [3] that inhibition of adenosine deaminase  
65 with deoxycoformycin potently reduced [<sup>3</sup>H]-adenosine uptake by *P. berghei*. The  
66 resulting model of a crucial role for hypoxanthine transport in *Plasmodium* purine  
67 salvage was further validated by the study of Berman et al. [6] who showed that  
68 depletion of hypoxanthine from infected erythrocytes by xanthine oxidase reduced *P.*  
69 *falciparum* growth by ~90%.

70 While hypoxanthine transport clearly plays a pivotal role in an essential  
71 function of the malaria parasite, almost all available information on *Plasmodium*  
72 purine transporters concerns uptake of adenosine. Penny et al. [7] injected *Xenopus*  
73 oocytes with mRNA from *P. falciparum* and observed increased uptake of adenosine  
74 and hypoxanthine, sensitive to at least partial reciprocal inhibition, demonstrating the  
75 presence of at least one transporter capable of recognizing both substrates. Two  
76 groups have independently cloned the same *P. falciparum* gene of the Equilibrative  
77 Nucleoside Transporter family, which they termed *PfENT1* or *PfNT1*, respectively  
78 [8,9], and the PfNT1 protein was found to be localised in the parasite's plasma  
79 membrane [10]. Both groups expressed the cloned transporter in *Xenopus* oocytes for  
80 characterization, but they arrived at strikingly different conclusions. Carter et al. [9]  
81 described PfNT1 as a broad specificity nucleoside transporter of moderately high  
82 affinity for adenosine and inosine and no affinity for nucleobases. In contrast, Parker  
83 et al. [8] report a purine nucleoside/nucleobase transporter with a similar, but low,  
84 affinity for adenosine, adenine and hypoxanthine. The presence of a low affinity  
85 adenosine transporter was recently confirmed by Downie et al. [11] using *P.*  
86 *falciparum* trophozoites isolated after treatment with saponin. On the basis of the  
87 adenosine  $K_m$  value, they concluded that this activity must be PfNT1, and this seemed  
88 to be confirmed by expression of *PfNT1* in oocytes, which revealed a low affinity  
89 nucleoside transporter. However, in the current manuscript we will demonstrate  
90 conclusively that the low affinity adenosine transporter is not encoded by *PfNT1*.

91 Studies to date have not investigated the possibility of a very high affinity  
92 purine transporter, such as found in other intracellular [12-14] and extracellular  
93 [15,16] protozoan parasites. However, a recent report [17] did strongly suggest that  
94 *PfNT1* must encode a high affinity purine transporter, as wild-type *P. falciparum* were  
95 able to grow in very low levels of purines, but  $\Delta$ *PfNT1* parasites grew only in >10  $\mu$ M  
96 hypoxanthine, adenosine or inosine. The same study also showed that, at 1  $\mu$ M

97 concentrations, accumulation of those three purines was reduced in  $\Delta PfNT1$  but the  
98 characteristics of this transporter in terms of substrate affinity and specificity was not  
99 further investigated.

100 We report here the first systematic study of high affinity hypoxanthine,  
101 adenine and adenosine transport activities in *P. falciparum* trophozoites. We have  
102 identified and characterised a broad-specificity carrier that transports hypoxanthine,  
103 guanine, adenosine, guanosine and inosine, but not for adenine. In addition we  
104 characterised a low affinity adenosine transporter (which we denote PfLAAT) and a  
105 high affinity adenine transporter (denoted PfADET1) and demonstrate the existence  
106 of a further low affinity uptake route for adenine (PfADET2). To confirm our  
107 hypothesis that the high affinity transport activity is encoded by the previously  
108 reported *PfNT1*, we generated a mutant parasite clone with a disrupted *PfNT1* gene,  
109 designated  $\Delta Pfnt1^{GU}$  to distinguish itself from the clone generated by El Bissati et al.  
110 [17].  $\Delta Pfnt1^{GU}$  had completely lost both the high affinity adenosine uptake capacity  
111 and over 85% of hypoxanthine uptake, while low affinity adenosine uptake was  
112 unchanged and high affinity adenine uptake was slightly increased. We therefore  
113 conclude that *PfNT1* encodes a high affinity hypoxanthine/purine nucleoside  
114 transporter.

115 We present for the first time a comprehensive model of purine uptake by *P.*  
116 *falciparum*, in which the intraerythrocytic stages express at least four distinct purine  
117 transport activities. This model finally identifies the biochemical and genetic basis for  
118 the parasite's overwhelming reliance on hypoxanthine salvage as its main purine  
119 source and is in line with a recent analysis of the *P. falciparum* permeome that  
120 showed the presence of four genes of the Equilibrative Nucleoside Transporter (ENT)  
121 family in its genome [18]. This family includes all protozoan purine transporters  
122 known to date [1].

## 123 **Experimental**

124

### 125 ***Plasmodium* lines, human cells and chemicals.**

126 All transport experiments were performed with the standard 3D7 drug sensitive  
127 laboratory clone of *Plasmodium falciparum*, originally obtained from David Walliker,  
128 University of Edinburgh, UK. Human blood and serum used for *Plasmodium* culture  
129 was obtained from the Glasgow and West of Scotland Blood Transfusion Service.  
130 Standard chemicals including purines and pyrimidines were obtained from Sigma and  
131 of the highest purity available. The purine analogues JA-23 (2-Amino-N<sup>6</sup>-amino-N<sup>6</sup>-  
132 methyladenosine), JA-24 (2-Amino-N<sup>6</sup>-amino-adenosine) and JA-32 (N<sup>6</sup>-Hydroxy-  
133 9H-purin-6-amine) were kindly donated by Daniel Brown and David Loakes of the  
134 MRC Laboratory of Molecular Biology, Cambridge, UK.

135

### 136 **Culturing of *P. falciparum* for transport assays**

137 Asexual parasites of *P. falciparum* were maintained in continuous culture using  
138 slightly modified standard methods [19]. Briefly, parasites were grown in RPMI 1640  
139 medium supplemented with 5.94 g/l HEPES, 0.21% NaHCO<sub>3</sub> and 10% heat-  
140 inactivated normal human serum at 5% haematocrit. The culture was incubated at 37  
141 °C under a gas mixture of 1% O<sub>2</sub>, 3% CO<sub>2</sub>, 96% N<sub>2</sub> and medium was changed daily.  
142 Parasitaemia of the culture was routinely maintained below 5%, unless otherwise  
143 stated. Prior to use in experiments, parasite cultures were synchronised using a  
144 previously described method [20]. Transport experiments were performed with  
145 cultures of parasitaemia between 7 and 10% in order to maximise yield.

146

### 147 **Saponin permeabilization of *P. falciparum*-infected erythrocytes**

148 Permeabilised *P. falciparum*-infected erythrocytes were prepared by incubating  
149 parasitised red blood cells with 0.15% (w/v) saponin as described [21]. Briefly, the  
150 culture was centrifuged at 600 × g and the supernatant removed. The cell pellets were  
151 re-suspended in 5 volumes of 0.15% (w/v) saponin in phosphate-buffered saline (137  
152 mM NaCl, 27 mM KCl, 1.76 mM K<sub>2</sub>HPO<sub>4</sub>, 8 mM Na<sub>2</sub>HPO<sub>4</sub>) for one minute. The  
153 freed parasites were washed three times with purine-free RPMI 1640 culture medium  
154 without serum, and resuspended in the same medium. Parasitaemia was determined by  
155 microscopical examination of thin blood smears stained with Giemsa's stain, and red  
156 cell density was estimated using a Neubauer counting chamber.

157

### 158 **Transport assays with saponin-permeabilised *Plasmodium falciparum*- 159 infected erythrocytes**

160 Transport assays were performed with synchronised cultures in the trophozoite stage.  
161 After permeabilisation of the erythrocytes with saponin [22], which leaves the parasite  
162 plasma membrane intact [23,24], the cells were resuspended in purine-free RPMI  
163 1640 at a concentration of 5×10<sup>8</sup> cells/ml. Uptake of [<sup>3</sup>H]-hypoxanthine (GE  
164 Healthcare, 31 Ci/mmol), [<sup>3</sup>H]-adenosine (GE Healthcare, 16.0 Ci/mmol), or [<sup>3</sup>H]-  
165 adenine (Perkin-Elmer, 32.2 Ci/mmol) by *P. falciparum* trophozoites was measured  
166 using a rapid stop/spin method essentially as described previously for *Trypanosoma*  
167 *brucei* [25], *Leishmania* spp [14,26], *Toxoplasma gondii* [13] and human erythrocytes  
168 [27]. Transport was measured either at various time intervals to generate plots of  
169 uptake rate versus time, or over fixed time intervals in the presence of variable  
170 inhibitor concentrations to generate plots for the determination of K<sub>m</sub> and K<sub>i</sub> values,  
171 using non-linear regression (GraphPad Prism version 4). Briefly, equal volumes of a  
172 suspension of permeabilised-infected erythrocytes and a radiolabeled permeant at

173 twice its final concentration (as well as inhibitor at 2× concentration, where  
174 applicable) were mixed for a predetermined time. Influx of permeant into the cells  
175 was terminated by adding 1 ml ice-cold stop solution (unlabelled permeant at  
176 saturating concentrations) and immediately pelleting the cells by centrifugation at  
177  $13,000 \times g$  in a microfuge through an oil-mix (300µl of 5 parts dibutylphthalate  
178 (Aldrich):4 parts dioctylphthalate (Aldrich), v/v), thus preventing further uptake of  
179 permeant, which remains in the aqueous layer. Non-mediated influx of the respective  
180 permeant was assessed by determining the rate of uptake of the radiolabelled  
181 permeant in the presence of a saturating concentration of unlabelled permeant at both  
182 room temperature and 0 °C.

183 The parasite pellets were processed after the uptake using the method  
184 previously described by Saliba et al [28], with a slight modification. The aqueous  
185 phase was removed by aspiration and the inside walls of the tube carefully washed  
186 with distilled water. The water was aspirated out, followed by the oil-mix, and the  
187 inside of the tube wiped with folded tissue paper. The cells were resuspended in 200  
188 µl of 1% Triton X-100 in water (v/v) for 10 minutes at room temperature and proteins  
189 were precipitated with 200 µl of 5% trichloroacetic acid (TCA). The mixture was  
190 finally centrifuged for 7 minutes at  $13000 \times g$  and the supernatant transferred into a  
191 scintillation vial and mixed with 3.5 ml scintillation fluid (OptiPhase HiSafe, Perkin  
192 Elmer). Radioactivity was determined with a Perkin-Elmer 1450 Microbeta Wallac  
193 Trilux liquid scintillation counter. All uptake experiments were performed in  
194 triplicate.

195 For any given permeant, the linear phase of uptake was first determined, with  
196 a constant permeant concentration incubated for various times to generate a plot of  
197 uptake versus time. Linearity was assessed using linear regression and defined as a  
198 correlation coefficient  $>0.95$  and significant difference from zero uptake (F-test;  
199 GraphPad Prism version 4). When uptake was too rapid to obtain linearity of transport  
200 at room temperature, this was reassessed at 6 °C using a ThermoStat plus (Eppendorf,  
201 Germany), as indicated in Results. Inhibition studies were always performed well  
202 within the linear phase of uptake, and thus reflect true initial rates of transport across  
203 the *P. falciparum* plasma membrane rather than rates of metabolism or sequestration.

204

### 205 **Generation of *Plasmodium falciparum* clones with disrupted PfNT1**

206 Our strategy for knocking-out the *Plasmodium* gene *PfNT1* relies on single crossover  
207 homologous recombination and full details of the methods are provided in the on-line  
208 data supplement. Briefly, a plasmid was constructed containing the central sequence  
209 of *PfNT1* spanning the predicted transmembrane domains 3-6 and part of  
210 transmembrane domain 2. The construct was used to transfect *P. falciparum* parasites  
211 of clone 3D7 using standard methods [29], with control parasites treated similarly but  
212 with no construct present during electroporation. Transformed parasites were selected  
213 with blasticidin and cloned. Successful disruption of the gene was confirmed using  
214 PCR and Southern blot as described in the data supplement.

## 215 Results and Discussion

216

### 217 High affinity hypoxanthine transport in *P. falciparum* trophozoites

218 In order to study the transporters in the plasma membrane of *P. falciparum*, infected  
219 human erythrocytes were permeabilised with saponin, after which the erythrocyte  
220 plasma membrane ceases to be a barrier to the passage of solutes [22] but the parasite  
221 membranes are not significantly affected by this saponin treatment protocol [23,24].  
222 The parasitophorous vacuole membrane remains but this membrane is intrinsically  
223 permeable to small solutes [30]. Therefore, initial rates of transport measured in this  
224 system reflect uptake by the *Plasmodium*-encoded transporters located in the  
225 parasite's plasma membrane.

226 Uptake of 0.25  $\mu\text{M}$  or 0.1  $\mu\text{M}$  [ $^3\text{H}$ ]-hypoxanthine followed a hyperbolic  
227 curve, which was linear for up to 120 s at 20 °C, with a rate of  $0.62 \pm 0.04$  and  $0.40 \pm$   
228  $0.03 \text{ pmol}(10^7 \text{ cells})^{-1}\text{s}^{-1}$ , respectively, and was completely inhibited by 1 mM  
229 hypoxanthine (Figure 1A). The hypoxanthine thus accumulated would, in the absence  
230 of metabolism, reach very high intracellular concentrations: based on an intracellular  
231 volume of  $2.8 \times 10^{-7} \text{ l}/10^7$  parasites isolated by saponin treatment [28] uptake of 0.25  
232  $\mu\text{M}$  hypoxanthine would be over 1000-fold concentrated in the parasite – reaching a  
233 level of 260  $\mu\text{M}$  in 2 minutes. Transport rates remained constant during this time (i.e.  
234 linear phase of uptake) because the rate of metabolism was greater than the rate of  
235 transport, making transport the rate-limiting step. Alternatively, high affinity purine  
236 transport in *P. falciparum* may be energy-dependent, as it is in other protozoan  
237 species [31,16,25], allowing uptake against a concentration gradient – but this  
238 possibility was not further investigated in this study.

239 Determining [ $^3\text{H}$ ]-hypoxanthine transport at sub-micromolar concentrations  
240 revealed a very high affinity transporter for oxopurines, with a mean  $K_m$  value for  
241 hypoxanthine of  $0.34 \pm 0.05 \mu\text{M}$  ( $n = 6$ ; Figure 1B, inset), a  $V_{\text{max}}$  of  $0.36 \pm 0.12$   
242  $\text{pmol}(10^7 \text{ cells})^{-1}\text{s}^{-1}$  ( $n=6$ ) and a mean  $K_i$  value of  $0.11 \pm 0.01 \mu\text{M}$  for guanine ( $n=3$ ).  
243 In addition, the nucleosides adenosine (Figure 1B), inosine and guanosine displayed  
244 affinity in the low micromolar range (Table 1). This hypoxanthine transporter with  
245 high affinity for purine nucleosides was designated *Plasmodium falciparum*  
246 Nucleoside Transporter 1 (PfNT1) based on our later observations linking the *PfNT1*  
247 gene to this transport activity (see “Purine transport in the  $\Delta pfnt1^{\text{GU}}$  clone”).

248 PfNT1 exhibited low affinity for pyrimidines. Only uridine, uracil and  
249 thymidine inhibited transport of 30 nM [ $^3\text{H}$ ]-hypoxanthine significantly, at a  
250 concentration of 1 mM inhibitor, by  $36 \pm 9\%$  ( $P<0.05$ ),  $49 \pm 6\%$  ( $P<0.01$ ) and  $57 \pm$   
251  $6\%$  ( $P<0.02$ ) respectively (Paired Student's *t*-test against no-inhibitor control, based  
252 on four independent experiments in duplicate). Cytosine, thymine and cytidine (up to  
253 1 mM) did not significantly inhibit 30 nM [ $^3\text{H}$ ]-hypoxanthine transport (data not  
254 shown). This result is not surprising, in the light of the well-documented inability of  
255 *Plasmodium* spp to salvage preformed pyrimidine nucleobases and nucleosides [1,32].

256 Of all the natural purines tested, only adenine had very little effect on transport  
257 of 0.03  $\mu\text{M}$  [ $^3\text{H}$ ]-hypoxanthine, with less than 50% inhibition at 1 mM. However, the  
258 antiplasmodial adenine analogue JA-32 [33] displayed a  $K_i$  value of  $14 \pm 3 \mu\text{M}$  (Fig.  
259 1B), probably as a result of its ‘hypoxanthine-like’ conformation (i.e. lactam  
260 hydrogen on N1 and H-bond acceptor at the N<sup>6</sup> position, see figure 1B). Two N<sup>6</sup>  
261 substituted adenosine analogues, JA-23 and JA-24, with antiplasmodial activities in  
262 the low micromolar range [33], had little ( $K_i = 320 \pm 80 \mu\text{M}$  for JA-24) or no effect  
263 (JA23) on high affinity hypoxanthine transport (not shown).



264 The transporter appeared to display a preference for oxopurine nucleobases  
265 over aminopurine bases whereas this preference appears to be curiously absent in the  
266 binding of nucleosides: e.g. guanine is the highest affinity nucleobase whereas  
267 guanosine is the lowest affinity purine nucleoside (Table 1). Two testable hypotheses  
268 may be proposed to explain this apparent paradox: (i) the nucleosides are not actually  
269 substrates, but allosteric inhibitors of hypoxanthine transport, or (ii) the nucleosides  
270 and nucleobases assume a different orientation within the binding pocket, due to the  
271 presence of the ribose moiety. In order to distinguish between these alternatives, we  
272 assessed the uptake of low concentrations of [<sup>3</sup>H]-adenosine.

273

### 274 **High affinity adenosine transport in *P. falciparum* trophozoites**

275 Uptake of 0.25 μM [<sup>3</sup>H]-adenosine proceeded with a rate of 0.015 ± 0.002 pmol(10<sup>7</sup>  
276 cells)<sup>-1</sup>s<sup>-1</sup> over the linear phase (at least 30 s), and was completely inhibited by 1 mM  
277 adenosine (see figure S1 of the supplementary material). At the end of linear phase,  
278 cells had transported approx. 0.55 pmol [<sup>3</sup>H]-adenosine/10<sup>7</sup> cells, which would  
279 correspond to ~8-fold the extracellular concentration if unmetabolised. Both the rate  
280 of uptake and the level of concentration of adenosine were much less than for  
281 hypoxanthine.

282 Uptake of 0.25 μM [<sup>3</sup>H]-adenosine was potently inhibited by hypoxanthine  
283 (mean K<sub>i</sub> = 0.75 ± 0.18 μM; n=3) and unlabelled adenosine, but only by very high  
284 concentrations of adenine (Figure 2). Over three separate experiments, the average of  
285 the extrapolated values for an adenine IC<sub>50</sub> value would be ~1 mM – three orders of  
286 magnitude lower affinity than for hypoxanthine. In the same three experiments, the  
287 inhibition by hypoxanthine was not quite complete at 100 μM (6.1 ± 0.2%; P<0.05,  
288 paired Student's t-test), possibly the result of a minor contribution to 0.25 μM [<sup>3</sup>H]-  
289 adenosine flux from a second, low affinity adenosine transporter, that is insensitive to  
290 hypoxanthine (see “Low affinity transport of [<sup>3</sup>H]-adenosine in *P. falciparum*  
291 trophozoites”). The mean K<sub>m</sub> for adenosine on the high affinity transporter was  
292 determined to be 2.0 ± 0.2 μM and the V<sub>max</sub> as 0.18 ± 0.08 pmol(10<sup>7</sup> cells)<sup>-1</sup>s<sup>-1</sup> (n=3;  
293 Figure 2, inset). The strikingly similar reciprocal K<sub>m</sub> and K<sub>i</sub> values for adenosine and  
294 hypoxanthine uptake are consistent with high affinity uptake of both hypoxanthine  
295 and adenosine by PfNT1. However, the V<sub>max</sub>/K<sub>m</sub> ratio indicates an 11.9-fold higher  
296 efficiency of translocation for hypoxanthine compared to adenosine, consistent with  
297 the preference for hypoxanthine for optimal growth *in vitro*.

298 The different selectivity for aminopurine nucleobases and nucleosides noted in  
299 the previous section thus seems to indicate that nucleobases and nucleosides orient  
300 differently in the same binding site and the kinetic profiles presented here are  
301 consistent with competitive inhibition (Figures 1B and 2). There are precedents for  
302 this; for instance, there is good evidence for different binding orientations for xanthine  
303 and uric acid in the *Aspergillus nidulans* UapA purine transporter [34].

304

### 305 **Uptake of [<sup>3</sup>H]-adenine is mediated by a separate high affinity** 306 **transporter**

307 At 1 μM of [<sup>3</sup>H]-adenine, uptake was 0.0091 ± 0.0025 pmol(10<sup>7</sup> cells)<sup>-1</sup>s<sup>-1</sup> over the  
308 linear phase (6 s; r<sup>2</sup> = 0.93) and mostly inhibited by 1 mM unlabelled adenine (data  
309 not shown). At 120 s the accumulation ratio (intracellular/extracellular radiolabel  
310 concentration based on the intracellular volume of 2.8 × 10<sup>-7</sup> l/10<sup>7</sup> parasites cited  
311 above) had reached ~0.6, consistent with an equilibrative process and at best a slow  
312 rate of metabolism or no metabolism at all. We tentatively designate this novel

313 transport activity *Plasmodium falciparum* adenine transporter 1 (PfADET1), pending  
314 the identification of the encoding gene.

315 In order to determine the  $K_m$  value for this high affinity transporter, uptake of  
316 adenine was assessed at 50 nM of radiolabel. Uptake was linear and did not reach  
317 equilibrium for up to 240 s (accumulation ratio was 0.65) (Figure 3A). This allowed  
318 inhibition experiments at an incubation time of 150 s, although it was clear that there  
319 was a small uptake component that was not sensitive to 1 mM adenine (Figure 3A) –  
320 possibly resulting from the presence of a very low affinity transporter. Adenine  
321 transport was not inhibited by the oxopurines hypoxanthine (Figure 3B), guanine and  
322 inosine (not shown), though it was sensitive to adenosine, with a  $K_i$  value of  $2.0 \pm 0.2$   
323  $\mu\text{M}$  (Figure 3B). The sub-micromolar  $K_m$  value, similar to the hypoxanthine  $K_m$  for  
324 PfNT1, and very low  $V_{\text{max}}$  (Figure 3B), make PfADET1 a high affinity but low  
325 capacity transport system with a maximum uptake rate 2 – 3 orders of magnitude  
326 lower than that measured for high affinity transport of hypoxanthine and adenosine  
327 (Table 1).

328

### 329 **A very low affinity/high capacity transporter of adenine**

330 As the uptake of  $0.05 \mu\text{M}$  [ $^3\text{H}$ ]-adenine was consistently not fully inhibited by 1 mM  
331 unlabelled adenine (previous section) we investigated the possible presence of a low-  
332 affinity adenine transport activity in saponin-isolated *P. falciparum* trophozoites.  
333 Uptake of  $10 \mu\text{M}$  [ $^3\text{H}$ ]-adenine was linear for up to 12 s, with a rate of  $0.13 \pm 0.01$   
334  $\text{pmol}(10^7 \text{ cells})^{-1}\text{s}^{-1}$  ( $r^2 = 0.96$ ), reaching an accumulation ratio of 0.85 in 30 s. This  
335 could not be entirely attributed to simple diffusion as it was partly inhibited (~60%)  
336 by 1 mM adenine (Figure S2A, supplementary material). Attempts to establish a  $K_m$   
337 value for [ $^3\text{H}$ ]-adenine uptake on this transporter were unsuccessful – due in part to  
338 the high rate of diffusion of adenine at high concentrations, as reported for the related  
339 apicomplexan parasite *Toxoplasma gondii* [13], and because the limitations of adenine  
340 solubility prevented the determination of a complete inhibition curve (Figure S2B,  
341 supplementary material). However, the  $K_m$  for adenine was estimated to be  
342 approximately 1 mM, and the  $K_i$  value for adenosine  $>2.5 \text{ mM}$ . There was no clear  
343 inhibition by up to 1 mM hypoxanthine (data not shown). This observation, and the  
344 fact that these experiments were carried out at  $10 \mu\text{M}$  [ $^3\text{H}$ ]adenine – which fully  
345 saturates PfADET1 – as well as the low affinity for adenosine, clearly shows that this  
346 adenine transport phenomenon must be mediated by a low affinity transporter separate  
347 from either PfNT1 or PfADET1. The low affinity adenine transport activity was  
348 provisionally designated PfADET2.

349 Despite the presence of two adenine transporters, it is not at all clear whether  
350 adenine salvage is important to *Plasmodium* spp and there is considerable controversy  
351 about whether *Plasmodium* can utilise adenine at all, as several groups report the  
352 inability to identify genes encoding adenine phosphoribosyltransferase (APRT) and  
353 methylthioadenosine phosphorylase in any *Plasmodium* species [35,36] nor is there  
354 any evidence for the activity of adenine deaminase [35]. Yet, the characterisation of  
355 APRT from *P. chabaudi* and *P. falciparum* have been reported [37,38]. In addition, *P.*  
356 *falciparum* is reportedly able to grow in vitro on adenine as sole purine source [39]  
357 and *P. knowlesi* incorporated adenine into nucleic acids [32]. Although these latter  
358 observations could be explained by conversion of the adenine to hypoxanthine in the  
359 host cell, Van Dyke reported that the free parasite can also incorporate adenine into  
360 nucleic acids, albeit at 1% of the efficiency by which hypoxanthine is incorporated  
361 [40]. The report here of uptake of adenine by *P. falciparum* trophozoites, however  
362 inefficient, would be compatible with the view that adenine can be utilised in some

363 way but is unlikely to play any major role in the synthesis of nucleotides. It is  
364 conceivable that the parasite has a different use for low levels of adenine, which it  
365 cannot generate itself from other purines [35], or that adenine at high concentrations  
366 could be deaminated by adenosine deaminase.

367

### 368 **Low affinity transport of [<sup>3</sup>H]-adenosine in *P. falciparum* trophozoites**

369 Several groups have reported a much lower affinity adenosine transport process than  
370 the  $K_m$  value reported here for PfNT1 [8, 9, 11]. We therefore decided to reinvestigate  
371 the presence of such a transporter and found that 25  $\mu$ M [<sup>3</sup>H]-adenosine is extremely  
372 rapidly taken up at room temperature (22 °C) (see Figure S3A, supplementary data),  
373 as reported by Downie et al. [11], reaching an accumulation ratio of ~0.6 by 30 s. We  
374 therefore conducted our experiments at 6 °C and consistently found that uptake of 25  
375  $\mu$ M [<sup>3</sup>H]-adenosine was linear over a period of 4 – 6 s at this temperature (see Figure  
376 S3B, supplementary data), and that initial rates of transport could therefore be  
377 assessed over a three second interval. At 22 °C, 10 °C or 6 °C we found that transport  
378 was saturable by 1 mM unlabelled adenosine, although this never inhibited fully  
379 100% of transport (Figures 4 and S3), reflecting the low affinity of this transporter for  
380 the substrate. At 6 °C the rate of uptake of 25  $\mu$ M [<sup>3</sup>H]-adenosine was  $0.041 \pm 0.004$   
381 pmol ( $10^7$  cells)<sup>-1</sup>. The mean  $K_m$  value under these conditions was  $197 \pm 20$   $\mu$ M, with  
382 a  $V_{max}$  of  $0.19 \pm 0.03$  pmol( $10^7$  cells)<sup>-1</sup>s<sup>-1</sup> (n=3; Figure 4). This transporter was not  
383 sensitive to the nucleoside transport inhibitor dipyrindamole at concentrations up to 25  
384  $\mu$ M (data not shown). We have designated this activity the *P. falciparum* Low  
385 Affinity Adenosine Transporter (PfLAAT).

386 These observations are entirely consistent with the only other characterisation  
387 of adenosine transport in *P. falciparum* trophozoites, by Downie et al. [11]. They  
388 reported a low affinity adenosine transporter that mediated uptake of adenosine,  
389 inosine and thymidine very quickly, equilibrating within seconds, and insensitive to  
390 dipyrindamole.

391

### 392 **Disruption of the *PfNT1* gene.**

393 In order to gain insight into the possible role of PfNT1 in purine salvage, a parasite  
394 line with a disrupted locus was generated. Single crossover homologous  
395 recombination at the *PfNT1* locus was expected to generate a pseudodiploid  
396 configuration, with both truncated copies lacking some of the transmembrane domains  
397 of the protein predicted to be essential for the function of the protein (Supplementary  
398 data; Figure S4). As the parasite is haploid and *PfNT1* is a single copy gene, only one  
399 round of drug selection is necessary to obtain a null-mutant. Four parasite clones,  
400 denoted B11, D6, B7 and B9, were obtained, and successful disruption of the *PfNT1*  
401 gene was verified in each clone by PCR and by Southern blot (Figures S5 and S6,  
402 supplementary data). The mutant *PfNT1* clones were designated  $\Delta pfnt1^{GU}$ , in order to  
403 distinguish them from the independently derived  $\Delta pfnt1$  clone of El Bissati et al. [17].  
404

405

### 405 **Purine transport in the $\Delta pfnt1^{GU}$ clone.**

406 We next determined the purine uptake profile in  $\Delta pfnt1^{GU}$  clone D6, using as controls  
407 untransformed parasites (wild-type) that had undergone the same procedures. The  
408 transport assays were performed simultaneously on both clones, to assess which, if  
409 any, of the above identified purine transport activities was affected in the parasites  
410 with the disrupted gene. Uptake of 25  $\mu$ M [<sup>3</sup>H]-adenosine at 6 °C was not affected by  
411 the disruption of *PfNT1*, and identical rates of uptake were measured in the two lines:

412  $0.069 \pm 0.05$  and  $0.070 \pm 0.07$  pmol( $10^7$  cells) $^{-1}$ s $^{-1}$  for  $\Delta pfnt1^{GU}$  and control,  
413 respectively (Figure 5A).

414 In contrast, in several experiments uptake of  $0.25 \mu\text{M}$  [ $^3\text{H}$ ]-adenosine was  
415 reduced by 97 – 100% in  $\Delta pfnt1^{GU}$  ( $n = 3$ ; Figure 5B), clearly indicating the loss of  
416 high-affinity transport in the mutant line. Similarly, uptake of  $0.4 \mu\text{M}$  [ $^3\text{H}$ ]-  
417 hypoxanthine over 120 s was reduced by 82 – 98% ( $n = 3$ ; Figure 5C). The small  
418 residual hypoxanthine transport leads us to speculate that a low-affinity hypoxanthine  
419 activity might also be expressed in *P. falciparum*. This would be consistent with the  
420 observation of El Bissati et al. [17] that  $\Delta pfnt1$  *P. falciparum* are able to grow in the  
421 presence of high concentrations of hypoxanthine but not in low concentrations.

422 Transport rates of  $1 \mu\text{M}$  [ $^3\text{H}$ ]-adenine were not affected in the  $\Delta PfNT1$   
423 parasites: the initial rates of transport over 12 s were not significantly different in the  
424  $\Delta pfnt1^{GU}$  line and control cells ( $n = 3$ ; Paired t-test,  $P > 0.05$ ) (Figure 5D and inset).  
425 However, in three independent experiments the maximum level of [ $^3\text{H}$ ]-adenine  
426 transport in the  $\Delta pfnt1^{GU}$  line was double that of the control ( $0.16 \pm 0.06$  vs  $0.077 \pm$   
427  $0.022$  pmol( $10^7$  cells) $^{-1}$ s $^{-1}$ ;  $n = 3$ , non-linear regression). Rather than upregulation of the  
428 transporter we believe this may reflect an increased rate of adenine metabolism in the  
429 absence of a source for hypoxanthine.

430 The clear conclusion from these results is that *PfNT1* encodes the high affinity  
431 hypoxanthine/adenosine transporter. This conclusion is somewhat at odds with the  
432 reports of PfNT1 as a relatively low affinity transporter when expressed in *Xenopus*  
433 *laevis* oocytes [8,9,11]. However, these previous reports differ substantially from each  
434 other, producing adenosine  $K_m$  values of  $320 \mu\text{M}$ ,  $13.2 \mu\text{M}$  and  $1.86 \text{mM}$ ,  
435 respectively. It may be that the *Xenopus* expression system produces ambiguous  
436 results when expressing some *Plasmodium* transporters, possibly as a result of the  
437 extremely high A+T content of the *P. falciparum* genome [41]; *PfNT1* is 72% A+T.  
438 This would also explain the reported difficulties of expressing other *P. falciparum*  
439 ENT genes in this system [11]. Also, the Carter study used a different parasite clone  
440 than the Parker and Downie studies (W2 and 3D7, respectively), which had a single  
441 amino acid difference in their *PfNT1* gene (Leu or Phe, respectively, at position 385).  
442 This could perhaps explain some of the discrepancy, especially since the Phe385  
443 polymorphism is unusual: in a multiple alignment of 28 protozoan ENT family genes  
444 plus the four human ENT sequences, the equivalent amino acid residue was conserved  
445 in so far as it was never aromatic, amide, charged, cysteine or proline. However, it is  
446 unlikely that the 3D7 clone of *P. falciparum* encodes a partly deficient *PfNT1*, as this  
447 clone was also used in the study reported by El Bissati et al that first suggested that  
448 PfNT1 is essential for growth on low purine concentrations [17]. We also used the  
449 3D7 clone here and verified that its *PfNT1* sequence was 100% identical to the entry  
450 in PlasmoDB (PF13\_0252) and to the sequence reported by Parker et al. [8].

451

#### 452 **A new model for purine salvage by *P. falciparum*.**

453 Our results, establishing a central role for a high affinity hypoxanthine  
454 transporter in the purine salvage of *Plasmodium falciparum*, agree very well with the  
455 early studies of purine salvage in other *Plasmodium* species, which describe a  
456 transport system for hypoxanthine with secondary capacity for purine nucleosides and  
457 a separate uptake system for adenine [1]. It is also consistent with the current  
458 understanding that purine nucleotide synthesis in *Plasmodium* spp. is overwhelmingly  
459 through phosphoribosylation of nucleobases, particularly hypoxanthine, rather than  
460 nucleoside kinases [4,5,42] and they do not seem to encode an adenosine kinase in

461 their genome [35,36]. Furthermore, it brings purine uptake in *P. falciparum* very close  
462 to purine transport activities reported for other protozoa, which typically display  $K_m$   
463 values in the 0.2 – 5  $\mu\text{M}$  range for their substrates [1]. Earlier proposals that *P.*  
464 *falciparum* may rely exclusively on low affinity purine transporters seemed hard to  
465 reconcile with a free concentration of purine nucleosides and bases in the infected  
466 erythrocyte that is presumably at best in the low micromolar range. Both the hENT1  
467 nucleoside [43] and hFNT1 nucleobase [27] transporters of human erythrocytes are  
468 equilibrative, making it impossible for the free concentrations of substrates to exceed  
469 those in plasma. Nor is it likely that purine concentrations are much higher at the  
470 parasite plasma membrane, as the parasitophorous vacuole membrane is freely  
471 permeable to such low molecular weight solutes [30].

472 However, at adenosine concentrations that saturate the high affinity PfNT1  
473 transporter, a very rapid and saturable nucleoside transport activity, PflAAT, was  
474 observed. Our transport experiments with  $\Delta Pfnt1^{GU}$  prove that this is indeed a  
475 separate transporter from PfNT1. We conclude that *P. falciparum* trophozoites  
476 express both a high affinity purine nucleobase/nucleoside transporter and a low  
477 affinity/high capacity adenosine transporter in addition to a saturable transport system  
478 for [ $^3\text{H}$ ]-adenine. The  $K_m$  value of  $\sim 200$   $\mu\text{M}$  for PflAAT rules out the possibility that  
479 it might be identical to the high affinity adenine transporter PFADET1 ( $K_i(\text{adenosine})$   
480 = 2.0  $\mu\text{M}$ ) and equally appears to rule out that it is identical to the low affinity adenine  
481 transport activity PFADET2 that is almost insensitive to adenosine. However, this  
482 awaits formal proof, consisting of experiments with genetic deletion lines of the genes  
483 encoding these purine transporters – which are in progress.

484 Thus a model arises (depicted in Figure 6) that closely mirrors the model for  
485 purine transport in the only other apicomplexan parasite for which purine transport  
486 has been studied in detail. *Toxoplasma gondii* is known to express a low affinity  
487 adenosine transporter TgAT1, with a  $K_m$  value of  $\sim 110$   $\mu\text{M}$  [13,44,45], as well as a  
488 high affinity transporter TgAT2 ( $K_m$  is 0.49  $\mu\text{M}$  for adenosine and 0.77  $\mu\text{M}$  for  
489 inosine) [13]. The main difference between purine salvage in the two apicomplexan  
490 species is that, unlike *P. falciparum*, *T. gondii* expresses separate high affinity  
491 transporters for purine nucleosides (TgAT2) and oxopurine nucleobases (TgNBT1).  
492 Like PfNT1, TgNBT1 does not transport adenine [13].

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503

504

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660 **Figure 1. Hypoxanthine transport in saponin-isolated *Plasmodium falciparum***  
 661 **trophozoites.** (A) Uptake of 0.25  $\mu\text{M}$  [ $^3\text{H}$ ]-hypoxanthine (squares) and 0.10  $\mu\text{M}$  [ $^3\text{H}$ ]-  
 662 hypoxanthine (circles) into saponin-permeabilised *P. falciparum*-infected erythrocytes  
 663 in the presence (open symbols) or absence (filled symbols) of 1 mM unlabelled  
 664 hypoxanthine at 20 °C. The correlation coefficients ( $r^2$ ) were determined as 0.99 and  
 665 0.98 for 0.25  $\mu\text{M}$  and 0.1  $\mu\text{M}$  radiolabel, respectively, by linear regression over the  
 666 first 120 s. (B) Uptake of 30 nM [ $^3\text{H}$ ]-hypoxanthine in the presence or absence of  
 667 various concentrations of unlabelled hypoxanthine ( $\square$ ), adenosine ( $\bullet$ ) or JA32 ( $\Delta$ ).  
 668 Uptake rates are presented as percentage of no-inhibitor controls and combined from  
 669 two experiments with 100% values of 0.013 (adenosine and JA32) and 0.033  
 670  $\text{pmol}(10^7 \text{ cells})^{-1}\text{s}^{-1}$  (hypoxanthine), respectively. Inset: conversion of hypoxanthine  
 671 inhibition data to Michaelis-Menten plot for determination of  $K_m$  and  $V_{\text{max}}$ .  
 672 Representative experiments are shown, conducted in triplicate. Error bars indicate  
 673 SEM

674

675 **Figure 2. Uptake of low concentrations of [ $^3\text{H}$ ]-adenosine in isolated *Plasmodium***  
 676 ***falciparum* trophozoites.** Transport of 0.25  $\mu\text{M}$  [ $^3\text{H}$ ]-adenosine was measured over  
 677 30 s in the presence or absence of various concentrations of unlabelled adenosine ( $\square$ ),  
 678 adenine ( $\blacktriangle$ ) and hypoxanthine ( $\bullet$ ). Inset: conversion of the adenosine inhibition data  
 679 to a Michaelis-Menten plot. The adenine  $\text{IC}_{50}$  value was obtained by extrapolation to  
 680 100% inhibition. Representative experiments performed in triplicate are shown; error  
 681 bars represent SEM.

682

683 **Figure 3. Characterization of a high affinity adenine transporter in *P. falciparum***  
 684 **trophozoites.** (A) Transport of 0.05  $\mu\text{M}$  [ $^3\text{H}$ ]-adenine over 240 s, in the presence ( $\circ$ )  
 685 or absence ( $\blacksquare$ ) of 1 mM unlabelled adenine. In the absence of inhibitor the transport  
 686 rate was  $3.9 \pm 0.2 \times 10^{-5} \text{ pmol}(10^7 \text{ cells})^{-1}\text{s}^{-1}$  as calculated by linear regression ( $r^2 =$   
 687 0.98). At 240 s, uptake was inhibited 70% by 1 mM adenine. (B) Transport of 0.05  
 688  $\mu\text{M}$  [ $^3\text{H}$ ]-adenine was inhibited by increasing concentrations of unlabelled adenine ( $\circ$ )  
 689 or adenosine ( $\blacksquare$ ). Adenine uptake was expressed as % of control because two different  
 690 experiments were combined, with no-inhibitor values of  $2.4 \times 10^{-4}$  and  $1.5 \times 10^{-4}$   
 691  $\text{pmol}(10^7 \text{ cells})^{-1}\text{s}^{-1}$ . Inset: conversion of the inhibition data to a Michaelis-Menten  
 692 plot, yielding a  $K_m$  value of 0.12  $\mu\text{M}$  for this experiment. Units of uptake were  
 693  $\text{pmol}(10^7 \text{ cells})^{-1}\text{s}^{-1}$ . Error bars represent SEM.

694

695 **Figure 4. Low affinity transport of adenosine in saponin-isolated *Plasmodium***  
 696 ***falciparum* trophozoites.** Transport of 25  $\mu\text{M}$  [ $^3\text{H}$ ]-adenosine was determined over a  
 697 three second interval at 6 °C, in the presence of various concentrations of unlabelled  
 698 adenosine as indicated. Inset: conversion to a Michaelis-Menten plot. Error bars are  
 699 SEM; where not shown fall within the symbol.

700

701 **Figure 5. Purine transport in *P. falciparum* trophozoites lacking *PfNT1*.** Transport  
 702 in  $\Delta\text{PfNT1}^{\text{GU}}$  is indicated with open squares and in control 3D7 parasites with filled  
 703 circles. (A) 25  $\mu\text{M}$  [ $^3\text{H}$ ]-adenosine at 6 °C. (B) 0.25 [ $^3\text{H}$ ]-adenosine at 22 °C. (C) 0.4  
 704  $\mu\text{M}$  [ $^3\text{H}$ ]-hypoxanthine at 22 °C. (D). 1  $\mu\text{M}$  [ $^3\text{H}$ ]-adenine at 22 °C. Insets display the  
 705 linear phase of uptake ( $r^2 = 0.99$  for  $\Delta\text{PfNT1}^{\text{GU}}$  and 0.98 for control 3D7).

706

707 **Figure 6. Model of purine uptake into intraerythrocytic *P. falciparum***  
 708 **trophozoites.** Double-headed arrows indicate presumed equilibrative transport,

709 whereas the single headed arrow of PfNT1 indicates possible active transport.  
710 However, the assignment of active or equilibrative transport is speculative and was  
711 not investigated in the current study. hFNT1 and hENT1 are endogenous to the human  
712 erythrocyte whereas the origin of the NPP is unknown. The thickness of arrows for  
713 the *P. falciparum* transporters is meant to convey the relative flux of purines. NPP,  
714 New Permeation Pathways; PV, Parasitophorous vacuole; IEC, infected erythrocyte  
715 cytoplasm; Pf, *Plasmodium falciparum*.

716 **Table 1. Profile of purine transport in *P. falciparum* trophozoites.**  
717

|                                  | High affinity [ <sup>3</sup> H]-hypoxanthine uptake (PfNT1) |   | High affinity [ <sup>3</sup> H]-adenosine uptake (PfNT1)    |   | Low affinity [ <sup>3</sup> H]-adenosine uptake (PfLAAT)   |   | High affinity [ <sup>3</sup> H]-adenine uptake (PfAET1)     |   |
|----------------------------------|---|---|---|---|--|---|---|---|
|                                  | K <sub>i</sub> or K <sub>m</sub> value (at 22 °C; μM, ±SEM) | n | K <sub>i</sub> or K <sub>m</sub> value (at 22 °C; μM, ±SEM) | n | K <sub>i</sub> or K <sub>m</sub> value (at 6 °C; μM, ±SEM) | n | K <sub>i</sub> or K <sub>m</sub> value (at 22 °C; μM, ±SEM) | n |
| V <sub>max</sub> <sup>(a)</sup>  | 0.36 ± 0.12   | 6 | 0.18 ± 0.08   | 3 | 0.19 ± 0.03  | 3 | 0.0004 ± 0.0002   | 3 |
| V <sub>max</sub> /K <sub>m</sub> | 1.1   |   | 0.090   |   | 0.00096  |   | 0.0019  |   |
| Hypoxanthine                     | <b>0.34 ± 0.05</b>  | 6 | 0.75 ± 0.18   | 3 | ND   |   | >1000   | 3 |
| Guanine                          | 0.11 ± 0.01   | 3 | ND  |   | ND   |   | >50   | 3 |
| Inosine                          | 2.0 ± 0.2   | 3 | ND  |   | ND   |   | >1000   | 3 |
| Adenosine                        | 4.0 ± 0.67  | 3 | <b>2.0 ± 0.2</b>  | 3 | <b>197 ± 20</b>  | 3 | 2.0 ± 0.2   | 3 |
| Guanosine                        | 11.6 ± 2.7  | 3 | ND  |   | ND   |   | ND  |   |
| Adenine                          | >500  | 3 | 240 ± 70  | 3 | ND   |   | <b>0.23 ± 0.07</b>  | 3 |

718

719 Values in bold are K<sub>m</sub> values obtained with radiolabelled substrate; other values are K<sub>i</sub>  
720 values, obtained from dose-dependent inhibition of radiolabelled substrate. All values  
721 were determined using non-linear regression from experiments in duplicate or  
722 triplicate with a minimum of 8 points over the relevant range. Zero values were taken  
723 to be radiolabel associated with the cell pellet in the presence of saturating  
724 concentrations of unlabelled permeant. <sup>a</sup> units for V<sub>max</sub> are pmol(10<sup>7</sup> cells)<sup>-1</sup>s<sup>-1</sup>. ND,  
725 not determined.

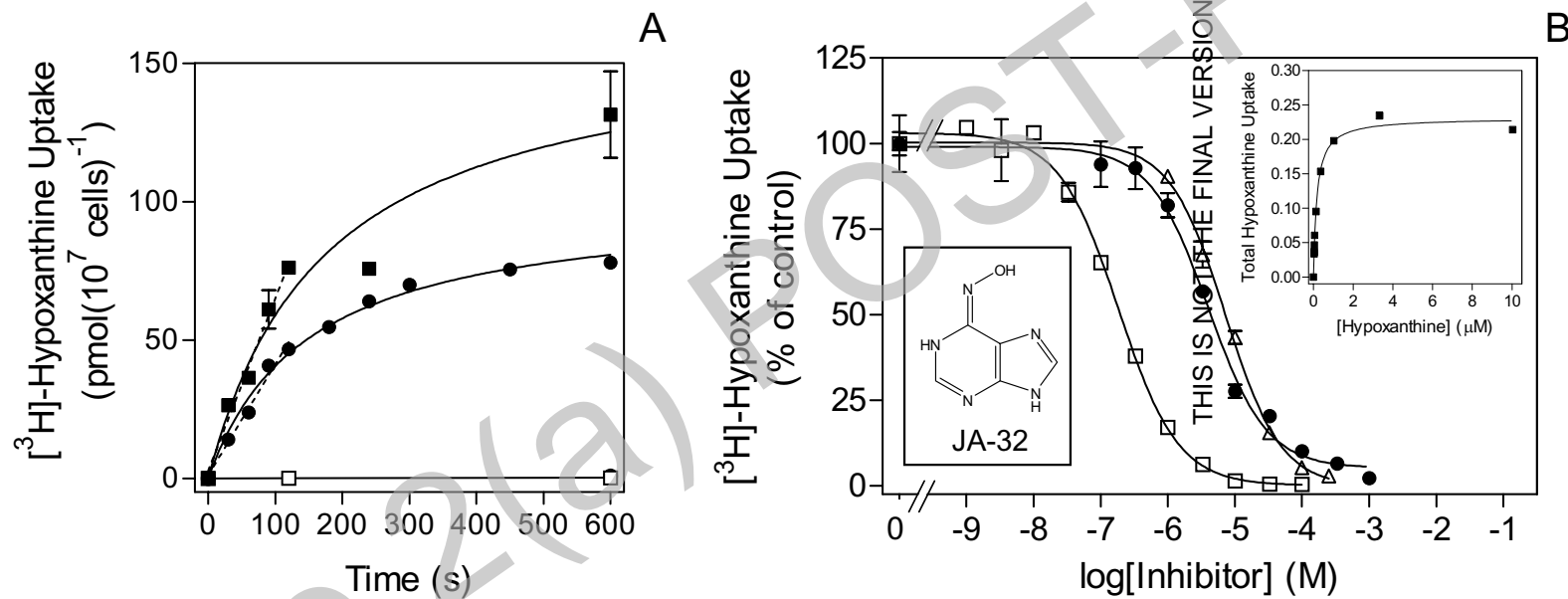
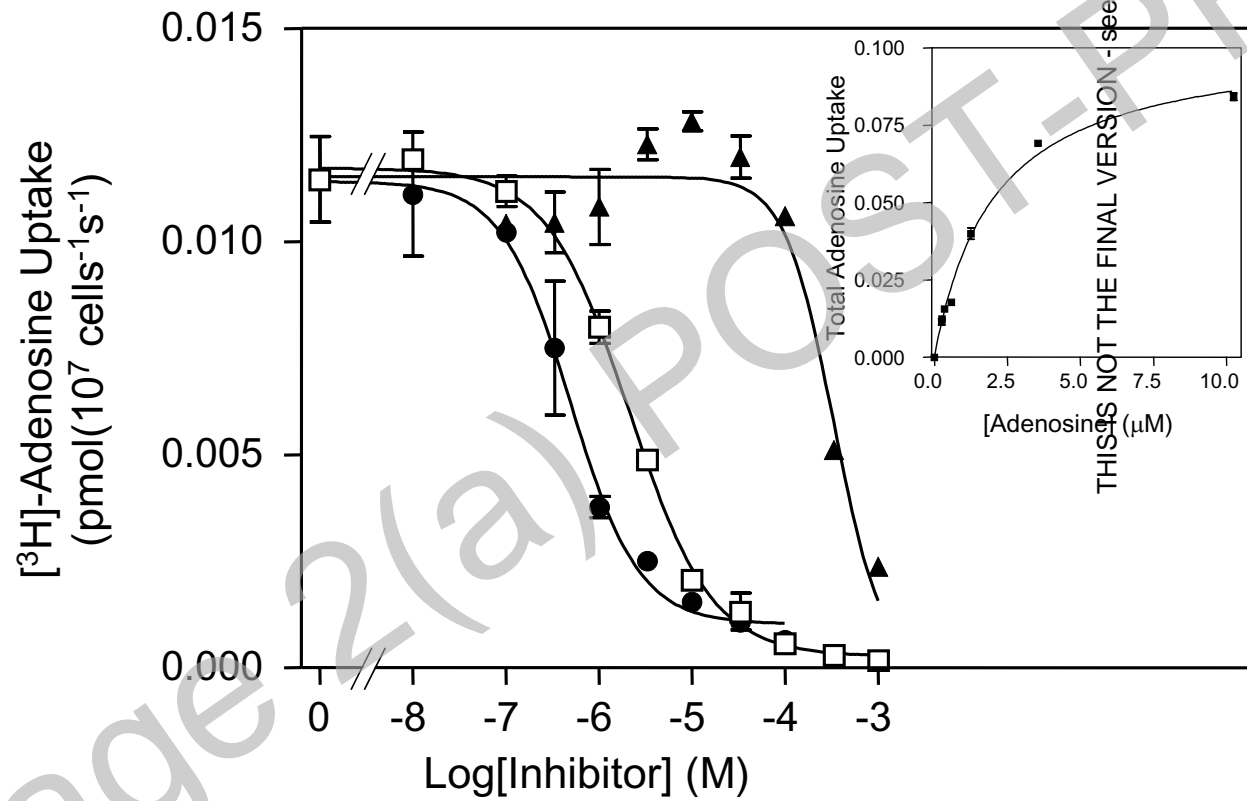


Fig. 1

Fig. 2



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Stage 2 (a) POST-PRINT

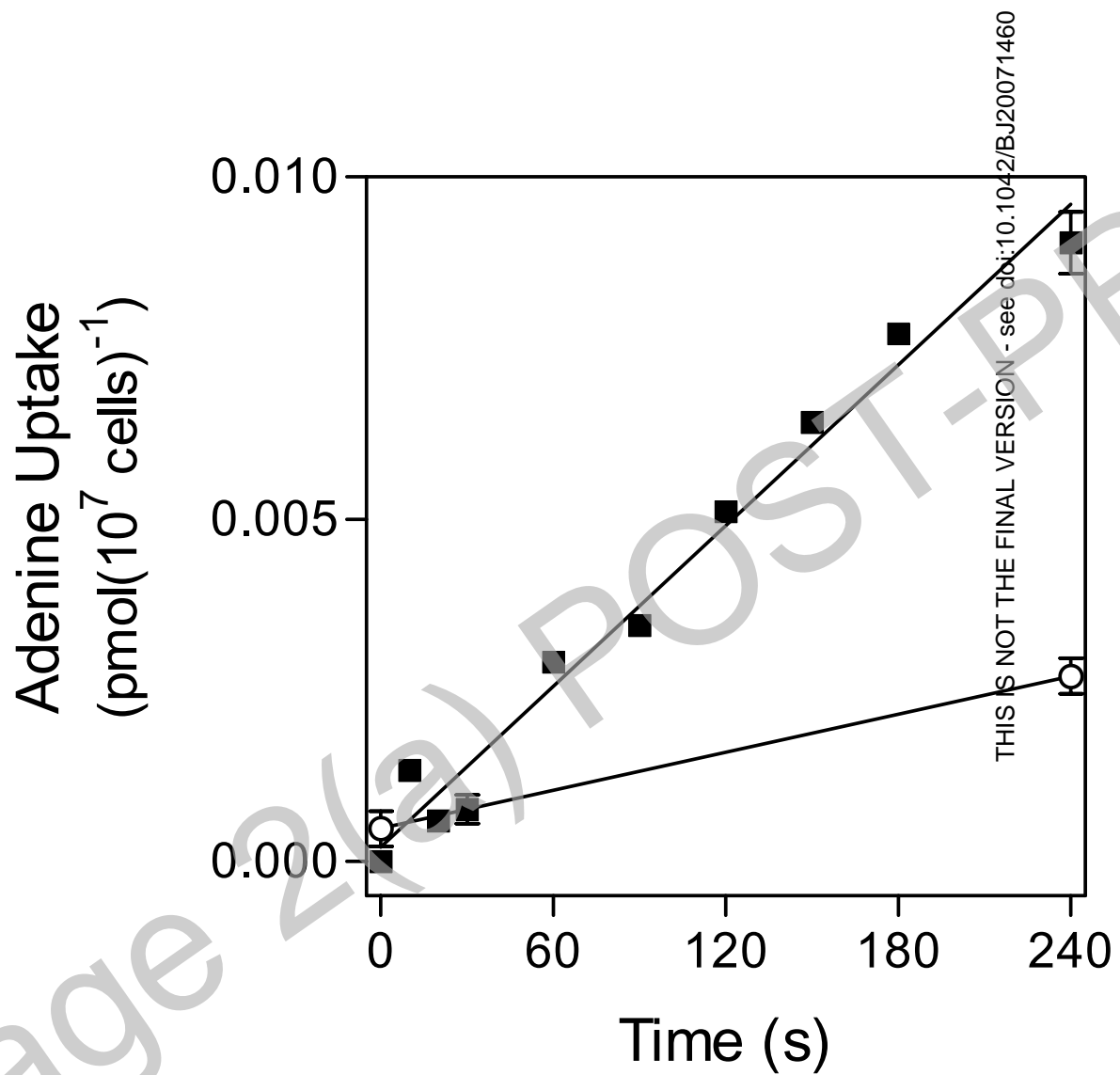


Fig. 3A



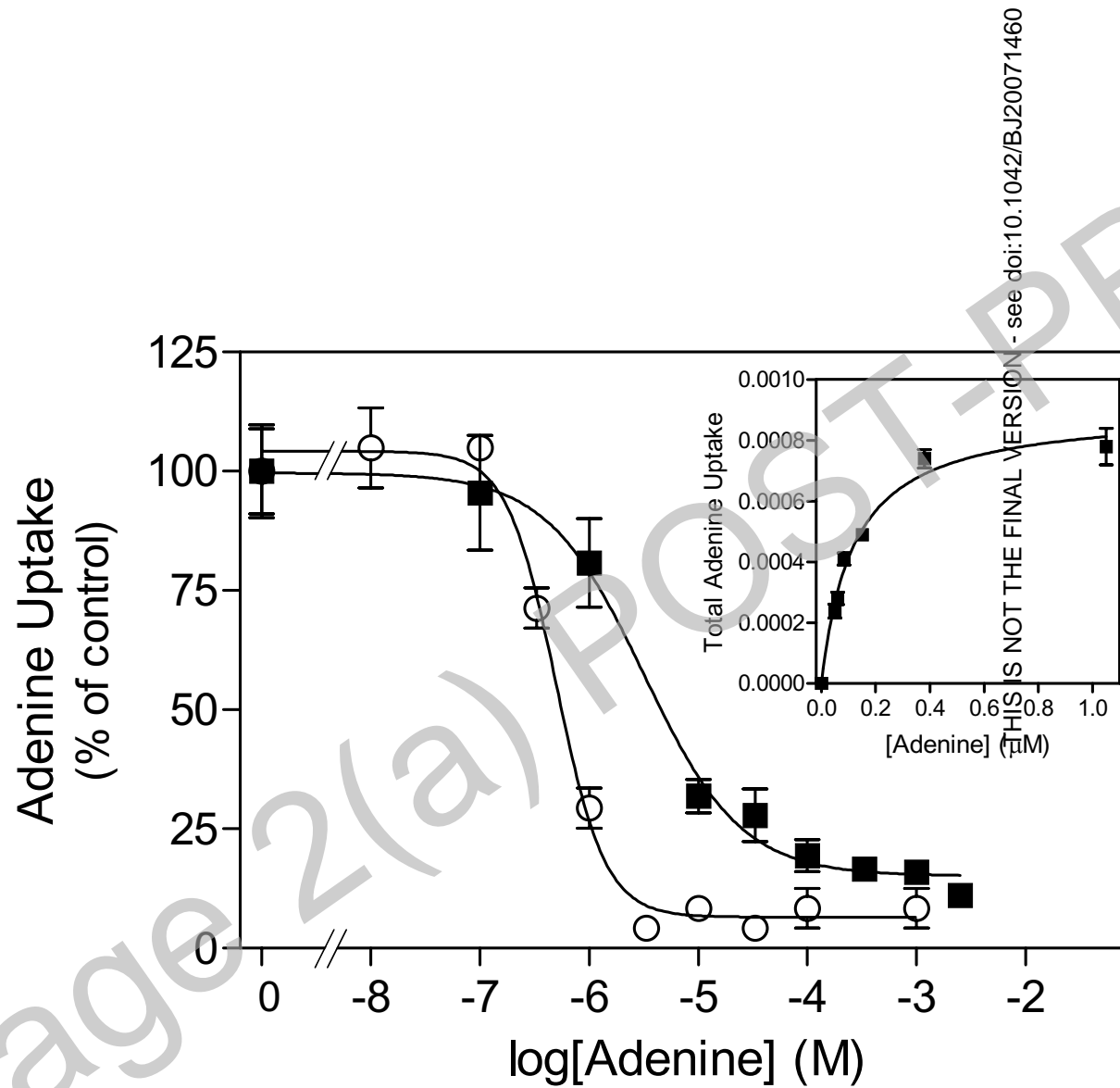


Fig. 3B

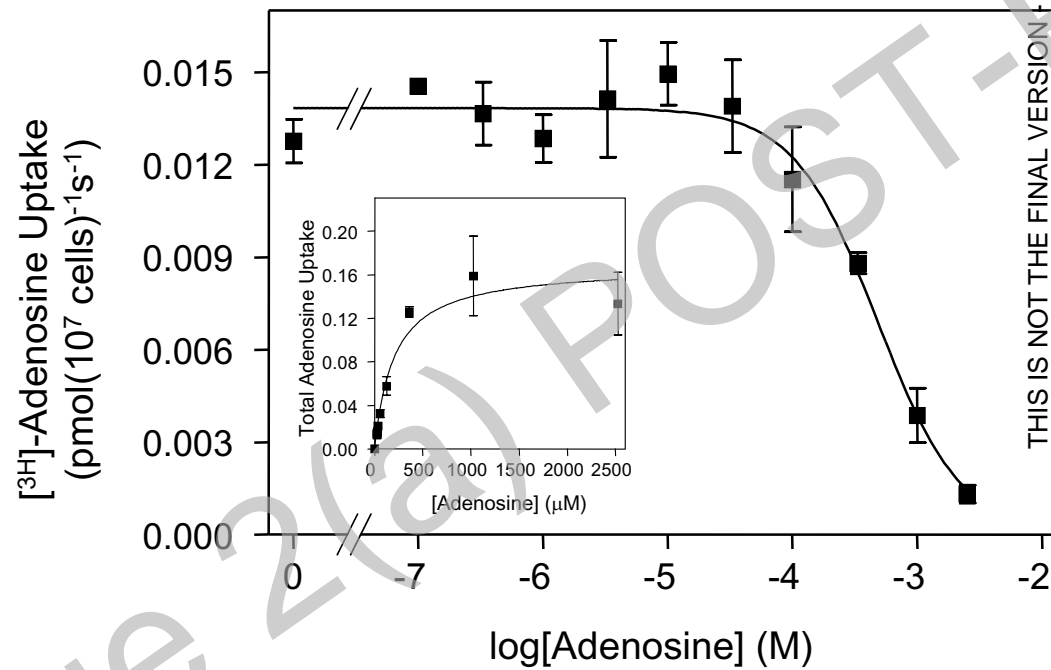
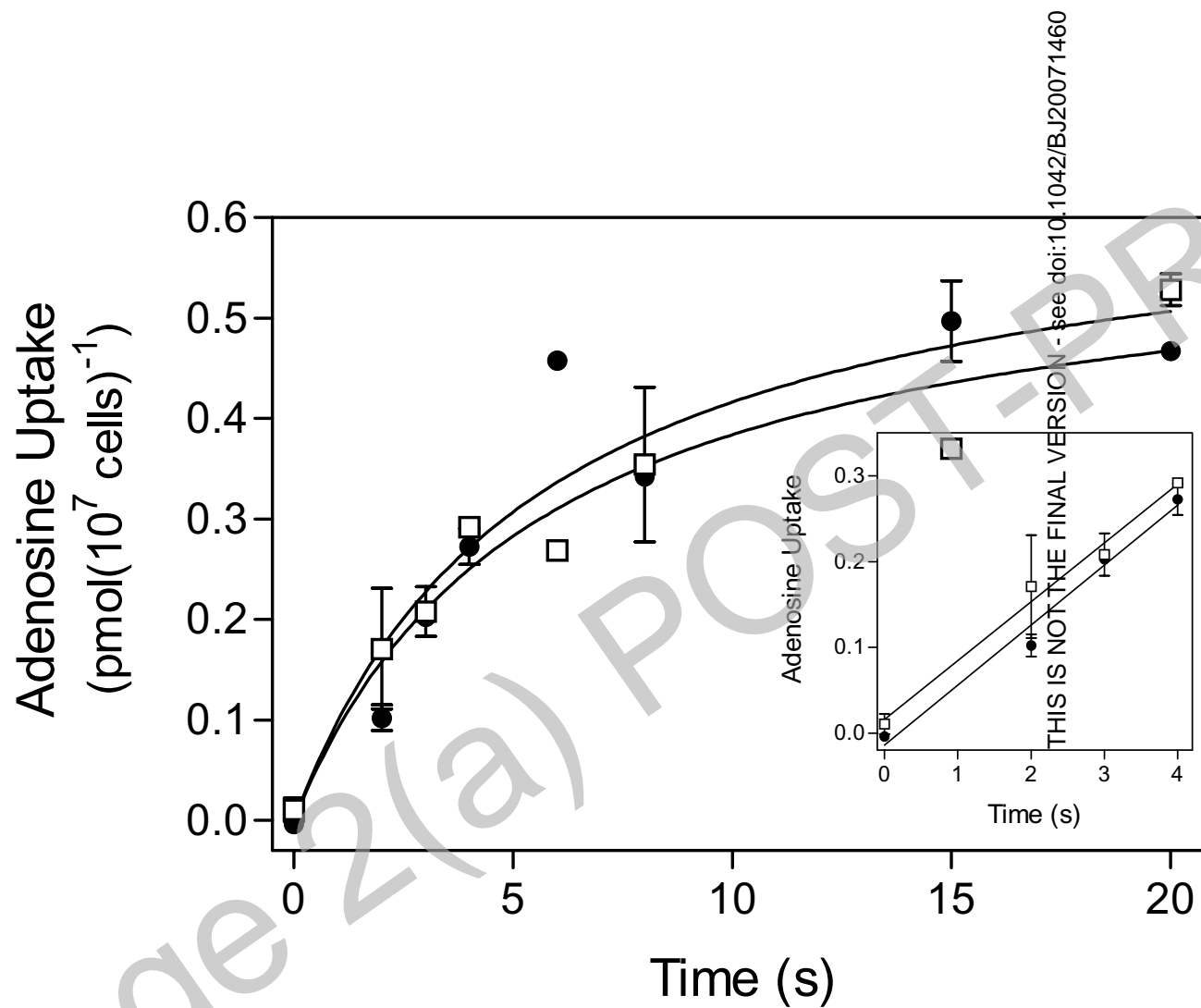


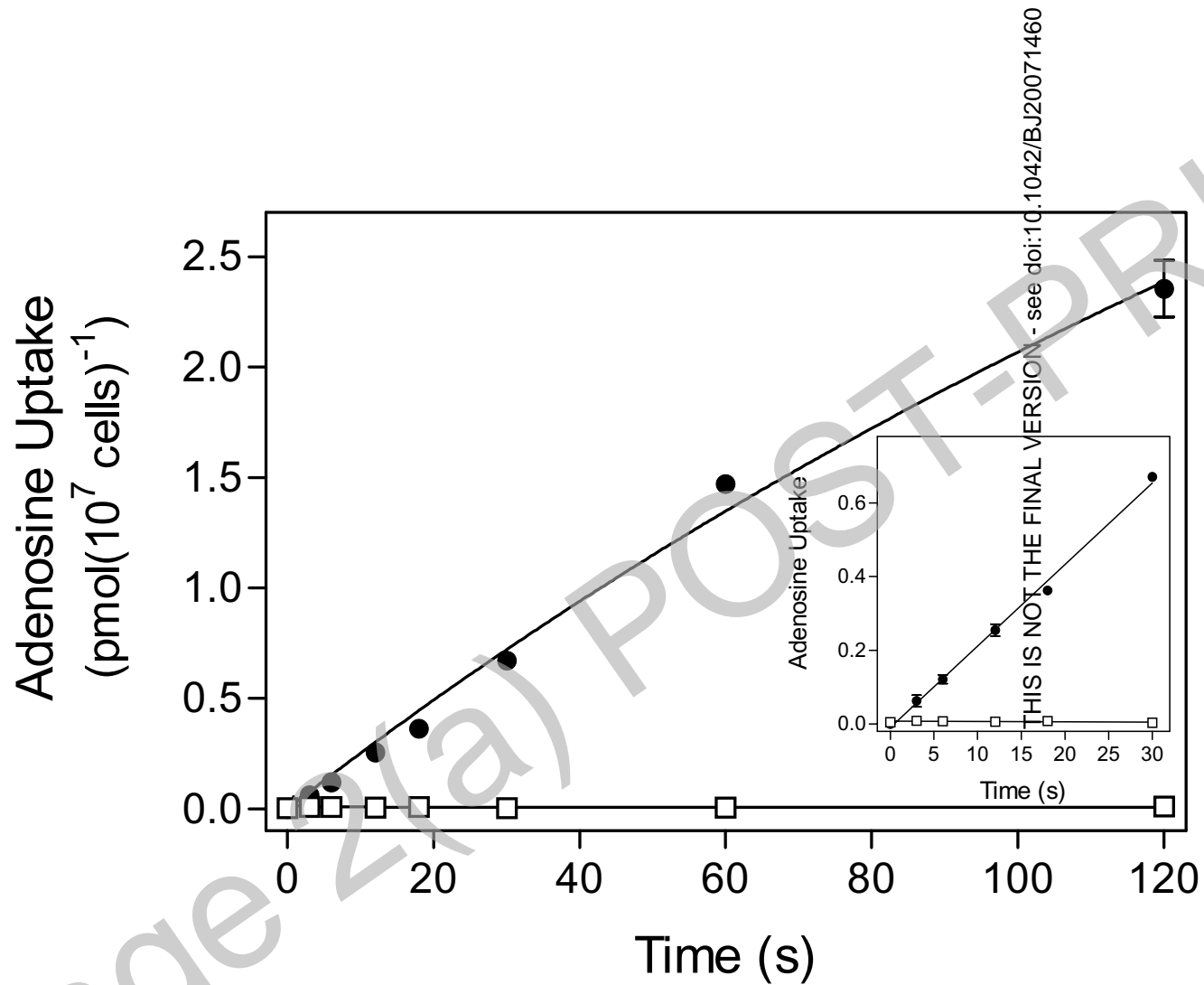
Fig. 4

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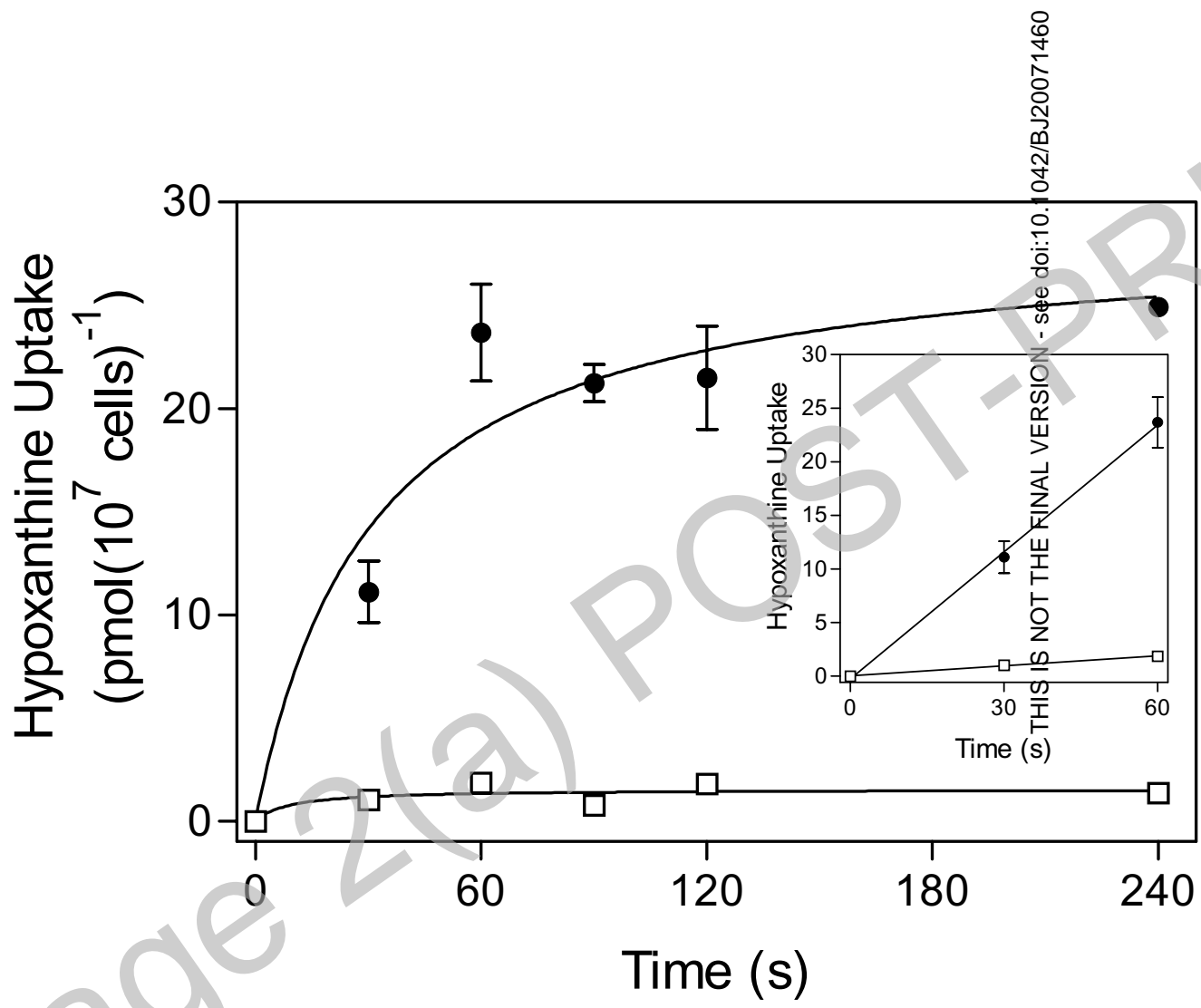
Stage 2



5A

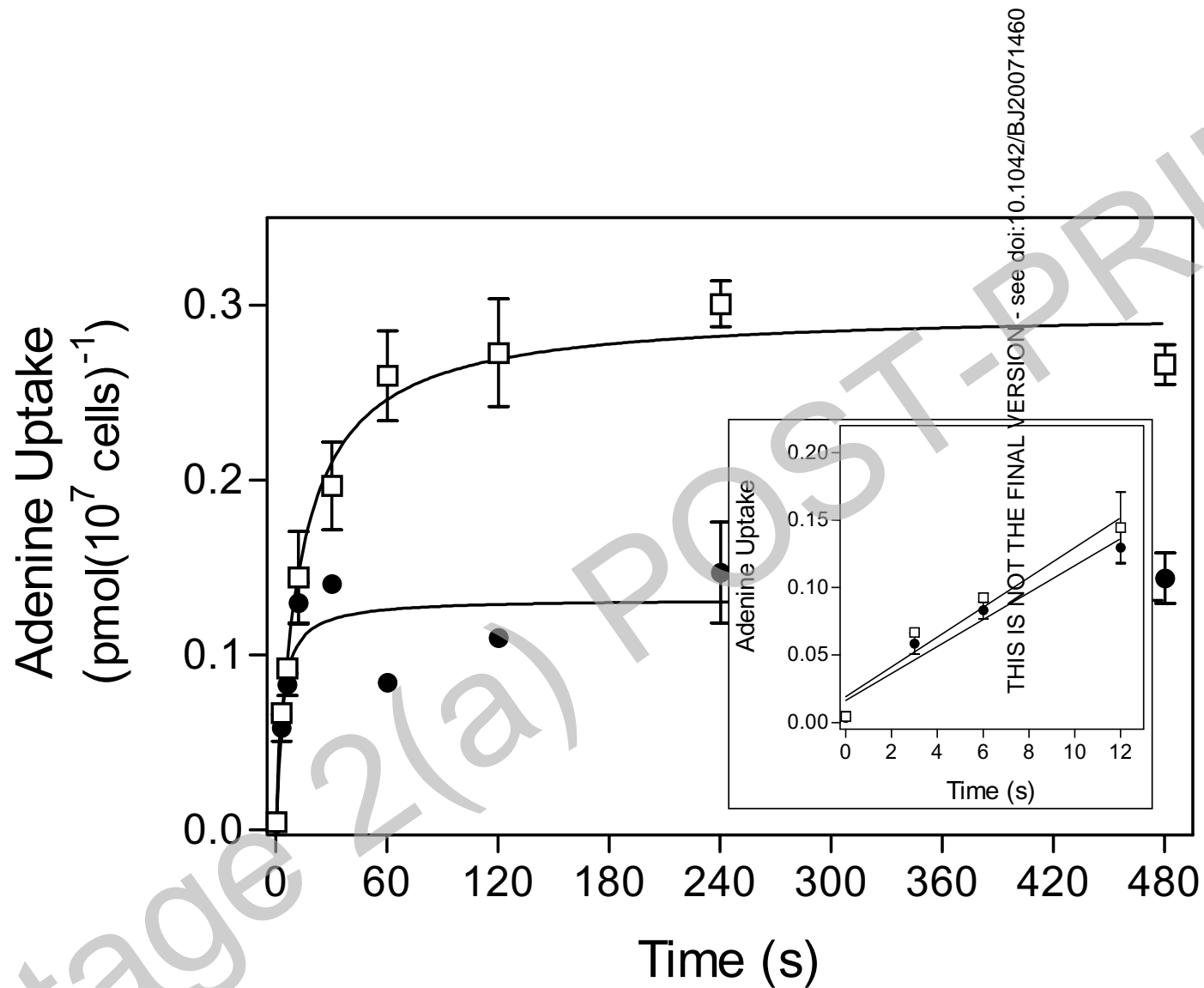


5B



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5C



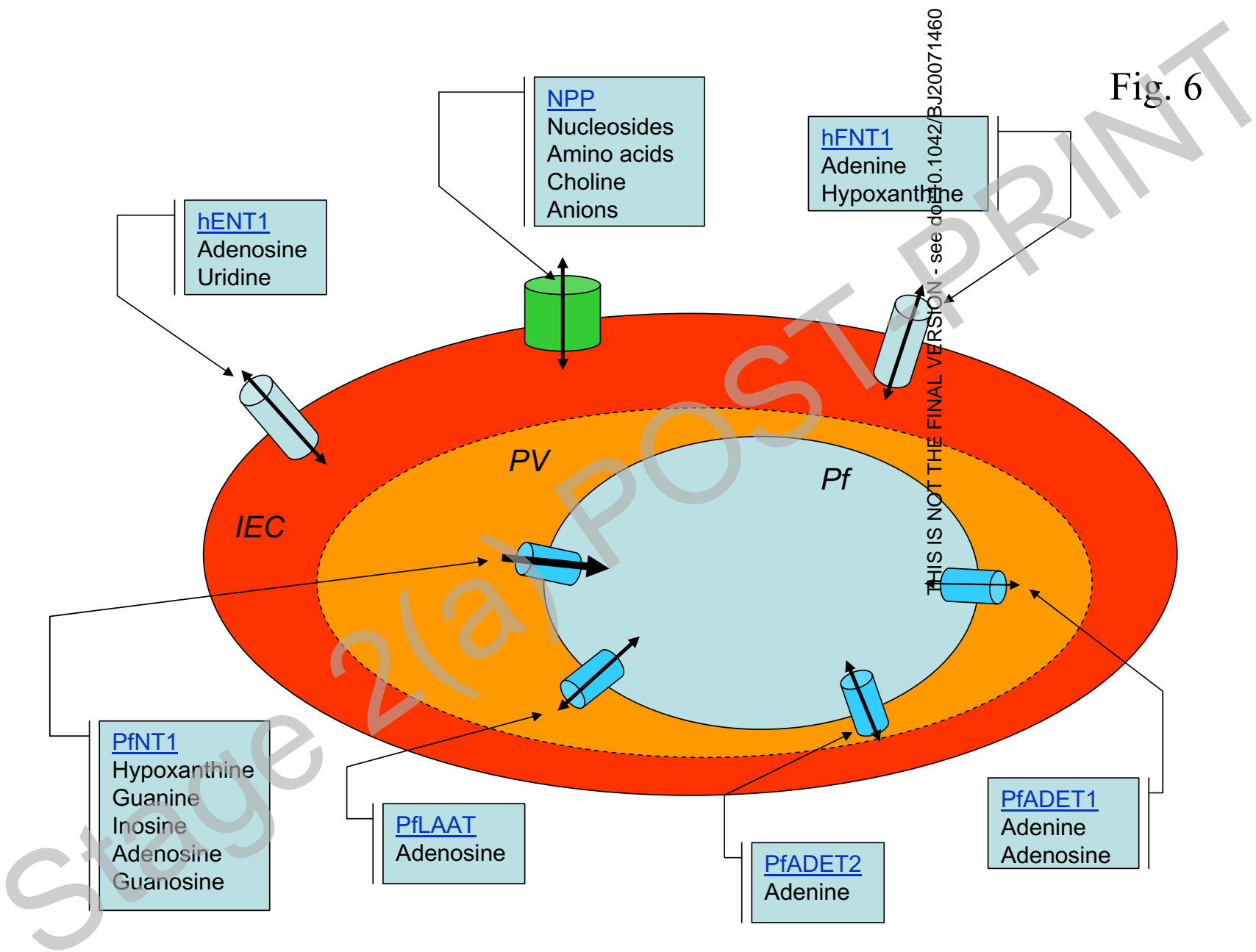


Fig. 6