Molecular Pharmacology manuscript #82321

# Pyrimidine salvage in Trypanosoma brucei bloodstream forms and the trypanocidal action of halogenated pyrimidines

Juma A. M. Ali, Darren J. Creek, Karl Burgess, Harriet C. Allison, Mark C. Field, Pascal Mäser, and Harry P. de Koning

## Supplementary methods

***Lectin blotting.*** Interaction of glycoproteins with lectins was visualized by separating proteins by reducing SDS-PAGE (using 10% gels and $1\times10^7$ cell equivalents/lane) and then Western blotting onto Immobilon-P transfer membranes (Millipore). Gels were stained with Brilliant Blue electrophoresis reagent (Sigma Aldrich) to check for equal cell loading and for visualization of protein bands. Membranes were stained using *Erythrina cristigalli* lectin (1:1,000) or *Ricinus communis* lectin (1:1,000) (both obtained from Vector Laboratories) and washed extensively before incubation with streptavidin-HRP (1:7,000) (Thermo Scientific) as in (Mehlert et al., 2012). Further extensive washing was followed by visualization of bands using ECL reagents (GE Healthcare).

## Supplementary Results

***Effect of 5-FU on glycosylation in T. b. brucei*.** To test whether the detection of significant quantities of 5F-UDP-hexoses and hexosamines contribute to trypanocidal action through interference with either protein glycosylation or glycosylphosphatidylinositol (GPI) anchor biosynthesis, we examined whether any major defects to glycosylation or GPI anchor synthesis took place under the influence of fluorinated pyrimidines. No detectable changes in VSG content were observed after treatment with 100 µM 5-FU or 5F-2'dUrd. Protein extracts of bloodstream trypanosomes, incubated for 12 h in the presence or absence of 100 µM 5-FU or 5F-2'dUrd, were separated by 1D SDS-PAGE and transferred onto Immobilon-P membranes. Two separate blots of the same samples were incubated with Ricinus communis lectin, which binds specifically to terminal β-galactose residues, or with Erythrina crystagalli lectin, which is specific for N-acetyl lactosamine modifications - both are hallmarks of mature N-glucan processing. This experiment was performed on three independent occasions but in no case was a difference in staining pattern or intensity observed between the extracts from treated and untreated trypanosome cultures (Supplemental Figure 8). Further, the intensity and migration position of the variant surface glycoprotein band, as detected by Coomassie staining of gels, was unaltered. We conclude that no major defects to glycosylation or GPI anchor synthesis took place under the influence of fluorinated pyrimidines. Although it is possible that glycosylation of a relatively rare glycoprotein could have been affected without this being apparent in the blot, it is clear that the bulk of N-glycan and GPI biosynthesis are unaltered.

**Supplementary figures**

Supplementary Figure 1. Transport of [$^3$H]-uridine by T. b. brucei bloodstream forms. (A) Bloodstream forms were incubated with 2.5 µM [$^3$H]-uridine for the indicated times in the presence (○) or absence (■) of 5 mM unlabelled uridine. Transport was terminated by the addition of 1 ml of ice-cold 10 mM uridine solution and immediate centrifugation through oil. Lines were calculated by linear regression analysis, with correlation coefficients of 0.98 (2.5 µM) and 0.63 (5 mM), respectively. (B) Transport of 2.5 µM [$^3$H]-uridine in bloodstream forms over 15 min in the presence of various concentrations of unlabelled uracil (■) or uridine (○). Lines were calculated using an equation for sigmoid curve with Hill slope set at -1 and bottom level at 0 for the purpose of extrapolation.

Supplementary Figure 2. Transport of pyrimidine nucleosides by T. b. brucei. (A) Transport of 5 µM [$^3$H]-2'-deoxyuridine by bloodstream T. b. brucei over 3 min at room temperature. (B) Transport of 5 µM [$^3$H]-thymidine by bloodstream T. b. brucei over 15 minutes at room temperature.
Both experiments were performed in triplicate and are representative of three identical experiments performed on different dates. Error bars are SE.

Supplementary Figure 3. Uptake of 0.25 µM [$^3$H]-inosine or 10 µM [$^3$H]-thymidine in bloodstream forms of *T. b. brucei*.

- **A.** Thymidine uptake was dose-dependently inhibited by inosine (■) using 15 min incubations. $K_i$ for three independent experiments was 1.6 ± 0.6 µM.
- **B.** Inhibition of [$^3$H]-inosine uptake by unlabelled inosine (○) and thymidine (■) at the indicated concentrations measured over 10 s.
- **C.** Conversion of the inosine inhibition curve to a Michaelis-Menten saturation plot. $K_m$ for this experiment was 0.53 µM. This experiment is representative of 4 identical experiments with highly similar outcomes.

Inosine uptake over 10 s is consistent with measurement of initial rates of transport as we previously reported linear uptake of [$^3$H]-inosine in *T. b. brucei* over 60 s (De Koning et al. (1998) J Biol Chem 273: 9486-9494).

Supplementary Figure 4. Non-saturable uptake of some pyrimidine nucleosides and nucleobases by T. b. brucei bloodstream forms..

- A. Uptake of 0.5 µM [$^3$H]-cytidine (■) was slow and only partly inhibited by 2.5 mM unlabelled cytidine (○).
- B. Uptake of 2.5 µM [$^3$H]-2'-deoxycytidine (■) was not inhibited by as much as 10 mM 2'-deoxycytidine (○).
- C. Uptake of 0.25 µM [$^3$H]-cytosine (■) was not significantly different from zero (F-test) when measured over 15 min, and [$^3$H]-cytosine accumulation was not reduced in the presence of 2.5 mM unlabelled cytosine (○).
- D. Uptake of 1 µM [$^3$H]-thymine in the presence (○) or absence (■) of 10 mM unlabelled thymine, measured over 15 min, was not significantly different from zero (F-test).

Supplementary Figure 5. Effect of fluorinated pyrimidines on growth of bloodstream form s427WT. Cultures containing $1\times10^5$ or $2\times10^5$ trypanosomes/ml were incubated with 500 µM of 5-FU (○), 5-FOA (▲), 5-fluoro,2'-deoxyuridine (■) or control (◊) . At various times samples were taken and counted microscopically using a haemocytometer. Each determination was in performed three times and the average is shown. The inset is the same data as in the main figure but including the control (no added drug) culture and on a different scale.

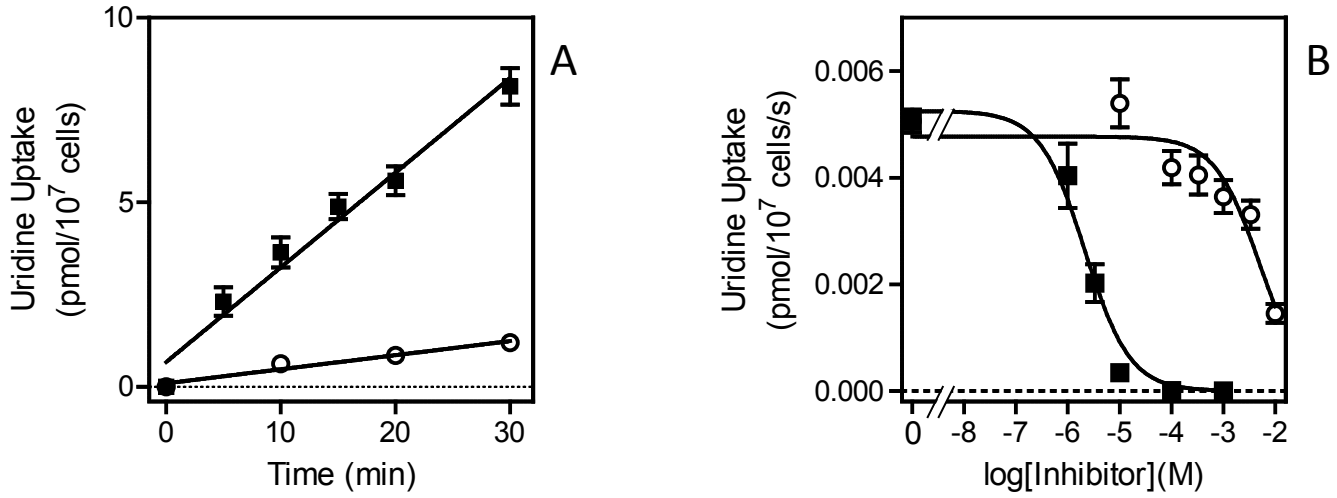
Supplementary Figure 6. Adaptation of s427 bloodstream T. b. brucei to high concentrations of fluorinated pyrimidine analogues during in vitro culturing. Concentrations indicated are the concentrations of analog added to the medium, in which the cells managed to survive and multiply. This was 5-fluorouracil to generate the adapted cell line 5FURes; this was 5F-2'-dUrd to generate the cell line 5F-dURes; and 5F-orotic acid to generate the cell line 5-FOARes. After adaptation of the

cultures the trypanosomes were cloned out by limiting dilution so that the eventual cell lines that were characterized were all grown from a single cell.

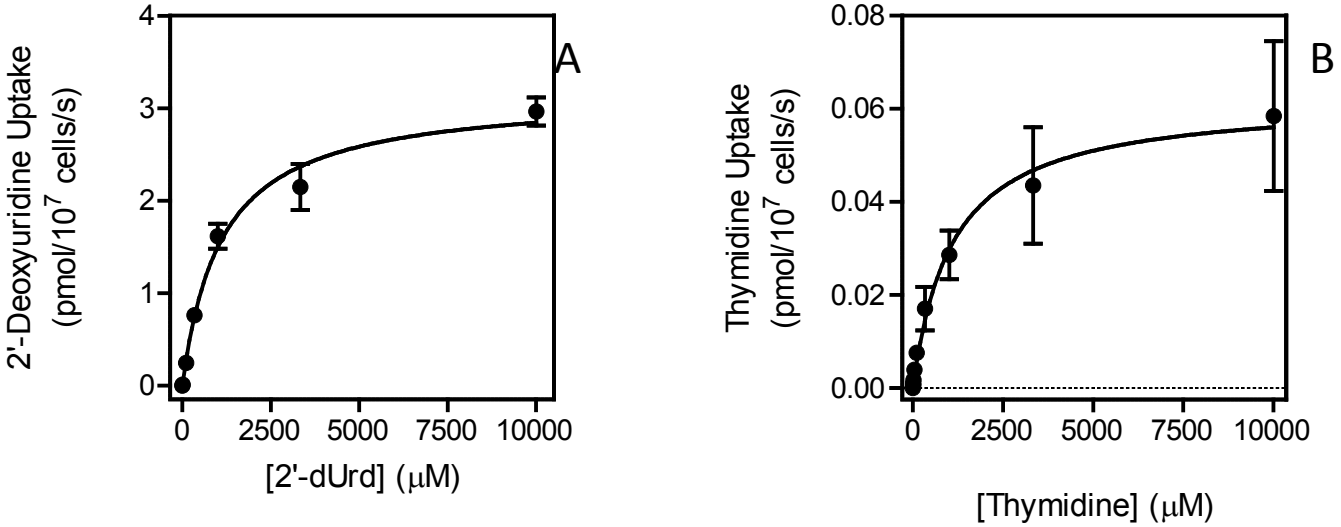
Supplementary Figure 7. Transport of 0.25 µM 5-fluorouracil by bloodstream forms of (A) T. b. brucei s427WT or (B) 5-FURes. Incubation time was 30 s for WT and 4 min for 5-FURes, well within the linear range of uptake (see figure 4B of main manuscript). The experiments were representative of three identical experiments with highly similar outcomes.

Supplementary Figure 8. Lectin blotting of T. b. brucei BSF protein samples after incubation with 5-fluorouracil or 5fluoro-2'deoxyuridine. Cultures of T. b. brucei bloodstream forms were incubated for 12 h in the presence or absence of 100 µM of either pyrimidine analog, under standard culturing conditions. Protein extracts were separated on 1D polyacrylamide gels (left hand-side, Coomassie Blue stained) and transferred to Immobilon-P membranes to be incubated with either Erythrina crystagalli lectin or Ricinus communis lectin (right hand-side). Lectin binding was visualized by using ECL reagents.

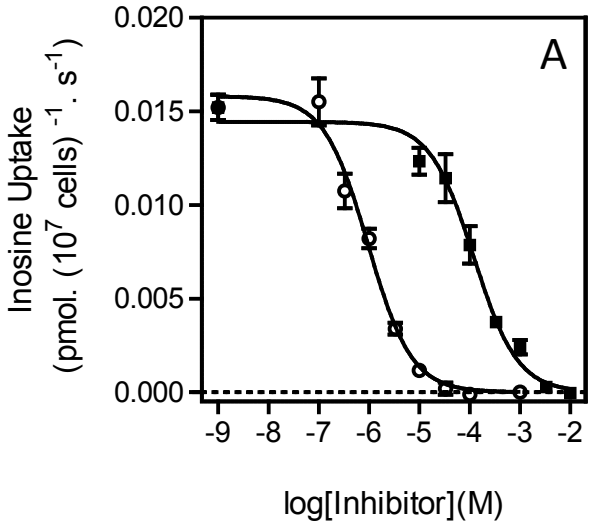
Supplemental Figure 1

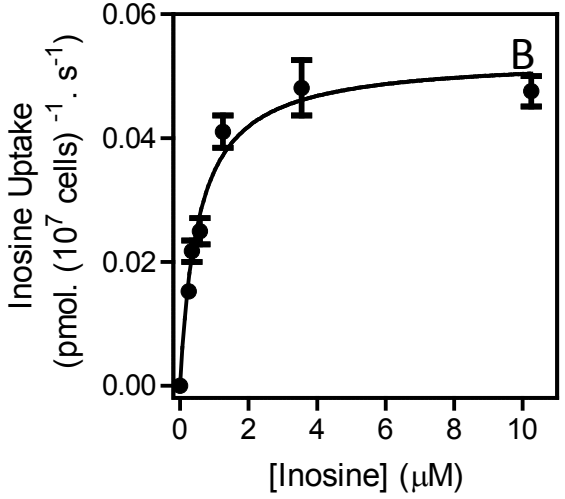


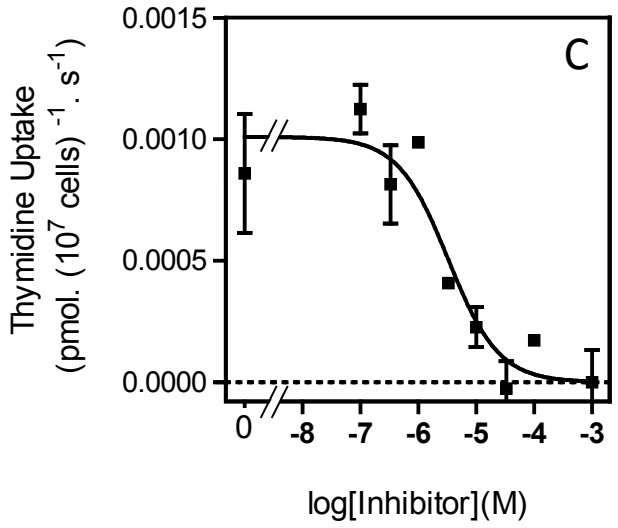
Supplemental Figure 2



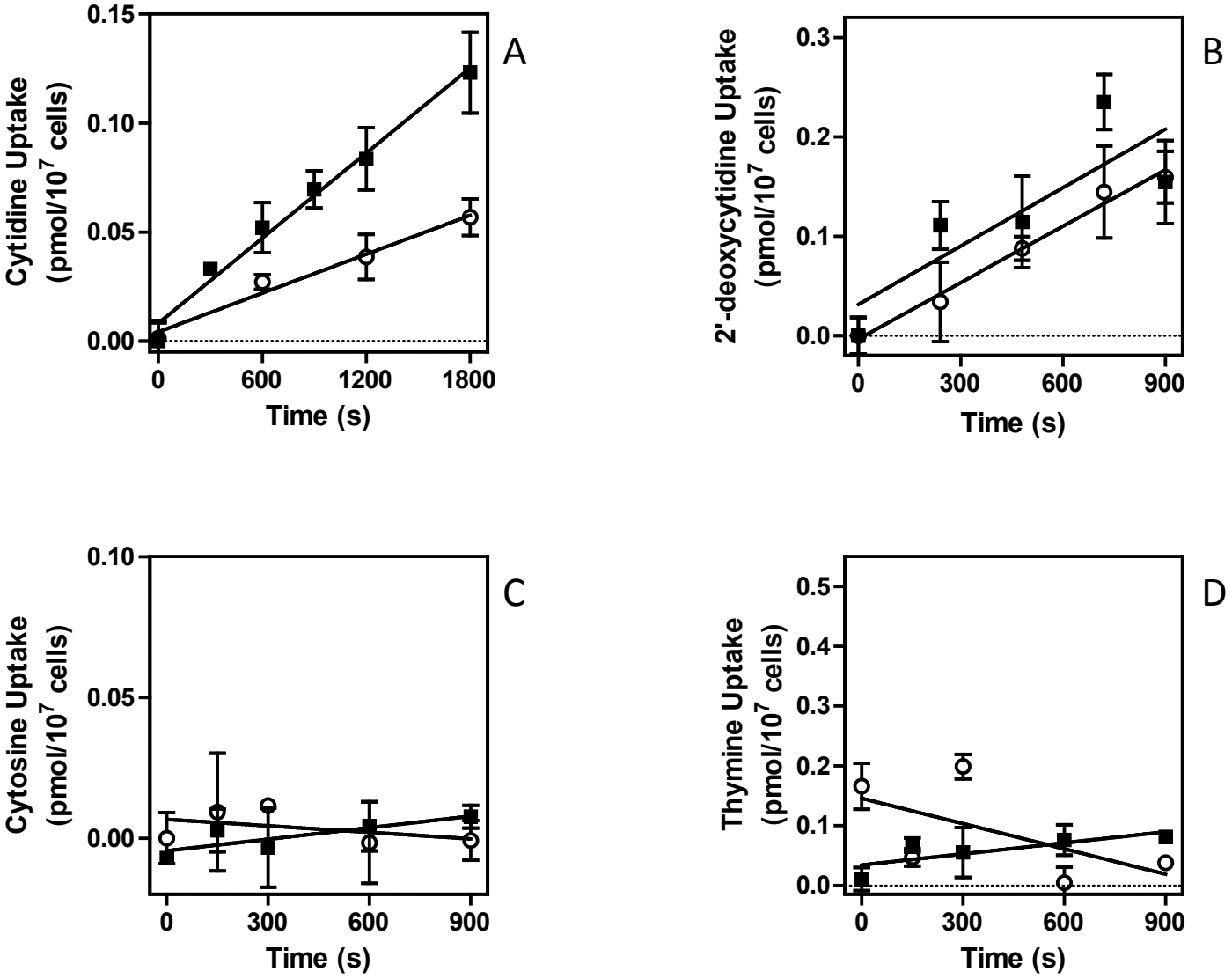
Supplemental Figure 3



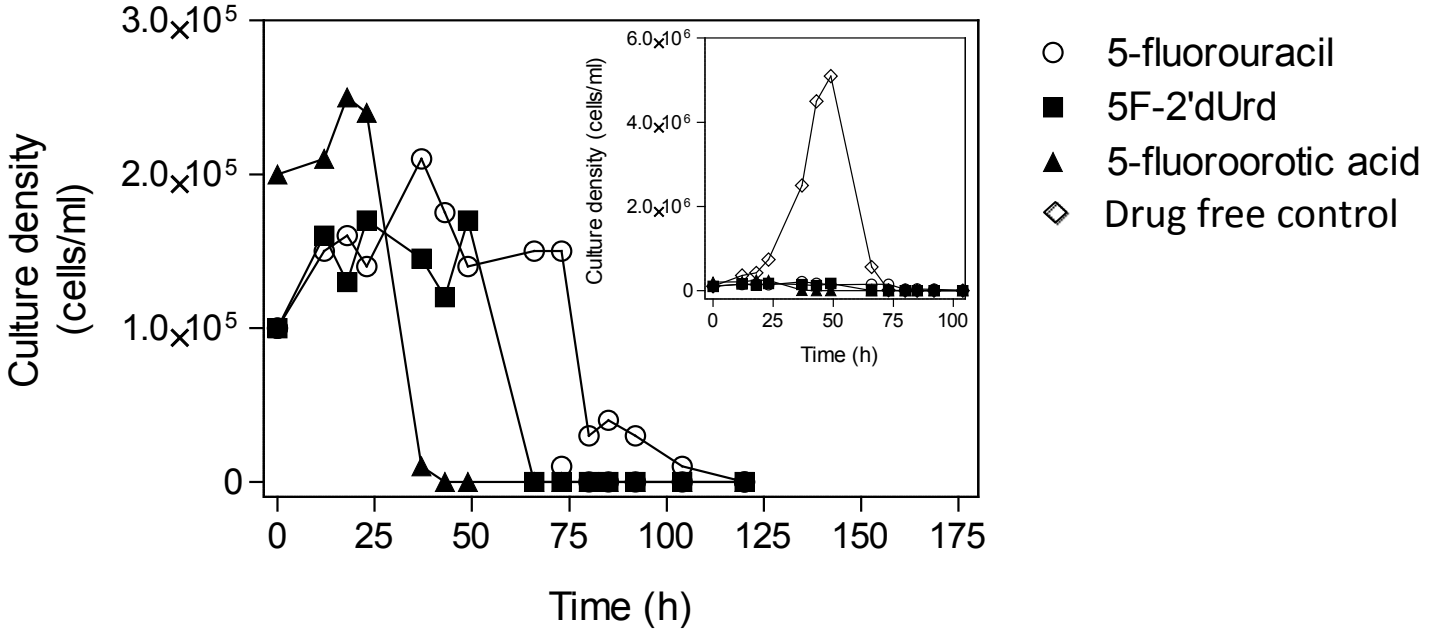



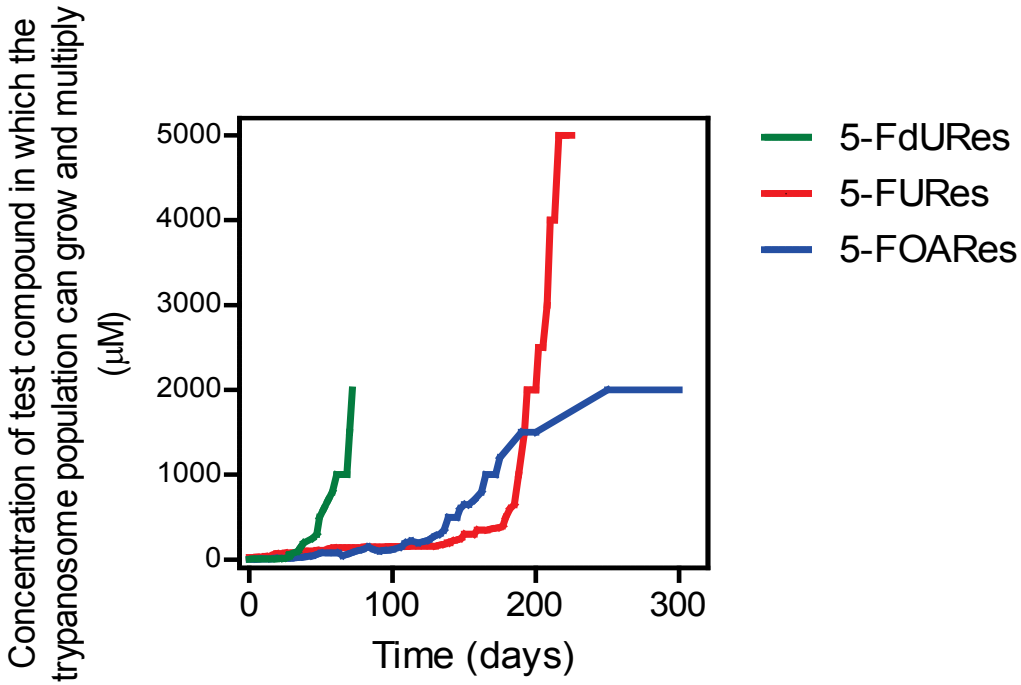
Supplemental Figure 4



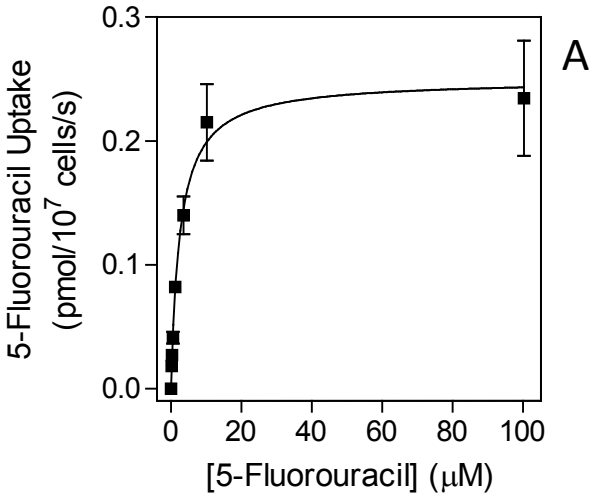
Supplemental Figure 5



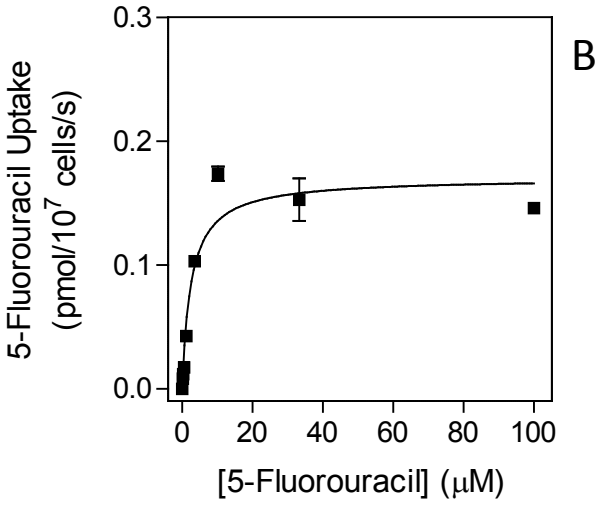
Supplemental Figure 6



Supplemental Figure 7




A




B

Supplemental Figure 8