Short communication

Negative selection using yeast cytosine deaminase/uracil phosphoribosyl transferase in *Plasmodium falciparum* for targeted gene deletion by double crossover recombination

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The ability to genetically manipulate *Plasmodium falciparum* has been established for a number of years and it is an important tool for functional analysis of parasite genes using gene disruption [1], allelic exchange [2] and transgene expression [3]. This was established using positive selection for maintenance of circular plasmids carrying a drug selectable marker such as *Toxoplasma gondii* dihydrofolate reductase (*TgDHFR*) [4,5], or the human *dhfr* gene [6] conferring resistance to pyrimethamine or WR99210 respectively although more recently other selection systems have been used [7]. Transfected *P. falciparum* parasites carrying copies of these plasmids integrated into the genome by homologous recombination were generally selected over time by cycles of growth on and off drug selection. The episomal plasmids are lost rapidly in the absence of drug selection as they are partitioned unevenly amongst the daughter progeny whereas the plasmids integrated into the genome are segregated normally with each chromosome [8]. This method ensured that after subsequent on/off drug cycles all transfected *P. falciparum* parasites obtained had integrated the plasmid by single crossover homologous recombination. Whilst this proved to be an extremely useful method for gene disruption it was limited because of the long periods of culture required to derive parasites with integrated copies of the plasmid due to persistence of the circular episomal forms. Another drawback is that with a single cross over the open reading frame is only disrupted, not deleted, and therefore drug pressure has to be maintained even after obtaining a pure population of single cross over parasites to prevent reversion to wild-type.

To overcome these limitations negative selection of transfected parasites was developed [9] using the thymidine kinase (Tk) gene of the Herpes simplex virus [10] and the cytosine deaminase (CD) gene of *Escherichia coli* [11] to increase the efficiency of gene targeting. Negative selection was utilised by expression of the enzymes encoded by these genes allowing conversion of a normally innocuous metabolite into a toxic form. The conversion of ganciclovir by thymidine kinase inhibits thymidylate synthase and DNA synthesis whilst the conversion of 5-fluorocytosine (5-FC) by cytosine deaminase into 5-fluoro-uracil (5-FU) inhibits RNA synthesis as well as thymidylate synthase. The use of thymidine kinase for negative selection in transfection plasmids for *P. falciparum* provided a selection for the rarer events such as double crossover homologous recombination and this decreased the time to obtain gene knockouts and allowed generation of parasite lines with decreased growth rates [12]. In contrast, *E. coli* cytosine deaminase was not particularly useful as it proved easy to select *P. falciparum* parasites resistant to the 5-fluorocytosine metabolite, even in the presence of the active enzyme [9].

The gene encoding a bifunctional chimeric protein, consisting of *Saccharomyces cerevisiae* cytosine deaminase (ScCD) and uracil phosphoribosyl transferase (ScUPRT), has been used as a suicide gene in human tumour cells and shown to be 1000-fold more potent than cytosine deaminase by itself [13]. This increased potency is due to a more efficient conversion of the 5-fluoro uracil into 5-fluoro uridine monophosphate (5-fluoro-UMP) by the ScUPRT. The following conversion of

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**Abbreviations:** CD, cytosine deaminase; DHFR, dihydrofolate reductase; 5-FC, 5-fluorocytosine; FCU, chimeric gene of *Saccharomyces cerevisiae* CD and URPT; TK, thymidine kinase; URPT, uracil phosphoribosyl transferase

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5-fluoro-UMP into the toxic 5-fluoro-UTP and 5-fluoro-dUMP is mediated by host cellular enzymes. 5-fluoro-UTP and 5-fluoro-dUMP is then incorporated into RNA and inhibits thymidylate synthase, respectively.

Additionally, this chimeric enzyme has recently been utilised in the rodent malaria model \textit{P. berghei} for hit and run mutagenesis allowing restoration of the original configuration of the gene and excision of the selectable marker by homologous recombination [14].

To test if the chimeric enzyme ScCD and ScUPRT (ScFCU) [13] could be used for selection of gene disruption in transfected \textit{P. falciparum} by double crossover homologous recombination we constructed the plasmid pHHT-FCU (Fig. 1). It contains human dihydrofolate reductase (hDHFR) [6] as a positive selectable marker driven by the calmodulin promoter and is based on the previous vector pHHi [15]. We inserted the ScFCU chimeric gene (amplified from the pCI-neoFCU plasmid [13]) in the Xho I site, so that it would be transcribed from the \textit{P. falciparum} hsp86 promoter with the \textit{P. berghei} dhfr terminator region (\textit{PhDT}). We then added two targeting regions from the gene \textit{PF11}_0037 locus that flanked the \textit{hDHFR} selectable marker cassette yielding plasmid pHHT-FCU-Δ\textit{PF11}_0037 (Fig. 2) (5’ flank: \textit{Eco} RI/Nco I, 3’ flank: Spe I/Sac II) [16]. \textit{PF11}_0037 is a 2-exon gene encoding for a protein with a theoretical molecular mass of 74 kDa expressed in trophozoites. The presence of a predicted protein export domain in the protein suggests its export into the cytosol of the infected red blood cell. The plasmid pHHT-FCU-Δ\textit{PF11}_0037 was inserted into CS2 [17] parasites by transfecting ring-stage parasites (~5% parasitemia) with 80 μg of purified plasmid DNA (Qiagen) as previously described [5] using modified electroporation conditions (0.310 kV, 950 μF) [6]. After 6 h the culture medium (RPMI-HEPES with 5% AlbumaxII (Invitrogen) and 5% heat inactivated human serum) was changed and 6 nM WR99210 (Jacobus Pharmaceuticals) was added.

Fresh media and WR99210 were added every 24 h for the next 5 days and every 48 h thereafter. After the establishment of WR99210 resistant parasites (25–32 days) 5-FC (Ancotil® ICN) was added whilst maintaining selection with WR99210. This procedure would allow positive selection for parasites that had integrated this cassette by double crossover recombination (cell line CS2-FCU) with WR99210 and negative selection with 5-FC against those that retained the episomal plasmid.

To determine if the ScFCU chimeric gene was functionally expressed CS2-FCU parasites were compared with the CS2 parent for the ability to grow in the presence or absence of the ‘suicide drug’ 5-fluorocytosine. The addition of 5-FC to parental CS2 cells had no effect on growth of these parasites; however, CS2-FCU parasites were rapidly lost in the presence of 5-FC consistent with expression of ScFCU (Fig. 1B). No parasites were obtained even after prolonged culture of CS2-FCU in 5-FC (up to 65 days) suggesting that selection of resistant mutant parasites was much less likely with the fused ScFCU gene than has been obtained using parasites transfected with the \textit{E. coli} CD enzyme alone [9]. Therefore, the ScFCU gene is useful for negative selection of \textit{P. falciparum} transfectants in the presence of the ‘suicide’ drug 5-FC.

![Fig. 1. The utility of ScFCU for negative selection using 5-FC in \textit{P. falciparum}.](image)

*Fig. 1. The utility of ScFCU for negative selection using 5-FC in \textit{P. falciparum}. (Panel A) The pHHT-FCU vector contains two cassettes the first containing \textit{hDHFR} for positive selection using \textit{WR99210}. The \textit{PhDT} gene is driven by the calmodulin promoter (5’ CAM) and has the histidine rich protein 2 terminator (3’ hrp2). The second cassette has the \textit{Saccharomyces cerevisiae} cytosine deaminase/araC1 phosphoribosyltransferase (ScFCU) chimeric gene for negative selection with 5-FC and is driven by the heat shock protein 86 promoter (5’ hsp86) and flanked by the \textit{P. berghei} dhfr terminator (3’ PbDT). The plasmid backbone contains the cassette for bacterial expression and selection (AMP). (Panel B) Testing of \textit{P. falciparum} parasites for sensitivity to growth on 5-FC when expressing ScFCU. Parental parasites CS2 are insensitive to 5-FC (50 μM) whilst CS2-FCU parasites containing the ScFCU gene are sensitive. The parasites were subcultured to 0.5% each time they reached 2–5% parasitemia as indicated by the drop every 48 h. Both WR99210 and 5-FC was added whilst changing the medium. (Panel C) Transfected parasites containing the plasmid pHHT-FCU-Δ\textit{PF11}_0037 were grown in \textit{WR99210} and 5-FC at concentrations of 0, 1, 10, 100 and 1000 μM were tested to determine the concentration allowing growth of parasites that had deleted the ScFCU cassette. The parasites were subcultured to 0.5% each time they reached 2–5% parasitemia as indicated by the drop every 48 h.*
expressing parasites metabolise the pro-drug 5-FC to the toxic metabolite 5-fluoro uracil and this could diffuse into the non-ScFCU containing parasites resulting in inhibition and killing [18,19]. In thymidine kinase and E. coli cytosine deaminase containing P. falciparum transfectants this bystander effect was observed in the presence of ganciclovir and 5-FC, respectively, which also resulted in significant killing of parasites lacking the negative selection cassettes [9]. CS2-FCU parasites were grown on different concentrations of 5-FC (from 1 to 1000 μM) in the presence of WR99210 to determine the concentration that allows the selection of parasites lacking ScFCU without being killed through the bystander effect from transfectants still expressing the enzyme and producing the toxic metabolite. Whilst parental CS2 were able to grow in all concentrations of 5-FC tested (not shown) the CS2-FCU transfectants showed growth inhibition for all concentrations tested. Only at a concentration of 1 μM 5-FC could parasites be recovered after approximately 10 days (Fig. 1C). To determine if these parasites had integrated the hDHFR selection cassette into the PF11_0037 gene we used Southern blot hybridisation on genomic DNA cut with the restriction enzymes Afl II and Eco RV (Fig. 2B). This indicated that PF11_0037 had indeed been disrupted with the hDHFR cassette integrated by homologous double crossover recombination across the 5’ and 3’ flanks. The lack of expression of the encoded protein in these parasites was confirmed using specific anti-rabbit antibodies (data not shown). As a result we have demonstrated that pHHT-FCU can be used for gene disruption in P. falciparum.

Previously E. coli cytosine deaminase had been tested in P. falciparum for use in negative selection with 5-FU and whilst parasites expressing it were inhibited, frequent mutants arose and it has not successfully been used for selection of transfectants with double crossover recombination [9]. This was most likely because the selection was not sufficiently potent as the IC₅₀ of 5-FC for transfected parasites expressing CD was approximately 250 μM. This was in contrast to transfected parasites expressing TK, where the IC₅₀ for ganciclovir was approximately 500 nM and parasites with double crossover homologous recombination integration were easily obtained. Although vectors containing the TK gene for negative selection have now been successfully used for many different gene disruptions in P. falciparum [12,20–26] (Maier and Cowman, unpublished data) some gene disruptions using TK have resulted in single cross over recombination with insertion of the full plasmid including the TK gene [27]. It has not been clear why this occurs but one reason may be the lack of sufficient TK enzyme expression from one copy of the gene for potent negative selection allowing some of these parasites to survive. Therefore, it was necessary to develop a more reliable negative selection system. Here we tested the ScFCU gene to determine its utility for negative selection in P. falciparum and it has proven to be much more useful than E. coli CD by itself. It would appear to be also much more potent than TK as the concentration used for negative selection is 1 μM. Higher concentrations result in inhibition of all cells in a mixture of parasites most of which express ScFCU [9]. Additionally we have successfully used the ScFCU gene to disrupt a number of P. falciparum genes by double crossover recombination and as yet have not seen any insertion by single recombination where the negative selection cassette is retained (Maier and Cowman, unpublished data).

The use of ScFCU in negative selection is an improvement in our ability to construct ‘loss of function’ mutants as it is more potent and increases the probability of obtaining gene disruption events that are deleterious to the growth of the parasite. It also opens the possibility of utilising ‘hit and run’ methodology as has been demonstrated in P. berghei whereby a gene disruption can...
be recovered by imposing negative selection to regain parasites that have deleted the plasmid previously inserted by homologous single crossover recombination [14]. The use of ScFCU has provided a new tool for potent negative selection allowing more efficient exploitation of the information provided by the P. falciparum genome.

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