

A Single Point Mutation in the *Plasmodium Falciparum* 3'-5' Exonuclease Does Not Alter Piperaquine Susceptibility

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Abstract

Background

The rise in *Plasmodium falciparum* resistance to dihydroartemisinin-piperazine (DHA-PPQ) treatment has been documented in the Greater Mekong Subregion with associations with mutations in the *P. falciparum* chloroquine resistance transporter (*pfcr*) and plasmepsin 2 (*pfpm2*) genes. However, it is unclear whether other genes also play a role with PPQ resistance, such as the E415G mutation in the exonuclease (*pfexo*) gene. The aim of this study was to investigate the role of this mutation in PPQ resistance by generating transgenic parasites expressing the *pfexo*-E415G mutant allele.

Methods

Transgenic parasite clones carrying the E415G mutation in *pfexo* of the B5 isolate were derived by CRISPR-Cas9 gene editing and verified using PCR and gene sequencing. Polymorphisms of *pfkelch-13*, *pfcr*, and *pfexo* were examined by PCR while the copy number variations of *pfpm2* were examined by both relative quantitative real-time PCR and the duplication breakpoint assay. Drug sensitivity against a panel of antimalarials, the ring-stage survival assay (RSA), the PPQ survival assay (PSA), and bimodal dose-response curves were used to evaluate antimalarial susceptibility.

Results

The transgenic line, B5-*rexo*-E415G-B8, was successfully generated. The PPQ-IC₉₀, %PPQ survival, and the bimodal dose-response clearly showed that E415G mutation in PfEXO of B5 isolate remained fully susceptible to PPQ. Furthermore, growth assays demonstrated that the engineered parasites grew slightly faster than the unmodified parental isolates whereas *P. falciparum* isolates harbouring *pfkelch-13*, *pfcr*, and *pfexo* mutations with multiple copies of *pfpm2* grew much more slowly.

Conclusions

Insertion of the E415G mutation in PfEXO did not lead to increased PPQ-IC₉₀ and % PPQ survival, suggesting that this mutation is not associated with PPQ resistance and unsuitable to be used as a molecular marker for monitoring PPQ-resistant parasites. The results also highlight the importance of monitoring and evaluating suspected genetic mutations with regard to parasite fitness and resistance.

Background

Artemisinin-based combination therapies (ACTs) remain the recommended first-line antimalarial therapy for uncomplicated *Plasmodium falciparum* infections in almost all endemic countries [1]. ACTs consist of two drugs: a semisynthetic artemisinin (ART) derivative and a longer lasting partner drug. Currently, six ACTs are available including artemether+lumefantrine (AL), artesunate+amodiaquine (AS+AQ), artesunate+mefloquine (AS+MQ), artesunate+sulfadoxine-pyrimethamine (AS+SP), dihydroartemisinin+piperazine (DHA+PPQ) and most recently artesunate+pyronaridine (AS+PND) [1]. However, the effectiveness of the ACTs is threatened by the emergence of parasites with decreased susceptibility to the ART derivatives and/or resistance to ACT partner drugs [2–4]. Soon after DHA-PPQ was recommended by WHO as a first-line therapy for uncomplicated *P. falciparum* malaria [5], the emergence of DHA-PPQ resistance was reported within the Greater Mekong sub region including Cambodia [6–9], Vietnam [10] and more recently in Thailand [11].

ART resistance is characterized by the presence of mutations in the *Pfkelch13* (*pfk13*) propeller domain which is associated with delayed *in vivo* parasite clearance times [2, 3] and with increased survival in the *in vitro* ring-stage survival assay (RSA) [12]. There are three molecular marker candidates for PPQ resistance: multiple copies of *P. falciparum* plasmepsin 2 (*pfpm2*) (PF3D7_1408000) [13–16], novel mutations in *P. falciparum* chloroquine resistance transporter (PfCRT) (PF3D7_0709000) [17–20], and a single mutation in *P. falciparum* exonuclease (PfEXO) (PF3D7_1362500) [13, 14, 21]. There have been eight previous publications reporting on the E415G mutation in the *pfexo* gene [13, 22–28]. In addition to the amplification of *pfpm2* and *pfpm3*, Amato R *et al.*, [13] identified a non-synonymous single nucleotide polymorphism (SNP) E415G substitution on an exonuclease-encoding gene from recrudescence isolates from DHA-PPQ treatment failures in Cambodia. The prevalence of DHA-PPQ treatment failures was at 2% in Ratanakiri, 16% in Preah Vihear, and 46% in Pursat. In Vietnam, neither PfEXO-E415G mutations nor *pfpm2* amplification was observed in parasites from south-central Vietnam between 2015 and 2016 [27], but *P. falciparum* samples collected from 2018 to 2019 in Vietnam's Central Highlands [25] were found to harbour the PfEXO-E415G mutation with a prevalence of 66.7% and 85.5% in Dak Nong and Dak Lak, respectively. Si, Y *et al.* [26] has recently reported that no parasites from the China-Myanmar border carried PfEXO-E415G, novel PfCRT mutations, or *pfpm2* amplification, even though some parasites with higher PPQ survival assay (PSA) values were detected, indicating different mechanisms of reduced PPQ susceptibility. In Africa, Robert MG *et al.*, [23] first investigated the presence of

both the PfEXO-E415G mutation and multiple copies of *pfpm2* in Senegalese parasites; however, no isolated parasites carried both molecular markers. A similar trend was observed in Sudan where no PfEXO-E415G mutations were observed [28]. However, in Mali, of 214 *P. falciparum* samples from Dangassa, two isolates exhibited the PfEXO-E415G mutation [22].

While the implication of multiple copies of *pfpm2* and novel PfCRT mutations on PPQ resistance have been elucidated [19, 20, 29], and the PPQ-resistance phenotype can be observed in the presence of novel PfCRT mutations with the PfEXO-E415G mutation [24], there is still a lack of confirmation as to whether the PfEXO-E415G mutation alone causes PPQ-resistance. To investigate whether the PfEXO-E415G is directly associated with PPQ resistance, in this study, we used CRISPR-Cas9 genome editing to generate *P. falciparum* Cambodian parasites harbouring PfEXO-E415G. The *in vitro* susceptibility to piperazine and other frontline antimalarials of these gene-edited parasites was determined and compared to those of validated PPQ-resistant *P. falciparum* clinical isolates of Cambodian origin.

Materials And Methods

pfexo gene and amino acid prediction

The nucleotide sequence of *pfexo* from 3D7 was obtained from PlasmoDB with the accession number PF3D7_1362500. The *P. falciparum* B5 line was obtained from the cloning of a Cambodian *P. falciparum* isolate [24]. This isolate is ART- and PPQ-sensitive, but CQ-resistant. The full-length *pfexo* gene of B5 was amplified from *P. falciparum* B5 genomic DNA using polymerase chain reaction (PCR) with primers PA_exon1 and Screening_3UTR_Rev (Table S1). The encoded amino acid sequences and the nucleotide sequences were aligned using Clustal Omega [30]. The signal peptide region was predicted using Signa-P.5.0 [31] and the InterPro program was used for protein family classification [32].

P. falciparum culture

Asexual blood-stages of *P. falciparum* were maintained in fresh human erythrocytes (O⁺) in RPMI1640 (Sigma-Aldrich, USA) supplemented with 5.94 gL⁻¹ HEPES, 2.1 gL⁻¹ sodium bicarbonate, 0.1 gL⁻¹ gentamycin sulphate, 0.5% (w/v) Albumax II, 4 gL⁻¹ dextrose 0.05 gL⁻¹ hypoxanthine, and 10-15% of human serum (complete medium) [33]. Human blood products (erythrocytes and serum) were obtained from the Thai Red Cross. Culture flasks were gassed with 5% CO₂, 5% O₂, 90% N₂ gas and incubated at 37 °C. For synchronisation, mature schizont stage parasites were isolated on cushions of 75% (v/v) isotonic Percoll cushion (GE Healthcare Life Science) as previously described [34, 35] whereas enrichment for ring stages following invasion was performed using 5% (w/v) D-sorbitol [36] The final ring cultures were washed and returned to culture or used as required.

Construction of plasmid constructs to genetically modify *P. falciparum*

PCR amplicons used in plasmid cloning were generated using Fusion, High Fidelity DNA polymerase (New England Biolabs) and purified using Qiagen PCR purification or Qiagen Gel extraction kits. For diagnostic PCR amplification, GoTaq (Promega) DNA master mix was used. All constructed plasmids were sequenced to verify authenticity (Biobasic, Canada). For parasite genomic DNA extraction, total cell pellets were first treated with 0.15% saponin in PBS for 10 min, then washed with PBS before DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen).

Construct for donor plasmids

A fragment of *exo* sequence with E415G mutation (1,441 bp) was commercially synthesised (GenScript, USA), comprising a stretch of native *P. falciparum* 3D7 *exo* sequence (covering residues Lys283, *exo* intron, and Asn408) followed by a stretch of recodonized *exo* gene sequence encoding the 3D7 amino acid residues Met409 to Glu514 with the point mutation at residue 415 from Glu to Gly but using a different codon usage, and finally a stretch of native 3D7 *exo* sequence covering amino acid residues 515 to the stop codon. This fragment was cloned into pUC57 by *EcoRV* generated pUC57-Exo-E415G plasmid. To generate a donor plasmid to introduce Glu415Glu wild-type, pUC57-Exo-E415E plasmid was derived from pUC57-Exo-E415G by Q5 site-directed mutagenesis (New England Biolabs) to alter the codon encoding at amino acid position 415 from GGC to GAA primers Q5SDM_G415E_F and Q5SDM_G415E_R were used (Table S1).

Construct for CRISPR-Cas9 plasmids

pDC2-Cas9-hDHFR-yFCU, containing a Cas9 expression cassette and the drug selection marker human dihydrofolate reductase (*hdhfr*) [37] was used as a template vector to generate pDC2-Cas9-bsd-yFCU. Since the *P. falciparum* B5 strain is resistant to pyrimethamine, the *hdhfr* gene was replaced with the *blastidin* gene (*bsd*). The *bsd* gene (399 bp) was amplified from PkpSKIP_Pk47 plasmid [38] using primers bsd_NcoI_F and bsd_SacII_R (Table S1) and was cloned into the NcoI/SacII-digested pDC2-Cas9-hDHFRyFCU, giving rise to pDC2-Cas9-bsd-yFCU.

Guide RNA sequences specific for targeting at amino acid position 415 were identified using Benchling (<https://www.benchling.com/crispr/>) (Fig. S1). A pair of complementary oligonucleotides (sgE415G-1F and sgE415G-1R) corresponding to the 19 nucleotides adjacent to the identified PAM sequences were phosphorylated using T4 polynucleotide kinase, annealed and ligated into pDC2-Cas9-bsd-yFCU predigested with *BbsI*, resulting in the guide vector pGuide1.4-bsd.

Generation of *P. falciparum* lines expressing *exo*-E415G mutant

The donor plasmid pUC57-Exo-E415G was linearized with *ScaI* prior to electroporation. Percoll-enriched mature schizonts of *P. falciparum* B5 were electroporated with 20 µg of pGuide1.4-bsd and 60 µg of linearized pUC57-Exo-E415G using either Amaxa P3 primary cell 4D Nucleofector X Kit L (Lonza) or Amaxa Basic Parasite Nucleofector Starter Kit as described [39, 40]. Twenty-four hours post-transfection, the electroporated parasites were treated with 5.45 µM blasticidin-S-hydrochloride (Sigma-Aldrich, USA) to select for transfectants harbouring pGuide1.4-bsd before returning the cultures to medium without drug. Detection of the *exo*-E415G modified locus was carried out by diagnostic PCR using primer pairs Exon1_K283_F and Screen_WT_Rev_V616, Exon1_K283_F and Recodon_R, and Recodon_F and Screen_3'UTR_R. The wild-type *pfexo* locus was detected by diagnostic PCR using primers Exon1_K283_F and Screen_WT_Rev_V616, with a PCR product of 766 bp. Transgenic parasite clones were obtained by limiting dilution cloning by plating a calculated 0.3 parasite per well (200 µL and 1% haematocrit) in flat-bottomed 96-well microplate wells as described [41]. Wells containing single plaques were identified after 10-14 days using an inverted microscope and the parasites subsequently expanded into round-bottomed wells for further analysis. Transgenic parasite clones were finally checked by diagnostic PCR for integration and modification of the endogenous *pfexo*^{E415G} gene. A *pfexo*^{E415E} transgenic line expressing the wild-type Glu415 codon was generated in the same manner of that for a *pfexo*^{E415G} transgenic line by transfecting *P. falciparum* B5 with 20 µg of pGuide1.4-bsd and 60 µg of linearized pUC57-Exo-E415E. Once established, all transgenic clones were maintained in medium without any drug.

pfk13, *pfcr1*, and *pfexo* genotyping

Master Cycler Nexus Gradient (Eppendorf) was employed to evaluate the propeller domain of the *P. falciparum* kelch13 (*pfk13*) (amino acid residues 442-727) [42, 43], *P. falciparum* exonuclease (*pfexo*) SNP at a codon corresponding to the amino acid position 415 [13], and the *P. falciparum* chloroquine resistant transporter (*pfcr1*) SNPs at codons corresponding to amino acid positions 93, 97, 145, 218, 343, 350, and 353 [19, 44]. Primers used to identify *pfk13*, *pfexo*, and *pfcr1* SNPs are shown in Table S1. *P. falciparum* reference DNAs from 3D7 and W2 clones (Malaria Research & Reference Reagent Resource, Manassas, VA) were used as positive controls, and all samples were performed in duplicate.

Plasmepsin (*pfpm*) 1, 2 and 3 Copy number variation assay

To determine copy numbers of *pfpm1* (PF3D7_1407900), *pfpm2* (PF3D7_1408000), and *pfpm3* (PF3D7_1408100) gene, real-time quantitative PCR (qPCR) was performed on genomic DNA as previously described [29]. The amplification reactions were performed according to Luna Universal qPCR master mix kit (New England Biolabs) with 200 nM of each forward and reverse primer (Table 3) and 2 ng of DNA template using Rotor-Gene Q (QIAGEN, Valencia, CA). For the housekeeping gene, b-tubulin (PF3D7_1008700), b-tubulin forward and reverse primers were designed and used as a reference control for all experiments with the same validated PCR conditions as target primers. *P. falciparum* 3D7 was used as a reference clone. All samples including the references clones were performed in triplicate. The average copy number values for each gene were calculated using $2^{-\Delta\Delta Ct}$ method where $\Delta\Delta Ct$ is $[Ct_{pfpm} - Ct_{pf\beta-tubulin}]_{sample} - [Ct_{pfpm} - Ct_{pf\beta-tubulin}]_{3D7}$. Parasites with copy number greater than 1.6 copies for *pfpm2* [15] were interpreted to contain multiple copies.

Plasmepsin 2/3 (*pfpm2/3*) duplication breakpoint PCR assay

The *pfpm2/3* breakpoint PCR assay was performed as previously described [45]. Three pairs of primers (Table 3) were used in this assay. Primers AF_for and AR_rev amplified a 623 bp product surrounding the breakpoint located at the 3' end of *pfpm1*. Primers BF_for and BR_rev amplified a 484 bp product surrounding the breakpoint at the 3' end of *pfpm3*. Primers BF_for and AR_rev amplified the junction between the breakpoint, producing a 497 bp product in parasite isolates with *pfpm2/3* amplifications. A *pfpm2/3* single copy isolate is not expected to have the PCR product with these primers. One copy isolate was only noted when the control primer sets amplified a product, and the duplication PCR was negative. Two or more copies were annotated as > 1 copy of *pfpm2/3* only when both the control and duplication primer sets generated a product. PCR reactions contained 12.5 µL GoTaq Green Master Mix (Promega), 1 µL of each primer (10 µM stocks), 3 µL of DNA up to 25 µL final volume with water. PCR conditions were as follow: 95°C for 2 min, followed by 30 cycles of 95°C for 45 s, 50°C for 30 s, 72°C for 1 min, followed by a 5-min extension at 72°C.

In vitro drug susceptibility

Drug susceptibility testing used HRP-2 ELISA to measure 50% or 90% inhibitory concentration (IC₅₀ and IC₉₀) performed as previously published [46, 47]. *In vitro* drug susceptibility testing was carried out for control reference clones (W2, D6, C2B) (Malaria Research & Reference Reagent Resource, Manassas, Vermont, USA) as described previously [48]. IC₅₀s and IC₉₀s were estimated by nonlinear regression analysis using GraphPad Prism version 6.0 program. Samples having poor growth rate, as perceived by obtaining an OD ratio of < 1.7 between the no-drug test wells and the maximum tested drug concentration, were excluded from data analysis.

Bimodal dose response curve

To determine a bimodal-dose response curve, the PPQ concentration (2.44 to 100,000 ng/mL) and the dilution series were increased from 8 to 24 points, according to previously published reports [16, 24]. Culture-adapted clinical isolates or engineered parasites were prepared in the similar manner as in *in vitro* drug susceptibility testing. The synchronized rings were grown for 72 hours in the presence of different concentrations of PPQ (24-point dilution) in 96-well plates at 1.5% haematocrit, 0.5% starting parasitaemia in 0.5% Albumax RPMI 1640. Growth at 72 hours was measured by HRP-2 ELISA. Assays were carried out in three biological replicates and the control reference clone W2 was tested along with each culture-adapted clinical isolate. The area under the curve (AUC) for the dose response curve at 0.01 - 100 μ M was calculated using GraphPad Prism 6.0.

Ring-staged survival assay (RSA)

In vitro RSA_{0-3 h} was performed on 0-3 h post-invasion rings obtained from selected culture-adapted clinical isolates following published methods [12] with slight modifications. Briefly, parasite cultures were tightly synchronized using 5% (w/v) D-sorbitol and 75% Percoll to obtain 0 to 3-h post-invasion rings which were adjusted to 0.5-1% starting parasitaemia with a 2% haematocrit in culture media. (0.5% Albumax RPMI 1640 with 2.5% AB serum) and cultured in a 48-well microplate with 700 nM DHA and 0.1 % DMSO in separate wells for growth control. The culture plate was then incubated for 6 hours at 37 °C in modular incubator chambers and gassed with 5% CO₂, 5% O₂ and 90% N₂ gas. Cells were then washed once, resuspended in drug-free medium, and cultured further for 66 hours. Susceptibility to DHA was assessed microscopically on thin films by estimating the percentage of viable parasites, relative to control (% survival rate). For the controls, the RSA_{0-3h} was also performed on *P. falciparum* reference clones W2 (ART-sensitive control), IPC-4884 and IPC-5202 (BEI Resources, NIAID, NIH, USA) as ART-resistant control lines. A survival rate > 1% was deemed resistant for RSA.

Piperaquine survival assay (PSA)

PSA_{0-3h} was performed on culture-adapted clinical isolates with 0–3-hour ring-stage parasite cultures following a previously published method [17]. Briefly, parasite cultures were tightly synchronized using 5% (w/v) D-sorbitol and 75% Percoll to obtain 0 to 3-hours post-invasion. Synchronized ring parasites at 0.5-1% starting parasitaemia and 2% haematocrit were incubated with 200 nM PPQ or 0.5% lactic acid in water at 37 °C for 48 hours in a 48-well microplate. The cultures were then washed once, resuspended in drug-free medium, and cultured further for 24 hours. Susceptibility to PPQ was assessed microscopically on thin films by estimating the percentage of viable parasites in the similar manner as RSA. A survival >10% was deemed resistant to PPQ.

Growth Assays

For longer-term replication assays, cultures were synchronized as described and resulting in ring stage cultures. The ring stage parasites were synchronized using 5% (w/v) D-sorbitol and parasitaemia levels were calculated, and cultures adjusted to 0.1% parasitaemia, 2% haematocrit in a final volume of 1 mL per well of a 12-well plate. Samples were then taken at t = 0, 24, 72, 120, and 168 h, fixed in 0.8% glutaraldehyde in PBS and stored at 4°C for flow cytometry analysis. Culture media was replaced at 48, 71, and 120 h. Giemsa-stained thin films were also prepared as required for microscopic analysis.

Flow cytometry for parasite quantification

Parasite samples were fixed in 0.8% glutaraldehyde in PBS and stored at 4°C. Cells were prepared for analysis by staining with 2X SYBR Green I nucleic acid gel stain (Invitrogen, Thermo Fisher Scientific) for 30 min at 37°C. Labelling was stopped with an equal volume of PBS and samples acquired using a CytoFlex (Beckman Coulter, USA) with CyEXpert software. Total RBC numbers were calculated using forward- and side-scatter while fluorescence was detected using the 530/630 blue detection laser. Fluorescence intensity was used to distinguish uninfected from infected RBCs, low fluorescence indicating uninfected cells and gating fixed accordingly. Data were analysed using FlowJo.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software, Inc., San Diego, CA, USA). The difference of the data between groups was assessed by nonparametric Mann-Whitney test, as appropriate. Statistical significance was defined as a P value of < 0.05.

Results

CRISPR-Cas9-mediated editing to introduce *pfexo*^{E415G} into *P. falciparum* parasites

The *pfexo* gene from *P. falciparum* 3D7 (PF3D7_1362500) consists of 2,274 bp with 2 exons and 1 intron. Exon 1 comprises nucleotides 1-949, while the exon 2 starts from nucleotides 1,082-2,274 (Fig. S1). PfEXO consists of 713 amino acids with a molecular mass of 86.7 kDa. The enzyme contains a peptide signal region (residues 1-70) and a 3' to 5' exonuclease domain (residues 445-618). The DNA sequence of the *pfexo* gene from *P. falciparum* B5 [24], the parental line for genome editing in this study, showed a single non-synonymous mutation, leading to a missense mutation from Lys to Asn at residue 614 (Figs. S2).

To evaluate the impact of the PfEXO-E415G mutation on parasite susceptibility to piperazine and other front-line chemotherapies, we used CRISPR-Cas9 editing to introduce the E415G mutation into the native *pfexo* gene (Fig. 1A). A *pfexo*^{E415G} was efficiently installed onto the *P. falciparum* B5 background [24]. In parallel, a *pfexo*^{E415E} transgenic line expressing wild-type E415 codon in the context of recodonization abating the Cas9 protospacer adjacent motif (PAM) site was also attempted to rule out a role for silent mutations in drug susceptibility. Several transfections were performed to introduce both *pfexo*^{E415E} and *pfexo*^{E415G} mutations, but only three transfections provided revived parasites. Two of the three transfections gave rise to the modified *pfexo* gene, and one transfection did not provide the modified parasites. Successful modification of the *pfexo* gene in the transfected population following the introduction of the targeting vector was observed around 21 days after transfection and confirmed by diagnostic PCR (Fig. 1B, Pre-cloning). Limiting dilution cloning of the modified parasites resulted in the isolation of parasite clone, B5-*rexo*-E415G-B8. It is noted that several clones obtained from the cloning did not contain the modified region at *pfexo* gene. Modification of the native *pfexo* locus was then confirmed in the transgenic line by diagnostic PCR (Fig. 1B, B5-*rexo*-E415G-B8) and genomic sequences of transgenic lines (Fig. 1C). Unfortunately, the *pfexo*^{E415E} transgenic line could not be obtained from this study.

Molecular genotyping of modified *pfexo* transgenic lines and clinical isolates with PfEXO-E415G mutation.

The genotypic profiling for piperazine and artemisinin molecular markers is outlined in Table 1. In addition to the reference clones and engineered parasite lines, two Cambodian isolates from a previous *P. falciparum* clinical efficacy study (ASAP-21 and ASAP-168) [49] were included as they harboured PfEXO-E415G with and without novel PfCRT mutations. As expected, the transgenic B5-*rexo*-E415G-B8 line carried a similar genotypic profile as the parental B5 line except for the E415G mutation in the *pfexo* gene. Regarding the two clinical isolates, both carried the PfK13-C580Y and PfEXO-E415G mutations. The ASAP-21 isolate harboured the novel PfCRT-F145I mutation while ASAP-168 did not. Two methods were carried out to determine the copy number variation of the *pfpm2* gene: the SYBR-green qPCR and breakpoint assays [45]. By using the qPCR assay, ASAP-21 was found to contain multiple copies of *pfpm2* while ASAP-168 carried a single copy of *pfpm2*. On the contrary, both ASAP-21 and ASAP-168 isolates were positive for *pfpm2/3* amplification as detected by the breakpoint assay (>1 copy) (Fig. S3). The non-concordant detection between the two methods were also reported by Ansbro, et al [45] and it was suggested that the breakpoint PCR assay is more sensitive than the qPCR assay for detecting minor clones containing the duplication in field isolates. Therefore, in this study, ASAP-21 and ASAP-168 isolates were treated as having multiple copies of *pfpm2*.

Table 1
Molecular genotyping of *pfexo*-modified, parental and clinical *P. falciparum* parasites.

Sample	PfK13 C580Y	PfEXO E415G	PfCRT							Copy Number Variation			<i>pfpm2/3</i> break point
			T93S	H97Y	F145I	I218F	M343L	C350R	G353V	pfpm1	pfpm2	pfpm3	
3D7	C	E	T	H	F	I	M	C	G	1.00	1.00	1.00	1
B5	C	E	T	H	F	I	M	C	G	1.37	0.93	1.99	1
B5- <i>rexo</i> - E415G- B8	C	G	T	H	F	I	M	C	G	1.96	0.77	2.71	1
ASAP- 21	Y	G	T	H	I	I	M	C	G	1.25	2.31	2.60	>1
ASAP- 168	Y	G	T	H	F	I	M	C	G	1.18	1.11	1.88	>1

Underline and bold letters indicate either mutations or multiple copy number. A cut-off copy number of 1.6 is used to define *pfpm2* multiple copy number.

Table 2
Piperazine (PPQ) susceptibility (mean ± SD)

Sample	PPQ-IC ₅₀ (nM)	PPQ-IC ₉₀ (nM)
B5	112 ± 54	172 ± 9
B5- <i>rexo</i> -E415G-B8	185 ± 54 ¹	243 ± 22 ¹
ASAP-21	5,207 ± 690 ²	N.D.
ASAP-168	111 ± 17 ¹	312 ± 37 ¹

¹No significant difference (*P* value > 0.05) compared to B5 as tested by Mann-Whitney Test

²Significant difference (P value = 0.0167) between ASAP-21 and B5 as tested by Mann-Whitney Test

N.D. for not determined

Drug sensitivity and survival assay of modified *pfexo* transgenic lines and clinical isolates

The transgenic parasites expressing E415G mutant of *pfexo* and the clinical isolates were tested for their PPQ susceptibility (Table 2). No significant difference in IC_{50} values of B5-*rexo*-E415G-B8 and ASAP-168 parasites compared to B5 parasites was observed, while PPQ- IC_{50} value of ASAP-21 parasites was significantly higher than that of B5 parasites. The same trend was observed for PPQ- IC_{90} values in that there was no significant difference in the IC_{90} values for B5-*rexo*-E415G-B8 and ASAP-168 parasites compared to B5 parasites. The PPQ- IC_{90} of ASAP-21 exceeded the highest PPQ concentration used in this study, so the IC_{90} could not be reported.

To evaluate if the modified *pfexo* transgenic parasites and clinical isolates with PfEXO-E415G mutation have cross-resistance, parasite drug sensitivity was also assessed against a panel of antimalarial drugs (Table 3). No significant difference in IC_{50} values between B5 and B5-*rexo*-E415G-B8 parasites were observed for most of antimalarial drugs except for atovaquone (ATG) and proguanil (PG), where higher IC_{50} values were detected in comparison with the B5 parental line. The ASAP-21 parasite had significantly higher IC_{50} values for quinine (QN), chloroquine (CQ), atovaquone (ATQ) and doxycycline (DOX) but lower IC_{50} value for pyronaridine (PND), while the ASAP-168 parasite had reduced drug sensitivity against dihydroartemisinin (DHA), mefloquine (MQ), QN, and ATQ. The reduced drug sensitivity of QN and ATQ was observed in both ASAP-21 and ASAP-168 parasites.

To gain a better understanding of the ART and PPQ resistance phenotypes, the survival assay (RSA_{0-3h} and PSA_{0-3h}) and PPQ-bimodal dose response curve were performed (Fig. 2). For RSA_{0-3h} (Fig. 2A), *P. falciparum* W2 was used as a control of ART-sensitive parasites, while IPC-4884 and IPC5202 parasites were controls for ART-resistant parasites. The ASAP-21 and ASAP-168 parasites, containing the PfK13-C580Y mutation, exhibited % RSA survival rate of greater than 1, while B5 and B5-*rexo*-E415G-B8 parasites, carrying wild-type PfK13, exhibited % RSA survival rate of less than 1, clearly validating the correlation between PfK13-C580Y mutation and ART resistance. The % PSA survival rate of the modified *pfexo* transgenic lines and clinical isolates were next examined (Fig. 2B). PPQ-sensitive parasites *P. falciparum* W2, IPC-4884 and IPC-5202 were used as controls. The transgenic B5-*rexo*-E415G-B8 parasites harbouring merely PfEXO-E415G mutation had a % PSA survival rate of less than 10 (a cut-off for PPQ resistance), similar to that of the parental B5 parasites. This suggested that the presence of PfEXO-E415G mutation alone could not confer the PPQ resistance phenotype. The ASAP-21 and ASAP-168 parasites, containing the PfEXO-E415G mutation in combination with either PfcRT mutation or multiple copies of *pfpm2*, exhibited a % PSA survival rate higher than 10, indicative of PPQ-resistance.

Table 3

In vitro susceptibility of *P. falciparum*, parental (B5), *pfexo*-modified (B5-rexo-E415G-B8) and adapted clinical parasites to dihydroartemisinin (DHA), artesunate (AS), mefloquine (MQ), quinine (QN), chloroquine (CQ), atovaquone (ATQ), lumefantrine (LUM), doxycycline (DOX), tafenoquine (TQ), cycloguanil (CYC), primaquine (PQ), proguanil (PG), and pyronaridine (PND). (mean \pm SD, nM)

Drug	IC ₅₀ (nM)				P-value
	B5	B5-rexo-E415G-B8	ASAP-21	ASAP-168	
DHA	9 \pm 4	14 \pm 6	5 \pm 0.2	2 \pm 1	0.121 ³
AS	7 \pm 5	4 \pm 1	3 \pm 0.4	3 \pm 1	-
MQ	161 \pm 58	82 \pm 37	61 \pm 23	50 \pm 20	0.048 ³
QN	546 \pm 180	564 \pm 165	71 \pm 3	138 \pm 32	0.017 ^{2,3}
CQ	562 \pm 218	416 \pm 219	64 \pm 12	256 \pm 59	0.017 ²
ATQ	48 \pm 38	114 \pm 1	5 \pm 0.6	4 \pm 1	0.017 ^{1,2,3}
LUM	10 \pm 8	4 \pm 1	2 \pm 0.2	3 \pm 1	-
DOX	13,523 \pm 2,337	14,649 \pm 1,071	8,527 \pm 349	10,920 \pm 1,061	0.024 ²
TQ	263 \pm 143	315 \pm 45	189 \pm 13	274 \pm 554	-
CYC	2,063 \pm 2,703	705 \pm 15	7,183 \pm 388	N.D.	-
PQ	3,094 \pm 2,015	6,387 \pm 218	5,720 \pm 1,160	2,458 \pm 61	-
PG	12,522 \pm 7,247	23,816 \pm 3,786	2,114 \pm 243	3,643 \pm 473	0.033 ¹
PND	28 \pm 16	25 \pm 4	50 \pm 2	12 \pm 6	0.033 ²

P-value calculated by Mann-Whitney Test

^{1,2,3} significant differences between data from B5-rexo-E415G-B8, ASAP-21, and ASAP-168 compared to B5 line, respectively.

N.D. for not determined

In addition to PSA_{0-3h}, PPQ-resistant parasites have been reported to exhibit a bimodal dose-response curve with a second peak between 0.01 and 100 μ M and that the AUC correlates with the degree of PPQ resistance [16]. B5 and B5-rexo-E415G-B8 parasites did not exhibit the bimodal dose-response curve, confirming the PPQ-sensitive phenotype, whereas both ASAP-21 and ASAP-168 parasites clearly showed the bimodal dose-response curve with the AUC of 1029 and 596, respectively (Figs. 3C-F). The AUC of these parasites was in good agreement with both IC₅₀ and IC₉₀ values in that ASAP-21 parasites are more resistant to PPQ than ASAP-168 parasites.

Growth Assay

To assess whether the introduction of E415G mutation into PfEXO affects the parasite fitness, growth assays of B5-rexo-E415G-B8 parasites was carried out in comparison with W2, 3D7, B5 and the other two culture adapted clinical isolates (ASAP-21 and ASAP-168) (Fig. 3). Parasite lines originating from Cambodia (i.e., B5, B5-rexo-E415G-B8, ASAP-21 and ASAP-168) grew much more slowly than W2 (an Indochina clone exhibiting CQ resistance) and 37D (African origin) lines. Parasite B5-rexo-E415G-B8, harbouring *pfexo*-E415G mutation, seemed to grow slightly faster than its parental line B5, suggesting this mutation may improve the parasite's fitness. In the presence of Pfk13-C580Y, PfEXO-E415G, novel PfcRT-F145I mutations and multiple copy number of *pfpm2*, ASAP-21 parasite had a severely retarded growth rate. ASAP-168, having the similar genetic background to ASAP-21 but without the PfcRT-F145I mutation, grew marginally better than ASAP-21 parasites. This observation implies an important contribution of the strain background toward parasite fitness, especially when Pfk13-C580Y, PfcRT-F145I mutations and multiple copy number of *pfpm2* were present.

Discussion

Validated molecular markers, such as *pfk13* for ART resistance, have been widely employed as identification and prediction tools for the emergence of drug-resistant *P. falciparum* [50]. While a molecular marker of PPQ resistant *P. falciparum* malaria has been reported [42], there is

still a pressing need for validating potential new or additive molecular markers to determine effects on PPQ as a partner drug in ACT. In this study, it is evident that the E415G mutation in a gene encoding exonuclease cannot alone reduce PPQ susceptibility nor improve parasite survival rates that are characteristic of PPQ resistant isolates circulating in the Greater Mekong Subregion [17]

Exonucleases are essential to genome stability, catalysing the removal of a single nucleotide monophosphate (dNMP) from the end of one strand of DNA and acting as a proof-reader during DNA replication [51]. Exonucleases are highly conserved and can be classified into families based on both sequence and functional homology, including the 5'-3' exo C-terminal domain (CTD) superfamily, the RNAaseH domain superfamily i.e., DnaQ-like family and other 3'-5' exonucleases. The *pfexo* gene from *P. falciparum* (PF3D7_1362500) encodes a protein with a mass of 86.7 kDa with a 3'-5' exonuclease domain at the C-terminus. In this study, the *P. falciparum* B5 line used for genome editing had one mutation (K614N) in exonuclease). Nucleases may be partially or fully redundant, depending on the pathway, and such redundancy might complement functional losses. Zhang Q et. al. [52] used bioinformatics to predict eight putative RNA exosome-associated proteins in the *P. falciparum* genome, including exoribonuclease functional domain-containing proteins Dis3 and Rrp6, the latter of which was found in PfEXO of this study. Even though, the genuine function of PfEXO has not been revealed, it is clear that the E415G mutation in PfEXO is unlikely to impact on the catalytic activity of PfEXO.

CRISPR/Cas9 approaches have transformed the speed and scale with which *Plasmodium* genome editing can be achieved, and the approach has been employed to introduce point mutations into several *P. falciparum* genes [53]. In this study, instead of using *P. falciparum* 3D7 or Dd2 lines, the Cambodia adapted-B5 line [24] was employed with the hypothesis that the B5 line has a close genetic background to the currently circulating *P. falciparum* in Cambodia where both ART- and PPQ-resistance parasites have been detected. It has been previously shown that different phenotypes were observed when using different parasite backgrounds. Targeted gene disruption of either *pfpm2* or *pfpm3* in the 3D7 genetic background caused a only slight decrease in PPQ susceptibility [54] and *pfpm2* and *pfpm3* overexpression in 3D7 did not alter the sensitivity of *P. falciparum* to PPQ [55]. However, when *P. falciparum* Dd2 parasites with copy number variation in *pfpm2* were generated [29], *pfpm2* amplification contributed to PPQ resistance, with a bimodal dose-response observed. In addition to a qPCR assay to detect *pfpm2* copy number variation, the breakpoint assay, as described by Imwong M, et. al. [56], was carried out. It was found that in parasites with a *pfpm2/3* copy number above the cut-off of 1.52, 88% were confirmed to have *pfpm2/3* amplification by the breakpoint assay, while for those <1.14 (the cut-off for a single copy number) and for an intermediate value between 1.14-1.52, the proportion of *pfpm2/3* amplified parasites using the breakpoint SNP were 4% and 38%, respectively. In this study, the ASAP-168 parasite was found to have *pfpm2* copy number variation of 1.11, lower than the cut-off for a single copy number, but the breakpoint assay confirmed the gene duplication of *pfpm2*. Therefore, it is important to also apply the breakpoint assay to isolates with copy number values < 1.52, to capture all isolates with *pfpm2/3* amplification.

IC₉₀ values, PPQ survival rates, and bimodal dose-response curves were used for assessing PPQ resistance *in vitro* [16, 17, 48]. All three assays confirmed that the engineered parasites harboring the E415G-PfEXO mutation (B5-rexo-E415G-B8) did not show a PPQ resistant phenotype. On the contrary, parasites with the combination of PfEXO-E415G mutation with either novel PfCRT or *pfpm2* multiple copies demonstrated reduced IC₉₀ susceptibility, high PPQ survival rates, and a second peak of bimodal curve (Fig. 3). If the PfEXO-E415G mutation does not alter the PPQ susceptibility, this finding also recapitulates the work of Silva, M et. al. [29] that parasites with the *pfpm2* multiple copy alone (i.e. ASAP-168) show reduced PPQ susceptibility and involved in PPQ resistance. Nonetheless, when the novel PfCRT was added on top of the *pfpm2* multiple copies, the level of PPQ resistance increased tremendously as judged by the IC₅₀ and IC₉₀ values as well as AUC between 0.01-100 μM of PPQ in the bimodal dose-response curve. Bopp, S et. al. [16] showed that when exposed up to 10 μM PPQ for 12 hours, PPQ resistance parasites could survive and complete their lifecycle. It was also previously evident that the introduction of novel PfCRT mutations resulted in a fitness cost for the mutations. Similar trends were observed in this study; parasites holding either novel PfCRT or *pfpm2* multiple copies grew much slower than those without mutations. It is likely that introducing PfEXO-E415G mutations may account for better fitness compared to parasites with the same genetic background. Even though the scope of this work could not address the function of PfEXO, it is evidently showed that the E415G mutation in PfEXO does not alter PPQ susceptibility, but rather affects parasite fitness.

Conclusion

In summary, this study suggests that the E415G mutation in PfEXO may not influence PPQ susceptibility and that caution should be used if assessing this mutation as a molecular marker to monitor drug resistance. The insertion of the PfEXO-E415G mutation did not lead to an increased PPQ-IC₉₀ or improve % PPQ survival, suggesting that this mutation is not associated with PPQ resistance and unsuitable to be used as a molecular marker for monitoring PPQ-resistant parasites. Additionally, this specific mutation resulted in parasites that grew better than those with the same background, highlighting the importance of genetic mutations toward parasite fitness and resistance.

Declarations

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Author's contributions

NB conceived, designed, and executed the study. BAV, MW, JSG, and NCW conceived, and supported the study. NB constructed all plasmids used in this study. NB and KK performed transfection, cloning and selection processes. KK, CT and CP performed cell culture, drug susceptibility assay, survival assay. CP performed cell growth assay and flow cytometry analysis. NB wrote the first draft. All authors edited, reviewed, and approved the final manuscript.

Disclaimer

Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation/publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense. The investigators have adhered to the policies for protection of human subjects as prescribed in AR 70-25.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing Interests

The authors declare that they have no competing interests.

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Figures

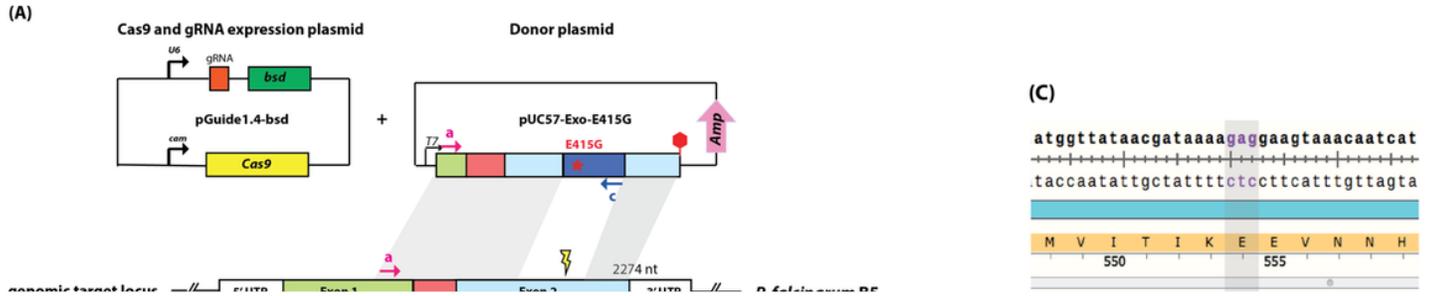


Figure 1

Gene editing of *pfexo*. (A) CRISPR-Cas9 editing was used to install the *pfexo*^{E415G} variant codon into the endogenous locus. Using a donor plasmid and Cas9-aided homologous recombination, the region of the *pfexo* encoding Met409 to Glu514 was replaced with a recodoned *pfexo* gene (navy blue) with the point mutation at residue 415 (a red star) from Glu to Gly. Stop codon is represented as a red hexagon. Position of hybridisation of primers used for confirmation of the integration event by diagnostic PCR are shown as coloured arrows. A schematic of the co-transfected plasmid for expression of Cas9 nuclease and the guide RNAs is also shown. (B) Diagnostic PCR analysis of genomic DNA of the control parental B5 line, the transfected parasites prior to limiting dilution cloning and clone parasite lines expressing *pfexo*^{E415G} (clone B8). Expected sizes of the various PCR amplicons are indicated on the right of the gel, whilst the left-hand lane contains double-stranded DNA ladder marker fragment (Quick-load Purple 1 kb Plus DNA ladder (New England Biolabs)). (C) Chromatograms of a region of exon 2 obtained from the PCR product amplified *pfexo* gene of B5 and B5-*rexo*-E415G-B8 lines. The codon “GAG” encoding Glu in B5 parasites is changed to “GGC” encoding Gly in B5-*rexo*-E415G-B8 clone.

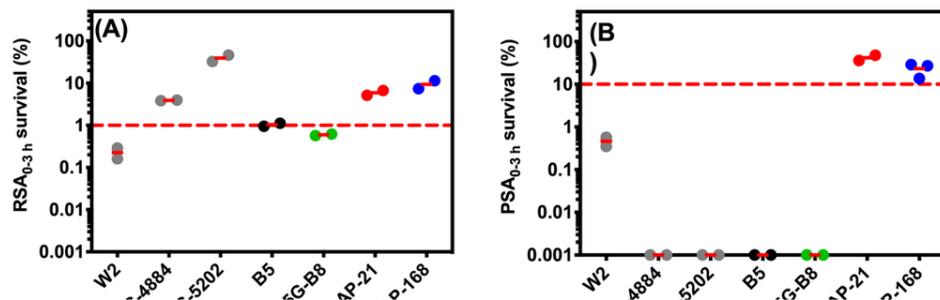


Figure 2

Phenotypic analysis of *pfexo* transgenic lines and clinical isolates. (A-B) *In vitro* RSA_{0-3h} and PSA_{0-3h} survival rates, respectively, for standard laboratory-adapted clones (W2 for an ART-sensitive control, IPC-4884 and IPC-5202 for ART-resistance control), B5 (parental line, B5-rexo-E415G-B8 (modified *pfexo* transgenic line) and culture-adapted clinical isolates (ASAP-21 and ASAP-168). The dashed line represents the 1% survival rate cut-off that differentiates ART-resistance ($\geq 1\%$) from ART-sensitive ($< 1\%$) parasites in RSAs or the 10% survival rate cut-off that distinguishes PPQ-resistance ($\geq 10\%$) from PPQ-sensitive ($< 10\%$) parasites in PSAs. Two independent biological replicates were performed. Zero values of % survival rate were plotted as 0.001 % in logarithmic scale. (C-F) PPQ-bimodal dose-response curves of B5, B8, IPC-4884, and IPC-5202, respectively. Increasing the starting concentration and number of data points (24 points) for HRP2 ELISA dose-response curve provided a bimodal distribution of parasite response to PPQ exposure for PPQ-resistant parasites. ASAP-21 and ASAP-168 shows a second peak in PPQ concentration 0.01- 100 μM . The concentration range used to calculate the area under the curve (AUC) is indicated. Data are shown as mean values from three biological replicates with S.D.

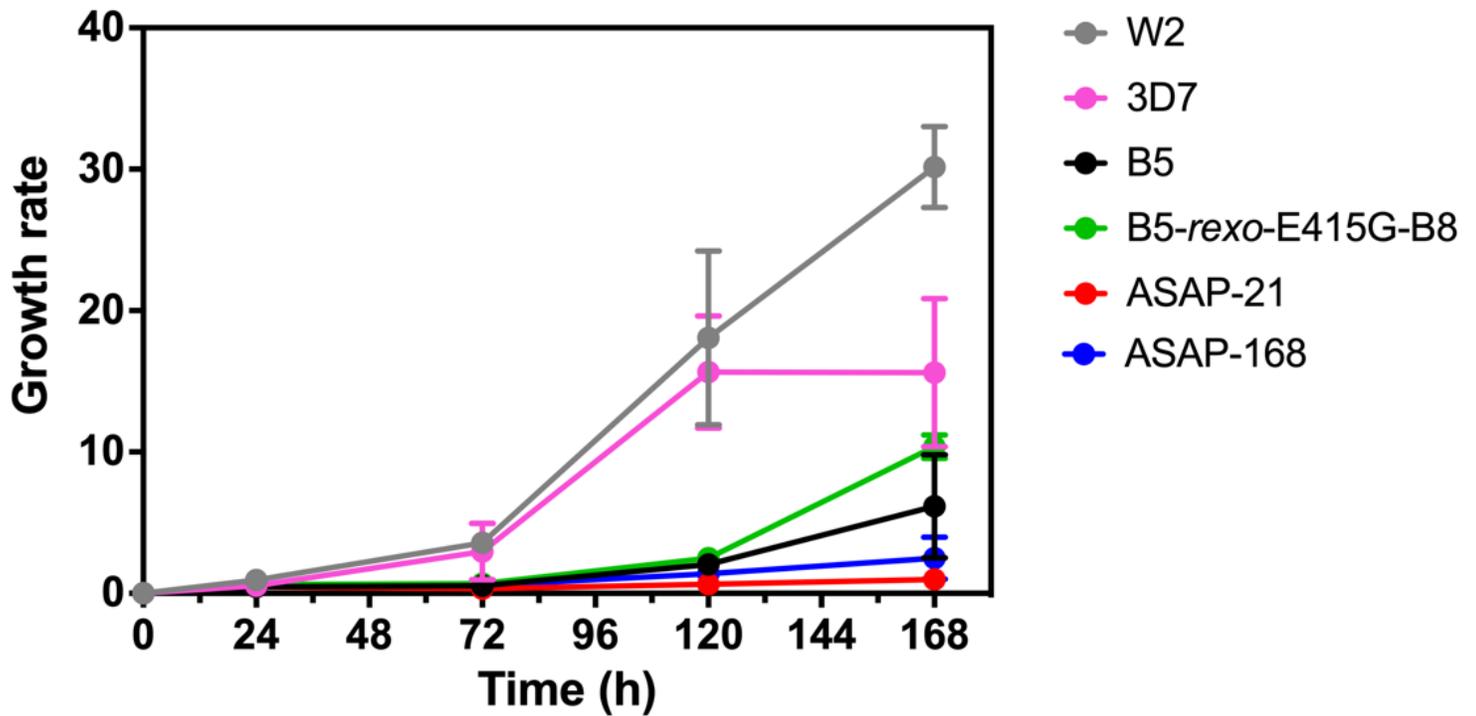


Figure 3

Analysis of growth rates. *P. falciparum* parasites with PfEXO wild-type (W2, 3D7 and B5), PfEXO-E415G transgenic line (B5-rexo-E415G-B8), and clinical isolates with the combination of PfEXO-E415G and multiple *pfp2* copies (ASAP-21 and ASAP-168) were synchronized (initiated at 0.1% parasitaemia ring stage, 2% haematocrit) and followed for 168 hours. Nucleic acids were stained with SYBR Green I and parasitaemia assessed by flow cytometry. Raw values were corrected, and data represent mean \pm S.D. from 3 independent experiments.

Supplementary Files

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- [20220104SupportingInfo.docx](#)