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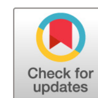
MECHANISMS OF RESISTANCE



An Alternative Autophagy-Related Mechanism of Chloroquine Drug Resistance in the Malaria Parasite

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ABSTRACT We generated highly chloroquine (CQ)-resistant (ResCQ) *Plasmodium yoelii* parasites by stepwise exposure to increasing concentrations of CQ and CQ-sensitive parasites (SenCQ) by parallel mock treatments. No mutations in genes that are associated with drug resistance were detected in ResCQ clones. Autophagy-related genes were highly upregulated in SenCQ compared to ResCQ parasites during CQ treatment. This indicates that CQ resistance can be developed in the malaria parasite by the inhibition of autophagy as an alternative drug resistance mechanism.

KEYWORDS malaria, *Plasmodium yoelii*, chloroquine, drug resistance, autophagy, drug resistance mechanisms

Due to its effectiveness, affordability, and relatively safe profile, chloroquine (CQ) was the mainstay of malaria treatment for many years. However, the heavy usage of chloroquine led to the development of drug resistance in Cambodia and Thailand, which lately has spread to many other countries (1). In CQ-resistant parasites, it was found that the CQ accumulation inside the digestive vacuole was decreased. Later, it was reported that the main reason for reduced CQ accumulation in digestive vacuole was mutation in the CQ-resistant transporter (*CRT*) gene (2, 3). Numerous mutations related to CQ resistance have been reported; however, the K76T mutation in the *CRT* gene is the widely reported mutation in all *in vitro* CQ-resistant parasites (4).

CQ is still the drug of choice to treat *Plasmodium vivax*. However, it is not known if there is any other mode of CQ action and therefore another resistance mechanism.

Here, in this study, we generated CQ-resistant and -sensitive *P. yoelii* parasite clones (ResCQ and SenCQ, respectively), which resemble *P. vivax* in growing in reticulocytes (5, 6), as a model to study an alternative CQ mode of action and drug resistance mechanisms.

6–8 weeks old female BALB/c mice were used in the study. The G_0 frozen stocks of *P. yoelii* 17X-NL *p230p(−)* clone A5 were stored at −150°C. Freshly thawed parasites were injected into naive donor mice. When the parasitemia reached 0.5 to 1%, the donor mice were bled and 20,000 infected erythrocytes mixed with incomplete RPMI were intravenously (i.v.) injected into recipient mice. On the second day of infection, when parasitemia reached 0.5%, the parasites were exposed to the stepwise ascending concentration of CQ for 7 consecutive days. The initial concentration of CQ was 0.1X or 10% (28.8 mg/L) the curative dose, which was gradually increased up to 2X (600 mg/L) the curative dose given orally in water and decided based on previous studies (7, 8).

The corresponding sequences of *P. yoelii* orthologues of the drug resistance mutation-bearing genes *PfCRT*, *PfMDR1*, *PfMDR2*, *PfKelch13*, and *PfDHFR* of the human

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malaria parasite *Plasmodium falciparum* were selected and analyzed using Molecular Evolutionary Genetics Analysis (MEGA) software.

For the characterization of drug-resistant loci, 1, 5, and 10 parasite doses of highly drug-resistant populations were obtained by limiting serial dilution and were intravenously (i.v.) injected into groups of mice (n = 10) to obtain clonal parasite populations. We did not observe any blood-stage parasitemia in mice injected with 1 and 5 parasites, and therefore we moved further to mice injected with 10 parasites, which showed blood-stage parasitemia at days 7 and 8 post injection, which we considered clonal parasite populations. At 2 to 3% parasitemia, mice were bled and the total genomic DNA was extracted using total genomic DNA extraction kit (Qiagen; catalog no. 69506). The coding sequences of targeted genes with known mutations were amplified from genomic DNA using gene-specific oligonucleotide primers and were subjected to sequencing (MCLAB, USA). The primer sequences used in this study are as follows: *CRT* gene, pair 1, forward, TGGTAGTAACACTACACCTTATATAGTACA, reverse, CTGTAGAAGCAT CTAATAATGAGATAAGGA (*PyK74* = *PfK76*), pair 2, forward, TGGGAGCATTCATTATACT TATTACTATAG, reverse, TTGTGCTCATTTCAGAGAATGGCATA (*PyA220* = *PfA220*), and pair 3, forward, TGTGTGATAACTGTGAGGGAGCATGGGT, reverse, CCAAATAAGTAGCCAAA CTGTAAGGAAGA (*PyN326*, *I356*, *R731* = *PfN326*, *I356*, *R371*); *MDR1* gene, pair, 1, forward, GAGTTAAATAAGAAATCTACCGTTGAGTTG, reverse, TCGGACTTTTTTATTACATATAGAGCTGCA (*PyN84*, *Y182* = *PfN86*, *Y184*), and pair 2, forward, GTTGGAGAAACTGGATGTGGTAAATCCACA, reverse, TGTGAATCAAGAGATGAAGTAGCTTCGTCT (*PyD1248* = *PfD1246*); *MDR2* gene, forward, CACCAATTTATCTTGGTTTAGCATCAACTG, reverse, TCTAATGAATCATGAGCTATACCATGA (*PyV514* = *PfT484*); *Kelch13* gene, forward, AGAAATCCGCTAACCATACCTATACCT, reverse, AAGCTGCCCCTGAACTTCTAGCTTCTA (*PyF458*, *R573 C592* = *PfF446*, *R561*,*C580*); and *DHFR* gene, forward, TCGATATATATGCAATTTGTGCATGTTG, reverse CGTTTATTTCTGGGAA TAAAACATCACA (*PyN48*, *S56*, *S108*, *I173* = *PfN51*, *C59*, *S108*, *I164*).

Total RNA was isolated using TRIzol reagent and Phase Lock Gel tubes (5-Prime; catalog no. 2302830) according to the manufacturer's instruction. Total RNA was reverse transcribed into cDNA using the oligo(dT) primer and SuperScript III first-strand synthesis system for reverse transcription-PCR (RT-PCR) (Invitrogen; catalog no. 18080-051). The real-time expression was checked using iTaq Universal SYBR green supermix (Bio Rad; cataog no. 172-5120) with the respective primer sequence of targeted genes. The primer sequences used in RT-PCR analysis are as follows: ATG5, forward, CTGCATGTGAATTTTCCCAC, reverse, AAGGAGATTTTAATGGAATTTG; ATG8, forward, CCCCTTTGAAAGTAGAGTTGCAG, reverse, CGCTTTTTCACACACCACTGG; ATG12, forward, TGGAAATGAAACACTAGTATCCA, reverse, TCCAAATTGGGTTTAATCGTGTT; Metacaspase1, forward, AAAACAGAAGGAGAAATAATTG, reverse, AATTTAACTCCATCTTTTAATGG; and green fluorescent protein (GFP), forward, GATACCCGGACCACATGAAG, reverse, GATCCT GTTGACGAGGGTGT.

Statistical analysis was done by one-way analysis of variance (ANOVA) with GraphPad Prism 5. Data are presented as the mean $\pm$ standard deviation (SD) of values from at least triplicate determinations. Significance is noted as follows: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

For the selection of CQ highly resistant (ResCQ) and CQ-sensitive (SenCQ) clones, we chose a parasite clone of *P. yoelii* wild-type-like (WT-like) *Pyp230p(−)* clone A5, expressing enhanced GFP (eGFP) constitutively under the control of *PyHSP70_1* promoter (9), as a starting G_0 clone. The average water uptake of mice is around 5 mL/day (7, 8). Therefore, this curative dose, the curative dose i.e. 288 mg/L, corresponds to a 1.44-mg/day CQ consumption. Although the plasma CQ level depends on different parameters like *Plasmodium* species, percentage of parasitemia, and duration of exposure, a study reported that a 50-mg/kg dose resulted in 1 μM and 14.2 μM plasma CQ levels after intraperitoneal (i.p.) injection and oral gavage, respectively (10). If we take 20 g as the average weight of a 7-week-old mouse, then a 50-mg/kg dose will correspond to 1 mg per mouse. Consequently, a 1.44-mg/day CQ consumption will result in 20.36 μM plasma CQ. The curative dose of 288 mg/L was determined in previous studies

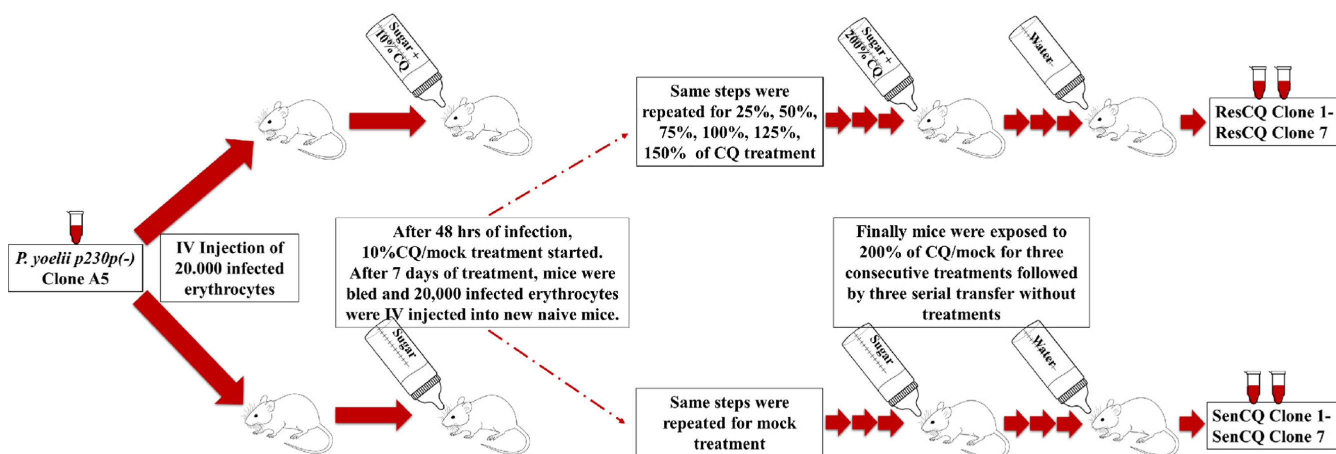

FIG 1 Schematic representation of the generation of ResCQ and SenCQ parasite clones from the *P. yoelii p230p(−)* A5 parasite clone. BALB/c mice were intravenously (i.v.) injected with 20,000 ($n = 3$) infected erythrocytes of the *P. yoelii p230p(−)* A5 parasite clone. On the second day post infection, when the parasitemia reached ≥0.5%, mice were exposed to 10% of the curative dose of CQ (which was determined to be 288 mg/L of drinking water) for 7 consecutive days. Mice were bled, 20,000 infected erythrocytes were serially i.v. transferred into naive mice, and frozen stocks were made. The concentration of CQ was gradually increased from 10% of the curative dose to over 200% of the curative dose (600 mg CQ/L of drinking water) over 7 more serial transfers (with 25, 50, 75, 100, 125, 150, and 200% of the curative dose of CQ) according to the same serial transfer and CQ treatment procedures. After two additional serial transfers with over 200% of the curative dose of CQ treatments, three consecutive transfers with no drug or sugar were applied to all groups to reduce or remove any effects of phenotypic resistance due to constitutive drug application. Thus, the parental ResCQ parasite line was established, which was used to generate multiple ResCQ parasite clones. The exact same serial transfers and procedures were done in parallel groups of mice that were mock treated with no drug in drinking water to get the parental sensitive CQ (SenCQ) strain, which was used to obtain several SenCQ parasite clones. All CQ- or mock-treated drinking water was provided with 10 g sucrose/L.

with the *P. berghei* ANKA strain in mice (7, 8). First, we confirmed that the A5 clone is sensitive to the curative CQ dose in drinking water. Thereafter, the stepwise exposure to ascending concentrations of CQ started with 10% of the curative dose for a period of 1 week of CQ treatment and continued by serially transferring parasites into new mice after each treatment cycle with 25, 50, 75, 100, 125, and 150% curative doses of CQ, and then we applied 600 mg/L of drinking water, which is more than 200% of the CQ curative dose, for three consecutive treatments, followed by three serial transfers without treatments to remove any effects of CQ phenotypic temporary resistance (Fig. 1). After these 13 serial transfers, we established ResCQ parental parasites, which were used to select 7 clones of ResCQ by limited dilution. We established SenCQ parasites by likewise 13 serial transfers with mock treatments, followed by selection of 7 clones of SenCQ by limited dilution (Fig. 1).

Initially, the 7 clones selected for ResCQ and the 7 clones selected for SenCQ showed similar growth to their respective parental parasites without CQ treatments (data are shown in Fig. 2 for only two clones from each set of ResCQ and SenCQ clones). The growth rates of the parental ResCQ and SenCQ parasites were compared to each other and to the starting A5 clone, and there was no significant difference observed in the parasitemia of any of the three parasite populations (data not shown). For easier continuation of the evaluation of the established resistant pattern, two clones were selected for each of the ResCQ and SenCQ parasites. The ResCQ parental and ResCQ clonal parasites grow similarly to each other with exposure to more than 2X of the curative dose or without drug treatment (Fig. 2A and B), However, the percentages of parasitemia in the SenCQ parental strain and SenCQ clonal parasites go down rapidly with exposure to more than 2X of the curative dose of CQ (Fig. 2C and D).

Moreover, the intraerythrocytic development and the expression of eGFP of ResCQ clone 1 (Fig. 3A) and SenCQ clone 1 (Fig. 3B) showed normal development of trophozoites at 6 h and schizonts at 12 h from the i.v. injection of merozoites of each clone into mice.

The *P. yoelii 17X* orthologs of the *P. falciparum* drug resistance mutation-bearing genes were compared to identify the possible mutation sites, if any (Fig. 4). Forward

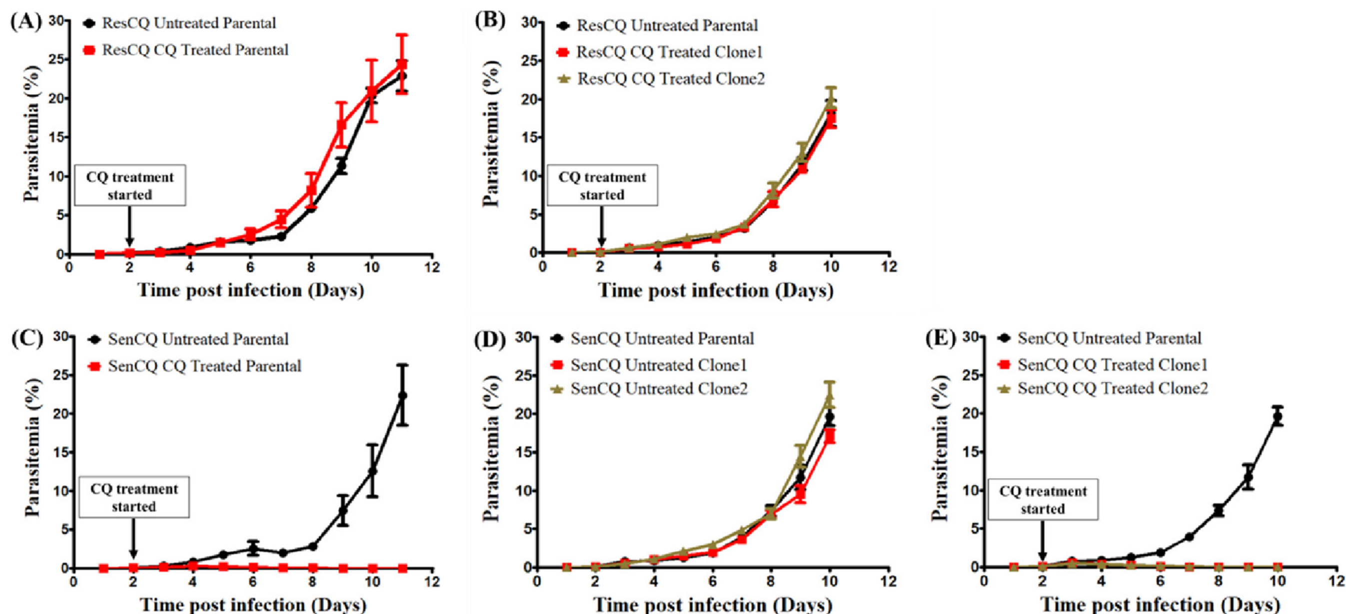

FIG 2 ResCQ parental and clonal parasites show normal growth under CQ or mock treatment, whereas SenCQ parental and clonal parasites are highly sensitive to CQ treatment. Groups of BALBc mice were i.v. injected with 20,000 infected erythrocytes per mouse with ResCQ parental parasites, ResCQ clones 1 and 2 ($n = 5$ mice) (A and B), or with SenCQ untreated parental parasites, SenCQ clones 1 and 2 ($n = 4$) (C, D, and E). ResCQ parental parasites showed similar growth under CQ or mock treatments, whereas in SenCQ parental parasites, parasites given mock treatment showed normal parasite growth, while those given CQ treatment quickly cleared parasitemia (A and C). The same results were obtained when ResCQ and SenCQ clonal parasites were treated with CQ or mock treated (B, D, and E).

and reverse primers were designed at about 250 bp upstream and downstream of the candidate mutation site, respectively, and were used to amplify these amplicons from ResCQ clones 1 and 2, which were extracted and purified from agarose gels and sequenced. We targeted known mutations in *PfCRT, PfMDR1*, *PfMDR2*, *PfKelch13*, and *PfDHFR.* Mutations in all of these genes have been confirmed to play important roles in generation of drug resistance against various antimalarials like CQ, pyrimethamine, and artemisinin (11–14). Reduction in the accumulation of CQ in the digestive vacuole because of *PfCRT* mutation is the widely accepted explanation of CQ drug resistance. However, in this study we did not find any mutation in any of the drug resistance-associated genes in ResCQ clonal parasites, which suggests that the prevention of CQ accumulation in the digestive vacuole is not the only mechanism of resistance to CQ. An earlier study showed that the CQ resistance in *Plasmodium* spp. does not depend only on CQ accumulation in digestive vacuole (15). Another study explains the differences in the CQ accumulation in digestive vacuoles in sensitive versus resistant parasites, the mechanism of resistance at cytostatic versus cytocidal CQ concentrations, and the role of autophagy in resistance against cytocidal CQ concentration (16). These observations confirm that the accumulation of CQ in the digestive vacuole is not the only mechanism of CQ resistance, and therefore an alternative CQ resistance mechanism needs to be explored.

To find out the alternative mechanisms of CQ resistance, we checked the regulation of the autophagy pathway. Autophagy is a well-known stress response mechanism in higher eukaryotes, used by the cells to cope with different physical or chemical stress responses (17). Autophagy is controlled by a group of autophagy-related proteins (ATGs) and in cancer is considered as a double-edged sword, where it can be upregulated or downregulated in favor of cancer treatment (18–21). The homologs of ATGs have been found in the *Plasmodium* parasite, yet their function in terms of autophagy is not clear (22). Here, we checked the mRNA expression of ATGs, including ATG5, ATG8, and ATG12, in SenCQ and ResCQ clonal parasites with or without CQ chemical

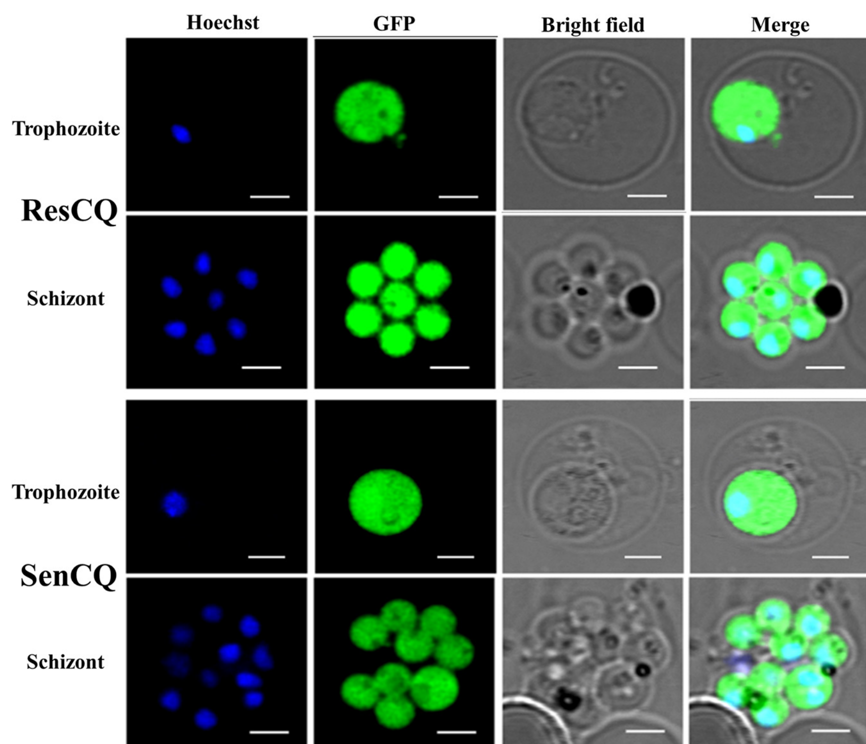

FIG 3 Live fluorescence microscopy shows normal development and viability of ResCQ and SenCQ clonal parasites. Confocal microscopy of live blood-stage parasites after 6 and 12 h of i.v. injection of merozoites showed that the serial transfers and the generation of CQ resistance are not causing any viability or developmental changes in blood-stage parasites. (Scale bar is 2 μm).

stress. We found that the expression of ATGs was highly upregulated in SenCQ parasites, whereas in ResCQ parasites, there were no significant change in the expression of ATGs compared to ResCQ or SenCQ parasites under no CQ treatment (Fig. 5). The expression of ATG5 was increased 9.8-fold ($P < 0.001$), that of ATG8 was increased 11.8-fold ($P < 0.001$), and that of ATG12 was increased 3.1-fold ($P < 0.05$) in CQ-treated SenCQ compared to untreated SenCQ parasites (Fig. 5A, B, and C). These results show the induction of autophagy in CQ-sensitive (SenCQ) parasite population. The upregulation of autophagy as a cell death mechanism has also been reported after CQ treatment in esophageal carcinoma cells (23). We also checked the expression of the gene coding for the apoptosis marker Metacaspase-1; however, no change in the expression of Metacaspase-1 was observed (Fig. 5D).

These results suggest the role of autophagy in CQ-mediated cell death of *Plasmodium* parasites. The results also supported by previous studies. A study reported the CQ toxicity against *P. falciparum* blood-stage parasites mediated by a process like autophagy (24). DNA fragmentation was observed, but the use of a caspase inhibitor did not show any effect on cytotoxicity, which excludes the role of apoptosis in mediation of cytotoxicity of CQ or SNAP. The study observes the cytoplasmatic vacuolization and concluded CQ, SNAP, or staurosporine toxicity is mediated by a process like autophagy (24). Another interesting study reported the role of autophagy in CQ drug resistance in malaria parasites which depends on the CQ concentration used for treatment (25). The study reported both cytostatic and cytocidal CQ resistance by calculating 50% inhibitory concentration (IC_{50}) and 50% lethal dose (LD_{50}) values. They identified that *PfCRT* mutation is the main cause of cytostatic CQ resistant, but this mutation has just a partial role in cytocidal CQ resistance. The quantitative trait loci (QTL) of the progeny of genetically crossed CQ-resistant versus CQ-sensitive parasites showed a unique genetic architecture for cytostatic CQ-resistant and cytocidal CQ-

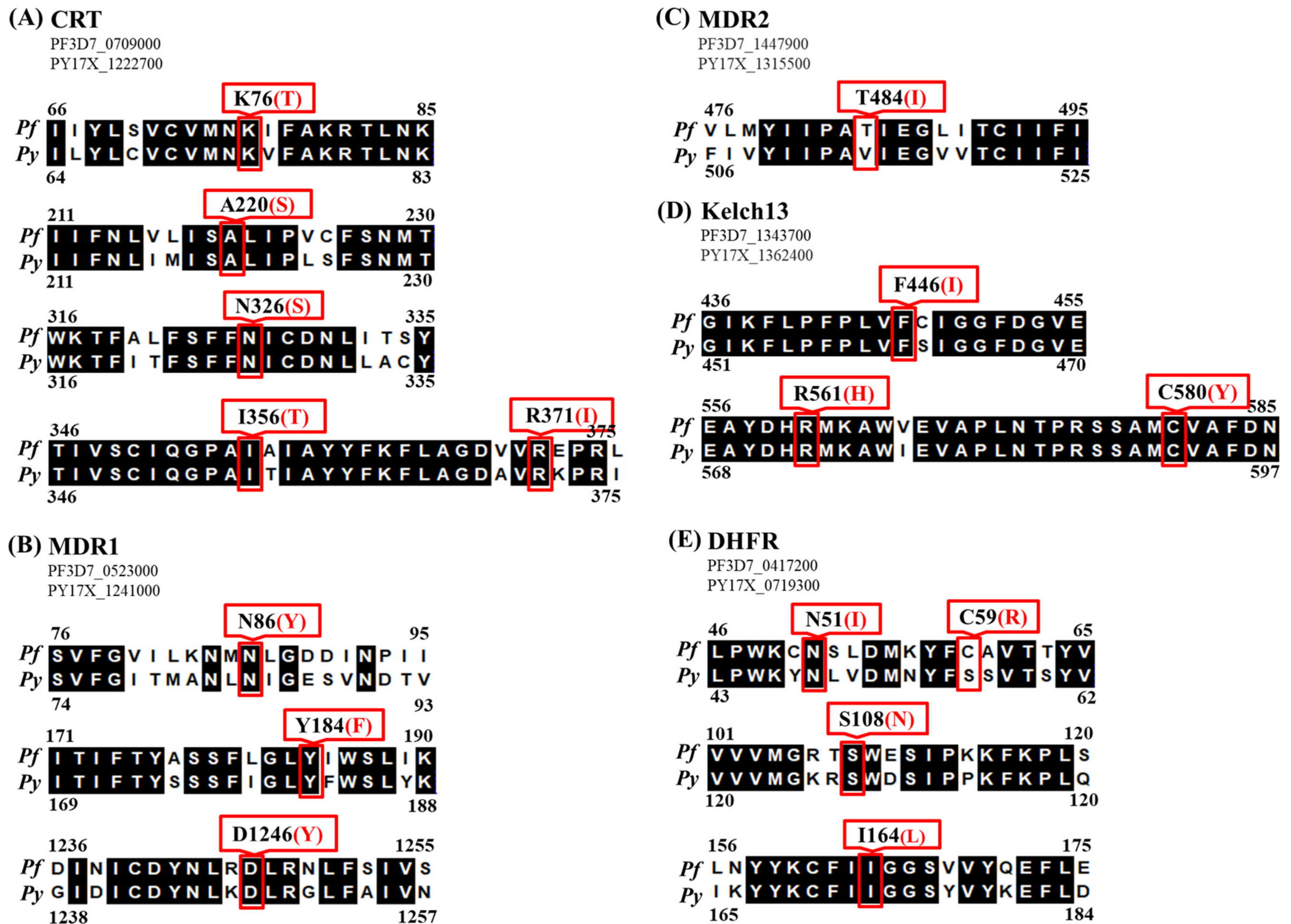

FIG 4 Alignment of *P. yoelii 17X* genes with their *P. falciparum* orthologs of drug resistance mutation-bearing genes. The orthologs of the *P. falciparum* drug resistance mutation-bearing sequences in *P. yoelii* were selected by aligning the *P. yoelii 17X* gene sequence with the targeted genes of *P. falciparum* using Molecular Evolutionary Genetics Analysis (MEGA) software. (A) *PfCRT* K76, A220, N326, I356, and R371 correspond to K74, A220, E271, N326, I356, R371 in *PyCRT*, respectively. (B) *PfMDR1* N86, Y184, and D1246 correspond to N84, Y182, and D1248 in *PyMDR1*, respectively. (C) *PfMDR2* T484 corresponds to V514 in *PyMDR2*. (D) *PfK13* F446, R561, and C580 correspond to F458, R573, and C592 in *PyK13*, respectively. (E) *PfDHFR* N51, C59, S108, and I164 correspond to N48, S56, S108, and I173 in *PyDHFR*, respectively.

resistant parasites. Here, the authors observed a consistent expression profile of genes involved in autophagy in the cytocidal CQ-resistant parasite population and confirmed it by the expression of the ATG8 protein in the cytocidal CQ-resistant parasitic population. Finally, the authors concluded that in addition to the mutation in *PfCRT*, autophagy play an important role in the generation of cytocidal CQ resistance in *P. falciparum* (25). A study discussed the importance of targeting autophagy as a cell death pathway in apicomplexan parasites. The role of autophagy in drug sensitivity and drug resistance was also discussed (26).

Our results are showing that the mutation is not the only reason for the generation of drug resistance. Other mechanisms of drug resistance like autophagy in *Plasmodium* parasites need to be investigated. The results presented here encourage further study to explore autophagy in *Plasmodium* parasites and develop it as a promising target for the new antimalarial compounds as a novel strategy to treat malaria.

Ethics statement. All procedures for animal care and handling of mice in this study were approved by the Experimental Animals Ethical Committee of Bezmialem Vakif University, Istanbul, Turkey (no. 2019/45).

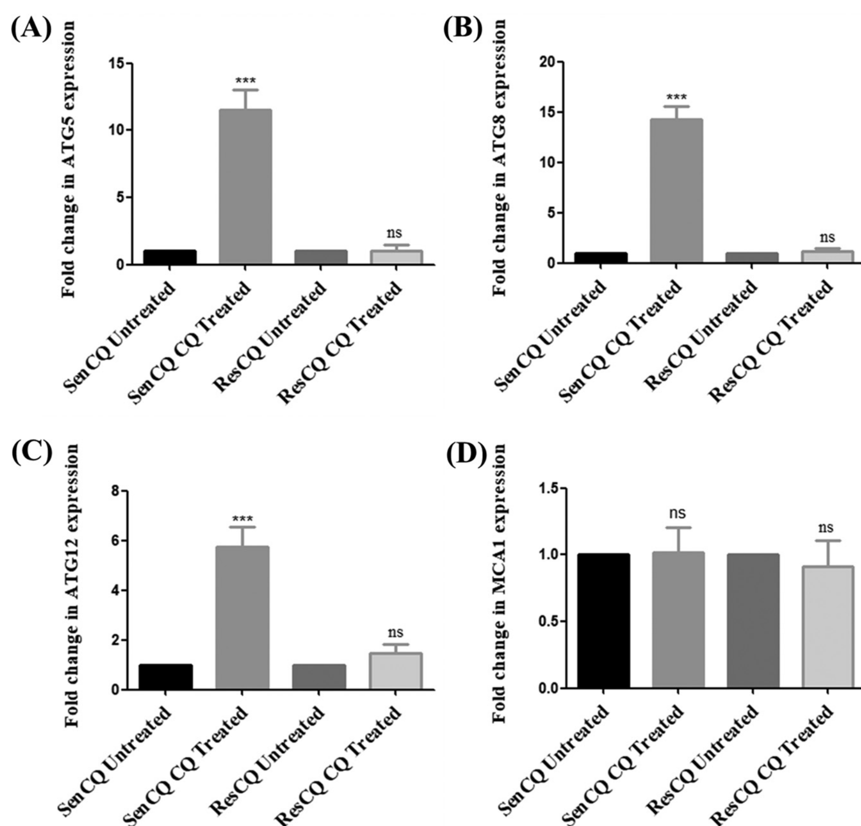

FIG 5 qRT-PCR analysis shows between 6- and 15-fold upregulation of the transcription of the central autophagy-related genes in SenCQ compared to ResCQ parasites during CQ treatment. BALB/c mice were injected with 20,000 infected erythrocytes of ResCQ clones 1 and 2 and SenCQ clones 1 and 2 (n = 4 for each group). When the percentage of parasitemia reached 2%, 2 mice of each group were treated with the curative dose of 300 mg/L of CQ for 2 days, and 2 mice were mock treated. When the parasitemia diminished to between 0.5% and 0.75% in the SenCQ parasite-infected CQ-treated mice, all groups of treated and untreated ResCQ- and SenCQ-infected mice were bled, and total RNA was extracted from all mice in the 4 groups. The extracted RNAs were converted into cDNAs using the first-strand cDNA synthesis kit. Each reaction mixture had an equal amount of RNA from both clones of each of the treated or untreated ResCQ and SenCQ parasites, and this was done in triplicate and repeated three times. The average threshold cycle (C_T) values for the qRT-PCR control were 22.3 (SenCQ untreated), 22.8 (SenCQ treated), 22.6 (ResCQ untreated) and 23.0 (ResCQ treated). ns, nonsignificant; ***, $P < 0.001$ versus mock-treated control.

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We declare no conflict of interest.

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