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Expression and function of *pvcrt-o*, a *Plasmodium vivax* ortholog of *pfcrt*, in *Plasmodium falciparum* and *Dictyostelium discoideum*

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Abstract

Chloroquine resistance in *Plasmodium vivax* threatens the use of this drug as first-line treatment for millions of people infected each year worldwide. Unlike *Plasmodium falciparum*, in which chloroquine resistance is associated with mutations in the *pfcrt* gene encoding a digestive vacuole transmembrane protein, no point mutations have been associated with chloroquine resistance in the *P. vivax* ortholog gene, *pvcrt-o* (also called *pvcg10*). However, the question remains whether *pvcrt-o* can affect chloroquine response independent of mutations. Since *P. vivax* cannot be cultured in vitro, we used two heterologous expression systems to address this question. Results from the first system, in which chloroquine sensitive *P. falciparum* parasites were transformed with *pvcrt-o*, showed a 2.2-fold increase in chloroquine tolerance with *pvcrt-o* expression under a strong promoter; this effect was reversed by verapamil. In the second system, wild type *pvcrt-o* or a mutated form of the gene was expressed in *Dictyostelium discoideum*. Forms of PvCRT-o engineered to express either lysine or threonine at position 76 produced a verapamil-reversible reduction of chloroquine accumulation in this system to ∼60% of that in control cells. Our data support an effect of PvCRT-o on chloroquine transport and/or accumulation by *P. vivax*, independent of the K76T amino acid substitution.

Keywords: Malaria; Chloroquine resistance; *Plasmodium vivax*; *Plasmodium falciparum*; *Dictyostelium discoideum*; Transgenic lines; *pfcrt*; *pvcrt-o*; Luciferase; Transgene expression

1. Introduction

Plasmodium vivax is the most widely distributed human malaria parasite and is responsible for 70–90 million cases annually [1]. Although the disease caused by *P. vivax* does not cause the high fatalities of its more malignant relative *P. falciparum*, the clinical symptoms of vivax malaria generate high morbidity and present a grave socioeconomic burden in countries such as Brazil where it is the most prevalent *Plasmodium* species.

Chloroquine (CQ) was introduced at the end of World War II as first-line treatment against malaria. The drug remained effective everywhere against all malaria for more than a decade until the first cases of *P. falciparum* CQ resistance (CQR) were reported in the late 1950s [2]. Although CQ-resistant *P. falciparum* spread in subsequent decades from a number of different foci [3], resistant *P. vivax* malaria was not reported until 1989 [4]. Cases of resistance have subsequently been reported in Indonesia, Myanmar, India, and South America [5].

CQR in *P. falciparum* relates to its ability to protect against the toxicity of ferriprotoporphyrin IX (FPIX), a byproduct of hemoglobin digestion in the digestive vacuole (DV) of intraery-

Abbreviations: CQ, chloroquine; CQS, chloroquine sensitivity; CQR, chloroquine resistance; DV, digestive vacuole; h, hour; *hdhfr*, human dihydrofolate reductase gene; IC_{50}, 50% drug inhibitory concentration value; *luc*, luciferase gene; min, minutes; PbEF1α, intergenic region of the elongation factor 1α from *P. berghei*; PCR, polymerase chain reaction; RT-PCR, reverse-transcriptase PCR; *pfcrt*, *P. falciparum* CQR transporter gene; *pvcrt-o*, *P. vivax* CQR transporter ortholog gene; RBC, red blood cells; RLU, relative luminescence unit; VP, verapamil; S.D., standard deviation

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throcytic parasite stages [6]. Malaria parasites normally avoid this toxicity by promoting the crystallization of FPIX into hemozoin [7]. CQ exerts its effect by accumulating inside the acidic environment of the DV and forming complexes with FPIX that disrupt essential cellular processes, killing the parasite. CQ-resistant *P. falciparum* parasites reduce CQ accumulation inside the DV and so can survive drug concentrations that sensitive parasites cannot; verapamil (VP) and other chemosensitization agents can partially reverse this resistance [8,9].

CQR in *P. falciparum* is linked to mutations in the vacuolar membrane transporter protein PfCRT [10–12], a member of a newly described family of transporters in apicomplexan parasites and *Dictyostelium discoideum* [13]. These transporters fall within a larger superfamily of drug/metabolite transporters [14,15]. The identification of an ortholog of *pfcrt* in *P. vivax* encouraged the search for point mutations also linked to resistance in this parasite [16]. However, no mutations of this ortholog (*pvcg10*, here termed *pvcrt-o*) have been associated with CQR in *P. vivax*, suggesting that the mechanism of resistance probably differs from that of *P. falciparum*.

In natural infections *P. vivax* CQ-sensitive parasites can be eliminated by the same standard treatment used for *P. falciparum* sensitive isolates (1.5 g CQ base). Treatment with a minimum dose of 0.3 g of CQ and plasma concentrations above 10 ng/ml (19 nM) have cured blood-stage CQ-sensitive *P. vivax*, and Baird [5] has suggested a baseline of 50 ng/ml (97 nM) for in vitro sensitivity. A number of published IC_{50}s suggest that *P. vivax* CQ-sensitive isolates might be able to survive in higher concentrations of the drug in vitro than *P. falciparum* sensitive parasites (50–100 nM versus 30 nM) [3,5,10,17–19], although Kocken et al. reported lower IC_{50}s of 3–5 nM [20,21]. It remains difficult to define clinical and biological CQR for *P. vivax*, particularly in the absence of effective in vitro culture systems and possible confounding effects in drug resistance assays.

Considering the fact that candidate genes for *P. vivax* CQ response are still lacking, we decided to further investigate a possible role of *pvcrt-o* in drug handling. Since *P. vivax* cannot be readily cultured in vitro, we decided to use two previously validated heterologous systems to investigate the effect of *pvcrt-o* expression in CQ response. Here we present evidence for a baseline effect of *pvcrt-o* on CQ accumulation and discuss potential implications of this finding.

2. Materials and methods

2.1. DNA constructs

Plasmid pHDEF1-luc contains the entire intergenic region of the elongation factor 1α of *Plasmodium berghei* (PbEF1α) that works as a bidirectional promoter to drive the expression of both the human dihydrofolate reductase gene (*hdhfr*), used as a selectable marker, and a luciferase (*luc*) cassette as a reporter gene [22]. To test the strength of the other side of the promoter sequence, we inverted the PbEF1α intergenic region from its orientation in the original pHDEF1-luc plasmid to generate pHDEF1i-luc (Fig. 1A). Briefly, plasmid pHDEF1-MH [22] was digested with *Sma*I and *Eco*RI to release the complete PbEF1α intergenic region. The resulting promoter-containing fragment was cloned between the *Sma*I and *Eco*RI sites of plasmid pBS-luc-hrp2 (not shown). Digestion of this resulting plasmid with *Sma*I and *Kpn*I yielded a full cassette containing the inverted promoter sequence, *luc* and the 3′-untranslated region (UTR) of *pfhrp2*. The pHDEF1i-luc construct was finally obtained by ligation of this full cassette with the plasmid backbone obtained from pHDEF1-luc by restriction with *Eco*RI, Klenow treatment, and a second restriction with *Kpn*I.

To amplify the cDNA of *pvcrt-o*, we used total RNA from a *P. vivax* CQ-sensitive isolate from Rondônia, Brazil. A pair of oligonucleotide primers (Fpvcrt-oMH 5′-GGACTAGTATGACCATCCTGAAAAAGAAG and Rpvcrt-oMH 5′-TCCCCCGGGGTGTGTTATTATCGAGTC) was designed to have a *Spe*I site in the forward sequence and a *Sma*I site in the reverse sequence to enable cloning of the complete *pvcrt-o* cDNA sequence in-frame with the myc-His tag present in all transfection plasmids. The vectors obtained were denominated pHDEF1-pvcrt-o, pHDEF1i-pvcrt-o (Fig. 1C and D).

2.2. Parasite transfection

Uninfected red blood cells (RBC) were electroporated according to Deitsch et al. [23] and transfected cells were selected with 5 nM WR99210. For luciferase activity measurements, parasites were harvested then processed according to the manufacturer's protocol (Promega) using a Berthold luminometer (Lumat LB 9507). Activity was recorded in relative luminescence units (RLU) normalized by parasitemia.

RT-PCR assays were used to verify *P. falciparum* transcription of the *P. vivax* transgene *pvcrt-o*. First-strand cDNA synthesis was performed using 1 μg of total RNA and the Superscript-II pre-amplification system (Invitrogen) with random primers. The following oligonucleotide primers were used to screen for the transcripts of *pvcrt-o*, *hdhfr* and a *P. falciparum* myosin gene control—F1pvcrt-o: 5′-ATGACCATCCTGAAAAAGAAG-3′; R1pvcrt-o: 5′-TCAGTGTGTTATTATCGAGTC-3′; Fhdhfr: 5′-ATGGTTGGTTCGCTAAAC-3′; Rhdhfr: 5′-TTAATCATTCTTCTCATATAC-3′; Fpfmyo: 5′-ATGGCTGTTACAAATGAAGA-3′; and Rpfmyo: 5′-TTATTGAGCTACCATTT-3′.

Real time RT-PCR assays were performed using the Syber green method and Rotor Gene machine (Uniscience). The following oligonucleotide primers were employed for the real time experiments—FpfcrtRT: 5′-ATTCCTGTATGCTTTTCAAACATGAC-3′; RpfcrtRT: 5′-AACAATTGGAAAAAGGATACCATAGG-3′; Fpvcrt-oRT: 5′-ATGTCCAAGATGTGCGACGAT-3′; Rpvcrt-oRT: 5′-CTGGTCCCTGTATGCAACTGAC-3′; FpmyoRT: 5′-ATTGTATCTGGTGAATCTGGTGCA-3′; RpfmyoRT: 5′-TCGCATTACCAAAA GCTTCAAG-3′. To analyze the relative amount of transcripts, we used the threshold cycle value (C_t) to calculate the ΔC_t of each sample compared with that of the myosin house keeping gene as well as the $\Delta\Delta C_t$ to determine the proportion of *pfcrt* transcript between 3D7 and the transgenic lines expressing *pvcrt-o* and the proportion of *pvcrt-o* transcript between 3D7/pHDEF1-pvcrt-o and 3D7/pHDEF1i-pvcrt-o [24].