



## *pfmdr1* Amplification Is Related to Increased *Plasmodium falciparum* *In Vitro* Sensitivity to the Bisquinoline Piperaquine

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**The 4-aminoquinoline bisquinoline piperaquine is an important partner drug in one of the presently recommended artemisinin combination therapies. Recent clinical trials have confirmed its high efficacy in combination with dihydroartemisinin. Resistance to piperaquine alone has, however, been documented. Amplification in copy number of the *Plasmodium falciparum* multi-drug resistance locus on chromosome 5, containing the *pfmdr1* gene, has been shown to confer resistance to structurally unrelated antimalarials. Through the determination of the 50% inhibitory concentrations (IC<sub>50</sub>s) and IC<sub>90</sub>s for piperaquine and chloroquine in a set of 46 adapted *P. falciparum* cultures originating from the Thai-Burmese border, we have characterized the regions around the *pfmdr1* gene and identified a significant association between the presence of *pfmdr1* duplications and enhanced sensitivity to piperaquine ( $P = 0.005$  for IC<sub>50</sub> and  $P = 0.002$  for IC<sub>90</sub>) and chloroquine, reaching statistical significance at IC<sub>90</sub>s ( $P = 0.026$ ). These results substantiate the potential importance of *pfmdr1* copy number amplifications in the efficacy of the combination therapy piperaquine-dihydroartemisinin. It supports the rational use of 4-aminoquinolines and artemisinin-based compounds, as they independently select for mutually incompatible combinations of mutations.**

Artemisinin combination therapy (ACT) is instrumental in the global decrease of *Plasmodium falciparum* malaria in recent years. However, *P. falciparum* resistance to several partner drugs, namely, artesunate-mefloquine (25), artemether-lumefantrine (26, 31), and artesunate-amodiaquine (15), has been documented. These early signs have been associated with decreased parasite responses to the slowly eliminated partner drugs, a phenomenon believed to be accelerated in circumstances of high transmission due to the exposure of reinfecting parasites to sub-therapeutic concentrations (14).

A more recent ACT, dihydroartemisinin-piperaquine (DHA-PPQ), has been widely used in Southeast Asia and is now ready to be launched in sub-Saharan Africa. PPQ is a bisquinoline, structurally a 4-aminoquinoline-based antimalarial like chloroquine (CQ). Piperaquine is not a recent newcomer, having been extensively used in monotherapy regimens in the southern regions of China in the 1970s and 1980s, as a response to the rise of CQ resistance. Later, it was adopted by the national Vietnamese malaria control program in several formulations (9). The most recent development of PPQ-based ACT is commercially known as Artekin (Hollekyn Pharmaceuticals, China) or Eurartesim (Sigma-Tau, Italy), each tablet containing 40 mg dihydroartemisinin plus 320 mg piperaquine phosphate.

DHA-PPQ has shown excellent efficacy in recent clinical trials in Africa (1, 4, 17), making it a promising fixed-dose formulation for malaria treatment on the continent. Nevertheless, the PPQ long elimination half-life of >4 weeks after the standard 3-day course (2.25 mg/kg [of body weight] DHA and 18 mg/kg of piperaquine phosphate per day) raises concerns about the long-term sustainability of its efficacy in African high-transmission settings (22). Furthermore, *P. falciparum* resistance to PPQ has long been documented, albeit in the context of monotherapy (9). In this

scenario, the search for molecular markers of early detection of PPQ resistance is increasingly important. Interestingly, the *pfmdr1* (multidrug resistance 1) N86Y and *pfprt* (chloroquine resistance transporter) K76T CQ resistance markers, known to confer different degrees of resistance to the common 4-aminoquinolines, have not been consistently associated with parasite response to PPQ either *in vitro* (5) or *in vivo* (32).

Copy number variation (CNV) polymorphism at the multi-drug resistance locus *pfmdr1* in chromosome 5 is well known in Southeast Asian settings to be associated with susceptibility to antimalarial drugs both *in vitro* (2, 7, 36) and *in vivo* (24–26). CNV events in this chromosomal region normally involve more than the *pfmdr1* gene. In fact, the amplification includes a genomic fragment that can reach 100 kb, encompassing multiple genes (12, 34). These amplified genome regions (amplicons) have been structurally characterized in field samples from the Thailand-Burma border (21), although the precise importance and impact of their diversity in the development of drug resistance are yet to be investigated.

Recently, it has been reported that the use of PPQ in Vietnam is associated with a significant decrease in the prevalence of *pfmdr1* gene duplications in the parasite population of this region (16). Trying to identify the genetic basis of PPQ resistance, a recent

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study showed that continuous *in vitro* exposure leads to a deamplification of the amplicon encompassing the *pfmdr1* gene and amplification of a neighboring upstream region on chromosome 5 (11). This, associated with previous observations of decreased copy number in parasites long exposed to chloroquine (2), led us to hypothesize that gene copy number amplifications in *pfmdr1* and its amplicon size might lead to an increased sensitivity to PPQ.

## MATERIALS AND METHODS

**Parasite adaptation and *in vitro* drug susceptibility assays.** We analyzed 46 strains previously culture adapted from the region of Mae Sot, in the Thai-Myanmar border, from clinical cases occurring between 2002 and 2008, specifically, 1 strain from 2002, 4 strains from 2007, and 41 strains from 2008 (36). The study was ethically cleared by the relevant institutions, the blood samples having been obtained upon informed consent provided in the local language.

AQ: A The maintenance medium for the parasite cultures contained 5% hematocrit (O<sup>+</sup> erythrocytes) in RPMI 1640 (GIBCO BRL; Invitrogen 42402-010) supplemented with 1 × L-glutamine (GIBCO BRL; Invitrogen 25030-032), 25 µg/ml gentamicin (GIBCO BRL; Invitrogen 15750-037), and 10% human serum. Piperaquine monophosphate (molecular weight [MW], 633.5) was obtained from AvaChem (San Antonio, TX), and chloroquine diphosphate (MW, 515.86) was purchased from Sigma-Aldrich (St. Louis, MO). The inhibitory concentrations (ICs) were determined through the histidine-rich protein 2-based double-site sandwich enzyme-linked immunosorbent assay (ELISA) (23). Briefly, 200 µl of synchronized culture at ring stage containing 0.05% parasitemia and 1.5% hematocrit was precoat in 96-well culture plates (titration of 1/2), with a PPQ concentration in row 8 of 200 nM and a CQ concentration of 2 µM. Cultures were incubated at 37°C in a candle jar (33) for 72 h, followed by the lysis of the cells by freeze-thawing for ELISA analysis. Four independent assays were performed for each field strain, and the 3D7 reference strain 50% IC (IC<sub>50</sub>) was measured for drug quality and efficacy control. The 3D7 strain was kindly provided by the late D. Walliker (Department of Animal and Population Genetics, University of Edinburgh, United Kingdom).

AQ: B **Molecular analysis.** Extraction of gDNA of the 46 Thai strains and its molecular characterization, including *pfmdr1* N86Y, *pfprt* K76T single nucleotide polymorphisms (SNPs), and *pfmdr1* gene copy number variation, were previously performed (36).

AQ: C In order to better understand the importance for drug resistance of the amplified regions at chromosome 5, all strains carrying *pfmdr1* increased copy number (24 strains), as well as the laboratory strains FCB, FCR3, F32, and Dd2, known to carry multiple amplification of this gene, were further characterized for their approximate amplicon size through real-time PCR of the *pfmdr1*-adjacent regions.

AQ: D The software program Primer Express 2.0 (Applied Biosystems, CA) was used for the design of 20 sets of TaqMan probes and primers to quantify the CNV at relevant chromosome 5 regions. This scanning approach covered a ca.157-kb fragment containing the *pfmdr1* gene (see Table S1 in the supplemental material).

The β-tubulin gene (PF10\_0084) was used as the single-copy endogenous control as previously described (25). We estimated the copy number of the target sequence in relation to a standard calibrator lab strain genome (3D7; single *pfmdr1* copy) by using the cycle threshold (ΔΔCT) method.

All assays were performed at least in triplicate on 96-well plates using the ABI PRISM 7000 sequence detection system (Applied Biosystems, Fresno, CA). The detection threshold was set above the mean baseline value for the first 6 to 15 cycles.

AQ: E Following the previous findings that *in vitro* exposure of PPQ in strain Dd2 leads to an amplification of the fragment (spanning from PFE1010w to the PFE1085w gene) identified by Eastman et al. at chromosome 5, we scrutinized all Thai samples (*n* = 46) for CNV polymorphism in the

PFE1010w and PFE1085W loci. Primers and probes as well as the real-time PCR conditions were as Eastman et al. described (11).

**Statistical analysis.** Piperaquine and chloroquine 50% and 90% inhibitory concentrations were calculated by nonlinear regression analysis (<http://malaria.farch.net>). Statistical analysis was carried out using Sigma-Plot for Windows, version 11.0. Pearson correlation was used to assess linear relations of PPQ and CQ. The associations between the *in vitro* PPQ susceptibility values (IC<sub>50</sub> and IC<sub>90</sub>) and the *pfmdr1* gene copy numbers were tested through the performance of *t* test, and when the normality test failed (Shapiro-Wilk), the Mann-Whitney rank sum test was applied.

## RESULTS

The 50% and 90% inhibitory concentrations (IC<sub>50</sub>s and IC<sub>90</sub>s) were successfully determined for PPQ and CQ in the 46 *P. falciparum* strains. A large range of sensitivities was recorded for PPQ, with a median IC<sub>50</sub> of 39.4 nM, from 13.8 to 108.2 nM (see Table S2 in the supplemental material). All strains were found to be highly resistant to CQ, with an IC<sub>50</sub> of >450 nM. The interaction between PPQ and CQ ICs was tested with Pearson correlation analysis. It was agonistic both for IC<sub>50</sub>s (correlation coefficient of 0.37; *P* = 0.01) and IC<sub>90</sub>s (correlation coefficient = 0.40; *P* = 0.006).

Out of the 46 strains analyzed, 24 carried increased *pfmdr1* copy numbers. Specifically, 16 strains carried two copies, 6 strains carried three copies, and 2 strains harbored four copies of the *pfmdr1* gene. A significant association was found between the *pfmdr1* gene copy number amplifications and a decrease in the IC<sub>50</sub>s for PPQ (geometric mean of 42.9 nM for strains with *pfmdr1* CNV of 1 versus geometric mean of 29.2 nM for the strains with *pfmdr1* CNV of >1; *P* = 0.002). The same pattern was observed for the IC<sub>90</sub>s for CQ (*P* = 0.026) (Fig. 1). Stratifying the strains with >1 *pfmdr1* copy (2, 3, or 4 copies) did not, however, reveal any trend of progressive enhanced PPQ or CQ sensitivity.

In order to find any association with the *in vitro* drug outcome phenotype and the type of amplified fragment containing the *pfmdr1* gene, the approximate amplicon size and structure (i.e., which loci were involved) were characterized in the 24 Thai strains and in the reference strains FCB, FCR3, F32, and Dd2. Six different types of amplicons were found in the Thai samples (types a to f [Fig. 2]). Two amplicon types were found in the reference lab strains, with amplicon size and position on chromosome 5 illustrated as vertical pink bars in Fig. 2. The amplicon varied in size from approximately 14 to 100 kb, spanning different genes. All the characterized amplicons in the Thai strains were found to be smaller than the structure present in the analyzed reference strains.

We found no significant associations between the types of amplicon (types a to f) and the determined ICs for PPQ and CQ. Considering the previously determined phenotypes (36), significance difference was detected where parasites carrying amplicon type a are associated with significantly higher mefloquine IC<sub>50</sub>s (*P* < 0.01) (Table 1). A trend in increased IC<sub>50</sub> is also detected for the same type of amplicon with artemisinin (ART) and lumefantrine (LUM), although statistically nonsignificant (Table 1).

A region from the PFE1010w gene to the PFE1085w locus, located ca. 72 kbp upstream of the *pfmdr1* gene (Fig. 2, dashed green box), has been previously associated with *in vitro* PPQ resistance (11). However, no increase in copy number for the PFE1010w and PFE1085w genes was found among our 46 field strains.

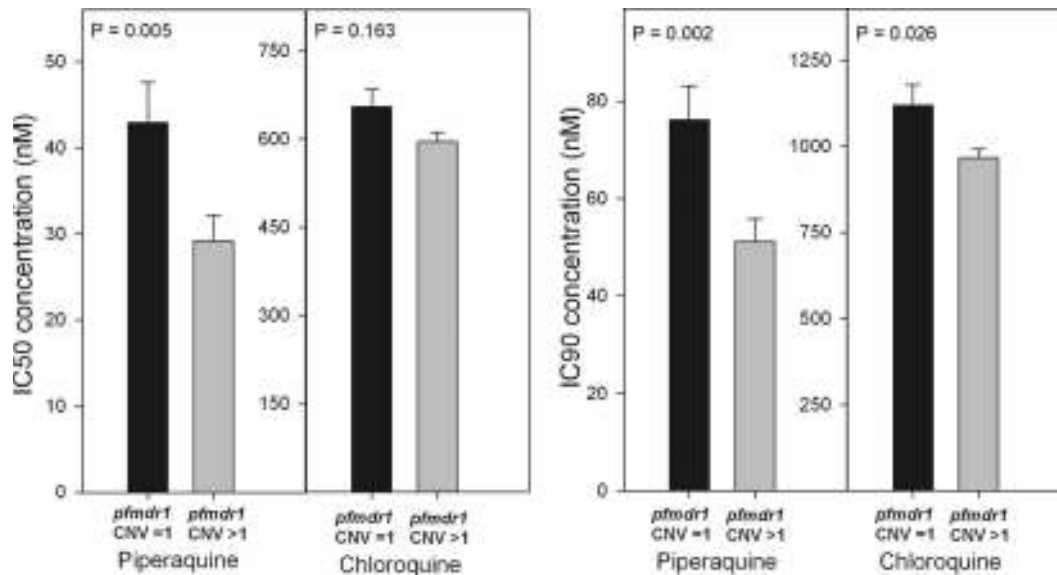


FIG 1 *pfmdr1* copy number variation (CNV) and *in vitro* IC<sub>50</sub>s and IC<sub>90</sub>s of PPQ and CQ. Shown is the association between the geometric mean of *in vitro* PPQ and CQ ICs and the *pfmdr1* CNV polymorphism. Data are described in Table S2 in the supplemental material. Black bars, single copy of the *pfmdr1* gene (22 strains); gray bars, more than 1 *pfmdr1* gene copy (24 strains). *t* test was applied to determine significant difference. Error bars represent standard errors of the means.

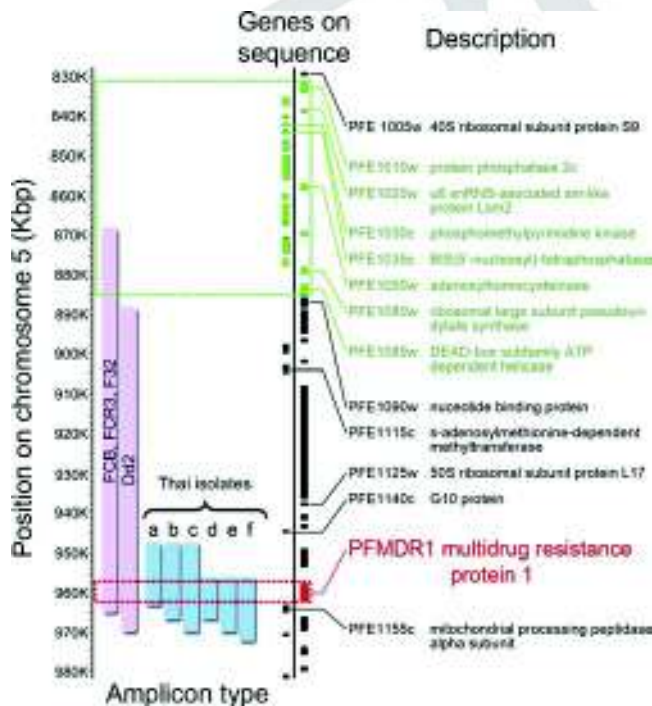


FIG 2 Genomic characterization of amplified regions. Vertical bars characterize amplicon size and position on chromosome 5 (left y axis), including information for genes in the sequence (right y axis). Pink bars illustrate amplicon type for the lab strains, and blue bars different amplicons found in the Thai strains ( $n = 24$ ) all carrying *pfmdr1* copy number amplifications (gene highlighted in red). The green box highlights a genomic region previously found to be associated with decreased susceptibility to PPQ (11) which is not found in the Thai strains. The left y axis shows the position on chromosome 5, and the right y axis shows the gene annotation relative to the 3D7 genome sequence. Genes with known or putative function described in the depicted genomic position were acquired from NCBI Map viewer at <http://www.ncbi.nlm.nih.gov>. The nonannotated genes represent hypothetical proteins with unknown function.

## DISCUSSION

The ACT DHA-PPQ has shown excellent efficacy in field clinical trials, making it a promising fixed-dose formulation for the control of malaria. The recent detection of ART resistance in Southeast Asia (10), in regions where the partner drug (mefloquine) is failing (6), supports the importance of maintaining the integrity of the efficacy of the long-standing partner drug. With its very long half-life, PPQ is potentially at risk in settings of high transmission, due to the possibility of a large posttreatment drug resistance selection window, akin to what has been seen with other ACTs (15, 31). This, associated with the knowledge that PPQ resistance has already emerged in the past (13), commands urgency regarding the research of its molecular mechanisms. Additionally, it should be noted that emerging drug resistance has even been suggested as an explanation for an unexpected low cure rate of the DHA-PPQ ACT in a clinical trial conducted in Papua New Guinea (18).

In the present study, a large range of sensitivities was registered for PPQ, covering almost 1 log of IC<sub>50</sub>s. According to the previous genotype data for the herein-analyzed parasites (36), this large range of sensitivities cannot be explained by the monomorphic *pfcr1* gene, nor by the SNPs found in the *pfmdr1* and *pfmrp1* genes. The noninvolvement of *pfmdr1* and *pfmrp1* SNPs in the parasite's response to PPQ mirrors recent similar studies conducted in Kenyan isolates (19) as well as other studies *in vitro* (5) and *in vivo* (32).

On the other hand, we have found for the first time evidence that increased copy number of *pfmdr1* sensitizes the parasite to PPQ. The P-glycoprotein homologue 1 (Pgh1) encoded by *pfmdr1* is almost exclusively located in the parasite food vacuole (FV) (8), facing the lumen of the organelle (29), consistent with the presently accepted model of this transporter being an importer of solutes toward the FV (28). Such function implies that the presence of increased *pfmdr1* copy numbers (expected to lead to an increase



TABLE 1 Association between the type of amplicon and the determined IC<sub>50</sub>s<sup>a</sup>

<i>pfmdr1</i> CNV or amplicon type <sup>b</sup>	No. of Thai strains	IC <sub>50</sub> , nM (SE)					
		ART <sup>c</sup>	DHA <sup>c</sup>	MQ <sup>c</sup>	LUM <sup>c</sup>	PPQ	CQ
<i>pfmdr1</i> CNV							
= 1	22	4.4 (0.6) <sup>d</sup>	1.3 (0.3)	49.1 (19.7) <sup>d</sup>	8.6 (1.8) <sup>c</sup>	49.7 (7.8)	676.1 (40.9)
>1	24	11.0 (1.1)	1.5 (0.5)	159.1 (26.6)	18.9 (2.7)	32.2 (5.2)	601.0 (25.8)
Amplicon type							
a	3	14.8 (4.7)	2.4 (1.2)	243.6 (16.5) <sup>e</sup>	26.0 (6.8)	35.5 (8.7)	585.3 (17.2)
b	6	8.1 (2.1)	0.9 (0.2)	113.3 (31.6)	13.6 (2.7)	28.2 (4.8)	569.2 (23.6)
c	2	11.3 (7.0)	1.0 (0.4)	130.4 (23.6)	21.1 (5.7)	35.3 (8.6)	592.9 (12.7)
d	3	6.7 (1.9)	1.0 (0.3)	135.2 (34.7)	10.4 (1.7)	32.5 (4.5)	588.7 (9.5)
e	8	12.7 (1.1)	1.8 (0.5)	170.9 (13.8)	21.9 (1.4)	35.0 (5.9)	638.8 (28.3)
f	2	12.9 (5.1)	2.6 (1.5)	186.4 (4.7)	22.8 (2.8)	25.1 (2.2)	594.9 (109.9)

<sup>a</sup> PPQ, piperazine; ART, artemisinin; DHA, dihydroartemisinin; MQ, mefloquine; LUM, lumefantrine; CQ, chloroquine. Pearson correlations (correlation coefficients [*r*]) were as follows: for ART, *r* = 0.03 (*P* > 0.05); for DHA, *r* = 0.21 (*P* > 0.05); for MQ, *r* = -0.11 (*P* > 0.05); for LUM, *r* = 0.14 (*P* > 0.05), and for CQ, *r* = 0.37 (*P* = 0.01). For pairs with *P* values greater than 0.05, there is no significant relationship between the two variables.

<sup>b</sup> Amplicon type is described in detail in Fig. 2, with the characterization of amplicon size and position of the amplified fragment carrying the *pfmdr1* gene.

<sup>c</sup> Inhibitory concentrations previously published (36).

<sup>d</sup> *P* < 0.001 (*t* test). Results previously published (36).

<sup>e</sup> *P* = 0.005 (unpaired *t* test between amplicon type a and amplicon type other than a).

of Pgh1 load in the FV membrane) is associated with an enhanced accumulation of PPQ in the organelle. The increased susceptibility to PPQ hence suggests that the target of this drug is located in the FV, as in the case of CQ, another 4-aminoquinoline. This hypothesis is supported by the positive correlation we found in the parasite IC response to PPQ and CQ. It is, however, important to note that although PPQ is structurally related to CQ, these two drugs must be associated with other relevant mechanisms of resistance, as PPQ remains active against CQ-resistant parasites (3).

As for CQ, all strains were highly resistant to this drug. *pfprt* mutations, in particular at amino acid position 76, are a central factor for CQ resistance. The *pfprt* gene in this set of Thai samples was found to be totally monomorphic of the Dd2 type, which explains the fact that all the parasites were highly resistant to this drug (i.e., IC<sub>50</sub> > 100 nM threshold) but does not explain the large variation that was observed between them. Interestingly, we could see a trend where decreased *pfmdr1* copy number polymorphism tends to coincide with higher ICs, reaching statistical significance at IC<sub>90</sub>s (Fig. 1). The inverse relation between CQ resistance and *pfmdr1* copy number has previously been documented *in vitro* (2) but not observed in highly controlled gene knockdown experiments (30), possibly indicative of an effect visible only in certain genomic environments (e.g., possibly the presence of the *pfmdr1* 86N allele).

It has long been known that the duplication events involving the *pfmdr1* vary in size while spanning different genes in different parasites (12, 20, 27, 34). We tried to further investigate if the type (i.e., which genes are included) and size of the amplicon influence drug susceptibility phenotypes. Overall, the field strains were found to carry smaller amplicons than were lab strains (Fig. 2), possibly due to a fitness cost, consistent with previous reports of intrahost population dynamics (35). Selection of parasites with smaller amplicons may be favored due to the reduced costs associated with the lower number of genes and/or replication of unnecessary DNA (20). This penalty might also be the underlying reason for the trend observed for the amplicon type a to be associated with significant decreased susceptibility to mefloquine and the observed trend for ART and LUM (Table 1). Further studies

are warranted to explore the relationship between amplicon size and drug activity.

To elucidate potential determinants of resistance to PPQ, a recent work based on continuous exposure to PPQ was able to select parasites with IC<sub>50</sub>s > 100-fold greater than that for the parent line (Dd2). Applying comparative genome hybridization procedures on these parasites allowed the detection of a new amplification fragment neighboring the amplicon containing the *pfmdr1* gene (11) (see Fig. 2, green dashed box). The Thai samples herein analyzed did not show events of genomic amplification in this region. It is possible that this has to do with the fact that PPQ has not been widely used in western Thailand, where these parasites originated. It is of particular interest to analyze this genomic region on parasites from locations where PPQ has been used in national malaria control programs—like Vietnam or Papua, Indonesia—in order to identify relevant DNA changes.

Our data reinforce the notion that *pfmdr1* copy number amplifications are selected out by the use of 4-aminoquinolines, in contrast with what is observed with amino alcohol quinolines (lumefantrine and mefloquine) (25, 26, 30). Such effects, which mirror those previously observed for the *pfmdr1* N86Y SNP (15, 31), support the rational use of 4-aminoquinoline and amino alcohol quinoline-based ACTs as alternative first- and second-line chemotherapies in malaria control programs, as potentially they independently select for mutually incompatible combinations of mutations.

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