

## Influence of primaquine and ritonavir interaction on CYP3A4 mRNA expression in HepG2 cell culture

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### Abstrak

**Latar Belakang:** Pengobatan antimalaria dan anti-HIV secara bersamaan merupakan tantangan baru dalam penanganan koinfeksi malaria dan HIV. Primaquin merupakan substrat sekaligus inhibitor bagi CYP3A4, sedangkan ritonavir merupakan substrat, inhibitor, sekaligus inducer bagi CYP3A4. Tujuan penelitian ini adalah untuk mengukur ekspresi mRNA CYP3A4 pada kultur sel HepG2 yang diinduksi oleh pemberian primaquin dan ritonavir secara bersamaan.

**Metode:** Pada penelitian pendahuluan, sel HepG2 diinkubasi dengan primaquin 30, 40, 50  $\mu$ M; ritonavir 2, 10, 20  $\mu$ M; DMSO <0,1 % sebagai kontrol negatif; atau rifampisin 20  $\mu$ M sebagai kontrol positif. Adapun pada penelitian dengan perlakuan kombinasi obat, sel HepG2 diinkubasi dengan primaquin 40  $\mu$ M+ritonavir 10  $\mu$ M; DMSO <0,1 %; atau rifampisin 20  $\mu$ M selama 72 jam. Sel dipanen menggunakan tripsin-EDTA dan RNA total diekstraksi menggunakan reagensia isolasi Tripure. Setelah jumlah RNA total dikuantifikasi menggunakan alat spektrofotometer, ekspresi mRNA CYP3A4 diukur dengan real-time reverse transcription polymerase chain reaction (RT-PCR).

**Hasil:** Terjadi peningkatan ekspresi mRNA CYP3A4 (1,22 kali lipat terhadap kontrol) pada sel HepG2 yang diinkubasi dengan primaquin dan ritonavir secara bersamaan. Hal ini menunjukkan bahwa efek induksi oleh ritonavir lebih dominan daripada efek inhibisi oleh primaquin.

**Kesimpulan:** Pemberian primaquin dan ritonavir secara bersamaan meningkatkan ekspresi mRNA CYP3A4 in vitro. (*Med J Indones* 2012;21:3-7)

### Abstract

**Background:** Concomitant treatment with antimalaria and antiretroviral drug is a new challenge in the management of malaria and HIV co-infection. Primaquine is a substrate and also an inhibitor of CYP3A4, while ritonavir is a substrate, an inhibitor, and also an inducer for CYP3A4. The objective of this study is to measure the CYP3A4 mRNA expression in HepG2 cell culture induced by primaquine and ritonavir co-treatment.

**Methods:** For the initial study HepG2 cells were treated with 30, 40, 50  $\mu$ M of primaquine; 2, 10, 20  $\mu$ M ritonavir; DMSO  $\leq$ 0.1 % for negative control; or 20  $\mu$ M rifampicin for positive control. While for the co-treatment study the cells were treated with 40  $\mu$ M primaquine+10  $\mu$ M ritonavir; DMSO  $\leq$ 0.1 %; or 20  $\mu$ M rifampicin for 72 hours. The cells were harvested using trypsin-EDTA and total RNA was extracted using the Tripure isolation reagent. After determining the quantity of RNA spectrophotometrically, CYP3A4 mRNA expression was quantified using real-time reverse transcription polymerase chain reaction (RT-PCR).

**Results:** The expression of CYP3A4 mRNA was up-regulated (1.22 fold over control) in HepG2 cells co-treated with primaquine and ritonavir. These data suggest that the induction effect of ritonavir was more dominant than the inhibitory effect of primaquine.

**Conclusion:** Concomitant administration of primaquine and ritonavir result in up-regulation of CYP3A4 mRNA expression in vitro. (*Med J Indones* 2012;21:3-7)

**Keywords:** CYP450 induction, CYP3A4, drug interaction, primaquine, ritonavir

Concomitant treatment with antimalaria and antiretroviral drug is a new challenge in the management of malaria and HIV co-infection. Essential aspect that can affect the achievement of a successful clinical cure is drug interaction. Drug interactions can cause altered drug exposures that may lead to serious drug toxicity or a reduction in pharmacological effects.<sup>1</sup> Therefore, mechanistic study to explore enzyme and gene expression contributing to drug interaction in the cellular level is needed to provide substantial information for ongoing efforts to optimize dosage recommendation in clinical practice.

Cytochrome P450 system (CYP) are the main enzyme system in the liver responsible for drug metabolism of nearly 70-80% of clinically used drugs.<sup>2</sup> Many drug interactions are a result of the inhibition or induction of these CYP enzymes.<sup>3</sup> CYP450 have many different isoform, including CYP1A1, CYP1A2, CYP2C19, CYP2D6, and CYP3A4.<sup>2</sup> However, CYP3A4 is responsible for approximately 60% of CYP450-mediated metabolism of drugs in therapeutic use today.<sup>4</sup> Thus, it is important to assess CYP3A4 in identifying drug interactions.

Primaquine (PRQ) is the only drug available for preventing relapse in malaria caused by dormant liver stage/hypnozoites form of *Plasmodium vivax* or *P. ovale*.<sup>5,6</sup> This drug is a substrate and also an inhibitor of CYP3A4.<sup>7,8</sup> In the other hand, ritonavir (RTV) is a protease inhibitor (PI) that frequently used as a booster to increase plasma concentration of other PI in the highly active antiretroviral therapy (HAART) regimen for HIV patients.<sup>9</sup> Ritonavir is a substrate, inhibitor, and also an inducer for CYP3A4.<sup>10</sup> Accordingly, it is a curiosity whether ritonavir would act as an inducer or inhibitor of CYP3A4 when given concomitantly with another drug.

Louisa et al. reported that concomitant administration of primaquine and ritonavir lead to significant decrease on plasma concentration of each drugs in rats in comparison to the single administration of either primaquine or ritonavir only.<sup>11,12</sup> To explain whether the decreased plasma concentration of both drugs was caused by the induction effect of primaquine or by the autoinduction effect of ritonavir, we measure the expression of CYP3A4 mRNA in HepG2 cell culture incubated with primaquine, ritonavir, and primaquine+ritonavir.

## METHODS

This in-vitro experimental study was conducted from August 2010 to April 2011. Cell culture was conducted at Makmal Terpadu Imunoendokrinologi, Faculty of Medicine. Universitas Indonesia. RNA isolation was performed at the Laboratory of Biochemistry and Molecular Biology, Faculty of Medicine. Universitas Indonesia. The purity and concentration of total RNA was measured at the Laboratory of Pharmacology and Therapeutic Department, Faculty of Medicine. Universitas Indonesia. Real-time RT PCR was performed at Eijkman-EOCRU Laboratory.

## Chemicals

Primaquine, ritonavir, rifampicin (RIF), and dimethylsulfoxide (DMSO) were purchased from Sigma- Aldrich Ltd (Singapore). Dulbecco minimal essential medium (DMEM) and foetal bovine serum (FBS) were purchased from Gibco Ltd (Singapore). Tripure isolation reagent was purchased from Roche Diagnostic (Singapore). Nuclease free water was purchased from Promega Corporation (Singapore). Primers for  $\beta$ -actin and CYP3A4 were purchased from 1<sup>st</sup> BASE Ltd (Singapore). iScript one-step RT-PCR kit with SYBR Green were purchased from Bio-Rad Laboratories Inc (Singapore). All other chemicals used were of the highest purity commercially available.

## Cell culture and drug treatment

HepG2 cells were purchased from BPPT (Serpong, Indonesia). The cells were cultured in DMEM supplemented with 10% FBS and maintained at 37°C

in a humidified incubator of 5% CO<sub>2</sub>. Cells were subcultured at confluence onto 12-well plates (seeding density 2.0 x10<sup>5</sup> cells/mL medium). Hepatocyte culture were maintained in culture medium for one days before incubation with the test-drug. In the second day, the medium were aspirated and replaced with fresh medium containing the test-drug. Drugs were dissolved for treatment in DMSO. For the initial study, cells were treated with one of the following: 30 uM PRQ, 40 uM PRQ, 50 uM PRQ, 2 uM RTV, 10 uM RTV, 20 uM RTV, DMSO  $\leq$ 0.1 % for negative control, or 20 uM RIF for positive control. While for the co-treatment study, cells were treated with one of the following: 40 uM PRQ+10 uM RTV, DMSO  $\leq$ 0.1 % for negative control, or 20 uM RIF for positive control. In all cases the concentration of DMSO was less than 0.1%. All treatments were done in duplicates. The cells were harvested at 72 hours after drug treatment.

## RNA isolation

Cells were harvested using trypsin-EDTA and total RNA was extracted using the Tripure isolation reagent according to the manufacturer's instructions. The RNA was quantified spectrophotometrically by measuring absorbance at 260 nm. The purity of the final preparations was determined by calculating the absorbance ratio at 260 and 280 nm.

## Real-time RT PCR

The sequences of the primers used for real-time PCR are shown in Table 1. The samples were prepared by mixing 30 mM of each primer, 12.5 uL SYBR Green RT PCR reaction mix, 0.5 uL iScript reverse transcriptase, 100 ng template RNA, and 9.5 uL RNase free water. The samples were then incubated in real-time thermal detection system with the following condition: 10 minutes at 50°C for cDNA synthesis, 5 minutes at 95°C for iScript reverse transcriptase inactivation, and PCR cycling and detection (40 cycles) which was 10 seconds at 95°C for denaturation stage and 30 seconds at 58°C for annealing and extension stage. Threshold values (Ct) were calculated automatically by the software. The Ct data was processed according to the method described by Pfaffl.

Table 1.  $\beta$ -actin and CYP3A4 primers

Gene	Primer	Sequence	Product size
$\beta$ -Actin	Forward	5'-TCAGCAAGCAGGAGTATG-3'	97
	Reverse	5'-GTCAAGAAAGGGTGTAAACG-3'	
CYP3A4	Forward	5'-ATCATTGCTGTCTCCAACCTTCAC-3'	103
	Reverse	5'-TGCTTCCCCTCAGATTCTC-3'	

A: adenine, C: cytosine, G: guanine, T: thymine

## RESULTS

HepG2 cells were not changed morphologically during the drug treatment period under an inverted phase contrast microscope. Figure 1 shows normalized CYP3A4 mRNA expression in various dose of primaquine. Exposure to 30, 40, and 50  $\mu\text{M}$  primaquine did not induce CYP3A4 mRNA expression, while exposure to 20  $\mu\text{M}$  rifampicin as positive control increased CYP3A4 mRNA expression by 3-fold in comparison to DMSO treated cell as negative control.

The normalized CYP3A4 mRNA expression in various doses of ritonavir is demonstrated in Figure 2. Exposure to 2  $\mu\text{M}$  ritonavir did not induce CYP3A4 mRNA expression. However, a slight increase (1.49 and 1.93 fold over control) of CYP3A4 mRNA expression was detected when ritonavir concentration was increased to 10 and 20  $\mu\text{M}$ .

The expression of CYP3A4 mRNA was slightly increasing (1.22 fold over control) in HepG2 cells co-treated with primaquine and ritonavir. These data suggest that the induction effect of RTV was more dominant than the inhibitory effect of PRQ.

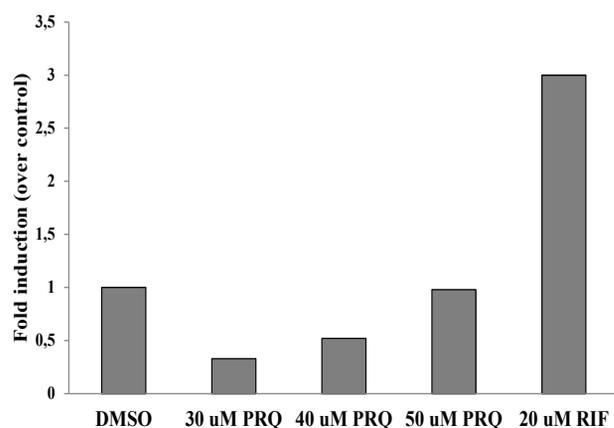


Figure 1. Concentration-response relationship of PRQ treatment on the expression of CYP3A4 mRNA in HepG2 cells.

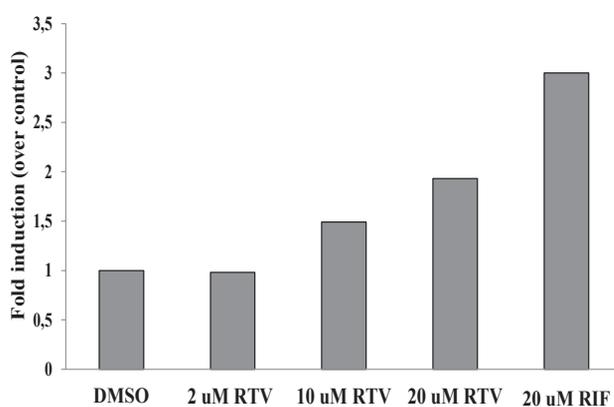


Figure 2. Concentration-response relationship of RTV treatment on the expression of CYP3A4 mRNA in HepG2 cells.

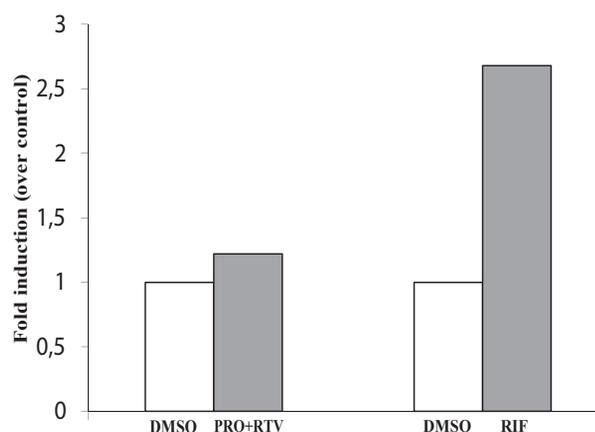


Figure 3. Effect of primaquine and ritonavir co-treatment on the expression of CYP3A4 mRNA in HepG2 cells.

## DISCUSSION

To our knowledge, this is the first study reporting the interaction between anti-malarial and anti-HIV drug at the mRNA level. From the initial study, it is confirmed that even with the highest concentration (50  $\mu\text{M}$ ), primaquine did not increase the CYP3A4 mRNA expression. The highest concentration of primaquine used in this study is much higher than the maximum concentration in blood ( $C_{\text{max}}$ ) following single administration of primaquine of a normal dose in human for radical cure or prevention of relapsing vivax or ovale Malaria.<sup>13</sup> Nevertheless, 30  $\mu\text{M}$  of primaquine was used by Backlund et al. to assess the induction effect of primaquine on CYP1A1 and 40  $\mu\text{M}$  of primaquine was used by Kanebratt et al. to assess HepaRG cells as an *in vitro* model for evaluation of CYP450 induction in human.<sup>14,15</sup> Consequently, it is unlikely that the induction of CYP3A4 mRNA expression by primaquine will happen under the *in vivo* circumstances. The results are in agreement with other studies that suggest primaquine as a substrate and also an inhibitor, but not an inducer of CYP3A4.<sup>7,8</sup>

In the other hand, the effect of ritonavir seemed to be dose-dependent, the higher ritonavir concentration, the higher CYP3A4 mRNA expression. The evidence corroborates the property of ritonavir as CYP3A4 inducer. However, the ability of ritonavir as an inducer was not as strong as rifampicin as a positive control. This probably due to lower dose used in this study. The  $C_{\text{max}}$  of ritonavir following a single dose administration in human is approximately 11.2  $\mu\text{g}/\text{mL}$  or equal to 155.351  $\mu\text{M}$ , while the maximum ritonavir dose used in this study is only 20  $\mu\text{M}$ .<sup>13</sup> Nevertheless, Luo et al. also used 2, 10, and 20  $\mu\text{M}$  of ritonavir in their study examining CYP3A4 induction by drugs using primary hepatocyte culture.<sup>16</sup> Therefore, ritonavir administration *in vivo* may possibly produce higher induction of CYP3A4.

From the co-treatment study, it is demonstrated that the net result of concomitant administration of primaquine and ritonavir is a slight increase of CYP3A4 mRNA expression, where ritonavir behave as an inducer. The behavior of ritonavir in this study is in contrast with other study reported by Ikezoe et al. Co-administration of 10  $\mu$ M ritonavir and  $10^{-3}$   $\mu$ M docetaxel in DU145 cells resulted in no increase of CYP3A4 mRNA expression compared to DMSO treated control group, although docetaxel alone increased CYP3A4 mRNA expression by 2.5-fold.<sup>17</sup>

The slight increase of CYP3A4 mRNA expression by ritonavir in this study can be explained by receptor-mediated induction mechanism. CYP3A induction commonly occurs via the binding of the inducer to pregnane X receptor (PXR) and/or constitutive androstane receptor (CAR). Once activated, these receptors form heterodimers with other factors, retinoid X receptor (RXR for both PXR and CAR) and then bind to the target xenobiotic response elements (XRE) located in both the proximal and distal P450 gene promoters, resulting in the elevation of transcription of the respective CYP gene.<sup>18</sup>

In the other hand, Ikezoe suggest that ritonavir may block docetaxel-induced expression of CYP3A4 by affecting co-regulators such as silencing mediator for retinoid and thyroid receptor and steroid receptor coactivator-1 that mediated basal and xenobiotic-induced transcriptional activity of CYP3A4.<sup>17</sup>

The increase of CYP3A4 mRNA expression reported in this study could lead to the increase of CYP3A4 enzyme that could decrease both plasma concentration of primaquine and ritonavir. The result from this study gives us a signal that both the efficacy of ritonavir in anti-HIV regimen and primaquine as an anti-malarial agent could be reduced if both drugs were administered concomitantly in human.

From the elimination half-life ( $t_{1/2}$ ) point of view, ritonavir has relatively short  $t_{1/2}$  which is 3-5 hours.<sup>9,13</sup> This fact possibly will cause the effect of ritonavir induction on primaquine would not last long. However, we should consider that the duration of induction of a drug does not solely depend on the  $t_{1/2}$ , but also depend on the time needed for CYP enzyme degradation. It is stated that  $t_{1/2}$  of CYP450 ranged between 1-6 days.<sup>3</sup> Therefore, ritonavir may only induce the CYP3A4 for 3-5 hours, but the induction effect would probably last for several days.

In addition, as a monotherapy or as a booster for other antiretroviral, ritonavir is given twice daily (every 12 hours).<sup>13</sup> For that reason, the induction effect of ritonavir would last a full day.

The elimination half-life of primaquine is 3.7-9.6 hours.<sup>13</sup> If primaquine is given concomitantly with

ritonavir, the  $t_{1/2}$  of primaquine will be shortened, so the duration of primaquine's therapeutic effect would also be shortened.

There are several limitations from this study. According to Hewitt et al, it was found that HepG2 cells have very low CAR, PXR, CYP3A4 expression, but relatively high CYP3A7 expression. This profile is in keeping with a fetal and not adult expression profile of hepatocytes. This lack or low abundance of PXR and CAR makes them unsuitable for induction assays involving these pathways.<sup>18</sup>

This probably explain why 10  $\mu$ M ritonavir in this study only gives slight increase (1.49 fold over control) of CYP3A4 mRNA expression but the same dose of ritonavir in the study using primary hepatocyte culture could produce 2.2 to 21 fold over control of CYP3A4 mRNA expression.<sup>16</sup> However, the mechanism controlling CYP3A4 expression in HepG2 cells are identical to those of human hepatocytes.<sup>19</sup>

Another limitation from this study is that we did not measure the CYP3A4 enzyme activity. Although there is a good relationship between mRNA levels and enzyme activities for most CYPs, the definitive endpoint is the activity of the enzyme rather than its expression because the overall change in enzyme activity will ultimately affect the clearance of the drug itself or of a co-administered drug.<sup>18</sup>

In conclusion, concomitant administration of primaquine and ritonavir result in a slight increase of CYP3A4 mRNA expression in vitro, where ritonavir act as an inducer. Nevertheless, the significance of this finding should be further clarified by performing CYP3A4 enzyme activity measurement.

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