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Cytochrome 1A1 Induction by Primaquine In Human Hepatocytes and HepG2 Cells: Absence of Binding to the Aryl Hydrocarbon Receptor

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ABSTRACT. Malaria remains the most prevalent infectious disease of tropical and subtropical areas of the world. It represents a crucial problem in public health care, affecting 750 million people annually, of whom at least two million die. Various antimalarials currently used were studied for their capability to induce expression of the cytochrome P450 1A1 (*CYP*1A1) gene, an enzyme that plays an important role in the activation of xenobiotics to genotoxic derivatives. Studies on human hepatocytes and HepG2 cell lines showed that primaquine was capable of dose dependently increasing both the ethoxyresorufin-*O*-deethylase activity and *CYP*1A1 mRNAs, suggesting a transcriptional activation of this gene. Moreover, α-naphthoflavone, a partial aryl hydrocarbon receptor (AhR) antagonist, and 8-methoxypsoralen, which interferes with the binding of activated AhR to the xenobiotic responsive element, were shown to suppress *CYP*1A1 induction when added to the cultures. However, neither primaquine nor its metabolites were able to displace [^{3}H]2,3,7,8-tetrachlorodibenzo-*p*-dioxin from AhR in competitive binding studies using 9S-enriched fractions of human cytosol. These data, together with the induction of *CYP*1A1 promoter-directed chloramphenicol acetyl transferase gene expression, suggest that *CYP*1A1 induction involves the participation of the AhR but not a direct primaquine–receptor interaction. This supports the notion that an alternative ligand-independent mechanism has to be considered. Given the pharmaco-toxicological significance of *CYP*1A1 induction, these findings may have important implications in the treatment of malaria with primaquine and new analogs. BIOCHEM PHARMACOL **57**;3:255–262, 1999. © 1998 Elsevier Science Inc.

KEY WORDS. *CYP*1A1; primaquine; hepatocytes; Ah receptor

Malaria is estimated to kill between 2 and 4 million people every year. Another 500 million to 1 billion people have the disease, and one-third of all humanity lives in zones where they risk catching it. The parasite's resistance is growing alarmingly, and a recent 7-fold increase in malaria deaths over five years in parts of Africa has been linked to the emergence of increased chloroquine resistance [1]. Primaquine, an 8-aminoquinoline [8-(4-amino-1-methylbutylamino)-6-methoxyquinoline] which was introduced in 1952, possesses a large spectrum of antimalarial activity and has remained the most effective agent for the radical cure of subsequent relapses of *Plasmodium vivax* and *ovale* blood stage infections [2]. The drug is well tolerated at standard dosages, but at higher doses, toxic effects are common, including methaemoglobinaemia and haemolytic anaemia due to membrane damage [3]. In spite of these drawbacks, primaquine remains of extreme importance in malaria chemotherapy, because extensive studies of many other compounds have not yet revealed any other class that equals or surpasses the 8-aminoquinolines in radical curative activity against the reviviscent forms of malaria parasite [4]. The objective of this paper was hence to address the question of the effect of this antimalarial on *CYP*1A1‡ gene expression, which is known to play an important role in metabolic activation of xenobiotics into toxic, mutagenic, and carcinogenic compounds. Indeed, induction of cytochromes P450 [5, 6] is considered to be one of the most sensitive and early biochemical cellular responses, as it generally takes place at much lower doses of a given chemical than those known to cause toxic effects. Compounds belonging to the major antimalarial families such as quinine (natural precursor), the amino-alcohol mefloquine, the amino-4-quinoleines chloroquine, amodiaquine, and quinidine, the antimetabolite pyrimethamine, arteflene, a yingzhaosu A derivative and primaquine, were compara-

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‡ *Abbreviations:* AhR, aryl hydrocarbon receptor; α-NF, α-naphthoflavone; CAT, chloramphenicol acetyl transferase; *CYP*1A1, cytochrome P450 1A1; EROD, ethoxyresorufin-*O*-deethylase; 3-MC, 3-methylcholanthrene; 8-MP, 8-methoxypsoralen; TCDD, 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin; TCDF, tetrachlorodibenzofuran; and FBS, fetal bovine serum.

tively studied for their direct cytotoxicity and *CYP*1A1 induction capability in hepatocyte primary cultures.

Among them, we report here that primaquine causes a dose-dependent increase in EROD activity which is correlated to a rise in the *CYP*1A1 mRNA level. This was confirmed by using an HepG2 cell line stably transfected with the CAT reporter gene, cloned under control of the human *CYP*1A1 promoter. We found that this induction was greatly suppressed by α-NF, and 8-MP, two compounds interfering with the AhR [7, 8] and the binding of activated AhR to DNA, respectively [9]. However, data from competition experiments showed that primaquine and its metabolites were unable to bind the human hepatic AhR.

MATERIALS AND METHODS

Chemicals

Williams' E medium, Dulbecco's modified Eagle's medium, Eagle's nonessential amino acids, FBS, sodium pyruvate, trypsine-EDTA, L-glutamine, and penicillin/streptomycin solution were obtained from Eurobio. Primaquine, βNADP, glucose-6P, glucose-6P-dehydrogenase, 3-MC, 8-MP, and DMSO were from Sigma. α-NF was from Aldrich. 7-ethoxyresorufin and resorufin were from Boehringer Mannheim Gmbh. [^{3}H]TCDD (35 Ci/mmol) and TCDF were purchased from Chemsyn Sciences Lab.

Cellular Models

HEPATOCYTES. Hepatocytes from human surgical liver biopsies were obtained by a reverse two-step collagenase perfusion [10]. Isolated cells were resuspended in medium I consisting of Williams E medium with 10% FBS, penicillin (50 units/mL), streptomycin (50 μg/mL) and insulin (0.1 unit/mL). Hepatocyte viability was determined using the erythrosin B exclusion test, and ranged between 80 and 85%. Cells were seeded in collagen type I-coated dishes and incubated for 4 hr at 37° under a humidified atmosphere with 5% CO_2. Afterwards, the medium was replaced with medium II which was identical to the first except that it did not contain serum and was supplemented with hydrocortisone hemisuccinate (1 μM) and BSA (240 μg/mL) for induction studies. When cryopreserved human hepatocytes were used, the cells, after thawing in a water bath at 37°, were resuspended in L15 medium supplemented with 7% DMSO and 5% FBS. The hepatocytes were then purified by a rapid centrifugation in a culture medium containing Percoll [11] and seeded as described previously.

CELL LINES. HepG2 cells and HepG2.241c.1 cells (stably transfected with the CAT reporter gene, under the control of the human *CYP*1A1 promoter) were also cultured, as an acceptable substitute for hepatocytes, in Dulbecco's Modified Eagle's Medium (10% FBS, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids and 2 mM glutamine) and Eagle's Minimum Essential Medium (10% FBS, 50 U/mL of penicillin 50 μg/mL of streptomycin, 0.4 mg/mL of G418 and 2 mM glutamine). Cultures were incubated at 37° in a humidified atmosphere with 5% CO_2. Cells were subcultured every 6 days and every week at a ratio of 1:2 and 1:5, respectively.

Cell Treatment

Cells were seeded on 100-mm diameter plates for Northern blot analysis and 96-well microtiter plates for the EROD assay. The HepG2 cell line grown to near confluence and hepatocytes were induced over a 3-day period (one treatment every 24 hr) by 1 μM 3-MC or 0.1 to 50 μM primaquine and coincubated with various inhibitors: α-NF (0.1–10 μM) and 8-MP (5–75 μM). Inducers were prepared as a DMSO stock solution and added directly to cultures at the time of culture medium change. The final DMSO concentration never exceeded 0.5% (v/v).

Monooxygenase Activity Assays

EROD activity (specific for *CYP*1A1/2 forms) was assayed in hepatocytes or HepG2 cells cultured on 96-well culture plates [12, 13]. After induction, cells were washed twice (15 min each time) with Williams' E medium. The medium was then discarded and 25 μL of TMB buffer (Tris–HCl 100 mM, pH 7.85, $MgCl_2$ 5 mM, BSA 0.06%) containing 1% glycerol was added per well before storing at −80°. After thawing, the buffer with glycerol was gently removed and 200 μL per well of TMB buffer with glucose-6-phosphate (G_6P, 3 mM), NADP (0.5 mM), dicumarol (10 μM), and G_6P dehydrogenase (0.1 U/mL) was added. Incubations were performed with ethoxyresorufin (2 μM) and the *CYP*-specific activity was measured at 37° by spectrofluorimetry. Kinetics of appearance of resorufin (λ_{ex} = 535 nm, λ_{em} = 600 nm) from ethoxyresorufin was followed.

mRNA Analysis

For mRNA analysis, medium was removed from 100-mm diameter plates and hepatocytes were washed twice with cold PBS before being scraped with 1 mL of guanidium solution (guanidine thiocyanate 4 M, sodium citrate 25 mM pH 7, sarcosyl 0.5%, and 2-mercaptoethanol 0.1 M [14]). The culture extract was kept at −80° until analysis. Total RNA was isolated using the acidic phenol extraction procedure. Twenty μg of mRNA was size-fractionated on a 0.9% agarose gel containing 10% formaldehyde, and was then transferred to a nitrocellulose membrane. Hybridization was performed with a 475 bp fragment of human *CYP*1A1 cDNA, corresponding to nucleotides +311 to +786.

CAT Assay

After the chemical incubation period, consisting of one 24-hr treatment, the HepG2.241c.1 cells (HepG2-CAT) were washed twice and lysed with a detergent-based buffer

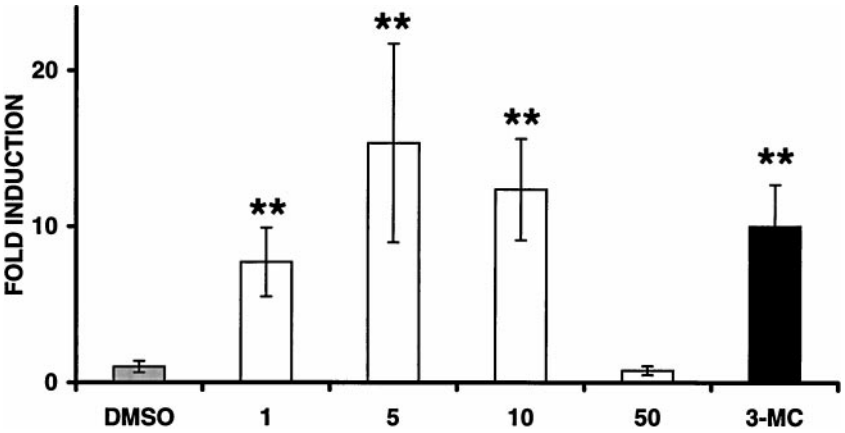

FIG. 1. Effects of primaquine treatments on EROD activity of thawed human hepatocytes. Cells were treated with DMSO (0.25%), 3-MC (1 μM), and with various concentrations of primaquine (1–50 μM) for 72 hr. EROD activity was monitored by kinetic apparition of resorufin at 37°. EROD activity at 5 μM primaquine was 2.908 pmoles.min^{-1} per well (±1.18, N = 3). **Significantly different ($P < 0.001$) from DMSO controls.

to release total cellular protein. An aliquot was used to measure the amount of protein present. The remaining cellular proteins were transferred to 96-well plates containing polyclonal anti-CAT antibodies. A standard sandwich ELISA test was performed and in the final step, horseradish peroxidase was used to catalyze a color change reaction that could be measured at optical density 408 nm.

Ligand Binding

Three hundred μL of enriched 9S fraction containing human hepatic AhR from HepG2 cells was incubated with 50 nM [^{3}H]TCDD in the absence or presence of either 50 μM TCDF or 100, 200, or 400 μM primaquine or 3-MC. [^{3}H]TCDD, TCDF, and primaquine were dissolved in DMSO. Then, cytosol samples were analyzed by velocity sedimentation in sucrose gradient (10–30%). Gradients were centrifuged at 4° for 2 hr (372,000 *g*), and 22 fractions (282 μL; 8 drops per fraction) were collected. The radioactivity of each fraction was determined by liquid scintillation counting [15].

Binding assays were also undertaken with primaquine metabolites that were generated with human hepatic microsomes at 2 mg · mL^{-1} protein. The preparation of metabolites was performed using kinetics for 30 min to 3 hr, with primaquine concentrations ranging from 100 to 500 μM in HEPES buffer (HEPES, 25 mM; EDTA, 1.5 mM; dithiothreitol, 1 mM; pH 7,6). The microsomes were characterized before and after the kinetics to make sure that there was still enzymatic activity. One hundred μL of enriched 9S fraction containing human hepatic AhR from HepG2 cells was incubated with 300 μL of microsomal incubates supplemented with glycerol 10% (v/v), 20 mM of Na_2MoO_4 (final concentration), and 50 nM [^{3}H]TCDD. These conditions led to a maximum concentration of 337.5 μM for primaquine and its metabolites, corresponding to 6750-fold the concentration of [^{3}H]TCDD.

RESULTS

Comparative Study of Cytotoxicity

Compounds belonging to the major antimalarial families such as quinine (natural precursor), mefloquine (amino-alcohol derivative), chloroquine, amodiaquine, and quinidine (amino-4-quinoleine derivatives), pyrimethamine (antimetabolite), arteflene (yingzhaosu A derivative) and primaquine, were comparatively studied for their cytotoxicity, using the neutral red test, in rat hepatocyte primary cultures. Chloroquine, mefloquine, amodiaquine, and arteflene (IC_{50}s ~40 μM) were shown to be more toxic than quinidine, primaquine, quinine, and pyrimethamine (IC_{50}s ~300 μM, data not shown).

Effect of Primaquine on EROD Activity

Among the eight antimalarials tested for their effects on CYP1A1 gene expression, only primaquine, a tissue schizontocidal amino-8-quinoleine, dose dependently induced EROD activity supported by CYP1A1/2 in human hepatocytes (Fig. 1) as well as in HepG2 cells (data not shown). A strong induction was observed at a concentration of 50 μM for the HepG2 cell line and at 5 μM for human hepatocytes. Under these conditions, primaquine increased the EROD activity ~15.3-fold (±6.3, N = 3) over control DMSO in thawed human hepatocytes, as compared to ~10-fold (±2.7, N = 3) for 3-MC. The cytotoxicity study showed that the various antimalarials used as references were devoid of any toxic effect (100% of the control value) in the range of concentrations used for induction (data not shown). At a higher dose, as already observed for 3-MC [16], primaquine decreased EROD activity although these concentrations were shown not to be cytotoxic.

Induction of CYP1A1 Transcription

To demonstrate the capability of primaquine to induce CYP1A1 at the level of transcription, a stably transfected HepG2-derived cell line (HepG2.241c.1 cells) was used which expressed CAT enzyme under the control of the 5′-end region (−1140 to +59) of the human CYP1A1 gene. When this cell line was challenged by primaquine, a dose-dependent increase in CAT expression was observed. Primaquine and 3-MC produced a ~2.25-fold (±0.11, N = 3) and ~3.75-fold (± 0.7, N = 3) increase in CAT

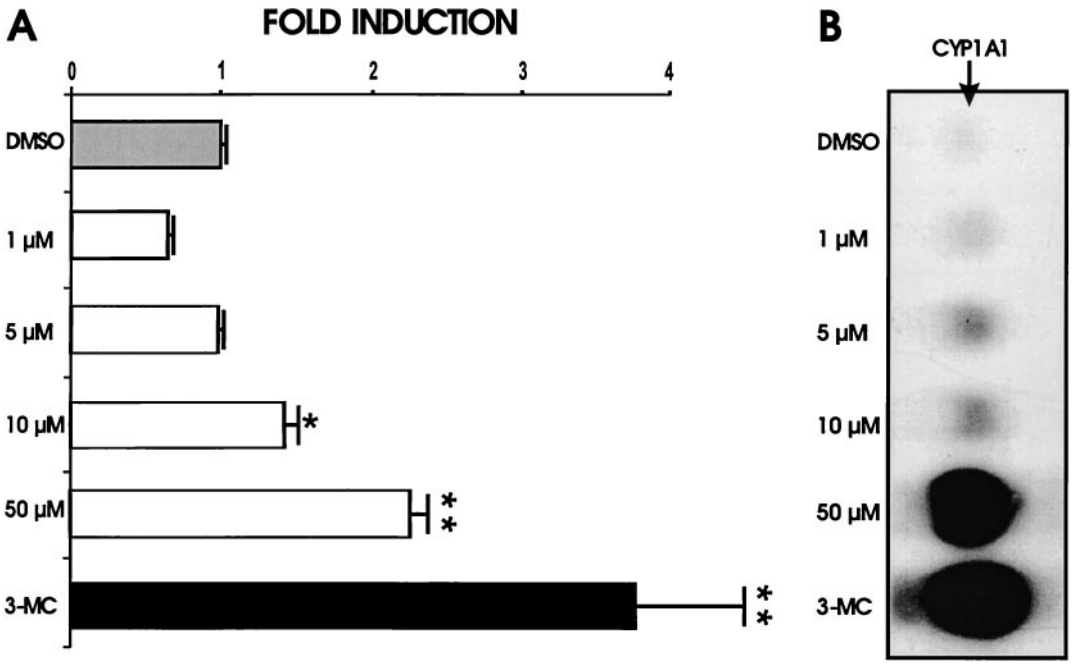

FIG. 2. Dose-dependent increase in CAT activity on the HepG2.241c.1 hepatic cell line steadily transfected with CAT reporter gene (HepG2-CAT) under the control of the human *CYP*1A1 promoter (A), and induction of *CYP*1A1 expression in cultures of fresh human hepatocytes exposed to primaquine (B). Cells were either treated with DMSO (0.25%), 3-MC (1 µM), or primaquine (1–50 µM). For the CAT activity assay, a standard sandwich ELISA was performed and, in the final step, horseradish peroxidase catalyzed a colored reaction measured at OD 405. For mRNA analysis, 20–30 µg of total RNA was analyzed and *CYP*1A1 mRNAs were revealed with radiolabeled *CYP*1A1/2 probe as described in Materials and Methods. *Significantly different ($P < 0.05$) from DMSO controls. **Significantly different ($P < 0.001$) from DMSO controls.

activity, respectively (Fig. 2A). Analysis of Northern blots of *CYP*1A1/2 mRNA from human hepatocytes treated for 72 hr (Fig. 2B) agreed with the trends obtained for CAT values (even after a 24-hr treatment only) and demonstrated that primaquine provoked an increased accumulation of *CYP*1A1 mRNA. However, the highest primaquine concentrations did not decrease *CYP*1A1 mRNA (Fig. 2B) in human. These data, in contradiction to those obtained for EROD activity (Fig. 1), suggest either an effect of primaquine at the translational level, or an enzyme inhibition by the substrate as already reported by Thabrew and Ioannides in rat liver microsomes [17].

Effects of α-NF and 8-MP on CYP1A1 *Induction*

To further examine the possible involvement of AhR in *CYP*1A1 induction [18, 19] by primaquine, we studied the effects of α-NF and 8-MP, two compounds interfering with the AhR and the binding of activated AhR to DNA, respectively. Cells were cotreated with primaquine (5 µM)

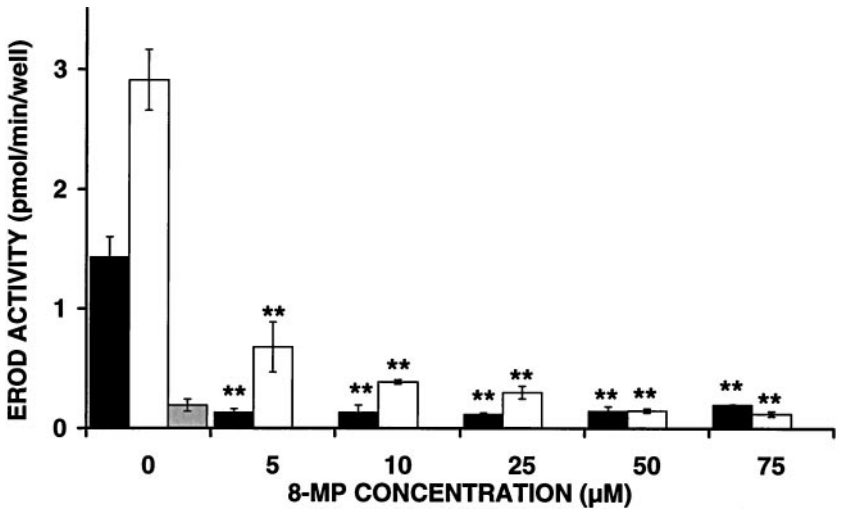

FIG. 3. Inhibition of primaquine and 3-MC EROD induction by 8-MP in thawed human hepatocytes. Cells were cotreated with primaquine (5 µM) or 3-MC (1 µM) and various concentrations of 8-MP (5 to 75 µM). After 72 hr of treatment (one every 24 hr), EROD activity was measured as described previously. **Significantly different ($P < 0.001$) from PQ or 3-MC controls without 8-MP. ■, 3-MC; □, PQ; ▨, DMSO.

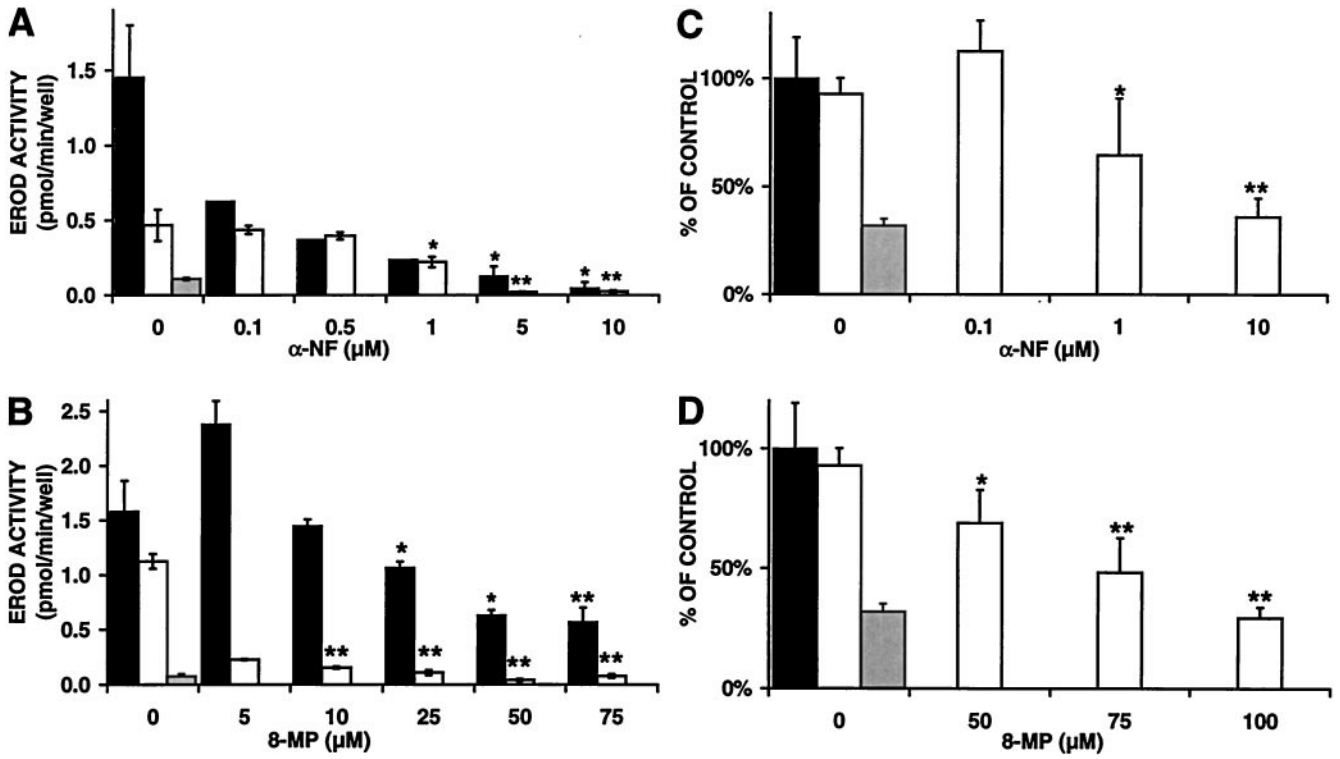

FIG. 4. Inhibition of primaquine and 3-MC CYP1A1 induction by α-NF (A/C) or 8-MP (B/D) in HepG2 (left) and hepG2-CAT (right) cells. Cells were cotreated with primaquine (50 μM) or 3-MC (1 μM), and various concentrations of α-NF (0.1 to 10 μM) or 8-MP (5 to 100 μM). After 72 hr of treatment (one every 24 hr) for EROD and one treatment of 24 hr for CAT, enzymatic activities and CAT levels were measured as described previously. *Significantly different ($P < 0.05$) from PQ or 3-MC controls without α-NF or 8-MP. **Significantly different ($P < 0.001$) from controls. ■, 3-MC; □, PQ; ▨, DMSO.

or 3-MC (1 μM), and α-NF (0.1 to 10 μM) or 8-MP (10 to 75 μM) at concentrations devoid of any toxicity. Figure 3 illustrates the dose-dependent effects of 8-MP on EROD induction by primaquine and 3-MC in thawed human hepatocytes. Figure 4 illustrates the effects of both antagonists on EROD induction in HepG2 (A-B) and HepG2-CAT (C-D) cells. Cell cotreatments with α-NF or 8-MP resulted in a concentration-dependent decrease in the EROD activity. These results suggested that primaquine could induce CYP1A1 via the AhR.

Lack of Effect of Primaquine and Its Metabolites on [^{3}H]TCDD Binding to Human Hepatic AhR

The ability of primaquine to bind the AhR was examined in competition experiments. The 9S-enriched fraction of human liver cytosol was incubated with [^{3}H]TCDD at 50 nM, in the absence or presence of a 2000-, 4000-, and 8000-fold excess of primaquine or 3-MC or a 1000-fold excess of TCDF (50 μM). As shown in Fig. 5B, no significant competition occurred with primaquine, whereas the same concentrations of 3-MC, a classic AhR ligand and potent CYP1A1/2 inducer, dose dependently displaced the radioligand (Fig. 5A). Similar results were obtained with the mouse liver AhR (data not shown). A strong competitive effect was obtained when a 1000-fold excess of TCDF was used as a positive control. The apparent inability of primaquine to bind the AhR suggests that this antimalarial would not be a ligand of the AhR. These results do not exclude an interference with the AhR, but direct interaction does not occur between unchanged primaquine and AhR.

Competitive binding assays were also performed with primaquine incubates obtained with human hepatic microsomes. TCDF and 3-MC controls strongly displaced the radioligand, while none of the primaquine incubates did so (data not shown).

DISCUSSION

We showed that primaquine, an 8-aminoquinoline, induces CYP1A1 gene expression in human hepatocytes and in parental and transfected HepG2 cell lines, whereas compounds belonging to other major antimalarial families do not exert any significant effect. The induction was characterized by a dose-dependent increase in the EROD activity in all cellular models (Fig. 1). This effect was in agreement with a concomitant rise in steady-state concentration of CYP1A1 mRNA and induction of CYP1A1 promoter-directed CAT gene expression (Fig. 2).

The transcriptional activation of CYP1A1 gene expression by the classical inducers such as polycyclic aromatic hydrocarbons is known to involve the AhR [5, 20–22] and

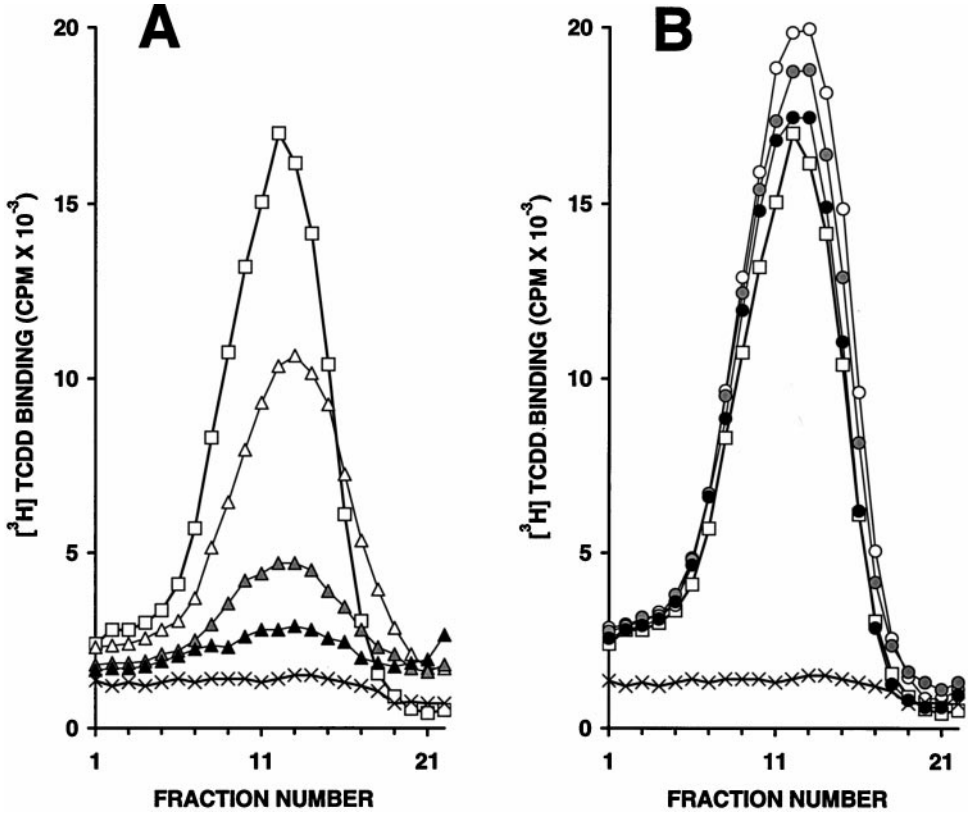

FIG. 5. Absence of competitive binding of primaquine on the AhR. The enriched fraction from human hepatocyte cytosols was incubated with 50 nM of [^{3}H]TCDD, in the absence or presence of control 3-MC (A) or primaquine (B) (100 to 400 μM) or TCDF 50 μM, for 2 hr at 4°. The samples were then analyzed by velocity sedimentation on 10–30% sucrose gradients as described in Materials and Methods. —□—, 50 nM of [^{3}H]TCDD; ∗, + 50 μM of TCDF; —○—, + 100 μM of PQ; —◎—, + 200 μM of PQ; —●—, + 400 μM of PQ; and —△—, + 100 μM of 3-MC; —◬—, + 200 μM of 3-MC; —▲—, + 400 μM of 3-MC.

the xenobiotic responsive element. Therefore, we evaluated the effects of two specific antagonists of the Ah-dependent cascade of events leading to *CYP*1A1 gene induction: i) α-NF, a partial AhR antagonist [7, 8] and ii) 8-MP, recently described in Hepa-1c1c7 cells [9] as an inhibitor of the *CYP*1A1 induction provoked by TCDD by interfering with the nuclear dioxin receptor binding to DNA. When 8-MP (5 to 100 μM) was added to the culture medium of primaquine-treated (5 μM) human hepatocytes (Fig. 3) or (50 μM) HepG2 cells (Fig. 4B), a dose-dependent inhibition of EROD activity was observed. Assays on HepG2-CAT cells gave the same result at the level of gene transcription (Fig. 4D). Similarly, cotreatment with primaquine (5 μM) and α-NF (0.1 to 10 μM) resulted in a concentration-dependent decrease in primaquine-induced EROD activity and CAT level, but only in HepG2 (Fig. 4A) and HepG2-CAT cells (Fig. 4C), respectively. α-NF did not act as an AhR antagonist on hepatocytes and dose dependently induced EROD activity (data not shown). Control experiments performed with 3-MC (1 μM), an AhR ligand greatly enhancing expression of *CYP*1A1 [18], led to the same results. However, in hepatocytes, the 8-MP inhibitory effect was stronger in the case of 3-MC than in that of primaquine (Fig. 3), whereas the opposite observation was found in HepG2 cells (Fig. 4B). This apparent contradiction could be due to the existence of some 8-MP metabolites that may be capable of inducing *CYP*1A1 expression, but which appear only in one of the cellular models. Thus, in HepG2 cells, this effect occurs in the case of 3-MC but not in that of primaquine (Fig. 4B). Since a previous study has shown that EROD activity was inhibited by primaquine in rat liver microsomes [17], a reasonable hypothesis could be that some primaquine remained in the cytoplasm of the treated cells despite the successive washings, thus inhibiting EROD activity induced by 8MP metabolites.

On the whole, these data suggest that primaquine-mediated *CYP*1A1 induction involves the AhR-dependent pathway, as already evidenced for polycyclic aromatic hydrocarbons. However, the absence of competitive binding of primaquine to the AhR suggests that unchanged primaquine is probably not a ligand of this receptor (Fig. 5B). This result was expected because primaquine is not a polycyclic aromatic chemical like 3-MC, which at the same concentrations strongly displaces the radioligand (Fig. 5A). It should be noted that, in an attempt to study the potential involvement of the AhR in *CYP*1A1 induction by primaquine, AhR-knockout mice (male AhR −/− C57BL/6 strain) and responsive mice were also used. These experiments were not conclusive, since primaquine appeared to

be devoid of any effect in knockout as well as responsive mice (data not shown). This result was already obtained in a previous study with some other *CYP*1A1 inducers (in the series of benzimidazole) which are active in human and rabbit hepatocytes, but are unable to induce *CYP*1A1 mRNA in mouse hepatoma cells or hepatocytes [23].

In summary, the data presented demonstrate that primaquine significantly induces *CYP*1A1 gene expression both in hepatocytes and HepG2 cells. The typical blood concentration of primaquine in humans during a normal daily administration of 15 mg over 14 days is approximately 50 to 60 ng/mL. This concentration actually corresponds to the micromolar range used for this study. Despite the fact that human liver *in vivo* expresses virtually no *CYP*1A1 mRNA nor *CYP*1A1 protein, there is an increasing amount of data available about *in vivo* *Cyp*1A1 induction in rats exposed to cigarette smoke or hyperoxia or fed diets containing β-carotene isomers or even after per os administration of caffeine [24–27]. In this context, considering the fact that primaquine is an even more potent *CYP*1A1 inducer than 3-MC itself in human hepatocytes, one can reasonably imagine the possibility that primaquine may induce *CYP*1A1 *in vivo* in human liver.

Finally, we have shown that primaquine is not a ligand of the AhR by itself; conversely, this receptor seems to be involved in *CYP*1A1 induction. Furthermore, competitive binding assays with primaquine metabolites generated by incubations with human hepatic microsomes did not displace TCDD from its binding site on the AhR (data not shown). This supports the notion that primaquine does not activate the AhR by direct interaction with the ligand binding site, but indirectly, via an alternative mechanism mediated by intracellular signal transduction systems, possibly involving oxidative stress [28]. Indeed, several recent studies have described some ligand-independent activation mechanisms of the AhR [29–33] which might explain these results. According to Lesca *et al.* [23], phosphorylation could be involved in the transformation and activation of the AhR without the intervention of any ligand. This hypothesis was recently developed by Backlund *et al.* [34] when they showed that casein kinase II could mediate these phosphorylations. The steroid receptors are also known to be indirectly activated without any ligand by phosphorylations, as reviewed by Weigel [35].

In conclusion, due to the biological significance of P450-dependent metabolism, changes (*e.g.* by induction) in metabolic activity are of clinical importance in the case of certain drugs such as primaquine, which may be coadministrated with other interfering molecules such as antibiotics. Furthermore, as *CYP*1A1 is known to play an important role in the chemical activation of xenobiotics to cytotoxic, mutagenic, and carcinogenic derivatives, this should be added to the list of major drawbacks of therapy that have already been described with this class of compounds, namely methaemoglobinaemia and haemolytic anaemia. Primaquine toxic side-effects and the recent apparition of primaquine-tolerant *vivax* malaria in Thailand [36] have led to the development of new, less toxic primaquine analogs that will probably be used at the same posologies as aminoquinoleines (quinine, chloroquine, etc.) and aminoalcohols (mefloquine), i.e. 500 to 750 mg/day. Thus, the possible induction of *CYP*1A1 by the most promising of these new primaquine analogs, i.e. 5-fluoroprimaquine, WR242511, and 238605 (Walter Reed Army Institute of Research), should be even more carefully considered given the pharmaco-toxicological importance of this induction [4, 37, 38].

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References

1. Miller LH, Time to put malaria control on the global agenda. *Nature* **386:** 535–541, 1997.
2. Hollingdale MR, Malaria and the Liver. In: *The Liver: Biology and Pathobiology*, Second Edition (Eds. Arias IM, Jakoby WB, Popper H and Schachter D), pp. 1195–1207. Raven Press, Ltd., New York, 1988.
3. Luzzi GA and Peto, TEA, Adverse effects of antimalarials, an update. *Drug Safety* **8:** 295–311, 1993.
4. O'Neill PM, Tingle MD, Mahmud R, Storr RC, Ward SA and Park BK, The effect of fluorine substitution on the haemotoxicity of primaquine. *Bioorg Med Chem Lett* **5:** 2309–2314, 1995.
5. Denison MS and Whitlock JP Jr., Xenobiotic-inducible transcription of cytochrome P450 genes. *J Biol Chem* **270:** 18175–18178, 1995.
6. Gonzalez FJ, Liu SY and Yano M, Regulation of cytochrome P450 genes: Molecular Mechanisms. *Pharmacogenetics* **3:** 51–57, 1993.
7. Merchant M, Kirshnan V and Safe S, Mechanism of action of α-naphthoflavone as an Ah receptor antagonist in MCF-7 human breast cancer cells. *Toxicol Appl Pharm* **120:** 179–185, 1993.
8. Merchant M and Safe S, *In vitro* inhibition of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced activity by α-naphthoflavone and 6-methyl-1,3,8-trichlorodibenzofuran using an aryl hydrocarbon (Ah)-responsive construct. *Biochem Pharmacol* **50:** 663–668, 1995.
9. Jeong HG, Yun CH, Jeon YJ, Lee SS and Yang KH, Suppression of cytochrome P450 (*Cyp*1a1) induction in mouse hepatoma hepa-1c1c7 cells by methoxsalen. *Biochem Biophys Res Commun* **208:** 1124–1130, 1995.
10. Nicolas F, de Sousa G, Thomas P, Placidi M, Lorenzon G and Rahmani R, Comparative metabolism of 3′-azido-3′-deoxythymidine in cultured hepatocytes from rats, dogs, monkeys, and humans. *Drug Metab Dispos* **23:** 308–313, 1995.
11. Dou M, de Sousa G, Lacarelle B, Placidi M, Lechene de La Porte P, Domingo M, Lafont H and Rahmani R, Thawed human hepatocytes in primary culture. *Cryobiology* **29:** 454–469, 1992.
12. Reiners Jr. JJ, Cantu AR, Pavone A, Smith SC, Gardner CR and Laskin DL, Fluorescence assay for per-cell estimation of cytochrome P-450-dependent monooxygenase activities in

keratinocyte suspensions and cultures. *Anal Biochem* **188:** 317–324, 1990.
13. Donato MT, Gomez-Lechon MJ and Castell JV, A microassay for measuring cytochrome P450IA1 and P450IIB1 activities in intact human and rat hepatocytes cultured on 96-well plates. *Anal Biochem* **213:** 29–33, 1993.
14. Chomczynski P and Shacchi N, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162:** 156–159, 1987.
15. Lesca P, Fernandez N and Roy M, The binding components for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and polycyclic aromatic hydrocarbons. *J Biol Chem* **262:** 4827–4835, 1987.
16. Delescluse C, Ledirac N, de Sousa G, Pralavorio M, Botta-Fridlund D, Letreut Y and Rahmani R, Comparative study of *CYP*1A1 induction by 3-methylcholanthrene in various human hepatic and epidermal cell types. *Toxicol* In Vitro **11:** 443–450, 1997.
17. Thabrew MI and Ioannides C, Inhibition of rat hepatic mixed function oxidases by antimalarial drugs: Selectivity for cytochrome P-450 and P-448. *Chem-Biol Interact* **51:** 285–294, 1984.
18. Okey AB, Riddick DS and Harper PA, Molecular biology of the aromatic hydrocarbon (dioxin) receptor. *Trends Pharmacol Sci* **15:** 226–232, 1994.
19. Swanson HI and Bradfield CA, The Ah-receptor: Genetics, structure and function. *Pharmacogenetics* **3:** 213–230, 1993.
20. Wanner R, Brömmer S, Czarnetzki BM and Rosenbach T, The differentiation-related upregulation of aryl hydrocarbon receptor transcript levels is suppressed by retinoic acid. *Biochem Biophys Res Commun* **209:** 706–711, 1995.
21. Roberts EA, Johnson KC, Harper PA and Okey AB, Characterization of the Ah receptor mediating aryl hydrocarbon hydroxylase induction in the human liver cell line Hep G2. *Arch Biochem Biophys* **276:** 442–450, 1990.
22. Takahashi Y, Itoh S, Shimojima T and Kamataki T, Characterization of the Ah receptor promoter in human liver cell line, HepG2. *Pharmacogenetics* **4:** 219–222, 1994.
23. Lesca P, Peryt B, Larrieu G, Alvinerie M, Galtier P, Daujat M, Maurel P and Hoogenboom L, Evidence for the ligand-independent activation of the Ah receptor. *Biochem Biophys Res Commun* **209:** 474–482, 1995.
24. Wardlaw SA, Nikula KJ, Kracko DA, Finch GL, Thornton-Manning JR and Dahl AR, Effect of cigarette smoke on *cyp*1A1, *cyp*1A2 and *cyp*2B1/2 of nasal mucosae in F344 rats. *Carcinogenesis* **19:** 655–662, 1998.
25. Moorthy B, Nguyen UT, Gupta S, Stewart KD, Welty SE and Smith CV, Induction and decline of hepatic cytochromes P4501A1 and 1A2 in rats exposed to hyperoxia are not paralleled by changes in glutathione *S*-transferase-alpha. *Toxicol Lett* **90:** 67–75, 1997.
26. Gradelet S, Leclerc J, Siess MH and Astorg PO, beta-Apo-8′-carotenal, but not beta-carotene, is a strong inducer of liver cytochromes P4501A1 and 1A2 in rat. *Xenobiotica* **26:** 909–919, 1996.
27. Goasduff T, Dreano Y, Guillois B, Menez JF and Berthou F, Induction of liver and kidney *cyp*1A1/1A2 by caffeine in rat. *Biochem Pharmacol* **52:** 1915–1919, 1996.
28. Vasquez-Vivar J and Augusto O, Oxidative activity of primaquine metabolites on rat erythrocytes *in vitro* and *in vivo*. *Biochem Pharmacol* **47:** 309–316, 1994.
29. Daujat M, Peryt B, Lesca P, Fourtanier G, Domergue J and Maurel P, Omeprazole, an inducer of human *CYP*1A1 and 1A2, is not a ligand for the Ah receptor. *Biochem Biophys Res Commun* **188:** 820–825, 1992.
30. Aix L, Rey-Grobellet X, Larrieu G, Lesca P and Galtier P, Thiabendazole is an inducer of cytochrome P4501A1 in cultured rabbit hepatocytes. *Biochem Biophys Res Commun* **202:** 1483–1489, 1994.
31. Gleizes-Escala C, Lesca P, Larrieu G, Dupuy J, Pineau T and Galtier P, Effect of exposure of rabbit hepatocytes to sulfur-containing anthelmintics (Oxfendazole and Fenbendazole) on cytochrome P450 1A1 expression. *Toxicol* In Vitro **10:** 129–139, 1996.
32. Gradelet S, Astorg P, Pineau T, Canivenc MC, Siess MH, Leclerc J and Lesca P, Ah receptor-dependent *CYP*1A induction by two carotenoids, canthaxantin and beta-apo-8′-carotenal, with no affinity for the TCDD binding site. *Biochem Pharmacol* **54:** 307–315, 1997.
33. Ledirac N, Delescluse C, de Sousa G, Pralavorio M, Lesca P, Amichot M, Bergé JB and Rahmani R, Carbaryl induces *CYP*1A1 gene expression in HepG2 and HaCaT cells but is not a ligand of the human hepatic Ah receptor. *Toxicol Appl Pharmacol* **144:** 177–182, 1997.
34. Backlund M, Johansson I, Mkrtchian S and Ingelman-Sundberg M, Signal transduction-mediated activation of the aryl hydrocarbon receptor in rat hepatoma H4IIE cells. *J Biol Chem* **272:** 31755–31763, 1997.
35. Weigel NL, Steroid hormone receptors and their regulation by phosphorylation. *Biochem J* **319:** 657–667, 1996.
36. Looareesuwan S, Buchachart K, Wilairatana P, Chalermrut K, Rattanapong Y, Amradee S, Siripiphat S, Chullawichit S, Thimasan K, Ittiverakul M, Triampon A and Walsh DS, Primaquine-tolerant *vivax* malaria in Thailand. *Ann Trop Med Parasitol* **91:** 939–943, 1997.
37. Marino MT, Peggins JO, Brown LD, Urquhart MR and Brewer TG, Pharmacokinetics and kinetic-dynamic modeling of an 8-aminoquinoline candidate anticyanide and antimalarial drug (WR242511). *Drug Metab Dispos* **22:** 358–366, 1993.
38. Idowu OR, Peggins JO, Brewer TG and Kelley C, Metabolism of a candidate 8-aminoquinoline antimalarial agent, WR 238605, by rat liver microsomes. *Drug Metab Dispos* **23:** 1–17, 1995.