

Impact of inflammation on chlorpromazine-induced cytotoxicity and cholestatic features in HeparG cells.

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The abbreviations used are:

BCRP, breast cancer resistance protein; BSEP, Bile salt export pump; CAR, Constitutive androstane receptor; CDFDA, 5(6)-Carboxy-2',7'-dichlorofluorescein diacetate; CPZ, chlorpromazine; CYP, cytochrome P450; DILI, Drug-induced liver injury; DMSO, dimethyl sulfoxide; FXR, Farnesoid X receptor; H₂-DCFDA, 2',7'-dichlorodihydrofluorescein ; HO1, Heme Oxygenase 1; iDILI idiosyncratic drug-induced liver injury; IL-1 β , interleukin-1; IL-6, interleukin-6; LPS, lipopolysaccharide; MDR1, multidrug resistance protein 1 ; MnSOD, Manganese Superoxide Dismutase; MRP, multidrug resistance-associated protein; MTT, methylthiazole tetrazolium; Nor-1 CPZ, N-desmethyl-CPZ; Nrf2, NF-E2-related factor; NTCP, Na⁺-dependent taurocholate co-transporting polypeptide; OATP, organic anion-transporting polypeptide; OCT-1, organic cation transporter 1; 3-OH CPZ, 3-hydroxyl CPZ; 7-OH CPZ, 7-hydroxyl CPZ ; PBS, phosphate buffered saline; PXR, Pregnane X receptor; ROS, Reactive Oxygen Species; RT-qPCR, real time-quantitative polymerase chain reaction; SO CPZ, sulfoxy-CPZ; TA, taurocholic acid.

Abstract

Several factors are thought to be implicated in the occurrence of idiosyncratic adverse drug reactions. The present work aimed to question as to whether inflammation is a determinant factor in hepatic lesions induced by chlorpromazine (CPZ) using the human HepaRG cell line. An inflammation state was induced by a 24 hour-exposure to pro-inflammatory cytokines IL-6 and IL-1 β ; then the cells were simultaneously treated with CPZ and/or cytokine for 24 hours or daily for 5 days. The inflammatory response was assessed by induction of C-reactive protein and IL-8 transcripts and proteins as well as inhibition of CPZ metabolism and down-regulation of CYP3A4 and CYP1A2 transcripts, two major CYPs involved in its metabolism. Most effects of co-treatments with cytokines and CPZ were amplified or only observed after 5 daily treatments; they mainly included increased cytotoxicity and overexpression of oxidative stress-related genes, decreased Na⁺-taurocholate cotransporting polypeptide (NTCP) mRNA levels and activity, a key transporter involved in bile acids uptake, and deregulation of several other transporters. However, CPZ-induced inhibition of taurocholic acid efflux and pericanalicular F-actin distribution were not affected. In addition, a time-dependent induction of phospholipidosis, was noticed in CPZ-treated cells, without obvious influence of the inflammatory stress. In summary, our results show that an inflammatory state induced by pro-inflammatory cytokines, increased cytotoxicity and enhanced some cholestatic features induced by the idiosyncratic drug CPZ in HepaRG cells. These changes, together with inhibition of CYP activities, could have important consequences if extrapolated to the *in vivo* situation.

Introduction

Drug-induced liver injury (DILI) is a major cause of attrition during both early and late stages of the drug development and marketing process (Meng, 2010). DILI is usually classified as dose-dependent which is typically reproducible, or unpredictable (idiosyncratic) occurring only in certain susceptible patients and being not overtly dose-dependent (Greer et al., 2010). The mechanisms underlying idiosyncratic DILI (iDILI) remain largely unknown. Several factors are thought to be implicated; they include genetic polymorphisms of drug metabolism-related or HLA genes, liver pathologies of various origins including viral infection, and/or environmental inflammatory stress. Among these factors, inflammation caused by agents such as bacterial lipopolysaccharide (LPS) and pro-inflammatory cytokines, has been shown to exacerbate toxicity of many hepatotoxic chemicals (Roth et al., 1997; Yee et al., 2000) (Barton et al., 2000) and to induce hepatotoxicity of various drugs associated with idiosyncratic reactions, such as trovafloxacin (Waring et al., 2006; Shaw et al., 2007) and chlorpromazine (CPZ) (Buchweitz et al., 2002).

The antipsychotic agent CPZ is known to cause hepatotoxicity which includes cholestasis (Hollister, 1957; Regal et al., 1987), hepatocellular necrosis and phospholipidosis, in some patients (Velayudham and Farrell, 2003). Various CPZ-induced liver disturbances have also been described in *in vivo* and *in vitro* animal models but they were mainly limited to hepatic lesions related to cell death, that were usually amplified by pre-treatment with inflammatory agents (Buchweitz et al., 2002; Gandhi et al., 2010; MacAllister et al., 2013). No evidence of changes in serum bile acid concentrations and alterations of bile canaliculi were observed in rats treated with LPS and CPZ (Buchweitz et al., 2002). Obviously, whether cholestatic effects of CPZ are aggravated by concomitant exposure to pro-inflammatory cytokines remains unclear.

LPS and pro-inflammatory cytokines have been reported to alter various liver functions, including drug metabolism and transport. In particular, they down-regulate activity of major cytochromes P450 (CYP) involved in xenobiotic metabolism and also markedly impair phase 2 conjugating enzymes as well as basolateral and canalicular transporters of bile acids and organic anions (Abdel-Razzak et al., 1993; Aitken et al., 2006). Recently, our group reported that CPZ generated an early oxidative stress, altered mitochondrial membrane potential and disorganized pericanalicular cytoskeletal F-actin distribution in human HepaRG cells. These effects were associated with inhibition of taurocholic acid (TA) efflux (Antherieu et al.,

2013). These results led us to question as to whether two major pro-inflammatory cytokines (IL-6 and IL-1 β) might enhance cytotoxic and cholestatic effects induced by CPZ in human hepatocytes. We showed that an inflammatory situation caused by both cytokines and resulting in a strong inhibition of cytochrome P450-mediated metabolism of CPZ, decreased cell viability and aggravated some cholestatic markers in hepatocyte-like HepaRG cell cultures treated with this cholestatic drug.

Materials and methods

Chemicals.

CPZ, methylthiazolotetrazolium (MTT) and 5(6)-carboxy-2',7'-dichlorofluorescein diacetate (CDFDA) were purchased from Sigma (St. Quentin Fallavier, France). Human recombinant IL-6 and IL-1 β were from Promokine, (Heidelberg, Germany). Human C-Reactive Protein (CRP) and CXCL8/IL-8 DuoSet kits were from R&D (Abingdon, United Kingdom). [3 H]-Taurocholic acid ([3 H]-TA) was from Perkin Elmer (Boston, MA). 2',7'-dichlorodihydrofluorescein (H2-DCFDA) was from Life Technologies-Molecular probes (Saint Aubin, France). Phalloidin fluoprobe SR101 (200 U/ml) was purchased from Interchim (Montluçon France). Hoechst dye was from Promega (Madison, Wisconsin, USA). CPZ metabolites [N-desmethyl-CPZ (Nor-1 CPZ), sulfoxy-CPZ (SO CPZ), 3-hydroxyl CPZ (3-OH CPZ) and 7-hydroxyl CPZ (7-OH CPZ)] were a gift from Sanofi (Alfortville, France).

Cell culture.

HepaRG cells were seeded at a density of 2.6×10^4 cells/cm 2 as previously described (Gripon et al., 2002). After two weeks, the medium was supplemented with 2% dimethyl sulfoxide (DMSO) for two further weeks in order to obtain confluent differentiated HepaRG cell cultures. At that time, these cultures contained hepatocyte-like and progenitors/primitive biliary-like cells in around an equal proportion (Cerec et al., 2007) and were ready for experimental use. All treatments were done with a medium supplemented with 2% fetal bovine serum and 1% DMSO.

Cell viability.

Cytotoxicity was evaluated by the MTT colorimetric assay. Briefly, cells were seeded in 24-well plates and treated with various concentrations of cytokines and CPZ. After medium

removal, 500 μ L serum-free medium containing MTT (0.5 mg/ml) was added to each well and incubated for 2 hours at 37°C. The water-insoluble formazan was dissolved in 500 μ l DMSO and absorbance was measured at 550 nm.

Measurement of Reactive Oxygen Species (ROS).

ROS generation was determined by the H₂-DCFDA assay. Cells were incubated for 2 hours at 37°C with 2 μ M H₂-DCFDA; then they were washed with cold phosphate buffered saline (PBS), and scraped in phosphate buffer (10 mM, pH 7.4) / methanol (v/v) added with 0.1% Triton X-100. Fluorescence intensity of cell lysates was determined by spectrofluorimetry using excitation/emission wavelengths of 498/520 nm.

CRP and IL-8 protein measurements.

CRP and IL-8 proteins were measured in cell supernatants after 1 and 5 days of co-treatment with cytokines and CPZ using the CRP and CXCL8/IL-8 DuoSet kits, according to manufacturer's instructions. Briefly, supernatants were collected at the appropriate time points and stored at -80°C until analysis; 96-well microplates were coated with capture antibody and incubated overnight. Samples and standards were diluted appropriately and added for 2 hours after a saturation step. Secondary antibody was added for 2 hours after washing. Streptavidin-horseradish peroxidase and its substrate were used for the revelation step. Optical density was read at 450nm with wavelength correction. All steps were performed at room temperature.

Real time-quantitative polymerase chain reaction (RT-qPCR) analysis.

Total RNA was extracted from 10⁶ HepaRG cells with the SV total RNA isolation system (Promega). RNAs were reverse-transcribed into cDNA and RT-qPCR was performed using a SYBR Green mix. Primer sequences are listed in Supplemental Table 1.

F-actin distribution.

After cell exposure to CPZ and/or cytokines, cells were washed twice with warm PBS, fixed with 4% paraformaldehyde for 20 minutes at 4°C and permeabilized with 0.3% Triton in PBS for 20 minutes. F-actin and nuclei were labeled simultaneously using the phalloidin-fluoprobe diluted at 1/100 and 5ng/ml Hoechst dye respectively, for 20 minutes (Pernelle et al., 2011). Imaging was done using the Cellomics ArrayScan VTI HCS Reader (Thermo Scientific).

Na⁺-dependent taurocholic cotransporting polypeptide (NTCP) activity.

Activity of the NTCP transporter was estimated through determination of sodium-dependent intracellular accumulation of radiolabeled TA substrate as previously described (Antherieu et al., 2013). Briefly, treated cells were incubated with radiolabeled TA for 30 minutes. Cells were then washed twice with PBS and lysed with 0.1 N NaOH. Accumulation of radiolabeled substrates was determined through scintillation counting. Taurocholate accumulation values in the presence of sodium minus accumulation values in the absence of sodium represented NTCP activity.

TA efflux activity.

Cells were first incubated with [³H]-TA, a substrate of bile salt pump export (BSEP), for 30 minutes, then washed with PBS and exposed to CPZ, cytokines or both for 2 or 4 hours in a standard buffer containing Ca²⁺ and Mg²⁺ ions. At the end of the incubation time, cells were washed with PBS and incubated for 5 minutes with a Ca²⁺ and Mg²⁺-free buffer in order to disrupt the canalicular tight junctions. Then, they were scraped in 0.1 N NaOH; the remaining radiolabeled substrate was measured through scintillation counting to determine TA efflux (Marion et al., 2012).

CDF excretion.

After 2 or 4 hours of exposure to CPZ and cytokines individually or simultaneously, cells were incubated with 3μM CDFDA, which is hydrolyzed by intracellular esterases to CDF, a substrate of multidrug resistance-associated protein 2 (MRP2) (Zamek-Gliszczynski et al., 2003) for 20 minutes at 37°C and then washed with PBS. Imaging was done using inverted microscope Zeiss Axiovert 200M and AxioCam MRm.

Quantification of CPZ and its metabolites.

After 1 and 5 days of treatment, incubation media were collected and stored at -20°C until analysis. Cells were washed twice with PBS and then lysed with 0.01N NaOH. An aliquot was used to determine protein concentration and the remaining volume was stored at -20°C until analysis. Concentrations of CPZ and four of its metabolites (Nor-1 CPZ, SO CPZ, 3-OH CPZ and 7-OH CPZ) were determined in both cell lysates and incubation media using an HPLC-MS-MS system. HPLC separation was performed on a C18 column (Hypersil Gold, C18; Particle Size: 1.9μm; 50 x 2.1mm, I.D., ThermoFisher Scientific). Mobile phase A

consisted of 10mM ammonium acetate with 0.1% formic acid. Mobile phase B was acetonitrile with also 0.1% formic acid. The gradient program was as follows: 0-2.50 min, 95% A; 2.51-3.70 min, 70% A; 3.71-5.50 min, 60% A; 5.51-6.60 min, 95% B; 6.61-8.00 min, 95% A. Under these conditions, retention times were, respectively, 3.16 min for SO CPZ, 3.57 min for 3-OH CPZ, 3.85 min for 7-OH CPZ, 4.60 min for Nor-1 CPZ and 4.70 min for CPZ. The flow rate was kept at 500 μ L/min. The injected volume was 5 μ L and the temperature of column was maintained at 35°C. Detection parameters were set in MRM mode, with the following transitions: 304.9/72.0 (collision energy 18) for Nor-1 CPZ, 334.9/231.9 (collision energy 37) for SO CPZ, 334.9/86.0 (collision energy 20) for both 3-OH CPZ and 7-OH CPZ, 319.1/86.1 (collision energy 20) for CPZ, and 388.0/315.0 (collision energy 23) for flurazepam, as internal standard.

Statistical analysis.

One-way ANOVA with Bonferroni's multiple comparison test (GraphPad Prism 5.00) was performed to compare data between CPZ-, cytokine-, cytokine and CPZ-treated cells and control cultures. Each value corresponded to the mean \pm standard deviation (SD) of at least three independent experiments. Data were considered significantly different when $p < 0.05$.

Results

Dose- and time-dependent effects of CPZ and cytokines on HepaRG cell viability.

First, the MTT assay was carried out to estimate cytotoxicity of CPZ, IL-6 and IL-1 β in HepaRG cells after one and 5 daily additions. At 5 and 20 μ M, CPZ elicited no toxicity regardless of treatment duration while at 50 μ M it caused 90% cell death after 3 additions (data not shown). IL-6 did not alter cell viability at concentrations ranging from 0.01 (1unit/ml) to 1ng/ml regardless of the duration of treatment while at 10ng/ml, it induced a 20% loss in cell viability after 5 daily treatments. IL-1 β also caused 20-30% decrease in cell viability at concentrations ranging from 0.1 (20 units/ml) to 1ng/ml after 5 days of treatment (data not shown). Based on these results, nontoxic concentrations of 20 μ M CPZ, 1ng/ml IL-6 and 0.01ng/ml IL-1 β were selected for 1 and 5 day co-treatment while 50 μ M CPZ was used for short-term co-treatment.

In order to verify whether their viability was affected by co-treatments with cytokines, HepaRG cells were first incubated with IL-6 or IL-1 β for 24h prior to simultaneous CPZ

addition for 1 or 5 days. Compared to untreated cells, a significant although modest decrease of cell viability was observed after 5 additions of CPZ with either cytokine (12% and 20% with IL-6 + CPZ and IL-1 β + CPZ respectively) (Fig. 1).

Effects of cytokines and CPZ on inflammation markers.

Generation of an *in vitro* inflammatory response with the selected cytokine concentrations was validated by measurement of CRP and IL-8 mRNA and secreted protein levels after 1 and 5 additions of either IL-1 β or IL-6. Both cytokines caused a statistically significant increase of CRP mRNA (8- to 95-fold) and protein (12- to 48-fold) levels at both time points compared to corresponding untreated cells (Fig. 2A, C), thereby demonstrating an inflammatory response of HepaRG cells. At 20 μ M CPZ did not cause any significant change in CRP expression, excluding any inflammatory effect of this neuroleptic drug at least up to this concentration. However, combination of CPZ and IL-6 or IL-1 β enhanced increase of CRP mRNAs (53-fold with IL-6 versus 80-fold with IL-6 + CPZ and 8-fold with IL-1 β versus 12-fold with IL-1 β + CPZ after 5 daily additions) (Fig. 2A). Although cytokine-mediated induction of CRP protein was also enhanced by CPZ after 5 simultaneous additions (41-fold by IL-6 versus 48-fold by IL-6 + CPZ and 12-fold by IL-1 β versus 14-fold by IL-1 β + CPZ), these variations were not statistically significant (Fig. 2C).

Regardless of treatment duration, IL-8 mRNA and protein levels were not affected by IL-6 and 20 μ M CPZ individually nor by their combination. By contrast, IL-8 mRNAs were increased by IL-1 β alone or associated with CPZ after 1 addition; this induction was lower after 5 additions (Fig. 2B). Similarly, IL-8 protein was also augmented (5- and 4-fold after 1 and 5 additions respectively) (Fig. 2D). Secreted IL-1 β and IL-6 proteins were undetectable in either untreated or 20 μ M CPZ-treated HepaRG cells even after 5 additions (data not shown).

Additional experiments were performed with 50 μ M CPZ, a concentration previously shown to induce early characteristic cholestatic features. At 50 μ M, CPZ increased IL-8 at both mRNA (3.15-fold) and protein (6.6-fold) levels after 24 hours; induction of IL-8 protein was observed as early as 2 hours. Moreover, IL-1 β and IL-6 transcripts were induced by 50 μ M CPZ (6.6- and 2.3-fold after 24h) and secreted IL-6 protein became detectable after 4h exposure to 50 μ M CPZ (Supplemental Figure 1).

Inhibition of CPZ metabolism by pro-inflammatory cytokines.

At the selected concentrations and regardless of the duration of treatment, both IL-6 and IL-1 β strongly repressed mRNA expression of CYP1A2 (a drop by 67% and 58% respectively) and CYP3A4 (a drop by 90% and 75% respectively), two major CYPs involved in CPZ oxidative metabolism, in HepaRG cells. While CYP1A2 and CYP3A4 transcripts were induced 4- to 6-fold in 20 μ M CPZ-treated HepaRG cells compared to untreated cells, their induction was abolished in the presence of cytokines (Fig. 3A). Of note, cytokine-induced inhibition of both cytochromes was attenuated or even completely abolished with IL-1 β after 5 additions in CPZ co-treated cells.

Moreover, four CPZ metabolites, Nor-1 CPZ, SO CPZ, 7-OH CPZ and 3-OH CPZ, were quantified by HPLC-MS-MS in culture media and cell lysates after 1 and 5 drug additions. Detectable amounts of Nor-1 CPZ, SO-CPZ and to a lesser extent 7-OH CPZ, were found in both cell lysates (Fig. 3B) and incubation media (Fig. 3C) of untreated and cytokine-treated HepaRG cells, whereas 3-OH CPZ was barely detectable in all tested conditions, preventing its accurate quantification (data not shown). After 1-day of simultaneous treatment with cytokines, 7-OH CPZ and Nor-1 CPZ amounts were decreased by up to 42% while CPZ content was statistically higher in cytokine-treated cells compared with values obtained in cells treated with CPZ alone. After 5 days, all detectable metabolites were decreased in the presence of IL-6 and most of them with IL-1 β (SO CPZ and Nor-1 CPZ). Accumulation of CPZ was nearly doubled in both cell lysates and supernatants of cytokine-treated cultures (Fig. 3B, C).

Time-dependent effects of CPZ and cytokines on phospholipidosis induction.

Intracytoplasmic vesicles, corresponding to lamellar bodies, hallmark of phospholipidosis, were detected in both hepatocyte-like and biliary-like cells after 3 days exposure to 20 μ M CPZ and their number increased during the following days. Co-treatment with cytokines did not seem to affect their accumulation (Fig. 4). This observation was confirmed by measurement of transcript levels of some genes related to phospholipidosis after one and five treatments. Indeed, Adipose Differentiation-Related Protein (ADFP) and Perilipin-4 (PLIN4), two genes involved in the formation of lamellar vesicles and Acyl-CoA Desaturase (SCD1) and Lipin-1 (LPIN1), both involved in lipid metabolism, were all up-regulated, especially after 5 daily treatments (Table 1). None of the cytokines did prevent up-regulation of these genes by CPZ.

By contrast, THRSP, a lipogenic gene encoding the thyroid hormone responsive spot 14 protein, was down-regulated and its induction by CPZ completely abolished with both cytokines (Table 1).

Effects of CPZ and cytokines on ROS generation and oxidative stress markers.

We have previously reported that oxidative stress plays a major role as both primary causal and aggravating factor in intrahepatic cholestasis induced by 50 μ M CPZ in HepaRG cells (Antherieu et al., 2013). To assess whether co-treatment of CPZ with inflammatory cytokines modulated ROS generation in HepaRG cells, H₂-DCFDA fluorescence intensity was measured after 1 and 5 additions of CPZ and/or cytokines. A 1.8-fold increase in ROS formation was observed with 20 μ M CPZ whereas none of the cytokines induced ROS nor altered their generation by CPZ at both time points (Fig. 5A). Since 50 μ M CPZ was previously shown to cause an early oxidative stress in HepaRG cells, ROS generation was also assessed after 24-hour exposure to IL-6 or IL-1 β followed by 2- or 4-hour co-treatment with 20 and 50 μ M CPZ. No aggravation of CPZ-induced oxidative stress was observed with cytokine pretreatment (Fig. 5B).

Next, we measured mRNA levels of three oxidative stress-related genes, heme oxygenase 1 (HO1), manganese superoxide dismutase (MnSOD), and NF-E2-related factor (Nrf2). HO1 and MnSOD were induced by both IL-6 and IL-1 β after 1 and 5 additions while CPZ caused a statistically significant increase of HO1 mRNA levels after 5 additions (2.4-fold) (Fig. 5 C, D). When HepaRG cells were co-exposed to CPZ and either IL-6 or IL-1 β , HO1 mRNA levels were statistically further augmented after 5 additions (2.7-fold with IL-6 versus 4.8-fold with IL-6 + CPZ and 1.6-fold with IL-1 β versus 3.3-fold with IL-1 β + CPZ) (Fig. 5C). MnSOD transcripts were also increased after 5 additions of cytokines and were further elevated by co-treatment with CPZ (1.7-fold with IL-6 versus 2.5-fold with IL-6 + CPZ and 2.2-fold induction with IL-1 β versus 3-fold with IL-1 β + CPZ) (Fig. 5D). Expression of NF-E2-related factor (Nrf2) was not affected by any of the treatment conditions (data not shown).

Effects of CPZ and cytokines on canalicular efflux membrane transporters.

Activity of two main apical efflux transporters, BSEP and MRP2, was estimated by using their prototypical substrates TA and CDF, respectively. To determine BSEP activity, no cytokine pretreatment was performed in order to avoid any inhibitory effect on NTCP activity, the main influx transporter of TA. CPZ at 20 and 50 μ M induced a time-dependent

intracellular accumulation of TA after 2 and 4 hours. Cytokines associated or not with CPZ, were ineffective on TA efflux (Fig. 6A). To estimate MRP2 activity, HepaRG cells were exposed to IL-6 or IL-1 β for 24 hours followed by 2- and 4-hour treatment with 20 μ M or 50 μ M CPZ and canalicular excretion of CDF, characterized by accumulation of green fluorescence in bile canaliculi, was analyzed. CPZ inhibited canalicular excretion of CDF only at 50 μ M while co-exposure of cells to 20 or 50 μ M CPZ and cytokines did not result in additional effects on CDF excretion (Fig. 6B), even if cytokine concentrations were increased by 10-fold (data not shown).

Effects of CPZ and cytokines on F-actin distribution.

Phalloidin fluoprobe labeling was used to assess whether 20 μ M CPZ and/or cytokines, affected the integrity of the pericanalicular F-actin microfilament network. Whatever the treatment, F-actin distribution was not disrupted even after 5 days (Fig.7 a, b, c). Consequently, additional experiments were performed with 20 or 50 μ M CPZ treatment for 2 or 4 hours. As expected from previous studies (Antherieu et al., 2013), at 50 μ M CPZ strongly altered distribution of cytoskeletal pericanalicular F-actin and bile canaliculi shape after 2 (data not shown) and 4 hours (Fig.7 d). These alterations were not aggravated by cytokine pre-treatment (Fig. 7 e, f).

Effects of CPZ and cytokines on NTCP activity.

In order to investigate whether CPZ and cytokines also affected NTCP-mediated uptake activity, HepaRG cells were exposed to CPZ and/or cytokines for 1 and 5 days and then incubated with [3 H]-TA for 30 minutes. NTCP-dependent activity was assessed by measuring intracellular accumulation of radiolabeled TA; it was found to significantly drop by 53 or 62% and 30 or 42% in the presence of IL-6 and IL-1 β after 1 or 5 days, respectively. CPZ was ineffective regardless of treatment duration. Combination of cytokines and CPZ led to a greater inhibition of NTCP activity that was statistically significant after 5 additions (83% with IL-6 + CPZ and 78% with IL-1 β + CPZ) (Fig. 8).

Effects of CPZ and cytokines on expression of cholestasis-related genes.

To further investigate interactions between pro-inflammatory cytokines and CPZ, expression of the main genes involved in bile acids transport and synthesis was assessed by RT-qPCR (Table 2). Several transporters were modulated by cytokines alone; the major changes were

represented by down-regulation of influx transporters, especially NTCP. mRNA levels of the uptake transporter NTCP dropped by 80 and 60% in cells treated with IL-6 and IL-1 β after either 1 or 5 days whereas 20 μ M CPZ did not cause any significant change. However, combination of CPZ with either cytokine resulted in a statistically significant additional decrease of NTCP transcripts after 5 additions (75% with IL-6 versus 91% with IL-6 + CPZ and 58% with IL-1 β versus 87% with IL-1 β + CPZ). In addition, IL-6 down-regulated other influx transporters, organic anion-transporting polypeptide (OATP)-B, and organic cation transporter 1 (OCT-1) mRNAs after 1 addition and OATP-C at both treatment time points. IL-1 β also down-regulated OCT-1 after the first addition. Increased expression of OATP-C by CPZ was observed after only one addition and was completely antagonized by both cytokines (Table 2).

Cytokines individually did not modulate expression of the canalicular transporter MDR3 which was down-regulated by CPZ. CPZ alone induced mRNA expression of breast cancer resistance protein (BCRP) and multidrug resistance protein (MDR1) after one addition; this increase was antagonized by both cytokines, which caused a 30-40% drop of BCRP at both time points and of MDR1 transcripts after one addition. Although neither CPZ nor IL-1 β had a significant effect on BSEP transcripts, their combination caused a statistically significant decrease of BSEP mRNA expression after 5 additions. CPZ alone or with cytokines did not significantly change the relative mRNA levels of MRP2.

By contrast, CPZ associated or not with cytokines, caused increased expression of the basolateral efflux gene MRP4 at both treatment time points. Noticeably, MRP3 expression was affected neither by CPZ nor by the cytokines individually, while their combinations caused a significant decrease after both 1 and 5 additions (CPZ+IL-6) and 5 additions (CPZ+IL-1 β).

Expression of cytochromes P450 genes involved in bile acid synthesis was also analyzed. CYP8B1 mRNA levels were reduced by CPZ + IL-1 β (44% drop compared to control) after 5 additions, whereas no change occurred in the presence of CPZ, IL-1 β or IL-6 alone. By contrast, CYP7A1 was slightly overexpressed by co-treatment with CPZ and IL-1 β after 5 additions while CYP27A1 was not affected by CPZ or cytokines, alone or in combination.

Transcripts of three major cholestasis-related nuclear receptors, the farnesoid X receptor (FXR), the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) were analyzed. Only IL-1 β , alone or in combination with CPZ, slightly induced FXR expression

after 5-day treatment. By contrast, PXR expression was down-regulated by both cytokines alone or in presence of CPZ after 1-day treatment and only by IL-6 and IL-6 + CPZ after 5 days. Expression of CAR was decreased by both cytokines and CPZ after 5 days. Combination of cytokines and CPZ did not exacerbate this repression.

Discussion

Inflammation is thought to play a role in the susceptibility to iDILI. Bacterial lipopolysaccharide and pro-inflammatory cytokines are frequently used to induce an inflammatory state in animals and *in vitro* cell models. In the present work we used the metabolically competent HepaRG cell line to evaluate the influence of an inflammatory stress induced by the two pro-inflammatory cytokines, IL-6 and IL-1 β , on the hepatotoxic and cholestatic effects of the idiosyncratic hepatotoxicant CPZ after either one or five daily treatments. Our data showed that in the presence of an inflammatory stress resulting in marked reduction of its metabolism, CPZ was more cytotoxic, amplified inhibition of NTCP activity and enhanced deregulation of expression of several other transporters. However, CPZ-induced TA efflux inhibition and bile canaliculi structures were not affected by cytokines co-treatment; most effects were amplified or observed only after 5 daily treatments.

HepaRG cells have been previously shown to exhibit an inflammatory response to the bacterial lipopolysaccharide (Aninat et al., 2008). We showed in the current study that 1ng/ml IL-6 and 0.01ng/ml IL-1 β strongly induced CRP mRNA and protein levels. IL-1 β also induced mRNA and protein levels of IL-8, a pro-inflammatory CXC chemokine that plays an important role in liver inflammation, regeneration and repair (Zimmermann et al., 2011). These data support the conclusion that, at these selected concentrations, both cytokines induced a chronic inflammatory stress in HepaRG cells. Noticeably, while at 20 μ M CPZ had no effect on CRP expression after 5 days, when associated with cytokine, it enhanced CRP transcript levels as well as protein content although to a lower extent.

Pretreatment of HepaRG cells with either IL-1 β or IL-6 followed by concomitant exposure to 20 μ M CPZ resulted in a significant although modest enhanced cytotoxic response after five repeated additions compared to treatments with the cytokines or the drug individually. These results agree with previous observations in rodents (Buchweitz et al., 2002) and primary mouse hepatocytes (Gandhi et al., 2010). This increased cytotoxicity did not appear to be directly related to the strong inhibition of CPZ metabolism and the reduction in the formation of non-toxic metabolites in the presence of cytokines. Indeed, HepaRG cells and HepG2 cells

which lack CYP3A4- and CYP1A2-mediated metabolic activity, have been reported to be nearly equally sensitive to CPZ (Aninat et al., 2006; Gerets et al., 2012). Since expression of two oxidative stress-related genes HO1 and MnSOD were significantly induced in co-treated cells, especially after five days, increased cytotoxicity could be related to generation of an oxidative stress although an increase in ROS content could not be evidenced by the H₂-DCFDA assay.

As expected, in agreement with previous reports (Abdel-Razzak et al., 1993), the two cytokines were also found to down-regulate CYP3A4 and CYP1A2, two major CYPs together with CYP2D6 involved in CPZ oxidative metabolism (Wojcikowski et al., 2010). Accordingly, CPZ metabolism was highly repressed in cytokine-treated cells. Noteworthy, a strong intracellular accumulation of CPZ was observed, especially after five daily treatments. As other cationic amphiphilic drugs, CPZ is able to induce phospholipidosis that is characterized by lamellar bodies formed by excessive accumulation of phospholipids to which CPZ and its metabolites can possibly bind (Fujimura et al., 2007). Accordingly, increasing accumulation of intracellular vesicles was observed in CPZ-treated cells starting after 2 days in the presence or absence of either cytokine. As previously reported (Antherieu et al., 2013) accumulation of these vesicles was associated with an induction of genes involved in lipid metabolism and phospholipidosis (ADFP, PLIN4, SCD1, LPIN1, THRSP) in CPZ-treated cells. In agreement with morphological observations, overexpression of ADFP, PLIN4, SCD1, LPIN1 was not prevented by co-treatment with either cytokine, suggesting that an inflammation stress does not affect induction of phospholipidosis by CPZ. Noteworthy, expression of the PXR/CAR target gene THRSP was down-regulated by cytokines, especially IL-6, as a consequence of their ability to down-regulate these nuclear receptors (Breuker et al., 2010).

At 50 μ M, CPZ was found to generate an early oxidative stress, which was associated with an inhibition of TA efflux, and shortly later, of its uptake, and alterations of bile canaliculi structures (Antherieu et al., 2013). Similarly, at 20 μ M CPZ also induced an oxidative stress and inhibited TA efflux; however, it did not alter pericanalicular cytoskeletal F-actin distribution and bile canaliculi structures, even in the presence of either cytokine after 5 daily treatments. Nevertheless, many genes related to cholestasis were differentially deregulated by pro-inflammatory cytokines individually or combined with CPZ. In agreement with previous studies (Le Vee et al., 2008; Le Vee et al., 2009), we found that an IL-1 β - and IL-6- induced inflammatory stress down-regulated mRNA and activity of NTCP and altered expression of

several additional influx and efflux transporters in HepaRG cells. Interestingly, NTCP down-regulation was amplified by co-treatment with CPZ, suggesting that inhibition of sodium-dependent bile acids uptake was exacerbated by CPZ in pro-inflammatory cytokine-stimulated hepatocytes. Cytokines did not induce any change in MRP2 and BSEP transcripts and activity. Discrepant data have been reported in the effects of pro-inflammatory cytokines on the main canalicular transporters BSEP and MRP2. Thus, transcripts of these two transporters were not affected by LPS in human liver slices (Elferink et al., 2004); by contrast, they were repressed by IL-1 β and IL-6 whereas BSEP protein, unlike MRP2, was induced in sandwich-cultured human hepatocytes (Diao et al., 2010). Such discrepancies are likely due to the use of different experimental models and conditions such as cytokine concentrations. Noteworthy, our results agree with those obtained in liver biopsies of patients with inflammation-induced cholestasis where no change of MRP2 and slight reduction of BSEP mRNA, were reported, while a strong down-regulation of NTCP mRNA and protein was observed (Zollner et al., 2001). Noticeably, mRNA expression of BSEP was down-regulated in IL-1 + CPZ- treated cells after 5 day-treatment although no effect was observed by each agent separately. Such data could explain the onset of cholestasis in patients treated chronically with CPZ.

In addition, SLC transporters, OATP-B, OATP-C and OCT-1, and ABC transporters, BCRP and MDR1, were down-regulated mainly by IL-6 after the first addition which is in agreement with previous studies (Le Vee et al., 2008; Le Vee et al., 2009). By contrast, most deregulated genes by 20 μ M CPZ were overexpressed, i.e. OATP-C, BCRP, MDR1, and MRP4, while only MDR3, which mediates biliary phospholipid excretion, was down-regulated by CPZ, whether in the presence or absence of cytokines. Co-treatment with cytokines and CPZ had an antagonist effect on the expression of genes induced by CPZ alone, including BCRP, MDR1, and OATP-C. By contrast, induction of MRP4 expression by CPZ was still increased, and to a higher level, after 5 days, even in the presence of cytokines. Overexpression of this basolateral transporter, together with down-regulation of NTCP, could be interpreted as a compensating mechanism aiming to reduce intra-hepatic accumulation of toxic bile acids (Zollner et al., 2006; Wagner et al., 2009).

Changes in the expression of cytochromes P450 which are involved in the formation of bile acids could represent another means to modulate intracellular accumulation of bile acids (Zollner et al., 2006). Accordingly, differential deregulation of CYP7A1, CYP8B1 and CYP27A1 transcripts were observed depending on the treatment and its duration. As expected

(Aitken et al 2006), CAR and PXR were down-regulated by IL-6 and IL-1 β in HepaRG cells. Noticeably, CAR was also down-regulated by CPZ after five daily treatments; this finding deserves further investigations.

An immune reaction has been evoked as an important determinant factor in CPZ-induced cholestasis but data, at least in animals, did not confirm an allergic hypersensitivity with CPZ (Mullock et al., 1983). An increased accumulation of neutrophils was observed in the liver of LPS+CPZ-treated rats but without evidence of cholestasis (Buchweitz et al., 2002). However, extrapolation of such data to the human situation is questionable. Co-culturing HepaRG cells with immune cells, inflammatory cells or non-parenchymal liver cells will be of interest for future investigations.

Taken together our findings suggest that pro-inflammatory cytokines, which induced an inflammatory stress and cholestatic features in HepaRG cells by themselves, in combination with CPZ increased cytotoxicity and aggravated some cholestatic features caused by this idiosyncratic drug. The decrease of NTCP activity, repression of CYP expression and inhibition of CPZ metabolism could have major consequences if extrapolated to the *in vivo* situation. First, since NTCP is now recognized as required for the entry of HBV and HDV into hepatocytes (Yan et al., 2012; Ni et al., 2013), it might be postulated that pro-inflammatory cytokine-stimulated and cholestatic hepatocytes are less susceptible to viral infection. Second, inhibition of the major CYPs involved in drug metabolism by pro-inflammatory cytokines led to postulate that CPZ- treated patients in an inflammation state would be more or less sensitive to other hepatotoxic drugs depending on whether these compounds are detoxified or activated by cytochrome P450-mediated metabolism. Finally, in the future, it would be of interest to evaluate in a similar inflammatory state other cholestatic drugs known to act by other mechanisms.

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Authorship Contributions.

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Wrote or contributed to the writing of the manuscript: Bachour-El Azzi, Abdel-Razzak, Guillouzo.

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Footnote

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Figure legends

Fig. 1. Cytotoxic effects of CPZ and/or pro-inflammatory cytokines in HepaRG cells.

Cells were pretreated with IL-6 (1ng/ml) or IL-1 β (0.01ng/ml) for 24 hours, then 20 μ M CPZ were added daily simultaneously with cytokines, for 1 or 5 days (1 and 5 additions respectively). Cytotoxicity was measured by the MTT colorimetric assay. Each point is the mean \pm SD of three independent experiments. [#] $p < 0.05$ compared with untreated cells.

Fig. 2. Effects of CPZ and pro-inflammatory cytokines on inflammatory stress markers.

Cells were pretreated with IL-6 (1ng/ml) or IL-1 β (0.01ng/ml) for 24 hours, then 20 μ M CPZ were added daily simultaneously with cytokines, for 1 or 5 days (1 and 5 additions respectively). (A) CRP and (B) IL-8 mRNA levels were assessed by RT-qPCR; (C) CRP and (D) IL-8 proteins were measured by ELISA in culture media. Data represent the means \pm SD of three independent experiments. All results are expressed relative to the levels found in control cells, arbitrarily set at a value of 1. [#] $p < 0.05$ compared with untreated cells, * $p < 0.05$ compared with cells treated with cytokine and CPZ individually.

Fig. 3. Effects of pro-inflammatory cytokines and/or CPZ on CYP3A4 and CYP1A2 mRNA levels and CPZ metabolism in HepaRG cells.

Cells were exposed to IL-6 (1ng/ml) or IL-1 β (0.01ng/ml) for 24 hours prior to daily co-treatment with 20 μ M CPZ for 1 or 5 days (1 and 5 additions respectively). (A) mRNA levels of CYP3A4 and CYP1A2 were estimated by RT-qPCR. Data represent the means \pm SD of three independent experiments. [#] $p < 0.05$ compared with untreated cells. (B, C) At each time point, cell lysates and incubation media were collected. Concentrations of CPZ and three metabolites (N-desmethyl CPZ (Nor-1 CPZ), sulfoxo-CPZ (SO CPZ), and 7-hydroxyl CPZ (7-OH CPZ)) were determined in cell lysates (B) and incubation media (C) by HPLC-MS-MS and were normalized relative to total protein content. * $p < 0.05$ compared with cells treated with CPZ alone.

Fig. 4. Phase-contrast micrographs of HepaRG cells.

Cells were either directly exposed to 20 μ M CPZ or pre-treated with IL-6 (1ng/ml) or IL-1 β (0.01ng/ml) for 24 hours before adding daily 20 μ M CPZ simultaneously with cytokines. After 3 CPZ additions, many intracytoplasmic vesicles are visible (arrow) in cells treated with CPZ alone or simultaneously with IL-6 or IL-1 β . Control: untreated cells ($\times 20$ magnification).

Fig. 5. Effects of CPZ and/or pro-inflammatory cytokines on ROS generation and expression of oxidative stress-related genes.

Cells were pretreated with IL-6 (1ng/ml) or IL-1 β (0.01ng/ml) for 24 hours prior to simultaneous addition of (A, C, D) 20 μ M CPZ for 1 or 5 days (1 and 5 additions respectively) or (B) 50 μ M CPZ for 2 or 4 hours. (A, B) ROS

generation: hydrogen peroxides were quantified by the H₂-DCFDA assay. A treatment with 25mM H₂O₂ for 2 hours was used as a positive control. (C, D) HO1 and MnSOD relative mRNA levels were assessed by RT-qPCR. Data represent the means \pm SD of three independent experiments. All results are expressed relative to the levels found in control cells, arbitrarily set at a value of 1. # $p < 0.05$ compared with untreated cells. * $p < 0.05$ compared with cells treated with cytokine and CPZ individually.

Fig. 6. [³H]-TA efflux and MRP2 activity in HepaRG cells treated with CPZ and pro-inflammatory cytokines. (A) Cells were exposed to [³H]-TA for 30 minutes to induce an intracellular accumulation of TA and then treated with IL-6 (1ng/ml), IL-1 β (0.01ng/ml) and/or CPZ (20 or 50 μ M) for 2 or 4 hours. TA efflux was determined at 2 and 4 hours in control and treated cells, by measuring intracellular TA accumulation. Efflux of TA was expressed relative to the levels found in control cells, arbitrarily set at a value of 100%. Data represent the means \pm SD of three independent experiments. # $p < 0.05$ compared with control cultures. (B) Cells were pretreated with IL-6 (1 ng/ml) or IL-1 β (0.01ng/ml) for 24 hours then CPZ (20 or 50 μ M) was simultaneously added for 4 hours. MRP2 activity was estimated using CDF, a substrate of MRP2 and was characterized by accumulation of green fluorescence into bile canaliculi. Untreated cells (a); unchanged CDF canalicular excretion with 20 μ M CPZ alone (b) or in combination with IL-6 (c) or IL-1 β (d) and loss of canalicular CDF excretion with 50 μ M CPZ (e).

Fig. 7. Pericanalicular cytoskeletal F-actin distribution in CPZ- and cytokine-treated HepaRG cells. Cells were pretreated with IL-6 (1ng/ml) or IL-1 β (0.01ng/ml) for 24 hours prior to simultaneous addition of 20 or 50 μ M CPZ for 5 days (5 additions) or 4 hours respectively, and then F-actin was localized by using the phalloidin-fluoprobe. Nuclei were stained in blue using Hoechst dye; cells treated with 20 μ M CPZ alone (a), simultaneously with IL6 (b) or IL-1 β (c); 50 μ M CPZ alone (d), simultaneously with IL6 (e) or IL-1 β (f). Constricted bile canaliculi are observed only in 50 μ M CPZ-treated cells in the absence or presence of cytokines.

Fig. 8. NTCP activity in CPZ- and/or pro-inflammatory cytokine-treated HepaRG cells.

Cells were pretreated with IL-6 (1ng/ml) or IL-1 β (0.01ng/ml) for 24 hours, then 20 μ M CPZ were added daily simultaneously with cytokines, for 1 or 5 days (1 and 5 additions respectively). Cells were then incubated with [³H]-TA for 30 minutes. NTCP activity was evaluated through measurement of intracellular accumulation of the radiolabeled substrate

TA. Data represent the means \pm SD of three independent experiments. Uptake of [^3H]-TA was expressed relative to the levels found in untreated cells, arbitrarily set at a value of 100%. # $p < 0.05$ compared with untreated cells, * $p < 0.05$ compared with cells treated with cytokine and CPZ individually.

Table 1. Effects of CPZ and/or cytokines on expression of mRNAs encoding genes related to phospholipidosis in HepaRG cells.

	1 addition					5 daily additions				
	CPZ	IL-6	IL-6 + CPZ	IL-1 β	IL-1 β + CPZ	CPZ	IL-6	IL-6 + CPZ	IL-1 β	IL-1 β + CPZ
ADFP	1.37 \pm 0.21	0.87 \pm 0.14	0.92 \pm 0.33	1.07 \pm 0.27	1.37 \pm 0.27	2.08 \pm 0.24 [#]	1.18 \pm 0.25	1.98 \pm 0.15 [#]	0.88 \pm 0.09	2.36 \pm 0.64 [#]
PLIN4	1.13 \pm 0.21	0.87 \pm 0.27	0.74 \pm 0.22	1.27 \pm 0.31	1.02 \pm 0.14	1.69 \pm 0.29 [#]	1.18 \pm 0.19	2.01 \pm 0.68 [#]	1.02 \pm 0.19	1.66 \pm 0.14 [#]
SCD1	1.97 \pm 0.46 [#]	0.75 \pm 0.17	1.04 \pm 0.56	1.01 \pm 0.10	1.79 \pm 0.47 [#]	2.11 \pm 0.26 [#]	0.69 \pm 0.21	1.21 \pm 0.24	0.87 \pm 0.11	1.50 \pm 0.57
LPIN1	1.37 \pm 0.08 [#]	0.70 \pm 0.12 [#]	0.72 \pm 0.04	1.01 \pm 0.09	1.40 \pm 0.29 [#]	1.78 \pm 0.43 [#]	0.86 \pm 0.18	1.43 \pm 0.26	0.97 \pm 0.11	1.41 \pm 0.45
THrsp	1.93 \pm 0.37 [#]	0.18 \pm 0.03 [#]	0.51 \pm 0.02 [#]	0.52 \pm 0.19	1.02 \pm 0.22	2.25 \pm 0.48 [#]	0.18 \pm 0.15 [#]	0.32 \pm 0.11 [#]	0.65 \pm 0.32	0.97 \pm 0.28

Cells were exposed to IL-6 (1ng/ml) or IL-1 (0.01ng/ml) and/or CPZ (20 μ M) for 24h or daily for 5 days. mRNAs were analyzed by RT-qPCR. Results are expressed as fold of the value found in control cells arbitrarily set at 1. Data are mean \pm SD of at least three independent experiments. # p <0.05 compared with untreated cells.

Table 2. Effects of CPZ and/or cytokines on the relative mRNA levels of hepatobiliary transporters, bile acid synthesizing enzymes and nuclear receptors in HepaRG Cells.

	1 addition					5 daily additions				
	CPZ	IL-6	IL-6 + CPZ	IL-1 β	IL-1 β + CPZ	CPZ	IL-6	IL-6 + CPZ	IL-1 β	IL-1 β + CPZ
Influx transporters										
NTCP	0.92 \pm 0.24	0.18 \pm 0.06 [#]	0.12 \pm 0.05 [#]	0.41 \pm 0.08 [#]	0.22 \pm 0.07 [#]	0.98 \pm 0.14	0.25 \pm 0.07 [#]	0.10 \pm 0.04 [*]	0.42 \pm 0.08 [#]	0.14 \pm 0.04 [*]
OATP-B	1.34 \pm 0.27	0.55 \pm 0.10 [#]	0.56 \pm 0.05 [#]	0.76 \pm 0.13	0.73 \pm 0.10	1.07 \pm 0.27	0.62 \pm 0.05	0.52 \pm 0.16	0.79 \pm 0.23	0.76 \pm 0.20
OATP-C	1.57 \pm 0.35 [#]	0.46 \pm 0.09 [#]	0.65 \pm 0.22	0.66 \pm 0.19	0.76 \pm 0.09	1.08 \pm 0.33	0.54 \pm 0.16 [#]	0.49 \pm 0.16 [#]	0.77 \pm 0.09	0.68 \pm 0.14
OATP-8	1.37 \pm 0.46	0.88 \pm 0.19	1.05 \pm 0.14	0.82 \pm 0.12	0.86 \pm 0.13	1.05 \pm 0.18	0.74 \pm 0.05	0.85 \pm 0.13	0.86 \pm 0.13	0.87 \pm 0.35
OCT1	1.37 \pm 0.29	0.49 \pm 0.16 [#]	0.52 \pm 0.20 [#]	0.61 \pm 0.15 [#]	0.65 \pm 0.13 [#]	1.36 \pm 0.39	0.57 \pm 0.21	0.48 \pm 0.31	0.92 \pm 0.08	0.83 \pm 0.17
Efflux transporters										
BSEP	1.21 \pm 0.36	0.90 \pm 0.15	0.96 \pm 0.11	0.90 \pm 0.17	0.81 \pm 0.04	0.76 \pm 0.23	1.11 \pm 0.37	0.90 \pm 0.48	0.77 \pm 0.20	0.51 \pm 0.27 [#]
BCRP	1.43 \pm 0.11 [#]	0.60 \pm 0.07 [#]	0.68 \pm 0.07 [#]	0.67 \pm 0.17 [#]	0.72 \pm 0.13 [#]	1.05 \pm 0.40	0.61 \pm 0.06 [#]	0.74 \pm 0.13	0.70 \pm 0.28	0.89 \pm 0.22
MRP2	1.21 \pm 0.39	0.72 \pm 0.11	0.84 \pm 0.08	0.74 \pm 0.06	0.72 \pm 0.26	1.38 \pm 0.29	1.28 \pm 0.45	1.28 \pm 0.45	1.06 \pm 0.22	1.01 \pm 0.33
MDR1	1.40 \pm 0.10 [#]	0.65 \pm 0.08 [#]	0.85 \pm 0.01	0.72 \pm 0.11	0.83 \pm 0.07	1.16 \pm 0.14	0.82 \pm 0.12	0.86 \pm 0.07	0.93 \pm 0.10	0.94 \pm 0.10
MDR3	0.59 \pm 0.21 [#]	0.81 \pm 0.18	0.59 \pm 0.17 [#]	0.98 \pm 0.12	0.69 \pm 0.15 [#]	0.52 \pm 0.20 [#]	0.69 \pm 0.29	0.46 \pm 0.16 [#]	1.16 \pm 0.25	0.40 \pm 0.13 [#]
MRP3	1.00 \pm 0.09	0.89 \pm 0.07	0.64 \pm 0.06 [*]	0.84 \pm 0.13	0.75 \pm 0.12	0.73 \pm 0.04	0.78 \pm 0.12	0.52 \pm 0.14 [*]	0.84 \pm 0.10	0.61 \pm 0.23 [#]
MRP4	1.41 \pm 0.22 [#]	1.25 \pm 0.06	1.42 \pm 0.10 [#]	1.28 \pm 0.11	1.51 \pm 0.13 [#]	2.02 \pm 0.12 [#]	1.42 \pm 0.15 [#]	2.19 \pm 0.17 [#]	1.37 \pm 0.33	1.78 \pm 0.25 [#]
Bile acid synthesis										
CYP7A1	1.21 \pm 0.18	0.79 \pm 0.13	0.95 \pm 0.10	0.96 \pm 0.12	1.10 \pm 0.12	1.62 \pm 0.30	0.83 \pm 0.11	1.36 \pm 0.24	0.98 \pm 0.20	1.90 \pm 0.24 [#]
CYP27A1	1.16 \pm 0.18	0.83 \pm 0.03	0.92 \pm 0.12	1.02 \pm 0.14	1.10 \pm 0.11	1.21 \pm 0.08	0.75 \pm 0.11	0.86 \pm 0.13	1.01 \pm 0.14	1.25 \pm 0.34
CYP8B1	1.13 \pm 0.24	0.68 \pm 0.20	0.64 \pm 0.23	0.94 \pm 0.12	0.82 \pm 0.04	1.07 \pm 0.35	0.84 \pm 0.17	0.78 \pm 0.38	0.78 \pm 0.15	0.56 \pm 0.23 [#]
Nuclear receptors										
FXR	0.96 \pm 0.14	1.07 \pm 0.17	1.09 \pm 0.10	1.12 \pm 0.17	0.98 \pm 0.10	1.02 \pm 0.07	0.98 \pm 0.04	1.03 \pm 0.19	1.44 \pm 0.24 [#]	1.36 \pm 0.16 [#]
PXR	1.08 \pm 0.21	0.52 \pm 0.16 [#]	0.43 \pm 0.09 [#]	0.55 \pm 0.13 [#]	0.69 \pm 0.17 [#]	1.19 \pm 0.26	0.53 \pm 0.08 [#]	0.50 \pm 0.03 [#]	0.84 \pm 0.25	0.95 \pm 0.28
CAR	1.21 \pm 0.35	0.59 \pm 0.14	0.33 \pm 0.18 [#]	0.67 \pm 0.17	0.53 \pm 0.10 [#]	0.43 \pm 0.15 [#]	0.36 \pm 0.13 [#]	0.39 \pm 0.09 [#]	0.58 \pm 0.05 [#]	0.48 \pm 0.12 [#]

Cells were pretreated with IL-6 (1ng/ml) or IL-1 β (0.01ng/ml) for 24 hours, then 20 μ M CPZ were added with a continuous treatment with cytokines, for 1 or 5 days (1 and 5 additions respectively). Total RNAs were extracted and mRNAs were analyzed by RT-qPCR. Results are expressed as fold of the value found in control cells arbitrarily set at 1. Data are mean \pm SD of at least three independent experiments. # p < 0.05 compared with untreated cells, * p < 0.05 compared with cells treated with cytokine and CPZ individually.

Figure 1

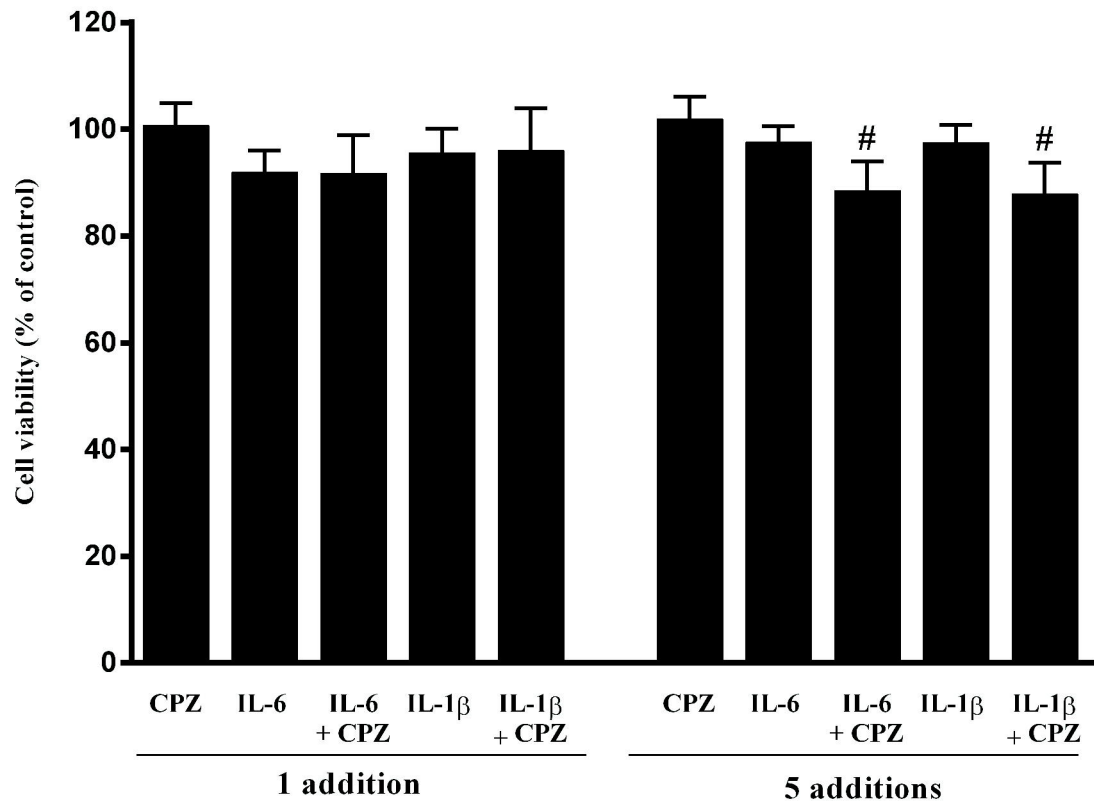


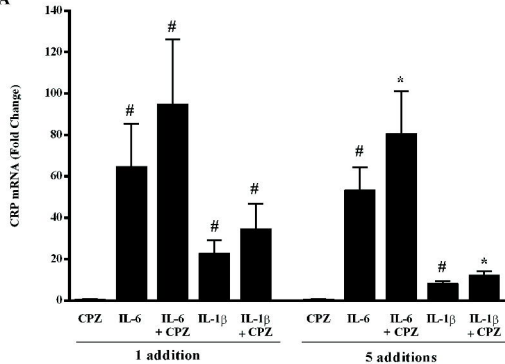
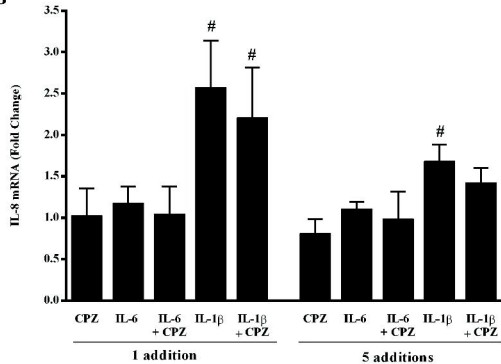
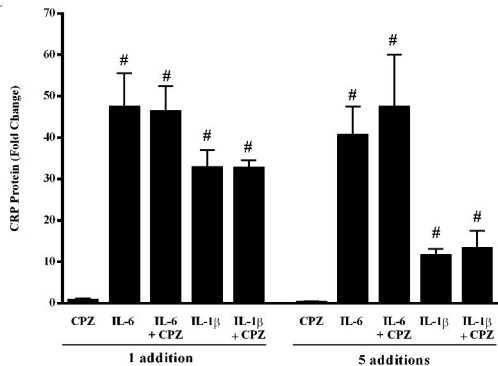
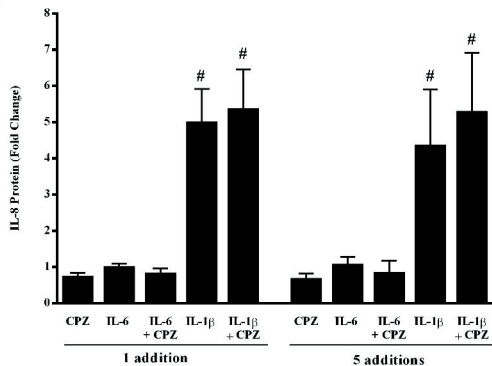
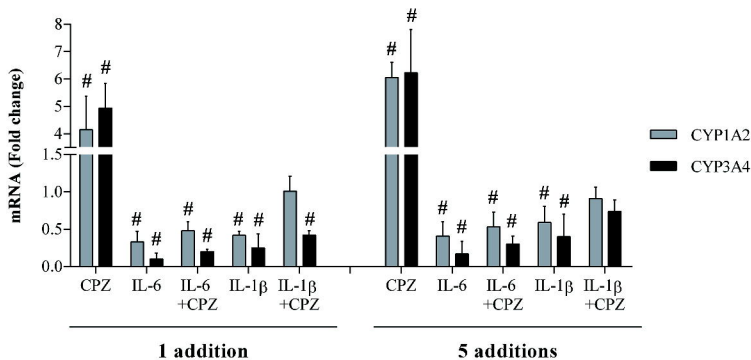
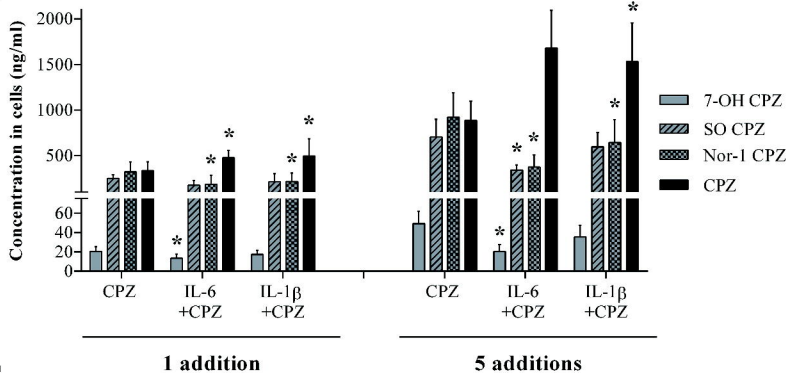
Figure 2**A****B****C****D**

Figure 3

A



B



C

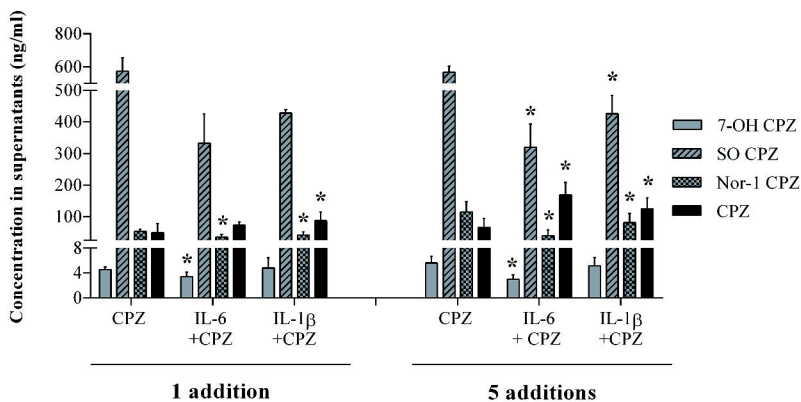
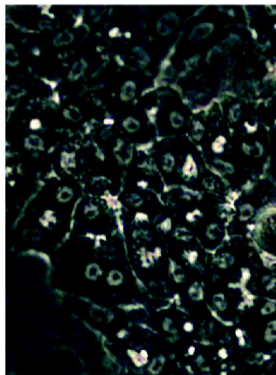
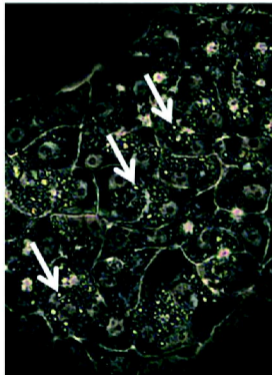


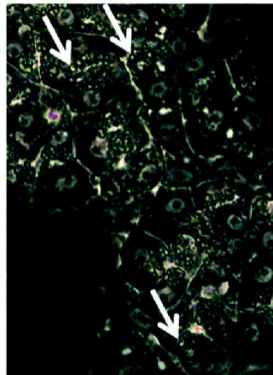
Figure 4



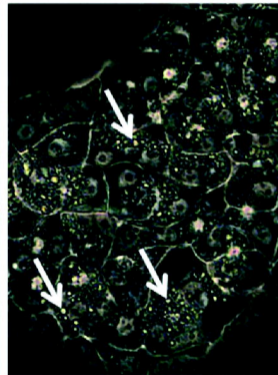
Control



CPZ



IL-6 + CPZ



IL-1 β + CPZ

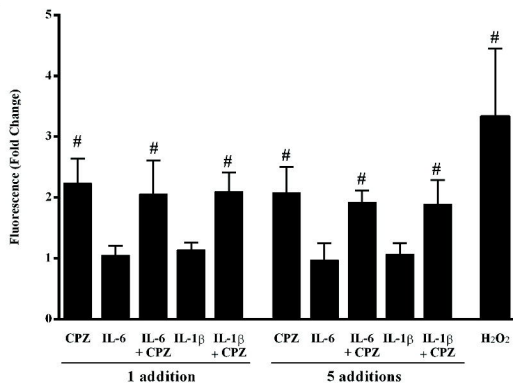
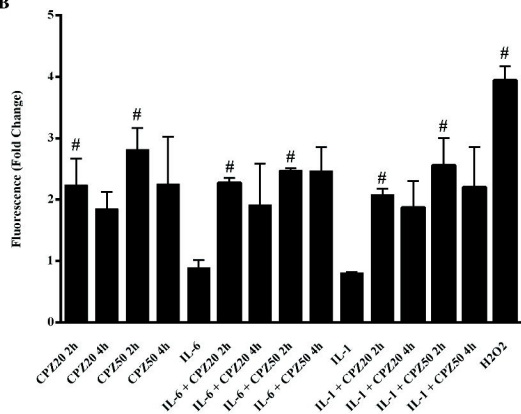
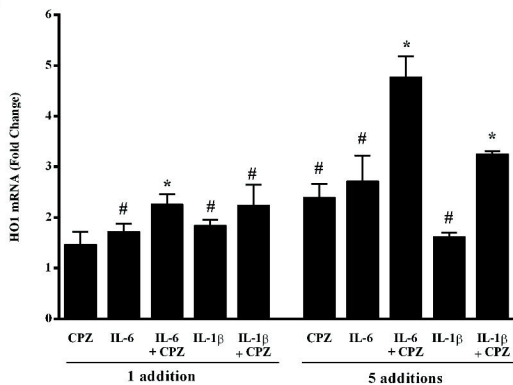
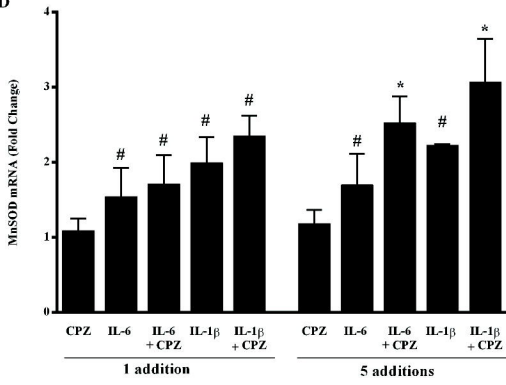
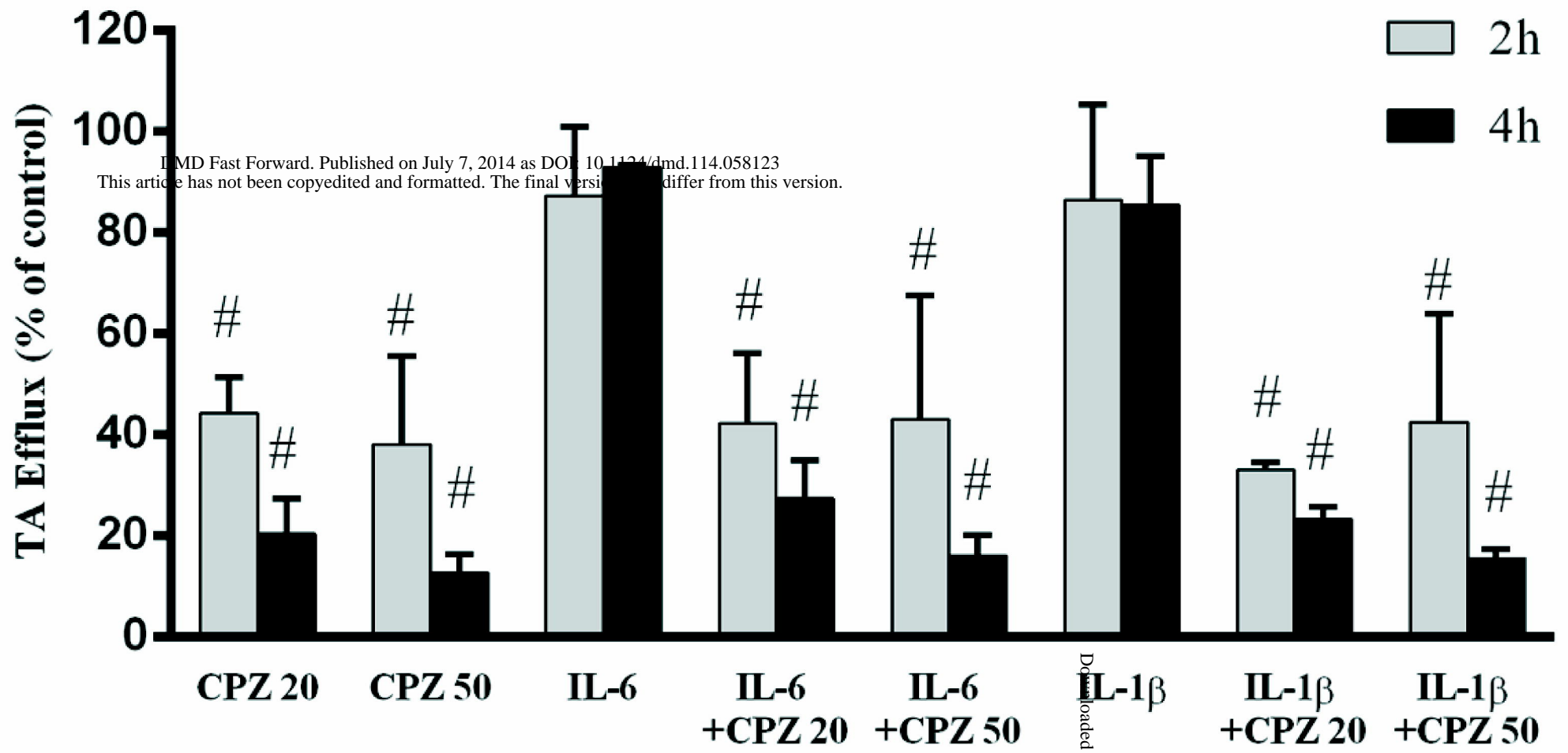
Figure 5**A****B****C****D**

Figure 6

A



B

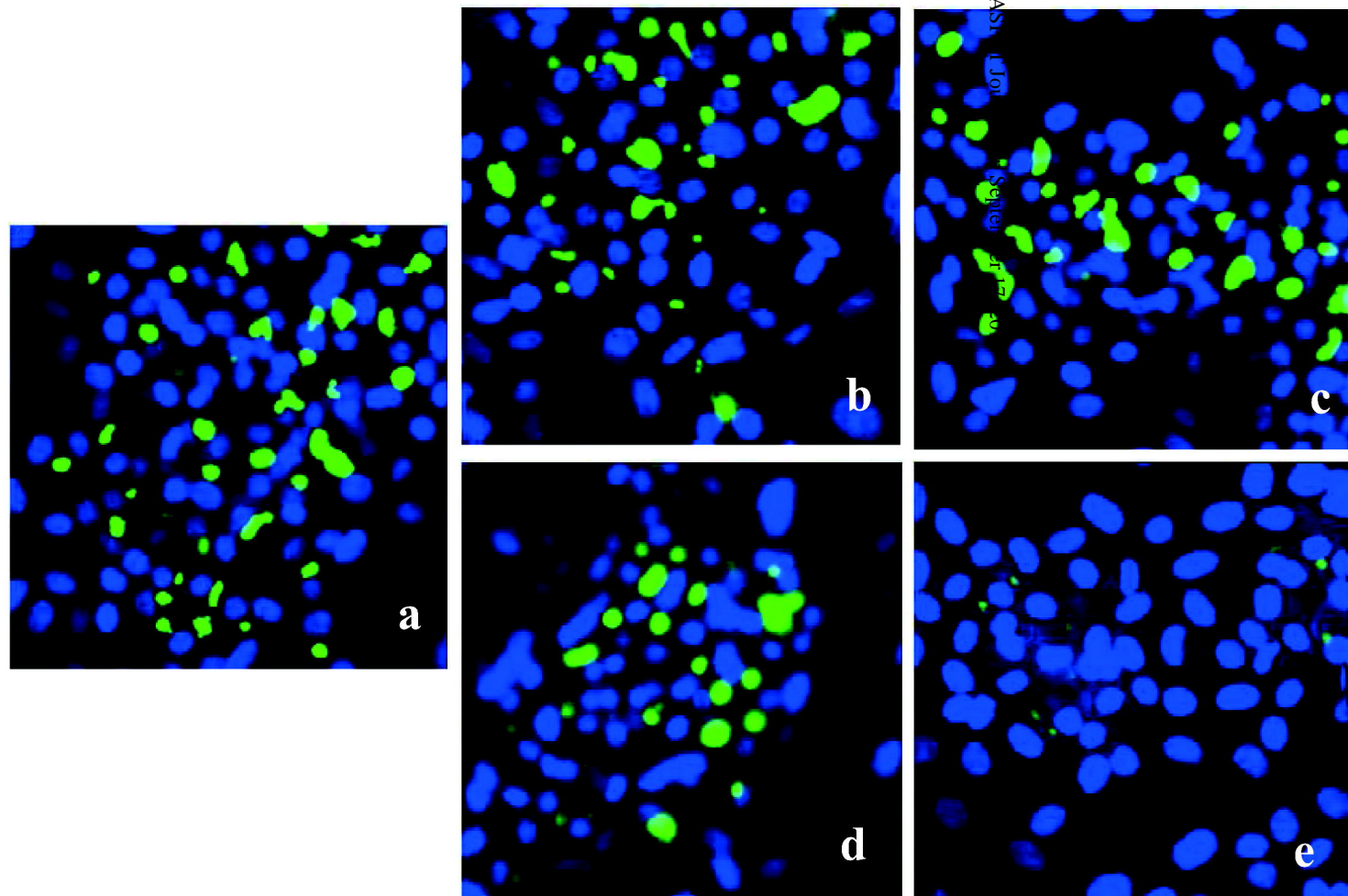


Figure 7

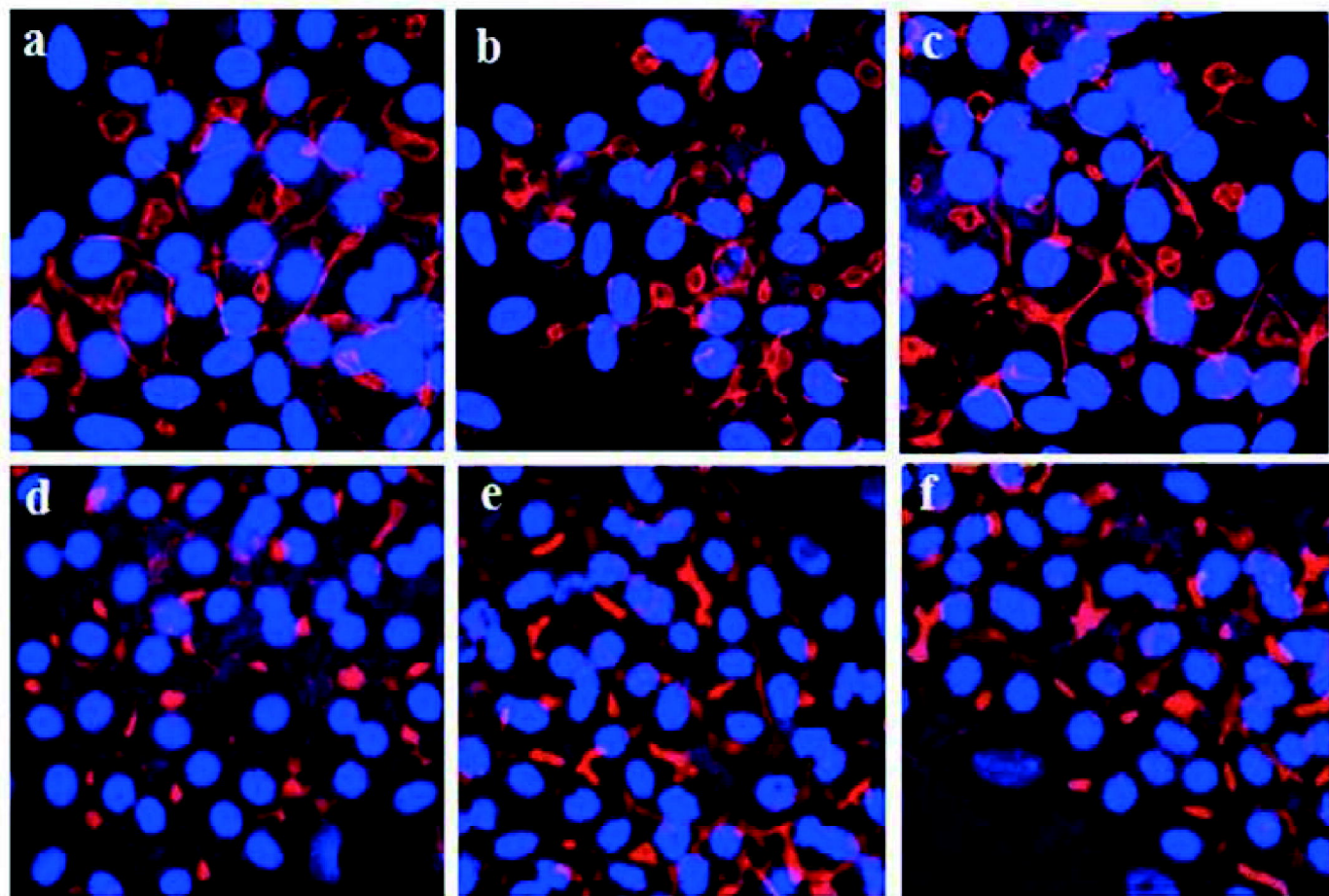


Figure 8

