

Combination Treatments of 1-(N-Acetyl-6-Aminoethyl)-3-Hydroxy-2-Methylpyridin-4-One (CM1) With Deferiprone and Desferrioxamine Reduced Labile Iron Pool and Protected Oxidative Stress in Iron-Loaded Cultured Hepatocytes

Kanokwan Kulprachakarn¹, Kanjana Pangjit^{1,2}, Chada Phisalaphong³, Suthat Fucharoen⁴, Robert C. Hider⁵ and Somdet Srichairatanakool^{1*}

¹Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Thailand

²College of Medicine and Public Health, Ubon Ratchathani University, Thailand

³Institute of Research and Development, Government Pharmaceuticals Organization, Ministry of Public Health, Thailand,

⁴Thalassemia Research Center, Institute of Molecular Biosciences, Mahidol University Salaya Campus, Nakornprathom, Thailand,

⁵Pharmaceutical Science Division, Franklin-Wilkins Building, King's College London, United Kingdom.

Abstract

Iron overload associated with oxidative stress is a serious problem in transfusion-dependent patients with β -thalassemia major. The increased iron overload in several organs may be caused by higher intestinal absorption along with less intensive chelation therapy. Liver iron overload could in turn facilitate the development or persistence of chronic progressive liver disease. Previous studies have shown that chelation with desferrioxamine (DFO) and deferiprone (DFP) substantially reduced body-iron scores in β -thalassemia patients with transfusional iron overload. We have synthesized and characterized a new bidentate iron chelator, 1-(N-acetyl-6-aminoethyl)-3-hydroxy-2-methylpyridin-4-one (CM1). The compound can efficiently scavenge iron from both ferrous and ferric salts and plasma non-transferrin bound iron (NTBI). In this study we have studied the efficacy of the CM1 treatment on the decrease of levels of the labile iron pool (LIP) and reactive oxygen species (ROS) in iron-loaded mouse hepatocyte and HepG2 cell cultures. The isolated hepatocytes were treated with DFP, DFO and CM1 at different concentrations. The treated cells were analyzed for intracellular LIP using the calcein fluorescent technique and ROS levels using the dichlorofluorescein (DCF) fluorescent method. It was found that CM1 reduced the levels of intracellular LIP and hydrogen peroxide-induced ROS in both treated cells in a concentration-dependent manner. The combination treatment of CM1 with 25 μ M DFP and DFO was demonstrated to decrease the levels of the LIP in both cells and tended to reduce the levels of ROS in HepG2 cells. Our findings support the evidence of iron-chelating and free radical-scavenging activities of CM1 in the livers with iron overload, which potentially can protect against oxidative liver inflammation and fibrosis. The efficacy of the CM1 treatment needs to be further investigated intensively under *in vivo* conditions.

Keywords: 3-Hydroxypyridinone; Hepatocyte; Iron overload; Labile iron; Reactive oxygen species

Introduction

Iron is vital for almost all living organisms by participating in a various metabolic processes, including oxygen transport, DNA synthesis, and electron transport. The total amount of body iron is approximately 3-4 g, two-thirds are red blood cell (RBC) iron, and recycled iron by destruction of RBC in the reticuloendothelial system (RES) and the both of the remainder is stored in tissue in form of ferritin/hemosiderin. Only 1-2 mg of iron per day are absorbed by the duodenal epithelial cells and circulated in the blood. Physiologically, iron is bound to transferrin (Tf) in plasma and most of the Tf-bound iron is utilized for bone marrow erythropoiesis. Since there is no active mechanism to excrete iron from primates, long-term repeated blood transfusions in anemic patients with genetic disorders such as thalassemia, sickle cell disease (SCD), Diamond Blackfan syndrome, and bone-marrow failures such as aplastic anemia (AA) and myelodysplastic syndromes (MDS) can result in iron overload [1]. Increased duodenal iron absorption can also lead to iron overload. The excess iron appears in various iron pools, namely the intracellular labile iron pool (LIP) and the extracellular non-transferrin bound iron pool (NTBI), a component of which is the labile plasma iron (LPI) [1-3]. Elevated levels of LIP lead to the increased accumulation of ferritin iron, and in extreme cases to the function of hemosiderin [4]. The ferrous ion participates in the Fenton reaction and catalyzes the conversion of hydrogen peroxide to the highly reactive hydroxyl radicals (HO[•]). The presence of hydroxyl radicals induces damage to DNA, proteins and lipids [5]. Consequently, elevated LIP causes damage to a variety of cells and tissues which accumulate NTBI including heart, liver, pancreas,

erythrocytes and endocrine glands resulting in organ dysfunction. Without treatment, such iron overload becomes fatal. Administration of effective iron chelators, desferrioxamine (DFO), deferiprone (DFP) and deferasirox (DFX) are used for treatment of β -thalassemia patients with iron overload [6-9]. Combined therapy with DFP and DFO can decrease severe iron overload in patients with β -thalassemia major [10] and resulted in greater iron excretion and decreased adverse effects [11]. The combination resulted in urine excretion equal to (in three patients) or even greater than (in two patients) the total excreted if the drugs were given on separate days. Presumably, there is an additive or even synergistic effect on total iron excretion in urine and feces rather than deviation of iron from feces to urine by DFP [12]. Recently, a novel orally active iron chelator, 1-(N-acetyl-6-aminoethyl)-3-hydroxy-2-methylpyridin-4-one (CM1) has been designed [13] and preliminary results have demonstrated that the CM1 is an effective bidentate chelator which is slightly more lipophilic than the DFP. The compound has been shown to reduce iron-induced

***Corresponding author:** Somdet Srichairatanakool, Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200 Thailand, E-mail: ssrichai@med.cmu.ac.th

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redox damage and to decrease the levels LIP in hepatocytes [14]. In the present study, we have investigated the effects of CM1 on LIP and ROS levels in primary hepatocyte and HepG2 cell cultures. In addition the combined treatment of CM1 with DFP and DFO has been investigated with the overall aim of detecting and enhanced iron excretion with the combination of two orally active chelators.

Materials and Methods

Chemicals and reagents

Calcein-AM solution (Invitrogen Corporation, CA, USA) and 2',7'-dichlorodihydrofluorescein diacetate (DCFHDA) (Sigma-Aldrich, St. Louis, MO, USA) is fluorescent probes. Collagenase type IV, Dulbecco's modified eagle medium (DMEM), Krebs-Ringer buffer (KRB), penicillin-streptomycin, 0.5% trypsin-EDTA solution and fetal bovine serum were purchased from GIBCO[®] Invitrogen, CA, USA. Insulin (Humulin[®]R) is a product of Health Central Network Inc., USA. Dexamethazone, dihydrogen phosphate potassium salt (KH₂PO₄), disodium hydrogen phosphate (Na₂HPO₄), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), and hydrogen peroxide (35%) were obtained from Sigma-Aldrich, St. Louis, MO, USA. Dimethyl sulfoxide (DMSO) (Fisher Scientific, UK), ferric ammonium citrate (FAC) (BDH, England) and Desferrioxamine mesylate (DFO) (Novatis, Switzerland) were purchased from a drug store in Maharaj Nakorn Chiang Mai Hospital, Faculty of Medicine, Chiang Mai University. Deferiprone (DFP) was kindly donated by Dr. Chada Phisalaphong, Government Pharmaceutical Organization Thailand. CM1 was synthesized by Dr. Kanjana Pangjit at Chiang Mai University. Ethylenediaminetetraacetic acid (EDTA) and nitrilotriacetic acid (NTA) were purchased from Sigma-Aldrich, St. Louis, MO, USA.

Animals

Wild type mice (strain C57BL/6) aged between 6-10 weeks and having a body weight 24-30 grams were kindly supplied by the Thalassemia Research Center, Institute of Molecular Biosciences, Mahidol University, Salaya Campus and used as a source of primary hepatocytes [15,16]. The animals were housed in polyethylene cages and maintained in a clean air-conditioned room under the controlled conditions of 12-h day/12-h night cycle at $23 \pm 1^\circ\text{C}$ and at 40-70% humidity. The study protocol has been approved by the Animal Ethical Committee of Medical Faculty, Chiang Mai University, Thailand (Reference Number -3/2554).

Isolation and cultures of mouse primary hepatocytes

Mice were anesthetized with vapor diethyl ether and their chests were opened. The liver was perfused in situ via the portal vein with the KRB pH 7.4 solution comprising 116 mM NaCl, 5.4 mM KCl, 25 mM NaHCO₃ and 0.63 mM EGTA at 37°C, a flow rate of 1 ml/min for 20 minutes and with the KRB buffer containing 1 mM CaCl₂, 0.025% (w/v) collagenase type IV for 20 minutes. Then, the livers were excised, teased apart, incubated at 37°C for a further 15 minutes in the collagenase solution, and isolated hepatocytes were harvested through nylon mesh (250-61 µm). Crude cells were sedimented by differential centrifugation (60 g) for 5 minutes at 25°C and resuspended in the 20 mM HEPES buffer containing 116 mM NaCl, 5.4 mM KCl, 1 mM CaCl₂, pH 7.4. Cell viability was assayed using trypan blue exclusion technique. Cell numbers were adjusted to 4×10^5 viable cells/ml and cultured in DMEM supplemented with 10% (v/v) FBS, 2.0 mM glutamine, 100 U/ml penicillin/100 U/ml streptomycin, 200 mU/ml insulin and 1 µM dexamethasone [17].

HepG2 cell culture

Human hepatoma (HepG2) cells were used to study the biochemical and toxicological properties due to their increased oxidative stress, loss of mitochondrial function, and loss of viability when challenged with prooxidants such as iron [18]. Cells were cultured in complete DMEM containing 2 mM glutamine, pyridoxine hydrochloride, 110 mg/l sodium pyruvate, 10 mM HEPES, 44 mM NaHCO₃, 10% (v/v) inactivated FBS and 0.01% (w/v) penicillin-streptomycin in a humidified incubator containing 5% CO₂ and 95% air at 37 °C [19].

Iron loading to cultured hepatocytes

The primary hepatocytes and HepG2 cells suspension (5×10^3 cells/well) were plated to a 96-well plate and incubated in an incubator (5% CO₂ atmospheric condition) at 37 °C for 24 hours. Then, the cells were incubated with sterile ferric ammonium citrate (FAC) solution (a final concentration of 0.5 mM) at 37 °C for 24 hours and washed three times with phosphate buffered saline (PBS) solution, pH 7.4 to remove the excessive iron [20,21].

Chelation of intracellular labile iron pool (LIP)

Solutions of DFP (0-100 µM), DFO (0-100 µM), CM1 (0-100 µM), CM1 (0-100 µM) with 25 µM DFP and CM1 (0-100 µM) with 25 µM DFO were freshly prepared in 50 mM HEPES buffer, pH 7.2 and filtered through a membrane (cellulose type 0.22 µm). The cells were incubated with the DFP, DFO, CM1, CM1 plus DFP and CM1 plus DFO solutions at 37°C for 12 and 24 hours [22]. The treated cells were washed three times with the culture medium and labeled with calcein-AM solution (1 µM in DMSO). Fluorescent intensity (FI), which was inversely proportional to the analyzed amount of LIP, was measured with a 96-well plate reader spectrofluorometer (excitation/emission wavelengths 485 nm/530 nm) [23]. Viability of studied cells was greater than 80% and was not changed during the assay.

Measurement of reactive oxygen species (ROS) levels

DCFH-DA can simply diffuse into the cells and be hydrolyzed by esterase in viable cells to produce 2',7'-dichlorofluorescein (DCFH), which will be subsequently oxidized by existing ROS to 2',7'-dichlorofluorescein (DCF). An increase of a green fluorescent signal indicates increased intracellular oxidative stress. The hepatocytes were incubated with the compounds as above at 37°C for 12 and 24 hours. The treated cells were washed three times with the culture medium, labeled with 10 µM DCFH-DA solution for 30 minutes, and challenged with H₂O₂ solution (125 µM) for 30 minutes. FI was measured using the spectrofluorometric technique (excitation/emission wavelengths 485 nm/530 nm) [24]. FI value is correlated with oxidative stress and directly proportional to the amount of free radicals (ROS) in the cells [25].

Statistical analysis

Data were presented as mean \pm SEM. Statistical significance was determined using one-way analysis of variance (ANOVA), for which $p < 0.05$ was considered significant.

Results

Dose-response chelation of LIP

The LIP levels of primary hepatocytes pretreated with FAC for 24 hrs was decreased markedly in the presence of DFP and CM1 chelators in a concentration-dependent manner at both 12 and 24 hrs (Figure 1a). A similar influence on LIP levels was observed for HepG2 cells

(Figure 2a). This decreasing in LIP levels was monitored by an increase in the FI of the intracellular calcein. DFP was found to cause a large decrease in LIP levels at concentrations up to 25 μM at both 12 and 24 hrs. There was a similar effect with CM1. Inversely, there was no marked loss of effect at both 12 and 24 hrs with DFO. In combination treatment, CM1 synergized the persisting chelation of 25 μM DFP tended to decrease the LIP concentrations at both 12 and 24 hrs in primary hepatocyte cultures. Nonetheless, this effect disappeared when used the 25 μM DFO (Figure 1b). Apparently, CM1 treatment (25-100 μM) with 25 μM DFP dose dependently reduced levels of LIP in HepG2 cells when incubated for 12 hrs; inversely, treatment with 25 μM DFO caused no marked loss of effect. However, CM1 co-treatment with 25 μM DFO significantly decreased levels of LIP in HepG2 cells when incubated for 24 hrs (Figure 2b). Importantly, DFP (12.5-100 μM) and 25 μM DFO effectively reduced the LIP levels in HepG2 cells when the cells were treated for 24 hrs (Figure 3).

Effect of chelators on intracellular ROS levels

DFP and CM1 treatment tended to reduce levels of hydrogen peroxide derived-ROS in the cultured primary hepatocyte in dose-dependent manner at 12 hrs, but not time-dependent manner. CM1 at concentration 100 μM decreased the ROS levels when incubated for 24 hrs; inversely, DFO was unable to decrease the ROS at any incubation

time (Figure 4a). A similar influence on ROS levels was observed for HepG2 cells, DFP and CM1 treatment tended to decrease levels of hydrogen peroxide derived-ROS at both 12 and 24 hrs. DFO (50 and 100 μM) treatment reduced levels of produced ROS in cultured HepG2 cells at 12 and 24 hrs (Figure 5a). Co-treatment with CM1 (100 μM) and 25 μM DFP significantly decreased the levels of ROS in primary hepatocyte cultures when incubated for 12 hrs. DFO did not enhance the activities of CM1 (12.5-100 μM) in scavenging generated ROS in the treated primary hepatocytes. The combined treatments were able to reduce the generated ROS in the cells when incubated for up to 24 hrs (Figure 4b). In HepG2 cells, CM1 treatment with 25 μM DFP tended to decrease the ROS levels at hour 12. At concentration of 100 μM CM1 combined with 25 μM DFP effectively reduced levels of ROS at 24 hrs. Above this concentration range, CM1 co-treatment with 25 μM DFO significantly diminished levels of hydrogen peroxide derived-ROS in both 12 and 24 hrs (Figure 5b).

Effect of various chelators on intracellular ROS levels

As result, DFP and EDTA treatments (25 μM) were found to cause a large reduced in the levels of ROS when compared to untreated HepG2 cells at 24 hrs. There was a similar effect with CM1 and NTA. Nevertheless, there was no marked loss of effect at 25 μM with DFO (Figure 6).

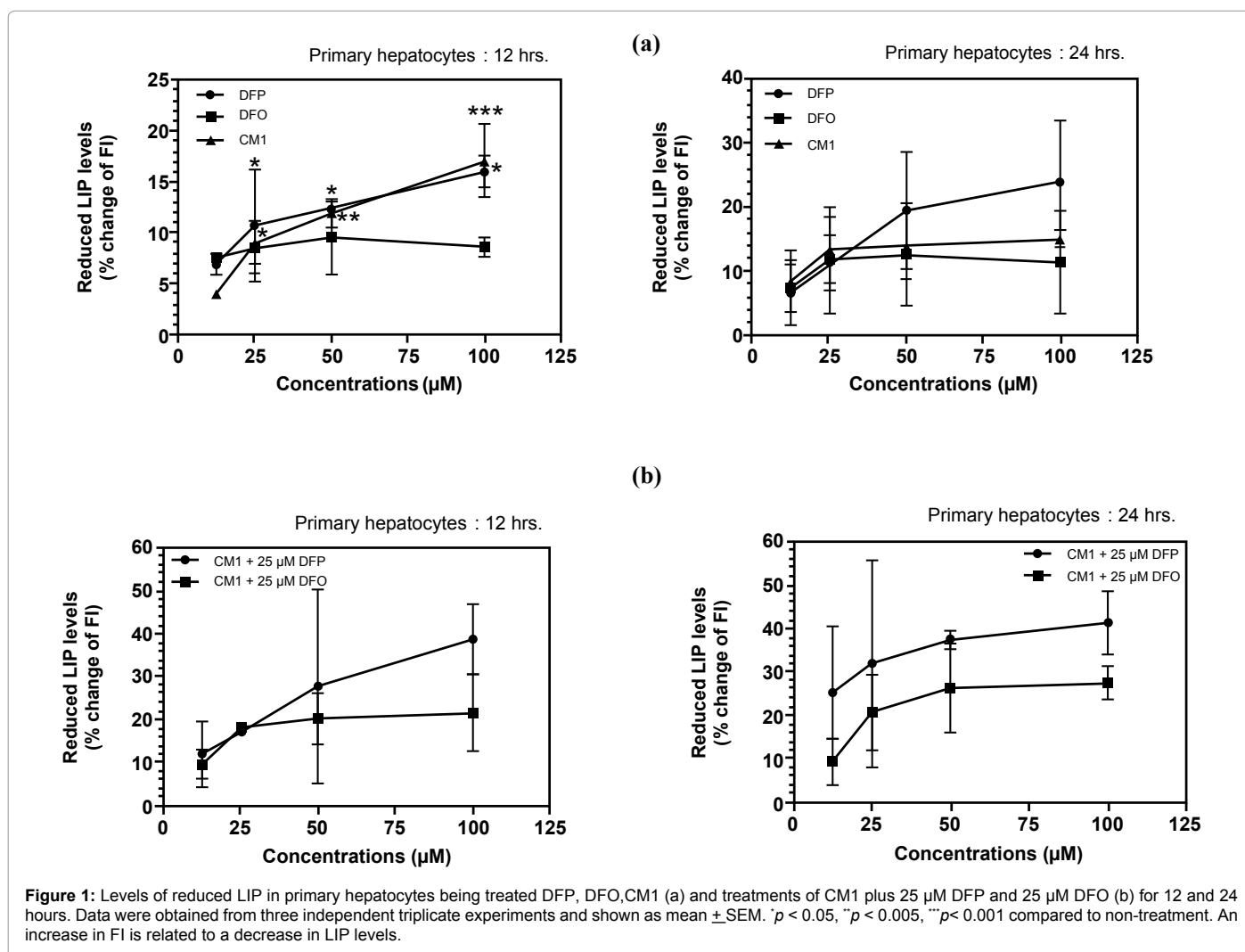


Figure 1: Levels of reduced LIP in primary hepatocytes being treated DFP, DFO, CM1 (a) and treatments of CM1 plus 25 μM DFP and 25 μM DFO (b) for 12 and 24 hours. Data were obtained from three independent triplicate experiments and shown as mean \pm SEM. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ compared to non-treatment. An increase in FI is related to a decrease in LIP levels.

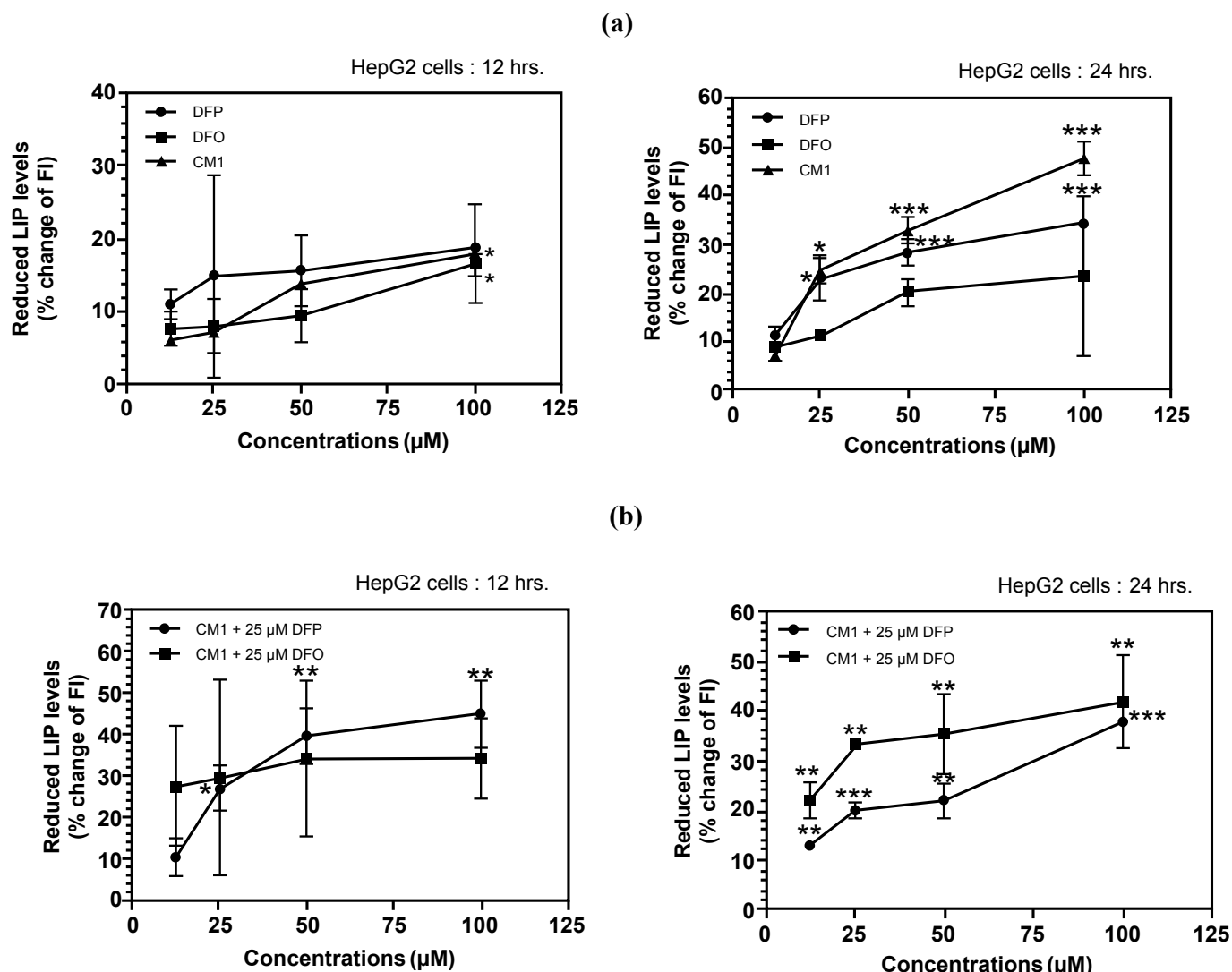


Figure 2: Levels of reduced LIP in HepG2 cells being treated with DFP, DFO, CM1 (a) and treatments of CM1 plus 25 μM DFP and 25 μM DFO (b) for 12 and 24 hours. Data were obtained from three independent triplicate experiments and shown as mean ± SEM. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ compared to non-treatment. An increase in FI is related to a decrease in LIP levels.

Discussion

The liver is chiefly responsible for taking up and storing excessive amounts of iron. The major hepatic toxicities of iron overload include damage to multiple cell types (hepatocytes, Kupffer cells, hepatic stellate cells) and to multiple subcellular organelles (mitochondria, lysosomes, and smooth endoplasmic reticulum) [26]. Examination of the hepatocellular iron content of liver specimens is required for the diagnosis of iron overload [27]. Under conditions of iron overload, non-specific albumin bound iron (NTBI) is transported in transferrin-saturated plasma and is rapidly cleared by the liver. Rat hepatocytes in primary culture has been demonstrated to possess a high capacity to absorb the NTBI in the form of ferric citrate and small-molecular-weight iron complexes [28,29]. Iron uptake into the cultured hepatocytes of wild type mice was found to be less than the cultured HepG2 cells because HepG2 cells as a human hepatoma cell line as the high proliferation rate of latter cells creates a higher demand for iron [30]. Both cell types have been investigated in this study. Our study has demonstrated that DFP and CM1 decreased concentration of transient iron as

LIP in both primary hepatocytes and HepG2 cells cultures at both 12 and 24-hour incubations. However, DFO slightly decreased the LIP, probably the chelator is hypophilic than DFP and CM1 [11]. *In vivo* studies have shown that DFP which enters cells is subsequently able to transfer intracellularly chelated iron to a stronger chelator DFO, if simultaneously present in plasma [11,31]. Both chelators can bind iron released from the RE macrophages during recycling of iron from senescent RBC and erythroblasts, the main source of the increased iron turnover. One study demonstrated that DFO penetrate into the hepatocytes and chelated cytosolic LIP slowly whereas DFP and DFX readily entered the cells and efficiently chelated the LIP [32]. Synthetic hydroxypyridinone chelators such as 1,2-dimethyl (DFP) and 1-ethyl-2-methyl derivatives of 3-hydroxypyrid-4-one are active in removing iron from the reticuloendothelial system and hepatocytes, and indeed are superior to DFO [33]. Moreover, in this study CM1 combination treatment with DFP was found to remove persisting LIP in target cells better than CM1 alone. Apparently, co-treatment with the standard iron chelators DFP and DFO lowered the LIP concentrations in the HepG2 cells. Combined therapy with DFO and DFP has demonstrated

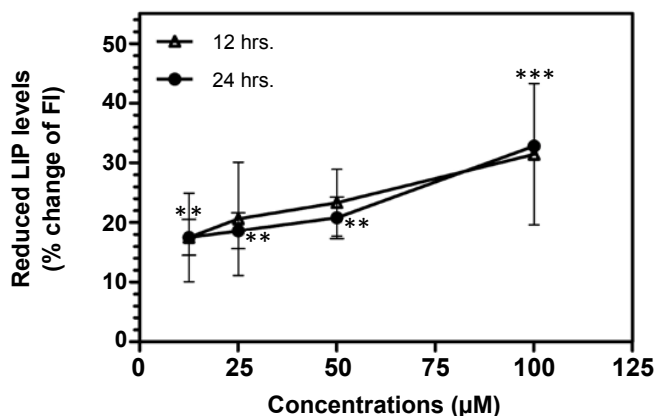


Figure 3: Effect DFP plus 25 µM DFO treatment on reduced LIP levels in HepG2 cells for 12 and 24 hours. Data were obtained from three independent triplicate experiments and shown as mean ± SEM. **p* < 0.005, ***p* < 0.001 compared to non-treatment. An increase in FI is related to a decrease in LIP levels.

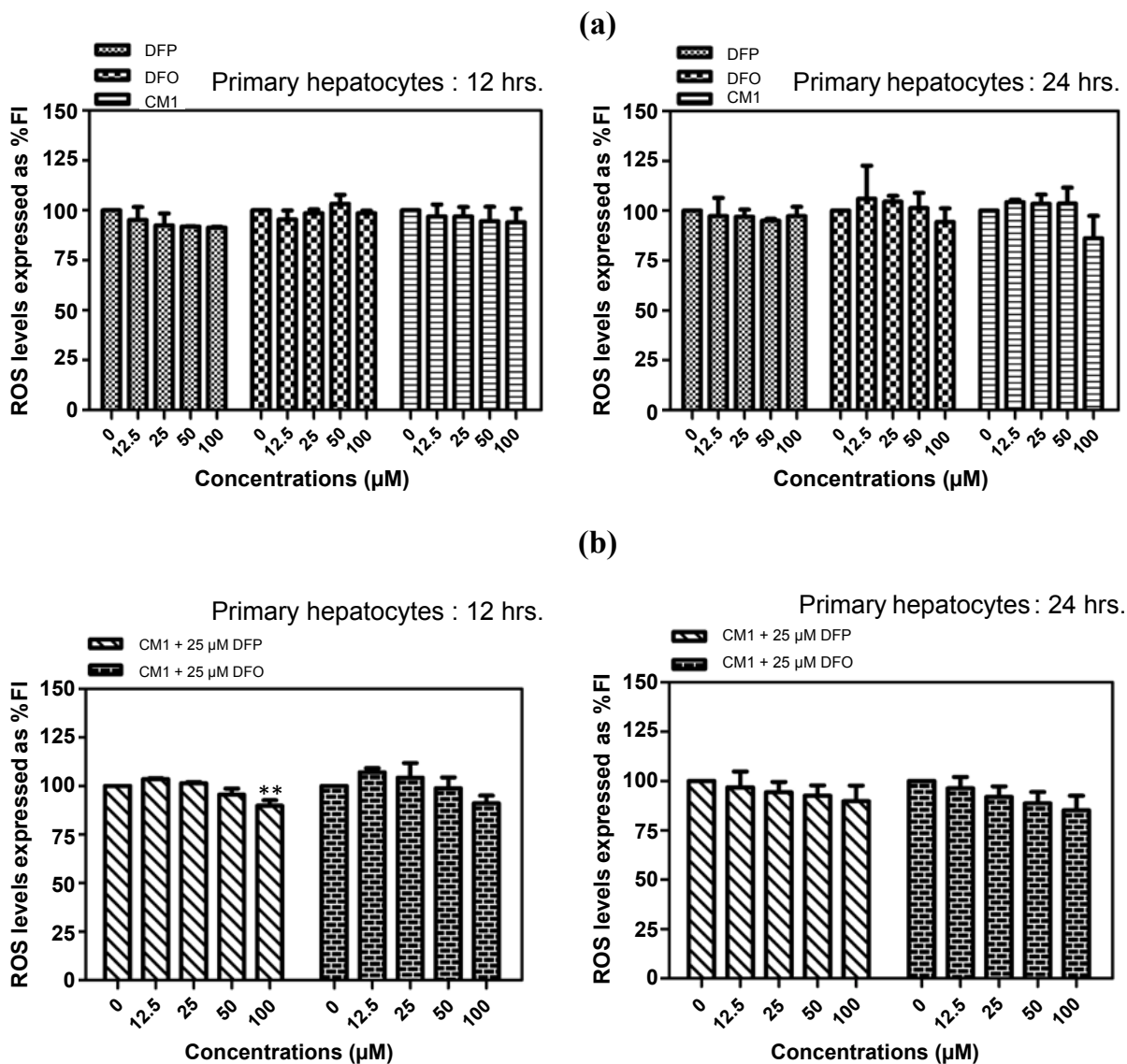


Figure 4: Levels of ROS in primary hepatocytes being treated with DFP, DFO, CM1 (a) and treatments of CM1 plus 25 µM DFP and 25 µM DFO (b) for 12 and 24 hours. Data were obtained from three independent triplicate experiments and shown as mean ± SEM. ***p* < 0.005 compared to non-treatment.

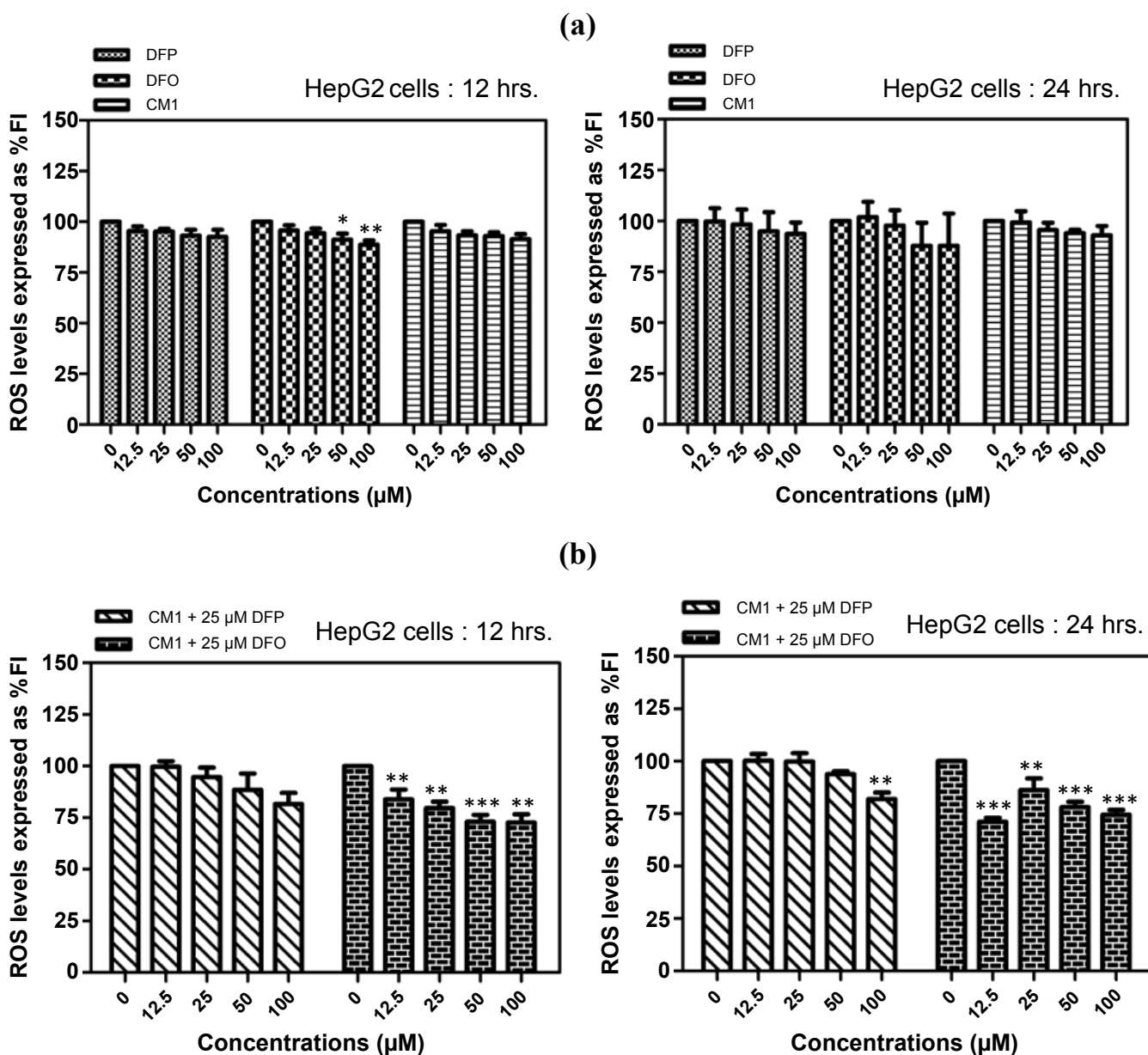


Figure 5: Levels of ROS in HepG2 cells being treated with DFP, DFO, CM1 (a) and treatments of CM1 plus 25 μM DFP and 25 μM DFO (b) for 12 and 24 hours. Data were obtained from three independent triplicate experiments and shown as mean ± SEM. **p* < 0.05, ***p* < 0.005, ****p* < 0.001 compared to non-treatment.

decreased serum ferritin levels of beta thalassemic patients [34]. CM1 has potential to chelate intracellular transient iron and to work together with DFP for iron chelation in liver cells with iron overload. DFP and CM1 tended to reduce levels of ROS in the cultured primary hepatocytes as well as HepG2 cells at both 12 and 24-hour incubation. DFO slightly changed levels of the ROS persisting in the HepG2 cells. Suggestively, DFP, CM1, EDTA and NTA exhibit not only iron-chelating but also antioxidative properties, which can penetrate into the cells and interact with intracellular reactive oxidants including redox-active iron and free radicals [35-37]. Combined treatment of CM1 with DFP and DFO tended to decrease the level of ROS at 24-hour incubation in primary hepatocytes. Interestingly, the combination treatment of CM1 with DFO at 12 and 24-hour incubation significantly lowered the level of ROS in HepG2 cells. Since HepG2 cells are highly metabolic human hepatoma cells and produce larger amounts of reactive oxidants,

their free-radical scavenging activity seems to be more apparent than that of primary hepatocytes. Previously study shows DFO exhibits the antioxidant and free radical scavenging activities in iron-loaded hepatocyte cultures [38].

Conclusions

As a result of this preliminary study it is clear that CM1 could reduce excessive redox-active, transient (labile) iron and reactive oxygen species in cytosolic compartment of *ex-vivo* mouse primary hepatocytes as well as HepG2 cell cultures. Collectively, our findings imply protective and therapeutic effects of CM1 on the liver with iron overload and oxidative stress. Most importantly, it needs designing the merit adjunctive study of CM1 and deferiprone and/or desferrioxamine to prevent liver pathogenesis in thalassemia patients with iron overload in the near future.

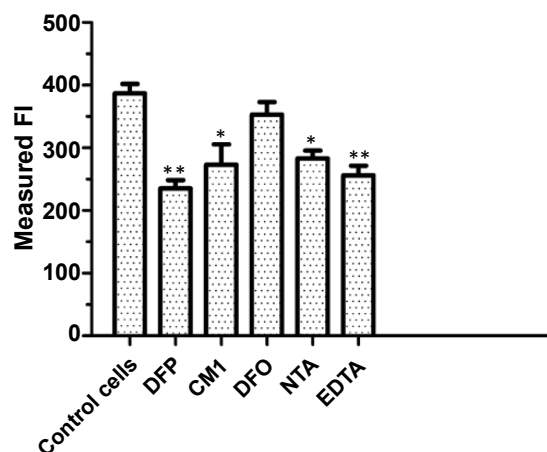


Figure 6: Treatments of 25 μ M DFP, CM1, DFO, NTA and EDTA on levels of ROS in HepG2 cells for 24 hours. Data were obtained from three independent triplicate experiments and shown as mean \pm SEM. * p < 0.05, ** p < 0.005 compared to non-treatment.

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