

The Target of Mitochondrial and Plasma Membrane Citrate Transporter Protein Inhibitors on De Novo Lipogenesis Pathway Inhibition Induces Apoptosis in HepG2 Cell

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ABSTRACT

The reprogramming of energy pathways in cancers by switching the major metabolism pathway from oxidative phosphorylation (OXPHOS) to rely on aerobic glycolysis or Warburg effect provides substrate precursors for the de novo lipogenesis (DNL). High rates of ATP generation and fatty acid synthesis from DNL ensure a high demand of energy and biomolecule synthesis in tumor progression. The intracellular citrate is the starting material of the DNL pathway in cancer cells. Two sources of intracellular citrate are identified which are mitochondrial and extracellular citrate. Citrate derived from a mitochondrial matrix of OXPHOS pathway is transported by the mitochondrial citrate transport protein (CTP) on the inner membrane of mitochondria. Citrate is also imported from the extracellular to the cytosol via plasma membrane citrate transporter (PMCT). Inhibition of DNL pathway has been found as one of the most efficient cancer therapies by suppressing cell viability and promoting cell apoptosis in cancers. Thus, this study investigated the anti-tumor effect of CTP inhibitor and PMCT inhibitor to decrease intracellular citrate and fatty acid levels leading to inhibition the DNL pathway and reduction cancer cell proliferation. It was found that after 24 h of treatment HepG2 with the CTP inhibitor and PMCT inhibitor resulted in decrease cell viability measured by MTT assay, enhanced apoptotic cell death associated with a disruption of mitochondrial membrane potential detected by flow cytometer. Moreover, CTP inhibitor and PMCT inhibitor inducing apoptosis were associated with decrease of intracellular citrate and fatty acid levels. Thus, we speculated that the mechanism of CTP inhibitor and PMCT inhibitor involved the deficiency of the intracellular citrate and fatty acid levels contributing to induction of HepG2 cell apoptosis. Collectively, our findings demonstrate that the CTP inhibitor and PMCT inhibitor will potentially assist in the development of novel cancer treatment that targets the inhibition of the DNL for HCC and other cancers.

Keywords: De novo lipogenesis, PMCT inhibitor, CTP inhibitor

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Introduction

Hepatocellular carcinoma (HCC) is a primary malignant cancer of liver. HCC is now the third most commonly diagnosed malignant tumor in worldwide. The incident rate of HCC is growing in Africa and South East Asia including Thailand (Ferlay et al., 2015). Independent risk factors HCC are Hepatitis B virus (HBV) infection and Hepatitis C virus (HCV) infection. Other risk factors are alcohol cirrhosis, non-alcoholic steatohepatitis, associated to obesity epidemics (Kim et al., 2016). Previous research has demonstrated that pathophysiology of HCC relates to metabolic syndrome with insulin resistance (Paschos & Paletas, 2009). The gene expression of IRS-1, TNF-alpha, PPAR, and PI3K signaling leading to free fatty acids flux and accumulation of the fatty liver can developed HCC (Rahman, Hammoud, Almashhrawi, Ahmed, & Ibdah, 2013). Induction of lipogenic enzyme expression by stimulating the release of insulin which activates protein kinase AKT2 and PI3K/AKT pathway is reported to be responsible for elevated lipogenesis in cancer (Hagiwara et al., 2012). Several studies have reported that therapeutic targeting via suppression of DNL enzymes including ACLY (ATP citrate lyase) (Hanai et al., 2012; Migita et al., 2014), FASN (fatty acid synthase) (Mullen & Yet, 2015; Yellen & Foster, 2014) and ACC (acetyl-CoA carboxylase) (Svensson et al., 2016; Wang et al., 2015) result in tumor regression both in vitro and in vivo. The present study we focused the intracellular citrate level which is a precursor of DNL pathway for synthesis of saturated long chain fatty acids (LCFAs). These fatty acid products are metabolized for the constituents of phospholipid cell membrane synthesis and acetyl CoA production via β -oxidation. Mitochondrial citrate and extracellular citrate are sources of citrate for DNL. Inhibition of mitochondrial citrate by mitochondrial citrate transport protein (CTP) inhibitor has been reported. The discovery of this compound has been reported via *in silico* screening of the ZINC database (Sun et al., 2010). The source of citrate derived from blood circulation is inhibited by plasma membrane citrate transporter (PMCT) inhibitor. Inhibition of citrate fluxes from plasma into the cytoplasm by PMCT inhibitor reduces and lowers intracellular citrate level leading to promotion the glycolysis pathway and reduction lipogenesis and gluconeogenesis. In previous studies, inhibition of citrate transporter NaCT (or PMCT) in vitro and in vivo by compound 2(PF-06649298) reduced hepatic lipid production and plasma glucose levels (Huard et al., 2015). Thus, we proposed to examine the mechanism of the CTP and PMCT inhibitors that might be involved in the suppression of the DNL pathway leading to induction apoptosis in human hepatocellular carcinoma, HepG2 cell.

Objective of the study

To investigate the potential effects and mechanisms of the inhibitors of mitochondrial citrate transport protein (CTP) and plasma membrane citrate transporter (PMCT) on suppression of DNL pathway which triggered apoptosis in HepG2 cell.

Materials and Methods

Cell culture

Human hepatocellular carcinoma, HepG2 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained at 37°C under a humidified 5% CO₂ with 95% humidifier incubator in

Eagle's Minimum Essential Medium (EMEM) (Corning, Manassas, VA, USA) supplemented with 10% FBS and 1% penicillin-streptomycin (Gibco, MA, USA).

MTT assay

Cell viability was determined using 3'-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (AMRESCO, Solon, OH, USA). After 24 h treatment with varies concentrations of 4-chloro-3-[(3-nitrophenyl) amino] sulfonyl} benzoic acid (mitochondrial citrate transport protein, CTP) inhibitor (ChemBridge Corporation #6652048) and 2-(benzylsulfonyl)-N-[(pyridin-2-yl) methyl] propanamide (Plasma membrane citrate transporter, PMCT) inhibitor (TimTec ID ST056138), MTT solution 5 mg/ml final concentration in phosphate-buffered saline (PBS) was added to each well and incubated for 4 h. Then formazan crystals were dissolved in DMSO and detecting the absorbance at 595 nm using Synergy HT Microplate Reader (BioTek Instruments, Inc, Winooski, VT, USA) and IC50 value was calculated using Graph Pad Prism version 5.

Detection of apoptosis

HepG2 cells were treated with CTP inhibitor and PMCT inhibitor at the indicated IC50 concentrations for 24 h. The apoptosis of treated cell was double stained with Alexa Fluor® 488 annexin V/ propidium iodide (PI) Dead Cell Apoptosis Kit (Merck Millipore, Germany) and measured apoptotic rate. The data was analyzed using Muse cell analyzer (Merck Millipore, Germany).

Determination of mitochondrial membrane potential ($\Delta\Psi_m$)

HepG2 cells were treated with CTP inhibitor and PMCT inhibitor for 24 h. Cells were harvested and incubated with JC-1 dye (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide) - Mitochondrial Membrane Potential Probe (Life Technologies, Thermo Scientific, NY, USA) at 37°C and 5% CO₂ for 45 min. The JC-1 aggregated forms accumulated inside mitochondria in response to changing of $\Delta\Psi_m$. A healthy mitochondria in polarized state exhibits red fluorescence while it remains monomeric form in cytoplasm with depolarized state of mitochondrial membrane and exhibits green fluorescence. The level of $\Delta\Psi_m$ in HepG2 cells was measured by FACSCalibur flow cytometry and the data were analyzed using CellQuset Pro software.

Intracellular citrate quantification assay

Intracellular citrates are substrates for DNL pathway. Conversion of citrate to pyruvate via oxaloacetate was quantified by citrate bioAssay kit (C5802: US Biological; Life Sciences, Salem, MA, USA) as described in the manufacturer's protocol. Briefly, HepG2 cells were incubated with the CTP inhibitor and PMCT inhibitors for 24 h. Subsequently, cells were harvested, homogenized with citrate assay buffer, followed by adding the reaction mix to each samples. Then, the pyruvate was quantified by fluorescence intensity measured at Ex/Em 535/590 nm using Synergy HT Microplate Reader with Gen5 Data Analysis software.

Intracellular long chain free fatty acid quantification assay

To evaluate intracellular long chain free fatty acid level, a product from DNL pathway was measured by free fatty acid bioAssay kit (F0019-94; US Biological; Life Sciences, Salem, MA, USA) as described in the manufacturer's protocol. Briefly, after incubated with the CTP inhibitors and PMCT inhibitor, HepG2 cells were

harvested and homogenized with 1% Triton-X 100 in pure chloroform and fatty acid assay buffer. Subsequently, the reaction mix in the assay kit was added. Long chain fatty acids were converted to CoA derivatives and then quantified by either colorimetric (spectrophotometry at 570 nm) or fluorometric (at Ex/Em = 535/587 nm) using the Synergy HT Microplate Reader (BioTek Instruments).

Statistical Analyses

One-way analysis of variance (ANOVA) with Turkey's post-hoc analysis was used to determine the statistical significant differences in all experimental results between treatment and control group. The vehicle was set as control cells treated with 0.2% DMSO. All data were presented as mean± SD of at least three independent experiments, $p < 0.05$ and analyzed using the Graph Prism Software version 5.

Results

The effects of CTP inhibitor and PMCT inhibitor on cell viability

HepG2 cells cultured with CTP and PMCT inhibitors resulted in decreased cell viability as shown in figure 1A and 1C. All the inhibitors induced reduction of the number of viable cells on a dose-dependent manner with IC50 value was approximately estimated to be 2.5 mM for both CTP inhibitor and PMCT inhibitor treatment, as shown in figure 1B and 1D, while the vehicle alone remained viable throughout the incubation period. Therefore, the results demonstrate that CTP inhibitor and PMCT inhibitor caused inhibition of proliferation in HepG2 cells.

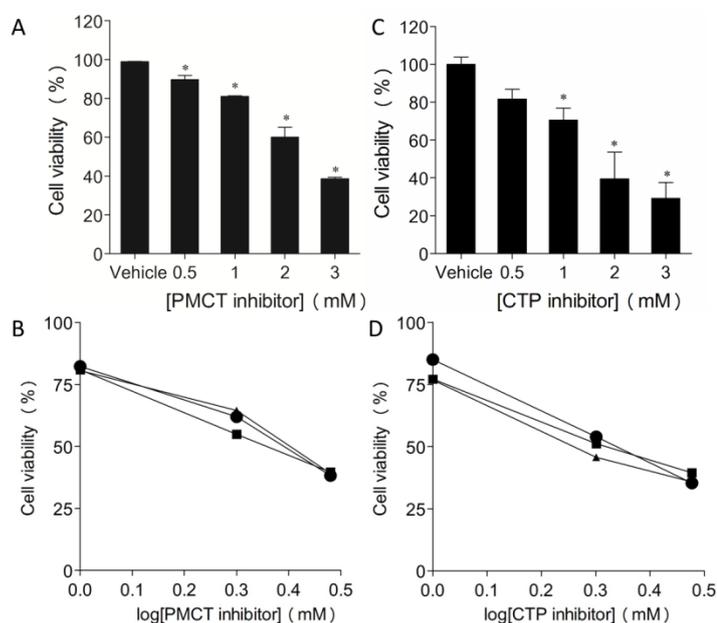


Figure 1 CTP inhibitor and PMCT inhibitor decreased viability of HepG2 cells. Cells were treated with different concentrations of CTP and PMCT inhibitors for 24 h. Cell survival was examined by MTT assay and expressed as percentage of cell viability compared with 100% of the vehicle control. Three independent

experiments were performed for statistical analysis and expressed as mean \pm SD. *denotes statistically significant difference from the vehicle at $P < 0.05$.

The effect of CTP inhibitor and PMCT inhibitor on cell apoptosis

Investigations were performed to determine the mechanisms CTP and PMCT inhibitors to reduced cell viability which occurred by apoptosis induction. HepG2 cells were treated with CTP and PMCT inhibitors at 2.5 mM, the IC₅₀ concentration of both inhibitors was obtained from the MTT result for 24 h. Apoptosis was detected by annexin V/PI staining and measured by flow cytometer as shown in figure 2. Therapy of 2.5 mM CTP inhibitor and 2.5 mM PMCT inhibitor significantly reduced viability of cells to approximately 51% and 66% (from 87% control) and increased apoptotic cells to approximately 45% and 33% (from 9% control), respectively. These results suggest that treatment with CTP inhibitor and PMCT inhibitor exhibits apoptotic induction effect on HepG2 cell.

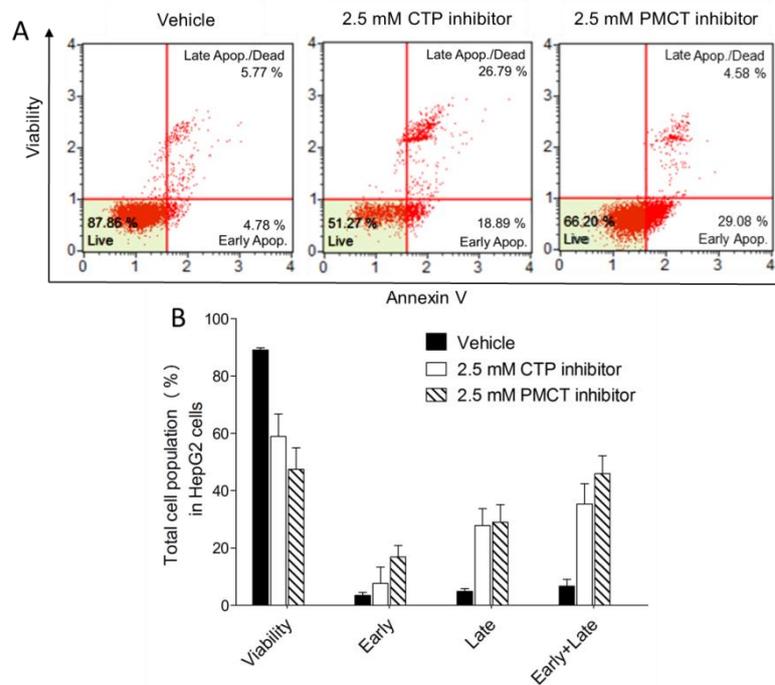


Figure 2 CTP inhibitor and PMCT inhibitor induced apoptosis on HepG2 cells. Cells were treated with IC₅₀ concentrations of CTP and PMCT inhibitors alone for 24 h, double stained with annexin V/ PI, and detected by flow cytometry. (A) Flow cytometry showed representative dot plot analysis. (B) Effect of CTP and PMCT inhibitors on apoptosis of HepG2 cell represent in bar graph. All data were mean \pm SD of at least three independent experiments. * $P < 0.05$ versus control group.

The effect of CTP inhibitor and PMCT inhibitor on mitochondria membrane potential (Ψ_m)

Depolarization of the mitochondrial membrane is a special characteristic of the initiation event of the intrinsic apoptotic pathway. In this study JC-1 staining was performed to investigate the effect of CTP and PMCT

inhibitors on induction of apoptosis through loss of $\Delta\Psi_m$ as shown in Figure 3. Cells treated with 2.5 mM CTP inhibitor and PMCT inhibitor showed a loss of $\Delta\Psi_m$ to approximately 54% and 33% compared to less than 10% of the control and to approximately 60% for a CCCP positive control, the depolarization of the mitochondrial membrane, respectively. The results suggest that the CTP and PMCT inhibitors induce mitochondria-dependent apoptosis in HepG2 cells.

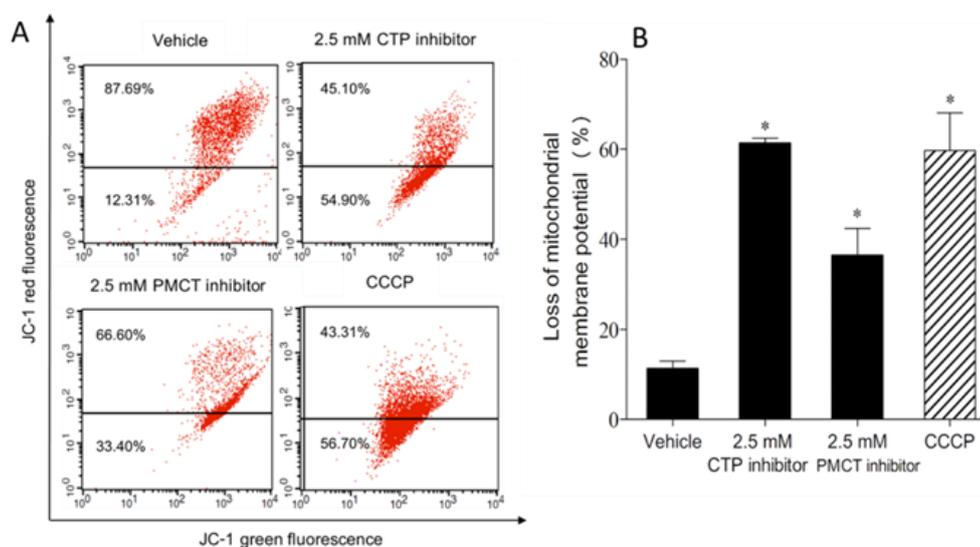


Figure 3 CTP inhibitor and PMCT inhibitor induced a damage of $\Delta\Psi_m$ on HepG2 cells. Cells were incubated at 2.5 mM of CTP and PMCT inhibitors for 24 h, stained with JC-1 fluorescence dye, and detected by flow cytometry. (A) $\Delta\Psi_m$ of HepG2 cells was evaluated by flow cytometry and showed as representative dot plot analysis. (B) The percentage of disruption of the $\Delta\Psi_m$ in HepG2 cells is expressed in bar charts. Data indicate mean \pm SD from at least three independent experiments performed in triplicates. * $p < 0.05$ significantly different from the control.

The effect of CTP inhibitor and PMCT inhibitor on intracellular citrate and fatty acid level

This study further investigated decreased of fatty acid levels in DNL pathway following inhibition of citrate transport could potentially exert apoptotic induction in HepG2 cells. Reduction of fatty acid synthesis is known to cause apoptosis in cancer cells (Li, Tian, & Ma, 2014; Puig et al., 2008; Puig et al., 2009). As shown in figure 4, after cells were treated with CTP and PMCT inhibitors for 24 h, the percentage of intracellular citrate level decreased to approximately 30% and 50%, respectively compared with 100% of the vehicle. An intracellular fatty acid level production of DNL pathway decreased to 40%, 55%, respectively compared to 100% of the vehicle. These results demonstrate that CTP and PMCT inhibitors inducing apoptosis was involved in a suppression of DNL pathway in HepG2 cells.

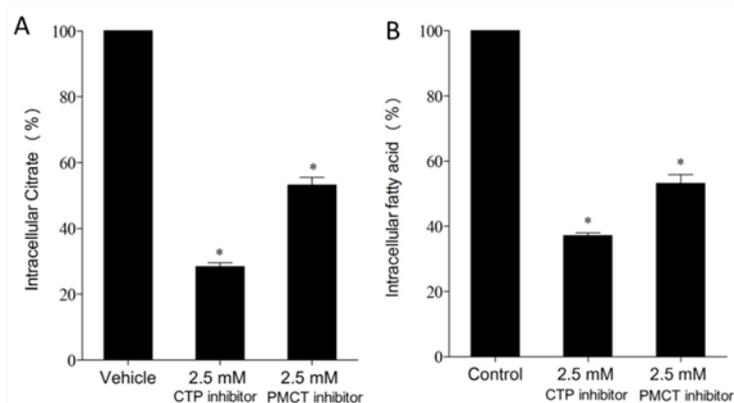


Figure 4 CTP inhibitor and PMCT inhibitor decreased intracellular citrate and fatty acid levels in HepG2 cells. Cells were treated with CTP and PMCT inhibitors at 2.5 mM concentrations of each for 24 h. Cell exposed to 0.2% DMSO was set as vehicle. (A) Intracellular citrate and (B) Intracellular fatty acid levels were quantified as described in materials and methods. The result showed mean±SD, n=3, *p < 0.05 from three independent experiments.

Discussion

Intracellular citrate is transported from the extracellular source into the cytoplasm via the action of PMCT or NaCT presented in the plasma membrane to serve as substrate for de novo fatty acid synthesis. Citrate is concomitantly transported out of mitochondria via CTP into the cytoplasm. Previous study has been reported that an inhibition of NaCT or PMCT activity decreases fatty acid and cholesterol biosynthesis that lead to prevention obesity and extending life-span (Sun et al., 2010). In addition, Inhibition of CIC or CTP activity inhibits tumor growth in breast, lung, and bladder cancers (Kolukula et al., 2014). However, the functional role of CTP inhibitor and PMCT inhibitor regulating of intracellular citrate level via targeting an inhibition of DNL pathway has not been further evaluated. Thus, a decreased level of citrate via inhibition of citrate transporters may provide alternative novel anticancer approach.

In this study, we found that CTP and PMCT inhibitors decreased cell viability and induced apoptosis in HepG2 cells. The apoptosis induction was a mitochondrial-dependent pathway. Moreover, we showed that citrate transporter inhibitors inducing apoptosis was associated with inhibition of fatty acid synthesis via DNL pathway suppression. Decreased intracellular citrate resulted in decrease of carbon sources for the de novo fatty acid synthesis after treatment with CTP and PMCT inhibitors. Thus, we suggest that inhibition of fatty acid synthesis by CTP and PMCT inhibitors might cause apoptosis in HepG2 cells. As a result of inhibition of fatty acid levels, apoptosis induction may be resulted from the depletion of fatty acid products required for the synthesis of membrane phospholipid during cell proliferation (Deepa, Vandhana, & Krishnakumar, 2013; Jackowski, Wang, & Baburina, 2000). Decreased fatty acid synthesis may suppress the mevalonate pathway for cholesterol synthesis required for

lipid posttranslational modification in malignancy development (Xin et al., 2016). Thus, this result provides promising development of novel anticancer agent targeting inhibition of *de novo* lipogenesis.

Conclusions

The inhibition of DNL pathway by suppression of citrate transport by CTP and PMCT inhibitors treatment. This inhibition contributes to induction apoptosis through the mitochondrial dependent pathway in HepG2 cells. This research will further suggest that the ability to selectively inhibit DNL pathway may be a novel therapeutic of cancers. In addition, targeting DNL by suppression of citrate transport pathways will be therapeutic intervention of metabolic disorders resulting from the synthesis of excess lipid, cholesterol, and glucose in human obesity, hyperlipidemia, hyper-cholesterolemia, and type 2 diabetes.

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