Chrysophanol localizes in mitochondria to promote cell death through upregulation of mitochondrial cyclophilin D in HepG2 cells

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Objective: Chrysophanol (Chry) displays potent anticancer activity in human cancer cells and animal models, but the cellular targets of Chry have not been fully defined. Herein, we speculated whether mitochondria were a target involved in Chry-induced cytotoxicity.

Methods: Human liver cancer cell line HepG2 was incubated. The cytotoxicity was evaluated by MTT assay. Mitochondria localization was evaluated by a confocal microscopy. Mitochondrial membrane potential $\Delta W_m$ was detected by TMRE staining and determined by the flow cytometer. The levels of ATP, mitochondrial superoxide anions, and GSH/GSSG were determined according to the assay kits. The apoptosis were evaluated through Hoechst33342/PI and Annexin V/PI staining, respectively. The expression of cyclophilin D (CyPD) was determined by immunoblot method, and the interaction between CyPD and Chry was analyzed by molecule docking procedure.

Results: Chry itself mainly localized in mitochondria to cause mitochondrial dysfunction and cell death in HepG2 cells. As regard to the mechanism, cyclosporin A as the inhibitor for the formation of mitochondrial permeability transition pore (mPTP) moderately suppressed cell death, indicating mPTP involved in the process of cell death. Further, Chry enhanced the protein expression of Cyclophilin D (CyPD) which is a molecular componentry and a modulator of mPTP, while antioxidant N-acetyl-L-cysteine inhibited the expression of CyPD. Molecule docking procedure disclosed two hydrogen-bonds existed in CyPD-Chry complex with $-11.94 \text{ kcal/mol}$ of the binding affinity value. Besides, the mtDNA-deficient HepG2-p0 cells were much resistant to Chry-induced cell death, indicating mtDNA at least partly participated in cell death. A combination of Chry and VP-16 produced the synergism effect toward cell viability and $\Delta W_m$, while Chry combined with Cis-Pt elicited the antagonism effect.

Conclusion: Taken together, enrichment in mitochondria and actions on mPTP, CyPD and mtDNA provides an insight into the anticancer mechanism of Chry. The combination therapy for Chry with clinical drugs may deserve to further explore.

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1. Introduction

Chrysophanol (Chry) is a naturally occurring anthraquinone that distributes across the plant and animal kingdoms as well as in the microbial world (Malik & Müller, 2016; Prateeksha et al., 2019; Xie, Tang, Song, Long, Zhang, & Li, 2019). Extraction from Rheum palmatum L. gave 6.030 mg/g Chry by reflux in water for 30 min after hydrolysis anthraquinones (Wei, Yao, Ji, Wei, & Peng, 2013). The pharmacological effects of Chry have been extensively explored, including anticancer, antioxidant, neuroprotection, antibacterial, antiviral and antiaging, which suggested that Chry might be used against various diseases (Malik & Müller, 2016; Prateeksha et al., 2019; Xie et al., 2019). Specific to anticancer activity, Chry could suppress malignant cell growth, induce apoptosis and necrosis, trigger endoplasmic reticulum stress, arrest cell cycle progression, and inhibit cell invasion, metastasis (Lim, An, Yang, Bazer, & Song, 2018; Lu et al., 2010; Ni et al., 2012). Some molecular targets in various types of human cancer cells have been reported to be affected by Chry, including phosphatidylinositol 3-kinase (PI3K)/Akt (Lim, Yang, Bazer, & Song, 2017), extracellular signal-regulated kinase (ERK) (Lim et al., 2017), mitogen-activated protein kinase (MAPK) signal pathway (Lim et al., 2018; Park, Lim, & Song, 2018), EGFR/mTOR pathway (Lee, Cha, Sul, Song, & Kim, 2011), NF-κB pathway (Ren et al., 2018), serine/threonine-protein kinase 1 (RIP1) (Choi, 2016) and DNA (Yang, Li, Huang, Li, & Yan, 2012). Because of its undoubted anticancer
Mitochondrial matrix-specific protein cyclophilin D (CypD), as the only isomer of human mitochondria cyclophilins, participates in the formation of mitochondrial permeability transition pore (mPTP) which leads to both apoptosis and necrosis (Li, Johnson, Capano, Edwards, & Crompton, 2004; Gutiérrez-Aguilar & Baines, 2015). Evidently, isolated mitochondria from a neuronal B50 cells stably overexpressing CypD was far more susceptible to Ca\(^{2+}\)-elicited mPTP opening and oxidative stress (Li et al., 2004); And mitochondria from CypD-null mice were more resistant to Ca\(^{2+}\)- and oxidative stress-induced mPTP opening than wild-type mice (Baines et al., 2005). Additionally, the cellular redox environment has a certain effect on the structure and functional characteristics of CypD. CypD in its reduced form might be responsible for refolding of the newly imported proteins in mitochondria and maintaining organelle integrity. Oxidized CypD might induce the opening of mPTP and lead to mitochondria-mediated cell death (Linnard et al., 2008). For instance, it has demonstrated that the higher expression of CypD was detected in necrocytosis caused by drugs such as salariomycin (Qin, Jia, Zhang, & Zhang, 2015), cisplatin (Chen et al., 2013), adriamycin (Lu, Shi, & Xu, 2014), and gemcitabine (Chen et al., 2014).

Additionally, human mitochondrial genome contains 16.5 kb DNA which encode 13 respiratory chain subunits. Respiratory chain regulates mitochondrial ATP generation, so drugs that damage mitochondrial DNA (mtDNA) probably will remarkably affect cell growth and cellular physiological function (Fliss, 2000). Moreover, because of the lack of protection by histones and the relatively weak DNA repair capacity in mitochondria, mtDNA is more susceptible to DNA damaging agents (Singh et al., 1999). Failure to repair mtDNA damage might facilitate to initiate cell death (Grishtko, Rachek, Muisijenko, LeDoux, & Wilson, 2005).

In the present study, we observed uptake of Chry in mitochondria and investigated how Chry affected mitochondrial components to trigger HepG2 cell death. And the combination efficacies of Chry with VP-16 or cisplatin (Cis-Pt) on HepG2 cell viability and mitochondrial membrane potential (ΔΨm) were also evaluated.

2. Materials and methods

2.1. Materials

Chrysophanol (Chry), Cyclosporin A (CsA), N-acetyl-L-cysteine (NAC), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma Aldrich Chemical Co. (Beijing, China). Fetal bovine serum (FBS) was purchased from Hyclone (Shanghai, China). Tetramethylrhodamine ethyl ester (TMRE) was purchased from Biolite Biotech (Tianjin, China). Annexin V-FITC/PI apoptosis detection kit was purchased from Nanjing jiancheng (Nanjing, China). Hoechst-33342 and propidium iodide (PI) was purchased from Beyotime (Shanghai, China). All antibodies were from Affinity Biosciences (OH, USA). Mito-Tracker deep red (MTDR) was obtained from Thermo Fisher (Shanghai, China).

2.2. Cell culture

HepG2 cells were obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science. The cells were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. They were maintained at 37 °C in a humidified incubator with 5% CO\(_2\).

2.3. Cell viability assay

HepG2 cells (1 × 10\(^4\) cells/well) were incubated with Chry (12.5, 25, 50, 100, and 120 µmol/L) in triplicate in 96-well plates for 48 h at 37 °C in a final volume of 100 µL. Additionally, to determine the combination effects of Chry with VP-16 and Cis-Pt, cells were incubated with Chry (25 µmol/L) and VP-16 (10, 20, 30, and 60 µmol/L) or Cis-Pt (5, 10, 20, and 40 µmol/L) for 48 h. At the end of the treatment, MTT assay was performed to assess the cell viability using a microplate reader (Thermo Scientific Multiskan GO, Finland).

2.4. Mitochondrial uptake assay of Chry

HepG2 cells (5 × 10\(^4\) cells/dish) were cultured on coverslips and treated with Chry (50 µmol/L) for 15 min followed by staining with 100 nmol/L Mito-Tracker deep red for another 15 min. Then the cells were washed three times with phosphate-buffered saline (PBS, pH = 7.4) and photographed using a confocal microscope (FV 1000MP, Olympus, Japan) by argon laser at 488 nm and helium–neon laser at 633 nm. The colocalization coefficient is analyzed by the software along with the confocal microscope.

2.5. Detection of ΔΨm, ATP, superoxide anion O\(_2^–\) and GSH/GSSG

After incubation of HepG2 cells (5 × 10\(^4\) cells/well) with Chry (0, 50, 100 µmol/L) for 6, 12, 24, 48 h, the cells were washed with PBS. For ΔΨm assay, 100 µL of fresh medium containing 200 nmol/L tetramethylrhodamine ethyl ester (TMRE) was added to each well and incubated for another 30 min at 37 °C. Cells were then washed with PBS three times. For ATP assay, it was detected by ATP Assay Kit following the manufacturer’s instruction. For superoxide anion O\(_2^–\) assay, 50 µL of 0.2 µmol/L MitoSOX probe working solution was added and incubation continued for another 45 min. Cells were then rinsed with PBS and the fluorescence intensity was determined by a microplate reader (Varioskan Flash, Thermo scientific, Finland) at 510 nm excitation and 580 nm emission. For GSH/GSSG assay, the assay was performed using GSH and GSSG Assay Kit according to the manufacturer’s instructions.

2.6. Determination of apoptosis and/or necrosis

After the cells were pretreated with CsA (1 µmol/L) for 1 h, Chry (0, 50, 100 µmol/L) was added and incubation continued for another 48 h. The percentages of apoptotic cells were determined by the AnnexinV-FITC/PI kit using FACS Canto flow cytometer (Canto, Becton Dickinson, USA). For Hoechst–PI double staining assay, cells were stained with 5 µL Hoechst 33,342 and 5 µL propidium iodide (PI) for 25 min at 4 °C. After staining, cells were washed by PBS and examined by fluorescence microscopy (Germany Leica).

2.7. Western blots

After treatment, mitochondrial fractions were isolated. Equal amounts of denatured proteins (20–40 mg) were separated by 12% SDS PAGE followed by electroblotting onto polyvinylidene difluoride membranes. Targeted proteins were detected with specific primary antibodies. Corresponding secondary antibodies were then utilized and immunoreactive bands were visualized by fully automatic chemiluminescence analysis system (Tanon, Shanghai, China).

2.8. Molecular docking between chrysophanol and CypD

The 3D structure of chrysophanol was drawn in SYBYL 6.9 software (SYBYL, Version 6.9, Tripos Inc., St. Louis, MO). Partial atomic...
charges were calculated using the Tripos force field with a convergence criterion of 0.01 kcal/mol. Then, the CDOCKER module of Discovery Studio 2.5 software was used to dock chrysophanol with CypD (PDB code: 2BIT) (Discovery Studio version 2.5, Accelrys Inc., CA, 2009). We defined the binding site sphere through receptor cavities, and set the radius of the sphere to 7 Å (Liu et al., 2015).

2.9. Statistical analysis

The data are expressed as the means ± SD of at least three independent experiments. Statistical differences between two groups were measured by one-way factorial analysis of variance (ANOVA), using Duncan’s post-hoc test (SPSS 19.0). P < 0.05 versus control was used as the criterion for statistical significance.

3. Results

3.1. Uptake of Chry in mitochondria

Having a planar polycyclic aromatic system, Chry displays the inherent fluorescence with \( \lambda_{ex} = 445 \text{ nm} \), and \( \lambda_{em} = 525 \text{ nm} \) (Fig. 1A). Thus, we mapped its cellular localization in HepG2 cells by laser confocal microscopy (Fig. 1B). Upon treatment with Chry, the cells exhibited bright green fluorescence in discrete subcellular distribution (A), likely to be mitochondria around the nucleus. In order to test this assumption, a recognized method for mitochondrial imaging by MTDNR-staining was performed (B). The merging of A and B presented a clear yellow colocalization signal (C) with a colocalization coefficient of 0.591, confirming that Chry could permeate into mitochondria and localize in and around mitochondria.

3.2. Induction of mitochondrial dysfunction and cell death

As expected, Chry decreased \( \Delta \Psi_m \) and ATP levels in dose- and time-dependent manner (Fig. 2A and B). Simultaneously, Chry-promoted \( \text{O}_2^- \) generation in mitochondria remained significantly higher than the controls. The maximum \( \text{O}_2^- \) production was triggered after exposure of the cells to 100 μmol/L Chry for 6 h (Fig. 2C). Due to the overproduction of reactive oxygen species (ROS), cellular GSH was oxidized to GSSG immediately. Hence, the ratio of GSH/GSSG was decreased in a dose- and time-dependent manner (Fig. 2D), showing an occurrence of redox imbalance. Next, it has reported that Chry triggered necrotic signaling in human hepatoma cells, such as J5 cells (Lu et al., 2010) and H3B cells (Ni et al., 2012). Herein, we also found that Chry killed HepG2 cells predominantly through the induction of necrosis. The IC\(_{50}\) value from the curve of cell viability was around \( 74.5 \pm 4.65 \) μmol/L (Fig. 2E). However, Chry did not show significant cytotoxicity toward normal liver cells L02 at 100 μmol/L after 48 h. The images of Hoechst/PI staining showed that Chry primarily induced cellular necrosis, appearing red and purple cells (Fig. 2F).

3.3. Involvement of CypD in cell death

Further, in order to determine whether CypD was involved in Chry-evoked cell death, CsA as CypD inhibitor was employed. Inclusion of CsA moderately attenuated cell death (Fig. 3A). On the other hand, Chry increased the expression of CypD in a dose-dependent manner (Fig. 3B), while CsA obviously attenuated the Chry-evoked protein expression of CypD (Fig. 3B). Besides, CypD is sensitive to redox conditions and oxidized CypD induces the opening of mPTP and leads to cell death (Linard et al., 2009). Then, we explored the function of antioxidant N-acetyl-L-cysteine (NAC) on CypD expression. Owing to exposure of the cells to 100 μmol/L Chry for 6 h led to a maximum production of ROS, we performed this condition for following assay. Pretreatment of the cells with NAC remarkably suppressed the CypD level (Fig. 3C), indicating that ROS participated in the relevant role of CypD. It was also found that NAC significantly inhibited Chry-induced cell death (data not shown). Finally, the molecular docking was used to evaluate whether Chry could bind to the active sites of CypD. Chry formed two hydrogen bonds with Gln-63 and Arg-82 respectively, including the binding affinity value of –11.94 kcal/mol (Fig. 4). Because of these hydrogen bonds, the effective interaction between Chry and CypD is relatively strong.

3.4. Involvement of mtDNA in cell death

Due to uptake of Chry by mitochondria, we determined whether mtDNA was also involved in cell death. The mtDNA-deficient HepG2 cells (termed p0 cells) was used. Obviously, HepG2-p0 cells were much resistant to the growth suppressive induction after exposure to Chry, suggesting that mtDNA damage was at least in part involved in the necrotic mechanism of action of Chry (Fig. 5).

3.5. Combination effects of Chry with VP-16- or Cis-Pt

Due to weak antiproliferative activity of Chry, we sought to investigate whether Chry could sensitize HepG2 cells to the antinecancer effect of VP-16 or Cis-Pt. VP-16 and Cis-Pt are two of the first-line chemotherapeutic drugs. First, the combination effects of VP-16 or Cis-Pt with Chry on cell viability were evaluated using MTT assay. As shown in Fig. 6A, Chry at 25 μmol/L alone showed no inhibitory activity; Chry at the above dose in combination with VP-16 decreased the cell viability more intensely relative to cell viabil-

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**Fig. 1.** Detection of Chry in mitochondria of HepG2 cells by laser confocal microscopy. (A) The chemical structure of Chry. (B) Fluorescent imaging of mitochondria. The cells were incubated with 50 μmol/L of Chry for 15 min, and then stained with MTDNR (an exclusive dye for mitochondria); for another 15 min.
ity only affected by VP-16 alone, showing the occurrence of synergistic effect. To our surprise, concomitant treatment of cells with Cis-Pt and Chry led to the higher cell viability than treatment with Cis-Pt alone, suggesting an antagonism effect existing between Chry and Cis-Pt. In order to confirm the combination effects, their effects on $\Delta \Psi m$ were evaluated. From Fig. 6B, VP-16 combined with Chry markedly collapsed $\Delta \Psi m$ relative to VP-16 alone; however, the decrease of $\Delta \Psi m$ was much less for Cis-Pt combined with Chry than that for Cis-Pt alone. Thus, the same conclusion was obtained from $\Delta \Psi m$ assay.

4. Discussion

Rhubarb is a well-known folk herbal medicine and has been widely used for treating gastrointestinal disease, acute hepatitis, blood diseases, chronic renal failure, diabetes, and especially, constipation due to its effective laxative activity (Cao et al., 2017; Zheng et al., 2013). In rhubarb, anthraquinones, including rhein, aloe-emodin, Chry, emodin, physcion and their glycosides are thought to be the major active components. These five anthraquinones may applied as lead compounds for the development of future drugs. Here, we observed that Chry mainly distributed in mitochondria of HepG2 cells analyzed by its inherent fluorescence (Fig. 1B). Next, anticancer drugs with anthraquinones skeleton, such as doxorubicin, mitoxantrone and daunorubicin act partly through induction of ROS, because quinones could be activated to the semiquinone radical intermediate, which in turn could react with oxygen to produce ROS. Once the level of cellular ROS over threshold, it will attack DNA strand, destroy unsaturated lipids, proteins and other macromolecules resulting in cell damage. On the other hand, semiquinone radicals were also found to equally contribute to cytotoxicity (Malik & Müller, 2016). We found that Chry enhanced $O_2^-$ accumulations within mitochondria as determined by its inherent fluorescence (Fig. 1B). Next, anticancer drugs with anthraquinones skeleton, such as doxorubicin, mitoxantrone and daunorubicin act partly through induction of ROS, because quinones could be activated to the semiquinone radical intermediate, which in turn could react with oxygen to produce ROS. Once the level of cellular ROS over threshold, it will attack DNA strand, destroy unsaturated lipids, proteins and other macromolecules resulting in cell damage. On the other hand, semiquinone radicals were also found to equally contribute to cytotoxicity (Malik & Müller, 2016). We found that Chry enhanced $O_2^-$ accumulation within mitochondria, consequently causing intracellular redox imbalance as determined by the level of GSH and GSSG (Fig. 2 C and D). Moreover, induction

Fig. 2. Effects of Chry on mitochondria function and cell viability toward HepG2 cells. (A) Determination of $\Delta \Psi m$ by TMRE probe. (B) Determination of ATP by ATP Assay Kit. (C) Determination of $O_2^-$ production by MitoSOX probe. (D) Determination of GSH/GSSG by GSH/GSSG Assay Kit. The concentrations and the action time were indicated on the figures. (E) Cell viability for 48 h was determined by MTT assay. (F) Analysis of cell death by nuclear condensation using Hoechst/PI staining. Scale bars of fluorescence images: 20 μmol/L. The values are expressed as the means ± SD; n = 3, *P < 0.05 vs control.

Fig. 3. Effects of CsA on Chry-induced cell death and CypD expression, including the effects of NAC on CypD expression. (A) Analysis of cell viability after 48 h. (B&C) Analysis of CypD level in the presence of CsA (1 μmol/L) or NAC (5 mmol/L) by Western blots. GAPDH was used as internal control. The values are expressed as the means ± SD; n = 3, *P < 0.05 vs control.
of diminished level of ATP, collapse of \( \Delta \Psi_m \) together with necrosis (Fig. 2) were also observed. Hence, we concluded that mitochondria could act as a primary cellular site for enrichment of Chry which induced mitochondria-mediated cell death.

Being an integral constituent of mPTP, CypD plays a decisive role in opening of mPTP which induce execution of cell death. The immunosuppressant CsA is the CypD ligand that has been available for studying the role of CypD in mitochondria-mediated cell death (Li et al., 2004; Gutiérrez-Aguilar & Baines, 2015). Here, we proposed CypD as a target of Chry in mitochondria and demonstrated Chry-induced necrosis through a previously uncharacterized mechanism by regulating this protein. First, CsA effectively attenuated both necrosis percentages and CypD expression triggered by Chry in dose-dependent manner, suggesting that the process of necrosis was closely associated with enhancement of CypD expression. Second, it has reported that oxidation of CypD has an impact on its conformation and activity, facilitating cell death (Linard et al., 2009). We found that antioxidant NAC almost completely inhibited CypD expression, suggesting that ROS production caused by Chry might participate in upregulation of CypD expression. Third, in CypD-Chry complex: Residues of two amino acids (Gln-63, Arg-82) are involved in H-bond interactions based on molecule docking procedure, showing an effective combination between Chry with CypD. On the other hand, promotion of DNA strand scission and alkylation, intercalation into DNA, inhibition on DNA topoisomerase I and II are recognized as the prominent mechanisms underlying anticancer activity of anthraquinones (Malik & Müller, 2016). Here, we found that involvement of mtDNA damage to the cytotoxicity of Chry was demonstrated by mtDNA-deficient HepG2-p0 cells. HepG2-p0 cells were more resistant than HepG2 cells to the growth suppressive induction after exposure to Chry (Fig. 5). Taken together, our data suggested that Chry might target CypD and mtDNA in HepG2 cells to induce cell death.

In principle, the biological significances of targeting mitochondria and its subunits, such as CypD and mtDNA in cancer cells are at least as following. First, mitochondria is considered as a drug target which has manifested the clinical effectiveness in combating relapsed or refractory cancer (Xia et al., 2019). More importantly, as mitochondria is a control center of cell death and an energy factory for ATP production, the drugs targeting mitochondria can directly perturb its function and therefore bypass multidrug resistance (Wang et al., 2018, 2020). Second, CypD regulates the formation of mPTP, leading to overproduction of ROS and alteration in cellular redox states. Meanwhile, CypD is also oxidized by ROS and oxidized CypD in turn promotes the opening of mPTP. Bigi et al. found that oncogenic Ras might enhance CypD protein through Raf-1/MEK/ERK pathway which has a deterministic role in tumor progression (Bigi et al., 2016). Third, the damage to mtDNA may elicit dysfunction of electron transport chain which will lead to an increase in mitochondria-generated ROS (Fliss, 2013).
ROS are involved in cell death by imposing an oxidative stress. We believe that all these factors contribute to the cellular function of Chry. However, Chry displayed the weak antiproliferative activity against HepG2 cells with the IC50 around 74.5 μmol/L. Then, we assumed whether Chry could be used as an ancillary drug for cancer treatment. Here, we found that Chry combined with VP-16 showed the synergistic effects on inhibition of cell growth and depolarization of ΔΨm, while Chry with Cis-Pt showing the antagonism effects (Fig. 6). As we known, it has reported that Chry could be inserted into the base pair of double-stranded DNA and damage DNA (Yang et al., 2012). Cis-Pt as a DNA crosslinking agent, its DNA binding mode is sensitive to DNA conformation (Bubley et al., 1996). We hypothesized that Chry could influence DNA topology which partly prevented the DNA crosslinks by Cis-Pt. For example, Daunorubicin (Dauno) is also an anthraquinone (9,10-dioxoanthracenes), binding to DNA and inhibiting topoisomerase II (Gewirtz, 1999). Recently, it has reported that Dauno at low concentration elicited the antagonism with Cis-Pt through the decrease of interstrand crosslinks of Cis-Pt and the diminished Dauno uptake in the presence of Cis-Pt (Erfaneh, Thibaut, László, Péter, Szabolcs, Zsolt, Frank, & Gábor, 2020). Hopefully, the antagonism mechanism of Dauno combined with Cis-Pt might help us to speculate antagonism of Chy with Cis-Pt.

5. Conclusion

In summary, we have discovered mitochondria as a main location of Chry in HepG2 cells and reported that Chry induced necrotic cell death at least partly through CypD upregulation and mtDNA damage. The observations of the combination effects of Chry with VP-16 or Cis-Pt provided a deep insight into understanding the anticancer action of Chry in vivo.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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