Published in final edited form as:

Mol Nutr Food Res. 2010 July ; 54(7): 967–976. doi:10.1002/mnfr.200900265.

Chrysophanol induces necrosis through the production of ROS and alteration of ATP levels in J5 human liver cancer cells

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Abstract

Anthraquinone compounds have been shown to induce apoptosis in different cancer cell types. Effects of chrysophanol, an anthraquinone compound, on cancer cell death have not been well-studied. The goal of this study was to examine if chrysophanol had cytotoxic effects and if such effects involved apoptosis or necrosis in J5 human liver cancer cells. Chrysophanol induced necrosis in J5 cells in a dose- and time-dependent manner. Non-apoptotic cell death was induced by chrysophanol in J5 cells and was characterized by caspase independence, delayed externalization of phosphatidylserine and plasma membrane disruption. Blockage of apoptotic induction by a general caspase inhibitor (z-VAD-fmk) failed to protect cells against chrysophanol-induced cell death. The levels of ROS production and loss of mitochondrial membrane potential ($\Delta\Psi_m$) were also determined to assess the effects of chrysophanol. However, reductions in ATP levels and increases in LDH activity indicated that chrysophanol stimulated necrotic cell death. In summary, human liver cancer cells treated with chrysophanol exhibited a cellular pattern associated with necrosis and not apoptosis.

Keywords

Chrysophanol; necrosis; ROS; ATP; mitochondrial; J5 human liver cancer cells

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The authors have declared no conflict of interest.

1 Introduction

Cell death can be divided into necrosis, apoptosis and autophagy [1]. Apoptosis, a programmed cell death, is a highly regulated process which is associated with several molecular events involving receptors, pro-apoptotic/anti-apoptotic proteins and signaling pathways leading to DNA fragmentation and eventually cell death [2]. On the other hand, necrotic cell death is dissimilar to apoptosis and does not include characteristics like chromatin condensation, DNA laddering and cell membrane blebbing. Furthermore, necrosis is not caspase-dependent and it is usually triggered by pathophysiological conditions such as inflammation, infection or ischemia [3]. Nonapoptotic machinery or necrotic cell death may be useful in cancer therapy.

Liver cancer is one of the major causes of malignancy-related deaths worldwide, and its incidence is on the rise [4]. Typical treatment approaches to liver cancer include surgery, radiotherapy, chemotherapy and transplantation [5] but cure rates are not satisfactory. Currently, investigators are focused on new agents and novel targets for liver cancer treatment. Chrysophanol (1, 8-dihydroxy-3-methyl-anthraquinone), a member of the anthraquinone family, is one of the components of a Chinese herb including *Rheum officinale* (rhubarb, Chinese name Da Huang) and *Polygonum cuspidatum* [6,7]. It was reported that chrysophanol did not induce cytotoxic effects including apoptosis in rat hepatocytes [8] and human leukemia HL-60 cells [9]. Other studies found that anthraquinone compounds including emodin (1,3,8-trihydroxy-6-methylanthraquinone), aloe-emodin (1,8-dihydroxy-3-hydroxyl-methyl anthraquinone) and rhein (1,8-dihydroxy-3-carboxyanthraquinone) induced apoptosis *in vitro* and *in vivo* [10–12]. However, there are no reports addressing effects of chrysophanol on necrosis or apoptosis in human liver tumor cells. The overall aim of this study was to determine if chrysophanol was cytotoxic in human liver cancer cells and the roles of necrosis and apoptosis in cell death.

2 Materials and Methods

2.1 Chemicals and reagents

Chrysophanol, PI and NAC were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Necrosis inhibitor (IM-54) was purchased from Merck & Co., Inc. (Whitehouse Station, NJ, USA). DCFH-DA, DiOC₆(3) and Indo 1/AM were purchased from Molecular Probes (Invitrogen Corp., Carlsbad, CA, USA). Dulbecco's Modified Eagle's Medium (DMEM), penicillin-streptomycin, trypsin-EDTA, fetal bovine serum (FBS) and L-glutamine were obtained from GIBCO BRL (Invitrogen Corp., Carlsbad, CA, USA). Caspase-3 activity assay kit (PhiPhiLux^R-G₁D₂) was bought from OncoImmunin, Inc. (Gaithersburg, MD, USA). ATP detection kit was purchased from Luminescence ATP Detection Assay by ATPliteTM kit (PerkinElmer, Waltham, MA, USA). LDH detection kit was obtained from LDH-Cytotoxicity Assay Kit (MBL, Nagoya, Japan). The general caspase inhibitor (z-VAD-fmk) was from R&D systems (Minneapolis, MN, USA).

2.2 Human liver cancer cell line (J5)

The J5 human liver cancer cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured and plated onto 75 cm² cell culture flasks and grown at 37°C (humidified 5% CO₂ and 95% air at one atmosphere) in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 Units/ml penicillin and 100 µg/ml streptomycin.

2.3 Morphological changes and viability assessed by phase-contrast microscopy and flow cytometry

Different concentrations (0, 25, 50, 75, 100 and 120 μ M) of chrysophanol or 1% DMSO as a solvent control were added to the cells. Cells were grown and incubated for indicated time periods. A phase-contrast microscope was used to determine morphological changes and a flow cytometry assay (Becton Dickinson FACS Calibur) was used to determine cell viability as previously described [13].

2.4 Flow cytometric analysis of cell death by Annexin V/PI double staining

Cells were incubated with 120 μ M chrysophanol for 0, 3, 6, 12, 16, 20 and 24 h. Cells were then trypsinized and harvested by centrifugation before incubation with Annexin V and PI for 15 min at room temperature. Necrosis was examined and rates were analyzed by flow cytometry using Annexin V-FITC/PI kit (BD PharMingen, San Diego, CA, USA). Annexin V binds to necrotic and apoptotic cells in which phosphatidylserine is exposed on the cell surface and the percentage of necrotic cells was determined [14].

2.5 Comet assay of DNA damage and DAPI staining analysis of apoptosis

Cells were treated with or without different concentrations of chrysophanol (0, 25, 50, 75, 100 and 120 μ M) for 48 h, then isolated and DNA damage determined using the Comet assay as previously described [15]. In a separate set of experiments cells were treated with different concentrations of chrysophanol for 24 h, then stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes/Invitrogen Corp., Carlsbad, CA, USA) and photographed using a fluorescence microscope as previously described [16].

2.6 ROS, cytosolic Ca^{2+} levels and $\Delta\psi_m$

Cells were incubated with chrysophanol (120 μ M) for 0, 6, 12, 24 and 48 h and changes in ROS production, cytosolic Ca^{2+} levels and $\Delta\psi_m$ were measured. In addition, cells were also pre-treated with or without the antioxidant NAC 3 h prior to treatment with 120 μ M chrysophanol to examine effects on ROS. Cells were harvested, washed twice by PBS, then were re-suspended in 500 μ l of DCFH-DA (10 μ M) for measurement of ROS levels, Indo 1/AM (3 μ g/ml) for cytosolic Ca^{2+} levels and DiOC₆(3) (4 μ mol/L) for $\Delta\psi_m$ in a dark room for 30 min at 37°C and assayed by flow cytometry as previously described [13,14,17].

2.7 Caspase-3 activity and cell death after chrysophanol treatment in the presence of a caspase inhibitor

Cells were pretreated with or without the cell permeable general caspase inhibitor (z-VAD-fmk) 3 h prior to treatment with chrysophanol (120 μ M) or DMSO alone. Caspase-3 activity and cell viability were determined using the PhiPhiLux^R-G₁D₂ (10 μ M) kit and analyzed by flow cytometry [17,18].

2.8 ATP and LDH levels

Cells were seeded in 100 μ l phenol red-free medium with a serial concentration of chrysophanol for 6 h onto 96-well white microplates and the intracellular ATP content level was measured using Luminescence ATP Detection Assay by ATPliteTM kit as previously described [19]. The resulting luminescence was monitored in a luminometer (SynergyTM HT Multi-Mode Microplate Reader, BioTek). Necrotic cell death was estimated by determining LDH released into the culture medium after a 24-h incubation with chrysophanol or DMSO as the solvent control. LDH released into the phenol red-free medium was determined using a LDH assay kit and procedures described by the manufacturer [20].

2.9 Cytochrome c release determined by confocal laser microscopy

Cells in 4-well chamber slides were treated with 120 μ M chrysophanol for 24 h and immunofluorescence staining was performed as previously described [21,22]. Slides were then incubated with anti-human cytochrome *c* antibody (1:100 dilution; Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) overnight and then exposed to the secondary antibody (FITC-conjugated goat anti-mouse IgG at 1:100 dilution; Santa Cruz), followed by mitochondrial counterstaining with Rhodamine 123 (Molecular Probes/Invitrogen Corp.). Photomicrographs were obtained using a Leica TCS SP2 Confocal Spectral Microscope.

2.10 Effects of chrysophanol on protein levels associated with cell survival

Protein levels were determined by Western blotting analysis [23,24]. Briefly, total proteins were collected and prepared from cells treated with or without chrysophanol (120 μ M) for 0, 6, 12, 24, 48, 72 h. The relative intensities of protein bands (levels of cytochrome *c*, Bax, AIF, Endo G, Apaf-1, caspase-3, -8, -9, -12, SOD (Cu/Zn), SOD (Mn), catalase and GST proteins)(Santa Cruz Biotechnology, Inc.) were analyzed using the ImageMaster 1D Elite v 4.00 densitometric analysis program (Amersham Biosciences).

2.11 Real time PCR of caspase-3 and AIF mRNA levels

Total RNA was extracted from cells after treatment with 120 μ M chrysophanol for 0, 24 and 48 h, using the Qiagen RNeasy Mini Kit (Qiagen, Inc, Valencia, CA, USA). Total RNA was reverse-transcribed with High Capacity cDNA Reverse Transcription Kit according to the standard protocol of the supplier (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was performed with the forward and reverse primers (caspase-3: F- CAGTGGAGGCCGACTTCTTG, R-TGGCACAAAGCGACTGGAT; AIF: F- GGGAGGACTACGGCAAAGGT, R-CTTCCTTGCTATTGGCATTTCG; GAPDH: F- ACACCCACTCCTCCACCTTT, R-TAGCCAAATTCGTTGTCATACC). Each assay was run on an Applied Biosystems 7300 Real-Time PCR system as previously described [23,25].

2.12 A necrosis inhibitor increases viability of chrysophanol-treated cells

Cells pre-treated with 10 μ M of IM-54 (Necrosis inhibitor, 2-(1H-Indol-3-yl)-3-pentylamino-maleimide) for 2 h followed by treatment with 120 μ M chrysophanol and 1% DMSO as a control. Cells were then harvested after a 24-h incubation and the percentage of viable cells was determined as previously described [26].

2.13 Densitometry and statistical analysis

All data were expressed as mean $\pm$ S.D. of at least three separate experiments. Statistical calculations of the data were performed using an unpaired Student's *t*-test. Statistical significance was set at **p*<0.05, ***p*<0.01 and ****p*<0.001.

3 Results

3.1 Chrysophanol effects on cell morphology and viability

Chrysophanol induced morphological changes and cell death as shown in Figure 1A and B. The results demonstrated that chrysophanol caused necrotic morphological changes and decreased the percentage of viable cells and these effects were dose- and time-dependent. In normal smooth muscle A10 cells, chrysophanol had no significant effect on cell viability (Supporting Information Fig. S1).

3.2 Chrysophanol increases necrotic cell death

Chrysophanol did not significantly alter cell cycle arrest at concentrations of 25, 50, and 75 μM when incubated for 48 h. However, cells exposed to higher concentrations (100 and 120 μM) for 48 h displayed S phase arrest and cell death (data not shown). Annexin V/PI assay examination indicated that cell death was due to necrosis (Fig. 2A and B) as the occurrence of a sub-G1 (apoptosis) population was not observed. These data are comparable with data in Figure 1 showing that chrysophanol decreased the percentage of viable cells and induced necrotic morphological changes.

3.3 Chrysophanol-induced non-apoptotic cell death and DNA damage

To further investigate whether the effect of chrysophanol on cell death and DNA damage was non-apoptotic, chromosome and DNA damage were examined by DAPI staining and Comet assay, respectively. The results indicated that at all chrysophanol concentrations DNA condensation (a kind of apoptotic character) did not increase and there was no effect on fluorescence intensity (Fig. 3A). Moreover, chrysophanol also induced DNA damage (Fig. 3B) in J5 cells after treatment. These effects were dose-dependent responses.

3.4 Chrysophanol altered ROS production, cytosolic Ca^{2+} levels and $\Delta\Psi_m$

Chrysophanol increased ROS production and cytosolic Ca^{2+} levels and reduced $\Delta\Psi_m$ as shown in Table 1. The antioxidant NAC decreased the chrysophanol-induced ROS production (Fig. 4A) and the percentage of viable cells was increased 18.2% in the presence of 20 mM NAC (Fig. 4B).

3.5 Effects of the caspase inhibitor (z-VAD-fmk) on chrysophanol-affected caspase-3 activity, mRNA gene expression and cell death

Chrysophanol did not stimulate caspase-3 activity (Fig. 5A). Real time PCR also showed that chrysophanol did not promote the expression of *caspase-3* mRNA (Fig. 5B) or another important apoptotic factor *AIF* mRNA as compared with control cells (Fig. 5B). Pretreatment of cells with the caspase inhibitor z-VAD-fmk did not increase the percentage of viable cells compared with cells treated with chrysophanol alone (Fig. 5C).

3.6 Chrysophanol alters ATP level, LDH activity and survival of cells pre-treated with a necrosis inhibitor

Chrysophanol decreased ATP levels (Fig. 6A) around 56–96% (** $p < 0.01$ and *** $p < 0.001$) but promoted LDH activity (Fig. 6B) about 377–516% (** $p < 0.01$ and *** $p < 0.001$). Pre-treatment with the necrosis inhibitor IM-54 inhibited the effects of chrysophanol. The percentage of viable cells was increased with IM-54 pretreatment compared with chrysophanol treatment alone (Fig. 6C).

3.7 Effects of chrysophanol on necrosis- and apoptosis-associated protein levels and cytochrome c levels

It can be seen in Figure 7A, B and C that chrysophanol decreased protein levels of AIF, Endo G, Apaf-1, Caspase-3, Caspase-8, Caspase-9 and Caspase-12 but promoted levels of cytochrome *c*, Bax, SOD (Cu/Zn), SOD (Mn), catalase and GST. Chrysophanol induced release of cytochrome *c* from mitochondria when compared to control cells as seen in Figure 8. These results are in agreement with other reports [27–29]. Even though in necrotic cells the cytochrome *c* also will be released from mitochondria, due to the decrease in total ATP cytochrome *c* can not interact with Apaf-1 and pro-caspase-9 to form apoptosome; thus the caspase cascade could not be activated for apoptotic occurrences.

4 Discussion

It is well documented that cell death can be divided into apoptosis and necrosis [30]. Most chemotherapy drugs induce apoptosis but some drugs can cause necrosis [14,31]. Necrosis is an irreversible inflammatory form of cell death, and it also known that therapy induced necrotic cell death initiates an immune response to tumor cells. However, whether or not the inflammation associated with necrosis is still unclear but therapeutics that target regulators of necrotic cell death are already in early-phase clinical trials [32]. It was reported that a cell can initiate its own demise by necrosis that initiates both inflammatory and/or reparative responses in the host [33].

The plant rhubarb (Da Huang) has been used as a traditional Chinese medicine for centuries. The major anthraquinones in rhubarb, *Rheum palmatum* including emodin, aloe-emodin, chrysophanol, danthron, physcion and rhein [6] have been shown with the exception of chrysophanol to induce apoptosis in animal and human cancer cells including human liver cancer cell lines [10,34–36]. Chrysophanol can induce cell death which may not involve apoptosis. There is no available information on effects of chrysophanol in human liver cancer cells. Therefore, we investigated the induction of cell death in human liver cancer J5 cells after exposure to chrysophanol. Results of this study indicated that chrysophanol not only induced necrotic cell death but also promoted S phase cell cycle arrest (Supporting Information Fig. S2).

Chrysophanol induced necrotic morphological changes in cells and decreased cell viability in a dose- and time-dependent manner. However, it did not induce cell death in human normal smooth muscle A10 cells. Chrysophanol also induced non-apoptotic cell death and DNA damage based on DAPI staining and Comet assay indicating that chrysophanol induced necrosis rather than apoptosis. In addition, caspase-3 activation did not differ between control and chrysophanol-treated groups. Caspase-3 activation is an important contributor to apoptosis [37,38]. Chrysophanol did not alter levels of proteins associated with apoptosis.

ROS production can cause cell death including involvement of necrosis [39,40]. Chrysophanol promoted the amounts of ROS and cytosolic Ca^{2+} and decreased $\Delta\Psi_m$ levels. Chrysophanol-induced ROS generation was reduced after pretreatment with the antioxidant NAC which increased the percentage of viable cells suggesting that chrysophanol-induced necrotic cell death is associated with ROS production. We also found that chrysophanol increased cytosolic Ca^{2+} levels but when cells were pre-treated with BAPTA (Ca^{2+} chelator) there was a reduction in cytosolic Ca^{2+} levels but the percentage of viable cells was not significantly increased (Supporting Information Fig. S3). Cell death due to necrosis is associated with decreases in $\Delta\Psi_m$ [28,41,42] and ATP levels [27,28,43] and we observed that chrysophanol did indeed reduce $\Delta\Psi_m$ and ATP levels. It has been shown that necrotic cell death involved the release of LDH [20,44]. In the present study, chrysophanol increased LDH levels. Our findings are in agreement with other reports [27,43] showing ROS production, loss of $\Delta\Psi_m$ and ATP depletion in cancer cells and that those cells undergo necrosis (necrotic cell death).

In summary, chrysophanol is shown to be a new molecular inducer of necrotic cell death which may have an application as a new drug candidate. A model of the mechanisms of chrysophanol-induced cell death is presented in Figure 9. We propose that chrysophanol stimulates ROS production, DNA damage, mitochondrial dysfunction, loss of ATP and promotion of LDH activity which result in cell necrosis.

Acknowledgments

This research was supported by Grants NSC 94-2745-B-039-002-URD and NSC 95-2745-B-039-002-URD from the National Science Council, Taiwan and NIH grants AG-23524 and AG-18357.

Abbreviations

AIF	apoptosis-inducing factor
ATP	adenosine triphosphate
$\Delta\Psi_m$	mitochondrial membrane potential
DCFH-DA	2,7-dichlorodihydrofluorescein diacetate
DiOC₆(3)	3,3'-dihexyloxacarbocyanine iodide
FITC	fluorescein isothiocyanate
LDH	lactate dehydrogenase
NAC	<i>N</i> -acetyl-cysteine
PI	propidium iodide
ROS	reactive oxygen species

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Figure 1.

Chrysophanol affected on cell morphology and percentage of viable cells in J5 human liver cancer cells. Cells were examined and photographed by phase-contrast microscopy (200x) (A) and for percentage of viable cells (B). Cells were cultured with various concentrations of chrysophanol for 24, 48 and 72 h. Each point is mean $\pm$ S.D. of three experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Significantly different from the control.

Figure 2.

Chrysophanol stimulated the necrotic cell death in J5 cells. Cells were exposed to chrysophanol for indicated time periods and the necrotic cells were determined (A and B) by flow cytometry. Data represents mean $\pm$ S.D. of three experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Significantly different from the control.

Figure 3.

Chrysophanol-induced non-apoptotic cell death and DNA damage in J5 human liver cancer cells. Cells were incubated with various concentrations of chrysophanol for 24 or 48 h. DNA condensation was determined by DAPI staining (A) (200x); FI: fluorescence intensity (folds of control). DNA damage was detected by Comet assay (B) (200x).

Figure 4. Effects of NAC (ROS scavenger) on chrysophanol-induced the production of ROS and viable cells in J5 cells. Cells were pre-treated with NAC then treated with 120 μ M chrysophanol before cells were harvested for ROS (A) and viable cells (B) determinations. Data represent mean $\pm$ S.D. of three experiments. * $p < 0.05$; ** $p < 0.001$. Significantly different from the control.

Figure 5.

Effects of the z-VAD-fmk on chrysophanol-induced caspase-3 activity, *caspase-3* and *AIF* gene expressions and cell death in J5 cells. Cells were pre-treated with z-VAD-fmk for 3 h then treated with or without 120 μM chrysophanol before cells were harvested for caspase-3 activity and cell death determinations. Caspase-3 activity (A); *caspase-3* and *AIF* mRNA expressions (B); cell viability after z-VAD-fmk treatment (C). Data shown represent the mean ± S.D. of three independent experiments. N.S.= Not significant.

Figure 6.

Chrysophanol affected the levels of ATP, LDH activity and cell survival in J5 cells. Cells were at various concentrations with chrysophanol at 0, 10, 25, 50, 75, 100 and 120 μM for 24 h then the total levels of ATP (A) and LDH (B) and Viable cells after treatment with IM-54 (C) were prepared and determined. Data represent mean ± S.D. of three experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Significantly different from the control.

Figure 7.

Representative Western blotting showing changes in the levels of associated proteins in necrotic cell death of J5 cells after exposure to chrysophanol. Cells were at various conditions with chrysophanol at 120 μ M for 0, 6, 12, 24, 48 and 72 h then the total protein were prepared and determined. The levels of associated proteins expressions (A: Cytochrome *c*, Bax, AIF, Endo G and Apaf-1; B: Caspase-3, Caspase-8, Caspase-9, Caspase-12; C: SOD (Cu/Zn), SOD (Mn), Catalase and GST) were estimated by Western blotting, as described in Materials and Methods.

Figure 8.

Effects of chrysophanol on cytochrome *c* release from mitochondria in J5 cells. Cells were incubated with chrysophanol at 120 μM for 24 h. Cells were stained with cytochrome *c* and FITC-labeled secondary antibodies were used (green fluorescence) and the proteins were detected by a confocal laser microscopic system. The mitochondrial was stained by Rhodamine 123 (red fluorescence). Areas of colocalization between cytochrome *c*, expressions and cytoplasm in the merged panels are yellow. Scale bar, 40 μm .

Figure 9.
The schematic proposed signaling pathways of chrysophanol-induced necrotic cell death in J5 human liver cancer cells.

Table 1

Flow cytometric analysis of ROS, $\Delta\Psi_m$ and cytosolic Ca^{2+} levels with or without chrysohanol (120 μM) treatment in J5 cells

Chrysophanol Time of incubation (h)	ROS	Ca^{2+}	$\Delta\Psi_m$
	(% of control)		
0	2.1 $\pm$ 0.5	5.7 $\pm$ 0.3	98.3.0 $\pm$ 5.1
6	14.9 $\pm$ 2.5 *	13.7 $\pm$ 1.8 *	65.7 $\pm$ 8.3 ***
12	18.1 $\pm$ 4.8 **	32.4 $\pm$ 13.9 **	68.2 $\pm$ 11.1 ***
24	30.7 $\pm$ 2.4 **	95.1 $\pm$ 3.7 ***	39.8 $\pm$ 3.3 ***
48	89.0 $\pm$ 3.4 ***	91.1 $\pm$ 3.5 ***	20.3 $\pm$ 16.2 ***

Cells were exposed to chrysohanol (120 μM) for various intervals of time. The zero concentration was defined as untreated-control. The percentage of cells in ROS production, cytosolic Ca^{2+} levels and loss of $\Delta\Psi_m$ were stained by specific dyes and determined by flow cytometry as described in the Materials and Methods. Values are means $\pm$ S.D. (n=3). Groups not sharing a same letter are significantly different to 0 h by Student's *t*-test (

* $p < 0.05$,

** $p < 0.01$ and

*** $p < 0.001$).