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

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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 reactive oxygen species production and loss of mitochondrial membrane potential (ΔΨm) were also determined to assess the effects of chrysophanol. However, reductions in adenosine triphosphate levels and increases in lactate dehydrogenase 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:
Adenosine triphosphate / Chrysophanol / J5 human liver cancer cells / Necrosis / Reactive oxygen species

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 cell injury or damage to cellular components. Necrosis is a passive process that leads to cell swelling and fragmentation, while apoptosis is a programmed and cell death. Necrosis is often associated with cell necrosis, whereas apoptosis is often associated with cell death. Necrosis is often associated with cell necrosis, whereas apoptosis is often associated with cell death.
by pathophysiological conditions such as inflammation, infection or ischemia [3]. Non-apoptotic 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, 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 (1,8-dihydroxy-3-methyl-anthraquinone), 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, propidium iodide (PI) and N-acetyl-cysteine (NAC) were obtained from Sigma Chemical (St. Louis, MO, USA). Necrosis inhibitor (IM-54) was purchased from Merck (Whitehouse Station, NJ, USA). 2,7-Dichlorodihydrofluorescein diacetate, 3,3-diaminocyanine (NAC) were obtained from Sigma Chemical (St. Louis, MO, USA). Necrosis inhibitor (IM-54) was purchased from Merck (Whitehouse Station, NJ, USA). Caspase-3 activity detection kit was purchased from Luminescence ATP Detection Kit (Gaithersburg, MD, USA). Adenosine triphosphate (ATP) assay kit (Becton Dickinson FACS Calibur) was used to determine morphological changes and a flow cytometry assay (Becton Dickinson FACS Calibur) was used to determine cell viability as described previously [13].

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% fetal bovine serum, 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 µM) 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 described previously [13].

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

Cells were incubated with 120 µM 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 4,6-diamidino-2-phenylindole dihydrochloride staining analysis of apoptosis

Cells were treated with or without different concentrations of chrysophanol (0, 25, 50, 75, 100 and 120 µM) for 48 h, then isolated and DNA damage determined using the Comet assay as described previously [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) and photographed using a fluorescence microscope as described previously [16].

2.6 Reactive oxygen species, cytosolic Ca²⁺ levels and ΔΨₘ

Cells were incubated with chrysophanol (120 µM) for 0, 6, 12, 24 and 48 h and changes in reactive oxygen species (ROS) production, cytosolic Ca²⁺ levels and mitochondrial membrane potential (ΔΨₘ) were measured. In addition, cells were also pre-treated with or without the antioxidant NAC 3 h prior to treatment with 120 µM chrysophanol to examine
effects on ROS. Cells were harvested, washed twice by PBS, then were re-suspended in 500 μL of 2,7-dichlorodihydrofluorescein diacetate (10 μM) for measurement of ROS levels, Indo 1/AM (3 μg/mL) for cytosolic Ca\(^{2+}\) levels and 3,3'-dihexyloxocyanine iodide (4 μmol/L) for ΔΨ\(_{m}\) in a dark room for 30 min at 37°C and assayed by flow cytometry as described previously [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 μM) or DMSO alone. Caspase-3 activity and cell viability were determined using the PhiPhiLux\(^{TM}\)-G1,D2 (10 μM) kit and analyzed by flow cytometry [17, 18].

2.8 ATP and LDH levels

Cells were seeded in 100 μL 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 ATPlite\(^{TM}\) kit as described previously [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].

Figure 1. Chrysophanol affected cell morphology and percentage of viable cells in J5 human liver cancer cells. Cells were examined and photographed by phase-contrast microscopy (200 ×) (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 ± SD 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 ± SD of three experiments. *p < 0.05; **p < 0.01; ***p < 0.001; significantly different from the control.
2.9 Cytochrome c release determined by confocal laser microscopy

Cells in 4-well chamber slides were treated with 120 μM chrysophanol for 24 h and immunofluorescence staining was performed as described previously [21, 22]. Slides were then incubated with anti-human cytochrome c antibody (1:100 dilution; Santa Cruz Biotechnology, 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). 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

Table 1. Flow cytometric analysis of ROS, ΔΨm, and cytosolic Ca2+ levels with or without chrysophanol (120 μM) treatment in J5 cells

<table>
<thead>
<tr>
<th>Chrysophanol Time of incubation (h)</th>
<th>ROS (%) of control</th>
<th>Ca2+ (%) of control</th>
<th>ΔΨm (%) of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.1 ± 0.5</td>
<td>5.7 ± 0.3</td>
<td>98.3 ± 5.1</td>
</tr>
<tr>
<td>6</td>
<td>14.9 ± 2.5*</td>
<td>13.7 ± 1.8*</td>
<td>65.7 ± 8.3***</td>
</tr>
<tr>
<td>12</td>
<td>18.1 ± 4.8**</td>
<td>32.4 ± 13.9**</td>
<td>68.2 ± 11.1***</td>
</tr>
<tr>
<td>24</td>
<td>30.7 ± 2.4**</td>
<td>95.1 ± 3.7***</td>
<td>39.8 ± 3.3***</td>
</tr>
<tr>
<td>48</td>
<td>89.0 ± 3.4***</td>
<td>91.1 ± 3.5***</td>
<td>20.3 ± 16.2***</td>
</tr>
</tbody>
</table>

Cells were exposed to chrysophanol (120 μM) for various intervals of time. The zero hour was defined as untreated control. The percentage of cells in ROS production, cytosolic Ca2+ levels and loss of ΔΨm were stained by specific dyes and determined by flow cytometry as described in Section 2. Values are means ± SD (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).
from cells treated with or without chrysophanol (120 mM) for 0, 6, 12, 24, 48 and 72 h. The relative intensities of protein bands (levels of cytochrome c, Bax, apoptosis-inducing factor (AIF), Endo G, Apaf-1, caspase-3, -8, -9, -12, SOD (Cu/Zn), SOD (Mn), catalase and GST proteins) (Santa Cruz Biotechnology) 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 μM chrysophanol for 0, 24 and 48 h, using the Qiagen RNeasy Mini Kit (Qiagen, 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-CAGTGGAGGCGACTTCTTG, R-TGGCA-CAAAGGACTGGAT; AIF: F-GGGAGGACTACGG-CAAAGGT, R-CTTCTTGTGAATGGGATCG; GAPDH: F-ACACCCACTCTCCACCTT, R-TAGCCAAAATTCGTTGTGATAC). Each assay was run on an Applied Biosystems 7300 Real-Time PCR system as described previously [23, 25].

2.12 A necrosis inhibitor increases viability of chrysophanol-treated cells

Cells pre-treated with 10 μM of IM-54 (necrosis inhibitor, 2-{1H-Indol-3-yl}-3-pentylamino-maleimide) for 2 h followed by treatment with 120 μM 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 described previously [26].

2.13 Densitometry and statistical analysis

All data were expressed as mean ± SD 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 Figs. 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 (Figs. 2A and B) as the occurrence of a sub-G1 (apoptosis) population was not observed. These data are comparable with data in Fig. 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 alters ROS production, cytosolic Ca\(^{2+}\) levels and ΔΨ\(_m\)

Chrysophanol increased ROS production and cytosolic Ca\(^{2+}\) levels and reduced ΔΨ\(_m\) as summarized 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).

**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 or 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 ± SD 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 μM 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 Section 2.
3.7 Effects of chrysophanol on necrosis- and apoptosis-associated protein levels and cytochrome c levels

It is shown in Figs. 7A–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 with control cells as shown in Fig. 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 cannot 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, R. 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 involving involvement of necrosis [39, 40]. Chrysophanol promoted the amounts of ROS and cytosolic Ca\(^{2+}\) and decreased ΔΨ\(_{m}\) 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 ΔΨ\(_{m}\) [28, 41, 42] and ATP levels [27, 28, 43] and we observed that chrysophanol did indeed reduce ΔΨ\(_{m}\) 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 ΔΨ\(_{m}\) 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 Fig. 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.

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.

The authors have declared no conflict of interest.

5 References


