

Selected flavonoids potentiate the toxicity of cisplatin in human lung adenocarcinoma cells: A role for glutathione depletion

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Abstract. Adjuvant therapies that enhance the anti-tumor effects of *cis*-diammineplatinum(II) dichloride (cisplatin, CDDP) are actively being pursued. Growing evidence supports the involvement of mitochondrial dysfunction in the anti-cancer effect of cisplatin. We examined the potential of using selective flavonoids that are effective in depleting tumor cells of glutathione (GSH) to potentiate cisplatin-mediated cytotoxicity in human lung adenocarcinoma (A549) cells. We found that cisplatin (40 μ M, 48-h treatment) disrupts the steady-state levels of mitochondrial respiratory complex I, which correlates with elevated mitochondrial reactive oxygen species (ROS) production and cytochrome *c* release. The flavonoids, 2',5'-dihydroxychalcone (2',5'-DHC, 20 μ M) and chrysin (20 μ M) potentiated the cytotoxicity of cisplatin (20 μ M), which could be blocked by supplementation of the media with exogenous GSH (500 μ M). Both 2',5'-DHC and chrysin were more effective than the specific inhibitor of GSH synthesis, L-buthionine sulfoximine (BSO, 20 μ M), in inducing GSH depletion and potentiating the cytotoxic effect of cisplatin. These data suggest that the flavonoid-induced potentiation of cisplatin's toxicity is due, in part, to synergetic pro-oxidant effects of cisplatin by inducing mitochondrial dysfunction, and the flavonoids by depleting cellular GSH, an important antioxidant defense.

Introduction

Cisplatin and its analogs are widely used in cancer chemotherapy for the treatment of testicular and lung tumors, despite their ability to injure healthy tissues such as renal tubular and auricular epithelial cells (1). Cisplatin forms DNA adducts, yet its ability to induce programmed cell death (apoptosis) in both cancer and normal cells has been associated with

the formation of reactive oxygen species (ROS) in the mitochondria, cytochrome *c* release into the cytosol, and subsequent activation of caspases (2-8). One report suggests that cytochrome *c* release is required for cisplatin-induced apoptosis, yet a caspase 3-independent pathway has been reported as well (8,9). The mechanisms by which cisplatin induces the formation of ROS remain unclear. Inhibition of all the mitochondrial respiratory chain complexes has been reported using high concentrations of cisplatin (100 μ M) in isolated mitochondria (10). Although the formation of mitochondrial DNA (mtDNA) adducts with cisplatin has been demonstrated, the toxicological relevance of such adducts remains poorly investigated (11-13).

Glutathione (GSH) depletion as a potential strategy to sensitize cancer cells has been under investigation for over two decades (14-22). Indeed, GSH and glutathione peroxidase (GPx) play central roles in cellular homeostasis by controlling the levels of ROS, and cancer cells tend to exhibit higher levels of ROS (22,23). Several studies have associated GSH depletion with mitochondrial dysfunction, but the precise mechanism remains unclear (24). GSH depletion is commonly achieved using the inhibitor of GSH synthesis, L-buthionine sulfoximine (BSO), which has been shown to sensitize cancer cells to cisplatin treatment (19,20). However, limitations of this strategy include an inherent lack of specificity towards cancer cells and subsequent side effects (21). A potentially more tumor specific approach to deplete GSH is by using substrates of multidrug resistance proteins (MRPs), a family of ATP-binding cassette (ABC) proteins that are known GSH co-transporters and tend to be over-expressed in cancer cells (14-17,25).

Flavonoids and their precursors hydroxychalcones (HCs) are natural plant products found in high abundance in fruits and vegetables. Although flavonoids are generally viewed as antioxidants, they can also generate ROS depending on their structure and molecular environment (26). In the cell, flavonoids can produce modulatory effects through alterations of protein and lipid kinase signaling pathways (27). Moreover, a number of flavonoids may exert direct and indirect prooxidant effects by inhibiting the mitochondrial respiratory chain complexes I and II and by inducing GSH depletion through MRP1 activation (14,28-31). Most MRP1 'inhibitors' are MRP1 substrates that are competitive inhibitors of drug efflux and are co-transported with GSH, yet verapamil and some flavonoids can induce GSH depletion without being expelled from the cell (31). We recently reported that, among a series of MRP substrates,

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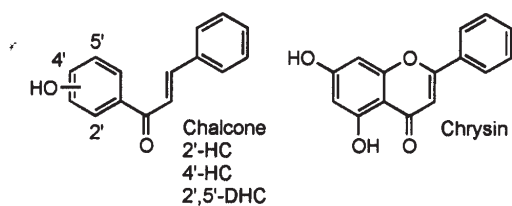


Figure 1. Molecular structures of chalcone (no hydroxyl group), 2'-hydroxychalcone (2'-HC), 4'-hydroxychalcone (4'-HC), 2',5'-dihydroxychalcone (2',5'-DHC) and chrysin.

2',5'-dihydroxychalcone (2',5'-DHC) and chrysin (Fig. 1) were the most efficient inducers of GSH depletion in several cancer cell lines (14). It is also worth noting that 2',5'-DHC, in particular, exerts interesting antiangiogenic properties (32).

In this study, we report that: i) cisplatin treatment in A549 cells disrupted the steady-state level of mitochondrial respiratory complex I, which in turn may account for some of the mitochondrial ROS formation; ii) cisplatin's cytotoxicity was potentiated in A549 cells by non-toxic concentrations of 2',5'-DHC and chrysin, and that these effects were attenuated by exogenously added GSH; and iii) the potentiation effect involved increased dysfunction of the mitochondria, as shown by the assessment of cytochrome *c* release.

Materials and methods

Chemicals and reagents. Chalcone, 2'-hydroxychalcone (2'-HC), 4'-hydroxychalcone (4'-HC), and 2',5'-dihydroxychalcone (2',5'-DHC) were purchased from Indofine Chemicals Company, Inc. (Hillsborough, NJ). Chrysin, *cis*-diammine-platinum (II) dichloride (CDDP or cisplatin), L-gluthathione (reduced), pyruvate (sodium salt), phosphoric acid, metaphosphoric acid, sodium phosphate (monobasic), Triton X-100, EDTA, NADH, K_2HPO_4 , KH_2PO_4 , HEPES, DMSO and DMF were from Sigma-Aldrich (St. Louis, MO). Tris-HCl, perchloric acid and methanol were from Fisher (Pittsburgh, PA). MitoSOX and JC-1 were from Molecular Probes (Eugene, OR). Phosphate-buffered saline (PBS) was from Cellgro (Herndon, VA). Protease inhibitor cocktail tablets supplemented with EDTA were from Roche Diagnostics (Indianapolis, IN).

Cell line and culture conditions. Human lung adenocarcinoma (A549) cells were purchased from ATCC (Manassas, VA). They were grown in Ham's F12 medium with 2 mM L-glutamine (ATCC) supplemented with 10% fetal bovine serum (FBS) and 1% pen/strep (10,000 unit, Cellgro) at 37°C and 5% CO_2 air atmosphere. T-75 or T-150 flasks were used for mitochondrial purification, and 24-well plates for flow cytometry studies, cytosolic GSH measurements, and percentage of LDH release assessment. 2',5'-DHC and chrysin were added from 10 mM stock solutions in DMF, and cisplatin from 10 mM stock solutions in H_2O /DMF (1:1).

Mitochondrial isolation. Isolation of A549 mitochondria was achieved through differential centrifugation as previously described (14). Following cisplatin and 2',5'-DHC treatments,

A549 cells were trypsinized (0.04% trypsin in Puck's EDTA), pelleted by centrifugation (2,000 x g for 10 min at 4°C), resuspended in PBS, and spun (2,000 x g for 10 min at 4°C) to yield a final cell pellet. The pellet was resuspended in 550 μ l of ice-cold hypotonic buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5). After 10 min, the cell suspension was homogenized (Kontes glass homogenizer, Fisher-Scientific, Fair Lawn, NJ). Immediately after homogenization, 400 μ l of 2.5-X mannitol-sucrose buffer was added (525 mM mannitol, 175 mM sucrose, 12.5 mM Tris-HCl, 2.5 mM EDTA, pH 7.5). Addition of 2 ml of ice-cold single-strength mannitol-sucrose buffer (210 mM mannitol, 70 mM sucrose, 5 mM Tris-HCl, 1 mM EDTA, pH 7.5) was added and contents divided into two 1.5 ml centrifuge tubes. Cellular debris was pelleted by centrifugation at 1,300 x g for 10 min at 4°C. Centrifugation was repeated twice and mitochondria from the supernatant were isolated by centrifugation at 17,000 x g for 15 min at 4°C. The mitochondrial pellet was washed and centrifuged at 17,000 x g for 15 min at 4°C to limit cytosolic contamination. Mitochondrial enrichment was determined by the relative activity of a cytosolic enzyme marker (LDH) and a mitochondrial enzyme marker (glutamate dehydrogenase, GDH) in the fractions as previously described (14).

Immunoblotting of mitochondrial respiratory complexes I and II. A549 mitochondria pellets were lysed in a ground glass homogenizer with 50 μ l of 50 mM HEPES, 0.5% Triton X-100, pH 7.0 lysis solution. After homogenization, total volume was brought to 10 μ l H_2O . Total protein concentration was measured at 595 nm on Spectra Max 340PC micro-plate reader (Molecular Devices Corp., Sunnyvale, CA) using Coomassie Plus (Pierce, Rockford, IL). PAGER[®] Gold Precast Polyacrylamide 4-20% Tris-Glycine (Cambrex Bio Science, Rockland, ME) were loaded with 10 μ g protein per well. Samples were run at 125 V for 75 min and transferred to PVDF-plus membrane (Osmonics Inc., Westborough, MA) at 100 V for 1 h. Blocking, washing, and stripping solutions were prepared as suggested by the manufacturer for optimal results with the ECL Plus Western Blot Detection Reagents Kit (Amersham Biosciences, Buckinghamshire, UK). All wash steps were performed in triplicate for 10 min in Tris-buffered-saline-Tween (TBS-T). Membranes were blocked for 1 h at room temperature in TBS-T and 10% horse serum. Complex I (0.25 μ g/ml) primary antibody (monoclonal 15 kDa antibody #A-21342; Molecular Probes, Eugene, OR) was applied for 2.5 h. Secondary antibody (peroxidase-conjugated AffiniPure goat anti-mouse IgG, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:40,000 in TBS-T, was applied for 30 min. ECL Plus Western Blot Detection Reagents were used to detect proteins. Following complex I detection membranes were submerged in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) and incubated at 55°C for 45 min with occasional agitation. Membranes were re-probed for complex II (Molecular Probes monoclonal 70 kDa antibody, #A-11142) using 0.125 μ g/ml antibody. Identical secondary antibody was diluted 1:40,000 in TBS-T.

Flow cytometry. MitoSOX is an analog of hydroethidine and has been used to detect mitochondrial superoxide by flow

cytometry (33). The results obtained with this method remain qualitative rather than quantitative (34), yet flow cytometry allows the study of specific groups of cells. The oxidation products of MitoSOX were detected using the red channel (FL2). The ratio of red/green fluorescences (FL2/FL1 channels) emitted by JC-1 following cell treatment has been widely used as a marker of mitochondrial depolarization and is based on the differential accumulation of this positively charged molecule into the mitochondria (33). Briefly, drug-treated A549 cells ($\sim 2 \times 10^5$) were exposed to 5 μ M MitoSOX or 20 μ M JC-1 (from 5 and 20 mM stock solutions in DMSO, respectively) for 20 min. The supernatant was removed and the cells scraped in 0.5 ml ice-cold PBS, centrifuged at $2,000 \times g$ for 15 min, and re-suspended in 0.5 ml ice-cold PBS. Cells were analyzed within 30 min using FACSCalibur flow cytometer (Becton-Dickinson Biosciences, San Jose, CA). The total number of cell counts was 10,000. The FL2/FL1 ratio was measured using the mean JC-1 fluorescence in each channel.

Cytosolic levels of GSH. Intracellular GSH levels were determined by HPLC-EC (35). Cultured cells from 24-well plates were washed once with 1 ml of PBS and then re-suspended in 0.5 ml of distilled water with 40 μ M digitonin (from a 2 mM stock solution in DMSO) for 30 min at room temperature. Next, 50 μ l of 10% meta-phosphoric acid was added (1% final concentration), the samples were sonicated for 2 min and centrifuged at $20,000 \times g$ for 10 min, and 0.2 ml of supernatant placed in vials for HPLC analysis. The HPLC column used was Synergi 4u Hydro-RP 80A (150x4.6 mm) from Phenomenex (Torrance, CA) and the mobile phase was sodium phosphate buffer (125 mM sodium phosphate monobasic, pH adjusted to 3 with phosphoric acid) and 0.9% methanol. The flow rate was $0.5 \text{ ml} \cdot \text{min}^{-1}$. The retention time for GSH in these conditions was 7.5 min. The HPLC instrument was from ESA, Inc. (Chelmsford, MA), equipped with an autosampler (model 540) and a Coul array detector (model 5600A). The potential applied was +0.75 V vs. H/Pd electrode, and the injection volume was 5 μ l.

Assessment of cytotoxicity. The MTT assay is commonly used to measure cancer cell survival, yet it has revealed artifacts when measuring the cytotoxicity of prooxidant agents (36). Another simple method to evaluate drug-induced cytotoxicity is using membrane integrity as an index, which is assessed by monitoring the release of cytosolic lactate dehydrogenase (LDH). LDH activity was measured in the culture medium and cell lysates (50 mM HEPES, Triton X-100 0.5%, pH 7.0) using a plate reader format as previously described (37). Briefly, 5 μ l of cell culture supernatant and lysates were incubated with 0.24 mM NADH in a Tris/NaCl pH 7.2 buffer in 96-well plates for 5 min at 25°C . The reaction was started by the addition of 9.8 mM pyruvate and the consumption of NADH followed at 340 nm for 5 min at 30°C . Percent LDH release was calculated as follows: (supernatant LDH / supernatant LDH + lysate LDH) $\times 100$.

Immunoblotting of cytochrome c. The cytosolic fractions resulting from the mitochondrial purification were concentrated using Centricon YM-10 filters (Millipore, Bedford, MA). A

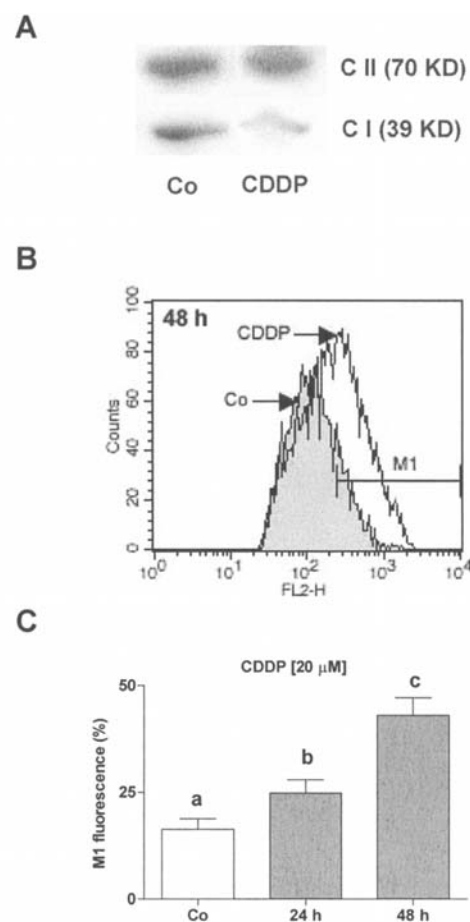


Figure 2. Cisplatin (CDDP) disrupted the steady-state level mitochondrial respiratory complex I and increased mitochondrial superoxide ($\text{O}_2^{\cdot -}$) formation in A549 cells. (A) Lower levels of the mitochondrial respiratory complex I were detected when compared to complex II as shown by immunoblotting in CDDP-treated A549 cells (40 μ M, 48-h treatment) versus control (Co). Each figure was representative of three samples and the experiment repeated twice. (B) CDDP (20 μ M, 48-h treatment) induced an increase of $\text{O}_2^{\cdot -}$ formation in the mitochondria as detected by flow cytometry using the mitochondrial $\text{O}_2^{\cdot -}$ sensitive dye MitoSOX. (C) Flow cytometry analysis of CDDP-mediated mitochondrial $\text{O}_2^{\cdot -}$ over time at 24 and 48 h. Bars with different letters are statistically different from one another ($n=3$, $P<0.05$).

precipitated gradient 4-20% SDS PAGE (Bio-Rad Laboratories, Hercules, CA) was loaded with 30 μ g protein per well. The samples were run and transferred, and the membranes blocked using the same conditions as described above for immunoblotting of complexes I and II. Monoclonal anti-cytochrome c primary antibody (1 μ g/ml, mouse IgG_{2b} isotype, BD Biosciences, San Jose, CA) was applied for 2.5 h with gentle rocking. Secondary antibody (peroxidase-conjugated AffiniPure goat anti-mouse IgG, Jackson ImmunoResearch Laboratories, Inc.) was diluted 1:40,000 in TBS-T and applied for 30 min with gentle rocking. Cytochrome c was detected using ECL Plus Western Blot Detection Reagents (Amersham Biosciences). Following cytochrome c detection, membranes were submerged in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.25 mM Tris-HCl, pH 6.7) and incubated at 55°C for 45 min with occasional agitation. Membranes were re-probed for glyceraldehyde 3 phosphate dehydrogenase (GAPDH) as loading standard using monoclonal anti-GAPDH antibody (0.2 μ g/ml, mouse IgG_{2b} isotype, Abcam, Cambridge,

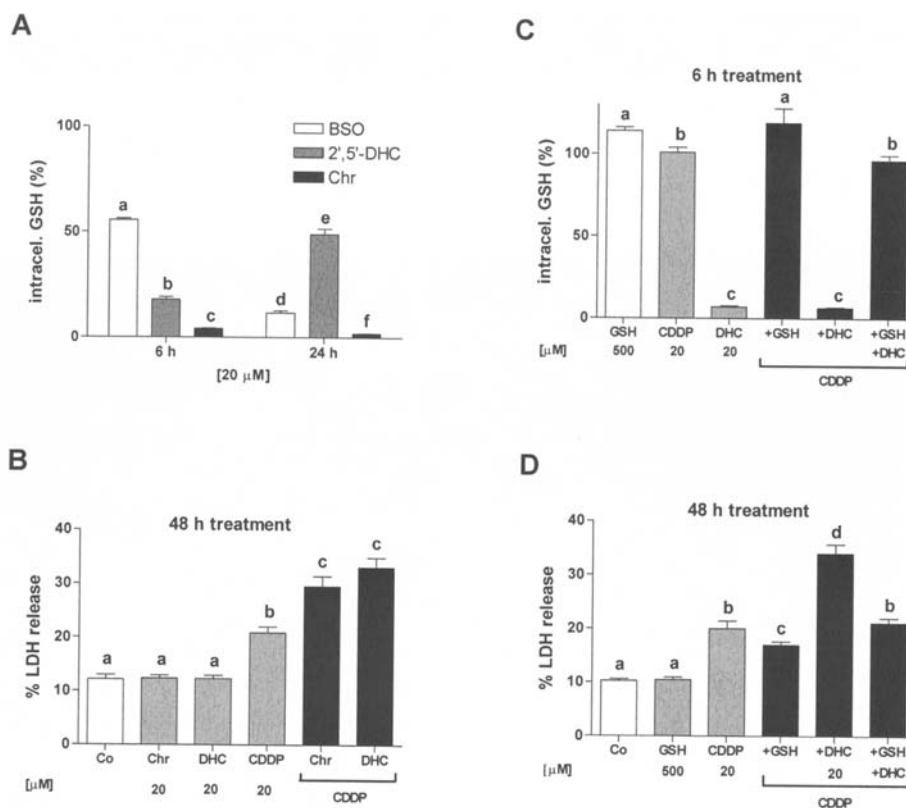


Figure 3. 2',5'-DHC and chrysin induced GSH depletion and potentiated cisplatin-mediated cytotoxicity in A549 cells. (A) At 6-h treatment, the abilities of 2',5'-DHC (DHC, 20 μ M) and chrysin (Chr, 20 μ M) to deplete GSH were more important compared to the inhibitor of γ -glutamylcysteine synthetase BSO (20 μ M) (n=3). In the case of 2',5'-DHC, however, a rebound of GSH levels was observed at 24 h. (B) 2',5'-DHC and chrysin potentiated the toxicity of cisplatin (CDDP, 20 μ M) in A549 cells. (C) Exogenous GSH (500 μ M) restored cytosolic GSH levels (n=3). (D) Exogenous GSH (500 μ M) partially protected the cells towards the toxicity of cisplatin and blocked the 2',5'-DHC-mediated potentiation of cisplatin's toxicity. Similar results were obtained with chrysin (data not shown). Bars with different letters are statistically different from one another (n=4, P<0.05).

MA). Identical secondary antibody was diluted 1:40,000 in TBS-T and applied for 30 min with gentle rocking.

Statistical analysis. Data are presented as means \pm standard error. Each experimental group consisted of an n \geq 3 and the results duplicated at least once. Data were subsequently analyzed for significant differences using ANOVA analysis coupled with a Tukey's range test where significance was preset at P<0.05 (Prizm v.4, GraphPad, San Diego, CA).

Results

Cisplatin disrupts the steady-state level of mitochondrial respiratory complex I. Although the formation of cisplatin-mtDNA adducts has repeatedly been demonstrated, little has been done to evaluate the toxicological relevance of such adducts (11-13). A number of apoproteins of mitochondrial electron transport complexes I, III, IV and V are encoded by mtDNA, whereas complex II is totally encoded by nuclear DNA. Immunoblots from isolated mitochondria demonstrated lower levels of the heavily mitochondria encoded respiratory complex I in cisplatin-treated A549 cells (40 μ M, 48 h) when compared to the nuclear encoded complex II (Fig. 2A). This effect was cisplatin dose-dependent (data not shown). Increased mitochondrial $O_2^{\cdot-}$ formation has been associated with the release of cytochrome *c* and apoptosis (2,8), yet cisplatin (20 μ M, 24- and 48-h treatment) induced an increase of mitochondrial $O_2^{\cdot-}$ in A549 cells as shown using flow cyto-

metry and the dye MitoSOX (Fig. 2B and C). These results add to the growing data implicating mitochondrial dysfunction in cisplatin's anti-tumor effects.

2',5'-DHC and chrysin potentiate the toxicity of cisplatin in A549 cells. 2',5'-DHC (20 μ M) and chrysin (20 μ M) induced GSH depletion in A549 cells (Fig. 3A). After 6-h treatment, this effect was greater when compared to the GSH synthesis inhibitor BSO (20 μ M). In the case of 2',5'-DHC, however, a rebound of GSH levels was observed after 24 h, possibly due to stimulation of GSH synthesis, as reported previously with some flavonoids (39). Using the same concentrations (20 μ M), 2',5'-DHC and chrysin were not cytotoxic, yet they potentiated the toxicity of cisplatin in A549 cells (Fig. 3B). Adding exogenous GSH to the media (500 μ M) restored intracellular GSH levels and significantly protected both the toxicity of cisplatin and the potentiation effect of 2',5'-DHC (Fig. 3C and D). Similar results were obtained using chrysin (not shown). BSO (20 and 50 μ M) significantly potentiated the cytotoxicity of cisplatin at 72-h treatment, but not at 48-h treatment (data not shown). Whereas chrysin was more efficient in depleting GSH than 2',5'-DHC in this cell line, the latter was slightly more efficient in potentiating the effect of cisplatin, showing that GSH depletion is only one of the factors involved. 2',5'-DHC is also known to interfere with the mitotic phase of the cell cycle and possibly with the mitochondrial respiratory chain (29,30). When 2',5'-DHC was compared to its analogs chalcone, 4'-hydroxychalcone (4'-HC) and 2'-hydroxychalcone

Table I. Structure/activity relationship of several hydroxyl derivatives of chalcone in A549 cells.

	Chalcone	4'-HC	2'-HC	2',5'-DHC
GSH levels (% Co) ^a	100	56	35	18
Toxicity (% Co) ^b	None	72	126	140
Potential effect (% CDDP) ^c	None	None	29	46

^aIntracellular GSH levels (% compared to control, Co) as measured by HPLC-EC and using 20 μ M of compound (6-h treatment). ^bToxicity (% compared to control, Co) as measured by percentage of LDH release and using 40 μ M of compound (48-h treatment). ^cPotential effect of the toxicity of cisplatin (% compared to 20 μ M of CDDP) as measured by percentage of LDH release and using 20 μ M of compound (48-h treatment). Margin of errors were less than $\pm 5\%$, except for 2',5'-DHC induced toxicity ($\pm 7\%$).

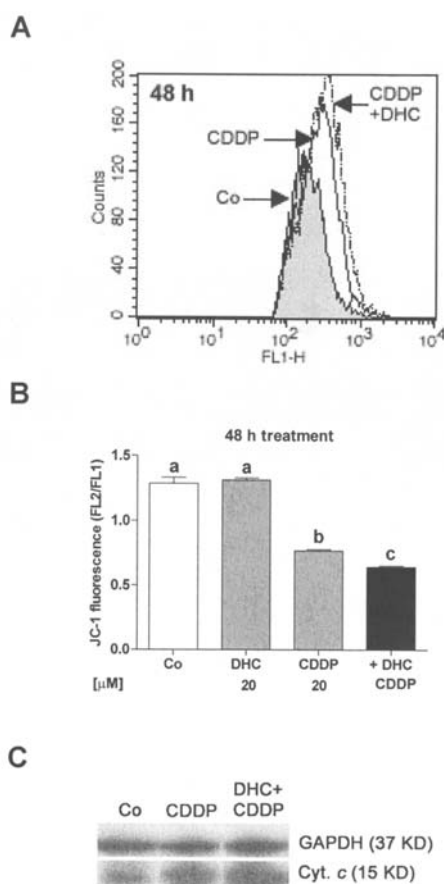


Figure 4. 2',5'-DHC increased the cisplatin-mediated depolarization of the mitochondrial membrane and cytochrome c release. (A and B) As shown using flow cytometry and JC-1 as dye, cisplatin treatment (CDDP, 20 μ M, 48 h) induced a depolarization of the mitochondrial membrane, effect that was amplified by adding 2',5'-DHC (DHC, 20 μ M). The FL2/FL1 ratio was measured using the mean fluorescence in each channel. Bars with different letters are statistically different from one another ($n=3$, $P<0.05$). (C) As shown by immunoblotting using GAPDH as internal standard, cisplatin treatment (CDDP, 20 μ M, 48 h) induced the release of cytochrome c (Cyt. c), which was increased by adding 2',5'-DHC (DHC, 20 μ M). 2',5'-DHC alone had no significant effect (not shown). Each figure was representative of three samples and the experiment was repeated at least twice.

(2'-HC), 2',5'-DHC was the most efficient in depleting GSH in A549 cells, in killing A549 cells, and in potentiating the effect of cisplatin (Table I). These results show an apparent correlation between the ability of hydroxychalcones to induce

GSH depletion with their ability to potentiate the toxicity of cisplatin.

2',5'-DHC and chrysin exacerbate cisplatin-induced mitochondrial dysfunction. The depolarization of the mitochondrial membrane and the subsequent release of cytochrome c are usually seen as the 'point of no return' in mitochondrial-mediated apoptosis. Cisplatin treatment (20 μ M, 48 h) in A549 cells induced a depolarization of the mitochondria as shown using flow cytometry and the dye JC-1 (Fig. 4A and B). Whereas 2',5'-DHC alone did not show a decrease in FL2/FL1 ratio, the mitochondrial membrane was further depolarized by adding 2',5'-DHC (20 μ M) to cisplatin. Also, cisplatin treatment (20 μ M, 48 h) induced a release of cytochrome c, which was increased by adding 2',5'-DHC (20 μ M) as shown by immunoblotting (Fig. 4C). A shorter cisplatin treatment time (24 h) induced a slight depolarization of the mitochondrial membrane, but no significant difference between cisplatin alone and cisplatin-2',5'-DHC-treated cells was detected (data not shown). Chrysin (20 μ M, 48-h treatment) induced an increase in cytochrome c release as well, but we were unable to detect a significant effect on the depolarization of the mitochondrial membrane using JC-1 (data not shown). This result may be due to the limits in the method sensitivity.

Discussion

ROS have been associated with carcinogenesis but also, paradoxically, with mitochondrial-mediated cell death in cancer cells. In fact, a number of anti-cancer drugs, including adriamycin and cisplatin, induce the formation of mitochondrial ROS, the depolarization of the mitochondrial membrane, and the release of cytochrome c (40). These attributes may play an important role for the side effects of such drugs (neuro, renal and cardiotoxicities) but also for their cytotoxic action in cancer cells. The chemopreventive properties of naturally occurring compounds may also be mediated by ROS, as in the case of 2-methoxyestradiol, an inhibitor of mitochondrial respiratory complex I (41,42). Formation of mitochondrial ROS appears to be increased by reversible glutathionylation of complex I as well (43). GSH depletion sensitizes cisplatin-resistant cancer cells (19,20). Conversely, treatments with *N*-acetyl-L-cysteine, a glutathione ester, or GSH are protective against cisplatin-induced toxicity (44,45). Yet, it is important to note the delayed

nature of cisplatin-induced mitochondrial ROS formation. Whereas inhibitors of the electron transport chain induce an oxidative burst within hours following treatment, the increase of ROS levels induced by cisplatin was significant only after 24 h of treatment (Fig. 2C). Moreover, cisplatin induced a decrease in the ratio of complexes I/II, which has previously been associated with the formation of mitochondrial superoxide ($O_2^{\cdot-}$) (38). These findings suggest that, at the concentration used in our studies (under 40 μ M), cisplatin's target is mtDNA rather than direct inhibition of the electron transport chain (10-13). mtDNA encodes for 13 proteins that are critical for the proper function of the electron transport chain, and the effects of cisplatin and other alkylating agents on mtDNA should not be underestimated. A variety of effects of cisplatin in the cell have been reported, yet the disruption of the assembly of the mitochondrial respiratory chain is, in our view, the most reasonable explanation for cisplatin-induced formation of mitochondrial ROS and subsequent mitochondrial-mediated cell death.

A continuing avenue of research is to combine agents to synergize the anti-tumor effects without increasing damage to normal tissue. Previous studies have shown that lowering tumor cell defenses against chemotherapeutic agents can synergize anti-tumor effects. GSH is an abundant and critical cellular thiol antioxidant. Cellular GSH levels are maintained by synthesis, transport, utilization, and degradation pathways. Early attempts to target these pathways involved the use of inhibitors to block GSH synthesis or glutathione reductase that recycles the oxidized form of glutathione (GSSG) back to the active reduced form (GSH). BCNU (carmustine) is an approved anti-cancer drug that is an alkylating agent and an inhibitor of glutathione reductase (46). BSO is an example of an inhibitor of cellular GSH synthesis that has been used in combination with mephalan or radiotherapy (21,22). A major problem associated with these approaches has been the lack of selectivity. In the past few years, several studies have associated the ability of verapamil to sensitize cancer cells by an MRP-mediated GSH depletion rather than inhibition of drug efflux (15-17). We showed recently that, in three separate cancer cell lines, simple molecules such as 2',5'-DHC and chrysin were more effective than verapamil in depleting cytosolic GSH (14).

We have chosen to target MRP-mediated GSH transport using 2',5'-DHC and chrysin as a new strategy to achieve GSH depletion and to sensitize cancer cells to cisplatin treatment. As shown using A549 cells, when compared to BSO treatment, both 2',5'-DHC and chrysin were more effective in inducing GSH depletion and in potentiating of toxicity of cisplatin. In order to explain this potentiation effect, inhibition of drug efflux cannot be discarded. However, adding GSH to the culture media protected the cells, thus confirming the importance of cytosolic levels of GSH. Also, whereas cisplatin is a substrate of MRP2, chrysin is a substrate of MRP1 rather than MRP2 (31,47), and preliminary results in our laboratory suggest that 2',5'-DHC is not a substrate for MRP2 (data not shown). Although the conjugation of 2',5'-DHC with GSH cannot be ruled out, it cannot account for the dramatic decrease in GSH levels induced by this compound. Overall, our studies suggest that the potentiation effect of cisplatin-induced toxicity results, in part, from synergetic prooxidant effects of

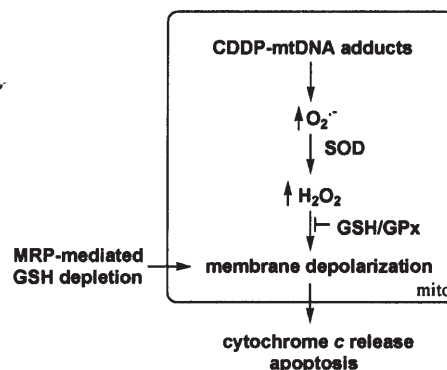


Figure 5. Working hypothesis. The formation of cisplatin-mitochondrial DNA (CDDP-mtDNA) adducts disrupts the assembly of the apoproteins of the mitochondrial respiratory chain, which in turn induces the formation of mitochondrial superoxide ($O_2^{\cdot-}$). The latter is converted by superoxide dismutase (SOD) into hydrogen peroxide (H_2O_2), which mediates the depolarization of the mitochondrial membrane and the release of cytochrome *c*, thus triggering the cascade of events leading to nuclear DNA fragmentation and apoptosis. Flavonoid-induced GSH depletion adds to the mitochondrial membrane depolarization by mechanisms that remain to be clarified.

cisplatin, by inducing a disruption of the assembly of the apoproteins of the mitochondrial respiratory chain, and the selected flavonoids, by depleting the cells of a key antioxidant defense.

Intracellular GSH depletion has been previously associated with mitochondrial dysfunction, including the increased production of ROS by mitochondrial respiratory complex III (24). Since GSH is synthesized in the cytosol and high concentrations are found in the mitochondria (48), cytosolic GSH depletion can indirectly translate into mitochondrial GSH depletion. Mitochondria mainly rely upon GSH and glutathione peroxidase to control hydrogen peroxide (H_2O_2) levels, and thus lower GSH levels could lead to higher mitochondrial steady-state H_2O_2 levels and trigger the formation of mitochondrial pores and the depolarization of the mitochondrial membrane. However, it is worth noting that the depolarization of the mitochondrial membrane is mediated by the oxidation of lipoproteins such as cardiolipin (50), which is located in the inner mitochondrial membrane, and its recycling (reduction) is not necessarily controlled by the pool of mitochondrial GSH. Although it is generally accepted that weakening antioxidant defenses sensitizes cancer cells to prooxidants such as cisplatin, the mechanistic relationship between thiol-redox status and pro- and anti-apoptotic signals is yet to be clarified (Fig. 5). As shown in this study, whereas chrysin was more effective than 2',5'-DHC in depleting cytosolic GSH, 2',5'-DHC was slightly more effective than chrysin in potentiating the toxicity of cisplatin (Fig. 3A and B), suggesting that other mechanisms are involved, possibly direct interference with the mitotic phase of cell cycle and/or with the mitochondrial respiratory chain (14,30). Non-toxic concentrations of 2',5'-DHC and chrysin (20 μ M) further increased the release of cytochrome *c* in cisplatin-treated A549 cells (Fig. 4), suggesting that the potentiation effect was also mediated by the mitochondria.

In summary, cisplatin-induced mitochondrial dysfunction is likely due to the disruption of the assembly of the apoproteins

of the mitochondrial respiratory chain and can be modulated by the ability of selected flavonoids in inducing GSH extrusion from the cell.

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