2,2’-Dihydroxychalcone, a glutathione transferase inhibitor, sensitises human colon adenocarcinoma cells to chlorambucil and melphalan, but not to actinomycin D

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Abstract. 2,2’-Dihydroxychalcone (2,2’DHC) is a potent inhibitor of glutathione S-transferases (GSTs). Pre-treatment of human colon cancer cells by a non-toxic concentration of this GST inhibitor significantly sensitised cancer cells to chlorambucil and melphalan, which are substrates of glutathione (GSH) conjugation. However, sensitisation to actinomycin D, which has not been shown to be detoxified by GSH-related mechanisms, was not observed. These results further confirm the contribution of GSH-related mechanisms to drug resistance by increased detoxification of drugs. 2,2’DHC inhibited GST activity and the transport of GSH conjugates by cancer cells. Its combined effects on GST and glutathione conjugate export (GS-X) pump may provide more potent sensitisation of cancer cells to chemotherapeutic drugs.

Introduction

Chemotherapy has been widely used as a strategy for the treatment of a variety of malignancies, including colon cancers (1-3). However, its clinical effectiveness is significantly affected by cellular drug resistance. Various biochemical and molecular mechanisms of cellular resistance to these chemotherapeutic agents have been proposed (4). Glutathione (GSH), GSH-related enzymes and glutathione conjugate export (GS-X) pump have been shown to participate in the detoxification of many anticancer drugs, including melphalan and chlorambucil (5-7). Previous studies have indicated that plant polyphenols sensitise human tumour cells to chlorambucil by inhibiting GST activity (8), glutathione reductase (GSH-RD) (9) and the transport of GSH conjugates (8,10). Increased expression of glutathione S-transferase (GST) (11) and GS-X pump (12) was also observed in melphalan-resistant tumour cells. Moreover, the inhibition of GST by ethacrynic acid (13-15) and the depletion of GSH by buthionine sulfoximine (16) potentiated melphalan cytotoxicity in tumour cells. These studies suggest that GSH, GSH-related enzymes and GS-X pump contribute to cell resistance to anticancer drugs such as melphalan and chlorambucil, both of which are substrates of the detoxification system. However, none of these GSH-related factors act independently; their modulation alone may be insufficient for the sensitisation of drug-resistant cells.

Studies of sensitivity to chemotherapeutic drugs that cannot be detoxified by GSH-related mechanisms may provide information useful for understanding other possible mechanisms of drug detoxification inhibition. Currently, this knowledge is quite limited. Actinomycin D, as a non-covalent DNA-binding drug inhibiting DNA-directed RNA and DNA synthesis, is used clinically to treat Wilms’s and Ewing’s tumours and soft tissue sarcomas (17,18). This anticancer drug is not metabolised in animals (19), and no report has shown its detoxification by GSH-related mechanisms (20,21). In our previous study, the activity of GSH-related factors was found to be correlated to the sensitivity of human cancer cells to chlorambucil and adriamycin (22), but not to actinomycin D (23). This suggests that GSH-related mechanisms contribute to drug resistance by means of increased drug detoxification. In the present study, we further examined the effect of 2,2’-dihydroxychalcone (2,2’DHC), a modulator of GSH-related mechanisms, on the sensitivity of human colon cancer cells to chlorambucil and melphalan, which are substrates of GSH-related mechanisms, and to actinomycin D, which has not been shown to be detoxified by the system. The aim of this comparative study was to provide additional valuable information on the subject.

Materials and methods

Chemicals. Melphalan, chlorambucil, actinomycin D, GSH, 1-chloro-2,4-dinitrobenzene (CDNB) and 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tissue culture flasks and multi-well plates were from Nunclon (Resklide, Denmark). RPMI-1640 medium, MEM medium and foetal calf serum (FCS) were Gibco products from Life
Assay of GST activities. Dose-dependent curves in the linear range. The reaction mixture contained 1 mM sodium pyruvate, 1 mM non-essential amino acid and 10% FCS. Cells were maintained in a humidified atmosphere of air/CO₂ (19/1) and were subcultured every 2-3 days. The GST activities were expressed as nmol of dinitrophenyl-S-glutathione (DNP-SG) formed/min/mg of protein at 340 nm. GST activities were determined spectrophotometrically by monitoring absorbance at 340 nm. The extinction coefficient was 9.6 mM⁻¹cm⁻¹ (25).

Assay of cytotoxicity. Cells (~1x10⁶) were seeded in each well of 96-well tissue culture plates and incubated in a CO₂ incubator for 24 h. The cells were pre-treated with 10 μM 2,2'DHC for 1 h. Stock solutions of chlorambucil, melphalan and actinomycin D were freshly prepared in absolute ethanol, and aliquots of the stock solutions were added to the wells of the plates at the desired concentrations. After 24 h incubation with chlorambucil, melphalan and actinomycin D, the number of surviving cells in the plate wells was determined by MTT assay as previously reported (24). To each well, 25 μl of the 5 mg/ml MTT stock solution was added; after 2 h incubation at 37˚C, 100 μl of the extraction buffer (20% SDS w/v in 50% N,N-dimethyl formamide v/v, 2.5% of 80% acetic acid and 2.5% 1 N HCl, pH 4.7) was added. After incubation overnight at 37˚C, optical densities at a wavelength of 570 nm were measured using a microplate reader (Dynatech Laboratories). Cell survival for each dose of chlorambucil, melphalan and actinomycin D was calculated as the ratio of absorbance in the wells containing the drug-treated cells to that of the control wells. IC₅₀ values were calculated from dose-dependent curves in the linear range.

Assay of GST activities. Cells (~1x10⁶) were harvested by trypsinisation. Cell homogenates prepared by a polytron homogeniser were then centrifuged at 105,000 x g for 45 min at 4°C. The GST activities of the cytosol preparations of human tumour cells were measured according to the method of Habig et al (25). The reaction mixture contained 125 mM potassium phosphate buffer, pH 6.5, 1 mM GSH and 1 mM CDNB. The reaction was activated by the addition of GST to the cytosol preparations of human tumour cells. The rate of formation of GSH and CDNB conjugate was determined spectrophotometrically by monitoring absorbance at 340 nm. The extinction coefficient was 9.6 mM⁻¹cm⁻¹ (25). For the inhibition study, inhibitor was added to the reaction mixture containing 1-40 nmol CDNB/ml in the presence of excess GSH and rat liver GSTs as a standard.

Protein determination. The protein contents of the cytosol preparations of human tumour cells were measured by the method of Bradford (27) using Bio-Rad reagent with bovine serum albumin (BSA) as a standard.

Statistical analysis. The differences between cells treated with drugs only and cells treated with both drugs and the sensitiser were statistically analysed using the Student’s t-test. p-values were obtained using Microsoft Excel (Redmond, WA, USA). A p-value <0.05 was considered statistically significant.

Results

2,2’-Dihydroxychalcone sensitises human colon tumour cells to melphalan and chlorambucil, but not to actinomycin D. Dose-dependent cytotoxicity of chlorambucil and melphalan was observed in the human colon adenocarcinoma cells. As shown in Figs. 1 and 2, pre-treatment of the cells with 10 μM 2,2’DHC significantly sensitised them to chlorambucil and melphalan. When the cells were treated with 40 μM melphalan, the toxicity of the drug was potentiated by 50%. Upon addition of 2,2’-Dihydroxychalcone, the toxicity of melphalan was potentiated by 60%. The DNP-SG content exported to the extracellular HBSS by the cells was quantified by the HPLC-UV method as described previously (10). Extracellular HBSS (0.5 ml) was passed through a 0.45-μm filter, then 100 μl of the filtrate was injected for HPLC-UV analysis using an HP 1100 liquid chromatographic system and a variable wavelength detector set at 340 nm. The column (100x4.6 mm) was packed with C₄ Hypersep ODS, and elution was carried out with a gradient of 40-90% (v/v) methanol in 0.01 M sodium phosphate, pH 6.0, for 6 min. The flow rate was 1 ml/min. The peak representing DNP-SG at the retention time of 2.1 min was quantified by comparison with DNP-SG standards formed from 1-40 nmol CDNB/ml in the presence of excess GSH and rat liver GSTs purified by S-hexylglutathione affinity chromatography (26). GS-X pump activity shown as the rate of DNP-SG efflux from the human tumour cells was calculated and expressed as nmol/10⁶ cells/h at 37°C.

Measurement of GS-X pump activities of human tumour cells. Cells were cultured on a 6-well plate at a density of 1.0x10⁶ cells/well/2 ml in a CO₂ incubator for 24 h. They were then incubated with 1 mM CDNB at 10˚C for 45 min for DNP-SG loading. The formation of DNP-SG inside human tumour cells was described in our previous study (10). Cells in the wells were washed three times with 1 ml Hank’s Balanced Salt Solution (HBSS), then incubated in HBSS at 37°C for up to 60 min. The DNP-SG content exported to the extracellular HBSS by the cells was quantified by the HPLC-UV method as described previously (10). Extracellular HBSS (0.5 ml) was passed through a 0.45-μm filter, then 100 μl of the filtrate was injected for HPLC-UV analysis using an HP 1100 liquid chromatographic system and a variable wavelength detector set at 340 nm. The column (100x4.6 mm) was packed with C₄ Hypersep ODS, and elution was carried out with a gradient of 40-90% (v/v) methanol in 0.01 M sodium phosphate, pH 6.0, for 6 min. The flow rate was 1 ml/min. The peak representing DNP-SG at the retention time of 2.1 min was quantified by comparison with DNP-SG standards formed from 1-40 nmol CDNB/ml in the presence of excess GSH and rat liver GSTs purified by S-hexylglutathione affinity chromatography (26). GS-X pump activity shown as the rate of DNP-SG efflux from the human tumour cells was calculated and expressed as nmol/10⁶ cells/h at 37°C.

Table I. Inhibition of glutathione S-transferase activity in human colon adenocarcinoma cells by 2,2’-dihydroxychalcone.

<table>
<thead>
<tr>
<th>Concentration of 2,2’DHC (μM)</th>
<th>GST activity (nmol/min/mg protein)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>82.0±1.5</td>
<td>(0.0)</td>
</tr>
<tr>
<td>10</td>
<td>71.1±1.4</td>
<td>(13.3)</td>
</tr>
<tr>
<td>20</td>
<td>58.8±2.0</td>
<td>(28.3)</td>
</tr>
<tr>
<td>40</td>
<td>22.2±0.5</td>
<td>(72.9)</td>
</tr>
<tr>
<td>80</td>
<td>17.9±0.3</td>
<td>(78.1)</td>
</tr>
<tr>
<td>160</td>
<td>6.2±0.0</td>
<td>(92.4)</td>
</tr>
</tbody>
</table>

Glutathione S-transferase (GST) was extracted from the cells, then its activity was determined spectrophotometrically using CDNB as an electrophilic substrate. Values are the means ± SD of at least three independent experiments.
treatment with 80 μM chlorambucil, a 30% increase in toxicity was achieved by the sensitiser. Statistical differences were observed at concentrations of 20, 40 and 80 μM in the case of both drugs. The IC50 values for chlorambucil and melphalan are 132.9 and 111.1 μM respectively; these values decreased to 76.4 and 52.5 μM respectively when the cells were pre-treated with 2,2'DHC. However, as shown in Fig. 3, the dose-response curve of the cells to actinomycin D was not affected by their pre-treatment with 2,2'DHC. These results suggest that 2,2'DHC sensitises human colon cancer cells to chlorambucil and melphalan, which are substrates of GSH-related detoxification mechanisms, but not to actinomycin D, which has not been previously shown to be detoxified by this system.

Inhibition of cancer cell glutathione S-transferases by 2,2'-dihydroxychalcone. To study the mechanisms of chemosensitisation as described above, the effects of 2,2'DHC on the drug detoxification enzyme GST were assessed. Table I shows the dose-dependent inhibition, by 2,2'DHC with an IC50 value of 28.9 μM, of the GSH transferase extracted from the human colon cancer cells. Inhibition of the activity of this drug detoxification enzyme by 2,2'DHC may contribute to its sensitisation of the cells.

Inhibition of GS-X pump activity in human colon cancer cells by 2,2'-dihydroxychalcone. The export of GSH conjugates of anticancer drugs such as melphalan and chlorambucil is a sequentially separated step of the detoxification system. The effects of 2,2'DHC on this export were therefore studied. As shown in Table II, the export of a typical GSH conjugate, DNP-SG, by human cancer cells was dose-dependently affected by 2,2'DHC. This inhibition by 2,2'DHC may also contribute to its sensitisation of the cancer cells.

Discussion

Chalcones are plant-derived polyphenolic substances widely found in human diets and traditional medicines (28,29). They

Table II. Inhibition of glutathione conjugate export pump activity in human colon adenocarcinoma cells by 2,2'-dihydroxychalcone.

<table>
<thead>
<tr>
<th>Concentration of 2,2'DHC (μM)</th>
<th>GS-X pump activity (nmol/10^6 cells/h (% inhibition))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>1.36±0.054 (0)</td>
</tr>
<tr>
<td>20</td>
<td>1.10±0.034 (19)</td>
</tr>
<tr>
<td>40</td>
<td>0.94±0.056 (31)</td>
</tr>
<tr>
<td>60</td>
<td>0.88±0.052 (36)</td>
</tr>
<tr>
<td>80</td>
<td>0.70±0.036 (49)</td>
</tr>
</tbody>
</table>

*Cells were pre-incubated with 2,2'DHC for 1 h then incubated with 1-chloro-2,4-dinitrobenzene at 10°C for 45 min for dinitrophenyl-S-glutathione (DNP-SG) loading. The DNP-SG-loaded cells were washed with and then incubated in HBSS for 60 min. DNP-SG exported to the extracellular HBSS was determined by HPLC-UV analysis. Values are the means ± SD of at least three independent experiments.

Figure 1. Sensitisation of human colon adenocarcinoma cells by 2,2'-dihydroxychalcone (2,2'DHC) to chlorambucil. Cells were pre-incubated with 2,2'DHC for 1 h then incubated with chlorambucil for 24 h. Cell survival was determined by MTT assay and expressed as a percentage of the control cells.

Figure 2. Sensitisation of human colon adenocarcinoma cells by 2,2'-dihydroxychalcone (2,2'DHC) to melphalan. Cells were pre-incubated with 2,2'DHC for 1 h then incubated with melphalan for 24 h. Cell survival was determined by MTT assay and expressed as a percentage of the control cells.

Figure 3. Sensitisation of human colon adenocarcinoma cells by 2,2'-dihydroxychalcone (2,2'DHC) to actinomycin D. Cells were pre-incubated with 2,2'DHC for 1 h then incubated with actinomycin D for 24 h. Cell survival was determined by MTT assay and expressed as a percentage of the control cells.
are generally considered non-toxic and have been shown to have various biological and pharmacological effects, including anticancer activity (30). In our previous study, chalcones were shown to inhibit GST activity; a structure-activity analysis showed that hydroxylation substitutions at the C-2’ and C-2 positions in ring A and B of the chalcone molecule, respectively, are required for the inhibition of GST activity (10,26). 2,2’DHC was therefore selected for this study so that its effects on GST activity in human colon cancer cells and its possible sensitisation of cancer cells to anticancer agents could be investigated.

2,2’DHC was revealed to be a potent inhibitor of GST activity in human colon cancer cells with an IC_{50} value of 28.9 μM. This inhibition may be the mechanism for its sensitisation of human colon cancer cells to chlorambucil and melphalan, which are substrates for GSH-related detoxification. Notably, the toxicity of actinomycin D to these cells was not affected by pre-treatment with 2,2’DHC. As actinomycin D has not been shown to be detoxified by GST conjugation, modulation of the GSH-related detoxification system in cancer cells should not affect its detoxification or cellular toxicity. Chlorambucil, melphalan and actinomycin D exert their cytotoxic effects on cancer cells through different mechanisms; numerous factors, operating from the time of drug administration to the final achievement of drug effects, may account for drug resistance. These include changes in the influx and efflux of drugs across the tumour cell membrane, alterations in drug activation and detoxification, modulations in the target site and the repair of drug-induced damage. Based on our current data, the possibility that other mechanisms play a role in the sensitisation of cancer cells by 2,2’DHC cannot be excluded yet. However, 2,2’DHC as a modulator of GSH-related detoxification mechanisms could sensitise cancer cells to only those drugs which are substrates of GSH conjugation. This further confirms the contribution of increased detoxification by GSH-related mechanisms to the drug resistance of these cells. Relatively non-toxic modulators of this system, such as chalcones, are therefore potential sensitisers for cancer cells resistant to these chemotherapeutic agents.

In this study, 2,2’DHC inhibited the transport of GSH conjugates and GST activity in human colon cancer cells. Although the relative contribution of each of these processes to the sensitisation of the cells was not assessed, they could conceivably provide synergistic effects as they are sequentially separated steps in the overall detoxification system. This property of the polyphenol adds to its potential as a sensitiser for cancer chemotherapy. However, further studies are required to ascertain the other biological and pharmacological effects of 2,2’DHC on cancer and non-malignant cells.

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References