Modulation by Acrolein and Chloroacetaldehyde of Multidrug Resistance Mediated by the Multidrug Resistance-associated Protein (MRP)

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ABSTRACT

Acrolein (AC) and chloroacetaldehyde (CHA) are metabolites of the non-multidrug resistance cytotoxic drugs cyclophosphamide and ifosfamide. It has previously been reported that both metabolites can induce extensive depletion of glutathione (GSH) in vitro and in vivo and that this depletion occurs at drug concentrations in the micromolar range. A link between the function of the multidrug resistance-associated protein (MRP) and the intracellular concentration of GSH has also been demonstrated. To determine whether AC and CHA can modulate the function of MRP by inducing GSH depletion, we used two human lung cancer cell lines overexpressing MRP: the large cell carcinoma cell line COR-L23/R and the adenocarcinoma cell line MOR/R0.4, along with their respective sensitive parental lines, COR-L23/P and MOR/P. We showed that micromolar concentrations of AC and millimolar concentrations of CHA are able to deplete GSH concentrations in the cell lines studied. In addition, concentrations of 50 mM AC and 5 mM CHA could completely reverse the daunorubicin (DNR) and vincristine accumulation deficit present in COR-L23/R and partially reverse the DNR accumulation deficit in MOR/R0.4. In contrast, AC and CHA did not reverse the drug accumulation deficit in the P-glycoprotein-overexpressing lung cancer cell line H69/LX4.

The effect of CHA and AC on drug accumulation was related to the GSH depletion, as we found a concentration-dependent relationship between the GSH levels and the reversal of the accumulation deficit for both AC and CHA. To substantiate further this correlation, we increased cellu-

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INTRODUCTION

CP and IF have widespread use in the treatment of various neoplastic diseases usually as part of combination chemotherapy protocols (1) and are also used, at high doses, as tumor-ablative conditioning agents in several bone marrow transplantation protocols (2, 3). Both agents are classified as nitrogen mustards and are prodrugs that require metabolic activation by hepatic mixed function oxidases. CP and IF undergo virtually identical biotransformations (4). CP is first hydroxylated to yield 4-OH-CP, which exists in equilibrium with its ring-opened tautomer, aldoephosphamide. Aldophosphamide spontaneously decomposes to the alkylation agent phosphoramide mustard and AC. Phosphoramide mustard and AC are both cytotoxic. However, the antineoplastic action of CP and IF is usually mediated by phosphoramide and ifosfamide mustard, respectively, and not by AC since it rapidly reacts with noncritical molecules in the immediate vicinity of its formation. The generation of phosphor-

The abbreviations used are: CP, cyclophosphamide; IF, ifosfamide; 4-OH-CP, 4-hydroxycyclophosphamide; AC, acrolein; GSH, glutathione; CHA, chloroacetaldehyde; MRP, multidrug resistance-associated protein; Pgp, P-glycoprotein; BSO, [DL]-buthionine(S,R)-sulfoximine; DNR, daunorubicin; VBL, vincristine; 4-OH-CP, 4-hydroperoxycyclophosphamide; MDR, multidrug resistance.

The effect of CHA and AC on drug accumulation was related to the GSH depletion, as we found a concentration-dependent relationship between the GSH levels and the reversal of the accumulation deficit for both AC and CHA. To substantiate further this correlation, we increased cellular GSH content in AC- and CHA-treated cells with the restoration of the DNR accumulation deficit. No significant effect of the GSH ethyl ester was detected on DNR accumulation for both AC and CHA. A link between the function of the multidrug resistance-associated protein (MRP) and the intracellular concentration of GSH has also been demonstrated. To determine whether AC and CHA can modulate the function of MRP by inducing GSH depletion, we used two human lung cancer cell lines overexpressing MRP: the large cell carcinoma cell line COR-L23/R and the adenocarcinoma cell line MOR/R0.4, along with their respective sensitive parental lines, COR-L23/P and MOR/P. We showed that micromolar concentrations of AC and millimolar concentrations of CHA are able to deplete GSH concentrations in the cell lines studied. In addition, concentrations of 50 μM AC and 5 mM CHA could completely reverse the daunorubicin (DNR) and vincristine accumulation deficit present in COR-L23/R and partially reverse the DNR accumulation deficit in MOR/R0.4. In contrast, AC and CHA did not reverse the drug accumulation deficit in the P-glycoprotein-overexpressing lung cancer cell line H69/LX4.

The effect of CHA and AC on drug accumulation was related to the GSH depletion, as we found a concentration-dependent relationship between the GSH levels and the reversal of the accumulation deficit for both AC and CHA. To substantiate further this correlation, we increased cellular GSH content in AC- and CHA-treated cells with the GSH ethyl ester. An increase in cellular GSH levels in CHA- and AC-treated COR-L23/R cells was accompanied by a restoration of the DNR accumulation deficit. No significant effect of the GSH ethyl ester was detected on DNR accumulation in COR-L23/P parental cells. In conclusion, treatment with AC or CHA can reverse the drug accumulation deficit of MRP-overexpressing cells, and this effect appears to be mediated by GSH depletion.
Acrolein and Chloroacetaldehyde as MRP Modifiers

... efflux pump, has been isolated from cell lines that display a MDR phenotype but do not overexpress Pgp (9). A link between MRP and the intracellular concentration of GSH has recently been demonstrated. A previous report from our laboratory showed a strong decrease in drug resistance in three MDR lung carcinoma cell lines overexpressing MRP (10), an inhibitor of γ-glutamylcysteine synthetase, the enzyme that catalyses the first step in GSH synthesis. Zaman et al. (11) demonstrated that depletion of intracellular GSH by BSO resulted in a complete reversal of resistance to several MDR agents in lung carcinoma cells transfected with a MRP cDNA expression vector. GSH depletion had little effect on MDR in cells transfected with MDR1 cDNA encoding Pgp. These experiments suggest that GSH is specifically required for the export of drugs from cells by MRP and not by Pgp.

The reported link between GSH and export of drugs by MRP prompted us to study the effects of the metabolites AC and CHA on MRP-MDR in two human lung carcinoma cell lines overexpressing MRP. The aim of the study was to explore whether the non-MDR agents CP and IF are able to modulate MRP-MDR through the induction of GSH depletion by their metabolites.

MATERIALS AND METHODS

Cell Lines. The human lung cancer cell lines and their doxorubicin-selected resistant sublines used in this study have been described elsewhere: the large cell lung cancer cell line COR-L23/P and the MDR variant COR-L23/R (12, 13), and the adenocarcinoma cell line MOR/P and the MDR subline MOR/R0.4 (14). None of the MDR sublines shows overexpression of the MDR-1 gene or its product, Pgp, but each overexpresses MRP (14). In addition, a Pgp-overexpressing small cell lung carcinoma cell line, H69/LX4, and its parental cell line, H69/P, were used (12). All of the cell lines were cultured in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum with 2 mm L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator in 8% carbon dioxide and 92% air. Cells of the COR-L23 and MOR lines grow as attached monolayers on plastic, but those of the H69 line grow as floating aggregates.

For growth inhibition experiments, cells were plated in 96-well plates (Falcon), and after 24-h incubation, different concentrations of AC or CHA were added to the wells. Cells were then grown for 6 days, and growth inhibition was determined in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (13).

Chemicals. [G-3H]Dauorubicin hydrochloride (specific activity, 3.6 Ci/mmol) was obtained from NEN-DuPont de Nemours (Bad Homburg, Germany). [G-3H]Vinblastine sulfate (specific activity, 8.3 Ci/mmol) was obtained from Amersham. The reduced form of the GSH ethyl ester, 5,5-dithiobis(2-nitrobenzoic acid), AC, and CHA were obtained from Sigma. Ethanol was used to prepare stock solutions of AC and CHA, which were used for all of the experiments.

GSH Content. Proteins from 4 × 10^5 cells were precipitated with 20% (w/v) trichloroacetic acid. The cellular GSH content of the supernatant was determined with Ellman’s substrate in cells treated with or without CHA or AC for 1 h at 37°C. Results (nmol/10^5 cells) are expressed as means ± SD from two to five experiments, each performed in triplicate.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Effect of CHA and AC on the intracellular GSH* levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Line</td>
<td>Control</td>
</tr>
<tr>
<td>COR-L23/P</td>
<td>1.44 ± 0.33 (100%)</td>
</tr>
<tr>
<td>COR-L23/R</td>
<td>0.92 ± 0.30 (100%)</td>
</tr>
<tr>
<td>MOR/P</td>
<td>1.38 ± 0.18 (100%)</td>
</tr>
<tr>
<td>MOR/R0.4</td>
<td>1.75 ± 0.18 (100%)</td>
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</table>

*Cellular GSH content was determined with Ellman’s substrate in cells treated with or without CHA or AC for 1 h at 37°C. Results (nmol/10^5 cells) are expressed as means ± SD from two to five experiments, each performed in triplicate.

Number of experiments.

Percentage of control.
Values were corrected for the amount of cell-associated drugs at time 0 at 0°C.

As measured using trypan blue exclusion, the cells remained viable during the drug accumulation studies with AC and CHA.

RESULTS

Intracellular GSH Levels. Since AC and CHA can induce GSH depletion in human peripheral blood lymphocytes (7, 8) and in some leukemia cell lines (6, 7), we determined the effects of both metabolites on cellular GSH content in COR-L23 and MOR cells (Table 1). Basal GSH levels did not predict for resistance because we found that COR-L23/R cells had a lower and MOR/R0.4 a similar or higher GSH content in comparison to their parental cell lines. The percentage of GSH depletion in the parental and resistant cells was similar for the same concentrations of either CHA or AC (Table 1).

The lowest concentration of CHA used was 200 μM. This concentration did not have an effect in the GSH levels of the two cell lines studied after either a 1-h exposure or a 20-h exposure (data not shown). The lowest concentration of AC used was 10 μM and only had a minor effect in the intracellular GSH levels (Fig. 1A).

Effect of CHA and AC on [3H]DNR and [3H]VBL Accumulation. First, we studied the accumulation of [3H]DNR in the two MRP-overexpressing lung cancer cell lines COR-L23 and MOR (Table 2). The addition of 5 mM CHA or 50 μM AC reversed the accumulation deficit for DNR completely in COR-L23/R and partially in MOR/R. The concentrations used are able to reduce GSH levels by more than 60% in both cell lines (Table 1). CHA and AC did not increase to a significant extent the accumulation of DNR in the parental cells of the two lung cancer cell lines (drug accumulation in the presence of CHA or AC versus control is not significantly different, P > 0.05, Student’s paired t test). This result suggests that the passive DNR transport was not affected by CHA or AC. We found that the SDs of the experiments carried out in MOR cells were larger than those in COR-L23. This fact is attributable to the difficulty in obtaining a single-cell suspension with MOR cells. To determine whether the effect of CHA or AC on DNR accumulation was specific for that drug or whether the same effect can also be observed on the accumulation of other MDR drugs, we used [3H]VBL in COR-L23, CHA (5 mM) and AC (50 μM) also reversed the VBL accumulation deficit of COR-L23/R, with little effect on the parental cell line COR-L23/P (the small increase observed in the parental cell line in the presence of CHA or AC was not significantly different).

We have also analyzed the effect of CHA and AC in a Pgp-overexpressing cell line, H69/LX4, and in its parental line H69/P (Table 3). CHA and AC had no effect on the decreased DNR accumulation in H69/LX4. The lack of effect of CHA and AC on the accumulation of DNR in a Pgp-overexpressing cell line suggests that the effect of those metabolites is specific for MRP-MDR.

Correlation between Cellular GSH Content and Drug Accumulation. To evaluate the effect of GSH depletion by CHA and AC on drug accumulation, we studied the concentration-dependent effects of AC and CHA in the resistant cell line COR-L23/R. A concentration-dependent effect of AC and CHA on DNR accumulation in this resistant cell line was found (Figs. 1 and 2).

The concentration-dependent effects of CHA and AC suggest that the effect of these compounds on drug accumulation in MRP-overexpressing cells is caused by GSH depletion. To exclude further a direct effect of AC and CHA on drug transport, we increased the cellular GSH content in AC- and CHA-treated cells with GSH ethyl ester (Table 4). Monoesters of GSH are
Table 2  Effect of CHA and AC in MRP-overexpressing lung cancer cell linesa

<table>
<thead>
<tr>
<th></th>
<th>DNR accumulation</th>
<th>VBL accumulation</th>
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<tbody>
<tr>
<td></td>
<td>COR-L23/P</td>
<td>COR-L23/R</td>
</tr>
<tr>
<td>Control</td>
<td>177 ± 1.2</td>
<td>50 ± 12.7</td>
</tr>
<tr>
<td>CHA (5 mM)</td>
<td>218 ± 18.0</td>
<td>181 ± 6.5a</td>
</tr>
<tr>
<td>AC (50 μM)</td>
<td>194 ± 12.0</td>
<td>145 ± 9.4a</td>
</tr>
<tr>
<td>MOR/P</td>
<td>87 ± 44.0</td>
<td>138 ± 50.0</td>
</tr>
<tr>
<td>MOR/R0.4</td>
<td>12 ± 7.6</td>
<td>61 ± 39.7</td>
</tr>
</tbody>
</table>

*Data shown are expressed in pmol/10^6 cells and are means ± SD from three experiments, each performed in triplicate. CHA concentration (mM) = 50 ± 12.0 218 ± 18.0 194 ± 12.0.

Table 3  Effect of CHA and AC on DNR accumulation in the Pgp-overexpressing cell line H69/LX4 and in its parental line H69/P

<table>
<thead>
<tr>
<th></th>
<th>H69/P</th>
<th>H69/LX4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>155 ± 37</td>
<td>17 ± 1.2</td>
</tr>
<tr>
<td>CHA (5 mM)</td>
<td>155 ± 30</td>
<td>29 ± 7.8</td>
</tr>
<tr>
<td>AC (50 μM)</td>
<td>139 ± 36</td>
<td>19 ± 0.9</td>
</tr>
</tbody>
</table>

*Results are expressed in pmol/10^6 cells and are means ± SD from three experiments, each one performed in triplicate. DNR accumulation in the presence of CHA or AC vs. control was not significantly different (P > 0.05, Student's paired t test).

DISCUSSION

Here, we show that depletion of cellular GSH levels by CHA and AC in two MRP-overexpressing human lung cancer cell lines results in an increase in drug accumulation. We have also shown that replenishment of cellular GSH levels in CHA- and AC-treated cells restores the DNR accumulation deficit in COR-L23/R (Table 4). In contrast to these effects on MRP-overexpressing cells, cellular GSH depletion had no effect on the decreased DNR accumulation present in the Pgp-overexpressing lung cancer cell line H69/LX4 (Table 3). Therefore, these data indicate that drug transport is regulated differently in MRP- and Pgp-overexpressing cells. These results are similar to those obtained using BSO to induce GSH depletion in MRP-overexpressing human cancer cells (10, 11). However, the mechanisms by which CHA and AC deplete intracellular GSH are different. It has been shown that AC can be inactivated by human GSH transferases by either conjugation with reduced GSH or covalent binding to the enzymes in the absence of GSH (18). Specifically, AC is among the most active substrates known for transferases π (18). AC can also react spontaneously with free sulphydryl groups of macromolecules (19). A conjugation reaction between CHA and GSH to form a thioether conjugate has been postulated as a possible molecular mechanism for the observed depletion of GSH with this compound (7, 20). In addition, CHA is also a competitive inhibitor of human placental GSH transferase (7).
eral blood lymphocytes, a 50% depletion of intracellular GSH levels by about 80% after a 1-h exposure (6). In human periph-

induced measurable GSH depletion, and 4 μM AC reduced GSH levels in cell lines and normal cells. In mouse P388 cells, 1 μM CHA in influx. The cells remained viable during the drug accumulation were higher than the IC50 values for these compounds in of CHA and AC used to induce more than a 50% depletion of another role for AC and CHA: the circumvention of MRP-MDR increased formation of phosphoramidemustard. In another re-

stabilizing influence of GSH on 4-OH-CP is removed, leading to the parent 4-OH-CP by depleting cellular GSH so that the spontaneous fission of 4-OH-CP to the ultimate toxic species, phosphoramidemustard. Thus, AC may promote the cytotoxicity of

Table 4 Modification of CHA and AC effects by GSH ethyl ester in COR-L23

<table>
<thead>
<tr>
<th>Intracellular GSH levels (nmol/10^6 cells)</th>
<th>DNR accumulation (pmol/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1.73 ± 0.24</td>
<td>Control 197 ± 10</td>
</tr>
<tr>
<td>Control + ester 2.55 ± 0.23</td>
<td>Control + ester 154 ± 31</td>
</tr>
<tr>
<td>CHA (5 mm) 0.30 ± 0.03</td>
<td>CHA (5 mm) 223 ± 13</td>
</tr>
<tr>
<td>CHA (5 mm) + ester 2.47 ± 0.29</td>
<td>CHA (5 mm) + ester 147 ± 7.2</td>
</tr>
<tr>
<td>AC (50 μM) 0.48 ± 0.12</td>
<td>AC (50 μM) 173 ± 27</td>
</tr>
<tr>
<td>AC (50 μM) + ester 2.66 ± 0.42</td>
<td>AC (50 μM) + ester 142 ± 4.1</td>
</tr>
</tbody>
</table>

“Cells were treated with either vehicle, CHA (5 mm), or AC (50 μM) for 15 min. Following incubation with 5 mM GSH ethyl ester for 1 hr, cellular GSH and DNR content were measured. Data shown are means ± SD of three experiments, each performed in triplicate.

Prior reports have implicated GSH as a determinant of the antitumour activity of CP. Lee (21) showed that GSH protects against the cytotoxicity of 4-hydroperoxycyclophosphamide (a stable precursor that rapidly gives rise to 4-OH-CP spontaneously under physiological conditions) by minimizing the spontaneouse fission of 4-OH-CP to the ultimate toxic species, phosphoramidemustard. Thus, AC may promote the cytotoxicity of the parent 4-OH-CP by depleting cellular GSH so that the stabilizing influence of GSH on 4-OH-CP is removed, leading to increased formation of phosphoramidemustard. In another report, GSH depletion of human leukemia cells resulted in a 5-fold potentiation of the cytotoxicity of CP (6). Our study shows another role for AC and CHA: the circumvention of MRP-MDR by the induction of GSH depletion. However, the concentrations of CHA and AC used to induce more than a 50% depletion of GSH were higher than the IC50 values for these compounds in COR-L23 and MOR cell lines. Nevertheless, the effects of CHA and AC on drug accumulation cannot be attributable to damage of the plasma membrane, which in turn could increase drug influx. The cells remained viable during the drug accumulation studies as measured using trypan blue exclusion.

It is interesting to note that AC was approximately 100-fold more potent than CHA to induce a similar degree of GSH depletion and reversal of the accumulation deficit. A complete reversal of the accumulation deficit of COR-L23/R was achieved with 5 mM CHA or 50 μM AC. Levels of CHA in a millimolar range are not achievable in the clinic. Concentrations of CHA up to 50 μM have been detected in the plasma of patients treated with IF (22). However, the concentration of AC needed to achieve a complete reversal of the accumulation deficit in COR-L23/R is in the micromolar range. Although a concentration of 50 μM AC is very toxic as a continuous exposure for the cell lines used in this study, it seems likely that lower micromolar concentrations of AC could be achieved in vivo. Indeed, intracellular depletion of GSH by activated CP has been reported in vivo (23), and this depletion of GSH is attributable to intracellularly released AC (6, 8). It has also been shown that a different range of AC and CHA concentrations in vitro is required to induce depletion of GSH in different tumor cell lines and normal cells. In mouse P388 cells, 1 mM CHA can reduce the GSH levels to 20% (7). In the human chronic leukemia cell line K562, AC concentrations as low as 1 μM induced measurable GSH depletion, and 4 μM AC reduced GSH levels by about 80% after a 1-h exposure (6). In human peripheral blood lymphocytes, a 50% depletion of intracellular GSH

was achieved by 16 μM AC and 30 μM CHA (8). Thus, it seems likely that, at least for some tumors (those in which the smallest micromolar doses of AC can induce an extensive depletion of GSH), intracellular depletion of GSH might be achieved in a clinical setting using CP or IF. According to our observations, an extensive GSH depletion (at least 70%) would be required to obtain significant effects on clinical MRP-mediated resistance. This possibility could allow the use of either CP or IF, not only as cytotoxic agents but also as MRP modifiers, in combination with MDR agents in tumors in which MRP is frequently over-expressed, such as chronic lymphocytic leukemia (24). Nevertheless, this approach will only be effective if the GSH levels are an important contributing factor in the perhaps multifactorial resistance displayed by those tumors in the clinic. Based on these observations, it is also tempting to speculate about a potential clinical role for an analogue of 4-OOH-CP as an MRP modifier in combination with MDR agents. Deschloro-4-OOH-CP generates AC but not phosphoramide mustard in the spontaneous fission reaction, is essentially nontoxic when compared with 4-OOH-CP, but is equally potent in depleting GSH (21). To test all of these hypotheses will require further in vitro and in vivo studies.

The fact that BSO, CHA, and AC could modulate MRP-MDR through GSH depletion suggests that any chemotherapy or drug treatment which depletes GSH, regardless of its mech-

anism, could affect MRP activity. In this regard, it is also important to bear in mind that normal tissues which overexpress MRP could be affected by the use of such chemotherapy sched-

ules. Moreover, it seems clear that more studies are needed to clarify the clinical relevance of MRP, which at present is not known.

The ability of AC and CHA to overcome MRP-MDR by depletion of intracellular GSH in MRP-overexpressing lung cancer cell lines is shown. In this experimental system, AC is more potent than CHA to induce GSH depletion and reversal of MRP-MDR. Potential clinical implications for these findings are discussed. In addition to their cytotoxic action, a new role as MRP modifiers for CP and IF, as well as other oxazaphospho-

rines, is suggested.

REFERENCES


Modulation by acrolein and chloroacetaldehyde of multidrug resistance mediated by the multidrug resistance-associated protein (MRP).

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