Restoration of Sensitivity to Oxazaphosphorines by Inhibitors of Aldehyde Dehydrogenase Activity in Cultured Oxazaphosphorine-resistant L1210 and Cross-Linking Agent-resistant P388 Cell Lines

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ABSTRACT

The sensitivity of cultured L1210 and P388 cells sensitive (L1210/0, P388/0) and resistant (L1210/OAP, P388/CLA) to oxazaphosphorines, to 4-hydroperoxycyclophosphamide, ASTA Z-7557, phosphoramid mustard, and acrolein was determined in the absence and presence of known (disulfiram, diethylidithiocarbamate, cyanamide) or suspected [ethylphenyl(2-formyl-ethyl)phosphinate] inhibitors of aldehyde dehydrogenase activity. The L1210/OAP cell line is resistant specifically to the oxazaphosphorines; P388/CLA cells are partially cross-resistant to other cross-linking agents. All four inhibitors of aldehyde dehydrogenase activity potentiated the cytotoxic action of the oxazaphosphorines, 4-hydroperoxycyclophosphamide and ASTA Z-7557, against L1210/OAP and P388/CLA cells; in the presence of a sufficient amount of inhibitor, sensitivity was essentially fully restored in both cases. The inhibitors did not potentiate the cytotoxic action of the nonoxazaphosphorines, phosphoramid mustard and acrolein, against these cell lines. The cytotoxic action of the oxazaphosphorines and nonoxazaphosphorines against L1210/0 and P388/0 cells was not potentiated by any of the aldehyde dehydrogenase inhibitors. Inhibitors of xanthine oxidase or aldehyde oxidase activities did not potentiate the cytotoxic action of the oxazaphosphorines against L1210/OAP cells. These observations strongly suggest that (a) aldehyde dehydrogenase activity is an important determinant with regard to the sensitivity of a cell population to the oxazaphosphorines; (b) L1210/0 and P388/0 cells lack the relevant aldehyde dehydrogenase activity; (c) the phenotypic basis for the resistance to oxazaphosphorines by L1210/OAP cells is aldehyde dehydrogenase activity; and (d) the major reason that P388/CLA cells are resistant to oxazaphosphorines is aldehyde dehydrogenase activity.

INTRODUCTION

The studies reported herein are part of our continuing effort to identify the phenotypic basis for the relatively favorable margin of therapeutic safety exhibited by the 2-chloroethylamido-oxazaphosphorines, e.g., cyclophosphamide.

Tumor cell populations frequently become resistant to a cytotoxic agent when repeatedly exposed to it. Identification of the phenotypic basis for this resistance often serves to identify one of the determinants influencing the sensitivity of a given cell population to the drug. Such an approach was taken in the present investigation. Cultured L1210 and P388 cells sensitive (L1210/0, P388/0) and resistant (L1210/OAP, P388/CLA) to oxazaphosphorines (36) were used for this purpose. The L1210/OAP cell line is resistant specifically to the oxazaphosphorines, i.e., it does not exhibit cross-resistance to other cross-linking agents; indeed, it is collaterally sensitive to such compounds. The P388/CLA cell line exhibits partial cross-resistance to other cross-linking agents.

Cyclophosphamide is a prodrug (Chart 1). It is first hydroxylated to 4-hydroxycyclophosphamide/aldophosphamide, which is also a prodrug. 4-Hydroxycyclophosphamide/aldophosphamide can then give rise to phosphoramid mustard; the cytotoxic action of cyclophosphamide is apparently due to the cross-linking of DNA by this metabolite. Alternatively, 4-hydroxycyclophosphamide/aldophosphamide can be oxidized to carboxyphosphamide. This metabolite is without cytotoxic activity and does not, under physiological conditions, give rise to cytotoxic metabolites. Oxidation to carboxyphosphamide is catalyzed by NAD-linked aldehyde dehydrogenases (6, 7, 15, 16, 20, 21, 34, 35). Thus, differentially greater aldehyde dehydrogenase activity could account for the relative insensitivity of some cells to cyclophosphamide, and inhibitors of this activity should confer sensitivity.

The ability of known (disulfiram, diethylidithiocarbamate, cyanamide) and suspected (EPP) inhibitors of aldehyde dehydrogenase activity (3, 9–12, 30, 33) to potentiate the cytotoxic activity of oxazaphosphorines (ASTA Z-7557, 4-hydroperoxycyclophosphamide) and nonoxazaphosphorines (phosphoramid mustard, acrolein) against cultured L1210/0, L1210/OAP, P388/0, and P388/CLA cells was tested.

MATERIALS AND METHODS

ASTA Z-7557 was supplied by Dr. P. Hilgard, Asta-Werke AG, Bielefeld, Federal Republic of Germany. 4-Hydroperoxycyclophosphamide was supplied by Dr. A. Takamizawa, Shionogi and Co., Fukushima, Japan. Phosphoramid mustard-cyclohexylamine was supplied by Dr. H. B. Wood, Jr., Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Acrolein, cyanamide, and pyridoxal hydrochloride were purchased from the Aldrich Chemical Company. Phosphoramid mustard, cyclohexylaminesalt. 1,3,2-oxazaphosphorine 2-oxo Jude cyclohexylamine salt. EPP, ethylphenyl(2-formyl-ethyl)phosphinate; ASTA Z-7557 and 4-hydroperoxycyclophosphamide are relatively stable preceeds to an irreversible reaction with the drug. Both precursors rapidly and spontaneously (without benefit of enzymatic involvement) give rise to the 4-hydroxy intermediate under physiological conditions (Chart 1).
REVERSAL OF ACQUIRED RESISTANCE TO OXAZAPHOSPHORINES

Parent

\[
\begin{align*}
\text{NH}_2 & \quad \text{R-P=O} \\
\text{O-CH}_2 &
\end{align*}
\]

\[
\begin{align*}
\text{Cl-CH}_2-\text{CH}_2 & \\
\text{N-} &
\end{align*}
\]

Cyclophosphamide

\[
\begin{align*}
\text{NADPH} + \text{H}^+ & \\
\text{oxidase} &
\end{align*}
\]

\[
\begin{align*}
\text{NADPH} + \text{H}^+ & \\
\text{oxidase} &
\end{align*}
\]

4-Hydroperoxycyclophosphamide

\[
\begin{align*}
\text{NH}_2 & \quad \text{R-P=O} \\
\text{O-CH}_2 &
\end{align*}
\]

ASTA Z 7557

4-Hydroxycyclophosphamide

\[
\begin{align*}
\text{OH} & \\
\text{H}_2\text{O} &
\end{align*}
\]

Transport

\[
\begin{align*}
\text{NH}_2 & \quad \text{R-P=O} \\
\text{O-CH}_2-\text{CH}_2-\text{CH}=\text{O} &
\end{align*}
\]

Aldophosphamide

\[
\begin{align*}
\text{CH}_2=\text{CH}=\text{CH}=\text{O} & \quad \text{NAD}^+ + \text{H}_2\text{O} \\
\text{aldehyde dehydrogenase} &
\end{align*}
\]

Phosphoramide mustard

\[
\begin{align*}
\text{CH}_2=\text{CH}=\text{CH}=\text{O} & \quad \text{NADH} + \text{H}^+ \\
\text{acrolein} &
\end{align*}
\]

Toxic

\[
\begin{align*}
\text{CH}_2=\text{CH}=\text{CH}=\text{O} & \quad \text{O-CH}_2-\text{CH}_2-\text{C}=\text{OH} \\
\text{carboxyphosphamide} &
\end{align*}
\]

Inactive

Chart 1. Metabolism of oxazaphosphorines.

ical Co., Milwaukee, WI. Disulfiram, diethylthiocarbamate, pargyline hydrochloride, menadione, and allopurinol were purchased from the Sigma Chemical Co., St. Louis, MO. EPP was supplied by Dr. L. A. Cates, College of Pharmacy, University of Houston, Houston, TX.

All drugs, except disulfiram and menadione, were dissolved in sterile, triple-distilled water; disulfiram and menadione were dissolved in 95% ethanol. All solutions except for those containing acrolein or 95% ethanol were sterilized by passage through 0.22-μm Millipore filters; all were used within 1 h of preparation and were kept at approximately 4°C prior to their use. Ethanol, at the concentrations used, was not cytotoxic to tumor cells.

Drug-exposure medium contained CaCl₂ (100 mg/liter), KCl (200 mg/liter), MgCl₂·6H₂O (100 mg/liter), KH₂PO₄ (42 mg/liter), Na₂HPO₄·7H₂O (453 mg/liter), and NaCl (8614 mg/liter). The pH was adjusted to a value of 7.4. Sterilization was effected by passage through a 0.22-μm Millipore filter.

Cultured mouse L1210 and P388 cells sensitive (L1210/0, P388/0) and resistant to oxazaphosphorines specifically (L1210/OAP) or to cross-linking agents in general (P388/CLA) (36) were obtained from the Southern Research Institute, Birmingham, AL through the courtesy of Dirs. R. F. Struck and L. J. Wilkoff. The resistant sublines were originally obtained in vivo by means of serial treatment with cyclophosphamide (13, 27). In our laboratory, the 4 cell lines were grown in static suspension culture at 37°C. RPMI 1640 culture medium supplemented with 10% horse serum and a humidified atmosphere of 5% CO₂ in air was used to grow the cells. Mean population-doubling times were approximately 9 h.

The back-extrapolation method of Alexander and Mikulski (1) was used, essentially as described previously (36), to determine the sensitivity of cultured tumor cells to drugs. The only departure from the protocol utilized previously was that the total drug exposure period was 90 min. Inhibitors of aldehyde dehydrogenase activity and/or vehicle were present during the entire 90-min period; ASTA Z-7557, 4-hydroperoxycyclophosphamide, phosphoramide mustard, acrolein, and/or vehicle were present during only the last 30 min of the drug exposure period.

RESULTS

The sensitivities of cultured L1210/0, L1210/OAP, P388/0, and P388/CLA tumor cells to fixed concentrations of 4-hydroperoxycyclophosphamide, ASTA Z-7557, phosphoramide mustard, acrolein, and/or vehicle were present during only the last 30 min of the drug exposure period.
REVERSAL OF ACQUIRED RESISTANCE TO OXAZAPHOSPHORINES

Chart 2. Sensitivity of cultured tumor cells to 4-hydroperoxycyclophosphamide, ASTA Z-7557, phosphoramide mustard, and acrolein in the presence of disulfiram. Tumor cells were incubated with vehicle or disulfiram for 60 min at 37°C. 4-Hydroperoxycyclophosphamide (A), ASTA Z-7557 (■), phosphoramide mustard (□), acrolein (○), or vehicle (×) was added, and incubation was continued at 37°C for an additional 30 min. Concentrations of cytotoxic agents were 12 (L1210/0), 35 (L1210/OAP), 1.5 (P388/0), and 36 (P388/CLA) μM 4-hydroperoxycyclophosphamide; 17 (L1210/0), 43 (L1210/OAP), 4.5 (P388/0), and 20 (P388/CLA) μM ASTA Z-7557; 90 (L1210/OAP) and 220 (P388/CLA) μM phosphoramide mustard; and 5 (L1210/OAP, P388/CLA) μM acrolein. Cells were harvested at the end of the incubation period and subsequently resuspended and grown in drug-free medium. Surviving fractions were determined by the back-extrapolation method described in “Materials and Methods.” Each point represents the mean of measurements on duplicate cultures.

Chart 3. Sensitivity of cultured tumor cells to 4-hydroperoxycyclophosphamide, ASTA Z-7557, phosphoramide mustard, and acrolein in the presence of diethyldithiocarbamate. Tumor cells were incubated with vehicle or diethyldithiocarbamate for 60 min at 37°C. 4-Hydroperoxycyclophosphamide (A), ASTA Z-7557 (■), phosphoramide mustard (□), acrolein (○), or vehicle (×) was added, and incubation was continued at 37°C for an additional 30 min. Concentrations of cytotoxic agents were 12 (L1210/0), 35 (L1210/OAP), 1.5 (P388/0), and 36 (P388/CLA) μM 4-hydroperoxycyclophosphamide; 17 (L1210/0), 43 (L1210/OAP), 4.5 (P388/0), and 20 (P388/CLA) μM ASTA Z-7557; 90 (L1210/OAP) and 220 (P388/CLA) μM phosphoramide mustard; and 5 (L1210/OAP, P388/CLA) μM acrolein. Cells were harvested at the end of the incubation period and subsequently resuspended and grown in drug-free medium. Surviving fractions were determined by the back-extrapolation method described in “Materials and Methods.” Each point represents the mean of measurements on duplicate cultures.

Chart 4. Sensitivity of cultured tumor cells to 4-hydroperoxycyclophosphamide, ASTA Z-7557, phosphoramide mustard, and acrolein in the presence of cyanamide. Tumor cells were incubated with vehicle or cyanamide for 60 min at 37°C. 4-Hydroperoxycyclophosphamide (A), ASTA Z-7557 (■), phosphoramide mustard (□), acrolein (○), or vehicle (×) was added, and incubation was continued at 37°C for an additional 30 min. Concentrations of cytotoxic agents were 12 (L1210/0), 35 (L1210/OAP), 1.5 (P388/0), and 24 (P388/CLA) μM 4-hydroperoxycyclophosphamide; 17 (L1210/0), 43 (L1210/OAP), 4.5 (P388/0), and 30 (P388/CLA) μM ASTA Z-7557; 90 (L1210/OAP) and 181 (L1210/OAP, P388/CLA) μM phosphoramide mustard; and 5 (L1210/OAP, P388/CLA) μM acrolein. Cells were harvested at the end of the incubation period and subsequently resuspended and grown in drug-free medium. Surviving fractions were determined by the back-extrapolation method described in “Materials and Methods.” Each point represents the mean of measurements on duplicate cultures.

Chart 5. Sensitivity of cultured tumor cells to 4-hydroperoxycyclophosphamide, ASTA Z-7557, phosphoramide mustard, and acrolein in the presence of ethylphenyl(2-formylethyl)phosphinate. Tumor cells were incubated with vehicle or ethylphenyl(2-formylethyl)phosphinate for 60 min at 37°C. 4-Hydroperoxycyclophosphamide (A), ASTA Z-7557 (■), phosphoramide mustard (□), acrolein (○), or vehicle (×) was added, and incubation was continued at 37°C for an additional 30 min. Concentrations of cytotoxic agents were 12 (L1210/0), 35 (L1210/OAP), 1.5 (P388/0), and 24 (P388/CLA) μM 4-hydroperoxycyclophosphamide; 17 (L1210/0), 43 (L1210/OAP), 4.5 (P388/0), and 30 (P388/CLA) μM ASTA Z-7557; 90 (L1210/OAP) and 181 (L1210/OAP, P388/CLA) μM phosphoramide mustard; and 5 (L1210/OAP, P388/CLA) μM acrolein. Cells were harvested at the end of the incubation period and subsequently resuspended and grown in drug-free medium. Surviving fractions were determined by the back-extrapolation method described in “Materials and Methods.” Each point represents the mean of measurements on duplicate cultures.

Two of the four inhibitors, namely, cyanamide and EPP, were themselves without cytotoxic action toward any of the 4 cell lines (Charts 2 and 3). Disulfiram and diethyldithiocarbamate were not toxic to L1210/0 or L1210/OAP cells (Charts 2 and 3). However, both were toxic to P388/CLA cells at low concentrations but not at relatively higher concentrations (Charts 2 and 3). A single concentration of each of these agents was used when...
4-HYDROPEROXYCYCLOPHOSPHAMIDE, µM

Chart 6. Sensitivity of cultured L1210/OAP tumor cells to 4-hydroperoxycyclophosphamide and ASTA Z-7557 in the presence of known or suspected inhibitors of aldehyde dehydrogenase activity. L1210/OAP cells were incubated with vehicle (X), 3 µM disulfiram (●), 3 µM diethyldithiocarbamate (■), 30 µM cyanamide (▲), or 100 µM ethylnaphthy(2-formyl)phosphinate (◇) for 60 min at 37° C. 4-Hydroperoxycyclophosphamide or ASTA Z-7557 was added, and incubation was continued at 37° C for an additional 30 min. Cells were harvested at the end of the incubation period and subsequently resuspended and grown in drug-free medium. Surviving fractions were determined by the back-extrapolation method described in "Materials and Methods." Each point represents the mean of measurements on duplicate cultures. The response of cultured L1210/OAP tumor cells to 4-hydroperoxycyclophosphamide or ASTA Z-7557 in the presence of known or suspected inhibitors of aldehyde dehydrogenase activity is not apparent and was not investigated further. A maximum of 10 µM disulfiram was used in these experiments because greater concentrations of this agent were highly toxic to all 4 cell lines.

Table 1

Sensitivity of cultured tumor cells to 4-hydroperoxycyclophosphamide and ASTA Z-7557 in the presence of known or suspected inhibitors of aldehyde dehydrogenase activity.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Inhibitor</th>
<th>LC99 (µM) 4-Hydroperoxycyclophosphamide</th>
<th>LC99 (µM) ASTA Z-7557</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>63</td>
<td>164</td>
</tr>
<tr>
<td>L1210/OAP</td>
<td>Disulfiram, 3 µM</td>
<td>19</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Disulfiram, 3 µM</td>
<td>14</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>Cyanamide, 30 µM</td>
<td>25</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>EPP, 100 µM</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>L1210/OAP</td>
<td>Disulfiram, 3 µM</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Disulfiram, 3 µM</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Cyanamide, 30 µM</td>
<td>19</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>EPP, 100 µM</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>P388/CLA</td>
<td>None</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Disulfiram, 3 µM</td>
<td>32</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Disulfiram, 3 µM</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Diethyldithiocarbamate, 10 µM</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Cyanamide, 30 µM</td>
<td>19</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>EPP, 100 µM</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>P388/0</td>
<td>None</td>
<td>4</td>
<td>6</td>
</tr>
</tbody>
</table>

LC99 values* were obtained from the data shown in Charts 6 and 7.

P388/0 cells were tested but, presumably, a similar biphasic response would have been obtained had more concentrations been examined. The biological basis for the biphasic response is not apparent and was not investigated further. A maximum of 10 µM disulfiram was used in these experiments because greater concentrations of this agent were highly toxic to all 4 cell lines.

The 4 inhibitors of aldehyde dehydrogenase activity effected little or no potentiation when the cytotoxic action of 4-hydroperoxycyclophosphamide or ASTA Z-7557 against L1210/OAP or P388/0 cells was determined (Charts 2 to 5). In contrast, all 4 inhibitors greatly potentiated the cytotoxic action of these agents against L1210/OAP and P388/CLA cells (Charts 2 to 7; Table 1). Serving to illustrate the point are the facts that, when L1210/OAP cells were exposed to 35 µM 4-hydroperoxycyclophosphamide, the log surviving fraction decreased from approximately −1 to approximately −5 if 3 µM disulfiram (Chart 2), 3 µM diethyldithiocarbamate (Chart 3), 100 µM cyanamide (Chart 4), or 40 µM EPP (Chart 5) were included in the drug exposure medium, and that the concentration of ASTA Z-7557 required to effect a 99% cell-kill of L1210/OAP cells was reduced from 164 µM to 29, 36, 36, and 11 µM when 3 µM disulfiram, 3 µM diethyldithiocarbamate, 30 µM cyanamide, or 100 µM EPP, respectively, were included in the drug exposure medium (Chart 6 and Table 1). Inclusion of a sufficient amount of inhibitor in the drug exposure medium, i.e., one that gave maximum potentiation (Charts 2 to 5), restored virtually full sensitivity to 4-hydroperoxycyclophosphamide and ASTA Z-7557 in the resistant sublines; i.e., the sensitivity of the resistant sublines L1210/OAP and P388/CLA to 4-hydroperoxycyclophosphamide and ASTA Z-7557 in the presence of inhibitors of aldehyde dehydrogenase activity was comparable to that exhibited by the respective parent lines L1210/OAP and P388/CLA (Charts 2 to 5, Table 1).

None of the inhibitors potentiated the cytotoxic action of acrolein against L1210/OAP or P388/CLA cells (Charts 2 to 5). Disulfiram, diethyldithiocarbamate, and cyanamide did not potentiate the cytotoxic action of phosphoramidate mustard against these cell lines (Charts 2 to 4). These results demonstrate the
specificity of the potentiation, i.e., that the cytotoxic action of only the oxazaphosphorines is potentiated. This is as expected, given that the current understanding of oxazaphosphorine metabolism and pharmacodynamics is correct and that disulfiram, diethyldithiocarbamate, and cyanamide effect their potentiating action by inhibiting aldehyde dehydrogenase activity. A small amount of potentiation was observed when L1210/0, L1210/OAP, or P388/CLA cells were exposed to phosphoramide mustard in the presence of EPP (Chart 5). This may mean that, in addition to inhibiting aldehyde dehydrogenase activity, thus potentiating the cytotoxic action of oxazaphosphorines specifically, EPP also exerts an action which leads to an increase in sensitivity to all nitrogen mustards.

Pargyline (100 μM), yet another inhibitor of NAD-linked aldehyde dehydrogenase activity (32), did not potentiate the antitumor activity of 4-hydroperoxycyclophosphamide or ASTA Z-7557 against L1210/OAP cells (data not presented). Inhibition of NAD-linked aldehyde dehydrogenase activity by pargyline has been shown to be effected by a metabolite of this agent, namely, propiolaldehyde (32). Apparently, L1210/OAP cells lack the enzyme required for this conversion.

Allopurinol (100 μM), a known inhibitor of xanthine oxidase activity (17), and pyridoxal (100 μM), believed to be a physiological substrate for aldehyde oxidase (36) and therefore expected to inhibit the oxidation of other aldehydes by this enzyme, also failed to potentiate the antitumor activity of 4-hydroperoxycyclophosphamide or ASTA Z-7557 against L1210/OAP cells (data not presented). Menadione (3 μM), yet another inhibitor of aldehyde oxidase activity (25), slightly potentiated the antitumor activity of 4-hydroperoxycyclophosphamide and ASTA Z-7557 against L1210/OAP cells, but it effected a potentiation of similar magnitude when phosphoramide mustard was used as the antitumor agent (data not presented), indicating that the basis for the potentiation was independent of its ability to inhibit aldehyde oxidase activity. Moreover, it effected a slight potentiation of oxazaphosphorine activity against L1210/0 cells as well (data not presented).

DISCUSSION

One suspected and 3 known inhibitors of aldehyde dehydrogenase activity potentiated the cytotoxic action of the oxazaphosphorines, 4-hydroperoxycyclophosphamide and ASTA Z-7557, against oxazaphosphorine-resistant L1210/OAP and cross-linking agent-resistant P388/CLA cells; they did not potentiate the cytotoxic action of the nonoxazaphosphorines, phosphoramide mustard and acrolein, against these cells, nor did they potentiate the cytotoxic action of the oxazaphosphorines or nonoxazaphosphorines against the corresponding sensitive L1210/0 and P388/0 parent cells. Potentiation was substantial; a 4- to 5-log increase in cell kill could be achieved.

Our findings that the inclusion of disulfiram in the drug-exposure medium restores the sensitivity of L1210/OAP and P388/CLA cells to the oxazaphosphorines confirm and extend an earlier report by Hilton and Cohen (21) on the same subject. These investigators also found that the inclusion of disulfiram in the treatment regimen restored the sensitivity of their cyclophosphamide-resistant L1210 cells to the oxazaphosphorines but had little effect on the sensitivity of cyclophosphamide-sensitive L1210 cells to these agents.

Potentiation by diethyldithiocarbamate, cyanamide, and EPP has not been reported previously. That diethyldithiocarbamate, a metabolite of disulfiram, did so is not surprising, since it is known to inhibit aldehyde dehydrogenase activity in vivo, even though it is a very poor inhibitor of these enzymes in cell-free systems (9, 15). It may be that the actual inhibitor is disulfiram and that diethyldithiocarbamate is oxidized to disulfiram in vivo (9).

Cyanamide is also an inhibitor of aldehyde dehydrogenase activity in vivo and without inhibitory action in cell-free systems (10, 15, 16). This is because inhibition of aldehyde dehydrogenase activity by cyanamide is dependent on its conversion to an active form; activation is known to be catalyzed by intact rat liver mitochondria (11, 12, 33). Our findings indicate that mitochondria of L1210/OAP and P388/CLA cells are also capable of activating cyanamide. Whether all cells are capable of activating cyanamide is not known. Given that the basis for the relatively favorable margin of safety exhibited by the oxazaphosphorines is due to differentially greater aldehyde dehydrogenase activity in critical normal cells ordinarily sensitive to nitrogen mustards analogues (see below), and that some neoplastic cells are relatively insensitive to oxazaphosphorines because of a large aldehyde dehydrogenase content, it would, from the viewpoint of therapeutic strategy, be fortuitous indeed if only the latter were able to activate cyanamide.

EPP is a phosphorylated aldehyde structurally resembling aldophosphamide. It is a demonstrated competitive inhibitor of aldehyde oxidase activity (3). Increases in the life span of L1210-implanted mice obtained when EPP was concurrently administered with cyclophosphamide were greater than those obtained when cyclophosphamide alone was given (3). EPP had no antitumor activity itself. Preliminary experiments in our laboratory indicate that, as expected in view of its chemical structure, EPP may also be a substrate for NAD-linked aldehyde dehydrogenases. In addition to inhibiting aldehyde dehydrogenase activity and thus potentiating the cytotoxic action of the oxazaphosphorines specifically, EPP may be acting at another site to potentiate the cytotoxic action of all nitrogen mustards in each of the sensitive and resistant cell lines used in this study. This is suggested by the observation that EPP slightly potentiated the cytotoxic action of phosphoramide mustard in both sensitive and resistant cell lines and would explain why the combination of 4-hydroperoxycyclophosphamide or ASTA Z-7557 and EPP was slightly more toxic to L1210/OAP cells than were the oxazaphosphorines alone to L1210/0 cells. It would also explain why virtually full sensitivity to the oxazaphosphorines was restored in P388/CLA cells when EPP was included. P388/CLA cells are not only resistant to oxazaphosphorines but they are also partially cross-resistant to nonoxazaphosphorine cross-linking agents (36). We have postulated that at least 2 changes in phenotype are operative, one effecting a decrease in sensitivity to all cross-linking agents and a second additionally effecting a decrease only to the oxazaphosphorines. Given the correctness of this postulate, full restoration of sensitivity in P388/CLA cells would not be expected if the sole action of EPP was to effect the latter by inhibiting aldehyde dehydrogenase activity.

Our observations and those of Hilton and Cohen (21) strongly suggest that (a) aldehyde dehydrogenase activity is an important

N. E. Sladek, unpublished observations.
determinant with regard to the sensitivity of normal and neoplastic cell populations to the oxazaphosphorines; (b) L1210/OAP cells lack the major determinant for the resistance to oxazaphosphorines; (c) the phenotypic basis for the resistance to oxazaphosphorines by L1210/OAP cells is aldehyde dehydrogenase activity; and (d) the major reason that P388/CLA cells are resistant to oxazaphosphorines is aldehyde dehydrogenase activity.

Several additional observations can be offered in support of these conclusions. Hilton and Cohen (21) found that extracts from cyclophosphamide-resistant L1210 cells contained much more aldehyde dehydrogenase activity than did extracts from cyclophosphamide-sensitive L1210 cells and that extracts from cells pretreated with disulfiram were unable to catalyze the formation of carboxyphosphamide from 4-hydroxycyclophosphamide. Recently, Hilton and Colvin (22) reported an approximately inverse correlation between aldehyde dehydrogenase activity and the sensitivity of several rodent tumor cell lines, human leukemia cell lines, and leukaemic cells derived from marrow aspirates of several patients, to activated cyclophosphamide; disulfiram potentiated the cytotoxic action of activated cyclophosphamide against those cells with relatively high amounts of aldehyde dehydrogenase activity. Initial results of ongoing experiments in our laboratory, in which aldehyde dehydrogenase activity (substrates were 4-hydroxycyclophosphamide, acetaldehyde, butyraldehyde, and propionaldehyde) was measured in extracts of L1210/OAP, P388/OAP, and P388/CLA cells, indicate that the sensitive cells lack the aldehyde dehydrogenase that is present in the 105,000 x g soluble fraction of resistant cells.

The presence of aldehyde dehydrogenase activity may explain why pluripotent hematopoietic stem cells are relatively insensitive to the oxazaphosphorines (26); differentiation of these cells to committed cells appears to be accompanied by a decrease in or even total loss of aldehyde dehydrogenase activity (22, 26). This observation is particularly supportive of the idea, first proposed a decade ago (4–7, 14, 35), that the relatively favorable margin of safety exhibited by the oxazaphosphorines, particularly cyclophosphamide, when used as immunosuppressive agents and in the treatment of certain neoplasms, may well have as its basis aldehyde dehydrogenase activity. Indirect evidence is provided by the observation of Cox et al. (7), who reported that disulfiram essentially did not potentiate the therapeutic action of cyclophosphamide against the ADJ/PC6 mouse plasma cell tumor but markedly potentiated its lethal action against the host mice; such responses would, according to our hypothesis, be expected if critical normal cells contained aldehyde dehydrogenase activity and the neoplastic cells did not. This is not to say that other oxazaphosphorine-specific determinants, e.g., bioactivation (2, 23, 28, 29, 37, 39), cannot also be operative, although such determinants have yet to be conclusively demonstrated as being so. Nonspecific determinants such as DNA repair and sulfhydryl content, i.e., those that would affect the cytotoxic action of all nitrogen mustards, would of course also be operative.

A number of aldehyde dehydrogenase isozymes have been identified in various subcellular fractions of normal mammalian cells (31). Which of these catalyze the oxidation of aldosphosphamide to carboxyphosphamide is not known. Also not known is the genotypic basis of acquired resistance to oxazaphosphorines resulting from increased aldehyde dehydrogenase activity. It could be due to increased steady-state levels of the enzyme, to the production of an isozyme not normally present, or the selection of a subpopulation of cells with relatively greater aldehyde dehydrogenase activity.

Acquired resistance to cyclophosphamide is often observed in humans. Whether it is occasionally/inevitably due to increased aldehyde dehydrogenase activity is not known. Chemosensitivity testing of the type described by Daniels et al. (8) could answer the question. In such experiments, the sensitivity of neoplastic biopsy material, taken prior to the initiation of cyclophosphamide therapy and again following relapse, to oxazaphosphorines in the presence and absence of aldehyde dehydrogenase inhibitors, would be quantified. The relevant aldehyde dehydrogenase activity could also be quantified directly.

Even though acquired resistance to the oxazaphosphorines might be prevented or reversed, inclusion of an aldehyde dehydrogenase inhibitor in the treatment regimen would not appear to offer any therapeutic advantage if aldehyde dehydrogenase activity is the basis for the margin of safety exhibited by the oxazaphosphorines; the same result would be achieved by simply increasing the dose of the oxazaphosphorine. The observations that disulfiram potentiates cyclophosphamide-induced leukopenia (19) and host lethality (7,18) without (7), or only slightly (19) increasing the antitumor action of cyclophosphamide lends credence to this conclusion.

Along the same vein, a cursory review of the medical literature reveals that more than 30 drugs in current clinical use produce a disulfiram-like effect, i.e., nausea, vasodilation, pulsating headache, etc., when taken prior to the ingestion of alcohol. Disulfiram is believed to induce these symptoms by virtue of its ability to inhibit aldehyde dehydrogenases, thus preventing the oxidation of acetdehyde, generated from ethanol, to acetic acid. Presumably, some or all of these drugs, many of which are used in cancer patients, also induce the disulfiram-like effect by inhibiting aldehyde dehydrogenases. Whether they inhibit the isozyme(s) catalyzing the oxidation of aldosphosphamide to carboxyphosphamide is not known, nor is it known whether they inhibit the relevant enzyme activity in all tissues. However, in the light of our findings and the foregoing discussion, the potential for clinically relevant drug interactions is obvious.

Several drugs, e.g., phenobarbital, are known to induce hepatic aldehyde dehydrogenase activity in rodents. It is not known if they induce this activity in humans, if they induce the activity of the relevant isozyme(s), or if they induce aldehyde dehydrogenase activity in all tissues. Again, the potential for clinically relevant drug interactions is obvious.

REFERENCES


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1554

C. L. Manthey and N. E. Sladek, unpublished observations.

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REVERSAL OF ACQUIRED RESISTANCE TO OXAZAPHOSPHORINES


Restoration of Sensitivity to Oxazaphosphorines by Inhibitors of Aldehyde Dehydrogenase Activity in Cultured Oxazaphosphorine-resistant L1210 and Cross-Linking Agent-resistant P388 Cell Lines

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