Alkylating Agents: In Vitro Studies of Cross-Resistance Patterns in Human Cell Lines

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ABSTRACT

The alkylating agents represent one of the most important classes of antitumor agents and play a major role in combination with other agents in the curative chemotherapy of selected human cancers. By repeatedly exposing cells to escalating doses of an alkylating agent, we have developed four human tumor cell lines which are relatively stably resistant to the drug with which the culture was treated. The response of these cell lines to a variety of alkylating agents was compared to the response of the parent cell lines to the same drug. The Raji/HN2 line was 7-fold resistant to nitrogen mustard and about 3-fold resistant to 4-hydroxycyclophosphamide, but it was not resistant to N,N'-bis(2-chloroethyl)-N-nitrosourea (BCNU), melphalan (MEL), busulfan, trimethylenemethanolophosphoramide, 4-hydroperoxyifosfamide, or cisplatin [cis-diaminedichloroplatinum(II)] (CDDP). The Raji/BCNU line was 5.3-fold resistant to BCNU and 4-fold resistant to both MEL and CDDP. The Raji/CP line was 7-fold resistant to CDDP and 3-fold resistant to both nitrogen mustard and BCNU, but it was not resistant to busulfan, trimethylenemethanolophosphoramide, or 4-hydroperoxyifosfamide. The SCC-25/CP line, which was 12-fold resistant to CDDP, was 5-fold resistant to MEL, and 3-fold resistant to 4-hydroxycyclophosphamide. The SCC-25 line was almost 24-fold resistant to methotrexate after 30-min treatment and about 7-fold resistant to methotrexate after continuous treatment. None of the other cell lines was resistant to methotrexate. The survival of SCC-25 and SCC-25/CP cells exposed to several antineoplastic agents was examined over several logs of survival. The SCC-25/CP cells are highly resistant to CDDP; the ratio of the slopes of the survival curves (SCC-25/CP to SCC-25) of the two lines was 43. At survivals of 1%, resistance to MEL and BCNU became evident in the SCC-25/CP line. At survivals of 0.1%, resistance to mitomycin C and, to a lesser degree, to Adriamycin and vincristine was evident. It is more difficult to produce resistance to alkylating agents, even with extended selection pressure, than to other antineoplastic drugs such as antimetabolites and natural products. We found no evidence of pleiotropic resistance in any alkylating agent-resistant cell line. Our results suggest that a judicious choice of alkylating agents given in sequential or concurrent combination may be a rational treatment strategy with potential applications in the clinic.

INTRODUCTION

The alkylating agents represent one of the most important classes of antitumor agents (1) and play a major role in combination with other agents in the curative chemotherapy of selected human cancers (2). The introduction of CDDP, a nonclassical alkylating agent, has further extended the efficacy of alkylating agents in the clinic (3). The clinically effective alkylating agents are bifunctional; that is, they have the capacity to form two covalent linkages. While they may combine with many nucleophilic sites within the cell, there is compelling evidence that the cytotoxicity of many alkylating agents is the result of DNA monoaadt formation followed by inter- or intranstrand cross-links in DNA (4–7). The kinetics of monoaadt and cross-link formation, the nature and sites of binding to nucleic acid bases, and the efficiency and kinetics of repair vary substantially among the alkylating agents (4–7). Similarly, other determinants of drug action, such as plasma membrane transport and intracellular biotransformation (activation or inactivation), vary substantially among alkylating agents (8–11). Nevertheless, it has been widely assumed that there is cross-resistance among the alkylating agents. If this were true, all alkylating agents would be essentially equivalent except for differences based on differing routes of administration and some differences, generally slight, in side effects and antitumor spectrum. On the other hand, absence of cross-resistance among the alkylating agents would have substantial therapeutic implications, among which the most important would be the possibility of using these drugs in sequence, in combination, or in high-dose combination in the bone marrow transplant setting.

Recently developed information concerning the mechanisms of action and resistance of alkylating agents indicates substantial heterogeneity among these agents (12). Resistance to individual alkylating agents in L1210 and P388 mouse leukemia by selection pressure in vivo was described in 1978 by Schabel and coworkers (13). In these systems, in general, there was no cross-resistance among the alkylating agents. More recently, we have used selection pressure in culture to develop alkylating agent resistance in two human tumor cell lines (12). In this paper, the cross-resistance patterns of four of the resistant cell lines to antineoplastic agents from the various classes of drugs are examined. The response of a CDDP-resistant head and neck squamous cell carcinoma cell line (SCC-25/CP) to several anticancer drugs is also examined in detail.

MATERIALS AND METHODS

Drugs. HN2, BCNU, MEL, ADR, FU, VCR, and thiotEPA were obtained from the Dana-Farber Cancer Institute pharmacy. HN2 as the hydrochloride salt was resuspended in 0.1 M HCl. In this form it remains stable for up to 1 yr at 0–8°C (12). Aliquots were thawed and used immediately. BCNU lyophilized powder was resuspended in 95% ethanol and stored, protected from light, at 4°C. This preparation results in 10% degradation in 78 days (14). MEL was dissolved in HCl-acidified ethanol and diluted in serum-free DME just before use. FU and VCR were diluted with DME just before use. CDDP pure powder was a gift from Johnson-Matthey, Malvern, PA. HO2-CPA was kindly provided in the powder form by M. Colvin of Johns Hopkins University and was prepared in DME just before use. MitoC was purchased from Sigma Chemical Co., St. Louis, MO. Both ADR and MitoC were resuspended in H2O and diluted with DME just before use. MTX was a gift from the Pharmaceutical Resource Branch, National Cancer Institute, Bethesda, MD. MTX and thiotEPA were dissolved in H2O and diluted with DME just before use. Busulfan, obtained from the Burroughs Wellcome Co., Research Triangle Park, NC, was dissolved in ethanol and diluted with DME just prior to use. HO2-IFA was obtained from M. Peukert of Asta-Werke AG, Federal Republic of West Germany, and prepared in DME just prior to use.
Cells. The Raji cell line, derived from a human Burkitt lymphoma, was obtained from H. Lazarus of the Miami Comprehensive Cancer Center. Cells were grown in suspension in DME supplemented with 10% fetal bovine serum, l-glutamine (292 μg/ml), penicillin (10 μg/ml), and streptomycin (100 μg/ml) in 8% CO2/92% air at 37°C. The SCC-25 cell line was derived from biopsy of a human squamous cell carcinoma of the tongue and was established and characterized initially by J. G. Rheinwald at this Institute (15). Monolayers were maintained in the DME supplemented with 20% fetal bovine serum and antibiotics. For the SCC-25 line, hydrocortisone (0.4 μg/ml) was included in the medium (12).

Production of Resistance. Two drug schedules were used to develop resistant cell lines: intermittent and continuous (daily). For the intermittent schedule, the SCC-25 cells (2 x 10⁶ cells per 100-mm dish) and Raji cells (5 x 10⁶ cells per ml) were treated for 30 min with the IC₅₀ concentration of the drug. The cultures were observed daily and allowed to grow until they reached the initial density or greater as determined by trypsin blue exclusion (Raji) or microscopic observation (SCC-25). For the Raji cells, the concentration was increased primarily on the basis of recovery time. For short times (1 to 2 wk) a 1.5- to 2-fold increase in concentration was made; for recoveries of 2 to 4 wk, the increase was smaller (10 to 50%); and for recoveries exceeding 4 wk, the concentration was either kept the same or decreased. For the SCC-25 cells, concentration changes were based on the reduction and recovery of colony number and size. For continuous (daily) exposure of Raji and SCC-25 cells, daily “pulsing” for 30 min with selected alkylating agents in serum-free medium was used. The resistant lines are treated weekly to maintain the resistance.

Cloning. The cloning of resistant populations of Raji cells was accomplished by the limiting dilution technique (16) and was performed at least twice to ensure clonal derivation. Daily microscopic observation confirmed that the clone was derived from a single cell. Cloning of SCC-25 monolayers was accomplished by isolation of a single large colony in a cloning chamber, followed by removal and trypan blue exclusion. Measurement of the IC₅₀ was made periodically with each clone to determine the degree of resistance.

Cytotoxicity. The drug concentration that reduced the number of viable cells to 50% of control (IC₅₀) was determined as follows. For the SCC-25 line, 1 x 10⁶ cells were plated on a 60-mm dish and allowed to attach overnight; 24 h later the medium was replaced with serum-free DME containing the drug. Treatment was terminated after 30 min by washing with DME and replacing with fresh medium. After 2 wk, the plates were fixed with phosphate-buffered 10% formalin, and the colonies were stained with 0.2% methylene blue. An appropriate concentration range was used. Raji cells were diluted to 5 x 10⁶ cells per ml in serum-free DME. Drugs were added, and the cells were incubated (30 min at 37°C), washed, and resuspended in fresh medium. The number of viable cells was determined on Day 3, initially by trypsin blue exclusion, then confirmed with a limiting dilution technique as a clonogenic assay for cytotoxicity (16).

Survival Curves. SCC-25 and SCC-25/CP cells in exponential growth were treated with various doses of the drugs described above. After exposure to the agent or vehicle for 1 h, the cells were washed 3 times with phosphate-buffered 0.9% saline solution and suspended by treatment with 0.25% trypsin/0.1% EDTA. The cells were plated in duplicate at 3 dilutions for colony formation. After 2 wk the colonies were visualized by staining with crystal violet, and colonies of 50 cells or greater were counted. The results were expressed as surviving fraction of treated cells compared to vehicle-treated control cells.

Data Analysis. Quantitative analysis of survival curves was performed using the log-probit iterative least-squares method of Litchfield and Wilcoxon (17) as revised by Tallarida (18). Calculations were performed on an Apple II+ microcomputer.

RESULTS

By repeatedly exposing cells to escalating doses of an alkylating agent, we have developed four human tumor cell lines which are relatively stably resistant to the drug with which the culture was treated. The response of these cell lines to a variety of alkylating agents was compared to the response of the parent cell lines to the same drug. The ratios of the drug concentrations needed to reduce the survival of the resistant lines and the parent lines to 50% are shown in Table 1. The Raji/BCNU cell line which was 5.3-fold resistant to BCNU was appreciably cross-resistant (4-fold) to both MEL and CDDP. The Raji/HN2 cells were almost 7-fold resistant to HN2. These cells were about 3-fold resistant to HO₂-CPA. Almost no resistance was observed with BCNU, MEL, busulfan, thiotEPA, HO₂-IFA, or CDDP. The Raji/CP cell line was about 7-fold resistant to CDDP; it was also about 3-fold resistant to both HN2 and BCNU. This cell line was not resistant to busulfan, thiotEPA, or HO₂-IFA. The SCC-25/CP cell line which was 12-fold resistant to CDDP was also 5-fold resistant to MEL and almost 3-fold resistant to HO₂-CPA. Most often cross-resistance developed to MEL and HO₂-CPA. Resistance to HO₂-CPA did not correlate with resistance to HO₂-IFA. Cross-resistance with busulfan, thiotEPA, or HO₂-IFA was not observed in any of the four cell lines.

Very little cross-resistance (pleiotropic resistance) was observed with any of the four alkylating agent-resistant cell lines to nonalkylating agent drugs (Table 2). All of the alkylating agent-resistant lines were more sensitive (collaterally sensitive) than the parent lines to ADR after short (30 min) or continuous exposure to the drug. To a lesser degree, all of the alkylating agent-resistant cell lines were also more sensitive to VCR than the parent cell lines. On the other hand, the SCC-25/CP cell line was almost 24-fold resistant to MTX (30-min treatment) and about 7-fold resistant to MTX (continuous treatment). None of the other cell lines including the Raji/CP line was resistant to MTX.

We have examined in greater detail the survival of SCC-25 and SCC-25/CP cells exposed for 1 h to a range of concentrations of drugs from various classes of antineoplastic agents. From the data in Fig. 1, it is evident that the SCC-25/CP cells are highly resistant to CDDP. At 100 μM of drug there was a 4-log kill in the SCC-25 cell line and only 50% kill in the SCC-25/CP cell line. Twenty % of the SCC-25/CP cells survived treatment with 500 μM CDDP. The ratio of the slopes of the survival curves (SCC-25/CP to SCC-25) of the two cell lines was 43. Thus the degree of resistance varies depending upon the assay method used. The curvilinear nature of the dose-response curve for the resistant line in Fig. 1, as compared to the sensitive line, leads to a determination of 12-fold resistance at the IC₅₀, but substantially greater resistance when slopes or survivals are compared at concentrations greater than the IC₅₀. The response of these cells to treatment with other alkylating agents is shown in Fig. 2. After 1-h exposure to 250 μM MEL, there was a 2-log kill of SCC-25 cells, and there was a 1-log kill of SCC-25/CP cells. This 1-log differential persisted at 500 μM MEL. The ratio of the survival curve initial slopes for this drug was 3, and the ratio of the terminal slopes was 1.1, indicating that, at high drug concentrations, the remaining populations of cells in both cell lines are equally sensitive to MEL. Although at low drug concentrations there was no difference in the survival of SCC-25 and SCC-25/CP cells exposed to BCNU, at higher drug concentrations the survival curves diverged, so that at 500 μM BCNU there was >1-log differential in cell kill between the SCC-25 and SCC-25/CP cells. The ratio of the terminal slopes of these survival curves was 3.6. Busulfan, after 1-h exposure, was not a very effective cytotoxic agent in these cell lines. There was no difference in cell survival between the SCC-25 and SCC-25/CP cells after treatment with busulfan. At low concentrations of MitoC, the ratio of the slopes of the

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Table 1 Resistance ratios to various alkylating agents

The resistance ratio equals the IC_{50} of the resistant line divided by the IC_{50} of the parental line. The IC_{50} (µM) for the Raji parental line were: HN2, 2.6; MEL, 20; BCNU, 30; HO2-CPA, 18; busulfan, 6.6; thiotePA, 2.4; CDDP, 30; HO2-IFA, 23.3; Mitoc^* 10.2; and Mitoc^* 0.042. The IC_{50} (µM) for the SCC-25 parent line were: HN2, 11; MEL, 8; BCNU, 200; HO2-CPA, 25; busulfan, 15.5; thiotePA, 2.4; CDDP, 17; HO2-IFA, 25; Mitoc^* 3.3; and Mitoc^* 0.004.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>HN2</th>
<th>MEL</th>
<th>BCNU</th>
<th>HO2-CPA</th>
<th>Busulfan</th>
<th>ThiotePA</th>
<th>CDDP</th>
<th>HO2-IFA</th>
<th>Mitoc^*</th>
<th>Mitoc^*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raji/BCNU</td>
<td>1.9</td>
<td>4.0</td>
<td>5.3</td>
<td>1.6</td>
<td>1.2</td>
<td>1.6</td>
<td>4.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Raji/HN2</td>
<td>6.6</td>
<td>1.4</td>
<td>1.9</td>
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<td>1.0</td>
<td>0.9</td>
<td>1.5</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Raji/CP</td>
<td>3.2</td>
<td>1.3</td>
<td>2.6</td>
<td>1.0</td>
<td>1.7</td>
<td>1.0</td>
<td>1.7</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>SCC-25/CP</td>
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<td>5.0</td>
<td>2.0</td>
<td>2.8</td>
<td>1.0</td>
<td>1.7</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
</tr>
</tbody>
</table>

* Drug exposure for 30 min.
* Drug exposure continuous.
* Drug exposure for 24 h.

Table 2 Resistance ratios to various antineoplastic agents

The resistance ratio equals the IC_{50} of the resistant line divided by the IC_{50} of the parental line. The IC_{50} (µM) for the Raji parental line were: ADR, 0.66; ADR, 0.04; VCR, 0.11; FU, 44; MTX, 41; and MTX, 0.022. The IC_{50} (µM) for the SCC-25 parental line were: ADR, 2.8; ADR, 0.013; VCR, 0.21; FU, 52; MTX, 34; and MTX, 0.006.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>ADR</th>
<th>ADR</th>
<th>VCR</th>
<th>FU</th>
<th>MTX</th>
<th>MTX</th>
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<tr>
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<td>0.3</td>
<td>0.7</td>
<td>1.0</td>
<td>1.4</td>
<td></td>
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<tr>
<td>Raji/HN2</td>
<td>0.3</td>
<td>0.5</td>
<td>0.7</td>
<td>3.4</td>
<td>1.2</td>
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<tr>
<td>Raji/CP</td>
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<td>0.5</td>
<td>0.2</td>
<td>1.9</td>
<td>1.6</td>
<td>1.5</td>
</tr>
<tr>
<td>SCC-25/CP</td>
<td>0.6</td>
<td>0.4</td>
<td>0.9</td>
<td>0.6</td>
<td>23.8</td>
<td>7.3</td>
</tr>
</tbody>
</table>

* Drug exposure for 30 min.
* Drug exposure continuous.
* Drug exposure for 24 h.

Fig. 1. Survival of SCC-25 (●) and SCC-25/CP (○) cells exposed for 1 h to various concentrations of CDDP. Survival was measured by colony formation. Points, mean for three independent experiments; bars, SE.

The survival curves for the SCC-25/CP and SCC-25 cell lines was 8. At a concentration of 50 µM there was a 2.5-log greater kill of the SCC-25 cells than of the SCC-25/CP cells. The ratio of the terminal slopes of the curves was 11.3, indicating continued resistance in the SCC-25/CP cells even at very high drug levels.

From the 50% inhibitory concentrations shown in Table 2, it appears that all of the alkylating agent-resistant lines are somewhat more sensitive to ADR than are the parent Raji and SCC-25 lines. When the survival of SCC-25/CP and SCC-25 cells treated with ADR was examined over several logs, the results shown in Fig. 3 were observed. Only after a 2-log kill did the survival curves begin to diverge, and then only slightly. The ratio of the terminal slopes of the survival curves (SCC-25/CP to SCC-25) is 1.5. As was indicated in Table 2 from the 50% inhibitory concentrations, the resistant cell lines are slightly more sensitive to VCR than are the parent cell lines. Through the first two logs of cell kill on Fig. 3 the survival of the VCR-treated SCC-25 and SCC-25/CP cells was identical. In the terminal phase of these dose-response curves, the SCC-25/CP cells showed less sensitivity to this drug than did the SCC-25 cells. The ratio of the terminal slopes was 2.3. As is evident from Fig. 4, there was no difference in the survival of SCC-25 and SCC-25/CP cells treated with FU. The SCC-25/CP cell line was highly resistant to MTX. At a dose of 150 µM, there was a 2-log kill in the SCC-25 cell line, and there was a 50% kill in the SCC-25/CP cell line. At 500 µM MTX, there was a nearly 3-log kill of SCC-25 cells while 30% of the SCC-25/CP cells survived. The ratio of the slopes of the survival curves at low drug concentrations was 14.4, and the ratio of the slopes of the terminal portions of the curve was 3.7. We have shown that a cell line which is highly resistant to CDDP (>10% of the cells are not killed by 500 µM CDDP) contains a population (about 3% of the cells) which is highly resistant to MEL, a population (about 3% of the cells) which is highly resistant to BCNU, a population (about 0.04% of the cells) which is...
highly resistant to MitoC, and a population (about 30% of the cells) which is highly resistant to MTX.

**DISCUSSION**

It is more difficult to produce resistance to alkylating agents than to other antineoplastic drugs, such as antimetabolites and natural products, under conditions of extended selection pressure. We have been able to produce only relatively low resistance (≤12-fold when measured by $IC_{50}$) to alkylating agents as compared to the many hundredfold resistance which can be produced to other antitumor agents (12). This has major therapeutic implications. It is well known experimentally and clinically that the dose-response curves for alkylating agents are steep (13). In the autologous marrow transplant setting, a 5- to 15-fold increase in alkylating agent dosage is possible (19). Thus, intensive treatment with alkylating agents (for example, a 10-fold increase in dose) ought to produce major, or perhaps total, cell kill, since drug-resistant clones in a heterogeneous population should rarely exceed 10-fold resistance. On the other hand, for nonalkylating agents, much higher levels of resistance are possible, suggesting that dose intensification designed for cytoreduction may be a less realistic goal than with the alkylating agents. This is borne out by the fact that a linear relationship between dose and log cell kill through some 4 to 5 logs is generally maintained for the alkylating agents in the drug-sensitive lines (compare Figs. 1 and 2), whereas it is generally curvilinear with the nonalkylating agents (note particularly MTX, FU, and VCR). Indeed, with MTX and VCR, for example, at 4-log and 2-log kill, respectively, further increase in dose would not be expected to provide greater cytoreduction. The lack of cross-resistance between HO2-CPA and HO2-IFA indicates that even small change in molecular structure can lead to alkylating agents with differing resistance mechanisms.

The observation that resistance to a given antitumor agent may impart pleiotropic resistance to a number of seemingly unrelated agents has major therapeutic implications (20). We examined the alkylating agent-resistant lines for cross-resistance to several nonalkylating agent antitumor agents including ADR, MTX, VCR, and FU. We found no evidence of pleiotropic resistance in any alkylating agent-resistant cell line except for the cross-resistance of the SCC-25/CP line to MTX. The fact that most of the alkylating agent-resistant lines are collaboratively sensitive to other antitumor agents was reassuring, since the latter are commonly used in combination with alkylating agents, either concurrently or sequentially (21).

Finally, the relationship of cell kill carried out over 3 to 4 logs with increasing concentration of drug may provide information not evident when 50% inhibitory concentrations determined over a 1-log range are used. For the SCC-25 line, there was generally a log linear relationship between cell kill and increasing concentration for the alkylating agents CDDP, BCNU, MEL, and MitoC. This is important, since for nonalkylating agents there is a tendency, at increasing doses, for a reduced fractional cell kill that may occur as early as 1 or 2 logs. The data for the parent SCC-25 cell line follow the classical steep dose-response relationship for alkylating agents and thus support the idea of dose intensification therapeutic strategies with these drugs. However, there was a consistent difference between the SCC-25 and SCC-25/CP cell lines. For the CDDP-resistant line, whether treated with CDDP or the other alkylating agents, there was a loss of linearity and, therefore, lower fractional kill with increasing concentrations of drug. For example, with CDDP, resistance was 20-fold at the 50% survival level, 40-fold at the 20% survival level, and much greater at lower levels in comparison with the parent cell line. The differences, while not nearly so great, were nevertheless present for the other alkylating agents, adding a measure of complexity to the interpretation of cross-resistance patterns and suggesting greater heterogeneity among tumor cell lines not only specifically with respect to alkylating agent resistance, but perhaps more generally with respect to cytokinetic, cytogenetic, and biochemical properties not directly linked to alkylating agent resistance. Since alkylating agents are mutagenic, cell lines made resistant to an alkylating agent might be expected to exhibit more heterogeneity than the parent lines (22).

Clinical cancer chemotherapy is now in a position to aim for the curative treatment of solid tumors. To achieve "cure" it is necessary to eradicate essentially all the neoplastic stem cells in the body. Multidrug regimens using combinations of alkylating agents with a low degree of cross-resistance offer considerable theoretical promise in this regard, but there has been, until recently, a notable lack of experimentally based guidelines for the design of such regimens. The present in vitro studies indicate that cross-resistance among some of the alkylating agents is very low, while for others partial cross-resistance may occur. In Raji cells, for example, CDDP resistance and HN2...
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resistance are associated with minimal cross-resistance to any of six other alkylating agents, while BCNU resistance is associated with cross-resistance to both CDDP and MEL. Moreover, the pattern of cross-resistance may not be the same in every type of tumor cell, as illustrated by the finding of cross-over, the pattern of cross-resistance may not be the same in six other alkylating agents, while BCNU resistance is associated with minimal cross-resistance to any of these agents. For example, there is a suggestion from our data that BCNU, HN2, and CDDP resistance in Raji cells is associated with enhanced sensitivity to ADR. On the other hand, CDDP resistance in SCC-25 cells seems to be associated with a decrease in sensitivity to MTX that would argue against a sequential use of these two agents with CDDP preceding MTX. Studies are currently in progress in our laboratory to elucidate the basis of these patterns of altered nonalkylating agent sensitivity in alkylating agent-resistant cell lines. In vitro studies of this type should make it possible to ultimately design new rational combinations of alkylating and nonalkylating drugs for the treatment of solid tumors in patients.

REFERENCES

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