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Development of alkylating agent-resistant human tumor cell lines*

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Summary. Survival curves and dose escalation studies of four representative human tumor cell lines exposed to the various alkylating agents are presented. With HN2, at a level of one log of cell kill there was a fivefold range in drug concentration required to achieve this degree of cell kill among the cell lines, from 4.5 μ*M* for the SL6 lung adenocarcinoma to 22 μ*M* for the SW2 small-cell lung carcinoma. Four logs of SCC-25 squamous carcinoma cells were killed by 100 μ*M* CDDP; however, there was only about one log of SL6 cells killed by 500 μ*M* CDDP. To kill one log of G3361 melanoma cells required 380 μ*M* 4-HC and to kill one log of SCC-25 cells required 24 μ*M*, approximately a 16-fold difference. The curves for cell kill by L-PAM appeared to be biphasic, with a break at about 100 μ*M*. There was about a threefold range in drug concentration required to achieve one log of cell kill with L-PAM, from 60 μ*M* in the SCC-25 cell line to 18 μ*M* in the SW2 line. To kill one log of SCC-25 cells required 295 μ*M* BCNU and to kill one log of SW2 cell required 120 μ*M*, about a 2.5-fold difference. The range of maximally tolerated HN2 concentrations were from 1200 μ*M* for the SL6 cell line, 48 times the initial concentration, to 300 μ*M* for the SCC-25 line, 16 times the initial concentration. The G3361 line tolerated the highest level of CDDP, 1900 μ*M*, 48 times the initial concentration. The SCC-25 line, on the other hand, tolerated only 600 μ*M*, 30 times the initial concentration. The SL6 cell line maximally tolerated 36 times the initial concentration of 4-HC (1450 μ*M*), whereas the SCC-25 cell line tolerated only 18 times the initial concentration (720 μ*M*). The G3361 melanoma tolerated 1555 μ*M*, 30 times the initial concentration of L-PAM, and the SCC-25 cell line tolerated 700 μ*M*, 14 times the initial concentration. The SL6 cell line tolerated the highest concentration of BCNU, 4200 μ*M*, 24 times the initial concentration. The SCC-25 cell line tolerated 1450 μ*M*, 8 times the initial concentration. In all cases, the SCC-25 cell line was least able to tolerate exposure to increasing concentrations of alkylating agents. The SL6 and G3361 cell lines showed the greatest tolerance for increasing concentrations of alkylating agents. With maximal selection pressure, in terms of intensity and duration, 5- to 15-fold resistance at best could be produced to these alkylating agents. This contrasts with other drugs, indicates that alkylating agents are more like X-rays, and has implications for high-dose clinical treatments. The importance of these findings to the clinical treatment of cancer is discussed.

Introduction

Alkylating agents are the oldest and, as a group, the most heterogeneous class of antineoplastic agents in clinical use. The advent of autologous bone marrow transplantation as a clinical strategy has allowed physicians to escalate doses of some alkylating agents manyfold beyond the doses achievable prior to bone marrow transplantation [13]. This methodology provides a potential for extending the spectrum and effectiveness of alkylating agents and has revived interest in these drugs in both the laboratory and the clinic [14, 15]. The tools of modern cell biology are being applied to questions regarding the action of alkylating agents in cells and the reaction of cells to exposure to these drugs. These studies, in addition to the classic work of earlier investigators, are providing a clearer picture of the extent, variety, and specificity of the responses which cells can mount upon exposure to an alkylating agent.

It is becoming more generally accepted that, in most cases, the resistance of cells to alkylating agents is multifactorial, i.e., that more than one change has occurred in the cellular biology in order to produce resistance and that DNA is the critical target for alkylation. The chemistry of alkylation has recently been reviewed [7, 29]. Goldie and Coldman have developed a mathematical model that describes the emergence of resistance in a tumor cell population and suggests that the use of sequential non-cross-resistant therapies would suppress the emergence of resistance and lead to an optimal treatment outcome [6, 20, 21]. Although the mathematical model cannot account fully for the diversity of the levels of resistance which may occur in tumors, it does lead to strategies that may be useful in circumventing drug resistance in the clinic [60]. Clinically, resistance may result from not only changes in cell biology but also physiological factors within the tumor, including

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Abbreviations: Nitrogen mustard (mustragen), HN2; *cis*-diamminedichloroplatinum(II) (cisplatin), CDDP; N,N'-bis(2-chloroethyl)-N-nitrosourea, BCNU; L-phenylalanine mustard (melphalan), L-PAM; 4-hydroperoxycyclophosphamide, 4-HC; Dulbecco's modified Eagle's medium, DMEM; fetal bovine serum, FBS; phosphate-buffered saline, PBS

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nutritional status, hypoxia, and heterogeneity of drug penetration into the tumor.

We have attempted to produce a panel of alkylating agent-resistant human tumor cell lines from four representative solid tumor types: melanoma, lung adenocarcinoma, small-cell lung carcinoma, and head and neck carcinoma. These cell lines were produced by a systematic dose escalation process over more than 1 year. We now report the results of our dose escalation protocol and discuss our findings in the context of other alkylating agent-resistant cell lines as well as of antineoplastic agent resistance in general.

Materials and methods

Drugs

HN2, BCNU, and L-PAM 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 year at −20° C [17]. 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 [9]. L-PAM was dissolved in HCl-acidified ethanol and diluted in serum-free DMEM just before use. CDDP as a pure powder was a gift from Johnson Matthey (Malvern, Pa, USA). 4-HC was kindly provided in powder form by M. Colvin of Johns Hopkins University (Baltimore, Md, USA) and was prepared in DMEM just prior to use.

Cell lines

G3361 melanoma. This cell line was derived from a biopsy of human melanoma, from a single cell that had been cloned in soft agar or methylcellulose. It has a 100% plating efficiency in soft agar. This line is heavily pigmented and has a human polyploid karyotype. A high level of tyrosinease activity in the melanin and melanin grain microstructure indicates that this cell line retains its differentiated phenotype [58]. The G3361 cell line was grown in RPMI 1640 medium (Grand Island Biological Co., Grand Island, NY, USA) supplemented with 10% FBS (Sterile Systems, Logan, Utah, USA) and antibiotics.

SCC-25 squamous cell carcinoma. This cell line retains an epithelioid appearance and grows without the aid of a feeder layer [38]. It has a plating efficiency of about 10% as judged by plating single colonies on plastic. The cells were grown in DMEM supplemented with 5% FBS and antibiotics. For the SCC-25 line, hydrocortisone (0.4 μg/ml) was included in the medium [17].

SW2 small-cell lung carcinoma. Pleural fluid was obtained from a patient with small-cell carcinoma by a pleural tap for symptomatic relief of breathlessness. The malignant cells were perpetuated as a continuous cell line [11, 12]. After more than 1 year of serial passages, the cells continued to grow as spheroids in suspension culture. The growth properties, enzyme activity, and response to dibutyryl cyclic AMP treatment have been reported [12]. The cells were characterized as small-cell type by the identification of cytoplasmic dense-cored vesicles by electron microscopy, as well as the presence of desmosomes and tight junctions between cells that distinguish small-cell carcinomas from lymphoblastoid and myelogenous cells. APUD (amine precursor uptake and decarboxylation) characteristics were noted. The cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (Sterile Systems) and antibiotics. They grew exponentially as enlarging spheroids with a doubling time of 2–4 days, eventually reaching a plateau by day 30. The spheroids were then dispersed as single cells, which began exponential growth after a lag period of about 2 days.

SL6 lung adenocarcinoma. The SL6 cell line was developed from a lung mass from a male patient with large-cell carcinoma who had no prior treatment. This cell line grew in RPMI 1640 medium supplemented with 10% FBS and antibiotics.

Survival curves

Cells in exponential growth were treated with various doses of the drugs. After exposure to the agent or vehicle for 1 h in media without serum, the cells were washed three times with 0.09% PBS solution and suspended by treatment with 0.25% trypsin/0.1% EDTA. The cells were plated in duplicate at three dilutions for colony formation. After 2 weeks the colonies were visualized by staining with crystal violet, and colonies of 50 cells or more were counted. SW2 cells were treated as single-cell suspensions and plated in 2% agar for colony formation. The results were expressed as the surviving fraction of treated cells compared to vehicle-treated control cells.

Data analysis

Quantitative analysis of survival curves was carried out using the log-probit iterative least-squares method of Litchfield and Wilcoxon [32] as revised by Tallarida [50]. Calculations were performed on an Apple II+ microcomputer.

Dose escalation experiments

Nearly confluent 100-mm^3 dishes were treated with approximately the concentration of each drug that would kill 90% of the cells for 1 h, were washed three times with 0.09% PBS, then covered with fresh medium plus serum. The dose of the alkylating agent treatment was escalated at a rate of 15%–20% per week, and the cells were treated weekly unless there was no evidence of cell growth between treatments. The cells were „rested" (i.e., not treated) only if there was danger of losing the line. Repeated attempts were made to escalate the drug treatment beyond the plateau concentrations. After 14 months of treatment, attempts were made to clone alkylating agent-resistant sublines from the treated cultures.

Results

The survival for four representative human tumor cell lines exposed to the various alkylating agents for 1 h over a concentration range is shown in Fig. 1. HN2, from 1–50 μ*M*, killed up to 3.5 logs of SCC-25 squamous carcinoma cells. At a level of one log of cell kill, there was a fivefold range in drug concentration required to achieve this degree of cell kill, from 4.5 μ*M* for the SL6 lung adenocarcinoma to 22 μ*M* for the SW2 small-cell lung carcinoma. There was a wide range of sensitivity among the four cell lines to CDDP, from 1–500 μ*M*. Four logs of SCC-25 squamous carcinoma cells were killed by 100 μ*M* CDDP;

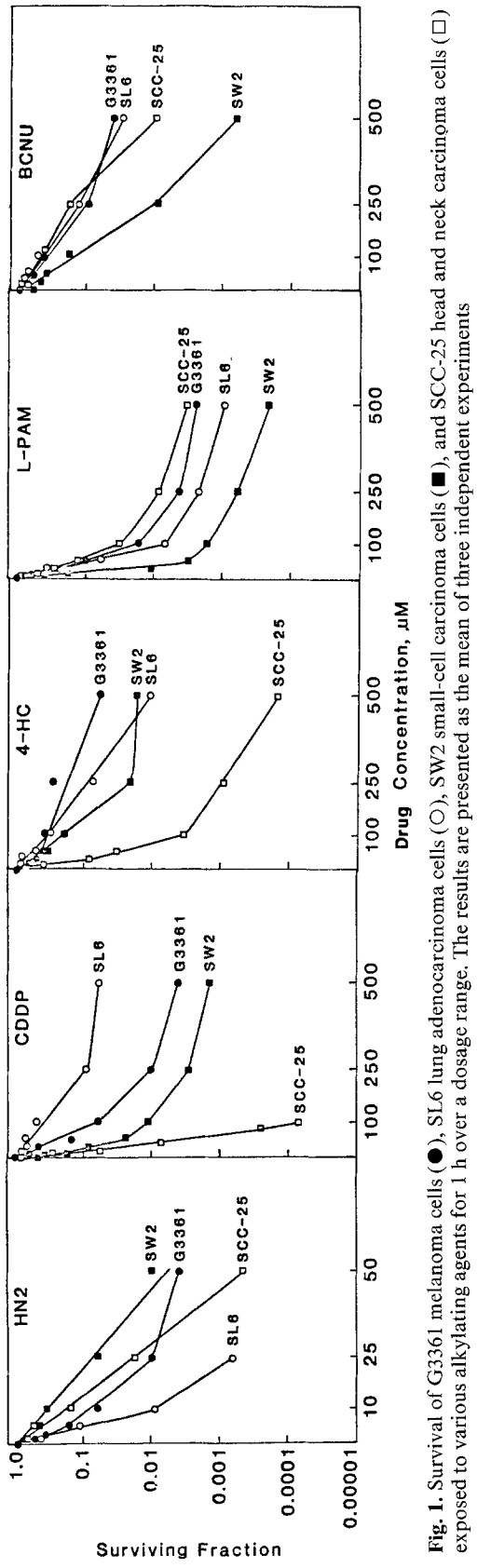

Fig. 1. Survival of G3361 melanoma cells (●), SL6 lung adenocarcinoma cells (○), SW2 small-cell carcinoma cells (■), and SCC-25 head and neck carcinoma cells (□) exposed to various alkylating agents for 1 h over a dosage range. The results are presented as the mean of three independent experiments

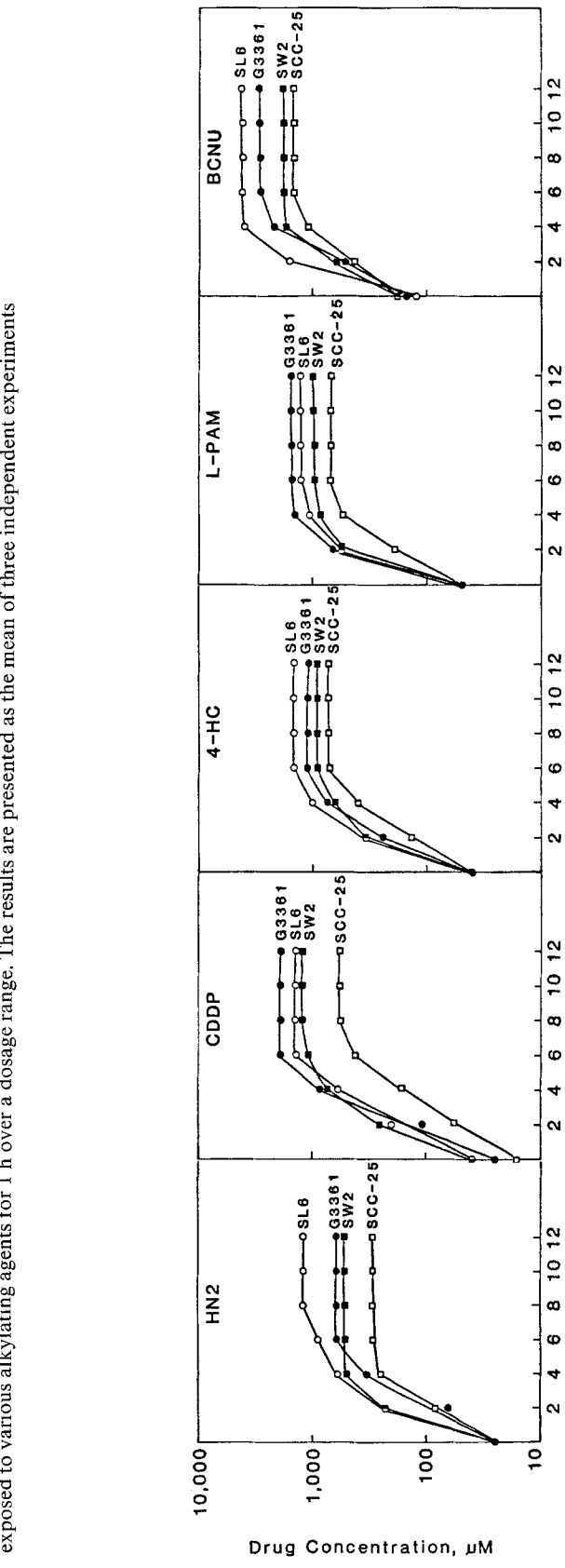

Fig. 2. Time course of drug concentration escalation for the G3361 melanoma cell line (●), SL6 lung adenocarcinoma cell line (○), SW2 small-cell carcinoma cell line (■), and the SCC-25 head and neck carcinoma cell line (□) exposed to the various alkylating agents

however, there was only about one log of SL6 lung adenocarcinoma cells killed by 500 μ*M*. At a level of one log of cell kill, there was about a 13-fold range in the CDDP concentration required to achieve this degree of cell kill, from 18 μ*M* for the SCC-25 cell line to 240 μ*M* for the SL6 lung adenocarcinoma. 4-HC, which is used as an activated analog of cyclophosphamide, killed almost four logs of SCC-25 squamous carcinoma cells at a concentration of 500 μ*M*. To kill one log of G3361 melanoma cells required 380 μ*M* 4-HC, and to kill one log of SCC-25 squamous carcinoma cells required 24 μ*M*, approximately a 16-fold difference. The SW2 small-cell carcinoma was most sensitive to L-PAM, with nearly four logs of cell kill at 500 μ*M*. The curves for cell kill by L-PAM appeared to be biphasic, with a break at about 100 μ*M*. At a level of one log of cell kill, there was about a threefold range in drug concentration required to achieve this degree of cell kill, from 60 μ*M* in the SCC-25 squamous carcinoma cell line to 18 μ*M* in the SW2 small-cell lung carcinoma line. The SW2 cell line was also more sensitive to the cytotoxic effects of BCNU than the three other cell lines. There were about three logs of kill of SW2 cells by BCNU with 500 μ*M* of the drug. To kill one log of SCC-25 squamous carcinoma cells required 295 μ*M* BCNU, and to kill one log of SW2 cells required 120 μ*M*, about a 2.5-fold difference.

The results of the dose escalation experiments are shown in Fig. 2. It took 4–8 months to reach a plateau concentration, beyond which dose escalation resulted in cell death, for the alkylating agent HN2. The final, maximally tolerated drug concentrations of 1200 μ*M* and 300 μ*M*, were, respectively, 48 times the initial concentration for the SL6 lung adenocarcinoma cell line and 16 times the initial concentration for the SCC-25 squamous carcinoma cell line. The four cell lines reached the plateau in dose escalation of CDDP after 6–8 months of treatment. The G3361 melanoma tolerated 1900 μ*M* CDDP, 48 times the initial concentration. The SCC-25 squamous carcinoma tolerated 600 μ*M* CDDP, 30 times the initial concentration. All of the cells reached the plateau in dose escalation of 4-HC 6 months after initiation of treatment. The SL6 lung adenocarcinoma cell line maximally tolerated 36 times the initial concentration, plateauing at 1450 μ*M*. The SCC-25 squamous carcinoma cell line maximally tolerated 18 times the initial concentration, plateauing at 720 μ*M*. Four to six months were required to escalate the dose of L-PAM to the maximally tolerated concentration. The G3361 melanoma tolerated 1555 μ*M* of the drug, 30 times the initial concentration. The SCC-25 cell line tolerated 700 μ*M* of the drug, 14 times the initial concentration. Plateau levels of BCNU dose escalation were reached in 4–6 months. The SL6 lung adenocarcinoma cell line tolerated 4200 μ*M* BCNU, 24 times the initial concentration. The SCC-25 squamous carcinoma cell line tolerated 1450 μ*M* BCNU, 8 times the initial concentration.

In all cases, the SCC-25 squamous carcinoma cell line was least able to tolerate exposure to increasing concentrations of alkylating agents. The SL6 lung adenocarcinoma and the G3361 melanoma showed the greatest tolerance for increasing concentrations of alkylating agents.

Discussion

The production of drug-resistant cell lines through increasing selection pressure has been a very successful means of producing resistance to antitumor antimetabolites such as methotrexate [16, 44] and antitumor antibiotics such as adriamycin (Fig. 3) [28, 55]. This methodology has been much more difficult to apply to the alkylating agents because of their generally short lifetimes in solution; however, a number of alkylating agent-resistant cell lines have been produced (Table 1). Goldenberg and Alexander have been able to isolate single clones from the L5178Y murine leukemia cell line with differing levels of sensitivity to

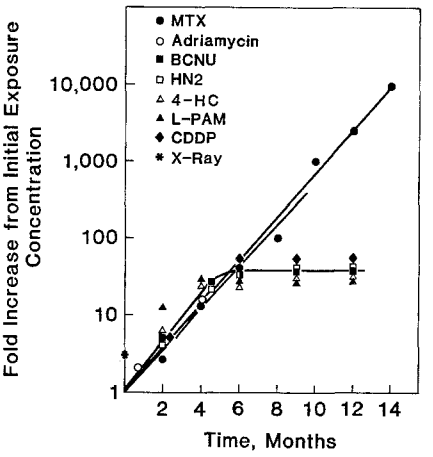

Fig. 3. The development of resistance to various antineoplastic agents using selection pressure. The methotrexate line is based on data from Frei et al. [16]. The adriamycin line is based on data from Twentyman et al. [55] and Vrignaud et al. [56]. The point shown for X-rays is under hypoxic conditions. The alkylating agent line is based on the results presented in this report

Table 1. Alkylating agent-resistant cell and tumor lines

Parent line	Species	Drug used for selection pressure	Refs
Lines maintained in culture			
L5178Y leukemia	mouse	HN2	[18, 19]
Walker carcinoma	rat	chlorambucil	[54]
Walker carcinoma	rat	CB 1954	[54]
AUXB1 ovary	hamster	L-PAM	[10]
L1210 leukemia	mouse	L-PAM	[40–42]
L1210 leukemia	mouse	cyclophosphamide	[8]
9L gliosarcoma	rat	BCNU	[3]
L1210 leukemia	mouse	CDDP	[5]
P388 leukemia	mouse	CDDP	[5]
Raji/Burkitt lymphoma	human	HN2	[17]
Raji/Burkitt lymphoma	human	BCNU	[17]
SCC-25 squamous carcinoma	human	CDDP	[51, 52]
Raji/Burkitt lymphoma	human	CDDP	[51]
A2780 ovarian carcinoma	human	L-PAM	[26, 27]
A2780 ovarian carcinoma	human	CDDP	[27]
mer-MM253 melanoma	human	L-PAM	[35, 36]
mer-MM253 melanoma	human	DTIC	[35, 36]
mer-MM253 melanoma	human	cyclophosphamide	[4]
HCT 116 colon carcinoma	human	mitomycin C	[59]
K562 leukemia	human	CDDP	[46]
lymphoblasts	human	L-PAM	[34]
KFr ovarian carcinoma	human	CDDP	[31]

HN2 and dimethyl myleran [18, 19]. The S63 subline of the L5178Y cell line exhibited an approximately twofold resistance to HN2, as determined at the IC_{90} or from the slopes of the survival curves. Tisdale and Philips have established a Walker carcinoma cell line in vitro and subsequently developed one series of three cell lines resistant to different concentrations of chlorambucil and another series of four cell lines resistant to CB1954 by dose escalation [54]. These cell lines have subsequently been used extensively to study the cell biology of resistance to nitrogen mustards and the nitrosoureas [53, 57].

Elliot and Ling have used a single-step selection method to establish L-PAM-resistant sublines of the AUXB1 Chinese hamster ovary cell line [10]. Parental AUXB1 cells in exponential growth were exposed for 1 h to concentrations of L-PAM sufficient to kill approximately 6–7 logs of cells. The cells were then plated at 10^6 cells/100-mm^3 dish, and surviving colonies were grown. Two resistant clones, MEL^R1 and MEL^R6, were obtained from two different selections, and they occurred at a frequency of about 10^{-7}. The IC_{90} values of the resistant lines were fourfold higher than that of the parental line. The resistance of these lines was stable for at least 2 months in the absence of the drug. The mechanism(s) of resistance of these lines have been studied [1, 10].

Several alkylating agent-resistant L1210 murine leukemia cell lines were established by in vivo passage with drug treatment during each passage for approximately 1 year [40–42]. Subsequently, some of these resistant L1210 lines were adapted to culture. Redwood and Colvin [37] and Suzukake et al. [49] have used the Southern Research Institute L1210/LPAM line to study the cell biology of L-PAM resistance. DeWys has established a series of sublines of L1210 leukemia with different degrees of cyclophosphamide resistance by treatment of tumor-bearing mice with cyclophosphamide and serial transplant passage [8]. These lines have been used extensively in mechanistic studies related to cyclophosphamide resistance [30, 47, 48].

Bodell et al. [3] have treated Fisher 344 rats bearing intracerebral 9L gliosarcoma with a single dose of BCNU sufficient to kill approximately three logs of tumor cells. Three sublines were established, 9L-2, 9L-7, and 9L-8, which showed, respectively, approximately 3.5-, 1.6-, and 1.6-fold resistance to BCNU at the IC_{90} compared to the parental line.

Burchenal et al. [5] have produced L1210 and P388 murine leukemia lines resistant to CDDP by passage in mice treated with single doses of 8 mg/kg of the drug 24 h after inoculation over successive generations until no increase in survival time was seen with any tolerated dose of CDDP. This cell line has been examined in studies related to CDDP transport [23, 46].

More recently, attention has focused on the development of human tumor cell lines resistant to various alkylating agents. Dose escalation in vitro has been the most common methodology applied to this task. Frei et al. [17] have succeeded in producing Raji/Burkitt lymphoma sublines resistant to HN2, BCNU, and CDDP. The same laboratory has produced a SCC-25 human squamous carcinoma cell line that is stably resistant to CDDP [51, 52]. A group at the National Cancer Institute has developed sublines of the A2780 ovarian carcinoma cell line with resistance to L-PAM and to CDDP. These lines were developed by stepwise exposure of the parental A2780 line to increasing concentrations of each drug, up to a level of 10 μM L-PAM or 20 μM CDDP [27]. The 2780^{ME} line showed a tenfold resistance to L-PAM, and the 2780^{CP} line showed a 14-fold resistance to CDDP. These lines have been used in extensive studies in the cellular biology of resistance to these drugs [2, 27, 33].

Parsons et al. [35, 36] have developed several human melanoma sublines of the *mer*-MM253 line that exhibit 1.5- to 12.5-fold resistance to L-PAM, microsomally activated DTIC, MTIC, and MNNG. Boon and Parsons [4] have produced a human melanoma cell line resistant to microsomally activated cyclophosphamide by treating the parental MM253C1 cell line with activated cyclophosphamide, then with 0.2 μM MNNG, and finally again with activated cyclophosphamide. After each treatment, the surviving cells were allowed to recover to the original number before the next treatment. This methodology resulted in the development of the MM253C1-4CG subline, which shows an approximately a 1.5-fold resistance to activated cyclophosphamide by survival curve slope analysis compared to the MM253 parental cell line [4].

Willson et al. [59] have treated the HCT116 human colon carcinoma cell line repeatedly with 3 μM mitomycin C. The HCT116R11 cell line treated with 11 cycles of mitomycin C exhibited a 2.2-fold resistance to mitomycin C at the IC_{50}. The HCRT116R22 cell line treated with 22 cycles of mitomycin C showed 4.7-fold resistance at the IC_{50}. Shionaya et al. [46] have developed a human leukemia cell line K562DDP from the parental K562 cell line by selecting a surviving colony from K562 cells cloned in soft agar with 16.5 μM CDDP. The K562DDP cells showed a 6.7-fold resistance to CDDP at the IC_{50}. The K562DDP cells were maintained by continuous exposure to 3.3 μM CDDP. Kikuchi et al. [31] established a human ovary carcinoma cell line, KFr, which is resistant to CDDP from the KF-1 parental line. The KFr line is maintained in 0.5 or 1.0 μg ml/CDDP.

As a result of efforts in many laboratories to improve the cloning efficiency of human tumor stem cells, numerous human tumor cell lines with a range of drug sensitivities have been developed [24, 25, 26, 39, 45]. Hamilton et al. [26] have reported a series of human ovarian cancer cell lines from nontreated patients and patients refractory to therapy. The IC_{50}s for four of these lines to L-PAM range from 0.6 μM in the A2780 line to 3.0 μM in the OVCAR-2 line, a fivefold difference in sensitivity. Maynard et al. [34] have established lymphoblastoid cell lines from patients with ovarian cancer by transformation of peripheral B lymphocytes with Epstein Barr virus. There was an approximately tenfold range in the sensitivity of these cell lines to L-PAM at the IC_{90}. In general, cell lines developed from the lymphocytes of patients previously treated with L-PAM were found to be more resistant to the drug than cells from normal controls or from previously nontreated patients.

While it was possible to escalate the exposure dose to alkylating agents of cells in vitro 30- to 40-fold over the course of several months, further increase of selection pressure was not possible (Fig. 2 and 3) and resulted in cell death. Even when cultures were maintained at a maximum exposure concentration for several weeks, in many cases resistance was unstable upon removal of selection pressure, perhaps due to back mutation [22]. Thus, there appears to be a limitation to the degree of resistance which

can be produced to alkylating agents, even under optimal conditions of selection pressure.

Although there was a range of sensitivity of the four cell lines to the various alkylating agents, there were no consistent patterns of sensitivity and resistance among the cell lines and the drugs. In the dose escalation experiments, there was also a range of 2- to 5-fold in the plateau or maximally tolerated drug concentrations, which reflected somewhat the responsiveness of these tumor types to chemotherapy. There was no significant difference in the rate at which the various cell lines tolerated dose escalation. The recent clinical trials of combined high-dose alkylating agent treatment with autologous bone marrow transplantation take advantage of the difficulty in surpassing even tenfold resistance to alkylating agents [13–15]. This resistance "ceiling" at relatively low drug levels contrasts with that of other agents, particularly methotrexate, with which, with continued selection pressure in culture, very high levels (10^4-fold increase) of resistance can be achieved [14, 43, 44], and with natural products such as adriamycin, with which levels of resistance achieved often closely match the level of selection pressure [28, 55, 56]. In contrast, the greatest level of resistance to ionizing radiation can be achieved through the application of hypoxia, in which case a maximum of threefold resistance is possible. The effects of alkylating agents on cells and the responses of cells to these agents are as varied as are the structures of the molecules which make up this class of anticancer agents. Although cells can respond to alkylating agent exposure by a variety of mechanisms, even at maximal levels of response of the mechanism further dose escalation appears to be able to overcome cellular defenses. The plateau of dose escalation may reflect an inability of the cells to activate a gene amplification mechanism through which greater drug resistance could be achieved. The ceiling on high-dose survival for even dose-escalated/alkylating agent-adapted cells has very important clinical implications and provides a strong rationale for high-dose combination alkylating agent clinical regimens. The multifactorial nature of drug resistance has major implications in the development of clinically relevant means of overcoming this problem.

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