0026-895X/90/020198-09$02.00/0

MOLECULAR PHARMACOLOGY, 38:198-206

Characterization of Auromomycin-Resistant Hamster Cell Mutants that Display a Multidrug Resistance Phenotype

FRANK J. RAUSCHER III,[1] TERRY A. BEERMAN, and RAYMOND M. BAKER

Department of Experimental Therapeutics, Grace Cancer Drug Center, Roswell Park Memorial Institute, Buffalo, New York 14263

Received December 6, 1989; Accepted May 1, 1990

SUMMARY

We have selected and characterized Chinese hamster ovary (CHO) cells resistant to auromomycin (AUR), an antitumor antibiotic composed of a protein moiety and a nonpeptide chromophore. AUR is cytotoxic as a consequence of DNA strand-scission activity associated with the chromophore. Initial single-step selections for clones resistant to AUR detected a subpopulation of phenotypically resistant colonies, but nearly all such clones failed to display heritable resistance. One isolate that did show somewhat increased resistance was selected further and yielded a clone designated AUR^R-R1 that exhibits stable 10-fold increased resistance to AUR. The R1 line is also resistant to the AUR chromophore and cross-resistant to the closely related agent neocarzinostatin (NCS) and to the NCS chromophore. For AUR-treated whole cells, resistance to AUR cytotoxicity was inversely correlated with DNA damage as measured by filter elution; by contrast, isolated nuclei from sensitive and resistant cells displayed similar levels of AUR-induced DNA damage. The R1 cell line was found to be cross-resistant to colchicine, Adriamycin, Daunomycin, and vinblastine. The resistance phenotype is expressed with incomplete dominance in cell hybrids and appears similar to the "classic" multidrug resistance of CHO cells selected with other agents. Indeed, we found the multidrug-resistant CHO line CCH^R-C5 to be about 5-fold cross-resistant to AUR and to NCS. We ascertained that AUR-resistant (AUR^R) isolates express elevated levels of the molecular weight 170,000 P-glycoprotein often associated with multidrug resistance and also contain amplified DNA sequences that contain the gene for P-glycoprotein. When multiple-step enrichment selections were carried out as an alternative approach for isolating AUR^R mutants, each of nine clonal isolates showed phenotypes resembling the AUR^R-R1 line. Thus, our findings imply that increased cellular resistance to AUR may frequently be associated with P-glycoprotein-mediated multidrug resistance.

The antitumor antibiotic AUR, isolated from culture filtrates of *Streptomyces macromomyceticus* (1), is composed of an apoprotein of molecular weight 11,700 to which is bound a nonpeptide chromophore of molecular weight 667 (2). All the biological activity of the drug is associated with the chromophore (2). The protein component, which may bind to the cell surface and release the chromophore into the hydrophobic environment of the membrane, is thought to serve as a carrier and stabilizer of the chromophore (3). Both the AUR holoantibiotic (apoprotein plus chromophore) and isolated chromophore degrade DNA by introducing SSB (4). This strand scission activity is observed both *in vitro* and *in vivo* (5, 6), and a number of investigators have presented indirect evidence that cell death is a result of AUR-induced cellular DNA damage (7, 8). AUR is an extraordinarily potent drug, causing cytotoxicity at concentrations as low as 10^{-11} M. NCS, another member of this family of antitumor agents (4), shares amino acid sequence homology with AUR, but the drugs differ in the three-dimensional structure of the apoproteins, structure of the chromophore, and detailed specificity for cleavage sites (9). Although considerable information is available concerning interaction of these drugs with cell-free DNA, little has been reported concerning cellular determinants of response and resistance to these drugs.

In the present study we took a genetic approach to investigating the cellular pharmacology of AUR by selecting and characterizing AUR^R variants of CHO cells, which we found to be also resistant to NCS and to the AUR and NCS chromophores. Further, these mutants selected with AUR are resistant to multiple unrelated drugs and express elevated levels of the molecular weight 170,000 membrane P-glycoprotein now known to be the principal determinant of classic MDR (10, 11). Characterization of the AUR^R cells in filter elution experiments

This research was supported by National Institutes of Health Grants CA13038 (R.M.B., T.A.B.), CA28495 (T.A.B.), CA40553, and CA21071 (R.M.B.), Training Grant CA09072 (F.J.R.), and Core Grant CA28853.

[1] Present address: The Wistar Institute, 36th and Spruce Sts., Philadelphia, PA 19104.

ABBREVIATIONS: AUR, auromomycin; AUR^R, auromomycin-resistant; CCH^R, colchicine-resistant; CHO, Chinese hamster ovary; DSB, double-strand breaks (DNA); EMS, ethyl methanesulfonate; MDR, multidrug resistant (resistance); MNNG, *N*-methyl-*N'*-nitro-nitrosoguanidine; NCS, neocarzinostatin; SSB, single strand breaks (DNA); CCH, colchicine; SDS, sodium dodecyl sulfate; kb, kilobase; ADR, Adriamycin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

indicated that increased resistance to cytotoxicity correlated with reduced SSB and DSB DNA damage in whole cells, demonstrating directly for the first time corresponding alterations in biological response and molecular damage at physiological AUR doses.

Preliminary descriptions of this work have appeared (12–14).

Materials and Methods

Drugs

Crude AUR (NSC 170105) was obtained from the National Cancer Institute. This preparation was further purified virtually free of non-chromophore-containing protein by the procedure previously described (15). AUR activity was assayed by determination of conversion of PM2-form I DNA to PM2-form II DNA (6). The drug was stored at a concentration of 10^{-6} M in 10 mM Tris·HCl, pH 7.9, at −70°. The nonprotein chromophore of AUR was extracted from the holoantibiotic immediately before use, as described (2, 16). The recovery of activity varied between 25 and 50%, as determined by PM2 assay. The concentration of chromophore utilized in experiments was adjusted by this factor and resulted in cytotoxicity virtually identical to that of the holoantibiotic. In control experiments, we ascertained that the cytotoxic activity of AUR is lost within 1 hr of incubation in cell culture medium.

Highly purified NCS was a generous gift from Dr. W. Bradner, Bristol Laboratories (Syracuse, NY). Sterile vials containing 1.0 mg/ml NCS in 0.15 M Na acetate buffer, pH 5.0, were stored at −70°. Separately frozen aliquots of this stock were thawed immediately before dilution in 10 mM Tris·HCl, pH 7.9, and use. NCS chromophore was extracted and quantitated as described for AUR. All manipulations with AUR and NCS were performed in a darkened room or hood.

CCH and vinblastine were from Sigma Chemical Co. (St. Louis, MO) and ADR and daunomycin were gifts from Adria Laboratories (Columbus, OH). Bleomycin and cisplatin were gifts from Bristol Laboratories.

Cell Lines and Culture

The CHO cell line CHO-K1 (17) was used for selection of drug-resistant sublines in the present work. The AUXB1 cell line, which is auxotrophic for glycine, adenosine, and thymidine (18, 19), is the wild-type CHO line from which CCH^R-C5 was selected by Ling and colleagues (10, 20). CCH^R-B30 is a CCH^R line later obtained by further selection of CCH^R-C5 (20). CHO-WTH is a ouabain-resistant clone selected from an $HPRT^-$ isolate AG^R-B11 (21). The cell culture procedures have been described (19, 22, 23). Cells were grown in monolayer culture in α-minimum essential medium (GIBCO), containing nucleosides as necessary for the AUXB1 line and derivatives, supplemented with 5% fetal calf serum and antibiotics.

Selection of Resistant Cell Lines

The procedure for mutagen treatment of CHO-K1 before selection was analogous to those previously described (19, 23). Briefly, 1×10^7 exponentially growing cells/150-cm^2 flask were treated with appropriate concentrations of MNNG or EMS (Sigma) for 18 hr. Survival was then determined by assay of an aliquot of cells for plating efficiency in comparison with an untreated control culture, while the remainder were maintained in culture for a 7–9-day expression and recovery period before selection with drug. Cultures that had been treated with mutagen such that survival was reduced to 20–30% of control were used for selection platings.

Single-step selections. Single-step mutant selection experiments were designed as follows. Dishes for selection of resistant colonies were seeded at 10^4, 10^5, and 10^6 cells/60-mm or 100-mm dish in quadruplicate for each desired drug concentration and cell density. For quantitative assessment of cell survivals, duplicate 60-mm dishes were seeded at 10^2, 10^3, and 10^4 cells/5 ml at each drug concentration. Following cell attachment, 1 ml of medium containing drug at 6 times the desired concentration was added and thoroughly mixed. After 8–10 days of incubation, survival curve dishes and two of the four selective dishes at each concentration were stained with methylene blue (19). Remaining selective dishes were examined for colonies (based on staining of duplicates) or were incubated for up to 3 additional weeks. Colonies appearing at selective doses of AUR (see description in Results) were isolated and removed from the dish using glass cloning rings, expanded to mass population in the absence of drug, and tested for drug sensitivity.

In some experiments, selection plates were treated on 3 successive days of the initial drug treatment with fresh selective agent. This was done by rinsing the monolayers with phosphate-buffered saline and refeeding with growth medium containing new drug. In some instances, colonies arising 2–3 weeks after initial single-dose treatment were challenged with additional selective doses of drug, followed by reincubation and subsequent colony isolation. Some isolates were cloned by limiting dilution in 96-well multiwell dishes.

Multiple-step selections. For multiple step selections, CHO-K1 cells that had been previously mutagenized with EMS were seeded at 5×10^6 cells/T150 flask and treated with a drug dose designed to decrease relative survival by about 100-fold. Twenty four hours later, cells were trypsinized and an aliquot was diluted and plated for survival. The remaining cells were split into two new flasks and allowed to proliferate in the absence of drug. When these reached near confluence (approximately 3–6 days), they were pooled and two new flasks were reseeded at 5×10^6/flask for another cycle of treatment. One flask was retreated with drug, while the other served as a control. A portion of the population obtained following eight cycles of AUR treatment was utilized for cloning and further discrete step selection experiments, as above.

Assay of Relative Resistance and Cross-resistance Phenotypes

Presumptive drug-resistant cell lines were plated in 60-mm or 24-well Linbro dishes as a function of drug dose (19, 23). The CHO-K1 wild-type was included in all experiments for direct comparison of parental and resistant cell dose-response curves. Colonies were stained and scored after 8–10 days of incubation at 37°. The D_{10}, defined as the dose that decreases the plating efficiency to 10% of that observed in untreated control dishes, was determined for each cell line. Relative resistance is defined as the ratio of the D_{10} for resistant cells to the D_{10} for the wild-type parental cell line.

Derivation of Cell Hybrids

Intraspecific cell hybrids were formed between CHO cell lines containing different auxiliary genetic markers (22, 24). Nascent cell hybrids in a fused population were isolated in selective media in which neither parental cell type was able to survive but in which hybrids could proliferate because of complementation of recessive proline and GAT^- auxotrophies or because of dominant expression of both $HPRT^+$ (HAT resistance) and ouabain resistance markers (21). Cell lines to be fused were mixed 1:1 in suspension, seeded to multiwell dishes at densities of 5×10^5 cells/cm^2/ml, incubated for 18–24 hr to allow for attachment and maximum cell contact, and treated for 1 min with 50% polyethylene glycol (molecular weight 6000; Koch Light) as described (25). The treated cells were then rinsed repeatedly and incubated overnight in nonselective medium before trypsinization and inoculation to hybrid-selective medium. The systems employed, including auxiliary markers on the cell lines and necessary selective media, have been previously described (21, 24). The frequencies of spontaneous mutants of the parental cells that were able to form colonies in the hybrid-selective media were monitored in appropriate control platings, to confirm that occurrence of such mutants was rare (much less than 1%) compared with the induced hybrids.

For assays of drug response, the fused hybrid populations were plated at 2×10^5 and 5×10^4/100 mm dish in 18 ml of hybrid-selective medium

and dosed with various AUR concentrations to generate a curve for relative plating efficiency of hybrids as a function of AUR dose. For comparison, parental cells of each type that had been treated with polyethylene glycol were plated concurrently for dose response to the drug in medium not selective for hybrids. Plates were stained and scored after 7–10 days of incubation. In some cases, individual clones were isolated from dishes containing hybrids, expanded, and cloned by limiting dilution. The quasitetraploid character of putative hybrid cells was confirmed by monitoring cell size with a Coulter counter and subsequent chromosome spreads.

Filter-Elution Analyses

These experiments were performed essentially as described by Kohn *et al.* (26, 27). Cells were labeled with [^{14}C]thymidine (56 mCi/mmol) at 0.025 μCi/ml for 18–24 hr, followed by a chase period of 4 hr. Cells in suspension (5×10^5/ml, 1.5 ml) were treated with drug for 15 min at 37°, after which 1 ml of suspension was diluted into 10 ml of ice-cold phosphate-buffered saline and gently deposited on 2-μm pore size polycarbonate filters. Cells were lysed with a solution of 2% SDS, 0.02 M EDTA, pH 9.7, containing 0.25 mg/ml proteinase K (Boehringer Mannheim) for 30 min. Filters were washed once with 5 ml of 0.02 M EDTA, pH 10.3, followed by addition of 30 ml of 2% tetrapropylammonium hydroxide (Fisher)/0.02 M EDTA/0.2% SDS, pH 12.1. Elution was carried out at a flow rate of 0.028 ml/min and 90-min fractions were collected. Radioactivity remaining in the filter, in the filter holder, and in each fraction was determined, and the fraction retained on the filter at each time point of elution was calculated. A procedure described by Glisson *et al.* (28) was adapted for isolation of nuclei. First, 10^7 [^{14}C] thymidine-labeled and chased cells were washed twice with buffer A (1 mM KH_2PO_4, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM EGTA, pH 7.2). Cells were then resuspended in 1 ml of buffer A followed by lysis with 9 ml of buffer A containing 0.3% Triton X-100. After 30 min on ice, nuclei were pelleted at $2000 \times g$ for 10 min and resuspended in buffer A. Nuclei were treated with drug and assayed for DNA damage as described above. Comparison of DNA damage induced in drug-sensitive and -resistant cells and nuclei was performed by calculating a relative value of strand breaks, as described (29).

P-Glycoprotein Detection

Western immunoblot experiments were performed in the laboratory of Dr. V. Ling, Ontario Cancer Institute (Toronto, Canada, with the generous assistance of Dr. J. Gerlach. Results were subsequently confirmed independently in this laboratory.[2] The materials and methods utilized in this procedure have been described (20, 30). Briefly, membrane proteins prepared from wild-type and AUR^R cells were size fractionated on polyacrylamide gels according to the method of Fairbanks *et al.* (31) and electroblotted onto nitrocellulose paper according to the method of Towbin *et al.* (32). Blots were probed with directly iodinated C219 monoclonal antibody (20) against P-glycoprotein and then autoradiographed using Kodak XAR-5 film to visualize binding.

DNA Isolation and Southern Hybridization

DNA from appropriate cell lines was purified (33) and 10 μg of each DNA were digested to completion with a 3-fold excess of *Bam*HI (Boehringer Mannheim) under conditions recommended by the manufacturer. Fragments were separated in 1.0% agarose gels and transferred to nitrocellulose membranes (Schleicher and Schuell) by the method of Southern (34). The pDR7.8 probe used was originally isolated from the ADR^R-LZ cell line (35) and was kindly provided by Drs. Croop and Housman, MIT. This plasmid was labeled by nick translation to a specific activity of $1–2 \times 10^8$ cpm/μg (33). Membranes were prehybridized for 4 hr in 5× concentrated Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrolidone, 0.02% bovine serum albumin), 0.1% SDS, 300 μg of salmon sperm DNA. Probe was added (100 ng, $1–2 \times 10^7$ cpm) and hybridization continued for 18 hr. Membranes were washed in 4 liters of 15 mM NaCl, 1.5 mM Na citrate, 0.1% SDS, at 65°, dried, and placed onto Kodak XAR-5 film with intensifying screens. Exposed films were scanned with a Helena densitometer interfaced to an Apple IIe Computer.

Results

Selection of AUR^R cell lines. Measurements of the survival of CHO-K1 wild-type cells as a function of AUR dose indicated a D_{10} of approximately 20 pM (see Fig. 1 below). For doses in excess of 100 pM, where relative plating efficiency was reduced to approximately 10^{-5} (not shown), there appeared to be a "plateau" of surviving cells for a culture that had been previously treated with the mutagen EMS, suggesting the presence of a subpopulation of drug-resistant cells whose frequency had been elevated by the mutagenesis. Based on these observations, four separate experiments were carried out to isolate AUR^R cells in a single selection step. Each experiment involved a total of at least 10^8 cells of unmutagenized or mutagenized CHO-K1 cultures, employing selective drug doses of 200 to 500 pM AUR. Surviving colonies (which generally appeared only after prolonged incubation) were isolated, grown to mass population in the absence of selection, and retested for resistance to AUR.

Of 22 putative AUR^R isolates obtained in this manner, 21 displayed AUR sensitivity indistinguishable from that of the wild-type parent. Similar results were obtained when more stringent selection tactics were applied, involving multiple drug treatments on consecutive days at the time of initial selection and/or periodic rechallenging of developing colonies on selection dishes by fresh drug. Thus, a form of unstable or nonheritable resistance occurs reproducibly, although at low frequency, in this AUR treatment system.

One of the selected colonies, which was recovered at a survival of 10^{-5} from a single-step AUR selection of EMS-mutagenized CHO-K1 cells, yielded a clone that did exhibit stable 3-fold resistance to AUR. This clone was further mutagenized with MNNG and selected with AUR. A colony that developed within the first week was isolated and cloned again in the presence of 40 pM AUR, to confirm drug resistance. A subclone obtained in this manner, designated AUR^R-R1, showed stable 10-fold resistance to AUR, compared with the K1 wild-type (Fig. 1).

The resistance phenotype of AUR^R-R1 has been stable during serial culture of the cell line in the absence of AUR for up to 2 months. The doubling time of AUR^R-R1 is approximately 22 hr, as compared with a doubling time of approximately 13 hr for the parental CHO-K1; the modal chromosome number for both cell types is 21.

Because of the difficulty apparent in selecting stable AUR^R variants in a single step, a multiple-step selection strategy was also employed, with a view to enabling progressive enrichment for rare variants with modest resistance. This was accomplished by cycling a large population of mutagenized CHO-K1 cells through drug doses that would be toxic to approximately 99% of wild-type cells but would allow outgrowth of drug-resistant mutants, as described in Materials and Methods. The population obtained following eight cycles of AUR selection displayed a markedly increased resistance to AUR (data not shown). Each of nine clones obtained from this population showed some degree of stable resistance to AUR. For example, the isolate

[2] J. Sopher, W. Fredericks, Y. Chen, and R. M. Baker, unpublished data.

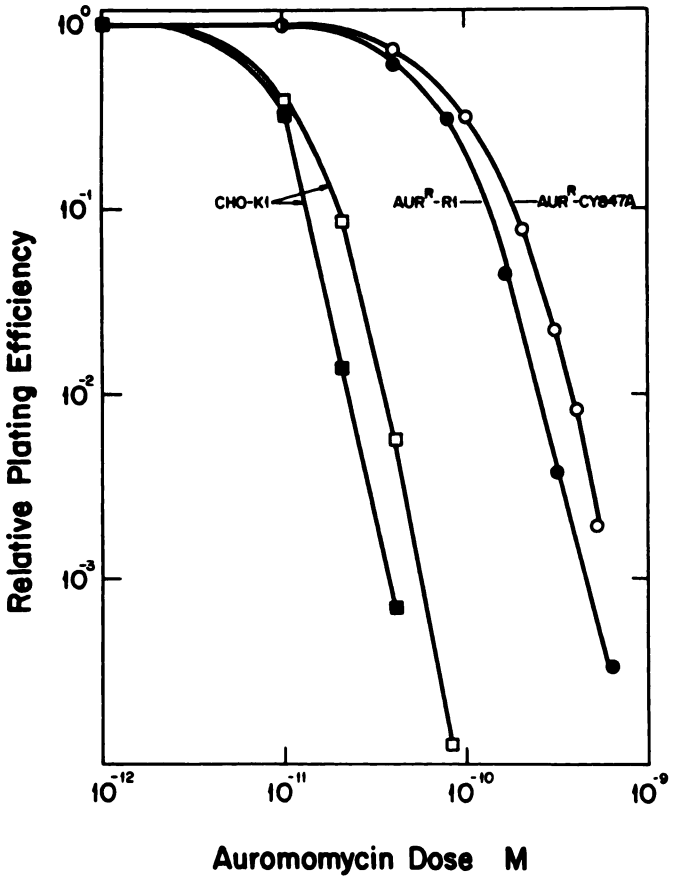

Fig. 1. Relative plating efficiencies as a function of AUR concentration for the CHO-K1 parental line and the AUR^R-R1 and AUR^R-CY847A mutant isolates. Cells were seeded into 60-mm dishes, and 2 hr later drug was added to achieve the indicated concentrations. Dishes were stained and scored for colonies with at least 30 cells after 8–10 days of incubation. Data from two experiments are shown, one comparing K1 (■) and R1 (●) and the other comparing K1 (□) and CY847A (○).

designated AUR^R-CY847A has 13-fold increased resistance to AUR (Fig. 1).

DNA damage in AUR^R cells and nuclei. We used the sensitive "filter-elution" technique to ascertain whether resistance to the cytotoxic effects of AUR was reflected in decreased amounts of cellular DNA damage. Both SSB and DSB damage were assessed, utilizing alkaline and neutral elution procedures, respectively (26, 27).

Induction of SSB by AUR in CHO-K1 wild-type cells was detectable at a dose of 25 pM and was dose dependent, as indicated by increasing slopes of the elution profiles with increasing doses. (Examples of elution profiles are shown below in Fig. 4.) An identical analysis showed the AUR^R-R1 cell line to be much less sensitive to AUR-induced SSB damage, evidenced by minimal elution even at high doses. To compare the cell lines, a relative value of SSB (29) was calculated for each profile and plotted as a function of AUR dose (Fig. 2A). A 4- to 5-fold increase in AUR dose is required to produce a similar relative value of SSB (i.e., to produce an equivalent elution profile) in AUR^R-R1 as compared with the CHO-K1 wild-type.

Nuclei from wild-type and AUR^R-R1 cell lines were isolated to determine whether resistance to AUR-induced damage was retained in this subcellular system. If resistance were due solely to extranuclear (e.g., membrane or permeability) factor(s), one would expect equivalent DNA damage in drug-treated nuclei from sensitive and from resistant cells. In fact, the dose responses for AUR-induced SSB damage in nuclei from CHO-K1 and AUR^R-R1 cells were virtually identical (Fig. 2B), in contrast to the results when whole cells were treated. Moreover, SSB damage in nuclei occurred for drug doses similar to those that were effective for CHO-K1 whole cells.

AUR^R-R1 cells also show marked resistance to AUR-induced DSB, compared with the parental CHO-K1 line. Assay of DSB in the neutral elution system required higher AUR doses, because of the need for two SSB in proximity on opposite strands of the DNA to produce a DSB. Fig. 3 shows curves for calculated relative values of DSB, from which it is evident that the resistant line requires about 7-fold higher AUR doses than the wild-type for comparable induction of DSB. By comparison of drug doses that gave approximately similar elution profiles in neutral and alkaline elution procedures, the ratio of DSB to SSB for both wild-type CHO-K1 and AUR^R-R1 cells was estimated to be 1:5.

Cross-resistances to AUR chromophore and to NCS and NCS chromophore. The AUR^R variants, selected for resistance to the AUR holoantibiotic, were tested for cross-resistances to cytotoxicities of the AUR chromophore and of NCS and the NCS chromophore (Table 1). The AUR^R-R1 line was found to have acquired 10-fold increased resistance to the AUR chromophore and 4.5-fold increased resistances to both NCS and the isolated NCS chromophore. The isolates obtained via multiple-step enrichment selections displayed cross-resistances analogous to AUR^R-R1, as exemplified by the 4-fold resistance of the AUR^R-CY847A clone to NCS. On the other hand, neither AUR^R-R1 nor AUR^R-CY847A cells were resistant to the DNA strand-scission drug bleomycin, which is a glycopeptide structurally unrelated to AUR and NCS (Table 1). Filter-elution analyses of DNA damage in drug-treated whole cells showed that the relative resistances of AUR^R-R1 to SSB induced by NCS and bleomycin corresponded to the relative resistances to these agents in cytotoxicity assays (data not shown).

Further cross-resistances of AUR^R and of MDR cell lines. AUR^R isolates were tested further for cross-resistances to structurally and functionally diverse cytotoxic agents (Table 1). AUR^R-R1 was cross-resistant to the anthracyclines ADR (16-fold) and Daunomycin (4-fold), to CCH (13-fold), and to vinblastine (6-fold). Also of note is the 4-fold collateral sensitivity exhibited by AUR^R-R1 to cisplatin. Each of the nine other AUR^R lines that we obtained via multiple-step selection, including CY847A, also showed varying degrees of cross-resistance to NCS, CCH, and ADR. These AUR^R isolates appear to be cross-resistant to the same spectrum of drugs as previously described for MDR hamster cell lines originally selected with Actinomycin D (22, 36) or CCH (10, 20). The cross-resistance phenotype of the CHO line CCH^R-C5, isolated by Ling (10), is shown in Table 1 as an example of classic MDR for comparison.

We tested the MDR CCH^R-C5 line (in comparison with its parent AUXB1) for sensitivity to AUR and NCS to determine whether the previously recognized MDR phenotype includes resistance to AUR and NCS. As shown in Table 1, the CCH^R-C5 line displayed a 5-fold cross-resistance to both AUR and NCS. It, thus, appears that the AUR^R isolates we have identified are independently derived examples of a classic MDR phenotype, and the drug resistances of our isolates may be due to decreased drug accumulation associated with elevated P-

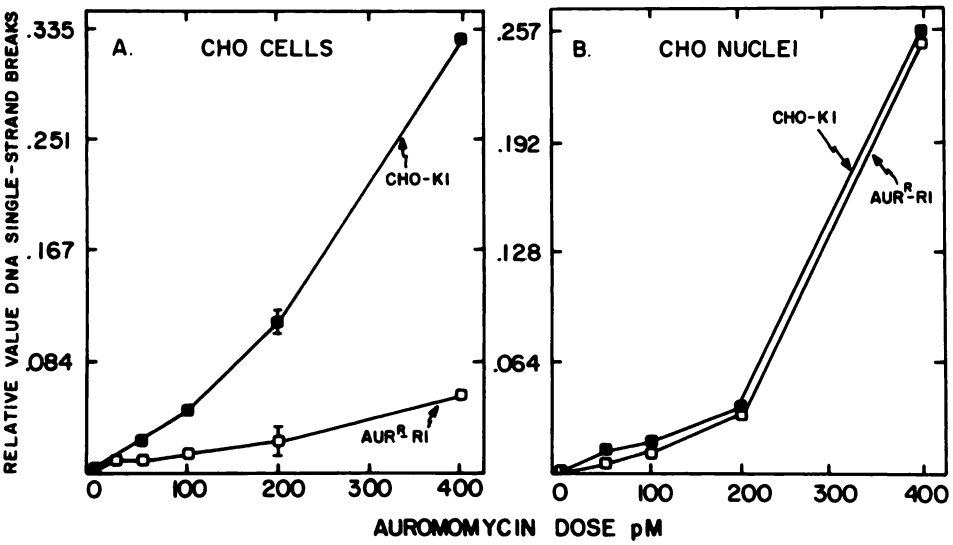

Fig. 2. Relative values of AUR-induced SSB as a function of dose for CHO-K1 and AUR^R-R1 whole cells (A) and corresponding isolated nuclei (B). [^{14}C]Thymidine-labeled cells (5 × 10^5 cells/ml in 1.5 ml of growth medium) were treated with the indicated concentrations of drug for 15 min at 37°, followed by alkaline elution analysis as described in Materials and Methods. Nuclei were treated with drug in their isolation buffer and assayed by alkaline elution exactly as for whole cells. The averaged midpoints of elution curves at each dose were used to calculate the relative value for SSB (29). The *data points* represent three experiments for whole cells (means ± standard errors) and one experiment for isolated nuclei.

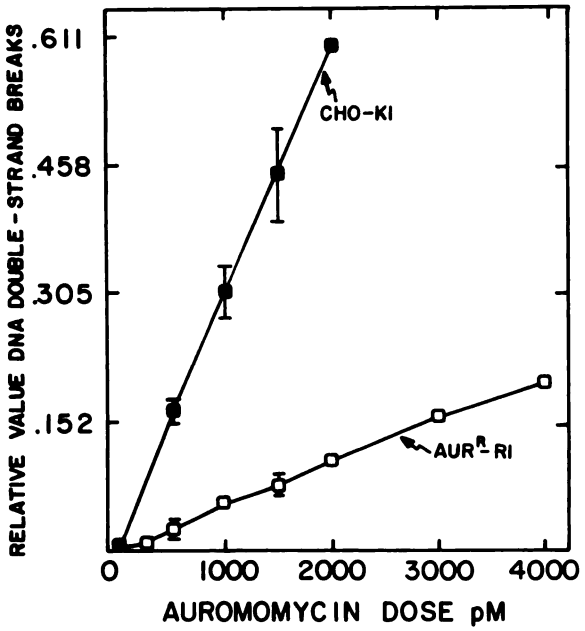

Fig. 3. Relative values of AUR-induced DSB in CHO-K1 and AUR^R-R1 cells. The relative value for DSB was calculated from the relative retentions at the 12-h time point of elution for each AUR dose, in view of a biphasic nature of the elution curves (29). The data points represent the means ± standard errors of three experiments for each cell line except for the two highest doses for AUR^R-R1, which were from a single experiment.

TABLE 1

Cross-resistance characteristics of AUR^R and CCH^R hamster cell lines

For each cell line and drug, the dose that decreases plating efficiency to 10% of that observed for the untreated control (D_{10}) was determined as described in Materials and Methods. Relative resistance is defined as the ratio of the D_{10} for the mutant isolate to the D_{10} for the appropriate parental wild-type. Thus, a value of >1.0 indicates cross-resistance, whereas a value of <1.0 indicates collateral sensitivity. Values shown are the averages of two or three independent determinations of the ratios. For wild-type CHO-K1 cells, the D_{10} values for the drugs were as follows: AUR, 20 pM; NCS, 12 nM; CCH, 0.075 μg/ml; ADR, 0.05 μg/ml; Daunomycin, 0.1 μg/ml; vinblastine, 0.02 μg/ml; bleomycin, 2 μM; and cisplatin, 1 μM.

Drug	Relative resistance		
	AUR^R-R1	AUR^R-CY847A	CCH^R-C5
AUR	10	13	5.0
AUR chromophore	10	—[a]	—
NCS	4.5	4.0	5.0
NCS chromophore	4.5	—	—
CCH	13	16	160[b]
ADR	16	16	25[b]
Daunomycin	4.0	—	76[b]
Vinblastine	6.0	—	30[b]
Bleomycin	1.0	1.0	1.0[b]
Cisplatin	0.25	—	—

[a] —, not assayed.
[b] Value taken from Ling (10).

glycoprotein expression, as previously described for other MDR lines (11).

Dominance of resistances in hybrid cells. In order to confirm whether the AUR^R-R1 trait is expressed with intermediate dominance in the presence of wild-type alleles, as previously described for other MDR CHO cells (22, 24), somatic cell hybrids between AUR^R-R1 and AUR-sensitive CHO lines were characterized. In view of the readily measurable difference in the amount of AUR-induced SSB between the CHO-K1 and AUR^R-R1 cell lines, we applied filter elution to assay directly for AUR resistance as manifested by a reduction in DNA damage. Clonal hybrids derived from fusion of CHO-K1 or AUR^R-R1 with an AUR-sensitive, azaguanine-resistant, ouabain-resistant (CHO-WTH) line were analyzed for sensitivity to AUR-induced SSB, in comparison with suitable controls.

In the course of these experiments, we discovered that, regardless of the AUR sensitivity specified by the genotype of the hybrid, the physical presence of tetraploid DNA content caused an apparent decrease in the AUR sensitivity of that hybrid in the elution assay. The influence of tetraploid DNA content in hybrid cells could be precisely compensated for by increasing the AUR concentration by 2-fold or decreasing the cell density at the time of treatment by 2-fold (data not shown).

The filter-elution profiles in Fig. 4 furnish a suitably normalized comparison of AUR-induced SSB damage in the AUR^R-R1 cell line, in the AUR-sensitive CHO-K1, CHO-WTH, and hybrid K1×WTH cells, and in two independently derived R1×WTH clones. The latter hybrids, heterozygous for the AUR^R trait, displayed responses to drug that were intermediate between those of the resistant and sensitive controls.

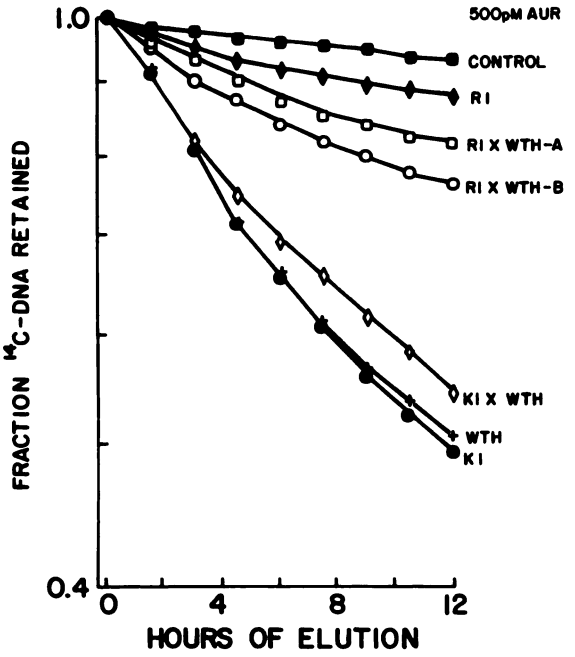

Fig. 4. Codominant expression of AUR^R manifested in alkaline elution assays. CHO-K1, -R1, and -WTH cell lines suspended at 5 × 10^5 cells/ml and hybrid cell lines (K1×WTH, R1×WTH-A, and R1×WTH-B) suspended at 2.5 × 10^5 cells/ml were exposed to 500 pM AUR for 15 min at 37° and then analyzed by alkaline elution. The control data for all cases were superimposable, represented by the *top curve*.

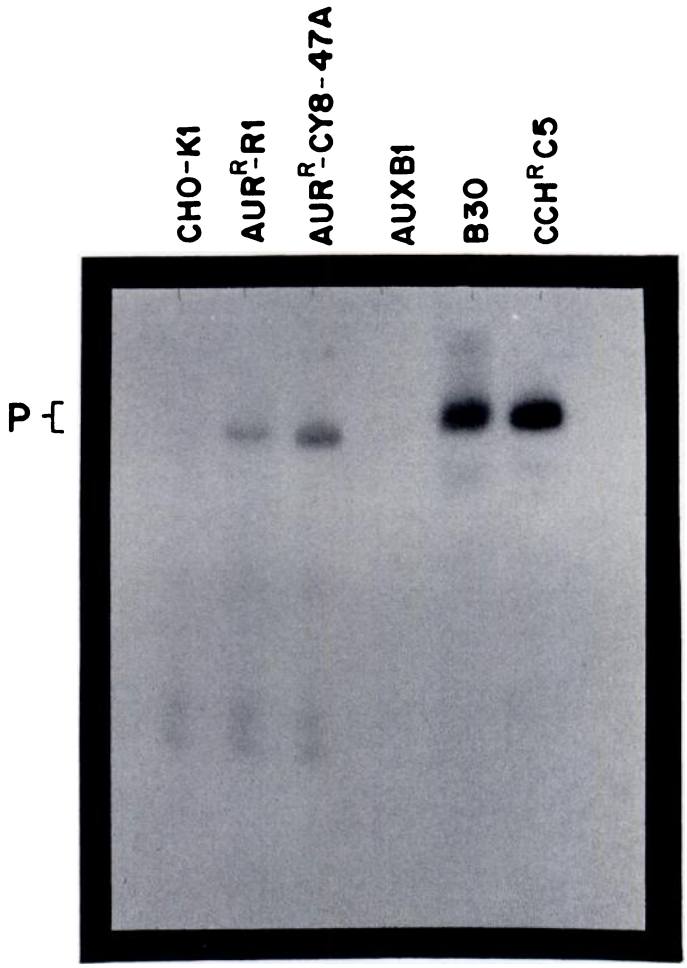

Fig. 5. Detection of P-glycoprotein in plasma membrane preparations from AUR^R and CCH^R cell lines. Equal amounts (50 μg) of membrane proteins were electrophoresed in polyacrylamide gels, electroblotted onto nitrocellulose, and reacted with an iodinated C219 monoclonal antibody specific for P-glycoprotein, as described in Materials and Methods. The control samples assayed from AUXB1, CCH^R-C5, and B30 lines were from sucrose gradient-enriched membrane preparations.

These data, thus, indicate intermediate (i.e., partial) dominance of the AUR^R at the biochemical level.

Dominance of cross-resistance to ADR was also tested, in this case by means of cytotoxicity assays. Using either the AUXB1 or WTH cell line as the fusion partner, recently fused hybrid populations and parental cell lines were tested for survival as a function of ADR dose (24). Hybrids displayed sensitivity to ADR intermediate between that of the R1 and wild-type cells, indicating intermediate dominance of the determinant for cross-resistance to ADR (data not shown). Independent hybrid clones were also isolated and tested with similar results. Taken together, our results thus imply that AUR^R and MDR are expressed with analogous intermediate dominance.

P-Glycoprotein expression in AUR^R cell lines. The various characterizations described above are all consistent with the idea that the AUR^R traits identified in the present study are manifestations of the previously described classic MDR (11). Increased expression of a molecular weight 170,000 plasma membrane glycoprotein, known as P-glycoprotein, has been shown to be responsible for the MDR phenotype in the CHO CCH^R-C5 and other cell lines (11). A monoclonal antibody specific for P-glycoprotein (20) was used to probe the expression of P-glycoprotein AUR^R cell lines by Western immunoblotting. Fig. 5 shows results for crude membrane protein preparations from the CHO-K1 wild-type, AUR^R-R1, and AUR^R-CY847A cells. Also included on this immunoblot as markers and positive controls are sucrose gradient-purified membrane preparations from the AUXB1 drug-sensitive CHO line, the MDR CCH^R-C5 line, and B30, a more highly resistant cell line selected from CCH^R-C5 (20). P-Glycoprotein is clearly elevated in the two AUR^R lines but is undetectable in the CHO-K1 parental line. The amount of P-glycoprotein is greater in the resistant CY847A line than in the slightly less resistant AUR^R-R1 cell line. The P-glycoproteins expressed by AUR^R cells have an increased mobility in polyacrylamide gels, as compared with the P-glycoproteins from the CCH-resistant cells C5 and B30. Others have reported variations in the apparent molecular weights of this protein in cells selected with different agents (11).

Detection of amplified DNA sequences in AUR^R cell lines. Southern blotting experiments were carried out to determine whether, like other MDR hamster cells, AUR^R cells contained amplified DNA sequences for genes encoding P-glycoprotein. Purified DNA from the AUR^R-R1 and AUR^R-CY847A cells and their parental CHO-K1 wild-type and from the CCH^R-C5 and B30 cells and their parental AUXB1 line analyzed by Southern blotting. These blots were hybridized to a 7.8-kb probe (pDR 7.8) known to detect 8- and 16-kb fragments from a 120-kb DNA domain amplified in the MDR hamster cell lines CCH^R-C5 and ADR^R-LZ and a 4.5-kB mRNA species detectable in resistant but not sensitive cells (37). As shown in Fig. 6, *Bam*HI fragments of 8- and 16-kb size are present but barely detectable in both wild-type parental lines. Identical sequences are amplified in CCH^R-C5 DNA and are even more elevated in the more highly resistant B30 cell line.

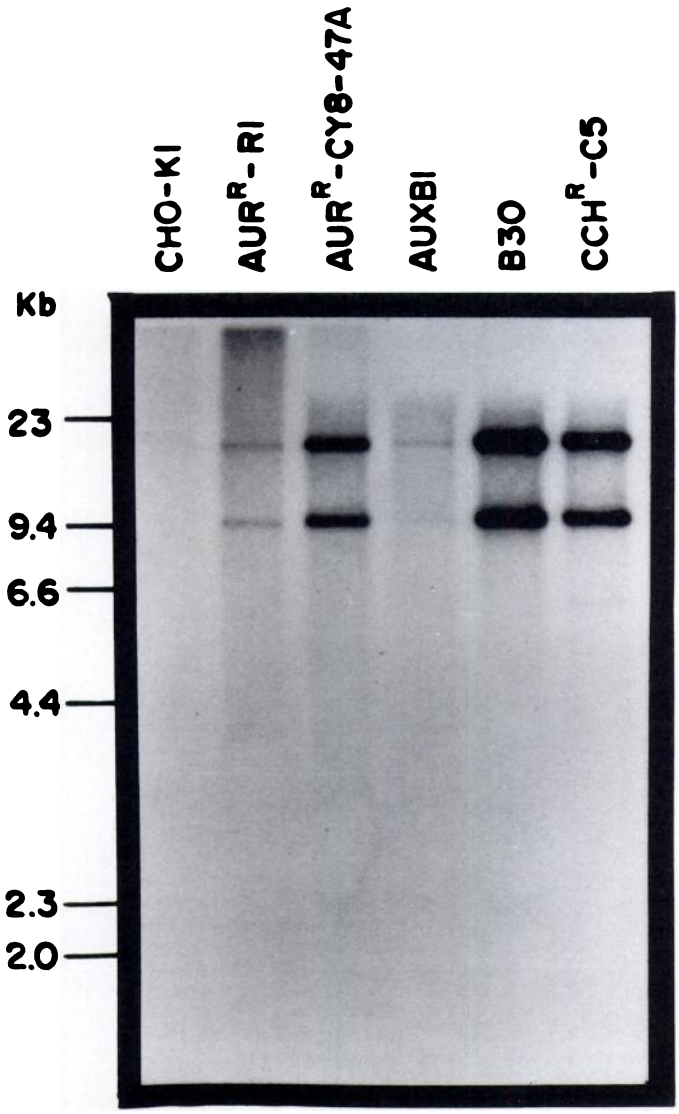

Fig. 6. Detection of amplified DNA sequences in MDR cell lines selected by AUR and by CCH. *Bam*HI-digested DNAs were electrophoresed on agarose gels (5 μg/lane for CHO-K1 and AUR^R lines; 2 μg/lane for AUXB1 and CCH^R lines), Southern blotted, and probed with the ^{32}P-labeled pDR7.8 plasmid.

These sequences are also amplified in DNA from AUR^R-R1 and AUR^R-CY847A cells, to a greater extent in the latter. Longer exposures of this film resulted in detection of a weakly hybridizing fragment of 2.1-kb, which is present in both R1 and CY847A and the two CCH^R cell lines. Dot blots (not shown) indicate the increase in copy number over CHO-K1 is 2- to 4-fold for AUR^R-R1 and 10- to 20-fold for AUR^R-CY847A. The increase in copy number is 20- to 30-fold for CCH^R-C5 and 60-fold for B30 relative to the AUXB1 parental line.

Discussion

The findings reported above represent the initial description of hamster cell mutants resistant to the antitumor antibiotic AUR. A notable feature of the AUR^R isolates was marked cross-resistance to CCH, the anthracyclines ADR and daunorubicin, and vinblastine, reminiscent of the phenotype previously described for MDR cells obtained using other selective agents (11). Other data consistent with these observations have been reported for mouse L5178Y cells selected *in vivo* for resistance to macromomycin, a less potent preparation of AUR (38). For two independently selected AUR^R clones, we determined that the gene for the molecular weight 170,000 membrane P-glycoprotein commonly associated with MDR (11, 37) is overexpressed and amplified. Conversely, we observed that the well characterized CCH^R-C5 line of CHO cells (20), whose MDR phenotype is attributable to elevated P-glycoprotein, shows significant resistance to AUR and to NCS. The variants that we obtained by selecting for increased resistance to AUR, thus, appear to be fresh examples of the P-glycoprotein-mediated pleiotropic MDR phenotype.

Our observations that MDR isolates selected with AUR holoantibiotic are resistant also to AUR chromophore and to NCS holoantibiotic and its chromophore (Table 1) imply that the mechanism of resistance affects both forms of both antibiotics in a comparable manner. Thus, the correlated effects on the markedly different forms of the drugs are consistent with chromophore-apoprotein dissociation before chromophore-specific interaction with the determinant of resistance, presumably P-glycoprotein. The chromophores share physicochemical characteristics of a number of agents that are affected by and select for P-glycoprotein-mediated MDR, being hydrophobic natural products of relatively low molecular weight with heterocyclic ring regions.

The data in Table 1 show other interesting features in the cross-resistance patterns of the cell lines tested. In contrast to the CCH^R-C5 line, which displays 5-fold cross-resistance to AUR but 160-fold resistance to CCH, the AUR^R-R1 and -CY847A isolates that are 10–13-fold resistant to AUR show only 13–16-fold cross-resistances to CCH. The main causes for such differences in resistance profiles between MDR cell lines are not well understood at present. One must consider the possibility that, in the course of multistep selections, particular MDR lines may have also acquired additional resistance mechanisms more specific to the selecting agent (22). The AUR^R-R1 and -CY847A isolates are exceptions to the common observation that MDR cell lines are most resistant to the primary selecting agent, however, because their levels of resistances to ADR and to CCH are somewhat greater than to AUR. Also noteworthy in Table 1 is the collateral sensitivity to cisplatin shown by the AUR^R-R1 line. Although sensitivity to cisplatin is not considered a consistent feature of the classic MDR phenotype, it has been noted in some other MDR cell lines isolated using different selecting agents (e.g., Ref. 39).

The filter-elution analyses presented here demonstrate that AUR-induced SSB damage can be detected in the same range of drug doses that cause reduction in CHO cell viability. Furthermore, the AUR^R-R1 cell line that was selected for resistance to the cytotoxic effects of AUR displays a correlated resistance to AUR-induced strand breakage, furnishing more direct evidence than previously available that the DNA damage is indeed the cause of cytotoxicity. We also applied filter-elution analyses to confirm that the resistance to molecular damage is expressed with intermediate (i.e., partial) dominance in somatic cell hybrids of sensitive and resistant cell lines, in agreement with results for this and other MDR systems using cell viability assays (22, 24). In these characterizations, it was established that DNA dosage is an important stoichiometric factor in analyses of AUR action, a technical observation that will be noteworthy for future work with this agent.

In contrast to results for whole cells, isolated nuclei from CHO-K1 and R1 cells showed identical dose responses for AUR-induced SSB, consistent with a resistance mechanism attributable to elevated plasma membrane P-glycoprotein and involving decreased cytoplasmic drug accumulation. The fact that very similar drug concentrations induced equivalent levels of SSB in the isolated nuclei and in sensitive whole cells indicates that normal mechanisms for AUR access through cells to act on DNA are very efficient, consistent with the extraordinarily low doses of AUR sufficient for cytotoxicity.

A phenomenon of nonheritable phenotypic resistance was observed during initial attempts at selection of AUR^R CHO cell lines. High-dose selections revealed surviving colonies at low frequencies consistent with genetic variation (10), but these colonies were very slow to develop and did not breed true for resistance when they were grown up in nonselective medium and retested. From previous studies of macromomycin (i.e., AUR) effects on HeLa cells, Hidaka *et al.* (40) suggested possible induction of a nongenetically mediated resistance state by the drug itself and presented evidence for cell cycle-related changes in drug cytotoxicity. A similar phenomenon of nonheritable resistance has also been reported for macromomycin in a microbial system (41).

Because it was evident that single-step selections would be of limited utility for isolating AUR^R CHO cells, we designed a multiple-step procedure to enrich for stable mutants that might be rare initially or might exhibit only low levels of resistance. This enrichment method involved repeated cycles of treatment with a single drug dose of modest toxicity. It proved quite effective; following eight cycles of AUR treatment, each of nine clones tested was stably drug resistant. Because the enrichment selections did not involve escalating drug doses, we expect that they minimized bias toward recovery of mutants with resistance mechanisms requiring progressive changes or of mutants with multiple resistance mechanisms. As it happened, the AUR^R mutants identified here proved to be independent isolates of a well known MDR type, which we infer to be the most commonly occurring type of CHO variant with reduced sensitivity to AUR.

A different result was obtained in analogous experiments employing the closely related protein antibiotic NCS as the selective agent, however. Although cell lines displaying P-glycoprotein-mediated MDR are cross-resistant to NCS and its chromophore, as shown in Table 1 and discussed above, another and apparently specific type of NCS-resistant mutant was revealed by multiple-step enrichment selections utilizing NCS as the selective agent (14). Of three clones obtained in that way that were tested, each displayed 4–6-fold NCS resistance but no cross-resistance to AUR or any of the other drugs tested.[3] These contrasting results with NCS indicate that further studies will enable characterization of resistance mechanisms specific to the action of such protein antibiotic drugs.

Acknowledgments

We thank J. Sczeklik-Safee for general technical assistance and V. Ling, J. Gerlach, and N. Kartner for generous collaboration on the initial Western blotting experiments.

References

1. Lippman, M. M., W. R. Laster, B. J. Abbot, J. Vendetti, and M. Barrata. Antitumor activity of macromomycin B (NSC 170105) against murine leukemias, melanoma and lung carcinoma. *Cancer Res.* **35**:939–945 (1975).
2. Kappen, L. S., M. A. Napier, and I. H. Goldberg. Roles of chromophore and apoprotein in neocarzinostatin action. *Proc. Natl. Acad. Sci. USA* **77**:1970–1974 (1980).
3. Kappen, L. S., and I. H. Goldberg. Mechanism of the effect of organic solvents and other protein denaturants on neocarzinostatin activity. *Biochemistry* **18**:5647–5653 (1979).
4. Goldberg, I. H., T. Hatayama, L. S. Kappen, M. A. Napier, and L. F. Povirk. Protein antibiotics as DNA-damaging agents, in *Molecular Actions and Targets for Cancer Chemotherapeutic Agents* (A. C. Sartorelli, ed.). Academic Press, New York, 163–191 (1981).
5. Suzuki, H., T. Nishimura, and N. Tanaka. DNA strand-scission *in vivo* and *in vitro* by auromomycin. *Cancer Res.* **39**:2787–2791 (1979).
6. Beerman, T. A. Strand-scission of superhelical and linear duplex DNA by the antitumor protein macromomycin: relationship of in *vitro* damage to cell growth inhibition. *Biochim. Biophys. Acta* **564**:361–371 (1979).
7. Beerman, T. A. Cellular DNA damage by the antitumor protein macromomycin and its relationship to cell growth inhibition. *Biochem. Biophys. Res. Commun.* **83**:908–914 (1978).
8. Suzuki, M., T. Nishimura, K. Muto, and N. Tanaka. Mechanism of action of macromomycin: DNA synthesis and mitosis. *J. Antibiot. (Tokyo)* **31**:875–883 (1978).
9. D'Andrea, A. D., and W. A. Haseltine. Sequence-specific cleavage of DNA by the antitumor antibiotics neocarzinostatin and bleomycin. *Proc. Natl. Acad. Sci. USA* **75**:3608–3612 (1978).
10. Ling, V. Genetic basis of drug resistance in mammalian cells, in *Drug and Hormone Resistance in Neoplasia* (N. Bruchovsky and J. M. Goldie, eds.), Vol. 1. CRC Press, Miami, FL, 1–19 (1982).
11. Gerlach, J., N. Kartner, D. Bell, and V. Ling. Multidrug resistance. *Cancer Surv.* **5**:25–46 (1986).
12. Rauscher, F. J., III, T. A. Beerman, and R. M. Baker. A mutant resistant to the antitumor protein auromomycin which displays a multidrug resistant phenotype. *Proc. Am. Assoc. Cancer Res.* **25**:1346 (1984).
13. Rauscher, F. J., III, R. M. Baker, and T. A. Beerman. Characterization of DNA damage in auromomycin-resistant pleiotropic multidrug-resistant hamster cells. *Proc. Am. Assoc. Cancer Res.* **27**:1058 (1986).
14. Rauscher, F. J., III. Isolation and characterization of somatic cell mutants resistant to auromomycin, neocarzinostatin, and bleomycin. Ph.D. dissertation. Department of Pharmacology, Roswell Park Division, State University of New York at Buffalo (1987).
15. Woynarowski, J. M., and T. A. Beerman. Purification and characterization of macromomycin 1. A protein antibiotic containing a chromophore. *Biochem. Biophys. Res. Commun.* **94**:769–776 (1980).
16. Napier, M. A., L. S. Kappen, and I. H. Goldberg. Effect of non-protein chromophore removal on neocarzinostatin action. *Biochemistry* **19**:1767–1773 (1980).
17. Kao, F. T., and T. T. Puck. Genetics of somatic mammalian cells. VII. Induction and isolation of nutritional mutants in Chinese hamster cells. *Proc. Natl. Acad. Sci. USA* **60**:1275–1281 (1968).
18. McBurney, M. W., and G. F. Whitmore. Isolation and characterization of folate deficient mutants of Chinese hamster cells. *Cell* **2**:173–182 (1974).
19. Thompson, L. A., and R. M. Baker. Isolation of mutants of cultured mammalian cells. *Methods Cell Biol.* **6**:209–281 (1973).
20. Kartner, N., D. Evernden-Porelle, G. Bradley, and V. Ling. Detection of P-glycoprotein in multidrug-resistant cell lines by monocloanl antibodies. *Nature (Lond.)* **316**:820–823 (1985).
21. Baker, R. M., D. M. Brunette, R. Mankowitz, L. H. Thomspon, G. F. Whitmore, L. Siminovitch, and J. E. Till. Ouabain-resistant mutants of mouse and hamster cells in culture. *Cell* **1**:9–21 (1974).
22. Baker, R. M., and V. Ling. Membrane mutants of mammalian cells in culture. *Methods Memb. Biol.* **9**:337–384 (1978).
23. Baker, R. M., C. B. Hirschberg, W. A. O'Brien III, T. E. Awerbuch, and D. Watson. Isolation of somatic cell glycoprotein mutants. *Methods Enzymol.* **83**:444–458 (1982).
24. Ling, V., and R. M. Baker. Dominance of colchicine resistance in hybrid CHO cells. *Somat. Cell Genet.* **4**:193–200 (1978).
25. Davidson, R. L., K. A. O'Malley, and T. B. Wheeler. Polyethylene glycol-induced mammalian cell hybridization: effect of polyethylene glycol molecular weight and concentration. *Somat. Cell Genet.* **2**:271–280 (1976).
26. Kohn, K. W., R. A. G. Ewig, L. C. Erickson, and L. A. Zwelling. Measurement of strand breaks and crosslinks in DNA by alkaline elution, in *DNA Repair: A Laboratory Manual of Research Techniques* (P. C. Friedberg and P. C. Hanawalt, eds.). Marcel Dekker, New York, 379–401 (1981).
27. Kohn, K. W. DNA as a target in cancer chemotherapy: measurement of macromolecular DNA damage produced in mammalian cells by anticancer agents and carcinogens. *Methods Cancer Res.* 291–345 (1979).
28. Glisson, B. S., S. E. Smallwood, and W. E. Ross. Characterization of VP-16-induced DNA damage in isolated nuclei from L1210 cells. *Biochim. Biophys. Acta* **783**:74–79 (1984).
29. Bradley, M. O., and K. W. Kohn. X-ray induced DNA double strand break production and repair in mammalian cells as measured by neutral filter elution. *Nucleic Acids Res.* **7**:793–804 (1979).
30. Gerlach, J. H., D. R. Bell, C. Karakousis, H. K. Slocum, N. Kartner, Y. M. Rustum, V. Ling, and R. M. Baker. P-Glycoprotein in human sarcoma: evidence for multidrug resistance. *J. Clin. Oncol.* **5**:1452–1460 (1987).
31. Fairbanks, G., T. L. Steck, and D. H. F. Wallach. Electrophoretic analysis of

[3] Unpublished data.

the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10**:2606–2617 (1971).
32. Towbin, H., T. Staehelin, and J. Gordon. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354 (1979).
33. Maniatis, T., E. F. Fritsch, and J. Sambrook. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 109–112 (1982).
34. Southern, E. M. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503–517 (1975).
35. Gros, P., J. Croop, I. B. Robinson, A. Varshavsky, and D. E. Housman. Isolation and characterization of DNA sequences amplified in multidrug-resistant hamster cells. *Proc. Natl. Acad. Sci. USA* **83**:337–341 (1986).
36. Biedler, J. L., and H. Riehm. Cellular resistance to actinomycin D in Chinese hamster cells *in vitro*: Cross-resistance, radioautographic and cytogenetic studies. *Cancer Res.* **30**:1174–1184 (1970).
37. Riordan, J. R., K. Deuchars, N. Kartner, N. Alon, J. Trent, and V. Ling. Amplification of P-glycoprotein genes in multidrug-resistant mammalian cell lines. *Nature (Lond.)* **316**:817–819 (1985).
38. Suzuki, H., Y. Sugimoto, K. Tanaka, T. Nishimura, and N. Tanaka. Isolation, drug sensitivity, and some biochemical and genetical properties of macromomycin-resistant mouse lymphoblastoma L5178Y cells. *J. Antibiot. (Tokyo)* **37**:602–609 (1984).
39. Keizer, H. G., G. J. Schuurhuis, H. J. Broxterman, J. Lankelma, W. G. E. J. Schoonen, J. van Rijn, H. M. Pinedo, and H. Joenje. Correlation of multidrug resistance with decreased drug accumulation, altered subcellular drug distribution, and increased P-glycoprotein expression in cultured SW-1573 human lung tumor cells. *Cancer Res.* **49**:2988–2993 (1989).
40. Hidaka, T., Y. Yano, K. Yamashita, and K. Watanabe. Biological activity of macromomycin. *J. Antibiot. (Tokyo)* **32**:340–346 (1979).
41. Buckmire, F. L. A., and T. L. Winkelhake. Bacteriocidal activity of macromomycin, an antitumor antibiotic. *Antimicrob. Agents Chemother.* **11**:1010–1016 (1977).

Send reprint requests to: Dr. R. M. Baker, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, NY 14263.