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MITOMYCIN C RESISTANT L1210 LEUKEMIA CELLS: ASSOCIATION WITH PLEIOTROPIC DRUG RESISTANCE

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Abstract—A mitomycin C-resistant (MMC_R) strain of L1210 mouse leukemia was developed by continuous drug exposure *in vitro*. MMC concentrations were increased in a stepwise fashion beginning at 0.033 μM and ending at 0.34 μM. This produced a 10-fold resistant cell line over the parental line. Resistance simultaneously developed to anthracene and anthracycline DNA intercalators, to vinca alkaloids and epipodophyllotoxins but not to cisplatin, bleomycin, fluorouracil or ionizing X-rays. MMC resistance was reversed using the membrane-active agent verapamil. The level of non-protein sulfhydryls was increased 2-fold in the MMC_R cells. Intracellular uptake of unchanged MMC was reduced by 40% in the MMC_R cells. Cytogenetic analyses demonstrated no recognizable clonal chromosomal alterations unique to the resistant subline and no evidence of double minutes or homogeneously staining regions in the DNA. Gel renaturation analysis failed to document the presence of an amplified DNA domain. Southern blotting of parental and MMC_R DNA using a cDNA probe (CHP1) for the P-glycoprotein gene also failed to demonstrate amplification or rearrangement of P-glycoprotein-related homologous sequences. However, an M_r 180,000 glycoprotein was detected in the plasma membranes from MMC_R cells. This protein also specifically reacted with a monoclonal antibody (C219) to the P-glycoprotein of Ling and co-workers [Kartner *et al.*, *Nature, Lond.* **316**, 820 (1985)]. These results suggest a pleiotropic drug resistance pattern in the MMC_R cells, associated with membrane glycoprotein alterations, enhanced non-protein sulfhydryl levels, and reduced MMC accumulation. This is a novel observation for a resistant cell line selected with an alkylating agent.

Cellular resistance to antineoplastic agents is a major problem in current clinical practice. It can be mediated by several different mechanisms including impaired drug accumulation via active extrusion or reduced uptake [1, 3], amplified gene sequences for target enzymes [3, 4], and enhanced detoxification of active drug species [5]. In addition, pleiotropic drug resistance is now a well-described phenomenon for different natural products such as the anthracycline antibiotics and the vinca alkaloids [6–8]. This pattern of simultaneous loss of sensitivity to separate classes of drugs has been associated with the presence of a unique M_r 170 membrane-glycoprotein termed "P"-glycoprotein [9–12]. Another mechanism of drug resistance involves enhanced intracellular detoxification of active drug species. This has been demonstrated recently in murine leukemia cells [13, 14] or rat mammary carcinoma cells [5], and in human ovarian cancer cells cultured for resistance to the bifunctional alkylating agent melphalan [15, 16]. In these latter instances, drug resistance appears to be correlated with enhanced intracellular levels of reduced GSH [13–16] and/or specific glutathione *S*-transferase (GST) detoxification enzymes [5].

Mitomycin C (MMC) is another bifunctional alkylating agent which crosslinks DNA in a dose-dependent fashion at pharmacologic drug levels [17, 18]. Previous studies of MMC resistance in a human colon carcinoma cell line (HCT 116) showed that MMC resistance correlates with altered membrane proteins and DNA binding, but that there is no simultaneous resistance to other natural products [19]. A subsequent report on this cell line described several unique cytosolic proteins that are elevated in the MMC-resistant cells [20]. One of the unique cytosolic proteins had physicochemical properties similar to those of the anionic form of GST. This suggested that augmented sulfhydryl-based detoxification pathways could be a factor mediating MMC resistance.

In the current report, we describe significant resistance to MMC in a murine leukemia cell line that developed elevated GSH levels and also demonstrated pleiotropic drug resistance *in vitro*. A preliminary report of the data has been published previously [21].

MATERIALS AND METHODS

Cell line. L1210 cells were maintained in suspension culture in 75 cm^2 flasks containing 90% RPMI 1640 growth medium supplemented with 1% glutamine, and 10% (v/v) heat-inactivated fetal bovine serum (all from GIBCO, Grand Island, NY). One percent (v/v) penicillin (100 units/ml) and streptomycin (100 μg/ml) were routinely included.

Drugs and chemicals. Antineoplastic drugs were obtained in commercial clinical formulations of: doxorubicon (Adriamycin) and fluorouracil (Adrucil), Adria Laboratories, Columbus, OH; mitomycin (Mutamycin), bleomycin (Blenoxane), cisplatin (Platinol), etoposide (Vepesid, VP-16), and methotrexate (Mexate) all from Bristol Laboratories, NY; mechlorethamine (Mustargen) and dactinomycin (Cosmegen) from Merck Sharpe & Dohme, Philadelphia, PA; vinblastine (Velban) and vincristine (Oncovin) from Eli Lilly, Indianapolis, IN; and

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bisantrene and mitoxantrone from American Cyanamid, Pearl River, NJ. Melphalan was obtained in pure powdered form from the Sigma Chemical Co., St. Louis, MO. It was solubilized using an established acid alcohol-phosphate buffer system. Verapamil solution (2.5 mg/ml) was obtained from Knoll Pharmaceuticals, Whippany, NY.

Cell culture methods. Resistance to MMC was developed by continuous exposure of log phase L1210 cell cultures to increasing concentrations of MMC beginning at 0.033 μM which is approximately 2% of the IC_{50} for continuous exposure MMC in the parental L1210 strain (about 0.15 μM [17]). After several weekly passages, MMC concentrations were increased by 50% until an IC_{30} was reached. This was then maintained until doubling times were again within 100–150% of the original 12-hr period for this cell line. The *in vitro* MMC concentration was thusly escalated over 3.5 months and maintained as above. After this time the cells were subcloned in the absence of MMC.

Cytotoxicity was assessed using a standard two-layer, soft-agar colony-forming assay [22]. Colonies of greater than 60 micron size (50+ cells each) were counted in 35-mm plastic petri dishes using an automated image analysis system optimized for tumor cell culture [23]. After plating at cell densities of about 1.25×10^4 ml, colonies were normally counted after 7 days of incubation in a humidified incubator under a 10% CO_2, 90% room air mixture. Resistance to a particular drug was arbitrarily defined as a molar ratio of 50% inhibitory concentrations (IC_{50} MMC_R/MMC parental) of greater than 2.0. The calcium channel antagonist verapamil (Knoll Pharmaceuticals) was also evaluated for cytotoxicity alone and in combination with MMC.

Sulfhydryl and MMC determinations. The concentration of reduced non-protein sulfhydryl (NPSH) levels within mid log-phase cells of equal passage number was measured using the spectrophotometric method of Sedlak and Lindasay [24]. For these analyses, $1–5 \times 10^6$ cells in logarithmic growth were lysed and sonicated in water. Proteins were removed by precipitation in 5% sulfosalicylic acid. NPSH were then complexed with a pH 8.0 solution of Ellman's reagent (dithio-bis-nitrobenzoic acid, DTNB, Sigma), and absorbance was measured at 412 nm (Perkin Elmer, Lambda 3A). Standards were prepared using reduced glutathione. Absorbance values for NPSH determinations include slight (1–5%) contributions from oxidized glutathione (GSSG [25]).

HPLC of MMC. After a 1-hr exposure to MMC concentrations of up to 750 μM, intracellular drug levels were determined using HPLC analysis of iced cell sonicates. Detection was facilitated by absorbance at 365 nm. The chromatographic conditions were similar to previously published methods [26]. These included a C-18 reverse phase column, 5–10 μM particle size. The elution on a 15-cm column (Altex Associates) was isocratic using a methanol: water (65:35) mobile phase pumped at 1.2 ml/min. The assay can accurately quantitate MMC levels of 1 ng/ml (on column).

Cytogentic and molecular biologic analysis. Chromosomal analysis was performed using G- and Q-banding techniques as described previously [27]. DNA was extracted from MMC_S and MMC_R cells, run on agarose gels, and blotted to nitrocellulose using previously reported techniques [28]. A cDNA probe for P-glycoprotein (termed CHP1) representing a 500 base pair cDNA encoding a portion of the Chinese hamster P-glycoprotein gene was used to determine if rearrangement or amplification of this gene had occurred [29]. Finally, in an attempt to detect amplified sequences (other than P-glycoprotein), DNA renaturation in agarose gels was performed using the technique of Roninson [30]. Plasma membranes from 2×10^8 cells were isolated after swelling and homogenization in 1.0 mM $CaCl_2$. The membrane vesicles were then purified by ultracentrifugation (100,000 *g* for 18 hr) in sucrose gradients [10]. Gel electrophoresis of proteins (10–50 mg/lane) was performed in 2% (w/v) sodium dodecyl sulfate (SDS) polyacrylamide gels according to the method of Fairbanks *et al.* [31] as modified by Debenham *et al.* [32]. Proteins were electrophoretically transferred from the gels to nitrocellulose filter paper using the method of Towbin *et al.* [33]. The filters were then probed with a ^{125}I-labeled antibody (C219) raised against pleiotropically resistant Chinese hamster ovary cells expressing the M_r 180,000 P-glycoprotein of Kartner *et al.* [34] (supplied by Dr. V. Ling, Ontario Cancer Institute).

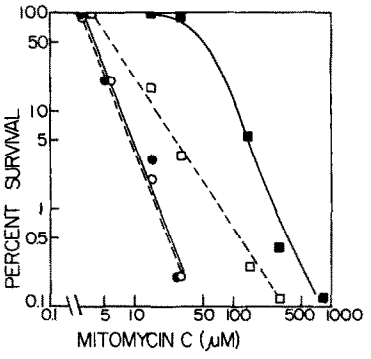

Fig. 1. Percent survival of L1210 colony-forming units in soft agar as a function of a 1-hr exposure to mitomycin C (solid symbols and lines). Each point represents the mean of six determinations (three determinations each from two experimental runs). Verapamil (2 μM) was co-incubated with MMC for 1 hr (open symbols and dotted lines) for both sensitive L1210 cells (circles), or MMC_R L1210 cells (squares).

RESULTS

Development and drug sensitivity characteristics of MMC_R variant in vitro. After six *in vitro* passages (each about 1.5 weeks), a non-cytotoxic MMC concentration of 0.34 μM was achieved in the L1210 cells. This concentration of drug was then maintained in continuous culture for 4 weeks. Afterwards the cells were subcloned in the absence of MMC, and the MMC_R cells were serially studied for drug sensitivity patterns *in vitro*.

Figure 1 shows the dose–response patterns for the two cell lines. The MMC_R cells displayed a 10-fold

Table 1. Drug sensitivity and resistance patterns for parental and MMC_R L1210 cells

Drug class-Agent	Drug exposure method*	IC_{50} values (μM)		Resistance index*
		Parental	MMC_R	
Alkylating agents				
Cisplatin	1.10†	2.16	3.33	1.5
Mechlorethamine	1.0	0.08	5.1	6.4
Melphalan	1.0	4.91	81.9	16.7
Mitomycin C	1.0	4.2	42.0	10.0
Antimetabolites				
Fluorouracil	C†	0.4	0.13	0.3
Methotrexate	C	0.97	22.0	22.6
DNA intercalators				
Dactinomycin	1.0	0.12	0.79	6.6
Daunomycin	1.0	0.12	8.86	71.4
Doxorubicin	1.0	0.21	3.96	15.3
Bisantrene	1.0	0.21	19.5	92.0
Mitoxantrone	1.0	0.096	1.64	17.0
Miscellaneous				
Bleomycin (munits/ml)	C	1.14	1.50	1.3
Etoposide	C	0.001	0.01	10.0
Vinca alkaloids				
Vinblastine	1.0	0.33	2.09	6.3
Vincristine	C	0.011	0.54	49.0
Radiation (cGy, X-rays)	‡	175	175	1.0

* Ratio of IC_{50} MMC_R divided by IC_{50} of parental strain.
† 1.0 = 1-hr incubation at 37°; C = drug added to final plating medium.
‡ Irradiation at 0.3 cGy/min at room temperature.

increase in resistance to a 1-hr MMC exposure. In the absence of MMC in the incubation medium, the MMC_R cells maintained the same level of drug resistance for up to 3 months with weekly passaging. This figure also shows that sensitivity to MMC can be restored with concurrent exposure to 2 μM verapamil, a drug known to perturb cellular plasma membranes and reverse multidrug resistance *in vitro* [35].

Table 1 shows the drug sensitivity patterns for this cell line exposed to a variety of different antineoplastic agents. The MMC_R line displayed marked resistance to the alkylators mechlorethamine and melphalan as well as the antimetabolite methotrexate. However, the greatest degree of collateral resistance was to agents derived from natural cytotoxic products. These included the anthracycline antibiotics (especially daunomycin), the vinca alkaloids (especially vincristine), and the epipodophyllotoxin derivative etoposide (VP-16). Interestingly, the MMC_R cells were not significantly more resistant to cisplatin or to irradiation with X-rays. The MMC_R cells also demonstrated enhanced sensitivity to the fluoropyrimidine antimetabolite 5-fluorouracil (5-FU).

Cytologic characteristics. Compared to the parental L1210 cells, MMC_R cells exhibited prolonged doubling times (28 hr versus 12 hr), larger cytoplasmic volumes, and marked pleomorphism. Interestingly, MMC_R cells had equivalent plating efficiences of about 2% in soft agar compared to parental cells. The distribution of colony sizes was also not different between the two lines with the majority of colonies (>80%) being between 60 and 80 microns in size 7 days after plating.

Cytogenetic and molecular biologic analysis. G- and Q-banding analysis showed that the modal chromosome number was 37 in both the sensitive and resistant cells. No clonal numeric or structural chromosomal alterations were recognized that were unique to the resistant subline. Furthermore, no cytologic evidence for gene amplification (double minute particles or homogenously staining regions of DNA) was observed in any cell examined.

To determine whether an amplified DNA domain was present in MMC_R cells (but went unrecognized by conventional cytogenetic analysis), *in-situ* gel renaturation analysis was performed [29]. Within the sensitivity of this technique (> 20-fold amplification for detection [10]), no homologous repeated sequences indicative of an amplified domain were observed. Southern blotting analysis using a ^{32}P-labeled cDNA to glycoprotein-homologous sequences was also not positive (results not shown).

However, membrane protein analyses using the MMC_R cells did demonstrate the presence of a M_r 180,000 glycoprotein which was not observed in the parental cells. Subsequent immunoblot analysis demonstrated that this protein reacted strongly with the C219 antibody to the P-glycoprotein ([34], Fig. 2). These data show that the P-glycoprotein is present

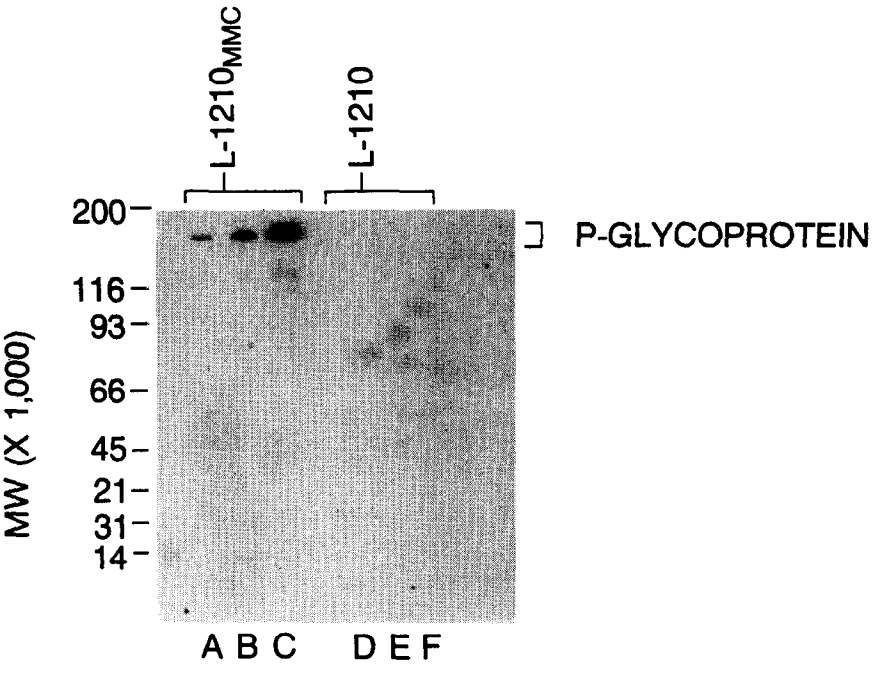

Fig. 2. Immunoblot analysis of membrane proteins from L1210 cells. Proteins were purified from vesicles [10] of parental L1210 (lanes A–C) and resistant (MMC_R) cells (lanes D–F). Different molecular weight proteins were separated by SDS-polyacrylamide gel electrophoresis [31, 32], transferred to nitrocellulose, and then probed with a monoclonal antibody specific to the M_r 180,000 P-glycoprotein [34]. Protein content was determined by the method of Bradfors [36] and various concentrations were loaded onto each lane of the gel (lanes A and D, 2.8 μg; B and E 5.6 μg; and C and F, 14 μg).

in the plasma membranes of the MMC_R cells in the absence of detectable gene amplifications.

Mitomycin C uptake studies. Figure 3 compares the intracellular MMC concentrations in the parental (sensitive) and MMC_R cells. It is apparent that there is reduced accumulation of free MMC in the MMC_R variant. Again, it should be emphasized that this HPLC assay will only detect free (unreacted) MMC. Thus, any intracellular drug that is covalently bound to DNA or other cellular nucleophiles will not be quantitated. Notwithstanding this limitation, the MMC_R cells accumulated only about 60% of the free MMC level present in the parental cells. At the lowest MMC exposure level of 75 μM (or fifteen times the IC_{50} for parental L1210), no drug was detected in the MMC_R cells.

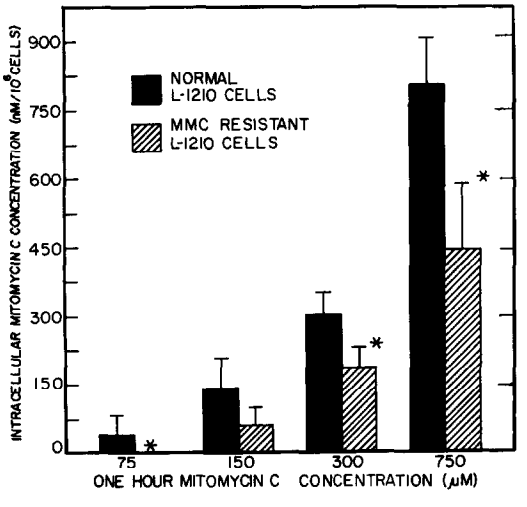

Fig. 3. Concentration of intracellular mitomycin C in L1210 cells exposed to drug for 1 hr at 37°. Each point represents the mean of triplicate HPLC determinations (bars represent one SD). * Significantly different values by the paired *t*-test.

NPSH level determinations. The parental L1210 cells had an NPSH level of 1.9 (± 0.32) nmol/10^6 cells (mean and SD of five determinations). These analyses were consistently performed on cells that were 4 days post passaging. Higher levels may be obtained at earlier post-passage times [37]. In the MMC_R cells, the NPSH level was elevated approximately 2-fold to 3.7 nmol/10^6 cells (± 0.31). The difference between the two values was statistically significant ($P < 0.05$ by Student's *t*-test).

DISCUSSION

In this study we have shown that experimentally-induced resistance to the antibiotic MMC is associated with (1) the presence of membranal P-glycoprotein, (2) reduced drug accumulation, (3) pleiotropic resistance to natural products, (4) partial reversal of resistance by verapamil, and (5) increased levels of reduced thiols. To our knowledge, this is the first description of pleiotropic resistance obtained

by selection with an alkylating agent. Other drugs that were collaterally resistant to MMC included anthracycline and vinca alkaloid natural products, as well as the alkylating agent melphalan, and the antimetabolite methotrexate.

Similar "pleiotropic" patterns of resistance have been observed in a variety of cell lines selected for resistance to specific anthracycline antibiotics and mitotic spindle poisons [6–12]. These cell lines have typically expressed an M_r 180,000 membrane glycoprotein. Similarly, our MMC_R cells demonstrated significant P-glycoprotein levels in the plasma membranes. Since we did not observe amplification of the MDR gene, it follows that enhanced expression of this protein without amplification has occurred. This has been seen previously with human cell lines selected for the MDR phenotype with a variety of agents [38]. In this regard, Beidler and co-workers [39] have also described a poor correlation of MDR gene amplification, but a good correlation of MDR gene expression to multidrug-resistance in CHO cells. We are currently examining for this pattern in MMC_R cells by probing the level of expression of P-glycoprotein mRNA via Northern blotting.

Because the MMC_R cells maintain their resistant phenotype (e.g. MMC resistance) for up to 3 months under no MMC pressure, the phenotype appears to represent a stably-altered variant of the parental cells. The cytologic characteristics of this variant include longer doubling times, larger cells with reduced nuclear/cytoplasm ratios (increased cytoplasm), and marked structural pleomorphism.

Another intriguing observation in the current report is the elevation of reduced sulfhydryls in the MMC_R cells. Since the majority of the NPSH level is due to GSH [40], this means that mid log-phase cultures of MMC_R cells have relatively twice the GSH level of the parental line. Elevated intracellular sulfhydryl levels in the MMC_R cells may also explain the resistance to melphalan and the depressed accumulation of free MMC in the resistant phenotype. This is compatible with the observation that membrane perturbation by verapamil did not restore drug sensitivity completely. Both observations suggest that multi-drug resistance is not explained entirely by membrane-based phenomena.

A number of other pleiotropically resistant tumor cell lines have also demonstrated enhanced thiol-based detoxification systems [15, 40]. These observations include the detection of a unique anionic GSH-transferase isoenzyme in rat hyperplastic liver nodules [41] and in MCF-7 human breast cancer cells selected for doxorubicin resistance *in vitro* [42]. The findings suggest that the association of multidrug resistance with GSH-based detoxification systems is not spurious and may indeed constitute a functional component of the pleiotropic response of mammalian cells to natural product toxins. Robson and Hickson [43] have also demonstrated that resistance to MMC in CHO cells is associated with at least four different genetic complementation groups, suggesting that cellular resistance to MMC is mediated by several mechanisms.

In summary, we have observed a pleiotropic drug resistance pattern associated with P-glycoprotein expression and reduced drug accumulation in an MMC-resistant L1210 cell line. This occurred in the absence of detectable amplification of the MDR gene coding for P-glycoprotein. It is intriguing to speculate that the elevated thiol levels and the membrane glycoprotein alterations are linked events in the overall response of L-1210 cells to MMC. Why pleiotropic resistance occurs with a natural product alkylator such as MMC, and not to the synthetic cross-linking agents such as busulfan, cisplatin or HN_2 [44] also remains to be elucidated. Furthermore, it will be interesting to evaluate whether the collateral sensitivity seen to 5-FU *in vitro* in this study is reproducible *in vivo*. Other more lipophilic compounds will also be tested for collateral sensitivity in the MMC_R cells. These studies are in progress and include agents such as corticosteroids, calmodulin inhibitors, and membrane-active adjuvants such as Tween-80. Of importance, our results extend the pleiotropic drug resistance pattern to a model alkylating agent and suggest that gene amplification is not a prerequisite to significant drug resistance *in vitro*.

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REFERENCES

1. M. Inaba and R. K. Johnson, *Biochem. Pharmac.* **27**, 2123 (1978).
2. D. Kessel and H. B. Bosmann, *Cancer Res.* **30**, 2695 (1970).
3. R. T. Schimke, F. W. Alt, R. E. Kellems, R. Kaufman and J. R. Bertino, *Cold Spring Harbor Symp. Quant. Biol.* **42**, 649 (1978).
4. J. T. Trent, R. N. Buick, S. Olson, R. Horns and R. T. Schimke, *J. clin. Oncol.* **2**, 8 (1984).
5. A. L. Wang and K. D. Tew, *Cancer Treat. Rep.* **69**, 677 (1985).
6. N. T. Bech-Hansen, J. E. Till and V. Ling, *J. cell. Physiol.* **88**, 23 (1976).
7. K. Dano, *Cancer Chemother. Rep.* **56**, 701 (1972).
8. J. L. Biedler and H. Riehm, *Cancer Res.* **30**, 1174 (1970).
9. V. Ling, *Can. J. Genet. Cytol.* **17**, 503 (1975).
10. J. R. Riordan and V. Ling, *J. biol. Chem.* **254**, 12701 (1979).
11. W. T. Beck, *Cancer Treat. Rep.* **67**, 875 (1983).
12. N. Kartner, J. R. Riordan and V. Ling, *Science* **221**, 1285 (1983).
13. K. Suzukake, B. J. Petro and D. T. Vistica, *Biochem. Pharmac.* **31**, 121 (1982).
14. S. Somfai-Relle, K. Suzukake and D. T. Vistica, *Biochem. Pharmac.* **33**, 485 (1984).
15. J. A. Green, D. T. Vistica, R. C. Young, T. C. Hamilton, A. M. Rogan and R. F. Ozols, *Cancer Res.* **44**, 5427 (1984).
16. T. C. Hamilton, B. J. Foster, K. R. Grotzinger, W. McKoy, R. C. Young, and R. F. Ozols, *Proc. Am. Ass. Cancer Res.* **24**, 313 (1983).
17. R. T. Dorr, G. T. Bowden and D. S. Alberts, *Cancer Res.* **45**, 3510 (1985).
18. J. W. Lown, in *Mitomycin C: Current Status and New Developments* (Eds. S. K. Carter and S. T. Crooke), pp. 5–26. Academic Press, New York (1979).
19. J. K. V. Willson, B. H. Long, M. E. Marks, D. E. Brattain, J. E. Wiley and M. G. Brattain, *Cancer Res.* **44**, 5580 (1984).

20. C. W. Taylor, M. G. Brattain and L. C. Yeoman, *Cancer Res.* **45**, 4422 (1985).
21. R. T. Dorr, J. D. Liddil and J. M. Trent, *Proc. Am. Ass. Cancer Res.* **26**, 344 (1985).
22. S. E. Salmon, A. W. Hamburger, B. Soehnlen, B. G. M. Durie, D. S. Alberts and T. E. Moon, *New Engl. J. Med.* **298**, 1321 (1978).
23. C. J. Herman, O. E. Pelgrim, W. J. Kirkels, R. Verheijen, F. M. J. Debruyne, P. Kenemans and G. P. Voojis, *Cytometry* **3**, 439 (1983).
24. J. Sedlak and R. H. Lindasay, *Analyt. Biochem.* **25**, 192 (1968).
25. A. Meister, in *Current Topics in Cellular Regulations* (Eds. R. W. Estabrook and P. Srere), Vol. 18, pp. 21–58. Academic Press, New York (1981).
26. R. G. Buice, P. Sidhu, B. J. Gurley and H. B. Niell, *Ther. Drug Monit.* **6**, 113 (1984).
27. J. M. Trent and F. H. Thompson, *Methods in Enzymology*, in press.
28. P. S. Meltzer, Y-C. Cheng and J. M. Trent, *Cytogenet. Cell Genet.* **17**, 289 (1985).
29. J. R. Riordan, K. Deuchars, N. Kartner, N. Alon, J. Trent and V. Ling, *Nature, Lond.* **316**, 817 (1985).
30. I. B. Roninson, *Nucleic Acids Res.* **11**, 5413 (1983).
31. G. Fairbanks, T. L. Steck and D. H. F. Wallach, *Biochemistry* **10**, 2606 (1971).
32. P. G. Debenham, N. Kartner, L. Siminovitch, J. R. Riordan and V. Ling, *Molec. cell. Biol.* **2**, 881 (1982).
33. H. Towbin, T. Staehelin and J. Gordon, *Proc. natn. Acad. Sci. U.S.A.* **76**, 4350 (1979).
34. N. Kartner, D. Evernden-Porelle, G. Bradley and V. Ling, *Nature, Lond.* **316**, 820 (1985).
35. L. M. Slater, S. L. Murray, M. W. Wetzel, R. M. Wisdom and E. M. Duvall, *J. clin. Invest.* **70**, 1131 (1982).
36. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
37. G. Batist, B. C. Behrens, R. Makuch, T. C. Hamilton, A. G. Katki, K. G. Louie, C. E. Myers and R. F. Ozols, *Biochem. Pharmac.* **35**, 2257 (1986).
38. D. Shen, A. Fojo, J. Chin, I. Roninson, N. Richert, I. Pastan and M. Gottesman, *Science* **232**, 643 (1986).
39. K. W. Scotto, J. L. Biedler and P. W. Melera, *Science* **232**, 751 (1986).
40. M. S. Moron, J. W. de Pierre and B. Mannervik, *Biochim. biophys. Acta* **582**, 67 (1979).
41. K. H. Cowan, G. Batist, A. Tulpule, B. Sinha and C. E. Myers, *Proc. natn. Acad. Sci. U.S.A.* **83**, 9328 (1986).
42. G. Batist, A. Tulpule, B. K. Sinha, A. G. Katki, C. E. Myers and K. H. Cowan, *J. biol. Chem.* **261**, 15544 (1986).
43. C. N. Robson and I. D. Hickson, *Mutation Res.* **163**, 201 (1986).
44. B. A. Teicher, C. A. Cucchi, J. B. Lee, J. L. Flatow, A. Rosowsky and E. Frei, *Cancer Res.* **46**, 4379 (1986).