0026-895X/89/030360-06$02.00/0

MOLECULAR PHARMACOLOGY, 36:360–365

1-β-D-Arabinofuranosylcytosine Enhancement of Resistance to Several Antineoplastic Drugs in Mammalian Tissue Culture Cells

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Received April 17, 1989; Accepted June 23, 1989

SUMMARY

This report describes the enhancement of drug resistance by 1-β-D-arabinofuranosylcytosine at four individual loci and gene amplification at one locus in a hamster cell line. This drug has been used chemotherapeutically in the treatment of neoplasia and has documented effects on DNA synthesis. Our results in this paper demonstrate that the incidence of resistance to methotrexate, *N*-(phosphonoacetyl)-L-aspartate, and 5-fluoro-2′-deoxyuridine was appreciably increased after pretreatment with 1-β-D-arabinofuranosylcytosine and they show that an increase in the incidence of gene amplification is one of the mechanisms by which drug resistance is increased. In contrast, the incidence of vincristine resistance was minimally enhanced by this drug. Possible reasons for this differential enhancement are discussed.

The generation of drug resistance in common neoplasias has been the limiting factor in successful chemotherapy. Although some cancers (especially leukemias and lymphomas) can be erradicated by aggressive chemotherapy, many solid tumors rapidly become resistant to drug treatment, thereby precluding effective chemotherapy. Gene amplification is a common cellular mechanism by which mammalian cells become resistant to toxic chemicals and chemotherapeutic drugs (for recent reviews see Refs. 1 and 2). Biedler and Spengler (3) were the first to describe the association of MTX drug resistance with chromosomal abnormalities and postulated that an increased copy number of specific gene sequences could be responsible. Molecular verification of this hypothesis was provided by the discovery that MTX resistance could result from amplification of the *dhfr* gene (4).

Studies in recent years have helped to define variables that control the incidence of gene amplification. Evidence supports the view that anticancer drugs or treatments themselves may enhance the emergence of drug resistance; pretreatment of mammalian cells with hydroxyurea (5), hypoxia (6), and even MTX itself (7) enhanced the emergence of MTX resistance 10- to 1000-fold. Molecular analyses of the bases for MTX resistance (altered transport of MTX, altered affinity of the DHFR enzyme for MTX, and amplification of the gene locus coding for the DHFR enzyme) showed that the frequency of all three were increased (5, 8, 9). The mechanistic details by which agents cause these increases are still unknown.

This report describes the enhancement of the incidence of drug resistance and gene amplification at multiple loci by araC, a compound that has been used chemotherapeutically and has known effects on DNA synthesis (10, 11). Enhancement of MTX resistance by various agents has been reported (see above); however, this report extends this phenomenon to additional genetic loci (*cad*, *ts*, and *mdr*). The list of loci that may respond to selection pressure through amplification of the targeted gene is long and includes the genes used in this study. Exposure of mammalian cells to PALA selects for the amplification of the gene coding for a multifunctional enzyme known as CAD; this enzyme catalyzes the first three steps of *de novo* pyrimidine biosynthesis through the action of its carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase activities (12). Selection of cells in FdUrd can result in cells that contain amplification of the *ts* gene and overexpression of its protein product (13). More recently, resistance to VCR, as well as to a number of other vinca alkaloids and nonrelated drugs, has been correlated with the overexpression and, oftentimes, amplification of a specific gene region (14–18). This region, termed *mdr*, codes for several protein products, including one called the p170 glycoprotein. Transfection of the gene that codes for the p170 glycoprotein into drug-sensitive

This research was supported in part by a Pharmaceutical Manufacturers Association Research Starter Grant, an American Cancer Society Institutional Subgrant (ACSIS IN-15-28) and a Public Health Service grant from the National Institutes of Health to T.D.T., an ACSIS Subgrant (IN-15-4) to P.L.C., and The G. Harold and Leila Y. Mathers Charitable Foundation grant and a North Carolina United Way Medical Research Fund grant to B.G.

ABBREVIATIONS: MTX, methotrexate; araC, 1-β-D-arabinofuranosylcytosine; CAD, multifunctional enzyme for carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase; DHFR, dihydrofolate reductase; FdUrd, 5-fluoro-2′-deoxyuridine; *mdr* gene, multidrug resistance gene; PALA, *N*-(phosphonoacetyl)-L-aspartate; TS, thymidylate synthetase; VCR, vincristine; PBS, phosphate-buffered saline.

cells confers the multidrug resistance phenotype. Our results show that the incidence of resistance to MTX, PALA, and FdUrd was increased appreciably after araC pretreatment, whereas the incidence of VCR resistance was relatively minimally enhanced by this drug. Possible reasons for this differential enhancement will be discussed.

Materials and Methods

Cell culture. SV28 cells were used in all experiments. This line was originated by *in vitro* SV40 transformation of BHK 21/C13 cells (19). Kempe *et al.* (20) used these cells (designated C13/SV28) to generate the PALA-resistant subclone 66-6, used as a control in the quantitation of *cad* amplification in this study (Fig. 3). The cells were free of *Mycoplasma* contamination, as determined by the procedure of McGarrity and Carson (21). The cells were grown in complete medium, Eagle's minimum essential medium with 10% fetal bovine serum (GIBCO).

Plating efficiencies of untreated cells and cells after araC treatment were determined by inoculating the treated or untreated cells into 75-cm^2 flasks that contained complete medium and then, 8 to 9 days later, counting the number of colonies containing greater than 50 cells. Before counting, the cells were fixed with 50% ethanol and stained with methylene blue. The diminution in the number of viable cells after treatment with araC (Table 2) was used in the estimation of drug resistance (relative plating efficiency).

Drug selection. For a given experiment, cells were seeded into a combination of 225-cm^2 (or 150-cm^2) and 25-cm^2 flasks, at a density of approximately 2×10^3 cells/cm^2. The cells in the 25-cm^2 flasks were treated in parallel with those in the 150-cm^2 flasks and used to follow cell growth and ara-C toxicity, up to the point at which the selective drug (MTX, PALA, FdUrd, or VCR) was added. Thus, the cells in the 225- or 150-cm^2 flasks, once seeded, were not disturbed except for the addition of drug and the careful changing of the medium. To determine the effect of araC pretreatment on the generation of drug resistance, cells were plated in the flasks, allowed to attach for 24 hr, and incubated with or without araC for 16 hr. This treatment was terminated in the 225-cm^2 flasks by removal of the medium, a wash with PBS, and addition of fresh medium (lacking araC) with the test drug (MTX, PALA, FdUrd, or VCR) at the indicated concentrations. The medium was changed once, at 6 days. The incidence of drug-resistant cells was determined by the number of colonies in the 225- or 150-cm^2 flasks after 12 days of continuous exposure to the drug. AraC and FdUrd were purchased from Sigma Chemical Co. (St. Louis, MO). PALA was obtained from the Drug Synthesis and Chemistry Branch of the Division of Cancer Treatment in the National Cancer Institute. Mr. William R. Fields of the Lilly Research Laboratories supplied vincristine sulfate and Dr. Robert Capizzi, Bowman-Grey School of Medicine, the MTX.

Assessment of gene amplification. To propagate resistant cells (for gene copy quantitation), individual colonies were selected at random from independent flasks and were isolated by gently scraping the plate with a sterile pipet tip. The cells were transferred to individual 25-cm^2 flasks, where they were propagated in the presence of the selection agent and ultimately expanded to approximately 2×10^6 cells. Half of these cells were stored frozen in glycerol (for future characterization) and the rest were trypsinized, rinsed in Hanks' balanced salt solution that contained 1% serum, pelleted, and stored at −80° until they were processed for determination of the gene copy number.

Gene copy number was quantitated by the slot hybridization assay, as previously described (11). Briefly, cells were lysed by the addition of a solution of 0.01 M Tris·HCl, 0.01 M EDTA, 0.01 M NaCl, and 0.5% SDS and were treated with proteinase K and RNase A. Each sample was extracted with phenol/chloroform (1:1) and then chloroform alone. The DNA was denatured by the addition of NaOH, neutralized by the addition of ammonium acetate, and immediately applied to nitrocellulose. After application, the filter was briefly rinsed in 5× standard saline phosphate-buffered EDTA and baked *in vacuo* for 2 hr at 80°. The filter was then hybridized with various cloned ^{32}P-labeled probes (specific activities, $\geq 10^8$ cpm/μg) under formamide conditions. The filters were washed, dried, exposed to X-ray film, and developed. Quantitation was done by densitometry. To detect the *cad* gene, we used probes derived from pCAD142, a plasmid containing a 6.5-kilobase cDNA insert (22). The mouse α-fetoprotein plasmid was obtained from A. Dugaiczyk , University of California, Riverside (23).

Results

Enhancement of drug resistance by pretreatment with araC. Although the increase in the number of MTX-resistant colonies that may emerge after pretreatment of mammalian cells with agents such as hydroxyurea, ethylmethane sulfonate, and UV light has been documented (5, 8, 9), the effect of these or other agents on other loci has not been reported to date. In this study, hamster cells were tested for their ability to form drug-resistant colonies, both with and without pretreatment with araC. Four different loci were tested; three of the loci code for proteins that are important for the synthesis of nucleic acid precursors (*dhfr*, *cad*, and *ts*) and one, *mdr*, codes for a membrane protein that mediates multidrug resistance probably through altered transport (15, 24). Overproduction of each of the first three proteins can be achieved by step-wise selections of cells in increasing concentrations of drugs that inhibit the action of each of these proteins; hence, MTX resistance can result from DHFR overproduction (4), PALA resistance can result from CAD overproduction (20), and FdUrd resistance can result from TS overproduction (13). Overproduction of the p170 glycoprotein is achieved by selection in VCR; the resultant effect is alteration in cell permeability (24).

We exposed asynchronously growing populations of SV28 cells to 0–20 μM araC for 16 hr (the equivalent of one cell cycle), gently rinsed them with PBS, and added fresh medium that contained either 300 μM PALA, 200 nM MTX, 300 nM FdUrd, or 0.65 μM VCR. The flasks were incubated for 12 or more days (with a change every 6 days with fresh medium containing the indicated drug) and colonies were fixed, stained, and counted. The absolute numbers of colonies obtained after selection in PALA or VCR are presented in Table 1, along with the incidence of drug resistance calculated both before and after correction for cell viability. Fig. 1 shows that the incidence of resistance to MTX, PALA, and FdUrd was greatly enhanced by araC, in a dose-dependent manner.

Unlike the effects on resistance to PALA, MTX, and FdUrd, araC pretreatment did not markedly enhance the frequency of resistance to VCR. Enhancement of VCR resistance frequency was relatively modest, as may be seen by comparing it with the effect of araC on PALA resistance, shown in Table 1 and Fig. 1. Four experiments are documented in Table 1. Frequencies are calculated by both total cells plated or with a correction for the cells killed by the araC pretreatment, which shows a dose-dependent decrease in cell viability from the 16-hr pretreatment itself (Table 2). As also depicted in Fig. 1 and Table 1, the frequency of PALA resistance increases dramatically in each experiment as higher concentrations of araC are used for pretreatment (the wide range of control PALA resistance frequencies are likely due to differences in serum lots used). In each experiment, however, the VCR resistance frequency increases with araC concentrations up to 2–5 μM but then either increases no further, decreases, or reaches a level undetectable with the number of cells plated for selection. Possible interpretations

TABLE 1

Enhancement of PALA or VCR resistance by AraC pretreatment

Selection was in 350 μM PALA (Expts. 1 and 4), 300 μM PALA (Expts. 2 and 3), 0.65 μM VCR (Expts. 1–3) or 0.87 μM VCR (Expt. 4).

AraC pretreatment (16 hr)	Colonies/ 10^6 cells plated		Incidence of drug resistance calculated from total cell number		Incidence of drug resistance calculated from viable cell number	
	PALA^r	VCR^r	PALA^r	VCR^r	PALA^r	VCR^r
μM						
Expt. 1						
None	32	3.8	3.2×10^{-5}	3.8×10^{-6}	9.4×10^{-5}	1.1×10^{-5}
2	25	58	2.5×10^{-5}	5.8×10^{-5}	2.1×10^{-4}	5.0×10^{-4}
5	304	96	3.0×10^{-4}	9.6×10^{-5}	5.4×10^{-3}	1.7×10^{-3}
10	735	12	7.4×10^{-4}	1.2×10^{-5}	2.4×10^{-2}	3.8×10^{-4}
20	982	<5.9[a]	9.8×10^{-4}	$<5.9 \times 10^{-6}$	1.1×10^{-1}	$<6.7 \times 10^{-4}$
Expt. 2						
None	4.1	15	4.1×10^{-6}	1.5×10^{-5}	7.0×10^{-6}	2.6×10^{-5}
2	8.3	58	8.3×10^{-6}	5.8×10^{-5}	1.3×10^{-4}	8.8×10^{-4}
5	246	35	2.5×10^{-4}	3.5×10^{-5}	5.8×10^{-3}	8.2×10^{-4}
10	900	7.7	9.0×10^{-4}	7.7×10^{-6}	3.3×10^{-2}	2.9×10^{-4}
20	3678	<11	3.7×10^{-3}	$<1.1 \times 10^{-5}$	1.7×10^{-1}	$<5.3 \times 10^{-4}$
Expt. 3						
None	0.6	3.1	6.3×10^{-7}	3.1×10^{-6}	1.5×10^{-6}	7.4×10^{-6}
2	8.5	4.2	8.5×10^{-6}	4.2×10^{-6}	2.3×10^{-4}	1.2×10^{-4}
5	56	<1.8	5.6×10^{-5}	$<1.6 \times 10^{-6}$	3.9×10^{-3}	$<1.1 \times 10^{-4}$
10	308	<1.7	3.1×10^{-4}	$<1.7 \times 10^{-6}$	7.7×10^{-2}	$<4.2 \times 10^{-4}$
20	812	<2.4	8.1×10^{-4}	$<2.4 \times 10^{-6}$	4.3×10^{-1}	$<1.3 \times 10^{-3}$
Expt. 4						
None	2.5	5.0	2.5×10^{-6}	5.0×10^{-6}	5.9×10^{-6}	1.2×10^{-5}
2	60	<5.0	6.0×10^{-5}	$<5.0 \times 10^{-6}$	$<6.7 \times 10^{-4}$	$<5.6 \times 10^{-5}$
5	116	<5.3	1.2×10^{-4}	$<5.3 \times 10^{-6}$	3.8×10^{-3}	$<1.7 \times 10^{-4}$
10	845	<9.1	8.5×10^{-4}	$<9.1 \times 10^{-6}$	4.4×10^{-2}	$<4.8 \times 10^{-4}$
20	2158	<8.3	2.2×10^{-3}	$<8.3 \times 10^{-6}$	7.2×10^{-1}	$<2.8 \times 10^{-3}$

[a] Values were calculated from estimations of one colony/given cell number.

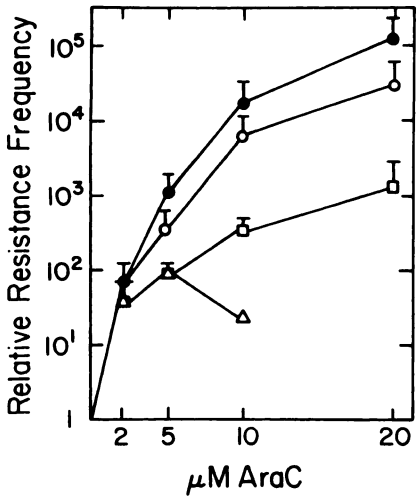

Fig. 1. Effect of araC concentration on the incidence of drug resistance. About 24 hr after plating, exponentially growing SV28 cells at the appropriate density were pretreated for 16 hr with 2–20 μM araC. After the pretreatment, the flasks were rinsed with PBS and fresh medium containing either 300 μM PALA (●), 200 nM MTX (○), 100 nM FdUrd (□), or 0.65 μM VCR (△) was added. Selection was for a minimum of 12 days with a change of medium every 6 days thereafter. Drug-resistant colonies were counted after the cells were fixed and stained. Resistance frequencies were corrected for the reduced viability of cells due to pretreatment with araC. The values on the *ordinate* are the fold increase in resistance frequency by araC relative to nonpretreated controls. The *vertical bars* indicate the standard deviations. The resistance frequencies for the controls were $2.7 \pm 3.9 \times 10^{-5}$ for PALA, $3.2 \pm 2.5 \times 10^{-5}$ for MTX, $2.2 \pm 0.48 \times 10^{-4}$ for FdUrd, and 1.9×10^{-5} for VCR (the average of two experiments).

TABLE 2

Toxicity of AraC as measured by colony formation

About 24 hr after plating exponentially growing SV28 cells, the cells were treated for 16 hr with 0–20 μM araC. The cells were removed from the flasks, counted, and added to flasks with drug-free medium at appropriate cell numbers. After 9 days, colonies (>50 cells) were fixed, stained, and counted. Values are the average plating efficiencies ± the standard deviations.

AraC concentration	Plating efficiency
μM	%
0	40 ± 10
2	6.1 ± 3.5
5	2.7 ± 1.8
10	1.4 ± 1.3
20	0.60 ± 0.73

for this difference in enhancement of VCR resistance frequency are discussed later.

Enhancement of *cad* gene amplification by pretreatment with araC. Enhancement of MTX resistance after pretreatment has predominantly been examined with respect to amplification of the *dhfr* gene (5, 9). To date, enhancement of gene amplification at other loci has not been reported. For this reason, as well as the fact that the only reported mechanism for PALA resistance is amplification of the *cad* gene, we investigated the molecular basis of the increase in PALA resistance. PALA-resistant colonies were isolated and the *cad* gene copy number was determined after hybridization of slot blots to ^{32}P-labeled cDNAs for the *cad* gene and the α-fetoprotein gene. Differential densitometry indicated the extent of the increase in *cad* gene copy number. In all cases, an increase in the *cad* gene copy number was observed in PALA-resistant colonies; estimations of the extent of this amplification ranged from 3- to 11-fold after single-step selection to resistance to 100 μM

PALA. Representative data from the slot hybridizations are shown in Fig. 2 and quantitation of gene copy number is shown in Table 3. Although VCR resistance was either not increased or slightly increased after treatment with araC, preliminary analysis of VCR-resistant colonies that were obtained in this study has demonstrated that amplification of the *mdr* locus is responsible for the resistance in five of six resistant colonies analyzed (data not shown). Also, all VCR-resistant cells were as sensitive to VCR as wild-type cells when tested in the presence of verapamil, a drug known to reverse multidrug resistance (15).

Duration of araC pretreatment and the enhancement of PALA resistance. To further characterize the enhancement of PALA resistance by araC, we investigated the minimal duration of exposure needed to elicit a response. Asynchronous populations of SV28 were exposed to 5 μM araC for 1–16 hr, rinsed with PBS, and immediately placed in 100 μM PALA. Fig. 3 shows the increase in the incidence of PALA resistance as a function of the time of exposure to araC.

Does PALA rescue cells pretreated with AraC? It may be argued that the enhancement of drug resistance by araC is only apparent and that drugs such as PALA block incorporation of araC into DNA and thus prevent the lethal effect of araC. To test this, cells were pretreated with 20 μM araC for 16 hr,

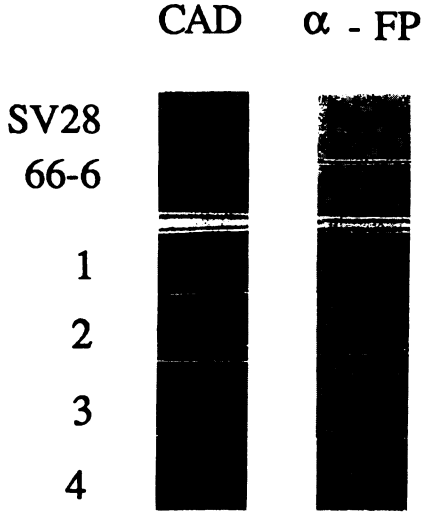

Fig. 2. Determination of *cad* gene copy number. PALA-resistant colonies described in Table 3 were analyzed for their *cad* gene copy number and α-fetoprotein (*α-FP*) gene copy number, as described in Materials and Methods.

TABLE 3

Gene amplification in PALA-resistant colonies

Subclone	LD_{50}[a]	AraC pretreatment[b]	Relative *cad* gene copy number[c]
SV 28 (parent)	5.8 μM	–	1
SV 66.6	2.8 mM	–	15
1	0.40 mM	–	4–6
2	0.45 mM	–	10–12
3	0.60 mM	+	4–5
4	0.73 mM	+	10–11

[a] For PALA.

[b] AraC pretreatment was in 5 μM for 16 hr before the cells were placed in 100 μM PALA for selection.

[c] Independent colonies of sensitive cells (SV28 parent), highly PALA-resistant cells (SV66.6; Ref. 20), and SV28 cells selected in 100 μM PALA (clones 1–4) were randomly picked and expanded as described in Materials and Methods. The degree of amplification was calculated after normalizing the DNA content by using the hybridization to α-fetoprotein.

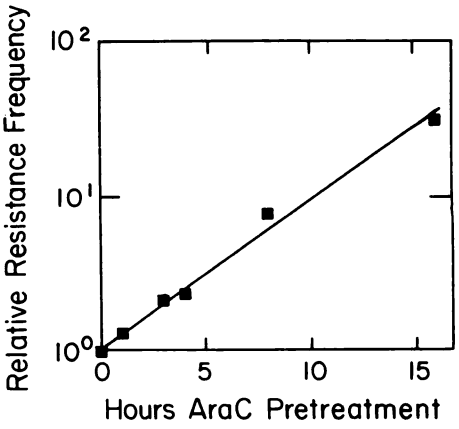

Fig. 3. Increase in incidence of PALA resistance as a function of the duration of araC pretreatment. SV28 cells were pretreated with 10 μM araC for 1 to 16 hr, rinsed in PBS, and immediately placed in selection medium containing 100 μM PALA. The results are the average of three experiments. The control values for the incidence of PALA resistance (without pretreatment with araC) in these experiments were 2.8×10^{-4}, 5.2×10^{-4}, and 6.2×10^{-4}. The incidence of PALA resistance after pretreatment with araC is relative to the control value based on viable cell number.

washed with PBS, incubated for another 24 hr with or without 300 μM PALA, and assayed for plating efficiency. In three such experiments, PALA failed to decrease the lethal action of araC. Control plating efficiency was 29 ± 1.4% and for PALA alone, 13 ± 2%. Plating efficiency for cells treated with both araC and PALA (0.038 ± 0.024%) was less than that of cells treated with araC alone (0.1 ± 0.042%). Thus, PALA does not prevent araC lethality and, therefore, cannot account for the enhancement of the frequency of resistance to PALA. In addition to these data, consider that Table 1 shows an enhancement of PALA resistance frequency even when not corrected for the viability decrease after araC pretreatment. Lastly, we have found[1] that PALA treatment for 24 hr after exposure to radiolabeled araC for 16 hr does not decrease the extent of araC incorporation into DNA.

Discussion

Actively growing mammalian tissue culture cells are able to develop drug resistance by a number of molecular mechanisms. Cells may alter transport of the drug, which results in a non-inhibitory intracellular concentration, a mechanism that has been documented for MTX and FdUrd resistance (1, 13). Alternatively, a reduction in the binding affinity of the enzyme for the drug can also provide resistance (1). A third possible mechanism for the development of resistance to a chemotherapeutic drug is the overproduction of the target enzyme (1). Previous studies on MTX-resistant cells demonstrated that this overproduction could be due to amplification of the *dhfr* gene within mutant murine cell lines (4), an observation that has been extended to hamster and human cell lines (3). The generation of MTX resistance has also been examined *in vivo*, in patient populations. Several clinical studies have demonstrated increased *dhfr* gene copy number in tumor cells after relapse and the emergence of MTX-resistant cells following chemotherapy (25–31). As in the studies of MTX resistance, overproduction of the TS enzyme in FdUrd resistance can be

[1] Unpublished observations.

the result of amplification of the target gene, in this case *ts* (13). PALA resistance differs from MTX and FdUrd resistance in that only one mechanism (overproduction of the multifunctional CAD protein, which contains the aspartate transcarbamylate activity) has been reported to date. Overproduction of this activity is due to amplification of the *cad* gene (2, 12).

MTX resistance can be enhanced by pretreatment of the cells before placing them in selection. Hydroxyurea (5), UV light (9), ethyl methane sulfonate (8), hypoxia (6), and MTX (7) are a few of the agents that have been reported to increase the incidence of MTX-resistant colonies. In this study we show that pretreatment of tissue culture cells with araC can enhance the generation of MTX-resistant colonies. Each of the agents that enhance the generation of MTX-resistant colonies exhibits a plethora of cellular effects. Cytotoxicity, inhibition of DNA synthesis, introduction of strand breaks into DNA, and the stimulation of chromosomal abnormalities are a few of the reported effects. The agent used in this study also evokes many of the responses observed with the other agents mentioned above (11, 32–36). Detailed studies have shown that araC is phosphorylated to the triphosphate, araCTP, which acts in an S phase-dependent manner to inhibit DNA synthesis in bacterial, viral, and mammalian systems (11), in addition to a multitude of other effects (32–34). The biochemical mechanism of action of this drug remains unclear despite intensive study.

Although amplification of the *ts* gene and *cad* gene is known to confer resistance to the respective drugs, it is not known whether this resistance can be enhanced in a fashion similar to that seen with MTX resistance and *dhfr* gene amplification. In this study, we show that pretreatment of tissue culture cells with araC can enhance the generation of MTX-, PALA-, and FdUrd-resistant colonies. Previous studies have attempted to influence the incidence of PALA resistance by pretreatment with ethylmethane sulfonate. A 2-fold increase in the number of drug-resistant colonies was obtained, leading to the conclusion that mutagens had little effect on the emergence of PALA resistance (20). Because of the carefully defined conditions that are required for observation of enhancement of MTX resistance (such as the duration of pretreatment, transient nature of the response, and the dose dependence), we believe that the lack of enhancement of PALA resistance in the previous study was a reflection of the conditions that were used. In support of this interpretation, it should be noted that the same cell line was used in the present study and that we have obtained enhancement of PALA resistance in three additional cell lines by using conditions similar to those described in this report.[1] In other studies reported by Bojan *et al.* (37), it was concluded that a phorbol ester could enhance MTX, PALA, and cadmium resistance in mouse cells but that the effect was not observed in hamster cells. In our studies, the enhancement of resistance to MTX, PALA, or FdUrd by araC in the hamster cell line SV28 is substantial and similar for these three selective drugs.

The molecular basis for the enhanced PALA-resistant colonies was investigated and we confirm that PALA resistance is due to amplification of the *cad* gene. In this, as well as previous, reports (2, 12), the sole mechanism for PALA resistance remains amplification of the *cad* gene. All PALA-resistant colonies tested demonstrate an increase in *cad* gene copy number. Table 3 shows representative data for the determination of *cad* gene copy number in some PALA-resistant colonies. Estimations of the extent of *cad* gene amplification in cells resistant to 100 μM PALA ranged from 3- to 12-fold, values similar to those reported in the literature (12, 38). The extent of *cad* amplification in the colonies that emerged after pretreatment with araC was no greater than the extent of *cad* amplification that was observed in those PALA-resistant colonies that emerged without treatment. In these experiments, while the incidence of PALA resistance and *cad* amplification increased significantly after pretreatment, the extent of *cad* amplification remained the same. These data are consistent with those presently and previously obtained in the enhancement of *dhfr* gene amplification (1, 5, 9) (data not shown). Comparison of the *cad* locus with the *dhfr* locus shows that the extent of amplification at both loci after a single-step selection is similar (5- to 10-fold) and that the extent of amplification in individual colonies is independent of the pretreatment used.

We also examined the generation of resistance to VCR, an agent that inhibits tubulin polymerization. VCR selection by itself (no pretreatment) yields resistant cells in a frequency range that is similar to that seen with other drugs (see Fig. 1 and Table 1). However, in contrast to the other three drugs, VCR resistance is not appreciably enhanced by araC pretreatment (see Table 1). Recent reports have indicated that multidrug resistance can be achieved by amplification of the *mdr* locus in hamster cell lines (15–17, 29) as well as overexpression of the locus in the absence of amplification in human cell lines (14, 18). Our preliminary analysis indicates that five of the six VCR-resistant colonies we examined were amplified.[1]

In this study, the cellular resistance to four different chemotherapeutic agents has been investigated. Resistance to three of these agents (MTX, PALA, and FdUrd) can be significantly increased by pretreatment with araC. Resistance to the fourth, VCR, was relatively minimally increased. What could be the basis for the difference in araC enhancement seen between the two different classes of genetic loci? One explanation could involve the genomic position of the *mdr* locus. Studies with the *cad* gene (39) as well as the *dhfr* gene (40) transfected into various genomic sites have led to the appreciation that different genomic loci may be highly permissive or relatively nonpermissive for amplification of the transfected gene. Amplification of the same gene at different loci can vary by 3 orders of magnitude. Similar conditions could be applicable in the enhancement of gene amplification, leading to the differences noted in this study.

A second explanation could relate to the nature of the selective drug. Each of the first three agents (MTX, PALA, and FdUrd) limits the amount of precursors available for the synthesis of DNA, thereby halting the cell cycle in S phase. Resistance to each of these drugs can be significantly increased by pretreatment with araC. In contrast, VCR is known to bind specifically to tubulin and treatment of mammalian cells with VCR results in a G2 block within the cell cycle (41). Polymerized tubulin is necessary for chromosome separation and cell division as well as other cellular processes that utilize microtubular structures. VCR resistance is not dramatically enhanced by pretreatment with araC. Could it be that both pretreatment and selection drugs must block in S phase in order to enhance drug resistance? If so, then a lack of direct inhibition of DNA replication by VCR could be responsible for the inability of araC to strongly enhance the *mdr* locus. This explanation can be tested by using other drugs to select the multidrug-resistant phenotype.

As a third explanation, it should be noted that *dhfr*, *cad*, and *ts* are all housekeeping genes and, as such, are transcriptionally active in each cell cycle. Obviously, amplification of a transcriptionally active gene can lead to overexpression of that gene product and the resultant drug resistance. Although the *mdr* gene product is thought to be involved in a membrane alteration that results in decreased intracellular drug accumulation (24), it is known that expression of *mdr* is not required for cycle progression. Transcriptional activity of the *mdr* gene locus is not detectable in several drug-sensitive cell lines (16–18). Amplification of a transcriptionally inert sequence would not necessarily result in drug-resistant colonies unless it were transcriptionally activated. Thus, transcriptional activity may be a governing factor in the ability of a genomic locus to respond to agents that enhance gene amplification. Further studies on the difference between the *mdr* locus and the other three loci with regard to their ability to generate drug-resistant colonies after pretreatment may give us insights into the genomic or metabolic variables that govern the enhancement of gene amplification.

During resistance can come into play at two levels in patient treatment. The first is in the inherent resistance of the tumor mass to initial chemotherapy; the second is during a patient relapse, when a tumor that was previously sensitive to a drug is now resistant. Gene amplification is involved in both levels. The results presented in this paper are particularly relevant to the second level of patient treatment, in that we demonstrate that chemotherapeutic agents themselves can significantly enhance the generation of drug-resistant cell populations.

Acknowledgments

We thank J.-M. Vos, V. Stewart, and B. Neely for helpful discussions and comments on the manuscript and S. McCord for assistance with the slot blot hybridizations.

References

1. Schimke, R. T. Gene amplification in cultured animal cells. *Cell* **37**:705–713 (1984).
2. Stark, G. R., and G. M. Wahl. Gene amplification. *Annu. Rev. Biochem.* **53**:447–491 (1984).
3. Biedler, J. L., and B. A. Spengler. Metaphase chromosome anomaly: association with drug resistance and cell-specific products. *Science* (*Wash. D. C.*) **191**:185–187 (1976).
4. Alt, F. W., R. E. Kellems, J. R. Bertino, and R. T. Schimke. Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. *J. Biol. Chem.* **253**:1357–1370 (1978).
5. Brown, P. C., T. D. Tlsty, and R. T. Schimke. Enhancement of methotrexate resistance and dihydrofolate reductase gene amplification by treatment of mouse 3T6 cells with hydroxyurea. *Mol. Cell. Biol.* **3**:1097–1107 (1983).
6. Rice, G. C., C. Hoy, and R. T. Schimke. Transient hypoxia enhances the frequency of dihydrofolate reductase gene amplification in Chinese hamster ovary cells. *Proc. Natl. Acad. Sci. USA* **83**:5978–5982 (1986).
7. Tlsty, T. D., P. C. Brown, R. Johnston, and R. T. Schimke. Enhanced frequency of generation of methotrexate resistance and dihydrofolate reductase gene amplification in cultured mouse and hamster lines, in *Gene Amplification* (R. T. Schimke, ed.). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 231–238 (1982).
8. Flintoff, W. E., S. V. Davidson, and L. Siminovitch. Isolation and partial characterization of three methotrexate-resistant phenotypes from Chinese hamster ovary cells. *Somat. Cell. Genet.* **2**:245–261 (1976).
9. Tlsty, T. D., P. C. Brown, and R. T. Schimke. UV radiation facilitates methotrexate resistance and dihydrofolate reductase gene amplification in cultured murine cells. *Mol. Cell. Biol.* **4**:1050–1056 (1984).
10. Frei, E., J. N. Bickers, J. S. Hewlett, M. Lane, W. V. Leary, and R. W. Talley. Dose schedule and antitumor studies of arabinosyl cytosine. *Cancer Res.* **29**:1325–1332 (1969).
11. Kufe, D. W., D. Munroe, D. Herrick, E. Egan, and D. Spriggs. Effect of 1-β-D-arabinofuranosylcytosine incorporation on eukaryotic DNA template function. *Mol. Pharmacol.* **26**:128–134 (1984).
12. Wahl, G. M., R. A. Padgett, and G. R. Stark. Gene amplification causes overproduction of the first three enzymes of UMP synthesis in *N*-(phosphoacetly)-L-aspartate-resistant hamster cells. *J. Biol. Chem.* **254**:8679–8689 (1979).
13. Rossana, C., L. G. Rao, and L. F. Johnson. Thymidylate synthetase overproduction in 5-fluorodeoxyuridine-resistant mouse fibroblasts. *Mol. Cell. Biol.* **2**:1118–1125 (1982).
14. Fuqua, S. A. W., I. M. Moretti-Rojas, S. L. Schneider, and W. L. McQuire. P-glycoprotein expression in human breast cancer cells. *Cancer Res.* **47**:2103–2106 (1987).
15. Riordan, J. R., and V. Ling. Genetic and biochemical characterization of multidrug resistance. *Pharmacol. Ther.* **28**:51–75 (1985).
16. Roninson, I. B., H. T. Abelson, D. E. Housman, N. Howell, and A. Varshavsky. Amplification of specific DNA sequences correlates with multi-drug resistance in Chinese hamster cells. *Nature* (*Lond.*) **309**:626–628 (1984).
17. Scotto, K. W., J. L. Biedler, and P. W. Melera. Amplification and expression of genes associated with multidrug resistance in mammalian cells. *Science* (*Wash. D. C.*) **232**:751–755 (1986).
18. Shen, D.-W., A. Fono, J. E. Chin, I. B. Roninson, N. Richert, I. Pastan, and M. M. Gottesman. Human multidrug-resistant cell lines: increased *mdr1* expression can precede gene amplification. *Science* (*Wash. D. C.*) **232**:643–645 (1986).
19. Wiblin, C. N., and I. A. MacPherson. The transformation of BHK21 hamster cells by Simian virus 40. *Int. J. Cancer* **10**:296–309 (1972).
20. Kempe, T. D., E. A. Sward, M. Bruist, and G. R. Stark. Stable mutants of mammalian cells that overproduce the first three enzymes of pyrimidine nucleotide biosynthesis. *Cell* **9**:541–550 (1976).
21. McGarrity, G. J., and D. A. Carson. Adenosine phosphorylase-mediated nucleoside toxicity. *Exp. Cell Res.* **139**:199–205 (1982).
22. Shigesada, K., G. R. Stark, J. A. Maley, L. A. Niswander, and J. N. Davidson. Construction of a cDNA to the hamster *cad* gene and its amplification toward defining the domain for aspartate transcarbamylase. *Mol. Cell. Biol.* **5**:1735–1742 (1985).
23. Law, S., T. Tamoaki, F. Krevzaler, and A. Dugaiczyk. Molecular cloning of DNA complementary to mouse α-fetoprotein RNA sequence. *Gene* **10**:53–61 (1980).
24. Ling, V., and L. Thompson. Reduced permeability in CHO cells as a mechanism of resistance to colchicine. *J. Cell. Physiol.* **83**:103–116 (1974).
25. Bertino, J. R., M. D. Carman, H. L. Weiner, A. Cashmore, B. A. Moroson, S. Srimatkandada, J. H. Schornagel, W. D. Medina, and S. K. Dube. Gene amplification and altered enzymes as mechanisms for the development of drug resistance. *Cancer Treat. Rep.* **67**:901–904 (1983).
26. Cardman, M. D., J. H. Schornagel, R. S. Rivest, S. Srimatkandada, C. S. Portlock, T. Duffy, and J. R. Bertino. Resistance to methotrexate due to gene amplification in a patient with acute leukemia. *J. Clin. Oncol.* **2**:16–20 (1984).
27. Chabner, B. A., N. J. Clendeninn, and G. A. Curt. Symposium on cellular resistance to anticancer drugs. Cancer Treat. Rep. **67**:855–857 (1983).
28. Curt, G. A., D. N. Carney, K. H. Cowan, J. Jolivet, B. D. Bailey, J. C. Drake, C. S. Kao-Shan, J. D. Minna, and B. A. Chabner. Unstable methotrexate resistance in human small-cell carcinoma associated with double minute chromosomes. *N. Engl. J. Med.* **308**:199–202 (1983).
29. Curt, G. A., K. H. Cowan, and B. A. Chabner. Gene amplification in drug resistance: of mice and men. *J. Clin. Oncol.* **2**:62–64 (1984).
30. Horns, R. C., W. J. Downer, and R. T. Schimke. Gene amplification in a leukemic patient treated with methotrexate. *J. Clin. Oncol.* **2**:2–7 (1984).
31. Trent, J. M., R. M. Buick, S. Olson, R. C. Horns, and R. T. Schimke. Cytologic support for gene amplification in methotrexate-resistant cells obtained from a patient with ovarian adenocarcinoma. *J. Clin. Oncol.* **2**:8–15 (1984).
32. Crowther, P. J., I. A. Cooper, and D. M. Woodcock. Biology of cell killing by 1-β-D-arabinofuranosylcytosine and its relevance to molecular mechanisms of cytotoxicity. *Cancer Res.* **45**:4291–4300 (1985).
33. Fram, R. J., and D. W. Kufe. DNA strand breaks caused by inhibitors of DNA synthesis 1-β-D-arabinofuranosylcytosine and aphidocolin. *Cancer Res.* **42**:4050–4053 (1982).
34. Kufe, D. W., P. P. Major, E. M. Egan, and G. P. Beardsley. Correlation of cytotoxicity with incorporation of araC into DNA. *J. Biol. Chem.* **255**:8997–9000 (1980).
35. Woodcock, D. M., J. K. Adams, and I. A. Cooper. Relationship between aberrant DNA replication and loss of cell viability in Chinese hamster ovary CHO-K1 cells. *Cancer Res.* **42**:4744–4752 (1982).
36. Woodcock, D. M., and I. A. Cooper. Aberrant double replication of segments of chromosomal DNA following DNA synthesis inhibition by cystosine arabinoside. *Exp. Cell Res.* **123**:157–166 (1979).
37. Bojan, F., A. R. Kinsella, and M. Fox. Effect of tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate on recovery of methotrexate-, *N*-(phosphonoacetly)-L-aspartate-, and cadmium-resistant colony-forming mouse and hamster cells. *Cancer Res.* **43**:5217–5221 (1983).
38. Zieg, J., C. E. Clayton, F. Ardeshir, E. Giulotto, A. El Swyrd, and G. R. Stark. Properties of single-step mutants of Syrian hamster cell lines resistent to *N*-(phosphonoacetyl)-L-aspartate. *Mol. Cell. Biol.* **3**:2089–2098 (1983).
39. Wahl, G. M., R. A. Padgett, and G. R. Stark. Effect of chromosomal position on amplification of transfected genes in animal cells. *Nature* (*Lond.*) **307**:516–520 (1984).
40. Gasser, C. S., and R. T. Schimke. Cell cycle regulation of transfected murine dihydrofolate reductase gene. *J. Biol. Chem.* **261**:6938–6946 (1986).
41. Mujagic, H., S.-S. Chen, R. Geist, S. J. Occhipinti, B. M. Conger, C. A. Smith, W. H. Schuette, and S. E. Shackney. Effects of vincristine on cell survival, cell cycle progression, and mitotic accumulation in asynchronously growing sarcoma 180 cells. *Cancer Res.* **43**:3591–3597 (1983).

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