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Selective Cytotoxicity of Haloethylnitrosoureas in a Carcinoma Cell Line Resistant to Bifunctional Nitrogen Mustards

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SUMMARY

A Walker 256 rat carcinoma cell line (WR) has been shown to be resistant to a broad spectrum of bifunctional nitrogen mustards (NM) in cell culture. The parent cell line (WS) from which the WR cells were selected retains marked sensitivity to this class of drugs. Karyotype analysis showed that the parent WS had a chromosome content which was significantly higher than the WR. A number of chromosome marker bands were also distinguishable, indicative of distinct nuclear structural differences. A lack of collateral resistance to haloethylnitrosoureas was demonstrated for the WR cell line. In some cases the sensitivity of the WR to nitrosoureas was greater than that of WS. In addition, the WR could be sensitized to NM by a concomitant addition of a water-soluble carbamoylating agent, *N,N'*-bis(*trans*-4-hydroxycyclohexyl)-*N'*-nitrosourea, which possessed no intrinsic alkylating activity. Since NM and nitrosoureas differ pharmacologically, mainly by the latter's potential to carbamoylate, this reaction would appear to be critical to the cytotoxic properties of nitrosoureas against WR cells. Heretofore, carbamoylation has been considered of little importance to the antitumor properties of nitrosoureas. Moreover, 1-(4-amino-2-methylpyrimidine-5-yl)-methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU) has been reported as possessing only minimal carbamoylating potential, as measured *in vitro* by a lysine assay. While remaining an accurate assay for the modification of the ε-amino groups of lysine, it is possible that the quantitative lysine assay may not predict the physiological carbamoylating potential of ACNU in the Walker cells, since marked cytotoxicity was achieved in both WR and WS by ACNU.

INTRODUCTION

A major problem in the successful eradication of tumor cells by conventional chemotherapy remains the ultimate selection of tumor cell subpopulations which either have intrinsic or acquire resistance to anticancer agents. In many cases, the precise molecular mechanism by which a tumor cell acquires resistance is unknown, although factors such as impaired drug uptake, inability to activate drug, and increased repair potential have been implied. In addition, differences in nuclear structure have been linked with differential drug response and drug resistance for antimetabolites such as methotrexate (1–3), but there is no definitive link between resistance to nuclear-reactant drugs and nuclear structural alterations. Earlier considerations of the resistance phenomenon suggested that, because of the similar pharmacological properties of most nuclear-reactant drugs, cross-resistance to a variety of bifunctional alkylating agents was assured (4). Since then, specific examples of animal tumors which exhibit no cross-resistance between aromatic nitrogen mustards and chloroethylnitrosoureas have been discovered and reviewed (5). The possible therapeutic synergism of drugs such as L-phenylacetic mustard and bis(chloroethyl)nitrosourea have been discussed (5), as well as increased cytotoxic potential of the nitrosoureas chlorozotocin and CCNU[1] in combination (6). From such

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[1] The abbreviations used are: CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; CLZ, 2-[3-(2-chloroethyl)-3-nitrosoureido]-2-deoxy-D-glucopyranose; STZ, 2-(3-methyl)-3-nitrosoureido-2-deoxy-D-glucopyranose; ACNU, 1-(4-amino-2-methylpyrimidine-5-yl)-methyl-3-(2-chloroethyl)-3-nitrosoureahydrochloride; HN_2, mechlorethamine (nitrogen mustard); (4-OH)-BCYNU, *N,N'*-bis-(*trans*-4-hydroxycyclohexyl)-*N'*-nitrosourea; PM, phosphoramide mustard; D-SM, 2,3,4,6-tetra-*O*-acetyl *N,N*-bis(2-chloroethyl)-D-glucopyranosylamine; L-SM, 2,3,4,6-tetra-*O*-acetyl *N,N*-bis(2-chloroethyl)-L-glucopyranosylamine; *cis*-DDP, *cis*-diamminedichloro-platinum; MNU, 1-methyl-1-nitrosourea; CNCC, di[(2-chloroethyl)-2*N*-nitroso-*N*-carbamoyl]-*N,N*-cystamine; FCCNU, 1-(2-chloroethyl)-3-(1*H*-decafluoro-cyclohexyl)-1-nitrosourea; HSR, homogeneously staining regions.

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studies it is apparent that, even though designated alkylating drugs possess similar pharmacological properties, their potential to induce tumor cell death may not be mechanistically identical.

Nitrosoureas are known to possess carbamoylating activity, as measured by modifications of [^{14}C]lysine *in vitro* (7). Low carbamoylating potential for the glucose nitrosoureas, CLZ and STZ, is attributable to intramolecular reactions, resulting in the formation of six 5-membered ring carbamate sugars (8, 9). In common with CLZ and STZ, ACNU has been shown to possess negligible potential to carbamoylate lysine (10); however, several enzyme systems are modified *in vivo* by this drug (11). It has been possible, in this study, to consider a number of nitrosoureas with diverse carbamoylating potential (see Tables 1 and 3) in order to estimate the cytotoxic potential of this covalent drug modification. By overcoming the resistance of Walker carcinoma cells to bifunctional alkylating agents, the importance of carbamoylation to nitrosourea antitumor activity has been established.

MATERIALS AND METHODS

Cell cultures. Walker 256 carcinoma cells were obtained through Dr. K. R. Harrap from the Chester Beatty Research Institute, London. Resistant (WR) cells had been previously developed through selective exposure of the parent cell line (WS) to chlorambucil (12). Both cultures were maintained in Dulbecco's minimal essential medium with glucose (4.5 g/liter) supplemented with 10% horse serum (MA Bioproducts, Walkerville, Md.) under 5% CO_2. Resistance is maintained biannually by exposure to chlorambucil (20 μg/ml) and by periodic i.p. passage in Wistar rats.

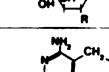
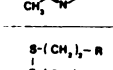
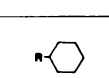
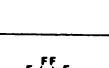
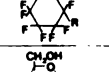
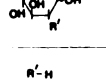
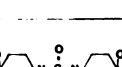
Colony-forming assay. Log-phase cells (2×10^5 cells/ml) were treated for 1 hr [HN_2, (4-OH)-BCyNU] or 2 hr (all others) at 37° with various concentrations of the drugs shown in Table 1. Following incubation, 0.15 ml of cells were transferred into 19.85 ml of complete medium and Noble agar, final agar concentration 0.3% (Difco Scientific, Detroit, Mich.). This step effectively diluted out unincoporated drug. After gentle mixing to ensure minimal cell clumping, four 5-ml aliquots were poured onto 15 × 60 mm Petri dishes. Plates were allowed to solidify at ambient temperature for 15 min prior to being transferred to a humidified incubator at 37° under 5% CO_2. After 6 days, colonies of 32 cells or greater (represents more than five cell divisions) were scored as viable, and data were expressed as percentage of control cultures. Plating efficiency was >70% for all experiments.

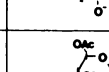
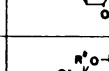
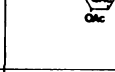
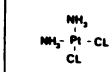
Karyotype analysis. Incubation of log-phase WR and WS cultures (2×10^5 cells/ml) with colcemid (0.5 μg/ml) (2 hr at 37°) was followed by a 10-min cell lysis at room temperature with a hypotonic solution of 0.075 M KCl. Subsequent fixation was achieved by using three changes of methanol-acetic acid (3:1). Chromosome banding was achieved by the trypsin-Giemsa method (13).

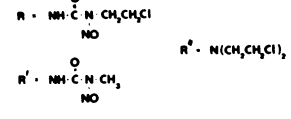
Drugs. Information concerning the drugs used and their abbreviated names is shown in Table 1. HN_2, PM, D-SM, L-SM, *cis*-DDP, CLZ, ACNU, CCNU, STZ, and MNU were supplied by Dr. V. L. Narayanan, National Cancer Institute (Bethesda, Md.); (4-OH)-BCyNU was a gift from Dr. J. Montgomery, Southern Research Institute (Birmingham, Ala.); FCCNU was a gift from Dr. A. Foster, Chester Beatty Research Institute (London, Eng-

TABLE 1

Chemical structure of alkylating agents and nitrosoureas

COMMON NAME (ABBREVIATION)	STRUCTURE	CHEMICAL NAME	NSC
CLZ		2-[3-(2-CHLOROETHYL)-3-NITROSOUREIDO]-2-DEOXY-D-GLUCOPYRANOSE	178248
ACNU		1-(4-AMINO-2-METHYLPYRIMIDINE-5-YL)-METHYL-3-(2-CHLOROETHYL)-3-NITROSO-UREA HYDROCHLORIDE	245382
CCNU		DI[(2-CHLOROETHYL)-2N NITROSO N-CARBAMOYL] N,N-CYSTAMINE	TCIG-1525
FCCNU		1-(2-CHLOROETHYL)-3-CYCLOHEXYL-1-NITROSOUREA	79037
FCCNU		1-(2-CHLOROETHYL)-3-(1H-DECAFLUORO-CYCLOHEXYL)-1-NITROSOUREA	301741
STZ		2-(3-METHYL-3-NITROSOUREIDO-2-DEOXY-D-GLUCOPYRANOSE	85998
MNU		1-METHYL-1-NITROSOUREA	23909
(4-OH)-BCyNU		N,N'-BIS-(TRANS-4-HYDROXYCYCLOHEXYL)-N''-NITROSOUREA	305715

COMMON NAME (ABBREVIATION)	STRUCTURE	CHEMICAL NAME	NSC
HN_2		MECHLORETHAMINE (NITROGEN MUSTARD)	762
PM		PHOSPHORAMIDE MUSTARD	69945
D-SM		2,3,4,6-TETRA-O-ACETYL N,N-BIS (2-CHLOROETHYL)-D-GLUCOPYRANOSYL-AMINE	342215
L-SM		2,3,4,6-TETRA-O-ACETYL N,N-BIS (2-CHLOROETHYL)-L-GLUCOPYRANOSYL-AMINE	342216
CIS-DDP		CIS-DIAMMINEDICHLORO-PLATINUM (II)	119875



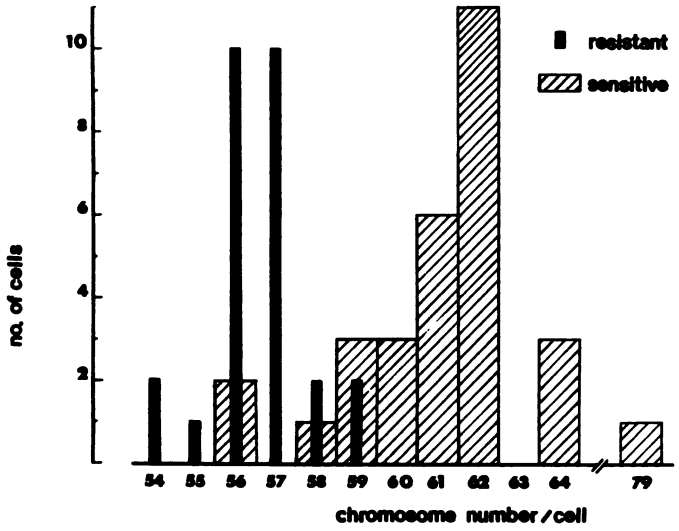


FIG. 1. *Chromosome number heterogeneity in WR and WS cells*
Mitotic cells were prepared and chromosome numbers per cell were estimated. Modal chromosome per cell values were different for WR and WS. Mean chromosome numbers per cell were significantly different as judged by parametric (Student's t-test, $p < 0.01$) and nonparametric (Kruscal-Wallis test, $p < 0.005$) analyses.

land); CNCC was a gift from Dr. G. Mathe (Villejuif, France) and was supplied under the auspices of the United States-France Scientific Exchange Programme. Non-water-soluble compounds (D-SM, L-SM, *cis*-DDP, CCNU, MNU, FCCNU, CNCC) were dissolved in absolute ethanol, which was found to have no effect upon WR and WS cell survival. All others were dissolved in culture medium.

RESULTS

Karyotype analysis. Initial characterization of the nuclear structure and karyotype of these cell lines revealed that significant differences existed in chromosome number per cell. Figure 1 shows the numerical chromosomal heterogeneity between WR and WS. The mean chromosome per cell values were 56.5 for WR and 61.5 for WS. Both parametric and nonparametric analyses of these data confirmed that the populations were significantly different from each other and were aneuploid (polysomic) with respect to the normal diploid rat cell, where $2n = 42$. Other nuclear differences are reported in Table 2. The extra chromosomes are reflected in the DNA and RNA ratios between WS and WR: WS contained 1.49 pg of DNA and 19.1 pg of RNA per cell, and WR contained 1.19 pg of DNA and 13.7 pg of RNA per cell. A number of marker chromosomes were present, and distinct differences between WR and WS were apparent (Table 2). The precise relevance of these marker regions is unclear; however, HSR have been reported in other tumor cells which possess resistance to antimetabolites (2). In addition to these specific chromosomal differences, initial data reported elsewhere (14) suggested that there were differences between structural proteins of the nucleus (nuclear matrix) in WR and WS.

TABLE 2
Karyotype analysis of WR and WS cells

Analysis	Type of cell	
	WR	WS
Polyploid percentage	10	10
Modal chromosome number	56	62
DNA ratio	1	1.25
RNA ratio	1	1.4
No. of marker chromosomes	9–11	8–11
Specific markers		
Large metacentric	++	++
Large acrocentric	–	+
Acrocentric H_2 with terminal arm HSR	+[a]	+[b]
Submetacentric H_1	–	+
Submetacentric H_3	+	–
Submetacentric variable banding	++	++

[a] Short HSR.
[b] Long HSR.

Structure-activity relationships. The cytotoxic potential of a diverse range of nuclear-reactant drugs was determined, and the data are presented in Figs. 2–10. The drugs have been classified into four groups based on their molecular interactions with nucleophilic sites as outlined in Table 3.

A comparison of the D- and L-forms of the sugar mustard (Fig. 2) showed that on a micromolar basis D-SM was more toxic to both WR and WS. The attachment of the glucopyranosylamine residue had no effect upon the resistance properties of WR cells, suggesting that possible differences in carbohydrate transport were not of relevance to the resistance phenomenon. Other bifunctional mustards, such as PM (Fig. 3) and HN_2 (Fig. 4), were more cytotoxic at similar molarity to both WR and WS, but similar degrees of resistance were found in the WR cells. Although *cis*-DDP is not a true bifunctional mustard, it has been shown to exchange chloride ions for nucleophilic groups and form covalent bonds. In an aqueous medium, both chloride ions can be replaced by nucleophiles with or without a hydrated intermediate (18), thus creating bifunctionality similar to that of the mustards. The cytotoxic properties of *cis*-DDP are shown in Fig. 3, with a degree of resistance again demonstrated in WR cells.

A variety of haloethylnitrosoureas were screened against WR and WS (Figs. 4–7). For all of these drugs the resistance properties of the WR cells to drugs of Group 1 were overcome by those of Group 2 (cf. Table 3). The attachment of 10 fluorine molecules to the cyclohexyl ring of CCNU was originally conceived to prevent the metabolism of CCNU (22). As is apparent in Figs. 4 and 6, micromolar ID values were lower for FCCNU than for CCNU, suggesting that the extra halogen groups increased the cytotoxic potential of the drug. The inclusion of cystamine into the nitrosourea CNCC was designed to modify the transport and metabolism of the drug (21). Figure 5 demonstrates that CNCC cytotoxicity to WR and WS is consistent with that of the other haloethylnitrosoureas. Both CLZ and ACNU have been shown to possess low carbamoylating potential as measured by lysine modification *in vitro* (7, 10). Of the nitrosoureas tested, chlorozotocin was found to be the least cytotoxic, as a function of concentration (Fig. 6), or less cytotoxic than CCNU, as a function of time after

TABLE 3

Possible lesions caused by nuclear-reactant drugs

Group 1, bifunctional nitrogen mustards (+ *cis*-DDP); Group 2, haloethylnitrosoureas; Group 3, methylnitrosoureas; Group 4, carbamoylating agent. The potential to form specific adducts is signified by a plus sign.

Drug	Reaction				References
	Monoadduct	Strand breaks	Cross-links	Carbamoylation	
Group 1					
HN_2	+	+	+	–	15, 16
PM	+	+	+	–	17
D-SM	+	+	+	–	*,[a] *[b]
L-SM	+	+	+	–	*,[a] *[b]
Cis-DDP	+	+	+	–	18, 19
Group 2					
CLZ	+	+	+	–[c]	8, 10, 16, 20
ACNU	+	+	+	–[c]	10
CNCC	+	+	+	+	21, *[b]
CCNU	+	+	+	+	7, 16, 20
FCCNU	+	+	+	+	20, 22
Group 3					
STZ	+	+	–	–[c]	9, 20
MNU	+	+	–	+	16, 20
Group 4: (4-OH)-BCyNU	+[d]	–	–	+	23, 24

[a] Experimental evidence unavailable. Reactions are based upon expectation from structural analysis.

[b] K. D. Tew and A. L. Wang, unpublished observations.

[c] Carbamoylation of lysine measured *in vitro* as approximately 3% of CNU, i.e., considered quantitatively negligible.

[d] In addition to amino acids, nucleophilic sites on nucleic acids and other macromolecules are susceptible, but are likely to have minimal effects on the cytotoxic properties of (4-OH)-BCyNU.

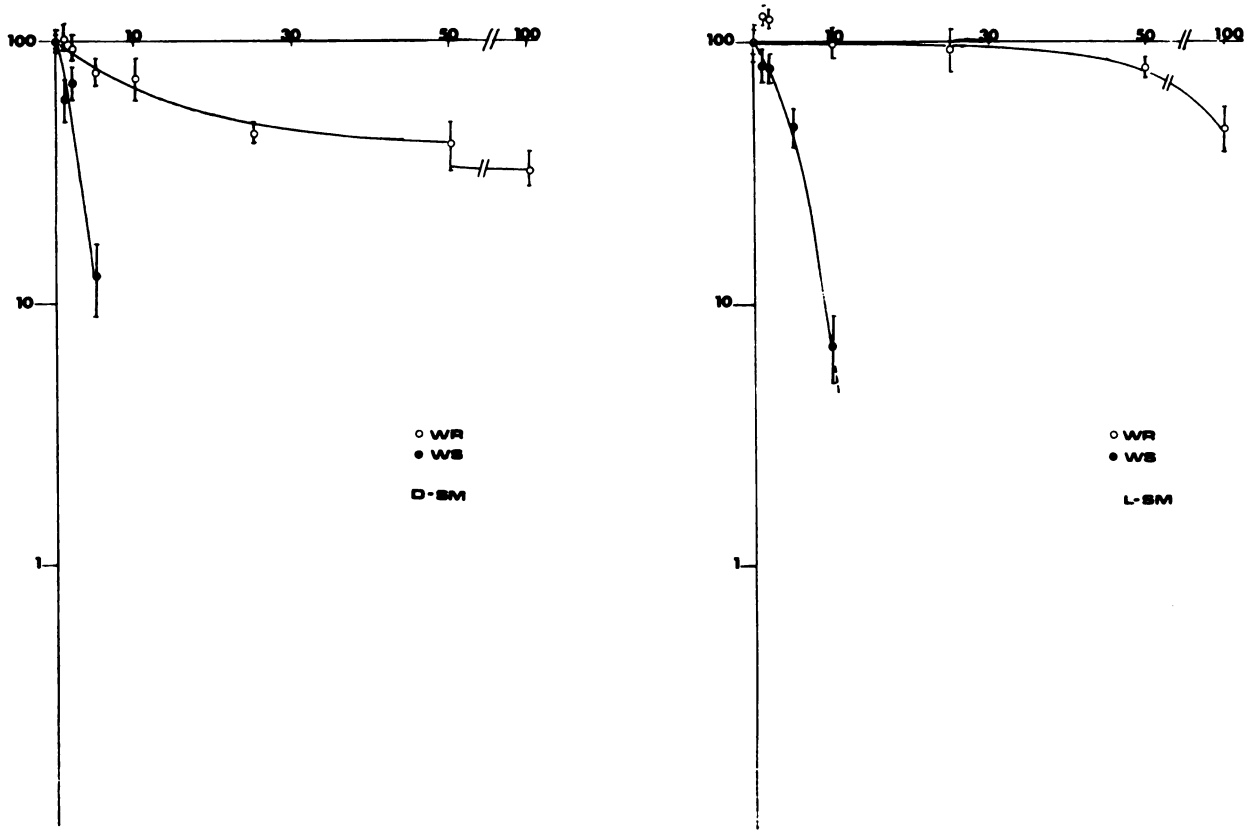


FIG. 2. *Effect of various concentrations of* D*-SM and* L*-SM on colony-forming ability of WR and WS cells*

Drug concentrations are expressed in micromolarity (abscissa). The percentage log colony-forming units is plotted on the ordinate axis. Colony-forming assays were performed as outlined under Materials and Methods. Each point represents the mean of quadruplicate experiments ± standard deviation.

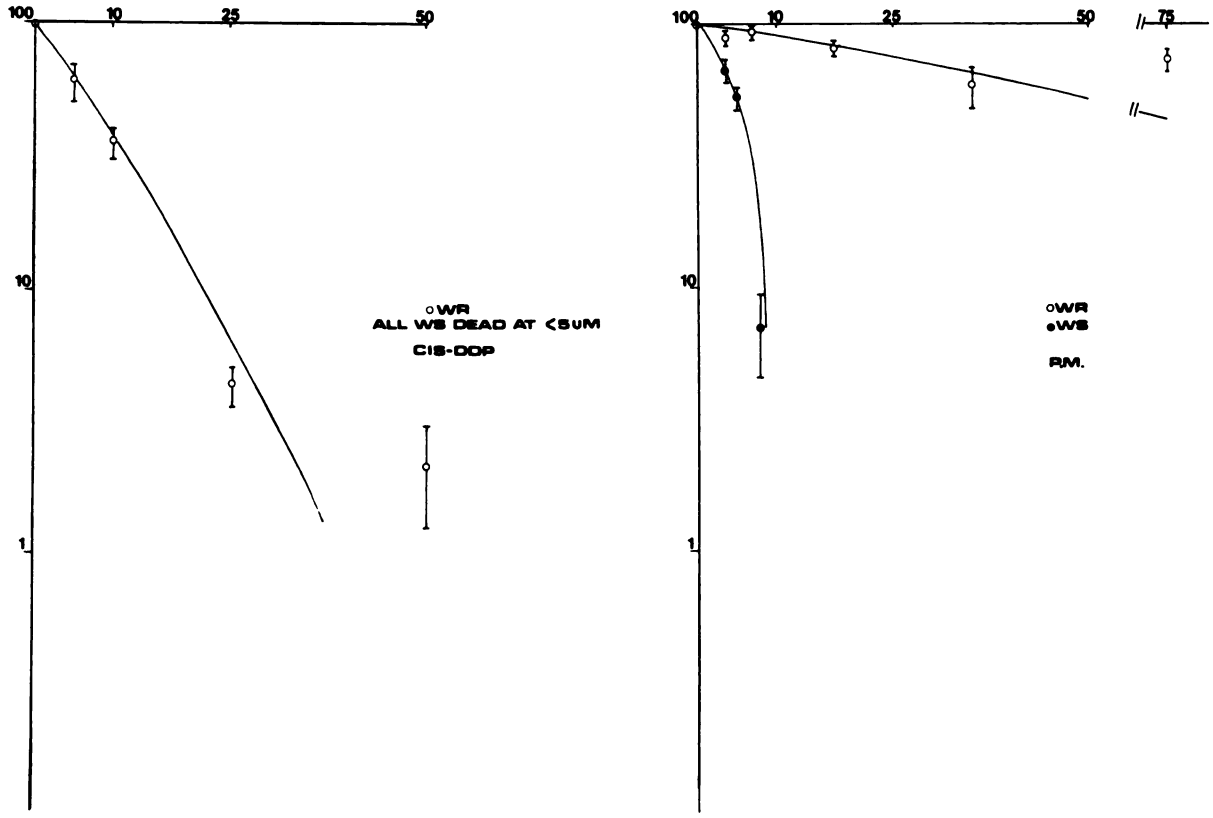


FIG. 3. *Effect of various concentrations of cis-DDP and PM on colony-forming ability of WR and WS cells*
See legend to Fig. 2 for further details.

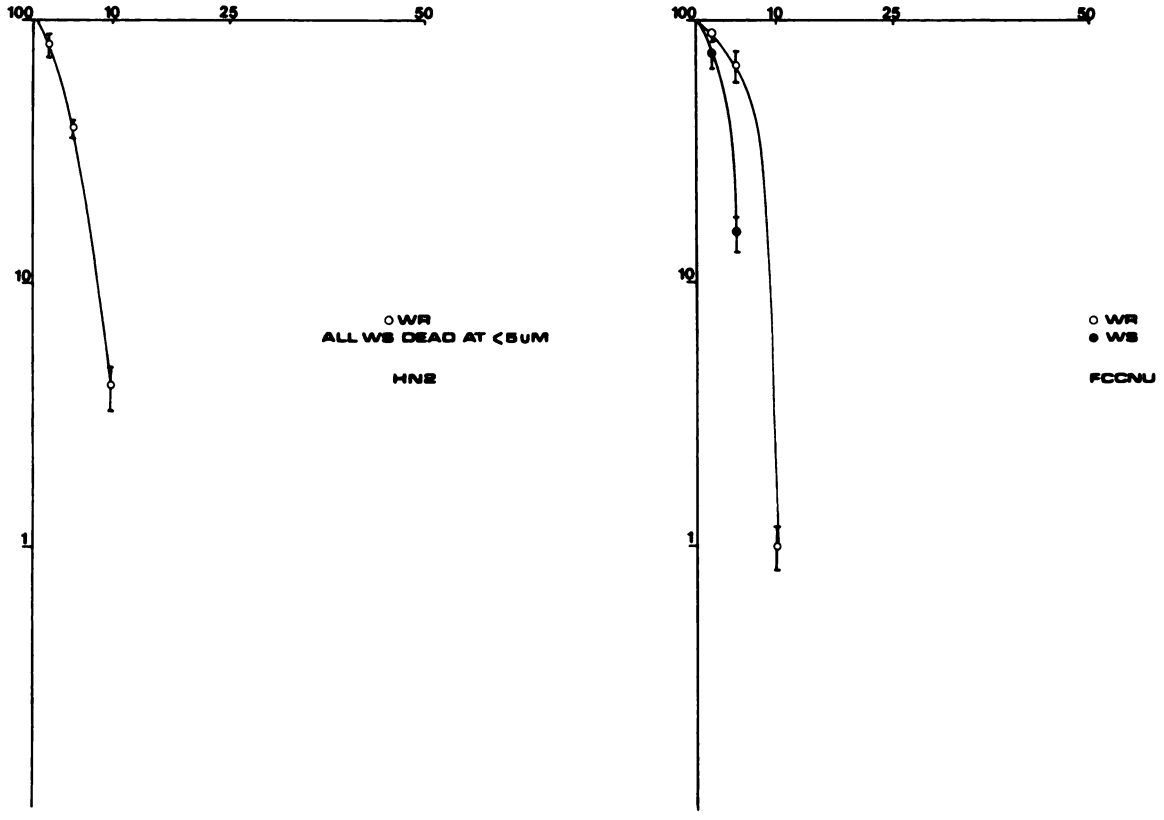


FIG. 4. *Effect of various concentrations of HN_2 and FCCNU on colony-forming ability of WR and WS cells*
See legend to Fig. 2 for further details.

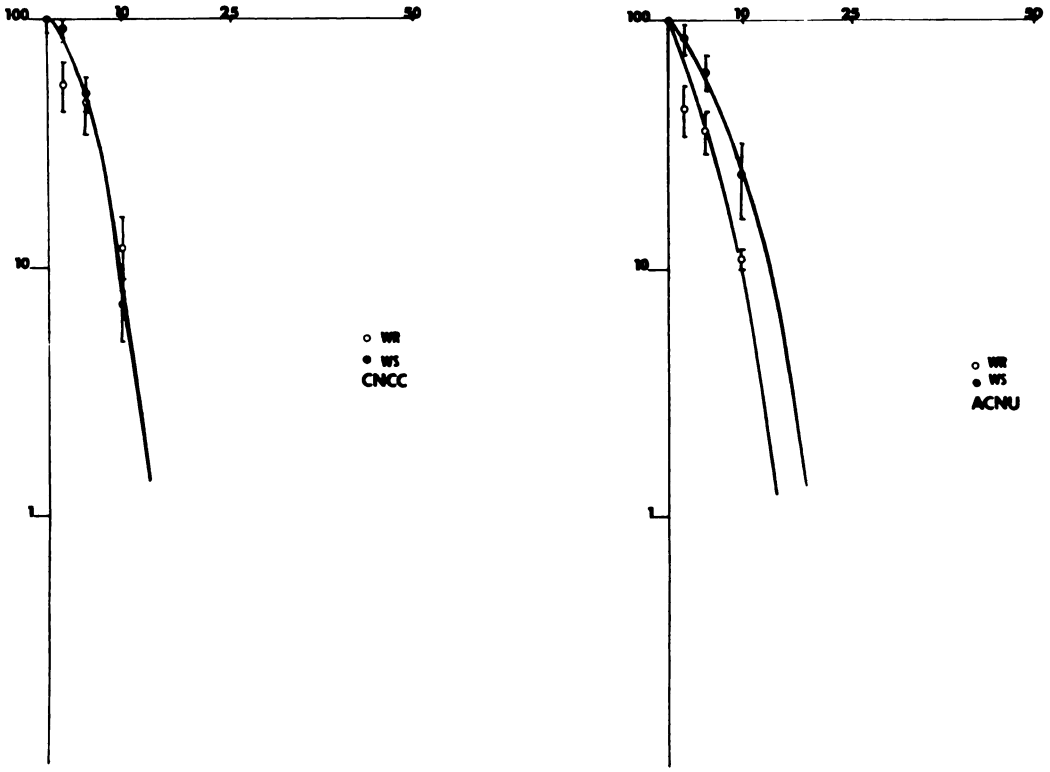


FIG. 5. *Effect of various concentrations of CNCC and ACNU on colony-forming ability of WR and WS cells*
See legend to Fig. 2 for further details.

administration (Fig. 7). At a concentration of 10 μM, CCNU killed all cells between 12 and 24 hr after administration. At the same molarity, CLZ caused complete cell kill between 24 and 48 hr. On a molar basis, CLZ was less cytotoxic than CCNU or the other chloroethylnitrosoureas. This would be consistent with the reported low carbamoylating activity of CLZ *in vitro* or could be indicative of either reduced drug uptake or reduced in-

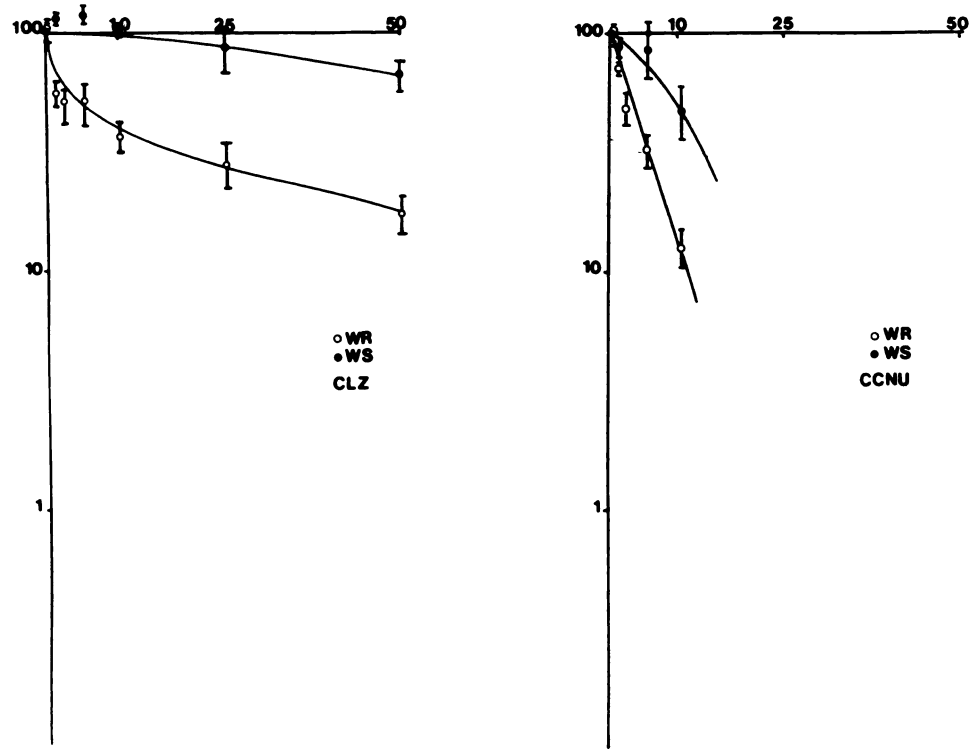


FIG. 6. *Effect of various concentrations of CLZ and CCNU on colony-forming ability of WR and WS cells*
See legend to Fig. 2 for further details.

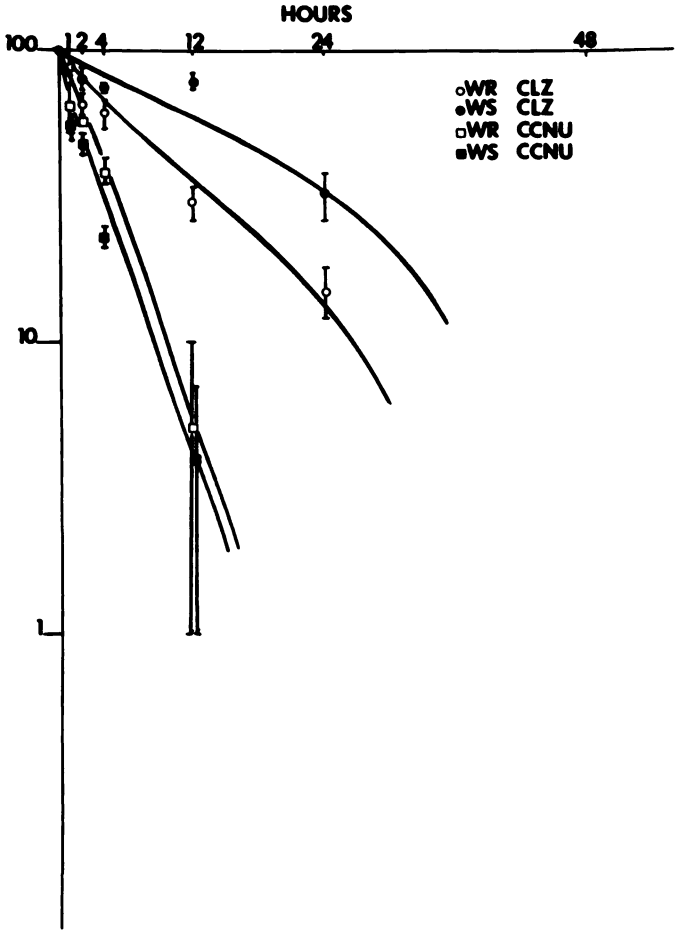


FIG. 7. *Effect of 10 μM CLZ or CCNU on colony-forming ability of WR and WS cells as a function of time*

The abscissa shows time after administration of nitrosourea in hours. Colony-forming assays were carried out as described in legend to Fig. 2.

tracellular decomposition. Indications that quantitative carbamoylation values *in vitro* may be anomalous were provided by ACNU (Fig. 5), which was effective on both WR and WS at molarities equivalent to the other nitrosoureas even though it is reported to have carbamoylating activity equal to that of CLZ. Such data may emphasize the importance of qualitative as opposed to quantitative effects of carbamoylation.

The reduced cytotoxic properties of methylnitrosoureas are shown in Fig. 8. Streptozotocin is the methyl equivalent of CLZ, with MNU as the active part of the drug. The sugar moiety reduces the carbamoylating potential by intramolecular carbamate formation (9). Approximately 10-fold higher concentrations of the methylnitrosoureas were required to achieve cytotoxicity similar to that of the chloroethylnitrosoureas. The increased efficacy of MNU as compared with that of STZ was consistent with its increased potential to form isocyanates, with no internal carbamate formation.

Conclusive evidence of the role of carbamoylation in sensitizing the WR cells was achieved by using *trans*-(4-OH)-BCyNU (Figs. 9 and 10). This urea has no alkylating potential, but it may carbamoylate proteins through isocyanate formation (23). The administration of (4-OH)-BCyNU alone caused death of the WR cells with little toxicity in the WS cells. When concentrations of D-SM were combined with the carbamoylator, no increase in the toxicity of D-SM was observed in WS cells (Fig. 9). However, a combination of the two drugs decreased WR cell survival synergistically (Fig. 10). The carbamoylator was capable of sensitizing the WR cells to D-SM.

DISCUSSION

Although carbamoylation of a number of structural and functional nuclear proteins has been reported (25–29), the importance of this drug modification to the cytotoxic properties of nitrosoureas has been considered

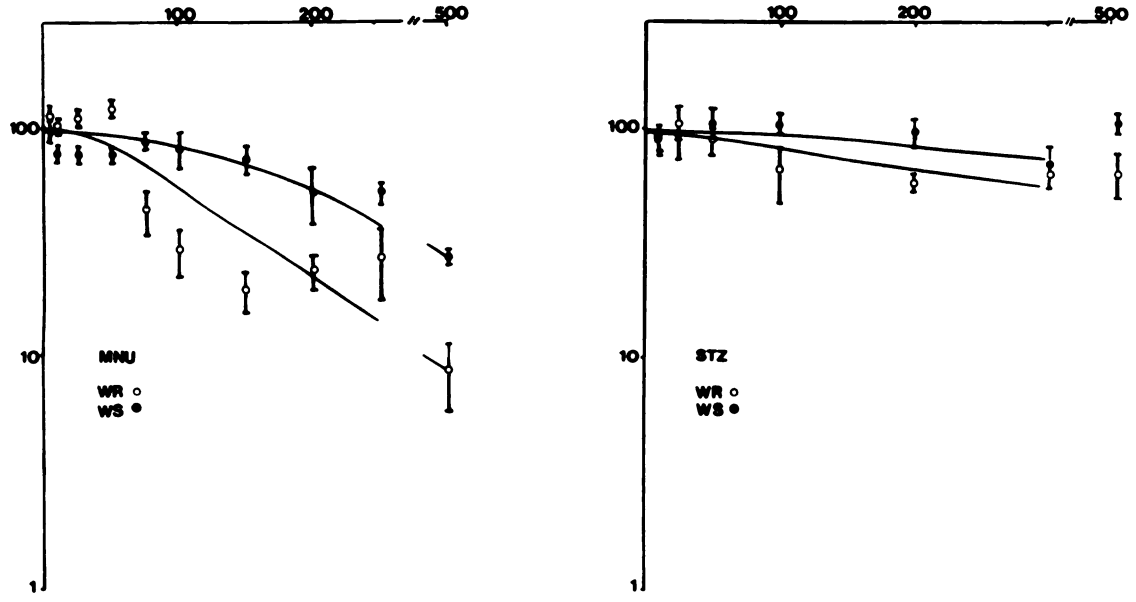
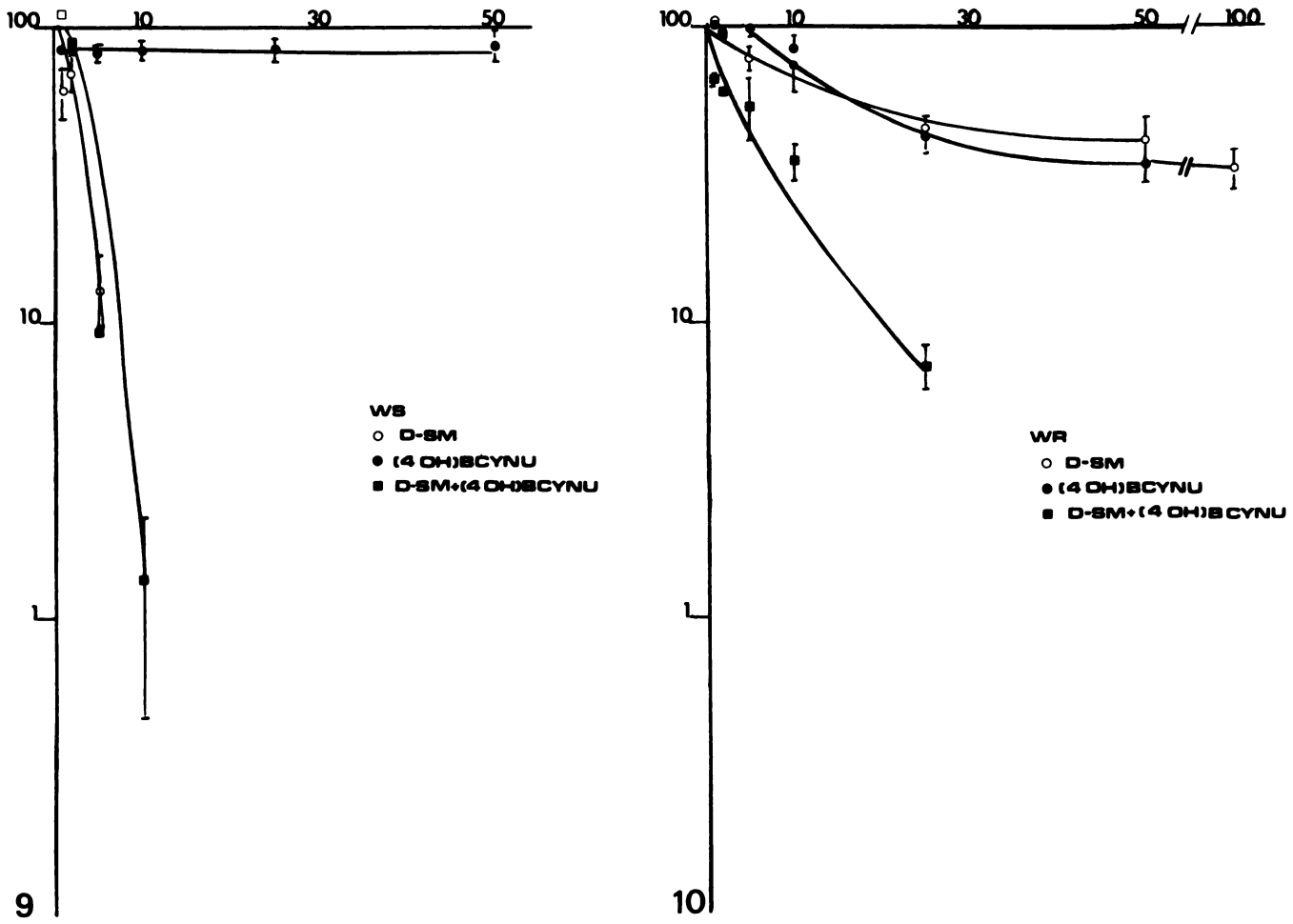


FIG. 8. *Effect of methylnitrosoureas on colony-forming ability of WR and WS cells*

The abscissa shows micromolarity of the drugs. Colony-forming assays were carried out as described in legend to Fig. 2.



FIGS. 9 AND 10. *Effect of concomitant administration of* D*-SM and (4-OH)-BCyNU on colony forming ability of WS (Fig. 9) and WR (Fig. 10) cells*

The abscissa shows the micromolar concentration of either D-SM or (4-OH)-BCyNU alone or in combination. Incubations were carried out for 2 hr. With combinations, identical micromolar concentrations of each drug were employed. Colony-forming assays were carried out as described in legend to Fig. 2.

minimal with the exception of those in a TLX5 lymphoma cell line (30). The present data suggest that, in Walker 256 cells, acquired resistance to bifunctional alkylating agents can be overcome by the presence of carbamoylating agents, whether these are part of a haloethylnitrosourea or separate compounds given concomitantly with a nitrogen mustard. Two nitrosoureas with reportedly low carbamoylating potential, CLZ and STZ, are less effective than related drugs in killing the WR cells at equimolar concentrations. However, these drugs still kill WR cells more efficiently than WS, suggesting that important carbamoylating potential may be qualitative rather than quantitative. ACNU has been shown to possess no more carbamoylating activity *in vitro* than do CLZ and STZ (approximately 3% that of CNU). A rationale of non-enzymatic, intramolecular carbamoylation by ACNU has been proposed (31); however, significant carbamoylation of glutathione reductase has been reported (11). The cytotoxic properties of ACNU against WR and WS would suggest that modification of lysine residues may be of limited value in predicting the physiological significance of qualitative carbamoylation of nucleophilic sites on other amino acids. The equivocality of this assay has been considered previously (32), and it is possible that certain crucial enzyme systems in WR cells may be inhibited by the isocyanate moieties of ACNU, CLZ, or STZ.

The precise mechanism by which carbamoylation contributes to cytotoxicity in the Walker cells is unclear at present. The following possibilities are being considered: (a) Specific site inactivation of enzymes may occur within the glutathione metabolic pathways. A lowering of protective thiols would result from such an enzyme inactivation which could mimic the kinetics of an antimetabolite treatment. (b) It is known that isocyanates are capable of inhibiting repair of X-ray damage in certain cell lines (33). It is possible that similar effects are mediated by the carbamoylator inhibiting the repair of alkylations and their ultimate conversion to cross-links. (c) The observed differences in nuclear structure must be accompanied by quantitative and/or qualitative differences in nuclear and cytoplasmic proteins. In fact, preliminary evidence (14) suggests that there are differences between WR and WS in the macromolecules which

constitute the structural nuclear framework—the nuclear matrix, which has been shown to be a preferential target for chloroethylnitrosoureas (34, 35).

A consideration of the survival data for Group 3 drugs confirmed that the lack of bifunctionality of methylnitrosoureas reduced their cytotoxic potential, requiring approximately 10 times the molar concentration (compared with chloroethylnitrosoureas) to achieve similar cell kill. Thus, the cross-linkage of nucleic acids is a more cytotoxic phenomenon than is monoadduct alkylation in the Walker cells. Such a situation has been observed in other mammalian cell lines (20).

The survival curve for *cis*-DDP mimics that of other Group 2 drugs, confirming the bifunctional potential for the two Cl^- ions in the platinum complex.

The presence of sugar moieties in drugs of Group 1, 2, or 3 did not alter the relative cytotoxicity of these compounds in WR and WS cells. In every case the resistance to the mustards was overcome by the nitrosoureas. The reduced cytotoxicity of the L-isomer as compared with the D-isomer of the sugar mustards (NSC 342215 and 342216) is presumably indicative of a reduced cellular uptake or increased catabolism of the L-SM.

The presence of karyotypic aneuploidy in these cell lines is consistent with karyotypic heterogeneity of many tumor cell lines (36) and in tumors resistant to antimetabolites such as methotrexate, where resistance has been correlated with amplification of the dihydrofolate reductase gene (1–3). HSR have been found in human tumors (37, 38), but the length of the region varied between cells. The short HSR in WR cells probably has a common origin with the long HSR in WS, but was selected for by drug treatment during the isolation of the resistant clone. The precise function of the HSR (or the other markers) is not clear, but such anomalous regions have been previously associated with drug resistance in tumor cells (2). The nuclear differences between WR and WS cells is represented by a reduction of nuclear chromatin in WR. Superficially, this situation is discordant with possible gene amplification of, for example, an enzyme of the glutathione pathway. However, such evidence does not discount the possibility of selective amplification of useful genes with concomitant reduction of redundant chromatin. The isolation and characterization of tumor cell populations resistant to certain nuclear-reacting drugs may present an interesting correlate with cell populations resistant to antimetabolites, since, at least in this case, distinct nuclear structural properties exist. Whether such differences account for, or are the inconsequential result of, drug resistance remains to be established.

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