Altered Ribonucleotide Reductase Activity in Mammalian Tissue Culture Cells Resistant to Hydroxyurea

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Received August 21,1974

Summary: Four Chinese hamster ovary cell lines and one mouse L cell line have been isolated which are resistant to the cytotoxic effects of hydroxyurea and guanazole. These five cell lines contain an altered ribonucleotide reductase activity as judged by a decreased sensitivity to the inhibitory action of both drugs. This is strong evidence that ribonucleotide reductase is one of the lethal sites of action for these two antitumour agents. The results are also consistent with the view that mammalian cell variants can arise from structural gene mutations.

Hydroxyurea and guanazole (3,5-diamino-1,2,4-triazole) are specific and potent inhibitors of DNA synthesis in mammalian cells (1,2). Consequently, they have been under investigation as cancer chemotherapeutic agents for some time (3,4). Hydroxyurea has also been widely used as an inhibitor of DNA synthesis during biochemical studies and as a cell synchronizing agent (5). However, their exact molecular modes of action have not been fully elucidated. There have been several reports that hydroxyurea (6) and guanazole (1) inhibit the *in vitro* activity of ribonucleotide reductase. Therefore, this enzyme may be the lethal target for both drugs. However, there exists much evidence to support other sites of action (7,8,9,10).

In order to investigate the proposed sites of action, we have isolated one mouse L cell variant and four Chinese hamster ovary (CHO) cell variants resistant to the cytotoxic effects of hydroxyurea. Previously we reported that CHO cell lines selected for resistance to hydroxyurea or guanazole also exhibit a high level of resistance to the nonselective agent (11). If, as this suggests, both antitumor agents share a common site of action; then an alteration making the site less sensitive to hydroxyurea should also make it correspondingly less sensitive to guanazole. We wish to report here that cell lines selected for resistance to hydroxyurea contain an altered ribonucleotide reductase activity

which is less sensitive in vitro to inhibition by the drug than parental-type enzyme. Furthermore, the enzyme activity exhibits resistance to the inhibitory action of guanazole as well. This is strong evidence that ribonucleotide reductase is at least one of the lethal sites of action for both hydroxyurea and guanazole.

Materials and Methods: CHO cells (12) or mouse L cells were routinely maintained in α-minimal essential medium (Flow Laboratories, Inc.) or CMRL 1066 (Microbiological Associates) as previously described (11). Cultures were found to be negative for Mycoplasma contaminants by routine plating techniques (13).

A procedure previously described (11) was used in isolating the various cloned and independent drug-resistant lines. The isolation conditions are summarized in Table 1. Preliminary analyses of karyotypes indicated that all cell lines have a modal chromosome number of 21.

Cells to be assayed for ribonucleotide reductase activity were grown in suspension culture at 37°. Exponentially growing cells from 2 to 4 litres of culture were collected by centrifugation and washed with phosphate buffered saline. Two volumes of 0.02M Tris-Cl buffer (pH=7.0) containing 1.0 mM dithioerythritol were added. The suspension was homogenized, centrifuged at 100,000 g for 1 hour and the supernatant was dialysed an additional hour against 1000 volumes of the same Tris buffer. The dialysed preparation was used as the enzyme source. The protein content varied from 8 to 13 mg/ml as determined by the method of Lowry et al (14) after precipitation on membrane filters (15).

Guanazole (NSC1895) was provided by Dr. Harry B. Wood, Jr. as a gift from the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. Dowex 1-X8 (200-400 mesh) was purchased from Bio-Rad Laboratories, Richmond, California. (^3H) thymidine and (^{14}C) cytidine diphosphate (CDP) were purchased from Amersham/Searle. All remaining chemicals were obtained from Sigma Chemical Co., St. Louis, Mo.

Results: *Drug resistance at the cellular level* - The four CHO variant cell lines isolated all exhibited high levels of resistance to hydroxyurea, having plating efficiencies of close to 1 up to a concentration of 50 μg/ml (results not shown). This was twice the concentration required to reduce the plating efficiency of the wild-type population to 10^{-5}. As expected (11) all hydroxyurea-resistant cell lines showed a striking cross-resistance to guanazole. Also, the variant cell lines exhibited the same sensitivity as the wild-type population to a number

TABLE 1 Isolation conditions for CHO cells resistant to hydroxyurea

cell line	medium	temperature	selection steps
HU^R-1	α-MEM	37°	1 (25 μg/ml)
HU^R-2	α-MEM	34°	2 (25 & 75 μg/ml)
HU^R-3	CMRL 1066	37°	2 (25 & 35 μg/ml)
HU^R-4	α-MEM	34°	1 (25 μg/ml)

TABLE 2 (^{3}H) thymidine incorporation into acid precipitable material in the presence of hydroxyurea or guanazole.

cell line	hydroxyurea	guanazole
wild-type CHO	11	20
HU^R-1	105	99
HU^R-2	79	103
HU^R-3	64	125
HU^R-4	75	116

Hydroxyurea at a final concentration of 25 µg/ml or guanazole at 200 µg/ml was added to a logarithmically growing cell suspension containing 2 x 10^5 cells/ml. After 15 minutes incubation at 37°, (^{3}H) thymidine (0.5 µg/ml, 1.0 µCi/ml) was added. After an additional 30 minutes incubation, the cells were collected on a glass fibre filter (Gelman #61630), washed with phosphate buffered saline, extracted 5 minutes with 10% ice-cold trichloroacetic acid and washed with 70% ethanol. Dried filters were solubilized with 1.0 ml of NCS (Amersham/Searle) overnight before the addition of a toluene-based scintillation fluid. The figures are given as per cent of uptake in the absence of drug.

of unrelated agents. For example, 0.22 µM arabinofuranosylcytosine reduced the plating efficiency of the wild-type and the four drug resistant lines to 63% ± 13%.

The variant cell lines exhibited resistance to hydroxyurea or guanazole even after a short period of exposure to hydroxyurea or guanazole. From Table 2 it can be seen that 25 µg/ml hydroxyurea or 200 µg/ml guanazole markedly inhibited the incorporation of labelled nucleoside into the wild-type population after a 45 minute exposure to the drug. Incorporation in the drug-resistant cell lines was not affected by the presence of guanazole and only marginally affected by the presence of hydroxyurea.

Drug resistance at the enzyme level- As with enzyme preparations from other mammalian sources (16) activity is optimal upon addition of ATP and dithioerythritol; while 0.12 mM $FeCl_3$ stimulates the reaction over two-fold.

The effect of hydroxyurea upon ribonucleotide reductase activity is shown in Figure 1a. All the enzyme preparations from cell lines selected for resistance to hydroxyurea showed significantly more activity than the wild-type in the presence of various concentrations of the drug. For instance, at a concentration of 15 µg/ml

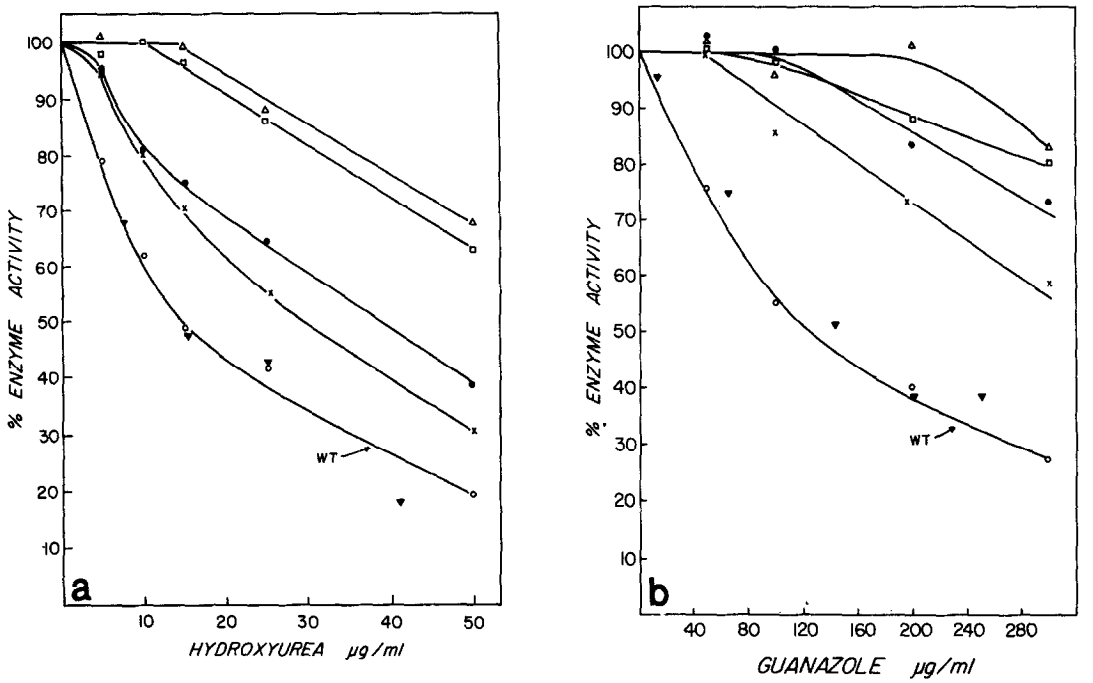

Figure 1. The inhibition of ribonucleotide reductase activity by hydroxyurea (1a) or guanazole (1b).
The enzyme was assayed by slightly modifying the standard procedure of Moore (16). Briefly, 40 µl of dialysed extract was incubated with 0.4 mM (^{14}C) cytidine diphosphate (5000 cpm/mµmole), 4.4 mM ATP, 6.2 mM dithioerythritol, 2.7 mM magnesium acetate, 0.12 mM $FeCl_3$ and 8.3 mM potassium phosphate buffer (pH=7.0) in a total volume of 150 µl. After incubation at 37° for 40 minutes, the reaction was terminated by heating at 100°. 0.3 µmoles of carrier dCMP was added to each tube and the deoxycytidine phosphates were converted to deoxycytidine by the action of Crotalus adamanteus venom as described by Cory (30). After centrifugation, the supernatant was passed over a column of Dowex 1-borate (31). The column was washed with 4.0 ml of water, and the eluant containing the deoxycytidine was collected. Absorption at 270 nm was determined to calculate per cent recovery, and the eluant was then added to 15 ml of Aquasol (New England Nuclear) for liquid scintillation counting. The specific activities of the various enzyme preparations varied from 2 to 6 mumoles of CDP converted per mg protein in 40 minutes. The points for wild-type CHO are the average of duplicate points from three different experiments utilizing separate enzyme preparations. The points for the remaining cell lines are the average of duplicate points obtained from a single enzyme preparation of each cell line. Wild-type CHO (O), HU^R-1 (●), HU^R-2 (△), HU^R-3 (□), HU^R-4 (X) and PHA^R-3 (▼).

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the wild-type preparation showed less than 50% activity while the hydroxyurea-resistant cell lines exhibited activities ranging from 70 to 100%. When guanazole was used to inhibit the reaction (Fig. 1b), the drug-resistant cell lines once

again exhibited increased resistance when compared to the wild-type. At 200 µg/ml guanazole, which reduced the wild-type activity to 40%, the variant cell line activities ranged from 75 to 100%. As an added control, enzyme preparation from CHO cells (PHA^R-3) selected for resistance to the cytotoxic effects of the lectin phytohemagglutinin-P (17) was also tested and was indistinguishable from wild-type activity in response to both drugs (Figs. 1a & 1b).

The possibility of an increased degradation of hydroxyurea and guanazole during the enzyme assay was ruled out by two experiments. In the first, enzyme preparation from HU^R-2 cells was incubated with the complete reaction mixture containing 50 µg/ml hydroxyurea. After 40 minutes the concentration of hydroxyurea remaining was determined by the colorimetric method of Levine and Kretchmer (18). By this assay, the reaction mixture still contained 50 µg/ml hydroxyurea, indicating no degradation had occurred. The colorimetric method utilized is specific enough to differentiate hydroxyurea from such possible breakdown products as urea, hydroxylamine and N-carbamoyloxyurea. In the second experiment, the HU^R-2 preparation was again incubated with the complete reaction mixture containing 50 µg/ml. After 40 minutes incubation the reaction mixture was dialysed overnight at 4° against an equal volume of distilled water. An aliquot of the dialysed water was then added to the standard reaction mixture containing the enzyme preparation from wild-type cells. The resulting reduction in enzyme activity was used to calculate the amount of hydroxyurea present. For example, an aliquot representing a 1/5 dilution reduced the wild-type enzyme activity to 62% of controls which is a value equivalent to that obtained in the presence of 10 µg/ml freshly prepared hydroxyurea. This indicates that even after incubation the HU^R-2 reaction mixture contained the entire 50 µg/ml hydroxyurea.

Drug resistant mouse L cells- A two step selection procedure (25 µg/ml and 100 µg/ml hydroxyurea) was used to isolate one mouse L cell line resistant to the drug. This clone exhibited an increased plating efficiency in the presence of both hydroxyurea and guanazole when compared to wild-type L cells (Table 3). When the enzyme preparation from the drug-resistant L cell line was assayed for

TABLE 3 Hydroxyurea-resistant cell line derived from mouse L cells.

	Plating Efficiency		Ribonucleotide Reductase Activity*	
Cell Line	hydroxyurea	guanazole	hydroxyurea	guanazole
Wild-type L cell	10^{-5}	10^{-5}	29% ± 7%	47% ± 3%
Hydroxyurea-resistant L cell line	1	1	69% ± 5%	83% ± 5%

Procedures for determination of ribonucleotide reductase activity were the same as described for CHO cells. The concentrations of the drugs in both sets of experiments were 25 μg/ml hydroxyurea and 200 μg/ml guanazole.
* The ribonucleotide reductase activity figures are given as per cent of activity in the absence of drug and are the average of duplicate samples of 3 saparate experiments.

ribonucleotide reductase activity, it showed a 40% increase in activity as compared to the parental L cell at a hydroxyurea concentration of 25 μg/ml. As expected, the enzyme activity of the variant cell line also exhibited increased resistance to the inhibitory action of guanazole as shown in Table 3.

Discussion: Our results strongly suggest that ribonucleotide reductase is a primary target for these two antitumor agents. Of course, we have not ruled out the possibility that additional sites of action exist or that drug permeability changes in the resistant cells (19) may contribute to the resistance at the cellular level.

The molecular mechanism responsible for the enzyme inhibition in the presence of these drugs is not understood. However in E. coli (20) and in mammalian systems (21) there is some evidence that hydroxyurea may inactivate the iron containing subunit of the enzyme. These variant cell lines will be useful in our investigation into the mode of action of these drugs. Furthermore, ribonucleotide reductase is responsive to a complex pattern of allosteric control. Perhaps some other enzyme properties, such as the allosteric interactions, may

be altered in the resistant cell lines. Therefore, a detailed biochemical comparison of wild-type and altered enzyme activities is now in progress.

Finally, we feel this report is of interest in respect to general somatic cell theory. Many phenotypic variants have been selected in mammalian cell tissue culture systems. Several reports bring into question the genetic origin of these phenotypic alterations (22,23). We have shown here, that cell lines selected for resistance to hydroxyurea contain an altered ribonucleotide reductase activity. Although other explanations are possible the simplest one is that the resistant cell lines contain a structural gene mutation. Our observations add to the growing number of reports suggesting that mammalian somatic cell variants with altered gene products can be selected in tissue culture (24,25,26,27,28,29)

Acknowledgements: Financial support of the National Research Council of Canada and the National Cancer Institute of Canada is gratefully acknowledged. W.H.L. is a recipient of a Fellowship from the National Cancer Institute of Canada.

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