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HYDROXYUREA DERIVATIVES—1-METHYL-1-HYDROXYUREA AND 1-ETHYL-1-HYDROXYUREA*

GLEN R. GALE

Veterans Administration Hospital and Department of Pharmacology, Medical College of South Carolina, Charleston, S.C., U.S.A.

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Abstract—Certain aspects of the biochemical pharmacology of 1-methyl-1-hydroxyurea (MHU) and 1-ethyl-1-hydroxyurea (EHU) were investigated and were contrasted with the actions of hydroxyurea (HU). Assay of inhibitory action against DNA synthesis in an ascites tumor cell system *in vitro* revealed an order of relative potency of MHU > HU > EHU. Slopes of the regression lines of dose response relationships were numerically similar. The action of each was reversible after removal of the drug by washing the cells. In addition, the inhibitory action of each on DNA synthesis was antagonized by the presence of exogenous deoxyribosides. No effect was demonstrated on the synthesis of RNA or of protein. Cross resistance to HU and MHU was shown in a microbial system, but EHU was without effect on the bacteria at the concentrations used.

RECENT reports have described certain pharmacological properties of two hydroxyurea (HU) derivatives with antitumor activities equal to or greater than that of the parent compound. 1-Methyl-1-hydroxyurea (MHU) at 250 mg/kg/day induced a 53 per cent inhibition of a transplantable mammary tumor in rats; 1-ethyl-1-hydroxyurea (EHU) at 350 mg/kg/day conferred 60 per cent inhibition. MHU at 500 mg/kg/day produced a 24 per cent elevation of the RNA/DNA ratio in the tumor, but the alteration was principally due to an enhanced RNA content; the DNA content was virtually unaltered. EHU at 700 mg/kg/day caused a 29 per cent increase in this ratio, with a slight reduction of the DNA content accompanied by a 12 per cent increase in the RNA content.[1] Inhibition by both agents of the growth of several other transplantable tumors has also been shown.[2] A comparison of the actions of HU, MHU, and EHU on the peripheral blood of rats showed that while HU reduced the hematocrit, erythrocyte (RBC) and white blood cell (WBC) count, EHU reduced the WBC count without a notable alteration of the other two parameters. MHU reduced the hematocrit and RBC count, but more markedly depressed the WBC count.[3] The work of these latter authors indicates that a depression of the rate of WBC production, which is the basis of the effectiveness of HU in treatment of leukemia, may be evoked by closely related derivatives which do not induce a concomitant depression of RBC formation.

The following study was initiated to delineate certain aspects of the biochemical pharmacology of MHU and EHU, and to contrast these with the actions of HU.

EXPERIMENTAL

HU, MHU, and EHU were supplied by Miss Barbara Stearns of the Squibb Institute for Medical Research. Eagle's minimum essential medium with Hank's

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balanced salt solution (MEM) was from Microbiological Associates, and isotopes were from New England Nuclear Corp. The Ehrlich ascites tumor was maintained in BALB/c male mice (Dublin Farms) by weekly passage. Deoxyribosides were from Calbiochem or Nutritional Biochemicals Corp.

The synthesis of DNA, RNA, and of protein was measured by incorporation of thymidine-^{3}H, uridine-^{3}H, and L-leucine-^{14}C (uniformly labeled), respectively, into the trichloroacetic acid (TCA) insoluble fraction of the cells *in vitro*. Mice were sacrificed by cervical dislocation. Cells were removed, washed twice with MEM, and finally suspended to 1% (v/v) in MEM. Each reaction tube usually contained 4·5 ml of cell suspension, 0·5 ml of the appropriate isotope in saline, and 0·5 ml of the drug in saline. The final isotope concentration in each case was 0·18 μc/ml. At intervals of incubation at 37° with gentle agitation, an aliquot of each suspension was added to an equal volume of 10% TCA at 5°. The insoluble material was washed three times with 5% TCA by centrifugation and resuspension, and solubilized in 2·0 ml hydroxide of Hyamine (1·0 M in methanol). This was then transferred to 15 ml of phosphor (PPO-POPOP, Packard Instrument Co.) in toluene, and radioactivity was measured with a Mark I liquid scintillation spectrometer (Nuclear-Chicago Corp.).

Growth of *Pseudomonas aeruginosa* was assessed by O.D. measurements and by cell enumeration in a Coulter model B cell counter after incubation of the cultures for 18 hr at 37°.

RESULTS

Fig. 1 shows the effects of HU, MHU, and EHU, each at a single concentration, on incorporation of thymidine into Ehrlich ascites tumor cells *in vitro*. At this concentration, which conferred approximately 80–90 per cent inhibition throughout the course of measurement, no striking dissimilarities among the three compounds were evident.

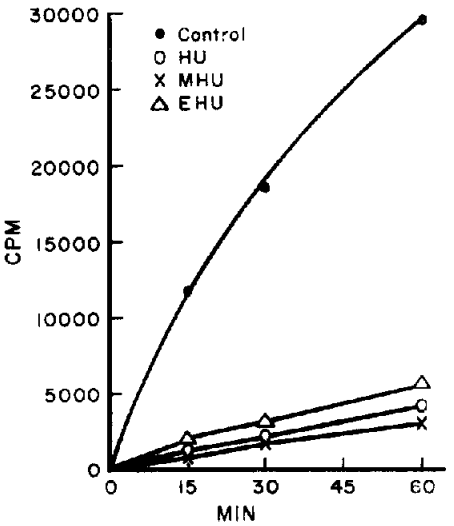

FIG. 1. Effects of a single concentration (0·001 M) of hydroxyurea (HU), 1-methyl-1-hydroxyurea (MHU), and 1-ethyl-1-hydroxyurea (EHU) on DNA synthesis in Ehrlich ascites tumor cells. Cells were preincubated with each drug for 30 min prior to addition of isotopic thymidine. Two-ml samples of each incubation mixture were removed and prepared for counting at the times indicated.

To evaluate more precisely the relative potency of each agent, dose response relationships of DNA synthesis were assessed over a 100-fold concentration range (10^{-5} to 10^{-8} M) of each compound. Linearity was achieved by plotting probit of per cent control against the logarithm of the concentration of each. Least squares analysis of the results of five experiments yielded the data presented in Table 1, and shows MHU to be the most active compound, and EHU the least. Slopes of the regression lines were quite similar. Day to day variations in the absolute values were noted, and probably were due to differences in ages of the cells. However, the values within each experiment were not widely different. At 10^{-3} M final concentration, each compound was totally without effect on the synthesis of RNA and of protein. The previously demonstrated selectivity of HU for DNA synthesis[4–7] is thus shared by each of the present congeners.

TABLE 1. REGRESSION COEFFICIENTS (b) AND 50 PER CENT INHIBITORY CONCENTRATIONS OF HYDROXYUREA (HU), 1-METHYL-1-HYDROXYUREA (MHU), AND 1-ETHYL-1-HYDROXYUREA (EHU) AS CALCULATED FROM DOSE RESPONSE EXPERIMENTS ON DNA SYNTHESIS*

Experiment	HU		MHU		EHU	
	b	IC_{50}(M)	b	IC_{50}(M)	b	IC_{50}(M)
A	− 1·67	$2·2 \times 10^{-4}$	− 1·36	$1·3 \times 10^{-4}$	− 1·62	$3·2 \times 10^{-4}$
B	− 1·86	$3·0 \times 10^{-4}$	− 1·72	$2·3 \times 10^{-4}$	− 2·22	$3·8 \times 10^{-4}$
C	− 0·88	$3·9 \times 10^{-5}$	− 0·85	$3·3 \times 10^{-5}$	− 0·82	$6·2 \times 10^{-5}$
D	− 0·93	$4·5 \times 10^{-5}$	− 0·66	$1·3 \times 10^{-5}$	− 0·80	$3·2 \times 10^{-5}$
E	− 1·52	$1·1 \times 10^{-4}$	− 1·48	$1·0 \times 10^{-4}$	− 1·21	$1·3 \times 10^{-4}$
Average	− 1·37	$1·4 \times 10^{-4}$	− 1·21	$1·0 \times 10^{-4}$	− 1·33	$1·8 \times 10^{-4}$

*Cells were preincubated with each drug for 30 min prior to addition of isotopic thymidine. Samples were removed and prepared for counting 20 min later. Values in each "b" column represent the changes in probit units for each 10-fold increase in drug concentration.

Reversibility of inhibition of DNA synthesis upon removal of each inhibitor was assessed by exposing a 1% cell suspension in MEM to each compound at a final concentration of 0·001 M for 30 min at 37°. Parallel control suspensions received no inhibitor. After incubation, each suspension to which either HU, MHU, or EHU had been added was divided into two equal aliquots. The cells in one sample were washed three times with MEM, and those in the other were washed three times with MEM containing the appropriate inhibitor at the same concentration as was originally present. After restoring each suspension to the original volume, thymidine uptake was measured as before. Fig. 2 shows total restoration of the DNA-synthesizing capacity of the cells upon removal of each compound.

A number of reports have implicated an inhibition of the reduction of ribonucleotides to deoxyribonucleotides as a major metabolic defect conferred by HU[7–11]. Partial[12] and complete[13] annulment of the inhibitory action of HU on mammalian test systems *in vitro* has been demonstrated after supplementing the incubation medium with a mixture of purine and pyrimidine deoxyribosides. A similar antagonism was sought with MHU and EHU. Table 2 shows the alterations by exogenous deoxyribosides of the inhibitory action of all three drugs on the ascites tumor test system, and indicates that the methyl and ethyl derivatives have modes of action similar to that of

the parent compound. The restoration by deoxyribosides of the rate of DNA synthesis to greater than the control value was reproducible.

HU inhibits the growth of *P. aeruginosa* approximately 80 per cent at 0·003 M;[14] at somewhat higher concentrations, activity has been demonstrated against other

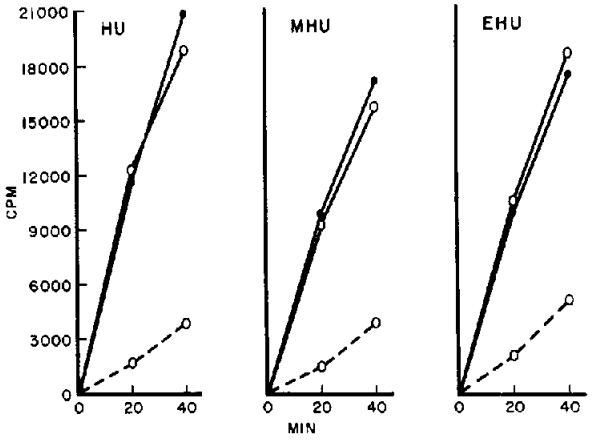

FIG. 2. Reversibility of the inhibitory action of hydroxyurea (HU), 1-methyl-1-hydroxyurea (MHU), and 1-ethyl-1-hydroxyurea (EHU) on the synthesis of DNA by Ehrlich ascites tumor cells. Solid lines, cells washed after initial drug exposure with Eagle's medium (MEM) alone; dotted lines, cells washed with MEM containing the indicated drug at 0·001 M. Closed circles, no drug; open circles, drug present during 30-min preincubation period. See text for experimental details. Two-ml samples were removed and prepared for counting at the times indicated.

TABLE 2. ANTAGONISM BY DEOXYRIBOSIDES OF THE INHIBITORY ACTION OF HYDROXYUREA (HU), 1-METHYL-1-HYDROXYUREA (MHU), AND 1-ETHYL-1-HYDROXYUREA (EHU) ON DNA SYNTHESIS IN ASCITES TUMOR CELLS*

Additions	cpm	per cent of control
	2488	
HU	81	3
MHU	57	2
EHU	125	5
Deoxyribosides	1595	
Deoxyribosides + HU	1819	114
Deoxyribosides + MHU	1816	114
Deoxyribosides + EHU	1766	111

*All tubes were supplemented with thymidine-^{3}H at 2×10^{-5} M. Inhibitors were present at 10^{-3} M. Deoxyribosides were added to yield final concentrations of deoxyguanosine, deoxyadenosine, and deoxycytidine of 0·002 M, 0·007 M, and 0·0001 M, respectively, and were added 10 min prior to addition of the inhibitors. After further incubation for 15 min, thymidine-^{3}H was added, and the extent of its incorporation into 4-ml aliquots was measured after 40 min.

gram-negative bacteria.[15] In each case, growth inhibition as measured by optical density or viable unit counts is accompanied by marked elongation of individual cells. It was therefore considered that cross resistance of bacteria to HU, MHU, and EHU would suggest more clearly identical modes of action of the three compounds.

Consequently, a wild strain of *P. aeruginosa* from a clinical source was subjected to 33 sub-cultures in tryptic soy broth (Difco) with HU present initially at 0·005 M and being gradually increased to 0·05 M; control cultures without HU, derived from the same isolate, were transferred in parallel. Initial assay of this strain showed the 90 per cent inhibitory concentration to be about 0·005 M, and unbalanced growth was quite evident microscopically. After the thirty-third subculture, the cells grown in the presence of 0·05 M HU approximated 100 per cent of control growth with little or no cell elongation. Attempts to evaluate the dose response relationships of both sub-cultured strains to each compound were unsatisfactory, since the strain propagated without HU had also acquired considerable resistance coupled with a loss of pigment production (the enhanced pigment production of recent isolates of *P. aeruginosa* which occurs in the presence of HU has been noted previously[14]).

Microscopic observations of gram-stained preparations were of interest from two aspects. First, the strain resistant to HU was pleomorphic, whereas the strain maintained without HU retained its usual morphological appearance (Fig. 3). The initial impression was that the culture of the resistant strain had become contaminated. However, the staphylococcal-like forms were distinctly gram-negative, and each subculture, upon streaking onto blood agar plates, consistently emerged as a pure culture of *P. aeruginosa*. This pleomorphism was readily reversible on blood agar plates, but was retained for 1 to 2 transfers in tryptic soy broth in the absence of HU. Second, MHU at 0·01 M induced marked unbalanced growth in the sensitive strain just as did HU, but the resistant strain was unaffected (Fig. 3). At least from this aspect, cross resistance would seem to be evident, implying similar action mechanisms of the two compounds. The sensitive strain was morphologically unaltered by EHU at the concentrations tested.

DISCUSSION

The current data support the conclusion that in a tumor cell test system *in vitro*, the mode of action of 1-methyl-1-hydroxyurea and of 1-ethyl-1-hydroxyurea is substantially similar to that of the parent hydroxyurea. This action is characterized as being highly selective for DNA synthesis and having no notable actions on RNA or protein synthesis within the concentration range tested. Restoration of the rate of DNA biosynthesis to near control values ensues promptly upon removal of the drug by washing the cells. These actions are not limited only to the compounds herein described, but are shared by other alkyl[16] and aryl[17] hydroxamic acids and certain derivatives thereof.[18]

Unpublished experiments in this laboratory have shown that all of these and other hydroxamic acids that selectively inhibit the synthesis of DNA *in vitro* are, like hydroxyurea, antagonized partially or completely by exogenous deoxyribosides, suggesting that at least part of the pharmacological action of these agents is mediated through an interference with riboside reduction. The practical benefits that have accrued through the use of hydroxyurea in certain chronic leukemias should be adequate rationale for the evaluation of an extensive series of hydroxamic acids in experimental leukemias with an aim toward molecular additions to the hydroxamate functional group which may predispose toward preferential distribution into the central nervous system.

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