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PROPERTIES OF *N*-HYDROXY-*N'*-AMINOGUANIDINE DERIVATIVES AS INHIBITORS OF MAMMALIAN RIBONUCLEOTIDE REDUCTASE*

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Abstract—In previous studies, *N*-hydroxy-*N'*-aminoguanidine (HAG) derivatives were demonstrated to suppress growth and clonogenicity of tumor cells which correlated with the inhibition of ribonucleotide reductase and DNA synthesis. The present work has focused on the properties of five HAG derivatives as inhibitors of the ribonucleotide reductase from Ehrlich ascites tumor cells. HAG derivatives acted as non-competitive inhibitors of ribonucleotide reductase with respect to the substrates CDP and ADP. The apparent K_i values for the various HAG derivatives as inhibitors of CDP reductase ranged from 3.4 to 543 μM. However, the apparent K_i values for these inhibitors with respect to ADP reductase were 2- to 10-fold lower than the respective values for CDP reductase. After a preincubation of HAG derivatives and ribonucleotide reductase in the absence of substrates, an increased inhibition was observed. The activity of the inhibited enzyme could be restored by passage over a Sephadex G-25 column and subsequent incubation with dithioerythritol. The addition of either the non-heme iron subunit or the effector-binding subunit to the intact enzyme in the assay mixture resulted in a diminished inhibition of ADP reduction. Inhibition by HAG derivatives of ribonucleotide reductase activity in the test tube was not enhanced by iron chelators. However, a combination of HAG compounds and iron chelators synergistically inhibited the growth of L1210 cells.

Inhibitors of ribonucleotide reductase (EC 1.17.4.1) suppress tumor cell growth [1–3]. They include inhibitors of the non-heme iron subunit, e.g. hydroxyurea [4] or the thiosemicarbazones [5], and inhibitors of the effector-binding subunit like dATP, dGTP and dTTP [6]. Since the reduction of ribonucleoside 5'-diphosphates to deoxyribonucleoside 5'-diphosphates is the rate-limiting step in the *de novo* synthesis of dNTPs, this reaction provides a critical control point in the synthesis of DNA and, hence, the proliferation of tumor cells.

N-Hydroxy-*N'*-aminoguanidine (HAG‖) derivatives represent a new class of ribonucleotide reductase inhibitors. Synthesized recently [7–9], they were shown to be cytostatic and cytotoxic in L1210 mouse leukemia cell cultures [10, 11]. The inhibition by HAG derivatives of intracellular ribonucleotide reductase activity correlates with a decrease in DNA synthesis [10, 11]. Like hydroxyurea [12], HAG derivatives synchronize cultured L1210 cells by transiently arresting them in the late G_1/early S-phase of the cell cycle [11]. HAG derivatives differ in the nature of the ring attached to the *N*-hydroxy-*N'*-aminoguanidine core. The HAG isoquinolylmethylene compound is the most potent inhibitor in the series [10, 11, 13] (Fig. 1).

In the present investigation into the inhibitory properties of five HAG compounds, we have characterized the type of inhibition, the K_i values, and the modulating effects of iron chelators. These five derivatives were chosen for further study since they represent over a 100-fold range in sensitivity toward ribonucleotide reductase and L1210 cell growth.

MATERIALS AND METHODS

Materials. The HAG derivatives were synthesized and provided by E. J. Lien's group [7–9]. The nucleotides and biochemicals were purchased from the Sigma Chemical Co., St. Louis, MO. Desferal was a gift from the CIBA Pharmaceutical Co., Summit, NJ. [2,8-^{3}H]ADP (25.3 Ci/mmol) and [U-^{14}C]CDP (482 Ci/mol) were supplied by NEN Research Products, Boston, MA. Horse serum, RPMI 1640 culture medium, and sodium bicarbonate were bought from the Grand Island Biological Co., Grand Island, NY. EDTA (free acid) came from Matheson Coleman & Bell, Norwood, OH, and was neutralized with NaOH before use.

Determination of K_i *values*. The CDP and ADP reductase assays were similar to those described previously [14, 15]. For the assay of CDP reductase, the reaction mixture (0.15 ml) contained 1 mM ATP, 4 mM magnesium acetate, 6 mM dithioerythritol, 13 mM sodium phosphate and 13 mM Tris·HCl, pH

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‖ Abbreviations: DMSO, dimethyl sulfoxide; HAG, *N*-hydroxy-*N'*-aminoguanidinetosylate; IMPY, 2,3-dihydro-1*H*-pyrazolo-[2,3-*a*]imidazole (NSC 51143); IQ, 1-formylisoquinoline thiosemicarbazone; and MAIQ, 4-methyl-5-amino-1-formlyisoquinoline thiosemicarbazone.

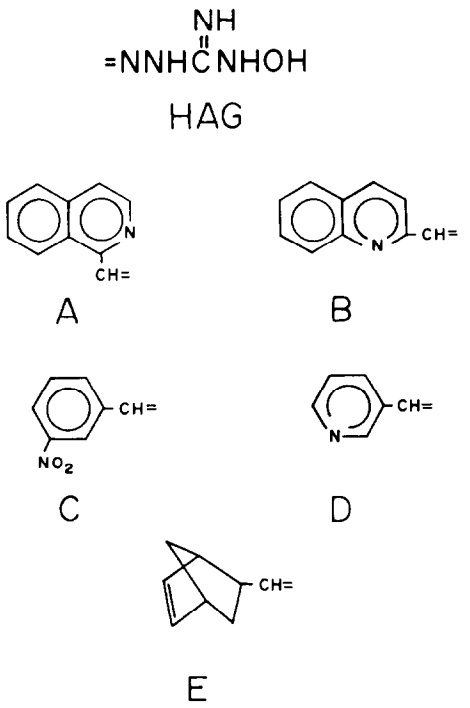

Fig. 1. Structures of the HAG derivatives characterized in this paper. The ring groups A to E are attached to the HAG core via a double bond. HAG, *N*-hydroxy-*N'*-aminoguanidine; A, 1-isoquinolylmethylene; B, 2-quinolylmethylene; C, 3-nitrobenzylidene; D, 3-pyridylmethylene; and E, 5-norbornen-2-ylmethylene.

7.0. The concentration of [^{14}C]CDP (0.03 μCi/assay) was varied between 10 and 50 μM in the presence of a fixed concentration of the respective HAG derivative. Each assay contained 0.3% DMSO, which was required to dissolve the HAG compounds. CDP reductase from Ehrlich tumor cells was purified through the ammonium sulfate step [16] and added to the test mixture at a final concentration of 4 mg/ml. After initiation of the reaction by addition of substrate and activators, the incubation was carried out for 30 min at 37° and stopped by immersing the test tubes into boiling water for 4 min. Snake venom from *Crotalus atrox* (1 mg in 50 μl H_2O) as well as magnesium acetate and dCMP (1 and 0.25 μmol, respectively, in 50 μl of 0.1 M Tris·HCl, pH 9.0) were added prior to a second incubation at 37° for 90 min. The reaction mixture was immersed into boiling water for 4 min and applied to 1-ml columns of Dowex-1 X8-400 (borate-form). Elution was performed with 4 ml H_2O, and the amount of [^{14}C]deoxycytidine was determined by liquid scintillation counting. Experiments were set up in triplicate.

The ADP reductase assay was similar to the one described above. Instead of ATP, it contained 1 mM dGTP. [^{3}H]ADP (0.5 μCi/assay) was varied between 8 and 43 μM. The snake venom treatment was performed in the presence of dAMP as carrier. For the separation of [^{3}H]adenosine and [^{3}H]deoxyadenosine, the Dowex-1 columns were pretreated with 2 ml of 1 mM sodium borate, pH 9.2, prior to sample application and elution with the same buffer (14 ml).

Reactivation of ribonucleotide reductase after incubation with HAG derivatives. For the inactivation of the enzyme, 1 ml of the ammonium sulfate fraction [16] containing ribonucleotide reductase was mixed with the inhibitors dissolved in 0.2 ml of H_2O–DMSO (98:2, v/v). The final concentrations of the HAG isoquinoline derivative, hydroxyurea and dATP were 0.03, 2.0 and 1 mM respectively. As a control, the enzyme was mixed with H_2O–DMSO lacking any inhibitor. Thus, the concentration of DMSO was 0.3% throughout. The concentrations of the other HAG derivatives tested were: 2-quinolylmethylene, 0.11 mM; 3-nitrobenzylidene, 0.5 mM; 5-norbornen-2-ylmethylene, 1.0 mM; and 3-pyridylmethylene, 1.0 mM. These mixtures were incubated for 20 min at 37° and then split into two aliquots. One aliquot (1 ml) was passed over a 40-ml column of Sephadex G-25 using 0.02 M Tris·HCl, pH 7.0, as an eluent at 4° and a flow rate of 20 ml/hr. Fractions of 1 ml were collected. In the second aliquot (immediately after inactivation) and in the peak fraction eluting from the Sephadex column, the enzyme activity was determined as described above [14, 15]. The assay mixture contained 0.05 mM [^{14}C]CDP (0.1 μCi/150 μl). To further activate the enzyme, 0.18 ml of the peak fraction was mixed with 0.02 ml of 0.1 M dithioerythritol and again incubated for 5 min at 37°.

In vitro L1210 *cells.* RPMI 1640 medium was used for the cultivation of L1210 mouse leukemia cells. The culture medium was supplemented with horse serum (10%, v/v), sodium bicarbonate (2 g/l) and gentamicin sulfate (50 mg/l) [10]. In 24-well (2 ml) tissue culture plates, the cells were added to the drugs at a seeding concentration of 1.0 to 1.5 × 10^5 cells/ml. The concentration of DMSO was 0.05% throughout. For 48 hr the plates were kept at 37° in a humidified atmosphere of CO_2–air (10:90). Cell densities were determined with a Coulter cell counter (model ZBI).

RESULTS

Inhibition of CDP reductase and ADP reductase activity by N-hydroxy-N'-aminoguanidine derivatives in vitro. In parallel experiments, the substrate dependency of the reaction velocity of CDP and ADP reductase was determined in the presence of each of the five HAG derivatives shown in Fig. 1. The resulting apparent K_i values are listed in Table 1. The HAG isoquinoline derivative was the most potent inhibitor of CDP reductase, the apparent K_i value being 3.4 μM. With 3-pyridylmethylene attached to the HAG core, the apparent K_i value was 160-fold higher. All agents tested were markedly more inhibitory for ADP reductase than for CDP reductase. The 5-norbornen-2-ylmethylene and the 3-pyridylmethylene derivatives of HAG exhibited the biggest difference in that the apparent K_i values of CDP reductase were more than 10-fold higher than those of ADP reductase.

A non-competitive type of inhibition was sug-

Table 1. Apparent K_i values of mammalian ribonucleotide reductase for *N*-hydroxy-*N'*-aminoguanidine derivatives (HAG)*

HAG derivative	CDP reduction	ADP reduction
	K_i (μM)	
1-Isoquinolylmethylene	3.4	1.2
2-Quinolylmethylene	36.5	19.5
3-Nitrobenzylidene	77.0	13.0
5-Norbornen-2-ylmethylene	150.0	14.0
3-Pyridylmethylene	543.0	53.3

* The K_i values were obtained from double-reciprocal plots as shown in Fig. 2 using the assay procedures described under Materials and Methods.

gested by the pattern of lines in the double-reciprocal plot (Fig. 2). Since an identical pattern results from an irreversible inhibition, the reversibility of the inhibition by HAG derivatives was studied (Fig. 3). After exposure of ribonucleotide reductase to the HAG isoquinoline derivative at 37°, the enzymic activity was reduced by approximately 75%. Although passage over a Sephadex G-25 column substantially increased reductase activity, a subsequent incubation in the presence of dithioerythritol was required for almost full reactivation. Hydroxyurea and dATP, ribonucleotide reductase inhibitors of well defined properties, were subjected to the same procedure (Fig. 3). Hydroxyurea acted very similarly to the HAG isoquinoline compound; dATP, however, was different in that a complete restoration of enzymic activity was achieved by the column step alone. Under the same conditions, the other four HAG derivatives (Fig. 1) gave results like the HAG isoquinoline compound in Fig. 3. No difference in the degree of reactivation was observed when dithioerythritol was replaced by 10 mM dithiothreitol and 10 μM $Fe(NH_4)_2(SO_4)_2$. Like the column step, dialysis of the inhibited ribonucleotide reductase yielded only partial restoration of the activity.

Figure 4 shows the extent of the inhibition as a function of the time period the reductase was preincubated with the inhibitor prior to assaying. Over the 20-min preincubation period, the degree of inhibition by the HAG derivatives was increased.

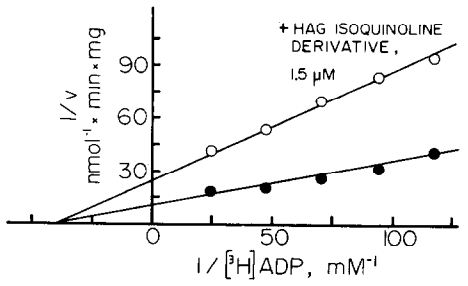

Fig. 2. Double-reciprocal plot of the reaction velocity of ADP reduction versus substrate concentration. The ADP reductase assay [14, 15] was run in the absence (●) or in the presence (○) of a 1.5 μM concentration of the HAG isoquinoline compound. Values are the mean of triplicate experiments.

The rate of loss of reductase activity appeared to be greater with the HAG quinoline derivative, although the inhibition by the HAG isoquinoline derivative was greater on a molar basis. To ensure that the enhanced inhibition was due to the preincubation, the time period of the enzyme assay was reduced from 30 to 5 min and the same result was obtained.

Reversal of inhibition by addition of either of the two subunits of ribonucleotide reductase. Ribonucleotide reductase consists of two non-identical subunits, the non-heme iron and the effector-binding subunits. In an attempt to determine if the HAG isoquinoline compound specifically inhibited one of the reductase subunits, aliquots of exogenous non-heme iron subunit or effector-binding subunit were added to the ADP reductase assay. As seen from the data in Fig. 5, neither subunit was preferentially

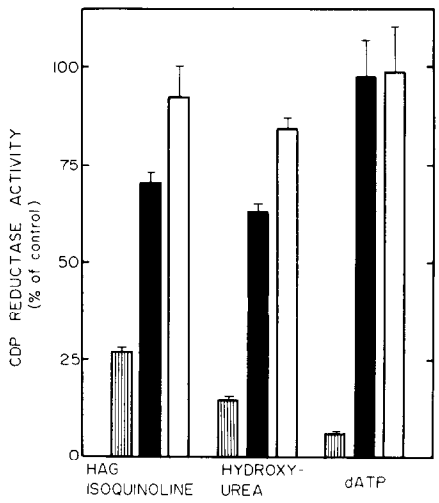

Fig. 3. Inhibition of CDP reductase and reactivation by gel chromatography and dithioerythritol. CDP reductase was incubated at 37° in the presence of the HAG isoquinoline drug, hydroxyurea or dATP. The activity of the uninhibited CDP reductase was 6.4 nmol·mg^{-1}·hr^{-1}. The loss of enzymic activity by this treatment is indicated by the hatched bars. The black bars show the CDP reductase activity after passage of the inhibited enzyme over a Sephadex G-25 column. After gel chromatography and an additional exposure to 10 mM dithioerythritol, the activity was measured that is indicated by the white bars. Values (±SD) represent the mean of three to five determinations.

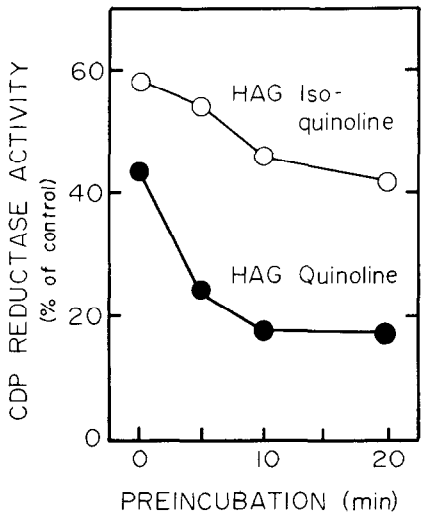

Fig. 4. Effect of the preincubation of CDP reductase with HAG derived compounds on the degree of inhibition. Inhibitors and enzyme were mixed and incubated at 37°. At the times indicated, aliquots of this mixture were withdrawn and added to a solution containing activators and [^{14}C]CDP to initiate the regular CDP reductase assay [14, 15]. The concentrations of the HAG isoquinoline and the HAG quinoline derivative were 18 and 150 μM during the preincubation and 3 and 25 μM in the assay respectively. Control incubations were carried out in the presence of 0.3% DMSO during the preincubation. The activity of CDP reductase in the control was 6 nmol·mg^{-1}·hr^{-1}. The values are the mean of triplicate experiments.

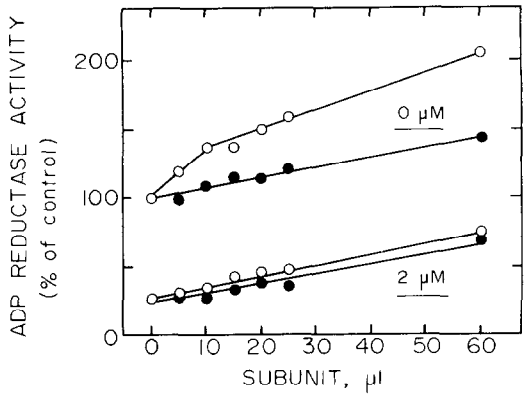

Fig. 5. Effect of the HAG isoquinoline compound on the activation of ADP reductase by exogenous non-heme iron and effector-binding subunit. In the absence or in the presence of a 2 μM concentration of the HAG isoquinoline drug, the regular ADP reductase assay, additionally containing increasing amounts of either non-heme iron (●) or effector-binding subunit (○) was carried out [14, 15]. The two subunits of the enzyme were obtained by separation of the ammonium sulfate fraction [16] on a blue dextran column eluted with 0.25 M NaCl [17]. They were devoid of intrinsic ADP reductase activity. The protein concentrations of the suspensions containing the non-heme iron subunit and the effector-binding subunit were 10.7 and 7.1 mg/ml respectively. The values are the mean of triplicate experiments. The activity of ADP reductase in the absence of inhibitor or exogenous subunit was 5.4 nmol·mg^{-1}·hr^{-1}.

effective in reversing the effects of the HAG isoquinoline derivative. In the absence of an inhibitor, the effector-binding subunit activated the reduction of ADP more strongly than the non-heme iron subunit did.

Modulating effects of iron chelators on the inhibition by HAG derivatives. Iron chelators can enhance or decrease the effect of ribonucleotide reductase inhibitors [18]. In this study, EDTA and Desferal were used to modulate the inhibition of both ADP and CDP reduction by HAG derived compounds (Table 2). EDTA and Desferal were effective in decreasing the inhibition of ADP reductase by the HAG isoquinoline derivative. The I_{50} values for the HAG isoquinoline derivative were increased 5.4- and 3.4-fold by EDTA and Desferal respectively. Inhibition of CDP reductase activity

Table 2. Effects of iron chelators on the inhibition by *N*-hydroxy-*N'*-aminoguanidine (HAG) derivatives of mammalian ribonucleotide reductase

HAG derivative	Substrate tested	I_{50} value* (μM)		
		No addition	EDTA†	Desferal†
1-Isoquinolylmethylene	ADP	0.5 (100)‡	2.7 (540)	1.7 (340)
	CDP	6.0 (100)	8.5 (142)	6.5 (108)
2-Quinolylmethylene	ADP	18.0 (100)	17.3 (96)	14.9 (83)
	CDP	23.2 (100)	33.1 (142)	30.0 (129)

* I_{50} value, concentration of drug required to inhibit CDP or ADP reductase by 50%. The I_{50} values were estimated from Dixon-type plots [19]. Five concentrations were used for each drug. The assay was carried out as described under Materials and Methods [14, 15].

† The concentrations of EDTA and Desferal were 0.166 and 0.1 mM respectively.

‡ The values in parentheses are expressed as percent of the K_i values listed in the first column (no addition).

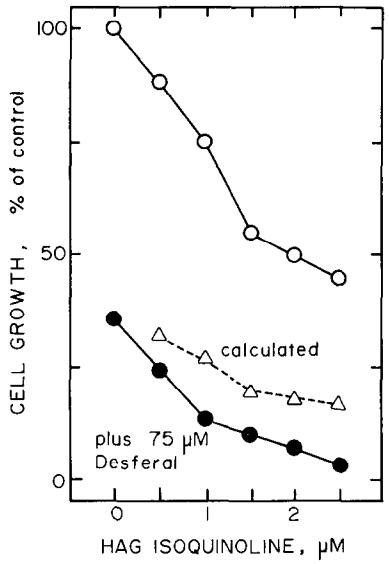

Fig. 6. Synergistic growth inhibition by the HAG isoquinoline compound plus Desferal. L1210 mouse leukemia cells were exposed to the drugs for 48 hr. The open circles represent the growth in the absence of Desferal; the solid circles show the growth in the presence of 75 μM Desferal. The values represented by the triangles were obtained according to the equation $(G_1 \times G_2)/100 = G_3$. G_1, growth (% of control) in the presence of 75 μM Desferal; G_2, growth (% of control) in the presence of the HAG derivative alone; G_3, calculated growth in % of control. In the control, the cell concentration was 5.55×10^5 cells/ml. The values are the mean of triplicate experiments.

was essentially unaffected by the presence of the iron chelating agents in agreement with a previous report [10]. Neither EDTA nor Desferal had any effect on the inhibition of ADP or CDP reductase activities by the HAG quinoline compound.

Combinations of the HAG isoquinoline compound and Desferal were tested for their antiproliferative action in L1210 mouse leukemia cell cultures (Fig. 6). These combinations were slightly synergistic. No change in the growth inhibition was observed when Desferal was administered 2 hr before or 2 hr after the HAG isoquinoline drug. Using EDTA in combination with the HAG isoquinoline compound resulted also in a synergistic antiproliferative effect.

DISCUSSION

The inhibition studies presented here allow a further insight into the properties of ribonucleotide reductase and its inhibitors of the HAG series. ADP reductase was more susceptible than CDP reductase to the HAG inhibitors (Table 1). A difference between both reductase activities was also observed earlier [20]: magnesium ion concentrations greater than 0.1 mM or the presence of organic solvents like dimethylformamide inhibit ADP reductase but not CDP reductase. In line with this, IMPY inhibits ADP reductase stronger than CDP reductase [21]. Whether these results suggest two different entities with either ADP or CDP reductase activity awaits further investigation. However, the ranking by the apparent K_i values in Table 1 also indicates similarities, i.e. the HAG isoquinoline drug was the strongest inhibitor and the HAG pyridine drug was the weakest inhibitor of both CDP and ADP reductase. In line with studies on L1210 cell growth, DNA synthesis and intracellular ribonucleotide reductase activity [10, 11], the 1-isoquinolylmethylene HAG drug was markedly more inhibitory than the structurally related 2-quinolylmethylene counterpart.

The reversibility studies (Fig. 3) lead to a better understanding of the nature of the inhibition. Since gel chromatography or dialysis restored enzyme activity only partially, the mechanism of the inhibition by HAG derivatives may be comparable to that characteristic of hydroxyurea. The latter was shown to destroy the tyrosyl-free radical in the non-heme iron subunit of ribonucleotide reductase [22]. Restoration of the radical was achieved by a short exposure to dithiothreitol and Fe^{2+} [22]. Under this condition, a reactivation was also observed for ribonucleotide reductase after removal of HAG derivatives (Fig. 3).

Regarding the reversal effect of the non-heme iron subunit or the effector-binding subunit on the inhibition by the HAG isoquinoline compound (Fig. 5), two explanations seem possible. The reversal may be due either to direct activation of the uninhibited fraction of the reductase or to the interaction of inhibitor and exogenous subunit resulting in a scavenger effect. However, if the first possibility applies, one would expect different slopes of the activation curves of the two subunits as it was demonstrated for the uninhibited enzyme (Fig. 5). Results similar to those of Fig. 5 have been shown for CDP reductase isolated from L1210 cells after exposure to the HAG isoquinoline derivative [10]. Thus, a possible conclusion from these studies is that both subunits interact with the HAG derivatives.

In earlier studies it was shown that iron chelating agents potentiate the inhibition of ribonucleotide reductase by hydroxyurea, IMPY and guanazole whereas the inhibition by MAIQ and IQ is essentially abolished [18]. Since the HAG derivatives represented a new class of ribonucleotide reductase inhibitors, experiments were carried out to determine the effects of iron chelating agents on the inhibition of ribonucleotide reductase by the HAG derivatives. With the combinations of HAG derivatives and iron chelators (Table 2), differences were found between CDP and ADP reductases as well as between the HAG isoquinoline and the HAG quinoline derivatives. In line with previous results [10], EDTA or Desferal did not affect the inhibition by HAG derivatives of CDP reductase. However, these iron chelators counteracted the effect of the HAG isoquinoline derivative on ADP reductase. Differences between CDP and ADP reductases were also observed with the K_i values, as mentioned above. The antagonistic action of iron chelators on the inhibition of ribonucleotide reductase by the HAG isoquinoline derivative is comparable to that for iron chelators on MAIQ [5]. The iron chelate of MAIQ is a much more potent inhibitor of ribonucleotide reductase than free MAIQ [23]. Since the iron chelators had no effect on the inhibition caused

by the HAG quinoline drug (Table 2), the latter appears to be independent of iron ions. Thus, the differences observed between the isoquinoline and quinoline derivatives could be due to differences in the metal chelating properties of the two agents. A suitable explanation is not now available. In the cell growth experiments, the HAG isoquinoline compound in combination with iron chelators gave a synergistic cytostatic effect, which was not predictable from the enzymic studies. The difference between the results with the isolated enzyme and the intact cells may be due to the fact that, in the intact cells, ribonucleotide reductase is a rate-limiting enzyme which upon further "stressing" through interactions with iron chelating agents and the HAG derivatives leads to much more pronounced metabolic imbalance in this critical deoxyribonucleotide pathway. These data may also suggest different or additional targets of Desferal in cellular systems. Hydroxyurea plus Desferal represents another synergistic drug combination in L1210 cultures [18].

In conclusion, HAG derivatives were potent inhibitors of ribonucleotide reductase and affected ADP reduction markedly stronger than CDP reduction. The effects of these inhibitors on the isolated enzyme and the growth of L1210 cells were partially similar to those of MAIQ, hydroxyurea or IMPY.

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