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MECHANISM OF INHIBITION OF RIBONUCLEOSIDE DIPHOSPHATE REDUCTASE BY α-(*N*)-HETEROCYCLIC ALDEHYDE THIOSEMICARBAZONES*

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Abstract—Methyl and benzo derivatives of 2-formylpyridine thiosemicarbazone were shown to be potent inhibitors of the ribonucleoside diphosphate reductase of the Novikoff hepatoma. Blockade of enzyme activity correlated with the ability of the various agents to retard the incorporation of ^{3}H-thymidine into DNA of Sarcoma 180 ascites cells *in vitro*. Structure–activity relationships suggested that position six of 2-formylpyridine thiosemicarbazone and position three of 1-formylisoquinoline thiosemicarbazone are comparable with respect to orientation at the inhibitor binding site and that the enzyme has little bulk tolerance at this locus.

α-(*N*)-HETEROCYCLIC carboxaldehyde thiosemicarbazones with the potential to form coordination compounds with certain transition metals inhibit the growth of a number of transplanted rodent neoplasms,[1–9] spontaneous lymphomas of dogs[10] and DNA viruses of the Herpes family.[11] 1-Formylisoquinoline thiosemicarbazone (IQ-1) and 2-formylpyridine thiosemicarbazone (PT), the parent compounds of two of the most potent of the heterocyclic ring series, cause marked inhibition of the incorporation of ^{3}H-thymidine into the DNA of several tumor cell lines.[12–17] The syntheses of RNA and protein are considerably less sensitive to these agents, although inhibition of these processes is seen at higher concentrations.

The site of the metabolic lesion on the DNA biosynthetic pathways is at the conversion of ribonucleotides to deoxyribonucleotides.[11–13,15,16,18–20] Studies, conducted with a partially purified ribonucleoside diphosphate reductase from the Novikoff rat tumor,[18,19] indicated that both IQ-1 and PT produce inhibition which is noncompetitive with respect to the nucleoside diphosphate substrate, the allosteric activator and the cation Mg^{2+}. The thiosemicarbazone inhibitors exhibited partial competition with dithioerythritol or dithiothreitol, model hydrogen donors used in place of the natural substrate, thioredoxin. IQ-1 and PT have great affinity for ferrous ion; nevertheless, inhibition of the enzyme by these agents was not reversed, and in certain conditions was enhanced by increasing concentrations of this cation. These findings are consistent with models in which IQ-1 and PT bind to an iron-charged enzyme, or the iron chelates of the thiosemicarbazones interact with the enzyme at or adjacent to a site occupied by the dithiol substrate. The need for an intact formyl thiosemicarbazone side-chain α to the heteroaromatic nitrogen atom for inhibition of tumor

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growth[3,8] suggested additionally the involvement of these functions in the mechanism of action of these compounds.

To gain further information as to the structural requirements for the hypothetical fit of these agents on the ribonucleoside diphosphate reductase molecule, various methyl- and benzo-substituted derivatives of PT were tested as inhibitors both of the reductase enzyme and of the incorporation of ^{3}H-thymidine into the DNA of Sarcoma 180 cells.

MATERIALS AND METHODS

IQ-1, PT, 6-methyl PT, 3-formylisoquinoline thiosemicarbazone and 2-formylquinoline thiosemicarbazone were supplied by Mr. Frederic A. French, Cancer Chemotherapy Research Department, Mount Zion Hospital and Medical Center, Palo Alto, Calif., and Dr. Harry B. Wood, Jr., Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md.

The 3-, 4-, and 5-methyl-substituted derivatives of PT and IQ-1 were synthesized in this laboratory.[21]

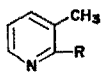
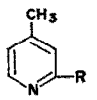
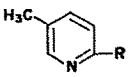
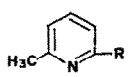
CDP labeled in the α-phosphate with ^{32}P was prepared from α-^{32}P-CTP purchased from Schwartz BioResearch or International Chemical and Nuclear Corp. The labeled CTP was incubated with partially purified nucleoside diphosphate kinase (EC 2.7.4.6),[22] and an excess of ADP. The product was isolated by the method of Hurlbert and Furlong.[23] The ribonucleoside diphosphate reductase was purified from the Novikoff rat tumor and assayed as previously described,[24] except for variations in the incubation mixture. $Fe(NH_4)_2(SO_4)_2$ was substituted for $FeCl_3$ in most experiments at a concentration of 4×10^{-6} M, unless otherwise stated.

^{3}H-thymidine (160 μg, $6{\cdot}7 \times 10^3$ counts/min/μg) incorporation into DNA was measured in 6-day-old Sarcoma 180 ascites cells by incubating approximately $1{\cdot}5 \times 10^8$ cells with isotope and inhibitors for 30 min at 37° in a total volume of 12 ml of Fischer's medium[25] minus horse serum. The reaction was terminated by the addition of perchloric acid (PCA) to give a concentration of 0·4 M. The precipitate was washed twice with 0·2 M PCA, extracted with 0·4 M PCA at 90° for 15 min, and aliquots were analyzed both for radioactivity with a Packard Tri-Carb liquid scintillation spectrometer and for deoxyribose[26] using deoxyadenosine as the standard. The 50 per cent inhibitory concentration (ID_{50}) was determined graphically.

RESULTS AND DISCUSSION

The effects of PT, IQ-1 and methyl- and benzo-substituted derivatives on the activity of ribonucleoside diphosphate reductase and ^{3}H-thymidine incorporation into DNA are shown in Tables 1 and 2. PT required a concentration of $4{\cdot}4 \times 10^{-7}$ M to cause 50 per cent inhibition of reductase activity. The 3-, 4-, and 5-methyl-substituted derivatives of PT were slightly more active in this regard, whereas 6-methyl PT was approximately ten times less active. Within the isoquinoline series (Table 2), IQ-1 was the most powerful inhibitor, requiring only $7{\cdot}5 \times 10^{-8}$ M for 50 per cent inhibition. 3-Methyl IQ-1 was about six times less active than IQ-1, whereas 4-methyl and 5-methyl IQ-1 and 3-formylisoquinoline thiosemicarbazone were only slightly less potent. 2-Formylquinoline thiosemicarbazone was the least active member of this series, requiring $2{\cdot}5 \times 10^{-6}$ M for 50 per cent inhibition of ribonucleoside diphosphate reductase activity. In general, the effects of these compounds on the synthesis of DNA

TABLE 1. INHIBITION OF RIBONUCLEOSIDE DIPHOSPHATE REDUCTASE AND ^{3}H-THYMIDINE INCORPORATION INTO DNA BY 2-FORMYLPYRIDINE THIOSEMICARBAZONE AND RELATED DERIVATIVES

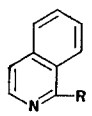
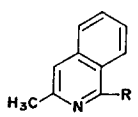
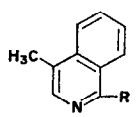
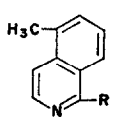
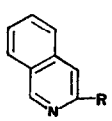
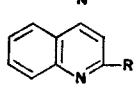
Compound	Structure*	$ID_{50} \times 10^7$ M	
		Ribonucleoside diphosphate reductase	^{3}H-thymidine into DNA
2-Formylpyridine TSC† (PT)		4·4	22
3-Methyl-2-formylpyridine TSC		2·6	14
4-Methyl-2-formylpyridine TSC		1·8	20
5-Methyl-2-formylpyridine TSC		1·8	11
6-Methyl-2-formylpyridine TSC		43	265

* R = (—CH=NNHCSNH$_2$).
† TSC = thiosemicarbazone.

as measured by the incorporation of ^{3}H-thymidine into acid-insoluble material, agreed well with the enzymic data, supporting the concept that blockade of ribonucleoside diphosphate reductase activity is responsible for inhibition of the synthesis of DNA. The quantitative relationship of the data obtained with the whole cell studies to that of the enzymatic series, however, was obscured by the differing solubilities of the various derivatives.

From previous studies on the mechanism by which these agents inhibit the activity of ribonucleoside diphosphate reductase, it was postulated that IQ-1 and PT interacted with the enzyme by coordination of the iron in the metal-bound enzyme[16] or that the iron chelate of these agents was the active inhibitory form;[18,19] kinetic analyses suggested that this occurred at the site occupied by the dithiol substrate.[13,15,16,18,19] Employing in part these data, French *et al.*[27] have arrived at a similar conclusion. The need for an intact formyl thiosemicarbazone side-chain in a position α to the heterocyclic ring nitrogen atom for tumor inhibitory activity implicated this portion of the molecule in its mechanisms of action.[3,8] A hypothetical interaction scheme illustrating these requirements is depicted in Fig. 1. The model postulates that the orientation of PT and IQ-1 at the inhibitor binding site is comparable. The finding that IQ-1 was a considerably more potent inhibitor than PT suggests that a hydrophobic interaction

TABLE 2. INHIBITION OF RIBONUCLEOSIDE DIPHOSPHATE REDUCTASE AND ^{3}H-THYMIDINE INCORPORATION INTO DNA BY 1-FORMYLISOQUINOLINE THIOSEMICARBAZONE AND RELATED DERIVATIVES

Compound	Structure*	$ID_{50} \times 10^7$ M	
		Ribonucleoside diphosphate reductase	^{3}H-thymidine into DNA
1-Formylisoquinoline TSC† (IQ-1)		0·75	2·6
3-Methyl-1-formylisoquinoline TSC		5·0	20
4-Methyl-1-formylisoquinoline TSC		1·0	3·1
5-Methyl-1-formylisoquinoline TSC		1·2	2·4
3-Formylisoquinoline TSC		1·2	7·0
2-Formylquinoline TSC		25	400

* R = (—CH=NNHCSNH$_2$).
† TSC = thiosemicarbazone.

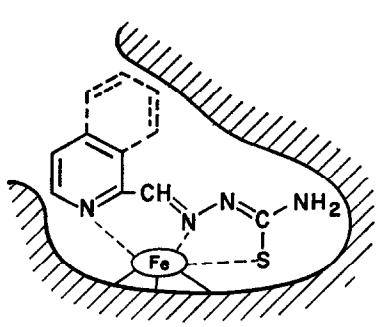


FIG. 1. Hypothetical interaction of α-(*N*)-heterocyclic aldehyde thiosemicarbazones with iron-charged ribonucleoside diphosphate reductase.

occurs between the benzenoid portion of the molecule and the enzyme. Such interaction might explain the fact that 3-methyl, 4-methyl and 5-methyl substitutions on the PT molecule render this compound more active as an inhibitor of the enzyme. 6-Methyl PT, 3-methyl IQ-1 and 2-formylquinoline thiosemicarbazone are considerably less active inhibitors of reductase activity than the parent member in each series. Although it is conceivable that the decreased activity of these derivatives may be due to electronic changes caused by the substituents, the finding that 4-methyl PT, which would be expected to have an electron-donating effect of the methyl group equal to or greater than that of the 6-methyl substituent due to hyperconjugation, is more potent as an enzyme inhibitor renders it more likely that the decreased inhibitory potencies are expressive of low bulk tolerance by the enzyme to substituted groups on position six of PT and position three of IQ-1, which are analogous sites with respect to the postulated interaction with the reductase molecule.

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