INHIBITION OF NUCLEOTIDYL TRANSFERASE ENZYMES BY METAL IONS IN COMBINATION WITH 5-AMINO-1-FORMYLISOQUINOLINE THIOSEMICARBAZONE†

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Inhibition of urchin polymerases by the metal ions Fe^{+2} and Cu^{+2} is modulated by exogenous ligands and the resultant inhibition is further a function of the polymerase class. The DNA polymerase β is singularly resistant to Fe^{+2} and Cu^{+2} metal ions alone and in combination with the title ligand. DNA polymerase α and the RNA polymerases II and III show varying degrees of sensitivity to the title inhibitor.

Several N-heterocyclic thiosemicarbazones have demonstrated potential as both antineoplastic and antiviral agents (1). N-methyl isatin β-thiosemicarbazone inhibits the growth of several groups of viruses including RNA tumor viruses (2,3). Thiosemicarbazones are strong chelators of copper and other metal ions and a role for the cupric ion in antiviral and antitumor activity of such compounds has been postulated and supported experimentally (4,5). In addition, Levinson, et al. have reported that either N-methyl isatin β-thiosemicarbazone or inorganic cations can inhibit the RNA dependent DNA polymerase of Rous sarcoma virus and that this inhibition parallels inactivation of the transforming ability of the virus (3); synergism between the thiosemicarbazone and Cu^{+2} ions was also observed for both polymerase inhibition and inactivation of the transforming ability.

Several α-(N)-heterocyclic carboxaldehyde thiosemicarbazones of French and Sartorelli have also shown potential as both antineoplastic and antiviral agents.‡ The primary biochemical lesion induced by these agents is on the synthesis of DNA, with the metallo-enzyme, ribonucleoside diphosphate reductase, being the major site of blockade in the biosynthetic pathways leading to deoxyribonucleotides. Studies indicate

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that inhibition is due to coordination of the Fe^{+2} cofactor by the compounds (7). However, several investigations have suggested that the α-(N)-heterocyclic carboxaldehyde thiosemicarbazones cause a second metabolic alteration on the DNA biosynthetic pathways distinct from reductase blockage (6,8).

RNA biosynthesis is also inhibited by most of the heterocyclic derivatives but to a much lesser extent than in the case of DNA synthesis (6). Clearly, this would not be a direct effect of ribonucleoside diphosphate reductase inhibition. In particular 4'-diethyleneoxy substituted α-(N)-heterocyclic carboxaldehyde thiosemicarbazones show a large increase in the inhibition of RNA relative to DNA synthesis (9). A number of reports of polymerase inhibition by compounds of either a well known, or potential ability to coordinate metal ions have appeared in the literature (10-16). These observations, coupled with (17) the metallo-nature of nucleotidyl transferase enzymes prompted the present investigation of the effect of metal ions (Cu^{+2}, Fe^{+2}), 5-NH_2-IQ-1,* and other physiological ligands on partially purified RNA and DNA nucleotidyl transferases.

MATERIALS AND METHODS

DNA-dependent RNA polymerases II and III from Strongglocentratus purpuratus embryos were purified by gradient sievorptive elution on DEAE-Sephadex A-25 (Pharmacia) as previously described (16). Fractions containing the peak activities of RNA polymerase II and III were individually stored in liquid N_2 until use; because only the peak fractions of each enzyme were used in these experiments, the cross-contamination of any RNA polymerase activity by a cognate RNA polymerase is less than 2% as determined by α-amanitin titrations. DNA polymerases α and β were prepared from urchin eggs by sucrose density gradient centrifugation (17). Extracts of urchin embryos prepared as previously described (16) were fractionated on 5-20% sucrose gradients constructed in 50 mM Tris-HCl, pH 7.9 at 4°, 0.1 mM EDTA, 0.5 mM dithiothreitol, 25% glycerol, and 0.4 M NaCl. Centrifugation was for 22 h at 10° and 60,000 rpm in the Spinco SW 60Ti rotor. All Enzymes were assayed by the previously described procedure (16).

Aliquots of each polymerase were preincubated for 4 min at 0° with the metal ion-ligand complex prior to starting the polymerization reaction by addition of the assay mixture and transfer to a 30° water bath. Stock solutions of 5-NH_2-IQ-1 were prepared in 2-methoxyethanol and then diluted into the metal ion solutions. The final concentration of 2-methoxyethanol was 2% v/v in all assays; controls showed that 2-methoxyethanol alone had no effect on any of the enzyme activities. All data reported in this manuscript were obtained with 0.6×10^{-4} M metal ion, 1.2×10^{-4} M citrate, glutamate, aspartate, or EDTA, and 10^{-4} M 5-NH_2-IQ-1. Where used, the chelating agents are in molar excess over the metal ion. Ternary mixtures were prepared by adding the complexed metal ion to the 5-NH_2-IQ-1; all complexes were prepared

*5-Amino-1-formylisoquinoline thiosemicarbazone (18) kindly supplied by Paul Mooney, Oklahoma College of Oesteopathy and Surgery.

Table I. Effect of Fe^{+2}, Cu^{+2}, and 5-NH_2-IQ-1 on DNA Polymerase α.

Ligand	No Metal	% Inhibition Cu^{+2}	Fe^{+2}
None	0	89	0
EDTA	6	26	-
Citrate	6	92	19
5-NH_2-IQ-1	6	35	-
Aspartate	-	-	38
Citrate, 5-NH_2-IQ-1	-	-	12
Aspartate, 5-NH_2-IQ-1	-	-	34

See Materials and Methods for details.

prior to admixture with the enzyme preparation. The data presented are representative of observations made at several concentrations of metal ion or ligand.

RESULTS AND DISCUSSION

DNA Polymerase β. The low molecular weight DNA polymerase showed no detectable inhibition by either Fe^{+2} or Cu^{+2} alone or in combination with 5-NH_2-IQ-1. Further, the binary complex of either metal ion with EDTA, aspartate, or citrate as well as the ternary mixture of either metal ion plus EDTA, aspartate, or citrate plus 5-NH_2-IQ-1 does not show any inhibition of the DNA polymerase β. This is quite unlike the observations with the other polymerases in this study and with the Rous sarcoma viral reverse transcriptase which Levinson, *et al*. (3) found to be inhibited by either Cu^{+2} or Fe^{+2} at 4×10^{-4} M. The reverse transcriptase inhibition by Cu^{+2} was found to be potentiated by N-methyl isatin β-thiosemicarbazone. The observed resistance of the DNA polymerase β to inhibition by Cu^{+2}, Fe^{+2}, and 5-NH_2-IQ-1 is conconant with previous data that the β-polymerase is catalytically dissimilar from the DNA polymerase α (19), and the viral encoded DNA polymerase.

DNA Polymerase α. As shown in Table I the DNA polymerase α preparation is quite sensitive to the Cu^{+2} ion and this inhibition is tempered by premixing metal with the EDTA or 5-NH_2-IQ-1 ligands before assay. However, complete restoration of activity is not achieved even with the potent sequestering agent EDTA. On the other hand, citrate affords little protection against the toxic copper ion. The great tenacity with which EDTA chelates Cu^{+2} metal ions and the fact that the metal is preincubated with the sequestering agent before introduction

Table II. Effect of Fe^{+2}, Cu^{+2}, and 5-NH_2-IQ-1 on RNA Polymerase III.

Ligand	No Metal	% Inhibition Cu^{+2}	Fe^{+2}
None	0	72	0
EDTA	0	80	20
5-NH_2-IQ-1	0	89	11
EDTA, 5-NH_2-IQ-1	0	76	32

See Materials and Methods for details.

into the enzyme preparation makes it rather unlikely that the residual inhibition is due to free metal and suggests that the complex itself may have inhibitory properties. This is further suggested in the case of Fe^{+2} which alone shows no detectable inhibition of DNA polymerase α but in the presence of citrate, and especially aspartic acid, significant inhibition is observed. Addition of 5-NH_2-IQ-1 in competition with the citrate or aspartate ligands showed insignificant inhibition beyond that of a single ligand.

RNA Polymerase III. The general trend of inhibition of metal ion-ligand combinations on RNA polymerase III (Table II) is similar to the DNA polymerase α, although apparent quantitative differences exist. For all the Cu^{+2}:ligand combinations tested none really protected the enzyme against the inhibition seen with the Cu^{+2} and, in fact, the data for the 5-NH_2-IQ-1:Cu^{+2} combination indicates possible enhanced inhibition over the uncomplexed Cu^{+2}. The effect of the Fe^{+2}: ligand combinations on the RNA polymerase III appear quite similar to the DNA polymerase α with each showing some degree of inhibition of [^{3}H]-UTP incorporation into RNA, while metal ion or ligands alone have little or no effect.

RNA Polymerase II. The data in Table III shows that RNA polymerase II responds quite differently to the metal ion-ligand combinations than does either DNA polymerase β, DNA polymerase α, or RNA polymerase III. Addition of uncomplexed Cu^{+2} to the RNA polymerase II results in 73% inhibition, and like DNA polymerase α and RNA polymerase III, addition of Cu^{+2} as the complex with EDTA, citrate, or aspartate is considerably less inhibitory than the metal alone. However, addition of the binary Cu^{+2}:5-NH_2-IQ-1 complex or the ternary mixture results in 82-92% inhibition of the RNA polymerase II activity. It must be emphasized that

Table III. Effect of Fe^{+2}, Cu^{+2}, and 5-NH_2-IQ-1 on RNA Polymerase II.

Ligand	No Metal	% Inhibition Cu^{+2}	Fe^{+2}
None	0	73	0
EDTA	0	23	-
EDTA, 5-NH_2-IQ-1	-	85	-
Citrate	0	15	8
Citrate, 5-NH_2-IQ-1	-	82	61
Aspartate	0	25	17
Aspartate, 5-NH_2-IQ-1	-	87	53
Glutamate	0	51	4
Glutamate, 5-NH_2-IQ-1	-	92	38
5-NH_2-IQ-1	0	62	70

See Materials and Methods for details.

based on stability constants (Cu^{+2}:EDTA, 1:1 ratio, log K_1 = 18.9 (22); Cu^{+2}:IQ-1, 1:1 ratio, log β_2 = 11-12#) the concentration of free Cu^{+2} is expected to be vanishingly low, and the active inhibitor is probably a Cu^{+2} complex. The extensive inhibition seen with the ternary mixtures (Cu^{+2}, 5-NH_2-IQ-1, and another ligand) in contrast to the Cu^{+2}:5-NH_2-IQ-1 binary complex suggests that the most active inhibitor may be a three component complex.

In the case of Fe^{+2}, the 5-NH_2-IQ-1 again is seen to be requisite for significant inhibition of RNA polymerase II (Table III). Whereas the Fe^{+2} alone or complexed with EDTA, aspartate, or glutamate gives little inhibition, the addition of 5-NH_2-IQ-1 potentiates this inhibition The stability constants again insure extensive chelation of the Fe^{+2} ion (Fe^{+2}:EDTA, 1:1 ratio, log K_2 = 14.4 (22); Fe^{+2}:IQ-1, 1:2 ratio, log β_2 = 28#).

DISCUSSION

The compound, 5-amino-1-formylisoquinoline thiosemicarbazone, currently of interest as an antineoplastic and antiviral agent, is known to be a strong chelator of metal ions and may well inhibit ribonucleoside diphosphate reductase (7) by chelation of the Fe^{+2} cofactor. We report here preliminary observations showing that 5-NH_2-IQ-1 alone is ineffective in the rapid inhibition of any of the nucleotidyl trans-

#The dissociation constants for the parent 1-formylisoquinoline:Cu^{+2} and Fe^{+2} complexes were kindly supplied by Professor Krishna Agrawal.

ferases examined. We reasoned that the introduction of 5-NH_2-IQ-1 into any cellular system would result in the formation of chelates with metal ions present in the cells. Such chelates could then potentially interact with the nucleotidyl transferases. Our results substantiate these speculations.

The DNA polymerase β is seen to be insensitive to any combination of 5-NH_2-IQ-1 or metal ion tested. The remaining three polymerases (as well as RNA polymerase I and calf thymus RNA polymerase II, data not shown) are observed to be inhibited by the metal ion:5-NH_2-IQ-1 combinations. The effect of Fe^{+2} upon RNA polymerase II is particularly interesting in that Fe^{+2} chelates with EDTA, citrate, glutamate, or aspartate are weak inhibitors at best, whereas, the addition of 5-NH_2-IQ-1 results in more potent inhibition. The inhibition of DNA polymerase α and RNA polymerase III is not markedly potentiated by 5-NH_2-IQ-1. The strong inhibition with uncomplexed Cu^{+2} probably has little meaning since such an uncomplexed metal ion is unlikely to exist in the cellular environment.

These results and the ubiquitous presence of iron in living systems may account for the previously observed *in vivo* inhibition of total RNA synthesis by the α-(N)-heterocyclic carboxaldehyde thiosemicarbazone class of compounds (6) and the inhibition of viral reverse transcriptases (4). Further, our results suggest that metal ion complexes with specific ligands represent an avenue toward selective inhibition of nucleotidyl transferases. It is clear that the chemical structure of the ligand is important to the potency of the metal ion chelate. Judicious development of the ligand structure and combination with the appropriate metal ion may result in inhibitors with considerable specificity among the nucleotidyl transferases.

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