Antiviral Research, 2 (1982) 291–300
Elsevier Biomedical Press

INHIBITION OF AVIAN MYELOBLASTOSIS VIRUS REVERSE TRANSCRIPTASE AND VIRUS INACTIVATION BY METAL COMPLEXES OF ISONICOTINIC ACID HYDRAZIDE

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(Received 16 March 1982; accepted 15 June 1982)

The cupric and ferric complexes of isonicotinic acid hydrazide (INH) inhibit the DNA synthesis catalysed by avian myeloblastosis virus (AMV) reverse transcriptase. The inhibition was to the extent of 95% by 50 μM of cupric-INH complex and 55% by 100 μM of ferric-INH complex. These complexes have been found to bind preferentially to the enzyme than to the template-primer. Kinetic analysis showed that the cupric-INH complex is a non-competitive inhibitor with respect to dTTP. The time course of inhibition has revealed that the complexes are inhibitory even after the initiation of polynucleotide synthesis. In vivo toxicity studies in 1-day-old chicks have shown that the complexes are not toxic up to a concentration of 500 μg per chick. Infection of the 1-day-old chicks with AMV pretreated with 150 μg of either of the complexes prevented symptoms of leukemia due to virus inactivation.

isonicotinic acid hydrazide (INH) metal complexes of INH reverse transcriptase inhibition AMV infection inactivation

INTRODUCTION

The molecular processes leading to the manifestation of neoplastic transformation by RNA tumor viruses can be inhibited by blocking either the synthesis of proviral DNA or its expression. The DNA synthesis catalysed by the reverse transcriptase offers a unique target for developing specific inhibitors of RNA tumor virus multiplication. A number of studies have been made to investigate compounds which may block the proviral DNA synthesis by interacting with the viral genomic RNA or the reverse transcriptase [7,16, 21]. Among a host of inhibitors of the reverse transcriptase, heavy metal-binding agents such as thiosemicarbazide and *N*-methyl-beta-thiosemicarbazone are strong inhibitors of the enzyme [13,24]. Recently it has been shown that metal complexes of a number of ligands possess inhibitory activity against neoplasms [8,18,19]. We have earlier reported

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0166-3542/82/0000–0000/$02.75

that the copper complex of INH can inactivate, on contact, the ability of Rous sarcoma virus (RSV) to malignantly transform chick embryo cells in vitro [4].

In the present report we examined the inhibitory effect of cupric and ferric–INH complexes on AMV reverse transcriptase, in vivo cytotoxic effects of the complexes, and the effects of these complexes on AMV infection in 1-day-old chicks.

MATERIALS AND METHODS

Chicks

One-day-old leukosis virus free white Leghorn chicks were obtained from the Karnataka State Poultry Farm, Bangalore, India.

Virus

Avian myeloblastosis virus (BAI, strain A) was provided by Dr. M.R. Das (Centre for Cellular and Molecular Biology, Hyderabad, India). The virus was injected intravenously into 1-day-old chicks and blood samples were examined daily for hematological changes and the symptoms of AMV infection in chicks were also followed as described by Beard [5]. Virus was purified from plasma from leukemic chickens.

Enzyme

The AMV reverse transcriptase was purified by the method described by Houts et al. [12]. Purified AMV reverse transcriptase obtained through the viral oncology program (National Cancer Institute) and provided by Dr. J.W. Beard (Life Sciences, Inc., St. Petersburg, FL), was also used in some experiments.

Chemicals

The synthetic template-primers were obtained from P-L Biochemicals (Milwaukee, WI) and the deoxynucleoside triphosphates from Sigma Chemical Co. (St. Louis, MO). [^{3}H]dTTP (47 Ci/mmol) was from the Radiochemical Centre (Amersham, U.K.). All the other chemicals used were of analytical grade.

Metal complexes of INH

Cupric- and ferric-INH complexes were prepared by mixing equal volumes of equimolar solutions of $CuSO_4$ or $FeCl_3$ with INH as described earlier [22]. The precipitate obtained in each case was washed extensively with distilled water, dried and stored at 4°C. Fresh stock solutions of the complexes (5 mM) were prepared by dissolving them in a minimum volume of 1 N HCl and then diluting with 20 mM Tris-HCl buffer, pH 7.8. Stock solutions were diluted to desired concentration with the same buffer before use.

Reverse transcriptase assay

The assay was carried out in a final volume of 100 μl and contained the following components: 50 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol, 50 μg of bovine serum

albumin, 20 μM [^{3}H]dTTP (final specific activity of 1000 c.p.m./pmol), 0.5 μg of template-primer, 100 mM KCl, 10 mM $MgCl_2$ and 2 units of enzyme (one unit of enzyme is defined as that amount which incorporates one nanomole of dTMP into an acid-insoluble product in 10 min at 37°C) [12]. Incubation was done at 37°C for 30 min. The reaction was stopped by the addition of 5% (w/v) trichloroacetic acid (TCA) containing 0.01 M sodium pyrophosphate. The acid-insoluble precipitate was collected on Whatman GF/C filters, washed extensively with TCA-containing pyrophosphate, water and finally with ethanol, dried and counted in toluene-based scintillation fluid [17].

In vivo toxicity studies

Different amounts of cupric- and ferric-INH complexes in Tris-HCl buffer, pH 7.8 were injected intravenously into 1-day-old chicks. The chicks were observed for one month for any toxic symptoms and lesions.

Antiviral property of the complexes

One-day-old chicks were injected intravenously with AMV plasma (1 : 100) which had been pretreated separately with various concentrations of the complexes, INH, $CuSO_4$ and $FeCl_3$ for 15 min at 4°C. The chicks were examined for symptoms of AMV infection [5] for a period of 3 weeks. The symptoms of leukemia observed were loss of weight, muscular weakness, ruffled feathers, general dullness, sluggish response when disturbed, development of gradual anaemia followed by increase in the number of myeloblasts.

RESULTS

Inhibition of proviral DNA synthesis

The effect of increasing concentrations of cupric- and ferric-INH complexes, $CuSO_4$, $FeCl_3$ and INH on proviral DNA synthesis was studied. At 100 μM concentration cupric- and ferric-INH complexes showed 97% and 55% inhibition respectively, whereas $CuSO_4$, $FeCl_3$ and INH showed 32%, 30% and 20% inhibition respectively in poly(rA) · $(dT)_{12-18}$-dependent reaction (Fig. 1A). At the same concentration the cupric-INH complex showed 69% inhibition, whereas $CuSO_4$ and INH showed 43% and 21% inhibition respectively in poly(dA) · $(dT)_{12-18}$-dependent reaction. The inhibition of poly(dA) · $(dT)_{12-18}$-dependent reaction by $FeCl_3$ and ferric-INH complex was found to increase progressively up to 40 μM and decreased thereafter (Fig. 1B).

Time course of inhibition

To determine whether cupric- and ferric-INH complexes could inhibit the polynucleotide synthesis even after the reaction was initiated, these complexes were added to the ongoing reaction system at different time intervals and the enzyme activity was

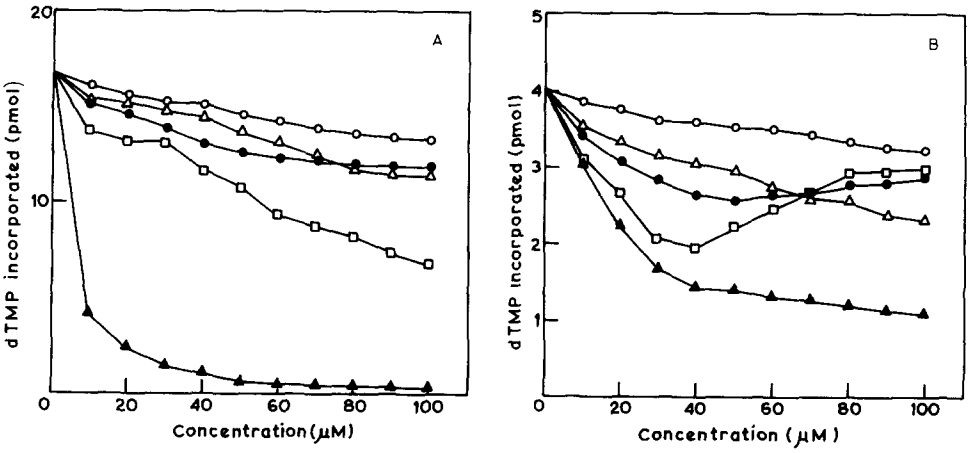

Fig. 1. Effect of INH, $CuSO_4$, $FeCl_3$, cupric-INH complex and ferric-INH complex on AMV reverse transcriptase activity. DNA synthesis was followed using poly(rA) · $(dT)_{12-18}$ (A) and poly(dA) · $(dT)_{12-18}$ (B) as template-primers. The assay conditions were described in the text. The dTMP incorporated into polynucleotide was measured as picomoles at various concentrations of INH (○), $CuSO_4$ (△), $FeCl_3$ (●), cupric-INH complex (▲) and ferric-INH complex (□). The molecular weights of the complexes were calculated with the assumption that cupric-INH forms a 1 : 2 complex (469.30) and ferric-INH forms a 1 : 3 complex (576.00).

monitored. Both complexes caused an instantaneous inhibition whether they were added at the time of initiation or after the initiation of the reaction (Fig. 2). The inhibition by ferric-INH complex was found to be less than that by cupric-INH complex.

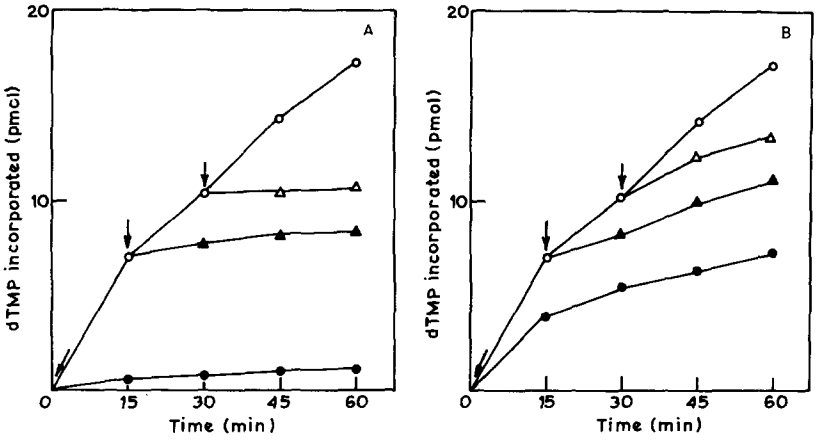

Fig. 2. Time course of cupric-INH (A) and ferric-INH complex (B) inhibition of AMV reverse transcriptase. The complexes were added at a final concentration of 50 μM at various time intervals after the initiation of the enzyme reaction. The enzyme assay conditions were the same as described in the text. The complexes were not added in the case of control reaction (○).

Site and target of inhibition

Two types of experiments were conducted to find out whether the inhibition was due to the direct binding of the complexes to the enzyme, or to the template or both. In the first type, an additional 1 μg of the template-primer poly (rA) · $(dT)_{12-18}$ was added to the complex inhibition system after the 5 min preincubation period and in the second type, an additional 4 units of enzyme were added. Table 1 shows that the addition of enzyme to the reaction system, where template-primer was preincubated with the cupric-INH and ferric-INH complexes increases the activity by 73% and 20% respectively. Addition of enzyme in the reaction system where enzyme was preincubated with the cupric-INH and ferric-INH complexes increases the activity by 64% and 26% respectively. On the other hand, the addition of template-primer did not show any appreciable increase in the activity. It is therefore suggested that the complexes inhibit the reverse transcription by binding to the enzyme and not to the template-primer.

Effect of substrate concentration on the cupric-INH complex inhibition

The nature of the inhibition was further characterized by examining the inhibition with increasing concentrations of dTTP. When the data were plotted by the method of

TABLE 1

Site and target of inhibition of reverse transcriptase by cupric- and ferric-INH complexes

System[a]	dTMP incorporated (pmol)			
	No addition	Addition of 1 μg template-primer	Addition of 4 units enzyme	Addition of 1 μg template-primer and 4 units enzyme
Control reaction	12.3 (100)	–	–	–
Template-primer preincubated with:				
a) cupric-INH complex	1.6 (13)	1.7 (14)	10.6 (86)	10.3 (84)
b) ferric-INH complex	8.1 (66)	8.9 (72)	10.6 (86)	10.8 (88)
Enzyme preincubated with:				
a) cupric-INH complex	0.6 (5)	0.9 (7)	8.5 (69)	8.2 (67)
b) ferric-INH complex	6.1 (50)	6.3 (51)	9.3 (76)	10.7 (87)

[a] Additional enzyme, template-primer or both were added to the reaction mixture in which either the enzyme or the template-primer had been preincubated separately with 25 μM of cupric-INH or 50 μM of ferric-INH complexes. The assay conditions were the same as described in the text. The complexes were not added in the case of control reaction. Values in parentheses are percentage of enzyme activity.

Lineweaver and Burk [15], straight lines could be drawn intersecting on the abscissa. The results (Fig. 3) indicate that the inhibition was non-competitive with respect to dTTP and the apparent K_i for cupric-INH complex was approximately 2 μM.

Fractionation of cupric-INH complex bound AMV reverse transcriptase

Hundred units of the AMV enzyme were added separately to 25 μM and 250 μM of cupric-INH complex and incubated at 37°C for 5 min. The complex-bound enzyme was loaded onto the Sephadex G-25 column (0.8 × 18.5 cm) and eluted as 1 ml fractions with assay buffer containing 10% glycerol. The absorbance of the fractions was recorded at 280 nm using a double beam spectrophotometer. Alternate fractions were assayed for the enzyme activity and the copper content of the fractions was measured at 3242 Å using an atomic absorption spectrophotometer. The results indicate partial enzyme activity when the enzyme was bound to 250 μM of cupric-INH complex (Fig. 4A), whereas significant enzyme activity was detected in the case of 25 μM of cupric-INH complex (Fig. 4B). Incomplete binding of the enzyme with the complex was observed at 25 μM concentration of the cupric-INH complex, while at 250 μM concentration free complex was detected even after binding with the enzyme.

In vivo toxicity of the cupric- and ferric-INH complexes

The cupric- and ferric-INH complexes were injected intravenously into 1-day-old chicks at amounts varying from 100 μg to 4 mg per chick and the chicks were observed over a period of 1 month for toxic symptoms and lesions. The chicks which were in-

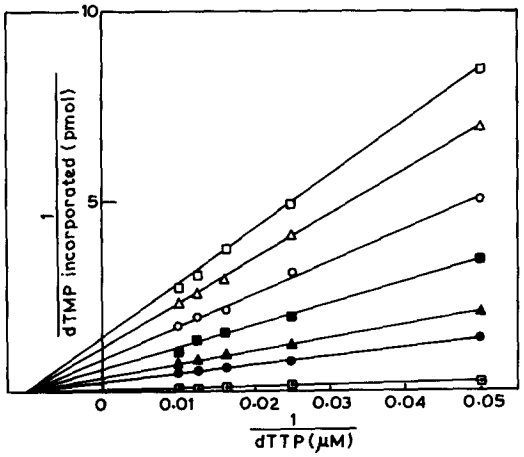

Fig. 3. Double reciprocal plot of the reaction rate in the presence of various concentrations of cupric-INH complex as inhibitor and dTTP as substrate. The assay was carried out as described in the text. The velocity is expressed as picomoles of dTMP incorporated into polynucleotide. The cupric-INH concentrations were 0 (◉), 1 (●), 2 (▲), 4 (■), 8 (○), 12 (△) and 16 (□) μM.

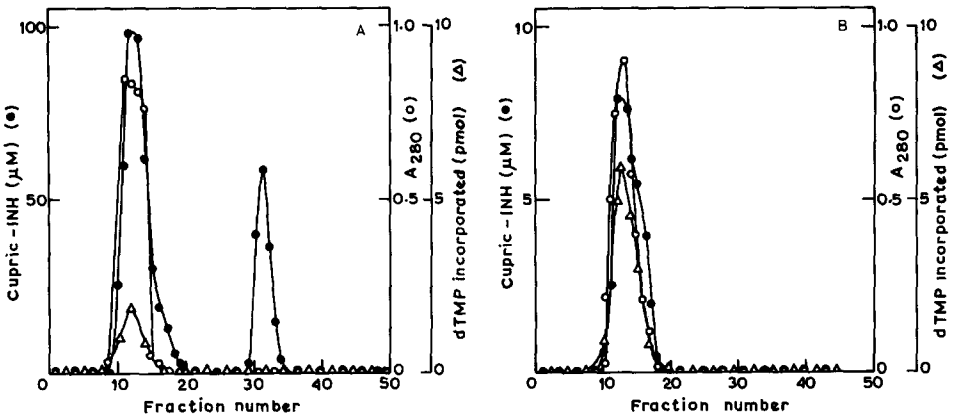

Fig. 4. Fractionation of 250 μM (A) and 25 μM (B) cupric-INH complex bound AMV reverse transcriptase. The experimental conditions were the same as described in the text. The dTMP incorporated into polynucleotide (Δ), absorbance (○) and the copper content (●) were measured in the fractions.

jected with 750 and 1000 μg of the complexes showed local inflammation, edema and induration that heals up in 7–10 days, whereas the chicks which received up to 500 μg of the complexes did not show any toxic symptoms and appeared normal. Chickens injected with 2 and 4 mg died after 24 h and 30 min respectively due to acute toxicity of the complexes.

Antiviral property of cupric- and ferric-INH complexes

One-day-old chicks were injected with AMV plasma which had been pretreated for 15 min at 4°C with various amounts of the complexes, INH, $CuSO_4$ or $FeCl_3$. The chicks were examined for signs of leukemia. Those chicks which were injected with AMV showed acute leukemia on day 16, and those which were injected with pretreated AMV with cupric- and ferric-INH complexes showed no signs of leukemia and behaved like control chicks. Pretreatment of AMV with 150 μg of the complex and not INH, $CuSO_4$ or $FeCl_3$ resulted in the complete protection of the chicks from leukemia (Table 2).

DISCUSSION

Although the copper-INH complexes were shown to inhibit the growth of RSV upon contact with the virus [4], the molecular mechanism underlying the inhibition was not clearly studied. The present work was undertaken to investigate the mechanism of inhibition of AMV multiplication by cupric- and ferric-INH complexes in order to evaluate their antiviral properties. Copper complex of INH was reported to enhance the in vitro activity of INH against *M. tuberculosis* [14]. The complex formation occurs through the interaction of metal ions with the hydrazide side chain of INH [1,2,10,11].

TABLE 2

Effect of INH, $CuSO_4$, $FeCl_3$, cupric-INH complex and ferric-INH complex on AMV infection in chicks

Dosage (μg)	INH	$CuSO_4$	$FeCl_3$	Cupric-INH complex	Ferric-INH complex
0	0	0	0	0	0
50	0	0	0	1	1
100	0	0	0	3	3
150	0	0	0	6	6
250	0	1	1	6	6
500	0	2	2	6	6

AMV plasma pretreated separately with various amounts of INH, $CuSO_4$, $FeCl_3$, cupric-INH complex and ferric-INH complex was injected intravenously into 1-day-old chicks. The results indicate the number of chicks surviving out of total number (6) of chicks injected in each case.

The results (Fig. 1) showed that the inhibition of reverse transcriptase activity by cupric- and ferric-INH complexes was dose-dependent. Similar inhibitory activities on reverse transcription have been reported using other compounds such as phosphonoacetic acid (PAA) [3], phosphonoformate (PFA) [23], derivatives of rifamycin [9] and fagaronine, an alkaloid from *Fagara zanthoxyloides* [20]. The 50% inhibition for cupric and ferric-INH complexes was observed at 7 and 90 μM respectively in the poly(rA) · $(dT)_{12-18}$-dependent DNA synthesis. The inhibition of the poly(dA) · $(dT)_{12-18}$-dependent reaction by these complexes was found to be less than that obtained in the poly(rA) · $(dT)_{12-18}$-dependent reaction. However, $FeCl_3$ and ferric-INH complex showed consistently a decrease in inhibition above 40 μM concentration.

The data on site and target of inhibition indicates that both cupric- and ferric-INH complexes have more affinity towards the enzyme than towards the template-primer. However, the inhibitor binding to the template-primer cannot be completely ruled out because in the presence of an excess inhibitor addition of suboptimal concentration of template-primer may not cause a marked change in the cpm. Other compounds which preferentially bind to the enzyme are rifamycins, streptovaricins and alkaloids [7]. The nature of inhibition by cupric-INH complex was found to be non-competitive with respect to dTTP which is in agreement with similar observations reported for PAA [3] and PFA [23]. The apparent K_i value for the cupric-INH complex was 2 μM, which is less than the value (9 μM) calculated for PFA [23] showing that the cupric-INH complex is a stronger inhibitor of AMV polymerase than PFA. It was found that these complexes inhibit the reverse transcription even after the initiation of polynucleotide synthesis which is in agreement with the results reported for PAA [3], PFA[23] and fagaronine [20]. The results indicate that both the cupric- and ferric-INH complexes inhibit the reverse transcription by their interaction with the enzyme. The DNA polymerases α and β, partially purified from chick embryos [6], were not inhibited by cupric-INH complex even up to a concentration of 100 μM (data not shown).

The inhibition of the AMV infection by these complexes on contact with the virus even at a very low and non-toxic concentration of 150 μg warrants further studies on the possibility of using them as antiviral agents against RNA tumor viruses.

ACKNOWLEDGEMENT

We are grateful to Dr. T. Ramakrishnan for his encouragement and helpful discussions. We thank Mary Samuel for her excellent technical assistance. This work was supported by a grant from the Department of Science and Technology, Government of India.

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