Biochemical Pharmacology, Vol. 27, pp. 579–584. Pergamon Press, 1978. Printed in Great Britain.

MOLECULAR MECHANISM FOR THE SPECIFIC INHIBITION OF REVERSE TRANSCRIPTASE OF ROUS SARCOMA VIRUS BY THE COPPER COMPLEXES OF ISONICOTINIC ACID HYDRAZIDE

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(*Received* 13 *June* 1977; *accepted* 31 *August* 1977)

Abstract—Isonicotinic acid hydrazide (isoniazid), one of the most potent antitubercular drugs, was recently shown, in our laboratory, to form two different complexes with copper, depending upon the oxidation state of the metal ion. Both the complexes have been shown to possess antiviral activity against Rous sarcoma virus, an RNA tumor virus. The antiviral activity of the complexes has been attributed to their ability to inhibit the endogenous reverse transcriptase activity of RSV. More recent studies in our laboratory indicate that both these complexes inhibit both endogenous and exogenous reactions. As low a final concentration as 50 μM of the cupric and the cuprous complexes inhibits the endogenous reaction to the extent of 93 and 75 per cent respectively. Inhibition of the exogenous reaction varies with the templates. The inhibition can be reversed by either β-mercaptoethanol or ethylene-diamine-tetra-acetic acid. The specificity of this inhibition has been ascertained by using a synthetic primer-template, -(dG)$_{\sim 15}$-(rC^{m})$_n$, which is highly specific for reverse transcriptases. The inhibition is found to be template specific. The studies carried out, using various synthetic primer-templates, show the inhibition of both the steps of reverse transcription by the copper complexes of isoniazid.

The discovery of the enzyme RNA dependent DNA polymerase (reverse transcriptase) by Temin and Baltimore [1, 2] has opened up the possibility of using a specific inhibitor of this enzyme as a chemotherapeutic approach to malignancy. Among a host of inhibitors of reverse transcriptase available, thiosemicarbazide, a heavy metal chelating agent, is a strong inhibitor of the enzyme [3]. Thiosemicarbazide is of additional interest since it is antitubercular, a property it shares with yet another heavy metal chelating agent—isonicotinic acid hydrazide (isoniazid). We, therefore, made an attempt to investigate whether isoniazid or its metal complex possessed any inhibitory activity against reverse transcriptase, isoniazid being a relatively non-toxic drug in wide-spread use.

The results of our studies on the inhibition of both endogenous and exogenous reverse transcriptase activity of Rous sarcoma virus (RSV) by the copper complexes of isoniazid are presented in this paper. We also suggest a molecular mechanism of action of this new antiviral drug.

MATERIALS AND METHODS

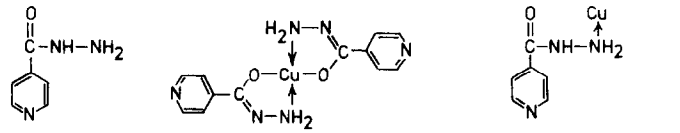
Chemicals. Isoniazid, deoxynucleoside triphosphates, calf thymus DNA Grade I, primer-templates poly (dA-dT) and poly (dI-dC) were purchased from Sigma Chemical Co., St. Louis, U.S.A. [^{3}H]TTP (Sp. Act. 47 Ci/mmol) and [^{3}H]dGTP (Sp. Act. 15 Ci/mmol) were obtained from Radiochemical Centre, Amersham, U.K. Primer-templates (dG)$_{\sim 15}$-(rC^{m})$_n$ and (dT)$_{\sim 15}$-(rA)$_n$ were generously provided by Dr. H. S. Allaudeen, Yale University, CT, U.S.A. Eagle's minimum essential medium (EMEM) with Earle's salts and non-essential amino acids was obtained from Centron Research Laboratory, Bombay, India. Tryptose phosphate broth (TPB) was purchased from Bios, Bombay, India. All the other reagents used were of analytical grade.

Drugs. The cuprous and the cupric complexes of isoniazid were prepared as described earlier [4]. 5 mM stock solutions of the drugs were prepared in 1% HCl and diluted appropriately with Tris–saline (20 mM Tris, 150 mM NaCl, 5 mM KCl and 55 mM glucose), pH 7.4. The solutions were stored at 4°.

Templates. The templates were dissolved separately in a template buffer containing 0.01 M Tris–HCl buffer with 0.1 M NaCl, pH 7.5 and stored frozen at −20° before use.

Propagation and purification of RSV. Primary chick embryo fibroblast cells, obtained from 9 to 11 days old leukosis virus-free White Leghorn chick embryos (State Poultry Farm, Bangalore, India) were grown in EMEM supplemented with 5% heat inactivated goat serum, 1% heat inactivated chick serum and 10% TPB as described earlier [5]. The confluent primary cultures were passaged according to the conventional procedure [6]. The secondary cultures were infected with non-defective Schmidt–Ruppin strain of RSV (subgroup A), 4–6 hr after seeding as described earlier [7], and grown in complete medium without chick serum. The cultures were incubated at 38.5° in a humidified atmosphere with 5% CO_2. The culture

Abbreviations used: (dG)$_{\sim 15}$-(rC^{m})$_n$ = Oligodeoxyguanylate- poly2′-O-methylribocytidylate; (dT)$_{\sim 15}$-(rA)$_n$ = oligodeoxythymidylate-polyriboadenylate; poly (dA-dT) = Polydeoxyadenylate-polydeoxythymidylate; poly (dI-dC) = Polydeoxyinosinate-polydeoxycytidylate; NP40 = Nonidet P40; RSV = Rous Sarcoma Virus.



Isoniazid | Cupric - isoniazid complex | Cuprous - isoniazid complex

Fig. 1. The chemical structures of isoniazid and its two copper complexes. For details see [4].

medium was collected every 24 hr and pooled after the cells got transformed. The virus was then precipitated from the pooled medium by 50% ammonium sulfate saturation. The precipitate was dissolved in STE (0.1 M NaCl, 0.02 M Tris and 10^{-3} M EDTA) and centrifuged twice on a 15–60 per cent (w/w) sucrose cushion gradient at 24,000 r.p.m. at 4° for 2 hr in the SW 25.1 rotor of a Beckman Model L2 Ultracentrifuge. The purified virus was stored frozen before use.

Reverse transcriptase assay

Endogenous reaction. The assay was done essentially as described by Levinson *et al.* [8], with slight modifications. 15 μl (25 μg) of purified RSV, suspended in 50 μl of a solution containing 80 mM Tris-buffer, pH 8.1, 8 mM $MgCl_2$, 100 μM each of dATP, dGTP and dCTP was incubated at 37° for 15 min. After the addition of 5 μl of 1% NP40 and 10 μl of [^{3}H]TTP (330 μCi/ml) the reaction mixture was incubated at 37° for additional 60 min. The reaction was stopped by adding 0.5 ml of 2% sodium pyrophosphate, 50 μg calf thymus DNA and 2 ml of 10% TCA. The acid insoluble precipitate was filtered through Whatman GF/C glass fibre filter paper, washed, dried and the radioactivity was determined in a Beckman LS-100 Liquid Scintillation Spectrometer at 4.5 per cent efficiency.

Exogenous reaction. The assays were done essentially as described by Sarngadharan *et al.* [9], with slight modifications. 50 μl of the assay mixture contained 25 μg of purified RSV, 80 mM Tris buffer, pH 8.1, 8 mM $MgCl_2$, 0.3 μg of the template and appropriate deoxynucleoside triphosphates depending upon the template used. After incubating the reaction mixture for 15 min at 37°, 5 μl of NP40 and appropriate [^{3}H]deoxynucleoside triphosphate (330 μCi/ml) were added. All the other steps were same as those used in the endogenous reaction.

Reverse transcriptase inhibition studies. 15 μl (25 μg) of purified RSV, irrespective of the template used, was exposed to various final concentrations, ranging from 20–50 μM, of cupric sulfate, isoniazid, cuprous-isoniazid or cupric-isoniazid complex separately and preincubated at 37° for 15 min. All the other steps were same as described above. In one set of experiments, 5 μl of NP40 was added along with the drugs and then the assay mixture was incubated for 15 min. For the reversal of inhibition, either β-mercaptoethanol or EDTA was added at a final concentration of 10^{-3} M. In later experiments, 50 μM final concentrations of the drugs were used.

RESULTS

The chemical structures of isoniazid and its copper complexes are given in Fig. 1.

Inhibition of endogenous reaction by the copper complexes of isoniazid. The data for the inhibition of the endogenous reverse transcriptase activity by the copper complexes of isoniazid are presented in Table 1. Omission of NP40 or RSV or [^{3}H]TTP results in near total inhibition of the enzyme activity. It can be seen that at 50 μM final concentration, isoniazid alone has no significant effect, whereas, the copper complexes of isoniazid bring about considerable amount of inhibition of the enzyme activity. The data in Table 1 also compares and contrasts the inhibitory effects of the complexes before and after the treatment and pre-incubation with NP40. There is a slight increase of inhibition when the virus is pretreated with the non-ionic detergent. The inhibitory activity of the compounds can be rated in the following order: cupric-isoniazid complex > cuprous-isoniazid complex > cupric sulfate > isoniazid, or per cent inhibition-wise: 93, 75, 71 and 6 per cent respectively.

Inhibition of exogenous reaction by the copper-complexes of isoniazid. The inhibition pattern was also studied using an exogenous primer-template, $(dG)_{\sim 15}$-$(rC^m)_n$, which is known to be specific for reverse transcriptases [10]. The data in Table 2 clearly demonstrate that the observed inhibition is specific for reverse transcriptase and the same order of rating is obtained, the maximum inhibition at a final concentration of 50 μM being 45.2, 42.7, 28.0 and 10% respectively.

Reversal of inhibition by β-ME and EDTA. Table 3 shows the reversal of inhibition by β-ME and EDTA. EDTA at a final concentration of 10^{-3} M increases the activity from 15 to 70 per cent for cupric sulfate inhibited system, from 13 to 92 per cent for cuprous-isoniazid complex inhibited system, and from 6 to 79 per cent for cupric-isoniazid complex inhibited system. Similarly, β-ME also increases the activity from 15 to 62 per cent for cupric sulfate inhibited system, from 13 to 90 per cent for cuprous-isoniazid complex inhibited system and from 6 to 64 per cent for cupric-isoniazid complex inhibited system.

Site and target of inhibition. We carried out experiments to find out whether the inhibition was due to the direct binding of the copper-isoniazid complexes with the enzyme or with the template or both. Two types of experiments were done. In the first type, additional 0.3 μg of the primer-template $(dT)_{\sim 15}$-$(rA)_n$ was added to the complex inhibited system after the 15 min preincubation period and in the other, additional 15 μl of RSV was added as the enzyme

Table 1. Inhibition of endogenous reverse transcriptase reaction by the copper complexes of isoniazid and the effect of NP40 pre-treatment

System	[^{3}H]TMP incorporation into DNA (CPM)	% Inhibition	Pre-treatment with NP40 [^{3}H]TMP incorporation into DNA (CPM)	% Inhibition
1. Minus NP40	90	93.1		
2. Minus RSV	52	96.0		
3. Minus [^{3}H]TTP	47	96.4		
4. Complete	1300	0.0	1300	0.0
5. Complete + copper sulfate	383	70.5	101	92.2
6. Complete + isoniazid	1225	5.8	1200	7.7
7. Complete + cuprous-isoniazid complex	325	75.0	156	88.0
8. Complete + cupric-isoniazid complex	88	93.2	60	95.4

Final concentration of the drugs used = 50 μM for 50 μl assay mixture.
Average background counts = 45.
Zero time control counts = 328.
CPM given in the Table represent final values after subtraction of background and zero time control counts.

Table 2. Inhibition of exogenous reverse transcriptase reaction by the copper complexes of isoniazid

System	[^{3}H]dGMP incorporation into DNA [$(dG)_{\sim 15}$-$(rC^m)_n$ primer-template] (CPM)	% Inhibition
1. Complete	572	0.0
2. Complete + copper sulfate	412	28.0
3. Complete + isoniazid	511	10.1
4. Complete + cuprous-isoniazid complex	328	42.7
5. Complete + cupric-isoniazid complex	314	45.2

Final concentration of the drugs used = 50 μM for 50 μl assay mixture.
Average background counts = 125.
Zero time control = 199.
CPM given in the Table represent final values after subtraction of background and zero time control counts.

Table 3. Reversal of inhibition by EDTA and β-ME

System	No addition	% Activity 10^{-3} M EDTA	10^{-3} M β-ME
1. Complete + copper sulfate	15	70	62
2. Complete + cuprous-isoniazid complex	13	92	90
3. Complete + cupric-isoniazid complex	6	79	64

Final concentration of the drugs used = 50 μM for 50 μl assay mixture.

Table 4. Inhibition site and target evaluation

System	[^{3}H]deoxynucleoside monophosphate incorporation into DNA (c.p.m.) $(dT)_{\sim 15}$-$(rA)_n$ No addition	Enzyme	Template	$(dG)_{\sim 15}$-$(rC^m)_n$ No addition	Enzyme	Template
1. Complete + cuprous-isoniazid complex	272(69.8)	325(63.9)	387(57.0)	328(42.7)	333(41.8)	362(36.8)
2. Complete + cupric-isoniazid complex	264(70.7)	355(60.6)	417(53.7)	314(45.2)	395(30.9)	482(15.8)

Final concentration of the drugs used = 50 μM for 50 μl assay mixture.
Figures in parentheses indicate % inhibition.
Average background counts = 98.
Average zero time control counts = 200.
CPM given in the Table represent final values after subtraction of background and zero time control counts.

source. Same experiment was also done using the primer-template $(dG)_{\sim 15}$-$(rC^m)_n$. Data in Table 4 clearly demonstrate that the addition of $(dT)_{\sim 15}$-$(rA)_n$ increases the activity from 30 to 43 per cent in cuprous-isoniazid complex inhibited system and from 29 to 46 per cent in cupric-isoniazid complex inhibited system. On the other hand, the addition of enzyme increases the activity by only 6–10 per cent respectively. Similarly, addition of $(dG)_{\sim 15}$-$(rC^m)_n$ to the cuprous-isoniazid complex inhibited system increases the activity from 57 to 63 per cent and from 55 to 84 per cent in the case of cupric-isoniazid complex inhibited system. Addition of enzyme brings about an increase of only 1–14 per cent respectively.

Since the addition of either the template or the enzyme to a control assay mixture brings about slight inhibition, it is suggested from these results that the complexes inhibit the reverse transcription mainly by forming complexes with the template because the addition of templates brings about significant reversal of inhibition as compared to the partial reversal of inhibition on addition of the enzyme. Inhibition of reverse transcriptase activity by the exogenously added RNA has already been reported [11].

Inhibition step evaluation. Reverse transcription is accomplished in two steps. The enzyme first catalyses the synthesis of a single stranded DNA on the RNA template to form a RNA:DNA hybrid. RNA is degraded by the RNase H activity to release a single stranded DNA which is then utilised by the same enzyme for the synthesis of a double stranded DNA.

We carried out experiments to find out which step of the reverse transcription was being inhibited by the copper complexes of isoniazid. The exogenous reactions with three primer-templates viz. $(dT)_{\sim 15}$-$(rA)_n$, poly (dA-dT) and poly (dI-dC) with and without drugs were carried out by the procedure described by Chandra *et al.* [12]. The results are given in Table 5.

It is evident from the data that all the reactions utilizing $(dT)_{\sim 15}$-$(rA)_n$, poly (dA-dT) and poly (dI-dC) are highly sensitive to the action of the copper complexes of isoniazid. The inhibition observed in the second step, where poly (dA-dT) primer-template is used, is more than that in the first step, where the primer-template $(dT)_{\sim 15}$-$(rA)_n$ is used. In all the cases the order of inhibition follows the same pattern viz. cupric-isoniazid complex > cuprous-isoniazid com-

Table 5. Inhibition step evaluation

System	[^{3}H]deoxynucleoside monophosphate incorporation into DNA $(dT)_{\sim 15}$-$(rA)_n$ CPM	% Inhibition	Poly(dA-dT) CPM	% Inhibition	Poly(dI-dC) CPM	% Inhibition
1. Complete	900	0.0	3548	0.0	394	0.0
2. Complete + copper sulfate	468	48.0	2924	17.6	192	51.3
3. Complete + cuprous-isoniazid complex	272	69.8	513	85.6	173	56.1
4. Complete + cupric-isoniazid complex	264	70.7	500	86.0	120	69.5

Final concentration of the drugs used = 50 μM for 50 μl assay mixture.
Average background counts = 104.
Average zero time control counts = 408.
CPM given in the Table represent final values after subtraction of background and zero time control counts.

plex > cupric sulfate. These results clearly indicate that both the copper complexes of isoniazid inhibit both the steps of reverse transcription.

DISCUSSION

Although several inhibitors of reverse transcriptases have been reported, the molecular mechanism underlying the inhibition is known only for a few of them [3, 12]. Since we attributed the inhibitory action of the two copper complexes of isoniazid, prepared in our laboratory, against RSV [4], to their ability to inhibit the reverse transcriptase activity of the virus, we made an attempt to study the mechanism of action of the copper complexes of isoniazid at molecular level.

Our results indicate that though isoniazid alone has no significant effect, both cuprous and cupric complexes of isoniazid are strong inhibitors of both endogenous (using genome RNA as the template) as well as exogenous (using several synthetic primer-templates) reverse transcriptase activity of RSV. The cupric-isoniazid complex is more active than the cuprous-isoniazid complex. Although the cupric ions alone bring about inhibition of enzyme activity, the observed inhibition by the copper complexes of isoniazid is not due to the degradation of the complexes. Both the complexes retain their structural integrity under the assay conditions. Structural integrity of the complexes was established by carrying out melting temperature studies. It is well documented that at low concentrations, copper ions increase the Tm of the native DNA but at higher concentrations, the Tm is decreased to a great extent [13]. The cuprous-isoniazid complex when incubated with DNA in 0.01 M Tris–HCl buffer, pH 8.1, for 12 hr, did not alter the Tm of the native DNA, whereas, a progressive increase in the Tm was observed with increasing concentration of the cupric-isoniazid complex, under similar conditions. One would expect a significant decrease in the Tm value in case the complexes are dissociated resulting in the release of copper ions. Further, it is shown that isoniazid alone has no effect on the Tm profile [14].

Although copper sulfate inhibits the *in vitro* reverse transcriptase activity, it cannot be used as an antiviral drug because of its adverse toxicity. The copper-isoniazid complexes, on the other hand, are relatively non-toxic to the normal chick embryo fibroblast cells. Moreover, cupric ions alone have little inhibitory effect on the focus formation in chick embryo fibroblast cells by RSV, whereas the copper-isoniazid complexes have a significant effect on the focus formation by the virus.* Using a specific synthetic primer-template, $(dG)_{\sim 15}$-$(rC^m)_n$, it has been established that the inhibition by the copper-isoniazid complexes is very specific for reverse transcriptase, since no other known DNA polymerase can utilize this primer-template [10].

Since isoniazid alone has no effect on the enzyme activity, the possibility that it is capable of removing the integral metal part of the polymerase, by chelation, is ruled out. However, the observed inhibitory effect by the cupric ions can be attributed to the direct binding of the ions to the sulfahydryl groups of the enzyme. On the other hand, any such direct effect on the enzyme by the preformed complexes seems to be secondary. These complexes presumably bind to the template, blocking the movement of the polymerase along the template, since addition of excess template to the inhibited system brings about a considerable reversal of inhibition. The fact that the addition of enzyme also brings about partial reversal of inhibition, suggests that the drug has a small inhibitory effect on the enzyme also, but the main action is by binding to the template. Increased inhibition after the pre-treatment with NP40 further strengthens this point. The cupric-isoniazid complex has been shown to bind to the purified 70S RNA of RSV.† Using model system also, the complexes have been shown to bind to both DNA and RNA.* Since the binding to the nucleic acids is reversible by either EDTA or β-ME, the inhibition is also reversed by these reagents.*

Using different polyribonucleotide and polydeoxyribonucleotide strands as primer-templates, it is demonstrated that both the complexes inhibit both the steps of reverse transcription. Inhibition of the second step, catalyzed by a polydeoxyribonucleotide primer-template, is more than that of the first step, catalyzed by a polyribonucleotide primer-template.

It is interesting to note that even in the model system, these complexes bind to DNA more than they do to RNA.* A few antibiotics like daunomycin and adriamycin have been shown to inhibit the reverse transcription in the second step only [12], whereas, the copper complexes of isoniazid inhibit both the steps of reverse transcription, suggesting that these complexes may prove to be very potent inhibitors of RNA tumor viruses. The cupric-isoniazid complex has also been shown to inhibit the *in vivo* tumor growth in chicken by RSV.†

The recent report by Antony *et al.* [14] that a variety of DNA and RNA animal viruses are inhibited by isoniazid and its copper complexes suggests that these compounds specifically interact with the viral nucleic acids, and thus raises the possibility of an effective antiviral chemotherapy against both DNA and RNA animal viruses.

Acknowledgements—We wish to express our sincere thanks to Professor H. Hanafusa, Rockefeller University, New York, U.S.A., for his kind gift of RSV. One of us (A.S.) gratefully acknowledges the financial assistance from the University Grants Commission, New Delhi, India, in the form of a Senior Research Fellowship.

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