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Inhibition of RNA-Dependent DNA Polymerase of Rous Sarcoma Virus by Thiosemicarbazones and Several Cations

(RNA tumor viruses/DNA synthesis/viral inactivation/chelation)

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ABSTRACT **The RNA-dependent DNA polymerase of Rous sarcoma virus is inhibited by *N*-methyl isatin β-thiosemicarbazone and by thiosemicarbazide, but not by semicarbazide. These inhibitors also inactivate, upon contact with the virion, the transforming ability of Rous sarcoma virus. Sulfhydryl donors, such as 2-mercaptoethanol, can prevent these effects. The RNA-directed activity of the purified polymerase is inhibited to a greater degree than is the DNA-directed activity.**

Two cations, Cu^{++} and Hg^{++}, can inhibit RNA-dependent DNA polymerase and inactivate the transforming ability of the virus. Synergism between *N*-methyl isatin β-thiosemicarbazone and Cu^{++} occurs, since treatment of the virus with a low dose of either *N*-methyl isatin β-thiosemicarbazone or Cu^{++} has little effect; however, when the two compounds are mixed together, significant inactivation occurs. This observation supports the hypothesis that the antiviral action of thiosemicarbazones is a function of their ability to act as a ligand for metallic ions.

Several cations (Ag^{+}, Co^{++}, Zn^{++}, Cd^{++}, and Ni^{++}) significantly inactivate the RNA-dependent DNA polymerase, but have little effect on the transforming ability. In view of this result, the conclusion that the enzyme activity is required for transformation remains open to question.

N-methyl isatin β-thiosemicarbazone (Me-IBT) inhibits the growth of pox viruses (1, 2), and is effective in the prophylaxis of smallpox (3) and in the treatment of eczema vaccinatum, a complication of smallpox vaccination (4). Additional *in vitro* studies have extended the spectrum of action of this drug to include other groups of viruses, such as adenovirus, herpesvirus, picornavirus, reovirus, arbovirus, and myxo- and paramyxovirus viruses (5, 6). In these cases, inhibition of the virus was obtained by treatment of the cells after infection.

In contrast, we have recently reported that Rous sarcoma virus (RSV) can be inactivated by exposure to the drug before infection (7). Other RNA tumor viruses, such as mouse sarcoma virus, and leukemia virus, and feline sarcoma virus, are similarly inactivated (unpublished observations). However, RNA-containing cytopathic viruses, including Newcastle disease virus, poliovirus, and vesicular stomatitis virus, are not affected (ref. 7, and unpublished results).

In our previous paper (7), we described several negative attempts to determine the mechanism of inactivation (including inhibition of the RNA-dependent DNA polymerase). Subsequent experiments, reported here, have demonstrated that the RNA-dependent DNA polymerase of RSV is, indeed, inhibited by Me-IBT and that the presence of 2-mercaptoethanol prevented the action of IBT in our original experiments. The observation that a drug that inhibits the RNA-dependent DNA polymerase also inactivates the transforming ability of the virus provides evidence for the importance of this enzyme to malignant transformation by RSV.

Since thiosemicarbazones are strong chelators of copper (8, 9), a role for the cupric ion in the antiviral and antitumor activity of these drugs has been postulated and supported experimentally (10, 11). Therefore, we tested the effect of copper and several other cations on the transforming ability and on the RNA-dependent DNA polymerase activity of RSV. It was found that, at a concentration of 40 μM, Cu^{++} and Hg^{++} inhibited both the transforming ability and the enzyme activity. In addition, low doses of Cu^{++} and Me-IBT, which had little effect on the virus, caused significant inhibition of enzyme activity and inactivation of transforming ability when mixed together. These findings lend support to the previously stated hypothesis that the action of thiosemicarbazones is related to their ability to chelate metallic ions (16).

Other cations (Ag^{+}, Co^{++}, Zn^{++}, Cd^{++}, and Ni^{++}) can significantly inactivate the RNA-dependent DNA polymerase but have a minor effect on transforming ability. This could be interpreted as evidence that the enzyme is not required for transformation but can be explained in other ways (see below). In addition, a third group of cations that has a smaller or no effect on enzyme activity and on transformation was demonstrated.

MATERIALS AND METHODS

Virus. The Schmidt–Ruppin strain (Group A) of RSV was propagated and purified as described (12).

Chemicals. The drugs were obtained from the following sources: Me-IBT and methyl isatin from K & K Rare Chemicals, thiosemicarbazide and semicarbazide from Eastman Kodak, thiourea from Calbiochem, and the $(CH_3)_2SO$ used as the solvent from Matheson, Coleman and Bell. Stock solutions at 40 mM in $(CH_3)_2SO$ were prepared. Reagent grade inorganic compounds from either Baker or from Matheson, Coleman and Bell were used. Stock solutions at 40 mM were prepared in double-distilled H_2O.

RNA-Dependent DNA Polymerase Was Assayed in one of two solutions. Solution A, used when the thiosemicarbazones were being tested, was composed of 100 mM Tris·HCl (pH 8.1), 10 mM $MgCl_2$, and 0.1 mM dATP, dGTP, and dCTP.

Abbreviations: RSV, Rous sarcoma virus: Me-IBT, *N*-methyl isatin β-thiosemicarbazone.

Since it was found that both Tris buffer and deoxynucleoside triphosphates decreased the antiviral activity of the cations, Solution B, composed of 150 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 10 mM $MgCl_2$, and 25 μM dATP, dGTP, and dCTP, was used when the cations were being tested. After suitable incubation in the presence of the drug, [^{3}H]TTP (17 Ci/mmol) and 0.05% v/v (final concentration) NP-40 (Nonidet P40, Shell Chemical Co.) were added. The reaction mixtures were incubated at 37° for 30 min and acid-precipitable counts were measured (13).

Transformation Assay. The focus-forming ability of the virus was assayed on chick-embryo cells in culture as described (14). A 1:200 dilution of the virus–drug mixture was made before infection, ensuring that the remaining drug had no effect on focus formations (unpublished results).

Partial Purification of RNA-Dependent DNA Polymerase. This procedure and the assay of the enzyme with RNA and DNA template have been described (15).

RESULTS

In the initial experiment, either Me-IBT or one of several analogues was added to a suspension of RSV at a final concentration of either 400 μM or 40 μM, and the mixture was incubated at 37° for 60 min. After samples were taken for transformation assay, [^{3}H]TTP and NP-40 were added, the reaction mixture was incubated at 37° for an additional 30 min, and the activity of RNA-dependent DNA polymerase was determined. In this experiment, the 2-mercaptoethanol (1% v/v) was added after Me-IBT had interacted with RSV. In contrast, the experiments previously reported (7) were performed with 2-mercaptoethanol (2.5% v/v) present when the Me-IBT was added. The data in Table 1 demonstrate the inhibition of RNA-dependent DNA polymerase activity by Me-IBT and by the thiosemicarbazide side chain. Me-IBT is clearly the more effective compound. Methyl isatin, semicarbazide, and thiourea analogues have no activity. The infectivity data demonstrate that those compounds that inhibit the enzyme activity also inactivate the transforming ability of RSV.

TABLE 1. *Inhibition of RNA-dependent DNA polymerase of RSV by Me-IBT*

Compounds	Enzyme*	Transforming*
None	100	100
$(CH_3)_2SO$ (1%)	100	100
Me-IBT (40 μM)	2	1
Me-IBT (4 μM)	31	30
Thiosemicarbazide (400 μM)	14	10
Thiosemicarbazide (40 μM)	88	63
Semicarbazide (400 μM)	100	100
Thiourea (400 μM)	90	100
Methyl isatin (400 μM)	88	100

10 μl of purified RSV, suspended in 200 μl of solution A, was exposed to different concentrations of the various compounds by addition of a 2-ul aliquot of the substance to be tested. After 60 min at 37°, a 5-μl sample was taken for transformation assay and 2 μl of 2-mercaptoethanol, 2 μCi of [^{3}H]TTP, and 10 μl of 1% NP-40 were added. The reaction mixture was incubated at 37° for 30 min, and acid-precipitable counts were obtained. 34,811 cpm and 116 foci were assayed in the untreated control sample.

* % Activity.

TABLE 2. *Effect of 2-mercaptoethanol on the interaction between Me-IBT and RSV*

Compounds	Enzyme*	Transforming*
$(CH_3)_2SO$	100	100
Me-IBT	4	1
Me-IBT + 0.1% 2-mercaptoethanol	92	100
Me-IBT + 0.01% 2-mercaptoethanol	46	33

Purified RSV, suspended in 200 μl of solution A, was exposed to 40 μM Me-IBT in the presence or absence of 2-mercaptoethanol. The mixture was incubated at 37° for 30 min, a 5-μl sample was taken for transformation assay, and 2 μCi of [^{3}H]TTP and 10 μl of 1% NP-40 were then added. After incubation at 37° for an additional 30 min, acid-precipitable counts were measured. 15,770 cpm and 214 foci were assayed in the untreated control sample.

* % Activity.

In the experiment just described, 2-mercaptoethanol was added after a 60-min exposure to the drugs. If 2-mercaptoethanol is omitted from the reaction entirely, the incorporation of [^{3}H]TTP is 2- to 4-fold lower, but the inhibition and inactivation by the various compounds remain relatively the same (data not shown). In the following experiment, we demonstrate directly that 2-mercaptoethanol prevents the inhibition by Me-IBT. As seen in Table 2, there is no inhibition of either enzyme activity or transforming ability of the virus that received 0.1% (14 mM) 2-mercaptoethanol at the same time as the Me-IBT, but inhibition did occur when Me-IBT alone was added. When 0.01% (1.4 mM) 2-mercaptoethanol is used, the protective effect is incomplete. Two other sulfhydryl compounds, dithiothreitol and glutathione, have a similar protective effect.

The effect of Me-IBT on the purified DNA polymerase from RSV was also determined, with both RNA and DNA templates. The data in Table 3 demonstrate that with 400 μM

TABLE 3. *Inhibition of the purified RNA-dependent DNA polymerase of RSV by Me-IBT*

	RSV DNA Polymerase Template		*E. coli* Polymerase I Template
Compounds	RNA	DNA	DNA
$(CH_3)_2SO$	100*	100	100
Me-IBT (400 μM)	12	50	3
Me-IBT (40 μM)	44	82	54

Enzyme reactions were performed with a 500-fold purified preparation of the RSV DNA polymerase, with purified 70S RSV-RNA and calf-thymus DNA templates at a concentration of 2 μg/ml. The enzyme–template combination, in 200 μl of solution A, was exposed to either 400 μM or 40 μM Me-IBT and incubated for 15 min at 37°. 2 μl of 2-mercaptoethanol and 5 μCi of [^{3}H]TTP were then added. The reaction was incubated for 1 hr at 37° and acid-precipitable counts were obtained. The cpm incorporated in the $(CH_3)_2SO$-treated control samples of the RNA-directed and DNA-directed RSV polymerase and of the *E. coli* polymerase were 923, 1,194, and 11,417, respectively.

* % Enzyme activity.

TABLE 4. *Effect of EDTA on the interaction between Me-IBT and RSV*

Compounds	Enzyme*	Transforming*
None	100	100
Me-IBT	15	19
EDTA	280	100
Me-IBT + EDTA	120	110

Purified RSV, suspended in 100 μl of a solution composed of solution A without $MgCl_2$, was exposed to 40 μM Me-IBT in the presence or absence of mM EDTA. After 30 min at 37°, a 2-μl sample was taken for transformation assay. The solution was then made 10 mM with respect to $MgCl_2$, and 1 μCi of [^{3}H]TTP and 0.05% NP-40 were added. The reaction mixture was incubated at 37° for 30 min and acid-precipitable counts were obtained. 5687 cpm and 183 foci were assayed in the untreated control sample.

* % Activity.

Me-IBT the RNA-directed activity of the purified DNA polymerase of RSV is inhibited by 88%, while the DNA-directed activity is inhibited by 50%. One explanation for the difference in the degree of inhibition is that the DNA-directed activity may be more efficiently protected by 2-mercaptoethanol, another is that the polymerase may contain a different active site for each of the two activities. Since 1% 2-mercaptoethanol was necessary in order to obtain efficient activity of the purified RNA-dependent DNA polymerase, it is probable that the 400 μM concentration of Me-IBT needed to demonstrate significant inhibition was due to the protective effect of 2-mercaptoethanol. In order to determine whether the action of Me-IBT was specific for the DNA polymerases of RSV, we exposed *Escherichia coli* DNA polymerase I (Kornberg polymerase) to the drug and found its activity to be markedly inhibited.

Since thiosemicarbazide inhibits the enzyme activity and inactivates the infectivity of RSV, but semicarbazide and thiourea do not, it appears that the activity requires the presence of both the thio and the hydrazine moieties. These groups are also necessary for the strong binding of transitional metal ions exhibited by thiosemicarbazide, and there is data implicating copper in the biological action of this compound (8–10). The hypothesis that metal ions are involved in the action of these drugs is supported by the observation (Table 4) that the presence of 1 mM EDTA can prevent the inhibition of enzyme activity and the inactivation of infectivity by Me-IBT. It was observed also that EDTA alone enhances the activity of the RNA-dependent DNA polymerase but does not affect the transforming ability of the virus. It is possible that low concentrations of cations inhibitory to the enzyme may be present in the reaction mixture, and these cations are removed by EDTA.

In view of this result, we investigated the effect of copper and several other cations on the activity of RNA-dependent DNA polymerase and the transforming ability of RSV. The data in Table 5 demonstrate that two cations, Cu^{++} and Hg^{++} (40 μM each), have the ability to inactivate the transforming ability and inhibit the enzyme activity of the virus (Group A). Cu^{+} ion has no effect on either function (Group C).

Several other cations (Ag^{+}, Co^{++}, Zn^{++}, Cd^{++}, and Ni^{++}) have the ability to inhibit RNA-dependent DNA polymerase but have little effect on the transforming ability of RSV (Group B). This finding can be interpreted in several ways. One conclusion is that the activity of RNA-dependent DNA polymerase is not necessary for transformation. However, since the sample for transformation assay must be taken before the addition of the detergent NP-40, it is possible that these cations cannot affect the enzyme in the intact virion but are capable of inhibiting the enzyme activity of the disrupted particle. We have tested several polyene detergents (Tween 20, 40, 60, 80, 85, Brij 35, 56, and 58) but were unable to retain infectivity in a concentration of detergent that allowed enzyme activity.

We therefore performed an experiment in which the intact virion was exposed to the Co^{++} ion for 30 min at 37°, diluted 4-fold, then treated with NP-40, and assayed for enzyme activity in the usual manner. The *extent* of inactivation of the diluted suspension was the same as the inactivation of the undiluted suspension; the extent of inactivation of the suspension treated with one-fourth as much Co^{++} was significantly less. This indicates that the Co^{++} ion does affect the enzyme within the virion. The effect of this group of cations cannot, therefore, be explained on the basis of an inability to penetrate the inact virion.

One additional possibility that makes the interpretation of the effect of this group of cations difficult is that the cations could be removed from the site of enzyme inhibition during the infection of the chick embryo cell. In contrast, Cu^{++} and Hg^{++} ions would remain bound during infection and transformation would be inhibited. In view of this possibility we cannot conclude that the activity of RNA-dependent DNA polymerase is not required for transformation.

TABLE 5. *Inhibition of the RNA-dependent DNA polymerase of RSV by cations*

Compounds	Enzyme*	Transforming*
None	100	100
Group A		
$CuSO_4$	5	4
$HgCl_2$	1	1
Group B		
$AgNO_3$	5	41
$Co(NO_3)_2$	7	100
$ZnCl_2$	6	50
$CdSO_4$	5	82
$NiCl_2$	5	100
Group C		
CuCl	91	93
$PbCl_2$	23	100
$FeSO_4$	21	63
$FeCl_3$	98	69
$InCl_3$	50	97
$RhCl_3$	23	34
$PtCl_4$	24	83

Purified RSV, suspended in 200 μl of solution B, was exposed to 40 μM of the various compounds. The mixture was incubated at 37° for 30 min, a 5-μl sample was taken for transformation assay, and 4 μCi of [^{3}H]TTP and 10 μl of 1% NP-40 were then added. After incubation at 37° for an additional 30 min, acid-precipitable counts were obtained. 1127 cpm and 293 foci were assayed in the untreated control sample. The enzyme activity is lower in this experiment than in previous ones because phosphate buffer (solution B) is less efficient than Tris buffer (solution A).

* % Activity.

Other cations (Group 4) that have a smaller or no effect on enzyme activity and on transformation were also found. These include indium, rhodium, and platinum, which were found to inhibit vaccinia growth in mice (10), and ferrous, ferric, lead, and, as mentioned above, cuprous ions.

At a lower concentration (4 μM), only Hg^{++} has a significant effect on the enzyme activity and transforming ability; all the other ions have little effect on either function. The activity of Hg^{++} can no longer be demonstrated at a concentration of 0.4 μM.

In an experiment performed in the same manner as that described in Table 3, it was found that copper sulfate inhibited the purified polymerase activity in a similar manner as did Me-IBT, namely, the RNA-directed reaction was significantly more inhibited at 400 μM than the DNA-directed reaction and little inhibitory activity remained at 40 μM. The *E. coli* polymerase I had 1% activity after treatment with 400 μM copper sulfate and 20% activity when exposed to 40 μM $CuSO_4$ (data not shown).

The nature of this antiviral activity was investigated further by determining whether a synergistic effect occurs between Me-IBT and Cu^{++}. In this experiment, the virus was exposed to low doses of the compounds (1 μM), separately as well as mixed together. It can be seen in Table 6 that there is a minor effect on the virus when the compounds are used by themselves, but a significant inactivation of the transforming ability and inhibition of the enzyme activity occurs when they are present together.

TABLE 6. *Synergistic effect of Me-IBT and Cu^{++} on the RNA-dependent DNA polymerase of RSV*

Compounds	Enzyme*	Transforming*
$(CH_3)_2SO$	100	100
Me-IBT	42	50
$CuSO_4$	86	90
Me-IBT + $CuSO_4$	1	1

Purified RSV, suspended in 200 μl of solution B, was exposed to either 1 μM Me-IBT, 1 μM $CuSO_4$, or both. The mixture was incubated at 37° for 30 min, a 5-μl sample was taken for transformation assay, and 4 μCi of [^{3}H]TTP and 10 μl of 1% of NP-40 were then added. After incubation at 37° for an additional 30 min, acid-precipitable counts were obtained. 3370 cpm and 300 foci were assayed in the untreated control sample.

* % Activity.

DISCUSSION

We have demonstrated that two classes of compounds, the thiosemicarbazones and the inorganic cations, can inhibit the RNA-dependent DNA polymerase activity and the transforming ability of RSV. The correlation between the effect of these agents on the enzyme activity and transformation supports the contention that the product of the RNA-dependent DNA polymerase is necessary for transformation. This correlation is not complete since several cations inhibit the enzyme but do not inactivate transformation. However, for the reason previously mentioned, it is difficult to conclude on the basis of this finding that the enzyme activity is not required for transformation.

One interpretation of the synergistic effect seen when Me-IBT and Cu^{++} are mixed is that a chelate compound is the active material. This possibility is supported by the known affinity of thiosemicarbazones for Cu^{++} (8, 9) and other cations of the first transition series (9). Biologically, Cu^{++} has been shown to be necessary for the antitumor activity of a thiosemicarbazone derivative (11) and is suspected to be involved in the antitumor activity of several other derivatives (16, 17). More speculatively, the inhibition of the recently purified DNA polymerase of chick embryo cells by *o*-phenanthroline (18) could be due to a chelate since this compound strongly binds cupric ions.

Another explanation of the synergistic effect of Me-IBT and Cu^{++} is that the action of one predisposes to the action of the other. It is also possible that the Me-IBT could act by the removal of the metal component of one of the virion enzymes and the Cu^{++} could further inhibit either that enzyme or a different one.

If the chelate is the active compound then an explanation is required for the activity of the Me-IBT when used alone, as seen in Table 1. It is possible that sufficient copper ion is provided as a trace contamination in the solutions or in the glass test tube to make the active complex. This possibility is supported by the observation that EDTA can increase the activity of the enzyme and can inhibit the action of the Me-IBT.

It is not at all clear that the site of action of these compounds is the enzyme itself since there is evidence that Cu^{++} and other cations bind to and degrade synthetic polyribonucleotides (19) and inactivate the infectivity of tobacco mosaic virus RNA (20). There is also evidence that Cu^{++} binds to DNA (21, 22). This interaction reduces the stability of the DNA, as demonstrated by a marked decrease in the melting temperature, and is thought to occur specifically at the guanine and cytosine residues (23). There are no data regarding the relationship between thiosemicarbazones and RNA or DNA. There is evidence that derivatives of thiosemicarbazide such as *1*-formyl isoquinoline thiosemicarbazone and *2*-formyl pyridine thiosemicarbazone can inhibit DNA synthesis in tumor cells and *in vitro* by the inhibition of ribonucleoside reductase (24, 25). Clearly, however, this cannot be the mechanism of action in the case of RNA-dependent DNA polymerase since the deoxynucleoside triphosphates are provided as substrates.

Sulfhydryl compounds, such as 2-mercaptoethanol, protect the virus from the effect of Me-IBT (and Cu^{++}, unpublished results). This type of protective effect has been reported previously; namely, dithiothreitol can protect ribonucleotide reductase from inhibition by *1*-formyl-isoquinoline thiosemicarbazone (24). In either case, the mechanism of this protection is unknown; it could be due to the preservation of the reduced (functional) state of the enzyme or to the binding, by the thiol, of a cation required for the action of the thiosemicarbazone.

The inhibition of RNA-dependent DNA polymerase activity and the inactivation of transforming ability of mouse sarcoma virus has been reported recently with derivatives of the drug rifamycin (26). The experiments with mouse sarcoma virus were performed in a similar fashion to those described in this paper with RSV. Similar results were obtained in so far as a direct correlation between the inhibition of the enzyme activity and the inactivation of transformation is concerned. However, no protection with sulfhydryl compounds was observed and no effect of cations was tested. It

will be of interest to determine whether the relationship of these factors is the same for rifamycin as it is for the thiosemicarbazones.

NOTE ADDED IN PROOF

In experiments performed in the same manner as those in Table 5, we found that *o*-phenanthroline (40 μM), a heavy-metal chelating agent, inactivates RNA-dependent DNA polymerase activity, but not the transforming ability of RSV. This finding supports the contention that the enzyme activity is not required for transformation, but is subject to the same criticism as mentioned with results.

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