Isolation and Characterization of a 
γ-Thiochromanone-4-Thiosemicarbazone-Resistant Mutant of Vaccinia Virus

EHUD KATZ,* EVA MARGALITH, AND BELA WINER

The Chanock Centre for Virology, Hebrew University, Hadassah Medical School, Jerusalem, Israel

Received for publication 2 October 1974

γ-Thiochromanone-4-thiosemicarbazone (TCT) inhibits the growth of vaccinia virus in BSC1 cells by interfering with viral maturation. A mutant of the virus (TCTμ) which is resistant to this drug was isolated. This mutant also exhibits resistance to another thiosemicarbazone related compound, isatin β-thiosemicarbazone (IBT). There is a good correlation between the cross-resistance of the two mutants IBTμ and TCTμ to TCT and IBT, respectively, and the similar antipoxvirus activity of these two thiosemicarbazone-related compounds.

Isatin β-thiosemicarbazone (IBT) is an effective antipox drug (15); its methyl derivative (Marboran) is clinically used in Africa, India, and in South America. Tsunoda et al. (17) reported that γ-thiochromanone-4-thiosemicarbazone (TCT) is also an efficient inhibitor of poxvirus in chicken cells in vitro and in the mouse. Thiosemicarbazone-related compounds, which inhibit the growth of poxviruses, are able to do so either by inhibiting deoxyribonucleic acid (DNA) synthesis or by blocking a maturation event (5).

In the present study, we determine whether the cross drug resistance of the mutants IBTμ (6) and TCTμ to TCT and IBT, respectively, indicates a similar mode of inhibition of the wild-type (WT) strain by these two drugs.

MATERIALS AND METHODS

Compounds and reagents. [3H]thymidine (18.6 Ci/mmol) and [35S]methionine (144 Ci/mmol) were obtained from Radiochemical Centre, Amersham, England. TCT was kindly provided by H. Isoyama, Meiji Seika Kaisha Ltd., Tokyo, Japan. IBT was purchased from Mann Research Laboratories, New York, and 4-formyl-acetanilide thiosemicarbazone was from K & K Laboratories, Inc., Plain View, N.Y. 5-Cyanothiophene-2-carboxaldehyde thiosemicarbazone (Hoe 105) was kindly provided by H. Rolly, Farwerke Hoechst AG, Frankfurt, Germany. Antivaccinia human immunoglobulin was obtained from Povite, Poviet Production N.V., Amsterdam, Holland.

Cell cultures and viruses. HeLa S-3 and BSC1 cells were grown as monolayer cultures in M199 medium supplemented with 10% inactivated calf serum, and chicken embryo cells in M199 containing 5% calf serum. Stocks of WR strain (WT), IBT-resistant (IBTμ) and IBT-dependent (IBTδ) mutants of vaccinia virus were prepared in HeLa cells and titrated on BSC1 monolayers, as previously described (9).

Infection. Monolayers of BSC1 cells were washed with saline and infected with virus at an input multiplicity of 5 plaque-forming units per cell. After incubation for 45 min at 37°C, the cultures were washed, and Eagle medium (MEM, I) containing 2% inactivated calf serum was added.

Titration of virus infectivity. BSC1 cell monolayers in 60-mm plastic petri dishes (Nunc, Denmark) were infected with serial end-point dilutions of virus sample. After 45 min at 37°C, the monolayers were overlayed with Eagle medium containing 1% Special Agar Noble (Difco Laboratories, Detroit, Mich) and 5% inactivated calf serum. Neutral red (0.0025%) was added 4 days after infection and plaques were counted on the following day.

Polyacrylamide gel electrophoresis. Gel electrophoresis was performed essentially as described by Summers et al. (16). Gels (10 by 0.6 cm) were prepared using 7.5% acrylamide 0.2% N,N-methylene bisacrylamide in 0.1 M sodium phosphate (pH 7.1) and 0.1% sodium dodecyl sulphate. Prior to use, excess catalyst was removed from the gels by electrophoresis at 5 mA/gel for 1 h. Cell cytoplasm was dissociated with 2% sodium dodecyl sulfate and 1% mercaptoethanol for 1 min at 100°C. Sucrose was added to the solubilized sample to 10% and 200 μlitters were applied to each gel. Electrophoresis was continued at 3.5 mA/gel for 17 h. After electrophoresis, the gels were placed in 10% trichloroacetic acid, stained with 0.1% Coomassie blue in 10% trichloroacetic acid, and washed in 7.5% acetic acid. The gels were sliced longitudinally, dried, and placed in contact with X-ray film (2).

RESULTS

Nature of the inhibition of vaccinia virus by TCT. The conditions of the inhibition of
vaccinia virus growth by TCT and the step in virus replication which is affected by the drug were first studied:

**Effect of TCT on WT strain, IBT<sup>a</sup>, and IBT<sup>T</sup> mutants of vaccinia virus.** Since TCT is an IBT-related compound, we examined its effect on two mutants of vaccinia virus which were recently isolated (6, 9). One mutant was able to grow in the presence of IBT (IBT<sup>a</sup>) and the other needed IBT for its growth (IBT<sup>T</sup>). The results (Table 1) indicate that the IBT<sup>a</sup> mutant is also resistant to TCT and that the IBT<sup>T</sup> mutant can use TCT instead of IBT for its growth. The behavior of WT strain and IBT<sup>T</sup> mutant in the presence of increasing concentrations of TCT in the cultures indicated that WT strain is inhibited by 99% in the presence of 3.5 \( \mu \text{M} \) of TCT, and that 1.75 \( \mu \text{M} \) of TCT is enough to enable the maximal growth of the IBT<sup>T</sup> strain (Fig. 1). The inhibition of WT strain by TCT can be reversed after the removal of the drug. When the drug was removed by washing the treated cultures at 7 h after infection, virus growth occurred (Fig. 2).

Vaccinia DNA is synthesized in HeLa cells mainly between 2 and 5 h after infection and then starts to acquire deoxyribonuclease resistance (4, 12). Since vaccinia virus multiplies in the cytoplasm of the cell, it is possible to follow viral DNA synthesis, by determination of the incorporated thymidine, into trichloroacetic acid precipitable material after short pulses (4). Infected BSC1 cells were pulse labeled with 4 \( \mu \text{Ci} \) of radioactive thymidine per ml for 10 min at different times after infection. The cells were washed and the nonionic detergent Nonidet P-40 (0.5%) was added to the cells. Nuclei were removed by centrifugation at 1,000 \( \times g \) for 2 min (18). The quantity of radioactively labeled DNA in the cytoplasmic fraction was measured after trichloroacetic acid precipitation. DNA synthesis in the infected cells started between 1

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Virus titer (plaque-forming units per ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>WT</td>
<td>2.0 ( \times 10^7 )</td>
</tr>
<tr>
<td>IBT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.0 ( \times 10^7 )</td>
</tr>
<tr>
<td>IBT&lt;sup&gt;T&lt;/sup&gt;</td>
<td>&lt;10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Vaccinia virus strains, WT, IBT<sup>a</sup>, and IBT<sup>T</sup> were titrated on BSC1 monolayers as described. IBT (14 \( \mu \text{M} \)) or TCT (14 \( \mu \text{M} \)) were supplemented in the agar overlay.

**Table 1. Growth of WT strain of vaccinia virus, IBT<sup>a</sup>, and IBT<sup>T</sup> mutants in the presence of IBT and TCT**

![Fig. 1. The effect of different concentrations of TCT on the growth of WT strain and IBT<sup>T</sup> mutant in BSC1 cells. Virus growth is plotted as a ratio of the yield at 22 h to the virus present after washing the cultures at the end of the 45-min adsorption period. Symbols: ●, WT; ○, IBT<sup>T</sup>.

![Fig. 2. Growth curves of WT strain in BSC1 cells without (○) or with (●) TCT (14 \( \mu \text{M} \)). TCT was removed from one set of cultures by washing the cells with saline at 7 h post infection (△).](http://aac.asm.org/Downloaded from http://aac.asm.org/)
and 2 h after infection, reached the highest level at 3 h, and then declined (Fig. 3). In TCT-treated cells, the rate of synthesis of the viral DNA was similar to that of untreated, infected cells (Fig. 3).

We then studied the fate of newly synthesized vaccinia viral DNA made in the presence of TCT, using deoxyribonuclease digestion, after sucrose gradient centrifugation. In the presence of TCT, no virus band was detected in WT-infected cells; most of the radioactively labeled DNA was still deoxyribonuclease-susceptible and located at the top of the gradient (Fig. 4). However, in IBT\textsuperscript{R}-infected cells treated with TCT, \textsuperscript{3}H\textsuperscript{[}H\textsuperscript{]}thymidine appeared in a deoxyribonuclease-resistant band, coincident with infectious virus, in the middle of the sucrose gradient (Fig. 4).

**Synthesis of viral proteins.** The failure of vaccinia viral DNA to become resistant to deoxyribonuclease in the presence of TCT might result from the absence of structural proteins. Host protein synthesis is progressively inhibited after vaccinia virus infection, thus permitting the specific labeling of viral polypeptides (3, 10, 11, 13, 14). The viral proteins are divided into two classes: (i) “early” proteins, which are synthesized at the early period after infection, and which are produced even when the replication of the viral genome is inhibited; and (ii) “late” proteins, which are formed only after the synthesis of viral DNA. BSC1 cells were labeled with \textsuperscript{3}S\textsuperscript{[}S\textsuperscript{]}methionine between 1 and 2 h after infection with WT and IBT\textsuperscript{R} mutant of vaccinia virus. The proteins in the cytoplasm were solubilized with sodium dodecyl sulfate and analyzed by polyacrylamide gel electrophoresis. The normal gradual transition

---

**Fig. 3.** Effect of TCT on viral DNA synthesis. Symbols: \(\bullet\), infected cells in the presence of TCT (14 \(\mu\)M); \(\bigcirc\), infected cells in the absence of TCT; \(\Delta\), uninfected cells in the presence of TCT (14 \(\mu\)M); \(\triangle\), uninfected cells in the absence of TCT.

**Fig. 4.** Fate of virus DNA in the presence of TCT. BSC1 cells treated with TCT (14 \(\mu\)M) were incubated with \textsuperscript{3}H\textsuperscript{[}H\textsuperscript{]}thymidine (8 \(\mu\)Ci/ml) between 1.5 and 3 h after infection with WT or IBT\textsuperscript{R}. The cells were then washed and medium containing unlabeled thymidine (10\(\times\) \(\mu\)M) was added. At 21 h after infection, the cells were washed, resuspended in 1 ml of 1 mM sodium phosphate (pH 7.0), disrupted with a Dounce homogenizer, and layered on a 25 to 40% (wt/vol) sucrose gradient. After centrifuging in an SW50.1 rotor at 13,000 rpm for 35 min, fractions (0.3 ml) were collected from the bottom of the tube. One portion of each fraction was directly precipitated with trichloroacetic acid and another was first adjusted to 10 mM MgCl\textsubscript{2} and incubated with deoxyribonuclease (50 \(\mu\)g/ml; Worthington) for 30 min at 37 C. The fate of DNA in infected cells treated with TCT (14 \(\mu\)M) was similarly determined. Symbols: \(\bigtriangleup\), WT total; \(\bigcirc\), WT, deoxyribonuclease resistant; \(\blacklozenge\), IBT\textsuperscript{R}, deoxyribonuclease resistant.
from synthesis of host proteins to specific early viral proteins took place also in the presence of TCT and IBT. The effect of TCT and IBT on the formation of late proteins was studied by the addition of [35S]methionine to the cultures between 4 and 7 h after infection and the analysis of the labeled polypeptides in polyacrylamide gels. Similar amounts of labeled methionine were incorporated into the cells. Late viral proteins were produced by WT and IBT strains of vaccinia virus, also in the presence of IBT and TCT (Fig. 5).

A major vaccinia virus structural polypeptide was previously shown by pulse chase experiments to form from a higher-molecular-weight precursor; this process appears to be a late step associated with virus maturation (7, 8). We followed the formation of this structural polypeptide in the presence of TCT and IBT. At 7 h after infection, infected BSC1 cells, treated with TCT and IBT, were labeled with [35S]-methionine for 30 min. The polypeptides in the cytoplasm of the cells, at the end of the pulse period and after 4 h of chase, were examined by polyacrylamide gel electrophoresis. The precursor polypeptide (P4a) was formed in the presence of TCT and IBT (Fig. 6, pulse). However, the cleavage, which produces the structural polypeptide 4a, occurred very efficiently in vaccinia-infected BSC1 cultures (control) but was partially inhibited in IBT-treated, infected cultures and even more in TCT-infected cells.

**TCT-resistant mutant.** A TCT-resistant mutant was isolated in the presence of iodode-
oxyuridine and TCT. The neutralization of the mutant by antivaccinia immunoglobulin and its resistance to other thiosemicarbazone-containing compounds were studied:

**Induction and isolation of a TCT-resistant mutant (TCT<sup>8</sup>).** A vaccinia mutant (TCT<sup>4</sup>) which is resistant to TCT was isolated from WT-infected cultures treated with the mutagenic agent iododeoxyuridine in the presence of TCT. Chicken fibroblast monolayers were infected with WT strain of vaccinia virus at an input multiplicity of 1 plaque-forming unit per cell. After 45 min at 37 C, the culture was washed and Eagle medium containing 2% calf serum, iododeoxyuridine (5 μg/ml), and TCT (14 μM) was added. The culture was harvested 2 days later, and the virus was used for infection of HeLa and BSC1 cultures in the presence of TCT. Virus from one of these cultures formed plaques in agar-overlaid cultures in the presence of TCT. The TCT<sup>8</sup> mutant was plaque purified and virus stock was prepared in HeLa cell monolayers in the absence of the drug.

**Neutralization of WT, IBT<sup>8</sup>, and TCT<sup>8</sup> strains of vaccinia virus by immunoglobulin.** The degree of neutralization of WT, IBT<sup>8</sup>, and TCT<sup>8</sup> strains of vaccinia virus by antivaccinia human immunoglobulin was compared. Dilutions of the virus suspensions were incubated at 37 C for 30 min with a constant amount of antivaccinia human immunoglobulin. Although all three virus strains were neutralized by immunoglobulin, there were minor differences in the degree of neutralization (Table 2).

**Resistance of TCT<sup>8</sup> virus to several thiosemicarbazone-containing compounds.** TCT<sup>8</sup> virus was examined for its resistance to several thiosemicarbazone-containing compounds. The three drugs tested were IBT, 4-formylacetanilide thiosemicarbazone, and 5-cyanothiophene-2-carboxaldehyde thiosemicarbazone. Similarly to IBT<sup>8</sup>, TCT<sup>8</sup> showed resistance toward these three compounds which inhibit the growth of the WT strain (Table 3).

**DISCUSSION**

The two antipoxvirus drugs IBT and TCT contain one common component (thiosemicarbazone) in their molecule. The thiosemicarbazone structure by itself lacks any antiviral activity. During the present study we compared the two mutants, IBT<sup>8</sup> and TCT<sup>8</sup>. The mutant
TABLE 2. Neutralization of vaccinia virus (WT strain), IBT*, and TCT* mutants by antivaccinia immunoglobulin

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Virus titer (plaque-forming units per ml)</th>
<th>Neutralization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control + Immunoglobulin</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>8.0 \times 10^7</td>
<td>1.8 \times 10^6</td>
</tr>
<tr>
<td>IBT*</td>
<td>2.1 \times 10^8</td>
<td>1.2 \times 10^6</td>
</tr>
<tr>
<td>TCT*</td>
<td>1.1 \times 10^7</td>
<td>1.9 \times 10^6</td>
</tr>
</tbody>
</table>

*Dilutions (0.5 ml) of the three vaccinia virus strains WT, IBT*, and TCT* were incubated with 0.5 ml (8.5 mg) of antivaccinia human immunoglobulin for 30 min at 37°C. Virus dilutions without immunoglobulin were also incubated. The virus was then titrated on BSC1 monolayers.

IBT* was induced and selected in the presence of IBT, whereas the mutant TCT* was isolated independently in the presence of TCT. Each of these mutants was found to be resistant to both IBT and TCT. We wished to find out whether the phenomenon of cross-resistance of the mutants reflects similarity of the mechanisms by which these two drugs inhibit the growth of the WT strain of vaccinia virus.

It was found that the stage in virus growth which is affected by TCT is later than DNA synthesis. Both early and late viral polypeptides are produced but the viral DNA remains susceptible to deoxyribonuclease, unless the drug is removed. Similar findings were obtained with vaccinia-infected cells treated with IBT. In the presence of IBT, partial cleavage of the polypeptide precursor P4a to the core polypeptide 4a is detected; but in infected cells treated with TCT this process is almost completely inhibited. This might result from a more efficient inhibition by TCT in BSC1 cells than by IBT. It is possible to conclude that there is a good correlation between the cross-resistance of the two mutants IBT* and TCT* and the similar antipox activity of IBT and TCT.

ACKNOWLEDGMENTS

We would like to thank N. Goldblum for his advice and encouragement.

This work was supported by a grant from the Ministry of Health of the State of Israel.

LITERATURE CITED