Inhibition of Vesicular Stomatitis Virus by Kethoxal Bis (Thiosemicarbazone)

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SUMMARY

Kethoxal bis (thiosemicarbazone) (KTS) inhibited replication of, and plaque formation by, vesicular stomatitis virus (VSV) in chick embryo cells. No other thiosemicarbazones tested were effective. Virus-specific m-RNA and protein synthesis were inhibited by KTS. However, virion RNA-dependent RNA synthesis was not inhibited by the drug. Treatment of VSV virions directly with KTS produced enhancement, rather than inactivation, of plaque formation.

KTS inhibited cellular DNA and RNA synthesis by 67 and 25% respectively. Since cellular DNA and RNA synthesis are not required for VSV replication, the inhibition of these processes is probably unrelated to the antiviral activity of KTS. Cellular protein synthesis was inhibited 24% by KTS. Unexpectedly, synthesis of four proteins was induced in KTS-treated uninfected cells.

INTRODUCTION

The antiviral activity of thiosemicarbazones is well documented in several systems (Levinson, 1973). The N-methyl isatin derivative (M-IBT) is well known for its ability to inhibit vaccinia virus (Bauer & Sadler, 1960) and for its clinical use in the treatment of smallpox vaccination complications (Turner, Bauer & Nimmo-Smith, 1962). M-IBT or analogues of it have been shown to inhibit several DNA or RNA viruses (Pearson & Zimmerman, 1969; Bauer, Apostolov & Selway, 1970). This compound also inactivates, on contact, the ability of Rous sarcoma virus (Levinson, Woodson & Jackson, 1971) and other RNA tumour viruses (Levinson et al. 1973a; Levy, Levy & Levinson, 1976) to transform cells malignantly. This inactivation is due to inhibition of the RNA-dependent DNA polymerase in the virion (Levinson et al. 1973b). The genome RNA may be the site of action of this compound since we have shown that M-IBT copper complexes bind to RNA and DNA (Mikelens, Woodson & Levinson, 1976). Other viruses, such as herpes simplex types I and II (Levinson et al. 1974), RNA slow viruses (Haase & Levinson, 1973), arenaviruses (Logan et al. 1975) and lambda phage (Levinson & Helling, 1976) are also inactivated.

Another thiosemicarbazone, the kethoxal bis derivative (KTS), has anti-tumour activity in both mice (Booth & Sartorelli, 1967) and rats (Crim & Petering, 1967; Van Giessen & Petering, 1968), but no antiviral action has been reported. In this paper, we show that KTS inhibits the replication of vesicular stomatitis virus (VSV). In addition to its antiviral effect, we found that KTS enhances the synthesis of four proteins in uninfected cells.

The structure of KTS is shown in Fig. 1.
METHODS

Virus and cells. Stocks of VSV (Indiana) grown on primary cultures of chick embryo cells (titre 5 × 10^7 to 2 × 10^8 p.f.u./ml) were used. Primary and secondary cultures of chick embryo cells were prepared as previously described (Levinson, 1967). Vero cells were propagated in the same manner as the chick embryo cells.

Chemicals. The drugs were obtained from the following sources: KTS and Kethoxal from Nutritional Biochemicals, Cleveland, Ohio. M-IBT from K and K Rare Chemicals, Plainview, New York and thiosemicarbazide from Eastman Kodak, Rochester, New York. 2-Formyl pyridine thiosemicarbazide was a gift from Dr Fred French (Mount Zion Research, Palo Alto, Calif.). Dimethyl sulphoxide (spectral quality), obtained from Matheson, Coleman and Bell, Los Angeles, Calif., was used as the solvent for the 2 mg/ml stock solutions.

Plaque assay. Confluent monolayers of chick embryo cells, which were grown in medium 199 (Grand Island Biological) with 4% calf serum and 4% of 0.2 M-sodium bicarbonate, were infected with 0.2 ml of an appropriate dilution of virus. The virus was allowed to adsorb for 45 min at 38 °C and an agar overlay consisting of 46% of 1.8% agar (Difco Bacto), 46% 2 × medium 199, 4% calf serum and 4% of 0.2 M-sodium bicarbonate was added. The cultures were incubated at 38 °C in a humidified, 5% CO₂ atmosphere for 48 h. One ml of a 1:10000 solution of neutral red was added and the plaques counted several hours later.

Analysis of virus m-RNA and protein. At an appropriate time after exposure of the cells to the drug, ³H-uridine (sp. act. 23 Ci/mmol) at a final concentration of 50 μc/ml or ³⁵S-methionine (sp. act. 275 Ci/mmol) at a final concentration of 2 μc/ml was added. The cultures were incubated for 1 h at 38 °C. The medium was discarded, the cultures washed once with medium 199, and the cell monolayer was scraped into 2 ml medium 199. The dish was washed once with 2 ml medium 199, and the wash pooled with the previously obtained cells. The cells were centrifuged at 1500 rev/min for 10 min at 4 °C in a Sorvall RC-3 centrifuge and the supernatant fluid completely removed. The tube was allowed to drain and wiped dry. Fifty μl of solubilizing buffer containing 0.07 M-tris, pH 6-8, 10% glycerol, 2% sodium dodecyl sulphate (SDS) and 5% 2-mercaptoethanol was added. For the RNA extraction, the solubilizing buffer contained 5% phenol to inhibit ribonuclease. The sample was boiled for 2 min and stored at −70 °C. RNA and protein samples were analysed by electrophoresis on polyacrylamide slab gels (14 × 15 × 0.15 cm³ with 1 cm wells) using an apparatus described by Studier (1973). Gels were run with constant current of 30 mA for approx. 4 h, then processed for fluorography (Bonner & Laskey, 1974). Proteins were separated on 12% gels (Crosslinker = 1/20) in a discontinuous buffer system (Laemmli, 1970). RNAs were separated on 2.5% gels (Crosslinker 1/7) in buffer containing 0.1% SDS, 36 mm-tris, 30 mm-NaH₂PO₄, 1 mm-EDTA, pH 7.7 (Loening, 1969). To allow handling of the RNA gels after the run, they were transferred directly from the glass plates on to Whatman filter paper.

Cellular DNA, RNA and protein synthesis. Chick cells were seeded at 1 × 10⁶ cells per...
Table 1. Inhibition of VSV plaque formation by KTS

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molarity</th>
<th>Plaque formation</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>46</td>
<td>1.0</td>
</tr>
<tr>
<td>KTS</td>
<td>7 × 10^-8</td>
<td>2</td>
<td>0.94</td>
</tr>
<tr>
<td>KTS</td>
<td>3.5 × 10^-8</td>
<td>42</td>
<td>0.07</td>
</tr>
<tr>
<td>KTS (Vero cells)</td>
<td>7 × 10^-8</td>
<td>3</td>
<td>0.91</td>
</tr>
<tr>
<td>M-IBT</td>
<td>4 × 10^-6</td>
<td>40</td>
<td>0.87</td>
</tr>
<tr>
<td>2-PY-TSC</td>
<td>7 × 10^-7</td>
<td>48</td>
<td>1.0</td>
</tr>
<tr>
<td>TSC</td>
<td>4 × 10^-7</td>
<td>45</td>
<td>0.98</td>
</tr>
<tr>
<td>Kethoxal</td>
<td>6 × 10^-7</td>
<td>44</td>
<td>0.96</td>
</tr>
<tr>
<td>DMSO (0.1%)</td>
<td>—</td>
<td>40</td>
<td>0.87</td>
</tr>
</tbody>
</table>

* Five µl of an appropriate concentration of drug stock solution was added to the 5 ml agar overlay of the plaque assay described in the Methods section. The assays used duplicate chick embryo cells except for the Vero cell experiments.

RESULTS

Inhibition of plaque formation by KTS

The data in Table 1 demonstrate that KTS at a concentration of 7 × 10^-8 M (20 ng/ml) inhibits plaque formation by VSV. This activity is specific since neither M-IBT nor 2-formyl pyridine thiosemicarbazone (PY-TSC), which has significant antitumour activity (Booth, Moore & Sartorelli, 1970), inhibit even at higher concentrations. The side chain, thiosemicarbazide (TSC), and the kethoxal component are not active. The solvent, dimethyl sulphoxide (DMSO), is inactive. KTS also inhibited VSV plaque formation in monkey (Vero) cells.

The dose–response of plaque formation to the drug is quite striking. At 7 × 10^-8 M, 96 % inhibition is seen, at one half that concentration, no inhibition is seen (Table 1). At higher concentration, visible cell toxicity is not observed after exposure to 28 × 10^-8 M but is seen when 56 × 10^-8 M is applied. The effect of KTS on the host cell is examined in detail below:

Inhibition of replication by KTS

The data in Fig. 2 shows that exposure of VSV infected cells to 28 × 10^-8 M-KTS (80 ng/ml) causes a 1 h increase in the latent period and a greater than 90 % inhibition in the amount of virus produced. The drug must be added directly after the absorption period in order to be maximally effective; if added 1 h later only 50 % inhibition is obtained (data not shown). If KTS is removed after 1 h of exposure, no inhibition occurs. If the drug is removed after 2 h, 66 % inhibition of virus production occurs.
The data shown in Fig. 3 demonstrate that 2 h after exposure to KTS the synthesis of three VSV m-RNA classes (13 to 19S) within the infected cell is completely inhibited. No RNA can be seen in the actinomycin treated uninfected cells. Chick cell ribosomal RNA is included as a marker.

**Inhibition of virion RNA-dependent RNA polymerase**

In view of the inhibition of virus m-RNA, it was of interest to determine whether this was due to inhibition of transcription by the virion polymerase or to subsequent transcription. It was found that the virion polymerase activity is not inhibited by as high a concentration as \( 1 \times 10^{-4} \text{ M-KTS} \).

**Inhibition of VSV protein synthesis by KTS**

The data depicted in Fig. 4 demonstrate that 2 h after exposure to KTS the synthesis of the L, G, N, and M proteins of VSV is inhibited. The effect of KTS on NS protein is unclear since it migrates so close to actin. The synthesis of the cellular protein actin is somewhat lower in the presence of KTS but the virus proteins are inhibited to a much greater extent.

We were surprised to find four new or induced proteins in the uninfected cells treated with KTS (arrows on left side of Fig. 4). The three lower mol. wt. proteins do not appear to be synthesized in the untreated cell whereas the highest mol. wt. species is produced in untreated cells in small amounts. These new proteins are unlikely to play a role in the antiviral activity of KTS since actinomycin treatment of VSV infected cells prevents the synthesis of these proteins but has no effect on the inhibitory action of KTS.
Inhibition of VSV by KTS

Fig. 3. Inhibition of VSV m-RNA synthesis. Confluent monolayer of chick embryo cells were infected with VSV (m.o.i. = 5), adsorbed for 45 min at 38 °C, then washed twice with growth medium. Growth medium (2.5 ml) containing actinomycin (4 µg/ml) was replaced and KTS (80 ng/ml) was added. After 1 or 2 h at 38 °C, ³H-uridine (50 µc/ml) was added, and the cultures incubated for an additional hour. The RNA was extracted and analysed by polyacrylamide slab gel electrophoresis as described in the Methods section. (a) Uninfected, no KTS; (b) uninfected, with KTS; (c) VSV infected, 1 h, no KTS; (d) VSV infected, 1 h, with KTS; (e) VSV infected, 2 h, no KTS; (f) VSV infected 2 h, with KTS; (g) empty; (h) ribosomal RNA with arrows designating 28S and 18S species.

Effect of KTS on chick cells

The effect of KTS on cell growth and the synthesis of DNA, RNA and protein in chick cells was determined. It was found that KTS (7 x 10⁻⁸ M) inhibits cell growth by 25 % when exposed for 24 h and by 66 % when exposed for 48 h. KTS (28 x 10⁻⁸ M) has approximately the same effect. However, the visual appearance of the treated cells cannot be distinguished from the untreated controls.

Since significant inhibition of VSV replication by 28 x 10⁻⁸ M was observed at 3 h after infection, the effect of this dose on synthesis in uninfected cells was studied. It was found that cellular DNA synthesis was inhibited 67 %, RNA synthesis was inhibited 25 % and protein synthesis was inhibited 24 %. We performed control experiments with other inhibitors of DNA and RNA synthesis to determine whether the inhibition of cellular DNA and RNA synthesis contributed to the inhibition of VSV replication. The exposure of VSV
infected cells to $2 \times 10^{-6}$ M-cytosine arabinoside, which inhibited DNA synthesis by 92% at 3 h after exposure, had no effect on plaque formation. This confirms previous results that DNA synthesis is not required for VSV replication (Follett et al. 1974). The previous report that cytosine arabinoside inhibits VSV synthesis used 50 $\mu$g/ml (Campbell et al. 1968) compared to 0.5 $\mu$g/ml used by us. Similarly, the exposure of VSV infected cells to 0.8 $\mu$g/ml actinomycin for 3 h, which inhibited RNA synthesis by 99%, had no effect on the amount of VSV produced in a growth curve experiment. This confirms previous results (Shincariol & Howatson, 1970). It appears that the inhibition of cellular DNA and RNA synthesis caused by KTS did not contribute to the inhibition of VSV replication. It seems unlikely
that the inhibition of protein synthesis observed could account for the profound inhibition of VSV production.

**Effect of actinomycin on the inhibition of VSV by KTS**

It is possible that the inhibitory effect of KTS requires the synthesis of a cellular protein, for example, interferon. To test this possibility we determined the effect of actinomycin on the inhibition of VSV by KTS. It was found that the presence of \(0.8 \mu g/ml\) actinomycin, which inhibits cellular RNA synthesis by 99\% at 3 h, had no effect on the inhibition of VSV replication by KTS.

**Failure of KTS to inactivate VSV**

The ability of KTS to inactivate the infectivity of VSV, on contact, by direct exposure of the virion to the drug prior to infection was tested. It was found that \(3.5 \times 10^{-8} \text{M-KTS}\) and \(7 \times 10^{-6} \text{M-KTS}\) enhance plaque formation eight and threefold respectively. Since a fourfold enhancement was also achieved by the chelating agent, ethylene diamine tetraacetic acid (\(7 \times 10^{-6} \text{M: EDTA}\)), we speculate that this enhancement may be due to the removal of a toxic heavy metal cation.

**DISCUSSION**

In this report, we have shown that KTS, but not two other thiosemicarbazones, inhibits VSV. The reason for the specificity of the response is unknown. It is not the only example of specificity, however, since \(N\)-methyl isatin \(N\)'-\(N\)-butyl thiosemicarbazone, but not the unbutylated compound, inhibits poliovirus replication apparently by interfering with RNA synthesis (Pearson & Zimmerman, 1969). We could speculate that lipid solubility may be a factor but there is no evidence to support this hypothesis.

We have shown that KTS inhibits virus m-RNA synthesis within the infected cell, although virion polymerase is not inhibited. The question which remains unanswered is whether the inhibition of virus m-RNA synthesis is due to an effect of KTS on subsequent m-RNA transcription or on translation, which has been shown to be required for late–early VSV m-RNA synthesis (Repik, Flamand & Bishop, 1974; Flamand & Bishop, 1974).

The induction of four proteins in KTS-treated, uninfected cells is of interest. Since actinomycin inhibits their induction, DNA-dependent RNA synthesis is required which implies that they are host cell in origin. However, it is possible that they may be the proteins of an integrated, endogenous virus. Since actinomycin does not block the anti-VSV effect of KTS, it appears that these induced proteins are not required for its antiviral action.

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