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Inactivation of Herpes Simplex Virus by Thiosemicarbazones and Certain Cations

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We demonstrated that herpes simplex virus types 1 and 2, including a type 2 strain which transforms hamster cells in vitro, and *Herpesvirus saimiri* are inactivated by exposure to thiosemicarbazones. Because thiosemicarbazones are thought to interact with heavy metals in this inactivation process (9), we tested and found some of these herpesviruses to be susceptible to exposure to certain heavy metals. A virion polymerase was sought because the ribonucleic acid (RNA)-dependent deoxyribonucleic acid (DNA) polymerase of Rous sarcoma virus and the DNA-dependent RNA polymerase of vaccinia virus are inhibited. However, neither DNA nor RNA polymerase activity could be demonstrated in herpes simplex virions. The ability of thiosemicarbazone to ameliorate the course of herpes simplex virus infection in rabbit eyes was observed, but was considered insufficient to be of clinical importance.

N-methyl isatin β-thiosemicarbazone (Me-IBT) inactivates, upon contact, the ribonucleic acid (RNA)-dependent deoxyribonucleic acid (DNA) polymerase and transforming ability of Rous sarcoma virus (8, 9). Several other RNA tumor viruses, such as Rous-associated virus I, mouse sarcoma virus, mouse leukemia virus, mouse mammary tumor virus, and feline sarcoma virus, are susceptible to this drug as assayed either by infectivity, transformation, or polymerase activity (W. Levinson et al., Proc. ICN-UCLA Symp. Virus Res., in press). In addition, the cytopathic effect and RNA-dependent DNA polymerase activity of Visna virus are inactivated after exposure of the virion to Me-IBT (7). Not all viruses are susceptible; however, vaccinia virus, Newcastle disease virus, reovirus, vesicular stomatitis virus, poliovirus, and poliovirus infectious RNA are not affected despite the fact that some of these possess a virion transcriptase (9). This lack of susceptibility may be a function of the ability of the drug to penetrate the virion, for we have demonstrated that the DNA-dependent RNA polymerase activity of the vaccinia virus core is markedly inhibited by exposure to 40 μM Me-IBT (W. Levinson et al., Proc. ICN-UCLA Symp. Virus Res., in press). No effect of Me-IBT on herpes simplex virus (HSV) in rabbit eyes was observed.

MATERIALS AND METHODS

Viruses and cells. HSV type 1 Ph strain (4) and two strains of HSV type 2, the MS strain (4) and a strain (HSV-2-333) which transforms hamster cells in vitro (5) (kindly supplied by Fred Rapp), were used. The cytopathic effect of these viruses was determined on Vero cells. Titers of the stocks were 2×10^7, 2×10^6, and 2.5×10^5 plaque-forming units (PFU)/ml, respectively.

Exposure of virus to the drug. In most experiments, a 1:200 dilution of the virus stock in 0.01 M phosphate-buffered saline (PBS), pH 7.4, was made, and 1 ml of the virus suspension was exposed to the 40 μM drug. Stocks of the organic compounds and of the cations were prepared in 100% dimethylsulfoxide (DMSO) or water, respectively, at a concentration of 40 mM. Control viral suspensions were exposed to an appropriate concentration of DMSO or water. After incubation at 37 C for varying times, a 1:100 dilution into medium 199 plus 5% calf serum was made, and a 0.2-ml sample was assayed for surviving infectivity on Vero cells.

Chemicals and solutions. The drugs were obtained from the following sources: Me-IBT and methyl isatin were from K and K Rare Chemicals; unsubstituted IBT and ethyl-IBT were from Nutritional Biochemicals; thiosemicarbazone (TSC) and semicarbazide were from Eastman Kodak; and DMSO was from Matheson, Coleman and Bell. Reagent grade inorganic compounds from either Baker or from Matheson, Coleman and Bell were used. All tissue culture media and calf serum were obtained

from Grand Island Biological Co. Polyethylene glycol (Carbowax 4000) was obtained from Union Carbide.

RESULTS

Inactivation by certain TSCs and their analogues. In the initial series of experiments, HSV type 1 was exposed to various compounds at a 40 μM concentration as described previously and assayed for infectivity remaining after the treatment. Me-IBT and ethyl-IBT are the most effective inactivators of plaque formation (Table 1). The activity resides in the TSC portion of the molecule; methyl isatin is ineffective. The thiol group which is required for semicarbazide did not inhibit infectivity. Both Me-IBT and TSC inactivate greater than 90% at 4 μM and retain the capacity to inactivate 20 to 30% at 0.4 μM. Exposure of the virus to either 0.1% DMSO or to as high a concentration as 1% DMSO caused no inactivation of the infectivity. Two strains of HSV type 2 were also exposed to some of these compounds at a concentration of 40 μm. One of the type 2 strains was HSV-2-333 which transforms hamster cells in vitro (5). The results were qualitatively and quantitatively similar to those presented in Table 1. *Herpesvirus saimiri* treated with 40 μM Me-IBT under similar conditions was inactivated 81%.

In order to determine whether this effect was due to the inactivation of the virion itself or to intracellular inhibition of viral replication, the cells were infected with untreated HSV and exposed to either 0.4 μM Me-IBT or to 0.4 μM TSC in the methocel overlay. This represents the concentration of drug present in the overlay used in the experiment described in Table 1 as a result of a 1:100 dilution of a 40 μM solution of drug. No inhibition of plaque formation was seen, which indicates that the loss of infectivity is not the result of inhibition of intracellular viral replication.

TABLE 1. *Effect of isatin β-thiosemicarbazones and their analogues on plaque formation by herpes simplex virus*[a]

Compounds	Inhibition (%)
None	—
DMSO (0.1%)	0
Me-IBT (40 μM)	99
Ethyl-IBT	99
Unsubstituted IBT	75
Thiosemicarbazide	95
Semicarbazide	0
Methyl-isatin	0

[a] One μliter of a 40 mM solution of the compounds was added to 1 ml of a 1:200 dilution of herpes simplex virus type 1 in phosphate-buffered saline. After incubation at 37 C for 15 min, a 1:100 dilution of the virus in medium 199 plus 5% calf serum was made, and a 0.2-ml sample was assayed for plaque-forming ability.

Brockman et al. have reported that herpes simplex cytopathic effect was inhibited by exposure of the infected cells to pyridine, isoquinoline, and purine (but not isatin or *N*-methyl isatin) derivatives of TSC (3). In order to determine whether these derivatives also inactivate extracellular virus, we exposed HSV to 40 μM concentrations of five of the compounds which Brockman et al. found to be effective and to two compounds, 3-formyl pyridine TSC and 4-formyl pyridine TSC, which was ineffective. The pyridine and purine derivatives of TSC do not inactivate the infectivity of HSV (Table 2), but isoquinoline TSC has a marked effect.

Protection of HSV by components of tissue culture medium from inactivation by Me-IBT. In contrast to the results in Table 1, when HSV was exposed to Me-IBT in tissue culture medium little, if any, inactivation was observed (9). Suspension of the virus in PBS was required in order to demonstrate significant susceptibility of the virus to the drug. We explored the basis for this protection by exposing the virus to 40 μM Me-IBT in the presence of various components of the medium. The results of these experiments, described in Table 3, indicate that minimal Eagle medium (MEM) offers significant protection and that most of that protection resides in the essential amino acids portion of the MEM. Scherer maintenance medium also provides protection. There are three amino acids in common in these two solutions, namely tryptophan, cysteine, and histidine, which were tested at the concentration at which they are present in Scherer medium (0.02 mg/ml) and at 10 times that concentration. It can be seen that

TABLE 2. *Effect of several thiosemicarbazide derivatives on plaque formation by herpes simplex virus*[a]

Compounds	Inhibition (%)
DMSO	—
1-Formyl isoquinoline TSC	97
5OH-1-formyl isoquinoline TSC	35
2-formyl pyridine TSC	14
5-OH-2-formyl pyridine TSC	14
3-formyl pyridine TSC	5
4-formyl pyridine TSC	15
6-formyl purine TSC	15

[a] The experimental procedure was the same as that described in Table 1.

TABLE 3. *Effect of culture medium components on inactivation of herpes simplex virus by N-methyl isatin β-thiosemicarbazone*[a]

Media or components	Inhibition (%)
None	99
Medium 199	40
MEM	22
MEM essential amino acid	54
MEM nonessential amino acid	96
MEM vitamins	98
Scherer	23
Tryptophan (0.2 mg/ml)	86
Tryptophan (0.02 mg/ml)	99
Cysteine (0.2 mg/ml)	99
Histidine (0.2 mg/ml)	40
Histidine (0.02 mg/ml)	81
Imidazole (0.2 mg/ml)	97

[a] A 1-ml amount of a 1:10 dilution of herpes simplex virus type 1 in phosphate-buffered saline was exposed to 40 μM Me-IBT plus 1 × final concentrations of the above media or components as indicated. After incubation at 37 C for 30 min, a 1:100 dilution of the virus in medium 199 plus 5% calf serum was made, and a 0.2-ml portion was assayed for plaque-forming ability.

histidine possesses significant protective ability, but not sufficient to account for the protection offered by Scherer medium. Tryptophan has a slight protective effect at the higher concentration, and cysteine has none at all. Imidazole at 0.2 mg/ml gives no protection.

Inactivation by certain cations. TSCs are known to form strong complexes with metals of the first transition series (14). We have shown that copper can act synergistically with Me-IBT to inactivate RSV and that copper and other cations can inactivate the RNA-dependent DNA polymerase activity and the transforming ability of RSV (8). In view of these findings, we tested the effect of several cations on the infectivity of HSV type 1. The data in Table 4 indicate that 40 μM copper, silver, rhodium, platinum, and mercury ions inactivate HSV infectivity 99% or more, whereas the cations in group B have a 50% or less inactivating ability. Approximately 50% inactivation was obtained by using 4 μM copper, 4 μM mercury, and 0.4 μM silver ions. Variable inactivation was obtained with iron ($FeCl_3$) and indium ($InCl_3$). The addition of 0.02 M tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride, pH 7.4, a frequently used buffer and known chelating agent (1), completely prevents the inactivation of HSV by copper (data not shown).

Failure to demonstrate virion polymerase in HSV. Because Me-IBT inhibits the RNA-dependent DNA polymerase of RSV and inactivates the biological activity of that virus and because herpesvirus infectivity is inactivated by Me-IBT, we postulated that a virion polymerase may be present in the herpes virion and may be the site of action of the drug. We attempted to demonstrate the presence of this enzyme by using herpes simplex virus type 1 purified by isopycnic centrifugation in either CsCl or sucrose, in a reaction mixture composed of 0.1 M Tris buffer, pH 8.0, either Mg^{2+} or Mn^{2+}, the appropriate unlabeled ribo- or deoxyribonucleotide triphosphates, 0.1 or 1% β-mercaptoethanol, and the detergent Nonidet P-40 in concentrations ranging from 0 to 1%. Many combinations of these components, with and without calf thymus DNA as a primer, were tested for their ability to support the incorporation of either ^{3}H-labeled uridine triphosphate or ^{3}H-labeled thymidine triphosphate into acid-precipitable material. No significant incorporation was observed under any of these conditions, indicating an absence of a virion DNA or RNA polymerase. The locus of Me-IBT action on herpesvirus remains unknown.

Treatment of herpes keratitis in rabbits by Me-IBT or TSC. In view of the marked inactivation of the herpes simplex type 1 infectivity by Me-IBT and TSC, we tested the ability of these drugs to ameliorate the course of herpes keratitis in rabbits. The infection was initiated by instilling 2 drops of a herpes simplex (pH strain) suspension (titer: 2×10^7 PFU/ml) in

TABLE 4. *Effect of several cations on plaque formation by herpes simplex virus*[a]

Cations	Inhibition (%)
Group A	
$CuSO_4$	99
$AgNO_3$	99
$HgCl_2$	98
$RhCl_3$	99
$PtCl_4$	99
Group B	
$ZnCl_2$	43
$PbCl_2$	50
$BaCl_2$	35
LiCl	50
$MnCl_2$	28
$Co(NO_3)_2$	21
$CdSO_4$	7
$NiCl_2$	17

[a] A 1-ml amount of a 1:200 dilution of herpes simplex virus type 1 in phosphate-buffered saline was exposed to 40 μM concentrations of the above cations. After incubation at 37 C for 15 min, a 1:100 dilution of the virus in medium 199 plus 5% calf serum was made, and a 0.2-ml portion was assayed for plaque-forming ability.

both eyes of 15 New Zealand White rabbits. Seventy-two hours later, when corneal lesions were visible by slit lamp examination, treatment of one eye of each rabbit with either 2% (wt/vol) Me-IBT in petrolatum ophthalmic ointment or 2% (wt/vol) TSC in 50% polyethylene glycol (two drops every 2 h for 16 h) was begun and continued for 5 days. The control eye of each animal was treated with the suspending vehicle alone. In these experiments treatment was begun when lesions appeared because that is clinically the important time. Our experiments were designed to determine whether the drug could inactivate the virus during its extracellular passage from cell to cell, thereby causing more rapid resolution of the lesions. Different vehicles were used for the two drugs because Me-IBT is not soluble in 50% polyethylene glycol, but TSC is. Because polyethylene glycol was thought to be the superior vehicle, it was used when feasible. The course of the disease was followed by daily slit lamp examinations graded on a "nonblind" basis and by HSV infectivity assays three times a week (4). Although a statistically significant decrease (by using the Student's t test) in the number and severity of dendritic lesions occurred on 1 day in the TSC-treated eyes compared with the control eyes, this effect was much less than that observed with either iododeoxyuridine or proflavine plus light treatments tested in the same model system by us using the same virus dose. The recovery of virus from the TSC-treated and untreated eyes was the same during and after therapy. No effect of Me-IBT on the course of the infection or on the amount of herpesvirus produced could be demonstrated.

DISCUSSION

We have demonstrated that Me-IBT inactivates the infectivity of herpesviruses. The nature of the inactivation process remains unclear, because we failed to demonstrate a virion polymerase which, putatively, could be the site of action of the drug. We have recently observed that the Me-IBT-copper complex binds to and precipitates both single- and double-stranded DNA and RNA (P. Mikelens, W. Levinson, and B. Woodson, manuscript in preparation). It seems possible, therefore, that the drug binds to herpesvirus DNA within the virion, thereby interfering with subsequent intracellular, replicative steps. Whether DNA replication or RNA transcription, or both, are affected was the subject of our present investigation.

Me-IBT inactivated strains of both type 1 and type 2 HSV, including a strain of HSV type 2 which transforms hamster cells in vitro. The rates of inactivation of the type 1 and 2 strains appear to be quite similar in contrast to previous findings from this laboratory and others (4, 6, 11, 12) with other compounds such as silver nitrate, cytosine arabinoside, and iododeoxyuridine.

Me-IBT, a compound in which the TSC is attached to the second carbon atom from the ring N (B position), is effective as an inactivating agent, as reported here, but is inactive as an inhibitor of viral cytopathic effect when added to the cell after infection (3). Other compounds (α-TSCs), such as 2-formyl pyridine TSC and 6-formyl purine TSC, can inhibit intracellular replication, but cannot inactivate it. Despite this apparent correlation of structure and inhibitory activity, no conclusion can be drawn for two reasons: (i) one compound, 1-formyl isoquinoline TSC, is an α-TSC, but has both extra and intracellular activity; and (ii) the α-TSCs, although covalently bound to the α carbon of isatin, are attached via a formyl group which separates the ring N from the N1 atom of the TSC by two carbon atoms, which is the same structural relationship as with the β-TSCs. This places the chelating portion of the TSC (potentially) in the same relationship to the ring N in both types of compounds. A more subtle and precise analysis of the three-dimensional structure of these compounds is apparently required before a satisfactory explanation of these different effects on HSV can be obtained.

Several of the α-thiosemicarbazones tested (Table 2) are powerful inhibitors of ribonucleotide reductase (3). For example, 2-formyl pyridine TSC and 1-formyl isoquinoline TSC cause 50% inhibition of the enzyme at 4×10^{-7} and 1.7×10^{-7} M, respectively. In contrast, Me-IBT requires 1×10^{-5} M for 50% inhibition. It is postulated that inhibition of this enzyme plays a role in the inhibition of HSV cytopathic effect when added to the cell following infection (3). It seems unlikely, however, that this enzyme is the site of action of the in vitro inactivation reported here for two reasons: (i) the pyridine and isoquinoline are both approximately equally effective inhibitors of the enzyme, yet isoquinoline TSC inactivates the infectivity while pyridine TSC does not; and (ii) there is no evidence that ribonucleotide reductase is present in herpes virions or that it is necessary for herpesvirus replication.

The observation reported in Table 2 that isoquinoline TSC is a powerful inactivator of HSV leads to the speculation that the inhibition of cytopathic effect seen by Brockman et al. (3) with this drug could have been due to the

inactivation of virus in transit to other cells. This speculation should be viewed with caution, however, because the Brockman studies were carried out in Eagle (BME) medium with 2× amino acids which could interfere with the inactivation by isoquinoline TSC just as it does with the inactivation by Me-IBT. It should be noted also that Brockman et al. reported inhibitory concentrations which varied from 3.2 to 320 μg/ml, depending upon the activity of the drug. It is possible, therefore, that had higher concentrations of the drugs been used (Table 2), inactivation of HSV would have been observed. We, however, chose to test these compounds at 40 μM (approximately 10 μg/ml) in order to compare their inactivating ability with that of Me-IBT and TSC as reported in Table 1.

We found that exposure of HSV to Me-IBT suspended in tissue culture medium resulted in striking protection of the infectivity of the viruses against the action of the drug. This protection was shown to be due, in part, to histidine. The ability of histidine to protect may be correlated to its ability to form much more stable complexes with heavy metals such as copper, cobalt, zinc, and manganese than the other amino acids tested (10). Because it is known that Me-IBT also forms stable complexes with heavy metals (8, 14), competition may result.

The involvement of heavy metals in the action of TSCs prompted us to investigate the effect of the metal ion alone on HSV plaque formation. Because several of them possess powerful inactivating ability, it is possible that the function of the Me-IBT or TSC is to act as a carrier for heavy metal, thereby increasing its ability to penetrate the viral envelope. The ability of TSCs to increase the intracellular copper concentration has been reported (2).

The inactivation of HSV by cations has been reported (13). In this previous study, mercury was found to be the most effective (10^{-6} M) inactivator with silver and uranium active at 10^{-5} M. At higher concentrations (10^{-4} M), copper, bismuth, gold, cadmium, and lead are potent inactivating agents. In approximate terms, the data presented in Table 4 of this report confirm and extend the former study. The reason for the inconsistencies in the data between the two studies is not apparent.

The ability of one molar solution of magnesium or calcium to enhance the inactivation of herpes simplex virus at 50 C has been described (15). Although it seems unlikely that the mechanism of this enhanced inactivation is the same as the inactivation caused by the 40 μM solutions of cations incubated at 37 C and reported in this present study, it is important to be aware that several factors may play a role in the inactivation of a virus and that they may interact in a complex manner.

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