Acta virol. **30** : 192—198, 1986

INHIBITION OF VACCINIA VIRUS REPLICATION IN RK-13 CELLS BY N,N′-BIS(METHYLISATIN-BETA--THIOSEMICARBAZONE)-2-METHYLPIPERAZINE

A. BAŃKOWSKI, K. FILCZAK, M. KORBECKI

Serum and Vaccine Research Laboratory, 30/34 Chelmska Str., 00-725 Warsaw, Poland

Received July 9, 1985

Summary. — N,N′-bis(methylisatin-beta-thiosemicarbazone)-2--methylpiperazine in a 100 μmol/l concentration inhibited the reproduction of vaccinia virus in RK-13 cells by about 90%. This compound (bis-IBTMP) had no influence on virus adsorption and on early stages of virus multiplication, but affected virus reproduction from 12 to 24 hr post-infection (p.i.). The incorporation of ^{3}H-thymidine into infected cells increased during first 10 hr p.i., decreasing gradually afterwards. In the infected cells treated with bis-IBTMP the same tendency was observed up to 10 hr p.i., but later on the incorporation level remained unchanged. The uptake of ^{14}C-amino acids in the presence of bis--IBTMP was reduced both in vaccinia virus-infected and non-infected RK-13 cells.

Key words: vaccinia virus, RK-13 cells, bis-IBTMP, mechanism of action

Introduction

The antiviral action of thiosemicarbazones against viruses from the smallpox group has been known for over 30 years. Isatin thiosemicarbazone and its N-methyl derivatives were found to be most active (Bauer and Sadler, 1960; Tilles, 1974). Further studies were conducted on synthesis of new compounds from this group with higher antiviral activity (Gatti and Cavrini, 1980). Such studies were performed also in Poland. Łucka-Sobstel and Zejc (1973) obtained through aminoalkylation a number of derivatives of isatin beta-thiosemicarbazone. One of these derivatives — N,N′-bis(methylisatin--beta-thiosemicarbazone)-2-methylpiperazine — showed a strong action against vaccinia virus both in vitro (Zgórniak-Nowosielska *et al.*, 1973; Borysiewicz and Łucka-Sobstel, 1978) and in vivo (Zgórniak-Nowosielska *et al.*, 1976, 1980).

The purpose of the present studies was to investigate the mechanism of action of N,N′-bis(methylisatin-beta-thiosemicarbazone)-2-methylpiperazine (bis-IBTMP) in vaccinia virus-infected RK-13 cells.

Materials and Methods

Cells. A continuous line of rabbit kidney cells (RK-13) was used. The cells were grown in Parker's (199) medium supplemented with 5% of heat-inactivated calf serum and antibiotics. In the maintenance medium applied after infection, the content of calf serum was reduced to 1.5%.

Virus. The Lister strain of vaccinia virus was adapted to RK-13 cells and used in an initial titre of 10^8 PFU/ml. Cell cultures were infected using 0.01 PFU/cell input multiplicity (IM). Titrations of infectious virus in all experiments were performed by the standard PFU method in tube cultures of RK-13 cells (Madaliński *et al.*, 1977).

Chemicals. N,N´-bis-(methylisatin-beta-thiosemicarbazone)-2-methylpiperazine was obtained from Professor Zgórniak-Nowosielska from the Institute of Microbiology, Medical Academy in Cracow. The initial 25 mmol/l solution of the compound was prepared in hot N-methylacetoamide (NMA). The working solutions for virological investigations were obtained by adequate dilution of the initial solution in the maintenance medium for RK-13 cells. They were prepared immediately before use and sterilized by ultrafiltration through a Millipore 0.22 μm filter. For control cultures the solutions of the diluent (NMA) in the maintenance medium were prepared in an identical way.

^{3}H-thymidine (methyl-^{3}H-thymidine) with a specific activity of 7.14 GBq/mmol and a radioactive concentration of 37 MBq/ml (Amersham) and ^{14}C-amino acids (U-^{14}C-protein hydrolysate) with a specific activity 2.18 GBq per milliatom carbon and radioactive concentration of 1.85 MBq/ml (Amersham) were used.

The cells were dissolved in NCS fluid (Amersham-Searle), Scintillator solutions: Ready-Solv HP (Beckman) or 8 g of butyl-PBD and 0.5 g PBBO (Beckman) per liter of toluene.

Cytotoxic effect was determined using tube cultures of RK-13 cells. After removal of the growth medium, 1 ml of maintenance medium lacking the tested substance and NMA (control) or 1 ml of maintenance medium containing bis-IBTMP in concentrations from 10 μmol/l to 200 μmol/l or NMA in concentrations from 0.04% to 0.8% were added to the test tubes. The cell cultures were incubated for 48 hr at 37 °C. Thereafter the morphological features of the cultured cells were evaluated microscopically, and the total number of cells in a culture as well as the per cent of dead cells were determined by trypan blue staining. Maximum non-toxic dose of the compound (or of NMA) causing neither evident changes of cell morphology nor increase in the proportion of dead cells was determined.

Plaque reduction test. It was used to test the effect of bis-IBTMP on virus replication, the virucidal action of the compound and the efficiency of its action in relation to the dose of the infecting virus. With regard to the aim of these studies bis-IBTMP was present in the maintenance medium prior to infection only, during virus adsorption only, or for a definite time after the end of adsorption. At each interval the percentage of plaque reduction was calculated in the cell cultures treated with bis-IBTMP or with NMA in relation to untreated controls.

Incorporation studies. The cultures of RK-13 cells were infected with vaccinia virus in a dose of 2 PFU/cell. One hr after adsorption, the cultures were washed thrice with PBS and the maintenance medium containing 100 μmol/l of bis-IBTMP was added. Non-infected cells maintained in the presence of bis-IBTMP served as a control. At definite time intervals of incubation at 37 °C the medium was removed and 1 ml aliquots of the maintenance medium or PBS were added. The medium contained 37 kBq of ^{3}H-thymidine, the PBS contained 7.4 kBq of ^{14}C--amino acids. The cultures were labelled for 30 min at 37 °C, then washed rapidly with two portions of cold (4 °C) PBS (each portion 10 ml) and finally, the fluid was removed. The cultured cells were disrupted in 1 ml NCS, the samples were transferred into glass scintillation vials each containing 12 ml of liquid scintillator. The radioactivity was measured with a Beckman LS-3150 counter. The activity in d.p.m. for ^{3}H and ^{14}C was counted by the method of external standard channel ratio (ESCR).

Results

Effects of bis-IBTMP on non-infected cells

Testing of the cytotoxicity of bis-IBTMP for RK-13 cells demonstrated that in the concentration range from 0 to 100 μmol/l the compound did not cause any evident changes in cell morphology and did not reduce signifi-

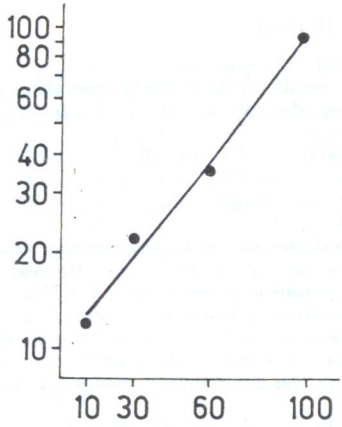

Fig. 1.
Relationship between bis-IBTMP concentration and vaccinia virus plaque reduction
Abscissa: bis-IBTMP concentration in μmol/l; ordinate: plaque reduction (%)

cantly the total number of cells in culture or the percentage of viable cells. In the concentration of 200 μmol/l bis-IBTMP exerted a cytotoxic effect, which was evidenced by vacuolization of the cytoplasm of a part of cells, reduction of the total number of cells in the cultures by about 30% ($p < 0.05$) and a rise in the percentage of dead cells by about 17%. The dead cells stained with trypan blue showed a marked damage to the cell membrane. It is possible that this phenomenon was caused by the action of the solvent (NMA), since it was observed also in cultures maintained in the presence of the solvent alone in a 0.8% concentration. At this solvent concentration a significant ($p < 0.05$) reduction of the total number of cells in culture was observed. As follows from these results, the maximum non-toxic dose of bis-IBTMP under given experimental conditions was 100 μmol/l non-toxic concentration of NMA being 0.4%.

Effect of bis-IBTMP on vaccinia virus yield in RK-13 cells

Fig. 1 shows the dose-dependent effect of bis-IBTMP, when the compound had been present in the culture medium throughout the whole period of virus replication. It is evident that the reduction of plaque number showed a linear correlation with the concentration of bis-IBTMP. At 60 μmol/l concentration the inhibition was about 40%, and at the 100 μmol/l concentration it reached 90%.

Neither bis-IBTMP nor NMA alone inactivated the virus.

Further investigations concerned the phase of the replication cycle of the virus sensitive to bis-IBTMP. In these investigations bis-IBTMP was used in 100 μmol/l concentration, and the concentration of NMA for corresponding control cultures was 0.4%.

The examined substance or the solvent were present in the culture medium for only 2 hr prior to infection with the virus (preincubation), for only one hr during virus adsorption, or from the end of virus adsorption to the end of the experiment, i.e. for 48 hr. The results of plaque reduction test were

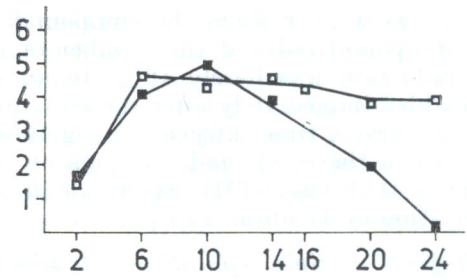

Fig. 2.
Incorporation of ^{3}H-thymidine into vaccinia virus infected RK-13 cells
Abscissa: hr p.i.; ordinate: ^{3}H (dpm $\times 10^{3}$)
■——■ infected cultures untreated with bis-IBTMP
□——□ infected cultures treated with bis-IBTMP (100 μmol/l)

compared with those obtained in virus-infected cultures in the absence of bis-IBTMP or NMA. Bis-IBTMP present at the time of preincubation or virus adsorption exerted no antiviral effect as similar as NMA alone. An antiviral action of bis-IBTMP against vaccinia virus was observed, however, when the compound was present in the culture medium from the end of adsorption to 48 hr post-infection (p.i.). The plaque reduction in relation to controls was about 90% and this difference was highly significant ($p < < 0.01$). NMA used alone in the 0.4% concentration showed no effect.

The effect of bis-IBTMP in concentration of 100 μmol/l depended only slightly on the virus infecting dose. At doses 0.001 to 0.1 PFU/cell the virus yield was reduced by nearly 90%, while at 10 PFU/cell it was reduced by about 85%.

In successive experiments bis-IBTMP in 100 μmol/l concentration was added to the cultures at various time intervals p.i., or immediately after the end of virus adsorption, and then the compound was removed by washing-out at various time intervals, the cultures being further maintained in a medium free from bis-IBTMP. Forty-eight hr p.i. the plaques were counted and the per cent of plaque number reduction was calculated in relation to controls (infected cultures free from bis-IBTMP throughout the whole incubation time). The compound added to the culture even 12 hr p.i. was as effective as that added immediately after the end of virus adsorption. The

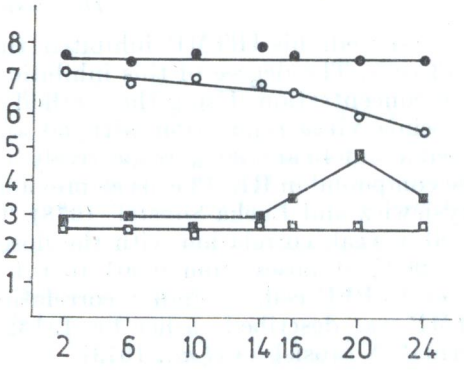

Fig. 3.
Incorporation of ^{14}C-amino acids into vaccinia virus infected and noninfected RK-13 cells
Abscissa: hr p.i.; ordinate: ^{14}C (dpm $\times 10^{3}$)
●——● noninfected cultures untreated with bis IBTMP
○——○ noninfected cultures treated with bis-IBTMP (100 μmol/l)
■——■ infected cultures untreated with bis-IBTMP
□——□ infected cultures treated with bis-IBTMP (100 μmol/l)

effect was weaker when the compound was added later, e.g. at 24 hr p.i. the compound reduced the number of plaques by 60%, and at 36 hr p.i. the reduction was barely 12%. In an alternative experiment bis-IBTMP was added immediately after the end of adsorption period and then washed out at various time intervals; a significant plaque reduction was observed only when the compound was present at least up to 12 hr p.i. These data indicate that bis-IBTMP exerts an inhibiting effect on late stages of the replication cycle since 12 hr p.i.

Stages of vaccinia replication cycle sensitive to the action of bis-IBTMP

Further data on the stages of the replication cycle sensitive to bis-IBTMP were obtained from the experiments in which the cultures were subjected to pulse labelling with ^{3}H-thymidine and ^{14}C-amino acids. Fig. 2 shows the kinetics of ^{3}H-thymidine uptake by the cells infected with the virus and maintained in a medium containing 100 μmol/l bis-IBTMP or devoid of this compound. The quantitative values obtained directly in these investigations were transformed subtracting the counts of radioactivity in non-infected (control) cultures from the counts obtained in the infected cell cultures. It is evident that the kinetics of ^{3}H-thymidine uptake was similar in the infected cultures with the presence of bis-IBTMP and in its absence up to about 10 hr p.i.; but later on the ^{3}H-thymidine uptake by the cell cultures not containing bis-IBTMP decreased gradually while in those containing the compound the incorporation of ^{3}H-thymidine remained practically unchanged.

Fig. 3 presents the incorporation of ^{14}C-amino acids. The uptake of ^{14}C by the noninfected cell cultures was maintained at the same level throughout the whole period of observation. In the noninfected cultures treated with bis-IBTMP the uptake of ^{14}C-amino acids was slightly lower and decreased gradually. The incorporation of ^{14}C into the infected cells was smaller than into the noninfected cells, however, an evident peak of this incorporation was observed between 14 and 24 hr p.i. In the presence of bis-IBTMP the incorporation of ^{14}C into infected cells was relatively low and remained unchanged throughout the whole observation period.

Discussion

As observed, bis-IBTMP inhibited the replication of vaccinia virus in RK-13 cells. The degree of this inhibition was a linear function of the substance concentration. Using the method of plaque reduction, the inhibition of vaccinia virus replication with 60 and 100 μmol/l doses of bis-IBTMP reached about 40 and 90%, respectively, thus requiring higher concentrations of the compound in RK-13 cells as previously described in chicken fibroblasts (Borysiewicz and Łucka-Sobstel, 1978). The degree of replication inhibition showed a weak correlation with the dose of the infecting virus, which was about 90% at doses from 0.001 to 0.1 PFU/cell, and about 85% at the dose of 10 PFU/cell. A similar correlation for lower concentrations of bis-IBTMP was described earlier for L-132 cells infected with vaccinia virus (Zgórniak-Nowosielska *et al.*, 1973).

Information on the mechanism of bis-IBTMP action is scant. Recently Rada and Zgórniak-Nowosielska (1984) found that this substance inhibits the late function or the synthesis of a late component in the replication cycle of vaccinia virus. More detailed data have been published for methisazone. Appleyard *et al.* (1965) found absence of at least five late viral proteins in the cells infected with rabbitpox virus and maintained in presence of methisazone. According to Woodson and Joklik (1965) late viral m-RNAs appeared in the presence of methisazone but their life time was short leading to a premature breakdown of polyribosomes and a failure of the appearance of late viral proteins.

In our own experiments bis-IBTMP caused no inactivation of vaccinia virus and had no effect on the process of its adsorption on RK-13 cells. Inhibition of the virus replication was observed, on the other hand, when bis-IBTMP was added to culture medium after virus adsorption. The studies, in which the compound was added or removed at various time intervals after the end of adsorption period, showed that the stage of vaccinia virus replication in RK-13 cells sensitive to the effect of bis-IBTMP began about 12 hr p.i.

Radioisotope investigations revealed that the maximum quantities of ^{3}H-thymidine in infected cells were demonstrated between 6 and 10 hr p.i. At later intervals this uptake decreased gradually. In the same studies higher uptake of ^{14}C-amino acids was noted between 14 and 24 hr p.i. This observation correlated well with the earlier data on the vaccinia virus replication cycle. It is known, for example, that in the infected cell late viral m-RNAs coding for structural virus proteins appear after beginning of viral DNA synthesis (Oda and Joklik, 1967). Then synthesis of viral DNA is blocked probably due to accumulation of late, structural viral proteins (Kates and McAuslan, 1967). In vaccinia virus-infected RK-13 cell cultures treated with bis-IBTMP a prolonged enhancement of ^{3}H-thymidine uptake was observed, but no increased incorporation of ^{14}C-amino acids was noted between 14 and 24 hr p.i. This seems to suggest that the synthesis of late viral proteins was blocked in these cells. As a result of this inhibition no infectious viral particles were formed despite of the prolonged viral DNA synthesis.

References

Appleyard, G., Hume, V. B. M., and Westwood, J. C. N. (1965): The effect of thiosemicarbazones on the growth of rabbitpox virus in tissue culture. *Ann. N.Y. Acad. Sci.* **130**, 92—104.

Bauer, D. J., and Sadler, P. (1960): The structure-activity relationships of the anti-viral chemotherapeutic activity of isatin-beta-thiosemicarbazone. *Brit. J. Pharmacol.* **15**, 101—110.

Borysiewicz, J., and Łucka-Sobstel, B. (1978): The effect of certain Mannich bases derivatives of isatin-beta-thiosemicarbazone on the replication of vaccinia virus in vitro studies. *Acta Microbiol. Pol.* **27**, 111—121.

Gatti, R., and Cavrini, V. (1980): Über eine virustatische Cyclopropananaloge des Methisazons. *Arch. Pharmaz.* **313**, 89—91.

Kates, J. R., and McAuslan, B. R. (1967): Relationship between protein synthesis and viral deoxyribonucleic acid synthesis. *J. Virol.* **1**, 110—114.

Łucka-Sobstel, B., and Zejc, A. (1973): Mannich bases derivatives of isatin and its beta-thiosemicarbazone, potential antiviral agents. *Acta Microbiol. Pol.* **22**, 197—198.

Madaliński, W., Bańkowski, A., and Korbecki, M. (1977): Purification of vaccinia virus by zonal centrifugation and analysis of viral protein composition. *Acta virol.* **21**, 104—108.

Oda, K., and Joklik, W. K. (1967): Hybridization and sedimentation studies with "early" and "late" vaccinia m-RNA. *J. molec. Biol.* **27**, 395—419.

Rada, B., and Zgórniak-Nowosielska, I. (1984): Site of action of N,N′-bis(methylisatin-β-thiosemicarbazone)-2-methylpiperazine in the vaccinia virus replication cycle. *Acta virol.* **26**, 428—432.

Tilles, J. G. (1974): Antiviral agents. *Ann. Rev. Pharmacol.* **14**, 469—489.

Woodson, B., and Joklik, W. K. (1965): The inhibition of vaccinia virus multiplication by isatin-beta-thiosemicarbazone. *Biochemistry* **54**, 946—953.

Zgórniak-Nowosielska, I., Marciszewska, M., Pastuszek, J., and Lucka-Sobstel, B. (1973): The effect of isatin-beta-thiosemicarbazone derivatives on vaccinia virus multiplication in L-132 cell line (human embryonic lung). *Acta Microbiol. Pol.* **22**, 199—204.

Zgórniak-Nowosielska, I., Gatkiewicz, A., and Poteć, Z. (1976): The ativiral activity of isatin--beta-thiosemicarbazone derivatives on vaccinia virus infection in mice. *Arch. Immunol. Ther. Exp.* **24**, 597—601.

Zgórniak-Nowosielska, I., Gatkiewicz, A., Veckenstedt, A., and Béládi, I. (1980): Effect of N,N′-bis(methylisatin-β-thiosemicarbazone)-2-methylpiperazine against virus induced encephalitis in mice. *Acta virol.* **24**, 439—444.