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EFFECTS OF SURAMIN, HPA-23 AND 3′-AZIDOTHYMIDINE TRIPHOSPHATE ON THE REVERSE TRANSCRIPTASE OF BOVINE LEUKAEMIA VIRUS

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Summary. — We used purified bovine leukaemia virus (BLV) to test the inhibitory effects of suramin, HPA-23, and 3′-azidothymidine triphosphate (N_3dTTP) on the reverse transcriptase activity. The 50 % inhibitory concentrations (ID_{50}) of the compounds were determined to be 2.8 μmol/l (suramin), 8.0 μmol/l (HPA-23), and 0.17 μmol/l (N_3dTTP). Kinetic analyses of suramin and HPA-23 inhibition are discussed. The observed inhibitory effects emphasize the suitability of BLV as a model virus for investigations of retrovirus chemotherapy.

Introduction

The discovery of human immune deficiency virus (HIV), the causative agent of AIDS and the association of human T-cell leukaemia /lymphoma virus types I and II (HTLV-I, HTLV-II) with adult leukaemias and lymphomas have revived the scientific interest in antiretroviral compounds. Because of the key function of the reverse transcriptase in replication of retroviruses and in the pathogenesis of these malignant diseases, this enzyme is considered for the potential target of antiviral activity. In the last years various inhibitors of reverse transcriptase were tested both *in vitro* and *in vivo*.

Suramin, a drug which has been widely used in the treatment of African trypanosomiasis and onchocerciasis, was the first compound found to inhibit HIV replication (Mitsuya *et al.*, 1984). De Clecq (1979) demonstrated the inhibitory effect of suramin on the reverse transcriptase activity of diverse avian and murine retroviruses. Mitsuya *et al.* (1984) found that suramin blocks the replication and cytopathic effect of HIV in T-cell cultures. Clinical studies showed no clearcut benefit in AIDS patients despite of the drug effects *in vitro* (Broder *et al.*, 1985; Rouvroy *et al.*, 1985; Collins *et al.*, 1986).

HPA-23 (ammonium-21-tungsto-9-antimoniate) was found active against a broad spectrum of RNA and DNA viruses *in vivo* (Jasmin *et al.*, 1974; Werner *et al.*, 1976; Kimberlin *et al.*, 1983) and it was proved to inhibit the

viral polymerases *in vitro* (Chermann *et al.*, 1985), including the reverse transcriptases of human and simian AIDS viruses (Dormont *et al.*, 1985). HPA-23 was also tested clinically in the treatment of AIDS patients (Rozenbaum *et al.*, 1985; Buimovici-Klein *et al.*, 1986).

The thymidine analogue 3′-azido-3′-deoxythymidine (N_3TdR) is phosphorylated by cellular enzymes to the triphosphate form, which was demonstrated by Mitsuya *et al.* (1985) to inhibit the reverse transcriptase, viral replication, and the cytopathic effect of HIV *in vitro*. Chain terminating incorporation was discussed as the key mechanism of this activity (Mitsuya *et al.*, 1985; Furman *et al.*, 1986; Nakashima *et al.*, 1986; Öberg, 1986). Matthes *et al.* (1987) assume that 3′-azidothymidine triphosphate (N_3dTTP) as well as 3′-fluorothymidine triphosphate (FdTTP) act primarily as competitive inhibitors rather than as chain terminators. In the first clinical trials 3′-azidothymidine showed clinical efficacy but also severe side effects (Yarchoan *et al.*, 1986). Further potential retrovirus inhibitors, especially of HIV, were tested, for example phosphonophormic acid (Sandstrom *et al.*, 1985; Balzarini *et al.*, 1986*a*), anionic dyes (Balzarini *et al.*, 1986; Balzarini *et al.*, 1986a), nucleoside analogues (McCormick *et al.*, 1984; Balzarini *et al.*, 1986*a*; Balzarini *et al.*, 1986*b*; Baba *et al.*, 1987; Matthes *et al.*, 1987), heparin and dextran sulphate (Ito et al., 1987). From all these 3′-azidothymidine and 3′-fluorothymidine are the most potent inhibitors of HIV replication described so far. In a double blind trial, Fischl *et al.* (1987) reported a beneficial effect of 3′-azidothymidine treatment in 145 drug-treated versus 137 placebo-treated patients. One treated patient died in comparison to 19 placebo recipients during the study. Considerable clinical improvements (less oportunistic infections, increase in the proportion of CD4 cells, reversion of skin anergy) were observed in the drug-treated group. In contrast, suramin did not show any clinical efficacy (Kaplan *et al.*, 1987).

Bovine leukaemia virus (BLV) belongs to the retrovirus family. Although more related to the human retroviruses HTLV-I and HLTV-II, BLV shares also some biological, pathogenetic and clinical features with HIV. In our study we used purified BLV for testing the inhibitory effects of suramin, HPA-23, and 3′-azidothymidine triphosphate on the reverse transcriptase activity. The inhibitory effects found and the kinetic analyses emphasize the favourable model character of BLV for antiretroviral investigations.

Materials and Methods

Purification of bovine leukaemia virus. The pooled culture supernatants of BLV-infected foetal lamb kidney cells were concentrated about 10—20 fold by a hollow-fiber system (cartridge type H1MP01-43). The virus was then sedimented onto a cushion of 39 % sucrose in TEN buffer (20 mmol/l Tris-HCl, pH 7.4, 1 mmol/l EDTA, 100 mmol/l NaCl) through a 10—15 ml layer of 20 % sucrose in TEN using a Beckman SW 27 rotor at 20 000 rev/min for 1.5 hr at +4 °C. The virus-containing interphase fraction was obtained by a gradient collector, diluted 1 : 4 with 8.5 % sucrose in TEN and sedimented again through 20 ml of 20 % sucrose in TEN. The pelleted virus was resuspended in TEN, aliquoted and frozen at −20 °C.

Assay for BLV-associated reverse transcriptase. The optimal conditions for detection of BLV reverse transcriptase described by Rössler *et al.* (1980) were used with small modifications. The assay mixture contained the following components in a total volume of 100 µl: 30 mmol/l Tris-

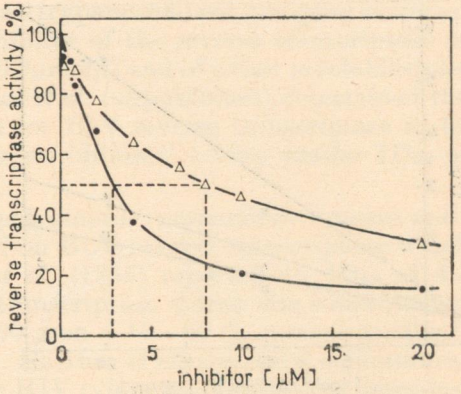

Fig. 1.
Effects of different concentrations of suramin (●) and HPA-23 (△) on the activity of bovine leukaemia virus reverse transcriptase after 60 min incubation

HCl, pH 8.0, 50 mmol/l KCl, 8.4 mmol/l $MgAc_2$, 0.1 % mercaptoethanol, 0.05 % Triton X-100, 1.6 % glycerol, 0.02 OD poly rA-oligo dT, 10 μmol/l dTTP (185 kBq ^{3}H dTTP) and 1 μl BLV concentrate. The mixtures were incubated at +25 °C for 60 min. dTTP and poly rA-oligo dT were purchased from Boehringer (Mannheim), ^{3}H-dTTP from the Radiochemical Centre (Amersham).

Inhibitors. HPA-23 was a gift from Dr. J. C. Chermann, Paris. Azidothymidine triphosphate was kindly provided by Dr. M. v. Janta-Lipinski, Berlin. Suramin was obtained from Bayer, Leverkusen. Aqueous solutions of suramin and HPA-23 were freshly prepared for each assay.

Results

The effects of increasing concentrations of suramin, HPA-23, and N_3dTTP on the reverse transcriptase activity using poly rA-oligo dT as template primer and dTTP as substrate are shown in Figs. 1 and 2. The 50 % inhibitory concentrations (ID_{50}) of the compounds were determined graphically to be 2.8 μmol/l (suramine), 8 μmol/l (HPA-23) and 0.17 μmol/l (N_3dTTP), respectively. In each case mean values of 4 experiments are given.

Varying concentrations of template primer poly rA-oligo dT and of the compounds were used to characterize the mechanism of reverse transcriptase inhibition. The double-reciprocal Lineweaver-Burk plots for the kinetics of

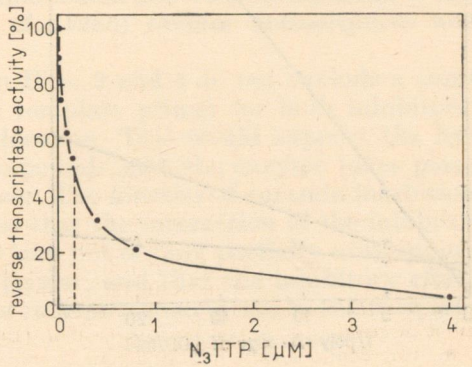

Fig. 2.
Effects of different concentrations of 3′-azidothymidine-5′-triphosphate (N_3 dTTP) on bovine leukaemia virus reverse transcriptase activity after 60 min incubation

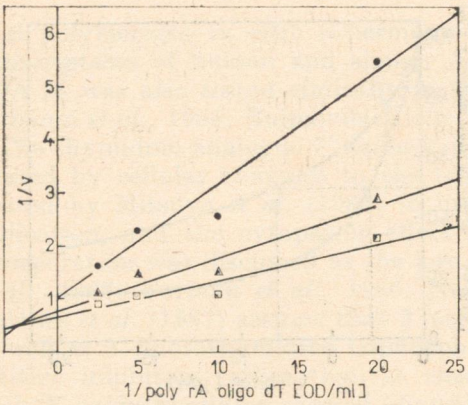

Fig. 3.
Double-reciprocal plot for the kinetics of inhibition of bovine leukaemia virus reverse transcriptase by suramin. Concentrations of suramin: 0 μmol/l (□), 0.8 μmol/l (△), and 4 μmol/l (●).v = pmol dTMP incorporated in 30 min at 25 °C.

inhibition of BLV reverse transcriptase activity by suramin and HPA-23 are shown in Fig. 3 and Fig. 4. The points of intersection with the ordinate were obtained by linear regression analysis. The results are not unambiguous. The deviation of the points of intersection from the ordinate could result from experimental errors, or — especially in the case of suramin — reflect a complex, not fully competitive mechanism of action.

Discussion

We investigated the inhibitory effects of suramin, HPA-23 and 3′-azidothymidine triphosphate on the reverse transcriptase of BLV. De Clercq (1979) reported that suramin, a complex polyanionic compound which has

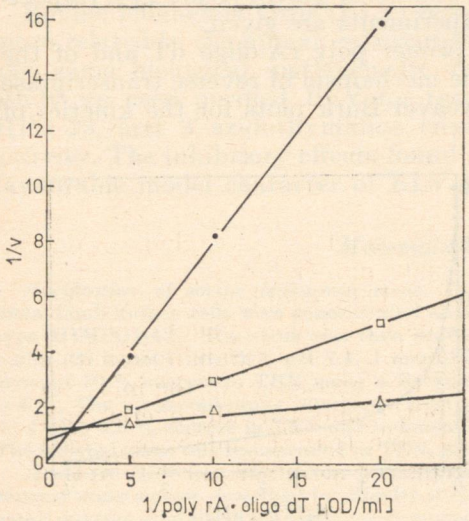

Fig. 4.
Double-reciprocal plot for the kinetics of inhibition of bovine leukaemia virus reverse transcriptase by HPA-23 Concentrations of HPA-23: 0 μmol/l (△), 4 μmol/l (□), and 20 μmol/l (●). v = pmol dTMP incorporated in 30 min at 25 °C.

long been used in the treatment of African trypanosomiasis (sleeping sickness) and of onchocerciasis, was a potent inhibitor of the reverse transcriptase of Moloney murine leukaemia virus (ID_{50} 0.7μmol/l) and of avian myeloblastosis virus with an ID_{50} of 0.1 μmol/l. Matthes *et al.* (unpublished) determined the 50 % inhibitory suramin concentration for HIV reverse transcriptase to be about 3.5 μmol/l. Under our conditions we obtained a very similar ID_{50} of 2.8 μmol/l for the BLV enzyme.

In contrast, HPA-23, a cryptate polyanionic ammonium-tungsto-antimoniate, revealed an ID_{50} of 8.0 μmol/l on BLV reverse transcriptase which was higher than for HIV. Dormont *et al.* (1985) reported an ID_{50} of 4.5 μmol/l for non-purified HIV reverse transcriptase (virus was concentrated by 10 % PEG and centrifuged at 55,000 g on a 10—60 % sucrose gradient) and 1.6 μmol/l for the purified enzyme. Matthes *et al.* (personal communication) obtained an ID_{50} of 2.0 μmol/l for HIV (virus was purified as described for BLV in our experiment).

From the data recorded for the inhibition of BLV reverse transcriptase by 3′-azido-3′-deoxythymidine triphosphate we calculated an ID_{50} of 0.17 μmol/l. Matthes *et al.* (1987) found an ID_{50} of 0.05 μmol/l for the inhibitory effects of both 3′-azidothymidine triphosphate as well as 3′-fluorthymidine triphosphate on the HIV enzyme. The comparison of methods and results of the referred investigations suggests that the data do not only reflect the different susceptibilities of the various reverse transcriptases, but that they are also influenced by the purity of the enzyme. Though the results are not fully comparable due to partially different purification procedures and assay conditions, it appears that BLV reverse transcriptase might be less sensitive to the above-mentioned inhibitors than the enzymes of other retroviruses, particularly of HIV.

To characterize the nature of the inhibitory effects on BLV reverse transcriptase by the complex inhibitors suramin and HPA-23 we tested varying concentrations of template primer poly rA-oligo dT at different suramin and HPA-23 concentrations. De Clecq (1979) described a competitive mechanism for the inhibition of avian myeloblastosis virus reverse transcriptase by suramin, with respect to the template primer concentration. For HPA-23 a competitive inhibition of Moloney murine leukaemia virus (Chermann *et al.*, 1985) as well as of HIV (Dormont *et al.*, 1985) reverse transcriptase was observed.

The Lineweaver-Burk plots shown in Figs. 3 and 4 do not exclude a competitive inhibition with respect to the template primer for both inhibitors, taking experimental errors into consideration. This would support the hypothesis that the interaction of the compounds with the enzyme takes place at the binding site of the template primer. The kinetics of suramin inhibition tion (Fig. 3) could also be interpreted so that the interaction of the inhibitor with the enzyme takes place at another site, not or only partially overlapping with the binding site of the template primer, and that the inhibitory effect is due to a modification of the affinity of reverse transcriptase to the template primer.

It has been suggested that effects other than competitive inhibition of reverse transcriptase may be of importance for the antiviral efficacy of suramin and HPA-23 (Broder et al., 1985; Öberg, 1986; De Clercq, 1987). Our results let us conclude that bovine leukaemia virus is suitable as a model for the development of antiretroviral compounds. These *in vitro* investigations can be supplemented by *in vivo* experiments with BLV infected sheep as suggested by Burkhardt *et al.* (1987). In this animal model suramin treatment led to a drastic decrease in the expression of p24 antigen on short-term cultivated leucocytes of BLV infected sheep.

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