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The mechanism of action of the anti-herpes virus compound 2,3-dimethyl-6(2-dimethylaminoethyl)-6H-indolo-(2,3-b)quinoxaline

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Summary

The compound 2,3-dimethyl-6(2-dimethylaminoethyl)6H-indolo-(2,3-b)quinoxaline (B-220) has been shown to exhibit potent antiviral activity against herpes simplex virus type 1 (HSV-1), varicella-zoster virus (VZV) and cytomegalovirus (CMV). The mechanism of antiviral action of B-220 against HSV-1 has been studied; from the results it appears that B-220 binds by intercalation into the DNA helix and then disturbs steps that are vital for viral uncoating.

Herpes simplex virus; Indoloquinoxaline; Uncoating

Introduction

Several condensed-ring systems containing indole display antiviral activity (Gwaltney, 1970). We have previously tested a number of analogs of 6H-indolo-(2,3-b)quinoxaline (Harmenberg et al., 1988); the most active compound, 2,3-dimethyl-6(2-dimethylaminoethyl)6H-indolo-(2,3-b)quinoxaline (B-220), has been shown to exhibit potent antiviral activity against herpes simplex virus type 1 (HSV-1), varicella-zoster virus (VZV) and cytomegalovirus (CMV). B-220 was

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shown to exert its antiviral activity early in the virus cycle, before the initiation of viral DNA synthesis. B-220 inhibited the expression of CMV early antigens as measured by immunofluorescence (IF), whereas this inhibition could not be achieved with either acyclovir (ACV) or foscarnet (PFA; Harmenberg et al., 1988). The aim of the present study was to analyse the effects of B-220 on the early steps of the HSV-1 virus cycle.

Materials and Methods

Compounds

B-220 (Fig. 1) was synthesized at the Department of Chemistry, Royal Institute of Technology, Stockholm, Sweden. Tritiated thymidine and tritiated leucine were from DuPont, NEN Research Products (Boston, MA, U.S.A.). Human immunoglobulin (Kabi AB, Stockholm, Sweden) had a high anti-HSV neutralizing titer.

Virus

HSV type 1 strain C42 has been previously described (Gronowitz and Källander, 1980).

Cells

Human embryonic lung fibroblasts (HL) and African green monkey kidney (GMK) cells were maintained in Dulbecco's modified Eagle's medium with 5% fetal calf serum and antibiotics (penicillin, 100 IU/ml; streptomycin, 100 μg/ml). All cell cultures were repeatedly tested for mycoplasma contamination, using the Hoechst 33258 staining method (Chen, 1977).

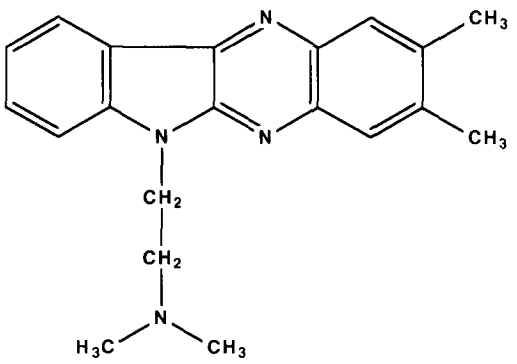

Fig. 1. Structure of B-220.

Virus susceptibility assay

Susceptibility of HSV-1 to B-220 was determined as described in detail elsewhere (Wahren et al., 1983a; Wahren et al., 1983b). After 48 to 72 h of culture of HSV-1 in the presence of different concentrations of B-220, viral protein production was quantified by a virus protein-specific enzyme-linked immunosorbent assay (ELISA; Sundqvist and Wahren, 1981).

Adsorption of [^{3}H]thymidine-labelled virus

Adsorption was analysed using modifications of previously published procedures (Fox et al., 1986; McSharry et al., 1979). HSV-1 was grown to high titers in HL cells in the presence of [^{3}H]thymidine (100 μCi/ml). The virions were purified using previously published procedures (Sundqvist and Wahren, 1981). The HL cells were incubated with virus in the presence or absence of 20 μM B-220 at 37°C for 60 min. Following the incubation period, the cells were washed twice with phosphate-buffered saline (PBS). The cells were then disrupted and the radioactivity was quantified using liquid scintillation. All experiments were performed in quadruplicate.

Virus penetration studies

Virus penetration was studied using previously published procedures with some modifications (McSharry et al., 1979; Eggers 1977). HSV-1 (multiplicity of infection, MOI: 5–10) was incubated with HL cells in the presence or absence of 7.5 μM B-220 for 60 min at 4°C. Subsequently, the temperature was shifted to 37°C and neutralizing immunoglobulin was added at indicated times. The antisera were washed away after 30 min incubation. Twenty-four hours after infection, the virus protein production was analysed with ELISA. All experiments were performed in quadruplicate.

Uncoating of [^{3}H]thymidine- and [^{3}H]leucine-labelled virus

The uncoating process of HSV-1 was studied by a procedure adopted from Feldman and coworkers (Feldman et al., 1981). HSV-1 was grown in HL cells in the presence of [^{3}H]thymidine or [^{3}H]leucine. Labelled virions were subsequently purified from the medium according to previously published procedures (Sundqvist and Wahren, 1981). HL cells were preincubated with cycloheximide (200 μg/ml) for 1 h. The cells were then infected with labelled virions in the presence or absence of 20 μM B-220 and an excess of unlabelled thymidine (100 μg/ml) in the medium. Three hours post-infection the cells were washed with PBS and the cytoplasmic and nuclear fractions were separated according to previously published procedures (Mark and Kaplan, 1971). The amount of radioactivity of each fraction was quantified using liquid scintillation. All experiments were performed in duplicate.

Optical spectroscopy of DNA interactions

Calf thymus DNA was obtained from Sigma (Sigma Chemical Company, St. Louis, MO, U.S.A.) and used without further purification. Optical spectra were recorded on a Cary 219 spectrophotometer; fluorescence spectra on a Shimadzu RF 510 spectrofluorimeter. All measurements were performed at room temperature (22°C). In the fluorescence titrations, every measurement was performed on a freshly prepared sample. The fluorescence intensity was recorded at a fixed time after mixing in the cuvette to minimize the systematic errors arising from drug adsorption on the walls of the quartz cuvette.

The fraction of B-220 bound to DNA, X_b, was evaluated from:

$$X_b = \frac{(F_0/F) - 1}{(F_{max}/F) - 1}$$

where F_0 and F are fluorescence emission intensities of B-220 in the absence and presence of DNA, respectively, and F_{max} is the intensity at a high DNA concentration beyond which no change of fluorescence intensity was observed (cf. Zegar et al., 1989). The excitation wavelength was 370 nm and the emission wavelength was 470 nm.

Results

Pretreatment of HL cells and HSV-1

To analyze the antiviral mechanism of B-220, HL cells were preincubated with different concentrations of B-220 for 3 days (Fig. 2). Before infection, the B-220-containing medium was removed and the cells were carefully washed with medium without B-220. The cells were then infected with HSV-1 without any B-220 present. Two days later the viral protein production was quantified using ELISA. The results indicate that pretreatment of cells with 1–10 μM of B-220 did not affect the virus protein production. Thirty μM B-220 was, however, clearly toxic for the cells (data not shown).

Cell-free HSV-1 suspension was preincubated with 20 μM B-220 for 1 or 4 h at 4°C or 37°C (Table 1). Before infection the virus samples were diluted 100 times so that the remaining concentration of B-220 (0.2 μM) would not in itself affect viral growth. Two days after infection, the viral protein production was quantified using ELISA. The results indicate that preincubation of the virus suspension with 20 μM B-220 for 1 or 4 h at 37°C decreased the viral protein production by 31 and 95%, respectively. Preincubation of the virus with 20 μM B-220 at 4°C did not affect viral growth. The results suggest that B-220 interacts with the virus at 37°C but not at 4°C, resulting in a diminished infectivity.

The antiviral activity of B-220 was studied following removal of the drug at

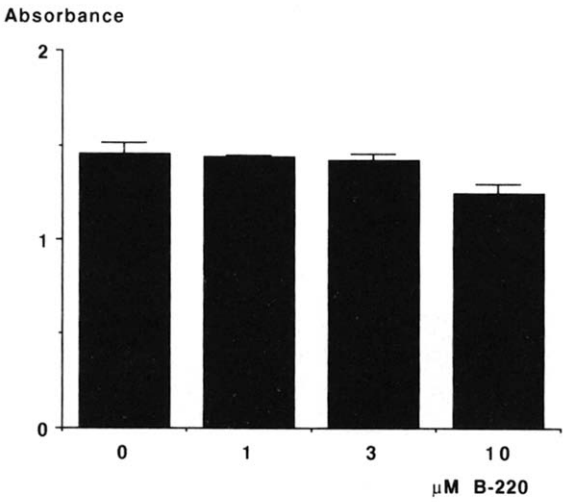

Fig. 2. Three-day pretreatment of HL cells with B-220 before infection with HSV-1 (MOI = 0.1). Procedures as in Materials and Methods. Experiments were performed in quadruplicate. Standard deviations are shown in the figure.

TABLE 1

Antiviral activity (measured by ELISA) of B-220 when preincubated with virus[a]. Results are shown as an average of quadruplicate experiments with standard deviations, and as a % of the virus control (in parentheses).

	Absorbance	
Preincubation temperature	4°C	37°C
0 μM B-220		
1 h preincubation	1.755 ± 0.003 (104%)	1.745 ± 0.023 (103%)
	1.762 ± 0.020 (104%)	1.670 ± 0.067 (99%)
4 h preincubation		
20 μM B-220		
1 h preincubation	1.732 ± 0.025 (103%)	1.169 ± 0.224 (69%)
	1.744 ± 0.015 (103%)	0.084 ± 0.086 (5%)
4 h preincubation		
Virus control (no preincubation)		1.688 ± 0.041 (100%)

[a]Virus (MOI = 5–10) was preincubated for the designated times and temperatures with and without B-220. Subsequently, the samples were diluted 100 times to lower the B-220 concentration to inactive levels and incubated on the cells.

various times. HL cells were infected in the presence or absence of 7.5 μM B-220. The drug was then removed at various times by 5 successive washings with drug-free medium (Fig. 3). Finally, the virus protein production was quantified by ELISA. The results indicate that removal of B-220 60 min after infection did not restore the full virus antigen production. B-220 therefore appeared to inactivate the virus in a rapid fashion.

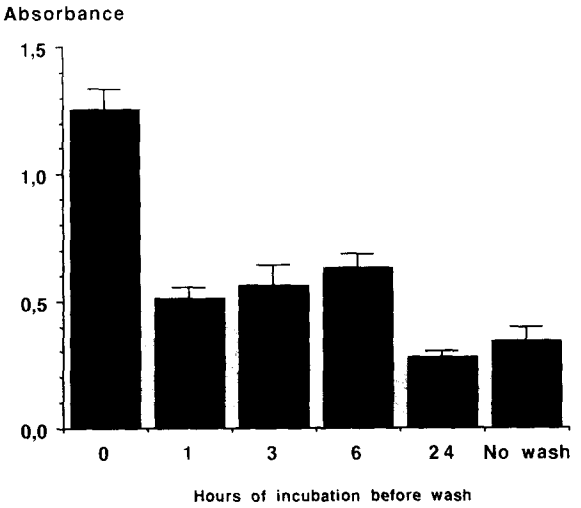

Fig. 3. The antiviral activity of B-220 following removal of the drug at various times. HL cells were infected at time 0, when medium containing 7.5 μM B-220 was added. At indicated times, the B-220-containing medium was removed and, after repeated washings, medium without B-220 was added. Procedures as in Materials and Methods. Experiments were performed in quadruplicate. Standard deviations are shown in the figure.

The influence of B-220 on the adsorption of HSV-1

In order to determine the effect of B-220 on attachment of HSV-1 virions to the cell surface, HL cell monolayers were incubated for 60 min at 37°C with [^{3}H]thymidine-labelled HSV-1 virions. Following the incubation period, the cells were washed and the radioactivity was quantified using liquid scintillation. B-220 treatment resulted in 3310 ± 683 cpm compared with 3132 ± 631 in the control. The results thus indicate that 30 μM B-220 did not appear to exhibit any effect on virus attachment to HL cells.

The influence of B-220 on the penetration of HSV-1

The effect of B-220 on penetration of HSV-1 into HL cells was determined by the ability of immunoglobulin, containing neutralizing antibody, to neutralize the virus after adsorption to the cell surface. Virus on the external surface of cells is regarded to be susceptible to neutralization by a specific antibody, whereas virus that has penetrated the cell is not. The virus was absorbed to the cells at 4°C in the presence or absence of 7.5 μM B-220. At the end of the adsorption period 91% (B-220-treated) and 80% (untreated controls) of the virus, as measured by ELISA, could be inhibited by the addition of antibody. This indicates that B-220 exhibited some inhibition on virus penetration at 4°C. Addition of antibody, at various times following the temperature shift to 37°C, inhibited decreasing fractions of virus in

TABLE 2

Distribution of parental virus DNA and virus proteins in the cytoplasmic and nuclear fractions of HSV-1 infected cells[a]

	20 μM B-220 treated	Nuclear fraction (cpm ± SD)	Cytoplasmic fraction (cpm ± SD)
HSV-1 ([^{3}H]thymidine)	–	11,485 ± 2,373 (19%)	49,575 ± 3,175 (81%)
HSV-1 ([^{3}H]thymidine)	+	1,820 ± 1,371 (3%)	66,661 ± 3,734 (97%)
HSV-1 ([^{3}H]leucine)	–	13,485 ± 2,850 (67%)	6,523 ± 1,235 (33%)
HSV-1 ([^{3}H]leucine)	+	240 ± 650 (2%)	14,083 ± 3,903 (98%)

[a]The nuclear fraction was separated from the cytoplasmic fraction at 3 h post infection (MOI = 5–10), as described in Materials and Methods, and the amount of radioactivity associated with each fraction was determined. Experiments were performed in quadruplicate. Results are shown as average cpm ± standard deviation, and as % of total intracellular radioactivity (in parentheses).

both groups (Fig. 4). Both groups followed similar curves, indicating that HSV-1 penetrated the HL cells at a similar rate in the B-220 treated group compared to controls at 37°C.

The influence of B-220 on viral uncoating of HSV-1

The effect of B-220 on viral uncoating was analysed using [^{3}H]thymidine- and [^{3}H]leucine-labelled purified virions (Table 2). The results indicate that 67% of the

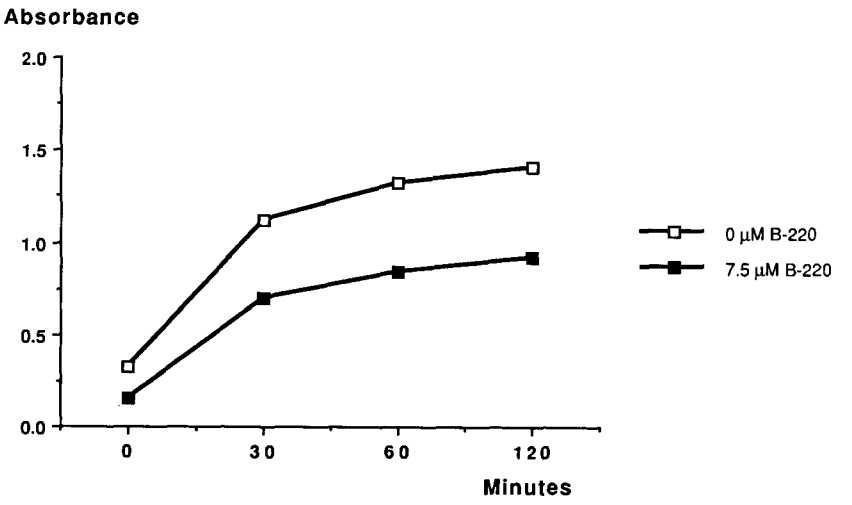

Fig. 4. Influence of B-220 on the penetration of HSV-1. Procedures as in Materials and Methods.

[^{3}H]leucine-labelled virion proteins became associated with the nuclear fraction of the HL cells. Following B-220 treatment only 2% was found in the nuclear fraction. Virions with [^{3}H]thymidine-labelled DNA showed that 19% of the DNA became associated with the nuclear fractions during the three-hour incubation period. After B-220 treatment only 3% of the radioactivity was found in association with the nuclear fraction. The experiments indicate that B-220 treatment induced a substantial reduction in the appearance of labelled material from the virions in the nuclear fractions of the HL cells. The results suggest that B-220 inhibits an event after virus penetration but before transfer of viral DNA to the nucleus of the cell.

It is therefore possible that B-220 inhibited steps of the viral replication cycle which are essential for viral uncoating. The procedure for studying viral uncoating was adopted from Feldman and coworkers, who investigated temperature-sensitive uncoating mutants of pseudorabies virus (Feldman et al., 1981).

The physical interaction of B-220 with DNA

Fig. 5 shows the optical absorption spectra of B-220 alone and in a 1:60 (drug: nucleotide ratio) complex with calf thymus DNA. Under these conditions the drug was almost completely bound to the DNA, as is evident from the unchanged optical absorption spectrum when the DNA concentration was further increased. Upon binding to DNA, the drug exhibits an 8-nm bathochromic shift in its UV optical absorption as well as a 17% decrease of the maximum extinction in this band (from $\varepsilon_{364}^{max} = 18000$ to $\varepsilon_{372}^{max} = 15000$).

The 8-nm bathochromic shift is indicative of intercalative drug binding to the DNA, as previously observed for the closely related compound 6-(2-dimethylaminoethyl)6H-indolo-(2,3-b)quinoxaline (B-125; Gräslund et al., 1988; Zegar et al., 1989).

When B-220 binds to DNA, its fluorescence intensity increases by about a factor

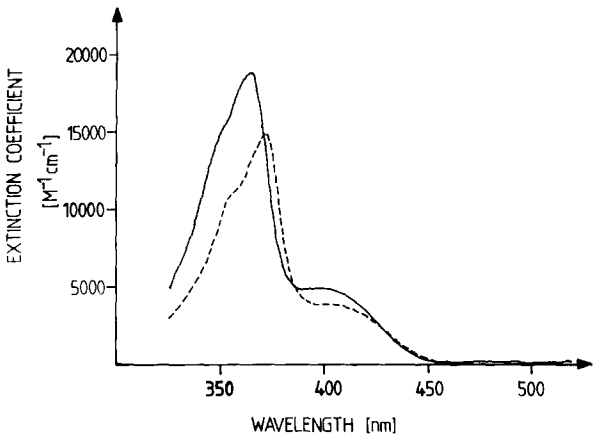

Fig. 5. Optical absorption spectra of 6×10^{-6} M B-220 (——) alone in 10 mM phosphate buffer solution, pH 6.0; (---) mixed with DNA in a 1:60 drug/nucleotide ratio at 23°C.

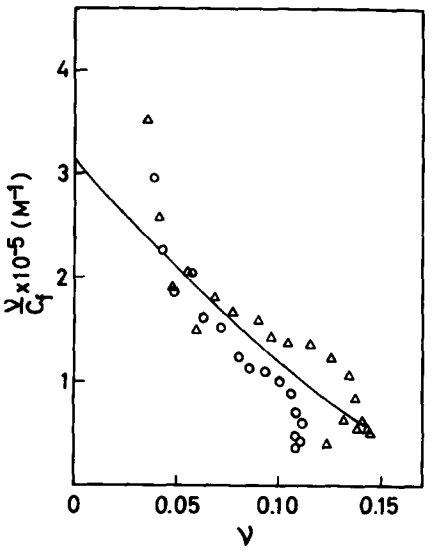

Fig. 6. Scatchard plot of B-220 binding to DNA (ν/C_f versus ν, where ν is the concentration of the bound B-220 per DNA nucleotide unit and C_f is the concentration of free B-220 in solution). The amounts of free and bound drug were evaluated from fluorescence spectra at 23°C for different samples with different total B-220 to DNA nucleotide ratios. Two independent sets of experiments were performed, indicated by (0) and (Δ), with a drug concentration of 3.34×10^{-6} M and DNA in the concentration range 1.54×10^{-6} M to 98.1×10^{-6} M in 10 mM phosphate buffer solution, pH 6.0. (——) is a simulated binding curve, obtained by a minimum least squares fit of a model allowing for neighbor exclusion as described in Zegar et al. (1989).

of 19 compared to the drug alone in aqueous solution. The binding of the drug to DNA was studied by a fluorescence titration technique (cf. Zegar et al., 1989). Fig. 6 shows the results of two separate titration experiments, evaluated as Scatchard plots. Fitting the data to a model allowing for neighbor exclusion (Zegar et al., 1989) gave a binding constant $K = 3.2 \times 10^5$ M^{-1} and the number of bases excluded per binding site (n) = 4. These parameters are also close to those previously observed for B-125 (Zegar et al., 1989).

Discussion

Indoloquinoxaline has previously been shown to have antiviral activity against vaccinia virus (Furusawa et al., 1964). Several different analogs of 6H-indolo-(2,3-b)quinoxaline have also been synthesized and tested for antiviral activity (Harmenberg et al., 1988). The most active compound was shown to be B-220. This compound was originally believed to be a DNA polymerase inhibitor but previous studies (Harmenberg et al., 1988) have indicated that it inhibits a step in the virus cycle before the initiation of viral DNA synthesis. In the present paper, we present evidence that virus replication is substantially reduced by preincubation of HSV-1

with B-220 at 37°C (but not 4°C) and subsequent removal of drug. One explanation of this might be decreased penetration of the virus envelope by B-220 at 4°C. Preincubation of uninfected cells before infection did not affect the virus replication. The results indicate that B-220 interacts with cell-free virus rather than with the host cell, thus distinguishing its mechanism of action from that of nucleoside analogs like acyclovir (ACV), where preincubation of the virus before cell infection does not influence viral growth (J. Harmenberg, unpublished observations). Incubation of B-220 with virus-infected cells and subsequent removal of the drug after 60 min did not restore the virus replication, thus indicating a fast and irreversible antiviral activity of B-220. This property also distinguishes B-220 from ACV, which shows a higher degree of reversibility (Harmenberg and Wahren, 1982). In our experiments B-220 did not show an effect on virus absorption.

B-220 may show some effect on viral penetration at 4°C, at least as measured by the addition of neutralizing antibodies. At 37°C, no effect of B-220 on virus penetration could be shown. The results suggest that the anti-penetration effect of B-220 is limited and does not appear to account for its strong antiviral effect.

The fate of virions labelled in the DNA or proteins was followed. The respective radioactivities associated with nuclear and cytoplasmic fractions were analysed separately. The results indicated that treatment with B-220 arrested both the DNA-associated ([^{3}H]thymidine) and protein-associated ([^{3}H]leucine) radioactivity in the cytoplasmic fractions and only minor amounts reached the nuclear fractions in B-220-treated cells. This is consistent with inhibition of some steps vital for the normal uncoating of the virus.

Previous studies using the closely related drug B-125 have shown this compound to be a DNA intercalating agent (Gräslund et al., 1988; Zegar et al., 1989). Here we show that B-220 exhibits DNA binding characteristics quite similar to B-125. B-220 shows an intercalative binding mode with nearest neighbor site exclusion and an equilibrium DNA binding constant $K = 3.2 \times 10^5\ M^{-1}$ at 22°C, which is close to that observed for B-125 ($K = 2.3 \times 10^5\ M^{-1}$) under similar conditions. The binding constant shows that B-220 intercalation is readily reversible if B-220 is removed from the solution.

DNA binding compounds have traditionally been divided into DNA intercalating and DNA nonintercalating compounds (Zimmer and Wähnert, 1986). Some of the DNA nonintercalating compounds have shown antiviral activity. Examples of these are netropsin and distamycin (reviewed by Zimmer and Wähnert, 1986). These two compounds appear to be too toxic for clinical use. Analogues of netropsin and distamycin appear to be more promising (Lown et al, 1989; Debart et al., 1989).

Of the DNA intercalating substances, tilorone hydrochloride has shown considerable broad-spectrum antiviral activity (Chandra et al., 1979). The mechanism of action is unclear because tilorone hydrochloride also has potent interferon inducing activity in addition to its DNA intercalating properties (Chandra et al., 1979).

B-220 has shown rather prominent toxicity in cell culture (Harmenberg et al., 1988). This toxicity appears to be related to exposure time. At 10–30 μM B-220 reduced cellular DNA synthesis by 50% during a 3-day experiment. A decreased

exposure time decreased the toxicity in cell culture. B-220 exhibited prominent antiviral activity even when the incubation time was as short as 60 min. The cellular toxicity during a 60-min incubation should be minimal.

The acute lethal toxicity of B-220 has been studied in mice and rats after oral and parenteral administration. The maximum non-lethal dose in rats was 800, 1600 and 100 mg/kg after oral, subcutaneous and intravenous administration respectively. Studies on the general toxicity of B-220 after repeated administration for 28 days have been performed in rabbits following dermal application and in rats following intravenous injection. No serious side effects were observed (Malmfors, manuscript in preparation). The in vivo toxicity may therefore be regarded as being low and manageable.

At 12 h after a single intravenous or oral dose of [^{3}H]B-220 at 10 mg/kg, the plasma drug concentration (based on radioactivity) was about 3 μM. This concentration is well above the level producing antiviral activity in vitro.

In conclusion, B-220 appears to be a potent antiviral drug. The putative mechanism of action involves inhibition of steps vital to the viral uncoating process, since our results show that the drug prevents the intracellular release of viral DNA as well as viral protein.

The intercalative DNA binding of B-220 might be connected to these observations, assuming that the DNA-bound drug interferes with the proper interaction between the viral DNA and the viral capsid, leading to disturbances in the uncoating process. Our results have shown that intercalated B-220 rapidly leaves the calf thymus DNA if removed from the medium (binding constant $K = 3.2 \times 10^5\ M^{-1}$). However, B-220 inhibited virus replication in an irreversible manner. Differences in B-220 intercalation between cellular and viral DNA might account for this observation. Further research is needed to elucidate the basis for the antiviral selectivity in the mechanism of action of B-220.

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