VIROLOGY 218, 389–395 (1996)
ARTICLE NO. 0208

SHORT COMMUNICATION

Derived from Hydroquinone: Mechanism of Inhibition

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Received September 5, 1995; accepted February 13, 1996

Humic acids are natural constituents of soil and ground water and mainly consist of mixtures of polycyclic phenolic compounds. A similar complex of compounds with a mean size of about 1000 Da, designated HS-1500, was synthesized by oxidation of hydroquinone. HS-1500 inhibited HIV-1 infection of MT-2 cells with an IC_{50} of 50–300 ng/ml and showed a mean cell toxicity of about 600 μg/ml. Inhibition of HIV-induced syncytium formation was observed at 10–50 μg/ml. Treatment of free and cell-attached HIV with HS-1500 irreversibly reduced its infectivity, whereas the susceptibility of target cells for the virus was not impaired by treatment prior to infection. The HIV envelope protein gp120SU bound to sepharose-coupled HS-1500 and could be eluted by high salt and detergent. HS-1500 interfered with the CD4-induced proteolytic cleavage of the V3 loop of virion gp120SU. Furthermore, binding of V3 loop-specific antibodies was irreversibly inhibited, whereas binding of soluble CD4 to gp120SU on virus and infected cells was not affected. In conclusion, our data suggest, that the synthetic humic acid analogue inhibits the infectivity of HIV particles by interference with a V3 loop-mediated step of virus entry.

The progression of the HIV infection toward AIDS appears to be correlated to the virus burden which is sustained by continuously high virus replication and reinfection of target cells (*1, 2*). Thus, virus-receptor binding and internalization represent promising targets for antiviral therapy. Antibodies to the viral envelope proteins, gp120SU and gp41TM (*3, 4*), to the receptor CD4 as well as soluble forms of the receptor itself (*5*) inhibit primary steps of infection *in vitro.* Yet the type-specificity of V3 loop-reactive neutralizing antibodies (*6, 7*) and the low efficiency of soluble CD4 against primary virus isolates (*8*) limit their therapeutic applicability. In contrast, polyanionic compounds with various chemical structures inhibit a broader spectrum of HIV subtypes (*9*). Here we report on the inhibition of HIV-1 by HS-1500, a stable complex that was synthesized by oxidation of hydroquinone at high pH as described in the European patent No. 0 537 430 B1. HS-1500 consists of aliphatically linked phenolic rings with carboxylic functional groups. The pattern of bands obtained by isoelectric focussing of HS-1500 was remarkably similar to that of natural humic acid complexes (*10*). The lyophilized product has a virtually unlimited shelf life. Neutral aqueous solutions retain their virus inactivating capacity at 4° for at least three months. The compound is related to the previously described polyhydroxy-carboxylates (PHCs) by its complexity, basic chemical structure, and functional groups (*11*). However, HS-1500 is distinct from the group of PHCs by its lower mean molecular mass, a significantly higher efficiency against HIV-1, and an inhibition mechanism that mainly affects virus–cell fusion.

We have first observed the inhibitory potential of HS-1500 in a syncytium assay with MT-2 cells (*12*). The potential mode of inhibition was elucidated by inhibition of virus antigen production and the demonstration of direct influence of HS-1500 on the fusion of infected with uninfected MT-2 cells. The inhibitory concentrations in these assays, which were derived from repeated dose response experiments (not shown) are summarized in Table 1. Upon pretreatment of virus and continuous presence of HS-1500 an inhibitory concentration 50 (IC_{50}) of 0.3 μg/ml was observed in the syncytium assay. The complete inhibition at 10 μg/ml appeared to be irreversible, since syncytium formation was not resumed, when cells of the completely protected microcultures were further subcultured in the absence of HS-1500 for up to 50 days. In the virus antigen assay, an IC_{50} of 0.05 μg/ml was observed, indicating inhibition of virus production. Fusion of infected with uninfected MT-2 cells was sup-

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0042-6822/96 $18.00

TABLE 1

Efficient Inhibition of HIV-1 in Cell Culture by HS-1500

Assay	Syncytia[a]	Antigen[b]	Cell fusion[c]
IC_{50} [μg/ml]	0.3	0.05	10–30
IC_{90} [μg/ml]	2–5	0.3	50–100

[a] 1.25×10^4 MT-2 cells (*33*) were incubated in triplicate for 5 days with 150 syncytium-inducing units of HIV-1_{IIIB} from H9 cells (*34*) and appropriate dilutions of HS-1500 in the wells of microtiter plates. Dose response curves were obtained by counting of syncytia with an inverted microscope 5 days later.

[b] Inhibition of virus replication was measured in the cell-free supernatant by the p24 core protein ELISA (DuPont).

[c] To determine syncytium formation by fusion of infected with uninfected lymphocytes, 10% of HIV-1_{IIIB}-infected and 90% of uninfected MT-2 cells were cocultivated for 2 days with varying concentrations of HS-1500 before syncytia counting.

pressed with an IC_{50} of 10–30 μg/ml and completely inhibited at 300 μg/ml. Thus, for direct interference with HIV-induced cell fusion about 30- to 100-fold more HS-1500 was needed than for inhibition of fusion as a consequence of infection by free virus. The differences in the two syncytium assays suggest that incubation of HIV with HS-1500 may affect an early step of the virus replication cycle, which is more sensitive to the synthetic humate, than the virus-induced syncytium formation itself. Similar differences have been observed with other inhibitors (*13, 14*). HIV-induced cell fusion may be less sensitive to inhibition by HS-1500 because of the higher multiplicity of g120-CD4 interactions and additional cellular proteins such as LFA-1 that are exclusively involved in cell to cell fusion (*15*). The 50% anticellular toxicity (TC_{50}) of HS-1500 was found to be about 600 μg/ml in MT-2 cells by the MTT assay (*16*). Comparison of this result with the IC_{50}-values (Table 1) yields a selectivity index (SI; ratio of IC_{50} to TC_{50}) of 2,000 (syncytium assay) to 12,000 (antigen assay).

In quantitative terms HS-1500 resembles the most efficient polyanionic polysaccharides such as dextran sulfate (*13*) and curdlan sulfate (*17*). In particular, HS-1500 inhibits HIV at least 10 times more efficiently and selectively than the PHCs (*11*), although the compounds are structurally related. Due to the chemical complexity of these two groups of inhibitors, no correlations between their chemical structure and efficacy can be drawn at present.

To study the influence of the synthetic humate on the infectivity of HIV particles, three dilutions of the virus stock in cell culture medium were treated with HS-1500 and then separated from the inhibitor by sedimentation. Separate sedimentation of either untreated virus or of HS-1500 only served as controls. Reduction of the viral infectivity was visualized by the syncytium assay. As shown in Fig. 1A, pretreatment of the virus for 30 min with 0.4 μg/ml of HS-1500 reduced its syncytium inducing activity by more than 50% as compared to the virus stock that was sedimented without prior treatment. HS-1500 could not be concentrated by sedimentation. Because almost the same IC_{50} was observed, when cells and virus were treated simultaneously for 5 days (Table 1), we are tempted to conclude that HS-1500 rapidly inactivated the HIV particle in an irreversible mode. Thus, the virion appeared as the primary and most sensitive target. Inhibition of syncytium formation by continuous presence of HS-1500 in the cell culture can be explained by inactivation of the virus, because it follows the same dose response as the inactivation of cell-free virus. Inactivation of the input virus would also suffice to explain suppression of virus antigen production.

Pretreatment of MT-2 cells with HS-1500 concentrations of 1.5 to 44 μg/ml and washing prior to infection resulted in a 20–30% increase of syncytium formation (data not shown). Therefore, CD4 and other cell surface structures relevant for virus attachment and entry are not significantly involved in the inhibition by HS-1500.

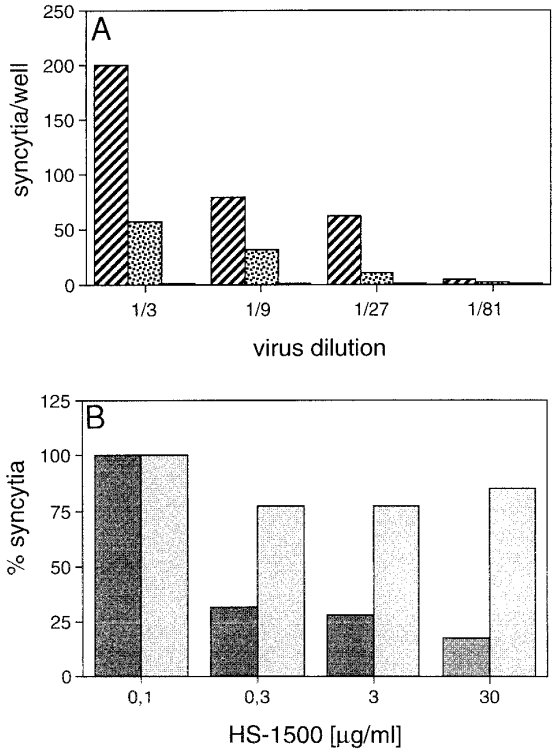

FIG. 1. Inactivation of HIV-1 virions before and after binding to target cells. (A) Pretreatment of the virus. The indicated dilutions of the HIV-1_{IIIB} virus stock were incubated with two concentrations of HS-1500 for 15 min at room temperature (▨, mock-treated; ▫, 0.4 μg/ml; and ■, 40 μg/ml HS-1500). Virus was washed twice before determining the infectious units in aliquots of the resuspended virus pellet by the syncytium assay. (B) Postbinding inhibition of HIV by HS-1500. MT-2 cells were incubated with 200-fold dilutions of virus stock at 0° (■ or 37° (□). Unbound virus was removed by washing of the cells. These were then incubated with the indicated concentrations of HS-1500 for 12 hr and washed again. 1.25×10^4 cells were plated in triplicates into wells of microtiter plates and incubated without HS-1500 for 6 days followed by counting of syncytia.

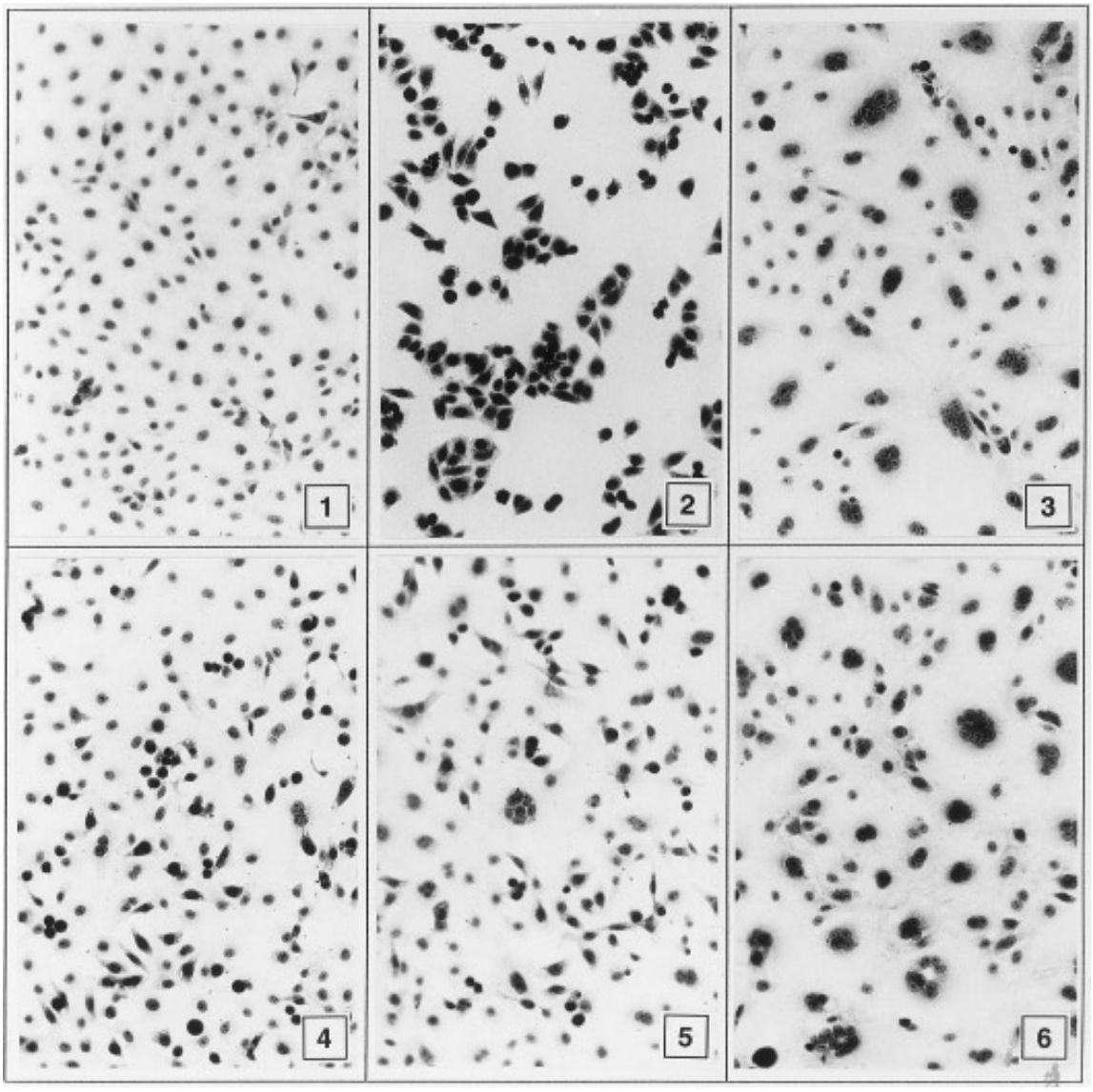

FIG. 2. Inhibition of HIV-envelope-induced syncytium formation. Gp120SU expressing CL4-cells (section 1) and HeLa-CD4 cells (section 2) were incubated alone or mixed at a 2/1 ratio (section 3) and cultured for 20 hr without HS-1500 (sections 1–3). In the other mixed cultures (sections 4–6) HS-1500 was present at 25, 12.5, or 6.25 μg/ml from the start of the coculture. Staining with hemocolor (Merck, Darmstadt) facilitated the detection and counting of multinucleated giant cells.

To discriminate between interference at pre- and post-binding events, virus was incubated with MT-2 cells for 1 hr at 0°, thus, allowing binding without entry (*18*). After removal of unbound virus, cells were treated with HS-1500. The cells were further cultured at 37° for 12 hr in the presence and then 6 days in the absence of inhibitor. Incubation of cells and virus in the cold vs 37° reduced the mean number of syncytia/well from 140 to 45 in the inhibitor-free control cultures (not shown). As illustrated in Fig. 1B, syncytium formation was suppressed to about 25% of the inhibitor-free cultures, when HS-1500 was applied after attachment of virus to the cells in the cold. In contrast, incubation of the cells with virus for 1 hr at 37° prior to treatment with HS-1500 only weakly affected syncytium formation. This temperature shift experiment showed that a step of the infection process occurring within the first hour after virus–cell attachment is inhibitor sensitive. The presence of HS-1500 for 12 hr was sufficient for inhibition, indicating, that the majority of cell-attached virions were irreversibly inactivated. With regard to IC_{50}, HS-1500 is equally effective irrespective of its addition before or after binding of the virus to the target cell (see Table 1). However, cell-attached virus is incompletely inhibited by 30 μg/ml of HS-1500, a concentration sufficient to render cell-free virus preparations noninfectious. Because in contrast to membrane fusion, the CD4-induced conformational change of gp120SU is not influenced by low temperature (*19*), HS-1500 most probably affects a later step of virus internalization.

To further examine a potential direct effect of the inhibi-

tor on the HIV-induced cell to cell fusion, interference was studied in a virus-free model system (*20*). CD4-expressing HeLa cells (*35*) fuse with the HIV envelope protein expressing CL4 cells to form multinucleated syncytia within 20 hr (Fig. 2, sections 1–3). Formation of syncytia was completely inhibited at 25 μg/ml of HS-1500 (section 4). Sporadic syncytia were observed at 12.5 μg/ml (section 5), and the inhibitory effect was diluted out at 6.25 μg/ml (section 6). Counting syncytia/well yielded an IC_{50} of about 10 μg/ml. This value is in the range of the IC_{50} (10–30 μg/ml) estimated for fusion of infected with uninfected cells (Table 1). Thus, fusion-inhibition is independent of virus replication and cell type. Because in infection experiments treatment of the CD4-bearing target cells failed to inhibit syncytium formation, this experiment suggested an interaction of HS-1500 with the viral envelope protein complex.

We therefore studied the binding of gp120SU to an affinity matrix consisting of HS-1500 covalently coupled to sepharose. Culture supernatants of radiolabeled H9/HIV-1_{IIIB} cells served as source of gp120SU. HIV-specific polypeptides were immunoprecipitated by an HIV-specific serum (*36*) from the respective culture supernatants before and after adsorption to HS-1500-sepharose. The autoradiograph in Fig. 3A shows that serum from an AIDS-patient immunoprecipitated polypeptides of about 120, 24, and 18 kDa from the unabsorbed supernatant (lane 5), which represent the respective viral polypeptides gp120SU, p24CA, and p18MA of HIV. Absorption of the culture fluid with HS-1500-bound sepharose preferentially removed gp120SU (lane 1). The bands of the other *bona fide* virion proteins of 18, 24, and 65 kDa are also decreased, suggesting a certain degree of unspecific binding. Absorption with humate-free sepharose had no effect (lane 2). Normal control serum did not recognize any polypeptide in the absorbed or unabsorbed culture supernatants (lanes 3 and 4). Thus exhausting absorption demonstrated that gp120SU selectively bound to HS-1500, whereas other immunoreactive HIV-polypeptides mainly remained in the supernatant. The excess of the serum proteins from cell culture medium present during binding supports the notion of selective binding of gp120SU to HS-1500. To exclude a potential proteolysis or unspecific loss of gp120SU by incubation with HS-1500-sepharose, gp120SU was eluted from the affinity matrix and subsequently immunoprecipitated. Gp120SU could be eluted from HS-1500-sepharose by high salt washing buffer (Fig. 3B, lane 2), by its constituents NP-40 and desoxycholate (lane 4), and to a lesser extent by 0.5 *M* sodium chloride (lane 5), but not by 30% sucrose (lane 3). Altogether these experiments demonstrate, that soluble gp120SU binds reversibly to humate without impairment of major antigenic determinants. The fact that binding was reverted by detergents and high salt suggests a hydrophobic and/or ionic nature of interaction.

The inactivation of the virion was neither caused by interference with gp120-CD4 binding, nor by forced shedding of gp120SU, since pretreatment of virus particles with HS-1500 did not impair attachment of a recombinant form of soluble CD4 (*5*) to virus or detection of gp120SU in virus pellets (data not shown). However, two indepen-

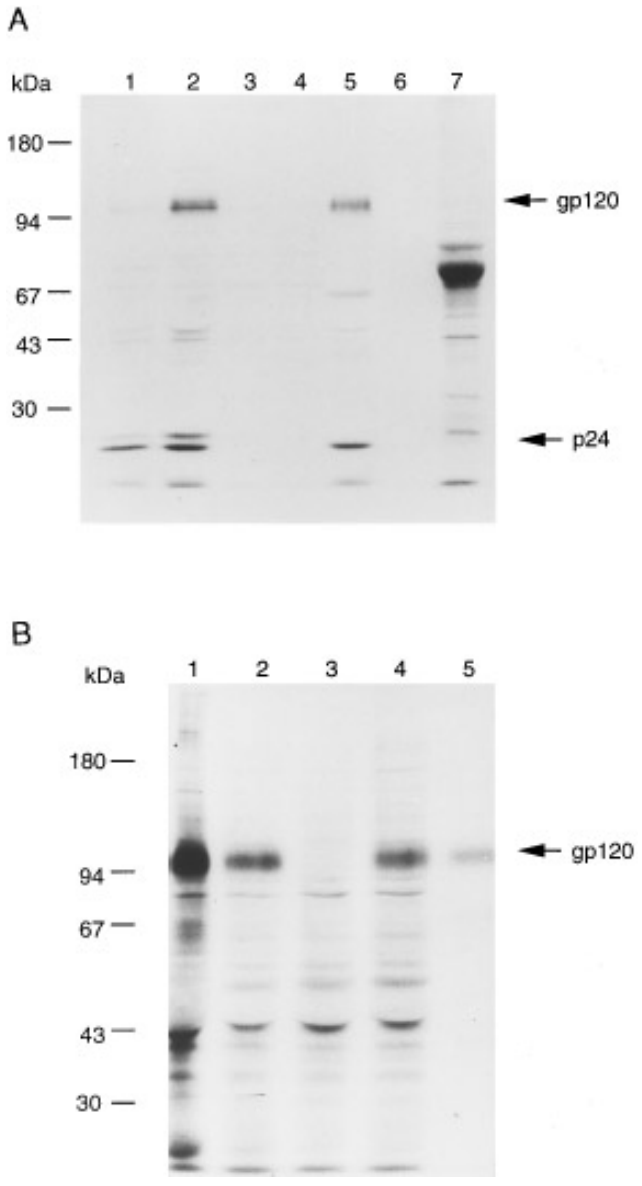

FIG. 3. Specific adsorption of gp120SU to HS-1500 sepharose. HS-1500 was covalently coupled to epoxy sepharose (Pharmacia, Freiburg, Germany) as described by the manufacturer. Culture supernatant of ^{35}S-cysteine-labeled HIV-1_{IIIB}-infected H9 cells was preabsorbed with HS-1500-sepharose (A, lanes 1 and 3) or uncoupled sepharose (A, lanes 2 and 4). Unabsorbed HIV-polypeptides were immunoprecipitated with an AIDS-patient serum (A, lanes 1 and 2) or a negative control serum (A, lanes 3 and 4). For control, supernatant was immunoprecipitated with the same sera prior to absorption (A, lanes 5, 6; B, lane 1). HIV-polypeptides were eluted from HS-1500-sepharose by high salt washing buffer (B, lane 2), 30% sucrose (B, lane 3), 0.5% desoxycholate, and 0.5% NP-40 (B, lane 4), and 0.5 *M* NaCl (B, lane 5). Immunoprecipitates and a sample of the culture supernatant (A, lane 7) were analyzed by SDS–PAGE and autoradiography.

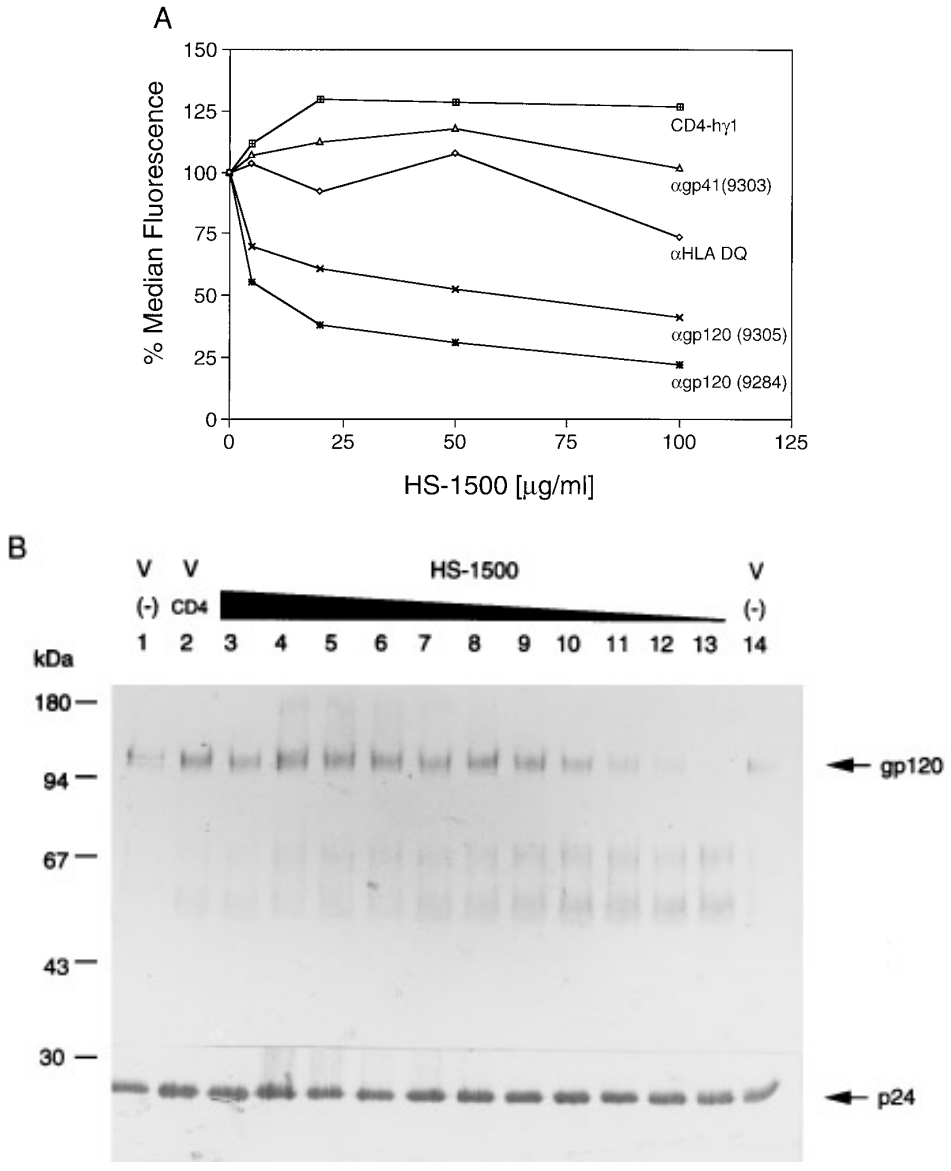

FIG. 4. Interaction of HS-1500 with the V3 loop of gp120SU. (A) Permanent and dose-dependent inhibition of V3 loop-specific antibody binding. HIV-1_{IIIB}-infected H9 cells were treated with the indicated serial dilutions of humate, washed, and incubated individually with mouse monoclonal antibodies specific for either the N-terminal part (NEA9284) or the β-turn of the V3 loop (NEA9305), for gp41 (NEA9303) or HLA-DQ (0416, Dianova Hamburg); (NEA antibodies: DuPont, Bad Homburg). After staining with a FITC-labeled goat-anti-mouse antibody respective FITC-labeled donkey-anti-human antibody for CD4-hγ 1 (*5*), cells were characterized by FACS analysis. Medians of respective histograms are provided as a function of HS-1500 concentrations. (B) Inhibition of the proteolytic cleavage of the V3 loop by HS-1500. HIV-1_{SF2} was incubated with rsCD4 and HS-1500, followed by immunoblot analysis of the proteins gp120SU and p24CA as described previously. Lane 1, untreated virus; lane 2, virus with 20 ng of rsCD4; lane 3 virus with 20 ng of rsCD4 and 20 μg/ml HS-1500; lanes 1–3 no incubation; lanes 4–13, virus with 20 ng of rsCD4 and 20, 10, 5, 2.5, 1.25, 0.6, 0.3, 0.15, 0.075 μg/ml HS-1500, or without inhibitor; lane 14, virus incubated without rsCD4 and HS-1500.

dent observations support the notion that HS-1500 inactivated HIV mainly by binding to the V3 loop of gp120SU: HS-1500 specifically prevented (i) the binding of antibodies to the V3 loop and (ii) the proteolytic cleavage of the V3 loop. HS-1500 significantly inhibited the binding of the V3 loop-specific antibodies 9284 (*21*) and 9305 (*22*) to HIV-infected H9 cells. In this assay, binding of recombinant soluble CD4 was slightly enhanced, the binding of the gp41TM-specific antibody 9303 (*23*) was not affected, and the reaction of the HLA-specific antibody was only weakly impaired at 50 μg/ml of HS-1500 (Fig. 4A). These data illustrate that HS-1500 specifically suppressed the recognition of V3-specific epitopes on infected cells in a dose-dependent manner. Since the gp41TM-specific and the V3 loop-specific antibodies are of the same murine IgG subclass, a direct impairment of the reactivity of antibodies by HS-1500 appears most unlikely. Because the inhibitor-treated cells had been washed prior to incubation with the antibodies, HS-1500 seems to bind permanently to the native form of gp120SU in the multimeric

envelope complex at the cell surface. The results of this and the temperature shift experiment (Fig. 1B) suggest that HS-1500 by binding to the V3 loop interferes with the same function of gp120SU as V3 loop-specific antibodies (*24*).

Incubation of highly purified virions with soluble CD4 resulted in cleavage of the V3 loop by a protease inherent to these virus preparations. The cleavage could be blocked by monoclonal antibodies to the crest of the V3 loop (*25*). Incubation of virus with soluble CD4 and HS-1500 and subsequent analysis of viral proteins by immunoblotting as described (*25*) demonstrated that the presence of HS-1500 interfered with the cleavage of gp120SU into fragments of approximately 70 and 50 kDa (Fig. 4B). Spontaneous cleavage was observed in the purified particles (Fig. 4B, lane 1) prior to incubation, and cleavage was enhanced by incubation at 37° even without CD4 (lane 14). Due to this background, the efficacy of the CD4-induced cleavage can be evaluated more precisely from the decrease of the gp120SU band rather than from the appearance of the cleavage products. Incubation of the virus preparation with 20 ng of recombinant soluble CD4 (rsCD4) yielded complete cleavage (lane 13). Addition of HS-1500 in twofold serial dilutions inhibited the cleavage in a dose-dependent manner with an IC_{50} between 0.3 and 0.075 μg/ml. Thus, inhibition of the cleavage and inhibition of HIV infectivity are observed at about the same concentration of HS-1500, which further supports the hypothesis that interaction of HS-1500 with the V3 loop represents the predominant inhibition mechanism of the HS-1500 complex.

In further support of a direct interaction of HS-1500 with the V3 loop, we observed a positive correlation between the surplus of basic vs acidic amino acids in the V3 loop and the sensitivity of individual virus isolates to inhibition. Along this line, HS-1500 inactivated HIV-1_{IIIB} and HIV-1_{NDK} with similar efficiency. The surplus of 9 basic amino acids is conserved in their V3 loops, while in the V3 loop amino acid sequences only 17 of 38 positions are identical (*26, 27*). In agreement, HIV-1_{BaL}, characterized by a surplus of only 3 basic amino acids in the V3 loop, is inhibited at a 30–100 times higher IC_{50} although the V3 loop sequences of HIV-1_{BaL} and HIV-1_{IIIB} are more closely related than those of HIV-1_{NDK} and HIV-1_{IIIB}. These data also suggest that HS-1500 inhibits a broader range of HIV-isolates than the mainly type-specific V3 loop-reactive antibodies (*28*). Interestingly, the high cytopathogenicity and efficient replication in lymphocytes of HIV-1 isolates appears to be correlated to a certain excess of basic vs acidic amino acids in the V3 loop (*29, 30*). Therefore, development of resistance to HS-1500 may select for HIV variants with low cytopathogenicity.

For other polyanionic HIV-inhibitors interaction with the V3 loop is suggested as one of several potential mechanisms of HIV-inhibition. However, all polyanionic polysaccharides tested so far fail to inactivate free virus (*17, 31, 32*). Even the PHCs, which resemble HS-1500 structurally, inhibit HIV by a different mechanism. Most PHC preparations efficiently interfere with binding of gp120SU to CD4 and block the binding of virus to cells completely (*11*), whereas HS-1500 slightly increases the binding of recombinant soluble CD4 to HIV-infected cells and mainly inactivates the virus particle.

In conclusion, we describe the inhibition of HIV-1 by a synthetic complex of compounds related to natural humic acids. Isolation of the active component(s) of the mixture by affinity binding to V3 loop oligopeptides could yield a valuable tool to study the functions of the V3 loop within the process of virus–cell fusion. Therapeutic approaches involving a mixture of closely related compounds rather than a singular compound of defined chemical structure may represent a suitable strategy against a variable target such as HIV. The low toxicity of HS-1500 in various mammalian species (A. Haase, personal communication) and lack of mutagenicity (U. N. Riede, unpublished) will facilitate its evaluation in animal models for human AIDS.

ACKNOWLEDGMENTS

We thank Monika Konanz for excellent technical assistance. We are indebted to R. C. Gallo, M. Popovic, V. Bosch, K. Karjalainen, A. Traunecker, and H.-J. Schlaeger for providing cell lines and to O. Haller and O. Herchenröder for critical reading of the manuscript. This work was supported by the Forschungsschwerpunkt Baden-Württemberg (AZ II-7532).

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