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Efficiency of Nanoparticles as a Carrier System for Antiviral Agents in Human Immunodeficiency Virus-Infected Human Monocytes/Macrophages In Vitro†

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Polyhexylcyanoacrylate nanoparticles loaded with either the human immunodeficiency virus (HIV) protease inhibitor saquinavir (Ro 31-8959) or the nucleoside analog zalcitabine (2′,3′-dideoxycytidine) were prepared by emulsion polymerization and tested for antiviral activity in primary human monocytes/macrophages in vitro. Both nanoparticulate formulations led to a dose-dependent reduction of HIV type 1 antigen production. While nanoparticle-bound zalcitabine showed no superiority to an aqueous solution of the drug, a significantly higher efficacy was observed with saquinavir-loaded nanoparticles. In acutely infected cells, an aqueous solution of saquinavir showed little antiviral activity at concentrations below 10 nM, whereas the nanoparticulate formulation exhibited a good antiviral effect at a concentration of 1 nM and a still-significant antigen reduction at 0.1 nM (50% inhibitory concentrations = 4.23 nM for the free drug and 0.39 nM for the nanoparticle-bound drug). At a concentration of 100 nM, saquinavir was completely inactive in chronically HIV-infected macrophages, but when bound to nanoparticles it caused a 35% decrease in antigen production. Using nanoparticles as a drug carrier system could improve the delivery of antiviral agents to the mononuclear phagocyte system in vivo, overcoming pharmacokinetic problems and enhancing the activities of drugs for the treatment of HIV infection and AIDS.

In addition to the $CD4^+$ T lymphocytes, which are a major target for infection by the human immunodeficiency virus (HIV), cells of the mononuclear phagocyte system also play a decisive role as a reservoir for HIV. In tissues such as the lung and the brain, HIV is located primarily in macrophage-like cells (i.e., alveolar macrophages and microglia, respectively) (24, 51). In contrast to $CD4^+$ T cells, in which HIV replication is proliferation dependent and finally leads to cell death, macrophages in a mature nonproliferating but immunologically active state can be productively infected with HIV type 1 (HIV-1) and HIV-2 (10, 22, 31, 48). Altered cellular functions in the macrophage population may contribute to the development and clinical progression of AIDS. In addition, aberrant cytokine secretion may lead to a cascade of secondary events that are likely to cause the wasting syndrome, neurological manifestations of disease, and changes in T-cell responses (i.e., switching from a T helper 1 to a T helper type 2 activity) (4, 9, 26). A large proportion of AIDS patients show severe HIV encephalitis with diffuse neuronal damage which is thought to be mediated by viral proteins and/or factors with neurotoxic activity (i.e., cytokines). This can occur through proinflammatory cytokines or by HIV-1-specific proteins secreted from cells of the mononuclear phagocyte system, including brain macrophages, microglia, and multinucleated giant cells, which have been shown to be productively infected with HIV (9, 11).

There is evidence that, apart from having a function in the pathogenesis of the disease, cells of the macrophage lineage are vectors for the transmission of HIV. The placental macrophage is likely to be the primary cell type responsible for vertical (maternofetal) transmission of HIV (27). For mucosal transmission it was found that an important property of the transmitted HIV variant is its ability to infect macrophages (28). The phenotypic characterization of virus populations which were transmitted sexually or vertically revealed a selection of variants with a predominant tropism for macrophages in the recipient (46). Because of the important role of cells of the monocyte/macrophage (Mo/Mac) lineage in the pathogenesis of HIV, fully effective anti-HIV therapy must reach Mo/Mac in addition to other target cells.

Each step in the retroviral infection cycle represents a potential target for antiviral chemotherapy (29). Various substances with different chemical structures and mechanisms of interference with the replication of HIV have already been described as antiviral agents (15, 45). One basic requirement for the successful use of any drug against retrovirus-related diseases is sufficient bioavailability. However, many promising antiviral agents are unfortunately compromised by disadvantageous physicochemical properties which lead to poor biodistribution and insufficient cellular uptake (38, 39). Hence, the failure of existing approaches may include a failure to maintain adequate drug levels at the site of viral replication over extended periods of time (15, 32). Moreover, the well-known adverse reactions and side effects of antiviral treatment are often related to the accumulation of the drug at inappropriate sites (53).

Special drug carrier systems and dosage forms, such as nano-

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† Dedicated to Ernst Mutschler on the occasion of his 65th birthday.

particles and liposomes, hold the promise of overcoming these pharmacokinetic obstacles to bring about successful therapy (35, 39, 43). Nanoparticles are stable, solid colloidal particles consisting of macromolecular material and ranging in size from 10 to 1,000 nm. Drugs can be adsorbed on the particle surface or can be entrapped or dissolved in the particle matrix (18). Various techniques for the preparation of drug-loaded nanoparticles have been described and successfully used for a large number of substances (1, 19). Following intravenous administration, nanoparticles are known to accumulate in the tissues of the MPS because of phagocytosis by Mo/Mac (21, 44, 49). This results in a specific enrichment in macrophage-containing organs like the liver and spleen. Infection of these cells with HIV does not abrogate their phagocytic activity in vitro or in vivo (42). Therefore, nanoparticles represent an interesting carrier system for the specific transport of antiviral agents to Mo/Mac in an attempt to reduce the required dose, to minimize toxicity and side effects, and to improve the delivery of substances which have insufficient intracellular uptake. With this drug targeting technology, substances whose development has been halted because of their unfavorable pharmacokinetic properties could potentially be made available for the treatment of HIV-related diseases.

Nucleoside analogs were the first substances approved for anti-HIV treatment. They have shown significant antiviral activity with high selectivity in vitro as well as clinical activity on a large scale. After intracellular activation, these compounds act as DNA chain terminators or competitive inhibitors of the virus-associated reverse transcriptase. However, all such drugs have limited effectiveness in vivo because of the rapid emergence of viral resistance (23, 40). While zidovudine has been regarded as the primary anti-HIV treatment, zalcitabine and didanosine, which are both 2′,3′-dideoxynucleosides, are utilized for patients that are no longer responding to zidovudine or are intolerant of it, as well as in combination therapy (16). Dideoxynucleosides in general exhibit effective pharmacokinetic properties (15, 45, 53). Their in vitro activities vary depending on the cell culture system used for testing and on the intracellular phosphorylation rate (34).

Whereas nucleoside analogs are only effective when present before the onset of infection, inhibitors of the virus-encoded protease can also block the replication of HIV in chronically infected cells (25). The first protease inhibitor approved for clinical treatment was saquinavir, a peptide-based hydroxyethylamine transition state mimetic. Saquinavir displays extreme potency in vitro against HIV-1 and HIV-2 in various cell culture systems, with 50% inhibitory concentrations (IC_{50}s) in the nanomolar range (6, 36, 37). Unfortunately, the solubility and bioavailability of this drug are relatively poor, and high doses have to be administered in order to achieve antiviral effects in patients. In clinical trials in which saquinavir was administered as monotherapy as well as in combination with zidovudine alone and with both zidovudine and zalcitabine, positive effects on surrogate markers, such as an increase in the $CD4^+$ cell count and a reduction of the plasma viral load, were demonstrated (17, 47, 52).

In this study, nanoparticles prepared by emulsion polymerization from polyhexylcyanoacrylate were loaded with the nucleoside analog zalcitabine or the HIV protease inhibitor saquinavir. These nanoparticulate preparations of anti-HIV drugs were tested for antiviral activity in primary human Mo/Mac in vitro, and the results were compared with those obtained in studies with the free drugs.

MATERIALS AND METHODS

Isolation and cultivation of Mo/Mac. Fresh human mononuclear cells from healthy blood donors were separated on a Ficoll density gradient (density, 1.077 g/ml) by centrifugation at 400 × *g* 30 min at room temperature. Mononuclear cells (3×10^6/ml) were introduced in rectangular bags made of hydrophobic Teflon membranes (Biofolie 25; Heraeus, Hanau, Germany) and cultivated in RPMI 1640 medium supplemented with 5 μM 2-mercaptoethanol, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 4 mM L-glutamine, 1 mM sodium pyruvate, minimal essential medium vitamins, minimal essential medium nonessential amino acids, and 5% (vol/vol) heat-inactivated AB group serum. The cells were cultivated until day 6 and then plated out into 24-well plates. After 30 min of incubation to allow adherence, the nonadherent cells were washed off by vigorous pipetting with 2 ml of medium. The adherent cell layer consisted of up to 95% macrophages (2×10^5 to 3×10^5 macrophages per well) as judged by morphology, nonspecific esterase staining, and expression of the CD14 antigen. The adherent Mo/Mac were further cultivated in 1.5 ml of supplemented RPMI 1640 medium.

Preparation and characterization of nanoparticles. Polyhexylcyanoacrylate nanoparticles were prepared by emulsion polymerization of the monomer in an acidic medium. For preparation of zalcitabine-loaded nanoparticles, 100 μl of hexyl-2-cyanoacrylate (Sichelwerke, Hannover, Germany) was added to an aqueous solution of 10 mM hydrochloric acid (10 ml, pH 2.0) containing 10 mg of zalcitabine (a kind gift from H. Schott, University of Tübingen, Tübingen, Germany) and 1% poloxamer 188 (Erbslöh, Düsseldorf, Germany), a nonionic surfactant. After 6 h of polymerization at room temperature at a stirring speed of 500 rpm, the nanoparticulate preparation was neutralized with an aqueous solution of 1.0 N sodium hydroxide. This raw nanoparticle suspension was ultracentrifuged at 30,000 rpm for 60 min (type L8-60M centrifuge, SW-41 rotor, Beckman), and the resulting pellet was resuspended by sonication in supplemented RPMI 1640 medium for incubation in cell culture.

For the preparation of saquinavir-loaded nanoparticles, 100 μl of hexyl-2-cyanoacrylate was added to a clear aqueous solution of 0.1 M hydrochloric acid (5.0 ml, pH 1) in 5.0 ml of ethanol (96%) containing 5 mg of saquinavir (Roche Products Ltd., Welwyn Garden City, Hertfordshire, United Kingdom), as well as 1% sodium sulfate and 2% poloxamer 188 for stabilization. After 2 h of polymerization at room temperature at a stirring speed of 650 rpm, the nanoparticulate preparation was neutralized (see above), and stirring was then continued for another 60 min. This nanoparticle suspension was ultrasonicated and diluted in supplemented RPMI 1640 medium for incubation in cell culture. Unloaded nanoparticles were prepared in the same manner but with no drug being added to the polymerization medium.

The entrapment efficacy (which indicates the proportion of drug incorporated during the manufacturing process) was evaluated for both drugs, zalcitabine and saquinavir, by UV photometry. Nanoparticles were separated by ultracentrifugation, and the amount of unbound drug in the resulting supernatant was measured photometrically. As a measure of size distribution, particle size and polydispersity were determined by photon correlation spectrometry with a BI-200 SM goniometer, version 2.0 (Brookhaven Instruments Corp., Holtsville, N.Y.).

Prophylactic effect of zalcitabine-loaded nanoparticles on HIV infection of Mo/Mac. Mo/Mac were incubated on day 6 with zalcitabine-loaded nanoparticles at 37°C for 24 h. All doses of the drug were tested in triplicate. Excess nanoparticles were removed after the incubation period. The cells were infected with 100 μl of HIV-$1_{D117III}$ stock virus per well; this virus, a monocytotropic strain of HIV-1, replicates to high titers in Mo/Mac (41). This stock virus suspension consisted of a free-virus-containing supernatant derived from an HIV-infected macrophage culture (reverse transcriptase activity, 9.0×10^5 cpm/ml/90 min). The virus production in Mo/Mac was monitored over a 24-h period in each well on day 17 by using a commercially available HIV antigen enzyme-linked immunosorbent assay (ELISA) (Innotest; Innogenetics, Antwerp, Belgium). Day 17 was selected since cultures consistently produced high levels of antigen by this time point. The prophylactic effect of the zalcitabine-loaded nanoparticles was compared with that of an aqueous solution of the drug.

Antiviral activity of saquinavir-loaded nanoparticles in acutely HIV-infected Mo/Mac. One day after being transferred to 24-well plates (i.e., on day 7 of culture), Mo/Mac were infected with 100 μl of stock virus suspension per well (see above). On day 10, the medium was changed and the Mo/Mac were exposed to various concentrations of drug-loaded nanoparticles. All doses of the antiviral compound were tested in triplicate. Each culture medium was replenished with the identical medium every 3 days during antiviral treatment of the cells. The virus production was monitored on day 17 as described above. The antiviral activity of drug-loaded nanoparticles was compared with that of an aqueous solution of the drug and that of a simple mixture of an aqueous solution of the drug and unloaded nanoparticles.

Antiviral activity of saquinavir-loaded nanoparticles in chronically HIV-infected Mo/Mac. Mo/Mac were infected on day 7 with 100 μl of stock virus per well (see above), and the medium was changed every 3 days. On day 25 of the culture, the Mo/Mac were exposed to drug-loaded nanoparticles for 24 h. The virus production in Mo/Mac was monitored in 24-h increments in each well by using an HIV antigen ELISA (see above) on days 25 through 29. The antiviral activity of the drug-loaded nanoparticles was compared with that of an aqueous solution of the drug (free drug).

TABLE 1. Antiviral activity of zalcitabine and saquinavir in HIV-infected Mo/Mac

Antiviral agent	$IC_{50} \pm SD$ (nM)		
	Free-drug solution	Nanoparticulate preparation	Free-drug solution plus unloaded nanoparticles
Zalcitabine[a]	91.5 ± 48	117 ± 80	ND[b]
Saquinavir[c]	4.23 ± 0.15	0.39 ± 0.23	5.31 ± 0.75

[a] Prophylactic treatment of Mo/Mac 24 h before HIV infection.
[b] ND, not determined.
[c] Antiviral treatment of acutely infected Mo/Mac 3 days after HIV infection.

Influence of unloaded nanoparticles on viability and HIV replication in Mo/Mac. The colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to determine the viability of Mo/Mac; this assay measures the amount of MTT formazan produced as a marker for cell viability (30). This test was performed under the same conditions as described for determination of antiviral activity in freshly infected Mo/Mac (see above).

In each experiment in which the antiviral activity of a nanoparticulate preparation of an antiviral drug was tested, unloaded particles were tested at corresponding concentrations to determine their influence on HIV replication (antigen production).

RESULTS

Characteristics of nanoparticulate preparations. Drug-loaded nanoparticles were prepared by an emulsion polymerization technique. While 50 to 60% of the saquinavir that was added was found to be trapped by the carrier system, only 15 to 20% of the more hydrophilic drug zalcitabine could be bound to nanoparticles. Drug-loaded nanoparticles possessed mean diameters of approximately 200 nm (with a polydispersity of 0.22) for zalcitabine and 475 nm (with a polydispersity of 0.05) for saquinavir.

Unloaded nanoparticles, when tested at concentrations corresponding to those of the nanoparticle preparations of both drugs, had no effect on the cell viability and virus production of HIV-infected Mo/Mac, as determined by comparison with cell viability and virus production results for untreated cells (data not shown).

Prophylactic effect of zalcitabine-loaded nanoparticles on HIV infection of Mo/Mac. To determine the prophylactic effect of zalcitabine in Mo/Mac, cells were preincubated with the free drug or drug-loaded nanoparticles for 24 h before HIV infection. HIV antigen production was monitored in the cell culture supernatant on day 17 of cultivation. Table 1 shows the antiviral activity of zalcitabine-loaded nanoparticles in HIV-infected Mo/Mac expressed as the calculated IC_{50} of 117 nM. When this value was compared with the free-drug IC_{50} of 91.5 nM, it was concluded that there was no benefit to the administration of the nanoparticulate preparation of this drug.

Antiviral activity of saquinavir-loaded nanoparticles in acutely HIV-infected Mo/Mac. For assessment of antiviral efficacy, treatment of the culture with various formulations of saquinavir was started 3 days after HIV infection of Mo/Mac, and antigen production over a 24-h period was measured in each culture on day 17. Optimal antiviral activity (inhibition up to 100%) was observed in a concentration range from 10 to 100 nM for the free-drug solution, the nanoparticle-bound drug, and the simple mixture of the free-drug solution and unloaded nanoparticles. Whereas the free-drug solution and the simple mixture of nanoparticles with a free-drug solution did not show any antiviral activity at concentrations between 1 and 0.1 nM, the nanoparticle-bound drug showed antiviral activity at 1 nM (80% inhibition) and a still-significant inhibitory effect at 0.1 nM (Fig. 1). This indicates a more than 10-fold increase in antiviral activity; an IC_{50} of 0.39 nM was determined for the nanoparticulate preparation, whereas the free-drug solution and the simple mixture of a free-drug solution and unloaded nanoparticles had IC_{50}s of 4.23 and 5.31 nM, respectively (Table 1).

Antiviral activity of saquinavir-loaded nanoparticles in chronically HIV-infected Mo/Mac. On day 25 after culturing, chronically infected Mo/Mac that had reached their highest level of virus production (approximately 15 ng of antigen per ml over a 24-h period) were incubated for 24 h with the nanoparticulate preparation or a free-drug solution containing 100 nM saquinavir. Whereas the free-drug solution had no inhibitory effect under these conditions, a 35% reduction of HIV antigen production was obtained with the particle-bound drug (Fig. 2). It is noteworthy that after termination of treatment under these conditions, a sharp increase of antigen production occurred which exceeded production in the untreated control culture. This might be related to an accumulation of precursor proteins when the viral protease is partially blocked. When the inhibitor is removed from a culture, the accumulated polyproteins are processed and mature virions can be released from the cells.

DISCUSSION

Cells of the Mo/Mac lineage have been recognized as major targets for HIV in the body and represent an important virus reservoir in a wide range of tissues in HIV-infected individuals. Therefore, they play a crucial role in the pathogenesis and progression of HIV-induced disease, which must be taken into account for any novel antiviral therapy.

The rationale for our study was based on the specific delivery of antiviral agents to the site of viral replication in human Mo/Mac. We evaluated the use of nanoparticles as a colloidal carrier system for two drugs with different modes of action against HIV in an antiviral assay in vitro. With a nanoparticle-

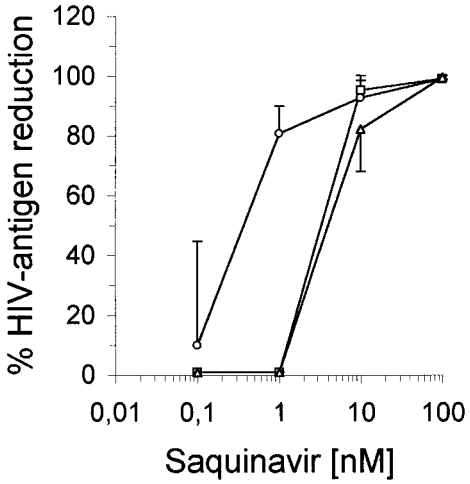

FIG. 1. Antiviral activity of saquinavir-loaded nanoparticles in acutely HIV-infected Mo/Mac. Cells cultured in 24-well plates were infected with HIV-1 and, 3 days later, exposed to various concentrations of drug-loaded nanoparticles (○), an aqueous solution of saquinavir (□), or a simple mixture of unloaded nanoparticles and an aqueous drug solution (△). On day 17 of cell culture, virus production over a 24-h period was monitored by measuring the HIV antigen content in the cell culture supernatant by ELISA. At this time point, virus production in untreated controls has reached a level of approximately 8 ng of antigen per ml within 24 h. The results are presented as percent reductions of HIV antigen compared with that in infected, untreated controls. The bars indicate errors of the mean ($n = 3$).

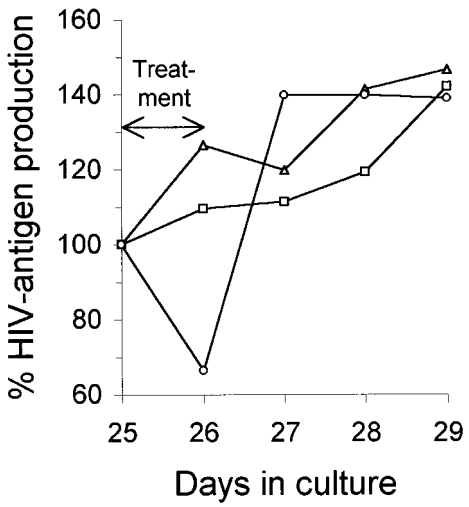

FIG. 2. Antiviral activity of saquinavir-loaded nanoparticles in chronically HIV-infected macrophages. Cells cultured in 24-well plates were infected with HIV-1 on day 7 after the culture was started. When the mature macrophages had reached their highest level of virus production, on day 25 after the start of the culture (approximately 15 ng/ml within 24 h), they were incubated for 24 h with the nanoparticulate preparation or a free-drug solution containing 100 nM saquinavir. The virus production over a 24-h period was monitored daily for 4 days by measuring the HIV antigen content in the cell culture supernatant by ELISA. The resulting HIV antigen values for saquinavir-loaded nanoparticles (○), an aqueous solution of saquinavir (□), and an infected, untreated control (△) are expressed as the percentage of total HIV antigen production in the 24-h period before antiviral treatment on day 25 of the culture.

bound inhibitor of the reverse transcription process, the nucleoside analog zalcitabine, we were able to show good antiviral activity in Mo/Mac, but there was no advantage to the nanoparticulate delivery of the drug in relation to the use of the free drug. In contrast to the nucleoside analog, up to 10-fold improvement in potency was achieved when the nanoparticle-bound HIV protease inhibitor saquinavir was tested in acutely infected cells.

As is to be expected with a nucleoside analog, only protection of uninfected cells is possible because such compounds act at a preintegration stage of the viral replication cycle (29). Infected cultures cannot be treated effectively. Zalcitabine displayed an IC_{50} in the nanomolar range when present before infection of Mo/Mac in culture. This level of activity is in accordance with the data of other groups who used comparable cell culture systems for testing antiviral agents in human Mo/Mac (33). However, no enhancement of activity was seen when the drug was bound to nanoparticles. Nucleoside reverse transcriptase inhibitors obviously do not profit from this drug carrier since they apparently already show maximal cellular uptake. These findings with zalcitabine correlate well with the data of our previously published zidovudine studies (3). Zalcitabine enters the cell not only by nonfacilitated diffusion through the cellular membrane; distinct transport mechanisms for nucleosides and nucleobases are also at least partially utilized (5, 8). Moreover, the limiting factor for the availability of the active antiviral drug is its intracellular conversion to the corresponding 5′-mono-, di-, and triphosphate derivatives (5, 12). Therefore, the efficiency of biological activation of nucleoside analog prodrugs may be of greater importance for antiretroviral activity than the affinity of resultant nucleoside triphosphates for the reverse transcriptase. In addition, the phosphorylation pattern of 2′,3′-dideoxynucleosides is also believed to differ from one cell type to another (7).

Inhibitors of the virus-encoded protease act at a postintegration stage of the viral replication cycle by blocking the maturation of infectious virions. Therefore, they should be suitable for HIV inhibition in cells that already contain proviral DNA, especially chronically and productively infected cells. Furthermore, in acutely infected cells, protease inhibitors are effective at reducing virus spread throughout the culture, which is analagous to the in vivo situation, where the rapid turnover and permanent passage of virions in lymphoid tissue to newly differentiating immune cells from bone marrow (i.e., $CD4^+$ T lymphocytes) must be interrupted (14, 50).

Saquinavir has been described as a very potent protease inhibitor with a high binding affinity for this HIV-encoded enzyme (the K_i for HIV-1 protease is approximately 0.12 nM) (36). This compound has been found to be active in various cell culture systems, including primary human Mo/Mac (32, 36). In clinical studies to date, doses of up to 600 mg of saquinavir administered three times per day have been used to achieve antiviral effects in treated patients (17, 52). The physicochemical properties of this substance, including its poor solubility in both water and lipids, may limit its availability at the cellular level (38, 52), and concentrations that are active in chronically infected Mo/Mac under in vitro conditions may not be reached in some affected tissues and target cells in HIV-infected patients. Therefore, the possibility of improving the antiviral efficacy of a protease inhibitor by using nanoparticles could be of great importance, especially in relation to reaching cells that are already productively infected. As we were able to show in our study, within a certain concentration range, an antiviral effect in chronically infected Mo/Mac could be obtained only by treatment with nanoparticulate-bound saquinavir. These findings indicate that the cellular uptake of protease inhibitors is the limiting factor for their antiviral activity in Mo/Mac. Thus, in contrast to nucleoside analogs, protease inhibitor effectiveness profits from the use of a nanoparticulate formulation.

These promising in vitro results, however, do not measure the influence of a carrier system on the biodistribution of antiviral drugs in vivo. That will have to be analyzed in animal models. The approach of modifying the pharmacokinetic properties of a compound by entrapment in a colloidal carrier, i.e., increased delivery to specific organs and tissues, offers the opportunity for optimized antiviral therapy and gives additional support to this concept. With specific nanoparticulate preparations of drugs, enhanced peroral bioavailability as well as passage of peptides through the blood-brain barrier resulted (2, 20). Furthermore, in a recent study with liposome-encapsulated didanosine, effective targeting of this substance to lymphoid tissue has been described (13). This might be of importance for an improved efficacy of anti-HIV agents since the lymph nodes play a major role in the immune pathogenesis of HIV infection.

In conclusion, nanoparticles hold the promise of improving the potency of anti-HIV agents by enhancing cellular uptake and by enrichment of these agents in HIV target cells and relevant tissues.

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