Biomolecular and pharmacokinetic studies on drug delivery preparations with dual activity on HIV-1

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11 SUMMARY

The work described in this thesis focuses on the features of negatively charged human serum albumins (NCAs) with the aim to produce intrinsic active drug carriers for anti-HIV-1 therapy. The NCAs, earlier developed in our laboratory, are proteins in which extra negative charges are introduced by modification of the ε-NH_2 groups of the lysines either by succinylation or aconitylation. This results in the prototypes Suc-HSA and Aco-HSA, modified proteins that displayed a high potency against HIV-1 replication with IC_{50} values in the low nanomolar range. Preliminary studies already indicated that the mechanism of antiviral action was explained by disruption of the early virus binding/fusion events in the replication cycle of HIV-1. To characterize the multiple mechanisms of the NCAs involved in inhibition of HIV-growth and to investigate the possibilities of using these antivirally active proteins as a drug-targeting device for the antiviral drug AZT the studies described in this thesis were performed. The project was financially supported by the Dutch Organization for Scientific Research (NWO). The studies were predominantly performed in the laboratories of the section of Pharmacokinetics and Drug Delivery of the Groningen Institute of Drug Studies (GIDS) as part of the research school Groningen Utrecht Institute for Drug Exploration (GUIDE), the Netherlands. Through structural collaborations with the Central Laboratory of the Netherlands Red Cross Blood Tranfusion Service, Amsterdam (Dr. J.G. Huisman and Dr. H. Schuitemaker and coworkers), the department of Virology of the Erasmus University, Rotterdam (Drs. M. Schutten and Prof.Dr. A.D.M.E. Osterhaus) and the Rega Institute for Medical Research, University of Leuven, Belgium (Dr. R. Pauwels, Dr. M-P de Bethune, Dr. M. Witvrouw and Prof.Dr. E. de Clercq and coworkers) the antiviral studies, described in this thesis, could be conducted.

Chapter 1 starts with a summary of some of the advantages of using drug targeting including "dual targeting" in antiviral therapy. Dual targeting implies the use of carriers that, apart from delivering adequate amounts of the coupled drug to the wanted cell type in the body, also contributed themselves to the required therapeutic effect. In addition to

the presently known aspects of the AIDS syndrome and the description of the replication cycle of HIV-1, the possibilities and limitations of drug intervention in this disease are discussed. Special attention is given to antiviral compounds acting at the early phases in the replication cycle of HIV. The properties and potentials of soluble CD4, monoclonal antibodies, certain synthetic peptides, polyanionic compounds and the NCAs are described. Furthermore the potential use of these compounds as dual targeting devices is discussed.

The mechanism of antiviral action of the NCAs is described in the **chapters 2, 3 and 4**. Components of the viral particle, especially the viral envelope proteins gp120 and gp41, as well as receptors on the target cells are involved in the penentration of HIV-particles in the particular target cells. In the **chapters 2 and 3** we describe the studies on the interaction of the NCAs with gp120 employing synthetic peptides composed of 15 up to 36 amino acids and corresponding to different parts of the gp120 envelope protein of HIV-1. Among the gp120 peptides tested, high affinity binding of the NCAs was observed with the so called "V3 loop" (amino acids 296-330) and the C-terminal part of gp120, domains both involved in post-binding steps of virus and cells. A higher number of negatively charged residues in the albumins resulted in higher binding affinities. The NCAs mainly bound to the tip of the V3 loop (GPGRAF sequence), a sequence that is strongly positively charged. Yet, apart from the overall charge, the actual amino acid composition appeared to be important for binding as well, since less positively charged fragments at the C-terminal part of the loop showed a stronger affinity for the NCAs as compared to the N-terminal part of the loop. After the initial gp120-CD4 binding, but prior to viral entry, the V3 loop is suppossed to be cleaved by a cellular protease. In order to mimic this situation *in vitro*, cleaving of the V3 loop at the GPGRAF domain was performed using thrombin, and this resulted in a complete loss of affinity for the NCAs as compared with the uncleaved loop. Binding of the NCAs to the V3 and C-terminal oligopeptide was competitively inhibited by heparin and dextran sulfate. We conclude that the mechanism of antiviral activity of the NCAs can (at least partly) be explained by the interaction with the gp120 envelope protein and is dependent on the electrostatic attraction of both components, the presence of hydrophobic amino acids and the circular conformation of the V3 loop.

In **chapter 4** the influence of the NCAs on the entry process of the virus at the cellular level was investigated. We earlier observed that preincubation of the NCAs with the target cells seemed to cause an additional inhibitory effect of virus replication. Therefore MT-4 cells, commonly used in *in vitro* screenings assays, and freshly isolated human peripheral blood mononuclear cells (PBMCs), used as a representative of the target cells for HIV *in vivo*, were used to identify a possible membrane component, other than the CD4 receptor, that could be involved in this aspect of the antiviral activity of the NCAs. We observed that the NCAs avidly bind to MT-4 cells. Two binding sites were inferred for both NCAs. The binding of the NCAs to MT-4 cells could be inhibited by polyinosinic acid, heparin and dextran sulfate, strongly suggesting the involvement of a binding site for polyanionic substrates. Interestingly the binding of the NCAs to PBMCs was greatly influenced by the activation status of the PBMCs. Phytoheamagluttin-(PHA) prestimulated PBMCs were used in this study since the activated cell type is more characteristic for the situation in AIDS patients. Binding of NCAs to prestimulated cells was significantly higher as

compared to unstimulated PBMCs. These results suggest that PHA-prestimulation increases the expression of polyanionic binding sites on PBMCs. We therefore conclude that binding to lymphocytes-receptors may represent one of the components in the multi-mechanistic antiviral activity of the NCAs. Furthermore, these binding sites for the NCAs on the target cells for HIV are attractive target sites for the NCAs as a drug-carrier for the specific delivery of anti-retroviral compounds to lymphocytes.

In order to investigate whether Suc-HSA is able to preserve its potency in an *in vivo* situation, in **chapter 5** we analyzed the antiviral effect of Suc-HSA on HIV-1 replication in the presence of whole human plasma instead of commonly used tissue culture medium. We observed no changes in the antiviral effect of Suc-HSA when tested in tissue culture medium, 30% plasma, or whole blood when CPDA-1 (citrate-phosphate-dextrose-adenine 1) was used as the anticoagulant. However, a dramatic decrease (greater than 99%) in the antiviral activity was observed when these experiments were performed in plasma prepared from blood using heparin as anticoagulant. In the present study we demonstrate that heparin largely reduces Suc-HSA activity on HIV replication in the same concentration in which if affects binding of Suc-HSA to the envelope protein gp120 and in particular its V3 domain. In the same concentration range, heparin reduced binding of Suc-HSA to MT-4 cells, an other HTLV-1 transformed cell line. We concluded that heparin can displace Suc-HSA from its binding sites on the hybrid lymphoid cells as well as on HIV-1 particles.

In **chapter 6** additional studies involving the multi-mechanistic antiviral activity of the NCAs are described. We demonstrated that syncytium formation of HIV infected and uninfected T-cells is inhibited at concentrations of 1-4 μg/ml of the NCAs. The gp120 mediated virus/cell binding process however was inhibited by the NCAs at about a 10 fold higher concentrations. Addition of Suc-HSA to target cells incubated with HIV-1 could be delayed until 50 minutes after infection before loss of activity occurred. These experiments demonstrate that in addition to the direct influence of the NCAs on the virus entry, inhibitory effects on syncytium formation and gp120-CD4 binding can be expected, that is if sufficiently high enough concentrations are attained in the whole organism.
Furthermore we investigated whether the NCAs give rise to the production of NCA resistant HIV-1 strains. Interestingly Suc-HSA displayed an unchanged activity against a Suc-HSA pressured strain as compared with the control HIV-1 IIIB strain. In contrast, chronic exposure of Aco-HSA to HIV-1 resulted in ten fold reduced ability of HIV-growth inhibition by Aco-HSA, indicating a moderate development of drug resistance to Aco-HSA. These results imply that Suc-HSA should probably be preferred in long term treatments in which a minimal development of drug resistance is required.
In order to develop a dual-targeting device using AZT and the NCAs, we first checked whether both types of antiviral agents did antagonize each other. We rather observed a moderate synergistic activity against HIV-1 with the combination of Suc-HSA and AZT and also with Aco-HSA and AZT. This result favours a combined use of the drugs: lower doses of both drugs can be anticipated to be effective whereas the severity of side effects and toxicity of such a combination could be lower. At the same time, using AZT/NCA combinations or conjugates, the emergence of resistant virus strains could be postponed. When AZT (in its monophosphate form) was covalently coupled to the NCAs, and added 30 or 60 minutes after infection of the target cells, an additional anti-HIV-1 activity of

NCA-AZTMP conjugates was demonstrated as compared to NCAs alone. This afforded a clear illustration of a dual activity of these preparations.

In order to optimize the coupling efficiency of the monophosphate derivative of AZT to proteins using watersoluble carbodiimide (EDCI) the use of *N*-hydroxy-sulfosuccinimide (NHS) in the conjugation reaction was investigated in **chapter 7.** The couplings efficiency was greatly improved by the use of this second activator of the conjugation reaction. The hydrolysis of the activated AZTMP intermediate, responsible for the low degree of conjugation in the earlier method, was decreased considerably since the activated phosphate group was converted into an activated ester by addition of NHS. In order to minimize the use of compounds needed for the preparation of AZTMP-protein conjugates, we used an experimental design to determine the reaction conditions in which a conjugate with two AZTMP-molecules per molecule of neo-glycoprotein would be produced. In addition a low proportion of cross-linked conjugates was desired. We demonstrated that the the optimal conditions for coupling 1-2 mole of AZTMP to 1 mole of protein were the use of 50 mg protein, 4 mg of AZTMP, an incubation time of 30 h, and an NHS amount of 8 to 15 mg in the reaction mixture.

Chapter 8 describes the antiviral activity of the NCAs if tested against patient HIV-isolates versus HIV-laboratory strains. The phenotypically distinct patient HIV-1 isolates, so called primary isolates, are more representative for the virus strains present in HIV-infected persons compared to the commonly used laboratory HIV-strains. The latter usually undergo multiple passages in the particular cell lines and have been shown to loose some important viral-specific characteristics. In the present study both Suc-HSA and Aco-HSA were shown to potently inhibit replication of primary HIV-1 variants, independent of the SI capacity of the HIV-1 variant. A distinct activity was found against clinical isolates albeit in about a 10 to 100 fold higher concentrations as compared to results earlier found against the laboratory strains. In addition in this chapter the effect on virus/cell fusion by the NCAs was demonstrated since inhibition of the syncytium formation and the absence of proviral DNA products in cells inoculated with HIV-1 in the presence of Suc-HSA or Aco-HSA was observed.

In **chapter 9** we investigated whether the NCAs could also exhibit an anti-HIV efficacy *in vivo*. We therefore used a recently developed chimeric, human to mouse, HIV infection model, in which CBA/N mice were intraperitoneally grafted with human lymphocytes, resulting in an acute graft versus host reaction (GvHD), with highly activated immune competent cells. In the present study we analyzed the plasma disappearance and organ distribution of both compounds after i.v. and i.p. injections. This pharmacokinetic screening enabled us to establish a dosage regimen by which a significant antiviral activity in mice should be expected. I.v. injections of 10 mg to 300 mg/kg for both NCAs showed a first order kinetic profile, in which a linear correlation between the area under the curve (AUC) and the dose was observed. This in contrast to the saturation kinetics of the NCAs earlier found in rats. Blood concentrations of the NCAs were significantly influenced by preinjections of an excess of formaldehyde treated albumin (Form-HSA), a known inhibitor of scavenger receptor mediated uptake. Organ distribution studies showed an appreciable accumulation of the NCAs in liver. Treatment with Form-HSA however lowered the tissue to plasma concentration ratio's of liver and lungs in the mice only moderately. This

indicates that in the mouse scavenger-receptor mediated uptake is not the sole mechanism for accumulation in these organs. Suc-HSA was rapidly absorped from the peritoneal space into the general circulation after intraperitoneal injections of 300 mg/kg Suc-HSA, resulting in a final bioavailability of about 0.45.
Dosages of 0.3 to 300 mg/kg Suc-HSA were evaluated for their *in vivo* neutralizing capacity in this human to mouse chimeric model for. HIV-1 IIIB challenge was performed approximately one hour after grafting of the human PBMC and long before the characteristic activation of the human PBMC graft takes place. Intraperitoneal injection of 3 mg/kg Suc-HSA, given within 30 to 15 minutes before the mice were challenged with the virus, was sufficient to protect these mice against infection with the HIV-1 IIIB-strain. These results imply for the first time that the NCAs are not only effective in anti-HIV-1 screenings tests *in vitro* but also can exhibit potent anti-HIV-1 effect *in vivo*.

Finally in **chapter 10** the results of the abovementioned studies are discussed and further perspectives for drug delivery of antivirals are mentioned. We concluded that the NCAs are able to serve as intrinsically active drug carriers to target other anti-retroviral compounds, such as AZT, to the target cells of HIV, while the NCAs can interfere with early events in the HIV replication cycle themselves. Future studies will be focused on the exploration of the NCAs for *in vivo* efficacy studies in animal infection models and HIV-patients.
In addition, by exploring the necessary features to obtain a potent anti-HIV compound, such as the introduction of negatively charged as well as hydrophobic parts, even more versatile anti-HIV compounds can potentially be developed. Within this scope, the characterization of the binding sites on HIV target cells for negatively charged components is of much relevance, since the general knowlegde may provide more optimal drug carriers with higher affinities for these cells. In addition the cellular processing of the particular drug targeting preparations and the efficay of dual-targeting devices of NCAs and other antiviral conjugates should be further investigated to optimize targeted therapy for HIV infections.