Anti-HIV Activity of Modified Milk Proteins and Fragments Thereof

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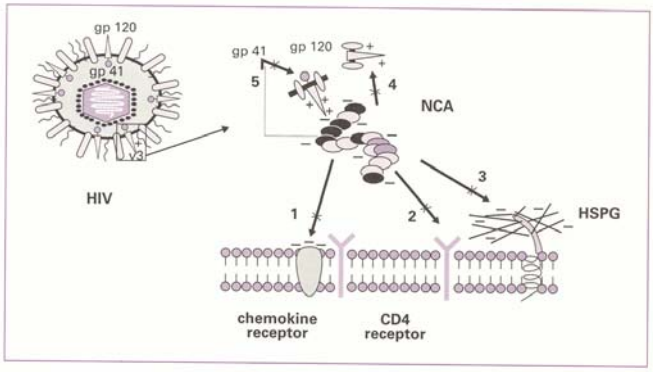
René Floris has studied chemistry at Leiden University and obtained his PhD degree at Amsterdam University in the field of biochemistry/enzymology. Currently he is working at NIZO food research where he is responsible for research activities on (milk) proteins, bioactivity and enzymology. Anti-HIV activities of milk proteins is an area of focus in his research.

I work with NIZO Food Research, which is a Dairy Research Institute has been for some 50-plus years.

First, I would like to say something briefly about HIV. We all know about it, and I would like to into it with a bit more detail. I think we started off with basics and now I would like to go to the level of the virus and molecules. Then we will touch upon the anti-HIV activity of milk proteins, modified milk proteins, and peptides derived from milk proteins. Then I would like to spend some time on lactoferrin and I would also like to say something about virus transmission.

So we all know that from the early 80s on that AIDS was caused by a virus. After that time we learned much more about the molecular details, and for that reason I would like to speak about the virus. HIV-1 and HIV-2 are the basically the two major viruses and at this moment I would like to focus on the HIV-1 type. We all know the number of infected individuals has been increasing very rapidly, and that as of the beginning of 2004 the estimated numbers were 12 million deaths and 31 million infected. The realistic numbers were probably much higher. So I think there is a really great need for cheap and efficient therapies, and is actually the reason we began to conduct HIV studies with milk proteins.



Because I would like to touch on the molecular details, we need to understand how the HIV virus interacts with cells, like T-cells. Here is a graphic that shows the HIV virus the object in the model has some peptides and proteins—and very important proteins are gp41 and gp120, which are involved in the cellular attachment and also in the transmission of the virus. The virus then interacts with receptors on the cell's surface, the CD4 receptor is a very important receptor, and also co-receptors like chemokine receptors are used. The target of most drugs that are available on the market affect processes that occur after infection, like the synthesis of DNA or the spread of the virus. But what may be preferred are drugs or molecules that block the entry of the virus.

For that reason we were going to do some research on modified and native milk and plasma proteins. What I am going to discuss is some research on modified proteins which are milk proteins that have been modified by introducing charges into the molecule to become negatively charged. This was introduced on the outside of the molecule, which in this case were modified by heparin. In the second part of the talk I would like to say something about native proteins from milk, charged and amphipathic fractions and lactoferrrin.

Interaction HIV-1 and host cell: electrostatic interactions

Virus:
- gp120: V3-loop is strongly positively charged
- gp 41: harbours clusters of lysines

Host cell:
- CXCR4 co-receptor: negatively charged
- CCR5 co-receptor: (less) negatively charged

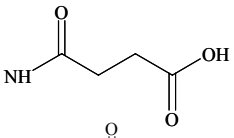
Modified and native milk and plasma proteins

Modified proteins:
- Negatively charged proteins (NCP's)
- Heparin modified albumins

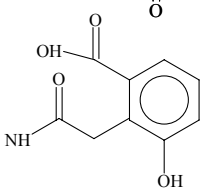
Native proteins:
- Charged and amphipathic milk protein fragments
- Lactoferrin

The modification of the milk proteins was done by introducing salicylic acid, which has a negative charge. If you put a lot of these molecules on the surface of milk molecules you can make them more negatively charged. This is a class of molecules that we made. The other class was the introduction of 3-hydroxyphthalic acid, which in effect introduces a negative charge on the outside of the protein, but also a hydrophobic carrot in that this introduces the aromatic nucleus. Here's a stretch of a piece of heparin where you can see there are numerous negatively charged receptors on the outside of the molecule.

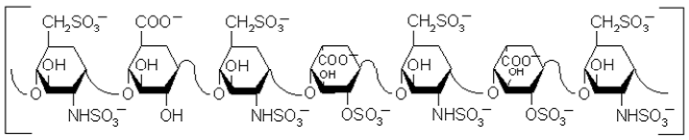
NCP's



Succinic acid (Suc)



3-hydroxyphthalic acid (3-HP)



Heparin

Anti-HIV activity of NCP's

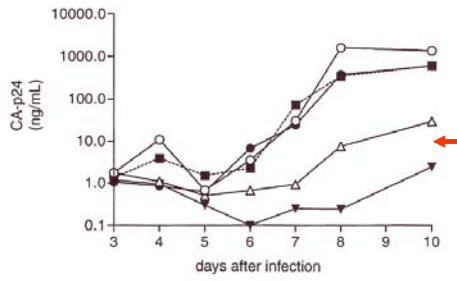


Fig. 2. Anti-HIV activity of Suc–HSA (–▼–), Hep3 kD–HSA (–○–), Hep13 kD–HSA (–△–) and HSA (–●–) as compared to a control infection (···■···) of SupT1 T-cells with the LAI isolate. Virus replication was monitored by CA-p24 measurement in the culture supernatant.

- Suc/3-HP-proteins: effective inhibitors
- Heparin-proteins: inhibition increases with size heparin

We made these molecules to see how effective are they in preventing the virus to spread. We used an assay in which supT1 T-cells were taken and virus was added to the cells and then we just measured how much virus was produced. So if you then add molecules, for example, succinylated human serum albumin (Suc-HAS) as we did in this case, or if you add a molecule like heparin modified human serum albumin (Hep3 kD-HAS), then you can see that the spreading was blocked. So basically what we can prove from this is that introduction of these types of charges in this type of molecule made a quite effective inhibitor of viral replication. And, moreover, if you look at heparin proteins, you can see that the mode of inhibition became better when the heparin molecule became bigger. In other words, inhibition increases with the size of the heparin molecule.

That's also depicted here. The inhibitory concentration 50% (IC50) value, which is really just an indication of how well it inhibits viral replication, is depicted here, you take the native molecule, it's hardly an inhibitor, but if you modify it, for instance, by introducing negative charges, you really bring down the IC50 value. As depicted here, introducing a cluster of negative charges only on certain places on the native molecule, that really increases the inhibitory character. If you introduce bigger stretches of heparin, the resultant molecule is a really potent inhibitor. So that's the conclusion here.

Anti-HIV activity of NCP's

Compound	IC_{50} (mg/mL)	IC_{50} (nM)
HSA	>250	>3731
Suc-HSA	0.11	1.57
Hep3kDa-HSA	>250	>2660
Hep6kDa-HSA	79.89	660
$(Hep13kDa)_3$-HSA	20.18	190
$(Hep13kDa)_{12}$-HSA	5.36	36.9

- Anti-HIV activity Suc-HSA is higher than Hep-HSA activity

If we look at how these molecules are inhibiting the viral replication, we should look at the gp120 molecule. What I forgot to say in the beginning is that the gp120 molecule binds itself to the V3 loop, and the V3 loop is highly positively charged. For that reason we were looking at how well are these molecules was able to bind to this V3 loop. We conducted an experiment in which the labeled Suc-HSA was bound to the V3 loop, and we just added the other molecules to see how well they displayed the label. It turns out, as shown here, of course, that the Suc-HSA worked out fairly well. And if you increase the size of the heparin molecule, the way in which you can splice this molecule also increases it becomes a much better inhibitor. We have shown here that both the Suc-HSA as well as the heparin-modified versions of that binds to the gp120 molecule on the surface of the virus.

Another interesting question was, "What are the pharmacokinetics of these types of molecules?" What we did here was to take a rat and inject it these molecules into the rat and looked at the plasma lymphatic concentrations as a function of time, as shown here.

Pharmacokinetics

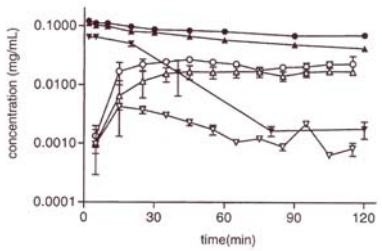


Fig. 5. Plasma (closed symbols) and lymphatic (open symbols) concentrations 2 hr after injection of 4 mg/kg ^{125}I-labeled Hep3 kD–HSA (–○–), Hep13 kD–HSA (–△–), and Suc–HSA (–▽–). Means ± SD (N = 3).

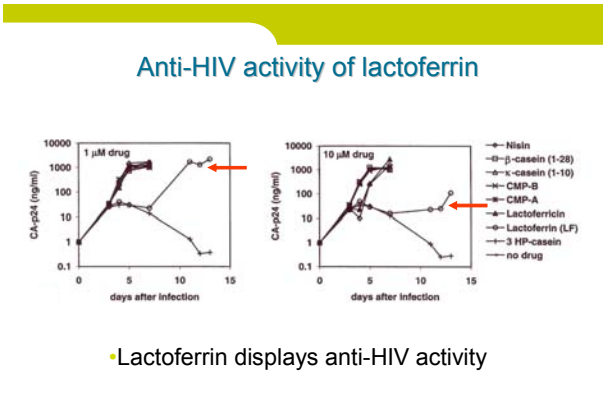
- Higher plasma and lymphatic concentrations for Hep-HSA than Suc-HSA
- Efficiency determined by IC50 and kinetics

In the open circle- and triangle-connected lines are the plasma lymphatic concentrations of the heparin version of the Suc-HSA. It indeed shows you that the as a function of time they remain high and pretty constant. If you look at the concentration of the succinylated version as a function of time, you see that the concentration of these molecules decrease rather rapidly. But, of course, we've been measuring the IC50

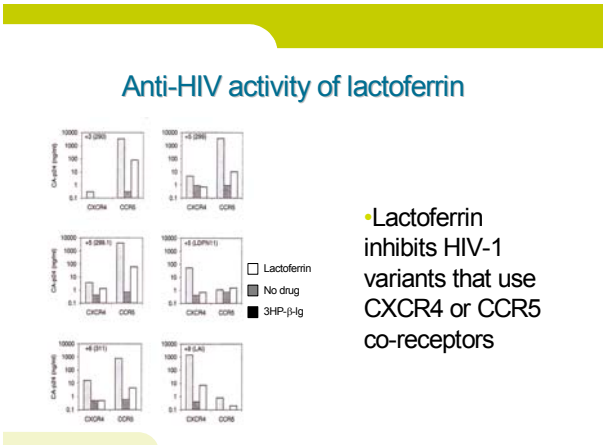
values, that's its inhibitory power, but also the kinetics. The efficiency of these molecules is determined by the IC50 value, but also that the kinetics of the heparin-sized molecules are, in fact, more favorable.

With this research I think what we can say about modified milk plasma proteins is that an inhibition of HIV activity is really at the virus entry level, because they really bind to the virus at the outside of the gp120 molecule V3 loop. The heparin-modified molecules result in a much higher plasma and lymphatic concentrations as a function of time, which is a situation that is really desired for *in vivo* activity. If you modify whey proteins, in this case lactoglobulin, it turns out that these molecules are very good inhibitors of the HIV virus because they, in fact, bind at the cellular level of the CD4 receptor. So now we have two types of molecules: one that binds with the virus, and one that binds to the cell. My suggestion is to see what a combination of these types of molecules will do.

If you look at the milk proteins you will see that stretches buried inside of the molecule which may a different charge. For example, maybe very negatively charge or maybe very positively charged. What we did here was to select a number of peptides can be enzymatically prepared from milk proteins which have a certain characteristic. For example, a positively charged peptide could be isolated for fabricating and a negatively charged peptide could also be isolated. All the peptides were tested in the assay that I showed you before, and it turned out that at least five peptides do not seem to be very potent inhibitors—possibly because they are not big enough. There is only one exception, lactoferrin.



What I would like to stress now is that bovine lactoferrin was a good inhibitor of HIV activity. So this will be the topic of the next few slides. Here you see an experiment in which lactoferrin was added to the assays. The virus was measured, and you can see here that lactoferrin could inhibit HIV replication. Although, after some time HIV particles are still being produced, but at least it shows us that lactoferrin is inhibitory.



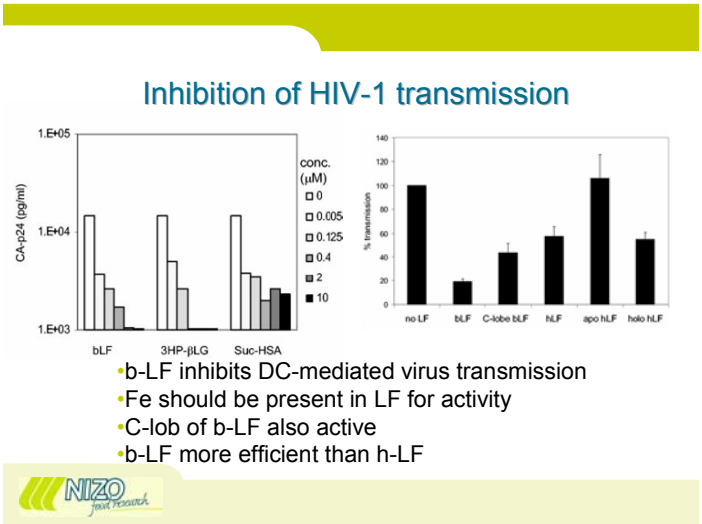
We know that the V3, which is positively charged, is important here. We know that the virus, which attaches to the cell surface on the receptor (a negative CD4 receptor) also uses CXCR4 or CCR5. In this experiment we have taken viral isolates which have different charges on the V3 loop, some affect the variance of the virus, which has preference for either the CXCR4

receptor or CCR5. We tested whether lactoferrin could inhibit these types of interactions. It turns out that lactoferrin, which is the white bar here, you have to compare it to the no drug group.

Lactoferrin may inhibit interaction of the virus with the cellular surface. So, in effect, it inhibits viral replication and plays a very broad activity, because not only is it inhibits the HIV variants, but it also inhibits variants that use both co-receptors. I have to say here that we were interested in how it works, so what we did was to try to find isolates that had resistance to lactoferrin. By replicating it over and over again, we could find a viral variant that was not inhibited a lot by lactoferrin. By looking at what type of mutations have occurred there you could figure out of all the mutations that have occurred that the interaction had something to do with the co-receptors. It really shows us that lactoferrin is interacting with the attachment of the virus with the co-receptors of the cell.

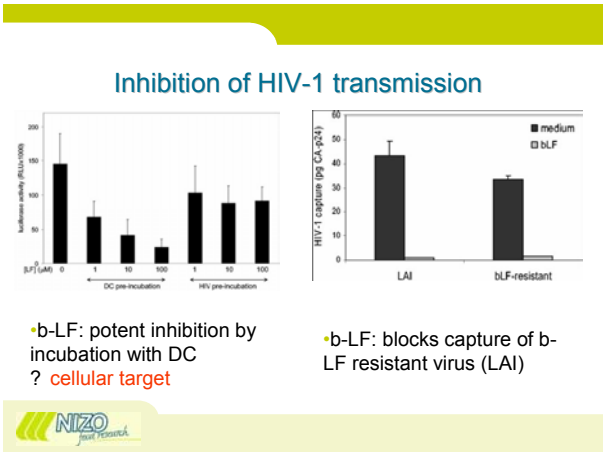
I would like to switch to the last subject, which is the inhibition of HIV transmission. Dendritic cells are cells that are very important in viral spread. In effect, dendritic cells are thought to be the first cells that will be encountered by the HIV-1 virus. So once the HIV-1 virus is in the dendritic cells, they will sit there for a few days then the dendritic cells will migrate to the lymph nodes and through the body. The HIV virus is very clever, of course, because in the lymph node it cannot transmit itself to the T-cells, and by that through the T-cells to the whole body. So the question here was, "Can lactoferrin prevent the dendritic cell mitigated HIV-1 transmission?"

We set up an assay where transmission can be measured, and the dendritic cells were used to infect T-1 cells. And if you add molecules like, for example, bovine-lactoferrin, modified with lactoglobulin or Suc-HSA, you can see that all these types of molecules are able to, in fact, inhibit transmission. But the lactoferrin was very effective in dong so. For that reason we began to study lactoferrin (and also human lactoferrin). We also looked at a transmission, and as you can see, of course, if that is you don't add lactoferrin the virus will be transmitted. If you add, for example, bovine-lactoferrin we inhibit the transmission—a much better effect than human lactoferrin. And if we look at lactoferrin, as we already said before, lactoferrin is, in fact, a molecule that consists of two lobes; each lobe contains an iron molecule. So the question was, does the iron saturate or inhibit the transmission, or is it something else? So we removed the iron molecules and as you can see it became a very bad inhibitor. You have to have the iron's presence, or apparently there is something about the structure which also is important-not only the amino acid sequence.



We looked at bovine-lactoferrin where we were able to isolate one lobe, the C-terminal lobe, and we were then able to see if that inhibited transmission. The C-lobe of lactoferrin is able to inhibit the transmission, although not as well as the bovine intact molecule. Apparently you really need the intact molecule for efficient inhibition.

We looked to see at what level the inhibition takes place. Does it take place at the cellular level, or does it take place at the viral level? We took the dendritic cells, pre-incubated it with lactoferrin, plus the lactoferrin whey to see how the transmission was inhibited. What you can see is that if you add enough lactoferrin and wash it away; in fact the transmission can be inhibited. You can do the same trick with the virus, the pre-incubated virus, and then add it to the cells. You see that inhibition does not occur. This tells that, in fact, lactoferrin transmits the assay as a cellular target. As was indicated by the speaker before, the cellular target is a target on the dendritic cells.



In last experiment here we were able to isolate the lactoferrin-resistant virus. Well, suppose we have that resistant virus and the person is infected by that. Can lactoferrin still block the transmission? We took a normal virus and a bovine lactoferrin-resistant virus; you can see that if you add bovine lactoferrin you can see that, indeed, transmission has been blocked completely.

In conclusion, lactoferrin and acylated milk proteins are potential HIV-inhibitors. They work, in effect, at the level of virus entry. If you use heparin to modify milk proteins, you can reach higher plasma lymphatic levels concentrations, so this is also important for therapy. The combination of 3HP-modified milk protein and, for instance, lactoferrin or acylated milk proteins may result in even more potent HIV-1 inhibition as you work at both the cellular level and the viral level. What is very interesting is the bovine lactoferrin or lactoferrin, as such, can prevent the dendritic cells mitigated to HIV-1 transmission. For example, we have had the persistent infection. Also we know that bovine lactoferrin inhibits the transmission of a resistant variant, so it has nothing to do with the virus itself, because it works at the cellular level. I think in the end we all know that there are still, compared to the drugs, relatively cheap molecules.

I did not include only the work of my own group and would like to thank others, with whom I cooperated very intensely. Included are the group at University of Amsterdam, Department of Human Retrovirology and the University of Groningen, Department of Pharmacokinetics and Drug Delivery.