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The potential for heparin and its derivatives in the therapy and prevention of HIV-1 infection

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Heparin is one of several sulphated polysaccharides which potently inhibit replication of the human immunodeficiency virus type 1 (HIV-1) in cultures of $CD4^{+ve}$ human cells. The EC_{50} value is around 5 μg ml^{-1}. We have demonstrated that heparin binds to recombinant gp120, the envelope glycoprotein of HIV-1, at a site termed the V3 loop, or principle neutralizing domain, which consists of a disulphide-bridged loop of 32–35 amino acids particularly enriched with basic residues. Using a series of chemically modified heparins we have shown that there is structural specificity in the anti-HIV activity of heparin. Heparin is routinely used clinically as an anticoagulant, and has proved essentially non-toxic and well tolerated. Low anticoagulant derivatives of heparin which retain high anti-HIV-1 activities *in vitro* may be generated by several routes. Such preparations are ideal candidates for clinical investigation as potential novel therapeutic agents for use in combination with other drugs in the management of AIDS and HIV infection.

***Keywords*: heparin, human immunodeficiency virus, HIV-1, AIDS, CD4, gp120, V3 loop, sulphated polysaccharides, dextran sulphate**

The anti-HIV-1 activity of sulphated polysaccharides

A number of sulphated polysaccharides have been shown to inhibit the replication of human immunodeficiency virus type 1 (HIV-1) in cultures of human $CD4^{+ve}$ cells. The active compounds include curdlan sulphate [1], dextran sulphate [2, 3], dextrin sulphate [4], fucoidan [5], heparin [2, 6] and pentosan polysulphate [5]. These macromolecules are potently active, with EC_{50}s, the concentrations giving 50% inhibition of viral replication, in the region of 5 μg ml^{-1}. The polyanionic nature of these compounds appears to be essential for their *in vitro* anti-HIV-1 activities as their nonsulphated counterparts are inactive. However other sulphated polysaccharides, such as chondroitin sulphate and dermatan sulphate are inactive, implying that structural features beyond merely the possession of multiple sulphate groups on a polysaccharide backbone are important for activity.

The structure and biological activity of heparin

Sulphated polysaccharides have multiple biological activities, most notably they are anticoagulant. Indeed heparin has been used extensively as an anticoagulant for several decades. This usage has established that heparin is essentially non-toxic, and has moreover given rise to a considerable understanding of the pharmacology and biochemistry of heparin.

Heparin is a linear polysaccharide of alternating glucosamine and hexuronic residues. The major anticoagulant activity of heparin results from its binding to and activation of the anticoagulant protein, antithrombin (for recent review, see [7]). This interaction is one of high affinity and specificity, involving a particular pentasaccharide sequence. The glucosamine residue at the centre of this pentasaccharide motif bears an unusual 3-*O*-sulphate group, which together with additional sulphate groups and other structural features, is essential for antithrombin binding [8]. Some two thirds of the heparin chains in a typical preparation lack this pentasaccharide sequence and thus show no interaction with antithrombin.

During biosynthesis, the hexose residues in the nascent heparin chains are subject to a variety of post-incorporational modifications (for review see [7]). These include *N*-deacetylation of the *N*-acetylglucosamine residues, their *N*-sulphation, and *O*-sulphation at several potential sites on either the glucosamine or hexuronic acid residues. Each of these modifications is incomplete such that only a proportion of the potential sites on the heparin chain are altered. Therefore a high degree of variably modified

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oligosaccharide sequences is elaborated along the heparin chains.

The long-established clinical use of heparin has stimulated a large body of work on the structural and functional characterization of heparin and the related glycosaminoglycan, heparan sulphate. Chemical and enzymic methods have been established for the selective and controlled fragmentation of heparin chains [9, 10]. Moreover there are numerous chemical methods which enable particular chemical moieties to be removed or modified [11]. A large body of work has shown that such selective chemical modifications alter the biological properties of heparin. By comparison, the more restricted structural diversity of other sulphated polysaccharides, such as dextran sulphate, combined with more limited approaches to their structural characterisation prevent such manipulation of their biological properties.

The mode of anti-HIV-1 action of sulphated polysaccharides

(i) Binding to CD4

Many polyanions, in particular dextran sulphate, exert their anti-HIV-1 activity by blocking the binding of the virion envelope glycoprotein, gp120, to its major cell surface receptor, CD4. Expression of CD4 is largely but not exclusively restricted to helper T cells. CD4 is a member of the immunoglobulin superfamily. The high resolution crystallographic structure of its two amino terminal domains, which include the HIV-1 gp120 binding site, is available [12, 13]. Studies involving both CD4-specific monoclonal antibodies and site directed mutagenesis have revealed that a single loop, residues 40–60, is the binding site for gp120 (for review see [14]). This loop is exposed on the face of the aminoterminal domain, and corresponds structurally to the second complementarity determining region of an immunoglobulin light chain.

The surface of the amino terminal domain of CD4 is relatively enriched in basic amino acids, many of which occur in sequence clusters typical of polyanion binding sites. Mapping of the polyanion binding site on CD4 through the use of monoclonal antibodies directed at epitopes in the aminoterminal domain, has shown that polyanions do not bind at the gp120 binding site, but are in close proximity to it [15]. It would seem likely that occupancy of the polyanion binding site blocks the subsequent interaction of CD4 with gp120. Unfortunately gp120 has thus far proved resistant to crystallization, and the lack of a detailed structural model means that this postulate cannot be fully examined.

One report suggests that heparin blocks the binding of gp120 to CD4 [16]. However other work [15], including our own [17] indicates that this is not the case and that heparin acts via the virus envelope glycoprotein, gp120, rather than its cellular receptor. In terms of developing novel therapeutics, there is an obvious advantage in studying compounds which bind to the pathogen rather than its target cell. This is particularly the case in HIV infection, where depletion and dysfunction of the CD4 helper cell population are underlying causes of the resulting immunodeficiency. CD4 is an important component of the cellular immune response and compounds binding to it are likely to either block its interaction with natural ligands and may also result in transduction of inappropriate cellular signals.

(ii) Binding to the V3 loop of gp120

A second site for the anti-HIV-1 activity of sulphated polysaccharides is a functionally important domain of the virus envelope glycoprotein, gp120. This is a disulphide-bridged loop delineated by invariant cysteines 303 and 338, and is termed the V3 loop. The functional importance of this domain is demonstrated by the fact that antibodies binding to the V3 loop are potently neutralizing. Since most antibodies recognizing epitopes on the surface of HIV-1 are non-neutralizing, the V3 loop is often referred to as the principle neutralizing domain (PND). Virions incubated with anti-V3 loop antibodies are still able to bind to CD4 but are unable to undergo fusion with the plasma membrane. This leads to the conclusion that the V3 loop is not involved in the initial binding of viral gp120 to CD4, but has an essential role in post-binding membrane fusion events (for review see [18]).

A number of groups have provided evidence that the V3 loop is a binding site for both dextran sulphate and heparin [17, 19–22]. In our studies we showed that heparin was able to block the binding of six different V3-loop specific monoclonals to recombinant gp120, whereas it had no effect on the binding of anti-gp120 monoclonal antibodies specific for epitopes outside the V3 loop [17] (and Harrop *et al.* unpublished data). More recently we have established that radiolabelled heparin binds to recombinant gp120 with high affinity and in a saturable manner (Harrop and Rider, unpublished observations). Overall the simplest interpretation is that heparin binds to the V3 loop of gp120 and in doing so, blocks the binding of monoclonal antibodies at that site. The alternative explanation, that heparin binds to the idiotypic region of all V3 loop monoclonals, seems unlikely.

In our ELISA we employed recombinant gp120 from three different isolates of HIV, and obtained inhibition of binding of V3-loop specific monoclonals in each case (Harrop and Rider, unpublished data). For gp120 from HIV strain IIIB, recombinant gp120s expressed by CHO cells and baculovirus expression systems were used. Despite the probability that the glycosylation of these two forms of the recombinant protein is very different, identical results were obtained. There is thus no evidence for the direct involvement of gp120 glycans in these interactions.

As discussed by Callahan *et al.* [19], the V3 loop sequence has the highest density of positively charged amino acids of any region of gp120. Moreover our analysis of 245 V3 loop sequences shows that positions 3, 11, 12, 22 and 36 are almost invariably occupied by either arginine or lysine, the

two amino acids with basic side chains [23]. The remarkable degree of conservation of these basic residues in a viral genome which is subject to rapid mutation strongly implies that they serve an essential function. Given that monoclonal antibodies binding to the V3 loop are able to block virion fusion with the cell surface, it is not surprising that sulphated polysaccharides which bind to the V3 loop also block viral replication.

An important issue is which structural elements within heparin are responsible for its V3-loop binding activity. We have addressed this by studying a range of heparins with selective chemical modifications [23]. These studies show that *N*- desulphation of heparin markedly reduces its ability to displace V3-loop monoclonals from recombinant gp120. However, subsequent *N*-reacetylation almost completely restores the activity. Thus substitution of the amino function of the glucosamine residues appears to be essential, and may be necessary either to mask the positive charge carried here, or to maintain an appropriate secondary structure in the heparin chain. Either way it would appear unimportant as to whether the substituent is uncharged, *ie* acetyl moieties, or negatively charged as in the case of *N*-sulphates. A second modification resulting in minimal effect on V3-loop binding is carboxyl reduction. By contrast *O*-desulphation, even when performed selectively to remove preferential 6-*O*-sulphates, results in a marked loss of binding [23]. Overall these findings indicate that net negative charge density is of little significance in the interaction between heparin and the V3 loop of gp120. Instead particular structural features, *ie* the *O*-sulphate groups, are important whereas others, such as the *N*-sulphates and carboxyl groups, have little involvement. Thus, the interaction with the V3 loop exhibits structural specificity.

In this study we also examined the ability of these various chemically modified heparins to inhibit HIV-1 replication *in vitro*. We found that this correlates with V3-loop binding activity as measured in ELISA. Thus, all preparations exhibiting high levels of V3 loop binding also were effective in inhibiting viral replication and *vice versa*. Conversely those heparins which were poor inhibitors of replication also bound only weakly to the V3 loop [23]. The complete correlation between these two activities strongly suggests that the binding of heparin to the V3 loop of gp120 is indeed its mode of anti-HIV-1 activity. In these studies of HIV replication *in vitro*, the virions are grown in dividing C8166 T lymphoid cells. There is no evidence that any of these heparin preparations were cytotoxic for either infected or uninfected cultures of these cells.

Dissociation of anti-HIV-1 and anticoagulant activities

Because of the latency displayed by HIV-1, any potential therapeutic agent would be administered over a prolonged period. An obvious limitation of using heparin in this way is its potent anticoagulant activity. This is particularly so with AIDS patients who tend to develop haematological abnormalities. Fortunately it is now clear that the well defined pentasaccharide sequence responsible for the major anticoagulant activity of heparin, activation of antithrombin [7], has no particular importance in anti-HIV-1 activity. Indeed there appear to be a number of routes to obtaining low anticoagulant heparins which lack high affinity for antithrombin yet retain good anti-HIV-1 activity.

For example, Barzu *et al.* [24] have shown that selective *O*-acylation of both heparin and periodate-cleaved heparin result in preparations with low anticoagulant activities but high anti-HIV-1 activities *in vitro*. Moreover, elsewhere it has been shown that *N*-succinylation of heparin results in preparations have reduced anticoagulant activity, yet remain active inhibitors of HIV-1 induced syncytia in cultures of $CD4^{+ve}$ cells [25]. In our studies of chemically modified heparins, the carboxyl-reduced and *N*-desulphated, *N*-reacetylated heparins retain high anti-HIV-1 activity [23]. Both of these modifications are known to abolish high affinity for antithrombin. More recently we have shown that when heparin is fractionated by affinity chromatography on immobilized antithrombin, both the retained, high-anticoagulant, and unbound, low-anticoagulant fractions, obtained are indistinguishable from each other and the parent heparin in their anti-HIV-1 activities *in vitro* [26].

Conclusions

1) Heparin inhibits HIV-1 replication in cultures of $CD4^{+ve}$ CD4 cells, with an EC_{50} value in the range of $5\,\mu g\,ml^{-1}$. Such a concentration is readily achieved during the therapeutic usage of heparin as an anticoagulant. In current clinical use, heparin is essentially a non-toxic and well tolerated therapeutic.

2) Unlike other polysaccharides, notably dextran sulphate, which may act in part by binding to the HIV-1 receptor protein CD4, heparin is more selective. Its mode of action is by binding to the V3-loop, a major neutralizing epitope of the viral envelope glycoprotein, gp120. Thus heparin is unlikely to interfere with the function of CD4 in the declining $CD4^{+ve}$ cell populations. Moreover given the current interest in developing combination drug therapy for HIV infection, the V3 loop has yet to be exploited as a therapeutic target.

3) A number of strategies exist for removing the major anticoagulant activity of heparin with no or minimal effect on anti-HIV-1 activity *in vitro*. Such modified heparins should now be investigated clinically as novel anti-HIV-1 agents.

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