Carbohydrate-binding agents (CBAs) inhibit HIV-1 and it is proposed that therapy with such agents may have important implications for the future of anti-HIV therapy. Examples of CBAs include the procaryotic cyanovirin-N (CV-N), plant lectins such as HHA, GNA, NPA, CA and UDA, the monoclonal antibody 2G12 directed against a glycan-containing epitope on HIV envelope gp120, and the mannose-specific non-peptidic antibiotic Pradimicin A, which inhibits the entry of HIV-1 into its target cells. CBAs prevent not only virus infection of susceptible cells, but also inhibit syncytia formation between persistently HIV-infected cells and uninfected lymphocytes. In addition, CBAs may also prevent DC-SIGN-mediated transmission of HIV to T-lymphocytes. Therefore, CBAs qualify as potential microbicide drugs. Long-term exposure of HIV to CBAs in cell culture results in the progressive deletion of N-glycans of HIV gp120 in an attempt of the virus to escape drug pressure. In this respect, the CBAs are endowed with a high genetic barrier. Multiple mutations at N-glycosylation sites are required before pronounced phenotypic drug resistance development becomes evident. CBA treatment of HIV may consist of a novel chemotherapeutic concept with a dual mechanism of antiviral action: a direct antiviral activity by preventing HIV entry and transmission to its target cells, and an indirect antiviral activity by forcing HIV to delete glycans in its gp120 envelope. The latter phenomenon will result in creating ‘holes’ in the protective glycan shield of the HIV envelope, whereby the immune system may become triggered to produce neutralizing antibodies against previously hidden immunogenic epitopes of gp120. If this concept can be proven in vivo, low-molecular-weight non-peptidic CBAs such as Pradimycin A may become the cornerstone for the efficient treatment of infections of those viruses that require a glycosylated envelope (that is, HIV, but also hepatitis C virus) for entry into its target cells. In addition, influenza virus and coronavirus infections may also qualify to be treated by CBAs.

Keywords: carbohydrate-binding agents, drug resistance, gp120 envelope, HIV entry, microbicides

Introduction

The entry of enveloped viruses is often mediated by viral envelope glycoproteins. For HIV, the non-covalently bound gp120 and gp41 are involved. Five variable regions (V1–V5) interspersed by more conserved amino acid sequences (C1–C5) have been identified in HIV gp120 (Gallaher et al., 1995; Leonard et al., 1990). The conserved gp120 regions form structures that are important for interacting with the gp41 ectodomains and the cellular (co-) receptors CD4, CXCR4 and CCR5. Both conserved and variable regions of gp120 are extensively glycosylated (Geyer et al., 1988; Mizuochi et al., 1988a,b). It is assumed that the variability of glycosylation of the gp120 envelope surface modulates the immunogenicity of gp120. This envelope glycoprotein is the main target for neutralizing antibodies that appear during natural infection (Losman et al., 2001; Olofsson and Hansen, 1998). Continuous changes in the glycan shield during infection allows the virus to persist in the presence of an evolving antibody repertoire (Wei et al., 2003).

For human hepatitis C virus (HCV), the envelope E1 and E2 glycoproteins, which are non-covalently linked as heterodimers, are the major determinants for HCV entry in its target cells (Deleersnyder et al., 1997). They interact with at least two, if not more, different receptor molecules. The CD81 tetraspanin and the scavenger receptor SR-BI are proven receptors for HCV (Bartosch et al., 2003a,b; Hsu et al., 2003; Zhang et al., 2004). Whereas E1 contains four or five N-glycosylation sites, at least 11 glycosylation sites have been identified on E2, most sites being well conserved (Voisset & Dubuisson, 2004). The glycans play...
a major role in the folding of these proteins (Choukhi et al., 1998; Dubuisson & Rice 1996; Merola et al., 2001).

Influenza viruses are another example of enveloped viruses in which the envelope proteins hemagglutinin (HA) and neuraminidase (NA) are glycosylated, and play an instrumental role in the entry (and release) process of these viruses. HA is a homotrimeric glycoprotein with an ectodomain composed of a globular head and a stem region, both carrying N-linked glycan chains (Wilson et al., 1981). Five N-linked glycosylation sites on the stem region are strictly conserved in A/H3N2 viruses (Abe et al., 2004), and there are at least six or seven glycosylation sites on the globular head of HA present in several influenza A2N2 virus strains. The acquisition of new oligosaccharides is an important mechanism underlying the antigenic drift of HA to prevent neutralization by antibodies (Abe et al., 2004). Several studies have revealed that new glycosylation sites on HA of A2N2 viruses may occur in escape mutants selected under pressure by monoclonal antibodies (Tsuchiya et al., 2001, 2002a,b).

Finally, coronavirus (that is, SARS-CoV) entry into cells is mediated through interactions between the viral spike (S) glycoprotein and angiotensin-converting enzyme 2 (ACE2; Kuhn et al., 2004). The S glycoprotein is often cleaved post translationally into a heterodimer consisting of an extracellular binding subunit, S1, and a membrane-anchored S2 subunit, responsible for mediating membrane fusion (Simmons et al., 2004). The SARS-CoV S glycoprotein contains 23 predicted N-linked glycosylation sites of which at least 12 appear to be used (Krokhin et al., 2003; Rota et al., 2003). Thus, it is clear that glycoproteins in the envelope of many different viruses play an instrumental role in virus entry and, therefore, should be considered as attractive targets for antiviral intervention.

### CBAs

There are a broad variety of CBAs, which are nearly all exclusively protein in nature (that is, lectins) and can be derived from several distinct species in nature, including procaryotics, sea corals, algae, fungi, higher plants, invertebrates and vertebrates (that is, mammalians; Botos & Wlodawer, 2005; Balzarini 2006a,b, 2007a). CBAs are characterized by different carbohydrate specificities, including, amongst others, mannose, glucose, galactose, N-acetylgalactosamine (GlcNAc), N-acetylglucosamine (GlcNac), sialic acid and fucose. Interestingly, those CBAs that are endowed with prominent anti-HIV activity have a predominant mannose-specificity, although CBAs with GlcNAc specificity have also been shown to have anti-HIV potential in cell culture (Balzarini et al., 1991, 1992, 2004a, 2005b). Recently, a β-galactose-specific CBA from the seaworm Chaetopterus variopedatus (CVL) to be endowed with anti-HIV activity has been reported (Wang et al., 2006). For an overview of plant lectins with anti-HIV activity, see Van Damme et al., (1998), Sharon & Li (2003) and Balzarini (2006a,b) (Table 1).

The most well-known lectin from procaryotic origin is undoubtedly cyanovirin-N (CV-N) isolated from the cyanobacterium Nostoc ellipsosporum (Boyd et al., 1997). This lectin is the best studied with regard to its structural properties (Bewley et al., 1998), carbohydrate specificities (Bolmstedt et al., 2001) and antiviral activities (Balzarini et al., 2006; Boyd et al., 1997). Among the plant lectins endowed with anti-HIV activity, the mannose-specific agglutinin from Galanthus nivalis (snowdrop; GNA), Hippeastrum hybrid (Amaryllis; HHA), Cymbidium sp. (an orchid; CA), Narcissus pseudonarcissus (daffodil; NPA),

### Table 1. CBA with proven anti-HIV activity in cell culture

<table>
<thead>
<tr>
<th>CBA</th>
<th>Origin species</th>
<th>M.W. monomer, dalton</th>
<th>Presumed active form</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNA</td>
<td>Galanthus nivalis</td>
<td>12,500</td>
<td>Tetramer</td>
<td>α(1,3)Man</td>
</tr>
<tr>
<td>HHA</td>
<td>Hippeastrum hybrid</td>
<td>12,500</td>
<td>Tetramer</td>
<td>α(1,3):α(1,6)Man</td>
</tr>
<tr>
<td>NPA</td>
<td>Narcissus pseudonarcissus</td>
<td>12,500</td>
<td>Dimer/trimer/tetramer</td>
<td>α(1,6)Man</td>
</tr>
<tr>
<td>CA</td>
<td>Cymbidium hybrid</td>
<td>12,500</td>
<td>Dimer</td>
<td>Man</td>
</tr>
<tr>
<td>LOA</td>
<td>Listera ovata</td>
<td>12,500</td>
<td>Dimer</td>
<td>α(1,3)Man</td>
</tr>
<tr>
<td>EHA</td>
<td>Epipactis helleborine</td>
<td>12,500</td>
<td>Dimer</td>
<td>Man</td>
</tr>
<tr>
<td>APA</td>
<td>Allium porrum</td>
<td>13,000</td>
<td>Tetramer</td>
<td>Man</td>
</tr>
<tr>
<td>CV-N</td>
<td>Nostoc ellipsosporum</td>
<td>11,000</td>
<td>Dimer</td>
<td>α(1,2)Man</td>
</tr>
<tr>
<td>PRM-A</td>
<td>Actinomadura hibisca</td>
<td>833</td>
<td>*</td>
<td>α(1,2)Man</td>
</tr>
<tr>
<td>UDA</td>
<td>Urtica dioica</td>
<td>8,500</td>
<td>Monomer</td>
<td>GlcNac</td>
</tr>
<tr>
<td>2G12</td>
<td>Monoclonal antibody</td>
<td></td>
<td></td>
<td>Man-containing glycans on gp120</td>
</tr>
</tbody>
</table>

*PRM-A is thought to interact as Ca ++-bound dimer with α(1,2)-mannose oligomers. CBA, carbohydrate-binding agent; Man, mannose; M.W., molecular weight
Epipactis helleborine (EHA) and Listera ovata (twayblade; LOA), and the GlcNAc-specific agglutinin from Urtica dioica (UDA) are well-studied (Balzarini et al., 1991, 1992, 2004a, 2005b).

It should be mentioned that the innate immune system of mammals also makes use of carbohydrate-binding proteins (Rudd & Dwek, 1997; Rudd et al., 2001) such as the Ca ++-dependent mannos-binding serum lectin (MBL) to opsonize pathogens (Ji et al., 2005; Klein, 2005), the macrophage mannos receptor that plays a role in the binding and transmission of HIV by macrophages (Nguyen & Hildreth, 2003), and DC-SIGN (dendritic cell-specific ICAM-3 grabbing non-integrin) that functions as a mannos-specific C-type lectin in dendritic cell recognition and uptake of pathogens (that is, HIV) leading to antigen presentation to T-cells (Geijtenbeek et al., 2000; Feinberg et al., 2001).

In addition, a variety of other carbohydrate-binding proteins (that is, defensins) that are part of the innate and/or adaptive immune system also exist (Chang & Klotman, 2004). Finally, mention should be made of the monoclonal antibody (mAb) 2G12 that belongs to one of the very few neutralizing anti-HIV antibodies directed against an epitope on HIV-1 gp120 that lay around the C4/V4 region. The 2G12-recognized epitope on gp120 contains high-mannose glycans at several N-glycosylation sites (Calarese et al., 2003, 2005; Scanlan et al., 2002; Trkola et al., 1996). Generation of an antibody like 2G12 is very unusual, and has been found to have a unique and unprecedented structure caused by V H domain swapping (Calarese et al., 2005). This results in the generation of a total of four distinct carbohydrate binding sites, which may contribute to the multivalent binding of 2G12 to HIV-1 gp120.

In contrast with the abundant literature on CBAs that are protein in nature, there are only very few reports on non-peptidic CBAs. Interestingly, the actinomycete strain Actinomadura hibisca was found to produce pradimicin A (PRM-A), showing activity against systemic fungal infections (Oki et al., 1988). In addition, the fungicidal action of the closely related benanomycin derivatives have been reported (Watanabe et al., 1996). PRM-A has an unique structure containing a D-alanine, the carbohydrates D-xylene and 4,6-dideoxy-4-methylamino-D-galactose, and a substituted 5,6-dihydrobenzo[a]naphtacenequinone (Figure 1). This drug was shown inhibitory to HIV in the late 80s/90s (Tanabe et al., 1988; Tanabe-Tochikura et al., 1990, 1992). The antifungal activity could be ascribed to binding with mannoside residues in the obligatory presence of calcium (Tanabe-Tochikura et al., 1990). More details on the mechanism of biological activity and molecular carbohydrate interactions of PRM-A are reported (Ueki et al., 1993; Walsh & Giri, 1997; Igarashi & Oki, 2004; Hiramoto et al., 2005). These findings prove that CBAs can also be non-peptidic (low molecular weight). The existence of small-size CBAs may have important repercussions from a therapeutic viewpoint and provides the rational basis of exploring the field of small-molecule CBAs for their antiviral activity.

**Antiviral activity of CBAs**

The mannos-specific plant lectins HHA, GNA, NPA, CA, LOA and EHA show pronounced anti-HIV-1 activity in CEM cell cultures (Balzarini et al., 1991, 1992, 2004a) (Table 2). Their 50% effective concentration (EC 50) ranged between 0.004–0.018 μM for HIV-1(IIIb). The procaroytic mannos-specific CV-N is even more inhibitory (EC 50; 0.003 μM; Boyd et al., 1997; Balzarini et al., 2006). The GlcNAc-specific UDA derived from U. dioica is less inhibitory (EC 50; 0.140 μM; Balzarini et al., 2005b). Interestingly, the mannose-specific small-size (molecular weight: 833 D) non-peptidic antibiotic PRM-A (Figure 1) is also endowed with pronounced anti-HIV activity although its EC 50 value is in the lower micromolar range, which is far below its toxicity threshold (>50 μM; Balzarini et al., 2007a) (Table 2). There is generally a close correlation for the antiviral activity of the CBA against HIV-1, HIV-2 and simian immunodeficiency virus (SIV) in CEM and MT-4 cell cultures. Whereas a consistent pronounced activity of these CBAs is observed for the laboratory HIV-1(IIIb) strain in CEM cell cultures, a higher degree of variation in antiviral activity was noticed against a variety of HIV-1 clade isolates in peripheral blood mononuclear cells (PBMCs). Especially, the antiviral potency of the Amaryllidaceae-derived HHA and GNA could vary substantially depending on the nature of the clades (Balzarini et al., 2005b). In contrast, the Orchidaceae derived CA and LOA
show much less variation in their antiviral activity against the different HIV-1 clades. Also CV-N showed a pronounced and consistent suppression of the different viral clades in PBMCs. Interestingly, although the GlcNAc-specific UDA was clearly less potent against the HIV-1 clades than CV-N, it also showed a limited variation in activity against the HIV-1 clades as noticed for CV-N (Balzarini et al., 2005b). Whereas HHA and GNA are tetramers of 50,000 D, CA and LOA are dimers of 25,000 D, CV-N has a molecular weight of 11,000 D and UDA is active as its monomer of 8,700 D. It is felt that the higher the size of the CBA, the more the variation in antiviral potency exists when a broad variety of viral strains were evaluated. This phenomenon can perhaps be explained by an easier penetration capability of the lower-sized CBA into the glycan shield on HIV gp120.

For example, pradimicin-A, the smaller, non-peptidic CBA is consistently inhibitory against the broad variety of HIV-1 clades (Balzarini et al., 2007a).

An interesting property of CBAs is that they also efficiently prevent syncytia formation between persistently HIV-1-infected cells and uninfected T-lymphocytes (Balzarini et al., 2004a). This is an important property of CBAs that is shared with other entry inhibitors (that is, adsorption inhibitors such as dextran sulphate [DS-5000], fusion gp41 inhibitors such as enfuvirtide [Matthews et al., 2004] or the CXCR4 and CCR-5 antagonists), but absent in drugs that target a site in the replication cycle of HIV located at a time point after viral entry (that is, reverse transcriptase, integrase, protease, etc.). Such a property is beneficial if CBAs are to be used as potential microbical agents (see below) (Balzarini & Van Damme 2005, 2007). Whereas the plant lectins HHA and GNA are generally less inhibitory in such co-cultivation assays than in the virus infection assays (Balzarini et al., 2004a), other CBAs such as PRM-A kept a similar suppressive activity in both assay systems (Balzarini et al., 2007a).

Finally, and maybe even more importantly from a microbicidal viewpoint, we have very recently shown that CBAs in general are able to prevent the capture of HIV-1 particles to DC-SIGN-expressing cells such as dendritic cells and Raji/DC-SIGN cells that are engineered to express DC-SIGN in their membrane (Balzarini et al., 2007b). Both the peptidic lectins and the non-peptidic (PRM-A) CBAs were able to inhibit HIV-1 capture by Raji/DC-SIGN cells as well as subsequent transmission of the virus to co-cultured T-lymphocytes in a dose-dependent manner (Balzarini et al., 2007b). These observations demonstrate that CBAs may potentially also play an important role in preventing HIV transmission at the very early stage of virus infection after sexual intercourse.

### Potential of CBAs as microbicide drugs

CBAs may be attractive agents for microbicidal use because of their inhibitory potential against both infection by cell-free viruses and transmission of HIV by virus-infected cells (Balzarini et al., 2004a). Moreover, CBAs may also have the potential to block the binding (capture) of the virus by disseminating cells such as DC-SIGN-expressing dendritic cells (Balzarini et al., 2007b). It has recently also been shown that mannose- and GlcNAc-specific plant lectins inhibit HIV-1 infection of dendritic cells and dendritic cell-directed HIV-1 transfer albeit with a differential potency (Turville et al., 2005). Furthermore, binding of CBAs to HIV gp120 may prevent HIV from interacting with the macrophage mannose receptor (Nguyen & Hildreth, 2003). An important property of an ideal microbicide is its resistance to acidic (vaginal) pH and stability at higher (tropical) temperatures. In addition to being odourless, colourless and tasteless, several CBAs such as GNA and HHA also retain full

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**Table 2. Antiretroviral activity of CBA in cell culture**

<table>
<thead>
<tr>
<th>CBA</th>
<th>HIV-1(IIIb) EC_{50} μM</th>
<th>HIV-2(ROD) EC_{50} μM</th>
<th>SIVmac EC_{50} μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNA</td>
<td>0.018 ±0.0</td>
<td>0.011 ±0.007</td>
<td>0.042 ±0.012</td>
</tr>
<tr>
<td>HHA</td>
<td>0.006 ±0.003</td>
<td>0.016 ±0.0</td>
<td>0.010 ±0.0008</td>
</tr>
<tr>
<td>NPA</td>
<td>0.009 ±0.0</td>
<td>0.013 ±0.005</td>
<td>0.020 ±0.006</td>
</tr>
<tr>
<td>CA</td>
<td>0.031 ±0.010</td>
<td>0.018 ±0.011</td>
<td>0.034 ±0.009</td>
</tr>
<tr>
<td>LOA</td>
<td>0.004 ±0.0</td>
<td>0.003 ±0.001</td>
<td>0.030 ±0.015</td>
</tr>
<tr>
<td>EHA</td>
<td>0.004 ±0.0</td>
<td>0.001 ±0.0007*</td>
<td>0.075 ±0.036</td>
</tr>
<tr>
<td>CV-N</td>
<td>0.003 ±0.0</td>
<td>0.002 ±0.0001</td>
<td>0.017 ±0.0009</td>
</tr>
<tr>
<td>PRM-A</td>
<td>3.4 ±1.2</td>
<td>1.8 ±0.0</td>
<td>5.0 ±0.30</td>
</tr>
<tr>
<td>UDA</td>
<td>0.140 ±0.040</td>
<td>0.330 ±0.090</td>
<td>0.162 ±0.034</td>
</tr>
<tr>
<td>mAb 2G12†</td>
<td>1.7 ±0.46</td>
<td>&gt;50/≥20‡</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

*Antiviral data obtained in MT-4 cell cultures. †Data expressed in μg/ml. ‡Antiviral data obtained in PBMC for HIV-2 (BV-5051W). CBA, carbohydrate-binding agent; EC<sub>50</sub>, Effective concentration, or compound concentration required to inhibit virus-induced cytopathicity in cell culture.
activity after being exposed to high temperatures (that is, several days at 50°C) and a low pH environment (that is, several days at pH 5.0) (Balzarini et al., 2004a).

Cyanovirin has been investigated for microbicidal action in vivo (Tsai et al., 2003, 2004) and shown to efficiently inhibit SIV/HIV (SHIV) infections in a vaginal transmission model using female Macaca fascicularis monkeys. These data provide a proof-of-concept that CBA can efficiently prevent virus infection in the in vivo setting, and thus validate virus envelope glycans as a novel target for microbicidal drugs. Efforts have also been devoted to expressing cyanovirin in bacteria such as Escherichia coli, Streptococcus gondii or Lactobacillus jensenii (Colleluori et al., 2005; Giomarelli et al., 2002; Liu et al., 2005). In the latter case, CV-N was expressed either attached to the bacterial cell surface, or secreted in soluble form in the culture medium. L. jensenii also has the advantage that it belongs to the commensal vaginal flora, where it helps acidify the cervicovaginal environment and maintains the host’s natural defences. So far, no microbicidal experiments have been performed using bacteria expressing CBAs in a (pre)clinical (in vivo) setting.

CBA resistance development

It is assumed that every drug that acts at a target in the replication cycle of a virus will, sooner or later, select for virus strains that are less sensitive to the inhibitory effect of the drug through generation of mutations in virus-encoded proteins. Such phenomenon is well-documented for a variety of viruses and antiviral drugs (that is, reverse transcriptase inhibitor (NRTI) and non-NRTI (NNRTI); protease of HIV for protease inhibitors; gp41 of HIV for the entry inhibitor enfuvirtide; hemagglutinin and neuraminidase of influenza virus for zanamivir and oseltamivir; thymidine kinase, DNA polymerase or UL97 encoded by members of the herpesvirus family for the nucleoside analogues acyclovir or ganciclovir; DNA polymerase of hepatitis B virus for lamivudine, etc.). Therefore, it could also be expected that exposure of CBA to enveloped viruses will eventually result in the selection of drug-resistant virus strains.

When exposed to escalating CBA concentrations, a variety of HIV strains can indeed be isolated which showed decreased sensitivity to the CBA. This phenomenon could be demonstrated for HIV-1 strains exposed to the mannose-specific HHA, GNA and ConA plant lectins (Balzarini et al., 2004b, 2005a; Witvrouw et al., 2005), the mannose-specific proaryotic cyanovirin (Witvrouw et al., 2005; Balzarini et al., 2006), the glycans-recognizing anti-gp120 mAb 2G12 (Huskens et al., 2007), the mannose-recognizing low-molecular-weight antibiotic Pradimicin A (Balzarini et al., 2007a) and the GlcNAc-specific UDA plant lectin (Balzarini et al., 2005b). Except for the mAb 2G12, a relatively long time-period of drug exposure is required to afford marked phenotypic resistance against the CBA. In particular, virus resistance development against CV-N and UDA occurred very slowly and took a long time period of selection. The virus isolates that emerge under CBA pressure predominantly contained amino acid changes in HIV envelope gp120, in particular at N-glycosylation sites (Balzarini et al., 2004b, 2005a, b, 2006, 2007a). Either the asparagine or the serine or threonine in the NXS/T glycosylation motif was mutated. An increasing degree of resistance to the CBA was generally correlated with an increasing number of glycosylation site mutations. However, with exception of 2G12, a few glycan deletions in gp120 proved not sufficient to provoke significant phenotypic CBA resistance. Indeed, at least four or five glycan deletions were often found to be required to result in significant drug resistance (Balzarini et al., 2004b, 2005a, b, 2006, 2007a). This means that CBA are intrinsically endowed with a high genetic barrier.

Interestingly, up to at least 30 to 40% of the glycans in HIV-1 gp120 can be deleted under prolonged CBA pressure in one single virus particle. The affected N-glycosylation sites are predominantly containing high-mannose-type glycans, and seems not to appear clustered in gp120. Instead, they occur rather scattered over gp120 although glycosylation site mutations in the V1/V2 region (containing 6 complex-type glycans) appear rather sporadically (Balzarini et al., 2004b, 2005b, 2006) (Figure 2). Whereas in a very few cases we noticed that a certain configuration of glycan deletions resulted in a mutant virus with a higher infectivity potential and fitness (Balzarini et al., 2005a), several highly mutated virus strains were shown to rather have a compromised infectivity (Balzarini et al., 2005b). The reason might be that certain glycans on gp120 are indispensable for keeping a pronounced viral fitness. Alternatively, it is possible that such highly mutated virus strains contain envelope (gp120) molecules that were not properly folded or processed anymore. Further research is needed to adequately address this issue.

As mentioned above, significant phenotypic 2G12 resistance development occurs faster than observed for the other CBA and one or a very few (well-defined) glycan deletions suffice to result in pronounced resistance of the mutated virus to 2G12 (Huskens et al., 2007). This means that, in contrast with plant or proaryotic lectins, antibodies might be too specific in their interaction with gp120 resulting in a rather easy circumvention of the antiviral activity by the appearance of only one or two single amino acid mutations.

CBA: a new therapeutic concept?

Vaccine development faces huge problems, mainly because of the low antigenicity and immunogenicity of the HIV
envelope glycoprotein gp120 and the efficient hiding of highly immunogenic epitopes by the glycans present on gp120 (Burton, 1997). Thus, the immune system is not able to efficiently suppress HIV because gp120 does not elicit an efficient neutralizing antibody response in infected individuals. The high degree of protein glycosylation (approximately 50% of HIV-1 gp120 consists of glycans) is mainly responsible for this. In addition, the high mutational rate of HIV results in a high degree of viral variation in the envelope, which further compromises the efficient neutralization of a broad variety of continuously emerging virus strains by the immune system. Interestingly, it was shown that the glycan shield of HIV gp120 evolves during the course of infection in the presence of a continuously changing antibody repertoire (Wei et al., 2003). Such continuous changes in glycan packing efficiently prevent neutralizing antibody binding. Thus, it is clear that the abundant number of glycosylation sites at the gp120 glycoprotein surface helps to protect the virus against human immune responses to gp120 epitopes that are critical for HIV infectivity and/or transmission.

Importantly, Reitter and colleagues (1998) have demonstrated that rhesus monkeys infected with mutant SIV strains that lack only two N-glycosylation sites in their external envelope protein showed markedly increased antibody binding to specific peptides from the SIV env region. The mutant viruses were also substantially neutralized by the immune system. These observations illustrate that deletion of only two glycosylation sites in the viral env gene of SIV is sufficient to provoke a pronounced neutralizing antibody response. Very recently, Reynard et al. (2006) demonstrated that HIV gp120 envelope-selective deglycosylation may represent a valuable strategy to improve elicitation of neutralizing antibodies. These conclusions were based on characterization of the antibody response elicited by HIV-1 envelope glycomutant in rabbits.

These observations and considerations led us to propose that CBAs may represent a novel therapeutic approach of HIV treatment that substantially differs from all current treatment modalities (Balzarini, 2005) for the following reasons: (i) CBAs do not directly target viral peptides, proteins or enzymes. They predominantly target the glycans present on the gp120 glycoprotein. Such interaction affects the conformation and/or efficient functioning of the envelope molecule during viral entry. It cannot be excluded that some CBAs may also afford peptide binding in addition to their glycan binding. The mAb 2G12 is an (exceptional) example of a gp120-specific antibody that interacts with a glycan epitope on gp120. In addition protein-protein interactions also contribute to the overall specificity and binding affinity of 2G12. (ii) CBAs are the very first therapeutic molecules that do not interact in a stoichiometric manner to its target (glyco)protein. Whereas a HIV reverse transcriptase, integrase, protease inhibitor or gp41 bind to its viral target protein in a 1:1 ratio, CBA have the potential to bind with several molecules to one single target envelope glycoprotein molecule due to the abundant presence of glycans on gp120. This may explain the high genetic barrier of several CBAs for HIV. One or a few glycan deletions would not be expected to markedly affect phenotypic sensitivity of the virus to the CBA, and this has indeed been experimentally demonstrated in our cell culture studies (Balzarini et al., 2004b, 2005a,b; 2006; 2007a). (iii) CBA drug pressure forces the virus to progressively delete glycans on gp120 and thus weaken its glycan shield. As mentioned earlier, CBAs keep a pronounced antiviral suppressive effect even upon deletion of a few glycans on gp120. Further presence of CBA results in an increasing amount of glycan deletions enlarging the number and size of the ‘holes’ in the viral glycan shield. Such ‘hypermutated’ virus strains may now increasingly induce an immunotherapeutic reaction. Deletion of the glycosylation sites in gp120 may trigger the production of neutralizing antibodies against the previously hidden strong immunogenic epitopes of gp120. Although still to be proven, CBAs may become the first chemotherapeutic agents that exert a dual mechanism of antiviral action in vivo. Beside their direct purely antiviral effect by binding

Figure 2. Structure of the HIV-1 envelope gp120 according to Kwong et al. (1998)

Balls indicate the glycans on N-glycosylation sites. Red balls represent those glycans that were found deleted under CBA pressure in CEM cell cultures. (Overview of the mutations found in virus strains isolated under pressure of HHA, GNA, CV-N, PRM-A, 2G12 and UDA). Image courtesy of Ir. Katrien François, Rega Institute for Medical Research, Leuven, Belgium.
to the gp120 glycans and ‘freezing’ the entry process of the
virus after viral adsorption to its target cells, CBA may
likely trigger a strong immune response against the
mutated (glycan-deleted) virus strains as well. Initiating
production of neutralizing antibodies will result in a kind of
’self-vaccination’ of the infected individual. If this premise
turns out to be the case, CBA may become the first
chemotherapeutics that provoke a pronounced
immunomodulatory activity in addition to its direct
antiviral effects and may become a cornerstone in future
anti-HIV therapy. (iv) Glycan deletions in gp120 may
likely also delay the spread of virus from DC-SIGN-
containing dendritic cells to T-lymphocytes due to a less
efficient capture of an ‘incomplete gp120’ of the HIV
particle by the C-type DC-SIGN lectin present on
dendritic cells. (v) Given the high degree of similarity
between several enveloped viruses in terms of the presence
and role of high-mannose glycans on their envelope glyco-
proteins, the CBA approach can likely be extended to other
enveloped viruses that cause chronic infections such as
HCV or hepatitis B virus. In fact, very recently, cyanovirin
was demonstrated to markedly inhibit HCV in cell culture,
due to its glycan binding to the HCV envelope glycopro-
teins (Helle et al., 2006). These findings show that CBA
may indeed be able to block enveloped viruses other than
HIV in their entry process. Coronaviruses (that is, SARS
coronavirus) and influenza viruses are other examples of
enveloped viruses that may be highly susceptible to the
antiviral action of CBAs. Although, it may be questioned
whether the potential immunomodulatory activity of the
CBAs may play a significant, if any, role in their eventual
antiviral activity to the latter viruses, due to the fact that
these viruses are not chronic. Indeed, the virus infection
may become under control upon CBA administration
before glycan deletions occur and the immune system can get
involved in the further clearance of the virus. Nevertheless,
we feel that CBA must be thoroughly investigated against
these, and other, enveloped viruses as well.

Potential pitfalls of CBA to be used as
therapeutic agents

With the exception of Pradimicin A derivatives, all the
known antiviral CBAs are protein in nature. This may have
several disadvantages. The CBA proteins may be expensive
to produce, to scale-up and to purify. A commensal bac-
terial expression system as exemplified by the vaginal
commensal Lactobacillus sp. can be an interesting alternative
approach to deliver such CBAs as a microbicide in the
vaginal environment (Lagenauer et al., 2005). However,
many lectins are also endowed with a variety of unfavourable
properties such as immunogenicity, mitogenic stimulation
of human peripheral lymphocyte cells, hemagglutination of
human red blood cells, inflammatory activity, cellular toxici-
ty, stimulation of differentiation markers (Van Damme et
al., 1998; Sharon and Li, 2003). It is realistic to assume that
the plant lectins, and also the proacryotic cyanovirin – if
they should be ever applied as antiviral drugs – may elicit
an antibody response. However, these CBAs have been
investigated so far from a conceptual viewpoint, rather than
from a therapeutic viewpoint. Therefore, the observation
that the non-peptidic low-molecular-weight antibiotic
pradimicin A behaves as an ‘artificial lectin’ is an important
finding. It demonstrates that it may be possible to develop
synthetic non-peptidic CBAs for which an immunologic
response is highly unlikely. We have recently shown that,
beside its potent antiviral activity, cyanovirin is also
endowed with a pronounced mitogenic activity and cyto-
toxicity. It also stimulates the expression of activation
markers in PBMCs such as CD25, CD69 and HLA-DR
(Balzarini et al., 2006a). However, whereas the antiviral and
in vitro antiproliferative activity of CV-N can be efficiently
reversed by mannan (by competing with the CBA for its
mannose-binding site), the pronounced mitogenic activity
of CV-N on PBMCs seemed to be independent from
mannan competition with the lectin, indicating that some
side effects observed for CV-N may be unrelated to its
carbohydrate specificity. It is likely that these type of unde-
sired side effects may therefore be absent in some other
CBAs, and this has already been demonstrated for several
plant lectins such as HHA and GNA (Balzarini et al.,
2004a, 2006). Also, the human red blood cell hemagglu-
tinating activity of many lectins was also found to be absent
in HHA, GNA and UDA, showing that each CBA
deserves to be considered as separate drugs and to be inves-
tigated on their own right. The challenge would be to find
or choose the right CBA being endowed with minimal
unfavourable and optimal pharmacokinetic (that is, oral
bioavailability, frequency of dosing, etc.) properties.
The greatest concern, however, might be an unwanted
interaction of the CBA with cellular glycoproteins, and thus,
the degree of selectivity of the CBA and their potential to
discriminate between cellular and viral glycans. Although all
glycosylated proteins acquire their glycans from the cellular
glycosylation machinery, there exist, however, clear differ-
ences in the content of the glycans on glycoproteins from
pathogens versus cellular glycoproteins. It is indeed evident
that the manner in which oligosaccharides are presented
provides a mechanism for distinguishing ‘self’ from ‘non-self’.
Pathogens which present specific oligosaccharides as repeti-
tive arrays can, if these arrays have a suitable geometry, acti-
vate multivalent receptors. One example is MBL, which is
involved in the recognition and subsequent elimination of
pathogens through interaction with carbohydrates on the
pathogen (Gettins et al., 1981; Weis et al., 1998; Rudd et al.,
2001). This means that selectivity of CBA to pathogens, in
Figure 3. Multiple interactions of CBA with HIV transmission and infection of target cells

HBV infection of T-lymphocytes & macrophages

HIV infection of T-lymphocytes

CBA

Selection of HIV strains with glycan deletions in gp120

HIV transmission to T-lymphocytes through HIV capture by DC-SIGN

HIV neutralization by triggering neutralizing antibody production to uncovered gp120 epitopes

particular viruses, may become an achievable goal. In fact, the HIV envelope gp120 carries a remarkable high number of high-mannose-type-containing mannans (Leonard et al., 1990), a phenomenon that is rarely seen in mammalian glycoproteins. This may already be a rational basis of discrimination between both types of glycoproteins, and can be exploited among CBAs to afford a marked degree of selectivity. High-mannose-type glycans contain several terminal α-1,2-mannose oligomers that are usually absent in cellular glycoproteins and cyanovirin, but also the non-peptidic antibiotic PRM-A are known to selectively recognize and bind to α-1,2-mannose oligomers. We should keep in mind that many CBAs do not simply recognize monosaccharides, but, instead, often interact with quite well-defined three-dimensional carbohydrate oligomer structures. It would be challenging to select or design those CBAs that specifically recognize oligosaccharide structures that are uniquely present in the glycoproteins of the pathogen. Given the existence of low-molecular-weight non-peptidic CBAs (such as PRM-A) behaving amazingly similar to a mannos-specific lectin (Balzarini et al., 2007a), an intensive search for other non-peptidic low-molecular-weight CBAs would seem justified to allow further exploration of the novel CBAs concept as potential ‘broad-spectrum’ drugs against (glycosylated) enveloped viruses.

Conclusion

CBAs are endowed with interesting antiviral properties (Figure 3). They bind to the glycans of the viral envelope glycoprotein thereby inhibiting viral entry into its target cells. CBAs may have the potential to inhibit infection with cell-free virus and giant cell formation between virus-infected cells to uninfected cells, and DC-SIGN-directed virus capture and subsequent transmission to T-lymphocytes. CBAs may therefore qualify as potential microbicide drugs. Systemic CBA use may select for mutant virus strains with deleted glycans in its gp120 envelope, resulting in the active involvement of the immune system through neutralizing antibody production against previously hidden epitopes on gp120. Finally, CBAs may also be applied in the treatment of other enveloped viruses such as, but not limited to, coronaviruses, influenza viruses and flaviviruses such as hepatitis C virus.

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