ABSTRACT: The infection of humans by lethal pathogens such as Ebola and other related viruses has not been properly addressed so far. In this context, a relevant question arises: what can chemistry do in the search for new strategies and approaches to solve this emergent problem? Although initially a variety of known chemical compounds – for other purposes – have been disappointingly tested against Ebola virus infection, more recently, specific molecules have been prepared. In this Perspective, we present a new approach directed to the design of efficient entry inhibitors to minimize the development of resistance by viral mutations. In particular, we focused on dendrimers as well as fullerene C60 – with a unique symmetrical and 3D globular structure – as biocompatible platforms for the multivalent presentation of carbohydrates. The antiviral activity of these compounds in an Ebola pseudotyped infection model were in the low micromolar range for fullerenes with 12 and 36 mannoses. However, new tridecafullerenes – in which the central alkyne scaffold of [60]fullerene has been connected to 12 sugar-containing [60]fullerene units (total 120 mannose-based leads for practical applications.

INTRODUCTION

Ebola virus (EBOV) is among the most lethal pathogens for humans. Since its initial description in 1976 in Zaire (now DRC), several outbreaks have been reported mainly in Central Africa1. EBOV belongs to the Zaire Ebolavirus species within the Ebolavirus family where three additional varieties of highly pathogenic agents: Sudan, Tai forest and Bundibugyo viruses, have also been described2. There is a fifth variety known as Reston virus endemic in certain areas in Asia that apparently is not pathogenic for humans3. Although some fruit bats have been identified to be carriers of EBOV genetic sequences, still the natural reservoirs of EBOV are not clear and this is a crucial information for surveillance and prevention of future outbreaks4.

The recent Ebola outbreak in West Africa (2013-2016) has been caused by EBOV and it has been unprecedented in the number of infected cases. Thus, over 28000 cases and a toll of more than 11000 fatalities have been officially reported4 in the main affected area of Guinea, Sierra Leone and Liberia and also some cases in neighbor countries such as Nigeria, Mali and Senegal. Apart from the devastation in West Africa, this outbreak fueled a health emergency of international concern since Ebola infected patients travelled or were evacuated out of Africa and were treated in USA, UK, Spain, France, Germany, Italy, Switzerland and Norway5.

There is no specific treatment for EBOV and supportive therapy based in treatment of complications and active fluid and electrolyte replacement, remained as the basis for patient’s management during this outbreak6,7. A variety of experimental compounds, such as EBOV specific monoclonal antibodies (ZMap), interference RNA (TKM) or inhibitors of the viral RNA polymerase (Brincidofovir, Favipiravir (T-505) and GS-5734) (Figure 1) have shown therapeutic potential in experimental animal models of infection, rodents and non-human primates (NHP). However, thus far none of them has demonstrated clinical efficacy since they have only been anecdotally used in selected patients or in relatively small non-controlled clinical trials10.

At the beginning of the aforementioned last outbreak, there were also two prototypes of vaccines to prevent EBOV infection that had been tested in NHP with encouraging results but never used in human beings11. In an accelerated process to fulfill the safety and immunogenicity requirements in humans, the two vaccines, both virally vectored in chimpanzee adenovirus (ChAD) and in vesicular stomatitis virus (VSV), respectively, were ready to be administered in the affected area in early 2015, at a time where the epidemic was weaning and new cases started to be significantly reduced. Only results of efficacy from the VSV-based vaccine are available at this moment and, although involving a relative small group of individuals, the vaccine showed a 100% protection against EBOV when immediately administered to contacts of infected patients as compared with a control group that was vaccinated with a delay of 3 weeks12. Despite these promising results, questions remain on the duration of protection and the potential coverage against other members of the ebolavirus family such as Sudan or Bundibugyo viruses.

Under this situation, new programs and actions have been launched and funded by public institutions and foundations in order to speed up the discovery of new and effective drugs as soon as possible. However, the output of these strategies has...
This is a well-known inhibitor of the enzyme \( \alpha \)-glycosidase by the FDA to treat type I Gaucher disease (GD1) (Figure 1). Miglustat, an aminosugar derived of the D-glucose approved and its anti-viral activity by the modification of the proved15. However, a recent study using these iminosugars as composition of viral envelope glycoproteins has been shown the capacity of these molecules to inhibit Ebola infection in a murine Ebola infection model16.

Another example is the \( \alpha \)-butyl-deoxynojirimycin (Miglustat), an aminosugar derived of the D-glucose approved by the FDA to treat type I Gauche disease (GD1) (Figure 1). This is a well-known inhibitor of the enzyme \( \alpha \)-glycosidase and its anti-viral activity by the modification of the \( N \)-glycan composition of viral envelope glycoproteins has been proved15. However, a recent study using these iminosugars as inhibitors of Ebola infection in a guinea pig model showed very limited protection16.

The lack of information on the mechanism of infection, the need to use BSL4 laboratories to manipulate the wild virus – with the implications in terms of cost and accessibility that this means – as well as the scarce chances to carry out clinical studies on the ground with infected patients – usually concentrated in remote areas in Central Africa – provide a complicated scenario to progress in this field. In this context, a relevant question arises: what can chemistry do in the search for new strategies and approaches to achieve some success in this emergent problem? Actually, new treatment alternatives are desperately needed.

**DC-SIGN AS TARGET MOLECULE**

EBOV has a broad cell tropism although macrophages, monocytes and dendritic cells are major targets for infection. There are different possibilities in terms of checkpoints for antiviral actions during the infection of EBOV: entry inhibitors; viral inhibitors of viral replication; suppression of viral assembly and particles release and vaccination (activation of the immune system because infected antigen presenting cells are not activated by the Ebola virus and they should not be able to induce an immunological response). Among all these strategies, the design of entry inhibitors appears as a good approach with the advantage to minimize the development of resistance by viral mutations. Entry inhibitors can be focused on different targets involved in the interaction of the envelope viral glycoprotein (GP) with cell receptors, membrane fusion, endosome formation and viral particles release into the target cell cytoplasm.

The GP protein is the sole molecule of EBOV responsible to interact with target cells. In 2002, a new gate for Ebola infection of cells was discovered, the lectin DC-SIGN. DC-SIGN (Dendritic Cells Specific ICAM-3 Grabbing Non-integrin) present at the surface of dendritic cells (DCs) is a lectin of the C-type able to recognize in a calcium dependent manner, highly glycosylated envelope proteins such as gp120 of HIV or GP of Ebola virus. This lectin facilitates the entrance of the virus into DCs, cells presented in mucosal surfaces and potentially facilitating viral infection and systemic dissemination. Since its discovery in 2000 and the demonstration of its role in the recognition of a large family of pathogens including viruses, bacteria, fungi and parasites such as HIV, Ebola, Citomegalovirus, Hepatitis C virus, *Mycobacterium tuberculosis*, *Schistosoma*, *Candida spp*., etc., this lectin has been considered as an universal pathogen receptor and a potential new therapeutic target. This discovery opened the door for the development of new antiviral compounds based on carbohydrates mimicking the presentation of the glycans presented in the viral glycoproteins. To achieve this aim, a multivalent presentation of carbohydrates is required to reach high affinities for this lectin and to compete efficiently with the virus for this receptor.

Recently, a potential role of DC-SIGN expressing cells has been reported in animal models of infection suggesting that, in fact, these dendritic cells have a key role in the initial steps of EBOV infection and dissemination. The GP protein is the sole molecule of EBOV responsible to interact with target cells. In 2002, a new gate for Ebola infection of cells was discovered, the lectin DC-SIGN. DC-SIGN (Dendritic Cells Specific ICAM-3 Grabbing Non-integrin) present at the surface of dendritic cells (DCs) is a lectin of the C-type able to recognize in a calcium dependent manner, highly glycosylated envelope proteins such as gp120 of HIV or GP of Ebola virus. This lectin facilitates the entrance of the virus into DCs, cells presented in mucosal surfaces and potentially facilitating viral infection and systemic dissemination. Since its discovery in 2000 and the demonstration of its role in the recognition of a large family of pathogens including viruses, bacteria, fungi and parasites such as HIV, Ebola, Citomegalovirus, Hepatitis C virus, *Mycobacterium tuberculosis*, *Schistosoma*, *Candida spp*., etc., this lectin has been considered as an universal pathogen receptor and a potential new therapeutic target. This discovery opened the door for the development of new antiviral compounds based on carbohydrates mimicking the presentation of the glycans presented in the viral glycoproteins. To achieve this aim, a multivalent presentation of carbohydrates is required to reach high affinities for this lectin and to compete efficiently with the virus for this receptor.

**CHEMICAL STRUCTURES**

Several carbohydrate multivalent systems have been developed and tested as good inhibitors for DC-SIGN-dependent viral infections. A variety of platforms have been evaluated as useful scaffolds for conjugation of carbohydrates to create different carbohydrate multivalent systems where the size, shape and valency (number of carbohydrates) have been modulated at will. Dendrimers are monodisperse highly branched polymers. The stepwise synthesis of dendrimers allows a fine control of the structure as well as the physical and chemical properties of
The functional groups present at the periphery can be manipulated to modulate these properties. Furthermore, these functional groups can be used for the conjugation of ligands, thus creating multivalent systems. In other words, dendrimers can be considered as ideal scaffolds for a multivalent presentation of ligands.

Carbohydrate multivalent systems based on dendrimers have been synthesized for the development of molecules capable to block DC-SIGN. More than a decade ago, mannosylated dendritic polymers based on the 2nd and 3rd generations of the commercially available Boltorn hyperbranched polymer were synthesized. The good activity of the 3rd generation glycodendrimer to inhibit the interaction of pathogens to DC-SIGN opened the door for the development of more active compounds using the same scaffold but more sophisticated and efficient ligands for DC-SIGN, in particular a pseudomannobiose and a pseudomannotrioside (Figure 2). These glycodendrimers have been prepared in a divergent way, building up the dendrimer from the central core to the periphery. As a final step, carbohydrate ligands were conjugated by a classical amide formation.

With the aim to mimic better the multivalent presentation of carbohydrates at the surface of pathogens to compete for DC-SIGN, a virus like particle (VLP) was used as core to conjugate trivalent and nonavalent dendrons of mannoses in a convergent synthetic strategy. Using direct mutagenesis, an unnatural aminoacid, homopropargylglycine, replacing a methionine, was introduced in the peptidic sequence of the monomer QB protein. After a self-assembly process, a sphere-like icosahedron particle with a diameter of around 28 nm was obtained with 180 alkyne groups at the surface. These alkyne groups were conjugated via click copper-catalyzed alkyne cycloaddition (CuAAC) reaction with mannosylated dendrons of 1st and 2nd generation containing azido group at the focal position to create the corresponding particles with 540 and 1620 mannoses, respectively (Figure 3). This glycodendriproteins have a size in the same order of magnitude as the pathogens to be mimicked and display in a precise way a large number of ligands (mannoses) to interact in a very strong way to DC-SIGN.

In the search for innovative scaffolds, we focused on unexplored 3D fullerene C_{60} since it has a unique symmetrical and globular structure (~1 nm of diameter) which makes it an interesting biocompatible carbon platform for the multivalent presentation of carbohydrates. Its high hydrophobicity can be avoided by the preparation of hexakis-adducts of [60]fullerene, as these compounds present six organic addends with a T_{d}-symmetrical octahedral addition pattern. Hexakis-adducts of [60]fullerene are generally easily obtained by the Bingel-Hirsch addition of malonates to C_{60}. One limitation of this synthesis is, however, the low yields obtained when the size of the added malonates increases. To overcome this problem, hexakis-adduct bearing six malonates appended with alkynes was synthesized. This compound allowed the formation of “sugar balls” by using the efficient click CuAAC reaction. In this way, it is possible to obtain the carbohydrate-containing carbohydrates at the focal position to create the corresponding particles with 540 and 1620 mannoses, respectively (Figure 3). This glycodendriproteins have a size in the same order of magnitude as the pathogens to be mimicked and display in a precise way a large number of ligands (mannoses) to interact in a very strong way to DC-SIGN.

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[60] Fullerene derivatives in two synthetic steps, as depicted in Scheme 1 with good to excellent yields.33-34

The characterization of these compounds was carried out by the common analytical and spectroscopic techniques, being $^{13}$C NMR especially helpful. The $^{13}$C NMR spectra of hexakis-adducts of C$_{60}$ show only two signals in the sp$^2$ region ($\delta \sim 141$ and 145) and only one signal for the sp$^3$ carbons ($\delta \sim 69$), thus allowing to have evidence of the formation of the highly symmetric octahedral derivative. For the completion of the click reaction, FTIR analyses were also valuable, as the typical bands for alkyne and azide groups (at $\sim 2117$ and 2092 cm$^{-1}$ respectively) observed in the starting products are not present in the sugar derivatives, indicating the efficiency of the cycloaddition step.

To check if this presentation of carbohydrates shows a multivalent effect, interaction of compounds 4a-b,d with Concanavalin A (Con A), a mannose recognizing lectin present in jack-beans, was studied by isothermal titration calorimetry (ITC). Indeed, an increase in the $K_a$ was observed when going from the monovalent reference ligand (Me-$\alpha$Man, $K_a \sim 1.2 \times 10^4$ M$^{-1}$) to the hexakis-adducts 4a ($K_a \sim 421 \times 10^4$ M$^{-1}$) and 4d ($K_a \sim 137 \times 10^4$ M$^{-1}$) bearing 24 mannoses (4b substituted with galactoses was used as negative control and showed no interaction with Con A). Interestingly, if the $\Delta G$ values for 4a ($\sim -9$ kcal·mol$^{-1}$) and 4d ($\sim -8.4$ kcal·mol$^{-1}$) are examined, the binding with Con A seemed to be more favorable in the case of 4a with a lower valency. These experimental findings were accounted for by the great entropic cost that has the binding between 4d and the lectin, and indicates that not only the number of ligands is important but also the distribution around the spherical platform of fullerene and the size of the linker between C$_{60}$ and the sugars.

The dynamics of glycofullerenes 4a and 4f has been investigated by NMR translational diffusion and quantitative $^{13}$C relaxation studies.35 These studies show that the better activity of 4f in comparison to 4a (which differ in one order of magnitude for the IC$_{50}$ values) may find explanation in its slower translational diffusion, thus facilitating rebinding to the receptor, together with the bigger spatial extension of the molecule, which would allow chelate binding to a second receptor site.

Scheme 1. Synthesis of fullerene sugar balls from alkyne appended derivative 2.
To increase dramatically the valency and the size of the fullerene derivatives, new tridecafullerenes (5) – in which the central alkyne scaffold of [60]fullerene has been connected to 10 sugar-containing [60]fullerene units – have been synthesized (Figure 4).16 The synthesis of these compounds implies obtaining new [60]fullerene based building blocks carrying the carbohydrate moieties as well as an azide group for further functionalization, and they have been prepared with two different spacers between the central fullerene and the peripheral C60 units. These azide containing glycofullerenes can be added under CuAAC conditions to the previously obtained hexakis-adduct 2 to yield the “super balls” formed by 13 [60]fullerene moieties covalently linked and surrounded by 120 carbohydrate ligands! This reaction has been described as the fastest dendrimer growing reported to date.37

To test the inhibitory properties of compounds on EBOV DC-SIGN-mediated infection we have used a system in which a T-lymphocyte cell line, one of the few cell population resistant to EBOV infection19, is rendered susceptible by the expression of DC-SIGN21a. In this system cell infection with an EBOV GP-pseudotyped viral particle is completely dependent on DC-SIGN since T-lymphocytes lack any other attachment receptors to facilitate EBOV cell entry. On one hand, this model is very appropriate to have clean experiments and to demonstrate that the inhibition of tested molecules is based exclusively on the blockage of the receptor DC-SIGN. On the other hand, this model permits the use of BSL-2 laboratories, more accessible and

BIOLOGICAL ASSAYS

Figure 4. Molecular structure of tridecafullerenes bearing 120 sugar moieties (left). Representative 13C NMR signals for compound 5a (right).
available all around to obtain results concerning the activity of the new candidates to inhibit Ebola infection at a reasonable cost and in a short time. As a control of the infection we used viral particles pseudotyped with the envelope glycoprotein of the vesicular stomatitis virus (VSV) that does infect T-lymphocytes in a DC-SIGN-independent manner. The 50% of inhibition of the infection (IC$_{50}$) is typically calculated with 95% confidence intervals (IC$_{95}$). Recombinant viruses are produced by co-transfection in producer cells of the viral glycoprotein along with the retroviral backbone expressing firefly luciferase as the reporter gene. The assay based on GP-pseudotyped viral particles has been extensively used for pathogenesis and antiviral screening by our group and others for different purposes and it has been compared with live viruses assays showing strong correlation.

Proof of concept of the capability of multivalent mannose glycoconjugates to compete with DC-SIGN for viral entry of EBOV-GP particles was obtained with relative simple dendrimeric compounds such as Boltorn derivates exhibiting 32 carbohydrate molecules with IC$_{50}$ in the micromolar to nanomolar range (depending on the ligand used). Subsequently, in order to gain mannose multivalency, the highly polyvalent bacteriophage-based glycodendrin nanoparticle displaying 1620 copies of mannose on its surface achieved IC$_{50}$ in the high picomolar range. More recently, the synthesis of giant globular glycofullerenes decorated with mannoses has also shown antiviral activity with IC$_{50}$ in the subnanomolar range.

**OUTLOOK AND PERSPECTIVES**

Emergent pathogenic viruses represent nowadays one of the most dangerous causes for diseases in the human being. In particular, Ebola virus since its first detection in 1976 has been responsible for several outbreaks resulting in a great proportion of deaths. The unprecedented magnitude of the last outbreak in West Africa (2013-6) and its global impact make it necessary to establish preparedness for future epidemics.

In the search for new pathways for addressing Ebola virus infections, we have focused on the well-known carbohydrate–protein interactions since they govern a wide variety of biological processes. Importantly, carbohydrate–protein interactions usually occur by means of the multivalent effect. In this regard, different chemical and bio-inspired scaffolds have been used in the literature in the search for new glycoconjugates bearing larger units of carbohydrates located in the right spatial arrangement.

Within the development of our project, we have prepared mannosylated fullerenes containing several copies of carbohydrates in a globular presentation. These multivalent systems have a good solubility in aqueous media and a low cytotoxicity against several cell lines. Preliminary binding studies using the model lectin Concanavalin A demonstrated the potency of these glycodendrofullerenes to interact with lectins in a multivalent manner. In this regard, a variety of carbohydrate multivalent systems based on dendrimers have been synthesized for the development of molecules capable to block DC-SIGN. In order to get a 3D globular scaffold thus resembling the most usual virus geometry, we were able to prepare, for the first time, new glycodendrofullerenes as antiviral agents. The antiviral activity of these compounds in an Ebola pseudotyped infection model were in the low micromolar range for fullerenes with 12 mannoses, a very promising data. Interestingly, the increase of valency to 36 mannoses in glycofullerene induced a loss of antiviral effect. This could be probably related to steric congestion of sugars at the surface of the fullerene. One important factor to achieve high affinity in binding processes is not only the spatial presentation of the ligand but also the adequate accessibility of these ligands to interact with the corresponding receptor. Using a glycodendrofullerene showing the same valency as compound 4e, but including a longer spacer, we have increased remarkably the inhibitory activity of these compounds with IC$_{50}$ values in the low micromolar range, probably due to a more efficient interaction with DC-SIGN. This result highlights the importance to combine an adequate scaffold to achieve the multivalency (the spherical [60]fullerene) with the right ligand presentation in terms of accessibility and flexibility. The valency of the compound is an important factor to obtain good affinities in a carbohydrate–lectin interaction but, as it has been shown in these experiments, it is not the only factor to be taken into account.

More recently, the synthesis of giant globular glycofullerenes decorated with mannoses (sugar superballs) have been synthesized and characterized by a variety of techniques. Interestingly, the so-called tridecafullerenes decorated with 120 mannoses have also shown antiviral activity with IC$_{50}$ in the subnanomolar range. These experimental findings make these sugar superballs the most active molecules to inhibit the Ebola virus infection reported so far.

Based on the aforementioned results, fullerenes should be considered as very attractive and compatible 3D scaffolds for a globular multivalent presentation of sugars. These promising results prompt us to the search for new approaches for the design and preparation of glycodendritic key building blocks to conjugate on fullerenes. An important concept is, however, a fine control of the congestion between carbohydrates to prevent unfavorable steric hindrances in the search for better antiviral activities.

Needless to say that currently there are a great variety of known carbon nanoforms which could also be used as potential scaffolds for the multivalent presentation of carbohydrates, namely single and multiwall carbon nanotubes, graphene or graphene quantum dots where the control of the carbohydrate functionalization and distribution still represents a future scientific challenge. This is also applicable to the variety of potential carbohydrates, both in terms of their own nature (monosaccharides, disaccharides, polysaccharides, etc.) and number of units (monomer, dimer, trimer, etc.). A combination of more efficient ligands and more adequate presentation on different scaffolds should eventually afford a variety of lead hybrid molecules with higher specificity and efficiency on Ebola virus infection.

A final consideration is that, although some vaccines are currently under study for the Ebola virus, the availability of chemical compounds able to fight against Ebola virus at different stages of the infective process are scarce and
preliminary results of clinical efficacy have been limited. The multivalent presentation of specific carbohydrates by using 3D fullerences as controlled bio-compatible carbon scaffolds represents a real advance, which, however, requires more studies to determine the optimized leads for practical purposes. The pathway is open and the only limitation to this goal is the imagination of the chemists.

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REFERENCES

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