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Multimeric glycotherapeutics: New paradigm

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The general principle of anti-adhesion therapy is the inhibition of microorganism adhesion to the host cell with the help of a soluble receptor analog. Despite an evident attractiveness of the concept and its long existence, the therapeutics of the 'post-antibiotic era' have not yet appeared. This can be explained by the contradictoriness of requirements for anti-adhesion drugs: to be efficient a drug must be multivalent, *i.e.* large molecule, but to obtain FDA approval it should be a small molecule. A way to overcome this contradiction is self-assembly of glycopeptides. The carbohydrate part of glycopeptide is responsible for binding with the lectin of microorganisms, whereas a simple peptide part is responsible for an association to the so-called tectomers. Depending on the structure, tectomers are formed either spontaneously or upon promotion of a microorganism. In particular, sialopeptide, which is capable of converting to a tectomer only in the presence of the influenza virus, has been obtained. Thus, the new strategy of anti-adhesion therapy can be formulated as follows: (1) identification of oligosaccharide-receptor for a particular virus (bacteria); (2) optimization of the peptide part; (3) conventional trials. The expected advantages of this strategy are the following: (i) no polymer; (ii) a virion completely covered with a tectomer, *i.e.* blocking is both complete and irreversible; (iii) rapid and rational lead identification and optimization; (iv) minimum side effects; (v) potential for microorganism resistance to natural receptor is lower than in the case of mimetics.
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Abbreviations: **CBS, carbohydrate-binding site; HA, hemagglutinin; PAA, polyacrylamide; SA, sialic acid; SL, sialyllactose; SLN, sialyl(N-acetyl)lactosamine.**

Introduction: Principle of anti-adhesion therapy

Carbohydrate chains on the human cell are primary receptors for many microorganisms. The corresponding lectin (adhesin) of a microorganism ensures the binding to these chains. The anti-adhesion therapy means inhibition or, better yet, complete blocking of lectin-mediated adhesion (Figure 1) [1]. Antibiotics exert strong evolution pressure on pathogens, thus increasing the probability that resistance will develop. In contrast, an anti-adhesion strategy could minimize evolutional pressure on a pathogen; the potential for mutational escape or development of resistance to this type of therapeutic may be lessened [2].

Discussion concerning this approach was started more than 30 years ago; followed by experimental studies, and even clinical trials were performed during the last decade. However, practical application of anti-adhesion therapeutics still seem to be a distant matter. Why? This can be explained by the fact that monomeric oligosaccharides are incapable of effective competition with the same oligosaccharides on the cell surface due to a low affinity of 1:1 interaction. Microorganism interaction with a cell is multipoint and generally cooperative; moreover, the primary carbohydrate-protein recognition is rapidly followed by further cascade events, making the whole process irreversible. Thus, an adhesion blocker as a drug must have a greater affinity toward microorganisms than to the natural receptor. An obvious way of affinity increase is the synthesis of multivalent receptor analogs, *i.e.* the way that inevitably leads to high m.w. compounds. In this study we demonstrate, *in vitro* and *in vivo,* the efficiency of large molecules as influenza virus blockers as compared to monomers. The further development of this approach is the design of self-assembled glycoconjugates, *i.e.* non-covalent polymers as a new generation of polyvalent blockers.

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Choice of the ligand: 6'SLN as common receptor for all human influenza A and B viruses

Influenza virus infection is initiated by specific interactions between the viral envelope glycoprotein hemagglutinin (HA) and host cell surface receptors [5–7]. Terminal sialic acid residue

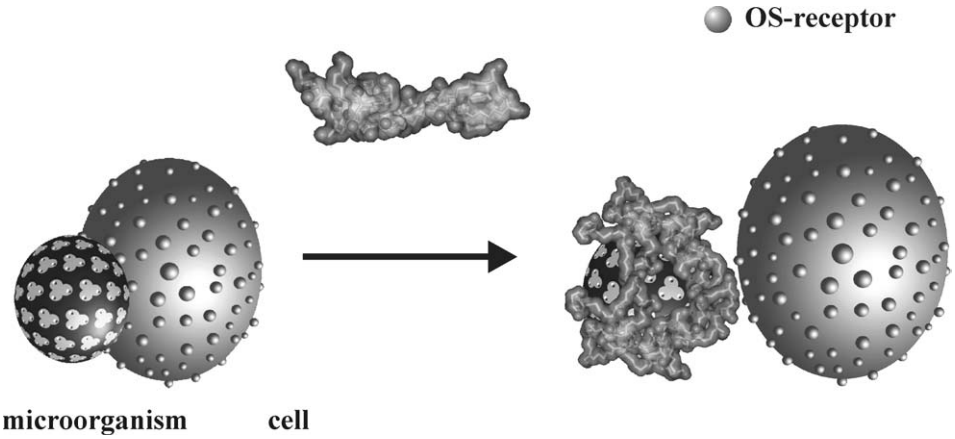


Figure 1. Anti-adhesion principle. OS-receptor of a target cell can competitively inhibit binding of a microorganism to the cell. Bulky multimeric conjugates of this OS are generally more effective blockers due to elevated affinity and the so-called steric stabilization effect [3,4].

of sialoglycoconjugates is known to be the minimum binding determinant of these receptors. Virus binding also depends on the type of the SA linkage to penultimate galactose and on the structure of the more distant parts of the sialyl oligosaccharides [5,6,8–11]. Human viruses bind to fragment Neu5Acα2-6Gal, whereas avian ones bind to Neu5Acα2-3Gal. More recent data [9] show evidence that the next residue of the carbohydrate chain, namely GlcNAc, also takes a significant part in reception, the shared receptor for all real human A and B strains (non egg adapted) is trisaccharide Neu5Acα2-6Galβ1-4GlcNAc (6'SLN). It should be noted that in contrast to 6'SLN trisaccharide, 6'SL and disaccharide Neu5Acα2-6Gal bind viruses H1N1 by 1 to 2 orders of magnitude weaker. This study was performed on viruses grown on MDCK cell culture [9]. Identical results were obtained using the viruses grown on another cell culture, Vero, of monkey origin [12]. Modern (within the last 10 years) virus strains display the most pronounced specificity towards 6'SLN. Although, in contrast to embryonic eggs, the structure of carbohydrate chains on the surface of Vero and MDCK cells resembles that of human cell targets of the influenza virus, there still has been no evidence that virus specificity remained unchanged during cultivation on these cultures. So, it was principally important to test specificity of viruses taken directly from patients without any passage *in vitro*. Such studies have not been done earlier, because the virus amount that is possible to isolate from one patient is minute. Sensitive assay developed by us [13] made way to study hospital material directly. Such experiments confirmed the 6'SLN specificity of clinical viruses. Finally, viruses H9N2, which have recently caused several viral outbreaks in Hong Kong, also demonstrated binding to 6'SLN [14]. Interestingly, 6'SLN specificity is realized by virus hemagglutinins with amino acid substitutions at the carbohydrate-binding site (CBS); moreover, specificity is modulated by carbohydrate chains of HA near CBS [12]. This conservatism of virus HA towards 6'SLN looks surprising and unexplainable: it seems that it is more advantageous for the virus as a population to have a variety of receptors.

Anyhow, according to the experimental data mentioned above, the selection of 6'SLN trisaccharide as the ligand for the design of multivalent therapeutic-blocker is quite obvious.

Lessons of multimeric PAA-conjugates

Understanding very well that therapeutics on the base of polyacrylamide (PAA) or similar polymers do not have real chances to gain FDA approval, we have been working with PAA glycoconjugates as a convenient model for comparison of monomeric *vs*. multivalent virus blockers. Experiments with PAA glycoconjugates showed a drastic increase of blocking potency by the increase of polymer size; also, polymer ability to hamper cell culture infection, and efficiency of polymers *in vivo*.

In the *in vitro* inhibition assay (fetuin-binding assay [15]), the dependency was the following: 30 kDa Neu5Ac-PAA was at least three orders of magnitude more potent than the monomer, whereas 1000 kDa polymer was two orders more active than the 30 kDa substance [12,16]. Experiments on inhibition of virus infectivity were performed with 30 kDa 3'SL-PAA, 6'SL-PAA, 6'SLN-PAA, and YDS-PAA. The latter is the conjugate of biantennary N-glycan, 11-oligosaccharide, bearing 6'SLN fragments in each of the antennae. MDCK cells were infected by human viruses; the activity of the polymeric inhibitors in this system coincided with those in the fetuin-binding inhibition assay; *i.e.*, 3'SL did not inhibit infectivity, 6'SL worked poorly with H1N1 strains, but both 6'SLN derivatives displayed the same high activity towards all the tested strains [9] of types A and B, see Table 1.

Finally, the experiments on a murine model where the same set of polymeric blockers was tested demonstrated drastic increase of survival of infected (five semi-lethal doses of virus) experimental animals when 6'SLN polymer was administrated. Moreover, all of the three schemes, *i.e.*

Table 1. Inhibition of virus infectivity by PAA sialoglycoconjugates. Concentration of 90% suppressing of the virus infectivity in MDCK cell culture, μM Neu5Ac

	Concentration of 90% suppressing virus infectivity, μM Neu5Ac			
	Avian strain	*Human strains*		
Inhibitor	*H9N2 A/Mallard/3/82*	*B B/NIB/15/88 M**	*H1N1 A/NIB/23/89 M*	*H3N2 A/NIB/3/90 M*
3′SL-PAA	4	ND	ND	ND
6′SL-PAA	ND	1	>20	0.2
6′SLN-PAA	ND	1	0.5	0.2
YDS-PAA	ND	1	0.5	0.2

*M means that the virus strain was cultured solely on MDCK cells, without the embryonated eggs stage.

treatment before infection, simultaneously with infection, and after infection proved to be efficient [17]. Thus, all of the model experiments evidenced, firstly, about the dramatic advantage of large molecules over monomers and, secondly, about the principal possibility of therapy by reasonable doses of polyvalent sialoside.

Self-assembled molecules instead of true polymers

Increasing interest in the self-assembly of small molecules [18–21] into complex supramolecular structures has arisen from the design of nano-materials and molecular devices. The self-assembly is largely governed by the simultaneous formation of hydrogen bonds between complementary fragments, resulting in either β-sheet-like structures or nanotubes [22–25]. In addition to hydrogen bonding these supramolecular structures require additional van der Waals (hydrophobic) and/or ionic interactions between side chains to provide the required stability. Most of the structures described in the literature are stable in non-polar organic solvents and in the solid phase. Only the classic amphiphils, such as micelles, liposomes, and aggregates of carbohydrate-substituted porphyrines [26], are able to form non-covalent structures in aqueous solutions. Our aim therefore was to design a stable inaqueous solution, *non-covalent* polymer instead of true polymers (Figure 2).

In our case, the main requirement for the self-assembling entities was the absence of hydrophobicity, thus avoiding any nonspecific interaction with the cell membrane, while maintaining their chemical simplicity. We have found that glycine-based peptides (Figure 3) meet these requirements.

These structures were expected to possess a dramatically higher activity when compared to a monovalent carbohydrate ligand due to its multivalent interaction with virion as in the case of the previously discussed polymeric conjugates.

Actually, the attachment of Neu5Ac to the non-covalent polymer, tectomer (the term *tecton* [27] is used to describe the monomeric form, while the assembled structures are referred to as *tectomers*), enhanced its effect. The non-assembling glycopeptides of a general formula [Neu5Acα-linker-Gly$_n$-NHCH$_2$]$_4$C ($n = 1 \div 6$) (Figure 3) did not show a substantial increase in activity relative to monovalent Neu5AcαOBn. However, an increase in chain length by elongation of the oligoglycine fragment up to Gly$_{7-9}$ enhanced the activity by at least three orders of magnitude (Table 2). Electron microscopy (EM), atomic force microscopy (AFM), and light scattering data showed that all active compounds were actually high molecular weight aggregates. The specificity of binding was confirmed by EM: Neu5Acα tectomer demonstrated binding to virus particles, while the Neu5Acβ tectomer showed no binding [28]. The antiviral activity of Neu5Acα tectomers ($K_d \sim 10^{-7} - 10^{-8}$ M

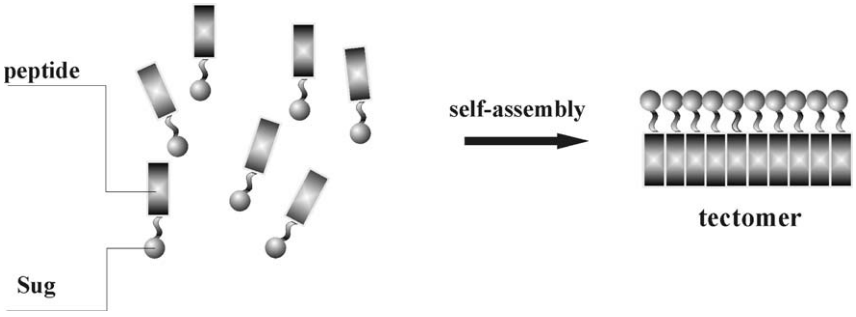


Figure 2. Biologically active non-covalent polymers (tectomers) consist of saccharide capable of binding to microbial lectin and oligopeptide capable of self-assembling due to formation of multiple hydrogen bonds.

Table 2. Relative potency of glycopeptides as inhibitors of the influenza virus adhesion to the glycoprotein fetuin (based on a previously described binding assay (15]). Virus strain A/NIB/44/90M (H3N2). Bn, benzyl; linker, —$OCH_2C_6H_4NHCOCH_2NH$—$CO(CH_2)_4CO$—.

Glycopeptide	*Relative activity*
Non-assembling	
Neu5AcαOBn (reference compound)	1
[Neu5Acα-linker-Gly_n-$NHCH_2]_4$C ($n = 1 \div 6$)	1–3
Assembling (tectomers)	
[Neu5Acα-linker-Gly_7-$NHCH_2]_4$C	1000
[Neu5Acα-linker-Gly_8-$NHCH_2]_4$C	1400
[Neu5Acα-linker-Gly_9-$NHCH_2]_4$C	3300

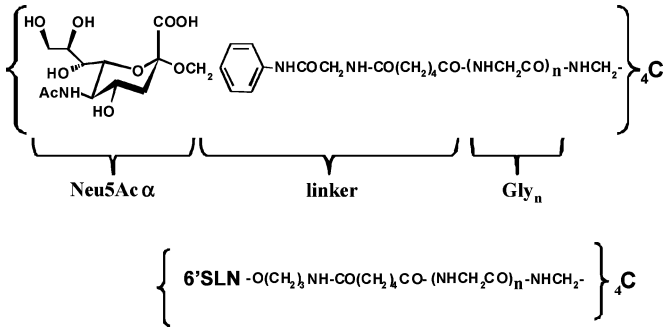


Figure 3. Chemical structure of glycopeptides (tectons). Four identical oligoglycine antennae are attached to the central carbon atom, sialic acid or sialooligosaccharide is connected to terminal Gly moiety through the linker group, $n = 1$ to 10.

by sialic acid) approached that of the corresponding polyacrylamide derivatives.

The so-called polyglycine II structure was identified in the tectomer peptide chains and was distinct from canonical α-helix and β-sheet formations. Previously, the polyglycine II was found in the crystalline glycine polymer [29], bolaamphiphils [30], and nylons [31], whereas all the NH and CO groups of the 3_1 helices ($\phi = -76.9^\circ$, $\psi = 145.3^\circ$) form intermolecular hydrogen bonds with the six surrounding chains although no intra-chain H-bonds are formed. Contrary to all cited examples where polyglycine II was only observed in a solid phase, the tectomers are stable in aqueous solution. The polyglycine II structure of tectomers is evidenced from Raman spectra. The spectrum profiles of both the peptide and glycopeptide tectomers comprise a banding pattern with the position, form and relative intensity consistent with crystalline polyglycine II [32]. The shape and size of tectomers was investigated by AFM (Figure 4) and electron microscopy [28].

These studies showed the tectomers as thin, flat sheets (Figure 4). According to AFM, the experimentally determined thickness of the glycopeptide tectomer [Neu5Acα-linker-Gly_7-$NHCH_2]_4$C is 74 ± 5 Å, whereas the [Gly_7-$NHCH_2]_4$C peptide tectomer is 45 ± 5 Å.

The tectomer is a two-dimensional crystal (Figure 5), which is rigid due to a high-cooperative system of hydrogen bonds; any deviation from the flat shape, even on a macro-level, appears to be unfavorable. The unusual stability of the tectomer in aqueous media can be explained by the participation of all CO and NH glycine groups in H-bonding, and the exclusion of any H-bond interactions with the aqueous solvent inside the two-dimensional crystal. Moreover, each Gly_n element of the crystal is covalently bound with one of its neighbours via a central –$NHCH_2]_4$C fragment; this is also the basis for an elevated stability of the system.

Aqueous tectomers are stable at room temperature in the presence of salts at physiological concentrations, while a reversible disintegration occurs in the presence of concentrated solutions of lithium bromide, trifluoroacetic acid, or upon heating.

Synthesis

The tetra-antennary peptides and glycopeptides (see Figure 3) were synthesised by a conventional peptide chemistry using tetra(aminomethyl)methane as the starting material. Glycine residues were inserted individually or *en block* as hydroxysuccinimide-activated Boc-derivatives [33]. Carbohydrate groups were coupled through acylation of amino groups of terminal Gly residues by Neu5Acα-linker-COONp (Np = 4-nitrophenyl) in two different ways: (a) the addition of 1 M

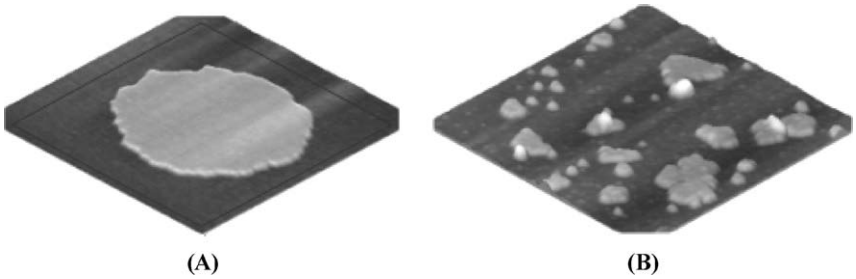


Figure 4. Peptide and glycopeptide tectomers as probed with atomic force microscopy (AFM). AFM data. (A) AFM imaging (1035×1035 nm, mica) of water insoluble tectomer [Gly_7-$NHCH_2]_4$C formed from the corresponding hydrochloride by adding four equivalents of sodium bicarbonate; the tectomer thickness is 45 Å. (B) AFM imaging (1035×1035 nm, pyrographite) of the water soluble tectomer [Neu5Acα-linker-Gly_7 -$NHCH_2]_4$C; the tectomer thickness is 74 Å (or sporadically as a double sheet with thickness 148 Å).

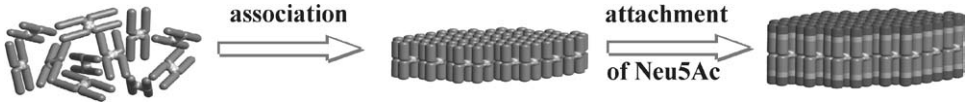


Figure 5. Chart showing supramolecular organisation of symmetrical tetraantennary tectons into one molecule-thick tectomers.

$NaHCO_3$ to aqueous $[HCl \cdot Gly_7\text{-}NHCH_2]_4C$ (which exists as a monomer) resulting in the assembled $[Gly_7\text{-}NHCH_2]_4C$ followed by acylation with Neu5Acα-linker-COONp; (b) alternatively, the glycopeptide was derived by acylation of monomeric $[Gly_7\text{-}NHCH_2]_4C$ in concentrated LiBr in the presence of Li_2CO_3. The degree of 'glycosylation' by method **a** was ~75%, whereas the synthesis by method **b** permits achievement of 100% substitution by Neu5Ac.

Virus-promoted association instead of self-assembly

While the above-presented results have focused on the activity of pre-formed tectomers, a more promising approach has to be the specific generation of an extended assembly *only in the presence of virus* (Figure 6).

Examples of surface-promoted assembly include molecules possessing two different binding sites; the first one is for homotypic binding, and the second one is for an interaction with a surface [34–36]. One of the well-known examples is the multivalent mode of interaction of formally monovalent galectin-3 with laminin [36]. From the perspective of an antiviral therapy the assembly of small molecules into tectomer on virion has clear advantages as compared to the administration of the same, but pre-formed, tectomer. Results of the first positive experiments demonstrating the feasibility of this concept, virion-promoted assembly, are shown in Figure 7.

According to electron microscopy data, the micrometer size tectomer was formed from smaller entities (dynamic mixture of tectons with small aggregates) only upon contact with the influenza virus. Importantly, this virus-promoted assembly is ligand-specific; in the same conditions an analogue with beta-connected Neu5Ac demonstrated no aggregation when in contact with the virus (Figure 7). Thus, the microorganism-promoted self-assembly is a novel mode offering to surmount general problems in the design of multivalent therapeutics, namely both inconsistency and poor biodegradation of true polymers.

Structural features of material capable of virus-promoted assembly (Figure 7)

Carbohydrate ligand was coupled to tecton in the presence of LiBr. The synthesis in the presence of LiBr enables the achievement of 100% substitution by Neu5Ac (see above), glycopeptides synthesized in these conditions are incapable of *spontaneous* assembly into extended flat tectomers due to spatial hindrance by Neu5Ac groups. Although tetrasubstituted tectons are finally capable of forming tectomer, a potential barrier of the aggregation is too high for spontaneous step-by-step process; this is why tectomer formation has not been observed in the absence of the virus. Viruses play a role of priming; HA trimers densely situated on virion serve as a scaffold which forms and stabilizes tectomer germs. Thus, the driving force of virus promotion seems to be double cooperativity of tectons assembly on the one hand, and tectons binding to HA on the other hand. The presence of a neuraminidase inhibitor, 4-amino-4-deoxy-Neu5Ac2en (3 μM), does not affect the phenomenon; thus the virus-promoted assembly cannot be explained by the action of neuraminidase, *i.e.* by viral neuraminidase-promoted splitting off of the "extra" Neu5Ac residues constraining the assembly.

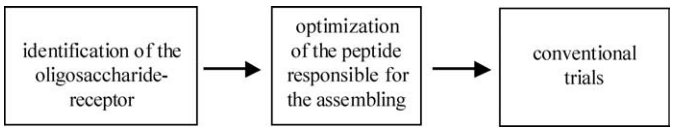
Anti-adhesion as a general approach

Association described here is not limited by sialic acid derivatives; we have prepared glycopeptide tectomers bearing other saccharides. Thus, it is possible to synthesize diverse tectomers with pendant-specific saccharides for blocking viruses and bacteria. However, we expect a limitation, not related to the nature of a pendant saccharide. Lectin density on the microorganism surface must be high in order to promote tectomer assembly by the mechanism shown in Figure 6. In the case of the influenza virus, HA density on the viral surface is high; this protein occupies more than a half of the total surface. We expect other targets for virus-promoted therapy also have to have similar lectin density as well.

Another important matter is closely related to lectin density on the viral particle: would a tectomer be assembled on human cells, in particular, due to the presence of siglecs? This question should be answered 'no' because siglec density on any cell is much lower than HA density on the virus, whereas the binding of non-associated sialoside (tecton) with siglec is not strong. So, there is low probability of side effects due to drug interaction with siglecs and other sialo-binding lectins of the human cell.

Another question is whether the *natural* saccharide-receptor is mandatory for the design of tectomers therapeutics or it is possible to replace OS with a small molecule-mimetic. We suppose that there is no need in mimicking; moreover, this is impossible in the case of antivirals and antibacterials due to three reasons.

(1) The first reason evidently follows from the principle of anti-adhesion in our interpretation, as shown below:



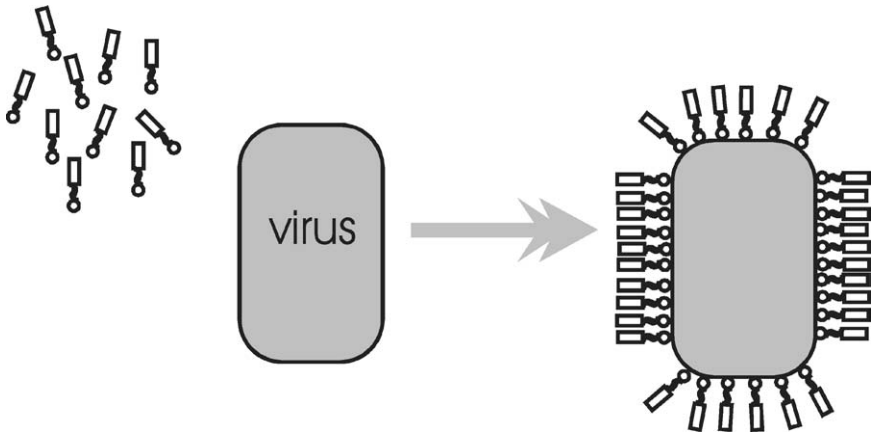


Figure 6. Principle of virus-promoted association of small molecules (tectons). In contrast to the above described molecules, these tectons are not able to assemble simultaneously.

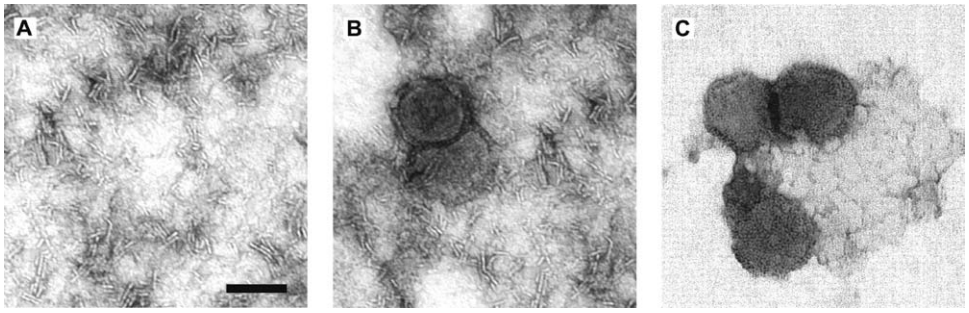


Figure 7. EM micrographs demonstrating the interaction of the influenza virus with glycopeptides synthesized in the presence of LiBr. A) [Neu5Acα-linker-Gly$_7$-NHCH$_2$]$_4$C exists as small tectomers and monomeric tectons; a similar picture was observed for the unnatural Neu5Acβ analogue. B) Addition of the virus does not affect assembly of [Neu5Acβ-linker-Gly$_7$-NHCH$_2$]$_4$C (compare with (A)). C) The virus promoted assembly of [Neu5Acα-linker-Gly$_7$-NHCH$_2$]$_4$C. The glycopeptide is fully assembled into a tectomer; the monomeric form has completely disappeared. Adapted from [28].

Just the natural receptor, which has been selected by a particular virus or bacteria during evolution, is selected by us as a specific oligosaccharide-ligand. Oligosaccharide or glycopeptide receptors have already been found for a wide range of microorganisms, so there is no need to waste time and resources for the invention of something artificial when the already optimal natural receptor is known.

(2) Why is a natural oligosaccharide-receptor *a priori* better than any analog or mimetic?

The main problem of the search for antivirals and antibacterials is the rapid change of a microorganism, *i.e.* mutations leading to the appearance of new, resistant strains. Understanding of the ability of microorganisms to become resistant to drugs that interfere with their biological activity is of great importance to the development of sustainable antimicrobial drug therapy; this conclusion follows from the lessons learned of relenza and related influenza neuraminidase inhibitors [37]. Relenza, a molecule little different from natural Neu5Ac (it preserves the most important for recognition: carboxy group, acetamide-, and glycerol tail) causes virus mutations very rarely. Other inhibitors (*e.g.*, with the replacement of glycerol tail to alkyl chain display comparable activity towards wild strains), in contrast to Relenza, cause mutations leading to persistent strains.

Viable variants, selected under drug pressure, should retain wild-type function. This requires the retention of binding interaction with the functional ligand (6'SLN-receptor in our case) at the same time as loss of binding interactions with the drug: such an outcome will be more likely if the drug and the natural ligand bind to the target in chemically different ways. Moreover, though each of the mutant proteins (lectins) recognizes the same natural ligand, it does it in its own way (in simpler words, from different positions (see Figure 8)), analogously to recognition of different epitopes of an antigen by polyclonal antibodies. A mimetic is capable of recognizing only one of the 6'SLN 'glycotopes' and, correspondingly, to neutralize only one of the HA variants. Only the natural oligosaccharide itself is capable to bind all the variants of the lectin, including wild and various mutant ones. This means that an optimum strategy for drug design in the face of high mutation frequency of the target is a minimal strategy that preserves as many structural features of the natural ligand as practicable. The best strategy, as mentioned above, is not to make any changes at all, *i.e.* to

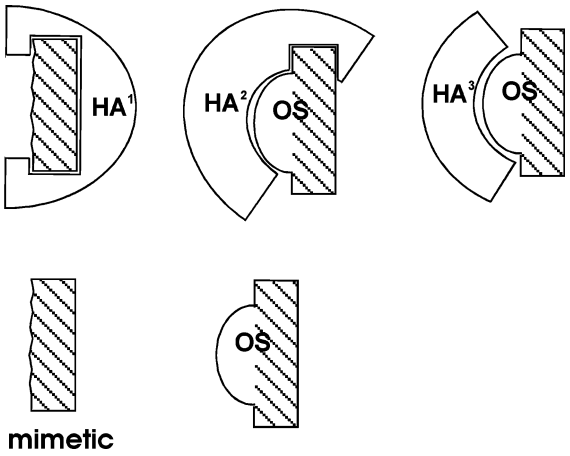


Figure 8. The same natural oligosaccharide-receptor (OS) is recognized by HA of different viral strains by different sites (glycotops). In contrast, a small molecule-mimetic is capable of binding only one or few (mutant) sorts of HA.

use the natural ligand as it is. So, one should aim to target drugs via chemical interactions that resemble, as closely as possible, those used by the natural ligand, and in which such a strategy minimizes the possibility of mutations [37].

Apparently, this principle is true in the case of designing not only antiviral therapeutics, but also blockers of other rapidly mutating cells, *e.g.* blockers of lectins of metastasing cells. This is even truer for proteins normally existing as multiple forms such as polyclonal antibodies.

(3) Finally, an advantage of a natural ligand compared to a mimetic is the expected low risk of side effects because a therapeutic will be, in fact, a variant of a molecule presented onto human cells, *i.e.* a molecule tolerant to its environment. This means there practically will not be any sorting out of the lead candidates due to their side effects; therefore, general efforts during the development of new therapeutics are decreased.

Thus, we propose a new strategy for antimicrobial drug design, based on a bifunctional blocker, the peptide part of which provides non-covalent assembly, whereas the saccharide part is to be as closely related as possible to natural ligands of viruses or bacteria.

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