CHARLES UNIVERSITY, PRAGUE
FACULTY OF SCIENCE

Ph.D. THESIS

DESIGN AND SYNTHESIS OF TN-BEARING GLYCODENDRIMERS AND THEIR INTERACTION WITH COMPONENTS OF INNATE AND ADAPTIVE IMMUNITY

PRAGUE 2006 PAVEL VEPŘEK

Prohlašuji, že jsem tuto disertační práci vypracoval samostatně pod odborným vedením Prof. RNDr. M. Tiché, CSc. a RNDr. J. Ježka, CSc., a že řádně cituji všechna převzatá data a skutečnosti.

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Table of contents

1. STATE OF THE ART

1.1 INTRODUCTION

The incidence of tumour diseases grows steadily every single year. In spite of this unfavourable trend the mortality due to cancer remains constant over extended period of time. This phenomenon demonstrates significant progress in treatment and diagnostics achieved in past decades. Key factors participating in this success are new treatment procedures and generation of new anti-cancer drugs together with the application of new information concerning the biology of tumour cell processes into the clinical practice.

New therapeutic approaches must take into account two relevant clinical facts: a tolerance of the organism to the malignantly transformed cells and immune insufficiency caused by radio- and chemotherapy. This situation reflects the present tendency in therapy of tumour disease to replace the classical chemotherapy by immunotherapeutic approaches or by combined immuno- and chemotherapy. Immunotherapy has been suggested as a tool for the treatment of disease residuals after the chemotherapy, because chemotherapy is usually ineffective at the elimination of minimal residual cancer cells due to multidrug resistance.

Human immune system should be able, in principle, to recognize and reject a tumour cell. Therefore immunotherapy including cancer vaccination represents very attractive therapeutic approach. In spite of the significant therapeutic effect of antitumour vaccines in experimental models, their efficiencies in randomized clinical tests are weak or sporadic. The main reasons are defects of the immune systems that have been proved at oncologic patients. These patients, owing to the disease as well as immunosuppressive effect of chemotherapy, represent the immunocompromised individuals and their successful complex immunotherapy should cover up the target modulation of both adaptive and innate immunity. These considerations have recently caused a considerable effort in the search of compounds which will be able to effectively modulate the individual components of the immune system.

Taking into account these facts, we focused our attention on compounds that could modulate both adaptive and innate antitumour immune response. Tn antigen (α-D-GalNAc-Ser/Thr) is an example of such molecule. It is very specific to human cancers and therefore it has a significant potential for the construction of synthetic antitumour vaccines based on branched structures or dendrimers. Simultaneously, the compounds of this type can play a significant role in modulation of innate immune response. It is known that N-acetyl-D-hexosamines and their glycoconjugates are able to modulate cytotoxic activity of natural killer

cells (NK cells) by interaction with their surface receptors. A systematic study of the lectin receptors of NKR-P family and human CD69 showed that complex natural oligosaccharide structures presented on the surface of carcinoma and infected cells exert binding affinity to these receptors. But these ligands are not suitable for practical use as immunotherapeutics due to their limited availability. This fact has led to the search for their simplified and hence better available mimetics, and the neoglycoconjugates of D-hexosamine type based on dendrimeric structures represent such group of compounds. To achieve above-specified goals dendrimeric structures bearing Tn antigen were chosen. Dendrimer carriers are generally non-immunogenic and enable multiplication of the structure of interest (antigens/ligands) and thus increase of molecular weight above the limit necessary for the construction of synthetic vaccines, i.e. parameters necessary for the successful modulation of immunogenicity towards the adaptive and innate immunity.

1.2 DENDRIMERS

Dendrimers, originally referred to as arborols, cauliflowers, cascade polymers or starburst polymers, were first as a structural group described by Vögtle in 1978.[1] The word dendrimer stems from Greek word dendron meaning „tree" or „branch", and meros meaning part.[2]

- Dendrimers are well-defined hyper-branched macromolecules with characteristic globular structures.[3]
- Dendrimers are versatile, derivatizable, well-defined, compartmentalized chemical polymers with sizes and physicochemical properties resembling those of biomolecules, e.g. proteins.[4]
- Dendrimers are highly branched three-dimensional macromolecules with highly controlled structures, a single molecular weight, a large number of controllable „peripheral" functionalities and a tendency to adopt a globular shape once a certain size is reached.[5]
- Dendrimers are chemically well-defined, non-linear, hyper branched polymeric structures formed by successive reactions of polyfunctional building blocks around a central (initiator) core possessing, in comparison with their linear analogues, unique spatial architecture and physico-chemical properties.[6]

Countless structural motifs with a variety of building blocks including PAMAM™, poly(propylene imine) dendrimers, peptide and glycopeptide dendrimers[7-11] have been used for the construction of bioactive dendrimers and branched structures. Today, dendrimers stand within the focus of an important interdisciplinary research with technical and biochemical applications in catalysis, polymer science, material science, bioorganic chemistry, biomimetics, biotechnology and biomedicine.[12-18]

1.2.1 Peptide dendrimers

Peptide dendrimers are radial or wedge-like branched macromolecules consisting of a peptidyl branching core and covalently attached surface groups (peptide antigens).[19]

Fundamental landmarks in the history of peptide dendrimers are works of J.P. Tam, and M. Mutter. Tam first used branched oligolysine core for the preparation of synthetic peptide vaccines, and called these structures Multiple Antigenic Peptides (MAPs), Fig. 1A.[20,21] Recent progress in carbohydrate chemistry and availability of glycosylated amino acids resulted in the development of glycopeptide analogues of MAPs, termed as Multiple Antigenic Glycopeptides (MAGs).[22,23]

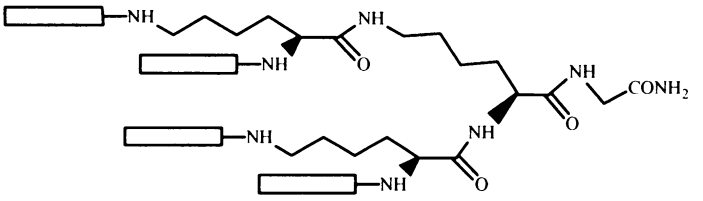


Fig. 1A Multiple Antigen Peptides by J.P. Tam

To overcome the well-known folding problem in the protein *de novo* design, Mutter et al. introduced Template-Assembled Synthetic Proteins (TASPs), Fig. 1B.[24] TASPs represent a novel class of artificial proteins assembled on a template with β-structural motif that is capable to induce folding of attached amphiphilic peptide blocks to complex protein-like arrangement obviating the need for extensive folding required for biochemical activity.

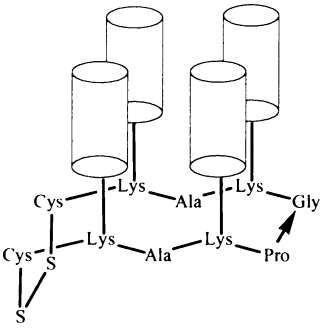


Fig. 1B Template-Assembled Synthetic Proteins by M. Mutter and G. Tuchscherer

These templates have been used in engineering of artificial proteins functioning as ion-channels, synthetic vaccines, adjuvants, enzymes, antibiotics, diagnostic reagents, and drug carriers.[25,26] This concept was further expanded, and Regioselectively Addressable Functionalized Templates (RAFTs), with orthogonally protected lysine side-chains, were introduced.[27]

A novel class of carriers, called Sequential Oligopeptide Carriers (SOCs), Fig. 1C, has been recently introduced by V. Tsikaris and C. Sakarellos.[28,29] SOCs are linear analogues of tripeptide Lys-Aib-Gly sequential motif, which is known to fold into defined helicoidal secondary structure (3_{10}-helix) and direct the arrangement of antigens into defined spatial conformation.[28,29] Other scaffoldings have also been used.[30-33]

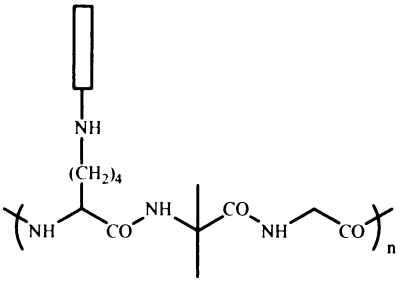


Fig. 1C Sequential Oligopeptide Carriers by C. Sakarellos and V. Tsikaris

1.2.1.1 Multiple antigenic peptides (MAPs)

MAPs were introduced in 1988 by J.P. Tam as a fully synthetic alternative to frequently used natural and semisynthetic vaccines.[21] A molecule of MAP consists of three structural features: (i) a simple amino acid such as glycine or β-alanine (an internal standard for amino acid analysis), (ii) a branched oligolysine core (a synthetic low-molecular biocompatible carrier), and (iii) multiple copies of a synthetic peptide antigen, Fig. 2. Their preparation is discussed further in the text.

Tetra- and octameric MAPs (Fig. 2 B and C) are the most frequently used in vaccine formulations. Higher analogues (e.g. hexadecameric, Fig. 2D) are (i) difficult to prepare, (ii) often heterogeneous and (iii) have equal of even worse immunological properties.[34]

However, if MAPs are used as inhibitors in immunoassays, their effectiveness grows with increasing number of branches (i.e. copies of antigen) i.e. $2 < 4 < 8 < 16$.[19] This is due to the increased number of available antigenic sequences for interaction.

The number of branches required to elicit strong immune response largely depends on the number of amino acid residues of the respective antigen. For >15-amino acid long peptides immunization with MAPs in tetrameric format gives satisfactory results.[35] In addition, MAPs were found to overcome the MHC class restriction in induction of humoral response, and to act as potent immunogens capable of inducing long-lasting immune memory.[36]

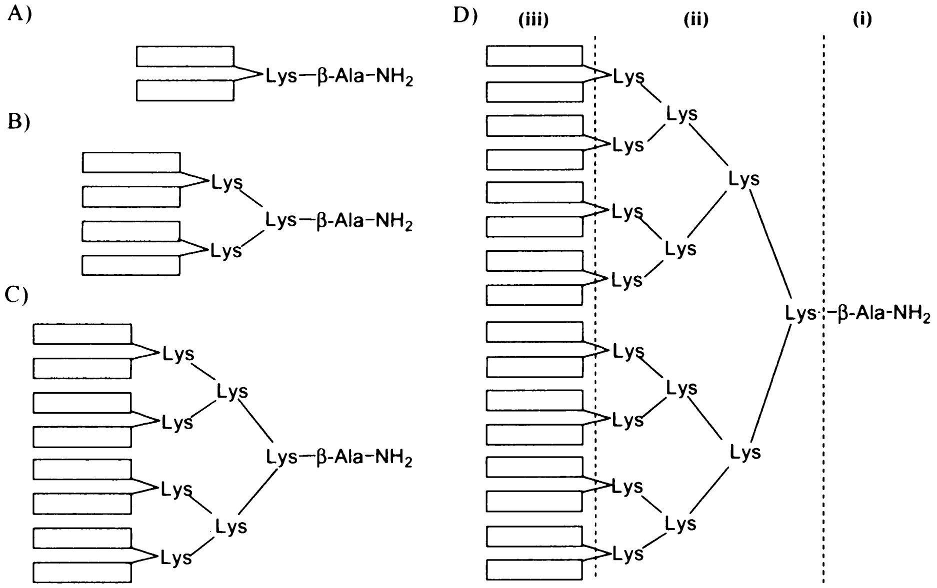
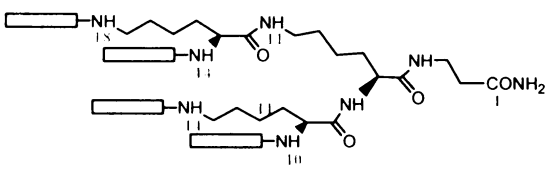


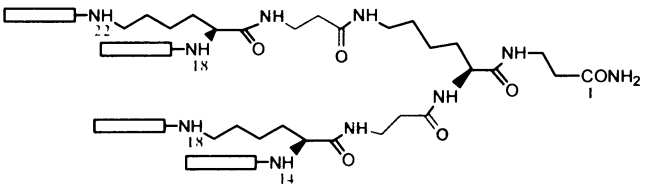
Fig. 2 Structures of (A) di-, (B) tetra-, (C) octa- and (D) hexadecameric MAPs: (i) an internal standard, (ii) branched oligolysine core, and (iii) multiple copies of peptide antigen.

Antibodies obtained by immunization with MAPs cross-react with corresponding native protein and are generally of higher titre than those obtained by immunization with peptide-protein conjugates.[37] However, in some cases, peptide-protein conjugates failed and only MAP constructs induced antisera capable of cross-reacting with native protein[38] and *vice versa.*[39] Reasons for such behaviour are unknown, and it is speculated that this behaviour is an intrinsic attribute of a given epitope sequence, or it is caused by the mode of presentation of the particular epitope to the immune system.

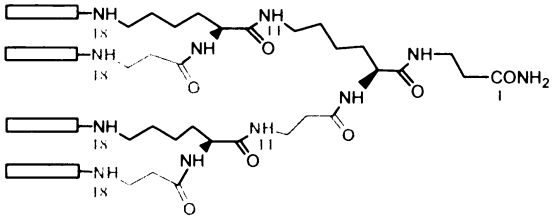
To improve immunological properties of MAPs, structures with modified cores were designed. A symmetrical core (using β-alanyl-lysine as a building block),[40] and cores with linear inserts: such as β-alanine[41] and γ-amino butyric acid,[42] were prepared, Fig. 3. However, no improvement has been observed and, surprisingly, structures with linear γ-Abu insert were difficult to prepare (*see further in the text*).



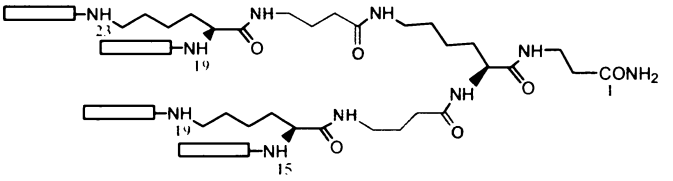
(A) Asymmetrical MAP core



(C) Asymmetrical MAP core with inserted β-Ala



(B) Symmetrical MAP core



(D) Asymmetrical MAP core with inserted γ-Abu

Fig. 3 Structural modifications of tetrameric oligolysine core. Asymmetry and symmetry is comprehended in terms of the number of bonds and atoms between the N-terminus and the C-terminus of the molecule of the core.

MAPs, and generally all "bio-compatible" dendrimers, offer several advantages over the peptide-protein conjugates: (i) synthetic availability, (ii) low or none immunogenicity of the carrier (oligolysine core), (iii) the defined and controllable number of copies of antigen (controlled by the core design), (iv) possibility of incorporation of two or more defined epitopes, using orthogonal protection of α- and ε-amino groups of lysines, and (v) bulk of the molecule is formed by antigens and carrier forms only minor part of the molecule, see also Tab. 1.

Tab. 1 Number of amino acids and their rations in tetrameric MAP and peptide-protein conjugate.

Length of peptide antigen	MAP			Peptide-protein conjugate	
	Synthetic Carrier		Peptide antigen (4 copies)	Protein**	Peptide antigen (20 copies)***
	Internal AA standard	Tetravalent oligolysine core			
10	1 (2.3%)*	3 (6.8%)	40 (90.9%)	583 (74.5%)	200 (25.5%)
15	1 (1.6%)	3 (4.7%)	60 (93.7%)	583 (66.0%)	300 (34.0%)

* absolute number of AA (% of AA),

** BSA (67 kDa, 583 amino acids)

*** the number of conjugated peptide copies is affected by the efficiency of conjugation; 20 copies of an antigen were taken into consideration

Biological and physico-chemical properties of peptide-protein conjugates compared to MAPs (representing fully synthetic structures) are summarized in Tab. 2.

Tab. 2 Comparison of some physico-chemical and biological properties of peptide-protein conjugates and MAPs.

Properties	Peptide-protein conjugates	MAPs
Physico-chemical properties		
Structure	Branched, not defined	Branched, defined
Composition	Variable, difficult to control	Defined
Antigen/carrier mass ratio	Variable and low	Defined and high
Stability	Unknown	High
Biological properties		
Possibility to incorporate two or more epitopes	Questionable but possible, ratio not controllable	Yes, defined ratio
Possibility to incorporate built-in adjuvant	Questionable	Yes, defined ratio
Immunogenicity of the carrier/core	Yes	No
Source of T_h epitopes	Yes	No, but can be introduced
Presence of undesired epitopes	High	Low

1.2.1.1.1 MAPs in the preparation of synthetic vaccines

Early studies employing monoepitopic MAPs bearing only B-cell epitope revealed that such constructs are not always successful in eliciting humoral response even if Freund's complete adjuvant (FCA) was used. Therefore diepitopic MAPs bearing both B- and T-cell epitopes have been designed and prepared.[43]

MAPs with various arrangements of B- and T-cell epitopes (Fig. 4) were prepared and their potential as immunogens was evaluated. The study, where mice were immunized with tetra- or octameric MAPs bearing either T- of B-cell epitopes or B-T or T-B chimeras and then challenged with *Plasmodium berghei* sporozoites, identified as the most potent construct the tetrameric MAP with B-T chimeras (80% protection to mice).[34] Other constructs used in this study, even octameric MAP with B-T chimeras, were less effective.

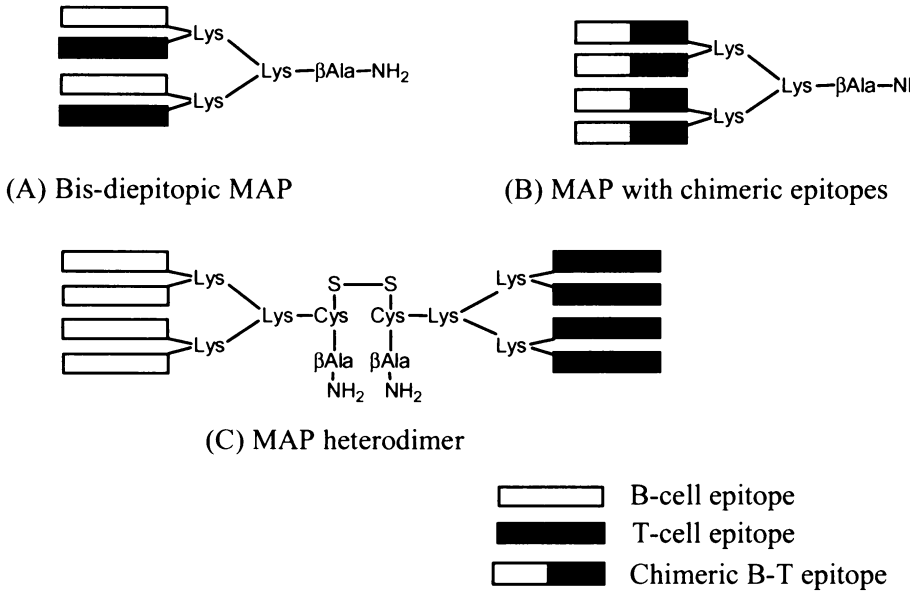


Fig. 4 Structural arrangements of diepitopic (B- and T-cell epitopes) tetrameric MAPs.

Combination of an immunogen with an immunoadjuvant in one molecule brings new perspectives in effective vaccine development. FCA is toxic, pyrogenic and induces uveitis (intraocular inflammation) in rabbits and arthritis in rats and therefore it cannot be used in human vaccines.[44]

N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-cysteine (tripalmitoyl-S-glyceryl-cysteine, P_3C) also known as Jung's peptide, derived from the bacterial lipoprotein of the outer membrane of *Escherichia coli*, has been successfully used in the preparation of complex MAP-based vaccine with built-in adjuvant, Fig. 5.[45-51]

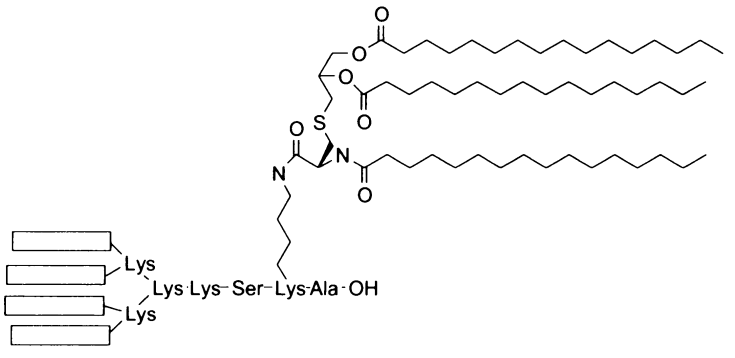


Fig. 5 Example of complex synthetic vaccine with built-in adjuvant. The structure of tetrameric monoepitopic MAP with P_3C is given.

P_3C has been shown to be a potent B-cell and macrophage activator, and inducer of CTL *in vivo*.[52] Its application in MAP constructs has been successfully demonstrated.

Tetrameric MAPs with four copies of 24-amino acid long sequence derived from gp120 of HIV-1 (residues 308-331) with P_3C at the C terminus were administered both in free and liposomal form to mice and guinea pigs. Elicited antiserum (i) neutralized a virus infectivity (as evaluated by the inhibition of syncytium* formation and reverse transcriptase activity), (ii) induced T response (IL-2 production), and (iii) elicited CD8+ CTL (lysis on syngeneic cells expressing gp160 on the cell surface). This construct also induced long-term T-cell memory (undiminished CTL response 5 months after the last boost immunization[53]). The cytolytic response already after one immunization was found to be superior to the response induced by a full immunisation cycle by corresponding monomer in FCA.

Studies with P_3CSK_4 (a peptapeptide analogue of Jung's peptide) showed that it (i) can act as potent adjuvant for human immune responses *in vitro*, (ii) is one of the most effective leukocyte activators[54] and (iii) that it does not require to be covalently bound to the antigen.[55,56] Replacement of palmitoyl chains with longer or shorter lipophilic chains significantly diminished the adjuvant effect.[55]

Minimization of complex structure of P_3C has led to the preparation of lipopeptide analogues of MAPs (lipoMAPs) with simple fatty acids attached to the structure.[57]

The major advantages of simple fatty acids are: (i) low costs of fatty acids, (ii) coupling of fatty acids can be performed in a peptide synthesizer, (iii) purification can be done by standard purification protocols, (iv) chemical stability of fatty acids.

LipoMAPs are chemically stable, non-toxic, non-pyrogenic, and do not cause any tissue damage, and can be prepared in large quantities and high purity.[58] Lipidation (mostly palmitoylation) affects not only immunogenicity but also biodegradation and transport through membranes.[59,60] The character of a bond between the lipophilic chain and the rest of the molecule and the site of acylation greatly affects immunogenicity.[61,62] In evaluated preparations, the lipoMAP with palmitoyl attached via thioester bond was at least as immunogenic as KLH-peptide conjugates.[61]

1.2.1.1.2 Other applications of MAPs

MAPs have been successfully used (i) in immunoassays, (ii) in serodiagnostics, (iii) as inhibitors of e.g. HIV interaction with CD4+ and CD4- cells, (iv) in epitope mapping, (v) in affinity purifications of antibodies and (vi) for extracellular targeting and delivery, Tab. 3.

* An epithelium or tissue in which there is cytoplasmic continuity between the constituent cells (Biology-Online.org). A mass of cytoplasm containing several nuclei and enclosed in a membrane but no internal cell boundaries (as in muscle fibers) (WordReference.com)

Tab. 3 Specific examples of various applications of MAPs.

Application	Reference
Immunoassays and serodiagnosis	
Systemic lupus erythematosus	Caponi et al. (1995), [64] Vlachoyiannopoulos et al. (2004), [65]
HIV-1	Vogel et al. (1994), [66] Kim et al. (2001), [67]
Hepatitis A virus	Firsova et al. (1996), [68] Gómara et al. (2000), [69]
EBV	Marchini et al. (1994), [70]
Inhibitors	
HIV-1 fusion and infection	Yahi et al. (1995), [71]
	Weeks et al. (1994), [72]
IL-6	Wallace et al. (1994), [73]
IL-2	Fassina et al. (1995), [74]
Fibronectin	Ingham et al. (1994), [75]
Anthrax	Pini et al. (2006), [76]
Intracellular delivery	
	Sheldon et al. (1995), [77]
	Flinn et al. (1996), [57]
Molecular recognition	
	Wiegandt et al. (1996), [78]
Ab recognition by retro-, inverso peptides	Verdoliva et al. (1996), [79]
Purification methods	
Affinity purification of antibodies	Fassina et al. (1996), [80] Verdoliva et al. (1995), [81]
	Butz et al. (1994), [82]

Multimeric format of MAPs enables good binding to plastic surfaces, but still preserves enough intact copies of antigen for effective interaction.[63] This is especially advantageous for solid-phase based immunoassays such as ELISA and RIA. The multimeric character also improves detection of low affinity antibodies and thus possibly identification of early stages of infections. As inhibitors, MAPs enable multiple-point contact and thus stronger binding compared to the linear analogue.

1.2.2 Glycodendrimers

Glycodendrimers are low molecular weight multiantennary biopolymers either carbohydrate-coated, or carbohydrate-centred, or carbohydrate-based.[83]

During recent years it has become clear that carbohydrate-protein interactions are essential molecular recognition events[84-87] and that multivalency plays an important role in this process.[88-90] Cell-surface carbohydrate interactions are involved in cell-cell interactions responsible for cell growth and metastasis, immune recognition processes (e.g. pathogen recognition and neutralization), interactions between leukocytes and the inflammatory epithelium, lymphocyte trafficking and mediation of natural killing[91] of various targets. The key role of cell-surface carbohydrates as markers of a healthy stage of a cell and targets for immunotherapy has also been recognized.[92] Simple mono- and oligosaccharide sequences have been identified as ligands in bacterial, toxin, mycoplasma, or even in viral infections, Tab. 4, and some as important tumour markers, Tab. 8 on page 21.

Lectins bind specifically various saccharide structures[93,94] and mediate many biological functions, including immune defence, clearing of glycoproteins and cell-cell adhesion[95] and therefore they became important targets in the study of roles of saccharides in these processes.[96] Glycodendrimers have become important molecular tools for the investigation and manipulation of carbohydrate-protein interactions[97-99] with regard to the observed multivalency effect[100,101] and, of course, as glycopeptide vaccines.[102]

Tab. 4 Sugar specificities of cell surface lectins, taken from the ref. 103.

Saccharide	Source of receptors
L-Fucose	*Vibrio cholerae*
Lactose	*Actinomyces spp.*
	E. Coli
	Lung tissues *(cancer metastasis)*
D-Mannose	*Salmonella typhimurium*
	HIV virus
	Pseudomonas aeruginosa
D-GalNAc	*E. coli*
D-GlcNAc	*E. coli*
	HIV virus
Sialic acid (NeuAc)	Mycoplasma
	Influenza virus (flu)
Sialyloligosaccharides	*E, P, L selectins* (Inflammation)
	H. pilori (Gastric ulcer)

1.2.2.1 Modulation of activity of rat natural killer cell receptors NKR-P1A and NKR-P1B

Natural killer (NK) cells are a major population of lymphocytes, distinguishable from other lymphocytes by the absence of antigen receptors found on B and T cells.[104] NK cells can spontaneously kill certain infected, stressed, or tumour cells, i.e. cells expressing low amounts of MHC class I molecules. NK cells play a role in a control of cell numbers during haematopoiesis, and in transplantation reactions (graft-versus-host disease).[105] On the other hand some tumours (e.g. ovarian tumour) can produce factors inhibiting activity of NK cells.[106]. Activity of NK cell can be also affected under certain pathological conditions or after application of some medicine that suppressed expression of activation receptors on the surface of the NK cell.[107]

NK cells evolved a variety of membrane-bound surface lectin receptors belonging to both immunoglobulin (killer cell receptor, KIR) and C-lectin type families.[108] These receptors transfer both activating and inhibitory signals that effectively regulate their (NK cells) killing potency, Fig. 6.

C-type lectins of lymphocytes belong mostly to the type II membrane proteins with a single carbohydrate recognition domain (CRD) and are one of the largest groups of vertebrate membrane proteins identified by a computer analysis of mouse, rat and human genome encoded in the NK cell gene complexes. [109,110] These gene complexes encode receptors that can trigger or inhibit target cell lysis by NK cells (e.g., NKR-P1, Ly49, NKG2 and CD94) or can activate various haematopoietic cells (e.g. CD69). These receptors participate in a wide range of signalling events regulating the effector functions of killer lymphocytes, Fig. 6.

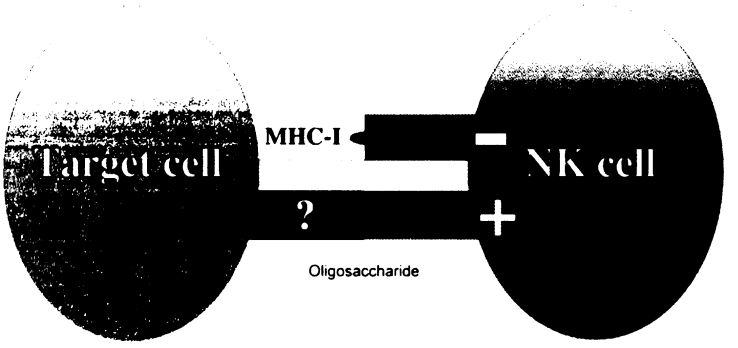


Fig. 6 The role of activation and inhibitory receptors in natural killing.

First identified in the rat, NKR-P1 protein is expressed by all NK cells as a disulfide-linked homodimer. Several isoforms, which occur simultaneously on NK cells of the rat and

mouse, have been cloned and sequenced.[111] While isotype A has activation properties (NKR-P1A), the B and D isotypes are inhibitory receptors (NKR-P1B, NKR-P1D).[112,113] In human NK cells only one isoform on NKR-P1 receptor has been identified so far.

Antibodies bound to NKR-P1 can induce antibody-dependent cytotoxicity of FcR^- target cells,[114] and crosslinking NKR-P1 with antibodies stimulated phosphoinositide turnover and mobilization of extracellular calcium.[115] For these reasons there has been considerable interest in identifying ligands for this protein and elucidating its involvement in the cascade of events that result in killing of target cells.

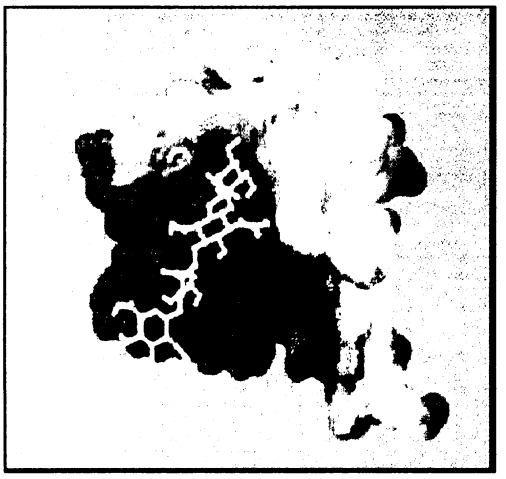


Fig. 7 Spatial view of the molecular model of the rat NKR-P1A receptor with the chitotetraose docked into oligosaccharide-binding groove.

Ligands of these receptors are complex linear and branched oligosaccharides that are difficult to prepare or isolate from natural sources. Recent studies have identified simple monosaccharide and oligosaccharide ligands for NKR-P1 receptors.[116] N-acetylhexosamines were shown to have good affinity growing from N-acetylglucosamine, N-acetylgalactosamine to N-acetylmannosamine. Chitotetraose, a linear oligosaccharide containing four N-acetylglucosamine units connected by $\beta(1\rightarrow4)$ glycosidic bonds has been identified as one of the best ligands so far, Fig. 7. Recent studies with glycodendrimers bearing glucose or mannose monosaccharides showed that these structural types are capable of differentiation between NKR-P1A and NKR-P1B receptors, the property not observed for simple monosaccharides, Fig. 8.[117,118] Octavalent polylysine-based (G8L) and PAMAM-based GlcNAc glycodendrimers (G8P) were identified as the most potent modulators of NKR-P1A receptor. *In vitro* studies demonstrated that GlcNAc, a structure presented in many cell

surface glycoconjugates, shows a high affinity for the rat NKR-P1A recombinant receptor.[119] Positive anticancer effects were observed *in vivo*, in a rat colorectal carcinogenesis model, after the use of ganglioside G_{M2} and heparin-related I-S oligosaccharides (the high affinity ligands for NKR-P1A) in liposomal preparations.[120]

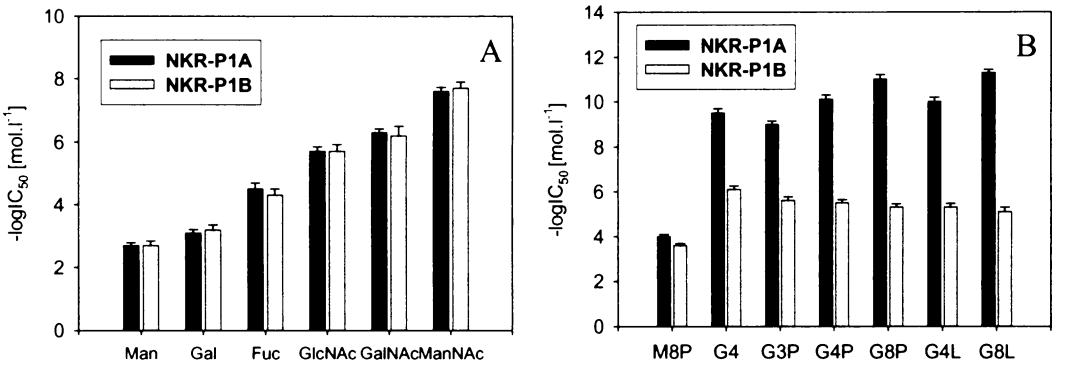


Fig. 8 Inhibition of binding of NKR-P1A and NKR-P1B receptors to GlcNAc17BSA-coated wells by (A) monosaccharides, (B) complex carbohydrates: chitotetraose (G4), polyamidoamine-based (G3P, G4P, G8P, M8P) and polylysine-based (G4L and G8L) glycodendrimers. G is β-D-GlcNAc, M is β-D-Man. Figure taken from ref. 117.

1.2.2.2 Glycodendrimers in anti-cancer vaccine design

Glycoconjugates (glycolipids and glycoproteins) are dominant molecules of animal cell membrane bilayer and play important roles in many physiological properties.[84-86] Aberrations of their structures and consequential appearance of novel or re-appearance of oncofetal structures have been established as a universal characteristic of malignant transformations of cells and cancer – in this respect - has been referred to as a molecular disease of the cell membrane glycoconjugates.[121] The investigation of the structural changes of cancer-related glycoconjugates is important not only for the understanding of the origin of these alterations, but also for the development of improved diagnostic and therapeutic agents of cancer.[122] Importantly, these novel structures can be recognized by the immune system and thus become perspective targets for treatment and prevention of a variety of diseases, particularly cancer.[123]

Glycoconjugates are a heterogeneous group of macromolecules that contain saccharides covalently linked to lipids or proteins. The protein-containing glycoconjugates can be divided into two major groups according to the nature of the linkage of a saccharide to proteins:[124] *N-glycosides*, a mannose-containing oligosaccharide conjugates with β-N-acetyl-D-glucosamine linked to the side chain of Asn residues (found in animals, plants and microorganisms), and

O-glycosides, a mannose-free oligosaccharide conjugates with (i) α-N-acetyl-D-galactosamine linked to the side chains of the serine/threonine residues (found in animals), also known as mucins; (ii) β-D-xylose linked to serine (proteoglycans, human thyroglobulin), (iii) β-D-galactose linked to 5-hydroxylysine (collagens), (iv) β-L-arabinose linked to 4-hydroxyproline (plant and algal glycoproteins).

1.2.2.2.1 Mucins

Mucins are the most abundant representatives of O-glycoconjugates and are of particular interest in tumour prevention and treatment.[125] Their general structure is given in Fig. 9. These large extracellular glycoproteins (over 10^6 Daltons) are heavily glycosylated by complex oligosaccharides that constitute about 50-80% of the total mass. They establish a selective molecular barrier at the epithelial surface and engage in morphogenetic signal transduction.[85,95] Mucins are expressed by various epithelial cell types that exist in relatively harsh environment, e.g. secretory epithelial surfaces of specialized organs such as the liver, pancreas, gall bladder, kidney, salivary glands, lacrimal glands and eye.

Their biochemical composition provides protection for the cell surface, and specific molecular structures regulate the local molecular microenvironment near the cell surface. Mucins serve as cell-surface receptors and sensors, and conduct signals in response to external stimuli that lead to coordinated cellular responses that include proliferation, differentiation, apoptosis and secretion of specialized cellular products.[126]

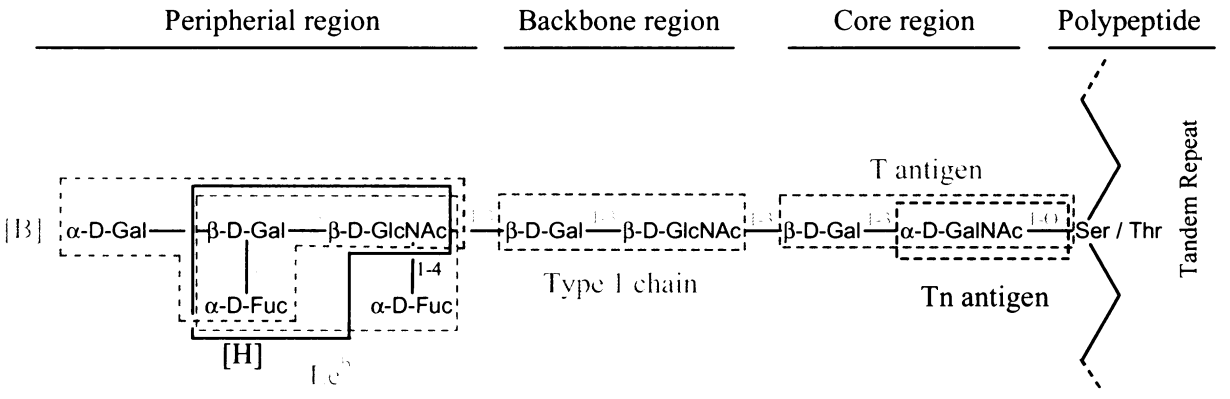


Fig. 9 Structure of complex oligosaccharides on apomucin polypeptide in healthy cell (adopted from ref. 127). In oligosaccharides, three regions can be found: core region responsible for attaching saccharide to apoprotein, backbone region, and peripheral region where most of the antigenic determinants can be found. In tumour cells both backbone and peripheral regions are not developed. [B], [H] are blood group determinants, Le^b is Lewis b antigen.

Two main classes of mucins exist: (i) secreted ones: MUC2, MUC5AC, MUC5B, MUC6, MUC7 - gel-forming and non-gel forming mucins – and, (ii) membrane-associated ones: MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13, MUC16 and MUC17 - bound to cells by an integral transmembrane domain and with relatively short cytoplasmic tails that associate with cytoskeletal elements, cytosolic adaptor proteins and/or participate in signal transduction. Protein parts of mucins are characterised by the presence of the so called tandem repeats (TRs); regions of several copies of identical or highly similar sequences rich in proline, serine and threonine, Tab. 5. O-glycosylation of later two amino acids is crucial to mucin structure and function. N-acetyl-D-galactosamine (GalNAc), N-acetyl-D-glucosamine (GlcNAc), N-acetyl-D-neuraminic acid (Neu5Ac, or sialic acid), D-galactose (Gal) and D-fucose (Fuc) were found in the structure of complex oligosaccharides.

Tab. 5 Examples of amino acid composition of tandem repeats of various mucins (**bold** indicates possible sites of O-glycosylation).

Source of the TR	Length of the TR	AA sequence of the TR
MUC1	20 mer	PD**T**RPAPG**ST**APPAHGV**TS**A
MUC2	23 mer	P**TTT**PI**TTTTT**V**T**P**T**P**T**P**TGTQ**T**
MUC3	17 mer	H**ST**P**S**F**TSS**I**TTT**E**TTS**
MUC4	16 mer	**TSS**A**ST**GHA**T**PLPV**T**D

Alterations in mucin expression or glycosylation accompany the development of cancer and influence cellular growth, differentiation, adhesion, invasion and immune surveillance. From this reason, mucins have been used as diagnostic markers in cancer, and are under investigation as therapeutic targets.[128,129]

Cancer cells, especially adenocarcinomas, express aberrant forms or different amounts of mucins.[130-133] These alterations can be grouped to five categories: (i) increased expression compared with non-malignant cells, (ii) reappearance of normal antigens usually present in fetal tissue (oncofetal antigens), (iii) expression of antigens incompatible with blood type, (iv) incomplete glycosylation, precursor accumulation or exposure of core carbohydrate structures as a result of impaired synthesis and (v) neosynthesis of novel and unique saccharide antigens.

Immunohistochemical studies have identified three major groups of Tumour-Associated Carbohydrate Antigens (TACAs), Tab. 6, expressed in most common human cancers; (i) lacto-series structures in lung, gastrointestinal tract, liver, breast, colorectal and pancreatic

cancers, (ii) ganglio- and (iii) globo-series on specific types of human cancer such as melanoma, Burkitt's lymphoma, neuroblastoma and small-cell lung carcinoma.[134,135]

Tab. 6 Types of TACAs and their localization on glycoconjugates, taken from reference 136.

TACA	Expression	Example
Lacto-series type 1 and type 2 chain*	On glycoproteins and glycosfingolipids	Le^a, Le^b, Le^x, dimeric Le^a, sialosyl-Le^a
Core structures of carbohydrates in mucins	Exclusively on glycoproteins	**Tn, T, sialosyl-Tn and sialosyl-T**
Precursor accumulated as a result of blocked synthesis	Only on glycosfingolipids	G_{M3}, G_{M2}, G_{D3}

* type 1 β-D-Gal (1-3)-β-D-GlcNAc(1-3)-D-Gal, type 2 β-D-Gal(1-4)-β-D-GlcNAc(1-3)-D-Gal

The expression of TACAs has been shown to be associated with a poor prognosis in a variety of adenocarcinomas, possibly by increasing metastatic potential through interactions between sialic acid residues and components of extracellular matrix.[137] Carbohydrate antigens expressed exclusively on proteins are therefore of particular interest and are discussed in the following section.

1.2.2.2.2 Thomsen-Friedenreich related antigens

Incomplete glycosylation of the protein part of the mucin molecule leads to the disclosure of otherwise cryptic core carbohydrate structures of the core, see Fig. 9. These core structures are generally referred to as Thomsen-Friedenreich-related (TF-related) antigens and include: Tn antigen (α-D-GalNAc-*O*→Ser/Thr), T antigen (β-D-Gal(1-3)-α-D-GalNAc-*O*→Ser/Thr), as well as their sialylated analogues (sTn and sT, respectively), Fig. 10. T antigen can be terminated by sialylation at 3-position or 6-position i.e. α-Neu5NAc-(2→3)-β-D-Gal(1→3)-α-D-GalNAc-*O*→Ser/Thr and β-D-Gal(1→3)-[α-Neu5NAc-(2→6)]-α-D-GalNAc -*O*→Ser/Thr), or at both positions.

These structures were found to be immunogenic as a result of lack of immunological tolerance, and they are therefore involved in host rejection.[140] The negatively charged sialic acids have been considered to shield against host recognition[141] and, therefore, an antigenic response against these abundant antigens elicited by a challenge with a glycopeptide vaccine could be an effective way of cancer treatment.

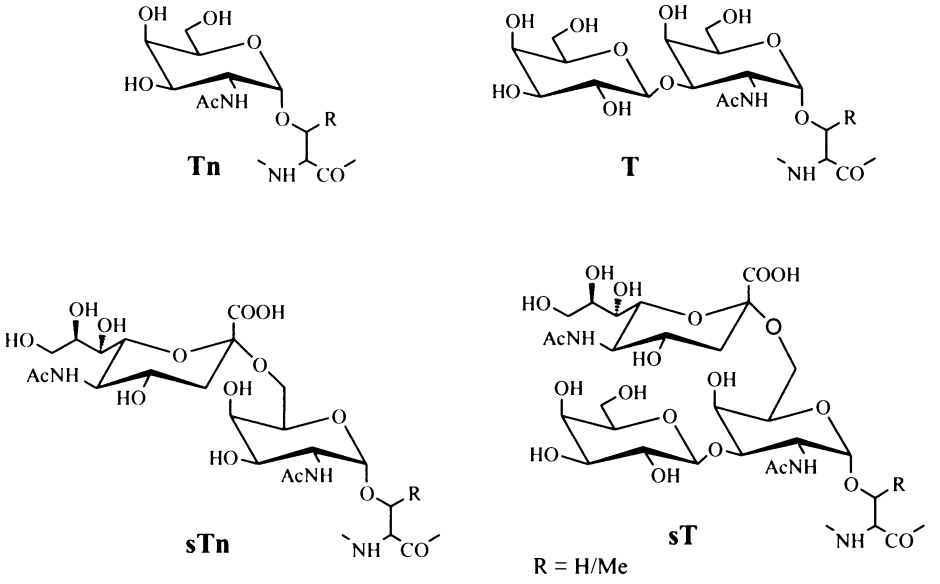


Fig. 10 Core carbohydrate structures found on mucins.

Tn and T antigens have been found on the external surface membranes of most primary carcinomas and metastases.[142,143] Interestingly, their existence has not been detected either in malignant mesenchymal and central nervous system tumours, or in benign tumours. Tn and T antigens are general and specific carcinoma markers, and for some types of carcinoma they permit prediction of the tumour's likely clinical progress. They may be involved in cancer invasion of healthy tissues as well.[144] Their biosynthesis depends on an orchestrated expression of several glycosyltranferases[145] and has often been correlated with cancer progression.[146,147] A simple down-regulation of core 2 GlcNAc transferase, as occurs in some breast cancer specimens, is consistent with the overexpression of the T-antigen, which correlates with progression and metastasis in this and other epithelial malignancies.[146] A molecular basis for the relationship between T-antigen expression and metastasis is provided by the observation that this carbohydrate structure can mediate docking of tumour cells to endothelial cells expressing galectin 3 (a galactose binding animal lectin).[148] sTn antigen is also expressed by breast cancer and correlates with a poorly differentiated state.[149] Expression of sTn is associated with poor prognosis in colonic[150] and gastric[151] cancers. Circulating sTn antigen has been detected in gastrointestinal and ovarian malignancies, and raised levels have also been associated with a worse prognosis.[154] Immunohistochemical studies showed that TF-related antigens are not normally expressed in colonic epithelium, while they are expressed by adenomatous polyps and carcinoma; e.g. 70% of colon cancers express T antigen,[134] Tab. 7.

Tab. 7 Expression of TF-related antigens in colon cancer tissues (taken from ref. 134).

Tn	T	sTn	No. of samples (total 24)
+	+	+	15 (62.5%)
+	-	+	6 (25.0%)
-	+	+	2 (8.3%)
-	-	-	1 (4.2%)

+ positive detection, - negative detection

Unlike T antigen, which is preferentially expressed by moderately and well-differentiated adenocarcinomas,[152] both Tn and sTn antigens are expressed by most of colon cancers, including poorly differentiated adenocarcinomas and mucous carcinomas, Tab. 8. The majority of cancers expressed both Tn and sTn, usually in association with T antigen. Fetal colonic mucosal cells expressed all three antigens, particularly in goblet cell mucin.[153]

Tab. 8 Expression of TF antigens in normal and neoplastic colon and pancreas (taken from ref. 127).

	Normal colon [%]	Hyperplastic polyps [%]	Adenomatous polyps [%]	Colorectal cancer [%]	Normal pancreas [%]	Pancreatic cancer [%]
Tn	14	100	100	72-81	100	100
T	0	-	-	71	53	44
sTn	0	35	63	93-96	0	97

On normal red blood cells (RBCs), T antigen can be unmasked by desialylation, whereas this treatment would unmask only small amount of Tn antigen.[155] In normal tissues the T antigen can be unmasked by desialylation in the urinary bladder,[156] but such treatment has no or only a limited effect on breast and colon mucosa. In spite of this difference, carcinomas of bladder, colon and breast have all been reported to express TF antigens.

Semisynthetic vaccines

Studies with TF antigens regarding the immunogenicity were initially conducted with natural sources of carbohydrate antigens including ovine submaxillary mucin (OSM) and bovine submaxillary mucin (BSM) - sources of sTn and after desialylation also of Tn antigen[123,157] - and semi-synthetic carbohydrate/glycopeptide-protein conjugates using KLH,[158,159] BSA,[160] or ovine submaxillary albumin (OSA).[161]

Immunization with desialoOSM (dOSM) provided mice with good protection against challenge by highly invasive Tn-expressing syngeneic mouse mammary tumour, TA3-Ha. dOSM induced *in vitro* proliferation of T-lymphocytes obtained from mice immunized with dOSM and delayed-type hypersensitivity (DTH) in mice in response to footpad injections with irradiated TA3-Ha cells.[123]

Studies with semisynthetic T vaccine composed of synthetic disaccharide T antigen covalently attached to KLH and injected with Ribi adjuvant showed that this preparation is able to induce production of IgM and IgG antibodies and DTH in mice against mouse mammary adenocarcinomas expressing the T antigen.[162]

OSA conjugates with mono-, di-, and trimer of sTn antigen were examined for their ability to stimulate Tn specific immune responses. All conjugates produced high titres of IgM antibodies. In addition, OSA with dimer and trimer of Tn antigen also induced IgG response.[163]

In semi-synthetic vaccine preparations, pure TACAs were also replaced for complex penta-O-glycosylated MUC1 peptide sequence and its KLH glycopeptide-protein carrier conjugate. Their binding activities were examined using MoAb SM3 and HMPV. Both structures (i) were equally recognized by both MoAbs and (ii) inhibited binding of both MoAbs to the breast cancer mucin. Since these antibodies did not recognize mucin from healthy tissues it has been hypothesized that they recognize glycosylated rather then pure peptide epitopes.[164]

However, in most cases these preparations elicit low levels of desired antibodies due to the low molecular ratio of the TACA over the carrier protein. The resulting irrelevant response against carrier, and the undefined composition of the molecule represent major limitations for their use in cancer therapy.

Synthetic vaccines

An alternative class of fully synthetic vaccines based on peptide or lipopeptide cores has been designed to circumvent some of these drawbacks. PAMAM (poly(amidoamine))[165],

poly(propyleneimine), self-assembling bipyridyl Cu(II+) complexes[166], oligolysine scaffolds,[167] RAFTs,[168] and many others[169] have been used as a nonimmunogenic carriers of mono- and oligosaccharides and glycopeptides. Complete control over design, utilization of automatic synthesizers, suitably protected glycosylated amino acid derivatives are another advantages.[170-172] Alternatively, on-resin glycosylation can be carried out after polypeptide chain assembly.[173]

Tetrameric MAG carrying the carbohydrate Tn antigen (B epitope) associated with a CD_4+ Th epitope (a known sequence from Poliovirus) was readily recognized by anti-Tn 83 D4 (IgM) and MLS 128 (IgG) MoAbs. These results corroborate previous findings obtained with dOSM and Tn-OSA semisynthetic constructs about specificity of MLS 128 MoAb for cluster of α-GalNAc-Ser/Thr antigen. This binding specificity may explain poor binding to MAG carrying only one Tn antigen.[23] As expected, tri-Tn MAG[174] as well as linear glycopeptides displaying tri- and hexa-Tn cluster[175] were well recognized by this MoAb. IgG antibodies induced by this tri-Tn construct recognized murine and human tumour cell lines expressing Tn antigen. Vaccination with this immunogen administered together with alum adjuvant increased survival of tumour-bearing mice. In immunotherapy study tri-Tn was found to provide better protection than the mono-Tn analogue. It was also found that linear glycopeptide was less efficient compared to the MAG analogue.[174]

RAFT scaffolding has also been recently used for the preparation of synthetic glycopeptide vaccine.[176] Structures bearing both B-cell (Tn) and T-cell (CD 4^+ helper T-cell peptide from type I poliovirus) antigens were prepared using oxime-based ligation approach combined with orthogonal protection of lysine side chain (Boc, Alloc). Antigenicity and immunogenicity of these constructs were investigated both *in vitro* and *in vivo* tests. GalNAc saccharide moieties were successfully recognized by anti-Tn MoAb 6E11 (IgG_3) and 83D4 (IgM) showing good availability to the immune system. In Balb/c mice, these constructs showed good IgG immune response. Moreover these antibodies were capable to recognize native Tn antigens on human Jurkat cell line as documented by FACS. Interestingly, almost no antibodies reacted with synthetic RAFT scaffold showing good perspective in multiepitope vaccine design.[176]

Surprisingly, opposite results obtained with Starburst dendrimers bearing Tn dimer are quite surprising. These constructs did not induce any anti-Tn antibodies despite high density of Tn antigen.[177]

1.3 STRUCTURAL CHARACTERIZATION OF DENDRIMERS BY COMPUTATIONAL ANALYSIS

The structure of dendrimers has been investigated from both theoretical and experimental point of view.

Experimental studies of structural properties of dendrimers have been carried out by means of different techniques including ^{1}H- and ^{13}C-NMR spectroscopy,[178,179] small-angle neutron scattering,[180,181] NMR spectroscopy using paramagnetic cores,[182] small-angle X-ray scattering.[183] However, these methods are not generally applicable and give only limited amount of information. It is due to the extreme flexibility of particular arms/branches of the molecule that allows adoption of a huge number of energetically similar conformations rapidly interchanging in solution. In addition, from the physico-structural point of view, particular branches of symmetric structures behave "identically" (e.g. PAMAM), and for non-symmetric structures "nearly identically" (e.g. MAPs, MAGs). Implications are that these structures cannot be characterized atom-wise but rather group- or statistic-wise.

For the characterization of homogeneity, gel permeation chromatography (used not only for the purification of products, but also for the assessment of molecular size)[184] and soft ionization mass spectrometry techniques, such as matrix-assisted laser desorption/ionization-time of flight and electrospray ionization methods,[185] velocity sedimentation, translational diffusion and viscosity measurements[186] have been applied. However, these techniques have only a limited applicability, generally giving reliable results for low-generation dendrimers.

Therefore the utilization of computational methods to explore conformational space of flexible dendrimers offers a great chance to model their conformational preferences.[187,188] To study geometrical characteristics of dendrimers, Monte Carlo methods[189] and molecular dynamics (MD) simulations[190,191] have been successfully employed. To obtain as much as precise characteristics of the conformational behaviour of a dendrimer, a long-time MD simulation with inclusion of explicit solvent molecules is necessary. Quite reasonable correspondence between theory and experiment was found when explicit water molecules were included.[192] In principal, these methods are suitable to explore conformational preferences if long conformational trajectory is calculated.[193]

Conformational space of dendritic molecules in solution can be, in principal, described by ensembles of energetically similar conformers. There are several factors affecting

conformational variability and behaviour of dendrimers including character of the core and character of terminal groups of the dendrimer.

One of the first reports, in which the positions of end groups in dendrimers were considered, showed that modelled dendrimers could freely grow up to a certain "predictable-limiting" generation.[194] This study also showed that with growing dendrimer generation inner part (the core) of the dendrimer has lower density than the outer part pointed towards solute. When ends of the particular branches were studied, they were found to be positioned not at the surface of the molecule but severely backfolded. It showed that the backfolding of the particular branches on the spatial arrangement of the dendrimer plays a substantial role with increasing dendrimer generation. Numerical calculation published later[195] showed contradictory results and predicted a monotonic decrease in density going from the core of the dendrimer to its periphery.

Boris and Rubinstein[196] described in their model of flexible dendrimers that the highest density is concentrated in a core and that end groups are evenly distributed throughout the volume of the dendrimer. However, subsequent simulation studies have shown that some backfolding of the terminal amino acids occurs, but not to such an extent that the dendrimer core is completely filled.[197] Depending on the force field used, there are some levels of backfolding that are more pronounced for the force field representing a "bad" solvent.

Welsch and Muthukumar[198] showed a dramatic change in dendrimer conformation depending on the ionic strength of the solvent. At high ionic strength, backfolding of the end groups took place and a "dense core" dendritic structure was formed. At low ionic strength, multiple charges in the dendrimer force the molecule to stretch out individual arms resulting in a dense shell structure.

1.4 PREPARATION OF MULTIPLE ANTIGENIC SYSTEMS

There are two approaches for the preparation of peptide and glycopeptide dendrimers: *Divergent Strategy* and *Convergent Strategy*, Fig. 11. In the divergent strategy the whole molecule is prepared stepwise on a solid support, whereas in the convergent strategy the molecule is divided into two or more fragments (building blocks) that are prepared separately and then assembled together. These strategies are generally applicable for all classes of peptide and glycopeptide dendrimers.

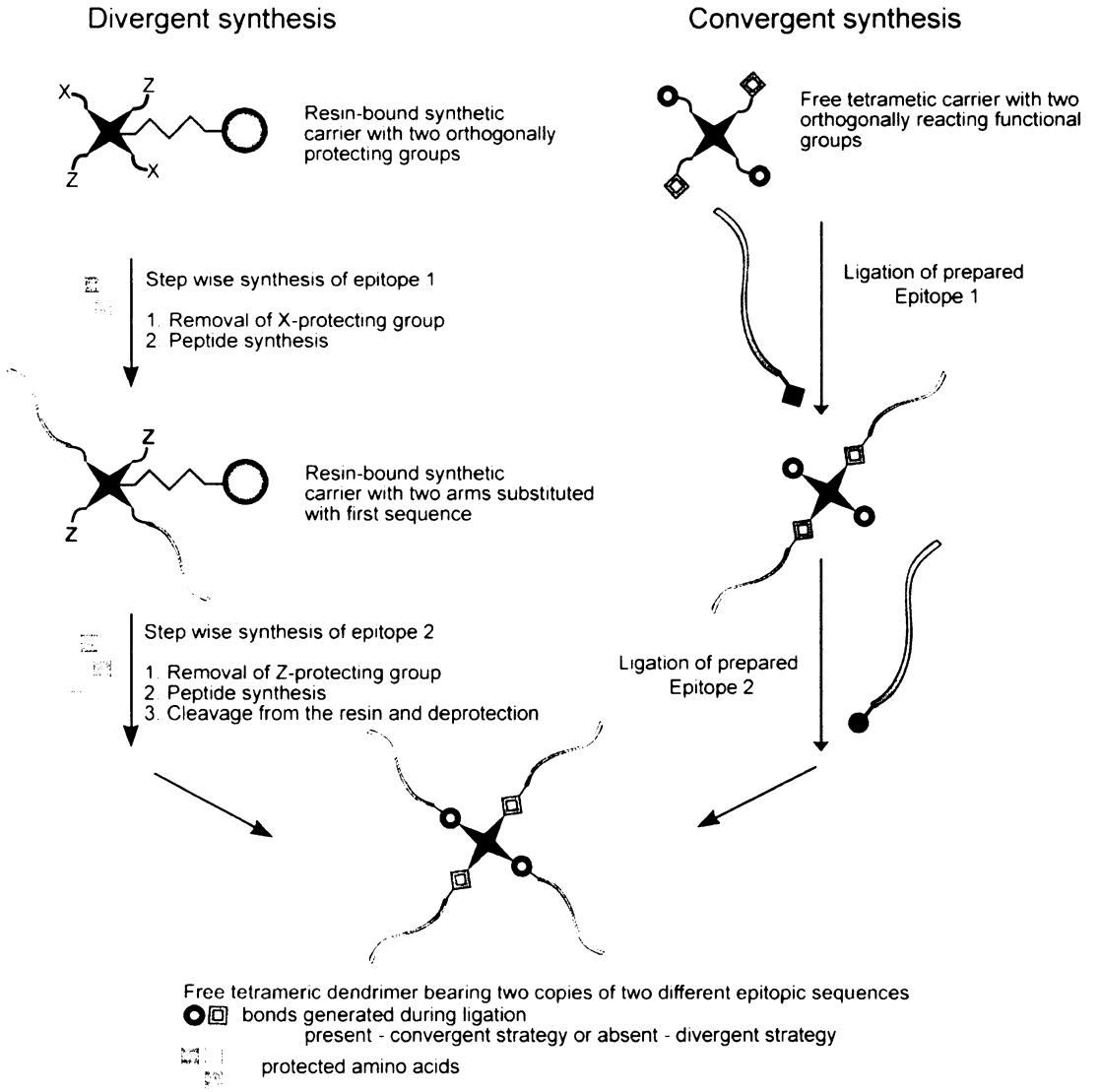


Fig. 11 Scheme of divergent and convergent synthetic approach for the preparation of tetrameric constructs with two different epitopes.

1.4.1 Divergent strategy

The synthetic availability, trivalency (*in terms of peptide bond*) and reactivity makes lysine one of the most frequently used branching units for the preparation of peptide dendrimers.

Both Boc and Fmoc chemistries have been successfully applied in the synthesis on a solid support. Monoepitopic MAPs carrying either B-, T- or chimeric B-T and T-B epitopes have been synthesized stepwise from the C terminus of the core to the N termini of antigens, employing either Boc-Lys(Boc) or Fmoc-Lys(Fmoc).

The synthesis of diepitopic and, generally, multiepitopic structures (two or more different peptide chains), takes advantage of the availability of orthogonally protected commercially available lysines.[199] There are several suitable combinations of protecting groups: (i) Boc-Lys(Fmoc) and Fmoc-Lys(Boc) used in Boc scheme,[200,201] (ii) Fmoc-Lys(Dde), Dde-Lys(Fmoc), Fmoc-Lys(Mtt), Mtt-Lys(Fmoc) used in Fmoc scheme,[202-204] (iii) Fmoc-Lys(Npys), Boc-Lys(Npys),[205] Fmoc-Lys(Alloc) and Boc-Lys(Alloc)[206] used for both Boc and Fmoc chemistry.

Characterization of commonly used protecting groups

Dde [1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene) ethyl] is cleavable by 2% hydrazine in DMF (v/v) and is stable during the Fmoc removal.[202] Recently described migration of this group from an α-NH_2 or an ε-NH_2 group to an unprotected ε-NH_2 during the Fmoc removal with piperidine or even in neat DMF by a direct nucleophilic attack of the free ε-NH_2[207] can be prevented by the use of 2% DBU [1,8-diazabicyclo[5.4.0]undec-7-ene] for the Fmoc removal (3 x 3 min).[208]

Mtt [4-methyltrityl] is highly acid-labile group removable under mild acidic conditions, 1% TFA in DCM or by the mixture of HOAc:TFE:DCM (1:2:7) (v/v/v), ensuring stability of the *tert*-butyl protecting group and an ester bond between the polypeptide chain and the resin.[204]

Npys [3-nitro-2-pyridine sulphenyl] group is stable towards strong acids such as TFA and HF, and can be removed by treatment with triphenylphosphines or thiols (e.g. dithiothreitol).[209] It is usually used for the side-chain protection of lysine residue, i.e. Fmoc-Lys(Npys) or Boc-Lys(Npys).

Alloc [allyloxycarbonyl] group is catalytically removable protecting group and is usually removed by $Pd(PPh_3)_4$ in the presence of nucleophile, e.g. Bu_3SnH, acting as an allyl group scavenger.[210] This chemistry is compatible with both Boc and Fmoc synthesis. For Fmoc chemistry a nucleophilic scavenger of low basicity has to be used.[211]

The synthesis of MAPs follows standard SPPS protocol, is technically straightforward and therefore can be automated.[19,212] However, there are several issues that must be particularly noted.

(i) Resins with low substitution have to be used in order to minimize inter-chain interactions leading to the chain clustering that mimics cross-linking, limits resin swelling, reagent permeability and coupling efficiency. Typically, for octameric MAPs, the loading of the first amino acid should not exceed 0.1 mmol/g of the resin (after built-up of octavalent oligolysine core, this theoretically represents 0.8 mmol/g substitution (8×0.1) of terminal amino groups).

(ii) Due to the branched character and close proximity of N-reactive ends of the growing molecule, a 10-fold excess of activated amino acid is generally necessary to minimize incomplete coupling.

(iii) Purification of MAPs is troublesome because of the branched character of the molecules and the presence of erroneous (deletion) sequences in individual branches. MAPs are usually purified by dialysis, gel permeation chromatography or repeated precipitation that remove low molecular weight species and salts; the remaining bulk is generally considered homogeneous.[40,213] The utilization of RP-HPLC technique has been rarely reported. However, if properly used, this method can provide very homogeneous product. Only a few studies have been devoted to the detailed characterization and purification of MAPs.[214,215] On the other hand, a wide variety of analytical methods can be employed for their characterization, i.e. RP-HPLC, HPCE, AAA, NMR and various modes of mass spectrometry. Recently described partial cleavage by trypsin-like endopeptidase combined with RP-HPLC analysis of cleavage products is another method of choice.[216]

1.4.2 Convergent strategy

The preparation of high molecular weight polypeptides, proteins or enzymes is one of the challenging tasks for peptide chemists. Despite the progress in peptide chemistry since the introduction of SPPS by Merrifield,[217] larger peptides (with more than 60 residues) are still difficult to synthesize by stepwise solid-phase synthesis due to the solvatation-aggregation problems and the accumulation of deletion, truncation and formation of epimerization side-products.[218] The idea of splitting the target molecule into several parts, their synthesis and purification, followed by the controlled linking has long been pursued. Three approaches have emerged: (i) chemical condensation of side-chain protected peptide segments (often associated with poor solubility of the segments and low coupling yields, slow coupling reaction, racemization),[219] (ii) enzyme-catalyzed coupling of peptide fragments, i.e. reverse proteolysis (limited number of substrates)[220-222] and (iii) chemical ligation of unprotected peptide fragments (*modified with selectively reacting groups*) in buffered aqueous solution (ligation techniques).[223,224]

Ligation strategy offers advantages over the traditionally used coupling procedures by not requiring carboxyl-group activation or protection of amino acids` side-chains. Generally, the target molecule is divided into functional fragments/building blocks that are modified with a mutually reactive pair that consists of a nucleophile and an electrophile. These fragments are synthesized and purified by conventional methods and then assembled under mild conditions give complex molecular target compound. High selectivity of ligation reaction and increased solubility of peptide fragments significantly improve handling and work-up, and consequently provide highly homogeneous product.

Two types of ligation exist: *Chemoselective Ligation* placing "non-peptide bond" and recently introduced *Native Chemical Ligation* producing "native peptide bond" in the site of ligation.

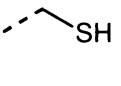
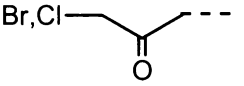
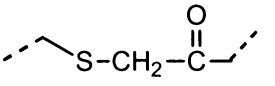
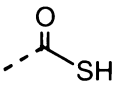
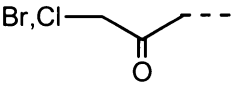
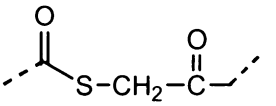
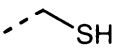
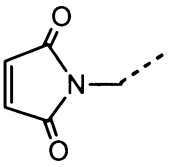
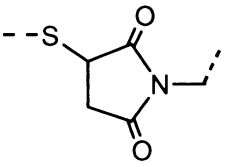
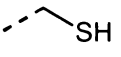
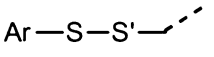
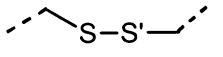
1.4.2.1 Chemoselective ligation

Two general methods based on thiol and carbonyl chemistries have been used to ligate unprotected peptides and cores to form MAPs with non-peptide bond in the site of ligation. To achieve the selectivity during ligation in aqueous buffers, the pKa of weak base used must be lower than that of functional groups presented on the peptide chains (Arg, His, Lys).

1.4.2.1.1 Thiol chemistry

Thiol chemistry makes use of the reactivity of sulphydryl groups with (i,ii) α-halocarbonyls, (iii) conjugated double bond, and (iv) sulphur-sulphur exchange with disulphides, Tab. 9.

Tab. 9 Thiol chemistry - types of reactants and character of generated bonds.

	Nucleophile	Electrophile	Product
(i) Alkylation - thioether			
(ii) Alkylation - thioester			
(iii) Addition to the activated double bond - thioether			
(iv) Disulphide exchange			

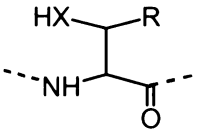
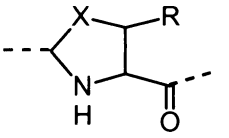
These reactions have been successfully applied in MAPs syntheses, e.g. (i,ii) linkage of chloroacetylated oligolysine core with synthetic peptide (with cysteine at N-terminus).[225,226] However, this method cannot be used for the preparation of glycopeptide dendrimers bearing O-glycosidically bound saccharides (β-elimination of saccharide moiety under conditions used for the ligation (pH ≅ 8.0)). (iii) Addition to an activated double bond of maleimide groups is well known from protein chemistry and has been successfully applied for the preparation of TASPs.[168] (iv) Thiol exchange has been also applied,[228] the thiolated core matrix reacted with activated S-(Npys)-cysteinyl peptide yielding MAP constructs with S-S linkage. However, due to the lack of the desired stability this method has not been used very often.

1.4.2.1.2 Carbonyl chemistry

Carbonyl chemistry works at the same principle as thiol chemistry, but exploits different types of weak bases. The selected weak base must react selectively with aldehyde group under acidic conditions. Under these conditions side-chain nucleophiles are protected by protonation so that they cannot be involved in ligation to form undesirable by-products.

There are two groups of nucleophiles used: (i) conjugated amines with basicity lowered by neighbouring electron-withdrawing groups, such as hydroxylamine and substituted hydrazines such as acylhydrazines and phenylhydrazines, (ii) bases with 1,2-disubstituted motif, such as 1,2-aminoethanol derived from serine or threonine and 1,2-aminoethanethiol group obtained from cysteine, Tab. 10. Aminooxyacetic function is usually introduced as a Boc derivative on the N-terminus of the growing peptide chain or on the side-chain of the lysine residue during the Fmoc synthesis and is stable to TFA or HF cleavage conditions. After ligation to the aldehyde it gives oxime bond that is stable in water at room temperature at pH 2-7.[229] The hydrazide-aldehyde ligation chemistry is usually carried out at pH 4.5-5.0 and gives a hydrazone bond. Alkyl hydrazone linkage is generally susceptible to hydrolysis and is usually reduced by a treatment with $NaBH_3CN$ to give hydrazine linkage.[230] In MAPs, the hydrazone linkage has been found to be sufficiently stable at neutral pH in comparison to the linear peptides, probably due to the branched character of the construct. Interestingly, phenyl hydrazone linkage is more resistant to hydrolysis and does not require the reduction step. Hydrazine can be introduced as a Boc-monohydrazide succinic acid, or as a 4-Boc-monohydrazinobenzoic acid (Hob) during the Fmoc synthesis. When 1,2-disubstitued amino acids are used for the ligation, a thiazolidine or an oxazolidine ring is formed at the site of ligation, i.e. the thio- and oxo- analogues of proline amino acid are formed, respectively.

Tab. 10 Carbonyl chemistry - types of reactants and character of generated bonds.

	Nucleophile	Electrophile	Product
(i) Oxime	---CHO	NH_2—O—CH_2---	--CH=N—O—CH_2--
(ii) Hydrazone/ Hydrazine	---CHO	NH_2—NH—CH_2---	---CH=N—NH-CH_2--- ↓ reduction --CH—N—NH-CH_2---
(iii) Thiazolidine/ Oxazolidine	---CHO	 R= H or CH_3 X = O or S	

Aldehyde function can be introduced both at the N-terminus and the C-terminus of the molecule. At the N-terminus or N_ε-amino group of lysine residue by (**i**) the $NaIO_4$ oxidation of coupled cysteine, serine or threonine under neutral conditions to yield α-oxoacyl moiety; (**ii**) the coupling of suitably protected aldehyde group, e.g. 5,5-dimethoxy-1-oxopentanoic acid and 2,2-dimethoxyacetic acid. At the C-terminus by (i) a special linker or/and reductive treatment, which is incompatible with aspartic or glutamic acid containing peptides; (ii) an enzymatic or chemical linkage of purified peptide to an amino acid containing masked aldehyde; (iii) a novel method based on the sensitivity of PAM resin anchor to aminolysis.[231]

1.4.2.2 Native chemical ligation

This approach is especially suitable for protein preparation because, unlike the chemoselective ligation, no artificial fragments or groups are introduced into the molecule.

It is applicable for any X-Cys sequence, where X is any of the 20 naturally occurring amino acids. In the first step of the process two fragments are joined head-to-tail by a fast chemoselective reaction such as thioester, disulphide or thiazolidine formation, which places the reacting amine and carboxyl-group in close proximity. The intermediate undergoes spontaneous rapid intramolecular reaction (S to N acyl shift), driven by a high "effective molarity", to form a peptide bond at the ligation site, Fig. 12. The major obstacle has been the introduction of thiocarboxylic acid at the C-terminus of the N-terminal peptide fragment. This is usually achieved during the solid-phase synthesis using special linkers. However, this

approach has been limited to the Boc-based synthetic protocols, due to the instability of thioester bond to the repeated exposure to a base during the Fmoc removal. Thus, this method cannot be used for the preparation of O-linked glycopeptides, due to the susceptibility of O-glycosidic bond to the acid-catalyzed cleavage by strong acids. This major obstacle has been recently overcome by using sulfonamide "safety-catch" linker, which can be easily converted to the suitable thioester.[232,233]

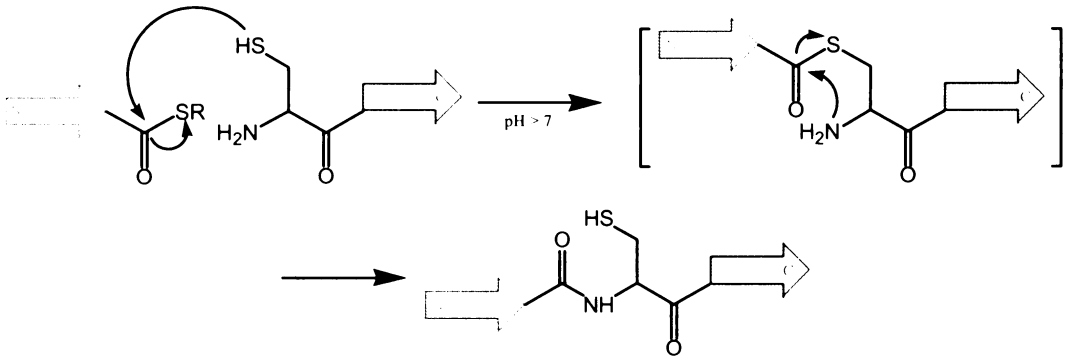


Fig. 12 Cysteine ligation via thiol-thioester exchange.

This approach suffered from critical limitations regarding the presence of cysteine residue at the site of ligation. The absolute need for cysteine residue has been recently overcome, and Gly, Ala, His and Met have been shown to be possible surrogates for cysteine.[234] The thiol capture scheme[235] and the Staudinger ligation[236] are other ligation approaches that are available and equally applicable. These approaches have not been used for the preparation of MAPs yet.

2. PROJECT AIMS

Synthetic part: synthesis of three types of α-D-GalNAc bearing glycodendrimers

i. Design and synthesis of MAGs attached to a biocompatible resin,

ii. Design and synthesis of MAGs of classical type,

iii. Design and synthesis of comblike multiple antigenic glycopeptides.

Biological part

i. Evaluation of the binding characteristics of immobilized MAGs and their potency to induce immune response in mice, in collaboration with MUDr. M. Písačka at the Institute of Haematology and Blood Transfusion,

ii. Evaluation of the potency of classical MAGs to induce humoral response in mice and correlation of these results with data on immobilized MAGs, in collaboration with MUDr. M. Písačka at the Institute of Haematology and Blood Transfusion,

iii. Evaluation of the binding characteristics of comblike glycodendrimers, in collaboration with RNDr. K. Bezouška, CSc. at the Institute of Microbiology,

iv. Evaluation of the potency of comblike glycodendrimers to modulate cytotoxic activity of NK cells, in collaboration with RNDr. K. Bezouška, CSc. at the Institute of Microbiology,

v. Evaluation of the potency of comblike glycodendrimers and the effect of individual modifications of parent structures on the character of the immune response in mice, in collaboration with MUDr. M. Hajdúch at the Dept. of Oncology, Palacký University.

Theoretical part

i. Evaluation - by the means of molecular dynamics – of the effect of the γ-Abu insert on the conformational behaviour of individual branches in MAGs as a methodical background for the design on novel structures, in collaboration with RNDr. J. Vondrášek at the IOCB.

3. RESULTS AND DISCUSSION

3.1 PREPARATION OF IMMOBILIZED AND CLASSICAL GLYCODENDRIMERS

3.1.1 Multiple antigenic glycopeptides attached to a biocompatible resin

Results are detailed in: Ježek J., Velek J., **Vepřek P.**, Velková V., Trnka T., Pecka J., Ledvina M., Vondrášek J., Pisačka M.: Solid Phase Synthesis of Glycopeptide Dendrimers with Tn Antigenic Structure and their Biological Activities. Part I. *J. Peptide Sci.* **1999**, 5, 46-55.

The idea behind the preparation of immobilized MAGs was a good immune response obtained against Tenta Gel-coupled peptides and assumption that the character of the selected resin would be suitable for the planned synthesis.

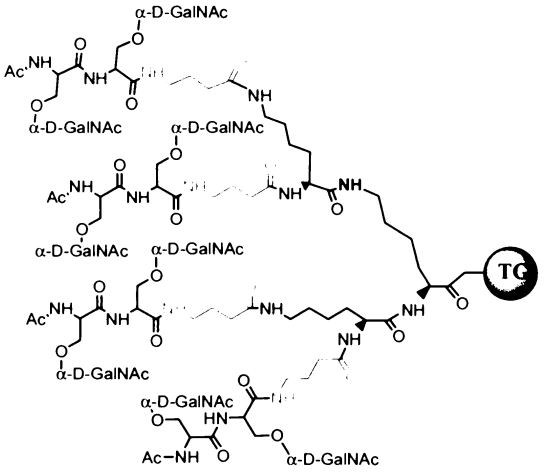
Tetra- and octameric MAGs with or without γ-Abu insert were designed and prepared to study the effect of valency and the insert on the antigenicity and immunogenicity of these compounds. MAGs immobilized on TentaGel resin were prepared by the divergent method using Boc/Bzl chemistry, 3-fold molar execs and DCC/HOBt or BOP/DIEA activation, see Tab. 11.

Tab. 11 Prepared immobilized peptides and glycopeptides.

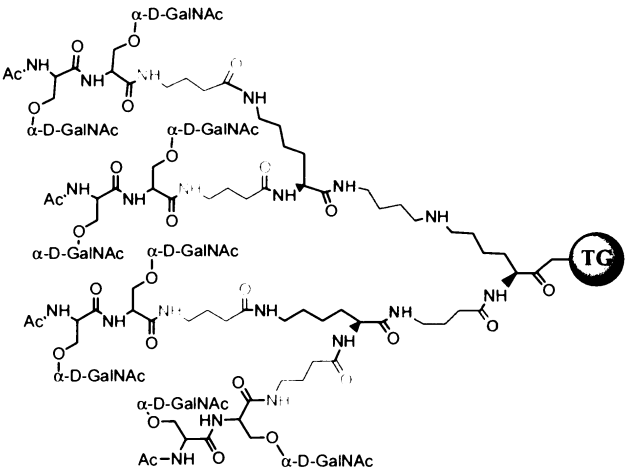
Comp.	Sequence
1	[Ac-Lys(Ac)]$_2$-Lys-β-Ala-NH-TG
2	[Ac-(**Tn**)$_2$-γ-Abu]$_4$-Lys$_2$-Lys-β-Ala-NH-TG
3	[Ac-Lys(Ac)-γ-Abu]$_2$-Lys-β-Ala-NH-TG
4	[Ac-(**Tn**)$_2$-γ-Abu]$_4$- (Lys-γ-Abu)$_2$-Lys-β-Ala-NH-TG
5	[Ac-Lys(Ac)]$_4$-Lys$_2$-Lys-β-Ala-NH-TG
6	[Ac-(**Tn**)$_2$-γ-Abu]$_8$-Lys$_4$-Lys$_2$-Lys-β-Ala-NH-TG
7	[Ac-Lys(Ac)-γ-Abu]$_4$-(Lys-γ-Abu)$_2$-Lys-β-Ala-NH-TG
8	[Ac-(**Tn**)$_2$-γ-Abu]$_8$- (Lys-γ-Abu)$_4$-(Lys-γ-Abu)$_2$-Lys-β-Ala-NH-TG

TG - Tenta Gel resin

Boc-Ser(3,4,6-tri-*O*-Ac-α-D-GalNAc)-OH was used as a glycosylated building block. Tetrameric compounds **2** and **4** were prepared using standard coupling procedures. In case of octameric compounds **6** and **8,** coupling difficulties during the loading of the second level of protected Tn antigens were observed. These difficulties were overcome by repeated coupling, the use of catalyst (DMAP) and prolongation of the reaction time until negative ninhydrin test.[237] The biocompatible Tenta Gel S NH_2 resin was selected as a solid support for the following reasons: i) swelling properties independent of the solvent (including water), ii) production of the resin with low loading suitable for the preparation of branched compounds without further modification, iii) high kinetic rates of coupling reactions, iv) possibility to inject Tenta Gel immobilized peptides into animals without negative side effects, v) no toxicity or other adverse effects on animals or cellular systems (biocompatibility), vi) ability to serve as a high-molecular carrier, vii) good immune response against Tenta Gel-coupled peptides reported in the literature. Fig. 13 shows structure of compounds **2** and **4** from Tab. 11.



Compound **2**



Compound **4**

Fig. 13 Tetrameric MAG without (**2**) and with (**4**) γ–Abu insert (blue colour) carrying Ac-Ser(Tn)-Ser(Tn)-γ–Abu B-cell epitope covalently attached to a polymeric carrier (Tenta Gel resin). γ-Abu is also present as a part of the glycopeptide epitope (red colour).

Due to the character of the linker between the branched peptide and resin (cleavable under conditions that would not prevent amide bond from fission) the only available analytical technique applicable was amino acid analysis, Tab. 12.

Tab. 12 Amino acid analyses of compounds **1-8** (theoretical values are given in parentheses)

Compound	β-Ala*	Lys	γ-Abu	Ser	$GalNH_2$
1	1 (1)	2.8 (3)	-	-	-
2	1 (1)	2.8 (3)	3.7 (4)	7.6 (8)	7.9 (8)
3	1 (1)	2.8 (3)	1.8 (2)	-	-
4	1 (1)	2.7 (3)	5.8 (6)	7.6 (8)	7.8 (8)
5	1 (1)	6.9 (7)	-	-	-
6	1 (1)	6.9 (7)	6.6 (8)	12.9 (16)	12.5 (16)
7	1 (1)	7.4 (7)	5.7 (6)	-	-
8	1 (1)	6.8 (7)	12.5 (14)	12.5 (16)	12.4 (16)

Conditions used for hydrolysis: 6 N HCl at 110°C for 20 h in sealed glass tube
* an internal amino acid standard to which all the values were calculated

3.1.2 Classical multiple antigenic glycopeptides

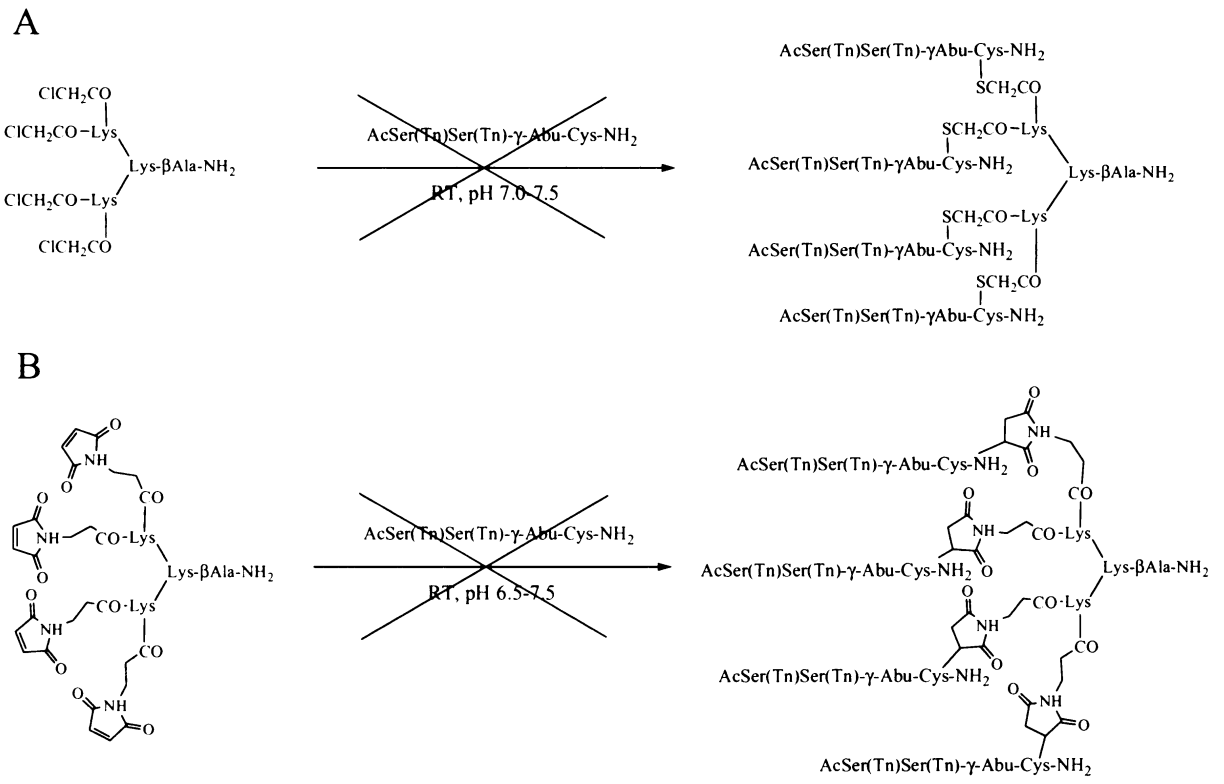
Preparation and evaluation of classical MAGs should enable comparison of results obtained with MAGs attached to a biocompatible resin.

For the preparation of "soluble analogues", Fmoc/tBu chemistry had to be adopted due to the reported instability of O-glycosidic bond to anhydrous hydrogen fluoride treatment.[238] This treatment is used for the removal of the side-chain protecting groups and cleavage from the solid support (resin) in Boc chemistry. Conditions used for the removal of Fmoc protecting group as well as the removal of side-chain protecting groups and detachment from the resin are compatible with the stability of O-glycosidically bound α-D-GalNAc residue.

Four different commercially available resins (RINK amide resin, AM resin and Sieber resin, and 2-chlorotritylchloride resin), three different Fmoc removing techniques (25% piperidine in DMF, 40% piperidine in DMF, 2% DBU + 4% piperidine in DMF), five different coupling techniques (DCC, DIPCI, BOP, HATU, TBTU), room and elevated (45°C) temperature, and coupling times ranging from 1 h - 1 day were used for the preparation of target compounds. Crude mixtures obtained after cleavage from the resin were highly complex with minimal presence of target structures, in case of octameric structures, no product was detected.

To understand the nature of these difficulties, a computational study of two tetrameric structures (with or without γ-Abu insert) was undertaken. Results of this study are discussed further in the text.

In the view of a failure of divergent method to produce soluble MAGs, the use of a chemoselective ligation (convergent strategy) seemed to be a logical step. The target structure was divided into two functional subunits: the carrier and the glycopeptide antigen. These subunits were modified according to the ligation technique selected and assembled in buffered aqueous solution.



Scheme 1 Scheme of the preparation of classical MAGs using chemoselective ligation: (A) thioether bond formation, (B) addition to an activated double bond that was **NOT WORKING**.

First, a nucleophilic replacement of halogen in a halomethylcarbonyl group was selected. The antigen Ac-Ser(Tn)-Ser(Tn)-γ-Abu was extended by a cysteine residue at the C-terminus, and chloroacetic acid was conjugated onto the carrier's N-terminal amino groups, Scheme 13A. However, this approach proved to be unsuccessful.

In second attempt, an addition to an activated double bond was selected. The antigen Ac-Ser(Tn)-Ser(Tn)-γ-Abu was extended by a cysteine residue at the C-terminus, and the 3-maleimidopropionyl group was attached onto the carrier's N-terminal amino groups, Scheme 13B. However, this approach also failed to provide target compound(s).

Conclusions

Transition from Boc to Fmoc chemistry, i.e. change of the protecting groups, resin and synthetic protocol had a negative effect on the synthetic availability of target branched compounds, *see also chapter 3.4, p. 59.* Attempts to prepare target compounds using Boc chemistry were unsuccessful. Application of two chemoselective ligation techniques did not improve the synthetic availability. We assume, based on the computational calculations mentioned further in the text, that this effect was caused by the presence of γ-Abu residues that might backfold and make sulfhydryl group sterically inaccessible. We also assume that the branched character of the oligolysine carrier causing close proximity of N-teminal functional groups also negatively influenced the synthetic availability of these compounds.

3.2 PREPARATION OF COMBLIKE MULTIPLE GLYCOPEPTIDE ANTIGENS

Results are detailed in **Vepřek P.**, Hajdúch M., Džurbák P., Kuklík R., Poláková J., Bezouška K.: Comb-like dendrimers containing Tn antigen modulate natural killing and induce the production of Tn specific antibodies. J. Med. Chem. **2006**, *49*, 6400-6407.

In the view of previous findings, a comb-like core/carrier was designed as a structural alternative to branched oligolysine core. Unlike branch-like MAGs, where the branching and binding of antigens is achieved by employing both α- and ε-amino groups of lysine residue, linear comb-like structures employ α-amino groups for the carrier construction and ε-amino groups for antigen attachment. Unlike linear SOCs, where the orientation of lysine side chains is uniform[28], the orientation of lysine side chains in comb-like structures has not been studied.

Compounds carrying glycopeptide antigens with monomer, dimer or trimer of Tn antigen i.e. Ser(α-D-GalNAc), on individual arms (**9-11**) were prepared. These structures were further modified and compounds having DNP group (**12-14**), or T-cell epitope[239] (**15**) were prepared, Fig. 14.

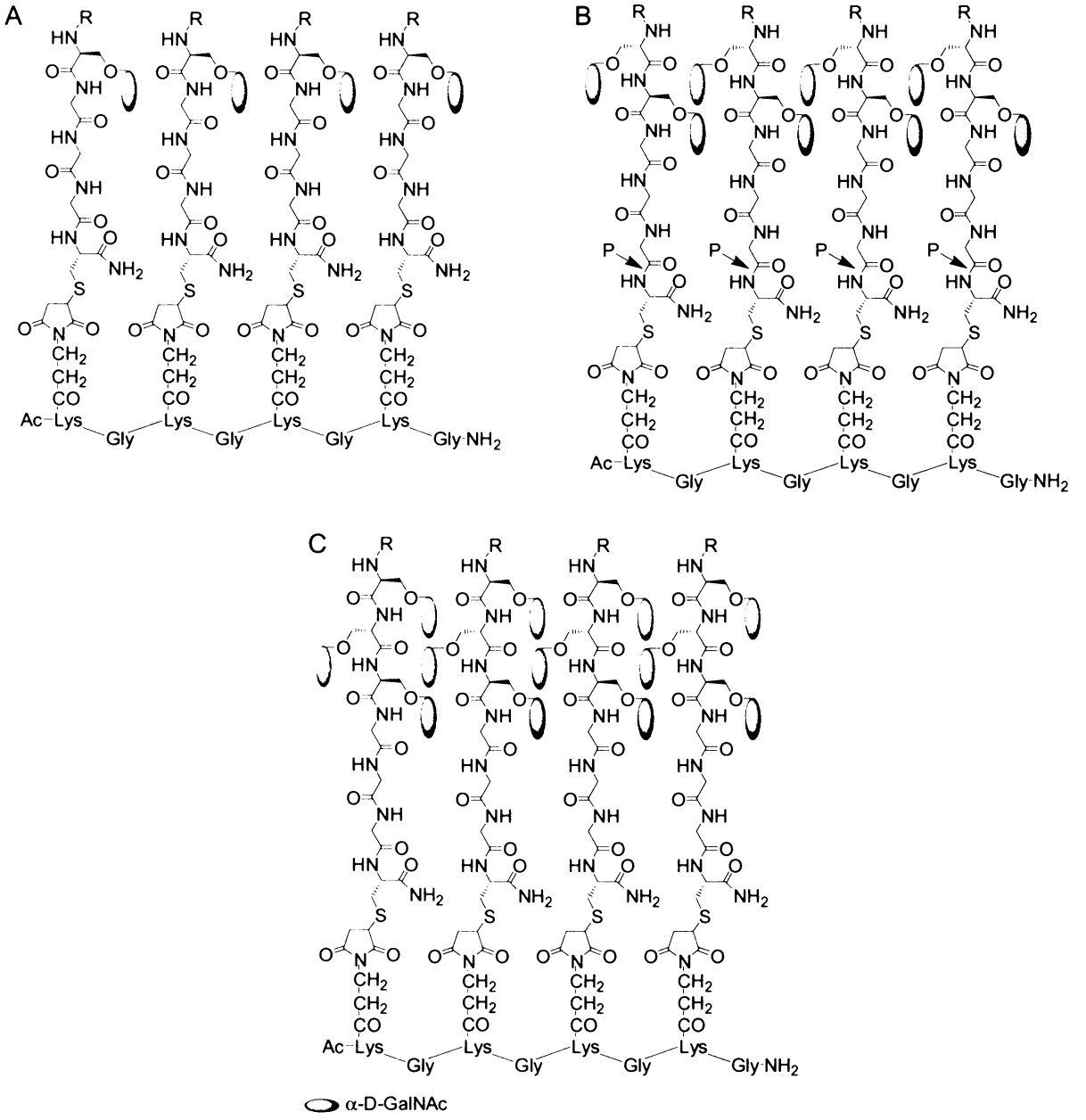
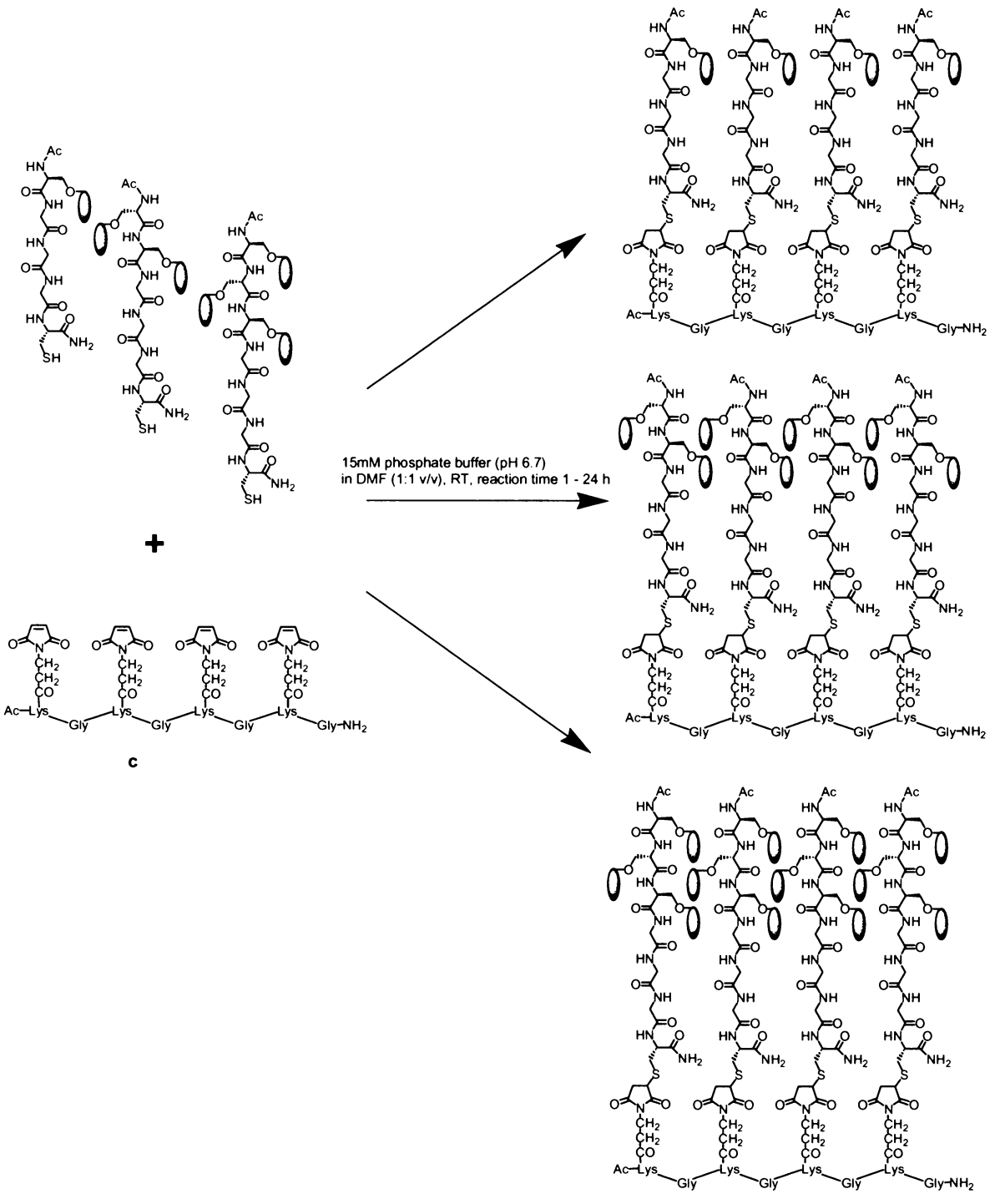


Fig. 14 Structure of comb-like dendrimers prepared by chemoselective ligation from Tab. 13: **9**: R = acetyl, **12**: R = DNP (A); **10**: R = acetyl P = 0, **13**: R = DNP, P = 0, and **15**: R = acetyl, P = PKYVKQNTLKLAT peptide sequence (B); **11**: R = acetyl, **14**: R = DNP (C).



Scheme 2. Scheme of the preparation of comb-like multiple antigenic glycopeptides by chemoselective ligation that **WAS WORKING**.

Compounds **9-15** were prepared by a combination of solid-phase synthesis using Fmoc/tBu strategy and chemoselective ligation technique exploiting selective reaction between sulfhydryl group and activated double bond.[227] Chemoselective ligation was carried out in the mixture of 15 mM phosphate buffer (pH 6.7) in DMF (1: 1, v/v), Scheme 2. The reaction was typically completed within 12-24 h. Identity and purity of purified compounds were confirmed by amino acid analysis, RP-HPLC, and MALDI-TOF mass spectrometry, see Tab. 13. Structures of compound **9-15** are given in Fig. 14.

Tab. 13 Physico-chemical data of prepared comb-like multiple antigens.

Comp.	Sequence	No. of Tn groups	Purity#	Retention time (min) ‡	Yield (%)	Molecular weight	
						Found+	Calc*
9	(Ac-Ser(**Tn**)-Gly_3-Cys-NH_2)$_4$*LC*	4	96.7 %	12.5 min	72 %	3896.35	3899.03
10	(Ac-[Ser(**Tn**)]$_2$-Gly_3-Cys-NH_2)$_4$*LC*	8	97.7 %	13.1 min	75 %	5057.98	5060.15
11	(Ac-[Ser(**Tn**)]$_3$-Gly_3-Cys-NH_2)$_4$*LC*	12	97.8 %	13.6 min	77 %	6218.07	6221.23
12	(DNP-Ser(**Tn**)-Gly_3-Cys-NH_2)$_4$*LC*	4	98.5 %	14.3 min	60 %	4393.10	4395.37
13	(DNP-[Ser(**Tn**)]$_2$-Gly_3-Cys-NH_2)$_4$*LC*	8	96.8 %	14.6 min	62 %	5554.15	5556.39
14	(DNP-[Ser(**Tn**)]$_3$-Gly_3-Cys-NH_2)$_4$*LC*	12	97.5 %	15.1 min	65 %	6714.12	6717.47
15	(Ac-[Ser(**Tn**)]$_2$-Gly_3-*P*-Cys-NH_2)$_4$*LC*	8	98.2 %	14.2 min	85 %	10999.98	11003.35

determined by RP-HPLC
‡ gradient 0-100 % in 30 min (solvent A: 0.1% TFA in water, solvent B: 0.1 % TFA in MeOH, column used: Vydac 208TP53 C_x 3.2 × 250mm, flow rate = 0.75 ml/min, λ = 218 nm.
+ ESI-MS
* monoisotopic mass
LC = linear tetravalent comb-like carrier
P = T-cell epitope (HA306-318, PKYVKQNTLKLAT) derived from influenza virus hemagglutinin

Amino acid analysis sheet

Comp.	D/N	T	S	E/Q	P	G*	A	C	V	L	Y	K
9			3.7 (4)			16 (16)		3.5(4)				3.8 (4)
10			7.7 (8)			16 (16)		3.4(4)				3.9 (4)
11			11.6 (12)			16 (16)		3.6(4)				4.0 (4)
12+			0.0 (4)+			16 (16)		3.4(4)				4.1 (4)
13+			3.8 (8)+			16 (16)		3.3(4)				3.9 (4)
14+			7.6 (12)+			16 (16)		3.5(4)				4.1 (4)
15	4.2 (4)	7.8(8)	7.7 (8)	4.3 (4)	4.5(4)	16 (16)	4.2(4)	3.6(4)	3.8(4)	7.5(8)	3.7 (4)	15.7 (4)

* amounts of all amino acids recalculated to the amount of glycine

3.3 BIOLOGICAL CHARACTERIZATION OF GLYCODENDRIMERS

3.3.1 Multiple antigenic glycopeptides attached to a biocompatible resin

3.3.1.1 *In vitro* study: Interaction with anti-Tn monoclonal antibodies

For the initial evaluation of accessibility on Tn antigens and recognizability of compounds **1-8** by MoAbs, a rosetting test[242] that gives qualitative information about the accessibility and specific discriminability of tested epitopes was used. Three MoAbs were tested: DAKO (anti-Tn), BRIC-66 (anti-Tn and anti-A), and Gamma (anti-A). MoAbs were incubated with Tn+ or type A red blood cells (RBCs) in the presence of compounds **1-8** and the results were determined under microscope, Tab. 14 and Fig. 15.

Tab. 14 Results of the rosetting test.

MoAb	Anti-Tn (DAKO)		Anti-Tn/A (BRIC 66)		Anti-A (Gamma)	
RBCs / Comp.	Tn^+	A	Tn^+	A	Tn^+	A
1	-	-	-	-	-	-
2	+	-	+	+	-	-
3	-	-	-	-	-	-
4	+	-	+	+	-	-
5	-	-	-	-	-	-
6	+	-	+	+	-	-
7	-	-	-	-	-	-
8	+	-	+	+	-	-

+ positive result, i.e. crosslinking of immobilized MAG structures with RBCs by MoAbs
- negative result of the rosetting test

Results of the rosetting test show good accessibility of Tn antigen on the surface of the Tenta-Gel beads for the monoclonal antibody binding. All Tn bearing dendrimers inhibited binding of Tn-specific MoAb to RBCs. Specific discrimination of Tn structure is important for the potential use of the glycodendrimers in affinity purification of antibodies. The cross-reactivity of compound **2, 4, 6** and **8** with blood group A antigen was shown to be related to the unique specificity of the anti-Tn/A antibody (BRIC 66) and not to the antigen itself.

An example of typical observation of rosetting test is given in Fig, 15. In positive rosetting test (Fig. 15A) the RBCs are cross-linked with resin beads by MoAbs covering almost entire surface of the beads. When rosetting test is negative (Fig. 15B) , the resin beads (big circles with thick black borders) are clear i.e. free from red cells.

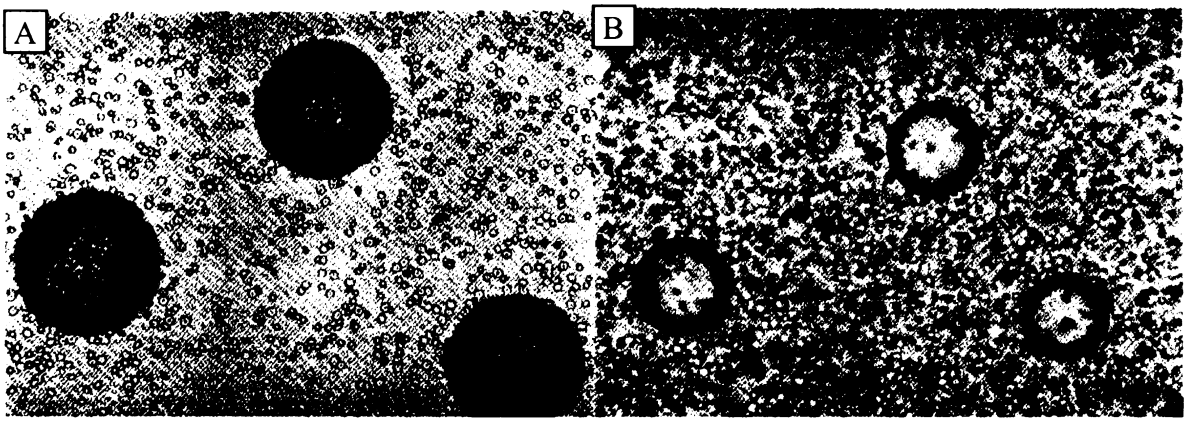


Fig. 15 Microscopic observation of MAG-resin beads (compound **4**) after incubation with Tn+ RBCs or blood type A RBCs: panel A) positive result - MoAb DAKO (anti-Tn) crosslinks compound **4** and Tn+ RBCs; panel B) negative result – no crosslinking by MoAb DAKO (anti-Tn) between compound 4 and blood type A RBCs observed.

3.3.1.2 *In vitro* study: Inhibition of agglutination reaction

The ability of compounds **1-8** to inhibit agglutination of Tn^+ RBCs by anti-Tn monoclonal antibody (DAKO) is shown in Fig. 16. This test should show if prepared compounds bearing Tn antigen can effectively compete with Tn antigen from natural source for the binding sites in anti-Tn MoAb.

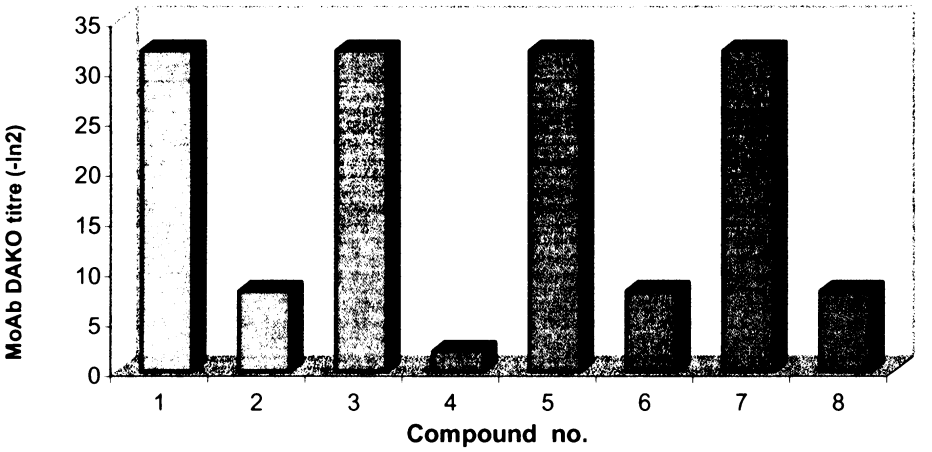


Fig. 16 Inhibition of agglutination reaction of Tn+ red blood cells with monoclonal anti-Tn antibody (DAKO) after preincubation of the monoclonal antibody with conjugates with Tn antigen (**2, 4, 6** and **8**) compared with negative controls (**1, 3, 5** and **7**). 100 μg of compound was incubated for 1 h with 100 μl antibody solution and supernatant was then tested in agglutination test. For structures see Tab. 11.

All structures with Tn antigen (**2, 4, 6** and **8**) showed good inhibition of agglutination reaction. Compound **4** gave the best results: MoAb DAKO titre = 2.

3.3.1.3 *In vivo* study: Production of anti-Tn specific antibodies

This study should prove the ability of tested compounds to modulate/activate adaptive immunity in tested animal model. Results of immunization of Balb/c mice with compounds **7** and **8** are given in Fig. 17. Immunization studies were carried out in Balb/c female six-week old mice. Two mice were immunized with **7** (C1-C2) as a control, and five mice with **8** (E1-E5). Five doses of 300 μg of conjugate were administered subcutaneously (without adjuvants) at 3-

week intervals. Sera and spleen cells were collected after the fifth immunization and analyzed for the presence of anti-Tn specific antibodies.

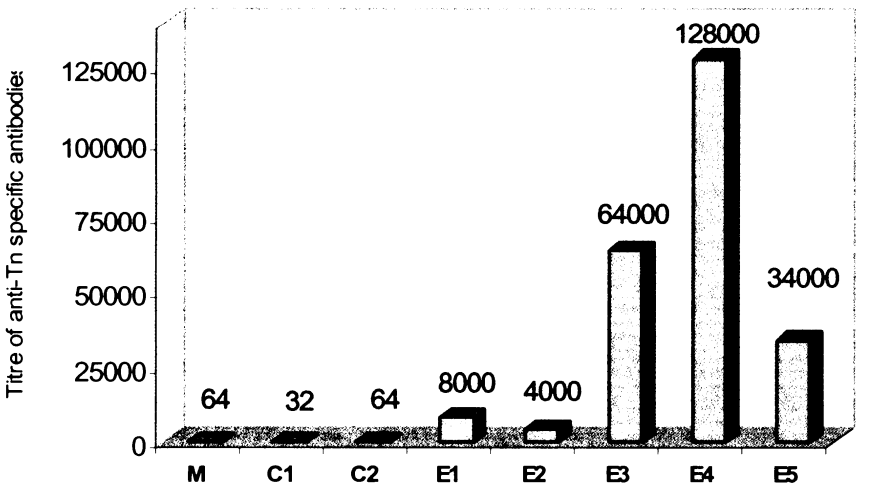


Fig. 17 Anti-Tn immune response to compounds **7** (C1,C2) and **8** (E1-E5); M represents a negative control, i.e. values for non-immunized mice serum.

Immunized mice did not exhibit any sign of adverse reaction to the administered conjugates. In all animals immunized with **8,** a remarkable increase in the level of anti-Tn (titre 2000-64000) and no change of anti-A levels (titre 8) was found. Neither non-immune nor immune sera showed any reactivity with T+, Cad+ and blood group O erythrocytes.

Conclusions:

We proved good surface accessibility of Tn antigen in all Tn-bearing immobilized MAGs (compounds **2**, **4**, **6**, **8**). Compound **4** was found to be the best candidate for the application in affinity purification of anti-Tn antibodies, other compounds were found to be a little less effective. Compound **8** (octameric MAG with γ-Abu insert) was found to be effective in modulation of adaptive immunity in mice (production of anti-Tn specific antibodies).

3.3.2 Comb-like multiple glycopeptide antigens

*Note: Comb-like multiple antigens were tested in completely different systems, therefore their potencies cannot be directly compared to results obtained with MAGs attached to solid support. The reasons were the unavailability of compounds **1-8** and change of the partner's laboratory where these tests were performed. Comparative study of both structural types is necessary to obtain directly comparable data. Such a study will be a part of the currently prepared project.*

3.3.2.1 *In vitro* study: Binding affinity to plant lectins

Binding properties of compounds **9-11** (Fig. 14 and Tab. 13) were first evaluated using four commercially available plant lectins (Sigma-Aldrich) with known specificity for D-GalNAc residue (*Bandeiraea simplicifolia*: α-D-Gal (major affinity), α-D-GalNAc (secondary affinity), *Codium fragile*: α-D-GalNAc (Tn) at the terminal reducing or non-reducing end, *Dolichos biflorus*: α-D-GalNAc (Tn) at the terminal non-reducing end, *Vicia villosa* B4: α-D-GalNAc at the terminal reducing end). This test was used to obtain the very first information about the accessibility and discriminability of Tn antigen or cluster of Tn antigen in the novel class of compounds without significant financial demand.

The results of ELISA binding assays (Fig. 18) demonstrate that the binding of compounds **9-11** was 10-10^5 times more effective than that of standard carbohydrate inhibitor D-GalNAc. Moreover, individual lectins differed in their ability to discriminate between tested dendrimers. *Bandeiraea simplicifolia* and *Dolichos biflorus* lectins (Fig. 18A and C) showed no or negligible discrimination. Some ability to differentiate among the tested compounds was found for the *Vicia villosa* lectin (Fig. 18D). This lectin discriminated between **9** and **10**; compound **11** showed the same binding properties as compound **10.** The *Codium fragile* lectin (Fig. 18B) differentiated among all three tested dendrimers and also showed the strongest binding affinity of all lectins tested. The ability of the latter two lectins to discriminate between the tested compounds was related to the density of carbohydrate coating, an effect that was most evident for compounds **9** and **10** (Fig. 18B and D). Altogether, the *Codium fragile* lectin displayed the best ability to discriminate between the tested compounds, and showed the highest difference between the binding of the

monosaccharide and the dendrimers (compound **11** was 10^5 times more effective than D-GalNAc monosaccharide).

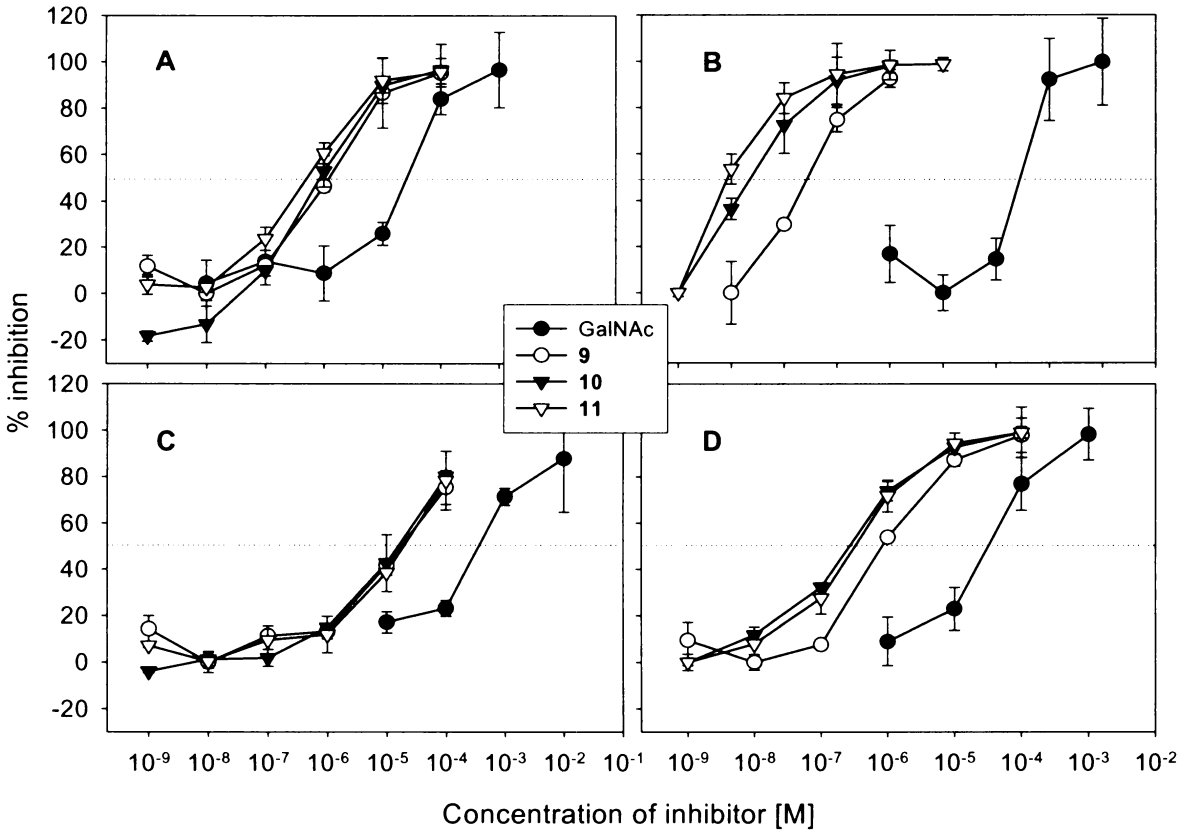


Fig. 18 Inhibitory activities of compounds **9-11** and D-GalNAc to inhibit binding of plant lectins to microtiter plates coated with dBSM. Plant lectins used: Bandeiraea simplicifolia (A), Codium fragile (B), Dolichos biflorus (C), and Vicia villosa B4 (D). Results are presented as average values +/- range from two experiments carried out in duplicates.

The results obtained for assays with the *Codium fragile* lectin correlate with previous findings about the ability of this lectin to bind α-D-GalNAc residue positioned on the reducing or non-reducing end of the saccharide moiety.[243] We assume that this is due to the structural complementarity of the Tn dimer and lectin-binding groove.[244] Similarly, our results obtained with the *Vicia villosa* lectin seem to agree with the affinities of Tn1-Tn3 glycopeptides measured previously using surface plasmon resonance; the affinity increased with increasing degree of substitution, but the increase was more dramatic from the mono-substituted to the di-substituted compound than from the di-substituted to the tri-substituted compound.[245]

3.3.2.2 *In vitro* study: Interaction with anti-Tn monoclonal antibody 83D4

To provide a preliminary biochemical assessment of the ability of compounds **9-11** to raise the production of anti-Tn antibodies, their abilities to be recognized by an anti-Tn MoAb in inhibition assay were tested. Compounds **9-11** were used as inhibitors of the binding of the anti-Tn monoclonal antibody 83D4[246] to plates coated with dOSM. The dependence of the inhibitory potencies of these glycodendrimers on the degree of sugar substitution was clearly proved (Fig. 19); while there has been very little inhibition by the monosaccharides in this experimental system, the dendrimers turned out to be efficient inhibitors with IC_{50} values ranging from 10^{-5} M for compound **9** up to 10^{-8} M for compound **11**. When compared to plant lectins, the MoAb 83D4 showed significantly better discriminability between compounds with different carbohydrate load.

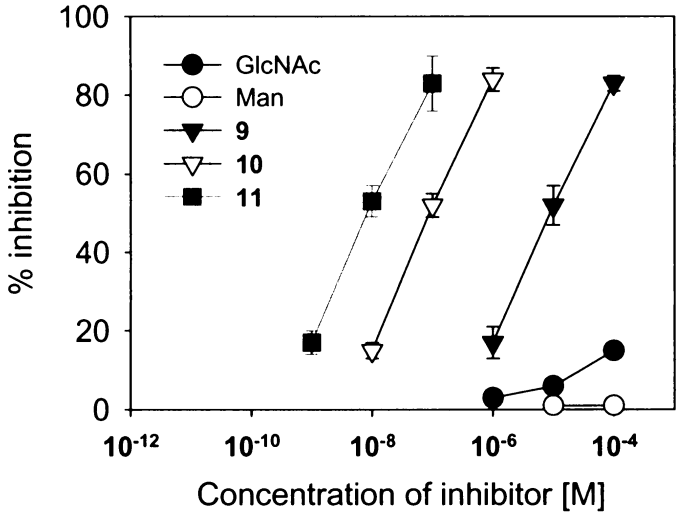


Fig. 19 Potencies of compounds **9-11** to inhibit interaction of monoclonal anti-Tn antibody 83D4 with dOSM-coated microtiter plates. GlcNAc and Man were used as a positive and a negative control, respectively. Results are presented as average values +/- range from duplicate experiments.

3.3.2.3 *In vitro* study: Interaction with NKR-P1A and NKR-P1B receptors and modulation of cytotoxic activity of NK cells

Major receptor of natural killer cells NKR-P1, which was shown to interact with D-mannose and D-glucose dendrimers,[247] is critical for the activation of cytotoxic tumour killing lymphocytes.[248] Comb-like dendrimers carrying α-D-GalNAc residue were evaluated as potential tools for modulation of the activity of NK cells via NKR-P1 receptor.

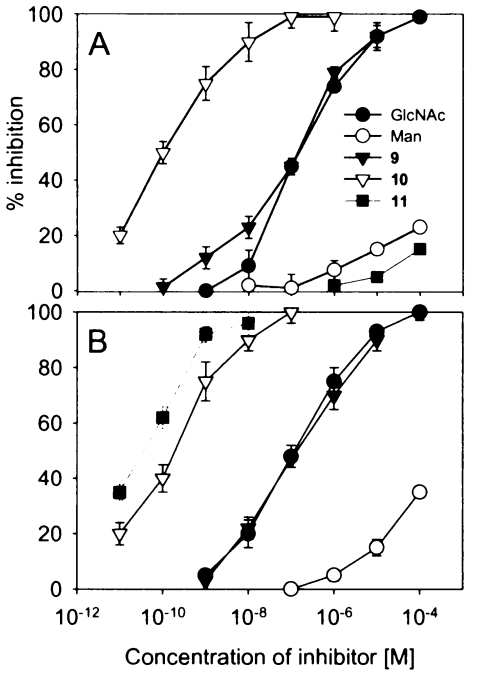


Fig. 20 Potencies of compounds **9-11** to inhibit interaction of two isoforms of the recombinant soluble rat NK cell receptors, NKR-P1A (A) and NKR-P1B (B) with dOSM-coated microtiter plates. GlcNAc and Man were used as a positive and a negative control, respectively. Results are presented as average values +/- range from duplicate experiments.

Compounds **9-11** were tested as the inhibitors of binding of two isoforms of rat NKR-P1, NKR-P1A (activating) and NKR-P1B (inhibitory), to their high affinity ligand, β-D-$GlcNAc_{23}BSA$ neoglycoprotein.[249] In assays with NKR-P1A, compound **9** had inhibitory activity comparable with the standard monosaccharide ligand, D-GlcNAc (Fig. 20A). However, compound **10** was much better inhibitor with IC_{50} as low as 10^{-10} M ranking this compound among the high affinity ligands for NKR-P1A. Notably, compound **11** was a very

poor inhibitor for NKR-P1A with inhibitory activity even weaker than the negative control, D-mannose (Fig. 20A). When similar tests were performed with rat NKR-P1B receptor, the results for compounds **9** and **10** were very similar to those obtained with the A isoform: compound **9** had activity comparable to D-GlcNAc monosaccharide, while compound **10** had a very high inhibitory activity (IC_{50} of 3×10^{-10} M). However, the binding of compound **11** to NKR-P1B was entirely different: not only there was specific binding, but the IC_{50} was about ten times lower than measured for compound **10** (IC_{50} of **11** = 3×10^{-11} M, see Fig. 20B). Compound **11** displayed a unique and selective affinity for NKR-P1B, which makes it one of the best ligands identified for NKR-P1B isoform to date.

These findings are very interesting especially due to the previously published binding specificities towards both simple and complex oligosaccharide structures that predicted poor interaction of these receptors with tested compounds. [250]

Since dendrimers **9** and **10** both had a high affinity for NKR-P1A and NKR-P1B receptors, a test of their ability to enhance cytotoxicity of NK cells *in vitro* was performed.[251] For this purpose, two rodent leukemic cell lines, an NK sensitive line (YAC-1) and NK resistant line (P815), were employed, Fig. 21. When compared to the control, compound **9** stimulated natural killing of both YAC-1 and P815 targets (Fig. 21A and D, respectively) in a dose-dependent manner, but the enhancement of natural killing was more profound in the case of the NK resistant P815 cell line (nearly three-fold increase in natural killing using 10^{-8} to 10^{-6} M concentration of compound **9** compared to control, Fig. 21D). The effect of compound **10** on natural killing was very similar, except that the activation potential was even higher (Fig. 21B and E) and there was a nearly four-fold increase in natural killing in the case of the NK resistant cell line P815 (Fig. 21E). As expected, the effect of compound **11** on natural killing was exactly opposite: this compound efficiently inhibited natural killing in both tested cell lines (Fig. 21C and F).

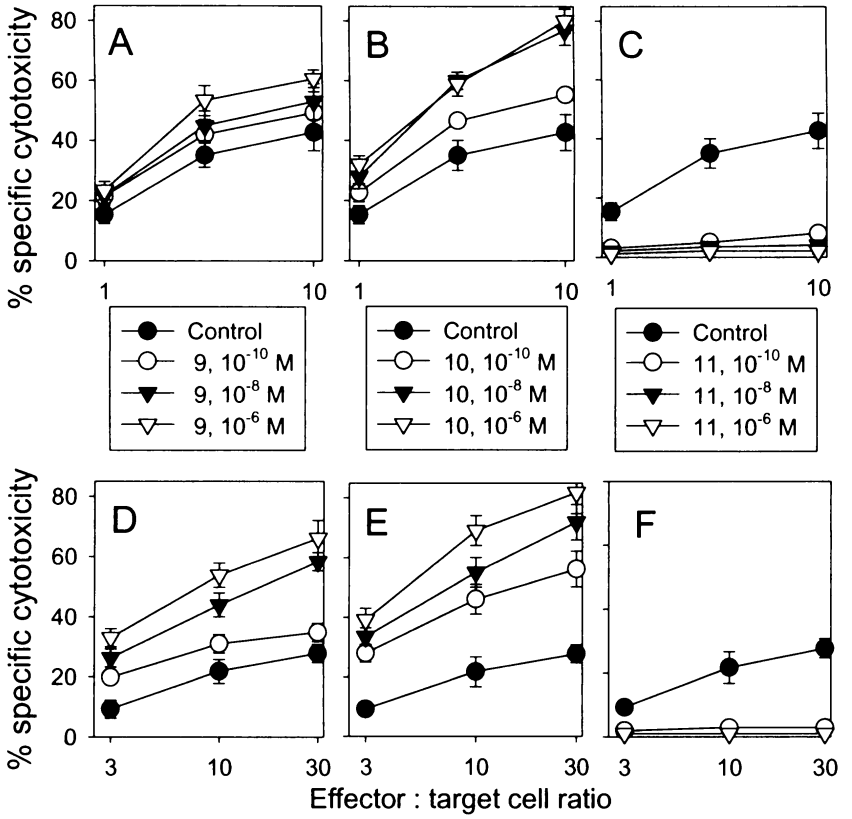


Fig. 21 Compounds **9** (panels A and D), and **10** (panels B and E) enhance, and compound **11** (panels C and F) inhibits natural killing of NK-sensitive tumour cell line YAC-1 (A-C), and NK-resistant tumour cell line P815 (D-F) by rat NK cells. Compounds were dissolved in PBS (pH 7.4), and tested at the indicated concentrations together with the control sample (PBS only) at the effector: target cell ratio given in each panel. As effector cells mononuclear cells isolated from spleen of Fischer F344 rats or human peripheral blood of were used.

Because of the different impacts our compounds had on natural killing, the effects of their combined applications were also investigated. The influence of individual compounds and their combinations on natural killing of NK resistant rat cell line P815 was tested at effector: target cell ratio 30: 1 using 10^{-8} M concentrations. It is evident that the inhibitory effect of compound **11** is predominant (Fig. 22A). This dendrimer inhibited natural killing efficiently in the presence of **9** and **10** at equimolar concentrations. Furthermore, in order to evaluate the immunological effects of the dendrimers from the standpoint of their potential use in human therapies, the latter test was repeated using the human NK resistant cell line RAJI, and very similar results were obtained for this human cell line (Fig. 22B).

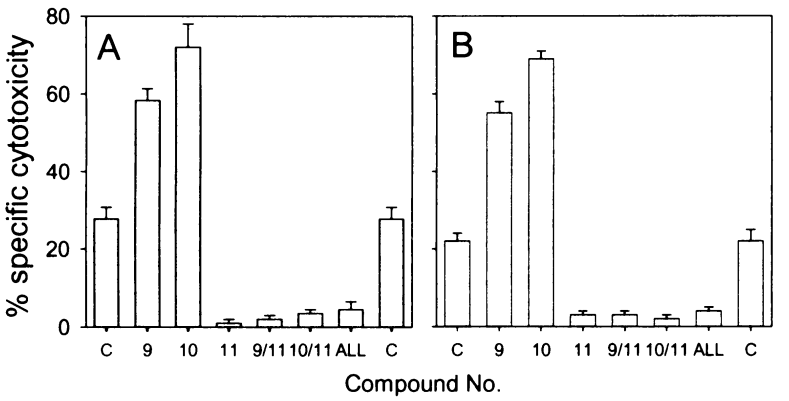


Fig. 22 Combined effects of compounds **9-11** on NK resistant mouse tumour cell line P815 (A) and human tumour cell line RAJI (B) at effector: target cell ratio 30: 1. Compounds were tested at 10^{-8} M concentrations. ALL means compounds **9-11**. As effector cells mononuclear cells isolated from spleen of Fischer F344 rats or human peripheral blood of were used.

Activation of innate immune responses, especially by T-cells, NK/T-cells, and NK cells, is of importance for permanent protection against cancer. Compounds **9-10** exerted significant activation potency for the killer lymphocytes as tested in the short-time cytotoxicity assays that measure mostly the activities of NK cells. The ability of these compounds to activate natural killing of the resistant tumour cell lines in both rodents and humans (P815 and RAJI, respectively - Fig. 22) is of particular importance. While the molecular mechanism for this activation in rodents and in humans may differ (in humans, the NKR-P1 is not an activating NK cell receptor, and the comb-like dendrimers tested here did not react with human NKR-P1; data not shown), these results point to the potential of the compounds **9-11** not only for use in animal experimental therapy models, but also for use in human cancer therapies. These findings open new possibilities for biological testing of this compound *in vivo*, which is interesting in light of the previously reported significant anti-tumour properties of GlcNAc-coated polyamidoamide dendrimers.[247] We also expect that combination of the effective humoral immune response with induction of NK cell activity, as demonstrated for **10**, should result in an effective antitumour immune response *in vivo*.

Our unique findings regarding the compound **11** (selective and strong interaction with the inhibitory receptor NKR-P1B and efficient inhibition of natural killing in rodent and human cells) open new avenues in the development of effective inhibitors of NK and NK/T cells in pathological situations connected with overactivation of these cells. In particular, recent findings demonstrate that iVα14 NK/T cells are responsible for allergen-induced airway inflammation,[252] a cardinal feature of allergic asthma.[253] Thus, the effective inhibition of NK cells by **11** may be exploited under these, and potentially other, pathological conditions.

3.3.2.4 *In vivo* study: Production of anti-Tn specific antibodies

Compounds **9-15** were tested for their ability to elicit humoral immune response in Balb/c mice models. Female Balb/c mice (eight weeks old) were treated with two doses of synthetic vaccines (100 μg/dose at 2-week intervals) adsorbed on 1 mg of aluminium hydroxide per dose as an adjuvant.

Modification of parent compounds **9-11** by replacing the N-terminal acetyl groups on the antigen chains for DNP group (**12-14**), or by incorporation of T-cell epitope and creation of chimeric B-T antigen (**15**) allowed the assessment of the role of three structural and functional characteristics: (i) the effect of the growing saccharide density (mono-, di- or tri-Tn cluster), (ii) the effect of additional hapten (DNP), and (iii) the effect of T-cell epitope (HA306-318, derived from Influenzae virus hemagglutinin).

3.3.2.4.1 Evaluation of the effect of the growing saccharide density

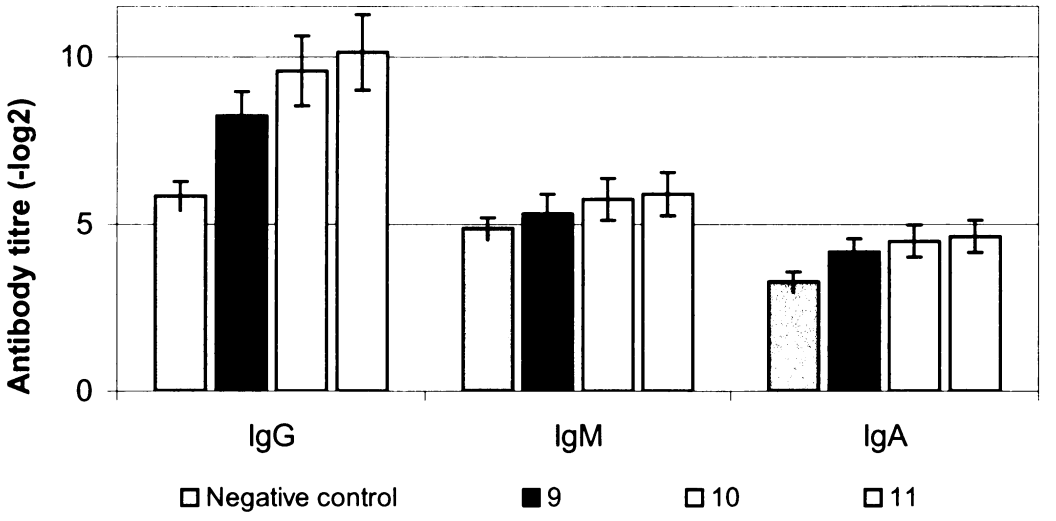


Fig. 23 The effect of growing saccharide density on the level of anti-Tn antibodies raised by comb-like multiple glycopeptide antigens with monomer, i.e. 4 copies (**9**), dimer, i.e. 8 copies (**10**) or trimer, i.e. 12 copies (**11**) of Tn antigen (5 eq. to dilution 1/32, 10 eq. to dilution 1/1024).

Effect of increasing Tn antigen "load" on immune response in Balb/c mice injected with compounds **9-11** is given in Fig. 23. Compounds **9-11** elicited anti-Tn IgG antibody production with antibody titre increasing with increasing number of Tn antigens in molecule.

The most significant effect is observed with IgG class of antibodies. Compounds **9-11** elicited anti-Tn IgG antibody production: with the highest titre for **11**: 1024 (-log2 = 10.12). These compounds also elicited low titres of anti-Tn IgM and IgA antibodies, but this production cannot be taken as significant.

These results corroborate previously published findings about the effectiveness of clustered Tn or sTn antigens to be recognized by MoAbs such as MLS-128 (anti-Tn) that preferably recognize cluster of three or four Tn antigens[175] or B72.3 (anti-sTn) that recognize cluster of three sTn antigens.[254]

3.3.2.4.2 Evaluation of the effect of additional hapten molecule

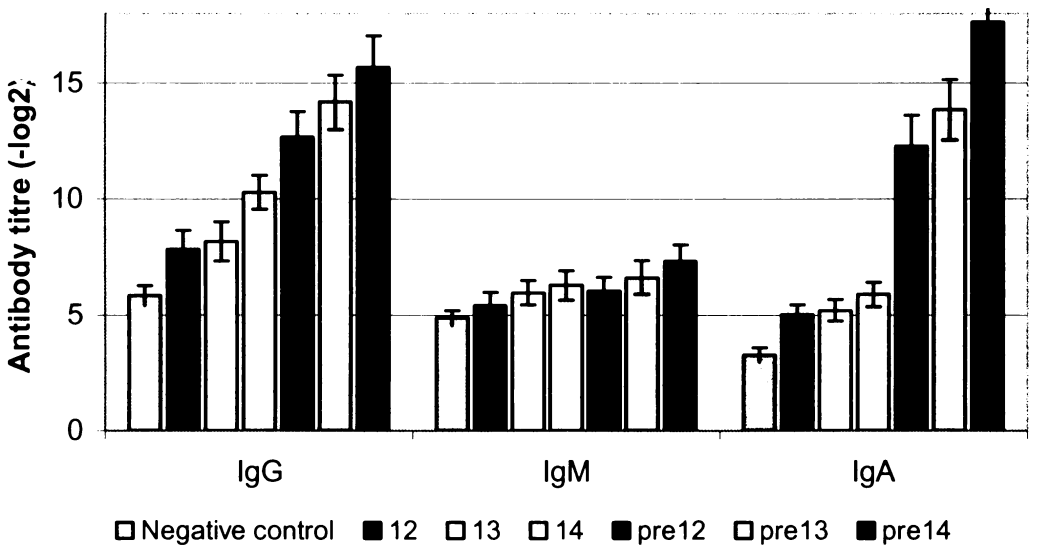


Fig. 24 The effect haptenization of the parent compounds on the level of anti-Tn antibodies (compounds **12-14**) and the effect of sensitization (preimmunization) on the level of anti-Tn antibodies (**pre12-pre14**); mice were sensitized (preimmunized) with five doses of 0.15% DNP in PBS at week intervals before performing the standard immunisation scheme, (5 eq. to dilution 1/32, 10 eq. to dilution 1/1024, 15 eq. to dilution 1/32768).

The effect of the incorporation of small hapten on the magnitude of anti-Tn antibody production and the effect of the preimmunization with DNP alone on anti-Tn antibody production is shown in Fig. 24. The rationale for this approach (DNP haptenization of parent molecule) is the well-established observation that immunization with a hapten-modified protein can induce an immune response to the unmodified protein even when that protein is a normal self antigen and thus tolerated by the immune system.[255]

Results obtained with compounds **12-14**, again, corroborate previous findings about the effect of increased Tn antigen "load" on the antibody production. Compound **14** (DNP analogue of **11**) showed the highest potency to elicit anti-Tn antibody production in order of magnitude equal to compound **11**.

The preimmunization of mice with DNP had a significant impact on the antibody production. There was a considerable increase of anti-Tn antibody titre of IgG class: for compound **14** from 1260 (-log2 = 10.3) to 52136 (-log2 = 15.67), i.e. approx. 40-fold increase.

The results also revealed a key effect of the preimmunization on the production of anti-Tn antibody of IgA class. Mice immunized with compound **14** showed negligible production of IgA class antibodies. However, when these animals were preimmunized with DNP there was a tremendous increase in the production of IgA class anti-Tn antibodies: for compound **14** from 59 (-log2 = 5.89) to 201440 (-log2 = 17.62), i.e. approx. 3400-fold increase.

3.3.2.4.3 Evaluation of the effect of the T-cell epitope

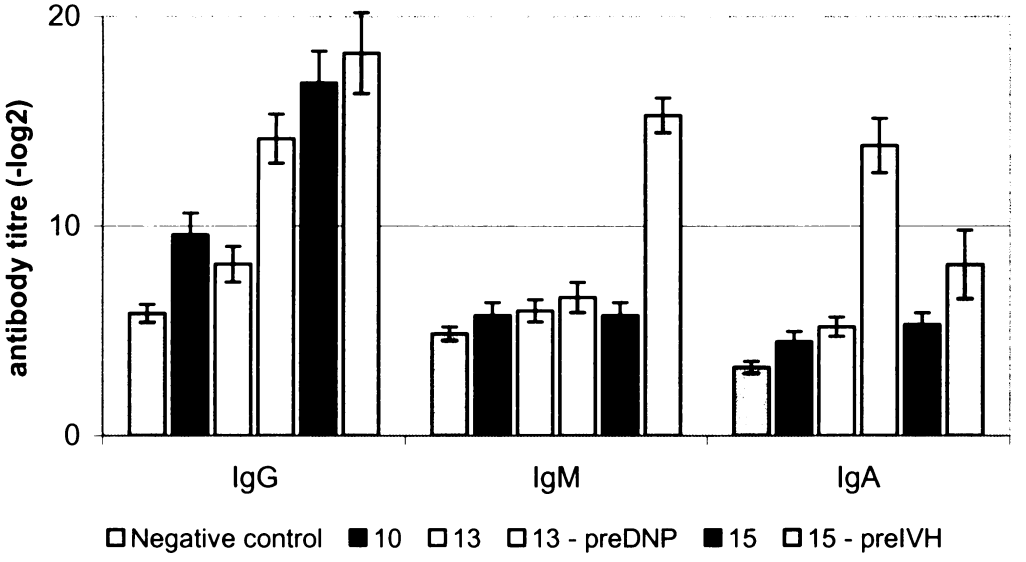


Fig. 25 The effect of T-cell peptide epitope raised by compounds with Tn dimer (**10**, **13**, **15**) and the effect of sensitization on the level of anti-Tn antibodies (**pre13**, **pre15**), for DNP sensitization see Fig. 24, IVH sensitization: two doses of 50µl of commercial influenza virus vaccine at two-week interval; (10 eq. to dilution 1/1024, 15 eq. to dilution 1/32768, and 20 eq. to dilution 1/1048576).

The effect of T-cell peptide epitope (IVH) vs. additional haptenization by DNP on the production and antibody class profile is given in Fig. 25. Incorporation of either DNP or T-

cell epitope had a positive effect on anti-Tn antibodies of IgG class production: for compound **15**: 116500 (-log2 = 16.83). Preimmunization with IVH also significantly increased the magnitude of the antibody titre and corroborated the results obtained with compounds **12-14**: compound **13**: 228 (-log2 = 8.17) to 18305 (-log2 = 14.16) i.e. 80-fold increase, compound **15**: 116500 (-log2 = 16.83) to 313900 (-log2 = 18.26) i.e. 2.7-fold increase.

Fluorescence-activated cell sorting (FACS)[257] was performed to demonstrate antibody binding to the cell surface of Jurkat cell line, a human T lymphoid leukaemia cell line known to express Tn, Tab. 15.[258]

Tab. 15 Flow cytometric analysis of anti-Tn antibodies (raised using compounds **9-15**) bound to surface of Tn positive Jurkat cells (taken from ref. 255).

Vaccination antigen	**Vaccination antigen**	**Anti-Tn antibodies FACS (% of gated cells)**			
		IgG (x±SD)		**IgM (x±SD)**	
		Pretreatment	**Posttreatment**	**Pretreatment**	**Posttreatment**
-	-	10.2±3.21	9.7±2.43	9.2±1.81	10.1±3.48
-	DNP	9.9±2.84	11.4±3.08	10.9±2.40	9.2±3.52
-	IVH	10.6±2.93	8.9±3.87	10.5±4.12	11.7±3.35
9	-	11.8±4.27	17.9±4.10	10.33±2.4	23.1±6.19
10	-	9.8±3.57	29.3±7.38	10.4±2.15	28.9±8.33
11	-	10.4±2.11	25.8±6.87	9.63±2.19	27.1±7.73
12	-	10.7±3.80	22.4±8.41	11.0±1.97	26.0 ±6.58
13	-	10.1±2.21	27.7±7.74	9.7±3.15	31.8±9.83
14	-	9.3±2.04	28.6±7.12	10.5±3.25	33.9±10.22
12	DNP	10.0±3.77	35.2±7.83	11.4±2.32	41.3±9.44
13	DNP	10.1±2.07	38.4±12.25	10.7±2.95	44.8±11.75
14	DNP	9.75±2.02	33.6±8.96	11.0±1.94	43.4±11.55
15	-	10.5±2.67	35.8±9.62	10.7±1.88	25.3±7.20
15	IVH	9.2±2.93	38.9±9.36	10.6±3.06	30.8±6.88

In all cases, elicited anti-Tn IgG and IgM antibodies recognized Tn structures on Jurkat cells. Cell staining capacity was generally higher for IgM anti-Tn antibodies, and further increased in the animals pre-sensitized with DNP/IVH and immunized with DNP/IVH-

conjugated Tn antigen. These results corroborate previous finding about the ability of comb-like compounds to elicit anti-Tn specific humoral response.

Conclusions from *in vitro, ex vivo* and *in vivo* tests

In vitro studies showed selective binding of compound **11**to NKR-P1B receptor and this compound has been identified as the most potent ligand so far. No binding to NKR-P1A receptor was observed.

Ex vivo tests showed that compounds **9** and **10** can effectively activate cytotoxic activity of NK cells in both NK-sensitive and NK-resistant cell lines. Tests with compound **11** confirmed *in vitro* results: this compound effectively inhibited cytotoxic activity of NK cell even in the presence of activatory ligands (compounds **9** and **10**).

In vivo studies identified compound **15** containing T-cell epitope as the most perspective candidate for further development of synthetic vaccine. In addition, this study also showed the importance (effect) of the preimmunization (with DNP or IVH) on the further increase of antibody titre (IgG) and induction of the production of another class of anti-Tn specific antibodies (IgA and IgM).

3.4 STRUCTURAL CHARACTERIZATION OF GLYCODENDRIMERS

Results are detailed in **Vepřek P.**, Ježek J., Trnka T. and Vondrášek J., Molecular dynamics study of the effect of the γ-Abu insert on the conformational behavior of the glycopeptide dendrimers based on the oligolysine scaffold in N, N'-dimethylformamide. *J. Biomol. Struct. Dyn.* **2004**, 22, 79-90.

As mentioned in chapter **3.1.1**, during the preparation of soluble MAGs serious coupling difficulties were observed. Modification of synthetic protocol did not improve the situation and thus this approach failed to provide target compounds. To find out the reason for the synthetic difficulties and to better understand the nature of this unexpected behaviour, a MD study of the behaviour of the growing glycodendrimer during the synthesis was undertaken.

Two effects were evaluated in the study: **i**) the effect of the γ-Abu insert; as backfolding of relatively long aliphatic chain that might make unreacted amino groups inaccessible was suspected, and **ii**) the effect of the Tn antigen to evaluate steric hindrance effect of the bulky side-chain.

MD simulations in DMF using Gromacs software package provided geometrical parameters to the problem of dendrimer flexibility during the synthesis. From these calculations, parameters and descriptors showing the direct influence of the length of the dendrimer branch on the spatial distribution of particular termini during the simulation were extracted: **i**) changes of distances among free and/or substituted N termini and a resin anchor (C atom of resin); **ii**) changes of distances among free and/or substituted N termini where-as one of the termini was a reference point; **iii**) radius of gyration; **iv**) mass density plot for the fully equilibrated dendrimers. The colours of the individual geometrical parameters during the MD simulations were utilized uniformly throughout all presented graphs as depicted in Fig. 26. Structures taken into MD were modelled on assumption that coupling is a sequential process. Under this assumption two rules were applied: **i**) preferential coupling to the α-NH_2 groups, **ii**) the most distant free amino terminus of the energetically minimized structure of the growing dendrimer as the most likely site of attachment, Fig. 27.

Data obtained allowed further analysis: i) inserted vs. non-inserted structures, Fig. 28, and **ii**) structures containing amino acid serine with hydroxyl side-chain occupied by the protected Tn antigen vs. a simple protecting *tert*-butyl group, Fig. 29. These results are further corroborated by data about the radius of gyration (Fig. 30) and mass density plot (Fig. 31).

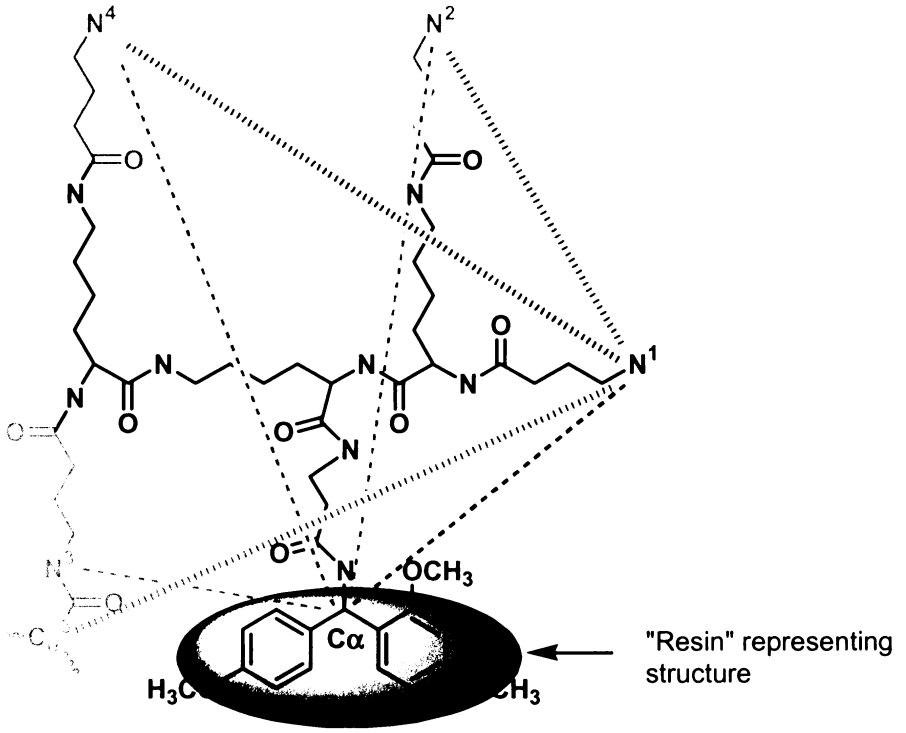


Fig. 26 Scheme of the DEND2_1EA structure (intermediate structure with N^3 amino terminus substituted) with marked interatomic distances used for the conformational characterization of the growing peptide-dendrimer over the span of 2ns.

--- line used for the characterization of distance between N termini or corresponding C_α atoms (in this figure C_α^3 atom to reflect the structural change i.e. attachment of protected amino acid) and C_α atom (1^{st} and 3^{rd} row in Fig. 28 and Fig. 29),

"""" line used for the characterization of distance between N_1 and N_2, N_3, N_4 termini, respectively, or respective C_α^x atoms), (2^{nd} and 4^{th} row in Fig. 28 and Fig. 29). Colours correspond with the colours of lines used in following figures.

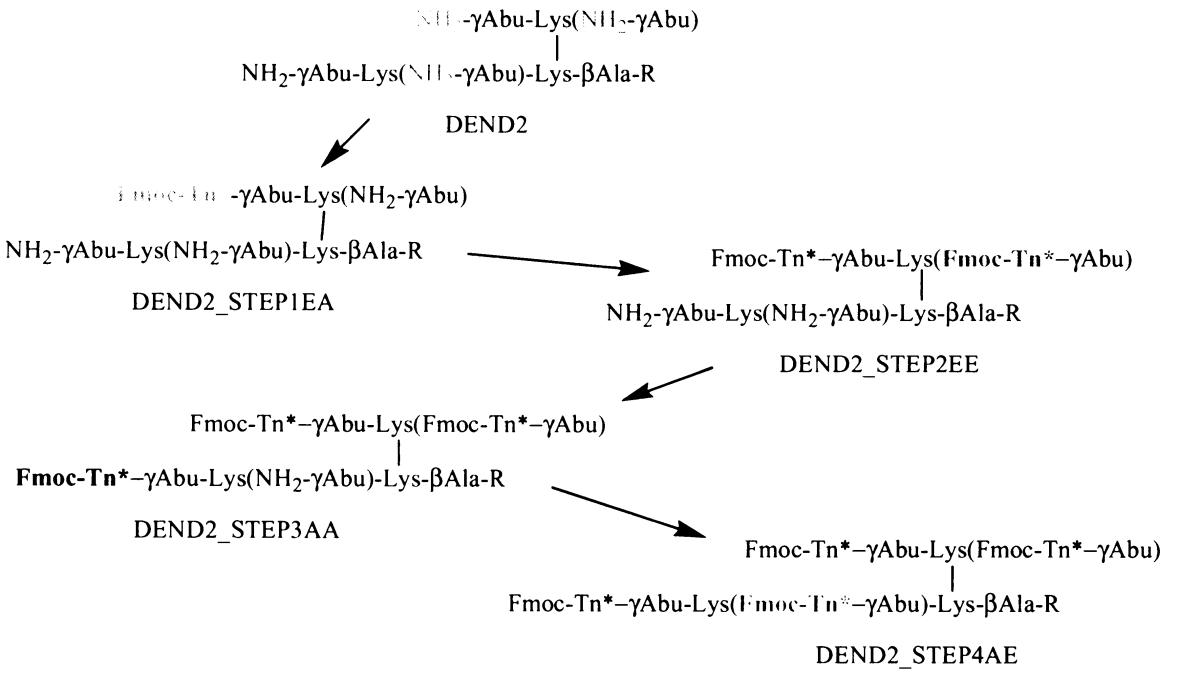


Fig. 27 Scheme of the structures taken in to the MD calculations. Colours correspond with the colours of lines used in following figures.

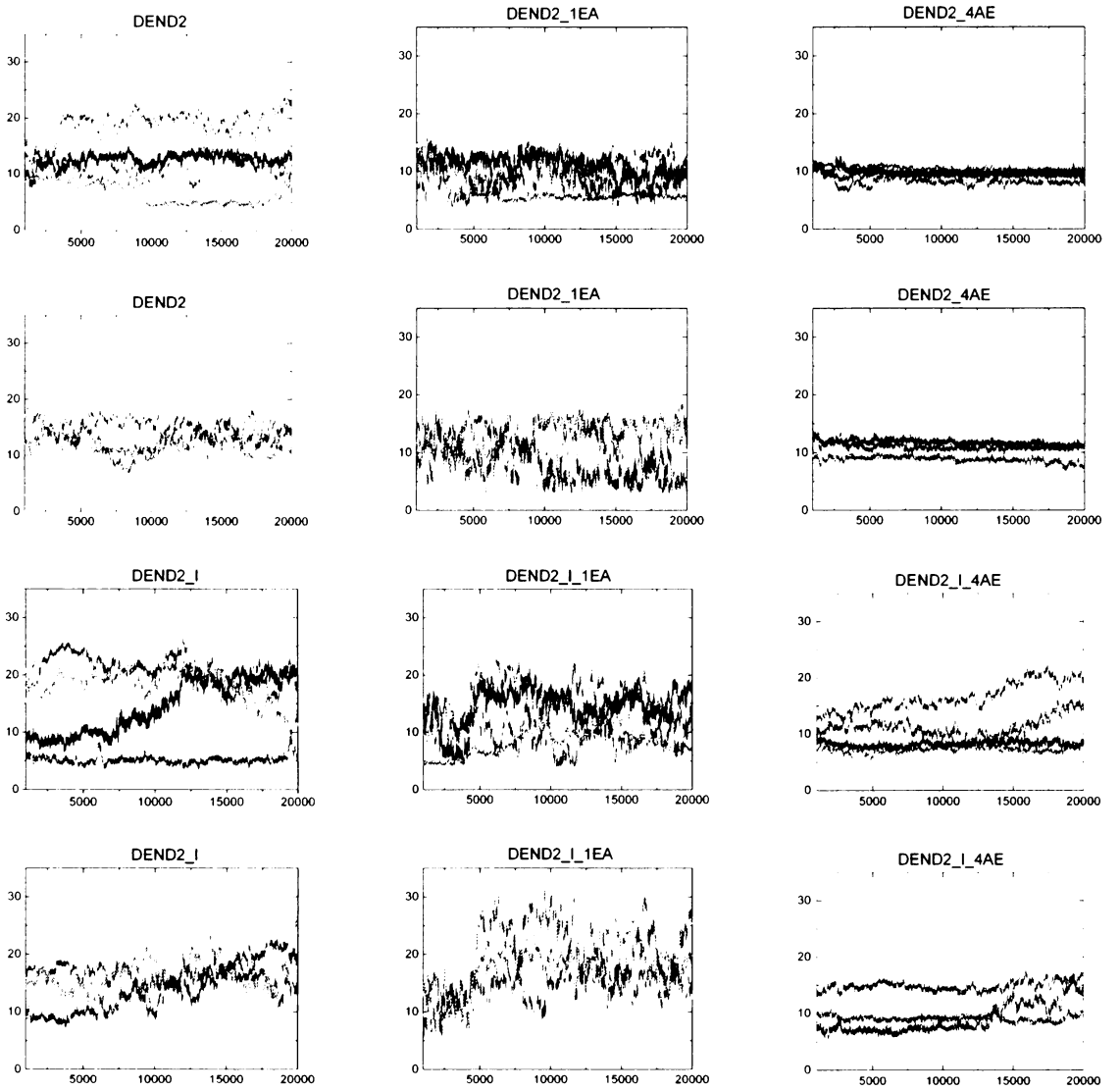


Fig. 28 Time course of the development of the interatomic distances between the N-termini and C_α - (the first and third rows) and distances between the N-termini (second and fourth rows) for the non-inserted DEND2 and the inserted DEND2_I structures (i.e. with γ-Abu insert, for illustration see Fig. 13), respectively.

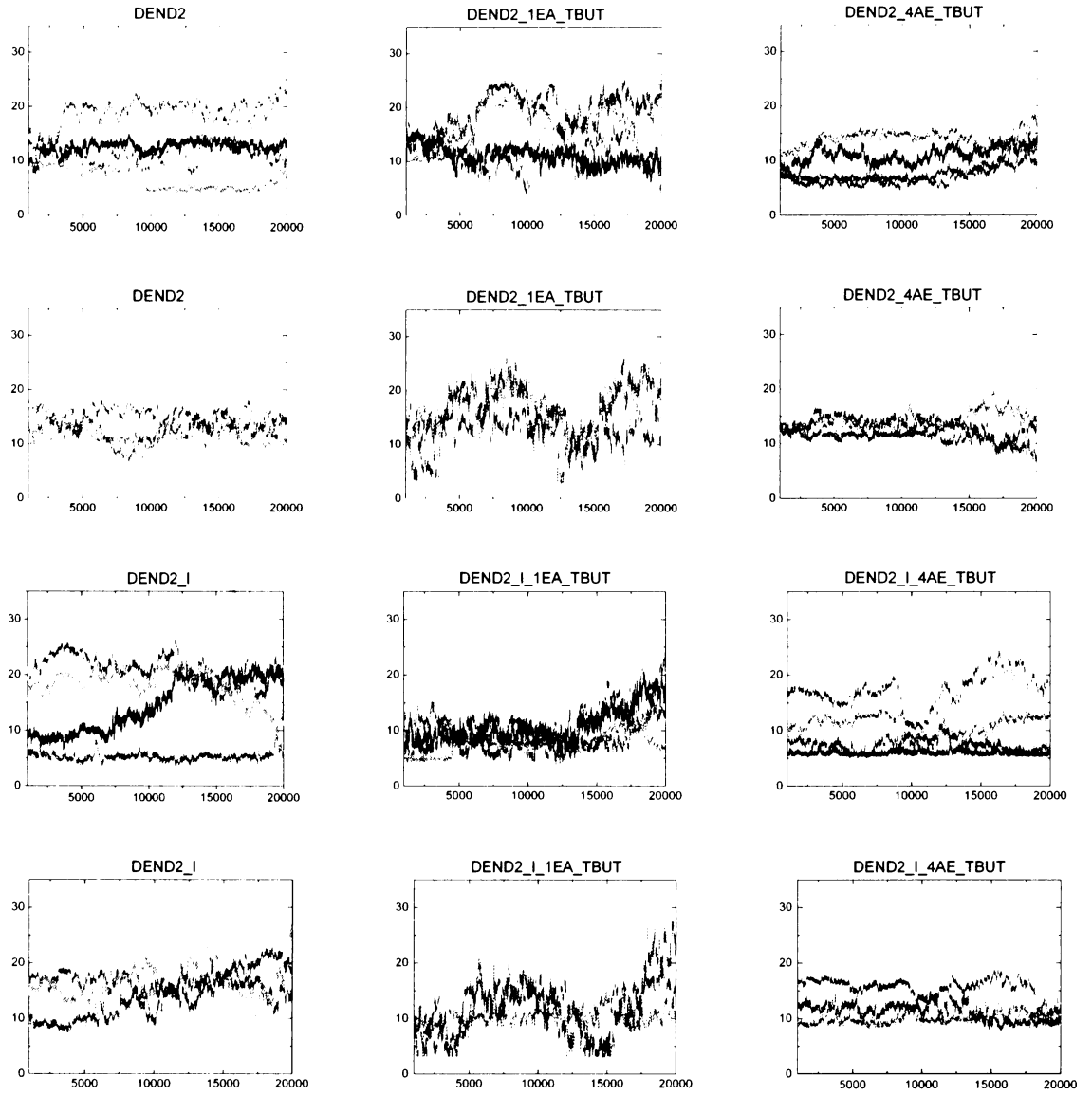


Fig. 29 Time course of the development of the interatomic distances between the N-termini and C_α - (the first and third rows) and distances between the N-termini (second and fourth rows) for the non-inserted DEND2 and the inserted DEND2_I structures, respectively, Fmoc-Ser(tBu)-OH was used instead of Fmoc-Ser(3,4,6-tri-*O*-Ac-α-D-GalNAc)-OH to evaluate the effect of bulky protected saccharide side-chain-

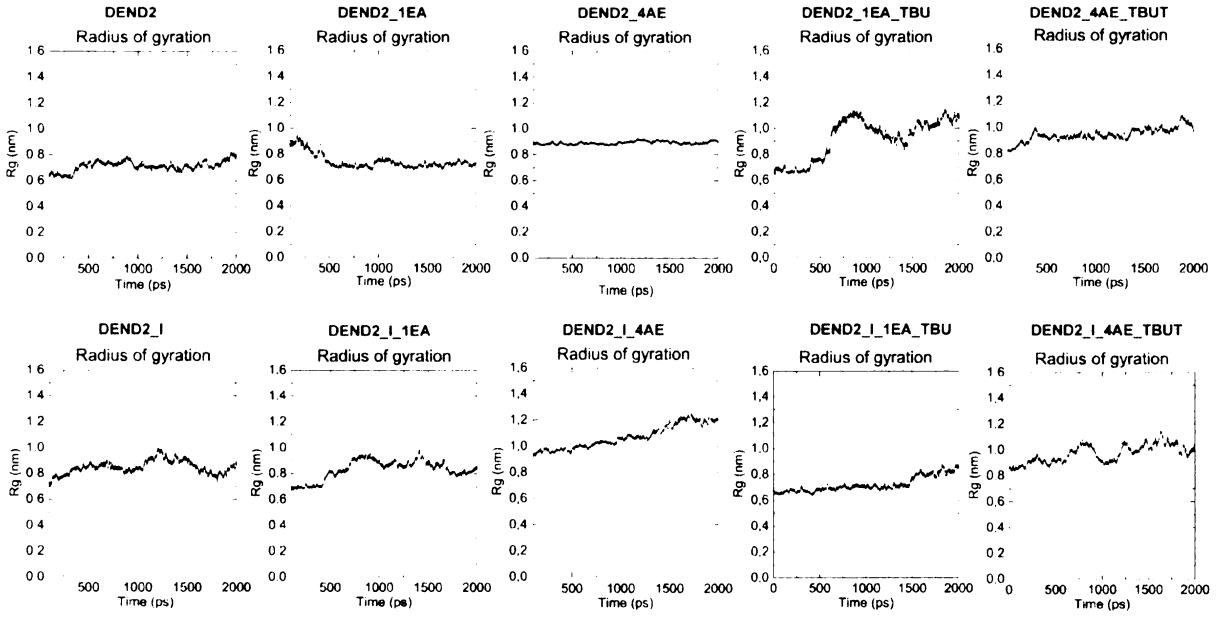


Fig. 30 Time course of the radius of gyration for modelled structures.

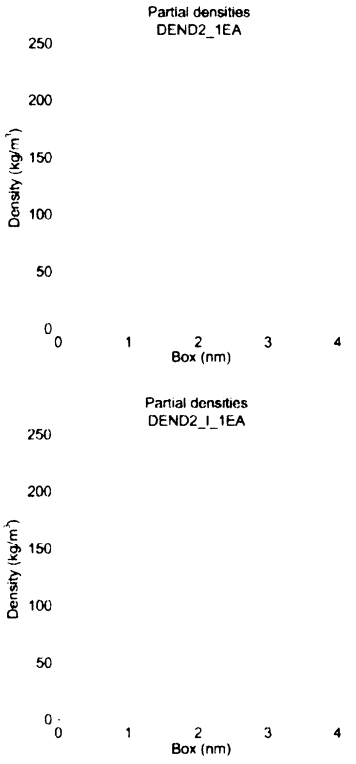


Fig. 31 Mass density plots of fully equilibrated structures.

Results of the MD calculations

DEND2_I structure is more flexible than DEND2 (Fig. 28, first column, first vs. third row). This is not surprising considering the length and character of the γ-Abu insert (approximately 6Å and free rotation of C-C bonds of this insert).

Substantial spatial rearrangement of the molecule takes place after the first substituent is introduced (Fig. 28, second column). Branches of non-inserted dendrimer (DEND2_1EA) show tendency to increase their conformational variability among N termini. The behaviour of the inserted ones (DEND2_I_1EA) can be characterized as shrinking because termini are fluctuating mainly towards to the C_α (where back-folding takes place), see also Fig. 31. Their N termini, on average, are closer to the resin anchor, have a tendency to keep the introduced compactness and move in a concerted way, compared to their non-inserted counterparts.

The substitution of all four N termini makes a great difference between non-substituted and fully substituted molecules in each family (Fig. 28, first vs. third column). The effect on the overall compactness is stronger for the non-inserted species than for those with the insert.

The whole effect is more obvious when looking at the radius of gyration, Fig. 30. They show that the very first substitution is very likely the crucial step for the following dendrimer modification in terms of its synthetic feasibility.

The replacement of the saccharide moiety by the *tert*-butyl group confirms the influence of the extension on the overall flexibility of the particular arms (Fig. 29). The saccharide plays an important role in the stability and maintaining compactness of the dendritic molecules. It is obvious that for fully extended dendrimers the effect of the Fmoc groups alone is not sufficient enough to maintain tight arrangement.

Dendrimers with the γ-Abu insert show a tendency to back-fold their extended termini into the interior (or core) of the dendrimer. The influence of the hydrogen bonds between O and N atoms of the lysine amino acids is negligible for the overall compactness of molecules because no principal hydrogen bond persists during the simulation. On the contrary, some of the polar atoms of lysines establish temporary hydrogen bonds with saccharide moieties. The formation of these bonds has an important effect on the compactness and we assume that the nature of the solvent also plays an important role in the orientation of the extended arms back to the core.

Calculation of the density of the groups (their masses were taken into account) gives a plot of density against the principal axes connected with the system. It is obvious that the mass distribution shown in Fig. 31 agrees with the above conclusions. The non-inserted species has lower density than the inserted ones and the density is preferably distributed towards termini. The maximum of density is located 1.5 -2.5 Å from the origin. Dendrimers with the insert show a significant peak between 1.0 - 1.5 Å and they are more compact. It confirms the change in behaviour between these two molecules after the first extension takes place. The shrinking tendency is obvious.

Conclusions from the MD calculations

Results of the MD study corroborate previously obtained experimental data and confirmed the negative effect of the γ-Abu residue on the spatial behaviour of the growing molecule. Instead of loosening the structure and making amino termini more accessible, the structures behave in opposite fashion (green and blue coloured lines should be the most distant). On the other hand, preliminary results of MD study with inserts with constrained flexibility show that incorporation of such inserts might improve not only the synthetic feasibility but also other properties important for targeting dendrimers (*data not shown*).[259]

4. CONCLUSIONS

The aim of my thesis was to prepare an "ideal" structure based on Tn antigen that would enable modulation of both adaptive and innate immunity. Tn antigen was selected because it is presented on the surface of tumour cells (as one of the tumour associated carbohydrate antigens) and because N-acetylhexosamines and their glycoconjugates were shown to modulate cytotoxic activity of natural killer cells.

To summarize:

Four types of immobilized MAGs were designed and successfully prepared:

- they showed high interaction potential with anti-Tn monoclonal antibodies
- they induced formation of high titre antisera specific to Tn antigen

Soluble MAGs of the same structure were not prepared and tested. The reason for it was partially disclosed by MD calculations. It showed that insertion of γ-Abu substantially (negatively) affected spatial behaviour of amino ends of individual branches of the growing molecule that hindered and prevented further "growth" on individual branches and thus preparation of target structures.

This situation resulted in the design and successful preparation of seven types of soluble comblike multiple antigenic glycopeptides. They differed by the number of Tn antigen on particular branch: 1, 2, or 3 groups for a branch, by an additional incorporation of a hapten group (DNP) or incorporation of a T-cell epitope. We proved that Tn antigen on this novel structural type of a carrier:

- was recognized by plant lectins giving the positive answer on the principal question about the steric accessibility of the presented Tn epitope in various arrangement on this dendrimeric structure
- was recognized by monoclonal anti-Tn antibody 83D4
- was recognized NKR-P1A and NKR-P1B receptors and showed unique and selective binding (compound **11**)
- effectively modulated cytotoxic activity of NK cells
- induced formation of high titre antisera specific to Tn antigen

4.1 FURTHER PERSPECTIVES OF THIS PROJECT

Original and promising results obtained on the modulation of adaptive immunity in mice model with compounds **13** and especially **15** as models of synthetic vaccine show that designed structures are perspective for further development as synthetic vaccine. In comparison with results published on synthetic vaccines with Tn antigen,[174, 176] this concept is advantageous because it offers higher flexibility for optimalization and tailoring of the immunogenic properties including the incorporation of safe adjuvant. In this respect the role of preimmunization on the production of IgM, IgG and IgA antibodies should be also clarified because obtained data indicate the immune system in the state of alertness give complex and stronger response. As a safe adjuvant we plan to use muramyldipeptide (MDP) analogues with minimized side-effects that are currently developed by RNDr. M. Ledvina's group at the IOCB. These structures are perspective because some of the analogues of MDP have been introduced into the clinical practice e.g. Romurtid™ and Likopid™, and hence the introduction of another analogue with better immunopharmacological profile would be less problematic.

Encouraging results obtained on the modulation of cytotoxic activity of NK cells via NKR-P1A and NKR-P1B receptors show that glycodendrimers might be selective ligands for individual receptors. Unfortunately, not enough data has been cumulated so far. Therefore, the extensive study focusing on the elucidation of the effect of spatial orientation and number and character of N-acetyl-D-hexosamine residues, and the effect of their clustering on the activation/inhibitory properties of these glycoclusters is necessary.

We also plan to use glycodendrimers for the preparation of mimetics of naturally occurring branched oligosaccharides. As, for example, in case of CD69 receptor we plan to continue in the work of A. Kovalová, Ph.D.[260] and use glycodendrimers bearing simple linear and branched oligosaccharides that she prepared to mimic natural pentaantenary oligosaccharide ligand derived from ovomucoid.[261]

4.2 POSTERS AND PUBLICATIONS

Most of the results have been presented at both domestic and international symposia and in recognized international journals. Following is the list of my work related to the subject of my thesis:

Related posters:

Ježek J., Velek J. , **Vepřek P.**, Vondrášek J., Velková V., Trnka T., Pecka J., Písačka M.; Multiple Antigenic Glycopeptide Carying Tn Antigens Structure-Activity Correlations. 25th European Peptide Symposium, August 30 – September 4, **1998**, Budapest, Hungary.

Velek J., Ježek J., **Vepřek P.**, Velková V., Trnka T., Pecka J., Písačka M., Synthetic Glycopeptide Dendrimers with Tn Antigenic Structure: Immunological Study II, p. 405-406, in Innovation and Perspectives in Solid Phase Synthesis & Combinatorial Libraries, London 2-6 Sept. 1997, ed. R. Epton , Mayflower Scientific Ltd., Birmingham, **1999**.

Ježek J., Velek J., **Vepřek P.**, Vondrášek J., Velová V., Trnka T., Pecka J., Písačka M., Stucture-activity correlations of multiple antigenic glykopeptides carrying Tn antigens. Cukrblik, 10 March, **1999**, Prague, Czech Republic.

Sejbal J., Vondrášek J., Velek J., **Vepřek P.**, Trnka T., Ježek J. Computational study of multiple antigenic glycopeptides (MAGs) with Tn antigens. 26th European Peptide Symposium, September 10-15, **2000**, Montpellier, France.

Vepřek P., Knytlová K., Ježek J., Trnka T., Synthesis of multivalent glycopeptide dendrimers with tumor antigens of the Tn type (In Czech). Biologically Active Peptides 7, 25 – 27 April, **2001**, Prague, Czech Republic.

Sejbal J., Vondrášek J., Velek J., **Vepřek P.**, Trnka T., Ježek J. Computational and NMR study of multiple antigenic glycopeptides (MAGs) with Tn antigens. Cukrblik, 22 March, **2003**, Prague, Czech Republic.

Trnka T., Ježek J., **Vepřek P.**, Syntéza glykopeptidů a glykopeptidových dendrimerů s Tn antigenem. Cukrblik 2001, Současná glykobiologie, chemie a biochemie sacharidů v českých zemích. Cukrblik, 22 March, 2003. Prague, Czech Republic.

Vepřek P., Kelkar S., Trnka T., Hajdúch M., Ježek J., Semi-combinatorial approach for the preparation of linear comb-like glycopeptide dendrimers with Tn antigen using maleimide ligation. 27th European Peptide Symposium, 31 October – 6 September **2002**, Sorrento, Italy.

Ježek J., Kelkar S., **Vepřek P.**, Hajdúch M., Sejbal J., Trnka T., Multiple antigen glycopeptides (MAGs) with Tn tumour antigens and incorporated anjuvant: synthesis and immunobiological activities. 27th European Peptide Symposium, 31 October – 6 September **2002**, Sorrento, Italy.

Vepřek P., Ježek J., Trnka T., Vondrášek J.; Molecular dynamics study of the conformational behavior of branched glycopeptides during the solid-phase synthesis. The effect of insert and modification of terminal group. 28th European Peptide Symposium, 5 – 10 September **2004**, Prague, Czech Republic.

Vepřek P., Poláková J., Ježek J. Bezouška K.; Modulation of activity of NKR-P1A and NKR-P1B receptors on NK cells by comb-like dendrimers. 29th European Peptide Symposium, 3-8 September **2006**, Gdansk, Poland.

Related publications:

Vepřek P. Ježek J.: Peptide and glycopeptide dendrimers. Part I. *J. Peptide Sci.* **1999**, *5*, 5-23.

Vepřek P., Ježek J.: Peptide and glycopeptide dendrimers. Part II. *J. Peptide Sci.* **1999**, *5*, 203-220.

Ježek J., Velek J., **Vepřek P.**, Velková V., Trnka T., Pecka J., Ledvina M., Vondrášek J., Písačka M., Solid phase synthesis of glycopeptide dendrimers with Tn antigenic structure and their biological activities. Part I, *J. Peptide Sci.* **1999**, *5*, 46-55.

Vepřek P., Ježek J., Trnka T., Vondrášek J.: Molecular dynamics study of the effect of the γ-Abu insert on the conformational behavior of the glycopeptide dendrimers based on the oligolysine scaffold in N, N'-dimethylformamide. *J. Biomol. Struct. Dyn.* **2004**, *22*, 79-90.

Vepřek P, Hajúch M., Džurbák P., Kuklík R., Poláková J., Bezouška K.: Comb-like dendrimers containing Tn antigen modulate natural killing and induce the production of Tn specific antibodies. *J. Med. Chem.* **2006**, *49*, 6400-6407.

4.3 ACKNOWLEDGEMENT

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I would like to express ma appreciation to Doc. RNDr. K. Bezouška, CSc. for the opportunity to test my compounds on NKR-P1 receptors and guidance through the lectin assays, MUDr. M. Hajdúch, Ph.D. for immunological testing, RNDr. J. Vondrášek, CSc. for the introduction to the molecular dynamics simulations, MUDr. M. Písačka for introduction of basic biological testing and Mr. J. Zbrožek and Mrs. V. Himrová for amino acid analyses.

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4.4 LIST OF ABBREVIATIONS

AAA	amino acid analysis
Alloc	allyloxycarbonyl
Boc	*tert*-butyloxycarbonyl
BOP	benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate
BSM	bovine submaxillary mucin
dBSM	desialo bovine submaxillary mucin
CEA	carcinoembryonic antigen
CRD	carbohydrate-recognition domain
CTL	cytotoxic T-cell lymphocytes
DBU	1,8-diazabicyclo-(5.4.0)-undec-7-ene
DCC	*N,N'*-dicyclohexylcarbodiimide
Dde	1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene) ethyl
DIEA	*N,N*-diisopropylethylamine
DIPCI	*N,N'*-diisopropylcarbodiimide
DMAP	4-(*N,N*-dimethyl amino) pyridine
DMF	*N,N*-dimethylformamide
DNA	deoxyribonucleic acid
DNP	2,4-dinitrophenol
dOSM	desialo ovine submaxillary mucin
DTH	delayed type hypersensitivity
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescent-activated cell sorting
FCA	Freund's complete adjuvant
Fmoc	9-fluorenylmethyloxycarbonyl
G_{M2}	G_{M2} ganglioside
HF	hydrogen fluoride
HATU	O-(7-azabenzotriazole-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate
HOBt	1-hyroxybenzotriazole
HPCE	high performance capillary electrophoresis
I-S	disaccharide produced by the action of heparinase I and II on heparin
IVH	influenza virus hemagglutinin
KIR	killer cell immunoglobulin-like receptor
KLH	keyhole limpet hemocyanin
MoAb	monoclonal antibody
MoAbs	monoclonal antibodies
MAG	multiple antigenic glycopeptide
MAP	multiple antigenic peptide
MD	molecular dynamics
MHC	major histocompatibility complex
Mtt	4-methyltrityl
NK	natural killer
NKR-P1	natural killer receptor protein 1
NK/T	natural killer T cell
NMR	nuclear magnetic resonance
Npys	3-nitro-2-pyridine sulphenyl
OSA	ovine submaxillary albumin

OSM	ovine submaxillary mucin
P_3C	tripalmitoyl-S-glycerylcysteine
PAMAM	polyamidoamine
PBS	phosphate buffered saline
RAFT	regioselectively addressable functionalized templates
RBC	red blood cell
RIA	radioimmunoassay
RP-HPLC	reverse phase high pressure liquid chromatography
SOC	sequential oligopeptide carrier
TAA	tumour-associated antigen
TACA	tumour-associated carbohydrate antigen
TASP	template assembled synthetic peptides
TBTU	O-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate
TF	Thomsen-Friedenreich
TR	tandem repeat
TT	tetanus toxin

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