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Carbohydrate Research 342 (2007) 1781–1792
Carbohydrate RESEARCH

N-Acetyl-D-glucosamine substituted calix[4]arenes as stimulators of NK cell-mediated antitumor immune response

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Received 9 February 2007; received in revised form 18 April 2007; accepted 26 April 2007
Available online 6 May 2007

This paper is dedicated to the memory of Professor Dr. Milan Pospíšil (Inst. of Microbiology, Prague) who made pioneering discoveries in the field of natural killer cells

Abstract—A series of calixarenes substituted with 2-acetamido-2-deoxy-β-D-glucopyranose linked by a thiourea spacer was prepared and tested for binding activity to heterogeneously expressed activation receptors of the rat natural killer cells NKR-P1, and the receptor CD69 (human NK cells, macrophages). In the case of NKR-P1, the binding affinity of β-D-GlcNAc-substituted calixarenes carrying two or four sugar units was in a good agreement with the inhibitory potencies of the linear chitooligomers (chitobiose to chitotetraose) reported previously. The influence of GlcNAc substitution of the calixarene skeleton on binding affinity for CD69 receptor was more profound and the 5,11,17,23-tetrakis[*N*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-thioureido]-25,26,27,28-tetrapropoxycalix[4]arene (cone) (**1**) proved to be the best CD69 ligand identified to date. Lower GlcNAc substitution led to dramatic decrease of the binding activity (by about 1.5 order of magnitude per one GlcNAc unit). The immunostimulating activity results with the newly synthesized GlcNAc tetramers on calixarene scaffolds exhibited stimulation of natural cytotoxicity of human PBMC in concentrations 10^{-4} and 10^{-8} M. These calix-sugar compounds were superior to the previously tested PAMAM-GlcNAc$_8$ **5**.

Keywords: Calix[4]arene; 2-Acetamido-2-deoxy-D-glucopyranose; Natural killer cell receptors; CD69

1. Introduction

Natural killer (NK) cells are important components of the innate immune response against tumors and early protection against viruses and other intracellular pathogens. They mediate natural cytotoxicity and produce chemokines and inflammatory cytokines. NK cells express a large number of surface receptors that activate or inhibit their effector function (Fig. 1).[1] Immunological studies performed on human, rat, and mouse lymphocytes provide evidence of the important role of C-type lectin-like receptors such as NKR-P1 (CD161) (rat) or NKG2D and CD69 (human) in activation of natural (NK cells, macrophages) and specific (cytotoxic

Abbreviations: CD69, very early T cell activation antigen; CD161 (NKR-P1), C-type lectin like receptor of NK cells; CRD, carbohydrate recognition domain; CTL, cytotoxic T lymphocytes; EDTA, ethylenediamine tetra-acetic acid; FCS, heat-inactivated fetal calf serum; FT-MS, Fourier transform-ion cyclotron resonance mass spectrometry; GalNAc, 2-acetamido-2-deoxy-D-galactose; GlcNAc, 2-acetamido-2-deoxy-D-glucose; K562, NK-sensitive human erythroleukemia cell line; LPS, lipopolysaccharide; NK-cells, natural killer cells; ManNAc, 2-acetamido-2-deoxy-D-mannose; PAMAM, polyamidoamine; PBMC, peripheral blood mononuclear cells; SEM, standard error of the mean quantifies; SI, stimulation index; TalNAc, 2-acetamido-2-deoxy-D-talose; THF, tetrahydrofuran

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doi:10.1016/j.carres.2007.04.026

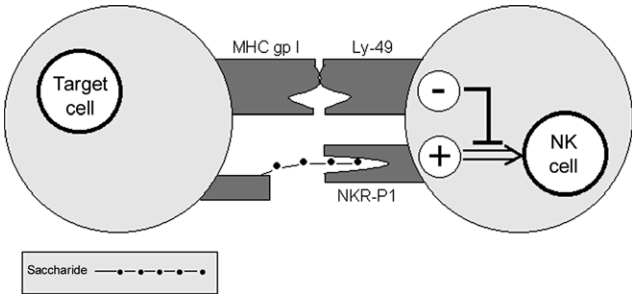

Figure 1. NK cell activation mechanism.

T lymphocytes—CTL) immune responses during the recognition of infected or transformed targets. Upon activation, NK cells are known to up-regulate triggering receptors such as CD69 on their surface, endowing them with the new recognition capabilities.[2]

Both major activating receptors, for example, NKR-P1 and CD69 were recently cloned and characterized in detail and although their physiological ligands have not been identified, high affinity carbohydrate ligands have been found.[3]

We have recently determined the structural requirements of the recombinant soluble dimeric form of NKR-P1 for its optimal carbohydrate ligands.[4] The best monosaccharide ligand known for NKR-P1 is ManNAc; the affinity of other *N*-acetyl-hexosamines decreases in the order GalNAc > GlcNAc ≫ TalNAc. GlcNAc was used in our studies for the following reasons: (1) GalNAc is approximately a thousand times more expensive than GlcNAc, while being nearly equally effective; (2) Substitution of calixarenes with GalNAc yields partly substituted structures, probably due to steric reasons (unpublished experiments); (3) The synthesis of β-ManNAc linkages is one of the most complicated tasks in carbohydrate chemistry and its attachment to complicated scaffolds would bring other problems. In addition, we use GlcNAc-containing glycoconjugates in all our studies, which enables us to compare the effectiveness and SAR of the respective scaffolds.

The length of the carbohydrate chain is crucial for the binding as determined by using a series of (β-(1→6)-GalNAc)$_n$ oligomers ($n = 2$–6) and a series of chitooligomers (β-(1→4)-GlcNAc)$_n$; $n = 2$–9.[4,5] The optimum length of the oligosaccharide chain is four carbohydrate units, with the β-(1→4)-glycosidic linkage being the best. The major structural feature of CRD (binding groove) of this lectin is the affinity of linear *N*-acetylhexosamine-based carbohydrates.[5]

The CD69 receptor also has a high affinity for GlcNAc, which is higher than for GalNAc. However, direct binding assays, site-directed mutagenesis, and molecular modeling revealed the details of the individual binding sites[6] demonstrating that this receptor is highly specific for multi-antennary, branched GlcNAc-containing structures (glycoproteins, neoglycoconjugates, and PAMAM-based glycodendrimers).

Multivalency was found to be crucial for the binding of carbohydrate structures to the NKR-P1 receptor in vitro.[7] This was also proven in ex vivo experiments with isolated NK cells against NK-resistant tumor cell lines[3] and in in vivo experiments by treatment of colon cancer with activated NK cells.[8] Here, trivalent, tetravalent, hexavalent, and octavalent glycodendrimers based on the PAMAM cores with thiourea-bridged β-GlcNAc units were used.[7] The activity of the glycoclusters was high, despite the use of rather suboptimal ligand for the clustering, for example, a 2-acetamido-2-deoxy-β-D-glucopyranosyl unit connected via a β-N-glycosidic linkage.

Despite the essential role of carbohydrates in a wide range of biological phenomena, there are relatively few glycodrugs (compounds, in which the sugar moiety carries the biological function).[9,10] The variety of core structures that can be constructed has the potential to organize and orient carbohydrate ligands in a multitude of topographic arrays, a feature that is becoming crucial in target selectivity.[11,12] A common feature of lectin binding to carbohydrate ligands is the intrinsic weak affinity. Presentation of the sugar epitopes as multiple copies on an appropriate scaffold (molecular, dendritic, polymeric) creates a multivalent display that can efficiently mimic the natural mode of affinity enhancement that arises from multiple interaction between the binding proteins and the carbohydrate ligands.[13,14] The lectin-binding efficiency and specificity of glycoclusters have been found to be dependent not only on the epitope density but also on the nature of the core and the geometrical characteristics of the multivalent assembly.

Our previous results using PAMAM-based glycoclusters with GlcNAc for therapeutic application to tumor-bearing animals (colorectal carcinoma in rats and melanoma in mice) showed decreased tumor growth and prolonged survival time of treated animals accompanied by enhancement of immune response (cytokine production, cytotoxicity, infiltration of tumor by activated lymphocytes).[15,16]

Calixarenes[17–20] are a versatile class of compounds, often being used as scaffolds in various supramolecular structures, in host–guest chemistry, etc. There are many advantages of using calixarenes, including well-defined geometrical conformation of functional groups and easy accessibility and workup, even in large-scale reactions. Besides this, the vicinity of the polyaromatic system often increases the affinity of glycosidic ligands to respective receptors via nonspecific interactions.[21] For our purposes, a major potential advantage of calixarene-based glycoconjugates is better steric control of the interaction of the attached carbohydrates with their respective receptor(s).

This study was initiated to determine differences in the binding capability of calix[4]arene and polyamidoamine based glycoconjugates to recombinant NKR-P1 and CD69 receptors, as well as their effects on proliferation and cytotoxic cell effector function. Some of these new compounds proved to be the best glycomimetic multivalent ligands for both proteins tested to date.

2. Results

2.1. Design of calix[4]arene glycoconjugates

A series of calix[4]arene–based glycoconjugates with varying geometry was prepared. The targets (Fig. 2) consist of a calix[4]arene skeleton with various number of GlcNAc units.[†] Both cone and 1,3-alternate (Fig. 3) conformers were prepared.

The variable conformation of the calix[4]arene scaffold and different number of sugar units define the geometrical arrangement of all GlcNAc subunits, which is crucial for the biological properties of each molecule. Appropriate substitution of the calix[4]arene phenolic function ensures fixation of the desired conformation indefinitely in time. The use of different spacers between sugar moieties and calix[4]arene scaffold can affect the nature of this linker by means of rigidity, electron density, flexibility, chemical and biochemical stability, etc.

2.1.1. Preparation of the calix[4]arene based glycoconjugates. Each respective amino-calix[4]arene (obtained by reduction from corresponding nitro derivative by $SnCl_2$) was coupled with 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl isothiocyanate. After removal of protecting acetyl groups with sodium methoxide in methanol, target compounds **1**–**3** were prepared.

2.2. Inhibitory activities for soluble recombinant NKR-P1 and CD69 receptors

The concentration of compounds required to cause 50% inhibition of binding of the soluble ^{125}I-labeled receptors to the high affinity ligand $GlcNAc_{23}BSA$ was determined (IC_{50}).[3] This is a suitable measure of affinity of the compounds for these receptors.

2.2.1. Reference compounds for plate binding and plate inhibition assays. 2-Deoxy-2-acetamido-D-glucopyranose (GlcNAc), used as a reference compound, gave typically $-\log IC_{50}$ of 6.7 (NKR-P1A) and 5.1 (CD69), which is in accordance with previously reported values.[3,6] Because the tetrapropoxycalix[4]arene itself is water insoluble, we used the corresponding hydroxycarbonylmethoxy (–O–CH_2–COOH) derivative **4** (Fig. 4) in the form of its salt as a reference compound for a nonsugar derivatized calix[4]arene, to confirm expected noninhibitory properties of calix[4]arene scaffold. Respective calix[4]arenes carrying carbohydrates that do not bind to NKR-P1A or CD69 receptors (e.g., mannose) cannot be used as negative control. These sugars, especially in the form of multivalent neoglycoconjugates, interact with other receptors on the cell surface and this could disturb the experiments with the whole cells.

2.2.2. Binding of calix[4]arene glycoconjugates to NKRP1A and CD69. First, inhibitory effects of the unsubstituted calix[4]arene skeleton of compound **4** were tested. This compound had rather low inhibitory activity for NKR-P1A ($-\log IC_{50}$ was 5.0; Fig. 5), but it was surprisingly active with CD69 reaching $-\log IC_{50}$ of 7.7 (Fig. 6). When tested as inhibitors of NKR-P1A, GlcNAc-substituted calix[4]arenes reached $-\log IC_{50}$ between 7.0 and 9.0. Interestingly, the cone structures were always better inhibitors than the alternate ones (Fig. 5).

The results obtained for CD69 were even more interesting. First, the tetrasubstituted aromatic derivative (**1**) displayed very potent inhibitory activity with $-\log IC_{50}$ as high as 9.3 making it to be one of the best ligand of this receptor described so far (Fig. 6).

2.3. Effect of glycoconjugates on proliferation of human PBMC in vitro

Compounds that exhibited the highest affinity to the recombinant proteins were further tested on the proliferation of human PBMC. Initial in vitro assays were performed to test the effect of those glycoconjugates that exhibited the highest affinity to recombinant proteins on proliferation of human PBMC either spontaneously (Fig. 8a) or in the presence of sub optimal concentration (1 μg/mL) of polyclonal mitogen lipopolysaccharide (LPS, Fig. 8b). This method also serves as a part of immunotoxicity studies of new pharmaceuticals. For this purpose a 72-h ^{3}H-thymidine incorporation assay was employed. The best of all new calix-glycoconjugates, **1**, and reference compounds **4** and **5**[7] (Fig. 7) were tested in concentrations from 10^{-4} to 10^{-9} mol/L at 10-fold dilution. No toxicity to PBMC by the glycoconjugates, or control calix[4]arene **4** was detected, even at high concentrations (10^{-4} mol/L).

The results (Fig. 8) showed a clear dose-dependent effect of glycoconjugates on the proliferation of PBMC

[†] An additional 'aliphatic set' of calixarene glycoconjugates was prepared. In these compounds, a methylene bridge is inserted between the calix[4]arene scaffold and every thiourea in the molecule. These compounds proved to be intrinsically unstable after deacetylation and, therefore, only partial structural data were obtained and preliminary biological tests were performed. These preliminary experiments are described in the Supplementary data to this paper.

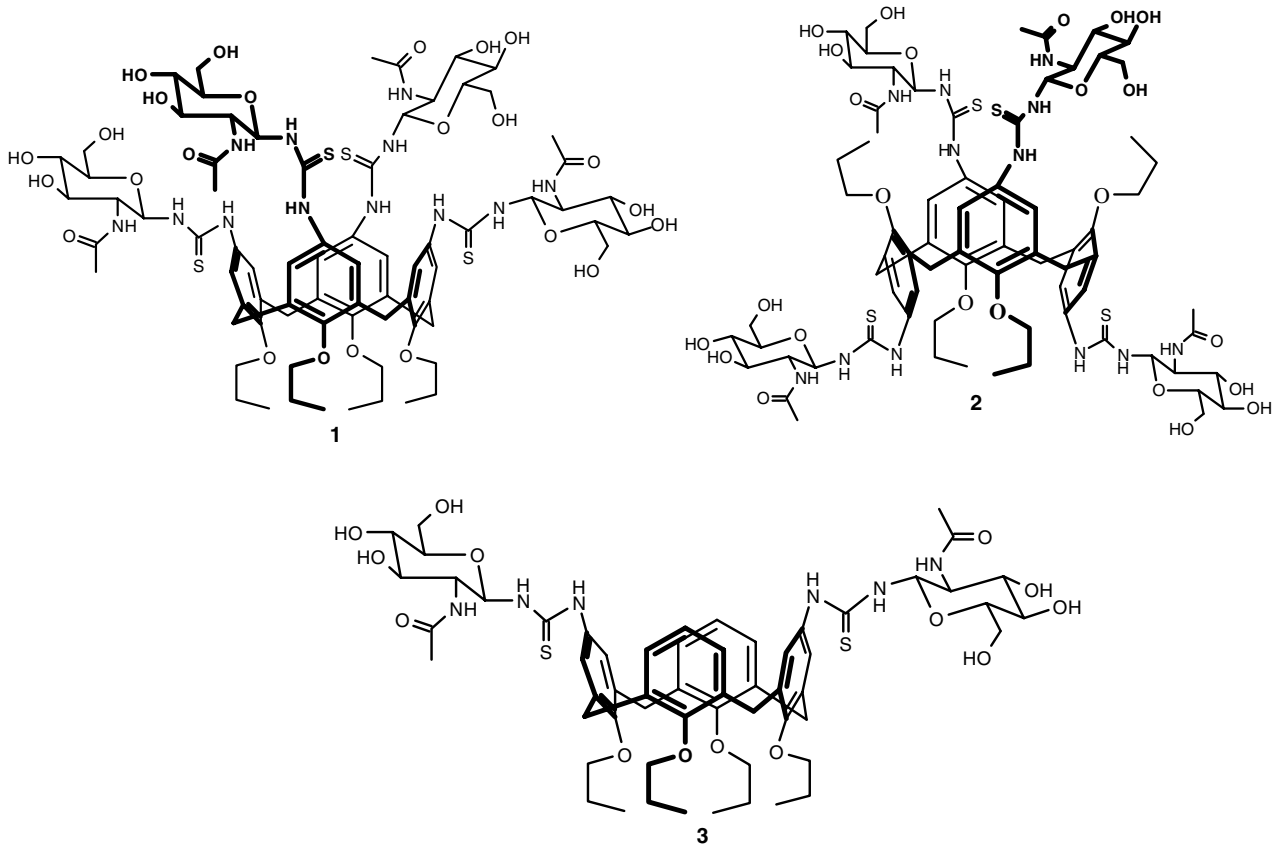

Figure 2. Set of calix[4]arene glycoconjugates.

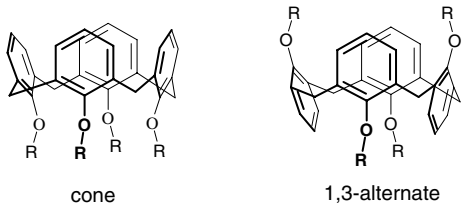

Figure 3. The two selected conformations of calix[4]arenes out of four possibilities.

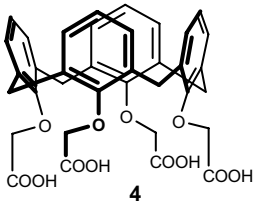

Figure 4. Hydroxycarbonylmethoxy calix[4]arene derivative.

according to the initial state of mitotic activity (spontaneous or LPS-activated). Surprisingly, all substances were ineffective at concentration of 10^{-6} mol/L (SI = 1, for SI definition see Section 4.3.3). Compound **1** at a concentration 10^{-8} mol/L induced PBMC proliferation, which was similar (reaching SI −2.0 at 10^{-7} and 10^{-9} mol/L) or higher (SI −2.5 at 10^{-8} mol/L) than previously used PAMAM-GlcNAc$_8$ **20** (Fig. 7).

When the compounds were added 30 min prior to the LPS, significant inhibition of mitogenic activity of LPS was noted in all calix[4]arene-based glycoconjugates compared to the PAMAM-based one. Due to the ineffectiveness of **4** and **5** at 10^{-7}–10^{-9} mol/mL, we can deduce that this activity is specifically mediated by the GlcNAc structure bound to calix[4]arene (rigid conformation), but not to PAMAM (flexible conformation), and not by calix[4]arene scaffold itself.

In summary, the glycoconjugates, particularly **1**, in optimal stimulatory concentration (10^{-8} mol/L) had a similar effect on the proliferation of human PBMC as LPS. The control calix[4]arene (**4**) and GlcNAc itself (data not shown) did not influence significantly the unstimulated PBMC, but they block the LPS-induced proliferation from 10^{-4} to 10^{-6} mol/L.

2.4. Effect of glycoconjugates on NK cell activity of human PBMC in vitro

The glycoconjugates with the highest affinity to the recombinant proteins NKR-P1A and CD69 in the binding experiments were tested for their immunomodulatory effect in a functional cytotoxicity assay in vitro.

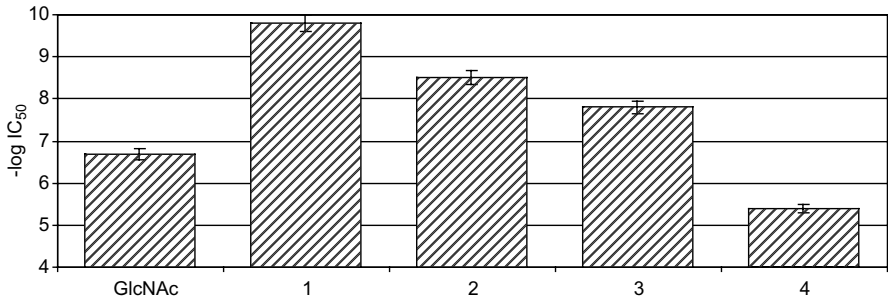

Figure 5. Inhibitory activity of calix[4]arene glycoconjugates toward NKR-P1A. Average values ± SD from three independent experiments are shown.

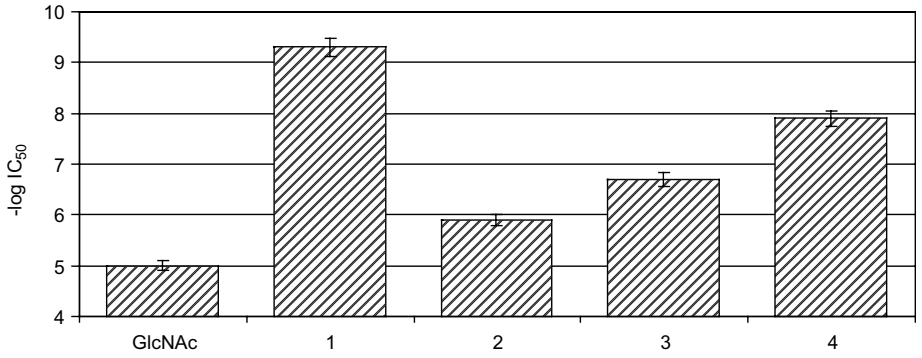

Figure 6. Affinity of calix[4]arene glycoconjugates toward CD69. Average values ± SD from three independent experiments are shown.

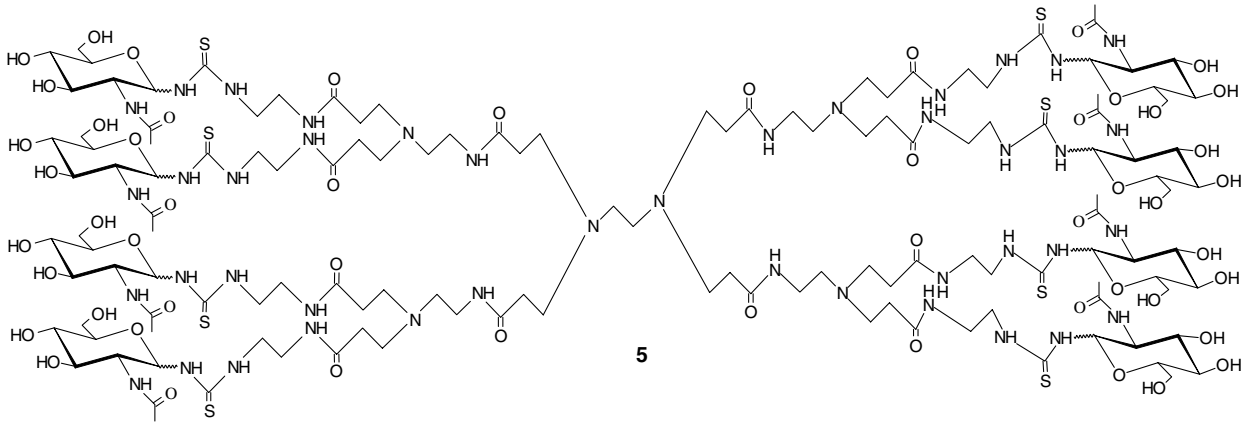

Figure 7. PAMAM dendrimer with eight GlcNAc substituents.

Human PBMC from healthy donors as effector cells were incubated 18 h in the presence of a each compound in concentration range 10^{-4}–10^{-9} mol/L, prior to the addition of target cells to allow better processing by effector cells and their subsequent activation. Based on preliminary experiments of different effectors to target cells ratios (64, 32, 16, and 8:1), a 16:1 ratio was chosen for further assays as optimal to assess the immunomodulatory action of glycoconjugates. The effect of newly synthesized $GlcNAc_4$-calix[4]arene **1** was compared with the previously described GlcNAc-substituted octamers on PAMAM scaffold (**5**),[22] the control calix[4]aren (**4**), and untreated control cells.

NK cell-mediated cytotoxicity of PBMC was measured in a standard 4-h ^{51}Cr-release assay against NK-sensitive human erythroleukemia cell line (K562). The best stimulatory effect was found by **1** (Fig. 9) in comparison to **4**, and previously used PAMAM-GlcNAc dendrimer **5**. Biological results with the GlcNAc tetramers on a calix[4]arene scaffold exhibited stimulation of NK cell-mediated cytotoxicity expressed as a percentage of an unstimulated control (100 ± 5) by **1** in concen-

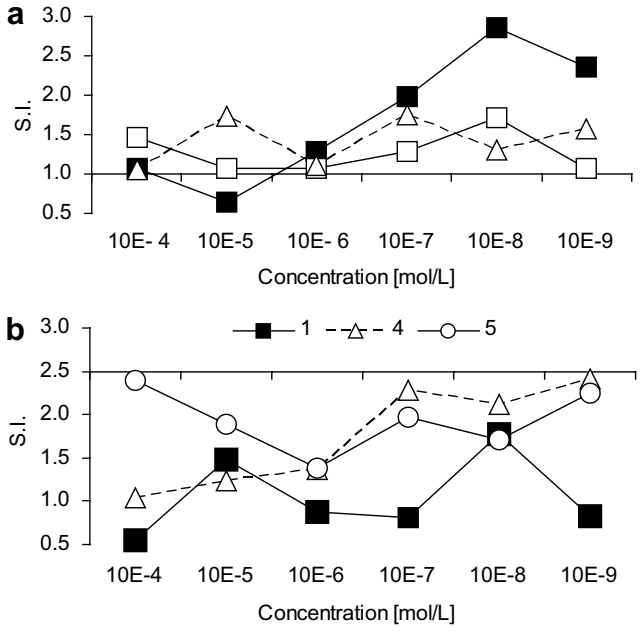

Figure 8. Influence of compounds on spontaneous (a) or LPS-induced (b) proliferation of human PBMC. The results are expressed as stimulation index (SI) of the control unstimulated cells.

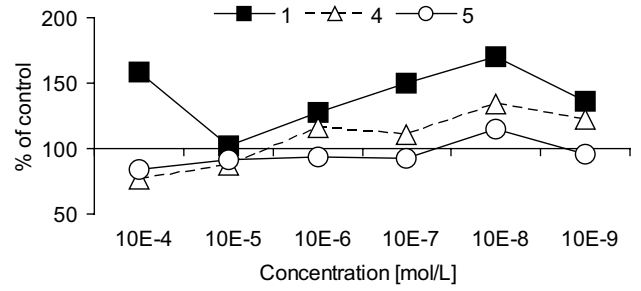

Figure 9. Influence of tested compounds on NK activity of human PBMCs.

trations 10^{-4} mol/L (158% ± 19, $p = 0.002$) and 10^{-8} mol/L (170% ± 21, $p = 0.004$) of higher extent than **5** (115% ± 9, $p = 0.04$). Moreover, compound **1** induces, at the concentrations 10^{-4} mol/L, the spontaneous death of tumor targets (not shown), which was not present in the case of control calix[4]arene **4**, and **5**.

3. Discussion

One of the possibilities of why immune effector cells are unable to recognize tumor cells is due to altered surface glycosylation, which masks the structural motifs of target molecules with concomitant changes in enzyme activities thus facilitating the growth and invasiveness of tumors. Previous studies have suggested that the target structures for NK cells are not only proteins, but also carbohydrates including *N*-glycans.[5,8,23]

We have recently determined the structural monosaccharide requirements of the recombinant soluble dimeric form of NKR-P1 for its optimal carbohydrate ligands.[21] Structural requirements of CD69 have shown to be similar,[24] but differ mostly in topological preferences of ligand arrangements.

The main structural preferences of NKR-P1 are as follows: (1) The best monosaccharide ligands are *N*-acetylhexosamines ranking as ManNAc > GalNAc > GlcNAc ≫ Glc > Man; (2) The optimal length of the linear oligosaccharided chain (chitooligomers) are four carbohydrate units (four binding sites in a linear CRD);[5] (3) The presence of aromatic subunits in the glycoconjugate enhances (unspecifically) the binding; (4) Significantly higher levels of activation are observed when a multivalent ligand is employed.[25]

The important differences in CD69 structural requirements are as follows: (1) ManNAc is not recognized at all. The rank of monosaccharide affinity is GlcNAc ~ GalNAc ≫ Glc > Man; (2) Branched oligosaccharide structures are strongly preferred to linear ones; (3) Compounds carrying groups with negative charge (carboxylate, etc.) have higher affinity.[25]

We have previously used glycoconjugates based on PAMAM dendrimers[26,27] to achieve high NKR-P1 binding affinity. These compounds proved to be highly active and effective stimulants for rat NK cells. The introduction of calix[4]arenes in glycoconjugates as a novel scaffold bearing sugar units enables better three-dimensional structural control. This can be achieved either by changing the calix[4]arene conformation or by variation of the calix[4]arene-sugar spacer.

During our study, Consoli et al. reported[31] lectin-binding ability of **1**. The ability to bind wheat germ agglutinin, a lectin that is *N*-acetyl-β-D-glucosamine specific, was described.

Inhibitory activities of calix[4]arene derivatives using recombinant soluble preparations of the major NK cell activation receptors served as the selection of the most active components for detailed immunological evaluations. Significant inhibitory activity of the carboxylated calix[4]arene skeleton for CD69 may be understood in view of other carboxylated inhibitors for this receptor, such as sialylated oligosaccharides or peptides rich in the acidic amino acids (K. Bezouška, unpublished observations). Further substitution with GlcNAc further reinforced inhibitory affinities of the calix[4]arene core. However, these effects were different for NKR-P1 compared to CD69. In the case of the NKR-P1, the inhibitory potencies of GlcNAc-substituted calix[4]arenes were those expected for compounds containing from two to four hexosamine residues, and were, in fact, in a good agreement with the inhibitory potencies of the linear chitooligomers (chitobiose to chitotetraose) reported previously.[5]

The influence that GlcNAc-substituted calix[4]arenes had on the inhibitory activities for CD69 was much more interesting, because the compounds turned out to be the best CD69 ligands identified to date. Our previous work indicated the existence of multiple binding sites for GlcNAc at the surface of CD69, and from this perspective the exact density and spacing of GlcNAc residues would be expected to be critical.[28] The critical importance of GlcNAc density, that we have previously reported for dendrimeric GlcNAc-containing structures,[7] can be clearly demonstrated on the inferior inhibitory activities of the disubstituted aliphatic derivative when compared to their tetrasaccharide counterparts.

As a conclusion we can state that the results of our work corroborate our previous results indicating structural preferences of the NK cell receptors NKR-P1 and CD69 for the GlcNAc-containing dendrimeric structures with aromatic spacers. In the case of the NKR-P1, the binding affinity of GlcNAc-substituted calix[4]arenes carrying two to four sugar units were in a good agreement with the inhibitory potencies of the linear chitooligomers (chitobiose to chitotetraose) reported previously. The influence of GlcNAc substitution of the calix[4]arene skeleton on binding affinity for CD69 receptor was more profound and the GlcNAc-terasubstituted calix[4]arene (**1**) proved to be the best CD69 ligand identified so far. Lower GlcNAc substitution led to substantial decrease of the binding activity (one GlcNAc unit ~ca. 1.5 order lowering). The immunostimulating activity results with the GlcNAc tetramers on calix[4]arene scaffolds exhibited stimulation of natural cytotoxicity of human PBMC by compound **1** in the concentrations 10^{-4} and 10^{-8} M. These calix-sugar compounds were superior to the previously tested PAMAM-GlcNAc$_8$ (**5**). Moreover, compound **1** used at the above concentrations induced an increase in the spontaneous death of tumor targets. This effect was not observable in the case of control calix[4]arene scaffold **4**, GlcNAc itself or PAMAM-GlcNAc$_8$ (**5**).

4. Experimental

Most of the NMR spectra were recorded on a Varian INOVA-400 spectrometer (399.89 MHz for ^{1}H, 100.55 MHz for ^{13}C) in $CDCl_3$ or DMSO-d_6 (see respective spectra) at 30 °C (unless otherwise stated). Chemical shifts were referenced to the residual solvent signal (δ_H 7.265, δ_C 77.00; δ_H 2.50, δ_C 39.60). Digital resolution used justified reporting the proton and carbon chemical shifts to three and two decimal places, respectively. All 2D NMR experiments (HOM2DJ, gCOSY, TOCSY, HMQC, HMBC) were performed using standard manufacturer's software. Some spectra were recorded on a Varian Gemini 300 spectrometer using tetramethyl silane as an internal standard at 20 °C.

Mass spectra were measured on a matrix-assisted laser desorption/ionization reflectron time-of-flight (MALDI-TOF) mass spectrometer BIFLEX (Bruker-Franzen, Bremen, DE) equipped with a nitrogen laser (337 nm) and gridless delayed extraction ion source. Ion acceleration voltage was 19 kV and the reflectron voltage was set to 20 kV. Spectra were calibrated externally using the monoisotopic $[M+H]^+$ ions of matrix peak 379.1 m/z. A saturated solution of α-cyano-4-hydroxy-cinnamic acid or 2,5-dihydroxybenzoic acid in MeCN/0.3% aq acetic acid (1:1) was used as a MALDI matrix. A 1 μL of matrix solution was mixed with a 1 μL of sample diluted in acetonitrile or THF and a 1 μL of premix was loaded on the target, the droplet was allowed to dry at ambient temperature. The MALDI-TOF spectra were collected in reflectron mode.

Flash chromatography was performed on silica gel (Merck Kieselgel 60, No. 9385, 230–400 mesh ASTM). TLC was carried out on silica gel plates (E. Merck 60 F_{254}); zones were detected visually by UV (254 nm) or by spraying with 5% H_2SO_4 in ethanol, followed by heating at 100 °C. Preparative TLC chromatography was carried out on 20 × 20 cm glass plates covered by Silica gel 60 GF_{254} or Al_2O_3 type G.

For the final purification size-exclusion chromatography using LH-20 gel as stationary and MeOH as mobile phase gave the best results. This method was applicable even on a large scale.

4.1. Preparation of glycoconjugates

4.1.1. 5,11,17,23-Tetrakis[*N*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-thioureido]-25,26,27,28-tetrapropoxycalix[4]arene (cone) (1a). 5,11,17,23-Tetraamino-25,26,27,28-tetrapropoxycalix[4]arene (cone)[29] 34 mg (0.05 mmol) and of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl isothiocyanate[30] 100 mg (0.83 mmol, 16 equiv) were dissolved in dry CH_2Cl_2 and stirred at room temperature for 3 days. The solvent was evaporated in vacuo and the crude reaction mixture was purified by column chromatography (EtOAc). The product was isolated as 110 mg (96%) of white solid. MALDI-TOF MS $[M+H]^+$ 2206.3, calcd 2205.8; for ^{1}H and ^{13}C NMR data see Table 1.

4.1.2. 5,11,17,23-Tetrakis[*N*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-thioureido]-25,26,27,28-tetrapropoxycalix[4]arene (cone) (1). Compound **1a** 166 mg (0.075 mmol) was dissolved in dry MeOH. A catalytic amount of Na was added to the solution, which was then stirred for 24 h at room temperature under a $CaCl_2$ tube. Sodium salts were removed by the addition of Dowex 50W in H^+ form. The resin was filtered after 24 h and the crude reaction mixture was purified by size-exclusion chromatography (Sephadex LH-20, MeOH). The product was isolated as 53 mg (41%) of a

Table 1. ^{1}H and ^{13}C NMR data (399.87 and 100.55 MHz, DMSO-d_6, 67 and 30 °C) in accordance with previously published results[31] of compound **1a**

	67 °C							30 °C	
Atom	δ_C	m	δ_H	n_H	m	J [Hz]	HMBC (C to H)	δ_C	δ_H
1	82.63	d	5.572	4	dd	9.9, 8.3	2	82.83	5.548
2	51.75	d	3.989	4	ddd	10.2, 9.9, 9.2	3, 2-NH	51.72	3.999
3	72.98	d	5.129	4	dd	10.2, 9.3	2, 4	73.04	5.108
4	68.90	d	4.833	4	dd	10.0, 9.3	3, 6[u]	68.76	4.827
5	72.06	d	3.764	4	ddd	10.0, 4.6, 2.7	4, 6[u]	72.13	3.761
6	61.79	t	4.187	8	dd	12.3, 4.6	4	61.84	4.205
			3.982		dd	12.3, 2.7			3.939
2-NH	—	—	7.946	4	d	9.2		—	8.117
2-Ac	22.38	q	1.802	12	s	—		22.65[a]	1.793
CO	169.90	s	—	0	—	—	2-NH, 2-Ac	170.06	—
3-Ac	20.08	q	1.925	12	s	—		20.37[b]	1.921[c]
CO	169.24	s	—	0	—	—	3, 3-Ac	169.55	—
4-Ac	20.15	q	1.969	12	s	—		20.45[b]	1.962[c]
CO	169.03	s	—	0	—	—	4, 4-Ac	169.34	—
6-Ac	20.26	q	2.004	12	s	—		20.58[b]	2.001[c]
CO	169.72	s	—	0	—	—	6, 6-Ac	170.06	—
1-NH	—	—	7.367	4	br s	—		—	7.487
CS	181.39	s	—	0	—	—	1	181.36	—
Ar–NH	—	—	9.513	4	s	—		—	9.637
Ar–CH_2	30.12	t	4.378	8	d	12.7		30.19[x]	4.348
			3.234		d	12.7			3.245
ortho	134.23	s	—	0	—	—	Ar–CH_2	134.42	—
meta	123.72	d	6.794	8	s	—	Ar–CH_2, *meta*,	124.04[x]	6.812
			6.824		s		2-NH		
para	131.87	s	—	0	—	—	2-NH	132.15[x]	—
ipso	153.42	s	—	0	—	—	Ar–CH_2, *meta*, OCH_2	153.48[x]	—
OCH_2	76.35	t	3.870	8	m	—	CH_2, CH_3	76.65	3.839
CH_2	22.49	t	1.945	8	m	—	OCH_2, CH_3	22.76[a]	1.95[d]
CH_3	9.93	q	1.005	12	t	7.4	OCH_2, CH_2	10.19	0.994

[a,b,c] Might be interchanged.
[d] HMQC readout.
[u] Upfield resonating proton.
[x] Broad signal.

white yellowish solid. MALDI-TOF MS $[M+H]^+$ 1703.5, calcd 1703.3; for ^{1}H and ^{13}C NMR data see Table 2.

4.1.3. 5,11,17,23-Tetrakis[*N*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-thioureido]-25,26,27,28-tetrapropoxycalix[4]arene (1,3-alternate) (2a). Same procedure as for **1a**. 5,11,17,23-Tetraamino-25,26,27,28-tetrapropoxycalix[4]arene (1,3-alternate)[32] 34 mg (0.05 mmol) and 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl isothiocyanate[30] 100 mg (0.83 mmol, 16 equiv) were used. The product was purified by column chromatography (petroleum ether/EtOAc, 2:3) and isolated as 60 mg (52%) of a white solid. MALDI-TOF MS $[M+H]^+$ 2205.5, calcd 2205.8.

4.1.4. 5,11,17,23-Tetrakis[*N*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-thioureido]-25,26,27,28-tetrapropoxycalix[4]arene (1,3-alternate) (2). Same procedure as for **1a**. Starting from **2a** 118 mg (0.053 mmol). The title product was obtained as 70 mg (77%) of a white yellowish solid. MALDI-TOF MS $[M+H]^+$ 1701.4, calcd 1701.7; for ^{1}H and ^{13}C NMR data see Table 3.

4.1.5. 5,17-Bis[*N*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-thioureido]-25,26,27,28-tetrapropoxycalix[4]arene (cone) (3a). Same procedure as for **1a**. 5,17-Diamino-25,26,27,28-tetrapropoxycalix[4]-arene (cone)[32] 134 mg (0.02 mmol) and 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl isothiocyanate[30] 250 mg (0.14 mmol, 6 equiv) were used. The product was purified by column chromatography (petroleum ether/EtOAc, 2:3) and isolated as 273 mg (91%) of a white solid. MALDI-TOF MS $[M+H]^+$ 1399.5, calcd 1399.7; for ^{1}H and ^{13}C NMR data see Table 4.

4.1.6. 5,17-Bis[*N*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-thioureido]-25,26,27,28-tetrapropoxycalix[4]arene (cone) (3). Same procedure as **1a**, starting from **3a** 194 mg (0.14 mmol). The product was isolated as 70 mg (65%) of white yellowish solid. MALDI-TOF MS $[M+H]^+$ 1148.3, calcd 1148.4, for ^{1}H and ^{13}C NMR data see Table 5.

4.1.7. 25,26,27,28-Tetrakis(hydroxycarbonylmethoxy)-calix[4]arene (cone) (4). 25,26,27,28-Tetrakis(ethoxycarbonylmethoxy)-calix[4]arene[38] (1 g, 1.3 mmol) and

Table 2. ^{1}H and ^{13}C NMR data (399.87 and 100.55 MHz, DMSO-d_6, 30 °C) of compound **1**

Atom	δ_C	m	δ_H	n_H
1	83.73	d	5.292	4
2	54.53	d	3.54[a]	4
3	74.13	d	3.32[a]	4
4	70.47	d	3.117	4
5	78.41	d	3.117	4
6	60.75	t	3.624	4
			3.486	4
2-NH	—	—	8.058	4
2-Ac	22.94	q	1.828	12
CO	170.77	s	—	0
3-OH	—	—	5.002	4
4-OH	—	—	5.002	4
6-OH	—	—	4.473	4
1-NH	—	—	7.320	4
CS	181.06	s	—	0
Ar–NH	—	—	9.579	4
Ar–CH_2	30.23	t	4.340	8
			3.26[a]	
ortho	134.49[x]	s	—	0
meta	124.16[x]	d	6.855	4
			6.687	4
para	132.06[x]	s	—	0
ipso	153.65	s	—	0
OCH_2	76.58	t	3.839	8
CH_2	22.77	t	1.924	8
CH_3	10.21	q	0.985	12

No coupling constants were resolved because of signal broadening.
[a] HMQC readout.
[x] Broad signal.

Table 3. ^{1}H and ^{13}C NMR data (399.87 and 100.55 MHz, DMSO-d_6, 30 °C) of compound **2**

Atom	δ_C	m	δ_H	n_H	m	J [Hz]
1	83.37	d	5.366	4	br s	[b]
2	54.23	d	3.626	4	m	[b]
3	74.50	d	3.33[a]	4	m	[b]
4	70.41	d	3.167	4	m	[b]
5	78.35	d	3.109	4	m	[b]
6	60.70	t	3.626	4	m	[b]
			3.485	4	m	[b]
2-NH	—	—	7.977	4	d	7.3
2-Ac	22.86	q	1.831	12	s	—
CO	170.18	s	—	0	—	—
3-OH	—	—	4.967	4	d	5.3
4-OH	—	—	4.982	4	d	6.7
6-OH	—	—	4.482	4	br s	—
1-NH	—	—	7.505	4	br s	—
CS	181.27	s	—	0	—	—
Ar–NH	—	—	9.679	4	br s	—
Ar–CH_2	37.64	t	3.730	8	br s	—
	37.43		3.670		br s	—
ortho	133.72[x]	s	—	0	—	—
meta	124.77[x]124.28[x]	d	7.128	8	br s	—
	124.28[x]					
para	132.22[x]	s	—	0	—	—
ipso	154.04	s	—	0	—	—
OCH_2	72.49	t	3.293	8	m	[b]
CH_2	22.61	t	1.327	8	m	[b]
CH_3	10.01	q	0.680	12	t	7.4

[a] HMQC readout.
[b] Unresolved.
[x] Broad signal.

NaOH (0.3 g) were heated at reflux in EtOH (30 mL) overnight. The reaction mixture was evaporated to dryness in vacuo and dissolved in 40 mL of water. By gradual addition of 2 M HCl the product was precipitated, filtered, and dried in air to give 770 mg (90%) of a white solid. NMR spectroscopic data were in compliance with literature.[39]

4.2. Plate binding and plate inhibition assays

The affinity of compounds **1**–**4** toward two representative NK cell activation receptors, rat NKR-P1A and human CD69 proteins, was tested. Due to their good availability and stability, monomeric soluble forms of these proteins, that is, NKR-391 and CD69CWTY, which encompass the ligand-binding domains, were used for the tests.[3,6] To verify the identity and homogeneity of the protein preparations, the technique of Fourier transform-ion cyclotron resonance mass spectrometry (FT-MS)[40] was employed in addition to the standard analytical techniques described.[3,6] This method showed the proteins to be homogenous with $m/z = 11965.4$ and 11630.1 for the native (nonreduced) NKR-P1A and CD69, respectively. The biological activity of the soluble receptors after ^{125}I iodination was verified using simple sugars. D-Mannopyranose served as a negative (noninhibitory) control.

All inhibition assays were performed three times in duplicates and the results for NKR-P1 and CD69 are given in Figures 8 and 9, respectively, as $-\log IC_{50}$ with standard deviations.

4.3. Immunological assays

4.3.1. Isolation of human peripheral blood mononuclear cells (PBMC). Human peripheral blood (citrate/EDTA) samples were collected from healthy donors from the Blood Transfusion Service (Prague, Czech Rep.). Peripheral blood mononuclear cells were separated by using a Ficoll-Paque density gradient (1.077), and after three washes used for functional assays.

4.3.2. Cell cultures. Established cell line of K562 (human chronic myelogenous leukemia, ATCC CCL-243) was maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, antibiotics (0.05 mg/mL gentamycin, 25 mg/mL amphotericin B), and 10% heat-inactivated fetal calf serum -FCS (Gibco, Grand Island, NY, USA). The cytotoxic activity and proliferation assay was performed in RPMI 1640 medium supplemented with L-glutamine, gentamycin and 5% FCS. Incubation was carried out at 37 °C in a

Table 4. ^{1}H and ^{13}C NMR data (399.87 and 100.55 MHz, DMSO-d_6, 30 °C) of compound **3a**

Atom	δ_C	m	δ_H	n_H	m	J [Hz]	HMBC (C to H)
1	82.72	d	5.600	2	dd	[a]	
2	51.79	d	4.029	2	ddd	[a]	3, 2-NH
3	73.10	d	5.139	2	dd	[a]	2, 4
4	68.80	d	4.848	2	dd	[a]	3, 6[u]
5	72.12	d	3.787	2	m	—	4, 6[u], 6[d]
6	61.87	t	4.206	4	dd	4.6, 12.3	4
			3.976		dd	[a]	
2-NH	—	—	8.148	2	d	9.3	
2-Ac	22.68	q	1.815	6	s	—	
CO	170.04	s	—	0	—	—	2-NH, 2-Ac
3-Ac	20.37	q	1.933	6	s	—	
CO	169.54	s	—	0	—	—	3, 3-Ac
4-Ac	20.44	q	1.974	6	s	—	
CO	169.35	s	—	0	—	—	4, 4-Ac
6-Ac	20.56	q	2.005	6	s	—	
CO	170.04	s	—	0	—	—	6, 6-Ac
1-NH	—	—	7.650	2	br s	—	
CS	181.46	s	—	0	—	—	
Ar–NH	—	—	9.731	2	br s	—	
Ar–CH_2	30.19	t	4.333	8	d	13.1	*meta*[N]
			3.177		d	13.1	
			3.158		d	13.1	
ortho[S]	135.50	s	—	0	—	—	Ar–CH_2
	135.58						
meta[S]	123.88[x]	d	7.008	4	br s	—	Ar–CH_2
para[S]	132.14[x]	s	—	0	—	—	
ipso[S]	154.12	s	—	0	—	—	Ar–CH_2, OCH_2[S]
OCH_2[S]	76.27	t	3.899	4	m	—	CH_2[S], CH_3[S]
CH_2[S]	22.66	t	1.935	4	m	—	OCH_2[S], CH_3[S]
CH_3[S]	9.95	q	0.933	6	t	7.5	OCH_2[S], CH_2[S]
ortho[N]	133.28	s	—	0	—	—	*para*[N], Ar–CH_2
	133.30						
meta[N]	127.60	d	6.425	4	m	—	*meta*[N], Ar–CH_2
	127.65						
para[N]	121.93	d	6.371	2	m	—	
ipso[N]	155.30	s	—	0	—	—	*meta*[N], Ar–CH_2, OCH_2[N]
OCH_2[N]	76.56	t	3.697	4	m	—	CH_2[N], CH_3[N]
CH_2[N]	22.93	t	1.874	4	m	—	OCH_2[N], CH_3[N]
CH_3[N]	10.47	q	1.038	6	t	7.4	OCH_2[N], CH_2[N]

[N] Not substituted moiety.
[S] Substituted moiety.
[a] Unresolved.
[d] Downfield resonating proton.
[u] Upfield resonating proton.
[x] Broad signal.

humidified atmosphere containing 5% CO_2 in a CO_2 incubator (Jouan, France).

4.3.3. ^{3}H-Thymidine proliferation assay. Peripheral blood mononuclear cells (2×10^5/100 μL/well) suspended in RPMI 1640 medium containing L-glutamine, gentamycin, and 10% FCS were plated on flat-bottomed 96-well plates (Costar) and cultured in the presence of indicated concentrations of glycoconjugates at 37 °C, 5% CO_2 in a humidified atmosphere. In lipopolysaccharide (LPS) co-stimulated cultures, 0.5 μg/mL of *Salmonella typhimurium* derived LPS (Sigma) was added 30 min before the addition of tested compounds. Two days later, 18.5 kBq/well of ^{3}H-thymidine (Amersham, UK) was added to each well and allowed to incubate for additional 16 h before cells were harvested using 96-well harvester (Tomtec, SF) on glass fiber filters (Wallac, SF) and analyzed on scintillation counter (Microbeta TriLux, Wallac, SF). All samples were tested in tetraplicates. Results are expressed as stimulation index (cpm experimental/cpm control = SI).

4.3.4. Cytotoxicity assay. The in vitro NK cell-mediated cytotoxicity was estimated using the standard ^{51}Cr-release assay with human PBMC; we utilized the NK-sensitive K562 target cell line for 60 min labeled by $Na_2{}^{51}CrO_4$. Effector cells at the indicated concentrations were incubated with 10/4 target cells in round-bottomed 96-well microtiter plates (NUNC), at 37 °C in a humidified atmosphere containing 5% CO_2.

Table 5. ^{1}H and ^{13}C NMR data (399.87 and 100.55 MHz, DMSO-d_6, 30 °C) of compound **3**

Atom	δ_C	m	δ_H	n_H	m	J [Hz]
1	83.77	d	5.330	2	br s	—
2	54.85	d	3.548	2	m	[b]
3	74.05	d	3.382	2	m	[b]
4	70.53	d	3.14[a]	2	m	[b]
5	78.52	d	3.130	2	m	[b]
6	60.80	t	3.643	2	dd	11.4, 4.8
			3.479	2	dd	[b]
2-NH	—	—	8.105	2	d	9.3
2-Ac	23.02	q	1.812	6	s	—
CO	170.81	s	—	0	—	—
3-OH	—	q	4.994	2	d	—
4-OH	—	q	5.018	2	d	—
6-OH	—	q	4.490	2	br s	—
1-NH	—	—	7.393	2	br s	—
CS	180.97	s	—	0	—	—
Ar–NH	—	—	9.557	2	br s	—
Ar–CH_2	30.21	t	4.339	8	d	12.9
			4.328		d	12.9
			3.18[a]		d	[b]
ortho^{S}	134.85[x]	s	—	0	—	—
meta^{S}	124.40[x]	d	6.865	4	br s	—
para^{S}	131.92[x]	s	—	0	—	—
ipso^{S}	153.79	s	—	0	—	—
$OCH_2{}^{S}$	76.45[c]	t	3.839	4	m	—
$CH_2{}^{S}$	22.78	t	1.910	4	m	—
$CH_3{}^{S}$	10.17	q	0.948	6	t	7.5
ortho^{N}	134.00[x]	s	—	0	—	—
meta^{N}	127.93	d	6.586	4	m	—
para^{N}	122.02	d	6.478	2	m	—
ipso^{N}	155.81	s	—	0	—	—
$OCH_2{}^{N}$	76.41[c]	t	3.767	4	m	—
$CH_2{}^{N}$	22.87	t	1.881	4	m	—
$CH_3{}^{N}$	10.34	q	0.993	6	t	7.4

[N] Not substituted moiety.
[S] Substituted moiety.
[a] HMQC readout.
[b] Unresolved.
[c] Might be interchanged.
[x] Broad signal.

Evaluation of NK cell activity was performed after 4 h of incubation as described before.[37] The cell free supernatants were harvested (0.025 mL/sample). Scintillation cocktail (SuperMix, Wallac, SF; 0.1 mL) was added, and radioactivity measured using scintillation counter Microbeta Trilux (Wallac, SF).

4.3.5. Statistical analysis. The sum of results was expressed as mean value ± SEM of individual experiments. Statistical significance of differences between groups was calculated by paired Student's *t*-test. *p* Values lower than 0.05 were considered as significant ($p < 0.05 = *$, $p < 0.01 = **$).

Acknowledgments

Support from the Grant Nos. IAA400200503 and IAA500200620 from Grant Agency of Academy of Sciences of the Czech Republic, COST D34 (MŠMT OC 136), GACR 203/06/1796, and MŠMT LC06010 are gratefully acknowledged.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2007.04.026. Refs. 33–36 are cited in the Supplementary data.

References

1. Raulet, D. H. *Nat. Immunol.* **2004**, *5*, 996–1002.
2. Pisegna, S.; Zingoni, A.; Pirozzi, G.; Cinque, B.; Cifone, M. G.; Morrone, S.; Piccoli, M.; Frati, L.; Palmieri, G.; Santoni, A. *J. Immunol.* **2002**, *169*, 68–74.
3. Bezouška, K.; Vlahas, G.; Horváth, O.; Jinochová, G.; Fišerová, A.; Giorda, R.; Chambers, W. H.; Feizi, T.; Pospíšil, M. *J. Biol. Chem.* **1994**, *269*, 16945–16952.
4. Krist, P.; Herkommenová-Rajnochová, E.; Rauvolfová, J.; Semeňuk, T.; Vavrušková, P.; Pavlíček, J.; Bezouška, K.; Petruš, L.; Křen, V. *Biochem. Biophys. Res. Commun.* **2001**, *287*, 11–20.
5. Bezouška, K.; Sklenář, J.; Dvořáková, J.; Havlíček, V.; Pospíšil, M.; Thiem, J.; Křen, V. *Biochem. Biophys. Res. Commun.* **1997**, *238*, 149–153.
6. Pavlíček, J.; Sopko, B.; Ettrich, R.; Kopecký, V., Jr.; Baumruk, V.; Man, P.; Havlíček, V.; Vrbacký, M.; Martínková, L.; Křen, V.; Pospíšil, M.; Bezouška, K. *Biochemistry* **2003**, *42*, 9295–9306.
7. Bezouška, K.; Křen, V.; Kieburg, C.; Lindhorst, T. K. *FEBS Lett.* **1998**, *426*, 243–247.
8. Pospíšil, M.; Vannucci, L.; Fišerová, A.; Krausová, K.; Horváth, O.; Křen, V.; Mosca, F.; Sadalapure, K.; Lindhorst, T. K.; Bezouška, K. *Adv. Exp. Biol. Med.* **2001**, *495*, 343–348.
9. Sears, P.; Wong, C. H. *Proc Natl. Acad. Sci. U.S.A.* **1996**, *93*, 12086–12093.
10. *Carbohydrate-Based Drug Discovery*; Wong, C.-H., Ed.; Wiley-VCH: Weinheim, 2003.
11. Roy, R.; Zanini, D.; Meunier, S. J.; Romanowska, A. *J. Chem. Soc., Chem. Commun.* **1993**, 1869–1872.
12. Roy, R.; Baek, M. *Rev. Mol. Biotechnol.* **2002**, *90*, 291–309.
13. Turnbull, W. B.; Stoddart, J. F. *Rev. Mol. Biotechnol.* **2002**, *90*, 231–255.
14. Roy, R. *Top. Curr. Chem.* **1997**, *187*, 241–274.
15. Bezouška, K. *Rev. Mol. Biotechnol.* **2002**, *90*, 269–290.
16. Vannucci, L.; Fišerová, A.; Sadalapure, K.; Lindhorst, T. K.; Kuldová, M.; Rossmann, P.; Horváth, O.; Křen, V.; Krist, P.; Bezouška, K.; Luptovcová, M.; Mosca, F.; Pospíšil, M. *Int. J. Oncol.* **2003**, *23*, 285–296.
17. Gutsche, C. D. In *Calixarenes: Monographs in Supramolecular Chemistry*; Stoddart, J. F., Ed.; The Royal Society of Chemistry: Cambridge, England, 1989; Vol. I.
18. Vicens, J.; Asfari, Z.; Harrowfield, J. M. *Calixarenes 50th Anniversary: Commemorative Issue*; Kluwer Academic: Dordrecht, 1994.
19. *Calixarenes: a Versatile Class of Macrocyclic Compound*; Vicens, J., Böhmer, V., Eds.; Kluwer: Dordrecht, 1991.

20. *Calixarenes*; Asfari, Z., Böhmer, V., Harrowfield, J., Vicens, J., Eds.; Kluwer Academic: Dordrecht, 2001.
21. Bezouška, K. *Collect. Czech. Chem. Commun.* **2004**, *69*, 535–563.
22. Pospíšil, M.; Vannucci, L.; Horváth, O.; Fišerová, A.; Krausová, K.; Bezouška, K.; Mosca, F. *Int. J. Oncol.* **2000**, *16*, 267–276.
23. Křen, V. In *Chemical Probes in Biology*; Schneider, M. P., Ed.; Kluwer: Amsterdam, 2003; pp 379–389.
24. Bezouška, K.; Pavlíček, J.; Ettrich, R.; Man, P.; Jedelský, P.; Pompach, P.; Švábová, L.; Fišerová, A.; Martínková, L.; Křen, V. *Immunol. Lett.* **2003**, *87*, 82.
25. Fialová, P.; Namdjou, D.-J.; Ettrich, R.; Přikrylová, V.; Rauvolfová, J.; Křenek, K.; Kuzma, M.; Elling, L.; Bezouška, K.; Křen, V. *Adv. Synth. Catal.* **2005**, *347*, 997–1006.
26. Semeňuk, T.; Krist, P.; Pavlíček, J.; Bezouška, K.; Kuzma, M.; Novák, P.; Křen, V. *Glycoconjugate J.* **2001**, *18*, 817–826.
27. Krist, P.; Vannucci, L.; Kuzma, M.; Man, P.; Sadalapure, K.; Patel, A.; Bezouška, K.; Pospíšil, M.; Petruš, L.; Lindhorst, T. K.; Křen, V. *ChemBioChem* **2004**, *4*, 445–452.
28. Böhmer, V. *Angew. Chem., Int. Ed.* **2003**, *34*, 713–745.
29. Matthews, S. E.; Saadioui, M.; Boehmer, V.; Barboso, S.; Arnaud-Neu, F.; Schwing-Weill, M.-J.; Carrera, A. G.; Dozol, J.-F. *J. Prakt. Chem./Chem.-Ztg.* **1999**, *341*, 264–273.
30. Camarasa, M. J.; Fernandez-Resa, P.; Garcia-Lopez, M. T.; de las Heras, F. G.; Mendez-Castrillon, P.; Felix, A. San *Synthesis* **1984**, *6*, 509–510.
31. Consoli, G. M. L.; Cunsolo, F.; Geraci, C.; Sgarlata, V. *Org. Lett.* **2004**, *6*, 4163–4166.
32. van Wageningen, A. M. A.; Snip, E.; Verboom, W.; Reinhoudt, D. N.; Boerrigter, H. *Liebigs Ann.* **1997**, 2235–2245.
33. Lang, K.; Cuřínová, P.; Dudič, M.; Prošková, P.; Stibor, I.; Šťastný, V.; Lhoták, P. *Tetrahedron Lett.* **2005**, *46*, 4469–4472.
34. van Wageningen, A. M. A.; Snip, E.; Verboom, W.; Reinhoudt, D. N.; Boerrigter, H. *Liebigs Ann. Recl.* **1997**, *11*, 2235–2245.
35. Conner, M.; Janout, V.; Regen, S. L. *J. Org. Chem.* **1992**, *57*, 3744–3746.
36. Pinkhassik, E.; Sidorov, V.; Stibor, I. *J. Org. Chem.* **1998**, *63*, 9644–9651.
37. Fišerová, A.; Kovářů, H.; Hajduová, Z.; Mareš, V.; Starec, M.; Křen, V.; Flieger, M.; Pospíšil, M. *Physiol. Res.* **1997**, *46*, 119–125.
38. Iwamoto, K.; Shinkai, S. *J. Org. Chem.* **1992**, *57*, 7066–7073.
39. Casnati, A.; Ting, Y.; Berti, D.; Fabbi, M.; Pochini, A.; Ungaro, R.; Sciotto, D.; Lombardo, G. G. *Tetrahedron* **1993**, *59*, 9815–9822.
40. Pavlíček, J.; Kavan, D.; Pompach, P.; Novák, P.; Lukšan, O.; Bezouška, K. *Biochem. Soc. Trans.* **2004**, *32*, 1124–1126.