APPENDIX 1.

Complete results of activation assays, natural killing and apoptosis assays

1.1. Inositolphosphate production

Peripheral blood mononuclear cells loaded with [^{3}H]inositol were incubated with the tested compounds for 0, 2, 4, 6, 8 and 10 minutes. The amount of Ins2P and Ins3P released into the cytoplasm of effector cell upon activation with the tested compounds was measured using liquid scintillation counter. Complete time course of inositolphosphate production for individual compounds of thiourea series can be seen on figure 1.1 - 1.4.

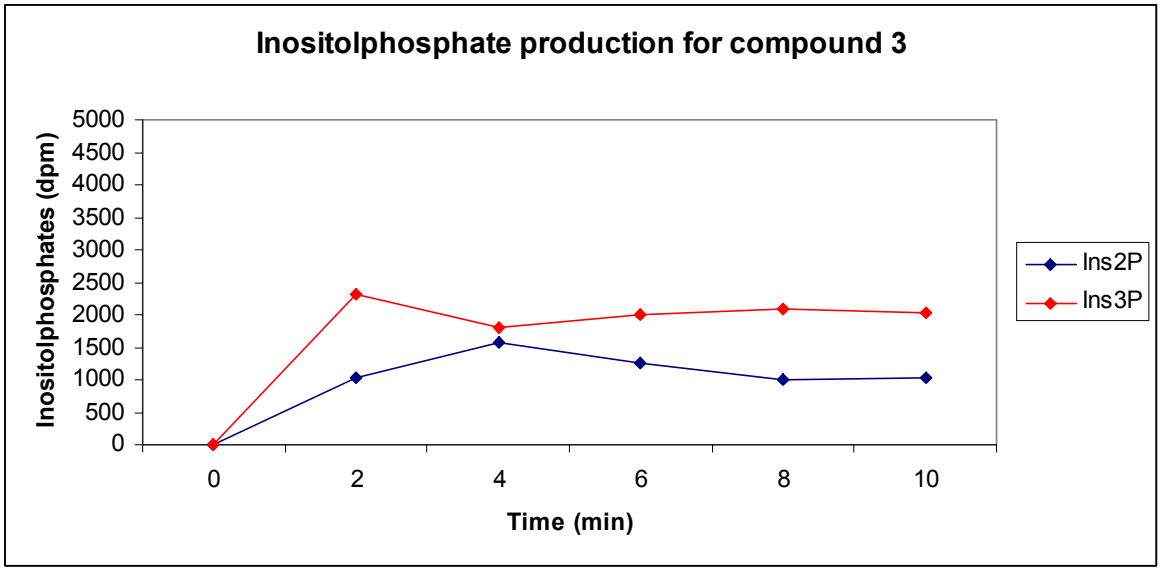

Figure 1.1. *Complete time course experiment regarding Ins2P and Ins3P production upon activation with compound 3.*

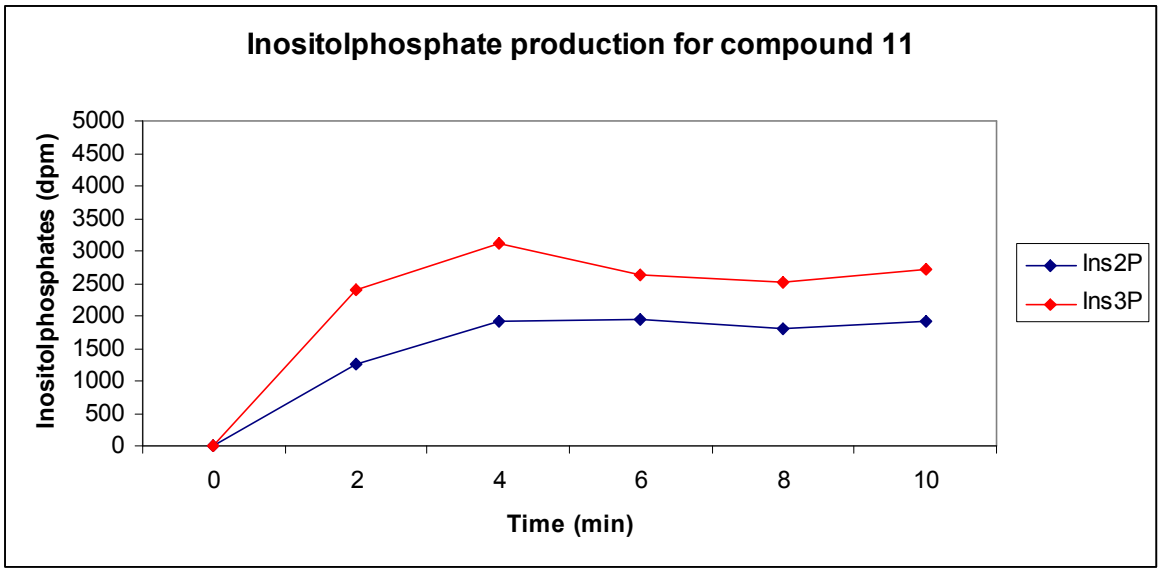

Figure 1.2. *Complete time course experiment regarding Ins2P and Ins3P production upon activation with compound 11.*

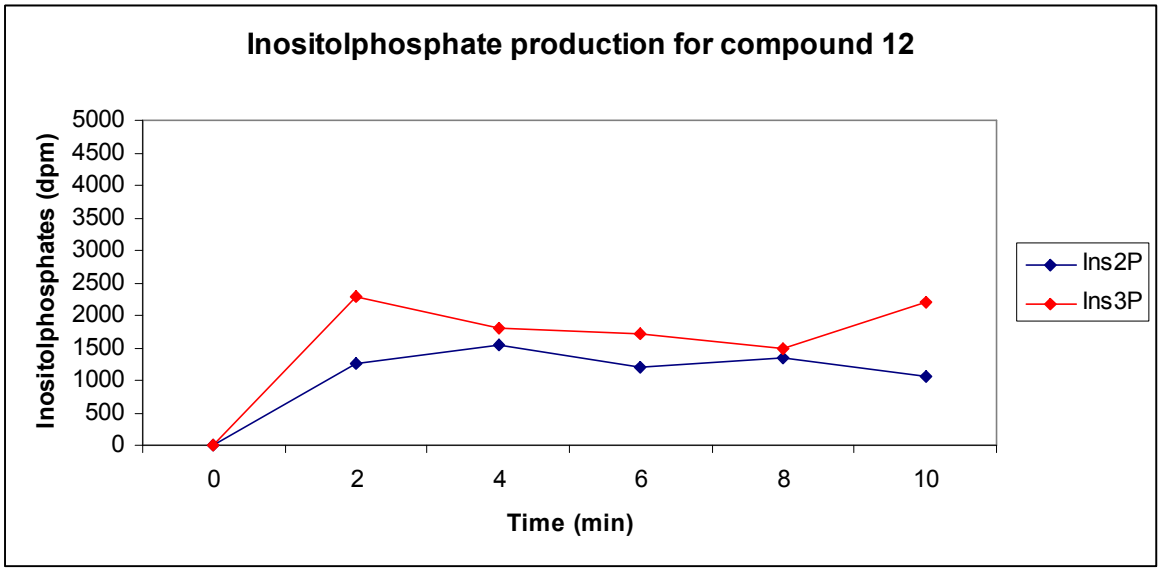

Figure 1.3. *Complete time course experiment regarding Ins2P and Ins3P production upon activation with compound 12.*

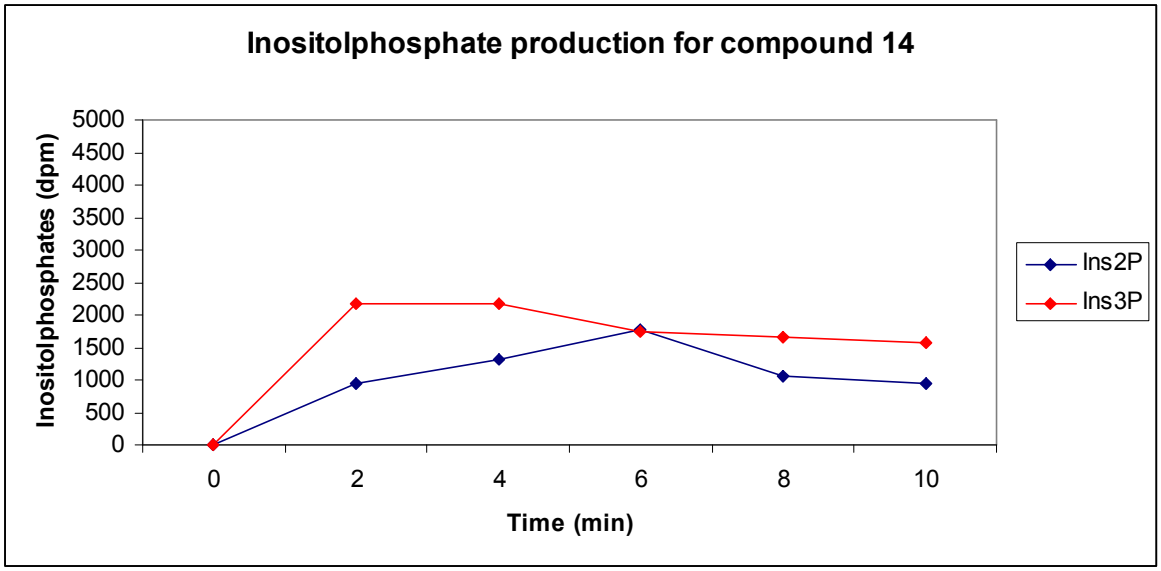

Figure 1.4. *Complete time course experiment regarding Ins2P and Ins3P production upon activation with compound 14.*

Maximum concentration of Ins2P is achieved later than maximum concentration of Ins3P what is in correspondence with proposition that Ins2P is created by dephosphorylation from Ins3P. Complete time course experiments of inositolphosphate production for compounds of triazole series can be found in figure 2.1 - 2.4.

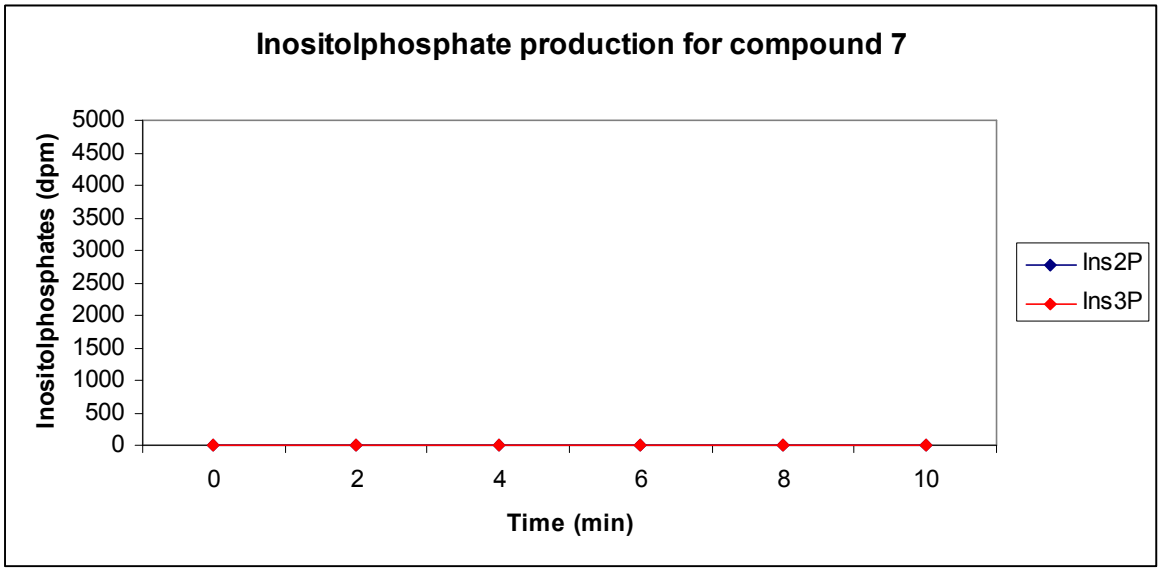

Figure 2.1. *Complete time course experiment regarding Ins2P and Ins3P production upon activation with compound 7.*

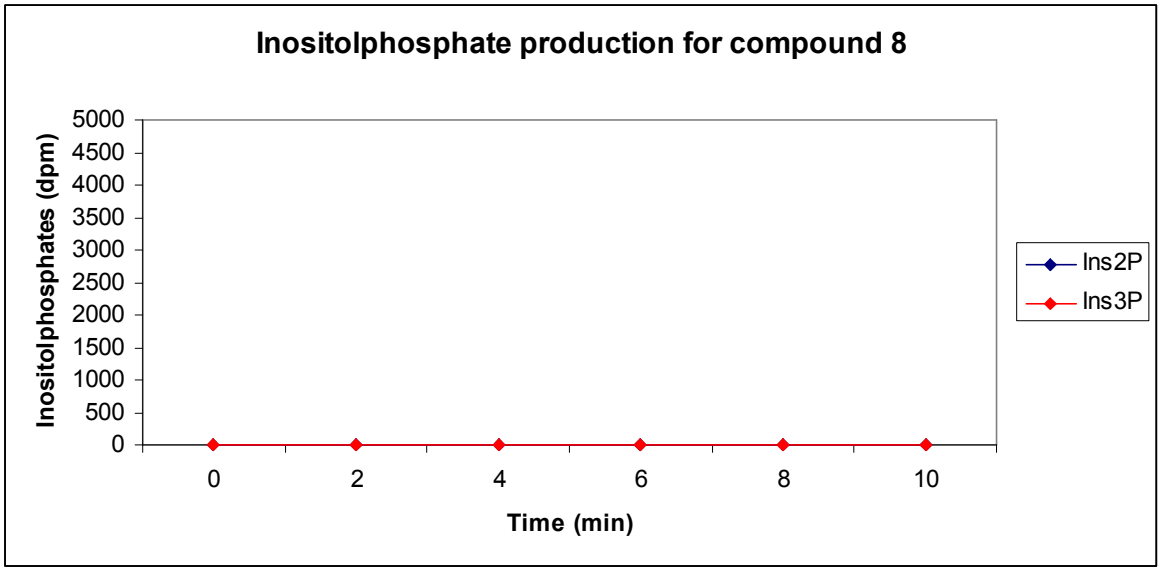

Figure 2.2. *Complete time course experiment regarding Ins2P and Ins3P production upon activation with compound 8.*

As we can see, compounds **7** and **8** had no effect at all on phosphoinositide signaling in peripheral blood mononuclear cells. On the other hand, compound **17** (figure 2.4) had great effect on production of inositolphosphates.

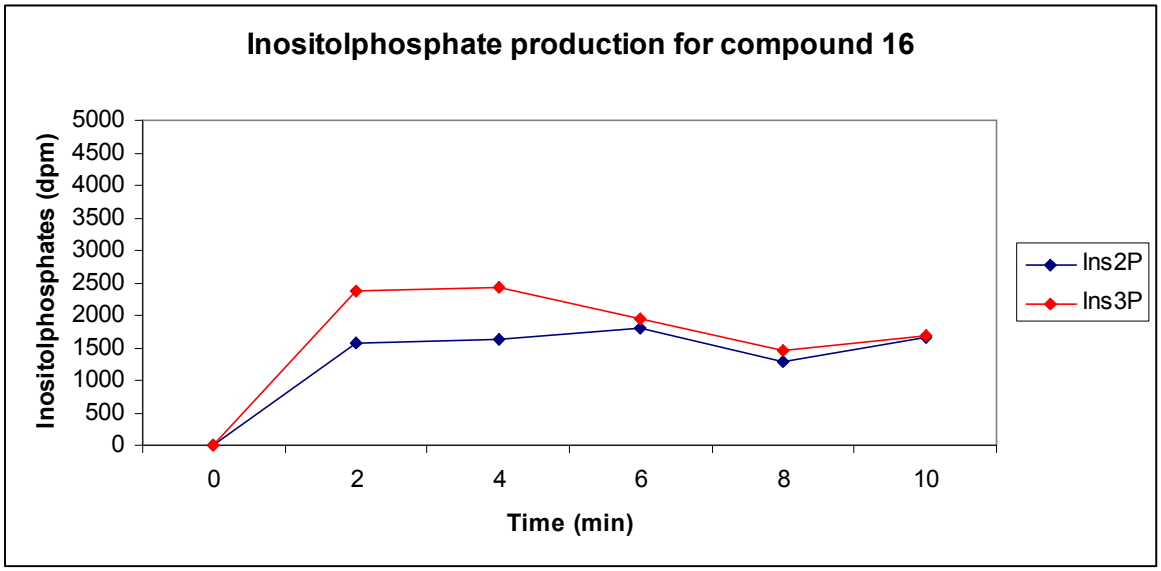

Figure 2.3. *Complete time course experiment regarding Ins2P and Ins3P production upon activation with compound 16.*

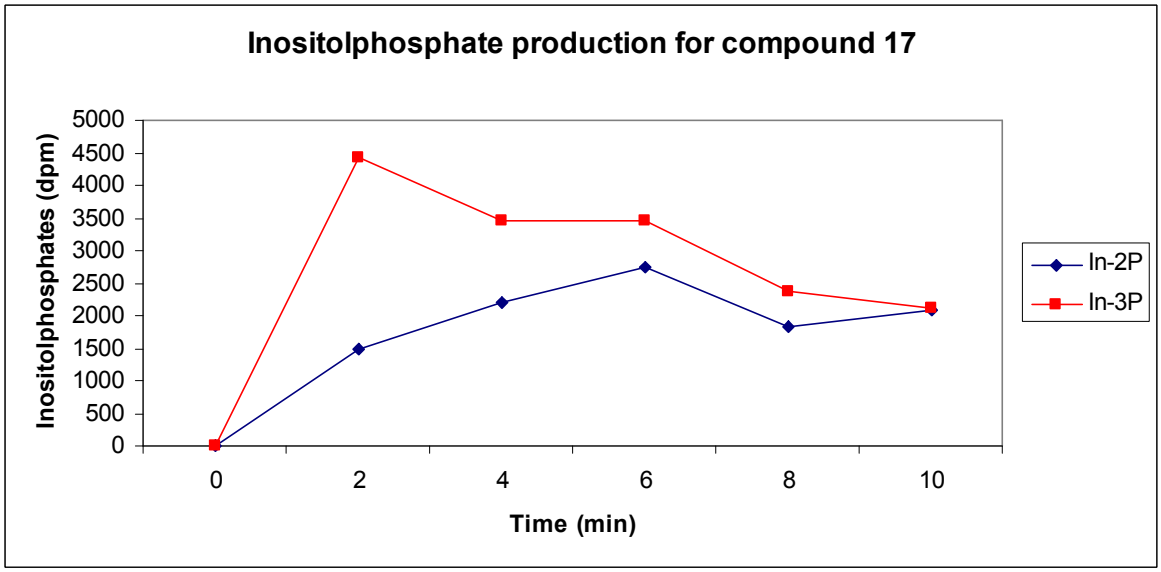

Figure 2.4. *Complete time course experiment regarding Ins2P and Ins3P production upon activation with compound 17.*

1.2. Intracellular calcium monitoring

Concentration of Ca^{2+} ions released in peripheral blood mononuclear cells upon activation with the tested compounds was counted using an equation introduced in section 3.2.3. and these data were used to create a plot showing a dependance of Ca^{2+} concentration on time for individual compounds (figure 3.1 and 3.2).

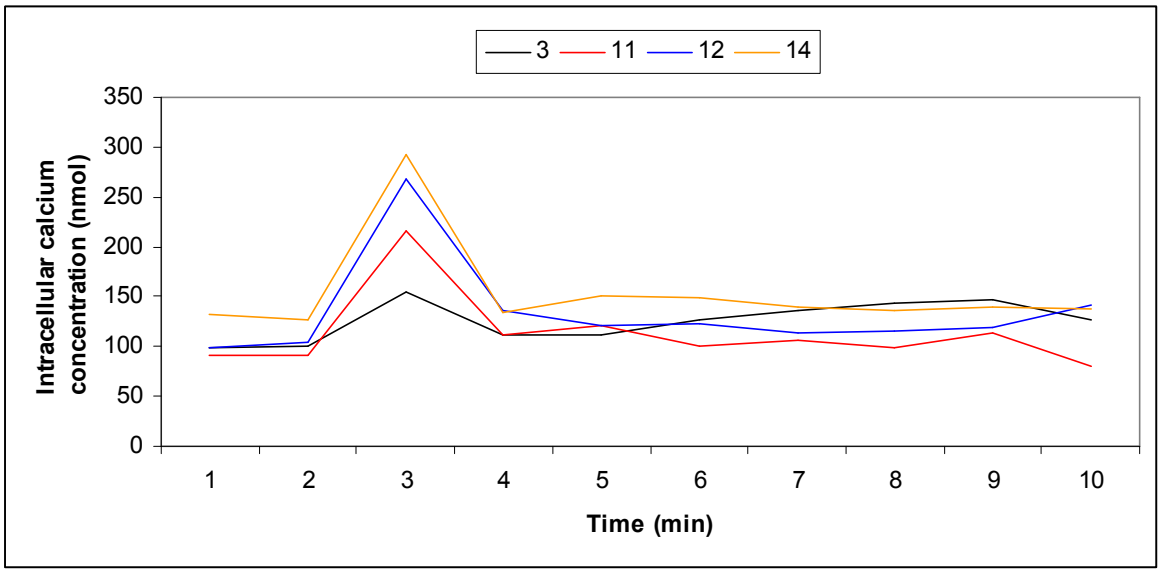

Figure 3.1. Time course experiments of intracellular calcium concentration for compounds of thiourea series: *Complete time course experiments for intracellular calcium level elevation upon activation with the compounds of thiourea series are shown.*

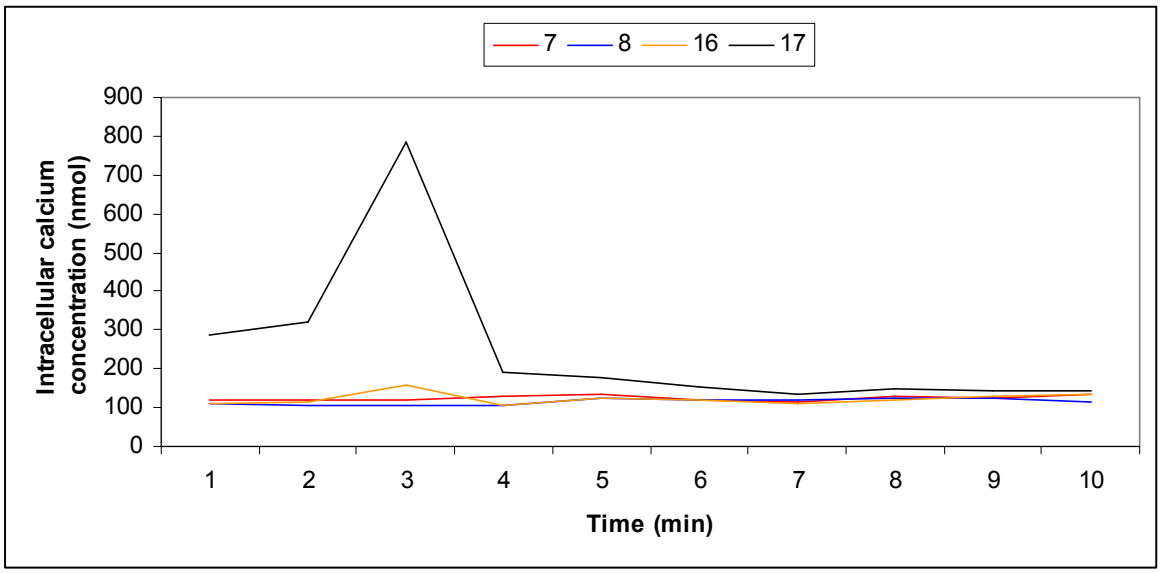

Figure 3.1. Time course experiments of intracellular calcium concentration for compounds of triazole series: *Complete time course experiments for intracellular calcium level elevation upon activation with the compounds of triazole series are shown.*

1.3. Natural killing

^{51}Cr radiolabeled cells of leukemic cell line were incubated with peripheral blood mononuclear cells activated by the tested compounds in ratio of effector to target cell E:T 30:1, 100:1, 300:1 and 1000:1. Specific cytotoxicity was counted using a formula introduced in part 3.2.4. and killing curves for individual compounds were constructed. These killing curves can be found on figure 4.1 and 4.2.

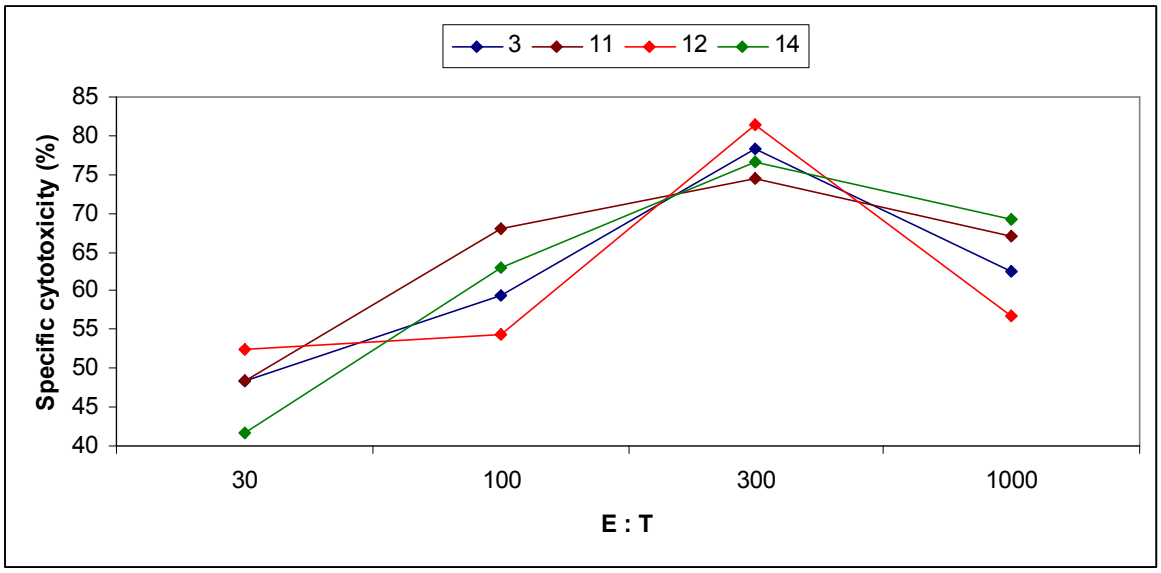

Figure 4.1. Killing curves for compounds of thiourea series: *Killing curves as a dependance of specific cytotoxicity on E:T ratio for compounds of thiourea series are shown.*

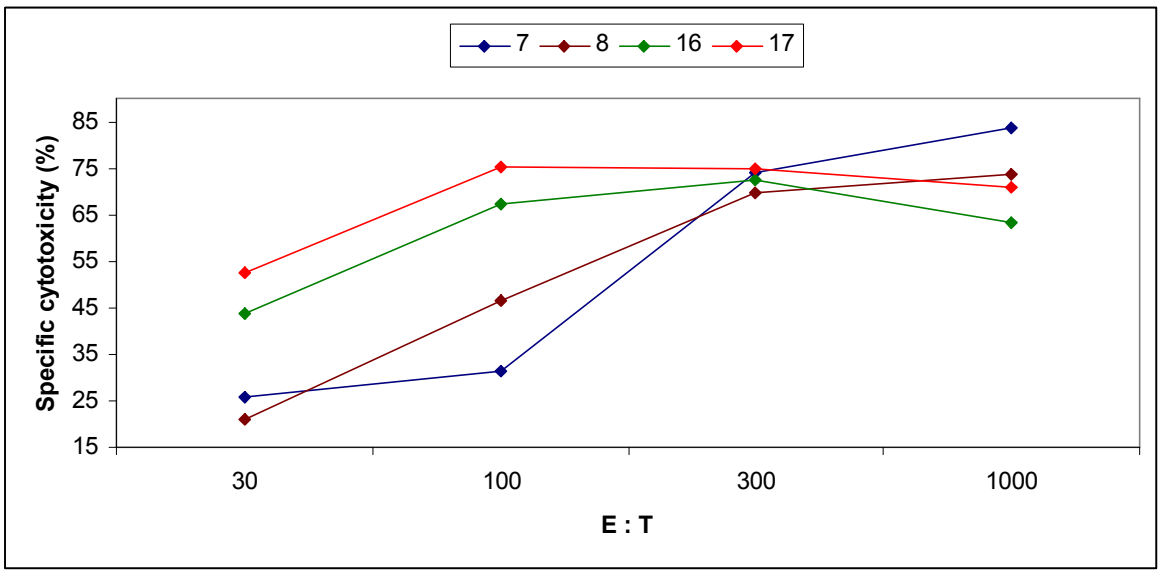

Figure 4.1. Killing curves for compounds of triazole series: *Killing curves as a dependance of specific cytotoxicity on E:T ratio for compounds of triazole series are shown.*

1.4. Apoptosis assays

Peripheral blood mononuclear cells were incubated with the tested compounds for 6 and 12 hours, respectively. After this time period, a percentage of early and late apoptotic cells was estimated using flow cytometry.

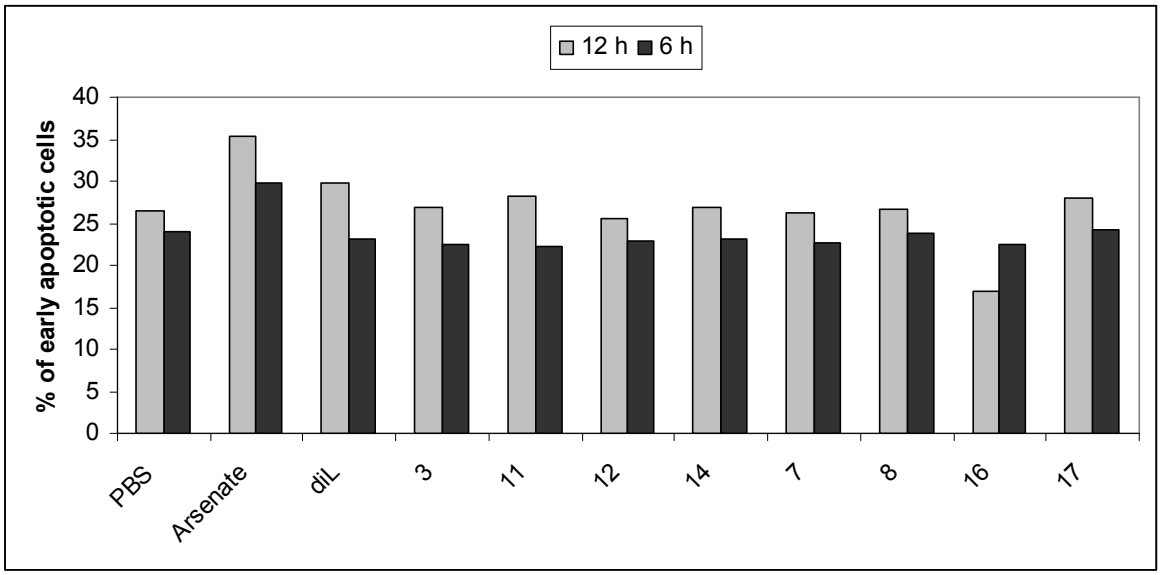

Figure 5.1. Percentage of early apoptotic cells after incubation of PBMC isolated from donor 1 with the tested compounds for given time periods: *PBMC from donor 1 were incubated with the tested compounds for 6 and 12 hours, respectively, and the amount of early apoptotic cells was measured using flow cytometry.*

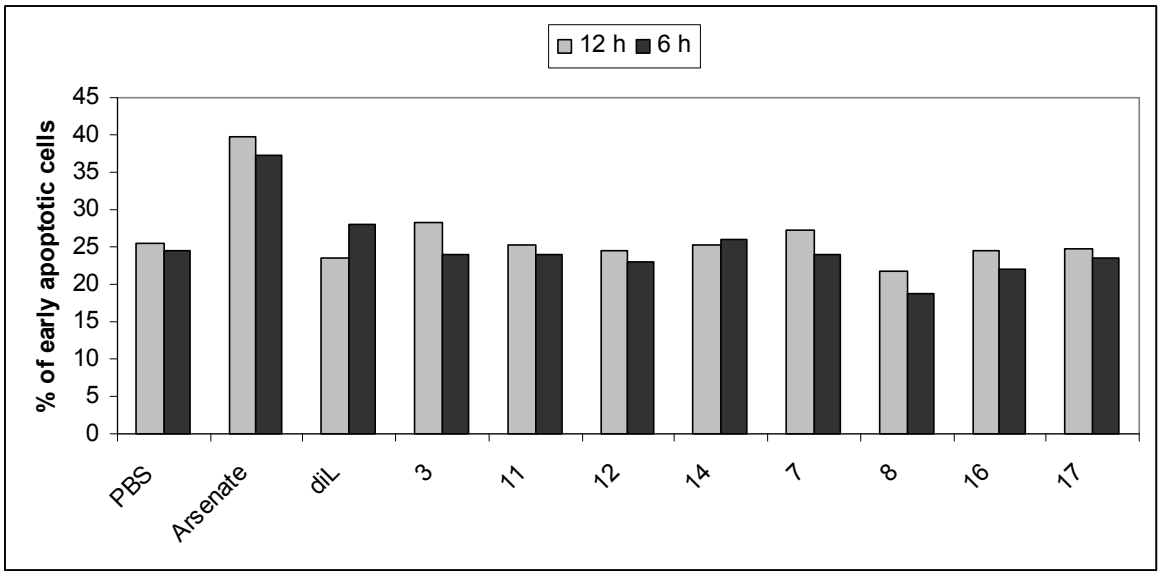

Figure 5.1. Percentage of early apoptotic cells after incubation of PBMC isolated from donor 2 with the tested compounds for given time periods: *PBMC from donor 2 were incubated with the tested compounds for 6 and 12 hours, respectively, and the amount of early apoptotic cells was measured using flow cytometry.*

Several parallel experiments were performed with PBMC obtained from donors with different CD69 expression. As a result, the obtained data were markedly heterogenous. Figure 5.1 and 5.2 (previous page) shows the percentage of early apoptotic cells in samples obtained from two different donors. Besides early apoptosis, cells undergoing late apoptosis were measured. However, the results of this estimation are not probative since all of the tested compounds together with positive and negative control caused approximately the same extent of late apoptosis in PBMC. This is illustrated on figure 6.1.

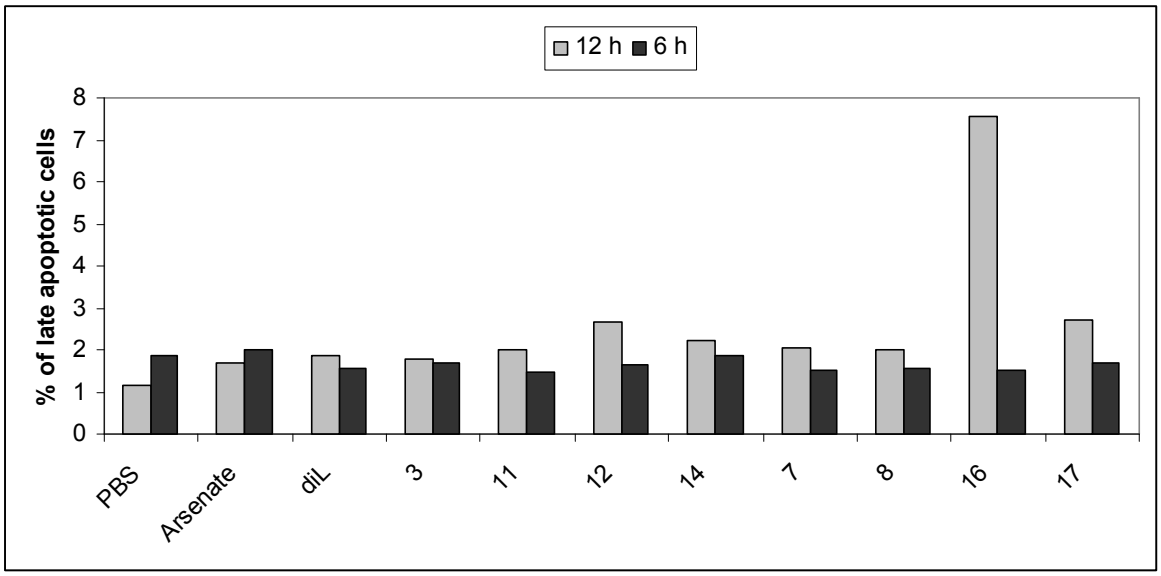

Figure 6.1. Percentage of late apoptotic cells after incubation of PBMC isolated from donor 2 with the tested compounds for given time periods: *PBMC from donor 2 were incubated with the tested compounds for 6 and 12 hours, respectively, and the amount of late apoptotic cells was measured using flow cytometry.*

APPENDIX 2.

An article published in Car. Res. discussing the data obtained by experiments with the tested compounds

Enzymatic synthesis of dimeric glycomimetic ligands of NK cell activation receptors

Anna Drozdová, Pavla Bojarová, Karel Křenek, Lenka Weignerová, Birgit Henßen, Lothar Elling, Helle Christensen, Henrik H. Jensen, Helena Pelantová, Marek Kuzma, Karel Bezouška, Monika Krupová, David Adámek, Kristýna Slámová, Vladimír Křen

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Enzymatic synthesis of dimeric glycomimetic ligands of NK cell activation receptors

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Abstract – This work reveal new structural relationships in the complex process of the interaction between activation receptors of natural killer cells (rat NKR-P1, human CD69) and novel bivalent carbohydrate glycomimetics. The length, glycosylation pattern and linker structure of receptor ligands were examined with respect to their ability to precipitate the receptor protein from solution, which simulates the *in vivo* process of receptor aggregation during NK cell activation. It was found that di-LacdiNAc triazole compounds show optimal performance, reaching up to 100% precipitation of the present protein receptors, and achieving high immunostimulatory activities without any tendency to trigger activation-induced apoptosis. In the synthesis of the compounds tested, two enzymatic approaches were applied. Whereas a β-*N*-acetylhexosaminidase could only glycosylate one of the two acceptor sites available with yields below 10%, the Y284L mutant of human placental β1,4-galactosyltransferase-1 worked as a perfect synthetic tool, accomplishing even quantitative glycosylation at both acceptor sites and with absolute regioselectivity for the C-4 position. This work insinuates new directions for further ligand structure optimisation and demonstrates the strong synthetic potential of the mutant human placental β1,4-galactosyltransferase-1 in the synthesis of multivalent glycomimetics and glycomaterials.

Keywords: β-*N*-Acetylhexosaminidase; Galactosyltransferase; Enzymatic glycosylation; Natural killer cell; CD69; Triazole

Abbreviations: Ins2P, inositolbisphosphate; Ins3P, inositoltrisphosphate; NK, natural killer; PBMC, peripheral blood mononuclear cells; PI, propidium iodide; GlcNAc-*t*Boc, 1-(β-D-2-acetamido-2-deoxyglucopyranosyl)-3-[2-(*terc*.butyloxycarbonylamino)ethyl]-thiourea; LacdiNAc, *N*-acetyl-β-D-galactosaminyl-(1→4)-*N*-acetyl-D-glucosamine; PBS, phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4.2H_2O$, 1.8 mM KH_2PO_4 pH 7.4); UDP-Gal, uridine 5`-diphosphogalactose; UDP-GalNAc, uridine 5`-diphospho-*N*-acetylgalactosamine; *p*NP-β-GlcNAc, *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide; *p*NP-β-GalNAc, *p*-nitrophenyl *N*-acetyl-β-D-galactosaminide.

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1. Introduction

Glycosylation processes set up platforms for numerous *in vivo* mechanisms. Two enzyme classes, glycosidases (EC 3.2.1.-) and glycosyltransferases (EC 2.4.-.-), are conveniently applied in the preparative synthesis of carbohydrate structures, targeted, *inter alia*, for fighting malignant diseases in the form of immunotherapeutics. Both of these enzyme classes can transfer glycosyl moieties from activated sugar donors (nitrophenyl glycosides or sugar nucleotides, respectively) to various acceptors.[1-3]

This study is primarily aimed at the preparation of divalent LacdiNAc ligands for the NK cell activation receptors and, secondly, at comparing the performance of two different enzymatic approaches for glycosylation of multivalent (divalent) acceptors. GlcNAc dimers containing various types of spacers and glycosidic linkages, *i. e.*, thiourea and triazoline linkers, were employed. Dimeric LacdiNAc glycomimetics represent a new generation of agonists of activation receptors of natural killer (NK) cells, primarily NKR-P1 and CD69.[4] The pioneer compound of this type, 1,2-bis[*N´*-(2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl)-thioureido]-ethane, could efficiently precipitate both soluble and cellular forms of CD69, and thus trigger leukocyte activation and cytokine secretion.[5,6] Since NK cells are a unique population of lymphocytes able to eliminate malignant, virally infected, or damaged cells,[7,8] this class of compounds may show a new way in experimental tumour therapy.[9,10]

The two enzymes used in the synthetic pathway are the β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066 (EC 3.2.1.52) and the Y284L mutant of human placental β1,4-galactosyltransferase-1 (EC 2.4.1.38).

The synthetic potential of β-*N*-acetylhexosaminidases has been demonstrated many times with a wide array of more or less peculiar glycosyl donors and acceptors.[11,12] In the transglycosylation synthetic mode, it is a key factor to optimise the reactant concentrations and the reaction time in order to eliminate the formation of unwanted products such as autocatalytic products and other regioisomers or eventual hydrolysis of products. The β-*N*-acetylhexosaminidase from *A. oryzae* has previously been shown to prefer the formation of β(1→4)-bound products,[13-15] which is an important factor in the proposed synthesis.

β1,4-Galactosyltransferase-1 (β4GalT-1) is a membrane-bound, Mn^{2+}-dependent glycoprotein. *In vivo*, it produces *N*-acetyllactosamine or lactose using UDP-Gal as a galactosyl donor and β-D-GlcNAc or β-D-Glc as acceptors, respectively.[16,17] It disposes over a broad substrate specificity, as abundantly demonstrated previously.[18-21] Despite this fact, its ability to utilize *N*-acetylhexosamine nucleotides (UDP-GalNAc) as glycosyl donors is negligible.[22] This is because an extra hydrogen bond is formed between the active-site Tyr and the substrate *N*-acetamido group, which hinders the transfer of GalNAc.[23] In bovine milk β4GalT-1, the mutation of Tyr289 to Leu increased the ratio of GalNAc/Gal transferase activity to quasi 1:1.[23] We have recently demonstrated the preparation of optimised fusion constructs of human placental β4GalT-1[24,25] and of its Y284L mutant[4] and their use in galactosylation reactions. In contrast to the bovine mutant β4GalT-1,[23] the mutant of the human enzyme uses exclusively UDP-GalNAc as a glycosyl donor and not UDP-Gal – thus, its specificity became much more pronounced through the mutation of the analogous Tyr residue, compared to the bovine mutant.

In this article, we compare the synthetic potential of both enzymatic glycosylations and we demonstrate the synthesis of three novel dimeric LacdiNAc structures based on thiourea- and triazole-linkers.

2. Results and discussion

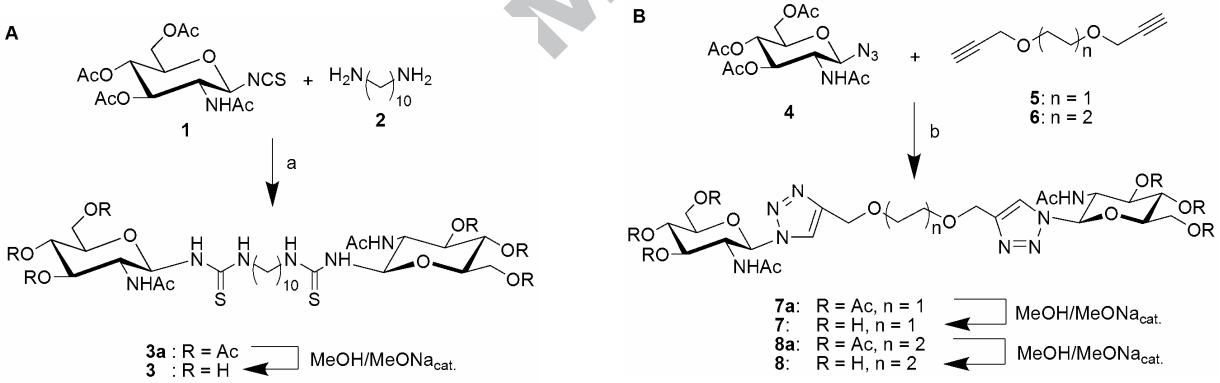
2.1. Synthesis of glycosyl acceptors 3, 7, 8

All three acceptors **3, 7** and **8** were designed in order to enable sufficient flexibility of the divalent linker for bridging two binding sites on the NK cell activation receptors and, at the same time, to be biocompatible and resistant to *in vivo* cleavage by glycosidases. The choice

of the triazole-linkers was also supported by our previous study on 2-acetamido-2-deoxy-β-D-glucopyranosyl-1*H*-1,2,3-triazole demonstrating that the attached triazole moiety promotes one to two order better binding compared with free GlcNAc.[26] Acceptor **3** contains an *N*-glycosidic thiourea bond and was prepared from the established GlcNAc isothiocyanate (**1**)[27] by reaction with diamine **2** (Scheme 1A). The protected acceptor **3a** was purified by silica gel chromatography and, after Zemplén deprotection, by gel permeation chromatography (Sephadex™ LH-20), to yield **3** (48%).

Dimeric β-GlcNAc 1,2,3-triazoles **7** and **8** (Scheme 1B) were prepared from azide **4**[28,29] and bis-propargyl spacers **5** or **6**, respectively. The assembling step occurred by the Huisgen azide-alkyne cycloaddition as reported by Meldal[30] and Sharpless[31] in the presence of a catalytic amount of $Cu(OAc)_2$ and sodium ascorbate, resulting in peracetates **7a** and **8a** in good yields (51% and 56%, respectively). Finally, Zemplén deprotection yielded **7** and **8** in quantitative yields.

The structures of all acceptors were verified by MS and NMR. They all displayed good solubility in water.



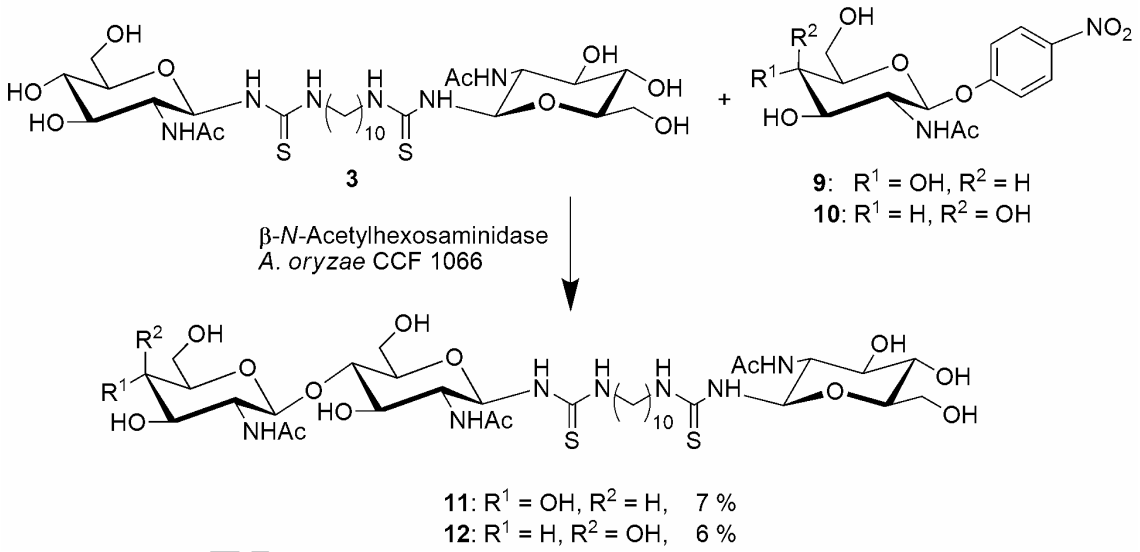
Scheme 1. *Synthesis of glycosyl acceptors.* ***A****, 1,10-bis[*N*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-thioureido]-decane (**3**), a) MeCN/DIPEA.* ***B****, Triazoles **7** and **8**, reagents and conditions: b) $Cu(OAc)_2$, sodium ascorbate, 1:1 acetone/water, r.t., Ar*(g)

2.2. β-*N*-Acetylhexosaminidase-catalysed glycosylation: Synthesis of dimers 11 and 12

The β-*N*-acetylhexosaminidase from *Aspergillus oryzae*[32] was selected for preparative glycosylation of acceptor **3** due to its documented ability to selectively - β(1-4) - glycosylate a range of acceptors including various complex compounds.[33] In addition, it is readily and in high activity available from the enzyme collection located in our laboratory. It is a robust and

stable enzyme with a balanced ratio of GlcNAc-ase/GalNAc-ase activities. For the aim of this reaction, the enzyme was purified as described previously.[34]

The enzyme was applied in two preparative reactions with acceptor **3**, namely the transfer of β-D-GlcNAc moiety (product **9**, *p*NP-β-GlcNAc donor) and of β-D-GalNAc moiety (product **10**, *p*NP-β-GalNAc donor) (Scheme 2). Though the ultimate aim was to prepare a di-LacdiNAc product, this was not possible to accomplish with a β-*N*-acetylhexosaminidase, even with enzymes from other fungal strains than *A. oryzae* (results not shown). Instead of glycosylating the other GlcNAc unit of the bivalent acceptor, the reaction tended to proceed towards side products *e. g*., β(1-6)-bound - especially in the case of *p*NP-β-GlcNAc as donor. As a result, we isolated and characterized asymmetric products **11** and **12** from the reactions with *p*NP-β-GlcNAc and *p*NP-β-GalNAc donors, respectively.

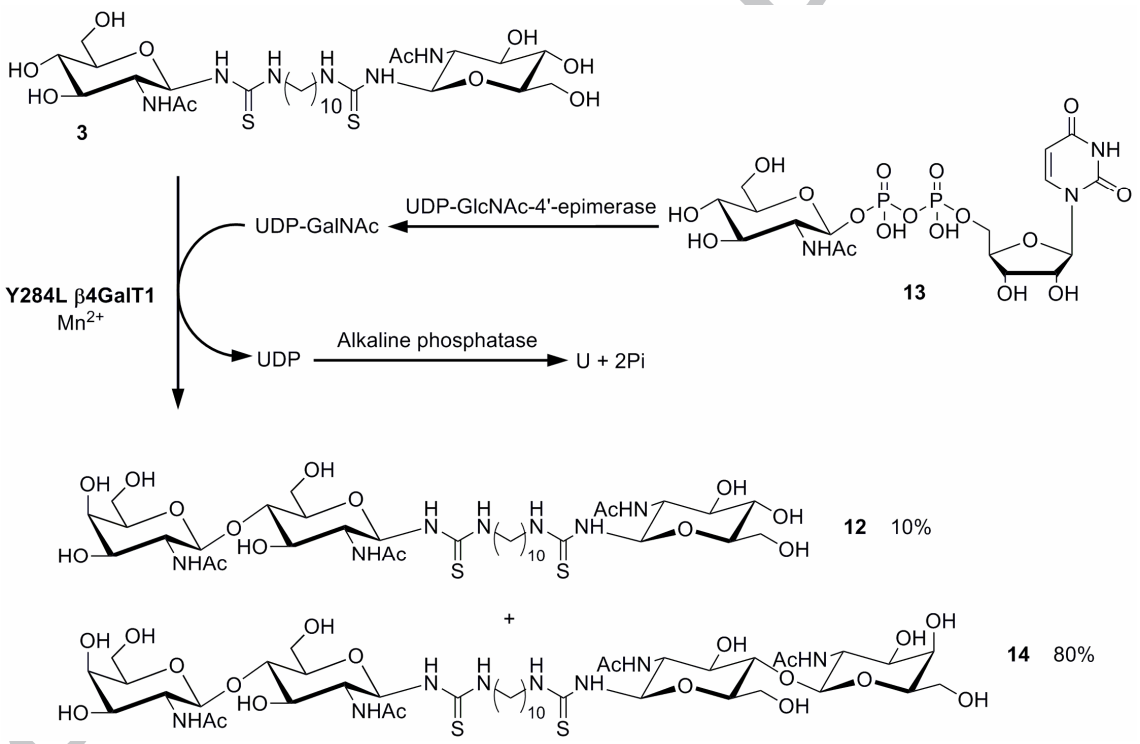


Scheme 2. *Synthesis of compounds **11** and **12**.*

2.3. β1,4-Galactosyltransferase-catalysed GalNAc-transfer: Synthesis of dimers 14-17

Since the glycosylation of the bivalent acceptor **3** at both ends failed with a β-*N*-acetylhexosaminidase, another approach was sought for. Finally, we opted for the Y284L mutant of human placental β1,4-galactosyltransferase-1 (EC 2.4.1.38), which previously has been described and successfully used in a synthetic reaction previously.[4] It can selectively transfer the β-D-GalNAc unit and its purification is straightforward thanks to the presence of a His-tag. The need of a relatively expensive UDP-GalNAc donor can be overcome by employing UDP-GlcNAc in combination with the UDP-GlcNAc-4'-epimerase from

Campylobacter jejuni in one pot.[35] Monitoring of the reaction course and optimisation of reaction conditions were performed by HPLC (Multokrom 100-5 C18 column, CS-Chromatographie Service GmbH, Cologne, DE). The glycosylation of acceptor **3** (Scheme 3) was very efficient. Apart from a small amount of the product **12** (yield 9%) and the starting material **3** (5%) the majority of the desired di-LacdiNAc product **14** (86%) originated in the reaction on an analytical scale (Figure 1). The kinetic characterization of the mutant β4GalT-1 (Y284L) revealed K_m and V_{max} values of 0.213 mM and 137 mU/mg, respectively, for compound **3**, and 0.395 mM and 134 mU/mg, respectively, for UDP-GalNAc. The di-LacdiNAc product **14** was synthesized on preparative scale in 2 days and isolated in a single-step purification by gel chromatography (Sephadex™ LH-20 column) with 80% overall yield (Scheme 3).



Scheme 3. *Synthesis of compounds* ***12*** *and* ***14****.*

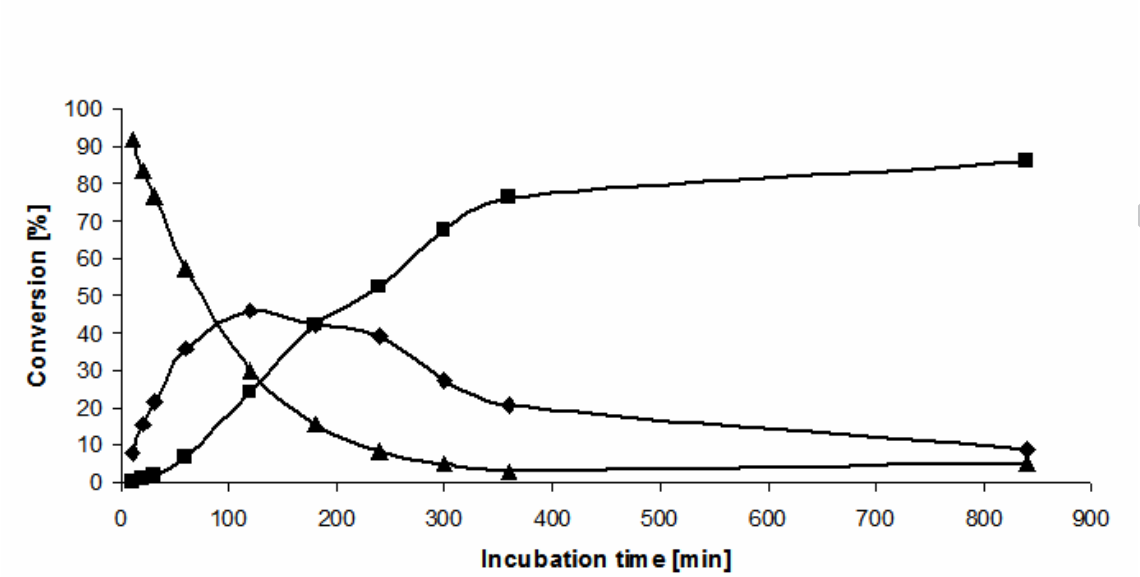
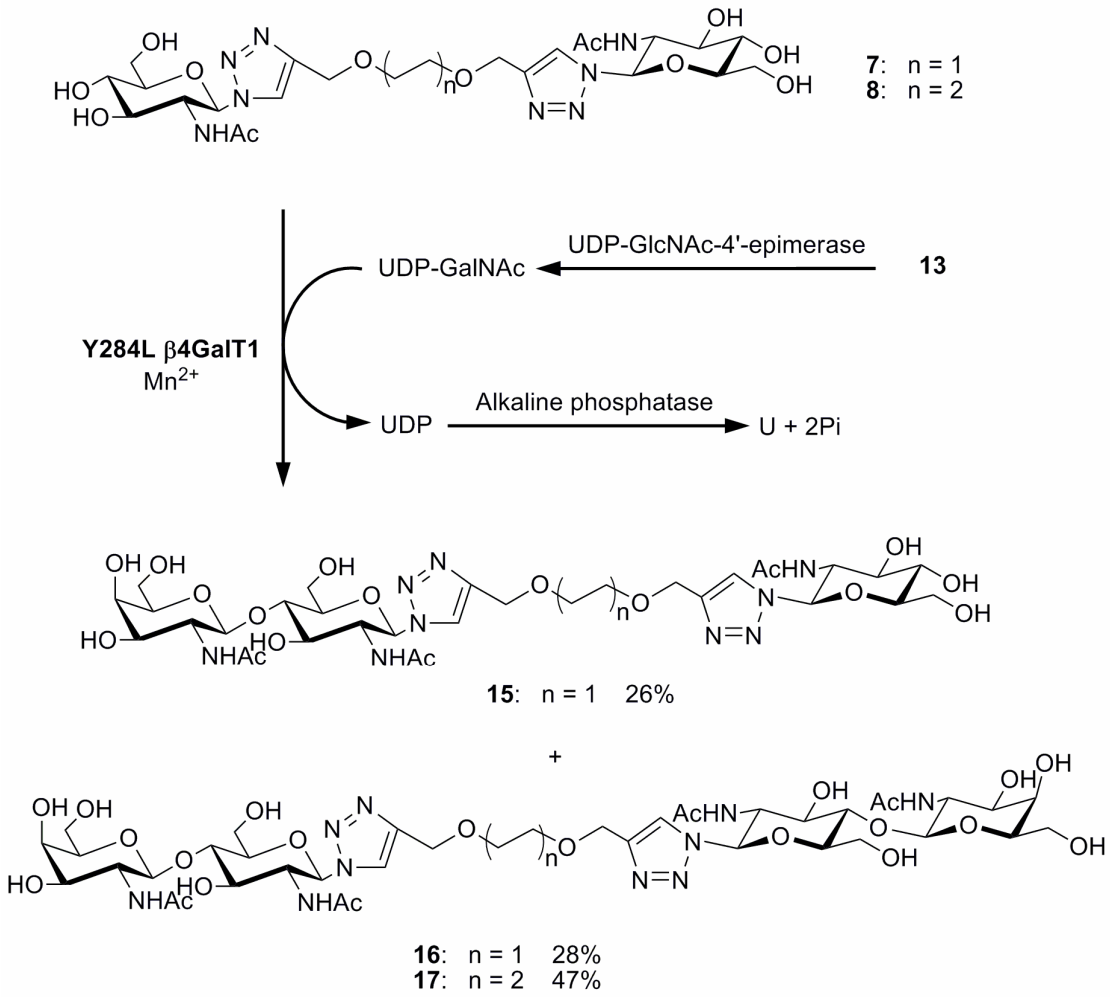


Figure 1. *Time course of the conversion of acceptor* ***3****(▲), intermediate* ***12*** *(♦), and the final product* ***14*** *(■) generation.*

Encouraged by this result, we decided to glycosylate two other GlcNAc dimers with an alternative linker structure, *i. e.* triazole-linked compounds **7, 8**. In both cases, the reactions led to the respective di-LacdiNAc products in good yields (**16** in 28% yield, **17** in 47% yield). During the glycosylation of acceptor **7**, the mono-LacdiNAc product was also obtained (**15**, 25% yield) (Scheme 4).



Scheme 4. *Synthesis of compounds* ***15-17.***

2.4. Binding and precipitation studies with NKR-P1 and CD69 activation receptors of NK cells

All prepared divalent compounds (acceptor **3** with products **11, 12** and **14**, acceptor **7** with products **15** and **16**, and acceptor **8** with product **17**) were subjected to binding and precipitation studies with two fluorescently-labelled activation receptors of NK cells, rat NKR-P1 and human CD69. The assays were essentially performed as described previously,[4] with D-mannose as a negative (non-inhibitory) control carbohydrate and GlcNAc as a positive control, providing IC_{50} values of 4.0×10^{-6} M (NKR-P1A) and 3.2×10^{-4} M (CD69). The results for NKR-P1 and CD69 receptors are summarized in Table 1 as $-\log IC_{50}$. It is apparent that both triazole compounds are weaker ligands than the thioureido compound **3** (generally in the range of 2-3 orders of magnitude). The extension of the ligand with one or two GalNAc

units did not essentially bring a notable increase in binding; the difference was mostly within one order of magnitude. Only in the case of the CD69 receptor, some more pronounced increase could be traced (*cf.* **8** and **17**). The acquired results are quite comparable with those found for similar structures we described previously.[4]

Table 1

Affinity of carbohydrate ligands to two NK cell activation receptors, rat NKR-P1A, and human CD69, expressed in the logarithmic scale (−log IC_{50} ± SD). The values express the ability of the studied compounds to inhibit binding between the respective protein and a standard high affinity ligand $GlcNAc_{23}BSA$

Compound	NKR-P1A	CD69
GlcNAc	5.4 ± 0.1	3.5 ± 0.1
3	9.3 ± 0.5	5.9 ± 0.1
11	9.3 ± 0.4	5.8 ± 0.1
12	9.5 ± 0.6	7.3 ± 1.5
14	8.3 ± 0.5	5.8 ± 0.1
7	5.9 ± 0.1	4.0 ± 0.1
15	6.3 ± 0.5	5.3 ± 1.6
16	7.4 ± 0.6	3.8 ± 0.1
8	7.2 ± 0.5	3.9 ± 0.1
17	7.9 ± 0.1	6.0 ± 0.2

We also performed precipitation studies (see Figure 2 and Supplementary Data, Figure S1) with the above set of compounds (Scheme 2-4 and Table 1). Contrary to the simple binding assays, precipitation assays are not competitive, and the amount of the formed precipitate reflects the ligand's ability to bind more receptor molecules at the same time, causing their cross-linking and finally precipitation from the solution. The receptor precipitation simulates the *in vivo* process of receptor aggregation on the cell surface in response to the presence of a target cell. This process is essential for signal amplification to trigger the killing action.[36,37] In the case of NKR-P1, the triazole compound **7** proved to be the best precipitation agent (71% of protein precipitated), whereas all derivatives of the thioureido compound **3** showed no notable precipitation effect with these soluble protein receptors. Interestingly, the extension of the bivalent acceptors with any GalNAc units did not bring any improvement in the precipitation effect, even *vice versa* in the case of triazoles – here, the additional GalNAc moiety even caused a decrease in the precipitation abilities (*cf.* **7**,

15 and **16**). In the case of the CD69 receptor, more significant results were observed – the extension with the GalNAc unit always brought a positive effect, importantly, leading to 100% protein precipitated with di-LacdiNAc triazole (n=1) **16** or to nearly 90% protein precipitated with di-LacdiNAc triazole (n=2) **17**. Like with NKR-P1, the triazoles showed an improved performance over the thioureido compounds.

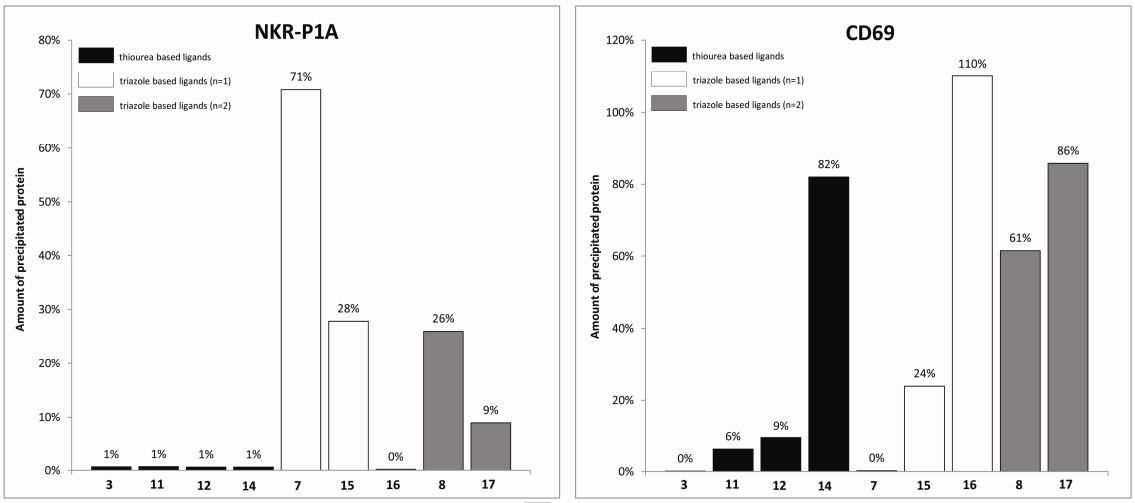


Figure 2. *Precipitation Assay: Ability of the compounds studied to precipitate respective proteins from solution (experimental error ≤ 10%).*

2.5. Immunological testing of the prepared compounds

In view of the low precipitation activities of the synthesized compounds for recombinant soluble rat NKR-P1A receptor, and considering the inhibitory role of human NKR-P1, the immunological testing focused on activation of human peripheral blood mononuclear cells (PBMC) through CD69 receptor that appeared promising in precipitation assays. The results of these tests performed with $CD69^{high}$ lymphocytes are shown in Figure 3a – 3c. In the thiourea series we have observed much broader activity in cellular assays compared to precipitation assays in which only compound **14** was reactive. The highest potential to activate human PBMC, and to enhance natural killing of tumour targets by these cells was found for compound **11** followed by compound **14**. In the triazole series of compounds, compound **7** and **8** remained completely inactive in cellular activation assays although they caused some increase in natural killing. Compound **17** that scored best in the precipitation of soluble CD69 also appeared to be the best activator in cellular activation assays as well as in enhancement of natural killing. On the other hand, the individual

compounds had only negligible effects when tested on CD69^{low} lymphocytes (Supplementary data, Figure S2a to S2c), except some lower effects on natural killing.

Principal differences were observed between the thiourea series dimers and the triazole series dimers with regard to their ability to induce activation-induced apoptosis. In the thiourea series, a notable and gradual increase in the ability of these compounds to cause activation-related programmed cell death could be observed: this tendency could be observed starting with the lactosyl-di-*N*-acetyl positive control, *i. e.* 1,2-bis[*N´*-(2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl)-thioureido]-ethane[4] and then gradually increased toward higher proapoptotic activity for compounds activating CD69^{high} lymphocytes (Figure 3d). However, the triazole series dimers were completely devoid of this proapoptotic activity, which related not only to inactive compounds **7** and **8,** but even to highly active compounds **16** and **17** (Figure 3d). Again, there has been very little effects of the tested compounds using CD69^{low} PBMC (Supplementary data, Figure S2d).

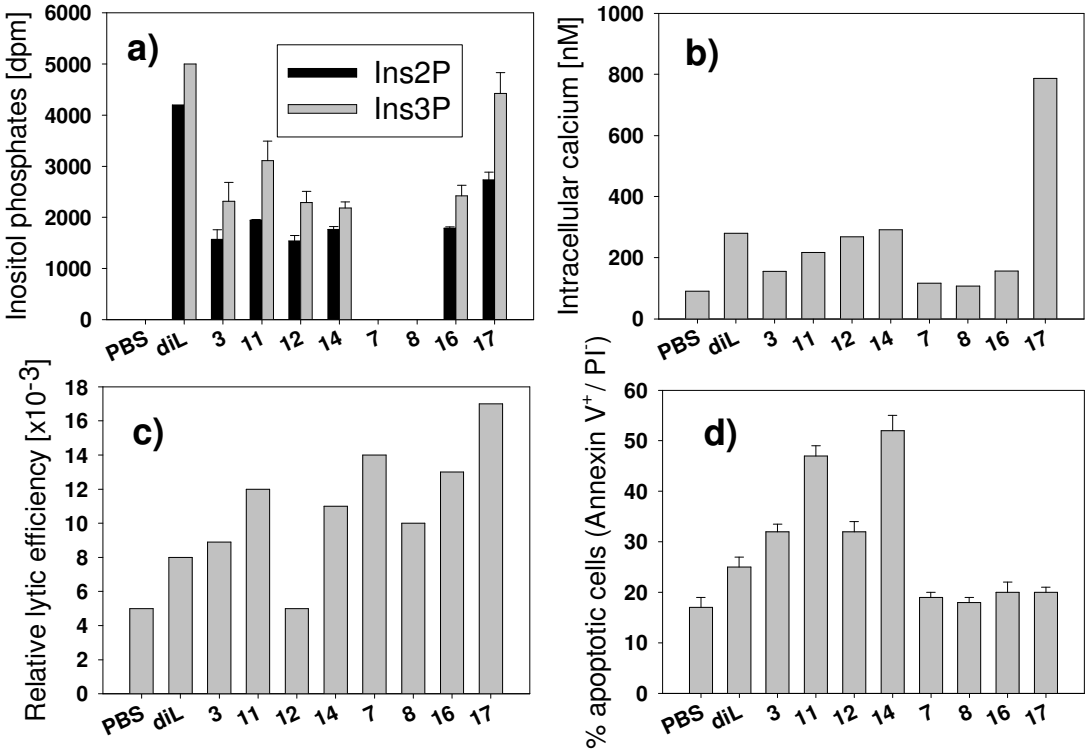


Figure 3. *Immunological testing using optimized (1 nM) concentrations of the synthesized compounds and PBMC from CD69^{high} donors. (a) Production of inositol phosphates. From the complete time course experiments, maximal values were extracted between 4 and 6 minutes (Ins2P) or between 2 and 4 minutes (Ins3P) after the addition of the tested*

compounds. Average values ± range from duplicate experiments are shown. (b) Intracelular calcium levels. were monitored using PBMC from CD69^{high} donors. From the complete time course curves, maximal values observed 1 min after the addition of the tested compounds are shown. The indicated results are averaged from two independent measurements. (c) Natural killing of K562 erythroleukemia by PBMC from CD69^{high} donors.. Lytic units were extracted from the complete killing curves, and expressed as a reciprocal value indicating relative lytic efficiency. Indicated values were averaged from triplicate measurements. (d) Apoptosis of PBMC from CD69^{high} donors was measured after incubation in the presence of individual compounds for 12 h. Results are average values ± range from duplicate experiments.

2.6. Concluding remarks

The present work demonstrates a comparison of two enzymatic approaches in the glycosylation of divalent complex acceptors. Whereas β-*N*-acetylhexosaminidase was unable to perform glycosylation at both acceptor sites and the yields were relatively poor (< 10 %), the Y284L mutant of human placental β1,4-galactosyltransferase-I proved to be a reliable synthetic tool, reaching practically quantitative glycosylations at both acceptor sites. Apart from the thioureido acceptor **3**, which was selected on the basis of previous experience,[4] two alternative bivalent triazole-bound acceptors with different linker length were glycosylated with good results (30-50% yield of di-LacdiNAc products). All prepared bivalent structures were subjected to binding and precipitation studies with two model activation receptors of NK cells, namely NKR-P1A (rat) and CD69 (human). Especially the precipitation study simulates the natural process of receptor aggregation in the presence of target cells. In this experiment, the prepared compounds proved to be very good precipitation agents, especially for the CD69 receptor, where the additionally introduced GalNAc units greatly improved the precipitation effect (100% protein precipitated with di-LacdiNAc triazole **16**). Triazoles generally exhibited better performance than the compounds with the thioureido linker. This refers particularly to compound **17** that proved to be highly efficient activator of CD69^{high} lymphocytes with a profound effect on natural killing yet turned out to be surprisingly inactive in activation induced apoptosis. The effects of compounds on CD69^{low} lymphocytes are generally much lower pointing to the critical participation of this particular receptor in the present assays. However, at this stage of our research it would be difficult to rule out completely the participation of other closely related receptors from CLEC2 family that might have been also upregulated by treatment used to enhance CD69. CD69 is not specific exclusively to NK

cells, it can be also expressed on activated T cells.[7,8] Although it is not probable that this specific cellular population participates during the short term killing assays performed here, their influence during long term experiments, and, in particular, during the prospective *in vivo* tumor therapy experiments (with the critical role of specific effector $CD8^+$ T-cells) will have to be taken into consideration. These characteristics are critical from the viewpoint of further development of this compound into potential antitumor and immunomodulatory drugs since the activation-induced apoptosis (of NK cells) represents a major drawback against the activity of such compounds in tumour microenvironment. Thus, the lack of proapoptotic activity in the studied compounds makes them perfect model compounds in the study of tumour treatment. In sum, this work sheds further light on the structure-affinity relationship in the complex process of activating NK cell receptors and offers new ideas for further optimisation of the ligand structure.

3. Experimental

3.1. General methods

All reagents unless stated otherwise were used as purchased without further purification. TLC was performed on aluminium sheets precoated with Silica Gel 60 (F_{254} Merck, DE), and the spots were visualized by UV light (254 nm) and by spraying with 5% H_2SO_4 in ethanol and charred with heat gun or with ceric sulfate/ammonium molybdate in 10% H_2SO_4 or $KMnO_4$ stain. Oven-dried glassware (120 °C) was used for reactions carried out under nitrogen atmosphere. Solvents were dried according to standard procedures prior to use. Flash chromatography was performed with Merck silica 60 (230-400 mesh) as stationary phase.

Compounds **4 - 8** were structurally characterized as follows: ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) were recorded on a Varian Mercury 400 spectrometer. The chemical shifts are reported in ppm downfield to TMS ($\delta = 0$), the chemical shifts for ^{1}H-NMR were referenced using either the residual $CDCl_3$ ($\delta = 7.26$) or the central CD_3OD resonance ($\delta =$ 3.31). For ^{13}C-NMR either the central $CDCl_3$ resonance ($\delta =$ 77.16) or the central CD_3OD resonance ($\delta =$ 49.00) was used as the reference peak.[38] Spectra were assigned based on *g*COSY and *g*HMQC experiments. MS spectra were recorded on a Micromass LC-TOF instrument by using electrospray ionization (ESI). High resolution spectra were recorded with either of the following compounds as internal standard: (Boc-L-alanine: $C_8H_{15}NO_4Na$: 212.0899; BzGlyPheOMe: $C_{19}H_{20}N_2O_4Na$: 363.1321; BocSer(OBn)SerLeuOMe: $C_{25}H_{39}N_3O_8Na$: 532.2635; erythromycin: $C_{37}H_{67}NO_{13}Na$: 756.4510).

All other compounds were structurally characterized as follows: NMR spectra were measured on a Bruker AVANCE III 600 MHz spectrometer (600.23 MHz for ^{1}H, and 150.93 MHz for ^{13}C) in D_2O (99.96 atom% D, Sigma-Aldrich, Steinheim, Germany) at 303 K. Residual signal of solvent was used as an internal standard (δ_H 4.508 ppm). Carbon chemical shifts in D_2O were referred to acetone (δ_C 30.50 ppm). ^{1}H NMR, ^{13}C NMR, COSY, HSQC, HMQC, HSQC-TOCSY, HMQC-TOCSY, HMBC, and 1D-TOCSY spectra were measured using standard manufacturers' software (Topspin 2.1, Bruker BioSpin GmbH, Rheinstetten, DE). The proton spin systems of each sugar unit were assigned by COSY, TOCSY, HSQC-TOCSY and 1D-TOCSY. The assignment was transferred to carbons by HSQC. The position of substitution of sugar moiety was confirmed by long-range heteronuclear correlations in the HMBC spectrum and configuration at C-1 was deduced from coupling constants $J_{H\text{-}1,\ H\text{-}2}$ observed in the ^{1}H NMR spectra. Several signals both in the ^{1}H and ^{13}C NMR spectra in the substances containing thiourea moiety were doubled due to their conformational behaviour.

Chemical shifts are given in δ-scale [ppm], and coupling constants in Hz. Digital resolution enabled us to report chemical shifts of protons to three and coupling constants to one and carbon chemical shifts to two decimal places. Some hydrogen chemical shifts were read out from HSQC or HMQC and are reported to two decimal places.

Mass spectra were measured on a MALDI-TOF/TOF ultraFLEX III mass spectrometer (Bruker-Daltonics, Bremen, DE). Positive spectra were calibrated externally using the monoisotopic $[M+H]^+$ ions of PepMixII calibrant or using average mass of ProtMixI calibrant (Bruker-Daltonics, Bremen, DE). A 50 mg/mL solution of 2,5-dihydrobenzoic acid in 50% MeCN/ 0.1% TFA was used as a MALDI matrix. The sample (0.4 µL) was dissolved in 50% MeOH and it was allowed to dry at ambient temperature and over-laid with the matrix solution (0.3 µL) on the target. The MALDI-TOF positive spectra were collected in reflectron mode.

3.2. Chemical synthesis of dimeric acceptors 3, 7, 8

*3.2.1. 1,10-Bis[*N'*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-thioureido]-decane (**3**)*

2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl isothiocyanate (**1**; 400 mg, 1.03 mmol)[27] and 1,10-diaminodecane (**2**; 90 mg, 0.52 mmol) were dissolved in dry acetonitrile (10 mL) with DIPEA (0.5 mL). After 24 h at 22 °C, the reaction mixture was purified by column chromatography on silica gel (chloroform/MeOH, 30:1). The dry peracetate **3a** was suspended in dry methanol (4 mL), sodium methoxide (2 M solution in methanol, 0.5 mL)

was added, and the mixture was stirred until the disappearance of the starting material as indicated by TLC (acetonitrile/H_2O, 5:1). The reaction mixture was neutralized with Dowex 50W-X2 (H^+ form), filtered, concentrated, purified by gel chromatography on Sephadex^{TM} LH-20 (Sigma, USA) in MeOH as a mobile phase, 12 mL/min), evaporated to dryness, dissolved in water and lyophilised to obtain **3** (121 mg, 48%) as white solid. For NMR data, see Tables S1, S2 (Supplementary material).

*3.2.2. 1,4-Bis(propynyloxy)butane (**6**)*

To the solution of 1,4-butanediol (557 mg, 6.18 mmol, 1 *eq*) in dry THF (10 mL) NaH (55-65% in mineral oil, 928 mg, 30.9 mmol, 5 *eq*) was added at 0 °C. The mixture was stirred at 0 °C under N_2 atmosphere for 15 min. Propargyl bromide (80% wt. in toluene, 1.7 mL, 15.5 mmol, 2.5 *eq*) was added. The resulting mixture was stirred overnight at room temperature. The mixture was diluted with CH_2Cl_2 followed by addition of water. The aqueous phase was extracted three times with CH_2Cl_2, the combined organic layers were dried over anhydrous $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel, EtOAc/pentane 1:20) yielding 698 mg (4.20 mmol, 68%) of **6** as bright yellow oil. R_f: 0.24 (EtOAc/pentane 1:20). ^{1}H-NMR (400 MHz, $CDCl_3$) δ_H 4.08 (d, 4H, *J* 2.4 Hz, H-3), 3.53-3.50 (m, 4H, H-4), 2.39 (t, 2H, *J* 2.4 Hz, H-1), 1.67-1.64 (m, 4H, H-5). ^{13}C-NMR (100 MHz, $CDCl_3$) δ_C 80.2 (C-2), 74.4 (C-1), 67.0 (C-4), 58.2 (C-3), 26.4 (C-5). HRMS (ES): Calculated for $C_{10}H_{14}O_2Na$ *m/z* 189.0891. Found *m/z* 189.0891.

*3.2.3. 1,6-Bis[1-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl)-1,2,3-triazole-4-yl]-2,5-dioxa-hexane (**7a**)*

1,2-Bis(propynyloxy)ethane **5**[39] (359 mg, 2.60 mmol, 1 *eq*) was dissolved in 50 mL of argon-degassed mixture acetone:water 1:1 (resulting in 0.05 M concentration of **5**). To this solution $Cu(OAc)_2$ (94 mg, 0.518 mmol, 0.2 *eq*), sodium ascorbate (206 mg, 1.04 mmol, 0.4 *eq*) and finally GlcNAc azide **4** (2.13 g, 5.72 mmol, 2.2 *eq*) were added. The reaction mixture was stirred under argon atmosphere at room temperature overnight. Acetone was removed from the mixture by rotatory evaporator. $CHCl_3$ and 5% aqueous ammonia were added to the residue. The aqueous phase was extracted three times with $CHCl_3$, the combined organic phases were washed with brine, dried over anhydrous $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by flash column chromatography (gradient eluent system: 1:3 acetone/EtOAc, then acetone) yielding 1.18 g (1.34 mmol, 51%) of **7a** as white powder. Mp (uncorr.): 102.2-104.4 °C. R_f: 0.29 (acetone/EtOAc, 1:4). ^{1}H-NMR (400 MHz,

$CDCl_3$) δ_H 8.00 (s, 2H, H-7), 7.18 (d, 2H, $J_{NH,2}$ 9.1 Hz, NH), 6.15 (d, 2H, $J_{1,2}$ 9.8 Hz, H-1), 5.53 (t, 2H, $J_{2,3} = J_{3,4}$ 9.6 Hz, H-3), 5.31 (t, 2H, $J_{3,4} = J_{4,5}$ 9.6 Hz, H-4), 4.66-4.56 (m, 6H, H-2, H-8), 4.31 (dd, 2H, $J_{5,6a}$ 4.8 Hz, $J_{6a,6b}$ 12.5 Hz, H-6a), 4.16 (dd, 2H, $J_{5,6b}$ 1.9 Hz, $J_{6a,6b}$ 12.5 Hz, H-6b), 4.13-4.09 (m, 2H, H-5), 3.68-3.61 (m, 4H, H-9), 2.05 (s, 18H, $OCOCH_3$), 1.70 (s, 6H, $NHCOCH_3$). ^{13}C-NMR (100 MHz, $CDCl_3$) δ_C 171.3, 171.0, 170.7, 169.7 (C=O), 145.5 (triazole-C), 122.5 (C-7), 85.9 (C1), 75.0 (C5), 72.7 (C3), 69.7 (C9), 68.5 (C4), 64.4 (C8), 62.1 (C6), 53.8 (C2), 22.9 ($NHCOCH_3$), 21.0, 20.9, 20.8 ($OCOCH_3$). HRMS (ES): Calculated for $C_{36}H_{50}N_8O_{18}Na$ *m/z* 905.3141. Found *m/z* 905.3144.

*3.2.4. 1,8-Bis[1-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl)-1,2,3-triazole-4-yl]-2,7-dioxa-octane (**8a**)*

Compound **8a** was prepared by the same procedure as **7a** using 1,4-bis(propynyloxy)butane **6** (310 mg, 1.86 mmol). The crude product was purified by flash column chromatography (gradient eluent system: EtOAc; then acetone/EtOAc, 1:3; then acetone) yielding 956 mg (1.05 mmol, 56 %) of **8a** as white powder. Mp (uncorr.): 220 °C (decomp.). R_f: 0.43 (1:4 Acetone/EtOAc). ^{1}H-NMR (400 MHz, $CDCl_3$) δ_H 7.94 (s, 2H, H7), 7.05 (d, 2H, $J_{NH,2}$ 9.2 Hz, NH), 6.16 (d, 2H, $J_{1,2}$ 9.9 Hz, H-1), 5.52 (t, 2H, $J_{2,3}$=$J_{3,4}$ 9.8 Hz, H-3), 5.27 (t, 2H, $J_{3,4}$=$J_{4,5}$ 9.8 Hz, H-4), 4.68-4.61 (m, 2H, H-2), 4.56 (s, 4H, H-8), 4.30 (dd, 2H, $J_{5,6a}$ 4.7 Hz, $J_{6a,6b}$ 12.5 Hz, H-6a), 4.15 (dd, 2H, $J_{5,6b}$ 2.0 Hz, $J_{6a,6b}$ 12.5 Hz, H-6b), 4.09 (ddd, 2H, $J_{5,6b}$ 2.0 Hz , $J_{5,6a}$ 4.7 Hz, $J_{4,5}$ 9.8 Hz, H-5), 3.49-3.43 (m, 4H, H-9), 2.06 (s, 6H, $OCOCH_3$), 2.04 (s, 6H, $OCOCH_3$), 2.03 (s, 6H, $OCOCH_3$), 1.73 (s, 6H, $NHCOCH_3$), 1.59-1.56 (m, 4H, H10). ^{13}C-NMR (100 MHz, $CDCl_3$) δ_C 171.2, 171.1, 170.9, 169.6 (C=O), 145.8 (triazole-C), 122.2 (C7), 85.9 (C1), 75.1 (C5), 72.7 (C3), 70.4 (C9), 68.4 (C4), 64.0 (C8), 62.0 (C6), 53.7 (C2), 26.5 (C10), 22.9 ($NHCOCH_3$), 20.9, 20.8, 20.7 ($OCOCH_3$). HRMS (ES): Calculated for $C_{38}H_{54}N_8O_{18}Na$ *m/z* 933.3454. Found *m/z* 933.3446.

*3.2.5. 1,6-Bis[1-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-1,2,3-triazole-4-yl]-2,5-dioxa-hexane (**7**)*

A catalytic amount of freshly prepared CH_3ONa in CH_3OH was added to a stirred solution of **7a** (312 mg, 0.353 mmol) in dry CH_3OH (4.5 mL). The reaction mixture was stirred at room temperature until TLC analysis indicated reaction completion. The reaction mixture was then concentrated under reduced pressure to afford 223 mg (0.353 mmol, 100%) of **7** as a white solid. For further work with enzymes a gel filtration purification step was necessary (Biogel P2, 100 × 2 cm, H_2O 10 mL/h). Mp (uncorr.): 125 °C (decomp.). R_f: 0.48 (CH_3OH/acetone,

2:3). ^{1}H-NMR (400 MHz, CD_3OD) δ_H 8.18 (s, 2H, H7), 5.81 (d, 2H, $J_{1,2}$ 9.8 Hz, H1), 4.62 (s, 2H, H8), 4.25 (t, 2H, J 10.0 Hz, H2), 3.90 (dd, 2H, $J_{6a,5}$ 1.5 Hz, $J_{6a,6b}$ 12.3 Hz, H6a), 3.78-3.68 (m, 4H, H3, H6b), 3.64 (s, 4H, H9), 3.59-3.54 (m, 4H, H4, H5), 1.77 (s, 3H, $NHCOCH_3$). ^{13}C-NMR (100 MHz, CD_3OD) δ_C 173.5 (C=O), 146.0 (triazole-C), 124.0 (C7), 88.2 (C1), 81.3 (C5), 75.8 (C3), 71.4 (C9), 70.6 (C4), 64.9 (C8), 62.4 (C6), 56.9 (C2), 22.6 ($NHCOCH_3$). HRMS (ES): Calculated for $C_{24}H_{38}N_8O_{12}Na$ *m/z* 653.2507. Found *m/z* 653.2511.

*3.2.6. 1,8-Bis[1-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-1,2,3-triazole-4-yl]-2,7-dioxa-octane (**8**)*

Starting from **8a** (908 mg, 0.997 mmol) following the same procedure as for **7**, reaction yielded 656 mg (0.997 mmol, 100%) of **8** as white solid. For further work with enzymes a gel filtration purification step was necessary (Biogel P2, 100 × 2 cm, H_2O 10 mL/h) to remove low molecular impurities inhibiting enzyme activity. Mp (uncorr.): 83.3-85.6 °C R_f: 0.54 (CH_3OH/Acetone, 2:3). ^{1}H-NMR (400 MHz, CD_3OD) δ_H 8.15 (s, 2H, H7), 5.82 (d, 2H, $J_{1,2}$ 9.9 Hz, H1), 4.56 (s, 4H, H8), 4.25 (t, 2H, J 9.9 Hz, H2), 3.90 (br d, 2H, $J_{6a,6b}$ 12.0 Hz, H6a), 3.78-3.69 (m, 4H, H3, H6b), 3.64-3.54 (m, 4H, H4, H5), 3.54-3.44 (m, 4H, H9), 1.78 (s, 6H, $NHCOCH_3$), 1.64-1.60 (m, 4H, H10). ^{13}C-NMR (100 MHz, CD_3OD) δ_C 173.4 (C=O), 146.2 (triazole-C), 123.8 (C7), 88.1 (C1), 81.3 (C5), 75.8 (C3), 71.4 (C9), 71.2 (C4), 64.5 (C8), 62.4 (C6), 56.8 (C2), 27.4 (C10), 22.7 ($NHCOCH_3$). HRMS (ES): Calculated for $C_{26}H_{42}N_8O_{12}Na$ *m/z* 681.2820. Found *m/z* 681.2819.

3.3. β-N-Acetylhexosaminidase-catalysed glycosylation

3.3.1. β-N-Acetylhexosaminidase from Aspergillus oryzae *CCF 1066*

The β-*N*-acetylhexosaminase (EC 3.2.1.52) from a filamentous fungus *Aspergillus oryzae* CCF 1066 (Culture Collection of Fungi (CCF), Department of Botany, Charles University in Prague, CZ) was produced in mineral medium consisting of [g/L] yeast extract (0.5), KH_2PO_4 (3), $NH_4H_2PO_4$ (5), $(NH_4)_2SO_4$ (2), GlcNAc (5), NaCl (15), pH 6.0. Flasks (1 L) with medium (200 mL) were inoculated with the suspension of spores in 0.1% Tween 80 and cultivated on a rotary shaker at 28 °C for 6 days. Then, the medium was filtered off and the supernatant was fractionally precipitated by $(NH_4)_2SO_4$ (0-80%). For purification, the precipitate was dissolved in 10 mM citrate-phosphate buffer, pH 3.5 (10 mM Na_2HPO_4/ 10 mM $C_6H_8O_7$), and dialysed against this buffer at 4 °C overnight. Cation-exchange chromatography was performed on Fractogel EMD SO_3^- (Merck, DE) equilibrated with 10 mM citrate-phosphate

buffer, pH 3.5 at 22 °C. Bound proteins were eluted with a linear gradient of 0–1 M NaCl in the same buffer at a flow rate of 2 mL/min. β-*N*-Acetylhexosaminidase activity was determined by standard assay[40] and positive fractions were pooled and concentrated. The final purification yield was 5 %.

3.3.2. Transglycosylation reactions on analytical scale

Analytical reactions for preparing compound **11** (total volume 100 µL) contained acceptor **3** (9-30 mg, 13-43 µmol) and *p*NP-β-GlcNAc (**9;** 2.5-5 mg, 7-15 µmol) in 50 mM citrate-phosphate buffer pH 5.0 (50 mM Na_2HPO_4/ 50 mM $C_6H_8O_7$). Analytical reactions for preparing compound **12** (total volume 200 µL) contained acceptor **3** (5-15 mg, 7-28 µmol) and *p*NP-β-GalNAc (**10;** 5-20 mg, 15-58 µmol) in 50 mM citrate-phosphate buffer pH 5.0.

The reactions were started by the addition of the β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066 (0.2-5 U) and were incubated at 35 °C with shaking for 24 h. The reaction course was monitored by TLC (acetonitrile/H_2O, 5:1).

*3.3.3. 1-[*N‘*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-thioureido]-10-[*N‘*-(2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl)-thioureido]-decane (**11**)*

The reaction mixture containing acceptor **3** (120 mg, 170 µmol), *p*NP-β-GlcNAc (**9,** 30 mg, 87 µmol), and the purified β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066 (0.5 U) in 50 mM citrate-phosphate buffer pH 5.0 (600 µL) was incubated under shaking at 35 ºC. After 4.5 h the reaction was stopped by adding methanol (1200 µL) and loaded onto Sephadex™ LH-20 column (1850 × 25 mm, mobile phase MeOH, flow rate 10 mL/h). The final purification (separation of the β(1→6) by-product) was performed by preparative HPLC on Chromolith SemiPrep RP-18e column (100 × 10 mm, Merck, mobile phase MeOH/H_2O 1:1, flow rate 2.5 mL/min). Compound **11** was obtained as a white solid (11 mg, 12 µmol, 7%). MS (MALDI-TOF) calculated for $C_{36}H_{65}N_7O_{15}S_2Na$, 923.086, found *m/z* 922.407. For NMR data, see Tables S1, S2 (Supplementary material).

*3.3.4. 1-[*N‘*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-thioureido]-10-[*N‘*-(2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-galactopyranosyl)-thioureido]-decane (**12**)*

The reaction mixture containing acceptor **3** (45 mg, 64 µmol), *p*NP-β-GalNAc, (**10**, 60 mg, 175 µmol), and the purified β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066

(0.5 U) in 50 mM citrate-phosphate buffer pH 5.0 (600 µL) was incubated under shaking at 35 ºC. After 4.5 h the reaction was stopped by adding methanol (1200 µL) and loaded onto Sephadex^{TM} LH-20 column (1850 × 25 mm, mobile phase MeOH, flow rate 10 mL/h). The final purification was performed by preparative HPLC on MAG 5 100 C18 column (25 × 250 mm, Labio a.s., Prague, CZ) in a mobile phase of 40% methanol in H_2O, flow rate 2.5 mL/min. Compound **12** (12 mg, 13 µmol, 6%) was obtained as white solid. MS (MALDI-TOF) calculated for $C_{36}H_{65}N_7O_{15}S_2Na$, 923.086, found *m/z* 922.510; for NMR data, see Tables S1, S2 (Supplementary material).

3.4. Synthesis by mutant β1,4-galactosyltransferase-1

3.4.1. Mutant β1,4-galactosyltransferase-1 (His$_6$-Tag-propeptide-catβ4GalT1 Y284L)

The construction, expression, and purification of the Y284L mutant of human placental β1,4-galactosyltransferase-1 (EC 2.4.1.38; His$_6$-Tag-propeptide-catβ4GalT-1 construct) was performed as described previously.[4]

E. coli BL21(DE3) cells (11.3 g) were suspended in lysis buffer (18.3 mL; 50 mM NaH_2PO_4/ 300 mM NaCl/ 10 mM imidazole, pH 7.5), and benzonase nuclease (10 µL; 25 U/µL) was added. The mixture was sonicated on ice (3 × 20 s with 60 s breaks, sonotrode MS 72, 52% pulsion). The supernatant obtained after centrifugation (20 min, 19,000 rpm, 4 °C) was purified by immobilized metal affinity chromatography on Ni-NTA column (90 × 16 mm), pre-equilibrated with lysis buffer. The column was washed with washing buffer (50 mM NaH_2PO_4/ 300 mM NaCl/ 20 mM imidazole, pH 7.5; linear flow rate 1.5 mL/min), and then with elution buffer (50 mM NaH_2PO_4/ 300 mM NaCl/ 200 mM imidazole, pH 7.5; linear flow rate 1.5 mL/min). Fractions with the highest galactosyltransferase activity were pooled and concentrated. Addition of 20% (v/v) glycerol stabilized the final enzyme solution, which had more than 40 mU/mL activity.

3.4.2. His$_6$-Tag-propeptide-catβ4GalT1 Y284L activity assays

The spectrophotometric assay was described previously.[4] The transferase activity was determined by HPLC as follows. The reaction mixture (total volume 100 µL) consisting of 100 mM MOPS/ 25 mM KCl, pH 6.8; GlcNAc-*t*Boc (1 mM), UDP-GalNAc (1.2 mM), alkaline phosphatase (10 U/mL), $MnCl_2$ (2 mM), and 10 µL of enzyme sample was incubated at 30°C with shaking. Two aliquots (20, 40 min) were taken, the reactions were stopped by boiling (5 min) and after centrifugation (10 min, 10,000 rpm), the supernatant was loaded onto Multokrom 100-5 C18 column (250 × 4 mm; mobile phase was a 36 min linear gradient

of 11-42.2 % v/v CH_3CN in H_2O, 0.5 mL/min, UV detection at 205 nm and 254 nm). Enzyme activity was calculated as a ratio of peak areas depending on time of the reaction, A [U/mL] = [1 mM*(Area LacDiNAc-*t*Boc/(Area LacDiNAc-*t*Boc+Area GlcNAc-*t*Boc))*0.1/(incubation time*0.01)].

The kinetic constants of compound **3** were determined at a constant concentration of UDP-GalNAc donor (5 mM) with 0-6 mM of **3**. The kinetic constants of the donor substrate were measured at a constant concentration of acceptor **3** (5 mM) with 0-20 mM UDP-GalNAc. Donor and acceptor substrates were mixed in 100 mM MOPS-KOH buffer (100 µL final volume), pH 6.8, containing 2 mM $MnCl_2$, 25 mM KCl, alkaline phosphatase (10 mU), and the enzyme sample (10 µL). The mixture was incubated at 30 °C. Aliquots of the reaction mixtures were taken at set time intervals when substrate conversion was linear over time, and subsequently stopped by heating at 95°C for 5 min. The product formation was analyzed by HPLC as described above using 240 nm for detection of compound **3**, and products **12** and **14**. Kinetic constants were obtained by nonlinear regression of the data points using Sigma Plot 10 (SPSS GmbH Software, München, DE) following the Michaelis-Menten kinetic.

3.4.3. GalNAc transfer on analytical scale

The reaction mixture (total volume 100 µL) containing UDP-GlcNAc donor (**13**; 0.3-0.7 mg, 0.5-1 µmol) and acceptor **3** (0.2-0.4 mg, 0.3-0.6 µmol) **7**, or **8** (both 0.3 mg, 0.5 µmol), 100 mM MOPS/ 25 mM KCl, pH 6.8, 2 mM $MnCl_2$, alkaline phosphatase (10 U/mL), UDP-Glc(NAc)-4'-epimerase from *Campylobacter jejuni* (1 U/mL, prepared as described previously)[35,41] and the Y284L mutant of His_6-propeptide-catβ4GalT-1 (10 -20 mU/mL) was incubated at 30 °C with shaking for 72 h. Reactions were stopped by boiling (5 min) and after centrifugation (10 min, 10,000 rpm) they were analysed by TLC and HPLC. The TLC mobile phase was $CH_3CN/H_2O/HCOOH$, 4:1:0.1 for **3** or $CH_2Cl_2/MeOH/EtOH/H_2O/HCOOH$, 5:4:1:1:0.1 for **7** and **8**. HPLC was performed on Multokrom 100-5 C18 column (250 × 4 mm). Mobile phase was a 36-min linear gradient of 11-42.2 % v/v CH_3CN in H_2O for **3** or of 5-26% CH_3CN in H_2O for **7** and **8**, flow rate 0.5 mL/min, UV detection at 240 nm for **3** or 230 nm for **7** and **8**.

The conversion of acceptor **3** and formation of products **12** and **14** was followed over time and quantified by HPLC as described above. The reaction mixture (100 µL) consisted of 5 mM compound **3**, 12 mM UDP-GalNAc, 100 mM MOPS-KOH buffer, pH 6.8 with 25 mM KCl, 2 mM $MnCl_2$, alkaline phospatase (10 mU), and the enzyme sample (1.39 mU). The

mixture was incubated at 30 °C. Aliquots of the reaction mixture were taken at indicated time intervals and stopped by heating at 95 °C for 5 min.

*3.4.4. 1-[*N'*-2-Acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl)-thioureido]-10-[*N'*-(2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-galactopyranosyl)-thioureido]-decane (**14**)*

Acceptor **3** (24 mg, 34 µmol) and UDP-GlcNAc, (**13**; 54 mg, 90 µmol) were dissolved in 100 mM MOPS/ 25 mM KCl, pH 6.8 and 2 mM $MnCl_2$. Alkaline phosphatase (70 U), UDP-GlcNAc-4'-epimerase (7 U) and the Y284L mutant of His_6-propeptide-catβ4GalT-1 (175 mU) were added (total volume 7 mL). The reaction mixture was incubated at 30 °C with shaking. The reaction progress was monitored by HPLC and TLC. After 48 h, the reaction was stopped by boiling (5 min), centrifuged (10 min, 10,000 rpm), the supernatant was evaporated to dryness, dissolved in 80% MeOH in H_2O (2 mL) and loaded onto Sephadex™ LH-20 column (800 × 25 mm, mobile phase 80% MeOH in H_2O, flow rate 9 mL/h). Compound **14** was eluted pure and after lyophilization it was obtained as white solid (32 mg, 29 µmol) in 80% yield. For NMR data, see Tables S1, S2 (Supplementary material). MS (MALDI-TOF) calculated for $C_{44}H_{78}N_8O_{20}S_2Na$, 1125.437, found *m/z* 1125.462.

*3.4.5. 1-[1-(2-Acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl)-1,2,3-triazole-4-yl]-2,5-dioxa-6-[1-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-1,2,3-triazole-4-yl]-hexane (**15**) and 1,6-bis-[1-(2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl)-1,2,3-triazole-4-yl]-2,5-dioxa-hexane (**16**)*

Acceptor **7** (19 mg, 30 µmol) and UDP-GlcNAc (**13**; 47 mg, 77 µmol) were dissolved in 100 mM MOPS/ 25 mM KCl, pH 6.8 and 2 mM $MnCl_2$. Alkaline phosphatase (60 U), UDP-Glc(NAc)-4'-epimerase (6 U) and the Y284L mutant of His_6-propeptide-catβ4GalT1 (120 mU) were added (total volume 6 mL). The reaction mixture was incubated at 30 °C with shaking. The reaction progress was monitored by HPLC and TLC. After 72 h, the reaction was stopped by boiling (5 min), centrifuged (10 min, 10,000 rpm) and the supernatant was evaporated to dryness, dissolved in 80% MeOH in H_2O (2 mL) and loaded onto Sephadex™ LH-20 column (800 × 25 mm, mobile phase 80% MeOH in H_2O, flow rate 9 mL/h). Two compounds, **15** and **16**, were eluted pure. After lyophilization, compound **15** (6.5 mg, 8 µmol) was obtained as white solid in 26% yield, compound **16** as white solid (9 mg, 8.6 µmol) in

28% yield. For NMR data, see Tables S1, S2 (Supplementary material). MS (MALDI-TOF) calculated for $C_{32}H_{51}N_9O_{17}Na$ (**15**), 856.330, found *m/z* 855.517; calculated for $C_{40}H_{64}N_{10}O_{22}Na$ (**16**), 1059.409, found *m/z* 1059.462.

*3.4.6. 1,8-Bis-[1-(2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl)-1,2,3-triazole-4-yl]-2,7-dioxa-octane (**17**)*

Acceptor **8** (24 mg, 36 µmol) and UDP-GlcNAc (**13**; 56 mg, 92 µmol) were dissolved in 100 mM MOPS/ 25 mM KCl, pH 6.8 and 2 mM $MnCl_2$. Alkaline phosphatase (72 U), UDP-Glc(NAc)-4'-epimerase (7.2 U) and the Y284L mutant of His_6-propeptide-catβ4GalT-1 (144 mU) were added (total volume 7.2 mL). The reaction mixture was incubated at 30 °C with shaking. The reaction progress was monitored by HPLC and TLC. After 74 h, the reaction was stopped by boiling (5 min), centrifuged (10 min, 10,000 rpm) and the supernatant was evaporated to dryness, dissolved in 80% MeOH in H_2O (2 mL) and loaded onto Sephadex™ LH-20 column (800 × 25 mm, mobile phase 80% MeOH in H_2O, flow rate 8 mL/h). After lyophilization, compound **17** was obtained as white solid (19 mg, 18 µmol) in 47% yield. For NMR data, see Tables S1, S2 (Supplementary material). MS (MALDI-TOF) calculated for $C_{42}H_{68}N_{10}O_{22}Na$ (**17**), 1087.440, found *m/z* 1087.525.

3.5. Competitive inhibition assay

Inhibition assays were performed as described previously[42,43] with the following modification: the soluble NKR-P1 and CD69 protein receptors were labelled with fluorescent labels (fluorescein and rhodamine, respectively). The concentration of bound protein receptors in the microtiter wells were determined by fluorescence measurement ($\lambda_{ex}/\lambda_{em}$ 496/519 nm and $\lambda_{ex}/\lambda_{em}$ 546/577 nm, respectively) using a Safire 2 spectrophotometer (Tecan, AT). The results are given as a negative logarithm of the ligand concentration required to cause 50% inhibition of the receptor's binding to the standard high-affinity ligand $GlcNAc_{23}BSA$ ($-\log IC_{50}$). Proteins were labelled by the covalent attachment of fluorescent labels, using *N*-hydroxysuccinimide fluorescein and *N*-hydroxysuccinimide rhodamine (both from Pierce Biotechnology, USA) for rat NKR-P1A and human CD69 receptors, respectively.

The 96-well round-bottomed plate was coated with $GlcNAc_{23}BSA$ ligand, blocked with 2% BSA and after incubation at 4 ºC for 2 h, the plate was washed three times with PBS (Phosphate Buffered Saline: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). The labelled proteins and serial dilutions of the inhibitor (GlcNAc, **3, 7, 8, 11, 12, 14, 15, 16, 17**) were put in each well, incubated at 4 ºC for 1 h, then the plate was

washed three times with PBS and incubated at 4 ºC overnight with 0.1 M sodium acetate buffer supplemented with 0.1% octyl β-glucoside and 0.1% Triton X-100. Then, the solution was transferred to 96-well flat-bottomed UV transparent plates and the results were obtained by fluorescence measurement. Complete inhibition curves were constructed and the IC_{50} values were calculated from at least two independent experiments.

3.6. Precipitation assay

Each ligand was dissolved in water at concentrations of 200, 60, 20, 6, and 2 nM. The fluorescein-labelled NKR-P1A or rhodamine-labelled CD69 in 2 × PBS (20 nM, 50 μL)[37,43] was added to each sample (50 μL) in 96-well microtiter plates. Mixtures were incubated at 4 °C for 30 min, and then 20% (v/v) solution of PEG 8000 in PBS was added (100 μL). The mixture was left to precipitate for 1 h at 4 °C. After centrifugation (10 min, 4 °C, 1800 rpm), the supernatant was carefully removed, and 10% (v/v) solution of PEG 8000 in PBS (100 μL) was added. This procedure was repeated two more times to wash the precipitate. After additional centrifugation and supernatant removal, the precipitates were incubated overnight in acid/ detergent dissociation buffer (100 μL), transferred to UV Star plates, and fluorescein/ rhodamine fluorescence was measured under standard conditions. Each compound was measured in duplicate. The maximum experimental error is 10%.

3.7. Immunological tests

Peripheral blood mononuclear cells were obtained from standard blood fraction enriched in leukocytes (buffy coats from the local Blood Transfusion service), after dilution with RPMI1640 medium, and centrifugation over Ficoll-Paque. Cells were incubated overnight in complete RPMI1640 in plastic cell culture dishes to allow the adherent cells to attach. Collected non-adherent fraction of PBMC (N-PBMC) contained mostly lymphocytes (T, B, and NK cells). Lymphocytes from donors expressing less than 5% of CD69 were designated as $CD69^{low}$. Lymphocytes from donors with more than 20% CD69 positive cells were further activated by incubation at a density of 2×10^6 cells/mL in complete RPMI1640 medium for 4 h with PMA (50 ng/mL) and ionomycin (500 ng/mL). This procedure increased the surface expression of CD69 to 75-85%, as analyzed by flow cytometry using monoclonal antibody against CD69 labeled with phycoerythrin. Such lymphocytes were designated as $CD69^{high}$. Individual compounds were dissolved in PBS, and tested at 10^{-9} M final concentrations found to be optimal in the initial dose dependence assays using both $CD69^{low}$ and $CD69^{high}$

lymphocytes. Cellular activation assays and test of natural killing were performed essentially as described previously.[6] For apoptosis assays cells were resuspended at 2×10^6/mL in complete RPMI1640 medium, aliquoted (50 μL) into duplicate wells in round-bottomed 96-well plates, and the tested compounds were added in 50 μL to provide the final (10^{-9} M) tested concentrations. Individual tested compounds were added 12 and 6 h before the estimation of the percentage of apoptotic cells using Annexin V-FITC/Hoechst 33258 staining and flow cytometry. Percentage of apoptotic cells (Annexin V^+/Hoechst 33258^-) observed in the presence of PBS only or in the presence of 5×10^{-6} M arsenite was used as a negative and as a positive control, respectively.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at *doi.*

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