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Synthesis of Multivalent Glycoconjugates Containing the Immunoactive LELTE Peptide: Effect of Glycosylation on Cellular Activation and Natural Killing by Human Peripheral Blood Mononuclear Cells

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Abstract: Pentapeptide diacidic sequence LELTE, derived from the mycobacterial heat shock protein hsp65, has been recently identified as a "danger" signal of the immune system effective via specific binding to the universal leukocyte triggering receptor CD69. This sequence is not active per se, only after its presentation within the multivalent environment of its parent protein, or after artificial dimerization using a standard bifunctional reagents. Here we describe an entirely new way of presenting of this peptide based on its attachment to a cyclopeptide RAFT scaffold $(K\text{-}K\text{-}K\text{-}P\text{-}G)_2$ through the ε-amino group of lysine residues, alone or in combination with the carbohydrate epitope αGalNAc. The ability of such RAFT scaffolds to precipitate the target CD69 receptor or to activate CD69-positive cells is enhanced in compounds **2** and **4** possessing combined peptide/carbohydrate expression. Compounds **2** and **4** are highly efficient activators of natural killer lymphocytes, but they are completely inactive from the point of view of activation-induced apoptosis of lymphocytes by the target cells. These unique properties make the combined peptide/carbohydrate RAFTs highly suitable for future evaluation in animal tumor therapies in vivo and predict them to be readily available and efficient immunoactivators.

1. Introduction

Natural killer (NK) cells are effector lymphocytes of the innate immune system that control tumors and microbial infections.[1,2] Since NK cells do not possess a single "major" activating receptor of the T-cell receptor or B-cell receptor type, a large repertoire of sophisticated activating and inhibitory receptors has evolved to regulate NK cell activities, ensuring that they protect the host against pathogens, yet prevent deleterious NK-cell-driven autoimmune responses.[3] The large array of receptor−ligand pairs clustering at the NK cell−target cell interface known as "zippers" involve ligands such as sialic acids, collagen, MHC (major histocompatibility complex) glycoproteins, as well as receptors: members of the immunoglobulin, integrin, viral hemagglutinin, and calcium-dependent (C-type lectin) families.[1]

Lymphocyte receptors belonging to the C-type lectin receptor family have evolutionarily diverged to recognize not only carbohydrates (the classical ligands for C-type lectins), but also calixarene derivatives[4] and peptide or protein molecules.[5] Prominent among these receptors is NKR-P1A (CD161) shown previously to represent one of the major activating receptors on NK cells of rodents.[6] NKR-P1A receptor appears to be a genuine lectin interacting both with simple sugars such as GlcNAc or GalNAc[7] and with certain oligosaccharide sequences.[8] GlcNAc conjugates based on glycodendrimeric[9] or calixarene[4] cores have also been identified as efficient NK cell activators and compounds active in experimental tumor

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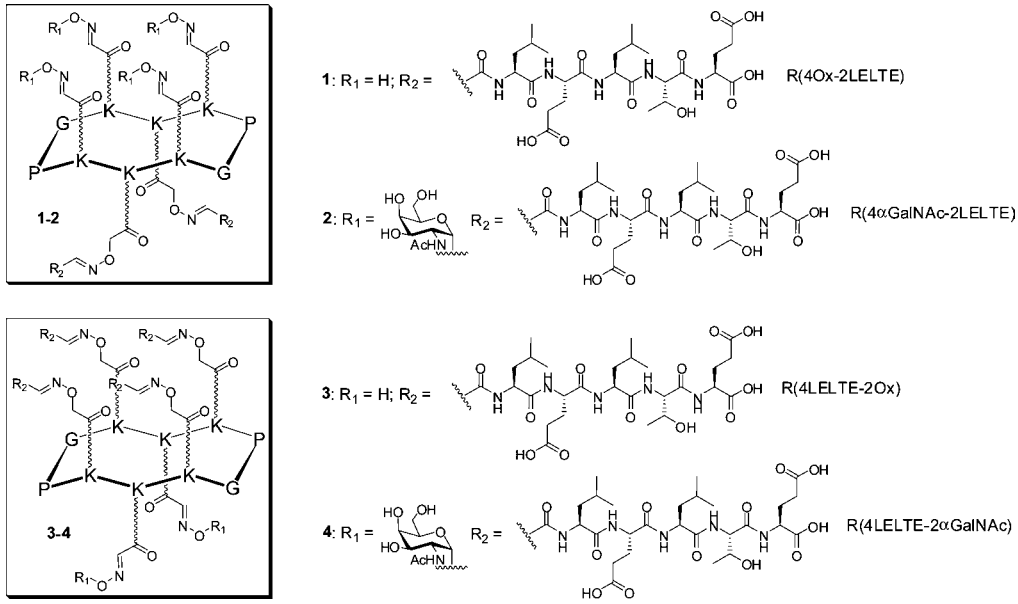
Scheme 1. Structure of Divalent or Tetravalent LELTE Conjugates and the Corresponding Glycoconjugates **1**−**4**



therapies.[10,11] On the other hand, not only is the related CD69 receptor expressed on the surface of NK cells, but it also represents the universal and rather widespread leukocyte signaling molecule.[12] It exhibits much more promiscuous reactivity that involves not only GlcNAc, GalNAc, and related carbohydrates but also carboxylated as well as sulfated oligosaccharide sequences.[13] Most of the carbohydrate ligands for NKR-P1 and CD69 receptors have been shown to contain β-linked *N*-acetylhexosamines. However, studies using complex comblike dendrimers also revealed the reactivity of these receptors with α-linked GalNAc.[14]

We have recently identified another class of high-affinity ligands for the CD69 receptor derived from the mycobacterial heat shock protein hsp65 (known to be one of the most immunogenic proteins) and consisting of the LELTE pentapeptide that has been shown to react with CD69 in its free form as well as with part of the extended protein sequence.[15] An important prerequisite for this pentapeptide ligand to be able to cross-link the cellular form of CD69 and thus to have significant lymphocyte proliferation and immunostimulatory activities is its oligomerization. Thus, the native hsp65 protein bearing the internal LELTE sequence only activates the cells of the immune system in the form of oligomers or at least dimers and not those in monomeric form.[15] Similarly, the monomeric peptide LELTE had very little effect on immune cell activation but had acquired a very potent ability to induce the proliferation of resting human lymphocytes and production of lymphokines after a simple chemical dimerization through its unique N-terminal amino groups using bifunctional reagents such as disuccinimidyl tartrate or disuccinimidyl glutarate.[15,16]

Here we describe the synthesis and evaluation of the biological activities of another class of LELTE conjugates in which the LELTE peptides were coupled onto a cyclodecapeptide scaffold (namely RAFT) using an oxime-based ligation strategy.[17−19] Two classes of these compounds have been prepared (Scheme 1): in one class, we attached two or four LELTE peptide sequences to the cyclic peptide core together with free oxime groups as the controls (R(4Ox-2LELTE) **1** and R(4LELTE-2Ox) **3**, respectively), while the second class contains an additional substitution with either four or two αGalNAc monosaccharides attached through an oxime ether linkage (R(4αGalNAc-2LELTE) **2** and R(4LELTE-2αGalNAc) **4**, respectively). When we tested the ability of these compounds to activate $CD69^+$ lymphocytes, the activity of LELTE-modified RAFTs was comparable with that exhibited by the previously tested LELTE dimers. However, the additional conjugation with αGalNAc resulted in a significant increase in their activation abilities, possibly due to cross-linking CD69 with another class of lymphocyte receptors such as NKR-P1. Compound **4**, a RAFT tetrasubstituted with LELTE and glycosylated with two αGalNAc residues, increased the natural killing of human peripheral blood mononuclear cells approximately 3-fold compared to the control, making it an interesting compound for future use in experimental therapies of tumors. Interestingly, the binding of RAFTs including compound **4** induced only minimal activation-induced apoptosis of the $CD69^+$ effector cells.

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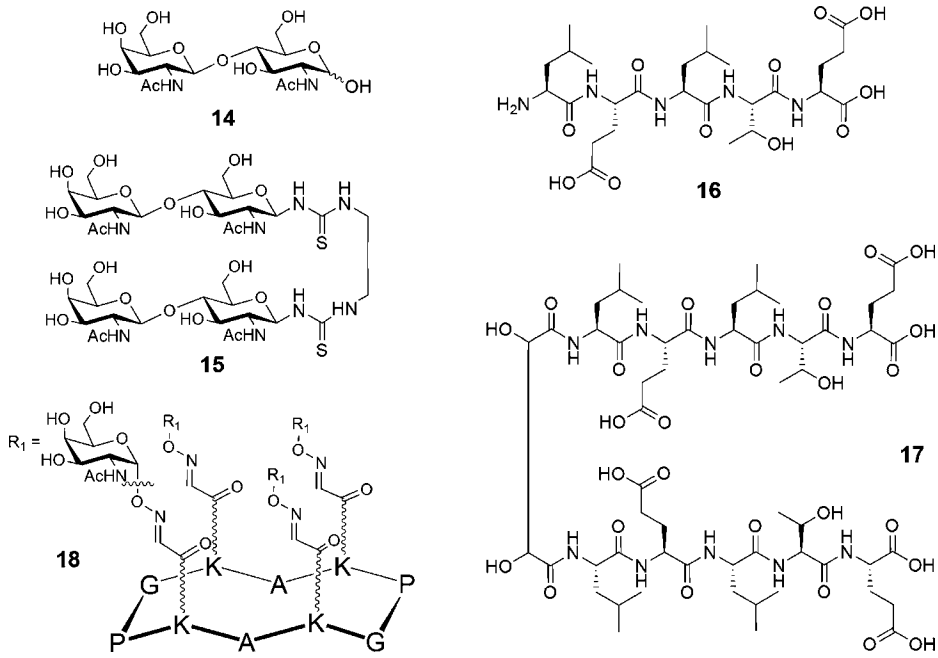

Figure 1. Structures of compounds **14**−**18**.

2. Materials and Methods

2.1. Materials and General Procedures. Protected amino acids and resins were obtained from Advanced ChemTech Europe (Brussels, Belgium), Bachem Biochimie SARL (Voisins-Les-Bretonneux, France) and France Biochem S.A. (Meudon, France). PyBOP was purchased from France Biochem and HBTU from Biosolve B.V. The other reagents were obtained from either Aldrich (Saint Quentin Fallavier, France) or Acros (Noisy-Le-Grand, France). Solid-phase peptide synthesis was performed using Fmoc/tBu chemistry on an automated synthesizer 433A (Applied Biosystem). Linear peptide sequences precursor of the RAFT cyclopeptides **5** and **11** and the LELTE peptide were prepared using the preloaded Fmoc-Gly-SASRIN resin and 2-chlorotrityl resin respectively, on a 0.25 mmol scale in NMP with HBTU as coupling reagent. The 0.25 mmol scale program (SynthAssist version 2) was used with a single coupling. The instrument was coupled to a Perkin-Elmer 200S UV/vis detector ($\lambda = 301$ nm, absorbance range = 0.025*A*, rise time = 2 s). Reverse-phase HPLC analyses were performed on Waters equipment using C_{18} columns. The analytical (Nucleosil 120 Å 3 μm C_{18} particles, 30 × 4.6 mm^2) was operated at 1.3 mL/min and the preparative (Delta-Pak 300 Å 15 μm C_{18} particles, 200 × 25 mm^2) at 22 mL/min with UV monitoring at 214 and 250 nm using a linear A/B gradient (buffer A: 0.09% CF_3CO_2H in water; buffer B: 0.09% CF_3CO_2H in 90% CH_3CN). Routine mass spectra were obtained for each intermediate by electrospray ionization on a VG Platform II. Accurate mass spectra were measured for conjugates **1**−**4** on a MALDI-APEX Qe-FT-ICR mass spectrometer equipped with a 9.4 T superconducting magnet and a ion Combi Source (Bruker-Daltonics, Bremen, Germany). All spectra were acquired in positive ion mode with a broad *m*/*z* range (850−5000) and calibrated externally using the monoisotopic $[M + H]^+$ ions. A 5 mg/mL solution of α-cyano-4-hydroxycinnamic acid or 10 mg/mL solution of 2,5-dihydrobenzoic acid in 50% CH_3CN/0.3% CH_3CO_2H was used as a MALDI matrix and was premixed with dissolved sample before loading on the target. The preparation of *N*-acetyl lactosamine disaccharide **14** and its dimeric form **15** has been described previously.[13] The control LELTE pentapeptide **16** was purchased from Xaia (Lund, Sweden), and dimerized (compound **17**) using a DST cross-linker as described previously.[16] Preparation of the compound R(4αGalNAc) (**18**) has been described previously (Figure 1).[17b]

2.2. Synthesis of R(4BocSer(tBu)-2Dde) (5). The linear decapeptide precursor of **5** (0.25 mmol) was cyclized while highly diluted in CH_2Cl_2 (500 mL) with PyBOP (156 mg; 0.3 mmol) and DIPEA (82 μL; 0.5 mmol; pH 8). After stirring for 1 h at room temperature, the solution was evaporated to dryness and peptide **5** recovered by precipitation in Et_2O. Analytical RP-HPLC of the crude reaction mixture shows complete conversion of the linear decapeptide into **5**. $R_t = 14.9$ min (5 to 100% B in 15 min, 214 nm); ESI-MS (positive mode): calcd for $C_{118}H_{201}N_{20}O_{30}$ 2378.5; found: *m*/*z* 2378.1 $[M + H]^+$.

2.3. Synthesis of R(4BocSer(tBu)-2Eei) (7). Protected cyclodecapeptide **5** (0.25 mmol) was treated for 1 h at room temperature with a solution of 3% hydrazine in DMF (50 mL). After evaporation and precipitation in Et_2O, Eei-Aoa-OSu **6** (129 mg; 0.5 mmol) and DIPEA (83 μL; 0.5 mmol; pH 8) were added, and the solution was stirred in DMF (25 mL). The coupling reaction was monitored by analytical HPLC and reached completion after 2 h. The solvent was then evaporated using a high-vacuum pump and the excess of **6** removed by precipitation in Et_2O. Compound **7** was used without further purification. Yield: 52% (300 mg; 0.13 mmol) from the corresponding linear peptide sequence (three steps); analytical RP-HPLC: $R_t = 14.1$ min (5 to 100% B in 15 min, 214 nm); ESI-MS (positive mode): calcd for $C_{110}H_{195}N_{22}O_{32}$ 2336.4; found: *m*/*z* 2336.2 $[M + H]^+$.

2.4. Synthesis of R(4Ser-2ONH$_2$) (8). Crude compound **7** (196 mg; 0.084 mmol) was treated with a cocktail of TFA/TIS/H_2O (50 mL; 95:2.5:2.5 v/v/v). After 2 h of stirring at room temperature, the solution was evaporated, and fully deprotected cyclopeptide **8** was recovered by precipitation in Et_2O as a white TFA salt. Analytical RP-HPLC of the crude reaction mixture shows complete conversion of the protected sequence **7** into **8**. $R_t = 5.7$ min (5 to 60% B in 15 min, 214 nm); ESI-MS (positive mode): calcd for $C_{66}H_{119}N_{22}O_{22}$ 1571.9; found: *m*/*z* 1571.9 $[M + H]^+$.

2.5. Synthesis of R(4Dde-2BocSer(tBu)) (11). Cyclodecapeptide **11** was synthesized by following the procedure described for **5**. Analytical RP-HPLC of the crude reaction mixture shows complete conversion of the corresponding linear decapeptide into **11**. $R_t = 12.9$ min (5 to 100% B in 15 min, 214 nm); ESI-MS (positive mode): calcd for $C_{114}H_{183}N_{18}O_{26}$ 2220.4; found: *m*/*z* 2220.1 $[M + H]^+$.

2.6. Synthesis of R(4Eei-2BocSer(tBu)) (12). Compound **12** was synthesized by following the procedure described for **7**. After

Dde removal with hydrazine, the incorporation of an aminooxy linker was achieved using Eei-Aoa-OSu (387 mg; 1.5 mmol) and DIPEA (165 μL; 1 mmol; pH 8) in DMF (25 mL). Yield: 65% (340 mg; 0.16 mmol) from the corresponding linear peptide sequence (three steps); analytical RP-HPLC: R_t = 12.7 min (5 to 100% B in 15 min, 214 nm); ESI-MS (positive mode): calcd for $C_{98}H_{171}N_{22}O_{30}$ 2136.3; found: *m*/*z* 2136.1 $[M + H]^+$.

2.7. Synthesis of R(4ONH$_2$-2Ser) (13). Compound **13** was synthesized by following the procedure described for **8**. Analytical RP-HPLC of the crude reaction mixture shows complete conversion of the protected sequence into **13**. R_t = 5.7 min (5 to 60% B in 15 min, 214 nm); ESI-MS (positive mode): calcd for $C_{64}H_{115}N_{22}O_{22}$ 1543.8; found: *m*/*z* 1543.6 $[M + H]^+$.

2.8. Synthesis of R(4Ox-2LELTE) (1). Peptide **8** (14 mg; 0.0069 mmol) was dissolved in a mixture of CH_3CN/H_2O/TFA (2 mL; 1:1:0.1 v/v/v), and LELTE-CHO **9** (27 mg; 0.041 mmol) was added to the solution. The mixture was stirred at 37 °C overnight, and then acetone (1 mL) was added. The resulting LELTE conjugate R(4Ser-2LELTE) (analytical RP-HPLC: R_t = 12.6 min (5 to 40% B in 15 min, 214 nm); ESI-MS (positive mode): calcd for $C_{122}H_{205}N_{32}O_{46}$ 2854.5; found: *m*/*z* 2854.3 $[M + H]^+$) was used without further treatment. Sodium periodate (59 mg; 0.28 mmol) was then added to the crude solution of R(4Ser-2LELTE) and the mixture purified by RP-HPLC R_t = 13.1 min, (5 to 100% B in 30 min, 214 nm) after 1 h to isolate the corresponding R(4CHO-2LELTE) in 95% yield (18 mg; 0.0066 mmol) from **8** (two steps). Analytical RP-HPLC: R_t = 12.9 min (5 to 60% B in 15 min, 214 nm). This compound (9 mg; 0.0033 mmol) was finally stirred at 37 °C with hydroxylamine hydrochloride (1.8 mg; 0.026 mmol) in CH_3CN/H_2O/TFA (2 mL; 1:1:0.1 v/v/v). After RP-HPLC purification (R_t = 13.9 min, (5 to 100% B in 30 min, 214 nm), compound **1** was obtained with a 70% yield (6.5 mg; 0.0023 mmol). Analytical RP-HPLC: R_t = 7.6 min (5 to 100% B in 15 min, 214 nm); MALDI-FT-ICR HRMS data are given in Supporting Information, Table 1.

2.9. Synthesis of R(4αGalNAc-2LELTE) (2). Compound **2** was obtained from R(4CHO-2LELTE) (9 mg; 0.0033 mmol) and GalNAc-αONH$_2$[20] **10** (8 mg; 0.033 mmol) in CH_3CN/H_2O/TFA (2 mL; 1:1:0.1 v/v/v) following the procedure described for **1**. Yield: 80% (9.5 mg; 0.0026 mmol); semi-preparative RP-HPLC: R_t = 17.8 min (5 to 60% B in 30 min, 214 nm); analytical RP-HPLC: R_t = 7.1 min (5 to 100% B in 15 min, 214 nm); MALDI-FT-ICR HRMS data are given in Supporting Information, Table 1.

2.10. Synthesis of R(4LELTE-2Ox) (3). Compound **3** was prepared following the three-step procedure described for **1**. The R(4ONH$_2$-2Ser) **13** (12 mg; 0.0068 mmol) was first treated with LELTE-CHO **9** (28 mg; 0.041 mmol). The resulting R(4LELTE-2Ser) (analytical RP-HPLC: R_t = 8.3 min, 5 to 100% B in 15 min, 214 nm; ESI-MS (positive mode): calcd for $C_{176}H_{287}N_{42}O_{70}$ 4109.0; found: *m*/*z* 4109.5 $[M + H]^+$) was then oxidized with sodium periodate (29 mg; 0.14 mmol) in water (7 mL). The R(4LELTE-2CHO) was purified by RP-HPLC. Yield: 91% (25 mg; 0.006 mmol) from **13** (two steps); semipreparative RP-HPLC: R_t = 14.6 min (5 to 100% B in 30 min, 214 nm); analytical RP-HPLC: R_t = 8.3 min (5 to 100% B in 15 min, 214 nm). This oxidized compound (11 mg; 0.0027 mmol) was reacted with hydroxylamine hydrochloride (0.8 mg; 0.011 mmol) in CH_3CN/H_2O/TFA (2 mL; 1:1:0.1 v/v/v) to obtain **3** after purification. Yield: 68% (7.5 mg; 0.018 mmol); semi-preparative RP-HPLC: R_t = 14.9 min (5 to 100% B in 30 min, 214 nm); analytical RP-HPLC: R_t = 8.4 min (5 to 100% B in 15 min, 214 nm); MALDI-FT-ICR HRMS data are given in Supporting Information, Table 1.

2.11. Synthesis of R(4LELTE-2αGalNAc) (4). Compound **4** was obtained from R(4LELTE-2CHO) (14 mg; 0.0034 mmol) and GalNAc-αONH$_2$[20] **10** (3 mg; 0.014 mmol) in CH_3CN/H_2O/TFA (2 mL; 1:1:0.1 v/v/v) following the procedure described for **3**. Yield: 66% (10 mg; 0.0022 mmol); semi-preparative RP-HPLC: R_t = 14.2 min (5 to 100% B in 30 min, 214 nm); analytical RP-HPLC: R_t = 8.1 min (5 to 100% B in 15 min, 214 nm); MALDI-FT-ICR HRMS data are given in Supporting Information, Table 1.

2.12. Plate Binding and Plate Inhibition Assays. Inhibition assays were performed as described previously[21,22] with the difference that the soluble NKR-P1 and CD69 protein receptors were labeled with fluorescent labels (fluorescein and rhodamine, respectively). The concentration of bound protein receptors in the microtiter wells was determined by fluorescence ($\lambda_{ex}/\lambda_{em}$ = 496/519 nm and $\lambda_{ex}/\lambda_{em}$ = 546/577 nm, respectively) using a Safire 2 spectrophotometer (Tecan, Austria). 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 labeled 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. Briefly, 1 mg of the activated dye was dissolved in 50 μL of methanol/DMSO mixture (50:50, v/v), and 10 μL of this solution was mixed with 90 μL of protein (10 mg/mL) in 10 mM PIPES buffer pH 6.8 with 49 mM NaCl and 1 mM NaN_3. The reaction mixture was incubated at 37 °C for 2 h, and the excess of unreacted dye was removed using repeated cycles of dilutions with the above buffer, and concentrations using a Centricon 10 device. Finally, the labeled protein was repurified, using reverse-phase chromatography and gel filtration,[21,22] and stored at 4 °C as a 10 mg/mL solution before use in the binding assay. The labeling resulted in the attachment of four molecules of fluorescein and five molecules of rhodamine per mol of NKR-P1A and CD69, respectively, as determined by quantitative spectrophotometry, MALDI-TOF, and ion cyclotron FT-MS.[22] Preliminary binding curves were performed with the starting and the labeled proteins using microtiter wells coated with GlcNAc$_{23}$BSA in order to verify that the labeling procedure did not have any adverse effects on the binding activity of the receptors.

2.13. Precipitation Assays. Each ligand was dissolved in water at concentrations of 200, 60, 20, 6, and 2 nM. The ^{125}I-labeled protein (50 μL, 20 nM) was added to each sample (50 μL) in 96-well microtiter plates. Mixtures were incubated at 4 °C for 30 min, and then a 20% (v/v) solution of PEG 8000 was added (100 μL). The mixture was left to precipitate for one hour at 4 °C. After centrifugation (10 min, 4 °C, 1800 rpm), the supernatant was carefully removed, and a 10% (v/v) solution of PEG 8000 (100 μL) was added. This procedure was repeated three times to wash the precipitate. After additional centrifugation and supernatant removal, the precipitates were dried overnight at 37 °C. The assay was continued as described previously.[23]

2.14. Inositolphosphate Production. [^{3}H]Inositol phosphates were separated and quantified by the methods described previously.[24] Incorporation of [^{3}H]inositol into the phospholipid was achieved by incubating human peripheral blood mononuclear cells (PBMC) (10^7 cells/ml) with 100 μL of [^{3}H]inositol (1.48 TBq/mol, 37 MBq/ml; GE Healthcare) for 3 h at 37 °C, followed by extensive washing, and resuspension at 10^8 cells/mL. 50 μL of this suspension containing 5×10^6 cells in complete RPMI 1640 with 10 mM Hepes pH 7.4 was mixed with 50 μL of the tested compounds, and incubated at 37 °C for the indicated times. The reaction was stopped by rapid transfer of the reaction mixture to 100 μL of 10% trichloroacetic acid. The reaction was neutralized by the addition of 50 μL of triethylamine; 20 μL of 50% aqueous slurry of Dowex l-X8, 100−200 mesh (Sigma) in formate form was then added. The supernatant was collected, and inositolbisphosphates and

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inositoltrisphosphates were eluted by the addition of 50 μL of 0.3 and 0.6 M ammonium formate pH 7.0, respectively. The eluent was dried in a thin-walled 96-well plate, and the radioactivity was counted in 100 μL of Biodegradable Counting Scintillant (GE Healthcare) using the Microbeta counter (Wallac).

2.15. Intracellular Calcium Monitoring.[24] Human PBMC were loaded with the calcium-sensitive fluor Indo-1 by incubating 10^7 cells/mL with 5 pM Indo-1, AM (Molecular Probes, Eugene, OR) in complete RPMI 1640 with 25 pM 2-ME at 37 °C. Cells were washed twice and resuspended at $5 \times l0^6$ cells/mL in fresh medium. The fluorescence of the cell suspension was monitored with a Safire2 spectrofluorimeter using an excitation wavelength of 349 nm and emission wavelength 410 nm. The signal was calibrated for each experiment by lysing the Indo-1-loaded cells with Triton X-100 (0.07%) for maximum fluorescence. The minimum fluorescence was determined after the addition of 10 mM EGTA and sufficient Tris base to raise the pH to >8.3. Intracellular calcium concentration was calculated using the formula: $[Ca^{2+}]$ (nmoles) $= 250 \times [(F - F_{min})/(F_{max} - F)]$ where F is the measured fluorescence, and 250 (nM) is the dissociation constant of Indo-1. Signaling was measured in the absence of extracellular calcium in medium containing 1 mM EGTA. Where indicated, ionomycin (Sigma) was added to a final concentration of 1 μM.

2.16. Natural Killing. The standard ^{51}Cr release test was performed as described previously.[25] Briefly, 10^4 chromium-labeled leukemic cells K562 in 100 μL of complete RPMI 1640 was mixed in triplicates with the tested compounds in 50 μL of RPMI 1640 in round-bottomed 96-well plates. Thereafter, the appropriate amount of effector cells (PBMC obtained by Ficoll-Isopaque separation)[26] was added in 100 μL of complete RPMI 1640, and the plate was placed into a tissue incubator and incubated at 37 °C for 3 h. Triton X-100 (50 μL of 1%) was added into the maxima release wells, and the incubation continued for another 1 h. Plates were cooled on an ice bath, and 100 μL of the supernatant was used for radioactivity measurements. Specific lysis (%) was calculated using the formula % = [(exp − spont)/(max − spont)] × 100, where exp are the counts in experimental wells, spont counts in wells containing medium instead of the effector cells, and max are the counts in wells containing 1% Triton X-100. Complete killing curves were constructed, from which the lytic unit counts for individual experiments were calculated. Lytic efficiency was defined as the inverse of the lytic unit count.

2.17. Apoptosis Assays. Cells were resuspended at 2×10^6/mL in complete RPMI 1640 medium and aliquoted into round-bottomed 96-well plates, and then the tested concentrations of compounds were added into duplicate test wells. 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. The percentage of apoptotic cells (Annexin V^+/Hoechst 33258^-) observed in the presence of PBS alone or in the presence of 5×10^{-6} M arsenite were used as the negative and positive controls, respectively.

3. Results and Discussion

3.1. Synthesis and Analysis of LELTE Conjugates and Glycoconjugates 1−4. Unglycosylated and glycosylated LELTE conjugates **1**−**4** were fully synthesized following the highly efficient iterative oxime procedure reported earlier.[27,28] For this purpose, two different cyclodecapeptide cores **5** and **11** were prepared. In the first RAFT core **5**, four Fmoc-Lys(BocSer-(tBu))OH building blocks[29] and two orthogonally protected lysines with 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde)[30] were used, whereas two building blocks and four Fmoc-Lys(Dde)OH were used for RAFT core **11** (Scheme 2). In both cases, the selective removal of Dde by treatment with hydrazine was followed by the incorporation of the ethoxyethylidene aminooxy acetic acid linker as the *N*-hydroxysuccinimidyl ester **6** (Eei-Aoa-OSu)[31] on the free lysine side chain to produce protected RAFT cores **7** and **12**. Final acidic treatment with a solution of trifluoroacetic acid and carbocation scavengers yielded **8** and **13**, displaying either four or two serines as aldehyde precursor and two or four aminooxy groups.

The complete assembly of each LELTE conjugate was performed by successive oxime bond formation and serine oxidation steps.[28] A pentapeptide LELTE-aldehyde **9** was first synthesized by oxidative cleavage from the corresponding hexapeptide sequence containing a serine residue at the *N*-terminal end.[32] An excess of **9** was further used for coupling to the aminooxy-containing RAFT cores **8** and **13** under mild aqueous acidic conditions. We thus quantitatively obtained two classes of conjugates displaying two and four copies of the LELTE peptide, respectively. These synthetic intermediates were next treated with sodium periodate to generate the aldehyde functions required for further coupling of the second addressable domain of the RAFT core with hydroxylamine or aminooxy αGalNAc **10**.[20] As illustrated in Supporting Figure S2 for **1** and **2**, analytical HPLC monitoring during the coupling reaction showed that reactions were complete after a few hours and yielded pure products in the reaction mixtures. The excess of aminooxy reagents was finally removed, and hydroxylamine and αGalNAc LELTE conjugates **1**−**4** were isolated by RP-HPLC in good yields (66−80%). High-resolution mass spectra measured by MALDI-FT-ICR confirmed the well-defined structure of the synthesized LELTE conjugates (Supporting Information, Table 1).

3.2. Affinity of Control and Glycosylated LELTE Conjugates for CD69 and NKR-P1 Receptors. We tested the binding of the tested compounds to both recombinant rat NKR-P1 specific for GlcNAc and GalNAc, and recombinant human CD69 that binds both GlcNAc and GalNAc, as well as the LELTE pentapeptide. As the initial test, we used plate inhibition assays in which the evaluated compounds are tested as the inhibitors of binding of the above receptors to their high affinity ligands, $GlcNAc_{23}BSA$ neoglycoprotein. A summary of the inhibition results is given in Table 1. The results clearly confirm the anticipated specificities of the two receptors. Thus with rat NKR-P1, high values of IC_{50} were only recorded for the glycosylated LELTE conjugates (compounds R(4αGalNAc-2LELTE) **2** and R(4LELTE-2αGalNAc) **4**), since the LELTE peptide is not recognized by this receptor. On the other hand, CD69 recognizes both *N*-acetylhexosamines and the LELTE peptide, and thus the highest activities are attained by compounds having both of these substitutions. In particular, R(4LELTE-2αGalNAc) **4** is one of the best ligands for the human CD69 receptor identified so far with an IC_{50} as low as 10^{-11} M (Table 1). Compound R(4αGalNAc) (**18**) carrying only carbohydrate moieties was

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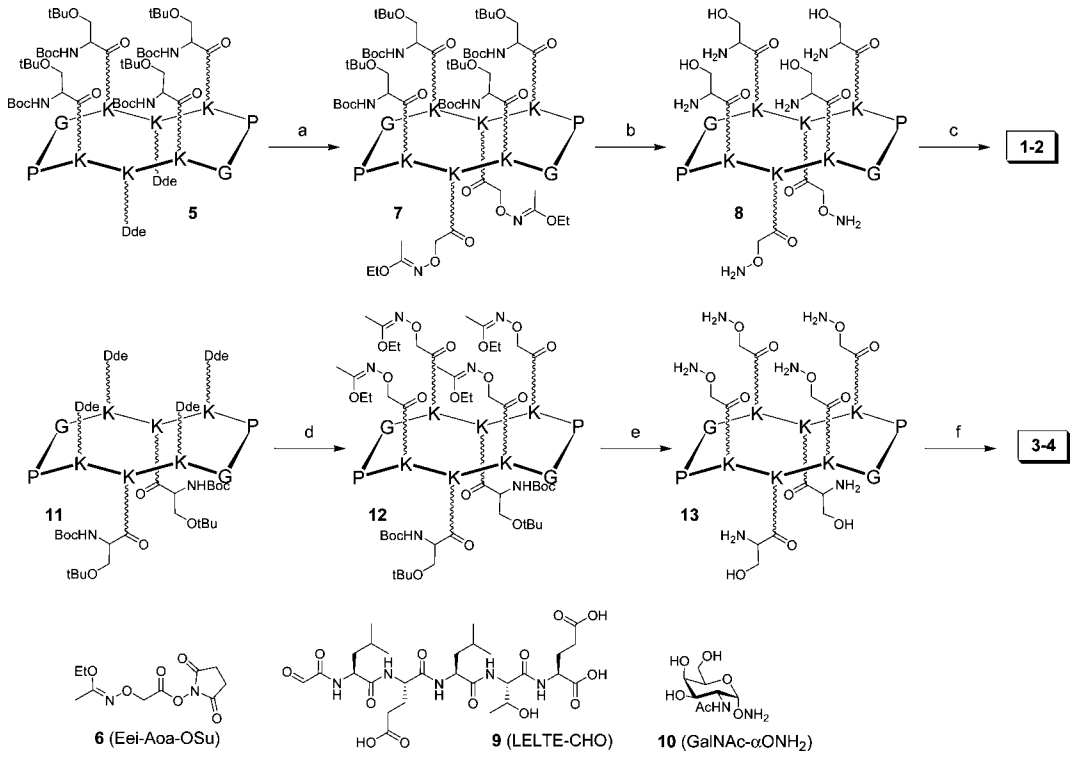
Scheme 2. Synthesis of LELTE Conjugates **1**−**4**[a]



[a] Reagent and conditions: (a) i: hydrazine/DMF (3:97, v/v); ii: **6**,[31] DIPEA, DMF; (b) TFA/TIS/H_2O (95:2.5:2.5, v/v); (c) i: **9**, CH_3CN/H_2O/TFA (1:1:0.1, v/v:v); ii: $NaIO_4$, H_2O; **10**,[20] CH_3CN/H_2O/TFA (1:1:0.1, v/v/v); (d) i: hydrazine/DMF (3:97, v/v); ii: **6**,[31] DIPEA, DMF; (e) TFA/TIS/H_2O (95:2.5:2.5, v/v/v); (f) i: **9**, CH_3CN/H_2O/TFA (1:1:0.1, v/v/v); ii: $NaIO_4$, H_2O; **10**,[20] CH_3CN/H_2O/TFA (1:1:0.1, v/v/v).

Table 1. Summary of the Plate Inhibitions Assays Performed with the Tested Compounds and NKR-P1A and CD69 Receptors

compound	−log IC_{50}
NKR-P1A (rat)	
GlcNAc	5.8 ± 0.1
18 R(4αGalNAc)	5.6 ± 0.2
1 R(4Ox-2LELTE)	5.8 ± 0.9
2 R(4αGalNAc-2LELTE)	8.8 ± 0.2
3 R(4LELTE-2Ox)	6.6 ± 0.2
4 R(4LELTE-2αGalNAc)	8.5 ± 0.4
CD69 (human)	
GlcNAc	4.2 ± 0.5
18 R(4αGalNAc)	—[a]
1 R(4Ox-2LELTE)	7.1 ± 0.1
2 R(4αGalNAc-2LELTE)	9.8 ± 0.1
3 R(4LELTE-2Ox)	8.7 ± 0.5
4 R(4LELTE-2αGalNAc)	11.0 ± 0.1

[a] No inhibition detected at all.

used as a control to demonstrate importance of the LELTE peptide: it had no inhibitory activity with CD69 receptors and only limited activity with NKR-P1A that was comparable to a monosaccharide (Table 1).

In addition, we also tested the ability of the evaluated conjugates to precipitate the soluble recombinant receptor in a reaction that is more relevant to biologically relevant receptor cross-linking that serves as a prerequisite for membrane signaling and cellular activation. The precipitation reactions with rat NKR-P1 proceeded in accordance with the surface density of the preferred ligand, and thus the highest activities were achieved with R(4αGalNAc-2LELTE) **2**, having four GalNAc residues (Figure 2, upper). Similarly, the highest precipitation activities for CD69 could be achieved with the compounds R(4LELTE-2Ox) **3** and R(4LELTE-2αGalNAc) **4**, i.e. those that had high

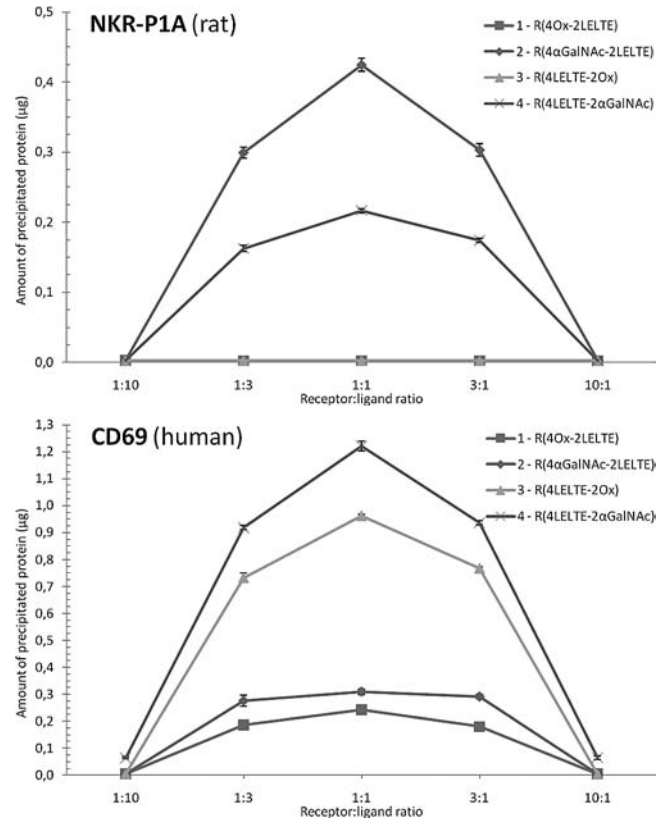


Figure 2. Precipitation curves for the interaction of the tested compounds with recombinant soluble rat NKR-P1A (upper) and human CD69 (lower). Results are the means of duplicate determinations from three representative experiments.

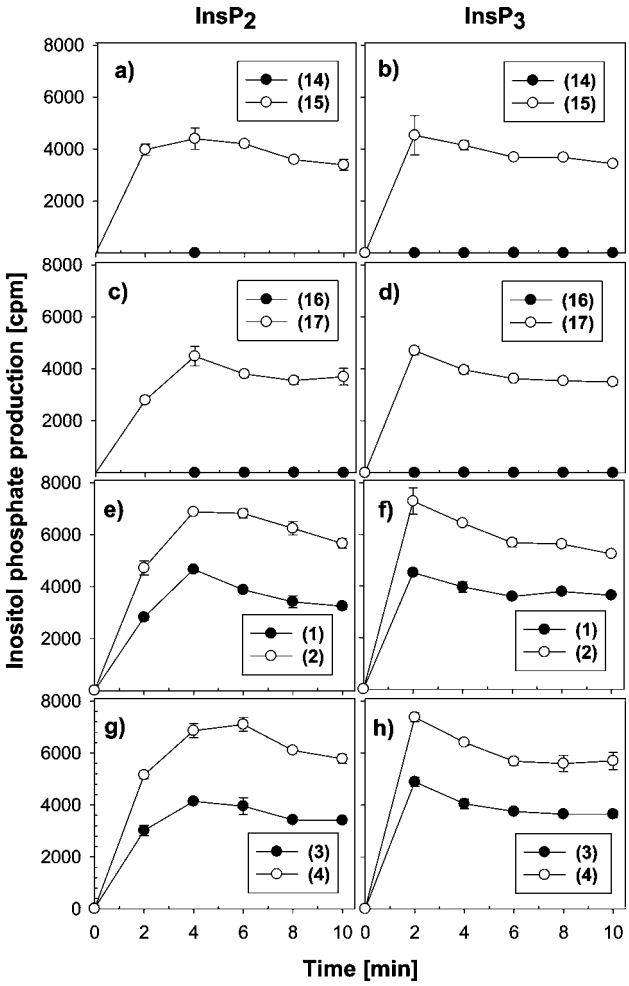


Figure 3. Inositolphosphate production in human PBMC incubated in the presence of tested compounds. The production of inositolbisphosphates (left column) and inositoltrisphosphates (right column) was followed for 10 min after the addition of compounds. Values are mean ± range.

substitution with the LELTE peptide (Figure 2, lower). These results are understandable, since the affinity of the LELTE peptide for the CD69 receptor is much higher than that of the GalNAc monosaccharide.[15]

3.3. Effect of LELTE Conjugates Glycosylation on Cellular Activation of Human PBMC. To evaluate the effect of the glycosylation of LELTE conjugates on receptor-mediated activation, two different cellular activation assays routinely used for studies on other leukocyte receptors were performed. While monomeric ligands **14** and **16** had no effects on inositolphosphate production, dimeric forms **15** and **17** were active (Figure 3, panels a and b). The efficiency of leukocyte activation by LELTE conjugate **1** containing dimeric LELTE is comparable to **17**, and no additional effect could be seen using the LELTE conjugate R(4LELTE-2Ox) **3** with tetrameric LELTE (Figure 3, panels e−h). However, the glycosylated LELTE conjugates R(4αGalNAc-2LELTE) **2** and R(4LELTE-2αGalNAc) **4** displayed a clear synergistic effect on cellular signaling, resulting in significantly increased levels of inositolphosphate production (Figure 3, panels e−h).

The inositol phosphate data were further corroborated by monitoring intracellular calcium in the absence of an extracellular calcium source. Under these conditions, the generated inositolphosphates activate their specific receptors at the intracellular side of the endoplasmic reticulum (ER) membranes,

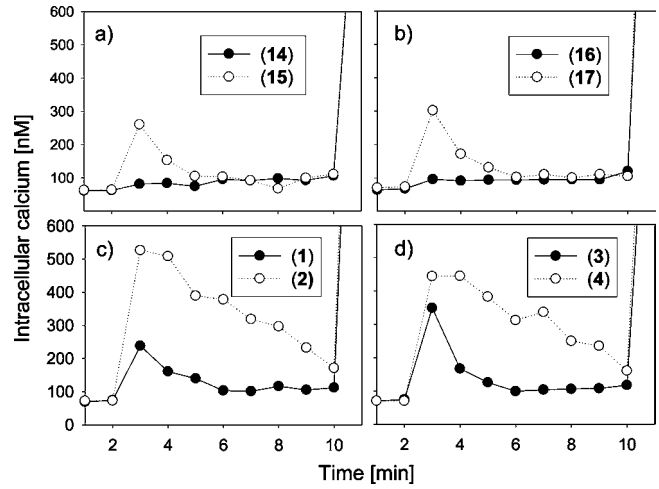


Figure 4. Effect of tested compounds on intracellular calcium levels in human PBMC incubated in the presence of tested compounds. Intracellular calcium was monitored for 2 min before the addition of tested compounds, and then for an additional 8 min in the presence of the tested compounds after which the ionomycin positive control was added. The experiment was conducted in the absence of extracellular calcium.

resulting in the opening of calcium channels and transfer of calcium from the ER into the cytoplasm. While monomeric ligands **14** and **16** had no effect on intracellular calcium levels, the dimeric forms of these ligands, **15** and **17**, resulted in short-term (more than 3 min) calcium signals (Figure 4, panels a and b). Using conjugate R(4LELTE-2Ox) **3** with tetrameric LELTE, the calcium signal was increased, but remained short-term (Figure 4, panel d). In the combined glycosylated LELTE conjugates, R(4αGalNAc-2LELTE) **2** and R(4LELTE-2αGalNAc) **4**, the calcium signal was significantly increased and sustained over a period of 8 min (Figure 4, panels c and d).

These results indicate that (1) CD69 receptor is very efficiently engaged by LELTE dimerized via a linear or RAFT scaffold and (2) further clustering of LELTE into tetrameric form does not have additional effects due to the saturation of the CD69 receptor signaling system. The addition of glycosylation to LELTE conjugates initiated the engagement of additional receptors (such as NKR-P1) triggering significantly increased signaling caused most probably by cross-talk of the two different receptors, each equipped with its own signal amplification cascade.[1,12]

3.4. Effect of LELTE Conjugates Glycosylation on Natural Killing. In order to evaluate the immunological activities of the tested compounds related to their potential use in tumor therapies, we followed their effect on natural killing of leukemic tumor targets. PBMC were used as a source of effector cells using effector/target (E/T) ratios from 30:1 to 300:1. Monomeric ligands **14** and **16** had little effect on natural killing or were slightly inhibitory (Figure 5, panels a and b). Dimeric ligand **15** caused an approximately 2-fold increase in natural killing, but dimeric ligand **17** was inhibitory, as were LELTE conjugates R(4Ox-2LELTE) **1** and R(4LELTE-2Ox) **3** (Figure 5c). The glycosylated LELTE conjugates R(4αGalNAc-2LELTE) **2** and R(4LELTE-2αGalNAc) **4** were prominent in significantly increasing natural killing, especially **4**, which increased the killing about 3-fold (Figure 5c).

High-affinity ligands for CD69 and other immune receptors, when multivalent, often initiate an extensive cross-linking of the target receptor resulting in cell hyperactivation, which may lead to activation-induced apoptosis.[23] Therefore, it appeared

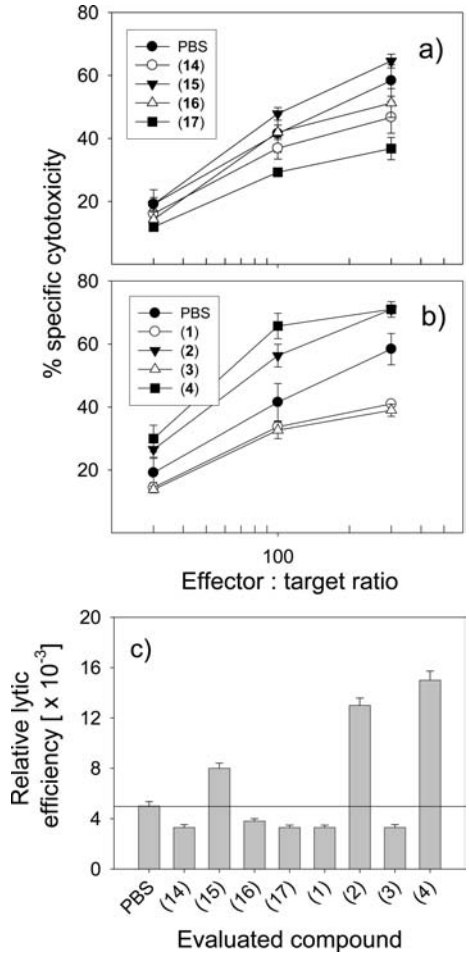


Figure 5. Natural killing of YAC-1 lymphoma tumor cells by human PBMC in the presence of tested compounds. In (a) and (b), experiments were performed in triplicate at the indicated *E*/*T* ratio. Values are mean ± SD. (c) Lytic efficiency of human cells, defined as the reciprocal value of the number of lytic units.

critical to test the effect of LELTE and combined RAFTs on lymphocytes expressing varying degrees of cell surface expression of the antigen. When we tested the influence of these compounds on human lymphocytes displaying a low surface level of CD69 expression, none of the tested compounds had significant effects on these cells apart from the arsenite positive control (Figure 6, left panel). Surprisingly, however, compounds **1**−**4** also had very little effect on the apoptotic killing of lymphocytes with a high surface expression of CD69 (Figure 6, right panel), despite the fact that dimerized *N*-acetylhexosamine disaccharide **15**, and dimerized LELTE antigen **17** had significant effects. These results were further corroborated by the apoptosis tests using DNA laddering that fully confirmed these effects (for details see Supporting Information, Figure S1). These observations greatly increase the attractiveness of LELTE RAFT conjugates for tumor therapies, in which the activation-induced apoptosis of the killer cells bearing the target receptors represents one of the major problems within the aggressive tumor microenvironment.[27]

4. Conclusions

As summarized in Figures 5 and 6, we demonstrated for the first time that carbohydrates and immunoactive peptides may cooperate as part of a single complex RAFT-based compound in the activation of killer cells of the immune system, while at

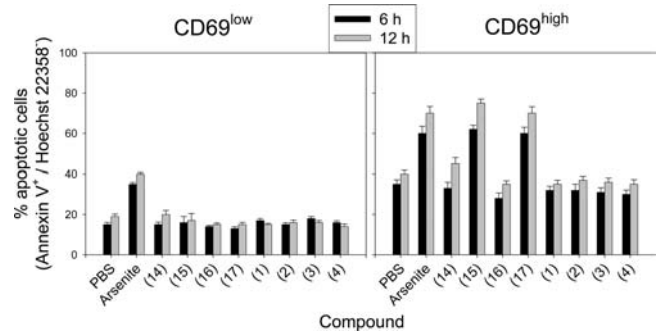


Figure 6. Effects of LELTE RAFT conjugates and control compounds on apoptosis of CD69^{low} and CD69^{high} lymphocytes using 10^{-10} M concentrations of the tested compounds. Percentage of apoptotic cells (Annexin A^+/Hoechst 33258^-) was measured 6 or 12 h after the addition of compounds. PBS, negative control; arsenite, positive control. The results are the means ± standard deviations calculated from three independent determinations performed in duplicate.

the same time displaying minimal undesired effects such as activation-induced apoptosis.[23] Before initiating complex immunological tests, the synthesized compounds were tested for their binding to the two most studied lectin-like receptors of natural killer cells, NKR-P1, and CD69 proteins.[5] The reactivity with these receptors tested in standard plate inhibition assays was in accordance with previous knowledge on the specificity of the two receptors, clearly indicating that the two ligands, GalNAc and LELTE pentapeptide, were readily available for interaction with the target receptors, even when covalently bound to the complex RAFT scaffold. Also, the ability of the RAFT conjugates to cross-link the relevant target receptors was tested based on their ability to precipitate the soluble recombinant proteins, which is a good measure of their abilities to interact with the membrane-bound forms of the receptors. In cellular activation assays based on measurements of inositol phosphate production and the increase in the intracellular calcium levels, the combined peptide/carbohydrate RAFTs proved much more efficient compared to RAFTs substituted only with the peptide, indicating efficient cross-talk between several receptor families. In those instances where it proved difficult to correlate the plate inhibition data with receptor precipitation results and cellular activation assays, we favor the results of those assays during which the relevant receptor could work in its natural environment to those obtained recombinant bacterially produced forms of the receptor.[23] Moreover, we prefer results obtained in the precipitation assays performed under conditions more relevant to receptor biology to results coming from plate inhibition assays employing somewhat artificial evaluation system.[13] The efficient activation of the leukocytes was paralleled in the ability of these compounds (in particular compound **4**) to efficiently activate natural killing against the established cell lines with a significant (up to 3-fold) increase in specific cytotoxicity. Interestingly, at the same time the mode of presentation of the active peptide in these compounds efficiently prevented the development of critical unwanted side activities, such as activation-induced apoptosis of $CD69^+$ lymphocytes that often poses a problem for efficient activators of these cells. During a natural killing assay, the cellular receptors and their ligands work in a complex network of cellular interactions that may result in killing both the tumor target and the effector lymphocyte. The primary interaction of effector lymphocyte receptors with target structures at the surface of a tumor generally results in the activation of an effector cell that is able to kill the tumor cell more efficiently, but the hyperactivation of the effector cell may also

result in its susceptibility to apoptosis and premature elimination. The in vitro natural killer assays described here provide evidence that cross-linking CD69 with other surface receptors specific for GlcNAc or GalNAc carbohydrates results in more efficient killing of the tumor while at the same time sparing the activated killer cells delivering the lethal attack. These results thus provide an encouraging starting point for the prospective use of these compounds in experimental tumor therapies in vivo.

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Supporting Information Available: Complete ref 22; Supporting Figure S1 and Supporting Methods describing apoptosis using DNA laddering; Supporting Figure S2. RP-HPLC profile of crude reaction mixture of **1** and **2** and Supporting Table 1 with MS data of **1**−**4** to further support the observations reported herein. This material is available free of charge via the Internet at http://pubs.acs.org.

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