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Carboxylated calixarenes bind strongly to CD69 and protect CD69$^+$ killer cells from suicidal cell death induced by tumor cell surface ligands

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ABSTRACT

We have recently identified a new class of high affinity ligands for CD69 leukocyte membrane receptor, carboxylated calixarenes. Of the three compounds investigated here, thiacalix[4]arene had the highest affinity for CD69 in direct binding assays, and proved to be the most specific inhibitor of CD69 identified so far in receptor precipitation and cellular activation experiments. Carboxylated calixarenes also proved effective at protection of CD69^{high} lymphocytes from apoptosis triggered by a multivalent ligand or antibody. Thus, carboxylated calixarenes set a new paradigm for noncarbohydrate ligands for CD69 making them attractive for protection of killer cells in combined animal tumor therapies.

1. Introduction

Receptor-mediated immune therapies have been established as important tools within the current strategies against the malignant disease. Until now, few of them rely on the manipulation of the cellular arm of the innate immunity by altering the balance between signals transmitted through inhibitory and activating receptors of killer cells.[1–3] Among these receptors, CD69, a universal leukocyte triggering molecule and an earliest lymphocyte activation antigen has a specific role. Unlike the prototype NKR-P1 receptor[4] cloned earlier, CD69 is also expressed by most other leukocytes in addition to killer cells,[5] and its role in tumor killing remains controversial.[6] Originally, it was reported as an important activation receptor of NK cells by Moretta et al.[7] However, it was recently shown by Sanchez-Madrid and co-workers that CD69$^{-/-}$ mice have increased resistance to MHC class I$^-$ lymphomas.[8] One reason for this controversy may be the hyperactivation of CD69$^+$ killer cells upon their contact with tumors, followed by their increased sensitivity to apoptotic death. However, until now such CD69 dependent activation-induced cell death has been described only in monocytes or eosinophils.[9–11]

CD69 is expressed at cell surface as homodimeric receptor belonging to C-type lectin family.[5] The physiological ligand for this receptor is not known, although our results indicate that CD69 is a functional lectin able to bind calcium and *N*-acetylhexosamines.[12–14] Introduction of charged groups into *N*-acetylhexosamine sequences increases significantly the affinity for CD69.[15,16] Sialylated oligosaccharides bearing negatively charged groups in Siaα(2→6)-Gal or Siaα(2→6)GalNAc (SiaTn) sequences are among such high affinity ligands. Since these sequences form integral part of multivalent sialomucins at carcinoma surfaces,[17–19] they may be among the ligands initiating activation-induced apoptosis in killer cells. This process could be inhibited by monomeric SiaTn. However, this disaccharide is expensive, not easily available, and unstable. Therefore, we have been searching for other high affinity ligands for CD69.

We have recently identified additional classes of non-carbohydrate ligands for CD69. Among them, carboxylated calix[4]arenes have been standing out as efficient ligands for CD69.[20] Here, we describe the properties of three additional compounds in this series.

2. Results

2.1. Design and synthesis of carboxylated calix[4]arenes

We have previously described the development of efficient ligand mimetics for lectin-like receptors, rat NKR-P1A and human CD69, based on their reactivity with *N*-acetyl-D-glucosamine

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(GlcNAc) that has been arranged into clustered form via covalent attachment to various chemical scaffolds. Of the tested compounds, the tetrasubstituted calix[4]arene 1 (Scheme 1) proved to be a very specific ligand for recombinant human CD69.[20] The development of a compound suitable as negative control in these experiments turned out to be difficult since the corresponding calix[4]arenes without GlcNAc substitution had very poor solubility in water. Thus, calix[4]arene and thiacalix[4]arene derivatives bearing carboxyl groups represented a promising alternative. Surprisingly, these 'non-reactive' controls showed a remarkable inhibitory activity for CD69 receptor with an IC_{50} as low as 1.2×10^{-8} M.[20] However, such high inhibitory potency could not be observed for the other lectin-like receptor examined (rat NKR-P1A protein), which indicated a high degree of specificity of these compounds for the human CD69 antigen.[20] These findings motivated us to test their reactivity with both the soluble and the cellular form of CD69 receptor under in vitro conditions. The structures of the tested compounds used in the present study are given in Scheme 1 and Supplementary Figure S1.

2.2. High affinity binding of carboxylated calixarenes to CD69

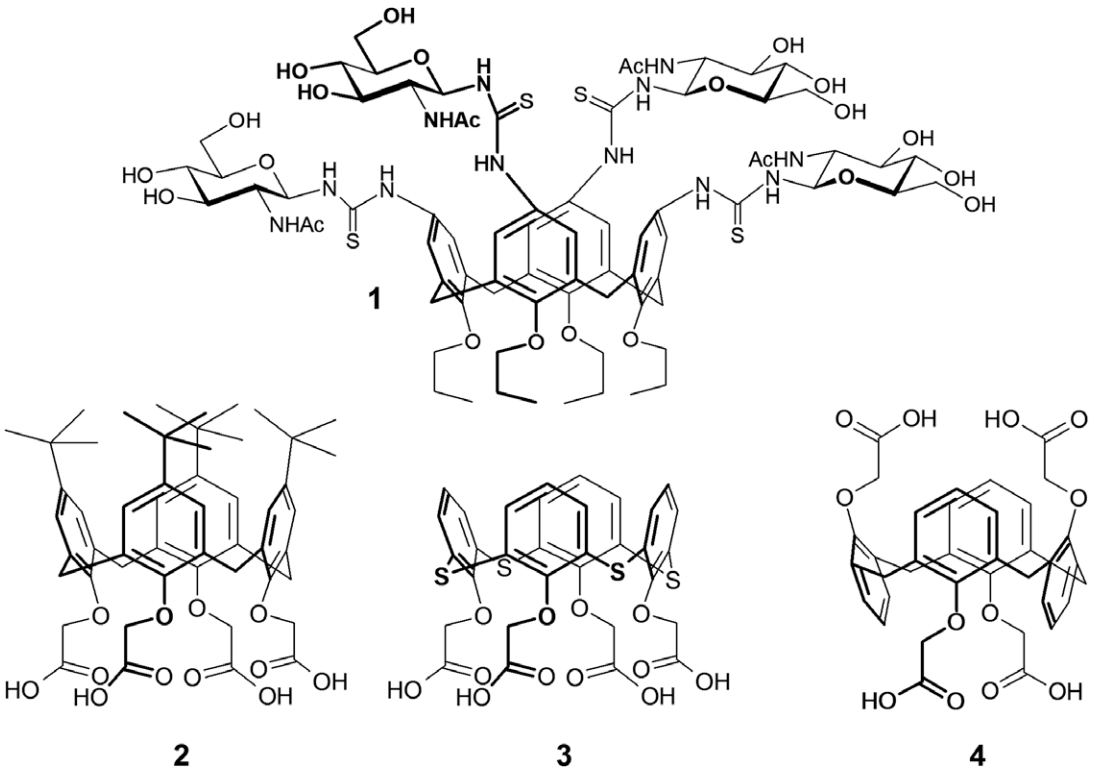
The available carboxylated calixarenes (see Supplementary Fig. S1) were initially screened in standard plate inhibition assays using recombinant soluble human CD69.[14] Their inhibitory potencies, expressed in a logarithmic scale as $-\log IC_{50} \pm SD$, varied in the range 3.4–7.9 compared to GlcNAc standard (3.0 ± 0.4) (Supplementary Table S1). Calixarene **1** substituted with *N*-acetyl-D-glucosamine (GlcNAc) monosaccharide that was described previously[20] to have one of the highest affinities for human CD69 ($-\log IC_{50} = 9.3$) was selected for detailed binding and functional studies together with the three new carboxylated calixarenes, compounds **2**, **3** and **4**, exhibiting IC_{50}'s in the nM range, and thus being among the most potent CD69 ligands identified so far (Table 1). The newly evaluated compounds included the *t*-butylated form of the hydroxycarbonylmethoxy calix[4]arene (**2**) as well as thiacalix[4]arenes, in cone (**3**) and 1,3-alternate (**4**) arrangement (Scheme 1).

Table 1
Affinity of carboxylated calixarene ligands for human CD69 receptor expressed on a logarithmic scale as $-\log IC_{50} \pm SD$

Compound	Affinity
GlcNAc (standard)	3.0 ± 0.4
1[20]	9.3 ± 0.3
2	7.6 ± 0.5
3	7.9 ± 0.4
4	7.3 ± 0.3

2.3. Direct binding of carboxylated calixarenes to soluble human CD69

In order to confirm the affinity of calixarenes for CD69 using another independent assay, we measured their direct binding to the recombinant soluble form of the receptor using tryptophane fluorescence quenching. No decrease in fluorescence could be detected in the control, CD69 related receptor NKR-P1,[21] at any of the tested concentrations of the three carboxylated calixarenes **2**, **3**, and **4**. With CD69, all three compounds displayed a concentration-dependent decrease in tryptophane fluorescence that could be observed at concentrations 10^{-10} M with **3**, 10^{-9} M for **2**, and 10^{-8} M for **4** (Supplementary Fig. S2). These results were further corroborated in the detailed tryptophane fluorescence quenching measurements presented in Figure 1. With all three tested compounds, the binding curves were linear with slopes (n) close to 2, indicating the existence of two binding sites for carboxylated calixarenes per receptor dimer.[14] The highest affinity was achieved for **3** with K_d



Scheme 1. Design of the calix[4]arene derivatives used in the study. The current calix[4]arene set included the *N*-acetylglucosamine-substituted derivative used previously[12] (**1**, upper panel), as well as the three new carboxylated derivatives **2** (lower left), **3** (lower middle), and **4** (lower right).

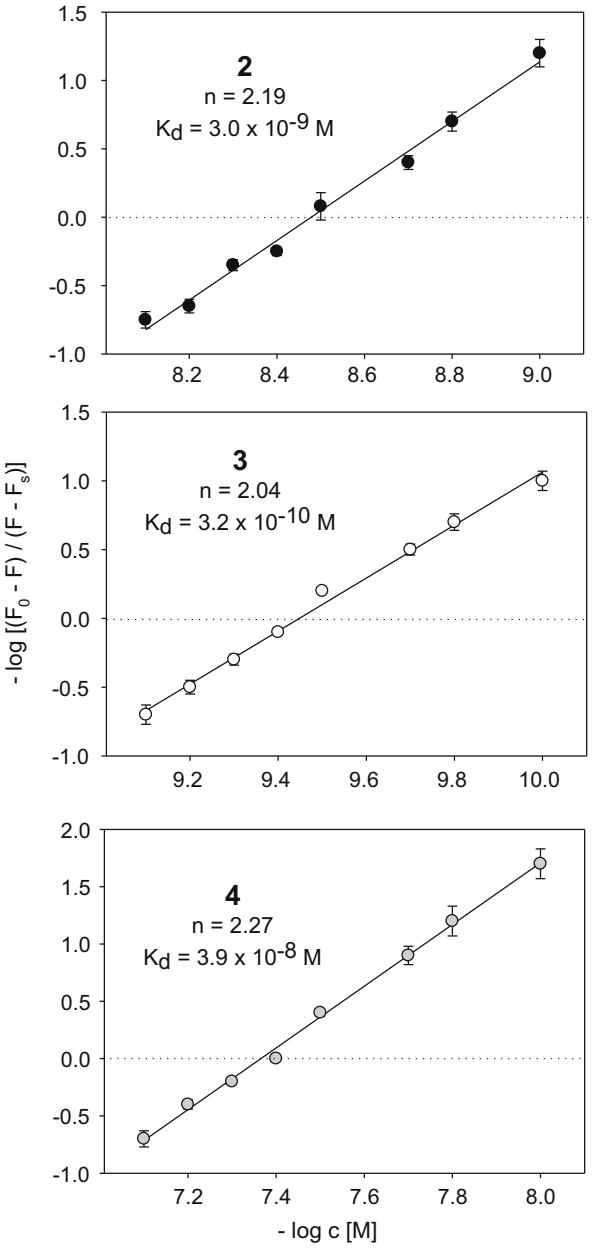


Figure 1. Direct binding assays for interaction of the tested carboxylated calixarenes (compounds **2**, **3**, and **4**) with recombinant human CD69. The corrected fluorescence was plotted against the concentration of individual compounds. Results are the means from three independent determinations with standard deviations indicated by error bars.

of 3.2×10^{-10} M, indicating the existence of a highly specific interaction.

2.4. Carboxylated calixarenes inhibit the precipitation of soluble CD69 by multivalent ligand

Additional data about the binding affinity of calixarenes to CD69 could be obtained from the inhibition of the binding of this receptor to its high affinity ligand or from the inhibition of the precipitation of the receptor by its multivalent ligand. We used the latter approach since it resembles the natural interaction with the receptor.[16] The soluble CD69 receptor precipitated efficiently at concentrations as low as 10^{-8} M using an equimolar concentration of the multivalent carbohydrate ligand SiaTnTRI2. Under the conditions of the assay, the precipitation of the receptor is very efficient as more than 95% of the added receptor protein is recovered in the precipitate.[16] We therefore tested the ability of carboxylated calixarenes to inhibit this precipitation in the concentration range 10^{-5}–10^{-10} M. The IC_{50} values for the tested carboxylated calixarenes were in accordance with their binding affinities for CD69: **3** proved to be the best inhibitor with IC_{50} as low as 1.5×10^{-8} M, followed by **2** and **4**, which had IC_{50}'s of 1.5×10^{-7} M and 1.3×10^{-6} M, respectively (Fig. 2A). Moreover, these compounds are also able, with identical hierarchy, to inhibit the precipitation of CD69 by specific monoclonal antibody (mAb), albeit at concentrations two orders of magnitude higher (Fig. 2B).

2.5. Carboxylated calixarenes inhibit the activation of human lymphocytes following CD69 cross-linking

The effect of the tested calixarenes on lymphocytes bearing the membrane-bound form of CD69 was investigated using cellular activation assays. We screened blood donors for the expression of CD69 at the surface of non-adherent fraction of peripheral blood mononuclear cells (N-PBMC) obtained by density gradient centrifugation. This cellular fraction is composed mostly of lymphocytes (on average 65% T-cells, 30% B-cells, and 5% natural killer (NK) cells), and is therefore referred to below as 'human lymphocytes'. The percentage of $CD69^{+}$ lymphocytes in donors with >20% positivity was further enhanced to 75–85% by 4 h stimulation with phorbol myristate acetate (PMA) and ionomycin[22] to obtain cell population designated as $CD69^{high}$ lymphocytes. Human lymphocytes from donors with less then 5% positivity were used as $CD69^{low}$ lymphocytes. When $CD69^{low}$ lymphocytes were incubated with a PBS control, or with mAb against CD69, or with the multivalent SiaTnTRI2 ligand, or with carboxylated calixarenes, very little cellular activation could be observed using any of these

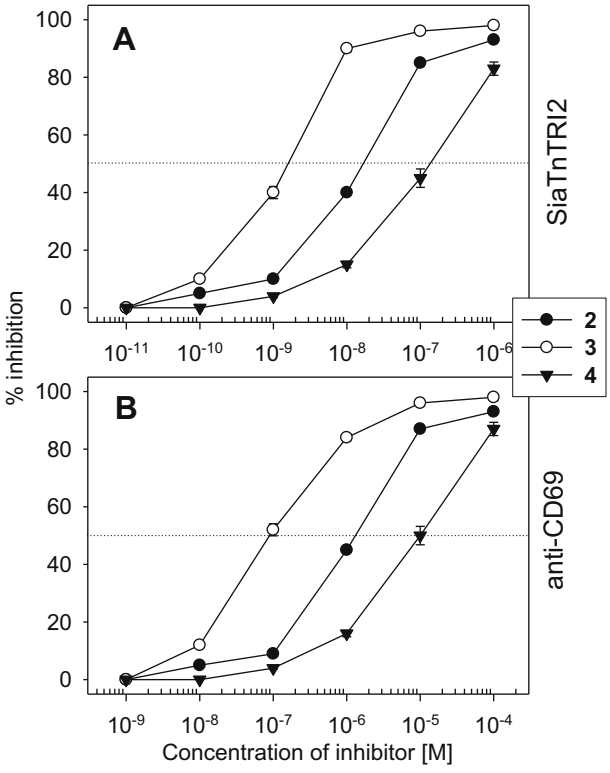


Figure 2. Inhibition of precipitation of soluble CD69 by SiaTnTRI2 (A) and mAb against CD69 (B) using the tested calixarenes (compounds **2**, **3**, and **4**). The results are the means ± standard deviations calculated from three independent measurements performed in duplicates.

reagents (Supplementary Table S2). On the other hand, CD69^{high} lymphocytes were readily activated by both ligand and mAb cross-linking, although the activation achieved by the ligand was somewhat smaller compared to that achieved by mAb (Supplementary Table S2). Essentially no activation could be observed after the addition of the three tested carboxylated calixarenes alone. We used the above optimized cellular assays to examine the ability of the tested carboxylated calixarenes to inhibit cellular activation through CD69 cross-linked by either SiaTnTRI2 or mAb. The inhibition of cellular activation occurred under conditions that precisely matched the inhibition of receptor precipitation (cf. Fig. 3 and Supplementary Fig. S3). Thus, compound **3** turned out to be the best inhibitor followed by **2** and **4**. The inhibition of cellular activation measured with two forms of inositol phosphates, inositol bisphosphate (InsP2), and inositol trisphosphate (InsP3) were identical. The IC_{50} values calculated from the complete inhibition curves are summarized in Table 2.

2.6. Carboxylated calixarenes protect CD69^{high} lymphocytes from apoptosis induced by tumor ligands or antibody cross-linking

Since the carboxylated calixarenes proved to be efficient inhibitors of the interaction of the CD69 receptor with its ligands using both the soluble form and the cell-bound form of the receptor, we wanted to evaluate the ability of these compounds to protect CD69^{high} lymphocytes from apoptosis induced by specific antibodies or cross-linking by tumor cell sialomucin type ligands. In these experiments we compared early apoptosis in CD69^{low} and CD69^{high} lymphocytes using 6 h and 12 h apoptosis assay.[23] The effect of carboxylated calixarenes was tested at three different concentrations reflecting the inhibitory concentrations in the assays for the inhibition of precipitation and for the inhibition of cellular activation (see the respective sections), as well as the concentrations two and four orders of magnitude higher.

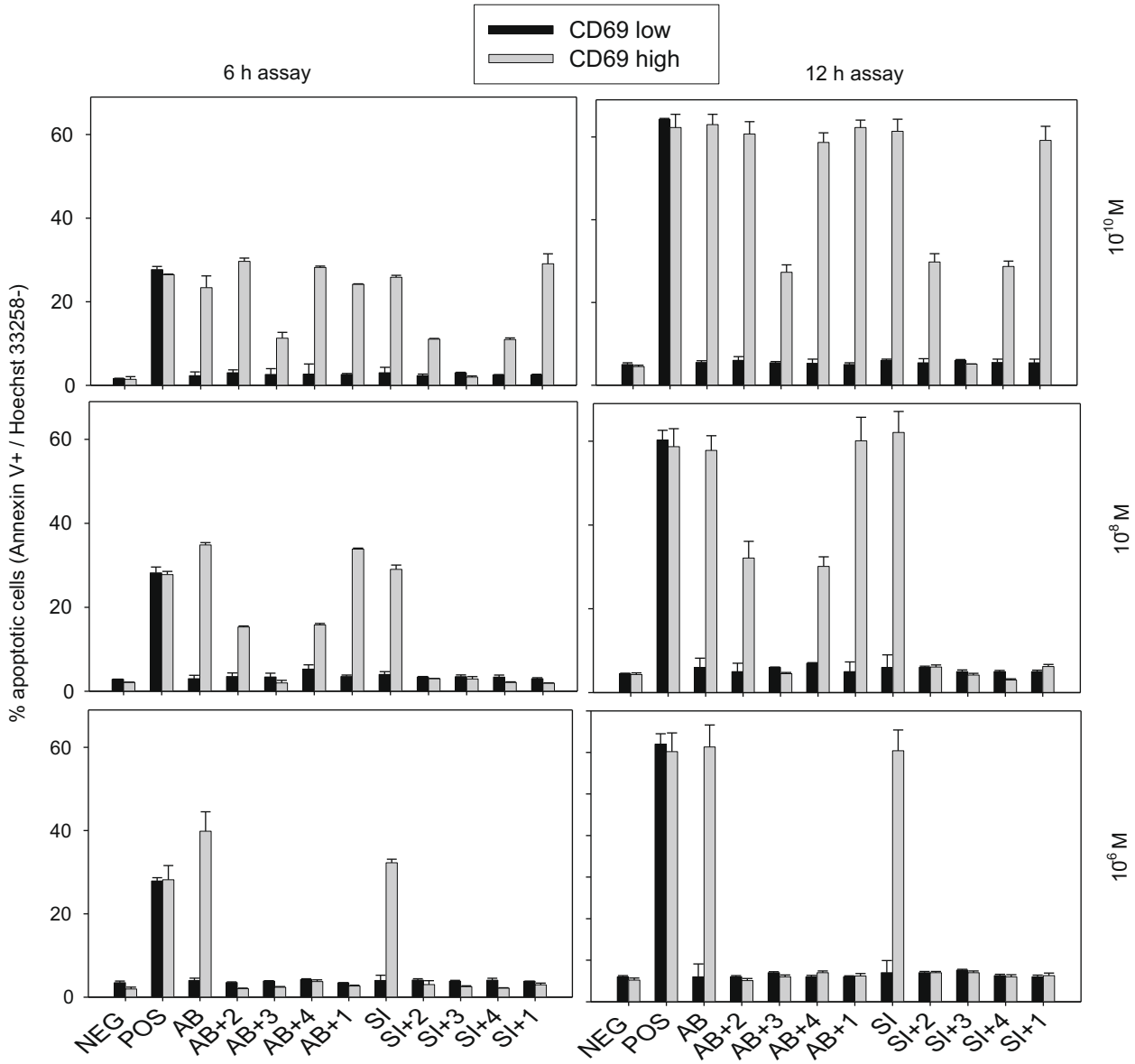


Figure 3. Protection of CD69^{high} lymphocytes from apoptosis induced by mAb (AB) or SiaTnTRI2 (SI) using 10^{-10}–10^{-6} M concentrations of the tested carboxylated calixarenes (compounds **1**–**4**). Percentage of apoptotic cells (annexin V+/Hoechst 33258^{-}) was measured in CD69^{low} or CD69^{high} lymphocytes 6 h or 12 h after the addition of compounds. NEG, PBS negative control; POS, arsenite positive control. The results are the means ± standard deviations calculated from three independent determinations performed in duplicates.

Table 2
Inhibition of activation of $CD69^{high}$ lymphocytes by SiaTnTRI2 using carboxylated calix[4]arenes based on the production of InsP2[(a)] and InsP3[(b)]

Compound	IC_{50} (M) for	
	InsP2	InsP3
2	3.7×10^{-9}	4.3×10^{-9}
3	6.5×10^{-10}	7.2×10^{-10}
4	1.4×10^{-8}	2.1×10^{-8}

The values of IC_{50} shown were calculated from the complete inhibition curves based on five experimental points from three independent experiments measured in duplicates (see Supplementary Fig. S3).

In control experiments, the direct effect of carboxylated calixarenes on the apoptosis of both cell types was measured, and found to be negligible compared to the negative control (results not shown). Using both 6 h and 12 h assays, mAb against CD69 as well as SiaTn TRI2 ligand-induced efficient apoptosis that was dependent on the surface concentration of this antigen. Apoptosis exerted in lymphocytes with a high surface expression of CD69 ($CD69^{high}$) was at the level of the positive control, while in $CD69^{low}$ cells it only reached the level of the negative control (Fig. 3), clearly confirming the dependence on CD69 engagement. This apoptosis induced by CD69 cross-linking could be inhibited by all tested carboxylated calixarenes as well as by the control GlcNAc-conjugated calixarene, but at very different concentrations (Fig. 3). Thus, at the lowest concentration tested (10^{-10} M), only compound **3** was able to provide complete protection from apoptosis induced by the CD69 ligand, as well as partial protection from apoptosis induced by the antibody. At higher calixarene concentrations, less specific protection could be observed, but it was only at 10^{-6} M concentrations that complete protection from CD69 dependent apoptosis could be observed for all tested compounds.

3. Discussion

We have described here an extensive evaluation of water-soluble carboxylated calixarenes as inhibitors (antagonists) of the leukocyte surface molecule and universal activation trigger of lymphocytes, CD69. The dual role of this antigen in tumor immunology, namely its ability to both activate natural killer cells and cytotoxic T-cells as well as to induce apoptosis in these cells through CD69 engagement[8] that has been documented in monocytes and eosinophils,[9–11] requires the development of new chemical tools capable of selective disengagement of this receptor while still leaving the highly similar lectin-like receptors of NK cells available for interaction. The ability of hydroxycarbonylmethoxy calix[4]arenes to selectively inhibit the binding of CD69 to its high affinity ligand while not affecting NKR-P1 binding[21] provided a suitable platform for further optimization of these compounds. The best of the new series of compounds turned out to be carboxylated thiacalix[4]arene **3**, which contains sulfide groups connecting the phenyl rings and a cluster of carboxymethyl groups positioned on one side of the molecule (Scheme 1). Among the four compounds tested, compound **3** proved to be the best ligand for CD69 in direct binding assays with K_d as low as 3.2×10^{-10} M. Since the tested carboxylated calixarenes are monovalent (their carboxylate groups are too close to bind simultaneously to two binding sites in CD69[14]), they are not able to crosslink the cellular form of CD69, and thus act as pure antagonists of the receptor. In order to test the efficiency of the examined compounds under experimental conditions relevant to the biology of CD69 receptor, we precipitated either the soluble form or the cellular form of CD69 using both monoclonal antibodies against this antigen, and the multivalent high affinity ligand SiaTnTRI2 mimicking the tumor surface sialomucins. Using the soluble form of the receptor, CD69 could be efficiently precipitated by the above reagents in a reaction that is reversible, and could be inhibited by carboxylated calixarenes as soluble inhibitors. Much higher concentrations were needed to inhibit antibody-dependent precipitation compared to ligand-induced precipitation, which can be explained by the different mechanisms of the inhibition reactions. While in ligand-induced precipitation the carboxylated calixarene antagonist is directly competing for the binding site with the sialylTn ligand, the disruption of the antibody precipitate is most probably a result of an altered CD69 conformation, for which much higher concentrations of the inhibitor may be needed. Notably, carbohydrate-substituted calixarene **1** is slightly less potent in the apoptosis protection assay, despite its high affinity for CD69 measured in the plate inhibition assay. These differences may reflect somewhat different effects of the tested compounds in the three assays used (plate inhibition assay, direct binding to the receptor, and the effects on apoptosis).

The shift in the binding paradigm for CD69 receptors from ligands based on neutral carbohydrates to those based on charged carbohydrates such as SiaTn continued into a complete departure from the carbohydrate skeleton based structures towards the chemical structure in which the scaffold for the attachment of the charged groups is materialized by entirely different compounds such as calixarene structures described here. Results presented in this paper clearly indicate the high affinity of these noncarbohydrate structural mimetics. Other groups of receptors belonging to C-type lectin family provide strong parallels with these findings. The best example is provided by the cell adhesion molecules selectins that are type I membrane proteins that express the carbohydrate-recognition domain characteristic of C-type lectins in the most membrane distal portion of their molecule. Here, also, the history of ligand identification for this group of receptors[24] went from neutral $Lexis^x$-derived carbohydrate sequences to various sialylated and sulfated carbohydrates to the identification of sulfated tyrosine sequences as specific ligands for these receptors.[25–27]

The optimized compound **3** is now available for in vivo testing using experimental tumor models.[28,29] These tests should reveal if the inhibition of CD69 by the mechanism of selective antagonist decoupling will prove efficient in enhancing the survival of $CD69^+$ killer lymphocytes, and thus increase their efficacy in killing of tumors similar to that observed for $CD69^{-/-}$ mice.[8] Moreover, the carboxylated calixarenes described here may help to explain the role of CD69 in the interaction between the tumor cell and the killer cell,[30] compared to other activation and inhibiting killer cell receptors as well as the Fas–Fas ligand interactions.[3] As a first aim along this line of research, it would appear interesting to repeat the present experiments with purified cells constituting the N-PBMC cellular fraction, in particular using highly enriched populations of human NK cells and cytotoxic T-cells critical for the antitumor immune response.

During the recent years, calixarene derivatives have become widely available scaffolds to which various ligands interacting weakly with their target receptors may be attached to secure multivalency necessary for biologically meaningful interactions. Thus, calixarene-based multivalent ligands have found a use in presenting carbohydrates for their interactions with lectins,[20] in DNA condensation and cell transfection, protein surface recognition, self-assembly, crystal engineering, and nanofabrication.[31] Ungaro and co-workers have shown that calixarene-based glycoclusters are efficient inhibitors of binding of medially relevant lectins and may influence adhesion and growth regulation by interfering with galectin activities.[32] Hamilton and co-workers used the combination of hydrophobic and acidic groups to construct calixarene derivatives that turned to be potent antagonists inhibiting plate-

let-derived growth factor-stimulated phosphorylation of the corresponding specific receptor.[33] Particularly relevant to our results are calixarene derivatives developed by de Mendoza and co-workers having the outer rim decorated with quanidinium groups.[34,35] Detailed investigations of the interactions of these compounds with several proteins indicated the existence of ionic interactions between the basic guanidinium groups on the calixarene-derived ligands and acidic groups of the target proteins. Such specific interactions have led to important biological applications of such compounds that could be used to stabilize the tetrameric domain of p53 tumor suppressor protein, and as probes for structural investigations of the voltage dependent potassium channels, respectively.[34,35]

4. Conclusions

We present here a series of carboxylated calixarenes that are specific antagonists of the leukocyte surface receptor CD69, and that provide complete protection against CD69 dependent apoptosis induced both by multivalent carbohydrate ligand and antibody cross-linking. Protection of $CD69^{high}$ killer lymphocyte from ligand-induced or antibody-induced apoptosis by carboxylated calixarenes proceeded specifically, and efficiently at concentrations as low as 10^{-10} M. The ability of these compounds to protect against apoptosis mediated by the cross-linking of CD69 by the multivalent ligand SiaTnTRI2 is of particular interest, since this ligand may mimic tumor surface sialomucins that are characterized by high accumulation of SiaTn disaccharide sequences. Thus, specific CD69 receptor antagonists based on carboxylated thiacalix[4]arenes may become attractive tools in the protection of CD69 receptors in vivo, and thus protect $CD69^{high}$ cytotoxic lymphocytes from apoptotic death and inactivation by the tumors. In vivo experiments that would incorporate carboxylated calixarenes into the combined treatment protocols (in combination with the carbohydrate-derived activators of killer cells) in animal tumor therapy experiments are currently underway.

5. Experimental

5.1. Materials

PMA, bovine serum albumin (BSA), and ionomycin were from Sigma. Media for cell isolation and cultivation were from the Institute of Molecular Genetics, Prague, Czech Republic. Monomeric SiaTnTRI was from Lectinity, Finland, and was dimerized as described previously[18] to yield SiaTnTRI2. Monoclonal antibody against CD69 BL-KFB/B1 has been described previously.[12] PBS (phosphate buffered saline) was prepared as described previously.[36] Ficoll-Paque was from GE Healthcare. Fetal calf serum was from GIBCO.

5.2. Mass spectrometry

Mass spectra were measured on a BIFLEX matrix-assisted laser desorption/ionization reflectron time-of-flight (MALDI-TOF) mass spectrometer (Bruker-Franzen, Bremen, Germany) equipped with a nitrogen laser (337 nm) and gridless delayed extraction ion source. The ion acceleration voltage was 19 kV and the reflectron voltage was set to 20 kV. Spectra were calibrated externally using the monoisotopic $[M+H]^+$ ion with matrix peak at *m/z* 379.1 and human angiotensin I with *m/z* at 1296.69. A saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.3% acetic acid was used as a matrix. One microlitre of matrix solution was mixed with 1 μl of the aqueous solution of sample and 1 μl of premix was loaded on the target, the droplet was then allowed to dry at ambient temperature. The MS spectra were collected in reflectron mode. Alternatively, high resolution ion cyclotron resonance mass spectrometry has been used as described previously.[14]

5.3. Synthesis of compounds 2–4

Compound **2** was obtained according to the published procedure by alkylation of *tert*-butylcalix[4]arene with ethyl bromoacetate in the presence of K_2CO_3, and subsequent hydrolysis of the tetraester with NaOH in water/ethanol mixture.[37] Thiacalix[4]arene derivatives **3** and **4** were obtained by similar procedure using alkylation with ethyl bromoacetate in the presence of Na_2CO_3 or Cs_2CO_3,[38] respectively, and subsequent hydrolysis.[39]

5.4. Preparation of soluble CD69 receptor

Recombinant soluble human CD69 (hCD69) was prepared as noncovalent dimers.[14] The protein stock was concentrated to 10 mg/ml and kept at 4 °C in 10 mM HEPES pH 7.4, 49 mM NaCl and 1 mM NaN_3. The quality of hCD69 preparations was routinely checked by SDS electrophoresis and mass spectrometry using the previously described protocols.[14]

5.5. Plate binding and inhibition

Soluble CD69 protein was labeled by covalent attachment of a fluorescent label through reaction with *N*-hydroxysuccinimide rhodamine (Pierce, Rockford, IL, USA) as described previously.[16] Five moles of rhodamine were attached per mole protein as confirmed by quantitative spectrophotometry, MALDI-MS, and ion cyclotron FT-MS.[16] Inhibition assays were performed essentially as described previously[16,21] except that rhodamine-labeled CD69 was used instead of the radiolabeled receptor. Flexible 96-well round-bottomed polyvinyl chloride plates (BD Biosciences) were coated with $GlcNAc_{17}BSA$ ligand, blocked with 2% BSA, and then incubated in the presence of rhodamine-hCD69 (0.1 μg/ml corresponding to half-saturation under the given experimental conditions) and serial dilutions of the inhibitor. After incubation at 4 °C for 2 h, plates were washed four times with PBS, and drained. The ligand-bound receptor was dissociated by overnight incubation in 100 μl of 0.1 M sodium acetate buffer supplemented with 0.1% octyl-β-glucoside and 0.1% Triton X-100, the solution was transferred to 96-well flat-bottomed UV transparent plates (UV Star, BioOne, Greiner), and the amount of bound protein was determined by fluorescence ($\lambda_{ex}/\lambda_{em}$ = 546/577 nm) using a Safire[2] spectrofluorometer (Tecan). Complete inhibition curves were constructed using SigmaPlot software, and the IC_{50} values were calculated from at least three independent experiments.

5.6. Direct binding assays

The assay was performed in total volume of 100 μl in triplicate wells of a UV Star flat-bottomed 96-well plate (BioOne, Greiner). PBS (80 μl) was pipetted into the triplicate wells, together with 10 μl of protein (stock solution of hCD69 diluted 1000× with PBS, final concentration in the assay was 1.0 μg of protein/ml), and 10 μl of the solution of the tested carboxylated calixarene using tenfold concentration compared to the final indicated values. The mixture was incubated for 1 h at 22 °C, and then the plate was screened for tryptophane fluorescence using a Safire[2] plate reader (Tecan) with the following settings: λ_{ex} 278 nm, λ_{em} 330 nm, excitation slit 5, emission slit 20, bottom fluorescence was read with the gain set to 80. The results were calculated as described previously.[40]

5.7. Precipitation assays with recombinant soluble CD69

The precipitation assays with recombinant CD69 protein were performed essentially as described previously.[16]

5.8. Preparation of CD69^{low} and CD69^{high} cells

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 then 5% of CD69 were designated as CD69^{low}. Lymphocytes from donors with more then 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}.

5.9. Cellular activation assays

These assays were performed essentially as described in previous publications.[41,42]

5.10. Apoptosis assay

Cells were resuspended at 2×10^6/ml in complete RPMI1640 medium, aliquoted into round-bottomed 96-well plates, and the tested concentrations of compounds were added into duplicate 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. 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 Table S1 and S2, Supplementary Figures S1–S3, and Supplementary References S1–S4 are available. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.01.015.

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