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Metabolic labelling of cancer cells with glycodendrimers stimulate immune-mediated cytotoxicity†

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The recruitment of antibody naturally present in human blood stream at the surface of cancer cells have been proved a promising immunotherapeutic strategy to fight cancer. Antibody recruiting molecules (ARMs) combining tumor and antibody binding modules have been developed for this purpose, however the formation of the interacting complex with both antibody and cell is difficult to optimize to stimulate immune-mediated cytotoxicity. To circumvent this limitation, we report herein a more direct approach combining cell metabolism of azido-sugar and bio-orthogonal click chemistry to conjugate at the cell glycocalyx structurally well-defined glycodendrimers as antibody binding module (ABM). We demonstrate that this strategy allows not only the recruitment of natural antibody at the surface of isolated cells or solid tumor models but also activate a cytotoxic response with human serum as unique source of immune effectors.

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Introduction

The exploitation of endogenous antibodies naturally present in the blood stream of all individuals has recently emerged as an alternative immuno-strategy to fight cancer.^{1–3} To this aim, synthetic antibody recruiting molecules (ARMs) combining two binding modules, one for tumor cell (TBM) and the other for the antibody recruitment (ABM, typically dinitrophenol, α Gal or Rha) have been demonstrated to successfully trigger immune cytotoxicity against cancer cells by CDC or ADCC mechanisms.^{4–7} From the first generation of ARMs to the more sophisticated antibody recruiting polymers (ARPs)⁸ or glycodendrimers (ARGs),⁸ significant advances have been made in the understanding of functional and structural requirements to improve immunological effects. If the multivalent presentation of ABM was clearly demonstrated as a key element to recruit endogenous antibodies,^{10,11} the major shortcoming of this approach concerns the TBM which has to ensure the binding of the cell surface without promoting internalisation to maintain the ABM exposure and accessibility at the cell surface. By doing so, the recruiting molecule can promote the formation of a reversible ternary interacting complex with antibodies and cancer cell. When suitable conditions are used to control this complex

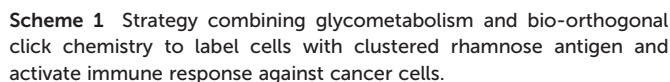
equilibrium process,¹² the immune-mediated cytotoxic effect can be activated against the cancer cell line.^{13,14} To avoid the utilization of TBM and the problems associated with interacting systems involving three partners, the insertion of dinitrophenol or carbohydrate haptens in the cell membrane either with a lipid anchor or by covalent conjugation was proved to be a valuable alternative as a simplified antibody recruiting system.^{8,15–19}

In this regard, the use of well-known substrates for cell surface engineering, such as the tetraacetyl-*N*-azidoacetyl-mannosamine (Ac₄ManNAz), represents a powerful and reliable method to modify the glycocalyx with unnatural recognition moieties. Once internalized, the intracellular metabolism of Ac₄ManAz into azido sialic acid indeed leads to azido group expression onto extracellular glycans that can be engaged in bio-orthogonal reaction to further decorate the cell membrane by copper-free strain-promoted azide-alkyne cycloaddition (SPAAC).^{20–25} Several groups used similar approach to conjugate diverse haptens on the membrane of different cells to promote immune-mediated cytotoxicity.^{15,16,26} In this study, we reasoned that the conjugation of clustered ABM at the surface of cancer cells would represent a robust antibody recruiting approach. We recently identified an ARG composed of a tetravalent cluster of cRGD as TBM and an hexadecavalent dendrimer of Rha as ABM to redirect natural antibodies against cancer cells expressing $\alpha_v\beta_3$ integrins.¹¹ This compound has been shown to stimulate immune-mediated cytotoxicity against this cell line. Among the variety of the tested compounds, we demonstrated that ARGs presenting the high rhamnose

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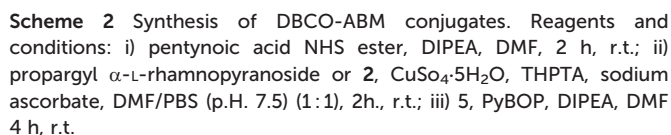
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Results and discussion

ABMs were synthesized as previously reported.¹¹ Briefly, mono- **1** and tetraazido **2** cyclodecapeptides were functionalized *via* CuAAC using propargylated α -L-Rha to



The influence of the Rha density on the recruitment of serum antibodies was next evaluated. Cells pre-incubated with or without Ac₄ManNAz were treated with 10 μM of mono, tetra- and hexadecaivalent DBCO-ABMs. The anti-Rha IgM recruitment was revealed by immuno-fluorescence as described earlier and analysed by flow cytometry and

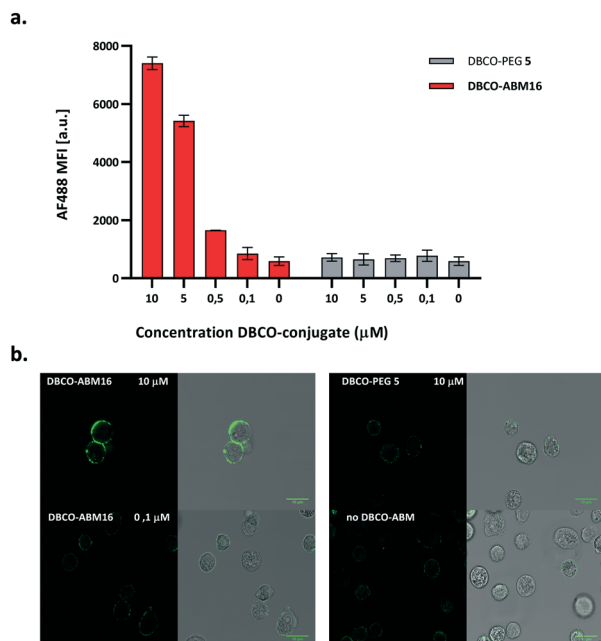


Fig. 1 DBCO-ABM16 coupling to azido groups exposed at the cell surface and anti-Rha recruitment. BT-549 cells were incubated with Ac_4ManNAz for azido labelling before treatment with DBCO-ABM16 or DBCO-PEG 5 (10–0.1 μM). Azido labelled cells untreated with DBCO-conjugate were used as control. SPAAC coupling of the DBCO-conjugates to the cell surface was revealed using anti-Rha IgM naturally present in HS and a fluorescent secondary antibody. Cell fluorescence was analysed by flow cytometry (a. data are presented as mean \pm SD for $n = 3$ measurements) and confocal microscopy (b. scale bar: 10 μm).

confocal microscopy. As shown by the histogram overlays reported in Fig. 2a, antibodies present in HS are efficiently recruited by all modified cell surface with DBCO-derivatives with a significantly higher efficiency for cells displaying the higher Rha density at their surface. Confocal microscopy confirmed this result since the highest fluorescence and homogenous dye labelling was observed with the ABM16 (Fig. 2b).

Interestingly, mono- and tetravalent ABM1 and ABM4 covalently anchored to the cell membrane were able to recruit antibodies present in HS while ARGs previously reported with low Rha density failed.¹¹ These results demonstrate the interest of the metabolic labelling strategy to recruit antibody onto cell surface. In addition, flow cytometry analysis and confocal microscopy experiment with cells untreated with Ac_4ManNAz or DBCO conjugates revealed negligible fluorescent intensity, thus suggesting the binding specificity of anti-Rha antibodies (Fig. 2b).

Antibody recruitment being dependent on the presence and the persistence of ABMs on the cell surface, we next studied the stability of the cell labelling with ABM. Cells pre-treated with or without Ac_4ManNAz were cultured for 1–8 h with 10 μM of DBCO-ABM16 prior to being incubated with HS and the secondary antibody. The cell fluorescence was then analysed by flow cytometry. As shown in Fig. 3

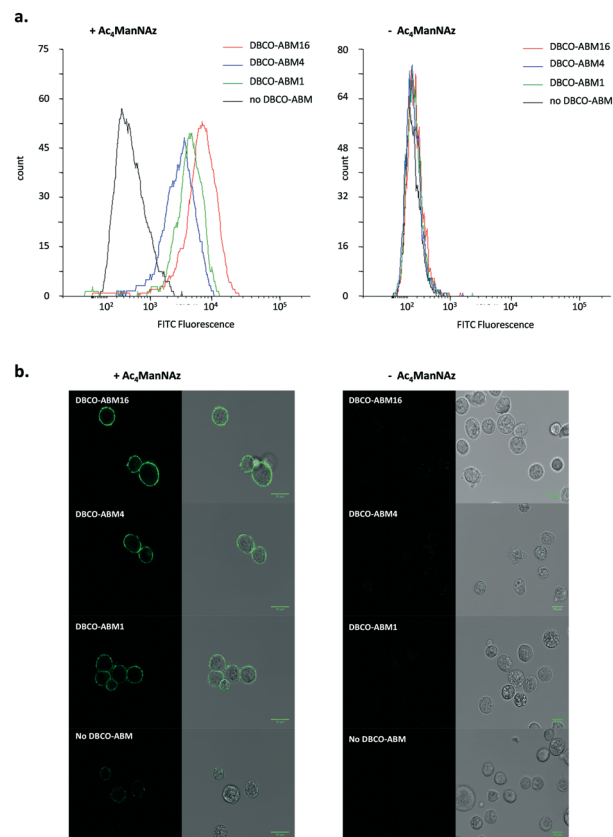


Fig. 2 a. Flow cytometry analysis of anti-Rha recruitment by labelled BT-549 cells with mono-, tetra- and hexadeca-antibodies. Azido cells were treated with 10 μM of DBCO-conjugates before incubation with HS. The antibody recruitment was revealed with AF488 conjugated secondary antibody. For both cases, non-labelled cells were used as control. b. Corresponding confocal microscopy images (scale bar: 10 μm).

persistent cell labelling with ABM16 is observed after 8 hours for cells pre-treated with Ac_4ManNAz and an increase of the fluorescent intensity is also measured with increasing incubation time. These results demonstrate the stability of cell labelling with ABM over the time. However a fluorescent

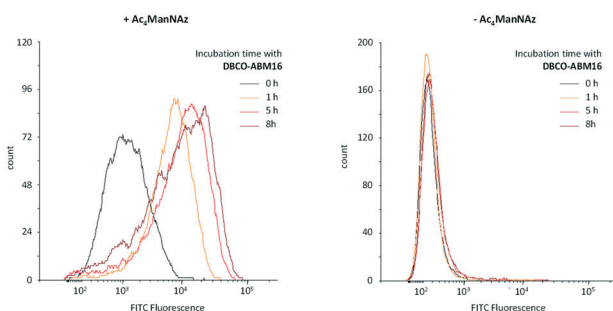


Fig. 3 Stability test of the ABM16 displayed on the cell surface. BT-549 cells metabolically labelled with Ac_4ManNAz or unlabelled cells (used as negative control) were incubated for different time (0, 1, 5 and 8 h) with DBCO-ABM16 (10 μM , final concentration) prior being treated successively with HS and the AF488 conjugated secondary antibody. The fluorescence of the cells was analysed by flow cytometry.



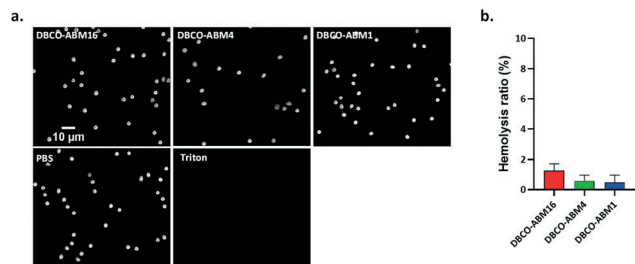


Fig. 4 *In vitro* human blood compatibility of DBCO-ABMs (1, 4 and 16). hRBCs were incubated with DBCO-ABMs at 100 μ M (final concentration). Red blood cells treatment with Triton 1% or phosphate-buffered saline (PBS) was used as positive and negative controls respectively. a. Morphology of treated hRBCs was observed under a light microscopy. b. Hemolysis ratio (%) of hRBCs after incubation with DBCO-ABM conjugates.

signal similar to the autofluorescence of the cells was observed for non azido-labelled cells incubated with the DNCO conjugate even after 8 hours showing one more time the specific ABM labelling for cells pre-treated with $Ac_4ManNAz$.

Since DBCO-ABM conjugates were attached onto the cells for antibodies recruitment in the human serum, we also evaluated their blood compatibility in an *in vitro* assay. This was performed by determining the effect of **DBCO-ABMs** (1, 4 and 16) on human red blood cells (hRBC). Like saline treatment, incubating hRBC with all DBCO-ABMs in saline did not cause detectable changes on morphology of hRBC under light microscopy even at a concentration 10 time higher than which was showing cytotoxic effect (100 μ M) (Fig. 4a). The hemolysis ratios for each DBCO-ABM treatments was calculated. Less than 2% of hemolysis was obtained for all conjugates, indicating a good blood compatibility of the DBCO conjugates (Fig. 4b).

We next evaluated the ability of the recruited antibodies to promote immune response leading to cancer cell destruction. Azido tagged cells treated with or without DBCO-ABMs were incubated with human serum as unique source of antibodies and protein complement and the cell viability assay which measures the complement-dependent cytotoxicity (CDC) was examined as reported previously. The cytotoxicity effect was found to increase with the ABM valency (Fig. 5), the lowest effect being observed for cells modified with the monovalent **ABM1** (20%) and the highest with the hexadecaivalent **ABM16** (30%). This result is in good correlation with the antibody recruitment observed by flow cytometry. By comparison with previous studies reported by other groups,^{16,27} the conjugation of ABM16 promote similar cytotoxic effect at much lower concentration, thus suggesting the importance of a dense presentation in hapten to the cell surface.

In order to evaluate the ability of ABMs to recruit antibody in solid tumor context, we cultured 3D-spheroid which are commonly used as *in vitro* model of such tumor.^{28,29} Spheroids grown 4 days were cultured with or without $Ac_4ManNAz$ (untreated spheroids were used as control) prior to be treated with **DBCO-ABM16** and HS (as 2D culture). As described before,

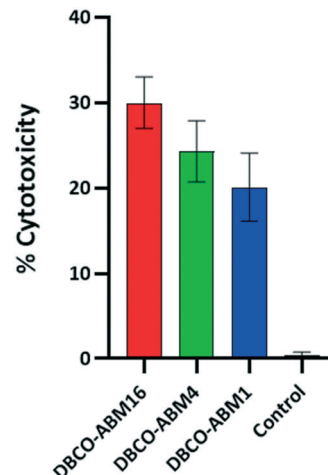


Fig. 5 Cytotoxicity of BT-549 cells induced by ABMs displayed to the cell surface following the metabolic labelling process in the presence of HS (average value of three independent experiments). Azido cells untreated with DBCO-ABMs exposed to HS were used as control. Data are presented as mean \pm SD ($n = 3$).

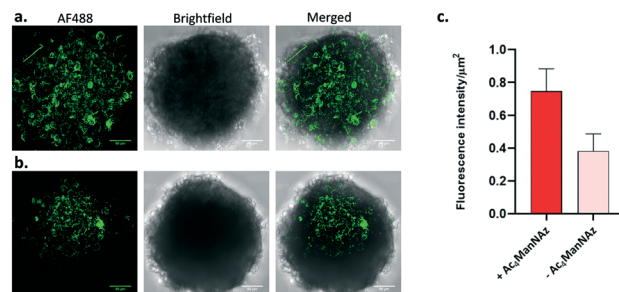


Fig. 6 Confocal microscopy images (maximum intensity projections) of BT-549 human breast cancer spheroids, cultured (a.) or without (b.) $Ac_4ManNAz$ and treated with 10 μ M of **DBCO-ABM16**, HS and AF488-secondary antibodies. Scale bar is 50 μ m. (c.) Average AF488 fluorescence intensity of BT-549 spheroids following the same treatment as described in (a) and (b.).

the recruitment of endogenous anti-Rha was revealed with AF488-conjugated anti-human secondary antibody.

Confocal microscopy images (Fig. 6) showed high fluorescence for spheroids cultured with $Ac_4ManNAz$ and treated with **DBCO-ABM16** compared to the control spheroids. The measure of the ratio of the Fluorescence intensity reported to the spheroid surface revealed a fluorescence intensity 2 times higher for cells incubated with $Ac_4ManNAz$ compared to the control spheroids. These results clearly indicate the effectiveness of the spheroid labelling and the accessibility of Rha antigens for IgM recruitment at the cell surface to induce a targeted immune response against BT-549 tumor cells.

Conclusions

In summary, we have demonstrated the robustness of cell glycometabolism and bio-orthogonal click chemistry to



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R_{sample} was the absorbance ratio of OD₅₇₀/OD₆₀₅ for the cells labelled with ARMs and incubated with HS.

R_{ctrl} was the absorbance ratio of OD₅₇₀/OD₆₀₅ for unlabelled cells incubated with HS.

R_o was the averaged background (non-cell control) absorbance ratio OD_{570}/OD_{605} .

Spheroids were generated by plating BT-549 cells at 5000 cells per well into ultralow adherence-96-well plates (Corning). Spheroids grew in complete medium as in 2D-cultures in the final volume of 200 μ L. After 72 h, spheroids were treated with 50 μ M Ac₄ManNAz and 10 μ M DBCO-ABMs in the same manner as in 2D-cultures. Spheroids were imaged using confocal microscope (TCS SP8 CSU, Leica, laser excitation at $\lambda = 448$ nm and fluorescence emission collected between $\lambda = 495$ and 545 nm) equipped with a 40 \times objective and analysis was performed with the ImageJ software.

Human red blood cell hemolysis assay

All experiments were performed in accordance with the Guidelines of the “Etablissement Français du Sang (EFS) Auvergne-Rhône Alpes 2017-2958”, and Experiments were approved by the ethics committee at the EFS AURA 21-033. Informed consents were obtained from human participants of this study. Human red blood (hRBC) were obtained by centrifugation of 2 mL of whole blood collected in heparin tubes, from healthy donors (EFS Grenoble) at 800 g for 10 min at room temperature. After removal of the plasma, hRBC were further purified by washing three times with PBS and resuspended in PBS at a final concentration of 2%. The hRBC suspensions (180 μ L) were incubated with 20 μ L of DBCO-ABM 1, 4 or 16 (1 mM in PBS) while parallel assays were performed using PBS as negative control and a 1% (w/v) solution of Triton X-100 as positive control. After incubation at 37 °C in 5% CO₂ for 2 h, each sample was centrifuged for 10 min at 800 g and 100 μ L of the supernatant was transferred to a 96-well microplate. Absorbance at 540 nm was measured using a microplate reader and the results were expressed as a percentage of hemoglobin released relative to the positive control. All treatments were performed in triplicates and the hemolysis ratio (%) was calculated using equation:

Hemolysis ratio(%)

$$= \frac{A(\text{sample}) - A(\text{negative control})}{A(\text{positive control}) - A(\text{negative control})} \times 100$$

The integrity of the precipitated hRBC was checked by morphological observations under light microscopy.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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