Cancer Vaccines

DOI: 10.1002/anie.201406897

A Cancer Therapeutic Vaccine based on Clustered Tn-Antigen Mimetics Induces Strong Antibody-Mediated Protective Immunity**

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Abstract: *Tumor-associated carbohydrate antigens (TACAs) are key components of cancer vaccines. A variety of vaccines based on native TACAs such as α-Tn have shown immunogenicity and protection in preclinical animal studies, however, their weak immunogenicity, low in vivo instability, and poor bioavailability, have discouraged their further evaluations in clinical studies. A new improved vaccine prototype is reported. It is composed of four clustered Tn-antigen mimetics and a immunogenic peptide epitope that are conjugated to a cyclopeptide carrier. The immunization of mice with this vaccine 1) was safe, 2) induced a strong and long-lasting Tn-specific response with IgM/IgG antibodies able to recognize native carbohydrate antigens; 3) produced high titers of IgG1, IgG2a, and IgG3 antibodies; and 4) produced a significant antibody-dependent regression of tumors and conferred protection. Altogether, these findings pave the way for the clinical development of safe and effective therapeutic vaccines against Tn-expressing cancers.*

Cancer cells undergo significant modifications in terms of carbohydrate expression. These alterations, mainly aberrant glycosylation, are known as tumor associated carbohydrate antigens (TACAs),[1,2] and can be used as diagnostic tumoral markers or therapeutic targets.[3] The most common TACA is the α-Tn antigen, an *N*-Acetylgalactosamine (GalNAc) residue that is α-linked to a serine or threonine residue (α-GalNAc-*O*-Ser/Thr).[4] α-Tn is detected in up to 90% of human breast, ovary, and colon carcinomas.[5] The induction of IgG antibodies (Abs) against TACAs is known to be a difficult task[3] and this is not surprising because several TACAs are self antigens and are therefore well tolerated by the immune system.[6–10] The shedding of TACAs by growing tumors exacerbates this tolerance.[6,9,11] Conversely, under appropriate conditions, α-Tn can induce tumor-specific IgG antibodies in mice and in nonhuman primates.[12] Furthermore, the level of Tn expression is statistically higher in tumors compared to healthy tissue.[13,14] These observations have raised confidence that TACAs, and Tn in particular, might be essential components in the design of humoral-mediated cancer vaccines. It is known that TACAs need to be covalently linked to a T-helper epitope to induce a strong and long-lasting production of high-affinity IgG Abs.[15] The cross reaction of these IgG Abs with tumor-expressing TACAs, with or without the cooperation of other immune cells, ultimately leads to regression of the cancer.[3,16–20] A wide variety of such immunogenic constructs have been synthesized and have shown promising immunogenicity in preclinical animal studies. However, to date, none of them have succeeded in clinical trials, as observed with the promising Theratope vaccine, for which neither an overall benefit nor increased survival were observed for patients in phase III trials.[21] One reason for these failures is the sensitivity of TACAs to endogenous glycosidases, which reduces their in vivo bioavailability.[22–24] As a consequence, structural modifications to native TACAs, including the use of C- and S-glycosides,[25–27] deoxyfluoroglycosides,[28–30] truncated antigens,[31] or thioether-bridged mimetics,[32] have been proposed to provide structures more stable than those of the parent antigens without interfering with their B-cell immunogenicity.[33,34]

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[**] This work was supported by Labex Arcane (ANR-11-LABX-003), the ANR-12-JS07-0001-01 "VacSyn"; by Ente Cassa di Risparmio di Firenze and AIRC, IG Grant 2012 to CN, the COST action CM1102 to OR and CN, by the PHC Galileo Program 2013 to OR and CN, and by Public Health Service Research NIH grants EY14900 and EY019896 to LBM.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201406897.

In this study, we hypothesized that TACA-based vaccines displaying mimetics instead of native Tn antigens could be more resistant to enzymatic degradation. We expected that this resistance might translate into increased in vivo bioavailability and hence lead to stronger and longer-lasting immunogenicity and protective efficacy. To test this hypothesis, we focused on previous vaccine prototypes[35] based on a cyclopeptide carrier termed regioselectively addressable functionalized template (RAFT),[35] which was decorated with clusters of GalNAc, the saccaridic epitope of the Tn antigen, and with either T-helper[12] or chimeric T-helper/cytotoxic T-cell peptide epitopes.[16,17,36] Although these constructs were able to promote tumor regression and improved survival in mice,[16,17,36] their sensitivity to enzymatic degradation may compromise clinical studies. We thus prepared a new prototype of a fully synthetic antitumor vaccine (**8**) based on the same model (Figure 1) but containing four residues of a Tn-

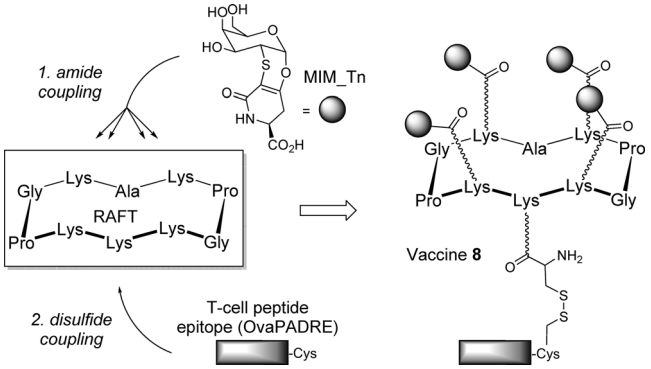
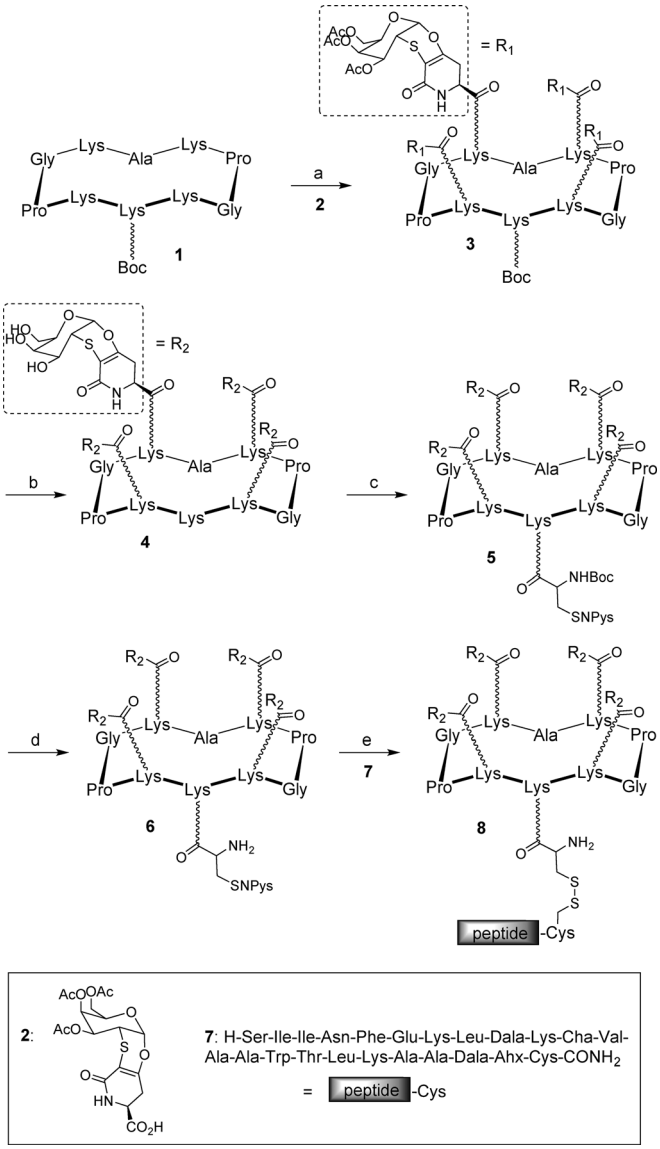


Figure 1. General strategy for the construction of vaccine **8**, which displays the α-Tn antigen mimetic MIM_Tn.

antigen mimetic (MIM_Tn). This bioactive epitope is a 2-deoxy-2-thio-α-*O*-galactoside that retains the $^4C_1$ chair conformation of the native antigen.[37] As depicted in Figure 1, MIM_Tn presents a carboxylic residue that can be used for conjugation through an amidic linkage to the four Lys residues of the RAFT. This cyclopeptide carrier also displays an immunostimulant peptide epitope (OvaPADRE) linked to the Lys residue on the lower face of the scaffold. The safety, immunogenicity, and protective efficacy of the resulting clustered Tn-antigen mimetic based construct (**8**) were assessed in mice.

The synthesis of **8** started with the conjugation of **1** with the acetylated mimetic **2** in the presence of PyBOP in DMF (Scheme 1). The conjugate **3** was afforded in 62% yield after precipitation in diethyl ether. Acetyl and Boc groups were removed by treatment with trifluoroacetic acid and sodium methoxide, respectively, to provide **4** in 93% yield. An activated cysteine with an S-3-nitro-2-pyridinesulfenyl (NPys) group was next coupled to the free Lys of **4** and the resulting compound **6** was reacted with the peptide epitope **7**. The complete conversion of **6** was observed by HPLC in 1 h, nevertheless, construct **8** was obtained in a moderate yield (22%) after HPLC purification.



Scheme 1. Preparation of compound **8**. a) **2**, PyBOP, DIPEA, DMF, RT, 1 h, 62%; b) 1: TFA/CH_2Cl_2 (1/1, v/v), RT, 30 min; 2: MeONa, MeOH, RT, 4 h, 93% for two steps; (c) BocCys(NPys)CO_2Su, DIPEA, DMF, RT, 1 h; d) TFA/CH_2Cl_2 (1/1, v/v), RT, 30 min, 79% for two steps; e) **7**, *i*PrOH/AcONa 25 mM pH 5 (1:1 v/v, 1 mM), RT, 2 h, 22%. PyBOP = benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate, DIPEA = *N*,*N*-diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, Boc = *tert*-butyloxycarbonyl, Su = succinimidyl , TFA = trifluoroacetic acid.

First, the in vivo safety and immunogenicity of **8** were evaluated. B10.D1 mice were immunized subcutaneously with **8** in CpG_{1826} adjuvant three times at 14-day intervals (Group 1, 10 mice/group). To control the response specificity and evaluate the effect of nonspecific immune responses induced by CpG_{1826}, a second group of mice (Group 2) was treated with CpG_{1826} alone. Ten days after the final immunization, post-immune sera were collected and the titres of IgG/IgM Abs were determined by enzyme-linked immunosorbent (ELISA) assay (Figure S2A in the Supporting Information). It is noteworthy that no adverse effects (e.g. local inflamma-

tion, systemic reactions, weight loss, or death of the treated mice) were observed during or after the course of immunization, thus confirming the safety of the construct **8** formulation. Ten days after the third immunization, significant levels of mucin-specific IgG/IgM Abs were induced in the immunized mice (Group 1), unlike in Group 2 and Group 3 (mice injected with PBS). The longevity of the IgG/IgM Abs was determined and significant amounts of IgG/IgMAbs were still present in the serum 240 days after the last immunization (Figure S2B). More interestingly, high amounts of the IgG1, IgG2, and IgG3 antibody subclasses were observed, which suggests that a broad and balanced IgG immune response had been elicited (Figure S3). Furthermore, the ratio of IgG2a/IgG1 indicated a higher induction of type 2 than type 1 T-helper response.

Flow cytometry was next used to analyze the binding of the immune serum Abs to mouse (NT2 and TA3HA) and human (MCF7) cancer cell lines that express native TACAs. When NT2, TA3HA, and MCF7 cells were treated with sera from immunized mice, we observed a significant enhancement of the fluorescence intensity, whereas sera from the control groups did not show any interaction (Figure S4). These results demonstrate that Abs generated by the Tn-mimetic-based vaccine **8** recognize tumor cells expressing the native antigen at their surface, which clearly confirms the tumoral specificity of the antibody response.

The immunotherapeutic efficacy of **8** was also determined by assessing tumor growth and survival rate in mice. Female B10.D1 mice were implanted subcutaneously with NT2 cells to create tumors and were then treated with: 1) **8** in CpG_{1826} adjuvant (Group 1, 10 mice/group); 2) CpG_{1826} adjuvant alone (Group 2); or 3) PBS alone (mock, Group 3). Tumor volume and mouse survival were recorded for up to 60 days after the final immunization. As shown in Figure 2A, the

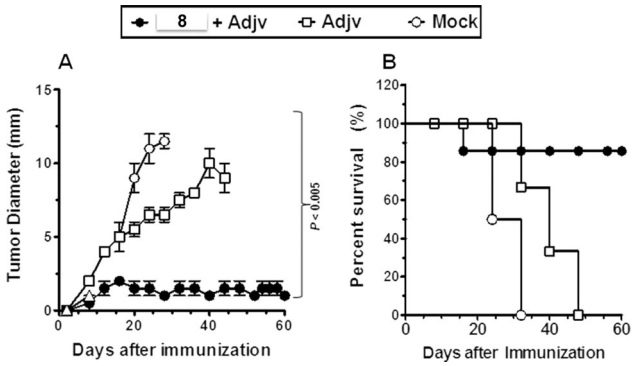


Figure 2. Immunotherapeutic efficacy of **8**. A) Tumor progression; B) Survival (see the Supporting Information for details). Adjv = adjuvant.

tumor diameter was significantly reduced in mice from Group 1 compared to mice from Group 2 ($p < 0.005$) and Group 3 ($p < 0.002$).

The strong immunotherapeutic effect of **8** is also evident from Figure 2B. Out of ten mice vaccinated with **8**, seven were alive eight weeks after tumor inoculation, whereas only one or none of the ten survived in Groups 2 and 3, respectively. To verify the involvement of B cells in the observed protection, in vivo depletion of B cells, $CD4^+$ cells, or $CD8^+$ T cells was performed in immunized mice by using specific monoclonal antibodies (mAbs). Interestingly, only the depletion of B cells significantly abrogated the protection induced by **8** against tumor progression (Figure 3A) and death (Figure 3B), thus suggesting that the protection is mainly due to B cells.

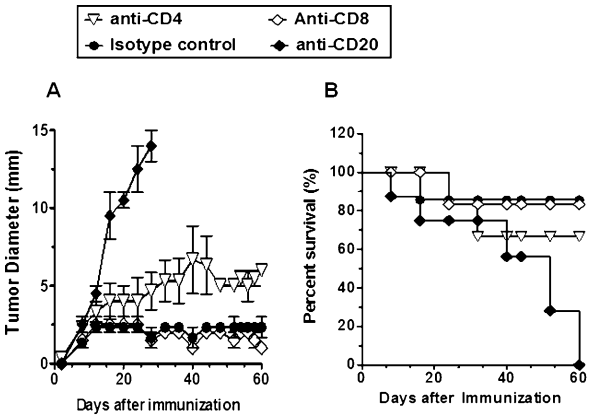


Figure 3. The effect of in vivo depletion of B cells, $CD4^+$ cells, or $CD8^+$ T cells on the suppression of tumor progression (A) and increase in survival (B) conferred by **8**. Vaccinated mice were treated through intraperitoneal injection with six doses of 100 μL of PBS containing mAb GK1.5 (anti-$CD4^+$), a mAb 2.43 (anti-$CD8^+$), mAb CD20-1 (anti-B cell), or hamster immunoglobulin control on day −7, −1, 0, 2, and 5 post tumor transplantation. Depletion of B and T cells was assessed by flow-cytometry analysis of splenocytes at the end of the experiment (days 12–13 post-inoculation).

In conclusion, although the discovery of a potent carbohydrate-based cancer vaccine remains a challenging goal, tailored synthetic immunogenic constructs offer safety, reliability, and cost advantages over traditional methods (e.g., live vectors, tumor-cell–APC fusions, and genetic immunization).[3,20,36]

Herein, we report the first use of a simple and structurally stable mimetic of the mucin antigen α-Tn for the synthesis of an unprecedented fully synthetic vaccine. We demonstrated that this vaccine prototype elicits a robust and long-lasting IgG/IgM antibody response and induces protection in mice through a mechanism mediated by B cells. Interestingly, these antibodies were shown to bind to MCF-7 human breast cancer cell lines expressing the native carbohydrate antigens on their surface, thus suggesting that biologically relevant antibody specificities were induced.[11] Although the in vivo mechanism by which **8** raises a carbohydrate-specific response is still unclear, our findings represent a step forward in the development of synthetic therapeutic vaccines against cancers. This fully synthetic approach addresses the problems associated with the use of large carrier proteins to deliver weakly antigenic carbohydrate molecules and removes the necessity for booster injections to convert the initial transient IgM response into a strong, durable IgG response. This new vaccine would not only address self-tolerance (a central problem in cancer immunotherapy) but also boost the

carbohydrate B-cell response, two effects that are essential to overcome low immunogenicity and enhance protective adaptive immunity against cancers.

Received: July 4, 2014
Published online: August 28, 2014

Keywords: antitumor agents · cancer · immunology · synthetic vaccines · Tn antigen

[1] S. Hakomori, *Adv. Exp. Med. Biol.* **2001**, *491*, 369.
[2] K. N. Syrigos, A. J. Karayiannakis, A. Zbar, *Anticancer Res.* **1999**, *19*, 5239.
[3] A. A. Chentoufi, A. B. Nesburn, L. BenMohamed, *Arch. Immunol. Ther. Exp.* **2009**, *57*, 409.
[4] X. Zhang, A. Issagholian, E. A. Berg, J. B. Fishman, A. B. Nesburn, L. BenMohamed, *J. Virol.* **2005**, *79*, 15289.
[5] G. F. Springer, *J. Mol. Med.* **1997**, *75*, 594.
[6] S. Ingale, M. A. Wolfert, J. Gaekwad, T. Buskas, G. J. Boons, *Nat. Chem. Biol.* **2007**, *10*, 663.
[7] M. A. Tarp, H. Clausen, *Biochim. Biophys. Acta Gen. Subj.* **2008**, *1780*, 546.
[8] A. L. Sørensen, C. A. Reis, M. A. Tarp, U. Mandel, K. Ramachandran, V. Sankaranarayanan, T. Schwientek, R. Graham, J. Taylor-Papadimitriou, M. A. Hollingsworth, J. Burchell, H. Clausen, *Glycobiology* **2006**, *16*, 96.
[9] G. Curigliano, G. Spitaleri, E. Pietri, M. Rescigno, F. de Braud, A. Cardillo, E. Munzone, A. Rocca, G. Bonizzi, V. Brichard, L. Orlando, A. Goldhirsch, *Ann. Oncol.* **2006**, *17*, 750.
[10] M. A. Tarp, A. L. Sørensen, U. Mandel, H. Paulsen, J. Burchell, J. Taylor-Papadimitriou, H. Clausen, *Glycobiology* **2007**, *17*, 197.
[11] S. F. Slovin, S. J. Keding, G. Ragupathi, *Immunol. Cell Biol.* **2005**, *83*, 418.
[12] S. Grigalevicius, S. Chierici, O. Renaudet, R. Lo-Man, E. Deriaud, C. Leclerc, P. Dumy, *Bioconjugate Chem.* **2005**, *16*, 1149.
[13] C. Vazquez-Martin, E. Cuevas, E. Gil-Martin, A. Fernandez-Briera, *Oncology* **2004**, *67*, 159.
[14] J. C. Manimala, Z. Li, A. Jain, S. VedBrat, J. C. Gildersleeve, *ChemBioChem* **2005**, *6*, 2229.
[15] F. Berti, R. Adamo, *ACS Chem. Biol.* **2013**, *8*, 1653.
[16] O. Renaudet, G. Dasgupta, I. Bettahi, A. Shi, A. B. Nesburn, P. Dumy, L. BenMohamed, *PLoS One* **2010**, *5*, e11216.
[17] I. Bettahi, G. Dasgupta, O. Renaudet, A. A. Chentoufi, X. Zhang, D. Carpenter, S. Yoon, P. Dumy, L. BenMohamed, *Cancer Immunol. Immunother.* **2009**, *58*, 187.
[18] X. Zhu, T. V. Ramos, H. Gras-Masse, B. E. Kaplan, L. BenMohamed, *Eur. J. Immunol.* **2004**, *34*, 3102.
[19] R. Lo-Man, S. Vichier-Guerre, R. Perraut, E. Deriaud, V. Huteau, L. BenMohamed, O. M. Diop, P. O. Livingstone, S. Bay, C. Leclerc, *Cancer Res.* **2004**, *64*, 4987.
[20] S. Vichier-Guerre, R. Lo-Man, L. BenMohamed, E. Deriaud, S. Kovats, C. Leclerc, S. Bay, *J. Pept. Res.* **2003**, *62*, 117.
[21] D. Miles, H. Roché, M. Martin, T. J. Perren, D. A. Cameron, J. Glaspy, D. Dodwell, J. Parker, J. Mayordomo, A. Tres, J. L. Murray, N. K. Ibrahim, the Theratope® Study Group, *Oncologist* **2011**, *16*, 1092.
[22] C. Ohyama, *Int. J. Clin. Oncol.* **2008**, *13*, 308.
[23] H. Chen, L. Jiao, Z. Guo, X. Li, C. Ba, J. Zhang, *Carbohydr. Res.* **2008**, *343*, 3015.
[24] H. Paulsen, I. Brockhausen, *Glycoconjugate J.* **2001**, *18*, 867.
[25] B. Kuberan, S. A. Sikkander, H. Tomiyama, R. J. Linhardt, *Angew. Chem. Int. Ed.* **2003**, *42*, 2073 – 2075; *Angew. Chem.* **2003**, *115*, 2119.
[26] D. R. Bundle, J. R. Rich, S. Jacques, H. N. Yu, M. Nitz, C. C. Ling, *Angew. Chem. Int. Ed.* **2005**, *44*, 7725; *Angew. Chem.* **2005**, *117*, 7903.
[27] L. Awad, R. Madani, A. Gillig, M. Kolympadi, M. Philgren, A. Muhs, C. Gerard, P. Vogel, *Chem. Eur. J.* **2012**, *18*, 8578.
[28] A. Hoffmann-Röder, A. Kaiser, S. Wagner, N. Gaidzik, D. Kowalczyk, U. Westerlind, B. Gerlitzki, E. Schmitt, H. Kunz, *Angew. Chem. Int. Ed.* **2010**, *49*, 8498; *Angew. Chem.* **2010**, *122*, 8676.
[29] F. Yang, X. J. Zheng, C. X. Huo, Y. Wang, Y. Zhang, X. S. Ye, *ACS Chem. Biol.* **2011**, *6*, 252.
[30] T. Oberbillig, C. Mersch, S. Wagner, A. Hoffmann-Roder, *Chem. Commun.* **2012**, *48*, 1487.
[31] H. K. Guttormsen, L. C. Paoletti, K. G. Mansfield, W. Jachymek, H. J. Jennings, D. L. Kasper, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 5903.
[32] a) A. Arcangeli, L. Toma, L. Contiero, O. Crociani, L. Legnani, C. Lunghi, E. Nesti, G. Moneti, B. Richichi, C. Nativi, *Bioconjugate Chem.* **2010**, *21*, 1432; b) L. Toma, E. Di Cola, A. Ienco, L. Legnani, C. Lunghi, G. Moneti, B. Richichi, S. Ristori, D. Dell'Atti, C. Nativi, *ChemBioChem* **2007**, *8*, 1646.
[33] E. Lazoura, V. Apostolopoulos, *Curr. Med. Chem.* **2005**, *12*, 1481.
[34] E. Lazoura, V. Apostolopoulos, *Curr. Med. Chem.* **2005**, *12*, 629.
[35] M. C. Galan, P. Dumy, O. Renaudet, *Chem. Soc. Rev.* **2013**, *42*, 4599.
[36] O. Renaudet, L. BenMohamed, G. Dasgupta, I. Bettahi, P. Dumy, *ChemMedChem* **2008**, *3*, 737.
[37] J. Jiménez-Barbero, E. Dragoni, C. Venturi, F. Nannucci, A. Arda, M. Fontanella, S. Andre, F. J. Canada, H.-J. Gabius, C. Nativi, *Chem. Eur. J.* **2009**, *15*, 10423.

