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Toward Self-Adjuvanting Subunit Vaccines: Model Peptide and Protein Antigens Incorporating Covalently Bound Toll-Like Receptor-7 Agonistic Imidazoquinolines

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Abstract

Toll-like receptor (TLR)-7 agonists show prominent Th1-biased immunostimulatory activities. A TLR7-active *N*¹-(4-aminomethyl)benzyl substituted imidazoquinoline **1** served as a convenient precursor for the syntheses of isothiocyanate and maleimide derivatives for covalent attachment to free amine and thiol groups of peptides and proteins. **1** was also amenable to direct reductive amination with maltoheptaose without significant loss of activity. Covalent conjugation of the isothiocyanate derivative **2** to α -lactalbumin could be achieved under mild, non-denaturing conditions, in a controlled manner and with full preservation of antigenicity. The self-adjuvanting α -lactalbumin construct induced robust, high-affinity immunoglobulin titers in murine models. The premise of covalently decorating protein antigens with adjuvants offers the possibility of drastically reducing systemic exposure of the adjuvant, and yet eliciting strong, Th1-biased immune responses.

Keywords

Imidazoquinoline; Toll-like receptor; TLR7; Self-adjuvanting vaccine; ELISA; Adjuvant

Toll-like receptors (TLRs) are pattern recognition receptors that recognize specific molecular patterns present in molecules that are broadly shared by pathogens, but are structurally distinct from host molecules.¹ There are 10 TLRs in the human genome.² The ligands for these receptors are highly conserved microbial molecules such as lipopolysaccharides (LPS) (recognized by TLR4), lipopeptides (TLR2 in combination with TLR1 or TLR6), flagellin (TLR5), single stranded RNA (TLR7 and TLR8), double stranded RNA (TLR3), CpG motif-containing DNA (recognized by TLR9), and profilin present on uropathogenic bacteria (TLR 11).³ The activation of TLRs by their cognate ligands leads to activation of innate immune effector mechanisms, including the production of pro-inflammatory cytokines, up-regulation of MHC molecules and co-stimulatory signals in antigen-presenting cells, resulting in amplification of specific adaptive immune responses involving both T- and B-cell effector functions.^{4–6} Thus, TLR stimuli serve to link innate

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Supplementary Material

Experimental methods and characterization of compounds (¹H, ¹³C, and mass spectra).

and adaptive immunity⁴ and can therefore be exploited as powerful adjuvants in eliciting both primary and anamnestic immune responses.

One aspect of our work in the area of evaluating TLR agonists as vaccine adjuvants⁷⁻⁹ focuses on developing self-adjuvanting vaccine constructs, i.e., antigen covalently coupled to a suitable adjuvant. The premise of covalently decorating protein antigens with potential adjuvants offers the possibility of drastically reducing systemic exposure of the adjuvant, and yet maintaining relatively high local concentrations at the site of vaccination.¹⁰ Most self-adjuvanting vaccine constructs to date have utilized TLR-2 agonistic 2,3-bis-(palmitoyloxy)propyl-cysteinyl peptides as the adjuvant.¹¹⁻¹⁶ The conjugation of the poorly soluble lipopeptide adjuvant to antigen has limited this approach to peptide^{12-15;17} or glycopeptide¹⁶ antigens, since native proteins are often irrevocably denatured under the coupling conditions employed, with potential loss of key epitopes. These limitations have recently been addressed by appending to the lipopeptide a long, water-solubilizing polylysine or polyethylene glycol moiety, and terminating in a free thiol.¹⁸ However, in addition to the potential problem of oxidation of lipopeptide thiol to the disulfide, free exposed thiols in proteins are rare.¹⁹ Furthermore, TLR2 ligation has been associated with Th2 and Th17 responses^{20;21} which may, in many instances, be undesirable.

We have surveyed TLR agonists in a series of hierarchical assays including primary TLR-reporter gene assays, secondary indices of immune activation such as cytokine induction and activation of lymphocytic subsets in whole human blood, and tertiary screens characterizing transcriptomal activation patterns with a view to identifying optimal immunostimulatory chemotypes.⁷ Of all the innate immune stimuli examined, we found that TLR7 agonists were extraordinarily potent, stimulating virtually all subsets of lymphocytes, and yet without inducing dominant proinflammatory cytokine responses.⁷ Desirous of specifically identifying chemotypes with strong Th1-biased immunostimulatory signatures, we implemented additional screens examining the induction of Type I²²⁻²⁴ and Type II^{25;26} interferons (IFN- α/β and IFN- γ , respectively), Interleukin-12 (IL-12),^{27;28} and Interleukin-18 (IL-18) using human PBMCs,²⁹⁻³¹ all of which are strongly associated with dominant Th1 outcomes. These experiments were most instructive in that they enabled us to determine that of all of the diverse chemotypes of our rapidly expanding libraries of TLR agonists,^{8;9;32;33} an *N*¹-(4-aminomethyl)benzyl substituted imidazoquinoline **1** displayed a prominent Th1 bias (Fig. 1).

Because the aqueous solubility of **1** and several of its congeners were excellent, we elected to evaluate direct covalent coupling to free amines and thiols on protein antigens via the introduction of conventional isothiocyanate and maleimide electrophilic handles on the imidazoquinoline scaffold. Our earlier attempts at incorporating the electrophilic isothiocyanate functionality on the C2-sidechain for covalent adduction to peptides and proteins had met with failure in that such adducts were bereft of TLR7-agonistic activity,³² and it occurred to us that not only could the free primary amine on the *N*¹ substituent of **1** be converted directly to the corresponding isothiocyanate (**2**, Scheme 1), but also an additional, thiol-specific maleimide electrophile could be attached via a spacer group (**5**, Scheme 1). The isothiocyanate derivative **2** reacted well with a tri-glycine methyl ester model peptide, yielding the adduct **3** (Scheme 1), which was purified to homogeneity, and found to be active (Fig. 2). Facile adduction of the maleimide derivative **5** with glutathione (reduced) also afforded the **6a** in near-quantitative yields.

The imidazoquinolines themselves are small, non-polar, and basic, and therefore gain access to the endolysosomal compartment in which TLR7 is predominantly sequestered. The human embryonic kidney reporter cell lines stably transfected with TLR7 (and reporter secreted alkaline phosphatase genes) that we employ in our primary screen are not

professional phagocytic cells, and we were concerned if the trans-membrane permeability of the bulky, dianionic adduct **6a** would be sufficient to trigger activation, and we therefore also obtained the conjugate of **5** with the dimethyl ester of reduced glutathione (**6b**). As could be predicted, the adducts **3** and **6b** retained activity (EC_{50} : 269 nM and 2.2 μ M), while **6a** was completely inactive (Fig. 2), indicating that trans-cellular transport of the polar adduct with two net negative charges was insufficient.

We elected to work with bovine α -lactalbumin as a model antigen for self-adjuvanting vaccine constructs not only because it lent itself eminently well to rigorous characterization by electrospray ionization mass spectrometry (ESI-MS) methods (Fig. 3), but also because it is being evaluated as a potential antigen for breast cancer vaccines.³⁴

We reacted 5 equivalents of **2** with bovine α -lactalbumin in isotonic aqueous buffer at pH 8.5. Direct LC-ESI-MS evidence was obtained for covalent adduction of **2** with the protein, indicating a remarkably beautiful and precise Gaussian distribution of adducted species, with the preponderant conjugate corresponding to a 1:5 molar ratio of protein:**2** (Fig. 3).

Encouraged by these results, we also attempted to conjugate **5** with human serum albumin (HSA), a 66 kDa protein with a single free thiol. We elected to use clinical grade (meant for human parenteral use, formulated with amino acids) HSA rather than the purer, 'essentially fatty acid free' protein available commercially. Furthermore, HSA is a carrier protein which binds promiscuously to a vast range of ligands including heavy metals, bilirubin, fatty acids, etc. We anticipated for these reasons, that deconvoluted direct electrospray-time-of-flight mass (ESI-TOF) spectra would be polydisperse and microheterogeneous. In the HSA-alone control sample, we found two major peaks corresponding to 66439.9819 and 66558.3027 Da (Fig. 4). Reaction of HSA with **5** produced a shift in one of the peaks with a Δ_{mass} of 508.814 Da which corresponds to the maleimide derivative **5** within instrument error (1.424 Da at 66kDa = 21.2 parts per million). The other peak at 66558 Da had remained unaltered upon addition of excess thiol-specific maleimide analogue **5**, suggesting that the thiol was unreactive (Fig. 4). An examination of the difference between the species with the free, reactive thiol (66439 Da) and the unreactive thiol (66558 Da) in the control sample suggests that the 'blocking' group is cysteine (expected exact mass of cysteine – 1 proton [disulfide] = 118.02; observed Δ_{mass} = 118.321 Da).

Aside from engineering otherwise feebly immunogenic peptide and subunit protein vaccines for the induction of strong CTL responses, we are also interested in polysaccharide vaccines which have proved enormously useful in the prevention of infections by bacterial pathogens such as *N. meningitidis* and *H. influenzae*.^{35–37}

Bacterial polysaccharides, unlike conventional protein antigens, have been considered classic T cell-independent antigens that do not elicit cell-mediated immune responses but rather elicit non-anamnestic responses characterized by low-affinity IgM and restricted classes of IgG immunoglobulins without the recruitment of T cell help. Conversion to canonical, T lymphocyte-dependent responses require their covalent coupling to immunogenic 'carrier proteins' such as diphtheria toxoid.^{38;39} This appears not to be the case for zwitterionic polysaccharides, however, which elicit potent CD4⁺ T cell responses.^{40;41} We are keen to re-examine the structural determinants of T-dependent and -independent humoral responses, especially in light of recent findings of intrinsic TLR2 activation by zwitterionic polysaccharides,^{42;43} and we are currently testing the hypothesis that polysaccharides with covalently linked TLR stimuli may be superior to carrier proteins. The free primary amine on the N^1 substituent of **1** lent itself eminently well to direct reductive amination with maltoheptaose, a model oligosaccharide with a reducing terminal maltose unit (**7**, Scheme 2) which, gratifyingly, was found to be active in TLR7 assays

(EC₅₀: 528 nM; Fig. 5). We are currently exploring conjugating *N. meningitidis* Group C polysaccharide⁴⁴ with **1**.

It was of particular interest to us to evaluate the well-characterized **2**: α -lactalbumin conjugate (Fig. 3) as a test-case for self-adjuvanting subunit vaccine construct. We specifically asked whether the conjugation procedure would preserve antigenicity of the protein, and whether the covalently-adducted construct would be superior to a physical mixture of α -lactalbumin and **1**. Cohorts (5 per group) of outbred CF-1 mice were immunized with 50 μ g per animal of α -lactalbumin, or 50 μ g of α -lactalbumin covalently conjugated with 5 equivalents of **2**, or a mixture of 50 μ g of α -lactalbumin and 5 equivalents of **1**. The animals were boosted once after two weeks following the priming dose, and bled after an additional week. α -lactalbumin-specific IgM, IgG, as well as IgG1 and IgG2a (isotypes characteristic of Th2 and Th1 responses,⁴⁵ respectively) were quantified by ELISA. No apparent systemic or local adverse effects were noted in animals. Weight gain and general health of the vaccinated cohorts were comparable to unvaccinated controls. As shown in Fig. 6, dramatic enhancements in antibody titers were observed with both covalently- and non-covalently adjuvanted protein (relative to α -lactalbumin alone).

We also observed modest, but consistent, and statistically significant differences in titers between the covalently coupled self-adjuvanting construct, and mixture of antigen and adjuvant, indicating that self-adjuvanting subunit protein vaccines may indeed be generated with full preservation of antigenicity.

Examination of the affinity of antigen-specific IgG using conventional chaotropic ELISA^{46;47} also indicates higher quality IgG (Fig. 7) elicited by the self-adjuvanting construct.

In conclusion, our continuing exploration of the TLR7-agonistic imidazoquinoline chemotype in tandem with expanded secondary and tertiary screens designed to specifically evaluate Th1-orienting immunostimulatory profiles have enabled the identification of **1**, whose free amine group can be conveniently exploited in constructing covalent conjugates with peptides, proteins, as well as polysaccharides with preservation of immunostimulatory activity.

The feasibility of covalently coupling a small-molecule TLR7 agonist to proteins under mild, non-denaturing conditions to yield self-adjuvanting subunit vaccines is evident, and may have considerable practical value in significantly reducing systemic exposure of the adjuvant, and yet inducing high innate and adaptive immune responses. We are currently examining CD8+ CTL responses elicited by such constructs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Kawai T, Akira S. *Semin.Immunol.* 2007; 19:24–32. [PubMed: 17275323]
2. Kumagai Y, Takeuchi O, Akira S. *J.Infect.Chemother.* 2008; 14:86–92. [PubMed: 18622669]
3. Akira S. *Immunol.Rev.* 2009; 227:5–8. [PubMed: 19120470]
4. Akira S, Takeda K, Kaisho T. *Nature Immunol.* 2001; 2:675–680. [PubMed: 11477402]

5. Cottalorda A, Verschelde C, Marçais A, Tomkowiak M, Musette P, Uematsu S, Akira S, Marvel J, Bonnefoy-Berard N. *Eur.J.Immunol.* 2006; 36:1684–1693. [PubMed: 16761317]
6. Kaisho T, Akira S. *Biochim.Biophys.Acta.* 2002; 1589:1–13. [PubMed: 11909637]
7. Hood JD, Warshakoon HJ, Kimbrell MR, Shukla NM, Malladi S, Wang X, David SA. *Hum.Vaccin.* 2010; 6:1–14.
8. Shukla NM, Kimbrell MR, Malladi SS, David SA. *Bioorg.Med.Chem.Lett.* 2009; 19:2211–2214. [PubMed: 19285861]
9. Warshakoon HJ, Hood JD, Kimbrell MR, Malladi S, Wu WY, Shukla NM, Agnihotri G, Sil D, David SA. *Hum.Vaccin.* 2009; 5:381–394. [PubMed: 19270494]
10. Bachmann MF, Jennings GT. *Nat.Rev.Immunol.* 2010; 10:787–796. [PubMed: 20948547]
11. Zeng W, Ghosh S, Lau YF, Brown LE, Jackson DC. *J.Immunol.* 2002; 169:4905–4912. [PubMed: 12391202]
12. Bettahi I, Zhang X, Afifi RE, BenMohamed L. *Viral Immunol.* 2006; 19:220–236. [PubMed: 16817765]
13. Abdel-Aal AB, Batzloff MR, Fujita Y, Barozzi N, Faria A, Simerska P, Moyle PM, Good MF, Toth I. *J.Med.Chem.* 2008; 51:167–172. [PubMed: 18072728]
14. Moyle PM, Toth I. *Curr.Med.Chem.* 2008; 15:506–516. [PubMed: 18289006]
15. Zeng W, Horrocks KJ, Robevska G, Wong CY, Azzopardi K, Tauschek M, Robins-Browne RM, Jackson DC. *J.Biol.Chem.* 2011
16. Wilkinson BL, Day S, Malins LR, Apostolopoulos V, Payne RJ. *Angew.Chem.Int.Ed Engl.* 2011; 50:1635–1639. [PubMed: 21308921]
17. Zeng W, Ghosh S, Lau YF, Brown LE, Jackson DC. *J.Immunol.* 2002; 169:4905–4912. [PubMed: 12391202]
18. Zeng W, Eriksson EM, Lew A, Jackson DC. *Mol.Immunol.* 2011; 48:490–496. [PubMed: 21056473]
19. Hill BG, Reily C, Oh JY, Johnson MS, Landar A. *Free Radic.Biol.Med.* 2009; 47:675–683. [PubMed: 19527783]
20. Wenink MH, Santegoets KC, Broen JC, van Bon L, Abdollahi-Roodsaz S, Popa C, Huijbens R, Remijn T, Lubberts E, van Riel PL, van den Berg WB, Radstake TR. *J.Immunol.* 2009; 183:6960–6970. [PubMed: 19915052]
21. Aliahmadi E, Gramlich R, Grutzkau A, Hitzler M, Kruger M, Baumgrass R, Schreiner M, Wittig B, Wanner R, Peiser M. *Eur.J.Immunol.* 2009; 39:1221–1230. [PubMed: 19350551]
22. Bracci L, La SV, Belardelli F, Proietti E. *Expert.Rev.Vaccines.* 2008; 7:373–381. [PubMed: 18393607]
23. Tovey MG, Lallemand C, Thyphronitis G. *Biol.Chem.* 2008; 389:541–545. [PubMed: 18953720]
24. Rajagopal D, Paturel C, Morel Y, Uematsu S, Akira S, Diebold SS. *Blood.* 2010; 115:1949–1957. [PubMed: 20065291]
25. Berenson LS, Ota N, Murphy KM. *Immunol.Rev.* 2004; 202:157–174. [PubMed: 15546392]
26. Pulendran B. *Immunol.Res.* 2004; 29:187–196. [PubMed: 15181281]
27. Gately MK, Brunda MJ. *Cancer Treat.Res.* 1995; 80:341–366. [PubMed: 8821585]
28. Scott P, Trinchieri G. *Semin.Immunol.* 1997; 9:285–291. [PubMed: 9327523]
29. Eberl M, Beck E, Coulson PS, Okamura H, Wilson RA, Mountford AP. *Vaccine.* 2000; 18:2002–2008. [PubMed: 10706961]
30. Tough DF, Zhang X, Sprent J. *J.Immunol.* 2001; 166:6007–6011. [PubMed: 11342616]
31. Marshall DJ, Rudnick KA, McCarthy SG, Mateo LR, Harris MC, McCauley C, Snyder LA. *Vaccine.* 2006; 24:244–253. [PubMed: 16135392]
32. Shukla NM, Malladi SS, Mutz CA, Balakrishna R, David SA. *J.Med.Chem.* 2010; 53:4450–4465. [PubMed: 20481492]
33. Wu W, Li R, Malladi SS, Warshakoon HJ, Kimbrell MR, Amolins MW, Ukani R, Datta A, David SA. *J.Med.Chem.* 2010; 53:3198–3213. [PubMed: 20302301]
34. Jaini R, Kesaraju P, Johnson JM, Altuntas CZ, Jane-Wit D, Tuohy VK. *Nat.Med.* 2010; 16:799–803. [PubMed: 20512124]

35. Rappuoli R. *Vaccine*. 2001; 19:2319–2322. [PubMed: 11257355]
36. Mawas F, Peyre M, Beignon AS, Frost L, Del Giudice G, Rappuoli R, Muller S, Sesardic D, Partidos CD. *J.Infect.Dis.* 2004; 190:1177–1182. [PubMed: 15319869]
37. Broker M, Dull PM, Rappuoli R, Costantino P. *Vaccine*. 2009; 27:5574–5580. [PubMed: 19619500]
38. Falugi F, Petracca R, Mariani M, Luzzi E, Mancianti S, Carinci V, Melli ML, Finco O, Wack A, Di Tommaso A, De Magistris MT, Costantino P, Del Giudice G, Abrignani S, Rappuoli R, Grandi G. *Eur.J.Immunol.* 2001; 31:3816–3824. [PubMed: 11745403]
39. Costantino P, Viti S, Podda A, Velmonte MA, Nencioni L, Rappuoli R. *Vaccine*. 1992; 10:691–698. [PubMed: 1523880]
40. Avci FY, Kasper DL. *Annu.Rev.Immunol.* 2010; 28:107–130. [PubMed: 19968562]
41. Kalka-Moll WM, Tzianabos AO, Bryant PW, Niemeyer M, Ploegh HL, Kasper DL. *J.Immunol.* 2002; 169:6149–6153. [PubMed: 12444118]
42. Gallorini S, Berti F, Mancuso G, Cozzi R, Tortoli M, Volpini G, Telford JL, Beninati C, Maione D, Wack A. *Proc.Natl.Acad.Sci.U.S.A.* 2009; 106:17481–17486. [PubMed: 19805031]
43. Gallorini S, Berti F, Parente P, Baronio R, Aprea S, D'Oro U, Pizza M, Telford JL, Wack A. *J.Immunol.* 2007; 179:8208–8215. [PubMed: 18056364]
44. Costantino P, Viti S, Podda A, Velmonte MA, Nencioni L, Rappuoli R. *Vaccine*. 1992; 10:691–698. [PubMed: 1523880]
45. Kaplan C, Valdez JC, Chandrasekaran R, Eibel H, Mikecz K, Glant TT, Finnegan A. *Arthritis Res.* 2002; 4:54–58. [PubMed: 11879537]
46. Pullen GR, Fitzgerald MG, Hosking CS. *J.Immunol.Methods.* 1986; 86:83–87. [PubMed: 3944471]
47. Macdonald RA, Hosking CS, Jones CL. *J.Immunol.Methods.* 1988; 106:191–194. [PubMed: 3339255]

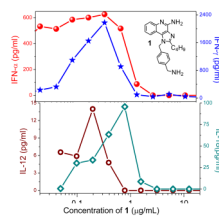


Figure 1. Induction of IFN- α , IFN- γ (top) and IL-12 and IL-18 (bottom) by **1** in human PBMCs. IFN and cytokine levels were quantified by ELISA. Results of a representative experiment are shown.

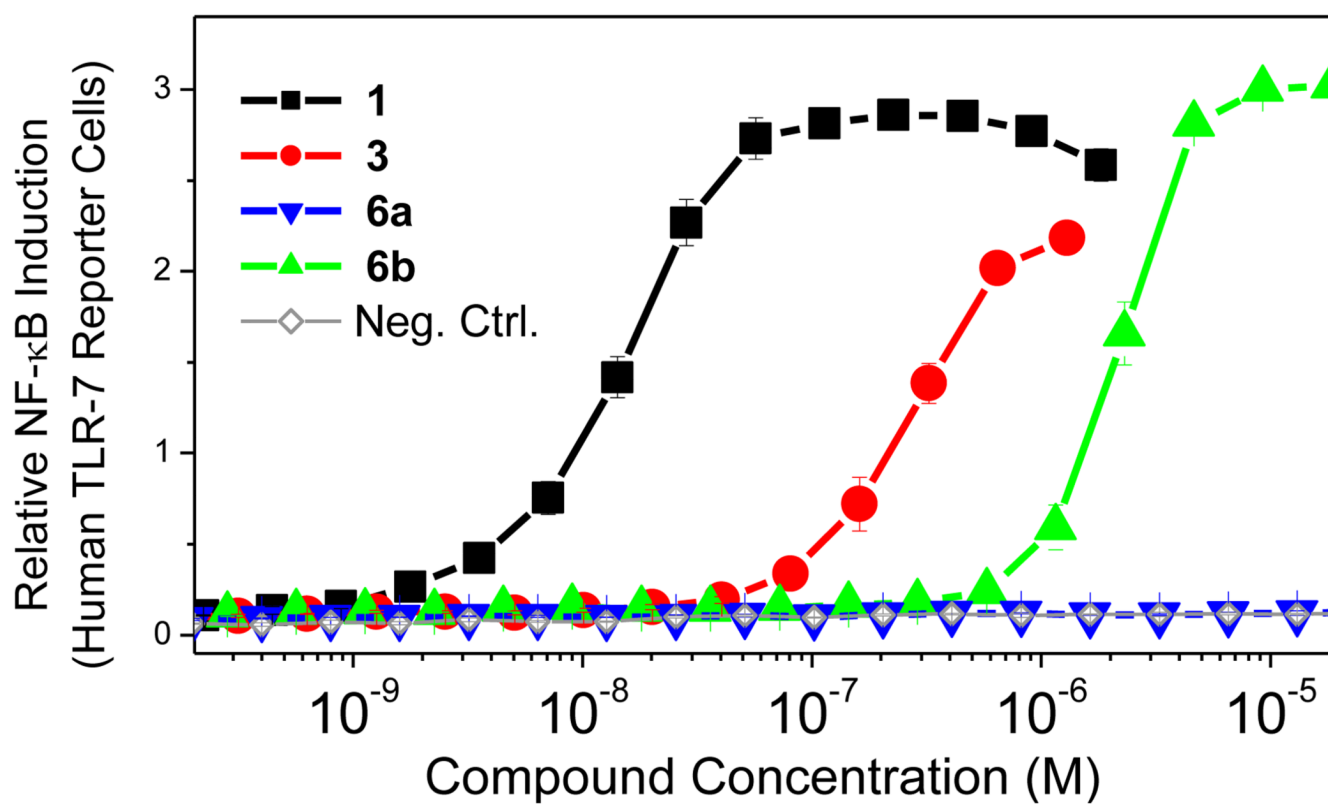


Figure 2. TLR7-agonistic activities of imidazoquinoline analogues in a human TLR7-specific reporter gene assay.

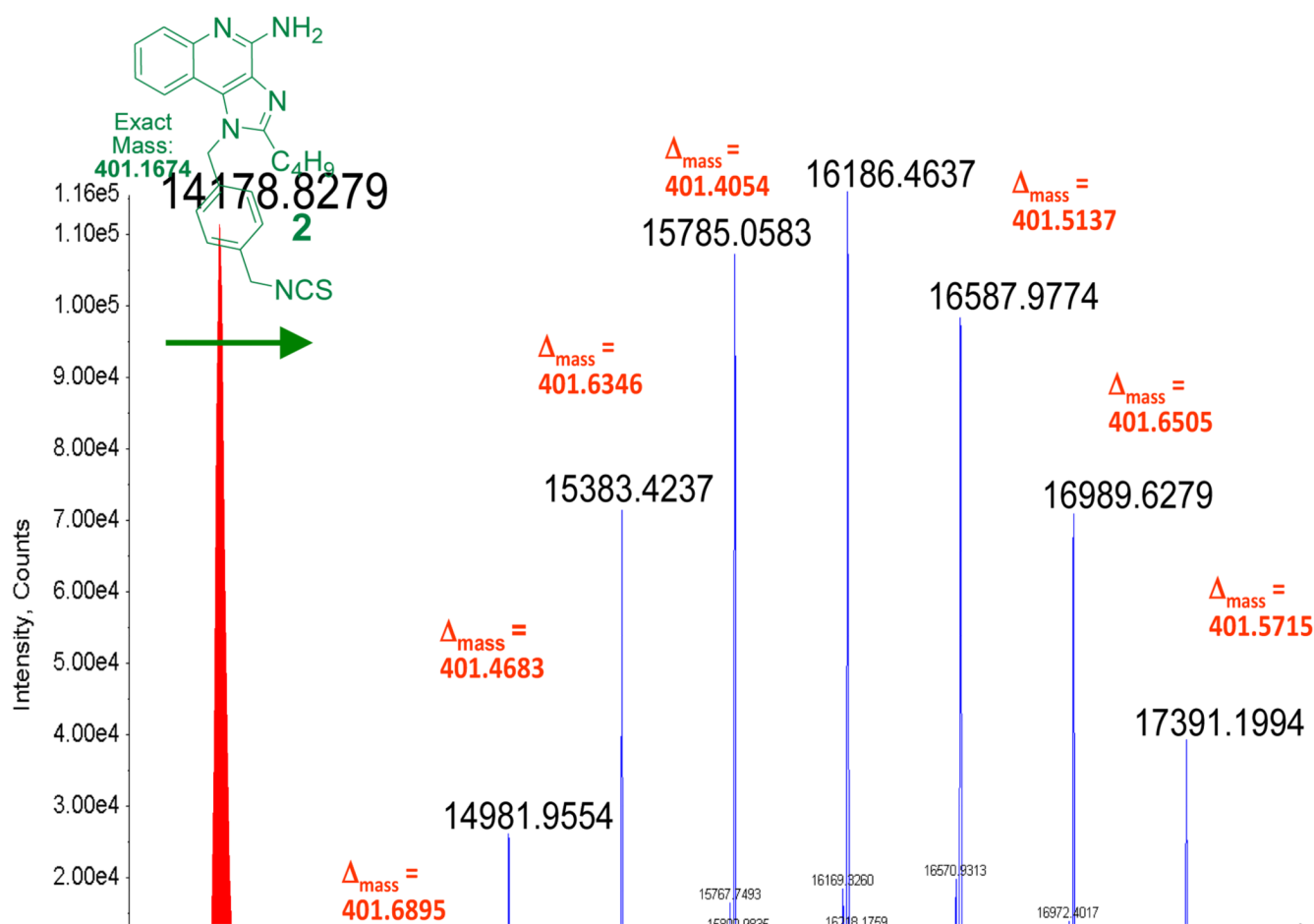
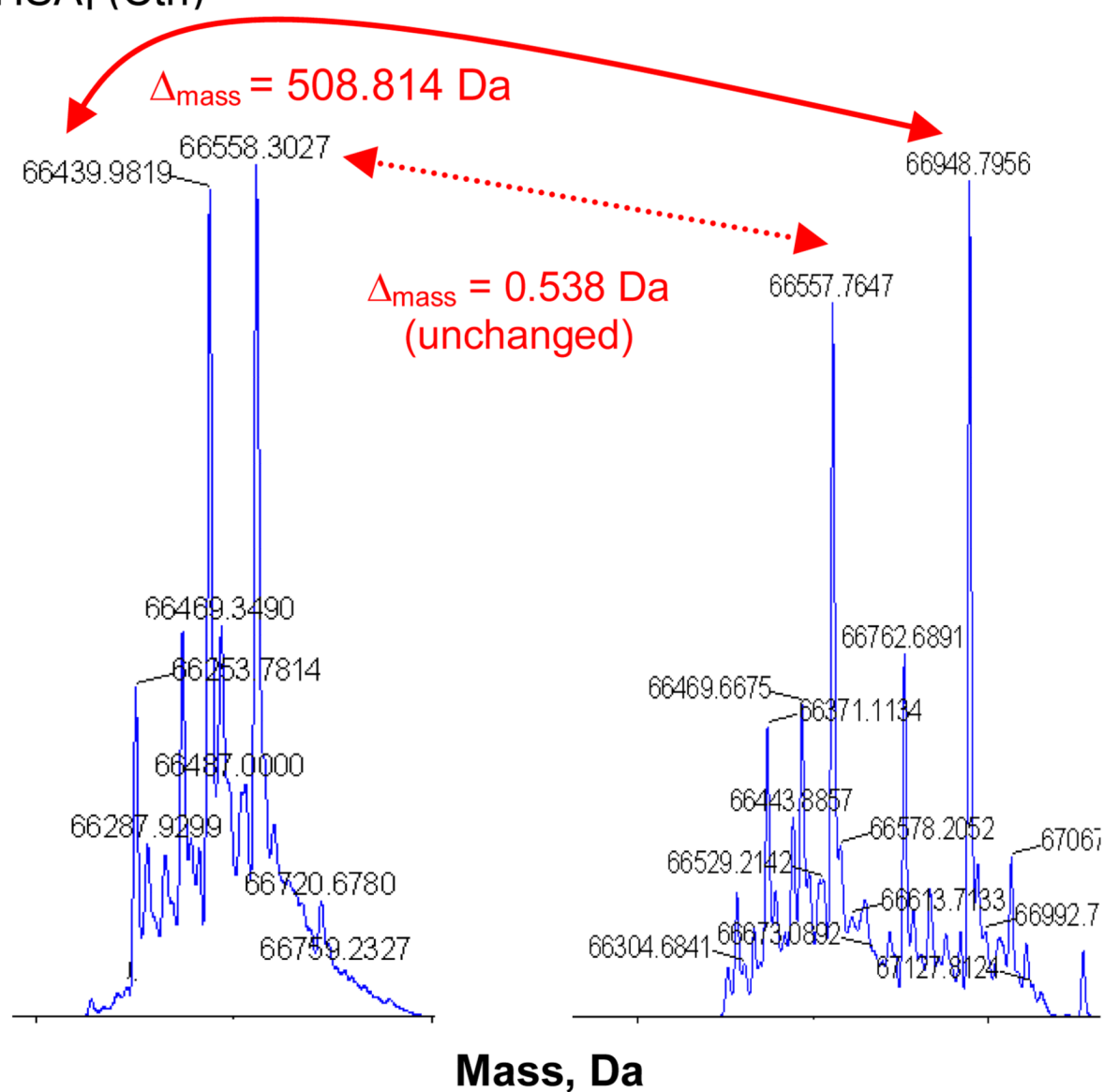


Figure 3. Deconvoluted positive-mode ESI-MS spectra of native bovine α -lactalbumin (left) showing a mass of 14178.83 Da, and α -lactalbumin reacted with 5 eq. of **2**, resulting in a stochastic coupling of the adjuvant with the centroid of the mass distribution corresponding to exactly 5 units of imidazoquinoline per protein molecule.

Human Serum Albumin
[HSA] (Ctrl)HSA + **5** (Mass: 510.238 Da)**Figure 4.**

Covalent coupling of the thiol-specific maleimide derivative **5** with human serum albumin showing addition of a single equivalent of **5** to albumin, as examined by LC-ESI-TOF. An excess (5 equiv.) of **5** was used.

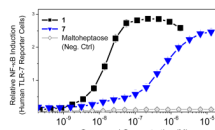


Figure 5. TLR7-agonistic activities of imidazoquinoline-maltoheptaose conjugate in a human TLR7-specific reporter gene assay.

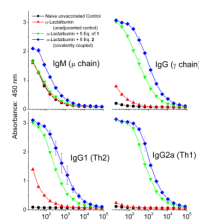


Figure 6.

Immunoglobulin profiles in outbred CF-1 mice immunized on Day 0 with 50 $\mu\text{g}/\text{animal}$ of α -lactalbumin, or α -lactalbumin covalently coupled with 5 equivalents of **2**, or α -lactalbumin mixed with 5 equivalents of **1**. Animals (5 per cohort) were boosted once on Day 14 exactly as mentioned above, and bled on Day 21. α -lactalbumin-specific immunoglobulin levels were quantified by standard antibody-capture ELISA, performed in liquid handler-assisted 384-well format.

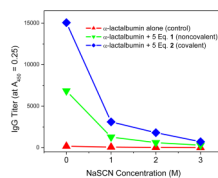
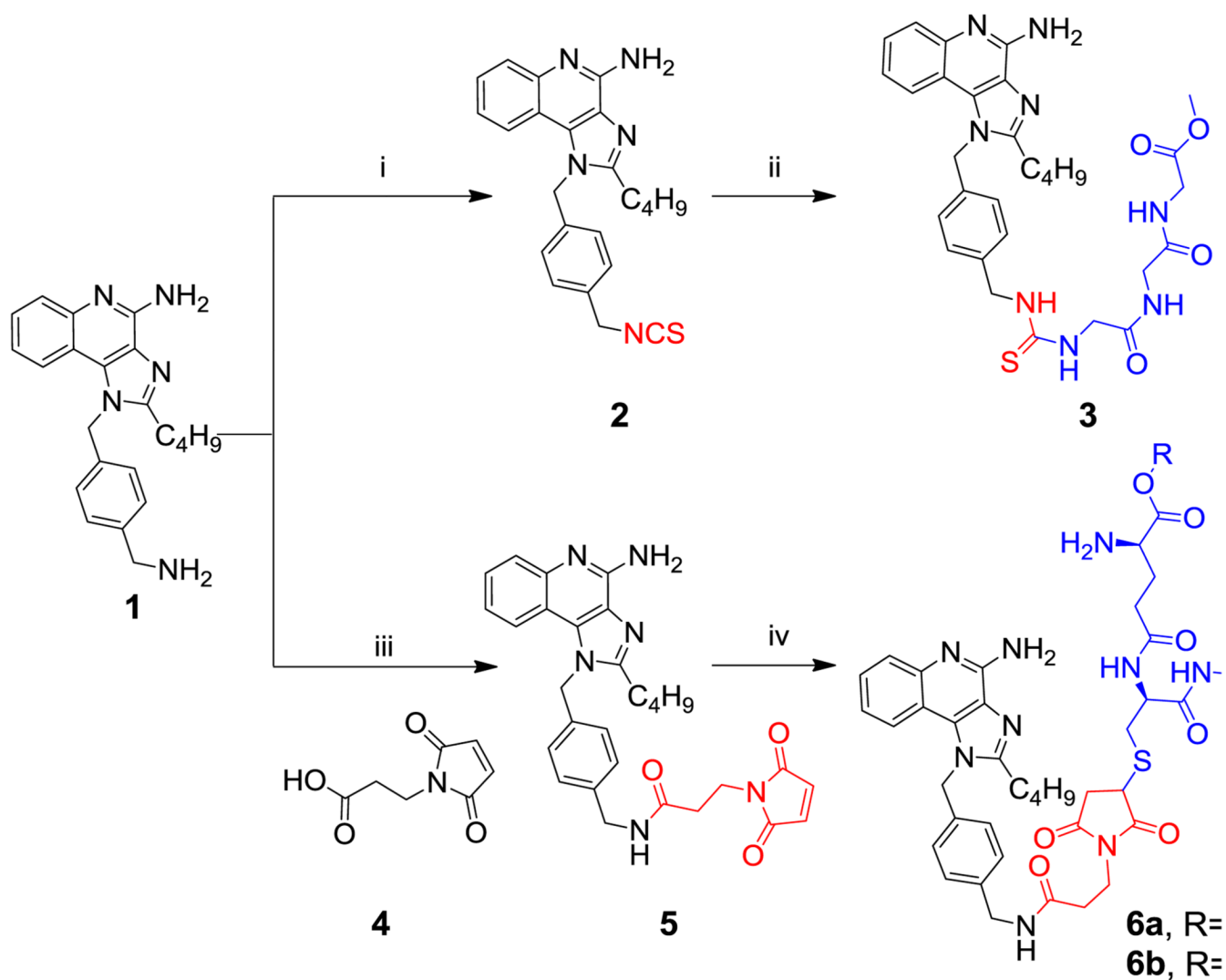


Figure 7. Affinity IgG ELISA showing antibody titer as a function of chaotrope (NaSCN) concentration. IgG titers on the ordinate axis were calculated from absorbance values at 0.25 (which corresponds to 3σ above that of naïve controls).



Reagents: i. CS_2 , Et_3N , DMAP, $(\text{Boc})_2\text{O}$, CH_2Cl_2 ; ii. Tri-glycine methyl ester, Et_3N , MeOH;
 iii. HRTU, Et_3N , DMAP, DMF; iv. glutathione-reduced (GSH) or GSH dimethyl ester, Et_3N

Scheme 1.

Syntheses of isothiocyanate and maleimide derivatives of **1**, and their corresponding model peptide conjugates.



Scheme 2.
Syntheses of the maltoheptaose conjugate.