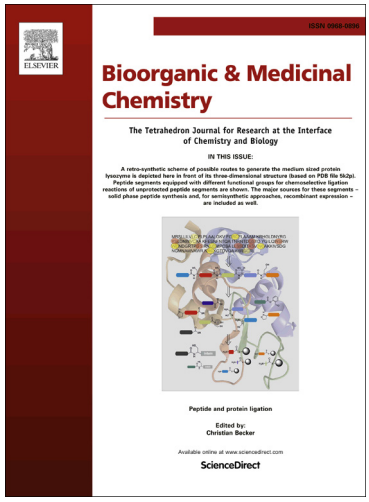

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Graphical Abstract

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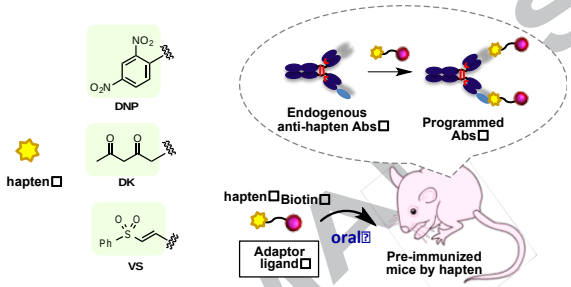

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In vivo Programming of Endogenous Antibodies via Oral Administration of Adaptor Ligands

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ABSTRACT

Vaccination is a reliable method of prophylaxis and a crucial measure for public health. However, the majority of vaccines cannot be administered orally due to their degradation in the harsh gut environment or inability to cross the GI tract. In this study, we report the first proof-of-concept study of orally producible chemically programmed antibodies via specific conjugation of adaptor ligands to endogenous antibodies, *in vivo*. Pre-immuniztion with 2,4-dinitrophenyl (DNP), or the reactive hapten, 1,3-diketone (DK), or a novel reactive hapten, vinyl sulfone (VS) in mice, followed by oral administration of adaptor ligands composed of the hapten and biotin to the pre-immunized mice resulted in successful *in vivo* formation of the biotin-hapten-antibody complexes within 2 hours. Pharmacokinetic evaluations revealed that apparent serum concentrations of programmed antibodies were up to 144 nM and that the serum half-lives reached up to 34.4 h. These findings show promise for the future development of orally bioavailable drug-hapten-antibody complexes as a strategy to quickly and easily modulate immune targets for aggressive pathogens as well as cancer.

1. Introduction

Currently, active immunization is the most powerful method to provide immunity against disease-related viruses and pathogens and is said to prevent 6 million deaths worldwide every year.[1] Unlike antibody therapies using passive immunization (e.g. hyperimmune globulin, therapeutic monoclonal antibodies and antibody-drug conjugates), active immunization elicits endogenous antibodies with a variety of immunoglobulin isotypes as well as long-lasting adaptive immunity via memory B-cells.[2] However, usage of common vaccine antigens (i.e. weakened or inactive forms of pathogens or their surface proteins) often take days to weeks to develop immunity to their corresponding pathogens, thus, limiting their efficacy against aggressive pathogens and rapidly acting toxins.

To address the relatively slow process of vaccine-mediated acquisition of immunity, a combination of elements from active and passive immunization have been explored to expedite the process of pathogen clearance.[3] This approach first induces an immune state for a designated immune-response eliciting small molecule, or hapten, via the injection of a synthetic vaccine composed of the hapten conjugated to a carrier protein.[4] Once endogenous anti-hapten antibodies are produced, injection of a pathogenic target protein-binding compound conjugated to the hapten (adaptor ligand) results in instant formation of adaptor ligand-antibody complexes (immunoconjugates) *in vivo* which can be used to target pathogens or even cancer cells (Figure 1a).

There have been successful examples for formation of anti-cancer immunoconjugates *in vivo*. Mice pre-immunized with fluorescein or 2, 4-dinitrophenyl (DNP) as haptens followed by the intravenous (i.v.) administration of the adaptor ligands composed of cancer-targeting small molecules linked to corresponding haptens resulted in successful *in vivo* formation of immunoconjugates.[5,6,7,8] Further, a treatment for renal cell carcinomas where immunization with the synthetic vaccine, fluorescein-conjugated KLH (keyhole limpet hemocyanin) followed by the injection of folate-conjugated fluorescein adaptor ligand had reached Phase II clinical trials (although terminated due to low subject accrual).[9] Our group has previously shown that reactive immunization with the hapten, 1,3-diketone (DK), conjugated to KLH enables the production of catalytic aldolase antibodies[10,11,12] These antibodies were able to covalently bind *in*

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‡C.F. Barbas III passed away on June 24th, 2014, during the preparation of the manuscript.

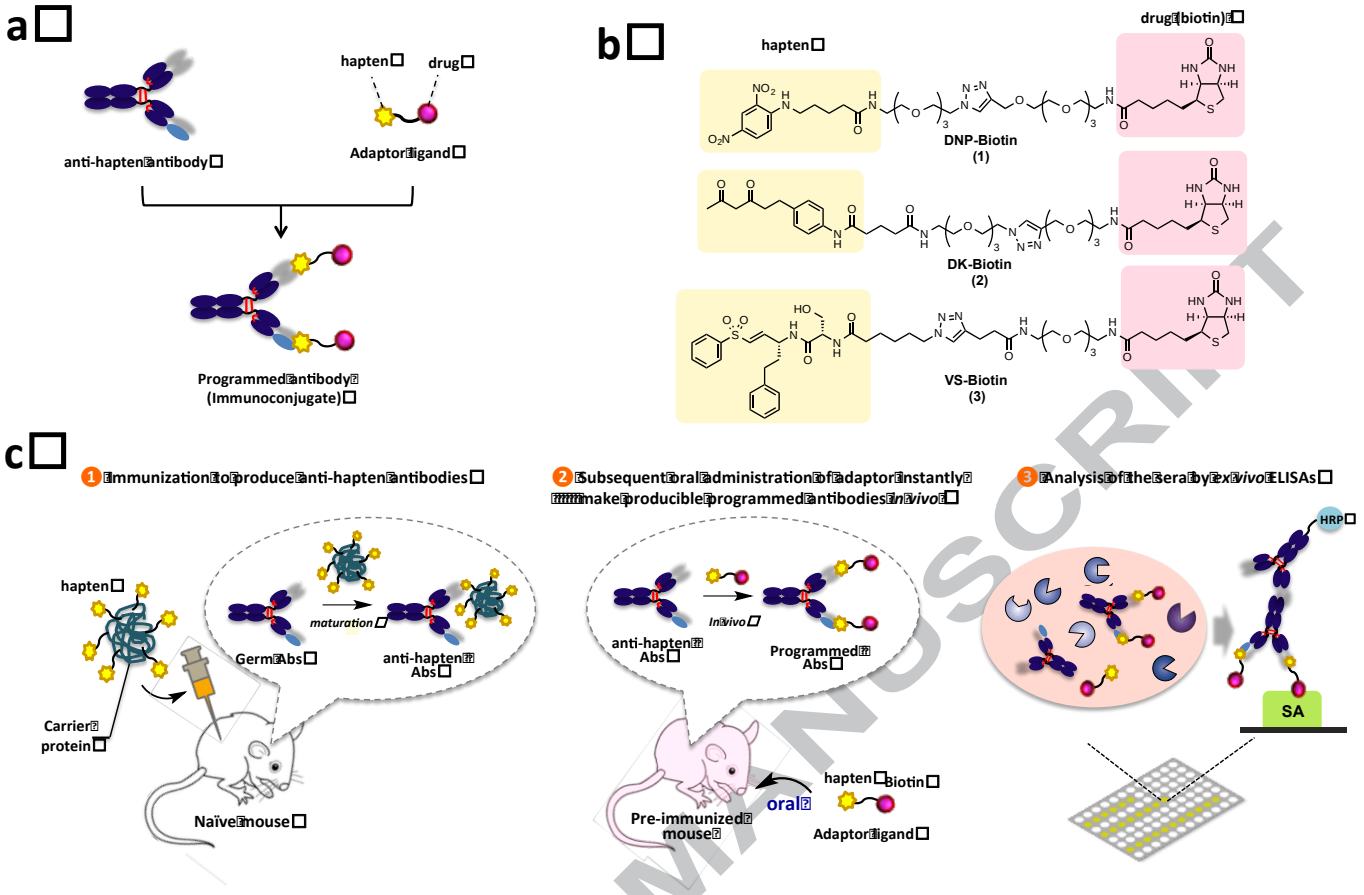


Figure 1. (a) A composition of programmed antibody. (b) Structures of adaptor ligands, DNP-biotin (**1**), DK-biotin (**2**) and VS-biotin (**3**). (c) The concept of this study. Active immunization against haptens followed by oral administration of the hapten-biotin adaptor ligand instantly produces programmed antibodies as anti-streptavidin antibodies *in vivo*.

vitro various adaptor ligands composed of DK-fused drugs against flu, HIV or cancer, in order to chemically program antibody specificity and thus biological activity.[13] Furthermore, the injection of DK fused integrin-binding compounds into mice pre-immunized against DK successfully led to the *in vivo* formation of programmed polyclonal antibodies that significantly reduced melanoma size by targeting cancer-related integrins.[14]

Thus the development of instant chemically induced immunity by way of hijacking the immune system through programming of endogenous antibodies with adaptor ligands is a significant leap from current vaccinnology. However, administration of adaptor ligands requires multiple injections over a given time course depending on the ailment to be treated. Such treatments would require multiple or prolonged visits to the clinic. Furthermore, orally bioavailable adaptor ligands would offer a number of significant advantages over current biological vaccination methodologies, such as instant immunity, targeting non-mucosomal pathogens, suitability for long term storage, a needless for trained personnel, low cost and suitable for mass production.

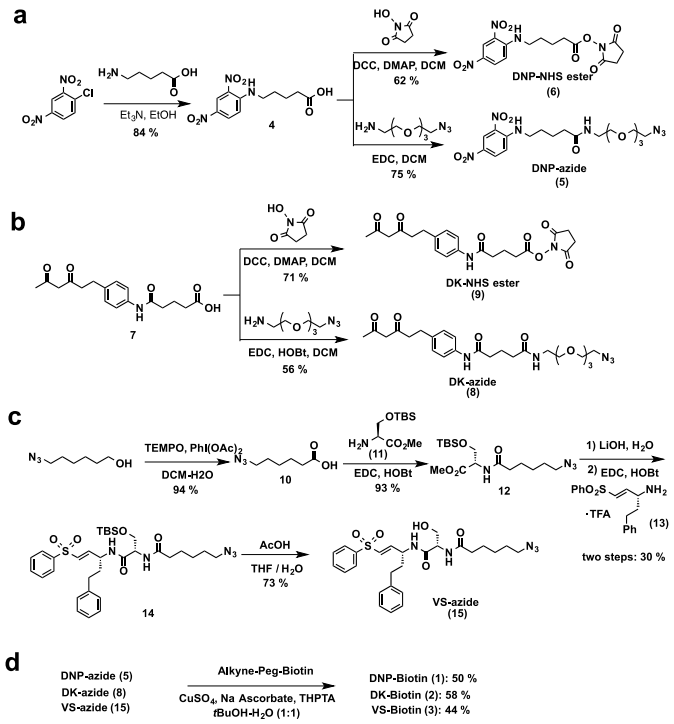
Here, we report a proof-of-concept study demonstrating the *in vivo* production of immunoconjugates via oral administration of hapten-biotin adaptor ligands in mice actively immunized by these adaptor ligands (Figure 1b). Further, to investigate the potency of immunoconjugates formed *in vivo*, the previously reported haptens DK and DNP as well as a novel reactive hapten, vinyl sulfone (VS), were chosen and chemically conjugated to biotin (Figure 1c). These adaptor ligands were subsequently orally administered to mice pre-immunized to the corresponding hapten. After 2 h, *in vivo* formation of the corresponding biotin-hapten immunoconjugates were detected at sub-micromolar concentrations in serum. Further pharmacokinetic analysis of these novel immunoconjugates revealed that, of the 3 haptens tested, DNP produced the most stable immunoconjugates with apparent serum half-lifes of up to 34.4 h. To the best of our knowledge, this is the first report of orally programmed antibodies to rapidly modify antigen specificity and studies of hapten potency forming stable immunoconjugates *in vivo*. Future studies using these haptens linked to potent pathogen or cancer-cell targeting small molecules may open new avenues for the modulation of active immunity via oral administration.

2. Results and discussion

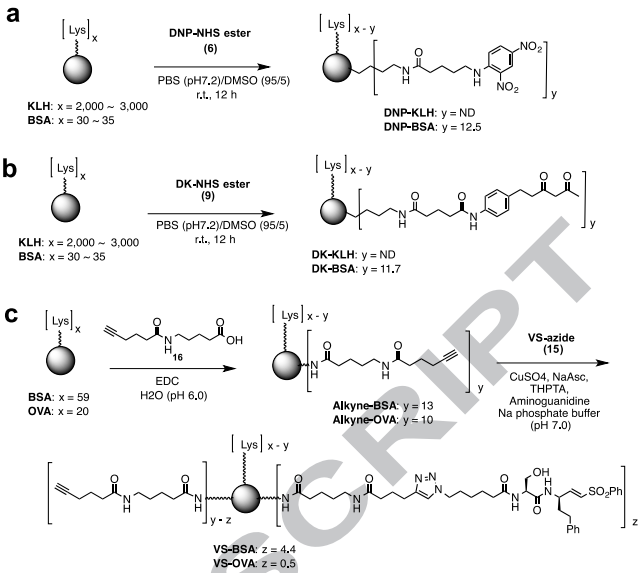
2.1 Synthesis of the haptens and adaptor ligands

As hapten moieties, we have chosen DNP, DK and VS. Humans have naturally occurring anti-DNP antibodies and these are expected to bind DNP in a non-covalent manner.[15] As described above, reactive immunization with DK is able to generate aldolase antibodies and are therefore expected to produce covalently bound antibody-hapten conjugates through enaminone formation. The α,β-unsaturated sulfone structure of VS is known to irreversibly inhibit cysteine proteases via Michael addition[16,17] and it was expected that reactive immunization with VS would behave similarly to the DK and produce covalent antibody-hapten conjugates. In this study, we chose to synthesize the aforementioned haptens conjugated to biotin as adaptor ligands due to the wide variety of detection and quantification methods via streptavidin.

The adaptors, DNP-biotin (**1**), DK-biotin (**2**) and VS-biotin (**3**) and *N*-hydroxy succinimide esters of these haptens were prepared for the programming of endogenous antibodies and conjugations



Scheme 1. Synthesis of hapten derivatives. (a) Synthesis of DNP (b) Synthesis of DK (c) Synthesis of VS (d) Synthesis of biotinylated haptens.



Scheme 2. Preparation of hapten-carrier protein conjugates as antigens. (a) Preparation of DNP antigen (b) Preparation of DK antigen (c) Preparation of VS antigen. X represents predicted number of lysines on a surface of protein from Thermo Scientific. ND, not determined.

to carrier proteins. Aromatic substitution of 2,4-dinitiro-1-chlorobenzene by 5-aminopetanoic acid, followed by condensation with azide-peg-amine or *N*-hydroxysuccinimide gave DNP-azide (**5**) or DNP-NHS ester (**6**), respectively (Scheme 1a). DK-azide (**8**) or DK-NHS ester (**9**) were prepared in the same manner starting from an acid of 1,3-diketone (**7**)[10] (Scheme 1b). Synthesis of VS was successfully prepared by slight modifications to the procedure reported previously for the preparation of the cysteine protease inhibitor, K-777 (Supplementary Figure 1).[16,18,19] Based on reported SAR information, the chirality of homophenylalanine at the P1 site was inverted and the phenylalanine at the P2 site, which is essential for binding to proteases, was replaced with a less inhibitory serine to avoid the undesired reaction of K-777 to cysteine protease homologs. Additionally, the P3 site was replaced with a PEG linker to increase solubility in water (Scheme 1c). Oxidation of 6-azidehexanol, using iodobenzene diacetate, followed by amidation with a TBS protected serine methyl ester (**10**) gave TBS protected VS-azide (**11**). Hydrolysis of **11** rendered an acid, and was subjected to coupling with a phenyl vinyl sulfonated compound (**13**), obtained from (*R*)-homophenylalanine to afford TBS-protected VS-azide (**14**). Acid catalyzed deprotection of the silyl group yielded VS-azide (**15**). Finally, the azide conjugated haptens were linked with biotinylated alkynes using copper catalyzed cycloaddition in the presence of THTPA[20], to give the desired adaptor ligands, DNP-biotin (**1**), DK-biotin (**2**) and VS-biotin (**3**) (Scheme 1d).

2.2 Preparation of hapten-carrier protein conjugates

To induce an immune responses against haptens, conjugation with appropriate carrier proteins is required. Therefore, for both DK and DNP, KLH was selected as the carrier protein for immunization purposes while bovine serum albumin (BSA) was selected as the carrier protein for downstream antibody-binding studies. Conjugates of DNP and DK were produced by coupling the amines of lysines on the surface of KLH and BSA via the hapten-NHS esters **6** and **9**, followed by gel filtration to give the desired BSA and KLH conjugates (Scheme 2a and 2b). The hapten density of BSA conjugates were estimated by MALDI-TOF mass spectrometry and gave roughly 12.5 and 11.7 modifications per protein for the DNP and DK derivatives, respectively. Since the hapten density of oligomeric KLH is difficult to measure by MALDI-TOF due to its high molecular weight (4-8 MDa), enzyme-linked immunosorbent assays (ELISAs) were performed for both DNP-KLH and DK-KLH to determine whether the haptens were successfully conjugated to KLH. Specific binding of anti-DNP and anti-DK monoclonal antibodies to DNP-KLH or DK-KLH, respectively, were detected and both conjugates showed comparable levels of hapten-conjugation (Supplementary Figure 2).

For VS, BSA was selected as the carrier protein for immunization studies while ovalbumin (OVA) was chosen as the carrier protein for downstream antibody-binding studies. To prepare the VS-carrier protein conjugates, BSA and OVA were coupled with the alkyne moiety **16** by EDC to give, 13.2 and 9.9 alkyne modificaitions, respectively (Scheme 2c). Subsequent click reactions of conjugates with VS-azide (**15**) gave the desired VS-BSA or VS-OVA with 4.4 and 0.5 hapten densities, respectively, as determined via MALDI-TOF MS analysis.

2.3 Active immunization with hapten-carrier protein conjugates

To produce anti-hapten antibodies against DNP, DK and VS, balb/c mice were interperitonealy (i.p.) injected with DNP-KLH, DK-KLH and VS-BSA, respectively, on days 1, 22 and 43 (Figure 3a). On days 29 and 50, serum samples were obtained by tail vein bleeds and analyzed by ELISA using DNP-BSA, DK-BSA and VS-OVA (Figure 3b) to determine antibody titers. Anti-DNP titers peaked at bleed-1 at over 20,000, while anti-DK titers reached a peak of 20,000 on bleed-2. Anti-VS titers showed a value of only 5,000 at bleed-2, thus, a fourth booster shot was administered and after 77 days (bleed-3) a maximum titer of near 25,000 was reached. In addition, anti-sera from DNP-KLH and DK-KLH immunized mice showed negligible levels of anti-KLH titers. In addition, anti-BSA titers were undetectable from VS-BSA immunized mice. Next, to investigate the cross-reactivity of the anti-hapten antibodies, we performed ELISAs using the

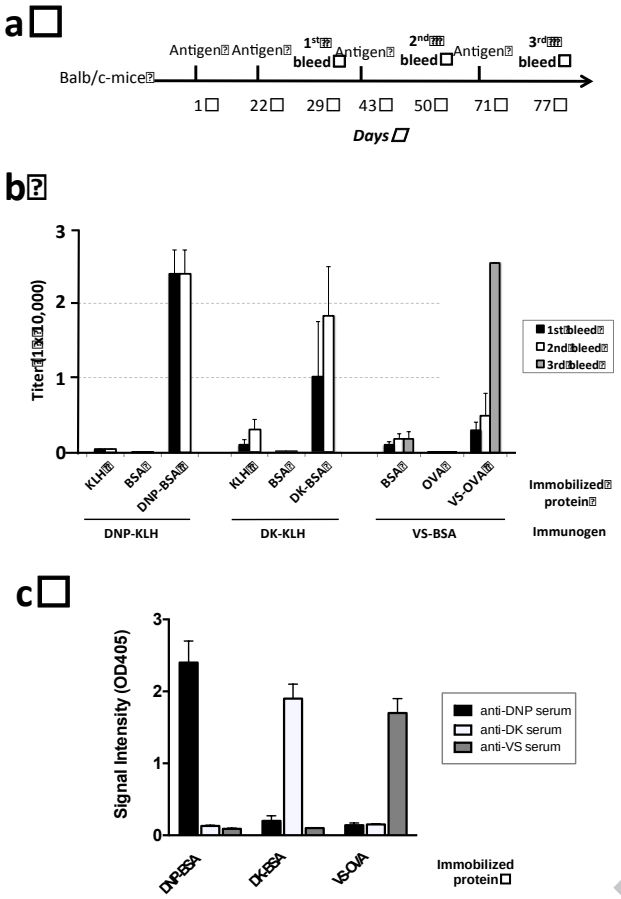


Figure 2. Active immunization with synthetic vaccines. (a) Immunization schedule. Only VS-BSA required an additional booster. (b) Anti-serum titers derived from vaccinated mice (n = 4 - 6) were individually determined by ELISA. Dilution range is up to 25,600. Error bars, s.d. (n = 4 - 6). (c) Cross reactivity checked by ELISA. The individual anti-serum derived from the last bleed were pooled. Error bars, s.d. (n = 3). The anti-serum was diluted to 1/2,500 in PBS.

haptens conjugated to carrier proteins that were not used for immunization (Figure 3c). When pooled DNP anti-sera were incubated with DNP-BSA, DK-BSA or VS-OVA coated wells, only DNP-BSA showed high absorbance while low signal intensities were detected for DK-BSA and VS-OVA. Additionally, DK and VS antisera showed specific binding to DK-BSA and VS-OVA, respectively. It should be noted that although anti-DNP antibodies are known to exist in mammals independent of immunization, DK and VS anti-sera did not show significant binding to DNP-BSA.[7] Overall, immunization with hapten-carrier protein conjugates successfully elicited the production of anti-hapten antibodies specific to each hapten.

2.4 Evaluation of the binding modes of anti-DK and VS antibodies

Next, we assayed for the presence of covalent antibodies against DK and VS. Previously, we have demonstrated that aldolase monoclonal antibodies that bind covalently to DK through the formation of enaminone are stable at low pH (pH 2.0), whereas non-covalent complexes readily dissociate.[14] Therefore, this acid-stable binding provides a means to detect the presence of covalent antibodies from sera. Thus, we used acid-wash ELISA to determine whether covalent antibodies were produced in DK and VS immunized mice (Supplementary Figure 3). When using the catalytic mAb 38C2 against DK-BSA as a positive control there was no significant loss of signal intensity after washing with acid due to covalent bond formation between 38C2 and DK on BSA. Next, anti-DK and anti-VS sera against DK-BSA and VS-OVA were tested. The signal intensity of both anti-DK and -VS sera drastically decreased when exposed to acidic conditions, in comparison to the neutral wash at pH 7.4. These results suggest that the main binding of anti-DK and VS antibodies produced via reactive immunization is non-covalent rather than covalent. It has been reported in a previous study that reactive immunization with DK resulted in only 2 aldolase antibodies out of the 20 highest affinity anti-DK antibodies produced in the study.[10,11] This may explain the low abundance of covalently-binding antibodies in this study.

2.5 In vitro programming of endogenous anti-hapten antibodies from immunized mice with adaptor ligands

Since it was shown that high titers against the haptens were achieved, we proceeded to investigate *in vitro* programming of anti-hapten antibodies in the antisera of the corresponding immunized mice. Anti-sera were incubated with different quantities of the corresponding adaptor ligands (**1**-**3**) followed by incubation in streptavidin coated wells for ELISA analysis (Supplementary Figure 4). Treatment of anti-DNP sera with DNP-biotin (**1**) resulted in strong absorption signals confirming successful antibody programming while treatment of sera from non-immunized naïve mice showed no significant adsorption in ELISA. Similarly, treatment with DK-biotin (**2**) and VS-biotin (**3**) detected a specific signal only when the respective anti-sera were used. Thus, findings from these studies indicate that all anti-hapten antibodies produced by immunized mice were capable of being programmed as desired biotinylated immunoconjugates by the adaptors in an *in vitro* manner.

2.6 Pharmacokinetic analysis of an intraperitoneally injected biotinylated hapten-monoclonal antibody

To assess the pharmacokinetic properties of immunoconjugates formed via oral administration (p.o.) of adaptor ligands, it is essential to determine the level of immunoconjugates *in vivo* without the bias of antibody titer and oral availability of the adaptors. To achieve this, immunoconjugates formed *in vitro* were intraperitoneally (i.p.) injected into non-immunized mice to analyze their pharmacokinetics. Since anti-DNP mouse mAb (9H8.1) and catalytic anti-DK mouse mAb (38C2) are commercially available, we prepared pre-immunoconjugates with the adaptor ligands DNP-biotin (**1**) and DK-biotin (**2**), respectively. It is important to note that both monoclonal antibodies are of the IgG isotype which are primarily induced by active immunization.[14] To construct the immunoconjugates, the monoclonal antibodies were incubated with 2.2 equivalents of the adaptors **1** and **2** in phosphate-buffered saline solution (pH7.4) at room temperature for 2 h. Quantitative conversion to the DK-biotin (**2**)-38C2

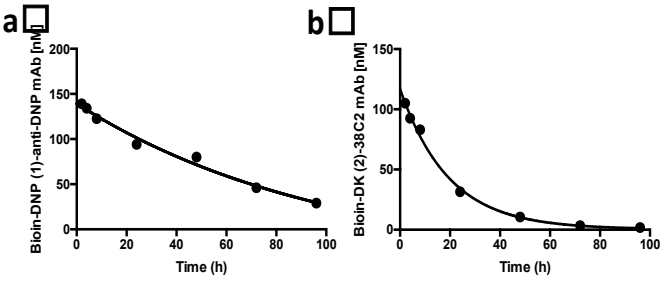


Figure 4. IP injection of *in vitro* programmed antibodies to non-immunized mice. (a) Biotin-DNP (**1**)-anti-DNP mAb (b) Biotin-DK (**2**)-38C2. The circles (●) are from averaged values (n = 2). The graphs were created by GraphPad Prism version 6.

complex was observed via methodol assay by monitoring the lack of retro-aldol catalytic activity in 38C2, while unreacted 38C2 showed high retro-aldol activity resulting in a strong fluorescence signal (Supplementary Figure 5). [21] MALDI-TOF mass spectrometry confirmed the complete conversion to the DK-biotin (**2**)-38C2 complex by an increase in mass that was indicative of the conjugation of two DK-biotins per antibody (one DK-biotin per active-site lysine) (Supplementary Figure 5b). ELISA assays against immobilized streptavidin with both DNP and DK conjugates gave similar signal intensities when using the same quantities of each complex (Supplementary Figure 5c). These results suggest that both DNP and DK conjugates near-quantitatively bound the adaptor ligands. For the quantification of immunoconjugates formed *in vivo*, standard curves were prepared using these *in vitro*-synthesized immunoconjugates for both DNP and DK by ELISAs (Supplementary Figure 6). These conjugates were used without purification for further *in vivo* experiments since only negligible amounts of the adaptor ligands remained. The immunoconjugates were i.p. injected into non-immunized balb/c mice with a single dose of 1 mg/kg each. Tail vein blood samples were collected at 2, 4, 8, 24, 48 and 72 h after injection. For each time point serum concentrations were determined by ELISA and the data was fitted to a one phase decay ($y = y_0 - y_{plateau}*\exp(-k*x) + y_{plateau}$), The apparent half-life can serve as an indicator of serum stability of the immunoconjugates. According to this method, we determined the apparent half life of DNP-biotin monoclonal immunoconjugate to be 55.5 h while DK-biotin exhibited an apparent t-half of 14.7 h (Figure 4).

2.7 Oral programming of endogenous anti-hapten antibodies via oral administration of adaptor ligands

Based on the success with *in vitro* programming using anti-sera, even though the molecular features of the adaptor ligands do not adhere to Lipinski's rule of 5,[22] we examined if oral administration of the hapten-biotin adaptor ligands could result in *in vivo* formation of immunoconjugates. The adaptor ligands **1-3,** formulated with PEG300, were orally administered at a single dose of 100 mg/kg to pre-immunized or non-immunized mice, which had been rested for 6 months after their final injection. Tail vein blood was collected at 2, 4, 8, 24, 48, 72 and 96 h after oral administration and the quantity of immunoconjugates at each time point was evaluated by ELISA (Figure 5). Intriguingly, ELISA analysis showed that all three adaptor ligands were able to successfully program endogenous anti-hapten antibodies forming immunoconjugates *in vivo* without the addition of any adjuvants. In contrast, oral administration of the adaptor ligands to non-immunized mice were undetectable. These promising results suggest that the combination of active immunization followed by the oral administration of adaptor ligands can lead to formation of immunoconjugates.

These results suggest that the tested biotin-hapten adaptor ligands were able to cross the membrane of GI tract, however, it still remains unclear how these adaptor ligands permeate through

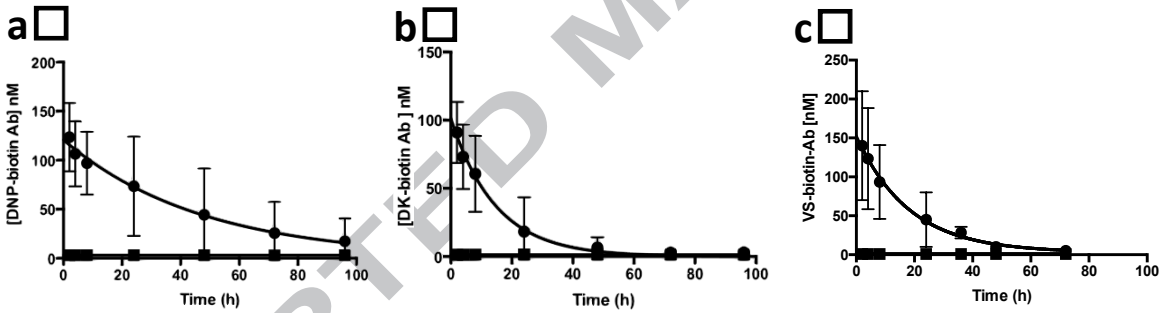


Figure 5. In vivo Programming of endogenous Abs by oral administration of adaptor lingands (**1**−**3**). (a) Biotin-DNP (**1**) (b) Biotin-DK (**2**). (c) Biotin-VS. The circles (●) represent injections to immunized mice (n = 4-6) and the squares (■) represent injections to naïve mice (n = 4-6). All data points were acquired from each individual mice and individual time points were acquired in triplicate (n = 4-6). Formation of biotin-VS (**3**)-antibody conjugates was approximately quantified by applying the standard curve of biotin-DK (**2**)-38C2 conjugate. See supporting information.

Table 1
Properties of biotinylated immunoconjugates prepared *in vivo* or *in vitro.*

	DNP-biotin (**1**)	DK-biotin (**2**)	VS-biotin (**3**)	DNP-biotin (**1**) -anti-DNP mAb[b]	DK-biotin (**2**) -38C2 mAb[b]
Administration	p.o.	p.o.	p.o.	i.p.	i.p.
Formulation	PEG300	PEG300	PEG300	PBS (pH 7.4)	PBS (pH 7.4)
Dose (mg/kg)	100	100	100	1	1
Immunogen	DNP-KLH	DK-KLH	VS-BSA	–	–
Titer before injection[a]	12,800 ± 4,525	3,466 ± 1,222	3,466 ± 1,093	N.D.	N.D.
Programmed Ab Cmax (nM)	127 ± 31	93 ± 24	144 ± 69	141 ± 9	122 ± 20
Programmed Ab apparent half-lives (h)	34.4	10.4	19.2	55.5	14.7

[a] The titers were measured 30 min before the time of injection. C-max values were acquired at 2 h after injection.
[b] Monoclonal antibodies conjugated in PBS were administered via I.P. injection (1 mg/kg) to naïve mice (n = 2). N.D. means Not Determined.

the gut. It has been reported that biotinylated compounds utilize carrier-mediated uptake in the gut through sodium-dependent multiple vitamin transporter (SMVT). Unlike the biotin transporter, which has narrow substrate specificity, SMVT has broad substrate specificity and, therefore, has been used to increase the oral bioavailablity of biotinylated drugs-of-interest.[23,24,25,26] Encouraged by the success of oral formation of immunoconjugates with biotin-hapten adaptor ligands, we investigated instant oral HIV vaccination in which adaptor ligands composed of the same haptens but linked to the HIV entry inhibitor, apraviroc,[27] instead of biotin, however, no detectable sign of forming anti-HIV immunoconjugates were observed (Data not shown). Given this evidence, SMVT may have contributed to the oral bioavailability of the adaptors used in this study. In addition, several mechanisms exist for the transport of mucosal IgAs between the gut and bloodstream.[28] However, results from an ELISA using an alkaline-phosphatase-conjugated anti-mouse IgA antibody as secondary antibody against anti-DNP serum showed no detectable signal, therefore, excluding the possibility of the haptens in this study getting into the bloodstream via binding mucosal IgAs (Fig S7 in SI).

Analysis of the pharmacokinetics of the biotin bound immunoconjugates (Table 1) revealed that the apparent t-halfs of the *in vivo*-synthesized DK-based immunoconjugates (10.4 h) were shorter than the apparent t-half of DNP-based immunoconjugates (34.4 h) and this tendency was consistent with the aforementioned studies comparing the apparent t-halfs of *in vitro*-synthesized DK and DNP immunoconjugates. Of note, *in vitro*-synthesized pre-conjugate experiments were based on high affinity monoclonal antibodies, in contrast to *in vivo*-synthesized immunoconjugates which were based on polycolonal antibodies.

Intriguingly, injection of the *in vitro*-synthesized DK-based immunoconjugates (apparent t-half = 14.7 h), in which the ligand and the catalytic monoclonal antibody were reversibly connected via enaminone bond, showed very similar apparent t-halfs with the DK-based immunoconjugates formed *in vivo* which were shown to be mostly non-covalently conjugated (apparent t-half = 10.4 h). We previously reported that an i.v. injection of *in vitro*-synthesized anti-Flu-immunoconjugate which 38C2 was irreversibly conjugated to a β-lactam hapten-zanamivir adaptor ligand through an amide bond, was found to have a t-half of 72 h.[29] This result suggests that the reversibility of enaminone bond formation with catalytic antibody greatly affects the stability of the conjugate *in vivo*.[30] Thus, we postulate that the dissociation of the DK-based immunoconjugates *in vivo* may have also led to promiscuous binding of reactive DK to nucleophilic amino acid residues of other proteins. To evaluate the off-target reactivity of DK to proteins in blood, we conducted SDS-PAGE analysis of rat serum mixed with DK (Fig S8 in SI). DK-azide (**9**) was incubated in rat serum at 37 ^{o}C for 2h, and then reacted with TAMRA-alkyne by click reaction. This result shows that the DK reacted with various serum proteins, especially albumin, thus depleting DK-biotin (**2**) concentration in the blood stream.

Next we focused on the orally programmed VS-based immunoconjugates. As described in Fig S3 in SI, the binding mode of reactive VS hapten to anti-VS antibodies in mice were mainly non-covalent. The apparent t-half of orally programmed VS-based immunoconjugates (t-half = 19.2 h) were longer than those of DK-based immunoconjugates (t-half = 10.4 h), even though their titers were similar prior to oral administration. As seen with DK, the off-target reactivity of the VS was also analyzed and showed similar tendencies of promiscuous binding to off-target proteins even though the structure of VS was based on a relatively inert antiparasitic agent K-777 (Fig S1 in SI). Thus, the VS-immunoconjugates could result in short serum half-life due to the non-existence of a covalent reservoir and high reactivity of VS, similar to DK-immunoconjugates. Nonetheless, VS showed adequate immunogenicity as well as *in vivo* formation of immunoconjugates with apparent t-halfs longer than that of DK validating it as a novel *bona fide* hapten.

Orally produced DNP-based immunoconjugates showed the longest apparent t-half (34.4 h) of all tested conjugates and compared to the DK-immunoconjugates, the DNP-immunoconjugates showed longer lasting t-halfs for both *in vitro* and *in vivo* synthesized immunoconjugates. DNP is known to interact nonspecifically with membranes and albumin due to its hydrophobicity.[31,32] However, DNP may lack the strong off-target interactions which can be considered with covalently binding DK and VS. It is worth noting that among the tested haptens, DNP was the most immunogenic and DNP-immunized mice maintained relatively high titers prior to oral administration of adaptor. According to Le Chatelier's principle, high titers lead to a shift in the binding equilibrium, leading to reduced clearance of DNP antigen base adaptor ligand and thus longer apparent t-half. These hydrophobic, less reactive and high immunogenic factors of DNP may have contributed to the prolonged half-life of the DNP-immunoconjugates. We expect that further engineering of hapten structures will lead to more suitable adaptor ligands beyond DNP, which is capable of conferring better oral availability and more serum longevity to *in vivo*-synthesized immunoconjugates. Thus, we demonstrated the potential of *in vivo* programming of endogenous antibodies by orally administrated reactive and non-reactive haptens, and believe that further investigation of oral availability of the adaptor ligands can be generalized to a drug possessing adaptor ligand toward to an instant oral vaccination.

3. Conclusion

Here, we report the successful generation of biotin bound immunoconjugates by using a combination of active immunization and oral administration of adaptor ligands as well as analysis of their pharmacokinetic properties. This is proof-of-concept study demonstrate the feasibility of programming endogenous antibodies via oral administration of a synthetic molecule and brings us closer to the realization of a novel methodology of oral vaccination. Modification of the adaptor with other orally bioavailable therapeutic drug moieties for the oral production of drug-conjugated antibodies is currently underway. We believe these findings could lead to new innovative approaches in vaccinology.

4. Experimentals

4.1 Chemical synthesis

Detailed information on synthesis as well as analytical data can be found in the Supporting Information.

4.2 Preparastion of hapten-carrier protein conjugates

DNP-KLH: 14 mg of KLH (Thermo) was dissolved in 1.4 mL of H_2O to have a concentration of 10 mg/mL in PBS, subsequently 5.6 mL of 100 mM sodium phosphate buffer (pH 7.2) was added. To 6 mL of resulting 2.0 mg/mL KLH was added 600 uL of 1mg/mL DNP-NHS (**6**) in DMF and the whole was incubated at rt for 16 h, and then purified through PD-10 column. Concentration was determined by BCA assay and 4.8 mg/mL 500 µL yellow suspended conjugate was used as an antigen for immunization.

DNP-BSA: To 1.0 mL of 5 mg/mL BSA (Calbiochem) in H_2O (pH6.0) was added 40 µL of 66 mM DNP-NHS (**6**) in DMSO and incubated at rt for 16 h. The resulting solution was purified

by PD-10 column and concentrated with ultra filtration (Amicon Ultra MWCO10,000) and centrifuged (3,000 rpm, 10 min) to less than 500 µL. The concentration of remaining solution was measured by BSA assay, then 5.7 mg/mL yellow suspended solution was used for immunizations. The number of modified residues were determined by MALDI-TOF MS and found to have 12.5 Lys modified.

DK-KLH and DK-BSA were prepared as described.[10] DK-KLH: 14 mg of KLH (Thermo, #77600) was dissolved in 1.4 mL of H_2O to be 10 mg/mL in PBS, then added to 5.6 mL of 100 mM Sodium phosphate buffer (pH 7.2). To 6 mL of resulting 2.0 mg/mL KLH was added 600 µL of 1mg/mL DK-NHS (**9**) in DMF and incubated at rt for 16 h, and then purified through a PD-10 column. Concentration was determined by BCA assay and 4.2 mg/mL 500 µL desired conjugate was used as an antigen for immunization.

DK-BSA: To 1.0 mL of 5 mg/mL BSA (Calbiochem) in H2O (pH 6.0) was added 40 uL of 66 mM DK-NHS (**9**) in DMSO and incubated at rt for 16 h. Resulting solution was purified by PD-10 column and concentrated with ultra filtration (Amicon Ultra MWCO10,000) and centrifuged (3,000 rpm, 10 min) to less than 500 µL. The concentration of the remaining solution was measured by BSA assay, then a 5.6 mg/mL solution was used for immunization. The number of modified residue was determined by MALDI-TOF MS and showed that 11.7 Lys were modified.

BSA-alkyne: 25 g of BSA was dissolved 5 mL of H_2O (pH 6.0), and filtered through Whatman PURADISC (25 mm, 0.45 µm). To the filtrate was added mixture of 4.83 mg of EDC-HCl in 100 □L of H_2O (pH 6.0) and 2.66 mg of alkyne-acid (**16)** in DMSO (5 □L), and then mixture was kept at RT for 24h. The reaction mixture was filtered through Whatman PURADISC (25 mm, 0.45 µm), and filtrate was freeze-dried to afford 5.0 mg of BSA-alkyne. MALDI-TOF: 68,973 (MALDI-TOF of BSA starting material; 66,435). Molecular weight increase: 2538, Molecular weight alkyne: 211, 2538/(211-18)=13.2, The BSA-Alkyne has 13.2 residues of alkyne.

VS-BSA: 5.0 mg of BSA-alkyne obtained above was dissolved in 5.0 mL of 100 mM sodium phosphate (pH 7.0), and mixed well. The mixture was centrifuged (10,000 rpm, 5 min), and then supernatant was filtered through Whatman PURADISC (25 mm, 0.45 µm). The protein concentration of this filtrate was 1.0 mg/mL (Nanodrop, A280, BSA mode). To 1297.5 µL of this BSA-alkyne solution was added 30 µL of 20 mM compound VS-azide (**15**) in DMSO, 22.5 µL of premixed solution of 20 mM $CuSO_4$-$5H_2O$ (36 □L) and 50 mM THPTA (72 □L), 75 µL of 100 mM aminoguanidine hydrochloride in H_2O and 75 µL of sodium L-ascorbate in H_2O, and then mixed well, kept at rt for 1h. The reaction mixture was applied to ultra filtration (Amicon ultra MWCO 10,000) and centrifuged (3,000 rpm, 10 min). 3.5 mL of H_2O was added to this solution, and centrifuged (3,000 rpm, 20 min) to remove small molecule reagents. This procedure was repeated 3 times, and the desired BSA-VS solution in H_2O (13.7 mg/mL, 118 µL) was obtained. MALDI-TOF MS gave molecular weight of VS-BSA = 71,313. Molecular weight increase from BSA-Alkyne: 4,828, Molecular weight VS-azide: 527, 4,828/527=4.4. The VS-BSA has 4.4 residues of VS.

VS-OVA: VS-OVA was prepared with the similar manner with VS-BSA via OVA-alkyne. Briefly, MALDI-TOF of OVA: 44,411, OVA-Alkyne: [M + H] = 46,318. Molecular weight increase: 1907, Molecular weight alkyne: 211, 1,907/(211-18)=9.9, The OVA-Alkyne has 9.9 residues of alkyne.

VS-OVA MALDI-TOF: 46,571, Molecular weight increase of mono VS-OVA from OVA-Alkyne: 591, Molecular weight VS-azide: 527, 591/527=1.1. The VS-OVA has 1.1 residues of VS.

4.3 BCA assay for determination of protein concentration

Protein concentrations were determined by a modification of Lowry's method, using bicinchonic acid (Pierce BCA Protein Assay Kit, Thermo Fisher), with BSA standards. Samples were incubated at 37 ^{o}C for 30 min before analysis on TECAN plate reader at 562 nm.

4.4 Animal experiment

All procedures were approved by The Scripps Research Institute Animal Care and Use Committee and were performed according to national and institutional guidelines for the humane treatment of animals. See supporting info for the details.

4.5 Active immunization and ELISAs

7 week old Balb/c mice (n = 4 for DNP-KLH, DNP-KLH, and KLH, n = 6 for VS-BSA and BSA) were intraperitoneally injected 100 µg/mouse in Sigma Adjuvant System, with 200 µL/mouse as a primary injection. Subsequently, 3 weeks later, the same amount of antigen were injected as a secondary injection (The day 22). A third injection was performed with 100 µg/mouse with ALUM at 200 µg/mouse (The day 43). For the immunization of VS, the fourth injection was performed with 100 µg/mouse with ALUM, 100 µL/mouse (The day 71). Individual DK-antiserum from DK-KLH-immunized mice was collected on days 28, and 49 (and 77 for VS) and used for in vitro assays. For ELISA, Costar 96-well ELISA plates (Corning) were coated with 200 ng of DNP-BSA, DK-BSA and VS-OVA in 50 µL PBS and incubated overnight at 4 °C. After blocking with 150 uL of PBS/3% BSA for 2 h at 37 °C, 50 µL of different dilutions (from 1 : 500 to 1 : 64,000) of individual sera (4 or 6 mice for hapten) was added into each well and the plates were incubated for 2 h at 37 °C. Washing and detection were performed as described using HRP-conjugated goat anti-mouse IgG antibody (diluted 1:3,000 in PBS/1 % BSA). In some experiments, additional incubation with 50 µL of 0.05 M citric acid, pH 2.5 (acid wash) for 15 min at rt was performed after the initial washing step. From individual bleed samples, the titers were measured by making 8 serial 2-fold dilutions starting at 1 : 200 through 1 : 25,600, and calculated as the dilution where 50 % of the max absorbance value was achieved.

4.6 Cross reactivity ELISA

PBS containing 0.1 % tween and 5% BSA in TBS containing 0.1 % tween were used as washing buffer or binding buffer. 200 ng/well/50 µL of corresponding BSA-DNP, BSA-DK, OVA-VS conjugate was coated to half well ELISA plates (Corning) overnight at 4 ^{o}C. After wells were rinsed with washing buffer and subsequently blocked with 5% BSA in TBS containing 0.1 % tween for 1.5 h at 37 ^{o}C, sample conjugates diluted in binding buffer were added and incubated for 1 h at 37 ^{o}C. After washing, secondary antibody Anti-Mouse IgG whole molecule alkaline-phosphate conjugate were added and incubated for 1h at 37 ^{o}C. Subsequently, the bound sample was detected according to the manufactures (Sigma) recommendations.

4.7 In vitro Programming of anti-hapten antibodies in anti-serum by adaptor ligands

50 µL of diluted anti-serum in PBS (1 : 500) was mixed with 10 µL of the adaptors (**1**, **2** or **3**) in PBS (0 to 50 ng/10 □L), and incubated for 3 h at rt. Coastar 96-well ELISA plate (Corning) were coated with 200 ng of Streptavidin in 50 µL PBS and incubated overnight at 4 ^{o}C. After blocking with 150 µL of 3% BSA in PBS for 2 h at 37 ^{o}C, 60 µL of anti-serum treated with the adaptors was added into each well and the plate was incubated for 2 h at 37 ^{o}C. Washing and detection were

performed essentially as described in our previous paper[14] using alkaline phosphatase-conjugated goat anti-mouse IgG antibody (diluted 1 : 1,000 in 1 % BSA/PBS).

4.8 In vitro programming of monoclonal antibodies with adaptor ligands and their i.p. injections

The in vitro programming of mAb 38C2 with DK-biotin (**2**) was performed as previously described.[21] anti-DNP monoclonal antibody (9H8.1) was purchased from Merk-Milllipore (MAB2223). The 9H8.1 antibody with the adaptors DNP-biotin (**1**) were incubated with 2.2 equivalents of the adaptors (**1**) in phosphate-buffered saline solution (pH 7.4) at rt for 2 h. ELISA assays against immobilized streptavidin with both DNP and DK conjugates. To estimate a concentration of biotin-hapten antibody conjugate *in vivo*, the standard curves were prepared for both DNP and DK by ELISAs. These conjugates were used without purification for further *in vivo* experiments. The immunoconjugates were i.p. injected into non-immunized balb/c mice with a single dose of 1 mg/kg each, respectively. Tail vein blood samples were collected at 2, 4, 8, 24, 48 and 72 h after injection. Formation of serum immunocomplexes were quantified by ELISA. PBS containing 0.1 % tween and 5% BSA in TBS containing 0.1 % tween was used as washing buffer or binding buffer. 200 ng/well of streptavidin was coated to half well ELISA plates (Corning) overnight 4 ^{o}C. After wells were rinsed with washing buffer and subsequently blocked with 5 % BSA in TBS containing 0.1 % tween for 1 h at 37 ^{o}C, sample diluted in binding buffer were added and incubated for 1 h at 37 ^{o}C. After washing, secondary antibody Anti-Mouse IgG whole molecule alkaline-phosphate conjugate were added and incubated for 1h at 37 ^{o}C. Subsequently, the bound sample was detected according to the manufactures (Sigma) recommendations.

4.9 Oral administration

The adaptors **1-3** were solubilized in PEG 300 at a dose of 100 mg/kg against 30 g weight mouse. Then, these soluble adaptors were orally administrated to the pre-immunized or non-immunized mice, which were rested for 6 months after the final injection. Tail vein blood was collected at 2, 4, 8, 24, 48, 72 and 96 h after oral administration and the amount of biotinylated immunoconjugates at each time point was evaluated by ELISA. For ELISAs, PBS containing 0.1 % tween and 5 % BSA in TBS containing 0.1 % tween were used as washing buffer and binding buffer, respectively. 200 ng/well of streptavidin was coated to half well ELISA plates (Corning) overnight 4 ^{o}C. After wells were rinsed with washing buffer and subsequently blocked with 5 % BSA in TBS containing 0.1 % tween for 1 h at 37 ^{o}C, sample diluted in binding buffer were added and incubated for 1 h at 37 ^{o}C. After washing, secondary antibody Anti-mouse IgG whole molecule alkaline-phosphate conjugate were added and incubated for 1 h at 37 ^{o}C. Subsequently, the bound sample was detected according to the manufactures (Sigma) recommendations.

4.10 Isotyping-ELISA

Water and 3 % BSA in PBS were used as washing buffer and binding buffer, respectively. 25 µL of 2.5 mg/ml BSA-DNP conjugate solution was coated to half well ELISA plates (Corning) overnight at 4 ^{o}C. After wells were rinsed with washing buffer they were blocked with 3 % BSA in PBS for 1.5 h at 37 ^{o}C. The 2nd bleed titer samples diluted in 1 % BSA in PBS buffer were added and incubated for 1.5 h at 37 ^{o}C. After washing, Anti-mouse IgA-alkaline phosphate antibody produced in goat in 1% BSA in PBS were added and incubated for 1 h at 37 ^{o}C. Subsequently, the bound sample was detected according to the manufactures (Sigma) recommendations.

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Supplementary Material

Supplementary material （details for chemical synthesis, figures related *in vitro* animal study, NMR and MALDI-TOF MS spectra） associated with this article can be found.

References and notes

1. Ehreth, J. *Vaccine* **2003**, *21*, 596–600.
2. Baxter, D. *Occup. Med. (Chic. Ill).* **2007**, *57*, 552–556.
3. Mcenaney, P. J.; Parker, C. G.; Zhang, A. X.; Spiegel, D. A. *ACS Chem. Biol.* **2012**, *7*, 1139–1151.
4. Jones, L. H. *Nat. Chem.* **2015**, *7*, 952–960.
5. Lu, Y.; Low, P. S. *Cancer Immunol. Immunother.* **2002**, *51*, 153–162.
6. Lu, Y.; You, F.; Vlahov, I.; Westrick, E.; Fan, M.; Low, P. S.; Leamon, C. P. *Mol. Pharmaceurics* **2007**, *4*, 2432–2443.
7. Dubrovska, A.; Kim, C.; Elliott, J.; Shen, W.; Kuo, T. H.; Koo, D. I.; Li, C.; Tuntland, T.; Chang, J.; Groessl, T.; Wu, X.; Gorney, V.; Ramirez-Montagut, T.; Spiegel, D. A.; Cho, C. Y.; Schultz, P. G. *ACS Chem. Biol.* **2011**, *6*, 1223–1231.
8. Rullo, A. F.; Fitzgerald, K. J.; Muthusamy, V.; Liu, M.; Yuan, C.; Huang, M.; Kim, M.; Cho, A. E.; Spiegel, D. A. *Angew. Chemie - Int. Ed.* **2016**, *55*, 3642–3646.
9. Amato, R. J.; Shetty, A.; Lu, Y.; Ellis, P. R.; Mohlere, V.; Carnahan, N.; Low, P. S. *J. Immunother.* **2014**, *37*, 237–244.
10. Wanger, J.; Lerner, R. A.; Barbas, C. F., Ⅲ *Science* **1995**, *270*, 1797–1800.
11. Zhong, G.; Lerner, R. A.; Barbas, C. F., Ⅲ *Angew. Chemie Int. Ed.* **1999**, *38*, 3738–3741.
12. Zhong, G.; Shabat, D.; List, B.; Anderson, J.; Sinha, S. C.; Lerner, R. A.; Barbas, C. F., Ⅲ *Science* **1998**, *273*, 2481–2484.
13. Rader, C. *Trends Biotechnol.* **2014**, *32*, 186–197.
14. Popkov, M.; Gonzalez, B.; Sinha, S. C.; Barbas, C. F., Ⅲ *Proc. Natl. Acad. Sci.* **2009**, *106*, 4378–4383.
15. Ortega, E.; Kostovetzky, M.; Larralde, C. *Mol. Immunol.* **1984**, *21*, 883–888.
16. Palmer, J. T.; Rasnick, D.; Klaus, J. L.; Brömme, D. *J. Med. Chem.* **1995**, *38*, 3193–3196.
17. Renslo, A. R.; McKerrow, J. H. *Nat. Chem. Biol.* **2006**, *2*, 701–710.
18. Choe, Y.; Brinen, L. S.; Price, M. S.; Engel, J. C.; Lange, M.; Grisostomi, C.; Weston, S. G.; Pallai, P. V.; Cheng, H.; Hardy, L. W.; Hartsough, D. S.; McMakin, M.; Tilton, R. F.; Baldino, C. M.; Craik, C. S. *Bioorganic Med. Chem.* **2005**, *13*, 2141–2156.
19. Jaishankar, P.; Hansell, E.; Zhao, D. M.; Doyle, P. S.; McKerrow, J. H.; Renslo, A. R. *Bioorganic Med. Chem. Lett.* **2008**, *18*, 624–628.
20. Hong, V.; Presolski, S. I.; Ma, C.; Finn, M. G. *Angew. Chemie - Int. Ed.* **2009**, *48*, 9879–9883.
21. Sinha, S. C.; Das, S.; Li, L.; Lerner, R. A.; Barbas, C. F., Ⅲ *Nat. Protoc.* **2007**, *2*, 449–56.
22. Lipinski, C.A.; Lombardo, F.; Dominy, B.W.; Feeney, P. J. *Adv. Drug Deliv. Rev.* **2012**, *64*, 4–17.
23. Al-Hilal, T. A.; Alam, F.; Byun, Y. *Adv. Drug Deliv. Rev.* **2013**, *65*, 845–864.
24. Ramanathan, S.; Pooyan, S.; Stein, S.; Prasad, P. D.; Wang, J.; Leibowitz, M. J.; Ganapathy, V.; Sinko, P. J. *Pharm. Res.* **2001**, *18*, 950–956.
25. Chae, S. Y.; Jin, C.; Shin, H. J.; Youn, Y. S.; Lee, S.; Lee, K. C. *Bioconjugate Chem.* **2008**, *19*, 334–341.

26. Janoria, K. G.; Boddu, S. H. S.; Wang, Z.; Paturi, D. K.; Samanta, S.; Pal, D.; Mitra, A. K. *J. Ocul. Pharmacol. Ther.* **2009**, *25*, 39–49.
27. Ban, H.; Nagano, M.; Gavrilyuk, J.; Hakamata, W.; Inokuma, T.; Barbas, C. F., Ⅲ *Bioconjug. Chem.* **2013**, *24*, 520–532.
28. Corthésy, B. *Front. Immunol.* **2013**, *4*, 1–11.
29. Hayakawa, M.; Toda, N.; Carrillo, N.; Thornburg, N. J.; Crowe, J. E.; Barbas, C. F., Ⅲ *ChemBioChem* **2012**, *13*, 2191–2195.
30. Bhat, A.; Laurent, O.; Lappe, R. In *Fusion Protein Technologies for Biopharmaceuticals: Applications and Challenges, Chapter 38*; **2013**.
31. Jung, H.; Yang, T.; Lasagna, M. D.; Shi, J.; Reinhart, G. D.; Cremer, P. S. *Biophys. J.* **2008**, *94*, 3094–3103.
32. Zhang, A. X.; Murelli, R. P.; Barinka, C.; Michel, J.; Cocleaza, A.; Jorgensen, W. L.; Lubkowski, J.; Spiegel, D. A. *J. Am. Chem. Soc.* **2010**, *132*, 12711–12716.