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Prescreening of Nicotine Hapten Linkers in Vitro To Select Hapten-Conjugate Vaccine Candidates for Pharmacokinetic Evaluation in Vivo

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Abstract

Since the demonstration of nicotine vaccines as a possible therapeutic intervention for the effects of tobacco smoke, extensive effort has been made to enhance nicotine specific immunity. Linker modifications of nicotine haptens have been a focal point for improving the immunogenicity of nicotine, in which the evaluation of these modifications usually relies on in vivo animal models, such as mice, rats or nonhuman primates. Here, we present two in vitro screening strategies to estimate and predict the immunogenic potential of our newly designed nicotine haptens. One utilizes a competition enzyme-linked immunoabsorbent assay (ELISA) to profile the interactions of nicotine haptens or hapten-protein conjugates with nicotine specific antibodies, both polyclonal

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Notes

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Supporting Information

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Procedures for the synthesis and characterization of HPs 1 and 3–9 in Figures S1–S7 and Schemes S1–S6 (PDF)

and monoclonal. Another relies on computational modeling of the interactions between haptens and amino acid residues near the conjugation site of the carrier protein to infer linker-carrier protein conjugation effect on antinicotine antibody response. Using these two in vitro methods, we ranked the haptens with different linkers for their potential as viable vaccine candidates. The ELISA-based hapten ranking was in an agreement with the results obtained by in vivo nicotine pharmacokinetic analysis. A correlation was found between the average binding affinity (IC_{50}) of the haptens to an anti-Nic monoclonal antibody and the average brain nicotine concentration in the immunized mice. The computational modeling of hapten and carrier protein interactions helps exclude conjugates with strong linker-carrier conjugation effects and low in vivo efficacy. The simplicity of these in vitro screening strategies should facilitate the selection and development of more effective nicotine conjugate vaccines. In addition, these data highlight a previously under-appreciated contribution of linkers and hapten-protein conjugations to conjugate vaccine immunogenicity by virtue of their inclusion in the epitope that binds and activates B cells.

Graphical Abstract

Keywords

pharmacokinetics; nicotine hapten linkers; vaccine candidates; computational modeling; B cells

INTRODUCTION

Nicotine (Nic) vaccines have been explored as an intervention strategy to improve smoking cessation. The rationale for this therapeutic strategy is based on the production of anti-Nic antibodies that could sequester Nic within the blood circulation and block its entry into the brain, thereby reducing Nic dependence. However, the success of this intervention is dependent on the ability of the vaccine to generate high quality anti-Nic antibody responses that produce high levels of antibodies with high-binding affinity to Nic. To date, the vaccines tested in clinical trials or investigated in preclinical studies have failed to induce sufficient antibody responses to enable their use as adjuncts for smoking cessation.^{1,2}

Given its small size, Nic is not immunogenic on its own. Like many small molecules, the immunogenicity of Nic is realized by making linker-containing Nic haptens (HPs) for their conjugation to a carrier protein. The protein serves as sources of antigenic epitopes for engaging T cells to induce T-cell dependent antibody responses. To improve the immunogenicity of Nic vaccines, various strategies have been attempted, ranging from linker modification, carrier selection, conjugation optimization, adjuvant addition, and multivalent vaccine combination, to nanoparticle packaging.¹ Unlike the conformational rigidity of

cocaine and heroin structures, which are believed to confer good immunogenicity of these molecules, the flexibility of Nic structure has been suggested to account for the poor immunogenicity of Nic HPs.³ To increase the Nic immunogenicity, Janda and co-workers developed several strategies, including design of conformationally constrained Nic analogues, use of multivalent scaffolds to increase HP density, and isolation of enantiopure HPs, aiming to direct Nic epitopes to antigen-specific B cells.⁴⁻⁶ In addition, a comprehensive study conducted by Pryde et al.,⁷ has surveyed various linkers that differ in length, rigidity, and polarity, as well as the position of attachment of Nic to the linkers. In this study, rigid linkers were found to reduce the immunogenicity of Nic diphtheria toxoid conjugates, as well as functional activities of elicited responses, which is consistent with the finding reported by de Villiers et al.⁸ Yet, variations in linker length or polarity appeared to exert minimal effect on the antibody level, affinity, or functionality of the antibody responses.⁷ Thus, it remains elusive as to what features of linker structures confer high quality anti-Nic antibody responses.

Recently, the selection of appropriate Nic HPs has also been coupled to the enumeration of HP-specific naïve B cells present in the host.⁹ Using two different Nic HPs that are conjugated to keyhole limpet hemocyanin (KLH), Laudenbach et al. reported that a higher number of HP-specific naïve (preimmunization) B cells was correlated with a stronger anti-Nic antibody response elicited by that particular HP-KLH conjugate.⁹ Although profiling of HP-specific naïve B cells may facilitate the selection of optimal HPs for B cell engagement, this strategy is not well suited to screening a large number of HPs, and may not serve to select those HPs that favor antibody maturation toward high affinity and high specificity to free Nic.

Instead of examining Nic binding of naïve B cells present in animals, we posited that anti-Nic antibodies with high binding affinities for free Nic may serve as a surrogate for Nic specific B cells, since the Nic-binding specificity of these antibodies and the surface immunoglobulins (Igs) expressed on the B cells are clonally related. This idea was inspired by the success of “vaccine reverse engineering”, first demonstrated in the development of HIV vaccine.¹⁰ The reverse engineering focuses on the interactions between neutralizing monoclonal antibodies (mAbs) and antigens of an interest, which infers the structures of the epitopes that fit into neutralizing mAbs.¹¹ Essentially, neutralizing mAbs were used as a bait to search for and capture mAb-specific “immunogenic” epitopes. Based on this principle, we asked whether high-affinity Nic-binding antibodies could be exploited as a screening bait to search for Nic-like epitopes. Because free Nic itself is not immunogenic, anti-Nic antibodies were actually raised against Nic HPs (Nic moiety plus linker) conjugated to a protein carrier. Emphasis in the past has been placed on designing HPs with nonimmunogenic linker sequences (e.g., using linkers with minimal structural complexity) to minimize the production of antibodies directed against linker rather than Nic. However, while it is the binding of free Nic that is desired for therapeutic purposes, Nic-specific B cells are usually engaged by linker-containing HPs for their activation. We hypothesized that effective HPs would use both their Nic and their linker components to bind and engage B cells while generated antibodies from these B cells could recognize free Nic as well as the complete HP. Thus, according to the reverse engineering principle, by using a known Nic-binding

antibody as a screening bait, we could search for those HPs that can best fit within the Nic-binding site of the antibody.

Anti-Nic antibodies can be distinguished by HP linker positions attached to the Nic moiety, for example, the antibodies raised against Nic HPs linked via 1' position do not bind HPs with linkers attached to different Nic positions, like 3'-aminomethyl-Nic conjugate and 6-(carboxymethylur-eido)-Nic conjugate, respectively.¹² Interestingly, although the antibodies raised against these different HPs that vary in linker attachment positions fail to cross-react among these different families of HPs, they can all bind to free Nic, which highlights the dual-binding features of Nic-specific B cells, i.e., nicotine and linker attachment. Thus, the antibody-based screening strategy could only be applied in HPs with the same linker attachment position as the HP originally used to elicit the screening antibodies.

To test our hypothesis, we focused on new HPs with linkers attached to Nic at the 1' position employing the pyrrolidyl methyl group, since using this position for attachment has been shown to allow generation of high affinity Nic-specific antibodies, and preserves the chirality of Nic as the *S*(-) enantiomer, the main form found in cigarette smoke.¹³ Using 1'Nic HP, that was first reported by Janda,¹⁴ as our prototype, we introduced different degrees of linker modifications and tested their binding activities to both polyclonal and monoclonal anti-Nic antibodies. Our strategy was to screen HPs by measuring their binding to Nic-specific antibodies via competition ELISA, in which the HPs serve as competitors for the binding of antibody to wells coated with 1'Nic HP linked to a protein unrelated to the carrier protein. We recognize the dilemma that the linker would not contribute to altering the pharmacokinetics of free Nic although it affects the binding of the antibody to Nic epitope in the competition ELISA. Nevertheless, we reasoned that linker binding is not entirely irrelevant nor is it necessarily undesirable because it is a determinant of how well an immunogen containing this HP will bind to B cell receptors having specificity similar to that of the screening antibody and activate those B cells.

Besides HP linkers, the amino acids near the HP-protein conjugation site could also interact with conjugated Nic HPs and influence their access to the same anti-Nic antibody, possibly accounting for linker-carrier protein conjugation effect. To address this issue, we applied computational molecular modeling to profile the display of Nic HPs on streptavidin, a carrier protein, by analyzing hapten-streptavidin (HP-SA) interactions near the conjugation site. The goal of this modeling was to estimate the extent to which HPs would interact with adjacent amino acids. Such an interaction may have multiple outcomes. On one hand, it may help orient and constrain the Nic moiety for better engagement with Nic-specific B cells. On the other hand, too much interaction might bury the epitope, limit recognition by Nic-specific B cells, or possibly, create new antigenic epitopes unrelated to Nic. Therefore, we hypothesized that high HP-protein interaction would interfere with the generation of Nic-focused antibody responses. This effect could also be assessed by competition ELISAs to examine and compare the binding activity of the anti-Nic antibody to free HPs vs HP-SA conjugates that contain the same HPs. The combination of computational modeling and competition ELISA of HP-SA conjugates was intended to offer an independent evaluation of the steric accessibility of conjugated HPs to the Nic-binding sites of anti-Nic antibodies.

The predictions derived from these in vitro experimental analyses were tested through immunization of mice and analysis of antibody effects on Nic distribution in serum and brain. Here, we showed a good agreement between the in vitro binding of HP candidates to Nic-specific antibodies and nicotine pharmacokinetics demonstrated in vivo. In addition, our analyses revealed a previously under-appreciated role of linkers and HP-protein conjugations in modulating the access of Nic-conjugate vaccines to Nic-specific B cells and subsequent production of anti-Nic antibody from these B cells. Taken together, computational modeling and competition ELISA approaches offer a simple in vitro prescreening strategy to facilitate the testing of new HPs with linker modifications that may improve Nic vaccine immunogenicity.

RESULTS

Experimental Rationale

We hypothesized that the accessibility of a given HP to its cognate Nic specific B cell clone could be assessed by its binding activity to known anti-Nic antibodies. To test this hypothesis, we took advantage of both polyclonal and monoclonal anti-1'Nic antibodies derived from mice immunized with 1'Nic-KLH conjugates mixed with CpG oligonucleotide (ODN) adjuvant. We conducted a competition ELISA to estimate interactions of various HPs with anti-Nic antibodies. Microplates were coated with HP 1'Nic conjugated to bovine serum albumin (Nic-BSA) to capture anti-1'Nic antibody. Various HPs were added to the antibody prior to their addition to the Nic-BSA coated wells and the extent to which they inhibited antibody binding to the Nic-BSA was measured. In the absence of inhibitors, the antibody titers that were predetermined in a linear range served as a control for 100% binding activity. IC₅₀ value, which is the inhibitory concentration of HPs required to inhibit binding of the antibody to plate-coated Nic-BSA by 50%, were used to estimate the relative binding affinities, with lower the IC₅₀ values indicating higher affinity. To make IC₅₀ comparisons across different microplates, samples, and experiments, free Nic was used as an internal competition control for each microplate. Thus, ratios of IC₅₀ of HPs to IC₅₀ of free nicotine, which is HP-IC₅₀/Nic-IC₅₀, were used to compare the antibody binding activities of HPs among different experiments, as well as between different antibodies (e.g., monoclonal and polyclonal anti-Nic antibodies). From these comparisons, we can estimate the relative binding affinities of the HPs to the anti-1'Nic antibodies, inferring their likelihood of interacting with 1'Nic-specific B cells.

HP Synthesis

In this study, our HP design was focused on the linkers attached to the 1' pyrrolidyl methyl group. Using 1'Nic HP, that was first reported by Janda's group, as our prototype for comparison, we synthesized a series of new Nic HPs (i.e., HP 1–9), which are chemically stable and hydrophobic in nature with lengths of 12–15 Å when fully extended. Given the previous reports on inferior responses of HPs with some rigid linkers,⁷ we wanted to use this feature to test our in vitro screening system to investigate whether HPs with rigid linkers could be selected out by our in vitro assays. In HPs presented here, rigidity of the linker was increased by incorporating different rings like pyrrolidine in HP 4, piperidine in HP 3 and piperazine in HP 5 in the linker region, which were distant from the Nic core, while other

parts of the linker close to the Nic core were kept unchanged. In HPs **1**, **2**, **5**, and **6**, we used various ring systems and reduced alkyl chain length toward the Nic core, while keeping the overall linker length closer to the linker used in HP **1'**Nic. A cyclohexane ring was introduced in the linker with trans and cis conformations in HPs **1** and **2**, respectively, and a cyclopentane ring was introduced into the linker of HP **6**. An aromatic ring (HP **7**) was introduced into the linker to confer more rigidity than the aliphatic ring of other HPs. Additional rigidity was also achieved by incorporating unsaturation in the aliphatic chain of HP **8**. The position of carbonyl group and nitrogen atom of an amide functionality was reversed in HP **9** in the linker region and the length of the linker was longer when compared to other linkers (except **3** and **5**), as shown in Figure 1. The synthesis of HP **2** is illustrated in Scheme 1 and described below. The synthetic routes and characterization of all of the remaining eight Nic HPs (**1**, **3–9**) are available in the Supporting Information.

The syntheses of the nine Nic HPs illustrated in Figure 1 all involved the presence of key intermediate **16**, the synthesis of which is illustrated in Scheme 1. For each of the HPs, a linker was subsequently added, as exemplified for Nic HP **2** in Scheme 1. The synthesis of Nic HP **2** started from commercially available 5-bromonicotinic acid (Scheme 1), which was converted to its ethyl ester **11** as a pale yellow solid in 89% yield. Ethyl 5-bromonicotinate (**11**) underwent base-mediated condensation with *N*-vinylpyrrolidinone to afford an intermediate β -keto-*N*-vinylactam. Acid-catalyzed hydrolysis, decarboxylation and cyclization during basic workup afforded 5-bromomyosmine (**12**) as a yellow solid in 70% yield. Imine **12** was then reduced with sodium borohydride in methanol–acetic acid to afford racemic 5-bromonornic (**13**) as a pale yellow liquid in 90% yield. Racemic **13** was resolved⁷ using α -methoxy- α -(trifluoromethyl)phenylacetic acid (MTPA). Recrystallization gave compounds **14** and **15** as colorless needles in 54% and 51% yields, respectively. Base washing of (*S*)-5-bromonornic (+)-MTPA salt (**15**), followed by catalytic reductive debromination afforded (*S*)-nornicotine (**16**) as a pale yellow liquid in 69% yield. Compound **16** was then alkylated with methyl 4-iodobutanoate affording compound **17** as a pale yellow oil in 78% yield, which upon hydrolysis gave acid **18** as a pale yellow oil (64% yield). Acid **18** was coupled with methyl 4-aminocyclohexanecarboxylic acid by using HBTU. This afforded **19** as a pale yellow liquid in 36% yield which, upon hydrolysis, gave the desired Nic HP **2** as a pale yellow liquid in 52% yield.

Interactions Between Nic HPs and 1'Nic Specific Antibodies

Polyclonal anti-1'Nic antiserum isolated from mice immunized with 1'Nic-KLH conjugates mixed with CpG adjuvant were used to conduct competition ELISA, in which various HPs varying in linker structures were used as competitors to block the antiserum from binding to plate-bound 1'Nic-BSA. In a representative competition analysis with pooled anti-1'Nic antiserum, the HPs displayed a wide range of IC₅₀ binding profiles, some HPs showing much lower IC₅₀ than that of free nicotine whereas others had IC₅₀ values close to or higher than Nic (Figure 2). The ratios of HP-IC₅₀ to Nic-IC₅₀ is summarized in Figure 2B. The ratios close to or larger than 1.0 mean that the antibody binding activity of these HPs is comparable to or worse than the binding of free nicotine to the antiserum, like HPs **1**, **2**, **6**, **7**, and **8**. On the other hand, the lower IC₅₀ ratios represent the linkers that confer higher binding activity of HPs to the antiserum, such as HPs **3**, **4**, and **5**, which appeared to have the

binding activity higher than HP **1'Nic** that serves as our positive control. These new HPs, which have linkers with a ring structure, did not seem to interfere with, but slightly increased their binding activities to the anti-**1'Nic** antibodies. However, we could not rule out the possibility that the increased binding activities observed in HPs **3–5** might have been due to the interactions of these HPs with linker-specific antibodies, which are present in the polyclonal antiserum and unrelated to Nic specific B cell clones.

To identify Nic-specific interactions without possible interference from linker-specific antibodies, we used a monoclonal anti-**1'Nic** antibody, mAb-5F3, as a surrogate for a high affinity **1'Nic**-specific B cell clone. This mAb was derived from mice immunized with **1'Nic**-KLH mixed with CpG and was found to have high binding affinity to free Nic with K_d of 66 nM (Figure S1A), which was estimated by an equilibrium dialysis assay, as previously described.¹⁵ This binding affinity is comparable to the one of Nic-311, another well-characterized monoclonal anti-Nic antibody (Figure S1A). Nic-311 was derived in mice immunized with 3'-aminomethylnicotine conjugated to recombinant *Pseudomonas* exoprotein A¹⁶ and had been shown to reduce brain nicotine levels if used as passive immunization.¹⁷ With the availability of mAb-5F3, we wanted to profile its interactions with our new panel of HPs, thereby getting inference about the HP interactions with the **1'Nic**-specific B cell clone that produces this particular antibody.

Using the same ELISA competition strategy described above (Figure 2), we examined HP binding to mAb-5F3. Surprisingly, the binding profile of mAb-5F3 followed a ranking order (Table 1) similar to the one seen with polyclonal anti-**1'Nic** antiserum (Figure 2), except HPs **1** and **9**. Similar to the finding observed with polyclonal antiserum (Figure 2), HPs **3**, **4**, and **5** have relatively higher binding affinities (i.e., low IC_{50} ratios), and HPs **2**, **6**, **7**, and **8** much lower affinities than that of HP **1'Nic** to the monospecific anti-Nic antibody. This finding suggests that antibody responses after multiple immunizations with **1'Nic**-KLH is either dominated by the mAb-5F3 like B cell clone, and/or clones closely related to mAb-5F3. To support this argument, we made sequence comparison between mAb-5F3 and another monoclonal anti-**1'Nic** antibody (NIC9D9),^{14,18} generated by Janda's group which was also raised specifically to **1'Nic**-KLH.¹⁴ We found high homology between these two mAbs at the variable region of the immunoglobulin heavy- (IgH) and light- chain (IgL). In particular, both clones possess the same germline V_{H1} and $V_{\kappa 1}$, similar D2, and somewhat different J_H and J_{κ} gene segments (Figure S1B). The high degree of sequence similarities at the variable regions of the two independent anti-**1'Nic** mAbs points to a limited repertoire of B cell clones involved in the **1'Nic**-specific B cell responses. Meanwhile, the use of mAb-5F3 in our analysis alleviates the ambiguity of HP binding to linker-specific antibodies, as well as antibodies targeted to linker-protein conjugation sites, which are all present in the polyclonal antiserum raised against Nic conjugate vaccines.

Conjugation of Nic HP to Streptavidin (SA)

We used streptavidin (SA) as a protein carrier for these Nic HPs, owing to the following properties: (i) well-characterized crystal structure and known residues (i.e., K80, K121, K132, and K134) for Nic conjugation on the protein surface; (ii) relatively small monomeric structure and tetrameric forms to make it feasible to control and characterize HP conjugation

efficiency, and to assess the binding valence and activity of HP-SA conjugates to Nic-specific antibodies, (iii) highly immunogenic nature,^{19–21} and (iv) ability to link antigen and CpG adjuvant together as a complex for enhanced immunity due to codelivery of antigen and CpG adjuvant to the secondary lymphoid tissue for an induction of T-cell-dependent B cell responses, for example, by inducing antibody affinity maturation and production of memory B cells.^{22,23} These features help facilitate the design and modification of HP-SA conjugates for enhanced immunogenicity. In our initial analysis, the **1'**Nic-SA-CpG vaccine served as a comparator and elicited good Nic-specific antibody responses at a level comparable to the one induced by the same HP linked to KLH, which is a more commonly used carrier protein (Figure S2). The conjugation of HPs to SA was made via *N*-hydroxysuccinimide (NHS) esters to lysine residues presumably displayed on the surface of SA. The average number of HPs conjugated to monomeric SA ranges from 2.5 to 3.7 (Figure S3), therefore 10–15 per SA tetramer, according to the MALDI analysis of conjugated SA monomer, as summarized in Table 2.

An ideal Nic vaccine should lead to the production of antibodies primarily targeted to free Nic. One criterion for generating such a response is an appropriate display of the Nic moiety on the HP-SA conjugates for their access to Nic-specific B cells. To examine the display of HPs on HP-SA conjugates with different linker structures, we applied both a computational modeling approach to assess the interactions between the HP and its carrier protein, SA, and the competition ELISA to determine the accessibility of the HPs displayed by HP-SA conjugates to mAb-5F3 and compare to one of the free HPs to the same antibody (Table 1).

Computational Modeling of Molecular Interactions Between HPs and Streptavidin

Upon conjugation, the interaction of the HP with neighboring amino acids near the conjugation site of the carrier protein may impact subsequent interactions with Nic-specific B cells, possibly accounting for linker-carrier conjugation effect. Depending on the level of their interactions, they could either increase or decrease the accessibility of the Nic moiety to Nic-specific B cell clones. On the other hand, strong interactions may block the access of the Nic moiety to the B cells or create immunogenic epitopes to induce responses not focused on Nic. On the basis of these considerations, we reasoned that conjugates with strong HP-SA interactions might be associated with poor immunogenicity whereas those with low and moderate interactions might exert little adverse effect to the quality of antibody responses. Given the availability of structural information on SA and various HPs, we conducted computational modeling to estimate the interaction strength between HPs and SA to predict possible linker-carrier conjugation effect. Specifically, we modeled the conformation of different HPs conjugated to SA using a rigid docking algorithm in the AutoDock4 program, as illustrated in Figure S4, and predicted the binding energy of these HPs with SA. The lowest binding energy was calculated at all four lysine positions for each HP (Table S1). The sum of the binding energy predicted for HP-SA interactions of the conjugates was ranked from high to low, as presented from left to right, respectively, on Figure 3. The low binding energy reflects a strong interaction between HP and SA, therefore, exerting high linker-carrier conjugation effect. For example, conjugates **1**, **2**, **6**, **7**, and **8** display lower binding energy than the other conjugates, reflecting their possible strong interactions with SA, and therefore, possibly a high linker-carrier conjugation effect.

associated with these conjugates. Interestingly, among these five HP-SAs, four contain HPs (i.e., HPs **2**, **6**, **7**, and **8**) that have more rigid linkers (Figure 1) and they also demonstrate relatively poor binding activities to mAb-5F3 (Table 1 and Figure 2). Thus, the rigid linkers of these HPs appear not only to block HP accessibilities to **1'**Nic-specific mAb-5F3 and polyclonal antiserum, upon conjugation, they might also confer a strong linker-carrier carrier conjugation effect, which may compromise the immunogenicity of these conjugates for generating Nic-focused responses.

Interactions Between HP-SA Conjugates and Anti-**1'**Nic mAb

To estimate the antibody binding activities of the HPs displayed by the HP-SA conjugates, we conducted the competition ELISA using mAb-5F3 on all the HP-SA conjugates to derive their antibody binding IC_{50} . As compared to the IC_{50} values of their free HPs, the HP-SA conjugates clearly showed reduced IC_{50} among all the conjugates, indicating their increased binding activities to the antibody. The extent of the increases in binding activities was expressed by the ratio of HP- IC_{50} to conjugate- IC_{50} , i.e., HP- IC_{50} /HP-SA- IC_{50} ratios (see the fifth column of Table 1). Two factors could contribute to the enhanced binding activity: (1) avidity increase due to increased Nic valences on the conjugates and (2) affinity increase intrinsic to the HP-SA conjugates. The increased avidity of the conjugate should be approximate to the sum of binding affinity of a free HP to the antibody, or slightly higher, presumably owing to synergized interactions between multivalent HPs on the conjugates and the bivalent IgG antibody. The increased affinity of the HP linked to SA may result from structural changes of the HP and new epitopes created near the HP-SA conjugation sites, which could reduce an elicitation of Nic-focused responses. According to the avidity scenario, the increased binding activity of the HP-SA conjugates determined by the HP- IC_{50} /HP-SA- IC_{50} ratio should match to the HP valence, which is the number of HPs (average 10–15) linked to one SA tetramer, as estimated by MALDI analysis. Such a match was indeed found in conjugates **3**, **4**, and **1'**Nic as their HP- IC_{50} /HP-SA- IC_{50} ratios (i.e., 16–19) were not too different from the number of HPs per SA conjugate (see Table 1 and 2). This finding suggests that the interaction of these conjugates with mAb-5F3 is dominated by HP-antibody interactions intrinsic to the HPs and the carrier protein has little influence over these interactions. The binding ratios for conjugates **5** and **9** are somewhat higher. However, conjugates **1**, **2**, **6**, **7**, and **8** exhibited very high HP- IC_{50} /HP-SA- IC_{50} ratios, ranging from 97 to 662, which is much higher than the estimated valence values and therefore likely resulted from an increase in the antibody binding affinity of the conjugates. Interestingly, all these conjugates showed lower binding energy predicted from the computational modeling (Figure 3), which implies strong interactions between conjugating HPs and the residues near the conjugation site of the carrier protein. This agreement is not unexpected since strong HP-SA interactions are likely to alter the display of HPs on the conjugates, dramatically changing their interactions with Nic-specific antibodies, which, however, may be irrelevant to ultimate binding to free nicotine. On the basis of these two lines of analyses, as well as HP binding to anti-**1'**Nic antibodies, we predicted that conjugates **3**, **4**, **1'**Nic, and **5** could function as good candidates, since they have high binding energy and thereby low HP-SA interactions predicted by computational modeling (Figure 3), low and moderate HP- IC_{50} /HP-SA- IC_{50} ratios (Table 1), as well as low HP- IC_{50} /Nic- IC_{50} ratios that reflect high binding affinities of these HPs to the anti-**1'**Nic antibodies (Figure 2 and Table 1). On the contrary, conjugates

2, 6, 7, and 8 were considered as inferior candidates as they were at the opposite spectrum of the three measurements listed above. To validate this prediction, we tested these conjugates directly in vivo using Nic pharmacokinetic analysis to determine their functional efficacy in blocking the Nic entry into the brain.

Nic Pharmacokinetics Conferred by HP-SA-CpG Vaccines

Upon binding to biotinylated CpG, the HP-SA-CpG complexes were used as Nic vaccines as the complexes render codelivery of antigen and adjuvant for better initiation of T cell dependent antibody responses.²³ Mice were immunized 3 times with HP-SA-CpG complexes on days 1, 21, and 42. Two months after the third immunization, a final injection of HP-SA was given to the mice to further enhance antibody production, in which CpG was not included since adjuvants were found to reduce maintenance of antibody-producing long-lived plasma cells.²⁴ Ten days later, the mice received Nic 0.1 mg/kg s.c., which is approximately equivalent on a mg/kg basis to the Nic absorbed from 7 cigarettes by a smoker. Serum and brain Nic levels were measured 4 min after Nic dosing. In this assay, vaccine efficacy was estimated primarily by the level of Nic present in the brain and compared to the one of the control group. As shown in Figure 4, the mice immunized with vaccines **1, 3, 4, 5, 6, and 9**, as well as **1'Nic**, presented significant reduction in brain Nic levels. The serum Nic levels in these groups were also elevated compared to the control. However, except group 4 that shows statistically significant increase in the serum nicotine levels than the control group, the differences observed in the other groups lack statistical significance, possibly because of high variabilities within each group or reduction of nicotine as a result of excretion or distribution of nicotine to other tissues. Nevertheless, reduction of nicotine accumulation in the brain is a good indicator in evaluating vaccine efficacy. In contrast, mice immunized with vaccines **2, 7, and 8** showed little reduction in brain Nic levels (Figure 4A). This finding indicates that these vaccines failed to induce functional anti-Nic antibody responses, which is also consistent with our prediction based on the in vitro analyses. In particular, we found a positive linear correlation between the average HP-IC₅₀/Nic-IC₅₀ ratios and the average brain Nic concentration in the immunized mice with $R^2 = 0.51$, $p = 0.02$ (Figure 4C). Taken together, this simple screening strategy helps exclude the inferior HP-SA conjugates (i.e., vaccines **2, 7, and 8**), and identify viable candidates.

Immunogenicity of the HP-SA-CpG Conjugates: Anti-Nic Antibody Titers and Their Nic-Binding Affinity (IC₅₀)

The levels of anti-Nic antibodies were measured by ELISA with HP-BSA conjugates as coating antigens, in which the same HP was used in both the vaccination and ELISA-based detection. The Nic binding affinity of the antiserum was estimated by the competition ELISA with free Nic. Variable levels of anti-Nic antibodies were detected in the immunized mice that showed significant changes in brain Nic levels, i.e., those with vaccines **1, 3, 4, 5, 6, 9, and 1'Nic** (Figure 5A). As compared to the group 7 that is a nonresponder, in term of changing nicotine pharmacokinetics (Figure 4B), groups **1, 3, 4, 5, 6, and 8** showed elevated antibody titers although the antibodies made in group **8** had an extremely high IC₅₀ value (>50,000 nM), therefore were rather ineffective. Interestingly, the antibody titers generated from vaccine **1'Nic** were quite variable, and therefore, they showed no statistically

significant difference from the antibody titer in group 7 (Figure 4). The lower immunogenicity of vaccine 1'Nic than the newly designed HPs was observed in several independent experiments; one representative experiment is shown in Supporting Information, Figure S5.

At the other extreme, low antibody titers were detected in the mice immunized with vaccines 2 and 7. This finding is consistent with their Nic pharmacokinetics profiles (Figure 4), indicating the lack of Nic-neutralizing antibodies in these immunized mice. Although the vaccination group 8 showed high antibody titers, the mice failed to change the Nic distribution (Figure 4). The extremely high IC_{50} value ($>50\,000\text{ nM}$) of the antiserum in this group explains the lack of functional efficacy of the generated antibodies, therefore, reflecting the poor quality of the anti-Nic antibody response elicited by vaccine 8. In comparing the immunogenicity and functional activity among all the immunization groups, we did find an inverse correlation between the average of antibody titers and the average of brain Nic concentration per immunization group with $R^2 = 0.55$, $p = 0.014$ (Figure 5C). In addition, the antibody titers of individual immunized mice were also inversely correlated with the brain concentrations in these same mice with $R^2 = 0.31$, $p = 10^{-7}$ (Supporting Information, Figure S6).

DISCUSSION

In this study, by applying two in vitro screening strategies, that is, competition ELISA and computational molecular modeling, we analyzed a group of newly synthesized Nic HPs with different linkers attached to the 1' position of Nic for evaluating their potential as vaccine candidates. The prediction ranked by the ratios of HP IC_{50} /Nic- IC_{50} was supported by the in vivo Nic pharmacokinetic analysis. In particular, we observed a positive correlation between the averages of HP IC_{50} /Nic- IC_{50} ratios in binding to mAb-5F3, and the reduction of nicotine concentration in the brain in various immunization groups ($R^2 = 0.51$, $p = 0.02$, Figure 4C). The strength of this correlation is comparable to that of the inverse correlation between the averages of serum anti-Nic antibody titers elicited by these vaccines in vaccinated mice and their in vivo pharmacokinetic efficacy ($R^2 = 0.55$, $p = 0.014$, Figure 5C). This finding lends support for the in vitro antibody-based profiling as a preimmunization tool in HP selection.

For HP-SA conjugates, given both positive and negative influences possibly exerted by linker-carrier carrier conjugation effect on Nic-specific antibody response, we were not surprised to find the lack of linear correlations between the total binding energy values of HP-SA interactions calculated from the computational modeling and brain Nic concentrations (Figure S7A), and between their antibody binding ratios of HP- IC_{50} /HP-SA- IC_{50} derived from competition ELISA and brain Nic concentrations (Figure S7B). Nevertheless, these two analyses help exclude HP-SA conjugates with strong linker-carrier carrier conjugation effect and offer some insight into the contribution of HP-protein interactions to the immunogenicity of conjugate vaccines. The conjugates 2, 7, and 8 that were predicted as inferior ones by both modeling (high HP-protein interaction) and competition ELISA were indeed poor vaccines. The lack of functional efficacy of these vaccines may be due to their inability to interact with Nic-specific B cells (e.g., conjugates 2

and **7**) or their reactions directed toward non-Nic epitopes, like vaccine **8** that induced a high antibody titer with very low Nic-binding affinity (Figure 5A and B). On the other hand, conjugates that showed low and moderate degrees of HP-SA interactions and exhibited low or moderate antibody-binding ratios of HP-IC₅₀ to HP-SA-IC₅₀ might be more viable candidates.

One exception in our prediction is vaccine **6**, which was predicted to be inferior by both competition ELISA and computational modeling (see Table 1 and Figure 3). This vaccine actually turned out to be effective in inducing the production of Nic-binding antibodies (Figure 5) and reducing Nic distribution to brain (Figure 4). This finding points out that factors other than the parameters measured in this study may also influence the immunogenicity of vaccine **6**. Nevertheless, the functional activity of vaccine **6** may be at a borderline since their serum Nic concentration appeared to be the lowest among all the vaccination groups that demonstrated functional effect in Nic neutralization (Figure 4A). Therefore, the exclusion of Nic-conjugate **6** by our in vitro screenings should not impact the enrichment and selection of good candidates.

For these new newly synthesized HPs, we introduced different degrees of rigidity in the linker. Among them, HPs **7** and **8** are the most rigid. This rigidity may account for their low binding affinities to the anti-**1'**Nic antibodies and strong interactions with carrier protein upon conjugation (i.e., high IC₅₀, see Tables 1 and Figure 2B). It has been reported by several groups that HPs with rigid linkers are poor vaccines.^{7,8} However, many of our good candidates, including HPs **1**, **3**, **4**, **5**, and **9**, possess greater rigidity than the control **1'**Nic. These vaccines induced no less or but possibly better (like vaccine **4**) responses than the one elicited by vaccine **1'**Nic (Figures 4, 5, and S5). In particular, HPs **3–5**, possess a linker structure very similar to the one of HP **1'**Nic, except an inclusion of different ring structures near the conjugation site. This linker modification seems to enhance the binding activities of these HPs to the anti-**1'**Nic antibody over HP **1'**Nic that was used to raise the antibody (Table 1). It is possible that the linker with some degree of rigidity can help orient the Nic epitope for HP interactions with **1'**Nic-specific B cells for elicitation of anti-**1'**Nic antibody responses. Our screening methods also revealed some subtle differences in the impact of linker structures. For example, HPs **1** and **2** differed only in isomeric conformation, trans in **1** but cis in **2**. These two conjugates demonstrated different immunogenicity and functionality in vivo (Figures 4 and 5), which seems correlated with the prediction made by the in vitro screening strategies (Table 1 and Figure 3).

In summary we found that an in vitro reverse engineering approach, using either Nic-specific antiserum or a Nic-specific mAb to screen and rank HPs, correlated well with the in vivo pharmacokinetic efficacy of immunogens containing these HPs. This approach may allow rapid in vitro screening of candidate HPs to identify those most likely to produce good immunogens when conjugated to carriers. A limitation of the study is that it involved only one drug (Nic) and linkers attached to Nic at just one position. Further study of different HPs and linkers will determine whether this screening method can be more widely applied to the development of therapeutic vaccines against other drugs and small chemical compounds.

EXPERIMENTAL PROCEDURES

General Procedures

Reagents and solvents for chemical synthesis were purchased from Aldrich Chemical, Co., or Sigma Chemical, Co., and were used without further purification. All reactions involving air- or moisture-sensitive reagents or intermediates were performed under an argon atmosphere. Analytical TLC was performed using Silicycle silica gel 60 Å F254 plates (0.25 mm), and was visualized by UV irradiation (254 nm). Flash chromatography was performed using Silicycle silica gel (40–60 mesh). Products were concentrated under diminished pressure. ^1H and ^{13}C NMR spectra were obtained using a Varian 400 MHz NMR spectrometer. Chemical shifts are reported in parts per million (ppm, δ) referenced to the residual ^1H of the solvent (CDCl_3 , δ 7.26). ^{13}C NMR spectra were referenced to the residual ^{13}C resonance of the solvent (CDCl_3 , δ 77.16). Splitting patterns are designated as follows: s, singlet; br s, broad singlet; d, doublet; br d, broad doublet; dd, doublet of a doublet; t, triplet; q, quartet; m, multiplet. High resolution mass spectra were obtained at the Arizona State University CLAS High Resolution Mass Spectrometry Laboratory.

HP Synthesis

Ethyl 5-Bromonicotinate (11).⁵—To an ice-cooled solution containing 15.0 g (74.6 mmol) of 5-bromonicotinic acid in 250 mL of ethanol was added dropwise 4 mL of conc. sulfuric acid, and the reaction mixture was heated at reflux under argon for 18 h. Ethanol was removed under diminished pressure, and the resulting white residue was dissolved in 100 mL of water. The aqueous solution was made basic with saturated aq NaHCO_3 and extracted with two 150 mL portions of ether. The combined organic extract was washed with 100 mL of brine, dried over anhydrous MgSO_4 , and concentrated under diminished pressure to afford **11** as a pale yellow solid: yield 15.2 g (89%); ^1H NMR (CDCl_3) δ 1.39 (t, 3H, J = 7.2 Hz), 4.40 (q, 2H, J = 14.8 and 7.6 Hz), 8.40 (t, 1H, J = 2.0 Hz), 8.81 (d, 1H, J = 2.4 Hz), 9.10 (d, 1H, J = 1.6 Hz); ^{13}C NMR (CDCl_3) δ 14.3, 62.0, 120.7, 127.6, 139.5, 149.0, 154.5, 164.1.

3-Bromo-5-(4, 5-dihydro-3H-pyrrol-2-yl)pyridine (12)—Sodium hydride (3.44 g, 86.0 mmol, 60% dispersion in oil) in a three-necked flask was washed with three 20 mL portions of hexane. The flask was fitted with a reflux condenser, flushed with argon, and charged with 70 mL of THF. To this was added a solution of 15.2 g (66.1 mmol) of **11** and 7.89 g (71.0 mmol) of 1-vinyl-2-pyrrolidinone in 15 mL of THF in one portion. The reaction mixture was stirred and heated at reflux for 60 min and then allowed to cool to room temperature. A solution of 12 mL of conc HCl in 18 mL of water was added, and the THF was concentrated under diminished pressure. An additional 18 mL of conc HCl and 36 mL of water were added, and the reaction mixture was heated at reflux overnight. The reaction mixture was cooled to 0 °C and made basic with conc. aq NaOH and the aqueous phase was then extracted with three 75 mL portions of CH_2Cl_2 . The combined organic extract was washed sequentially with 100 mL of water and 75 mL of brine, then dried over anhydrous MgSO_4 , and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 5 cm). Elution with 19:1 CH_2Cl_2 –acetone afforded **12** as a pale yellow solid: yield 10.3 g (70%); ^1H NMR (CDCl_3) δ 2.00–2.08 (m, 2H), 2.86–2.92 (m,

2H), 4.02–4.08 (m, 2H), 8.31 (t, 1H, $J = 1.6$ Hz), 8.67 (d, 1H, $J = 2.0$ Hz), 8.83 (d, 1H, $J = 1.6$ Hz); ^{13}C NMR (CDCl_3) δ 22.6, 34.9, 61.8, 121.0, 131.7, 137.2, 147.1, 152.2, 169.9.

3-Bromo-5-(2-pyrrolidinyl)pyridine (13).⁵—A solution of 2.00 g (8.88 mmol) of **12** in 80 mL of 4:1 methanol–acetic acid was cooled at -40 °C in a dry ice–acetonitrile bath. To this solution was added 747 mg (19.8 mmol) of NaBH_4 portionwise over a period of 10 min with vigorous stirring. During the course of the addition, the temperature rose to -20 °C. The reaction mixture was allowed to warm to ambient temperature; most of the solvent was removed under diminished pressure, and 200 mL of water was added and made basic with aq NaOH. The aqueous layer was extracted with three 90 mL portions of CH_2Cl_2 . The combined organic extract was washed with 100 mL of brine, dried over anhydrous K_2CO_3 , and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (10×4 cm). Elution with 1:1 ethyl acetate–methanol afforded racemic **13** as a yellow oil: yield 1.8 g (90%); ^1H NMR (CDCl_3) δ 1.56–1.66 (m, 1H), 1.81–1.95 (m, 3H), 2.16–2.25 (m, 1H), 3.00–3.06 (m, 1H), 3.11–3.17 (m, 1H), 4.15 (t, 1H, $J = 7.6$ Hz), 7.88 (t, 1H, $J = 1.6$ Hz), 8.46 (d, 1H, $J = 2.0$ Hz), 8.50 (d, 1H, $J = 2.4$ Hz); ^{13}C NMR (CDCl_3) δ 25.6, 34.7, 47.1, 59.3, 120.9, 136.8, 142.9, 146.8, 149.2.

Resolution of Racemic 5-Bromonornicotine—To a stirred solution of 2.33 g (10.3 mmol) of racemic **13** in 16 mL of ethyl acetate was added a solution of 1.20 g (5.15 mmol) of (–)- α -methoxy- α -(trifluoromethyl)phenylacetic acid [(–)-MTPA] in 4 mL of ethyl acetate. The reaction mixture was maintained at room temperature for 15 min, and the formed crystalline product was collected by filtration to give (*R*)-isomer enriched crystals. Three recrystallizations from boiling acetonitrile yielded 1.28 g (54%) of **14** [(*R*)-5-bromonornicotine(–)-MTPA salt] as colorless needles.

The filtrate was extracted with two 15 mL portions of 1 N sulfuric acid. The combined aqueous layer was washed with 20 mL of ether. The aqueous layer was made basic with aq NaOH, and extracted with three 20 mL portions of CH_2Cl_2 . The combined organic extract was dried over anhydrous K_2CO_3 and concentrated under diminished pressure to give an oil enriched in the (*S*)-isomer. The oil was dissolved in 9 mL of ethyl acetate and combined with a solution of 1.20 g (5.15 mmol) of (+)- α -methoxy- α -(trifluoromethyl)phenylacetic acid [(+)-MTPA] in 4 mL of ethyl acetate with stirring. The reaction mixture was maintained at room temperature for 15 min, and the crystallized product was collected by filtration. Three recrystallizations from boiling acetonitrile yielded 1.20 g (51%) of **15** [(*S*)-5-bromonornicotine (–)-MTPA salt] as colorless needles.

(S)-Nornicotine (16).⁵—A suspension of 812 mg (1.76 mmol) of (*S*)-5-bromonornic (+)-MTPA salt in 90 mL of diethyl ether was shaken vigorously with 30 mL of 1 M KOH in a separatory funnel. The organic layer was washed with 15 mL of 1 M KOH, dried over anhydrous K_2CO_3 , and concentrated. The residual oil was dissolved in 30 mL of ethanol, and this solution was treated with 600 μL of Et_3N and 100 mg of 10% Pd/C. Hydrogen gas was bubbled through the reaction mixture for 60 min; then the solution was filtered through a Celite pad, and the pad was washed with 3 mL of ethanol. The filtrate was poured into 90 mL of 1 M aq K_2CO_3 and extracted with two 100 mL portions of CH_2Cl_2 . The combined organic extract was washed with 50 mL of brine, dried over anhydrous K_2CO_3 and concentrated

under diminished pressure. The residue was purified by flash chromatography on a silica gel column (10 × 1 cm). Elution with 7:1 CH₂Cl₂–methanol afforded **16** as a pale yellow oil: yield 179 mg (69%); ¹H NMR (CDCl₃) δ 1.56–1.66 (m, 1H), 1.78–1.91 (m, 2H), 1.99 (br s, 1H), 2.11–2.20 (m, 1H), 2.96–3.02 (m, 1H), 3.11–3.17 (m, 1H), 4.10 (t, 1H, *J* = 7.6 Hz), 7.17–7.20 (m, 1H), 7.64–7.67 (m, 1H), 8.42 (dd, 1H, *J* = 4.8 and 1.6 Hz), 8.54 (d, 1H, *J* = 2.0 Hz); ¹³C NMR (CDCl₃) δ 25.5, 34.4, 47.0, 60.1, 123.4, 134.1, 140.3, 148.3, 148.6. This compound had an optical rotation the same as that reported previously.⁷

(S)-Methyl 4-(2-(pyridin-3-yl)pyrrolidin-1-yl)butanoate (17)—A solution of 227 mg (1.21 mmol) of methyl 4-iodobutanoate in 400 μL of acetonitrile was added to a stirred solution of 150 mg (1.01 mmol) of **16** and 0.53 mL (392 mg; 3.03 mmol) of diisopropylethylamine in 800 μL of acetonitrile at room temperature. After it was stirred for 18 h, the reaction mixture was concentrated under diminished pressure, and the residue was purified directly by flash chromatography on a silica gel column (15 × 2 cm). Elution with 5% methanol in CH₂Cl₂ afforded **17** as a pale yellow oil: yield 196 mg (78%); silica gel TLC *R*_f 0.35 (5% methanol in CH₂Cl₂); ¹H NMR (CDCl₃) δ 1.55–1.62 (m, 1H), 1.63–1.89 (m, 4H), 2.04–2.19 (m, 4H), 2.25–2.35 (m, 1H), 2.38–2.43 (m, 1H), 3.19–3.30 (m, 2H), 3.52 (s, 3H), 7.18 (dd, 1H, *J* = 7.6 and 4.8 Hz), 7.62 (d, 1H, *J* = 8.0 Hz), 8.42 (s, 1H), 8.47 (s, 1H); ¹³C NMR (CDCl₃) δ 22.7, 23.9, 31.7, 35.2, 51.4, 53.1, 53.2, 67.5, 123.5, 134.9, 139.6, 148.5, 149.5, 174.0; mass spectrum (APCI), *m/z* 249.1606 (M + H)⁺ (C₁₄H₂₁N₂O₂ requires *m/z* 249.1603).

(S)-4-(2-(Pyridin-3-yl)pyrrolidin-1-yl)butanoic Acid (18)—A solution of 63 mg (0.25 mmol) of **17** in 1 mL of methanol was combined with 30.4 mg (0.76 mmol) of NaOH in 100 μL of H₂O with stirring at room temperature. After 18 h, the reaction mixture was concentrated under diminished pressure; 2 mL of acetone was added to the residue, and the pH was adjusted to 7 using acetic acid. The acetone was concentrated, and the crude product was purified directly by flash chromatography on a silica gel column (10 × 2 cm). Elution with 1:1.5 methanol–CH₂Cl₂ afforded **18** as a pale yellow oil: yield 38 mg (64%); silica gel TLC *R*_f 0.3 (1:1.5 methanol–CH₂Cl₂); ¹H NMR (CDCl₃) δ 1.70–1.80 (m, 3H), 1.82–2.08 (m, 2H), 2.17–2.29 (m, 2H), 2.30–2.42 (m, 2H), 2.54–2.61 (m, 1H), 3.44–3.51 (m, 3H), 7.32 (d, 1H, *J* = 5.6 Hz), 7.84 (d, 1H, *J* = 8.0 Hz), 8.50 (s, 1H), 8.55 (s, 1H), 11.30 (br s, 1H); ¹³C NMR (CDCl₃) δ 22.4, 23.2, 33.8, 34.3, 53.3, 53.6, 67.7, 124.1, 135.9, 137.7, 148.3, 148.9, 176.6; mass spectrum (APCI), *m/z* 235.1452 (M + H)⁺ (C₁₃H₁₉N₂O₂ requires *m/z* 235.1447).

Methyl 4-(4-((S)-2-(pyridin-3-yl)pyrrolidin-1-yl)-butanamido)cyclohexanecarboxylate (19)—To an ice-cooled, stirred solution of 38.0 mg (0.16 mmol) of **18** in 700 μL of dry DMF was added 67.5 mg (178 μmol) of HBTU. After 10 min, a solution of 37.8 mg (195 μmol) of methyl 4-amino-cyclohexanecarboxylate hydrochloride and 53.6 μL (49.3 mg; 487 μmol) of *N*-methylmorpholine in 400 μL of dry DMF was added to reaction mixture at 0 °C. The reaction mixture was allowed to warm to ambient temperature and was then stirred for 18 h. The reaction mixture was poured into 30 mL of water and extracted with three 20 mL portions of ethyl acetate. The combined organic extract was washed with 25 mL of brine, dried over anhydrous MgSO₄, and concentrated under

diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 1 cm). Elution with 5% methanol–CH₂Cl₂ afforded **19** as a pale yellow oil: yield (22 mg, 36%); silica gel TLC *R_f* 0.31 (5% methanol–CH₂Cl₂); ¹H NMR (CDCl₃) δ 1.39–1.51 (m, 2H), 1.61–1.81 (m, 7H), 1.82–2.02 (m, 4H), 2.03–2.10 (m, 1H), 2.14–2.39 (m, 4H), 2.44–2.52 (m, 2H), 3.32–3.45 (m, 2H), 3.68 (s, 3H), 3.80–3.90 (m, 1H), 5.85 (br s, 1H), 7.25–7.31 (m, 1H), 7.69 (d, 1H, *J* = 6.4 Hz), 8.49 (s, 1H), 8.55 (s, 1H); ¹³C NMR (CDCl₃) δ 22.5, 24.2, 24.9, 29.0, 34.7, 34.9, 39.7, 46.0, 51.6, 53.4, 53.8, 67.8, 123.5, 135.0, 138.7, 148.6, 149.4, 172.2, 175.4; mass spectrum (APCI +), *m/z* 374.2443 (M + H)⁺ (C₂₁H₃₂N₃O₃ requires *m/z* 374.2444).

4-(4-((S)-2-(Pyridin-3-yl)pyrrolidin-1-yl)butanamido)-cyclohexanecarboxylic Acid (2**)**—A solution of 22.0 mg (59.0 μmol) of **19** in 600 μL of methanol was added 11.8 mg (295 μmol) NaOH in 100 μL of H₂O with stirring at room temperature. After 36 h, the reaction mixture was concentrated under diminished pressure; 1 mL of acetone added to the residue, and the pH was adjusted to 7 using acetic acid. The acetone was concentrated, and the crude product was purified directly by flash chromatography on a silica gel column (10 × 1 cm). Elution with 2:3 methanol–CH₂Cl₂ afforded **2** as a pale yellow oil: yield 11 mg (52%); silica gel TLC *R_f* 0.25 (2:3 methanol–CH₂Cl₂); ¹H NMR (CDCl₃) δ 1.32–1.39 (m, 1H), 1.42–1.51 (m, 2H), 1.53–1.72 (m, 4H), 1.74–1.88 (m, 3H), 1.91–2.05 (m, 3H), 2.11–2.29 (m, 5H), 2.40–2.49 (m, 2H), 3.32 (t, 1H, *J* = 8.0 Hz), 3.39–3.42 (m, 1H), 3.79 (t, 1H, *J* = 4.4 Hz), 6.63 (d, 1H, *J* = 8.0 Hz), 7.30 (s, 1H), 7.68 (d, 1H, *J* = 7.6 Hz), 8.46 (s, 1H), 8.75 (s, 1H), 10.97 (br s, 1H); ¹³C NMR (CDCl₃) δ 22.8, 24.0, 25.7, 29.6, 29.8, 33.9, 35.1, 46.4, 53.6, 53.8, 68.1, 123.8, 136.9, 140.2, 146.8, 148.1, 172.2, 179.0; mass spectrum (APCI+), *m/z* 360.2279 (M + H)⁺ (C₂₀H₃₀N₃O₃ requires *m/z* 360.2287).

Preparation of Free Nic Solution

Nic bitartrate (Sigma) was dissolved in phosphate buffered saline (PBS) and the absorbance was measured at 260 nm using a NanoDrop. The Nic extinction coefficient of 2,695 L/(mole·cm) was used to calculate the molar concentration of the solution. Concentrations are expressed as those of the base.

HP Conjugation

To ensure consistency of HP synthesis and HP conjugation to the carrier protein, the effort was made to prepare all HPs within a short period of time (less than 30 days). Moreover, all the newly synthesized HPs were kept in carboxylic acid form at –20 °C to minimize degradation. Right before their conjugation, the HPs were converted into active form, succinimidyl ester for conjugation. For conjugation, HPs were dissolved in anhydrous DMSO at concentrations around 60 mM. Streptavidin (SA, MP Biochemicals, Cat #08623001) and bovine serum albumin (BSA) were dissolved in 0.1 M sodium bicarbonate buffer, pH 8.3, at a concentration of 2 mg/mL, and keyhole limpet hemocyanin (KLH, Sigma Cat # H7017) was reconstituted in PBS at a concentration of 10 mg/mL and diluted in 0.1 M sodium bicarbonate buffer, pH 8.3, at a final concentration of 2 mg/mL. NHS ester activated Nic HPs were added to the carrier protein solution, with the molar ratio of HP: carrier protein at 200:1 for SA, 100:1 for BSA and 1,000:1 for KLH. The HP/carrier protein mixture was then shaken at room temperature overnight. Unreacted Nic HP was removed by

dialysis in 1× PBS with 6,000 Da dialysis membrane (Spectrum Laboratories). The HP conjugation ratio was estimated by SA protein mass change before and after the conjugation reaction (Figure S3), which was analyzed on a MALDI-TOF instrument in the Proteomics Laboratory of Arizona State University.

Immunization

Female BALB/c mice were obtained from Charles River Laboratories and maintained in a pathogen-free animal facility at the Arizona State University Animal Resource Center. All mice were handled in accordance with the Animal Welfare Act and Arizona State University Institutional Animal Care and Use Committee (IACUC). Before experimental treatment, the mice were randomly distributed in cages and allowed to acclimate for at least 1 week prior to vaccination. Each 6–8 week old mouse was immunized s.c. with 5 μ g (100 pmole) HP-SA incubated with 2 μ g (300 pmole) biotinylated CpG ODN 1826 (5′/5biosg/T*C*C* A*T*G* A*C*G* T*T*C* C*T*G* A*C*G* T*T 3′, * indicates phosphothioate backbone modification), or a mixture of 18 μ g Nic-KLH and 2 μ g CpG ODN 1826 (5′ T*C*C* A*T*G* A*C*G* T*T*C* C*T*G* A*C*G* T*T 3′, * indicates phosphothioate backbone modification), at weeks 0, 3, and 6. Blood was collected at various times from facial veins in accordance with the Arizona State University and Minneapolis Medical Research Foundation IACUC guidelines and centrifuged at 2000 × *g* for 10 min to collect serum. Two months after the third immunization, the mice received a final i.p. injection of HP-SA (without CpG adjuvant) to further enhance the antibody levels and 7 days later mouse serum were collected for titer and affinity analysis.

Nic Pharmacokinetics

Ten days after the final challenge with HP-SA, mice were injected s.c. with 0.1 mg/kg Nic (weight of the base) dissolved in PBS. Mice were decapitated 4 min later, and the brain and blood were collected for Nic concentration analysis by gas chromatography.^{8,12} Brain Nic concentrations were corrected for brain blood content.²⁵

Assessment of Antibody Titers and Relative Affinity

Nunc MaxiSorp 96-well plates (Thermo Fisher Scientific) were coated overnight at room temperature with Nic-BSA using the same Nic HP as for HP-SA (Nic-BSA, 1 μ g/mL). The conjugation and purification of Nic-BSA and HP-SA were performed in parallel. The antigen-coated plates were washed and blocked with blocking buffer (1% BSA, 0.1% NaN₃, and 0.25% Tween-20 in PBS) at 37 °C for 1 h and then washed. Mouse serum was serially diluted, loaded onto the plates, incubated at 37 °C for 2 h, and washed. Alkaline phosphatase-conjugated antimouse IgG antibody (Sigma-Aldrich, A1418) was diluted in blocking buffer and incubated on the plate at 37 °C for 1 h, and washed. Afterward, 4-nitrophenol-4-nitrophenyl phosphate disodium salt hexahydrate (Alfa Aesar) was added and allowed to react until the standard mAb-5F3 control produced an optical density at 405 nm (OD₄₀₅) of about 0.6. Then 25 μ L of 0.3 M NaOH was used to stop the enzymatic reaction. The plates were read on an Epoch plate reader (BioTek) at 405 nm. The anti-Nic titer was defined as the dilution of mouse serum at which OD₄₀₅ reached two folds higher than the background wells on the same plate.

Two types of competition ELISA were conducted. The first one was designed to assess HP binding affinity to anti-1'Nic antibodies, in which free HPs or HP-SA conjugates were used as competitors to block antibody (either polyclonal antiserum or monoclonal antibody) from binding to 1'Nic-BSA conjugates coated microplates. The free HPs were prepared at concentrations of 100 μ M and HP-SA conjugates were prepared at concentration of 1 μ M, then serially diluted 1 to 10-fold, to the plates that contain equal volumes of anti-Nic polyclonal antiserum or mAb-5F3 antibody with predetermined titers. The same amount of antiserum or antibody was also included in the absence of inhibitors to serve as a control of O.D₄₀₅ for 100% binding. The IC₅₀ values, defined by the concentration of inhibitors (HPs, HP-SA conjugates, or free Nic) that result in 50% reduction in O.D₄₀₅, were compared among various inhibitors. Each plate contains the analysis of the IC₅₀ of Nic, which serves to normalize IC₅₀ of all the inhibitors and the ratios of HP-IC₅₀/Nic-IC₅₀ were compared across different plates and experiments.

The second competition ELISA on antiserum of various vaccination groups was intended to characterize the relative binding affinity of the antiserum to free Nic. Plates were coated with 1 μ g/mL HP-BSA conjugates for corresponding HP-SA immunized mouse serum. Free Nic was prepared at a concentration of 100 μ M and then serially diluted 10 folds. Diluted free Nic solution premixed with diluted mouse serum at 1:1 volume ratio were added to the plate, where the mouse serums were prediluted based on their titer calculated from ELISA. The same amount of diluted mouse serum was also included in the absence of free Nic to serve as a control of OD₄₀₅ for 100% binding. For serum with very low binding affinity, where IC₅₀ was higher than 50 μ M, an approximation was made and IC₅₀ of 50 μ M were used for statistical analysis.

Statistical Analysis

GraphPad Prism 5.0 was used for statistical analysis. Two-tailed unpaired *t* test with Welch's correction and one-way ANNOVA Tukey's test was used. * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Figures 4A, 4B, 5B: one-way ANNOVA Tukey's test; Figure 5A: two-tailed unpaired *t* test with Welch's correction. We used linear regressions to model the relationships between nicotine concentration in brain and other in vitro or in vivo measurements (Figures 4C, 5C, S6, and S7A and S7B). For each linear regression model, we reported the sample-size-adjusted R^2 value (i.e., the percentage of the variation of brain nicotine concentration explained by a linear model), and the *p*-value of the F test (i.e., the overall significance of the linear model). *p*-Value less than 0.05 was regarded as significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ELISA	enzyme linked immunosorbent assay
HPs	haptens
HP-SA	hapten-streptavidin
Igs	immunoglobulins
mAb	monoclonal antibody
Nic	nicotine
SA	streptavidin

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Figure 1.

Chemical structures of Nic HPs and their linkers. The 1'-Nic structure has been reported previously¹² and served as the comparator for this study. Estimated rigidity based on their chemical structures: $8 > 7 > 6 > 2 = 1 > 4 > 3 > 5 > 9 \gg 1'\text{Nic}$.

Figure 2.

Competition ELISA of polyclonal anti-**1'**Nic antiserum by Nic haptens (HPs) and free Nic (Nic). (A) Representative competition profiles of HPs in blocking the antiserum from binding to the **1'**Nic-BSA coated plates. Diluted serum without any competitor was included as 100% binding control, and various competitors were serially diluted to generate a binding competition curve. The concentration of competitors at which 50% binding was inhibited was designated as IC_{50} . (B) Summary of HP- IC_{50} /Nic- IC_{50} ratios. IC_{50} values for each hapten were normalized to the IC_{50} value of free nicotine on the same ELISA plate, which was included in all the competition assays. The averages of HP- IC_{50} /Nic- IC_{50} ratios with standard deviation were presented.

Figure 3. Molecular modeling of nicotine-streptavidin interactions. The binding energy (kcal/mol) values were predicted using the AutoDock4 docking algorithm for the nicotine HPs at four lysine positions of streptavidin (see Figure S4 and Table S1). The sum of the lowest binding energy at the four positions was ranked from high to low, as displayed from left to right, respectively.

Figure 4.

Pharmacokinetic assay for functional analysis of Nic vaccine. Mice were injected with 0.1 mg/kg free Nic s.c. at 10 days post the final boost, and Nic level in serum (A) and brain (B) were measured with gas chromatography methods as described previously.^{8,12} Statistical differences were analyzed by one-way ANOVA, Dunnett test in Graphpad Prism 6.0. * represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$ comparing to the negative control group. (C) Correlation between HP-IC₅₀/Nic-IC₅₀ ratios and brain nicotine concentrations. The mean brain nicotine concentration was plotted against the mean HP-IC₅₀ ratios both in log scales. A linear regression line was fit to these data showing significant correlation. Black triangle represents the control 1'Nic vaccine. Green dots depict good vaccine candidates, vaccines 3, 4, and 5. Purple dots depict moderate ones, vaccines 1 and 9, and orange dots depict poor candidates, vaccines 2, 6, 7, and 8.

Figure 5.

Antihapten titer (A) and relative binding affinity (B) to free Nic of serum from 7 days post final boost. Antihapten titer was analyzed by ELISA using corresponding hapten-BSA conjugates as coating antigens. Relative Nic binding affinity is represented by IC₅₀ of the antiserum using competition ELISA. Statistical differences were analyzed by two-tailed unpaired *t* test with Welch's correction (A) and one-way ANOVA Tukey's test (B) in Graphpad Prism 5.0. * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Asterisks in (A) represents statistical differences compared to group 7 (nonresponder); Asterisks in (B) represents statistical differences compared to group 9. # indicates that all IC₅₀ values in group 8 are above the detection limit. (C) Functional efficacy of nicotine HP-SA conjugate vaccines with nicotine pharmacokinetics. The immunogenicity of each vaccine was tested in vivo with 8 mice per group. The mean nicotine concentration in brain was plotted against the mean anti-Nic antiserum (in reverse order), both in log scale. A linear regression line was fit to these data showing significant correlation. See Figure 4C for the symbols denoting all the samples.

Scheme 1.
Synthetic Route for Nic HP 2

Table 1

Binding Activities of Monoclonal Anti-1'Nic Antibody to Free HPs and HP-SA Conjugates

hapten	HP-IC ₅₀ (nM) ^a	HP-IC ₅₀ /Nic-IC ₅₀ ^b	HP-SA-IC ₅₀ (nM) ^c	HP-IC ₅₀ /HP-SA-IC ₅₀ ^d
free Nic	1412 ± 250	1.0		
3	8.6 ± 3.9	0.006	0.56 ± 0.06	15
5	9.1 ± 5.7	0.006	0.17 ± 0.08	54
4	10 ± 3.5	0.007	0.53 ± 0.15	19
1'Nic	23.2 ± 7.2	0.016	1.37 ± 0.17	17
1	174 ± 39	0.12	1.38 ± 0.96	129
9	379 ± 56	0.27	6.89 ± 2.50	55
2	802 ± 75	0.57	1.21 ± 0.50	662
6	2,191 ± 660	1.6	6.70 ± 1.10	327
7	5,973 ± 2,095	4.2	61.47 ± 1.20	97
8	60,490 ± 17,161	42.8	164.15 ± 4.95	368

^aIC₅₀ derived from antibody binding to free HPs.^bIC₅₀ ratio of HP-IC₅₀ to IC₅₀ of free nicotine on the same ELISA plate.^cIC₅₀ derived from antibody binding to HP-SA conjugates.^dIC₅₀ ratio of HP-IC₅₀ to IC₅₀ of HP-SA conjugates.

Table 2

HP-SA Conjugation Efficiency Quantified by MALDI

HP-SA	no. per tetramer ^a
1	12
2	11
3	12
4	15
5	10
6	15
7	12
8	11
9	10
1'Nic	11

^aNumber of conjugated HPs per SA tetramer was estimated by mass changes in Figure S3.