Published in final edited form as:

Chem Biol Drug Des. 2014 September ; 84(3): 354–363. doi:10.1111/cbdd.12326.

Hapten Optimization for Cocaine Vaccine with Improved Cocaine Recognition

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Abstract

In the absence of any effective pharmacotherapy for cocaine addiction, immunotherapy is being actively pursued as a therapeutic intervention. While several different cocaine haptens have been explored to develop anti-cocaine antibodies, none of the hapten was successfully designed which had a protonated tropane nitrogen as is found in native cocaine under physiological conditions, including the succinyl norcocaine (SNC) hapten that has been tested in phase II clinical trials. Herein, we discuss three different cocaine haptens: hexyl-norcocaine (HNC), bromoacetamido butyl- norcocaine (BNC), and succinyl-butyl- norcocaine (SBNC), each with a tertiary nitrogen structure mimicking that of native cocaine which could optimize the specificity of anti-cocaine antibodies for better cocaine recognition. Mice immunized with these haptens conjugated to immunogenic proteins produced high titer anti-cocaine antibodies. However, during chemical conjugation of HNC and BNC haptens to carrier proteins, the 2 β methyl ester group is hydrolyzed and immunizing mice with these conjugate vaccines in mice produced antibodies that bound both cocaine and the inactive benzoylecgonine metabolite. While in the case of the SBNC conjugate vaccine hydrolysis of the methyl ester did not appear to occur, leading to antibodies with high specificity to cocaine over BE. Though we observed similar specificity with a SNC hapten, the striking difference is that SBNC carries a positive charge on the tropane nitrogen atom, and therefore it is expected to have better binding of cocaine. The 50% cocaine inhibitory concentration (IC₅₀) value for SBNC antibodies (2.8 μ M) was significantly better than the SNC antibodies (9.4 μ M) when respective hapten-BSA was used as a substrate. In addition, antibodies from both sera had no inhibitory effect from BE. In contrast to BNC and HNC, the SBNC conjugate was also found to be highly stable without any noticeable hydrolysis for several months at 4°C and 2-3 days in pH 10 buffer at 37°C.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

Keywords

Cocaine; benzoylecgonine; substance abuse; addiction; TA-CD vaccine; vaccine; hapten; carrier protein; outer membrane protein complex; mice; antibody; specificity

INTRODUCTION

Cocaine abuse and addiction continues to be a health threat and creates socioeconomic problems throughout the world (1). Conventional pharmacological treatment strategies that aim to target the drug sites of action using different small molecule agonists and antagonists have not produced any effective treatment options for cocaine addicts thus far (2, 3). In response to these limitations, immunotherapy has been actively pursued as a different treatment strategy that targets the cocaine molecule directly, aiming to keep the target drug below its minimum effective concentration (MEC) at its sites of action (3). Since the site of pharmacological effect against cocaine is in the pleasure circuit inside the brain, it was hypothesized that antibodies against cocaine would sequester much of the cocaine in the peripheral circulation before it cross the blood brain barrier (4). Since the small cocaine molecule is not itself immunogenic, anti-cocaine antibodies were produced using conjugate vaccines in which a cocaine hapten is chemically linked to an immunogenic carrier protein and used to induce the host immune system to produce anti-cocaine antibodies (4, 5).

In 1994, the development of a cocaine conjugate vaccine, TA-CD, was started with support from the National Institute of Drug Abuse.(4) The vaccine was produced by covalently linking a cocaine derivative, succinyl norcocaine (SNC), to an immunogenic carrier protein, cholera toxin B subunit, and combining the conjugate with the human adjuvant alum. Using this vaccine, Phase I and Phase II clinical trials were completed and showed a proportion of vaccinated subjects produced clinically relevant quantities of anti-cocaine antibodies without any significant side effects (6-8). These antibodies were characterized as being highly specific to the pharmacologically active cocaine molecule without any interaction with BE (9, 10). We estimated that to be successful, the vaccine needed to generate concentrations of anti-cocaine antibodies exceeding 280 nM in order to block peak plasma cocaine concentrations of 500 nM, a concentration previously observed following typical recreational cocaine use (9, 11, 12). In the Phase IIA study, most but not all of the patients who attained that level of anti-cocaine antibody had more cocaine free urines when these high levels of antibody were present in circulation (8). However, only one third of the patients produced the levels of anti-cocaine antibody required for this effect. Thus, there is an unmet need to reengineer the existing vaccine formulation for increased immunogenicity and improved cocaine binding in order to treat cocaine addiction using immunotherapy.

In designing a cocaine hapten, both the *N*-methyl and 2 β methyl ester group of cocaine (see Figure 1) have been widely explored as positions for a linker that would attach the hapten to the carrier protein (4, 13, 14). The SNC hapten that was taken for human clinical trials was synthesized by reacting succinyl anhydride with norcocaine leading to an amide bond at the tropane nitrogen and a carboxylic acid group, which could be used to attach the hapten to the cholera toxin B used for the vaccine. This hapten preserved the integrity of the methyl ester

group of cocaine, so that the vaccine would bind cocaine preferentially, compared to benzoylecgonine (BE). This hypothesis was strongly supported by the unique specificity observed in vaccinated patients and rodent models as antibodies against BE were not observed (4, 10). Other haptens have been designed where the hapten was attached to the 2 β carboxylic acid group of cocaine that produced anti-cocaine antibodies with excellent behavioral data in rodents (13, 14). However, the interaction of antibodies with BE was not established. With BE as a major metabolite of cocaine(15), the production of such antibodies capable of binding both BE and cocaine would significantly reduce the clinical benefit(10) by having binding sites in the available antibodies bound by BE that would otherwise be able to bind the active drug.

Cocaine is a weak base with a pK_a of 8.6. Under physiological conditions, the tropane nitrogen atom accepts a proton and forms a positive charge, which facilitates the hydrolysis of labile ester group converting cocaine to BE (see Figure 1), e.g., in phosphate buffer at pH 7.4 (16, 17). This does not happen when the tropane nitrogen has an amide form as in SNC (18). However, despite the ability of the SNC hapten to produce antibodies specific to cocaine, it lacks the protonated nitrogen that exists under physiological conditions. In this study we aimed to design a novel cocaine hapten with protonated nitrogen in order to modulate vaccine potency to give enhanced cocaine-antibody recognition, and we also show the importance of retaining the 2 β methyl ester group during the hapten synthesis in developing immunotherapy for cocaine addiction.

METHODS AND MATERIALS

The chemicals used in the synthesis of the haptens were obtained from the Sigma-Aldrich Company, St Louis MO. The chemicals for the conjugation of the haptens to the proteins were obtained from Thermo-Fisher, Rockford, IL. Cocaine, norcocaine, and benzoylecgonine were obtained from the National Institute on Drug Abuse, Baltimore MD. BSA-SNC conjugates were prepared using the method described by Hrafnkelsdottir *et al* 2005 (19). The purity of the compounds was checked by thin layer chromatography and NMR. Monoclonal antibodies (mAb) mAb08, mAb09, and mAb11 were all purchased from US-Biologicals, location. The specificity of these antibodies to cocaine and BE was characterized earlier in our previous studies using highly sensitive isothermal titration calorimetry (10) and the percentage cross reactivity for each mAb was provided in the Certificate of Analysis received from the vendor. Antibody mAb08 recognizes both BE and cocaine, whereas mAb09 and mAb11 recognize BE with 1 and 2% cross reactivity rates to cocaine, respectively.

Synthesis of Succinyl Norcocaine (SNC)

A solution of 382 mg (1.3 mmol) norcocaine 264 mg (2.6 mmol) succinic anhydride and 0.5 mL diisopropylethylamine, or Hünigs base (DIPEA) in 10 mL of CH₂Cl₂ (DCM) was heated at reflux overnight. The resulting solution was washed with 1 M HCl and sat. NaCl, and dried over anhydrous. Na₂SO₄. The solvent was removed under vacuum and the resulting solid product was purified by column chromatography using 10% methanol in DCM and identified by NMR.

Synthesis of Bromoacetamido Butyl-norcocaine (BNC)

A mixture of 336 mg (1.3 mmol) norcocaine, 385 mg (1.3 mmol) (BOC)amino butyl bromide and 450 mg NaHCO_3 (4 meq.) in 10 mL toluene was heated at 80° C overnight with stirring. The toluene was removed under vacuum. The product was dissolved in ethyl acetate, washed with water and saturated NaCl and purified by column chromatography using 25% hexane in ethyl acetate to give 432 mg of (BOC)aminobutyl norcocaine (72% yield). The product was stirred overnight in 15 mL DCM and 1.5 mL trifluoroacetic acid, washed with sat. NaHCO_3 and sat. NaCl. Removal of the solvent gave 330 mg of aminobutyl norcocaine. To an ice-cooled solution of 130 mg (0.36 mmol) of this product in 1 mL DCM and 185 μL DIPEA (3 eq.) was added 30 μL (57 mg) bromoacetyl chloride, and stirring was continued at room temperature overnight. The solution was washed with sat. NaHCO_3 and sat. NaCl and the solvent removed under vacuum. Purification by column chromatography gave 130 mg of product, which was identified by NMR.

Synthesis of Succinyl Butyl Norcocaine (SBNC)

Aminobutyl norcocaine was synthesized as above and succinylated as for SNC.

Synthesis of carboxypentyl norcocaine (HNC)

The benzyl ester of 6-bromohexanoic acid was produced by the standard reaction between 6-bromohexanoyl chloride and benzyl alcohol. This ester was reacted with norcocaine as described above for (BOC)amino butyl norcocaine followed by standard hydrogenation to remove the benzyl alcohol group.

Conjugation of Haptens to Proteins

The conjugation of SNC to BSA was used as an example of the stoichiometry for the conjugation of the carboxylic acid functionalized haptens to the immunogenic proteins. A solution of 10 mg (0.00015 mmol) of BSA in 800 μL of phosphate buffered saline pH7.4 (PBS) and a solution of 11 mg SNC (0.03 mmol, 200 eq to BSA) in 10 μL dimethyl formamide (DMF) were prepared. To 200 μL PBS was added 10 mg sulfo-N-hydroxysuccinimide (sulfoNHS, 1.5 eq to SNC), and 8 mg N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, 1,1 eq to SNC) followed by the SNC solution. The pH was adjusted to ~6.0 if necessary, and stirring was continued for 1 hr. This solution was added to the BSA solution, the pH was adjusted to 7.4-7.6 and stirring was continued overnight. The conjugate solution was passed over a size-exclusion NAP-10 column (GE Healthcare, Piscataway NJ) and the final solution (1.5 mL, 6.67 mg/mL BSA) was sterile filtered through a 0.45 μm Millex syringe filter (Millipore, Billireca, MA).

In order to conjugate haptens functionalized with a bromoacetate group, the protein was first treated with SATA according to the manufacturer's directions to create free -SH groups, followed by application of a DMF solution of a hapten containing a bromoacetamide functionality (such as butyl bromoacetamide norcocaine, BNC). After overnight stirring, the conjugate solution was passed over a NAP column and filtered as above.

Using the same methods as above, conjugate vaccines were prepared using ovalbumin (OVA), keyhole limpet hemocyanin (KLH) and the outer membrane protein complex from *N. meningitidis* (OMPC) as the immunogenic carrier proteins.

Immunization with Hapten-conjugate vaccine

BALB/c male mice, 4–6 week old (breeding pairs originally obtained from Jackson Laboratory, Bar Harbor, ME) were bred and housed under pathogen-free conditions and were used at 7–8 week of age. The mice were immunized by intramuscular (IM) injection with a vaccine consisting of 50 µg of conjugate vaccine in 100 µl PBS mixed with 750 µg of alum. Three weeks later they received a booster injection with the same vaccine with alum as adjuvant. The same tissue site was used for both the prime and boost vaccine administrations. Blood was collected at weeks 2, 4, 6, 8, 12, 16 after initial immunization and allowed to clot at room temperature, and then centrifuged at 10,000 g for 20 minutes. The resulting serum was stored at –80°C. All animal studies were conducted under protocols reviewed and approved by Baylor's Animal Care and Use Committee.

Enzyme-linked Immunosorbent assay (ELISA)

To measure specific anti-cocaine antibody, ELISA plates (Immulon 2HB, Daigger, Vernon Hills, IL) were coated overnight in carbonate buffer (pH 9.5) using cocaine hapten conjugates prepared with a heterologous carrier protein (bovine serum albumin, BSA). The experiments related to the stability of different cocaine conjugates, the coating was also performed in PBS buffer (pH 7.4). Background antibody binding to the carrier alone (which was very low in most samples) was subtracted from every sample to ensure that the results reflect antibodies specific for the cocaine hapten. In order to quantify the hapten specific antibody, a standard curve of non-specific purified mouse IgG was included in each ELISA plate. Samples were added to plates in two or three fold serial dilutions starting at 1:1,000 in PBS–Tween (1%) and incubated for 3 hours. After washing with PBS–Tween, goat anti-mouse IgG conjugated to horseradish peroxidase (MP Biomedicals, Irvine, CA) was then incubated in the plates for 60 minutes. The plates were again washed and then substrate (tetramethylbenzidine, Sigma, St. Louis, MO) was incubated in the plates for 30 minutes. Reactions were stopped with 75 µl of 1M HCl and the optical density (OD) was measured at 450 nm on a microplate reader (TELAC, Research Triangle Park, NC). Background for the ELISA with buffers only was ~0.1 OD in all assays. OD readings in the linear range of the standard curve were used to calculate the concentration of antibody from appropriate dilutions.

Competitive Inhibition Assay

In order to measure the 50% inhibitory concentration (IC_{50}) with cocaine and BE, competitive inhibition assays were performed with the pooled sera at dilutions that fell well within the acceptable linear range of ELISA measurements to different hapten-BSA conjugates. The antibodies were preincubated for 30 minutes with a range of concentrations of cocaine or BE. Inhibition was evaluated by comparing the OD produced in the ELISA when drugs were added to the ELISA to the OD produced when the same dilution was tested

in wells with added PBS. The IC_{50} values were calculated with a 3 parameter nonlinear curve fitting analysis using Graphpad Prism version 5.

RESULTS

Hapten conjugate vaccine induces high level of anti-cocaine antibody with different immunogenic carriers

As observed in the past and in on going human clinical trials with various nicotine and cocaine vaccines, vaccinated patients produced nicotine or cocaine specific antibodies (8, 20). However, only moderate levels of antibodies were attained and thus there is a clear need to reengineer the existing vaccine formulations to increase immunogenicity, binding affinity, and the proportion of patients who respond well. Traditionally, in preclinical studies using rodents BSA, OVA and KLH-conjugates with complete or incomplete Freund's adjuvant were used, and in humans, *cholera* toxin B subunit vaccine mixed with alum was used.

Here we have tested OMPC from *Neisseria meningitidis* along with other carrier proteins. Since the availability of OMPC was limited, some vaccine constructs were made only with BNC and SNC haptens, and tested with other traditional constructs in BALB/c mice with a primary immunization and then a booster vaccination at week 3. The immune response was studied using the sera collected from the vaccinated mice at different time points (Fig. 2). Figure 2 A, B, C, D show the ELISA titer values of anti-sera IgG antibody binding to a BSA-BNC target and Figure 2 E, F, G, H show the anti-sera IgG antibody binding to a fish gelatin-SNC target. The background binding, which is the nonspecific IgG binding to the carrier target protein BSA (Fig. 2A-D) or fish gelatin (Fig 2 E-H) showed low ELISA absorbance background values in the range of 0.1-0.2 at 450 nm. While all the conjugate vaccines produced anti-cocaine antibodies, the OMPC conjugates produced substantially higher antibody titer values than other conjugates. It also maintained high antibody titers from 4-12 weeks before slowly declining.

With KLH, OVA and BSA conjugates, moderate levels of specific antibody titers were observed from week 4 onwards increasing to peak levels at week 8 and then declining from 8 to 12 weeks. We have also compared the immune response of the cholera toxin conjugate (TA-CD) to other carrier constructs. The antibody titers with CTB conjugates were comparable to BSA conjugates and higher than ovalbumin conjugates; however, the OMPC construct maintained high titer values compared to all other conjugates in our studies.

The stability of different cocaine conjugates and their binding to representative mAb's

Further ELISA experiments were performed with the constructs made with the different haptens. Using the distinct specificities of mAb binding (mAb08 antibody binds to both cocaine and BE, while mAb09 and mAb11 antibodies bind very preferentially to benzoylecognine over cocaine), we explored the binding characteristics of the different cocaine hapten conjugates by ELISA. The differences in mAb binding specificity can be explained by suggesting that the mAb08 antibody binding pocket does not require the methyl group from the 2 β methyl ester of cocaine and hence it binds to both cocaine and BE. However, for the other two mAbs mAb09 and mAb11, it requires carboxyl group as present

in BE and the presence of the methyl ester group of cocaine clearly hinders antibody binding activity. As shown in Fig 3, all the protein conjugated cocaine haptens (e.g. SNC, SBNC, BNC, HNC) showed high ELISA activity to mAb08 indicating the presence of cocaine or BE hapten on the conjugated carrier protein. Furthermore, the BNC and HNC conjugated haptens showed high activity to mAb09 as well as mAb11, indicating that the methyl ester group of these haptens was at least partially or completely hydrolyzed. In order to check the effect of pH induced hydrolysis, the conjugates were coated on the ELISA plate using PBS pH 7.4 (regular storage buffer) and bicarbonate pH 9.5 buffer (regular ELISA buffer) and found no significant change in the binding activity under these conditions. We also observed similar results even with the fresh conjugates suggesting that hydrolysis occurred during the conjugation with a carrier protein (PBS pH 7.4 at 25°C). Interestingly however, the SNC and SBNC conjugated haptens do not show any binding activity to mAb09. We also attempted to do a forced hydrolysis of SBNC conjugates by increasing pH to 8.5 in bicarbonate buffer or incubation at 37°C for 48 hrs at pH 10 in borate buffer and found no evidence of hydrolysis. These data suggests that the conjugates made using BNC and HNC haptens lost their 2 β methyl ester group (hydrolyzed conjugates) during chemical synthesis over conjugates prepared from SNC and SBNC haptens (nonhydrolyzed conjugates).

ELISA titers of sera obtained from mice immunized with hydrolyzed (BNC, HNC) and nonhydrolyzed (SNC, SBNC) conjugates and antibody interactions with cocaine and BE

The hapten-protein conjugates that lost the critical 2 β methyl ester group by hydrolysis (BNC, HNC) and the conjugates that retained the 2 β methyl ester group (SNC, SBNC) protein conjugates were used to immunize BALB/c mice. To characterize the specificity of anti-drug antibodies, drug conjugates were prepared by coupling haptens to a heterologous carrier protein (e.g., BSA) as binding targets for ELISA. Figure 4 shows high titer values for sera obtained from OMPC and KLH conjugates. In addition, sera from mice immunized with either KLH-BNC or OMPC-HNC showed a predominant binding activity to the hydrolyzed target BSA-BNC and very little activity to the nonhydrolyzed targets (BSA-SNC, BSA-SBNC). However, sera from nonhydrolyzed antigens such as KLH-SNC, OVA-SBNC did not show significant binding to hydrolyzed targets, but showed relatively higher binding to nonhydrolyzed targets. Though KLH-SNC sera showed high binding to its own target, its binding to another nonhydrolyzed target, BSA-SBNC is significantly lower. This is likely due to differences in the antibody binding pockets. Especially since SNC lacks the positive charge on the nitrogen, the antibodies produced with this hapten shows a moderate titer values to BSA-SBNC target, which carries a protonated positive charge on the nitrogen. However, OVA-SBNC sera shows nearly identical titers to both nonhydrolyzed targets.

We have also studied the specificity of different antibody binding to cocaine and BE by incubating the diluted sera with increasing concentrations of drug and looked at the competitive ELISA binding activity to both nonhydrolyzed BSA-SNC (Fig 5A and 5C) and hydrolyzed BSA-BNC (Fig 5B and 5D) targets. As shown in Figures 5A and 5B a complete inhibition of cocaine binding and no interaction to BE was observed with the antisera obtained from mice immunized with nonhydrolyzed cocaine conjugates (OVA-SNC, OVA-SBNC). Clearly the IC₅₀ values for cocaine with these antisera is much better for the nonhydrolyzed target. In addition, the lack of positive charge on the BSA-SNC target also

worsens antibody binding, as reflected by an increase in the cocaine IC₅₀ value of OVA-BSNC antibody binding to 48.2 μ M compared to the 2.8 μ M binding to the host BSA-BSNC target (Table I). However, the absence of the 2 β methyl ester group on the hydrolyzed target significantly increases the IC₅₀ values of both OVA-SNC and OVA-BSNC antibodies. In the case of antisera obtained from mice immunized with hydrolyzed cocaine conjugates (KLH-BNC, OMPC-HNC), antibodies clearly interact with both cocaine and BE with nearly equal IC₅₀ values in the range of 3-7 μ M to the hydrolysed BSA-BNC target (Figures 5C and 5D). The presence of 2 β methyl ester group of BSA-SNC and cocaine significantly increases the IC₅₀ to 70-161 μ M and also to lesser extent with BE (IC₅₀ 10-14 μ M).

DISCUSSION

Substance abuse drugs are molecules that are too small to elicit immune recognition on their own. To mobilize the B-lymphocytes to generate an antibody response to the target drug (e.g., cocaine, methamphetamine), the drug molecules must be chemically modified and covalently linked to an immunogenic carrier protein. In 1974, Bonese et al. demonstrated the feasibility of this approach by producing anti-morphine antibodies in rhesus monkey (21). However, subsequent research efforts were focused only in the mid 90's, mostly on cocaine addiction. (4, 5, 13) The principle reason behind this focus is the lack of therapeutic options and also the rapid metabolism of cocaine to inactive benzoylecgonine and ecgonine. As a consequence of the extensive metabolism of cocaine to inactive, but longer lived, derivatives such as BE, the specificity of the therapeutic antibodies should be oriented towards the recognition of the parent molecule. More than 80% of cocaine is metabolized to inactive, non-toxic compounds (benzoylecgonine, ecgonine methyl ester and ecgonine) (15) which should not be recognised by the ideal therapeutic anti-cocaine antibody, since such recognition would reduce the effective concentration of the therapeutic binding sites for the active drug. Hence it is imperative to develop haptens that mimic the *in vivo* chemical structure of cocaine. In addition to this it is also critical to understand the *in vivo* stability of hapten conjugates and how they are recognized by the immune system.

As shown in Figure 1, in addition to the existing SNC haptens, we prepared three alternative cocaine haptens and screened them for anti-cocaine antibodies with improved cocaine recognition. This was accomplished by direct ELISA binding assays, utilizing the distinct specificities of commercially available monoclonal antibodies (mAb08, mAb09, mAb11). Since these commercial mAb's were developed for forensic analysis to detect the presence of benzoylecgonine, all of them showed high recognition of BE. Recently, using isothermal titration calorimetric analysis we found that mAb08 showed higher affinity to cocaine (K_D = 2.5 nM) than BE (K_D = 19 nM)(10), than mAb09 and mAb11 which showed selectivity towards BE with extremely low cross reactivity to cocaine, confirming the Certificate of Analysis provided by the manufacturer. Among the new haptens studied here, both BNC and HNC BSA conjugates were bound by all the mAb's studied (Fig. 3). This suggests that at least a portion of the protein-conjugated BNC and HNC haptens had lost the critical 2 β methyl ester group by hydrolysis. Furthermore, vaccination with these constructs produced antibodies that bound to both cocaine and BE (Fig 5, Table 1). Interestingly, we also found that similar hydrolysis was not observed in SNC and SBNC protein conjugates as evidenced by high binding titers to mAb08 and very low binding titers to mAb09 and mAb11 (Fig. 3).

In addition, those constructs produced antibodies specific to cocaine as supported by the competitive ELISA inhibition assay (Fig 5, Table 1). The IC_{50} value for SBNC-OVA antibodies was 2.8 μ M when BSA-SBNC was used as a substrate, and for OVA-SNC antibodies was 9.4 μ M when BSA-SNC was used as a substrate. Furthermore, when these antibodies were tested with the nonhydrolyzed SNC target, anti-SBNC-IgG does not bind well with BSA-SNC. However, anti-SBNC-IgG and anti-SNC-IgG do bind well to the BSA-SBNC target. This shows that the antibodies produced from SNC and SBNC immunization are different and the positive charge on the SBNC hapten does produce antibodies with better affinity to cocaine than SNC hapten. In addition, the competitive interaction of these antibodies to the hydrolyzed BSA-BNC clearly increases the IC_{50} values significantly, which is due the absence of critical 2 β methyl ester group in BSA-BNC target. Furthermore, in the case of the hydrolyzed conjugates KLH-BNC and OMPC-HNC produced high affinity antibodies, but it binds equally well to cocaine and BE. As expected the presence of 2 β methyl ester group in non-hydrolyzed BSA-SNC target significantly increases the IC_{50} values in comparison to the BSA-BNC target (Table 1).

Even though the hapten structures of BSNC and BNC closely resemble each other, the vaccine constructs prepared from these haptens produced different anti-cocaine specificities, due to the loss of 2 β methyl ester group during the synthesis of BNC. Both BNC and BSNC haptens were prepared from aminobutyl norcocaine. In an attempt to increase the hapten density during the synthesis of BNC vaccine constructs, we introduced several free thiol (–SH) groups into the carrier proteins using N-succinimidyl S-acetylthioacetate (SATA) followed by deacetylation with hydroxylamine before covalently linking butyl bromoacetamide norcocaine, BNC. As expected the nucleophilic thiolated groups on the protein efficiently cross-linked with BNC leaving HBr. However, the excess free nucleophilic –SH groups from the carrier likely hydrolyzed the critical 2 β methyl ester group. The strong immune response and specificity of anti-sera to both cocaine and BE strongly support this hypothesis. The carbodiimide chemistry has been used successfully for covalently linking the hapten to the side chain amine groups present in the protein (8, 14, 22). In this case the hapten, usually with COOH group, acts as a nucleophile and forms a stable active-ester, which reacts to the free amine groups present in the protein.

In order to test the stability of SBNC conjugates, the ELISA binding activity of BSA-SBNC conjugates were tested after incubating in different conditions (37°C, basic pH 10 for 2-3 days) and the conjugates were found to be highly stable without any noticeable change in the binding activity to mAb09 and mAb11 (Fig 3). Recently Cai et al 2013 also addressed the stability issues in developing cocaine vaccines (23). The authors studied the stability issues by labeling their hapten with a chromophore. They incubated the labeled hapten in rat sera at different time points and extracted with methanol/acetic acid mixture and tracked the hydrolysis by measuring absorbance of the chromophore at 325 nm using HPLC. Incidentally the authors prepared a hapten similar to our HNC hapten, but with a butyl linker and found that hapten stability is less than 16 hrs in presence of mouse sera. This is also consistent with our observation on HNC hapten, and notably upon vaccination the rodents produced antibodies which bound both to cocaine and BE (Fig. 5).

In addition to hapten design changes, to increase the immunogenicity of cocaine vaccine, we have tested different carrier proteins such as BSA, OVA, KLH and OMPC. We found that OMPC and KLH conjugates produced higher titer antibodies than BSA, and OVA conjugates; however, OMPC constructs produced antibodies for longer duration than other carriers. This effect of OMPC has been observed with other conjugated antigens, and is thought to be due to the structure and composition of the complex, which forms a liposomal complex with porins and a small amount of retained lipopolysaccharide (24). This combination results in a large release of cytokines in the microenvironment of the vaccine (25), resulting in markedly enhanced T helper cell activation (26) to elicit high level antibody responses.

Besides the importance of hapten design and immunogenicity of carrier proteins, the production of antibodies from cocaine vaccination relies on multiple other factors. While the antibody titer values are used here to compare the immunogenicity of different constructs and for hapten optimization, the antibody titer values need to be converted to stoichiometric quantities (e.g. $\mu\text{g/ml}$) of cocaine binding antibodies and understand its binding kinetic parameters to cocaine. Currently, this issue has been widely addressed using equilibrium dialysis experiments with radioactive cocaine (23, 27, 28), however, these experiments still need to be validated. Since cocaine is a weak base with pK_a of 8.6, it converts to BE even in regular phosphate buffer and its half-life in normal plasma is only 154 min (29). This raises some ambiguity in the data obtained from ED assays, especially in dealing with anti-cocaine antibodies which might also bind to BE, which may be produced during the 6-8 hour or longer equilibration period of antibody and radiolabelled cocaine. These quantification challenges are not only present in the preclinical studies, but also in the clinical trials with large variation between the ELISA quantification and clinical outcome (8). Although vaccine development challenges remain for cocaine vaccines as demonstrated in this paper regarding cocaine hydrolysis effects, the potential of vaccines to treat abusers of cocaine and other substance abusers who are motivated to escape their addiction needs to be realized by additional research efforts.

Acknowledgments

We would like to thank Yan Wu and Bangyi Mao for expert technical assistance. This work was supported by the National Institute on Drug Abuse (NIDA) grants R21 DA035591 and R01 DA030338, the Michael E. DeBakey Veterans Affairs Medical Center Research Program, and the Department of Veterans Affairs Merit Review Program. Materials for this work were provided by the NIDA Drug Supply Program and OMPC from Merck Research Laboratories (West Point, PA).

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Figure 1. Schematic structures of cocaine, benzoylecgonine and different cocaine haptens
 SNC: Succinyl norcocaine; SBNC: Succinyl butyl norcocaine; BNC: Butyl bromo acetamide norcocaine; HNC: hexyl norcocaine. During the conjugation of haptens to the proteins the highlighted (square) functional groups such as –OH and –Br in the haptens were functionalized to the –NH₂ and –SH groups of proteins, respectively.

Figure 2. Hapten conjugate vaccine induces high titer anti-cocaine antibody with different immunogenic carrier

BALB/c mice were immunized i.p. with 100 μ g of BNC-carrier conjugate (A, B, C, D) or SNC-carrier conjugates (E,F,G,H) in alum and were given booster injection with the same dose in incomplete Freund's adjuvant on 21st day after immunization. The immune response was tested with the sera collected at different time lines after immunization using BNC conjugated to BSA (A, B, C, D) and SNC conjugated to fish gelatin (E, F, G, H) targets. Among the different carriers we have tested, the conjugates prepared with OMPC from *Neisseria meningitidis* carrier provided a robust immune response when combined with

alum. (OMPC: Outer membrane protein complex, KLH: Keyhole limpet hemocyanin, CTB: *cholera* toxin B subunit; OVA: ovalbumin; BSA: Bovine serum albumin).

Figure 3. ELISA binding of representative mAb's to different cocaine conjugates and its stability mAb08 binds to both cocaine and BE, whereas mAb09 and mAb11 binds only to BE. Both BSA-SNC and OVA-SBNC targets binds specifically to mAb08, but not to mAb09 and mAb11 indicating that the haptens on both conjugates represent cocaine and not BE. Furthermore, the methyl ester group of SBNC conjugate is stable without any noticeable hydrolysis (6 months at 8°C in PBS pH 7.4) even at basic pH 10 for 2-3 days. However, BSA-BNC and BSA-HNC conjugates get at least partially hydrolyzed immediately (PBS pH 7.4 at 8°C) with high binding to all three mAb's. In addition, the other two haptens BSA-BNC and BSA-HNC clearly bind to mAb08, mAb09 and mAb11 indicating that the methyl ester group of hapten was also hydrolyzed. The conjugate that was coated with PBS was also tested in bicarbonate pH 9.5 buffer and found no significant change in its binding activity.

Figure 4. ELISA titers of diluted mAbs and sera from immunized mice with hydrolyzed (BNC, HNC) and nonhydrolyzed (SNC, SBNC) conjugates

While mAb09 and mAb11 (specific to BE) binds only to the hydrolyzed target, mAb08 (specific to cocaine and BE) clearly binds to both hydrolyzed and nonhydrolyzed conjugates. Sera from hydrolyzed vaccine constructs (OMPC-HNC, KLH-BNC) specifically binds to the hydrolyzed conjugate indicating that it produced BE binding antibodies. Sera from nonhydrolyzed vaccine constructs (KLH-SNC, OVA-SBNC) specifically binds to the nonhydrolyzed conjugate indicating that it produced antibodies specific to cocaine.

Figure 5. Inhibition of ELISA activity with cocaine or BE to different targets

Non-hydrolyzed BSA-SNC (Panel A, C) and hydrolyzed BSA-BNC (Panel B, C) were coated in the ELISA plates. Diluted antisera from mice immunized with non-hydrolyzed (OVA-SNC, OVA-SBNC) and hydrolyzed (OMPC-HNC, KLH-BNC) conjugates were incubated with varying concentrations of cocaine (solid symbols) and BE (open symbols) and its competitive inhibition was studied to the non-hydrolyzed BSA-SNC (Panel A, C) and hydrolyzed BSA-BNC (Panel B, C). Similar studies were also performed with non-hydrolyzed BSA-SBNC target but not shown in the figure. The IC_{50} values were calculated from the individual data points with a three parameter nonlinear curve fitting analysis using Graphpad Prism version 5. All the data are summarized in Table 1. The data demonstrates that the non-hydrolyzed conjugates produced IgG, which are specific to cocaine and not to BE. Whereas the hydrolyzed conjugates produced IgG which binds to both cocaine and BE.

Table 1
Inhibitory constant (IC₅₀) values of antisera binding to cocaine and BE in presence of different targets

BSA-SNC and BSA-SBNC targets are the non-hydrolyzed target, which retains the 2 β methyl ester group, and BSA-BNC is the hydrolyzed target.

Hapten	Carrier	BSA-SNC target		BSA-BNC target		BSA-SBNC target	
		Cocaine (μ M)	BE (μ M)	Cocaine (μ M)	BE (μ M)	Cocaine (μ M)	BE (μ M)
SNC	OVA	9.4	N/I	85	N/I	9.2	N/I
SBNC	OVA	48.2	N/I	57.01	N/I	2.8	N/I
BNC	KLH	66.9	10.1	5.6	4.4	N/D	N/D
HNC	OMPC	161.3	14.2	3.1	2.5	N/D	N/D

N/I: No inhibition, N/D: Experiment was not done