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Modulating Cocaine Vaccine Potency through Hapten Fluorination

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Supporting Information

ABSTRACT: Cocaine addiction is a long-lasting relapsing illness characterized by cycles of abuse, abstinence, and reinstatement, and antibody-based therapies could be a powerful therapeutic approach. Herein, we explored the possibility of using halogenated cocaine haptens to enhance the immunological properties of anti-cocaine vaccines. Three fluorine-containing cocaine haptens (GNF, GNCF and GN5F) and one chlorine-containing cocaine hapten (GNCl) were designed and synthesized, based upon the chemical scaffold of the only hapten that has reached clinical trials, succinyl norcocaine (SNC). Hapten GNF was found to retain potent cocaine affinity, and also elicit antibodies in a higher concentration than the parent structure SNC. Our data suggests that not only could strategic hapten fluorination be useful for improving upon the current cocaine vaccine undergoing clinical trials, but it may also be a valuable new approach, with application to any of the vaccines being developed for the treatment of drugs of abuse.

Cocaine abuse and addiction remains a major medical and public health problem.[1] To date, no pharmacotherapies have been approved for the treatment of cocaine dependence. However, a number of direct/indirect agonists and antagonists aimed to modulate or disrupt the drug's effect at its site of action have been investigated, but these have achieved very limited success.[2] In view of these limitations, interest has turned to strategies that target the drug molecule itself, aiming to keep the drug below its effective concentration at its site of action. One such tactic using this approach is termed active vaccination, wherein a cocaine vaccine is used to elicit antibodies (Abs) for drug neutralization.[2,3] We view active vaccine design to be dependent upon three basic elements: a drug-like hapten, immunogenic carrier and adjuvant.[3,4] During the past two decades, a small but intensive effort has been devoted to exploring cocaine-like haptens to produce cocaine-specific antibodies; yet only one cocaine vaccine, termed TA-CD, which consists of succinyl norcocaine (SNC, Figure 1) coupled to a recombinant cholera toxin B subunit using an aluminum hydroxide gel as adjuvant, has reached clinical trials.[5] Furthermore, this vaccine has offered limited therapeutic efficacy for cocaine abstinence as a result of the high subject-to-subject variability in antibody titers among participants. Thus, there is an unmet need to engineer vaccine formulations with improved immunogenicity that will validate vaccination as a therapeutic strategy to treat cocaine abuse and addiction.

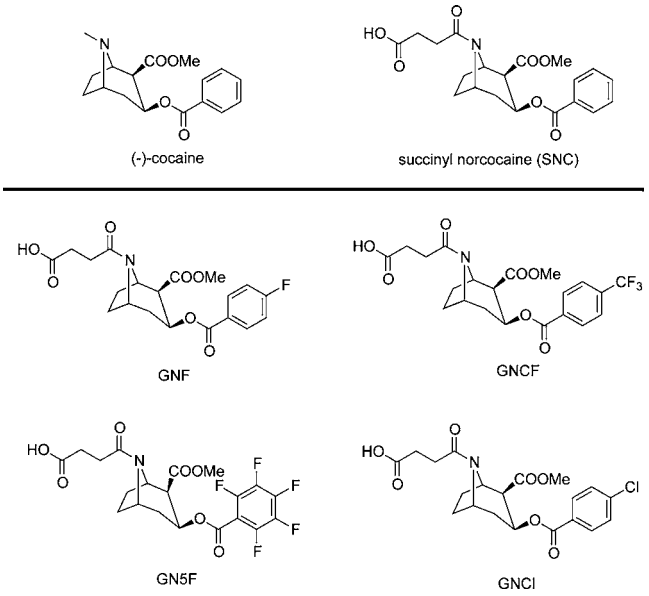

Figure 1. Structures of cocaine, hapten SNC and halogen-containing cocaine haptens.

Fluorine substitution is an established tool in bioorganic and medicinal chemistry due to the unique properties of fluorine, such as the comparable size of fluorine to hydrogen, the superhydrophobicity of fluorocarbons, and fluorine's unique inductive effect and "polar hydrophobicity".[6] Incorporation of fluorine atoms or fluorine-containing substituents is often used to enhance ligand-binding affinity, and has recently been found to enhance immune recognition.[7] Indeed, immune response in part is based on the T cell receptor (TCR) recognition of antigenic molecules bound and presented by the major histocompatibility complex (MHC). A weak interaction of TCRs with antigen-MHC may fail to evoke a significant immune response. There is increasing evidence that rationally modified antigens can boost TCR binding and thereby overcome the poor antigenicity of native antigens. Antigen-fluorination has become one means to enhance TCR affinity without significantly perturbing the composition or structure of the antigen.[8] Thus, various laboratories have prepared fluorine-modified carbohydrates, peptides or glycopeptides, and in some cases, these variants showed significant improvement in the immunogenicity of the vaccine.[9]

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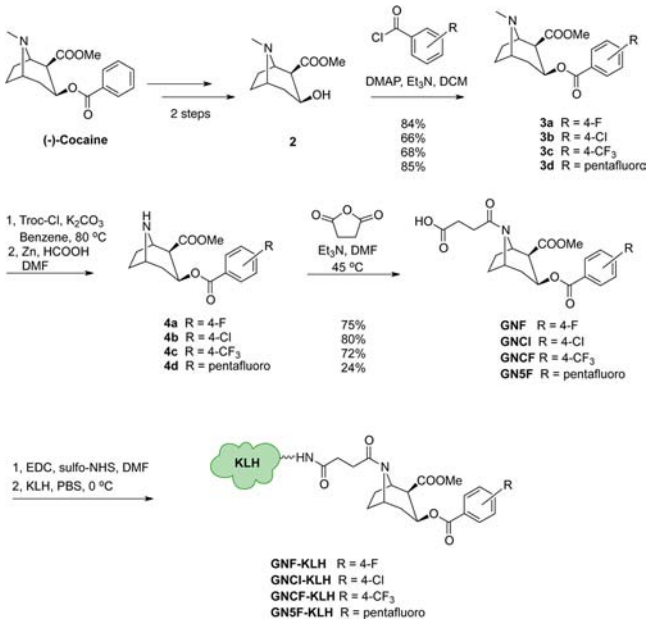
An Achilles' heel seen with vaccines against drugs of abuse has been poor immunogenicity. Herein, we detail using cocaine as a drug of abuse platform scaffold to show how hapten-halogenation can be implemented as a new tool for modulating vaccine potency. The succinyl norcocaine hapten was chosen as a starting structure to examine this hypothesis, as modification on this structure could be carefully controlled and immunological consequences readily accounted for. Three fluorine-containing homologues of SNC termed GNF, GNCF and GN5F were judiciously chosen and synthesized (Figure 1), in which the anticipated benzoyl ester dominant epitope was substituted with fluorine(s) or a trifluoromethyl group. Last, we also prepared a chlorine-containing cocaine hapten, GNCl, to probe the importance of the halogen atom itself.

The synthesis of the halogenated cocaine haptens is illustrated in Scheme 1. SNC was synthesized using a similar

Scheme 1. Synthesis of Halogenated Cocaine Haptens



strategy as reported,[10] while the synthesis of each halogenated hapten commenced with ecgonine methyl ester **2**, which was prepared from (−)-cocaine hydrochloride in two steps.[11] Benzoylation of **2** was achieved by the use of halogenated benzoyl chloride, Et_3N and DMAP in dichloromethane, furnishing compounds **3a**−**3d** in moderate to high yields. The demethylation of each of these tropane alkaloids compounds was accomplished by forming a carbamate intermediate before treatment with zinc dust, providing norcocaine derivative **4a**−**4d**. Finally, the target compounds GNF, GNCl, GNCF and GN5F were obtained through N-acylation of amine **4a**−**4d** with succinic anhydride in the presence of Et_3N in DMF. To generate the immunoconjugates for vaccination, the obtained haptens GNF, GNCl, GNCF and GN5F as well as SNC were coupled to the carrier protein keyhole limpet hemocyanin (KLH) under standard protein conjugation conditions.[12] Prior to administration, the resultant protein conjugates were formulated with SAS (Sigma Adjuvant System), a stable oil-in-water emulsion derived from bacterial and mycobacterial cell walls. Finally, each hapten was conjugated to bovine serum albumin (BSA) for ELISA microtiter plate coating and monitoring of the coupling efficiency using MALDI-TOF MS. Importantly, no significant difference in hapten coupling efficiency was observed (see Table S1).

The efficacy of SNC-KLH, GNF-KLH, GNCl-KLH, GNCF-KLH and GN5F-KLH immunoconjugates was assessed by vaccination of groups of $n = 5$ Swiss Webster mice. Test groups included a KLH negative control and the five cocaine hapten-KLH conjugates. Each unique vaccine was administered on days 0, 21, and 42 with bleeds taken one week post-vaccination. Success of active vaccination is contingent upon both the magnitude of the response as well as the affinity and specificity of the antibodies generated.[12] The magnitude of the immune response was initially assessed by ELISA on microtiter plates coated with SNC-BSA conjugate. While higher titers to each individual hapten could have been observed through examination of each hapten-BSA conjugate, we opted to use SNC-BSA as "generic" hapten for all vaccines tested, as we felt this recourse would provide a quick metric for assessing cocaine-antibody fidelity.

The KLH control group showed no significant titer to SNC, and thus, it was discarded and not investigated further. As seen in Figure 2, the titers of the second bleed increased significantly

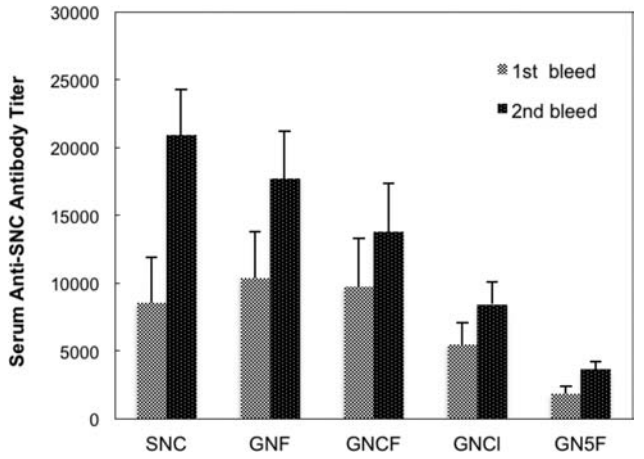


Figure 2. Total SNC-specific antibody titers from the sera of the first and second bleeds of mice immunized with SNC-KLH, GNF-KLH, GNCF-KLH, GNCl-KLH and GN5F-KLH conjugates, respectively. Each column represents the average antibody titer in the sera of five replicate mice.

compared to that of the first bleed. As expected, the titers elicited by SNC-KLH were significant; however, GNF-KLH also induced a strong titer to SNC-BSA. Further, analysis of this ELISA data presented us with additional observations that were not readily predictable. Thus, fluorine appears to be a "guiding" immune modulator as GNF and GNCF provided excellent titers. GN5F titers were greatly diminished, and we suspect this was due to hydrolytic lability of the pentafluorobenzoyl ester. Interestingly, titers induced by GNCl were vastly reduced in comparison to GNF. While steric effects could play a role (r_w = 1.35 Å for fluorine versus r_w = 1.75 Å for chlorine),[13a] this appears not to be the central tenet controlling titer magnitude as the titer induced by GNCF (r_w = 2.20 Å)[13b] was not altered dramatically. Furthermore, DFT calculations provided no evidence that the confirmation of SNC was perturbed by any of these halogen substitutions (see Figure S2). Intriguingly, this lack of GNCl immunogenicity is in contrast to a recent publication presenting sialylated tumor-associated carbohydrate

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antigens containing unnatural phenylacetyl derivatives where a *p*-chlorophenyl immunogen was found to be the most promising candidate.[9a]

To obtain a more accurate account of each vaccine's ability to bind cocaine, we conducted a soluble radioimmunoassay (RIA), which was then normalized thus allowing for direct comparison of values between test groups. As shown in Table 1, polyclonal

Table 1. Average Affinity of Antisera from Immunized Mice against Cocaine As Determined by Equilibrium Dialysis[a]

hapten	K_d (mean ± SD, nM)	[Ab] (mean ± SD, μg/mL)
SNC	5.81 ± 0.48	148.47 ± 3.61
GNF	**7.97 ± 0.22**	**181.53 ± 6.95**
GNCF	9.86 ± 0.074	107.31 ± 14.69
GNCl	101.91 ± 12.97	75.86 ± 1.06

[a]All assays were performed in triplicate. SD, standard deviation.

Abs elicited by SNC, GNF and GNCF displayed comparable affinity to cocaine between 5.8 and 9.8 nM. Unanticipated, vaccination with GNCl produced Abs with a rather poor cocaine-binding affinity (~102 nM). To begin to rationalize these findings, we note that GNF and GN5F haptens contain fluorine, the most electronegative atom among our tested compounds, followed by the GNCF hapten possessing the trifluoromethyl group and finally GNCl possessing chlorine, which has the least electron-withdrawing effect.[14] These results, while thought-provoking, do not establish a complete physicochemical basis for the binding affinity of the induced Abs. However, the correlation seen between the electronegativity of the substituents and the antibody binding affinity suggests that the electronic properties of the 4′-substituent on the benzoyl ring could be one critical determinant for affinity seen with these unique cocaine vaccines. Last, for hapten GN5F, the elicited antibodies were found to have no binding affinity for cocaine. Again, we assume this was due to hapten instability.

The benefits of active vaccination upon a drug's pharmacodynamics should be judged by not only antibody affinity, but also antibody concentration. Cocaine-specific IgG concentration can also be determined from RIA analysis, as shown in Table 1.[15] Excitingly, the highest cocaine-specific IgG concentration in sera was observed with Abs induced by GNF, which was found to be approximately 181 μg/mL. It is noteworthy that this is an increase of about 20% in antibody concentration compared with the clinical hapten SNC. Again, GNCl-KLH demonstrated the lowest immunogenicity among these four viable immunoconjugates; the antibody concentration in sera was about 60% lower than GNF. Remarkably, fluorination at the 4′-position of the benzoyl ring of SNC was able to not only maintain cocaine affinity, but also improve upon antibody concentration to cocaine. This latter finding is extremely pertinent, as documented active immunization regimens for abused substances have elicited poor or variable immune stimulation, and it is this failure to generate therapeutic antibody concentrations that has thwarted the approval of anti-drug vaccines for pharmacotherapy. The fact that monofluorination of hapten SNC provided equivalent binding affinity, yet superior antibody concentrations, supports the idea of using strategically fluorinated haptens for the development of cocaine vaccines with robust immunogenicity. In this regard, fluorination at alternative positions, either on the aromatic ring or the aliphatic scaffold of cocaine structure, or multiple-fluorination upon cocaine's scaffold is worthy of further investigation.

In summary, we have synthesized a series of halogenated cocaine haptens, including GNF, GNCF, GN5F and GNCl, as well as their KLH conjugates. The immunological properties of the synthetic immunoconjugates as vaccines were evaluated and compared to the SNC hapten undergoing clinical trials. Monosubstitution with fluorine at the 4′-position on the benzoyl ring of SNC resulted in a novel hapten GNF, which was found to elicit antibody concentrations to cocaine greater than the parent structure SNC and still retain potent cocaine affinity. TCR recognition of fluorinated antigenic peptides has been shown as a way to selectively modulate TCR binding affinity. Whether such a basis of immune recognition is operative for these fluorinated haptens will require additional research to elucidate hapten-immune molecular recognition. However, what is clear is that fluorine's unique properties[6] provide a promising new hapten design strategy for the development of active vaccines against all drugs of abuse.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic protocols, assay methods, additional biological assays and characterization of new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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