Efficient Syntheses of Cocaine Vaccines and their In Vivo Evaluation

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Efficient Syntheses of Cocaine Vaccines and their *In Vivo* Evaluation

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ABSTRACT: Though cocaine abuse and addiction continue to have serious implications for health and society, no FDA-approved interventions have been developed. Anticocaine conjugate vaccines offer an attractive opportunity for addiction treatment; however, vaccines have thus far failed in clinical trials. As a result, anticocaine vaccines must be further optimized to achieve clinical translation. Herein, we report a study on the relationship between vaccine efficacy and hapten stability towards hydrolysis. Two haptens developed by our laboratory, GND and GNE, were conjugated to tetanus toxoid (TT) and formulated with alum and cytosine-guanine oligodeoxynucleotide 1826 (CpG ODN 1826) adjuvants, the optimal formulation in anticocaine vaccine design. GND, a diamide, is more hydrolytically stable than GNE, a monoamide, towards butyrylcholinesterases. Ultimately, both vaccines induced antibodies with high affinity for cocaine. In hyperlocomotion testing, GND-TT and GNE-TT vaccinated mice exhibited robust blockade of cocaine's stimulatory effects at all tested doses. Overall, antibodies raised against both haptens were highly effective in protecting mice from cocaine-induced psychostimulation.

Cocaine abuse and addiction remain grave health and societal problems in the United States, with 900,000 individuals meeting the criteria for substance use disorder and nearly 11,000 overdose deaths in 2015.[1] The Drug Abuse Warning Network (DAWN) reported that approximately one in three drug-related visits to emergency departments were attributed to cocaine in 2011.[2] Moreover, according to the National Survey on Drug Use and Health (NSDUH), cocaine use in the United States has remained comparatively high, with an estimated 1.5 million past-month users in 2014.[3] Despite the high prevalence of cocaine use, no FDA-approved therapeutic for treating cocaine addiction has been achieved. Current treatment methods are limited to the alleviation of withdrawal symptoms.[4,5]

In view of the limited treatment options, interest has turned towards antibody-based pharmacokinetic (PK) strategies, which modulate receptor signaling by binding the drug in circulation, preventing its passage across the blood-brain-barrier (BBB).[6,7] In this strategy, administration of a protein-drug immunoconjugate prompts the immune system to generate highly specific antibodies that minimize free drug concentrations at their site of action.[8] While this targeted PK approach has been effective in many preclinical studies, only a single anticocaine vaccine, termed TA-CD, has reached clinical trials. These trials failed in phase III due to inconsistent vaccine efficacy among trial participants.[8-12] As a result, next generation anticocaine vaccines need to be optimized in hapten, adjuvant and carrier protein formulation to induce broadly efficacious and highly specific anticocaine antibodies in wide-ranging patient populations.

In the course of anticocaine vaccine development in the Janda Laboratory, we disclosed that hydrolytic stability of the cocaine hapten is a crucial determinant for vaccine efficacy.[13,14] Cocaine (**1**) itself has a half-life ($t_{1/2}$) of 1 – 2.5 h in humans
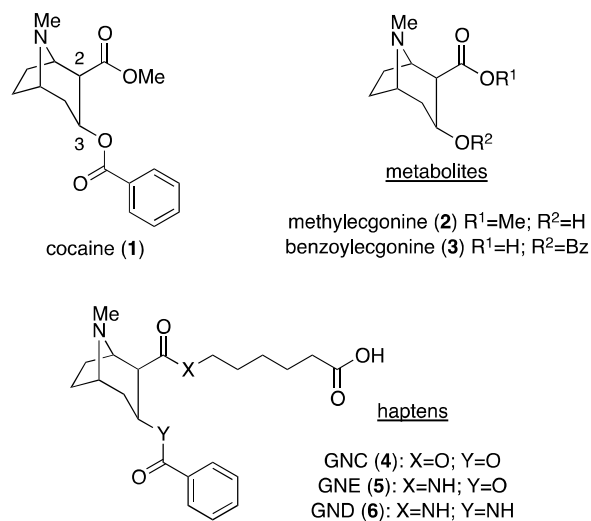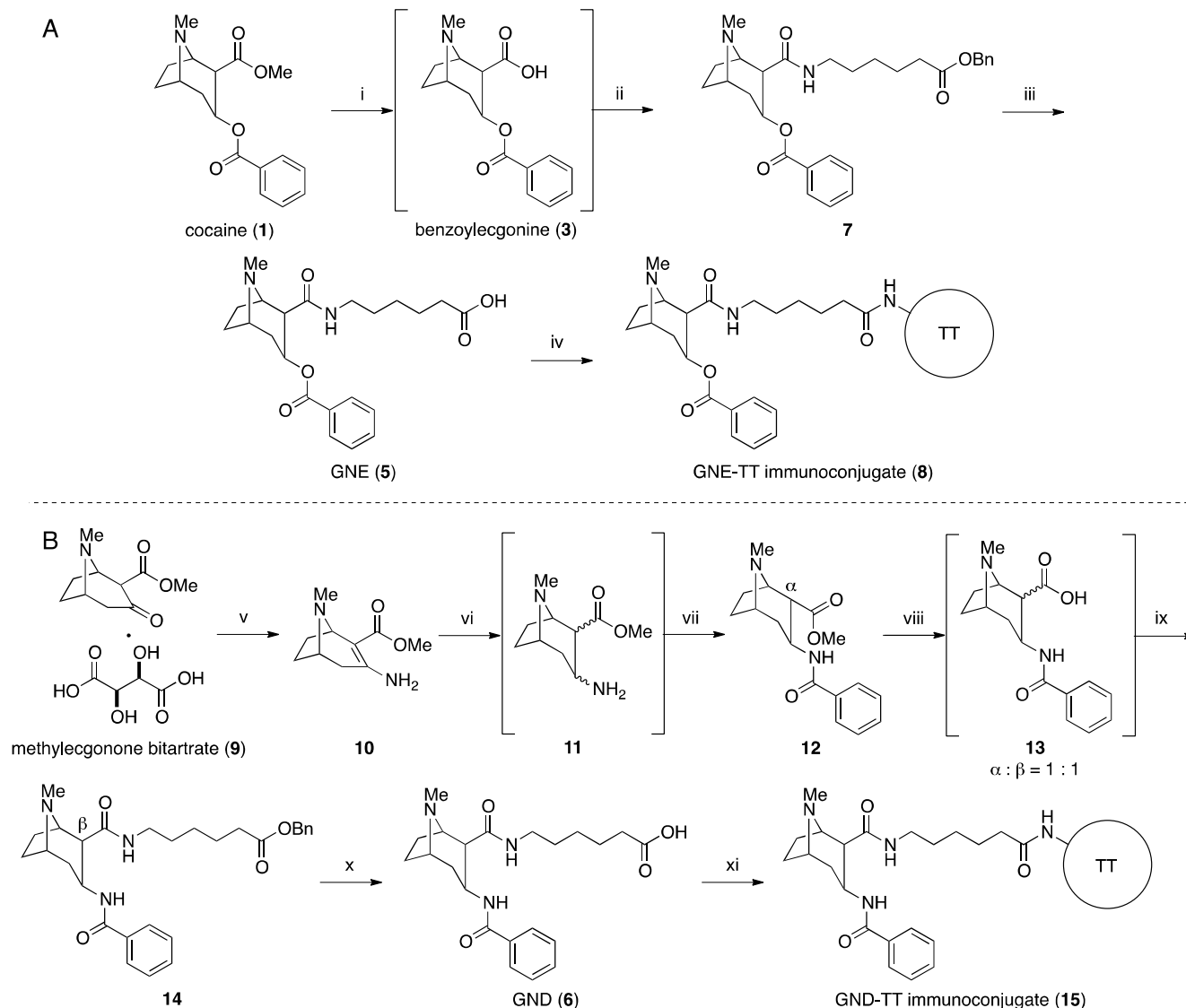

Figure 1. Chemical structures of cocaine, cocaine metabolites, and haptens previously developed in the Janda Laboratory.

with the most prevalent metabolites, methylecgonine (**2**) and benzoylecgonine (**3**), resulting from ester hydrolysis (Figure 1).[15,16] Hydrolysis of the benzoyl ester is catalyzed by plasma butyrylcholinesterases (BChE), while hydrolysis of the methyl ester occurs spontaneously under basic biological conditions.[15,16] Accordingly, cocaine haptens, which are designed with maximum congruence to the native drug, are likely susceptible to both spontaneous alkaline and enzymatic hydrolysis. To date, we have reported a number of cocaine haptens including GNC (**4**), GNE (**5**), and GND (**6**); a series characterized by increasing stability towards ester hydrolysis, respectively (Figure 1).[13,14,17] Our design emphasized replacing the implicated esters with amides.

Scheme 1. [a]Reagents and Conditions: (i) H$_2$O, 1,4-dioxane, MW, 40 W, 90 psi, 130 ^{o}C, 2 h; (ii) benzyl 6-aminohexanoate, DMTMM, Et$_3$N, THF, rt, 52% (one-pot); (iii) H$_2$, Pd/C, EtOH, rt, 68%; (iv) sulfo-NHS, EDCI, DMF/H$_2$O, rt, 12 h, then TT, PBS, 4 ^{o}C, 24 h; (v) sat. NH$_3$ in MeOH, MW, 300 W, 150 psi, 100 ^{o}C, 4 h, 59%; (vi) NaBH$_3$CN, 2 M HCl, 2 M HCl in 1,4-dioxane, MeOH; (vii) BzCl, NaHCO$_3$, 1,4-dioxane/H$_2$O, rt, 20% (one-pot); (viii) H$_2$O, reflux; (ix) benzyl 6-aminohexanoate toluenesulfonic acid salt, EDCI, Et$_3$N, DMAP, CH$_2$Cl$_2$, 16% (one-pot); (x) H$_2$, Pd/C, EtOH, rt, 94%; (xi) sulfo-NHS, EDCI, DMF/H$_2$O, rt, 12 h, then TT, PBS, 4 ^{o}C, 24 h.

We rationalized the enhanced stability of GNE, and especially GND, toward hydrolysis would make for a more effective cocaine vaccine as a result of sustained antigen concentration and fewer unwanted, competing antigens, the latter resulting from hydrolytic degradation.[13,14]

GNC, which incorporates ester linkages at C-2 and C-3, is the most structurally faithful to cocaine.[17] While GNC does induce antibodies with the highest absolute titer value and affinity for cocaine, the hydrolytic instability of GNC translates to a lessened blockade of cocaine-induced psychomotor behavior.[13] Early studies with GND, which incorporates amide linkages at C-2 and C-3, demonstrated longer-lasting titer values that translated to improved blockade of cocaine-induced stimulant effects when compared to GNC.[13] Despite this promising finding, the low-yielding synthesis of GND limited further optimization of anticocaine vaccine formulations with this hapten. Accordingly, we disclosed a mixed amide:ester (C3:C2, re-

spectively) hapten, termed GNE, with hydrolytic susceptibility only to BChEs to optimize additional vaccine components including the carrier protein and adjuvant(s).[18-26] Employing GNE, we identified tetanus toxoid (TT) as a highly effective carrier protein and the adjuvants alum and cytosine-phosphodiester-guanine oligodeoxynucleotide (CpG ODN 1826) to be the optimal vaccine formulation.[27] Our GNE-TT vaccine formulated with alum and CpG ODN 1826 reproducibly elicits high affinity anticocaine antibody production and robust blockade of cocaine-induced hyperlocomotor activity in mice and non-human primates.[27]

With an optimized vaccine formulation defined, we endeavored to evaluate a GND-TT vaccine formulated with alum and CpG ODN 1826, to compare this vaccine to the GNE-TT vaccine for the first time. Although we have previously reported syntheses for GNE and GND, these routes were particularly

low yielding. Accordingly, we introduce herein refined syntheses to GNE and GND (Scheme 1).

The synthesis of GNE began with selective hydrolysis of the methyl ester in cocaine (**1**) using a microwave reactor to yield benzoylecgonine (**3**). Notably, this microwave-assisted hydrolysis was completed after 2 h, while conventional conditions required 7 days.[28] After solvent removal, crude **3** was condensed with benzyl 6-aminohexanoate using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholium chloride (DMTMM) in the same pot to afford benzyl ester **7** in good yield. The benzyl ester was reduced upon treatment with H_2 and Pd/C to give GNE (**5**) in 35% overall yield, improving on our first generation synthetic effort by three-fold. Having GNE in hand, we pursued hapten-protein conjugation. The carboxylic acid of **5** was coupled with *N*-hydroxysulfosuccinimide (sulfo-NHS) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) and treated with TT to afford the desired GNE-TT immunoconjugate (**8**).

Our GND synthesis commenced with microwave-assisted one-pot desalinization/condensation of methylecgonone bitartrate (**9**) to afford enamine **10**. Reduction of **10** with $NaBH_3CN$ under ca. pH 4 gave a highly polar amine (**11**). After solvent removal, crude **11** was converted to benzamide **12** using BzCl and $NaHCO_3$ in the same pot. Although the stereochemistry of the methyl ester in **12** was undesired, epimerization occurred under aqueous refluxing conditions to afford a mixture of the desired α-carboxylic acid and the undesired β-carboxylic acid in a 1:1 ratio. This mixture of carboxylic acids was immediately subjected to amidation conditions using EDCI, Et_3N and 4-dimethylaminopyridine (DMAP) to give a mixture of stereoisomers. Benzyl ester **14** was isolated from this mixture by preparative TLC. Finally, the benzyl ester in **14** was cleaved under standard hydrogenation conditions to afford GND (**6**) in 1.8% overall yield. The significant improvement to this route includes the incorporation of multiple one-pot reactions, reducing the need for work-up and purification steps. With the requisite **6** in hand, we undertook hapten-protein conjugation. After the activation of **6** with sulfo-NHS using the conditions described above, activated **6** was treated with TT to afford the desired GND-TT immunoconjugate (**15**).

Mice were intraperitoneally administered the immunoconjugates GNE-TT or GND-TT. Alum and CpG ODN 1826 were employed as adjuvants in all vaccines. Antibody titers were assessed by ELISA against a corresponding hapten-BSA conjugate, namely GNE- or GND- BSA. For all bleeds (21, 35, 70, and 84 days), mice vaccinated with GND-TT elicited higher antibody titers than the GNE-TT vaccinated mice, though no significant difference was observed (Figure 2). The highest GND-TT induced titers were observed after the second bleed, while the GNE-TT induced titers peaked at the first bleed and steadily decreased. In general, both vaccines successfully induced high anticocaine antibody titers throughout the course of this study.

While ELISA titers are traditionally used to measure vaccine efficacy, this methodology can misrepresent the affinity and avidity of the generated antibodies for the native drug because ELISA evaluates binding to a coated antigen.[29] To further quantify the antibody concentrations and anticocaine affinities raised by GNE-TT and GND-TT, a competitive radioimmunoassay (RIA) was conducted (Table 1). Mice in all groups were

found to possess antibodies with high affinity for cocaine: $K_d =$ 19 nM (GND) and 56 nM (GNE). The GND-TT vaccine induced antibodies with higher affinity for cocaine, although these antibodies were raised in lower concentrations. Demonstrating the opposite trend, serum from GNE-TT vaccinated mice exhibited higher antibody concentrations, though the affinities for cocaine were lower compared to the GND vaccine. This is a particularly interesting result because the GNE hapten is more structurally similar to cocaine; despite this, lower anticocaine antibody affinities were observed. The reduced affinities presumably result from the hydrolytic instability of GNE (verses GND). In other words, GNE can more
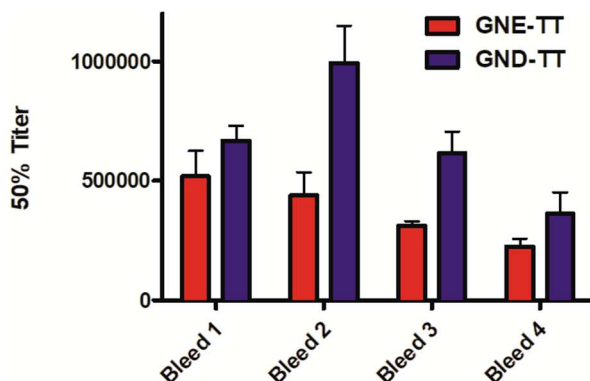

Figure 2. Anticocaine antibody midpoint titers measured by ELISA using serum from vaccinated mice (n = 6/group) on days 21 (bleed 1), 35 (bleed 2), 70 (bleed 3), and 84 (bleed 4) for each study group. Bars denote means ± SEM.

Table 1. Anticocaine antibody K_d values and concentrations measured by competitive RIA.

Vaccine[a]	K_d (nM)	Ab (μM)
GNE-TT	55.8 ± 1.4	59.1 ± 5.0
GND-TT	18.8 ± 2.0	11.8 ± 3.8

[a]Serum samples from vaccinated mice (n = 6/group) on day 21 were pooled for analysis. Data is reported as means ± SEM.
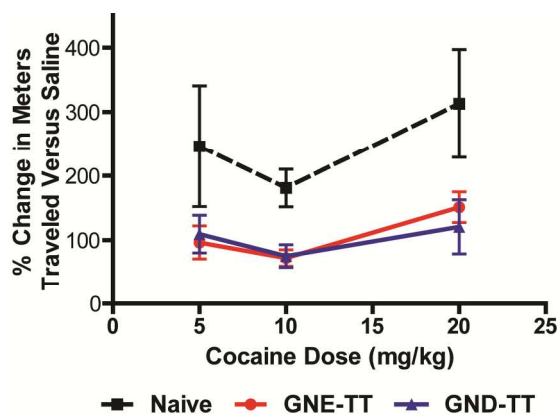

Figure 3. Cocaine-induced hyperlocomotor activity in vaccinated mice (n = 6/group) at 0, 5, 10, and 20 mg/kg on days 42 (0, 5 mg/kg), 44 (10 mg/kg), and 46 (20 mg/kg), respectively. Bars denote means ± SEM. Significance was observed.
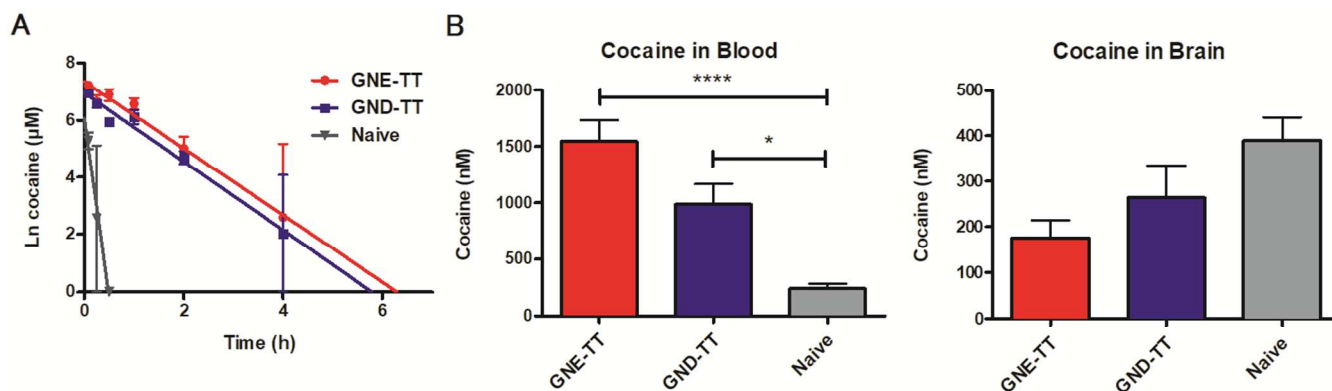
Figure 4. Serum pharmacokinetic results for vaccinated and naive mice (n = 6 total, n = 2 per time point) on day 81 (A); Concentration of cocaine in blood and brain of vaccinated and naive mice (n = 6) on day 101 (B). Bars denote means ± SEM. *$P < 0.05$; ****$P < 0.0001$ determined by one-way ANOVA.

readily undergo metabolic decomposition *in vivo* following immunization. Decomposition produces new antigenic motifs and thus, the generation of non-specific antibodies for cocaine. An alternative explanation is the increased hydrogen bonding capability of GND. Amides function as one of the best hydrogen bond donors/acceptors in molecular recognition.[30] Because the GND hapten incorporates two amide functionalities in the correct stereochemical configuration (of cocaine), the antibody affinity for cocaine may be improved as a result of the increased hydrogen bonding interactions of GND in the antibody/antigen complex.[13]

Being a stimulant, cocaine induces a quantifiable increase in rodent locomotor activity upon exposure. To assess vaccine performance, vaccinated mice were subjected to hyperlocomotion testing using ANY-Maze video tracking software (Stoelting Co.). Vaccine efficacy is measured in its ability to block cocaine-induced hyperlocomotion. As expected, naïve mice exhibited elevated hyperlocomotor activity with increasing doses of cocaine (Figure 3). Alternatively, GND-TT and GNE-TT exhibited robust blockade of cocaine's psychostimulatory effects at 5, 10, and 20 mg/kg doses. Despite the higher induced titers and superior anticocaine antibody affinities of GND-TT, both vaccines were comparably effective in stunting cocaine-induced behavior.

To evaluate the effect of GNE-TT and GND-TT vaccines on long-term drug metabolism, 20 mg/kg of cocaine was administered to all vaccinated and naive mice. Blood from each mouse was serially collected over the course of 24 h.[31] The amount of cocaine remaining in the blood at 0, 2, 4, and 6 h time points was quantified using LCMS (Figure 4A). In naïve mice, cocaine was found to have a serum half-life of 3.3 minutes. Vaccination was able to prolong cocaine's half-life to 34.5 (GND) and 35.6 (GNE) minutes; a 10.4 to 10.7-fold increase over naive animals, respectively (SI Table 1). As designed, the GNE-TT and GND-TT vaccines increased the peak concentration of cocaine in serum with comparable efficacy, demonstrating successful sequestration of free drug in circulation/outside the CNS.

To confirm these findings, biodistribution of the drug was investigated. After treating vaccinated and naïve mice with an additional 20 mg/kg cocaine, mice were sacrificed after 15 min by decapitation. Drug concentrations in the blood and brain were quantified by LCMS. As with the PK data dis-

cussed *vide supra*, the biodistribution data demonstrated that vaccine-generated antibodies effectively scavenged large amounts of free drug in the blood (Figure 4B). More specifically, mice vaccinated with GNE-TT appropriated nearly six times more cocaine in the blood than naïve mice, while GND-TT sequestered about four times more cocaine. Positively, both GNE-TT and GND-TT vaccines effectively lowered the cocaine concentration in the brain.

Comparing the results of ELISA, RIA and behavioral testing in this study revealed that hydrolytically stable haptens, GNE and GND, are extremely effective anticocaine vaccines when conjugated to TT and adjuvanted with alum and CpG ODN 1826. Both GNE-TT and GND-TT elicited antibodies that effectively blocked the drug's psychostimulatory activity at all tested concentrations. Accordingly, both the GND-TT and GNE-TT vaccines exhibited successful sequestration of cocaine outside the CNS. The results of this comparative analysis suggest that both GND-TT and GNE-TT present viable candidates for further consideration in more advanced rodent and non-human primate preclinical models, including self-administration studies to determine the surmountability and clinical-viability of these next-generation anticocaine vaccines.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website.

　　Experimental details; standard curve for LCMS quantification, half-life table, immobility times, RIA-generated binding curves

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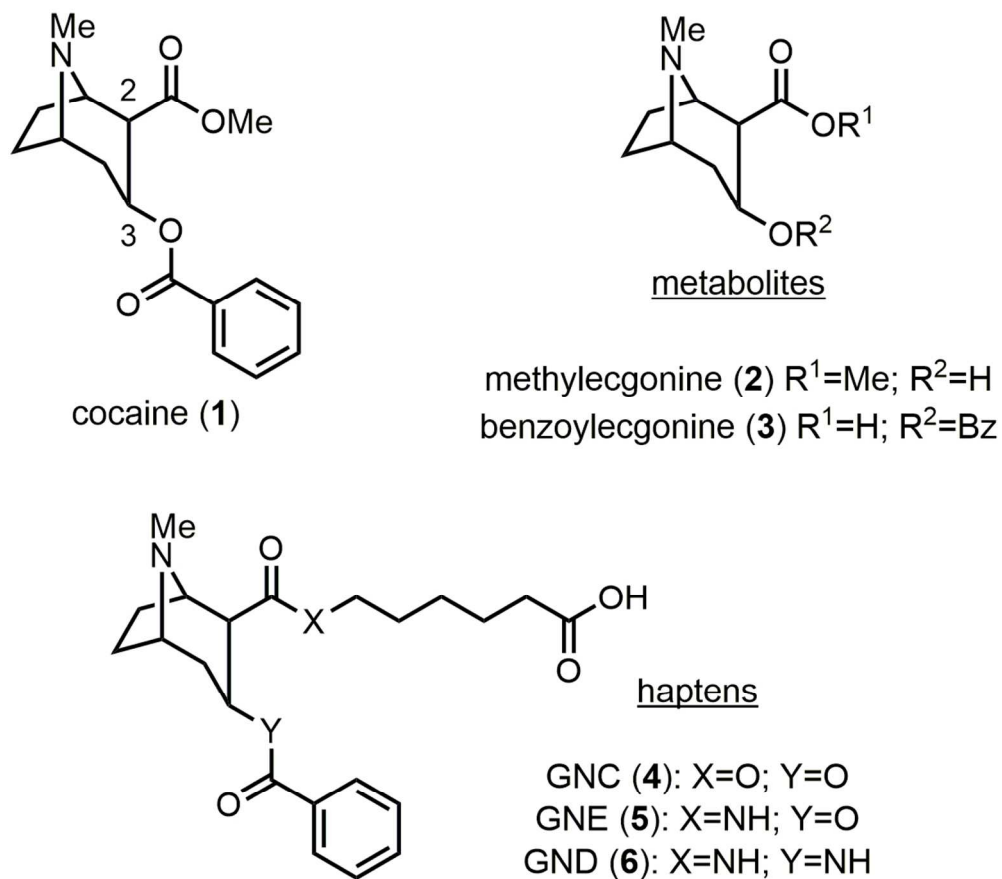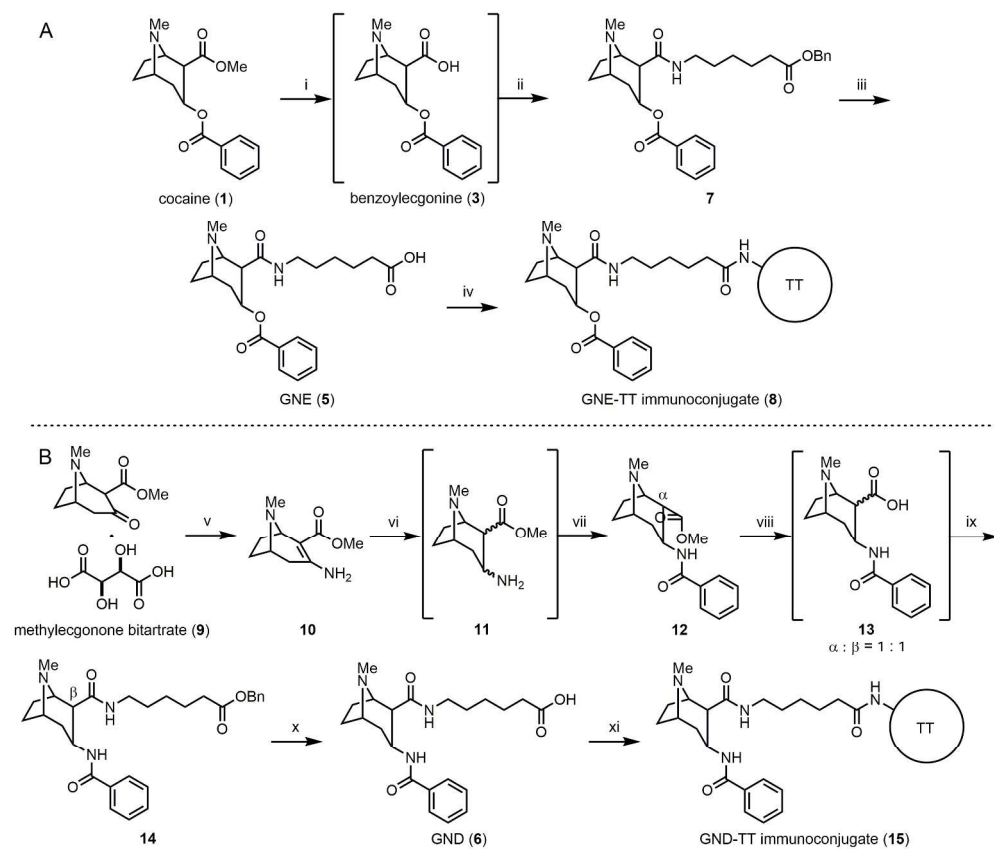
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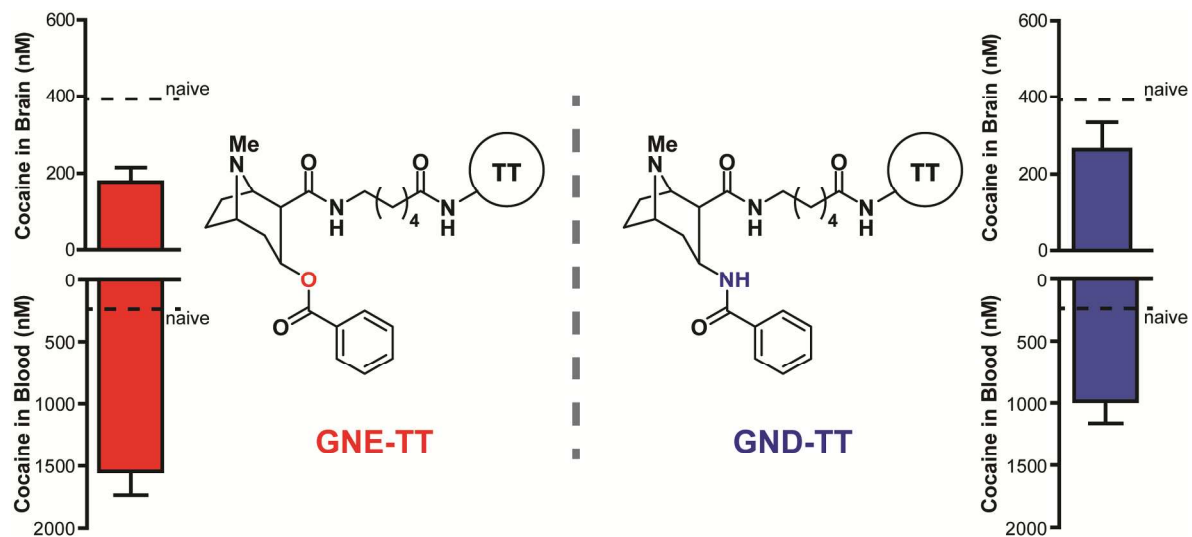
Figure 1. Chemical structures of cocaine, cocaine metabolites, and haptens previously developed in the Janda Laboratory.

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Scheme 1. aReagents and Conditions: (i) H2O, 1,4-dioxane, MW, 40 W, 90 psi, 130 oC, 2 h; (ii) benzyl 6-aminohexanoate, DMTMM, Et3N, THF, rt, 52% (one-pot); (iii) H2, Pd/C, EtOH, rt, 68%; (iv) sulfo-NHS, EDCI, DMF/H2O, rt, 12 h, then TT, PBS, 4 oC, 24 h; (v) sat. NH3 in MeOH, MW, 300 W, 150 psi, 100 oC, 4 h, 59%; (vi) NaBH3CN, 2 M HCl, 2 M HCl in 1,4-dioxane, MeOH; (vii) BzCl, NaHCO3, 1,4-dioxane/H2O, rt, 20% (one-pot); (viii) H2O, reflux; (ix) benzyl 6-aminohexanoate toluenesulfonic acid salt, EDCI, Et3N, DMAP, CH2Cl2, 16% (one-pot); (x) H2, Pd/C, EtOH, rt, 94%; (xi) sulfo-NHS, EDCI, DMF/H2O, rt, 12 h, then TT, PBS, 4 oC, 24 h.
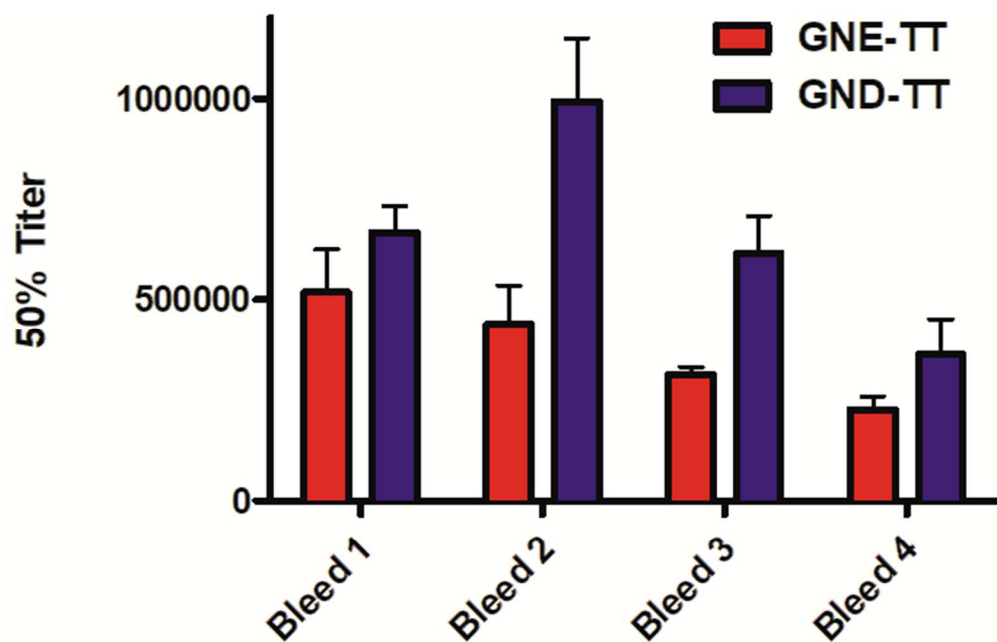
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Figure 2. Anticocaine antibody midpoint titers measured by ELISA using serum from vaccinated mice (n = 6/group) on days 21 (bleed 1), 35 (bleed 2), 70 (bleed 3), and 84 (bleed 4) for each study group. Bars denote means ± SEM.

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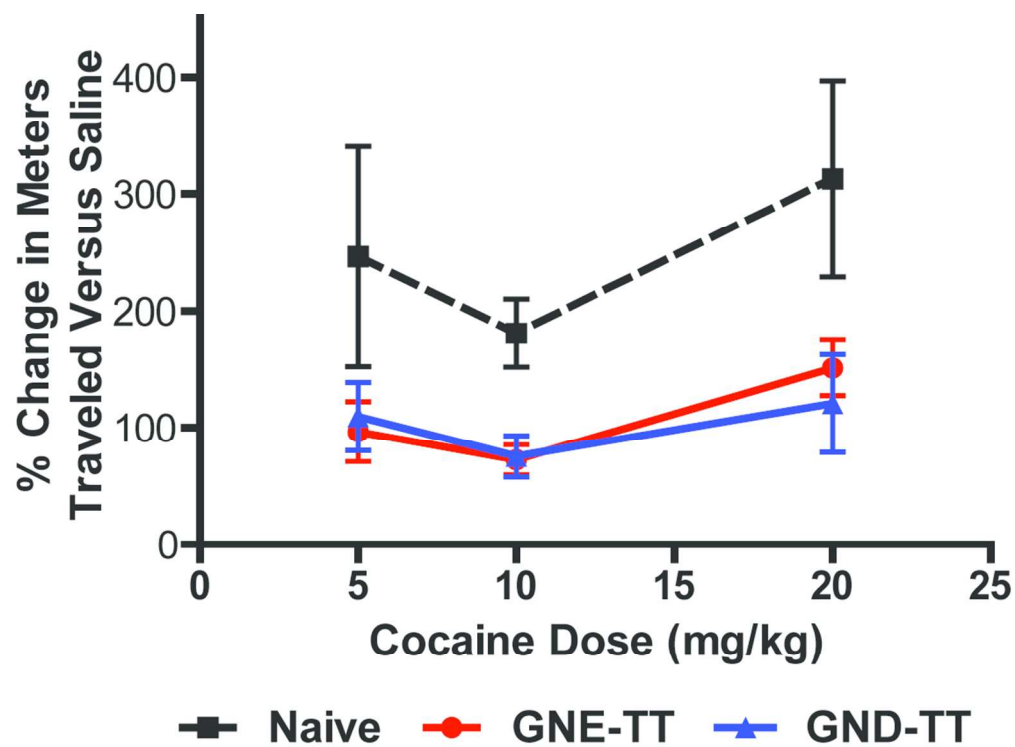Figure 3. Cocaine-induced hyperlocomotor activity in vaccinated mice (n = 6/group) at 0, 5, 10 and 20 mg/kg on days 42 (0, 5 mg/kg), 44 (10 mg/kg), and 46 (20 mg/kg), respectively. Bars denote means ± SEM. Significance was observed.
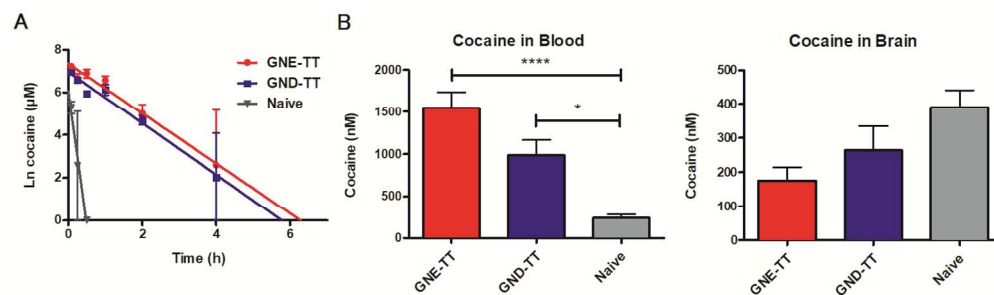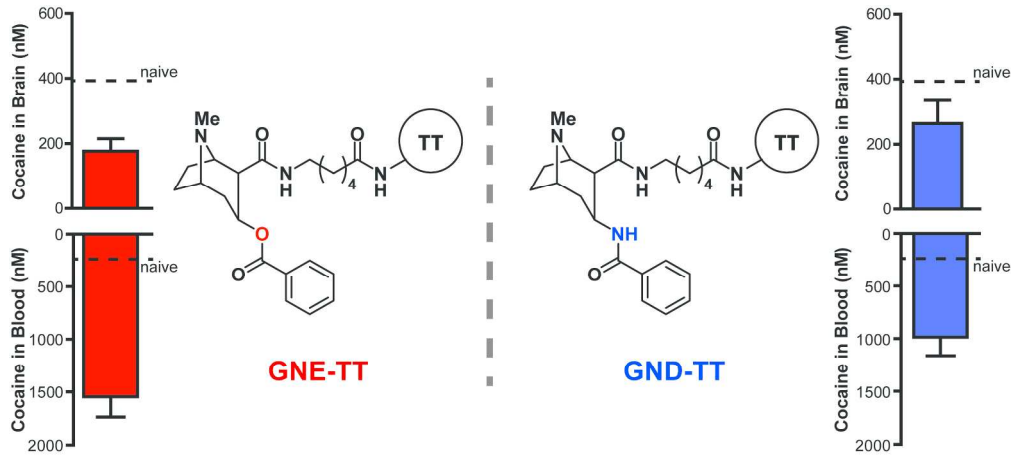
96x71mm (300 x 300 DPI)

Figure 4. Serum pharmacokinetic results for vaccinated and naive mice (n = 6 total, n = 2 per time point) on day 81 (A); Concentration of cocaine in blood and brain of vaccinated and naive mice (n = 6) on day 101 (B). Bars denote means ± SEM. *P < 0.05; ****P < 0.0001 determined by one-way ANOVA.

136x40mm (300 x 300 DPI)

TOC Graphic

222x100mm (300 x 300 DPI)