Conjugate Vaccine Immunotherapy for Substance Use Disorder

Paul T. Bremer and Kim D. Janda

Departments of Chemistry and Immunology, The Scripps Research Institute, La Jolla, California

Abstract—Substance use disorder, especially in relation to opioids such as heroin and fentanyl, is a significant public health issue and has intensified in recent years. As a result, substantial interest exists in developing therapeutics to counteract the effects of abused drugs. A promising universal strategy for antagonizing the pharmacology of virtually any drug involves the development of a conjugate vaccine, wherein a hapten structurally similar to the target drug is conjugated to an immunogenic carrier protein. When formulated with adjuvants and immunized, the immunoconjugate should elicit serum IgG antibodies with the ability to sequester the target drug to prevent its entry to the brain, thereby acting as an immunoantagonist. Despite the failures of first-generation conjugate vaccines against cocaine and nicotine in clinical trials, second-generation vaccines have shown dramatically improved performance in preclinical models, thus renewing the potential clinical utility of conjugate vaccines in curbing substance use disorder. This review explores the critical design elements of drug conjugate vaccines such as hapten structure, adjuvant formulation, bioconjugate chemistry, and carrier protein selection. Methods for evaluating these vaccines are discussed, and recent progress in vaccine development for each drug is summarized.

This work was supported by National Institutes of Health National Institute on Drug Abuse [Grants R01DA008590, R01DA026625, R21DA039634, and UH2DA041146].

https://doi.org/10.1124/pr.117.013904.

Address correspondence to: Dr. Kim D. Janda, Departments of Chemistry and Immunology, The Scripps Research Institute, 10550 North Torrey Pines Road, BCC-582, La Jolla, CA 92037. E-mail: kdjanda@scripps.edu
I. Introduction

Substance use disorder is a considerable detriment to society worldwide, and, in recent years, opioid abuse has become one of the most significant public health problems in the United States. Traditional pharmacological therapies such as μ-opioid receptor (MOR) agonists methadone and buprenorphine can assist in treating opioid dependence; furthermore, MOR antagonists naltrexone and naloxone are effective at reversing opioid overdoses. As another example, the nicotinic receptor partial agonist varenicline is Food and Drug Administration (FDA) approved for smoking cessation therapy. Although these pharmacotherapies can be effective, they have fallen short at mitigating the alarming surge in recent opioid abuse and can have significant side effects. Moreover, the polypharmacology of other drugs of abuse, e.g., cocaine and methamphetamine, presents a significant challenge for the development of small-molecule addiction therapeutics. As an alternative strategy for combating substance use disorder, conjugate vaccines hold promise due to their ability to generate high-affinity anti-drug antibodies. These serum antibodies can neutralize drug doses, thus preventing the target drug from acting on receptors in the brain (Fig. 1). Moreover, conjugate vaccines function as immunoantagonists to mitigate the pharmacodynamics of the target drug. Because the anti-drug antibodies themselves do not modulate drug receptors in the brain or even in the periphery, their side effect profile is minimal, but also they cannot ameliorate drug cravings or withdrawal symptoms. Vaccine-generated IgG antibodies have long half-lives; therefore, their duration of action is considerably longer than small molecules. Lastly, conjugate vaccines are versatile and can theoretically be designed to target any drug or combination of drugs.

The first reports of generating drug-specific antibodies were published in the early 1970s (Spector and Parker, 1970; Spector, 1971; Van Vunakis et al., 1972; Spector et al., 1973), in which anti-morphine antibodies were used in a radioimmunoassay for the detection of opioids. The therapeutic potential for anti-drug antibodies was first demonstrated in 1974, wherein a morphine-bovine serum albumin (BSA) conjugate formulated with complete Freund’s adjuvant was able to mildly attenuate heroin self-administration in a single rhesus monkey (Bonese et al., 1974). Since then, active vaccination was not further investigated as a substance abuse therapy until 20 years later when cocaine vaccines (Carrera et al., 1995; Fox et al., 1996) and nicotine vaccines (Hieda et al., 1997, 1999) were pioneered. Further investigations in clinical trials did not observe a significant effect of cocaine and nicotine vaccines on drug abstinence as a primary endpoint and did not measure patient antiserum drug affinity (a critical determinant of vaccine efficacy) (Cornuz et al., 2008; Martell et al., 2009; Hoogsteder et al., 2014; Kosten et al., 2014). Despite these failures, the trials have suggested that the vaccines could be effective if sufficient titer levels are achieved in each patient. Because efficacy is dependent on proper vaccine design to produce antibodies with adequate concentration and drug affinity, relatively recent advancements in drug conjugate vaccine design have increased the likelihood that these vaccines will one day succeed in the clinic. This review will explore these developments in relation to each aspect of vaccine design.

II. Drug Conjugate Vaccine Design

The design strategy of drug conjugate vaccines is illustrated in Fig. 2. Because small-molecule drugs inherently lack immunogenicity, they must be attached to an immunogenic entity in order for the immune system to recognize and generate antibodies against the target drug. By employing organic synthetic chemistry, a chemical linker can be attached to the drug to create what is known as a hapten. Bioconjugation of the drug hapten to a carrier protein results in the formation of a drug immunoconjugate. Formulation of this conjugate with immunostimulatory molecules, i.e., adjuvants, completes the production process of an anti-drug

**ABBREVIATIONS:** APC, antigen-presenting cell; AUC, area under the curve; BCR, B cell receptor; BSA, bovine serum albumin; DC, dendritic cell; DT, diphtheria toxoid; ELISA, enzyme-linked immunosorbent assay; FDA, Food and Drug Administration; HerCOOH, heroin carboxylic acid hapten; HerSH, heroin thiol hapten; IL, interleukin; KLH, keyhole limpet hemocyanin; LogP, partition coefficient; mAb, monoclonal antibody; MDPV, methylenedioxypyrrolidinone; MHC, major histocompatibility complex; MOR, μ-opioid receptor; MPLA, monophosphoryl lipid A; ODN, oligodeoxyribonucleotide; PAMP, pathogen-associated molecular pattern; αPVP, α-pyrrolidinopropenophenone; rEPA, recombinant Pseudomonas aeruginosa exoprotein A; RIA, radioimmunoassay; SMA, N-succinyl methamphetamine hapten; SNC, N-succinyl norecocaine; Th, T-helper; THC, tetrahydrocannabinol; TLR, Toll-like receptor; TT, tetanus toxoid.
vaccine, and injection of this vaccine into a subject will elicit antibodies against the drug hapten.

A. Drug Selection

Certain drugs are more amenable to targeting by a conjugate vaccine due to properties such as size, pharmacokinetics, and biodistribution. Size, i.e., mol. wt., and structural complexity are critical considerations because lower mol. wt. drugs do not possess a sufficient number of atoms to constitute an immune epitope. Furthermore, the presence of nitrogen and oxygen atoms is essential for forming hydrogen bonds between small-molecule drugs and antibodies. For example, ethanol is a frequently abused, small mol. wt. drug (mol. wt. = 46 g/mol) that is incompatible with the conjugate vaccine strategy. As ethanol only contains three heavy atoms, it would present as a poorly immunogenic epitope; furthermore, antibodies could not be produced with great enough affinity to ethanol because potential binding interactions are limited to only one hydrogen bond. Regardless, anti-ethanol antibodies would most likely cross-react with endogenous molecules such as serine side chains. As a result, morphinan haptens can act as effective immune epitopes, and anti-hapten antibodies have shown 10⁻¹⁰ to 10⁻⁹ M affinity for the target drugs (Torres et al., 2016; Kimishima et al., 2017). Similarly, antisera to a fentanyl conjugate vaccine showed affinity in the 10⁻⁹ M range for fentanyl (three heteroatoms, mol. wt. = 336 g/mol) (Bremer et al., 2016). A related reason for the success of opioid conjugate vaccines in producing high-affinity antibodies is that the structurally complex nature of opioids increases the foreignness of opioid epitopes. In contrast, methamphetamine and ethanol are structurally similar to endogenous amino acids phenylalanine and serine, respectively, possibly lending to diminished immunogenicity.

Drug pharmacodynamics and pharmacokinetics also play a major role in assessing the suitability of a drug for active vaccine targeting. The dose–effect curve of an exceptionally potent drug in behavioral assays can be shifted much more dramatically by vaccine-generated antibodies compared with a weak drug, so long as the antibodies possess sufficient affinity for the target drug. A good example of this phenomenon is a recently reported fentanyl vaccine, which shifted the fentanyl dose–effect curve by greater than 30-fold in assays for opioid-induced antinociception (Bremer et al., 2016). The ED₅₀ of fentanyl in mice was approximately 0.1 mg/kg, but in the presence of the vaccine that ED₅₀ increased to >3 mg/kg. In contrast, the ED₅₀ of morphine in antinociception assays is almost 10 mg/kg; therefore, assuming anti-morphine antibodies possess equal anti-drug affinity, a significantly larger amount of antibody would be needed to shift the morphine dose–effect curve.

Fig. 2. Design and production of drug conjugate vaccines. Using synthetic chemistry, a linker is appended to the target drug containing a cross-linking functionality at the linker terminus. Activation of this functionality enables the coupling of the drug hapten to a carrier protein, yielding an immunoconjugate. Formulation of the drug immunoconjugate with depot and immunostimulatory adjuvants generates the complete vaccine. Multiple immunizations of this vaccine coax the subject’s immune system into producing drug-neutralizing IgG antibodies.
Drug pharmacokinetics is crucial for designing the hapten structure because drug metabolites often possess differing psychoactive properties than the parent drug. For instance, cocaine is hydrolyzed to the mostly inactive metabolite benzoylecgonine, whereas heroin hydrolysis produces the primary psychoactive metabolite 6 AM; therefore, effective cocaine and heroin vaccines should target cocaine and 6 AM, respectively (Fig. 4). Drug half-life should also be considered, which will influence the amount of time in which the drug will occupy the antibody. It is known that antibody–drug binding increases the half-life of the target drug (Hill et al., 1975), presumably by shielding the drug from enzyme-mediated degradation, resulting in reduced drug clearance. For example, heroin, nicotine, and oxycodone vaccines produced fold increases in drug half-life of 3–5, 7, and 68–268, respectively (McCluskie et al., 2015a; Bremer et al., 2017; Kimishima et al., 2017). A possible explanation for the lower half-life shifts of heroin, i.e., 6 AM, is that the carboxylesterase enzymes responsible for 6 AM hydrolysis are ubiquitous in the blood, whereas oxycodone is metabolized via liver-localized cytochrome p450 enzymes; therefore, 6 AM experiences a greater degree of exposure to metabolizing enzymes. Vaccine-generated antisera that produces exceptionally long drug half-life would be anticipated to become easily saturated upon repeated drug administration.

A relevant pharmacokinetic parameter that serves as a good metric for vaccine efficacy is area under the curve (AUC), which is a measurement of drug plasma concentration over time. A larger AUC indicates increased antibody sequestration of drug in the blood, leading to lower distribution of drug to tissues and organs, most importantly the brain. As an example, a monoclonal cocaine antibody increased drug AUC in plasma by 26-fold, leading to a 4.5-fold decrease in brain AUC (Norman et al., 2007). The same phenomenon applies to vaccines: a Pfizer nicotine vaccine led to a 90-fold nicotine AUC increase (McCluskie et al., 2015a) and a heroin vaccine led to a 19-fold heroin AUC increase (Bremer et al., 2017). These studies in nonhuman primates did not permit quantification of drug concentrations in the brain, although it is reasonable to assume that the large serum AUC increase corresponded to a decrease in brain AUC.

Lipophilicity (LogP) of the target drug may also play a role in vaccine compatibility. For example, although a tetrahydrocannabinol (THC) vaccine was able to effectively generate anti-THC antibodies (Qi et al., 2005), the vaccine showed poor efficacy in blunting THC psychoactivity in vivo. This may be a result of the especially high LogP of THC (−7), causing increased tissue and blood brain barrier penetration of the drug, thus shifting the equilibrium away from antibody–drug–bound state in the blood. Compared with most drugs, fentanyl is relatively lipophilic (LogP ≈ 4), yet it is still 1000 times less lipophilic than THC and has been successfully targeted using conjugate vaccines (Bremer et al., 2016).

B. Immunologic Considerations

1. Injection Route. Injection of the vaccine formulation into biologic subjects causes a complex cascade of immunologic events whereby the conjugate is taken up, processed, and displayed for eventual production of antibodies with affinity for the drug hapten (Fig. 3). Drug vaccines are typically administered subcutaneously (s.c.) in rodent models, which translates well to the intramuscular (i.m.) route used in humans. Additionally, the vaccines have been introduced intradermally (i.d.) (Chen et al., 2012) and intraperitoneally (i.p.), the latter of which has been shown to elicit superior immunogenicity over other routes (Bremer et al., 2014). When vaccines are administered via the i.m. and s.c. routes, immunogens are channeled to local lymph nodes [either in free form or antigen-presenting cell (APC)–associated form], where T and B cell activation occurs. In the case of the i.p. route, antigens filter into the spleen, whereas i.d. administered immunogens encounter the skin-associated lymphoid tissue.

2. Adjuvant Formulation. Formulation of vaccines with depot adjuvants such as alum (colloidal aluminum salts), liposomes, oil-in-water, e.g., Sigma or Ribi adjuvants system, and water-in-oil emulsions, e.g., Freund’s adjuvant, is essential for vaccine efficacy due to the ability of these mixtures to form large particle sizes. Sufficient size of antigen-containing particles is crucial for holding the antigen at the site of injection and for uptake of the antigen by APCs such as dendritic cells (DCs) (Bachmann and Jennings, 2010). Our group has observed that alum, the oldest known adjuvant (Glenny et al., 1931), shows excellent performance—a fortunate result given that alum is cost-effective, widely used, and one of the only FDA-approved adjuvants. Alum is particularly well suited for use in combination with drug conjugates because it induces T-helper (Th)2-type immunity, which favors drug-neutralizing IgG antibody production in contrast to Th1-type, cell-mediated immunity (Gavin et al., 2006). Electrostatic and/or ligand exchange interactions between proteins and alum cause adsorption of the proteins onto alum, allowing for the depot effect. Moreover, antigens mixed with alum are retained at the site of injection and form large particle sizes for uptake of the particles by DCs (Morefield et al., 2005). However, many studies have found that the adjuvancing effects of alum are unrelated to antigen adsorption and retention (Romero Méndez et al., 2007), and degree of adsorption was even found to reduce antigen immunogenicity (Hansen et al., 2007, 2009; Noe et al., 2010). In all studies, coadministration of antigen and alum was necessary for antibody formation regardless of adsorption, and it has been proposed that alum aggregates can trap nonadsorbed antigen to cause
Immunologic mechanisms related to drug conjugate vaccines. Depot-forming adjuvants such as alum \([\text{Al(OH)}_3]\), liposomes, or oil-in-water emulsions form large particle sizes for uptake of drug conjugates by DCs, a type of APC. Immunostimulatory PAMPs activate pattern recognition receptors (PRRs) to stimulate DC and B cell maturation. For example, CpG ODNs bind to TLRs, i.e., TLR9, whereas alum stimulates NOD-like receptors, i.e., NALP3, to form a protein complex known as an inflammasome, and, in both cases, the adaptive immune response to the drug conjugate is enhanced. Important cytokines that mediate these processes are type I interferons and IL-1 family of cytokines. The function of DCs is to endocytose drug conjugates, process them intracellularly, and display them as the drug hapten linked to a carrier peptide on MHCII. Naive Th cells can be recruited to the DC via the T cell receptor for activation. B cells already exposed to the hapten conjugate via BCR cross-linking are activated by Th cells within lymph nodes. Following clonal expansion and affinity maturation in lymph node germinal centers, the activated B cells differentiate into memory B cells and plasma cells for induction of humoral (antibody-based) immunity to the target hapten.

Fig. 3. Immunologic mechanisms related to drug conjugate vaccines. Depot-forming adjuvants such as alum \([\text{Al(OH)}_3]\), liposomes, or oil-in-water emulsions form large particle sizes for uptake of drug conjugates by DCs, a type of APC. Immunostimulatory PAMPs activate pattern recognition receptors (PRRs) to stimulate DC and B cell maturation. For example, CpG ODNs bind to TLRs, i.e., TLR9, whereas alum stimulates NOD-like receptors, i.e., NALP3, to form a protein complex known as an inflammasome, and, in both cases, the adaptive immune response to the drug conjugate is enhanced. Important cytokines that mediate these processes are type I interferons and IL-1 family of cytokines. The function of DCs is to endocytose drug conjugates, process them intracellularly, and display them as the drug hapten linked to a carrier peptide on MHCII. Naive Th cells can be recruited to the DC via the T cell receptor for activation. B cells already exposed to the hapten conjugate via BCR cross-linking are activated by Th cells within lymph nodes. Following clonal expansion and affinity maturation in lymph node germinal centers, the activated B cells differentiate into memory B cells and plasma cells for induction of humoral (antibody-based) immunity to the target hapten.

Interestingly, MPLA alone or a combination of MPLA + alum induced lower anti-opioid antibody titers than alum alone (Pravetoni et al., 2014b). In contrast, a PAMP that has shown robust immunogenic synergy with alum is B-class CpG (ODN). These approximately 20 nucleotide oligos act on TLR9 to stimulate B cell and to a lesser extent DC maturation, ultimately resulting in enhanced antibody production (Hartmann et al., 2000; Hartmann and Krieg, 2000; Krug et al., 2001). A possible explanation for the synergy between alum and CpG ODN could be the fact that alum causes the release of host DNA due to cell death, which can also act on TLR9 to induce IL-1β and type-I interferon production (Marichal et al., 2011). The immunostimulatory nature of CpG ODN has prompted the investigation of it as an adjuvant, specifically sequence 7909, in clinical trials for multiple types of vaccines (Cooper et al., 2004; Valmori et al., 2007; Mullen et al., 2008; Brody et al., 2010; Sogaard et al., 2010). For drugs of abuse vaccines, rodent-specific CpG ODN 1826 has been successfully used as an adjuvant in combination with alum (Pryde et al., 2013; Bremer et al., 2014, 2016; Collins et al., 2016; Kimishima et al., 2017). Compared with alum alone, CpG ODN 1826 + alum could boost hapten-specific titers of Th2-associated (IgG1) and especially Th1-associated (IgG2a) antibody isotypes, resulting in enhanced mitigation of heroin potency (Bremer et al., 2014). In nonhuman primates, B-class CpG ODN combined with alum has been used as a nicotine and heroin vaccine adjuvant, also with increased success compared with alum alone (McCluskie et al., 2013, 2015a,b; Bremer et al., 2017). Given the wealth of data demonstrating the safety and efficacy of the alum + CpG ODN adjuvant formulation across many species, alum + CpG ODN appears to be a promising drug vaccine adjuvant for use in clinical trials.

3. Dosing and Schedule. Vaccines against drugs of abuse demand a relatively high titer level to achieve
efficacy due to the large molar quantities of drug that must be sequestered to block drug psychoactivity. In contrast, vaccines against pathogens have a lower titer requirement to block infection. As a consequence, not only is adjuvant formulation of utmost importance but also vaccine dosing and immunization schedule. Conjugate vaccination courses usually consist of three priming injections spaced 2–4 weeks apart. Titer levels peak 2–4 weeks after a given injection, at which point they will decay until vaccine boosters are administered, typically 2–3 months after the last priming injection (Martell et al., 2005; Hatsukami et al., 2011; Maoz et al., 2013; McCluskie et al., 2015b). In most cases, subsequent booster injections could not raise titers above initial peak titer levels. Few studies have compared different vaccination schedules; however, one clinical nicotine vaccine study found that five injections versus four injections administered within the same 26-week period produced higher titers (Hatsukami et al., 2011). Although vaccines for infectious diseases can remain efficacious for years without boosters, drug vaccines would most likely require a much shorter interval between boosters of 1 year or less. A critical question that has been asked in relation to vaccination schedule is whether immunizations can occur during drug administration. Two rodent studies have compared vaccine efficacy with and without concurrent drug administration during immunization, and results have shown no impairment of vaccine efficacy due to drug exposure (Hieda et al., 2000; Byrnes-Blake et al., 2001). A clinical implication of these studies is that vaccines could be used not only to maintain drug abstinence but also to induce drug abstinence in active drug users. In terms of immunoconjugate dosing, both cocaine and nicotine clinical studies (typically using doses of 200 or 400 μg per injection) have found convincing evidence that conjugate dosing even up to 2 mg per injection correlates positively with titer levels (Hatsukami et al., 2005, 2011; Martell et al., 2005). Because no ceiling effect has been observed, these results suggest that the optimum conjugate dose is the highest possible dose at which no side effects occur, and to date no significant adverse effects to drug conjugate vaccines have been observed in clinical conjugate vaccine studies (Hatsukami et al., 2005; Martell et al., 2005).

C. Hapten Design

The primary goal in designing drug haptens is to preserve the native chemical structure of the drug as much as possible to generate antibodies with the highest degree of drug affinity. Obviously, addition of a linker will alter the structure, but it can be done so strategically to minimize disruption of key drug functionalities. For example, drugs containing a terminal alkyl group can simply be elongated for installment of the linker and has to date been the most successful hapten design strategy (Fig. 5) (Carrera et al., 1995; Isomura et al., 2001; Moreno et al., 2011; Stowe et al., 2011; Bremer et al., 2016; Kimishima et al., 2017). Linker installment off of an aryl ring has also been shown to be an effective hapten design strategy. For example, haptens substituted at the 3 and 4 positions of the pyridine in nicotine and the phenyl ring in methamphetamine (Figs. 9 and 10) have shown success as immunoconjugates in generating antibodies with high affinity for their target drugs (Peterson et al., 2007; Pryde et al., 2013). Although hapten design via alkyl chain elongation or aryl substitution can equally preserve the chemical functionalities present in the target drug, it is unclear which strategy produces antibodies with better affinity. However, a key influence of hapten linker position is that it will most likely permit greater structural feature flexibility at the corresponding position of antibody-recognized small molecules. For example, a fentanyl hapten linked off the propanoyl group (Fig. 5) produced antibodies that could bind fentanyls containing acetyl, propanoyl, and butanoyl groups with equally high affinity, whereas binding affinity to fentanyls with methyl groups at other positions was lower (Bremer et al., 2016).

As mentioned in the previous section, drug metabolism is a critical consideration for hapten design. For example, the in vivo hydrolysis of cocaine and heroin generates less psychoactive metabolites in the former case, but more psychoactive metabolites in the latter case (Fig. 4). Attempts to stabilize heroin haptens by means of isosteric replacement of the 3- and 6-position esters to amides have failed to garner increased vaccine efficacy in blocking heroin psychoactivity (Fig. 6). This is most likely due to the failure of these haptens to properly mimic the most active heroin metabolite, 6 AM. Previously reported 3-AcN, DiAm, 6-PrOxy, and DiPrOxy haptens (Fig. 6) cannot present as a 6 AM-like epitope due to hydrolytically stable 3-position functionalities (Bremer and Janda, 2012; Li et al., 2014). The MorHap, while containing the proper 3-position phenol, possesses incorrect stereochemistry at the 6-position (Matyas et al., 2014). In contrast, M-6-HS, M(Gly)₄, and hydrolyzed heroin haptens HerSH and HerCOOH (Fig. 6) can properly mimic 6 AM and have shown efficacy in heroin self-administration models (Bonese et al., 1974; Anton and Leff, 2006; Stowe et al., 2011; Schlosburg et al., 2013; Raleigh et al., 2014). In a comparison of heroin, 6 AM, and morphine haptens, the corresponding heroin and 6 AM vaccines, but not the morphine vaccine, was effective at blocking heroin psychoactivity (Stowe et al., 2011; Schlosburg et al., 2013; Bremer et al., 2017). Given these results and the poor performance of the 3-AcNHerSH hapten (Bremer and Janda, 2012), the presence of a 3-acetyl group in the hapten is most likely unnecessary for heroin vaccine potency, whereas the 6-acetyl group is critical.

Stabilization of cocaine haptens via amide replacement has shown much greater success compared to
heroin hapten was observed (Fig. 7). A correlation was observed between cocaine hapten stability and vaccine efficacy (Carrera et al., 2001; Cai et al., 2013). The amide modification had a small effect on decreasing antibody affinity for cocaine, as one might expect; however, the small loss in affinity was greatly outweighed by an increase in antibody concentration. The success of amide cocaine hapten may also be related to the fact that the amide is positioned at the linker attachment point and thus plays less of a role in determining antibody–drug binding.

Simultaneous immunization of multiple hapten protein conjugates with distinct hapten structures has been explored (Keyler et al., 2008; Pravetoni et al., 2012a; Cornish et al., 2013; de Villiers et al., 2013). In these studies, the hapten linkers were installed at different positions on the nicotine molecule to presumably produce distinct B cell populations specific to each hapten. The resulting antiserum produced by the heterogeneous collection of B cells showed enhanced nicotine-neutralizing capacity compared with using only one immunizing hapten. Because many different permutations exist in experimental design, further research is needed to explore this approach. For example, structurally distinct hapten conjugates can be simultaneously coupled to one carrier, individually coupled to the same carrier, or each coupled to different carriers. Furthermore, the selection of haptenes and adjuvants are also important factors. Combination vaccines have also been explored for producing antibodies against multiple drug targets. Coadministration of 6 AM and oxycodone conjugates did not compromise efficacy against either drug compared with the response of individual vaccines (Pravetoni et al., 2012c). These opioids are structurally similar; however, it is unclear whether this approach would work for structurally dissimilar drugs. Heroin and fentanyl are structurally dissimilar, and a combination vaccine against them would be of great value given that the two are often mixed together by drug dealers.

Another strategy for hapten design involves conformationally constraining haptenes. Drugs such as methamphetamine and nicotine are fairly flexible; therefore, mimicking the most stable conformational isomers with the analogous haptenes could enhance antibody binding to these drugs (Meijler et al., 2003). A study comparing a conformationally constrained nicotine hapten (CNI; Fig. 9) with a corresponding unconstrained hapten (NIC; Fig. 5) showed that the constrained CNI hapten generated higher concentrations of anti-nicotine antibodies, possibly due to enhanced immunogenicity of the bulkier CNI hapten. Additionally, the CNI hapten as a conjugate vaccine showed greater antagonism of nicotine antinociception and greater alteration of nicotine self-administration versus the unconstrained hapten (Moreno et al., 2012). In contrast, methamphetamine vaccine efficacy did not benefit from the use of conformationally constrained haptenes (Moreno et al., 2011).

D. Linker Design and Bioconjugate Chemistry

Bioconjugation of drug haptenes to proteins is a critical step in forming an immunologically active conjugate that will stimulate the production of anti-drug antibodies. Studies have shown that higher hapten loading
Structures of drugs of abuse haptens designed with extended alkyl chains for linker incorporation. The target drugs are shown in black, and the linker moieties are shown in green. References for haptens depicted in figure are as follows: heroin (HerSH/COOH haptens) (Stowe et al., 2011; Bremer et al., 2017); oxycodone (Kimishima et al., 2017); fentanyl (Bremer et al., 2016); methamphetamine (MH6/COOH haptens) (Moreno et al., 2011; Collins et al., 2016); cocaine (GNC hapten) (Bremer et al., 2016); methamphetamine (MH6/COOH haptens) (Collins et al., 2016). The target drugs are shown in black, and the linker moieties are shown in green. References for haptens depicted in figure are as follows: heroin (HerSH/COOH haptens) (Stowe et al., 2011; Bremer et al., 2017); oxycodone (Kimishima et al., 2017); fentanyl (Bremer et al., 2016); methamphetamine (MH6/COOH haptens) (Moreno et al., 2011; Collins et al., 2016); cocaine (GNC hapten) (Bremer et al., 1995); nicotine (NIC hapten) (Isomura et al., 2001); and cathinones (Nguyen et al., 2017a). The latter requires the presence of a sulfhydryl group on the hapten as well as maleimide-loaded carrier protein (Fig. 8). The major histocompatibility complex (MHC)II loading is an essential aspect of the linker, recent studies have suggested that incorporation of peptidic spacers can enhance vaccine efficacy, and the only linker feature that markedly reduced vaccine efficacy was the presence of bulky squaramide or cyclohexyl groups (Pravetoni et al., 2012b, 2013). These two groups as well as maleimide are large enough to interfere with the drug-like epitope. Although length and lipophilicity do not appear to be an important aspect of the linker, recent studies have suggested that incorporation of peptidic spacers can enhance vaccine responses. For example, the addition of a diglycine unit into the linker of a nicotine vaccine improved the antibody response (Collins and Janda, 2014), and recent studies have shown a similar result with a methamphetamine hapten (Collins et al., 2016). Addition of a tetruglycine spacer was found to enhance the immunogenicity of an oxycodone hapten, giving higher titers than a similar vaccine without the tetruglycine (Pravetoni et al., 2012b, 2013). In preliminary experiments, we investigated the use of a di-β-alanine linker; however, this showed inferior results compared to a shorter linker without the di-β-alanine (Bremer et al., 2017). We postulate that the failure of the β-alanine linker could be attributed to the unnatural nature of the linker. The major histocompatibility complex (MHC)II loading is an essential process in recruiting T cell help for the generation of anti-hapten antibodies (Fig. 3), and, although nonpeptidic fragments of MHC ligands are permitted in some cases, these elements have to be designed carefully so as not to substantially disrupt the MHC–ligand interaction (Weiss et al., 1995; Bianco et al., 1998). Furthermore, antigen processing of hapten–protein conjugates results in hapten–peptide fragments, and the peptidic portion of these fragments is essential for anchoring the hapten to the MHCI (Avci et al., 2011), which may explain why peptidic linkers can influence hapten immunogenicity. Interestingly, in a recent study of
methamphetamine vaccines, addition of a mono-L-alanine spacer to the linker was superior to other peptidic spacers, including di-glycine, di-L-alanine, L-proline-glycine, and L-proline-L-alanine (Gooyit et al., 2017) (Fig. 10). The introduction of amino acids into hapten linkers has to date proven promising; however, future studies should seek to rationalize this strategy and establish whether it can translate to other drug haptens.

E. Carrier Protein Selection

The carrier protein is an essential component of drug immunoconjugates because it serves as a source of T cell epitopes. These epitopes are necessary for activation of T cells via APCs, and activated T cells can then stimulate B cells, i.e., T cell help (Fig. 3). The cognate interactions between these cells occur via the MHCII loaded with the hapten linked to the T cell epitope (~15 amino acids derived from the carrier protein). MHCII loading occurs first through uptake and intracellular proteolytic digestion of the immunoconjugate. An essential part of immunoconjugate processing by B cells is the cross-linking of multiple BCRs by the hapten antigen (Fig. 3). For this reason, an adequate degree of carrier protein haptenation, i.e., epitope density, is desired to induce efficient BCR cross-linking for downstream recruitment of T cell help.

Few studies have compared the efficacy of various proteins as carriers for small-molecule haptens. In nicotine and heroin vaccine studies from our group, we have uncovered a trend in immunogenicity in the interactions between these cells occur via the MHCII loaded with the hapten linked to the T cell epitope (~15 amino acids derived from the carrier protein). MHCII loading occurs first through uptake and intracellular proteolytic digestion of the immunoconjugate. An essential part of immunoconjugate processing by B cells is the cross-linking of multiple BCRs by the hapten antigen (Fig. 3). For this reason, an adequate degree of carrier protein haptenation, i.e., epitope density, is desired to induce efficient BCR cross-linking for downstream recruitment of T cell help.

Few studies have compared the efficacy of various proteins as carriers for small-molecule haptens. In nicotine and heroin vaccine studies from our group, we have uncovered a trend in immunogenicity in the interactions between these cells occur via the MHCII loaded with the hapten linked to the T cell epitope (~15 amino acids derived from the carrier protein). MHCII loading occurs first through uptake and intracellular proteolytic digestion of the immunoconjugate. An essential part of immunoconjugate processing by B cells is the cross-linking of multiple BCRs by the hapten antigen (Fig. 3). For this reason, an adequate degree of carrier protein haptenation, i.e., epitope density, is desired to induce efficient BCR cross-linking for downstream recruitment of T cell help.

Few studies have compared the efficacy of various proteins as carriers for small-molecule haptens. In nicotine and heroin vaccine studies from our group, we have uncovered a trend in immunogenicity in the interactions between these cells occur via the MHCII loaded with the hapten linked to the T cell epitope (~15 amino acids derived from the carrier protein). MHCII loading occurs first through uptake and intracellular proteolytic digestion of the immunoconjugate. An essential part of immunoconjugate processing by B cells is the cross-linking of multiple BCRs by the hapten antigen (Fig. 3). For this reason, an adequate degree of carrier protein haptenation, i.e., epitope density, is desired to induce efficient BCR cross-linking for downstream recruitment of T cell help.
following order: keyhole limpet hemocyanin (KLH) < DT < TT (Moreno et al., 2010; Bremer et al., 2017). Proteins BSA and ovalbumin are usually considered to be less immunogenic carriers than KLH. In many cases, DT is used as a detoxified mutant of diphtheria toxin (G52E) known as CRM197. The typical detoxifying procedure for diphtheria and tetanus toxins to produce the corresponding toxoids involves formaldehyde treatment; in contrast, CRM can simply be expressed in bacteria and used directly following purification without further treatment. Of the aforementioned carriers, CRM, DT, and TT have been approved for clinical use in polysaccharide conjugate vaccines (Pichichero, 2013). However, KLH has yet to be approved despite extensive use as a carrier. Native KLH exists as two distinct ~400-kDa subunits that form multidecameric quaternary structures, the size and complexity of which hamper characterization of KLH immunoconjugates. Drug conjugate vaccine studies have mostly used native KLH, although monomeric KLH has been successfully used (Ruedi-Bettschen et al., 2013; Stevens et al., 2016).

In nicotine vaccine studies, other carriers such as the outer protein capsid of bacteriophage Q-β and recombinant Pseudomonas aeruginosa exoprotein A (rEPA) have been explored. Q-β has been directly compared with CRM and has been found to be slightly less...
effective (McCluskie et al., 2015a). An assessment of KLH and rEPA as carriers has revealed higher antinicotinic antibody concentrations when vaccinating with the KLH conjugate, although different haptens on each carrier were used (Cornish et al., 2013). Another protein derived from the cholera B toxin (rCTB) has been used in a cocaine vaccine, which has been tested with limited success in clinical trials (phases I–III) (Kosten et al., 2002, 2014; Martell et al., 2005).

Liposomes have been used as carriers for heroin and nicotine haptenics, in which the haptenics are directly linked to lipids (Lockner et al., 2013; Matyas et al., 2013); however, a lack of evaluation in animal behavioral models has tempered enthusiasm for these vaccines. In a related strategy, a nicotine nanoparticle vaccine (SEL-068) has been developed containing a fully synthetic polymer matrix containing a TLR agonist and Th peptide, and nicotine haptenics are covalently linked to this matrix (Pittet et al., 2012). Results from monkey studies evaluating vaccine immunogenicity and effects on nicotine discrimination have proved promising (Fraser et al., 2014; Desai and Bergman, 2015), although key structural information regarding the vaccine components has not been disclosed.

III. Methods for Evaluating Drug Conjugate Vaccines

A. Immunochemical Methods

Characterizing vaccine-generated antibodies via immunochemical methods is essential for judging vaccine efficacy. Both antibody concentration and drug affinity are key determinants of how well antisera can neutralize drug doses. Enzyme-linked immunosorbent assay (ELISA) is an important tool for measuring antibody responses to any vaccine, whereas radioimmunoassay (RIA) can accurately quantify small-molecule affinity and concentration of antibodies. In the context of drugs of abuse vaccines, ELISA plates are typically coated with the immunizing hapten linked to a nonimmunizing carrier protein (often BSA). Serum collected from immunized animals is then serially diluted across the ELISA plates, and the serum antibodies bound to the plate are detected with an enzyme-conjugated secondary antibody (horseradish peroxidase secondary antibodies are often used). The antibody dilution that gives either 50% signal (midpoint) or no signal (endpoint) is reported as the antibody titer. Typically, the greater the affinity and concentration of polyclonal antibodies for the coating antigen, the higher this titer value will be (Friguet et al., 1985). For drug vaccines, our group prefers determining the 50% titer by means of a nonlinear fit of titer data because increased objectivity is obtained by interpolating the 50% value via curve fitting, in contrast to “eye-balling” the final well that produces signal for endpoint titer determination. ELISA has many limitations that include lack of consistency (repeat assays may give different results) and the fact that results will only show relative differences between samples run at the same time. Many different factors can influence output signal, such as incubation times and secondary antibody quality. A particularly important factor is the structure of the hapten used in the coating antigen. Binding of antiserum to immobilized hapten does not necessarily translate to binding to actual drug; therefore, the hapten should be highly congruent in structure to the target drug. Assuming that the appropriate hapten is used for ELISA, the titer results should correlate to the degree of antibody-mediated drug sequestration and potency attenuation in vivo, as observed in previous studies (de Villiers et al., 2010; Pravetoni et al., 2012c; Miller et al., 2013; Bremer et al., 2017).

To assess actual drug affinity of antiserum produced from vaccination, competitive ELISA can be used. In this variant of the ELISA, the limiting dilution of antiserum is first determined, which is the dilution that gives ~70% signal, and increasing concentrations of free drug are added to antiserum at the limiting dilution in the presence of immobilized hapten. Free drug will compete with the hapten for antibody binding and will decrease ELISA signal as a function of drug concentration. The resulting IC50 values from this assay will represent relative antiserum affinity for the specific drug. This competition experiment has been adapted to a surface plasmon resonance platform for increased ease and reproducibility (Bremer et al., 2016; Kimishima et al., 2017). Instead of competition with immobilized hapten, RIA can be employed, involving the use of isotopically labeled drug tracers in an equilibrium dialysis setup for determination of antibody $K_d$. In RIA, a scintillation counter must be used for detection of the tritium-labeled tracer. As a nonradioactive alternative, deuterated drug tracer can be used and detected by liquid chromatography–mass spectrometry (LCMS) (Torres et al., 2016). Using the $K_d$ value discerned from the assay as well as the tracer concentration, the antibody concentration can be calculated using Müller’s method (Muller, 1983). Assays directly measuring antibody interactions with drug tracers (RIA) compared with immobilized drug haptens (ELISA) are considerably more accurate and represent antibody binding to the actual drug. However, caution should be used in assigning absolute affinity and concentration to a polyclonal antibody mixture as the values represent an averaged result; vaccination produces a distribution of B cell populations with varying antigen affinities.

B. In Vivo Evaluation of Drug Vaccines

Because the level of antibody titer, concentration, and affinity needed for an efficacious vaccine is not well known and depends on the target drug, methods for measuring the pharmacokinetics and pharmacodynamics
of the drug in vaccinated subjects are essential. As mentioned before, drug-sequestering antibodies will increase half-life and AUC in the blood of the target drug. These parameters can be measured by administering a drug dose to vaccinated animals and extracting blood at various time points. Despite being a terminal procedure, brain tissue can be harvested and analyzed for determination of central nervous system drug exposure. Following sample preparation, LCMS is typically used to quantify both free and antibody-bound drug concentrations. Higher blood concentrations of drug as a result of antibody sequestration in vaccinated animals have consistently correlated with lowered distribution of drug to the brain (Fox et al., 1996; Pentel et al., 2000; Pravetoni et al., 2012c; Miller et al., 2013). Although alteration of drug pharmacokinetics and biodistribution is predictive of vaccine efficacy, an assessment of pharmacodynamics is arguably more important. Behavioral models are an important tool in measuring drug pharmacodynamic effects. It is known that most addictive drugs (opioids or psychostimulants) elicit their rewarding effects by increasing dopamine levels in a brain region known as the nucleus accumbens (Di Chiara and Imperato, 1988). In vivo voltammetry has been used to observe vaccine-mediated blockade of nicotine-induced dopamine release in this brain region (de Villiers et al., 2002, 2010). With the exception of producing downstream dopamine increases, opioids and psychostimulants have different pharmacodynamic profiles: opioids activate MORs, whereas psychostimulants target the dopamine transport system. In considering these differing mechanisms, behavioral models can be specifically tailored to measure the effects of either drug type. For example, opioids like heroin are potent analgesics, and their effects can be readily quantified by nociception (acute pain) assays such as the tail-flick and hot-plate tests (Bannon and Malmberg, 2007). In these assays, the animals’ response time to nociception induced by noxious stimuli (in this case heat) is measured as a function of increasing doses of opioid to generate a dose–response curve. In the presence of the vaccine, the dose–response curve will be right shifted, and the degree to which it is shifted serves as an excellent quantitative metric for assessing vaccine efficacy (Schlosburg et al., 2013; Bremer et al., 2014). Psychostimulants, in contrast, do not necessarily possess analgesic activity but produce high levels of locomotor activity that can be readily measured with infrared photobeam or video-tracking systems (Di Chiara and Imperato, 1988; Riviere et al., 1999). Vaccination against psychostimulants such as cocaine, methamphetamine, and cathinones can attenuate drug effects on locomotor activity, and the degree to which this occurs is indicative of vaccine efficacy (Carrera et al., 2001; Byrnes-Blake et al., 2003; Miller et al., 2013; Nguyen et al., 2017a,b). The locomotor and antinociception assays are the most basic and easy to perform behavioral tests for evaluating the immunoantagonistic action of drug vaccines and are predictive of efficacy in more sophisticated models for drug abuse such as conditioned-place preference, drug discrimination, and self-administration (Carrera et al., 2001; LeSage et al., 2006; Miller et al., 2013; Schlosburg et al., 2013; Desai and Bergman, 2015; Nguyen et al., 2017a,b). Most vaccine studies have used mice [BALB/c (inbred) or Swiss Webster (oubred) strains] or rats (Wistar or Sprague Dawley strains). Immune and drug response variation as a result of strain and species differences are likely to impact apparent vaccine efficacy, although no studies to our knowledge have investigated this directly. Male rodents have been more commonly used over female rodents, and it is unclear whether sex differences can affect vaccine responses as well. However, nicotine vaccine studies in pregnant female rats have shown that vaccine-generated antibodies can protect fetuses from nicotine (Keyler et al., 2003, 2005). Furthermore, a methamphetamine vaccine (MH6-KLH) was recently shown to be equally as effective in female rats, as was previously shown in male rats (Nguyen et al., 2017b).

IV. Recent Progress in Drug Conjugate Vaccine Research

A. Psychostimulants

1. Nicotine. Nicotine is an addictive natural product found in tobacco and acts as a nicotinic acetylcholine receptor agonist. Because the metabolites of nicotine (half-life of nicotine = 1–2 hours) are less physiologically active than the parent compound, drug vaccine haptens have sought to replicate the structure of nicotine (Fig. 5) (Hieda et al., 1997; Isomura et al., 2001; Pryde et al., 2013). The 3’AmNic hapten as a rEPA conjugate, also known as NicVax, has been tested in clinical trials, and results have demonstrated only mild efficacy (Hatsukami et al., 2005, 2011; Wagen et al., 2008; Hoogsteder et al., 2014). Improvements have been made to this vaccine that include the use of DT carrier, addition of CpG ODN (McCluskie et al., 2013), and an enanitopure version of the hapten mimicking the naturally occurring (-)-nicotine enantiomer (Lockner et al., 2015). The second-generation nicotine hapten known as NIC7 has shown promise in generating anti-nicotine antibodies in nonhuman primates, which could reduce brain concentrations of the drug (Fig. 9) (Pryde et al., 2013; McCluskie et al., 2015a,b). Another second-generation nicotine known as N4N (Fig. 9) has displayed superior performance when compared with 3’AmNic in attenuating nicotine-induced antinociception and hypothermia (Jacob et al., 2016). A fully synthetic nicotine vaccine (SEL-068) containing an unknown hapten has been shown to block nicotine discrimination in nonhuman primates (Fraser et al., 2014; Desai and Bergman, 2015).

2. Cocaine. Cocaine is a relatively short-acting (half-life = 30 minutes) drug and has complex inhibitory effects on the uptake of monoamine neurotransmitters
in the central nervous system. Importantly, it modulates the dopamine transporter, increasing synaptic dopamine—a key pharmacodynamic property that lends to the rewarding and reinforcing effects of cocaine (Ritz et al., 1987). Considering the metabolism of cocaine (Fig. 4), hapten design strategies have involved the stabilization of labile cocaine esters via isosteric replacement of the esters with amides (Fig. 7). Although the amide hapten generated antibodies with slightly less affinity for cocaine, a large boost in antibody concentration was supposedly responsible for a greater attenuation of cocaine-induced hyperlocomotion (Carrera et al., 2001; Cai et al., 2013). In terms of vaccine formulation, GNE hapten (Fig. 7) conjugated to carrier proteins flagellin and TT coadministered with alum and CpG ODN has proven effective in the mouse locomotor assay (Kimishima et al., 2016). The GNC hapten but also the more promising GNE hapten conjugated to a disrupted adenvirus formulated with Adjuplex (proprietary combination of lecithin and carbomer homopolymer) has also shown therapeutic potential (Hicks et al., 2011; Wei et al., 2012). In nonhuman primate studies, the GNE vaccine reduced cocaine occupancy of the dopamine transporter (Maoz et al., 2013), mitigated biodistribution of cocaine to the brain among other organs through serum antibody-mediated drug sequestration (Hicks et al., 2014), and attenuated cocaine self-administration and reacquisition (Evans et al., 2016). Another cocaine vaccine consisting of N-succinyl norcocaine (SNC)-conjugated rCTB formulated with alum has been tested in clinical trials through phase III, and results have shown a lack of efficacy (Kosten et al., 2002, 2014; Martell et al., 2005). These trials may have benefitted from the inclusion of CpG ODN in the adjuvant formulation, but more importantly the redesign of the SNC hapten to exclude the non-native amide at the cocaine nitrogen (Fig. 7). Efforts to convert the amide to an alkyl chain have been reported; however, the newer hapten contains labile esters that are unlikely to be stable in vivo (Ramakrishnan et al., 2014).

3. Methamphetamine. Methamphetamine, also known as meth, and its related parent compound amphetamine are in a large class of drugs known as substituted phenethylamines. These drugs produce their effects by modulating monoamine neurotransmitter systems. Meth potently activates trace amine-associated receptor 1, thus inhibiting dopamine uptake to increase extracellular dopamine levels (Harris and Baldessarini, 1973; Borowsky et al., 2001; Xie and Miller, 2009). This pharmacodynamic action of meth lends to its psychoactive and addictive effects, and, of the two isomers, the dextrorotatory isomer [(+)-methamphetamine] has been shown to be at least four times more potent than (-)-meth (Harris and Baldessarini, 1973). Initial immunotherapeutic approaches to meth abuse have focused on the creation of anti-meth monoclonal antibodies (mAbs), and phenyl-substituted hapten such as (+)-MET P4 and P6 (Fig. 10) could generate mAbs with low nanomolar $k_d$ affinity for meth (Peterson et al., 2007). Passive immunization of these mAbs in rodents reduced meth distribution to the brain and attenuated the effects of meth in self-administration and locomotor assays (Byrnes-Blake et al., 2003; McMillan et al., 2004). A first-generation vaccine containing a (+)-meth hapten known as MH6 (Figs. 5 and 10) conjugated to KLH and formulated with alum mitigated meth-induced thermoregulation, hyperlocomotion, and self-administration in rats (Moreno et al., 2011; Miller et al., 2013, 2015). Second-generation haptenes have focused on terminal sulfhydryl to carboxyl conversion (MH6-COOH; Fig. 10) (Collins et al., 2016). As mentioned previously, peptidic spacers have been incorporated into meth haptenes for increased anti-hapten antibody responses (Collins et al., 2016; Gooyit et al., 2017). A shortened glycine-terminal hapten (sGly; Fig. 10) conjugated to TT and formulated with alum + CpG ODN attenuated meth-induced hyperlocomotion in mice (Collins et al., 2016). Because a diglycine-containing hapten (Gly2) showed promise, a survey of peptidic spacers was performed and identified a monoalanine (Ala1) hapten (Fig. 10) that significantly reduced brain concentrations of meth in vaccinated mice (Gooyit et al., 2017). Other efforts in meth vaccine development include a succinyl methamphetamine hapten (SMA; Fig. 10)–KLH conjugate formulated with MPLA, which reduced meth-induced locomotor effects in mice (Shen et al., 2013). A SMA-TT conjugate formulated with alum also showed efficacy in the methamphetamine conditioned place preference model (Haile et al., 2015). However, the SMA hapten has been found to elicit antibodies with poor binding affinity for methamphetamine, presumably due to the substitution of the secondary amine in methamphetamine for a
secondary amide in the SMA hapten (Fig. 10) (Collins et al., 2016). Phenyl-substituted meth haptens have also been reported for active vaccination such as SMO9 and (+)METH P4 (Fig. 10). A SMO9-KLH conjugate formulated with alum elicited antibodies with high affinity for meth and ameliorated meth-induced impairment of behavioral responding for food (Carroll et al., 2011; Ruedi-Bettschen et al., 2013). The same conjugate formulated with TLR4 agonist glucopyranosyl lipid A/oil-in-water emulsion also elicited high-affinity anti-meth antibodies (Stevens et al., 2016). Lastly, a fully synthetic peptide construct linked to the (+)METH P4 hapten (Byrnes-Blake et al., 2001) generated meth-binding antibodies that could cause alteration of meth self-administration in rats (Duryee et al., 2009).

4. Cathinones. Cathinones are a class of psychostimulant drugs similar in structure and pharmacology to amphetamines but contain an aryl ketone (Fig. 5). Two cathinones, in particular 3,4-methylenedioxypyrovalerone and α-pyrrolidinopentiophenone (αPVP), have shown significant potential for abuse because of the observed rewarding and reinforcing effects of these drugs in rodent models (Watterson et al., 2014; Aarde et al., 2015a,b). These drugs were relatively unknown until reports of their recreational use first appeared in the 2000s, and a decade later they have been designated as schedule I drugs. Recently, vaccines against both 3,4-methylenedioxypyrovalerone and αPVP have been developed (Fig. 5), which consisted of the corresponding hapten–KLH conjugates formulated with alum and CpG ODN. In rats these vaccines could attenuate cathinone-induced hyperlocomotor activity, and the αPVP vaccine reduced self-administration of αPVP (Nguyen et al., 2017a).

B. Opioids

1. Heroin. Heroin is a highly addictive opioid that causes more overdose deaths in the United States than any other drug (Warner et al., 2016). As shown in Fig. 4, heroin is a prodrug that rapidly deacetylates to metabolites 6 AM and morphine, both of which are MOR agonists (Selley et al., 2001; Andersen et al., 2009). Since the early report of a 6 AM-like hapten known as M-6-HS (Fig. 6) (Bonese et al., 1974), a similar hapten containing a longer linker conjugated to TT formulated with alum showed efficacy in a rat self-administration model (Anton and Leff, 2006). Further development of 6-position–linked haptons resulted in the inclusion of a tetraglycine spacer to yield M(Gly)₄ (Fig. 6) (Pravetoni et al., 2012c). This hapten was conjugated to KLH and formulated with complete and incomplete Freund’s adjuvants (complete Freund’s is forbidden for human use), and the vaccine formulations mitigated heroin biodistribution, antinociception, and self-administration (Raleigh et al., 2013, 2014). A hapten known as MorHap (Fig. 6), which is the opposite epimer of heroin at the 6-position (Matyas et al., 2013, 2014), has been shown to reduce heroin antinociception as a TT or CRM conjugate formulated with MPLA-containing liposomes (Jalah et al., 2015). In a different hapten design strategy, the linker has been attached to the heroin nitrogen (HerSH and HerCOOH) (Figs. 5 and 6). When HerSH was conjugated to KLH and formulated with alum, the resulting vaccine could attenuate heroin reinstatement, self-administration, conditioned place preference, and antinociception (Stowe et al., 2006; Schlosburg et al., 2013; Bremer et al., 2014). In contrast to other reported vaccines, the HerSH conjugate vaccine containing alum and CpG ODN could reduce heroin antinociception at a dose of >5 mg/kg (Bremer et al., 2014) compared with the single dose of 1 mg/kg used in other studies (Raleigh et al., 2013; Jalah et al., 2015). Furthermore, a second generation HerCOOH-TT conjugate formulated with the same adjuvants reduced heroin antinociception at a doses of >10 mg/kg in mice and mitigated heroin-induced decreases in operant responding in nonhuman primates (Bremer et al., 2017).

2. Fentanyl. Fentanyl is a powerful MOR agonist (50–100 times more potent than morphine) and is
responsible for a recent surge in overdose deaths (Frank and Pollack, 2017). Fentanyl or various analogs of fentanyl such as acetylfentanyl are often mixed with heroin, thus enhancing the potency and danger of presumed heroin use (Centers for Disease Control and Prevention (CDC), 2013; Stogner, 2014). To date, one report of a fentanyl-TT conjugate formulated with alum + CpG ODN has been disclosed. This vaccine shifted the fentanyl dose–response curve by 30-fold in mouse antinociceptive testing and showed significant cross-reactivity with fentanyl derivatives that differed by one methyl group (Bremer et al., 2016). Furthermore, mouse serum antibodies bound fentanyl with single-digit nanomolar affinity.

3. Oxycodone and Hydrocodone. Oxycodone, also known as OxyContin, and hydrocodone, also known as Vicodin, are some of the most commonly prescribed opioids for pain relief, yet they are in the top 10 drugs involved in overdose deaths in the United States (Warner et al., 2016). The first oxy and hydrocodone haptenso were reported relatively recently and consisted of the tetragnocine spacer found in M(Gly)₄ linked to oxycodone via an oxime [OXY and HYDROC(Gly)₄; Fig. 11]. Immunization of the corresponding KLH conjugates formulated with Freund’s adjuvant could block oxycodone and hydrocodone biodistribution and antinociception (Pravetoni et al., 2012b,c, 2013). Interestingly, the OXY(Gly)₄ hapten showed much greater efficacy, even against hydrocodone, compared to the HYDROC(Gly)₄ hapten (Pravetoni et al., 2013). Further investigation of OXY(Gly)₄ revealed that TT and alum could be used with similar efficacy in place of KLH and Freund’s, respectively, whereas MPLA addition did not increase efficacy (Pravetoni et al., 2014b). The OXY(Gly)₄-KLH conjugate immunized with Freund’s adjuvant blocked acquisition of i.v. oxycodone in a rat self-administration procedure and mitigated oxycodone-induced modulation of brain gene expression (Pravetoni et al., 2014a). Investigation of oxy and hydrocodone haptenso linked through the nitrogen similar to heroin hapten HerSH (Fig. 6) showed significant right shifting (5- to 10-fold) of opioid dose–effect curves in antinociceptive testing of vaccinated mice (Kimishima et al., 2017). Additionally, the vaccinated mice showed resistance to opioid overdose mortality and reduced opioid biodistribution. Isolated serum IgG antibodies possessed subnanomolar affinity for both oxycodone and hydrocodone regardless of immunizing hapten, with minimal cross-reactivity to other opioids (Kimishima et al., 2017).

V. Conclusions and Outlook

In the last few decades, a wealth of studies has been published disclosing efforts to develop conjugate vaccines against a variety of harmful opioids and psychostimulants. First-generation cocaine and nicotine vaccines have failed in clinical trials, which may have dampened enthusiasm for the therapeutic viability of conjugate vaccines to ameliorate substance use disorder. We remain optimistic that these vaccines will one day meet clinical endpoints because of the major scientific advances that have been made in the design and development of the vaccines. Arguably, the most critical design element is the necessity of the hapten to possess maximal structural congruence to the target drug. A lack of adherence to this principle has most likely played a key role in the failure of the aforementioned vaccines in clinical trials. Specifically, stereochemistry should be preserved: racemic nicotine haptenso will not generate antibodies with optimal specificity to (-)-nicotine found in tobacco. Furthermore, hydrolytically labile esters in cocaine haptenso and inappropriately placed amides in both cocaine and methamphetamine haptenso in place of native amines will reduce antibody affinity to the target drugs, thus hampering vaccine performance. Second-generation haptenso have mostly addressed these shortcomings and will likely experience greater success in the clinic. Improvements to adjuvant formulation are also anticipated to boost efficacy of future vaccines entering clinical trials. For example, addition of TLR9 agonist CpG ODN to virtually any immunoconjugate–alum vaccine formulation has enhanced anti-hapten antibody responses.

An emerging trend in vaccine evaluation is the generation of drug dose–response curves in an appropriate behavioral assay. The degree to which a vaccine shifts these dose–response curves gives a quantitative measurement of the vaccine’s immunoantagonistic capacity as a relevant representation of efficacy. However, it is currently not clear what minimum fold-shift benchmark is required for efficacy against substance use disorder, and the benchmark will most likely depend on the type of drug and the severity of an individual’s substance use disorder. First-generation vaccines were not subjected to rigorous preclinical testing and were most likely prematurely entered into clinical trials. Before pursuing clinical evaluation of second-generation vaccines, they should first show robust production of antibodies with high titer and drug affinity and should markedly shift drug dose–effect

---

![Fig. 11. Oxime-linked oxycodone and hydrocodone haptenso (Pravetoni et al., 2013).](image-url)
curves; preferably, this should be demonstrated in non-human primates to maximize the potential for translational efficacy in humans. Despite the failure of the first-generation vaccines in clinical trials, the vaccines proved to be safe and mitigated drug intake in the highest responding individuals. These studies provide theoretical grounds that redesigned vaccines, which meet the aforementioned preclinical requirements could perform well in clinical trials.

Because no vaccines against drug abuse have been FDA-approved, the specific clinical applications of the vaccines have not been determined. One could imagine conjugate vaccines being used to prevent first-time drug use, drug overdose, or drug dependence. Furthermore, it could be used to assist active drug users in halting drug intake or to help former drug users in maintaining abstinence. A key limitation of immunotherapies for substance use disorder is that antibodies cannot ameliorate drug cravings and may precipitate symptoms of withdrawal from blunting drug potency. Fortunately, the exquisite selectivity of antibodies enables coadministration of pharmacotherapies that would quell cravings; in the case of opioid abuse, methadone and/or buprenorphine could be administered while vaccinating against heroin and/or fentanyl. Because substance use disorder occurs on a spectrum, vaccine therapy would most likely require case-specific tailoring of vaccine dosage, immunization schedule, and administration of adjunct pharmacotherapies.

References
Hatsukami DK, Jorenby DE, Gonzales D, Rigotti NA, Glover ED, Oncken CA, Harris JE and Baldessarini RJ (1973) Uptake of (3H)-catecholamines by homogenate of brain. 


Le CT, and Pentel PR (2005) Reduced nicotine distribution from mother to fetal brain. 


Martell BA, Fortin PL, Levesque K, and Janda KD (2006) Integration of nicotine into a vaccine against HIV-1 to enhance protective immunity. 


Hatsukami DK, Jorenby DE, Gonzales D, Rigotti NA, Glover ED, Oncken CA, Harris JE and Baldessarini RJ (1973) Uptake of (3H)-catecholamines by homogenate of brain. 


Harris JE and Baldessarini RJ (1973) Uptake of (3H)-catecholamines by homogenate of brain. 


Hogstede PL, Katoz D, van Spiegl PJ, Viechtbauer W, and van Schayck OCP (2014) Efficiency of the nicotine vaccine 3-AmmNic-HPA (NicVAX) co-administered with varicenine and counselling for smoking cessation: a randomized placebo-controlled trial. 


