Developing a Vaccine Against Multiple Psychoactive Targets: A Case Study of Heroin

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Abstract: Heroin addiction is a wide-reaching problem with a spectrum of damaging social consequences. Currently approved heroin addiction medications include drugs that bind at the same receptors (e.g. opioid receptors) occupied by heroin and/or its metabolites in the brain, but undesired side effects of these treatments, maintenance dependence and relapse to drug taking remains problematic. A vaccine capable of blocking heroin's effects could provide an economical, long-lasting and sustainable adjunct to heroin addiction therapy without the side effects associated with available treatment options. Heroin, however, presents a particularly challenging vaccine target as it is metabolized to multiple psychoactive molecules of differing lipophilicity, with differing abilities to cross the blood brain barrier. In this review, we discuss the opiate scaffolding and hapten design considerations to confer immunogenicity as well as the specificity of the immune response towards structurally similar opiates. In addition, we detail different strategies employed in the design of immunoconjugates for a vaccine-based therapy for heroin addiction treatment.

Keywords: Heroin, 6-acetyl-morphine, morphine, addiction, drug dependence, immunoconjugate, treatment, therapy.

1. INTRODUCTION

Heroin, (3,6-diacetylmorphine/diamorphine/morphine diacetate) was originally synthesized from morphine by the chemist Charles Alder Wright in 1874 [1]. The pharmacological potency of heroin in frogs and rabbits was later examined by Dott and Stockman [2], followed by a study in 1890 by the British Medical Association that found heroin was more effective in depressing the spinal cord and respiratory center in frogs and rabbits with a weaker narcotic action [3]. The pharmacology of heroin was studied by the physician von Mering [4] (who discovered hypnotic barbiturates [5]), the chemists Hoffman and Eichengrun (who were instrumental in the discovery of aspirin [6]), and Dreser [7] before being marketed as a cough suppressant by the German chemical company Bayer. Although not initially regarded as an addictive substance, the abuse properties of heroin quickly became apparent with intravenous use, particularly in the United States, where its use was restricted to prescriptions by the Harrison Act in 1914 and banned outright in the United States in 1924 [7]. Heroin is currently regarded as a drug of abuse included in the United Nations list of narcotic drugs under international control, but has not been completely outlawed for medicinal use in some countries [8].

Despite its potential usefulness in providing pain relief, heroin is a highly addictive substance with high negative costs and impact on society. Heroin is abused in most countries worldwide, with an estimated 8 million people (0.14% of the world's population) using heroin each year [9]. Even though heroin use makes up a small portion of total illegal drug use (less than 5%) [10], it was assessed as the most harmful abused drug based on physical damage to the user, the tendency to induce dependence and deleterious effects on families, communities and society [11]. It is the illegal drug with the highest mortality and emergency room visits according to the United Nations [12], with medical care, loss of productivity, crime and social welfare costs estimated at roughly $22 billion per year in 1996 [10]. Heroin is frequently administered by injection, with heroin and other opiates accounting for 83% of hospital admissions for injection drug abuse in the United States in 1999 [13]. As such, intravenous heroin use can be viewed as a driving force in the spread of HIV/AIDS, with an estimated ten percent of new cases worldwide attributed to injection drug abuse [14]. This situation is further exacerbated in developing and transitional countries: in 1999, injection drug abusers accounted for 69% of HIV infections in China, 66% of AIDS cases in Vietnam and 82% of HIV/AIDS cases in Central and Eastern Europe [15].

2. HEROIN AND ITS METABOLISM

After intravenous injection of heroin, users describe an intense 'rush' followed by a prolonged feeling of tranquility, reduced apprehension and euphoria lasting for several hours [16-18]. These pharmacological effects are generally attributed to heroin's action at opioid receptors in the brain, with the μ-opioid receptor considered as the major target. In comparison with other drugs of abuse such as nicotine [19], cocaine [20, 21] and methamphetamine [22-24], heroin is generally regarded as a prodrug that acts through its host of psychoactive metabolites that are more potent μ-opioid receptor agonists than heroin itself [25-27]. After injection, the half-life of heroin in humans is approximately 4 minutes before conversion to 6-acetylmorphine (6AM), a result of rapid enzymatic hydrolysis of heroin's 3' phenolic ester, predominately in the blood by erythrocyte acetylcholinesterase (AChE) [28]. The enzymes serum butyrylcholinesterase (BuChE) [29] and human carboxylesterase 1 [30] and 2 [31] (hCE1, hCE2) are also capable of hydrolyzing heroin's 3' ester. 6AM has a measured half-life of

approximately 22 minutes in humans before it is hydrolyzed to morphine by hCE1 and hCE2 in addition to erythrocyte AChE [28, 31]. In humans, morphine has a half-life of around 176 minutes, and is further metabolized to the non-psychoactive morphine-3-glucuronide (M3G) and psychoactive morphine-6-glucuronide (M6G), each of which possess half-lives of approximately 276 and 267 minutes, respectively (Fig. **1**) [32]. In comparing area under curve concentrations for heroin and all of its major metabolites, M3G is the major metabolite, followed by M6G, morphine, 6AM and heroin [32]. It should be noted that while M3G is a heroin metabolite in humans, guinea pigs, mice and rabbits, M6G is only a metabolite of heroin in humans, guinea pigs and rabbits, not rats or mice [33, 34].

Of particular immunopharmacological interest with respect to heroin and its psychoactive metabolites is that while they are all structurally similar to heroin, they vary in lipophilicity, and thus ability to cross the blood brain barrier (BBB). The two acetyl groups present on heroin's scaffold, in addition to heroin's pKa of 7.6 [35], confer lipophilicity, making heroin readily capable of crossing the blood brain barrier (BBB) [36]. It has been suggested that the brain uptake of heroin is controlled by the flow of blood into the brain [37], helping to create the intense pharmacodynamic effect felt by heroin users. This intense effect, or 'rush', is a major contributing factor to the highly addictive nature of heroin, as well as other drugs of abuse [38-40]. Interestingly, previous research has shown that decreasing the 'rush' felt after administration of cocaine by slowing entry into the brain can be as effective at minimizing the reinforcing effects of the drug as reducing the amount in the brain [41-43].

Heroin's first psychoactive metabolite, 6AM, also possesses a high degree of lipophilicity and readily crosses the BBB [36, 44]. The lack of a 3' blocking group of 6AM confers a much greater degree of analgesic potency in comparison with heroin. The literature strongly suggests that heroin is a prodrug acting primarily through its psychoactive metabolites [26], with 6AM being the causative agent for heroin's acute psychoactive effects after injection [27].

The fate of heroin and 6AM once across the BBB has been the subject of some debate. Thus, it was hypothesized in 1972 by Oldendorf *et al.* that after injection, heroin rapidly traverses the BBB and is hydrolyzed to 6AM and subsequently morphine [36]. However, it was found by Soreq *et al.* in 1999 that the form of acetylcholinesterase present in the brain did not significantly hydrolyze heroin or 6AM *in vitro*, leading to the hypothesis that once inside the brain heroin and 6AM are protected from the rapid enzymatic hydrolysis found in blood [28]. This hypothesis has been supported by the work of Andersen *et al.*, who found much higher brain concentrations of 6AM in comparison to morphine after injection of heroin or 6AM in mice [45], and by Karinen *et al.*, who determined that heroin is more stable in brain than blood [46]. Thus, despite the original predictions of Oldendorf, recent research provides significant evidence that once across the BBB, heroin and 6AM are more resistant to the rapid enzymatic hydrolysis occurring in blood.

The acute effects of the highly lipophilic heroin/6AM can be compared with the less lipophilic psychoactive heroin metabolites morphine and M6G. With respect to morphine, the work of Oldendorf *et al.* determined that 15 seconds after heroin injection in rats, the percentage of morphine brain uptake in relation to a highly diffusible reference substance was below the limits of measurability, while heroin brain uptake was 68 percent of the reference compound [36]. In addition, Andersen *et al.* determined the brain morphine concentration after injection of mice with heroin was too low to explain the immediate behavioral response
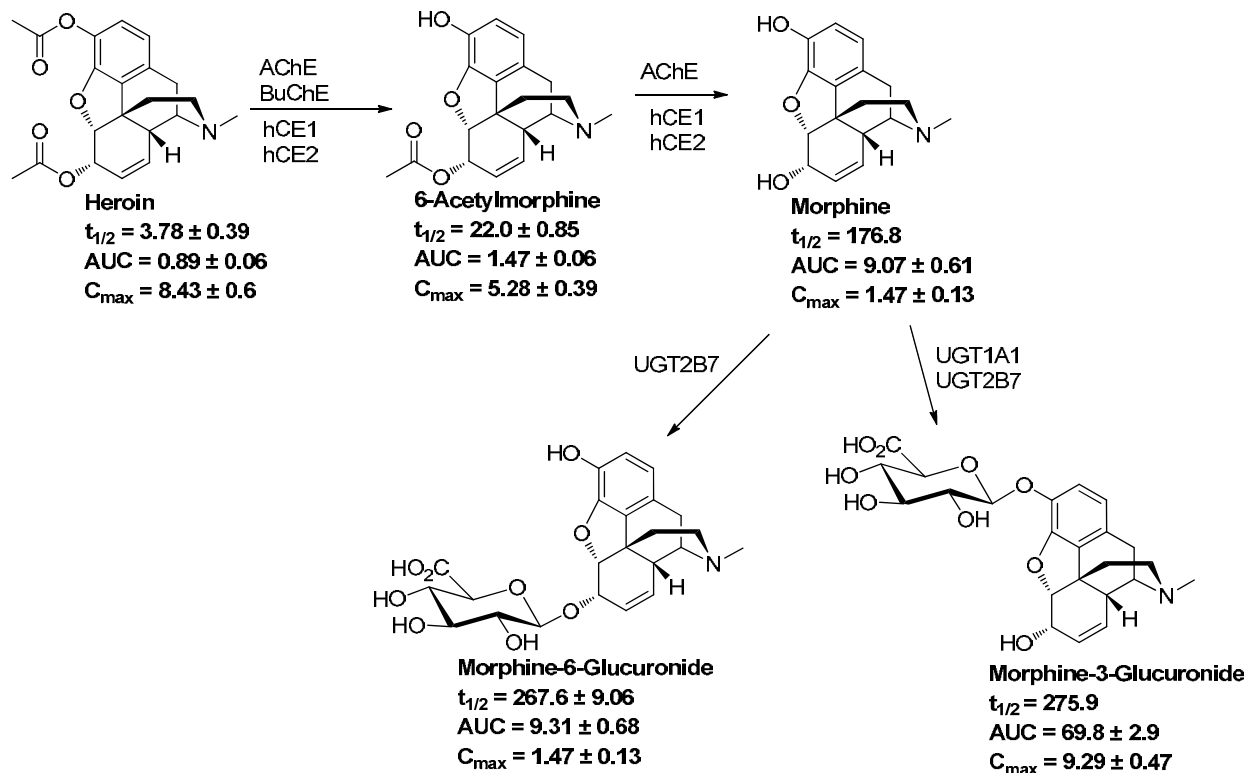

Fig. (1). Heroin metabolic pathway in humans. All half-lives units are minutes, C_{max} units are μmol/L, AUC units are hour · μmol/L. Adapted from *Rook et al.* 2006 [32].

generated. However, the concentration of brain morphine after disappearance of 6AM from the brain was sufficient to explain the prolonged behavioral effects observed after heroin injection [45].

M6G, heroin's phase II metabolite, has been shown to possess potent analgesic qualities in both rodents and humans, being slightly more effective than morphine after intravenous injection in rats [47]. But, it is also the least lipophilic psychoactive metabolite of heroin, and does not readily cross the BBB [48-50]. As a result, it has been found by many researchers to be ineffective for pain relief and have a lack of analgesic activity in humans. Thus, when M6G was injected in humans by Lötsch *et al.* to yield plasma concentrations equivalent to those following an analgesic morphine dosage, no analgesic activity was observed [51]. Motamed *et al.* also found injection of M6G was ineffective for postoperative pain relief in humans [52].

Given the data available, it can be proposed that heroin acts as a masked form of 6AM, which is the dominant force in heroin's immediate psychoactive effects, while the less lipophilic morphine is responsible for the longer lasting psychoactive effects of heroin. Since heroin and 6AM contribute to the 'rush' experienced after heroin injection, they could also be viewed as the major contributing forces towards heroin's highly addictive nature. The impact of M6G on heroin's prolonged psychoactive effects is debatable. While it has been shown to possess psychoactive efficacy similar to that of morphine, its inability to cross the BBB and lack of analgesic effects in many studies involving direct M6G injection provide evidence that it has no impact on the acute pharmacological effects of heroin, and may not be formed in sufficient amounts to enter the brain and contribute to heroin's long lasting psychoactive properties.

3. HEROIN ADDICTION AND REHABILITATION THERAPY OPTIONS

Drug addiction, also known as substance dependence, is a chronically relapsing disorder that is characterized by a compulsion to seek and take the drug, loss of control in limiting drug intake and the emergence of a negative emotional state (e.g. dysphoria, anxiety, irritability) when access to the drug is prevented [53, 54]. Memories related to drug use persist in the addict well into abstinence, during which time stress/drug associated cues, and the drug itself, can trigger craving or relapse, even many years after drug use has ceased [55].

In relation to heroin dependence, the alleviation of physical and emotional symptoms during acute withdrawal is currently accomplished by a number of mechanisms including treatment with the synthetic opioid methadone, a combination of clonidine and naltrexone, or buprenorphine [56, 57]. Once the primary withdrawal symptoms dissipate, drug treatment is gradually reduced. Unfortunately, while alleviation of the initial opiate withdrawal symptoms is well documented and can be accomplished with high reliability, it is only the starting point on the road to recovery. Long-term methods for the treatment of heroin addiction commonly rely upon pharmacotherapeutic interactions with μ-opioid receptors [55]. Many of these methods can be considered as 'harm reduction', wherein the patient is not encouraged to remain completely abstinent from all opioids, but instead given access to alternative substances that can help to reduce the cravings associated with heroin abstinence and allow the patient to live a productive lifestyle [58]. The success of these programs has been found to be dependent upon age of addiction onset and lifestyle of the addict after initiating treatment; however relapse is common [59].

For example, methadone is a long-lasting μ-opioid agonist commonly administered in 'harm reduction' heroin rehabilitation programs [60]. In the year 1999, there were about 115,000 heroin addicts maintained on methadone treatment in the United States [61]. While long-term methadone treatment can be an effective rehabilitation therapy, methadone is a potent opiate, and addiction

and overdose are possible; methadone-related poisoning deaths in the United States in 2005 reached a total of 4,462 [62]. Other μ-opioid agonist treatments such as buprenorphine also have side effects, some of which can be limiting [40]. Finally, methadone treatment is often unavailable in many areas of the developing world due to supply constraints or a lack of cultural acceptance [63, 64].

The μ-opioid antagonist naltrexone is also employed in heroin addiction rehabilitation, but it is not preferred by the majority of recovering addicts because it does not provide the opioid-like effects associated with methadone [65]. Opioid antagonists such as naltrexone have also been shown to block endogenous opioid function, resulting in potential dysphoric symptoms for the patient, and compliance can be an issue [55].

4. HEROIN IMMUNOPHARMACOTHERAPY AS A TREATMENT OPTION

Given the possible side-effects and limited efficacy of chemical agonist/antagonist treatments, additional treatment options could be useful to assist heroin addicts in their road to recovery. As an alternative strategy, the body's own immune system could be used to induce an antibody-based antagonism of heroin's psychoactive effects. Since antibodies do not cross the blood brain barrier (BBB) [66], a sufficiently high immune blockade could peripherally bind heroin and blunt its psychoactive effects without the negative side-effects associated with traditional chemical treatment options. The immune response should also be preferentially tuned to not bind endogenous opioids and other therapeutic compounds.

The feasibility of an immune system based drug blockade was first shown by Bonese *et al.* for the active vaccination of a single rhesus monkey against heroin, and has been subsequently employed by other groups for the construction of vaccines against cocaine, methamphetamine and nicotine [19, 20, 67-71]. These drugs of abuse are all relatively small molecules that do not illicit an immune response on their own, however the methodology used to construct all of these vaccines is similar. By covalent linkage of these small molecules to a carrier protein, an immune response can be generated *via* T and B cell activation that can be tuned towards the desired drug. This covalent linkage of a small drug molecule, known as a hapten, to carrier protein, generally relies upon condensation of a reactive organic functional group with free lysine or arginine amino acid residues on the protein. Since different proteins contain varying amounts of free lysine/arginine residues, the immune response can often be optimized by variation of carrier protein. The nature of the linker can also be tailored when optimizing immune response. Finally, an often underappreciated, but highly important, aspect to vaccine design is found in the attachment point of hapten to carrier protein. As such, variation of the hapten attachment point can vastly alter immune response quality, even when using the same linker and carrier protein.

5. LINKAGE IMPORTANCE FOR THE GENERATION OF OPIATE BINDING ANTIBODIES AND IMMUNE RESPONSE SPECIFICITIES

The opiate scaffolding to which heroin belongs presents a variety of moieties for appendage of a linker for carrier protein attachment to generate opiate binding antibodies. The positions used include the 2', 3', 6' and bridgehead nitrogen positions (Fig. **2**), with the antisera produced used primarily in immunoassays for the detection and quantification of opiates in urine and blood. The utilization of these functional groups is presented here in chronological order starting from the first known example.

In 1970, Spector *et al.* reported the synthesis of 3-O-carboxymethyl morphine **1** by reaction of morphine with sodium-β-chloroacetate in ethanol, followed by carbodiimide coupling of this compound to bovine serum albumin (BSA) and immunization of rabbits (Fig. **3**) [72]. Antibodies produced from this attachment

point were specific for morphine as determined by radioimmuno-assay measuring the displacement of ^{3}H dihydromorphine. However, codeine was even more effective at ^{3}H dihydromorphine displacement than morphine. This result was rationalized on the basis that the 3-O-carboxymethyl morphine hapten more closely resembles codeine than morphine.
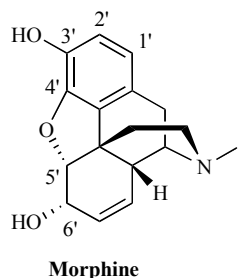
Morphine

Fig. (2). Numbering system for the 1' to 6' positions of the opiate scaffold.

In 1972, Wainer *et al.* described the synthesis of morphine-6-

hemisuccinate (M6H) **2** by reaction of morphine with succinic anhydride in refluxing pyridine (this reaction was initially thought to install the succinate at the morphine's 3' position) [73]. Morphine-6-hemisuccinate was also prepared independently by Simon *et al.* in the same year by reaction of morphine with succinic anhydride in refluxing benzene [74]. After preparation of M6H, anhydride coupling was used by Wainer *et al.* for BSA conjugation, followed by rabbit immunization (Fig. **3**). Antibodies generated from this attachment point bound to heroin, morphine and codeine with roughly equal affinity (as determined by inhibition of ^{14}C morphine binding inhibition) but did not bind the structurally similar opiate naloxone. The M6H BSA conjugate was also used by Koida *et al.* in 1974 for rabbit vaccination, with similar results for antibody specificity [75].

In 1973, Spector *et al.* coupled diazotized *p*-aminoacetanilide to the 2' position of morphine to give **3**, followed by carbodiimide coupling of the free amine to BSA [76]. The resulting immunoconjugate was used for the vaccination of rabbits, with the antibodies produced binding roughly equivalently to morphine,
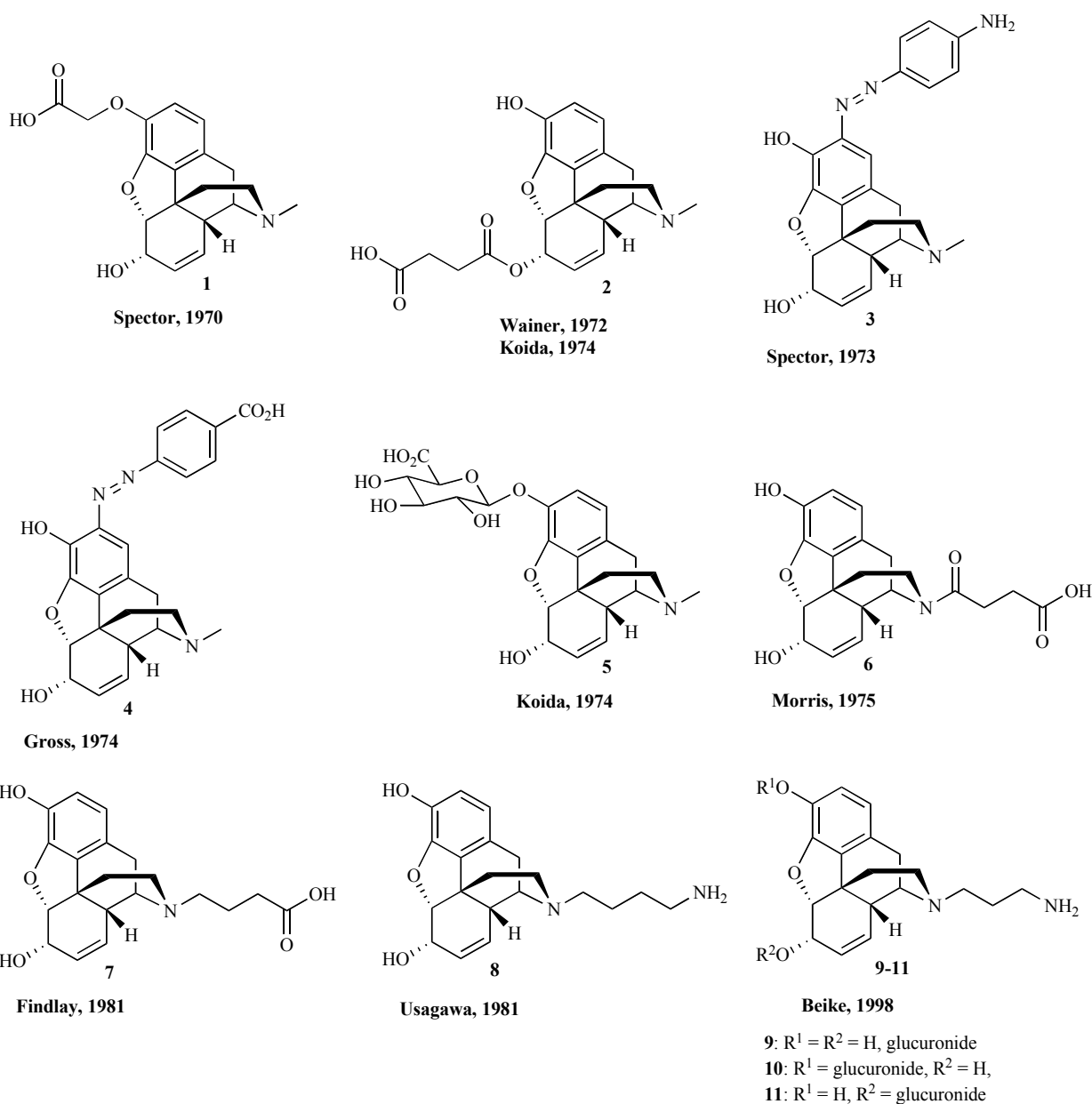

1
Spector, 1970

2
Wainer, 1972
Koida, 1974

3
Spector, 1973

4
Gross, 1974

5
Koida, 1974

6
Morris, 1975

7
Findlay, 1981

8
Usagawa, 1981

9-11
Beike, 1998

9: R[1] = R[2] = H, glucuronide
10: R[1] = glucuronide, R[2] = H,
11: R[1] = H, R[2] = glucuronide

Fig. (3). Linker attachment points for previous haptens.

heroin and codeine (Fig. **3**). The authors rationalized this cross-reactivity based on shielding of morphine's 3' hydroxyl group by the azo linker. In 1974, Gross *et al.* coupled diazotized ethyl *p*-aminobenzoate with morphine's 2' position, followed by benzyl ester hydrolysis of the resulting azomorphine to yield **4**, and carbodiimide coupling of the free carboxylic acid to keyhole limpet hemocyanin (KLH) [77]. This immunoconjugate was used for rabbit immunization, with purified IgG binding morphine with high affinity and showing little cross-reactivity for codeine or heroin. This result was surprising, given the extreme structural similarity of this immunoconjugate to that of Spector (Fig. **3**). Also in 1974, Koida *et al.* conjugated BSA to M3G through the 6 position carboxylic acid of the glucuronide *via* carbodiimide coupling, followed by rabbit immunization [78]. Antisera produced by this immunoconjugate bound codeine, morphine and M3G with roughly equal specificity.

In 1975, Morris *et al.* synthesized *N*-succinyl-normorphine by carbodiimide coupling of normorphine with succinic acid to give **6**, followed by carbodiimide coupling with BSA (Fig. **3**) [79]. Sheep, rats and rabbits were immunized with this immunoconjugate; antibodies produced were reported to have no cross-reactivity with codeine in sheep and rats and no cross-reactivity with heroin with antibodies from sheep. In 1975, Findlay *et al.* synthesized N-carboxypropyl morphine **7**, followed by conjugation to BSA and rabbit immunization (Fig. **3**). Similar to the findings of Morris, antisera from this protocol had minimal cross-reactivity with codeine, while heroin cross-reactivity was not measured [80]. In 1993, Usagawa *et al.* prepared bridgehead nitrogen linked N-aminobutyl immunoconjugate **8** by reaction of normorphine with N-(4-bromobutyl)phthalamide (Fig. **3**), followed by amine deprotection and carbodiimide coupling to BSA [81]. After immunization of mice, this immunoconjugate produced morphine specific antibodies with little cross-reactivity for codeine, M6G and M3G. Finally, in 1998, Beike *et al.* prepared bridgehead nitrogen linked N-aminopropyl immunoconjugates **9-11** resembling morphine, M3G and M6G (Fig. **3**) which were specific only for morphine, M3G and M6G, respectively, and showed little cross-reactivity codeine, codeine-6-glucuronide and dihydrocodeine [82].

From the data available, the synthesis of an immunoconjugate linked at the bridgehead nitrogen yields the most specific immune response. This finding is in accord with the hypothesis of Gross, who suggested that when designing a hapten, functionality that is necessary for biologic activity should remain in its natural steric configuration [77]. Conversely, linker attachment at morphine's 3' position produces an immune response binding codeine in preference to morphine, while linker attachment at morphine's 6' position produces antibodies with equivalent specificity for heroin, morphine and codeine. Linker attachment at morphine's 2' position induces an immune response of greater specificity, with the location of the amide bond vital for success.

6. SYNTHETIC APPROACHES AND RESULTS FROM VACCINES FOR HEROIN ADDICTION THERAPY

There is, as we have discussed (vide supra), a large amount of literature data on linker attachment points for the construction of opiate binding antibodies. Hence, in a logical fashion, similar strategies were used for carrier protein attachment to construct vaccines for heroin therapy. Thus, in the initial groundbreaking work of Wainer and Bonese in 1974, BSA-M6H conjugate **12** was used for the vaccination of a single rhesus monkey (Fig. **4**). After immunization, heroin self-administration in this monkey was blocked; however, the antibody induced blockade was shown to be dose dependent, being overcome by increasing the heroin dosage [83]. Despite the promising results of this study, no additional work on a heroin vaccine was performed for over 30 years, possibly due to the introduction of small molecule addiction therapies that looked more promising at the time.

In 2006, Anton and Leff revisited the work of Bonese, this time using linker extended tetanus toxoid (TT)-M6H immunoconjugate **13** [84]. Synthesis of this immunoconjugate was accomplished *via* construction of M6H using the procedure of Bonese, followed by carbodiimide coupling with derivatized TT (Fig. **4**). Vaccination of rats with this immunoconjugate produced a satisfactory immune response, with peak titer levels of ~ 1:100,000 occurring after the fourth biweekly boost. In comparing antibody specificities from this immunoconjugate, as determined by ELISA, heroin, 6AM, morphine, M6G and M3G were all bound equally. Since competition ELISA indicated heroin and its psychoactive metabolites were bound with equal affinity, a ^{14}C morphine radioimmunoassay was used to determine morphine specific antibodies, and by extrapolation heroin specific antibodies, in a concentration of 0.6-0.8 mg/mL. Competition ELISA indicated methadone, naltrexone, naloxone, Leu-enkephalin and β-endorphin were not bound by antibodies generated from this immunoconjugate. This vaccination protocol was successful, as the reinforcing effects of heroin were blocked in vaccinated rats, and reacquisition of heroin self administration after extinction was prevented.
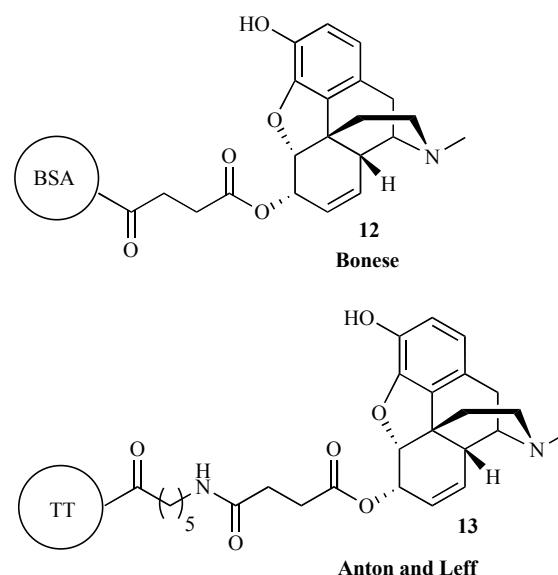

Fig. (4). Immunoconjugates synthesized by Bonese and Anton and Leff.

In constructing immunoconjugates for the stimulation of an optimal immune response, we have contended that proper hapten design is a critical component for immune recognition, and thus quality of the immune response [23, 85, 86]. In keeping with this postulate, we have recently completed a study towards a heroin addiction vaccine wherein the carrier protein was attached at the bridgehead nitrogen, rather than 6' position, of the opiate scaffold (Fig. **5**) [87]. Our design strategy was rationally thought out based upon the background literature available for the generation of opiate binding antibodies to potentially induce an enhanced degree of selectivity from the polyclonal immune response.

Hence, we visualized heroin and its metabolites not as a single entity that should be bound with equivalent specificity, but rather as a 'moving target', with the structural variations of the opiate scaffold resulting from heroin's metabolism yielding lipophilicity differences, thus modifying the ability to contribute to heroin's rewarding, and highly addictive, nature. Specifically, we hypothesized that in order to construct a more effective heroin vaccine, the two most lipophilic components (heroin and 6AM) should be the primary targets for antibody binding since: 1) heroin and 6AM readily cross the BBB and are reported to be responsible for the immediate rewarding effects ('rush') of heroin and 2) due to
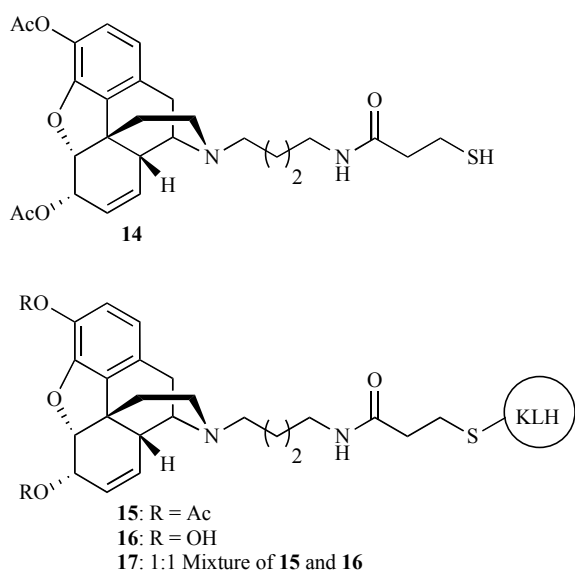
14

15: R = Ac
16: R = OH
17: 1:1 Mixture of **15** and **16**

Fig. (5). Bridgehead nitrogen linked heroin like hapten **14** and immunoconjugates **15-17**.

their lipophilic nature and increased stability in the brain *vs* blood, a vaccine designed to bind heroin/6AM could generate a concentration gradient, removing unbound heroin/6AM from the brain while also sequestering it from the blood's periphery [67]. Due to its intermediate lipophilicity, and thus intermediate capacity to traverse the BBB, we reasoned that the immune response should also be directed towards morphine. Given the low penetration of M6G into the brain, we did not consider it as a vaccine target.

We emphasize that this honed immune response to heroin's key psychoactive metabolites is in contrast to previous heroin vaccines, which have bound heroin and all of its metabolites, both psychoactive and non-psychoactive, with equal affinity, thus potentially decreasing the immune response towards the molecules most responsible for heroin's potency and highly addictive nature.

The synthetic procedure for our bridgehead nitrogen linked heroin-like hapten **14** started from heroin, followed by reaction of **14** with maleimide activated KLH carrier protein to yield heroin-like immunoconjugate **15** (Fig. **5**). KLH was selected based on its relatively low cost, with any positive findings being readily translatable to a different protein due to our modular hapten design. In terms of linker design, our hapten linker was appended to carrier protein by two amide bonds and a sufficient chain length to place it in free space away from the carrier protein, strategies we have previously found to generate a quality, high titer, immune response [86].

To optimize our tailored heterologous immune response for heroin and 6AM, we planned to take advantage of the protected environment created once the immunoconjugate is in formulation with alum adjuvant [88]. Adjuvants are vaccine additives that are combined in conjunction with the antigen to promote a strong, long lasting immune response with a minimum amount of antigen and reduced number of immunizations, and have been the recent subject of intense research [89, 90]. Of the available adjuvants, alum is the only one accepted worldwide and is generally regarded as safe, having been employed in hundreds of millions of vaccine formulations with few cases of severe adverse reactions [91]. Widespread use and safety led us to consider alum as the logical choice for our heroin vaccine with the potential for use in the developing and developed world.

The immunostimulatory activity of alum adjuvant was initially demonstrated by Glenny, who observed an increased immune response with a vaccine consisting of diphtheria toxin precipitated onto aluminum potassium sulfate [92]. Glenny postulated that alum

acted as a depot, keeping the vaccine at the injection site and slowly releasing it over a period of weeks or months to yield prolonged immune system activation [93]. This theory was supported by the findings of other researchers at the time [94], but has been challenged by others as the primary reason for alum's immunostimulatory effect [91, 95]. However, despite the debate surrounding the depot effect of alum, it has been shown to retain antigen at the site of injection in a protected environment for a period longer than injection of antigen alone [96, 97].

Thus, we reasoned that while heroin-like immunoconjugate **15** was in formulation with alum, it would be protected from the enzymatic hydrolysis that leads to the rapid degradation of heroin *in vivo*. As the immunoconjugate is slowly desorbed from adjuvant, we anticipated its metabolic degradation would present an immunostimulant mirroring heroin's degradation. We expected an immune response directed towards heroin and 6AM, since a continuous source of heroin immunoconjugate would be desorbed from adjuvant. We also anticipated an immune response directed towards morphine, although to a lesser degree, since some 6AM-like immunoconjugate would be metabolically degraded before immune system recognition (Fig. **6**).
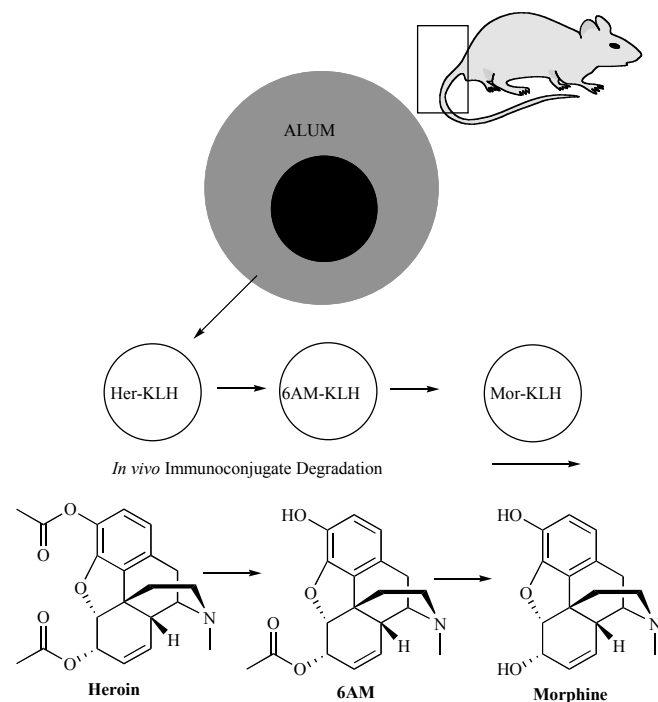

Fig. (6). Usage of Alum to create a selective immune response for opiates of interest. Her-KLH = Heroin-KLH, 6AM-KLH = 6-acetylmorphine-KLH, Mor-KLH = Morphine-KLH.

In addition to our metabolically degradable heroin-like immunoconjugate **15**, we vaccinated rats with morphine-like conjugate **16**, and a 1:1 mixture of heroin and morphine conjugates **17**. Based upon literature data, we reasoned morphine-like immunoconjugate **16** would produce antibodies primarily specific for morphine. Given heroin's rapid hydrolysis in the blood, we thought morphine specific antibodies could be useful to block heroin's psychoactive effects. Finally, based upon a pilot study performed in our laboratory (unpublished data), we hypothesized the 1:1 immunoconjugate mixture **17** could yield a truly heterologous immune response for heroin, 6AM and morphine (Fig. **5**).

Using a standard vaccination protocol, rats were immunized with the three immunoconjugates, as well as KLH protein alone, in formulation with alum adjuvant. A rapid, high titer immune response of ≈ 1:122,000 and 1:160,000 was observed for the

heroin- and morphine-like vaccines, respectively, while the immune response of ≈ 1: 39,000 was significantly decreased for the mixture **17** (determined by ELISA, Fig. **7**). As expected, heroin binding affinity was improved for the heroin-like *vs* morphine-like immunoconjugate. In addition, as determined by competition ELISA, heroin-like immunoconjugate **15** bound 6AM in the nanomolar range, while morphine-like immunoconjugate **16** did not bind 6AM. In line with our expectations, morphine-like immuno-conjugate **16** bound morphine significantly better than heroin-like immunoconjugate **15**. The 1:1 mixture of immunoconjugates **17** produced antibodies of roughly similar binding for heroin, 6AM and morphine as heroin-like immunoconjugate **15** (Table **1**).

To obtain more precise measurements, a radioimmunoassay was conducted using ^{3}H morphine for the heroin- and morphine-like vaccines **15-16**. The radioimmunoassay was not performed using antibodies from 1:1 vaccine mixture **17** due to its reduced titer level. From the radioimmunoassay, morphine specific antibody of 0.31 ± 0.01 and 2.84 ± 0.84 mg/mL was observed for the heroin- and morphine-like immunoconjugates, respectively, roughly equivalent to the morphine specific antibody of Anton and Leff. Since competition ELISA indicates heroin and 6AM are bound with greater affinity than morphine for the heroin-like vaccine, it is likely that specific antibody quantities for these substances are higher for this vaccine. However, due to a lack of both synthetic and commercial availability of ^{3}H heroin and [3]H 6AM, these values were not determined.
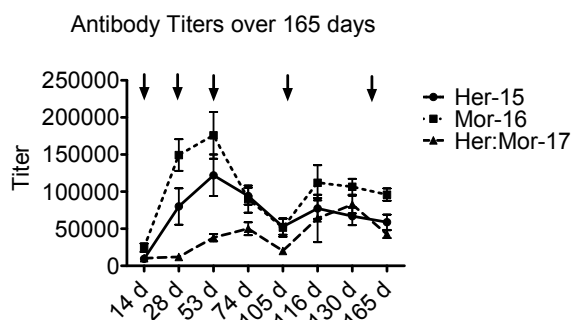
Antibody Titers over 165 days

Fig. (7). Vaccine titer levels over the course of 165 days. Vertical arrows represent booster injections at t = 14, 28, 53, 108 and 151 days. Data represented are the pooled mean value ± SEM.

Table 1.   Competition ELISA Data Obtained from Immunoconjugates 15-17

Entry	Heroin K_d[1,2]	6AM K_d	Morphine K_d
15	4.19 ± 1.01	0.035 ± 0.001	11.20 ± 1.11
16	14.18 ± 6.62	>100	1.18 ± 0.19
17	4.15 ± 1.22	0.037 ± 0.001	10.8 ± 1.00

[1]All K_d values are reported in μM.
[2]Data is the pooled mean sera value ± SEM.

To assess the effectiveness of the immune response from immunoconjugates **15-17**, the blockage of both thermal and mechanical antinociceptive effects of heroin were tested. Thus, the thermal and mechanical antinociceptive effects were blocked in rats vaccinated with the heroin-like immunoconjugate **15** and 1:1 mixture **17**. However, rats vaccinated with morphine-like immunoconjugate **16** were only blunted against heroin's antinociceptive effects as measured by thermal tests, although mechanical sensitivity did not show a change in antinociception from baseline levels (Fig. **8**). The same rats were then given access to heroin, with acquisition of heroin self-administration prevented in rats vaccinated with the heroin-like immunoconjugate **15**.

Both morphine-like and 1:1 mixture immunoconjugates **16** and **17** showed similar acquisition ability to the KLH control vaccine (Fig. **9**). As the next step towards a clinically viable therapy, we are currently testing the ability of our heroin-like vaccine **15** to prevent heroin-induced reinstatement of heroin-seeking, as well as blockage of the transition to heroin dependence otherwise produced by extended access to heroin [98].
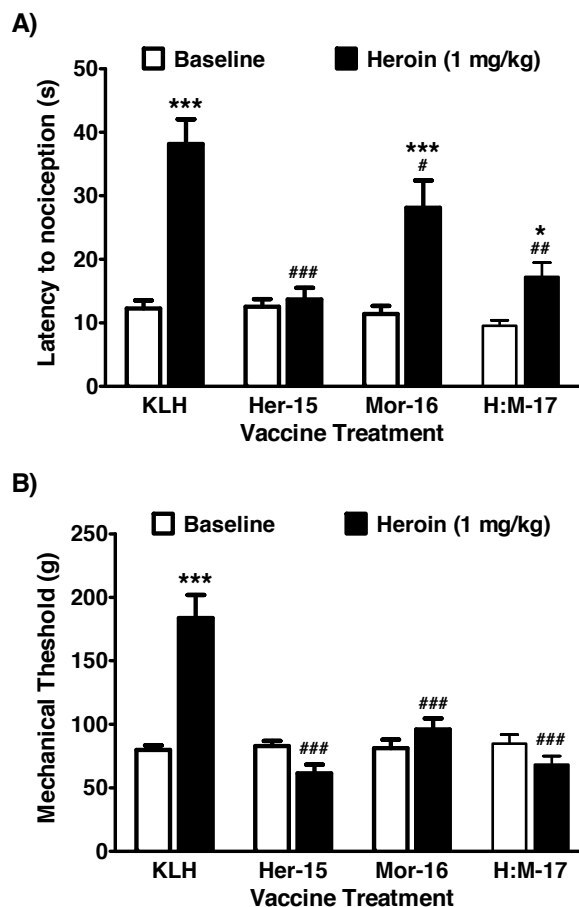
Fig. (8). Heroin vaccination selectively blocks the thermal and mechanical antinociceptive effects of heroin. Systemic injection of heroin (1 mg/kg, s.c.) produced robust decreases in both thermal (**A**) nociceptive sensitivity as measured by hot plate, and mechanical sensitivity (**B**) as measured by von Frey filament testing. This was fully reversed in the heroin-like **15** vaccine group. Morphine-like vaccine **16** significantly blunted the thermal nociceptive effects of heroin compared to control, but was still significantly elevated from baseline, while able to fully block mechanical effects of heroin. The combined vaccine **17** produced a partial blockade of heroin's thermal antinociception, while fully blocking the any changes in mechanical sensitivity due to heroin. N = 7-8 per group, *$p < 0.05$, ***$p < 0.001$, 30 min post-drug versus baseline; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, versus KLH response post-drug. Portions of this fig. were adapted from *Stowe et al.* 2011 [87].

While a comparison of peak titer levels from different experimental protocols is difficult to validate, we can state that our peak titer levels were achieved after only two boosts (three total injections) and in a minimum time period (53 days) in contrast to the vaccine of Anton and Leff which required four boosts (five total injections) and 68 days to reach peak titers. The significant decrease in titer levels generated from combination vaccine **17** could be due to an immune system diversion when different haptens are used simultaneously. It should also be noted that our vaccine did not
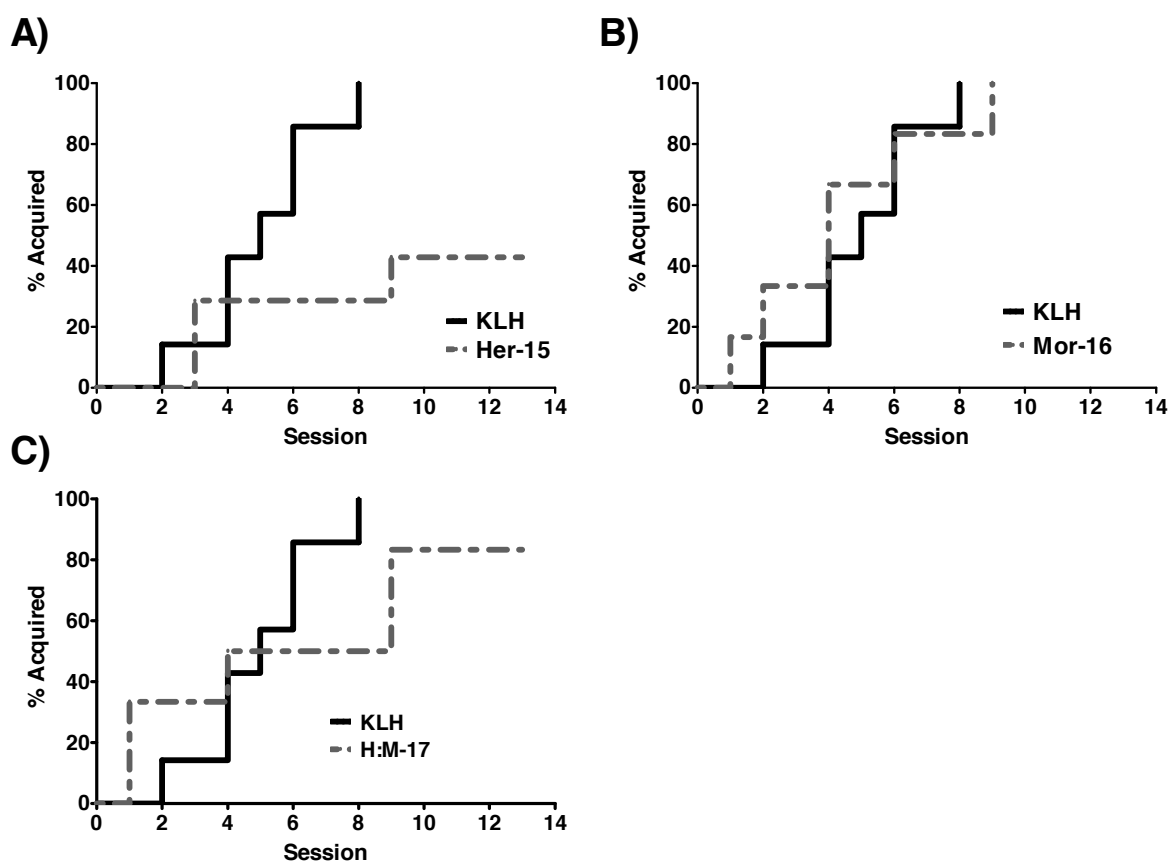
Fig. (9). Acquisition of heroin self-administration is prevented in a subset of rats vaccinated with heroin-like immunoconjugate **15**, but not morphine-like **16** or immunoconjugate mixture **17**. The percentage of animals that obtain the acquisition criteria, maintaining at least 3 consecutive sessions of 3 infusions (60 μg/kg/infusion) or more, is presented in the survival analysis graphs below. (**A**) The number of animals that acquired heroin self-administration was significantly lower in a group of rats receiving heroin-like vaccine **15** compared to KLH controls ($p < 0.05$). Only 3 of 7 rats receiving heroin-like vaccine **15** achieved criteria. (**B**) Conversely, the morphine-like vaccine **16** did not alter acquisition of heroin self-administration ($p = 0.96$). (**C**) Simultaneous vaccination **17** produced a delayed, but insignificant effect on heroin acquisition ($p = 0.25$). N = 7-8 per group. Portions of this figure were adapted from *Stowe et al.* 2011 [87].

engage an optimized carrier protein for immunostimulation, and other, more immunogenic, carrier proteins could be easily employed in subsequent studies. In contrast, the vaccine of Anton and Leff required significant optimization of carrier protein, as KLH, ovalbumin, tyroglubulin and BSA yielded inferior drug-specific antibody concentrations.

7. IMPROVING UPON HEROIN ADDICTION VACCINES

We believe that all vaccines currently under development against drugs of addiction can be improved, including our own heroin vaccine. Thus, what we view to be a logical step for the improvement of our current vaccine **15** would be a heroin-like immunoconjugate stabilized against enzymatic hydrolysis, but still significantly resembling the heroin scaffold. We envision that such a hapten could provide an immune response honed just to heroin. If this could be achieved, then we posit that such a highly specific immune response could be more beneficial as heroin is the primary opiate encountered upon injection. An immune response directed at sequestering only heroin before its crossing the BBB would essentially blunt the opiate's chain of neurochemical processing. As a possible additional benefit, any heroin that might escape the initial antibody induced blockade and enter the more hydrolytically stable environment found within the brain could be removed *via* Le Chatelier's principle. Since heroin is generally considered a prodrug acting through its psychoactive metabolites, this added antagonistic ability could prove highly useful.

To fulfill the goal of a more metabolically stable heroin-like hapten, conversion of heroin's 3' ester to an amide-based hapten **18**

is anticipated to facilitate a more robust vaccine, as the amide functionality is known to be more stable *in vivo* than an ester [99] (Fig. **10**). This conclusion can be reached based upon empirical evidence from our studies with heroin-like hapten **15**, and our documented success with cocaine-like amide **20** [100]. With respect to our results from **15**, our generated immune response has the highest affinity for 6AM, allowing the conclusion to be drawn that 6AM is the dominant antigen presented for immune recognition. This is not surprising, given the relative *in vivo* stability of 6AM. Thus, while heroin's 3' ester presents a liability in hapten-vaccine design, it may not be incumbent to convert heroin's 6' ester to an amide in order to confer additional stability. By analogy, cocaine-like amide hapten **20**, in which both of cocaine's labile esters have been converted to amides, was shown to produce a greater and more enduring suppression of cocaine-induced psychomotor behavior than cocaine-based hapten **19**, lending further support to this approach [20, 70].

8. CONCLUSION

Significant progress has been made in the pursuit of a clinically viable heroin vaccine candidate since the initial groundbreaking work of Bonese, and subsequent hapten-linker alterations conducted by Anton and Leff. Most recently, we have departed from the immunopharmacotherapy 'norm' designed to bind heroin and all of its related metabolites, but instead create a focused immune response directed towards heroin and its key lipophilic metabolites. In comparison to previous researchers, we contend that our vaccine represents a significant advance forward in the field of

immunopharmacotherapy. We believe the ability of our heroin- and morphine-like vaccines to rapidly generate peak titer levels from a minimum number of inoculations could be aided by the documented immunostimulatory properties of small quantities of compounds possessing the general opiate scaffold [101, 102]. However, it must be stated that the vaccine's ability to prevent excessive heroin self-administration and the dose dependency of the antibody-induced blockade must be determined. We are currently conducting these studies in our laboratory towards this goal.
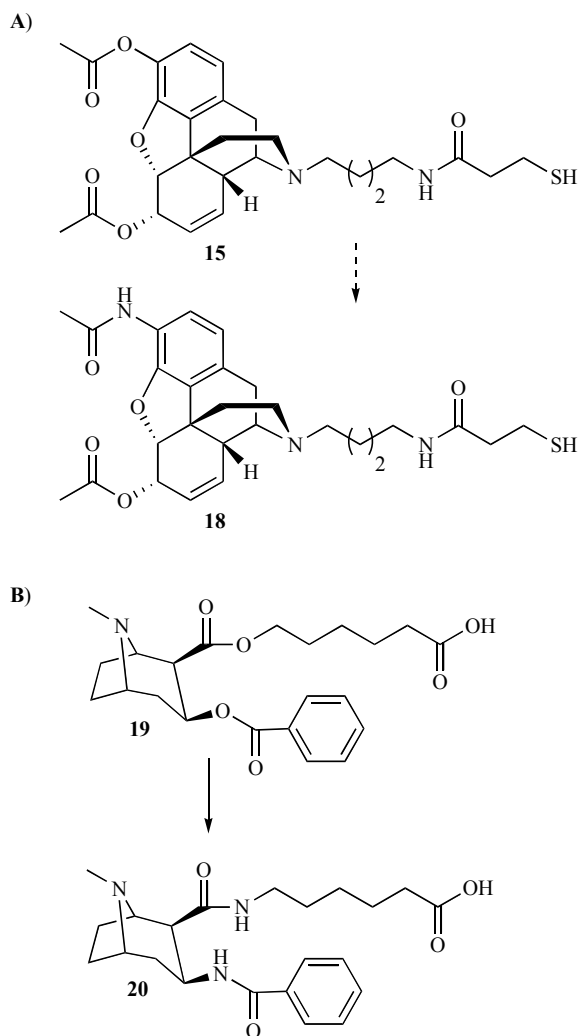
ACKNOWLEDGEMENTS

Fig. (10). A) Proposed conversion of heroin to stabilized amide derivative. **B)** Conversion of cocaine to stabilized amide derivative.

ABBREVIATIONS

BSA	=	Bovine Serum Albumin
KLH	=	Keyhole Limpet Hemocyanin
TT	=	Tetanus Toxoid
6AM	=	6-Acetyl Morphine
M6G	=	Morphine-6-Glucuronide
M3G	=	Morphine-3-Glucuronide
M6H	=	Morphine-6-Hemisuccinyl
BBB	=	Blood Brain Barrier
AChE	=	Erythrocyte Acetylcholinesterase
BuChE	=	Butyrylcholinesterase
hCE1	=	Human Carboxylesterase 1
hCE2	=	Human Carboxylesterase 2

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