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# Antibody production and pharmacokinetics of METH in rats following vaccination with the METH vaccine, IXT-v100, adjuvanted with GLA-SE

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ABSTRACT

*Background:* Methamphetamine use disorder continues to be inadequately treated, but improvements are being made in the field of immunotherapeutics, including vaccines, which could provide new options for treatment. Cocaine and nicotine vaccines have been tested clinically, but have yet to elicit the necessary antibody concentrations required to be effective. Methamphetamine vaccines have been tested in multiple nonclinical models and appear promising. Improved adjuvants have the potential to further stimulate the immune system to reach effective levels of antibodies. Previously, the methamphetamine vaccine IXT-v100 was administered with GLA-SE, a toll-like receptor 4 agonist, in mice to produce higher levels of antibodies than when it was administered with two other widely used adjuvants, Alhydrogel and Sigma Adjuvant System.

*Methods:* The purpose of this research was to evaluate IXT-v100, given in combination with the adjuvant GLA-SE, to determine its efficacy in antagonizing methamphetamine disposition in a rat pharmacokinetic study. Additional rat studies were conducted to compare the ability of IXT-v100 manufactured with greater hapten densities to elicit higher antibody levels.

*Results:* wAs expected based on prior studies with anti-methamphetamine monoclonal antibodies, the antibodies resulting from vaccination with IXT-v100 altered methamphetamine pharmacokinetics by increasing serum concentrations and extending the half-life. Furthermore, intentional variations in the ratio of components during manufacturing led to production of vaccines with higher hapten densities. The higher hapten densities resulted in production of antibodies that maintained the ability to bind methamphetamine with high affinity.

*Conclusions:* The results support continued development of IXT-v100 for the treatment of methamphetamine use disorder.

## 1. Introduction

According to the National Survey on Drug Use and Health, about 900,000 people in the US used methamphetamine (METH) regularly in 2015, and most were considered to have a METH use disorder (Center for Behavioral Health Statistics and Quality, 2016). While the absolute numbers fluctuate over time, METH use remains a significant problem worldwide for individuals and families, and for public health and judicial systems (Courtney and Ray, 2014). Currently available psychosocial treatments suffer from low rates of treatment initiation and also retention; pharmacologic treatments are in clinical trials, but none has yet been approved by the US FDA (Courtney and Ray, 2014).

Immunotherapy, both active and passive in the forms of conjugate vaccines and monoclonal antibodies (mAbs), is a promising pharmacologic strategy for combating substance abuse disorders. These therapies result in specific antibodies which antagonize and prevent the effects of the abused drug. In studies of mAb7F9, METH and amphetamine (AMP) serum concentrations were significantly elevated in antibody treated groups, resulting in increased area under the concentration-time curve (AUC), half-life, and reduced volume of METH

*Abbreviations:* AMP, amphetamine; ANOVA, analysis of variance; AUC, area under the concentration-time curve; CRL, Charles River Laboratories; GLA, glucopyranosyl lipid adjuvant; IM, intramuscular; KLH, keyhole limpet hemocyanin; mAb(s), monoclonal antibody(ies); METH, methamphetamine; PK, pharmacokinetic; SD, standard deviation; SE, stable emulsion; TLR4, Toll-like receptor 4

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**Table 1**
METH PK Experimental Design (study UAMS3127).

| Group[a] | Sub-group | Dose and Adjuvant | Regimen | Immuno Sampling | METH Dosing followed by PK sampling |
|---|---|---|---|---|---|
| 1 | a | NA | NA | NA | Wk 6 – 0.56 mg/kg,<br>Wk 7 – 1.68 mg/kg(Wks relative to 1b) |
| | b | 100 µg IXT-v100 + 5 µg GLA-SE | Intramuscular injection on Wk 0, 3, 9 | Wk -1, 3, 5, 9, 11, 12, 13 | Wk 11 – 0.56 mg/kg;<br>Wk 12 – 1.68 mg/kg;<br>Wk 13 – 3.0 mg/kg |
| 2[b] | a | SE only | Intramuscular injection on Wk 0, 3, 9 | NA | Wk 15 – 3.0 mg/kg<br>Wk 16 – 5.6 mg/kg |
| | b | 100 µg IXT-v100 + 5 µg GLA-SE | Intramuscular injection on Wk 0, 3, 9 | Wk -1, 3, 5, 9, 11, 12, 13, 14, 16 | Wk 15 – 5.6 mg/kg |

[a] Groups 1 and 2 were started two weeks apart. Within each group, subgroups a and b were run concurrently.
[b] Group 2 animals received additional METH challenges during weeks 5, and 11-14. These extra METH doses were used to measure a separate outcome that is not reported here.

distribution (Laurenzana et al., 2014). Further, a human-mouse chimeric version of the antibody, ch-mAb7F9, is safe in healthy human adults in doses up to at least 20 mg/kg (Stevens et al., 2014), and is currently undergoing Phase 2 clinical evaluation (STAMPOUT; NCT03336866).

Conjugate vaccines have been developed and tested for multiple psychostimulants, including METH vaccines from our group and at least 3 others, and opioids as recently reviewed by Bremer and Janda (Bremer and Janda, 2017). Results from previous clinical studies of cocaine and nicotine vaccines indicate that the most common reason for failure is a lack of sufficient antibody levels in most subjects (Bremer and Janda, 2017; Gorelick, 2012). Importantly, an adequate antibody response is a primary requirement for efficacy (Shen et al., 2012). More recently, adjuvants based on lipopolysaccharides that are Toll-like receptor 4 (TLR4) agonists have been developed and used in human studies with positive improvements in antibody titers (Duthie et al., 2011).

Immune Design Corp has developed a purely synthetic monophosphoryl Lipid A analog called glucopyranoside lipid adjuvant (GLA) which retains strong immunostimulatory characteristics, and has an excellent safety profile. Importantly, GLA is synthetically manufactured which provides advantages such as homogeneity (Coler et al., 2011). GLA is already in the clinic and is used in multiple Immune Design and partnered vaccine programs (Immune Design website and (Matthews et al., 2013; Treanor et al., 2013)). GLA-SE, made of GLA in an oil-in-water stable emulsion (SE) produces an enhanced antibody response to multiple targets (e.g., influenza HA, tuberculosis, and nicotine) (Coler et al., 2010, 2018; Duthie et al., 2011; Miller et al., 2014).

This work builds on previous published studies of the METH vaccine IXT-v100 (previously called $IC_{KLH}$-SMO9). In rats, IXT-v100 adjuvanted with Alhydrogel prevented METH-induced impairment of food maintained behavior caused by high METH doses (Rüedi-Bettschen et al., 2013). Next, doses of IXT-v100 adjuvanted with GLA-SE given to mice generated higher levels of antibodies than when adjuvanted with Alhydrogel or Sigma Adjuvant Systems (Stevens et al., 2016). Therefore, the current studies have used GLA-SE as an adjuvant. It was hypothesized that IXT-v100 adjuvanted with GLA-SE would also elicit high antibody levels in rats and thereby show favorably altered METH pharmacokinetics following METH challenge dosing.

## 2. Materials and methods

### 2.1. Drugs and reagents

(+)-Methamphetamine hydrochloride (METH) and the tritiated form of METH ([$^3$H]METH) were obtained from the NIDA Drug Supply Program. GLA-SE adjuvant was from Immune Design Corp. (Seattle, WA). Sulfo-SMCC (Sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate) crosslinker and TCEP − HCl (Tris(2-carboxyethyl)phosphine hydrochloride) were purchased from Thermo Fisher Scientific Inc. (Pierce, Rockford, IL). Vacmune®, two purified monomers of keyhole limpet hemocyanin (KLH), was obtained from Biosyn Corp. (Carlsbad, CA).

### 2.2. Vaccine production

IXT-v100 is a METH conjugate vaccine manufactured by linking KLH subunits, branded as Vacmune®, to a METH-like hapten called SMO9, via a chemical crosslinker. Early batches of the vaccine (originally called $IC_{KLH}$-SMO9) were produced at the University of Arkansas for Medical Sciences (UAMS) as previously described (Stevens et al., 2016) and hapten densities were determined by the method in Peterson (Peterson et al., 2014); see structure of vaccine and hapten in this reference). The METH pharmacokinetic (PK) study (designated UAMS3127) used vaccine produced at UAMS.

More recently, a commercial manufacturing organization, MilliporeSigma (St. Louis, MO), was contracted to produce IXT-v100 using the same conjugation chemistry at larger scale; batches used in this publication were up to 3 g. The three hapten density comparison studies reported here used IXT-v100 made by MilliporeSigma. To make batches with different hapten densities, the molar ratio of METH hapten to Vacmune® was intentionally adjusted during conjugation. Hapten densities were determined by hydrolysing the protein in acid with heat and quantifying the released METH hapten fragment by HPLC-UV; this method is usable over time for stability studies while the previous method (Peterson et al., 2014) can only be used at the time of production. Results are reported as mol hapten per mol KLH (mol/mol).

The final drug substance produced by MilliporeSigma is formulated as a 2 mg/mL solution in 50 mM sodium phosphate, pH 7.4, 5% sucrose and 0.1% polysorbate 80.

### 2.3. Pharmacokinetic study (study UAMS3127)

Male Sprague Dawley rats were purchased from Charles River Laboratories (n = 6/subgroup). Group 1a rats were not vaccinated (controls; Table 1). Group 1b and 2b animals were immunized with 100 µg IXT-v100 at weeks 0, 3 and 9. Just prior to dosing, IXT-v100 with a hapten density of 23 mol/mol was mixed with 5 µg GLA-SE adjuvant, then administered by intramuscular (IM) injection. Group 2a control animals were injected with the adjuvant carrier alone (SE).

Blood samples for immunogenicity testing were collected per the immuno sampling schedule (Table 1) by tail vein (or trunk blood at the end of schedule only), and allowed to clot at room temperature. Samples were centrifuged at 14,000 × *g* for 10 min, then the serum was transferred to a new tube and stored at < −30 °C. Immunogenicity testing by interpolated titer ELISA is described below. Anti-METH affinity (Kd) determinations were performed as in (Stevens et al., 2016).

METH doses of 0.56, 1.68, 3.0, and 5.6 mg/kg were administered subcutaneously to animals on the weeks indicated in Table 1; doses were calculated using free base weight. Blood samples for METH concentration analysis were taken after METH doses of 0.56 mg/kg at 0.0833, 0.5 and 2 h, and after METH doses of 1.68–5.6 mg/kg at

**Table 2**
Hapten Density Comparison Study Designs.

| Study Number | Group | # of Males | Hapten density (mol/mol) | Dose and Adjuvant | Regimen | Immuno Sampling |
|---|---|---|---|---|---|---|
| **CRL8623** | 1 | 5 | 7 | 100 µg | Intramuscular | Wk 0, 2, 5, 11 |
| | 2 | 10 | 14 | IXTv100 + 5 µg | injection on Wk | |
| | 3 | 10 | 27 | GLA-SE | 0, 3, 9 | |
| | 4 | 10 | 38 | | | |
| | 5 | 10 | 69 | | | |
| **CRL9226**[a] | 1 | 10 | 32 | | | |
| | 2 | 10 | 39 | | | |
| | 3 | 10 | 37 | | | |
| | 4 | 10 | 49 | | | |
| | 5 | 10 | 54 | | | |
| **CRL3126** | 1 | 10 | 40 | | | |
| | 2 | 10 | 41 | | | |
| | 3 | 10 | 43 | | | |

Each study was conducted separately and in the order listed. [a]Due to a protocol deviation in the second study (CRL9226), animals received only half the intended dose on the correct day in Week 3; the second half of the dose was administered two days later.

0.0833, 0.5, 2, 4, 6, 10, 24, 48, and 72 h. Serum was prepared and stored as above.

### 2.4. Hapten density comparison studies

Three sequential hapten density comparison studies were conducted at Charles River Laboratories (CRL; Shrewsbury, MA; Table 2). In each study, groups of male Sprague Dawley rats were immunized with IXT-v100 adjuvanted with GLA-SE using the same route and regimen developed at UAMS (Stevens et al., 2016). The dose was split into two equal aliquots, and injected into a rear thigh muscle in Weeks 0, 3, and 9. Due to a protocol deviation in the second study (CRL9226), animals received only half the intended dose on the correct day in Week 3; the second half of the dose was administered two days later. Each group received a different lot of IXT-v100 produced by MilliporeSigma with different hapten densities (see Table 2 for hapten densities tested). Sequential studies in separate groups of rats tested a narrowing range of hapten densities. Blood samples were taken for immunogenicity testing two weeks after each dose. METH affinity (Kd) determinations were performed as in (Stevens et al., 2016).

All animal experiments were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals under the oversight of the appropriate institutional animal care and use committees.

### 2.5. Interpolated titer ELISA

The concentration of anti-METH polyclonal antibodies in rat serum samples from UAMS3127 was determined by an interpolated titer ELISA technique similar to that used previously (Stevens et al., 2016). It used a mixture of purified IgG from polyclonal antibody sera taken from male Sprague Dawley rats immunized with the IXT-v100 vaccine made at UAMS as the calibration standard. Plates were coated with ovalbumin crosslinked with a METH-like hapten, then blocked with SuperBlock (Pierce Biotechnology, Inc.). Next, rat serum samples, standard curve, quality control, and blank samples were added in duplicate. The detection reagent, goat-anti-rat (Fc-specific) antibody labeled with alkaline phosphatase, was added last. Each step was incubated at room temperature for an hour and concluded by inverting the plates and shaking out the solution in the wells, followed by washing the plate to remove unbound material. Finally, the chromogenic substrate solution was added to the wells, the plate was incubated at 37 °C for 30 min and then read with a BioTek Epoch plate reader. Interpolated titers were back calculated against a 4-parameter standard curve generated using GraphPad Prism software (La Jolla, CA). Immunogenicity samples from the CRL studies used a very similar method, but a different calibration standard made from affinity-purified anti-METH antibodies isolated from rat polyclonal antibody serum since the previous calibration standard was used up. A correction factor has been applied to make the results comparable to those from the UAMS study.

### 2.6. METH and AMP quantitation

METH and its metabolite AMP were quantified in serum samples as previously described (Hambuchen et al., 2015) using an Acquity ultra-performance liquid chromatography system interfaced with a Quattro Premier XE mass spectrometer (Waters Corp, Milford, MA). The lower limit of quantification for both analytes was 1 ng/ml.

### 2.7. Statistical methods

METH and AMP concentrations, AUC, Cmax, and half-life values (Figs. 2 and 3, Tables 3 and 4) were compared by two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. Antibody concentrations from hapten density studies (Fig. 4) within each study were log transformed, then compared by one-way ANOVA followed by Tukey's multiple comparisons test. Statistical analysis was performed with GraphPad Prism software version 7.0 (La Jolla, CA). Differences were considered to be statistically significant at $p < 0.05$.

## 3. Results and discussion

### 3.1. METH PK studies in IXT-v100-vaccinated rats

Studies of anti-METH mAbs have previously shown that the serum concentration of METH is increased in the presence of mAbs compared

**Table 3**
METH and AMP half-life harmonic means ± pseudo SD (hours).

| | | METH Dose (mg/kg) | | |
|---|---|---|---|---|
| Analyte | Treatment | 1.68 | 3.0 | 5.6 |
| **METH** | IXT-v100 | 9.1 ± 3.0* | 10.2 ± 2.0*,[a] | 8.7 ± 1.3* |
| | Control | 1.6 ± 0.3 | 2.0 ± 0.7 | 2.1 ± 0.6 |
| **AMP** | IXT-v100 | 5.2 ± 3.1[a] | 5.6 ± 2.0*,[a] | 5.4 ± 2.1* |
| | Control | 1.9 ± 0.5 | 2.5 ± 0.8 | 2.5 ± 0.7 |

Asterisks indicate statistical significance compared to the Control group given the same METH dose. Half−life values for one animal in each group marked with superscript 'a' were not included because there were not enough data points to calculate half−life.

**Table 4**
METH area under the curve and Cmax are increased by vaccination with IXT-v100 (study UAMS3127).

| METH Dose (mg/kg) | AUC (ng/mL*hr) | | Average AUC/Dose | | AUC IXT-v100/Control |
|---|---|---|---|---|---|
| | IXT-v100 | Control | IXT-v100 | Control | |
| 1.68 | 5959 ± 3723* | 414 ± 81 | 3547 | 247 | 14.4 |
| 3.0 | 8070 ± 4891* | 733 ± 84 | 2690 | 244 | 11.0 |
| 5.6 | 9486 ± 5162* | 1365 ± 284 | 1694 | 244 | 6.9 |
| **METH Dose (mg/kg)** | **Cmax (ng/mL)** | | **Average Cmax/Dose** | | **Cmax IXT-v100/Control** |
| | IXT-v100 | Control | IXT-v100 | Control | |
| 0.56 | 605 ± 196* | 60 ± 14 | 1080 | 107 | 10.1 |
| 1.68 | 1093 ± 303* | 245 ± 56 | 650 | 146 | 4.5 |
| 3.0 | 1401 ± 390* | 392 ± 61 | 467 | 131 | 3.6 |
| 5.6 | 1784 ± 781* | 642 ± 197 | 319 | 115 | 2.8 |

Asterisks indicate statistical significance compared to the Control group given the same METH dose. The AUC from one animal in the IXT-v100 group given 3.0 mg/kg METH was excluded from the calculation because there were not enough data points to calculate half-life.

to controls, which coincides with decreased METH volume of distribution and brain concentration (Byrnes-Blake et al., 2003; Laurenzana et al., 2003). Therefore, a METH serum PK study (UAMS3127) was performed with IXT-v100 immunized rats to determine whether the anti-METH antibodies generated would be sufficient to alter METH concentrations. Groups of rats were immunized with three IM injections comprising 100 µg of IXT-v100 and GLA-SE adjuvant (Table 1).

An interpolated titer ELISA was used to determine the relative concentrations of anti-METH IgG in serum samples. Anti-METH IgG concentrations were low (8.7 ± 7.1 µg/mL) following the first dose (Week 3). Following the second dose, concentrations increased as shown at Week 5 (77 ± 28 µg/mL), but were highest following the third dose, during weeks 11 onward (Fig. 1). At week 11, the average serum concentration was 133 ± 65 µg/mL and the Kd for METH was 22 ± 10 nM, indicating that high affinity antibodies were produced in all rats.

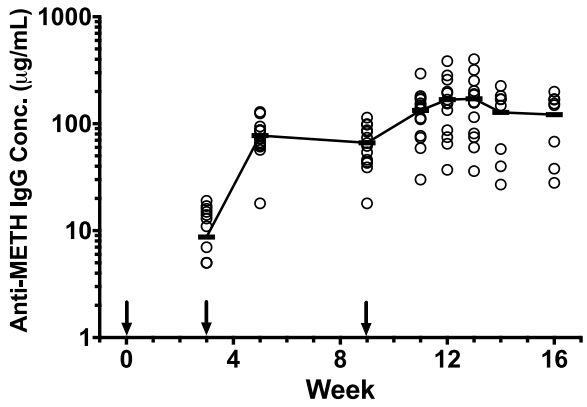



**Fig. 1.** Concentrations of anti-METH IgG over time following vaccination with IXT-v100 (study UAMS3127).
Individual animal data from vaccinated male rats are represented by open circles; horizontal bars indicate the mean. Responses from groups 1 and 2 were very similar and have been combined for weeks 3-13. In weeks 14 and 16, only group 2 data are shown since termination of group 1 occurred in week 13. Four samples from week 3 were below the limit of quantitation and were reported as 0; while not shown on the semi-log plot, the calculation of mean included them. Down arrows indicate vaccination time points.

Immunized rats (n = 6/group) were given METH challenge doses between weeks 11–15, when antibody concentrations were high, and sampled to analyze the serum concentrations of METH as detailed in Table 1 (Fig. 2). Control animals were given METH challenges starting at times convenient to the experimental schedule. At a low METH dose of 0.56 mg/kg, METH concentrations were significantly higher in immunized animals vs controls at all time points measured, from 5 min through 2 h post-dose. Doses of 1.68 and 3 mg/kg METH resulted in significantly higher METH serum concentrations for the first 4 h, and at 5.6 mg/kg, serum METH was significantly elevated for the first 2 h post-dose. At the three highest doses, samples from control animals were below the limit of quantitation at the last two time points whereas vaccinated animals still had detectable concentrations. As suggested by these figures, the serum elimination half-life of METH was extended by vaccination (Table 3); the increase was approximately 5-fold irrespective of METH dose. AMP concentrations were similarly elevated in animals vaccinated with IXT-v100 (Fig. 3) with the half-life also extended at least 2-fold (Table 3).

Frequent sampling allowed us to estimate the area under the concentration-time curve (AUC; Table 4). Individual animal METH AUC values were calculated from 5 min after the dose and extrapolated to infinity. The highest three doses resulted in significantly greater METH AUC in IXT-v100 treated animals compared with controls. There were not enough samples collected following the lowest dose to complete the calculations. Similarly, AMP AUCs were significantly higher following the 3.0 and 5.6 mg/kg METH doses (not shown). As previously reviewed (Bremer and Janda, 2017), AUC is a good metric for vaccine efficacy as sequestration of METH in serum reduces the dispersal of METH to tissues and organs, crucially the brain, in order to reduce detrimental health and behavior effects.

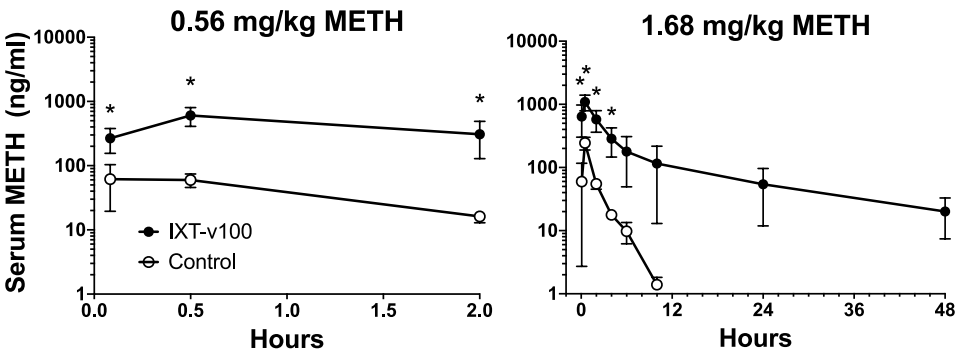
Normalizing the AUC to METH dose administered shows that, as expected, METH AUC increases proportionally to dose in controls (Table 4). This is not true for vaccinated groups; instead the AUC/Dose ratio is higher at lower METH doses, suggesting that the anti-METH antibodies are more effective at altering METH disposition when there is less METH present. While this is not surprising, even at the high METH dose of 5.6 mg/kg (equivalent to ~400 mg in a typical human), vaccination still increases METH AUC by 7-fold compared to the control value. The antibodies' ability to change AUC of METH doses between 1.68–5.6 mg/kg is only diminished by half even though the METH dose was increased over 3-fold.

Maximal METH concentrations (Cmax) were also increased by the presence of anti-METH antibodies (Table 4, lower half). At each concentration tested, the Cmax (30 min) was significantly greater in vaccinated groups compared to controls. Similar to the AUC data, normalization of the Cmax data to METH dose shows that the antibody increases Cmax better at lower doses, but continues to alter early METH concentrations by about 3-fold even over the range of a 10-fold dose increase.

Previous studies in rats with different METH vaccines have also indicated that anti-METH antibodies were capable of significantly increasing plasma or serum METH concentrations, generally in the range of ~2-3-fold, following an intraperitoneal (i.p.) or inhaled dose in female rats (Nguyen et al., 2017), i.p. dose in male mice (Gooyit et al., 2017), or subcutaneous challenge in rats e.g., (Miller et al., 2015). However, in each of the studies, METH concentrations were analyzed at only one or two selected time points. Our results are the first full investigation of METH PK in rats immunized with a METH vaccine. These PK data show that antibodies generated by vaccination with IXT-v100 plus GLA-SE are very effective at changing the disposition of METH, much like their mAb predecessors.

### 3.2. Hapten density comparison studies

Following initial efficacy testing of IXT-v100, a manufacturing platform was developed for large scale production. This work led to the ability to generate vaccines with a broader range of hapten densities.



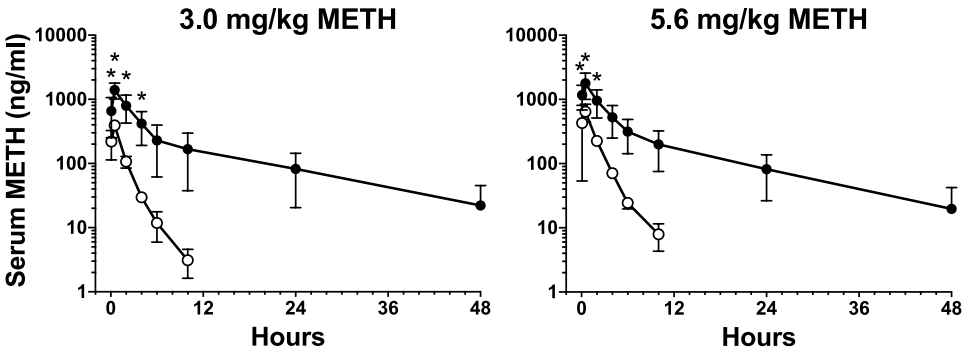
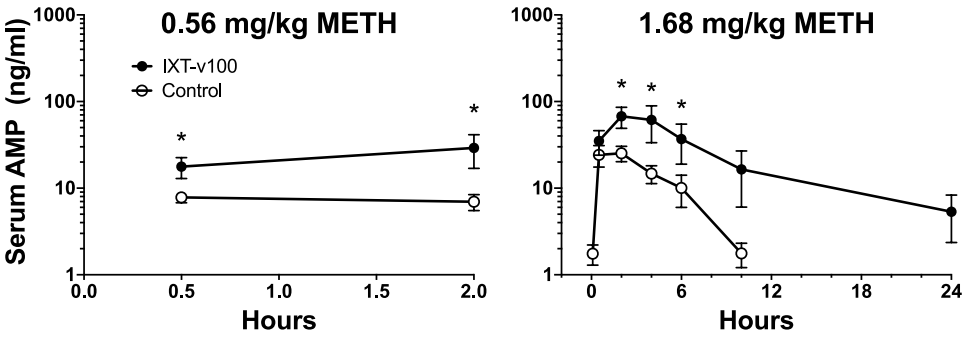




**Fig. 2.** Serum METH concentrations over time following METH challenge doses (study UAMS3127).
IXT-v100 vaccinated (black circles) or control (open circle) rats (n = 6) were administered the indicated METH doses followed by blood sampling for total METH concentration analysis (mean ± SD). Note the difference in x-axis scale for top left panel. Asterisks indicate significant differences between IXT-v100 treated and control groups at $p < 0.05$.



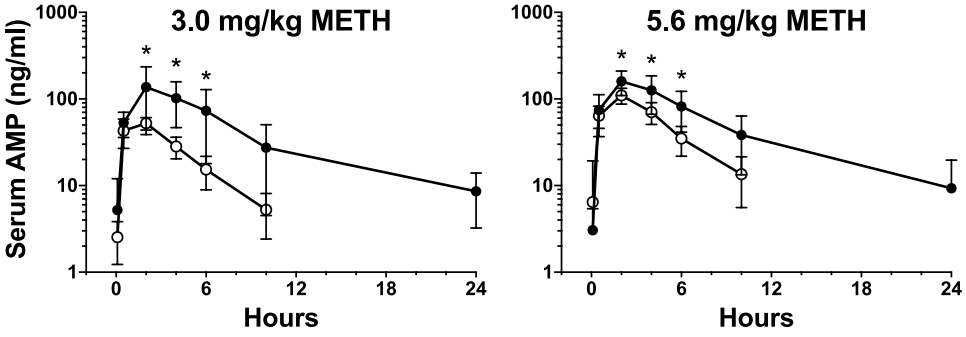




**Fig. 3.** Serum AMP concentrations over time following METH challenge doses (study UAMS3127).
IXT-v100 vaccinated (black circles) or control (open circle) rats (n = 6) were administered the indicated METH doses followed by blood sampling for AMP concentration analysis (mean ± SD). Note the difference in x-axis scale for first panel. Asterisks indicate significant differences between IXT-v100 treated and control groups at $p < 0.05$.

Previous studies have shown that increasing hapten density often results in greater antibody production, for instance, in nicotine vaccines using KLH as a carrier protein which is then attached to a liposomal nanoparticle (Zhao et al., 2017). We therefore tested IXT-v100 with multiple hapten densities in rats, and compared the results by measuring the antibody responses. Three studies were conducted at Charles River Laboratories (CRL; Shrewsbury, MA). The first two studies, using mg size lots of IXT-v100, examined a broad range of hapten densities. The third study compared three larger lots of vaccine all manufactured with a target hapten density of 40 (Fig. 4).

In the first study, CRL8623, a broad range of hapten densities, from 7 to 69 mol/mol, were tested. Increasing hapten density resulted in generally higher average anti-METH IgG production until the density reached an apparent maximum above which no significant amount of additional IgG was produced. Overall, group 4, with a hapten density of 38 mol/mol, generated the highest average (216 ± 135 μg/mL at week 11), and most uniform concentrations. The week 11 antibody concentrations from study CRL8623 group 4 were on average 160% higher than those from the UAMS3127 study at the same time point. Group 5 results averaged slightly lower; the IXT-v100 lot administered to this group also appeared cloudy and size exclusion chromatography suggested an increased level of aggregates. It was decided not to pursue

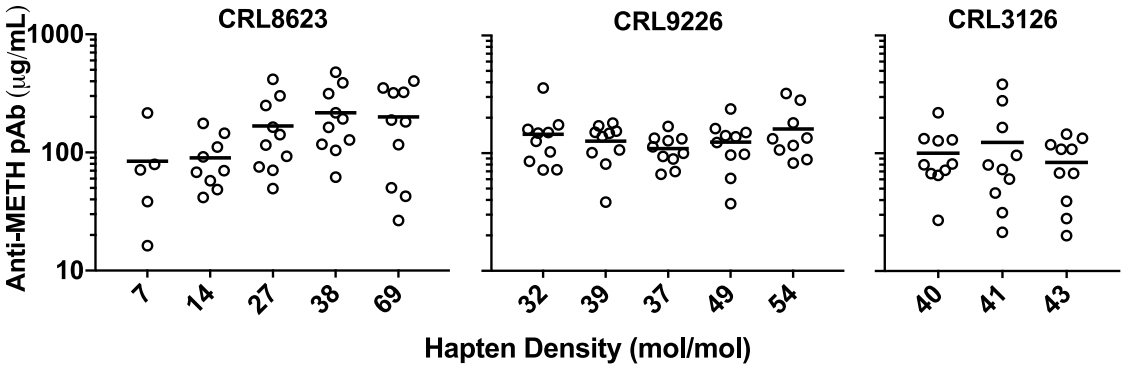



**Fig. 4.** Concentrations of anti-METH IgG produced by vaccination with IXT-v100 with varying hapten densities.
Rats were immunized with lots of IXT-v100 produced with different hapten densities. Relative anti-METH IgG concentrations from Week 11 derived from an interpolated titer ELISA are shown. Individual animal data are represented by circles; lines indicate the mean (n = 10 except for the group marked 7 in study CRL8623 which was n = 5). Due to a protocol deviation in the second study (CRL9226), animals received only half the intended dose on the correct day in Week 3; the second half of the dose was administered two days later. Statistical differences between groups were not identified within any study, however the adjusted p value for 7 vs 38 in study CRL8623 was 0.075.

such high hapten density manufacturing because the presence of aggregates makes it more difficult to manufacture a drug product to standards that are acceptable by regulatory agencies. Together, the results suggested that more moderate target densities were optimal for manufacturing and produced higher than previously reached antibody concentrations in rats.

To ensure that the antibody response retained the high affinity for binding METH expected from previous studies (Rüedi-Bettschen et al., 2013), we determined the Kd for each Group 4, Week 11 sample in study CRL8623 by testing for the ability to bind radiolabeled METH in the presence of increasing concentrations of unlabeled METH (inhibitors). The average Kd was 13 ± 1.5 nM indicating that the high affinity expected from this vaccine was maintained (Rüedi-Bettschen et al., 2013; Stevens et al., 2016).

Study CRL9226 was conducted using IXT-v100 lots with a hapten density range around 40 mol/mol. It appears to confirm that average IgG production remains approximately the same when similar hapten densities are compared; the averages ranged from 110 to 161 µg/mL. We also examined affinity for METH in each Group 2, Week 11 sample in this study. The average Kd was 15.7 ± 2.8 nM.

As noted in the methods, there was an unplanned protocol deviation during study CRL9226 which resulted in the Week 3 dose being administered at half the intended amount, with the remaining amount administered about 48 h later. It is unknown how this event may have affected IgG production relative to the prior study. However, since all groups still had high antibody responses, the target density for manufacturing continued to be 40 mol/mol for further development.

Three larger lots of IXT-v100 were compared in CRL3126, including two demonstration batches (≥1 g each) and a toxicology lot (3 g). Each lot was produced with a target hapten density of 40. On average, the three produced relatively similar responses (84–124 µg/mL average). This study confirmed that 40 mol/mol was repeatable on a gram scale manufacturing platform and resulted in similar anti-METH IgG production across multiple lots. Across the three hapten density studies, the overall average antibody concentration at week 11 in groups of rats (N = 99) given vaccines with a hapten density > 30 mol/mol was 139 ± 93 µg/mL.

### 3.3. Discussion of antibody concentrations

Other METH conjugate vaccines have been developed and tested in preclinical models of efficacy (reviewed in Bremer and Janda, 2017). However, each group has its own method for measuring antibody levels, i.e., many groups (Collins et al., 2016; Miller et al., 2015; Shen et al., 2013) report rat or mouse titers from a dilution series of serums which were allowed to bind to ELISA plates coated with different METH-like reagents; some also report pooled titers rather than individual results. Certain groups also report values determined by competitive radioimmunoassay with a back calculation to get antibody concentrations (Collins et al., 2016; Gooyit et al., 2017). It is unclear how antibody concentrations were derived from the mouse titers examined in Arora et al. (2019). Our assay compares individual serums in an ELISA against a calibration curve made from a pool of isolated polyclonal rat anti-METH IgG to determine an interpolated titer in units of µg/mL rather than a dilution value. These values are related to both the affinity and specificity of the particular pool of antibodies used. Somewhat similarly, Duryee et al. (2009) used a rat IgG as a standard curve to determine anti-METH antibody concentrations. The highest concentrations reported were between 1.5–2 µg/mL, but their standard curve was created from a specific pool of rat IgG, thus making the comparators different. Finally, there are differences in antibody production between mice and rats, with mice typically producing higher levels of antibody than rats. These rat anti-METH antibody values are therefore not comparable across laboratories due to significant differences in combinations of reagents, methods, and species.

### 3.4. Future work and conclusions

Further work is necessary to conclude that IXT-v100 will be effective at reducing human METH use. A study of the duration of antibody levels is currently ongoing in our laboratory; data through 24 weeks indicates that the response has leveled off at ~60% of maximum and could last for several months longer in rats. Studies using models such as METH self-administration could provide further efficacy data to support initial clinical trials. However, it is still difficult to predict from nonclinical data how well a vaccine may work in humans. In our case, this partially stems from species differences in response to toll-like receptor 4 (TLR4) activation by the adjuvant GLA-SE, a synthetic version of lipid A. TLR4 is naturally activated by lipopolysaccharide. While there are similarities, extrapolation of mouse data to humans is difficult due to differences in human and murine TLR4 sequences and responses, and rat TLR4 sequences are similarly divergent (reviewed in Vaure and Liu, 2014). These studies were undertaken in rats because of the blood volumes and frequency of sampling necessary for PK analysis. Due to greater sensitivities of human TLR4 to lipopolysaccharide relative to rats, it is possible that the anti-METH response in humans will be significantly higher.

It is yet unproven what concentration of antibodies, and minimum affinity values, may be required in humans to reduce METH use. Calculations done for a monoclonal anti-METH antibody have

suggested that ~50 μg/mL may be sufficient because there would be enough binding sites to capture all of the METH in the blood following a 30 mg IV dose of METH (Stevens et al., 2014). If this holds true, then our current vaccine candidate, which produces ~139 μg/mL in rats, appears to be likely to surpass this threshold and maintain it for many months. Exploratory trials in human subjects to optimize the dose and regimen will be the only definitive path to testing efficacy in reduction of METH use.

In conclusion, these current studies provide support for nonclinical efficacy of the METH vaccine, IXTv100, adjuvanted with GLA-SE. The vaccine elicits high levels of anti-METH IgG in rats and METH PK studies following vaccination (with a vaccine hapten density of 23 mol/mol) show that the polyclonal antibodies generated are capable of significantly changing METH distribution by sequestering it in the blood. This distribution change is expected to reduce brain METH levels soon after dosing as proven for anti-METH mAbs (Byrnes-Blake et al., 2003; Laurenzana et al., 2003) and other vaccines (Gooyit et al., 2017; Miller et al., 2013). A large scale manufacturing platform was developed which can repeatably produce vaccines at a target hapten density of 40 mol/mol. Altogether these data provide the rationale for pursuing continued development of IXT-v100 for use in the treatment of METH use disorders.

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## Contributors

All authors contributed to the design and conduct of the research and have approved the final version of this article.

## Declaration of Competing Interest

SMO has financial and fiduciary interests in InterveXion Therapeutic LLC, a pharmaceutical company. UAMS has licensed intellectual property developed by SMO to InterveXion Therapeutics LLC.

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