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Design, Synthesis and Biological Evaluation of a Bi-specific Vaccine Against α -Pyrrolidinovalerophenone (α -PVP) and 3,4-Methylenedioxypyrovalerone (MDPV) in Rats

Samantha J. McClenahan^a, Chad M. Kormos^b, Melinda Gunnell^a, Michael D. Hambuchen^a, Pamela Lamb^b, Frank Ivy Carroll^b, Anita H. Lewin^b, Eric C. Peterson^a, Samuel Michael Owens^a

^aDepartment of Pharmacology and Toxicology University of Arkansas for Medical Sciences, Little Rock, Arkansas, 72205, USA

^bResearch Triangle Institute, Research Triangle Park, NC, 27709, USA

Abstract

α -PVP (α -pyrrolidinovalerophenone) and MDPV (3,4-methylenedioxypyrovalerone) are potent abused stimulants that are members of the synthetic cathinone class of drugs. Although these drugs are taken with recreational intent, high doses can lead to unintended adverse effects including agitation, cardiovascular effects, sympathomimetic syndromes, hallucinations, and psychoses. One possible treatment is the use of a vaccine to block or attenuate adverse medical effects. These studies report the preparation of a vaccine that generates high affinity antibodies specific for both drugs and the pharmacological testing of this vaccine in male rats. Alkylation of a hydroxy- α -PVP analog with an appropriate thiol-bearing linker afforded the hapten. When hapten-conjugated carrier protein was mixed with adjuvant, the resulting vaccine stimulated production of antibodies in male Sprague Dawley rats that were found to significantly reduce α -PVP- and MDPV-induced hyperlocomotion as well as to significantly reduce the concentrations of MDPV drugs in critical organs. The novel vaccine produced high affinity antibodies against MDPV, (*R*)-MDPV, (*S*)-MDPV, and α -PVP. Cross-reactivity testing against nine structurally similar cathinones showed very limited binding, and no binding to off-target endogenous and exogenous

*Corresponding Author: S. Michael Owens, Phone: 501-686-5487. Fax: (501) 526-4618. mowens@uams.edu.

PRESENT/CURRENT AUTHOR ADDRESSES

Michael H. Hambuchen, Pharm.D., Ph.D., Assistant Professor, Pharmaceutical Science and Research, Marshall University School of Pharmacy, 1 John Marshall Drive, Huntington, WV 25755

AUTHOR CONTRIBUTIONS

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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compounds. Antibodies generated by this bi-specific vaccine also significantly shortened the duration of locomotor activity induced by both drugs up to a dose of 5.6 mg/kg in male rats.

Keywords

3,4-methylenedioxypyrovalerone; α -pyrrolidinovalerophenone; hapten; vaccine; affinity; pharmacokinetics

1. Introduction

The abuse and large number of synthetic psychoactive cathinones is a continuing concern in the United States and around the world [1]. These cathinones produce a range of pharmacological effects with mechanisms of action that are difficult to predict from their structures [2]. To add to the problem, drug users purchase these illegal drugs with common names like “bath salts” or “plant food” without knowledge of their potentially lethal chemical composition.

Structurally derived from natural cathinone (structure **1** in Figure 1), synthetic cathinones contain structural modifications at the aromatic ring, α side chain, and/or β side chain. A review of 30 new synthetic cathinone derivatives discovered from 2014–2017 grouped the derivatives into four main structural classifications (Figure 1): *N*-alkyl (**2**), 3,4-methylenedioxy-*N*-alkyl (**3**), *N*-pyrrolidinyl (**4**), and 3,4-methylenedioxy-*N*-pyrrolidinyl (**5**) derivatives [3]. The review noted that the most common cathinone-related designer drugs are *N*-pyrrolidinyl substituted analogs **4** and **5**.

Two synthetic cathinones commonly found in “bath salt” mixtures are 3,4methylenedioxypyrovalerone (MDPV) and α -pyrrolidinovalerophenone (α -PVP) (Figure 2) [4]. Both drugs are *N*-pyrrolidinyl substituted but fall into separate structural classes due to the methylenedioxy-substituent of MDPV. MDPV and α -PVP have similar potency, in vivo and in vitro, as norepinephrine and dopamine reuptake inhibitors [5–8]. Both drugs are commonly self-administered as racemates [i.e., (*R,S*)-MDPV or (*R,S*)- α -PVP], but the (*S*)-enantiomer is significantly more potent than the (*R*)-enantiomer in mice and rats [5,9,10].

Abuse of MDPV and α -PVP can have serious medical consequences including abnormal behaviors, profound ataxia, convulsions, severe neuropsychiatric reactions and adverse cardiovascular effects [11–14]. One treatment approach is by development of a conjugate vaccine, which would function by stimulating the production of anti-drug polyclonal antibodies (pAb) that would bind the drug in the bloodstream and limit its distribution into critical tissues like the brain [15–18]. For example, Pravetoni et al.[19] has reported that immunization with conjugate vaccines for oxycodone and morphine increases serum levels of the heroin metabolites 6-monoacetylmorphine (6-MAM), morphine, and oxycodone while reducing levels of 6-MAM and oxycodone in the brain of rats. Similar results have been shown in preclinical studies for other drug-conjugate vaccines including cocaine, methamphetamine, nicotine, and other opioids [17,18,20–22]. Thus, the disease target for vaccine treatment(s) for substance use disorders is the drug of abuse as opposed to small molecule treatments that mediate effects at a receptor or neurotransmitter uptake site.

The development of two separate vaccines, one for α -PVP and another for MDPV, has been reported [23]. The haptens chosen to produce these vaccines were conjugated via extension of the R_1 alkyl chain of structures **4** ($R_3 = R_4 = H$) and **5** (Figure 1), respectively. Each vaccine produced drug-specific pAb with little cross reactivity with the other drug. In rats, immunization with the vaccine derived from structure **5** reduced MDPV-induced wheel running while immunization with the vaccine derived from structure **4** ($R_3 = R_4 = H$) reduced α -PVP-induced wheel running as well as α -PVP intravenous self-administration [23]. However, the reported pAb affinities constants for MDPV and α -PVP were in the micromolar range, which is very low affinity, and the antibody titers dropped during testing requiring an additional booster immunization.

The goal of our study was to develop a novel bi-specific MDPV/ α -PVP vaccine to produce long-lasting, high affinity antibodies against both MDPV and α -PVP. A key component was the synthesis of a drug-conjugate vaccine with a hapten that preserved the common chemical structures of MDPV and α -PVP at the α and β side chains when conjugated at sites distal to the carrier protein. To this end, we designed a hapten bearing a linker attached at the aromatic ring, leaving the core structure of MDPV and α -PVP unperturbed. We hypothesized that treatment with this bi-specific conjugate vaccine would produce high affinity pAb capable of mitigating the pharmacological effects of MDPV and α -PVP. This report describes the preparation of the target hapten and the high affinity immune response in male Sprague Dawley (SD) rats, which attenuated the MDPV- and α -PVP-induced horizontal motion and reduced the distribution of the both drugs into organs through pAb binding of MDPV and α -PVP in serum.

2. Methods

2.1 General biological evaluation, drugs and chemicals

(*R,S*)-MDPV, (*R*)-MDPV, (*S*)-MDPV, α -PVP, MDMA (($\pm$)-3,4-methylenedioxymethamphetamine), methamphetamine, and phencyclidine were procured from the National Institute on Drug Abuse drug supply program (Rockville, MD). All other drugs and chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. The following radiochemicals used for characterizing immunochemical properties of the pAb were synthesized at the Research Triangle Institute: for studies of racemic MDPV, 1-(1,3-benzodioxol-5-yl)-2-[1-pyrrolidinyl-3,4- t_2]-1-pentanone hydrochloride, (MDPV- t_2 , specific activity 24.6 Ci/mmol); for studies of (*R*)-MDPV, (*R*)-MDPV- t_2 , 24.6 Ci/mmol; for studies of (*S*)-MDPV, (*S*)-MDPV- t_2 , 24.6 Ci/mmol; for studies of racemic α -PVP, α -PVP- t_2 , 25.1 Ci/mmol). Radiochemical (97%) and chemical (95%) purity for all radiochemicals was determined by HPLC. A report of these syntheses is in progress.

MDPV was extracted from rat serum and quantified by a chiral HPLC Mass Spectrometry/Mass Spectrometry (LC-MS/MS) method as previously described for MDPV [24]. Serum concentrations of racemic α -PVP were determined at the Pharmaceutical Sciences Research Institute (McWhorter School of Pharmacy, Samford University, Birmingham, AL). The method involved extraction and precipitation of rat serum (20 μ L) proteins with ice cold acetonitrile and analysis of the supernatant in positive ion mode by LC-MS/MS with 10 ng/mL of α -pyrrolidinopentiophenone- d_8 hydrochloride (α -PVP- d_8 ; Cayman Chemical,

Ann Arbor, MI) as internal standard. The lower limit of quantitation (LLOQ) across the validated range was set at 0.5 ng/mL and the upper limit of quantitation (ULOQ) was set at 1000 ng/mL. The accuracy and precision of the method was established by producing averages in the range of 85–115% and coefficients of variation of less than 15% for the quality control concentrations. Recovery of α -PVP from rat serum was consistent and reproducible, and averaged 91.7% compared to the analyte spiked into extracted rat serum over a concentration range of 1–1000 ng/mL. α -PVP was stable in extracted serum for at least 59 h at 4 °C, through three freeze thaw cycles, and when kept cold (approximately 4 °C) for 2 h. Similar methods were developed and validated for determining tissue (brain, heart and kidney) concentrations of MDPV and α -PVP, except that MDPV-containing organs were homogenized with a 1:4 ratio of water. The drug was extracted, and drug concentration was determined by LC-MS/MS as described for serum (see above). For α -PVP containing tissues, rat brains were homogenized with 4 volumes (1:5) of cold 5 mM ammonium acetate buffer. All other tissues (heart and kidney) were homogenized with 9 volumes (1:10) of ammonium acetate buffer.

2.2 Synthesis of MDPV-IC_{KLH} and Determinization of Hapten Epitope Density

Immunocyanin keyhole limpet hemocyanin (IC_{KLH}) was used as the carrier protein for the development of an α -PVP/MDPV bi-specific vaccine, which is a single vaccine that produces polyclonal antibodies that binds both MDPV and α -PVP. IC_{KLH}, obtained from Biosyn Corp. (Carlsbad, CA) is unlike native KLH because it is a highly purified form consisting of two stable subunit monomers with masses of ~360 and ~390 kDa [25]. The racemic α -PVP/MDPV-like hapten **15** (Supplemental Scheme 2) was conjugated to maleimide-activated IC_{KLH} to provide the conjugate used to evaluate the immunological response in adult male Sprague Dawley (SD) rats [25]. The validated method for determining epitope densities utilizes carbon-14 labeled cystine and reduced carbon-14 labeled cysteine as a radiometric tracer for estimating α -PVP/MDPV-like hapten epitope densities of the α -PVP/MDPV-IC_{KLH}.

2.3 Animals

Adult male SD rats (9 weeks, n=22) were purchased from Charles River Laboratories (Raleigh, NC) and housed 2 per cage with a 10-h light/14-h dark cycle, 22 °C environment. All testing was conducted during the light cycle. They were fed enough food pellets to maintain body weights at 300–350 g and had free access to water. Any procedures that caused potential pain or stress for the animals were conducted while under isoflurane anesthesia. Adequate depth of anesthesia was determined by response to a paw pinch and by monitoring respiration. Animals were housed in facilities that are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The use of rats was approved by the Institutional Care and Use Committee of the Universities of Arkansas for Medical Sciences in accordance with the “Guide for the Care and Use of Laboratory Animals” (National Research Council 2011).

2.4 Testing of Immune Response to α -PVP/MDPV-IC_{KLH} Vaccination in Male SD Rats

One week after arrival, rats were randomly assigned to groups (Sigma Adjuvant System (SAS) (Sigma-Aldrich) or IC_{KLH} vaccine). Rats in the SAS group were injected sc with 150 μ L of SAS; and rats in the vaccine group were immunized sc with 100 μ g of α -PVP/MDPV-IC_{KLH} plus 150 μ L SAS. Booster injections were administered at 3 and 9 weeks. For rats in studies of MDPV (n=5/group) or α -PVP (n=6/group), blood samples were collected via tail vein immediately prior to immunization (weeks 0, 3, and 9) and at weeks 2, 5, 11, and 16 after immunization. Blood samples were collected at the same time points. After centrifugation, the serum was collected and stored at -20°C . Optimal scheduling of immunization was based on previous experiments in our laboratory with a methamphetamine-conjugate vaccine [26].

Rapid Equilibrium Dialysis (RED; Thermo Fisher Scientific, Rockville, IL) and the MDPV- t_2 radioligand were used to determine pAb functional titers. While enzyme-linked immunosorbent assay (ELISA) can be used to follow α -PVP/MDPV pAb titers, we think it is preferable to assess pAb titers using an assay that measures binding to the ligand of interest (MDPV or α -PVP) instead of the hapten bound to carrier protein, because the free drug, not the hapten, is the functional target to be neutralized. For measurement of functional titers, each rat serum was diluted 1:50 in 0.1 M sodium phosphate with 0.15 M NaCl, pH 7.35 buffer, as described in Rüedi-Bettschen et al. [22]. An aliquot of each sample and 20 nM MDPV- t_2 was added to one chamber and buffer was added to the other chamber of the RED device with an 8,000 molecular weight cut-off membrane separating the two chambers. After gentle shaking at 4°C overnight to achieve bound and free equilibrium, the amount of MDPV- t_2 in each chamber was quantified by liquid scintillation spectrophotometry using 4 mL of Ecoscint A (National Diagnostics, Atlanta, GA) liquid scintillation fluid. The percent bound MDPV- t_2 in each serum sample was calculated from these data.

2.5 Determination of pAb affinities for MDPV and α -PVP, and cross-reactivity with selected cathinones and other drugs

Rat serum (n=5 for MDPV and n=6 for α -PVP) collected 11 weeks after the initial immunization was used to determine the ability of the pAb to bind endogenous and exogenous molecules. A competitive, magnetic bead-based radioimmunoassay (RIA) specific for IgG binding with tritium-labeled drug as a tracer was used to construct complete inhibition curves for MDPV, its two enantiomers, and α -PVP. The inhibitory concentration at 50% (IC₅₀) was determined for each drug. For testing of cross reactivity of structural analogs and other ligands, each ligand was tested in an RIA at two relatively high inhibitory concentrations of 10 and 100 μ M with 5 nM of MDPV- t_2 as the tritium labeled tracer.

2.6 Locomotor activity and duration of activity after administration of 0.3–5.6 mg/kg of MDPV and α -PVP in SAS control and vaccinated rats

Thirteen weeks after initial immunization, rats (n=5–6/group) were placed into open-field polyethylene chambers (60 cm $\times$ 45 cm $\times$ 40 cm) lined with cat litter (for contrast and absorption of urine) 60 min prior to drug or saline injection. Movement was recorded with overhead video cameras and analyzed using Ethovision 8 software (Noldus Information

Technology, Inc., Sterling, VA). After 60 min, rats were injected sc with saline or drug (MDPV or α -PVP in separate studies at 0.3–5.6 mg/kg) and movement was recorded for 6 h. Drug was administered in ascending concentrations with 2–3 days between injections. Horizontal movement for the 6 h after drug administration was summed for each rat and reported as total distance traveled (mean $\pm$ SD). The duration of drug-induced horizontal distance traveled was determined as the time required for each rat to return to their baseline activity for two consecutive 4-min bins. Baseline activity was defined as each rat's averaged saline-induced horizontal distance traveled plus one standard deviation.

2.7 MDPV and α -PVP serum and tissue concentrations after a sc 0.56 and 3.0 mg/kg MDPV or α -PVP challenge dose

Twelve weeks after initial immunization, serum drug concentrations from blood samples taken from the tail vein were determined at 30 min and 2 h following a 0.56 mg/kg sc dose of MDPV (n=5/group) and α -PVP (n=6/group) in the SAS and α -PVP/MDPV-IC_{KLH} rats. Three days later, the experiment was repeated with 3.0 mg/kg MDPV and α -PVP in respective rat groups. Blood samples were also collected 24 h after each drug administration to ensure clearance of drug from the serum. Blood samples were kept on ice for approximately 1 h to allow clotting, spun at $14,000 \times g$ for 7 min, and serum was stored at -80°C .

MDPV and α -PVP distribution into tissues was determined 20 weeks after initial immunization of rats. Rats within treatment groups were randomly assigned (0.5 or 2 h sacrifice times) and challenged with 3.0 mg/kg MDPV or α -PVP (sc). At 0.5 or 2 h, rats were anesthetized with isoflurane and decapitated. Serum, brain, heart and kidney were collected and stored at -80°C until analysis by LC-MS/MS as described above.

2.8 Statistical analysis

Statistical analysis, area under the curve (AUC_{30–120}) values and creation of figures was performed using GraphPad Prism software (V7, La Jolla, CA, USA). RIA IC₅₀ values were determined from plots of RIA dose response curves using a 4-parameter logistic regression model. All data were reported as mean $\pm$ SD. Results from the MDPV studies (functional titers, locomotor assay, serum and tissue concentrations) were analyzed using a two-way analysis of variance (ANOVA) with treatment as the between-subject factor and enantiomer, time, or dose as the within-subject factor. Following a statistically significant ANOVA, Holm-Sidak multiple comparisons tests (or simple main effect tests in the case of a statistically significant interaction) were performed for all pairwise comparisons. Results from the α -PVP locomotor assay were analyzed using a two-way ANOVA with treatment as the between-subject factor and dose as the within-subject factor. Following a statistically significant ANOVA, Holm-Sidak multiple comparisons tests (or simple main effect tests in the case of a statistically significant interaction) were performed for all pairwise comparisons. Results from the α -PVP serum and tissue concentration studies were first analyzed using an F-test. If an F-test revealed a statistically significant inequality between variance, an unpaired Welch's t-test was performed. In cases of equal variances, data were analyzed with an unpaired t-test.

3. Results and discussion

3.1 Strategy for Hapten Design

A major goal of hapten design was to produce anti-hapten antibodies with bi-specificity to two of the more potent psychoactive synthetic cathinones, MDPV and α -PVP (Figure 2). We chose to call this bi-specific vaccine α -PVP/MDPV vaccine or α -PVP/MDPV-IC_{KLH} (Figure 3). To maximize immune recognition, the common molecular features of these two cathinones were designed at the site distal to the point of carrier protein conjugation. This hapten design was accomplished by attaching the hapten linker group off the meta position of the aromatic ring of α -PVP. Use of a 10-methylene spacer group for the linker, by analog to the strategy successfully used for synthesis of a (+)-methamphetamine hapten [27], allowed for flexibility of the hapten on the carrier protein.

3.2 Functional Titers of the α -PVP/MDPV-Conjugate Vaccine

The α -PVP/MDPV vaccine, with epitope density of 26 mol of hapten per mol of IC_{KLH}, was mixed with Sigma Adjuvant System (SAS) and tested in two groups of male SD rats; one group was challenged with MDPV (n=5/treatment) and the second group was challenged with α -PVP (n=6/group). Functional serum titers of anti-hapten pAb binding by MDPV-*t*₂ after vaccination with α -PVP/MDPV-IC_{KLH} was variable at week 5 (Figure 4). Over time, variability in the binding decreased within each experimental group. The highest average binding and lowest variance of MDPV-*t*₂ binding occurred for all rat groups during weeks 11–16.

3.3 Affinity and Specificity of pAb

Competitive radioimmunoassay (RIA) binding studies were conducted to assess the binding specificities of the anti-hapten antibodies produced in the rats challenged with MDPV and α -PVP. The testing of pAb cross-reactivity was conducted with MDPV, α -PVP, nine structurally similar cathinones (Table 1), and other endogenous and exogenous ligands. The concentration of unlabeled drug required to inhibit fifty percent binding of radiolabeled drug (IC₅₀) to pAb in α -PVP/MDPV-IC_{KLH} immunized rats was determined using 11-week post-immunization rat sera from the MDPV study group. For MDPV, (*R*)-MDPV, (*S*)-MDPV and α -PVP, the IC₅₀ values were determined by using the corresponding tritium labeled [i.e., MDPV-*t*₂, (*R*)-MDPV-*t*₂, (*S*)-MDPV-*t*₂, and α -pyrrolidinovalerophenone-*pyrrolidino-3,4-t*₂ (α -PVP-*t*₂)] and unlabeled drugs. For all other compounds, MDPV-*t*₂ served as the radiolabeled drug. The IC₅₀ values for racemic MDPV (n=5) and α -PVP (n=6) were 6.6 ± 2.1 nM and 4.6 ± 3.7 nM (Figure 5, Table 1). For the individual (*R*)- and (*S*)-MDPV enantiomers, the IC₅₀ values were 3.9 ± 0.8 nM and 6.6 ± 2.3 nM, respectively (Table 1); however, the IC₅₀ values between the racemic drugs and the MDPV enantiomers were not significantly different.

Of the nine additional cathinones tested, only two were able to inhibit MDPV-*t*₂ binding at the high ligand test concentrations of 10 or 100 μ M. These cathinones were pyrovalerone (68% inhibition of a 10 μ M concentration) and 3,4-methylenedioxy- α -pyrrolidinobutiophenone (50% inhibition of a 100 μ M concentration). None of the tested neurotransmitters (dopamine, norepinephrine, epinephrine, serotonin), over the counter

drugs (pseudoephedrine, tyramine), or drugs of abuse (methamphetamine, MDMA, cocaine, phencyclidine) showed >50% inhibition of MDPV- t_2 binding at 100 μ M; indicating that there would likely be no clinically significant effects associated with pAb binding to these compounds in vivo. Overall, the binding specificity of anti-hapten antibodies suggests greatest affinity for MDPV and α -PVP with some mild cross reactivity with two structurally similar cathinones.

3.4 Effects of Vaccination on Locomotor Activity

α -PVP/MDPV-IC_{KLH} immunization protected against the stimulant effects of increasing MDPV and α -PVP (sc) doses in a locomotor activity assay. In SAS control rats, horizontal distance traveled progressively increased with increasing doses of MDPV (0.3–5.6 mg/kg), and α -PVP induced increases in horizontal movement with peak effects at 1.0 mg/kg α -PVP (sc) (Figure 6A and B). At doses greater than 1.0 mg/kg, α -PVP produced a decrease in horizontal distance traveled in SAS rats. Similarly, α -PVP-induced horizontal movement in α -PVP/MDPV-IC_{KLH} vaccinated rats was significantly lower at 0.56 and 1.0 mg/kg (sc) doses than in SAS rats (Figure 6B). Notably, the duration of MDPV- and α -PVP-induced horizontal motion was significantly shorter in α -PVP/MDPV-IC_{KLH} vaccinated rats than in SAS rats across all doses (0.3–5.6 mg/kg, sc) (Figure 6C and D).

3.5 Effects of Vaccination on Drug Pharmacokinetics

Serum, brain, heart and kidney drug concentrations following MDPV and α -PVP administration were measured at 30 and 120 min after dosing and then used to calculate the area under the concentration-time curve (AUC_{30–120}). This presumably post-absorption time period was chosen, since it included some of the greatest changes reported [28] in locomotor activity and stereotypic behavior after sc administration of MDPV. The same parameters were chosen for α -PVP for direct comparison to MDPV.

MDPV and α -PVP serum and tissue distribution studies were consistent with reductions in MDPV- and α -PVP-associated behavioral effects observed in vaccinated rats. Serum pharmacokinetic changes were evaluated at two doses, 0.56 and 3.0 mg/kg of MDPV or α -PVP, one week prior to the locomotor assay. Tissue distribution studies were conducted in rats after administration of 3.0 mg/kg MDPV or α -PVP following the locomotor assay. At 12 weeks post-immunization, α -PVP/MDPV-IC_{KLH} and SAS rats were challenged with 0.56 and 3.0 mg/kg MDPV (sc) or α -PVP (sc) (Figure 7). During the serum pharmacokinetic challenges, blood samples were collected at 30 min, 2 h, and 24 h after drug administration and used to determine the serum concentration of drug. MDPV, (*S*)-MDPV and (*R*)-MDPV were quantified by chiral LC-MS/MS as previously described [29]. Since a validated chiral LC-MS/MS method for determining concentrations of (*S*)- and (*R*)-enantiomers of α -PVP was not available, racemic α -PVP concentrations were quantitated by a LC-MS/MS method validated for these studies (see experimental section). After the acute challenge of 3.0 mg/kg MDPV (sc), the serum MDPV AUC_{30–120} was significantly increased in vaccinated rats compared to SAS control rats (Figure 7C). Serum AUC_{30–120} of α -PVP after acute challenges of 0.56 and 3.0 mg/kg were significantly greater in vaccinated rats than SAS rats at both challenge doses (Figure 7B, D). There was no significant

difference in AUC_{30–120} values between (*R*)- and (*S*)-MDPV enantiomers at either dose (Figure 7A, C).

Importantly, serum samples collected 24 h after drug administration showed MDPV and α -PVP concentrations in SAS and vaccinated rats to be below the lower limit of quantitation of the LC-MS/MS methods. Thus, the antibodies appear to regenerate their binding capacity for subsequent drug challenges in less than 24 hrs. The mechanism for this regeneration of binding capacity is presumed to be through in vivo clearance of unbound MDPV and α -PVP.

Rat tissue distribution studies at 20 weeks post-immunization, were carried out by administration of 3.0 mg/kg MDPV or α -PVP (sc) to SAS and vaccinated rats followed by collection of tissue samples at 30 min or 2 h post-dose. Significant effects of treatment were observed in the brain, kidney and heart AUC of (*R*)- and (*S*)-MDPV (Figure 8A). Results from the α -PVP tissue study were less pronounced (Figure 8B) than the MDPV tissue study (Figure 8A), showing a decreasing, but non-significant trend towards lower concentrations of α -PVP in brain, kidney, and heart (Figure 8B). The lack of significant differences in tissue concentrations after a 3.0 mg/kg α -PVP dose was consistent with the locomotor data where no significant difference was noted in horizontal movement at doses greater than 1.0 mg/kg of α -PVP, (Figure 6B). Nevertheless, the similarity between reductions in locomotor and pharmacokinetic effects supports the bi-specificity of the polyclonal anti-hapten antibodies for MDPV and α -PVP.

The development of a vaccine capable of producing pAb with high affinity for MDPV and α -PVP was achieved through a hapten design that maintained key structural similarities in the α and β side chains of MDPV and α -PVP. The pAb response to the immunization of rats with the MDPV IC_{KLH} vaccine showed remarkably similar high affinity binding for both compounds with little or no binding to nine structurally similar cathinones containing alterations at the α and/or β side chains. Antibody functional titers were reproducible across separate experimental groups (Figure 4). Interestingly, despite similar titers with higher affinity for α -PVP, the α -PVP/MDPV vaccine protected to higher doses of MDPV than α -PVP in the rats (Figure 6 and 8). While the relationship between antibody concentrations and affinity with the in vivo efficacy of drug conjugate vaccines is not well established, it may be possible that, although titers were not significantly different, the concentration of higher affinity pAb was greater in the MDPV challenged rats than the α -PVP challenged rats.

In pAb cross-reactivity studies, we found that while removal of the methylenedioxy group from MDPV to create α -PVP had virtually no effect on the reduced anti-hapten antibody affinity, replacement of the pyrrolidine ring greatly reduced anti-hapten antibody affinity (see Table 1). Modification at the α and β side chains essentially abolished anti-hapten antibody affinity; the exception was 3,4-methylenedioxy- α -pyrrolidinobutiophenone, for which very low affinity was noted. Since clandestine laboratories generate new structural analogs to circumvent drug laws, and their efforts frequently result in street preparations that are heterogeneous cathinone mixtures, it would have been beneficial to generate antibodies with more “global” reactivity toward cathinones. However, the high affinity of the pAb

produced in the current study (Figure 5) for two of the most potent and medically problematic synthetic cathinones, MDPV and α -PVP, provides an advantage over the separate MDPV and α -PVP vaccines previously reported, in which haptens conjugated off the α side chain (R_1 in Figure 1) did not generate pAb with bi-specificity for MDPV and α -PVP [23].

A secondary goal of the hapten design was to develop a hapten that would produce stereospecific antibodies targeted to the more potent (*S*)-enantiomers of MDPV and α -PVP [5,9]. Efforts to synthesize a stable (*S*)-MDPV hapten were unsuccessful due to rapid racemization which, although reversed-phase chiral resolution of the hapten **15** was developed, afforded partially racemized material, and to formation of a byproduct that, based on mass spectral analysis, appeared to be a thiazoline (**16**) formed by condensation of the thiol with the amide of **15** (Supplementary Scheme 3). At the same time, studies using the racemic α -PVP/MDPV vaccine demonstrated that the anti-hapten antibody affinities for (*R*)- and (*S*)-MDPV were not significantly different. Furthermore, the concentrations of (*R*)- and (*S*)-MDPV in serum following low- and high-dose challenges of MDPV were not significantly different (Figure 7A and C). Moreover, the vaccine was able to blunt MDPV-induced hyperlocomotion and to decrease organ concentrations of MDPV (Figure 6A and C and 8A).

Evaluation of the efficacy of the α -PVP/MDPV vaccine against α -PVP and MDPV in vivo, showed that the vaccine was able to attenuate hyperlocomotion elicited by MDPV and α -PVP up to doses of 5.6 mg/kg and 1.0 mg/kg, respectively, and to significantly reduce the duration of stimulant effects up to 5.6 mg/kg of both drugs. Additionally, MDPV concentrations were lower in organs of vaccinated rats than in organs of SAS rats after 3.0 mg/kg MDPV challenge. Recreational use of MDPV in humans is associated with blood concentrations of 10–50 ng/mL with intoxication and overdose occurring around 50–300 ng/mL [30]. At 3.0 mg/kg MDPV (sc) in male SD rats, serum concentrations were in the range of human intoxication and overdose suggesting the vaccine could potentially reduce MDPV-induced effects at doses used in humans.

4. Conclusions

Development of a bi-specific hapten capable of producing high affinity antibodies against both MDPV and α -PVP was achieved through a carefully planned chemical design of the racemic hapten. The resultant racemic vaccine produced antibodies with similar affinity for (*R*)-MDPV and (*S*)-MDPV (Table 1) that equally blocked drug disposition into organs, particularly the brain (Figure 8). Immunization with the α -PVP/MDPV vaccine in male SD rats led to significantly reduced locomotor distance traveled associated with sc challenges of MDPV and α -PVP up to doses of 5.6 and 1.0 mg/kg (respectively). Importantly, the duration of action of both drugs was significantly reduced at all doses up to the 5.6 mg/kg dose (Figure 6C and D). Cross reactivity with structurally similar compounds was limited, and there was no binding with off target endogenous neurotransmitters and exogenous compounds. The vaccine and immunization protocol produced a long-lasting and stable immune response over 16–20 weeks of testing. Overall, these data suggest that this α -PVP/MDPV vaccine could be potentially useful for treatment of MDPV and α -PVP substance

use disorders. However, the incidence of MDPV and α -PVP use and associated medical problems would need to justify the cost of vaccine development and clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS (INCLUDING SUPPLEMENTAL MATERIAL)

α-PVP	α -pyrrolidinovalerophenone
α-PVP-t_2	α -pyrrolidinovalerophenone- <i>pyrrolidino-3,4-t_2</i>
ANOVA	analysis of variance
AUC_{30–120}	area under the concentration-time curve from 30–120 min
DMA80	DCM:CH ₃ OH:conc NH ₄ OH (80:18:2)
IC₅₀	fifty percent inhibition of antibody binding of radiolabeled drug
IC_{KLH}	immunocyanin
KLH	keyhole limpet hemocyanin
LC–MS/MS	liquid chromatography with tandem mass spectrometry
LLOQ	lower limit of quantification
MDMA	($\pm$)-3,4-methylenedioxymethamphetamine
MDPV	3,4-methylenedioxypyrovalerone
MDPV-t_2	3,4-methylenedioxypyrovalerone- <i>pyrrolidino-3,4-t_2</i>
NMR	Nuclear magnetic resonance
ppm	parts per million
MS	mass spectra
pAb	polyclonal antibody
RED	rapid equilibrium dialysis
RIA	radioimmunoassay

SAS	Sigma Adjuvant Systems
SD	Sprague-Dawley
sc	subcutaneous
sulfo-SMCC	sulfosuccinimidyl-4-[N-maleimidomethyl]cyclohexane-1-carboxylate
TLC	thin-layer chromatography
ULOQ	upper limit of quantification
6-MAM	6-monoacetylmorphine
DCM	CH ₂ Cl ₂
THF	tetrahydrofuran
DMF	dimethylformamide
TFA	trifluoroacetic acid

REFERENCES

- [1]. UNODC. Global Synthetic Drugs Assessment: Amphetamine-type stimulants and new psychoactive substances; E.14.XI.6; 2014; 76.
- [2]. Horsley RR, Lhotkova E, Hajkova K, Feriencikova B, Himl M, Kuchar M, et al. Behavioural, pharmacokinetic, metabolic, and hyperthermic profile of 3,4-methylenedioxypyrovalerone (MDPV) in the wistar rat. *Front Psychiatry* 2018; 9. doi:10.3389/fpsy.2018.00144.
- [3]. Majchrzak M, Celi ski R, Ku P, Kowalska T, Sajewicz M. The newest cathinone derivatives as designer drugs: an analytical and toxicological review. *Forensic Toxicol* 2017; 36 (1), 33–50. doi: 10.1007/s11419-017-0385-6. [PubMed: 29367861]
- [4]. Leffler AM, Smith PB, de Armas A, Dorman FL. The analytical investigation of synthetic street drugs containing cathinone analogs. *Forensic Sci Int* 2014; 234, 50–56. doi:10.1016/j.forsciint.2013.08.021. [PubMed: 24378302]
- [5]. Kolanos R, Partilla JS, Baumann MH, Hutsell BA, Banks ML, Negus SS, et al. Stereoselective actions of methylenedioxypyrovalerone (MDPV) to inhibit dopamine and norepinephrine transporters and facilitate intracranial self-stimulation in rats. *ACS Chem Neurosci* 2015; 6 (5), 771–777. doi:10.1021/acschemneuro.5b00006. [PubMed: 25688761]
- [6]. Marusich JA, Antonazzo KR, Wiley JL, Blough BE, Partilla JS, Baumann MH. Pharmacology of novel synthetic stimulants structurally related to the “bath salts” constituent 3,4-methylenedioxypyrovalerone (MDPV). *Neuropharmacology* 2014; 87, 206–213. doi:10.1016/j.neuropharm.2014.02.016. [PubMed: 24594476]
- [7]. Simmler LD, Buser TA, Donzelli M, Schramm Y, Dieu L-H, Huwyler J, et al. Pharmacological characterization of designer cathinones in vitro. *Br J Pharmacol* 2012; 168 (2), 458–70. doi: 10.1111/j.1476-5381.2012.02145.x.
- [8]. Gannon BM, Rice KC, Collins GT. Reinforcing effects of abused “bath salts” constituents 3,4-methylenedioxypyrovalerone and α -pyrrolidinopentiophenone and their enantiomers. *Behav Pharmacol* 2017; 28 (7), 578–581. doi:10.1097/FBP.0000000000000315. [PubMed: 28570297]
- [9]. Gannon BM, Williamson A, Suzuki M, Rice KC, Fantegrossi WE. Stereoselective effects of abused “bath salt” constituent 3,4-methylenedioxypyrovalerone in mice: drug discrimination, locomotor activity, and thermoregulation. *J Pharmacol Exp Ther* 2016; 356 (3), 615–623. doi: 10.1124/jpet.115.229500. [PubMed: 26769917]

- [10]. Nelson KH, López-Arnau R, Hempel BJ, To P, Manke HN, Crissman ME, et al. Stereoselective effects of the second-generation synthetic cathinone α -pyrrolidinopentiophenone (α -PVP): assessments of conditioned taste avoidance in rats. *Psychopharmacology (Berl.)* 2018; 1–11. doi: 10.1007/s00213-018-5070-x. [PubMed: 29178009]
- [11]. Wojcieszak J, Andrzejczak D, Kedzierska M, Milowska K, Zawilska JB. Cytotoxicity of α -pyrrolidinophenones: an impact of α -aliphatic side-chain length and changes in the plasma membrane fluidity. *Neurotox Res* 2018; 34 (3), 613–626. doi:10.1007/s12640-018-9923-1. [PubMed: 29951896]
- [12]. Zawilska JB, Wojcieszak J. α -Pyrrolidinophenones: a new wave of designer cathinones. *Forensic Toxicol* 2017; 35 (2), 201–216. doi:10.1007/s11419-016-0353-6.
- [13]. Baumann MH, Solis E, Watterson LR, Marusich JA, Fantegrossi WE, Wiley JL. Baths salts, spice, and related designer drugs: the science behind the headlines. *J Neurosci* 2014; 34 (46), 15150–15158. doi:10.1523/JNEUROSCI.3223-14.2014. [PubMed: 25392483]
- [14]. Woloshchuk CJ, Nelson KH, Rice KC, Riley AL. Effects of 3,4-methylenedioxypyrovalerone (MDPV) pre-exposure on the aversive effects of MDPV, cocaine and lithium chloride: implications for abuse vulnerability. *Drug Alcohol Depend* 2016; 167, 121–127. doi:10.1016/j.drugalcdep.2016.08.008. [PubMed: 27520883]
- [15]. Hambuchen MD, Carroll FI, Rüedi-Bettschen D, Hendrickson HP, Hennings LJ, Blough BE, et al. Combining active immunization with monoclonal antibody therapy to facilitate early initiation of a long-acting anti-methamphetamine antibody response. *J. Med Chem* 2015; 58 (11), 4665–4677. doi:10.1021/acs.jmedchem.5b00220. [PubMed: 25973614]
- [16]. Carroll FI, Blough BE, Pidaparathi RR, Abraham P, Gong PK, Deng L, et al. Synthesis of mercapto-(+)-methamphetamine haptens and their use for obtaining improved epitope density on (+)-methamphetamine conjugate vaccines. *J Med Chem* 2011; 54 (14), 5221–5228. doi:10.1021/jm2004943. [PubMed: 21682289]
- [17]. Bremer PT, Kimishima A, Schlosburg JE, Zhou B, Collins KC, Janda KD. Combatting synthetic designer opioids: a conjugate vaccine ablates lethal doses of fentanyl class drugs. *Angew. Chem.* 2016; 128 (11), 3836–3839. doi:10.1002/ange.201511654.
- [18]. Bremer PT, Schlosburg JE, Banks ML, Steele FF, Zhou B, Poklis JL, et al. Development of a clinically viable heroin vaccine. *J Am Chem Soc* 2017; 139 (25), 8601–8611. doi:10.1021/jacs.7b03334. [PubMed: 28574716]
- [19]. Pravetoni M, Raleigh MD, Le Naour M, Tucker AM, Harmon TM, Jones JM, et al. Co-administration of morphine and oxycodone vaccines reduces the distribution of 6-monoacetylmorphine and oxycodone to brain in rats. *Vaccine* 2012; 30 (31), 4617–4624. doi: 10.1016/j.vaccine.2012.04.101. [PubMed: 22583811]
- [20]. Fox BS, Kantak KM, Edwards MA, Black KM, Bollinger BK, Botka AJ, et al. Efficacy of a therapeutic cocaine vaccine in rodent models. *Nat Med* 1996; 2 (10), 1129. doi:10.1038/nm1096-1129. [PubMed: 8837612]
- [21]. McCluskie MJ, Thorn J, Mehelic PR, Kolhe P, Bhattacharya K, Finneman JJ, et al. Molecular attributes of conjugate antigen influence function of antibodies induced by anti-nicotine vaccine in mice and non-human primates. *Int J Immunopharmacol* 2015; 25 (2), 518–527. doi:10.1016/j.intimp.2015.02.030.
- [22]. Rüedi-Bettschen D, Wood SL, Gunnell MG, West CM, Pidaparathi RR, Carroll FI, et al. Vaccination protects rats from methamphetamine-induced impairment of behavioral responding for food. *Vaccine* 2013; 31 (41), 4596–4602. doi:10.1016/j.vaccine.2013.07.038. [PubMed: 23906885]
- [23]. Nguyen JD, Bremer PT, Ducime A, Creehan KM, Kisby BR, Taffe MA, et al. Active vaccination attenuates the psychostimulant effects of α -PVP and MDPV in rats. *Neuropharmacology* 2017; 116, 1–8. doi:10.1016/j.neuropharm.2016.12.005. [PubMed: 27956054]
- [24]. Hambuchen MD, Hendrickson HP, Gunnell MG, McClenahan SJ, Ewing LE, Gibson DM, et al. The pharmacokinetics of racemic MDPV and its (R) and (S) enantiomers in female and male rats. *Drug Alcohol Depend* 2017; 179, 347–354. doi:10.1016/j.drugalcdep.2017.07.011. [PubMed: 28844011]
- [25]. Peterson EC, Hambuchen MD, Tawney RL, Gunnell MG, Cowell JL, Lay JO Jr., et al. Simple radiometric method for accurately quantitating epitope densities of hapten–protein conjugates

- with sulfhydryl linkages. *Bioconjug Chem* 2014; 25 (12), 2112–2115. doi:10.1021/bc500456z. [PubMed: 25426820]
- [26]. Stevens MW, Gunnell MG, Tawney R, Owens SM. Optimization of a methamphetamine conjugate vaccine for antibody production in mice. *Int J Immunopharmacol* 2016; 35, 137–141. doi:10.1016/j.intimp.2016.03.028.
- [27]. Peterson EC, Gunnell M, Che Y, Goforth RL, Carroll FI, Henry R, et al. Using hapten design to discover therapeutic monoclonal antibodies for treating methamphetamine abuse. *J Pharmacol Exp Ther* 2007; 322, 30–39. doi:10.1124/jpet.106.117150. [PubMed: 17452421]
- [28]. Novellas J, López-Arnau R, Carbó ML, Pubill D, Camarasa J, Escubedo E. Concentrations of MDPV in rat striatum correlate with the psychostimulant effect. *J Psychopharmacol (Oxford)* 2015; 29 (11), 1209–1218. doi:10.1177/0269881115598415. [PubMed: 26253621]
- [29]. Hambuchen MD, Hendrickson HP, Owens SM. Chiral determination of 3,4-methylenedioxypyrovalerone enantiomers in rat serum. *Anal Methods* 2017; 9 (4), 609–617. doi: 10.1039/C6AY03176E. [PubMed: 28286575]
- [30]. Hastedt MRC, Baselt (ed.): *Disposition of toxic drugs and chemicals in man*, 10th edition. *Forensic Sci Med Pathol* 2014; 11 (1), 147. doi:10.1007/s12024-014-9602-3.

Highlights

- An α -PVP/MDPV hapten was conjugated to an antigen to make a vaccine for both α -PVP and MDPV.
- The α -PVP/MDPV vaccine produced racemic α -PVP and MDPV antibodies in male rats.
- The α -PVP/MDPV vaccine reduced hyperlocomotion and organ drug concentrations.
- The vaccine shortened the duration of activity up to 5.6 mg/kg of α -PVP or MDPV.

E.g. R¹=Me, Et, Pr, Bu; R²=Me, Et; R³ and R⁴ = H, Me

Figure 1.
Structures of cathinone and of synthetic cathinone classes.

Figure 2.
Structures of synthetic cathinones MDPV and α -PVP commonly found in bath salt mixtures.

Figure 3.
α-PVP/MDPV hapten conjugated to IC_{KLH}.

Figure 4.

Changes in the percentage of MDPV- t_2 (20 nM of radioligand) bound to pAb at a 1:50 dilution of antiserum in individual male rats used for MDPV-challenge (open circles) and α -PVP-challenge (closed circles) experiments over time after immunization with α -PVP/MDPV-IC_{KLH} (n=5–6/group). Serum samples were obtained before each immunization, two weeks after each boost, and at week 16. Binding of MDPV- t_2 was corrected for non-specific binding as determined with pre-immunization serum samples collected for each rat.

Figure 5. Anti-MDPV (n=5) and anti- α -PVP (n=6) serum antibody IC_{50} values (mean $\pm$ SD) in α -PVP/MDPV- IC_{KLH} immunized male rats determined by RIA.

Figure 6.

Total distance traveled by male rats over 6 h (mean $\pm$ SD) after administration of ascending (sc) doses of MDPV (A) or α -PVP (B) spaced 2–3 days apart (MDPV: $n=5$ /group; α -PVP: $n=6$ /group). Duration of MDPV-induced (C) and α -PVP-induced (D) effects (mean $\pm$ SD) on horizontal distance traveled. Asterisks indicate significant difference from SAS rats as determined by two-way ANOVA, with Holm-Sidak's multiple comparisons test where appropriate ($p<0.05$).

Figure 7.

Effects of immunization on MDPV and α-PVP serum concentration 20 weeks after initial immunization. Area under the serum concentration time curve for MDPV enantiomers (A and C) and racemic α-PVP (B and D) from 30 to 120 min (AUC₃₀₋₁₂₀) after (sc) doses of 0.56 mg/kg (A, B) and 3.0 mg/kg (C, D) of drug in male rats. Asterisks indicate significant differences from SAS rats (p < 0.05) determined for MDPV enantiomers by a two-way ANOVA (Figure 7C) and for racemic α-PVP by a Welch's t-test (Figure 7B and D).

Figure 8.

Studies of tissue concentrations after MDPV and α -PVP dosing conducted 20 weeks after the start of immunizations. Tissue AUC₃₀₋₁₂₀ values for (*S*)-MDPV and (*R*)-MDPV enantiomers in brain, kidney, and heart after administration of 3.0 mg/kg racemic MDPV (sc) in male rats (Figure 8A). Figure 8B shows AUC₃₀₋₁₂₀ serum values for α -PVP in brain, kidney, and heart after administration of 3.0 mg/kg racemic α -PVP (sc). Significant differences ($p < 0.05$) between treatment (A) for each organ using a two-way ANOVA is indicated by an asterisk. Tissue AUC₃₀₋₁₂₀ values were the same for MDPV enantiomers in both SAS and vaccinated rats (A) indicating no enantioselectivity. No significant differences were found between SAS and vaccinated rats in the α -PVP AUC₃₀₋₁₂₀ for brain, kidney, or heart using an unpaired t-test ($p < 0.05$) (B).

Table 1.

Anti- α -PVP/MDPV polyclonal antibody affinity for MDPV, (*R*)- and (*S*)-MDPV enantiomers, α -PVP and nine structurally similar cathinones. Each structurally related cathinone was tested in an RIA at 10 μ M and 100 μ M with MDPV- t_2 as the radiolabeled tracer.

MDPV and α -PVP	IC ₅₀ (nM)	Structure
3,4-Methylenedioxypyrovalerone (MDPV)	6.6 $\pm$ 2.1 ^a
(<i>R</i>)-MDPV	3.9 $\pm$ 0.8 ^b
(<i>S</i>)-MDPV	6.6 $\pm$ 2.3 ^c
α -Pyrrolidinovalerophenone (α -PVP)	4.6 $\pm$ 3.7 ^d
Structurally related cathinones	IC ₅₀ (μ M)	
Pyrovalerone	10 (68% inhibition)
3,4-Methylenedioxy- α -pyrrolidinobutylphenone	100 (50% inhibition)
3,4-Methylenedioxy- α -pyrrolidinopropylphenone	>100
Mephedrone	>100
Methylone	>100

Pentedrone	>100
Pentylone	>100
(<i>S</i>)-Cathinone	>100
(<i>S</i>)-Methcathinone	>100

^aIC₅₀ data from Figure 5. Determined using racemic MDPV-*t*₂ radioligand and inhibition with a range of unlabeled racemic MDPV concentrations.

^bDetermined using (*R*)-MDPV-*t*₂ radioligand and inhibition with a range of unlabeled (*R*)-MDPV concentrations.

^cDetermined using (*S*)-MDPV-*t*₂ radioligand and inhibition with a range of unlabeled (*S*)-MDPV concentrations.

^dIC₅₀ data from Figure 5. Determined using racemic α-PVP-*t*₂ radioligand and inhibition with a range of unlabeled racemic α-PVP concentrations.