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Engineering and Characterization of a Mouse/Human Chimeric Anti-Phencyclidine Monoclonal Antibody

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Abstract

Previously, our laboratory produced a high affinity, anti-phencyclidine (PCP) murine monoclonal antibody (mAb6B5) that also binds other PCP-like arylcyclohexylamines. In this project, mAb6B5 is engineered into a mouse/human chimera (ch-mAb6B5) to assess the feasibility of developing it into a medication for PCP and PCP-like drug abuse. To create ch-mAb6B5, the light and heavy chain constant regions of mAb6B5 were replaced with human κ and IgG₂ constant regions in order to decrease its potential immunogenicity in humans. To be an effective anti-PCP medication, ch-mAb6B5 must retain the critical immunochemical binding properties of mAb6B5. Expression vectors containing ch-mAb6B5 light chain and heavy chain cDNA were constructed and expressed in the murine myeloma cell line P3X63-Ag8.653. Immunoassays confirm that ch-mAb6B5 is indeed a chimera, composed of mAb6B5's PCP-binding variable domains and human κ and IgG constant regions. Radioimmunoassays show that ch-mAb6B5 has the same drug-binding profile as mAb6B5. Ch-mAb6B5 and mAb6B5 bind PCP with a K_D of 0.67 nM and 1.17 nM (respectively) and bind PCP-like arylcyclohexylamines 1-[1-(2-thienyl)cyclohexyl]piperidine and N-ethyl-1-phenylcyclohexylamine with similar specificity. Additionally, ch-mAb6B5 and mAb6B5 have the same calculated isoelectric points and molecular weights, critical properties in antigen-antibody interactions. These data demonstrate that mouse/human ch-mAb6B5, a "more human" version of murine mAb6B5, retains mAb6B5's unique drug-binding properties. This work supports our continued efforts to develop ch-mAb6B5 into a medication for PCP and PCP-like drug abuse – introducing the intriguing possibility of using a single therapeutic mAb for treating a class of abused drugs.

Keywords

Phencyclidine; Arylcyclohexylamines; Substance abuse; Therapeutic antibody; Chimeric antibody

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S. Michael Owens has a financial interest in and serves as Chief Scientific Officer of InterveXion Therapeutics, LLC, a pharmaceutical biotechnology company, whose main interest is in developing new monoclonal antibodies for treatment of human diseases, including drug abuse.

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1. Introduction

A promising, new strategy for substance abuse treatment is the use of anti-drug antibodies. Whether administered passively as a monoclonal antibody (mAb) or produced actively by immunization, anti-drug antibodies offer major advantages over current drug abuse treatments [1,2]. Conventional treatments seek to use small molecule agonists or antagonists to target brain receptors. Unfortunately, these treatments often produce adverse side effects. Anti-drug antibodies avoid many of these adverse effects because they target only the drug. Acting as peripheral pharmacokinetic antagonists, anti-drug antibodies capture and hold the targeted drug in the vascular compartment, decreasing the amount of drug available to brain receptors. Additionally, the rate of drug clearance across the blood-brain barrier is reduced, which helps reduce the euphoric and reinforcing effects of the drug [3]. Passively administered anti-drug mAb have the additional advantages of immediate protection, long lasting effects, less frequent need for patient compliance, and lack of addiction liability [2].

There is a problem in using mAbs as therapeutic medications. By necessity mAbs are produced in species other than humans - most commonly mice. Mouse antibodies, particularly the constant regions, are highly immunogenic in humans. A human anti-murine immune response can render a therapeutic mAb ineffective by neutralization and/or can produce an allergic reaction. Scientists have addressed this problem by making murine mAbs “more human” with genetic engineering techniques such as chimerization or humanization [4–6].

Our laboratory has produced and rigorously tested a high affinity ($K_D = 1.3$ nM) murine anti-PCP mAb, named mAb6B5 (IgG₁ heavy chain, κ light chain) [7–11]. In preclinical testing with rat models of human drug abuse, anti-PCP mAb6B5 effectively reverses and prevents detrimental pharmacological effects of PCP. A single equimolar dose of mAb6B5 provides long-term reductions in PCP brain concentrations and PCP-induced locomotor activities in models of chronic, high dose, intravenous PCP use [8,9]. Remarkably, a single dose of mAb6B5 1/100th the molar equivalent of the PCP body burden prevents death, reduces PCP concentrations in the brain and decreases PCP-induced locomotor activity in mAb6B5-treated animals. Additionally, the general health of the animals significantly improves [10]. Finally, mAb6B5 has a long functional life (15 days in rats) [8,11]. Scaling of these rat data to human use indicates that a single 1 g dose of mAb6B5 would have the capability of significantly reducing the adverse effects of a 1.2 g/day binge use of PCP for up to 6 weeks [10]. Collectively these data strongly support the aim of developing mAb6B5 into a medication for PCP abuse treatment.

mAb6B5 is unique in that it cross reacts with other potent, structurally related arylcyclohexylamines such as TCP (1-[1-(2-thienyl)cyclohexyl]piperidine) and PCE (N-ethyl-1-phenylcyclohexylamine). In rats receiving high doses of PCP, PCE or TCP (3 mg/kg), mAb6B5 rapidly reversed locomotor activities resulting from these three drugs [12]. The effectiveness of mAb6B5 as an antagonist for multiple arylcyclohexylamines is exciting. It introduces the concept of using one therapeutic mAb as a treatment for a group of structurally related abused drugs.

The purpose of this study is to assess the feasibility of developing mAb6B5 into a medication for human PCP abuse. To be a safe medication for humans, mAb6B5 must be genetically engineered into a protein that is more “human” in its amino acid sequence. However, to be an effective medication, the engineered version must retain the unique drug-binding characteristics of mAb6B5 that made it so effective in preclinical animal studies. To convert mAb6B5 into a more safe and yet effective form, we genetically engineered it into a mouse/human chimera (ch-mAb6B5) that contains the PCP-binding domains of mAb6B5 [the light chain (LC) and heavy chain (HC) variable regions] attached to the constant regions of a human

κ LC and IgG₂ HC, respectively. Herein, we describe the genetic engineering, expression and characterization of anti-PCP ch-mAb6B5 in regard to structure and drug-binding characteristics.

2. Materials and methods

2.1. Cell lines

The anti-PCP mAb6B5-producing hybridoma cell line was created using the PCP-like hapten 5-[N-(1-phenylcyclohexyl)amino]pentanoic acid (PCHAP) covalently bound to bovine serum albumin (BSA) [7]. Murine, non-producing myeloma cell line P3X63-Ag8.653 (CRL 1580) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cell lines were maintained at 37°C under 10% CO₂ conditions in Dulbecco's modified Eagle Medium (Gibco, Carlsbad, CA) supplemented with 10% fetal calf serum (HyClone, Logan, UT).

2.2. Construction of ch-mAb6B5 LC and HC genes – overall strategy

The genes (cDNA) for ch-mAb6B5 LC and HC were created by inserting cloned cDNA of mAb6B5 LC variable region (V_L) and HC variable region (V_H) into mammalian expression vectors containing the cDNA of either human κ constant region (vector *pLC-huC_k*) or human IgG₂ constant region (vector *pHC-huC_{G2}*).

2.3. Cloning of mAb6B5 V_H and V_L cDNA by RT-PCR

The mAb6B5 V_L and V_H were cloned by reverse transcription (RT) and two rounds of polymerase chain reaction (PCR). The cDNA sequences of mAb6B5 V_L and V_H from leader sequence to J-C junction were unknown. To obtain these sequences, a first round of PCR was performed that amplified full length (including leader sequences) mAb6B5 LC and HC cDNA. For this PCR step, we used degenerate primers designed by Coloma *et al.* [13]. After cloning and sequencing full length HC and LC cDNA, we designed primers for a second round of PCR to amplify V_L and V_H with their corresponding leader sequences.

Total RNA was isolated from mAb6B5 hybridoma cells using an RNeasy Mini Kit (Qiagen, Valencia, CA). RT was performed on 1.0 µg total RNA using an oligo(dT)₁₅ primer (Promega, Madison, WI) and Moloney murine leukemia virus reverse transcriptase (Promega) according to manufacturer's recommendations. The resulting cDNA was used in PCR to amplify the full length open reading frame (including the leader sequences) of mAb6B5 LC [717 base pairs (bp)] and HC (1383 bp). Upstream PCR primers consisted of sets of degenerate primers described by Coloma *et al.* [13]. Design of downstream PCR primers was based on conserved C-terminal sequences of murine κ LC or IgG₁ HC as follows: HC 3' primer: 5'-GGGGCGGCCGACAGGGCTCCAAGGACACTGGGATCATTT-3' (*NotI* site underlined); LC 3' primer: 5'-GGGGCGGCCGCGCGTCTCAGGACCTTTGTCTCTAAC-3' (*NotI* site underlined). Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) and were designed with 5' restriction enzyme sites to facilitate directional cloning into vectors. *Pfu* polymerase (Stratagene, La Jolla, CA) was used in all PCR reactions to assure sequence fidelity. PCR amplifications were carried out in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). LC mAb6B5 cDNA was amplified with the following conditions: 35 cycles at 94°C for 1 min (denaturation), 57°C for 1 min (annealing), 72°C for 3 min (extension), and a final 10 min extension at 72°C. HC mAb6B5 cDNA was amplified using the same conditions except for an annealing temperature of 62°C. Amplified LC and HC cDNA were agarose gel purified and ligated into separate pcDNA3 vectors (Invitrogen, Carlsbad, CA). DH5 α competent cells (Invitrogen) were transformed with the constructs. Multiple clones of mAb6B5 HC and LC were sequenced using a 3100 Genetic Analyzer (Applied Biosystems).

Based on the sequences of cloned mAb6B5 LC and HC, we designed the following primers to amplify the V_L and V_H of mAb6B5: V_L 5' primer: 5'-CCCCGCTAGCC**CACCATGAAGTTGCCTGTTAGGCTGTTG**-3' (*NheI* site underlined; ribosome binding site in bold); V_L 3' primer: 5'-TATAGCGGCCG**CAGTTTTTATTTCCAGCTTG**-3' (*NotI* site underlined); V_H 5' primer: 5'-GGGGATATCC**CACCATGGAATGCAGCTGTGTAATGCTCTT**-3' (*EcoRV* site underlined; ribosome binding site in bold); V_H 3' primer: 5'-GGGGCTAGCT**GAGGAGACTGTGAGAGTGGT**-3' (*NheI* site underlined). The templates for V_L and V_H PCR were the cloned full length cDNA of mAb6B5 LC and HC. V_L was amplified using the following conditions: 35 cycles at 94°C for 1 min (denaturation), 62°C for 1 min (annealing), 72°C for 3 min (extension), and a final 10 min extension at 72°C. Amplification conditions for the V_H were the same except for an annealing temperature of 59°C. Amplified mAb6B5 V_L (393 bp) and V_H (408 bp) were agarose-gel purified and cloned into *pCR-Script Amp* cloning vector (Stratagene) for DNA sequence analysis.

2.4. Construction of ch-mAb6B5 LC and HC expression vectors

The mammalian expression vectors used to construct and express ch-mAb6B5 LC and HC were designed [14] and graciously provided by Dr. Gary R. McLean (University of British Columbia, Vancouver BC, Canada). Designed specifically for expressing recombinant chimeric immunoglobulin in mammalian cells, the LC vector *pLC-huC_k* contains human κ constant region cDNA and the HC vector *pHC-huC_{G2}* contains human IgG₂ constant region cDNA. Both vectors contain the cytomegalovirus (CMV) promoter.

To construct the ch-mAb6B5 LC expression vector, mAb6B5 V_L cDNA was cloned directionally (utilizing *NheI* and *NotI* restriction sites) into *pLC-huC_k* downstream of the CMV promoter and upstream of the cDNA sequences for human κ constant regions, with no intervening sequences. The ch-mAb6B5 LC construct was used to transform DH5α competent cells (Invitrogen). Multiple clones were sequenced on both strands to confirm the accuracy of the ch-mAb6B5 LC sequence.

Construction of the ch-mAb6B5 HC expression vector utilizing *pHC-huC_{G2}* was more complex. In an initial attempt to engineer and express ch-mAb6B5, we used a different pair of mammalian expression vectors constructed [13] and graciously provided by Dr. Sherie L. Morrison (UCLA, Los Angeles). Transfections with these vectors were unsuccessful in expressing an assembled ch-mAb6B5. However, a ch-mAb6B5 HC was transcribed (assayed by RT-PCR) and expressed [assayed by a sandwich enzyme linked immunosorbent assay (ELISA)]. This ch-mAb6B5 HC was subcloned by RT-PCR using the same 5' PCR primer used to amplify mAb6B5 V_H, (described above), except that a *NheI* site replaced the *EcoRV* site. Design of the 3' primer (5'-GGGCTCGAGTCATTTACCCGGAGACAGGGAG-3'; *XhoI* site underlined) was based on C-terminal conserved sequences of human IgG₂ HC. Sequence analysis of this ch-mAb6B5 HC showed it to be a perfect match for the cDNA sequence of mAb6B5 V_H and human IgG₂ constant regions. Therefore, instead of inserting only the V_H region of mAb6B5 into *pHC-huC_{G2}*, we inserted the subcloned ch-mAb6B5 HC cDNA. The procedure was as follows. First, the human IgG₂ constant region cDNA in *pHC-huC_{G2}* was enzymatically removed with *NheI* and *XhoI* restriction enzymes. Then, the cDNA of ch-mAb6B5 HC was inserted directionally (utilizing *NheI* and *XhoI* restriction sites) into *pHC-huC_{G2}* immediately downstream of the CMV promoter. The construct was used to transform DH5α competent cells (Invitrogen). Multiple clones were sequenced on both strands to confirm the accuracy of the ch-mAb6B5 LC sequence.

2.5. Co-transfection and expression of ch-mAb6B5

The HC and LC expression constructs were linearized with *PvuI*, then co-transfected into P3X63-Ag8.653 cells using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions. Following transfection, cells were diluted in growth medium and plated into 96-well tissue culture plates at 8×10^3 cells per well in 200 μ l media. Cells were re-fed after 24 hr with media containing 3.0 μ g/ml puromycin dihydrochloride for selection (Sigma-Aldrich, St. Louis, MO). After 13 days, supernatants from growing colonies were screened by ELISA to identify clones that produce chimeric antibodies.

2.6. Screening for transfectants by ELISA

A sandwich ELISA was designed to screen for cells producing assembled antibodies that have human IgG₂ and κ constant regions. Briefly, flat bottomed, 96-well plates were coated with 0.5 μ g/well of "capture" antibody [goat anti-human IgG (γ chain specific) (Southern Biotechnology Associates, Inc., Birmingham, AL)] overnight at 4°C. Plates were washed with phosphate-buffered saline (PBS), blocked with 3% BSA in PBS, and washed again. Supernatant (50 μ l) from transfected cells was added to wells and incubated overnight at 4°C. After washing, 100 μ l of "detection" antibody, [alkaline phosphatase-labeled goat anti-human κ (Southern Biotechnology Associates, Inc.)] diluted 1:1000 with 1% BSA in PBS was added and incubated for 1 hr at 37°C. Samples were run in duplicate. After thorough washing, 100 μ l of substrate (p-nitrophenyl phosphate) was added to wells. Plates were incubated at 37°C for 15 min, and OD₄₀₅ readings were measured with an ELISA plate reader. All positive clones were retested to confirm a positive response.

A quantitative ELISA was run to determine the amount of chimeric antibody produced by clones. The procedure was the same as described above with the inclusion of a standard (a human IgG₂, κ isoform antibody of unknown specificity) of varying concentrations (1, 10, 25, 50, 100, 250, 500, 1000 ng/ml). An absorbance vs. concentration standard curve was created from the standard's absorbance readings. The curve was used to convert absorbance readings of test samples into antibody concentration.

2.7. Purification of ch-mAb6B5

Ch-mAb6B5 was purified from culture media using a Protein G Sepharose column. Culture media from ch-mAb6B5 producing clone was diluted 1:5 (v/v) with deionized H₂O. After adjusting the pH to 7.0 with hydrochloric acid, the solution was loaded onto a chromatography column containing Protein G Sepharose 4 Fast Flow beads (Amersham Biosciences, Piscataway, NJ). The column was washed with 20 mM phosphate buffer (pH 7.0), then ch-mAb6B5 was eluted with a 0.1 M glycine buffer. Purified ch-mAb6B5 was then concentrated and buffer-exchanged with sterile PBS using an ultra-filtration device. Protein concentration for ch-mAb6B5 was 0.33 mg/ml, as determined by spectrophotometry. Purity was determined by HPLC and SDS-PAGE reducing gel electrophoresis.

2.8. Immuno-slot blot analysis of ch-mAb6B5

Immuno-slot blot analysis was used to confirm that ch-mAb6B5 contains human κ and IgG constant regions. Equal concentrations of purified ch-mAb6B5 or mAb6B5 in PBS were loaded onto nitrocellulose using a Millipore slot blot manifold (Millipore, Bedford, MA). After blocking overnight with 3% BSA in PBS, membranes were incubated with either alkaline-phosphatase-labeled goat anti-human IgG (Fc specific) (Caltag), murine mAb anti-human κ (Southern Biotechnology) or goat anti-mouse IgG (Fc specific) (Sigma-Aldrich). Membranes were then thoroughly washed with PBS. An alkaline phosphatase conjugate substrate kit (Bio-Rad, Hercules, CA) was used to detect binding of an alkaline-phosphatase labeled antibody.

2.9. PCP-binding ELISA

To test the antigen specificity of antibodies produced by transfected clones, cell culture supernatants from clones testing positive by screening ELISA were further tested with a PCP-binding ELISA. Briefly, flat bottomed 96-well plates were coated with 100 ng/well PCHAP-ovalbumin (a PCP-like hapten covalently bound to ovalbumin) diluted in coating buffer (0.1 M carbonate buffer pH 9.6), washed, and blocked overnight at 4°C with Super Block (Pierce, Rockford, IL). After adding 50 µl supernatant to wells in duplicate, plates were incubated overnight at 4°C and washed. Then 100 µl of detection antibody [alkaline phosphatase-labeled goat anti-human IgG (Fc specific) (Caltag, Burlingame, CA)] diluted 1:1,000 was added and incubated for 1 hr at 37°C. After washing, substrate was added and OD₄₀₅ readings measured. Purified mAb6B5 (5 ng) was analyzed as a control. The ELISA was repeated on the same supernatants using a different detection antibody - an alkaline phosphatase-labeled goat anti-mouse (Fc specific) antibody (Sigma-Aldrich).

2.10. Measuring drug-binding affinity and specificity

The IC₅₀ and K_D of purified ch-mAb6B5 and mAb6B5 for PCP were determined by RIA as described in Owens *et al.* [15]. Basically, a 100 µl aliquot of [³H]PCP (30,000–50,000 dpm) in RIA buffer (50 mM Tris-HCl adjusted to pH 7.6, 0.15 M NaCl, 0.1% BSA and 0.2% NaN₃) was added to 50 × 14 mm sample tubes (Sarstedt, Princeton, NJ). Then 100 µl of the appropriate concentration of PCP was added to duplicate tubes. The antibodies being tested were diluted in RIA buffer to a concentration which would bind 15 to 20% of the [³H]PCP in the absence of PCP. MAb6B5 (1 mg/ml) was diluted 1:3200 for 15 % binding. Purified ch-mAb6B5 (0.33 mg/ml) produced 17% binding, so it was used undiluted. A 100 µl aliquot of appropriately diluted antibody was then added to all the tubes except the tubes for nonspecific binding. To nonspecific binding tubes, 100 µl of RIA buffer was added. After vortexing, the tubes were incubated at 4–8°C overnight. Next, 1 ml of RIA buffer containing 2% goat anti-mouse IgG (whole molecule) (v/v; Pel-Freez Biologicals, Rogers, AR) and 5% polyethylene glycol 8000 (J.T. Baker Chemical Co.) was added. (The goat anti-mouse IgG antibody was used in procedures for both mAb6B5 and ch-mAb6B5.) The tubes were centrifuged for 15 min at 2000 × g to precipitate the antibody-bound radioactivity. The supernatant was aspirated and the pellet resuspended in the same test tube with 2 ml scintillation fluid. Tubes were placed in 7 ml scintillation vials and the concentration of [³H]PCP was determined by liquid scintillation spectrometry. Two sources of [³H]PCP [(1-(1-[phenyl-³H](n)cyclohexyl)piperidine)] were used. [³H]PCP with a specific activity of 44.7 Ci/mmol was purchased from NEN Life Sciences Products (now PerkinElmer, Waltham, MA), and [³H]PCP with a specific activity of 12.68 Ci/mmol was provided by the National Institute of Drug Abuse (NIDA)(Bethesda, MD). Two, six-point standard curves were run for ch-mAb6B5 (one using [³H]PCP from NIDA and one using [³H]PCP from NEN). Only one standard curve was run for mAb6B5 (using [³H]PCP from NIDA), since the K_D of mAb6B5 for PCP was previously established and published [7]. IC₅₀ values were obtained from the standard curves, and K_D values were calculated by subtracting the [³H]PCP molar concentration from the IC₅₀ value.

As a second method to compare the drug-binding affinity of ch-mAb6B5 and mAb6B5, an inhibition ELISA was designed to determine the IC₅₀ of PCP. IC₅₀ was defined as the concentration of drug that decreased ch-mAb6B5 or mAb6B5 binding to PCHAP-ovalbumin by 50%. Briefly, a PCP-binding ELISA was performed as described above except PCP was diluted to various concentrations and added to the wells along with 100 µl of supernatant from clone ch-mAb6B5 (10–11 ng/well) or purified mAb6B5 (10 ng/well). To detect ch-mAb6B5, an alkaline phosphatase-labeled goat anti-human IgG (Fc specific) (Caltag) was used. To detect murine mAb6B5, an alkaline phosphatase-labeled goat anti-mouse (Fc specific) antibody (Sigma-Aldrich) was used. Calculation of the percent bound for each PCP standard was

determined by dividing the average absorbance reading for that particular concentration by the average absorbance reading of the wells with no competing PCP, then multiplying by 100.

To measure and compare binding affinities of ch-mAb6B5 and mAb6B5 for the PCP-like drugs PCE and TCP, the IC_{50} for both drugs was determined using the [3H]PCP RIA protocol described above. The IC_{50} was defined as the concentration of PCE or TCP that decreased antibody binding to [3H]PCP by 50%. Because this procedure was not a homologous binding competition (*i.e.* PCE/TCP was competing against [3H]PCP-binding), a K_D value for these ligands could not be calculated.

2.11. Calculation of isoelectric point and molecular weight of ch-mAb6B5 and mAb6B5

The theoretical isoelectric point (pI) and molecular weight (MW) of ch-mAb6B5 and mAb6B5 were calculated from the predicted amino acid sequences using two software programs: ProtoPrm software program available online at ExPASy Proteomic Tools, and pI/MW Prediction Tool program developed by the LAQUIP Proteome Group.

2.12. Statistical analysis

Statistical analysis was performed with a two tailed, paired Student's t-test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Construction of ch-mAb6B5 LC and HC genes

The strategy for constructing ch-mAb6B5 LC and HC genes (cDNA) is summarized in Figure 1. The mammalian expression vectors *pLC-huC_k* and *pHC-huC_{G2}*, used to engineer and express ch-mAb6B5, were specifically designed to express functional recombinant antibodies [14]. LC vector *pLC-huC_k* contains the cDNA of human κ constant region, and HC vector *pHC-huC_{G2}* contains the cDNA of human IgG₂ constant region. The cDNA of mAb6B5 V_L and V_H was inserted into expression vectors *pLC-huC_k* and *pHC-huC_{G2}*, respectively, immediately upstream of human constant region cDNA. These constructs were then cloned and characterized by DNA sequence analysis. Ch-mAb6B5 LC has 100% sequence identity for mAb6B5 V_L joined to human κ constant region, and ch-mAb6B5 HC has 100% sequence identity for mAb6B5 V_H joined to human IgG₂ constant region. The nucleotide sequences and deduced amino acid sequences of ch-mAb6B5 LC and HC are presented in Figures 2 and 3.

An amino acid sequence alignment of ch-mAb6B5 and mAb6B5 light chains (Fig. 4A) and heavy chains (Fig. 4B) show that the LC and HC variable regions of ch-mAb6B5 are identical to those of mAb6B5, but the constant regions are different.

3.2. Expression of ch-mAb6B5

Following co-transfection of ch-mAb6B5 LC and HC expression vectors into the P3X63-Ag8.653 murine myeloma cell line, transfected cells were screened for chimeric antibody expression using a sandwich ELISA. Three positive clones were identified. One clone with a very slow growth pattern was eliminated from further testing. The two remaining clones were screened for antibody production levels using a quantitative sandwich ELISA. The clone producing the most antibody (160 μ g/liter) was chosen for further work and designated ch-mAb6B5.

3.3. Analysis of ch-mAb6B5 with immunoassays

To establish that the antibody expressed by clone ch-mAb6B5 is a mouse/human chimera of anti-PCP mAb6B5, a PCP-binding ELISA was performed. As seen in Figure 5, ch-mAb6B5

binds PCP and is detected by an Fc specific anti-human IgG antibody, but not by Fc specific anti-mouse IgG, demonstrating that ch-mAb6B5 contains the PCP-binding domains of mAb6B5 and human IgG constant region.

To confirm that the antibody produced by clone ch-mAb6B5 contains human κ and IgG constant regions, an immuno-slot blot was performed. As shown in Figure 6, ch-mAb6B5 is detected with anti-human κ and Fc specific anti-human IgG antibodies, but mAb6B5 is detected only with an anti-mouse IgG. There was a very low level of cross-reaction of the anti-mouse IgG antibody with ch-mAb6B5, which can be faintly seen in this reproduction. This is not surprising since IgG constant regions have high homology across species. The goat anti-mouse IgG antibody used in this experiment was adsorbed against human antibodies by the manufacturer (Sigma-Aldrich) to remove cross-reacting antibodies, but the absorption was ~98% efficient. This allowed a small amount of cross-reactivity, consistent with our results. In summary, the PCP-binding ELISA data (Fig. 5) and the immuno-slot blot data (Fig. 6) confirm that ch-mAb6B5 is a chimera of mAb6B5 - consisting of the PCP-binding variable regions of mAb6B5 and the constant regions of human κ and IgG.

3.4. Evaluating the drug binding profile of ch-mAb6B5

To determine if ch-mAb6B5 retains the high PCP-binding affinity of mAb6B5, RIA (Fig. 7A) and inhibition ELISA (Fig. 7B) were used to determine the IC_{50} and K_D values of both antibodies for PCP. By [3H]PCP RIA (Fig. 7A), the IC_{50} of mAb6B5 and ch-mAb6B5 for PCP are 4.6 nM and 3.6 nM, respectively, which are virtually the same. The K_D values are calculated to be 1.17 nM for mAb6B5 and 0.67 nM for ch-mAb6B5, also virtually the same. These results were confirmed by repeating the RIA for ch-mAb6B5, in which the IC_{50} was 3.0 nM and the calculated K_D was 1.31 nM (data not shown); and by the previously published K_D of mAb6B5, which is 1.3 nM [7]. By inhibition ELISA (Fig. 7B) the IC_{50} values are 2.0 nM for mAb6B5 and 3.2 nM for ch-mAb6B5, which are statistically the same ($p=0.21$). In performing these experiments, we found it unusual for the RIA and inhibition ELISA results to match so closely. For this reason, the inhibition ELISA was performed three times independently. Each time the results were similar. Taken together, these data demonstrate that ch-mAb6B5 retains the high PCP-binding affinity of mAb6B5.

The cross reactivity of murine mAb6B5 with structurally similar arylcyclohexylamines like PCE and TCP suggests that mAb6B5 might be used as a medication for several abused drugs in this class [12]. To determine if ch-mAb6B5 retains the unique specificity profile of mAb6B5, the IC_{50} values of ch-mAb6B5 and mAb6B5 for TCP and PCE were determined with a [3H] PCP RIA. To compare the results to previously published data [12], the IC_{50} values are expressed as relative potency to PCP (a ratio of the IC_{50} for PCP/ IC_{50} for test drug). Results and chemical structures of the three structurally related arylcyclohexylamines are shown in Table 1. Ch-mAb6B5 retains the binding specificity of mAb6B5. It binds TCP with the same relative potency as mAb6B5 (0.14 and 0.19, respectively). Ch-mAb6B5 also binds PCE, like mAb6B5, though somewhat less efficiently (0.16 and 0.75, respectively). Nevertheless, these data indicate that ch-mAb6B5 retains mAb6B5's broad binding specificity for multiple arylcyclohexylamines. Collectively, the data shown in Figure 7 and Table 1 demonstrates that mouse/human ch-mAb6B5 retains the drug-binding profile of murine mAb6B5.

3.5. Calculated isoelectric point and molecular weight of ch-mAb6B5

Alterations in the isoelectric point (pI) of an antibody can affect antigen binding [16], formulation, and biodistribution [17]. To determine if the process of engineering mAb6B5 into ch-mAb6B5 altered the pI or MW, two software programs were used to determine the theoretical pI and MW of ch-mAb6B5 and mAb6B5. As shown in Table II, ch-mAb6B5 retains the same pI and basically the same MW as mAb6B5.

4. Discussion

The goal of this project was to assess the feasibility of converting murine anti-PCP mAb6B5 into a medication for PCP abuse. We needed to determine if conversion of murine mAb6B5 into a more human-compatible form would decrease its drug-binding capabilities, thus eliminating its usefulness as an anti-drug medication. To address both criteria we chose to genetically engineer mAb6B5 into a mouse/human chimera (ch-mAb6B5) consisting of mAb6B5's variable regions and the constant regions of human κ and IgG₂ (Figures 1, 5 and 6). Collectively, these data confirm that ch-mAb6B5 is indeed a mouse/human chimera of mAb6B5, containing the PCP-binding regions of mAb6B5 and human constant regions. Additionally, and significantly, we demonstrated that the conversion of mAb6B5 into ch-mAb6B5 did not alter its unique drug-binding profile (Table 1 and Figure 7).

For an anti-drug antibody to be successful as a medication, it must have a high affinity for its target drug. Work in our laboratory supports the concept that a mAb with high binding affinity makes a better immunotherapeutic drug. Specifically, two anti-methamphetamine mAbs with different binding affinities (10 nM and 250 nM) were compared in rat models of methamphetamine overdose [18]. The 10 nM antibody more effectively antagonized behavioral effects and had a longer duration of action than the 250 nM antibody. This same concept is true for therapeutic antibodies that target cell-surface molecules. For example, three mAbs specific for vascular endothelial growth factor receptor (VEGFR), but with different binding affinities (50 pM, 200 pM and 250 pM), are all efficacious in inhibiting leukemia cell growth in NOD-SCID mice models, but with varying degrees of efficacy [19]. The investigators found a direct correlation between antigen-binding affinity and biological effectiveness both *in vitro* and *in vivo*, demonstrating that the higher the affinity, the more effective the antibody. Our extensive preclinical testing of mAb6B in rat models of acute and chronic drug abuse has shown mAb6B5 to be highly effective in reversing the detrimental effects of PCP [8–11]. We believe a key reason for its success is its high binding affinity for PCP ($K_D = 1.3$ nM).

In order to maintain the affinity and drug class specificity of murine mAb6B5, we thought it important to retain a unique three-dimensional feature that occurs at the PCP-binding site. X-ray crystallography of mAb6B5 Fab reveals a tryptophan flap that appears to help hold PCP in the binding site [20]. When a PCP molecule is in the antigen-binding pocket, this flap appears to close over it much like a trash can lid. The close interplay between ligand and tryptophan at this site suggests an important role for this feature in defining mAb6B5 drug-binding characteristics. Consequently, to avoid altering the three dimensional structure of the drug-binding site, we used an engineering strategy that retains the entire amino acid sequence of mAb6B5 V_L and V_H.

In deciding on a strategy to convert murine mAb6B5 into a human-compatible form, there were two established techniques from which to choose, chimerization and humanization. Both techniques are based on making a mouse mAb substantially more human in its sequence, and both have been used successfully by other scientists in converting an existing rodent mAb into an FDA-approved therapeutic mAb [5]. It is important to make mouse antibodies more human because murine HC constant regions induce very strong anti-isotype immune responses in humans even after just one exposure [6,21]. An anti-isotype response can cause an anaphylactoid reaction and render a therapeutic mAb ineffective due to neutralization and accelerated clearance [6]. In chimerization, the variable regions of the mouse mAb are coupled to human constant regions, resulting in an antibody that is about 33% the original murine sequence (containing the antigen binding site) and 67% human sequence (containing the constant regions). In essence, chimerization produces a mAb with antigen binding characteristics of the original mAb while eliminating the strongly immunogenic murine

constant regions. In humanization, less of the mouse sequence is preserved. Only the complementarity determining regions (regions in the antigen binding site that directly contact the antigen) are grafted into the framework of a human antibody, resulting in an antibody that is about 95% human and 5% mouse. Although humanization produces a more human antibody, the process often decreases the antigen binding affinity of the antibody [22–24].

Although it is intuitive to think that a humanized mAb is less immunogenic than a chimeric mAb, this idea is not always supported by clinical trials reported in the literature. Data collected from clinical trials of humanized and chimeric therapeutic mAbs document that both types decrease anti-isotype immune responses, and both may induce what is called an anti-idiotypic response. In some individuals, specific conformational arrangements of amino acids in the variable domains of a mAb, whether it is a mouse or human mAb, can cause an immune response. Thus, the immunological outcome of using a humanized versus a chimeric mAb appears to depend on each individual mAb [25–29]. For example, there were clinical trials which compared Infliximab, an FDA approved mouse/human chimeric anti-TNF α mAb (Centocor, USA) and CDP571, a humanized anti-TNF α mAb (Celltech Chiroscience, Slough, UK) for treatment of Crohn disease and rheumatoid arthritis. In these trials, 28% of patients treated with chimeric Infliximab and 35% of patients treated with humanized CDP571 developed immune responses to the respective mAb [30]. Finally, it is of interest to note that the success rates for chimeric and humanized mAbs completing Phase I–III clinical trials (for safety and therapeutic success) and ultimately achieving FDA approval are the same, 24% and 25%, respectively [31].

Considering the evidence cited above, we chose to convert mAb6B5 into a mouse/human chimera. However, decreasing the immunogenicity of a chimeric mAb depends on more than just replacing mouse constant regions with human constant regions. [4]. The isotype of the human IgG heavy chain is important. A therapeutic mAb is more immunogenic if it binds Fc receptors [32] or activates complement [33]. Of the four human IgG isotypes (IgG₁–IgG₄), IgG₂ and IgG₄ are the least efficient in complement activation and Fc receptor binding [34, 35]. Additionally, IgG₂ has only one known allotype, which reduces the potential of an anti-allotypic immune response [36]. Therefore, to avoid the possibility of activating unwanted effector pathways and to eliminate a possible anti-allotypic immune response; we chose to use an IgG₂ constant domain to create ch-mAb6B5.

The data presented herein demonstrates that we have succeeded in our goal - to make anti-PCP mAb6B5 more human in its sequence without changing its drug-binding capabilities. Now, the true extent of ch-mAb6B5's immunogenicity in humans needs to be determined. This can only be done in humans with tests reserved for FDA approved phase I clinical trials. However, we predict that ch-mAb6B5 would have low immunogenicity, based on the success of FDA approved mouse/human therapeutic antibodies currently in use. These medications include Rituxan (Genentech, Inc.) an anti-CD20 chimeric approved for non-Hodgkin lymphoma, chronic lymphocytic leukemia and rheumatoid arthritis; Infliximab (Centocor, Inc.) an anti-TNF- α chimeric approved for Crohn disease and rheumatoid arthritis; and Simulect (Novartis Pharm) an anti-CD25 chimeric approved for treating allograft rejection.

Another step in developing anti-PCP ch-mAb6B5 into a medication for human use involves comparing its bioequivalence to murine mAb6B5. Side-by-side testing of ch-mAb6B5 and mAb6B5 in animal models of PCP and PCP-like drug abuse will help determine if a ch-mAb6B5 can function as well as mAb6B5 *in vivo*. These experiments require large quantities of ch-mAb6B5. Unfortunately, the ch-mAb6B5 producing murine myeloma clone described in this paper is a low producer of mAb, producing only 160 μ g/liter in static culture. In spite of our valiant efforts to obtain more ch-mAb6B5 from this particular clone, we have only been able to generate enough ch-mAb6B5 for this initial project.

In conclusion, this study demonstrates that it is feasible to continue our efforts to develop ch-mAb6B5 into a medication for treating PCP abuse. Ch-mAb6B5 has the same antigen-binding profile as the original murine mAb6B5 and is as human in its amino acid sequence as several FDA-approved mouse/human chimeric therapeutic mAbs. Also, an interesting attribute of this particular antibody is its broad specificity for pharmacologically potent arylcyclohexylamines. Ch-mAb6B5 offers the intriguing prospect of using a single therapeutic mAb to treat abuse of multiple, structurally related drugs.

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Figure 1. Strategy for construction of ch-mAb6B5 HC and LC expression vectors (*pHC-C_{G2}* and *pLC-C_κ*, respectively).

1 - ATGAAGTTGCCTGTTAGGCTGTTGGTGCTGATGTTCTGGATTCTGCTTCCAGCAGTGAT - 60
 - M K L P V R L L V L M F W I P A S S S D
 61 - GTTTTGATGACCCAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCCATC - 120
 - V L M T Q T P L S L P V S L G D Q A S I
 121 - TCTTGACAGATCTAGTCAGACCATTGTACATAGTAATGGAAACACCTATTTAGAATGGTAC - 180
 - S C R S S Q T I V H S N G N T Y L E W Y
 181 - CTGCAGAAACCAGGCCAGTCTCAAAGCTCCTGATCTACAAAGTTTCCAACCGATTTTCT - 240
 - L Q K P G Q S P K L L I Y K V S N R F S
 241 - GGGGTCCCAGACAGGTTTCAGTGGCAGTGGATCAGGGACAGATTTCACTCAAGATCAGC - 300
 - G V P D R F S G S G S G T D F T L K I S
 301 - AGAGTGGAGGCTGAGGATCTGGGAGTTTATTACTGCTTTCAAGGCACACATGCTCCGTAC - 360
 - R V E A E D L G V Y Y C F Q G T H A P Y
 361 - ACGTTTCGGAGGGGGACCAAGCTGGAAATAAAAACTGCGGCCGCACCATCTGTCTTCATC - 420
 - T F G G G T K L E I K T A A A P S V F I
 421 - TTCCCGCCATCTGATGAGCAGTTGAAATCTGGAAGTGCCTCTGTTGTGTGCCTGCTGAAT - 480
 - F P P S D E Q L K S G T A S V V C L L N
 481 - AACTTCTATCCCAGAGAGGCCAAAGTACAGTGGGAAGGTGGATAACGCCCTCCAATCGGGT - 540
 - N F Y P R E A K V Q W K V D N A L Q S G
 541 - AACTCCCAGGAGAGTGTACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGC - 600
 - N S Q E S V T E Q D S K D S T Y S L S S
 601 - ACCCTGACGCTGAGCAAAGCAGACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACC - 660
 - T L T L S K A D Y E K H K V Y A C E V T
 661 - CATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTTCAACAGGGGAGAGTGTGA
 - H Q G L S S P V T K S F N R G E C *

Figure 2.

cDNA and amino acid sequence of ch-mAb6B5 LC (714 nt/237 AA with leader sequence).

Murine mAb6B5 variable region (underlined) is nt 1–393 (leader sequence double-underlined). Human κ constant region is nt 394–714 (***NotI*** restriction site in **bold**).

1 - ATGGAATGCAGCTGTGTAATGCTCTTCCTCCTGTCAGGAAGTGCAGGTGTCTCTCTGAG - 60
 - M E C S C V M L F L L S G T A G V L S E
 61 - GTCCAGCTGCAACAGTCTGGACCTGAGTTGGTGAAGCCTGGGGCTTCAGTGAAGATGTCC - 120
 - V Q L Q Q S G P E L V K P G A S V K M S
 121 - TGCAAGGCTTCTGGCTACACTTTCACTGACTACTACATACACTGGATGAAGCAGAGCCAT - 180
 - C K A S G Y T F T D Y Y I H W M K Q S H
 181 - GGAAAGAGCCTTGAGTGGATTGGATATATTTATCCTAACACGGTGGTAATGGCTACAAC - 240
 - G K S L E W I G Y I Y P N N G G N G Y N
 241 - CAGAAGTTCAAGGGCAAGGCCACATTGACTGTAGACAAGTCTCCAGCACAGCCTACATG - 300
 - Q K F K G K A T L T V D K S S S T A Y M
 301 - GAGCTCCGCACCCTGACATCTGAGGACTCTGCAGTCTATTACTGTGGAAGATCTACCTGG - 360
 - E L R T L T S E D S A V Y Y C G R S T W
 361 - GACGACTTTGACTACTGGGGCCAAGGCACCCTCTCACAGTCTCCTCAGCTAGCACCAAG - 420
 - D D F D Y W G Q G T T L T V S S A S T K
 421 - GGCCCATCGGTCTTCCCCCTGGCGCCCTGCTCCAGGAGCACCTCCGAGAGCACAGCGCC - 480
 - G P S V F P L A P C S R S T S E S T A A
 481 - CTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCTGGAAGTCAAGC - 540
 - L G C L V K D Y F P E P V T V S W N S G
 541 - GCTCTGACCAGCGCGTGCACACCTTCCCAGCTGTCTACAGTCTCAGGACTCTACTCC - 600
 - A L T S G V H T F P A V L Q S S G L Y S
 601 - CTCAGCAGCGTGGTGACCGTGCCTCCAGCAACTTCGGCACCCAGACCTACACCTGCAAC - 660
 - L S S V V T V P S S N F G T Q T Y T G T N
 661 - GTAGATCACAAAGCCCAGCAACCAAGGTGGACAAGACAGTTGAGCGCAAATGTTGTGTC - 720
 - V D H K P S N T K V D K T V E R K C C V
 721 - GAGTGCCCAACCGTGCCAGCACCACTGTGGCAGGACCGTCAGTCTTCTCTCCCCCA - 780
 - E C P P C P A P P V A G P S V F L F P P
 781 - AAACCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACGTGCGTGGTGGTGAC - 840
 - K P K D T L M I S R T P E V T C V V V D
 841 - GTGAGCCACGAAGACCCCGAGGTCCAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCAT - 900
 - V S H E D P E V Q F N W Y V D G V E V H
 901 - AATGCCAAGACAAAGCCACGGGAGGAGCAGTTCAACAGCACGTTCCGTGTGGTCAGCGTC - 960
 - N A K T K P R E E Q F N S T F R V V S V
 961 - CTCACCGTTGTGCACCAGGACTGGCTGAACGGCAAGGAGTACAAGTGAAGGTCTCCAAC - 1020
 - L T V V H Q D W L N G K E Y K C K V S N
 1021 - AAAGGCCTCCAGCCCCATCGAGAAAACCATCTCCAAAACCAAGGGCAGCCCCGAGAA - 1080
 - K G L P A P I E K T I S K T K G Q P R E
 1081 - CCACAGGTGTACACCCTGCCCCATCCCGGGAGGAGATGACCAAGAACCAGGTACGCCTG - 1140
 - P Q V Y T L P P S R E E M T K N Q V S L
 1141 - ACCTGCCTGGTCAAAGGCTTCTATCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGG - 1200
 - T C L V K G F Y P S D I A V E W E S N G
 1201 - CAGCCGGAGAACAACCTACAAGACCACACCTCCCATGCTGGACTCCGACGGCTCCTTCTTC - 1260
 - Q P E N N Y K T T P P M L D S D G S F F
 1261 - CTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGC - 1320
 - L Y S K L T V D K S R W Q Q G N V F S C
 1321 - TCCGTGATGCATGAGGCTCTGCACAACCACTACACGAGAAGAGCCTTCCCTGTCTCCG - 1380
 - S V M H E A L H N H Y T Q K S L S L S P
 1381 - GGTAAATGA
 - G K *

Figure 3.
 cDNA and amino acid sequence of ch-mAb6B5 HC (1389 nt/462 AA with leader sequence).
 Murine mAb6B5 variable region (underlined) is nt 1–408 (leader sequence double-
underlined). Human IgG₂ constant region is nt 409–1389 (*NheI* restriction site in **bold**).

A

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ch-mAb6B5_LC      MKLPVRLVLVLMFWIPASSSDVLMQTPLSLPVSLGDAQASISCRSSQTIVHSNGNTYLEWY
mAb6B5_LC         MKLPVRLVLVLMFWIPASSSDVLMQTPLSLPVSLGDAQASISCRSSQTIVHSNGNTYLEWY
*****

ch-mAb6B5_LC      LQKPGQSPKLLIYKVSNRFSGVDPDRFSGSGSGTDFTLKISRVEAEDLGVYYCFQGTAPY
mAb6B5_LC         LQKPGQSPKLLIYKVSNRFSGVDPDRFSGSGSGTDFTLKISRVEAEDLGVYYCFQGTAPY
*****

ch-mAb6B5_LC      TFGGGTKLEIKTA-AAPSVFIFPPSDEQLKSGTASVCLLNFFYPREAKVQWKVDNALQS
mAb6B5_LC         TFGGGTKLEIKRADAAPTVISIFPPSDEQLTSGGASVVCFLNFFPKDINVKWKIDGSRQ
***** *  ***: *  ****.***.*  *****:*****: :*:***.*.: :.

ch-mAb6B5_LC      GNSQESVTEQDSKDYSLSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
mAb6B5_LC         NGVLNSWTDQDSKDYSLSTLTLTDEYERHNSYTCEATHKSTSTSPIVKSFNRENC
..  :*  *:*****.******.*  :*:***: *.**.*:  :*:..****.*

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B

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ch-mAb6B5_HC      MECSCVMLFLLSGTAGVLESQLQSGPELVKPGASVKMSCKASGYTFDYIHWKQSH
mAb6B5_HC         MECSCVMLFLLSGTAGVLESQLQSGPELVKPGASVKMSCKASGYTFDYIHWKQSH
*****

ch-mAb6B5_HC      GKSLEWIGYIYPNNGNGYNQKFKGKATLTVDKSSSTAYMELRLTSED SAVYYCGRSTW
mAb6B5_HC         GKSLEWIGYIYPNNGNGYNQKFKGKATLTVDKSSSTAYMELRLTSED SAVYYCGRSTW
*****

ch-mAb6B5_HC      DDFDYWGQGTTLTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSG
mAb6B5_HC         DDFDYWGQGTTLTVSSAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVWNSG
*****.*  ***:*** *  ::*  .:*****.*****:****

ch-mAb6B5_HC      ALTSGVHTFPAVLQSSGLYSLSSVTVTPSSNFGTQTYTCNVDHKPSNTKVDK-TVERKC-
mAb6B5_HC         SLSSGVHTFPAVLQS-DLYTLSSSVTVTPSSWPSQTVTCNVAHPASSTKVDKKIVPRDCG
:*.*****. **:*** *****.: :** ***** *  .***** *  *.

ch-mAb6B5_HC      CVECPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVE
mAb6B5_HC         CKPC-ICTVPEVS--SVFIFPPKPKDVLITITLTPKVTCTVVVDISKDDPEVQFSWFVDDVE
*  *  *..* *:  ***:*****.* *:  **:*****.*:*****.*:***

ch-mAb6B5_HC      VHNATKPREEQFNSTFRVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKGQP
mAb6B5_HC         VHTAQTKPREEQINSTFRSVSELPIMHQDWLNGKEFKCRVNSAFAPIEKTISKTKGRP
*.*:*****.****** ** *.::*****:***:.. .:*****:*.

ch-mAb6B5_HC      REPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPMLDSDGS
mAb6B5_HC         KAPQVYTIPTPKEQMAKDKVSLTCMITNFFPEDITVEWQWNGQPAENYKNTQPIIMDTDGS
:  *****.*.:*:*:*****:..*.*.***:***  :***.* *:***

ch-mAb6B5_HC      FFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
mAb6B5_HC         YFVYSKLVNPKSNWEAGNTFTCSVLHEGLHNHTEKLSLSPGK
*:***.*:***.*: **.*:***.*.***.*:*** **

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Figure 4.

Amino acid sequence alignments of LCs (A) and HCs (B) of mouse/human ch-mAb6B5 and murine mAb6B5 show that the V_L and V_H sequences of ch-mAb6B5 and mAb6B5 are identical, while the human C_k and C_{G2} sequences of ch-mAb6B5 are different from the murine C_k and C_{G1} of mAb6B5. (*) represents identical amino acids; (:), (.), and () represents decreasing similarity between AA in regard to biochemical properties of side chains. The alignments were produced using Clustal W software tool at <http://myhits.isb-sib.ch/cgi-bin/clustalw>.

Figure 5.

A PCP-binding ELISA confirms that ch-mAb6B5 has the PCP-binding variable domains of murine mAb6B5 and the constant regions of a human IgG isotype. Cell culture medium supernatant from clone ch-mAb6B5 was analyzed in duplicate PCP-binding ELISAs that varied only in the detection antibody used: either goat anti-human IgG (Fc specific) (closed circles) or goat anti-mouse IgG (Fc specific) (open circles). Ch-mAb6B5 binds PCP and is detected by the anti-human IgG antibody, but not with the anti-mouse IgG. Data shown is representative of two independent experiments.

Figure 6.

Immuno-slot blots confirm that ch-mAb6B5 has human κ and IgG constant regions. Equivalent amounts of purified ch-mAb6B5 and mAb6B5 were loaded onto three nitrocellulose membranes. Each membrane was probed with one of three detection antibodies: either murine monoclonal anti-human κ , goat anti-human IgG (Fc specific), or goat anti-mouse IgG (Fc specific). Data shown is representative of two independent experiments.

Figure 7.

Ch-mAb6B5 and mAb6B5 have the same binding affinity for PCP as determined by radioimmunoassay (A) and inhibition ELISA (B). (A) By RIA, using [³H]-PCP from NIDA, the IC_{50} of mAb6B5 and ch-mAb6B5 for PCP are 4.6 nM and 3.6 nM, respectively. K_D values were obtained by correcting for the amount of [³H]-PCP in the assay and are 1.17 nM for mAb6B5 and 0.67 nM for ch-mAb6B. Upon repeating the RIA for ch-mAb6B5, using [³H]-PCP from NEN, the IC_{50} was 3.0 nM with a calculated K_D of 1.31 nM (data not shown). The published K_D of mAb6B5 for PCP is 1.3 nM [7] (B) By inhibition ELISA, the IC_{50} values of mAb6B5 and ch-mAb6B for PCP are 2.0 nM and 3.2 nM, respectively. Three independent inhibition ELISA experiments were performed and the data is expressed as the mean $\pm$ 1 S.D.

Statistical analysis with a paired Student's t-test shows no difference ($p=0.21$) between inhibition ELISA IC_{50} values of mAb6B5 and ch-mAb6B.

Table 1

Characterization of binding specificities of mouse/human ch-mAb6B5 and mouse mAb6B5 for three potent arylcyclohexylamines

Drug	Chemical Structure	Relative Potency to PCP ^a (by RIA)		
		mAb6B5 ^b	mAb6B5	ch-mAb6B5
PCP		1.0	1.0	1.0
PCE		0.89	0.75	0.16
TCP		0.11	0.19	0.14

^aRelative potency to PCP = (IC₅₀ value for PCP)/(IC₅₀ value for test drug).

^bData from Hardin *et al.*, 1998.

Table 2

Physio-chemical parameters of mouse mAb6B5 and mouse/human ch-mAb6B5

	Amino acids (number)	Isoelectric Point, ^{ab}	Molecular Weight (Da), ^{ab}
mAb6B5 LC	219	6.48/6.48	24158/24158
ch-mAb6B5 LC	218	6.48/6.48	23805/23805
mAb6B5 HC	441	7.27/7.27	48813/48813
ch-mAb6B5 HC	443	7.24/7.24	48940/48718

^aDerived using the ProtoPrm software program available at ExPASy Proteomics tools (<http://www.expasy.org/tools/#proteome>).

^bDerived using the pI/MW Prediction software program developed by LAQUIP Proteome Group (<http://proteome.ibi.unicamp.br/tools/ct.htm>).