Using Hapten Design to Discover Therapeutic Monoclonal Antibodies for Treating Methamphetamine Abuse

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ABSTRACT

When generating monoclonal antibodies (mAb) against small molecules, the chemical composition and molecular orientation of the drug-like hapten on the antigen is a crucial determinant. This is especially important when attempting to discover therapeutic mAb against the drugs of abuse (+)-methamphetamine [(+)-METH], (+)-amphetamine [(+)-AMP], and the related compound (+)-3,4-methylenedioxyamphetamine [(+)-MDMA, the plus isomer in the racemic mixture known as MDMA or ecstasy]. The goal of these studies was to design and synthesize (+)-METH-like haptens with structural attributes that could make them effective for generating monoclonal antibodies for treating medical problems associated with these stimulant drugs of abuse. Five prototype (+)-METH-like haptens, which mimic structural aspects of these drugs, were synthesized and used to generate mAb. After screening for anti-(+)-METH IgG antibodies in more than 25,000 potential mouse hybridoma cell lines, one prototype mAb from each of the five haptens was selected and studied in detail for molecular properties and preclinical efficacy. The amino acid sequences of the IgG variable regions, structural models, affinity, and ligand specificity of each mAb were then used to help elucidate important therapeutic characteristics. Four of these antibodies exhibited high affinity and specificity to (+)-METH and (+)-MDMA; whereas one antibody (designated mAb4G9) exhibited high affinity and specificity to (+)-METH, (+)-MDMA, and (+)-AMP, without significant cross-reactivity against other METH-like ligands, over-the-counter medications, or endogenous neurotransmitters. Considered together, discovery of mAb4G9 and the other antibodies in this report represent an important step in understanding the process for custom design of drug class-specific therapeutic antibodies for the treatment of drug addiction.

(+)-Methamphetamine [(+)-METH] abuse has become America’s number one drug threat (NACo, 2005), and effective treatment strategies for abuse of (+)-METH and related stimulants are greatly needed. Current pharmacotherapies for managing the acute cardiovascular system, central nervous system, and toxic effects are greatly needed (Sato, 1992; Albertson et al., 1999; Richards et al., 1999); they do nothing to remove the drug from its sites of action in the brain. Also lacking are medications that can reduce or treat the medically crippling effects of (+)-METH addiction. Monoclonal antibodies (mAb) provide an attractive potential medication that can target the drug instead of the site of action (Kosten and Owens, 2005). These high-affinity protein-based medications act as so-called pharmacokinetic antagonists, sequestering the drug in the bloodstream away from medically vulnerable tissues, such as the brain and heart.

Unlike nicotine and cocaine where the effects are caused by a single, specific compound, drugs such as opiates (e.g., mor- phine), arylycyclohexylamines (e.g., phencyclidine) and amphetamines [e.g., (+)-METH] are starting structures from which many pharmacologically similar compounds can be synthesized. These so-called “designer drugs” can be chemically modified to alter their effects. Thus, for a mAb therapeutic to have the broadest medical applicability, it should...
have high affinity and specificity for other medically important members of this drug class [i.e., (+)-METH, (+)-AMP, and (+)-MDMA] (Fig. 1).

There are other medication design issues that further complicate the development of effective treatments for (+)-METH-like stimulants. First, (+)-METH is one of several stimulant drugs of abuse with similar or overlapping effects. In particular, (+)-AMP is both a pharmacologically active metabolite of (+)-METH and a frequently used drug of abuse that could be substituted for (+)-METH. Second, (+)-MDMA can produce life threatening effects at high doses (Cho and Segal, 1994; Farre et al., 2004). In addition, all of these drugs are stereoisomers, with the minus isomers having a significantly different pharmaceutical profile of effects. For example, (+)-mescaline is commonly used as a bronchodilator in over the counter medications. The minus isomers of these drugs could potentially be purposely taken by drug abusers to neutralize mAb medications with high affinity binding for both plus and minus stereoisomers. In a related way, there are many structurally similar compounds like ephedrine and pseudoephedrine that could be used to lessen the efficacy of mAb therapies if the mAb is not highly specific for (+)-METH-like structures.

Previous reports have described the generation of mAb against (+)-METH for diagnostic use (Faraj et al., 1976; Usagawa et al., 1989; Sutitjitaapisal and Ratanabanangkoon, 1992), but none have reported the high affinity and class specificity that would be required to make a broadly effective antagonist for (+)-METH-like drugs. Our group (McMillan et al., 2002; Laurenzana et al., 2003; Byrnes-Blake et al., 2005; Gentry et al., 2006; Pitas et al., 2006) and others (Danger et al., 2006) have reported the generation of potential mAb antagonist for (+)-METH or (+)-MDMA as a racemic mixture (Danger et al., 2006). Our laboratory has also reported that some of these anti-(+)-METH mAb medications are effective for treating METH overdose (Byrnes-Blake et al., 2005) and for blocking (+)-METH self-administration (McMillan et al., 2004) in rat models of human drug use. Nevertheless, generating a high-affinity antibody to a broader group of (+)-METH-like drugs is especially challenging due to the simple structure of (+)-METH, the medical use of plus and minus isomers of METH-like drugs, and their very small molecular size (151 Da for (+)-METH).

Here, we report the design of five (+)-METH-like haptens that were specifically engineered to mimic important chemical features of (+)-METH, (+)-AMP, and (+)-MDMA. We hypothesized that a key to generating high-affinity, drug class-specific antibodies is careful design of both the drug-like haptens and the chemical linkers between the drug and the protein carriers. We used systematic hapten design, combined with immunization and hybridoma technology to generate novel, class-specific, therapeutic monoclonal antibodies against three medically important (+)-METH-like drugs. We also determined the mAb affinity, specificity, and common sequence and structural features that contribute to (+)-METH-like drug binding. The antibodies generated in these studies represent an important step in custom design of class-specific therapeutic antibodies for treatment of drug addiction or for other medical applications requiring broader specificity without sacrificing target selectivity.

Materials and Methods

Chemicals and Drugs. All chemicals and protein antigens were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise noted. Enzymes and Escherichia coli strains were purchased from Invitrogen (Carlsbad, CA). (+)-2,6-3H2[150Ci/mol)]methamphetamine ([(+)-3H]METH; 23.5 Ci/mmol) and ([±]-2,6-[3H2]N)amphetamine ([([±]-3H]AMP; 45 Ci/mmol) were obtained from the National Institute on Drug Abuse (Bethesda, MD) after synthesis at the Research Triangle Institute (Research Triangle Park, NC). Other METH-like drugs used in this study were also obtained from the National Institute on Drug Abuse.

[(+)-3H]METH was used as sent, but the [([±]-3H]AMP was chromatographically separated to obtain [([±]-3H]AMP for use in our studies of (+)-AMP specificity. The separation was performed on a 150 x 4 mm (i.d.), 5-μm CrownPak CR column (Chiral Technologies, Inc., Exton, PA). The mobile phase consisted of 0.1 M perchloric acid (Fisher Scientific Co., Pittsburgh, PA) containing 10% (v/v) methanol. The column temperature was maintained at 13°C. The flow rate was 1.0 ml/min, and the injection volume was 50 μl. Chromatographic peaks were detected using ultraviolet absorption detection at a wavelength of 210 nm. The retention times for [([±]-3H]AMP and [([±]-3H]METH were 20.1 and 24.4 min, respectively.

Haptens and Hapten-Protein Conjugation. Five different stereospecific (+)-isomer (+)-METH-like haptens were synthesized. All haptens were synthesized as HCl salts to aid in solubility, and they were stored as solids or powders until used. The chemical structures are shown in Table 1. The complete synthesis of one of the haptens, (+)-METH P6, was reported previously (Byrnes-Blake et al., 2001). The detailed description of the synthesis of the other four haptens is beyond the scope of this article, and it is not reported here. The chemical names and abbreviations of the five haptens are as follows: (S)-(+)-4-(3-carboxypropyl)methamphetamine, (+)-METH P4; (S)-(+)-4-(5-carboxypropyl)methamphetamine, (+)-METH P6; (S)-(+)-4-(5-carboxypropyl)oxymethamphetamine, (+)-METH P6; (S)-(+)-3-(5-carboxypropyl)oxymethamphetamine, (+)-METH M06; and (S)-(+)-3-(4-carboxynonyloxy)methamphetamine, (+)-METH M010.

Each hapten was initially covalently bound to at least two to three different protein antigens and used for immunization of mice to test for anti-METH IgG response. The individual mouse and hapten-protein antigen combination that yielded the highest anti-(+)-METH

Fig. 1. Structures of (+)-AMP, (+)-METH, and (+)-MDMA, members of the amphetamine class of abused drugs.
IgG titers were chosen for production of monoclonal antibodies (see details below). The following is a list of the hapten-protein conjugates that produced the mAb listed in Table 1: (+)-METH P4 and (+)-METH P6 conjugated to bovine serum albumin, (+)-METH PO6 and (+)-METH MO6 conjugated to Inject Supercarrier Immune Modulator (catorized BSA; Pierce Chemical, Rockford, IL); and (+)-METH MO10 conjugated to ovalbumin.

All chemical reactions for covalent binding of the hapten to protein antigens followed the same general procedure. The haptns were first solubilized in either 0.1 M 2-[N-morpholino]ethanesulfonic acid buffer, pH 4.5, or dimethylformamide and then the pH was adjusted to 4.5 with HCl. All haptns were coupled to their respective protein antigens by a carbodiimide reaction using the cross-linker 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (Pierce Chemical). This chemical synthesis forms a peptide bond between the carboxyl group of the hapten linker arm and free amino groups of lysine side chains in the respective proteins. The reactions were conducted with continuous stirring under dark conditions at room temperature for 18 h. At the end of the reaction, all antigens were purified as described by Byrnes-Blake et al. (2003). This purification involved dialysis against distilled water, phosphate-buffered saline, pH 7.4, and a final purification of the soluble fraction on a gel filtration column in phosphate-buffered saline, pH 7.4. Purified antigens were stored at −80°C until needed.

**Immunization, Screening, and Hybridoma Generation.** Female BALB/c mice (Charles River Laboratories, Wilmington, MA) were used for all immunizations. For production of the (+)-METH P6 mAb, mice were immunized subcutaneously in the hindquarters with 100 μg of the (+)-METH P6 antigen emulsified 1:1 (v/v) in TiterMax adjuvant (CytRx Corporation, Norcross, GA) and boosted monthly with 50 μg of the antigen until a favorable titer was reached. For all other antigen immunizations, the mice were initially immunized in the hindquarters subcutaneously with 20 to 100 μg of antigen emulsified in Freund’s complete adjuvant. The initial immunization was followed by a boost with 20 to 50 μg of antigen emulsified in Freund’s complete adjuvant. The initial immunization was followed by a boost with 20 to 50 μg of antigen emulsified in Freund’s incomplete adjuvant 3 weeks later followed by three boosts at 6-week intervals, until a favorable titer level was reached. Serum samples were taken via tail bleed periodically to measure anti- (+)-METH IgG. Titers were measured by enzyme-linked immunosorbent assay using 96-well microtiter plates coated with the original hapten conjugated to a different protein. For example, if the original antigen was (+)-METH-MO6 conjugated to thyroglobulin was used to avoid selecting carrier protein-reactive antibodies. The screening for anti- (+)-METH IgG response was conducted by a [(+)-3H]METH radioimmunoassay (RIA), using (+)-METH and (+)-AMP as the inhibitors. After sufficient anti- (+)-METH IgG titers were achieved, conventional hybridoma technology was used as described previously by our laboratory (Valentine et al., 1994). The hybridoma fusion partner for mouse B cells was cell line P3 X 63Ag8.653 (American Type Culture Collection, Manassas, VA). IgG isotype and light chain identity were determined with a mouse antibody isotyping kit (Roche Diagnostics, Indianapolis, IN).

**Production and Purification.** Monoclonal antibodies were produced in either a Cell-Pharm System 2500 hollow fiber bioreactor (Unisyn Technologies, Inc., Hopkinton, MA) (Valentine et al., 1996) or in a Biostat B 10 liter bioreactor (Sartorius Corp., Edgewood, NY). All antibodies were harvested and stored at −80°C until purification.

**TABLE 1**

<table>
<thead>
<tr>
<th>Hapten Structure and Abbreviated Name</th>
<th>mAb Name (Isotype and Light Chain Type)</th>
<th>Key Psychostimulant</th>
<th>Preclinical Testing</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)-METH P4</td>
<td>Anti-METH/MDMA mAb6EH8 (IgG1, κ)</td>
<td>(+)-METH</td>
<td>mAb-PCKN, CV, DD</td>
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<tr>
<td>(+)-METH P6</td>
<td>Anti-METH/MDMA mAb6H4 (IgG2, κ)</td>
<td>(+)-MDMA</td>
<td>mAb-PCKN, CV, DD, SA</td>
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<tr>
<td>(+)-METH PO6</td>
<td>Anti-METH/MDMA mAb6EH7 (IgG2a, κ)</td>
<td>(+)-AMP</td>
<td>mAb-PCKN, CV, DD, SA</td>
</tr>
<tr>
<td>(+)-METH MO6</td>
<td>Anti-METH/MDMA mAb9B11 (IgG1, λ)</td>
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<td></td>
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<tr>
<td>(+)-METH MO10</td>
<td>Anti-METH/MDMA/AMP mAb4G9 (IgG2a, κ)</td>
<td>(+)-MDMA</td>
<td>mAb-PCKN, CV, DD, SA</td>
</tr>
</tbody>
</table>

**Key Psychostimulant**

- CV, cardiovascular; DD, drug discrimination; L, locomotor; mAb-PCKN, antibody pharmacokinetics; M-PCKN, METH pharmacokinetics; and SA, self-administration.

* The superscript numbers after abbreviations refer to the following references: 1 Byrnes-Blake et al. (2003); 2 Laurenzana et al. (2003); 3 McMillan et al. (2002); 4 McMillan et al. (2004); 5 Byrnes-Blake et al. (2005); and 6 Gentry et al. (2006). No reference indicates manuscript is in preparation.
mAb were purified either by affinity chromatography using protein G-Sepharose (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK), or ion exchange chromatography using SP Sepharose (GE Healthcare) as described in Hardin et al. (1998), or a combination of the two methods. After purification, all antibodies were concentrated, and buffer was exchanged into 15 mM sodium phosphate containing 150 mM sodium chloride, pH 6.5 to 7.5, as described in McMullan et al. (2002).

**Determination of Immunochemical Specificity.** The cross-reactivity profiles of each mAb for methamphetamine, structurally related compounds, and unrelated compounds was determined by RIA in a manner similar to that described by Owens et al. (1988). An IC\(_50\) value for inhibition of \(+\)-METH and \(\text{AMP}\) was determined for each ligand after fitting a sigmoidal curve to the data points. \(K_D\) values for mAb were determined by the method of Akera and Cheng (1977).

cDNA Cloning and Sequencing of mAb. For these studies, five prototype anti-METH mAb ranging in METH affinities from 11 to 250 nM were analyzed (Table 1). A single prototype mAb resulting from each of the haptenst was chosen for detailed studies. The light chains cDNA of the mAb were cloned by reverse transcription-polymerase chain reaction using SuperScript II reverse transcriptase (Invitrogen) with an exact reverse primer matching C terminus of the light chain named MLEN1.Dnot (\(5'\)-GGG GCG GCC GCG CGT CTC AGG ACC TTT GTC TCT AAC-3'). The light chains of mAb6H4, mAb6H8, and mAb6H7 were amplified in the forward direction with the degenerate primer ML2, and the light chain of mAb6G9 was amplified in the 5' direction with the degenerate primer ML4 (Coloma et al., 1992). The light chain of mAb6B11 was amplified in the forward direction with the primer sequence 5'AGG GCCCTTGA/TCCTCACTATTACCTCCTCCTCGTGC-3'. The resulting cDNA was blunt-ligated into the Smal site of the cloning vector pGEM-3Z.

The heavy chain cDNA of all IgG1 (from \(+\)-METH P4, \(+\)-MDMA and \(-\)-MDMA MO6) mAb were amplified using reverse transcription-polymerase chain reaction as described above with an exact reverse primer to the C terminus of the heavy chain, named MHEN.Dnot 1 (\(5'\)-GGG GCG GCC GCA GGG CTC CAA GGA CAC TGG GAT CAT TT-3'), and a mixture of three degenerate primers based on the MHALT primers from Coloma et al. (1992). The primers were modified from the originally published sequence only by the substitution of a NheI restriction site for the original restriction site. The IgG2 mAb (from \(+\)-METH P6 and \(-\)-MDMA MO10) were amplified with the reverse primer 5'CCCTTCGGTCCTCCGGTAAA-3'.

The forward sequence of the heavy chain of mAb6H8 was amplified with primer MHALT1 (Coloma et al., 1992). The forward primers for mAb6H4, mAb6H7, mAb6B11, and mAb6G9 were designed from the results of N-terminal sequencing of the mature proteins (see Fig. 2 for protein sequence). The primer sequences used were 5'-GAGGTACCGCTAGATCTACAGACCTA-3' for mAb6H4, 5'-GATCTAGAATCCGAACGTGATCGATT-3' for mAb6H7, 5'-GAGGTACCGCTAGATCTACAGACCTA-3' for mAb6B11, and 5'-GAGGTACCGCTAGATCTACAGACCTA-3' for mAb6G9. The cDNA was then blunt-ligated into the Smal site of cloning vector pGEM-3Z. The resulting plasmids of all mAb were transformed into E. coli strain DH5\(\alpha\) and sequenced at University of Arkansas for Medical Sciences DNA Core Sequencing Facility (Little Rock, AR).

All sequences were submitted to the GenBank database. The GenBank-assigned accession numbers of the heavy chains of mAb6H8, mAb6H4, mAb6H7, mAb6B11, and mAb6G9 are 774083, 786626, 877567, 881246, and 877579, respectively. The GenBank-assigned accession numbers of the light chains of mAb6H8, mAb6H4, mAb6H7, mAb6B11, and mAb6G9 are 774081, 774071, 881226, 877571, and 877573, respectively. The germline use of the different mAb was determined by comparing the DNA sequences to those in the ImMunoGeneTics database using the web-based program V-QUEST tools (http://imgt.cines.fr) and by visual examination of the sequences (Giudicelli et al., 2004).

**IgG Variable Region Structural Modeling and Analysis.** Molecular modeling of the three-dimensional structure of the variable regions of three of the mAb was performed using the WAM antibody modeling algorithm (Whitelegg and Rees, 2000). mAb6H4, mAb6H8, and mAb6G9 were chosen for more detailed analysis because they exhibited the full range of affinities for \(+\)-METH and a broad range of ligand specificities for other important METH-like drugs. The primary amino acid sequences of the variable regions of the heavy chain and light chain were first submitted to the WAM antibody modeling site for alignment. The program aligned the sequences against known sequences in the database and searched for canonical classes of complementary-determining regions (CDRs). Based on these classifications, the program assigned a three-dimensional structure to the framework and CDR regions by fitting the main chain to that of the closest known structures.

**Structure Validation.** The resulting structure files from WAM modeling were analyzed for structure quality. The structure file was submitted to the program RAMPAGE to analyze the \(\Phi\) and \(\Psi\) angles of the main chain atoms (Lovell et al., 2003; http://raven.bioc.cam.ac.uk/rampage.php). Side chain orientations were analyzed by the program PROBITY (Lovell et al., 2003; http://kinemage.biochem.duke.edu/molprobity/index.html). Based on this analysis, small adjustments were made to side chain orientations and missing hydrogen atoms were added by the computer program Sybyl (Tripos, St. Louis, MO).

**Ligand Docking.** For docking simulation, the FlexX (Tripos) program was used. First, a deep pocket was identified at the interface of the CDR regions from surface modeling and electrostatic calculations in Pymol (Delano Scientific, San Carlos, CA) and Sybyl (Tripos). To define this region as a putative active site, residues within an area 6 Å around F L94 (for mAb6H4) or Y L94 (for mAb6G9) were selected. The METH ligand was assigned formal charges by Sybyl, and the molecule was allowed partial flexibility. The program was set to find the 30 best docking conformations and return these confirmations in a consensus scoring table.

**Results**

**Overall Strategy.** Our goal was to conduct structure-activity studies of the molecular features of haptenst that would stimulate immune cell production of high-affinity mAb for medical applications. Although high affinity for \(+\)-METH was our foremost goal, we also hoped to generate a single mAb with high affinity for \(+\)-METH, \(+\)-AMP, and \(+\)-MDMA. Inasmuch as antibodies selected by immuniza- tion with hapten-protein conjugates are directed at parts of the molecule projecting from the protein, the molecular ori- entation of the hapten on the protein carrier was considered critical to the specificity of the antibodies (Landsteiner, 1962). The \(+\)- or \(-\)-isomers of METH-like compounds produce significantly more psychomimetic effects, locomotor activity, stereotyped behavior, and monoamine oxidase inhibi- tion than the \(-\)- or \(\text{t}\)-isomers (Marquardt et al., 1978; Caldwell, 1980). Thus, all METH-like haptenst were synthesized to mimic the molecular features of \(+\)-isomers of the drugs.

**Hapten Design and Antibody Selection.** For these studies, the hapten spacers were progressively lengthened from four to 10 atoms to increase the potential for greater interaction of the METH-like structures with the antibody binding site and to increase the flexibility of the spacer. We hypothesized that a progressive lengthening of the spacer...
arm would lead to increases in affinity due to improved access to the entire METH-like structure; and the different immobilized conformations could elicit antibodies having different conformational selectivity for \((+/H11001)\)-METH-like compounds. We also attempted synthesis of an eight-molecule spacer hapten to complete our series, but synthesis proved more difficult than expected, so this hapten was abandoned. The haptens were conjugated to the terminal amino groups of lysines in bovine serum albumin or ovalbumin by carbodiimide chemistry, which forms a peptide bond with the available carboxylic acid on the hapten. There are 59 lysines in bovine serum albumin, 20 in each of the four subunits of ovalbumin and even more conjugation sites are available on cationized bovine serum albumin (i.e., Imject Supercarrier Immune Modulator). However not all of the lysines or conjugation sites are available at the surface of the protein for coupling to the haptens. Preliminary optimization experiments showed that a ratio of hapten to protein of 30:1 to 90:1 yielded the best incorporation rates for the syntheses. Although we were unable to precisely determine the hapten incorporation rate for the antigens, initial mass spectrophotometry studies indicated that an average of four haptens was conjugated to each molecule of protein (data not shown).

Because our primary goal was to select for high-affinity mAb, the antigen dose was kept relatively low (e.g., 10–20 \(\mu g\)). Although immunization with higher hapten-antigen doses (e.g., 50–100 \(\mu g\)) sometimes led to higher titers, the affinity for \((+/H11001)\)-METH was often too low. Thus, we usually immunized with a minimal dose of antigen. This strategy routinely led to immunological response in only 40 to 70% of animals. In more recent studies, we discovered that a primary reason for 100% immunological response was the low incorporation rate of the hapten on protein antigens, which we in part overcame by judicious use of Freund’s complete and incomplete adjuvants to boost and sustain immunological response.

We routinely screened each mouse serum from each group of immunizations (typically six to 10 mice) after each boost to determine the maturity of the immune response and the relative immunochemical characteristics of the polyclonal serum (titer, affinity, and specificity). For this assay, we used a \(^{[3]H}(+/)\)-METH RIA. The screening assay always involved

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**Fig. 2.** Amino acid sequence alignment of variable regions of five moderate to high-affinity anti-METH/MDMA and anti-METH/MDMA/AMP mAb. The sequence is in single letter amino acid notation and numbered according to Kabat and Wu (1991). Location of the framework and CDR residues are indicated.
inhibitions of \((+)^3\text{H}\)METH binding with increasing doses of \((+)^3\text{H}\)METH and \((+)^3\text{H}\)AMP to determine the relative affinities for each ligand. The final choice of a specific mouse for use in generating hybridomas was based primarily on the animal with the highest titer and affinity for \((+)^3\text{H}\)METH. From this process of screening immune serum, we generally found three to 10 unique monoclonal antibodies from each fusion. Most importantly, we did not discover a polyclonal antiserum that was positive for \((+)^3\text{H}\)AMP until we started using the MO10 hapten.

For producing the hybridomas, we chose mice that had been immunized with Freund’s complete adjuvant and boosted with Freund’s incomplete adjuvant. The one exception was the immunizations with \((+)^3\text{H}\)METH P6, which used TiterMax as the adjuvant. In preliminary optimization experiments (data not shown), we tried immunizations with alum-precipitated antigens, TiterMax adjuvant, and Ribi’s adjuvant on several occasions. Although these adjuvants generally produced high titers, we found that our highest affinity antibodies were generated with Freund’s adjuvants.

After screening more than 25,000 potential hybridoma cell lines for mAb production, five mAb with the most favorable immunochromatographic characteristics were extensively studied for molecular properties and preclinical efficacy (Table 1). The rest of the hybridoma cell lines were stored frozen in case of future need. The selection of a mAb for more extensive in vitro and in vivo testing was based on the desire to have a range of affinities, a range of drug specificities, and a high level of mAb production from the parent hybridoma cell line. This final criterion was needed to increase the feasibility of large-scale mAb production for in vivo testing. In most cases, there was one or more similar affinity or specificity mAb that were produced from the same fusion. For example, the separate fusions that produced mAb6H4 and mAb4G9 (Table 1) also produced mAb with virtually the same affinity and specificity but with slightly different amino acid sequences (data not shown). These two particular antibodies were chosen because the parent hybridoma cell line produced significantly more mAb.

mAb Cross-Reactivity Studies. RIA was used to determine the relative affinity and cross-reactivity profile of each mAb (Tables 1 and 2). Because mAb4G9 was the only mAb to significantly cross-react with \((+)^3\text{H}\)AMP (Tables 1 and 2), we wanted to better understand its affinity for \((+)^3\text{H}\)AMP. Thus, we conducted an RIA analysis using \([(+)^3\text{H}]\text{AMP}\) [in addition to an RIA with \([(+)^3\text{H}]\text{METH}\)] and AMP as the inhibitor. These data showed the actual affinity for AMP was 51 nM (Table 1), demonstrating that this mAb has virtually the same \(K_D\) value for \((+)^3\text{H}\)AMP and \((+)^3\text{H}\)METH. \([(+)^3\text{H}]\text{MDMA}\) was not available for determining a more accurate \(K_D\) value for \((+)^3\text{H}\)MDMA binding, but it seems likely that the true \(K_D\) value would be significantly lower than the value indicated by MDMA inhibition of \([(+)^3\text{H}]\text{METH}\) binding in the RIA.

Antibody Sequence Analysis. To gain a better molecular understanding of how the primary amino acid sequence affected mAb affinity for \((+)^3\text{H}\)METH, we analyzed the related and unrelated sequence features in each mAb variable regions (Fig. 2). Three of the mAb were IgG1 subclass and two were IgG2 subclass (Table 1). Except for anti-METH/MDMA mAb9B11 (λ light chain), all of the mAb possessed a κ light chain.

An analysis of complementary determining regions revealed a high degree of diversity in both composition and length. The first light chain CDRs (L1) varied in length from 10 to 14 residues, and with the exception of mAb4G9, they possessed a large number of serine residues. The only conserved residue in CDR L1, or any of the light chain CDRs, was the serine at position L26. The L2 CDRs were seven residues in length except for mAb6H7, which possessed only five amino acids. The L3 CDRs were all nine residues in length except mAb4G9, which had 10 residues. The CDRs of the heavy chain regions exhibited similar lengths in CDRs H1 and H2, but little homology. CDR H1 has a conserved threonine at position H30 and either a tryptophan or tyrosine five residues apart, with the second tyrosine before the tryptophan at H103.

Although comparisons of CDR sequences are important, differences in CDR can be attributed to differences in germline sequences of particular V-region genes and to somatic mutation within the CDRs of these V-region genes. To better understand the relative importance of the germline and somatic mutations, we analyzed the sequenced genes using the IMGT database (Giudicelli et al., 2004). The analysis showed that each antibody was unique and not clonal. That is, rather than coming from one germline gene arrangement early in B cell development, they resulted from unique VDJ recombination events. These unique germline gene rearrangements then underwent somatic DNA mutations that were often silent, but some resulted in amino acid changes that differed from the original germline gene. Thus, we found no clear pattern of response.

Molecular Modeling. Based on the results of the primary sequence alignment, we chose three mAb (mAb6H4, mAb6H8, and mAb4G9) for structural modeling. Each CDR

<table>
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<tr>
<th>Drug</th>
<th>Antibody Specificity (Relative Potency to METH)</th>
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<tbody>
<tr>
<td></td>
<td>11 nM mAb6H4(^a)</td>
</tr>
<tr>
<td>(+)^3\text{H})METH</td>
<td>1.00</td>
</tr>
<tr>
<td>(+)^3\text{H})AMP</td>
<td>0.001</td>
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<tr>
<td>(+)^3\text{H})MDMA</td>
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<td>(+)^3\text{H})MDMA</td>
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\(^a\)Relative potency to METH = (RIA IC\(_{50}\) value for METH/RIA IC\(_{50}\) value for test ligand). See Table 1 for the structures of the haptons used to generate these antibodies.

\(^b\)IC\(_{50}\) value for METH binding from Table 1.
variable region was assigned and given a canonical classification (Al-Lazikani et al., 1997), except for the H3 CDRs, which do not possess canonical classes. The three dimensional models exhibited classical antibody β-sheet fold conformation (Fig. 3). In general, all models showed conformity with geometrical constraints throughout the structures. The analysis indicated that less than 2% residues had main chain Ψ and Φ angles in outlier regions. All three models seemed to conform reasonably well to known protein structural features and constraints, and they presented an appropriate foundation to conduct base docking analysis.

All CDRs fell within canonical classes except L3 of mAb4G9 and the H3 CDRs, which do not have canonical classes. The CDR H3 regions of all three antibodies were predicted to form a kinked or “hairpin” rather than extended conformation. Comparison of the models revealed conserved structural elements and some potentially important differences in the root mean square deviations (RMSD) of the CDR loop configurations (Fig. 3). The loop structure of mAb6H4 was arbitrarily chosen as a reference point to compare the differences from the other two antibodies, because it had the highest affinity for (+)-METH. The L2 CDRs of all three antibodies occupied nearly the same spatial positions. The L3 regions of mAb6H4 and mAb6H8 were very similar, even though they differed in affinity for (+)-METH by approximately 25-fold.

Docking. Based on our modeling results, we chose to perform docking simulation with mAb6H4 and mAb4G9. According to our models (Fig. 4), a deep pocket is formed by the interaction of CDR loops H1, H2, L1, and L3 for both antibodies, with a wider pocket formed in the binding region of mAb4G9 due to a shorter H3 CDR. We created a theoretical docking of (+)-METH into these mAb pockets and identified residues within 8 Å of the ligand as possible sites for ligand-mAb interaction (Fig. 4). The results of this FlexX-based docking indicated that the (+)-METH molecule was generally oriented with the hydrophobic phenyl group toward the interior of the pocket. In mAb6H4 and mAb4G9, the charged nitrogen of (+)-METH was in proximity to a histidine at position L32 and H35, respectively.

Discussion

In this article, we report the generation of a potentially therapeutic antibody with custom specificity to three major drugs of abuse and hapten design criteria that could be broadly applicable to generation of antibodies against small molecules. During this multiyear research effort, we have reported some of the initial characteristics of two of the haptons and two of the resulting mAb (i.e., hapten structure, affinity, and binding properties for mAb6H8 and mAb6H4). A list of the publications for these mAb is included in the legend of Table 1. Data for PO6, MO6, and MO10 have never been reported. In addition, new is the sequences analysis of all five mAb and structural modeling of mAb6H4, mAb6H8, and mAb4G9. It is noteworthy that this is the first time we have published the interpretation of years of research aimed at understanding the hapten structure requirements to produce therapeutic antibodies. This type of overview is uncommon in the literature. After extensive in vitro screening, we determined that mAb4G9 exhibited features that we think are best in a potential therapeutic candidate for (+)-METH treatment. These features are high-affinity binding to (+)-METH, (+)-AMP, and (+)-MDMA, little or no cross-reactivity with (-)METH-like isomers, and no significant cross-reactivity with endogenous compounds or structurally similar common medications (Tables 1 and 2). We attribute the selection of these features to the design of the hapten (+)-METH MO10 (Table 1). No other hapten/linker/antigen combination yielded an antibody with high affinity for all three of these drugs of abuse.

Attaching the linker of the hapten distal to the chiral center of the molecule yielded a refined specificity for (+)-isomers. Antibody medications that are highly reactive against (-)-isomers would not be therapeutically advantageous because these isomers have no known addiction liabil-

![Fig. 3. Top, stereo view of superposed molecular models of anti-METH mAb. The variable regions of the three mAb were modeled, structurally aligned, and represented in cartoon format. The framework residues are represented in blue. The CDR regions are colored according to mAb: mAb6H4, blue; mAb6H8, red; and mAb4G9, green. The heavy chain, light chain, and CDR regions are labeled. Bottom, RMSD (angstroms) of CDRs from the main chain conformation of mAb6H4.](image-url)
ity and produce no significant medical threat. We hypothesized that all haptens might generate mAbs with significant cross-reactivity with (+)-METH and (+)-MDMA because of the para- or meta-positioning of the spacer arm on the phenyl ring of the hapten. The relatively short length of spacer arms of haptens (+)-METH P4 and (+)-METH P6 (4- and 6-carbon linkers, respectively), coupled with attachments at the para-carbon of the (+)-METH phenyl ring (Table 1), hindered the flexibility of haptens. This probably forced the immune system to recognize the presence of the methyl group on the nitrogen molecule of (+)-METH and (+)-MDMA and its absence in (+)-AMP. Thus, mAb affinity was high for (+)-METH and (+)-MDMA but low for (+)-AMP. The hapten (+)-METH PO6, like (+)-METH P6, was designed with a linker attached to the para-carbon of the phenyl ring, but an oxygen was included to influence localized charge and solubility and to mimic the presence of an oxygen atom at the para or meta positions, respectively, of the methylenedioxy group of (+)-MDMA (Table 1). For this reason, we included an oxygen attached to the meta-position of phenyl ring structure was included in the next series of haptens, (+)-METH MO6 and (+)-METH MO10, which also facilitated synthesis. We think this strategy helped improve immune presentation. Specifically, the oxygen of the (+)-MDMA-like structure was presented along the same spatial plane as the chiral center of the (+)-METH molecule. The longer (+)-METH MO10 spacer was used to allow more flexibility of the hapten on the protein in hopes of discovering mAb with broader recognition of (+)-METH-like structures. In the end, these combined strategies resulted in the best balance of affinity and led to the discovery of mAb4G9.

From these studies, we learned that 1) linkers located distal to the chiral center of this very small molecule favor generation of stereospecific antibodies, 2) a longer flexible linker arm such as (+)-METH MO10 favors generation of antibodies with broader selectivity for (+)-METH-like compounds, and 3) spacers equal to or greater than six atoms produce higher affinity mAb. It is noteworthy that discovery of mAb4G9 is not an isolated event. We have since discovered other MO10-derived mAbs with similar specificities for (+)-METH and (+)-AMP.

We performed sequence analysis to understand differences and commonalities of our most promising antibodies. This analysis elucidated unique sequence differences in the antibody CDRs. A common feature is a conserved proline at position 95 or 95a of all CDR L3 regions, except for mAb9B11 (Fig. 2), which has a serine residue. Because of their ability to form “hinges”, proline residues often lend flexibility in main
Important for interaction with the phenyl ring of (+)-METH or an aromatic residue. We reasoned these residues could be immediately followed by either a hydrophobic amino acid (i.e., leucine or valine as in mAb6H4 and mAb9B11, respectively) or an aromatic residue. We reasoned these residues could be important for interaction with the phenyl ring of (+)-METH-like compounds via hydrophobic or π-π interactions, and the preceding proline could lend flexibility to adapt to different conformations.

Based on our molecular modeling analyses, the interface between (+)-METH and the mAb is relatively small [the surface area of (+)-METH is 174 Å²] with small shifts in protein conformation producing large changes in binding. As can be seen in Fig. 3, the most striking deviations occurred in the H3 CDR region, with more than 6- and 7-Å RMSD in mAb6H8 and mAb4G9, respectively. The diversity in the positions of the CDRs suggests that each of these antibodies exhibits a binding paradigm to (+)-METH-like drugs that is somewhat independent of loop configuration. The surface rendering of the models exposed a deep pocket at the CDR interface of mAb6H4. This pocket seemed to be approximately the size of (+)-METH and would probably accommodate docking of the ligand. By contrast, the potential binding pocket of mAb4G9 was wider and shallower. We hypothesize that the longer linker arm of (+)-METH MO10 combined with the changed dihedral angle of an oxygen at the meta position of the phenyl ring contributed to the formation of a larger pocket. Analysis indicated that only five of the six CDR loops might be directly involved in binding of (+)-METH-like drugs, with L2 showing little contact. The (+)-METH docking simulation with FlexX indicated that the potential binding pockets are dominated by aromatic residues with some capability of making hydrogen bonds (i.e., histidine and tyrosine).

The discovery of mAb4G9 is an important step toward developing a mAb-based antagonist for the treatment of (+)-METH-like drugs of abuse. From a medication development approach, this new class of antibody has several advantages. First, it might be used for treating overdose of (+)-METH or (+)-AMP or (+)-MDMA and in relapse prevention for (+)-METH and (+)-AMP addiction. We have already demonstrated the proof of principle that some of these mAb medications can be used to treat (+)-METH addiction and overdose (see multiple references in Table 1), and reports of successful mAb4G9 effects in several preclinical models of diseases of addiction are in preparation. Second, having a single mAb medication that can bind (+)-METH, (+)-AMP, and (+)-MDMA means the regulatory approval process could be streamlined to some degree, because one antibody would have multiple interrelated indications. Third, if the mAb was used as a long-acting pharmacokinetic antagonist for treating (+)-METH or (+)-AMP addiction, and if the patient then switched to the other drug, the medication would still be effective. We purposely excluded (+)-MDMA (commonly called ecstasy) because we could find no evidence in the literature that the racemic mixture is considered an addicting drug. Finally, (+)-AMP is a pharmacologically active metabolite of (+)-METH. Thus, the same antibody would be active against either parent or metabolite in reversing adverse effects.

From our study, we have determined that 1) linkers located distal to the chiral center of the small molecule favor the generation of stereospecific antibodies, and 2) a longer linker arm favors the generation of antibodies with broader specificities to similar compounds. The longer linker might allow access to the molecule along the longest side or “face” of the molecule, maximizing the number of molecular interactions with the antibody. The hapten that generated mAb4G9 should also prove useful as the ligand in the selection process for in vitro affinity maturation techniques (e.g., phage, ribosome, and yeast display technologies; Hoogenboom, 2005), where haptenas are a critical component in the selection process of higher affinity antibodies and in choosing custom specificity to a class of small molecules.

In conclusion, we presented the hapten design criteria needed for generating antibody medications for (+)-METH-like drugs. Although all five of our haptenas produced anti- (+)-METH moderate-to-high affinity antibodies against (+)-METH and (+)-MDMA, only one hapten generated high-affinity mAb for (+)-METH, (+)-AMP, and (+)-MDMA. The hapten was (+)-METH-MO10, and the antibody was designated anti-METH/AMP/MDMA mAb4G9. The primary weakness of mAb4G9 is its affinity. We hypothesize that an even lower Kₐ value (in the picomolar range) with the same specificity profile could further improve clinical performance, but this hypothesis has not been tested. If preclinical testing of its ability to reduce drug dependence (currently in progress) proves its effectiveness in animal models, it would then be necessary to produce a chimeric or humanized version of this mAb for use in humans. Although other haptenas and hapten-antigen combinations could possibly produce even better mAb for therapeutic applications, we think these comprehensive studies help to focus on the key design elements needed to produce medically important therapeutic antibodies.

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References


Gentry WB, Laurenzana EM, Williams DK, West JR, Berg RJ, Terlea T, and Owens SM (2006) Safety and efficacy of an anti- (+)-methamphetamine monoclonal...
antibody in the protection against cardiovascular and central nervous system effects of (+)-methamphetamine in rats. *Int Immunopharmacol* **6**:968–977.


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