Development and Preclinical Testing of a High-Affinity Single-Chain Antibody against (+)-Methamphetamine


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Received November 16, 2007; accepted January 10, 2008

ABSTRACT

Chronic or excessive (+)-methamphetamine (METH) use often leads to addiction and toxicity to critical organs like the brain. With medical treatment as a goal, a novel single-chain variable fragment (scFv) against METH was engineered from anti-METH monoclonal antibody mAb6H4 (IgG, \( \kappa \) light chain, \( K_d = 11 \) nM) and found to have similar ligand affinity (\( K_d = 10 \) nM) and specificity as mAb6H4. The anti-METH scFv (scFv6H4) was cloned, expressed in yeast, purified, and formulated as a naturally occurring mixture of monomer (~75%) and dimer (~25%). To test the in vivo efficacy of the scFv6H4, male Sprague-Dawley rats (\( n = 5 \)) were implanted with 3-day s.c. osmotic pumps delivering 3.2 mg/kg/day METH. After reaching steady-state METH concentrations, an i.v. dose of scFv6H4 (36.5 mg/kg, equimolar to the METH body burden) was administered along with a \(^{[3H]}\text{scFv6H4} \) tracer. Serum pharmacokinetic analysis of METH and \(^{[3H]}\text{scFv6H4} \) showed that the scFv6H4 caused an immediate 65-fold increase in the METH concentrations and a 12-fold increase in the serum METH area under the concentration-time curve from 0 to 480 min after scFv6H4 administration. The scFv6H4 monomer was quickly cleared or converted to multivalent forms with an apparent \( t_{1/2,z} \) of 5.8 min. In contrast, the larger scFv6H4 multivalent forms (dimers, trimers, etc.) showed a much longer \( t_{1/2,z} \) (228 min), and the significantly increased METH serum molar concentrations correlated directly with scFv6H4 serum molar concentrations. Considered together, these data suggested that the scFv6H4 multimers (and not the monomer) were responsible for the prolonged redistribution of METH into the serum.

There are currently nearly 20 monoclonal antibody (mAb) medications approved by the United States Food and Drug Administration and over 20 more in early clinical or preclinical trials (Holliger and Hudson, 2005). These medications include full-length IgG mAbs, along with five mAb fragments as Fab, F(ab')\(_2\) (antigen binding fragments of IgG), or single-chain variable fragment (scFv) proteins. IgG mAbs are typically chimeric, humanized, or fully human proteins and are administered to patients requiring a long-acting antagonist with minimal extravascular penetration (Bazin-Redureau et al., 1997). IgG mAb is best for this purpose because it has a terminal elimination half-life (\( t_{1/2,z} \)) ranging from approximately 3 to 26 days. The longest \( t_{1/2,z} \) values are usually achieved when the antibody does not bind to tissue sites and is not prematurely cleared due to antigenicity. When a short duration of action and greater extravascular penetration are needed, a significantly smaller fragment like Fab (\( t_{1/2,z} \) ranging from 0.5–21 h; Trang, 1992) or scFv (\( t_{1/2,z} \) ranging from minutes to hours; Goel et al., 2000) is used. In particular, rat pharmacokinetic studies of an antianthrax toxin scFv report a \( t_{1/2,z} \) of ~5 min (Maynard et al., 2002). In addition, PCKN studies of a scFv in mice report \( t_{1/2,z} \) values of 2.7 and 1 min (Pavlinkova et al., 2000; Willuda et al., 2001).

It is possible that a short-acting scFv could be used to

Abbreviations: mAb, monoclonal antibody; scFv, single-chain variable fragment; \( t_{1/2,z} \), terminal elimination half-life; PCKN, pharmacokinetic; \( V_H \), variable heavy region; \( V_L \), variable light region; METH, (+)-methamphetamine; \(^{[3H]}\text{METH} \), (+)-\(^{[3H]}\text{H} \) methamphetamine; SEC, size exclusion chromatography; METH, (-)-\( \text{P} \)6, S(-)-6-[4-[(N-methylamino)propyl]phenyl]hexanoic acid; METH, (-)-\( \text{MOS} \), S(-)-6-[3-[(methylamino)propyl]phenoxyl]hexanoic acid; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; RIA, radioimmunoassay; HPLC, high-performance liquid chromatography; CLs, systemic clearance; (+)\( \text{MDMA} \), (+)-3,4-methylenedioxymethamphetamine; BMGY, buffered minimal glycerol-complex medium.
rapidly clear the body of small-molecule toxins. For example, Shelver et al. (1996) reported anti-desipramine scFv favorably altered rat serum desipramine concentrations. For these studies, a recombinant desipramine-specific scFv fragment (~27 kDa) was generated with a 15-amino acid (Gly₃Ser)₃ linker, using an amino terminus-variable heavy region (VH)-variable light region (VL)-carboxyl terminus orientation. For the preclinical test of this scFv, male rats were administered a tracer dose of [³H]desipramine (17 nmol), followed 15 min later with an equimolar dose of mouse monoclonal anti-desipramine scFv. One minute after scFv dosing, serum [³H]desipramine concentrations increased 7.3-fold, whereas control treatment with anti-desipramine monoclonal IgG and Fab increased 13.3- and 10-fold, respectively. These antibody-induced early increases in the [³H]desipramine serum concentrations appeared to be inversely related to the apparent volume of distribution of the three proteins (i.e., scFv > Fab > IgG). Although there were significant limitations in the study design (e.g., blood sampling was for only 15 min after antibody administration, and the doses of desipramine were extremely small), these data suggest larger doses of an anti-desipramine scFv could be a helpful antidote for desipramine toxicity.

A unique property of scFv is the ability of the heavy and light chains to form noncovalent multimeric derivatives including divalent, trivalent, and tetravalent proteins (Hudson and Kortt, 1999). Although these monomeric and multimeric proteins are denoted by several names in the literature, for the purposes of the discussion in this manuscript, we will use the terms monomeric/monovalent and multimeric/multivalent interchangeably. These configurations are believed to result from the heavy- or light-chain variable regions naturally associating with the corresponding light and heavy chains, respectively, of another scFv molecule. This multimer formation in vitro is at least partially dependent on the length of linker group between heavy- and light-chain Fv proteins (Hudson and Kortt, 1999). Amino acid sequences linking the heavy and light chains that are >12 residues yield a predominance of monomers, whereas shorter linkers yield noncovalently bound multivalent scFv proteins. The in vitro ratio of monomer to multimers is often dynamic and dependent on protein concentration and buffer conditions (Lee et al., 2002; Huang et al., 2005).

There have not been many studies elucidating the detailed pharmacokinetics of antibody fragments that target small molecules. Although the effects of dimerization on in vivo tumor binding and tissue localization are reported for one scFv (Kubetzko et al., 2006), no studies have extensively examined the pharmacokinetic fate of a scFv in the presence of an antigen target that would not affect its disposition. A potential solution to this issue is to use a small-molecule antigenic target like (+)-methamphetamine (METH; 151 Da), which would not be expected to affect the disposition of the much larger scFv (an ~27,000-Da protein).

In this study, we describe the design, production, and functional characterization of a high-affinity anti-METH scFv (designated anti-METH scFv6H4). We also report in vivo pharmacokinetic studies in rats, which show scFv6H4 quickly and dramatically increases serum concentrations of METH over an extended period of time (>5 h). Data from size exclusion chromatography (SEC) showed that in serum and in the presence of METH, scFv6H4 showed time-dependent in vivo conversion of the protein from monomeric to multimeric complexes without forming aggregates. We found that both monomer and multimeric complexes were functional, but the multimeric forms seemed to be primarily responsible for the prolonged redistribution of METH into the serum. These data suggest the effectiveness of scFv could be customized for specific medical indications by altering the scFv (e.g., linker length, pegylation) to maximize naturally occurring in vivo pharmacokinetics properties of the scFv.

Materials and Methods

Chemicals and Drugs. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Enzymes and Escherichia coli strains were purchased from Invitrogen (Carlsbad, CA). [³H]METH (23.5 Ci/mmol) labeled at two metabolically stable sites on the aromatic ring structure was obtained from the National Institute on Drug Abuse (Bethesda, MD) after synthesis at the Research Triangle Institute (Research Triangle Park, NC). Haptens METH (+)P6 and METH (+)MO6 that were used for generating and screening anti-METH scFv were synthesized by Drs. Ivy Carroll and Philip Abraham at the Research Triangle Institute. Other METH-like drugs were also obtained from the National Institute on Drug Abuse.

ScFv6H4 Cloning. The generation, characterization, and sequence determination of murine mAb6H4 (IgG1, κ light chain, Kᵢₐ = 11 nm) was previously reported (Byrnes-Blake et al., 2003; Peterson et al., 2007). General molecular and genetic techniques used for plasmid construction and transformation of scFv6H4 were described by Sambrook and Russell (2001). The coding sequences for the VH and VL regions of mAb6H4 were PCR amplified from the mAb6H4 cDNA (Peterson et al., 2007) heavy and light chains with the following primers synthesized by Integrated DNA Technologies (Corvalle, IA) in two separate PCR reactions with Pfu Turbo polymerase: primer 1 VH forward, GGAATTCCATGGATTACAAGGATGACGAC; primer 2 VH reverse, GACGAC; primer 3 VH reverse, GGCGGAGGTGGCTCTGGCGGTGGCGGATCCCAATTGGG. The amplification reaction was set to 30 cycles of 94°C for 15 s, 53°C for 30 s, 68°C for 1 min, and a final extension at 68°C for 7 min. To produce the coding sequence of the single-chain antibody, the products of the two PCR reactions were combined with primers 1 and 4 in a second reaction, which linked the two domains with the coding sequence for a (Gly₃Ser)₃ linker. The PCR product was gel-purified using the QIAquick gel purification kit (QIAGEN, Valencia, CA). The product was then restricted with enzymes EcoRI and HindIII and ligated into the amino terminus of the VH domain (Fig. 1). A His₆ affinity tag was encoded at the carboxyl terminus to aid in scFv protein purification. In brief, the VH and VL regions of mAb6H4 were PCR amplified from the mAb6H4 cDNA (Peterson et al., 2007) heavy and light chains with the following primers synthesized by Integrated DNA Technologies (Corvalle, IA) in two separate PCR reactions with Pfu Turbo polymerase: primer 1 VH forward, GGAATTCCATGGATTACAAGGATGACGAC; primer 2 VH reverse, GACGAC; primer 3 VH reverse, GGCGGAGGTGGCTCTGGCGGTGGCGGATCCCAATTGGG; primer 4 VH reverse, GTCAGACCTCCCGGGAGCG TTCCTCACCCAGTCTCC; and primer 4 VL reverse, GTCAA GCTTCCCGGGAGCGTTTATTTCC. The amplification reaction was set to 30 cycles of 94°C for 15 s, 53°C for 30 s, 68°C for 1 min, and a final extension at 68°C for 7 min. To produce the coding sequence of the single-chain antibody, the products of the two PCR reactions were combined with primers 1 and 4 in a second reaction, which linked the two domains with the coding sequence for a (Gly₃Ser)₃ linker. The PCR product was gel-purified using the QIAquick gel purification kit (QIAGEN, Valencia, CA). The product was then restricted with enzymes EcoRI and HindIII and ligated into matching sites of cloning vector pGEM-4Z (Promega, Madison, WI). The circularized product was then transformed into E. coli strain DH5α, and the sequence was checked to assure integrity of the transformed product (University of Arkansas for Medical Sciences DNA Core Sequencing Facility).

Subcloning and Large-Scale Expression of Anti-METH scFv ScFv6H4. For protein expression, the coding sequence of scFv6H4 was cloned into the yeast Pichia pastoris. The coding sequence was amplified by PCR with primers PP6H4.rev, GCCTTA AGACTAA TGGT-GATGGTGATGTGGGAGCCCGTTTATCC. The PP6H4.rev product (University of Arkansas for Medical Sciences DNA Core Sequencing Facility).
primer included a coding sequence for a 6-histidine tag for use in purification after protein expression. The resulting PCR product was gel purified, restricted with EcoRI and XbaI, and ligated into the plasmid pPICZa-A (Invitrogen) making the plasmid PPscFv6H4. This plasmid was transformed first into E. coli strain DH5α and then sequenced (University of Arkansas for Medical Sciences DNA Core Sequencing Facility). After this sequence confirmation, the plasmid was linearized with SacI and used to transform P. pastoris strain GS115 (Mut+) by electroporation according to the manufacturer’s instructions (Invitrogen). Potential zeocin-resistant colonies were selected on yeast extract/peptone/dextrose agar plates [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, and 2% agar] containing either 1 or 2 mg/ml zeocin. The starter culture was grown for 2 days at 30°C in a baffled flask shaken at 325 rpm. A baffled 2-liter starter culture and incubated at 30°C for 24 h, a fed batch phase was initiated to encourage cell growth. This included addition of aliquots of 50% glycerol based on cell weight reached 70 g/l, at which point glycerol was discontinued. After all glycerol was exhausted (~6 h), the yeast cell culture was converted to methanol metabolism by the introduction of 50% methanol at 5 ml/h. Methanol was then added at a rate of 10 ml/h for the next 120 h. After induction, the fermentation culture pH was adjusted to 7.0 by the addition of ammonium hydroxide. The culture was centrifuged at 4000g, and the supernatant was decanted and filtered through a 0.2-μm membrane filter. The clarified supernatant was stored at −80°C until the scFv6H4 was purified.

ScFv6H4 was purified by metal affinity chromatography using an AKTA Explorer 100 FPLC system and a 20-ml HisPrep 16/10 metal affinity column (GE Healthcare, Little Chalfont, Buckinghamshire, UK) in five 1-liter batches. In brief, the column was equilibrated with 5 column volumes of binding/wash buffer (20 mM NaPO₄, pH 7.4, 500 mM NaCl, 20 mM imidazole) at 7 ml/min. The supernatant from the fermentation was loaded onto the column, and the unbound sample was washed out with 3 column volumes of binding/wash buffer. The scFv6H4 was eluted from the column with elution buffer (binding/wash buffer plus 500 mM imidazole) and collected in 12-ml fractions. Fractions were analyzed for scFv6H4 content by SDS-PAGE and probing with anti-His6 antibody (data not shown).

Large-Scale Production and Purification of scFv6H4. To obtain sufficient scFv6H4 for in vivo pharmacokinetic studies, we expressed scFv6H4 in several large fermentation batches. Briefly described, a single P. pastoris yeast colony expressing scFv6H4 was picked from a freshly streaked yeast extract/peptone/dextrose plate containing 100 μg/ml zeocin and used to inoculate a starter culture of 40 ml of BMGY containing 100 μg/ml zeocin. The starter culture was grown for 2 days at 30°C in a baffled flask shaken at 325 rpm. A baffled 2-liter flask containing 500 ml of BMGY was inoculated with 10 ml of the starter culture and incubated at 30°C for 24 h, shaking at 325 rpm. Permutations were carried out in a 10-liter working volume BiostatB bioreactor (Sartorius BBL, Bethlehem, PA) interfaced with the Foxylogic Fermentor Control Program (version 4.3 software, http://www.foxylogic.com). This allowed automated bioreactor control and recording of production run data. Throughout the batch and induction phases, temperature was maintained at 28°C, and a pH of 6.0 was maintained by automatic addition of a 30% solution of ammonium hydroxide. Dissolved oxygen was maintained at 30% of saturation and was controlled by a dissolved oxygen cascade of pure oxygen addition followed by an appropriate increase or decrease in the stir rate. Air supplementation was kept constant at 5 l/min.

Fig. 1. Top panel, graphical representation of the scFv6H4 expression construct. From left to right, the amino terminus containing the FLAG epitope for protein detection, the VH and VL chains connected by a 15-amino acid (Gly-Ser)₅ linker, and carboxyl terminus fused to a six histidine tag for use in purification. FLAG, epitope for anti-FLAG antibody; 6His, six-histidine affinity tag. Bottom panel. Amino acid sequence of scFv6H4 labeled with the appropriate regions for framework and complementarity determining region residues. The sequence is in single-letter amino acid notation. The Kabat numbering scheme is written above the sequence.
lyzed in situ, in SEC under three different conditions. First, the scFv6H4 was anal-
alyzed as a tracer. In all cases, the SEC profile remained relatively constant
the in vivo studies, the [3H]scFv6H4 was included as an experimen-
tation of 4.6 mg/ml was near the limit of solubility of the scFv. As for
ranging from 0.25 to 4.6 mg/ml unlabeled scFv6H4. The concentra-
tions of 4.6 mg/ml was near the limit of solubility of the scFv. As for
the in vivo studies, the [3H]scFv6H4 was included as an experimen-
tal tracer. In all cases, the SEC profile remained relatively constant
and similar to the results shown in Fig. 2.
The monomer to multimer ratios of scFv6H4 were analyzed by SEC
under three different conditions. First, the scFv6H4 was ana-
alyzed in situ, in Pichia expression medium. Second, the purified
and concentrated form was analyzed after in vitro incubation at 4, 25,
and 37°C for 1 h. Finally, 5 μl of [3H]scFv6H4 was incubated in 200 μl
of administration buffer or rat serum at 37°C for 1 h. After the
incubation periods, 20 μl of each of the samples was analyzed by SEC
to determine whether there were changes in monomer to dimer ratio.

Bead-Based Radioimmunoassays for Determination of Kd
Values for METH and METH-Like Drugs. Radioimmunoassays
(RIAAs) were performed similarly to the method of Owens et al. (1988)
with the following changes. A 20-μl aliquot containing 100 ng
of purified scFv6H4 was incubated with 50,000 dpm of [3H]METH
in the presence of varying concentrations of unlabeled METH. A 20-μl
aliquot of a 50% slurry of TALON beads (Clontech, Mountain View,
CA) in RIA buffer (0.05 M Tris, pH 7.6, 150 mM NaCl, 2% bovine
serum albumin, 0.2% NaN3, 0.05% Tween 20) was added, and the
final volume was adjusted to 220 μl with RIA buffer. The solutions
were incubated overnight at 4°C with end-over-end rotation. The
next day, the tubes were centrifuged at 1000 g, and the beads were
washed twice with 1 ml of ice-cold RIA buffer and resuspended
in 2 ml of scintillation fluid for quantification by liquid scintillation
spectrometry.

Pharmacokinetic Studies of METH and scFv6H4 in Rats. For METH
and scFv6H4 PCKN studies, adult male Sprague-Dawley
rats (approximately 250 g) were purchased from Charles River Lab-
oratories (Raleigh, NC) with surgically implanted dual jugular vein
 catheters (Silastic medical-grade tubing, 0.020 in inner diameter and
0.037 in outer diameter; Dow Corning, Midland, MI) that were used
or drug administration and blood sampling, respectively. Catheters
were placed in the s.c. tissue for transport from the vendor and were
kept there until the day before the first experimental procedure,
when catheters were exposed under halothane anesthesia. Catheter
patency was maintained by daily injection of saline containing 25 U
of heparin.

Animals were housed individually in a light-controlled environ-
ment (12-h light/dark cycle). They received water ad libitum and were fed approximately 20 g of food pellets daily, which maintained
their body weights between 250 and 280 g. All experiments were
conducted in accordance with the (Institute of Laboratory Animal
Resources, 1996), and they were performed with the prior approval of
the Animal Care and Use Committee of the University of Arkansas
for Medical Sciences.

On day 2 of the study, pre-METH blood samples were taken, and
the rats were implanted with Alzet 3-day micro-osmotic pumps (Du-
rect Corp., Cupertino, CA) delivering 3.2 mg/kg/day METH. On day
−1, blood samples (200 μl) were taken at 10:00 AM, 1:00 PM, and
5:00 PM to allow determination of METH control steady-state con-
centrations. On day 0, the rats were given either 36.5 mg/kg anti-
METH scFv6H4 at 1.77 mg/ml containing a tracer dose of 1 × 10^6
dpm of [3H]scFv6H4 in antibody administration buffer or an equal
volume of buffer without scFv6H4 (controls). Immediately after ad-
ministration of the scFv6H4 or buffer, blood samples (200 μl) were
taken at 1, 5, 10, 30, 60, 90, 120, 240, 480, and 1440 min. After each
blood collection, the cannula was filled with saline or heparinized
saline (if the time between blood collections was greater than 1 h).
The saline was removed before collection of the next sample to
prevent volume dilution of the blood sample.

ScFv6H4 blood/plasma ratios were determined using blood sam-
ple collected during the PCKN studies. The blood samples were collected at time points from 1 to 120 min from the rats. A small
aliquot of whole blood was immediately used to determine the he-
matocrit in heparinized hematocrit tubes by standard procedures.
Duplicate whole-blood samples (10 μl) were then added to scintilla-
tion fluid, and the [3H]scFv6H4 concentration was determined by
liquid scintillation spectrometry. After centrifugation of the
remaining blood, duplicate 10-μl aliquots of the plasma were used to
determine the [3H]scFv6H4 concentration in plasma, as just de-
scribed for the whole blood. The ratio of the concentration in the
whole blood to the concentration in the plasma (the so-called blood/
plasma ratio) was used to represent the distribution of scFv6H4
between red blood cells and plasma.

The concentration of [3H]scFv6H4 in serum and urine samples
was determined by SEC similar to a previous method developed in
our laboratory (Proksch et al., 1998). For the analysis of [3H]scFv6H4
protein concentrations, a TSK-GEI G2000SWxl 30-cm size exclusion
column (Toso Haas, Montgomeryville, PA) was connected to a Waters
HPLC system (Waters, Milford, MA) consisting of an autoinjector in
series with a multisolvent delivery system, a UV absorbance detec-
tor, and a fraction collector. The Millenium software package (Wat-
ers) was used to control the HPLC system and to collect all HPLC
chromatography data. The column was equilibrated with buffer (50
mM NaPO4, 100 mM NaCl, pH 6.7) at a flow rate of 1 ml/min. The
column elution profile was determined using size exclusion standards
(Sigma-Aldrich) consisting of blue dextran (2000 kDa, to
determine void volume), β-amylase (200 kDa), bovine serum albumin
(66 kDa), carbonic anhydrase (29 kDa), cytochrome C (12.4 kDa),
and phenol red (to determine the column inclusion volume). Samples
were centrifuged at 20,000g for 10 min before chromatography to
remove any precipitated material or debris. The UV Abs280 was
used to detect the real-time elution of serum proteins in each
sample. Careful monitoring of the profile allowed detection of any
potential elution shift either related to column degradation or instru-
ment malfunction. In addition, reproducibility was moni-
tored using blank serum and urine samples spiked with a known
amount of [3H]scFv6H4. These quality control scFv samples were
analyzed with each batch of urine and serum samples. Liquid
scintillation spectrophotometry was used to quantitate the radioactivity in each 0.25-ml HPLC fraction.

To validate our analytical method, the serum protein SEC elution profile was monitored by Abs280 for each sample. The serum profile of the rats exhibited at least four characteristic UV280 peaks, the most prominent eluting at 3.8-8.07 min. Based on our standard curve generated with molecular mass standards, this peak was calculated to be 0.67 kDa, corresponding in abundance and size to rat serum albumin. Because this protein was in the molecular mass range of scFv monomers and multimers (25-150 kDa), this peak was used as an internal reference for elution times, and, based on this, the analytical retention times were quite reliable (elution time coefficient of variation < 0.3% for all samples, n = 49). The relative concentrations of scFv6H4 monomer and dimer were calculated and plotted against the time of collection (see Figs. 5 and 6). [$^{3}$H]scFv6H4 concentrations in the SEC fractions eluting after the monomer peak (at ~9 min) were considered to be small molecular mass degradation products that did not contribute to METH binding. Thus, they were not considered further.

To determine the molar concentration of unlabeled scFv6H4 in the serum after SEC fractionation, the known amount of [$^{3}$H]scFv6H4 radioactivity in the serum or urine was converted to the relative molar concentration of scFv6H4. The calculations were based on a 1 x 10^{9} dpm [$^{3}$H]scFv6H4 radiolabeled dose and a 36.5 mg/kg dose of unlabeled scFv6H4 per rat. For the calculation, a molecular mass of 27,000 g/mol for scFv6H4 was assumed. METH and amphetamine concentrations in serum and urine were determined by liquid chromatography-tandem mass spectrometry as described previously (Hendrickson et al., 2004).

**Data Analysis.** An IC_{50} value for METH binding to scFv6H4 was determined from the RIA [$^{3}$H]METH percent bound versus METH concentration curve (Aker and Cheng, 1977) using the computer program Origin (OriginLab Corp., Northampton, MA). Pharmacokinetic analysis was performed with the software package WinNonlin (Pharsight, Mountain View, CA). PCKN parameters for scFv6H4 were derived from model-independent analysis of the concentration versus time curves of scFv6H4 monomer and dimer. The PCKN calculations included terminal elimination rate constant (λ_{n}), t_{1/2,\text{ns}} systemic clearance (CLs), volume of distribution at steady-state (calculated as a product of the mean residence time and CLs), and area under the concentration-time curve.

To assess the effect of the presence or absence of scFv6H4 on METH serum concentrations at each time point, a repeated measures two-way analysis of variance statistical analysis was performed. If significant differences were found, then a Tukey’s post-hoc test was performed. Statistical differences were set at the P < 0.05 level. All tests were performed using SigmaStat computer software (Systat Software, Inc., San Jose, CA).

**Results**

**Cloning and Expression of Anti-METH scFv6H4.** The scFv6H4 was initially expressed in E. coli, but during purification, it was found to form >90% insoluble inclusion bodies. Attempts at refolding the insoluble protein only modestly improved the yield of functional scFv6H4; thus, an alternative yeast P. pastoris expression system was used.

The scFv6H4 coding sequence was ligated into a Pichia expression vector so that the coding sequence was in-frame behind the cleavable α-mating factor. This allowed protein secretion into the medium during methanol-induced protein expression, thereby decreasing the potential for formation of insoluble protein inclusion bodies. The scFv6H4 yielded after a 98-h yeast fermentation production run and downstream purification was approximately 12.4 mg/l.

The combination of a single gene-derived protein product, a medium-secreted protein, and an affinity tag on the protein allowed us to purify the scFv6H4 to over 98% homogeneity in one purification step (Fig. 2, SDS-PAGE, inset). Although the SDS-PAGE analysis showed a single protein band of <30 kDa, SEC analysis indicated that the purified and buffer-exchanged scFv6H4 product migrated as three peaks (Fig. 2, chromatogram). When plotted against molecular mass protein standards, the apparent molecular masses for the monomer and dimer were 34 and 73 kDa. These estimated values were in agreement with actual molecular size of these two species, calculated from the protein sequence analysis of the monomeric (27.4 kDa) and dimer (54.8 kDa) of scFv6H4 (Fig. 1).

**Bead-Based RIAs for Determination of IC_{50} Values for METH and METH-Like Drugs.** To determine the IC_{50} values and specificity of the purified scFv6H4 for METH-like analogs, we performed a bead-based RIA. Initially, we used an enzyme-linked immunosorbent assay to determine IC_{50} values of scFv6H4. However, we found these assays to be less accurate and less reproducible when compared with the results from a double antibody-based RIA used to characterize mAbs in our laboratory. Therefore, we developed a new RIA procedure that used the His6 affinity tag at the carboxyl terminus of the Vδ region of scFv6H4 (Fig. 1). Binding an affinity bead to this region oriented the scFv6H4 METH binding site distal to the bead, allowing sterically unhindered access to METH and possibly helping to stabilize the protein. This bead-based assay also allowed rapid separation of bound and free [$^{3}$H]METH by centrifugation and excellent signal/noise ratios for the measurement of [$^{3}$H]METH binding. The results of the ligand binding characterization showed the apparent IC_{50} for METH (10 nM) was nearly identical to that previously determined for the parent mAb6H4 (11 nM; Bynes-Blake et al., 2003) (Fig. 3, top panel). The scFv6H4 also retained the same stereospecificity for (+)-isomers of METH-like compounds as found with the parent IgG mAb6H4. For instance, there was a 70-fold greater affinity for (+)-METH than for (-)-methamphetamine. It is noteworthy that scFv6H4 had almost equal affinity for (+)-MDMA (17 nM) and (+)-METH (10 nM). We also attempted to determine the IC_{50} values of structurally related compounds (i.e., pseudoephedrine, norepinephrine, dopamine, and serotonin) and found that scFv6H4, like the parent antibody, had no significant cross-reactivity with these compounds, even at 100 μM concentrations. IC_{50} values from the scFv6H4 RIA (Fig. 3, top panel) were compared with IC_{50} values of mAb6H4 IgG (Bynes-Blake et al., 2003) using linear regression analysis (Fig. 3, bottom panel). These data indicated a strong linear relationship for the binding specificities of the two antibodies.

Single-chain antibodies have been reported to lose activity when incubated in serum or buffer at the physiologically relevant temperature of 37°C (Benhar and Pastan, 1995; Helfrich et al., 1998); thus, we were concerned with potential instabilities of the scFv6H4 during rat in vivo studies. To determine the in vitro functional stability of the scFv6H4 in buffer and serum, a bead-based RIA was performed with METH at 4, 22, and 37°C after 24-h incubation. The binding curves and IC_{50} values appeared unaffected by these temperatures (data not shown), indicating that scFv6H4 was stable in vitro in serum and buffer over a wide range of temperatures, including physiological temperatures.
Fig. 3. scFv6H4 binds to METH and (+) or (−) isomers of METH-like compounds with same affinity and specificity as parent IgG. Top panel, [3H]METH inhibition curves using the parent IgG monoclonal antibody mAb6H4 (open circle, designated “mAb6H4 IgG”) or scFv6H4. For these studies, METH and five other METH-like drugs were used as inhibitors of [3H]METH binding. (+)METH, (−)-methamphetamine; (+)AMP, (−)AMP; (−)MDMA, (−)-3,4-methylenedioxymethamphetamine. Bottom panel, relationship between anti-METH scFv6H4 binding specificity versus mAb6H4 binding specificity. These data are derived from the IC50 values for [3H]METH binding to scFv6H4 (see top panel) and IC50 values for [3H]METH binding to mAb6H4 IgG (data from Byrnes-Blake et al., 2003). Symbols for the test drugs correspond to those in the top panel. Values for the linear regression best-fit line and r2 value are shown on the graph.

Serum Pharmacokinetics of METH with and without scFv6H4. We used [3H]scFv6H4 as a tracer for determining scFv6H4 PCKN. Before adding the [3H]scFv6H4 to the unlabeled scFv6H4, the stability of the radiolabeled protein was characterized in vitro. SEC indicated that the [3H]scFv6H4 elution profile was unchanged after incubation in buffer or rat serum for 24 h at 37°C; however, after a similar incubation in rat urine, the [3H]scFv6H4 was largely degraded (data not shown).

To determine the ability of anti-METH scFv6H4 to alter serum concentrations of METH, s.c. osmotic minipumps were used to deliver METH at 3.2 mg/kg/day (Fig. 4, top panel), which resulted in an average steady-state serum METH concentration of 25 ng/ml after 24 h (Fig. 4, bottom panel). Administration of anti-METH scFv6H4 led to dramatic changes in serum METH concentrations. The serum METH concentration after scFv6H4 administration was significantly greater than vehicle-treated controls from 1 to 240 min (P < 0.05). The area under the METH serum concentration versus time curve (area under the concentration-time curve) from 1 to 480 min (AUC1 to AUC480) increased from 11,120 ng/min/ml in vehicle-treated controls (without scFv6H4) to 133,144 ng/min/ml for the scFv6H4-treated animals.

Pharmacokinetics of Monovalent and Multivalent Forms of scFv6H4. When we initially analyzed the raw serum counts of the [3H]scFv tracer, the concentration-time profile seemed to follow a traditional biphasic distribution and elimination process. However, after SEC separation of the scFv6H4 in serum samples (Fig. 5), we realized neither a two-compartment model nor a model-dependent analysis would be an acceptable interpretation. We reasoned a model-dependent pharmacokinetic analysis would only be appropriate if we had found a single, unaltered monomeric scFv6H4 species, but our data showed there is a diversity of monomers and multimers that appear progressively over time (Figs. 5 and 6). Therefore, we conducted model-independent analysis of these data sets, which does not make compartmental assumptions about the data that may not be correct. By analogy, a model-dependent analysis would not be appropriate to follow total parent drug and active metabolite concentrations as if they were one species. We also did not assume that the various phases of the scFv6H4 disposition were simply distribution followed by elimination. This is because our data showed some of the apparent elimination or clearance of monomer and dimer was possibly a reformation into larger multimers, which were also able to redistribute METH into the serum through high-affinity binding.

For the monomer form of the scFv6H4, only the concentra-
longer serum kinetic profile. It is interesting to note that, in min. The multimeric forms of scFv6H4 exhibited a much 2000) and 1 (Pavlinkova et al., 2000; Willuda et al., 2001) serum samples are represented with different ordinate scales for clarity.

After 30 min, the concentration of the \([^{3}H]\text{scFv6H4}\) was too low to accurately measure. These data indicated the monomer had a \(t_{1/2az}\) of 5.8 \pm 0.8 min (see Table 1 for complete PCKN parameters). This monomer \(t_{1/2az}\) is in agreement with rat PCKN of an anti-anthrax toxin scFv \(t_{1/2a}\) phase of \(\sim 5\) min (Maynard et al., 2002). In addition, PCKN studies in mice report even faster \(t_{1/2a}\) values of 2.7 (Pavlinkova et al., 2000) and 1 (Pavlinkova et al., 2000; Willuda et al., 2001) min. The multimeric forms of scFv6H4 exhibited a much longer serum kinetic profile. It is interesting to note that, in four of the five rats, the concentrations of the divalent form did not rapidly decrease in the first 10 min (Fig. 6). After 30 min, the multivalent scFv6H4 concentrations appeared to more rapidly decrease and exhibited an apparent biphasic curve. This biphasic shape was probably due to changes in scFv6H4 distribution and elimination, as well as formation of new scFv6H4 multimers.

Because the native scFv6H4 was administered as a mixture of monomer and dimer (see Fig. 2), we assessed the PCKN profile of the scFv6H4 monomeric (alone) and other multivalent forms (as a composite). Fractionation of the serum samples from each time point showed that the monomer was almost completely eliminated from the serum in \(<30\) min, but the divalent and other apparent multivalent larger proteins persisted for \(>240\) min (Figs. 5 and 6). Due to the complexity of these multivalent changes, we calculated the individual PCKN values for the monomer and calculated the PCKN values of the multimers (i.e., dimer and trimer) together. Based on the analysis of the terminal elimination data in Fig. 6, the overall \(t_{1/2az}\) of multivalent scFv6H4 forms was 228 \pm 38 min.

Studies of in vitro scFv6H4 stability at different protein concentrations, incubation times, and temperatures (for specific details, see Materials and Methods) indicated that there was excellent stability and a constant ratio of monomer to dimer, similar to the profile shown in Fig. 2. Studies of the in vivo blood to plasma concentration ratios of scFv6H4 indicated an average ratio of 0.52 \pm 0.04 and a hematocrit of 0.42 \pm 0.01. Because the average packed red blood cell volume (i.e., hematocrit) for each milliliter of blood was 0.42 ml, this would have resulted in an average plasma volume of 0.58 ml (1 minus the red blood cell volume). This estimated average value of 0.58 ml for the plasma volume was in very close agreement with the scFv6H4 blood/plasma ratio of 0.52 ml; therefore, these data suggested the scFv6H4 was confined to the plasma compartment, with little or no protein distributed into the red blood cells.

When METH concentrations in the presence of scFv6H4 (Fig. 7, top panel, solid circles) and total scFv6H4 protein concentrations (Fig. 7, top panel, open squares) were plotted as their respective molar concentrations, the relationship was nearly one-to-one. When the concentrations of METH and scFv6H4 were plotted on opposite axes, the resulting curve is sigmoidal (Fig. 7, bottom panel), with maximal METH concentrations occurring from 1 to 10 min after scFv6H4 dosing.

TABLE 1

<table>
<thead>
<tr>
<th>ScFv6H4 form</th>
<th>Pharmacokinetic Parameters</th>
<th>(\lambda_s)</th>
<th>(t_{1/2az})</th>
<th>(V_{ss})</th>
<th>(CL_s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monovalent</td>
<td>(0.07) (\text{min}^{-1})</td>
<td>(5.8) min</td>
<td>(780) ml/kg</td>
<td>(9.4) ml/min/kg</td>
<td></td>
</tr>
<tr>
<td>S.D.</td>
<td>(0.0004)</td>
<td>0.8 min</td>
<td>234 ml</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>CV (%)</td>
<td>22.9</td>
<td>13.7</td>
<td>30</td>
<td>29.6</td>
<td></td>
</tr>
<tr>
<td>Multivalent</td>
<td>(0.0031)</td>
<td>228</td>
<td>346 ml</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>S.D.</td>
<td>0.0004</td>
<td>0.8</td>
<td>234 ml</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>CV (%)</td>
<td>14.1</td>
<td>16.8</td>
<td>9.6</td>
<td>21.0</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 5. A representative plot of SEC time-lapsed analysis of monomeric and multimeric forms of scFv6H4. Serum samples separated by SEC show the proportion of monomeric and dimeric \([^{3}H]\text{scFv6H4}\) at the indicated time points. Dotted lines indicate the approximate retention time for the monomeric and dimeric scFv6H4 species. Early and later serum samples are represented with different ordinate scales for clarity.

Fig. 6. Individual plots of scFv6H4 concentrations in rat serum versus time after an i.v. bolus dose of anti-METH scFv6H4 (37 mg/kg) along with a tracer dose of anti-METH \([^{3}H]\text{scFv6H4}\) (1 \(\times\) 10\(^6\) dpm). After analysis of scFv6H4 by SEC, disintegrations per minute peak heights of monomeric (open circles) and multimeric (closed circles) forms were plotted.

Average PCKN values from rats (\(n = 5\)) for monovalent and multivalent forms of scFv6H4 determined by SEC and liquid scintillation spectrophotometry quantitation of the \([^{3}H]\text{scFv6H4}\). See Fig. 7 for the scFv6H4 serum concentration-time data.

See Fig. 7 for the scFv6H4 serum concentration-time data.
Although the serum PCKN data suggested profound redistribution of METH in the presence of scFv6H4, we found no intact scFv6H4 in the urine, and the METH renal clearance was not affected by scFv6H4. This finding was unexpected because our preliminary in vitro studies showed scFv6H4 was not stable when incubated in urine for 24 h at 37°C. However, we plan to address METH and scFv6H4 renal clearance in METH overdosage studies using i.v. bolus doses of METH followed by scFv6H4 treatment. We have performed similar studies to determine anti-phencyclidine Fab elimination (Proksch et al., 1998), and this experimental design will be more appropriate for studying METH elimination because continuous replacement of METH during the infusions limited our ability to reliably and accurately monitor METH renal clearance during discrete time intervals.

**Discussion**

The genetic re-engineering of mAb6H4 IgG into scFv6H4 changed the protein from an ~150-kDa protein with two anti-METH binding sites to an ~27.4-kDa protein with one METH binding site. It also converted the original IgG from a two-gene product (from heavy- and light-chain coding sequences) into a single gene product, without affecting its affinity or specificity for METH and other ligands (Fig. 3).

We constructed the scFv6H4 by joining the light- and heavy-chain variable domains of the parent mAb6H4 with a 15-amino acid linker (Fig. 1). Previous studies suggest that linkers shorter than 12 amino acids can result in multimeric complexes (diabody, triabody, etc.) due to “domain swapping” and that transition between monovalent and divalent scFv can be somewhat controlled by linker length (Atwell et al., 1999; Volkel et al., 2001). Considering these findings, it was our intention to design a predominately monomeric scFv. However, we found the purified and formulated scFv6H4 existed in situ as a mixture of monomer (~75%) and dimer (~25%), with traces of a possible trimer (Fig. 2).

Like the parent mAb6H4, the scFv6H4 had nearly the same affinity for (+)-MDMA and (+)-METH with little cross-reactivity against (-) isomers (17 versus 10 nM; Fig. 3). (+)-MDMA, or ecstasy, is abused as a racemic mixture, with the (+) form acting predominately on the dopaminergic system and the (−) form acting predominately on the serotonergic system (Cho and Segal, 1994). Some studies suggest (+)-MDMA is the more potent of the stereoisomers in vivo (Nichols and Oberlender, 1989; Nichols and Oberlender, 1990). Thus, the scFv6H4 we developed has potential clinical use for treatment of medical problems caused by two abused drugs, METH and MDMA.

To test the ability of scFv6H4 to alter METH disposition in vivo, we first chronically infused METH (Fig. 4, top panel). The 3.2 mg/kg/day dose allowed us to have mild METH pharmacological effects and easily measurable METH PCKN changes in the presence or absence of scFv6H4. We did not measure behavioral changes because it would have interfered with the PCKN experiments; however, we did note modest increases in locomotor activity in the METH-treated rats.

We calculated that a dosing regimen of 3.2 mg/kg/day METH equates to a METH body burden of 0.2 mg/kg at steady state. On day 2, after steady-state METH concentration was achieved, we administered a single bolus dose of scFv6H4 (36.5 mg/kg) that was equimolar in binding sites to the steady-state METH body burden. In comparison, this dose was one third of the dose of IgG (102 mg/kg) that would be needed to achieve the same number of METH antibody binding sites. Thus, use of scFv6H4 allowed a significantly lower protein load for the equivalent number of antibody binding sites. It is important to note that a single dose of scFv6H4 was administered, but the METH infusion continued to replace the drug at the rate of 50% of the body burden per hour based on a 1-h METH t1/2a (Rivière et al., 2000).

Because METH was infused to steady-state before scFv6H4 was administered, METH was already equilibrated in tissues (Rivière et al., 2000). Nevertheless, the scFv6H4 quickly bound METH in serum and caused a statistically significant redistribution of the drug for at least 4 h (Fig. 4, bottom panel). Indeed, (+)-METH serum concentrations increased 65-fold within 1 min, and the METH AUC increased 13-fold, compared with the control group. The nearly 1:1 ratio of scFv6H4 and METH concentration in the presence of scFv6H4 over time (Fig. 7, top panel) suggested that the scFv6H4 was responsible for the dramatic increases in METH concentrations during the 8-h study.
When scFv6H4 in the serum samples was separated by SEC, it was apparent that monomer and other multivalent scFv6H4 forms continuously changed over time (Fig. 5). Because both the monovalent (27.4 kDa) and dimer (54.8 kDa) scFv6H4 are below or near the molecular mass cut-off point for glomerular filtration (~50 kDa; Arend and Silverblatt, 1975), we expected a fairly rapid clearance of both proteins. Unexpectedly, there was an apparent time-dependent clearance of monovalent scFv6H4 and a progressive formation and clearance of other multivalent forms of scFv6H4 (Fig. 5). Early time changes in the monovalent form of scFv6H4 suggested the $t_{1/2,\text{SA}}$ for the monomer was only 5.8 min (see Fig. 5 for time-lapsed comparisons and Fig. 7 and Table 1 for PCKN analysis). These time-dependent changes in scFv6H4 suggested the clearance of monomeric scFv6H4 resulted from a combination of loss and conversion to dimers and other multivalent forms of scFv6H4.

This hypothesis is supported by the fact that the concentration of the dimer from 1 to 30 min changed much slower than the monomer (Fig. 5) and that the multivalent scFv6H4 concentration-time profile (without the monomer; Fig. 6, solid circles) showed peak concentration at approximately 5 to 10 min. We think that during the first 10 min after scFv6H4 delivery, the monomer was undergoing simultaneous elimination, distribution, and conversion to dimer and other multivalent forms. At the same time, the dimer was undergoing elimination and formation of larger multivalent isoforms. After 10 min, the monomer concentrations were below accurate detection levels (Figs. 5 and 6, top panel), and the remaining multivalent pool of scFv6H4 then followed an apparent biphasic PCKN profile. Because preliminary studies in rat serum (pH 7.35 and 37°C) showed no change in the ratio of monomer to dimer during a 24-h incubation, the in vivo conversion of scFv6H4 to larger multivalent forms was apparently due to in vivo mechanisms. We also hypothesize that the presence of METH helped to stabilize scFv6H4 and promote continued binding.

Although these suggested changes are primarily based on the time-dependent scFv6H4 concentrations, they are also supported by the time-dependent changes in METH concentrations (Fig. 4, bottom panel; Fig. 7, top and bottom panels) in the presence of scFv6H4. When we plotted the micromolar concentrations of METH (in the presence of scFv6H4, determined by liquid chromatography/tandem mass spectrometry) versus the micromolar concentrations of scFv (determined by SEC), there was a complex sigmoidal relationship (Fig. 7, bottom panel). From 1 to 10 min, the serum METH concentrations (solid circles) were very high and did not substantially change. This was also the time period in which monomeric and dimeric scFv6H4 concentrations were highest (Figs. 5 and 6). From approximately 30 to 120 min, the relationship between the METH and scFv6H4 micromolar concentration was fairly linear (Fig. 7, bottom panel). From 240 to 480 min, as the METH and scFv6H4 concentrations significantly decreased, the termination of the sigmoidal curve was found.

Dimer formation is common in scFv proteins, and relative amounts of dimer and monomer can vary depending on linker length, pH, ionic strength, and presence or absence of antigen (Arndt et al., 1998). Based on structural evidence, there are two major conformations that scFv dimers can adopt. The first conformation is the result of domain interactions between the $V_L$ of one scFv to the $V_H$ of another (Hudson and Souriau, 2003). After the protein is translated and folded this conformation, requires the “opening” of the scFv molecule around the linker and binding to another unbound scFv, requiring a transition state that is unable to bind to antigen. The other conformation that scFv dimers can adopt is a “back-to-back” conformation. Because the constant regions of the intact IgG that are normally adjacent to the variable region are absent in scFv, these regions are free to form other protein-protein interactions. Because transition from monomer to dimer does not require intra-$V_L$ and $V_H$ domains to dissociate, these dimers can theoretically associate and dissociate without perturbing the antigen binding site.

Residues involved in back-to-back dimer formation of an anti-tumor scFv, MFE-23, have been compared with several other dimerizing scFv (Lee et al., 2002). When we compared the salient dimer-forming sequence features of the MFE-23 and other back-to-back dimers to scFv6H4, we found scFv6H4 possesses the same linker sequence and framework residues that favor back-to-back dimer formation, specifically Pro-L40 and Gly-L41 in the $V_L$ domain and Pro-H41 and Glu/Gly-H42 in the $V_H$ domain (Fig. 1). In light of these structural features, the rapid transition from monomer to dimer and other multivalent scFv forms in serum (Fig. 5), along with no apparent loss of binding to METH (Fig. 7), we hypothesize that scFv6H4 forms a back-to-back dimer configuration.

In conclusion, these studies show the design, expression, purification, and preclinical characterization of a high-affinity therapeutic scFv6H4 against METH. The scFv was stable for an extended period of time in vivo and was able to significantly redistribute METH into serum for at least 4 h. PCKN data suggested that the multivalent forms of scFv6H4 were primarily responsible for the longer term METH binding in serum, even though the scFv6H4 dose was primarily composed of monomer (75%). More studies are needed to confirm our hypotheses, and we have begun crystal structure studies and behavioral studies that will hopefully add to our understanding of the pharmacological consequence of multivalent forms of scFv6H4. However, we think anti-METH scFv6H4 is a promising step toward developing a clinical therapy for treatment of METH overdose.

Acknowledgments

We thank Jon Hubbard, Rachel Tawney, Sally Huey, Sherri Wood, and Melinda Gunnell for valuable technical assistance.

References


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