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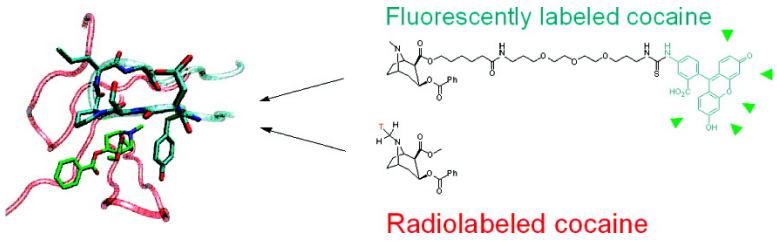

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Fluorescent Cocaine Probes: A Tool for the Selection and Engineering of Therapeutic Antibodies

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Abstract: Cocaine is a highly addictive drug, and despite intensive efforts, effective therapies for cocaine craving and addiction remain elusive. In recent years, we and others have reported advances in anti-cocaine immunopharmacotherapy based on specific antibodies capable of sequestering the drug before it reaches the brain. In an effort to obtain high affinity therapeutic anti-cocaine antibodies, either whole IgGs or other antibody constructs, fluorescence spectroscopic techniques could provide a means of assisting selection and engineering strategies. We report the synthesis of a series of cocaine−fluorophore conjugates (**GNC**−**F1**, **GNC**−**F2**, **GNC**−**I**) and the functional evaluation of these compounds against single-chain Fv antibodies obtained via crystallographic analysis/engineering and against commercially available anti-cocaine monoclonal antibodies with a wide range of cocaine-binding affinities. From these studies, we determined that the **GNC**−**F2** fluorophore reproduced affinity constants obtained using [^{3}H]-labeled cocaine. We anticipate that the readily synthesized and nonradioactive **GNC**−**F2** will find use both as a tool for bioimaging and in the high-throughput selection and engineering of potential therapeutic antibodies against cocaine.

Introduction

Cocaine **1** is one of the most widely abused drugs, and cocaine addiction continues to be prevalent throughout the world (Figure 1). Recent surveys for cocaine abuse in the United States alone have indicated that more than 30 million people have tried cocaine, including 1.7 million considered as regular users.[1] Cocaine inhibits the dopamine reuptake transporter in the pleasure/reward center of the brain, and this blockade results in an excess of dopamine in the synapse leading to an amplification of the pleasure sensation.[2,3] Despite extensive investigative efforts, the biochemistry of this addiction is still cryptic, and effective pharmacotherapies for cocaine craving and addiction remain elusive.[4,5]

Recently, we and others have shown how immunopharmacotherapy for cocaine addiction could provide an alternative and potentially viable treatment strategy for cocaine abuse as demonstrated in animal models measuring both locomotive behavior and relapse.[6−16] Immunopharmacotherapy is based on the in vivo generation or administration of antibodies that are capable of sequestering the targeted drug before it reaches the brain.[17−19] A series of studies from this laboratory have shown that active immunization (vaccination) of rats with the stable cocaine immunoconjugate **GNC**−keyhole limpet hemocyanin (KLH), which is hapten **2** conjugated to the carrier protein KLH, reduced the psychoactive effects of cocaine and lowered the cocaine levels in rat brains.[6−8]

The hapten **2**, a cocaine analogue, was designed to elicit a highly specific immune response to cocaine. Our strategy entailed the conjugation of the tropane framework to a carrier protein through a linker at the position occupied by the methyl

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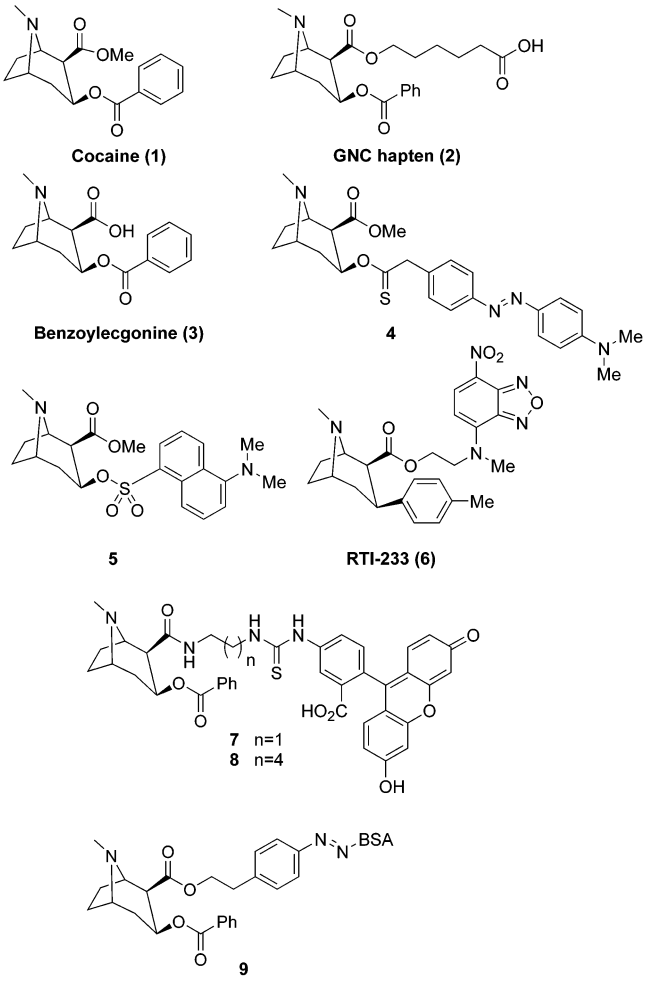

Figure 1. Structures of cocaine, haptens, and fluorescent cocaine probes.

ester in the cocaine molecule. This regiopositioning of the linker circumvents the problem associated with the hydrolysis of the methyl ester observed under physiological conditions, thus preventing an immune response against the hydrolysis product benzoylecgonine **3**. As anticipated, high titers of anti-cocaine antibodies were achieved, and competitive binding studies demonstrated that the immune response was highly specific for cocaine.[6] A murine monoclonal antibody (mAb), GNC92H2, was also generated using the **GNC**−KLH immunoconjugate.[7] GNC92H2 binds free cocaine with both excellent specificity and good affinity, and passive immunization with GNC92H2 effectively reduced cocaine relapse in rats.[7] This mAb and our animal studies provide evidence that passive administration could be useful both in a short-term emergency setting in the event of drug overdose and in a long-term rehabilitation program. Toward the ultimate goal of obtaining a humanized or fully human anti-cocaine mAb of suitable specificity and affinity for potential clinical applications, we recently solved the crystal structure of a GNC92H2 murine-human chimeric Fab.[20] On the basis of this structure, we were able to successfully achieve framework humanization of a single-chain Fv (scFv) format of GNC92H2.[21,22] Furthermore, we have also demonstrated the therapeutic potential of phage-displayed GNC92H2 scFvs.[23] Phage particles displaying scFv GNC92H2 on the phage major coat protein pVIII were delivered intranasally and shown to inhibit the psychoactive effects of cocaine.[24]

The mAb GNC92H2 has excellent affinity ($K_d \sim 40$ nM) for cocaine. However, we believe the use of an antibody with an affinity even higher than that of GNC92H2 is necessary for clinical applications, particularly for cases of cocaine overdose. A lower K_d would allow the use of less antibody in a smaller infusion volume with shorter infusion time. Patients intoxicated with cocaine may present serum levels up to 60 μM.[25,26] However, it has not been established what fraction of cocaine would need to be "neutralized" under these conditions in order to stabilize the patient. We believe that micromolar serum levels of an IgG having a low nanomolar K_d would bind sufficient cocaine and drive partitioning from the tissues into the bound state in circulation and, therefore, alleviate cardiac dysfunctions, seizures, and prevent death.

In an effort to isolate therapeutically applicable anti-cocaine mAbs in whole IgG, Fab, and scFv formats, the use of fluorescence spectroscopy could provide a means of implementing selection and engineering strategies.[27,28] Recently, Wittrup and co-workers reported the selection of an anti-fluorescein scFv with a $K_d = 48$ fM using fluorescence-activated cell sorting (FACS) methodology.[29] Furthermore, fluorescently labeled ligands represent a widely applicable tool and can be used to (i) substitute for radioligands in binding assays, (ii) visualize their target protein directly in/on living cells, and (iii) characterize the biophysical microenvironment of a binding site. Several fluorescent cocaine analogues have been reported as molecular probes of monoamine transporters and as potentially useful agents for the diagnosis of neuroblastoma cells. Bonisch and co-workers synthesized a series of fluorescent compounds structurally related to the neuronal norepinephrine transporter (NET) ligands, including cocaine analogues **4** and **5** (Figure 1).[30] These compounds bound to NET tightly and inhibited the uptake of [^{3}H]-norepinephrine in human neuroblastoma cells with IC_{50} values of 0.06 and 1.32 μM, respectively. Gether and co-workers synthesized RTI-233 **6**, a cocaine analogue that contained the environmentally sensitive fluorescent moiety 7-nitrobenzo-2-oxa-1,3-diazole (NBD).[31] RTI-233 maintained a high affinity ($K_I = 6$ nM) to the serotonin transporter (SERT) expressed in Sf-9 cell membranes and was used as a molecular reporter to probe the microenvironment of the cocaine binding site of SERT.[32] Although each retained the tropane framework of cocaine, the methyl ester and/or benzoate, which are both

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major recognition elements for cocaine-specific mAbs, were grossly modified with these special fluorophores.

In other studies, several fluorescent biosensing methods have been reported to detect cocaine and its metabolite, benzoylecgonine **3**. Eldefrawi and Wemhoff separately developed immunosensors for cocaine which relied on the ability of cocaine to displace fluorophore-labeled benzoylecgonine analogues **7** and **8**, respectively, from immobilized antibodies.[33,34] Eldefrawi employed mAb raised against a BSA conjugate **9**, and the affinity of the mAb for cocaine (K_I = 30 nM) was 4-fold poorer than that for **7** (K_d = 7.6 nM).[33] Wemhoff did not disclose the origin of the mAb and its affinities for cocaine and **8**.[34] Stojanovic and Landry developed a DNA aptamer-based fluorescent sensor for cocaine.[35] An anti-cocaine aptamer was engineered to obtain a partially folded structure with a ligand-induced binding pocket. A double end-labeled aptameric sensor with ligand-induced stem formation permits optical detection of the ligand through fluorescence change.

With the aim of developing selection-engineering protocols for specific and tight-binding anti-cocaine mAbs from cell cultures and from phage-display antibody libraries, we herein report the synthesis, properties, and analysis of several cocaine−fluorophore conjugates. One of these probes showed highly reproducible affinity measurements with values similar to those of unmodified cocaine using both whole IgG mAbs, as well as scFvs selected from a phage-display library.

Results and Discussion

Modeling of GNC92H2 for Fluorescent Probe Development. Our goal was to develop a reliable method for the high-throughput selection of anti-cocaine mAbs with excellent specificity and high affinity using a fluorescence-based assay. We designed a set of ligands based on the X-ray crystal structure of the mouse−human chimeric Fab GNC92H2 (Protein Data Bank accession code 1I7Z).[20] The crystal structure of GNC92H2 reveals a binding pocket with exquisite shape and electrostatic complementarity to cocaine (Figure 2). Remarkably, no direct hydrogen bonds are formed between the antibody and cocaine. However, a key framework glutamate residue at the base of the antibody combining site provides a complementary negative charge for the positively charged bridgehead nitrogen of the tropane framework. As shown in Figure 2, the cocaine scaffold is oriented such that the benzoate and methyl ester substituents point out of the binding pocket. From this structure, the **GNC** hapten could be reliably superimposed on the crystal structure of GNC92H2 (Figure 3). The ester linker mimics the alkyl character of the methyl ester of cocaine, which is important for recognition of this part of the molecule. The terminal carboxyl group in **2** is located outside the binding pocket and appeared to be a suitable position for the conjugation of fluorophores.

Fluorescent Probe Synthesis. On the basis of these models, we designed cocaine−fluorophore conjugates **GNC−F1**, **GNC−F2**, and **GNC−I**, in which the **GNC** hapten and fluorescent moieties are attached through appropriate spacers. As fluorophores, we chose fluorescein (**GNC−F1** and **GNC−F2**) and

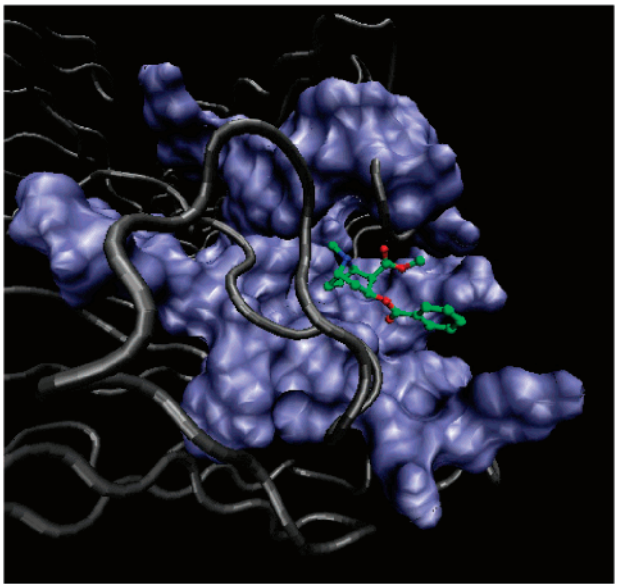

Figure 2. X-ray structure of cocaine bound to Fab GNC92H2.[20] Cocaine is shown in green. The Fv portion of the antibody (gray) is shown with the binding pocket surface (purple). The figure was made with VMD[36] and rendered in POV-ray.[37]

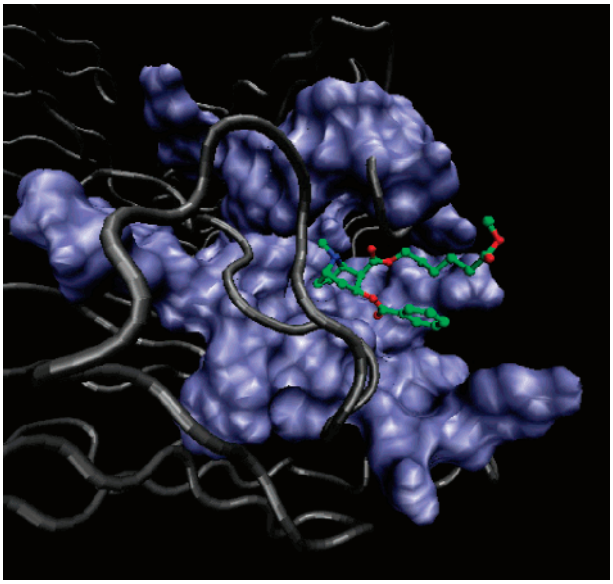

Figure 3. **GNC** hapten (green) superimposed onto the crystal structure of GNC92H2. The Fv portion (gray) is shown with the combining site (purple).

the *N*-methyl−isatoic amide group (**GNC−I**). The hydrophilic PEG linker (**GNC−F2** and **GNC−I**) as well as the hydrophobic alkyl linker (**GNC−F1**) was introduced to connect **GNC** and the fluorophores. According to our modeling studies, these linkers were deemed long enough to ensure that the fluorescent probes would be displayed outside of the antibody binding pocket.

Scheme 1 outlines the synthesis of cocaine−fluorophore conjugates **GNC−F1**, **GNC−F2**, and **GNC−I**. All compounds possess the same 2β,3β configuration as natural cocaine. The fluorescein derivatives, **GNC−F1** and **GNC−F2**, were synthesized starting from 9-fluorescein isothiocyanate **10**. Reaction of **10** with excess α,ω-diamino PEG **11** in the presence of Et_3N afforded thiourea **12**. The **GNC** hapten **2** was prepared following literature procedures.[6] Coupling of **12** with **2** using 1-ethyl-4-

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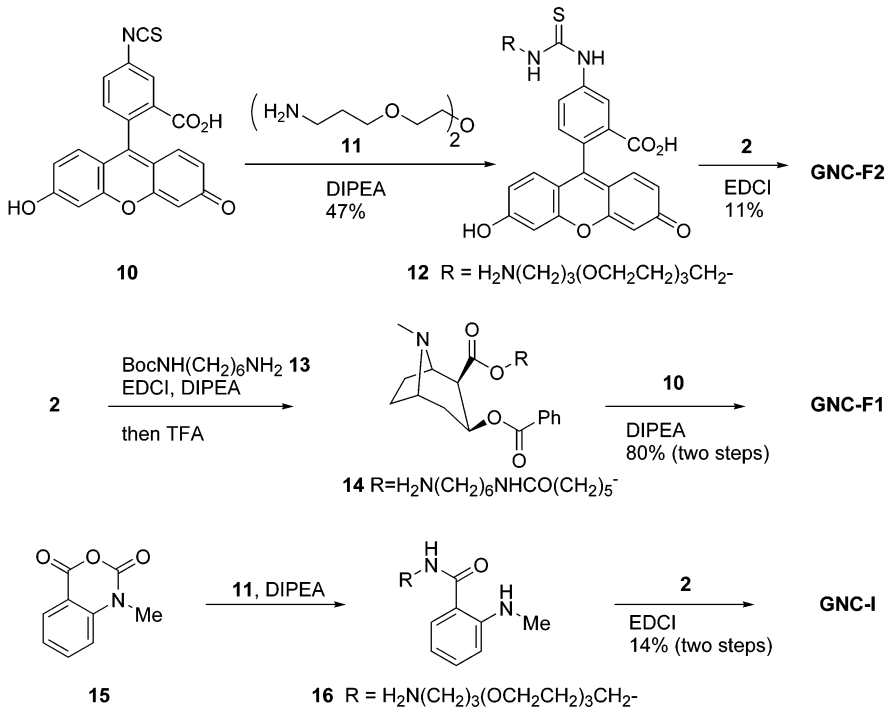
Scheme 1



dimethylaminoethyl carbodiimide (EDCI) followed by reversed-phase HPLC purification (C_{18} column, 30% CH_3CN in H_2O) gave **GNC−F2**. On the other hand, coupling of **2** and mono-*N*-Boc-hexanediamine **13** gave **14**, which was reacted with **10** to afford **GNC−F1**. Synthesis of **GNC−I** commenced from *N*-methyl−isatoic anhydride **15**. Reaction of **15** with α,ω-diamino PEG **11** gave amide **16**. Coupling of **16** with **2** and purification of the mixture by silica gel chromatography afforded **GNC−I**.

Engineering of GNC92H2. Further analysis of the crystal structure of the Fab fragment of GNC92H2 revealed that predominantly the light chain of the antibody and to a lesser extent the heavy chain interact with cocaine (Figure 5). The Fab mainly utilizes the complementarity determining regions (CDRs) L1, L2, and L3 of the light chain and CDRH3 of the heavy chain to bind cocaine. A total of 75 van der Waals contacts are made between Fab GNC92H2 and cocaine, 51 of which are derived from the light chain. The greater proportion of light chain contacts may be explained by the unusual conformation of V_L CDR3. Comparison to other antibodies in the protein data bank with the same V_L CDR3 insertion length (accession codes 1baF, 1ar1, and 1FL3) reveals a wide variety of bulged conformations that are probably influenced by prolines L95 and L95a. In CDRH3, the residues H95−H100 form a loop structure with proline H98 (ProH98) introducing a kink into the loop. Out of the six loop residues, only tyrosine H95 (TyrH95) interacts directly with the tropane moiety by forming a cation−π interaction.

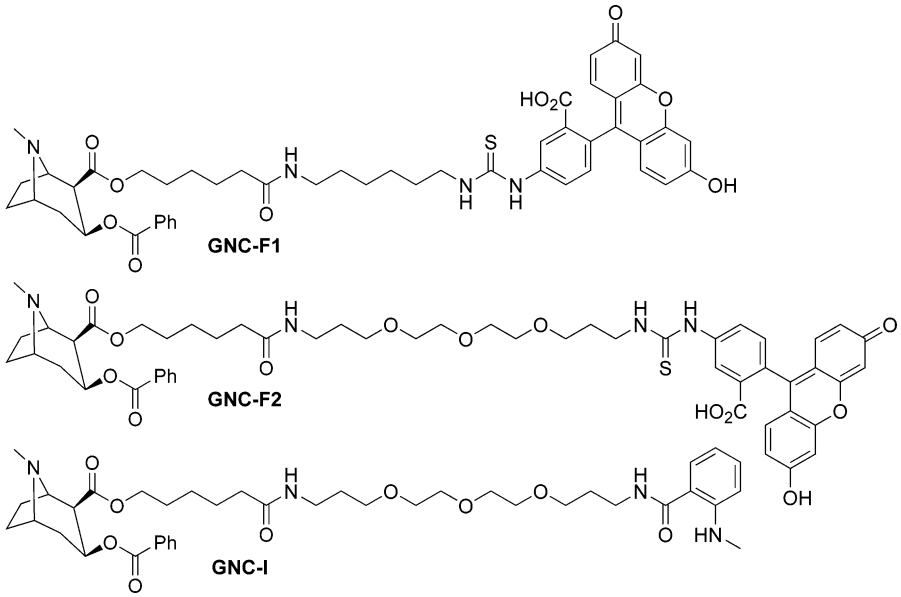


Figure 4. Structures of cocaine−fluorophore conjugates **GNC−F1**, **GNC−F2**, and **GNC−I**.

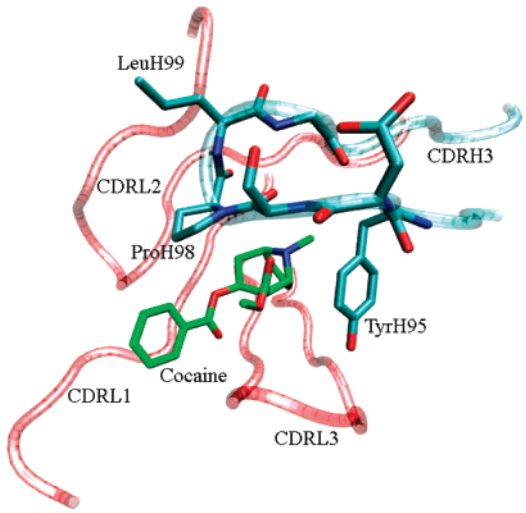


Figure 5. CDRs of GNC92H2.[20] Cocaine is shown in green. CDRs L1, L2, and L3 are shown in red. CDRH3 is shown in blue. TyrH95 in CDRH3 forms a cation−π interaction with the tertiary amine group in cocaine.

On the basis of these observations, our reasoning was to target residues in the CDRH3 loop that could potentially induce a variety of changes in the overall affinity of GNC92H2 to cocaine. We anticipated that hydrogen bonds could potentially be introduced, thus increasing the binding energy and lowering the dissociation constant, K_d. One could also envision that mutations in the loop region might change the overall structure of the loop introducing new interactions between the antibody and the cocaine molecule. In total, we hoped not only to select scFvs that possessed enhanced affinities for cocaine but also to validate our fluorescent probes by determining their affinities for GNC92H2.

Keeping these points in mind, our antibody engineering strategy was based on CDR-walking methodology. In particular, we focused on CDRH3 as this region has been shown (vide supra) to be responsible for high-energy interaction with other antigens, and such interactions seem to be consistent with the GNC92H2−cocaine X-ray structure. CDR walking is a direct technique that allows for the systematic randomization of 5−6 amino acids in a CDR followed by a selection process for affinity changes.[38] In accordance with the crystal structure and our CDR-walking strategy, six residues involved in the loop structure in the CDR3 of the heavy chain (H95−H100) were randomized, allowing the incorporation of any of the 20 naturally occurring amino acids. A scFv GNC92H2 phage-display library with a diversity of 3×10^7 independent members was generated following standard protocols.

CDR-Walking Library of GNC92H2. The library was panned against **GNC**−bovine serum albumin (BSA), in which **GNC** was covalently coupled to BSA according to our previous procedures.[24] The stringency of the washing steps during the panning was steadily increased. A 1000-fold enrichment was observed over the course of four rounds of panning. After the fourth round, 96 clones were picked and individually grown in a 96-well culture plate. Phage particles were produced and analyzed for their ability to bind **GNC**−BSA using enzyme-linked immunosorbent assay (ELISA).

Table 1. Protein Sequence of Residues H95−100 of GNC92H2 and Mutants

	Residue					
antibody	95	96	97	98	99	100
GNC92H2	Y	D	S	P	L	G
HB10	Y	L	R	C	I	R
HC3	Y	G	A	P	H	V
HD1	Y	A	E	A	Y	R
HD2	Y	A	E	A	V	R
HF1	W	E	R	A	D	R

From the ELISA (data not shown), 36 clones were judged appropriate for sequencing. Out of these 36 CDRH3 clones, only 2 showed the wild-type (WT) sequence, all other clones possessed altered CDRH3 sequences. On the basis of sequence analysis, five clones (HB10, HC3, HD1, HD2, and HF1; see Table 1) were chosen to be further analyzed. Clone HB10 was of particular interest as ProH98 was changed to a cysteine residue. Typically, cysteine residues are involved in inter- and intramolecular disulfide bonds which are utilized as inherent structural motifs for the basic immunoglobulin fold. However, we have previously shown that unique cysteine residues can be selected within the combining site of scFvs from synthetic combinatorial phage-display libraries.[39] Clone HC3 and two other clones out of the initially selected 36 mutants possessed the same altered CDRH3 sequence still retaining ProH98. Notably, aspartic acid (Asp) H96 was replaced with glycine, and leucine (Leu) H99 had been changed to histidine. These clones possessed a fairly nonpolar CDRH3 loop compared to other mutants and thus were selected (vide infra). Clones HD1 and HD2 were chosen as interesting because they (1) retained the key residue TyrH95, but ProH98 was substituted for an alanine residue and therefore potentially influenced the loop structure in CDRH3, and (2) gained a basic arginine (ArgH100) residue. The two clones differ only at position H99 wherein HD1 had an additional tyrosine (TyrH99) while HD2 has a valine (ValH99) residue at this position. These changes could have a fundamental impact on the antibody affinity toward cocaine. In HF1, TyrH95 was altered to a tryptophan, which should still enable cation−π interactions. Moreover, the three polar amino acids, ArgH97, ArgH100, and AspH99, were of interest, and ProH98 was mutated to an alanine.

Antibody−Cocaine Binding Studies. Using equilibrium dialysis, a powerful tool in the determination of physiologically relevant protein−ligand dissociation constants,[40] we studied the affinity of mAb GNC92H2 with fluorophores **GNC−F1**, **GNC−F2**, and **GNC−I**. We were unable to detect binding of **GNC−F1** to mAb GNC92H2 even at concentrations of up to 40 μM (using 5 nM **GNC−F1**). One hypothesis is that the hydrophobic linker in **GNC−F1** results in aggregation of this molecule in aqueous environments and thus lowers the observed affinities for GNC92H2. In contrast, binding was observed for **GNC−I**, but upon analysis of the ratio between the bound and unbound fluorescent probe, a perturbation was noticed that included a strong increase in fluorescence emission of *N*-methyl−isatoic amide upon interaction with the antibody.

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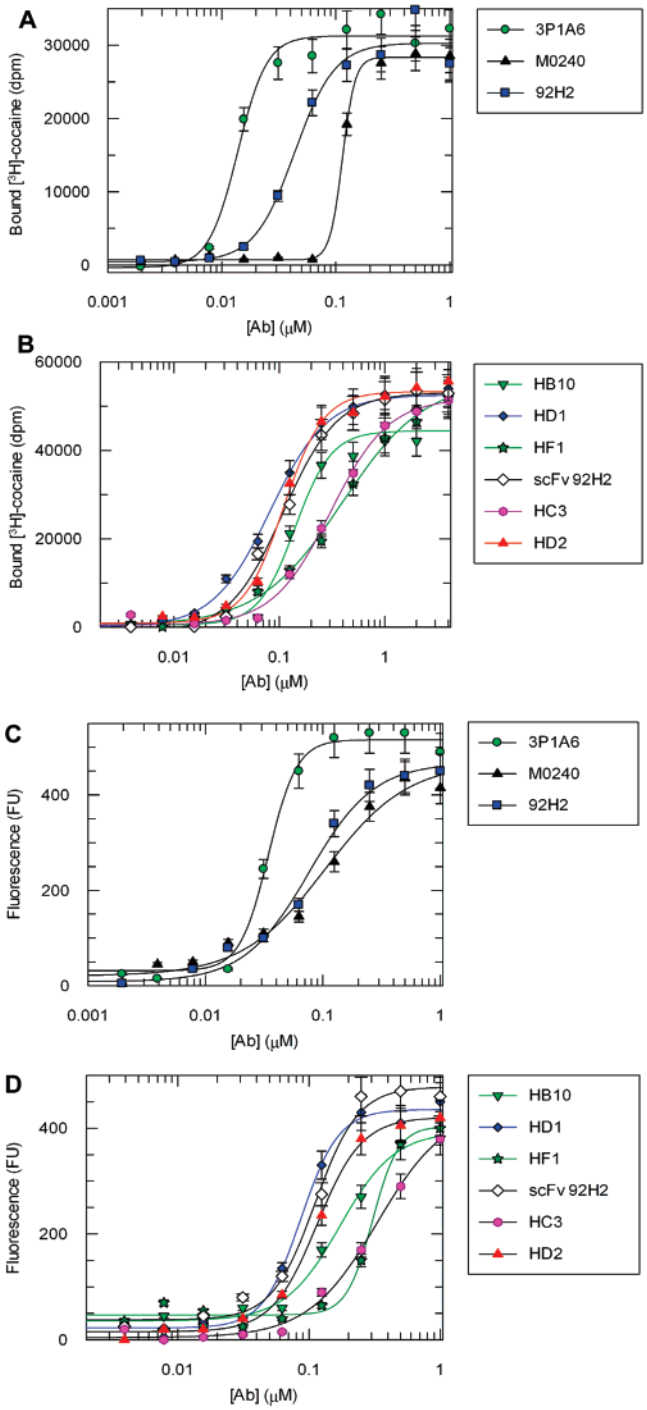

Figure 6. Binding assays for the interaction between various antibodies and cocaine, using either [^{3}H]-cocaine or **GNC−F2** as ligand. (A) Binding curves for mAbs 3P1A6, M0240, and GNC92H2 using [^{3}H]-cocaine. (B) Binding curves for scFv GNC92H2 and mutants HB10, HD1, HF1, HC3, and HD2 using [^{3}H]-cocaine. (C) Binding curves for mAbs 3P1A6, M0240, and GNC92H2 using **GNC−F2**. (D) Binding curves for scFv GNC92H2 and mutants HB10, HD1, HF1, HC3, and HD2 using **GNC−F2**.

Therefore, we were unable to determine the specificity of mAb GNC92H2 for **GNC−I**. Using the PEG-linked fluorescein-based probe **GNC−F2**, we were able to observe excellent binding to mAb GNC92H2. Determination of K_d values and comparisons between the various cocaine-binding antibodies could now be undertaken.

Equilibrium dialysis is inexpensive and relatively easy to perform with minimal instrumentation required to detect and quantify the ligand of interest. Since the results of this assay are obtained under equilibrium conditions, the physiological interaction can be studied and high affinity interactions that are not detectable with high reliability using other methods can also be accomplished. However, despite its simplicity, one drawback in this case has been the need for radiolabeled cocaine. This adds to the cost of these assays and also requires special storage, handling, monitoring of activity, and extensive cleanup/sanitary laboratory practices.

Table 2. Measured K_d Values for Cocaine with Various Antibodies using Either [^{3}H]-Cocaine (1 nM) or **GNC−F2** (5 nM) as Ligand

antibody	[^{3}H]-cocaine K_d (nM)	**GNC−F2** K_d (nM)
IgG GNC92H2	43 ± 1	76 ± 9
IgG 3P1A6	14 ± 1	34 ± 2
IgG M0240	114 ± 3	104 ± 23
scFv GNC92H2	108 ± 7	111 ± 9
scFv HB10	141 ± 18	172 ± 15
scFv HC3	305 ± 22	332 ± 47
scFv HD1	82 ± 6	95 ± 6
scFv HD2	110 ± 8	115 ± 5
scFv HF1	392 ± 40	305 ± 21

Figure 6a−d shows the results of equilibrium dialysis experiments with either [^{3}H]-cocaine[6] (Figure 6a,b) or **GNC−F2** (Figure 6c,d) as the ligand and commercially available mAbs 3P1A6 (Biodesign International) and M0240 (American Qualex), GNC92H2,[6] scFv GNC92H2 (wild-type),[21,22] and its mutants, HD1, HF1, HB10, HC3, and HD2.

From these binding curves, K_d values were derived. The results are listed in Table 2. From the eight antibodies that we screened using [^{3}H]-cocaine, commercially available mAb 3P1A6 had the highest affinity for cocaine ($K_d \sim 14$ nM), and the affinity of GNC92H2 for cocaine was approximately three-times less ($K_d \sim 43$ nM), while the affinity of commercially available mAb M0240 for cocaine was another three-times weaker ($K_d \sim 114$ nM). It should be noted that there is a discrepancy with respect to the affinities of mAbs 3P1A6 and M0240 for cocaine determined here and in a study by Paula et al. that reported K_d values of 0.22 and 11 nM, respectively.[41] We ascribe this 10- to 70-fold variability to the difference in methods used. Paula et al. used a radioligand binding assay based on antibody precipitation,[41] while our method maintains both the antibody and ligand in solution, which more accurately reflects the physiological interaction in solution and provides a more relevant K_d value. In accord with previous reports, scFv GNC92H2 had an affinity for cocaine weaker than that of its IgG counterpart,[42−44] although we did observe a slightly improved affinity with one of the mutants (HD1). Importantly, the observed trend in affinity determination using [^{3}H]-cocaine was largely the same when we used the fluorescent probe **GNC−F2**.

Conclusions

In this study, we have synthesized a series of fluorescently labeled cocaine analogues in the hope of ultimately developing

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a high-affinity fluorescent probe for the selection of cocaine-binding mAbs. From the standpoint of the selection of anti-cocaine mAbs, but also fluorescent labeling of bioactive small compounds in general, the effect incurred through a subtle change in the linker was of great interest. Whereas fluorescent probes **GNC−F1** and **GNC−I** failed to show high affinity for cocaine-binding mAbs, we did observe strong binding of several antibodies to **GNC−F2**. Notably, we obtained apparent K_d values that were close to the K_d values we obtained for these mAbs when [^{3}H]-cocaine was used in our equilibrium dialysis binding assays. Using these assays, we were able to determine K_d values for commercially available cocaine-binding mAbs, as well as for a panel of scFvs. The affinity probe **GNC−F2** could provide a powerful tool in selection and engineering strategies for the production of high-affinity anti-cocaine mAbs from a large panel of hybridoma cell cultures and phage-display libraries. In contrast with [^{3}H]-cocaine that presents problems with respect to cost, handling, and waste management, the use of the fluorescent probe **GNC−F2** is more practical for the characterization of large numbers of antibodies. In addition, optical bioimaging to investigate structure and function of proteins in cells and tissues continues to expand at a rapid pace. For the fluorescence imaging of bioactive protein−ligand interactions, the design of exogenous fluorescent labeling agents and linkers attached to ligands could provide selectivity and sensitivity of the imaging probes. **GNC−F2** might serve as a potential bioimaging probe to study the psychoactive and addictive effects of cocaine in living tissue.

Experimental Section

Construction of a CDRH3 Walking Library. The gene encoding the wild-type single chain variable fragment (scFv) antibody was used as template in polymerase chain reactions (PCR). The gene fragments containing the partly randomized CDRH3 were generated and amplified using the primers MHVsfi (5′-ttgttattactcgcggcccagccggccatggca-3′), CDRH3r (5′-ggtttcacagaaatatgtagccgtgtcc-3′),

CDRH3f (5′-gctacatatttctgtgaaaccnnknnknnknnknnknnknnkgactactggggccaaggcacc-3′), and MLJsfi (5′-gtcctcgtcgactggaattcggcccccgaggccac-3′). The scFv gene with randomized CDRH3 was subsequently assembled by overlap PCR using the gene fragments and finally amplified using the primers MVHsfi and MLJsfi. The fusion PCR products were digested with *SfiI* and ligated to *SfiI*-digested pCGMT phagemid vector. The ligation mix was ethanol precipitated and transformed into *Escherichia coli* XL-1 blue (Stratagene) by electroporation. After transformation, SOC (2% peptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose) was immediately added. The cells were allowed to recover at 37 °C for 1 h, then plated onto Luria−Bertani (LB) agar plates containing carbenicillin (100 μg/mL), tetracycline (10 μg/mL), and 20 mM glucose and incubated at 30 °C overnight. The library was determined to consist of 3×10^7 members. On the following day, glycerol stocks of the library were prepared and stored at −80 °C.

Panning of CDRH3 Walking Library. To amplify the CDRH3 walking library, 1 mL of glycerol stock was inoculated into a 1 L 2YT (1.6% peptone, 0.5% yeast extract, 1% NaCl) containing carbenicillin (100 μg/mL) and tetracycline (10 μg/mL). Upon reaching an optical density at the wavelength of 600 nm (OD_{600}) of ≈0.6, 1 mL of VCS-M13 helper phage suspension (titer > 10^{13}/mL) was added and incubated at room temperature for 30 min. The culture was transferred to a shaker (300 rpm) and shaken at 37 °C for 90 min. Finally, kanamycin (70 μg/mL) and 200 μL of IPTG (1 mM) were added to the culture. The temperature was adjusted to 30 °C and incubated overnight.

On the next day, the culture was spun down at 10 000*g* for 15 min. The supernatant was transferred to a clean bottle, and 4% (w/v) PEG 8000 and 3% (w/v) NaCl were added. The phage were precipitated on ice for 30 min. The precipitate was spun down at 8000*g* for 20 min. The supernatant was discarded. The pellet was drained for 10 min and then resuspended in 2 mL of PBS/1% BSA. The resuspended phage were spun down at 14 000 rpm (Eppendorf microcentrifuge 5415C) for 5 min. The supernatant contained the phage particles.

Star tubes (Nunc) were coated with GNC−BSA (100 μg/1 mL PBS/tube) at 4 °C overnight. On the next day, the tubes were washed three times with water and blocked with 5% dry milk in PBS for 1 h at 37 °C. The tubes were washed three times with dH_2O, and 1 mL of the phage suspension/tube was added. This was incubated at 37 °C for 1 h. After thorough washing with PBS/Tween 20 (0.05%), the bound phage were eluted with 1 mL of elution buffer (0.1 M glycine, pH 2.3). The eluate was removed and neutralized with 12 μL of 2 M Tris base (pH 8). Two milliliters of a fresh *E. coli* XL-1 blue culture (OD_{600} ≈ 1) grown with tetracycline (10 μg/mL) was infected with the eluant. After an incubation period of 30 min at room temperature, the culture was inoculated into 100 mL of 2YT medium containing carbenicillin (100 μg/mL) and tetracycline (10 μg/mL) and was incubated at 37 °C for 4 h. Two milliliters of VCS M13 helper phage and 200 μL of IPTG (1 mM) were added. After 30 min incubation at room temperature, the culture was transferred to a shaker and shaken (300 rpm) for 90 min at 37 °C. After the addition of 140 μL of kanamycin (70 μg/mL), the culture was incubated overnight at 30 °C. Phage particles were prepared the next day as described (vide supra).

Protein Expression and Purification. *Escherichia coli* BL21 (DE3) (Novagen) cells were transformed with the expression plasmid using chemical transformation. On the next morning, 5 L of SB (3% peptone, 2% yeast extract, 1% MOPS) containing carbenicillin (100 μg/mL) was inoculated. The cultures were incubated on a shaker (300 rpm) at 37 °C until an OD_{600} between 0.6 and 0.8 was reached. IPTG was added up to a final concentration of 1 mM, and the temperature was adjusted to 30 °C. The cultures were incubated overnight.

On the next morning, the cultures were chilled on ice for 30 min and spun down at 8000*g* for 15 min. The supernatant was transferred into clean flasks. In the next step, the supernatant was concentrated using a Millipore concentrator. The volume of the supernatant was reduced to approximately 250 mL. Affinity chromatography was applied to purify the antibody fragment from the supernatant. The supernatant was loaded onto a column containing M2-anti-FLAG resin (SIGMA). After the loading, the column was washed thoroughly with PBS. The scFv protein was eluted with elution buffer (0.1 M glycine, pH 2.3). The eluate was neutralized with 1 M Tris (pH 9). The neutralized eluate was concentrated with Centriprep YM10 concentrators (Amicon). The final concentration of scFv protein was 1 mg/mL, which was monitored as the absorption at 280 nm.

Fluorescence Spectroscopy. A FluoroMax-2 spectrofluorometer (Instruments S. A., Inc., Edison, NJ) equipped with a 150 W continuous xenon lamp was used to measure fluorescence emission spectra and quantum yields with both excitation and emission band-pass of 5 nm; a PMT voltage of 50 V was used, and measurements were performed at a scan rate of 1 nm/s. For the fluorescein-based probes, samples were excited at λ_{exc} = 485 nm and fluorescence was measured at λ_{em} = 515 nm. For the *N*-methyl−isatoic amide probe, measurements were performed at λ_{exc} = 350 nm and λ_{em} = 425 nm.

Binding Studies. Equilibrium dialysis was performed using either [^{3}H]-cocaine or a fluorescent cocaine probe as ligand and serial dilutions of IgG or scFv antibodies as hosts (80 μL per well). Wells (12 per sample) were then filled with another 80 μL of [^{3}H]-cocaine in PBS (2 nM per well). A second plate was prepared with 2 × 12 wells containing just PBS (160 μL/well). The two plates were tightly connected with filled wells facing each other and separated with a dialysis membrane (cutoff 6000−8000 Da). The plates were attached vertically to a shaker and were shaken for 24 h at room temperature, after which they were

carefully separated. The membrane was discarded, and from each well 100 μL was transferred to either a scintillation vial (for [^{3}H]-cocaine samples) or a quartz cuvette (for fluorescent samples). For radiation counting, 5 mL of scintillation fluid (ICN) was added to each vial, and radiation was counted for each sample for 5 min. The experiment was repeated twice for each sample. The average in differences in DPM (dosage per minute) or fluorescence between opposite wells was determined for each dilution of antibodies, yielding binding curves as shown in Figure 6a−d.

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Supporting Information Available: Synthesis and identification of new compounds by NMR and MS. This material is available free of charge via the Internet at http://pubs.acs.org.

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