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Cocaine Catalytic Antibodies: The Primary Importance of Linker Effects

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Abstract—Current treatments for cocaine addiction are not effective. The development of a catalytic monoclonal antibody (mAb) provides a strategy for not only binding, but also degrading cocaine, which offers a broad-based therapy. Hapten design is the central element for programming antibody catalysis. The characteristics of the linker used in classic transition-state analogue phosphonate haptens were shown to be important for obtaining mAbs that hydrolyze the benzoate ester of cocaine.

Cocaine abuse continues to be prevalent. Recent surveys for the United States indicate that more than 23 million people have tried cocaine and approximately 2.5 million are considered chronic abusers.[1,2] Numerous medical problems, including death, often accompany cocaine use and the association of the drug with the spread of AIDS is of concern.[3,4] Despite intensive efforts, the development of effective therapies for cocaine craving and addiction remain elusive. An improved pharmacotherapy would increase the effectiveness of rehabilitative programs. One alternative might rely on immunological reagents and the immune system. We, and others, have shown that the antibody-mediated binding of cocaine impeded passage of the drug into the central nervous system that resulted in a suppression of its characteristic actions.[5–7] Administration of a monoclonal antibody (mAb) endowed with not only binding, but also catalytic activity to metabolize cocaine, would have enhanced therapeutic effects if the kinetic properties of the mAb were sufficient.

Catalytic antibodies have emerged as a powerful tool at the interface of chemistry and biology.[8,9] In this regard, the hallmark reaction catalyzed by antibodies is ester hydrolysis. Since cleavage of the benzoate ester of cocaine **1** produces the nonpsychoactive metabolite ecgonine methyl ester **2**,[10] it is an excellent target for an immunopharmacological strategy (Fig. 1).

Landry and co-workers used a transition-state (TS) analogue approach for hapten design and reported several cocaine-hydrolyzing mAbs.[11,12] In this model, the benzoyl ester of the cocaine framework is replaced by a phenylphosphonate that approximates the TS for ester hydrolysis (Fig. 2). Subsequently, other workers also used a phosphonate analogue to obtain hybridomas which were subjected to high-throughput screening using cocaine benzoyl thioester.[13] However, it was more than 10 years ago that our laboratory applied the TS design to cocaine and prepared phosphonate haptens. The first two structures founded on this principle were **3** (code named **GNP**) and **4** (code named **GNN**) in which the site of a linker attachment for coupling to carrier proteins was different.

Yet, despite screening nearly 1000 clones, no mAbs with catalytic activity above the background rate were discovered. In an effort to elicit a cocaine esterase that will have utility for human use, we continue to examine TS-analogue designs, as well as other approaches. Herein, we report that a specific change in the linker composition of **3** and **4** is critical for obtaining cocaine catalytic mAbs, which provides a foundation for further advances.

Having tested a number of other modified phosphonate TS structures related to **3** without success, we decided to make a simple change in the linker. A β-alanyl unit was

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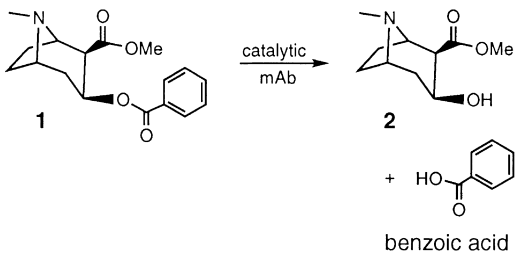

Figure 1. The antibody-catalyzed hydrolysis of cocaine.

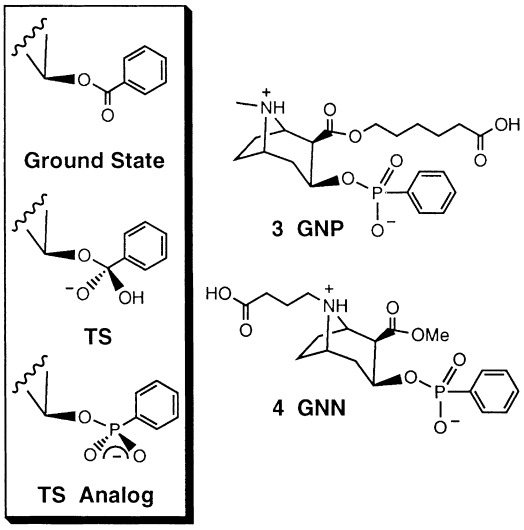

Figure 2. The principle of TS stabilization and haptens based on this concept.

appended at the linker terminus that afforded the **GNL** hapten **9** (Fig. 3).

We immunized mice with a **GNL**-KLH conjugate and the mAbs were screened for the hydrolysis of cocaine by following the release of benzoic acid using HPLC. Remarkably, we detected catalysis in ~25% of the total mAbs tested (>3-fold over background in the initial rate; 20 μM mAb, 500 μM cocaine), of which several were considered to have good activity (Table 1). Significantly, as facilitated by the low K_m value, the most efficient mAb, GNL3A6, was able to completely degrade all offered cocaine (20 μM mAb, 900 μM cocaine). Notably, the K_m is the lowest reported to date for any cocaine catalytic mAb at physiological pH. A low value for this parameter is an essential contributor to a high k_{cat}/K_m, the apparent second-order rate constant for the reaction of antibody and cocaine, that dictates mAb catalytic power.

Our mAbs are similar in activity to all mAbs reported by Landry, except one, in which k_{cat} was ~60-fold better than GNL23A6 and k_{cat}/K_m ~19-fold better than GNL3A6.[11] However, the conditions for the Landry mAbs were optimized, which required an increase to pH 8. Cashman et al. reported the most efficient mAb (k_{cat}/K_m ~10^3 M^{-1} s^{-1}),[13] however this was at pH 8.4 and 37 °C, so the value under our conditions would likely be reduced ~10-fold. What the results demonstrate is that, despite efforts by three laboratories involving numerous mAbs and methods, efficient clones are rare and new approaches will be required.

From the standpoint of the cocaine hydrolysis problem, but also catalytic antibody technology in general, the effect incurred through a subtle change in the linker was of great interest. Based on our experience with hapten designs for a variety of hydrolytic reactions, the linker lengths in **3** and **4** should be adequate to allow recognition of the cocaine framework, and certainly the phosphonate moiety. However, since the β-alanyl fragment not only introduced a new amide functionality, but also increased the linker length, it was necessary to separate these characteristics and determine which contributed to the efficacy of **9**. The hapten **13** (**GNK**) was synthesized in which the linker is an alkyl linker as in **3**, but of the same length as in **9** (Fig. 4).

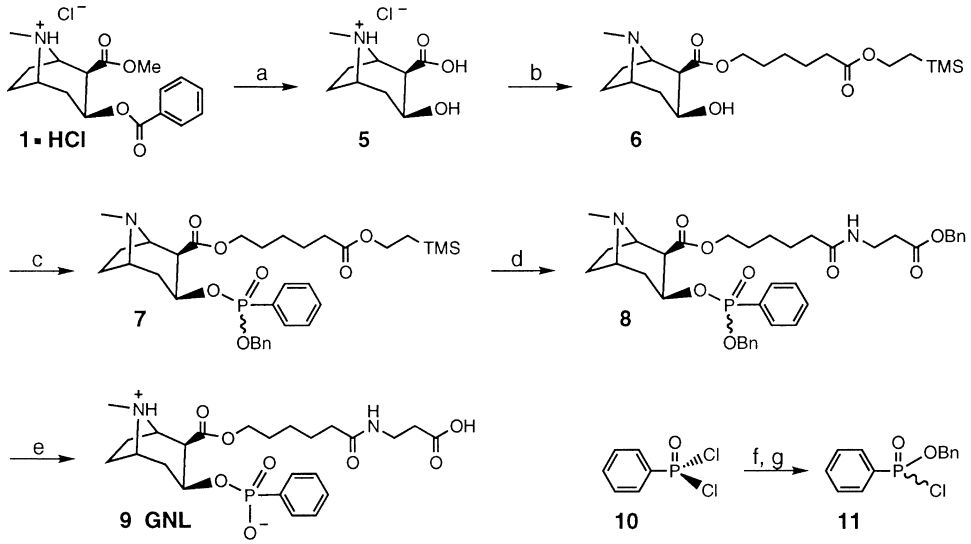

Figure 3. Reagents and conditions: (a) 1.25 M HCl, reflux; (b) 2-trimethylsilylethyl 6-bromohexanoate, NaOH, pyridine, 80 °C; (c) (i) LDA, (ii) **11**; (d) (i) TFA, (ii) β-alanine benzyl ester, EDC, HOBt; (e) H_2, Pd/C; (f) benzyl alcohol, NEt_3; (g) PCl_5, $CHCl_3$.

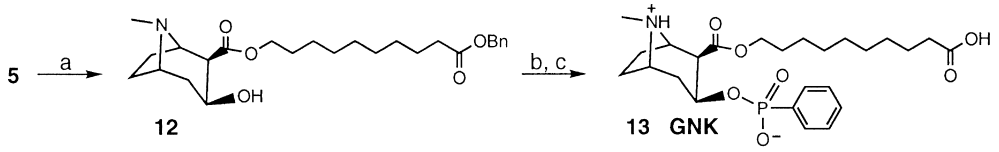

Figure 4. Reagents and conditions: (a) benzyl 10-bromoundecanoate, Bu_4NOH, Bu_4NI, DMF; (b) (i) LDA, (ii) **11**; (c) H_2, Pd/C.

Only one mAb from a panel of 19 clones derived from **GNK**-KLH was found with a significant rate above background (Table 1). Even though the activity was low, the one clone and its catalysis was more than we ever observed for **GNP** mAbs. Hence, the longer linker length possibly promotes some elicitation of catalytic activity. However, the internal amide bond seems principally responsible for the linker-directed effects that led to a 'switching on' of an immune response that resulted in catalytic mAbs. In order to provide a positive internal control and further support for the hypothesis, a new β-alanyl linker was introduced at the nitrogen atom as in **4** to give the **GNJ** hapten **17** (Fig. 5).

With **GNJ**-KLH three catalysts were found out of 24 tested (12.5%), fewer than with **GNL**-KLH. In addition, the best mAb, GNJ14G12, was less efficient than the **GNL** mAbs (Table 1). But again, this single panel of mAbs, derived from one fusion to produce a set of hybridomas, contained several catalysts, where before the **GNN** hapten yielded nothing from many fusions and a large survey of candidates.

Until further insight is gained from immunochemical and immunological experiments, we can only speculate as to the observed linker effect. One rationale is that an amide linkage might allow for more favorable hapten-peptide fragment presentation by MHC II and/or recognition by the T-cell receptor. Perhaps more tangible, the hydrogen bonding of the linker amide bond at the antibody binding site of B-cell surface immunoglobulin could elicit amino acid residues for chemical catalysis akin to the principle of 'bait and switch'.[14] Notably, the haptens of Landry et al. contain an amide-based linker, in which an amino terminus is capped with a succinyl unit, and their work has shown that mAb catalytic activity exceeds that expected from a correlation based only on TS stabilization.[11] Elucidation of catalytic mechanisms and further studies with new linkers will help shed light on our hypothesis.

Table 1. Data for some catalytic mAbs that hydrolyze cocaine[a]

mAb	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat}/k_{uncat}
GNL3A6	0.030	55	9.2	3705
GNL4D3	0.010	70	2.3	1210
GNL23A6	0.038	830	0.77	4690
GNJ14G12	0.023	5240	0.083	2840
GNK28C9	0.0060	2080	0.050	745

[a]Determined in 100 mM phosphate buffer, pH 7.4, 21 °C.

Both spontaneous[15–18] and esterase-catalyzed[16,19–21] hydrolysis of cocaine contribute to the short in vivo half-life of ~30 min in human blood, comparable to that determined in our laboratory in rats.[6] Yet, for an enzyme or catalytic antibody therapy to be effective, extensive clearance of cocaine must take place within seconds. The administration of purified human plasma cholinesterase reduced cocaine toxicity in mice.[22] Although the kinetic parameters for the enzyme are currently superior to the best cocaine mAb,[23] the efficiency is still not suitable to achieve cocaine clearance in the human condition at clinically manageable concentrations of enzyme. Landry et al. also reported some positive results in animal models using their best catalytic mAb.[24] However, high catalytic power is required to meet the demands of hydrolyzing cocaine rapidly enough to alter its pharmacokinetic profile and psychoactive effects in the human condition.

An estimate can be made as to the requirements of an anti-cocaine catalytic mAb during a period of rehabilitation from cocaine abuse. We suggest that an administered

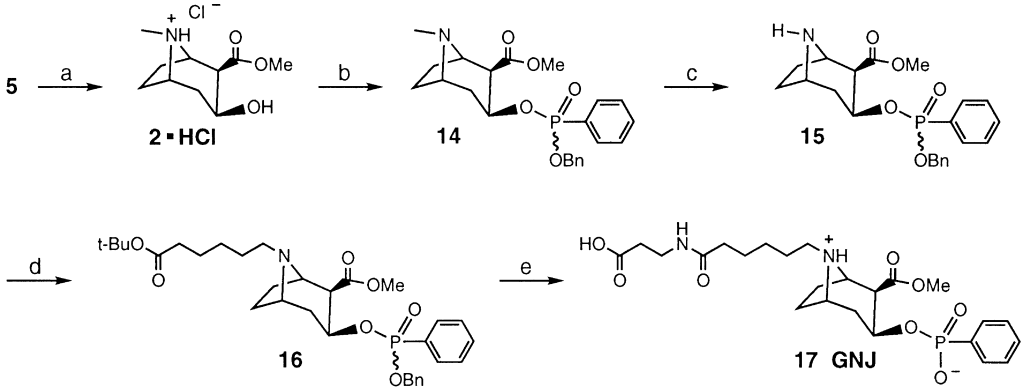

Figure 5. Reagents and conditions: (a) HCl, MeOH; (b) (i) LDA, (ii) **11**; (c) (i) Troc-Cl, NEt_3, (ii) Zn, formic acid; (d) *t*-butyl 6-bromohexanoate, NEt_3, CH_3CN; (e) (i) TFA, (ii) β-alanine benzyl ester, EDC, HOBt, (iii) H_2, Pd/C.

catalytic mAb, 'humanized' or even 'fully human' to minimize an immune response,[25,26] that is circulating at a practical, long-term clinical level of ~1 mg/mL (~15 μM in active sites for whole IgG) must have a minimum $k_{cat}/K_m \sim 10^4\ M^{-1}\ s^{-1}$. An mAb operating with this rate constant would afford clearance of a typical single dose of circulating cocaine (~10 μM) from the bloodstream within a few seconds before transit into the brain. This activity is in the range of the esterase family of enzymes studied using various ester substrates, other than cocaine, which again is indicative of the recalcitrant nature of cocaine as a substrate and for hapten programming. Even though our best mAb needs a 10^3-fold rate enhancement, this is not unrealistic considering a similar factor was attained in progressing from mAbs with no activity to those resulting from only a change in linker chemistry.

The use of novel hapten designs, immunization strategies such as reactive immunization,[27] and the advent of powerful protein engineering, computer modeling and X-ray crystallographic techniques should bring an antibody for cocaine degradation into the realm of therapeutic application. Expansion of the immunotherapeutic arsenal will provide benefits in the challenging battle against cocaine addiction.

Acknowledgements

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