Application of Artificial Enzymes to the Problem of Cocaine

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ABSTRACT: **Cocaine mediates its reinforcing and toxic actions through a "loss of function" effect at multiple receptors. The difficulties inherent in blocking a pleiotropic blocker pose a great obstacle for the classical receptor-antagonist approach and have contributed to the failure-to-date to devise specific treatments for cocaine overdose and addiction. As an alternative, we have embarked on an investigation of catalytic antibodies, a programmable class of artificial enzyme, as "peripheral blockers"—agents designed to bind and degrade cocaine in the circulation before it partitions into the central nervous system to exert reinforcing or toxic effects. We synthesized transition-state analogs of cocaine's hydrolysis at its benzoyl ester, immunized mice, prepared hybridomas, and developed the first anti-cocaine catalytic antibodies with the capacity to degrade cocaine to non-reinforcing, non-toxic products. We subsequently identified several families of anti-cocaine catalytic antibodies and found that out most potent antibody, Mab15A10, possessed sufficient activity to block cocaine-induced reinforcement and sudden death in rodent models of addiction and overdose, respectively. With the potential to promote cessation of use, prolong abstinence, and provide a treatment for acute overdose, the artificial enzyme approach comprehensively responds to the problem of cocaine.**

INTRODUCTION

Cocaine is abused in the United States by over two million hard-core addicts and in Europe by an additional one million.[1] Despite decades of effort, however, no useful antagonists of cocaine's reinforcing or toxic effects are available. This failure is due, in part, to the drug's mechanism of action as a competitive blocker of neurotransmitter re-uptake.[2] Acting at a synapse in the nucleus accumbens that is part of the reward pathway of central nervous system, cocaine blocks a presynaptic dopamine re-uptake transporter. This blockade allows dopamine to accumulate in the synapse, increasing neurotransmission to limbic centers and ultimately reinforcing self-administration.[3] The difficulties inherent in blocking a blocker appear to have hindered the development of antagonists for addiction. Further, dopamine appears to play such a general role in many types of behavior that dopamine receptor agonists

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and antagonist that might be expected to modify cocaine's actions do not act selectively.[4] For cocaine overdose, this problem is compounded by the binding of cocaine at high concentrations to multiple receptors in the CNS and the cardiovascular system, also with loss of function of these receptors. For example, blockade of norepinephrine re-uptake transporters and blockade of Na^+ channels on cardiac myocyte contribute to arrhythmias and sudden death.[5] Blockade of serotonin re-uptake transporters contributes to cocaine-induced convulsions[6] and dopamine re-uptake blockades and dopamine D_1 receptor binding[7] contribute to lethality. Thus, cocaine addiction and overdose pose severe challenges for the classical receptor antagonist approach.

ANTIBODIES AS ARTIFICIAL ENZYMES

As an alternative, we embarked on an investigation of catalytic antibodies, a programmable class of artificial enzyme,[8] as "peripheral blockers," i.e., as agents designed to bind and degrade cocaine in the circulation before it partitions into the central nervous system to exert reinforcing or toxic effects. The hydrolysis of cocaine at its benzoyl ester group yields the non-reinforcing, non-toxic products ecgonine methyl ester and benzoic acid.[9] This reaction proceeds through a tetrahedral intermediate and the species of peak energy in the transition from the ground state to this intermediate is the so-called "transition state." Catalysis, in simplest terms, reflects the acceleration in rate that follows from a binding interaction that preferentially stabilizes the transition-state over the ground state, thereby lowering the activation energy ($G^{\ddagger}$) for the reaction (FIG. 1, dotted path). According to transition-state theory, the structure and charge distribution of the transition state resembles that of the species on the reaction coordinate closest in energy, e.g., the tetrahedral intermediate. Natural esterases possess a binding pocket tailored by evolution to complement the negatively charged, tetrahedral intermediate relative to the neutral, trigonal planar ground state. In a seminal discovery, Lerner and Schultz independently demonstrated that such a binding pocket can also be generated artificially in the form of catalytic antibodies.[10,11]

ANTI-COCAINE CATALYTIC ANTIBODIES

The generation of anti-cocaine catalytic antibodies begins with the synthesis of a stable analog that mimics the evanescent transition state in charge distribution and geometry. Antibodies to such a transition-state analog (TSA) would possess a binding pocket that complements the actual transition-state structure and some of these antibodies will differentially stabilize the transition state over the ground state and function as artificial enzymes. On the basis of this analysis we constructed a transition-state analog of cocaine benzoyl ester hydrolysis that utilized the negatively charged, tetrahedral structure of a phosphonate monoester, the free TSA depicted in FIGURE 2.[12] Because small molecules do not typically elicit antibodies, the free TSA was tethered to a carrier protein to yield the immunogenic conjugate TSA-1. Balb/C mice were immunized with TSA-1 and a polyclonal anti-analog antibody response

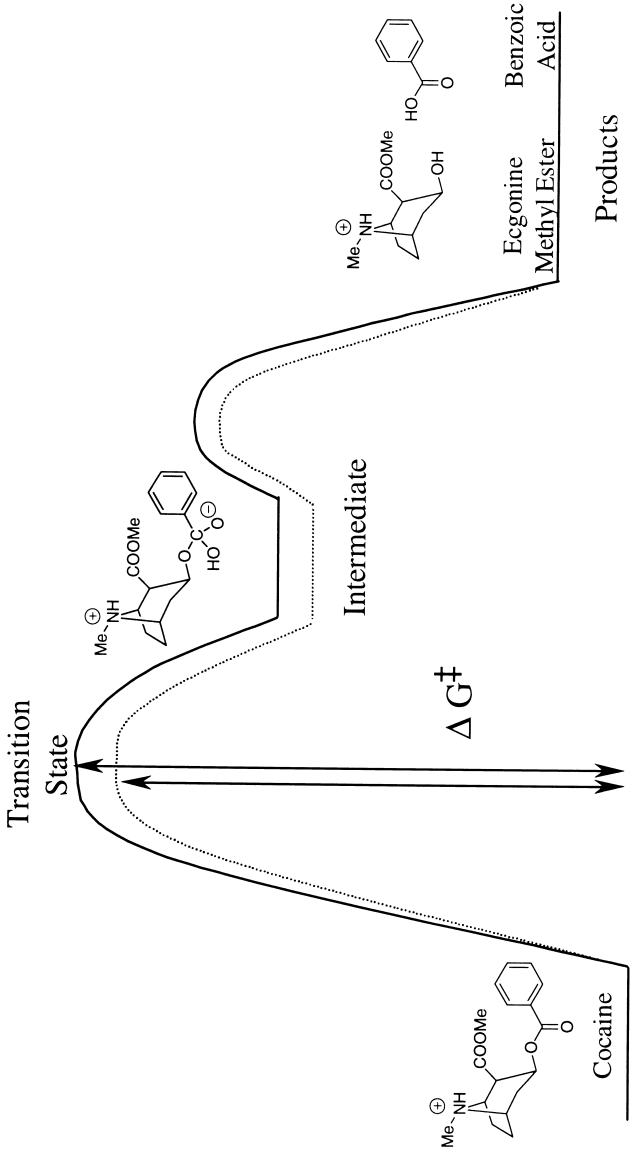

FIGURE 1. Reaction coordinate for hydrolysis of cocaine at the benzoyl ester.

was detected by ELISA. However, only a subset of anti-analog antibodies is typically catalytic and for this reason hybridomas were prepared and the resulting monoclonal anti-analog antibodies were characterized individually. We developed a simple assay for cocaine benzoyl ester hydrolysis based on the release of ^{3}H-benzoic acid from ^{3}H-phenyl-cocaine and using this assay, two catalytic antibodies were identified, Mab 3B9 and Mab 6A12. These antibodies demonstrated saturation kinetics with increasing concentrations of cocaine and a linear Lineweaver-Burke plot yielded kinetic constants (TABLE 1). As expected, the free transition-state analog was

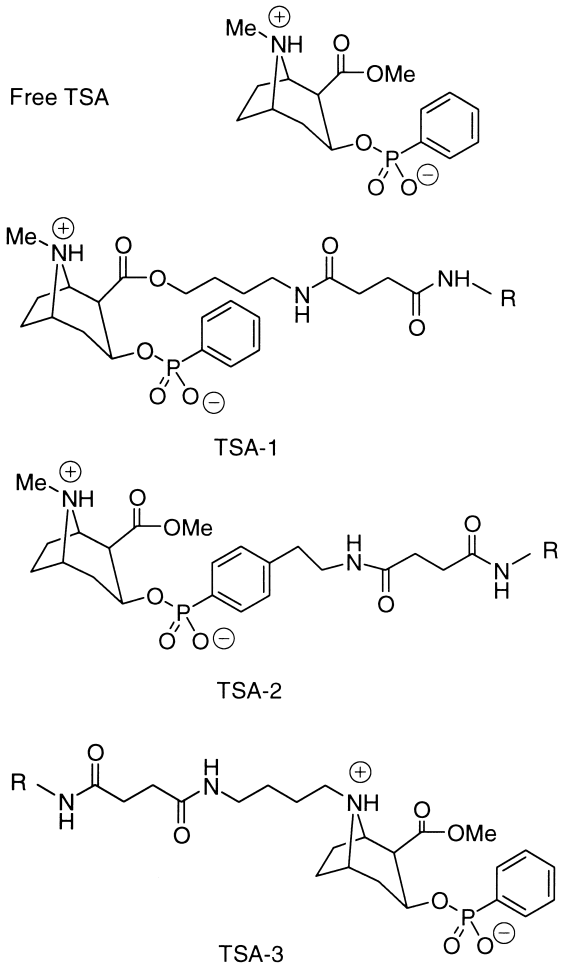

FIGURE 2. Structures of transition-state analogs for benzoyl ester hydrolysis.

TABLE 1. Kinetic Parameters for the hydrolysis of [^{3}H] cocaine by Mab's

Mab	TSA	k_m	k_{cat}	k_{cat}/k_o
3B9	1	490	0.11	1,110
6A12	1	1,020	0.072	880
2A10	1	3,000	0.011	420
15A10	1	220	2.3	23,000
9A3	1	270	0.015	140
19G8	1	900	0.091	830
12H1	2	150	0.16	1,500
8G4G	3	530	0.60	5,500
8G4E	3	1,200	0.12	1,100

a potent inhibitor and catalytic activity tracked with the F(ab) antibody fragment and not with the Fc fragment.

A SYNTHETIC APPROACH TO IMPROVED CATALYTIC ANTIBODY DIVERSITY

We expected Mab 3B9 and 6A12 to provide unique targets for optimization but upon pcr cloning and sequencing we found them to be 93% identical. This lack of diversity prompted us to consider a new strategy: to vary the placement of the immunogenic tether in order to vary the surfaces of the analog rendered inaccessible.[13] By presenting distinct epitopes for immunorecognition we hoped to elicit distinct classes of catalytic antibodies. Thus, analogs were prepared with the tether attached at the 4′-position of the phenyl group, TSA-2, or at the tropane nitrogen, TSA-3. From these three analogs, a total of nine catalytic antibodies were obtained from the 107 anti-analog antibodies elicited. TSA-1 yielded six out of 50 anti-analog antibodies; TSA-2 yielded one out of eight and TSA-3 yielded two out of 49. As before, all catalysts were inhibited by free TSA at 50 μM and not by an inhibitor of serum esterases, eserine at 1 mM. Antibodies demonstrated saturation kinetics and the first order rate constant (k_{cat} and the Michaelis constant (K_m) of the nine antibodies were determined and found to range from 0.011 to 2.3 min^{-1} and from 150 to 3000 μM, respectively, as shown in TABLE 1.

The nine catalytic antibodies were PCR-cloned and the variable regions of heavy and light chains were sequenced in order to assess the structural diversity of the group. The deduced primary amino acid structures of the antibodies were compared at each of the three complementarity determining regions (CDRs) of each chain. The pattern of homology defined discrete families and results are summarized in TABLE 2. TSA-1 yielded two structural families 3B9-96A12-2A10 and 15A10-9A3-19G8. The homology among light chain CDRs for pairings within the 3B9 family averaged 96% and among heavy chain CDRs averaged 90%, with 3B9 and 6A12 identical. Within the 15A10 family, light chain CDR homology averaged 93% and

TABLE 2. Homology (%) for CDRs of anti-cocaine catalytic antibodies

	3B9	6A12	2A10	12H1	15A10	9A3	19G8	8G4G	8G4E
A. Heavy Chain									
3B9	—								
6A12	93	—							
2A10	83	90	—						
12H1	93	93	83	—					
15A10	10	10	10	10	—				
9A3	10	10	10	10	87	—			
19G8	10	10	10	10	97	93	—		
8G4G	52	52	55	52	24	7	7	—	
8G4E	24	24	24	28	38	17	17	17	—
B. Light Chain									
3B9	—								
6A12	100	—							
2A10	70	70	—						
12H1	100	100	70	—					
15A10	23	23	27	23	—				
9A3	23	23	23	23	86	—			
19G8	29	29	27	29	80	80	—		
8G4G	26	26	20	26	23	31	23	—	
8G4E	17	17	17	17	23	37	23	11	—

heavy chain CDRs averaged 88%. The discrete nature of the families was evidenced by the low homology between 3B9 and 15A10 families: 14% for light chain CDRs and 32% for heavy chain CDRs. TSA-2 yielded a single antibody with 96% identity at the light chain CDRs of the 3B9 family and 100% identity for the heavy chain CDRs of 3B9 and 6A12. TSA-3 yielded two single-membered families.

We next sought to determine whether structural families of antibodies corresponded to functional groupings. Using a competitive inhibition enzyme immunoassay (CIEIA) we determined the relative affinity of TSA-1, TSA-2, and TSA-3 for each of the catalytic antibodies. As shown in TABLE 3, CIEIA identified two broad groups. The 3B9 family elicited by TSA-1 displayed a rank order of affinity of TSA-1 > TSA 2 > TSA-3. Interestingly, 12H elicited by TSA-2 had the same rank order as predicted by its amino acid homology to the 3B9 family and bound most avidly to TSA-1. In contrast, the 15A10 family bound TSA-1 and TSA-3 with high affinity but showed no affinity for TSA- 2, tethered at the 4′-position of the phenyl group. Thus, we concluded the structurally defined families were analogous in some respects to those functionally defined.

TABLE 3. Analysis of catalytic antibodies by competitive inhibition immunoassay of transition state analogs[a]

Mab	Free TSA (μM)	TSA-1 (μM)	TSA-2 (μM)	TSA-3 (μM)
3B9	0.01	0.02	3	100
6A12	0.01	0.01	4	90
2A10	0.5	3	20	150
12H1	0.001	0.01	2	60
15A10	0.009	0.003		0.0005
9A3	0.05	0.02		0.003
19G8	0.008	0.001		0.001
8G4G	0.003	0.001		0.001
8G4E	0.003	0.0005		0.003

[a]Competition was performed with TSA's tethered to *n*-amyl amine.

An unexpected result of this investigation was the finding that either TSA-1 or TSA-3 could have elicited any member of the 15A10 group. The effectiveness of TSA-1 and TSA-3 contrasts with the relatively poor performance of TSA-2, the tether of which precludes positioning the phenyl group of cocaine in a deep hydrophobic binding pocket. As a result of these findings, our work has focused on TSA-1 and TSA-3. Finally, the most potent antibody from this study, 15A10 was a candidate for preclinical trials.

CHARACTERISTICS OF Mab 15A10

Mab 15A10 was further evaluated for susceptibility to inhibition or inactivation that might impair its effectiveness *in vivo*. The finding that Mab 15A10 showed no affinity to TSA-2 suggested that the phenyl group of cocaine might occupy a deep binding pocket that was rendered inaccessible by the TSA-2 tether. A concern followed that the benzoic acid product of cocaine hydrolysis might bind avidly to the antibody and impair catalytic function through product inhibition. In fact, the alcohol product ecgonine methyl ester showed no inhibition up to 1 mM but benzoic acid inhibited with a K_i of 250 μM. Fortunately, in humans benzoic acid levels are markedly suppressed by reaction with glycine to form hippuric acid.[14] Perhaps owing to a difference in charge, the prominent, phenyl-containing metabolite of cocaine, benzoyl ecgonine, showed no inhibition of catalysis. A second concern was the possibility of deactivating side reactions in the course of repetitive turnover of the catalytic antibody. However, after 6 h and > 200 turnovers, the k_{cat} of Mab 15A10 remained > 95% of its baseline value. Thus Mab 15A10 possesses several characteristics essential for a useful *in vivo* catalyst.

THE EFFECT OF Mab 15A10 IN A MODEL OF ADDICTION

The reinforcing effect of cocaine in rat provided a convenient preclinical model in which to evaluate an agent that might modify cocaine's abuse liability. This measure reflects well the actions of cocaine that embody the behavioral and pharmacological basis of its abuse. Intravenous injection of cocaine and the smoking of crack cocaine have equivalent pharmacokinetics and, representative of both, an intravenous administration protocol was implemented.[15] Sprague Dawley rats were initially

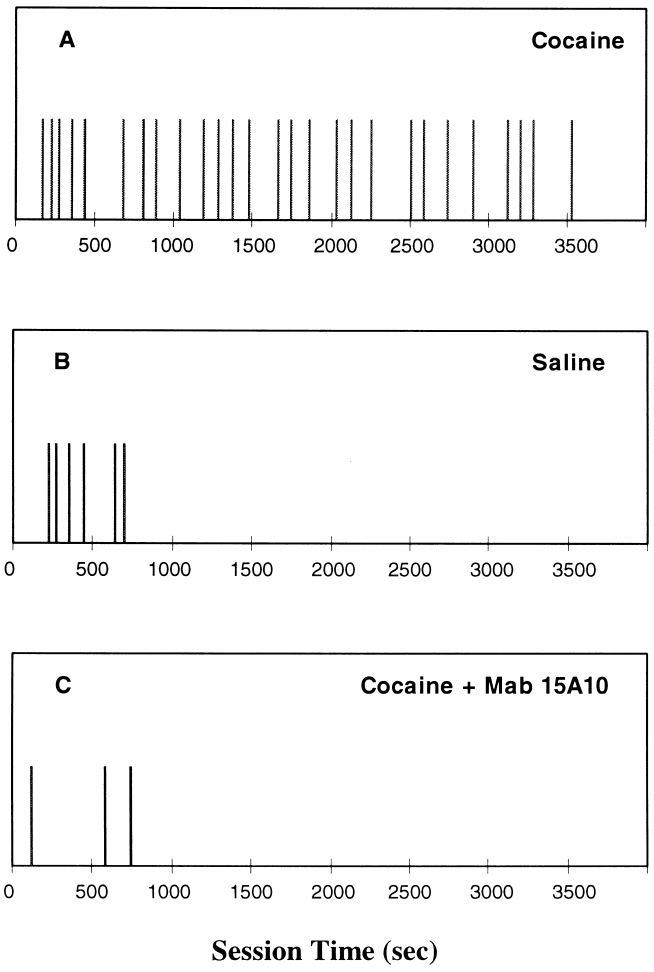

FIGURE 3. Pattern of intravenous self-administration of cocaine (**A**), saline (**B**), or cocaine + mAb 15A10 (**C**) in a single rat. Each vertical line indicates a single injection, obtained on a fixed ratio 5 time-out 10 sec schedule of cocaine delivery.The panels show infusion patterns from three consecutive sessions.

trained in operant conditioning chambers to press a lever for access to sweetened milk. A 10-sec timeout followed reinforcer delivery and the number of reinforcers was increased from 1 to 5 (fixed ratio 5). When the rats were responding with more than 50 milk reinforcers at fixed ratio 5 for three consecutive days, an intravenous catheter was implanted and the conditions were changed so that responding resulted in the intravenous delivery of cocaine at 0.3 mg/kg/injection. Cocaine maintained regular patterns of lever pressing during the one-hour sessions (FIG. 3, Panel A). When cocaine was replaced by saline, lever pressing decreased rapidly during the session (FIG. 3, Panel B). Returning cocaine to the infusion for the following session reestablished rapid responding. The administration of Mab 15A10 (9 mg) completely blocked the reinforcing effect of cocaine and despite the presence of cocaine in the intravenous line, the response was not different from the response to saline (FIG. 3, Panel C).

To exclude the possibility that this saline-like behavior was due to a nonspecific disruption of behavior, Mab 15A10 was tested in rats maintained on sweetened milk. As expected, no effect on rapid responding was observed.[15] Similarly, we sought to exclude the possibility that Mab 15A10 exerted a nonspecific effect on the dopaminergic reward pathway. Thus, we tested Mab 15A10 in rats that were maintained on bupropion and were rapidly responding in a manner similar to that induced by cocaine. Again Mab 15A10 did not alter the rapid responding to a non-cocaine stimulus.[15] These results indicated that Mab 15A10 is extremely selective in blocking behavior maintained by cocaine.

OTHER CONSIDERATIONS FOR A THERAPEUTIC ANTI-COCAINE CATALYTIC ANTIBODY

A small-molecule receptor antagonist blocks its target through a thermodynamic mechanism: a higher relative affinity for the target receptor. In contrast, a catalytic antibody such as Mab 15A10 blocks via a kinetic mechanism: a rate of turnover sufficient to decrease circulating target drug before it reaches its receptor. Thus, an effect by Mab 15A10 in a small animal, in which the transit time of cocaine between peripheral vein and central nervous system capillary is a few seconds, is a significant milestone.

However, a fair question is the potential for effectiveness of any blocker, regardless of mechanism, in the treatment of addiction. Here the experience with heroin antagonists is dispositive: the oral heroin antagonist naltrexone has only a limited use in treating heroin addiction since naltrexone requires daily administration, is easily discontinued and rapidly clears. A catalytic antibody that persisted in the circulation for weeks would diminish the problem of poor compliance much as long-acting, depot forms of antipsychotic medications have proved useful in the treatment of schizophrenia, a condition also complicated by a high rate of non-compliance with medication.

Nonetheless, what is the potential for developing a catalytic antibody sufficiently active for use in clinical practice? By means of a simple compartment model we previously estimated the activity and dose of catalytic antibody needed to neutralize typical street doses of cocaine.[12] Mab 15A10 remains short of these standards.

However, our model neglected the large volume of distribution of cocaine and thus it could provide only an upper limit on the required activity. The subsequent demonstration that mere binding antibodies can interfere with cocaine-induced reinforcement in rats[16] suggests that the parameters for a clinically useful catalytic antibody are well within reach and that improvements in substrate binding (k_m) might be sufficient. The clinical use of a catalytic antibody would have a critical advantage in that it would be much less susceptible to saturation than a binding antibody, either passively administered or actively elicited.

Finally, the use of anti-cocaine catalytic antibodies for the treatment of overdose can be considered. The pleiotropic blocking effect of high concentrations of cocaine argues strongly against the possibility of an antidote for cocaine intoxication based on receptor antagonism. Mab 15A10 blocks cocaine-induced seizures and lethality in a dose dependent fashion (data not shown) and thus a peripherally acting agent appears feasible. In contrast to butyrylcholinesterase, also under evaluation as a treatment for overdose,[17] administration of a long acting catalytic antibody in this setting would also commence treatment for addiction.

CONCLUSION

Using rationally designed transition-state analogs (TSA-1, 2 and 3) for the hydrolysis of cocaine's benzoyl ester, we elicited several distinct families of anti-cocaine catalytic antibodies. TSA-1 and TSA-3 were found to be particularly effective and are the subjects of further investigations. Mab 15A10, the most potent of the catalytic antibodies elicited, was found to be sufficiently active to prevent cocaine's reinforcing and toxic effects in rat. Mab 15A10 is a suitable candidate for humanization and optimization by mutagenesis in preparation for clinical trials.

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