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33. Tilts of negatively stained filaments showed that the RAD51 one-start helix is right-handed as is RecA. Minimal-dose procedures were used to avoid excessive radiation damage. Layer lines were indexed as arising from a structure with a repeat of 37 subunits in six turns of a 99 Å pitch helix. The layer lines used for the RAD51 reconstruction were: $l = 0$, $n = 0$; $l = 1$, $n = -6$; $l = 5$, $n = 7$; $l = 6$, $n = 1$; $l = 7$, $n = -5$; and $l = 12$, $n = 2$. The RecA reconstruction had, in addition, $l = 13$, $n = -4$. The threshold density for both the surface views and the contour plots was chosen so that the appropriate molecular volume was contained, assuming a partial specific volume of 0.75 $cm^3\ g^{-1}$. For the RAD51 reconstruction, a molecular volume corresponding to 30 kD, rather than 43 kD, was used because 120 additional NH_2-terminal residues were not visualized. The use of a larger molecular volume leads to a nearly isotropic enlargement of the reconstruction. In a comparison of the two polymers, several observations suggest that the RAD51 filament is even less regular than the very flexible RecA filament, and a comparison of layer line data suggests that the resolution of the RAD51 reconstruction is lower than that of the comparable RecA reconstruction. The transform of the crystal structure C_α backbone was multiplied by a Gaussian filter having a standard deviation of 1/(30 Å) and then back-transformed to match the electron microscopic resolution.
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Antibody-Catalyzed Degradation of Cocaine

Donald W. Landry,* Kang Zhao,† Ginger X.-Q. Yang, Michael Glickman, Taxiarchis M. Georgiadis

Immunization with a phosphonate monoester transition-state analog of cocaine provided monoclonal antibodies capable of catalyzing the hydrolysis of the cocaine benzoyl ester group. An assay for the degradation of radiolabeled cocaine identified active enzymes. Benzoyl esterolysis yields ecgonine methyl ester and benzoic acid, fragments devoid of cocaine's stimulant activity. Passive immunization with such an artificial enzyme could provide a treatment for dependence by blunting reinforcement.

Addiction to cocaine afflicts Western populations in epidemic proportions, and the exceptional reinforcing effect of cocaine renders abuse of this stimulant most resistant to treatment (*1*). Cocaine reinforces self-administration in relation to the peak serum concentration of the drug, the rate of rise to the peak, and the degree of change of the serum level (*2*). The drug rapidly partitions from serum into the central nervous system (CNS) and binds specifically to reuptake carriers for several monoamine neurotransmitters (*3*). The function of these presynaptic transporters appears to be the inactivation of released neurotransmitter (*4*). The receptor that mediates the reinforcing effect of cocaine corresponds to its binding site for competitive inhibition of dopamine reuptake (*5*). This reuptake inhibition is hypothesized to potentiate dopaminergic neurotransmission in mesolimbocortical pathways and ultimately result in reinforcement (*6*). Direct antagonists of cocaine-induced reinforcement do not exist currently, and agents that promote abstinence, such as desipramine, have an induction period of several weeks (*7*).

As an alternative to therapeutic approaches that are based on the pharmacology of the cocaine receptor, the delivery of cocaine to its receptor could be interrupted. Antibodies against opiates were found to antagonize the reinforcing effect of heroin in a paradigm of drug self-administration in a rhesus monkey (*8*). However, although successful in extinguishing heroin self-administration at low doses of heroin, these antibodies failed for repetitive high doses because of the depletion of circulating antibody by complex formation.

The recent development of catalytic antibodies (*9*) provides a potential solution to the problem of antibody depletion. Immunization with a stable analog of the evanescent transition-state structure of a chemical reaction can yield monoclonal antibodies with the capacity to catalyze the modeled reaction (*10*). A catalytic antibody could bind the target substrate, catalyze a deactivating transformation, and release the inactive products, and the antibody would be free for further binding.

Of all the commonly abused substances, cocaine is the best candidate for the catalytic-antibody approach. Hydrolysis of cocaine's benzoyl ester by a catalytic antibody would yield ecgonine methyl ester and benzoic acid, fragments that retain none of cocaine's stimulant or reinforcing activities (*11*). Antibodies with esterase activity approaching that of natural enzymes have been reported (*10, 12*), and cocaine's benzoyl ester side group, with its large hydrophobic surface, is particularly suited to elicit antibodies with strong binding and catalytic activity.

The transition state of the benzoyl ester cleavage reaction probably resembles the tetrahedral intermediate of second-order ester hydrolysis (*13*) and can be stably mimicked in terms of geometry and charge distribution by a suitably designed phosphonate monoester (*9, 14*) (Fig. 1). Transition-state analogs based on the phosphonate monoester functional group have yielded artificial esterases with the highest activities (*10, 12*); but these analogs can idiosyncratically fail to elicit catalytically active antibodies, and so the rules for analog construction must be empirically defined (*15*). Recently, investigators have described strategies to increase the frequency of obtaining enzymatic antibodies, such as the "bait and switch" (*16*) and substrate-attenuation (*15*) techniques. However, these approaches

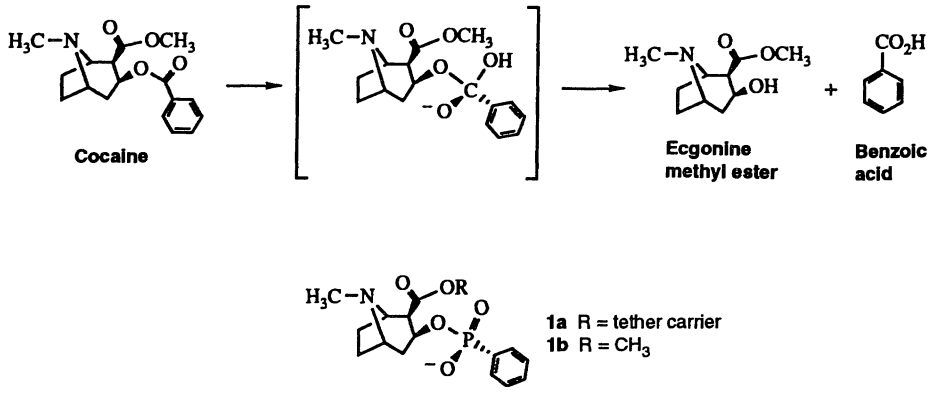

Fig. 1. Hydrolysis of the benzoyl ester of cocaine. Presumed tetrahedral intermediate formed along the reaction pathway is shown in brackets. General structure of a phosphonate monoester analog of the benzoyl ester is pictured immediately below the intermediate. The R substituent for **1a** is the tether depicted in Fig. 2.

Department of Medicine, Columbia University College of Physicians and Surgeons, New York, NY 10032.

*To whom correspondence should be addressed.
†Present address: Department of Chemistry, New York University, New York, NY 10003.

incorporate additional foreign structural elements into the analog, and divergence between analog and substrate results on average in enzymes with higher values for the Michaelis constant (K_m).

Thus, as a starting point, we chose to construct a high-fidelity analog differing from cocaine only by the phosphonate replacement and by the incorporation of a tether for the preparation of an immunogenic conjugate. The methyl ester group was chosen for the tether site because of its distance from the anticipated locus of hydrolysis and its ease of synthesis. On the basis of these considerations, we synthesized the transition-state analog **1a** from the readily available cocaine metabolite (−)-ecgonine (Fig. 2). We developed a new method (*17*) for phosphonate ester synthesis by 1*H*-tetrazole catalysis because available methods (*18*) failed to transform alcohol **2**, owing to its base lability and steric hindrance. Tetrazole selectively catalyzes the monoaddition of primary and secondary alcohols to phosphonic dichlorides to yield mixed phosphonate diesters (**2** → **3**) under mild conditions. A ^{14}C-label was incorporated into the tether (**3** → **4**) so that we could monitor the coupling efficiency of the activated ester, **5**, to the carrier protein.

Immunizations of mice with **1a** as a bovine serum albumin (BSA) conjugate elicited high-titer antisera, and monoclonal antibodies were prepared by standard protocols (*19*). Each fusion procedure yielded 10 to 30 hybridomas secreting analog-specific antibodies as determined by an enzyme-linked immunosorbent assay (ELISA). All immunoglobulin G (IgG) anti-analog antibodies were subcloned, propagated in ascites, and affinity purified by protein A column chromatography (*20*).

A simple method was devised to screen the monoclonal antibodies for hydrolytic activity against the benzoyl ester linkage of cocaine. We synthesized $^{14}C_{benzoyl}$-cocaine (*21*) and found that, upon acidification, it partitioned into an aqueous phase with >97% efficiency, whereas ^{14}C–benzoic acid partitioned with similar efficiency into an organic phase. The reaction of radiolabeled cocaine with carboxyl esterase (Sigma) served as a positive control, and we confirmed the production of benzoic acid with high-performance liquid chromatography (HPLC) (*22*). We applied the screening to the purified monoclonal antibodies, and 2 out of the 29 antibodies that were tested, 3B9 and 6A12, consistently released ^{14}C–benzoic acid above background. Both enzymes were completely inhibited by 50 μM free transition-state analog **1b** and were unaffected by the serum esterase inhibitor eserine (*23*) (1 mM); the Fab portion of each antibody retained catalytic activity (*24*).

Using $^3H_{phenyl}$-cocaine, which has a higher specific activity (*25*) (32 Ci/mmol), we determined the rate of hydrolysis in the presence and absence of each monoclonal antibody as a function of substrate concentration. Release of radiolabeled $^3H_{phenyl}$–benzoic acid at time points corresponding to <5% reaction provided initial rates. We observed saturation behavior consistent with Michaelis-Menten kinetics and obtained a linear Lineweaver-Burk plot for each enzyme (Fig. 3). The steady-state Michaelis-Menten parameters and the rate acceleration (k_{cat}/k_o) are presented in Table 1.

The activity of cocaine esterase monoclonal antibody (mAb) 3B9 is comparable with butyryl cholinesterase (*26*), the principal cocaine esterase in serum. Transition-state analog **1b** inhibited mAb 3B9 with an inhibition constant K_i < 2 μM (*27*), and the enzyme's 10^2 to 10^3 rate acceleration corresponds in magnitude to the relative stabilization of the transition-state to the ground-state (K_m/K_i). It should be possible to identify antibodies with more powerful catalytic mechanisms and rate accelerations of 10^5 to 10^6 through repetitive screening with **1a** and its congeners (*14*, *28*).

Animal studies of antibody-induced extinction of repetitive cocaine self-administration (*2*), previously not feasible because of antibody depletion (*8*), are now possible with mAb 3B9. An antibody that merely bound cocaine would be depleted with the first dose, whereas mAb 3B9, with one

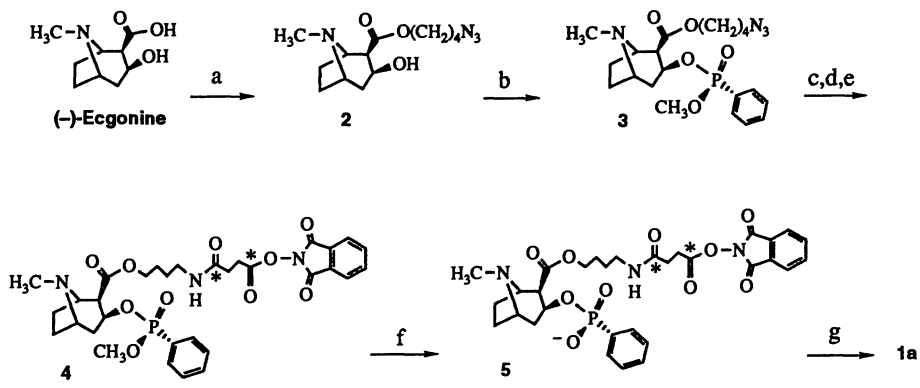

Fig. 2. Synthesis of transition-state analog **1a** from (−)-ecgonine. Reagents and conditions: (a) I-$(CH_2)_4N_3$, $(CH_3)_4NOH$, dimethylformamide (DMF), and CH_3OH at 50°C (92% yield); (b) $PhP(O)Cl_2$ (Ph, phenyl), tetrazole (0.1 eq), benzene, and diisopropylethylamine at room temperature (rt), then MeOH (Me, methyl) (80% yield); (c) $P(CH_3)_3$ and tetrahydrofuran (THF) CH_3OH-H_2O (9:9:2) at rt (62% yield); (d) ^{14}C–succinic anhydride (2.2 mCi/mmol) and THF at rt (purified as benzylester, regenerated with H_2-Pd on C, yield ~50%); (e) dicyclohexylcarbodiimide, *N*-hydroxyphthalimide, and DMF at rt (85% yield); (f) $(CH_3)_3SiBr$ and $CDCl_3$ at rt (unstable, ~90% yield); (g) BSA (coupling ratio 1:6) or ovalbumin (coupling ratio 1:15). No epimerization was observed at C-2 of the tropane nucleus by 300-MHz ^{1}H–nuclear magnetic resonance spectroscopy.

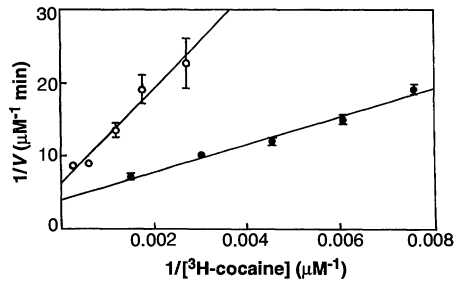

Fig. 3. Lineweaver-Burk plot, (1/*V*) as a function of (1/[S]), where *V* is the reaction velocity and S is the substrate, for hydrolysis of $^3H_{phenyl}$-cocaine by mAb 3B9 (closed circles) and mAb 6A12 (open circles). Artificial enzyme (2 μM) in phosphate-buffered saline was incubated with ^{3}H-cocaine at five concentrations between 100 and 2000 μM. At 10-min intervals, aliquots were acidified with cold HCl (aqueous) to a final pH of 2 and partitioned with hexane-diethyl ether (1:1), and we assayed the organic phase by scintillation counting. The optimal pH was determined and used for each enzyme: 3B9, pH 7.7, and 6A12, pH 7.0. Background hydrolysis was determined in otherwise identical reactions without antibody, and observed rates were corrected. Uncatalyzed-hydrolysis rates were determined under similar conditions. Assays were performed in triplicate, and SE is indicated (3B9, r = 0.99; 6A12, r = 0.98).

Table 1. Kinetic parameters for the hydrolysis of $^3H_{phenyl}$-cocaine by monoclonal antibodies 3B9 and 6A12 and butyryl cholinesterase (BChE). Michaelis constant, K_m; catalytic rate constant, k_{cat}; and spontaneous rate, k_o. The rate of release of $^3H_{phenyl}$–benzoic acid was determined by the assay described in Fig. 3. The parameters for BChE hydrolysis (pH 7.4)* were derived from published sources (*26*).

	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/k_o
3B9	490 ± 11	0.11 ± 0.01	540
6A12	1020 ± 500	0.072 ± 0.02	440
BChE	38	1.2	

turnover in 9 min, will be regenerated. A standard minimum dosing interval of 10 to 15 min will allow for turnover, but because of the modest activity of this first artificial cocaine esterase, near stoichiometric amounts of antibody will be required.

To estimate the characteristics of an antibody useful at a clinically practical dose of <1 g, we assumed a dose of smoked crack cocaine of 100 mg, a peak pulmonary-venous cocaine concentration of 10 to 30 μM (*29*), and a 20-s duration of reaction (the transit time from pulmonary to CNS capillaries). This simple model neglects the volume of distribution for cocaine and the threshold concentration for a biological effect, which if included would reduce the kinetic requirements. Under these constraints, a catalytic antibody against cocaine should ideally have a turnover number >2 s^{-1} and a K_m < 30 μM to deactivate cocaine before significant partitioning into the CNS can occur. However, the protection afforded by the artificial esterase may not need to be complete to be useful, and a significantly less potent enzyme could nonetheless diminish the reinforcing effect of cocaine by reducing the rate of rise and peak concentration of cocaine. By promoting cessation of use and maintenance of abstinence, passive immunization with an anticocaine catalytic antibody could provide a window for appropriate psychosocial and relapse-prevention interventions.

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19. Six BALB/c mice were immunized with the analog-carrier **1a** by both subcutaneous (30 μg) and intraperitoneal (120 μg) routes. Initial immunization was performed with 1:1 Freund's complete adjuvant, and boosting injections were administered with incomplete adjuvant at 2-week intervals. The animals were phlebotomized (200 μl) 14 days after the boost, and the plasma was separated from clot and stored at −78°C. In an ELISA, plastic 96-well plates were coated with ester **5** coupled to ovalbumin (4 μg per well) and incubated with dilutions of serum. Goat anti-mouse IgG coupled to horseradish peroxidase was used as the secondary antibody and indicator. Negative controls included ovalbumin without ligand, nonimmune test serum, omission of the test serum, and omission of the secondary antibody. By the third boost, several mice had developed antibody titers of 1:3000. One of these was boosted by tail-vein injection (50 μg, without adjuvant). On the fifth day after the boost, the spleen was harvested and hybridomas were prepared. Colonies positive for anti-analog antibodies by ELISA were plated to limiting dilution and subtyped (mAb 3B9 and 6A12 were both subtyped to IgG_1).
20. For each of the cell lines, 2 × 10^6 hybridoma cells were placed into a mouse peritoneum that had been pretreated with pristane. The harvested ascites were subjected to affinity chromatography on a preparative protein A HPLC column (Bio-Rad) (the purity of mAb 3B9 and 6A12 was >90% by SDS–polyacrylamide gel electrophoresis).
21. The $^{14}C_{benzoyl}$-cocaine was synthesized from methyl ecgonine and the acyl chloride of ^{14}C–benzoic acid. The specific activity was 111 μCi/mmol (Amersham).
22. The hydrolysis reaction mixture was analyzed by HPLC (Perkin-Elmer) with an analytical reverse-phase C_{18} column (VYDAC) with an acetonitrile-water (0.1% trifluoroacetic acid) gradient and the detector set at 220 nM. The methyl ester of cocaine spontaneously hydrolyzed to benzoyl ecgonine with a $t_{1/2}$ = 20 hours (pH 7). Thus, benzoyl ecgonine is not available as a benzoyl esterase substrate at the early reaction times (<1 hour) during which the detailed kinetics are studied, and the release of benzoic acid is attributed solely to cocaine hydrolysis.
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Translocation of TCRα Chains into the Lumen of the Endoplasmic Reticulum and Their Degradation

Jaekyoon Shin,* Songjae Lee,† Jack L. Strominger

After synthesis, the α chain of the T cell antigen receptor (TCRα) can form a complex with other TCR chains and move to the cell surface, or TCRα can undergo degradation in the endoplasmic reticulum (ER) if it remains unassembled. The mechanism of translocation and degradation in the ER is unclear. It was found that the putative transmembrane region of TCRα ($α_{tm}$) was incompetent on its own to act as a transmembrane region. Molecules that contained $α_{tm}$ were translocated into the ER lumen and then underwent either rapid degradation or secretion, depending on the sequence of the cytoplasmic domain. A specific signal for ER degradation within $α_{tm}$ does not appear to be present.

The T cell antigen receptor TCRαβ is a disulfide-linked heterodimer and is associated with nonpolymorphic components of the CD3 molecule (γ, δ, ε, ζ, and η) at the cell surface (*1*). In this oligomeric complex TCRα probably spans the membrane by a single transmembrane peptide domain (*2*). Partially assembled or unassembled TCR and CD3 components are retained, degraded, or both in the ER or are targeted for lysosomal degradation (*3*, *4*). In cDNA-transfected COS-7 cells, murine TCRα has been reported to be rapidly degraded in a nonlysosomal compartment before entering the Golgi apparatus (ER degradation) (*5*–

Division of Tumor Virology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115.

*To whom correspondence should be addressed.
† Present address: Department of Biochemistry, Kosin Medical School, Pusan, Korea.