The Therapeutic Potential for Catalytic Antibodies: From a Concept to a Promise

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Abstract: More than ten years have now elapsed since the first reports confirmed that antibodies not only label antigenic targets but can also perform catalytic functions. Much of the initial research in this area focussed on exploring the scope and utility of these biocatalysts both as enzyme mimics and as programmable protein catalysts. However, their potential in the biomedical field has also been probed. This review details the present perspective of catalytic antibodies as new tools for immunotherapy and specifically focuses on their application to prodrug activation and drug inactivation.

INTRODUCTION

The antibody is a remarkable adaptor molecule containing binding sites for both its antigen and an effector molecule that has evolved to bind a vast range of antigens. Binding alone may be sufficient to cause neutralization of certain antigens, such as viruses, tumor cell-surface markers and bacteria, but in most cases the antibody triggers the complement system and cell-mediated killing processes to assist the expulsion process. Recent years have seen tremendous strides toward both the generation of either recombinant human monoclonal antibodies [1,2] or humanized rodent antibodies [3,4] and their subsequent exploitation in immuno-pharmacotherapy [5-7]. This has culminated recently with the food and drug administration (FDA) approval of a calicheomycin-antibody immunotoxin conjugate for the treatment of certain leukemias. However, there may be both financial and medical disadvantages concerning the use of stoichiometric amounts of antibody *in vivo*. For example, the scale-up of antibody production coupled with the subsequent cost of the medical treatment may ultimately limit the scope and scale of immunotherapy. Additionally, the use of large amounts of immunoglobulin may be coupled with an increase in the number and potency of possible side effects due to antibody toxicity. Thus it is seductive to explore the development of antibody-based medical treatments that have a mode of action that is catalytic in nature.

Since the first reports by Lerner [8] and Schultz [9] that antibodies could not only label antigens but also be programmed to perform 'enzyme-like' biocatalytic processes the therapeutic potential of 'catalytic antibodies' has been a source of considerable speculation [10]. For example, antibodies that have the ability to recognise foreign antigens *in vivo*, such as in HIV infection or septicaemia, and destroy them could revolutionise the field of immunology. Conversely, antibodies that can perform the function of the complement system and phagocytes offer exciting possibilities in effector disease states where the immune system of the body is destructive, for example in autoimmune disorders or after organ transplant. In such cases, catalytic antibodies could be targeted to the body's own immune system components and destroy them, thus causing an amelioration of the disorder.

Before focussing on specific examples however, it is informative to have a cursory understanding of how antibody catalysts are isolated from the incredible diversity of the immune system. By far the most successful approach has involved an immunization protocol that utilizes a hapten that is a transition state analog (TSA) for the reaction in question [11,12]. In theory, antibodies that bind the TSA should stabilize, and hence lower, the energy of the transition state for the targeted reaction and be catalysts, (Fig. **1**) [13].

The TSA is conjugated to a carrier protein to ensure it will stimulate antibody production *in vivo* [14]. Typically the antibodies are then prepared in one of three main forms: polyclonal, monoclonal, or component fragments. Polyclonal antibodies are a mixture of the whole immune repertoire after hyperimmunization, with no attempt being made to purify the individual antibody clones that have specific affinity for the hapten. The benefits of this process are its time and cost efficiency. The problem however, is that accurate characterization of the specific antibody clones responsible for catalysis from within this heterogeneous mixture is very difficult. Furthermore, the drug administration agencies are unlikely to give approval to an antibody-based therapy where the sequence and structure of the core antibody component is unknown. For these reasons production of monoclonal antibodies, the individual members of the immune repertoire, in pure form is the standard approach exploited. This is achieved experimentally by hybridoma technology [15,16], which involves fusing the antibody-producing cells (B-cells) with a mouse cancer cell-line. The immortalized cell line thus formed secretes the monoclonal antibody programmed by the B-cell. The antibody preparations are homogeneous, can be produced in

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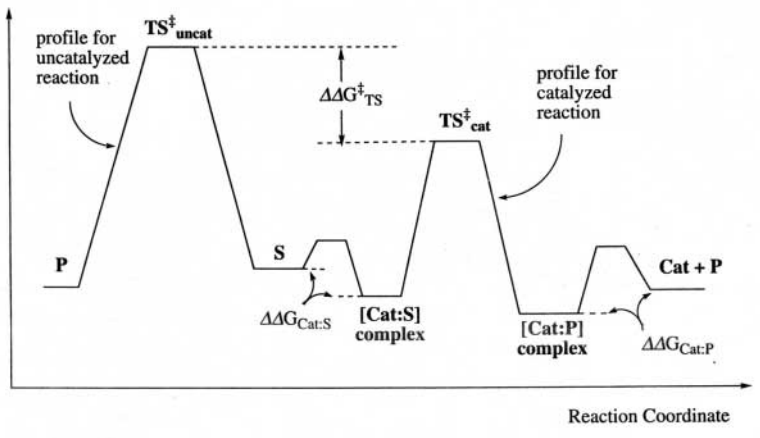


Fig. (1). Catalysis is achieved by reducing the free energy of activation of a process. A catalyst must bind the transition state more tightly than either the substrate or products: i.e. $\Delta\Delta G^{\ddagger} >> \Delta\Delta G_{Cat:S}$ and $\Delta\Delta G_{Cat:P}$.

large amounts, and can be rigorously purified to remove any potential enzymatic contaminant.

Antibody component fragments such as the Fab (antigen binding fragment) or scFv (single-chain variable fragment), are typically generated as combinatorial libraries and are expressed on phage-particals [17-19]. The major benefit of this approach is that, in principle, the time-consuming hyperimmunization protocol common to the methods *vide supra* can be side-stepped. A naive antibody library is simply tested for binding to the TSA. In practice however, to optimize the chances of success, the method includes an initial hyperimmunization protocol with the hapten that is followed by acquisition of the mRNA of the B-cells (obtained from the spleen). The combinatorial antibody library thus generated is a so-called focussed library, being biased toward antibody fragments with recognition for the hapten.

Whichever method is utilized, once a purified antibody sample is obtained initial screening is based on hapten recognition, measured by an enzyme-linked immunosorbent assay (ELISA) [20], followed by a screen to highlight catalysis of the target reaction.

Considerable progress has already been made in the application of antibody catalysts to a number of biomedically relevant areas. This review focuses on two such aspects: prodrug activation and drug inactivation.

PRODRUG ACTIVATION

The inherent short plasma half-lives and associated peripheral and non-specific toxicity of reactive therapeutics, particularly in the area of cancer and viral chemotherapies, is a serious limitation to their application and results in

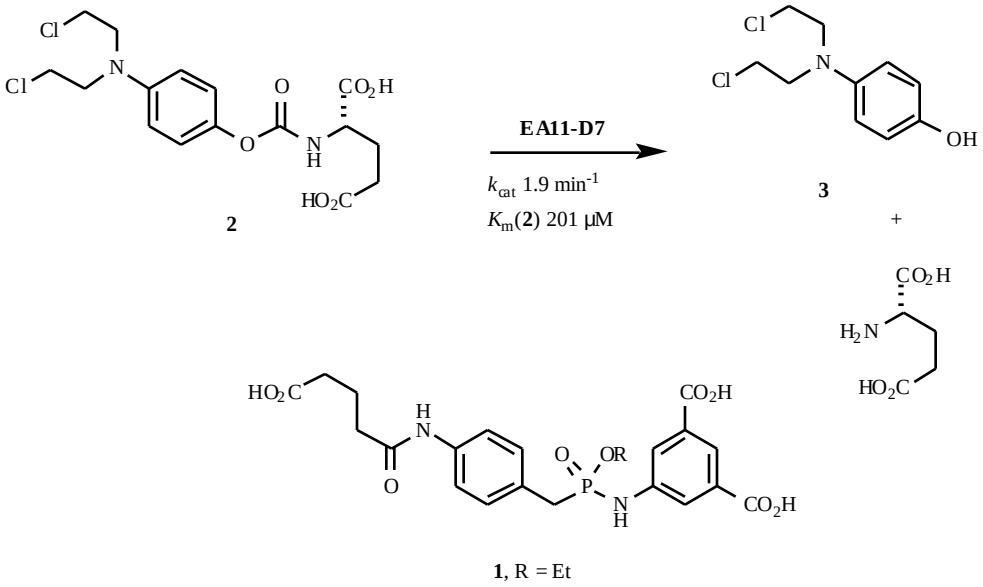


Fig. (2). Phosphonamidate ester **1** was utilized as a TSA hapten to elicit antibodies that could catalyze the hydrolysis of prodrug **2** to unmask the phenolic *N*-mustard **3**.

numerous undesirable side effects. Therefore, there are intensive efforts underway to improve the pharmacodynamic and toxicological profiles of such agents. One such approach, antibody-directed enzyme prodrug therapy (ADEPT), is a two component strategy that comprises of an antibody-enzyme conjugate and an anticancer prodrug [21-23]. The antibody binds to a tumor-associated antigen and thus shuttles the enzyme to the tumor surface. Following localization of the antibody-enzyme conjugate at the tumor site, a non-toxic prodrug of an anticancer agent is administered. The enzyme then, in principal, hydrolyzes (or converts) the inactive prodrug into its active form at the tumor surface. The benefits of the ADEPT approach compared to standard chemotherapy practices are reduced peripheral toxicity and improved efficacy of the therapeutic agent. This therapy can be limited however by the immunogenicity of the enzyme, usually of non-human origin, which triggers the destruction of the ADEPT conjugate, ironically, by the host's immune system. As a result, prodrug activation by a 'humanizable' catalytic antibody has been suggested as a viable solution to the problems associated with the enzyme component of ADEPT [24]. This has lead to the generation of a number of prodrug/antibody catalyst systems designed to improve the profile of a range of anticancer drugs.

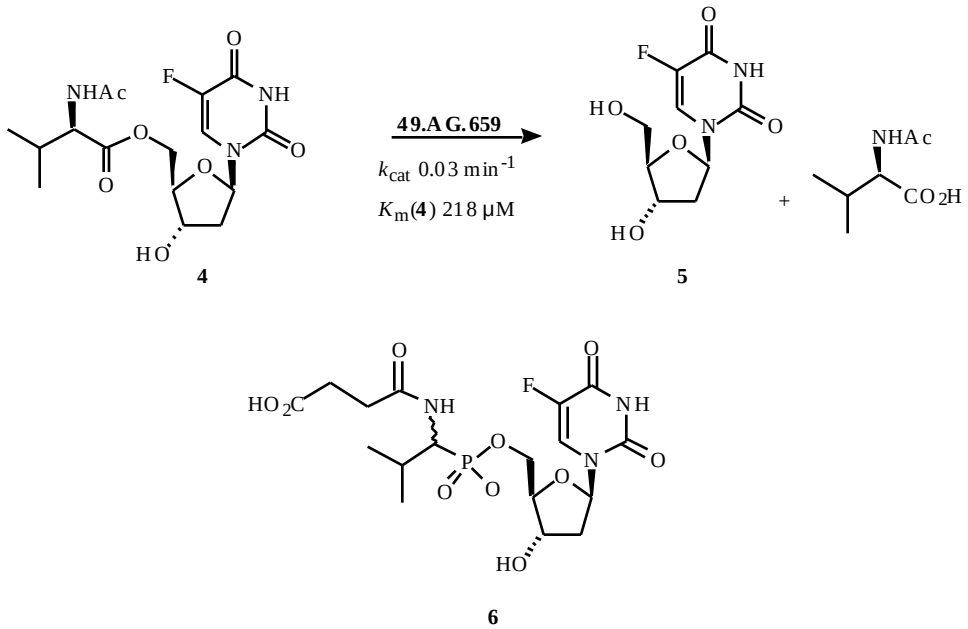
Blackburn and co-workers utilized the phosphonamidate immunogen **1** to elicit antibody catalysts that could unmask the L-glutamate carbamate ester prodrug **2** and reveal the nitrogen mustard **3**, (Fig. **2**) [24-26]. The design of hapten **1** included a number of key aspects: the antibodies generated had to be specific for L-Glu-containing carbamates to allow a direct comparison of antibody performance with that of the bacterial enzyme carboxypeptidase-G2 (presently exploited as an ADEPT component) [27,28]. Second, the locus of the nitrogen mustard in prodrug **2** was selected to define the position of the linker moiety in order to minimize prodrug alkylation of any catalysts. Third the phosphonamidate tetrahedral center was included to mimic the high-energy intermediate for a $B_{AC}2$ cleavage process.

A number of monoclonal antibodies that bound to **1** were generated. One monoclonal antibody, EA11-D7, catalyzed the efficient hydrolysis of prodrug **2**. This was investigated further with an *in vitro* cytotoxicity screen. Incubation of EA11-D7 (1 μM) and prodrug **2** (100 μM) lead to significant *in vitro* cell-kill of a human colorectal tumor cell line (LoVo), whereas incubation of **2** alone resulted in no cytotoxicity.

Despite the high selectivity for concentration of the ADEPT conjugate at the tumor site, the high ratio of circulatory volume to tumor volume means that a significant amount of the ADEPT conjugate remains in the circulation. Therefore the potential for peripheral prodrug activation is very real. The catalytic activity of EA11-D7 [catalytic rate constant (k_{cat}) = 1.9 min^{-1}; second-order specificity constant k_{cat}/Michaelis-Menten constant (K_m) = 156 $M^{-1}s^{-1}$] is significantly lower than the bacterial enzyme carboxypeptidase-G2 (k_{cat} = 32 s^{-1}; k_{cat}/K_m = 6.4 a 10^6 $M^{-1}s^{-1}$) [27,28]. However, low turnover may well be advantageous *in vivo* because this may both prolong the duration of drug delivery and increase the selectivity for tumor activation, *vide supra*.

In a similar approach, Schultz and co-workers produced an antibody, 49.AG.659.12, that catalyzes the hydrolysis of the 5'-D-valyl ester of prodrug **4** to release 5-fluorodeoxyuridine **5** a mechanism based inhibitor of thymidylate synthetase, (Fig. **3**) [29]. Phosphonate TSA **6** was utilized as the immunogen and 49.AG.659.12 catalyzes the hydrolysis of **4** with a k_{cat}/k_{uncat} = 968.

The free cytotoxic agent **5** completely inhibits the growth of *Eschericia coli* HB101 at a concentration of 20 μM, whereas prodrug **4** does not affect the growth of bacteria at a



4 **5** **6**

Fig. (3). Phosphonate ester **6** was utilized as a TSA hapten to elicit antibodies that could catalyze the hydrolysis of the 5'-D-valyl ester of prodrug **4** to release 5-fluorodeoxyuridine **5**.

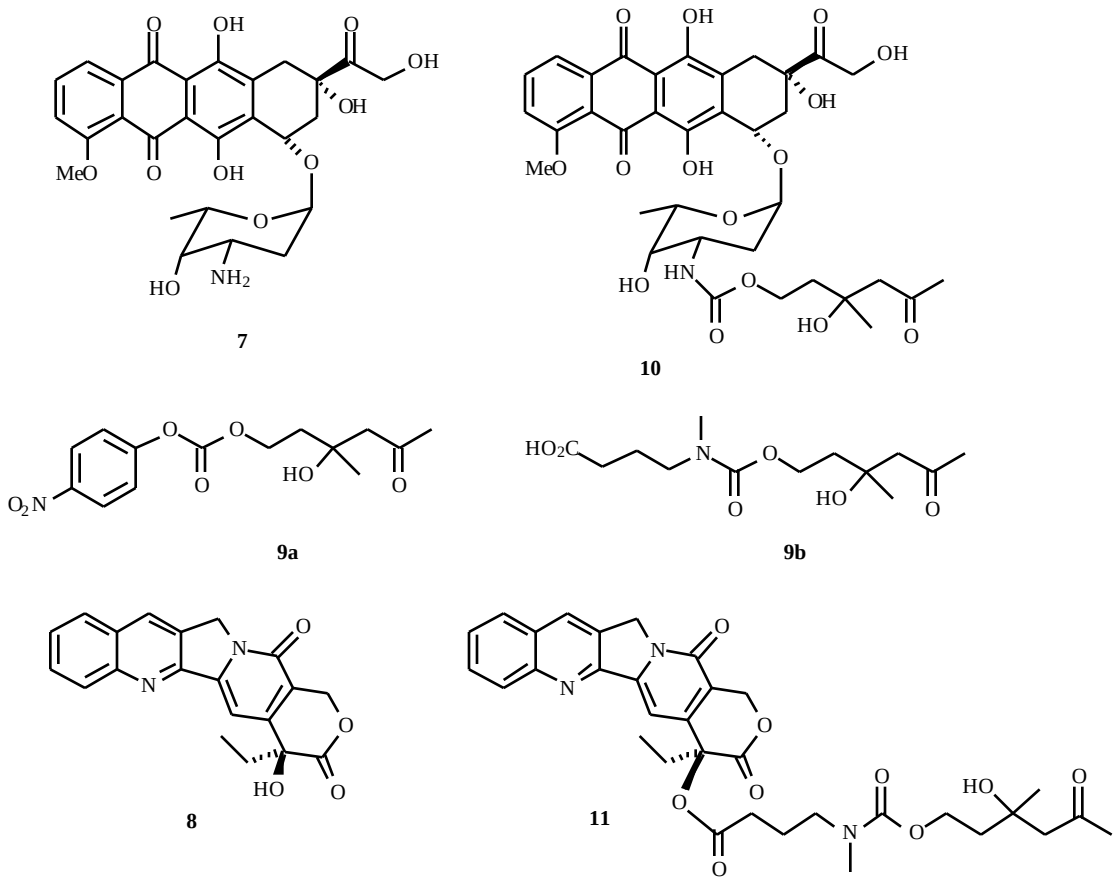


Fig. (4). Topoisomerase inhibitors doxorubicin **7** and camptothecin **8** were converted into inactive prodrugs **10** and **11** respectively. The linkers **9a** and **9b** install a tertiary aldol adduct which is the primary locus for attack by the 38C2 antibody, which catalyzes a *retro*-aldol *retro*-Michael cascade with **10** and **11** to unmask **7** and **8**.

concentration of 400 μM. This result confirmed that both esterification at the 5'hydroxyl of **5** is a key locus for drug inactivation and the D-valyl ester of **4** is not hydrolyzed by endogenous enzymes. Subsequent incubation of **4** (400 μM) and 49.AG.659.12 (20 μM) leads to complete inhibition of of *E. coli* HB101 growth, showing the effective activation of the prodrug in this system.

Conceptually the catalytic antibody component of the ADEPT conjugate can be programmed, by its hapten, to be either very specific or non-specific for its prodrug substrate. The relative merit of a highly-specific catalyst *in vivo* is that the risk of hydrolysis, cleavage or destruction of key chemical mediators and/or cellular components that possess structural homology with its substrate are slight. In the examples *vide supra*, EA11-D7 and 49.AG.659.12 were designed to be very specific catalysts. A potential detraction of this approach however, is that any significant change within the prodrug structure will render it a non-substrate and thus require the generation of a new antibody-catalyst. In an attempt to produce a generic antibody-prodrug trigger system that has a broad substrate tolerance Lerner, Barbas and co-workers utilized the 38C2 antibody that catalyzes aldol and retroaldol reactions with a wide range of donor and acceptor aldehydes and ketones [30].

Doxorubicin **7** and camptothecin **8**, topoisomerase I and II inhibitors, were exploited as the cytotoxic agents, (Fig. **4**). Each has previously been used in ADEPT systems since their use as chemotherapeutic agents is limited by non-specific toxicity [31].

The key to the generic approach was the construction of the linkers **9a-b**, which facilitate attachment to a wide range of drugs *via* either amino, hydroxyl or thiol residues. The resultant prodrugs are unmasked in a cascade that incorporates a sequential retro-aldol retro-Michael process [32]. The prodrugs, prepared as tertiary aldol adducts **10** and **11**, should have high *in vivo* stability because no known enzyme can catalyze this particular retro-aldol reaction. However, the carboxyl ester linkage of **11**, by which camptothecin **8** and linker **9b** are adjoined, may be susceptible to hydrolysis *via* plasma esterases.

Antibody 38C2 catalyzes both the retro-aldol reaction **10** and the retro-Michael reaction of the ketone intermediate **12**, resulting in the overall release of doxorubicin **7**, (Fig. **5**). Catalysis follows Michaelis-Menten kinetics with an enhancement ratio (k_{cat}/k_{uncat}) of greater than 10^5. Similarly, camptothecin **8** is released from its prodrug **11** *via* the same reaction sequence, resulting in formation of amine **13** that undergoes a spontaneous lactamization.

38C2 exhibits long-lived activity *in vivo* and efficiently unmasks the designed prodrugs **10** and **11**. Moreover, the release of doxorubicin **7** and camptothecin **8** by 38C2 is

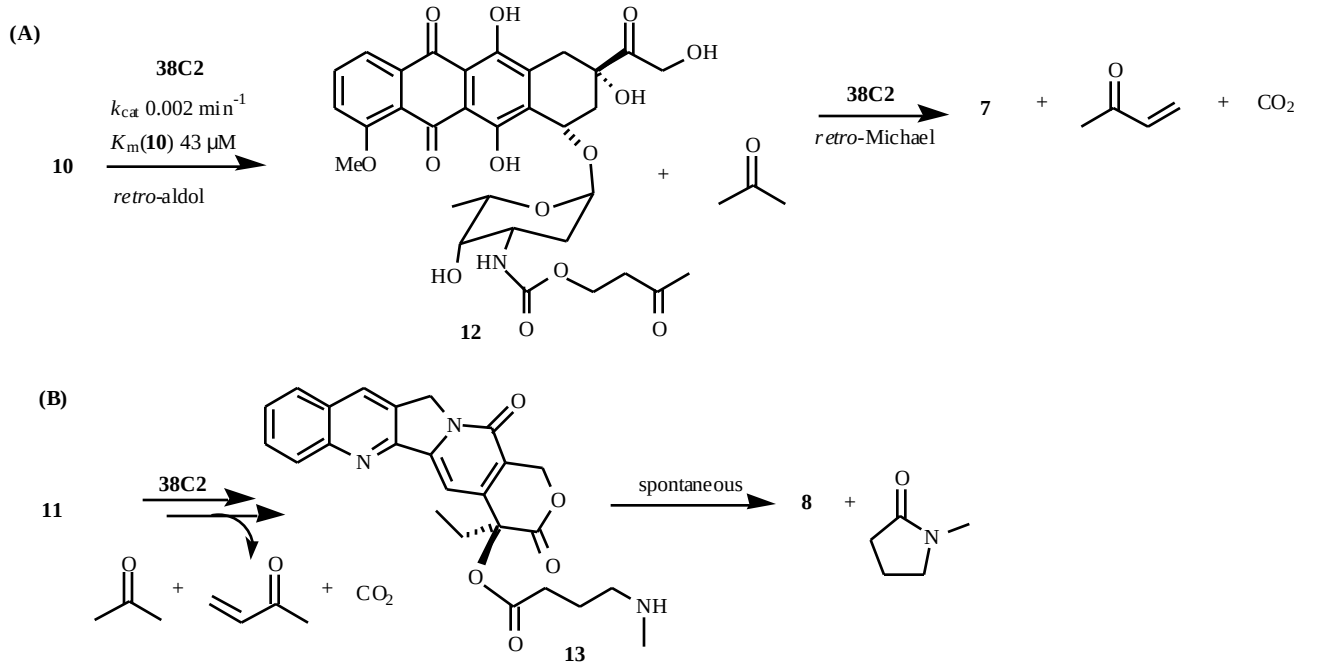


Fig. (5). (A) Cascade reaction with the doxorubicin prodrug **10** catalyzed by the 38C2 antibody, which proceeds *via* ketone intermediate **12**. **(B)** Cascade reaction with the camptothecin prodrug **11** catalyzed by the 38C2 antibody, which proceeds to the amine product **13**, which liberates the active drug **8** *via* a spontaneous lactamization reaction.

efficacious when applied to the inhibition of human prostate and colon cancer cell growth *in vitro*.

An attractive prospect for antibody catalysts is to control key chemical mediator concentration *in vivo*. This has been exemplified by Reymond, Lerner and co-workers who elicited antibody 9D9 that catalyses the retro Diels-Alder reaction of the anthracene-HNO cycloadduct **14** (k_{cat}/k_{uncat} = 233), (Fig. **6**) [33]. In aqueous media the acridinium hapten **15** was found to be in equilibrium with its conjugate Lewis base 9-hydroacridane **16**. This is thought to be a more faithful mimic of the transition state **17** for the reaction, in terms of the change in dihedral angle (α) between the two phenyl rings in reactant and product.

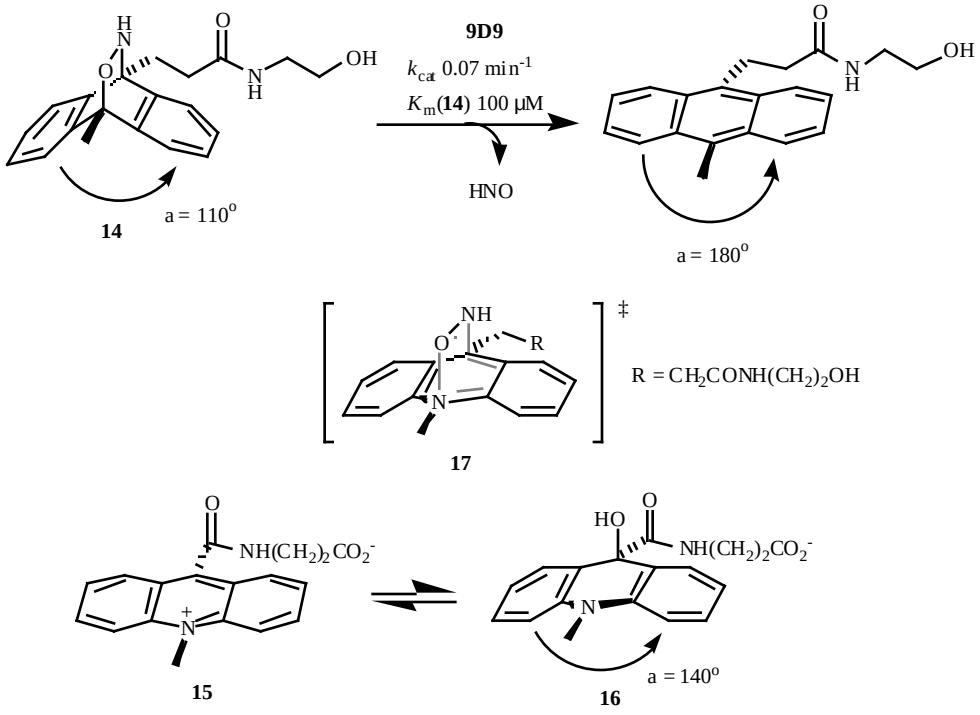


Fig. (6). An equilibrium mixture of acridinium **15** and its 9-hydroacridane **16** was used to elicit antibodies that would catalyze the retro Diels-Alder reaction of **14** to release nitroxyl. Acridane **16** is a more faithful structural mimic of the transition state **17** for the reaction based on the angle (α) between the two pendant aromatic rings.

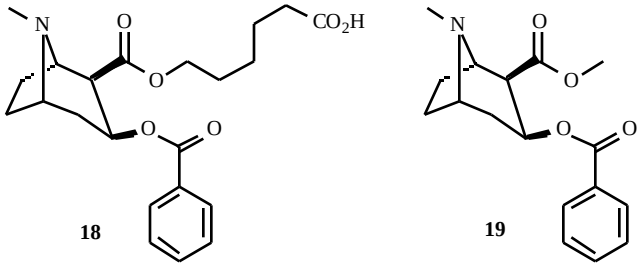


Fig. (7). Cocaine analog **18** was successfully used as its KLH conjugate in an active immunization protocol in rats to blunt the psychotropic effects of the tropane alkaloid cocaine **19**.

This system has medical relevance in that nitroxyl, released during the antibody catalyzed reaction, can be converted into nitric oxide (NO) by superoxide dismutase (SOD), a ubiquitous *in vivo* enzyme. Nitric oxide is a key regulator of numerous biological processes including vasodilation, neurotransmission and cytotoxicity [34]. NO-releasing drugs such as nitroglycerine, suffer from rapid decomposition and therefore require high doses. A nitroxyl synthase catalytic antibody-SOD conjugate with the nitroxyl produg **14** may circumvent this problem. Another attractive feature of this system is that no known enzyme can catalyze the retro Diels-Alder reaction, thus allowing for site-specific NO release, which may also be used to help probe the exact biochemical effects of nitric oxide.

DRUG INACTIVATION

A major problem associated with existing antibody-mediated small-molecule inactivation is *in vivo* depletion of circulating antibody *via* complex formation. For example, a study with a morphine immunized rhesus monkey showed that antibodies were effective at blocking the effects of small doses of heroin on the CNS [35]. However, at repetitive higher doses of heroin there was significant antibody depletion causing the blockade to self-administration to be overcome. It seems therefore that catalytic antibodies may be ideally suited as tools for clearance of small-molecules, such as drugs etc., from the plasma because they do not form long-lived complexes and hence are not cleared from the blood stream when they encounter their target.

An antibody-based approach to the medical treatment of cocaine dependence has recently attracted intense interest in the scientific community and the media. Cocaine, a naturally occurring tropane alkaloid, was widely used in medicine during the 19th century as an antidepressant and topical anaesthetic and its more recent abuse as a euphoric has reached near epidemic proportions in some countries. The addictive properties and reinforcing effects of cocaine abuse are attributed to its ability to block dopamine reuptake by the dopamine transporter in mesolimbocortical systems, resulting in activation of dopaminergic pathways. Various medical treatments for cocaine dependence have been investigated and are focused on modifying dopamine function. However, the risk of modifying *in vivo* dopamine levels on biological functions such as locomotion and motivation mean that alternative approaches are being sought [36].

Janda and co-workers recently reported a successful application of active immunization as a means of suppressing the psychoactive effects of cocaine in rats [37]. The cocaine analogue **18** was coupled to keyhole limpet hemocyanin and the resulting conjugate used for rat vaccinations, (Fig. **7**). The active immunization protocol was successful at suppressing the stereotypical behavior induced by cocaine **19** in rats with concomitant levels of cocaine in cerebral tissue being reduced. More importantly, cocaine self-administration reinstatement in rats was inhibited [38]. This vaccination approach is now being implemented as an alternative approach for cocaine abuse treatment.

Landry and co-workers are concentrating on generating monoclonal catalytic antibodies that hydrolyze cocaine [39].

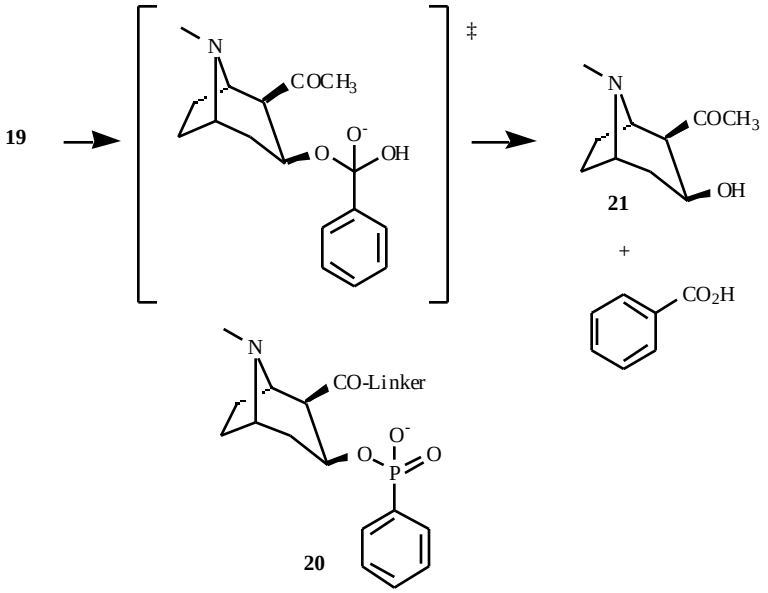


Fig. (8). Phosphonate ester **20** was used as a TSA hapten to elicit antibodies that would hydrolyze the benzoyl ester of cocaine **19** to generate non-psychotropic products **21** and benzoic acid.

The therapeutic application is different to the vaccination approach above, in that the antibody preparation will be administered only as required. However, the aim is to generate a new treatment for blunting reinforcement of the drug *in vivo*. Phosphonate monoester **20** was used as a mimic of the transition state for the hydrolysis of the benzoyl ester functionality of cocaine **19**, (Fig. **8**). This acyl transfer reaction generates ecognine methyl ester **21** and benzoic acid, both of which exhibit none of the stimulating or reinforcing activity of cocaine. Hapten **20** elicited a number of antibodies that catalyze the hydrolysis of cocaine [39]. One monoclonal antibody, 15H10, exhibits a rate enhancement of 2.3×10^4 over the background hydrolysis, an activity considered sufficient to begin pre-clinical trials [40].

CONCLUSIONS

Although the exploitation of catalytic antibodies in the therapeutic sphere is still in its infancy, the potential is quite clear. Being able to design and target a catalyst, that remains invisible to the host's immune system, to either an *in vivo* macromolecule or small-molecule target will undoubtedly stimulate a renaissance in chemotherapy. However, before such lofty aims are realized, there are fundamental issues that researchers in the field are seeking to address. Primarily these include both expanding the scope of chemical transformations that antibodies can catalyze and improving their kinetic profiles. While greater than sixty chemical reactions have been catalyzed by antibodies [11,12,41], this is still a small percentage of the plethora of accessible transformations. Furthermore, difficult reactions that are performed routinely *in vivo*, such as amide bond hydrolysis (and formation) and glycosyl transfer have proven difficult to mimic efficiently by antibodies. In addition, at present there are only a few examples of antibodies catalyzing reactions for which there are no known biocatalysts and it is arguably this ability that offers the greatest immediate potential for their exploitation *in vivo*. However, it is worth reiterating that the catalytic antibody research commenced only 14 years ago and has made tremendous advances in that time. The above stated boundaries of this exciting and challenging field are without doubt going to be pushed back rapidly and within the next decade the potential of catalytic antibodies in immunotherapy will become more clearly defined.

LIST OF ABBREVIATIONS

FDA	=	Food and drug administration
TSA	=	Transition state analog
HIV	=	Human immunodeficiency virus
Fab	=	Antigen binding fragment
ScFv	=	Single chain variable fragment
mRNA	=	Messenger ribonucleic acid
ELISA	=	Enzyme-linked immunosorbent assay
ADEPT	=	Antibody-directed enzyme prodrug therapy
L-Glu	=	L-glutamic acid
k_{cat}	=	Catalytic rate constant
K_m	=	Michaelis-Menten constant
SOD	=	Superoxide dismutase
CNS	=	Central nervous system

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