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Antibody-catalyzed activation of a model tripartate prodrug by a tandem hydrolysis–1,6-elimination reaction†

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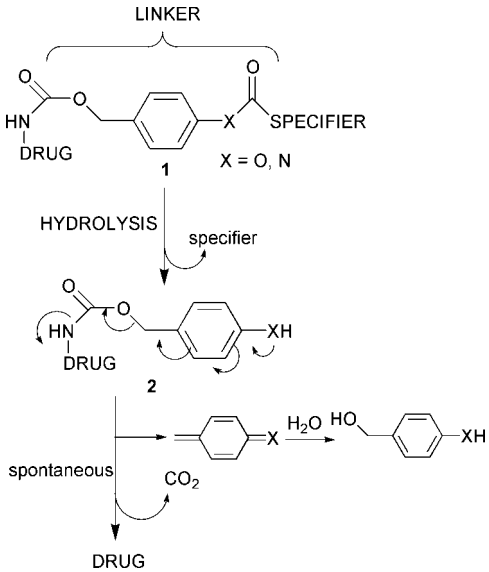
Activation of a model tripartate prodrug was achieved by abzyme-catalyzed hydrolysis of an *N*-methylcarbamate moiety followed by a spontaneous 1,6-elimination reaction.

Researchers have long been searching for ways of delivering drugs specifically to cancer cells. One such tactic is known as antibody-directed enzyme prodrug therapy (ADEPT).[1] In this approach, an enzyme–antibody conjugate is used to target an enzyme to the surface of tumour cells. An inactive prodrug is administered which is converted into the drug only by the tumour-bound enzyme. This results in a high concentration of the drug in the vicinity of the cancer cell while minimizing its presence at healthy cells. One of the key requirements for a clinically useful ADEPT system is the absence of an equivalent endogenous enzyme in humans. Consequently, the enzyme component has commonly been of non-human origin. However, the immunogenicity of the non-human enzymes has severely limited the clinical potential of ADEPT. To overcome this problem, researchers have suggested replacing the enzyme component with a catalytic antibody (antibody-directed abzyme prodrug therapy—ADAPT).[2a–d] Abzymes can be designed to act upon substrates that are not readily acted upon by human enzymes. In addition, antibodies can be humanized thus reducing the serious problem of immunogenicity.[3]

A number of abzymes have been developed that convert prodrugs into drugs.[2a–d] In most instances, the transition state analogues (TSA's) to which these abzymes were raised contained the drug or a structural analogue of the drug.[2a–c] Such abzymes were designed to activate bipartate prodrugs in which the moiety acted upon by the abzyme (known as the specifier[4]) is directly attached to the drug. An alternative to using bipartate prodrugs is to use tripartate prodrugs. In tripartate prodrugs, the specifier is separated from the drug by a linker. After cleavage of the specifier from the linker, the linker–drug product is designed to spontaneously fragment to release the drug. An example of a tripartate prodrug system is outlined in Scheme 1. Here, specific hydrolysis of the moiety separating the specifier and linker in **1** releases unstable intermediate **2** which undergoes a spontaneous 1,6-elimination reaction to release the drug. This prodrug design was first described by Katzenellenbogen and coworkers.[4] Prodrugs based upon this general design have been used extensively in ADEPT and for other applications.

Several years ago, we suggested that abzymes that are designed to activate tripartate prodrugs might be more versatile for ADAPT than those that act upon bipartate prodrugs.[5] If an abzyme could be designed to recognize only the specifier and linker of a tripartate prodrug, then a single abzyme could be used to activate a variety of different prodrugs.[5] This would be advantageous both in terms of economy and for dealing with tumours that have developed resistance to a certain drug. We reasoned that this could be accomplished by raising antibodies



Scheme 1

to TSA's in which the drug was *not* incorporated into the TSA.[5] Instead, the carrier protein (necessary for antibody production) could be attached to a position on the TSA that is equivalent to the point of attachment of the drug to the prodrug. Since abzymes are usually insensitive to changes in the region of the substrate that corresponds to the part of the TSA that is attached to the carrier protein,[6] then abzymes raised to such TSA's might be capable of acting as generic activators of tripartate prodrugs.

To test this approach we constructed the model tripartate prodrug **3**† (Scheme 2). In this system, drug release is triggered by abzyme-catalyzed hydrolysis of the *N*-methylcarbamate moiety to release *p*-nitrophenol (PNP, the specifier), CO_2 and intermediate **4**. Intermediate **4** then undergoes the spontaneous 1,6-elimination reaction to produce the amino acid L-tryptophan (Trp) as the model 'drug'.[7] Abzymes were raised to TSA-hapten **6**,[8] which mimics the rate-determining transition state for the hydrolysis of the *N*-methylcarbamate moiety in **3**. We recently reported that an antibody raised to **6**, called ST51, is capable of catalyzing the hydrolysis of *N*-methylcarbamate **7**.[8] Since the carrier protein in **6** was attached to a position on the TSA that corresponds to the point of attachment of tryptophan to **3**, we reasoned that ST51 should be capable of activating model prodrug **3**.

Model prodrug **3** was examined as a substrate for ST51 by removing aliquots from solutions containing ST51 (5 μM) and varying amounts of **3**, in buffer at pH 9.0 (100 mM bicine, 100 mM NaCl, 5% DMSO, 25 °C), at various time intervals, and then examining the aliquots for production of both PNP and Trp by HPLC. Under these conditions, we were unable to detect any PNP or Trp in the absence of ST51 after 40 hours. However, in the presence of ST51, both of these products were readily detectable. The antibody-catalyzed reaction obeys saturation

† Electronic supplementary information (ESI) available: the synthesis and characterization of **3**, and **9**–**11**, and experimental details for kinetic studies and Lineweaver–Burk plots. See http://www.rsc.org/suppdata/cc/b1/b103971g/

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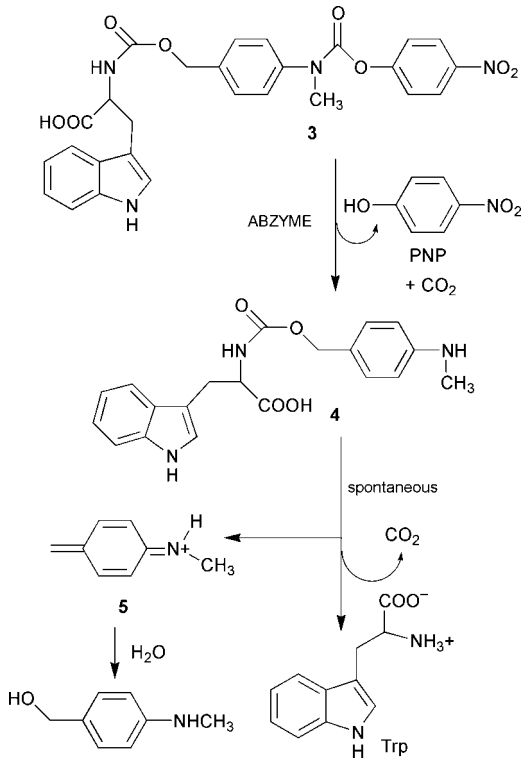
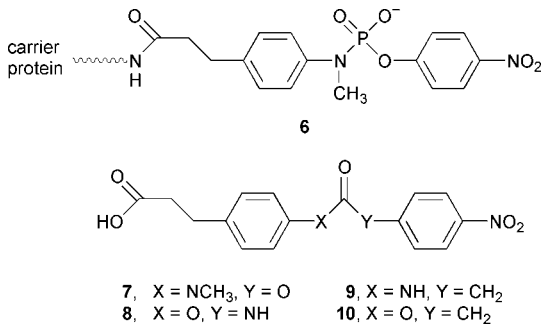


Scheme 2



kinetics, and approximately equimolar quantities of PNP and Trp were detected for the duration of the time the reactions were monitored. When following the production of PNP, ST51 exhibited a k_{cat} = 0.075 h^{-1} and a K_m = 137 μM. Similar values were obtained from data following the production of Trp. We also found that ST51 was unable to catalyze the hydrolysis of Z-Trp. Taken together, these results indicate that the production of Trp is not a result of hydrolysis of the *N*-H carbamate moiety, but rather initial hydrolysis of the *N*-methylcarbamate followed by the spontaneous fragmentation reaction and that the abzyme-catalyzed step is slow compared to the fragmentation reaction. We also found that ST51 was capable of catalyzing the activation of **3** with multiple turnover. This indicates that **5**, which is produced as an intermediate during the reaction, does not inactivate the abzyme after a single turnover by reacting with crucial residues in the active site.

Assuming that the spontaneous rate of hydrolysis of the *N*-methylcarbamate moiety in **3** is similar to that of **7** ($t_{\frac{1}{2}}$ = 5.7 years in 100 mM bicine, 100 mM NaCl, 5% DMSO, pH 9.0) then the rate enhancement (k_{cat}/k_{uncat}) with **3** is about 5000-fold. This is only slightly less than the rate enhancement found for ST51 using substrate **7** (6500-fold).[8] It is also important to note that **3** and **7** exhibit similar K_m values (266 μM for **7** and 137 μM for **3**). These results indicate that the 'drug' portion of the prodrug is not an important recognition site for the antibody-catalyzed reaction and this is consistent with our hapten design.

In summary, we have reported the first example of antibody-catalyzed activation of a model tripartate prodrug of type **1** by a tandem hydrolysis–1,6-elimination reaction.[9] We have demonstrated that it is possible to obtain an abzyme that can activate a tripartate system without incorporating the 'drug' into the TSA by attaching the carrier protein to a position on the TSA that corresponded to the point of attachment of the 'drug' to the tripartate prodrug. Although ST51 can catalyze the hydrolysis of *N*-methylcarbamates at physiological pH, the rate of the reaction is too slow for an accurate kinetic analysis and effective prodrug activation. It has been estimated that for ADEPT systems, a k_{cat} value of about 1.0 s^{-1} is required.[2c] Nevertheless, the approach outlined here should be readily applicable to tripartate prodrugs of type **1** bearing moieties that are more amenable to antibody–catalyzed hydrolysis, under physiological conditions, than *N*-methylcarbamates, which are highly challenging substrates for antibody catalysis.[8] An *N*-H carbamate linkage between the specifier and linker, of the type recently exploited by Blackburn and coworkers for abzyme catalysis,[2c] should be very suitable to the approach reported here.[11] However, tripartate prodrugs of type **1** bearing such a moiety cannot be used as substrates for ST51 since ST51 will not hydrolyze *N*-H carbamate **8**, amide **9**, or even ester **10**. Studies to elucidate the mechanism of ST51 are in progress and we are continuing our work to develop abzymes that can trigger tripartate prodrugs of type **1** under physiological conditions.

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7 Since the primary objective of these studies was to determine if ST51 could trigger the tripartate system, it was not necessary to use an actual drug to make the model 'prodrug'. Indeed, for these studies any amine-bearing molecule would have sufficed for the 'drug' so long as it (1) had no resemblance to the specifier (PNP) portion of the prodrug, (2) was relatively easy to detect and quantitate, (3) had a charged moiety that would enhance the solubility of the prodrug and, (4) was readily available and allowed for economic and facile synthesis of the model prodrug. Tryptophan was chosen since it satisfied all of these requirements.

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9 While our work was in progress, Shabat *et al*. reported abzyme-catalyzed activation of tripartate prodrugs by a tandem retro-aldol–retro-Michael reaction (see ref. 2*d*). Although the abzyme was not originally designed to act as a prodrug activator (see also ref. 10), generic tripartate prodrug activation was achievable because of the broad substrate specificity of the abzyme.

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