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The medical potential of catalytic antibodies

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Abstract: Catalytic antibodies are relatively slow catalysts with turnover numbers some 106 less than is common for enzymes. However, they have the advantage of high affinity for a pre-selected substrate and the ability to carry out a pre-determined chemical transformation with an efficiency that is adequate for medical application. In a feasibility study, we have chosen to investigate antibody catalysis of carbamate ester cleavage and apply it to achieve cell-kill in a system that is a paradigm for ADAPT: Antibody Directed Abzyme Prodrug Therapy.

We have differentiated the two alternative pathways for hydrolysis of an aryl carbamate ester by the synthesis of a tetrahedral phosphonamidate ester transition state analogue for the disfavoured BAc2 pathway and its use as a hapten to generate antibody catalysts. Such abzymes can lower the activation energy of the BAC2 pathway relative to that for the normal ElcB hydrolysis. Of the antibodies thus elicited, DF8-D5 proved to be the best catalyst, showing good Michaelis–Menten kinetics and strong inhibition by the hapten. Hammett analysis with a range of substrates gave $\rho = +0.53$ for the DF8-D5 hydrolysis and $\rho = 2.63$ for the hydroxide mediated reaction of a range of *p*-substituted carbamates, which confirms the mechanistic switch. When a carbamate ester of a phenolic mustard is used as substrate, a cognate antibody EA11-D7 can cause cell kill of human colorectal carcinoma cells in tissue culture as a result of the same catalytic cleavage process to release a cytotoxic phenolic mustard.

INTRODUCTION

Antibodies are mammalian proteins whose function in immune defence is to bind strongly to alien materials (antigens). The concept that selected antibodies may have the ability to act as biocatalysts has been expressed in terms of transition state theory. How did that idea originate?

In 1946 Linus Pauling proposed (1) that enzymes achieve catalysis through being tuned to complement and thereby stabilise the high energy transition state of a reaction; their binding to reactants and products should be much weaker (Figure 1). Bill Jencks built on that platform in 1969 by advocating the possibility that one can "synthesise an enzyme" by employing antibodies. He laid the foundation for the whole field of catalytic antibodies when he said (2):

"One way to do this is to prepare an antibody to a haptenic group which resembles the transition state of a given reaction."

The arrival of this possibility was delayed for 18 years, mainly because of technical problems associated with isolation and purification of single-species proteins from the immune repertoire. Fortunately, these were solved in 1976 by Köhler and Milstein's invention of hybridoma

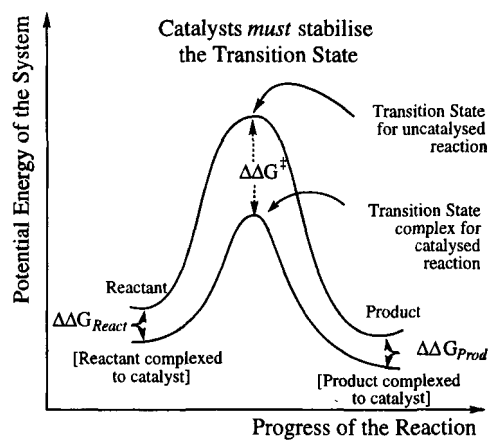


Fig.1 Energy profile of a general reaction. Catalysis is achieved by lowering the free energy of activation for a process, *i.e.* a catalyst must bind more strongly to the transition state ($TS^{\ddagger}$) of the reaction than to either reactants or products.

technology, which today has made it possible both to screen rapidly the 'complete' immune repertoire and to produce relatively large amounts of a chosen monoclonal antibody *in vitro* (3).

In 1986, Richard Lerner and Peter Schultz independently reported antibody catalysis of the hydrolysis of aryl esters and carbonates respectively (4,5). Such reactions involve an unstable tetrahedral intermediate, which is closely related to the transition state of the reaction. This sort of transition state (TS‡) has been mimicked effectively by a range of stable analogues (TSAs), including phosphonate esters, α-difluoroketones, and hydroxymethylene functional groups (6,7). Lerner's group elicited antibodies to a tetrahedral anionic phosphonate hapten [**1**] whilst Schultz's group isolated a protein which had a high affinity for *p*-nitrophenyl choline phosphate [**3**] (Figure 2).

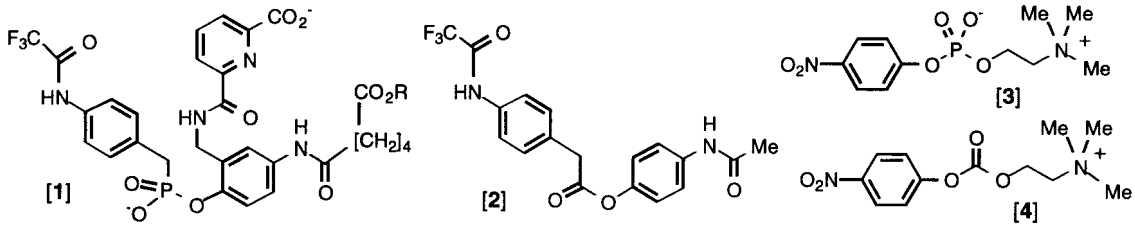


Fig.2 Lerner's group used phosphonate [**1**] as the hapten to raise an antibody which was capable of hydrolysing the ester [**2**] shown alongside it [**4**]. Schultz found (5) that naturally occurring antibodies using phosphate [**3**] as their antigen could hydrolyse the corresponding *p*-nitrophenyl choline carbonate [**4**].

This methodology, *i.e.* design of stable TS‡ analogues, has served as the bedrock of the field of abzyme research and has been the major route for the expansion of the subject, which has now led to over 70 species of catalytic antibody (8,9). New methods have been developed within the field and current advances use alternative strategies, notably through charge-charge complementarity as the basis for a '*bait and switch*' strategy (10-13) and *via* the control of entropy in the transition state. The latter is classically exemplified for enzyme catalysis by chorismate mutase, which catalyses the isomerisation of chorismic acid [**5**] into prephenic acid [**6**] with an acceleration of 3 x 10^6. A catalytic antibody generated using the '*entropic trap*' strategy (14-16) has achieved a rate enhancement of 10^4 and there is now an X-ray crystal structure (17) for the Fab' fragment of a second catalytic antibody (18) in binary complex with the same TSA (**7**). It shows that amino acid residues in active site of the antibody catalyst faithfully complement the components of the conformationally-ordered transition state analogue (Figure 3).

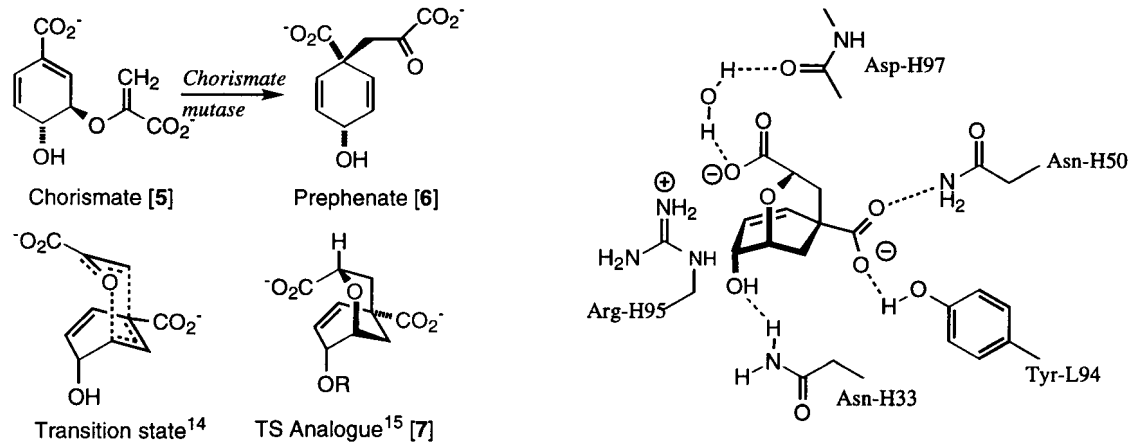


Fig.3 X-ray analysis[17] of a binary complex between the Fab' of abzyme 1F7 and TSA[15] shows hydrogen bonding (dashed lines) and electrostatic interactions of amino acid side chains in the binding site with TSA (**7**).

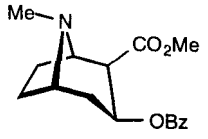
In abzyme catalysed reactions studied to date, which range from hydrolyses to carbon-carbon bond forming reactions, typical Michaelis constants (K_m) lie in the range 10 mM to 1 μM and binding selectivity for the TSA over the substrate (K_m/K_i) is in the range 10 to 10^5 fold. It therefore appears that antibodies have fulfilled expectations that they would be capable of similar reactant discrimination as that shown by

enzymes but over a wider range of substrate types. The nature of reactions that may be catalysed by antibodies appears to be dictated only by a sufficient knowledge of the transition state for any given reaction and the synthetic accessibility of a stable TSA.

In contrast to enzymes, abzymes are able to accelerate reactions by rates that at best are only some 10^7 times that of the spontaneous process (19). It has to be said that scientists generally are looking for a major step forward in antibody catalysis to achieve rate accelerations in the range 10^7 to 10^9 that would establish abzymes as a feature of synthetically useful biotransformations. It would appear to the optimist that an improvement in abzyme performance of only one or two orders of magnitude is needed before they can be put to work in a bioreactor and expand production into the kilogram range (20).

Medical Potential of Abzymes

Two medical targets readily offer themselves to antibody applications. The first is in the clearance of toxic substances from the blood stream by specific abzyme hydrolysis. That is well illustrated by Landry's study (21) of the hydrolysis of the ester function of cocaine [**8**]. The second is the conversion of a prodrug into the active agent in a process that is designed to improve either its pharmacokinetic, pharmacological, or toxicological profile. At least three laboratories have worked on this application of antibody catalysts.



[8]

Antibody mediated prodrug activation was first exemplified by Fujii's group (22). He isolated an active clone, 6D9, capable of hydrolysis of the acyl-chloramphenicol prodrug [**9**] with a rate enhancement, k_{cat}/k_{uncat}, of 1.8 x 10^3. Fujii then demonstrated the possibility of prodrug therapy by achieving inhibition of growth of *Bacillus subtilis* cells which were inhibited by the ester ([**0**] *only* in the presence of the abzyme 6D9, which can effect hydrolysis of [**9**] to give chloramphenicol. More recently, Campbell and co-workers have reported (23) abzymes of similar activity for releasing an anticancer drug, 5-fluorodeoxyuridine, from D-valyl ester prodrug [**11**] using TSA [**12**] (Figure 4).

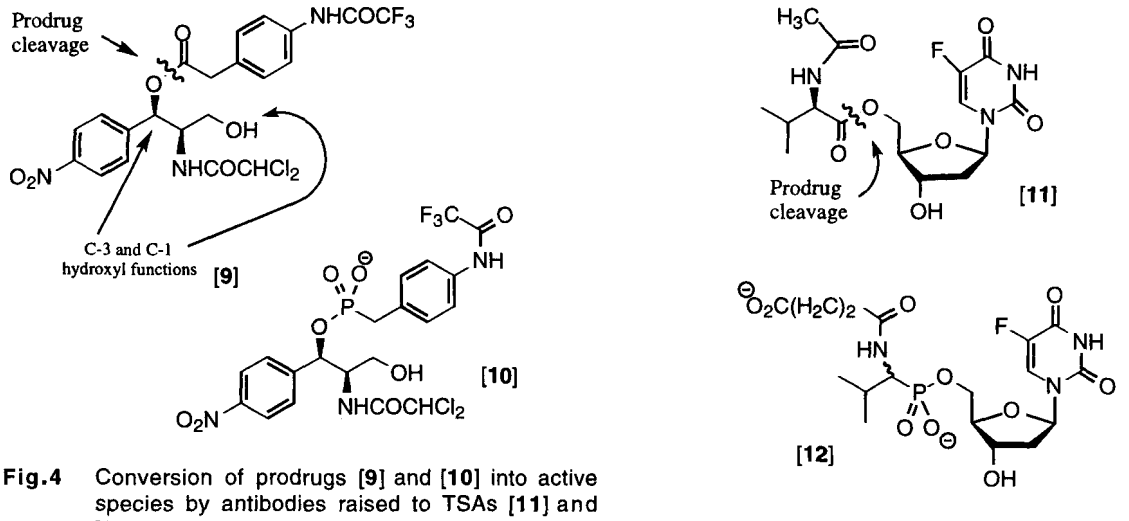


Fig.4 Conversion of prodrugs [**9**] and [**10**] into active species by antibodies raised to TSAs [**11**] and [**12**] respectively.

The choice of ester hydrolysis for abzyme prodrug-drug conversion is a happy one as there are many known examples, but it has drawbacks for *in vivo* application. The rate of spontaneous hydrolysis of esters is rather high and human serum contains a wide range of proteins that can effect such hydrolyses. We therefore selected carbamate cleavage as the Sheffield target for prodrug activation and chose to apply it to cancer chemotherapy through the ADEPT concept.

Antibody Directed Enzyme Prodrug Therapy (ADEPT) is a cancer therapeutic programme pioneered by Keith Bagshawe (24) in the 1980s. It builds on the discovery that tumours have specific antigens associated with their surface and that these are capable of eliciting an immune response. An effector function, such as

an enzyme, can be concentrated in the vicinity of the tumour by simply conjugating it to antibodies which recognise the antibody-specific surface epitopes (Figure 5). Administration of a cytotoxic agent in the form of a prodrug of greatly diminished activity reduces the adverse effects that arise from destruction of fast-dividing, healthy cells in the body.

If the effector function, typically an enzyme, can convert the prodrug into the parent cytotoxic agent at the locus of the tumour, then a high concentration of that drug will be delivered selectively to primary and secondary tumour cells.

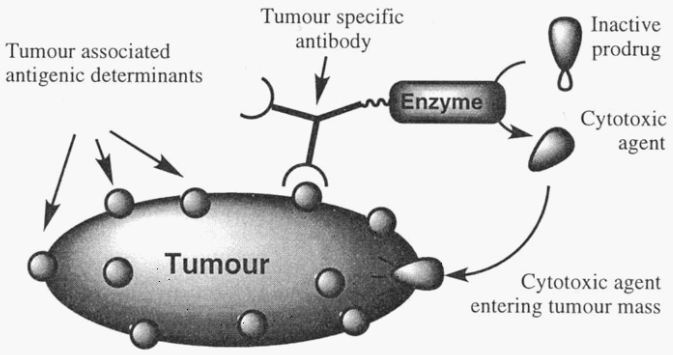


Fig.5 ADEPT: Mab-enzyme conjugates targeted at the tumour surfaces convert less toxic prodrugs into more active cytotoxic agents.

Problems have been encountered with this system because the enzyme component, usually of bacterial origin, is foreign to the mammalian host which brings into operation its immune system to try to neutralise the enzyme. The objective of our research is the production of a non-immunogenic biocatalyst, specifically a *humanisable* catalytic antibody, capable of activating a prodrug of choice to release a toxic, aryl nitrogen mustard agent. We have designated such a strategy ADAPT: **a**ntibody **d**irected **a**bzyme **p**rodrug **t**herapy.

FITTING MECHANISM TO APPLICATION

The abzyme catalysed breakdown of aryl carboxylate and carbonate esters had been demonstrated repeatedly by 1991 but, while this prior knowledge made such linkages suitable for incorporation into potential prodrugs, it seemed that serum lability might limit their use. Aryl carbamates constitute a class of acyl compound with stability intermediate between that of the corresponding esters and amides and whose mechanism of breakdown is well documented. They hydrolyse spontaneously by an elimination-addition process where proton abstraction triggers an intramolecular expulsion (E1cB) of the leaving group (25).

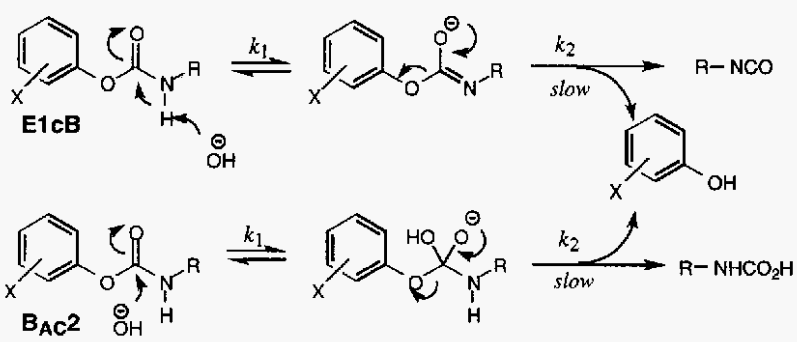


Fig.6 Two routes for the carbamate ester hydrolysis: the spontaneous E1cB (upper) and the disfavoured $B_{AC}2$ (lower) pathways.

The $B_{AC}2$ process, typical of carboxylate esters, is an alternative but higher energy route to the same products. We thus sought to catalyse the *disfavoured* mechanism of aryl carbamate ester hydrolysis by the use of a tetrahedrally-centred hapten to mimic attack of water on the C=O group (Figure 6).

A characteristic and definitive difference between the E1cB and $B_{AC}2$ mechanisms is the Hammett slope, which is large ($\rho \geq +2.5$) for the former and shallow ($\rho \leq +0.8$) for the latter processes (Figure 7). Consideration of this feature led us to identify four potential benefits from seeking to catalyse the disfavoured $B_{AC}2$ process over the spontaneous E1cB mechanism.

Firstly, as we had selected a phenolic mustard as the cytotoxic agent, the nitrogen mustard function in the prodrug [13] would have a value of the Hammett substituent constant $\sigma \leq -0.7$ while the substrate chosen for *screening* Mabs, the corresponding *p*-nitrophenol carbamate, would have $\rho \approx +1.0$. It follows that the optimum advantage for antibody catalysed cleavage of a phenolic mustard would be attained by screening for the $B_{AC}2$ mechanism using a *p*-nitrophenyl carbamate [14]. Secondly, it appeared likely that

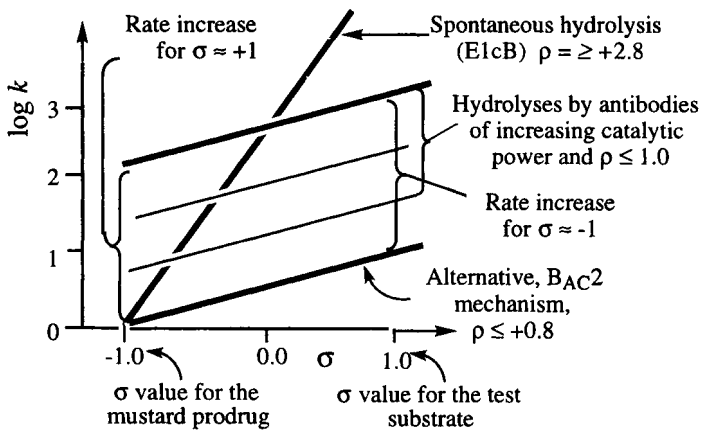
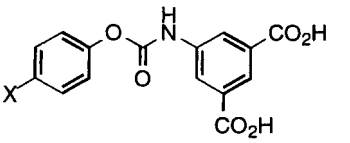


Fig.7 Scheme to show the effective rate enhancement for the abzyme hydrolysis of an aryl carbamate by the $B_{AC}2$ mechanism compared to the spontaneous E1cB mechanism is a function of the Hammett substituent constant, σ.



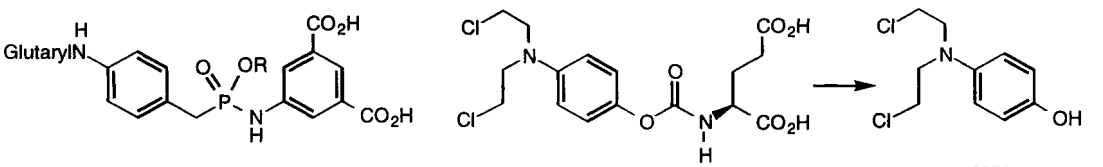
[13] X = $N(CH_2CH_2Cl)_2$, σ = -0.7;
[14] X = NO_2, σ = +1.0.

additional substitution in the phenolic ring could be introduced to stabilise differentially the abzymatic $B_{AC}2$ process relative to the spontaneous E1cB route. Thirdly, the relative insensitivity of the $B_{AC}2$ process to substituents in the aniline ring of **[14]** would permit its modification to change the characteristics of the prodrug for therapeutic advantage.

Lastly, catalysis of the $B_{AC}2$ process could also be applied to the family of *N*-methyl prodrugs, again creating opportunity for pharmacological diversity.

Hapten Design

We focused our initial research on the phosphonamidate haptens **[15a,b]** based on 5-aminoisophthalic acid, which were designed to create a more stable, entropically-limited mimic of one rotamer of the glutamic acid prodrug **[16]** (Figure 8) (26).



[15a] R = H; **[15b]** R = Et **[16]** **[17]**

Fig.8 ADAPT: TSAs **[15]** designed to generate abzymes for conversion of prodrug **[16]** into the cytotoxic phenolic mustard agent **[17]**.

The synthesis of these haptens was accomplished by standard organophosphorus chemistry and they were linked to BSA and KLH prior to immunisation. We analysed the success of this conjugation to the carrier proteins in three ways.

(a) SDS-polyacrylamide gel electrophoresis gave a qualitative verification of hapten conjugation to BSA but the KLH conjugates were too large to permeate the gel (27).

(b) The trinitrobenzenesulphonic acid (TNBS) assay provides a spectrophotometric method of assessing the number of free lysine residues remaining on the carrier proteins after conjugation of the hapten (28). This gives an average value for the percentage of potential sites to which the hapten has coupled.

(c) Matrix assisted laser desorption mass spectrometry, MALDI-MS, permits the

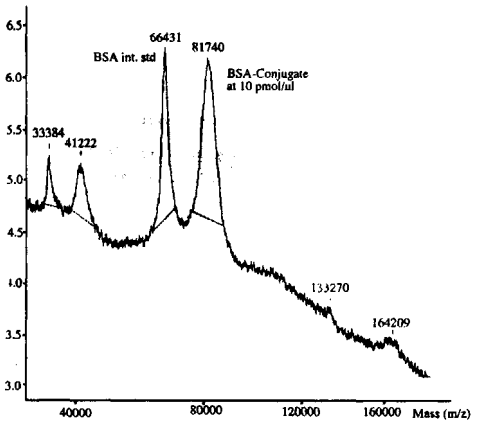


Fig.9 An LD-MS analysis of the BSA-hapten **[15b]** conjugate. Key peaks to note are for m/z at 81,740 (conjugate) and 66,431 (BSA).

accurate measurement of large macromolecules such as proteins up to MM 200 kDa (29). Using an internal BSA standard, the conjugates of this protein were measured to give a *modal* number of haptens conjugated per BSA molecule (Figure 8).

Thereafter, immunisation protocols using the KLH conjugates from **[15a]** and **[15b]** yielded over 50 monoclonal antibodies which bind strongly to their respective BSA-hapten conjugates.

Assessment of Binding Affinity

All purified cell lines obtained from these immunisations were rescreened using both an inhibition ELISA (30) and a surface plasmon resonance (SPR) procedure (31). The labour-intensive inhibition ELISA is somewhat prone to inaccuracies because the antibodies become bound to a solid phase and therefore their natural avidity contributes to their interaction with the BSA immunoconjugate bonded on that surface. By contrast, the SPR method involves immobilising a modified TSA which lacks the glutaryl spacer arm to a hydrogel that is not a true solid phase. Therefore the observed antibody affinity is usually not perturbed by avidity. By comparing results from the ELISA and SPR methods, we could identify and discard those clones which recognise only the spacer arm. In the event, the results from both techniques appeared to be satisfactorily comparable: the rank order of affinities were similar and the majority of values were within 25 %, the one of the other.

By siting the linker-spacer in the hapten in the same locus as the mustard function of **[14]**, we expected both to minimise recognition of the bischloroethylamino function in the prodrug-drug conversion and also permit some variety in our choice of functionalisation of the substrate for screening purposes. In the first instance we elected to screen with the activating *p*-nitro substituent to provide a simple UV assay. Additional functionalisation in the *para*-position can also be used to support the identification of the actual mechanism by which the carbamate substrates are hydrolysed by their effect on leaving group pK_a.

The initial assays using **[14]** were run at pH 7 and 37 °C, measuring the production of *p*-nitrophenol. Only antibodies selective for catalysing the hydrolysis of the *p*-nitroaryl carbamate were detected under these conditions and DF8-D5 was developed for further investigation. We next sought to establish the hydrolysis mechanism by using several *para*-substituted-phenyl carbamate esters as substrates for this antibody to determine the susceptibility of the process to the leaving group . The abzyme reaction has a ρ value of +0.5, typical of tetrahedral intermediate formation as the rate determining step in hydrolysis and fully consistent with operation of the disfavoured $B_{AC}2$ mechanism In particular, this compares well with the hydrolysis of *N*-methylated carbamates (which cannot form a conjugate base and so are excluded from the E1cB mode of breakdown) which has ρ = +0.8. We are presently examining the *N*-methylated derivatives of our substrates to see whether DF8-D5 is capable of hydrolysing these compounds also. At this time, DF8-D5 has not shown any capability to cleave the related amides or ureas, although the screening conditions we have used so far may require some modification.

Antibody Activation of a Prodrug:- Cell Kill in vitro

We have obtained several abzymes which can hydrolyse the carbamate ester bond in the nitrogen mustard prodrug **[16]** to generate the potent cytotoxic agent **[17]** (Figure 8) from the large number of antibodies with a high affinity for the TSAs **[15a,b]** (32). These antibody catalysts have been found to reduce the viability of a human tumour cell line *in vitro* when incubated with the prodrug **[16]**, as shown by use of the sulforhodamine B, SRB, assay (33). This rapid and inexpensive *in vitro* cytotoxicity assay is highly reproducible

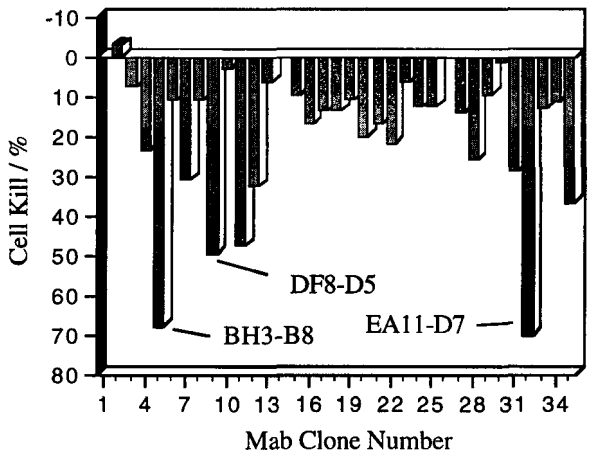


Fig.10

Cell viability in the presence of 20 ml of Mab (0.5 mg ml^{-1}) and 10 mM prodrug **[16]** in Dulbecco's modified Eagles' medium. The Mabs were all elicited to the phosphonamidate ester **[15b]**.

and appears to be practicable for the rapid screening of positive clones, as shown in preliminary binding studies for turnover of prodrug **[16]** (Figure 10). However, we have not yet been able to screen for cell-kill activity by use of the hybridoma cell supernatants directly, to which end a further increase in sensitivity of the assay is desirable.

The IgG_1 antibody EA11-D7 raised to hapten **[15b]** emerged as the most active catalyst for turnover of the carbamate prodrug **[16]** and its catalytic activity has been characterised in further

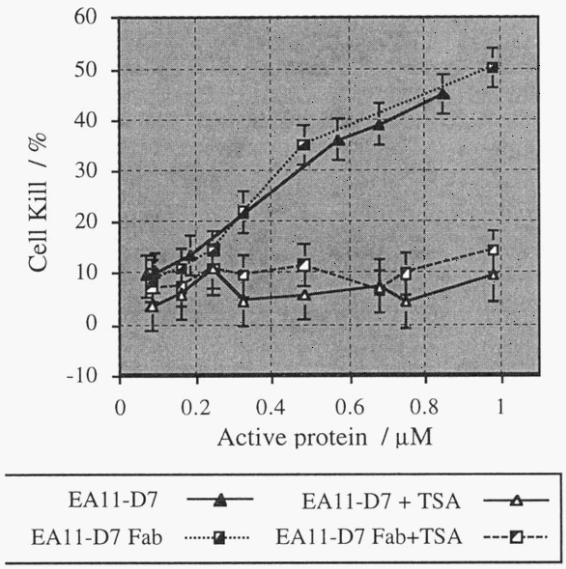


Fig.11 In this assay a comparison between the activity of parent monoclonal EA11-D7 and its derived Fab in the SRB was determined. In addition, the activity of both EA11-D7 and EA11-D7 Fab were studied in the presence of an equimolar concentration of TSA **[15b]**. The concentration of Fab and parent Mab have been corrected to allow for the two catalytic sites on the parent IgG antibody.

detail (34). By using increasing concentrations of EA11-D7, (0.06 - 1.00 μM), we have measured dose response values and these show good linearity up to 70 % cell kill. At the same time, papain-derived Fab fragments of the EA11-D7 cloned protein have shown catalytic activity comparable to that of the parent antibody (Figure 11). Most significantly, the catalytic activity is fully and stoicheiometrically inhibited by the TSA **[15b]** (Figure 8). This is also consistent with the affinity of EA11-D7 for a truncated form of **[15b]** of 5×10^{-9} M, determined by BIAcore™ assay.

The kinetics of hydrolysis of the prodrug **[16]** by 0.64 μM EA11-D7 were measured by following the UV absorbance change at 266 nm for a range of substrate concentrations. Initial slope measurements provided values of K_m = 201 μM and k_{cat} = 1.88 min^{-1} using non-linear regression analysis. From these data, it can be calculated that the abzyme EA11-D7 turns over *ca.* 3 equivalents of substrate (per site) in one hour in the SRB assay and transforms a net 4.18 μM of prodrug **[16]**, which is rather more than 2 x IC_{50}. That analysis correlates very well indeed with the best net cell kill we have observed of > 70 % for antibody EA11-D7.

CONCLUSION

The results described above adequately exemplify the ADAPT concept. Nonetheless, some issues remain to be examined further before the full potential of antibodies applied to the target-specific delivery of drugs can be attained. Abzymes remain slow catalysts, they may exhibit slow release of products, the affinity of the Mabs for the prodrug is capable of further improvement, and the full integration of pharmacokinetics and pharmacodynamics within the ADAPT system has to be developed for this approach to reach its full potential *in vivo*. What we present here is a first step: there is much work still to be done to make abzymes indisplaceable tools of clinical application.

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