Catalytic Antibodies as Magic Bullets

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Abstract: A reagent capable of detecting and selectively destroying tumor cells while leaving healthy cells intact would be a powerful tool for cancer therapy. This concept of the *magic bullet* has been approached by a number of strategies. Here we present a recent approach based on catalytic antibodies.

Keywords: antibody catalysis · bifunctional antibodies · cancer therapy · prodrugs · selective chemotherapy

Magic bullets

Based on the development of monoclonal antibody technology by Köhler and Milstein in the 1970s,[1] and rapid advances in antibody engineering technology since then,[2] antibodies have gained importance for the treatment of a variety of diseases as reflected by an analysis of pharmaceuticals in the biotechnology R&D pipeline.[3] In particular in cancer therapy, which is today mainly based on a triangle of surgery, radiotherapy, and chemotherapy, antibodies have demonstrated their potential as key tools in the anticancer arsenal. The potential of antibodies was first recognized by Ehrlich in the early twentieth century,[4] who suggested the role of the immune system in the defense against cancer. Ehrlich also coined the term *magic bullet* ("Zauberkugel"), a reagent capable of detecting and selectively destroying tumor cells. Here we consider a few of the features that make the antibody molecule well suited as a magic bullet for cancer therapy. Successful clinical trials have led to the recent approval of two antibodies for cancer therapy by the US Food and Drug Administration,[3] that is Rituxan from IDEC Pharmaceuticals for the treatment of non-Hodgkin's lymphoma and Herceptin from Genentech for the treatment of breast cancer. These approvals were in concert with the German approval of antibody 17-1A that has been developed by Riethmüller and co-workers for the treatment of colon cancer.[3] While the number of clinical trials involving antibodies is on the rise, a new generation of antibodies and antibody conjugates under development already scores promising preclinical results in vitro and in vivo.

The concept of the magic bullet is based on the conjugation of a targeting moiety to a cytotoxic moiety. While the targeting moiety is usually an antibody (alternative peptidic and nonpeptidic formats are under development), a broad variety of molecules have been used as cytotoxic moieties. Avoiding the severe side effects of conventional chemotherapy, the conjugation of a targeting molecule to a cytotoxic molecule allows for the selective destruction of cells expressing the target molecule on their surface. In cancer therapy, the target molecule is an antigen that is selectively expressed by tumor cells or the vascular cells that support tumor growth.

The targeting moiety

Our ability to engineer antibodies has not only facilitated the generation of antibodies to virtually any antigen of interest but also their improvement in terms of affinity, specificity, and immunogenicity.[2] Nonhuman antibodies are highly immunogenic in humans thereby limiting their potential use for therapeutic applications, especially when repeated administration is necessary. In order to reduce their immunogenicity, nonhuman antibodies have been humanized using strategies that are based on rational design, in vitro evolution, or a combination of both.[5] An alternative route is the direct generation of human antibodies from transgenic mice containing human immunoglobulin loci or by selection from human antibody libraries displayed on phage.[5]

The cytotoxic moiety

Without further conjugation, antibodies themselves can exert cytotoxic effects by interfering with ligand–receptor interactions required for tumor survival or by evoking effector functions including cell-mediated and complement-mediated cytotoxicity. In fact, Rituxan and Herceptin as well as the majority of antibodies in advanced clinical trials are "naked" antibodies. However, for most tumor antigens, antibody binding is not sufficient to mediate cytotoxicity, and a number of approaches have been made to conjugate antibodies to more efficient cytotoxic moieties.[6] Interleukin-2 (IL-2), which enhances the proliferation and cytotoxic activity of

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cytotoxic T cells and natural killer cells, and other cytokines have been combined with antibodies to potentiate their cytotoxic effect. Alternative cytotoxic moieties that have been conjugated to antibodies include radioisotopes, such as the β-emitter ^{131}I and ^{90}Y, as well as chemotherapeutic drugs like doxorubicin. In immunotoxins, antibodies are coupled to toxic proteins, such as *Pseudomonas* exotoxin A, a bacterial toxin. Another approach is the use of bifunctional antibodies that are specific for both a tumor antigen and an effector cell antigen that acts to recruit cytotoxic effector cells to the tumor site.

Toward selective chemotherapy

In yet another strategy, antibodies are conjugated to enzymes capable of activating nontoxic prodrugs to toxic drugs. This approach is based on a two-step administration in which an antibody–enzyme conjugate (Figure 1A) is given first. Binding to a tumor antigen results in the accumulation of the antibody–enzyme conjugate at the tumor site. Subsequently, a prodrug is administered and activated by the targeted enzyme. This strategy toward selective chemotherapy has been termed antibody-directed enzyme prodrug therapy, ADEPT.[7] In addition to selectivity, stoichiometry is a key feature of this approach. One molecule of enzyme catalyzes the activation of many molecules of prodrug, achieving high drug concentrations at the tumor site. The ideal prodrug is nontoxic and, thus, can be administered in higher doses than drugs used in conventional chemotherapy. One example that has demonstrated the feasibility of this approach in pilot scale clinical trials utilizes the bacterial enzyme carboxypeptidase G2 (CPG2) conjugated to an antibody to carcinoembryonic antigen.[7] The prodrug **1** was a L-glutamyl amide of an alkylating nitrogen mustard derived from 4-amino benzoic acid. CPG2 specifically cleaves the amide bond of prodrug **1** to release the more toxic drug **2** (Scheme 1). Despite its elegance, these studies shed light on the main drawbacks of the ADEPT concept. The nonhuman conjugate led to severe immunogenicity in the patients, which complicated repeated administrations. In fact, the requirements for the enzyme component in the ADEPT system are difficult to achieve. On the one hand, enzymes of nonhuman origin are highly immunogenic. On the other hand, the use of human enzymes is limited due to unselective prodrug activation by endogenous enzymes in blood and normal tissue of the patient.

Catalytic antibodies

To overcome these limitations, it has been suggested that the enzyme component might be replaced by a catalytic antibody.[9–11] The potential of catalytic antibodies for selective chemotherapy is indeed compelling; both, reactions that are not catalyzed by human enzymes and low immunogenicity through antibody humanization are feasible. In this concept, a humanized bifunctional antibody consisting of a catalytic and an antigen-binding site (Figure 1B) replaces the antibody–enzyme conjugate.

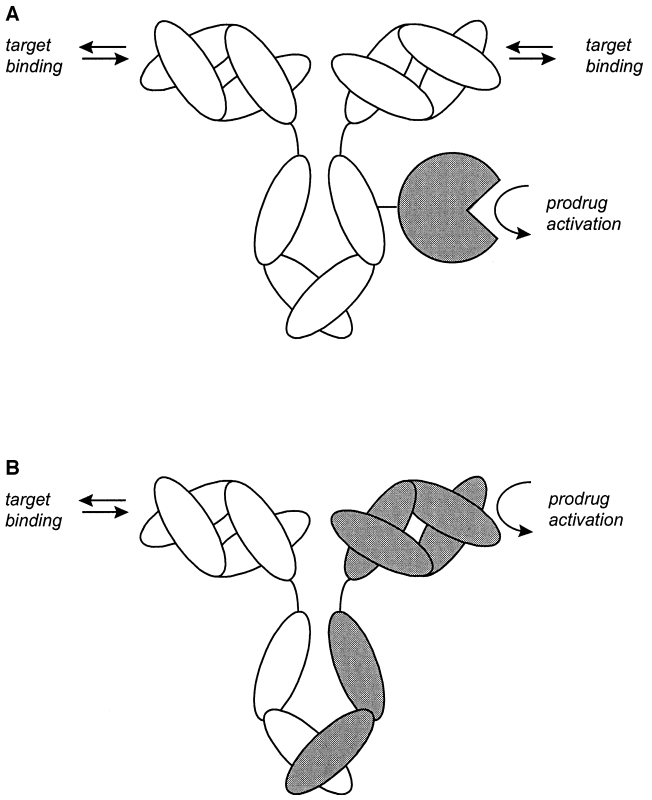

Figure 1. Constructs used for selective chemotherapy. A) Antibody–enzyme conjugate. The antibody component, here shown as bivalent Immunoglobulin G (IgG) molecule, localizes the conjugate at the target site where the enzyme component catalyzes the activation of a prodrug. Antibody and enzyme can be conjugated chemically or genetically. Alternatively, a bifunctional antibody consisting of an antigen-binding and an enzyme-binding site can be used for a noncovalent conjugation of the enzyme to the antibody. B) Bifunctional antibody that integrates a targeting antibody and a catalytic antibody. The catalytic antibody replaces the enzyme component in the original concept. The bifunctional antibody is shown as an IgG molecule. This and alternative formats of different size and valency can be readily engineered chemically or genetically[8].

Examples of antibody-mediated prodrug activation have been reported in the literature.[9–11] All of these reports use tetrahedral phosphonates as stable transition state analogues for antibody generation. Prodrug activation, that is release of the free drug, was achieved by hydrolysis of an ester or hydrolysis of an aromatic carbamate. Relatively low rate accelerations (10^3–10^4 over background hydrolysis) and potential catalysis by human enzymes has limited the application of these antibodies. Furthermore, immunization with transition state analogues generates antibodies that are generally highly specific for their designed substrates. Therefore, this approach is usually limited to the activation of a single prodrug. In addition, kinetic constrains have to be considered. The chemistry of the drug-release step is limited to low-background reactions. Rate accelerations (k_{cat}/k_{uncat})

Abstract in German: *Ein Reagenz, das in der Lage ist, Tumorzellen selektiv zu erkennnen und zu zerstören, wäre für die Krebstherapie äusserst nützlich. Verschiedene Strategien zu diesem Konzept, der sog. Zauberkugel, wurden bereits beschrieben. Wir präsentieren hier einen neuen Ansatz, der auf Antikörper-Katalysatoren beruht.*

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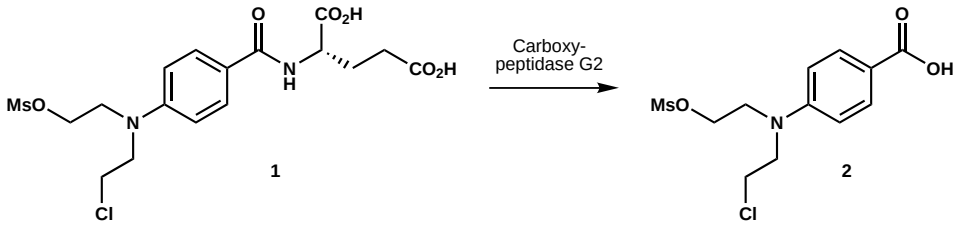


Scheme 1. Carboxypeptidase G2 catalyzed amide hydrolysis of L-glutamyl amide prodrug **1** to release active drug **2**.

should be as high as possible and k_{cat}'s should be in the range of 1 s^{-1}.[11] While rates of this magnitude have been achieved with a few catalytic antibodies, rate accelerations higher than 10^5 are rather rare.

The highest catalytic proficiencies have been observed with aldolase antibodies 38C2 and 33F12 and the recently discovered antibodies 84G3 and 93F3 that exhibit antipodal reactivity.[12, 13] These antibodies were generated by Barbas, Lerner, and co-workers using their reactive immunization technology and mechanistically mimic natural aldolase enzymes (Figure 2). The chemistry of these antibodies is unusual

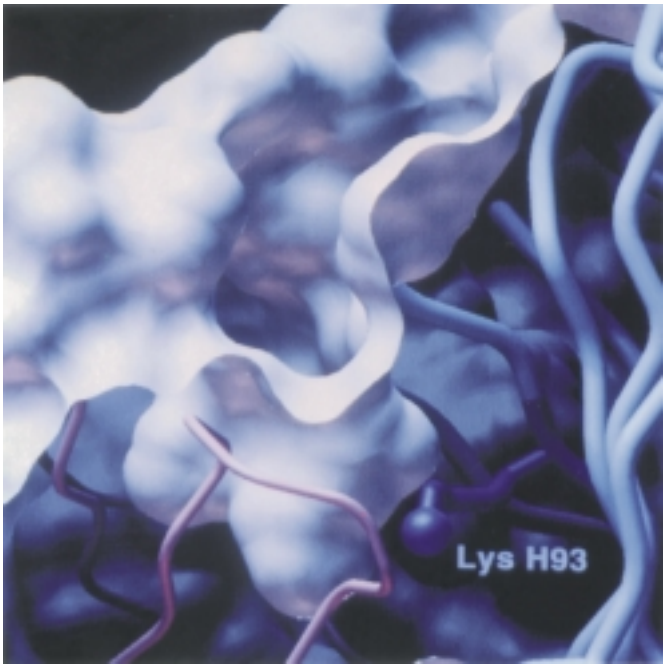
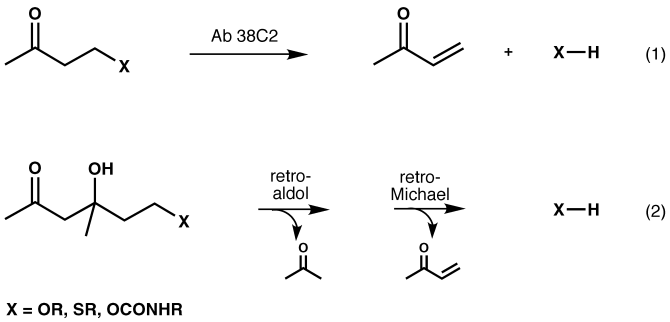


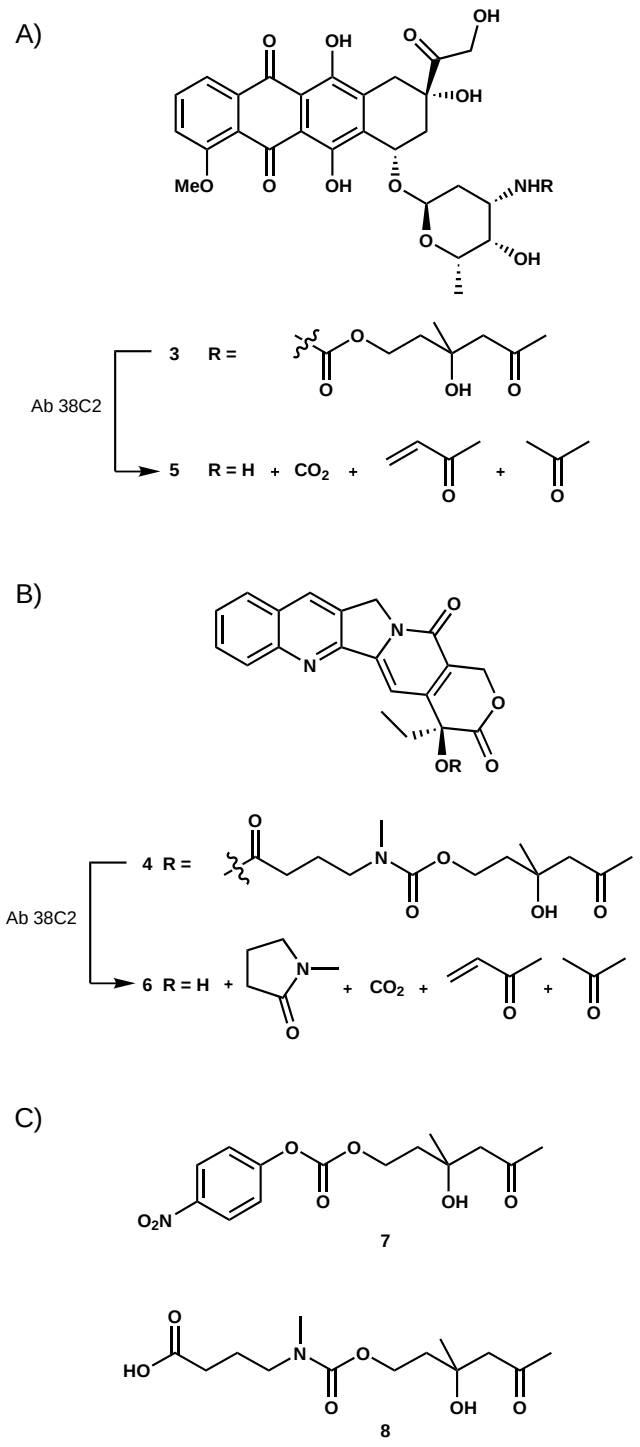
Figure 2. The active site of aldolase antibody 33F12.[12b] Through a reactive lysine (position 93 of the variable domain of the heavy chain) buried in a hydrophobic pocket at the base of the substrate binding site, 33F12 catalyzes aldol and retro-aldol reactions using the enamine mechanism of natural aldolases. The active site is conserved in aldolase antibody 38C2.

in many ways. First they are very efficient catalysts with rates (up to 2 s^{-1}) similar to natural aldolases. Further, their rate accelerations ($k_{cat}/k_{uncat} \approx 10^7 - 10^8$ fold) and catalytic proficiencies ($k_{cat}/k_{uncat} \cdot K_M$ up to $10^{13}\,M^{-1}$) are the highest ever observed with catalytic antibodies. The second unusual feature of these antibodies is their orthogonality to natural aldolase enzymes. Typically, aldolases use highly polar phosphorylated sugar derivatives, whereas most antibody substrates are hydrophobic (aromatic) compounds. In particular tertiary aldols, which have been demonstrated to be excellent substrates for aldolase antibody 38C2 are not used by natural enzymes.[12] It is likely that substrates common for both aldolase antibodies and endogenous aldolases do not exist. Because the aldolase antibodies were generated using reactive immunization, they are very broad in scope and accept hundreds of different substrates.[12, 13] This substrate tolerance is another attractive feature that eventually could lead to a multi-prodrug approach in analogy to already existing multi-drug cancer treatments. Despite efficiency, metabolic orthogonality and scope, the main drawback for the use of aldolase antibodies in this concept has been their limitation to aldol and retro-aldol reactions. A more general applicable chemistry would be strongly desirable. A breakthrough was achieved when it was discovered that the aldolase antibodies also catalyze the β-elimination (or retro-Michael reaction) of β-heterosubstituted ketones and aldehydes [Scheme 2, (1)].[14] While this



Scheme 2. The antibody-catalyzed β-elimination of β-heterosubstituted ketones (1) and the antibody-catalyzed tandem-retro-aldol-β-elimination (2).

reaction itself has a relatively high background, it can be linked to the retro-aldol reaction in a tandem process [Scheme 2, (2)].[15] This reaction sequence has proven to be well suited for drug-release chemistries and a number of heterosubstituted drugs, with modified amino, hydroxyl or sulfhydryl groups can be activated in this way. This chemistry has been successfully applied to anticancer drugs doxorubicin (**5**) and camptothecin (**6**) (Scheme 3),[16] both efficient topoisomerase inhibitors that are in clinical use. First, the drugs were chemically converted into their carbamate and ester prodrugs **3** and **4**, respectively, by treatment with readily available linkers **7** and **8**. It was then demonstrated that the rather nontoxic prodrugs are substrates for 38C2 and are converted into their parent toxic drugs **5** and **6**.[17] The reactions are effectively inhibited by the mechanism-based inhibitor 2,4-pentandione, which demonstrates that catalysis proceeds via involvement of a lysine amino group in the active site of the antibody (Figure 2). The rates of these reactions are relatively slow ($k_{cat} < 1\,min^{-1}$). However, a low K_M value



Scheme 3. A) The antibody-catalyzed activation of prodrug **3** to give doxorubicin (**5**). B) The antibody-catalyzed activation of prodrug **4** to give camptothecin (**6**). C) Linkers **7** and **8** were used to deactivate drugs **5** and **6** in one step.

(43 μM, for prodrug **3**) combined with good rate accelerations ($k_{cat}/k_{uncat} > 10^5$) and low toxicity of the corresponding prodrugs make this system quite effective in vitro. It could be demonstrated that only weakly toxic concentrations of prodrugs **3** and **4** can be activated by therapeutically relevant concentrations of antibody 38C2 to kill colon and prostate cancer cell lines (Figure 3). It was further shown that antibody 38C2 remains catalytically active over weeks after intravenous injection into mice and that sera from untreated mice are not catalytically active.

With these experiments, antibody 38C2 has clearly demonstrated its potential to become a useful tool in selective chemotherapy. Its advantages are a) its orthogonal chemistry to natural enzymes, b) the feasibility of its immunosilencing by humanization, c) its broad scope, d) its high efficiency, and e) the low background of the reactions it catalyzes. Future work will concentrate on engineering humanized bifunctional 38C2 constructs and the design and synthesis of novel and even more efficient prodrugs. Although great challenges and obstacles can be foreseen on our way toward these goals, this work may ultimately lead to a novel selective chemotherapy against cancer that would truly deserve the name "magic bullet therapy".

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[4] "[Der Stoff] würde in diesem Sinne genau den Immunprodukten des Organismus entsprechen, die ihrerseits nach Art von Zauberkugeln ihren Feind, die Parasiten, isoliert treffen. […] Die Tumorzellen sind mithin, im Gegensatz zu dem Parasiten, nichts dem Körper Fremdartiges, sondern es handelt sich gewissermaßen um feindliche Brüder, und es ist deshalb a priori viel schwerer, spezifische Heilstoffe aufzufinden, die nur die kranke Zelle treffen, ohne die gesunde zu schädigen. […]" Translation: "[The compound] would in this respect match exactly the immuno products of the organism, which themselves would target solely their enemy, that is the parasites, like magic bullets. […] The tumor cells are, contrary to

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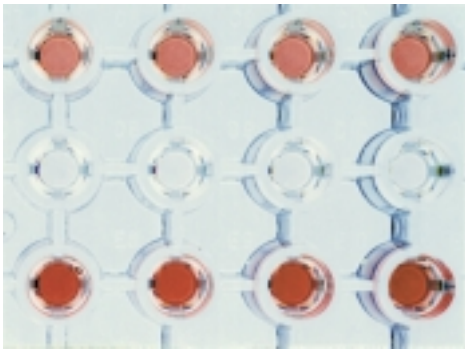

Figure 3. Inhibition of cancer cell growth by a combination of prodrug **3** ("prodoxorubicin"), and 38C2 IgG.[16] Cells in quadruplicate wells were lysed 120 h after drug addition, and the activity of the cytoplasmic enzyme lactate dehydrogenase released from the cells was detected by using a colorimetric assay. The intensity of the red color correlates with the number of surviving cells in the well. The combination of prodrug **3** and 38C2 (middle) strongly inhibits cell growth, whereas the prodrug alone (top) is far less potent. Antibody 38C2 alone has no effect (bottom). Top lane: 5μM prodoxorubicin, middle lane: 5μM prodoxorubicin+0.5μM 38C2 IgG, bottom lane: 0.5μM 38C2 IgG.

the parasites, not unfamiliar to the body, but are rather enemy brethren, and it is therefore a priori much more difficult to find specific drugs which would target only the infected cell without affecting the healthy cell. […]" Paul Ehrlich (1854–1915), Universität Göttingen and Königliches Institut für experimentelle Therapie, Frankfurt am Main. Cf. *Chemotherapie* (1913) in The Collected Papers of Paul Ehrlich, Vol. III, Pergamon Press, London, **1960**, pp. 443–455.

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[17] Antibody 38C2 catalyzed retro-aldol reactions are generally highly enantioselective. However, at 37 °C the enantioselectivity is lowered. With substrates **3** and **4** (which are diasteromeric mixtures) this allows for complete conversion.