Antibody Directed Enzyme Prodrug Therapy (ADEPT) and Related Approaches for Anticancer Therapy

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Abstract: In antibody directed enzyme prodrug therapy (ADEPT) an antibody bound enzyme is targeted to tumor cells. This allows for selective activation of a nontoxic prodrug to a cytotoxic agent at the tumor site for cancer therapy. Site-selective prodrug activation results in reduced side effects in remote tissue. This article reviews ADEPT with emphasis on the chemical viewpoint and recent developments.

INTRODUCTION

The search for selective anticancer therapeutics and hence safer drugs for combating malignant disease remains a challenging goal in oncology. One approach that seemed very promising for that aim is the use of monoclonal antibodies (mABs) [1, 2]. Those can be cytotoxic for cancer cells by themselves or are conjugated with drugs, toxins or radiopharmaceuticals in order to increase their efficacy. But disappointingly the clinical results of those approaches were often poor due to certain restrictions that apply to these protein derived drugs. One main problem is the poor uptake of the antibody resp. conjugate by the tumor cells. This is esp. limiting in larger and poorly perfused tumor tissue due to the size of those molecular entities. Further problems arise from proteolytic degradation of antibodies, the existence of antigen-negative cells and the formation of anti-mAB-antibodies. Maybe the most effective variation of that principle so far is radioimmunotherapy where due to the so called bystander-effect also antigen-negative cancer cells may be killed as well [3]. An example for a antibody-drug conjugate that was clinically approved recently is gemtuzumab oxogamicin (Mylotarg^{TM}) that consists of the enediyne anticancer antibiotic calicheamicin which is linked to an anti-CD33 antibody [4].

One interesting and promising form of targeted anticancer therapy is antibody directed enzyme prodrug therapy (ADEPT). In ADEPT an enzyme is linked to the antibody that targets the tumor cell. After application of this conjugate and clearance of non-bound circulating antibody a nontoxic prodrug of a cytostatic agent is administered. It is activated solely at the tumor site and exerts its activity also due to the bystander effect to antigen-negative cells. Such small molecule prodrugs resp. the activated forms are also able to diffuse better into larger tumor tissue. While initial studies originate some 30 years ago the concept was revived by the end of the 1980s [5, 6]. There have been several reviews throughout the last decade [7-10]. This article is intended to review all aspects of the ADEPT concept with a focus on the design of prodrugs for such an approach with an emphasis on recent developments. Concepts related to ADEPT such as gene- and polymer- resp. macromolecule-directed enzyme prodrug therapy (GDEPT, PDEPT, MDEPT) will be discussed as well. An update of the clinical situation and perspective will also be included.

TARGET ANTIGENS

As the target antigen has to be accessible to the antibody it is usually located at the outer cell membrane or secreted in the extracellular matrix [11]. Several antigens have been exploited for that aim. Examples are the p97 melanotransferrin antigen in melanoma cells [12], the L6 antigen on renal cell carcinomas [13], the carcinoembryonic antigen (CEA) [14] in colorectal cancer or the Ep-CAM1-antigen [15]. Esp. in the last few years a multitude of additional surface antigens with potential for targeted anticancer therapy has been recognized [16] which show potential for a use in ADEPT as well. E.g. approval for clinical use has been granted for a Her-2/neu antibody (trastuzumab, Herceptin^{TM}) [17].

ANTIBODY

The introduction of hybridoma technology and the possibility of the production of monoclonal antibodies were crucial to the introduction of targeted therapies. One of the problems associated with the classical mABs is that they are recognized as foreign proteins and the formation of anti-antibodies limits the therapeutic efficacy. With modern techniques it is possible to combine the regions of the murine antibody that are responsible for the antigen recognition with human antibody fragments. Such antibodies can be "chimerized" (murine variable region and human constant region; antibodies called –iximab) or "humanized" (additional replacement of the murine framework regions within the complementarity determining regions by human protein structures, antibodies called –umab) [18].

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Additionally the spectrum of the natural immune repertoire and the use of somatic cell affinity maturation has been superseded by large antibody display libraries and rapid molecular evolution strategies which will lead to a plethora of therapeutically useful recombinant antibodies in the near future [19]. Besides whole IgGs also single chains [20], F(ab)2 fragments [21] or variable region fragments (Fv) [22] can be used for the recognition process. Modern molecular biology also allows for the design of fusion proteins that combine the properties of both enzyme and antibody [23]. The use of catalytic antibodies (also called abzymes) that are created by immunization of mice with transition state analogues has been evaluated in several cases but so far the kinetics of the liberation require high or even almost stoichiometric amounts of the abzyme [24, 25]. This variation is also called ADAPT (antibody directed abzyme prodrug therapy) [26].

In MDEPT (also termed PDEPT) an enrichment of an enzyme-macromolecule conjugate in the tumor tissue is exploited for targeting. Certain tumors tend to have a much more permeable endothelium than normal tissue and on the other hand they are characterized by a limited lymphatic drainage. As a result of this so called EPR-effect (enhanced permeability and retention) macromolecules such as albumin, polyethylene glycols or polyacrylamides are enriched in the tumor. For the first example in MDEPT* a N-2-hydroxypropyl)methacrylamide was used [28].

ANTIBODY-ENZYME CONJUGATION

Linking antibody and enzyme is possible by chemical and other means. For chemical linking bifunctional reagents are used to connect the two peptides. Usually one acylating group is used to modify the side chain of lysines in the first protein and a thiol based bond provides the link to the second protein. For this aim maleimides which are subject to Michael additions resulting in thioethers are used as well as activated thiol compounds leading to mixed disulfide bonds with the second protein (Fig. **1**) shows some examples of possible bifunctional coupling agents.

Another possibility of linkage involves bispecific antibodies that recognize both the target antigen and the enzyme [29]. The most recent developments make use of the production of recombinant fusion proteins [20, 30-32]. The conjugation of the enzyme should not limit its ability the drug in a catalytic fashion and this is usually not the case, see e.g. [33].

CLEARANCE

The circulating antibody-enzyme conjugate that was not bound to the target tissue has to be eliminated from the bloodstream before the prodrug can be administered. Otherwise undesired prodrug activation takes place at sites remote from the tumor. The elimination may take days and the risk of adverse reactions by the immune system is increasing with the time of exposure. This can be limiting to therapy as only few cycles of treatment can be applied. Strategies which accelerate clearance of the conjugate therefore are an integrated part of most modern ADEPT approaches. One of the common tools to shorten the lag time before the prodrug can be administered is a second antibody which was raised against the active site of the enzyme and inactivates the fraction that is still circulating. Galactosylation of the second antibody provides an enriched absorption in the liver mediated by galactose receptors. This shortens the time of the circulating conjugate considerably and more cycles can be applied before e.g. inactivating antibodies are produced by the immune system. This approach was used in a recent clinical study [34].

ENZYMES AND PRODRUGS

The ideal enzyme is easily accessible, converts a suitable prodrug with a high selectivity and turnover at 37 °C and

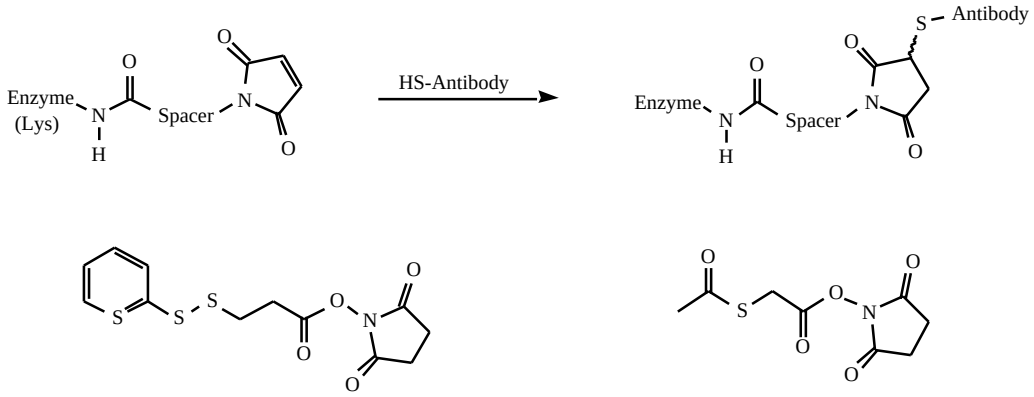


Fig. (1). Linker reagents for protein-protein coupling.

* MDEPT has also been used for Melanocyte-directed enzyme prodrug therapy. In this concept prodrugs that are converted by tyrosinase in tumor cells with a high expression level of that enzyme are used. No antibodies or other macromolecules are involved for targeting the cells [27].

near neutral pH in a non-reversible reaction. Nor should it be present normally in the human body neither induce a strong immune response. If the enzyme or a close homologue is found in humans then activation of the prodrug in remote tissue is possible or endogenous inhibitors may hamper the catalytic activity at the tumor site. On the other hand the risk of allergic reactions and therapy failure after few cycles increases if a non-human enzyme is used. The requirement of a cofactor might be a disadvantage as the presence of that factor might be the limiting step in the formation of the cytotoxic drug [10].

The prodrug should be converted by a suitable enzyme and its toxicity should be considerably less than that of the cytotoxic drug. The released agent should have steep dose-activity curves. This ensures a fast death of tumor cells even at the relatively low concentrations after release by the enzyme. A larger number of enzyme-prodrug combinations has been investigated and they are presented below. Structures are listed from new additions to the field in recent years. A good overview covering previous work is given in lit. [35].

Alkaline Phosphatase

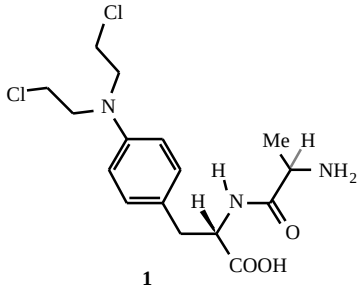
This enzyme cleaves phosphate groups from various prodrugs, e.g. of etoposide [6], mitomycin [36] or phenol mustards [37]. A high risk of negative side effects by endogenous enzyme activity is given here.

Amino Peptidases

Prodrugs of melphalan have been designed for the use of this class of bacterial enzymes. For example, **1** (Fig. **2**), has been synthesized for use in combination with D-aminopeptidase [38].

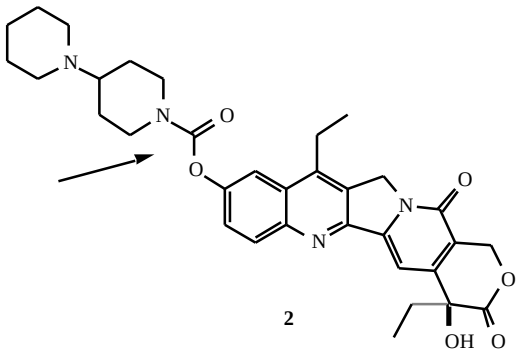
Aryl Sulfatase

The cleavage of sulfuric acid hemiesters by this bacterial enzyme was applied to prodrugs of etoposide and nitrogen mustards [39].



1

Fig. (2). Melphalan prodrug for D-aminopeptidase.



2

Fig. (3). Camptothecin prodrug for use with Carboxylesterase.

Carboxylesterase

Overexpression of this enzyme together with a camptothecin prodrug **2** (CPT-11, Irinotecan, Fig. **3**) was suggested for use in a GDEPT approach [40].

Carboxypeptidase A

This mammalian enzyme cleaves glutamate residues from prodrugs (see Fig. **4**) and has therefore been studied esp. with prodrugs of the glutamate containing antifolate methotrexate. Methotrexate-amino acid amides such as **3a** were shown to be cleaved by carboxypeptidase A and displayed anticancer activity [41]. The risk of conversion of the prodrug by human enzyme was circumvented by a most elegant approach using site-specific mutation of the enzyme. Bulky analogues such as the 3-cyclopentyltyrosine methotrexate derivative **3b** were shown not to be substrates for the wildtype enzyme. A threonine residue in the active site was discovered to be crucial for the steric repulsion. A mutant of the enzyme containing a glycine at this position was able to convert the bulky prodrugs and therefore seemed to be an ideal candidate for ADEPT concerning both selectivity and immunogenicity [42]. Unfortunately, in-vivo studies did not show an antitumor effect which was attributed to the instability of the conjugate [15]. A somewhat reversed ADEPT approach utilizes a folate rescue agent which is given together with an antifolate and is inactivated solely at the tumor site [43].

Carboxypeptidase G2 (CpG2)

This is a bacterial zinc metalloprotease which has been thoroughly studies in ADEPT approaches esp. in earlier work. Usually nitrogen mustards have been liberated from amide prodrugs [44] and a newer patent claims that 3-fluoro-benzamides such as **4a** (Fig. **5**) are especially useful and reactive for that purpose [45]. Newer self-immolative anthracycline prodrugs for that enzyme, e.g. **4b**, are found in lit. [46]. With so called self-immolative prodrugs the enzymatic reaction is followed by a second non-enzymatic degradation step which is fast, quantitative and guarantees

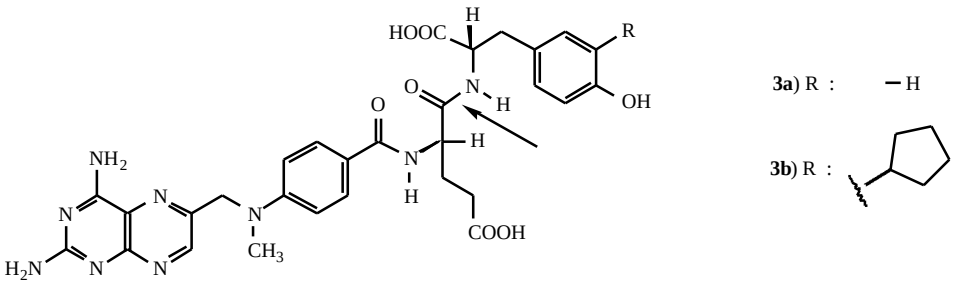


Fig. (4). Prodrugs for carboxypeptidase A.

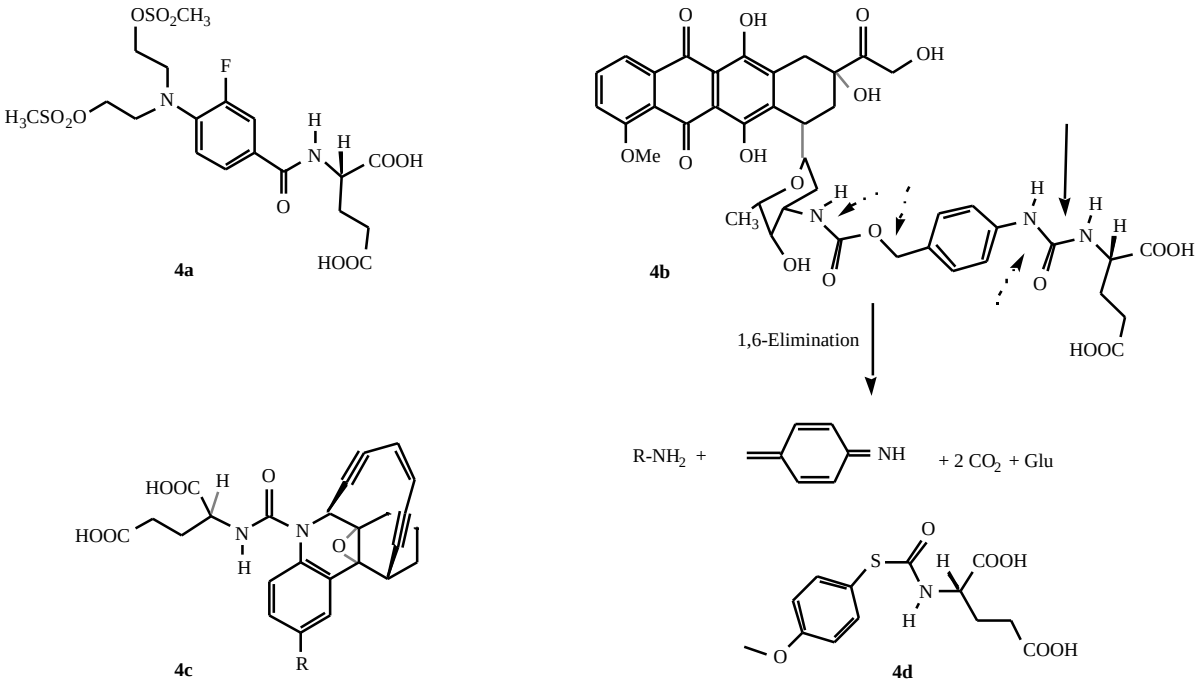


Fig. (5). Prodrugs for use with carboxypeptidase G2;dotted arrows pointing at bonds indicate the site of spontaneous non-enzymatic cleavage after the catalytic step.

for complete irreversibility. CpG2 is also one of the enzymes that have been studied for GDEPT approaches. In GDEPT a gene encoding an enzyme is targeted to the cells and expression in a fraction of the tumor cells may be sufficient for killing surrounding cells by the bystander effect. Usually the enzyme produced by the cancer cells has to be transported into the extracellular matrix to be active where it might be washed away. An alternative is the expression of a fusion protein that tethers the enzyme to the cell surface [46]. Enediyne prodrugs for CPGs **4c** have been recently disclosed in a patent [47]. There is also a report of competitive inhibitors of CpG2 such as **4d** [48]. They are supposed to inhibit the formation of drug on sites remote from the tumor tissue. Whether the pharmakokinetics will really work that way in a clinical setting remains to be determined. A fusion protein that consists of CpG and a single chain antibody against the carcinoembryonic antigen showed very good tumor/plasma levels in mice [31].

Catalytic Antibodies

A number of prodrugs have been evaluated with catalytic antibodies. A recent example involves prodrugs of anthracyclins **5a** and camptothecins **5b** [25]. The antibody catalyzes a cascade of a retro-aldol and a retro-Mannich fragmentation. With the modified spacer in **5b** an additional third chemical spontaneous reaction takes place.

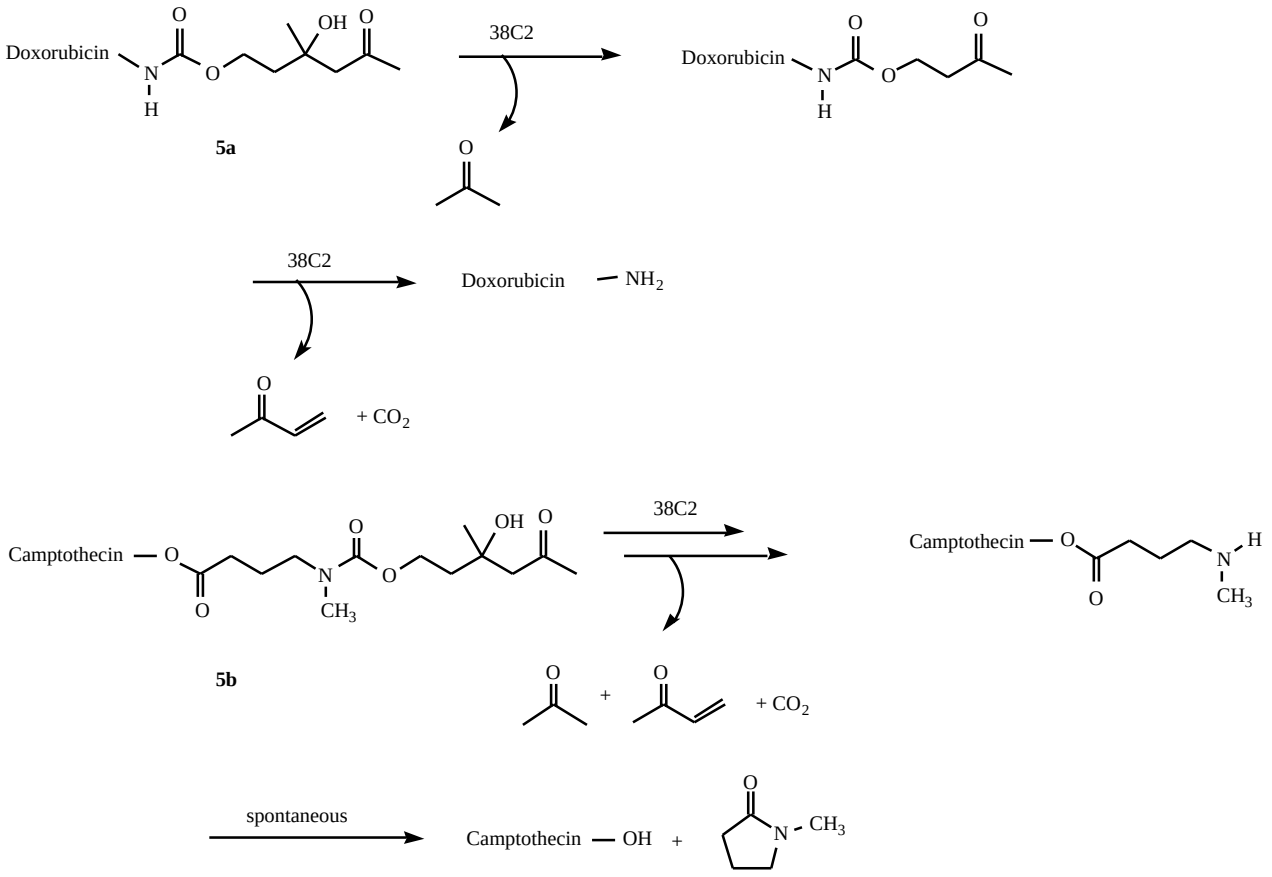


Fig. (6). Prodrugs for the catalytic antibody 38C2.

Cytosine Deaminase

Fluorouracil is generated by the deaminase from its prodrug 5-fluorocytosine which is a clinically approved agent for the treatment of fungal infections. The intratumoral conversion to the drug can be monitored by noninvasive magnetic resonance spectroscopic imaging [49]. This approach is also one the most favored ones in GDEPT strategies. The localization of a cytosine deaminase antibody conjugate could be optimized in an animal model of colon cancer when an increased dose of the conjugate was given followed by an anti-conjugate antibody 12 h afterwards to remove excess conjugate [50].

Galactosidases.

Anthracycline galactosides have been found suitable for the use with α-galactosidase [51] and ß-galactosidase has been used with that class of therapeutic agents as well [52].

β--Glucosidase

Cyanide can be enzymatically liberated from cyanogenous glucosides [53]. No resistance mechanisms of the cancer cells are known for this toxic principle. This approach has also been termed AGENT (antibody generated enzyme nitrile therapy).

β-Glucuronidase

The liberation of cytotoxic agents from glucuronides has also been used frequently. Recently developed prodrugs release anthracyclins, e.g. **6a** [54], camptothecins, e.g. **6b** [55] or phenol mustards [56]. In lit. [54] a bacterial glucuronidase is used which has a higher turnover number than the human homologue. 5-Fluorouracil-prodrugs such as **6c** have been realized as well [57, 58] and allow for real-time monitoring of the product release in-vivo by ^{19}F-NMR spectroscopy [57, 58]. A new spacer that relies on an enolether of arylmalondialdehydes is an alternative way to self-immolative prodrugs **6d** [59] as compared to the established carbamates that release quinonemethides resp. analogous imines. A different approach is the use of drugs that reduce multidrug resistance (MDR) in cancer cells and a prodrug of the MDR-modulator dipyridamole **6e** has been developed for that purpose [60].

β-Lactamase

Despite its bacterial origin this enzyme displays low immunogenicity in man. It is one of the best studied enzymes in ADEPT approaches and numerous anticancer agents have been investigated after suitable modification as prodrugs. Earlier work focussed on nitrogen mustards and doxorubicin and promising results from animal studies are already

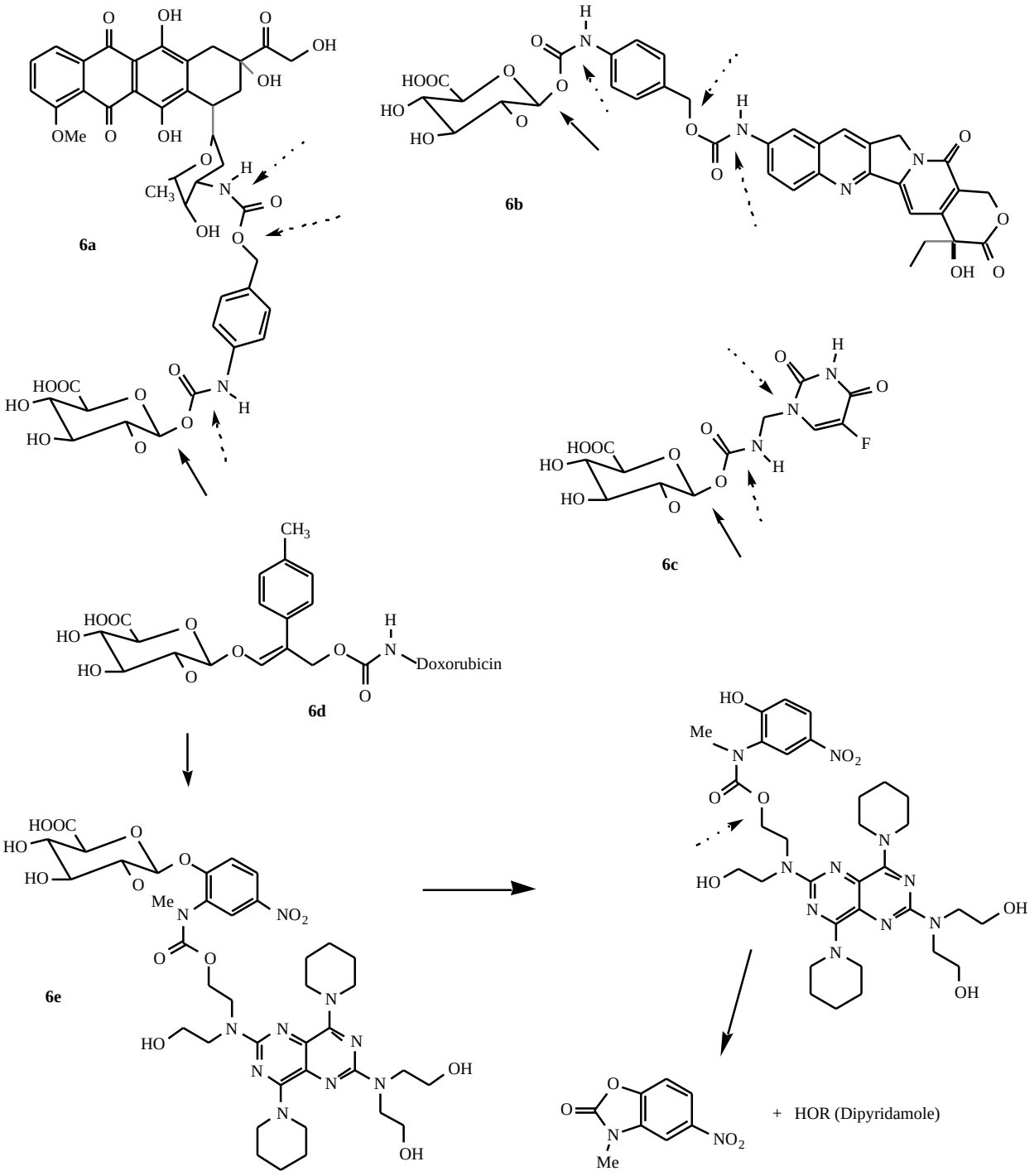


Fig. (7). Prodrugs for use with glucuronidases.

available [33]. More recent developments include prodrugs of mitomycin **7a** [61] and paclitaxel [62]. Additionally, fusion proteins with lactamase activity have already shown to be active in several animal models of cancer [32]. A novel concept involves the use of S-aminosulfeniminocephalosporins **7b** that are dual-release prodrugs and liberate an amine and a carboxylic acid salt (here acetate) which may be interesting for combinations of drugs in ADEPT approaches [63, 64].

Nitroreductase

This is another bacterial enzyme that has been investigated thoroughly for ADEPT and GDEPT approaches. It requires the presence of NADH cofactor for the reduction of the nitro group which may limit the efficacy in certain settings. Recent additions to the panel of nitroprodrugs are pyrrolobenzodiazepines **8a** [65], amino-CBI-derivatives **8b** [66], and enediynes **8c** [67]. The reduction converts the nitro

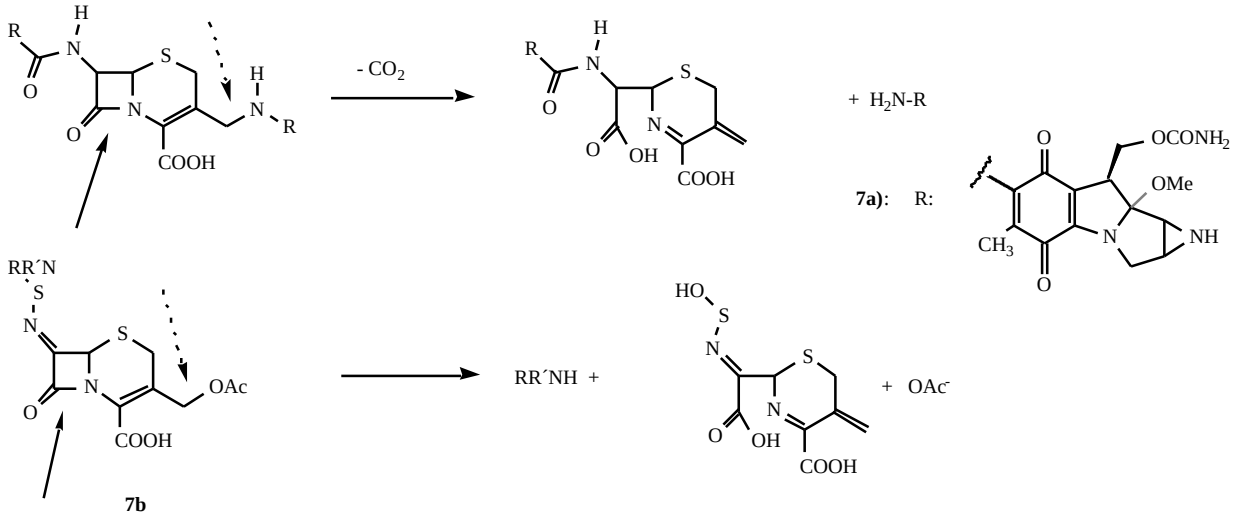


Fig. (8). Prodrugs for use with β-lactamase.

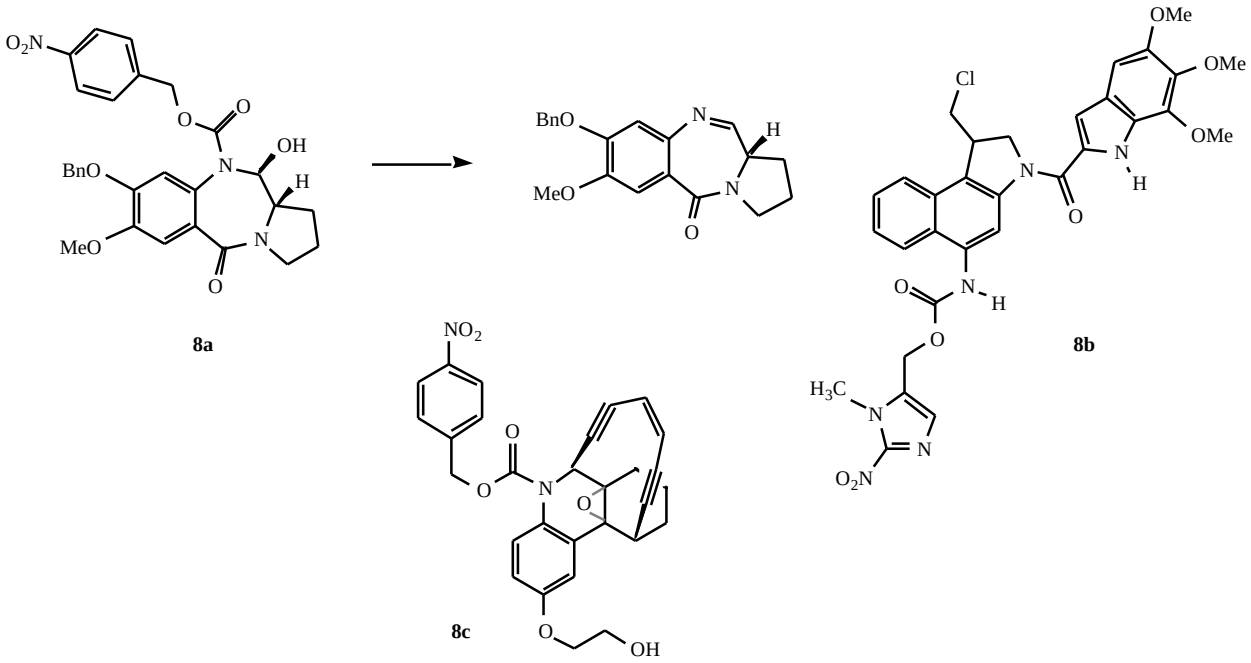


Fig. (9). Prodrugs for use with nitroreductase.

prodrugs to hydroxylamine compounds which then undergo facilitated hydrolytic cleavage, usually involving self-immolation.

Penicillin Amidase

The cleavage of a 4-hydroxyphenylacetamide prodrug releases palytoxin [68].

Xanthine Oxidase

Tumor targeted xanthine oxidase generates oxygen-based radicals which kill surrounding cells efficiently [69].

CLINICAL STUDIES

Besides initial pioneering studies there is only one recent clinical trial with an ADEPT system reported [34]. Carboxypeptidase G2 was linked to a Fab´$_2$-fragment of an antibody against the carcinoembryonic antigen. A galactosylated secondary antibody against the active site of CpG2 was used to accelerate the clearance of circulating conjugate. It was shown that the enrichment in the tumor vs. plasma was about 10.000:1. A N-lost benzoic acid was used as a glutamate prodrug. The K_M of the enzyme for this substrate was about 3 µM which can be achieved at the tumor site in a therapeutic setting. Ten patients with colorectal carcinoma were included in the study. Six patients showed no progression and one patient showed a partial

remission. It could be shown that neutropenia and thrombocytopenia occurred but to much lesser degrees as in conventional cytotoxic pharmacotherapy.

OUTLOOK

ADEPT has demonstrated significant activities in many preclinical cancer models, and initial clinical responses in early phase trials are encouraging. One of the important features of this promising approach is that it allows the use of therapeutic agents with novel mechanisms of action that are otherwise too toxic for systemic use. Since the drugs are selectively generated within tumor masses, the side-effects can be greatly reduced compared to systemic drug administration. Relatively high concentrations of the anticancer drug can be realised locally which may also help to prevent or overcome problems related to multi-drug resistance (MDR). This could be especially true in combinations with drugs that are too toxic for conventional application. ADEPT and related approaches such as GDEPT or MDEPT are still far from being a standard form of anticancer therapy, and despite many encouraging preclinical results, some significant challenges remain. These include the large scale production of antibody-enzyme conjugate, both from the cost and quality assurance perspective, immunogenicity of non-mammalian enzymes, and optimization of therapeutic protocols in people. A wider variety of prodrugs for the same enzyme will allow for additive and synergistic interventions. Therefore the next years of ADEPT research are anticipated with great interest.

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