Targeted Enzyme Prodrug Therapies

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Abstract: The cure of cancer is still a formidable challenge in medical science. Long-known modalities including surgery, chemotherapy and radiotherapy are successful in a number of cases; however, invasive, metastasized and inaccessible tumors still pose an unresolved and ongoing problem. Targeted therapies designed to locate, detect and specifically kill tumor cells have been developed in the past three decades as an alternative to treat troublesome cancers. Most of these therapies are either based on antibody-dependent cellular cytotoxicity, targeted delivery of cytotoxic drugs or tumor site-specific activation of prodrugs. The latter is a two-step procedure. In the first step, a selected enzyme is accumulated in the tumor by guiding the enzyme or its gene to the neoplastic cells. In the second step, a harmless prodrug is applied and specifically converted by this enzyme into a cytotoxic drug only at the tumor site. A number of targeting systems, enzymes and prodrugs were investigated and improved since the concept was first envisioned in 1974. This review presents a concise overview of the history and latest developments in targeted therapies for cancer treatment. We cover the relevant technologies such as antibody-directed enzyme prodrug therapy (ADEPT), gene-directed enzyme prodrug therapy (GDEPT) as well as related therapies such as clostridial- (CDEPT) and polymer-directed enzyme prodrug therapy (PDEPT) with emphasis on prodrug-converting enzymes, prodrugs and drugs.

Keywords: ADEPT, GDEPT, cancer, gene therapy, antibody.

PRINCIPLE OF DIRECTED ENZYME PRODRUG THERAPIES

In the early 1970s, antineoplastic chemotherapy began to became a successful treatment of cancers. At the same time, its often devastating side effects such as gut-tearing vomitus and diarrhea, alopecia, and fatal vulnerability to infections gained the focus, with the majority of patients still finally succumbing to the biology of metastatic cancer. Since these early days, the application of chemotherapeutics themselves and the development of drugs to alleviate the side effects have incessantly been refined, and today this modality on its own or in combination with surgery and radiotherapy has made a number of cancers curable and has improved duration and quality of life in many others.

Still, dose-limiting side effects and unsatisfactory efficacy which in pharmacological terms means a small therapeutic index, remains a concern today as it was as then. Thus, early in the era of chemotherapy, the search for specifically "activated" non-toxic prodrugs that could be used as cytotoxic chemotherapeutics began. The quest for a medical version of the "magic bullet", as Paul Ehrlich termed it after Weber's opera "Der Freischütz" [1], was on. In biology, at this time, enzymes were the latest focus of many research groups. Hence, the first approach to the magic bullet for chemotherapy was the use of enzymes to convert non-toxic prodrugs into an active, cytotoxic version specifically in tumor tissues. The discovery of suitable enzymes and syntheses of fitting prodrugs made progress, but tumor specificity remained an obstacle. While tumor tissue does provide some physical properties that can control an enzyme's catalytic activity, such as low oxygenation or high acidity, this proved to be insufficient to confer clinically efficient tumor specificity to therapeutic agents.

The development of the hybridoma method propelled monoclonal antibodies to the forefront, promising tailor-made solutions to almost everything. Clearly, their enormous specificity held the potential for a "magic bullet". In the field of tumor therapy, soon two routes were followed: (1) using monoclonal antibodies by themselves to mark tumor cells for destruction by the immune system, in either complement-dependent cytotoxicity or a - then little-understood - process called antibody-dependent cellular cytotoxicity, and (2) using antibodies as vehicles to specifically deliver a cytotoxic "payload", e.g. drugs or lethal cytokines, to tumor cells. While "the cure for cancer" was thought to be around the corner in the early 1980s (and repeatedly thence), it took both routes more than two decades to lead to approved clinical treatments.

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A third approach accompanied these efforts more quietly, trying to combine the specificities of enzymes with those of monoclonal antibodies and thus adding to the concept of enzymatic prodrug activation the hitherto missing tumor-specific targeting component. This concept was first envisioned by Philpott [2] and termed **antibody-directed enzyme-prodrug therapy** (**ADEPT**) by Bagshawe in 1987 [3]. The basic idea is as elegant and bold as its practical development has turned out to be complicated.

In ADEPT (Fig. **1**) a tumor-specific antibody (or antibody fragment) coupled to a prodrug-specific enzyme is systemically administered in a first step. Once this antibody-enzyme construct is bound to its cognate antigens in tumor tissue and the unbound remainder is cleared from the bloodstream, the prodrug is administered and circulates harmlessly through the body until it meets the tumor-bound antibody-enzyme construct, which then converts the prodrug into the corresponding active drug that kills the tumor cell.

Given the obvious problems with stability and immunogenicity when administering protein drugs, the concept of ADEPT has recently been adapted for gene therapy approaches (**gene-directed enzyme prodrug therapy**, **GDEPT**, Fig. **2A**), where the prodrug-converting enzyme is not directed to, but expressed by tumor cells. This requires delivery of the prodrug-converting DNA sequence to a large proportion of tumor cells and subsequent enzyme expression in order to mediate tumor-specific prodrug activation.

The theoretical advantages of such systems are obvious: Active cytotoxic drug will only be set free in tumor tissue, and despite imperfections such as leak-back of active drug into the circulation, the therapeutic index will in theory be increased by the factor of toxicity of drug over prodrug. Thus targeted prodrug-converting enzyme therapies should induce less side effects with the same efficacy or increased efficacy with the same toxicity – in short: increase the therapeutic index. Increased efficacy is probably the more important aspect, given that many drugs kill cancer cells *in vitro* at doses that are either impossible to achieve or lethal *in vivo*. Finally, the prodrug can be administered many times and yield the same effect as long as the prodrug-converting enzyme is still localized in the tumor – a potent advantage over direct antibody-drug conjugates. Thereby, separating the pharmacokinetics of antibody and drug, ADEPT is a pre-targeting strategy.

Unfortunately, theory leads to a number of problems in practice: The leak-back-effect may be substantial, and systemic activation of the prodrug can turn out to be massive. Immunogenicity of the enzyme is not easily overcome: as antibodies are essentially mammalian molecules, their humanization has been challenging but successful, whereas the enzyme must catalyze a reaction not present in the human repertoire, which makes it difficult to find (or create) an enzyme of "human-like" protein structure. Then there has been the technical issue of generating the fusion construct, which can be cumbersome at best when done by chemical conjugation. However, recombinant technology has opened the way to reproducible, high quality, and high quantity production. Finally, the application is complex: Before application of prodrug, the antibody-enzyme construct should be completely cleared from the bloodstream but be near its maximum concentration in tumor tissue. This window of opportunity can be small and easy to miss under clinical conditions. Similarly, gene therapy-based approaches need to achieve specific delivery of the enzyme-encoding DNA to tumor cells and high-level gene expression before administration of the prodrug.

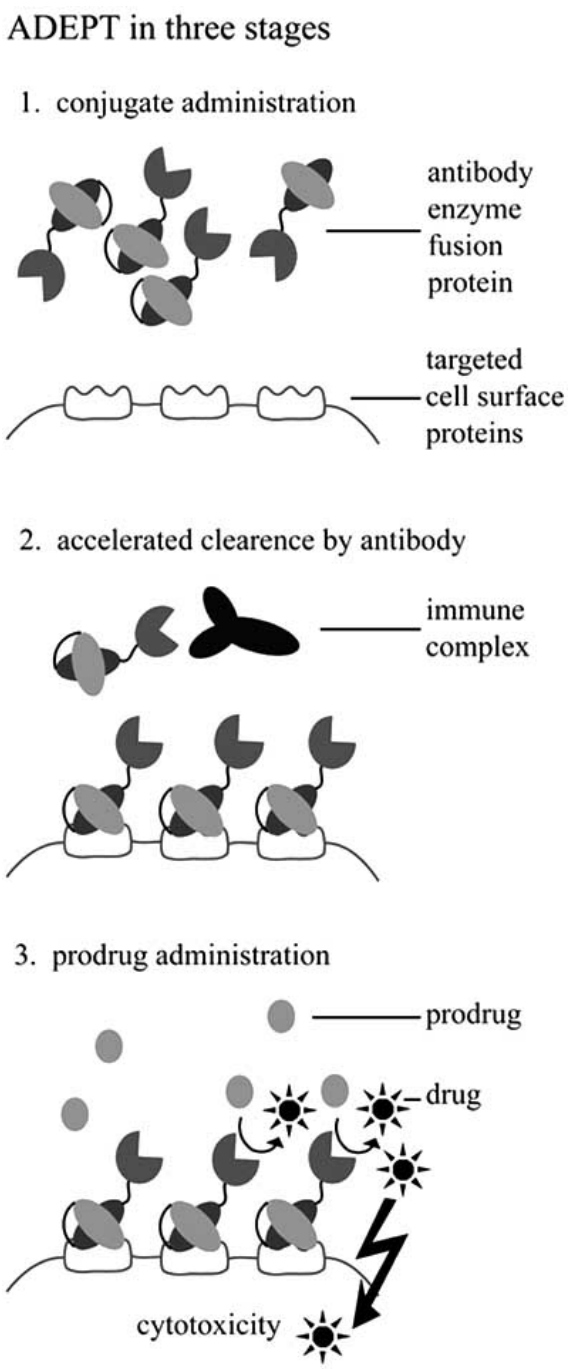


Fig. (1). Antibody-directed enzyme prodrug therapy (ADEPT) in three stages.

Despite these problems, a number of approaches to targeted prodrug-converting enzyme therapies have been developed, and ways to overcome the obstacles have been ex-

plored, namely by the workgroups of Bagshawe and Begent in London, who have performed the first and so far only successful clinical trials. This review tries to shine light on the current position of ADEPT and the related concepts that have evolved from it.

TARGETS OF ADEPT

The major theoretical requirements for a cellular antigen that could serve as target for ADEPT are tumor specificity and accessibility from the bloodstream. In addition, the antigen should ideally be constantly present on the cell surface, and should not be shed into the bloodstream. Either one of the two requirements is not or only partially fulfilled by the majority of the antigens described in more detail below. In principle, however, many antigens used or investigated as targets for antibody-based tumor therapy should be suitable for ADEPT, too. Indeed, a considerable number of antigens of various classes has been investigated in preclinical studies of ADEPT systems and *in vitro* (reviewed in [4]), and there are no good reasons why their number should not increase. Of these, especially oncofetal antigens have been the focus of continued efforts to establish ADEPT *in vivo* or even in clinical studies.

Oncofetal antigens are a very heterogeneous group whose members are expressed physiologically only during embryonic or fetal development. These antigens, – obviously associated with cell proliferation – are a pathological feature of various malignancies. Carcinoembryonal antigen is the prototypical oncofetal antigen which is widely seen in a circulating soluble form. This has proved beneficial for its use as a diagnostic tumor marker since the 1960's to monitor the course of adenocarcinomas and colon cancer in particular. Hence, despite the problem of circulation of the soluble antigen, which limits its tumor targeting ability, it has become the most intensively investigated antigen for ADEPT, thanks to the London research groups of Kenneth Bagshawe, Richard Begent and Kerry Chester. The studies of this group paved the way to clinical ADEPT with carcinoembryonal antigen. Preclinical studies with a chemical conjugate of an anti-carcinoembryonal antigen-antibody and carboxypeptidase G2, to activate the benzoic mustard prodrug CMDA found that such a system was feasible, but that circulating enzyme, not soluble carcinoembryonal antigen, led to non-specific prodrug activation in the bloodstream [5]. From this observation the concept of a clearing agent was developed, administered after tumor localisation of the antibody-enzyme construct to remove or inactivate its circulating portion from the bloodstream [6]. Upon administering the clearing antibody SB43-gal that binds and inactivates the enzymatic activity of carboxypeptidase G2, Napier *et al*. achieved an excellent tumor-to-blood and tumor-to-liver ratio of more than 10,000:1, respectively, [7]. This, however, made an already difficult two-phase pharmacokinetic system even more complex. One approach to circumvent the three compound treatment was the use of a glycosylated recombinant fusion protein as described in [8, 9], instead of non-glycosylated fusion proteins as described above. For pharmaceutical development, a mere two-step system is highly attractive and favorable. The glycosylated recombinant fusion protein, termed MFECP, has been produced in yeast to facilitate its rapid hepatic clearance *via* receptors detecting the glycosylation. Whether this will solve the problem of circulating unbound enzyme remains to be seen, but preliminary clinical data are promising. Another problem undermining the specificity of antibody-directed targeting is the leaking back of active drug from the tumor site into the bloodstream. While yet another clearing agent could be conceived, further adding to the complexity of the system, a more direct approach of solving this issue is reducing the half-life of the drug. With 36 minutes, the half-life of the bifunctional alkylating drug CJS11, released from the prodrug CMDA, was already shorter than that of most other cytotoxic drugs [10]. Recently, a bis-iodophenol prodrug, ZD2767, with an ultra-short half-life too short to be measured directly has been synthesized and submitted to the first clinical trials [11] (Table **1**).

Another well investigated antigen is the mucin-like tumor-associated glycoprotein 72 – alternatively classified as an oncofetal antigen – is expressed in adenocarcinomas, but except for secretory endometrium, no appreciable presence in normal tissues has been found. The original anti-glycoprotein 72 murine antibody, CC49 (minretumomab) is parent to chimerized, humanized, single chain and C_H2-domain-deleted versions which have been investigated at several institutions in numerous clinical phase I and II trials including patients with lung, colorectal, breast, ovarian, and prostate cancers. A thorough review of the clinical trials performed with CC49 and its derivatives has been compiled by Meredith *et al*. [17]. Recently, a recombinant single-chain fusion construct with β-lactamase to activate a geldanamycin prodrug has been reported [18], and a pharmacokinetic model for its dose distribution has been developed based on *in vivo* animal data [19].

The cognate antigen of the murine monoclonal antibody A33 has been sequenced and identified as a member of the immunglobulin superfamily [20]. It is now accepted as an adhesion molecule, although details of its function and possible natural ligand are still unknown. The antigen, recently termed glycoprotein A33 (gpA33), is expressed by gastrointestinal epithelia including pancreas and by more than 95% of colon cancers. Radioimmunoscintigrams confirmed the localization of antibody-bound radioisotopes to virtually all known primary or metastatic tumor sites. While there was initial uptake in normal bowel, time-dependent specificity became visible with a maximum at about two weeks after injection, when the antibody had almost completely cleared from normal tissue but tumor sites retained high activity [21]. Another study with ^{125}I-labeled A33 found modest anti-tumor activity with good tolerability in the absence of gastrointestinal or hematological toxicity [22]. The controlled studies encouraged by these results had to wait for the complementarity determining region-grafted humanized version. Even with humanized A33, however, immunogenicity remained a problem as human anti-human antibodies developed in 8 out of 11 patients, going along with strong infusion reactions [23]. Very similar results were observed in a parallel combination chemotherapy study with bis-chloronitrosourea, vincristin, fluorouracil, streptozocin in a fixed dose and huA33 dose escalation. Three out of 12 patients reached radiographic partial responses, but human anti-human immune reactions remained a serious problem in 7 out of 12 patients [24], although findings of another study were more favorable in this respect [25].

Table 1. Directed Enzyme Prodrug Therapies in Clinical Trails

Methods	Name	Prodrug	Enzymes	Indication	Reference
ADEPT	A5CP (A5B7-CPG2)	CMDA	CPG2	advanced colorectal carcinoma	[7, 10]
	A5CP (A5B7-CPG2)	ZD2767P	CPG2	advanced colorectal carcinoma	[11]
	MFECP1 (MFE-23-CPG2)	ZD2767P (= BIP)	CPG2	CEA-expressing tumors	[12]
VDEPT	Ad5-CD/TKrep	5-fluorocytosine, ganciclovir	cytosine deaminase, thymidine kinase	local recurrence of prostate cancer	[13]
	Adv.RSV-tk	ganciclovir	thymidine kinase	hepatic metastases from colorectal adenocarcinoma	[14]
	Adv-HSV-tk	ganciclovir	thymidine kinase	prostate cancer	[15]
	AdV-tk	valacyclovir	thymidine kinase	malignant glioma	NCT 00634231
				pancreatic cancer	NCT 00638612
	CTL102	CB1954	nitroreductase	liver cancer	[16]
	FP253	fludarabine	purine nucleoside phophorylase	prostate cancer	NCT 00625430

Abbreviations: A5B7, Fab'(2)-fragment of anti-carcinoembryonic antigen antibody; CB1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide; CD, cytosine deaminase; CPG2, carboxypeptidase G2; CTL102, bacterial nitroreductase; CMDA, 4-[(2-chloroethyl)(2-mesyloxyethyl)amino] benzoyl-l-glutamic acid; FP253, ovine atadenovirus-encoding PNP; HSV, herpes simplex virus; MFECP, anti-carcinoembryonic antigen single-chain fragment-carboxypeptidase G2 fusion protein; NTR, nitroreductase; PNP, purine nucleoside phosphorylase; ZD2767P, 4-[N,N-bis(2-iodoethyl)amino] phenoxycarbonyl L-glutamic acid.

Building on these results, a first gpA33-based ADEPT system was conceived with a chemical conjugate of the complete immunoglobulin G humanized A33 with bovine carboxypeptidase A, a pancreatic enzyme not normally found in the bloodstream that would cleave the amino acid off methotrexate-phenylalanine to yield active methotrexate. *In vitro* experiments – and, in a limited way, initial *in vivo* – experiments demonstrated the proof of principle and the feasibility of gpA33-based ADEPT [26]. To overcome the problem of immunogenicity even with the humanized A33, a single chain antibody fragment against gpA33 (A33scFv) has been developed in a phage display system [27]. Based on this A33scFv, first a recombinant fusion construct with green fluorescent protein was developed as a tool to further study the gpA33 receptor and to optimize the design and expression of A33scFv-based fusion proteins [28]. To realize an ADEPT concept based on this recombinant approach, an analogous fusion protein for pre-targeting bacterial cytosine deaminase, which converts the approved antifungal agent 5-fluorocytosine into the cytotoxic drug 5-fluorouracil, to tumor cells was produced and successfully analyzed *in vitro* [29]. However, *in vivo* testing was not possible due to an insufficient production yield of the protein. Hence, the yeast isoenzyme was chosen for further expression of the fusion protein in the yeast *Pichia pastoris* [30, 31]. This fusion protein was purified in high amounts, demonstrated gpA33-targeting and prodrug conversion of 5-fluorocytosine into 5-fluorouracil in cell culture systems and finally proved feasibility in promising tumor growth inhibition in early-stage animal experiments [32].

Human chorionic gonadotropin is expressed by choriocarcinomas. It has successfully been used as a target in initial xenograft studies of Bagshawe's research group [33], who later focused on the carcinoembryonal antigen target as outlined above. The same applies to human epidermal growth factor receptor 2, an antigen overexpressed in about a quarter of human breast cancers, which is also the target of the approved therapeutic antibody trastuzumab [34]. Studies on constructs containing carboxypeptidase G2 directed against these two targets led to valuable insight for further research, but currently appear not to be pursued.

Another approach was use of antibody-enzyme fusion proteins against CD20, an antigen of B-lymphocytes that is successfully targeted for lymphoma therapy by the antibody rituximab. However, there have been no further studies or reports using this approach [35]. γ-seminoprotein has been targeted in *in vivo* studies of a prostate carcinoma ADEPT model [36]. Generating antibody constructs directed against single-strand DNA that localize to necrotic tumor areas was a promising, new approach. The initial study published on this concept demonstrated good tumor localization of all

components required [37]. Whether specific binding to dead debris left behind the tumor growth zone will prove beneficial against cancer, however, remains to be seen.

TARGETING WITHOUT ANTIBODIES

The targeting of tumor cells is in no way restricted to antibodies or natural ligands. Various other types of targeting mechanisms for tumor-specific therapies have been described. In 2002, Ho *et al.* [38] presented the idea of an **enzyme-mediated insolubilization therapy** (**EMIT**, Fig. **2B**). The aim here is to accumulate radionuclides in the extracellular space of solid tumors by alkaline phosphatase-mediated cleavage of a radioactive water-soluble prodrug into a water-insoluble precipitate. Studies on the distribution detected the highest amount of radioactivity within the thyroid, presumably due to deiodination. Accumulation in the liver and the kidneys was thought to be caused by prodrug conversion since these organs are rich in alkaline phosphatase. Thus, to efficiently target cancer by EMIT, tumors have to express high levels of alkaline phosphatase or have to be pre-treated with a targeted alkaline phosphatase conjugate. Although EMIT is still in its infancy the method may develop future potential when applied to other prodrug-enzyme pairs.

The targeting of enzymes *via* carbohydrate-lectin interactions (**lectin-directed enzyme-activated prodrug therapy**, **LEAPT**, Fig. **2C**) has been described by Robinson *et al.* [39]. L-rhamnopyranose-capped doxorubicin is being used as prodrug. Due to the non-mammalian origin of L-rhamnopyranose, the prodrug is only cleaved by the pre-administered tumor targeted α-rhamnosidase. In a mouse model with hepatocellular carcinoma, LEAPT revealed 50% reduction in tumor burden. This method is similar to ADEPT but is based on engineered carbohydrates rather than antibodies for targeting. A similar targeting system has been termed **folate-directed enzyme prodrug therapy** (**FDEPT**, Fig. **2D**) where a folic acid enzyme conjugate is targeted to folate receptor-positive tumor cells [40, 41]. Compared to ADEPT and LEAPT, this method has the least flexibility in target adaptation. Another method belonging to this family of strategies is the targeting of tumor matrix, also referred to as **matrix attachment therapy** [42]. Central to this concept is a fusion protein consisting of the hyaluronan binding domain of tumor necrosis factor-α-induced protein 6 and cytosine deaminase as prodrug-converting enzyme. After administration of the prodrug 5-fluorocytosine, the authors observed a reduction in tumor growth in a colon adenocarcinoma mouse model.

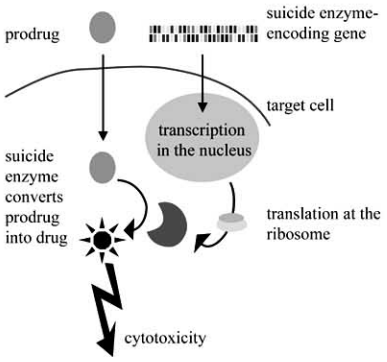
In **polymer-directed enzyme prodrug therapy** (**PDEPT**, Fig. **2E**), a polymeric prodrug and a polymer-enzyme-conjugate (e.g. a methacrylamide polymer may be conjugated to the prodrug and the enzyme) are targeted to solid tumors by the enhanced permeability and retention effect of macromolecules in tumor tissue [43, 44]. Due to the poorly organized vasculature within the tumor the permeability for circulating macromolecules is enhanced and the absence of a fully functional lymph system increases their tissue retention. In a melanoma mouse model, PDEPT resulted in decrease of tumor growth and showed no systemic toxicity due to non-specific prodrug activation. The authors emphasized that compared to ADEPT, the relatively short plasma residence time of the polymeric prodrug allows subsequent administration of polymer-enzyme without the problem of prodrug activation in the circulation. Moreover, the polymer-enzyme conjugates display a lower immunogenicity compared to antibody-enzyme constructs. Interestingly, in this system, physico-chemical rather than biological mechanisms confer the desired tumor specificity.

Huysmans *et al.* encapsulated nucleoside hydrolase from *Trypanosoma vivax* in liposomal nanocapsules (liposomes) containing porins for an efficient prodrug import and drug export through enhanced diffusion [45]. This method combines the benefits of ADEPT and liposome targeting strategies. As a major advantage compared to ADEPT, the encapsulation of the enzymes in liposomes efficiently solves the immunogenicity problem. However, the authors did not investigate the system for targeted liposomes, and no *in vivo* experiments have yet been presented.

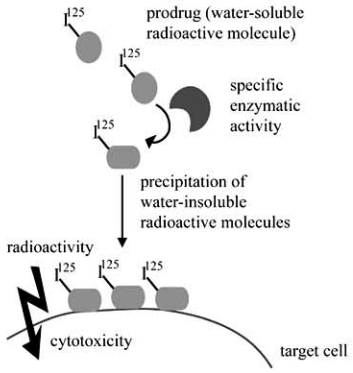
Targeting of prodrug-activating enzymes *via* activated natural killer cells was demonstrated by Yang *et al*. [46]. A prodrug-activating enzyme (alkaline phosphatase or β-galactosidase) was fused to a peptide transduction domain (also known as cell penetrating peptide) that enables cell membrane translocation resulting in loading of natural killer cells by alkaline phosphatase or β-galactosidase. In a lung metastasis model, based on melanoma cells, 24 h after injection of alkaline phosphatase-loaded natural killer cells alkaline phosphatase was found in fivefold higher concentration in the tumor compared to healthy tissue in mice. However, 48 h after injection of the loaded natural killer cells no alkaline phosphatase activity was detectable in the tumor anymore while the natural killer cells still reside within the tumor tissue. The authors claimed that this loss of enzymatic activity is only an experimental artifact due to proliferation of the natural killer cells, which would keep the enzyme within the tumor. Furthermore, based on the current data it is speculative whether the natural killer cells are killed by the approach and whether this may not even be counter-productive.

Another cell-based targeting system was published by Cavarretta *et al*. who used adipose tissue-derived mesenchymal stem cells because of their ability to track and engraft into tumors and micrometastases [47]. These cells were engineered for expression of cytosine deaminase/uracil phosphoribosyltransferase. The authors demonstrated substantial tumor growth inhibition in a prostate cancer mouse model. In essence, this system might be regarded a "mobile" variety of GDEPT, where not the tumor cell itself, but another cell type functioning like a circulating "Trojan horse" is genetically modified to express the prodrug-converting enzyme. In this, it overcomes the problem of specifically targeting the gene, which is the major limitation of GDEPT.

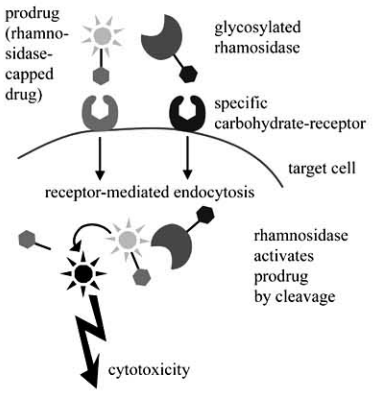
Genetically engineered, facultative anaerobic bacteria, such as *Salmonella typhimurium*, *Shigella flexneri* and *Escherichia coli* were examined for their use in cancer therapy because of their natural tumor targeting properties and their capability to express anti-tumor proteins, such as tumor necrosis factor-α [48], interleukin-2 [49] or cytosine deaminase. After the bacteria had accumulated at or inside the tumor, the expression of these effector proteins was



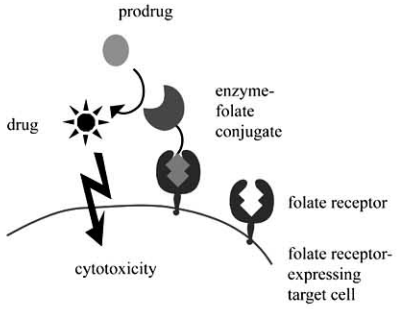
A. Gene-directed enzyme prodrug therapy (GDEPT)



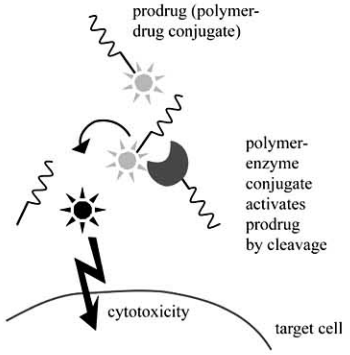
B. Enzyme-mediated insolubilization therapy (EMIT)



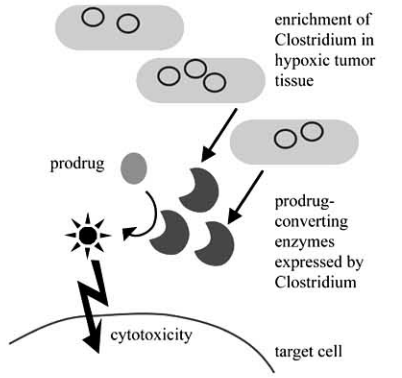
C. Lectin-directed enzyme-activated prodrug therapy (LEAPT)



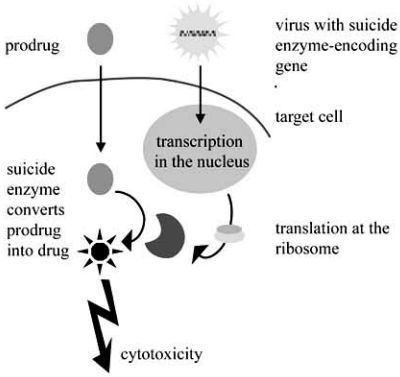
D. Folate-directed enzyme prodrug therapy (FDEPT)



E. Polymer-directed enzyme prodrug therapy (PDEPT)



F. Clostridial-directed enzyme prodrug therapy (CDEPT)



G. Virus-dependent enzyme prodrug therapy (VDEPT)

Fig. (2). Overview of directed enzyme prodrug therapies.

activated, dependent on the utilized promoter, by radiation [50, 51] or by non-toxic small molecules, such as L-arabinose, L-rhamnose or anhydrotetracycline [52, 53]. The targeting of the bacteria to the tumor and intratumoral growth likely results from the secretion of bacterial chemoattractants by quiescent tumor cells and from the presence of necrotic regions where bacteria preferably accumulate and replicate [54-56]. Detailed investigations on *S. typhimurium* demonstrated that it was attracted by chemotaxis to tumors that were excreting specific chemicals and the migration towards the tumor, the penetration and the accumulation in necrotic regions were dependent on its aspartate receptor, serine receptor and ribose/galactose receptor, respectively [57]. Furthermore, *S. typhimurium* grows preferentially in tumor tissue [54].

The administration of clostridial spores leads to accumulation and germination in hypoxic and necrotic tumor tissue, which are the basis of another promising tumor targeting strategy, **clostridial-directed enzyme prodrug therapy** (**CDEPT**, Fig. **2F**) which was first described by Minton *et al.* [58]. In mouse models the administration of attenuated bacteria of the species *S. typhimurium* and *Clostridium sporogenes* expressing prodrug converting enzymes or direct effectors resulted in marked anti-tumor efficacy [50, 59, 60]. However, in clinical phase I studies the treatment of patients with attenuated *S. typhimurium* failed to achieve tumor regression [61, 62]. Both bacterial concepts are remarkable for their built-in amplification system of the effector protein, which may resolve quantitative efficiency problems incurred with other pre-targeting strategies. Still, in our view it remains doubtful whether this biophysical targeting mechanism will be sufficiently precise, as other tissues of relative hypoxemia may also become harbors for these modified bacteria, leading to potentially devastating effects e.g. in patients with coronary heart disease and other conditions.

PRODRUG-CONVERTING ENZYMES

Tumor heterogeneity in patients expedited the development and choice of different prodrugs and their converting enzymes. In order to achieve high cytotoxicity specifically at the tumor site when administrating, targeted prodrug-converting enzyme approaches, many studies were performed with yeast or bacterial enzymes such as cytosine deaminase and β-lactamase, respectively [63, 32]. Non-human enzymes offer high specificity once directed to tumor tissue. By contrast, human enzymes can activate circulating prodrugs wherever they are naturally located [1]. To overcome the immunogenicity of non-human proteins, de-immunizing mutations of highly immunogenic epitopes have often been introduced as in other protein-based approaches such as immunotoxins [64]. In one such study in CB6F1 mice, a mutant β-lactamase induced five fold less β-lactamase-specific immunoglobulin G1 than the wild-type enzyme [65]. β-lactamase converts lactam-containing prodrugs into commonly used anti-cancer drugs (e.g. doxorubicin, melphalan). In ADEPT, β-lactamase-cleavable prodrugs showed promising results in the treatment of lung adenocarcinoma [66], melanoma [67], and colon carcinoma xenografts [63]. The high potential of bacterial enzymes is also demonstrated by the persistent β-lactamase activity detectable in mouse tumor xenografts one week after injection [63]. This offers the possibility to administer a single dose of the β-lactamase fusion protein followed by multiple prodrug injections, which should result in an enhanced cytotoxic effect on tumor cells.

The immunogenicity of other bacterial proteins, e.g. carboxypeptidase G2, was a limiting factor in earlier ADEPT studies [68]. Carboxypeptidase G2 converts prodrugs such as CMDA [69] or ZD2767P [70] into DNA cross-linking agents. Mayer *et al.* developed a carboxypeptidase G2 double mutant by modification of a known B cell-recognized epitope to reduce immunogenicity [71]. A fusion protein consisting of the single chain antibody fragment MFE-23 and mutant carboxypeptidase G2 led to a reduced immune response in BALB/c mice compared to a fusion protein with the wild-type enzyme, which may allow for multiple fusion protein administrations to enhance the cytotoxic effect. A strategy for improving the efficacy of carboxypeptidase G2 was pursued by Marais *et al.*, who genetically engineered bacterial carboxypeptidase G2 so as to achieve a fully active enzyme tethered to the surface of transfected cancer cells [69]. The treatment with the prodrug CMDA yielded tumor regression as well as cure in nude mice bearing breast cancer xenografts expressing membrane-bound carboxypeptidase G2. The promising anti-cancer efficacy is probably enhanced by a greater bystander effect and humoral immune response due to its extracellular localization.

Heterogeneity of antigen expression on tumor cells is an obstacle to immunologically targeted therapies that prodrug-converting enzyme therapies avoid by a potent bystander effect. An example is ADEPT with human β-glucuronidase in combination with the murine antibody 323/A3, which showed a regression of human ovarian cancer xenografts in mice after subsequent treatment with the glucuronide doxorubicin prodrug DOX-GA3 [72]. DOX-GA3 is hydrophilic, remains outside the cells and is only activated into a membrane-permeable drug if β-glucuronidase is administered extracellularly, because endogenous human β-glucuronidase is naturally restricted to microsomes and lysosomes. The generation of the cytotoxic drug in the extracellular space of the tumor enabled its diffusion into neighboring cells, constituting a bystander effect.

The herbal β-glucosidase cleaves the cyanogenic glycoside amygdalin, which results in the release of cytotoxic cyanide [73]. The efficacy of this enzyme/prodrug system in ADEPT was investigated in bladder cancer cells *in vitro* [74]. Linamarase, another herbal β-glucosidase, cleaves its native substrate linamarin and releases gaseous hydrogen cyanide. Linamarin is not hydrolyzed by most mammalian tissues [75], so this might be an appropriate prodrug system for tumor therapy. Link *et al.* used protein-transducing nanoparticles for the application of linamarase in rodent and human breast cancer cells (4T1 and MCF-7, respectively) and obtained a significant reduction of microtissue diameters and extensive cell death *in vitro*, using tumor spheroids as a model [76]. Moreover, the authors demonstrated a significant decrease in tumor growth in nude mice injected with linamarase-transduced 4T1-cells, and obtained a correlation between decrease in tumor growth and cyanide level. The effectiveness of this system is based on a substantial bystander effect of gaseous hydrogen cyanide that freely dif-

fuses through cell membranes. For instance, most HepG2 cells were killed in a cell culture experiment although only 10% of the cells were transfected with linamarase [77]. The linamarase/linamarin system causes inhibition of ATP synthesis by hydrogen cyanide-mediated inhibition of cytochrome C. Cell death mainly occurs by necrosis in this system [77], since ATP is required for apoptotic programs. This is a crucial advantage for the treatment of hepatocellular carcinoma, which is well known for apoptotic resistance [78].

Within the last years, protein-based targeted tumor therapies took a turn in that human proteins came into the focus of development to reduce immunogenicity. For instance, the expression of mutant human liver carboxylesterase instead of the homologous rat enzyme [79] has been accomplished for *in vivo* treatments [80]. Carboxylesterase converts camptothecin derivatives, such as CPT-11 into SN-38, a topoisomerase I inhibitor [81]. *In vivo* experiments with human astrocytoma cells transfected with mutant human carboxylesterase showed a complete elimination of tumors in SCID mice either with 5 or 10 mg CPT-11 per kilogram body weight [80]. This result supports the eligibility of mutant human enzymes for targeted tumor therapy. Nevertheless the use of human enzymes is challenging. If the prodrug is membrane-permeable or the enzyme is naturally located extracellularly, the risk of systemic toxicity is substantially increased. Jounaidi *et al*. developed a SCID-mice xenograft with rat gliosarcoma 9L cells transduced with six different human cytochrome P450 enzymes [82]. These enzymes convert oxazaphosphorines, such as cyclophosphamide into DNA cross-linking agents. The co-transduction of CYP450 2B6 and P450 reductase in 9L-xenografts resulted in a remarkable tumor growth delay after cyclophosphamide treatment. The bystander effect of cyclophosphamide in P450-gene therapy-approaches is immense and mediated by its membrane-permeable, cytotoxic 4-hydroxy-metabolites [83]. To overcome the obstacle of systemic toxicity by prodrug activation in human liver, Huang *et al*. utilized methimazole, an inhibitor that yielded an inhibition of human liver P450 reductase transcription of about 75% without affecting the tumor cells [84]. The use of low K_M P450 enzymes in combination with intratumoral prodrug injection and slow drug releasing cyclophosphamide polymers, is another possibility to decrease systemic cytotoxicity [85].

Furthermore, the amplification of several human enzymes by artificial and targeted overexpression in tumor cells became a promising opportunity. For instance the DT-diaphorase (also named NAD(P)H:quinone oxidoreductase), an obligate two electron reductase, which equally catalyzes the oxidation of both NADH and NADPH [86] was used in several gene therapy approaches [87-89]. However, the enzyme activities measured in lysates of DT-diaphorase - transduced tumor cells derived from *in vivo* studies did not achieve the activity seen *in vitro* [88]. DT-diaphorase converts quinones, such as mitomycin C into toxic agents, which can cause DNA alkylation. The human breast cancer cell line MDA468 showed a mitomycin C sensitization *in vitro* as well as in nude mice xenografts and the greatest sensitivity to DNA alkylating agents in comparison to all other investigated breast cancer cells in this study [88]. This indicates the necessity, as for any other modern pharmaceutical therapy approach, to adjust the enzyme/prodrug system to the type of tumor cells in order to achieve successful tumor regression.

The human zinc metalloproteinase carboxypeptidase A1 is synthesized and secreted by pancreas cells and needs to be cleaved by trypsin to gain its catalytic activity. To overcome the trypsin-dependency Hamstra *et al*. developed a mutant that includes a paired basic amino acid cleaving enzyme cleavage site [90]. The catalytic function of secreted, paired basic amino acid cleaving enzyme-activated carboxypeptidase A1 did not measurably alter in comparison to the activated wild type enzyme. As a result the methotrexate-α-peptide prodrug MTX-Phe was efficiently cleaved, which yielded MTX-Phe-sensitization. The diffusion of carboxypeptidase A1 into the tumor matrix may result in a less cytotoxic effect and increased systemic toxicity [91]. Therefore, the authors developed two mutants of carboxypeptidase A1 that are both activated by intracellular propeptidases, one secreted form and the other one located at the cell surface. However, the hypothesis on loss in cytotoxicity could not be corroborated since the bystander effect caused by secreted carboxypeptidase A1 appeared earlier and to a greater extent compared to the tethered carboxypeptidase A1 at equal prodrug concentrations [91]. This result was obtained by two-chamber, six-well tissue culture, which does not represent physiological tumor conditions *in vivo*. Therefore, additional studies in animal xenografts are necessary to clarify whether systemic effects occur and the bystander effect caused by secreted carboxypeptidase A1 is reliable.

One approach to increase the impact of prodrug-converting enzyme therapies is the fusion of various enzymes, which may lead to additive or even synergistic anti-tumor effects. Bernt *et al*. constructed a replication-activated adenovirus vector to express a secreted form of human β-glucuronidase and a fusion protein of bacterial cytosine deaminase and uracil phosphoribosyl transferase [92]. They demonstrated that the transgene expression, the activation of the prodrugs and the cytotoxicity to tumor cells are all adenovirus replication-dependent. Unfortunately, significant anti-tumor activity was only seen in combination with chemotherapy. However, the combination of cytosine deaminase and uracil phosphoribosyl transferase was successfully used in another approach for the expression in prostate cancer cells and applied in immunocompetent mice [93]. The authors showed that only 20% of the tumor cells have to express cytosine deaminase-uracil phosphoribosyl transferase to yield complete tumor regression, indicating a notable bystander effect. Cytosine deaminase converts the prodrug 5-fluorocytosine into 5-fluorouracil, which is converted by intracellular enzymes into the fluorinated nucleotides 5-fluorouridine-2-triphosphate or 5-fluoro-deoxyuridine monophosphate that inhibit the RNA- and DNA-synthesis, respectively. Uracil phosphoribosyl transferase directly converts 5-fluorouracil into cytotoxic fluorouridine monophosphate, which prevents 5-fluorouracil from degradation and detoxification. It is worthwhile to know this mechanism to utilize this enzyme/prodrug system for treatment of the proper tumor, because other than 5-fluorouracil, fluorouridine monophosphate can not cross membranes. Therefore, the de novo-synthesis of uracil phosphoribosyl

transferase may attenuate the bystander effect in tumors, whose cells exhibit a lack of gap junctions [94]. Only 2% of prostate cancer cells are dividing cells [95], therefore prodrugs that kill both dividing and non-dividing cells are required. 5-fluorocytosine meets this requirement and thus may contribute to success, e.g. one of the latest studies with yeast cytosine deaminase and uracil phosphoribosyl transferase expressed in mesenchymal stem cells demonstrated a complete regression of prostate tumors by targeting the fusion protein to prostate cancer cells [47].

Additionally, cytosine deaminase was fused to thymidine kinase from herpes simplex virus that activates the prodrug and guanine analogue ganciclovir. The recombinant adenoviral transduction of bacterial cytosine deaminase and herpex simplex virus thymidine kinase under control of the human heat shock protein 70 promoter into the prostate carcinoma cells PC-3 resulted in a heat-induced expression of the fusion protein and significantly reduced PC-3 survival [96]. At the highest multiplicity of infection (10 plaque-forming units/cell) the combined ganciclovir/5-fluorocytosine treatment showed much higher cytotoxicity than singular treatments. Furthermore, this treatment resulted in supra-additive enhancement of PC-3-radiosensitization [97]. Both treatments represent the approach of a tritherapy, where enzyme/prodrug systems were expanded by the use of heating or radiosensitization. Another promising tritherapy is the association of gene silencing to enzyme/prodrug systems. The gene silencing-targeted genes are coding for enzymes that detoxify the prodrug and thus may increase the success of a prodrug-converting enzyme therapy. Réjiba *et al*. used a fusion protein of the enzymes deoxycytidine kinase and uridine monophosphate kinase and combined it with two small interference RNAs for the treatment of pancreatic cancer [98]. The tritherapy decreased the IC_{50} of the prodrug combination down to 40-fold in Panc1 cells and reduced tumor volumes joined with significantly prolonged mice survival. All these results show that the use of different prodrug-converting enzymes and prodrugs in combination with other approaches, such as heating, radiosensitization, or gene silencing has a high potential for tumor therapy. Even though numerous directed enzyme prodrug therapy approaches have been developed (Table **2**) continuative studies have to be done to expand the knowledge of synergistic anti-tumor effects.

ENZYME DELIVERY BY TARGETED GENE THERAPY

The idea to use targeted gene therapy to treat cancer was intensively followed since the 1990s. This system is based on the delivery of a gene for a prodrug-converting enzyme to tumor cells. The terms commonly used for the genetic manipulation of tumor cells are GDEPT or suicidal gene therapy. When using a virus for gene delivery, some publications referred to **virus-dependent enzyme prodrug therapy** (**VDEPT**, Fig. **2G**). GDEPT is the superordinate concept and this term has been most commonly used in the last 10 years in the literature, even for those projects using viruses for the delivery of the prodrug-converting enzyme. However, we will use the proper term VDEPT in our review for suitable projects.

In numerous studies the cDNA encoding the prodrug-converting enzymes was transfected in tumor cells and the increased sensitivity of the enzyme-expressing cells upon incubation with the corresponding prodrug was analyzed. These studies were performed for the carboxypeptidases A1 and G2, herpes simplex thymidine kinase, tyrosinase and others [90, 104, 118, 109]. While these studies lack the relevance of gene therapy in a way to deliver the cDNA for the prodrug-converting enzymes specifically only to tumor cells, they provide systems to study the suitability of certain enzymes as prodrug-converting enzymes and allow efficacy studies on derivatives of prodrugs. Furthermore, these *in vitro* transfection studies provide the possibility to test systems for selective expression of prodrug-converting enzymes only in tumor cells.

Several studies were performed with prodrug-converting enzymes under the control of tumor-specific promoters. The expression of herpes simplex virus thymidine kinase was evaluated under the control of a promoter only active in p53 negative tumor cells [119]. Cytosine deaminase from yeast and *E. coli* were expressed under the control of a hypoxia-dependent promoter and a carcinoembryonic antigen-dependent promoter [120, 121]. The latter increased the sensitivity to 5-fluorocytosine only for cell lines that expressed the carcinoembryonic antigen after transfection. The authors thus concluded that this system has a low specificity. However, in responding cells up to 40-fold differences in prodrug sensitivity were observed. This should be a sufficient difference between target cells and non-target cell, since another publication demonstrates a 20-fold increased sensitivity, which is sufficient to achieve tumor growth inhibition in a mouse model [107]. Zhou *et al*. developed a GDEPT system for the expression of *E. coli* purine nucleoside phosphorylase under the control of human telomerase reverse transcriptase promoter and Plumb *et al*. used this promoter and the human telomerase RNA gene for the tumor-specific expression of *E. coli* nitroreductase and achieved substantial tumor shrinkage in a mouse model [122, 107]. Furthermore, the promoter for the transcription factor achaete-scute homolog 1 was successfully used to achieve tumor cell-specific expression of *E. coli* purine nucleoside phosphorylase and cytotoxicity upon prodrug application [123]. In another study the herpes simplex virus thymidine kinase was expressed under the control of a human papilloma virus-specific promoter to achieve only elimination of HPV-infected cancer cells [124].

Hayes *et al*. introduced another option for specific activation of prodrug-converting enzymes named plasmid-based splice-activated gene expression [125]. Its rationale is based on the transfection of a plasmid containing alkaline phosphatase, which is only transcribed in tumor cells that exhibit alternative splicing. The rather artificial cell culture systems were in addition used to evaluate the potential of combination of GDEPT and radiation therapy. This was either achieved by using radiation-sensitive promoters to activate prodrug-converting enzyme expression by radiation [126] or by combination of a hypoxia-sensitive promoter for the expression of the cytochrome P450 reductase and radiation of radiation-resistant tumors in mice [127, 126]. The combination resulted in 50% tumor-free mice after 100 days; however, the authors failed to analyze the curable effect

Table 2. Survey of Directed Enzyme Prodrug Therapies

Method	Enzymes	Fusion protein	Prodrug	Drug	Reference
ADEPT	CD	A33scFv::CDy	5-fluorocytosine	5-fluorouracil	[32]
	CPA	Anti-γ-SM-scFv/hCPA	MTX-∝-peptide	MTX	[36]
	CPA	huA33-CPA	MTX-∝-peptide	MTX	[26]
	CPG2	A5B7-CPG2	ZD2767P	ZD2767D	[71]
	CPG2	MFECP	ZD2767P	ZD2767D	[70]
	CPG2	ICR12-CPG2	CMDA	benzoic acid of CMDA	[34]
	β-galactosidase	HuCC49ΔCH2-β-ga-lactosidase	17-AG-C2-Gal	17-AG-C2	[99]
	β-glucosidase	HMFG1-β-glucosidase	amygdalin	benzaldehyde, cyanide	[74]
	β-glucuronidase	323/A3-β-glucuro-nidase	glucuronide derivative of doxorubicin	doxorubicin	[72]
	β-glucuronidase	chTNT-3 Fab/betaG	glucuronide derivative of doxorubicin	doxorubicin	[37]
	β-lactamase	L49-sFv-bL	C-Mel	melphalan	[67]
	β-lactamase	Tab2.5	GC-Mel	melphalan	[63]
GDEPT	CD		5-fluorocytosine	5-fluorouracil	[100]
	CD, UPRT	CD-URPT	5-fluorocytosine	5-fluorouracil, pyrimidine antimetabolites	[93, 101, 102]
	CPA	CPA 95	MTX-∝-peptide	MTX	[90]
	CPG2	CPG2	CMDA	benzoic acid of CMDA	[103]
	CPG2	CPG2*, stCPG2	CMDA	benzoic acid of CMDA	[69, 104]
	CYP450	CYP450 3A4	AQ4N	AQ4	[105]
	HRP		indole-3-acetic acid	3-methylene-2-oxindoles	[106]
	NTR		CB195	5-(aziridin-1-yl)-4-N-acetoxy-2-nitrobenzamide	[107]
	PNP		fludarabine phosphate	fludarabine	[108]
	tyrosinase	tyrosinase, truncated tyrosinases	HPP, NAsSCAP	quinones	[109]

(Table 2). Contd…..

Method	Enzymes	Fusion protein	Prodrug	Drug	Reference
VDEPT	CD, HSV-TK	CD-TK	5-fluorocytosine, ganciclovir	5-fluorouracil, ganciclovir triphosphate	[96, 97]
	CD, URPT	CD-URPT	5-fluorocytosine	5-fluorouracil, pyrimidine antimetabolites	[92]
	CE		CPT-11	SN 38	[80, 110]
	CPA	CPA ST3, CPA DAF	MTX-∝-peptide	MTX	[91]
	CPG2		ZD2767P	ZD2767D	[111]
	CYP450	CYP450 2B, 2C8, 2C9, 2C18, 2C19, and 3A4	cyclophosphamide, ifosfamide	phosphoramide mustard, isophosphoramide mustard	[82]
	dCK, UMK	dCK::UMK	gemcitabine	cytotoxic gemcitabine metabolites	[98]
	DTD		mitomycin C	cytotoxic mitomycin C metabolites	[88]
	HSV-TK		ganciclovir	ganciclovir-triphosphate	[112]
	linamarase		linamarin	cyanide	[77]
	NTR	CTL102	CB1954	5-(aziridin-1-yl)-4-N-acetoxy-2-nitrobenzamide	[113]
	P450R		mitomycin C	cytotoxic mitomycin C metabolites	[88]
			tirapazamine	reactive free radical intermediate	[114]
	PNP		6MPDR	6MP	[115]
			fludarabine phosphate	fludarabine	[116]
	β-glucuronidase		9-amino-camptothecin glucuronide	9-amino-camptothecin	[92, 117]

Abbreviations: 17-AG-C2, toxic geldanamycin derivate; 17-AG-C2-Gal, Galactose-amine derivate of geldanamycin at the C17-position; 323/A3, monoclonal antibody against pancarcinoma antigen epithelilal transmembrane glycoprotein; 6MPDR, 6-methylpurine deoxyriboside; A5B7, Fab'(2)-fragment of anti-carcinoembryonic antigen antibody; Anti-γ-SM-scFv/hCPA, anti-seminoprotein single-chain fragment/human CPA fusion protein; CB1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide; CD, cytosine deaminase; CE, carboxylesterase; chTNT-3 Fab, chimeric tumor necrosis therapy antigen binding fragment; CPA, carboxypeptidase A; CPG2, carboxypeptidase G2; CK, deoxycytidine kinase; CMDA, 4-[(2-chloroethyl)(2-mesyloxyethyl)amino] benzoyl-l-glutamic acid; CPT-11, irinotecan-7-ethyl-10-[4-(1-piperidina)-1-piperidino]carbonyloxycamptothecin; CTL102, bacterial nitroreductase; CYP450, cytochrome P450; dCK, deoxycytidine kinase; DOX-GA3, N-[4-doxorubicin-N-carbonyl (oxymethyl) phenyl] O-β-glucuronyl carbamate; DTD, DT-diaphorase; GC-Mel, glutaryl cephalosporin melphalan; HuCC49ΔCH2, anti-TAG 72 antibody fragment; HMFG1, monoclonal antibody against polymorphic epithelial mucin; HPP, hydroyphenyl-propanol; HRP, horseradish peroxidase; HSV, herpes simplex virus; ICR12, anti-human c-erbB2 monoclonal antibody; L49-sFv-bL, anti-human p97 melanotransferrin single-chain fragment-β-lactamase fusion protein; MFECP, anti-carcinoembryonic antigen single-chain fragment-carboxypeptidase G2 fusion protein; MTX, methotrexate; NAsSCAP, N-acetyl-4-S-cysteaminylphenol; NTR, nitroreductase; PNP, purine nucleoside phosphorylase; P450R, NADPH:cytochrome P450 oxidoreductase; SG, β-glucuronidase; SN-38, 7-ethyl-10-hydroxycamptothecin; Tab2.5, single-chain fragment (based on CC49 antibody)-β-glucuronidase fusion protein; UMK, uridine monophosphate kinase; UPRT, uracil phosphoribosyltransferase; ZD2767D, 4-[N,N-bis(2-iodoethyl)amino]phenol; ZD2767P, 4-[N,N-bis(2-iodoethyl)amino] phenoxycarbonyl L-glutamic acid.

mediated by GDEPT alone. A number of studies concentrated on cytochrome P450s and the NADPH-cytochrome P450 reductase. While one study achieved a weak increase of survival of tumor-bearing mice upon coexpression of both enzymes in combination with radiation or cyclophosphamide [128], two other studies of the same group revealed no additional effects of the coexpression in animal studies [105, 129]. These results demonstrate that the role of NADPH-cytochrome P450 reductase in GDEPT is being discussed and still uncertain. The benefit from NADPH-cytochrome

P450 reductase coexpression is most likely dependent on cytochrome P450, the cell line and the prodrug [130].

Cell culture-based studies allow efficient analyses of new improvements for GDEPT. Two different groups worked on ways to improve the impact of enzyme prodrug therapies by directing β-glucuronidase to the cell membrane. Heine *et al*. expressed a fusion of the lysosomal protease with the transmembrane domain of platelet-derived growth factor receptor and Chen *et al*. fused the same enzyme to immunoglobulin domains [131, 117]. The membrane localization of the enzymes increased prodrug conversion and resulted in efficient tumor growth inhibition in mice. Benouchan *et al*. studied the impact of *E. coli* nitroreductase expression in a 3D cell culture model and revealed detailed information on the bystander effect on non-target cells upon prodrug treatment [132]. In a following publication the authors demonstrated that the bystander effect is sufficient to inhibit tumor growth significantly by intratumoral injection of transfected prodrug-sensitive cells into developed non-sensitive tumors [133]. *E. coli* cytosine deaminase-transfected tumor cells were used to study more efficient delivery systems for the prodrug 5-fluorocytosine [100]. The authors demonstrated a liposomal formulation that decreased tumor growth more efficiently than free 5-fluorocytosine upon intravenous injection in a mouse model. Kang *et al*. recently proposed a more efficacious system for stable integration of prodrug-converting enzyme genes into the genome of tumor cells to increase GDEPT [134]. Furthermore, a number of prodrug-converting enzyme combinations were studied upon transfection of tumor cells with the enzyme-encoding cDNAs. Gopinath *et al*. demonstrated in 2008 the combined transfection of cells with *E. coli* cytosine deaminase and cytosine deaminase-uracil phosphoribosyltransferase, an enzyme that converts the nascent drug 5-fluorouracil into further toxic metabolites. The study demonstrated a slight increase in sensitivity upon expression of both enzymes [101]. In a recent study the cDNA for a fusion protein of deoxycytidine kinase and uridine monophosphate kinase was transfected into tumor cells and the prodrug sensitivity compared to cells additionally injected with small interference RNA against ribonucleotide reductase and thymidylate synthase [98]. The expression of this fusion protein resulted in efficient activation of the prodrug gemcitabine. *In vivo* studies revealed most potent tumor growth inhibition by a combination of the two transfected genes and the small interference RNA. An adenoviral system was used for the transduction of the tumor cells with the gene fusion. Adenoviral delivery systems were furthermore used in a study utilizing a fusion gene of cytosine deaminase and herpes simplex virus thymidine kinase [135]. The specific delivery of DNA to tumor cells is the main problem GDEPT currently faces. Although viral delivery systems usually lack tumor-specificity unless modified, viruses have been used due to their high transduction efficiency.

Replication-deficient adenoviruses are most often used for studies on VDEPT. The most common protocol for viral application is intratumoral injection, which limits the transduction events to the tumor and limits liver targeting of adenoviruses. However, in order to access metastatic cells or small tumors, systemic application would be desirable. Therefore highly tumor-specific delivery systems and/or expression systems are mandatory. A number of approaches were presented in the last years to overcome this limitation. Lipinski *et al*. described a β-catenin-dependent promoter for the expression of *E. coli* nitroreductase that resulted in highly tumor-specific nitroreductase expression [136]. Another group tried to combine the prostate-specific rat probasin gene promoter with the viral simian vacuolating virus 40 enhancer to increase the expression of *E. coli* purine nucleoside phosphorylase [115]. Although the expression was 20-fold increased in prostate cancer cell lines, the impact in a mouse model was rather weak. Cowen *et al*. delivered the NADPH-cytochrome P450 reductase under the control of a hypoxia-dependent promoter by adenoviruses, however, the overexpression of the reductase resulted only in a substantial and significant tumor growth inhibition upon combination of prodrug application and radiotherapy [114]. The expression of carboxylesterase under a hypoxia-dependent promoter resulted in a delayed tumor growth even without radiotherapy [110]. A number of studies revealed successful delivery of prodrug-converting enzyme-coding sequences to tumor cells and the tumor-specific expression of the enzymes under the control of promoters for carcinoembryonic antigen, prostate-specific membrane antigen and human telomerase reverse transcriptase [137-139].

In order to increase the immunologic response of the host on the side of tumor cell death, one group developed a combined expression of *E. coli* nitroreductase and heat shock protein 70 [140]. The coexpression induced a strong immune response in addition to the cell death mediated by nitroreductase upon prodrug application, preventing tumor outgrowth even more efficiently. Another approach involved the growth of cytosine deaminase- and uracil phosphoribosyltransferase-transfected tumor cells and subsequent adenoviral delivery of the genes for interleukin-12 and -18 [141]. The prodrug therapy in combination with adenoviral delivery for both interleukins resulted in efficient tumor growth reduction and significant reduction of lung metastases. Thus immunological tools support the positive effects of the prodrug therapy and should be considered for further studies.

Several publications in the last years presented ideas to target adenoviruses specifically to tumors or to use conditionally replicating adenovirus variants. These oncolytic variants can only replicate and lyse tumor cells when certain tumor-specific promoters are activated. The variant Onyx-017 replicates only in p53-deficient cells and was combined in a study with the coding sequence for a cytochrome P450 and NADPH-cytochrome P450 reductase [142]. This combination of enzymes and conditionally replicating adenoviruses inhibited tumor growth efficiently. Schepelmann *et al*. showed very clearly a gain in tumor growth inhibition by combining conditionally replicating adenoviruses and carboxypeptidase G2 expression in a mouse model [111]. Another comparable approach was presented by delivery of herpes simplex thymidine kinase or carboxylesterase genes by oncolytic adenoviruses [143]. Surprisingly the authors detected adverse effects of thymidine kinase expression and prodrug therapy, while carboxylesterase-mediated prodrug therapy augmented tumor cell killing. However, low prodrug concentrations inhibited adenovirus propagation, but higher

dosages resulted in strong enhancer effects of the prodrug therapy. Another issue with adenoviruses for gene therapy was reported in a study by Palmer *et al*. [113]. The authors observed activation of NF-κB and increased survival even after prodrug therapy. Only inhibition of NF-κB resulted in successful prodrug therapy.

Since the bystander effect is the most important advantage of the prodrug therapy, the following two studies analyzed this effect in more detail. The adenoviral-mediated expression of *E. coli* purine nucleoside phosphorylase inhibited tumor growth efficiently in a mouse model with tumor cells expressing high levels of the prodrug-converting enzyme [116]. However, analyses of the bystander effect with tumors consisting mainly of non-transformed cells resulted in comparable tumor growth inhibition. This is very remarkable, since these results demonstrate the potential of prodrug therapies *in vivo*. Furthermore, these observations prove that the successful transduction of a portion of tumor cells is sufficient to mediate tumor growth inhibition. Lee *et al*. followed a very interesting and successful idea to further increase the bystander effect. They fused yeast cytosine deaminase to a cell-penetrating peptide, the herpes simplex virus-1 tegument protein vp22, to allow adenoviral transduction of tumor cells and subsequent expression of the fusion protein [144]. The cell-penetrating peptide is able to leave the cells and delivers the prodrug-converting enzyme to cells in the vicinity, thus increasing the sensitivity of neighboring cells to the applied prodrug. The authors demonstrated the successful sensitizing of cells in a mouse model. The receptor responsible for initial adenovirus binding for subsequent uptake is the coxsackie virus-adenovirus receptor, and its low expression reduces transduction efficacies of VDEPT.

Gupta *et al*. followed an approach to develop adenoviruses exposing the fibroblast growth factor ligand to direct more efficient and specific transduction of fibroblast growth factor overexpressing tumor cells [112]. With this VDEPT approach using herpes simplex thymidine kinase, the authors achieved up to 80% successful transduction and reduced growth of transducted cells upon prodrug application in cell culture experiments. In a related very elegant work, Li *et al*. combined conditionally replicating adenoviruses (herpes simplex thymidine kinase expression dependent on cyclooxygenase-2 in tumor cells) with a fusion protein consisting of the ectodomain of the coxsackie virus-adenovirus receptor and a single chain antibody against carcinoembryonic antigen [145]. The fusion protein directed the viruses to hepatocarcinoma cells and efficiently reduced liver toxicity and tumor growth.

A number of further studies utilized other viral delivery systems like herpes viruses [146], sindbis viruses [147] or retroviral systems [148]. However, since viral delivery systems are still thought to be unsafe, further improvements for site-directed means of gene delivery are urgently needed to allow a better progress of GDEPT. Aoi *et al*. presented a viral-free local tumor transfection method by using ultrasound to generate microbubbles [149]. However, this method is only useful for local tumors and will not transfect metastases.

CONCLUSION

Within the last twenty years many approaches have been followed to develop safe and effective strategies for the treatment of cancer. Along the way the directed enzyme prodrug therapy based on ADEPT or on GDEPT kept the temper of progress. The basic ideas for targeted prodrug-converting enzyme therapies have already been developed some decades ago. However, severe problems have slowed down the progress some years ago. While the first therapeutic approaches failed and resulted in disappointment in the idea of designing "magical bullets" for tumor therapy, a better understanding of the highly complex requirements for successful anti-tumor drugs has been gained. The main problems for ADEPT and other extracellular targeting approaches are immunogenicity of the proteins and insufficient tumor killing. On the other side, GDEPT and other gene-therapeutic approaches often lack the ability to deliver the encoding DNA for the prodrug-converting enzymes specifically to tumor cells *in vivo*. Many new approaches were presented to overcome these problems, and we expect further developments in the near future. We anticipate clinical studies for ADEPT or GDEPT to be initiated soon to propose efficient, innovative, tumor-specific and less toxic anti-tumor therapies.

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ABBREVIATIONS

A33scFv = a single chain antibody fragment against gpA33

ADEPT = antibody-directed enzyme prodrug therapy

CDEPT = clostridial-directed enzyme prodrug therapy

EMIT = enzyme-mediated insolubilization therapy

FDEPT = folate-directed enzyme prodrug therapy

GDEPT = gene-directed enzyme prodrug therapy

gpA33 = glycoprotein A33

LEAPT = lectin-directed enzyme-activated prodrug therapy

PDEPT = polymer-directed enzyme prodrug therapy

VDEPT = virus-dependent enzyme prodrug therapy

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