



Review

Prodrugs: A challenge for the drug development

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Abstract:

It is estimated that about 10% of the drugs approved worldwide can be classified as prodrugs. Prodrugs, which have no or poor biological activity, are chemically modified versions of a pharmacologically active agent, which must undergo transformation *in vivo* to release the active drug. They are designed in order to improve the physicochemical, biopharmaceutical and/or pharmacokinetic properties of pharmacologically potent compounds. This article describes the basic functional groups that are amenable to prodrug design, and highlights the major applications of the prodrug strategy, including the ability to improve oral absorption and aqueous solubility, increase lipophilicity, enhance active transport, as well as achieve site-selective delivery. Special emphasis is given to the role of the prodrug concept in the design of new anticancer therapies, including antibody-directed enzyme prodrug therapy (ADEPT) and gene-directed enzyme prodrug therapy (GDEPT).

Key words:

prodrugs, drugs' metabolism, blood-brain barrier, ADEPT, GDEPT

Abbreviations: AADC – amino acid decarboxylase, ACE – angiotensin converting enzyme, ADEPT – antibody-directed enzyme prodrug therapy, BBB – blood-brain barrier, CNS – central nervous system, COMT – catechol-*O*-methyltransferase, GDEPT – gene-directed enzyme prodrug therapy, GPAT – gene prodrug activation therapy, LAT1 – large neutral amino acid transporter, L-DOPA – L-dihydroxyphenylalanine, LHRH – luteinizing hormone-releasing hormone, MCT – monocarboxylic acid transporter, PEPT – peptide transporter, PMSA – prostate membrane specific antigen, SDMT – sodium-dependent multivitamin transporter, VDEPT – virus-directed enzyme prodrug therapy

Introduction

During the last two decades, there has been a steady improvement in the physicochemical, biopharmaceutical and/or pharmacokinetic properties of pharmaco-

logically active compounds by the implementation of a prodrug strategy. It is estimated that currently about 10% of worldwide marketed drugs can be classified as prodrugs. Moreover, in 2008, one third of all approved small molecular weight drugs were prodrugs [35]. The first compound fulfilling the classical criteria of a prodrug was acetanilide, introduced (under the name of Antifebrin[®]) into the medical practice by Cahn and Hepp in 1867 as an antipyretic agent. In the body, acetanilide is hydroxylated (aromatic hydroxylation) to biologically active acetaminophen (paracetamol), the compound endowed with both antipyretic and analgesic activity. Acetaminophen can be also formed in the process of *O*-dealkylation of another analgesic drug – phenacetin (acetophenetidin), introduced into clinical use in 1887 by von Mering. Acetanilide and phenacetin were not originally designed as prodrugs, but their prodrug nature was de-

terminated later on. Another example of a historical prodrug is aspirin (acetylsalicylic acid), synthesized in 1897 by Felix Hoffman (Bayer, Germany), and introduced into medicine by Dreser in 1899 [12]. However, the prodrug concept was intentionally used for the first time in the middle of the 20th century by the Parke-Davis company during studies on modification of chloramphenicol structure in order to improve the antibiotic's bitter taste and poor solubility in water. As a result of this work, two prodrug forms of chloramphenicol were synthesized: chloramphenicol sodium succinate with a good water solubility, and chloramphenicol palmitate used in the form of suspension in children [71].

Prodrug concept

The basic aim of prodrug design is to mask undesirable drug properties, such as low solubility in water or lipid membranes, low target selectivity, chemical instability, undesirable taste, irritation or pain after local administration, presystemic metabolism and toxicity [54, 70, 74]. In general, the rationale behind the use of prodrugs is to optimize the absorption, distribution, metabolism, excretion, and unwanted toxicity (so-called ADMET properties) of the parent drugs [35]. Classically, the term prodrug, introduced in 1958 by Adrien Albert [3], relates to biologically inert derivatives of drug molecules that undergo an enzymatic and/or chemical conversion *in vivo* to release the pharmacologically active parent drug. The active drug is released from its inactive form before, during or after absorption of the prodrug. Some drugs are released only after reaching targets of their actions [54, 70]. A prodrug should increase the bioavailability and therapeutic effectiveness of a parent drug. Although the term prodrug is now standard, prodrugs have been also referred to as reversible or bioreversible derivatives or biolabile drug-carrier conjugates.

According to Testa [74] there are three basic, overlapping objectives in prodrug research:

1. Pharmaceutical: to improve solubility, chemical stability, and organoleptic properties; to decrease irritation and/or pain after local administration, to reduce problems related with the pharmaceutical technology of the active agent.
2. Pharmacokinetic: to improve absorption (oral and by non-oral routes), to decrease presystemic metabo-

lism, to improve time profile, to increase organ/tissue-selective delivery of the active agent.

3. Pharmacodynamic: to decrease toxicity and improve therapeutic index, to design single chemical entities combining two drugs (co-drugs strategy).

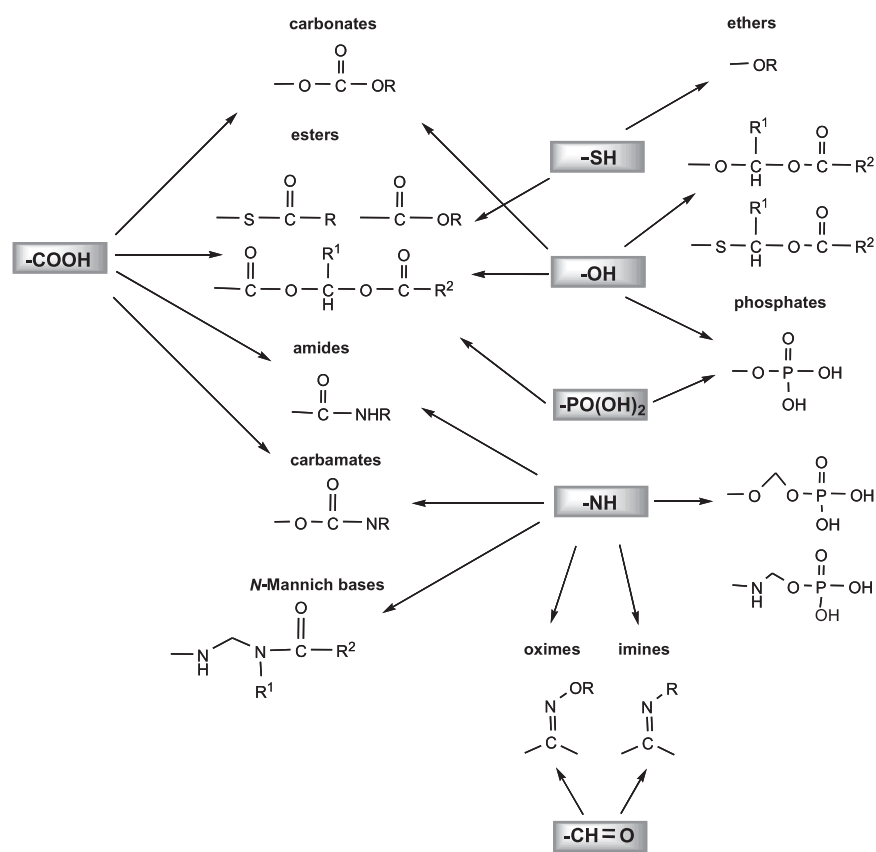
It should be noted that strategies to improve oral bioavailability and achieve brain- and tumor-specific targeting have been the most important developments in the prodrug design during the last decade.

Structure and classification of prodrugs

There are two main classes of prodrugs: (1) carrier-linked prodrugs, and (2) bioprecursor prodrugs. In the carrier-linked prodrugs, the active molecule (the drug) is temporarily linked to a carrier (also known as a promoiety) through a bioreversible covalent linkage. Once in the body, the carrier-linked prodrug undergoes biotransformation, releasing the parent drug and the carrier. Ideally, the carrier should be nonimmunogenic, easy to synthesize at a low cost, stable under the conditions of prodrug administration, and undergo biodegradation to nonactive metabolites [35, 63, 69]. In so-called co-drugs (mutual prodrugs, multiple prodrugs), a prodrug is formed from two pharmacologically active agents coupled together into a single molecule, and act as promoieties of each other. Examples of co-drugs include sulfapyridine – 5-aminosalicylic acid, indomethacin – paracetamol, L-DOPA – entacapone, gabapentin – pregabalin, 5-fluorouracil – cytarabine, 5-fluorouracil – dexamethasone triamcinolone, ampicilin – sulbactam, sulfamethoxazole – nalidixic acid [20]. The major groups of carrier-linked prodrugs are esters and amides; other groups include phosphates, carbamates, carbonates, oximes, imines and *N*-Mannich bases (Fig. 1). Bioprecursors do not contain a promoiety but result from a molecular modification of the active compound itself. The bioprecursor prodrug is transformed metabolically or chemically by hydration (e.g., lactones such as some statins), oxidation (e.g., dexpanthenol, nabumetone) or reduction (e.g., sulindac, platinum(IV) complexes) to the active agent [30, 42, 54, 61, 74].

Based on the site of conversion into the pharmacologically active agent, the prodrugs can be additionally classified into two groups:

Fig. 1. Common functional groups on parent drugs applied in prodrug design. Modified from [54]



• Type I – metabolized intracellularly. Type IA prodrugs (e.g., acyclovir, cyclophosphamide, 5-fluorouracil, L-DOPA, zidovudine) are metabolized at the cellular targets of their therapeutic actions. Type IB prodrugs (e.g., carbamazepine, captopril, molsidomine, primidone) are converted to parent drugs by metabolic tissues, namely by the liver.

• Type II – metabolized extracellularly. Type IIA – in the milieu of the gastrointestinal fluid (e.g., loperamide oxide, sulfasalazine). Type IIB – within the circulatory system and/or other extracellular fluid compartments (e.g., aspirin, bambuterol, fosphenytoin). Type IIC – near or inside therapeutic target/cells (ADEPT, GDEPT).

Some of the prodrugs, called mixed-type prodrugs, belong to more than one class [79].

Esters of active agents with carboxyl, hydroxyl or thiol functionalities, and phosphate esters of active agents with hydroxyl or amine functionalities are the most commonly used prodrugs. Approximately half of the prodrugs currently available on the market are activated *via* enzymatic hydrolysis by ubiquitous esterases (acetylcholinesterases, butyrylcholinesterases,

carboxylesterases, arylesterases) which are present throughout the body [35, 54]. Following the process of esterification, a desired chemical and biological stability of prodrug esters can be achieved by an appropriate manipulation of their electronic and steric properties [63]. An ideal ester prodrug should have chemical stability across a pH range, high aqueous solubility, exhibit good transcellular absorption, be resistant to hydrolysis during the absorption phase, and undergo rapid and quantitative breakdown to yield high circulating concentrations of the active component post absorption [10].

The main rationale underlying synthesis of prodrug esters is to (1) enhance lipophilicity, and thus the passive membrane permeability, of water soluble drugs, (2) increase aqueous solubility (phosphate esters). To increase the lipophilicity of hydroxyl group bearing pharmacologically active compounds, they can be acylated with aliphatic or aromatic carboxylic acids. Esterification of the hydroxyl group of the parent drug with carboxylic acid derivatives bearing additional functional groups, such as hydroxyl, amino or car-

boxyl, provides prodrug molecules with increased aqueous solubility (hydrophilicity) [35, 54].

Some esters are not suitable substrates for endogenous esterases, sulfatases or phosphatases, which, in turn, impose unfavorable, too slow kinetics of the prodrug hydrolysis. To optimize the rate of enzymatic hydrolysis, knowledge on the mechanisms of the specific processes involved is highly desirable. An increase in the rate of alkaline hydrolysis can be achieved by the attachment of electron-withdrawing groups, while in the case of acidic hydrolysis, tethering of electron-donating groups to the carboxylate part of esters is recommended. If the esters are too reactive, bulky substituents causing steric hindrance to hydrolysis or esters of long-chain fatty acids can be employed [71].

Prodrug approaches for enhancing administration, permeability, absorption, and distribution of drugs

Prodrugs with increased aqueous solubility

Poor aqueous solubility is considered as a serious problem limiting the therapeutic use of numerous drugs and drug candidates. Among various strategies used to overcome this drawback is the prodrug approach. One frequently employed means of improving the aqueous solubility of a drug is by the use of esters and amides of phosphoric acid, due to the ionic nature of the phosphate group. It should be noted that phosphate derivatives display high chemical stability, often even higher than the parent compound. Under physiological conditions phosphate prodrugs undergo rapid biotransformation by endogenous phosphatases, such as alkaline phosphatase, of the intestine, plasma, and the liver [15, 26, 44, 48, 54, 65, 74]. Examples of phosphoric acid ester prodrugs and their corresponding parent drugs are shown in Table 1.

Another approach to increase aqueous solubility is esterification with amino acids. Examples of such prodrugs are valacyclovir (Valtrex[®]) and valgancyclovir (Valcyte[®]), which are valine esters of the antiviral drugs acyclovir and gancyclovir, respectively [53, 67]. Both prodrugs are also good substrates of small peptide transporters (PEPT1) present in the intestine epithelium, a property that further increases their bioavailability [32, 48, 77].

A hemisuccinate group can be conveniently used to increase water solubility, as it contains a free carboxylic group, which is suitable for the formation of dissociated salts. A fine example of the utilization of hemisuccinate esters is cinazepam, a novel benzodiazepine anxiolytic drug suitable for intravenous injections [60].

Prodrugs with increased lipid solubility

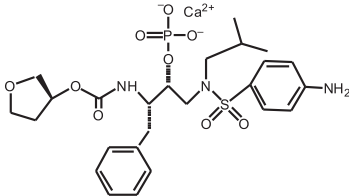
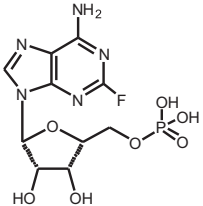
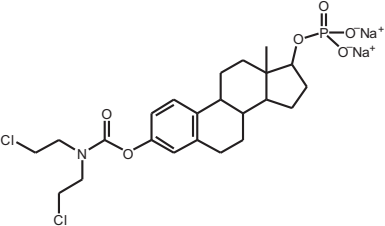
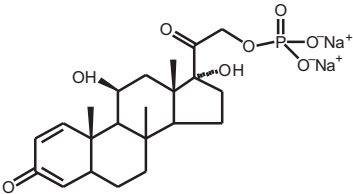
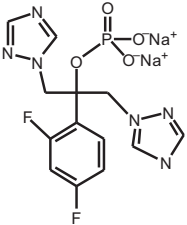
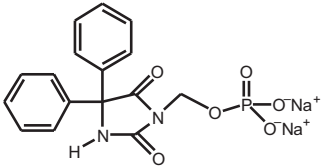
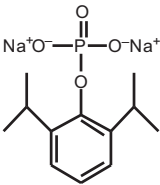
In order to improve lipophilicity, and thus passive transport through biological membranes, compounds containing polar or ionizable groups can be converted into ester prodrugs [26, 33, 36, 44, 54]. Examples of ester prodrugs with improved lipophilicity designed for oral administration are listed in Table 2.

Prodrugs with increased lipophilicity are also designed for topical administration. For example, esters of ketolac (a non-steroidal anti-inflammatory drug with potent analgesic activity) and fatty acids (stearic, linoleic, oleic) allow the drug to accumulate in the skin with concomitant low skin permeation, leading to increased therapeutic efficiency and reduced side effects of the parent drug [13]. Additional examples are latanoprost and travoprost, prodrugs of prostaglandin F_{2α} (PGF_{2α}) analogs, applied as eye drops for the treatment of glaucoma. Naturally occurring prostaglandins, including PGF_{2α}, are molecules which are relatively polar and hydrophilic due to their carboxylic acid moiety and several hydroxyl groups. They penetrate through biological membranes poorly. This problem has been solved by a modification of the chemical structure of PGF_{2α} analogs. Latanoprost (Xalatan[®]) and travoprost (Travatan[®]), isopropyl esters of latanoprost acid and travoprost acid, respectively, penetrate easily through the corneal epithelium due to their increased lipophilicity, where they are then hydrolyzed to active carboxylic acids (Fig. 2). The free acids of latanoprost and travoprost activate prostanoid FP receptors and reduce intraocular pressure by enhancing the uveoscleral and the trabecular meshwork outflow pathways [9].

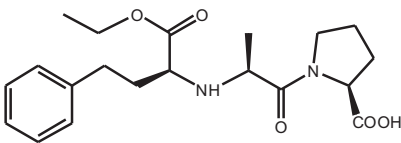
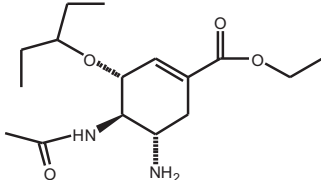
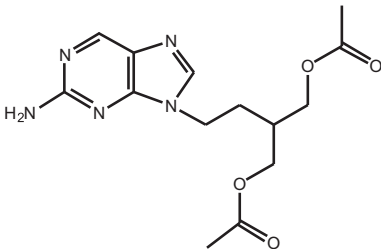
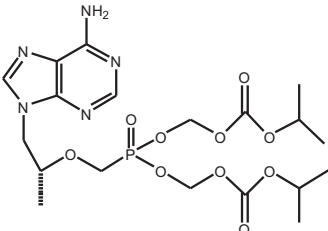
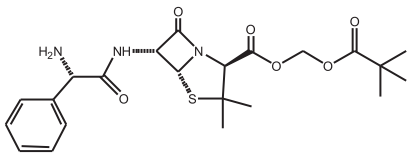
Prodrugs as substrates for GI membrane transporters

Gabapentin enacarbil ([(+)-1-(α -isobutanoyloxyethoxy)carbonyl]aminomethyl)-1-cyclohexane acetic acid; Horizant[®]), a prodrug of gabapentin (1-aminomethyl)-cyclohexane acetic acid), is a substrate for

Tab. 1. Phosphate esters as prodrugs for improved aqueous solubility. Modified from [54]

A. For oral administration		
Prodrug name; functional group	Structure	Action
Fosamprenavir (Telzir®); phosphate ester of amprenavir		Bioconverted by alkaline phosphatases to amprenavir, a HIV protease inhibitor. Antiviral, HIV infections.
Fludarabine phosphate (Fludara®); phosphate ester of fludarabine		Bioconverted by alkaline phosphatases to fludarabine. Fludarabine undergoes transformation to 2-fluoro-9-β-D-arabino-furanosyladenine, which after uptake into cells is converted to active 2-fluoro-9-β-D-arabino-furanosyladenine 5'-triphosphate. Antiviral.
Estramustine phosphate (Emcyt®); phosphate ester of estramustine		Bioconverted by alkaline phosphatases to estramustine, which is further transformed into estromustine. Antimitotic.
Prednisolone phosphate; phosphate ester of prednisolone (Inflamase®, Orapred ODT®)		Bioconverted by alkaline phosphatases to prednisolone. Anti-inflammatory, antiallergic.
B. For parenteral administration		
Fosfluconazole (Procif®); phosphate ester of fluconazole		Bioconverted by alkaline phosphatases to fluconazole. Antifungal.
Fosphenytoin (Pro-dilatin®, Pro-epanutin®, Cerebryx®); phosphate ester of phenytoin		Rapidly bioconverted by alkaline phosphatases to phenytoin. Anticonvulsant.
Propofol phosphate; phosphate ester of propofol		Rapidly bioconverted by alkaline phosphatases to propofol. Anaesthetic.

Tab. 2. Prodrugs with improved lipophilicity for oral administration. Modified from [54]

Prodrug name; functional group	Structure	Action
Enalapril (Innovace [®] , Vasotec [®] , Renitec [®]), monoethylester of enalaprilat		In the liver bioconverted by esterases to enalaprilat, an angiotensin-converting enzyme inhibitor. Used in the treatment of hypertension, ischemic heart disease.
Oseltamivir (Tamiflu [®]), ethyl ester of oseltamivir carboxylate		Bioconverted by carboxylesterase-1 to oseltamivir carboxylate – selective inhibitor of viral neuroamidase glycoprotein type A and B. Antiviral (anti-influenza).
Famciclovir (Famvir [®]), dimethyl ester of penciclovir		Bioconverted by esterases and aldehyde oxidase to penciclovir – inhibitor of Herpes DNA synthesis. Antiviral.
Adefovir dipivoxil (Viread [®]), bis-(pivaloyloxymethyl) ester of adefovir		Bioconverted by esterases and phosphodiesterases to tenofovir. In lymphocytes T tenofovir is converted to active metabolite, tenofovir diphosphate, an inhibitor of HIV virus reverse transcriptase. Antiviral (anti-HIV).
Pivampicillin (Alphacyllin [®]), pivaloylmethyl ester of ampicillin		Bioconverted by esterases to ampicillin (β-lactam antibiotic).

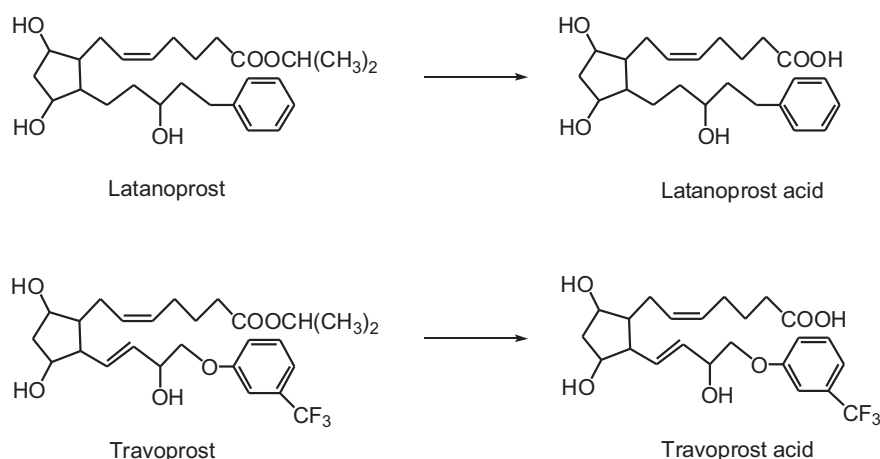
monocarboxylic acid transporter-1 (MCT-1) and sodium-dependent multivitamin transporter (SMVT), distributed throughout the intestine. Gabapentin enacarbil is stable at physiological pH and rapidly converted into gabapentin. *In vitro* and *in vivo* studies have demonstrated that gabapentin enacarbil is endowed with better absorption, bioavailability, and pharmacokinetic properties compared with gabapentin [78]. Gabapentin is approved for the treatment of epilepsy and postherpetic neuralgia. Phase II and III clinical studies indicate that gabapentin enacarbil administered in the form of the prodrug could be effective in the treatment of restless leg syndrome [80].

Several groups of prodrugs, such as ACE inhibitors (e.g., captopril, enalapril, fosinopril), antiviral (e.g., valcyclovir and valgancyclovir), and anticancer (e.g., ester analogs of fluridone and gemcitabine) are substrates for PEPT1 [32, 48, 58, 66, 76, 77].

Prodrug approaches for the CNS delivery

One of the major difficulties in the development of drugs acting at the central nervous system (CNS) is the inability of many therapeutic compounds to

Fig. 2. Chemical structures of prostaglandin $\text{PGF}_{2\alpha}$ analog prodrugs designed for improved ophthalmic delivery. Modified from [9]



cross the blood-brain barrier (BBB). The BBB is formed by endothelial cells of brain microvessels connected by extensive tight junctions. Combined with an absence of fenestrae and an extremely low pinocytotic activity, transport of molecules across the BBB is achieved either through diffusion as a passive process, or with the aid of special carrier systems involving intrinsic transporter proteins localized on the luminal (blood side) and abluminal (brain side) sides of the epithelial cells. The BBB is necessary to provide an optimal chemical environment for brain function. It restricts the passage of blood-borne drugs, neurotoxic substances and peripheral immune cells from entering the brain, while selectively facilitating the transport of nutrients into the brain. In addition, it prevents central neurotransmitters and neuromodulators from reaching the circulatory system [1].

Three basic prodrug strategies have been used to facilitate the transport of drugs into the CNS: (1) increasing passive diffusion by masking polar groups on the parent drug (so-called lipidization of molecules), (2) increasing carrier-mediated or receptor-mediated transport through the BBB, and (3) decreasing the active efflux of the drug from the brain into the blood. In order to achieve therapeutically effective doses in the CNS, the process of prodrug biotransformation should be slow in the peripheral tissues but fast in the brain itself. Furthermore, taking into account the anatomical complexity of the CNS, the biotransformation should be limited to selected brain structures [50, 51, 55].

Several specific endogenous influx transporters have been identified at the brain capillary endothelium which forms the BBB, including large neutral amino acid transporters (LAT1), glucose transporters

(GLUT1), peptide transporters (PEPT), monocarboxylic acid transporters (MCT), organic cation transporters (OCT), organic anion transporters (OAT), concentrative nucleoside and nucleotide transporters (CNT) [29, 31]. It is suggested that prodrugs with a very close structural resemblance to endogenous substrates of the BBB influx transporters will be recognized by them and transported across the BBB into the brain, where the release of an active drug will take place. L-DOPA, a dopamine prodrug used in the treatment of Parkinson's disease, is an example of compounds transported into the brain with the aid of influx transporters. Although dopamine is unable to cross the BBB due to its hydrophilic nature, L-DOPA, its amino acid analog and precursor, crosses the BBB *via* LAT1. The drug is then decarboxylated by amino acid decarboxylase (AADC) to dopamine. Additionally, catechol-*O*-methyltransferase (COMT) converts L-DOPA to 3-*O*-methyl DOPA. To minimize the metabolism of L-DOPA outside the CNS and to increase its half-life time, the drug is given in combination with peripheral inhibitors of AADC (carbidopa and benserazide) and COMT (tolcapone and entacapone) [23, 39].

How can prodrugs improve targetability and efficacy of chemotherapeutic agents?

Chemotherapy is a primary treatment for cancer. During the last several decades major progress has been made in the field of chemotherapy of various cancers. The vast

majority of anticancer drugs currently in use exert their oncostatic actions by inhibiting proliferation or by arresting cell cycle at a certain phase. As oncostatic drugs are endowed with poor selectivity, they affect not only neoplastic cells but also rapidly proliferating normal cells, such as, to name just a few, bone marrow, gut epithelia, hair follicles, lymphatic cells and gametes. The lack of selectivity of anticancer drugs, and associated toxicity, hampers their effectiveness and long term use. Hence, not surprisingly, there is an urgent need to improve their selectivity.

One of the promising approaches to achieve this goal is prodrug technology. An anticancer prodrug should be transported to neoplastic cells, where it will undergo transformation to a cytotoxic parent drug by native or recombinant enzymes [45]. Capecitabine (5'-deoxy-5-fluorocytidine carbamate; Xeloda[®]), a triple prodrug of 5-fluorouracil approved for the therapy of solid tumors including breast cancer and colorectal cancer, may be considered a pioneer of chemotherapy prodrugs [37, 43]. Following oral administration, capecitabine is rapidly and extensively absorbed. After adsorption, capecitabine is first metabolized by hepatic carboxyesterases, CES1 and CES2. 5'-Deoxy-5-fluorocytidine formed is then converted to 5'-deoxy-5-fluorouridine by cytidine deaminase in the liver and tumor tissues, and finally by thymidine phospho-

rylase in tumors to highly cytotoxic 5'-fluorouracil [47].

Anticancer prodrugs can be designed to target specific molecules (enzymes, peptide transporters, antigens) that are overexpressed in tumor cells in comparison to normal cells. The new promising chemotherapeutic prodrugs include:

1. Enzyme-activated prodrugs – antibody-directed enzyme prodrug therapy (ADEPT) and gene-directed enzyme prodrug therapy (GDEPT).
2. Targeting-ligand conjugated prodrugs – antibody-drug conjugates, peptide-drug conjugates, aptamer-drug conjugates, and folic acid-drug conjugates.
3. Enzyme-cleavable prodrugs.
4. Membrane transporter-associated prodrugs.
5. Polymeric prodrugs.

Enzyme-activated prodrugs

One approach toward improving the specificity of chemotherapy is enzyme-activated prodrug therapy in which a non-toxic drug is converted into a cytotoxic agent by an enzyme either extracellularly, at a site of tumor (ADEPT), or intracellularly in neoplastic cells (GDEPT). Both strategies utilize precursors of two groups of oncostatic drugs, i.e., antimetabolites and alkylating agents [59]. GDEPT is also known in the literature under other names, such as suicide gene therapy (SGT), virus-directed enzyme prodrug therapy (VDEPT), and gene prodrug activation therapy (GPAT) [4, 64]. Examples of enzyme-activated prodrugs are listed in Table 3.

The principle of ADEPT is to use an antibody directed at a tumor-associated antigen which localizes the enzyme in the vicinity of the tumor. A non-toxic prodrug, a substrate for the enzyme, is then given intravenously and converted to a cytotoxic drug only at the tumor site where the enzyme is localized, resulting in tumor cell death (Fig. 3). For ADEPT to be effective and safe the following criteria should be fulfilled:

- The prodrug should be easily dissolved in water, and marginally taken up by cells.
- The drug should be efficiently and rapidly transported into cancer cells.
- The antibody-enzyme complex should bind selectively to cancer cells.
- The enzyme should have high intrinsic activity.
- The enzyme cannot be inactivated by a prodrug or drug.

Tab. 3. Examples of enzyme-activated prodrugs

Antibody-directed enzyme prodrug therapy (ADEPT)			
Antibody	Prodrug	Drug	Tumor target
L6	Mitomycin C phosphate	Mitomycin C	Lung adenocarcinoma
BW413	Etoposide phosphate	Etoposide	Colon carcinoma
L6	Doxorubicin phosphate	Doxorubicin	Lung adenocarcinoma
Gene-directed prodrug therapy (GDEPT)			
Enzyme	Prodrug	Drug	
Cytochrome p450	Cyclophosphamide, ifosfamide	Phosphamide mustard, acrolein	
Cytosine deaminase	5-Fluorocytosine 5-Fluorouridine	5-Fluorouracil	
Nitroreductase	5-(Aziridin-1-yl)-2,4-dinitrobenzamide	5-(Aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide	

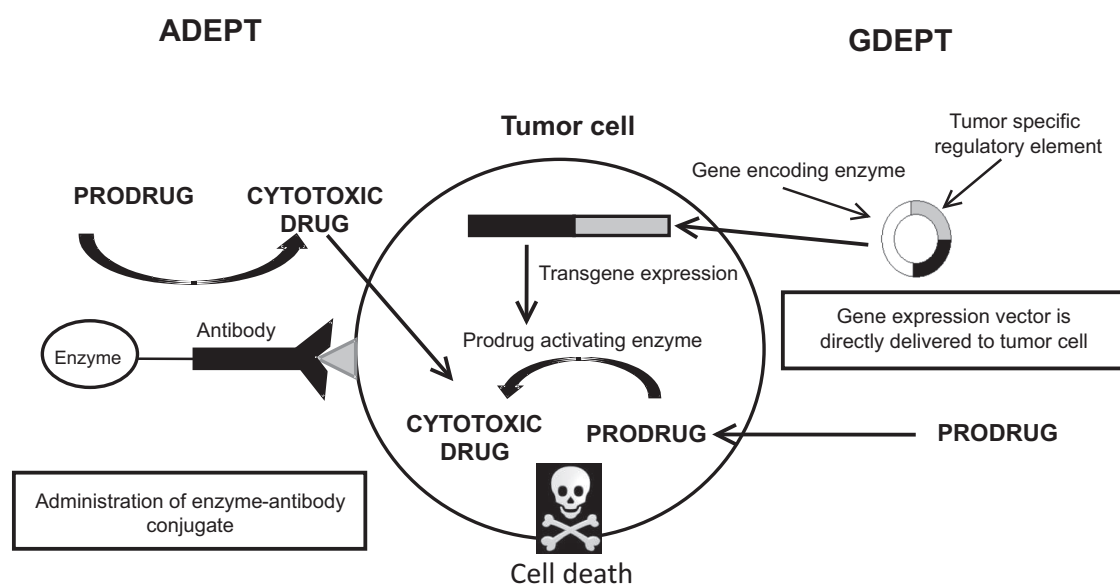


Fig. 3. Schematic diagram illustrating principles of the antibody-directed enzyme prodrug therapy (ADEPT) and gene-directed enzyme prodrug therapy (GDEPT). In the ADEPT approach, the activating enzyme is specifically delivered to tumor cells using a tumor-specific antibody, followed by administration of a prodrug. The prodrug is then activated by the conjugated enzyme to the active cytotoxic parent drug. In GDEPT, a gene expression vector encoding an activating enzyme is delivered to tumor cells. Next, a nontoxic prodrug is administered systemically and converted by the enzyme to the toxic parent drug. Modified from [55]

- Enzyme immunogenicity should be low.

Endogenously expressed human enzymes cannot be utilized for ADEPT, since the prodrug will be converted to a cytotoxic compound not only in the vicinity of the tumor, but also at sites where endogenous enzyme is expressed, causing systemic toxicity. Non-human enzymes which are currently used in ADEPT technology could, however, induce immune responses, which, in turn, will limit their therapeutic usefulness [2, 5, 75].

GDEPT, like ADEPT, is a two-step process. In the first step, the gene for a foreign enzyme is delivered to tumor cells. In the second step, a non-toxic agent is administered systemically and converted by the enzyme to its cytotoxic metabolite (Fig. 3) (e.g., [40, 46]). Enzymes used in GDEPT catalyze specific reactions that are not catalyzed by endogenous enzymes, and should be delivered in sufficiently high amounts to result in prodrug conversion. They can be divided into two categories. The first comprises foreign enzymes of nonmammalian origin (viral, bacterial, yeast), with or without human homologues. Examples are thymidine kinase (TK) from *Herpes simplex* or *Varicella zoster* viruses, cytosine deaminase (CD), uracil phosphoribosyl transferase (UPRT), purine nu-

cleoside phosphorylase (PNP), and nitroreductase (NR) from *Escherichia coli*. The second category consists of enzymes of human origin that are absent or are expressed only at low concentrations in tumor cells, such as deoxycytidine kinase (dCK) and cytochrome P450. The main disadvantage of nonmammalian enzymes is their potential immunogenicity. Enzymes of the second category are unlikely to induce immune responses, but their use will in most cases lead to some prodrug activation in normal cells [4, 6, 17]. Many viral and non viral gene delivery systems have been employed to deliver the therapeutic DNA into cancer cells. The most widely used are retrovirus vectors. To increase tumor selectivity of GDEPT, a tumor-specific regulatory element is incorporated in the gene expression vector. An example of such approach is using prostate specific antigen (PSA)-specific promoter or the prostate membrane specific antigen (PMSA)-specific promoter/enhancer to target GDEPT virus vector to prostate cancer cells. An important feature of GDEPT is so-called bystander or neighboring cell killing effect, which can be divided into two categories: (1) local – the active toxic drug spreads by passive, gap-junction independent diffusion to surrounding cells, including those that do not

express the enzyme, and kills them; and (2) distant – dying cells can stimulate systemic anticancer effects through the host immune responses mediated by natural killer cells, T cells and macrophages [19, 34]. A strong bystander effect could be seen even when only 1 in 100–1000 cells expresses the transgene [27]. Prodrugs for GDEPT must be considerably less cytotoxic than their corresponding active drugs, and must be suitable substrates for the activating enzyme under physiological conditions. In addition, they should have good pharmacological and pharmacokinetic properties, be chemically stable and diffuse readily in the tumor interstitium. The formed cytotoxic drug should be metabolically stable, and its retention time must be long enough to achieve desired therapeutic effects [40, 68].

Targeting-ligand conjugated prodrugs

Tumor-specific monoclonal antibodies (or fragments of antibodies) are conjugated to oncostatic drugs such as antifolates, anthracyclines, taxanes and vinca alkaloids. The antibody delivers the therapeutic agent to tumor cells. After reaching its target, the conjugate is internalized through a receptor-mediated pinocytosis, and the pharmacologically active compound is released in the cell. Tumor-specific antigens include, among others, CD19, CD22, CD33, CD56, MUC16, TAG-72, PMSA, epidermal growth factor receptor-2 (HER-2) and αV integrin. It should be emphasized that some of these antigens are preferably expressed at high levels in specific cancer types, e.g., MUC16 in ovarian cancer, PMSA in prostate cancer, TAG-72 in human adenocarcinomas [45]. Major problems related with this prodrug strategy that still have to be resolved include: immunogenicity, selectivity and permeability into the solid tumors [45, 72, 73]. Although at present, around twenty antibody-drug conjugates are in clinical trials, so far, only one such prodrug, i.e., Mylotarg[®] (Weyth), which consists of calicheamicin linked to the human anti-CD33 monoclonal antibody (gen-tuzumab) *via* a hydrolyzable bifunctional linker has been approved by FDA for the treatment of cancer (acute myeloid leukemia) [62]. However, due to severe side effects, the drug was withdrawn from the market in 2010 [45].

Peptide-conjugated prodrugs for cancer therapy utilize peptide ligands designed to bind with a tumor-specific antigen or a peptide transporter which is overexpressed in neoplastic cells. These ligands are

conjugated to a chemotherapeutic agent either directly or by a linker. Peptides have many advantages over antibodies as tumor-targeting moieties: particularly small molecular weight, very good cell permeability, and ease of production [45]. So far, promising results have been obtained with luteinizing hormone-releasing hormone (LHRH) conjugated to camptothecin *via* polyethylene glycol (PEG) as the spacer. LHRH is used as a targeting moiety to LHRH receptors that are overexpressed in the plasma membrane of breast, ovarian, and prostate cancer cells and are not expressed detectably in normal visceral organs [22].

Aptamers are single-stranded DNA or RNA oligonucleotides that bind to specific target molecules with high specificity and affinity. In general, they are stable, nonimmunogenic, and easy to synthesize and immobilize on chemical devices. Several types of aptamers have been selected against cancer-related proteins, such as platelet-derived growth factor (PDGF), vascular endothelium growth factor (VEGF), type 3 receptor for epidermal growth factor (HER3), nuclear factor κB (NF κB), tenascin-C or PSMA, by an *in vitro* process known as cell systematic evolution of ligands by exponential enrichment (cell-SELEX) [82]. There are now ongoing studies on aptamer-cytotoxic agent conjugates targeted against human acute lymphoblastic leukemia cells, prostate and breast cancer cells [21, 41, 81, 83].

Prodrugs in anticancer photodynamic therapy (PDT)

Photodynamic therapy (PDT) is a clinically approved method for the treatment of several types of cancers. The advantages of PDT over other conventional chemotherapies are its low systemic toxicity and ability to selectively destroy tumors accessible to light. PDT combines a drug with photosensitizing properties, a photosensitizer or photosensitizing agent, with a specific type of light to kill the photosensitized cells. Following administration of the photosensitizer and its accumulation in a malignant tissue, light of the appropriate wavelength is directed onto the tissue. The subsequent activation of the photosensitizer by light results in the transfer of its energy to molecular oxygen. This, in turn, generates reactive oxygen species, responsible for cytotoxicity of the cells [38].

Porphyrin derivatives are well known photosensitizers but their therapeutical use is limited by a low bioavailability and difficulties in administration [49].

To overcome these drawbacks, a prodrug strategy based on the administration of 5-aminolevulinic acid (ALA) was introduced in the 1990s [14, 25, 52]. ALA is an endogenous substrate used in the multistep synthesis of hemoglobin. *In vivo*, catalytic synthesis of ALA is the slowest step of the heme biosynthetic pathway, thus intake of exogenous ALA leads to accumulation of protoporphyrin IX in cancer cells. Furthermore, ALA can be administered by various routes, e.g., orally, intravenously or transdermally [49]. To increase ALA bioavailability even more, researchers designed cascade prodrugs: *double prodrugs*, *prodrug of a prodrug*. Lipophilicity, and thus bioavailability of ALA, can be improved by esterification with long chain alcohols such as hexanol or octanol [28], or by synthesis of dendritic derivatives [7, 8, 24, 56] which break down *in vivo* to the parent compound. Another approach is glycosidation of ALA, which gives a more cell-friendly derivative, free from potentially toxic carriers [18]. A further modification of the ALA molecule are short peptide conjugates, an important advantage of which is that enzymes and peptide transporters may be upregulated in cancer cells, thus increasing the efficiency of therapy and reducing the required concentration of the drug [11, 57].

Iron chelators in cancer therapy

Iron plays the role of both cancer initiator and promoter. Free iron ions participate in the Haber-Weiss and Fenton reactions leading to the formation of reactive oxygen species (ROS) that play an important role in carcinogenesis. Moreover, iron availability is necessary for rapidly proliferating tumors to convert protoporphyrin IX into hemoglobin. Therefore, reducing the pool of free iron ions using chelators inhibits ROS synthesis and facilitates protoporphyrin IX accumulation. This, in turn, increases the efficiency of ALA and its prodrugs. As iron bound within chelators can also produce ROS to some degree, chelators should not compete with proteins naturally binding iron *in vivo* [49]. This particular goal has been achieved by Charkoudian [16], who designed a prochelator by condensing isonicotinic acid hydrazide with pinacol ester of boronic acid. The synthesized compound is inactive *in situ* and does not compete with iron binding proteins. On the other hand, hydrogen peroxide formed during oxidative stress activates the prochelator to its active form: salicyl aldehyde isonicotinoyl hydrazone.

Concluding remarks

The prodrug strategy is one of the most promising approaches to enhance the therapeutic efficacy and/or reduce the adverse effects of the pharmacologically active agents *via* different mechanisms, including increased solubility, stability, improved permeability and bioavailability, prolonged biological half-life time, and tissue-targeted delivery. Hence, not surprisingly, prodrugs are becoming an integral part of the drug discovery paradigm. Their importance is supported by the increasing percentage of approved new drug entities that are, in fact, prodrugs. Despite the remarkable progress made in the field of prodrug design, more studies are clearly needed, especially at early stages of the drug discovery, for prodrugs to achieve the desired state of art and take their place in modern pharmacotherapy.

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