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ANTICANCER PRODRUGS - THREE DECADES OF DESIGN

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ABSTRACT

The conventional old treatment method for cancer therapy is associated with severe side effects along with several limitations. Therefore, searching and developing new methods for cancer became crucial. This mini review was devoted on the design and synthesis of prodrugs for cancer treatment. The methods discussed include targeted prodrugs which are depending on the presence of unique cellular conditions at the desired target, especially the availability of certain enzymes and transporters at these target sites, antibody directed enzyme prodrug therapy (ADEPT), gene-directed enzyme prodrug therapy (GDEPT) which is considered one of the important strategies for the treatment of cancer and prodrugs based on enzyme models that have been advocated to understand enzyme catalysis. In this approach, a design of prodrugs is accomplished using computational calculations based on molecular orbital and molecular mechanics methods. Correlations between experimental and calculated rate values for some intramolecular processes provided a tool to predict thermodynamic and kinetic parameters for intramolecular processes that can be utilized as prodrugs linkers. This approach does not require any enzyme to catalyze the prodrug interconversion. The interconversion rate is solely dependent on the factors govern the limiting step of the intramolecular process.

KEYWORDS: prodrugs, Targeted prodrugs, ADEPT, GDEPT, Cancer, Intramolecular process, Enzyme models.

Cancer is defined as uncontrolled growth of abnormal cells. The cancerous cells may invade the nearby tissues (cells) and spread to other parts of the body through the blood and lymph-systems. The anticancer agents used in chemotherapy are systemic anti-proliferative agents that kill the dividing cells. These cytotoxic agents include antimetabolites, alkylating agents; DNA-complexing agents, mitosis inhibitors and hormones, and they interfere with some aspect of DNA replication, cell division and cell translation or repair. These agents mainly rely on enhanced proliferative rate of cancer cells, which means that they are not truly selective for cancer cells. The prolonged use of chemotherapy results in lethal damage to proliferating non-cancerous cells and this is mainly true in the treatment of solid tumors. Studies have shown that cytotoxins use in patients having appreciable tumor burdens leads to remissions of varying degrees which is followed by re-growth and spread of more malignant forms of the cancer. Although extensive studies and trials have been carried out in the last several decades, the long-term outlook for patients with malignant cancer forms is still discouraging. Therefore, it is a must to invoke innovative approaches for the design of new anticancer drugs with reduced toxicity and better therapeutic indices.[1]

Prodrug therapy provides less reactive and cytotoxic form of anticancer drugs. The lack of selectivity of anticancer drugs results in significant toxicity to noncancerous proliferating cells. These toxicities along with drug resistance exhibited by the solid tumors are considered as a major challenge that results in poor prognosis for patients.[2]

The term "prodrug" or “predrug” was first used by Albert to define or describe therapeutically inactive molecule that can be utilized to modify the physicochemical properties of an active therapeutic drug for enhancing its effectiveness and eliminate or suppress its toxicity and/or its adverse effects. Prodrugs are chemically made by attaching a parent active drug to non-toxic promoiety and upon their exposure to physiological environment (in vivo) they undergo enzymatic or chemical cleavage to furnish the active form and a non-toxic linker (promoiety).[3- 32]

The aim of using prodrugs is to achieve optimized ADME (absorption, distribution, metabolism, and excretion) properties and to increase selectivity of drugs to their target sites. The prodrug approach has been utilized to overcome several drug’s barriers and optimize drug’s clinical application. Nowadays, prodrug design has succeeded to offer efficient and

selective drug delivery systems. For instance, targeted prodrug approach, with the aid of gene delivery and controlled expression of enzymes and carrier proteins has played a major role in providing a precise and efficient drug delivery which contributed much to the enhancement of the drug's therapeutic effect.[9-15]

The ways by which the prodrug approach can be utilized include: (1) an Improvement of the active drug's solubility and consequently its bioavailability. Statistics have shown that more than 30% of drug discovery compounds have low aqueous solubility,[33] (2) increasing the active drug's permeability and absorption,[21] (3) modifying the drug's distribution profile,[34-35] (4) prevention the active drug's fast metabolism and excretion,[36-39] (5) reducing the active drug's toxicity by altering one or more of the ADME barriers but more often is achieved by targeting drugs to desired cells and tissues via site-selective drug delivery.[40-42] and (6) prolong the active drug activity such as in the case of 6-mercaptopurine which is used to suppress the immune system (organ transplants), however, its elimination time is too fast. A prodrug that slowly is converted to the active drug allows a sustained release of the drug's active form.[9-15]

For synthesizing a prodrug from its parent active drug, the latter must contain a functional group that can be utilized to form a chemical linkage with a linker (promoiety) and this linkage should be labile and easy to cleave by enzyme catalyzed or un-catalyzed chemical cleavage or under a change in the physiological medium's pH.[43]

The commonly used linkages in prodrug design are carboxylic ester, phosphate ester, carbonate, carbamate, amide, oxime, imine or disulfide (Figure 1).

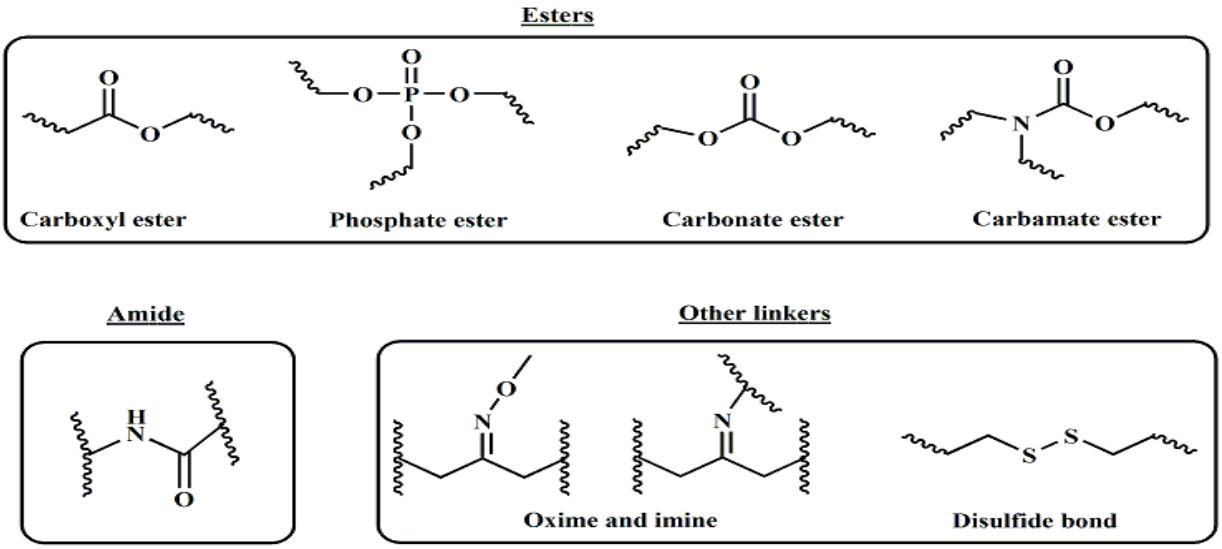

Figure 1: Commonly used linkages in prodrugs design.

Ester is the most common linkage used in prodrug design since it is easy to be synthesizes and its function groups, hydroxyl and carboxyl acid, are widely available in most parent active drugs.[44]

Amide bond is another commonly used linkage in prodrug design. It is derived from amine and a carboxyl group. The amide bond has higher enzymatic stability than ester bond. Several other types of linkers including oximes, imines, disulfide and uncleavable thioether bond have also been used in prodrug design.[45-50]

Anti-cancer prodrugs and conjugates design involves the synthesis of inactive moiety that is converted to its active form inside the body at the site of action. Targeting strategies of anti-cancer agents have attempted to take advantage of low extracellular pH, high enzymes levels in tumor tissues, the hypoxic environment inside the tumor, and tumor-specific antigens expressed on tumor cell surfaces.[41]

The drug release in most of the prodrugs is achieved by conjugating the drug to the carrier through a linker that incorporates a pre-determined breaking point, in which the drug can be activated on the target's active site. The general design of carrier-linked anti-cancer prodrug is shown in Figure 2.

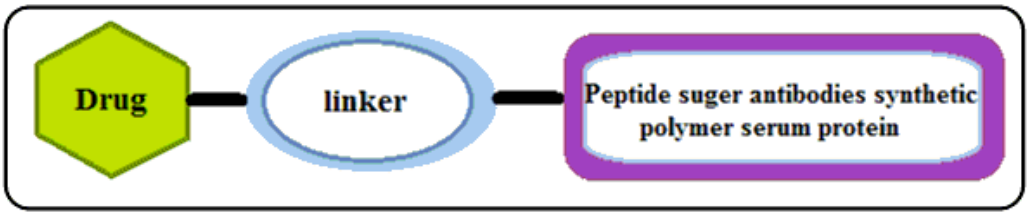

Figure 2: General design of carrier-linked anticancer prodrugs.

TARGETING STRATEGIES

The prodrugs can be targeted selectively to tumors either by active or passive targeting strategies.

ACTIVE TARGETTING

Tumor specific antigens or receptors—conjugate drug molecules to monoclonal antibodies (mAbs) or ligands.

In the period between 1998 and 2004, five chimeric or humanized antibodies including rituximab (Rituxan), trastuzumab (Herceptin), alemtuzumab (campath), bevacizumab

(Avastin) and cetuximab (Erbitux) was approved by the FDA for the treatment of hematological and solid tumors. A large number of anti-cancer drugs have been studied to be utilized in drug antibody conjugates. Among those agents are doxorubicin, CC-1065 (from Streptomyces zelensis), second-generation taxanes, monomethyl auristatin E, and geldanamycin. An important and prominent example used utilizing this approach is the cantuzumab mertansine conjugate of DM1 (Figure 3).[51-80]

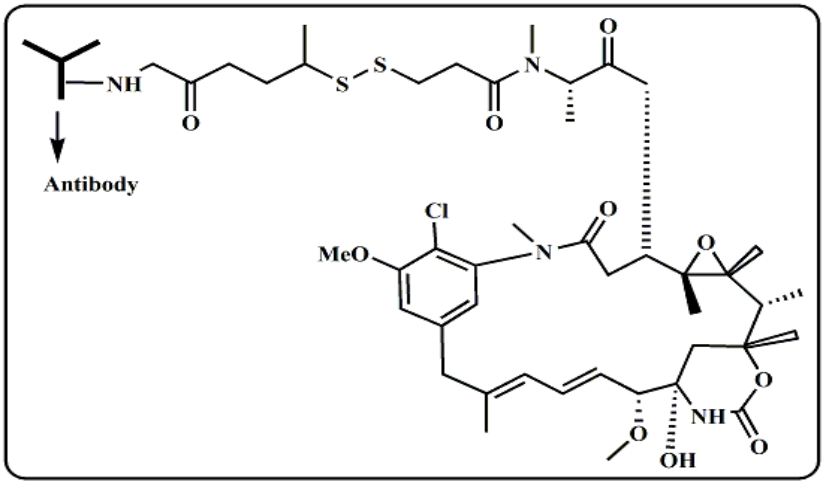

Figure 3: Cantuzumb mertansine (huC242-DM1)

Mylotarg (gemtuzumab, Wyeth) is the only immunoconjugate that was approved by the FDA for the treatment of cancer. This immunoconjugate consists of humanized anti-CD33 mAb linked to the cytotoxic antibiotic ozogamicin.[77] In addition, there are about twenty antibody-drug conjugates under clinical trials.

Mylotarg is antibody-drug conjugate for the treatment of acute myeloid leukemia (AML). This prodrug was approved in 2000 by the FDA, and a post-marketing study was begun in 2004. Unfortunately, this conjugate (Mylotarg) was withdrawn from the market in 2010 because of its ineffectiveness and severe side effects that were observed in post-approval clinical trial.

On the other hand, active targeting can be achieved by binding drugs to ligands that display high affinity for a particular receptor, (folic) the folate receptor (FR) which is over-expressed in many tumors, including those of the breast, lung, kidney and brain. FR binds folic acid (folate) with high affinity. Examples of such approach include folate conjugates of cytotoxic drugs such as camptothecin, taxol, mitomycin C, and folate-tethered protein toxins such as momordin and the Pseudomonas exotoxin.[51-80]

Antibody-directed enzyme prodrug therapy (ADEPT)

Antibody-directed enzyme prodrug therapy (ADEPT) is another approach for delivering anticancer drugs selectively to tumor cells. In this approach, there is a conjugation between an enzyme and tumor-specific antibody. Selective localization of the enzyme is achieved by the antibody and thus, reduced side effects are observed. An example of such approach is A CC-1065 analogue which was conjugated with a cephalosporin to provide a prodrug system. The resulting prodrug is expected to have reduced toxic effects when compared to its corresponding parent active drug. The prodrug system was designed such that it will undergo cleavage catalyzed by β–lactamases, localized on the tumor cell surface with the help of the conjugated antibody, to its active form (Figure 4). The selective activation of the mentioned prodrug at the core of the tumor site has the potential to lead to enhanced antitumor therapeutic efficacy.[70, 81]

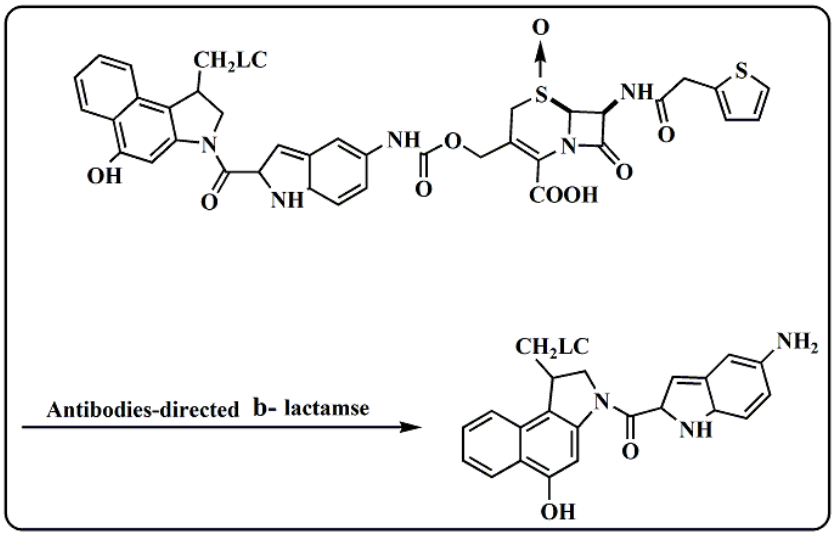

Figure 4: A prodrug consists of CC-1065 analogue conjugated to a cephalosporin and activated by β–lactamase.

ZD2767P (prodrug) is another example of prodrug was developed to investigate tumor targeting of the antibody-enzyme conjugate, and to study a new prodrug (bisiodophenol mustard, ZD2767P) whose activated form has a short half-life and is highly potent. ZD2767P was developed to reduce the problem associated with long-acting active drug (Figure 5).[82-84]

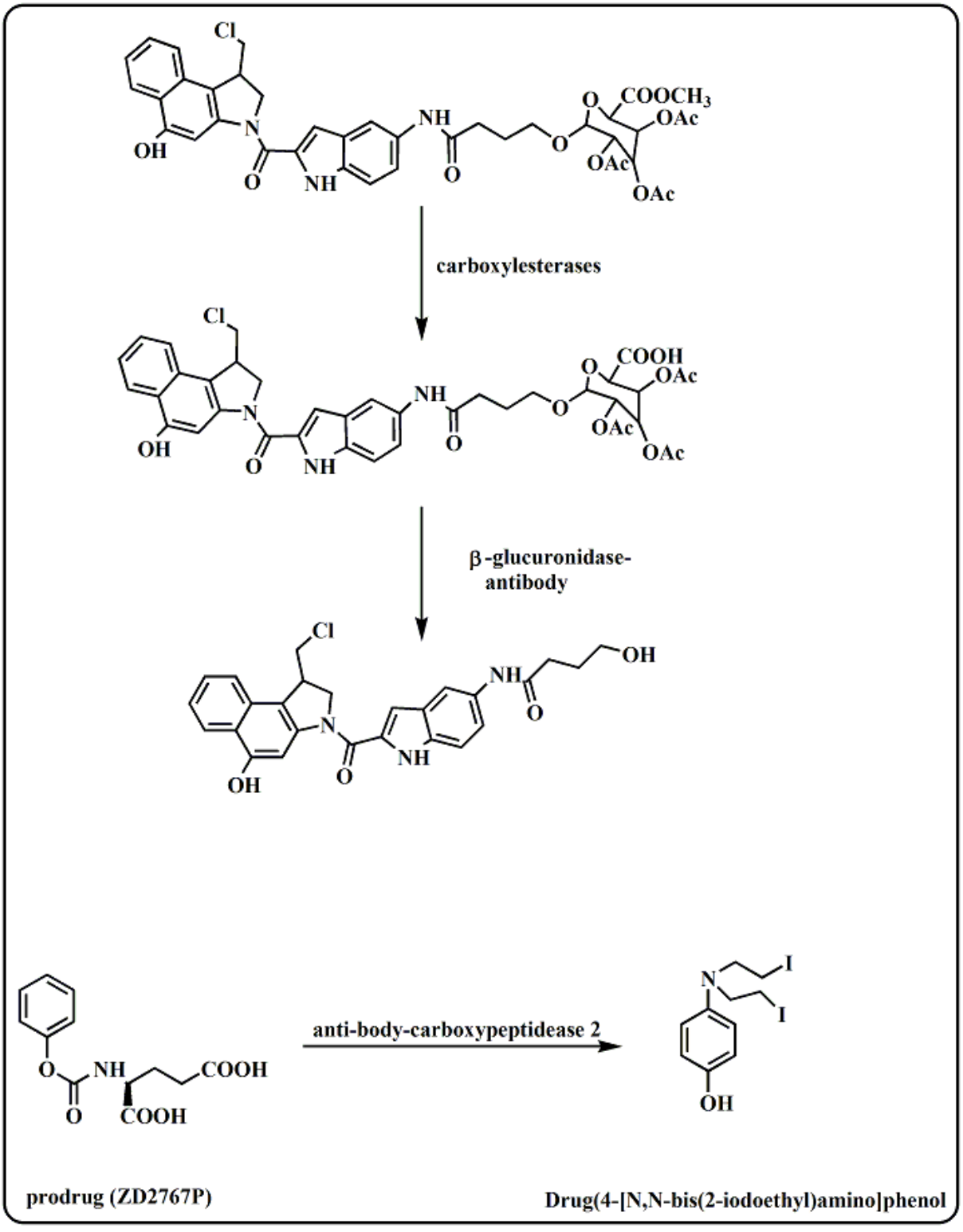

Figure 5: Representative example of prodrugs using the ADEPT system.

Gene-directed enzyme prodrug therapy (GDEPT)[85-101]

GDEPT known as suicide gene therapy involves a gene for a foreign enzyme delivery to the core of tumor cells without reaching the surrounding healthy cells. HSV TK with the

nucleoside analogue GCV is considered as the most well-investigated enzyme/prodrug strategy in cancer GDEPT therapy.

GCV and its related derivatives, mainly used in the treatment of HSV infection in humans, characterized by poor substrates for the mammalian nucleoside monophosphate kinase enzyme, but can be converted (1000-fold or more) efficiently to the monophosphate by TK from HSV 1 leading to a number of toxic metabolites; the most active metabolite is the triphosphates (Figure 6). The competition of GCV-triphosphate with deoxyguanosine triphosphate for incorporation into elongating DNA during cell division, results in inhibition of the DNA polymerase and consequently to a breakdown of single strand. These unique properties make the HSV TK/GCV combination perfectly suitable for the eradication of rapidly dividing tumor cells invading non-proliferating tissue.

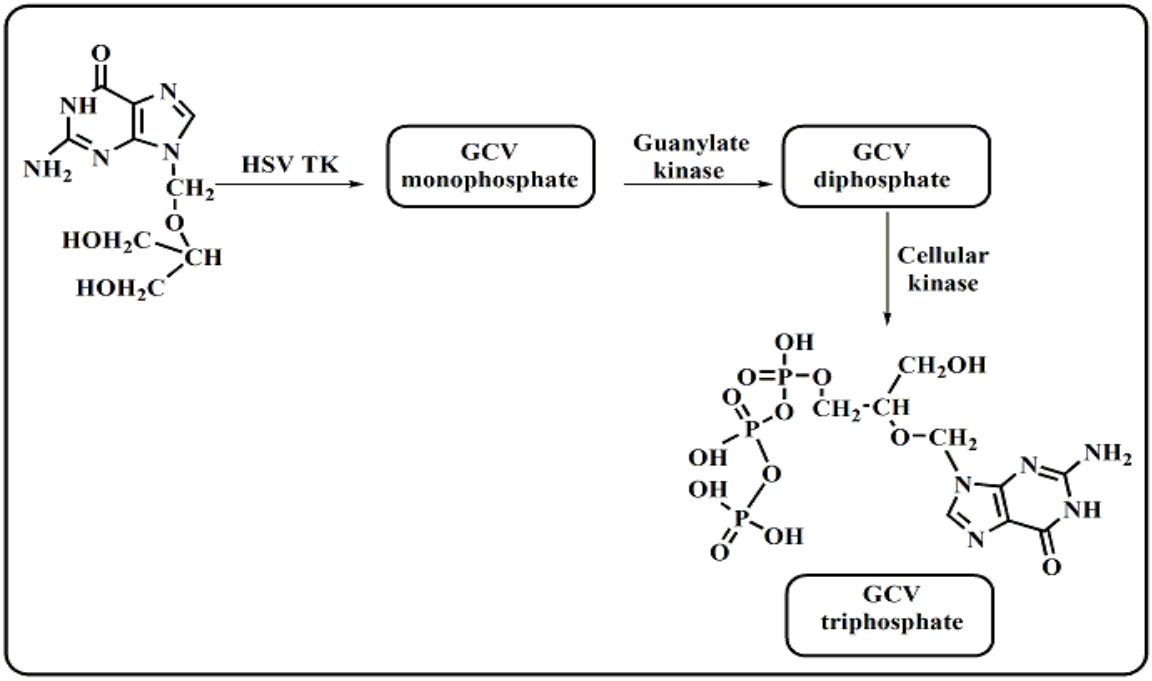

Figure 6. Metabolism of the prodrug ganciclovir (GCV).

GCV is specially phosphorylated by the herpes simplex virus 1 thymidine kinase (HSV TK) to its monophosphate. Subsequently, GCV-monophosphate is converted to the di- and triphosphate forms by guanylate kinase and other cellular enzymes and can be incorporated into elongating DNA, causing inhibition of the DNA replication and single strand breaks.

Several gene or treatment modalities were investigated to improve the GDEPT efficiency because it was realized that the treatment with a single GDEPT strategy might lead to partial

response and thus a combination of CD-HSV TK fusion genes was delivered followed by the prodrug GCV and 5-FC and as a result higher efficacy for the combined system was achieved. This system provided good results when was used in combination with radiotherapy.[85-101]

CYTOSINE DEAMINASE (CD)/5-FLUOROCYTOSINE (5-FC)

This system consisting of CD and 5-FC and relays on the production of a toxic nucleotide analogue. The enzyme CD, found in certain bacteria and fungi catalyzes the hydrolytic deamination of cytosine to uracil. Thus it can convert the non-toxic prodrug 5-FC to 5-fuorouracil (5-FU), which is then transformed by cellular enzymes to potent pyrimidine antimetabolites, 5-FdUMP, 5-FdUTP and 5-FUTP (Figure 7). 5-FU is the drug of choice in the treatment of colorectal cancer and it is widely used in cancer chemotherapy.[102]

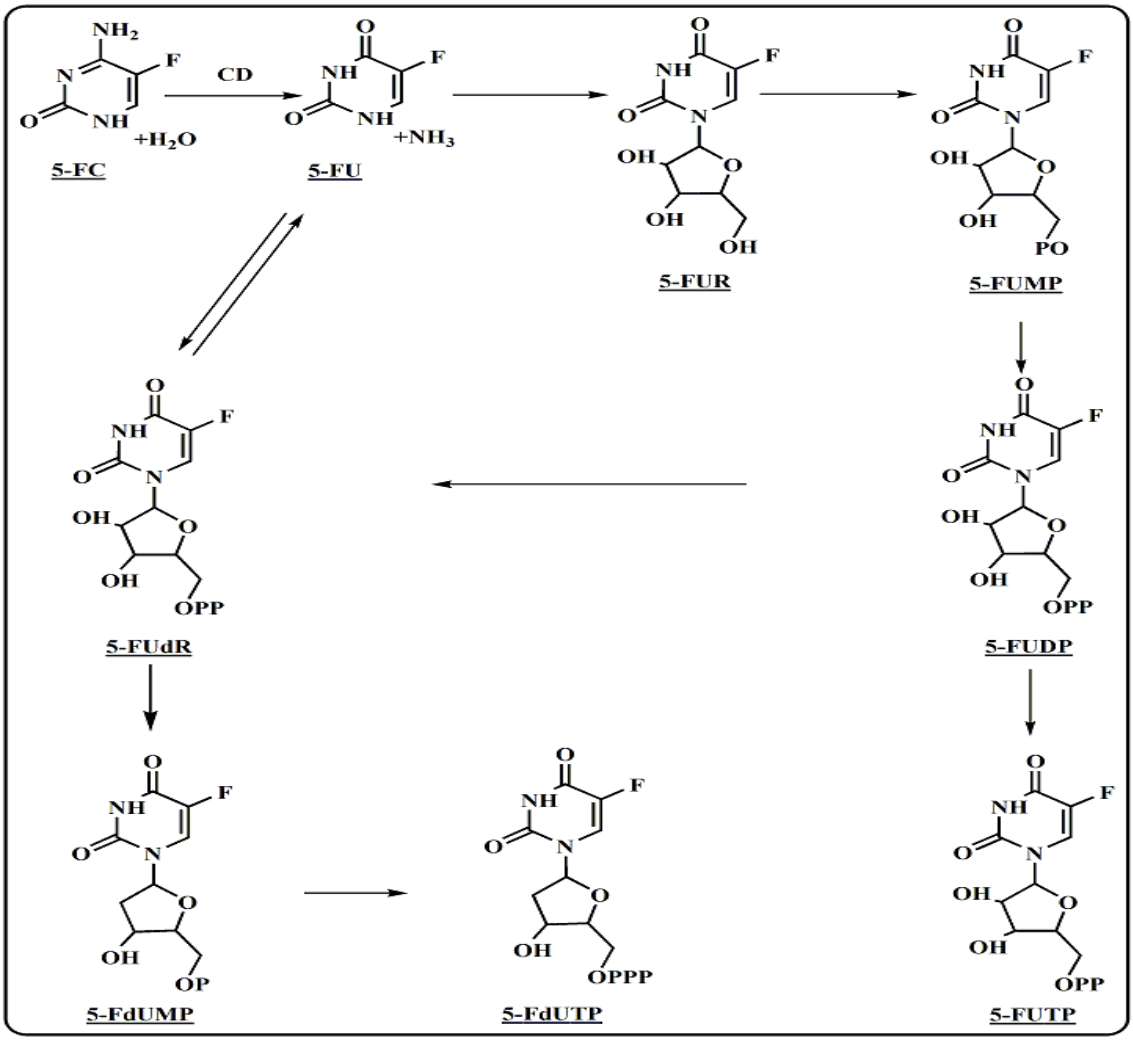

Figure 7: Conversion of 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU) by E. coli cytosine deaminase (CD). 5-FU is converted by cellular enzymes into 5-fluorodeoxyuridine-50-monophosphate (5-FdUMP), 5-uorodeoxyuridine-50-triphosphate (5-FdUTP) and 5-fluorouridine-50-triphosphate (5-FUTP).

***Membrane transporters*[103]**

Membrane transporters are integral membrane proteins that control the movement of amino acids, sugar, nucleosides, and peptides across cell membrane. It is known that, membrane transporters have been used to improve the bioavailability of polar drugs by the prodrug strategy.

Membrane transporters include glucose transporter, peptide and amino-acid transporter. Peptide transporter is the most attractive and widely used transporter for the prodrug design. Peptide transporters are divided into two categories: (i) peptide transporters PEPT1 and PEPT2; and (ii) peptide/ histidine transporters PHT1 and PHT2.

PEPT1 transporter is characterized by over-expression in many cancer cells including the malignant ductal pancreatic cancer cell lines AsPc-1 and Capan-2, and human fibrosarcomas cell line HT-1080, and this over expression is not seen in normal cell. The anticancer drug floxuridine used for metastatic colon cancer and hepatic metastases was linked to PEPT1 via an ester linkage to provide a prodrug based on the above mentioned approach. Studies have shown that this prodrug exhibited a higher uptake in PEPT1 over-expressing tumor cells. As a result, a selective growth inhibition was observed in tumor cells over-expressing PEPT1, but not in PEPT1-negative tumor cells.

Another example utilizing this approach was applied for Gemcitabine, a nucleoside analog compound that is used clinically as an efficient anti-neoplastic agent. Amino acid ester conjugates of Gemcitabine were shown to serve as substrate for either one or both of the peptide transporters PEPT1 and PEPT2.

Another important membrane transporter for targeted prodrug is the sodium-dependent multivitamin transporter (SMVT).

***PASSIVE TARGETTING*[103]**

Prodrugs can also be targeted to tumors by passive targeting. This is achieved by attaching the drug to large molecules or nanoparticles that act as inert carriers. This strategy depends on enhanced permeability and retention (EPR) effect of tumor environment.

***Drug Release at the Tumor Site*[103]**

When Prodrug is being inside the tumor, it must be activated to exert its antitumor activity. The activation of the free drug can occur intracellularly or extracellular.

***Enzymatic cleavage*[103]**

Prodrug activation can be achieved by tumor-associated enzymes, which are expressed either intracellularly or extracellular by cancerous cells. The drug release by enzymatic cleavage is achieved by the following mechanisms: (a) the active drug is directly linked to a peptide linker and the linkage between the two moieties is cleaved by the enzyme to provide the active drug and (b) an enzymatic cleavage to the peptide sequence is taking place to release the drug-peptide derivative, which is in a following step cleaved to the active drug. Another possibility is to attach self-immolative spacer to the peptide promoiety.[103]

***Acid sensitive linkers*[103]**

Acid sensitive linkages are used in the prodrug approach and they are intended to cleave under the acidic conditions present in tumors, lysosomes, and endosomes. The environment in tumor tissues is more acidic (0.5–1.0 pH units lower) than the normal tissues. These changes in pH can be used to cleave acid sensitive prodrugs extracellular, especially when the prodrug stays in tumor interstitium for long durations.

Examples of acid sensitive linkage used in prodrug and conjugate design are imine, hydrazone, carboxylic hydrazine, ketal, acetal, cis-aconityl and trityl bonds (Figure 8).

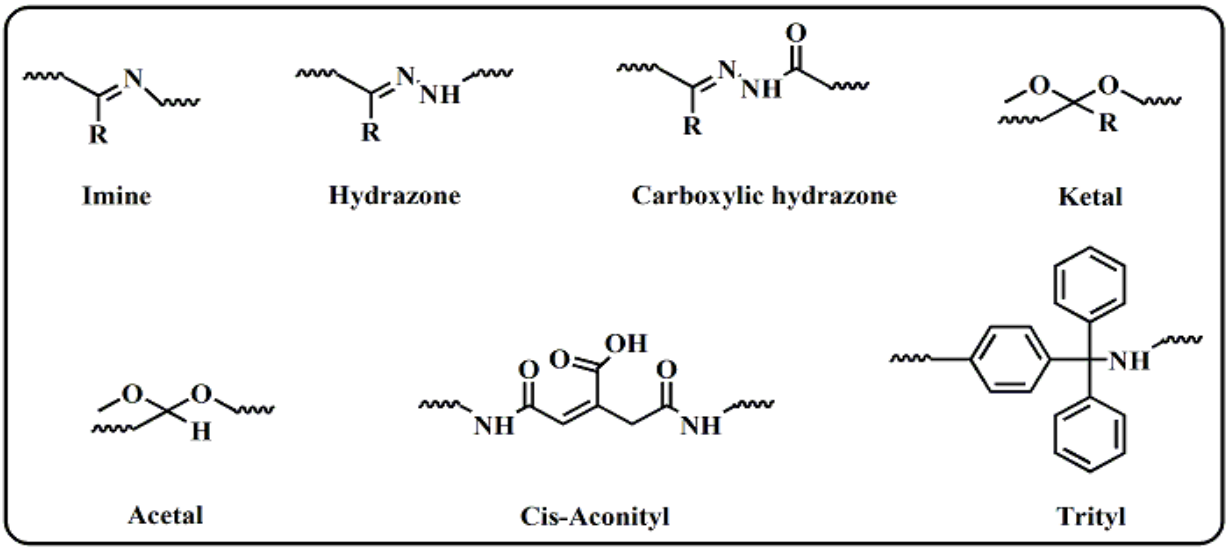

Figure 8: Acid sensitive linkages used in prodrug design.

***Hypoxia*[103-106]**

A common mechanism for converting non-toxic prodrug to a toxic drug in a hypoxic environment include reduction by one or two electrons of the prodrug to form a radical that becomes a substrate for back-oxidation by an oxygen to the original compound.

Examples of hypoxic prodrugs in clinical trials include: anthraquinone derivative (AQ4N). Three prodrug systems have been reported to be efficiently activated by ionizing radiations under hypoxia: nitrobenzyl quaternary ammonium salts, cobalt (III) complexes, and oxypropyl-substituted 5-fluoruracil derivatives.

Immunotoxins[103-107]

Antibody conjugates of highly potent drugs (DOX is frequently used) are called Immunotoxins. Immunotoxins contain a toxin made by insects, plants or microorganisms, Examples for Immunotoxins include *Pseudomonas* exotoxin A (PE), diphtheria toxin (DT), and ricin. Several Immunotoxins were constructed by conjugating mAbs to whole toxins via a disulfide linkage. The disulfide bonds are cleaved in the reducing environment present in endosomes/ lysosomes and the process usually involves thiol-exchange reaction. A widely investigated example is the BR96-DOX conjugate. Promising immune-toxins currently in clinical trials include TransMID 107 (transferrin-CRM107) and PRECISE (IL13-PEI-301-R03).

Self-immolative spacers[108]

The self-immolative spacers have three components: drug, linker, and trigger. A reaction takes place between trigger and the linker to form a drug-linker derivative, which then degrades spontaneously by cyclization or elimination to release the free drug (Figure 9).

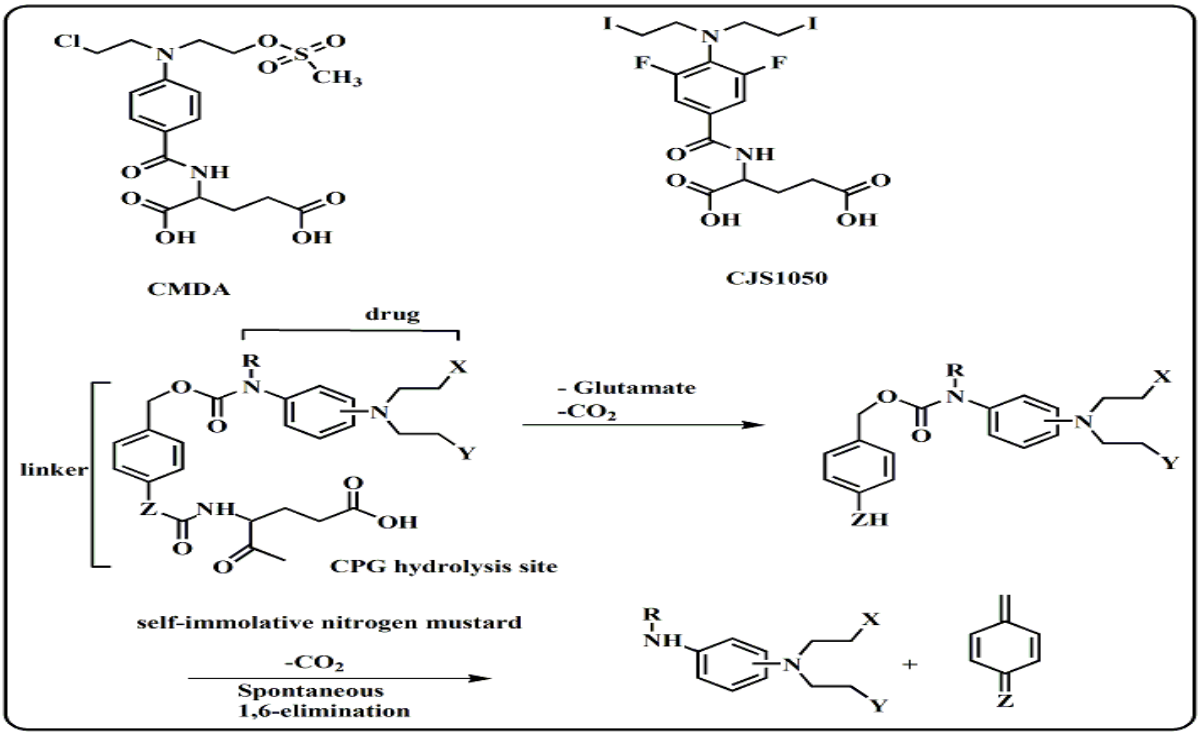

Figure 9: Self-immolative mustard prodrug.

ANTI-CANCER PRODRUGS BASED ON INTRAMOLECULAR PROCESSES[109-153]

Three myelodysplastic syndromes (MDS) agents were approved by the U.S. Food and Drug Administration: 5-azacitidine, decitabine and cytarabine (Figure 10). Chemotherapy with the hypomethylating agents, 5-azacytidine and decitabine resulted in a decrease of blood transfusion requirements and progression retard of MDS to acute myelogenous leukemia (AML). All three nucleoside agents have short half-life values ($t_{1/2}$). Design and synthesis of a slow degrading prodrug can provide sustained exposure to the drug during the treatment of MDS patients. This might result in better clinical outcome, more convenient dosing regimens and potentially less adverse effects.

Another example, decitabine has to be administered by continuous IV infusion, if a prodrug is designed to be breakdown in a slow release manner by SC route, optimum MDS maintenance treatment could be imminent.

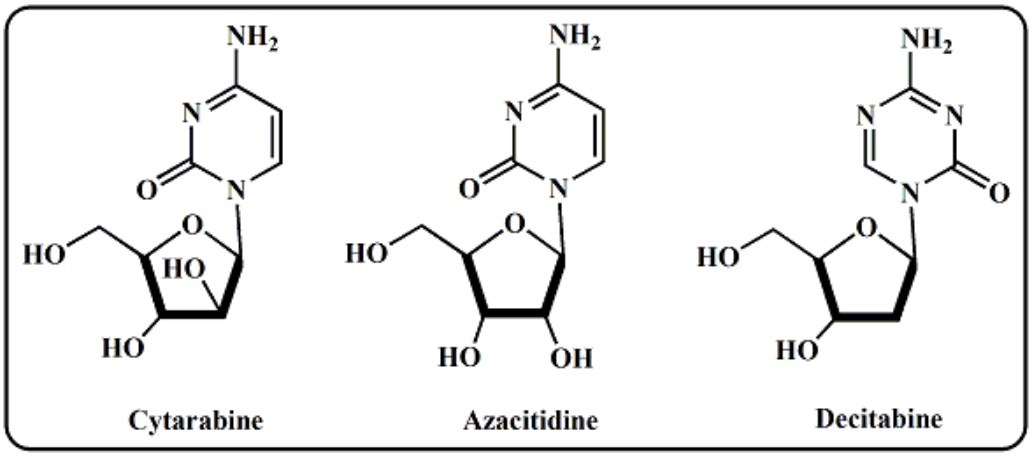

Figure 10: Chemical structures of the aza-nucleosides, cytarabine, azacitidine and decitabine.

By improving azacitidine, cytarabine and decitabine pharmacokinetic properties the drug absorption *via* a variety of administration routes, especially the SC injection route, can be facilitated. Utilizing a carrier-linked prodrug strategy by linking the aza nucleoside drugs to a carrier moiety can provide a chemical device capable of penetrating the membrane tissues and releasing the aza nucleoside in a controlled manner.

In the past five years, karaman's group has unraveled a respected number of intramolecular processes which were utilized as enzyme models. Based on DFT calculations on a proton transfer reaction in some of Kirby's enzyme models, Karaman's group have designed three

prodrugs of aza nucleoside. As shown in Figure 11, the aza nucleoside prodrugs **ProD 1-ProD 3** have N, N-dimethylanilinium group (hydrophilic moiety) and a lipophilic moiety (the rest of the prodrug), where the combination of both moieties secures a moderate HLB. Furthermore, in a physiologic environment of pH 5.5, SC, aza nucleoside prodrugs **ProD 1-ProD 3** may have a better bioavailability than their parent active drugs due to improved absorption. In addition, those prodrugs may be used in different dosage forms because of their potential solubility in organic and aqueous media due to the ability of the anilinium group to be converted to the corresponding aniline group in a physiological pH of 6.5.

The selection of Kirby's enzyme model to be utilized as carriers to aza nucleosides is based on the fact that those carriers undergo proton transfer reaction to yield an aldehyde, an alcohol and a hydroxy amine. The rate-limiting step in these processes is a proton transfer from the anilinium group into the neighboring ether oxygen. Furthermore, the proton transfer rate is strongly dependent on the strength of the hydrogen bonding in the reactions transition states. Therefore, the reaction rate is greatly affected by the structural features of Kirby's enzyme model system as evident from the different experimental rate values determined for the different processes.[144]

Karaman's DFT calculation results for intramolecular proton transfer reactions in Kirby's enzyme models revealed that the reaction rate is quite responsive to geometric disposition. For example, based on the calculated log EM, the cleavage process for prodrug **ProD 1** was predicted to be about 10^{10} times faster than for prodrug **ProD 2** and about 10^{4} times faster than prodrug **ProD 3**:$\text{rate}_{\mathbf{ProD1}} > \text{rate}_{\mathbf{ProD3}} > \text{rate}_{\mathbf{ProD2}}$. Hence, the rate by which the prodrug releases the aza nucleoside can be determined according to the structural features of the linker (Kirby's enzyme model). The three designed prodrugs were synthesized and characterized and in-vitro and in-vivo studies on their bioavailability are underway.[144]

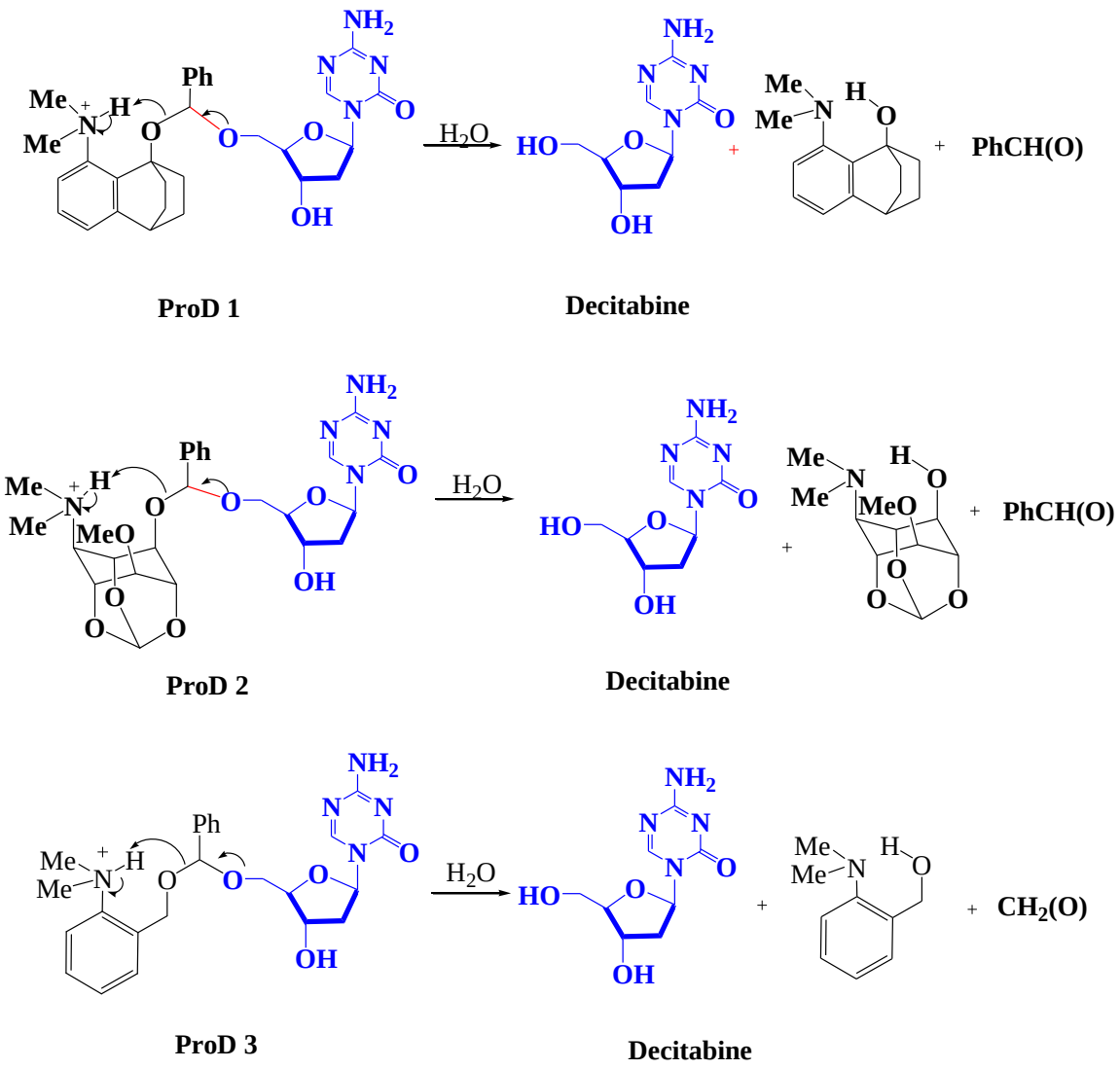

Figure 11: Intramolecular cleavage of aza-nucleoside prodrugs ProD 1-ProD 3 to their corresponding parent active drugs.

SUMMARY AND CONCLUSIONS

There are two major prodrug design approaches: the first is the targeted drug design approach by which prodrugs can be designed to target specific enzymes or carriers by considering enzyme-substrate specificity or carrier-substrate specificity in order to overcome various undesirable drug properties. This type of "targeted-prodrug" design requires considerable knowledge of particular enzymes or carriers, including their molecular and functional characteristics.

This approach has been accelerated after encouraging results emerged from several studies on targeted prodrugs that demonstrated better efficiency and safety profiles.

Active targeting of cancer cells can be achieved by targeting transporters present at these cells or by using chimeric/humanized mAbs. While passive targeting can be achieved by taking advantage of the EPR effect which is characteristic for tumor cells. Some conditions associated with tumors such as hypoxia and low pH are also considered as good methods for targeting. For prostate cancer specific linkers that can be cleaved by the highly expressed PSA were linked to a number of tested prodrugs. For targeting liver cancer HepDirect prodrugs and carbamate prodrugs were made, tested and are in use. In colon targeting all the developed prodrugs contain a labile bond that can be cleaved by the enzymes secreted by the colonic microflora, such as azo bond containing prodrugs or they are linked to specific conjugates that can be degraded only in the colon.

Redox chemical delivery systems that contain pyridine have shown a good efficacy for CNS targeting.

In targeting HIV, researchers have developed prodrugs to target macrophages by linking them to moieties that make the prodrug-conjugate capable of being internalized by receptor mediated endocytosis.

Antibody directed enzyme prodrug therapy (ADEPT) is relatively new method for cancer treatment. It is a two-step approach where an antibody-drug activating enzyme conjugate (AEC) is given first to be targeted and localized into the tumor and accumulates predominantly at the tumor cells that have the wanted tumor associated antigen. In the second step a nontoxic prodrug is injected systemically to be converted to its corresponding active form with high tumor concentration by the localized enzyme. This method has advantages over the older cancer therapy and is considered as a promising approach in the area of cancer treatment.

Alternative approaches designed to overcome the limitations of ADEPT are gene-directed enzyme prodrug therapy (GDEPT) and virus-directed enzyme prodrug therapy (VDEPT). In these approaches, genes encoding prodrug-activating enzymes are targeted to tumor cells followed by prodrug administration. In GDEPT, nonviral vectors that contain gene-delivery agents, such as peptides, cationic lipids or naked DNA, are used for gene targeting. In VDEPT, gene targeting is achieved using viral vectors, with retroviruses and adenoviruses being the most commonly used viruses. For both GDEPT and VDEPT, the vector has to be taken up by the target cells, and the enzyme must be stably expressed in tumor cells. This

process is called transduction. GDEPT and VDEPT effectiveness has been limited to date by insufficient transduction of tumor cells *in vivo*.

The second approach is the chemical design approach in which the drug is linked to inactive organic moiety which upon exposure to physiological environment releases the parent drug and a non-toxic linker which should be eliminated without affecting the clinical profile.

Unraveling the mechanisms of a number of enzyme models has allowed for the design of efficient chemical devices having the potential to be utilized as prodrug linkers that can be covalently attached to commonly used drugs which can chemically, and not enzymatically, be converted to release the active drugs in a programmable manner. For instance, exploring the mechanism for Kirby's acetals has led to the design and synthesis of novel prodrugs of aza-nucleosides for the treatment for myelodysplastic syndromes. In this example, the prodrug moiety was linked to the hydroxyl group of the active drug such that the drug-linker moiety (prodrug) has the potential to interconvert when exposed into physiological environments such as stomach, intestine, and/or blood circulation, with rates that are solely dependent on the structural features of the pharmacologically inactive promoiety (Kirby's enzyme model).

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