

# The prodrug approach to better targeting

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Prodrugs, the pharmacologically inactive derivatives of active drugs, are designed to maximize the amount of active drug that reaches its site of action, through manipulation of the physicochemical, biopharmaceutical or pharmacokinetic properties of the drug. But new developments are increasingly taking the concept beyond issues of availability to include targeting and enzyme activation.

Meeting	Prodrugs: Effective Solutions for Solubility, Permeability, and Targeting Challenges
Date	28-29 June 2004
	Location Philadelphia, PA, USA
Organizer	Pharmaceutical Education Associates
Website	<a href="http://www.pharmedassociates.com">www.pharmedassociates.com</a>

Prodrug design can be very effective in solving many of the stability, solubility, permeability and targeting problems that plague drug discovery and development. An inaugural two-day conference on prodrugs, organized by Pharmaceutical Education Associates and chaired by Howard Ando of Pfizer, covered the recent developments in prodrug techniques that are being used to solve delivery and targeting issues in R&D.

There are a number of criteria that should be met even before making a prodrug, began Kenneth Sloan of the University of Florida: (i) is there really a problem that is worth fixing, (ii) is the prodrug really transient, (iii) do the components of the prodrug cause extra toxicity, and (iv) is the prodrug cheap and simple to make?

Several common promoiety groups that can be used to make prodrugs of various functional groups, including acyl and 'soft alkyl' groups. The chemical stability of prodrugs may be different under enzymatic conditions. For example, the chemical stability of aliphatic carboxylic esters in buffered aqueous solutions increases with increasing chain length of the aliphatic acid. Enzymatic hydrolysis of esters increases initially with chain length, and then decreases as the chain lengthens beyond 6-7 carbons. Sloan explained several different hydrolysis mechanisms for the conversion of prodrugs to active moieties and the criteria used in the selection of the promoiety: avoid ones that generate toxic metabolites!

## Ester prodrugs

Oral absorption is a key component of oral bioavailability. Pfizer's Kevin Beaumont discussed issues involved in designing ester prodrugs including solubility, efflux and intestinal metabolism.

A prodrug moiety needs to be selected to balance lipophilicity and aqueous solubility, but increased lipophilicity increases the risk of P-glycoprotein-mediated efflux. Gut wall hydrolysis of ester prodrugs could limit oral bioavailability, while non-productive hepatic hydrolysis and biliary excretion could minimize the benefits expected of increased lipophilicity. Prodrugs of simple alkyl esters, cyclic carbonate esters and acyclic double esters often present their own advantages and disadvantages. Simple alkyl esters are not usually substrates of human blood esterases, tending to rely on hepatic hydrolysis, and have been used successfully for many ACE inhibitors. Cyclic carbonate esters and acyclic double esters can be activated by human blood borne esterases and have been used successfully for antibiotics, antivirals, and angiotensin II antagonists. But, there could be issues with their chemical stability and the formation of reactive aldehyde or ketone metabolites.

Esters, the most common type of prodrug, are converted back to the active parent via the ubiquitous esterases present in blood, tissues and organs. One theme throughout the conference was that these esterase enzymes exhibit broad and overlapping substrate specificity towards esters and amides and that their activity varies considerably between species. Aliphatic ester hydrolysis rates typically decrease in the order: rat > rabbit > dog > human. Rodents have some aliphatic esterases that are not present in humans and are believed to contribute to the large inter-species difference in drug disposition. For screening purposes, dogs should be used instead of rodents and whole blood should be used instead of just serum.

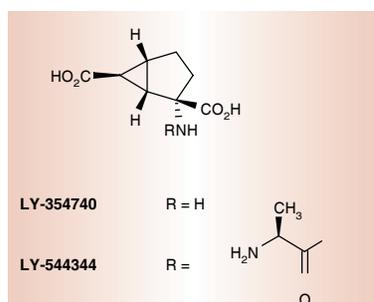
## hPepT1-targeted prodrugs

Everett Perkins of Eli Lilly discussed the use of peptide transporters in increasing membrane permeability. LY-354740 is an agonist for the type-2 metabotropic glutamate receptor (mGluR2), but its high water-solubility and zwitterionic nature result in very low membrane permeability and an oral bioavailability of only ~6% in humans. Simple ester modification of LY-354740 does not result in any viable candidate due to insufficient stability, although derivatization with L-alanine of the primary amine in LY-354740 makes it a good substrate of the

intestinal peptide transporter hPepT1. The resulting prodrug LY-544344 is enzymatically hydrolyzed to release molar equivalents of alanine and LY-354740 in vivo. In vitro, prodrug uptake is concentration-dependent following Michaelis-Menton kinetics and is inhibited by GlySar, a known substrate of hPepT1.

LY-544344 is rapidly absorbed and readily converted to the parent drug in rats and dogs, with systemic plasma exposure to the prodrug  $\leq 4\%$  of the corresponding values for the parent. The low concentrations of the prodrug in the portal vein of rats and dogs suggest that the primary site of prodrug hydrolysis is within the intestinal epithelium and mesenteric microcirculation. Pharmacokinetics of LY-354740 are generally dose-proportional following oral administration of the prodrug, with exposure to LY-354740 increased up to 16-fold in rats and up to 17-fold in dogs.

In clinical studies, the prodrug increased systemic LY-354740 exposure by approximately 13-fold compared with equivalent LY-354740 doses. Systemic exposure of prodrug was negligible as compared to LY-354740 ( $C_{max} < 3\%$  and  $AUC < 2\%$  of that achieved for LY-354740). LY-354740 pharmacokinetic parameters are dose-proportional following administration of LY-544344 up to 276.8 mg. Total LY-354740 exposure ( $AUC_{0-\infty}$ ) after a single oral dose of LY-544344 was equivalent after fasting, a high-fat or high-protein diet. High-fat and high-protein diets increased the exposure of prodrug slightly.



### Active transporters

Targeting active transport pathways can overcome a range of problems that limit drug development including poor intestinal permeability, narrow absorption window, short half-life, high first-pass metabolism, rapid drug efflux, poor absorption due to low solubility, poor CNS penetration, and poor tissue targeting.

Kenneth Cundy of XenoPort discussed two efforts to modify drugs into substrates of active transporter systems in the large intestine in order to improve the absorption and distribution characteristics of drugs.

Gabapentin is an anticonvulsant used for the treatment of epilepsy and post-herpetic neuralgia, but suffers from suboptimal pharmacokinetic properties including saturable absorption, high inter-patient variability, lack of dose proportionality and short half-life. To improve the PK properties of gabapentin, XP-13512 was developed as an oral prodrug. XP-13512 is a substrate of MCT-1, a monocarboxylate transporter (MCT) which is highly expressed in all segments of the colon as well as upper GI, and SMVT, and is a sodium-dependent transport system responsible for transfer and distribution of multiple vitamins from the various absorptive tissues. Oral bioavailability increased from 25% for gabapentin to 85% for XP-13512 in monkeys, and no saturation was observed for increasing prodrug doses. XP-13512 is cleaved

to gabapentin by non-specific esterases in the intestines, liver, and blood with low prodrug exposure ( $< 2\%$ ) in human clinical trials, while the tablet formulation of the prodrug allows twice-daily dosing. XP-13512 is currently in two phase IIa clinical trials for post-herpetic neuralgia and restless leg syndrome.

Cundy also discussed briefly XenoPort's effort to find a novel, patentable single isomer prodrug for baclofen, a GABA<sub>B</sub> agonist used as a muscle relaxant and antispastic. The company is focusing on XP-19986, which has demonstrated improved PK profiles in animals including a 15-fold increase in colonic absorption in monkeys. Xenoport plans to file in 2004 an IND application with FDA to start clinical trials.

### Ocular prodrugs

The complexity of the human eye presents unique challenges for drug delivery. Ocular bioavailability through topical administration (eye drops) is poor, usually  $< 5\%$ . Therapeutic levels of many drugs may be difficult to achieve in ocular tissues and systemic toxicities are of concern when the oral and intravenous routes of administration are used. Ashim Mitra of University of Missouri-Kansas City presented work on the molecular identification and functional characterization of P-glycoproteins in human and rabbit cornea. P-gps are efflux pumps that pump out the topically applied drugs that enter the cornea, contributing to low ocular bioavailability. An implantable microdialysis probe was used in the same eye of a rabbit to obtain ocular pharmacokinetics of erythromycin in the vitreous and aqueous chambers. The in vivo absorption data showed that P-gp is functional in the cornea and restricts drug absorption into the aqueous. Inhibition with various drugs resulted in increases in AUCs that was concentration-dependent.

One strategy was presented to overcome P-gp-mediated efflux through prodrug derivatization utilizing nutrient transporters expressed on the outer leaflet of cellular membranes. Quinidine, a well known substrate of P-gp, was conjugated to valine in the form of an ester. Val-quinidine does not interact with P-gp even at high concentrations. Competition with Gly-Sar and various model amino acid substrates indicates that Val-quinidine is a good substrate for the amino acid and peptide transporters present on the cornea. This led to the identification of various amino acid and peptide transporters on the cornea including a Na<sup>+</sup>-independent large neutral amino acid transporter LAT1, a neutral and cationic amino acid transporter B<sup>0,+</sup>, and oligopeptide transport system PepT1. To utilize the peptide transporters present on the cornea, the dipeptide-aciclovir conjugate, Val-Val-ACV, was synthesized and shown to be highly permeable across cornea (2.3-fold that of aciclovir). Though Val-Val-ACV is about 20% less permeable than valaciclovir, it is more stable. The dipeptide prodrug exhibited good affinity for hPepT1 in Caco-2 cells and was cleaved by the enzymes, specifically the dipeptidases, aminopeptidases and cholinesterases, present in the tissues to regenerate the active parent drug, ACV. Val-Val-ACV showed excellent in vitro antiviral activity against HSV1 and very good in vivo activity against HSV1 rabbit epithelial/stromal keratitis.

## Anticancer prodrugs

Folates are low-molecular weight vitamins required by all eukaryotic cells for one-carbon metabolism and de novo nucleotide synthesis. While most cells rely on a low-affinity ( $K_D \sim 1\text{--}5 \mu\text{M}$ ) membrane-spanning protein that transports reduced folates directly into the cell, a few cells also express a high affinity ( $K_D \sim 100 \text{ pM}$ ) receptor, generally referred to as the folate receptor (FR). FR preferentially mediates the uptake of oxidized forms of the vitamin (eg, folic acid) by receptor-mediated endocytosis. FR is known to be expressed on the surfaces of many malignant cells and is fully accessible to parenterally administered folate-drug conjugates. Philip Low of Purdue University and Endocyte has recently made progress in his laboratory on the use of folate receptor-mediated endocytosis in delivering imaging and therapeutic agents to tumor cells.

A radioactive indium probe in the form of indium-DTPA folate conjugate ( $^{111}\text{In}$ -DTPA-folate) was evaluated in phase I/II clinical studies in 1999 by Endocyte and has shown great promise in localizing tumor sites. Patients suspected of having ovarian cancer received a 5 mCi (2 mg) intravenous dose of the radiopharmaceutical, and whole-body single photon emission computerized tomographic (SPECT) images were taken 4 h later to identify the location of the probe. In addition to the kidneys (and to some extent the liver), the folate-targeted radiopharmaceutical probe accumulated in the widely-disseminated malignant tissue in ovarian cancer patients. A similar distribution pattern was also demonstrated in patients receiving a  $^{99\text{m}}\text{Tc}$ -folate conjugate. These clinical studies demonstrated that folate-mediated tumor targeting also occurs in humans and that folate-targeted radiodiagnostic imaging agents could be useful for non-invasively identifying the loci of pathological FR+ tissue within patients. Folate-conjugated imaging agents were also found to accumulate in inflamed joints in patients, dogs, and rats with rheumatoid arthritis, leading to the use of FR-targeted drug to eliminate activated macrophages involved in destruction of joint tissue in patients with rheumatoid arthritis and other autoimmune diseases.

A number of chemotherapeutic agents conjugated to folic acid for FR-targeted chemotherapy have shown improved therapeutic effects in animal models. A more interesting development is to use FR for immunotherapy. Since a substantial fraction of the occupied FR remains on the cell surface, it could be used for targeting highly immunogenic antigens to the cell surface of FR-expressing tumor cells. Treatment would begin with a series of subcutaneous inoculations of a hapten-based vaccine to stimulate

production of anti-hapten antibodies in the patient. After induction of an adequate antibody titer, a folate-hapten conjugate is administered to enable 'marking' of all FR-expressing tumor cells with the hapten. This process rapidly promotes the tumor cell's opsonization with the previously induced endogenous anti-hapten antibodies. For proof of concept, fluorescein isothiocyanate was conjugated to folic acid. Injection of folate-fluorescein into mice with metastatic tumor nodules shows bright green fluorescence in tumors with significantly less or no uptake in non-tumor tissues. This folate-targeted immunotherapy was effective in tumor-bearing rodent models without evidence of liver or kidney toxicity.

## Glutathione pathways

Glutathione S-transferases (especially the  $\pi$  isozyme) are elevated in many solid tumors and overexpressed in many drug resistant tumors. Kenneth Tew of the Medical University of South Carolina is working on three compounds as prodrugs at various stages of development. The first one is  $\gamma$ -glutamyl-S-(benzyl)cysteinyl-(*R*)-phenylglycine diethyl ester (TLK-199). The hydrolyzed diacid form of TLK-199 is a micromolar inhibitor of GST $\pi$ . It has been shown that the GST $\pi$  interacts with and suppresses the activity of c-Jun N-terminal kinase (JNK). GST $\pi$

deficient mice (GST $\pi^{-/-}$ ) have higher levels of circulating white blood cells than wild type. TLK-199 disrupts the interaction between GST $\pi$  and JNK and mimics in wild-type mice the increased myeloproliferation observed in GST $\pi^{-/-}$  animals. The JNK inhibitor, SP-600125, which by itself causes little inhibition of cytokine-induced myeloproliferation, prevented all myelostimulant effects of TLK-199, suggesting that GST $\pi$  plays a critical role in control of proliferation. TLK-199 is currently in phase I/II clinical trials.

The other two compounds discussed by Tew were TLK-286 and PABA/NO, both of which are activated by GST $\pi$ . TLK-286 is activated by GST $\pi$  to release the

alkylating phosphorodiamidate mustards while PABA/NO releases nitric oxide upon activation. TLK-286 showed mild dose-limiting toxicities in phase I trials and is currently under phase II clinical trial. PABA/NO has shown antitumor effects in a human ovarian cancer model grown in SCID mice. Using two dimensional gel electrophoresis with monoclonal antibodies directed against glutathionylated cysteine residues, PABA/NO produced a time- and dose-dependent glutathionylation of > 50 proteins within 5 minutes of treatment. Glutathionylation is emerging as an important post-translational modification that influences the function and stability of a variety of proteins.



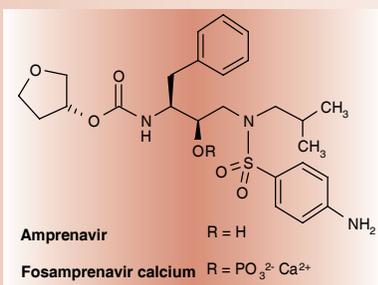
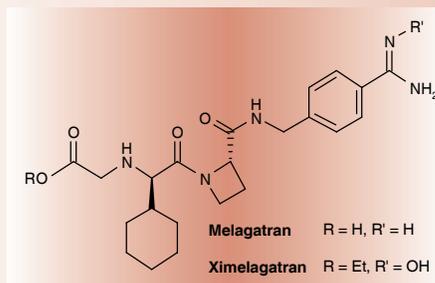
**Folic acid, found in vegetables like broccoli, is being used to target cancer.**

## Case studies

**Ximelagatran - a double prodrug**

Melagatran was identified in 1990s as a potent direct inhibitor of thrombin and platelet aggregation, although its oral bioavailability was only about 5% and was greatly reduced when dosed with food due to the presence of two strongly basic groups and one carboxylic acid group. It would be present as a zwitterion at intestinal pH. Ximelagatran is a double prodrug of melagatran designed to increase permeability while maintaining its good pharmacological properties.

According to Troy C Sarich of AstraZeneca, the carboxylic acid of melagatran was converted to an ester and the imidine was hydroxylated to reduce its basicity, leading to a compound that is not charged at intestinal pH, is 170-fold more lipophilic and 80-fold more permeable than the parent. Ximelagatran is virtually inactive towards thrombin and is rapidly converted to the active melagatran *in vivo* across a wide range of patient populations, leading to a much improved bioavailability of about 20% for the active melagatran.

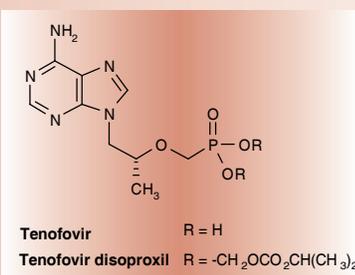
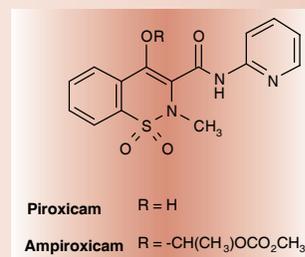
**Fosamprenavir: A soluble prodrug, a better product**

The HIV protease inhibitor amprenavir was approved by the FDA in 1999 for use in adults and children with HIV infection, but its limited water solubility requires the use of softgel formulation for delivery and multiple pills for a single dose. Dan Todd of GlaxoSmithKline presented fosamprenavir calcium as a prodrug of amprenavir with improved aqueous solubility. After screening 60 prodrugs in *in vitro* and *in vivo* assays, the phosphate prodrug, fosamprenavir calcium (GW-433908), was selected for its high water solubility, solution and solid-state stability, and rapid conversion to the parent drug on the apical side of epithelium. The prodrug is delivered from a solid dosage form with a lower pill

burden, two tablets replacing eight amprenavir softgels. Fosamprenavir calcium was approved by the FDA in October 2003 for use in combination with other antiretroviral agents for the treatment of HIV infection in adults.

**Amproxicam: A novel anti-inflammatory**

Piroxicam is an NSAID that can cause serious gastrointestinal bleeding, perforation and ulceration. Anthony Marfat of Pfizer discussed development of the prodrug amproxicam to reduce gastric irritation. Over 225 piroxicam derivatives were made and screened for COX inhibition, bioavailability in rat, solid state stability, and absorption in dogs. In a preclinical ulcerogenic study in rat, UD<sub>50</sub> for amproxicam was 38.6 mg/kg while UD<sub>50</sub> = 17.9 mg/kg for piroxicam, while in clinical trials, amproxicam showed comparable therapeutic efficacy

**Tenofovir disoproxil fumarate for HIV**

Tenofovir is an acyclic nucleoside phosphonate that undergoes phosphorylation to form tenofovir diphosphate, a potent and inhibitor of viral reverse transcriptase. But tenofovir is a dianion at physiological pH with a low partition coefficient, leading to low and erratic oral bioavailability. Reza Oliyai of Gilead discussed the design and development of tenofovir disoproxil fumarate as a prodrug to improve the permeability of tenofovir across biological membranes. Tenofovir disoproxil, the bis-isopropoxyl carbonate, was the most stable of several evaluated phosphonate prodrugs and quickly converted back to the parent tenofovir in the presence of tissue homogenates. Oral bioavailability as tenofovir in beagle

dogs is about 30%. <sup>18</sup>O incorporation studies indicate that non-enzymatic chemical hydrolysis of tenofovir disoproxil comes from nucleophilic attack on phosphorus resulting in initial P-O bond cleavage at pH 7.0 and 37°C. In solid state, the degradation product, formaldehyde, could further react with two molecules of tenofovir disoproxil resulting in the formation of geminal-diamine dimers. To minimize the extent of dimerization in solid state, the fumarate salt was selected. Tenofovir disoproxil fumarate is chemically stable in solid state, is non-hygroscopic up to 93% relative humidity at room temperature, and shows relatively rapid dissolution. Most importantly, it has an oral bioavailability of about 43% in humans, requiring only a once-daily dose.

## CYP450 prodrugs

Cytochrome P450 plays an important role in metabolism and detoxification of exogenous substances taken by mouth, including drugs. Knowledge of the CYP450 system is critical in understanding drug metabolism and drug interactions. Early identification of CYP450 substrates, inhibitors and inducers as well as possible reactive metabolites has become an integral part of the lead optimization process stated Rosa Sanchez of Merck in an overview of the biochemical and genetic aspects of CYP450 enzymes that included several examples of CYP450-mediated prodrug activation process.

Cyclophosphamide and ifosfamide are prodrugs activated by CYP450 in the liver. If tumors can be made to produce more of the activating CYP450 enzyme, it would greatly enhance the therapeutic effect of CYP450-activated prodrugs without increasing host toxicity caused by systemic distribution of active drug metabolites formed in the liver. This is the basis of CYP450 gene-directed enzyme prodrug therapy (P450 GDEPT) discussed by David Waxman of Boston University.

The chemosensitivity of tumors to CYP450-activated prodrugs can be dramatically increased by transfer of a rodent or human *CYP* gene, which increases the capacity of the target tumor tissue to activate the prodrugs. One way to further increase the therapeutic efficacy of P450 GDEPT is to increase the partition ratio for tumor:liver prodrug activation in favor of intra-tumoral metabolism by co-expression of the flavoenzyme NADPH-P450 reductase. Another approach is through the co-expression of an anti-apoptotic factor that would inhibit tumor cell apoptosis in a manner that prolongs the generation of cytotoxic metabolites but does not ultimately block tumor cell death. The cytotoxic metabolites would then diffuse to and kill the neighboring cancer cells, resulting in the so-called bystander effect.

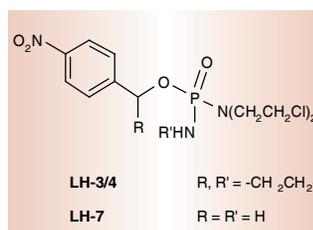
A third approach is anti-angiogenic scheduling of prodrug administration that substantially magnifies the antitumor effect and leads to major regression of established tumors. This is because the endothelial cells lining tumor vasculature are sensitive to low levels of phosphoramidate mustard and frequent low-dose treatment would preclude endothelial cell recovery and induce apoptosis of endothelial cells lining the tumor vasculature. In a phase I/II trial completed in 2002 in UK, P450 GDEPT showed some clinical benefit in late-stage breast cancer and melanoma and there was evidence of induction of systemic antitumor immune responses, which was confirmed in a second phase I/II study.

## Mustards

*E coli* nitroreductase is an example of an enzyme that has been investigated for use in GDEPT. CTL-102 from Cobra Therapeutics, a combination of virally-delivered nitroreductase and the commonly used prodrug substrate, CB-1954, is currently undergoing phase II trials in UK. While most efforts have been focused on structure modification of CB-1954 to find better prodrugs or on site-directed mutagenesis of the nitroreductase enzyme to optimize

the enzyme for better activation of the prodrug, my laboratory has been working on a new series of nitroaryl phosphoramidate mustards as better prodrugs for nitroreductase activation.

Among these are LH3/4, a 4-nitrophenyl-substituted cyclophosphamide, and LH7, an acyclic nitrobenzyl phosphoramidate mustard, with each with a strategically placed nitro group on the benzene ring in the para position to the benzylic carbon. The nitro group is strongly electron-withdrawing (Hammett  $\sigma$  electronic parameter = 0.78) and is converted to an electron-donating hydroxylamino group ( $\sigma = -0.34$ ) upon nitroreductase reduction. This large difference in electronic effect ( $\Delta\sigma = 1.12$ ) is exploited to effect the formation of the highly cytotoxic phosphoramidate mustard or like-reactive species which are excellent substrates of *E coli* nitroreductase and have high selective cytotoxicity against nitroreductase-expressing V79 and SKOV3 cells.



The cyclic compound LH3/4, was over 22,000-fold more cytotoxic in nitroreductase-expressing Chinese hamster V79 cells while the acyclic LH7 was 167,500-fold more cytotoxic in the same cell line with an IC<sub>50</sub> as low as 0.4 nM upon 72 h of drug exposure. This level of activity is about 100-fold more active and 27-fold more selective than CB-1954. Even when the V79 cells were exposed to each test compound for 1 h before the media were replaced with non-drug containing fresh media, the IC<sub>50</sub> was 10 nM, which is about 30-fold lower than CB-1954. LH7 also showed great bystander effect in cell culture assays comparable to, if not better than, CB-1954.

## Problem solving

This inaugural conference covered some of the basics in prodrug design and many of its applications in addressing drug delivery and targeting issues. A number of successful examples of prodrug design were presented that have resulted in pharmaceutical products already commercialized or in late-stage development. In addition to the more common uses in improving oral bioavailability, the conference demonstrated how prodrugs are increasingly being investigated for targeting purposes including site-specific activation and delivery of anticancer drugs to tumor tissues through transporters, tumor or tissue-specific enzymes and gene therapy.

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