

Prodrugs: Effective Solutions for Solubility, Permeability and Targeting Challenges

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This 2-day inaugural conference on prodrugs was presented by Pharmaceutical Education Associates and covered recent developments in prodrug techniques to solve delivery and targeting issues in drug discovery and development. The speakers were drawn from industry and academia, and the conference was attended mostly by researchers working in the pharmaceutical and biotechnology industries. A number of topics were presented at the conference, from basic prodrug design and functional group considerations to drug metabolism involving cytochrome P450 enzymes, from increasing water solubility, bioavailability, permeability and stability to tumor targeting, from the development of new anti-inflammatory agents to anti-HIV agents, and from the use of transporters and receptor-mediated endocytosis in prodrug delivery to the use of gene therapy for enzyme delivery to cancer cells and tissues. Several case studies were presented including improved pharmaceutical products in the clinic and at various stages of development.

Introduction

Approximately 50 delegates attended this 2-day conference, representing large pharmaceutical, small biotechnology, government and academic research laboratories. The conference was chaired by Howard Ando (Pfizer Inc, USA) and began with Kenneth Sloan (University of Florida, USA) discussing problems in the drug development process that can be solved through a prodrug approach and basic prodrug design functional group considerations. This was followed by 17 presentations on the application of prodrug approaches for addressing issues in drug delivery and targeting. Topics covered included: (i) the basics of prodrug design; (ii) increasing bioavailability with ester prodrugs; (iii) increasing permeability with hydroxyl amine prodrugs; (iv) creating water-soluble drugs with prodrugs; (v) prodrugs for topical administration; (vi) protecting therapeutics with PEGylation to increase stability; (vii) utilizing *in vivo* models of prodrug disposition; (viii) designing prodrugs to take advantage of intestinal transporters; (ix) reducing dosing and patient variability with nutrient transporters; (x) utilizing hPepT1 transporters for drug delivery; (xi) prodrug approaches to efflux transporters; (xii) cytochrome P450 (CYP450) gene therapy for cancer, using gene-directed enzyme prodrug therapy (GDEPT); (xiii) folate-receptor-targeted therapies for cancer and inflammatory diseases; (xiv) designing prodrugs for glutathione S-transferase activation; and (xv) new nitroaryl phosphoramides as anticancer prodrugs for nitroreductase activation in GDEPT.

Functional group considerations in prodrug design

The basic concepts of prodrugs and examples that are used clinically were reviewed by Kenneth Sloan. Several ACE inhibitors are currently marketed as ester prodrugs, including enalapril (Vasotec), ramipril (Altace), benazepril (Lotensin HCT) and fosinopril (Fozilec), for the treatment of hypertension. Other therapeutic classes that use a prodrug delivery method include antibiotic and antiviral agents. Before using a prodrug approach, the following criteria should be met: (i) there is a real problem that is worth addressing; (ii) the prodrug is transient; (iii) the components of the prodrug do not cause extra toxicity; and (iv) the prodrug should be cheap and simple to make. Several common pro-moieties that can be used to make prodrugs of various functional groups were reviewed, including acyl and 'soft' alkyl groups. It was noted that chemical stability of prodrugs may be different to enzymatic stability, 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, however, increases initially with chain length and decreases when the chain length is longer than six to seven carbons. Dr Sloan explained several different hydrolysis mechanisms for the conversion of prodrugs to active moieties and the criteria used in the selection of a pro-moiety, ie, chemical groups that generate toxic metabolites should be avoided.

Cytochrome P450 and its role

CYP450 plays an important role in metabolism and detoxification of exogenous substances, including drugs taken orally. Knowledge of the CYP450 system is critical in understanding drug metabolism and drug interactions, and early identification of CYP450 substrates, inhibitors and inducers, as well as possible reactive metabolites, has become an integral part of the lead optimization process. Dr Rosa Sanchez (Merck & Co Inc, USA) presented an overview of the biochemical and genetic aspects of CYP450 enzymes and discussed several examples of CYP450-mediated prodrug activation processes.

Ester prodrugs to enhance oral absorption

Oral absorption is a key component of oral bioavailability. Kevin Beaumont (Pfizer Ltd, UK) discussed issues involved in designing ester prodrugs including solubility, efflux and intestinal metabolism. Examples were used to illustrate that: (i) the appropriate prodrug moiety needs to be selected to balance lipophilicity and aqueous solubility, which is vital for prodrug delivery; (ii) increased lipophilicity may not lead to improved oral absorption due to the involvement of P-glycoprotein (P-gp)-mediated efflux; (iii) gut wall hydrolysis of ester prodrugs could limit oral bioavailability; and (iv) non-productive hepatic hydrolysis and biliary excretion could minimize expected benefits for the increased

lipophilicity. Prodrugs of simple alkyl esters, cyclic carbonate esters and acyclic double esters offer certain advantages and disadvantages. Simple alkyl esters are not usually substrates of human blood esterases, tend 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. There may, however, be issues with their chemical stability and the formation of reactive aldehyde or ketone metabolites.

Esters are a commonly used prodrug group. Conversion to the active parent drug involves the activity of ubiquitous esterases that are present in blood, tissues and organs. One theme throughout the conference was that these esterase enzymes show broad and overlapping substrate specificity toward esters and amides and 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 these are believed to contribute to the large interspecies difference in drug disposition. For screening purposes, dogs should be used instead of rodents and whole blood should be used instead of serum.

Prodrug design based on PEG linkers

An overview of Enzon Pharmaceuticals Inc's efforts to enhance therapeutic value by attaching PEG to therapeutic proteins and small molecules was given by Hong Zhao (Enzon Pharmaceuticals Inc, USA). PEG is a relatively non-reactive and non-toxic polymer that is frequently used to improve pharmacological characteristics, for example, increased stability, prolonged half-life and reduced toxicity, for both potential or existing macromolecular therapeutic agents. Several PEGylated versions of small molecules were presented including examples of camptothecin, paclitaxel, daunorubicin, vancomycin and ara-C, and several PEGylated prodrugs were shown to have improved efficacy in tumor xenograft models with an enhanced vascular permeability and retention effect resulting in passive tumor targeting. One example, pegamotecan (Enzon Pharmaceuticals Inc) is currently in pivotal trials.

Case studies

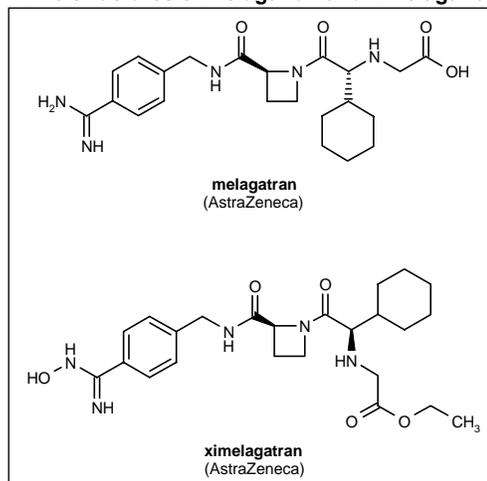
Ximelagatran as a double prodrug of melagatran to increase permeability

Melagatran (AstraZeneca plc; Figure 1) was identified in the 1990s as a potent direct inhibitor of thrombin and platelet aggregation. Its oral bioavailability is only approximately 5% and is greatly reduced when dosed with food, due to the presence of two strongly basic groups and one carboxylic acid group, meaning that it exists as a zwitterion at intestinal pH. Troy C Sarich (AstraZeneca plc, USA) presented the discovery and development of ximelagatran (Exanta, AstraZeneca plc; Figure 1), which was developed as a double prodrug of melagatran in order to increase permeability while maintaining good pharmacological properties. Thus, the carboxylic acid group was converted to an ester and the imidine moiety was hydroxylated to reduce its basicity.

Ximelagatran is not charged at intestinal pH and is 170-fold more lipophilic and 80-fold more permeable than its parent;

it is virtually inactive toward thrombin and is rapidly converted to active melagatran *in vivo* in a wide range of patient populations. Additionally, ximelagatran has a much improved bioavailability of approximately 20% for active melagatran and had a positive benefit/risk balance in phase II/III clinical trials as an oral anticoagulant. Ximelagatran has successfully completed the European Mutual Recognition procedure in 15 European countries and is currently under review by the FDA for the prevention of stroke and other thromboembolic complications associated with atrial fibrillation and long-term secondary prevention of venous thromboembolism.

Figure 1. The structures of melagatran and ximelagatran.



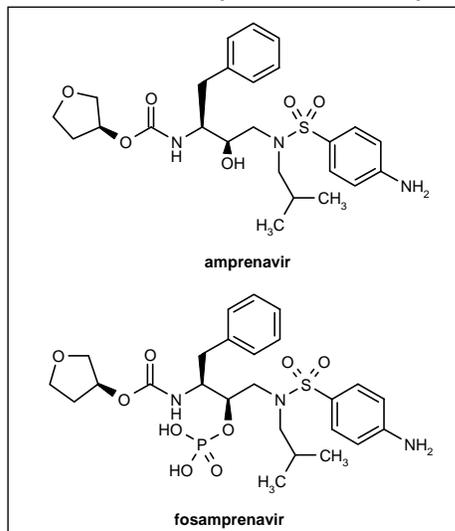
Fosamprenavir: A soluble prodrug leads to a better product

Amprenavir (Figure 2) is an HIV protease inhibitor approved by the FDA in 1999 for use in adults and children with HIV infection. Its limited water solubility requires the use of a softgel formulation and multiple pills for a single dose. Dan Todd (GlaxoSmithKline plc, USA) discussed Fosamprenavir (Figure 2) as a prodrug of amprenavir with improved aqueous solubility. After screening 60 potential prodrugs *in vitro* and *in vivo*, the phosphate prodrug fosamprenavir was selected for its high water solubility, solution and solid-state stability and its rapid conversion to the parent drug on the apical side of epithelium prior to absorption of the parent. The prodrug is delivered via a solid dosage form with a lower pill burden (two tablets replaced eight amprenavir softgels). Fosamprenavir was approved by the FDA in October 2003 for use in combination with other antiretroviral agents for the treatment of HIV infection in adults.

Ampiroxicam: A prodrug anti-inflammatory agent

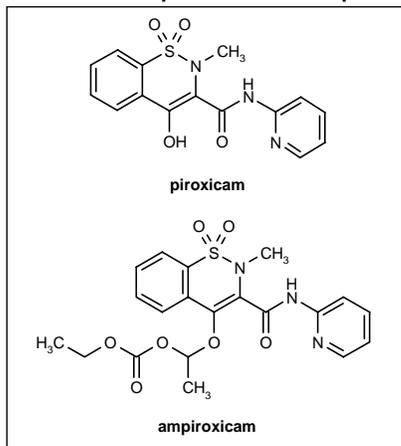
Piroxicam (Figure 3) is a non-steroidal anti-inflammatory drug (NSAID) that can cause serious gastrointestinal bleeding, perforation and ulceration. Anthony Marfat (Pfizer Inc, USA) discussed the use of the prodrug concept in the discovery of ampiroxicam (Figure 3) as an anti-inflammatory agent to reduce or eliminate the local or topical component of gastric irritation. Over 225 derivatives of piroxicam were made and screened for cyclooxygenase inhibitory activity, solution bioavailability in rats, and solid-state stability and absorption in dogs. Ampiroxicam was

Figure 2. The structures of amprenavir and fosamprenavir.



found to meet all decision criteria and was developed further. In a preclinical ulcerogenic study in rat, the UD_{50} value for ampiroxicam was 38.6 mg/kg compared with 17.9 mg/kg for piroxicam. In clinical trials, ampiroxicam was found to have comparable therapeutic efficacy to piroxicam but with less gastrointestinal side effects.

Figure 3. The structures of piroxicam and ampiroxicam.



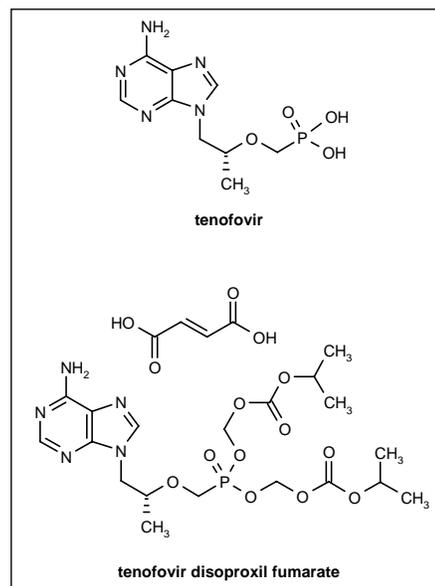
Tenofovir disoproxil fumarate: A successful anti-HIV agent

Tenofovir (Figure 4) is an acyclic nucleoside phosphonate that undergoes phosphorylation to form tenofovir diphosphate; the latter is a potent and selective inhibitor of viral reverse transcriptase that effectively blocks viral replication. However, tenofovir is a dianion at physiological pH with a low partition coefficient, and its oral bioavailability is low and erratic in animal studies. Reza Oliyai (Gilead Sciences Inc, USA) discussed the design and development of tenofovir disoproxil fumarate (Figure 4), which is a prodrug designed to improve the permeability of tenofovir across biological membranes.

Several ester prodrugs of tenofovir were evaluated. Tenofovir disoproxil, the bis-isopropoxyl carbonate

derivative, was the most chemically stable in solution and quickly converted back to tenofovir in the presence of tissue homogenates. The oral bioavailability of the prodrug for tenofovir in beagle dogs is approximately 30%. Isotope incorporation studies indicated that non-enzymatic chemical hydrolysis of tenofovir disoproxil results from nucleophilic attack on phosphorus, leading to initial P-O bond cleavage at pH 7 and 37°C. In the 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 this dimerization, the fumarate salt was selected; tenofovir disoproxil fumarate is chemically stable in the solid state, is non-hygroscopic at a relative humidity of up to 93% at room temperature and has a relatively rapid dissolution rate in aqueous solution. Tenofovir disoproxil fumarate formulated into a tablet has an oral bioavailability of approximately 43% in humans; a once-daily dose at 300 mg significantly reduced HIV RNA levels and is well tolerated.

Figure 4. The structures of tenofovir and tenofovir disoproxil fumarate.



Prodrug design targeting transporters

Abhijit Ray (University of Maryland, USA) discussed prodrug targeting of transporters. Solute transporters are membrane proteins that play a pivotal role in the cellular uptake of nutrients and drugs, but isolating and resolving their three-dimensional (3D) structure has been difficult. One approach to overcome this lack of structural information is to use computational methods such as pharmacophore modeling, comparative molecular field analysis, homology modeling and threading. Pharmacophore models were built for the intestinal nucleoside transporters P-gp and the human apical bile acid transporter.

Prodrugs targeting hPepT1: From concept to clinic

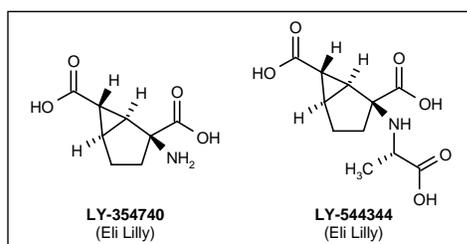
The use of peptide transporters for increasing membrane permeability was discussed by Everett Perkins (Eli Lilly &

Co, USA). LY-354740 (Eli Lilly & Co; Figure 5) is an agonist for metabotropic glutamate receptor 2 (mGluR2) and has two carboxylic acid groups and one basic amino group. The high water solubility and zwitterionic nature of LY-354740 results in low membrane permeability, while its oral bioavailability is only approximately 6% in humans. Simple ester modification of LY-354740 did not result in a viable prodrug candidate due to insufficient stability. Derivatization of the primary amine with L-alanine resulted in a LY-544344 (Eli Lilly & Co; Figure 5), a good substrate for the intestinal peptide transporter hPepT1. This prodrug has good water solubility and solution stability; it is enzymatically hydrolyzed to release molar equivalents of alanine and LY-354740 *in vivo*, while *in vitro* assays indicate that uptake of LY-544344 is concentration-dependent following Michaelis-Menten kinetics and is inhibited by GlySar, a known substrate of hPepT1. LY-544344 is readily hydrolyzed by rat and human jejunum S9 and plasma *in vitro*.

Preclinical studies indicated that LY-544344 was rapidly absorbed and readily converted to the parent drug in rats and dogs; systemic plasma exposure to prodrug was less than 4% of the corresponding values for the parent drug. 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. After oral administration of the prodrug, pharmacokinetic (PK) parameters of LY-354740 were generally dose proportional, and dogs and rats had up to 16- to 17-fold more exposure to LY-354740 compared with direct administration of the parent compound.

In clinical studies, the prodrug increased systemic LY-354740 exposure by approximately 13-fold compared with equivalent LY-354740 doses. Systemic exposure of prodrug is negligible compared with LY-354740 ($C_{max} < 3\%$ and $AUC < 2\%$ of that achieved for LY-354740). LY-354740 PK parameters are dose proportional following administration of LY-544344 up to a dose of 276.8 mg.

Figure 5. The structures of LY-354740 and LY-544344.

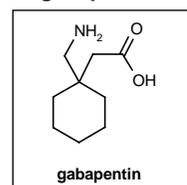


Prodrugs targeted to nutrient transporters in the large intestine

Targeting active transport pathways can overcome a range of problems that limit drug development including poor intestinal permeability, narrow absorption windows, 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 (XenoPort Inc, USA) discussed two efforts to modify drugs into substrates of intestinal active transporter systems in order to improve the absorption and distribution characteristics.

Gabapentin (Figure 6), an anticonvulsant used for the treatment of epilepsy and post-herpetic neuralgia, suffers from suboptimal PK properties including saturable absorption, high interpatient variability, lack of dose proportionality and a short half-life. To improve the PK properties of gabapentin, XP-13512 (XenoPort Inc) was developed as a prodrug to explore the use of intestinal monocarboxylate transporters (MCTs) to facilitate its absorption after oral administration. XP-13512 is a substrate of MCT-1, which is highly expressed in all segments of the colon, upper gastrointestinal tract and SMVT, a sodium-dependent transport system responsible for the transfer and distribution of multiple vitamins from various absorptive tissues. These active transport systems and better passive diffusion properties at low pH contributed to much improved PK properties. Oral bioavailability in monkeys increased from 25% for gabapentin to 85% for the prodrug, and XP-13512 showed dose-proportional exposure to active gabapentin in humans. No saturation was observed for increasing prodrug doses. XP-13512 is cleaved to gabapentin by non-specific esterases in the intestine, liver and blood, with low prodrug exposure (less than 2%) in clinical trials. A tablet formulation of the prodrug allows twice-daily dosing. XP-13512 has entered two phase IIa clinical trials for post-herpetic neuralgia and restless leg syndrome.

Figure 6. The structure of gabapentin.



XenoPort Inc's attempts to find a novel, patentable, single-isomer prodrug for baclofen, a GABA_B agonist used as a muscle relaxant and antispastic, were discussed briefly by Dr Cundy. The focus is on XP-19986, which has demonstrated an improved PK profile in animals including a 15-fold increase in colonic absorption in monkeys. XenoPort plans to file an IND for XP-19986 to start clinical trials in 2004.

Transporter and receptor-targeted prodrug delivery to the eye

The complexity of the human eye presents unique challenges for drug delivery. Ocular bioavailability after topical administration is usually less than 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 (University of Missouri, USA) presented research on the molecular identification and functional characterization of P-gp efflux pumps in human and rabbit cornea. An implantable microdialysis probe was used in the same eye of a rabbit to obtain ocular PK data for erythromycin in the vitreous and aqueous chambers. The *in vivo* absorption data indicate that P-gp is functional in the cornea and restricts drug absorption into the aqueous chamber. Inhibition with various P-gp inhibitors resulted in an increase in AUC

values which was concentration-dependent, and k_{abs} was increased by 6-fold with testosterone.

One strategy presented to overcome P-gp-mediated efflux was through prodrug derivatization using nutrient transporters expressed on the outer leaflet of cellular membranes. Quinidine, a well-known substrate of P-gp, was conjugated to valine (Val) through an ester link. Val-quinidine is not a P-gp substrate and 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 amino acid and peptide transporters present on the cornea. This led to the identification of various amino acid and peptide transporters including LAT1 (a sodium-independent, large, neutral amino acid transporter), B⁰⁺ (a neutral, cationic amino acid transporter), and PepT1 (an oligopeptide transport system). To use corneal peptide transporters, the dipeptide-aciclovir conjugate Val-Val-aciclovir was synthesized and shown to be highly permeable across the cornea (2.3-fold greater than aciclovir). Although Val-Val-aciclovir is approximately 20% less permeable than valaciclovir, it is more stable. The dipeptide prodrug showed good affinity for hPepT1 in Caco2 cells and was enzymatically cleaved, for example, by dipeptidases, aminopeptidases and cholinesterases, to generate aciclovir. Val-Val-aciclovir had excellent *in vitro* antiviral activity against HSV-1 and good *in vivo* activity against HSV-1 rabbit epithelial/stromal keratitis.

Receptor-targeted therapeutic and imaging agents for cancer and inflammation

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 ($K_d \sim 1$ to $5 \mu\text{M}$) 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). This receptor preferentially mediates the uptake of oxidized forms of the vitamin (eg, folic acid) by receptor-mediated endocytosis. FR is expressed on the surfaces of many malignant cells and is fully accessible to parenterally administered folate-drug conjugates. Philip Low (Purdue University, USA/Endocyte Inc) discussed recent progress in the use of FR-mediated endocytosis for delivering imaging and therapeutic agents to tumor cells.

An indium-DTPA folate conjugate probe (¹¹¹In-DTPA-folate) was evaluated in phase I/II clinical trials in 1999 by Endocyte Inc and has shown great potential in localizing to tumor sites. Patients suspected of having ovarian cancer received a 5-mCi (2 mg) intravenous dose of the radiopharmaceutical, followed by whole-body single photon emission computed tomography (SPECT) imaging after 4 h to identify the probe location. In addition to the kidneys and, partly, the liver, the folate-targeted radiopharmaceutical probe was found to accumulate in the widely disseminated malignant tissue. A similar distribution pattern was also demonstrated in patients receiving a ^{99m}Tc-folate conjugate. These clinical trials demonstrate that folate-mediated tumor targeting also occurs in humans and that

folate-targeted radiodiagnostic imaging agents could be useful to non-invasively identify the loci of pathological FR-positive tissue in patients. Folate-conjugated imaging agents were also found to accumulate in inflamed joints in patients and animals with rheumatoid arthritis. This led to the use of FR-targeted drugs to eliminate activated macrophages involved in the destruction of joint tissue in patients with rheumatoid arthritis and other autoimmune diseases.

Several chemotherapeutic agents have also been conjugated to folic acid for FR-targeted chemotherapy, which have improved therapeutic effects in animal models. A more interesting development is to use FR for immunotherapy because a substantial fraction of the occupied FR remains on the cell surface, which 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 in order to stimulate production of antihapten antibodies in the patient. After induction of an adequate antibody titer, a folate-hapten conjugate would be administered to enable 'marking' of all FR-expressing tumor cells with the hapten. This process rapidly promotes the opsonization of the tumor cell with previously induced endogenous antihapten antibodies. For a proof-of-concept study, fluorescein isothiocyanate was conjugated to folic acid, and was injected into mice with metastatic tumor nodules. Images showed green fluorescence in tumors, while significantly less (or no) uptake of the conjugate was noted in non-tumor tissues. This folate-targeted immunotherapy was effective in tumor-bearing rodent models without evidence of liver or kidney toxicity.

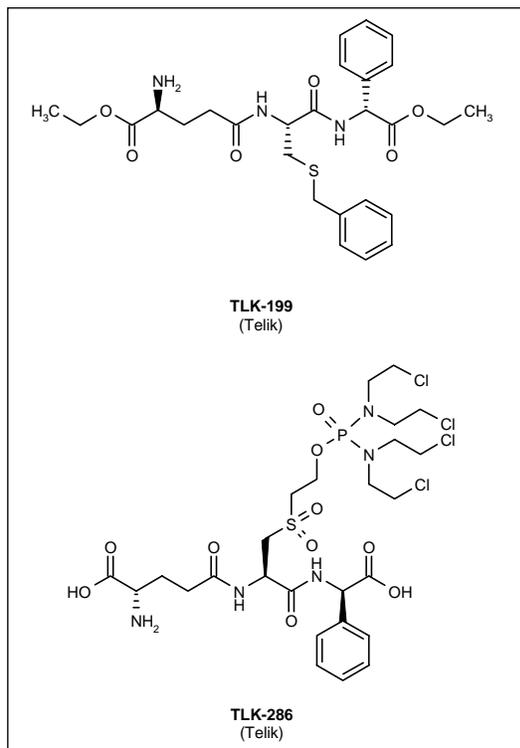
Glutathione pathways and prodrugs

Glutathione S-transferase enzyme levels (especially the π -isozyme) are elevated in many solid tumors and the enzyme is overexpressed in many drug-resistant tumors. Kenneth Tew (Medical University of South Carolina, USA) presented data on three prodrug compounds that are in various stages of development, beginning with TLK-199 (Telik Inc; Figure 7). The hydrolyzed di-acid analog of TLK-199 is a micromolar inhibitor of GST π . GST π interacts with, and suppresses the activity of, c-Jun N-terminal kinase (JNK). GST π -deficient mice have higher levels of circulating white blood cells than wild-type animals. In wild-type mice, TLK-199 disrupts the interaction between GST π and JNK and mimics the increased myeloproliferation observed in GST π -deficient animals. The JNK inhibitor SP-600125 (Signal Research Division of Celgene), which has low inhibitory activity against cytokine-induced myeloproliferation, prevents the myelostimulant effects of TLK-199, suggesting that GST π plays a critical role in control of proliferation. TLK-199 is currently in phase I/II clinical trials.

The other two compounds discussed by Dr Tew were TLK-286 (Telik Inc; Figure 7) and PABA/NO (*O*^2-*[2,4-dinitro-5-(N-methyl-N-4-carboxyphenylamino) phenyl]* 1-*N,N*-dimethylamino) diazen-1-ium-1,2-diolate), which are both activated by GST π . TLK-286 is activated to release an alkylating phosphorodiamidate mustard while PABA/NO releases nitric oxide. TLK-286 showed mild dose-limiting

toxicities in phase I clinical trials, and PABA/NO has antitumor effects in a human ovarian cancer model in SCID mice. Using two-dimensional (2D) gel electrophoresis with monoclonal antibodies directed against glutathionylated cysteine residues, PABA/NO produced a time- and dose-dependent glutathionylation of more than 50 proteins within 5 min of treatment. Glutathionylation is emerging as an important post-translational modification that influences the function and stability of a variety of proteins.

Figure 7. The structures of TLK-199 and TLK-286.



CYP450-prodrug-based gene therapy for cancer

Cyclophosphamide and ifosfamide are prodrugs that are activated by CYP450 in the liver. The therapeutic effect of CYP450-activated prodrugs would be greatly enhanced if tumors could be induced to produce more of the activating CYP450 enzyme without increasing host toxicity caused by systemic distribution of active drug metabolites formed in the liver. This is the basis of P450 GDEPT, discussed by David Waxman (Boston University, USA). Preclinical studies have shown that the chemosensitivity of tumors to CYP450-activated prodrugs can be dramatically increased by transfer of a rodent or human CYP gene that increases the capacity of the target tumor tissue to activate the prodrugs. Hypoxic conditions in tumor tissues do not seem to affect intratumoral CYP450 activity.

One approach that may further enhance the therapeutic efficacy of P450 GDEPT is to increase the partition ratio for tumor-liver prodrug activation in favor of intratumoral metabolism, by co-expression of the flavoenzyme NADPH-P450 reductase. In a xenograft tumor model, 9L solid tumors that are transduced with CYP2B6 and P450R *in vitro* became sensitized to cyclophosphamide *in vivo*. Another approach is

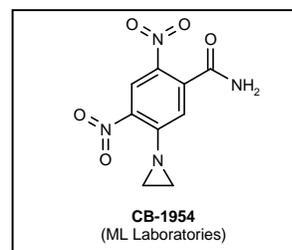
to use the co-expression of an anti-apoptotic factor that would inhibit tumor cell apoptosis in a manner that prolongs the generation of cytotoxic metabolites but does not ultimately block tumor cell death. The cytotoxic metabolites would then diffuse and kill neighboring cancer cells, resulting in the so-called 'bystander effect'. Baculovirus protein p35 is a caspase inhibitor with a broad specificity that may prevent cells from undergoing apoptosis. This p35 factor blocks cyclophosphamide-induced mitochondrial damage and thus prolongs the death of 9L/P450 cells, while p35 enhances the cytotoxicity of 9L/P450 cells to 9L/LacZ bystander cells.

The third approach discussed was to use 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 that line the tumor vasculature are sensitive to low levels of phosphoramidate mustard and frequent low-dose treatment would preclude endothelial cell recovery and induce endothelial cell apoptosis. In a UK-based phase I/II clinical trial completed in 2002, P450 GDEPT showed some clinical benefit in late-stage breast cancer and melanoma. Evidence of induction of systemic antitumor immune responses was confirmed in a second phase I/II trial.

Nitroaryl phosphoramidate mustards as prodrugs in nitroreductase-GDEPT

Escherichia coli nitroreductase has been investigated in GDEPT. The commonly used substrate CB-1954 (ML Laboratories plc; Figure 8), together with virally delivered nitroreductase (CTL-102; ML Laboratories plc), is currently in phase II trials in the UK. Most research efforts have been focused on structural modifications of CB-1954 to find better prodrugs or on site-directed mutagenesis of the nitroreductase enzyme to optimize activation of the prodrug. Longqin Hu (Rutgers University, USA) presented research on the discovery of a new series of nitroaryl phosphoramidate mustards that are expected to be better prodrugs for nitroreductase activation in GDEPT.

Figure 8. The structure of CB-1954.



Nitroaryl phosphoramidates LH3/4 and LH7 were designed and synthesized. LH3/4 is a 4-nitrophenyl-substituted cyclophosphamide analog, while LH7 is an acyclic nitrobenzyl phosphoramidate mustard. The nitro group is a strong electron-withdrawing group (Hammett σ_p electronic parameter = 0.78) that is converted to an electron-donating hydroxylamino group ($\sigma_p = -0.34$) after nitroreductase reduction. The large difference in electronic effect (change in $\sigma_p = 1.12$) is exploited to effect the formation of a highly cytotoxic phosphoramidate mustard-like reactive species.

The compounds were excellent substrates for *E coli* nitroreductase and had highly selective cytotoxicity in nitroreductase-expressing V79 and SKOV3 cells. Cyclic LH3/4 was over 22,000-fold more cytotoxic in nitroreductase-expressing Chinese hamster V79 cells than CB-1954, while acyclic LH7 was 167,500-fold more cytotoxic in the same cell line with an IC_{50} value of 0.4 nM after 72 h drug exposure. This level of activity is approximately 100-fold more active and 27-fold more selective than CB-1954. When V79 cells were exposed to each test compound for 1 h before the media was replaced with non-drug-containing fresh media, the IC_{50} value was 10 nM, which is approximately 30-fold lower than that of CB-1954. The high selectivity of these compounds was reproduced in SKOV3 human ovarian carcinoma cells infected with an adenovirus-expressing *E coli* nitroreductase. Enzyme kinetic analysis indicated that LH7 is a much better substrate for *E coli* nitroreductase than CB-1954, with a 20-fold better specificity

constant. LH7 also demonstrated a bystander effect in cell culture assays that was comparable to, if not better than, CB-1954.

Summary

Prodrug design can be highly effective for solving many of the stability, solubility, permeability and targeting problems in drug discovery and development. This inaugural conference covered some of the basics in prodrug design and many of its applications for addressing drug delivery and targeting issues. A number of successful examples of prodrug design were presented that resulted in pharmaceutical products, either already commercialized or in late-stage development. In addition to improving oral bioavailability, prodrugs are increasingly used 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.