Despite their broad spectrum activity, the clinical usefulness of cyclophosphamide and ifosfamide is limited by the formation of toxic byproducts. Both agents generate acrolein, a metabolite that has been implicated in kidney and bladder toxicity. In addition, ifosfamide gives rise to chloroacetaldehyde, a metabolite believed to cause CNS toxicity. To avoid toxicologic problems associated with the formation of such byproducts, we have investigated alternative strategies to deliver PM's into cells. Various prodrug formulations of PM's were prepared including compounds that are activated by carboxylate esterase, β-glucuronidase and β-galactosidase. GRAPH. In the absence of activating enzymes, most of the prodrugs were fairly stable and showed a low order of biological activity. In the presence of activating enzymes, however, the prodrugs were rapidly converted to PM's. The increased toxicity of the prodrugs to human tumor cells in the presence of activating enzymes varied from as little as 10-fold to as much as 500-fold. In addition to providing an alternative cell-delivery strategy for PM's, some of these prodrugs offer potential for use in conjunction with gene therapy approaches to tumor-selective drug activation. (Supported by grant CA RO1 89386).

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Design of a DNA damaging molecule "programmed" to release multiple high affinity inhibitors of EGFR tyrosine kinase under hydrolytic conditions: A novel antitumour drug combination strategy

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The altered protein expression and activity of receptor tyrosine kinases (TK) are implicated in the progression of various types of cancers. One such dysfunction is the overexpression of the epidermal growth factor receptor (EGFR) that correlates with aggressive tumor progression and poor prognosis. Recently, we developed a novel strategy that seeks to combine DNA damaging properties and EGFR TK inhibitory activities into single molecules termed "combi-molecules" designed to kill EGFR-expressing tumour cells (Matheson et. al., J. Pharm. Exp. Ther, 296, 832-840, 2001 Brahimi et al., ibid, 2002, in press). In order to enhance the EGFR inhibitory potency and stability of these compounds, we designed a novel strategy termed "cascade release" (CR) that seeks to mask the combi-molecule into a stable carrier "programmed" to release the antitumour species by hydrolytic cleavage. Since these molecules henceforth referred to as "cascade release molecules" (CRM) are also designed to retain EGFR affinity on their own, this principle leads to molecular systems whereby three generations of inhibitors can arise from the hydrolysis of the parent CRM. To study this model, we recently designed and synthesized RB24 (IC_{50} competitive binding=130 nM), which was a masked form of RB14 (IC_{50}=100 nM), a hydrolabile triazene capable of generating the combi-molecule ZR08 (IC_{50}=44 nM). The latter was found to further degrade into RB10, another potent inhibitor of EGFR (IC_{50}=40 nM). Kinetic studies using UV spectrophotometry demonstrated that the parent CRM, RB24, was hydrolyzed with a t1/2=42 min. Western blot analysis demonstrated potent inhibition of EGFR autophosphorylation by the CRM in the carcinoma of the vulva cell line, A431 (IC_{50}=2 uM). Studies on serum stimulated growth using a pair of isogenic cells [NIH3T3 and HER14 (engineered to overexpress EGFR)] showed that RB24 selectively induced approximately 5-fold stronger growth inhibitory activity in the EGFR-transfectant when compared with its parent NIH3T3, indicating significant EGFR selectivity. The results in toto suggest that RB24 is the first ever molecule capable of being an EGFR TK inhibitor (I), while being the parent of two other EGFR inhibitors (I2) and (I3), the latter being the precursor of another stable inhibitor (I4) + a DNA damaging fragment. Further studies are ongoing in our laboratory to determine the effects of the CR system on the sustainability and reversibility of EGFR TK inhibition.

Drug delivery

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Taxane-monoclonal antibody covalent conjugates for targeted chemotherapy of cancer

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In efforts to develop taxane derivatives capable of tumor-specific drug delivery (Safavy, US Patent 6,191,290 B1), we previously reported the synthesis and cytotoxicity results of a paclitaxel (PTX) monoclonal antibody (MAb) C225 (ErbituxTM, ImClone Systems, Somerville, NJ) conjugate (Figure 1) (Safavy et al., Eighth Conference on Radioimmunodetection and Radioimmunotherapy of Cancer, Princeton, NJ, 2000) with enhanced cytotoxicity of PTX against A431, UM-SCC-1, and UM-SCC-6 human cell lines.

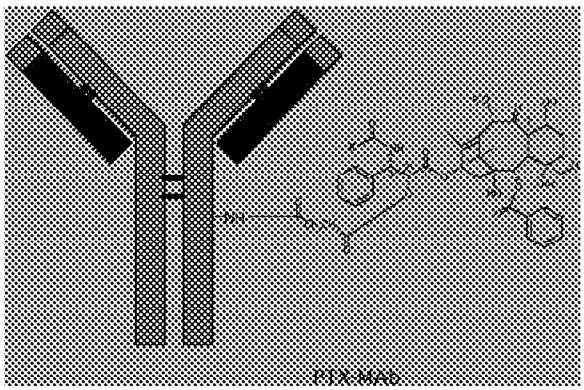
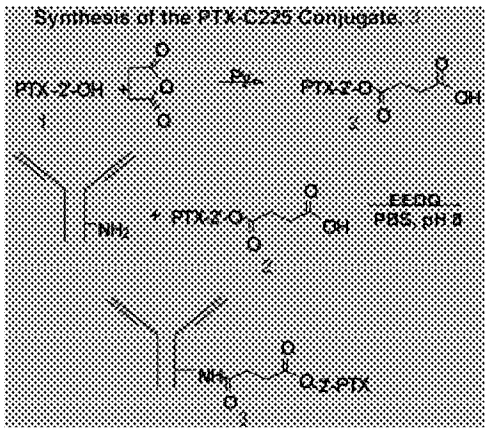


Figure 1

Here, conjugates of PTX with the anti-epidermal growth factor receptor antibody, C225 and anti HER2/neu antibody, Herceptin (Her, Trastuzumab, Genentech, South San Francisco, CA) were synthesized by the procedure shown in Scheme 1. The purity and number of drugs per antibody (PTX: MAb) were evaluated by HPLC and MALDI-TOF mass spectrometry, respectively.



Scheme 1

Purities of *98% and PTX: MAb of 2.5 were detected for both conjugates. These conjugates were then tested in cell binding and cytotoxicity experiments using MDA-MB-468 (human breast carcinoma) and LNCAP and DU145 (human prostate carcinoma) cell lines to determine the effect of receptor-targeted delivery in enhancing the drug efficacy. To demonstrate the retention of antigen-binding ability, the parent MAb and conjugates were radiolabeled with 125I and screened in binding inhibition assays. The percent cell binding (%B) of the PTX-C225 conjugate, as compared to the unconjugated MAb (parenthesized values) were 49 (88), 41 (87), and 40 (87) in MDA-MB 468, LNCAP, and DU145 cells, respectively. Herceptin and the PTX-Her conjugate showed a %B of 21 (36) to LNCAP cells with no appre-

ciable binding to the other two lines. Despite the relatively lower cell-binding activities of the conjugates compared to unconjugated MAb, the conjugate concentrations required to result in 50% cell death (IC_{50}s) were significantly lower than the free drug (Table 1).

Table 1. IC_{50} (nM) values of paclitaxel (PTX), PTXC225, and PTXHer conjugates in MDA-MB-468, LNCAP, and DU145 cell lines.

Cell Line	PTX	PTXC225	PTXHer
MDA-MB-468	13.5	3.9	3.7
LNCAP	3.0	0.9	N/A
DU145	8.3	3.3	3.2

Furthermore, preliminary therapy experiments show stabilization of A431 human epidermoid carcinoma tumors in athymic nude mice treated with PTXC225 as compared relative to the C225 treated control. Based on the above binding and IC_{50} results, a controlled therapy experiment with DU145-implanted nude male mice and using PTXC225, is underway. These results may point to a MAb-mediated tumor-specific paclitaxel delivery which may be advantageous to the conventional systemic administration of this important drug.

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Dynamics of tumor cell induced angiogenesis and microcirculation from tumor onset until late stage tumor disease: barriers to drug delivery

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Background: Tumor vasculature is characterized by a heterogeneous vessel distribution, morphology and physiology. These vascular irregularities are responsible for barriers of drug delivery and hinder successful therapies. However, due to inherit problems for continuous non-invasive monitoring of microvascular properties, the dynamics of these barriers during tumor onset and tumor growth are poorly understood.
Methods: A dedifferentiated Angiosarkoma cell line (SV40, GFP transfected, HBMEC-1) was implanted in cranial window for continuous non-invasive intravital microscopy in 12 weeks old male SCID mice (n=30). For 85 days vascular parameters such as functional vascular density, velocity, leukocyte endothelial interaction (LEI), tissue perfusion rate (TPR), branching pattern, vessel morphology and vascular permeability were obtained using fluorescence microscopy, as described elsewhere (Hansen-Algenstaedt et al. Cancer Research 2000, Yuan et al. Cancer Research 1994). To demonstrate histomorphologic aspects, immunhistochemistry was performed. Anti-laminin staining was used for basal membran visualization. Electronmicroscopy was performed for high resolution analysis.
Results: Tumor cell implantation was accompanied by an immediate and significant increase in permeability of pre-existing vessels. Although permeability peaked on day 12 the initial increase was significantly pronounced during the first 48 h, reaching a plateau phase after day 8. Blood flow in newly formed vessels was detected 3 days after tumor cell implantation. Tumor vessels demonstrated an increasing permeability from day 13 until day 61. No further increase until the end of observation period was observed. LEI increased significantly in tumor vessels. Increase of TPR was observed only during tumor onset. Later stages were characterized by a steady state while tumor size increased constantly and a slight decrease of TPR on day 85 respectively. During initial tumor growth the vascular branching pattern and blood flow velocity were less heterogeneous than during later stages. Anti-laminin staining revealed that tumor cells did not participate in endothelial lining of tumor vessels. Electronmicroscopy revealed intraluminal abnormalities such as multiple intercellular openings and transluminal bridging.
Conclusions: The vulnerable tumor onset period is characterized by regular vascular morphology but increased vascular permeability of host vessels. These characteristics can be utilized for the delivery of large molecules during tumor onset. Later stages with established tumors and tumor vessels are characterized by heterogeneous vessel distribution and irregular vessel morphology leading to impaired drug delivery. Therefore therapies that equalize blood supply, such as anti-vascular therapies, can be helpful to normalize drug delivery for combined therapies.

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Proteolysis of xyotax by lysosomal cathepsin B; metabolic profiling in tumor cells using LC-MS

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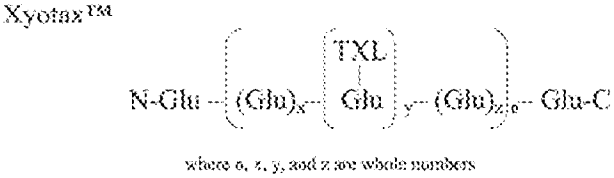
Xyotax(TM) (CT-2103) is a water-soluble polymer-drug conjugate that displays enhanced anti-tumor activity relative to paclitaxel (TXL) in a variety of preclinical models. Xyotax consists of a polydisperse poly-L-glutamic acid backbone, averaging 33 kD, in which paclitaxel molecules are esterified through the 2′ position on paclitaxel to the gamma-carboxylic acid residues of the PG polymer. Conjugation at 37% paclitaxel by weight results in approximately 1 paclitaxel molecule per 11 glutamic acid residues. It is currently under evaluation in Phase III clinical trials in multiple indications including colon, lung, and ovarian cancer. Tissue distribution studies in tumor bearing mice have led to the conclusion that Xyotax is biodegradable and undergoes degradation in part by proteolysis to form monoglutamyl-paclitaxel (2′-[L-gamma-Glu]-TXL), a chemically unstable species that is hydrolyzed to form paclitaxel and pyroglutamate. Although the specific mechanism(s) for this have not been fully elucidated, it has been proposed that principle uptake of amino acid polymers occurs by pinocytosis followed by transport to the lysosome for processing and degradation. Lysosomal cathepsin B, a cysteine protease which is highly expressed in a variety of tumor types and is associated with tumor cell invasion and metastasis, displays a high dipeptidase activity towards Xyotax resulting in abundant formation of diglutamyl-paclitaxel conjugates (i.e., 2′-[L- gamma -C-NH2-Glu-Glu]-TXL and 2′-[L-gamma-C-COOH-Glu-Glu]-TXL). We evaluated the ability of RAW264.7 (murine monocytic leukemia), HT-29 (human colon carcinoma), and NCI-H460 (human large cell lung) cell lines to metabolize Xyotax *in vitro*. Quantitative analysis was achieved using isotope labeling, reverse-phase HPLC, and electrospray ionization on a Micromass Quattro II mass spectrometer.



Time dependent generation of diglutamyl-paclitaxel, monoglutamyl-paclitaxel and free paclitaxel was observed for the cellular extracts over a 48 hour time period. Utilizing a cell permeable, selective inhibitor of cathepsin B, CA-074 Me, we find both limited and delayed proteolysis of Xyotax relative to control in the tumor cell lines studied. These data provide strong support for the biodegradability of Xyotax and suggest that release of free paclitaxel from CT-2103 may be increased in tumors with higher levels of cathepsin B expression.

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Increased sensitivity to chemotherapy during the window in time when tumor interstitial fluid pressure is lowered

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Chemotherapy against solid malignancies is often ineffective due to impaired transport of anti-cancer drugs into tumor tissue. This in part has been attributed to the pathologically increased tumor interstitial fluid pressure (IFP). We investigated the relevance of the pathologically high tumor IFP for efficacy of treatment with 5-fluorouracil (5-FU) in subcutaneous syngeneic PROb rat colonic carcinomas and chemically-induced rat mammary carcinomas. Prostaglandin E1 (PGE1) was used to acutely lower tumor IFP. IFP is transiently lowered following PGE1 administration, reaching a minimum after 10-15 minutes, and returning to the initial value after around 60 min. Lowering of IFP occurs without changes in blood flow or blood vessel permeability for albumin. 5-FU has a t1/2 of ~10-20 minutes in rats. By administering 5-FU at times when tumor IFP was lowered by PGE1, or alternatively, outwith those times, we could directly assess whether tumor IFP generates a functional barrier to chemotherapy. Lowering of tumor IFP with PGE1 increased capillary-to-interstitium transport of 5-FU as measured by microdialysis. A low dose of 5-FU had significant anti-tumor activity only