575 POSTER

Effect of silybin and docetaxel on LNCap prostate cell growth and total PSA levels

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Silybin, a flavonoid obtained from *Silybum marianum L.*, and its phospholipid complex IdB 1016 (silipide) have been shown to have antitumor activity and to potentiate the effects of cisplatin (Giacomelli et al., Life Sci. 70: 1447, 2002), and doxorubicin in mice implanted with DU145 human prostate tumor xenograft (Tyagi et al., Clin. Cancer Res. 8: 3512, 2002). The current study was performed to assess the effects of silybin on the growth of human prostate LNCap cells and their secretion of prostate specific antigen (PSA) *in vitro*. LNCap cells were plated at different densities in 96 well coated (poly-D-lysine) plates (Becton-Dickenson Biocoat) and incubated for 24h prior to the addition of various concentrations of silybin (0.2–200uM) or docetaxel (0.1–100nM). After 5 days, tumor cell mass was assessed using sulphorhodamine B assay and total secreted PSA was measured using a kit supplied by United Biotech. The concentration of agent to decrease tumor cell growth by 50% (IC50) was 55uM for silybin and 0.36nM for docetaxel. When human LNCap prostate tumor cells were treated with subtoxic concentrations of silybin (10–50uM) and docetaxel (0.1–0.3nM), dose dependent decreases in both cell number and PSA level were noted. However, when secreted PSA levels were normalized per cell number for docetaxel treated cells, there was no dose dependent change noted. This suggests that silybin not only inhibits LNCap cell growth but also directly decreases PSA secretion, supporting its potential use as a treatment for prostate cancer in man.

Prodrugs

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MVA-FCU1: a highly potent gene-based chemotherapy providing 5-FU local delivery

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Background: Direct transfer of pro-drug activation systems into tumours was demonstrated to be an attractive method for the selective *in vivo* elimination of tumour cells. Besides its local cytotoxic impact, this strategy was further demonstrated to enhance the host anti-tumour immune response through the local release of cellular debris that can be presented by the antigen presenting cells.
Material and methods: We describe a novel and highly potent suicide gene derived from the *Saccharomyces cerevisiae* cytosine deaminase (*FCY1*) and uracil phosphoribosyltransferase genes (*FUR1*). This suicide gene, designated *FCU1*, encodes a bifunctional chimeric protein that combines the enzymatic activities of *FCY1* and *FUR1* and efficiently catalyses the direct conversion of 5-fluorocytosine (5-FC), a non-toxic pro-drug, into the cytotoxic metabolites 5-fluorouracil (5-FU) and 5-fluorouridine-5′-monophosphate (5-FUMP). Interestingly, the cytosine deaminase activity is 10-fold higher in the chimeric protein compared to the natural protein.
Results: In this study we demonstrate that a MVA (Modified Vaccinia Virus of Ankara) engineered to express the *FCU1* gene significantly enhances the sensitivity of numerous human tumour cells to 5-FC (LD_{50} 5-FC = 1 μM in the FCU1 treated cells compared to LD_{50} 5-FC = 10mM in the CDase treated cells; $p<0.01$). Moreover, passive diffusion of the 5-FU ensures an impressive bystander effect with the ability to kill 100% of a *in vitro* tumour cell population with only 1% FCU1-transduced cells.
Intratumoral injections of MVA-FCU1 into human tumour-bearing mice, with concomitant systemic administration of 5-FC, led to a sustained control of tumour growth. The FCU1-induced tumour growth suppression was observed in different human colorectal tumour models whereas 5-FU administered IP at the maximum tolerated dose did not show any anti-tumor effect in the same model.
Finally, a 10-fold higher concentration of 5-FU is detected inside the tumour compared to a systemic administration of 5-FU while no detectable 5-FU is found in the circulation, ensuring a higher safety profile with no systemic toxicity.
Conclusions: The FCU1 suicide gene is a **unique combination** of an **innovative approach** and a **validate and secure chemotherapy** that makes it a novel and powerful candidate for treating all 5-FU sensitive tumours. A Phase I clinical trial is scheduled early 2005 in metastatic colorectal cancer patients.

577 POSTER

Bio-reductive prodrug approach to target angiogenesis in tumours

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Angiogenesis, the development of new blood vessels from existing ones, is a natural process occurring at many stages of life. A tumour cannot grow beyond a size of 1–2mm without being supplied by oxygen and nutrients. To overcome this problem, cancerous cells in hypoxic areas of tumours have the ability to trigger angiogenesis by a complex mechanism involving up and down regulations of transduction modulators. The subsequent vasculature of the tumours enables its growth and the formation of metastases.
In the last decade, many therapeutic compounds have been developed to inhibit angiogenesis. This work is focused on improving the delivery of such compound to hypoxic areas of tumours, using the unique properties of the tumour micro-vasculature. Endothelial cells within tumours are known to over-express reducing enzymes such as NQO1, NQO2 and cytochrome P450 reductase. We aim to design "bio-reductive prodrugs" which will, upon reduction by one of these enzymes, release the active anti-angiogenic drug. This project is particularly focused on the design and evaluation of nitroaromatic delivery systems.
Five nitroaromatic systems (**Figure 1**) were attached to a fluorescent compound (4-methyl-7-hydroxycoumarin) thus forming five "pro-fluorescent" prodrugs. These model-compounds enable the comparison by fluorescence spectroscopy of the behaviour of the delivery systems under chemical reductive conditions. The same delivery systems were then attached to two well-known anti-angiogenic compounds: the isoflavone biochanin A and an oxindole (SU5416®).

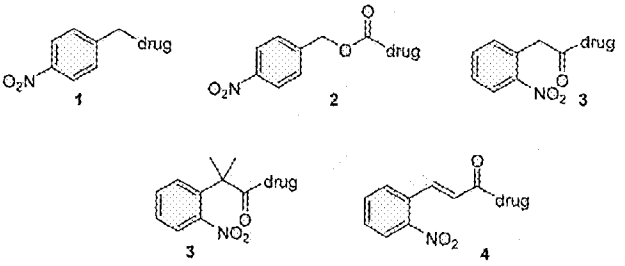

Figure 1. Delivery system–drug complexes.

The prodrugs prepared have been tested *in vitro* on a VEGF stimulated angiogenesis model. Endothelial cells in culture, in presence of VEGF, form a "vessel-like" structure. The intracellular enzymatic reduction of the nitro group into the hydroxylamine/amine is expected to trigger the release of the drug, which will inhibit the formation of this vessel-like structure.
While the prodrugs **1** and **5** did not lead to the delivery of the free drug upon chemical reduction and prodrug **2** was easily hydrolysed in aqueous conditions, prodrugs **3** and **4** seemed to be good systems under chemical reduction and *in vitro*, leading to very good inhibition of angiogenesis at concentrations of 10 and 100 μM.
Further research needs to be completed in quantifying the enzymes involved and optimising the delivery system–drug combination.

578 POSTER

PSA-activated prodrugs for the treatment of prostate cancer

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The construction and characterization of prodrugs activated by prostate specific antigen (PSA) – a protease – is described. A panel of recombinant, PSA-sensitive molecules was engineered from a template Type II ribosome-inactivating protein. The resultant prodrugs are latent toxins comprised of: an A chain with ribosome-inactivating N-glycosidase activity, and a B chain with lectin-binding activity and an interchain peptide linker. In previous studies, we successfully created prodrugs that could be activated by MMPs associated with solid tumors. In the current study, linkers were introduced to the protein template so as to regulate cytotoxicity by PSA cleavage. Linkers varied in length from 8 to 14 amino acids and contained variations of an hexapeptide PSA recognition sequence. Using Western blot analysis, prodrugs with longer linkers (10 to 14 aa) were efficiently cleaved by recombinant PSA; whereas no cleavage of prodrugs having 8 or 9 aa linkers was detected. Cleavage and activation of the prodrugs was PSA-specific insofar as the molecules could not be cleaved by tumor-associated MMPs or other selected proteases. The cytotoxicity of the PSA variants was measured using control cell line (COS-1) and two prostate cell lines (DU145 and LNCaP), which are PSA-negative and PSA-positive,

respectively. As anticipated, the PSA variants were activated to a greater extent by the PSA positive LNCaP cell line (LNCaP>DU145>COS-1). Data demonstrating the maximum tolerated dose and efficacy in mouse prostate cell xenograft models is presented.

579 POSTER

Design and synthesis of difluoromethylphosphonamidates for prodrugs of non-hydrolyzable phosphotyrosine peptidomimetics

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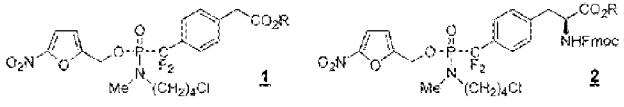
Our laboratory has reported previously a prodrug strategy to achieve intracellular delivery of nucleotides and phosphotyrosine peptidomimetics. However, the potential therapeutic benefit of such prodrugs is likely to be diminished by intracellular nucleotidases/phosphatases that can cleave the phosphate group from the drug species released in the cell. This difficulty can be circumvented for the phosphotyrosine peptidomimetics by incorporating the non-hydrolyzable difluoromethylphosphonate moiety in place of the phosphate group. We have now extended our prodrug strategy to the synthesis of aryl(difluoromethyl)phosphonate prodrug compounds **1** and **2** (R = H) that are suitable for incorporation into non-hydrolyzable phosphotyrosine peptidomimetics. The synthetic approach is based upon assembly of the analogous diethyl phosphonates and subsequent conversion of these intermediates to the nitrofuryl N-methyl-N-chlorobutyl phosphoramidates. The prodrug activation chemistry was verified for these difluoromethyl phosphonate analogs by hydrogenolysis of a model compound (**1** where the nitrofuryl group is replaced by benzyl) to the corresponding phosphonamidic acid. This intermediate was then dissolved in buffer and its conversion to the phosphonic acid monitored by ^{31}P nmr; a half life of 44 minutes (37ºC, pH = 7.4) was observed for this conversion, confirming the feasibility of this prodrug approach for the delivery of difluoromethyl phosphonates. The application of this chemistry to the synthesis of non-hydrolyzable phosphotyrosine peptidomimetics will be described, and approaches to the synthesis of analogous non-hydrolyzable phosphoserine peptidomimetics will also be presented.



580 POSTER

In vitro release of 17-demethoxy-17-allylaminogeldannamycin from its prodrugs

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Methods: In vitro stability procedure was established for both standard curves and actual plasma samples. A small amount of acetonitrile was used to help solublize prodrugs. The plasma samples were incubated at 37ºC over 24-hour period. Excess acetonitrile was used to stop possible enzymatic reactions in plasma at preset time points. Each prodrug was studied individually by HPLC to monitor the concentrations of both the remaining prodrug and the accruing 17-AAG at each time points. The conversion of each prodrug was investigated in mouse plasma, recovered, and fresh human plasma.

R	Name	NSC
-H	17-AAG	330507
-CO-(CH2)3NH2,HCL	11-(4'-amino)-butanoate HCl	683201
-CO-CH2NH2,HCL	11-(2'-amino)-acetate HCl	683661
-CO-CH2N(CH3)2,HCL	11-(2'-N,N-dimethylamino)-acetate HCl	683662
-CO-(CH2)2NH2,HCL	11-(3'-amino)-propionate HCl	683663
-CO-(CH2)2N(CH3)2,HCL	11-(3'-N,N-dimethylamino)-propionate HCl	683664
-CO-(CH2)4NH2,HCL	11-(4'-N,N-dimethylamino)-butanoate HCl	697886
-CO-(CH2)3N(CH3)2,HCL	11-(5'-amino)-pentanoate HCl	697866

Results: It was found that each individual prodrug has a comparable degradation pattern in three different plasmas. The 17-AAG is the major degrading compound that was observed for all seven prodrugs. In all plasma samples, NSC-683662 and NSC-683664 were the least stable: about 50% of these prodrugs released 17-AAG in the first five hours. Note that both of these prodrugs have a tertiary amine on the end of acyl chain. Though the NSC-697886 is similarly structured, its release rate is far slower: the release of 17-AAG was only about 10% after 24 hours. It appears that the number of $-CH_2-$ group affects the degradation rate: there are three $-CH_2-$ groups on the acyl chain in the NSC-697886 while only one in the NSC-683662 and two in the NSC-683664. It also appears that the prodrugs that have side chains with terminal primary amino group degrade to a lesser extent: the NSC-683201 almost has no degradation over 24 hours. In the first 6 hours NSC-683661, NSC-683663, and NSC-683666 only degrade marginally.

Conclusions: Stability profiles indicate that for each individual prodrug the degradation pattern is comparable in three different plasmas with 17-AAG as the major product. The NSC-683662 and NSC-683664 are least stable, thus are good candidates for further development.

Chemoprevention

581 POSTER

Lycopene, alone or combined with vitamin E, reduces orthotopic growth and plasma PSA release of PC-346C prostate tumors

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Background: Epidemiologic and clinical studies have indicated that high intake of lycopene or vitamin E is associated with a reduced prostate cancer risk, but no firm conclusions about protective effects can be drawn from these studies. The current study was undertaken to investigate whether lycopene or vitamin E, alone or in combination, may suppress orthotopic prostate tumor growth and whether blood PSA levels may serve as a surrogate marker for antitumor-efficacy.

Materials and Methods: The androgen responsive, PSA-releasing, human prostate cancer cell line PC-346C was injected into the dorsolateral lobe of the prostate of athymic nude mice. Three days after tumor inoculation, mice were supplemented on a once daily oral basis with synthetic lycopene (5 and 50 mg/kg BW), synthetic vitamin E (5 and 50 mg/kg BW), a mixture of lycopene and vitamin E (5 mg/kg BW each), or placebo. Tumor growth was followed weekly by transrectal ultrasonography of the mouse prostate and plasma was sampled for PSA analysis at 2-weekly intervals. Mice were sacrificed when tumor load exceeded 1000 mm^3 or at day 95, when the study was terminated. The prostate (including tumor) and liver were analyzed for the presence of lycopene isomers and α-tocopherol by HPLC-methodology.

Results: The low dose of 5 mg/kg BW lycopene significantly suppressed the growth of the prostate xenograft by 53% at day 42 and extended the tumor doubling time accordingly. All other single treatments, either with the high lycopene dose or with both vitamin E doses, had no significant tumor-inhibiting effect. Combined treatment with the low lycopene-vitamin E mixture gave by far the greatest tumor inhibition (73% at day 42). PSA values and PSA doubling times matched the tumor responses in all experimental groups. Vitamin E and lycopene were effectively taken up at nanogram levels in the prostate and liver. Although lycopene was mainly present in the *all-trans* conformation in the dietary supplement (90%), the lycopene in the tissues existed primarily as cis-isomers (70%), a pattern similar to that observed for humans and other species.

Conclusions: Synthetic lycopene in low doses may inhibit prostate cancer, but combining it with vitamin E may enhance its effects. The absence of a selective effect on PSA supports the usefulness of PSA as a surrogate marker for these supplements in clinical prostate cancer trials.

582 POSTER

Chemoprevention of ovarian cancer in primate model

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Ovarian cancer is the most lethal tumor among the gynecologic cancers and is associated with an extremely high mortality rate, partially due to the late stage of diagnosis and partially due to the lack of a durable response to cytotoxic chemotherapy. Primate models are ideal for developing strategies for both treatment and prevention because of the genetic similarity between primates and humans, such as hormonal regulation and menstrual cycle. 4-(N-hydroxyphenyl) retinamide (4HPR), a retinoid derivative, and the oral contraceptive (OCP) is currently being studied as chemopreventive agents for ovarian cancer but the mechanisms of their prevention activity are unclear.

We studied the effect of 4-HPR and OCP alone and in combination on the ovaries of 16 monkeys. The expression of retinoid receptors, hormone receptors, as well as apoptosis induction were tested in vivo. ERα was not detected in the primate ovaries, but ERβ, RARα and β, RXRα, and