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Novel customized releasable polyethylene glycol (PEG) linkers improve tumor delivery and efficacy of locked nucleic acids oligonucleotides

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Background: Locked Nucleic Acid (LNA) antisense oligonucleotides (LNA-ONs) represents a new generation of RNA antagonists. Unlike previous chemistry, each LNA monomer contains a methylene bridge between the 2'-oxygen and 4'-carbon of the ribose sugar. This fixes the LNA residue in a favorable RNA-like conformation and enables LNA-ONs to have much higher affinity, specificity, and resistance against degradation compared with other oligonucleotides. While unmodified LNA-ONs have activity in vivo, improved tumor targeting may further enhance efficacy. To address this goal, we have used Customized linker technology to attach polyethylene glycol (PEG) to LNA-ONs via releasable linkers. Different size PEGs were evaluated.
Methods: In vitro efficacy of PEG-LNA-ONs was evaluated in tumor cell lines after transfecting cells with lipofectamine. mRNA knockdown by PEG-LNA-ONs or unpegylated LNA-ONs were evaluated by qRT-PCR. Tumor and plasma distribution of PEG-LNA-ONs were measured in the A549 xenograft model. LNA-ON concentration in organs was evaluated by an ELISA hybridization assay. In vivo, knockdown of target mRNA was evaluated in liver and tumors derived of 15PC3 (prostate) and KB (epidermoid) cells implanted subcutaneously in nude mice.
Results: PEG-LNA-ONs showed good stability in buffers and could be prepared in high yield. In vitro, PEG-LNA-ON resulted in potent knockdown of target mRNA in various cells lines (IC50 < 10 nM). In mice, naked LNA-ONs had a very short circulation time. In contrast, PEG-LNA-ON had >50-fold higher concentration of LNA-ON in circulation (at 2 h and 4 h) compared to naked LNA-ON. At 24 h post injection, PEG-LNA-ON conjugates had 3-fold more accumulation in tumors compared to naked LNA-ONs. The higher molecular weight PEG (40 KDa) conjugates had better tumor accumulation than lower MW PEG (10 KDa) conjugates. Treatment with PEG-LNA-ON (q3dx4) increased knockdown of target mRNA by 2-fold in 15PC3 and KB xenografts models. Additionally, PEG-LNA-ON resulted in >85% knockdown of target mRNA in mouse liver.
Conclusions: Releasable PEGylation of LNA-ONs enhances the tumor targeting and efficacy of LNA-ONs. The improved effects may be due to the enhanced permeability and retention within the tumor, which has previously been observed with PEGylated molecules. Customized PEG linkers may enhance the in vivo delivery of RNA antagonists and subsequently improve efficacy.

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In silico modelling of doxorubicin penetration through multicell layers

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Background: Inadequate delivery of chemotherapeutic agents to solid tumours is a significant factor that limits curative potential. The factors which determine drug delivery to tumours are complex but the pharmacokinetic (PK) properties of the drug and its ability to leave the blood vessel and penetrate through avascular tissue are critically important factors. The aim of this study was to develop an in silico model based on these measurements which will predict how far a drug will penetrate from a blood vessel within its PK lifespan. The specific objective of this study is to develop a mathematical model for doxorubicin transport through multicellular layers and to assess the potential impact that efflux via P-Glycoprotein (PgP) may have on drug penetration.
Materials and Methods: Three cell lines were employed; DLD-1 (human colon carcinoma), MCF7 (human breast carcinoma) and MCF7-ADR (Doxorubicin resistant and PgP overexpressing derivative of MCF7). Cells were cultured on Transwell culture inserts to various thicknesses (20–145 μm) as determined by microscope analysis of histological sections. Doxorubicin at various concentrations (100, 50 or 25 μM) was added to the top chamber of the Transwell apparatus and the concentration of drug appearing in the bottom chamber was determined as function of time using HPLC-MS/MS analysis.
Results: In all cell lines, the rate of drug penetration was inversely proportional to the thickness of the multicell layer and the presence of PgP (MCF7-ADR) did not alter the rate of doxorubicin penetration compared to the wild type MCF7 cells. Initial studies have established a mathematical model which is based upon the fact that the transport of doxorubicin across cell membrane bilayers occurs by a passive flip-flop mechanism of the drug between two membrane leaflets. The mathematical model treats the Transwell setup as a series of compartments and the multicell layer is treated as a series of cell layers, separated by small intercellular spaces.
Conclusions: This initial model demonstrates good agreement between predicted and actual drug penetration rates in vitro. Further studies designed to incorporate PK parameters (both real and simulated) into the model are underway with the ultimate objective of making predictions of which schedule of drug administration (bolus vs infusion for example) is likely to be the most efficacious.

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Potent synergy of dual anti-tumor peptides for growth suppression of human glioblastomas using highly efficient peptide-delivery system

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Background: Molecular targeting agents have become formidable anti-cancer weapons which show much promise against the refractory tumors. Functional peptides are among the more desirable of these nanobio-tools. Intracellular delivery of multiple functional peptides forms a basis for potent, non-invasive mode of delivery, providing distinctive therapeutic advantages.
Materials and Methods: Here we examine growth suppression efficiency of human glioblastomas by dual-peptide targeting. We performed simultaneous introduction of two tumor-suppressor peptides, (p14ARF and p16INK4a, or p16INK4a and p21CIP functional peptides which substitutes for the core function of original genes), as compared with single peptide introduction, using highly efficient peptide/protein transporter (Wr-T)-mediated peptide delivery.
Results: Wr-T-mediated transport of both p14ARF and p16INK4a functional peptides (p14−1C and p16-MIS, respectively) into human glioblastoma cell line, U87deltaEGFR, reversed specific loss of p14 and p16 function, thereby drastically inhibiting tumor growth by >95% within the first 72 hours whereas the growth inhibition was approximately 40% by p14 or p16 single peptide introduction. Additionally, the combination of p16 and p21CIP1 (p21-S154A) peptides dramatically suppressed the growth of glioblastoma line Gli36deltaEGFR – which carries a missense mutation in p53 – by >97% after 120 hours. Significantly, our murine brain tumor model for dual peptide-delivery demonstrated a substantial average survival enhancement (P < 0.0001) for peptide-treated mice. Additonally, we tried our system to the other p14, p16 double negative-cancers, which also showed preferable anti-tumor effect.
Conclusions: Wr-T-mediated dual molecular targeting using anti-tumor peptides is highly effective against growth of aggressive glioblastoma cells, in comparison with single molecule targeting. Thus, jointly restoring multiple tumor suppressor functions by Wr-T-peptide delivery represents a powerful approach, with mechanistic implications for development of efficacious peptide-based molecular-targeting therapeutics against intractable human malignancies which lack tumor suppressor gene functions.

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Nanoparticles as drug delivery device in cancer therapy: investigation of nanodiamond internalization and cellular effects in endothelial and glioblastoma cells

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New drug delivery technologies must be designed to surmount biochemical and anatomical barriers and safely allow transport of biomolecules and protein-based drugs to specific intracellular targets. Due to their very low size, nanoparticles have become very attractive. Carbon-based nanomaterials present particular interest, since they are chemically inert, but can be surface functionalized for grafting of nucleic acids, peptides and proteins. This way may allow to specifically target cell compartments and lower drug concentration, reducing side effects. For all these reasons nanodiamond-based therapy may significantly improve cancer treatment.
Aim of the European project "Nano4drugs" was to develop peptide-grafted diamond nanoparticles, including nanodiamonds containing nitrogen-vacancy fluorescent color centers (NV), allowing single particle tracking into the cells. We have studied the uptake and the cellular effects of two kinds of nanodiamonds in endothelial and glioblastoma cells: irradiated fluorescent nanodiamonds (HPF2) and colloidal, functionalised detonation nanodiamonds (OND75). Dose-response (20–100 μg/ml) and

time course experiments were performed. Both types of nanodiamonds were efficiently internalized, as shown by optical, fluorescence and transmission electron microscopy and by flow cytometry. Internalized nanodiamonds did not produce cytotoxic effects (MTT assay), at doses lower than 100 μg/ml, and did not affect microtubular cytoskeleton and cell morphology. In particular, transmission electron microscopy showed that nanodiamonds were internalized by endothelial cells at higher extent than glioblastoma U87-MG cells. Internalized nanodiamonds accumulated in specific intracellular compartments. Further experiments are needed to identify these compartments and to better characterize the specific route of nanodiamonds.

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The antineoplastic activities of a novel oral formulation of interleukin-2 (IL-2)

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Background: A novel oral (mucosal) formulation for cytokine delivery is being developed for human administration: the tumour growth inhibition efficacy of oral mucosally (muc) administrated recombinant human (rh) IL-2 in mice implanted with melanoma B16, murine renal cancer RENCA, or H22 liver cancer was evaluated.

Materials and Methods: rhIL-2 for subcutaneous (sc) injection and in microencapsulated form (in solution) for oral muc administration were prepared by Kambridge Life Sciences (Melbourne, Australia). Well-grown H22 liver cancer, B16 melanoma or renal cancer clumps were isolated into single cell suspension in isotonic saline and 2×10^6 cancer cells (0.2 mL) inoculated sc dorsally into female Kunming, male C57BL/6 or male Balb/C mice, respectively. Mouse weight ranged 18−22 g. Animals were then randomly allocated (10 per group) to receive: no treatment; isotonic saline; 100 international units (IU) of rhIL-2 sc; 1, 10, 100 or 500IU oral mucosal rhIL-2 for 10 days (melanoma and liver) or 15 days (renal). Post sacrifice, body and extracted tumor weights were recorded. The studies were conducted in duplicate. Inhibition rate (IR) was mean tumour weight reduction compared to the respective control (%). Data were analysed by ANOVA. Significance level was $p < 0.05$ (denoted as *) or $p < 0.001$ (**) compared to control.

Results: Significant tumour growth inhibition of muc rhIL-2 occurred in a dose dependent manner (plateau between 10 and 100 IU) and was similar to sc rhIL-2 for all 3 cancer models. For H22 liver cancer, IR of 45.2**-47.5**% (results from duplicate studies) for 10 IU muc rhIL-2, 50.5**-59.5**% for 100 IU muc rhIL-2 while 100 IU sc rhIL-2 IR was 40.6**-42.8**%. For melanoma, IR of 10 IU muc rhIL-2 was 44.4**-71.6**% and 100 IU muc rhIL-2 was 67.0**-74.4**%. 100 IU rhIL-2 sc IR was 34.0**-58.5**%. In the renal cancer model, IR of 10 IU and 100 IU muc rhIL-2 were 37.0*-40.9**% and 44.7**-47.7**%, respectively. IR of 100 IU rhIL-2 sc was 32.3*-34.5**%. There was no evidence of toxicity in any animal.

Conclusions: rhIL-2 muc was well tolerated and resulted in significant growth inhibition of renal, melanoma and liver cancers in the murine model.

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Novel phage display-derived peptides for tumor- and vascular-targeted therapies against neuroblastoma

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Background: Disseminated neuroblastoma (NB) is refractory to most current therapeutic regimens. We showed that the therapeutic index of anticancer drugs is increased by liposome encapsulation and further improvements have been obtained by coupling tumor-specific ligands to the surface of the lipidic envelop. Phage display technology are used as a powerful tool in discovering novel ligands specific to receptor on the surface of tumor and tumor endothelial cells. The targeting of therapeutics to tumor blood vessels, using probes that bind to specific molecular addresses in the vasculature, combines blood vessel destruction with the expected anti-tumor activities of the drug, resulting in increased efficacy and reduced toxicity.

Methods and Results: In vivo selection of phage display libraries was used to isolate peptides binding specifically to the tumor blood vessel addresses aminopeptidase N (APN) and A (APA), expressed on endothelial and perivascular tumor cells, respectively. APN-targeted, doxorubicin (DXR)-entrapped liposomes displayed enhanced anti-tumor effects and prolonged survival in NB-bearing mice. In preliminary results APA-targeted, liposomal DXR displayed in vitro specific binding to APA-transfected cells and in vivo tumor growth delay in clinically relevant animal models of human NB. APN- and APA-targeted combination therapies are under investigation for their synergistic effectiveness on inducing NB tumor regression. To find more specific NB-targeting moieties, we established a protocol for the isolation of heterogeneous cell populations by tissue fractionation of primary tumors and metastases from orthotopic NB-bearing mice. By screening these mouse tissues with phage-displayed peptide libraries, we globally isolated 135 NB-binding peptides. Of these, 31 were selected for binding to the primary tumor mass, 16 to the metastatic mass, 63 to tumor endothelial cells, and 25 to endothelial cells of metastases. The binding enrichment in these experiments raised from 1.80 to 3.90 compared to healthy tissues and tumor cells. Based on their sequence homologies and conserved motifs, 3 peptides for each specific setting will be further validated.

Conclusions: The availability of novel ligands binding to additional tumor-associated antigens and to targets on both endothelial and perivascular tumor cells will allow to design more sophisticated liposomal targeted anticancer strategies that exhibit high levels of selective toxicity for the cancer cells.

Drug screening

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Development of potent water-soluble inhibitors of the DNA-dependent protein kinase (DNA-PK)

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The cellular response to DNA double-strand break (DSB) formation is an essential component of normal cell survival, following exposure to DNA-damaging chemicals (*e.g.* cisplatin and doxorubicin) and ionising radiation [1]. The serine/threonine kinase DNA-dependent protein kinase (DNA-PK) is a member of the phosphatidylinositol (PI) 3-kinase related kinase (PIKK) family of enzymes, and plays an important role in DNA DSB repair *via* the non-homologous end-joining (NHEJ) pathway [2]. DNA-PK inhibitors may, therefore, be useful as agents to improve the activity of radio- and chemo-therapy in the treatment of cancer [3]. Identification of the lead benzo[*h*]chromen-4-one DNA-PK inhibitor NU7026 ($IC_{50} = 0.23$ mM), guided the subsequent development of the potent and selective ATP-competitive chromenone NU7441 (DNA-PK $IC_{50} = 30$ nM) [4]. Although proof-of-principle studies with NU7441 confirmed promising activity *in vitro* as a chemo- and radio-potentiator in a range of human tumour cell lines [5], further biological studies with NU7441 were hampered by sub-optimal pharmaceutical properties.

Structure–activity relationship studies for DNA-PK inhibition by chromen-one-derivatives were conducted in conjunction with homology modelling. This approach predicted several positions on the pendant dibenzothiophen-4-yl substituent of NU7441 as tolerant to substitution, without detriment to DNA-PK inhibitory activity. The introduction of suitable functionality (*e.g.* OH, NH_2 CO_2H *etc*) at these positions provided a platform for the synthesis of focussed libraries of compounds bearing water-solubilising amine substituents. Interestingly, substitution with a methyl or allyl group (R) at the 3-position of the dibenzothiophen-4-yl ring enabled the separation by chiral hplc of atropisomers, as a consequence of restricted rotation about the dibenzothiophene-chromenone bond, albeit with a marked loss of potency (R = 3-Me, $IC_{50} = 2.5$ mM).

Library synthesis was undertaken employing a solution multiple-parallel approach, by *O*-alkylation or *N*-acylation of the appropriately substituted NU7441 derivatives, respectively, followed by reaction with a range of amines to afford the target compounds. These studies resulted in the identification of compounds that combined potent DNA-PK inhibition with excellent aqueous solubility (20−40 mg/mL as acid salts), without compromising cellular activity. Prominent amongst these derivatives is KU-0060648 (DNA-PK $IC_{50} = 8.6$ nM), which exhibits 20–1000 fold selectivity for DNA-PK over related PIKK enzymes and PI3K family members. The development of KU-0060648 and related water-soluble DNA-PK inhibitors will be described.