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Use of low molecular weight polyethyleneimine conjugated to transferrin for siRNA delivery

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Background: Since transferrin-conjugated polyethyleneimine (PEI) has previously been shown to mediate delivery of DNA in vivo, providing shielding against serum nucleases and uptake by rapidly dividing cells, we investigated its utility for delivery of siRNA to gastrointestinal cancer cells.
Material and Methods: siRNA or siRNA was complexed with linear PEI alone or with a mixture of linear PEI and transferrin-conjugated PEI (TfPEI), and transfected into colonic and gastric gastrointestinal (GI) cancer cells. Particle size and zetapotential were measured and correlated with transfection efficiency and knockdown in vitro over 72 hours. Transfection efficiencies were investigated by immunofluorescent microscopy of cells transfected with fluorescent siRNAs or a GFP-expressing plasmid and knockdown was quantified in cells expressing the luciferase reporter gene following transfection with luciferase-specific siRNAs. The most effective formulation was also investigated in a bioluminescent xenograft model.
Results: In vitro up to 93% knockdown was achieved. The ratio of PEI:nucleic acid was found to be the most important factor for determining efficacy of uptake for both siRNA and DNA, with optimal delivery achieved at intermediate ratios, correlating with intermediate particle size and charge. siRNAs were more sensitive than DNA to use of a low N:P ratio. At higher N:P ratios, whilst knockdown at 24 hrs was reduced, increased knockdown was observed at later time-points. The siRNA formulation which gave sustained knockdown in vitro was also effective in knocking down luciferase activity in the in vivo model with a 42.7% lower luminescence in the treated group compared with the control group 48 hours after treatment.
Conclusions: This study provides proof-of-principle that peptide-conjugated low molecular weight PEI has potential as a method for therapeutic delivery of siRNA provided that complex formulation is carefully optimised. This approach provides the possibility of specifically targeting siRNA to cancer cells as well as protecting siRNA from degradation by serum nucleases, and thus has potential to reduce toxicicity in normal cells as well as improving the efficacy of siRNA delivery.

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Identification of novel and potent RNA inhibitors of ErbB3, based on Locked Nucleic Acid (LNA) technology

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Background: As part of a drug discovery program, the aim of this study was to design, synthesize and screen a library of short single stranded Locked Nucleic Acid (LNA)-antisense oligonucleotides targeting human ErbB3 in order to identify potent RNA inhibitors as drug candidates for subsequent *in vivo* studies. LNA is a nucleic acid analogue that displays much increased binding affinity towards both DNA and RNA compared to other second and third generation nucleotide analogues and increases the resistance to nuclease degradation when incorporated into an oligonucleotide. We and others have previously shown that LNA enhances the potency of single stranded mRNA inhibitors.
Materials and Methods: A library of approximately 20 short LNA-oligonucleotides targeting human ErbB3 was designed. The library of ErbB3 oligonucleotides was screened at different concentrations in the cancer cell lines 15PC3 and HUH7 by lipofection. Target mRNA downregulation was measured by quantitative RT-PCR. The most potent oligonucleotides were evaluated in relation to IC50 values, plasma stability, thermal stability (Tm), and functional tumor responses of apoptosis (caspase 3/7 activity) and anti-proliferation (MTS assays).
Results: Several ErbB3 mRNA inhibitors were identified with IC50 values below 4 nM, with respect to ErbB3 mRNA downregulation measured 24 h after transfection. Apoptosis assays showed that the ErbB3 modulation led to apoptosis induction in prostate (15PC3) and hepatoma (HUH7) cancer cells after transfection with 5−25 nM of ErbB3 inhibitor. In addition, the ErbB3 oligonucleotides showed anti-proliferative activity in the cancer cell lines at 5−25 nM concentrations. A scrambled control LNA-oligonucleotide included in the screens displayed no effect on proliferation and showed no induction of caspase 3/7 activity. All LNA oligonucleotides had a Tm above 60 ºC against complementary RNA and showed high plasma stability, whereby more than 90% of the oligonucleotides remained intact after 24 h incubation in mouse plasma.
Conclusions: Based on the results from the *in vitro* screenings using a library of ErbB3 LNA oligonucleotides, several very potent new ErbB3 mRNA inhibitors were identified. These ErbB3 inhibitors downregulate ErbB3 at low nanomolar concentrations, inhibit proliferation of cancer cells and induce apoptosis. One of these LNA oligonucleotides, the 16-mer SPC3920, is currently being evaluated in pre-clinical pharmacology by Enzon Pharmaceuticals.

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Enhanced efficacy of therapy of anti-CD20 antibody with Locked Nucleic Acid antisense oligonucleotide targeting Bcl-2 in human Burkitt's lymphoma xenografts

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Background: Cell survival by abolishing programmed cell death in cancer cells has been closely linked to high Bcl-2 expression. The therapeutic potential of reducing Bcl-2 in cancer cells has been documented and resistance to existing cancer therapies have been linked to Bcl-2.
Materials and Methods: The RNA antagonist, SPC2996, is a 16-mer oligonucleotide incorporating Locked Nucleic Acid (LNA) with unique high-affinity binding to Bcl-2 mRNA and enhanced resistance to nuclease digestion. SPC2996 has completed a phase I/II trial in CLL where a dose response effect of SPC2996 was observed with higher doses giving improved effects on lymphocyte counts, lymph nodes, time to progression and overall responses
Results: Here we report on the anti-tumour activity of SPC2996 alone and in combination with Rituximab in SCID mice bearing disseminated Raji or Namalwa human Burkitt's lymphoma. SPC2996 was administered IV daily at 5 mg/kg for 14 days while Rituximab was dosed IV twice weekly for 3 weeks. A scrambled oligonucleotide was used as a negative control. In the Raji model the combination of SPC2996 plus Rituximab showed synergistic effect with significant longer survival than either treatment alone and a T/C value of 245 compared to Rituximab plus the scrambled control oligonucleotide.
Analysis of the bone marrow at day 18 after tumor cell injection showed a significant reduction in the percentage of human tumor cells from 27.7% in mice treated with the scrambled control oligonucleotide to 1.3% with SPC2996 alone and no signal above background level with the combination of SPC2996 plus Rituximab.
In the Namalwa model Rituximab alone had no significant effect on survival while SPC2996 alone showed significant prolonged survival with a T/C value of 143 compared to Rituximab and 195 compared to saline. The combination of SPC2996 plus Rituximab significantly prolonged the survival even further.
The percentage of human tumor cells in the bone marrow at day 14 showed a reduction from 14.5% in mice treated with the scrambled control oligonucleotide to 6.6% in the Rituximab treated mice while only background staining was observed with SPC2996 alone and in combination with Rituximab.
Conclusion: We have here presented data on the LNA containing RNA antagonist SPC2996 targeting Bcl-2 in two different human Burkitt's lymphoma xenograft models. SPC2996 shows highly significant prolonged survival in combination with Rituximab compared to Rituximab as monotherapy.

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Combination of a fusogenic glycoprotein, pro-drug activation and oncolytic HSV as an intravesical therapy for superficial bladder cancer

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Background: There are still no treatments for superficial bladder cancer (SBC) which alter its natural history where 20% of patients develop metastatic disease. SBC is often multifocal, has high recurrence rates after surgical resection and recurs after intravesical live BCG (bacillus Calmette-Guerin therapy). OncoVexGALV/CD, an oncolytic herpes simplex virus 1 (HSV-1), has shown enhanced local tumour control by combining oncolysis with the expression of a highly potent pro-drug activating gene (yeast cytosine deaminase/uracil phospho-ribosyltransferase fusion [Fcy::Fur]) and the fusogenic glycoprotein from gibbon ape leukemia virus (GALV). Previous studies with OncoVexGALV/CD have shown enhanced cell killing and tumour shrinkage (in vitro and in vivo) within tumours derived from

colon, pancreas, lung and glioma tissue. A version of this virus expressing GM-CSF has shown promising results in Phase I and II clinical trials. The present study aims to test OncoVexGALV/CD as an intravesical therapy for superficial bladder cancer.
Material and Methods: In vitro tumour cell killing by OncoVexGALV/CD was assessed by Fusion/Prodrug MTS assays. In vivo efficacy of the treatment was studies through histology and IVIS imaging.
Results: Treatment of three human bladder carcinoma cell lines with the virus, resulted in higher tumour cell killing through oncolysis, pro-drug activation and glycoprotein fusion. To further test OncoVexGALV/CD we have developed a rat orthotopic bladder tumour model to assess intravesical tumour control. Using luciferase-expressing tumours, we will further delineate the effects of individual properties of the virus on tumour growth using the IVIS imaging system.
Conclusions: Preliminary results on human bladder carcinoma cell lines indicate, that OncoVexGALV/CD may improve local tumour control within the bladder, and potentially alter its natural history.

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Structure–activity relationships for lipophilic dinitrobenzamide mustards as prodrugs for Escherichia coli NfsB nitroreductase

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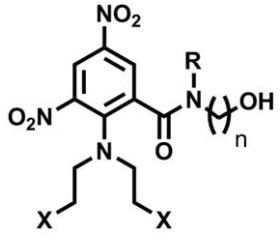
Background: A major weakness of cancer gene therapy protocols is the limited distribution of gene-delivery vectors within the tumour mass. Enzyme-prodrug activating systems (GDEPT) can potentially compensate by generating cytotoxic metabolites that diffuse locally to kill neighbouring vector-naive cells, creating what is known as a 'bystander effect'. E. coli nitroreductase *nfsB* (NTR) in combination with the prodrug CB1954 has been evaluated clinically but efficacy was constrained, at least in part, through inadequate bystander effects (Patterson, Can Res 2002;62:1425). New analogues of the dinitrobenzamide mustard (DNBM) class have demonstrably superior bystander efficiencies in vivo following *nfsB* activation (Singleton, Can Gen Ther 2007;14:953). We sought to maximise metabolite redistribution properties in the DNBM class by modifying the lipophilic nature of the prodrugs through placing additional alkyl groups at three available positions (R, n, X), as well as modification of mustard leaving groups (X).
Methods: A series of 14 novel lipophilic DNBM prodrugs were synthesised and characterised by HPLC, MS, NMR and combustion. Potency (24 h exp) was determined in vitro against $HCT116^{NTR}$ cells (relative to parental cells) in a 5 day proliferation assay. Nine candidates were advanced to mixed 3D tissue culture "bystander efficiency" testing in vitro containing only 1% NTR +ve cells with local toxicity transfer to cocultured WT cells being quantified by clonogenic survival.
Results: Prodrug Log P values spanned >4 orders of magnitude (0.14–4.4). All DNBM prodrugs were more dose-potent (2−60 fold) than CB1954 with $HCT116^{NTR}$ cell growth inhibition (IC_{50}) ranging from 0.05–1.4 μM (28-fold) in a manner that correlated with Log P ($r^2 = -0.82$). Increasing lipophilicity was paralleled with loss of NTR-dependent sensitisation of 2D monolayer cultures. However, only a weak correlation between 2D-IC_{50} and 3D-C_{10} values for $HCT116^{NTR}$ cells was seen ($r^2 = +0.58$), with no apparent relationship with WT-NTR potency ratios in the two systems ($r^2 = +0.24$). As expected, improvements in 3D bystander efficiency (% toxicity transfer from 1% NTR+ve cells) tracked with increasing lipophilicity ($r^2 = +0.69$), ranging from 14% for CB 1954 to 69% for the lead DNBM analogue.
Conclusion: This study establishes the importance of ranking prodrugs using in vitro models with appropriate tissue-like cell densities and identifies several promising leads for further development.



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Targeted suicide gene therapy for small cell lung cancer

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In suicide gene therapy, the introduced therapeutic gene encodes an enzyme capable of transforming a non-toxic prodrug into a cytotoxic drug. Utilizing cancer-specific promoters suicide gene expression can be selectively targeted to the cancer cells of interest. For that purpose we have identified several promotor regions, which are highly promising candidates for transcriptionally targeted gene therapy for small cell lung cancer (SCLC). The suicide gene yeast cytosine deaminase (YCD) converts the pro-drug 5-fluorocytosin (5-FC) into the known chemotherapeutic agent 5-fluorouracil (5-FU). YCD was cloned for regulated expression from the SCLC specific promoter Insulinoma-associated 1 (INSM1) and transiently transfected into different cell lines, which were exposed to increasing concentrations of 5-FC. Transfected SCLC cells were greatly sensitised to 5-FC and significant cell death was achieved while cancer cell lines of other origins were unaffected to treatment. Furthermore the YCD gene was fused with the yeast uracil posphoribosyltransferase (YUPRT) gene, which augments the conversion of 5-FU into active cytotoxins. The fusion construct (YCD-YUPRT) demonstrated significantly increased sensitivity towards 5-FC in treated SCLC cell lines inducing cytotoxicity comparable to treatment with the 5-FU toxin alone.
Due to limited efficiency of gene delivery in vivo an important feature of suicide gene therapy is the bystander effect where suicide gene/prodrug-produced toxins diffuse to untransfected neighbouring cells. In the cytosine deaminase-based suicide gene therapy 100% cell death was achieved after 5-FC treatment when only 50% of cells expressed the YCD or YUPRT gene. Further it was established that the YCD-YUPRT/5-FC strategy caused extensive cell death when as few as 10% cells expressed the transgene. This contrast previously obtained results with the suicide gene Herpes simplex virus thymidine kinase (HSVtk) and the prodrug penciclovir (PCV) where cell death was restricted to HSVtk-transfected cells.
As succesfull cancer treatment relies on multi-targeting treatment the combination of HSVtk and YCD-YUPRT suicide gene therapy was tested. At low prodrug concentrations an additive effect of the systems was obtained while the YCD-YUPRT mediated toxicity dominated at high 5-FC concentrations. Further testing of these and other suicide systems in vivo will conclude on the significance of combinatorial suicide gene therapy for SCLC.

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Effects of triple knockdown of cIAP-1, c-IAP-2 and XIAP on prostate cancer cell susceptibility to apoptosis

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Background: Treatments for hormone resistant prostate cancer are currently effective with resistance to apoptotic cell death a common mechanism of resistance in this advanced form of the disease. Manipulation of the apoptotic resistant phenotype represents an important strategy for increasing the response of hormone refractory prostate cancer cells to therapy. Previous studies in our laboratory have identified elevated expression of the inhibitors of apoptosis proteins (IAP) in prostate cancer cell lines and primary material. Knockdown of XIAP is associated with increased susceptibility to chemotherapy induced apoptosis. We hypothesis that simultaneous knockdown of cIAP-1, cIAP-2 and XIAP would further increase the sensitivity of both type 1 and type 2 triggers of apoptosis.
Material and Methods: PC-3 androgen independent prostate cancer cells were treated with optimum concentrations of siRNA to knock down cIAP-1, cIAP-2 and XIAP which was confirmed by western blotting. Following knock down these cells were treated with TRAIL, Etoposide, and Tunicamycin and assessed for apoptosis by PI DNA staining, Annexin V staining and PARP cleavage. Caspase 3 activity was assessed by western blotting and inhibition of apoptosis with the zVAD.fmk pan-caspase inhibitor. Clonogenic assays assessed the ability of the cells to recover following IAP knockdown and ID-1 protein expression was assessed by western blotting as a marker of proliferation.
Results: Triple knock of the IAP only sensitised for TRAIL induced apoptosis in the PC-3 cells with corresponding increases in caspase activity and PARP cleavage which was inhibited by ZVAD.fmk. Individual knock down of the IAP has no significant effects. Triple knock down alone decreases clonogeneic survival of the PC-3 cells which was correlated with a decrease in ID-1 expression.