cytotoxic than the parent PBD it releases. The N10-benzyl and SEM control molecules were significantly less cytotoxic in both NTR+ and NTR- A2780 cells, with IC_{50} values ranging from 1.9 to 3.3 mM. In preliminary *in vivo* experiments, the N10-(*p*-nitrobenzylcarbamate) prodrug was evaluated in a nude mouse human tumour xenograft model implanted with A2780 CMV-NTR cells. A clear response to the prodrug was observed at non-toxic doses.

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A potent PBD-heterocyclic polyamide conjugate targeting an ICB2 transcription factor binding site

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The binding of Nuclear Factor Y (NF-Y), a ubiquitous CCAAT-binding transcription factor, to five inverted CCAAT boxes (ICBs) within the promoter region of DNA topoisomerase IIa (topo IIa) results in control of cell proliferation. The regulation of NF-Y/topo IIa interactions by small molecules is of interest in relation to both the development of novel anticancer agents and also chemical tools and probes for use in cancer biology experiments. In this context, we have recently demonstrated that the pyrrolo[2,1-*c*][1,4]benzodiazepines (PBD) C8-*bis*-pyrrole conjugate, GWL-78, can interact specifically at CCAAT-box sites and block NF-Y binding (Kotecha, M. *et al*, *Molecular Cancer Therapeutics*, 7, 1319–1328, 2008).

To further explore this property of GWL-78, and to try to improve both selectivity and potency, a third heterocycle has now been added to the GWL-78 C8-side chain to produce examples of pyrrolo[2,1-*c*]-[1,4]benzodiazepines (PBD) C8-*tris*-heterocyclic conjugates which should span a slightly longer region of DNA upon interaction within the minor groove and offer additional molecular interactions (*e.g.,* hydrogen bonds, electrostatic interactions etc) that may further stabilize the adduct and modify sequence selectivity. On this basis, a library of fourteen PBD C8-*tris*-heterocyclic conjugates was synthesized by attaching a PBD capping unit to pre-constructed tri-heterocyclic polyamides comprising of a combination of pyrrole, imidazole and thiazole heterocycles assembled in a combinatorial fashion. The effect of the composition and length of the C8-heterocyclic polyamide side-chains on DNA binding was evaluated using a number of biophysical and cellular methods. Binding affinity was measured using a calf thymus DNA thermal denaturation assay, and sequence selectivity was evaluated using DNase I footprinting. Their ability to disrupt interaction of the NF-Y transcription factor with its cognate binding site was measured using an EMSA assay, and cytotoxicity was evaluated in the NCI 60 cell line panel.

One conjugate, RMH-41 (Py-Py-Im-PBD; Figure 1), which had the highest DNA binding affinity, also exhibited the ability to inhibit NF-Y transcription factor binding and had significant selective cytotoxicity in human tumour cells. Interestingly, the results of the footprinting experiments showed that, of all the novel conjugates evaluated, RMH-41 appeared to discriminate between two of the ICBs studied, binding to the ICB2 site at a lower concentration compared to that required to bind to ICB1.

In conclusion, the ability of low molecular weight ligands such as RMH-41 to recognize predetermined DNA sequences and prevent endogenous transcription factors from binding could be successfully exploited to modulate transcription and block cancer cell proliferation as part of a therapeutic strategy.

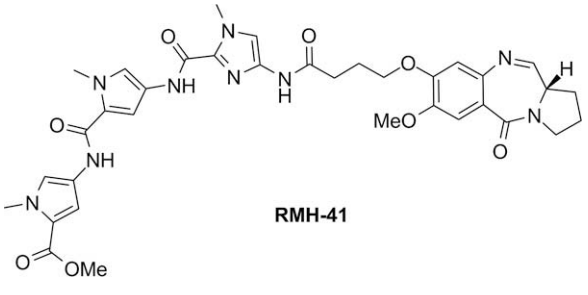

Figure 1. Structure of RMH-41

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siRNA targeting of thymidylate synthase and thymidine kinase for anti-cancer therapy

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Thymidylate synthase (TS) is the only *de novo* source of thymidylate (dTMP) for DNA synthesis and repair. Cytosolic thymidine kinase 1 (TK1) and mitochondrial TK2 are salvage pathways for producing dTMP. TS and TKs are often upregulated in human tumors, suggesting a role for both in malignancy. We have previously shown that antisense oligodeoxynucleotides (ODNs) targeting TS, as single agents, inhibit human tumor cell growth *in vitro* and *in vivo*. In addition, anti-TS ODNs and small interfering RNAs (siRNAs) enhance tumor cell growth inhibition by TS-targeting drugs. TS mRNA is a good target for development of antisense anticancer drugs. However, we hypothesize that when TS enzyme activity is inhibited, the ability of TKs to generate dTMP may mediate resistance to TS-protein targeting drugs.

To determine whether antisense to TK has potential therapeutic benefit, siRNAs targeting TK1 or TK2 were used *in* vitro in 3 different protocols against cultured tumor cell lines: (1) on their own; (2) in combination with TS siRNA; (3) in combination with TS siRNA and the anti-TS drugs 5-fluorodeoxyuridine (5FUdR) or pemetrexed. siRNAs targeting TS or TK1 or TK2, as single agents and in combination with each other, decreased TS or TK mRNA by more than 85% in human cervical carcinoma (HeLa) and human breast carcinoma (MCF7) cell lines (5 nM siRNA, 24 h post-transfection) and decreased TS and TK1 protein. siRNAs targeting TS, TK1 or TK2 did not independently decrease HeLa cell proliferation but did decrease TS, TK1 and TK2 mRNA and TS and TK1 protein levels (5 nM siRNA, 24 and 96 h post-transfection). The capacity of each siRNA to downregulate its target mRNA was unaffected by combination treatment with other siRNAs. HeLa cell TK2 protein after TK2 siRNA treatment was not measured. Treatment with TS siRNA sensitized HeLa cells to 5FUdR by approximately 50% and to pemetrexed by approximately 34% compared to non-targeting control siRNA. siRNA targeting TK1 or TK2 alone did not enhance tumor cell sensitivity to 5FUdR. In support of the concept that TK activity can reduce the ability of antisense TS to sensitize human tumor cells to TS-targeting drugs, we report that simultaneous treatment with TK2 siRNA and TS siRNA enhanced sensitivity to 5FUdR by approximately 25%, and adding TK1 siRNA to TS siRNA enhanced sensitivity to pemetrexed by approximately 20%, beyond the sensitization induced by TS siRNA alone. Enhanced sensitization to 5FUdR and pemetrexed by targeting both TS and TK with siRNAs suggests that the TK salvage pathways are potential targets for anticancer therapies. These data support the hypothesis that combined antisense targeting of TS and TK1/TK2 is more effective than either siRNA used alone to sensitize tumor cells to the effects of TS-targeting chemotherapeutic drugs.

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Observation of the reversibility of formation of a pyrrolobenzodiazepine (PBD) covalent DNA adduct using HPLC/MS and CD spectroscopy

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It has been previously demonstrated that exposure of a pyrrolobenzodiazepine (PBD) DNA adduct to heat and/or acidic conditions leads to the loss of up to 70% of covalently-bound ligand. For example, Hurley and co-workers have reported that anthramycin cleaves from DNA after heating in the presence of TFA, with simultaneous oxidative formation of a C11a-C1-dehydro product which is non-electrophilic at the N10-C11 position and so cannot re-react with DNA. Therefore, in principle, given the relatively labile nature of the aminal bond formed between PBDs and DNA, an adduct formed from a non-oxidizable PBD should be reversible upon exposure to conditions such as heat or low pH, although this has not been previously demonstrated. The PBD conjugate GWL-78, which is not prone to such oxidation, comprises a C-ring-unsubstituted PBD attached to a methyl ester terminated *bis*-(*N*-methylpyrrole) unit *via* a four-carbon linker between the C8-oxygen of the PBD A-ring and the N-terminus of one pyrrole unit. Using HPLC/MS and CD methodologies to monitor the interaction of GWL-78 with short oligonucleotides, we have now demonstrated for the first time that such reversibility occurs.

A GWL-78/DNA adduct was initially formed and characterized by HPLC/MS and CD. It was then heated to 90ºC at a rate of 10ºC/min which resulted

in 95% loss of covalently bound PBD. Interestingly, acidic conditions (*e.g.,* TFA) were not required as in the experiments conducted by Hurley and co-workers with anthramycin. Within a 10 minute cooling period at 0ºC, GWL-78 was observed to re-attach to the DNA. Furthermore, the rate of re-attachment was found to depend upon the sequence of the oligonucleotide. For example, with AT-rich oligonucleotides, 85% of the adduct had re-formed within 10 minutes, while only 30% had re-formed with GC rich sequences. Although PBDs generally prefer to bind to GC rich sequences of DNA, the latter result most likely reflects the relatively high affinity of GWL-78 for AT-rich DNA sequences due to the *bis*-(*N*-methylpyrrole) component it contains. Further investigations showed that although the rate of re-formation of the PBD-DNA adduct depended on DNA sequence, initial cleavage of the adducts by heat did not. These observations add to knowledge of PBD chemistry and mechanism of action, and may help in the design of future PBD-based gene-targeting agents and anticancer and antibacterial agents.

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Endocytosis and intracellular trafficking of cholesterol based cationic liposome for gene delivery

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Background: For the effective gene therapy or transfection, it is important not only to prepare the genetic materials such as plasmid DNA and siRNA but also to deliver these molecules to target cells. Using cationic liposome to mediate gene delivery is widely used because of the advantage of the safety and simplicity of use over viral gene therapy. Recently, we developed and published the cholesterol derived cationic lipids with ether linkers, and these showed more efficient gene delivery compared to commercial lipids such as DOTAP and lipofectamine. Therefore, we investigated the endocytosis and trafficking of this cholesterol based cationic lipids.
Material and Methods: We compared the endocytosis and trafficking of this cholesterol based cationic lipids with DOTAP as non-cholesterol based lipid in COS7 cells. We investigated the effects of several inhibitors of particular routes on the uptake of lipoplexs by flow cytometry and subsequent gene expression by luciferase expression assay and GFP observation using fluorescence microscopy. The involvement of late endosome and lysosome were verified by the colocalization of the fluorescence labeled plasmid and the antibody to EEA1 and LAMP1 in both lipids.
Results: Lipoplex internalization of both lipids was inhibited by clathrin pathway inhibitor chlorpromazine, but unaffected by caveolar pathway inhibitors such as filipin and genistein. The observation using confocal laser microscope showed the both of fluorescently labeled lipoplexes were co-localized with transferin known as clathrin pathway marker. The involvement of late endosome and lysosome were verified by the colocalization of the fluorescence lableled plasmid and the antibody to EEA1 and LAMP1 in both lipids. However, the decreased efficiency of transfection by lysosomal inhibition with chloroquine called the needs for further study. Subsequently, we found that the transfection efficiency of DOTAP was more inhibited by the depletion of membrane cholesterol with methyl-beta-cyclodextrin compared to newly synthesized cholesterol based lipids. Furthermore, the replenishment of cholesterol and our cholesterol based lipids restored the transfection efficiency of DOTAP.
Conclusions: In conclusion, we found that our cholesterol-based cationic lipids were internalized by clathrin-mediated pathway and the substitutive effects of cholesterols might be one cause of the superiority of this delivery vehicle.

530 POSTER

Activity of the TGF-beta 2 specific antisense oligodeoxynucleotide trabedersen in an orthotopic xenograft mouse model of metastatic pancreatic cancer

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Background: Transforming growth factor-beta (TGF-beta) regulates crucial cancer mechanisms such as cell proliferation, metastasis, angiogenesis, and immunosuppression. The isoform TGF-beta 2 plays a particular role in some cancers e.g. pancreatic cancer. Our novel therapeutic approach to treat cancers overexpressing TGF-beta 2 is based on the specific inhibition of its synthesis by the antisense oligodeoxynucleotide trabedersen (AP 12009). In the present study, the antitumor activity of trabedersen was investigated in an orthotopic xenograft mouse model of metastatic pancreatic cancer.
Material and Methods: BALB/C$^{nu/nu}$ mice were injected with 1×10^6 cells of the human TGF-beta 2 expressing cell line L3.6pl into the pancreatic tail. Intraperitoneal treatment with trabedersen or vehicle (controls) started 2 days after tumor implantation with an initial loading dose of 50 mg/kg and subsequent doses of 16 mg/kg thrice weekly. On day 29, mice received BrdU by intraperitoneal injection and were sacrificed 2 h thereafter. The incidence of liver and lymph node metastases as well as size and weight of the pancreatic tumors were determined. Tumor sections were stained with anti-BrdU antibody to determine tumor cell proliferation and with anti CD31/PECAM-1 antibody to determine vascularization.
Results: Tumor weight in trabedersen-treated mice was significantly reduced compared to control mice (mean tumor weight: 0.7 g vs 1.4 g, $p = 0.0084$). Concordantly tumor cell proliferation was significantly suppressed ($p = 0.028$). Lymph node metastases were detected in most (7 of 9) control mice but only in 2 of 10 trabedersen-treated mice ($p = 0.023$). Liver metastases were present in 5 of 9 control mice and in 4 of 10 trabedersen-treated mice (ns). Vessel area in tumor slices as readout for tumor angiogenesis was significantly reduced in trabedersen-treated animals to about a third of the vessel area of control animals ($p = 0.0001$).
Conclusions: In an orthotopic xenograft mouse model of metastatic pancreatic cancer trabedersen demonstrated potent antitumor activity. Key tumor promoting mechanisms such as tumor cell proliferation, metastasis and tumor angiogenesis were efficiently suppressed by trabedersen. This is in line with promising results from clinical studies of trabedersen in patients with advanced pancreatic cancer (phase I/II) or high-grade glioma (randomized, controlled phase IIb completed, phase III ongoing).

531 POSTER

Host cell specific responses to a novel rVSV oncolytic vector rNCP12.1

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Background: Vesicular stomatitis virus (VSV) is a -ssRNA virus known to be inherently oncolytic due to defects in tumor interferon (IFN) response pathways. This study addresses host specific responses and how they contribute to the oncolytic potential of rNCP12.1, a novel recombinant VSV shown to decrease intracranial tumors in an immunocompetent rat glioma model by 67%.
Methods: To determine tumor specificity of rNCP12.1, we performed MTS cell viability assays following viral infection from 6 hours (hpi)-1 week post-infection (wpi) in rat glioma cells (F98) and in primary rat astrocytes (PRAs). Supernatants were used to determine differences in replication between normal and tumor cells. To examine the differences in the ability of glioma and normal cells to elicit an antiviral IFN response, we performed IFN protection assays. IFN bioassays were used to test the host specific production of IFN in response to activation of both TLR7 and RIG-I pathways by transfected polyI:C (tpI:C) and viral infection, respectively.
Results: rNCP12.1 maintained wtVSV level cytotoxicity in F98 glioma, with majority of cell death seen by 48 hpi. rNCP12.1 displayed minimal cytotoxicity in PRAs throughout the study. Growth curve data supported these trends, showing highly permissive replication of both viruses in F98 and significantly restricted replication of rNCP12.1 in PRAs. Following IFN exposure, C6 glioma were partially protected from infection but required 4 times more IFN than PRAs; F98s were not protected with any amount of IFN. Following infection with rNCP12.1, rat fibroblasts (FR) produced high levels of IFN. C6s produced extremely low amounts of IFN while no IFN was detected from F98s. No IFN production was detected in wtVSV-infected cells of any type. tpI:C production of IFN paralleled results from IFN bioassays of virally infected cells but at 3−20 fold lower amounts for each cell type.
Conclusions: Though there are obvious host specific differences in IFN responses following rNCP12.1 infection among normal and tumor cells and even between similar glioma cell types, the overall inability of glioma cells to elicit a sufficient antiviral IFN response contributes to the oncolytic capacity of this vector. Based on these differences, we believe rNCP12.1 is an attractive candidate for treatment of glioma and further preclinical studies are being conducted.