Research Highlights

Highlights from the latest articles in nanomedicine

Mechanistic studies of Irinophore C™ lead to discovery of enhanced delivery of co-administered drugs

Evaluation of: Baker JG, Lam J, Kyle AH *et al.*: Irinophore C, a novel nanoformulation of irinotecan, alters tumor vascular function and enhances the distribution of 5-fluorouracil and doxorubicin. *Clin. Cancer Res.* 14(22), 7260–7271 (2008).

Irinophore C™ is a novel nanopharmaceutical that has been developed within the BC Cancer Agency (Vancouver, BC, Canada). Several studies demonstrating formulation attributes, enhanced efficacy and reduced toxicity in comparison to free irinotecan, on which the formulation is based, have been published [1,2], and have led to the planning for Phase I clinical testing as a single agent for patients who have failed standard care. However, cancer is a complex disease and virtually all chemotherapy regimens for treating cancer use drug combinations selected to affect several targets that contribute to cancer cell survival and disease progression. Although drug combinations are the standard of care for patients with advanced cancer, new anticancer drugs are first introduced typically as single agents and only after many years of clinical trials are these single agents combined with other drugs to determine their optimal role in cancer treatment. Preclinical studies that are designed carefully to elucidate the mechanism of action for enhanced therapeutic effect when drugs are given in combination can accelerate optimal combination therapeutic regimens in the clinic. The current paper by Baker *et al.* is an elegant example of this strategy.

The authors undertook a series of experiments to examine the effects of Irinophore C treatment on tumor vasculature, looking specifically for effects similar to those noted following metronomic dosing, as demonstrated by several researchers, most notably Robert Kerbel [3,4]. Female 129S6/SvEvTac-Rag2^{tm1Fwa} (Rag2M) mice bearing established subcutaneous HT-29 colorectal cancer tumors were used. Treatment was at a suboptimal dose of 25 mg/kg irinotecan (active pharmaceutical ingredient), once weekly for 6 weeks followed by 5-fluorouracil (5-FU) or doxorubicin. Parameters assessed were tumor growth, hypoxia, angiogenic markers, perfusion and vascularization, as well as tumor accumulation of secondarily administered drug.

Results confirmed the efficacy of Irinophore C in that, even at a suboptimal dose, tumor growth was halted with treatment in addition to overall lowering of cell density within the remaining tumor. Visualization of Hoechst 33342 following termination showed significantly higher levels in treated tumors, corresponding to increased perfusion. Further, the degree of CD31 staining in treated tissue was reduced. Together, the results indicate decreased overall vascularization; however, vessels present were more viable than in nontreated tumors. Not surprisingly, these results were supported by a finding of decreased hypoxia levels as assessed by EF5 in the treated tumors. This is of great importance when considering the use of radiotherapy and suggests that pretreatment with Irinophore C will enhance radiotherapy.

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The improvement in tumor oxygenation and functional vasculature led the researchers to assess uptake of a secondarily administered anticancer drug. In this paper, the drugs chosen for assessment were the commonly used 5-FU and doxorubicin. Accumulation of 5-FU was monitored using ^{19}F spectroscopy in a noninvasive manner whereas doxorubicin, being a natural fluorophore, was assessed using fluorescence microscopy. Overall, the results indicated a 1.5-fold increase in 5-FU in tumor tissue if preceded by Irinophore C and, similarly, a 2.7-fold greater distribution of doxorubicin.

To better understand the mechanism for these findings, the authors conclude the paper with an assessment of anti-angiogenic and antivascular markers and demonstrate downregulation of the proangiogenic factors vascular endothelial growth factor and IL-8 and upregulation of tissue inhibitor of metalloproteinase-1, an inhibitor of angiogenesis.

This work will have importance in determining the optimal clinical development of Irinophore C but, perhaps of more broad importance, will act as a template on which

other researchers can draw experiments for optimization of other drugs in preclinical development, thereby shortening the time to optimization in the clinic.

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Therapeutic potential of siRNA: building on lessons learned from cytotoxic and oligonucleotide delivery strategies: part I

Evaluation of: Sonoke S, Ueda T, Fujiwara K *et al.*: Tumor regression in mice by deliver of Bcl-2 small interfering RNA with pegylated cationic liposomes. *Cancer Res.* 68, 8843–8851 (2008).

The use of siRNA as a tool to knockdown expression of specific proteins implemented in disease processes is a relatively new technology, having its first demonstration in mammalian cells in 2001 [1]. The common means of transfection of cells is expression vectors to enable ongoing expression or lipid-mediated transfection. Although viewed initially as a tool to study molecular biology and signaling pathways, there is increased use of siRNA as a therapeutic tool in several disciplines, including sepsis

[2], hepatitis B [3] and cancer [4,5]. One of the problems associated with therapeutic use is that of degradation of the siRNA before delivery to the target, hence the use of several delivery mechanisms has been described, including lipid complexation, liposomes and block copolymers.

Lipids, whether in the form of lipid complexes or liposomes, have been used for delivery of antisense oligonucleotides by several research teams with varying success. Additionally, this technology is being pursued actively by several biotechnology and pharmaceutical companies. Given the success of liposomal encapsulation as a delivery method for antisense oligonucleotides, it is natural that researchers would apply this technique to the delivery of siRNA when being used as a therapeutic.

The authors have put these pieces together in a novel means of siRNA delivery that taps into the advantages gained by past research in drug-delivery technology of cytotoxics or antisense oligonucleotides, wherein the use of lipid complexation incorporating PEG or stealth technology, as first described by Allen and Chonn in 1987, enhances tissue distribution and cellular uptake [6].

In the present paper, properties of PEG lipids incorporated into the complexes were explored in relation to optimizing plasma and liver levels of siRNA, in which the authors found that longer, saturated acyl chains (C-18) yielded the highest plasma and lowest liver levels. This mirrors similar findings with liposomal delivery systems for cytotoxic agents [7]. The paper would have benefited from an

analysis of PEG lipid remaining in the formulation following administration because shorter chain lengths are associated with the PEG lipid exchanging out of the liposomal membrane. The authors do discuss the importance of linker type in forming the PEG lipid, comparing carbamate with phosphodiester. They propose use of phosphodiester on the basis of it being cleaved more readily *in vivo*, enabling formation of ethanolamine at the tumor site, which they suggest promotes inversion of the liposomes and thus endosomal escape of the siRNA, as opposed to degradation.

The authors have demonstrated the utility of complexation of siRNA with lipid in enhancing delivery of siRNA to tumors. This has translated into efficacy in two xenograft tumor models (PC3 prostate and MCAS ovarian) in mice with a dose response and enhanced survival noted in the two models, respectively. Furthermore, the authors demonstrated an effective decrease in Bcl-2 expression following administration of Bcl-2-specific siRNA complexed with the optimized lipid formulation. Overall, this research will serve to further the advancement of siRNA as a therapeutic option in the treatment of cancer as well as other diseases.

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Therapeutic potential of siRNA: building on lessons learned from cytotoxic and oligonucleotide delivery strategies: part II

Evaluation of: Mikhaylova M, Stasinopoulos I, Kato Y, Artemov D, Bhujwall AM: Imaging of cationic multifunctional liposome-mediated delivery of COX-2 siRNA. *Cancer Gene Ther.* (2008) (Epub ahead of print).

In this article, Mikhaylova *et al.* have built on previous work in their laboratory demonstrating the importance of COX-2 overexpression to the metastatic phenotype of MDA-MB-231 breast carcinoma

cells in mice [1]. This relationship has also been shown in other cancer types, such as osteosarcoma [2], colon [3] and breast [4], thus making it an attractive therapeutic target. The earlier work from this lab relied on direct cell transfection using a COX-2 short-hairpin RNA–coding plasmid under the control of the U6 promoter and lipofectamine, a commercially available transfection agent. Recognizing the limitations of using this system in an *in vivo* model, the authors have used a number of lipid complexes to develop an optimized system

for delivery. In this article, siRNA–lipid complexes formed with DOTAP/DOPE (DD) and DOTAP/DOPE/DOPE-PEG (DDP) are evaluated from an *in vitro* and *in vivo* perspective. The imaging agents gadolinium, Magnevist® and Feridex® were incorporated into the complexes to enable visualization of biodistribution following intravenous administration in mice bearing orthotopic MDA-MB-231 tumors, as well as to track metastatic events.

siRNA specific for COX-2 was incorporated successfully into several different

lipoplexes, as outlined earlier, and using the fluorophore Dy647, the authors showed uptake into cells within 30 min in tissue culture. Uptake may be attributed to the cationic properties of the complexes. In an elegant series of experiments, COX-2 siRNA effectively downregulated the target following cellular incorporation of the siRNA–lipid complexes; gadolinium or Magnevist incorporation into the lipoplexes resulted in nonspecific downregulation of protein expression inclusive of COX-2, thus leading to the use of Feridex for *in vivo* experimentation and image acquisition.

DDP lipoplexes with Feridex (DDPFe) were injected into severe combined immunodeficiency mice bearing orthotopic tumors to assess tumor uptake. This was demonstrated successfully using both fluorescence and iron-based imaging techniques in which the presence of the DDPFe–COX lipoplexes were seen to be tumor-tissue associated within 30 min of injection. As was typical for liposomal- or lipoplex-based delivery systems, there was also significant uptake into lung, spleen, kidney and liver, whereas no accumulation was noted in smooth or striated muscle.

In summary, Mikhaylova *et al.* have developed a lipoplex-based delivery system for siRNA that is nontoxic (results not discussed) and effective for tumor-specific uptake of the therapeutic in

addition to being amenable to visualization using fluorescence-based detection as well as MRI. The natural follow on to these experiments will be to examine tumor-tissue expression of COX-2 following administration of the DDPFe–COX-2 lipoplexes. This work supports the idea of using siRNA as a therapeutic option in the treatment of cancer and, more specifically, in reducing the incidence of metastatic lesions.

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Therapeutic potential of siRNA: building on lessons learned from cytotoxic and oligonucleotide delivery strategies: part III

Evaluation of: Villares GJ, Zigler M, Wang H *et al.*: Targeting melanoma growth and metastasis with systemic delivery of liposome-incorporated protease-activated receptor-1 small interfering RNA. *Cancer Res.* 68, 9078–9086 (2008).

The role of protease-activated receptor (PAR)-1 has been elucidated clearly in the arena of coagulation in addition to tumor progression [1], whereas its role in

tumor metastasis has only received attention recently [2]. The present paper by Villares *et al.* first demonstrates that using short-hairpin RNA (shRNA) the effects of PAR-1 downregulation both *in vitro* and *in vivo* and then reports on its efficient systemic delivery to mice bearing subcutaneous or systemic melanoma using a siRNA liposomal system.

Effective silencing of PAR-1 was achieved *in vitro* with a lentiviral shRNA system. The authors report almost complete silencing using this method, for

two highly metastatic melanoma lines: A375SM and C8161. On inoculation with the transfected lines, tumor growth and lung metastases in nude mice were inhibited significantly. The use of lentiviral systems is not a viable therapeutic option, however, owing to the potential for integration into the host genome, therefore, either naked siRNA or siRNA in a delivery system must be used.

Recognizing limitations of delivery of naked siRNA, such as degradation, poor bioavailability and intracellular delivery,

and building on reported successes in delivery of antisense oligonucleotides and siRNA to systemic models of disease, Villares *et al.* used neutral liposomes composed of DOPC to encapsulate siRNA specific for PAR-1. They examined uptake within tumor tissue using Alexa Fluor 555 as a liposomal marker and demonstrated successful uptake at a period of 2 days postintravenous injection to established subcutaneous melanomas.

The optimal scheduling of therapeutic delivery is often determined empirically through a series of clinical studies. In this article, the authors used real-time PCR of tumor tissue post-siRNA-DOPC administration and were able to show that downregulation peaked 4 days post-administration, but recovered by day 6, therefore treatment was optimized at twice weekly thereafter. With this schedule, the authors clearly showed not only decreased rate of tumor growth but also significant inability of the cells to form metastatic lung colonies (median of 62 lung colonies in mice

treated with nontargeting siRNA-DOPC vs only 12 in mice treated with the PAR-1 siRNA-DOPC). Furthermore, treatment resulted in an overall decrease in tumor blood vessels, therefore having an impact on angiogenesis.

To conclude, Villares *et al.* have presented a liposomal system of PAR-1-targeted siRNA that effectively reached subcutaneous tumor tissue and silenced PAR-1 therein. Furthermore, this formulation was nontoxic (results not discussed here) in the model system used and, following on the success of other liposomal therapeutics, holds promise for future clinical development.

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