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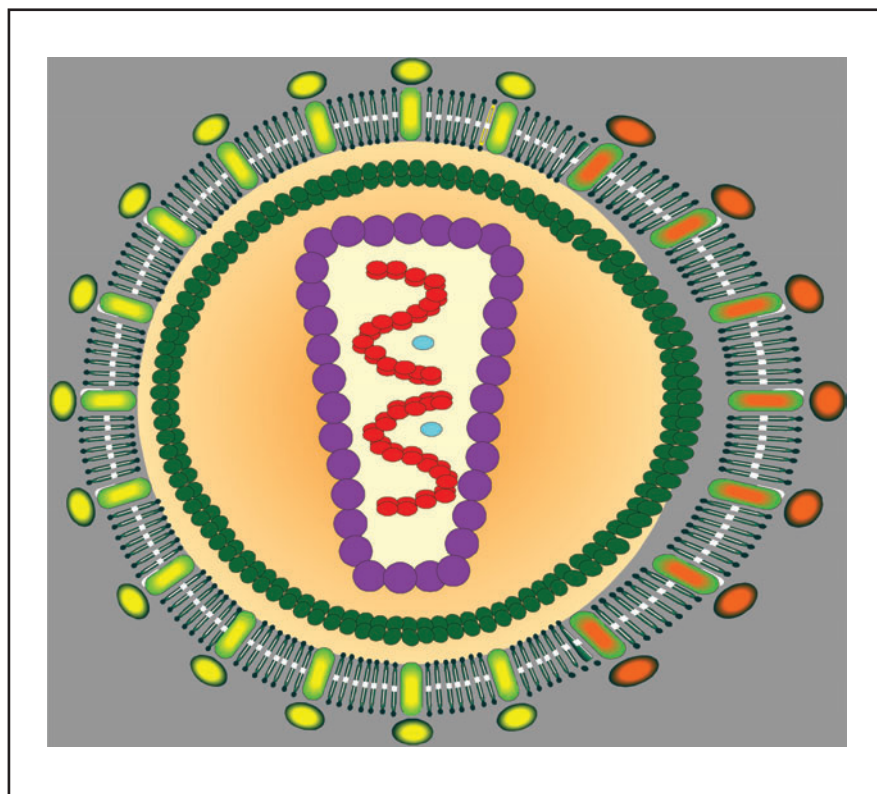
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Molecular Engineering of “Pathotropic” Targeting for Cancer Gene Therapy

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More than 70 percent of all prospective gene transfer/gene therapy protocols are designed to treat metastatic cancer.¹ A large number of such protocols involve strategies to attempt cancer immunization via cell-based gene transfer of cytokines or tumor antigens, while others involve the delivery of oncolytic viruses or vectors bearing prodrugs, chemoprotective agents, antisense constructs, or tumor suppressor genes. However, a major unresolved problem that has impeded the progress of cancer gene therapy to the clinic is that of inefficient gene delivery to target cells *in vivo*. In this regard, the advent of pathotropic targeting launches a new paradigm in cancer gene therapy. By targeting the histopathology of the cancerous lesion — rather than the cancer cells per se — to effectively concentrate the gene vector within primary and metastatic tumors, the safety and efficacy of intravenously administered vector nanoparticles were increased significantly in animal models of cancer. This article describes the development of the pathotropic Targeted Delivery System (TDS) that now serves as the guidance system for “smart” nanoparticles bear-



A pathotropic injectable nanoparticle [Illustrated by Bob Salazar]. The surface membrane glycoproteins of the viral-based vector have been modified by molecular engineering to provide a gain-of-targeting function. Guided by an enabling Targeted Delivery System (TDS), the pathotropic nanoparticles seek out and accumulate in cancerous lesions providing tumor site-specific gene delivery (Epeius Biotechnologies Corporation).

ing designer killer genes for cancer gene therapy (Table 1).

The Quest for a Targeted Delivery System: The Threshold of Genetic Medicine

From its very inception, gene therapy for cancer has sought a targeted delivery vehicle (or vector) that could be inject-

ed into the human blood stream, target cancer cells, and then deliver a cytotoxic “killer” gene to destroy cancer cells without causing undesirable side effects. Progress in the field of gene therapy, however, has been disappointingly slow and, despite its great promise, the applications of genetic medicine remain stalled at the brink of success.^{2–5} Numerous attempts to target specific

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Table 1. A decade of discovery by Hall & Gordon: From cloning to the clinic.

Date	Discovery	Publication
1994	Cloning of the human <i>cyclin G1</i> gene	Wu <i>et al.</i> , 1994
1995	Construction of antisense <i>cyclin G1</i> retroviral vector	Skotzko <i>et al.</i> , 1995
1996	Molecular engineering of Targeted Delivery System (TDS) for gene transfer to vascular lesions in balloon-injured rat arteries	Hall <i>et al.</i> , 1997
1997	Adaptation of TDS for gene therapy in humans	Hall <i>et al.</i> , 2000
1998	Cloning of designer mutant <i>cyclin G1</i> gene in TDS (creation/discovery of Rexin-G)	Xu <i>et al.</i> , 2001
1999-2000	Proof of concept/toxicology studies using Rexin-G in animal models of metastatic cancer	Gordon <i>et al.</i> , 2000 Gordon <i>et al.</i> , 2001
2000	U.S. FDA filing of Investigational New Drug and presentation to NIH Recombinant DNA Advisory Committee	Lenz <i>et al.</i> , 2002
2001-2002	GMP manufacture of clinical Rexin-G Vector	Gordon <i>et al.</i> , 2003
2002	U.S. FDA approval of Phase I/II clinical trial using Rexin-G for metastatic colon cancer	
2002	Philippine BFAD approval of compassionate use clinical trial using Rexin-G for end-stage pancreatic cancer	
2002-2003	First three patients with end-stage pancreatic cancer infused with Rexin-G in Manila, Philippines	Gordon <i>et al.</i> , 2003
2003	Rexin-G gains U.S. FDA orphan drug approval as treatment for pancreatic cancer	

cell types have focused on modifying the receptor-binding domain (SU) of the ecotropic Moloney murine leukemia virus (MuLV) envelope (*env*) protein.⁵⁻⁸ These cell-targeted vectors have all failed the acid test when evaluated *in vivo*, thus precluding their further progress into clinical trials.⁹ The consensus opinion has been that, “improved gene delivery methods were needed in order to give human tests a better chance of success.”¹⁰ Even though large amounts of research dollars have been invested in gene-based research and development programs during the last two decades, the first gene therapy product has yet to become commercially available in the United

States. As a result, many pharmaceutical companies have ceased the development of gene therapy products altogether.

The Advent of “Pathotropic” Targeting: An Enabling Platform Technology

Classical approaches to designing targeted gene therapy vectors for cancer have focused on varying properties of the cancer cells themselves (known as cell-specific targeting). Unfortunately, this strategy has proven to be far too inefficient to overcome the natural barriers of turbulence, flow, dilution, filtration, and inactivation encountered in

the human bloodstream and, thus, these strategies failed to advance to the clinic. By contrast, Hall and Gordon undertook a seemingly counterintuitive strategy of targeting a pervasive property of the diseased cancerous tissues, rather than the cancer cells themselves. The exploration of this “road less traveled” has made all the difference in terms of clinical utility. The resulting Targeted Delivery System (TDS) incorporates a disease-seeking guidance system into the vector, similar to a heat-seeking missile that targets a unique property of the aircraft rather than the pilot of the aircraft. When deployed intravenously, the TDS-guided nanoparticles seek out and concentrate in cancerous lesions,

thus dramatically improving the delivery of the genetic payload into the cancer cells and the growing blood vessels of the tumors.

Uniquely suited — by design — to function within the human circulatory system, and demonstrating a true “seek-and-destroy” surveillance function for treating cancerous lesions, the TDS-guided vectors readily find and accumulate in primary tumors and metastatic sites (where the cancer has spread), many of which may be otherwise inoperable, hidden, or undetectable by X-ray imaging or screening tests. Therefore, Hall and Gordon engineered a gene therapy vector incorporating a unique guidance system derived in principle from the proteomics and biochemistry of von Willebrand factor, which naturally guides platelets to injured or diseased vascular tissues.^{11,12} Von Willebrand factor (vWF) is a clotting factor normally found in circulating blood. vWF performs a vital surveillance function by mediating platelet adhesion to sites of vascular injury wherein collagenous proteins are exposed to circulating blood. Discrete domains of vWF bind with high-affinity to newly exposed collagen which, together with glycoprotein Ib and glycoprotein IIb-IIIa, promote the platelet-vessel wall interaction. The transposition of vWF-derived sequences into the retroviral envelope protein serves to direct retroviral recruitment and accumulation at sites of exposed collagen in cancerous lesions but not in healthy tissues wherein collagen is not normally exposed. This disease-seeking property of the therapeutic nanoparticles guided by our TDS technology is termed “pathotropic” targeting. Whether the seminal tissue disturbance is caused by: (1) the characteristic enzymatic activities of tumor invasion; (2) the remodeling of the extracellular matrices associated with tumor blood vessel formation (angiogenesis); or (3) the body’s reactive fibrotic attempts to wall off the tumor with a connective tissue capsule, the resulting nexus of tissue disturbance provides a biochemical Achilles heel for the TDS technology to target.^{13–15} The result of this is a biomedical platform technology that both enables and revo-

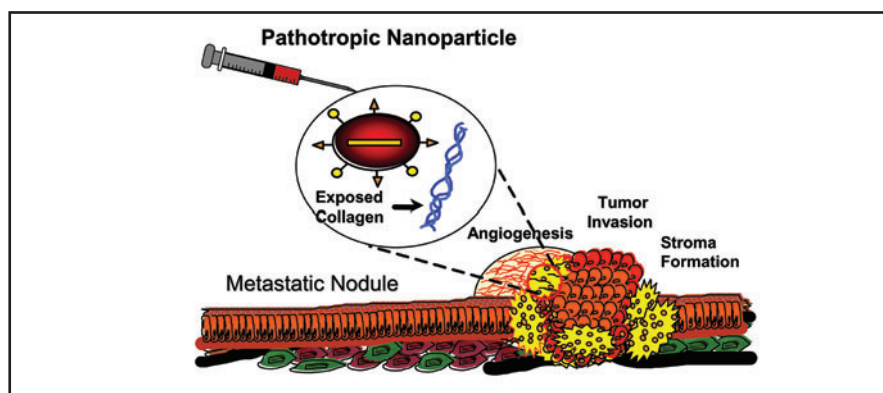


Figure 1. A pathotropic nanoparticle modified by genetic engineering to provide a gain-of targeting function [Illustrated by Bob Salazar]. When deployed intravenously, pathotropic nanoparticles seek out and accumulate in metastatic nodules wherein collagenous proteins are exposed to the circulation. The pathophysiologic processes involved in tumor invasion, tumor-associated angiogenesis, and stromal cell activation present a common nexus of collagen exposure which may be exploited to therapeutic advantage, as a potential Achilles heel of solid tumors.

lutionizes medical delivery to seriously diseased tissues, including metastatic cancer (Fig. 1).

Disrupting the Cancer Cell Cycle with a Cytocidal Designer Gene

The “cancer killing” designer gene in the targeted Rexin-G vector is a mutant form of the human *cyclin G1* gene, a growth-associated cell cycle control element that was first isolated (cloned) by Hall and co-workers in 1994.¹⁶ *Cyclin G1* is a prospective oncogene that favors the development of many types of cancer, including pancreatic, colon, breast, and prostate cancer, as well as numerous bone and soft tissue tumors such as osteosarcoma and Ewing’s sarcoma. To sabotage their cell cycle and destroy the cancer cells, a mutant form of *cyclin G1* was engineered that, when translated into protein, blocks the essential function of this cell cycle control element, thereby causing the cancer cells to die via biochemical mechanisms of apoptosis or programmed cell death. Further studies revealed that tumor-associated angiogenesis (i.e., the proliferative vascular cells associated with growing tumors) are also vulnerable to *cyclin G1* knockout.¹⁷ The destruction of tumor-associated angiogenic cells could by itself induce a disproportionate amount of tumor cell death by depletion of vascular supply or growth factor stimuli (known as a non-classical bystander

effect). A built-in safety feature of the Rexin-G targeted delivery system is that it only affects rapidly dividing cells, such as cancer cells and their attendant blood supply, thus selectively killing tumor cells while sparing the normal cells of the body.¹⁸ Therefore, it is not expected to cause untoward systemic toxicity such as hair loss, nausea and vomiting, bone marrow suppression, and liver or kidney dysfunction. This aspect of the genetic therapy would enhance the quality of life of patients with cancer, in addition to the obvious advantages of arresting, reducing, and/or eliminating the metastatic disease.

Translating Basic Science into Medicine: Proof of Concept/Toxicology Studies

A series of preclinical safety and efficacy studies using Rexin-G, the first pathotropic injectable retroviral vector for cancer gene therapy, formed the basis of an Investigational New Drug (IND) application which was submitted to FDA in November 2000.¹⁹ In gene marking studies, the infusion of a pathotropic vector, but not a non-targeted vector, bearing a β -galactosidase marker gene resulted in high level transduction of tumor cells and angiogenic endothelial cells ($35.7 \pm 1.4\%$) throughout the tumor nodules. To further investigate the high transduction effi-

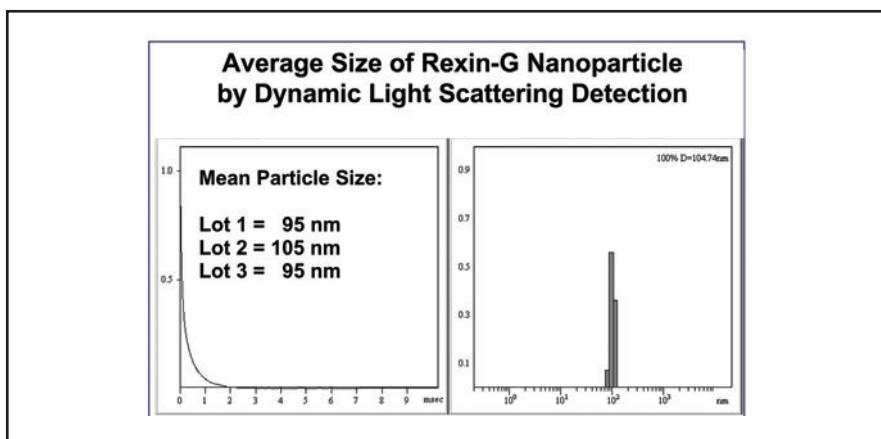


Figure 2: Average size of Rexin-G nanoparticles. Using a Precision Detector Instrument (Franklin, MA 02038 U.S.A.), the vector samples were analyzed using Dynamic Light Scattering (DLS) in Batch Mode for determining molecular size as the hydrodynamic radius (rh). Precision Deconvolve software was used to mathematically determine the various size populations from the DLS data. The average particle size of 3 Rexin-G clinical lots are 95, 105 and 95 nm respectively with no detectable viral aggregation.

ciency observed in the tumor nodules, a vector distribution study showed extensive accumulation of vector particles in angiogenic areas throughout the tumor nodule, one hour after intravenous infusion of the pathotropic vector. Consistent with these observations, *in vivo* efficacy studies showed that portal vein infusions of Rexin-G (regional delivery) induced a dramatic reduction in the size of tumor foci in Rexin-G-treated mice compared to those of control vector-treated animals.²⁰ In a more challenging study, peripheral vein infusions of Rexin-G arrested the growth of subcutaneous tumors in mice. In this case, the circulating vector necessarily transited the heart, passed through the microvasculature of the lungs, and returned to the heart before being distributed throughout the systemic circulation. Upon repeated intravenous injections of this targeted vector, long-term efficacy studies showed sustained inhibition of tumor growth and/or complete tumor regression of animals treated with Rexin-G compared to those treated with phosphate-buffered saline (PBS) placebo or a non-targeted vector.¹⁷ Using Kaplan-Meier plots, in a six-week follow-up period, the tumors quadrupled in all placebo-treated mice and in 75 percent of mice treated with the non-targeted control vector. In contrast, no tumors quadrupled in

mice treated with the targeted Rexin-G vector ($p = 0.004$; Tarone test for trend). In a 100-day follow-up period, comparative studies conducted with placebo-treated mice showed a rapid increase in tumor volumes to greater than five times their basal volumes within six weeks. In contrast, complete tumor eradication was achieved in 50 percent of the Rexin-G vector-treated mice while tumor growth was significantly inhibited in the remainder of the animals.²¹ It is important to note that the transduction efficiency and therapeutic efficacy observed under these formidable physiologic conditions are both unprecedented and clinically important.

Further, toxicology studies showed that vector doses equivalent to 2–10 times the highest dose proposed for a Phase I clinical trial did not cause bone marrow suppression or any significant alterations in liver or kidney function in mice, rats, and pigs.^{19,22} Biodistribution studies using a TaqMan PCR-based assay for vector integration showed that low-level positive PCR signals were detected only in the liver and spleen, which represent the major sites of viral clearance, one week after IV Rexin-G infusions. No pathology attributable to the vector was observed in non-target organs of vector-treated animals. These preclinical data indicate that Rexin-G

may be administered systemically with a wide margin of safety, providing the basis for an FDA-approved Phase I clinical trial for metastatic colon cancer in the United States and Bureau of Food and Drugs (BFAD)-approved clinical protocols for Stage IV pancreatic cancer and other solid tumors in Manila, Philippines.²²

Manufacturing and Validation of the Rexin-G Clinical Product: A Critical Step

Rexin-G (also known as Mx-dnG1 in FDA IND #9472) is a non-replicative “pathotropic” retroviral vector encoding an N-terminal deletion mutant construct of human cyclin G1 placed under the control of the MuLV long terminal repeat promoter.^{16,23,24} The clinical vector was produced by transient transfection of human embryonic kidney (HEK 293T) cells taken from a certified master cell bank in compliance with the Code of Federal Regulations on current good manufacturing practices (cGMP). The clinical vector was stored in volumes of 150 ml in 500-ml cryobags at -80°C . The fully validated product exhibits a viral titer of 3×10^7 colony forming units (Units) per milliliter; a biologic potency of 65–70% growth inhibitory activity in human breast, colon, and pancreatic cancer cells; a uniform nanoparticle size of ~ 100 nm with no detectable viral aggregation (Fig. 2); less than 550 bp residual DNA, indicating absence of intact oncogenes; no detectable E1A or SV40 large T antigen; and no detectable replication competent retrovirus (RCR) in five passages on *Mus dunni* and HEK 293 cells. The product is sterile with an endotoxin level of <0.3 endotoxin units (EU)/ml, and the end-of-production cells are certified to be free of mycoplasma and other adventitious viruses.

In summary, the integrated components of the targeted gene delivery system include:

(1) *The Therapeutic Designer Gene*: A mutant knockout construct of the *cyclin G1* gene, which is a human

growth control gene first isolated in 1994 by Hall and co-workers.¹⁶ This gene is highly expressed in many cancers and its knockout is lethal to both the cancer cells and their blood supply. This mutant *cyclin G1* gene integrates into proliferative cancer cells in which its product is cytotoxic. The cytotoxic designer gene is then degraded with the cell's nucleus via apoptosis and is eliminated, along with the cell itself, by the body's natural mechanism. Thus, the modified gene is not expected to be transmitted inadvertently to the recipient's offspring (germ-line transmission).

(2) *The Gene Delivery Vehicle:* A suspended nanoparticle based on FDA-approved molecular engineering of retroviral components, whose genetic payload is restricted to the therapeutic gene and whose gene delivery is restricted to proliferative cells (enhanced safety). The half-life of the delivery vehicle in human blood is approximately 50 minutes.

(3) *The Pathotropic Targeting System:* An integral disease-seeking surveillance system that concentrates gene delivery to cancerous lesions and enables intravenous infusion (enhanced safety and efficacy).

These integrated components of the TDS, along with FDA-approved bioprocessing and GMP manufacturing protocols for Phase I/II clinical trials, are combined in the formulation of Rexin-G, the first tumor-targeted injectable clinical product for cancer gene therapy.

Pioneering Cancer Gene Therapy in the Philippines: The First Human Experience

Phase I/II clinical trials for pancreatic cancer were conducted at the Makati Medical Center (Manila, Philippines) to evaluate the safety and efficacy of Rexin-G guided by TDS in three patients with end-stage pancreatic cancer who were refractory to conventional chemotherapy. The clinical study was led by Drs. Gerardo H. Cornelio and Conrado Lorenzo III of the University of the Philippines. An intra-patient

Table 2. Intra-patient dose escalation protocol for end stage pancreatic cancer

Treatment Day	Dose Level	Vector Dose per Day
Dose escalation (induction)		
Day 1–6	I	4.5 x 10 ⁹ Units
Day 7–8	II	9.0 x 10 ⁹ Units
Day 9–10	III	1.4 x 10 ¹⁰ Units
Rest one week		
High dose (intensification)		
Day 18–27	III	1.4 x 10 ¹⁰ Units

dose-escalation regimen was designed (to ensure safety), followed by an intensification regimen (to evaluate efficacy) (Table 2). In a second protocol, intravenous Rexin-G was given as front-line treatment followed by eight weekly doses of gemcitabine.

Intravenous infusions of Rexin-G arrested tumor growth in all three patients based upon caliper measurements, CT scan, and MRI imaging studies, and extended the patients' survival beyond their life expectancy of three to six months.²⁵ Remarkably, the Rexin-G infusions were *not* associated with nausea, hair loss, diarrhea, mucositis, hemodynamic instability, bone marrow suppression, or liver and kidney damage over a three-month observation period. No dose-limiting toxicity was noted in any patient receiving Rexin-G, indicating that additional vector infusions could conceivably be given for greater control of tumor growth and for induction of a remission. The only side effects associated with the vector infusions were minor: brief episodes of fever that were relieved by acetaminophen. In contrast to the deleterious side effects of chemotherapy, each of the patients reported a general feeling of well-being during the infusion period, indicating that Rexin-G would enhance the quality of life of cancer patients. Thus, although the initial clinical study involved a very small number of patients and a limited treatment regimen without maintenance therapy, the

results are exceedingly encouraging.

Preventing Cancer Recurrence: Toward a Cancer Cure and Beyond

An exciting potential use for Rexin-G is in preventive surveillance — finding and killing micrometastases long before they grow into tumor nodules large enough for imaging studies or screening tests to detect. The physiologic surveillance properties of Rexin-G offer the promise of seeking out and destroying these micrometastases before they become life-threatening. A second potential use is known as tumor sterilization, in which after surgical resection of a cancerous tumor, adjuvant treatment with Rexin-G may be given to destroy the residual cancer cells that could cause a recurrence of the cancer or could circulate and spread to vital organs. Intravenous deployment of Rexin-G would seek out the residual cancer cells in areas that are not surgically accessible, thus eradicating remote as well as occult tumors. Thirdly, Rexin-G could potentially be given in combination with other anti-cancer agents, for example, to assail and sensitize the tumors to subsequent chemotherapy, and/or to lessen the incidence and severity of chemotherapy-induced systemic toxicity (unpublished data). Finally, a novel TDS-targeted vaccination strategy is being developed wherein pathotropic nanoparticles bearing a cytokine gene (e.g., GM-CSF or inter-

leukin-2) would initially be infused to achieve local cytokine secretion and recruitment of immune cells into the tumor, followed by infusion of cytotoxic Rexin-G to expose tumor antigens and promote the induction of long lasting anti-tumor immunity.

Although enabling cancer gene therapy is the most compelling use of Rexin-G at present, the pathotropic vector may be useful for treating other proliferative disorders as well. Because the TDS technology is derived from a natural wound-seeking guidance system found within the human body, its potential clinical applications are extensive. In cardiovascular disease, for example, animal studies, ranging from rats to monkeys, have demonstrated effective inhibition of vascular restenosis in injured arteries following balloon angioplasty.²³ In studies of fibroproliferative eye disease in rabbits, the TDS-targeted nanoparticle, but not the non-targeted vector, prevented the development of corneal haze after laser injury.²⁶ In mice afflicted with experimental colitis, a TDS-guided construct of the epidermal growth factor (EGF) healed colonic ulcers more rapidly and more extensively than the commercially available non-targeted EGF.²⁷ The pathotropic platform technology is not limited to gene therapy applications, but may be used to target recombinant proteins and other pharmaceutical agents to injured tissues as well. Other applications of pathotropic TDS technologies are in areas of vascular disease, ischemia, stroke, inflammatory disease, and wound healing, suggesting an extensive potential pipeline of innovative products for improved medical delivery.

Perspectives

The development of Rexin-G spans a decade of basic and translational research, ranging from gene discovery to molecular engineering to GMP manufacturing, to clinical trials, to FDA Orphan Drug designation. Further demonstrations of the safety and efficacy of Rexin-G for treating metastatic cancer will extend the reach of the physician beyond the hands of the surgeon, beyond the lumen of the

smallest catheter, to the foundations of disease itself. Based upon the very fabric of our nature, the advent of pathotropic targeting serves as a versatile platform technology that enables the delivery of therapeutic nanoparticles as well as targeted pharmaceutical agents to injured and/or diseased tissues. The challenge of the future will be to build upon these newly established principles of molecular engineering and to further integrate these innovations into the practice of medicine.

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