AUCTORES
Globalize your Research

Journal of Cancer Research and Cellular Therapeutics
Erlinda M. Gordon *

Open Access

Review Article

Polypeptidic Taxol-Tropins: Targeting paclitaxel to the tumor microenvironment

Erlinda M. Gordon[1, 3*], Seiya Liu[2], Sant P. Chawla[1], Frederick L. Hall[1]

[1]Counterpoint Biomedica LLC, Santa Monica, California, USA

[2]Harvard University, Cambridge, Massachusetts, USA

[3]Aveni Foundation, Santa Monica, California, USA

***Corresponding Author:** Erlinda M. Gordon, Counterpoint Biomedica LLC 2700 Neilson Way, Suite 1227 Santa Monica CA 90405.

Received Date: July 07, 2021; Accepted Date: July 21 2021; Published Date: July 26, 2021

Citation: **E M. Gordon, S Liu, S P. Chawla, F L. Hall**. (2021) Polypeptidic Taxol-Tropins: Targeting paclitaxel to the tumor microenvironment. *J.Cancer Research and Cellular Therapeutics*. 5(3); DOI: 10.31579/2640-1053/089

Abstract

Background and Rationale: Although PTX is widely used as a single chemotherapeutic agent and in various combination regimens, its clinical utility is hindered by acquired drug resistance and serious dose-limiting side effects that result from the ungoverned biodistribution of the taxane.

Hypothesis: Conceptually, the precision, validity, and efficiency of paclitaxel delivery to tumor compartments might be substantially improved by "actively targeting" the exposed collagenous (XC-) proteins presented within the tumor microenvironment (TME)—XC-proteins physically exposed by the *pathologic* biochemical processes of tumor invasion, reactive stroma formation, and neo-angiogenesis.

Objective: An adaptive bioengineering approach aims to apply pathotropic tumor-targeting functionality to paclitaxel (PTX), a powerful cytotoxic taxane which exhibits anti-tubulin / anti-mitotic / anti-cancer activities against a broad range of solid tumors.

Materials and Methods: Synthetic peptide XC-targeting probes (< 40 aa) and polypeptide aptamers (40 to 53 aa), 85 - 99% purity, were prepared by 9-fluorenylmethyloxycarbonyl (Fmoc) solid phase peptide synthesis, purified by high performance liquid chromatography (HPLC), and verified by mass spectrometry and amino acid analysis, and the XC-targeting probes were FITC-labeled. Analysis of fluorescence in XC-binding assays was visualized with an Ultra Bright Blue Light trans-illuminator equipped with an amber filter; photo-documentation was provided by a Leica V-Lux 1 digital camera; and comparative fluorescence was quantified using a Quantus benchtop fluorimeter (Promega). The tumor-targeting properties of Taxol-Tropins were tested in vitro by Taxol-aptamer binding assays and collagen-agarose binding assays and the bioactivities of PTX bound non-covalently toTaxol-Tropin aptamers were tested on XC-agarose beads. Further, the tumor targeting property of the Taxol-Tropin aptamers was tested in vivo in a murine model of metastatic cancer.

Results: Here we report on the first actively targeted delivery of paclitaxel utilizing bifunctional polypeptide targeting onco-aptamers, called Taxol-Tropins, which: (i) bind PTX upon simple mixing with suitably high affinities and; (ii) bind exposed XC-proteins, thereby promoting enhanced partitioning and drug delivery into the TME. The bifunctional peptide sequence-optimized Taxol-Tropins bound tightly non-covalently to PTX and also exhibited high affinity and selectivity for XC-agarose beads *in vitro*. Importantly, the cytotoxic bioactivity of the Taxol-Tropin-bound-PTX molecule was well preserved *in cellulo*, as was demonstrated by cytocidal activity observed in MDA-MB-231 breast cancer cell cultures. Tumor-targeted PTX delivery by Taxol-Tropin onco-aptamers *in vivo* was modeled by subcutaneous xenografts of human pancreatic cancer in nude mice: where intense fluorescence of the PTX probe was observed in tumors of mice injected with the Taxol-Tropin-bound-PTX within minutes after intravenous injection, but not in untreated mice or mice treated with non-targeted PTX probe.

Conclusions: These results demonstrate the feasibility of pro-actively targeting PTX, a clinically important small molecule, using Taxol-Tropins: synthetic polypeptide onco-aptamers, revealing optimized drug binding sequences and structural modifications pertinent to further clinical development of the tumor-targeting platform which may indeed shift the Therapeutic Index of PTX to one of greater clinical efficacy at lower drug doses.

Keywords: Tumor-targeting; drug delivery; taxol; nab-paclitaxel; tumor microenvironment; extracellular matrix; anti-angiogenesis; chemotherapy; metastatic cancer

Introduction

The taxanes are among the most effective and widely used antineoplastic agents for cancer. Produced by an endophytic fungus found within the bark of *Taxus brevifola* (Pacific Yew Tree), the prototypical taxane, paclitaxel (also known as Taxol), was discovered in a National Cancer Institute study wherein the extracts of thousands of plants were screened for potential anticancer activity [1, 2]. Taxol, at sufficient doses, inhibits cell proliferation primarily by physical binding to microtubules, inducing a sustained mitotic block—associated with an incomplete metaphase plate and an abnormal organization of the spindle apparatus—which leads to apoptosis and cell death [3]. However, high affinity interactions with other cellular constituents: including the regulatory loop of the Bcl-2 anti-apoptotic protein [4] and the DNA-binding domain of the NFX1 transcriptional repressor [5], suggest additional molecular mechanisms of action. Exhibiting both cytotoxic and antiangiogenic properties, paclitaxel (PTX), formulated in organic solvents (*Taxo*l) or as albumin-bound nanoparticles (*nab-paclitaxel*, aka *Abraxane*), has demonstrated an exceedingly broad spectrum of antitumor activity: including cancers of the breast, ovary, lung, prostate, head and neck, bladder, and pancreas, as well as Kaposi's sarcoma [6,7].

The clinical benefits of paclitaxel-based chemotherapy are entirely dependent on achieving effective concentrations within the tumor microenvironment (TME), whereas the vast majority (>95%) of a given intravenous dose is known to accumulate in non-target organs—including the liver, spleen, and lungs [8,9]—resulting in serious dose-limiting toxicities. Thus, a major challenge and compelling medical need is to maximize the effective local concentrations, while minimizing the associated systemic toxicities, with a rational drug delivery system that is suitably and pro-actively tumor targeted. Indeed, the development of a nanoparticle delivery system for PTX, based on the physicochemical properties of human albumin: nab-paclitaxel, aka *Abraxane* [10,11], has served to lessen the limiting toxicities and hypersensitivity reactions associated with the earlier cremophor-based formulations [12,13], thereby permitting higher "equitoxic" doses of nab-paclitaxel (not lower drug doses) to be routinely administered [14]. However, despite the initial excitement and enthusiasm for this higher-cost and higher-dose nab-paclitaxel formulation from the medical community [15], more recent studies have had a sobering effect: Importantly, a large comparative phase III breast cancer study failed to demonstrate any superiority of nab-paclitaxel over standard *Taxol* formulations in either patient outcome or overall toxicity [15]. Moreover, the initial speculative claims for "active tumor targeting" of nab-paclitaxel via some selectivity for tumor SPARC (*secreted protein acidic and rich in cysteine*) proteins (16) have recently been refuted [17,18], confirming that the biodistribution of nab-paclitaxel to the tumor microenvironment is not based upon SPARC expression, and is largely, if not entirely, nab-paclitaxel dose-dependent [19,20].

When compared with the considerable potential for "smart or active tumor targeting"—i.e., synaphic (high-affinity) and/or pathotropic (disease-seeking) tumor targeting [21-23]—the essentially passive delivery of drug-bearing nanoparticles by so-called EPR effects (enhanced permeability and retention) was determined to be relatively meager [23-24]. Nab-paclitaxel delivery to tumors, in particular, can be enhanced significantly by the addition of active tumor targeting provided by tumor-homing peptides [25,26], which enabled clear demonstrations of anti-tumor activity with peptide-targeted abraxane at lower drug doses: doses at which non-targeted abraxane showed no significant effects [26]. Likewise, the development of a pathotropic tumor targeting platform based on high-affinity homing to abnormally exposed collagenous (or XC-) proteins—the signature of tumors invading the TME—provided for pro-active tumor targeting, which enabled the first targeted, injectable gene delivery vehicles to be validated in the clinic [24, 27-29]. The aim of the paper is to report the successful adaptation of this enabling pathotropic XC- or lesion-targeting platform—initially developed in the context of lesion-targeted growth factors for wound healing applications [30,31] and clinically in the context of tumor-targeted retroviral vectors for therapeutic gene delivery [32,33]—to small synthetic polypeptide targeting aptamers (called *onco-aptamers*) that are capable of: i) binding tightly yet, non-covalently to paclitaxel upon simple mixing and; ii) delivering the resulting complexes actively, selectively, and efficiently to tumors, potentially enabling lower effective paclitaxel doses to be routinely administered, albeit with greater clinical efficacy.

Materials and Methods

Materials. Synthetic peptide XC-targeting probes (< 40 aa) and polypeptide aptamers (40 to 53 aa), 85 - 99% purity, were prepared by 9-fluorenylmethyloxycarbonyl (Fmoc) solid phase peptide synthesis, purified by high performance liquid chromatography (HPLC), and verified by mass spectrometry and amino acid analysis. FITC-labeled XC-targeting probes and 'Taxol-Tropin' aptamers were synthesized by Biomatik; additionally, various Taxol-Tropin aptamers were synthesized by C.S. Bio Co. and Thermo-Fisher with similar purities. Polypropylene mini-columns, collagen-agarose chromatography matrix (1 mg/ml collagen type III; 4% beaded agarose) and paclitaxel were purchased from Sigma Aldrich. Paclitaxel, Oregon Green-488 Conjugate (Oregon Green-488-Taxol), aka Flutax-2 (Invitrogen/Life Technologies)—which enables fluorescent Taxol-labeling of tubulin filaments in live cells—was purchased from Thermo-Fisher. Analysis of fluorescence in XC-binding assays was visualized with an Ultra Bright Blue Light trans-illuminator equipped with an amber filter (New England Bio Group); photo-documentation was provided by a Leica V-Lux 1 digital camera; and comparative fluorescence was quantified using a Quantus benchtop fluorimeter (Promega).

Taxol-aptamer binding assays and collagen-agarose binding assays. For comparative Taxol and XC-binding studies, linear bifunctional polypeptide aptamers (5 mg by weight) were initially suspended in 200 µl of 50% dimethyl sulfoxide (DMSO) in PBS, pH 7.4, followed by stepwise dilutions: first to 5 mg/ml (10% DMSO) stock solutions, and then to 1 mg/ml (2% DMSO) as working aptamer concentrations, which were stored frozen at -20ºC. Varying amounts of the Taxol-Tropin aptamers were allowed to interact with Oregon Green-488-Taxol in PBS, pH 7.4, at room temperature for 30 minutes; the reaction mixtures were applied to pre-equilibrated mini-columns containing 250 ul bed-volumes of XC-agarose beads, followed by stringent washing (>10 column volumes) with PBS, PBST (PBS + 0.5% Tween-20), and 1.0 M NaCl in PBST, respectively. Following photo-documentation of the comparative fluorescence retained on the (XC) collagen-agarose columns, both the initial column pass-through eluates and the re-suspended XC-matrix retentates were analyzed by blue light fluorimetry.

Bioactivity of Paclitaxel Bound via Taxol-Tropin Aptamers to XC-agarose Beads. Human MDA-MB-231 breast adenocarcinoma cells (Sigma-Aldrich, #92020424), were maintained at 37ºC and 5% CO2 in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% Fetal Bovine Serum (D10 media). Documentation of cell growth in 24 well plates was obtained with an inverted microscope using phase contrast optics, followed by actual cell counts in the presence of Trypan Blue with a Bright Line Neubauer Hemocytometer. To verify that the characteristic biologic activity of paclitaxel is retained upon its non-covalent binding to the Taxol-Tropin targeting aptamer, which itself binds tightly to the exposed type III collagens of the collagen-agarose affinity matrix, the following experiment was performed: Clinically relevant concentrations of paclitaxel (low nM range; 34), which are toxic to MDA-MB-231 cells *in vitro* (5 ug/ml or 5.8 nM; 35) were prepared in 10 ml of D10 cell culture

media in the presence or absence of the selected Taxol-Tropin onco-aptamer (mw = ~5,000 kDa). The reaction mixtures were spiked with 1.0 ug Oregon Green-488-Taxol (to enable fluorescent monitoring of the washing stringency), mixed, and incubated for 30 minutes at 37ºC, prior to their application to the respective sterile collagen-agarose chromatography columns; followed by extensive column washes with sterile PBS. Parallel collagen-agarose beads, incubated with 5 ug/ml PTX minus the Taxol-Tropin onco-aptamer, followed by extensive column washing, served as a (PTX-negative) control. Washed collagen-agarose beads were collected in D10 medium and distributed into the wells of 24-well cell culture plates, which were subsequently over-laid with MDA-MB-213 breast cancer cells harvested in log-phase growth.

Tumor targeting of the Taxol-Tropin in a murine model of metastatic cancer. The following animal studies were performed in collaboration with AntiCancer Inc., San Diego, California, under a protocol approved by the University of California San Diego, Institutional Animal Care and Use Committee, Protocol Number 15-092. Five to six-week old NCR nu/nu mice received a subcutaneous injection of 5x10e6 MiaPaca-2 human pancreatic cancer cells into the right flanks. When the average tumor xenograft reached a volume of approximately 50 mm^3, test samples containing a fluorescent tumor-targeting probe or fluorescent Taxol (Oregon Green-488-Taxol) combined with the selected Taxol-Tropin onco-aptamer (Apt-2) were injected into the tail vein, followed by evaluation of tumor fluorescence over time using an Olympus OV 100 Fluorescent Imaging System (a closed-chamber system for high-sensitivity, high-speed imaging with minimal specimen damage). Following high-sensitivity analysis of the excised tumors, additional biopsies of the liver, heart, skeletal muscle, colon, and bone were similarly examined to assess the comparative biodistribution of Oregon Green-488-Taxol in tumors vs non-target organs.

Results

Adaptation of the vWF-derived (XC-) tumor-targeting platform to synthetic polypeptides. The conceptions and proofs-of-principle of pathotropic (XC) targeting—originally reduced to practice in the context of complex fusion proteins and strategic peptide insertions at permissive locations in retroviral envelope proteins—involved minimal XC-binding sequences flanked by linker designs that minimized steric hindrances which would impair either protein folding and/or expression in biologic production systems, including human producer cells. By contrast, the small synthetic polypeptides (<50 aa) described herein, enabled further experimentation and functional optimization of primary structures by varying the flanking sequences and orientation of the minimal collagen-binding decapeptides. The structure of a model XC-binding probe is shown in (Fig. 1A), where additional flanking sequences, a flexible linker, and a FITC reporter molecule are included in the polypeptide design, which was then used to compare the minimal decapeptide sequences derived from *human* versus *bovine* von-Willebrand Factor (vWF) *in vitro* and *in vivo*. Note: the native Cysteine at position 7 of the minimal decapeptide was conservatively substituted with a methionine (underlined) in both probes, in order to preclude the formation of disulfide bridges which are non-essential for high-affinity collagen-vWF (or collagen-aptamer) binding to occur.

A comparison of XC-binding properties of these model probes under stringent conditions, i.e., the theoretical "plates of separation" enabled by column chromatography (Fig. 1B), showed that both bovine (XC-BD-1) and human (XC-BD-2) sequences performed nearly as well in these assays, with the humanized vWF-derived decapeptide gaining a discernible edge in terms of XC-binding efficiency. Remarkably, this improved performance of the human decapeptide probe seen in stringent chromatographic assays translated into improved tumor-targeting efficiency observed *in vivo* in a nude mouse model of metastatic cancer. As shown in (Fig. 1C), the fluorescent tumor-targeting XC-binding probe (XC-BD-2) transited the heart, lungs, and systemic circulation upon intravenous infusion into the tail vein, and yet the probe was found to accumulate observably within minutes in the subcutaneous tumors, as shown by both non-destructive (Fig. 1C) and destructive (Fig. 1D) image analysis of the tumor xenogafts. The bovine vWF-derived probe was also found to accumulate rapidly in the subcutaneous tumors, albeit with a somewhat weaker fluorescent signal/noise ratio for a given i.v. aptamer dose.

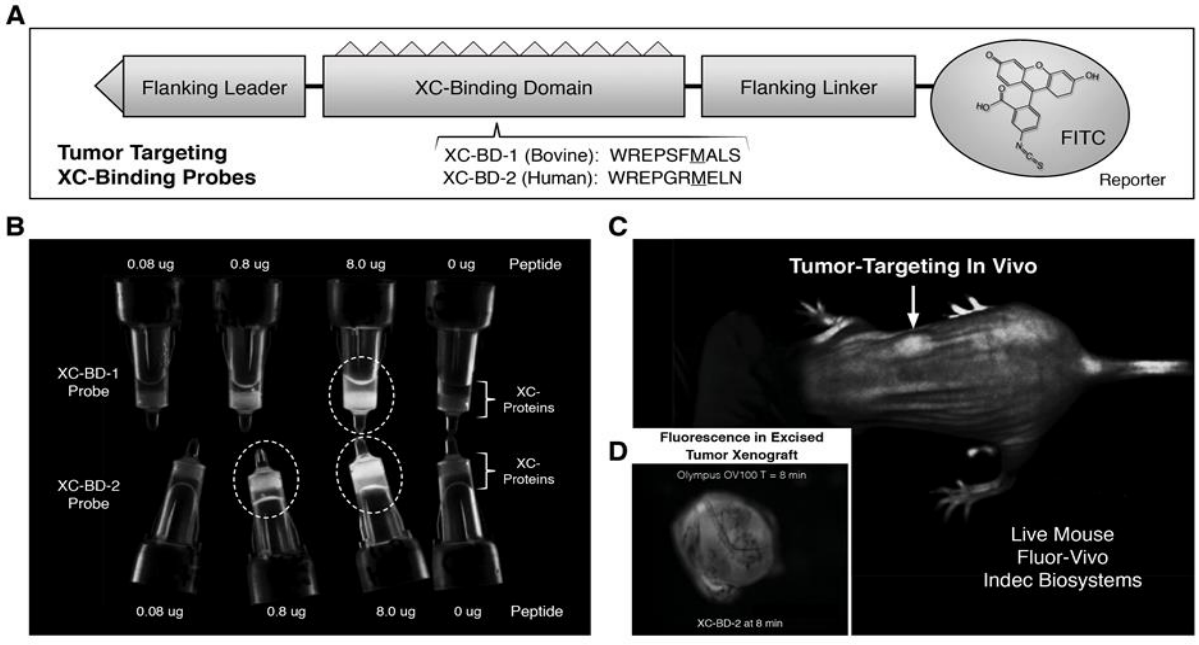

Figure 1: *Testing, selection and validation of optimized XC-binding domains.* **(A)** *Schematic diagram of fluorescent XC-binding probes, showing the minimal decapeptide sequences found in bovine and human vWF (conservative substitution of Methionine for the native Cysteine is underlined).* **(B)**

Comparative binding efficiency to collagen-agarose beads. **(C)** *Active targeting in vivo: Fluorescence imaging of a subcutaneous tumor xenograft in a nude mouse following tail vein injection of an XC-binding probe.* **(D)** *Confirmatory fluorescence imaging of an excised tumor, shown at t = 8 min.*

Molecular design engineering and selection of a taxol-binding tumor-targeting onco-aptamer. The next step was to develop a bifunctional XC-binding and drug-binding polypeptide, aka a tumor-targeting *onco-aptamer* that is additionally capable of high-affinity paclitaxel binding, thereby delivering paclitaxel (Taxol) more actively and selectively to tumors via the systemic circulation. This two-step binding function is embodied in the primary structure of a paclitaxel-binding onco-aptamer, referred to herein as a *Taxol-Tropin*, which is shown diagrammatically in (Fig. 2A). The structure of a prospective clinical *Taxol-Tropin* contains: i) a high affinity XC-binding domain and; ii) a Taxol binding domain and; iii) structural modifications that impart protease-resistance, increased molecular size, and/or enhanced serum stability.

The engineering challenge of discerning a small, linear, high-affinity taxol binding "domain" became obvious upon the failure of two model polypeptides based on the deduced paclitaxel-binding sites of b-Tubulin [36,37] to pass muster in the XC-column assays. Nor did a model peptide mimicking a deduced paclitaxel-binding region of Bcl-2 (4) provide sufficient binding affinity for further development (Table 1). Fortunately, the utility of phage display technology enabled the identification of NFX1 transcription factor as a potential physiological target of paclitaxel [5], providing insights into a singular linear paclitaxel-binding domain that could be mimicked in part by a synthetic peptide with identifiable homology to human NFX1 sequences.

Aptamer	Seq. Derivation	Amino Acid Sequences	Selected Tx-Apt	Tx-Binding
N34-G2-CBD	N-term, u-tubule	REIVHIQAGQMGNQIGAKFWEVISDEHGIDPT		negative
CBDG4TxBDi	Internal, u-tubule	RTLKLTTPTYGDLNHLVSATMSG		negative
NFX1(Human) PPCPAFMTKTCE**C**GRTRHTVRCGQAV ***** * T7 Phage CDSFARSCVRGVGIMKA**C**GRTRVTS (Aoki et al.) LFTDMALSGKVLVKA**C**GRTRVTS				
CBDTxPepPg	Phage / ~NFX1	RGVGIMKA**C**GRTRVTS	Tx-Tropin Apt 1	+++
CBD/KAC-HT	NFX1 (Human)	RGVGIMKA**C**GRTRHTVR[m]G	Tx-Tropin Apt 2	+++++
CBD/KAC-HT+	NFX1 (Hs) +pt	RGVGIMKA**C**GRTRHTVRMGpt	Tx-Tropin Apt 2F	++++
CBD/RAC-HT	NFX1(Hs)□K/R	RGVGIMRA**C**GRTRHTVR[m]G	Tx-Tropin Apt 3	++
CBD/KAS-HT	NFX1(Hs)□C/S	RGVGIMKASGRTRHTVR[m]G		+
CBD/KAA-HT	NFX1(Hs)DC/A	RGVGIMKAAGRTRHTVR[m]G		+/-
CBD/BCL-2	BCL-2, F49-T74	FSSQPGHTPHPAASRDPVARTSPLqt		negative

Table I. *Design and Selection of High-Affinity Paclitaxel (Tx)-Binding Sequences*

Further elaboration of this linear taxol-binding domain with extended NFX1 sequences and various structural modifications (Fig. 2B), followed by comparative XC-column chromatography (Fig. 2C) provided a series of optimized high-affinity Taxol-Tropins for analysis (Table 1). Conservative substitutions demonstrated selectivity for Lys over Arg within the deduced binding domain (T7 phage, underlined), along with importance of the central Cys residue (bold). While polyethylene glycol (PEG) of various sizes is often used for increasing the protease resistance and circulating time of synthetic peptides, there was a predictable tradeoff of reduced binding pharmacodynamics in the XC-column assays—which requires both high-affinity XC-binding and high-affinity Taxol-Green binding properties to produce a fluorescent signal (Fig. 2C). Alternatively, the use of D-amino acid stereoisomers on both the N-terminal and C-terminal ends of the optimized Taxol-Tropin (Apt-2) could potentially be used to increase protease resistance (38) with less steric interference with the requisite bifunctional binding affinities.

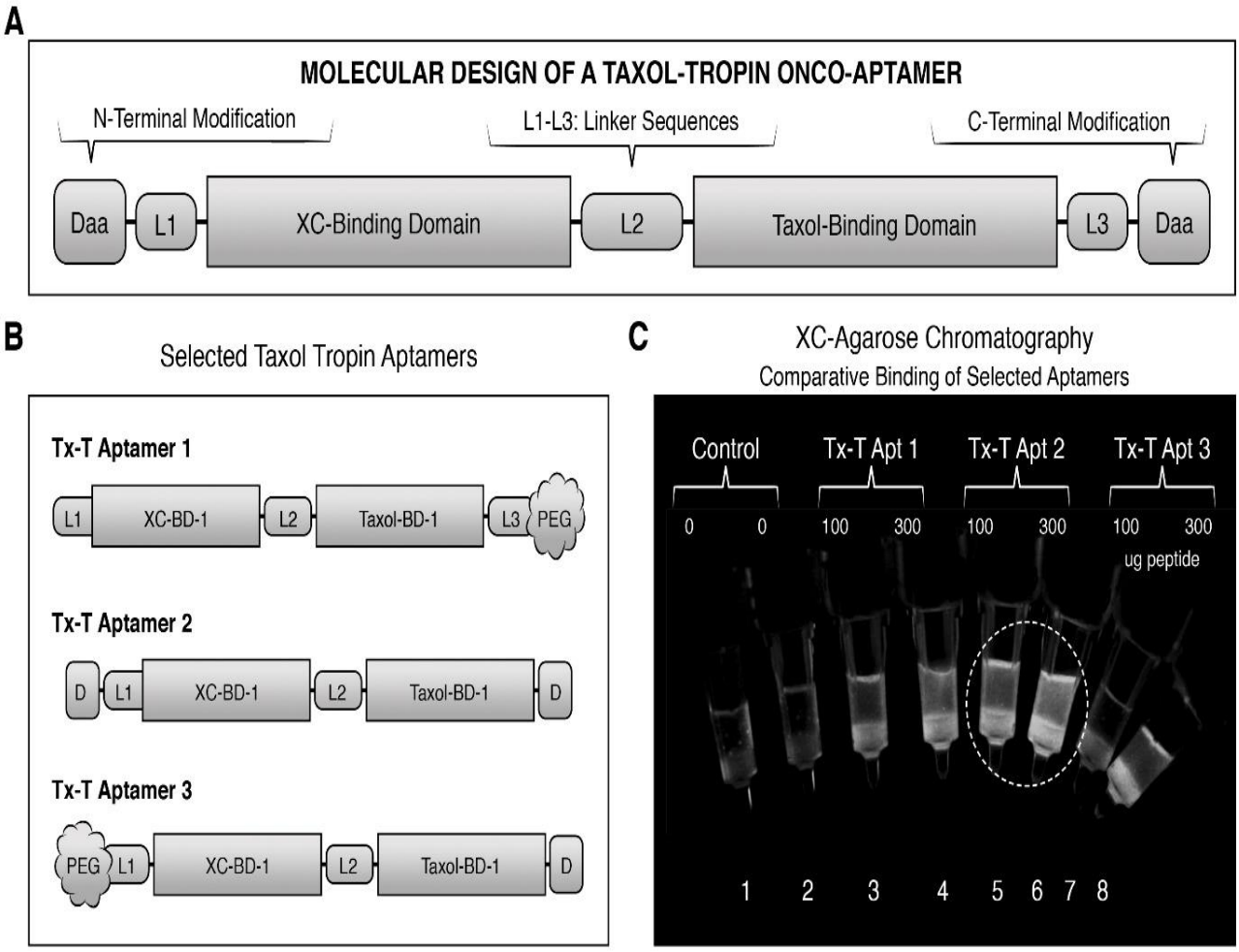

Figure 2: *Design engineering and selection of a Taxol-binding, XC-binding onco-aptamer. (A) Schematic diagrams of the structural features, including the binding domains, linker segments, and N-, C-terminal modifications. (B) Representative variations of polypeptides w/ or without pegylation (PEG). (C) Comparative collagen-agarose chromatography, using Taxol Green (fluorescent paclitaxel) as a reporter, identifies optimal bifunctional constructs with potential clinical utility in vivo.*

Using Taxol-Green (Flutax 2) as a fluorescent reporter for paclitaxel binding/delivery, the selectivity and efficiency of the optimized/selected Taxol-Tropin was further evaluated by differential column chromatography, wherein a layer of collagen-agarose was interposed between two layers of blank agarose beads (control) to serve as a molecular simulation of tumorous tissues vis-à-vis normal tissues *in vitro*. The results of these experiments represent a demonstration of feasibility: as described in Fig. 3B, Taxol Green, by itself, exhibits no affinity for either blank-agarose or collagen-agarose matrices under stringent washing conditions. In contrast, the selected Taxol-Tropin onco-aptamer (TxTApt 2) i) binds tightly to the fluorescent drug payload during a simple mixing step (note, discernable fluorescent quenching of the fluorophore observed in solution is indicative of tight binding affinity); and ii) binds tightly and selectively to the collagenous (XC-proteins) upon application of the PTX aptamer mixture to the three-layer matrices of the column. Mechanistically, the Taxol-Tropin binds tightly and selectively to the central layer of exposed collagens, thereby simulating *active* tumor-targeting functionality in the context of continuous systemic circulation (i.e., continuous column flow).

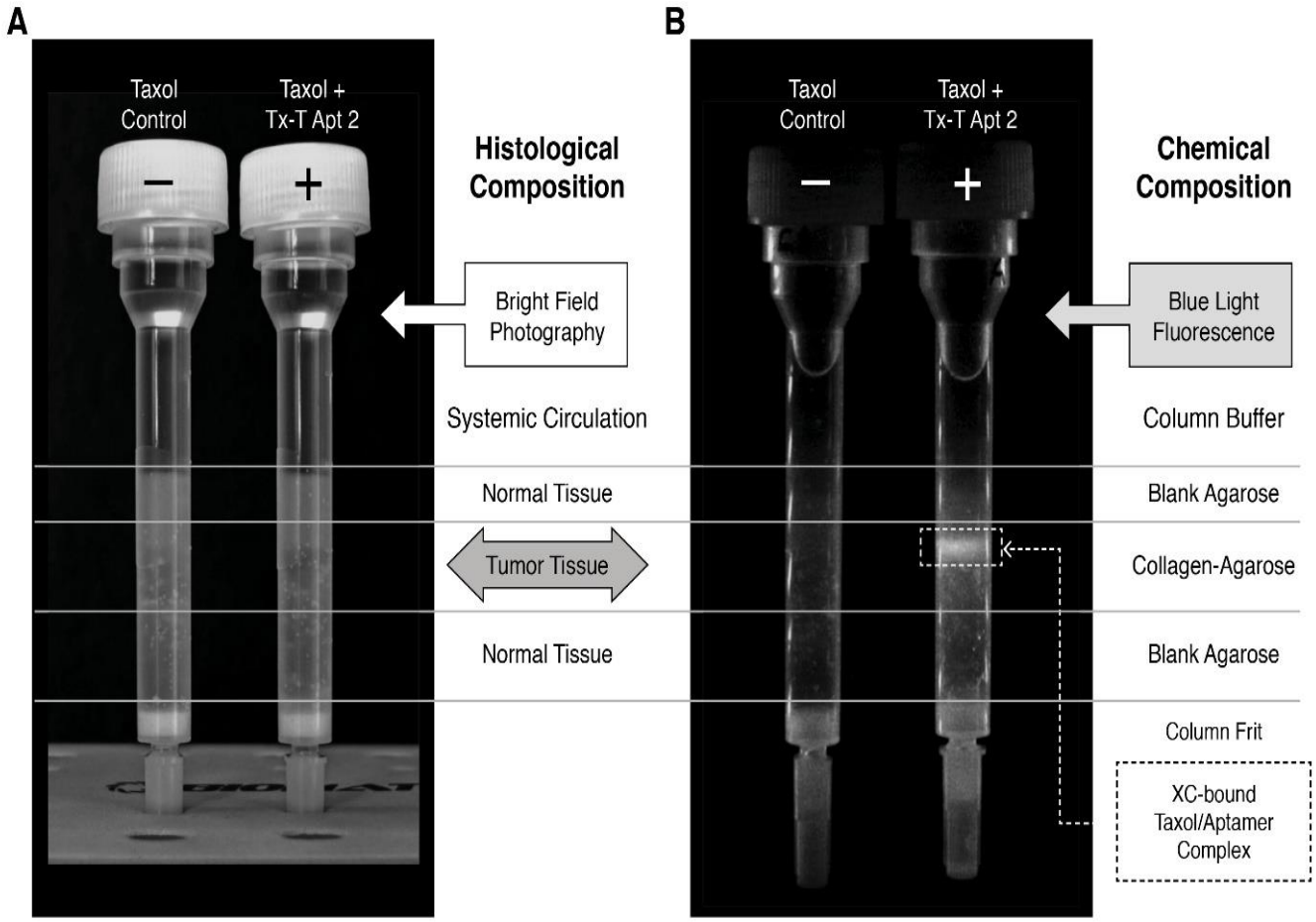

Figure 3: *Simulation of tumor-targeting in vitro with multi-layer column chromatography. In each of the columns, a layer of collagen-agarose (representing tumor tissue) is sandwiched between two layers of control agarose (normal tissues); Taxol-Green, the fluorescent paclitaxel reporter is applied, followed by stringent washing, which represents the flow and dilutions of blood in the systemic circulation. The paclitaxel reporter is mixed and then applied in the presence (+) or absence (-) of the Taxol-Tropin onco-aptamer.* **(A)** *Bright field photography.* **(B)** *Fluoresence imaging of the washed columns using a blue-light transilluminator. Note: Taxol-Green, by itself, passes through the chromatographic matrices without binding.*

XC-bound, onco-aptamer-tethered paclitaxel is biologically active as a cytotoxic mitotic inhibitor. To ascertain whether or not aptamer/XC-bound paclitaxel is rendered inactive by its physical association with either the high affinity taxol-binding aptamer and/or the XC proteins of the collagen agarose beads, column chromatography was performed with 50 ug Taxol Green in 10 ml PBS (5 ug/ml Taxol) in the presence or absence of the selected Taxol-Tropin Aptamer-2 (TxT-Apt2), using a 1.0 ug fluorescent paclitaxel spike to visually affirm PTX binding or non-binding to the collagen agarose column matrices, respectively. Fig. 4A shows binding of Taxol Green +Apt2 but not Taxol Green w/o Apt2 to collagen agarose beads. Both the washed collagen-agarose beads (Control Beads) and the Taxol Green + Apt2 aptamer/collagen-agarose beads (XC-Taxol beads) were harvested, re-suspended in D10 growth medium, distributed in separate wells of a 24-well cell culture plate in growth medium, and allowed to settle, followed by the addition (overlay) of human MDA-MB-231 breast cancer cells (Fig. 4B-E). As shown at both 24 and 48 hours after plating, the human breast cancer cells readily attached to the Control XC-agarose beads and began to proliferate uniformly, thereby increasing daily in number (Fig. 4B and D), whereas the cells plated onto the XC-Taxol beads (bound by Aptamer 2) exhibited overt signs of cytotoxicity within 24 hours, including mitotic arrest, apoptosis and necrosis (Fig. 4C), indicative of PTX bioactivity. By 48 hours, cell proliferation continued on the collagenous surfaces of the Control Beads (Fig. 4D); while only degenerative and necrotic remnants of cancer cells remained on the XC-Taxol beads (Fig 4E)—demonstrating that the PTX-bound to the selected onco-aptamer and localized to the exposed collagenous proteins of the XC-beads remains biologically active as an antimitotic drug.

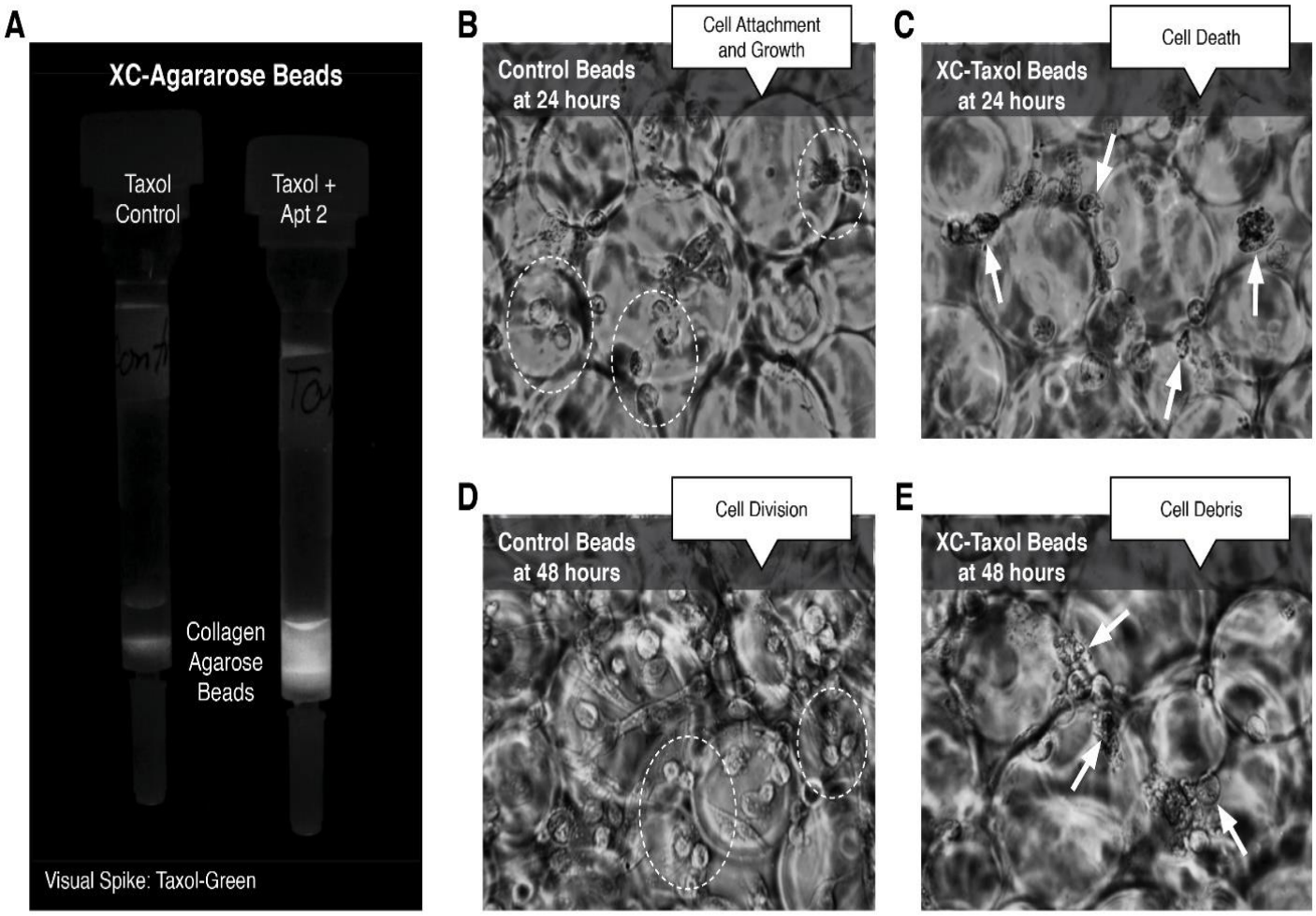

Figure 4: *Cytocidal bioactivity of Taxol (paclitaxel) is retained upon Taxol Green/aptamer binding to collagen-agarose beads. (A) Fluorescent imaging of collagen-agarose beads prepared with Taxol Green w/ Apt 2 (XC-Taxol) or w/o Apt2 (Control), followed by extensive washing, (a "spike" of Taxol Green was used to verify the washing and differential binding). The washed beads (Control or XC-Taxol beads) were then re-suspended in growth medium, added to cell culture plates, followed by an over-lay of MDA-MB-231 human breast cancer cells harvested in log phase growth. Control beads (large spheres) (B, D): demonstrating cell attachment, growth, and proliferation of at t = 24 hours; successful cell division (dashed ovals) and resulting cell multiplication is observable macroscopically at 48 hours. By contrast, (C, E): breast cancer cells plated onto the Taxol-charged XC-Aptamer beads (XC-Taxol) scarcely attached to the collagen-agarose beads (C), revealing an abundance of apoptotic, degenerating, and/or necrotic cells (arrows) at t = 24 hours; mostly dead, fragmenting, or degenerating cell debris are observed by t = 48 hours.*

Taxol Tropin Aptamer-dependent tumor-targeting in vivo in a murine model of metastatic cancer. To evaluate the potential for paclitaxel, which is bound non-covalently to the selected Taxol-Tropin onco-aptamer, to withstand the physical dilutions, filtration, turbulence, shear forces, and inactivating interactions encountered in the systemic circulation, while seeking out and accumulating in the exposed collagenous matrices of the tumor microenvironment, we employed the subcutaneous tumor xenograft model in nude mice (Fig. 5, upper left panel), using human MiaPaca-2 pancreatic cells and fluorescent paclitaxel (Taxol-Green) as the reporter. In the absence of the targeting onco-aptamer, thus solely dependent on the natural physiological binding to albumin and associated EPR-effects, there is little, if any, noticeable accumulations of fluorescent Taxol-Green within the tumors, beyond the faint background levels of auto-fluorescence (Fig. 5B). By contrast, the levels of Taxol-Green increased rapidly within the subcutaneous tumors, within 15 to 30 minutes (Fig. 5D), resulting in a brightness of fluorescence intensity (i.e., Taxol-Green) that appears to be orders of magnitude greater than that of the non-targeted fluorescent taxane applied intravenously at the same times and dosages.

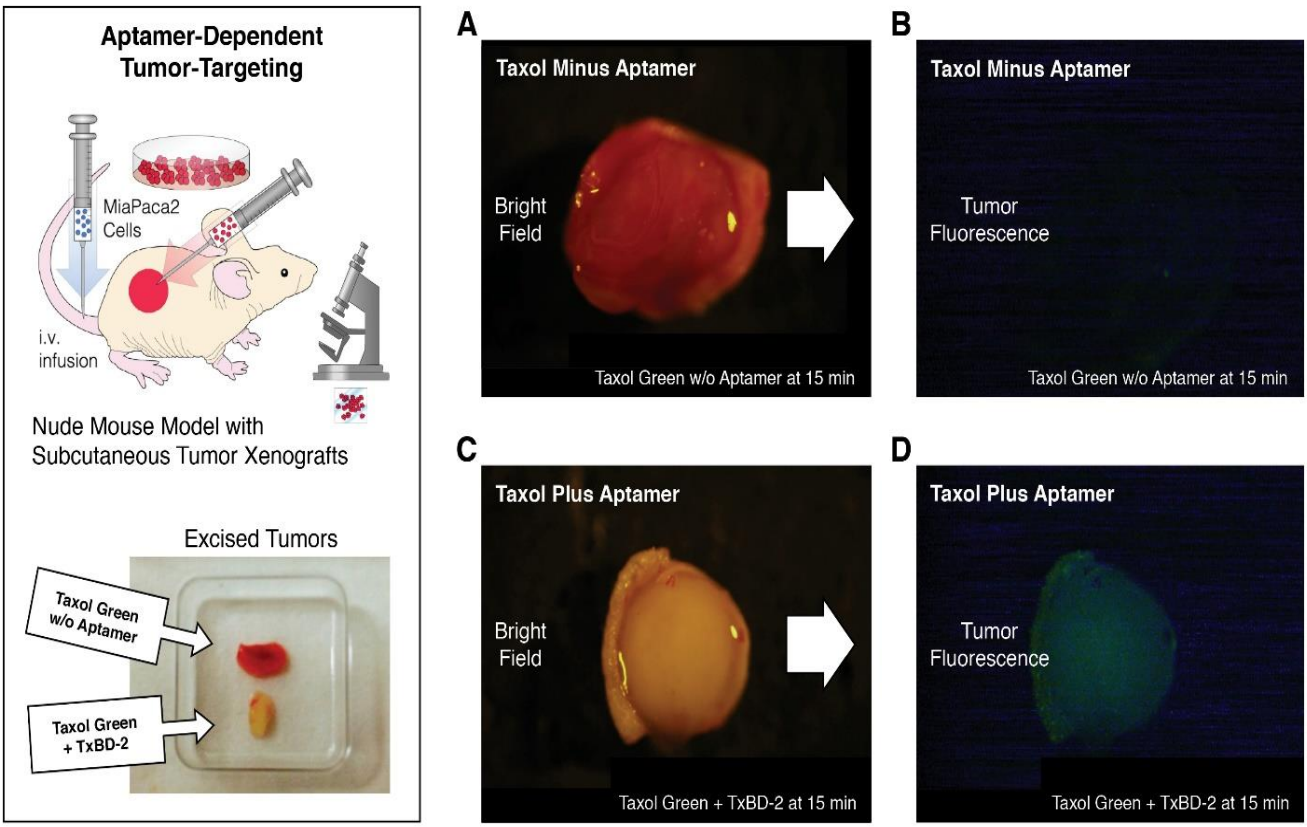

Figure 5: *Aptamer-dependent delivery of Taxol to tumors in a murine model of metastatic cancer. Left upper panel—Nude Mouse model of metastatic cancer: Following the establishment of subcutaneous tumor xenografts, Taxol-Green was administered alone (Taxol Minus Aptamer) or after simple mixing with an optimized Taxol-Tropin onco-aptamer (Taxol Plus Aptamer) via tail vein injections into tumor bearing mice; this initial treatment was followed 24 hours later (Day 2) by a second injection of Taxol-Green w/ or w/o TxBD-2 Aptamer, and analysis of the excised tumors (as a pilot study of potential low-dose efficacy). Lower Left panel: gross appearance of excised tumors of mice treated with Taxol-Green w/ or w/o Aptamer. Active tumor targeting vis-à-vis Passive tumor targeting (meager EPR effects) evaluated physiologically in vivo. Passive tumor targeting (Plates A, B: Taxol-Green Minus Aptamer): (A) bright field; (B), fluorescence imaging—note the absence of any fluorescent signal above background levels. Active targeting (Plates B, C: Taxol-Green Plus Aptamer): (C) bright field; (D, fluorescence—note the tumor fluorescence is largely dependent upon the Taxol-Tropin onco-aptamer.*

At 15 minutes post injection, fluorescent signals were detected transiently in the livers of both treatment groups (major site of PTX metabolism/excretion), but not at 30 or 60 minutes. No appreciable fluorescence was detected in the heart, muscle, bone, or colon of mice in either treatment group at any point in time as determined by comparative organ scans (not shown).

In an extended pilot study, an additional treatment group received a second infusion of Taxol-Green at T = 24 hours, with or without the targeting aptamer to evaluate potential histological indications of targeted efficacy. Remarkably, the excised tumors of the aptamer-targeted Taxol-Green treatment group versus the non-targeted treatment group appeared markedly different after only 2 days (Fig. 5, lower left panel). The tumors from the non-targeted treatment group appeared bright red in color, as was expected with the robust vascularization normally seen in these pancreatic cancer xenografts. In contrast, the excised tumors of the XC-/aptamer-targeted Taxol-Green treatment group appeared pale by comparison, indicative of a rapid and profound anti-angiogenic effect which is known to be associated with low-dose treatment with taxanes in the clinic (39). What makes this observation particularly remarkable (and largely unexpected for a mere marker study) was the finding that this striking anti-angiogenic effect was produced at an administered dose of 0.2 mg/kg of Aptamer-targeted paclitaxel, which is ~1/50th the standard effective dose commonly used in humans (10 mg/kg), and is ~1/10th the ultra-low "metronomic" doses of paclitaxel (2 mg/kg) that is commonly used to enhance the efficacy of anti-angiogenic chemotherapy in humans [40,41].

The implications of this macroscopic observation—*possible ultra-low-dose anti-angiogenesis* – prompted a closer histological examination of the excised tumors from these two pilot treatment groups; consequently, the excised tumors were fixed and embedded for histological examination: confirming that the low circulating dose levels of PTX (Taxol-Green) in the non-targeted treatment group had no observable effects on the exuberant angiogenesis of the pancreatic cancer xenografts, as these sections appeared to be indistinguishable from those of untreated controls: that is, flagrant tumors from non-treated mice. By contrast, there appeared to be a marked reduction in the focal zones and extent of vascularization within the tumors obtained from the XC-Aptamer-targeted treatment group at T=48 hours. While this first glimpse of possible ultra-low-dose anti-angiogenic activity is promising, along with the apparent orders of magnitude gained by *actively targeting* a bolus of PTX to cancerous ECM; it is also readily apparent that more advanced, confirmatory, quantitative, pharmacological studies of XC-targeted Taxol-Tropins are now needed for the expedient refinement and development of this precision PTX targeting biotechnology, in terms of prospective *Investigational New Drugs* (INDs

Discussion

The chemotherapeutic taxanes, paclitaxel *(Taxol)* and docetaxel *(Taxotere)* are highly effective antineoplastic agents that are used in the treatment of a wide variety of cancers. *Paclitaxel* and *Taxotere* were first approved in 1992 and 1996, respectively, and both of these taxanes are currently available as generic drugs. Exhibiting both cytotoxic and anti-angiogenic properties [39-41], paclitaxel in particular—delivered as either Taxol or albumin-bound nab-paclitaxel (*Abraxane*)—exhibits broad spectrum anti-cancer activity against ovarian, breast, lung, pancreas, head and neck cancers, Kaposi's sarcoma, and urologic malignancies [1-3]. Despite the initial enthusiasm for this improved nab-paclitaxel formulation, there still remains the systemic challenge of passive versus active delivery, largely ungoverned biodistribution, dose- and treatment-limiting toxicities, and acquired drug resistance, which encourages a clinical and mechanistic re-evaluation of current drug delivery systems [7-9]. Indeed, the recent failure of nab-paclitaxel to demonstrate any superiority over standard paclitaxel (*Taxol*) in a large-scale randomized Phase III trial for locally recurrent or metastatic breast cancer is both informative and cautionary [15]: given that the higher 'equitoxic doses' of the nab-paclitaxel formulation were associated with increased overall toxicity, a trend toward inferior effectiveness, and reduced palliation of disease-related symptoms, even though earlier phase II reports had suggested that nab-paclitaxel might be more efficacious than standard paclitaxel. Apparently, the higher 'equitoxic doses' of the nab-paclitaxel (*Abraxane*) used in the Phase III trial resulted in early discontinuation and dose reductions, which limited therapeutic exposure to the drug [15].

Physiologically, targeted drug delivery (or drug targeting) to tumors is divided into "passive" and "active" modalities. Albumin formulations like nab-paclitaxel accumulate passively in tumors with leaky vasculature: a discernable yet variable property of tumors referred to as the Enhanced Permeation and Retention (EPR) effect [7]. However, >95% of the administered nanoparticles are known to accumulate in non-target organs, including the liver, spleen, and lungs [8], which can hardly be considered either selective or efficient in terms of actual tumor targeting [9]. Accordingly, prior speculative claims of the active-targeting of nab-paclitaxel to tumors based on the expression of albumin-binding SPARC proteins [16] have been negated [17,18], thus affirming that the biodistribution of nab-paclitaxel to the tumor compartment is largely, if not entirely, dose-dependent [19,20]. By contrast, "active" (XC) tumor targeting described herein refers to high-affinity interactions between the drug carrier and a characteristic histopathology of the tumor microenvironment, which far exceeds the meager efficiencies of passive EPR effects and leads to significantly higher levels of accumulation at lower, rather than higher, injected drug doses [27-29]. The comparative performance of active versus passive (EPR-dependent) targeting of fluorescent paclitaxel upon systemic (tail vein) injection is clearly seen in Fig. 5, where it is found that the rapid accumulation of the Taxol-green in the tumor xenografts is dependent on the paclitaxel-binding (XC) tumor-targeting onco-aptamer (Fig. 5). Likewise, recent preclinical studies using fluorescent IgGs as a biomarker (and prospective drug cargo) have demonstrated little if any discernable EPR-mediated accumulation of IgGs in tumor xenografts in the absence of the IgG-binding (XC) tumor-targeting onco-aptamer, which served to provide active and efficient drug delivery: i.e., tumor-targeting of the marker IgGs [42].

In conclusion, given that Taxol is widely used and presently available as a generic and relatively inexpensive cancer drug, the development of a tumor-targeting Taxol-Tropin that is capable of favorably shifting the Therapeutic Index of PTX to one of greater clinical efficacy at lower drug doses—thereby reducing the confounding issues of systemic toxicity—represents a meaningful advance in terms of cancer drug delivery. In accordance with the modern concepts of metronomic anti-cancer therapy, where continuous lower doses of PTX over extended periods of time serve to provide better cancer control (43,44), the observed increase in PTX "delivery" to the tumor compartment *per se*—that is, the orders of magnitude gained in overall efficiency—by XC-targeting PTX pro-actively to the tumor compartments (see Fig 5C,D)—provides a new platform for ultra-low-dose metronomic protocols, which might well be conducted at PTX doses that are below the thresholds for systemic side effects. Indeed, the first glimpse of putative aptamer-dependent low-dose anti-angiogenic activity reported herein as a pilot study, in this initial report, represents the challenging first steps in the expedient translation of this applied/advanced molecular engineering from the bench to the bedside. Formal quantitative studies (pharmacokinetic and pharmacodynamic studies) are planned to characterize the performance, as well as the pharmaceutical safety and efficacy, of clinically-optimized Taxol-Tropins, with the anticipation that prospective clinical studies assessing active XC-targeted delivery of PTX will be forthcoming. Meanwhile, this purposeful demonstration of active and selective tumor-targeting, with yet another class of anticancer agents (small molecules, e.g., PTX), serves as an exemplar for a myriad of creative *pathotropically*-targeted onco-aptamers, realizing that delivery of potent cytotoxic drug payloads more actively and selectively to the pathologic TME remains a worthy and achievable goal of precision medicine [45].

Acknowledgements

The authors are also grateful to Heather C. Gordon, Art Consultant for Counterpoint Biomedica LLC, for graphic illustrations and assistance in manuscript writing.

Funding

Funded by Counterpoint Biomedica LLC

Availability of Data:

The authors understand that the materials included in the manuscript, including all relevant raw data, will be made freely available to any researchers who wish to use this for non-commercial purposes, while preserving any necessary confidentiality and anonymity.

Authors' Contributions and Approval

EMG conceived and designed the experiments, supervised the conduct of the preclinical studies, analyzed the results, reviewed the published literature, wrote the manuscript, reviewed and edited the final manuscript. SL reviewed the published literature, wrote sections of the manuscript and the reference section, and edited the final manuscript. SPC reviewed the published literature, analyzed the results, reviewed and edited the final manuscript. FLH designed the Taxol-Tropin oncoaptamers, helped design the experiments, co-supervised the conduct of the preclinical studies, analyzed the results, reviewed the published literature, wrote sections of the manuscript, reviewed and edited the final manuscript. All authors approved the submission of this manuscript for publication.

Ethics Approval and Consent to Participate: Not Applicable

Consent for Publication: Not Applicable

Disclosure of Competing Interests

Drs. Gordon, Chawla and Hall are co-founders of Counterpoint Biomedica LLC. Mr. Liu has no competing interest.

References

1. Rowinsky EK: (1997) The development and clinical utility of the taxane class of antimicrotubule chemotherapy agents. Ann Rev Med 48: 353-374.

2. Priyadarshini K and Keerthi Aparajitha U: (2012) Paclitaxel against cancer: A short review. Med Chem 2: 139-141.
3. Weaver B: (2014) How Taxol/paclitaxel kills cancer cells. Mol Biol Cell 25: 2677-2681.
4. Rodi DJ, Janes RW, Sanganee HJ, Holton RA, Wallace BA and Makowski L: (1999) Screening of a library of phage-displayed Peptides identifies human Bcl-2 as a Taxol-binding protein. J Mol Biol 285: 197-203.
5. Aoki S, Morohashi K, Sunoki T, Kuramochi K, Kobayashi S and Sugawara F: (2007) Screening of paclitaxel-binding molecules from a library of random peptides displayed on T7 phage particles using paclitaxel-photoimmobilized resin. Bioconjugate Chem 18: 1981-1986.
6. Khanna C, Rosenberg M and Vail DM: (2015) A review of paclitaxel and novel formulations including those suitable for use in dogs. J Vet Intern Med 29: 1006-1012.
7. Ma P and Mumper RJ: (2013) Paclitaxel Nano-Delivery Systems: A Comprehensive Review. J Nanomed Nanotechnol 4: 1-35.
8. Baea YH and Park K: (2011) Targeted drug delivery to tumors: myths, reality and possibility. J Control Release 153: 198-205.
9. Kwon IK, Lee SC, Han B and Park K: (2012) Analysis on the current status of targeted drug delivery to tumors. J Control Release 164: 1-17.
10. Desai N, Trieu V,Yao Z, Louie L, Ci S, Yang A, Tao C, De T, Beals B, Dykes D, *et al*: (2006) Increased Antitumor Activity, Intratumor Paclitaxel Concentrations, and Endothelial Cell Transport of Cremophor-Free, Albumin-Bound Paclitaxel, ABI-007, Compared with Cremophor-Based Paclitaxel. Clin Cancer Res 12: 1317-1324.
11. Miele E, Spinelli GP, Miele E, Tomao F and Tomao S: (2009) Albumin-bound formulation of paclitaxel (Abraxane® ABI-007) in the treatment of breast cancer. Intl J Nanomedicine 4: 99-105.
12. Hennenfent KL and Govindan R: (2006) Novel formulations of taxanes: a review. Old wine in a new bottle? Annals of Oncology 17: 735-749.
13. Yardley D: **(**2013) Nab-Paclitaxel mechanisms of action and delivery. J Control Release 170: 365-372.
14. Scripture CD, Figg WD and Sparreboom A: (2005) Paclitaxel chemotherapy: from empiricism to a mechanism-based formulation strategy. Therapeutics and Clinical Risk Management 1: 107-114.
15. Rugo HS, Barry WT, Moreno-Aspitia A, Lyss AP, Cirrincione C, Leung E, Mayer EL, Naughton M, Toppmeyer D, Carey LA, *et al*: (2015) Randomized phase III trial of paclitaxel once per week compared with nanoparticle albumin-bound nab-paclitaxel once per week or ixabepilone with bevacizumab as first-line chemotherapy for locally recurrent or metastatic breast cancer: CALGB 40502/NCCTG N063H (Alliance*).* J Clin Oncol 33: 2361-2369.
16. Desai N, Trieu V, Damascelli B and Soon-Shiong P: (2009) SPARC expression correlates with tumor response to albumin-bound paclitaxel in head and neck cancer patients. Translational Onclogy 2: 59-64.
17. Schneeweiss A, Seitz J, Smetanay K, Schuetz F, Jaeger D, Bachinger A, Zorn M, Sinn HP and Marmé F: (2014) Efficacy of nab-paclitaxel does not seem to be associated with SPARC expression in metastatic breast cancer. Anticancer Res 34: 6609-6615.
18. Hidalgo M, Plaza C, Musteanu M, Illei P, Brachmann C, Heise C, Pierce D, Lopez-Casas P, Menendez C, Tabernero J, *et al*: (2015) SPARC expression did not predict efficacy of nab-paclitaxel plus gemcitabine or gemcitabine alone for metastatic pancreatic cancer in an exploratory analysis of the phase III MPACT trial. Clin Cancer Res; 21:4811-4818.
19. Neesse A, Frese KK, Chan DS, Bapiro TE, Howat WJ, Richards FM, Ellenrieder V, Jodrell DI and Tuveson DA: (2014) SPARC independent drug delivery and antitumour effects of nab-paclitaxel in genetically engineered mice. Gut 63: 974-983.
20. Kim H, Samuel S, Lopez-Casas P, Grizzle W, Hidalgo M, Kovar J, Oelschlager D, Zinn K, Warram J and Buchsbaum D: (2016) SPARC-Independent Delivery of Nab-Paclitaxel without Depleting Tumor Stroma in Patient-Derived Pancreatic Cancer Xenografts. Mol Cancer Ther 15: 680-688.
21. Ruoslahti E, Bhatia SN and Sailor MJ: (2010) Targeting of drugs and nanoparticles to tumors. J Cell Biol 188: 759-768.
22. Ruoslahti E: **(**2012) Peptides as targeting elements and tissue penetration devices for nanoparticles. Adv Mater 24: 3747-3756.
23. Danhier F, Feron O and Préat V: (2010) To exploit the tumor microenvironment: Passive and active tumor targeting of nanocarriers for anti-cancer drug delivery. J of Controlled Release 148: 135–146.
24. Bertrand N, Wu J, Xu X, Kamaly N and Farokhzad OC: (2014) Cancer nanotechnology: The impact of passive and active targeting in the era of modern cancer biology. Adv Drug Deliv Rev 66: 2-25. Note Rex-G referenced
25. Karmali PP, Kotamraju VR, Kastantin M, Black M, Missirlis D, Tirrell M and Ruoslahti E: (2009) Targeting of albumin-embedded paclitaxel nanoparticles to tumors. Nanomed 5: 73-82.
26. Sugahara KN, Teesalu T, Karmali PP, Kotamraju KR, Agemy L, Girard OM, Hanahan D, Mattrey RF and Ruoslahti E: (2009) Tissue-penetrating delivery of compounds and nanoparticles into tumors. Cancer Cell 16, 510-520.
27. Waehler R, Russell SJ and Curiel DT: (2007) Engineering targeted viral vectors for gene therapy. Nat Rev Genet 8: 573-587.
28. Gordon EM and Hall FL: (2010) Rexin-G, a targeted genetic medicine for cancer. Expert Opin Biol Ther 10: 819-832.
29. Hall FL, Levy JP, Reed RA, Petchpud WN, Chua VS, Chawla SP and Gordon EM: (2010) Pathotropic targeting advances clinical oncology: Tumor-targeted localization of therapeutic gene delivery. Oncology Reports 24: 829-833.
30. Tuan T, Cheung DT, Wu LT, Yee A, Gabriel S, Han B, Morton L, Nimni M and Hall FL: (1996) Engineering, expression and renaturation of targeted TGF-beta fusion proteins. Connect Tissue Res 34: 1-9.
31. Hall FL, Kaiser A, Liu L, Chen ZH, Hu J, Nimni ME, Beardt RW and Gordon EM: (2000) Design, expression, and renaturation of a lesion-targeted recombinent epidarmal growth factor—von Willebrand factor fusion protein: Efficacy in an animal model of experimental colitis. Int J Exp Med 6: 635-643.
32. Hall FL, Liu LQ, Zhu NL, Stapfer M, Anderson WF, Beart RW and Gordon EM: (2000) Molecular engineering of matrix-targeted retroviral vectors incorporating a surveillance function inherent in von Willebrand factor. Hum Gene Ther 11: 983-993.
33. Gordon EM, Liu PX, Chen ZH, Liu L, Whitley MD, Gee C, Groshen S, Hinton DR, Beart RW and Hall FL: (2000) Inhibition of metastatic tumor growth in nude mice by portal vein infusions of matrix-targeted retroviral vectors bearing a cytocidal cyclin G1 construct. Cancer Res 60: 3343-3347.
34. Zasadil LM, Andersen KA, Yeum D, Rocque GB, Wilke LG, Tevaarwerk AJ, Raines RT, Burkard ME and Weaver BA: (2014) Cytotoxicity of paclitaxel in breast cancer is due to chromosome missegregation on mulitpoloar spindles. Sci Transl Med 6: 1-24.
35. Liebmann JE, Cook JA, Lipschultz C, Teaque D, Fisher J and Mitchell JB: (1993) Cytotoxic studies of paclitaxel (Taxol) in human tumor cell lines. Br J Cancer 68: 1104-1109.
36. Rao S, He L, Chakravarty S, Ojima I, Orr G and Horwitz S: (1999) Characterization of the Taxol Binding Site on the Microtubule: Identification of Arg^{282} in B-tubulin as the site of photo-

incorporation of a 7-benzophenone analogue of taxol. J Biol Chem 274: 37990-37994.
37. Rao S, Orr GA, Chaudhary AG, Kingston D and Horwitz S: (1995) Characterization of the taxol binding site on the microtubule: 2-(m-Azidobenzolyl) taxol photolabels a peptide (amino acids 217-231) of B-Tubulin. J Biol Chem 270: 20235-20238.
38. Tugyi R, Uray K, Ivan D, Fellinger E, Perkins A and Hudecz F: (2005) Partial D-amino acid substitution: Improved enzymatic stability and preserved Ab recognition of a MUC2 epitope peptide. PNAS 102: 413-418.
39. Bocci G, Di Paolo A, Danesi R: (2013) The pharmacological bases of the antiangiogenic activity of paclitaxel. Angiogenesis 16: 481-492.
40. Chen CA, Ho CM, Chang MC, Sun WZ, Chen YL, Chiang YC, Syu MH, Hsieh CY and Cheng WF: (2010) Metronomic chemotherapy enhances antitumor effects of cancer vaccine by depleting regulatory T lymphocytes and inhibiting tumor angiogenesis. Molecular Therapy 18: 1233-1243.
41. Kamat AA, Kim TJ, Landen Jr CN, Lu C, Han LY, Lin YG, Merritt WM, Thaker PH, Gershenson DM, Bischoff FZ, *et al*: (2007) Metronomic chemotherapy enhances the efficacy of antivascular therapy in ovarian cancer. Cancer Res 2007; 67: 281-288.
42. Hall FL, Chawla SP, Chawla NS and Gordon EM: (2016) Targeting monoclonal antibodies to the tumor microenvironment for cancer therapy/immunotherapy. J Immunol Serum Biol 2:1-7.
43. Pasquier E, Kavallaris M and André N: (2010) Metronomic chemotherapy: new rationale for new directions. Nat Rev Clin Oncol 7:455-465.
44. Romiti A, Cox MC, Sarcina I, Di Rocco R, D'Antonio C, Barucca V and Marchetti P: (2013) Metronomic chemotherapy for cancer treatment: a decade of clinical studies. Cancer Chemother Pharmacol 72:13-33.
45. André N, Carré M and Pasquier E: (2014) Metronomics: towards personalized chemotherapy? Nat Rev Clin Oncol 11:413-31.

DOI: 10.31579/2640-1053/089