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TumorSelect[®] Technology

Enhancing the Safety and Efficacy of Cancer Chemotherapy

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1. Abstract

Veiled Therapeutics has developed an anticancer technology, TumorSelect[®] Technology, which combines proprietary anticancer prodrugs and nanotechnology, which takes advantage from current knowledge of human physiology. Tumors have a voracious appetite for cholesterol which facilitates tumor growth and fuels their proliferation. We have transformed this need into a stealth delivery system to disguise and deliver anticancer drugs with the assistance of both the human body and the tumor cell. Veiled's designer prodrugs are assembled within pseudo-LDL nanoparticulates which carry them to tumor tissues where they are taken up, internalized and transformed into active drug and kill the cancer cells. This three-prong approach delivers the anticancer drug selectively to the tumors and thereby avoids or reduces the severe side effect toxicities associated with current chemotherapy. Reduction of side effect toxicity of cancer therapy by our technology will improve patient quality of life, patient retention in treatment regimes, more rapid patient recovery post treatment, and overall patient benefit.

A. Background

The costs of cancer, measured in terms of mortality, morbidity, direct costs of treatment, and costs of lost productivity are high.

B. Methods

ART-207 was synthesized; a pseudo-LDL lipid nanodispersion was formed; and mouse xenograft studies were performed.

C. Results

Preclinical toxicity, efficacy, and distribution data clearly show significant advantages of TumorSelect[®] paclitaxel over conventional Cremophor[®] formulations of paclitaxel. These advantages include:

- Increased suppression of tumor growth and regrowth
- Lower toxicity
- Increased survival
- Higher number of tumor free animals
- Significantly lower concentrations of paclitaxel in non-target tissues
- Significantly higher concentrations of paclitaxel in tumor tissue

Thus, data obtained demonstrated targeted drug delivery and support LDL-receptor dependent mechanism of selective cellular uptake by tumor tissue of TumorSelect[®] formulated paclitaxel.

D. Conclusions

Non-target tissue concentrations of paclitaxel are significantly lower in non-tumored and tumored mice injected with formulated TumorSelect[®] paclitaxel compared with the mice injected with Cremophor[®] EL/EtOH (ethanol) paclitaxel (<20%).

Tumor concentrations of paclitaxel are significantly higher in tumors of mice injected with formulated TumorSelect[®] paclitaxel compared with the mice injected with Cremophor[®] EL/EtOH paclitaxel (194%).

Plasma and heart concentrations of paclitaxel are significantly lower in tumored vs. non-tumored animals injected with formulated TumorSelect[®] paclitaxel (<80%).

Selective cellular uptake of TumorSelect[®] paclitaxel by tumors actively expressing LDL-receptors has been demonstrated.

Tumor suppression observed was sustained for 63 days after Q1Dx5 dosing with TumorSelect[®] paclitaxel.

TumorSelect[®] technology represents a potential major improvement in the clinical treatment of cancer through enhanced efficacy due to tumor-facilitated targeted delivery and reduced patient toxicity with its associated deleterious side effects.

2. Keywords

Nanoparticles; pseudo-LDL; tumor targeting; prodrugs;

3. Background

A. Costs of cancer

Despite the availability of diverse pharmacological¹ (as well as radiotherapeutic,² and surgical³) cancer treatments, cancer remains a heterogeneous disease with many unmet needs and is associated with very significant burdens measured in terms of morbidity, mortality, direct costs of care, and indirect costs of productivity losses. In its 2020 Report

on Cancer, the World Health Organization (WHO) reports that globally, in 2018, 9.6 million people are estimated to have died of cancer and that cancer was the cause of one in six deaths.⁴ In its 2021 Cancer Facts and Figures Report, The American Cancer Society (ACS) estimates that the United States will see nearly 2,000,000 million new cases of cancer in 2021 and over 600 thousand deaths due to cancer.⁵ In an assessment of US costs of cancer care, based on US National Cancer Institute (NCI) data, the US Centers for Disease Control and Prevention (CDC) estimates US costs of cancer care in 2020 to be \$174 billion.⁶ While a precise estimate of 2020 indirect costs of cancer in the US could not be found, it is useful to note that Jönsson, in 2019 stated, “indirect costs [in the European Union] related to the loss of production for persons with cancer are estimated to be of the same magnitude as the direct healthcare expenditures.”⁷ If Jönsson’s estimate of equivalence is applicable, it would suggest that in the US in 2020 the combination of the direct costs of cancer care and the costs of productivity lost to cancer morbidity and mortality approached \$350 billion.

B. Recent development

The enormous suffering and monetary costs of cancer are motivating significant progress in the understanding of cancer biology and the discovery, development, and implementation of new diagnostic tools, new drug therapies, and other new therapeutic modalities. In a recent analysis of the advancements in cancer therapy in the five years ending mid-2019,⁸ the characteristics of this progress are elaborated as: better understandings of tumor biology and of the mechanisms of action of cancer therapies; and the increased use of precision medicine. Advancements in cancer biology have led to a broad array of genomic^{9, 10, 11} and functional diagnostics^{12,13,14,15} that characterize a patient’s susceptibility to a particular cancer and a cancer’s susceptibility to a particular treatment (the bases of precision

1 medicine^{16,17}).

2 **C. Unmet needs**

3 Despite the significant progress that has been made in the development of cancer
4 therapeutics there are still very significant unmet needs. The unmet needs have been
5 enumerated: rare or unclassifiable tumors (no standard of care available), intractable tumor
6 types (standard of care ineffective), intractable tumor subsets (no targeted treatments),
7 intractable tumors (unexpectedly poor response to standard of care), and unfavorable benefit
8 /risk ratio (high sensitivity to treatment toxicity).¹⁸

9 **D. Cytotoxic chemotherapy**

10 Cytotoxic cancer chemotherapeutics^{19,20,21} were the first drugs used to treat cancer²² and
11 despite the development and approval^{23,24,25} of other classes of cancer chemotherapeutics
12 including immunotherapeutics,^{26,27} molecularly targeted therapeutics,²⁸ and antihormone
13 agents,²⁹ cytotoxics are very widely used. They are toxic to the rapidly dividing cells typical
14 of tumor tissues but are not highly selective and cause dose-limiting toxicities to rapidly
15 dividing normal tissues such as those of the bone marrow and the gastrointestinal tract.³⁰
16 Cytotoxics are typically dosed at previously determined drug-specific maximum tolerated
17 doses (MTD)³¹ so, by definition, their therapeutic indices (TI)³² are very low³³ (slightly
18 greater than 1), whereas most non-oncology therapeutics have TI's of much greater than 10.

19 Recently developed (and approved for clinical use) biologic programmed death
20 1/programmed death ligand 1 (PD-1/PD-L1) checkpoint-blockade cancer immunotherapy
21 agents^{34,35} hold great promise but are only effective in a low percentage of patients.^{36,37,38}

Recent studies have shown greater efficacy of these agents when co-dosed with cytotoxic agents.^{39,40,41,42}

E. TumorSelect® technology

The unmet needs outlined above might be addressed by a strategy that could deliver cytotoxic chemotherapeutics more selectively to tumors thereby shielding rapidly dividing normal systemic tissues from the ravaging side effects of the cytotoxic agents. Hundreds of thousands of cancer patients are treated each year with cytotoxic chemotherapeutic agents. A principal goal of chemotherapy drug development is to find strategies to make chemotherapeutics more selective and less toxic to normal tissues.

It was recognized decades ago that cancer patients (especially those with advanced disease) presented clinically with plasma lipid profiles significantly reduced in LDL concentration.⁴³ This observation suggests a strategy to deliver drug to tumors taking advantage of their high uptake of lipids to support their rapid and continuous cell division and growth.

Tumors are voracious consumers of cholesterol and triglycerides both as an energy source and as substrates for membrane production of rapidly dividing cells. As a result, tumors dramatically overexpress LDL receptors by as much as eight-fold relative to healthy tissues.⁴⁴ Incorporating chemotherapeutics into pseudo-LDL nanoparticles for delivery via overexpressed LDL receptors on tumor cells provides the opportunity for selective delivery of lipophilic chemotherapeutics to tumors compared with normal tissues. Pseudo-LDL particles can increase the utility of clinically approved chemotherapeutic agents by altering their PK/PD properties and dramatically raising their therapeutic indices.

1 TumorSelect[®] is a delivery system designed for tumor-targeting delivery of drugs thereby
2 overcoming the low therapeutic index limitation. TumorSelect[®] includes a prodrug of
3 sufficient lipophilicity to be entrapped in a lipid nanoparticle of composition similar to that
4 of the lipid core of human LDL particles. Previous work by Maranhão and coworkers has
5 demonstrated that after injection such a pseudo-LDL nanoparticle lipid nanoparticle adsorbs
6 a molecule of exchangeable apo-lipoprotein, the ligand which mediates internalization of the
7 particle by the LDL receptors (LDLR) that are overexpressed by rapidly dividing tumor
8 cells.^{45,46,47} This biological phenomenon is the rationale for TumorSelect[®] chemotherapeutic
9 delivery technology.

10 As a proof of concept of the technology, paclitaxel, a very widely clinically utilized
11 cytotoxic chemotherapeutic, was selected for evaluation for selective delivery via
12 TumorSelect[®] delivery.

13 The work described in this manuscript characterizes the preclinical performance of
14 TumorSelect[®] technology applied to the delivery of paclitaxel to demonstrate selective
15 delivery to tumors and thereby enhance its efficacy and reduce its toxicity.

16 Four questions were addressed:

- 17 1. Can a pseudo-LDL nanoparticle be created which is sufficiently similar to natural
18 human LDL particles to be physiologically recognizable and stable enough to serve
19 as a delivery vehicle for a cytotoxic payload?
- 20 2. Can a suitable paclitaxel derivative be prepared to be efficiently incorporated into the
21 delivery formulation?

1 3. Does the proposed TumorSelect[®] construct of cytotoxic derivative and delivery
2 vehicle improve TI of the cytotoxic and still retain efficacy?

3 4. Does the TumorSelect[®] technology selectively deliver cytotoxic to tumor tissue as
4 proposed?

5 Paclitaxel was chosen as the model for these development efforts as it is active in treatment
6 of numerous solid tumor types including: breast, ovarian, lung, esophageal, pancreatic,
7 prostate, melanoma, bladder, Kaposi's sarcoma, and other types of solid tumors. Current
8 paclitaxel therapy is limited by toxicity and resistance development. Commonly seen
9 toxicities include: leukopenia and neutropenia; nausea, vomiting, and diarrhea; hair loss;
10 peripheral neuropathy; hypersensitivity reactions; mouth sores; and arthralgias and
11 myalgias. Clinical utility of current paclitaxel therapy is limited by development of
12 resistance on the part of the tumors by expression of paclitaxel efflux by ABC transporters
13 (P-glycoprotein (Pgp), breast cancer resistance protein (BCRP), multi-drug resistance related
14 protein (MRP-1)), expression of mutated β 1 tubulin and aberrant tubulin isoforms, and
15 enhanced expression of cell survival mechanisms.⁴⁸

16 In order to incorporate the current clinically approved chemotherapeutic into the pseudo-
17 LDL particle, the hydrophilic chemotherapeutic was converted to a lipophilic acid-labile
18 prodrug which is capable of being retained within the pseudo-LDL particle until the particle
19 is internalized into the tumor cells by uptake by the LDL receptor and processed by the
20 endosome/lysosome cascade, ultimately releasing the active chemotherapeutic drug moiety.
21 This strategy is directly applicable to the majority of clinically approved and widely utilized
22 chemotherapeutics.

4. Materials and methods

To address question one – preparation of a pseudo-LDL delivery formulation vehicle we undertook efforts to prepare such a material via the technique of microfluidization.

A. Pseudo-LDL nanoparticle dispersion preparation

The TumorSelect[®] lipid nanoparticle formulation was prepared through extensive development efforts as previously described.⁴⁹

i. Homogenization

Quantities of the components which make up the nanoparticles (dispersed in 0.9% NaCl in 10 mM Acetate buffer, pH 5.5) of TumorSelect[®] paclitaxel are shown in Table 1 below.

1

Table 1 Composition of TumorSelect[®] paclitaxel nanoparticles

Ingredients	mg/mL in dispersion	%w/w in solids	Notes
Egg Yolk Phosphatidylcholine (PC) Lipoid, GMBH	42.5	59.1	Principal surfactant; mixture of fatty-acid diesters of glycerophosphocholine similar to that found in human LDL
Dimyristoylphosphocholine (DMPC) Avanti Polar Lipids, Inc.	11.2	15.6	Secondary surfactant; a specific diester of glycerophosphocholine; shorter fatty acids than those found in egg yolk PC
Soybean Oil (TG) Crisco	7.4	10.2	Triglyceride mixture in core of lipid particle; similar to that found in human LDL
ART-207	5.4	7.6	Prodrug payload; equivalent to 3.78 mg paclitaxel per mL
Poloxamer P188 (P188) Spectrum Chemicals	3.0	4.2	Secondary surfactant and surface modifier
Cholesterol Oleate (CE) Alfa Aesar	1.5	2.1	Cholesterol ester found in particle core and in human LDL
Cholesterol 95% (FC) Alfa Aesar	0.7	0.97	Lipid in particle core and in human LDL
Ubiquinol 10 (U) Kaneka Corpor	0.1	0.14	Antioxidant found in human LDL
Mixed Tocopherols (VitE) Swanson	0.1	0.14	Antioxidant mixture including α -tocopherol found in human LDL
Sum	71.9	100	

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The target amounts of formulation components (PC, DMPC, TG, FC, CE, U and Vit E) were weighed into a suitably sized beaker to form a lipid premix (LPM). The target amount of the acid labile, lipophilic prodrug (ART 207) was weighed into the beaker. The LPM was homogenized by dissolution in dichloromethane (DCM), 10 mL per 100 mL formulation. The DCM was removed by gentle heating in a chemical fume hood in a warm water bath with gentle agitation with nitrogen flow. Once the DCM had evaporated, the LPM was held in a 45°C vacuum oven at 100 μ m for at least 1 hour to

remove residual DCM. The LPM was stored at -5 °C prior to high-pressure homogenization.

The LPM was removed from the freezer and allowed to warm to RT; 100 mL of formulation buffer (0.9% NaCl in 10 mM Acetate buffer of pH 5.5 at 65 °C) was added and the mixture was homogenized with a hand blender with short pulses for 1 to 2 minutes to form a coarse emulsion. The coarse emulsion was transferred to a MicroFluidizer (MF) (MicroFluidics, Inc., Model 110P with temperature-controlled recirculation bath) and processed at 30,000 psi and 60 °C, monitoring particle size of processed material at 10 minutes intervals by dynamic light scattering until an intensity-weighted mean particle size of less than 65 nm was achieved. The nanoparticle dispersion was sterile filtered through a 0.22-μm polyethersulfone polymer membrane filter.

ii. Characterization of nanoparticle dispersions

The HPLC analytical method used to quantitate ART 207 in lipid emulsions was performed on an Agilent 1100 quaternary pump and single wavelength system. The column was a Phenomenex 4.6 x 50 mm Luna 5μm C18(2) 100Å, part number 00B-4252-E0. The method conditions were: flow rate: 1.5 mL/minute, detection: 230 nm, column temperature: 55°C, and injection volume: 3 μL. The gradient program is shown in Table 2 below.

Table 2 Reverse-phase HPLC gradient

<u>Time, minutes</u>	<u>% Acetonitrile</u>	<u>% 0.01 M aqueous</u> <u>H₃PO₄</u>
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0	75	25
7	100	0
9	100	0
10	75	25
11	75	25

Intensity-weighted mean particle sizes were determined by dynamic light scattering using a Nicomp Model 380 ZLS Zeta Potential/Particle Sizer from Particle Sizing Systems.

B. ART-207 synthesis

Discovery of a suitable cytotoxic derivative for incorporation (answering question 2 above) into the pseudo-LDL nanoparticle delivery vehicle recognized the derivative must be lipophilic, would readily release active cytotoxic moiety upon delivery to tumor tissue and be readily accessible via chemical synthesis.

The lipophilic paclitaxel prodrug ART-207 was synthesized as previously described.⁵⁰ The reaction sequence is shown in Figure 1 below.

Figure 1 Synthesis of ART-207

The reactions are described in Table 3 below.

Table 3 Synthesis of ART-207

Step	Reaction	Solvent	Purification by normal-phase chromatography
i	methyl oleate is reduced to oleyl alcohol with 4 mole equivalents of diisobutylaluminium hydride (DIBAL-H)	n-heptane	No
ii	oleyl alcohol is oxidized to oleyl aldehyde under with 1.2 equivalents of Dess-Martin periodinane (1,1,1-Tris(acetyloxy)-1,1-dihydro-1,2-benziodoxol-3-(1H)-one	dichloromethane	Yes
iii	Solketal is reacted with 4-nitrophenylchloroformate and pyridine (1.2 mol equivalent), catalyzed by dimethylpyridine (DMAP), to form the 4-nitrophenyl carbonate-solketal conjugate	dichloromethane	Yes

iv	The 4-nitrophenyl carbonate-solketal conjugate is reacted with oleyl aldehyde catalyzed by p-toluenesulfonic acid (p-TsOH) in toluene to form ART-206	toluene	Yes
v	ART-206 is reacted with paclitaxel and DMAP (1.5 equivalents) to form ART-207	dichloromethane	Yes

C. Biological Evaluation

Accepted *in vivo* animal models were selected to demonstrate TumorSelect[®] technology's potential.

i. ATL-1 and 2

To assess the toxicity and, preliminarily, the efficacy of TumorSelect[®] paclitaxel, two small studies were commissioned at Southern Research Institute, Birmingham, AL USA.

These studies were designed to address question 3 outlined above- does the proposed TumorSelect construct of cytotoxic derivative and delivery vehicle improve TI of the cytotoxic and still retain efficacy?

a. Experiment ATL-1

This study was to determine the toxicity of the TumorSelect[®] paclitaxel formulation.

The study consisted of six groups of five non-tumored mice per group for a total of 30 mice on Day 1 (January 22, 2013), the first day of treatment. All treatments

1 were administered intravenously (IV) once a day for five consecutive days
2 (Q1Dx5) beginning on Day 1. Thus, the total dose of drug administered was 5
3 times the dose injected per injection. Animals in Groups 1-4 were treated with
4 formulated ART 207 at doses of 72.6, 47.1, 35.7, and 23.7 mg/kg/injection,
5 respectively, administered to the mice by exact individual animal's body weight on
6 each day of treatment with the injection volume being 0.21 mL/20 g of body
7 weight. The formulated ART 207 doses of 72.6, 47.1, 35.7, and 23.7 mg/kg were
8 3.4x, 2.2x, 1.7x, and 1.1x molar equivalent of a paclitaxel dose of 15 mg/kg,
9 respectively, based on the molecular weight of formulated ART 207 of 1220.4 and
10 molecular weight of paclitaxel of 853.9. Animals in Group 5 (Emulsion control)
11 were treated with a drug free formulation (injection volume of 0.21 mL/20 g of
12 body weight). Animals in Group 6 were treated with paclitaxel at a dose of 15
13 mg/kg/injection (injection volume of 0.1 mL/10 g of body weight). (In this particle
14 experiment, the 15 mg/kg paclitaxel dose proved toxic to 4 of the 5 mice of the
15 paclitaxel group.)

16 ***b. Experiment ATL-2***

17 This study was commissioned to assess preliminary efficacy of the TumorSelect[®]
18 paclitaxel.

19 The study consisted of six groups of five mice per group for a total of 30 mice
20 bearing human MDA-MB-231 mammary tumor on the first day of treatment, Day
21 13 after tumor fragment implantation (January 22, 2013). All treatments were
22 administered IV on a Q1Dx5 schedule beginning on Day 13. Animals in Groups 1-

4 were treated with formulated ART 207 at doses of 72.6, 47.1, 35.7, and 23.7 mg/kg/injection, respectively, administered to the mice by exact individual animal's body weight with the injection volume being 0.21 mL/20 g of body weight. The formulated ART 207 doses of 72.6, 47.1, 35.7, and 23.7 mg/kg were 3.4x, 2.2x, 1.7x, and 1.1x molar equivalent of a paclitaxel dose of 15 mg/kg, respectively, based on the molecular weight of formulated ART 207 of 1220.4 and molecular weight of paclitaxel of 853.9. Animals in Group 5 (Emulsion control) were treated with a drug-free formulation (injection volume of 0.21 mL/20 g of body weight). Animals in Group 6 were treated with paclitaxel at a dose of 15 mg/kg/injection (injection volume of 0.1 mL/10 g of body weight).

c. ATL-3 Mouse breast-cancer xenograft safety and efficacy study

The purpose of the experiment ATL-3 (contracted for execution at Southern Research Institute, Birmingham, AL) was to evaluate and confirm the antitumor activity of the intravenous (IV) treatment with TumorSelect[®] paclitaxel when administered to female, athymic NCr-*nu/nu* mice implanted subcutaneously (SC) with human MDA-MB-231 mammary tumor xenografts and also to provide an indication of experimentally tolerated dose of TumorSelect[®] paclitaxel in murine models. Paclitaxel formulated in 50/50 Cremophor[®] EL/ethanol was included as a reference treatment. ATL 4&5 Mouse breast-cancer xenograft drug distribution studies

The purpose of the experiments ATL-4 and ATL-5 was to determine the concentration of ART 207 and paclitaxel in plasma, brain, heart, liver, lungs, and

tumor (experiment ATL-5 only) after a single intravenous (IV) injection of formulated ART 207 or paclitaxel to non-tumored female athymic NCr-*nu/nu* mice and female athymic NCr-*nu/nu* mice bearing a subcutaneous (SC) human MDA-MB-231 mammary tumor.

Plasma and tissue concentrations of paclitaxel were determined with a modification of a previously published LC-MS/MS method.⁵¹ Quantitative determination of Paclitaxel in mouse plasma or tissue homogenate was accomplished by protein precipitation and high-performance liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). ¹³C₆-paclitaxel was used as the internal standard (IS). Paclitaxel and IS were extracted from 50 µL mouse plasma or tissue homogenate by protein precipitation using acetonitrile. The chromatographic separation was achieved using an Ace 3 C₁₈ analytical column maintained at 40 °C under gradient conditions. Column effluents were analyzed by multiple-reaction monitoring (MRM) using a triple quadrupole mass spectrometer in positive-ion mode (AB Sciex 4000 Q Trap LC/MS/MS system, equipped with TurboV IonSpray®). The precursor/product transitions are 876.3→308.2 m/z for paclitaxel and 882.4→313.9 m/z for ¹³C₆-paclitaxel. The calibration curve was fit using weighted ($1/x^2$) linear regression analysis of the paclitaxel/IS peak area ratio versus the paclitaxel concentration from 5.00 – 5,000 ng/mL. Concentrations of incurred and quality control samples were calculated with the same regression analysis and results were reported in ng/mL or ng/g of paclitaxel.

Tumored mice were subdivided into 5 groups (3 mice per each group). All mice were bolus injected intravenously with either 70 mg/kg (49 mg/kg paclitaxel

equivalent) of TumorSelect[®] paclitaxel or 18.9 mg/kg of Chemophor[®]/ethanol formulated paclitaxel (the MTD for single bolus injection). Blood samples and organs were collected at 5, 30, 60, 240, and 480 min after single bolus injection. Each time point represents 3 mice.

Quantitative determination of ART-207 in mouse plasma and tissue homogenate was accomplished by protein precipitation and high-performance liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). ¹³C₆-ART-207 was used as the internal standard. ART-207 and IS were extracted from 50 µL mouse plasma or tissue homogenate by protein precipitation using acetonitrile. The chromatographic separation was achieved using an Ace 3 C₁₈ analytical column maintained at 40 °C under isocratic conditions. Column effluents were analyzed by multiple-reaction monitoring (MRM) using a triple quadrupole mass spectrometer in positive-ion mode (AB Sciex 4000 Q Trap LC/MS/MS system, equipped with TurboV IonSpray). The precursor/product transitions are 1220.7→652.5 m/z for ART-207 and 1226.7→658.5 m/z for ¹³C₆-ART-207. The calibration curve was fit using weighted (1/x²) linear regression analysis of the ART-207/IS peak area ratio versus the ART-207 concentration from 5.00 – 500 µg/mL. Concentrations of incurred and quality control samples were calculated with the same regression analysis and results were reported in µg/mL or µg/g of ART-207.

Tissue exposure is expressed as the area under the concentration-time curve (AUC) from 0 to the last time point at which the analyte was measured (8 hrs). Areas under the mean concentration-time curve from 0 to eight hours (AUC₀₋₈) were

calculated as previously described.^{52, 53}

ii. Mouse pancreatic tumor xenograft study

As a second comparison *in vivo* study, the pancreatic tumor cells MIA-PaCa-2 (ATCC, Manassas, VA) was implanted in NOD scid (NSG) female mice, and the animals were dosed with TumorSelect[®] paclitaxel with a comparison positive control of Abraxane[®]. Female, six-week-old, NOD.CG-PRKDC^{SCID} Il2RG^{TM1WJL}/SZJ mice known as NOD scid gamma mice (NSG 005557, Jackson Lab) were purchased from Jackson Laboratories (Bar Harbor, ME) and acclimated at the laboratory animal facility at the University of Mississippi Medical Center (Jackson, MS). All animals were housed in microisolator cages, up to five per cage, in a 12-hour light/dark cycle. The animals received filtered water and irradiated Teklad rodent diet (LM-7945, Envigo Inc.) *ad libitum*. No consumable enrichment was provided. Cages were changed twice weekly. The animals were observed daily and clinical signs were noted. At 8 weeks of age mice were injected in the flank with 10⁶ MIA-paca-2 human cells (ATCC, Manassas, VA) with 100 uL Matrigel (Corning). Tumor development was measured using an electronic caliper every 2 or 3 days through up to 105 days. The formula used for tumor volume calculation is

$$Tumor\ volume = \frac{4}{3}\pi \left(\frac{W}{2}\right)^2 \left(\frac{L}{2}\right)$$

where: *Tumor volume* is expressed in mm³; *W* is width (the shorter dimension) in mm; and *L* is length (the longer dimension) in mm. Mouse weights were measured every 2 or 3 days.

Beginning on day 24 after implantation (when the tumors reached 200-250 mm³), the mice were divided into three groups and were injected daily with Abraxane (15 mg/kg paclitaxel equivalent), TumorSelect vehicle, or TumorSelect paclitaxel (37.5 mg/kg paclitaxel equivalent) for 5 consecutive days (total Abraxane paclitaxel dose of 75 mg/kg, total TumorSelect paclitaxel dose of . Total Abraxane dose of 75 mg/kg and formulated ART- 207 dose of 187.5 mg/kg for a paclitaxel equivalent dose of 132.5 mg/kg.)

5. Results

A. Material characterization

The ART-207 content and mean particle-size data for TumorSelect[®] paclitaxel nanoparticle dispersions used in animal studies are shown in Table 4 below.

Table 4 Particle-size characterization of TumorSelect[®] paclitaxel nanoparticles

Lot#	Used in study	Intensity-weighted mean particle size, nm	ART-207 mg/mL	Particle size stability	
				Days past mfg	Particle size nm
002.151.8	ATL-3	62	5.26	42	67
002.156.3	ATL-4&5	62	4.80	39	61
06292018	Pancreatic xenograft	73	4.81	180	77

B. Biological evaluation

The results of the mouse safety, pharmacokinetics, and efficacy studies are shown below. In all displays, doses are expressed in paclitaxel equivalents.

1 **i. ATL-3 Breast-cancer mouse xenograft safety and efficacy study**

2 The following graphs show animal survival, animal weights, and tumor weights in the
3 breast-cancer mouse xenograft study. In all figures the TumorSelect[®] and Cremophor[®]
4 administered doses are expressed in paclitaxel equivalents per injection which provides
5 a total of 5-times the daily injection for the experimental drug exposure.

6 ***a. Animal survival data***

7 The survival of animals in Study ATL-3 is illustrated in Figure 2 below.

Figure 2 Animal survival in ATL-3

b. Mean animal weights

The mean animal weights in Study ATL-3 are illustrated in Figure 3 below.

Figure 3 Mean animal weights in ATL-3

c. Mean tumor weights

Figure 4 below shows mean tumor weights as a function of dose (expressed in paclitaxel equivalents).

Figure 4 Mean tumor weights in ATL-3

ii. ATL-4&5 Mouse breast-cancer xenograft drug distribution studies

a. Plasma and tissue pharmacokinetics

Table 5 below provides the derived plasma and tissue pharmacokinetic data for Study ATL-4.

1

Table 5 Plasma and tissue pharmacokinetics in ATL-4

Pharmacokinetic parameters calculated from plasma and tissue concentrations of ART 207 and paclitaxel Experiment ATL-4 / non-tumored mice								
Treatment	Tissue	Analyte	t _{max} ^a (hour)	C _{max} ^b (µg/mL or g)	AUC ₀₋₈ ^c (hr·µg/mL or g)	t _½ ^d (hr)	Cl ^e (mL/hr/kg)	V _{ss} ^f (mL/kg)
TumorSelect®	Plasma	ART-207	0.083	640	2794	2.8	22.0	88.0
		Paclitaxel	1	2.237	14.4	11.5	NA	NA
	Brain	ART-207	0.5	11.0	38.5	NA	NA	NA
		Paclitaxel	1	0.070	0.437	NA	NA	NA
	Heart	ART-207	1	156	928	NA	NA	NA
		Paclitaxel	8	2.08	13.6	NA	NA	NA
	Liver	ART-207	8	444	2354	NA	NA	NA
		Paclitaxel	8	13.2	80.6	NA	NA	NA
	Lungs	ART-207	0.083	111	614	NA	NA	NA
Paclitaxel		1	3.61	27.9	NA	NA	NA	
Cremophor®	Plasma	Paclitaxel	0.083	32.9	37.7	1.0	500	658
	Brain	Paclitaxel	0.5	0.301	1.31	NA	NA	NA
	Heart	Paclitaxel	0.083	19.5	33.9	NA	NA	NA
	Liver	Paclitaxel	1	81.4	283	NA	NA	NA
	Lungs	Paclitaxel	1	47.9	93.7	NA	NA	NA
<div>^a t_{max} – time at which maximum level seen</div> <div>^b C_{max} – maximum level seen</div> <div>^c AUC₀₋₈ – area under the 0-8 hour concentration-time curve</div> <div>^d t_½- plasma elimination half-life</div> <div>^e Cl – plasma clearance</div> <div>^f V_{ss} – volume of distribution at steady state</div>								

2

3 Table 6 below shows the derived plasma and tissue pharmacokinetic data for Study

4 ATL-5.

1

Table 6 Plasma and tissue pharmacokinetics in ATL-5

Pharmacokinetic parameters calculated from plasma and tissue/tumor concentrations of ART 207 and Paclitaxel Experiment ATL-5 / tumored mice								
Treatment	Tissue	Analyte	Tmax ^a (hour)	Cmax ^b (µg/mL or g)	AUClast ^c (hr·µg/mL or g)	t _{1/2} ^d (hr)	Cl ^e (mL/hr/kg)	V _{ss} ^f (mL/kg)
TumorSelect®	Plasma	ART-207	0.083	687	2309	2.6	26.0	97.0
		Paclitaxel	0.083	1.77	9.08	9.2	NA	NA
	Brain	ART-207	0.083	12.5	41.8	NA	NA	NA
		Paclitaxel	0.083	0.045	0.220	NA	NA	NA
	Heart	ART-207	1	171	926	NA	NA	NA
		Paclitaxel	0.5	1.39	10.1	NA	NA	NA
	Liver	ART-207	8	438	2106	NA	NA	NA
		Paclitaxel	0.083	14.2	68.1	NA	NA	NA
	Lungs	ART-207	0.5	109	631	NA	NA	NA
		Paclitaxel	1	3.61	27.6	NA	NA	NA
	Tumor	ART-207	4	41.6	279	NA	NA	NA
		Paclitaxel	4	6.06	32.0	NA	NA	NA
Cremophor®	Plasma	Paclitaxel	0.083	41.6	34.4	1.0	547	567
	Brain	Paclitaxel	0.083	0.600	1.51	NA	NA	NA
	Heart	Paclitaxel	0.083	18.8	27.9	NA	NA	NA
	Liver	Paclitaxel	0.083	92.9	254	NA	NA	NA
	Lungs	Paclitaxel	1	79.2	120	NA	NA	NA
	Tumor	Paclitaxel	8	3.97	18.9	NA	NA	NA
^a t _{max} – time at which maximum level seen ^b C _{max} – maximum level seen ^c AUC ₀₋₈ – area under the 0-8 hour concentration-time curve ^d t _{1/2} – plasma elimination half-life ^e Cl – plasma clearance ^f V _{ss} – volume of distribution at steady state								

2

3 ***b. Tissue distribution***

4 Figure 5 below shows the free paclitaxel eight-hour plasma, tissue, and tumor
5 AUCs obtained in Studies ATL-4 and ATL-5.

Figure 5 AUCs in ATL-4 and ATL-5

Difference between free paclitaxel levels of tumored mice injected with Taxol (paclitaxel) or TumorSelect[®] paclitaxel was significant for all assessed tissues ($p < 0.05$).

As illustrated in Figure 6 below, in Study ATL-5, tumor concentrations of free active paclitaxel are significantly higher from TumorSelect[®] paclitaxel than those from generic Cremophor[®] paclitaxel: (ATL5).

Figure 6 Paclitaxel tumor AUCs in study ATL-5

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As seen in Figure 7 below, plasma and heart concentrations of free paclitaxel are significantly lower (<80%) in tumored vs. non-tumored animals in those animals receiving TumorSelect[®] Paclitaxel.: (ATL4&5)

Figure 7 Plasma and heart AUCs in ATL-4 and ATL-5

c. TumorSelect® Paclitaxel vs Taxol® volume of distribution in the mouse model

Table 7 below shows the volumes of distribution of the TumorSelect® prodrug ART-207 and of paclitaxel delivered by the Cremophor®/EtOH formulation in Studies ATL-4 and 5.

Table 7 Volumes of distribution in ATL-4 and ATL-5

Volume of distribution mL/kg		
	TumorSelect® paclitaxel prodrug	Cremophor®/Ethanol paclitaxel
Non-tumored mice	88	658
Tumored mice	97	567

1
2 This parameter shows that TumorSelect[®] paclitaxel remains in the vasculature and
3 does not indiscriminately distribute the toxic chemotherapeutic to normal tissues
4 and organs as does Taxol[®].

5 **iii. Mouse pancreatic tumor xenograft**

6 In the pancreatic cancer model, the following was observed:

7 ***a. Animal weights***

8 Figure 8 below shows the mean animal weights from the day of implantation
9 through day 105. Each point on the curves represents mean animal weight ($\pm$ SE)
10 of each drug- and vehicle-treated group at the day of assessment. The mean group
11 cutoff is five animals per group.

Figure 8 Mean animal weights in pancreatic tumor xenograft study

b. Tumor growth

Figure 9 below shows the mean tumor volumes from the day of implantation through day 105. Each point on the curves represents mean tumor volume ($\pm$ SE) of each drug- and vehicle-treated group at the day of assessment. The mean group cutoff is five animals per group. It can be seen that tumors began to regrow at about 25 days post dosing following exposure to Abraxane[®] but continued to decrease in size for nearly an additional 30 days following the single exposure to TumorSelect[®] paclitaxel.

Figure 9 Mean tumor weights in pancreatic tumor xenograft study

6. Discussion

A. TumorSelect® Technology

“Cancer cells rewire their metabolism to meet the energetic and biosynthetic demands of their high proliferation rates and environment.”⁵⁴ They are voracious consumers of cholesterol, triglycerides and phospholipids.⁵⁵ Those essential nutrients are available to be taken up from LDL particles in the plasma. As a result, tumors dramatically overexpress LDL receptors (LDLR) by as much as eleven-fold relative to normal healthy systemic tissues.^{56,57} High LDLR expression is associated with rapid tumor growth^{58,59} and a poor prognosis for the patient.^{60,61,62,63} Incorporating chemotherapeutics into pseudo-LDL

1 nanoparticles for delivery via overexpressed LDL receptors provides the opportunity for
2 selective delivery of lipophilic chemotherapeutics to tumors compared with normal tissues.
3 Pseudo-LDL particles can increase the utility of clinically approved chemotherapeutic
4 agents by altering their PK/PD properties and dramatically raising their therapeutic indices.

5 In order to incorporate the current clinically approved chemotherapeutic into the pseudo-
6 LDL particle, the hydrophilic chemotherapeutic (selected to be hydrophilic in order to be
7 administered by intravenous infusion in aqueous infusion fluids) is necessarily converted to
8 a lipophilic metabolically labile prodrug which will be retained within the pseudo-LDL
9 particle until the particle is internalized into the tumor cells by uptake by the LDL receptor
10 and processed by the endosome/lysosome cascade. This strategy is directly applicable to the
11 majority of clinically approved and widely utilized chemotherapeutics.

12 The proposed mechanism for the cleavage of the promoiety from ART-207, and liberation
13 of paclitaxel, in the acidic environment of the endosome/lysosome cascade is shown in
14 Figure 10 below. This hydrolysis which takes place intracellularly delivers the active
15 cytotoxic, paclitaxel, and the lipophilic promoiety is cleaved to the nontoxic products,
16 carbon dioxide, glycerol, and oleoyl aldehyde which is rapidly transformed to the fatty acid
17 oleic acid.

Figure 10 Cleavage of TumorSelect® paclitaxel prodrug

Veiled Therapeutics has developed the strategy as TumorSelect® technology and licensed TumorSelect® Paclitaxel to Cloaked Therapeutics Development, LLC to demonstrate its utility of promise to improve both cytotoxic chemotherapy efficacy and tolerability when compared with standard generic Cremophor® as well as Abraxane® formulations of paclitaxel. Since many of paclitaxel's issues can be traced to its well-characterized PK/PD limitations, investigators expect that improvements in paclitaxel's PK/PD profile should translate to improved clinical outcomes. TumorSelect® paclitaxel has overcome the limitations of conventional paclitaxel delivery systems as demonstrated by its superior efficacy and apparent lack of toxicity in established preclinical *in vivo* mouse models of breast cancer and pancreatic cancer. These experimentally observed benefits support the validity of the TumorSelect® technology and strategy.

7. Conclusions

The future of traditional cytotoxic chemotherapy:

Clinical practice for the foreseeable future will continue to rely on cytotoxic chemotherapy with hundreds of thousands of patients treated annually. Also, it is clear from recently published studies, such as the NEJM atezolizumab plus Abraxane® in triple negative breast cancer study,⁴⁰

1 that traditional chemotherapy will remain a mainstay of cancer treatment for the foreseeable
2 future. In particular, there is evidence that taxanes can potentiate immunotherapy.⁶⁴

3 Abraxane[®] upon administration allows for a higher fraction of free paclitaxel than even generic
4 Cremophor[®] paclitaxel and thus drives wider tissue distribution than standard paclitaxel.⁶⁵

5 Albumin-bound drug may leave capillaries by active transport across vessel walls, but there is
6 little or no active uptake into tumors and thus little or no selectivity for tumor cells. In short,
7 with Abraxane[®] both normal and tumor tissue are non-selectively exposed to free paclitaxel.

8 Abraxane[®]'s pharmacokinetic "improvements" are the direct opposite of those that are
9 theoretically desired, and thus opposite to those seen with the superior TumorSelect[®] paclitaxel
10 formulation. TumorSelect[®] dramatically decreases normal tissue exposure while selectively
11 increasing tumor exposure.

12 Abraxane[®] and TumorSelect[®] paclitaxel were compared in both a pancreatic and an ovarian
13 model with results similar to the Cremophor[®] paclitaxel results. However, TumorSelect[®]
14 paclitaxel was better tolerated than standard generic Cremophor[®] paclitaxel or Abraxane[®] and
15 can be dosed more intensively (2.5 equivalents of the MTD in mice of either Cremophor[®]
16 paclitaxel or Abraxane[®]) than either the generic Cremophor[®] paclitaxel or the Abraxane[®]
17 formulation. In a tumored mouse model with various dose levels of TumorSelect[®] paclitaxel a
18 measure of weight loss post dosing, as an indication of toxicity, showed that TumorSelect[®]
19 paclitaxel could be dosed at 2.5 equivalents of paclitaxel with minimal weight loss in the
20 animals, demonstrating that the TumorSelect[®] paclitaxel formulation was less toxic than
21 Abraxane[®].

22 In addition to allowing more intense dosing due to lower systemic toxicity, the mechanism of

receptor mediated uptake will bypass the common mechanism of resistance of ABC transporter activity (PGP, MDR, etc.) which is membrane located. Both these attributes should enhance the efficacy and reduce the systemic side effect toxicity of the chemotherapeutic agents.

The demonstrated advantages of TumorSelect[®] paclitaxel include:

- Better efficacy—in the animals treated with Abraxane[®], the tumors continued their steady growth, while in those treated with TumorSelect[®] paclitaxel (at 2.5X the paclitaxel equivalent of Abraxane[®]) the tumors had nearly disappeared by the day 56 of study.
- Less toxicity—there was substantially less weight loss observed in the animals treated with TumorSelect[®] paclitaxel at 2.5X the paclitaxel-equivalent dose given to Abraxane[®] treated animals.

Unlike TumorSelect[®] paclitaxel, Abraxane[®] allows for a higher fraction of free paclitaxel and thus drives wider tissue distribution than Cremophor[®]-formulated paclitaxel. While tumor response rates are improved, the debilitating side-effects of paclitaxel are also made substantially worse. Nonetheless, Abraxane[®], which has never more than barely penetrated its total potential market, has become a blockbuster commercial success with sales of nearly \$1 billion.

TumorSelect[®] paclitaxel has the ideal and directly opposite PK/PD profile of Abraxane[®] and would therefore be expected to improve tumor responses while substantially decreasing side-effects.

Additional TumorSelect[®] Advantages

- It does not contain or require human proteins as part of its manufacturing process
- It is not a liposome technology and is comprised of well-known, safe, and easily sourced

1 components

2 **8. Availability of data and materials**

3 **9. Abbreviations**

4 μL Microliter

5 μm Micrometer

6 $\text{\AA}$ Angstrom

7 ACS American Cancer Society

8 AUC Area under the concentration-time curve

9 BCRP Breast cancer resistance protein

10 CDC Centers for Disease Control and Prevention of the U.S. Department of Health & Human
11 Services

12 CE Cholesterol oleate

13 DCM Dichloromethane

14 DMPC Dimyristoylphosphatidylcholine

15 EtOH Ethanol

16 FC Free cholesterol

17 IV Intravenous

1	kg	Kilogram
2	LC	Liquid chromatography
3	LDL	Low-density lipoprotein
4	LDLR	Low-density lipoprotein receptor
5	LPM	Lipid premix
6	m/z	Mass-to-charge ratio
7	MF	MicroFluidizer [®]
8	mfg	Date of manufacturing
9	mg	Milligram
10	mL	Milliliter
11	mm	Millimeter
12	MRP-1	Multi-drug resistance related protein
13	MS	Mass spectrometry
14	MTD	Maximum tolerated dose
15	NCI	National Cancer Institute of the US National Institutes of Health, U.S. Department of
16		Health & Human Services
17	nm	Nanometer

- 1 P188 Poloxamer P188
- 2 PC Phosphatidylcholine
- 3 PD Pharmacodynamics
- 4 PD-1/PD-L1 Programmed death ligand 1
- 5 Pgp P-glycoprotein
- 6 PK Pharmacokinetics
- 7 Q1Dx5 One dose every day for five days
- 8 SC Subcutaneous
- 9 SE Standard error
- 10 TG Soybean oil
- 11 TI Therapeutic index
- 12 U Ubiquinol 10
- 13 VitE Mixed tocopherols
- 14 WHO World Health Organization
- 15 **10. Declarations**

B. Ethics approval

All animal experimental procedures in Studies ATL 4 and ATL-5 were approved by the Institutional Animal Care and Use Committee of Southern Research. Animal laboratories of Southern Research are AAALAC accredited.

C. Consent for publication

Not applicable

D. Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

E. Competing interests

The authors declare that they have no competing interests.

F. Funding

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G. Author contributions

JDM conceived the manuscript and edited the final draft. SJB wrote the manuscript and edited the final draft. IN and DLR performed significant portions of the formulation

development work and edited the final draft. AW and MG designed and executed the chemical synthesis of ART-207, the lipophilic paclitaxel prodrug and edited the final draft of the manuscript. MC and DL prepared the TumorSelect[®] paclitaxel formulations used in the in vivo mouse model studies and edited the final draft of the manuscript. PPC and PP conceived and executed the in vivo mouse studies comparing TumorSelect[®] paclitaxel to Abraxane[®] and edited the final draft of the manuscript.

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