Article

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Bioconjugate Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.bioconjchem.6b00610 • Publication Date (Web): 07 Dec 2016

Pt-Mal-LHRH, a Newly Synthesized Compound Attenuating Breast Cancer Tumor Growth and Metastasis by Targeting Overexpression of the LHRH Receptor

Lindsay E. Calderon†, Jonathan K. Keeling‡, Joseph Rollins†, Carrie A. Black‡, Kendall Collins†, Nova Arnold†, Diane E. Vance‡, Margaret W. Ndinguri‡**

†*Department of Biology, Eastern Kentucky University, Richmond, KY, 40475, USA*

‡*Department of Chemistry, Eastern Kentucky University, Richmond, KY, 40475, USA*

**Corresponding Authors: Margaret W. Ndinguri: Margaret.Ndinguri@eku.edu and Lindsay E. Calderon: Lindsay.Calderon@eku.edu*

New Science Building 4126

521 Lancaster Avenue

Richmond KY 40475

Abstract

A new targeting chemotherapeutic agent, Pt-Mal-LHRH, was synthesized by linking activated cisplatin to luteinizing hormone releasing hormone (LHRH). The compound's efficacy and selectivity towards 4T1 breast cancer cells were evaluated. Carboplatin was selected as the comparative platinum complex since the Pt-Mal-LHRH malonate linker chelates platinum in a similar manner to carboplatin. Breast cancer and normal cell viability were analyzed by an MTT assay comparing Pt-Mal-LHRH with carboplatin. Cells were also treated with either Pt-Mal-LHRH or carboplatin to evaluate platinum uptake by ICP-MS and cell migration using an *in-vitro* scratch-migration assay. Tumor volume and metastasis were evaluated using an *in-vivo* 4T1 mouse tumor model. Mice were administered Pt-Mal-LHRH (carboplatin molar-equivalent dosage) through IP injection and compared to those treated with carboplatin (5 mg/kg/wk), no treatment, and LHRH plus carboplatin (unbound) controls. An MTT assay showed a reduction in cell viability ($p<0.01$) in 4T1 and MDA-MB-231 breast cancer cells treated with Pt-Mal-LHRH compared to carboplatin. Pt-Mal-LHRH was confirmed to be cytotoxic by flow cytometry using a propidium iodide stain. Pt-Mal-LHRH displayed a 20-fold increase in 4T1 cellular uptake compared to carboplatin. There was a decrease ($p<0.0001$) in 4T1 cell viability compared to 3T3 normal fibroblast cells. Treatment with Pt-Mal-LHRH also resulted in a significant decrease in cell-migration compared to carboplatin. *In-vivo* testing found a significant reduction in tumor volume ($p<0.05$) and metastatic tumor colonization in the lungs with Pt-Mal-LHRH compared to carboplatin. There was a slight decrease in lung weight and no difference in liver weight between treatment groups. Together, our data indicates that Pt-Mal-LHRH is a more potent and selective chemotherapeutic agent than untargeted carboplatin.

Introduction

In the United States breast cancer afflicts 1 in 8 women during their lifetime.[1] The National Cancer Institute's SEER database indicates the median overall survival rate for stage IV breast cancer is 23%. Despite recent advancements in the first-line treatments, many patients eventually relapse, their tumors become chemoresistant, and patients experience numerous debilitating side effects from chemotherapy treatment. Major issues of chemotherapy administration stem from the resulting severe side effects, which causes doctors to often resort to dose reduction, treatment delay or suspension of therapy.

The platinum drugs carboplatin and cisplatin have been used to treat breast, prostate, ovarian, bladder, lung, head and neck cancer.[2-3] Cisplatin is the most potent member of the platinum family; however, due to its poor targeting and formation of resistance, it is not an effective treatment option for breast cancer.[4-5] Carboplatin, an analogue of cisplatin has gained greater use due to its lower non-specific toxicity and higher activity against cisplatin-resistant tumors.[6] Platinum anticancer drugs promote cell death by interfering with cellular transcription and DNA replication mechanisms. Serious side effects resulting from the platinum family drugs include nephrotoxicity, myelotoxicity, neurotoxicity, vomiting and nausea.[7] Another possible concern is the ability of certain cancers to develop resistance mechanisms to cisplatin including efflux pumps.[8] To combat acquired and intrinsic multidrug resistance, as well the severe side effects exhibited during high dose treatment, recent cancer research is focused on developing targeted cancer delivery to decrease side effects while improving efficacy.

Systemic side effects result from anticancer drugs which are primarily designed to destroy rapidly dividing cells, including those found in healthy tissues. To decrease adverse side effects and limit nonspecific activity several methods of receptor directed chemotherapy have been developed using receptor ligands, lectins, antibodies, sugars, hormones and hormone analogs.[9-14] Luteinizing hormone releasing hormone (LHRH) receptor, also referred to as gonadotropin releasing hormone (GnRH)

receptor, has been found to be overexpressed in breast, prostate, endometrial and ovarian cancers in comparison to normal cells. This makes the LHRH peptide a good candidate for drug targeting.[15-17]

In many of these cancers, tumor proliferation can be inhibited by antagonist analogues of LHRH.[18-19] Previous studies have demonstrated that cytotoxic compound attachment at the D-lys moiety of LHRH does not change the intrinsic high binding affinity of the peptide to its receptors.[14, 20-21] For example, Schally and Nagy studied the potent antagonist on conjugates of [DLys6]-LHRH–DOX and [DLys6]-LHRH–2-pyrrolino-DOX revealing increased efficacy of doxorubicin.[22] The LHRH analogue maintained its high targeted binding affinity while the drug retained its cytotoxic effects on the tumor cells.[14] Aggarwal and coworkers showed that [DLys6]-LHRH–curcumin enhanced apoptosis of tumor tissue and was useful in the treatment of pancreatic cancer.[12] In another study, Hansel and coworkers showed that conjugates of lytic peptides and LHRH are very effective in destroying human breast and prostate cancer xenografts that express LHRH receptors.[23-26] Mudyiwa et al. also synthesized a [DLys6]-LHRH conjugate with phthalocyanine; however, the results of the biological testing have not been released.[27]

Although numerous breast cancers overexpress LHRH receptors compared to normal breast cells, few findings have been published regarding the role of LHRH and platinum drugs. This paper demonstrates the chemical synthesis of the new compound Pt-Mal-LHRH by attaching LHRH (targeting moiety) with activated cisplatin and reports data on the potency and selectivity of this chemotherapeutic agent towards breast cancer. Carboplatin was selected as the comparative platinum complex since the Pt-Mal-LHRH malonate linker chelates platinum in a similar manner to carboplatin (Figure 1).

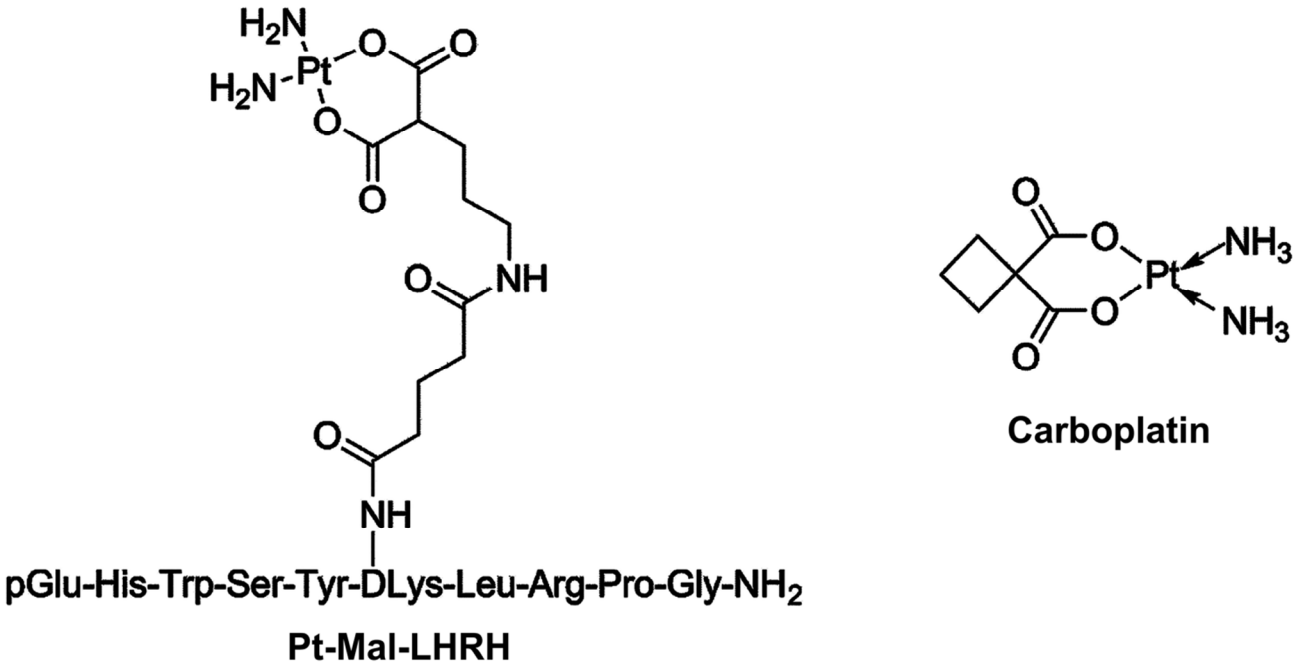

Figure 1: Chemical structure of Pt-Mal-LHRH and carboplatin. The bioactive region/structure of carboplatin is maintained in the Pt-Mal-LHRH compound.

Results

Synthesis of Pt-Mal-LHRH

For complete synthesis of Pt-Mal-LHRH, a malonate linker was first synthesized which contains a unique tumor-activated protease cleavage site.[12] Di-tert-butyl 2-(3-(4-Carboxybutanamido)propyl)-malonate (Mal) was synthesized as described in Ndinguri et al.[13] (Figure 1). Use of the malonate linker is advantageous as it will allow the platinum drug to be bioactive in the tumor cells. Starting from an amide based resin and using Fmoc chemistry, the peptide was assembled, the Alloc protecting group was removed followed by conjugation of the Mal. The fully assembled peptide was cleaved from the solid support using a cleavage cocktail to give target compound Mal-LHRH. The purity of the Mal-LHRH peptide was confirmed using analytical HPLC and its identity was confirmed by mass spectrometry. Platination with a 1.2-fold excess of activated cis-$[Pt\text{-}(NH_3)_2(H2O)_2]^{2+}(NO_3)_2$ using a standard procedure gave Pt-Mal-LHRH peptide shown in Figure 1. The platinum complexes could not be purified by HPLC

using either an acidic or a neutral buffer system because the platinum complex dissociated. The products were, therefore, purified by gel filtration to remove any excess platinum. Fractions containing the Pt-Mal-LHRH complex in water were identified by ESI-MS and lyophilized to Pt-Mal-LHRH give pure platinum in a 15-17% yield. Mass spectra revealed the expected $(M + Na)^+$ peak at 1756.7670 for the target compound (see Supporting Information). Any experiment with Pt-Mal-LHRH had to use freshly dissolved conjugate due to precipitates formed after storage, a phenomenon that has been reported before by Ndinguri, et al.[13] The Pt-Mal-LHRH compound was stable after the use of lyophilized powders at -20 °C. Before usage, fresh samples were made by reconstituting the dry samples to the desired concentration.

Mass spectrometry was used to verify the integrity of the redissolved Pt-Mal-LHRH. The final complex yields a chemical structure similar to carboplatin, in which the carboplatin bioactive region/structure is maintained (Figure 1).

Pt-Mal-LHRH increases breast cancer cytotoxicity through increased cellular uptake.

To examine the effects of Pt-Mal-LHRH on cell viability, 4T1 breast cancer cells were treated with carboplatin, LHRH, or Pt-Mal-LHRH from 0.1 μM to 100 μM. Pt-Mal-LHRH was found to significantly inhibit 4T1 viability compared to the untargeted carboplatin, concentration-dependently (Figure 2A). Both untargeted carboplatin and Pt-Mal-LHRH at 100 μM was found to significantly attenuate cell viability compared to the untreated control, however, this effect was more pronounced with Pt-Mal-LHRH. In addition, the enhanced potency of Pt-Mal-LHRH was also verified using a second cell line, the MDA-MB-231 breast cancer cell line. Pt-Mal-LHRH was found to significantly attenuate MDA-MB-231 breast cancer cell viability compared to carboplatin (see Supporting Information). Moreover, the induction of cytotoxicity by Pt-Mal-LHRH was confirmed by PI staining in which Pt-Mal-LHRH was found to significantly increase apoptosis compared to carboplatin (Figure 2B). Numerous experimental and clinical studies have shown overexpression of the LHRH receptor on human breast carcinomas, and specifically in the 4T1 and MDA-MB-231 cell lines.[28-29] To show the cytotoxic effect is induced by cellular entry of Pt-Mal-LHRH through

binding to the LHRH receptor a competition assay was conducted. Treatment with free LHRH antibody showed a significant attenuation in the ability Pt-Mal-LHRH to induce a cytotoxic effect (Figure 2C). Subsequently, due to targeting LHRH receptor overexpression, Pt-Mal-LHRH cellular uptake was found to be 20 fold higher than carboplatin in the 4T1 cell line (Figure 2D). Pt-Mal-LHRH uptake was also found to be significantly increased in the MDA-MB-231 breast cancer cells (see Supporting Information). These results suggest that Pt-Mal-LHRH is more potent and efficacious than carboplatin in mediating breast cancer cytotoxicity due to targeting aided by cellular overexpression of the LHRH receptor.

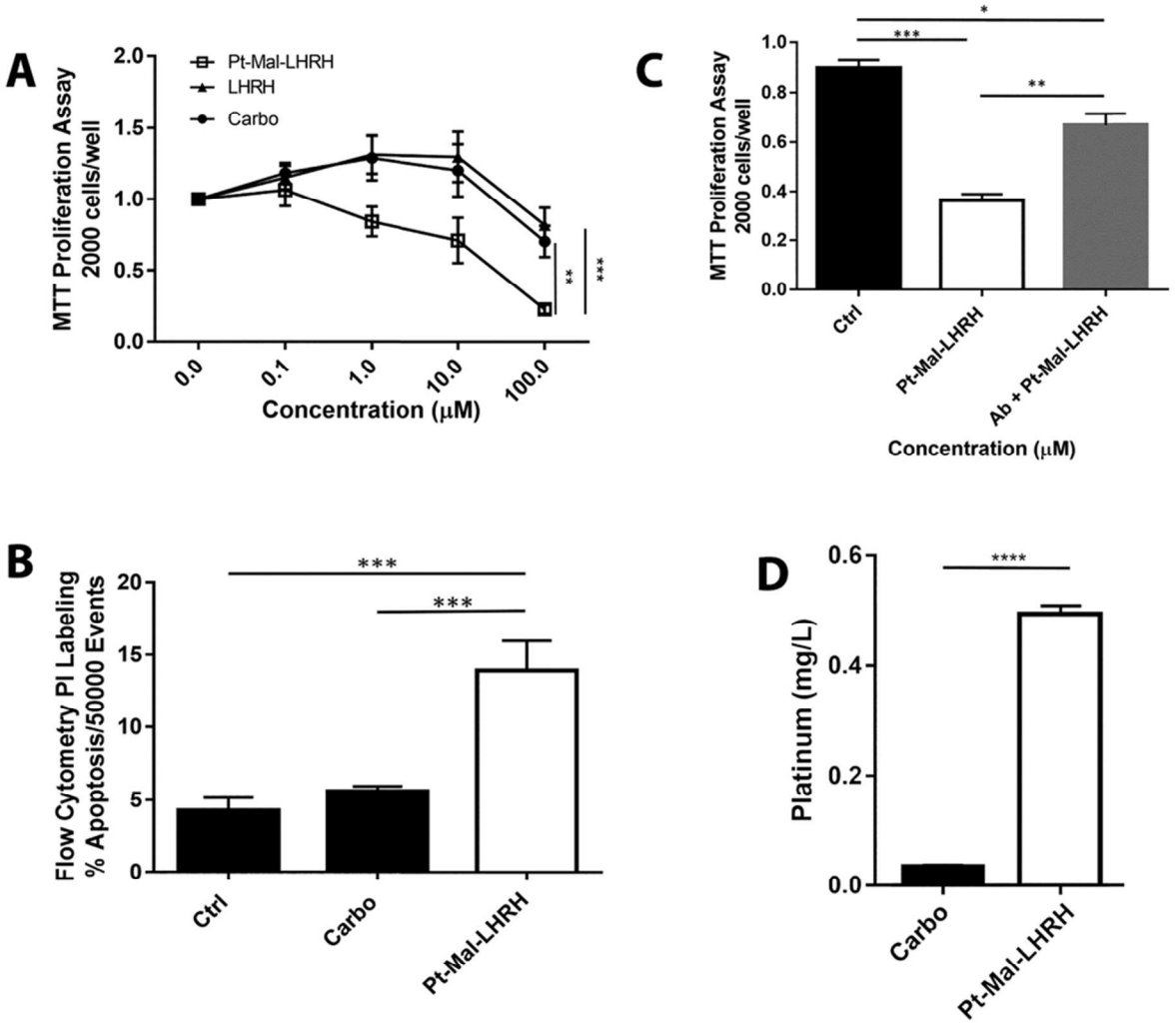

Figure 2: Pt-Mal-LHRH attenuates breast cancer cell viability through increased cellular uptake. 4TI breast cancer cells were treated with Pt-Mal-LHRH, LHRH, or carboplatin (carbo) from a range of 0.1 μM to 100 μM for 24 hours. Viability rates were analyzed by a MTT assay after the cells were

incubated for 48 hours **(A).** Pt-Mal-LHRH was Flow cytometric analysis of cell apoptosis was conducted by treating cells with Pt-Mal-LHRH or carboplatin (100 μM) for 4 hours, then washing the collected cells followed by incubating the cells in 20 mg/ml PI for 15 minutes **(B).** Free LHRH antibody decreases the cytotoxic effect of Pt-Mal-LHRH. Cells were pretreated with free LHRH antibody (100 μM) for 30 minutes and then concurrently with Pt-Mal-LHRH (100 μM) for 24 hours. Cellular viability was analyzed by MTT assay after 48 hours **(C)**. To measure drug uptake $1x10^6$ 4T1 cells were treated with Pt-Mal-LHRH or carboplatin (100 μM) for 24 hours. Cells were collected and metal (platinum) concentration mg/L was measured by ICP-MS **(D)**. (n=3); * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$; two-way ANOVA, one-way ANOVA and T-test.

Enhanced breast cancer cell selectivity is mediated by Pt-Mal-LHRH.

To determine the selectivity of Pt-Mal-LHRH towards breast cancer compared to normal fibroblast cells, a cell viability assay was conducted. Pt-Mal-LHRH was found to significantly attenuate cell viability in 4T1 cells compared to 3T3 normal fibroblast cells. This data indicates that the targeting ability of Pt-Mal-LHRH mediates selectivity towards breast cancer than normal cells (Figure 3).

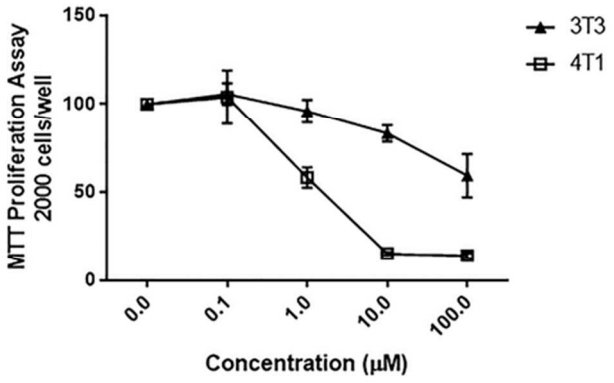

Figure 3: Pt-Mal-LHRH attenuates 4T1 breast cancer cell viability compared to normal cells (3T3). 4T1 and 3T3 cells were treated with Pt-Mal-LHRH from a range of 0.1 μM to 100 μM. Viability rates were analyzed by a MTT assay after 72 hours of treatment. (n=3); **** $p<0.0001$; Two-way ANOVA.

Pt-Mal-LHRH decreases breast cancer cell migration *in-vitro*.

The foremost cause of mortality in breast cancer patients is metastasis, in which 10-15% of women develop distant tissue colonization within 3 years after a primary tumor is diagnosed. [30] For this

reason we conducted an *in-vitro* scratch assay to investigate the ability of Pt-Mal-LHRH to retard cell migration. We found that Pt-Mal-LHRH significantly attenuated gap closure compared to untargeted carboplatin and LHRH (100 μM) treatments (Figure 4).

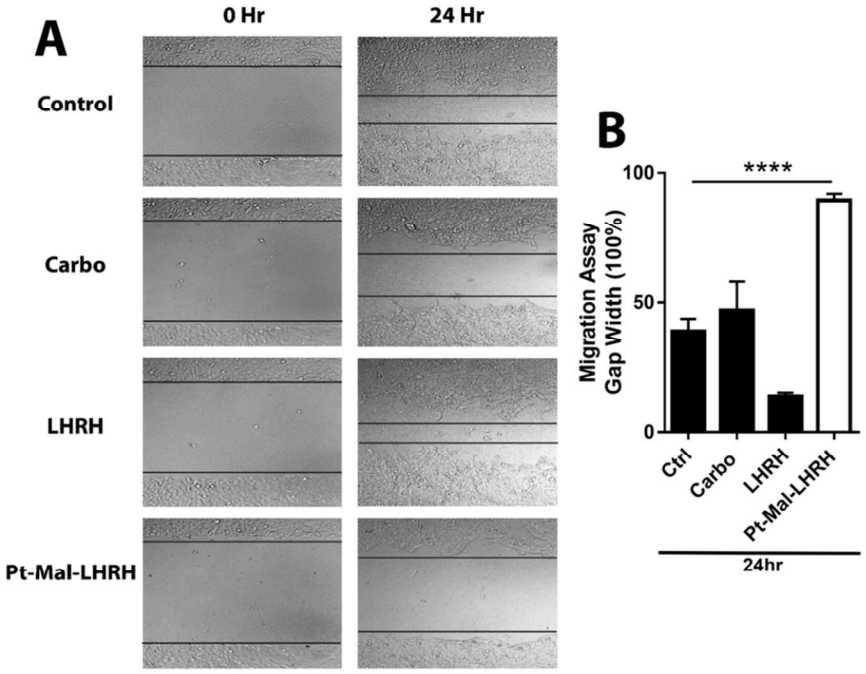

Figure 4: Breast cancer cell migration is decreased by Pt-Mal-LHRH. Cultured 4T1 cells were scratched under normal conditions (saline), carboplatin (carbo), LHRH, or Pt-Mal-LHRH (100 μM). Representative 10x images indicate Pt-Mal-LHRH treatment attenuated breast cancer cell migration **(A)**. Cell migration was quantitatively evaluated by measuring the distance between the scratch edges after 6 and 24 hours **(B)**. (n=3); **** $p<0.0001$; One-way ANOVA. Images were adjusted for brightness and contrast.

Pt-Mal-LHRH attenuates breast cancer tumor growth and lung metastasis *in-vivo*.

To support our *in-vitro* results, we examined the antitumor activity and the effects on body and liver weights of Pt-Mal-LHRH via *in-vivo* studies. Orthotopic 4T1 tumors grown in the right abdominal mammary fat pad were treated with saline, carboplatin, carboplatin plus LHRH (unbound), or Pt-Mal-LHRH by intraperitoneal injection of molar equivalent doses for two weeks. We purposefully chose a low treatment dose to optimally discern differences in the therapeutic effect of Pt-Mal-LHRH to carboplatin. Previous publications highlight the use of higher treatment doses of carboplatin to mediate a significant therapeutic effect on tumor growth.[31-34] As predicted no significant decrease in tumor growth was found

with carboplatin treatment compared to control mice; however, there was a significant decrease in tumor volume with Pt-Mal-LHRH treatment compared to control tumors (Figure 5A-B). Additionally, no difference in liver weights were found (Figure 5C) and cage-side observations did not detect any noticeable side effects from Pt-Mal-LHRH treatment. Lung examination revealed a significant decrease in lung tumor colonization in mice treated with Pt-Mal-LHRH compared to control and carboplatin treated mice (Figure 5D). Subsequently, Pt-Mal-LHRH mice showed a slight decrease in lung weight compared to control mice; however, significance was not reached (Figure 5E). Our *in-vivo* results indicate that Pt-Mal-LHRH results in regression of the breast cancer volume and metastasis.

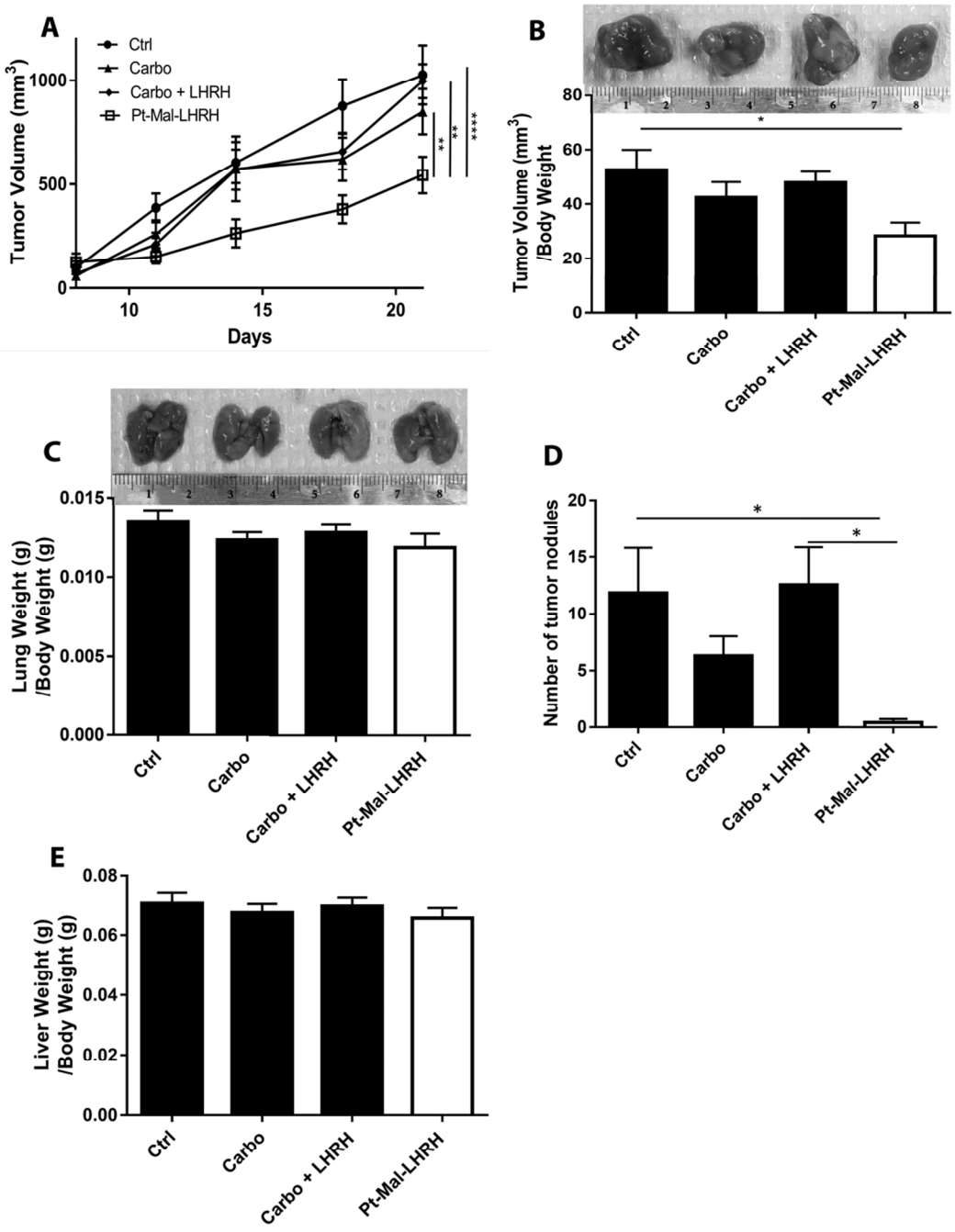

Figure 5: Breast cancer tumor growth is attenuated by Platinum-LHRH treatment. Female BALB/c mice were implanted with 4T1 cells ($1x10^6$) in the right second mammary fat pad. Tumors were grown for 7 days, distributed into treatment groups, and treated with carboplatin (carbo), carboplatin and LHRH, or Pt-Mal-LHRH (5mg/kg/wk) by intraperitoneal injection for 2 wks. Tumor volume was measured over the 2 wk injection period **(A)** along with end tumor weight **(B)**. Lung weight **(C)** Lung tumor nodule formation **(D)** and liver weight **(E)** was measured. (n=7); * $p<0.05$; ** $p<0.01$; **** $p<0.0001$; Two and One-way ANOVA.

Taken together, synthesis of Pt-Mal-LHRH produced a targeting chemotherapeutic agent which is more potent and selective towards breast cancer cells than carboplatin shown by our *in-vitro* results. Subsequently, our *in-vivo* results suggest Pt-Mal-LHRH attenuates breast cancer tumor growth and metastasis compared to control and carboplatin.

Discussion

The platinum family anticancer drugs are commonly used for chemotherapy of advanced carcinomas. Our research is to develop the platinum drugs into targeting agents, in order to increase the potency and efficacy while reducing systemic side effects. We synthesized the targeting chemotherapeutic agent Pt-Mal-LHRH to target the overexpression of the LHRH receptor on cancer cells relative to normal tissue. Studies have shown that LHRH receptors are overexpressed in breast, prostate, endometrial and ovarian cancers in comparison to normal cells making the LHRH receptor a good candidate for drug targeting.[14-17] Specifically, numerous experimental and clinical studies have shown overexpression of the LHRH receptor on approximately 50% of human breast carcinomas [35] and specifically in the 4T1 cell line.[28-29] Additionally, various non-reproductive cancers have been shown to overexpress the LHRH receptor including lung, bladder, pancreatic, among others.[36-38]

To form Pt-Mal-LHRH we have combined the two moieties (LHRH and activated cisplatin) using a malonate linker making the new conjugate target specific. In our synthesized platinum conjugate, we introduce an ester bond between the malonate linker and platinum. Prior published reports have shown that such a bond is hydrolyzed by cellular esterases, leaving the drug free to act at the cellular level.[12, 39-40] In our study we chose carboplatin as our untargeted platinum complex due to the fact that the malonate linker chelates platinum in a manner similar to that of carboplatin, as has been previously reported.[13] We

have conjugated activated cisplatin using the malonate linker on position 6 to ensure that the intrinsic properties of the LHRH peptide are maintained which would allow the delivery of the cytotoxic aquated platinum to the tumor cells.

One would expect that binding a platinum-based chemotherapeutic compound with a targeting agent should induce greater tumor toxicity with a concomitant reduction in systemic side effects. Chemotherapeutic agents which are given systemically without a significant targeting advantage should ideally have a low systemic toxicity or administered at a reduced dose to avoid complications. An example of this would be the use of BEL for the treatment of breast tumors, which in the animal model, has shown statistically significant reductions of tissue growth in both nicotine stimulated ($p< 0.01$) and non-nicotine stimulated breast cancer implants ($p < 0.05$).[41] We hypothesize an increase in the concentration of the drug Pt-Mal-LHRH in tumor cells as demonstrated in our *in-vitro* results would spare the normal cells from unnecessary exposure and advantageously allow for a higher dose of Pt-Mal-LHRH to be administered.

An examination of the current literature involving the use of LHRH with platinum drugs as a targeted treatment mechanism yields few results constituting effective or applicable targeted delivery. The few published studies utilizing platinum derivatives employ combinatory methods on cisplatin resistant tumors with LHRH and differ significantly in architecture and mode of delivery from the Pt-Mal-LHRH complex reported in this research.[42-43] For instance, LHRH-targeted nanogels show complications with free drug loading and slow release of the encapsulated cisplatin resulting in diminished cytotoxicity compared to free cisplatin.[43-44] However, our data demonstrated the successful synthesis of Pt-Mal-LHRH and its effectiveness on breast cancer. Additional research examining the effectiveness of Pt-Mal-LHRH on other cancers that express the LHRH receptor including but not limited to lung, bladder, and pancreatic will need to be performed.

Other modes of targeting platinum drugs that have been explored yielding limited results. Targeted platinum sugar complexes have been shown to have differences in activities between D and L - glucose conjugates; however, no increase in activity over the analogous carboplatin was found in either of the complexes.[45-47] Other complexes formed from a range of different sugars have been prepared, but the results of the biological testing have not been released.[47-48] Other studies targeting strategies for drug delivery vehicles against the estrogen receptor (ER) in breast cancer have included steroids as targeting units; however, these compounds did not fare better than cisplatin.[46, 49-52] In one study, the platinum conjugates cis-dichloro[N-(4-(17-ethynylestradiolyl)-benzyl)-ethylenediamine]platinum(II) and cis-diamino[2-(4-(17-ethynylestradiolyl)-benzoylamino)-malonato]platinum-(II) enhanced proliferation which made things worse and rendered them unsuitable for further investigation.[53] It has been shown that various cancer cell lines overexpress a glycoprotein that acts as a folate receptor. Cisplatin and carboplatin derivatives bearing folate units were prepared; however, the conjugates had low solubility in water which prohibited their use in biological experiments.[46, 54] Aronov et al. used a PEG spacer to enhance water solubility but the conjugate was found to be less cytotoxic than carboplatin.[55] In regards to peptide-based targeting, instances in which targeting has been successfully achieved with platinum(II) conjugates are few. One successful example is the use of cyclic peptide CNGRC sequence that targets the CD13 receptor overexpressed on the surface of certain cancer cells.[13]

The cytotoxic mechanism of action of Pt-Mal-LHRH mediates enhanced selectivity and efficacy through use of the LHRH moiety to bind to the LHRH receptors which leads to internalization of the compound. Our *in-vitro* data utilizing 4T1 cells supports the theory that Pt-Mal-LHRH increases cytotoxicity through enhanced cellular uptake by over 20- fold increasing the bioavailability of carboplatin and further augmenting its *in-vivo* tumoricidal properties. The increased tumor concentration may also allow a decrease in the systemic dosage of the chemotherapeutic agent with a concomitant decrease in system toxicity and side effects. This is an important consideration because of the carboplatin's narrow therapeutic index. Moreover, there are three cellular mechanisms which may impart

a resistance to platinum based drugs. These include decreased uptake, increased repair of DNA damage and increased drug deactivation.[56] It would be expected that the enhanced uptake of platinum with the Pt-Mal-LHRH compound may result in mitigating these resistance mechanisms, either directly or if saturation can be obtained. Subsequently, once, Pt-Mal-LHRH is internalized it will be cleaved releasing the aquated platinum to bind to the DNA forming DNA adducts resulting in cytotoxicity. We postulate the DNA adduct formation will be similar to carboplatin which preferentially attacks the sequences of GG>>AG>GA>GXG (X=an undefined residue), in that order.[57-58]

One possible effect of Pt-Mal-LHRH is the targeting of the ovaries, adrenal and pituitary gland. Chemical oophorectomy, adrenalectomy and hypophysectomy have been used in the treatment of advanced breast cancer.[59] If targeting of these organs take place, it is possible that it will be selective and possibly affect only some of the endocrine functions. Interestingly, one study demonstrates that the damaging effect to the pituitary gland by a targeted cytotoxic analog of LHRH was found to be reversible after 2 wks of treatment.[60] Further research into this area along with determining the exact mechanisms of tumorcidal activity, and potential risks and benefits is needed.

In addition, we observed that LHRH treatment modestly stimulated tumor growth and migration. This finding is expected since it is known that LHRH antagonists are effective breast cancer treatments.[61] It has been shown that LHRH treatment promotes proliferation through reducing the number of resting cells in the G0-phase.[62] Subsequently, our results demonstrate that tumor growth and metastasis stimulated by LHRH is slightly greater than the control. However, we were unable to demonstrate tumor cytotoxicity augmentation with the administration of un-linked carboplatin with LHRH treatment; therefore, the optimal timing, dosage and frequency of administration was not investigated.

We found Pt-Mal-LHRH significantly enhanced cellular cytotoxicity in 4T1 cells compared to the 3T3 normal cell line. Taken together, both our *in-vitro and in-vivo* data suggest Pt-Mal-LHRH elicits tumor-targeted drug delivery with increased potency, efficacy, and a possible reduction in

chemotherapeutic side effects which would allow a full or higher dose of chemotherapy to be used in a patient, as compared to other platinum drugs. Subsequently, our *in-vitro* scratch assay data demonstrated a reduction in migration. Importantly, *in-vivo* metastasis was investigated since the major cause of mortality in breast cancer patients is metastasis to distant sites including the lungs. Our *in-vivo* data supports our cellular data, as we found a significant decrease in tumor volume and lung tumor colonization through Pt-Mal-LHRH treatment.

The advantage of the Pt-Mal-LHRH conjugate is selectivity toward tumors that overexpress LHRH receptors locally at the site of their growth, while avoiding systemic distribution. Our approach to tumor-targeted drug/delivery will not be limited specifically to breast cancer, but could, in general, have applications in other metastatic cancers that overexpress the LHRH receptor such as bladder, melanoma, and pancreatic cancers, among others.

Conclusion

In summary, we have demonstrated the synthesis of a new chemotherapeutic agent, Pt-Mal-LHRH, designed to selectively target cancer cells overexpressing the LHRH receptor. Our *in-vitro* results using 4T1 breast cancer cells indicate increased cytotoxicity and uptake compared to carboplatin, along with selectivity towards breast cancer compared to normal cells. In addition, our *in-vivo* results indicate Pt-Mal-LHRH treatment significantly decreases tumor volume and metastasis to the lung. Our results indicate that Pt-Mal-LHRH has increased potency, efficacy, and selectivity towards breast cancer cells overexpressing the LHRH receptor compared to carboplatin. Pt-Mal-LHRH has the potential to be a clinically used chemotherapeutic agent.

Experimental Procedures

Reagents

All starting reagents listed below were obtained from commercial sources and used without further purification: *N*,*N*-diisopropylethylamine (DIEA), acetic acid, dichloromethane (CH_2Cl_2), trifluoroacetic acid (TFA), N-methylmorpholine (NMM), triisopropylsilane (Tips), dry solvents, $AgNO_3$, cis-diammineplatinum(II) dichloride (Cisplatin), were obtained from Fisher Scientific or Sigma Aldrich; all amino acids and activators were from Novabiochem or AGTC Bioproducts.

NMR Spectroscopy

The NMR spectroscopy was used to analyze and confirm the products obtained in each steps of linker formation. All ^{1}H NMR spectra were recorded on 400 MHz JEOL Eclipse+ NMR Spectrometer and processed with Delta™ NMR software.

Mass spectrometry

Analysis was done on a Thermo Scientific LTQ XL Mass Spectrometer using a DART (Direct Analysis in Real Time) or (Electrospray Ionization) ESI on an Agilent Technologies instrument processed with Analyst QS1.1 (Applied Biosystems) or Mass Hunter (Agilent)

HPLC

Analytical high performance liquid chromatography (HPLC) was performed to confirm purity of LHRH-Mal using an Agilent 1100 series multisolvent delivery system with a DAD detector controlled by Agilent Chem Station Plus with detection at 220 nm.

Inductive Coupled Plasma-Optical Emission Spectroscopy

Platinum uptake was analyzed using a Varian, Vista MPX CCD simultaneous spectrometer. PlasmaCal Pt (1000 μg/mL) from SCP Science was used for calibration by diluting it to standard solutions of known concentration.

Synthesis of Malonate (Mal) Linker

The malonate linker, Di-tert-butyl 2-(3-(4-Carboxybutanamido)propyl)-malonate (Mal) was synthesized as previously described by Ndinguri et al.[13] All analyses matched the reported ^{1}H NMR and mass spectra values.[13]

Synthesis of Malonate- LHRH (Mal-LHRH)

The Mal-LHRH decapeptide was synthesized using Fmoc solid phase chemistry techniques and then chelated to activated cisplatin. Briefly, Fmoc-Rink Amide-AM resin (0.4 mmol) was placed onto a reaction vessel. The resin was then washed with DMF and DCM in continuous-flow mode using a PS3 peptide synthesizer. All couplings employed four equivalents of amino acid and (2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) and sometimes (7-Azabenzotriazol-1-yloxy)tripyrrolidinophosphoniumhexafluorophosphate (PyAOP) dissolved in 0.4 M N-methylmorpholine (NMM) in DMF at room temperature. Coupling involved minimal preactivation times. The side chain-protected amino acid derivatives Fmoc-Gly-OH, Fmoc-Pro-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Leu-OH, Fmoc-DLys(Alloc)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-His(Trt)-OH and Boc-Glu(OtBu)-OH were used. The Alloc protecting group was removed using tetrakis(triphenylphosphine)palladium(0) along with a 37:2:1 mixture of methylene chloride, acetic acid, and NMM for 2 hours, followed by washing and double coupling of malonate linker (Mal). Intermediate products were washed between reactions with DMF. The Fmoc group was deprotected with 20% piperidine in DMF for 3 min. Using a cocktail of trifluoroacetic acid: water: triisopropylsilane (TFA:H_2O:TIPS) (90:5:5:) (15 mL), the peptide was cleaved from the resin, precipitated and dried under

vacuum to give the crude peptide conjugate Mal-LHRH. Mal-LHRH was purified by HPLC or gel filtration using Sephadex G-10. The purified samples were lyophilized before use in the next step. The crude peptide was purified using HPLC on reverse phase C18 column with a linear gradient from 5% to 80% B eluent in 15 min; t_R 5.5 min (see Supporting Information). The purity of the compound was also confirmed using mass spectrometry. Yield, 39%, ESI-MS $(M+H)^+$, calculated for $C_{70}H_{101}N_{19}O_{20}$, 1528.69 found 1528.7304 (see Supporting Information).

Synthesis of Pt-Mal-LHRH

Reaction of Mal-LHRH and activated cisplatin yielded Pt-Mal-LHRH, see Figure 6. Mal-LHRH was dissolved in water, and the pH of the solution was adjusted to 7 by titration with 1 M NaOH. A solution of cis-$[Pt\text{-}(NH_3)_2(H_2O)_2]^{2+}(NO_3)_2$ was made by stirring cis-$PtCl_2(NH_3)_2$ and $AgNO_3$ vigorously overnight in water in the dark. A precipitate of AgCl formed was filtered to give cis-$[Pt\text{-}(NH_3)_2(H_2O)_2]^{2+}(NO_3)_2$ (*35, 36*). A 1.2 fold excess of cis-$[Pt\text{-}(NH_3)_2(H_2O)_2]^{2+}(NO_3)_2$ (20 mmol) was reacted with Mal-LHRH (30 mg, 20 mmol) to form the Pt-Mal-LHRH. Yield, 15-17%, ESI-MS (TOF) gave a signal at 1756.760 $(M + Na)^+$ calculated for $C_{70}H_{101}N_{21}O_{19}Pt$ 1756.72

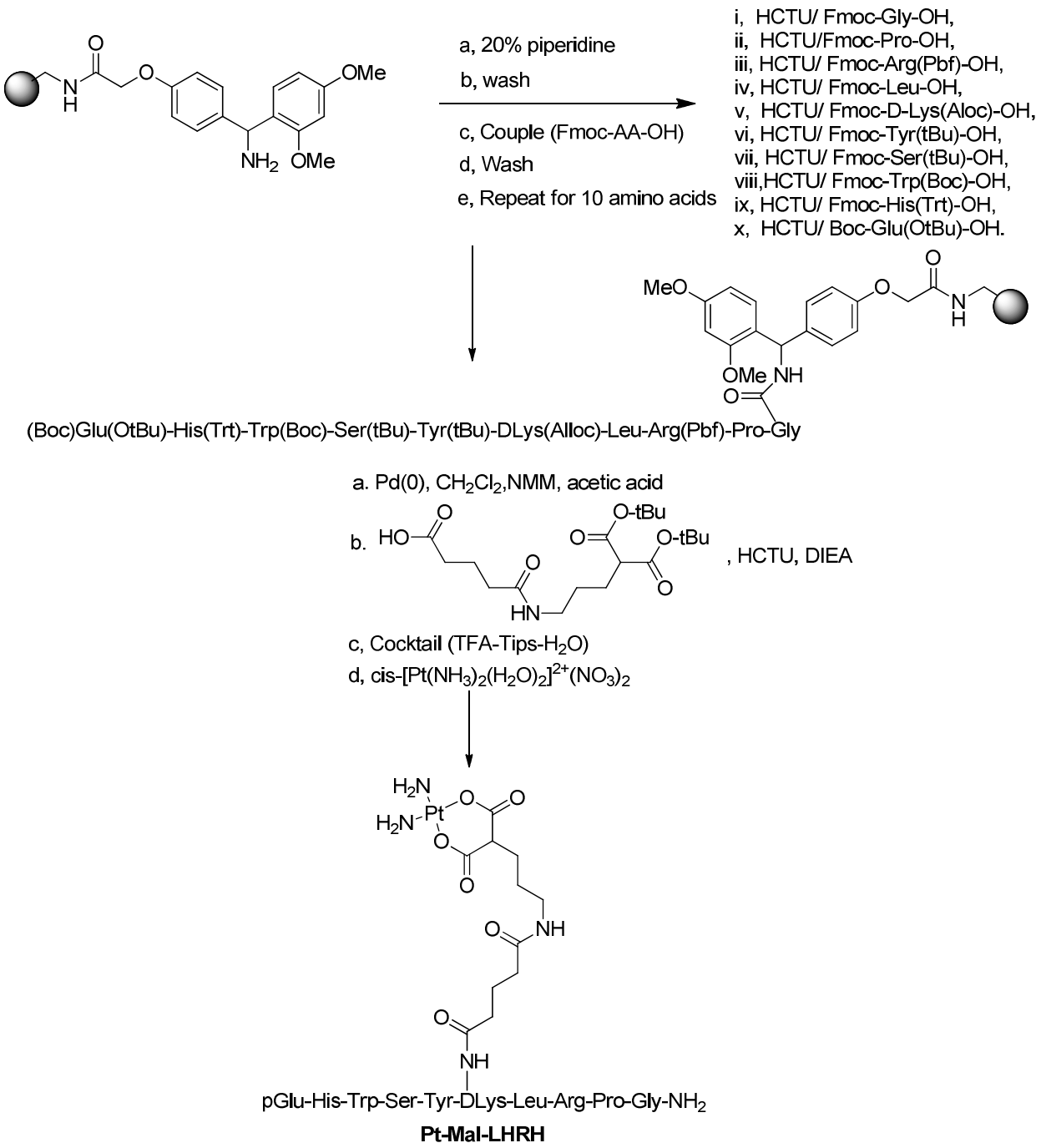

Figure 6: Synthesis of Pt-Mal-LHRH. Synthesis of Mal-LHRH decapeptide using solid phase chemistry and conjugation to activated cisplatin - Aminomethyl-ChemMatrix resin was coupled with 10 different amino acids in the sequence indicated above. The allyloxycarbonyl (Alloc) protecting group on the D-lys was selectively removed using palladium. The amine end of the D-lys was coupled with Mal and the conjugate cleaved from the resin using TFA-Tips-H_2O cocktail to yield LHRH-Mal. Reaction of LHRH-Mal and activated cisplatin yields Pt-Mal-LHRH

Cell Culture

The 4T1 mouse mammary tumor cell line and 3T3 mouse embryonic fibroblast cell line was obtained from ATCC and maintained as previously described.[41] In addition, the MDA-MB-231 cell line was provided by Dr. Michael Kilgore at the University of Kentucky (purchased from ATCC which authenticates the cell line through short tandem repeat (STR) profiling). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum and 100 U/ml penicillin and 100 μg/ml streptomycin.

Cell Viability Assay

To assess the ability of Pt-Mal-LHRH to affect cell viability an MTT Assay was utilized. 4T1 or MDA-MB-231 cells were seeded 2000 cells/100μl in a 96-well plate. The wells were treated with PBS, carboplatin, LHRH, or Pt-Mal-LHRH from a range of 0.1 μM to 100 μM for 24 hours. Cells were then washed with PBS and cultured in DMEM supplemented with 10% FBS for 48 hours. After this the cells were incubated at 37^{o}C for 4 hours in 10 μl MTT solution obtained from Vybrant MTT Cell Proliferation Assay Kit (Life Technologies). Cells were solubilized and mixed with SDS (sodium dodecyl sulfate) and the absorbance read at 595 nm on a Phenix Genios Tecon 96 well plate reader.

Cytotoxicity Assay

Apoptotic cells were detected by flow cytometry using propidium Iodide (PI) staining of DNA fragments to determine drug cytotoxicity. 4T1 cells were seeded 1X10^{6} cells/well and treated with 100 μM concentration of Pt-Mal-LHRH or carboplatin for 4 hours. Cells were collected, washed 3 times with PBS and incubated with 20 μg/ml PI for 15 minutes. Next, flow cytometry was carried out using a FACSCalibur^{TM} flow cytometer (Becton-Dickinson) at the University of Kentucky flow cytometry and cell sorting core facility. A total of 50,000 events were acquired and analyzed.

Competition Assay

To determine binding of Pt-Mal-LHRH to the LHRH receptor for entry into the cell a competition assay was conducted. 4T1 cells were seeded 2000 cells/100μl in a 96-well plate. Cells were pretreated with free LHRH antibody (EMD Millipore Corp. AB1567) 100 μM for 30 minutes and then concurrently

with Pt-Mal-LHRH 100 μM for 24 hours. Cells were then washed with PBS and cultured in DMEM supplemented with 10% FBS for 48 hours. A cell viability assay was then performed as described above.

Drug Uptake Assay

To determine cellular uptake of Pt-Mal-LHRH, 4T1 cells were seeded $1X10^6$ in 6 well plates and treated with either carboplatin (100 μM) or Pt-Mal-LHRH (100 μM) for 24 hours. Cells were washed 3 times with PBS, collected, and metal (platinum) concentration mg/L as measured by ICP-Mass spectrometry at Louisiana State University as previously described.[13]

Cell Migration

Cell migration was examined using a scratch assay. 4T1 cells were cultured in a 6-well plate until a confluent monolayer was formed. The wells were scratched with a 20 μl pipet tip, rinsed with PBS, and cultured in DMEM supplemented with 10% FBS. The wells were treated with PBS, carboplatin, LHRH, or Pt-Mal-LHRH. A concentration of 100 μM was used for all compounds. Four representative 10x images were taken at 0 and 24 hours and the gap width was quantified using an average of three leading edge measurements for each image.

***In-vivo* Syngeneic Isograft Model of Breast Cancer**

Female BALB/c mice, 10 weeks old with an average weight of 19.80g, were purchased from Jackson Laboratory (Bar Harbor, ME) and housed as previously described.[41] Animal protocols were approved by the committee on animal research care and use at Eastern Kentucky University.

4T1 cells ($1X10^6$) were suspended in 100 μl of DMEM not supplemented with FBS and injected into the right second mammary fat pad of female BALB/c mice as previously described in literature.[63] After 7 days of orthotopic tumor initiation (~$100mm^3$) the mice were treated with saline, carboplatin, carboplatin plus LHRH, or Pt-Mal-LHRH by intraperitoneal injection for two weeks. A molar equivalent dosage of 5mg/kg/wk was used for all compounds according to their molecular weights. Tumor growth was monitored daily and tumor volumes (mm^3) were calculated using the formula: $(width)^2 \times length/2$,

where width is the smaller of the two measurements. After 2 weeks of treatments the mice were sacrificed and tumor volume and organ weights were measured.

Statistics

Data are illustrated as mean ± SEM and statistical analyses were carried out using GraphPad, Prism 6 (San Diego, CA) using t-tests. One- or two-way ANOVAs were used where appropriate.

Acknowledgements: We would like to thank Dr. Thomas Blanchard for his help with the ICP analysis.

Funding Sources: Kentucky Biomedical Research Infrastructure Network (KBRIN) grants (P20GMI04489 and P20GM103436); EKU University Research Grant and National Institute of General Medical Sciences at the National Institutes of Health (grant # 1R25GM102776-01)

Supporting information: Supporting Information Available: MDA-MB-231 cytotoxicity graph, MDA-MB-231 uptake graph, HPLC analysis spectra and mass spectra analysis.

This material is available free of charge via the Internet at http://pubs.acs.org

Abbreviations: *N*,*N*-diisopropylethylamine (DIEA), dichloromethane (CH_2Cl_2), trifluoroacetic acid (TFA), N-methylmorpholine (NMM), triisopropylsilane (Tips), Dimethylformamide (DMF), cis-diammineplatinum(II) dichloride (Cisplatin), 2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU), 7-Azabenzotriazol-1-yloxy)tripyrrolidinophosphoniumhexafluorophosphate (PyAOP), Dulbecco's Modified Eagle Medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), American Type Culture Collection (ATCC), Fetal Bovine Serum (FBS)

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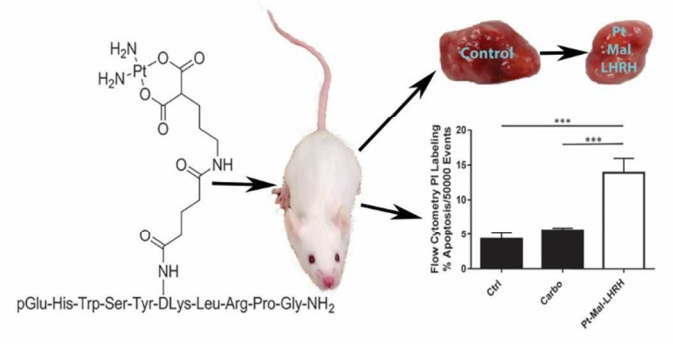
Control
Pt-Mal-LHRH
Flow Cytometry PI Labeling
% Apoptosis/50000 Events
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Ctrl
Carbo
Pt-Mal-LHRH
pGlu-His-Trp-Ser-Tyr-DLys-Leu-Arg-Pro-Gly-NH2

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