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Treating Triple Negative Breast Cancer with Targeted Nano-Therapy

Mikayla Bridges

PI: Katherine N. Weilbaecher, MD

Abstract

Bone metastasis in breast cancer patients is a prominent problem, with 70% of metastatic breast cancer patients having bone metastasis.^[1] The resulting complications are painful and decrease patient survival rate.^[1,3] This study is based on the previous lab work of Ross et al., who found that bone metastatic breast cancer cells over-express the integrin $\alpha\beta3$. Through a series of in vitro experiments, this study sought to investigate the degree to which the single lipid membrane $\alpha\beta3$ -targeted nanoparticles could function as a less toxic and more effective treatment to bone metastatic breast cancer.

Background

Bone metastasis in breast cancer patients is a prominent issue, with 70% of metastatic breast cancer patients having bone metastasis.^[1] The resulting complications are painful and can even cause pathological fractures and spinal cord compression, while also decreasing overall survival.^[1,3] Previous attempts to treat bone metastasis used hydroxyapatite-avid bisphosphonates; however, this method resulted in non-specific targeting in the bone matrix.^[1] More specific targeting was done by Ross et al., who exploited the fact that integrin $\alpha\beta3$ had low expression in most cells but is over-expressed in bone metastatic breast cancer cells. Their research used integrin $\alpha\beta3$ -targeted nanoparticles loaded with the chemotherapy drug docetaxel. The present study builds off of this work by using very similar nanoparticles that are instead loaded with a sphingosine kinase inhibitor. Sphingosine 1-phosphate (S1P) is a lipid that has been shown to support the growth and survival of tumor cells. S1P is formed through a process that requires the catalyzation of sphingosine phosphorylation.^[2] The molecules responsible for this catalysis are the sphingosine kinase. There are two sphingosine kinase isotopes, SK1 and SK2. SK1 is most prevalent and located in the cytosol, while SK2 is confined to the nuclei and mitochondria. Both SK1 and SK2 release a structurally identical S1P.^[4] Most research has focused on SK1 and found that its expression is higher in some tumor lines versus standard tissue cultures and has associated it with the survival of cancer cells.^[2] Through a series of in vitro experiments, this study sought to investigate the degree to which the single lipid membrane $\alpha\beta3$ -targeted nanoparticles could function as a less toxic and more effective treatment to bone metastatic breast cancer.

Materials and Methods

Cell Lines

The cells used in this study were 4T1 cells. These triple-negative breast cancer cells come from BALB/c mice. This study utilized wild type cells and a cell line that had been genetically modified using CRISPR to not express integrin $\alpha\beta3$. Both cell lines used were on their fourth passage. All cells were maintained at an optimal confluence with less than 2 million cells per flask in Dulbecco's Modified Eagle Medium (DMEM) was used with an added 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin (P/S).

MTT Assay

MTT assays were used to compare cell viabilities between experimental conditions. This study used 48-hour MTT assays. The reagent for the assays was the DMEM media with 1%

MTT reagent. DMSO was used as the solvent. The absorption spectrum was read at 570 nm and 630 nm. For the final analysis, the 630 nm wavelength was subtracted from the 570 nm wavelength to remove the ambient color.

Nanoparticles

The nanoparticles were a monolipid with an emulsified core. The emulsion contained polysorbate 80, the SK1 or SK2 inhibitor prodrug, and Dil, an orange-red fluorescent close in color to rhodamine. The prodrug is identical to the free drug versions of the SK1 and SK2 inhibitors but has been modified to be lipophilic. As a result, the prodrug is not active until it is cleaved by lipase in the cell, preventing toxic exposure in the body. The particles had a mean effective diameter of 12.9 nm and a mean zeta potential of 2.01 mV. The $\alpha\beta3$ -targeting ligand is a peptidomimetic coupled with MPB-PEG-DSPE for stability. This ligand can be seen in Figure 1. The procedure and material for synthesizing the nanoparticles have been left intentionally vague since it is proprietary information that has not yet been released to the public. The nanoparticles were designed by Dr. Gregory M. Lanza and made in association with Grace Cui. The nanoparticles used in this study were part of Batch XY13-104.

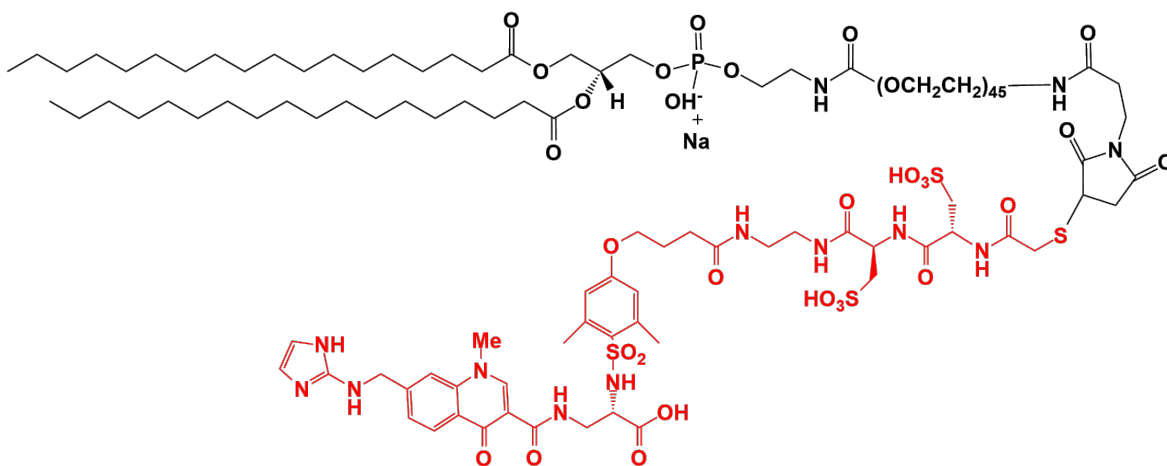


Figure 1. The $\alpha\beta3$ -targeting ligand is depicted here in red. The rest of the molecule functions as a stabilizer. These particles are the intellectual property of Gregory M. Lanza, MD, PhD, FACC and the Consortium for Translational Research in Advanced Imaging and Nanomedicine (C-TRAIN).

Statistical analysis

Statistical analysis was done using GraphPad's Prism 9 software. Statistical differences were determined using a one-way ANOVA with Tukey's test and multiple mean comparison. In addition, IC50 concentrations were determined using a dose-response four parameter non-linear regression. Occasionally the results of the regression were said to be

“unstable,” meaning that varying potential values had a nearly identical goodness of fit and curve, so an accurate IC50 value could not be determined.

Results

Experiment 1. Optimal Cell Concentration

This experiment aimed to determine the ideal concentration of cells to use in subsequent experiments. The concentrations tested were 1000, 2000, 3000, 4000, 5000, and 6000. Cells were plated and left untreated for the MTT assay. The resulting IC50 concentration was near 3000 cells per well. Since the subsequent experiments would use doxorubicin and additional treatment, a slightly higher cell concentration of 4000 cells per well was chosen.

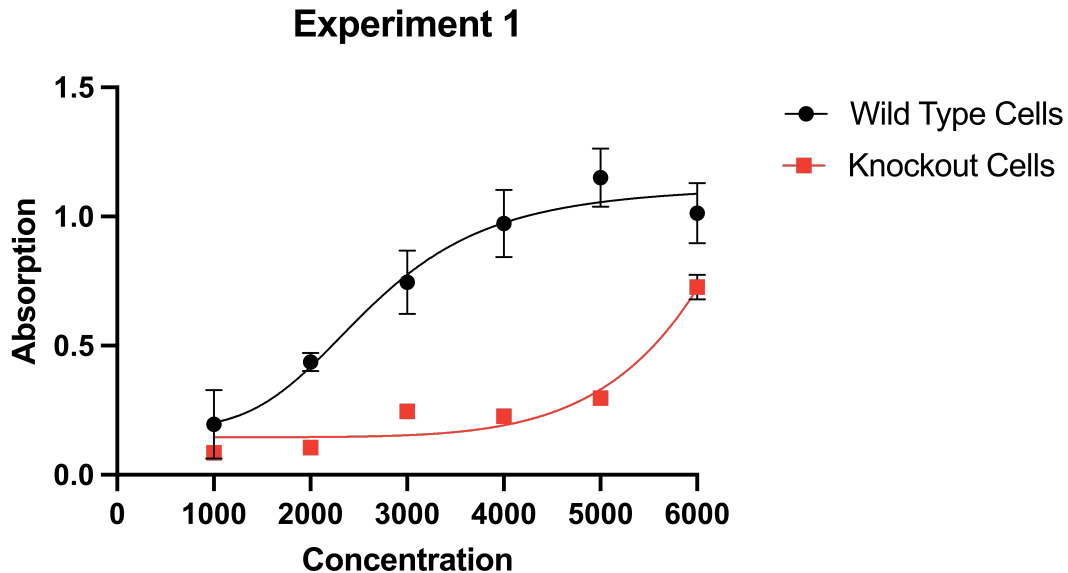


Figure 2. The resulting IC50 cell viability curve for untreated 4T1 wild type cells was 2627 cells per well and the regression was unstable for the $\alpha\beta3$ knockout cell line.

Experiment 2. Doxorubicin IC50 Curve

This experiment aimed to obtain a standard IC50 curve for the doxorubicin-treated cells. The cells were treated with concentrations of 0 nM, 10 nM, 20 nM, 30 nM, 40 nM, and 50 nM of doxorubicin for 48 hours. The results found that the IC50 value for wild type cells was 5.502 nM and 5.139 nM for knockout cells. The overall cell viability for the wild type cells were higher than the knockout cells.

Experiment 2

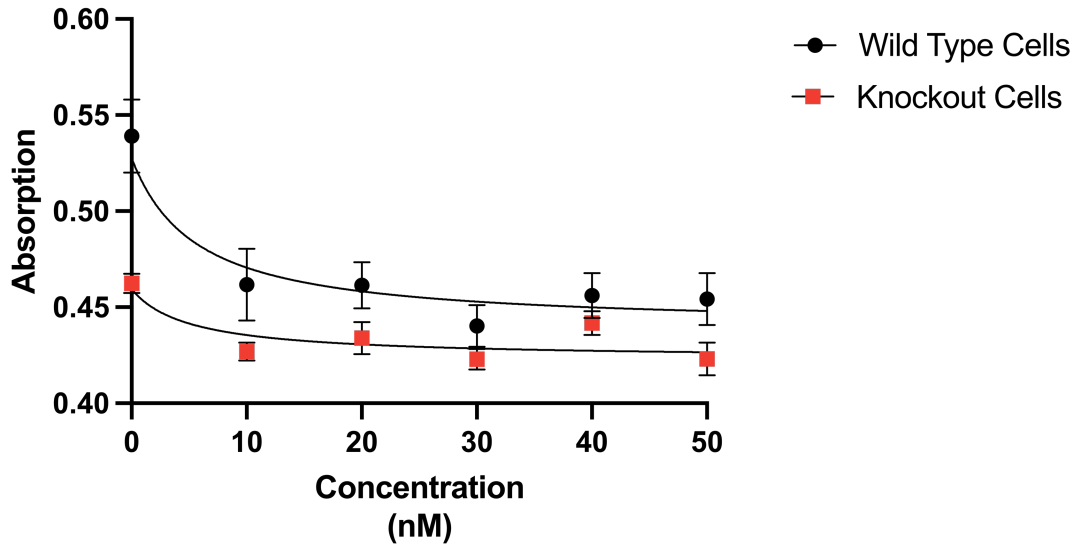


Figure 3. The resulting average cell viability with the associated IC50 curve from treating cells with varying concentrations of the chemotherapeutic doxorubicin.

Experiment 3. SK1 and SK2 Inhibitors

This experiment aimed to determine the effect of treating the cells with SK1 or SK2 inhibitor on the efficiency of doxorubicin treatment. The cells were treated for 48 hours with their respective inhibitor and doxorubicin given simultaneously. The resulting overall cell viability is nearly identical between the SK1 inhibitor and SK2 inhibitor treated cells. For the SK1 inhibitor, the IC50 value for the wild type control cells was 20.01 nM and the SK1 inhibitor treated wild type cells had a value of 19.40 nM; For the knockout cells, the control cells results were unstable and the SK1 inhibitor treated cells resulting IC50 value was 18.75 nM. Comparatively, there is a slight difference between the resulting cell viability when treating wild type and knockout cells with SK1 inhibitor. There is also a minor difference between the wild type control cells and the SK1 inhibitor treated cells. The data suggests that simultaneous treatment with SK1 inhibitor has a minimal effect on increasing the efficacy of doxorubicin. For the SK2 inhibitor, the IC50 value for the wild type control cells was 27.42 nM and 14.36 nM for the SK2 inhibitor treated cells; For the knockout cells, the IC50 value for control cells was 27.92 nM and the SK2 inhibitor treated cells had a resulting IC50 value of 22.58 nM. Comparatively, the IC50 value for the SK2 inhibitor treated cells was quite different between the wild type cells and the knockout cells. The results suggest that for SK2 inhibitor is more efficient at increasing chemotherapy susceptibility in wild

type cells than in the $\alpha\beta3$ knockout cells. The data also suggests that, for wild type cells, SK2 inhibitor is the best option in combination with doxorubicin for more efficient treatment. Overall, when comparing the treating of cells with doxorubicin to the treating of cells with doxorubicin and sphingosine kinase inhibitor, the IC50 value for the wild type cells is significantly lower with doxorubicin only than with the SK1 inhibitor combination treatment, while for SK2 inhibitor the difference was not significant. However, the cell viability is visually similar between Experiment 2 and Experiment 3.

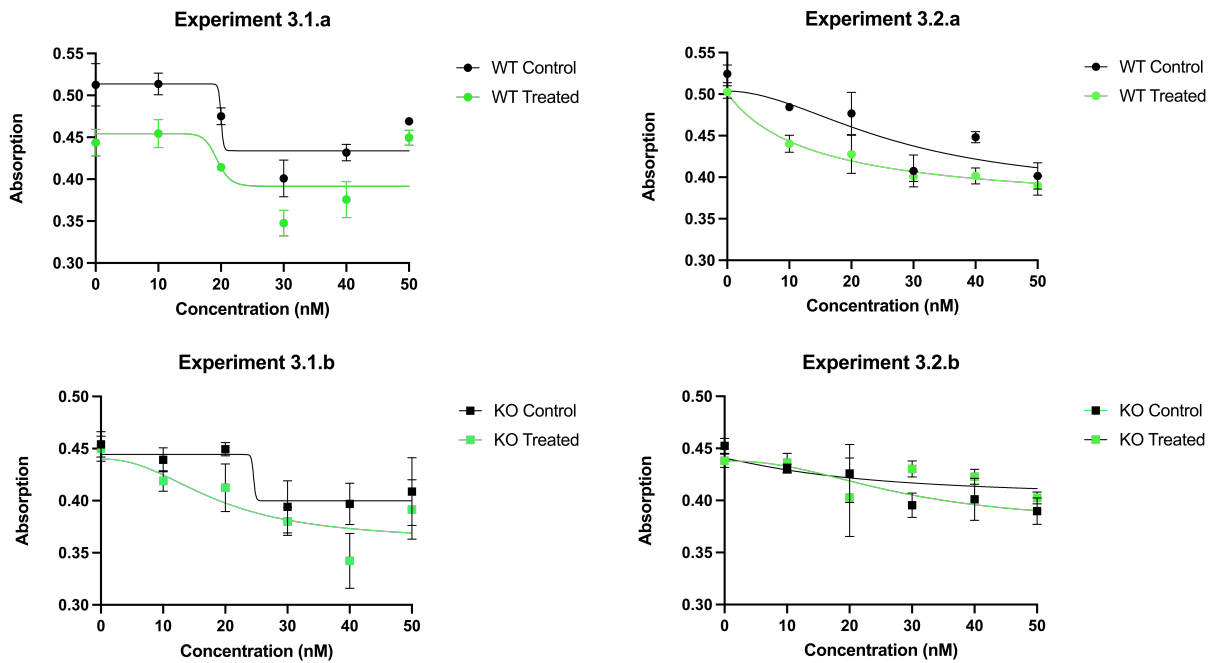


Figure 4. The results of an experiment determining the effect of treating cells with doxorubicin and sphingosine kinase inhibitors. The control cells received 0 moles of sphingosine kinase inhibitor. 3.1.x refers to treatments done with 1 μM of SK1 inhibitor and 3.2.x refers to treatments done with 5 μM of SK2 inhibitor.

Experiment 4. Nanoparticle Specificity

The purpose of this experiment was to verify that the nanoparticles were exhibiting targeting specificity to integrin $\alpha\beta3$. Cells were treated with their respective nanoparticles for 1 hour while being spun at 300 revolutions per minute. The spinning during treatment occurred to prevent incidental delivery since the nanoparticles could aggregate together, sink onto the 4T1 cells, and release their cargo through compression. The resulting IC50 value for the SK1 inhibitor loaded nanoparticles was 29.27 nM for the wild type cells and 18.41 for the knockout cells. For the SK2 inhibitor loaded nanoparticles, the IC50 value was 28.38 nM for the wild type cells and 25.52 nM for the knockout cells. The overall cell viability was similar for both SK1 and SK2 inhibitor treated cells. The data would suggest

that SK1 inhibitor nanoparticles had targeting in the knockout cells, which led to their high susceptibility to doxorubicin. However, since integrin $\alpha\beta3$ also plays a role in cell resistance to chemotherapy, these results cannot be concluded with only this information.^[2] Fluorescent microscopy did confirm that there were particle-knockout cell interactions. Figure 6 shows knockout cells with Dil in their periphery. This dye came from particle load release into these cells. Future research should prioritize a study that can quantify the number of non-specific interactions, like that which can be done through flow cytometry. This investigation could determine if the nanoparticles are binding significantly to the knockout cells, indicating they do not exhibit specific binding capabilities required for targeted delivery or that the supposed knockout cells still have a sufficient amount of integrin $\alpha\beta3$ expression. Additional research should be done on the optimal conditions for the nanoparticles to be the most efficient and have the most specific binding, starting with modifications to the duration of treatment time.

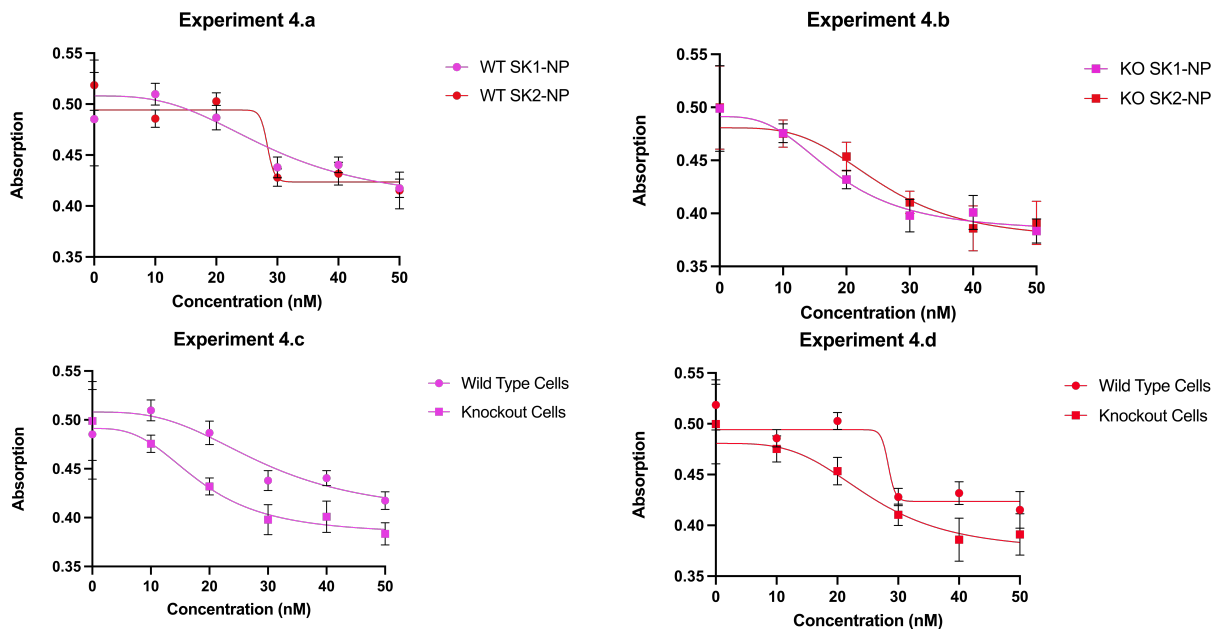


Figure 5. The IC50 curves for the SK1 and SK2 inhibitor loaded nanoparticles.

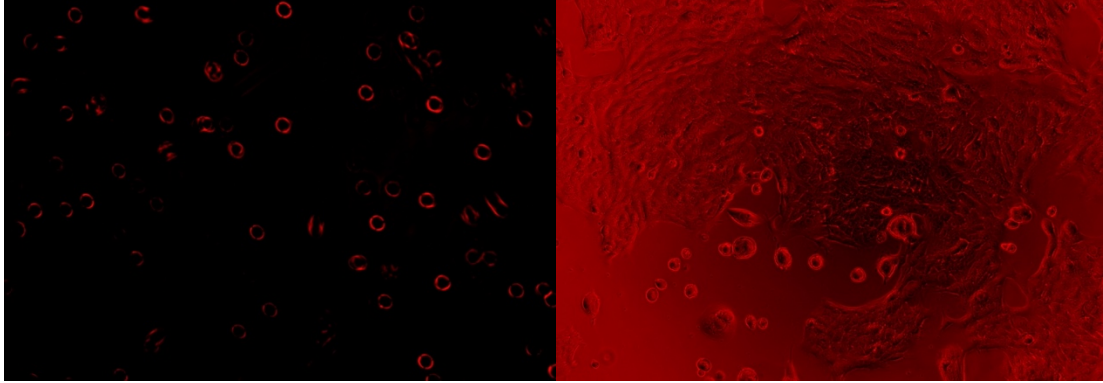


Figure 6. 4T1 cells genetically modified to not express integrin $\alpha\beta3$ demonstrating successful load transfer from the SK1 inhibitor nanoparticles. Similar was seen with the SK2 inhibitor nanoparticles.

Experiment 5. Efficacy of the Prodrug

The purpose of this experiment was to determine if the modifications used to make the sphingosine kinase lipophilic changed its efficacy. The term “free drug” refers to the original, non-lipophilic version of the sphingosine kinase inhibitors, while “prodrug” refers to the modified, lipophilic variation. The cells were treated for 48 hours with doxorubicin and 1 μM of either the free drug or prodrug of their respective inhibitor. For the SK1 inhibitor, the IC50 value for the wild type cells was 8.217 nM for the free drug treated cells and 14.34 nM for the prodrug treated cells; For the knockout cells, the resulting IC50 value was 53.73 nM for the free drug and 11.75 nM for the prodrug. The data indicates that the free drug is the more efficient treatment for the wild type cells, but not the knockout cells and that the prodrug is more efficient in the knockout cells than the wild type cells. For the SK2 inhibitor cells, the IC50 value for the wild type cells was 39.69 nM for the free drug and unstable for the prodrug; For the knockout cells, there was an IC50 value of 58.36 nM for the free drug and 16.00 nM for the prodrug. The data indicates that, like in the SK1 inhibitor cells, the prodrug is the most efficient treatment for the knockout cells and that the free drug is most effective in the wild type cells. For all the applicable conditions, the SK1 inhibitor prodrug and free drug had a lower IC50 value than its SK2 inhibitor counterpart, suggesting that SK1 is more vital to tumor cells' chemotherapy resistance.

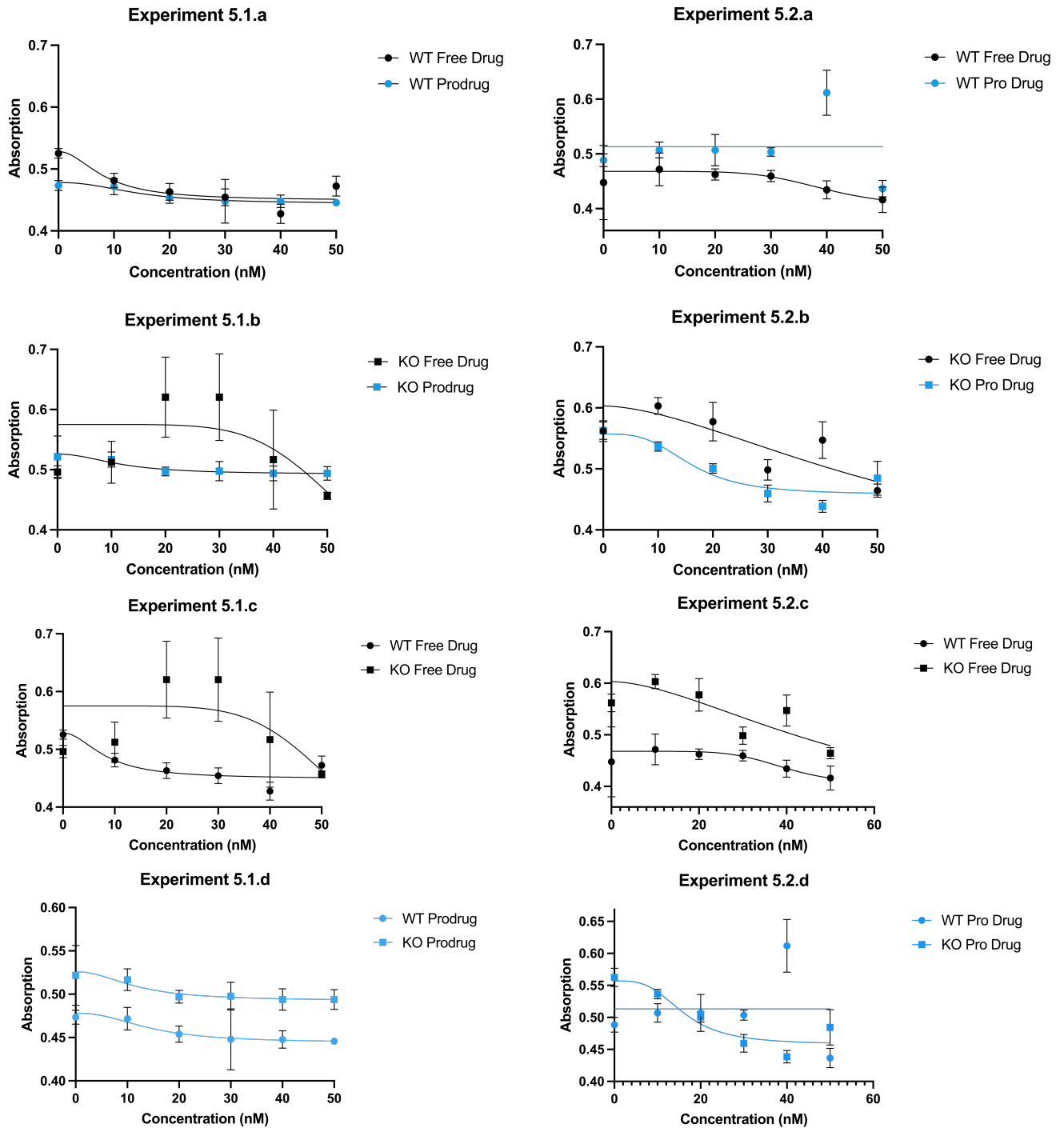


Figure 7. Comparison of the resulting IC50 curves from treating cells with doxorubicin and SK1 or SK2 inhibitor in free drug and prodrug form. 5.1.x refers to treatments done with SK1 inhibitor and 5.2.x refers to treatments done with SK2 inhibitor.

Experiment 6 Efficiency of Nanoparticles

The purpose of this experiment was to determine if the nanoparticles were more efficient than the prodrug. These cells were pre-treated with either a prodrug version of their respective inhibitor or the respective nanoparticle. Pre-treatment lasted for 1 hour. During this time, cells were spun at 300 revolutions per minute. After, the cells were treated with doxorubicin for 48 hours. For the SK1 inhibitor, the IC₅₀ value for wild type cells treated with the prodrug was unstable and 61.53 nM for those treated with the nanoparticles; For the knockout cells, the IC₅₀ value was 4.417 nM for the prodrug treated cells and unstable for the nanoparticle treated cells. For the SK2 inhibitor, the IC₅₀ value for the wild type was 48.67 nM for the prodrug treated cells and 19.81 nM for the nanoparticle treated cells; For the knockout cells, the IC₅₀ value was unstable for the prodrug treated cells and 25.55 nM for the nanoparticle treated cells. This data suggests that the SK2 inhibitor loaded nanoparticles were more efficient than SK1 inhibitor loaded nanoparticles at lowering chemotherapy resistance in the wild type cells. The data also indicates that the nanoparticles are the most efficient means of transmission for SK2 inhibitor.

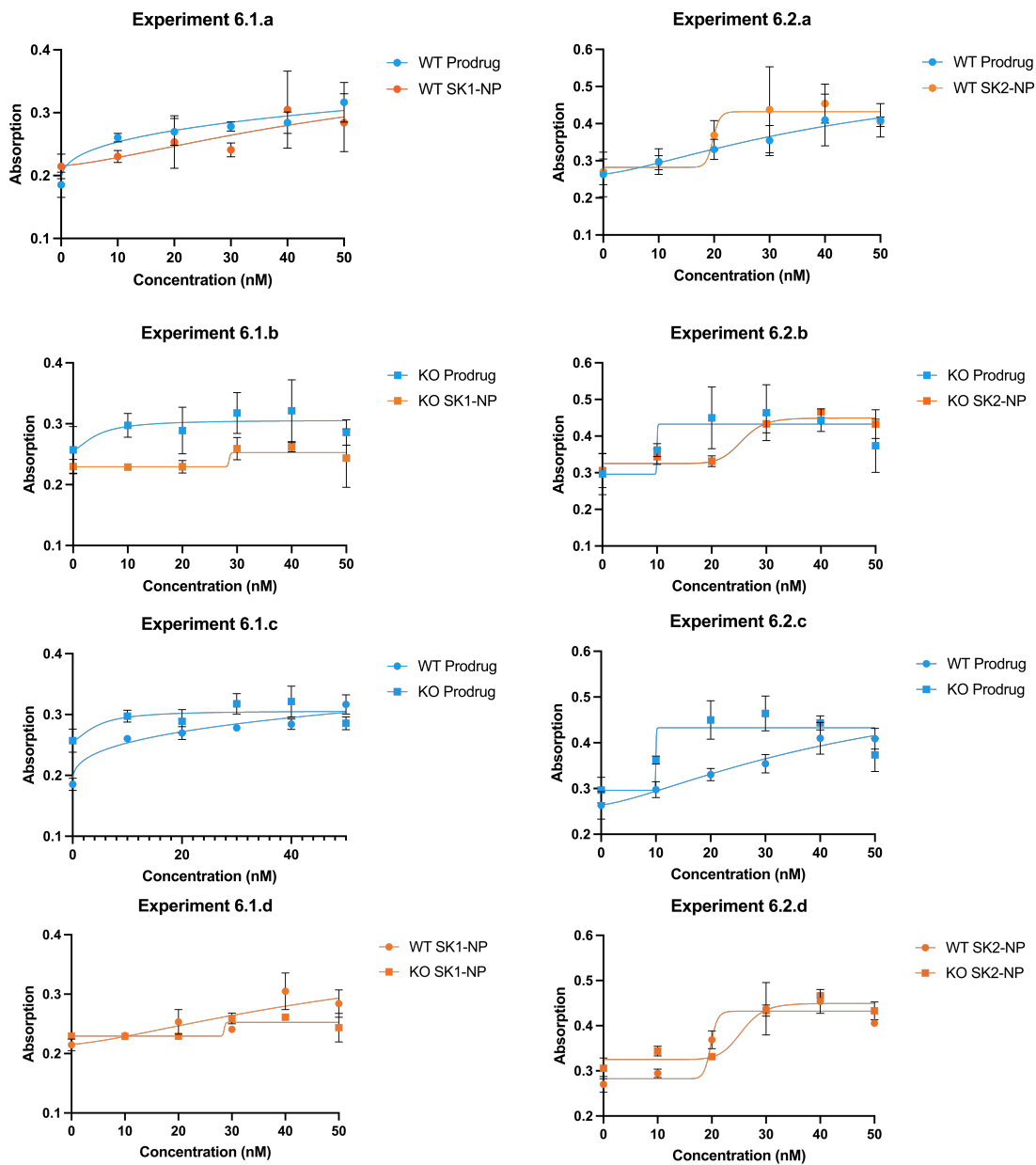


Figure 8. Comparison of the resulting IC50 curves from pre-treating cells with SK1 or SK2 inhibitor in either prodrug or nanoparticle form, then treating them with doxorubicin. 6.1.x refers to treatments done with SK1 inhibitor and 6.2.x refers to treatments done with SK2 inhibitor.

Conclusion

This study sought to determine if $\alpha\beta3$ -targeted nanoparticles loaded with sphingosine kinase inhibitor could be more efficient at treating breast cancer metastasizing in the bone. The series of experiments support previous lab findings that indicated that integrin $\alpha\beta3$ is

not only a marker but also has a role in tumor cells' resistance to treatment. The data of this study also indicate that pre-treating tumor cells with a sphingosine kinase inhibitor before treating them with doxorubicin has a more substantial effect on increasing doxorubicin susceptibility. This can be seen when comparing the overall cell viability in Experiment 6 versus Experiment 5 or Experiment 3, when the viability was higher. This study also showed that there was some degree of non-specific interactions with the integrin $\alpha\beta3$ knockout cells. As mentioned, future research should be done using flow cytometry to quantify these values and determine if either the knockout cells or the targeting mechanism of the nanoparticles is ineffective. Additionally, a future study that manipulates the treatment conditions could suggest if the nanoparticles exhibit specific binding and establish optimal conditions for in vitro nanoparticle administration.

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