Nanoparticle Retinoid Delivery: A Novel Functional Method for Inducing Cytotoxicity in Cancer

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Figures were constructed by the student researcher unless otherwise noted

Abstract

Retinoids, effective skin care drugs, have been shown to arrest cancer proliferation, leading to apoptosis. Retinoid delivery, however, remains faulty as the drugs are metabolized before reaching the targeted cells. Iron oxide nanoparticles in combination with four retinoids—All-trans retinoic acid, Adapalene, Tamibarotene, and Tazarotene—were used to overcome this limitation and were tested for efficiency in treating leukemia and breast cancer cell lines. Following the loading process, dynamic light scattering indicated that all nanoparticles maintained a hydrodynamic radius between 10-100 nanometers, therefore no aggregation occurred. Moreover, spectrophotometry confirmed that Adapalene, Tamibarotene, and All-trans retinoic acid were successfully loaded into the nanoparticles; nonetheless, Tazarotene was undetectable. Additionally, the CellTiter-Glo assay was completed to determine the effectiveness of individual retinoids and the retinoid-loaded nanoparticles in inducing cytotoxicity. In the leukemia cells, Post Hoc Tukey tests revealed that Adapalene across all concentrations exhibited immense cytotoxic effects ($p<0.0001$); likewise, Tamibarotene and All-trans retinoic acid at their highest concentrations elicited similar effects ($p<0.05$). It was also observed that the Adapalene and Tamibarotene loaded nanoparticles exhibited a decrease in cell viability ($p<0.05$). For the breast cancer cells, neither the retinoids nor retinoid-loaded nanoparticles were cytotoxic ($p>0.05$). This study is the first to provide strong evidence to support the use of Adapalene as a leukemia killing agent, as well establishes a foundation for retinoid-based nanotherapeutics. In addition, it is the first of its kind to successfully load retinoids into iron oxide nanoparticles to improve drug delivery, possibly aiding in the development of better, more effective treatments.

1.0 Introduction:

Cancer is the second leading cause of death in the United States today, accounting for an estimated 600,000 deaths annually[1]. Despite advancements in our understanding of cancer biology, the survival rate in patients has not dramatically improved, making it necessary to develop more efficacious treatments[2]. Furthermore, research has failed to create a treatment that specifically targets cancer cells[2-3].

Cancer often behaves like a stem cell, which is infinitely self-renewing[3]. Its proliferation is manifested by altered expression and/or the activity of cell cycle related proteins[3-4]. In addition, the survival of cancer stem cells may be in part due to alterations in cell metabolism[4]. Overall, cancer cells display abnormalities in the mechanisms that regulate normal cell proliferation, differentiation, and survival[4], thus making them increasingly difficult to eradicate. A major approach in cancer therapeutics is aimed at interrupting the cell cycle and breaking cancer proliferation.

Retinoids, natural and artificial compounds similar to vitamin A in structure and biological activity, typically used for skin disorders such as psoriasis or acne, have been found to promote cell differentiation and cancer cell death[5]. Tumor growth, angiogenesis, and metastasis are amongst the biological functions inhibited by retinoic acid; it is believed that these effects are achieved through the activation of the retinoic acid receptor (RAR) and retinoic X receptor (RXR)[6-7](Figure 1). Retinoids bind their nuclear receptors through ligand-binding domains[6-8]. These ligand-receptor and receptor-DNA interactions result in the modulation of direct retinoid target genes, which contain retinoid response elements in their promoter regions, becoming transcriptionally activated or repressed[7-9]. These cause alterations in gene expression that mediate biological effects, leading to cell differentiation, arrest, and apoptosis[10]. In particular, leukemia and breast cancer cells have exhibited such effects when treated with a retinoid known as all-trans retinoic acid (ATRA)[11-12]. However, the delivery of retinoids to targeted cells is challenging because of the rapid metabolism as well as somatic, genetic, or epigenetic changes causing cells to be retinoid-resistant[13-15].

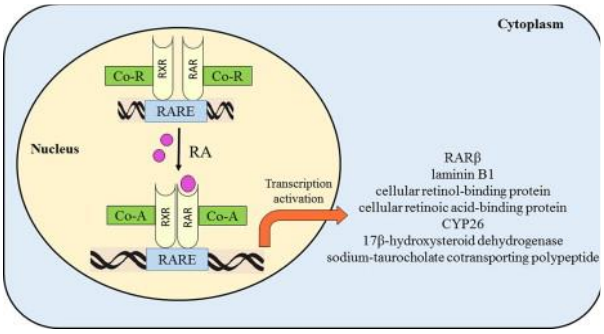

Figure 1. Retinoid signaling, (Ribiero et al 2014)

To confront these limitations, we turn to nanotechnology as recent advances in this field have shown to be quite promising for cancer treatments. Specifically, the use of Feraheme (FH) iron oxide nanoparticles as drug carriers has demonstrated potential and may provide better insight on therapy effectiveness[16-17]. Iron oxide nanoparticles are composed of an iron oxide core surrounded by a polymeric coating (Figure 2). For biomedical uses, nanoparticles are engineered to carry drugs within their polymeric coating, allowing them to travel unimpeded through the body to reach the targeted cancer cells[18-23]. Additionally, due to their nanoscopic size, nanoparticles have long retention time, are

biodegradable, and have low toxicity, allowing them to persist in various areas of the body without inflicting harmful side effects[24-29]. The intent of nanotechnology-based cancer research is to improve therapy by delivering drugs—capable of combating cancer or decreasing tumor size etc.—more efficiently via nanoparticles, therefore providing a potential method for the inclusion of retinoids in treatment.

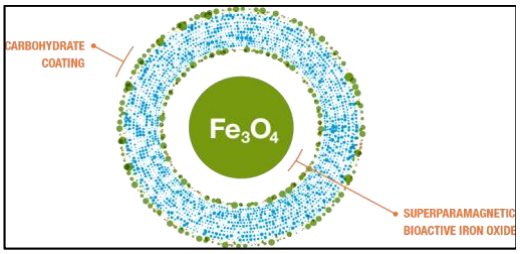

Figure 2. Above shows a diagram of a singular iron oxide nanoparticle. The iron oxide core (green) is surrounded by the polymeric coating (blue). The drug loading process causes the drug to diffuse through the carbohydrate outer coating and into the surrounding region (blue)[39]

Previous studies have utilized retinoid drug All-trans retinoic acid (ATRA) to kill both leukemia and breast cancer cells *in vitro*[11-12]. However, the obstacle of delivering the retinoids without being metabolized has not been addressed[13-15]. Therefore, to combat this problem, our study is the first to successfully load retinoids into iron oxide nanoparticles and show promising potential for inducing cancer cell death.

Consequently, we utilized Feraheme iron oxide nanoparticles to carry retinoid all-trans retinoic acid as it has been previously been shown to cause arrest in the cell cycle and lead to apoptosis[10]. Moreover, we tested three additional retinoid drugs—Tamibarotene, Adapalene, and Tazarotene—that have not been previously used to treat any cancer cell line. Both RAW 264.7 leukemia virus-induced tumor cells and T47D breast cancer cells were used in these experiments as they have been found to be retinoid sensitive[11-12]. Multiple concentrations of each drug were used to determine the ideal dosage to induce cell death. Dynamic light scattering was done to measure the hydrodynamic radius of the nanoparticle to judge the nanoparticle stability and solubility. Lastly, the Cell Titer-Glo Luminescent cell viability assay was completed to determine whether or not cell death occurred following treatment.

2.0 Purpose:

The purpose of this research was threefold:

1. Optimize the non-covalent loading of FDA-approved retinoids into Feraheme iron oxide nanoparticles.
2. Determine if All-trans retinoic acid, Adapalene, Tamibarotene, or Tazarotene can be used as a treatment for retinoid-sensitive cancers.
3. Determine if retinoid-loaded Feraheme iron oxide nanoparticles can elicit cytotoxicity in retinoid-sensitive cancers.

3.0 Hypotheses:

It was hypothesized that:

1. Retinoids can be successfully be loaded in Feraheme iron oxide nanoparticles.

2. All-trans retinoic acid, Adapalene, Tamibarotene, and Tazarotene can exhibit cytotoxic effects in leukemia and breast cancer cells.
3. Retinoid loaded Feraheme iron oxide nanoparticles can elicit cytotoxicity in leukemia and breast cancer cells.

4.0 Methods:

4.1 *Cell Culture*

Mouse leukemia virus-induced tumor cells (RAW 264.7) and breast cancer cells (T47D), purchased from the ATCC (Manassas, VA), were cultured in a tissue culture flask and passaged every three days. Cell passaging consisted of washing with Phosphate Buffer Saline (PBS), replacing the media (Dulbecco's Modified Eagle Medium and RPMI-1640), gently detaching cells with a cell scraper, transferring cell suspension into centrifuge tubes, centrifuging at 4000 rpm for 3-5 minutes, adding cell suspension into a new flask, and then being incubated at 37°C. Following incubation, cells were counted using the Thermo Fisher Scientific Countess Automated Cell Counter and were seeded in 96-well tissue culture plates based on number of live cells.

4.2 *Drug Loading*

The retinoid drugs All-trans retinoic acid, Tazarotene, Tamibarotene, and Adapalene were purchased from Sigma-Aldrich (St. Louis, MO). Drugs were diluted with dimethyl sulfoxide (DMSO) to the following two-fold concentrations: 10 μg/mL, 5 μg/mL, 2.5 μg/mL, 1.25 μg/mL, and 0.0625 μg/mL; each concentration received their own group. In addition, Feraheme iron oxide nanoparticles, purchased from AMAG Pharmaceuticals (Waltham, MA), were diluted with 500 μl of phosphate buffer saline (PBS) and then combined with 200 μl of the drugs each separately via dropwise addition while vortexing. The samples were run through the magnetic columns for purification and then washed with 300 μl of PBS (Figure 3). Iron particles are attracted to the magnets on the column therefore any unloaded drug is washed away. The nanoparticles were eluted by the removal of the magnetic field. All samples were stored at 4°C.

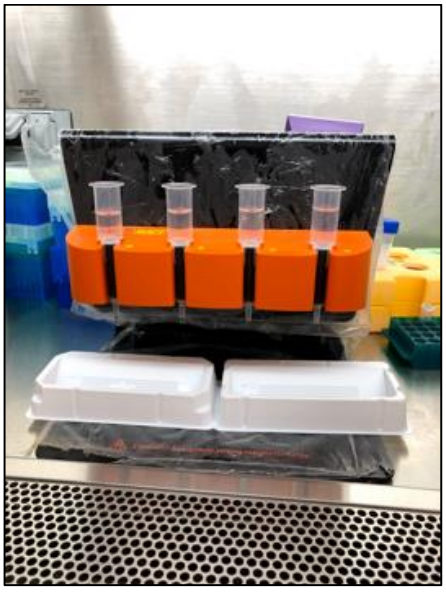

Figure 3.
Magnetic column for nanoparticle purification. Tubes were placed into the apparatus.

4.3 *Absorbance Spectrum*

Each retinoid as well as a DMSO blank was placed into a 96 well-plate and then into a SpectraMax iD5 spectrophotometer to determine the best wavelength to detect them in after they have been loaded into the nanoparticles. The absorbance spectrum was measured between 350nm and 1000nm.

Figure 4.
Nanoparticle samples were placed into a cannister and then lyophilized.

4.4 *Lyophilization*

All retinoid-loaded nanoparticle samples, as well the unloaded nanoparticles, were placed into vials (200µl per sample) and then into the lyophilization cannister and left overnight in the FreeZone 4.5 Liter Cascade Benchtop Freeze Dry System lyophilizer (Figure 3). This freeze-dries the samples to remove water so they can then be resuspended in DMSO to determine the drug concentration in the nanoparticles.

4.5 *Drug Detection*

The dried-out samples were removed from the lyophilizer and then diluted with 200µL of DMSO. From there, they were spun down in a centrifuge at 1400 rpm for 5 minutes, transferred into new 100µL tubes and then spun down again at 1400 rpm for 5 minutes**.** They were then put in a SpectraMax iD5 spectrophotometer and absorbance was measured at 350nm—the wavelength determined in Section 4.3. The iron quantification assay was also completed to measure the amount of iron present in the samples. From there, the ratio of drug to iron was mathematically computed using the values generated from drug absorbance and the iron quantification assays.

4.6 *Dynamic Light Scattering*

All retinoid-loaded nanoparticle samples, as well the unloaded nanoparticles**,** were diluted with 1000 µL of PBS and placed into a cuvette, to which was then placed in a Malvern Zeta Sizer to perform Dynamic Light Scattering. This measured the hydrodynamic radius of nanoparticles, from which we determined whether or not aggregation/clumping occurred as a result of the loading process to judge the particle stability and solubility.

4.7 *Experimental Trials*

Two separate 96-well tissue culture plates were seeded, one with the T47D breast cancer cells and the other with the RAW 264.7 leukemia virus-induced tumor cells. The All-trans retinoid acid loaded

nanoparticles, Adapalene loaded nanoparticles, Tamibarotene loaded nanoparticles, and Tazarotene loaded nanoparticles were placed into their own respective wells in a 96 well plate (Figure 5). Additionally, each concentration of the free All trans retinoic acid, Adapalene, Tamibarotene, and Tazarotene alone were placed into their own respective wells (Figure 5). A non-loaded nanoparticle group was added as a control, and untreated cells did not receive any form of treatment (Figure 5). All groups had duplicates. The plate was left to incubate at 37°C for 24 hours following treatment.

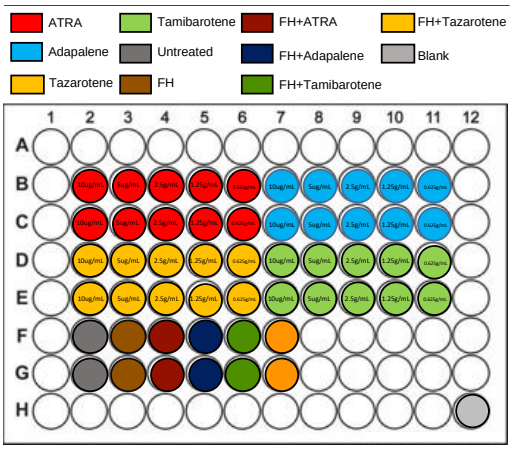

Figure 5.
Cells were treated with All-trans retinoic acid, Adapalene, Tazarotene, Tamibarotene, FH+ATRA, FH+Adapalene, FH+Tamibarotene, and FH+Tazarotene. In addition, there was an Untreated and FH group used for a control. All were done in duplicates. One well was left as a blank for the CellTiter Glo Assay.

4.8 *Cell Titer Glo Cell Viability Assay*

All media was aspirated from the wells and then replaced with 100 µL of the respective media. 100 µL of CellTiter-Glo reagent was added to each well and the plate was put into the SpectraMax iD5 spectrophotometer and luminescence was measured at 450nm. The addition of the CellTiter-Glo reagent results in cell lysis and the generation of a luminescent signal proportional to the amount of ATP present. From there, the amount of ATP is directly proportional to the number of cells present, allowing us to determine the cell viability; relative fluorescence was used as a measurement of cell viability.

4.9 *Data Analysis*

All data was analyzed in Microsoft Excel 2016 and GraphPad Prism 8. Differences between treatment conditions were measured using ANOVAs and Post Hoc Tukey tests. Statistical significance was set at $p \leq 0.05$.

5.0 Results:

5.1 *The structure of the retinoid loaded nanoparticles was not compromised by the loading process*

The dynamic light scattering determined the size distribution profile of the nanoparticles, more specifically the hydrodynamic radius. Any aggregation or clumping of the nanoparticles caused by the loading process indicates a change in the stability and properties of the nanoparticles, making them no longer viable for treatment. As a basis for comparison, the hydrodynamic radii of the unloaded nanoparticles were in the range of 10 nanometers to 100 nanometers (Figure 6). In the case of the retinoid-loaded nanoparticles, their hydrodynamic radii all remained in the same range of 10 nanometers to 100 nanometers (Figure 6). This ensured that the drug loading protocol did not alter the size or physical make-up of any of the retinoid loaded nanoparticles.

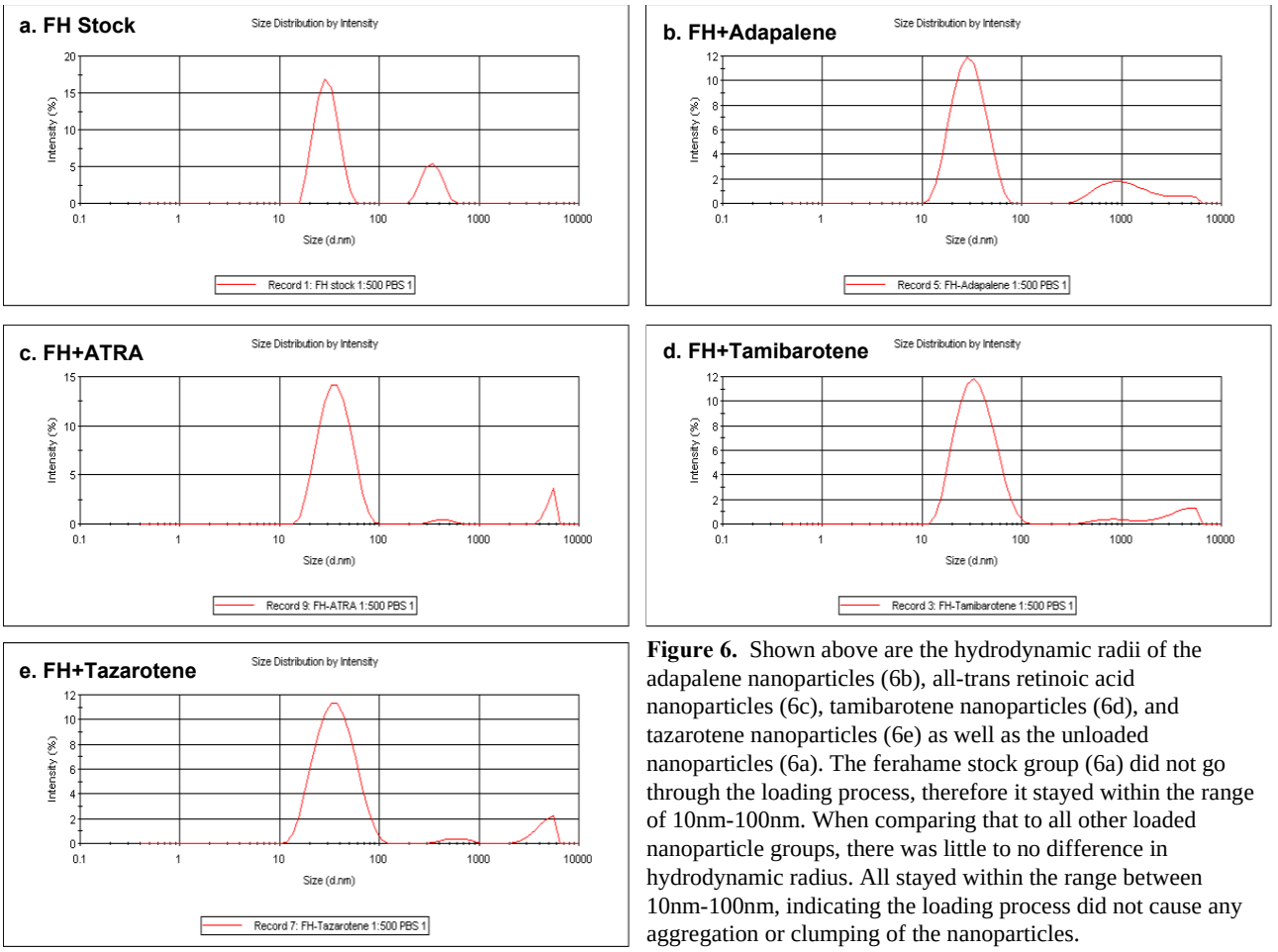

Figure 6. Shown above are the hydrodynamic radii of the adapalene nanoparticles (6b), all-trans retinoic acid nanoparticles (6c), tamibarotene nanoparticles (6d), and tazarotene nanoparticles (6e) as well as the unloaded nanoparticles (6a). The ferahame stock group (6a) did not go through the loading process, therefore it stayed within the range of 10nm-100nm. When comparing that to all other loaded nanoparticle groups, there was little to no difference in hydrodynamic radius. All stayed within the range between 10nm-100nm, indicating the loading process did not cause any aggregation or clumping of the nanoparticles.

5.2 *All-trans retinoic acid, Adapalene, and Tamibarotene were successfully loaded into iron oxide nanoparticles*

The lyophilization, dilution, and then the scanning of the samples via spectrophotometry allowed us to determine the amount of drug present in each of the retinoid-loaded nanoparticle samples. Moreover, the iron quantification assay provided us with the amount of iron present in each of these samples. From there, the ratio of these values was mathematically computed. Amongst the samples, the All-trans retinoic acid loaded nanoparticles held a drug payload of 1 nanogram per microgram of iron, the Adapalene loaded nanoparticles held a drug payload of 3 nanograms per microgram of iron, and the Tamibarotene loaded nanoparticles held a drug payload of 60 nanograms per microgram of iron

Table 1. Following lyophilization, the iron digest assay was completed to determine the amount of iron present in the sample. From there, we determined the amount of drug per iron in nanograms per micrograms. The all-trans retinoic nanoparticles had 1ng/µgFe, the adapalene nanoparticles had 3ng/µgFe, and the tamibarotene nanoparticles had 60ng/µgFe. However, tazarotene was undetectable.

Sample	Drug/Iron Ratio
FH+ATRA	1ng/µg Fe
FH+Adapalane	3ng/µg Fe
FH+Tazarotene	Undetectable
FH+Tamibarotene	60ng/µg Fe

(Table 1). This is the first study to succeed in loading retinoid class drugs into nanoparticles. However, Tazarotene was undetectable by the spectrophotometer in the tazarotene nanoparticle sample, indicating that it was not loaded (Table 1).

5.3 ***Adapalene induced strong cytotoxic effects in leukemia cells***

Since three of the four retinoids we tested had never been previously used on cancer cell lines, we first evaluated the potential of the drugs alone to induce cytotoxicity using the Cell Titer Glo cell viability assay. ANOVAs as well as Post Hoc Tukey tests were conducted to compare each of the samples with the untreated groups.

In the leukemia virus-induced tumor cells lines, we found that All-trans retinoic acid, Adapalene, and Tamibarotene all show potential as anti-cancer agents (Figures 7,8,10). Most consistently cytotoxic, was Adapalene—we saw significantly lower viability at all doses of Adapalene when compared to the untreated control group ($p<0.0001$; Figure 7). This is the first evidence that Adapalene can induce cell death in a leukemia cell line. Furthermore, All-trans retinoic acid exhibited cytotoxic effects at its highest concentration—10μg/mL. Post Hoc Tukey tests indicated that in the presence of a high concentration of All-trans retinoic acid, cell viability decreased ($p<0.05$; Figure 8) whereas the smaller concentrations failed to have a profound effect ($p>0.05$; Figure 8). Similarly, Tamibarotene also displayed cytotoxic effects only at the 10μg/mL concentration. Statistical analysis show that Tamibarotene in high amounts will also result in a decrease of cell viability ($p<0.05$; Figure 10), unlike any of its lower concentrations ($p>0.05$; Figure 10). On the other hand, Tazarotene was not cytotoxic, as there was no significant difference in cell viability amongst any concentration ($p>0.05$; Figure 9).

Conversely, our results relative to the breast cancer cells were not as promising. Adapalene was not cytotoxic under any of the conditions. Post Hoc Tukey tests indicated that there was no significant difference in cell viability when treated with adapalene across all concentrations ($p>0.05$; Figure 11). All-trans retinoic acid also showed no signs of cytotoxicity, as the 10μg/mL, 2.5ug/mL, 1.25μg/mL, and 0.625μg/mL concentrations were not significantly different from the untreated group ($p>0.05$; Figure 12). The 5μg/mL concentration did show a significant difference ($p<0.05$; Figure 12) however, this was an increase in cell viability caused by the lack of cytotoxic effects. Moreover, Tazarotene and Tamibarotene were ineffective in inducing cell death across all concentrations. Analysis confirmed that there was no significant difference in cell viability when comparing the untreated group with any concentration of Tazarotene or Tamibarotene ($p>0.05$; Figure 13+14).

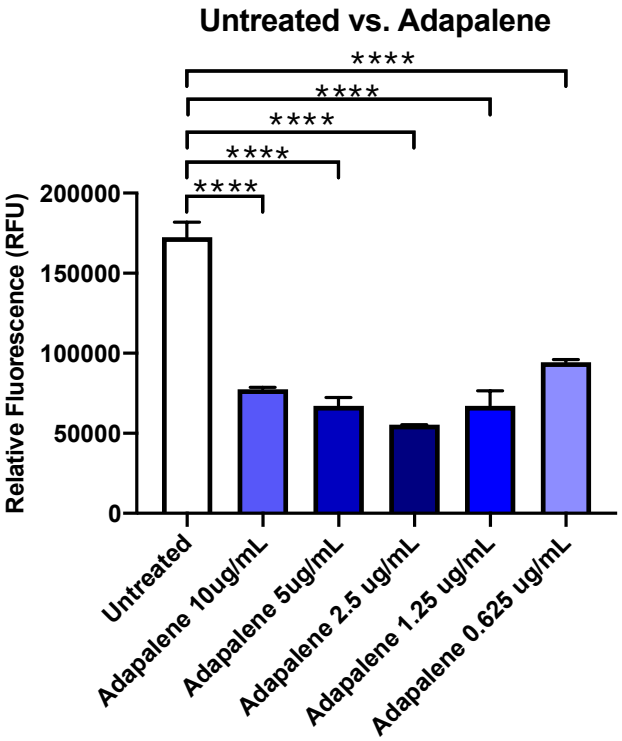

Figure 7. (Leukemia) In the RAW 264.7 leukemia cells, Adapalene exhibited the greatest/most consistent reduction in cell viability amongst all retinoid drugs. Post Hoc Tukey tests comparing the Adapalene 10ug/mL ($p<0.0001$), Adapalene 5ug/mL ($p<0.0001$), Adapalene 2.5 ug/mL ($p<0.0001$) , Adapalene 1.25 ug/mL ($p<0.0001$), and Adapalene 0.625 ug/mL ($p<0.0001$) groups with the Untreated group all exhibited a significant difference. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$

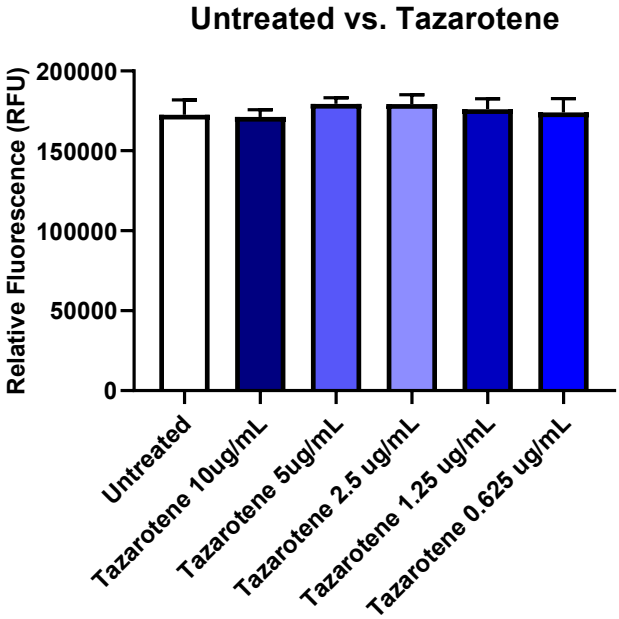

Figure 9. (Leukemia) In the Tazarotene-treated RAW 264.7 leukemia cells, no significant reductions in cell viability was observed. Post Hoc Tukey tests comparing the Tazarotene 10ug/mL ($p>0.9999$), Tazarotene 5ug/mL ($p=0.8930$), Tazarotene 2.5ug/mL ($p=0.9031$), Tazarotene 1.25 ($p=0.9921$), and Tazarotene 0.635ug/mL ($p=0.998$) with the Untreated group did not display a significant difference.

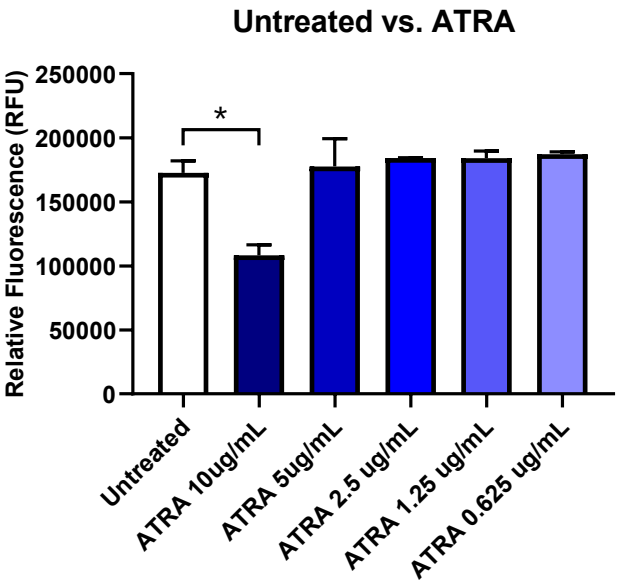

Figure 8. (Leukemia) In the ATRA-treated RAW 264.7 leukemia cells, Post Hoc Tukey tests comparing the ATRA 10ug/mL group and the Untreated group exhibited a significant difference ($p=0.0064$). Conversely, ATRA 5ug/mL ($p=0.9949$), ATRA 2.5ug/mL ($p=0.9949$), ATRA 1.25ug/mL ($p=0.8564$), and ATRA 0.625 ($p=0.7261$) did not display a significant difference. *$p<0.05$

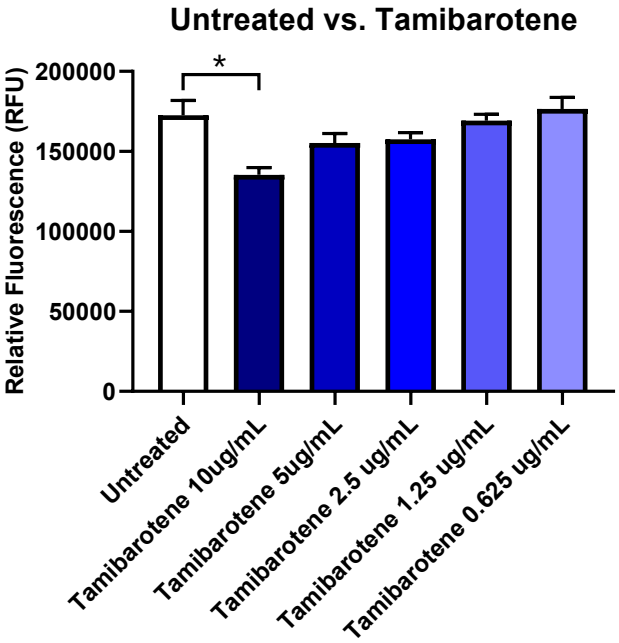

Figure 10. (Leukemia) In the Tamibarotene-treated RAW 264.7 leukemia cells, Post Hoc Tukey tests comparing the Tamibarotene 10ug/mL and the untreated group exhibited a significant difference ($p=0.0071$). Conversely, Post Hoc Tukey tests comparing Tamibarotene 5ug/mL ($p=0.0.1815$), Tamibarotene 2.5ug/mL ($p=0.2724$), Tamibarotene 1.25ug/mL ($p=0.9921$), and Tamibarotene 0.625 ($p=0.9802$) did not display a significant difference. *$p<0.05$

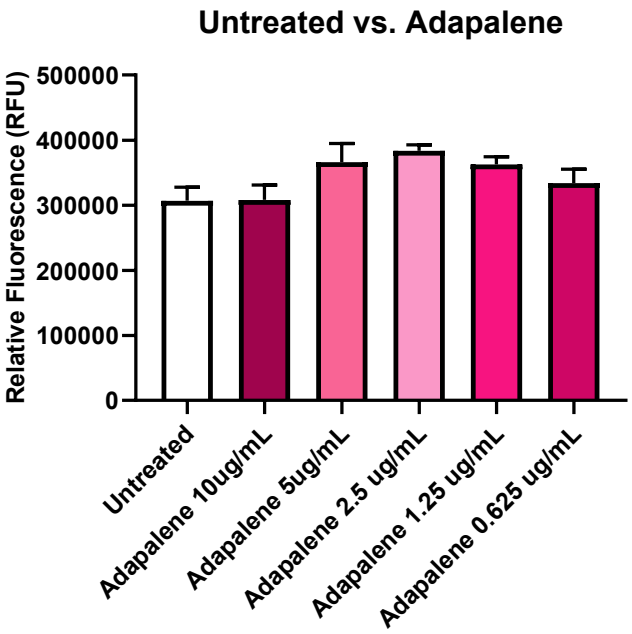

Figure 11. (Breast Cancer) In the Adapalene-treated T47D breast cancer cells, no significant reductions in cell viability were observed. Post Hoc Tukey tests comparing the Adapalene 10ug/mL (p>0.9999), Adapalene 5ug/mL (p=0.1605), Adapalene 2.5ug/mL (p=0.0623), Adapalene 1.25 (p=0.1941), and Adapalene 0.635ug/mL (p=0.7555) with the Untreated group did not display a significant difference.

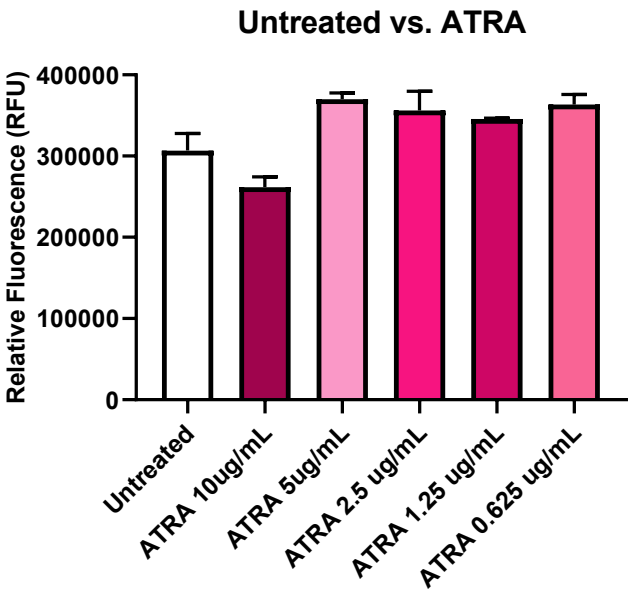

Figure 12. (Breast Cancer) In the ATRA-treated T47D breast cancer cells, Post Hoc Tukey tests comparing the ATRA 10ug/mL (p=0.1496, ATRA 1.25 (p=0.2359), and ATRA 0.635ug/mL (p=0.0652) with the Untreated group did not display a significant difference. The comparison between the ATRA 5ug/mL (0.0411) did exbibit a significant difference, however it was an increase of cell viability due to the lack of cytotoxic effects.

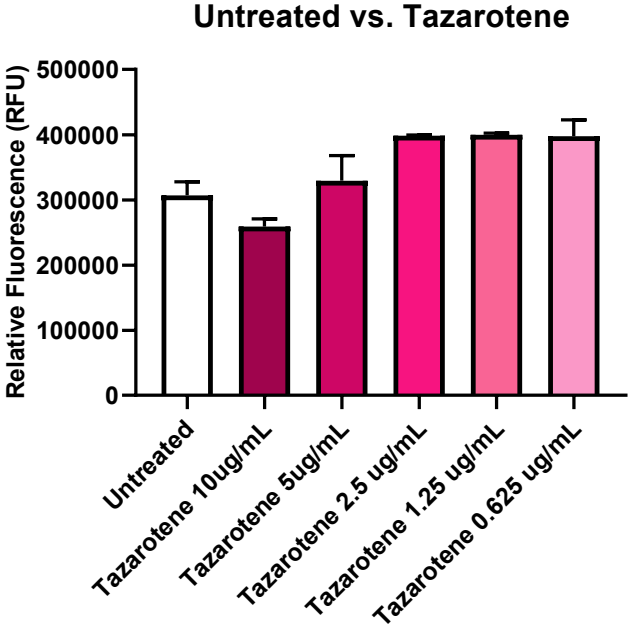

Figure 13. (Breast Cancer) In the Tazarotene-treated T47D breast cancer cells, Post Hoc Tukey tests comparing the Tazarotene 10ug/mL (p=0.3275) and Tazarotene 5ug./mL (0.8812) groups with the Untreated group did not display a significant difference. The comparisons between the Tazarotene 2.5ug/mL (p=0.0345), Tazarotene 1.25 (p=0.0321), and Tazarotene 0.625 (p=0.3157) did exhibit a significant difference, however it was an increase of cell viability due to the lack of cytotoxic effects.

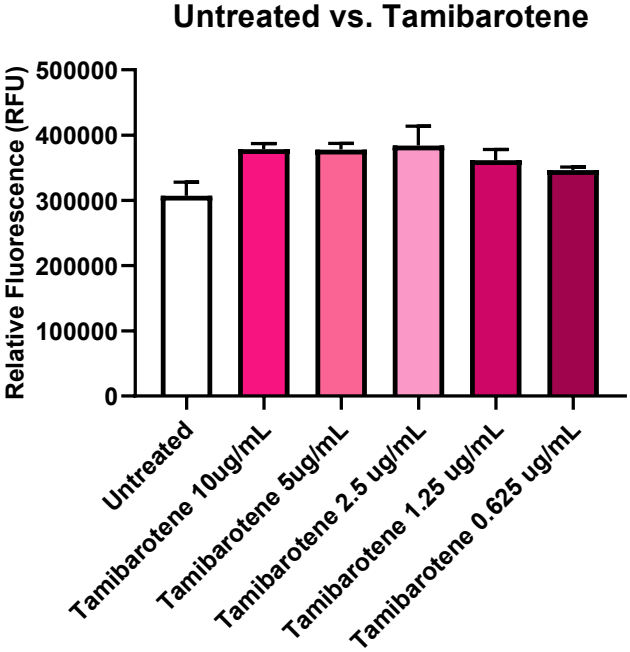

Figure 14. (Breast Cancer) In the Tamibarotene-treated T47D breast cancer cells, Post Hoc Tukey tests comparing the Tamibarotene 1.25ug/mL (p=1198) and Tamibarotene 0.625ug/mL (p=0.625) with the Untreated groups did not display a significant difference. The comparisons between the Tamibarotene 10ug/mL (p=0.400), Tamibarotene 5ug/mL (p=0.0426), and Tamibarotene 2.5ug/mL (p=0.0293) exhibited a significant difference, however it was an increase of cell viability due to the lack of cytotoxic effects.

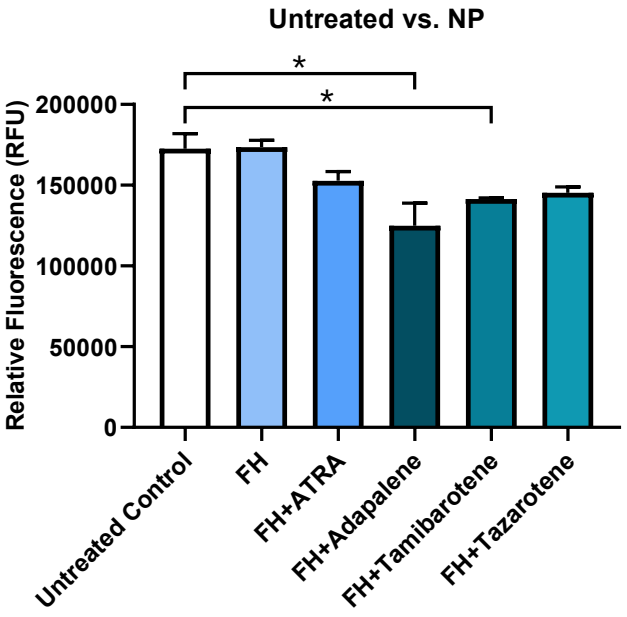

Figure 15. (Leukemia) All-trans retinoid acid, Adapalene, Tamibarotene, and Tazarotene were loaded into (FH) iron oxide nanoparticles and were used to treat RAW 264.7 leukemia cells. Post Hoc Tukey tests comparing the Untreated group with the FH (p>0.9999) FH+ATRA (p=0.2251), and the FH+Tazarotene (p=0.0748) groups did not exhibit a significant difference. Interestingly, FH+Adapalene (p=0.0057) and FH+Tamibarotene (p=0.0057) displayed a significant difference. *p<0.05

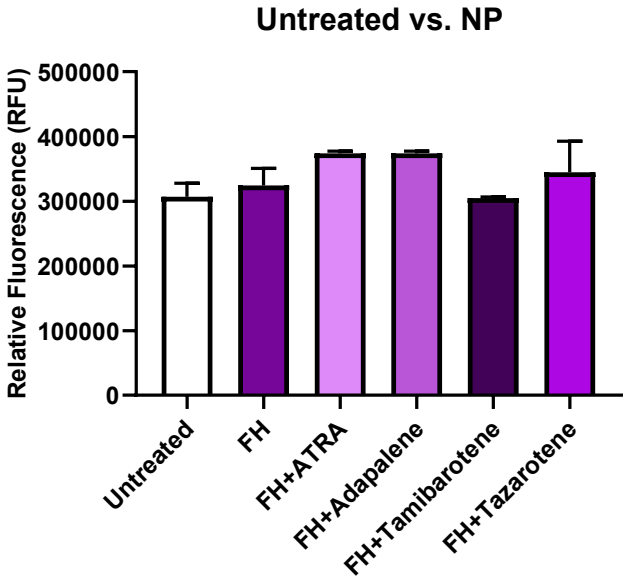

Figure 16. (Breast Cancer) All-trans retinoid acid, Adapalene, Tamibarotene, and Tazarotene were loaded into (FH) iron oxide nanoparticles and were used to treat T47D breast cancer cells. Post Hoc Tukey tests comparing the Untreated group with the FH (p=9697) FH+ATRA (p=0.1840), FH+Adapalene (p=0.1840), FH+Tamibarotene (p>0.9999) and the FH+Tazarotene (p=0.6408) groups did not exhibit a significant difference.

5.4 *Adapalene and Tamibarotene loaded nanoparticles induced strong cytotoxic effects in leukemia*

The main obstacle with using retinoids in present day cancer treatment is that they are typically quickly metabolized before reaching the targeted cells. Therefore, we assessed the ability of iron oxide nanoparticles to induce cytotoxic effects in cancer cell lines. ANOVAs as well as Post Hoc Tukey tests were conducted to compare each of the samples with the untreated groups.

In the leukemia virus-induced tumor cell lines, we found that two retinoid loaded nanoparticle showed promise as a potential cancer-killing agent. Interestingly, the Adapalene loaded nanoparticles exhibited the greatest cytotoxic effects, as there was a significant difference in cell viability when compared to the untreated group ($p<0.05$; Figure 15). Moreover, the Tamibarotene loaded nanoparticles also had cytotoxic effects, as a significant difference in cell viability was also observed ($p<0.05$; Figure 15). This is the first evidence supporting the use of iron oxide nanoparticles for the delivery retinoids to induce cytotoxic effects in a leukemia cell line. Additionally, the unloaded nanoparticles had no intrinsic cytotoxic effect, as there was no significant difference in cell viability ($p>0.05$; Figure 15), which was expected. However, neither the All-trans retinoic acid or Tazarotene loaded nanoparticles were cytotoxic as there was no significant difference in cell viability ($p>0.05$; Figure 15).

Yet, in the breast cancer cell line, the results again were not as positive. The unloaded nanoparticle did not show any signs of cytotoxicity as there was no significant difference in cell viability ($p>0.05$; Figure 16). In addition, the All-trans retinoic acid, Adapalene, Tamibarotene, and Tazarotene loaded nanoparticles were ineffective in inducing any sort of a cytotoxic effect. Statistical analysis confirmed this as there was no significant difference in cell viability when compared to the untreated group.

6.0 Discussion:

This research is the first to provide strong evidence to establish the retinoid drug adapalene as a promising anti-cancer agent and presents a novel method for retinoid delivery using Feraheme iron oxide nanoparticles. Our results show that adapalene is highly cytotoxic in leukemia cells and can elicit these same effects when carried via an iron oxide nanoparticle, providing for a more efficient drug delivery system.

Previous research has determined that retinoids have the ability to induce the differentiation of various types of cancer cells, causing arrest in the cell cycle and eventually apoptosis[10]. More specifically, multiple studies have used All-trans retinoic acid as a cancer killing agent and have observed these cyto-differentiating effects[11-12]. Tang et al. (2011), however, indicated that the delivery of retinoids to patients (*in vivo*) would be challenging due to the rapid metabolism of certain retinoids as well somatic, epigenetic, or genetic changes causing cancer cells to develop retinoid-resistance. Furthermore, that study claimed that successful cancer therapy with retinoids would require drugs that are capable of regulating the epigenome alongside chemotherapeutic agents. Although this may be a potential option, we show that nanotechnology is a viable alternative and should be further explored. Countless studies have shown that iron oxide nanoparticles—due to their microscopic size, long retention time, and low toxicity—have the ability to travel unaffected throughout the body and reach the targeted site, in this case cancer cells[18-23]. Moreover, they possess the ability to carry drugs within their polymeric coating providing for an ensured delivery and release to the cancer cells[24-29] (Figure 2). Therefore, our study took a novel approach by loading Feraheme iron oxide nanoparticles with retinoids to bridge the gap that divides retinoid drugs from being implemented into present day therapies. Not only was All-trans retinoic acid used, but retinoid drugs Adapalene, Tamibarotene, and Tazarotene were also included as they have not been mentioned in previous research as a cancer drug.

Kammari et al. (2017) asserted that the enhanced permeability and retention effects that iron oxide nanoparticles possess is limited by the condition of their biocompatible polymeric shell. With the intention of using iron oxide nanoparticles for drug delivery purposes, the loading protocol must not affect the stability or solubility of the particle. Any aggregation or clumping of the nanoparticles, caused

by the drug loading, is an indication of an alteration or disfigurement of the polymeric coating, making any efforts to deliver drugs not feasible. To dictate whether or not this occurred, dynamic light scattering was completed to measure the hydrodynamic radius of each of the nanoparticle samples. For comparison, the hydrodynamic radii of the unloaded nanoparticles—those that did not go through the loading process—was in the range of 10 nanometers to 100 nanometers (Figure 6). Likewise, the All-trans retinoic acid, Adapalene, Tamibarotene, and Tazarotene loaded nanoparticles also remained in the range of 10 nanometers to 100 nanometers (Figure 6). This confirmed that the drug loading process did not alter the shape and subsequent functionality of the nanoparticles.

Following this, and after lyophilization, spectrophotometry of the nanoparticle samples was done to determine the iron and drug concentrations. We calculated the ratio of drug in nanograms per microgram of iron, which allowed us to ascertain whether or not it is possible for retinoids to be loaded in iron oxide nanoparticles. Calculations revealed that the All-trans retinoic acid loaded nanoparticles held a drug payload of 1 nanogram per microgram of iron, the Adapalene loaded nanoparticles with 2 nanograms per microgram of iron, and the Tamibarotene with 60 nanograms per microgram of iron (Table 1). This is the first evidence of successful loading of retinoid class drugs into nanoparticles. In effect, this introduces a new method of delivery for retinoids as it provides for a vehicle that will not be metabolized prior to reaching the cancer cells. On the contrary, Tazarotene was undetectable in the tazarotene nanoparticle samples (Table 1). This, however, was not necessarily attributed to an error in the loading process, rather it was likely because of the chemical make-up of the drug itself. Pantapasis et al. (2017) reinforces that not all drugs are chemically comfortable in the iron oxide nanoparticle's coating. If future studies continue to research the cytotoxic ability of Tazarotene, selecting an alternative nanoparticle that is chemically suitable for tazarotene should be considered. Recent studies done by Krzysztof et al. (2019) and Jain et al. (2012) have seen success in the use of gold nanoparticles for cancer-related drug delivery; gold nanoparticles could possibly serve as the delivery vehicle for Tazarotene, as well as the other retinoids used in the study as they may have a larger drug capacity.

Additionally, when looking at the actual effectiveness of the retinoids alone as well as when they're loaded into iron oxide nanoparticles, one retinoid stands out in particular. In the RAW 264.7 leukemia virus-induced tumor cells, Adapalene displayed a strong cytotoxic effect across all dosages, yielding a significant difference in cell viability when compared to the untreated group. Both Chen et al. (2014) and Tang et al. (2011) hypothesized that the activation of retinoic acid receptor (RAR) and retinoic X receptor (RXR) by retinoid class drugs can cause apoptosis. Our study provided the first evidence of Adapalene's ability to induce cytotoxicity in leukemia cells, supporting the hypotheses those studies. We also observed that All-trans retinoic acid and Tamibarotene induced cytotoxicity only at their highest concentrations, thus implying their cytotoxic abilities at higher concentrations. All-trans retinoic acid was

expected to exhibit cytotoxic effects in the leukemia cells, as previous research has already established it as a cancer-killing agent[11-12]. As for Tazarotene, none of the concentrations seemed to have any profound effect, possibly indicating its inability to elicit cytotoxicity.

Interestingly, not only is Adapalene alone highly cytotoxic in the leukemia cells, when loaded into the iron oxide nanoparticles, the effects are similar, as cell viability significantly decreased. In addition, the Tamibarotene loaded nanoparticles also showed signs of cell death. Although recent research by Mou et al. (2019) has revealed a new form of programmed cell death dependent on iron—ferroptosis—it is unlikely that this is the primary cause of the decrease in cell viability in these groups, as the unloaded nanoparticles did not have any cytotoxic effects on the leukemia cells. Conversely, the All-trans retinoic acid and Tazarotene loaded nanoparticles did not cause a decrease in cell viability. The inability of the All-trans retinoic acid nanoparticles to induce cytotoxic effects could be caused by amount of drug in the nanoparticle itself, as it only contained 1 nanogram per microgram of iron (Table 1). Similarly, in the case of the Tazarotene nanoparticles, spectrophotometry did not detect any tazarotene in the samples, thus there was no effect.

Overall, our results in the T47D breast cancer cells were not promising, none of the retinoids alone or loaded nanoparticles were cytotoxic. We speculate that results in the breast cancer cells may be caused by the incubation time, as well-plates were incubated for only 24 hours following treatment. Similar results were seen in studies by Fettig et al. (2017) and Chen et al. (2014), as they suggested that although retinoids have cyto-differentiating effects on breast cancer cells, it was not to a great extent. Fettig et al. (2017), however, utilized the CK5+ breast cancer cell line as opposed to T47D, meaning there could possibly be a difference in receptor pathways. Upon reviewing these results, it is pertinent that one considers that this study was done *in vitro*, and that an *in vivo* study may display different, and possibly more promising results.

Additional research must investigate the modification of the loading protocol to improve retinoid capacity and increase drug payload, as in this study the loaded nanoparticles did not contain the same amount of drug in each group. Moreover, it could also be important to evaluate the effects of retinoids on T47D breast cancer and RAW 264.7 leukemia cell proliferation as an alternative to direct cytotoxicity. Dosage should also be taken into account, as tamibarotene and all-trans retinoic acid were effective only at their highest concentrations—optimal dosing may occur at higher concentrations. Even more so, future studies can also delve deeper into other cancer cell lines/types that are potentially retinoid-sensitive to see if they have a somewhat universal effect among cancer cells.

Overall, the findings of this study set a foundation for further research to effectively connect nanotechnology and retinoid-based cancer therapy. It expands our knowledge pertaining to the loading of retinoids into iron oxide nanoparticles and furthermore their effects on leukemia and breast cancer.

7.0 Conclusion:

This is the first study to provide evidence to establish the retinoid drug Adapalene as an efficient leukemia-killing agent. In addition, All-trans retinoic acid and Tamibarotene also showed promise at their highest doses. Furthermore, to evade the metabolic breakdown of retinoid drugs, this study also succeeded in taking the novel approach of using iron oxide nanoparticles as a delivery vehicle as All-trans retinoic acid, Adapalene, and Tamibarotene were successfully loaded into the nanoparticles. In doing this, both the Adapalene loaded nanoparticles and the Tamibarotene loaded nanoparticles elicited cytotoxic effects on the leukemia cells. Overall, this is the first study to display the potential of Adapalene as an anti-cancer agent as well as use nanotechnology to successfully overcome the limitation halting retinoid delivery.

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