INDUCING OXIDATIVE STRESS USING BIOTIN-RECEPTOR TARGETED ORGANOMETALLIC COMPOUNDS ON CANCER CELLS

by

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INDUCING OXIDATIVE STRESS USING BIOTIN-RECEPTOR TARGETED ORGANOMETALLIC COMPOUNDS ON CANCER CELLS

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Abstract

Cancer is one of the leading causes of death in the United States and is predicted to directly affect 38% of the population over the course of their life. Cancer is categorized as a collection of diseases primarily characterized by aberrant cellular proliferation. Many current cancer therapies, such as chemotherapy lack the ability to differentiate between cancer cells and normal cells resulting in several negative side effects. To minimize these side effects, there has been a huge push to develop targeted therapies, which exploit cancer-specific features to exhibit selective toxicity towards cancer cells. Due to their high proliferative and metabolic rate, some cancers overexpress vitamin receptors such as the biotin receptor. Biotin, also known as vitamin B_7, functions intracellularly as an important coenzyme for several carboxylase enzymes involved in fatty acid synthesis, amino acid metabolism and gluconeogenesis. Thus, by overexpressing the biotin receptor some cancers increase their overall absorption of biotin resulting in a higher metabolic and proliferation rate. Furthermore, the high metabolic rate in cancer leads these cells to have increased susceptibility to damage by reactive oxygen species (ROS) which can trigger apoptosis at high intracellular levels. Ferrocene is an organometallic compound with an iron-center that has been shown to generate ROS in cancer cells. Therefore, our project is exploring this overexpression of the biotin receptor as a potential avenue for targeted therapy against several cancers. Since certain cancers overexpress the biotin receptor and absorb biotin with a higher efficiency, we hypothesize that conjugating biotin to ferrocene will increase the efficiency of ferrocene entering cancer cells, resulting in selective toxicity. Therefore, we have produced a library of biotin-ferrocene conjugates to test their ability to selectively enter cancer cells and

generate ROS. Experiments were conducted utilizing ferrocene and a variety of conjugates (C1, C2, C3, 2) in both cancer (MCF-7) and non-cancer cells (HEK293).

Introduction

Cancer is a heterogenous disease that is one of the leading causes of death in the United States primarily characterized by the loss of cell cycle control resulting in aberrant cellular proliferation. The heterogeneity of cancer between cancer types as well as between tumors of the same cancer type make effective broad-spectrum therapies difficult to develop. In conjunction with surgery and radiation, one of the primary approaches to cancer treatment has been the use of chemotherapy. Chemotherapy utilizes cytotoxic drugs that kill rapidly dividing cells; however, these therapies mode of action does not differentiate between cancer and normal cells. This lack of cancer specificity results in numerous negative side effects including hair loss, fatigue, susceptibility to infection, anemia, nausea and potential infertility. To combat this lack of specificity, in part due to a better understanding of the molecular biology of cancer, the development of targeted therapies has been revolutionary in the advancement of cancer therapy (Chen, et al., 2010). One such targeted therapy that has revolutionized the treatment of some breast cancers is the monoclonal antibody trastuzumab, more commonly known as Herceptin, which targets human epidermal growth factor receptor 2 (HER2) found on the surface of some breast cancers (Nami, Maadi, & Wang, 2019).

To maintain their hyperproliferative nature, cancer cells have a more stringent metabolic requirement for certain dietary molecules including sugars and vitamins. One such important dietary vitamin in some cancers is vitamin B_7, more commonly known as biotin. Biotin is primarily taken as a dietary supplement to promote hair and nail growth; however, intracellularly biotin functions as a cofactor. Cofactors are molecules that associate with cellular enzymes and

play an important role in proper enzymatic function. Biotin functions as an important cofactor for several carboxylase enzymes involved in metabolic processes including gluconeogenesis, fatty acid synthesis and amino acid metabolism. Therefore, due to its important role in such metabolic processes, previous research has shown that many cancers overexpress the sodium-dependent multivitamin transporter (SMVT) which functions as the cell surface receptor for biotin (Tripodo, Mandracchia, Collina, Rui, & Rossi, 2014). Thus, cancers elevated metabolic requirement for biotin and subsequent overexpression of the biotin receptor provides a potential avenue for the development of targeted cancer therapies.

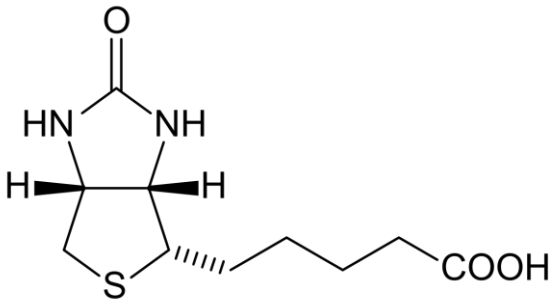

Figure 1:
Chemical structure of biotin
https://upload.wikimedia.org/wikipedia/commons/thumb/8/85/Biotin_structure.svg/1200px-Biotin_structure.svg.png

Another important process in cancer is the generation of reactive oxygen species (ROS). Reactive oxygen species are high energy molecules that possess an unpaired electron and thus exist as free radicals which can be damaging to cells. Elevated levels of ROS have been found across many different cancers and are primarily generated as byproducts of their elevated metabolic rate and potential mitochondrial dysfunction. However, moderate production of ROS

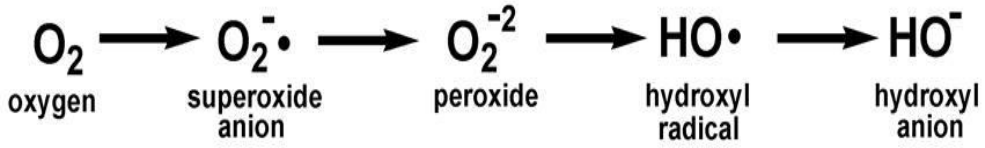

Figure 2: *Mechanism of ROS production*

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plays an important role during tumor formation by activating signaling pathways including the mitogen-activated protein kinase (MAPK)/ Erk1 pathway to stimulate cellular proliferation (Liou & Storz, 2009). Nevertheless, despite the important role ROS plays to stimulate cellular division during tumor formation, there appears to be a cellular limit above which ROS begins to induce DNA damage and interfere with protein function ultimately resulting in cell death.

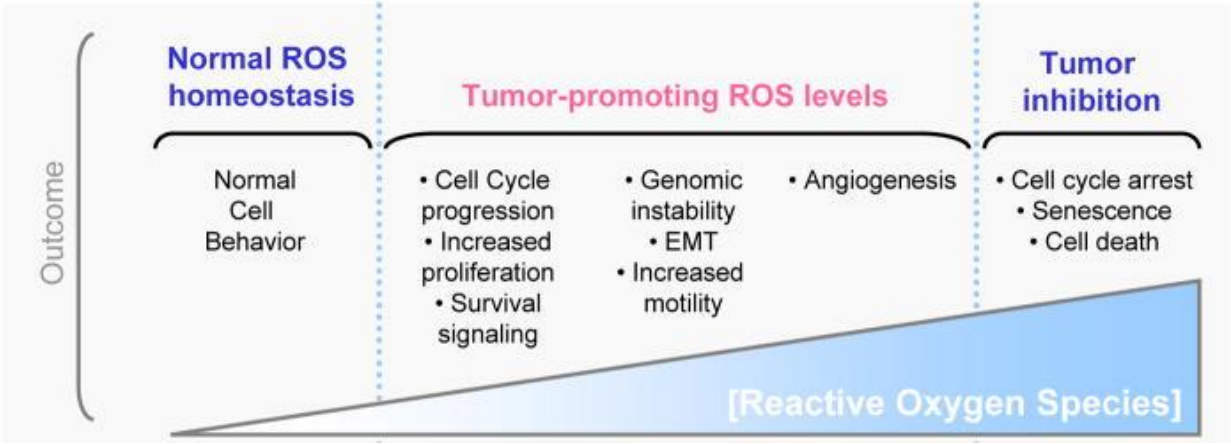

Figure 3: *The role of ROS in tumorigenesis*

(Liou & Storz, 2009)

Ferrocene is an organometallic compound with an iron center and two cyclopentadienyl rings which has previously been shown to generate ROS in cells via a Fenton reaction (Wang, Tian, & Ning, 2014). The Fenton reaction consists of the iron center of ferrocene donating an electron to oxygen resulting in a free radical species.

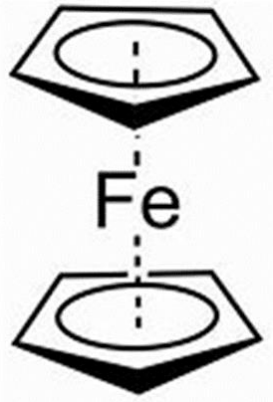

Figure 4:
Chemical structure of Ferrocene
https://upload.wikimedia.org/wikipedia/commons/thumb/0/0c/Ferrocene_Eclipsed_Conformer_Structural_Formulae_V.1.svg/399px-Ferrocene_Eclipsed_Conformer_Structural_Formulae_V.1.svg.png

Ferrocene has been analyzed as for its potential in treating many conditions including as an antimalarial and antitumor agent (Quirante, et al., 2011). As an antitumor agent, prior research has demonstrated that ferrocene's capability of generating oxidative stress in cancer cells can arrest the cell cycle particularly at the G2/M transition (Kowalski, Hikisz, Szczupak, Therrian, & Koceva-Chyla, 2014). Beyond arresting the cell cycle, the ROS production due to ferrocene has been investigated for its potential to generate DNA damage and cell death. (Perez, Soto, Ortiz, Matta, & Melendez, 2015). Furthermore, ferrocene has been explored as a potential mechanism to increase therapeutic efficacy of treatments against multi-drug resistant cancer cell lines (Renic-Podolski, et al., 2017). For example, conjugation of ferrocene with tamoxifen has been shown to increase the cytotoxicity of tamoxifen in breast cancer therapy; breast cancer cells were more sensitive to tamoxifen-ferrocene treatment compared to tamoxifen treatment alone (Wlassoff, et al., 2010). Current research is analyzing the potential for developing a ferrocene-

mediated targeted therapy by conjugating ferrocene to phospholipids for liposome delivery as well as other molecular targets for utilization as cancer therapy (Noyhouzer, et al., 2016).

In this project, we have analyzed the potential of utilizing ferrocene as an ROS-mediated targeted therapy by conjugating ferrocene to biotin. We were interested in biotin due to previous literature demonstrating that the biotin receptor (SMVT) was overexpressed more so than the folic acid receptor in some breast, cervical, lung, leukemia and renal cancers (Chen, et al., 2010). Our research group investigated a library of ferrocenyl-biotin conjugates to see if derivatization of ferrocene increased the toxicity of the conjugate by generating more reactive oxygen species or perhaps increased the bioavailability of the conjugate. Our model is that biotin will function as a trojan horse and thereby help increase cellular absorption of ferrocene. Based upon the overexpression of the biotin receptor in some cancers, we hypothesize that ferrocenyl-biotin conjugates will be absorbed with a higher efficiency in these cancer cells and result in an ROS-mediated targeted therapy.

Library of Ferrocene-Biotin Conjugates

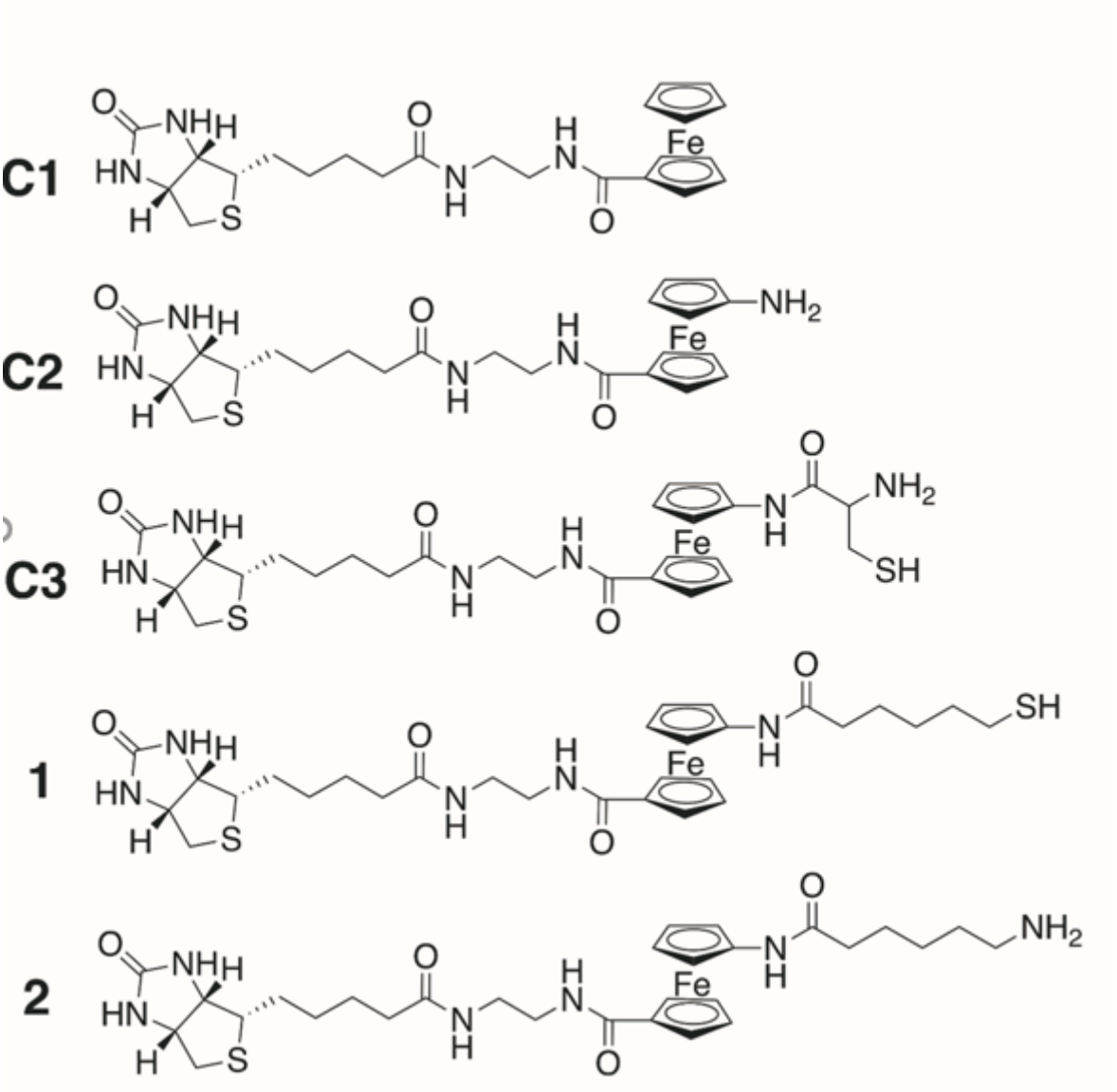

Figure 5:

Library of Ferrocenyl-biotin conjugates

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Methods

Cell Culture Technique:

Cells were grown in complete Dulbecco's modified eagle medium (10% fetal bovine serum, 1% nonessential amino acids, 2 mM L-Glutamine, 100 units of penicillin, and 1.7 mM of streptomycin) in a T75 tissue culture flask at 37°C, 5% CO_2, and 95% air. When cells reached confluency, the medium was aspirated, and the cells were washed with 5 mL of 1x PBS and then PBS was aspirated as well. Next, 3 mL of 0.05% trypsin was added to detach the cells from the surface of the flask and then quenched with 7 mL of DMEM. The trypsin-DMEM mixture was then triturated to further break up the cells and then cells were counted using a hemocytometer. After counting, cells were plated for experimentation in 96 well trays (MTT = 5,000 cells/well, DCFH-DA = 25,000 cells/well) and 10% of the cells were added to a new T75 flask containing 13 mL of DMEM.

MTT Cell Viability Assay:

Cancer (MCF-7) and non-cancer (293HEK) cells were plated in a 96-well tray at a concentration of 5,000 cells/well and incubated overnight at 37°C, 5% CO_2, and 95% air. The following day the cells were treated with concentration of compound dissolved in DMSO for 16 hours. The medium was then removed and 100 uL/well of 1mg/mL solution of thiazolyl blue tetrazolium bromide dissolved in serum-free medium was added and incubated for 4 hours. Afterwards, the medium was removed and 100 uL of DMSO was added and the cells were placed on a shaker for five minutes and then absorbance was measured in a spectrophotometer at 540 nm and the data was analyzed via a two-tailed t-test.

DCFH-DA ROS Generation Assay:

Cancer and noncancer cells were plated in a 96-well plate at 25,000 cells/well and incubated overnight at 37℃, 5% CO_2, and 95% air. Cells were then treated with 100 uL/well of 25 uM solution of 2'7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) dissolved in serum-free medium for 45 minutes. The medium was then removed and 100 uL of complete DMEM was added to the cells. Afterwards, cells were treated with various concentration of compound or DMSO as the negative control and incubated for 16 hours. After the 16-hour treatment, the fluorescent intensity was measured in a spectrophotometer with an excitation filter of 475 nm and emission filter of 520 nm and the data was analyzed with a two-tailed t-test.

Fluorescent Biotin Assay:

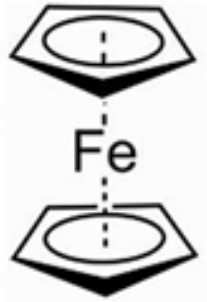
HCL-treated coverslips (1M soaked for 6 hours, 50-60° C) were added to a 6-well plate and allowed to dry. Cancer and noncancer cells were added to the coverslip within the 6-well plate at a concentration of 10,000 cells/well and incubated overnight at 37℃, 5% CO_2, and 95% air. Cells were treated with biotin-4-fluorescein (100 nM) for either 12 or 16 hours. The medium was removed, and coverslips were washed with 0.5 mL of 1x PBS and then 0.5 mL of paraformaldehyde was added for 30 minutes. Paraformaldehyde was then removed, and the cells were rinsed once again with 1x PBS prior to mounting the coverslips on slides. Coverslips were fixed to the glass slide with nail polish along all four edges and images were viewed using a fluorescencet microscope under a GFP filter.

Results

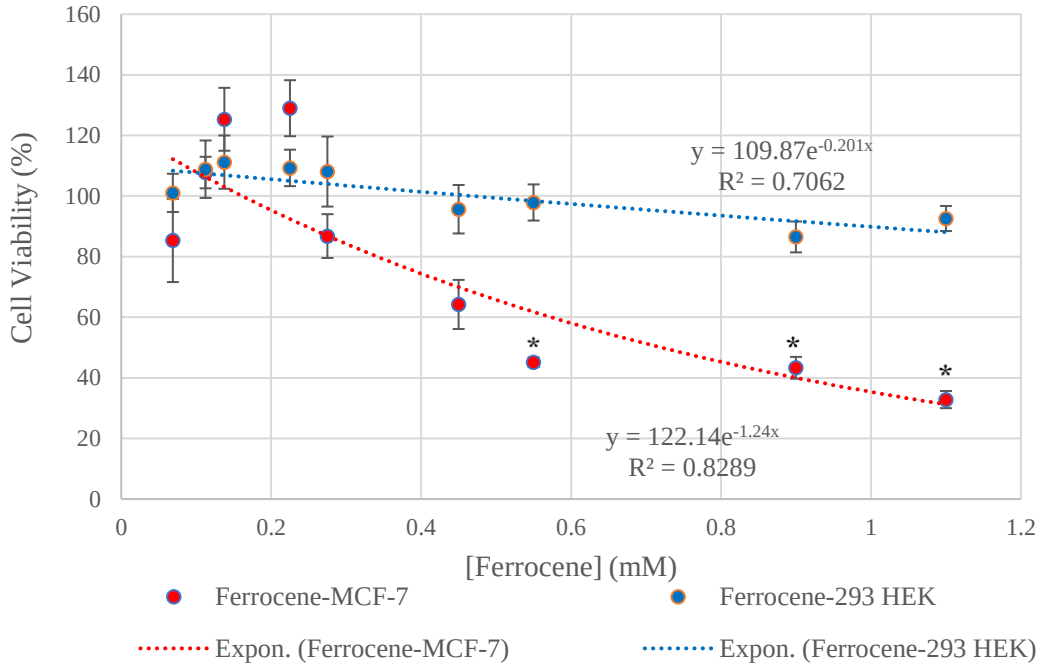
First, we analyzed the cytotoxicity of ferrocene (Figure 1A), the parent compound, in cancer and non-cancer cells to test if cancer cells were more susceptible to cell death via oxidative stress. Breast cancer cells (MCF-7) and noncancerous kidney fibroblasts (293 HEK) were treated for 16 hours with increasing concentration of ferrocene dissolved in DMSO prior to measuring cell viability and intracellular generation of ROS. The results demonstrated that ferrocene exhibited a significant dose-dependent toxicity towards cancer cells in comparison to minimal toxicity in non-cancer cells (Fig 3B, [t-value = 13.846 p-value < 0.001,). Data analysis showed that ferrocene resulted in an EC50, which is the concentration of a drug required to kill 50% of the cells, in cancer and non-cancer cells of 0.885 +/- 0.144 mM and 3.327 +/- 1.091 mM, respectively (Fig 3B, [t-value = 4.96], p-value < 0.01). Furthermore, this selective toxicity of ferrocene is correlated to an overall increase in the levels of intracellular ROS; Ferrocene resulted in an approximately 4.3-fold increase in ROS (Fig. 3C, [t-value =11.68], p-value < 0.001) specifically in MCF-7 and minimal increases in 293 HEK in comparison to controls.

Figure 6:

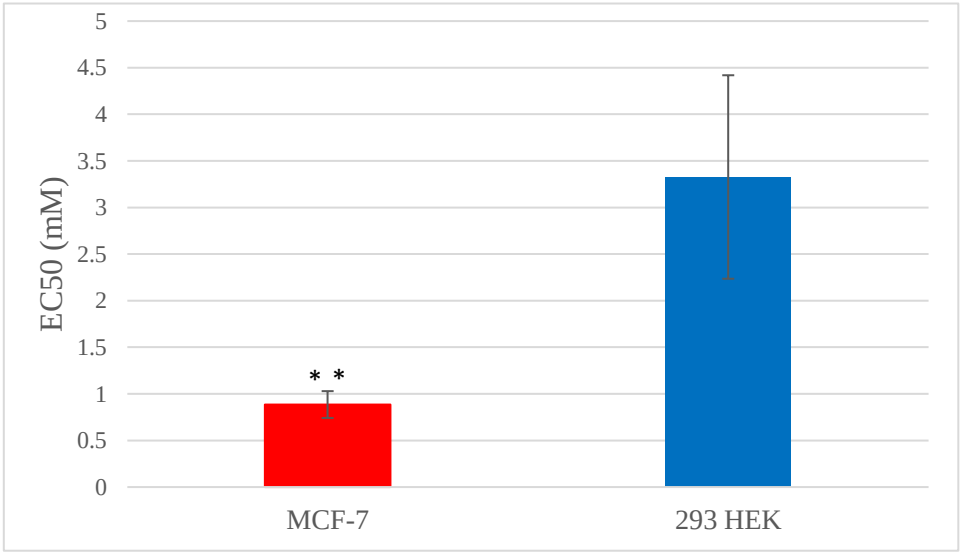
(a)



(b)


160
140
120
100
80
60
40
20
0
Cell Viability (%)
0
0.2
0.4
0.6
0.8
1
1.2
[Ferrocene] (mM)
y = 109.87e-0.201x
R² = 0.7062
y = 122.14e-1.24x
R² = 0.8289
*
*
*
Ferrocene-MCF-7
Ferrocene-293 HEK
Expon. (Ferrocene-MCF-7)
Expon. (Ferrocene-293 HEK)

(c)


5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
EC50 (mM)
* *
MCF-7
293 HEK

(d)

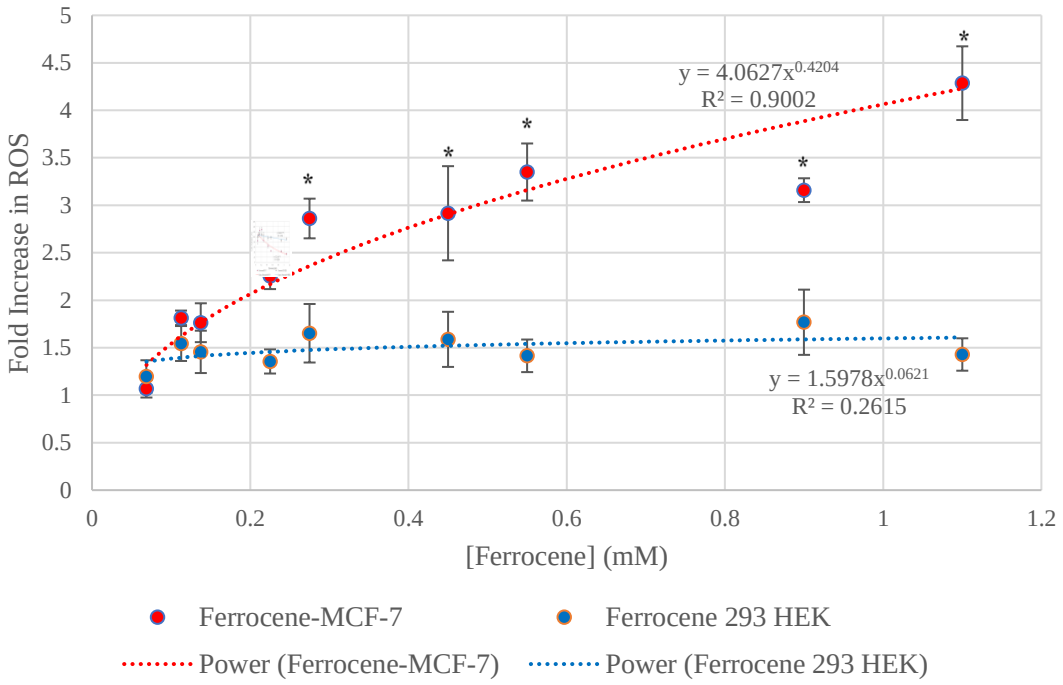


Figure 6:

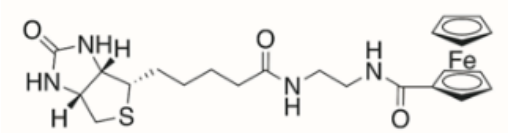
*(a) Chemical structure of Ferrocene (b)The cytotoxicity of ferrocene on MCF-7 (breast cancer) and 293 HEK (non-cancer) cells. (b) the EC50 of ferrocene on MCF-7 0.885 +/- 0.144 mM compared to 293 HEK 3.327 +/- 1.091 mM. (c) the DCFH-DA assay of ferrocene on MCF-7 (4.3-fold increase) and 293 HEK (1.5-fold increase). * p value < 0.05, ** p value < 0.01*

With ferrocene exhibiting selective ROS-mediated toxicity in MCF-7 breast cancer cells, we extended our experiment to include some of the biotinylated ferrocene derivates. According to our hypothesis, cancer cells overexpressing the SMVT transporter should absorb these conjugates, and therefore, ferrocene with a higher efficiency which should increase not only the selectivity, but also the toxicity and generation of reactive oxygen species. After analyzing the library of conjugates, the compound C1 (Figure 4A) was selected because its structure was biotin

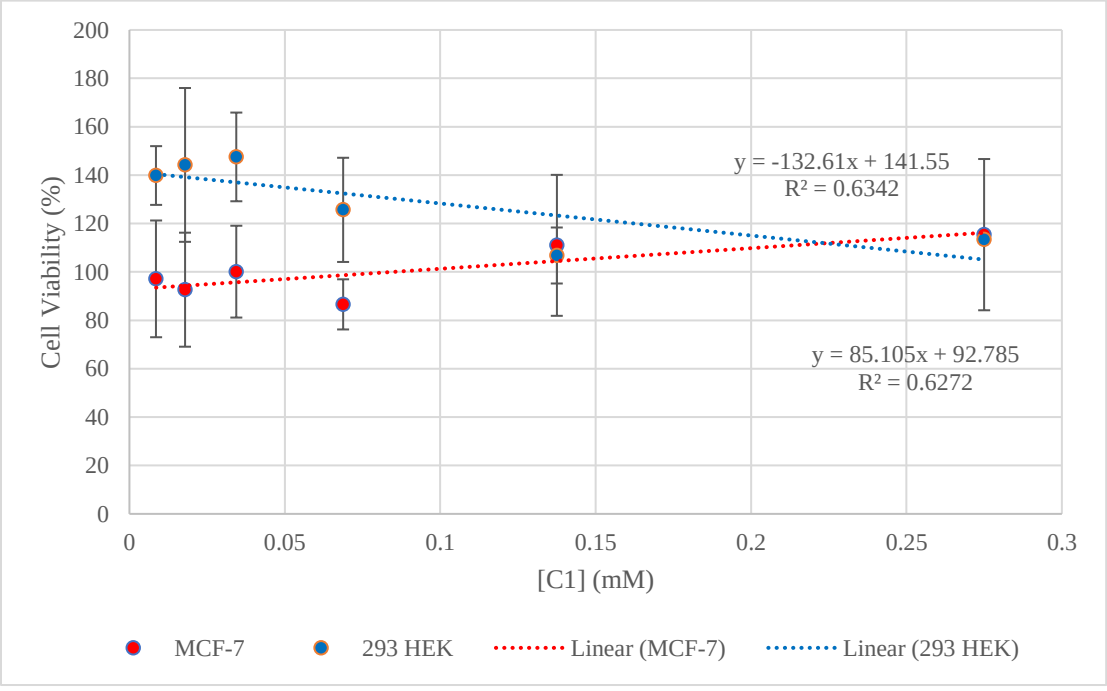
conjugated to ferrocene without any derivatization, allowing us to examine the effects of the addition of biotin. In similarity to the experiments with ferrocene, both MCF-7 and 293 HEK cells were treated for 16 hours with increasing concentration of C1.

Figure 7:

(a)



(b)



(c)

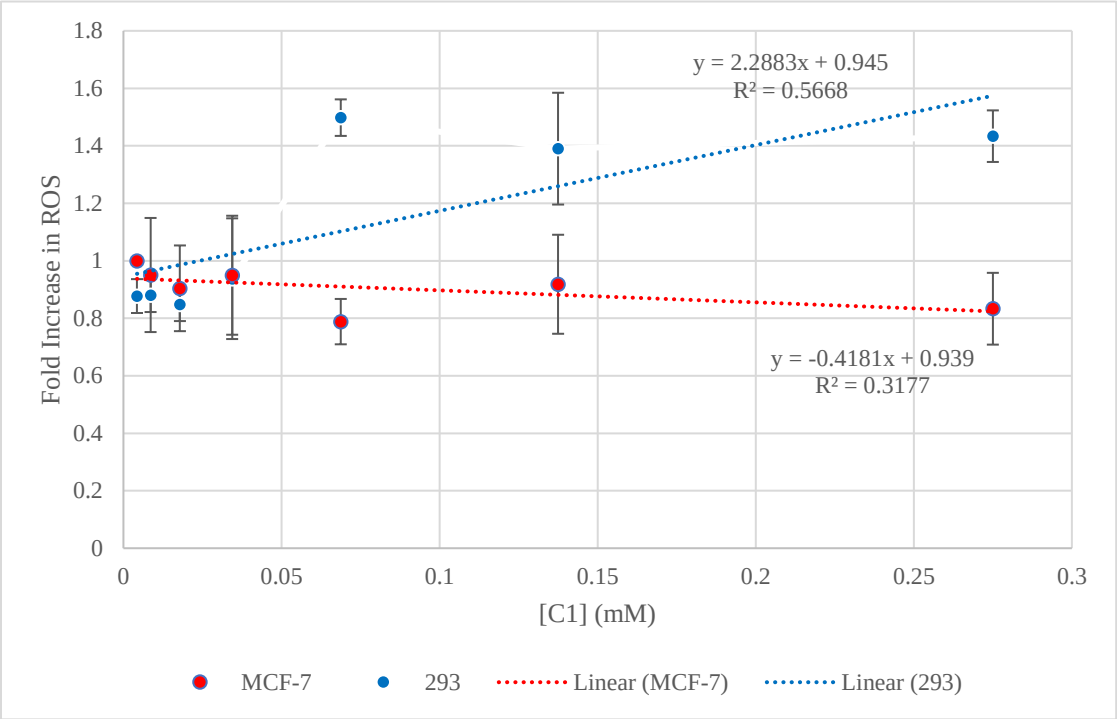


Figure 7:

(a) Chemical Structure of C1 (b) Cytotoxicity of C1 on MCF-7 and 293 HEK. (c) ROS Generation of C1 in MCF-7 and 293 HEK.

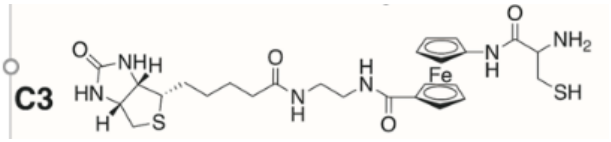
The cytotoxicity and ROS generation assay results demonstrated that C1 did not exhibit toxicity nor any substantial increase in intracellular ROS levels in neither cancer or non-cancer cells (Figure 4B and 4C). This data suggests biotinylating ferrocene does not increase the cellular absorption of ferrocene, contrary to our hypothesis. However, a potential explanation of these results could be the solubility of C1. At higher concentrations, C1 could be clearly viewed precipitating out of solution within the wells of the plate. This precipitation of C1 led to the

conclusion that solubility may be an issue and potentially could have impeded C1 from being absorbed by cells and thus affected both the cytotoxicity as well as the capability to generate ROS.

Based upon the results from the experiments utilizing C1, we analyzed our library of ferrocenyl-biotin conjugates to find a more soluble conjugate. The compound C3 which was one of the more soluble conjugates and contained the addition of the amino acid cysteine to one of ferrocene's cyclopentadienyl rings was selected for further testing (Figure 5A).

Figure 8:

(a)



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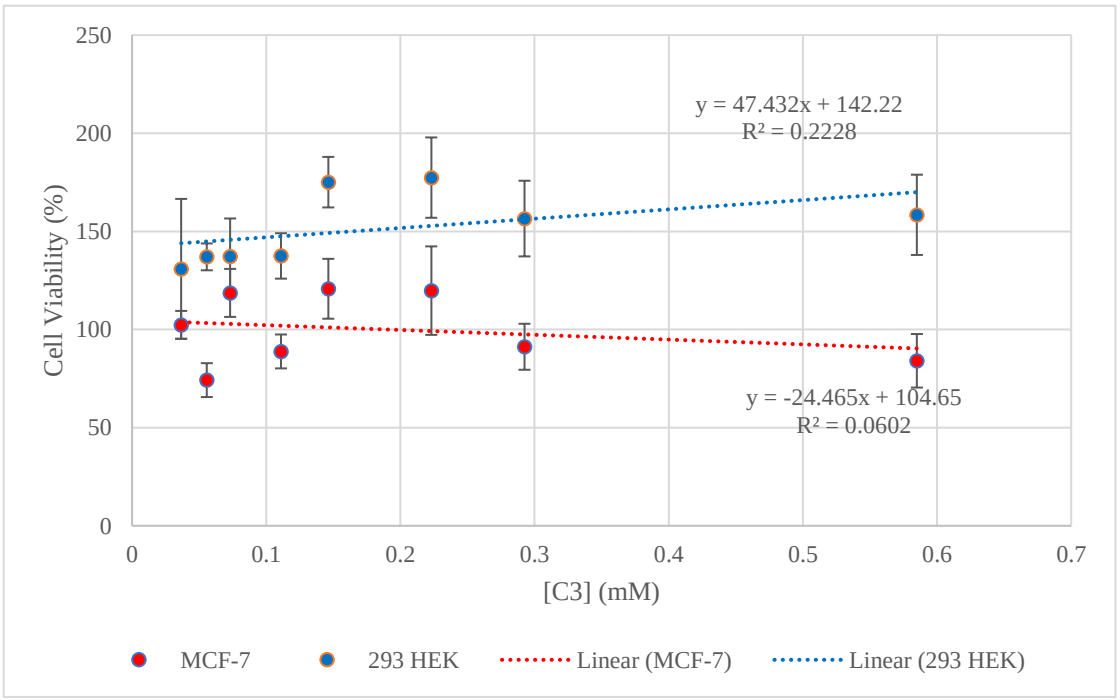

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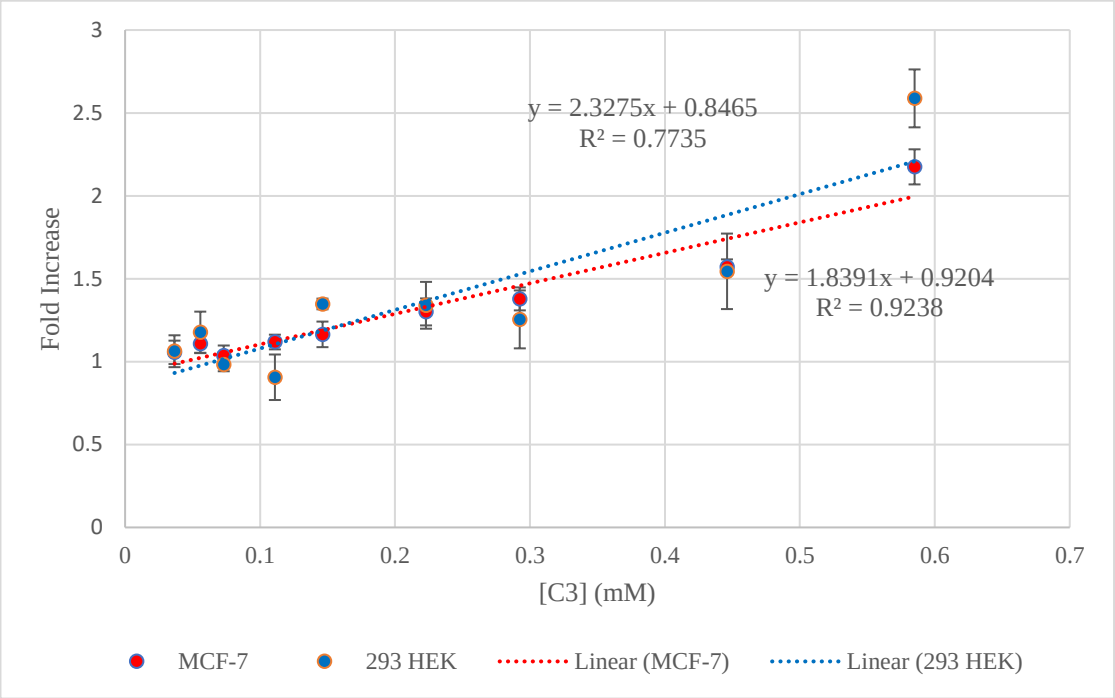


Figure 8:

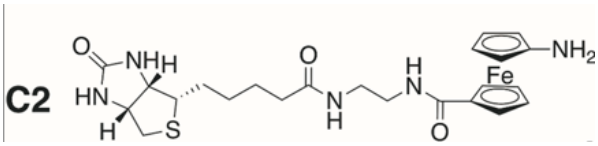
(a) Chemical Structure of C3 (b) Cytotoxicity of C3 on MCF-7 and 293 HEK. (c) DCFH-DA of C3 on MCF-7 and 293 HEK.

Despite the increased solubility, C3 exhibited no significant toxicity to both MCF-7 and 293 HEK cells (Figure 5B). Furthermore, the DCFH-DA assay did suggest an increase in intracellular ROS with a maximum 2.2-fold increase in comparison to a negative control (Figure 5C). However, this increase was determined to be insignificant due to the lack of corresponding cytotoxicity as well as similar increases in ROS generation in the 293 HEK non-cancer cells (Figure 5C).

We then tested the toxicity of C2 which contains the addition of an amine group to ferrocene (Figure 6A). According to the results, C2 was significantly more toxic towards cancer cells at higher concentrations with no apparent toxicity to non-cancer cells (Figure 6B, [t-value = 5.59], p-value <0.01). Using the data from the cytotoxicity assay, an EC50 for MCF-7 was calculated to 0.292 +/- 0.08 mM; we were unable to calculate an EC50 for 293 HEK due to the lack of toxicity. Further investigation of C2 should analyze the correlation of this selective toxicity and ROS generation.

Figure 9:

(a)



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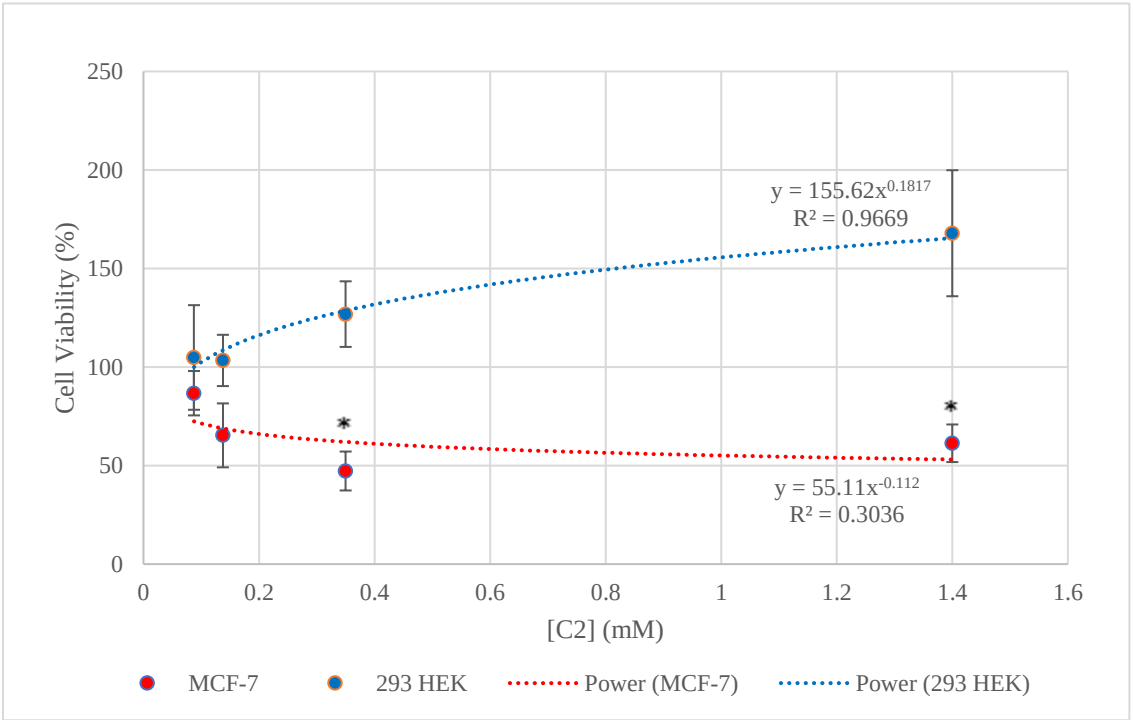

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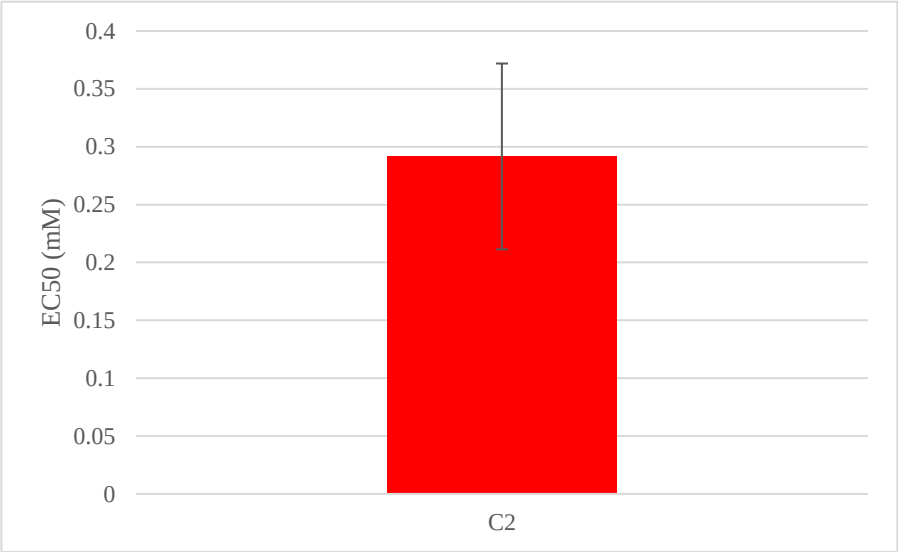


Figure 9:

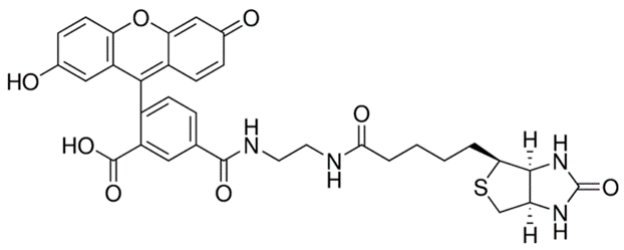
*(a) Chemical Structure of C2 (b) Cytotoxicity of C2 on MCF-7 and 293 HEK (c) EC50 of C2 on MCF-7 (0.292 +/- 0.08 mM); EC50 of 293 HEK could not be calculated. * p value <0.01*

Despite the selective toxicity exhibited by C2, the lack of toxicity by the other conjugates C1 and C3 led to questions about the original hypothesis on the importance of biotin in cancer cells. The entire project is centered around the overexpression of the SMVT biotin receptor in some cancer cells as a potential mechanism for targeted therapy. Our prediction is that cancer cells overexpressing this receptor increase their absorption of biotin; therefore, by conjugating biotin with ferrocene should also increase cancer cells' absorption of ferrocene. Due to the lack of expected results with most of the tested conjugates, we wanted to measure cellular absorption of biotin in both cancer and normal cells. We utilized a fluorescently-labeled biotin, biotin-4-

fluorescein, which could be visualized using a fluorescencet microscope and GFP filter (Figure 7A). According to our hypothesis, we would expect cancer cells that overexpress the biotin receptor would absorb more biotin-4-fluorescein than noncancer cells which would result in a larger fluorescent intensity.

Figure 10:

(a)



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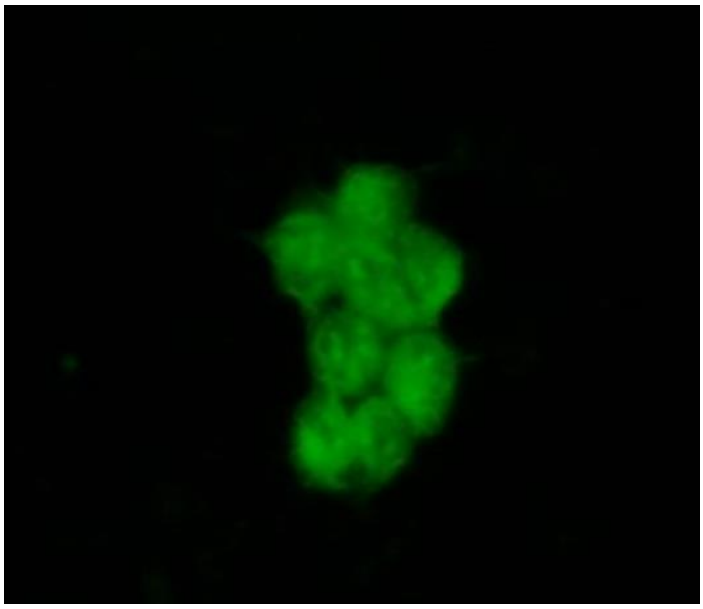
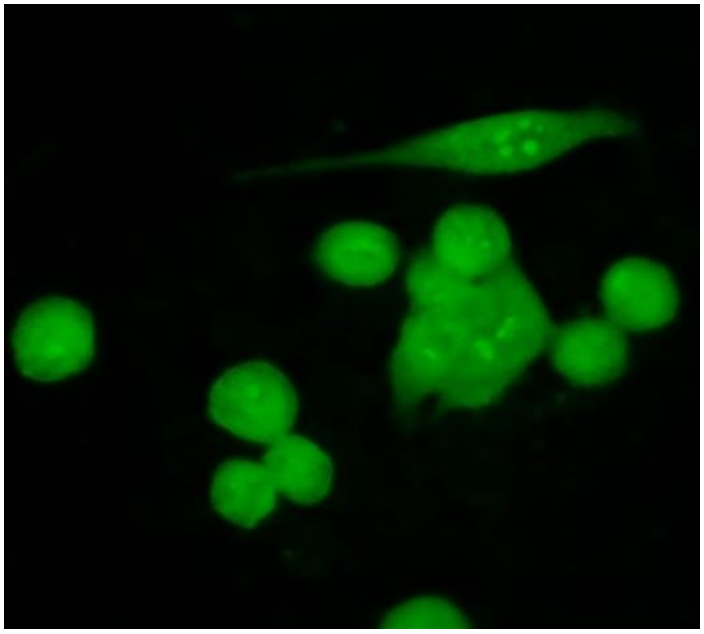
(b)



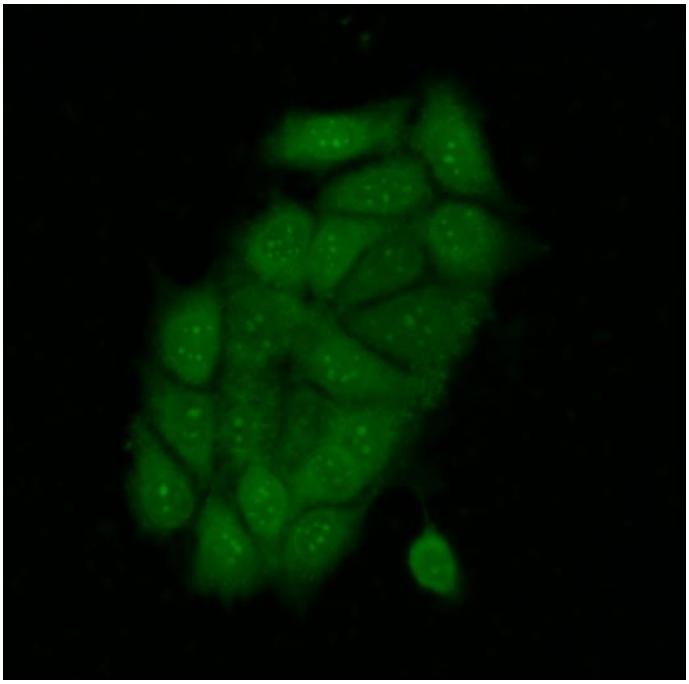
293 HEK

(c)



Hela

(d)



MCF-7

Figure 10:

(a) Chemical structure of biotin-4-fluorescein. Fluorescencet Imaging of fluorescent biotin absorption in (b) 293 HEK (c) Hela (d) MCF-7.

We utilized 3 different cell lines; a human embryonic kidney cell line (293 HEK), a cervical cancer cell line (Hela) and a breast cancer cell line (MCF-7). Both Hela and MCF-7 had previously been shown to overexpress the biotin receptor. Contrary to our hypothesis and the published literature, our fluorescent biotin assay yielded no significant difference in fluorescent intensity between non-cancer and cancer cells leading to the conclusion that biotin is not being absorbed by cancer cells with a higher efficiency (Figure 7B, 7C, 7D).

Discussion

The goal of our research project was to analyze the overexpression of the biotin receptor (SMVT) in some breast, leukemia, cervical and lung cancers as a potential novel mechanism for the development of targeted cancer therapy. Previous research had demonstrated that the elevated metabolic rate of cancer created a more stringent requirement for dietary molecules including sugars and vitamins. To increase their overall absorption of these vitamins, like biotin, many breast, cervical, renal and other cancer cells overexpress their cellular receptors. Furthermore, the increased metabolic rate found in cancer also leads to an increased production reactive oxygen species which can stimulate proliferation at moderate levels. However, when the level of ROS become too high, these molecules can induce DNA damage and cause cell death. Our group was interested in testing the ability of ferrocene to push cancer cells beyond the

cellular limit and induce cancer cell death and if conjugation of ferrocene with biotin further selected for cancer cells and increased the ROS production and ultimately toxicity of ferrocene and ferrocenyl derivatives.

First, ferrocene was tested on both breast cancer (MCF-7) and non-cancer cells (293 HEK) for levels of toxicity as well as the production of ROS. As expected, ferrocene exhibited significantly higher toxicity towards cancer cells. Further quantitative analysis demonstrated that cancer cells were approximately four times more sensitive to ferrocene in comparison to noncancer with an EC50 of 0.885 mM and 3.327 mM, respectively. Next, a DCFH-DA assay was performed to measure intracellular ROS levels; ferrocene exhibited a maximum of a 4.3-fold increase in cancer in comparison to 1.5-fold increase maximum in non-cancer cells. These results led us to the conclusion that ferrocene is selectively toxic towards cancer cells and this is correlated to the significant increase in ROS.

We then analyzed some of the biotinylated conjugates. We first selected C1 because this conjugate contained no derivatization of ferrocene except for the conjugation with biotin allowing for specific observation on the effect of biotin addition. According to our hypothesis, due to the overexpression of the biotin receptor in these cancer cells, conjugation between ferrocene and biotin should increase the overall absorption of ferrocene resulting in more ROS and selective toxicity. However, our data shows that C1 exhibited no toxicity in either cell line and this was correlated to no significant increase in ROS. We determined that a contributing factor to the lack of toxicity and ROS production of C1 could be the lack of solubility and the observed precipitation could have impeded the compound from entering cells efficiently. Therefore, we analyzed our library to find more soluble conjugates to see if increased solubility improved toxicity. After analysis, the conjugate C3 was selected which contained cysteine

connected to one of the cyclopentadienyl rings of ferrocene and was one of the most soluble compounds in our library. Despite the improved solubility, C3 exhibited no toxicity to either cancer or non-cancer cells. Concurrent with the cytotoxicity assay, ROS was measured and C3 did generate a maximum of a 2.2-fold increase in ROS in cancer, but this increase was determined to be insignificant due to similar effects observed in non-cancer cells and the lack of corresponding toxicity. Lastly, the toxicity of the conjugate C2, which has the addition of an amine to ferrocene, was tested and exhibited significant toxicity toward cancer cells with an EC50 of 0.292 mM. Furthermore, C2 exhibited no toxicity towards non-cancer cells and future studies should analyze ROS generation with increasing concentration of C2.

The lack of cytotoxicity and generation of ROS in cancer by most of the conjugates tested led to questions about the original hypothesis on the importance of biotin in cancer. According to our hypothesis, cancer cells that overexpress the SMVT biotin receptor should absorb biotin, and therefore our conjugates, with a higher efficiency. So, we decided to measure the cellular absorption of biotin in cancer and non-cancer cells using a fluorescently-labeled biotin that could be visualized using a fluorescent microscope. Upon treatment with biotin-4-fluorescein for 16-hours slides for 293 HEK, MCF-7 and Hela cells were prepared and viewed with a GFP filter. According to our results, there was no apparent difference in fluorescent intensities between the two cancer cells, which have been previously reported to overexpress the biotin receptor, and the non-cancer cell control. Potential explanations for these results could be nonspecific binding of the fluorescent biotin on the outside of the cells resulting in inaccurate measure of biotin absorption; this could be tested via a competition assay with co-treatment of fluorescent and non-fluorescent biotin. Future experiments should analyze the expression of the biotin receptor, via a western blot, to see if the cancer cells are in fact overexpressing the

receptor. However, with our current understanding it appears that biotin is not entering cancer cells with a higher efficiency.

The results of our research project indicate that ferrocene does have potential as an anticancer agent due to its selective toxicity and capability to significantly increase intracellular levels of ROS in cancer cells. However, contrary to our hypothesis, conjugation of ferrocene with biotin does not appear to increase the selectivity, toxicity, and efficiency of ferrocene being absorbed by cancer cells. This conclusion is supported by the lack of toxicity and generation of ROS by the ferrocenyl-biotin conjugates C1 and C3 as well as the result of the biotin absorption assay which demonstrated no significant difference in biotin absorption between cancer and normal cells. Despite the lack of toxicity of C1 and C3, the ferrocenyl-biotin conjugate C2 did exhibit substantial selective toxicity in cancer and future studies should repeat these toxicity assays as well as measure ROS generation. Furthermore, only two different cancers (breast and cervical) were tested with these conjugates and many different cancers have been previously reported to overexpress the biotin receptor; future studies should include a variety of cancers of different tissue origins. Additionally, cancer cells overexpress many different cellular receptors including folic acid and estrogen. In the case of breast cancer, and ferrocene could have potential therapeutic potential by conjugation with these molecular targets. In conclusion, ferrocene does have therapeutic potential against cancer due to its capability to generate ROS; however, conjugation with biotin appears not to be an effective way to develop an ROS-mediated targeted therapy.

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