# TESTING THE SPECIFICITY AND CYTOTOXICITY OF BIOTIN-FERROCENE COMPOUNDS ON CANCER CELLS

by

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# TESTING THE SPECIFICITY AND CYTOTOXICITY OF BIOTIN-FERROCENE COMPOUNDS ON CANCER CELLS

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## ABSTRACT

Cancer cells are characterized by loss of regulation of the cell cycle that results in uncontrolled proliferation. To drive this high rate of cellular division, cancer cells have mutated to increase uptake of vitamins by increasing the number of vitamin receptors, including biotin receptors, on their surface. Due to this difference in expression of biotin receptor between cancer and normal cells, research focusing on the use of biotin-conjugated molecules has gained attention as a method for anticancer drug delivery.

Another characteristic unique to certain cancer cells is that they exhibit dysregulation in normal cellular redox balance, such that certain redox reactions are favored that generate reactive oxygen species (ROS) and lead to oxidative stress. Many metal-based anticancer drugs have taken advantage of this feature of cancer cells in an attempt to increase the levels of ROS to the point that cell death occurs. Specifically, the iron atom of ferrocene has been shown to lead to the generation of damaging ROS upon oxidation from $Fe^{2+}$ to $Fe^{3+}$.

This research project focuses on testing the cytotoxicity of a variety of biotin-ferrocene compounds on cancer (HeLa and MCF7) and non-cancer (293HEK) cell lines. The tested compounds have three main features: a biotin moiety, a ferrocene core, and a variable side-chain covalently bound to the ferrocene moiety. We hypothesize that the biotin-containing compounds will enter HeLa and MCF7 cells more efficiently than 293HEK cells, allowing for the ferrocene moiety to reduce oxygen, leading to increased ROS generation and, ultimately, cell death.

Here, we demonstrate that ferrocene shows cytotoxicity specific to cancer cells. Three of the biotin-ferrocene compounds appear to be more toxic to cancer cells relative to non-cancer cells. Future studies are required to reveal how the differences in cytotoxicity are related to the differences in chemical moieties and exactly how these compounds cause specific cytotoxicity.

## Introduction

Cancer is the second-leading cause of death in the US[1]. Cancer cells are characterized by loss of regulation of the cell cycle that results in uncontrolled proliferation. Typical treatments of cancers include surgery, radiation therapy, and chemotherapy, often used in combination with one another[2,3]. Although historically effective to an extent, these approaches lack specificity and frequently kill non-cancerous cells, which can lead to the manifestation of negative side effects for cancer patients[1,3]. Furthermore, these treatments are not particularly effective in targeting and killing metastases.

In the past few decades, tremendous progress has been made in cancer research to develop more focused treatment options that aim to kill cancer cells specifically. Two examples of relatively recent treatment options include immunotherapy and targeted therapy, which exploit aspects unique to cancer cells as a means to target them for destruction[2,3,4,5,6]. Following successful treatment of chronic myeloid leukemia with the small molecule, protein-tyrosine kinase inhibitor Gleevec (imatinib; Fig 1), many targeted therapeutics have entered the pharmaceutical market and are currently used for treatment of a range of cancer types[2,3,6,7]. This research project aims to incorporate the ideology of targeted therapy as a mechanism to identify characteristics unique to cancer cells to lead to their specific destruction using small molecule drugs.

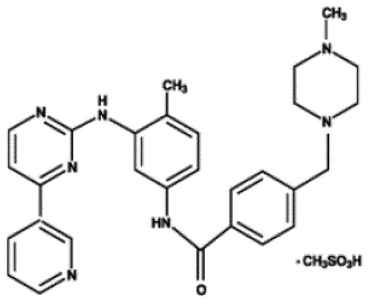



**Figure 1.** Chemical structure of Gleevec (imatinib), inhibitor of BCR-ABL tyrosine kinase found in Philadelphia chromosome positive chronic myeloid leukemia cancer cells[7].

To drive the high rate of cellular division, cancer cells have mutated to increase uptake of important nutrients including glucose by increasing the number of glucose transporters and to increase the intake of co-factors such as vitamins required for the function of metabolic enzymes by increasing vitamin receptors on their surface[8]. These overexpressed receptors allow the cancer cell to receive higher levels glucose for energy and metabolic processes and vitamins to drive high rates of enzymatic function relative to non-cancer cells. Biotin, or vitamin $B_7$, is known to bind to sodium-dependent multivitamin transporters (SMVTs), which is expressed at higher levels in certain cancer cells[9,10,11,12]. There has been considerable development in the use of biotin-conjugated molecules as a means for anticancer drug delivery systems[10]. In fact, *Ojima et. al.* provides precedent for utilizing biotin-conjugated cytotoxic agents as a mechanism to target and kill biotin receptor-overexpressing leukemia cells.

**Reaction 1**. The reduction of oxygen to water, through which several intermediate ROS are produced[14].

$$O_2 \rightarrow O_2^{\bullet -} \rightarrow H_2O_2 \rightarrow OH^{\bullet} \rightarrow H_2O$$

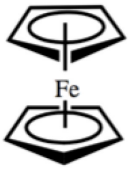
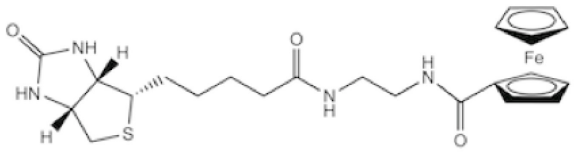
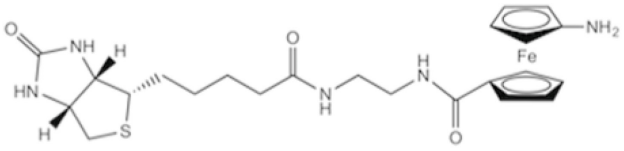
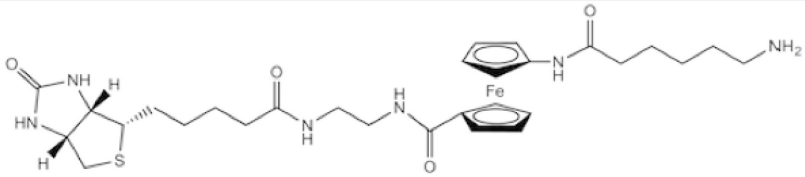
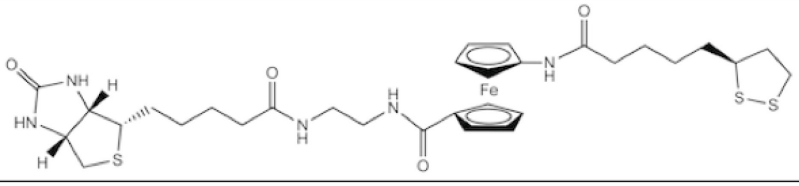
**Reaction 2**. Redox reaction between $Fe^{2+}$ and hydrogen peroxide, leading to the generation of hydroxyl radical[14].

$$Fe^{II} + H_2O_2 \rightarrow Fe^{III} + OH^{\bullet} + OH^{-}$$

Another interesting feature of certain cancer cells is that they exhibit dysregulation in normal cellular redox balance, such that the intracellular environment favors certain redox reactions, leading to oxidative stress[4,13,14]. Through Reaction 1, higher levels of harmful reactive oxygen species (ROS) are produced within cancer cells relative to non-cancer cells[4,14]. However, beyond a particular concentration threshold, high levels of ROS lead to cell death through damage to DNA, peroxidation of lipids, and oxidation of proteins[4,14]. Many metal-based

anticancer drugs have taken advantage of the abnormal redox environment of cancer cells to push intracellular levels of ROS beyond those which a cell can maintain, ultimately leading to cell death[4,14]. In particular, ferrocene has shown promising cytotoxic and anticancer properties related to its ease of oxidation from $Fe^{2+}$ to $Fe^{3+}$, which can generate elevated levels of ROS as depicted in Reaction 2[15,16,17,18]. Moreover, ferrocene has already been incorporated into a variety of anticancer drugs that have shown to be effective against breast cancer, leukemia, colon cancer, and lung cancer cells *in vitro*[15,16,17,18,19].

**Table 1.** Chemical structures of ferrocene (Fc) and biotin-ferrocene compounds.

| Compound | Structure |
|---|---|
| Ferrocene |  |
| C1 |  |
| C2 |  |
| 2 |  |
| 3A |  |

Here, we evaluate the cytotoxicity of several biotin-ferrocene compounds in cervical cancer (HeLa), breast cancer (MCF7), and non-cancer (293HEK) cell lines (Table 1). All these compounds have three main components to their structures: a biotin moiety, a ferrocene core, and a variable side-chain that alters hydrophobicity and the electronic properties of the molecule. The side-chains include: a hydrogen atom for **C1**; an amine group for **C2**; a long carbon chain with terminal amine group for **2**; and a lipoic acid group for **3A**. It is hypothesized that the biotin moiety will bind with greater frequency to vitamin receptors on cancer cells relative to non-cancer cells, resulting in preferable drug uptake. Upon entry, the ferrocene portion of the conjugates will act as an electron donor and generate high levels of ROS within the cell, ultimately leading to the death of cancer cells. Thus, by concentrating on two features unique to cancer cells, it is predicted biotin-ferrocene compounds will be more selective and, therefore, more toxic to cancer cells relative to non-cancer cells. Ultimately, these small molecule drugs have potential to serve as targeted therapeutics in cancer treatment regimens.

## Methods

### Cell Model and Culture

For the cancer cell model, both cervical cancer (HeLa) and breast cancer (MCF7) cell lines were used in cellular assays. As a non-cancer cell control, human embryonic kidney fibroblast (293HEK) cell line was utilized. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) complete medium (500 mL DMEM medium, 50 mL fetal bovine serum [FBS], 5 mL penicillin/streptomycin [50,000 units/50 mg], 5 mL glutamine [1.77 mM], 5 mL non-essential amino acids) and maintained in a humidified incubator set to 37°C and 5.0% $CO_2$.

### MTT Cytotoxicity Assay

On Day 1, cells were plated in a 96-well tray at a density of 5000 cells per well (100 µL/well) and incubated overnight at 37°C and 5.0% $CO_2$. On Day 2, drugs dissolved in DMSO were added into each well at the concentrations indicated in figures 2-6, run in quadruplicate. Cells were then incubated for 16-18 hours at 37°C and 5.0% $CO_2$. On Day 3, 100 µL of 1 mg/mL thiazolyl blue tetrazolium bromide (MTT dissolved in serum free medium) was added to each well after decanting supernatants. Cells were incubated for 4 hours, 37°C, 5.0% $CO_2$. Following incubation, supernatants were decanted and 100 µL DMSO was added to each well to solubilize the precipitate. Absorbance of the purple-colored precipitate was measured at a wavelength of 540 nm using a spectrophotometer. Protocol adapted from Sigma-Aldrich[20].

**Drugs and Compounds**

Ferrocene and all biotin-ferrocene derivatives were synthesized in the laboratory of Dr. Kayla Green (TCU, Department of Chemistry and Biochemistry). All derivatives were synthesized in house by graduate student Marianne Burnett. Ferrocene and biotin-ferrocene derivatives were dissolved in DMSO and stored at 4°C.

**Results**

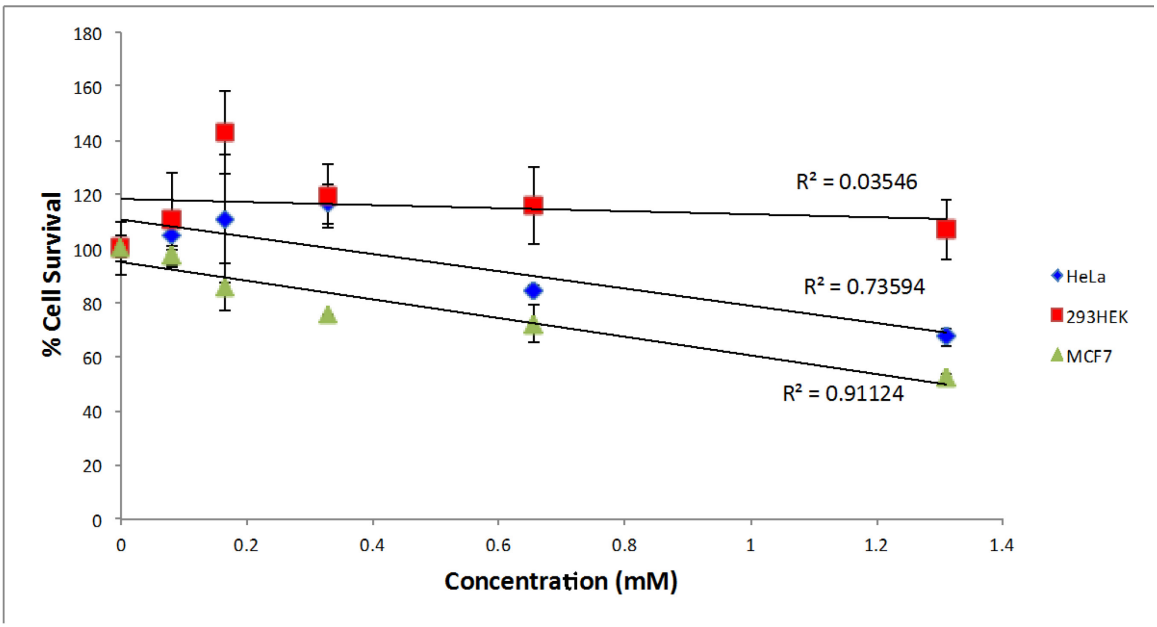



**Figure 2.** Cytotoxicity of ferrocene against 293HEK, HeLa, and MCF7 cell lines. Error bars indicate standard deviation of the mean. Trendlines represent linear best-fit lines.

To first assess the cytotoxicity of ferrocene alone, an MTT assay evaluating all three cell lines was carried out (Fig. 2). The results indicate that as the concentration of ferrocene increases, cell survival in 293HEK non-cancer cells remains constant. However, for both cancer cell lines, increasing concentration leads to lower levels of cell survival. These data suggest that ferrocene causes cell death in the cancer cells with certain specificity.

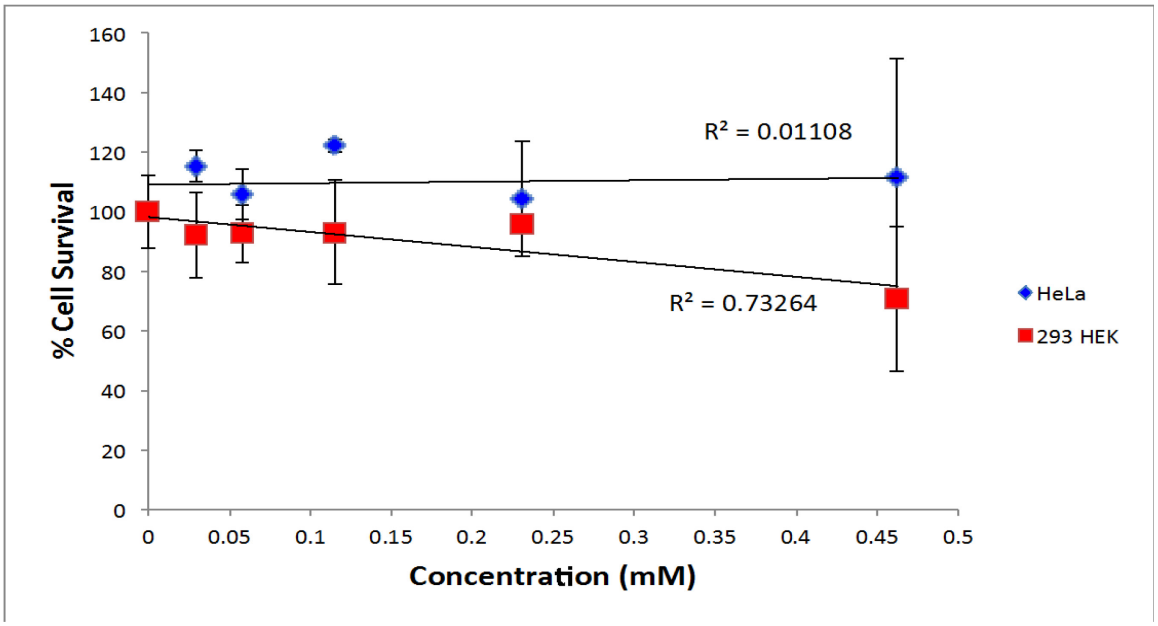



**Figure 3.** Cytotoxicity of **3A** against 293HEK and HeLa cell lines. Error bars indicate standard deviation of the mean. Trendlines represent linear best-fit lines.

Next, the cytotoxicity of **3A** was evaluated (Fig. 3). The results of this experiment were interesting in that the observed trend was opposite of that for ferrocene. Increasing concentrations of **3A** resulted in decreases in cell survival in 293HEK non-cancer cells, whereas there was no effect on cell survival in HeLa cancer cells.

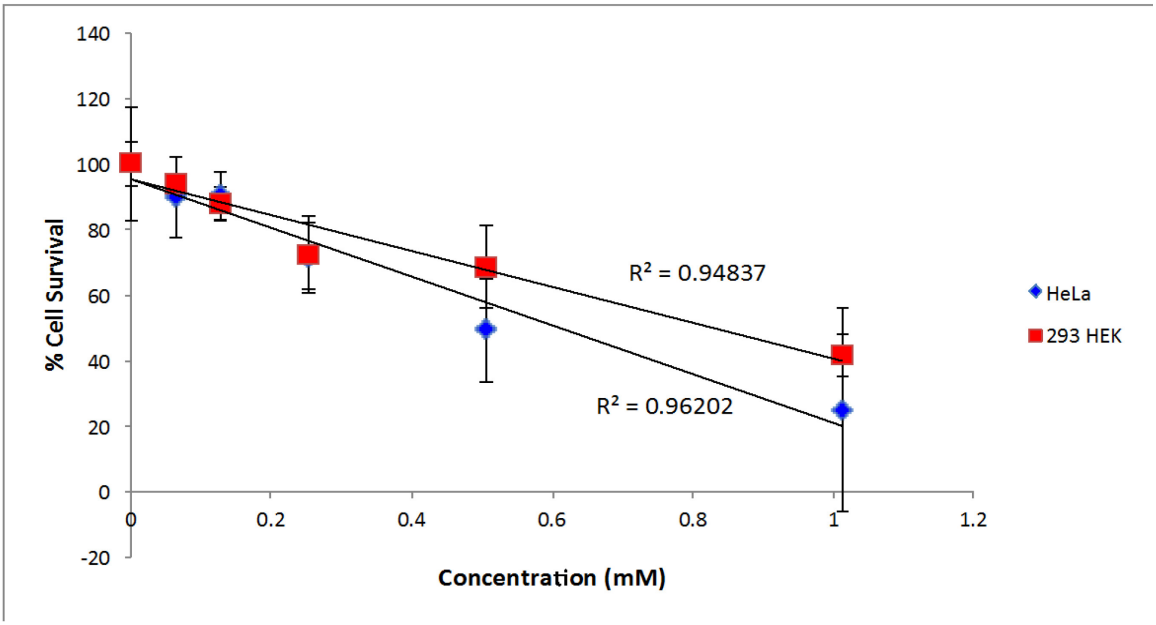



**Figure 4.** Cytotoxicity of **C1** against 293HEK and HeLa cell lines. Error bars indicate standard deviation of the mean. Trendlines represent linear best-fit lines.

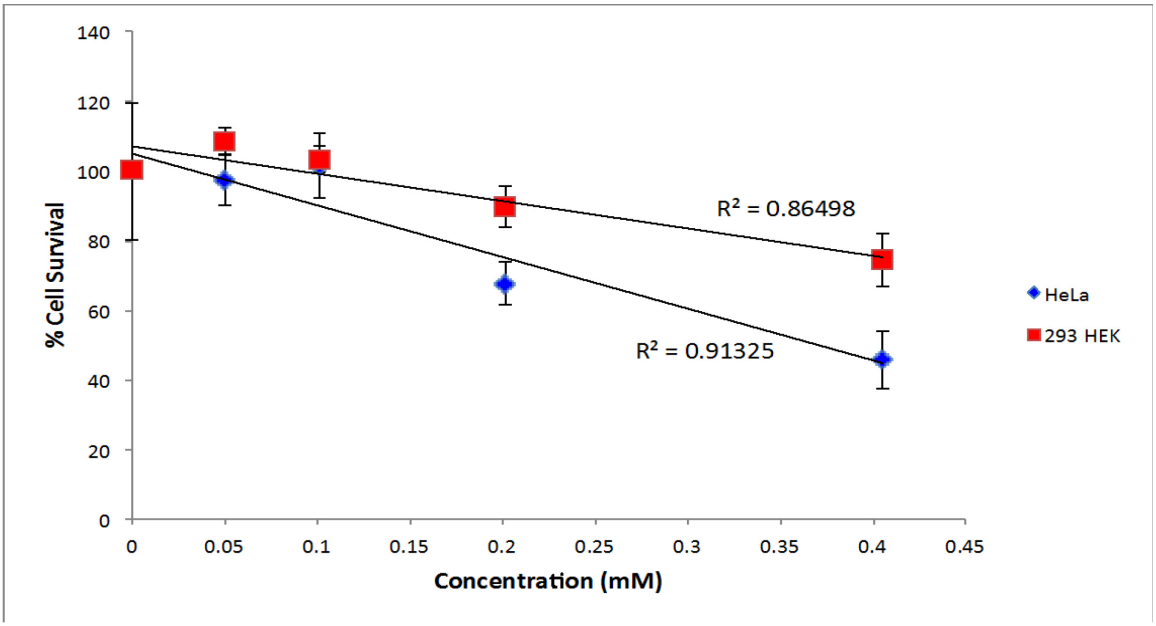



**Figure 5.** Cytotoxicity of **2** against 293HEK and HeLa cell lines. Error bars indicate standard deviation of the mean. Trendlines represent linear best-fit lines.

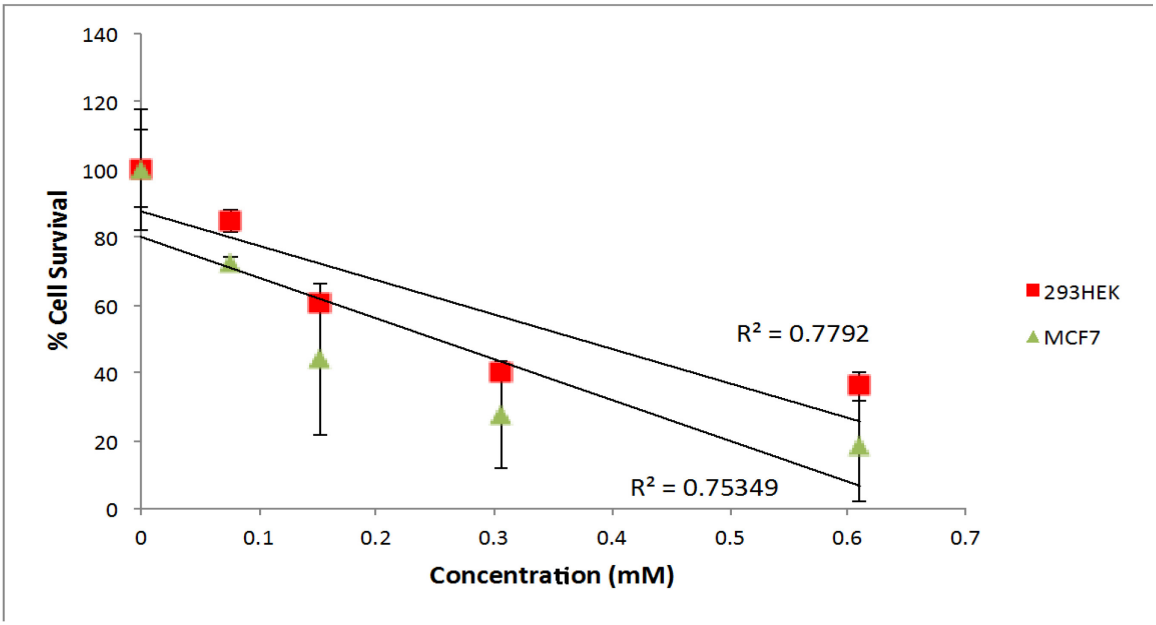



**Figure 6.** Cytotoxicity of **C2** against 293HEK and MCF7 cell lines. Error bars indicate standard deviation of the mean. Trendlines represent linear best-fit lines.

Upon testing the final three biotin-ferrocene compounds, a consistent dose-dependent cytotoxic trend was observed. Increasing concentrations of **C1**, **2**, and **C2** resulted in increased cell death for both cancer and non-cancer cell types (Fig. 4-6). It is worth noting that at any given concentration of **C1**, **2**, or **C2**, cell survival was always lower in cancer cells (HeLa, HeLa, and MCF7, respectively) relative to non-cancer cells.

**Table 2.** $EC_{50}$ values (mM) of ferrocene (Fc) and biotin-ferrocene compounds. "N.D." indicates not determined. "-" indicates the value could not be calculated.

| Cell Line | Fc | 3A | C1 | 2 | C2 |
|---|---|---|---|---|---|
| **293HEK** | 12.09 | 0.96 | 0.83 | 0.73 | 0.37 |
| **HeLa** | 1.91 | - | 0.61 | 0.37 | **N.D.** |
| **MCF7** | 1.30 | **N.D.** | **N.D.** | **N.D.** | 0.25 |

$EC_{50}$ values were determined for all tested compounds to compare cytotoxicity of ferrocene and biotin-ferrocene conjugates (Table 2). For ferrocene, six to ten times higher concentration was required to kill 50% of non-cancer cells (293HEK) relative to cancer cells. For **3A**, the $EC_{50}$ value in HeLa cells could not be calculated due to the positive trendline that is the best-fit line for those data. For the remaining three conjugates, higher concentrations were always required to kill 50% non-cancer cells relative to cancer cells. Moreover, all biotin-ferrocene compounds were more toxic than ferrocene alone, with **C2** being the most toxic compound.

**Discussion**

The cytotoxicity results appear to support the hypothesis that biotin-ferrocene compounds display higher cytotoxicity to cancer cells than non-cancer cells. The only exception to this trend is the specificity of **3A** for cancer cells, which does not support our hypothesis. However, it is worth noting that the error bars on that experiment were quite large and, therefore, those data are not as convincing as the others. Furthermore, statistical analysis of these data has yet to be performed; therefore, it cannot be confirmed that the cancer and non-cancer regression lines are truly different from one another.

Ferrocene appears to show cytotoxicity selective to cancer cells. It is predicted that the abnormal intracellular redox environment of cancer cells would favor the oxidation of the iron atom of ferrocene, thereby driving the generation of ROS leading to higher levels in cancer cells. Ultimately, an increase in intracellular levels of ROS beyond the cytotoxic threshold could explain the observed cell death. However, the lack of a localization experiment makes it difficult to conclude that the mechanism of cell death caused by ferrocene is indeed related to cellular uptake and subsequent intracellular ROS generation.

The remaining three compounds – **C1**, **2**, and **C2** – may be more toxic in cancer cells. These results support the hypothesis that the biotin portion of the conjugates would be more likely to bind to receptors on biotin receptor-overexpressing cancer cells relative to non-cancer cells, enter the cell, where ferrocene can reduce oxygen to generate ROS, and lead to cell death. Although it is an accepted notion in the literature that cancer cells overexpress biotin receptors (SMVTs), numerical data quantifying the degree of expression is scarce[9,10,11,12]. Furthermore, in searching the literature, there is considerable uncertainty and contradiction amongst sources that provide quantitative and qualitative information regarding the levels of SMVT expression and *slc5A6* (the gene that encodes SMVT) RNA expression in HeLa, MCF7, and 293HEK cell lines[1,21].

Among **C1**, **2**, and **C2**, it is difficult to explain why the cytotoxicity is variable among the compounds based solely on MTT assays and $EC_{50}$ values. Most likely, the variable side-chain conjugated to the cyclopentadienyl ring affects the cytotoxic properties of these compounds. **C2** has an amine group as its side-chain, which could donate electron density to the ferrocene cyclopentadienyl ring by resonance, thereby increasing the reductive potential of the iron atom. It is possible that an increase in the reductive potential of the iron atom would push Reaction 1 forward and generate higher levels of ROS, explaining why **C2** has the highest cytotoxicity. In this same vein of logic, **C1**, with a hydrogen atom as its side-chain, would display altered electronics such that the electron donating ability of the ferrocene core would not be as pronounced as that of **C2**, explaining its relatively lower cytotoxicity. The side-chain of compound **2** is a long chain amine, which theoretically would increase the hydrophobicity of the molecule. An increase in hydrophobicity could increase the affinity of the compound for the cellular membrane and promote cellular uptake by proximity. Furthermore, the amide group

directly off the ferrocene could act as an electron-withdrawing group, pulling electron density from the ferrocene moiety. These contrasting properties may explain why the cytotoxicity of **C2** and **2** are relatively equal in cancer cells. However, again it is worth mentioning that since statistical analysis of these data has yet to be performed, it cannot be confirmed that these differences in cytotoxicity are statistically significant and truly reflect differences in intrinsic toxicity of the compounds.

Alternatively, the observed data may simply be related to differences in solubility of the compounds. The addition of biotin to ferrocene results in a more polar molecule relative to ferrocene. Perhaps, upon treating cells with the drugs, the biotin-ferrocene compounds are simply more soluble and can enter the cells at a greater rate, regardless of biotin receptors, compared to the hydrophobic ferrocene. As a result, the enhanced cytotoxicity of the biotin-ferrocene compounds may actually be just as toxic as ferrocene but are more hydrophilic and, therefore, are more available to cells within the aqueous medium. Solubility data on these compounds and ferrocene in addition to a cytotoxicity experiment with ferrocene conjugated to a polar group such as polyethylene glycol would help to remove doubt.

Despite these interesting results, future experiments are required to understand the property-altering effect of the variable side-chain and to validate whether the biotin-ferrocene conjugates are entering cells. A competition experiment between free biotin and the biotin-ferrocene conjugates would shed light onto the mechanism by which these compounds act. It would be predicted that free biotin would compete for binding sites on biotin receptors, thereby decreasing entry of the cytotoxic agent and, ultimately, resulting in decreased cytotoxicity relative to experiments without free biotin. This proposed experiment would provide valuable information to determine if these compounds are binding to biotin receptors on the cell surface.

Additionally, a fluorescent tag would allow visualization of the conjugates upon addition to the cellular medium to further confirm the destiny of the compounds after treatment. Finally, the properties of these biotin-ferrocene compounds that promote greater cytotoxicity in cancer cells relative to 293HEK cells should be identified and exploited to develop more specific and effective anticancer drugs.

## Conclusion

Certain cancer cells are known to have both increased biotin receptors and dysregulated redox balances[4,9,10,11,12,13,14]. These characteristics unique to cancer cells appear to be feasible opportunities for the development of targeted anticancer therapeutics. Ferrocene is known to promote the generation of ROS within cells that can ultimately lead to cell death[15,16,17,18]. In this project, biotin – as a cancer targeting molecule – and ferrocene – as a cytotoxic agent – are combined in a series of conjugate molecules and evaluated for their toxicity to cancer (HeLa & MCF7) and non-cancer (293HEK) cells. The results indicate that three of these conjugates - **C1**, **2**, and **C2** – show higher cytotoxicity to cancer cells and that ferrocene alone shows cytotoxicity selective to cancer cells. It is hypothesized that the side-chain of these compounds can be chemically manipulated to generate highly cytotoxic drugs. Future efforts need to focus on confirming the mechanism of action and understanding the determinants of cytotoxicity and specificity by altering side-chains to have greater cytotoxicity to cancer cells relative to non-cancer cells. Ultimately, biotin-ferrocene compounds may prove to be an attractive anticancer therapeutic that targets two characteristics unique to cancer cells.

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