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Gd@C_{60}-(ZME-018) Immunoconjugate Targeting of A375 Melanoma Cells

by

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Abstract

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For the first time, C_{60}-monoclonal antibody (mAb) immunoconjugates have been determined to internalize into target cells using water-soluble Gd^{3+}-ion-filled fullerenes (Gd@C_{60}(OH)_{x}). Separate conjugations of Gd@C_{60}(OH)_{x} with the antibody ZME-018 and a murine antibody mixture (MuIgG) took place in a 1:5 mAb:Gd@C_{60} ratio. Quantitative analysis of the immunoconjugates was established using inductively-coupled plasma mass spectrometry (ICP-MS) and UV-Vis spectrometry (Gd@C_{60}+C_{60}). Enzyme-linked immunosorbent assays (ELISA) show little change in the specific binding of the ZME-018 once conjugated.

Each immunoconjugate was exposed to two cancer cell lines, A375m (a ZME-018-specific line), and T24, a bladder carcinoma line. Internalization of the immunoconjugate was measured at various timepoints, after which the cells were harvested and digested with 25% HClO_{3} for Gd^{3+} analysis by ICP-MS.

ICP-MS results show immunoconjugate internalization peaked in the first hour of exposure of the cells with a large dropoff occurring in the subsequent hour. These are the first results demonstrating the practicality of a cancer therapy based on Fullerene Immunotherapy (FIT).
Acknowledgements

While the work in this thesis is my own, none of it would have been possible without the enormous amount of help and effort from my friends and collaborators.

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To all the Wilsonites, both past and present. The best research group at Rice, they are less a group and more a family.

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Introduction

The birth of modern passive immunization began in two stages. First, in 1888, Émile Roux and Alexandre Yersin of the Pasteur Institute proved the existence of diphtheria toxin by injecting the cell-free sera of Corynebacterium diphtheriae into lab animals. Though no infection was possible, the lab animals soon developed symptoms of diphtheria, proving the cause of symptoms were not directly from the diphtheria bacteria, but instead from the toxins they release. The second stage was completed two years later when Roux and Yersin’s colleagues at the Pasteur Institute, Emil von Behring and Kitasato Shibasaburo, immunized lab animals with heat-attenuated forms of diptheria and tetanus. Sera collected from these animals was injected into infected animals with manifested symptoms. The result was surprising, for the antitoxins in the sera relieved symptoms and eventually led to recovery from an infection that would previously have been fatal.

By mass-producing the diphtheria antitoxin in horses, Behring’s “serum therapy” eventually became treatment for humans in 1896, as the first immunity-based treatment since Jenner’s smallpox vaccine developed a century before. As serum therapies appeared for other bacterial diseases, hope from this new therapy helped treat an entire generation. In the wake of this revolutionary therapy, Paul Ehrlich, a medical great who assisted Emil von Behring in his development of a diphtheria serum, made a prediction whose concept has been a goal of medical treatment for over a century. He noted that certain strains of infectious bacteria were susceptible to staining from different dyes, a precursor to Gram staining. He reasoned if dyes are capable of targeting and binding to
material in cell walls, it must be possible to find toxic materials that would preferentially target foreign bacteria in an infected patient over native tissue. This “magic bullet” would then provide a targeted therapy, capable of patient treatment with little to no side effects.

This magic bullet concept of a targeted therapeutic persists to this day. It is estimated that only 1 of every 100,000 molecules administered to a patient locally reaches its intended destination. The simple goal of targeted therapies is to produce high concentrations of therapeutic in the appropriate biological area, thus reducing patient side effects while allowing higher pharmaceutical doses to be administered. The development of cell-targeted agents for imaging and therapeutic applications in medicine is accelerating.

Cytokines, growth factor and kinase inhibitors and monoclonal antibodies (mAbs) all show promise for their ability to deliver payloads to the surface and the cytoplasm of targeted cancer cells, as well as other more exotic materials such as RGD (arginine-glycine-aspartic acid) peptides and chlorotoxin from the Deathstalker scorpion Leiurus quinquestriatus. However, by far the most versatile and successful class of targeting agents to show targeting capabilities for specific cancers currently are monoclonal antibodies (mAb). The study of antibodies and their role in immune response began with Emil von Behring and continued with Paul Ehrlich, but their potential as a therapy was not possible until the development of a method for producing large quantities of antibodies all recognizing the same cellular epitope.
By the early 1970s, research had revealed the role of antibodies in the immune system and many of the mechanisms by which B-cells identify antigens and stimulate immune responses. Investigation of paraproteins in multiple myeloma cases revealed, however, that cancerous B-cells deriving from a single sample produce monoclonal antibodies instead of the normal sample of polyclonal antibodies. In 1975, Niels Kaj Jerne, César Milstein and Georges Köhler successfully fused antibody-producing myeloma cells with spleen cells from immunized mice producing a specific antigen. The resulting hybridomas can be cloned, are immortal and after sufficient incubation, begin to secrete a single product: monoclonal antibodies binding to the specific antigen from spleen cells.

Antibodies, also known as immunoglobulins, are an integral components in the animalian immune system. These agents contain at least four subunits: two identical light chains (~23 kDa) and two identical heavy chains (mass ranging from 53-75 kDa). These subunits combine together through disulfide bonds and non-covalent interactions to form a Y-shaped symmetric molecule. It has now become regular laboratory practice to use the enzyme papain to proteolyze antibodies into the two identical Fab fragments, which serve as the antigen-binding “arms” of the Y-shaped antibody, from the single Fc fragment, which composes the antibody “stem”.
Using a patient's own cellular identification system to target cancer with immunoconjugates has the potential to become a potent anticancer therapy in personalized medicine. To date, three immunoconjugates have been FDA-approved for clinical use. Two murine mAbs target the B-cell glycoprotein CD20 to treat non-Hodgkin's lymphomas with $\beta$-emitting radionuclides. Ibritumomab tiuxetan is the IgG1-$\kappa$ mAb radiolabeled with either $^{90}$Y or $^{111}$In, while Tositumomab is the second agent, which is an IgG2a-$\lambda$ mAb radiolabeled with $^{131}$I. Gemtuzumab ozogamicin, a third immunoconjugate, is a humanized, anti-CD33 IgG4-$\kappa$ mAb covalently derivatized with cytotoxic calicheamicin for use in the treatment of acute myelogenous leukemia. These immunoconjugates must internalize effectively within target cells for optimal therapeutic efficacy.

Since the discovery of fullerenes in 1985 and carbon nanotubes in 1991, one of the most prominent areas of study for such carbon nanostructures has been for medical applications. Although their toxicity is still under debate, carbon nanostructures appear to
be an ideal scaffold for immunoconjugation. Properly derivatized, they are non-immunogenic, biologically stable, and eventually excreted from mammals. To date, water-soluble fullerenes have been studied for potential use as neuroprotective agents, HIV-1 protease inhibitors, bone-vectoring agents and x-ray contrast agents. Additionally, carbon nanomaterials can be internally loaded, either during initial synthesis or in post-production steps, with materials possessing useful properties for therapy or diagnosis, such as radionuclides or Gd ions for MRI.

The first fullerene (C₆₀)-antibody immunoconjugate was produced in 2005. Reacting 2-iminothiolane (Traut’s reagent) with the antibody ZME-018 labeled each antibody with an average of 5 free thiols for disulfide functionalization. The functionalized ZME-018 was then reacted with a water-soluble fullerene moiety attached with the reactive linker 3-(2-Pyridyldithio)propionic acid (SPDP). For a control, a separate sample of ZME-018 was treated with a simple water-soluble fullerene. To the researchers’ surprise, the water-soluble control conjugated more than the SPDP-fullerene, in 38:1 and 15:1 ratios respectively, in what is almost certainly a non-covalent attachment. The theory behind such an association is that the fullerenes, with a diameter of ~1 nm are able to non-covalently “hide” in hydrophobic pockets of the antibody, which measures roughly 7 nm x 8 nm x 10 nm.

Due to the possible low concentration of labeling agent used to determine the extent of antibody conjugation and internalization into cells, detection sensitivity is a key factor. In the past two decades, major strides have been made for the detection of certain chemical elements by inductively-coupled plasma mass spectrometry (ICP-MS) and
sensitivities on the order of parts per trillion have been achieved. However, due to current sample preparative methods, carbon is not detectable by ICP-MS, posing a problem for the detection of naked C60. A facile solution to this problem is to replace normally-derivatized fullerene with its endohedral metallofullerene analogue (M@C60) to monitor the amount of C60 internalized into target cells. Recent innovations in the preparation and purification of water-soluble endohedral Gd$^{3+}$-ion-filled fullerenes such as Gd@C60(OH)$_x$ (abbreviated here as Gd@C60) now make these materials readily accessible, and given our previous experiences with such "gadofullerenes" as MRI-contrast agents, they provide a well-characterized system.

In this study, Gd@C60 has been conjugated to both a melanoma antibody (ZME-018) and a control murine IgG antibody (MulgG). ZME-018 targets the gp240 antigen, found on the surface of >80% of human melanoma cell lines and biopsy specimens. Functionalized conjugates of ZME-018 have been utilized extensively, with studies ranging from in vitro fluorescent studies of surface antigens, to $^{111}$In-ZME-018 conjugate targeting as both a laboratory and clinical in vivo tumor imaging agent. ZME-018 shows great promise in clinical imaging trials for the delivery of toxins, cytokines and other therapeutic agents to melanoma cells both in vitro and in vivo. Immunoconjugates containing ZME-018 are known to rapidly internalize into melanoma cells and effectively localize into melanoma xenografts after systemic administration and demonstrate impressive cytotoxic effects against established tumors in orthotopic models. The reliable targeting properties of ZME-018 conjugates and its thorough
characterization in varied immunoconjugate systems make ZME-018 an ideal antibody for fullerene conjugate delivery.

Although just beginning, today’s human antibody-based therapies have many advantages over traditional therapies making this area of research very desirable. By working with a patient’s own immune system, monoclonal antibody therapies possess high specific binding to cells, negligible toxicity, multiple modes of function, and are capable of covalent functionalization. Currently twenty-one FDA-approved mAb therapies exist, treating everything from cardiovascular disease to autoimmune disorders to cancer, but current research is attempting to come closer to Ehrlich’s “magic bullet.” By conjugating therapeutics to mAbs, the research aim today is to use mAbs as targeting moieties to directly affect cells rather than rely on immune responses triggered by mAbs. The goal is that upon patient administration, these immunoconjugates bind to their specific cellular antigen and are internalized, delivering the specific therapies.
**Experimental**

Immunoconjugates of Gd@C\textsubscript{60} were prepared using a procedure similar to previous C\textsubscript{60}-based immunoconjugates, \textsuperscript{14} where conjugation is achieved by supramolecular chemistry rather than by conventional covalent attachment. Cell internalization studies were performed to determine the efficiency with which the cell-specific Gd@C\textsubscript{60}-immunoconjugates internalize into melanoma cells. Antigen positive (A375m; melanoma) and negative (T24; bladder carcinoma) cells were prepared on 100 mm\textsuperscript{2} tissue culture plates at \(2\times10^6\) cells/plate in Dulbecco's modified eagle medium. The cells were incubated overnight at 37°C, followed by addition of \(1\times10^{-6}\) g/ml (6.67 nM), 10 mL/plate, of the Gd@C\textsubscript{60}-immunoconjugates over various time frames. Incubation for 1, 2, 4 and 24 hour time points at 37°C allowed for cell internalization to occur. At the zero point, the media was removed and each cell sample washed with glycine buffer solution (0.05 M, pH 2.5 + 0.1 M NaCl), 10 mL, to strip off any non-internalized Gd@C\textsubscript{60}-immunoconjugate. The plates are then washed with a neutralizing buffer (0.15 M Tris, pH 7.4), 10 mL. Using a trypsin solution (0.25% Trypsin + EDTA [GIBCO]), the cells were then detached from the bottom of the plate and subsequently digested to determine if the Gd@C\textsubscript{60}-immunoconjugates internalize into target cells and to what extent over time.
Results and Discussion

Cell binding affinity was evaluated by calculating IC(50) values from enzyme-linked immunosorbent assay (ELISA) plots. Similar to an earlier C₆₀-immunoconjugate study,¹¹ dry A375m (antigen-positive) cell plates were utilized. However, to better understand binding efficiencies, T24 (antigen-negative) cells were also used for comparison with antigen positive cells. The Gd@C₆₀-(ZME-018) and Gd@C₆₀-(MuIgG) immunoconjugate ELISA binding curves and IC(50) values were determined for both cell lines (Figure 2). The IC(50) values, and hence binding efficiencies to A375m cells for the Gd@C₆₀-(ZME-018) immunoconjugate, was 44.8 ng/ml. This is practically identical to non-conjugated ZME-018, which demonstrated a IC(50) value of 43.9 ng/mL. When considered with negative MuIgG, which showed an IC(50) value of 1228 ng/mL for the Gd@C₆₀-(MuIgG) immunoconjugate (seven hundred seventy times less efficient), it is clear that Gd@C₆₀-based immunoconjugates retain cell specificity. The IC(50) value of the Gd@C₆₀-(ZME-018) immunoconjugate binding to T24 cells was 13.32 µg/mL. While this represents an increase in nonspecific binding over the unmeasurable IC(50) value of the naked ZME-018 mAb, the difference between specific and non-specific binding is still significant by a factor of almost 300. These findings are encouraging for the future development of fullerene immunotherapy (FIT), where fullerene/chemotherapeutic constructs could be antibody-targeted to specific cancer cells.
Since the Gd@C\textsubscript{60}(OH)\textsubscript{x} sample used for antibody conjugation contained both Gd@C\textsubscript{60}(OH)\textsubscript{x} and C\textsubscript{60}(OH)\textsubscript{x} species,\textsuperscript{26} ICP-atomic emission spectroscopy (ICP-AE) was used to determine the percent of Gd@C\textsubscript{60}(OH)\textsubscript{x} contained in the sample (41.9%). The concentration of the fullerene material in the immunoconjugate was determined using both UV-Vis spectrometry\textsuperscript{11} and ICP-MS. After fullerene-mAb conjugation, Bio-Rad protein assays were used to determine the antibody concentrations. Solutions of both the Gd@C\textsubscript{60}-(ZME-018) and Gd@C\textsubscript{60}-(MuIgG) immunoconjugates (0.667 nM) were prepared. ICP-MS then determined the Gd\textsuperscript{3+} concentration of each sample, whereupon the total fullerene concentration (only 41.9% of the sample is Gd@C\textsubscript{60}(OH)\textsubscript{x}) in the sample and the total fullerene (Gd@C\textsubscript{60}(OH)\textsubscript{x}+C\textsubscript{60}(OH)\textsubscript{x}):mAb ratio was determined, as shown in Table I.
Table I. ICP-MS results for the Gd@C_{60}(OH)_{x} monoclonal antibody immunoconjugates.

<table>
<thead>
<tr>
<th>mAb Conjugate</th>
<th>Gd@C_{60} Concentration (nM)</th>
<th>Gd@C_{60}+C_{60} Concentration (nM)</th>
<th>Fullerene:mAb Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gd@C_{60}-(ZME-018)</td>
<td>1.30</td>
<td>3.11</td>
<td>4.82</td>
</tr>
<tr>
<td>Gd@C_{60}-(MulgG)</td>
<td>1.29</td>
<td>3.08</td>
<td>4.77</td>
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</table>

Additionally, UV-vis spectra (Figure 3) of the naked antibodies and their Gd@C_{60}-immunoconjugates were obtained as a second, independent determination of the total fullerene:mAb ratio. Both of the immunoconjugates exhibited increased absorbance from 250-450 nm compared to the naked antibody, especially at the shorter wavelengths. Using Beer’s law to calculate these differences using a Gd@C_{60}(OH)_{x} standard solution, the ratio of the total fullerene:mAb for Gd@C_{60}-(ZME-018) and Gd@C_{60}-(MulgG) was found to be 5.97 and 5.23 respectively. The discrepancies between the total fullerene:mAb ratios by UV-Vis spectroscopy and ICP-MS data indicate a small preferential selection by the antibody for derivatized C_{60}(OH)_{x} over Gd@C_{60}(OH)_{x}.
Figure 3. UV-Vis Spectrum of Gd@C₆₀(OH)ₓ, the monoclonal antibodies and their respective immunoconjugates.

In order to help visualize the Gd@C₆₀ interaction with ZME-018, TEM images of the Gd@C₆₀ immunoconjugates were acquired (Figure 4). Similar to the previous C₆₀-based immunoconjugates,¹⁴ the ZME-018 mAb conjugate increased in size relative to unconjugated ZME-018 and contained aggregates of the Gd@C₆₀- and C₆₀-based nanomaterials, as evidenced by the uniform black spots in the image. These results show that the Gd@C₆₀ materials display similar interactions with ZME-018 as the previous C₆₀-SPDP and C₆₀-Ser derivatives,¹⁴ but that conjugation conditions still need to be optimized to increase the concentration of Gd@C₆₀ in the ZME-018 mAb conjugate.
Cell internalization studies for the Gd@C₆₀-immunoconjugates were performed in a manner similar to that for the previous C₆₀-immunoconjugates. The only deviation from the original procedure is that, instead of lysing the cells for fullerene analysis, cells were removed from the plate and spun down. Next, each sample was placed in an individual 20 mL scintillation vial and digested for ICP-MS analysis using 3 mL of 25% chloric acid (HClO₃) and 1 mL of 30% H₂O₂, while heating the vial to dryness at 100 °C to consume the cells and destroy the C₆₀ cage around the Gd³⁺ ion. After cooling, the resulting residues were dissolved in 3 mL of 2% nitric acid, filtered using a Millex-GP polyethersulfone 0.22 μm syringe filter, heated to dryness again, and then dissolved in 1 mL 2% HNO₃, the matrix material utilized for ICP-MS.

The Gd³⁺-ion concentration for both the Gd@C₆₀-(ZME-018) and Gd@C₆₀-(MuIgG) samples was sampled ten times using ICP-MS for the cell internalization studies using both immunoconjugates (Table II). The Gd@C₆₀-(ZME-018) immunoconjugate exhibited an increase in delivery of Gd@C₆₀ to the A375m cells, peaking in concentration
at the 2 hour timepoint at $7.06 \times 10^{13}$ Gd@C$_{60}$ molecules/cell and slowly declining to $1.26 \times 10^{13}$ Gd@C$_{60}$ molecules/cell after 24 hr (Figure 5).

<table>
<thead>
<tr>
<th>Cells, Antibody, Incubation Period</th>
<th>Gd Concentration in Sample (ppt)</th>
<th>Sample Size (Cells)</th>
<th>Gd@C$<em>{60}$+C$</em>{60}$ Per Cell</th>
<th>Antibodies Per Cell</th>
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<tbody>
<tr>
<td>A375m, No mAb, 0 hr</td>
<td>0.00</td>
<td>2.67E+06</td>
<td>0.00E+00</td>
<td>0.00E+00</td>
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<tr>
<td>A375m, ZME-018, 1 hr</td>
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<td>9.00E+05</td>
<td>4.05E+13</td>
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<td>A375m, ZME-018, 2 hr</td>
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<td>1.75E+06</td>
<td>7.06E+13</td>
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<td>A375m, ZME-018, 4 hr</td>
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<td>1.63E+06</td>
<td>5.71E+13</td>
<td>1.19E+13</td>
</tr>
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<td>A375m, ZME-018, 24 hr</td>
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<td>3.03E+06</td>
<td>4.15E+13</td>
<td>8.61E+12</td>
</tr>
<tr>
<td>A375m, MulgG, 1 hr</td>
<td>4.19</td>
<td>1.43E+06</td>
<td>2.68E+13</td>
<td>5.61E+12</td>
</tr>
<tr>
<td>A375m, MulgG, 2 hr</td>
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<td>4.66E+13</td>
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<td>3.77E+13</td>
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<td>A375m, MulgG, 24 hr</td>
<td>13.62</td>
<td>4.88E+06</td>
<td>2.55E+13</td>
<td>5.35E+12</td>
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<tr>
<td>T24, No mAb, 0 hr</td>
<td>0.00</td>
<td>1.15E+06</td>
<td>0.00E+00</td>
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</tr>
<tr>
<td>T24, ZME-018, 1 hr</td>
<td>1.11</td>
<td>1.24E+06</td>
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<td>1.70E+12</td>
</tr>
<tr>
<td>T24, ZME-018, 2 hr</td>
<td>2.28</td>
<td>5.40E+05</td>
<td>3.86E+13</td>
<td>8.01E+12</td>
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<td>T24, ZME-018, 4 hr</td>
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<td>8.00E+05</td>
<td>3.19E+13</td>
<td>6.63E+12</td>
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<td>T24, ZME-018, 24 hr</td>
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<td>4.01E+12</td>
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<td>T24, MulgG, 1 hr</td>
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<td>3.78E+13</td>
<td>7.93E+12</td>
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<td>T24, MulgG, 2 hr</td>
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<td>3.82E+13</td>
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<td>6.74E+12</td>
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<tr>
<td>T24, MulgG, 24 hr</td>
<td>8.28</td>
<td>2.07E+06</td>
<td>3.66E+13</td>
<td>7.66E+12</td>
</tr>
</tbody>
</table>

**Table II.** Cell internalization results for the Gd@C$_{60}$-(ZME-018) and Gd@C$_{60}$-(MulgG) immunoconjugates over time.
Figure 5. Internalization of Gd@C₆₀-mAb immunoconjugates into cells over time.

These initial internalization experiments demonstrate the feasibility of utilizing ICP-MS for determining [Gd³⁺] at very low concentrations after cell internalization of Gd@C₆₀-immunoconjugates into A375m cells and are the first such series to quantitatively determine antibody internalization using metal ions. For comparison, attempts to internalize the Gd@C₆₀-immunoconjugates into T24 antigen-negative cells were performed. These experiments showed lower numbers of Gd³⁺ ions in the T24 cells, demonstrating that the Gd@C₆₀-immunoconjugates retained their cell-specific targeting.
properties, as well as verifying successful internalization into A375m cells. These results suggest that Gd@C60-based immunoconjugates do, indeed, internalize into target cells, which is an encouraging result for the future development of FIT and the potential translation of FIT into the clinic.
Further Research

After proving that C$_{60}$-mAb immunoconjugates internalize into cells, the next logical step toward development of FIT is the construction of a water-soluble chemotherapeutic-fullerene antibody conjugate.

Camptothecin is a fluorescent cytotoxic and antiproliferative natural product first isolated in 1966 by Drs. Monroe Wall and Mansukh Wani of the Research Triangle Institute during a systematic screening of materials for their pharmacological properties. Camptothecin and its FDA-approved analogs, irinotecan and topotecan, form a class of chemotherapeutics that act by inhibiting the enzyme topoisomerase I, which plays an important role in DNA replication. DNA normally exists in a supercoiled form, unwinding during DNA replication and RNA transcription, allowing selected regions of the DNA duplex to separate and serve as templates. Rather than physically unwinding, topoisomerase I facilitates cleavage of one strand of DNA, allowing rotation of the broken strand around the intact strand to relieve torsional strain from replication and transcription bubbles. Once the torsional strain is relieved, topoisomerase I reseals the cleavage and reversibly dissociates from DNA. Camptothecin and its analogues hyperstabilize the DNA-topoisomerase I cleavage complex, inhibiting the religation step and causing irreversible breaking of the DNA double strand to ultimately induce cell apoptosis.
The two major problems that delayed camptothecin therapies are poor solubility of the molecule in water/blood and hydrolysis of the lactone ring at pH=7.4 (blood pH), which increases albumin binding, rendering the molecule therapeutically unavailable. Many current experimental therapies have sought to overcome these problems by covalently conjugating the camptothecin to water-soluble molecules via esterification, which stabilizes the lactone and reduces hydrolysis. Once these water soluble conjugates are internalized into a cancer cell, camptothecin is cleaved from the conjugate by one of the many ubiquitous cellular esterases, thus restoring the molecule and its bioactivity. Additionally, the lactic acidosis which is a characteristic of most cancers' high metabolism also reduces lactone hydrolysis of the internalized camptothecin, thus helping to preserve bioactivity.

The current chemotherapeutic of choice for advanced colorectal cancer and only one of two camptothecin analogues currently FDA-approved for clinical use, irinotecan (trade name: camptosar), utilizes a similar strategy to these experimental therapies. SN-38, a camptothecin analogue possessing a higher therapeutic index than the original molecule, is solubilized by conjugation of its phenolic hydroxyl to water-solubilizing groups to create a prodrug. Once dispersed in the fluids of the body, esterases cleave the bond creating the naked SN-38 and restoring its therapeutic activity.

By using fullerene-mAb conjugates as a delivery vehicle for camptothecin, the potential for a new modality is being introduced for targeted therapy. Fullerene immunotherapy (FIT) is a versatile tool capable of combining different potent pharmaceuticals in high ratios with different antibodies to custom make potent cocktails.
to attack cancer. Our strategy capitalizes on combining two current drug delivery methods: antibodies as a delivery vector and ester conjugations. As new technologies have increased the diversity and reduced the cost of clinical antibodies, research is heavily underway to use antibodies as delivery vectors. By using an ester bond to attach camptothecin to water-soluble C60 and then non-covalently “associating” the functionalized fullerene with an antibody, we are creating an entirely new, multifunctional moiety for drug delivery with a higher therapy:mAb ratio than is currently available. Upon cellular administration of a CPT-C60-ZME-018 immunoconjugate, either in vitro or in vivo, antibody internalization into the cell results in cellular digestion of the antibody, releasing the CPT-C60 into the cytosol. Cellular esterases, on which many cellular labeling and drug delivery techniques already rely, will release the camptothecin freeing it for therapeutic activity.

Having conclusively proven that C60-mAb immunoconjugates internalize into cells, the next logical step toward FIT is a drug-C60-mAb immunoconjugate for targeted chemotherapeutic drug delivery. For example, the proposed synthetic scheme below uses a fullerene scaffold and the camptothecin analogue SN-38 attached via a labile C60-based bond. It is proposed that prior to internalization, the lipophilic SN-38 resides between the fullerene and mAb hydrophobic pocket. Once the immunoconjugate is internalized into cells, protease digestion of the antibody releases the fullerene, and subsequent hydrolysis of the ester bonds will release SN-38, delivering the chemotherapeutic to its targeted cell.
Proposed Synthetic Scheme of a Water-Soluble SN-38-C₆₀ Conjugate

1. **HCl** + **DMAP** + **DCC**
   - Reaction conditions: Et₃N, THF, 3 hr, reflux
   - Result: HO₂C-CONH₂OMe

2. **HCl** + **Me₂CO**
   - Reaction conditions: DCC, DMAP, CH₂Cl₂
   - Result: MeO-CONH₂OMe

3. **Et₃N**, **THF**
   - Reaction conditions: MeO-CONH₂OMe
   - Result: MeO-CONH₂OMe

4. **Et₂O**, **TESO**
   - Reaction conditions: Pyridine, 18 hr, -20°C
   - Result: TESO-CONH₂OMe

5. **C₆₀ + 1**
   - Reaction conditions: Tol., CBr₄, DBU, 12 hr, rt
   - Result: Bingel Addition

6. **C₆₀ + 2**
   - Reaction conditions: Tol., 2, CBr₄, DBU, 12 hr, rt
   - Result: Bingel Addition

7. **H₂N-C₆₀-OH** + **diethyl malonate**
   - Reaction conditions: Et₃N, THF, 3 hr, reflux
   - Result: HO₂C-CONH₂OMe

8. **Et₂O**, **TESO**
   - Reaction conditions: Pyridine, 18 hr, -20°C
   - Result: TESO-CONH₂OMe

9. **C₆₀ + 1**
   - Reaction conditions: Tol., CBr₄, DBU, 12 hr, rt
   - Result: Bingel Addition

10. **C₆₀ + 2**
    - Reaction conditions: Tol., 2, CBr₄, DBU, 12 hr, rt
    - Result: Bingel Addition

11. **H₂N-C₆₀-OH** + **diethyl malonate**
    - Reaction conditions: Et₃N, THF, 3 hr, reflux
    - Result: HO₂C-CONH₂OMe

12. **Et₂O**, **TESO**
    - Reaction conditions: Pyridine, 18 hr, -20°C
    - Result: TESO-CONH₂OMe

13. **C₆₀ + 1**
    - Reaction conditions: Tol., CBr₄, DBU, 12 hr, rt
    - Result: Bingel Addition

14. **C₆₀ + 2**
    - Reaction conditions: Tol., 2, CBr₄, DBU, 12 hr, rt
    - Result: Bingel Addition

15. **H₂N-C₆₀-OH** + **diethyl malonate**
    - Reaction conditions: Et₃N, THF, 3 hr, reflux
    - Result: HO₂C-CONH₂OMe

16. **Et₂O**, **TESO**
    - Reaction conditions: Pyridine, 18 hr, -20°C
    - Result: TESO-CONH₂OMe

17. **C₆₀ + 1**
    - Reaction conditions: Tol., CBr₄, DBU, 12 hr, rt
    - Result: Bingel Addition

18. **C₆₀ + 2**
    - Reaction conditions: Tol., 2, CBr₄, DBU, 12 hr, rt
    - Result: Bingel Addition
References


**Appendix**

**Appendix I.** Intercoupled-Plasma-Mass Spectrometry (ICP-MS) Data and accompanying calculations.

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<tr>
<th>Cell Line</th>
<th>Antibody</th>
<th>Antibody Exposure Time</th>
<th>Gd Concentration in Sample (ppt)</th>
<th>Cells in Sample</th>
<th>Gd@C&lt;sub&gt;60&lt;/sub&gt; Concentration in Sample (nM)</th>
<th>Gd@C&lt;sub&gt;60&lt;/sub&gt;+C&lt;sub&gt;60&lt;/sub&gt; Concentration in Sample (nM)</th>
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