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Evaluation and In Vitro Studies of Folate PEG Biotin and Other PEG agents

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Evaluation and in vitro studies of folate PEG Biotin and other PEG agents

A Project

Submitted

To

Governors State University

By

Christopher E. Zmudka

In Partial Fulfillment of the
Requirements for the Degree

Of

Masters in Science

December, 2011

Governors State University

University Park, Illinois
Dedicated to my Family, Friends, Professors, and all others who have encouraged my academic pursuits
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My utmost gratitude and thanks to Dr. Walter Henne of Governors State University for his knowledge, support, funding, laboratory, and time throughout my work.

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Abstract

Folate receptor alpha is a membrane-bound protein displaying high affinity for folic acid. This receptor is believed to serve as a receptor-mediated transport system of folic acid into cancer and cells associated with inflammation. Interesting, most normal cells in the body have little if no high affinity folate receptor alpha. Based on these attributes, folate based drug delivery, imaging systems, and diagnostic systems are in several stages of development worldwide.[1-3]

Numerous methods have been explored in the literature in an attempt to detect low levels of free folate receptor and/or rare circulating cancer cells [4, 5]. In an attempt to further exploit these systems, we are currently exploring the use of a proprietary folate-fluorescein-PEG-biotin (FFPB) capture ligand synthesized by Dr. Walter Henne. In this current project, we have successfully purified and characterized FFPB capture ligand, which has been incorporated into a secondary detection scheme of captured folate receptor positive cells utilizing fluorescein microscopy. The inexpensive and previously produced folate probe was substituted for the more costly and cumbersome antibody based ligands that are typically used for this method. This method significantly reduces false positive events associated with non-specific binding and capture of non-targeted cells (a problem associated with the aforementioned affinity capture protocols).
Introduction

Folate

Folic acid (Figure 1) is an essential vitamin of the human diet due to the inability of mammals to synthesize folates de novo\[6\]. Folic acid, which occurs naturally as folate, is vital for the synthesis of multiple amino acids as well as nucleic acids \[7, 8\]. Folate vitamin is a water-soluble organic molecule with relatively low molecular weight (MW = 441.4 Da) \[2\]. Dietary folate has been studied and applied in many areas including, but not limited to its role as a dietary antianemia factor, cofactor in one-carbon metabolism, role of folate coenzymes in the synthesis of DNA precursors, and antiproliferative/antimicrobial agents\[6\]. Due to the importance of folate and the absence of synthesis, all mammalian cells contain at least one folate internalization pathway\[9\], which can be associated with the folate receptor (FR) or small membrane bound channel proteins\[10\].

The FR is classified as a glycosylphosphatidylinositol-anchored glycoprotein (GPI) with a high affinity (K\textsubscript{D}<10^{-9} M) for folic acid and the physiologic circulating form of the reduced folate vitamin, N\textsuperscript{5}-methyltetrahydrofolate \[11-13\]. GPI anchors have a highly conserved common core structure, but considerable variability can be found in the side chains and lipid moieties of these proteins\[7, 14, 15\] FR exists in multiple isoforms with FR-\(\alpha\) and FR-\(\beta\) being of particular interest as they have shown upregulation on both cancer cells and activated macrophages\[1\]. Due to most normal tissue virtually lacking FR\[10\], folate transport into nonmalignant adult cells relies heavily on transmembrane channel protein transportation, reduced-folate carrier, of the relatively small molecular weight N\textsuperscript{5}-methyltetrahydrofolate \[10\].
The FR, unlike channel transport, relies on a process of receptor-mediated endocytosis for cellular internalization and therefore added ability to transport larger molecules into the cell, illustration of which can be found in Figure 2[1, 18, 19]. Combining the upregulation of FR on certain detrimental cell types and most healthy cells lacking substantial FR expression, the FR has become an attractive candidate for a multitude of targeted agents.

Primarily of interest are the FR-α and FR-β isoforms due to the upregulation on various carcinomas and activated macrophages[1]. Cancers found to primarily upregulate FR-α include ovarian, lung, breast, kidney, brain, endometrial, colon, hematopoietic cells of myelogenous origin[1, 9], cervical, renal, nasopharyngeal[20], and mesothelioma[18]. In addition, FR expression may increase further with stage progression of various cancers including ovarian carcinomas[9, 21]. FR-β is present on activated macrophages, but not on their quiescent or resting counterparts[1] and therefore has promise concerning such diseases as rheumatoid arthritis, Crohn’s disease, psoriasis, ulcerative colitis, sarcoidosis, pulmonary fibrosis, atherosclerosis, systemic lupus erythematosis, multiple sclerosis, glomerulonephritis, organ transplant rejection[9], diabetes, osteoarthritis[1], and leukemia[10].

With the aforementioned carcinomas and diseases associated with upregulation of FR, many areas of research have utilized FR for targeted therapies and pharmaceutical agents. FR as a tumor-specific ligand with various covalently attached therapeutic agents has received much attention in the areas of imaging, drug delivery, and immunotherapies [1, 22]. Specific examples of these are radiopharmaceuticals, protein toxins, chemotherapeutic drugs, magnetic resonance imaging (MRI) contrast agents, fluorescent dyes, immunotherapeutic agents, neutron capture
therapeutic agents, gene therapy vectors, oligonucleotides (including small interfering RNA (siRNA)), liposomes with entrapped drugs, brain tumor nanocarriers, and enzyme constructs for prodrug therapy[1, 10, 12, 20, 22-29].

Affinity Capture

Traditional methodologies for cell separation involve various forms of centrifugation and/or filtration. These techniques, while useful, have many drawbacks including low specificity without costly instrumentation development[30], high levels of sheer force leading to decreased number of active cells post separation, costly consumables, and multiple time consuming steps when dealing with complex matrices, such as whole blood[31]. Modern techniques have been developed which utilize paramagnetic properties of metals. These techniques rely on the cells of interest containing either intrinsic magnetic properties or are tagged with a magnetic label for separation (for a thorough review of magnetic cell separation techniques see reference 28). Of the two techniques, cell types containing intrinsic magnetic properties are extremely limited with red blood cells (erythrocytes) and magnetotactic bacteria being the only cell types existing in nature. Magnetic labeling, however, is limited only by the existence of an appropriate cell surface target and some form of affinity ligand with bound paramagnetic particle. After successful attachment of paramagnetic particles, cells can then be separated using either flow-through or batch magnetic separators.

Flow-through magnetic separators have the advantage of typically high throughput, but are usually more expensive and require large sample volumes. Batch separators are preferred due to commercially available laboratory scale magnetic separators. Batch separators typically
consist of racks with tubes and a strong permanent rare earth magnet. Various forms of batch separators are designed to accommodate volumes ranging approximately from 5 µL to 1000 mL[31].

Magnetic labeling of cells is performed using two predominant methods consisting of direct or indirect methods. In the direct method, magnetic particles and ligand are coupled and then added directly to sample containing target cells. Incubation allows for direct binding of the magnetic particles, which can later be isolated using a magnetic separator. The indirect method relies on first incubating cells with primary affinity ligand followed by removal of excess unbound affinity ligand. Magnetic particles with an immobilized secondary affinity ligand targeted for the primary affinity ligand are added, will bind with target cells, and then can be isolated magnetically. While the direct method typically requires less incubation time, the indirect method generally is more efficient with higher purity due to “free” primary affinity ligand more readily finding their cell surface target[31, 32]. Many examples of selective magnetic separation targeting human cells subsets include, but are not limited to bone marrow cancers, B lymphocytes, endothelial cells, granulocytes, hematopoietic progenitor cells, Langerhans cells, leukocytes, monocytes, natural killer cells, reticulocytes, T-lymphocytes, spermatozoa[31], and breast cancer cells[33].

In this report, we describe the development of a folate fluorescein PEG biotin (FFPB), Figure 3, capture ligand that can be used for the indirect capture and detection of folate receptor positive cancer cells. By incorporating the fluorescein fluorophore into the folate PEG biotin chain, cancer cells can be captured followed by assessment and confirmation via a secondary
detection scheme (i.e. fluorescein microscopy or flow cytometry). This method significantly reduces false positive events associated with non-specific binding and capture of non-targeted cells (a problem associated with affinity capture protocols).

**Materials & Methods**

*Preparative HPLC of Folate-Fluorescein-PEG-Biotin*

The proprietary Folate-Fluorescein-PEG-Biotin molecule prepared by Dr. Walter Henne was purified with a Hewlett Packard, series 1050 HPLC equipped with a Diode array detector and ChemStation software.

The following instrument and method parameters were utilized:

- **Column**: Rigel 5µm C18 10X250mm from Stellar Phases Inc. (cat.#: 5C18-10-250)
- **Solvent A**: 10 mM Ammonium Bicarbonate Buffer (NH₄HCO₃)
- **Solvent B**: Acetonitrile (ACN)
- **Flow Rate**: 1ml/min
- **Run Time**: 60 min
LC/MS Analysis

The purified FPFB sample was analyzed using an Agilent Technologies, 1100 series LC/MSD Trap XCT for identity and purity confirmation utilizing Agilent ChemStation software and negative ion mode.

The following instrument and method parameters were utilized:

- Column: Eclipse XDB C18
- Solvent: Methanol and Water
- Sample Preparation: 1 to 5 dilution FFPB in Acetonitrile
- Injected Sample Volume: 5µl
- Flow Rate: 0.5ml
- Scan Range: 500-1500 m/z
- Gradient:

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**Preliminary FFPB Structure Modeling**

- Preliminary Modeling was performed using Hyperchem 8.0.3 with Amber setting and a hydrated environment.

**Capture of FR-α Cells with Streptavidin Coated Magnetic Beads**

- Magnabind™ Streptavidin from Thermo Scientific: Capacity- 2µg Biotin/ml resin with approximately 1.4µm average diameter
- Cells: L1210 mouse murine leukemia from Purdue University.
- RPMI Medium 1640 from Gibco/Invitrogen: [+]-L-Glutamine, [+]-Phenol Red, [-]Folic acid
- Phosphate Buffered Saline: 1X from Cellgro (Mediatech, Inc) without Calcium and Magnesium, Cat. No. 21-040-CV

**Assay Sequence**

Approximately one million L1210 mouse murine leukemia cells were incubated with 100 nM Folate-Fluorescein-PEG-Biotin (FFPB) bridging ligand for 30 minutes at 37°C. The sample was then centrifuged at 1000 g for 45 seconds. Supernatant was removed and cells were washed with RPMI Medium 1640 to remove unbound FFPB. Washing procedure was performed in triplicate. After final wash, the supernatant was again removed. Cells were then resuspended in cell media and incubated with 15 µL of Magnabind™ Streptavidin beads for 30 minutes at room temperature with intermittent
gentle swirling by hand. Captured cells were then isolated by utilizing a batch magnetic separator. Eppendorf tubes were placed in magnetic stand, supernatant removed, and cells washed with cell media to remove untargeted/uncaptured cells. Isolation procedure was performed in triplicate.

A control study was performed using approximately one million L1210 mouse murine leukemia cells were incubated with 15 µL of Magnabind™ Streptavidin beads for 30 minutes at room temperature with intermittent gentle swirling by hand to evaluate nonspecific binding events. Captured cells were then isolated by utilizing a batch magnetic separator. Eppendorf tubes were placed in magnetic stand, supernatant removed, and cells washed with cell media to remove untargeted/uncaptured cells. Isolation procedure was performed in triplicate.

Isolated cells from both FFPB assisted and non-FFPB assisted studies were observed utilizing light microscopy and fluorescent microscopy with a fluorescein filter.

**Results and Discussion**

The resultant chromatogram from preparative analysis of FFPB using a Hewlett Packard, series 1050 HPLC, Figure 4, resulted in a sharp, high intensity peak with a retention time of 14.74 minutes and a spurious peak at approximately 4.66 minutes when monitored at a wavelength of 280 nm. Monitoring at 365 nm gave rise to a well-defined peak attributed to
folate with a retention time of 14.74 minutes and a low intensity peak at 4.66 minutes attributed to column carrier over contamination (Figure 5).

Further analysis was performed using the diode array detector to confirm the aforementioned folate peak with a 14.74 minute retention time. The diode array spectrum gave rise to a typical double event with maximum absorbancies at approximately 280 and 360 nm, which is indicative of folate (Figure 6). In addition, a typical peak associated with fluorescein was observed at approximately 480-490 nm. Combining data from HPLC and DAD leads to confirmation of a relatively pure compound.

The purified FFPB was collected and analyzed using an Agilent Technologies, 1100 series LC/MSD Trap XCT for identity confirmation with aforementioned parameters. The LC chromatogram, Figure 7, gave rise to one prominent peak with a retention time of 5.3-6.0 minutes, assigned as FFPB, and a spurious peak due to column carryover between 6.1-6.5 minutes (Figure 7). Mass spectral analysis between 5.3-6.0 minutes yielded monoisotopic peaks for both the singly and doubly charged molecular ion forms of FFPB at m/z = 1385 and m/z = 692, respectively (Figure 8). These findings are consistent with the calculated FFPB molecular mass, 1386.6 Da, following a respective M$^+$ – 1 and M$^{**}$ – 1 scheme for the molecular ion species. Further, the doubly charged base peak was the predominant species observed and consistent with similar compounds.

A preliminary computer modeling was conducted using Hyperchem in a hydrated form. Data from this modeling supports the idea that addition of the fluorescein fluorophore does not appear to interfere with biotin or folic acid (Figure 9). While alteration of binding has been
noted by previous work using conjugates with shorter PEG, preliminary modeling suggests sufficient PEG length for minimization of binding interference.

We performed a cell capture studies with and without FFPB binding ligand followed by light and fluorescent microscopy. Select images can be found in Figure 10. Cells captured using FFPB binding ligand were observed to have a higher level of binding, Figure 10A, when compared to control cells experiencing non-specific binding and subsequent capture when incubated solely with Magnabind™ Streptavidin beads, Figure 10C, under light microscopy. Fluorescein microscopy was utilized to assess and confirmed successful FFPB capture in comparison to that of non-specific binding, therefore lacking fluorescein (Figures 10B and 10D respectively).

Conclusion

Folate fluorescein PEG biotin capture ligand has been successfully developed for isolation and detection of folate receptor positive cancer cells via an indirect Immunomagnetic assay. Characterization of the proprietary FFPB yielded a high purity compound with confirmed components of folate and fluorescein accompanied by structural confirmation via mass spectrometry. Preliminary structural modeling gave promise to the lack of fluorescein binding hindrance for both folate and streptavidin. Lastly, incorporation of a fluorescein fluorophore enabled assessment of specific and non-specific cell capture using secondary detection via
fluorescein microscopy. Overall, FFPB was shown to be a promising tool for the detection, capture, assessment, and confirmation of folate receptor positive cancer cells in vitro.

**Future Studies**

Further study of FFPB in the realm of characterization should include additional structural confirmation utilizing $^1$H-NMR, $^{13}$C-NMR, X-ray diffraction, positive ion-mode mass spectrometry, differential scanning calorimetry, and thermogravimetric analysis. Due to the success of FFPB with regards to cell capture, of interest are techniques based on using a handheld cytometer for fast comparison of relative captured cells on not only a qualitative level, but also quantitative. Lastly, while this study has proved successful, assay optimization and use with complex whole blood samples would be of much interest moving forward.

**Funding**

This work was supported by Dr. Walter Henne and Governors State University.
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Figure 2: Folate Receptor-Mediated Endocytosis[1]
Figure 3: Folate-Fluorescein-PEG-Biotin Bridging Ligand Structure (drawn using ChemBioDraw Ultra v. 11.0.1)

Chemical Formula: C_{67}H_{79}N_{13}O_{19}S_{2}
Molecular Weight: 1386.6

Figure 4: Purification Chromatogram for Folate-Fluorescein-PEG-Biotin at 280 nm
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Figure 10: Cell Capture Comparison with and without Folate-Fluorescein-PEG -Biotin

**Primary Cell Study:** FFPB binding ligand assisted cell capture

**Control Study:** non-specific cell capture without FFPB binding ligand