Synthesis and Analysis of Folate Histidine

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Synthesis and Analysis of Folate Histidine

In partial fulfillment of the requirements for the Masters in Science of Analytical Chemistry

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Abstract

A compound was specifically designed to further aid research on the detection and treatment of folate receptor positive cancer cells. A small but complex peptide chain was commercially produced to have a flexible glycine spacer and a six-histidine tag. Histidine tagging was chosen for its ability to bind to nickel. The compound in this report was purified and analyzed by HPLC and confirmed via mass spectrometry. This binding capacity and the ability of folate conjugates to bind to cancer cell receptors have both been explored for the past quarter of a century [1-3]. Folate conjugates have been successfully used as a transporter of cytotoxins and imaging agents through folate receptors (FRs), while histidine tagging has been used for imaging with dyes, separation, and purification with columns and metallic beads [4-14].
Introduction

Biochemists are constantly using life’s essential molecules for medical treatments and research. Both vitamin B₉ (folic acid) and histidine are two essential compounds that the human body must ingest [2, 12, 14]. The combination of these two compounds with a synthesis reaction may have important applications for treating malignant cells and eventually cancer patients, and the applications of this new compound should continue to be explored. There is hope that this new compound can be used for the detection and even capture of malignant cells with little adverse effects on the patient.

Numerous factors went into designing the specific EGGHHHHHH peptide, shown in figure 1. The fact that it is a peptide makes it unlikely to cause harm to surrounding tissue in the body. The chosen peptide was also found at an affordable price and is readily available. This commercially manufactured peptide resin was created for several reasons. Specifically, the histidine tags have the ability to bind with nickel chelators and the glutamic acid and glycine were also specifically chosen to form a spacer. The folate compound is also often connected to polyethylene glycol (PEG) to provide flexibility and expand the uses for folate conjugates [2, 5, 9]. Glycine is a flexible amino acid that is intended to be used as a spacer to aid in the delivery of the entire molecule.

Histidine is a unique amino acid that tends to bind to metals, and has an especially strong affinity towards nickel. For this reason, it has been used in ion chromatography as well as several separation and purification methods with the use of chelating agents [5]. Poly-histidine tagging entails attaching a chain of at least six histidine peptides to the N or C terminus of a protein or a peptide for attachment to a folate conjugate [6]. A six-histidine strand is the standard chain used for tagging, however, longer strands—a ten or fourteen histidine chain—
have increased bonding and can provide more definitive results due to their significant affinity [6]. Histidine tags only rarely interfere with protein’s function, and are a versatile tool for a wide range of protein purification purposes [15]. Histidine tagging has also been utilized in the medical field as a biomarker for cardiovascular disease [16].

A common chelating agent is nickel combined with nitrilotriacetic acid (Ni-NTA), which is used in many different processes. The Ni-NTA beads are used for the purification and separation of peptides and proteins with immobilized metal affinity chromatography. Ni-NTA is also applied to nano-particles and dyes that are better suited for diagnostic techniques. The dye still provides a simple method to determine if the compound was synthesized correctly and still has the function of a typical histidine tag molecule and can bring imaging agents to the target. Not only are the Ni-NTA compounds versatile but they have little to no effect on surrounding proteins or cells [17]. If this chelating agent does not work in vivo, there are several modified versions available [6, 7].

FRs have four different isomers: FR-α, FR-β, FR-γ, and FR-δ. FR-α and FR-β are glycosylphosphatidylinositol-anchored membrane-bound receptors that are necessary for DNA replication and single carbon pathways. The intake of folic acid and cofactors in a macrophagic manner with high affinity mediates the cellular uptake of this vitamin, and drug conjugates thereof, via receptor-mediated endocytosis [14, 18]. The significance of this receptor as a tumor marker was discovered in 1991 when the amino acid sequence analysis of a protein enriched on the surface of a human ovarian carcinoma cell line was shown to be the FR [12].

FR-β are generally found in hematopoietic tissues, macrophages, and fetal brain tissues. FR-γ are found in hematopoietic tissues and FR-δ are regulatory T-cells which travel throughout the body to fight infection, but neither have an affinity for absorbing folic acid at the same rate.
Folate conjugates have well-known interactions with folate receptors and have the ability to identify and attack malignant cells with folate cofactors attached to chemotherapeutics and imaging agents. Exploiting the fact that many cancers overexpress FRs (whereas most normal tissues express low to negligible levels) has proved to be of clinical significance, particularly regarding the current development of folate-targeted imaging and therapeutic agents [12]. These receptors are rarely found on healthy human cells outside the kidney, liver or bone marrow. The overexpression of FRs in malignant tissues provides a selectivity of toxicities to pathologic cells, thereby avoiding damage that accompanies the uptake by healthy cells [12].

Being able to produce a compound that can combine these technologies will open many new opportunities in biomedical research. Further testing is necessary to see if such a complex peptide can be absorbed into malignant cells in the same way as other FR targeting molecules employed in the past. In sum, the versatility of the histidine tag in the cell may be the next revolution in folate conjugate research.
Folate Peptide Synthesis

A one-step synthesis was conducted to conjugate the EGGHHHHHH peptide and folate.

Chemicals reagents; 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate, N-[(Dimethylamino)-1H-1,2,3-triazolo-[4,5-b]pyridin-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide (HATU), and 99% Diisopropylamine (DIPEA), Trifluoroacetic acid (TFA), triisopropylphosphatesulfonic acid sodium salt (TIPS), a 30 mL stock of dimethylformamide (DMF), and a stock of 20% piperidine in DMF solution made with 8 mL of DMF and 2 mL of piperidine were used to complete the synthesis. The peptide was prepared commercially by Peptide 2.0 (Chantilly, VA). All other reagents were purchased from Sigma/Aldrich (St. Lois, MO).

40.06 mg of resin was placed in a 3 mL peptide synthesis syringe then rinsed three times with 3 mL of DMF. An additional 3 mL of DMF was added to the syringe and it was placed on a digital vortex for mixing for 15 minutes, which allowed the resin to swell. Once the DMF was removed, 2 mL of the 20% piperidine was then added and placed back on the vortex three times for five minutes each. The piperidine was used to remove the Fmoc from our peptide. The resin was then ready for the addition of the pteroic acid, which attached a folate molecule to the peptide. The resin was then washed six times with the remainder of the DMF.

Meanwhile, a new vial containing 25.0 mg of pteroic acid was opened and 37.9 mg HATU was added followed by 22 mL of DIPEA. The contents of the vial were drawn into the syringe, and the syringe was then wrapped in parafilm and placed on the digital vortex overnight to insure the reaction was accomplished. The resin was then cleaved with 95% TFA, 2.5% TIPS and water, and precipitated in cold diethyl ether. The newly synthesized compound needed to be evaluated with high performance liquid chromatography/mass spectrometry (HPLC/MS) to
determine if it was produced properly. After verification of the molecule, the investigation of the applications of this new compound are soon to follow.
High Performance Liquid Chromatography Analysis

The HP 1050 HPLC was used both for the analysis and purification of the synthesized compound. The diode array detection (DAD) of folate’s known absorption pattern, with a sharp peak at 280 nm and a broad peak around 360 nm, was easily identified. Verification that the compound was synthesized successfully was performed, and an evaluation with HP 1050 HPLC was conducted to assure us that our compound was created without errors. A 5µL sample was injected into a reverse phase analytical column (Phenomenex 00G-4041-E0 Luna 5u C18 250x4.60 mm 5u micron 209 201-8). A single strong peak was found at 24.75 min during the HPLC separation. Figure 2 illustrates that the compound has been produced with little impurities. The DAD spectra shown in figure 3 was produced by our compound and matches the expected folate conjugate pattern. A 100 µL sample was then purified with the purification column Rigel 5µm C18 10x250 mm cat # 5c18-10-250 (column serial # 9-122A). The compound was dried with nitrogen gas to remove any remaining ammonia and a large sample was ready to be analyzed with MS.

The method for the analysis employed of a 0.1 nM ammonium bicarbonate buffer and acetonitrile is typically used for folate conjugates. However, due to the fact that histidine amino acids can easily become protonated, causing separate retention times, hydrochloric acid was added to the bicarbonate buffer to adjust it from pH 7.7 to pH 4.5 in order to prevent the peaks from splitting. The runs were performed at 1.0 ml/min starting with a 99% ammonium buffer, changing to 70% at 30 minutes, at which time the compound had already been eluted.
Mass spectrometry analysis

The folate histidine compound’s exact mass was calculated from chemdraw in figure 1, and was determined to have a mass of 1377.54 amu. The peak for the singly protonated compound has a m/z of 1378.54 and several informative peaks were expected to be found for the double (m/z=679.77), triply (m/z=460.18), and quadrupole (m/z=345.39) protonated molecules.

The instrument employed was Argilent 1260 MSD XCT trap, and the parameters were set as follows: nebulizer at 40.0 psi, dry gas at 8.0 l/min, and dry temp at 300.0° C. The sample was initially injected directly into the MS for positive ionization and analysis, producing figure 4. A second run was performed after maintenance using the HPLC’s auto sampler without a column, producing figure 5.

Both runs produced several peaks representing multiply charged species. Figure 5 shows strong peaks for the singly, doubly and triply charged molecules, while figure 4 contains the quadruple charged peak but not the singly charged. Due to the wide range of these peaks and the instruments used, all of the peaks could not be displayed on one mass spectrum. For these runs, the peaks were within the instrument’s normal error of m/z=0.5. This data clearly shows that the compound of interest was successfully synthesized.
Conclusion

The synthesis was completed successfully and verified with HPLC/MS. The purchase of a commercially manufactured peptide EGGHHHHHH resin provided a quick and affordable method of advancing studies in the field of folate targeting research. Due to the price of the peptide, alterations or the purchase of another peptide with different spacers or additional histidine, where necessary, can be an efficient way to continue research.

The development of a compound to be used in humans is a long and tedious process. In this research, a new compound was attached to a FR targeting molecule. The synthesis was verified with analytical procedures. Further extensive testing is required, however, to see if and what the synthesis can provide for medical research and treatment. Many steps and requirements will need to be passed before this molecule can be employed in the treatment of cancer. In particular, the Ni-NTA dyes will need to be investigated and their interactions with malignant cells are the next step.
References


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The structure of the synthesized molecule was drawn with Chemdraw Ultra 12 and is shown above. The software was used to determine the mass of the compound of 1377.54 amu. This mass was then used to verify that the molecule was synthesized correctly using mass spectrometry. The figure also shows the separate moieties of the compound.
Figure 2:

The chromatogram shows a single peak at 24.20 min. This indicates that there is a single compound with no impurities remaining in solution.
The diode array detector (DAD) produced during the analysis and purification of the compound shows a strong absorption at 280 nm and a broad peak at 360 nm. This is a sign that the compound being eluted contains folate moieties.
Figure 4:

The doubly, triply, and quadruple charged molecules can easily be found on this spectrum (at m/z= 689.9, 460.5, and 345.9 respectively). This shows better resolution of the molecule with more charges but loses some accuracy.
In this spectrum, the singly, doubly and triply charged molecules can easily be found (at m/z= 1378.7, 690.4, and 461.0 respectively).