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Synthesis of a Folate Conjugated Fluorescent Imaging Dye

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By

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Abstract: In recent years methods of selective targeting of pathologic cells have become increasingly important for both detection and treatment of various cancers. The use of folic acid as a selective therapeutic and targeting agent for cancer cells is becoming ever more significant. This is due to the well characterized up regulation of folate receptors for cancer cells.

In this experiment we focus on the development of a new folate conjugate dye by using a small fluorescent molecule known as Oregon Green 514 for fluorescent imaging of cancer cells. To obtain this objective we linked folate with Oregon green 514 dye via a solid phase synthesis technique. Purification and identification of the newly created fluorescent probe was accomplished by High Performance Liquid Chromatography and Mass Spectrometry. The folate-conjugated imaging probe was tested on L1210 leukemia cells, and was internalized by binding selectively to over expressed folate receptors on the surface of cancer cells. The folate receptor was confirmed to be responsible for conjugate internalization by a competition study with free folic acid.

Introduction

Folate receptor (FR), also known as a folate binding protein, is a glcosylphosphatidylinositol (GPI)-linked membrane glycoprotein with a molecular weight of 38- 40 kDa [2, 13]. In humans two membrane –bound form of FR have been identified one is designated α and the other is β [1-6,]. FR α-isoforms are the focus of studies for targeted imaging and drug delivery to cancer cells via folate. This is due to the fact that only α-isoforms bind folic acid (an oxidized form of folate) with high affinity [2, 15]. Knowledge of the up regulation of FR in human cancer cells became evident in the early 90 s with a series of research papers from various labs [1-2]. While elevated expression of FR has been observed in various cancer cells the receptor is generally expressed in low amounts in epithelial cells [1-14]. This is due to the fact that folate (or folic acid) is a vitamin used for biosynthesis of nucleotides at high levels for proliferating cells [8]. In addition, to folic acid being highly selective towards cancer cells it also allows

certain amount of flexibility when conjugated with therapeutics. Non-selective drugs can be specifically targeted to cancer cells. This method is typically less time consuming and more cost effective than designing a drug explicitly for a certain cancer type. In addition, ligand-targeted therapies are a preferred form of delivery for membrane-impermeable drugs due to the fact that the ligand can transport its cargo via specific receptor mediated endocytosis [7, 9, 12]. This will allow the membrane impermeable therapy to be transported effectively. Some examples of drugs that have been conjugated with folate and tested on animal cells and or human clinical trials with successful results are camptothecin, taxol, and mitomycin C [12].

To insure that folate conjugate therapies and imaging probes reach their destination and function properly there are design outlines that many researchers follow. Initially, researchers found that disulfide linkers placed in between folic acid and the compound being delivered to the cancer cell allow for easy release [3]. The use of a reducible disulfide bond allow for drug or imaging agents to be detached from folate while in the endosome [1, 2, 4]. Once outside the endosome these molecules can travel freely. Secondly, any group being attached to folic acid must have a derivatizable functional groups such as –NH2, -OH, or –COOH [3, 7]. This will allow for conjugation with the folic acid molecule. Finally, enhanced hydrophilicity is imperative to avoid passive diffusion of folate conjugates into random cells. By enhancing the polarity of the folate conjugate it increases the probability that the molecule will enter the cell via FR mediated endocytosis, thus decreasing the chances of targeting healthy cells. In the case of folate Oregon green 514 the dye molecule is polar.

Folate Oregon Green 514 conjugate was synthesized using a solid phase technique. Solid phase synthesis is a method typically used in the biochemistry field to produce peptides. In this method molecules are usually bound to polystyrene beads. The bead is coated with a compound that a molecule in this case folic acid can bind to. A protecting group 9-fluorenyl methyloxycarbonyl (Fmoc) or tert-butyloxycarbonyl (t-BOC) is used to prevent bound molecules from undergoing undesirable reactions.

Once the scientist is prepared to make an addition to the bead linked molecule the Fmoc group can be removed via piperidine and the t-Boc group can be removed using trifluoroacetic acid.

Materials and Instrumentation

Materials:

Dimethylsulfoxide (DMSO, from Sigma-Aldrich, Batch# 11696DK), 99% Diisopropylethylamine (DIPEA, from Sigma-Aldrich, Batch# 33396AK), 99.9% Methanol (MeOH, from Fischer Scientific, Batch# 085072), 99.9% Piperidine (from Sigma-Aldrich, Batch# 11329BJ), 99.9% N,N, Dimethylformamide HPLC Grade (DMF, from Sigma-Aldrich, Batch# 12067TH), High Purity Acetonitrile (CAN from Burdick & Jackson, Batch# 5953), High Purity Water (H_2O, from Burdick & Jackson, Batch# Z360), 98% Aldrithiol-4 (Sigma Aldrich), Oregon Green 514 Carboxylic Acid (from Invitrogen)

Instruments:

HPLC (Hewlett Packard series 1050) with Semi-preparative column (C_{18} 5 µm, 10x 250mm Serial 9-122A), Agilent LC-DAD-MS Ion-Trap 1050 series with Agilent Eclipse XDB-C_{18} column, Legend Micro 17 Centrifuge (Thermo Scientific), Rotary Evaporator (Wilmad Lab glass serial WG-EV311), Nikon Digital Microscope, Lyophilizer (GSU Science Dept.)

Methods:

Synthesis of Folate Oregon Green 514

A solid phase resin technique was performed in a 3Ml disposable syringe. The resin within the syringe contained pre-linked folate molecules. Dichloromethane (DCM) was used to swell the resin compound. The protecting group Fmoc was removed using a 20% piperidine solution in 10ml of DCM. The resin was the washed three times with DCM and three times with DMF. After the wash procedure the resin was swelled in DMF for 20 minutes. A solution containing 1.4mg of N, N-Diisopropylethylamine

(DIPEA) and 2.6 mg of Oregon green 514 in DMF was added to the resin. The syringe is placed in a shaker for 2 hours. The contents of the syringe were then washed with DMF, DCM and methanol three times. The folate Oregon green 514 conjugate was cleaved from the resin using a solution of 95% TFA, 2.5% H_2O, and 2.5% triisopropylsilyl (TIPS). Folate Oregon Green 514 was washed three times in cold ether, and dissolved in 10% ammonium hydroxide for 30 minutes. The vial containing the liberated Folate Oregon Green 514 was submerged in a canister of liquid nitrogen, then the cap was replaced with wax paper and the sample was lyophilized for 24 hours.

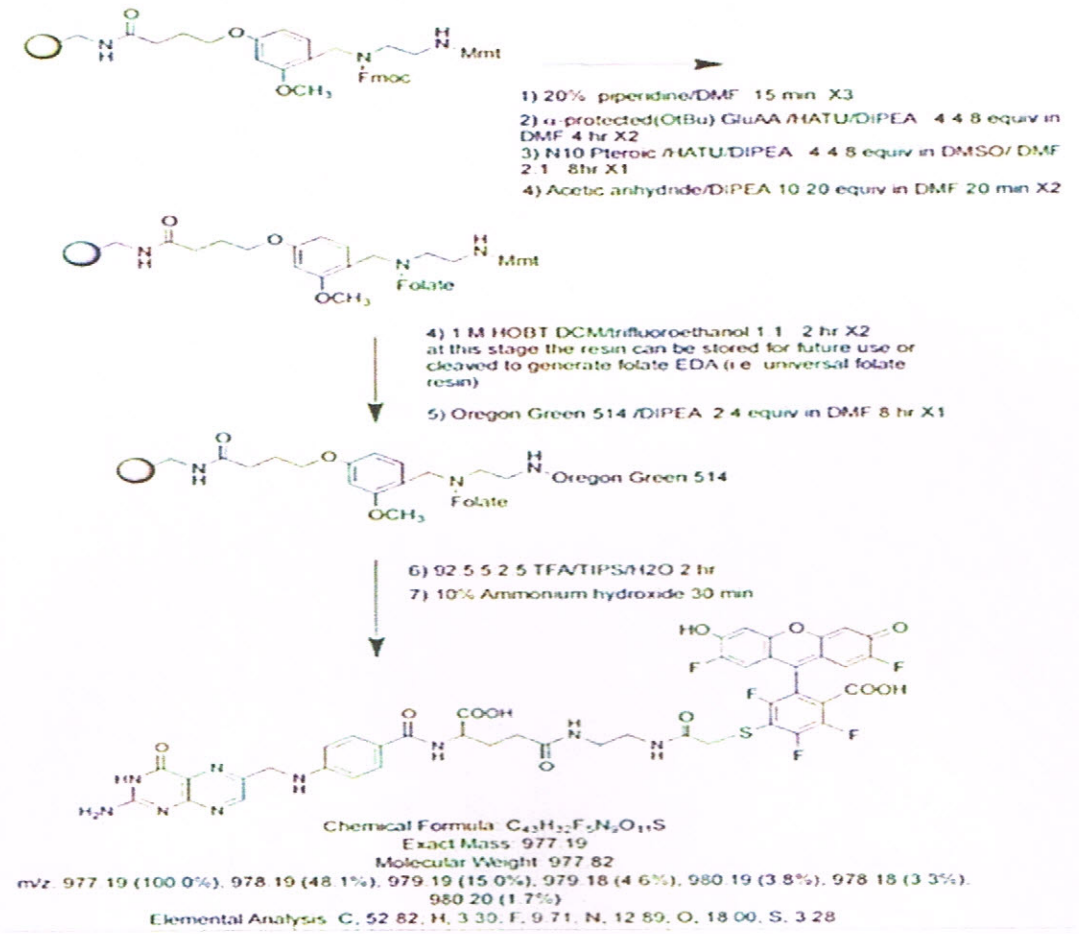


Figure 1: Solid Phase Synthesis of Folate Oregon Green 514

Purification and Identification by HPLC and HPLC-M/S

A 2 µL sample of Folate Oregon Green 514 was analyzed on a 1050 series Hewlett Packard HPLC with DAD using a semi-preparative column (C_{18} 5µm, 10x250mm). The mobile phases for this

analysis consisted of a 0.1mM ammonium bicarbonate buffer and acetonitrile. The change in the gradient with respect to time in minutes is shown in table 1. The same HPLC system was used to purify 100μL of Folate Oregon Green 514. The effluent which eluted at 23 minutes was collected into a vial, and used for qualitative analysis via an Agilent 1200 series LC-DAD-MS Ion-Trap. Twenty micro-liters of the purified Folate Oregon Green 514 in ammonium bicarbonate was analyzed by HPLC-M/S. Mass to charge ratios from 700 m/z to 1200 m/z was monitored in the negative ion mode. The DAD was set to monitor wavelengths 230, 254, and 280 nm. Chromatograms and total ion chromatograms for the purification and identification of Folate Oregon Green 514 are shown in the data and results section.

Table 1: Change in the mobile phase composition with respect to time.

Elapsed Time	0.1mM NH_4HCO_3	C_2H_3N	Flow Rate
0 minutes	99%	1%	1.0 ml/min
30 minutes	70%	30%	1.0 ml/min
40 minutes	50%	50%	1.0 ml/min
50 minutes	30%	70%	1.0 ml/min

Loading of Folate Oregon Green 514 into L1210 Leukemia Cells

To determine if the folate dye conjugate does in fact bind to cancer cells via folate receptors 0.5 mL of L1210 leukemia cells were employed. The concentration of folate dye conjugate diluted in 2Ml of phosphate buffer saline and used for cell loading was determined by using Beer's law. Control cells consisted of 0.5ml of L1210 cells in the presence of 10μL of folic acid and 8.3 μM of folate dye conjugate. Experimental cells consisted of 0.5Ml of L1210 cells in the presence of 8.3μM of folate dye conjugate. A Nikon Digital Microscope was used to take images of both control and experimental cells.

Results and Discussion

Purification and Identification

The purified sample of Folate Oregon Green 514 eluted from the column at 23.319 minutes and had a distinct orange color. DAD signals were also monitored at this time and showed an increase in absorption at 500nm. Refer to figure 2. Although absorption at the 500nm mark was a strong indicator that the dye conjugate was collected 23.319 minutes HPLC-MS was also performed to obtain a positive identification. A soft ionization technique in this case electrospray ionization was employed to avoid complication caused by fragmentation. The molecular weight of Folate Oregon Green 514 is 977.82 g/mol. The total ion chromatogram revealed a charged species at 975.7m/z and 997.7 m/z. The species which appears at 975.7 m/z indicates the loss of a hydrogen. The loss is most likely from one of the two carboxylic acid groups. The anticipation of deprotonating of carboxylic acid group is why monitoring was done in the negative ion mode. The species which appears at 997.7 m/z is due to sodium adducts. This adduct is quite common when using electrospray ionization and might be avoided by using better desalting techniques in the future.

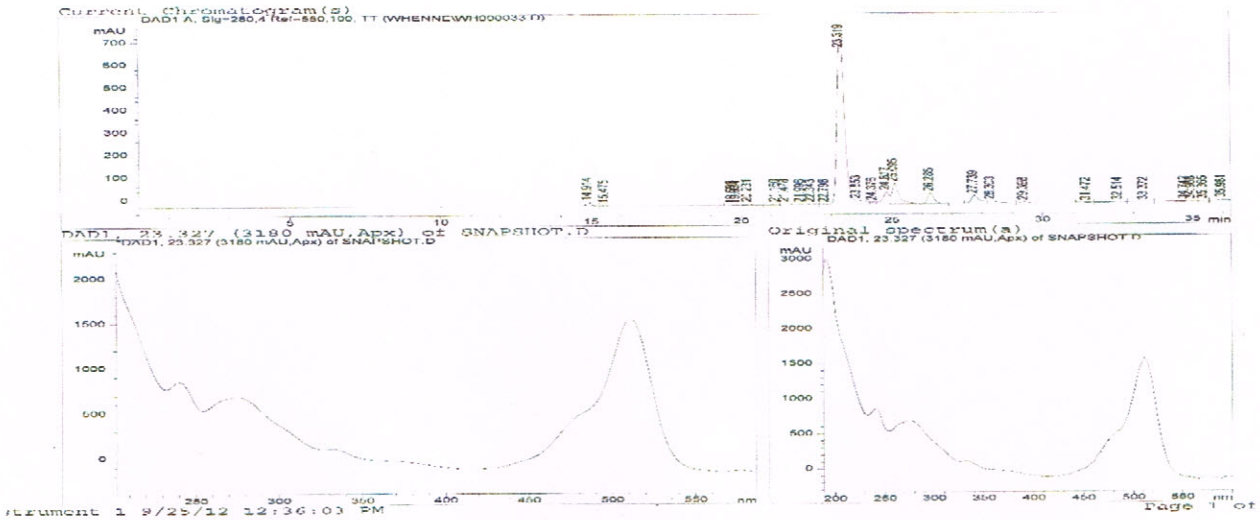


Figure 2: HPLC chromatogram for purification of Folate Oregon Green 514 and DAD data at 23.319 minutes.

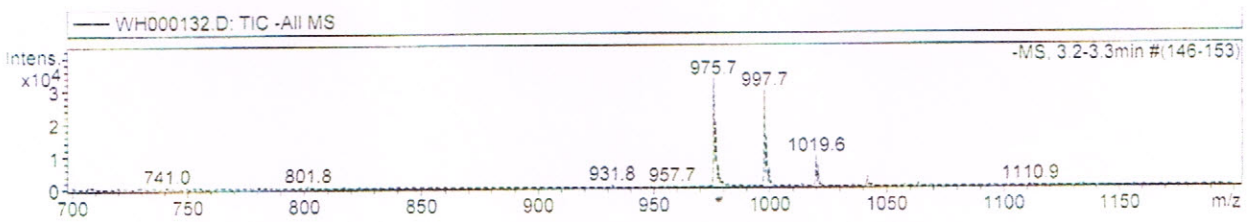
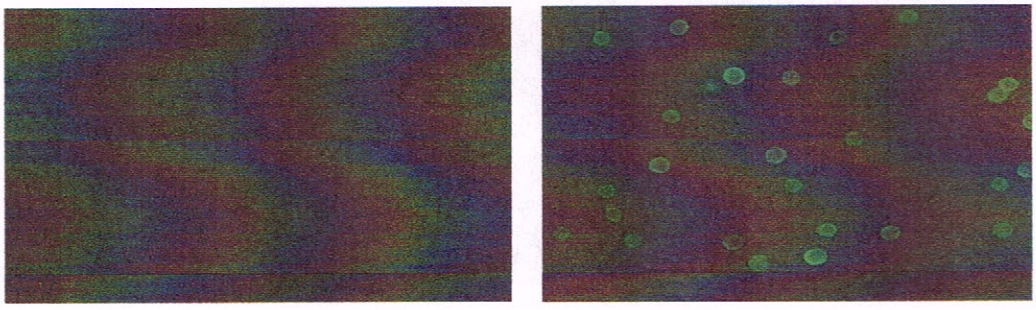


Figure 3: HPLC-MS data for the peak eluting at 3.2-3.3 minute region.

Cell Imaging and Competition Studies

As stated before a line of leukemia cells known as L1210 were used for imaging and competition studies. The purpose of this study was twofold. First, it was important to confirm that the dye conjugate would emit a visible light when loaded in cancer cells for imaging. Secondly, it was imperative to confirm that the folate dye conjugate targets and enters the cancer cell via the folate receptor. As stated previously cancer cells overexpress the membrane bound protein known as the folate receptor. This fact allows cancer cells to have abnormally high affinity for folic acid. If the folate dye conjugate does in fact enter the leukemia cells through use of the folate receptor control cells which contain 10μL of folic acid along with 2mL of dilute Folate Oregon Green 514 should not yield a fluorescent image. If you refer to the upper left hand corner of figure 4 below a lack of a fluorescent image helps confirm that Folate Oregon Green 514 does bind and enter the leukemia cells via membrane bound folate receptors. The right and lower left hand image of figure 4 shows pictures of the experimental cells in the absence of excess folic acid. When folic acid is absent the Folate Oregon Green 514 is in fact internalized and emits a green fluorescent light allowing the cells to be imaged. Folate conjugates can indeed be used to specifically target cancer cells.



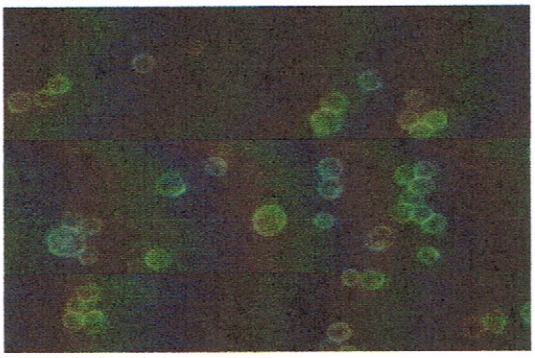


Figure 4: Images taken of the control cells and experimental cells. The upper left hand corner shows an image of L1210 cells in the presence of 10µl of folic acid and folate Oregon green 514. The right and lower left hand images shows L1210 cells in the presence of folate Oregon green 514 and absences of folic acid.

Conclusion

The synthesis of Folate Oregon Green 514 by solid phase synthesis method proved to be successful. Folic acid was linked to resin beads and the Oregon Green 514 dye was then connected to the carbonyl group of the folic acid to create the Folate Oregon Green 514 conjugate. This entire process was done inside a 3mL syringe. The Folate Oregon Green 514 was purified via HPLC. A peak eluting at 23.319 minutes had an absorption climax at 500nm, which was a good indication that a folate dye conjugate was created. To confirm that Folate Oregon Green 514 was produced HPLC-M/S was employed. The total ion chromatogram revealed two peaks with mass to charge ratio of 975.7 m/z and 997.7 m/z. The peak at 975.7m/z indicated a Folate Oregon Green 514 molecule minus two hydrogen atoms.

After confirming that Folate Oregon Green 514 was produced cancer cell targeting and transport of the dye conjugate into the cancer cells was assessed. This was done by placing leukemia cells in the presence of excess folic acid and no folic acid with Folate Oregon Green 514 present for both conditions. Imaging of the leukemia cells in the presence of excess folic acid yielded no fluorescent images. In

comparison, leukemia cell in the presence of Folate Oregon Green 514 and absence of folic acid showed fluorescent cells indicating an uptake of Folate Oregon Green 514. Refer to figure 4. In conclusion, the lack of internalization of Folate Oregon Green 514 when in competition with folic acid demonstrated receptor mediated cellular uptake of Folate Oregon Green 514.

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