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Synthesis and Analytical Evaluation of Folate Conjugates for Use in Cancer Cell Detection

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Synthesis and Analytical Evaluation of Folate Conjugates for Use in Cancer Cell Detection

A Project

Submitted

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By

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Dedicated to

My Parents, Friends & Family

Acknowledgements

I am thankful to Dr Walter Henne, who helped me to develop an understanding of the project, and who gave me encouragement, guidance and invaluable support from the start to the end of my research.

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Special thanks to my friends, Yogesh Radadiya, Rakesh Desai and Hardik Khared, who have offered support through out my project work and who have always been there whenever I had difficulties.

Also, this dedication goes to my parents whose encouragement has been with me all the time and without whose support I could not approach this work and could not pursue my degree.

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Abstract:

Folate-targeted dyes have been used for the detection of tumor tissue and isolated cancer cells in both the animal tumor models as well as in human cancer patients. Folate receptors (FR) are abundantly present on several cancerous cell types but they are found less in normal human cells[1]. There are several imaging agents that can be linked with folic acid and used for the detection of tumor[2-3]. These imaging agents could be protein toxins, chemotherapeutic agents, gene therapy vectors, oligonucleotides, radioimaging agents, magnetic resonance imaging contrast agents and liposomes with entrapped drugs, radiotherapeutic agents and/or immunotherapeutic agents[4]. This research showed that targeting one of these agents, the fluorescent dye, Dylight 488, produced more specific and sensitive imaging of malignant cells for diagnostics purpose of cancer detection[5].

In the present research, we synthesized folate-conjugate dye, Folate-DyLight^{TM}488, using simple methods and minimal efforts. The research involved purification of synthesized Folate-Conjugate, Folate-DyLight^{TM}488 by HPLC followed by the analysis of purified Folate-DyLight^{TM}488. The purified Folate-DyLight^{TM}488 was first analyzed using UV/VIS micro-plate reader as well as LC-DAD and finally with the LCMS. Mass Spectroscopy eventually confirmed the dye. Ultimately, the dye was lyophilized and used for the cancer cell detection by fluorescent microscope.

Introduction:

Folate and its role:

Folate belongs to the vitamin B group and generally termed as folic acid. It is involved the one carbon transfer reaction, which helps for DNA synthesis and replication, cell division and it also plays an important role in methylation[6-7]. Two types of folate binding proteins are present on the cell; one with low affinity binder, reduced folate carrier (RFC), responsible for the most of folate transport across the cell membrane and the other is high affinity binder, folate receptor (FR), which mediate folate uptake by endocytosis[6, 8-9]. Folate receptor mediated endocytosis is a simple strategy to transport folate dye because of the over expression of the folate receptor on cancer cells[3, 10].

Folate receptor pathway:

Two different systems are exists for the transportation of folates[11]:

i) Membrane bound Folate Receptor (FR-α and FR-β) – linked to cell surface via a glycosylphosphatydilinositol (GPI) anchor 20, which transport the folates by receptor mediated endocytosis[12-15].

ii) Reduced Folate Carrier (RFC) – Transport of folate was done by anion exchange into the cytoplasm. Unlike FR, RFC transport folates by binding it and carrying folate into the cell[16-17].

Folate-conjugate dye - DyLight™488:

For the screening of cancer cells we have used DyLight™488, which has a greater photostability than other commercial fluorescent dyes available in 488-range dyes. Also it has a pH insensitivity ranging from 4-9 and available at a lower cost. DyLight™488 is

available as labeling agent, used in fluorescent microscopy, and as a conjugates of secondary antibodies in western blotting and ELISA.

Mouse L_{1210} Leukemia model:

The first screening model used at national cancer institute (NCI) was in vivo L_{1210} and P_{388} murine leukemia. From 1955 until 1975, the mouse L_{1210} model has been used as a model for the treatment and detection of blood cancers. Moreover, the human leukemia has also been useful for prediction of the drugs that are effective against such diseases. However, this model is sometimes proved poor a predictor for cancers because of the difference in physiological activity of mouse and human tumor.

Materials and Instrumentation:

Materials:

DyLight^{TM}488 Maleimide (Thermo scientific, Lot# LC1294236), Folate Cysteine, Dimethylsulfoxide (DMSO, from Sigma-Aldrich, Batch# 11696DK), 99% Diisopropylethylamine- $[(CH_3)_2CH]_2NC_2H_5$ (DIPEA, from Sigma-Aldrich, Batch# 33396AK, MW:129.24 gm/mol), Sodium bicarbonate (buffer), L_{1210} Mouse leukemia cells, RPMI medium 1640 (GIBCO, Lot# 714204), Folic acid, Phosphate buffer saline (PBS)

Instruments:

HPLC (Hewlett Packard series 1050) with installed Zorbax column (3.5 µm, 4.6x 150mm) and C_{18} column (5µm, 10x 250 mm), Agilent LC-MSD trap 1100 series with Agilent Eclipse XDB-C_{18} column, Epoch Biotek microplate spectrophotometer, Water Jacketed Incubator (Fisher Scientific, Model 3326), Mini Centrifuge (Fisher Scientific),

Refrigerated condensation Trap RT100, Cole Parmer (Model 84000-00), epi-fluorescence microscope.

Method and Analysis:

Synthesis of Folate-DyLight™ 488

Initially 1 mg of DyLight 488 (stored at -20° C), and 2 mg of folate cysteine were weighed and mixed together. Then 200µl of dimethylsulfoxide (DMSO), a polar aprotic solvent, was added to dissolve the solid mixture. Finally, 2 µl of Diisopropylethylamne (DIPEA), an organic reagent, was added and the mixture was left to stir overnight. The sample mixture was analyzed through HPLC using Zorbax column and purification was done with column C_{18}.

For LCMS, Eclipse XDB-C18 column was used. The sample was run in negative mode with methanol and water as mobile phase.

Sample info: 10 µl of purified Folate-DyLight™488 (200 ppm)

Flow rate: 0.5 ml/min

Scan range: 600-1000 m/z

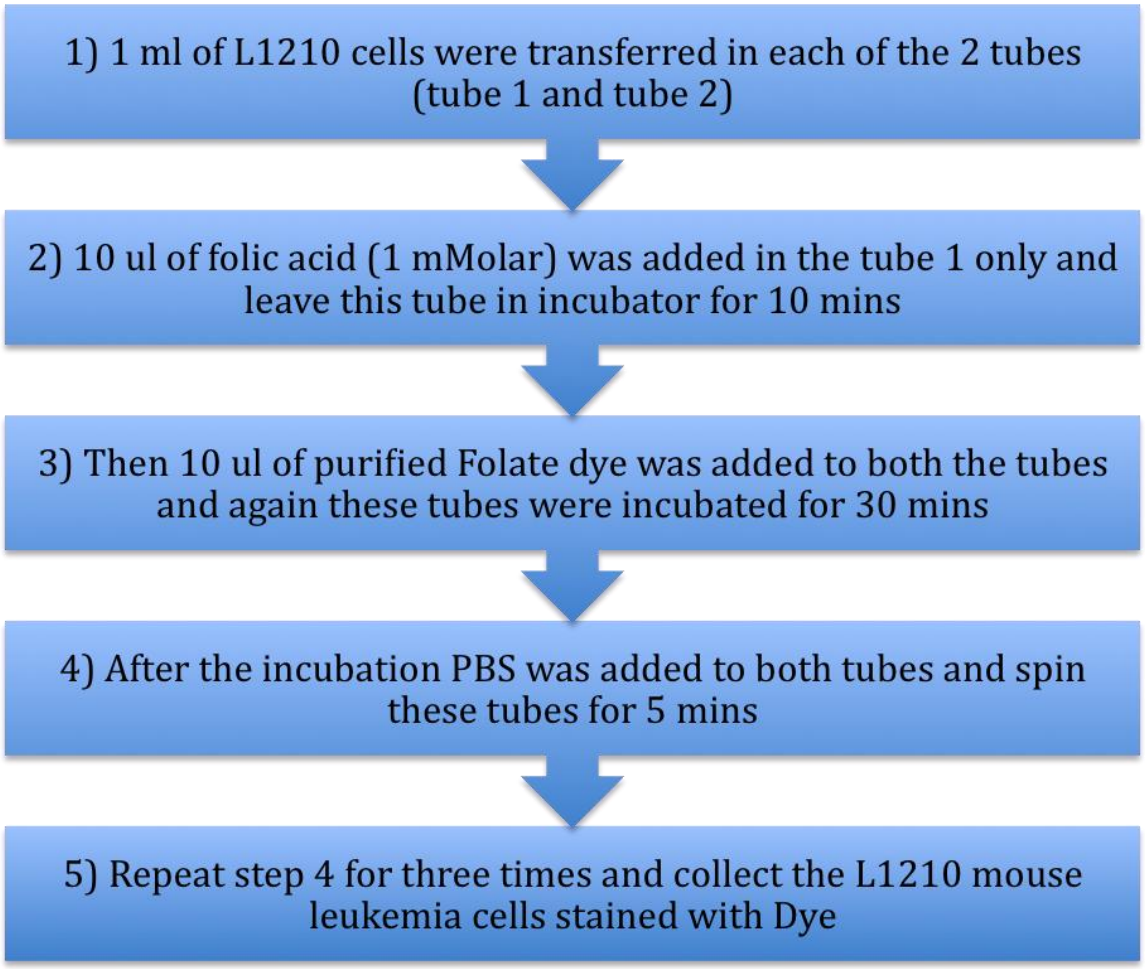
Method: waltLCDADMS.m

L1210 mouse leukemia cell development and observation:

L1210 mouse leukemia cells were grown and folate deficient RPMI (supplement with 10% fetal bovine serum and 1% streptomycin-penicillin) media to the level of 500,000 – 1 million cells per ml. The cells were incubated in the presence of 100 nmolar of folate-Dylight 488 with and without the presence of excess folic acid that will be 1000

fold excess. The incubation was done for 30 mins at the temperature of 37’C and samples were read on fluorescence microscope with fluorescence filters. 10-15 ul of cell suspension was mounted on glass slides for observation.

Binding of purified Folate DyLight 488 with incubated L1210 mouse Leukemia cells



Result and Discussion:

Analysis and purification of folate conjugated dye 488 by HPLC:

The analysis of Folate-Dylight 488 was done using HPLC. Sodium bicarbonate buffer (10 mM) and acetonitrile were used as a mobile phase solvent, flow rate was 1 ml/min and total run time was 60 mins. 10 ul of 5-fold sample was injected at the wavelength of 280 nm and initially the peak was seen at 16.85 min, which was DMSO. Then the other peak was seen at 23.25 min, which was the combination of our folate dye and folate cysteine. Purification of conjugated dye was then accomplished using preparative column to purify the suspected folate conjugate. We knew that our sample was eluted between 23.00 min and 25.00 min. At that time, the fraction was collected for LC-MS analysis.

Analysis of pure compound by LC-MS:

In LC-MS, we started with injection of purified conjugate sample (30 uL or 200 ppm) in positive ion mode, but we could not get any resultant peak after 1100 m/z. As a result, we tried in negative ion mode. In negative ion mode methanol and water were used as a mobile phase and we obtained a peak at 3.11 mins. The chromatogram in the figure 6 shows a sharp peak of purified folate-Dylight 488. For more confirmation, DAD absorption spectrum was obtained which confirm the peak at 3.11 mins in chromatogram. DAD spectrum has scan range of 200-800 nm and Figure 7 Shows the DAD absorption spectrum.

Mass spectrometry finally confirms the product by showing a peak at 447.0 m/z. Since Folate-conjugated Dylight 488 has molecular weight of 1343.0, the molecular ion peak was most likely a multi-charged species. Using the known molecular weight, we

calculated that we obtained a triply charged ion peak at 447.0 (m/3z, M-3H^{+}). We can confirm the molecular ion peak (1343.0 m/z) by multiplying triply charged ion peak (at 447.0 m/z) times three and adding 3 H atoms (3). This value was near the integer mass match. From the above analysis we confirm that Folate-Dylight 488 was present and ready for cancer cell detection.

After analysis of compound by LC-MS it was subjected to lyophilisation to remove excess water from the sample. Then the sample was diluted with 1 mL PBS and placed in the microplate reader to measure the absorbance at 493 nm. And from the absorbance (after path length corrected, 1.257), concentration of the sample was calculated as 1.62x10-4 using the known extinction coefficient of 70 M-1cm-1.Finally, L1210 mouse leukemia cells stained with the folate-conjugated dye was observed on epi-fluorescence microscope and the cancer cells were easily detectable with conjugated dye on it (See Figure 9).

Conclusion:

We have successfully completed the analysis and purification of folate Dylight 488 by reverse phase HPLC. LC-MS confirms the presence of folate conjugate dye by showing absorption spectrum and the triply charged (m/3z, M-3H+) ion peak in negative ion mode. The molecular ion was also monitored in positive ion mode but not seen at 1343 m/z. Finally, we demonstrated the binding of conjugated dye to L1210 mouse leukemia cells and confirmed the specificity by a control pre-incubated with excess free folic acid (See Figure 9).

Acknowledgement:

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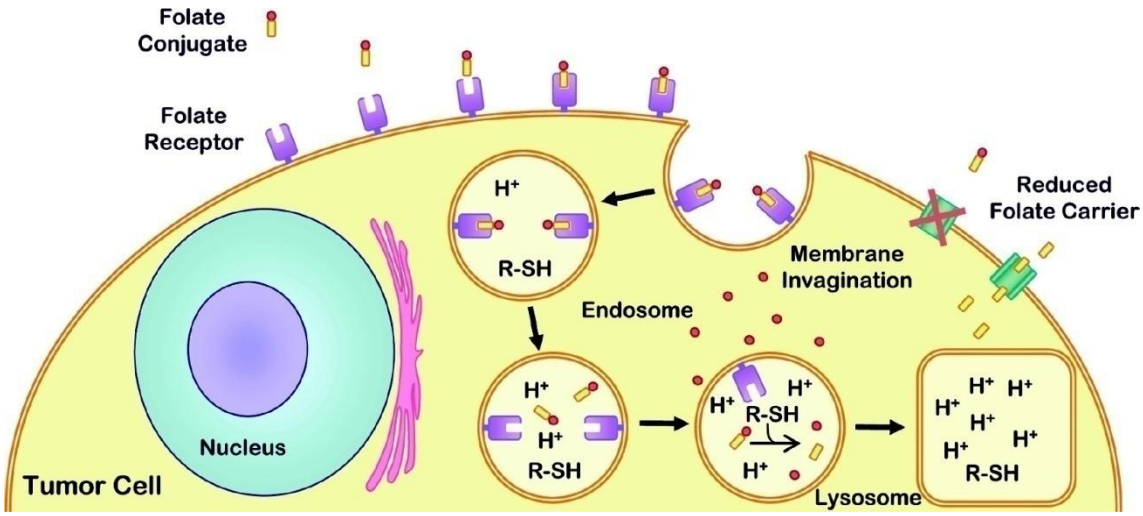

Figure 1: Figure demonstrates the high affinity folate conjugation on folate receptor (FR)+.

Figure 2: Mini centrifuge (Fisher Scientific)

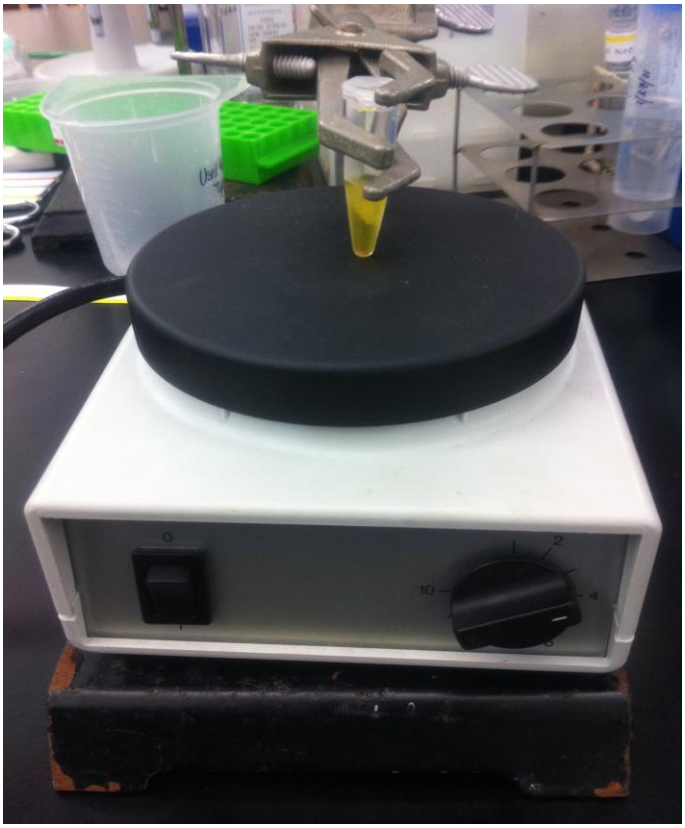

Figure3: Magnetic stirrer (Cole Parmer)

Figure 4: Water Jacketed Incubator for incubation of conjugated dye.

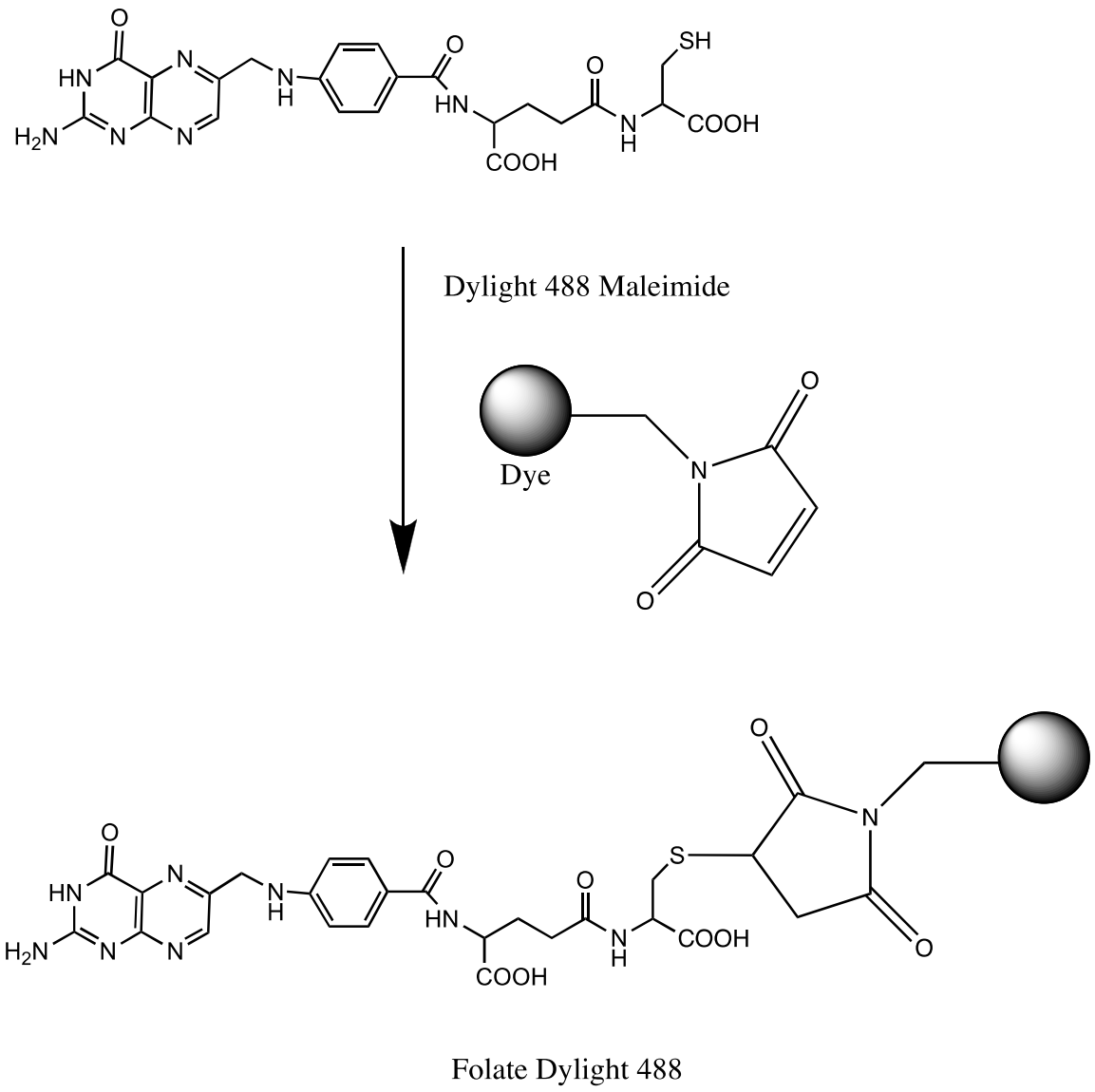

Figure 5: Schematic presentation of conjugation of folate cysteine with Dylight 488 Maleimide to yield Folate Dylight 488

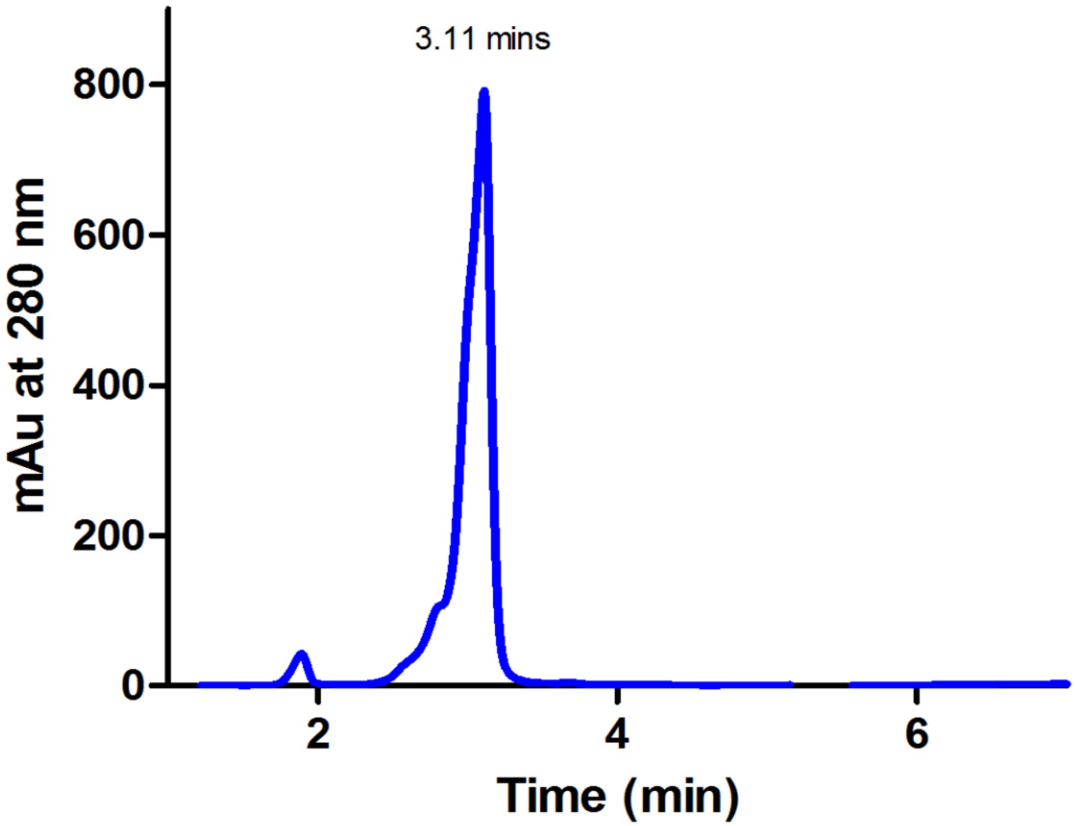

Figure 6: Chromatogram of Folate-DyLightTM 488. Peak shown at 3.11 mins indicates the measured absorbance at 280 nm on Agilent LC-MSD trap 1100 sereis.

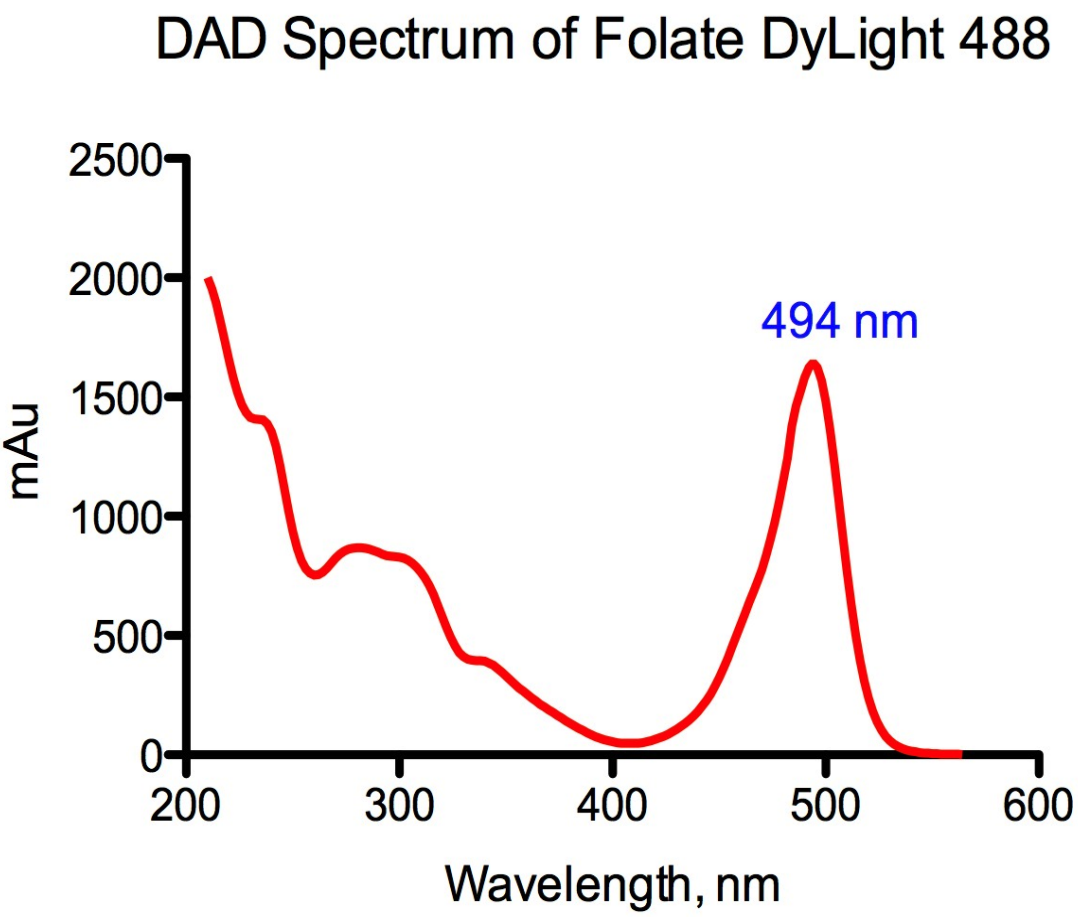

Figure 7: DAD absorption spectrum of folate DyLight 488.

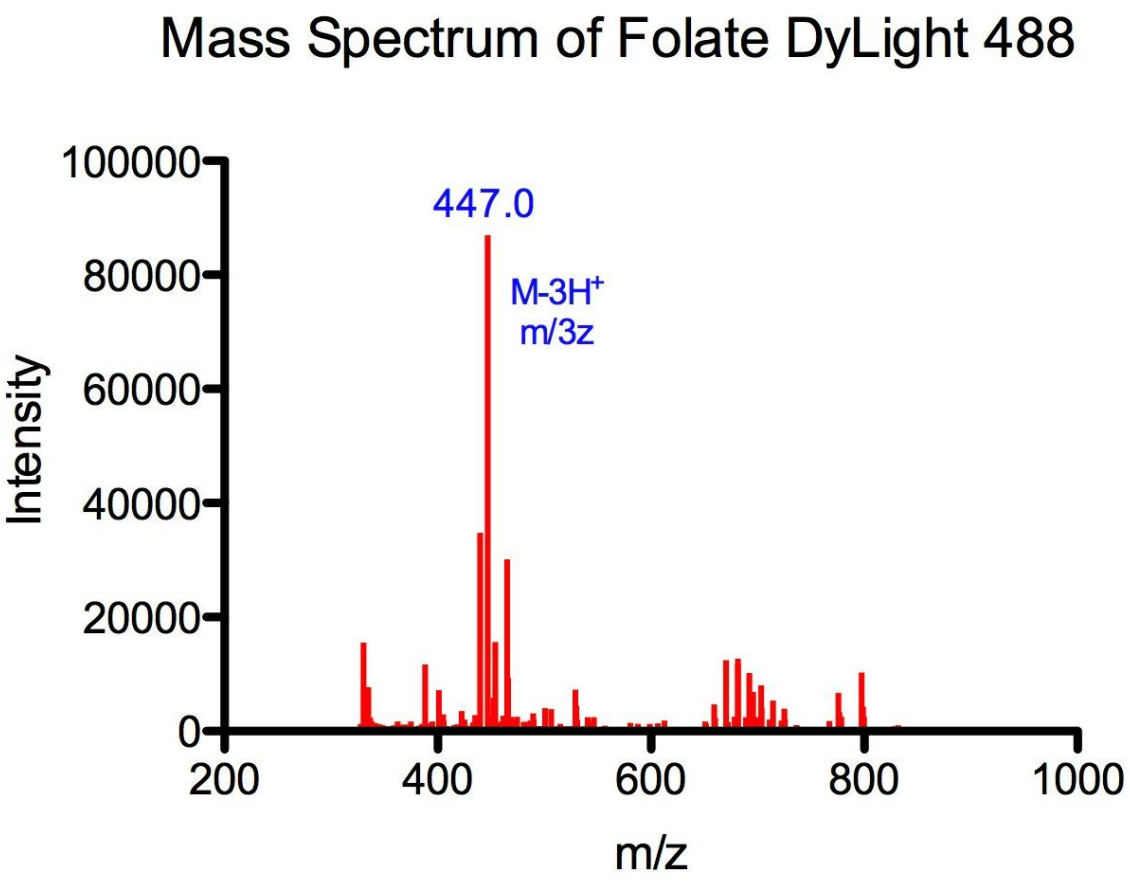

Figure 8: Mass spectrum of folate-DyLightTM 488. The data indicate the triply charged (m/3z, M-3+) molecular ion peak obtained by Agilent LC-MSD trap 1100 series using XDB C18 column.

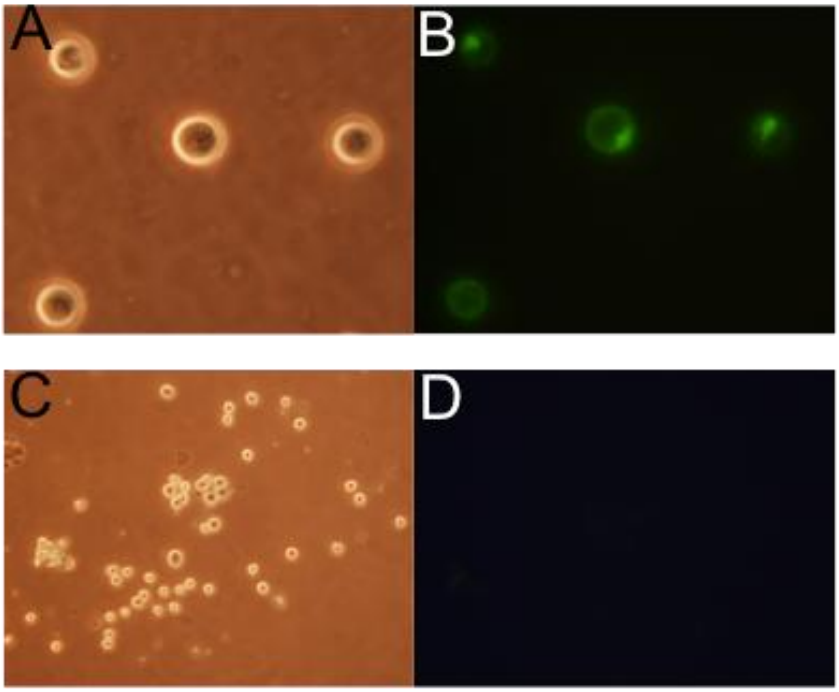

Figure 9: Establishment of FR+ L1210 mouse cancer cells. Cells were grown in folate deficient media for a total of 6 passages. Using a folate targeted dye (folate Dylight 488, developed "in Lab"). Cells were stained in absence and presence of an excess of folic acid competitor (epi-fluorescense panel B and D, respectively). Together, these data demonstrate both FR mediated uptake and folate targeted specificity. Panels A and C are the corresponding phase contrast images at 1000x and 250x magnification, respectively.