Evaluation of Coumarin Derivatives as Probes in Monitoring CYP2A6 Activity in Cancer Cells

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Abstract

Coumarins are fluorescent compounds that can be utilized as probes to monitor and detect certain types of CYP enzyme activity, specifically CYP2A6. Coumarins contain a benzene ring that gets hydroxylated at its seventh position when subject to CYP2A6 enzymes, and the result of this hydroxylation is an increase in fluorescence of these compounds—which allows for both qualitative and quantitative analysis of CYP2A6 activity. A fatty acid chain-linked coumarin was synthesized using an HOBt/EDC coupling reaction and subsequently tested as a probe in micro assays using HeLa cells, which do not express CYP2A6, and HL-60 cells, which do. When tested in assays, the coumarin derivative's 10-carbon fatty acid chain seemed to interfere with the structure of the cell membranes and was concluded to be cytotoxic to both the HeLa and HL-60 cell lines.

Background

Cytochrome P450

CYP2A6 belongs to the superfamily of metabolizing enzymes, Cytochrome P450. These enzymes exist throughout most of the cells in our bodies and significantly contribute to the pharmacokinetic properties of drugs. Xenobiotics are metabolized in human bodies by a variety of different enzymes—some contribute to Phase I metabolism whereas others contribute to

Phase II metabolism. Phase I metabolism typically involves reactions that introduce or expose a functional group like oxidation, reduction, and hydrolysis whereas Phase II metabolism involves conjugation reactions that significantly increase the drug's hydrophilicity to aid in excretion. CYP2A6 carries out Phase I metabolism on a variety of substrates including but not limited to nicotine, cotinine, quinoline, halothane, valproic acid, and coumarin. Because of its wide range of substrates and its application in metabolizing chemotherapeutic agents, the evaluation of the activity of CYP2A6 is the center of many research projects.

Coumarin

Coumarins are naturally occurring, polyphenolic compounds that are made up of two benzene rings in a structure very similar to quinoline (see comparison below). Coumarin and coumarin derivatives have gained importance in recent year because of their wide range of biological activities. In some studies, it was shown that certain coumarin derivatives display antitumor, photochemotherapy, and antimicrobial properties[2]. In addition, those studies showed that certain coumarins display cytotoxic activity while others display cytostatic activity. Specifically, the dosing of 7-hydroxycoumarin in cells from the HL-60 (human leukemia) cell line showed that the coumarin derivative displays cytostatic effects when evaluated against HL-60 cells that were not dosed with the coumarin[2]. As opposed to its other substrates, which are metabolized by other enzymes as well, coumarin remains a substrate that is exclusively metabolized by CYP2A6. Its wide range of applications in healthcare along with it being a major substrate of CYP2A6 made coumarin and its derivatives great compounds to utilize as potential probes to monitor CYP2A6 activity.

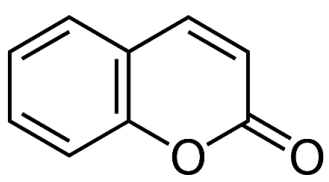


Figure 1.1. Coumarin[3]

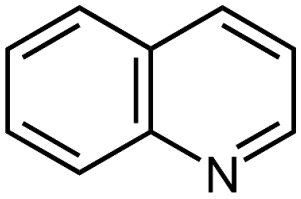


Figure 1.2. Quinoline[3]

Photophysical Chemistry

Along with its exclusive metabolism by CYP2A6, coumarin and its derivatives have photophysical properties that would allow for the visualization of the molecule once dosed in the cell. These properties can be altered depending on its substituents. Coumarin's pharmacophore gives it an emission range of ~410nm - ~470nm, and the addition of certain functional groups allows for the manipulation of both its absorbance and emission ranges. When coumarin gets metabolized by CYP2A6, a hydroxyl group is added to the seventh position on the benzene ring. The addition of a hydroxyl group provides coumarin with an electron-donating group, which gives 7-hydroxycoumarin a much greater fluorescence than coumarin has. This should allow for a better visualization using confocal microscopy and for an increase in relative fluorescence units (RFUs) using flow cytometry. On the other hand, the addition of a chloride group to coumarin should decrease the fluorescence because of chlorine's electron-withdrawing nature and properties.

Methods and Design

Synthesis and Testing Objectives

In this project, two coumarin derivatives were synthesized to be used as probes to monitor CYP2A6 activity in two different cell lines—HeLa cells, which do not express CYP2A6, and HL-60

cells, which have a high expression of CYP2A6. The coumarin derivatives were designed to contain a 10-carbon chain amine linker (decylamine) so they could enter the cells through diffusion through the cell membranes and localize to the ER. Coumarin 1, the test compound, was synthesized using HOBt/EDC amide coupling of coumarin-3-carboxylic acid with decylamine. Coumarin 2 was synthesized using HOBt/EDC amide coupling of 7-hydroxycoumarin-3-carboxylic acid with decylamine. When exposed to CYP2A6—a coumarin hydroxylase—in HL-60 cells, Coumarin 1 is expected to get hydroxylated at the seventh position. Thus, when evaluating the fluorescence of the compound in HL-60 cells, there should be a significant increase in fluorescence over a period of time and exposure to the hydroxylase. This should not occur in Coumarin 2, however. Instead, Coumarin 2 should show no change in fluorescence over a period of time regardless of the cell line used since it is already hydroxylated at the seventh position. Thus, Coumarin 2 acts as a positive control while Coumarin 1 is the test compound.

Organic Synthesis – Coumarin 1

The organic chemistry synthesis objective in this project was to synthesize multiple coumarin derivatives. The first derivative, the test compound 'Coumarin 1', was synthesized by linking the fatty-acid-linked amine 'decylamine' (Figure 2.2) to coumarin-3-caboxylic acid (Figure 2.1). Coumarin-3-carboxylic acid (0.0510g, 0.2644mmol) was dissolved in a 3:1 ratio of DCM:NMP (1.6615mL) and allowed to stir for five minutes. Then, EDC (0.0787g, 0.3965mmol) and HOBt (0.0616g, 0.3965mmol) were added and the mixture was allowed to stir for 30 minutes. Reaction was then sonicated for 10 minutes before slowly adding in the decylamine (0.11mL,

0.5288mmol, 0.78g/mL). The reaction was then allowed to run for 72 hours. After TLC analysis showed a new spot, crude was purified using a hand column run with 100% DCM. H NMR analysis was then conducted on Coumarin 1 (0.0510g, 329.424g/mol, 58.5% yield) to confirm intended product. Reaction scheme shown in figure 2.3 below.

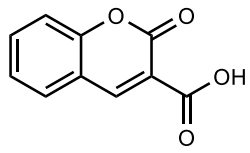


Figure 2.1. coumarin-3-carboxylic acid[3]

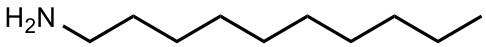
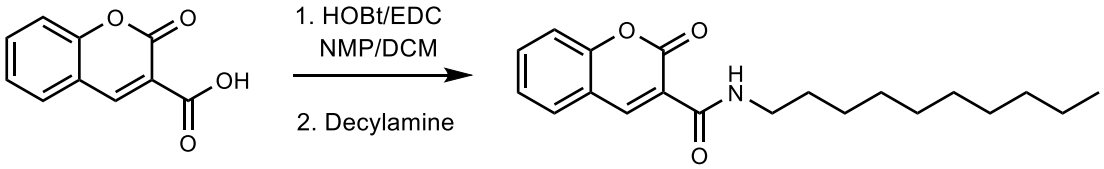


Figure 2.2. Decylamine (linker)[3]

Reaction Scheme – Coumarin 1



Coumarin-3-carboxylic acid Coumarin 1

Figure 2.3

Organic Synthesis – Coumarin 2

The second derivative, the positive control 'Coumarin 2', was synthesized by linking decylamine (Figure 2.2) to 7-hydroxycoumarin-3-carboyxlic acid (Figure 2.4). 7-hydroxycoumarin-3-carboxylic acid (0.0500g, 0.243mmol) was initially dissolved in DMF (1.67mL, 0.944g/mL). EDC (0.0468g, 0.291mmol) and HOBt (0.0559g, 0.291mmol) were then added and allowed to dissolve in the solution. The mixture was allowed to stir for 30 minutes before decylamine (0.097mL, 0.486mmol, 0.787g/mL) was slowly added. The reaction was run for 48 hours before purification. Purification was done through normal phase column chromatography using the CombiFlash (95% DCM/5% methanol). TLC analysis was then conducted on individual fractions and contents in test tube with product was placed on rotovap for 25 minutes. Product was

allowed to dry over 48 hours on a high vacuum line. H NMR analysis was then conducted on Coumarin 2 (0.0139g, 346.424g/mol, 16.6% yield) to confirm the intended product. Reaction scheme shown in figure 2.5 below.

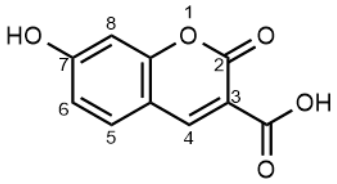
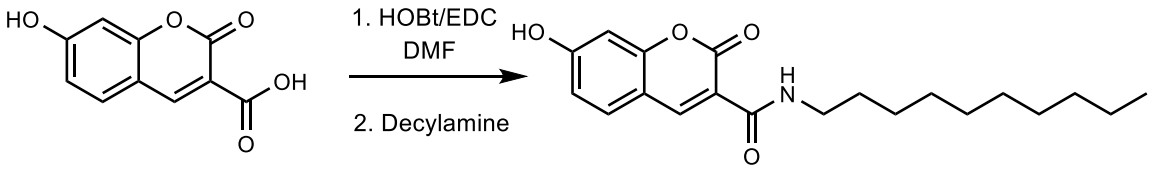


Figure 2.4. 7-hydroxycoumarin-3-carboxylic acid[3]

Reaction Scheme – Coumarin 2:



7-hydroxycoumarin-3-carboxylic acid Coumarin 2

Figure 2.5.

Cell Biology and Assay Development

HeLa cells were prepared and passaged in DMEM, then allowed to grow for 24 hours. HL-60 cells were prepared and passaged in DMEM and allowed to grow for 24 hours. The density and viability of both cell lines were evaluated to ensure a minimum cell count of 500,000 cells/mL. At 250uL/well, each well averaged about 125,000cells/well. Coumarins 1 and 2 were dosed in the HL-60 cells at varying concentrations using a serial dilution. Coumarin 1 stock solution was prepared using octanol and DMSO whereas Coumarin 2 10mM stock solution was prepared using just DMSO. The cytotoxicity assay was designed to include 3uM of propidium iodide. The addition of propidium iodide allowed for the visualization of the cells that were no longer viable as the stain cannot permeate live cells. The final concentration of solvent in the wells

containing Coumarins 1 and 2 was 0.1% of octanol and DMSO respectively. Coumarins 1 and 2 were both tested at 1uM and 10uM. Results shown in figure 4.5 below.

Results

H NMR – Coumarin 1

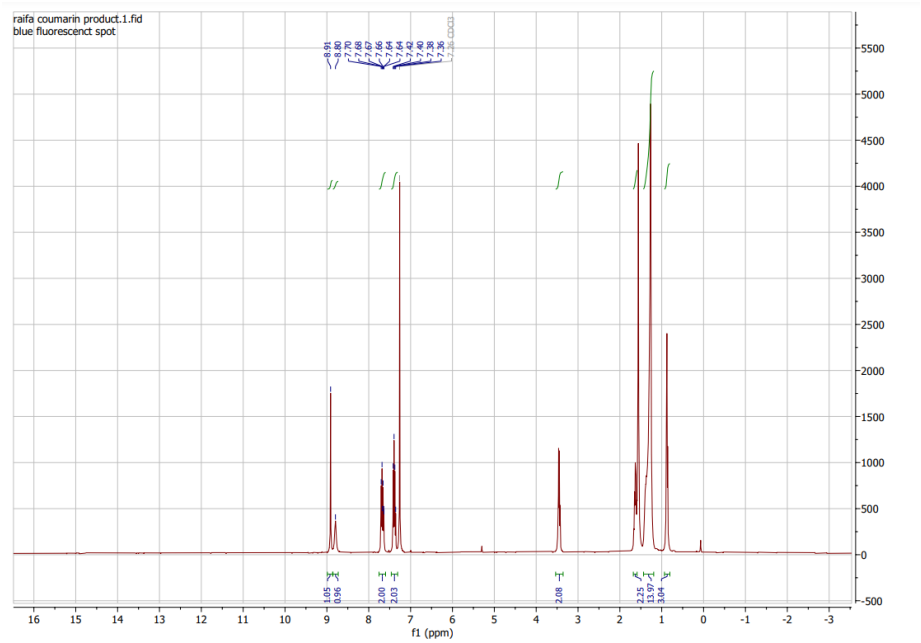


Figure 4.1. H NMR of Coumarin 1 in deuterated chloroform.

Beer-Lambert's Law – Coumarin 1 (abs. 296nm and 328nm in Octanol/Ethanol)

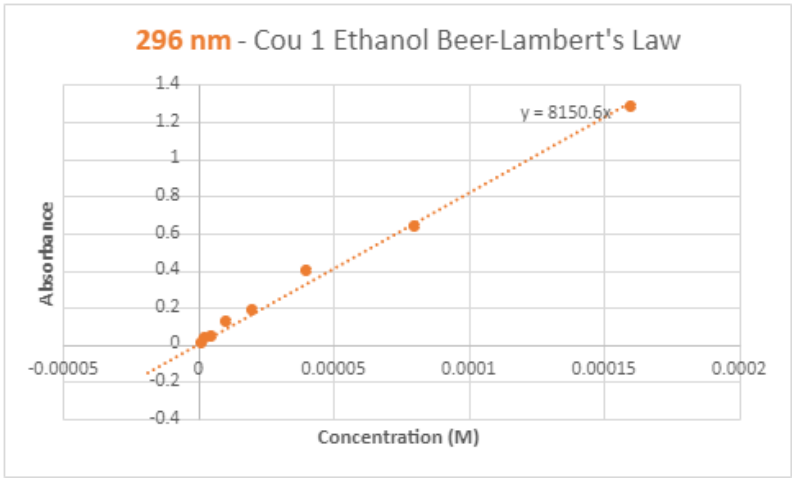


Figure 4.2.

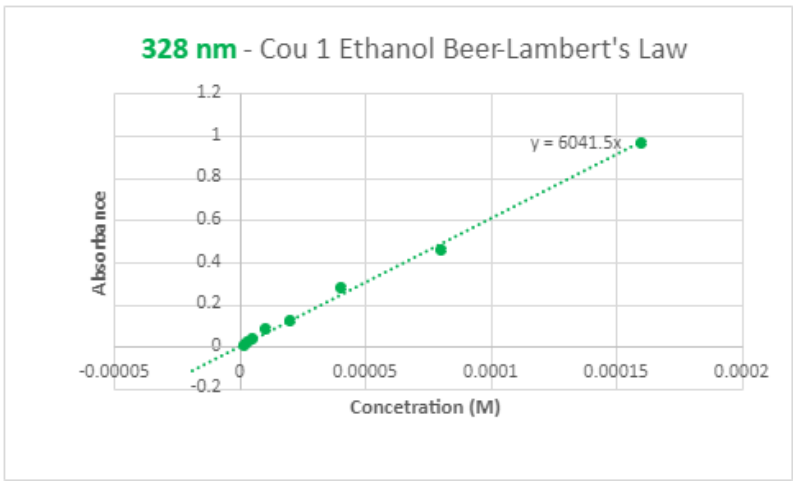


Figure 4.3.

Beer-Lambert's Law – Coumarin 2 (abs. 410nm in PBS/Ethanol)

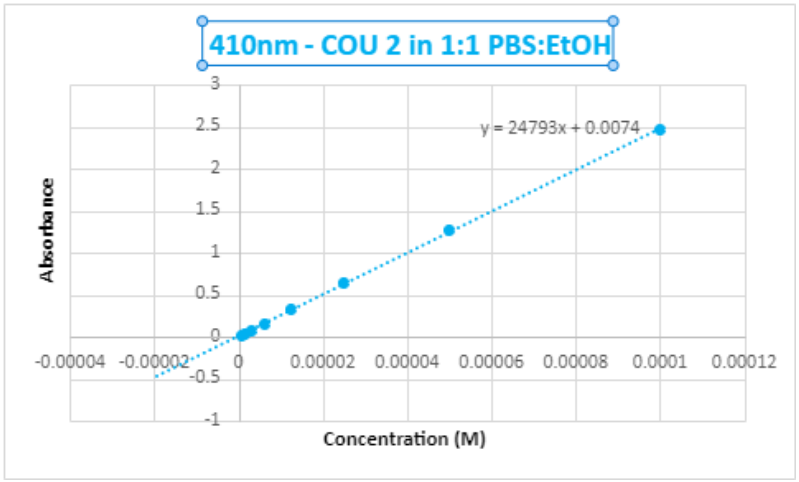


Figure 4.4.

Cytotoxicity Assay

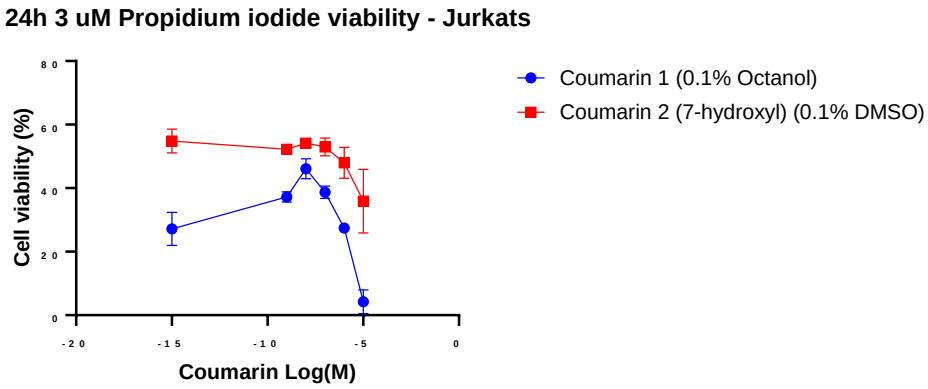


Figure 4.5.

Discussion

The presence of CYP2A6 in HL-60 cells and the induction of CYP2A6 using rifampin were expected to produce a significant increase in fluorescence in Coumarin 1 that would be comparable to the fluorescence in Coumarin 2, however, the results proved otherwise. When compared to the non-coumarin control, which had a measured fluorescence of 3000 RFUs, Coumarin 1's fluorescence after 24 hours of dosing at 1uM and 10uM was merely 4000 RFUs. This does not present a statistically significant result. While it was expected that the coumarin would become hydroxylated over 24 hours of exposure to the coumarin hydroxylase, the results showed otherwise. Because of that, a cytotoxicity assay (results in figure 4.5) supplementary to the primary assay (results not shown) was run to evaluate potential cytotoxicity. Figure 4.5 shows that cell viability significantly decreased over 24 hours for both coumarins 1 and 2 dosed in Jurkat cells. After 24 hours, viability decreased by ~40% in cells dosed with Coumarin 1, whereas viability decreased by ~20% in cells dosed with coumarin 2.

It can be hypothesized that the significant decrease in cell viability was caused by disruptions in the cell membranes of the HeLa, HL-60, and Jurkat cells. These disruptions were likely caused by the long-chain carbon linker attached to the coumarin backbones of both coumarin derivatives used in this paper and likely had cytotoxic effects on the cells. Instead of using decylamine as a linker, there could be promising results in using hexylamine or butylamine. The use of shorter carbon chains may allow the maintenance of the important hydrophobic property of the derivatives without compromising on cell membrane disruptions. The use of hexylamine or butylamine may also provide an opportunity to completely omit the use of

octanol in the experiment—which is known to be cytotoxic to cells at high concentrations. In addition, the decrease in cell viability may be caused by the coumarin itself. In the study mentioned above, coumarin was shown to exhibit cytostatic and cytotoxic properties when exposed to cancer cells—which makes it a molecule of interest in cancer therapeutics[2]. It may be beneficial to run a cytotoxicity assay using the starting materials and evaluating the differences in cell viability between the starting coumarins and the amide-linked coumarins for future experiments.

References

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Research Objectives

- To gain in-lab research experience in synthetic and analytical organic chemistry
- To gain in-lab research experience in molecular biology
- To learn sterile techniques in biology work
- To develop problem-solving and diagnostic skills in organic chemistry synthesis
- To highlight the intersection between pharmaceutics, molecular biology, and organic chemistry and recognize its place in medicine
- To understand, explore, and frequently utilize scientific literature as a resource in optimizing my in-lab techniques and conditions
- More specifically:
    - To synthesize three coumarin derivatives (coumarins 1-3)—each in a one-step synthesis
    - To test the purity of each synthesized coumarin compound
    - To perform cytotoxicity assays on each coumarin compound to determine effective in vitro concentrations
    - To use coumarin 1 as my test compound, coumarin 2 as my negative control, and coumarin 3 as my positive control in a variety of different cellular assays
    - To test my compounds in the HeLa cell lines—which is not known to express CYP2A6—as well as in HL-60 cell lines—which is known to highly express CYP2A6—and compare fluorescent activity
    - To apply the in-lab knowledge explored in the real-life context of cancer therapeutics

Outline:

Page 1: abstract and background (general about P450 enzymes in metabolizing

chemotherapeutic agents

Page 2: introduction – CYP2A6 substrates & coumarin metabolism (relate to therapeutics)

Page 3: introduction – introduce coumarin derivative, and why it was designed that way

Pages 4-5: methods

3.1 – Organic Chemistry Synthesis

3.2 – Cell Biology

3.3 – Cytotoxicity assay

Page 6: results – cytotoxicity assay and fluorescence values

Page 7: Discussion – what went wrong

Page 8: Discussion – potential fixes for what went wrong, potential control assays