Evaluation of D-Amino Acids as Probes for Molecular Imaging of Bacterial Infections

by

Tiffany Soomin Kwak

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

Biomedical Imaging

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Approved:

[redacted]

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Committee in Charge

Evaluation of D-Amino Acids as Probes for Molecular Imaging of Bacterial Infections

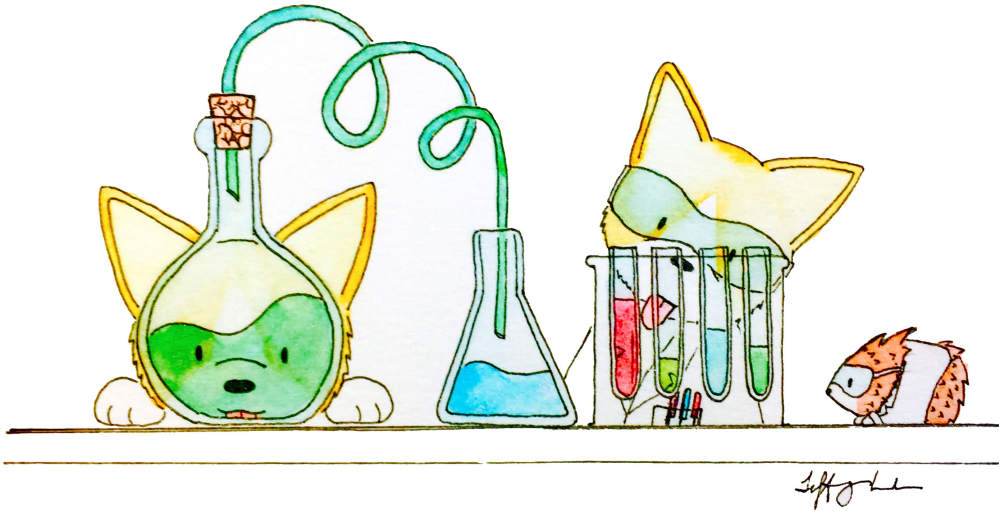
Tiffany Soomin Kwak

To my family and friends

(here and on yonder continents),

I dedicate this thesis to you.

Thank you for accompanying me on this journey.



Acknowledgments

"Not all who wander are lost" -J.R.R. Tolkien

It all started with a conversation. A few years back, the director of Lawrence Berkeley National Laboratory and I were chatting about my future. I told him that I felt pretty lost, but he reassured me. "Everything that you do," he said, "no matter how trivial it may seem, is actually building up to where you need to go. It's not always clear where you need to end up, but don't worry. You're getting there. And you *will* get there. Enjoy the journey." He was right. What an incredible journey this has been. There are too many people to thank individually, so I apologize for any omissions.

First, I want to say thank you to my family: my parents, Timothy and Stella, my two brothers, Michael and Thomas, and my extended family, 이모부, 이모, 고모부, 고모, 이병윤, 이윤원, 이두은, 김윤형, for their love and support. And also to my second family, the Gales family: Chris, Molly and David, who have followed my life ventures since my early years at Cal. And to Mama Jane Herrick for always cheering me on.

I could not have asked for a better team to work with. Go Team Science! Thank you to my wonderful advisors: Dr. Michael Ohliger, Dr. Dave Wilson, Dr. Oren Rosenberg, and Dr. Henry VanBrocklin for their insights and guidance. To Dr. Valerie Carroll and Dr. Renuka Sriram who have been invaluable to me in completing this project. And to Romelyn Delos Santos and the vibrant people of the Wilson Lab, VanBrocklin Lab, and Kurhanewicz Lab.

In addition, I am blessed to have such great friends: Jim Bui, Lesia Buckhalter, Will Chen, James Debalos, Alex Han, Stephanie Horiuchi, Richard Hsu, Eric Kim, Svet Kolev, Jonathan Kotker, Jerel Lim, Jeffrey Lin, Jon Lo, Jaron Lopez, Jed Mac, Mango Madness, Alicia Middleton, Coel Momita, Lisa Nguyen, Alex Probst, Diana Rios, Lena Salaita, Ashutosh Sheth, Crystal Villa, Matt Weldon, Spencer Wong, Julie Zamarripa, and Hilary Zheng.

And finally to you, my readers, I hope you find this thesis as entertaining and as worthwhile as watching splooting corgis.

Evaluation of D-Amino Acids as Probes for Molecular Imaging of Bacterial Infections

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Abstract

Purpose The goal of this study was to investigate a panel of D-amino acids and select potential probe candidates for imaging bacterial infections *in vivo*.

Methods Uptake of radiolabeled D-amino acids was tested in *E. coli*. Selection of candidates was based on the following criteria: (1) high uptake in *E. coli* and (2) ease of ^{18}F labeled analog synthesis. Selected D-amino acid candidates were then tested for uptake and specificity in *E. coli* at several time points and with coadministration of non-radioactive D-amino acid blocking dose. ^{18}F D-Phenylalanine was synthesized to test uptake in *E. coli* over time. ^{18}F-FDG was tested for uptake and specificity in *E. coli* at several time points and with coadministration of non-radioactive Cytochalasin B blocking dose.

Results D-Methionine showed the highest uptake in *E. coli*. D-Methionine uptake increased over time in *E. coli* and showed specific uptake. D-Phenylalanine uptake increased over time in *E. coli* and showed specific uptake. ^{18}F D-Phenylalanine showed higher uptake in *E. coli* and showed more bacteria specific uptake than ^{18}F-FDG.

Conclusion D-Methionine and D-Phenylalanine were selected as potential probe candidates for imaging bacterial infections *in vivo*.

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1 Background

1.1 Infection

Infection is a worldwide clinical problem, causing extensive morbidity and mortality. According to the *NIH National Institute of Allergy and Infectious Diseases*, the diversity of bacteria and their mechanisms to thrive pose a continuous threat to human health [1]. Early diagnosis is crucial for the prevention and treatment of bacterial infections. Despite advances in imaging technologies, there is currently no method to directly image bacterial infection *in vivo*. ^{18}F-FDG PET scans and radiolabeled white blood cells scans are commonly performed in the setting of infection, but these cannot distinguish true infection from sterile inflammation [2, 3, 4]. The current gold standard for diagnosing infection is microbial culture, which can take several days to yield results [5]. Also, invasive procedures may be required to obtain the proper specimens for testing; this is particularly true for spinal infections [5].

1.2 Peptidoglycan

One of the key features that differentiates bacteria from mammalian cells is the presence of peptidoglycan in the bacterial cell wall. The amount of peptidoglycan varies, depending on whether the bacteria are Gram-Positive or Gram-Negative as shown in Figure 1 [6]. Gram-negative bacterial cell walls contain lipopolysaccharides, lipoprotein, and little peptidoglycan. Gram-positive bacterial cell walls contain large quantities of peptidoglycan, polysaccharides, and teichoic acids or teichuronic acid [7]. Because peptidoglycan is present in all bacteria and not present in mammalian cells, this is a good target to explore for potential probe development.

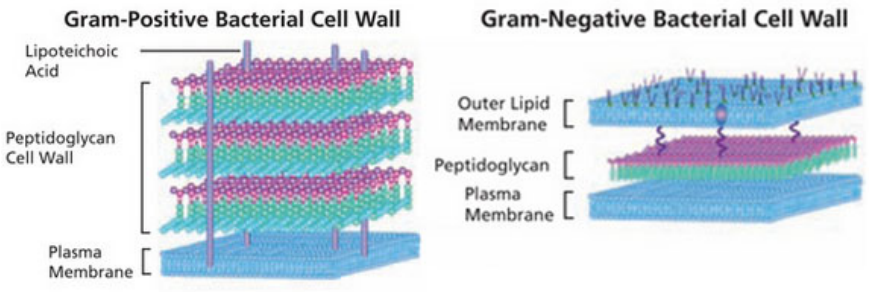

Figure 1: Peptidoglycan content in bacterial cell walls of Gram-positive (left) and Gram-negative (right) bacteria [6]

Looking more closely at the structure of peptidoglycan (Figure 2), we can see that it contains several D-amino acids, namely D-Alanine and D-Glutamate.

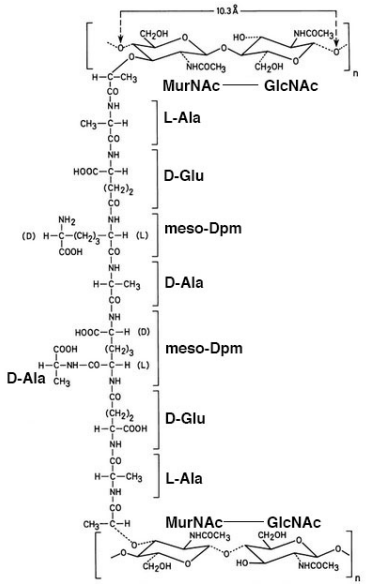

Figure 2: Peptidoglycan structure [8]

In nature, there is an abundance of L-amino acids and D-amino acids; however, D-amino acids are not readily incorporated into mammalian cells (as mammalian cells use almost exclusively L-amino acids) [9]. Therefore, uptake of D-amino acids is expected to be a specific marker of bacterial infection. Caparros *et al.* have demonstrated the incorporation of D-amino acids into peptidoglycan. They found that among the D-amino acids they tested, D-Methionine, D-Phenylalanine, and D-Valine were incorporated the most [10].

1.3 Positron Emission Tomography (PET)

Positron Emission Tomography or PET has become an integral part of nuclear medicine as it can provide functional information in the body. In PET, a radiolabeled probe is injected into the body. The probe then accumulates at the target location. A positron is emitted and interacts with a surrounding electron, causing complete annihilation. From the annihilation, a pair of 511 kEV photons are emitted in opposite directions, detected by a ring of scintillation detectors, and an image is generated [11].

1.4 Labeled Isotopes

Labeled compounds provide a good way to monitor processes within an organism. For our studies, we utilized various isotopes of the following elements: hydrogen, carbon, and fluorine. We considered ^{14}C, which contains 6 protons and 8 neutrons and has a long half-life of 5700 years [12]. ^{3}H contains 1 proton and 2 neutrons and has a half-life of 12.32 years [12]. Both ^{14}C and ^{3}H undergo beta decay, a process in which an electron is released, and the atomic number is increased by one. Because both of these have long half-lives and are commercially available, they were used for our experiments.

For PET imaging, ^{11}C and ^{18}F are commonly used. ^{11}C contains 6 protons and 5 neutrons. ^{11}C has a half-life of 20 minutes [12]. ^{18}F contains 9 protons and 9 neutrons. ^{18}F has a half-life of 110 minutes [12]. ^{11}C and ^{18}F undergo positron emission whereby a positron is emitted, and the atomic number is decreased by one. The advantage of using these elements is that they have relatively short half-lives, which reduces radiation exposure to the patient and allows for multiple studies. However, because they are not naturally occurring, they must be generated in a cyclotron [13]. ^{18}F can be generated remotely at a central site, but the short half-life of ^{11}C means it must be generated locally, requiring a cyclotron on-site [14].

Another isotope we are interested in is ^{13}C, a stable isotope, containing 6 protons and 7 neutrons. It is mainly used for research and makes up about 1.07% of all natural carbon

on Earth [15]. Currently, interest has risen in hyperpolarized ^{13}C for NMR, which aids in visualization of metabolism [16].

1.5 Liquid Scintillation Counter

Liquid scintillation counting is a method of quantifying the activity of a radioactive sample [17]. First, the sample is mixed with a scintillation cocktail, which contains a solvent and scintillators known as "fluors". As the sample emits beta particles, the solvent molecules are excited, and the energy is transferred to the "fluors". These "fluors" emit light, which is amplified by photomultiplier tubes (PMT) and then counted [17].

1.6 Probe Characteristics

The goal of this project is to determine a potential D-amino acid candidate that can serve as a probe for imaging of bacterial infections *in vivo*. In order to design a good probe, Chen *et. al* have determined that a good probe should have the following qualities: (1) high binding affinity to its target, (2) high specificity to its target, (3) high sensitivity, (4) high contrast ratio, (5) high stability *in vivo*, (6) low immunogenicity and toxicity, (7) easy production, and (8) low cost [18]. D-amino acids are available in nature and have been shown to be incorporated by bacteria; thus, they can be labeled and monitored for uptake.

2 Hypothesis

The hypothesis of this project is D-amino acids will have specific uptake by bacteria, which can be exploited to develop improved imaging probes.

3 Materials and Methods

3.1 Cell Lines and Culture Conditions

DH5-alpha *E. coli* was used for the bacterial cell line to monitor the uptake of D-amino acids. *Teknova* LB Broth containing 1.0% tryptone, 0.5% yeast extract, and 1.0% NaCl was used to culture all bacteria unless otherwise stated. *E. coli* were grown overnight on an orbital shaker (200 rpm) at 37°C. Bacterial suspensions were then transferred to *Teknova* MOPS Minimal Media supplemented with *Life Technologies* Vitamin MEM Solution for all experiments. J774A.1 (ATCC TIB-67) mouse macrophage cell line was used for the mammalian cell line to monitor the uptake of D-amino acids.

3.2 D-Amino Acids

Five D-amino acids were tested for this experiment: D-Alanine, D-Glutamate, D-Methionine, D-Phenylalanine, and D-Valine (Appendix A: Figure A1). All radiolabeled compounds were ordered from *Moravek Biochemicals and Radiochemicals*. For the ^{3}H D-amino acid studies, the following compounds were used: D-Alanine-2,3-^{3}H (MT-967) and D-Glutamic acid-^{3}H (MT-1577) were used. For the ^{14}C D-amino acid studies, the following compounds were used: D-Alanine-1-^{14}C (MC-2198), D-Methionine-1-^{14}C (MC-458), D-Phenylalanine-ring-^{14}C(U) (MC-2239), and D-Valine-1-^{14}C (MC-1383).

3.3 Preliminary *E. coli* Growth Studies

3.3.1 ^{13}C D-Alanine Uptake in *E. coli*

The following setup shown in Appendix B: Figure B1 was used. DH5-alpha *E. coli* was streaked out onto an LB agar plate from a glycerol stock. One colony was inoculated into 100 mL *Teknova* LB Broth and grown overnight to OD_{600} 1.0. The bacterial suspension was

centrifuged (1500 x g for 5 minutes) to pellet the bacteria. The supernatant was removed. The bacterial pellet was resuspended in 7.5 mL minimal media. Then the bacterial suspension was centrifuged (1500 x g for 5 minutes), and the supernatant was removed. This wash step was repeated two more times. The bacterial pellet was resuspended in 7.5 mL minimal media. 2 mL of the bacteria suspension was transferred into a new flask containing 47.5 mL minimal media and 2 mM ^{13}C D-Alanine.

OD_{600} was taken at the first time point. At each time point, 1 mL bacterial culture was removed for OD_{600} measurement, and 1 mL bacterial culture was transferred to an Eppendorf tube for future NMR analysis. The Eppendorf tube was centrifuged (13.2 rpm for 5 minutes). The supernatant was then transferred to a new Eppendorf tube. Both bacterial pellet and supernatant were stored at -80°C until NMR analysis.

Samples were thawed to room temperature (25°C). 540 µL sample was measured and transferred to an Eppendorf tube. 60 µL of *Sigma-Aldrich* Deuterium oxide (99.9 atom % D, contains 0.75 wt % 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid, sodium salt) was added to the sample. The sample was mixed by gentle pipetting and then transferred to an NMR sample tube. The samples were analyzed on the Bruker AVANCE 800 MHz Spectrometer.

3.3.2 ^{13}C Pyruvate Uptake in *E. coli*

The experiment described above was repeated for ^{13}C pyruvate (Appendix B: Figure B1). Bacterial suspensions were transferred into a new flask containing 47.5 mL minimal media and 2 mM *Sigma-Aldrich* sodium pyruvate-1-^{13}C.

3.4 *E. coli* Preparation for Uptake Studies

The experiment setup shown in Appendix Figure B2 was used. DH5-alpha *E. coli* was streaked out onto an LB agar plate from a bacterial glycerol stock. One colony was inoculated into 100 mL *Teknova* LB Broth and grown overnight to OD_{600} 1.0. The bacterial suspension was centrifuged (1500 x g for 5 minutes) to pellet the bacteria. The supernatant was removed.

The bacterial pellet was resuspended in 7.5 mL minimal media. Then the bacterial suspension was centrifuged (1500 x g for 5 minutes), and the supernatant was removed. This wash step was repeated two more times. The bacterial pellet was resuspended in 7.5 mL minimal media. 2 mL of the bacteria suspension was transferred into a new flask containing 47.5 mL minimal media. The bacteria were incubated for one hour to readjust to minimal media.

3.5 Macrophage Preparation for Uptake Studies

J774A.1 (ATTC TIB-67) mouse macrophage cell line stock was provided frozen from the lab of John Mackenzie, MD. The experiment setup is shown in Appendix Figure C1. *UCSF Cell Culture Facility* PBS Ca^{++} and Mg^{++} free and *Life Technologies* RPMI Medium 1640 with GlutaMAX were preheated to 37°C in a heated water bath for 1 hour before use. The media was removed from the flask, and the cells were washed with 10 mL PBS. Wash cycles were repeated three times. The cells were scraped from flask with a disposable rubber scraper. The detached cells were transferred into a 15 mL conical *Falcon* tube. Then 10 µL was transferred into an Eppendorf tube, and 10 µL *Thermo Fisher Scientific* Trypan Blue Solution 0.4% was added. The solution was mixed, and 10 µL was transferred to a hemocytometer. The number of viable cells were counted in the four corner quadrants, and the total number of viable cells/mL was calculated (Appendix C: Figure C3).

3.6 ^{3}H and ^{14}C D-Amino Acid Uptake Studies

3.6.1 ^{3}H and ^{14}C D-Amino Acid Uptake in *E. coli*

OD_{600} was measured. Four replicates were made per time point along with four blanks. Each vial per time point contained the following: 20 million *E. coli*, minimal media up to a total volume of 1 mL, and an equal mass of ^{3}H or ^{14}C D-amino acid probe. For blocking experiments, each vial contained the following: 20 million *E. coli*, 100 µL of 10 mM non-radioactive D-amino acid, minimal media up to a total volume of 1 mL, and an

equal mass of ^{3}H or ^{14}C D-amino acid probe. The vials were incubated at 37°C until the time point (Appendix B: Figure B2, B3).

3.6.2 ^{14}C D-Amino Acid Uptake in Macrophages

Cell viability counts were done using *Thermo Fisher Scientific* Trypan Blue assay. Four replicates were made per time point along with four blanks. Each vial per time point contained the following: 0.5 million macrophages, minimal media up to a total volume of 1 mL, and an equal mass of ^{3}H or ^{14}C D-amino acid probe. For blocking experiments, each vial contained the following: 0.5 million macrophages, 100 µL of 10 mM non-radioactive D-amino acid, minimal media up to a total volume of 1 mL, and an equal mass of ^{3}H or ^{14}C D-amino acid probe. The vials were incubated at 37°C until the time point (Appendix C: Figure C1, C2).

3.7 Liquid Scintillation Counting Sample Preparation

The washing procedure at each time point was the same for both *E. coli* and macrophages. At each incubation time point, the samples were centrifuged (13.2 rpm for 3 minutes). The supernatant was removed. 1 mL cold Phosphate-Buffered Saline (PBS) was added to the samples and vortexed gently to resuspend the contents. The suspension was centrifuged (13.2 rpm for 3 minutes). The supernatant was removed. 3 additional wash cycles were performed. The supernatant was removed. 500 µL 1 M NaOH was added to the bacterial pellet, vortexed, and incubated at 37°C for 5 minutes. 400 µL suspension was transferred to a scintillation vial. 4 mL *MP Biomedicals* Ecolite liquid scintillation cocktail was added to each scintillation vial. Samples were counted on the *Beckman* LSC 6500 scintillation counter. Cell counts and viability were monitored using *Promega* BacTiter-Glo for *E. coli* and *Thermo Fisher Scientific* Trypan Blue Assay for JA774.1 macrophages. Results were normalized for viable cell count (Appendix B: Figure B1, B4, Table B2, B3, B4, B5).

4 Results

4.1 Preliminary Studies

4.1.1 ^{13}C D-Alanine and ^{13}C Pyruvate Uptake in *E. coli*

We investigated the growth rate and uptake rate of ^{13}C D-Alanine and ^{13}C Pyruvate in *E. coli*. We conducted studies in triplicate and found the uptake rate of D-Alanine in *E. coli* was 2.48×10^{-4} +/- 2.03×10^{-5} mM/OD/min. OD_{600} of *E. coli* increased over time, and the concentration of ^{13}C D-Alanine uptake decreased over time as shown in Figure 3. We conducted studies in triplicate and found that the uptake rate of pyruvate in *E. coli* was 1.76×10^{-4} +/- 5.11×10^{-5} mM/OD/min. OD_{600} of *E. coli* increased over time, and the concentration of ^{13}C pyruvate uptake decreased over time as shown in Figure 4. From this study, we saw that D-Alanine was taken up at a higher rate than pyruvate in *E. coli* (p=0.021).

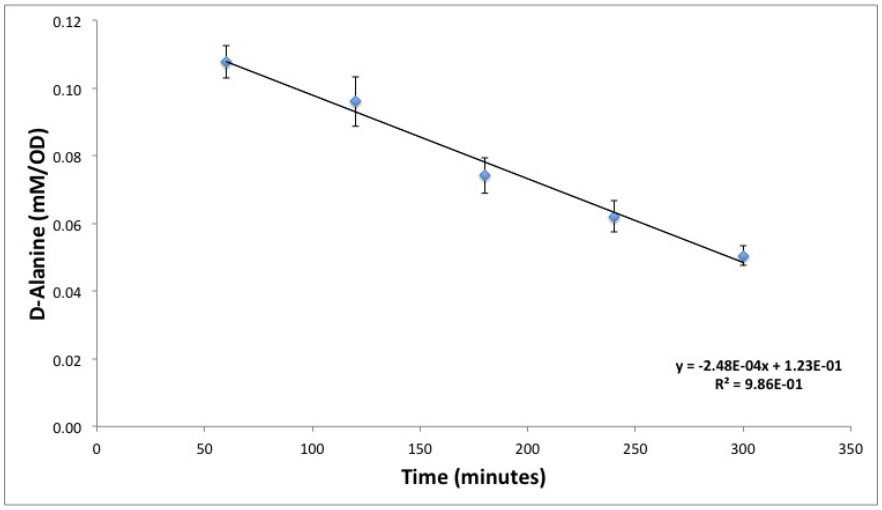

Figure 3: ^{13}C D-Alanine uptake in *E. coli* over time

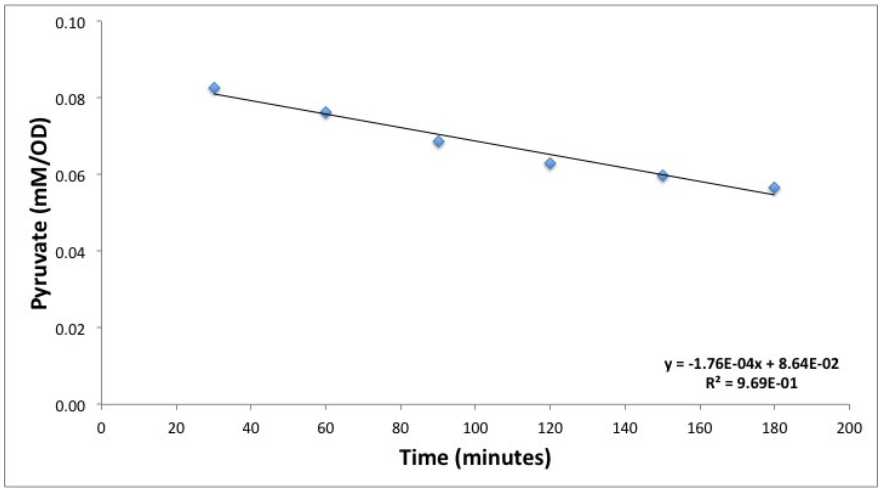

Figure 4: ^{13}C Pyruvate uptake in *E. coli* over time

4.2 D-Amino Acid Uptake Studies in *E. coli*

4.2.1 ^{3}H D-Alanine vs. ^{3}H D-Glutamate Uptake in *E. coli*

As previously mentioned, D-Alanine and D-Glutamate are present within the peptidoglycan structure. Due to the immediate availability of the compounds, ^{3}H D-Alanine and ^{3}H D-Glutamate were tested for uptake in *E. coli*. However, D-Glutamate was only commercially available as ^{3}H D-Glutamate, so we conducted cell uptake studies to assess the necessity of D-Glutamate for future studies. Table 1 shows the % cell associated activity per million cells and % error for ^{3}H D-Alanine. Table 2 shows % cell associated activity per million cells and % error for ^{3}H D-Glutamate.

Table 1: ^{3}H D-Alanine uptake in *E. coli*

	% Cell Associated Activity per million cells	% Error
1 hour	0.623	0.039
1 hour + block	0.045	0.002

Table 2: ^{3}H D-Glutamate uptake in *E. coli*

	% Cell Associated Activity per million cells	% Error
1 hour	0.423	0.031
1 hour + block	0.366	0.057

At 1 hour, ^{3}H D-Alanine showed high uptake and was blocked with coadminstration of 1 mM non-radioactive D-Alanine, indicating specific uptake. Whereas, ^{3}H D-Glutamate at the 1 hour showed high uptake but was poorly blocked with coadministration of 1 mM non-radioactive D-Glutamate, indicating non-specific uptake. Therefore, D-Glutamate was eliminated from further testing. Appendix D: Figure D1 shows the relative uptake of ^{3}H D-Alanine and ^{3}H D-Glutamate at 1 hour and at 1 hour with coadministration of 1 mM non-radioactive D-amino acid blocking dose.

4.2.2 ^{14}C D-Amino Acid Uptake in *E. coli*

The remaining four D-amino acids (D-Alanine, D-Methionine, D-Phenylalanine, and D-Valine) were commercially available as ^{14}C labeled compounds and were tested for uptake in *E. coli*. Table 3 shows the % cell associated activity per million cells and the % error.

Table 3: ^{14}C D-amino acid uptake in *E. coli*

	% Cell Associated Activity per million cells	% Error
^{14}C D-Alanine	0.813	0.152
^{14}C D-Methionine	1.400	0.147
^{14}C D-Phenylalanine	0.097	0.009
^{14}C D-Valine	0.104	0.010

^{14}C D-Methionine showed the highest uptake of the four D-amino acids, therefore, selected for future studies. The uptake of D-Phenylalanine was not as high as D-Methionine, but it was still considered as a potential candidate because of the feasibility of radiosynthesis of a PET analog (Appendix D: Figure D2).

4.2.3 ^{14}C D-Methionine Uptake in *E. coli*

^{14}C D-Methionine uptake was monitored at the following time points: 30 minutes, 1 hour, 2 hours, and 2 hours with coadministration of 1 mM non-radioactive D-Methionine as shown in Table 4.

Table 4: ^{14}C D-Methionine uptake in *E. coli*

	% Cell Associated Activity per million cells	% Error
30 minutes	0.186	0.037
1 hour	0.301	0.027
2 hours	1.094	0.097
2 hours + blocking	0.466	0.033

There was an increase in percent cell associated activity over time and was blocked at 2 hours, indicating specific uptake (Appendix D: Figure D3).

4.2.4 ^{14}C D-Phenylalanine Uptake in *E. coli*

As discussed above, D-Phenylalanine has a relatively straightforward synthesis as an ^{18}F labeled PET analog. Because of this practical issue, D-Phenylalanine was investigated further despite its lower uptake compared to D-Methionine. ^{14}C D-Phenylalanine uptake was monitored in *E. coli* over the following time points: 30 minutes, 1 hour, 2 hours, and 2 hours with coadministration of 1 mM non-radioactive D-Phenylalanine as shown in Table 5.

Table 5: ^{14}C D-Phenylalanine uptake in *E. coli*

	% Cell Associated Activity per million cells	% Error
30 minutes	0.007	0.001
1 hour	0.018	0.000
2 hours	0.059	0.005
2 hours + blocking	0.005	0.000

There was an increase in percent cell associated activity over time and was blocked at 2 hours, indicating specific uptake (Appendix D: Figure D4).

4.2.5 ^{18}F D-Phenylalanine vs. ^{18}F-FDG Uptake in *E. coli*

To test the feasibility of using a PET probe, an ^{18}F analog of D-Phenylalanine, (R)-2-amino-3-([4-^{18}F]-fluorophenyl)propanoic acid, was synthesized by another member of our group, Dr. Kiel Neumann (Figure 5). D-Phenylalanine was chosen for this initial test because it had a more straightforward radiosynthesis than D-Methionine. Because ^{18}F

D-Phenylalanine was chemically different from D-Phenylalanine, we tested the uptake of ^{18}F D-Phenylalanine in *E. coli* to see if the attachment of ^{18}F would change the uptake over the following time points: 30 minutes, 1 hour, 2 hours, and 2 hours with coadministration of 1 mM non-radioactive D-Phenylalanine dose as shown in (Table 6). ^{18}F-FDG uptake was monitored in *E. coli* over the following time points: 30 minutes, 1 hour, 2 hours, and 2 hours with coadministration of 10 µg/µL non-radioactive dose of Cytochalasin B as shown in Table 7. ^{18}F-FDG was chosen as our standard because all cells take up glucose. Cytochalasin B is a known glucose transport inhibitor so it was chosen as a blocking agent for ^{18}F-FDG [19].

Figure 5: Phenylalanine structures: ^{18}F D-Phenylalanine (left), D-Phenylalanine (right)

Table 6: ^{18}F D-Phenylalanine uptake in *E. coli*

	% Cell Associated Activity per million cells	% Error
30 minutes	0.054	0.004
1 hour	0.086	0.004
2 hours	0.156	0.021
2 hours + blocking	0.039	0.029

Table 7: ^{18}F-FDG uptake in *E. coli*

	% Cell Associated Activity per million cells	% Error
30 minutes	0.011	0.002
1 hour	0.010	0.002
2 hours	0.011	0.003
2 hours + blocking	0.071	0.080

^{18}F D-Phenylalanine showed an increase in percent cell associated activity and was blocked with 1 mM non-radioactive D-Phenylalanine, indicating specific uptake. ^{18}F-FDG

showed an increase in percent cell associated activity and was blocked by Cytochalasin B, indicating specific uptake. ^{18}F D-Phenylalanine showed a much higher uptake than ^{18}F-FDG, indicating more specific uptake of ^{18}F D-Phenylalanine in *E. coli*. There was large standard deviation for ^{18}F-FDG at 2 hours with blocking, which will need to be repeated for confirmation (Appendix D: Figure D5).

4.3 D-Amino Acid Uptake Studies in Macrophages

4.3.1 ^{14}C D-Amino Acid Uptake in Macrophages

We investigated whether labeled D-amino acids would be taken up in mammalian cells. Macrophages were used as a representative mammalian cell line. We tested the four D-amino acids (D-Alanine, D-Methionine, D-Phenylalanine, and D-Valine). We did not test D-Glutamate because it had shown non-specific uptake in *E. coli*. Table 8 shows the % cell associated activity per million cells and the % error.

Table 8: ^{14}C D-amino acid uptake in macrophages

	% Cell Associated Activity per million cells	% Error
^{14}C D-Alanine	13.257	0.417
^{14}C D-Methionine	5.319	0.092
^{14}C D-Phenylalanine	0.576	0.006
^{14}C D-Valine	1.746	0.015

^{14}C D-Alanine showed the highest uptake in macrophages, whereas the remaining D-amino acids had much lower uptake compared to ^{14}C D-Alanine (Appendix E: Figure E1).

4.3.2 ^{14}C D-Methionine Uptake in Macrophages

D-Methionine showed the best uptake in *E. coli*, so uptake was tested in macrophages over the following time points: 30 minutes, 1 hour, 2 hours, and 2 hours with coadministration of 1 mM non-radioactive D-Methionine as shown in Table 9.

Table 9: ^{14}C D-Methionine uptake in macrophages

	% Cell Associated Activity per million cells	% Error
30 minutes	0.128	0.029
1 hour	0.144	0.009
2 hours	0.141	0.011
2 hours + blocking	0.131	0.045

There was little change in uptake over time of D-Methionine in macrophages, and it showed unsuccessful blocking at 2 hours, indicating non-specific uptake (Appendix E: Figure E2). The percent cell associated activity of the D-Methionine in the D-amino acid panel experiment differed from the D-Methionine in the time course experiment. The reason for this difference is unknown, but it could be due the cell lines being handled by different people, cell lines being at different levels of confluency, or cells having different viabilities. These experiments will need to be repeated for confirmation.

4.3.3 ^{14}C D-Phenylalanine Uptake in Macrophages

D-Phenylalanine was also tested due to the ease of radiosynthesis. ^{14}C D-Phenylalanine uptake was monitored in macrophages over the following time points: 30 minutes, 1 hour, 2 hours, and 2 hours with coadministration of 1 mM non-radioactive D-Phenylalanine as shown in Table 10.

Table 10: ^{14}C D-Phenylalanine uptake in macrophages

	% Cell Associated Activity per million cells	% Error
30 minutes	0.027	0.004
1 hour	0.029	0.008
2 hours	0.025	0.004
2 hours + blocking	0.020	0.003

There was little change in uptake over time of D-Phenylalanine in macrophages, and it showed unsuccessful blocking at 2 hours, indicating non-specific uptake (Appendix E: Figure E3). The percent cell associated activity of the D-Phenylalanine in the D-amino acid panel experiment differed from the D-Phenylalanine in the time course experiment. The reason

for this difference is unknown, but it could be due the cell lines being handled by different people, cell lines being at different levels of confluency, or cells having different viabilities. These experiments will need to be repeated for confirmation.

5 Discussion

The goal of this project was to identify which D-amino acid candidates would be good probes for imaging bacterial infections *in vivo*. We selected D-Methionine as our ideal candidate for future PET probe development. In addition, we were able to successfully synthesize an ^{18}F analog of D-Phenylalanine and test its performance in *E. coli*. We found that ^{18}F D-Phenylalanine had higher uptake and good blocking in *E. coli* than ^{18}F-FDG, the standard for our experiment. Therefore, ^{18}F D-Phenylalanine had more bacteria-specific uptake than ^{18}F-FDG.

D-Methionine was our D-amino acid of choice, but because there is no literature on the radiosynthesis of ^{18}F D-Methionine, it was not able to be tested. However, ^{11}C L-Methionine is well documented in the literature and is currently being used for PET imaging of brain tumors in humans [20, 21]. Since ^{11}C L-Methionine synthesis is known, it will be feasible to synthesize ^{11}C D-Methionine with enough time. Then, ^{11}C D-Methionine uptake studies can be performed.

We also tested the uptake of ^{14}C labeled D-amino acids in macrophages. Macrophages are one of many different white blood cells present at the site of infection [22]. We expected to see low uptake in the macrophages because previous studies have shown that mammalians cells almost exclusively utilize L-amino acids. However, we saw some uptake of D-amino acids in macrophages and failure to block at the 2 hours for both D-Methionine and D-Phenylalanine. From this, we can infer that there is lack of specificity in uptake of D-amino acids in macrophages.

5.1 Limitations

In an ideal situation, we would have generated ^{18}F or ^{11}C labeled compounds and directly tested uptake *in vivo*. However, these compounds require a dedicated radiosynthesis, which will be the subject of future work. We instead chose surrogate isotopes: ^{3}H and ^{14}C labeled compounds, which were not only commercially available but also had long half-lives. Radionuclides with short half-lives would not have been suitable for the design of this thesis experiment. Nonetheless, the best gauge of performance is testing these future PET analogs *in vivo*.

Another difficulty was normalizing all of the tests. Ideally, we would directly compare the uptake in white blood cells with bacteria. We can compute the activity per cell. However, the more relevant comparison requires knowledge of how many cells are present in an active infection. This number is not well known. Therefore, the true test will require the use of an animal model.

5.2 Future Work

^{18}F D-Phenylalanine was tested because it has a straightforward synthesis and showed good uptake along with specificity in *E. coli*; therefore, it will proceed as the ideal candidate for future probe development for imaging bacterial infections *in vivo*. Next, a ^{11}C D-Methionine analog should be synthesized. Similar to the ^{18}F D-Phenylalanine experiment previously performed, we will test uptake of ^{11}C D-Methionine in *E. coli*, and it will also be compared against ^{18}F-FDG.

There is a possibility that the results from these *in vitro* experiments may not yield the same results as those *in vivo*. Performance of ^{11}C D-Methionine and ^{18}F D-Phenylalanine will need to be tested in a mouse infection model. We plan to follow an infection model proposed by Weinstein *et. al* in their study of imaging bacterial infections with ^{18}F-FDS. One thigh will be injected with an agent that will simulate sterile inflammation, while live bacteria

will be injected into the other thigh to simulate active infection [23]. One consideration is that small animals could potentially have different enzyme mechanisms for the radiolabeled probe than those present in humans. However, the information obtained from small animal imaging may help bridge the gap between *in vitro* and *in vivo* experiments [24].

In addition, this project can be expanded to encompass a wider selection of bacterial strains. Initially, we chose *E. coli* as a representative for Gram-Negative bacteria. However, bacterial infections are not limited to just Gram-Negative bacteria. We will need to test Gram-Positive bacteria as well, including *Staphylococcus aureus*, to cover a range of bacterial infections.

6 Conclusion

D-amino acids are specifically taken up by bacteria. We selected D-Methionine and D-Phenylalanine as potential probe candidates for imaging bacterial infections *in vivo* based on high uptake in *E. coli* and feasibility of radiosynthesis. Further testing needs to be done *in vivo* to confirm performance of these probes, but their potential looks promising.

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A Appendix A: Materials

A.1 D-Amino Acid Structures

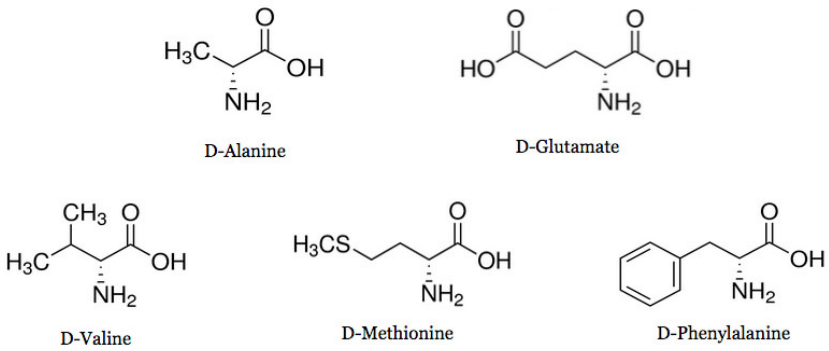

Figure A1: D-amino acid structures

B Appendix B: *E. coli* Experiment Setup

B.1 ^{13}C D-Amino Acid Uptake in *E. coli*

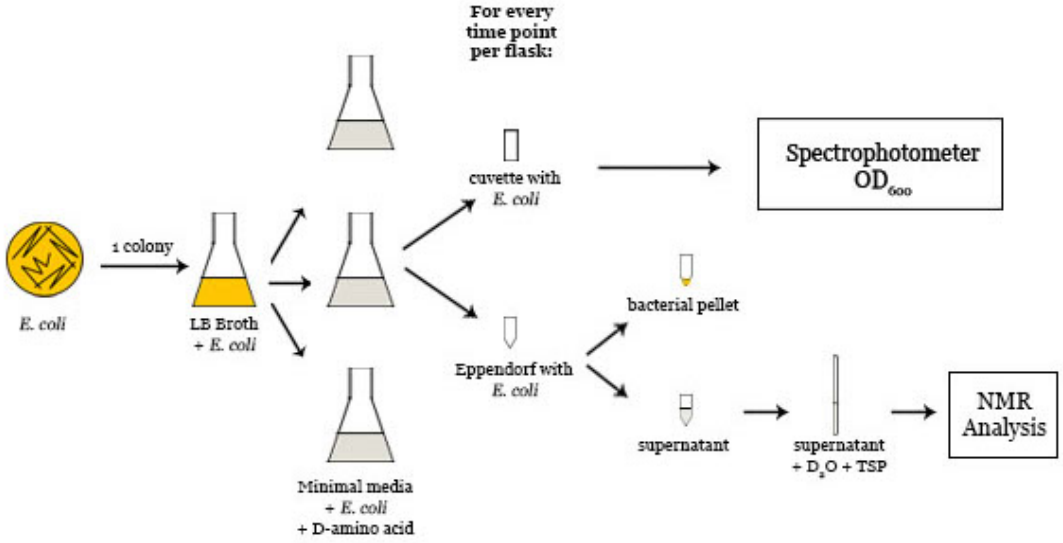

Figure B1: ^{13}C D-amino acid uptake in *E. coli* setup

B.2 ^{3}H and ^{14}C D-Amino Acid Uptake in *E. coli*

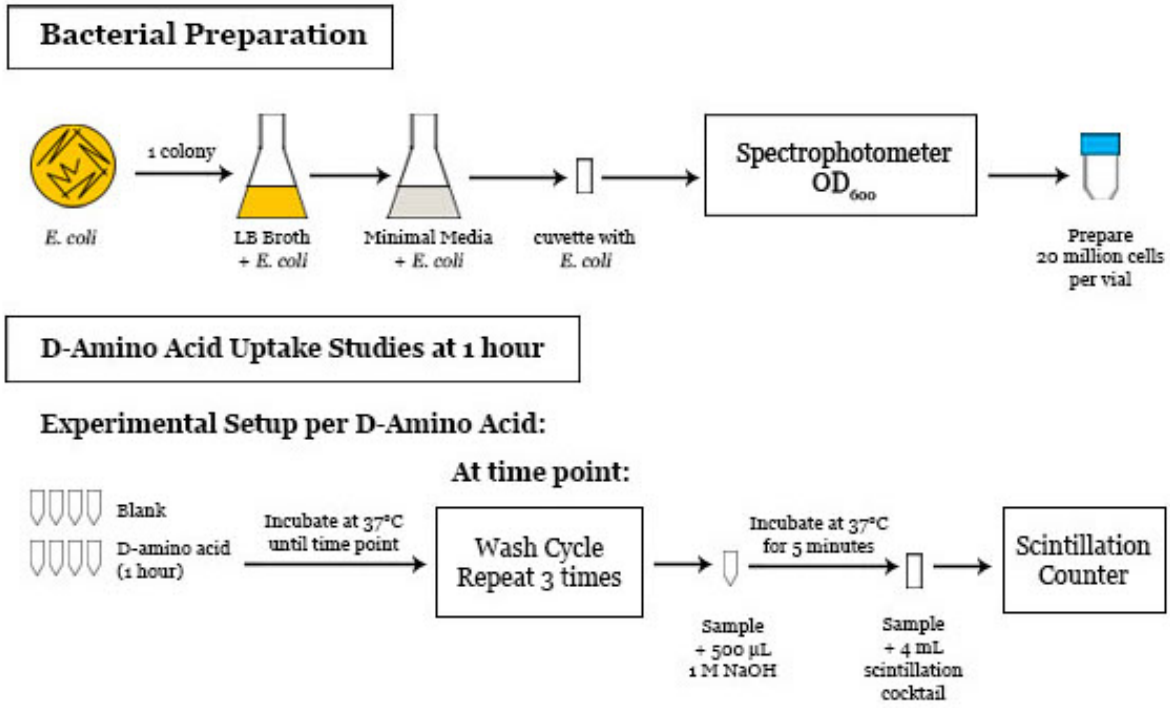

Figure B2: ^{3}H and ^{14}C D-amino acid uptake in *E. coli* setup

B.3 ^{14}C D-Amino Acid Candidate Uptake in *E. coli*

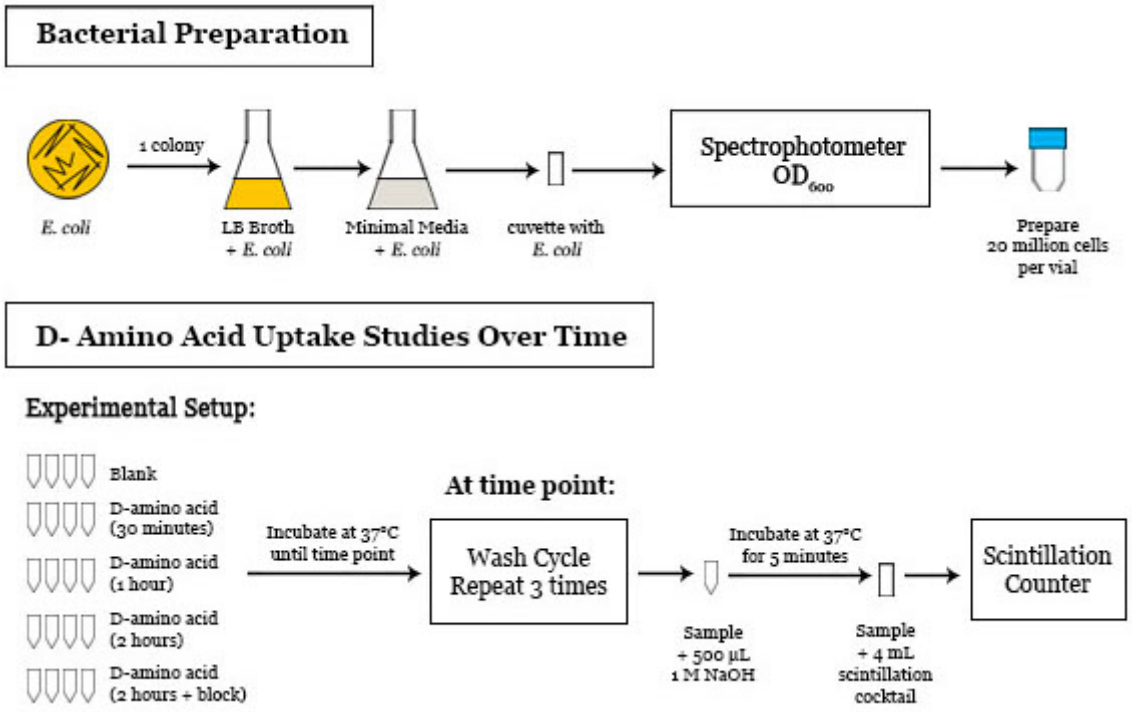

Figure B3: ^{14}C D-amino acid candidate uptake in *E. coli* setup

B.4 *Promega* BacTiter-Glo

B.4.1 BacTiter-Glo Standard Average Luminescence

Table B1: BacTiter-Glo standard curve

Bacteria Number	Average Luminescence
15 million	3672054
20 million	4912976
25 million	6200297
30 million	7758915
35 million	10208011
40 million	8868866
45 million	11156820
50 million	12418984

B.4.2 BacTiter-Glo Standard Curve

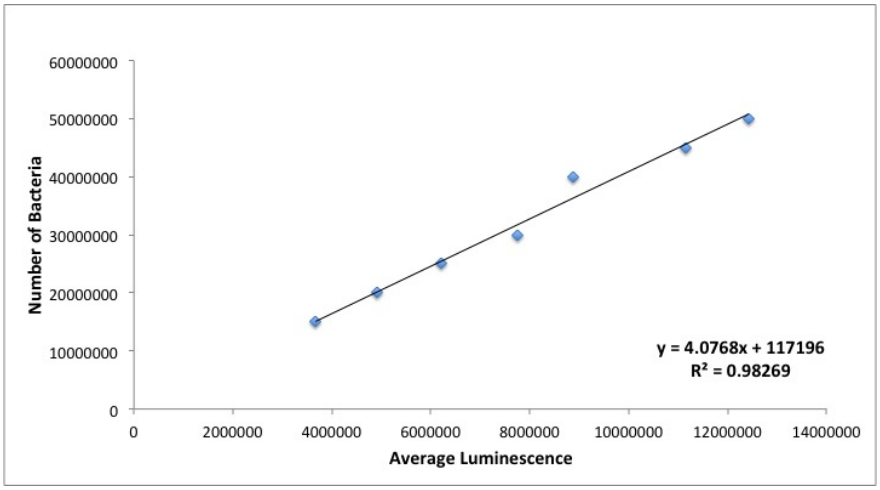

Figure B4: BacTiter-Glo standard curve: average luminescence vs. bacteria number

B.4.3 BacTiter-Glo: Average Luminescence in *E. coli* Over Time

Table B2: BacTiter-Glo: average luminescence of *E. coli* over time

Time	No Treatment	D-Alanine	D-Glutamate	D-Methionine
0 minutes	2514913	2755996	2851440	2496371
15 minutes	3456747	3571634	3650218	2925439
30 minutes	3716242	3751702	3983158	2410198
45 minutes	3316410	3369335	3845331	2224588
60 minutes	2706447	3144191	2978940	2171959
75 minutes	2343128	2959998	2723720	2106952
90 minutes	2140378	2845487	2359356	1878415
105 minutes	2020187	2526886	2194241	1629207
120 minutes	2155479	2636964	2464832	1813734

Table B3: BacTiter-Glo: average luminescence of *E. coli* over time (continued)

Time	D-Phenylalanine	D-Valine	Cytochalasin B
0 minutes	2767606	2875253	2720256
15 minutes	3674849	3775741	3730281
30 minutes	4044418	4108579	3856464
45 minutes	3828232	3884328	3773034
60 minutes	2829677	3000481	2957126
75 minutes	2645429	2709729	2623410
90 minutes	2550894	2434075	2342740
105 minutes	2517547	2269809	2410816
120 minutes	2898550	2592320	2679912

B.4.4 Number of Viable *E. coli* Over Time

Table B4: Number of viable *E. coli* over time

Time	No Treatment	D-Alanine	D-Glutamate	D-Methionine
0 minutes	1.04×10^7	1.14×10^7	1.17×10^7	1.03×10^7
15 minutes	1.42×10^7	1.47×10^7	1.50×10^7	1.20×10^7
30 minutes	1.53×10^7	1.54×10^7	1.64×10^7	9.94×10^6
45 minutes	1.36×10^7	1.39×10^7	1.58×10^7	9.19×10^6
60 minutes	1.12×10^7	1.29×10^7	1.23×10^7	8.97×10^6
75 minutes	9.67×10^7	1.22×10^7	1.12×10^7	8.71×10^6
90 minutes	8.84×10^7	1.17×10^7	9.74×10^6	7.78×10^6
105 minutes	8.35×10^7	1.04×10^7	9.06×10^6	6.76×10^6
120 minutes	8.90×10^7	1.09×10^7	1.02×10^7	7.51×10^6
Average Bacteria Number	1.12×10^7	1.26×10^7	1.24×10^7	9.02×10^6

Table B5: Number of viable *E. coli* over time (continued)

Time	D-Phenylalanine	D-Valine	Cytochalasin B
0 minutes	1.14×10^7	1.18×10^7	1.12×10^7
15 minutes	1.51×10^7	1.55×10^7	1.53×10^7
30 minutes	1.66×10^7	1.69×10^7	1.58×10^7
45 minutes	1.57×10^7	1.60×10^7	1.55×10^7
60 minutes	1.17×10^7	1.23×10^7	1.22×10^7
75 minutes	1.09×10^7	1.12×10^7	1.08×10^7
90 minutes	1.05×10^7	1.00×10^7	9.67×10^6
105 minutes	1.04×10^7	9.37×10^6	9.95×10^6
120 minutes	1.19×10^7	1.07×10^7	1.10×10^7
Average Bacteria Number	1.27×10^7	1.26×10^7	1.24×10^7

C Appendix C: Macrophage Experiment Setup

C.1 ^{14}C D-Amino Acid Uptake in Macrophages

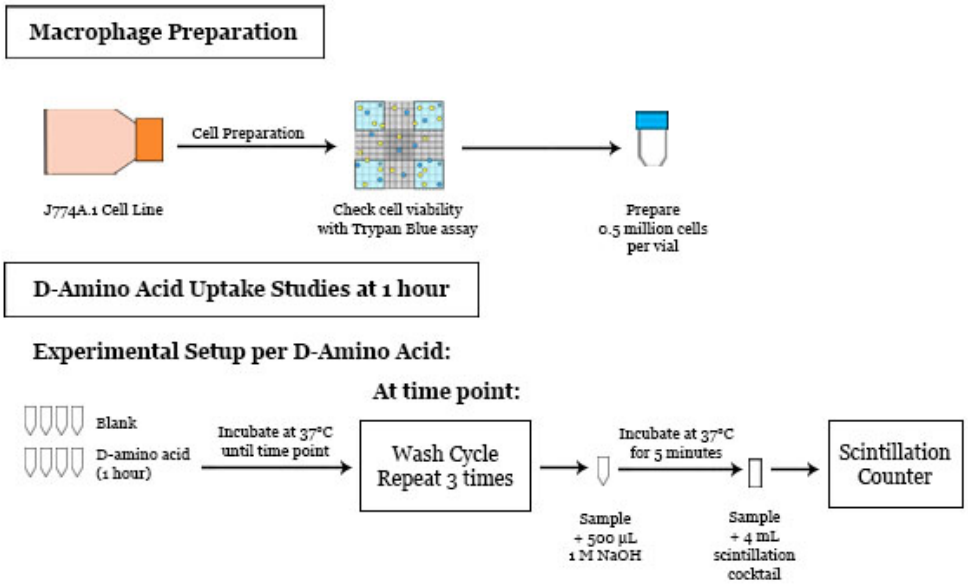

Figure C1: ^{14}C D-amino acid uptake in macrophages setup

C.2 ^{14}C D-Amino Acid Candidate Uptake in Macrophages

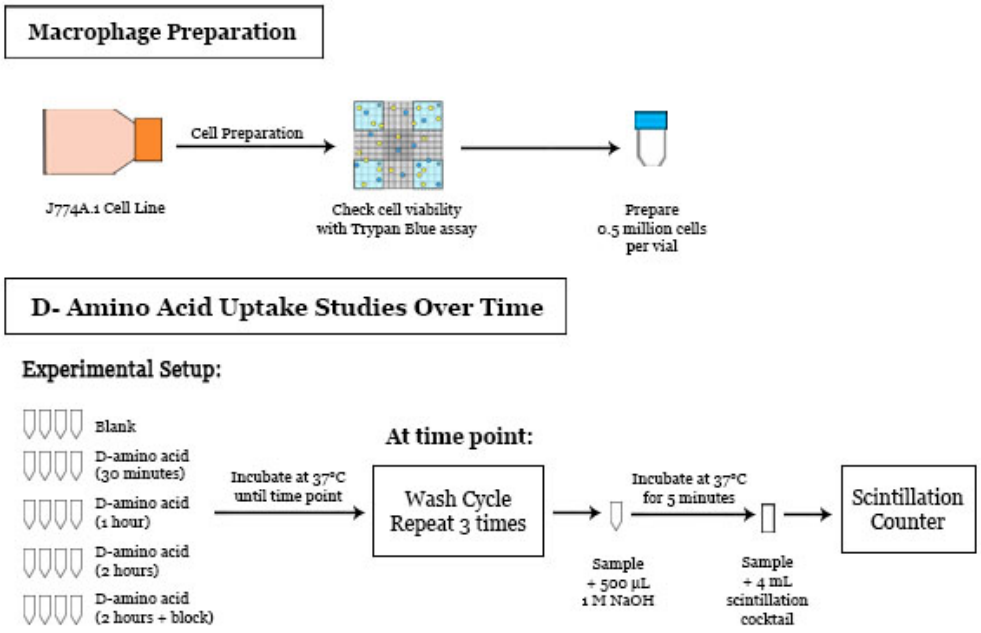

Figure C2: ^{14}C D-amino acid candidate uptake in macrophages setup

C.3 Hemocytometer

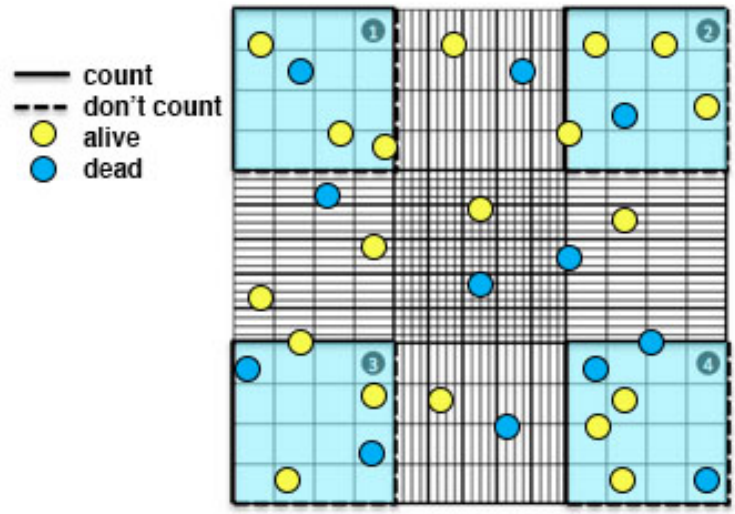

Figure C3: Hemocytometer: ratio calculated from counting viable cells (yellow) and dead cells (blue) in the four blue quadrants

D Appendix D: *E. coli* Experiment Results

D.1 ^{3}H D-Alanine vs. ^{3}H D-Glutamate Uptake in *E. coli*

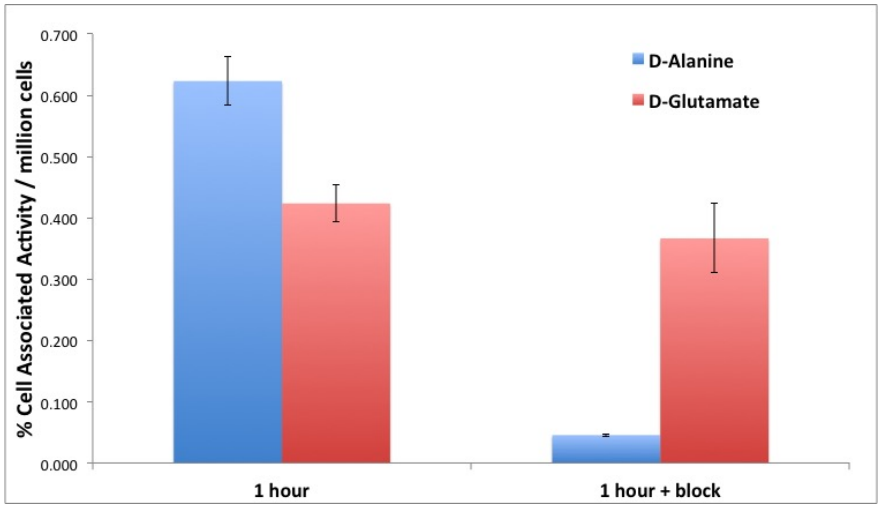

Figure D1: ^{3}H D-Alanine vs. ^{3}H D-Glutamate uptake in *E. coli*

D.2 ^{14}C D-Amino Acid Uptake in *E. coli*

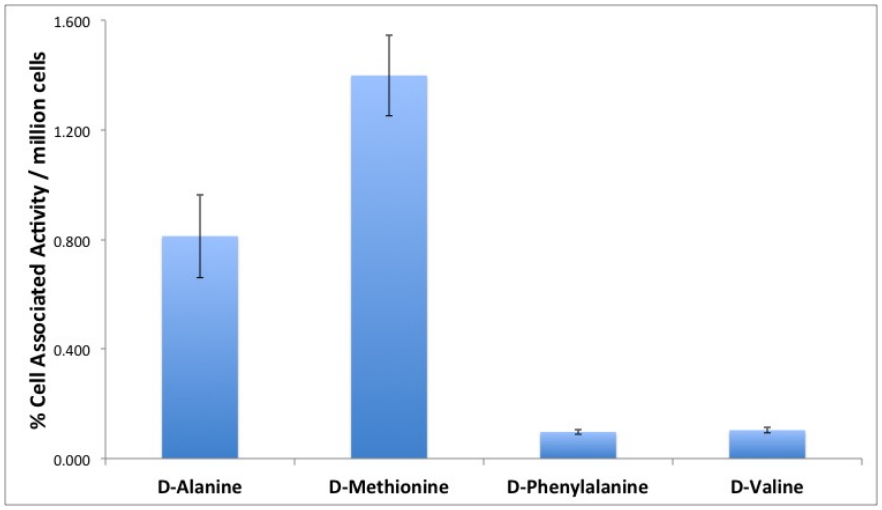

Figure D2: ^{14}C D-amino acid uptake in *E. coli*

D.3 ^{14}C D-Methionine Uptake in *E. coli*

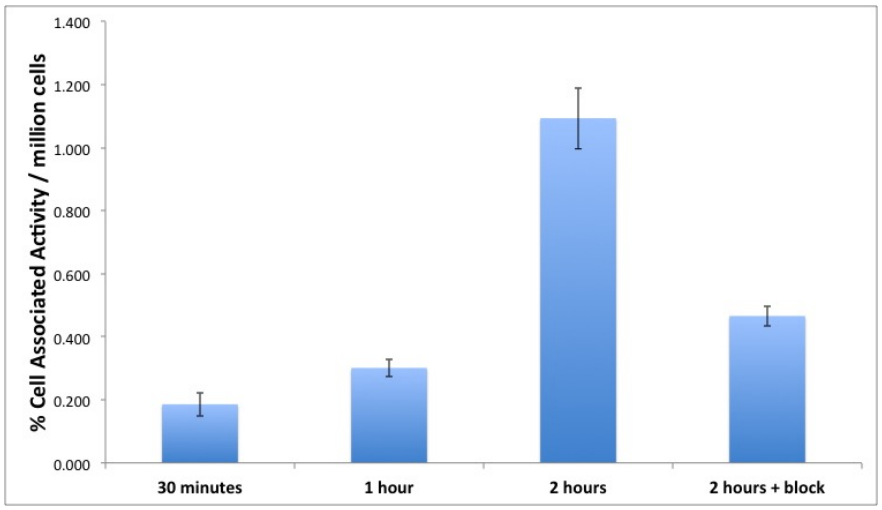

Figure D3: ^{14}C D-Methionine uptake in *E. coli*

D.4 ^{14}C D-Phenylalanine Uptake in *E. coli*

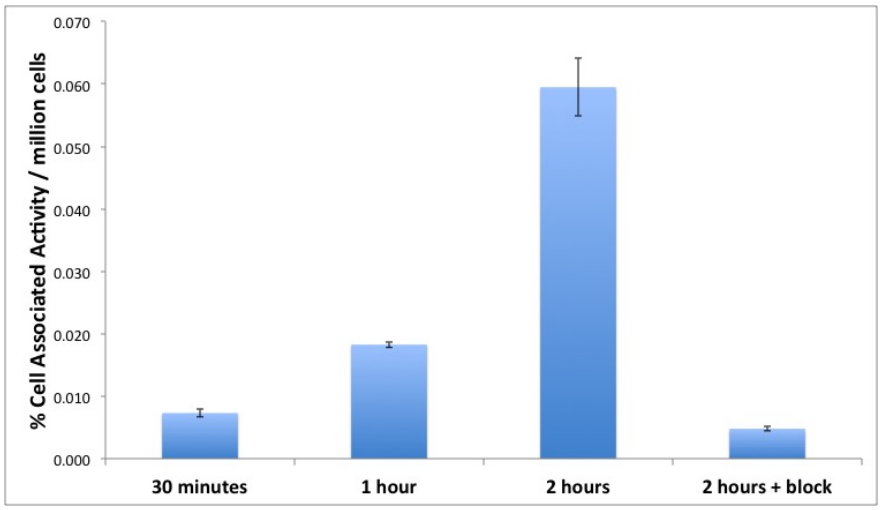

Figure D4: ^{14}C D-Phenylalanine uptake in *E. coli*

D.5 ^{18}F D-Phenylalanine vs. ^{18}F-FDG Uptake in *E. coli*

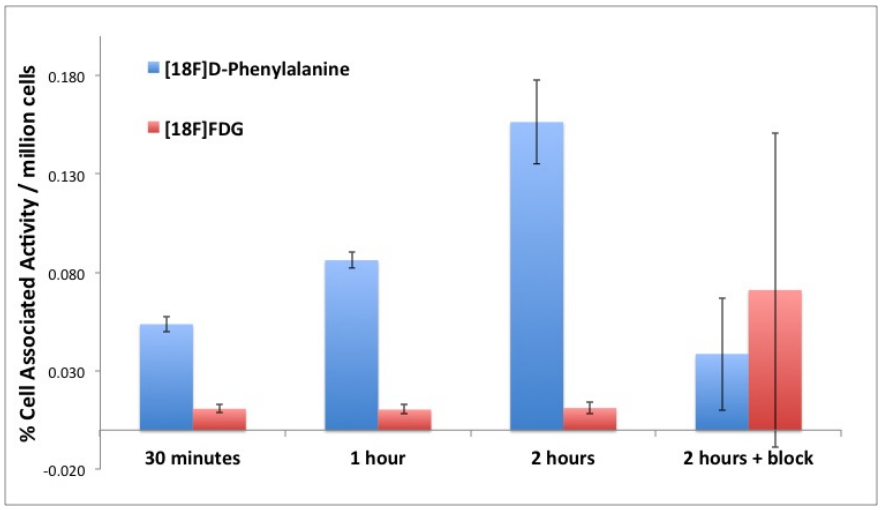

Figure D5: ^{18}F D-Phenylalanine vs. ^{18}F-FDG uptake in *E. coli*

E Appendix E: Macrophage Experiment Results

E.1 ^{14}C D-Amino Acid Uptake in Macrophages

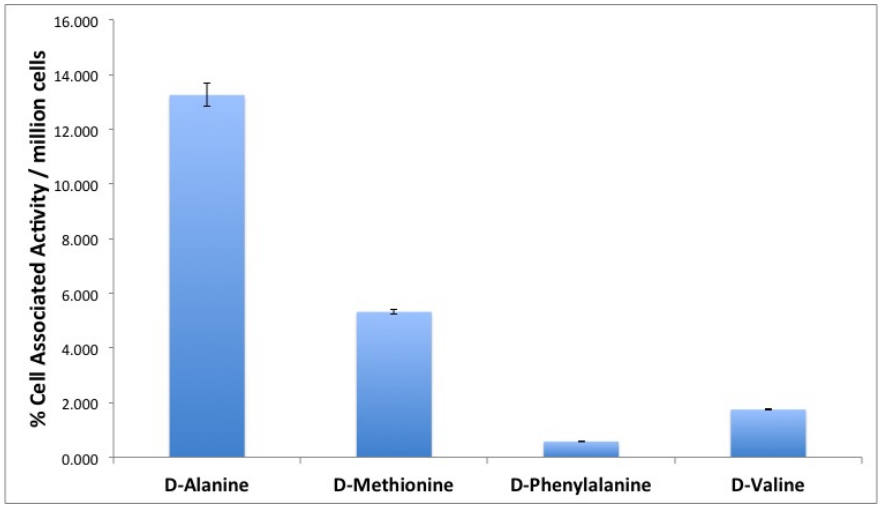

Figure E1: ^{14} D-amino acid uptake in macrophages

E.2 ^{14}C D-Methionine Uptake in Macrophages

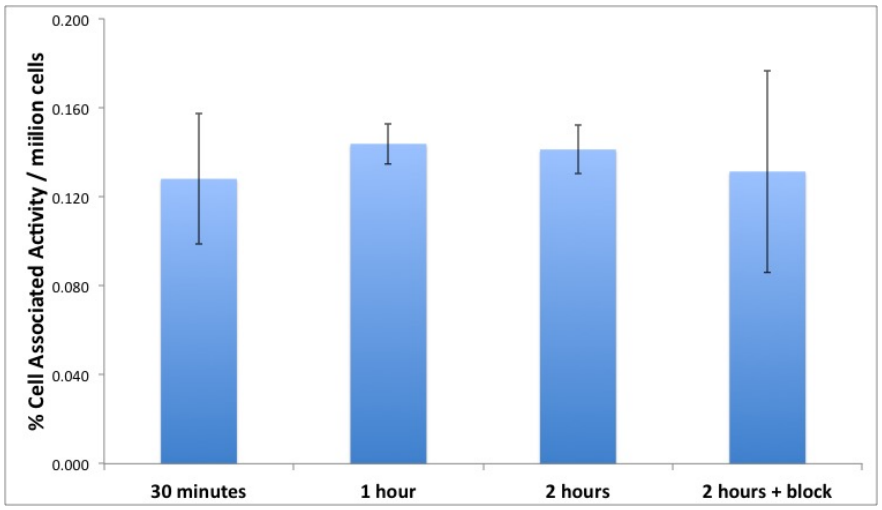

Figure E2: ^{14}C D-Methionine uptake in macrophages

E.3 ^{14}C D-Phenylalanine Uptake in Macrophages

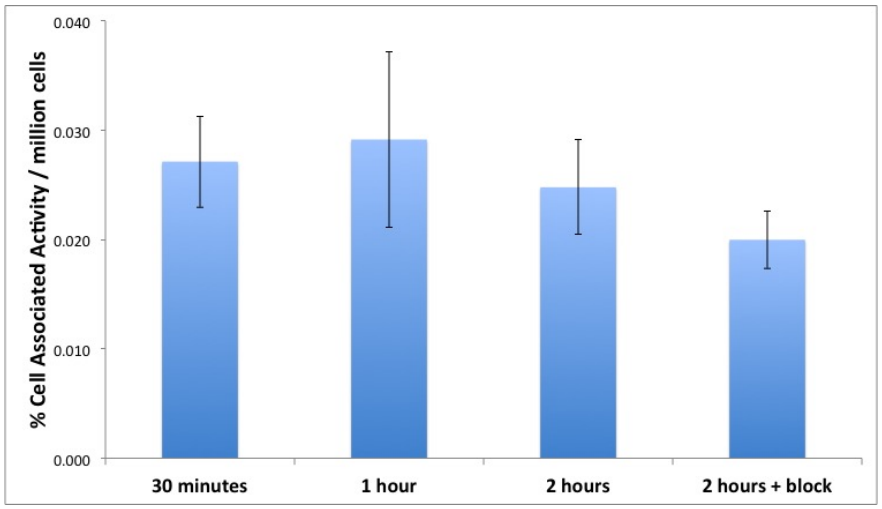

Figure E3: ^{14}C D-Phenylalanine uptake in macrophages

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