OXFORD JOURNAL
OF STUDENT
SCHOLARSHIP

Small Molecule Rescue of GLUT1DS

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ABSTRACT

GLUT1DS is a disease in the body that affects the human Glucose Transporter 1 protein (hGLUT1), it involves a missense mutation in the SLC2A1 gene and as a result one of the amino acids in the GLUT1 protein is translated incorrectly causing the structure to be misfolded and dysfunctional. GLUT1 transports glucose into the brain and acts as the primary gate of glucose into the brain. It controls the energy of the brain and thus it has a vital role in the function and development of the brain. As a result glucose is unable to be shuttled into through the blood brain barrier and causes a series of energy related failures in the brain of developing infants, paroxysms, reduced development of microvasculature, ataxia, epilepsy, and intellectual disability, GLUT1DS is be a disease that can greatly lower the quality of life of those afflicted. As a result the study aims to find a small molecule that can help address this deficient structure by acting as a structural stabilizer, restoring function, and thus alleviating symptoms. In order to accomplish this, a virtual screening software was used in order to simulate the interactions of molecules with each other. After compiling a library of compounds that could potentially be orally consumed by humans, the software simulated how these compounds would interact with a mutated form of the GLUT1 protein. The simulation showed how well each of the proteins bonded to the ligands, and thus it showed affinity. Then in order to validate this, the ligand that was identified sounded most favorably to the protein was chosen as a candidate. This candidate was a flavonoid known as glycycoumarin

Rather than using GLUT1 a structural homolog found in bacteria known as xylE was used instead. The bacteria were then put into multiple different petri dishes that had a nutrientless agar (attempt 1), and a nutrient rich agar (attempt 2) each was filled with xylose as a source of carbon. A portion of the petri dishes were put under a stressor, such as heat, PH, or under UV light. These stressors acted to simulate the mutations that GLUT1 faces. Then of this portion under stress, glycycoumarin was placed into another portion. Finally by counting how many E coli resided in each dish the effectiveness of the glycycoumarin in fixing the stressed xylE was found. If the amount of stressed dishes with glycycoumarin had a significant increase in the amount of E Coli then it can be said that the glycycoumarin was able to aid the xylE in consuming xylose.

It was found that the glycycoumarin was not able to make a significant difference in the survival of E Coli. The study also lacked rigor and scale meaning that a strong conclusion about glycycoumarin's effect on GLUT1 or XylE was not proven in this study. However further investigation that more directly explores

the interaction between glycycoumarin and GLUT1 without needing to use XylE as a representative of GLUT1 that can directly measure the function of GLUT1 are recommended.

INTRODUCTION

The brain runs on glucose, it requires a continuous supply of the sugar in order to function properly. It consumes around 20% of glucose derived energy[1]. Neurons and glial cells all utilize glucose as a primary energy course. But due to the blood brain barrier of the brain it is difficult for glucose to freely enter the brain. And as a result the brain developed a specialized protein known as hGLUT1(Human glucose transporter 1)(PDB:4PYP) to act as a facilitated diffusion protein for glucose through the blood brain barrier[2]. It is an integral, monogenic, protein composed of 12 transmembrane helices that functions as a transporter for glucose[(figure 1), 3]. GLUT1 primarily is located in the brain, as well as this there are also many other types of glucose transporter (GLUT1-4), that belong to the same genomic SLC2 family located in other areas of the brain and body[3]. This protein is integral to the brain, making up the majority of the glucose transporters in the blood brain barrier. It is a very prevalent and significant part of the function of both the brain and subsequently the human body. As a result a failure in GLUT1 can cause widespread damage to the brain and cause major failures in the body. This condition is known as GLUT1 Deficiency syndrome, or GLUT1DS.

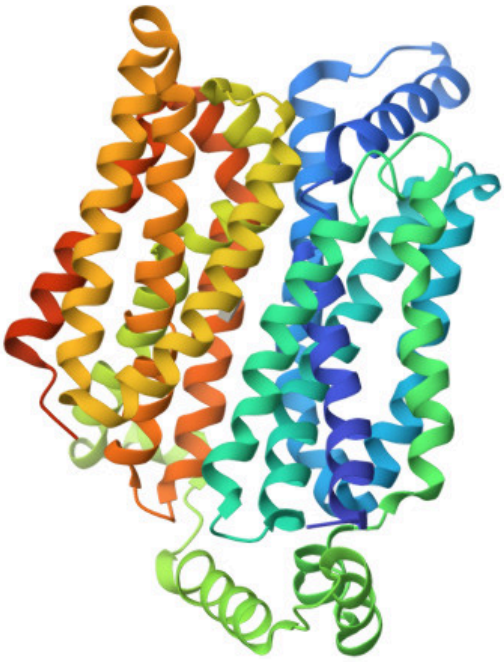


Figure 1: Crystal Structure of GLUT1 protein. It has 12 helices and has 2 conformations, inward where the helices shuttle out glucose and outward where GLUT1 open up to receive glucose[4].

GLUT1DS is a disease in the body that affects the human Glucose Transporter 1 protein (hGLUT1), it involves a missense mutation in the SLC2A1 gene and as a result one of the amino acids in the GLUT1 protein is translated incorrectly[5]. The function of the GLUT1 protein is to ferry glucose across the blood brain barrier, and this missense mutation causes the GLUT1 protein to function at a lower level, aggregate together, or be trapped in the endoplasmic reticulum[5]. As a result glucose is unable to be shuttled into through the blood brain barrier and causes a series of energy related failures in the brain of developing infants, paroxysms, reduced development of microvasculature [6], ataxia[(Figure 2), 7], epilepsy[8], and intellectual disability [7]. These manifestations of the disease occur due to the lack of energy the brain receives, causing a lack of growth and a lack of proper neuron formation. These diseases go on to affect people in their adult lives until they die. As a result GLUT1DS can be a disease that can greatly lower the quality of life of those afflicted. It has been shown to stabilize through adolescence, with the rate seizures dropping, however it is also accompanied with increasing or new levels of paroxysmal movement, such as exercise or stress induced dyskinesia occurring during and after the adolescent stage of life[9].

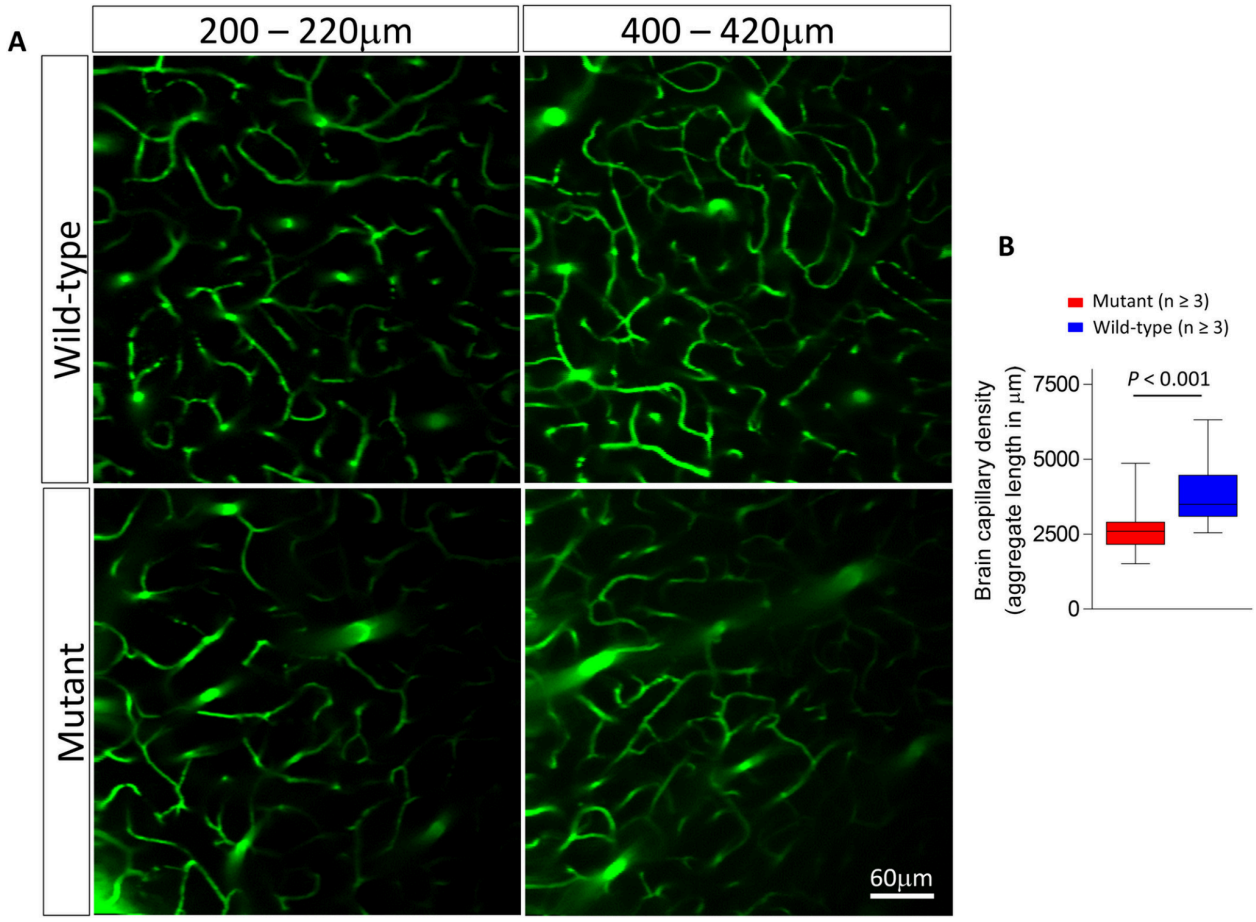


Figure 2:People shown to have the mutated form of GLUT1 have a lower amount of microvasculature in the brain, with smaller and less dense capillaries[11].

Because the disease occurs due to a mutation in the genome, the disease is congenital. It primarily affects children, and it manifests as repeated or paroxysmal eye movements, microcephaly, and a reduced level of glucose in the Cerebro Spinal Fluid or CSF[7]. 90% of GLUT1DS cases are either autosomal dominant or de novo[7]. GLUT1DS is a rare disease with Lopez et al. reporting that GLUT1DS has a prevalence of 1.65-2.22 out of 100,000 births. GLUT1DS occurs due to a variety of mutations in the SLC2A1 gene[10] It has been shown to be mainly a result of missense, frameshift, nonsense, and splice site mutations[11]. These mutations cause changes in the structure of the protein, preventing it from properly ferrying glucose into the brain.

Many treatments have been developed to alleviate the burden of GLUT1DS.The primary treatment for GLUT1DS is Ketogenic Diet Therapy or KDT [12]. Ketogenic diet therapy is a diet that primarily relies on high levels of fat and minimal levels of carbohydrates[(figure 3), 13]. This diet limits the amount of glucose that the body utilizes and instead forces the body to utilize fats instead. The lack of glucose forces the brain to start burning fat instead of carbohydrate stores, this is known as ketosis[14]. Ketosis is where the body uses fat as a primary energy source instead of carbohydrates. KDT works because of the functions of the liver in converting lipids, or fats, into ketone bodies which are eventually metabolized by the brain and rest of the body[15]. KDT has a pre-established clinical use in treating patients with drug-resistant epilepsy, having shown to drastically improve quality of life, reducing the amount of seizures in select populations of patients by 90%[16]. Due to the efficacy of KDT in treating epilepsy it has also been applied to GLUT1DS. KDT is the cornerstone of treatment for GLUT1DS, being shown to improve quality of life, alleviating many symptoms of the disease[17]. KDT accomplishes this by providing the brain with an energy source that does not need to be transported by GLUT1. Because the keto diet shifts the body's primary energy source into fats rather than glucose, the brain's energy source is changed as well. Rather than relying on glucose it relies on ketone bodies[18]. By using ketones instead of glucose the brain no longer requires GLUT1 to ferry its primary energy source. The brain instead relies on the Monocarboxylic Transporter 1 system, or MCT1 system which replaces GLUT1, by using an alternative source of energy and transport that properly functions the brain is able to utilize energy properly[19] Because of the proper usage of energy the energetic related effects of GLUT1Ds are mitigated. Similar to its effect on patients with epilepsy, KDT has been shown to decrease the rate of seizure and paroxysmal movements in patients with GLUT1DS[20]. The therapeutic strategy maintains that the diet is continued throughout childhood and adolescence in order to preserve the developmental stages of the brain[19]. There have been alternatives to this diet as well, including the modified Atkins diet which provides a more flexible alternative to KDT, which has been shown to decrease the amount of seizure but is unable to affect the rate of paroxysmal movement[21].

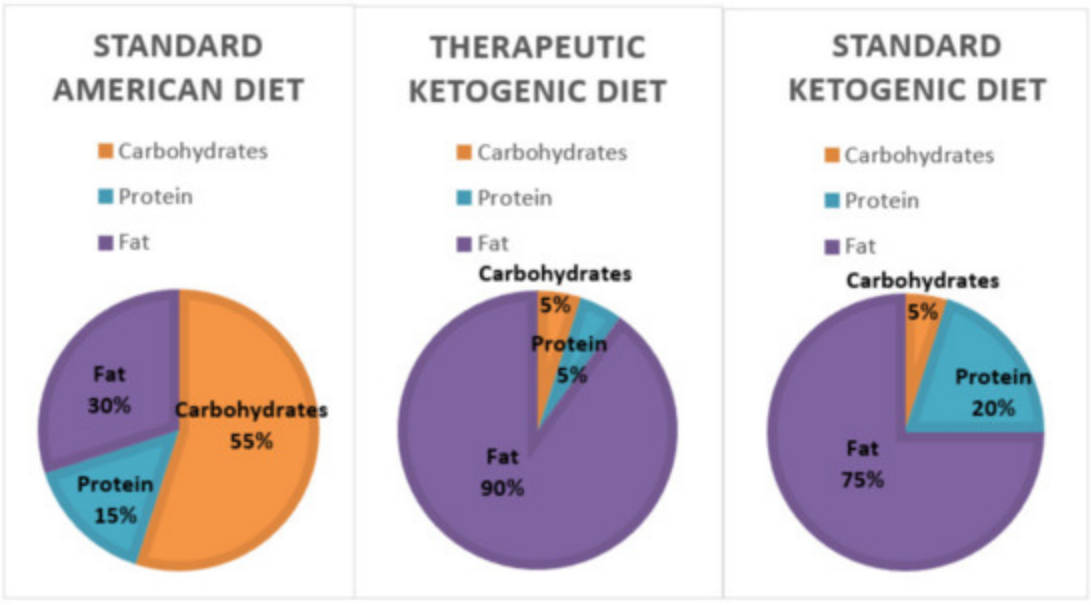


Figure 3: Representation of the ketogenic diets in comparison to standard diets. Ketogenic severely limits the amount of carbohydrates, therapeutic diets have an even higher emphasis on fats than the standard diet[22].

However despite the benefits of KDT, there are also potential drawbacks that have been observed with its long-term use. For instance, the usage of a ketogenic diet has shown to be correlated with an increase in the consumption of saturated fats in some patients[23]. The diet generally does not discriminate between the types of fat consumed during the diet, it emphasizes the restriction of carbohydrates and an increase in the amount of fats, because there is little discrimination in what type of fat is consumed during the course of the diet there is chance that the level of saturated fat in the body increases. Because of this increase in the consumption of saturated fats, there is also a chance that KDT can influence the level of low density lipoproteins in the blood[24]. Higher levels of low density lipoproteins have been shown to increase risk of coronary heart disease[25] as well as atherosclerosis[26]. Because KDT restricts carbohydrates dietary planning is a necessity when it comes to ensuring that all nutrients are being consumed in the right amounts, as nutrients commonly found in carbohydrates are no longer available [27]. Finally ketogenic diets have also been shown to be correlated with an increased risk of NAFLD(Figure 5), or non-alcoholic fatty liver disease. These metabolic, nutritional and clinical side effects of KDT should be evaluated when prescribing to GLUT1Ds patients, as some patients may require lifelong treatment.

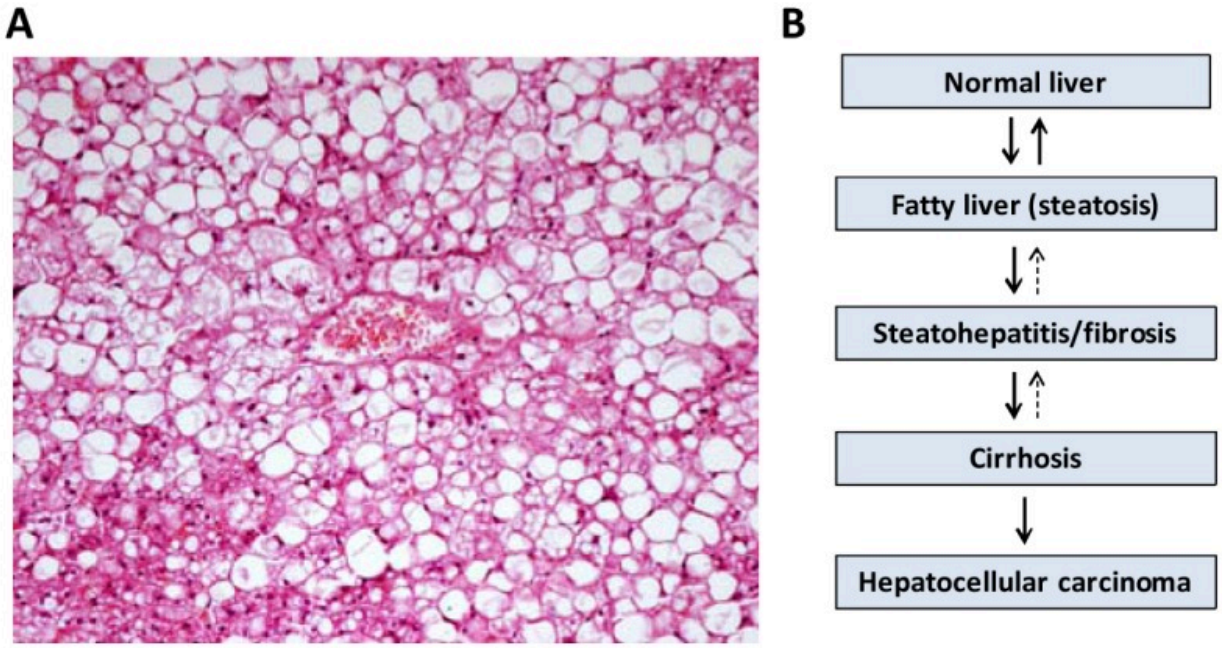


Figure 4: A slide of liver tissue taken from someone with a fatty liver, the cells are inundated with fat (white spots) and as a result the cells fall into disfunction, eventually becoming cancerous causing hepatocellular carcinoma[28].

Because of the problems associated with the current standard of treatment for GLUT1DS there has been an emerging field of science that is developing alternative treatments. This field is known as pharmacochaperone research, or small molecule rescue. Pharmacochaperone are small molecules developed with the specific purpose of binding to a misfolded or mutated protein in order to stabilize its form and eventually allow it to function properly[29], or by promoting a higher expression and translocation of a protein. They are small molecules derived or optimized from preexisting allosteric inhibitors, agonists, antagonists, and other small molecules that have already been shown to interact with the protein[30]. They act as a stabiliser and promoter to misfolded proteins; when a protein misfolds, its structure prevents it from carrying out its function. Pharmacochaperones bind to the protein and by doing so it can act as a substitute for the intramolecular interactions that occur in a protein, restoring its function[31]. Other mechanisms of rescuing a protein are also being explored such as using small molecules, present in the related processes in the synthesis and function of the protein, to induce greater translocation or expression[32], causing a higher amount of partially functioning proteins which offsets potential deficiencies in protein function. Structurally stabilizing pharmacochaperones are already an emerging part of the scientific landscape, currently they are being used as a treatment for cystic fibrosis, where certain small molecules allow the CFTR mutant F508del mutant to pass through the ER and subsequently be packaged for export and eventual usage[33]. This same approach is also being used on many other diseases where the structure of the protein is changed or mutated in some way such as this approach can also be applied to GLUT1DS as well, it is a disease similar to cystic fibrosis in that a protein is misfolded or

mutated in such a way that prevents its function or export from the ER. As a result there is strong evidence to suggest that GLUT1DS patients can benefit from being treated with pharmacochaperones.

Because GLUT1DS causes mutations that primarily affect its structure, GLUT1DS can be a good candidate for pharmacochaperone rescue. Missense mutations are point mutations in the genome that affect the protein's primary structure. They affect individual amino acids of the protein and by doing so they affect its structure[34]. They are also one of the most common types of mutation in GLUT1DS, they are also one of the most common forms of variant, according to Zhang Et al, the missense variants have been shown to make up 46 out 69 mutations in select cohorts[11]. As a result the problems GLUT1DS primarily faces are ones related to structure and function, rather than large scale deletions or mutations like those that occur with nonsense[35] and frameshift mutations[36]. As a result only small corrections need to be made to GLUT1 in order to restore it to its usual function and purpose. So if a pharmacochaperone is found that can rescue the GLUT1DS' structural deficiencies its function will return and symptoms will be alleviated.

There is currently research being done in finding small molecules to rescue GLUT1 function. Alpha Lipoic Acid is being considered as a candidate to alleviate GLUT1DS, this is because it has been shown to be a promoter in the export and translocation of GLUT1 and 4[37], meaning that it could potentially shuttle mutated GLUT1 proteins that have nondeleterious mutations rather than having them be trapped inside of the cytoplasm. Other forms of rescue, such as by stabilizing the activity of GLUT1, using small molecules to stabilize its structures, rather than its expression, will allow improvements in patient outcomes[12]. This form of research is currently ongoing, finding small molecules that have a high specificity and capacity to act as a stabilizing presence to the mutated GLUT1. Such as by finding relatives of the GLUT1 inhibiting molecule TXNIP which have demonstrated a rescuing effect on the structure of the protein accomplished by using interaction databases to find molecules that are related to GLUT1DS that can be modified to structurally fix the protein[38].

Pharmacochaperones will be a beneficial alternative treatment of GLUT1Ds due to the way that it can alleviate the cost related and health consequences of the KDT. KDT lasts for a very long time, often requiring continuous usage for 5 or more years[39]. KDT is also substantially more expensive than normal diets[40], as a result this can potentially cause financial strain due to the longevity and higher price of treatment. As well as this KDT's nutritional variability is also strained[26], this coupled with its chances of causing cardiovascular problems[23, 24, 25] can make KDT an unfeasible treatment option for those with preexisting conditions and financial burdens. However pharmacochaperones can alleviate this problem, they offer a promising alternative cost wise, being inexpensive to manufacture[41] and currently are enjoying increasing levels of research and advancement. They will allow GLUT1DS patients to consume normal and more diverse diets while remaining free from symptoms due to the restored and rescued functionality of the small-molecule-stabilized protein. The goal of this study is to find a small molecule that can stabilize GLUT1's mutated structure and restore its functionality.

MATERIAL AND METHODS:

This study was split into 3 parts: Finding a commercially available small molecule ligand that might be able to bind to a mutated GLUT1 protein, which was found by using the virtual screening software PyRx[42], by exposing E.Coli's XylE protein to a variety of stressors as a representation of GLUT1DS (this is because it is used as a structural homolog to model GLUT1 due to its high structural similarity making it a preliminary model of a mutated GLUT1 protein as long as GLUT1's mutations are properly mapped onto XylE), and then exposing the XylE to the sugar xylose and the chosen small-molecule and finding how coverage differs between the stressed bacteria that is and isn't exposed to the small molecule.

Virtual Screening:

The goal of this analysis was to model a mutation of GLUT1DS so that a group of ligands be modeled and docked to the mutated GLUT1DS. Various literature sources[42, 43, 44] were used to identify where the most common mutation sites for GLUT1DS were. After downloading a file from the PDB, the 3d model then needed to be mutated. This was done by using Mutation Explorer[45], a web-based server, the mutations were able to be modelled and converted into multiple separate PDB files. Then each of the mutated proteins were analyzed by comparing all of the different conformational changes the protein underwent relative to the wild-type protein. After comparing all of the proteins, it was found that a series of residues, from 200-270, were consistently amongst all of the mutations. That specific pocket was shown to have an unstable conformation based on their energies indicating weaker or unstable structures, leading to a conformational change from residue 200-270. Finally these mutated proteins were exported and taken into the docking preparation stage.

This was done by using Autodock tools[46] to clean the proteins, remove water, remove any hetero atoms (non amino-acids), and add the proper charges so that the proteins would be ready for docking. The next step was to find a library of ligands and dock them to protein. This was done by finding interactors of the GLUT1 proteins, specifically inhibitors. As inhibitors have a pre-established method of interaction with the protein, and as a result there is a possibility that their derivatives could be considered as potential candidates for interaction with GLUT1. The compounds were filtered using Pubchem's database using the guidelines for drug discovery as detailed in Lipinski's rule of 5 that determines whether or not a drug will be safe for consumption by humans[47]. The proteins and the library of ligands were then loaded into the virtual screening software, PyRx[48]. The ligands were then screened, this was done by selecting the mutations identified beforehand that were consistent among all of the mutated proteins (200-270) and by doing so a window was made where the ligands would be allowed to bind. PyRx then applied the ligands to the chosen window of residues, after running through multiple conformations of binding PyRx had an output of different binding energies for each ligand screened. This process identified molecules with favorable binding energies as potential candidates for further investigation in the in-vitro portion. The docking did not establish the effect of the ligands but whether or not they can bind to GLUT1 reliably.

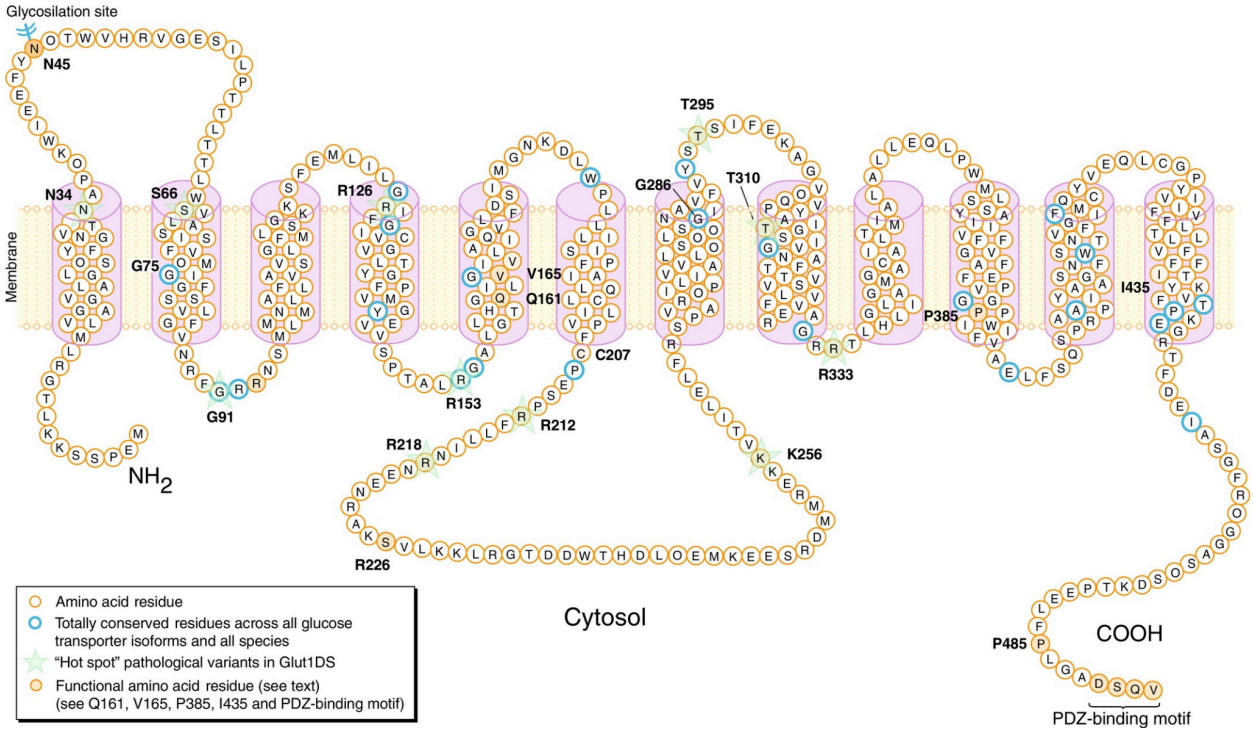


Figure 5: A map of common hotspot mutations in GLUT1DS, it shows the different amino acids and where they reside[5].

Expression model:

XylE is a protein in E.Coli that transports the 5-carbon sugar xylose, it is a symporter that uses the energy from H+ diffusion to power the transportation of xylose[43]. Despite mechanically acting in different ways it has been shown to be a protein that has a high degree of similarity with GLUT1 of 63% as well as having 12 identical binding sites to GLUT1[49]. XylE has widespread usage as a structural homolog of GLUT1, being used as a way to model if certain small molecules can interact with GLUT1 as well as to solve the different conformations GLUT1 can adopt[4, 50]. As a result this protein was chosen to represent GLUT1. It is not meant to be an exact representation, XylE is a symporter while GLUT1 is a facilitated diffusion transport protein, as a result there are a variety of differences, so this protein was chosen to represent GLUT1 in this preliminary stage of investigation.

In order to have a broad model of the loss of function that GLUT1 goes through under the mutations of GLUT1DS, the bacteria was exposed to slight levels of stresses that could potentially cause a decrease in protein function or structural deficiency. These included heat, acidity, alkalinity, and UV exposure. While these stressors were not precisely targeting the XylE protein their usage was meant to affect the entirety of

the bacteria's proteome, and, by proxy, affect the structure and function of the XylE molecule as well. Broadly disrupting the stability and structure of the protein instead of creating a specific mutation.

Because these stressors can cause changes in protein stability and function they may be able to affect the surface protein XylE as well. These stressors would more specifically affect the whole bacteria by exposing the proteins to suboptimal conditions affecting them in a negative manner. The potentially altered protein could then possibly be acted upon by glycycoumarin. This approach did not establish if XylE directly corrected the structure but created a way to observe an association between exposure to the compound and the growth of the bacteria under experimental conditions.

In-Vitro Testing:

This was done by creating 16 petri dishes. All had xylose as the only primary source of nutrition, the agar the bacteria was placed on had no nutrients. This was done in order to ensure that the survival of E Coli would primarily depend on the XylE's xylose uptake function and not on consuming any other nutrient.

One petri dish continued regular E coli, 3 contained E coli under stress, and of the 3 there would be one that was also exposed to glycycoumarin. After allowing the E coli to grow for a few days the petri dishes were then analyzed to find the differences in each dish. Specifically they were analyzed in order to find which petri dish contained more colonies. The changes in bacterial growth may be influenced by changes in xylose uptake. They can not directly measure the amount of xylose transported by the XylE or the restoration of function nor can this study fully isolate the function of XylE uptake. These steps were repeated twice between 2 trials.

Control/General prep:

I added 4 grams of agar to 200 ml of water. In trial 1 I added 0.9 grams of xylose to the agar solution and poured each plate until the bottom of the dish was fully covered. In the 2nd trial I added 2 grams of xylose to the agar, while maintaining the agar-water ratio. Finally, for each dish that needed glycycoumarin I added 100ul to the specific dishes, the glycycoumarin was suspended in the solution with a concentration of 2.5 ug/ml.

Out of the 16 dishes, 4 were chosen as controls, with no exposure to any stressor. The remaining 12 were split into 4 groups of 3 plates. With all 3 in each group being exposed to their designated stressor, and only one of that 3 was exposed to glycycoumarin as well.

UV (n=3, only one plate contained glycycoumarin):

In the first trial the UV dishes were exposed to 15 seconds of nm 312 radiation.
In the second trial the dishes were exposed to 30 seconds of 254 nm.

Heat (n=3, only one plate contained glycycoumarin):

In both trials the E Coli was exposed to 5 minutes of 45 celsius underneath a heat lamp. The temperature was measured using a thermometer.

Acid (n=3, only one plate contained glycycoumarin):

The acidic environment had a PH of 6. This was achieved by titrating 1.0 m hydrochloric acid with sodium hydroxide to get a pH of 6. In the first trial acid was pipetted on top of the inoculated bacteria immediately after plating, with a volume of 2 ml.

In the 2nd trial the acid was incorporated directly into the agar before it was autoclaved. With 5 ml being added to 75 ml of agar and water mixture.

Base (n=3, only one plate contained glycycoumarin):

The basic environment had a PH of 9. This was achieved by titrating ammonia with hydrochloric acid to get a pH of 9. In the first trial 1 ml of base was pipetted on top of the agar dish after the inoculation happened. In the 2nd trial the bases were added into the agar and then autoclaved.

2nd Attempt:

In this second attempt the only variation was the addition of a different form of agar. This version of the agar included beef broth, and peptone to fulfill the other nutritional needs of the bacteria.

In this attempt in order to quantify the amount of bacteria grown a colony counting software was used, imageJ helped calculate the percent coverage of the bacteria across the plate. In order to ensure that the proper amount of bacteria was picked up the images of the plates were processed in various ways, they were cropped so the window captured only the plate, smoothed over using the software, had the lighting corrected for, with the contrast increased, and converted into gray scale in order to analyze them.

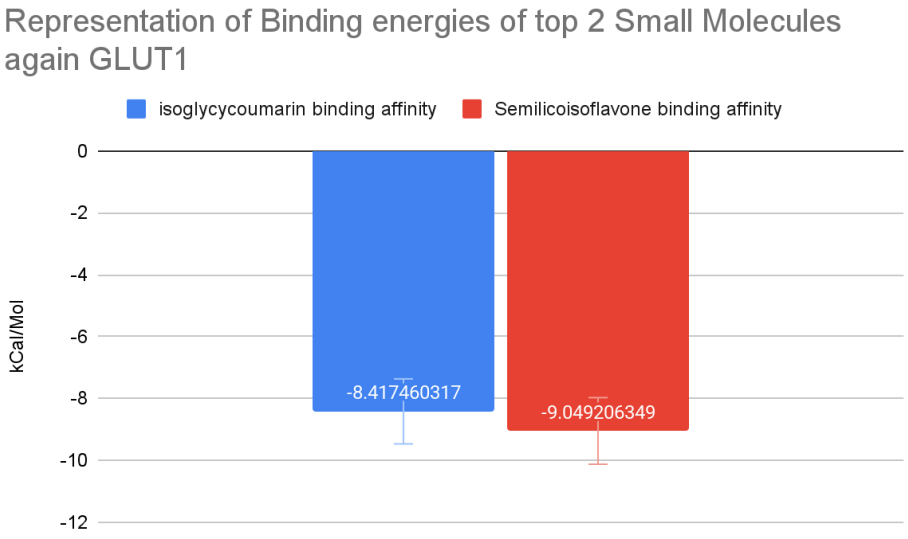
Because the make up for the agar was different in the 2 attempts they are treated as separate sets of data. They are not full replicates.

RESULTS

In Silico Results:

Based on the literature search it was found that flavonoids had a strong affinity to GLUT1[52, 53, 54]. Then using a database from Pubchem, a library of 151 compounds was gathered[55]. After running these proteins through the screening software it was found that certain compounds had a higher predicted affinity for the mutated proteins than any of the others. Specifically Semilicoisoflavone and Isoglycycoumarin. These two small molecules had the most favorable binding energies, with Semilicoisoflavone having predicted binding energies ranging from -8.2 to -10.4 kCal/mol across each of the mutated proteins, the average kcal/mol of this small molecule was -9.049+/-1.076 kcal/mol. While Isoglycycoumarin had an affinity

ranging from -7.5 to -9.6 kCal/mol the average kcal/mol of this small molecule was -8.417+/-1.051 kcal/mol(Graph 1). These binding affinities were all more favorable than any of the other compounds screened.



Graph 1: Graph of the average binding energies of the 2 different small compounds.

It was also found that Isoglycycoumarin is expensive and difficult to obtain, similarly with Semilicoisoflavone. It was then found that glycycoumarin has a very similar structure to isoglycycoumarin and is easier to purchase [56,57]. As a result glycycoumarin was chosen as a small molecule ligand instead of isoglycycoumarin or Semilicoisoflavone, due to the fact that it would be a feasible compound to test. Furthermore it was run through the screening software again in order to further test its predicted binding affinity to the GLUT1 protein, and it was found that it had binding energies comparable to isoglycycoumarin, with binding energies ranging from -8.5 to -9.5 kcal/mol. It had a favorable binding energy so this small molecule was chosen to be the potential candidate for interaction with GLUT1 in order to observe the effect it has on GLUT1 or its analog.

Furthermore in order to further validate the results of screening and ensure that these results would carry over to XylE screening was also done on the XylE compound, using similar mutations, and it was found the glycycoumarin has a similar binding affinity to the XylE as it had with the GLUT1. This supported glycycoumarin as a potential candidate as it displayed favorable predicted binding energies to GLUT1 and similarly mutated XylE.

Expected results:

In each of the differently stressed petri dishes it is expected to find different results in the amount of E coli that survive. This is because survival and growth of bacteria is dependent on whether the bacteria can

uptake and transport the necessary amount of nutrients. The trials were designed in order to isolate the function of XylE protein by making xylose the primary source of carbon. This would make it so that XylE's transport of xylose would become much more important to the survival and growth of E Coli as its main transporter of carbon. So it was assumed that if a bacteria has a dysfunctional XylE protein then it will not uptake xylose and presumably have a smaller amount of colonies than the normal E coli.

As a result, if an increase in bacterial growth in the plates exposed to glycycoumarin were observed, it would be consistent with the assumption that glycycoumarin might improve the function of a dysfunctional XylE, which would make it a candidate for further investigation to see if it can correct the structure of GLUT1.

In vitro results:

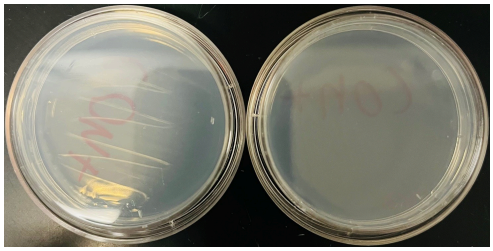
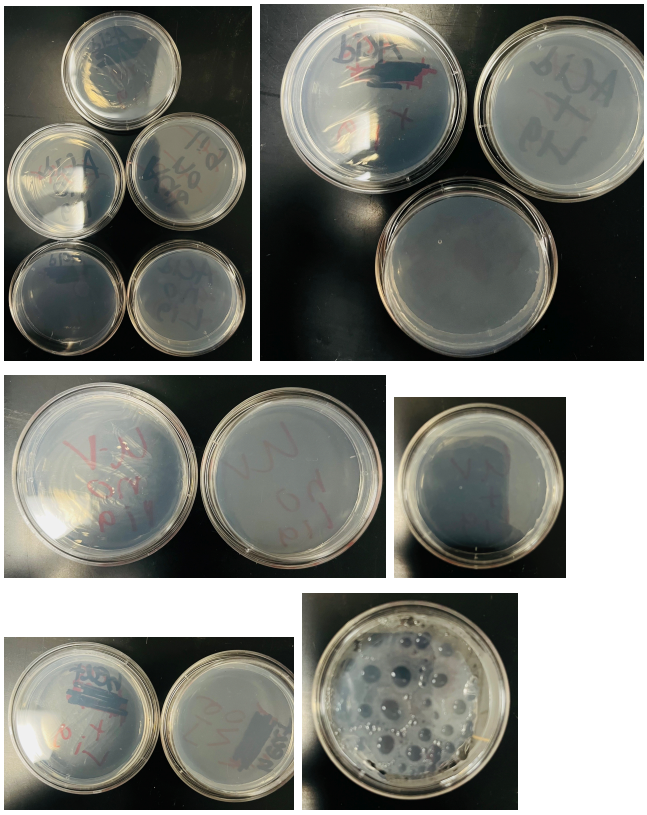


Figure 6: These were the control dishes. There are no discrete colonies in either dish.



Figures 7-12 (from left to right)
First row: Acidic environment with no glycycoumarin added, acidic environment with glycycoumarin added
Second row:UV without glycycoumarin, UV with glycycoumarin
3rd row: Heat exposure without glycycoumarin, Heat exposed with glycycoumarin(the water droplets are from condensation)



Figure 13: The 2 left most dishes are the dishes without a ligand but are exposed to acid. The rightmost dish has the ligand in the dish along with the acid. The image has increased contrast in order to aid visibility.

13 dishes in trial 1 exhibited no growth, and all 16 dishes in trial 2 exhibited no significant growth of bacteria. There were no discrete colonies in the dishes (figure 6-12). This included the control dishes as well. Because of this no data can be extracted from the research. Within these dishes, there are no quantitative observations to be made for either trial. As well as this the agar in the basic dishes was completely degraded so no plating was able to occur.

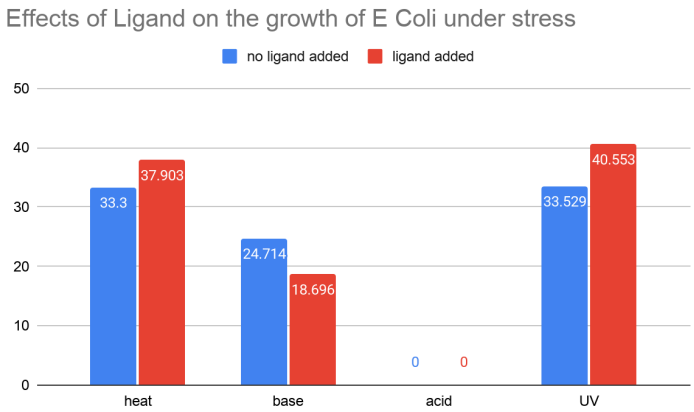
However there was one group of dishes that had growth displayed. In the first trial the acid stress test exhibited results. The non-glycycoumarin acidic dishes were observed to have a smaller amount of colonies growing in them than the glycycoumarin-exposed dish (Figure 13). Because of the sparseness of the data there was no T-test that could check for statistical significance. However in order to quantify this observation a colony counting method was used. The dish was split into 4 quadrants and the amount of colonies in 1 quadrant was counted and then multiplied by 4, this helped serve as an approximation to the amount of bacteria colonies that grew in the total plate. Without ligands one dish had 80 colonies and another had 102 colonies, this meant they had an average of 91+/-15.56 colonies. The dish with the ligand was found to have 304 colonies, there is no average as only 1 dish had the ligands and acid.

2nd Attempt

In this study it was found that there was visible growth of bacteria. However due to the high growth rate there was confluent growth, and no discrete colonies to be counted. So instead an image analysis

software: imageJ [58] was used instead. This software was used to estimate the percent of the plate that the bacteria streaks covered. The control group had an average percent coverage of 27.933% +/- 1.841% (n=4). For the group exposed to acid there was no growth (n=4). The group exposed to the bases had minimal growth in 2 plates, with the group exposed to the base without ligands having a mean of 24.174% coverage (n=2), and the bases and ligand exposed group had a mean of 18.696% coverage of confluent growth (n=2). The group exposed to heat without ligands had an average coverage of 33.300% +/- 1.413% (n=2), the group with ligands had an average coverage of 37.903% +/- 2.772% (n=2). The group exposed to the UV without ligands had an average coverage of 33.529% +/- 2.201% (n=2) the group with UV and ligands had a mean of 40.553% +/- 4.588% (n=2). (Figures 14-21 contain all images of the plates).

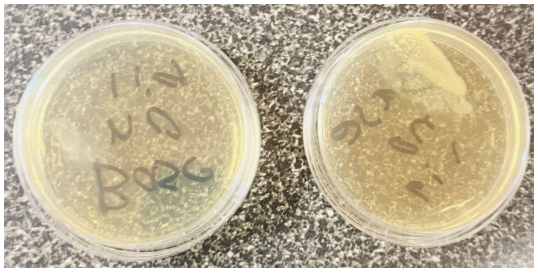
It was found that the groups with an addition of ligands had a higher percentage of plate coverage in 2 of the 3 groups with visible growth (heat and UV). There was an increase in coverage of 4% with the addition of ligands to the group exposed to heat. In the group exposed to UV there was an increase in coverage of ~7% with the addition of the ligand. In the group with the basic environment the group with the ligands had a decrease of ~6% in terms of coverage. However, because all of the data covers multiple different groups and the amount of groups in each category (n=2) is too low to be statistically valid, no ANOVA or T-test was run. Therefore the differences in plate coverage should be interpreted as observations rather than conclusive evidence of an effect from glycycoumarin on the function of XylE.

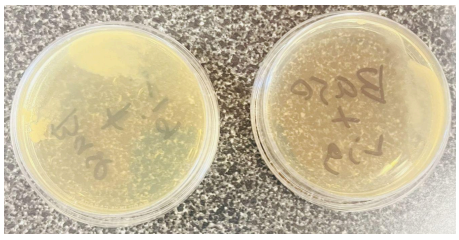


Graph 2: A graphical representation of the average percent of the plate that is covered by the bacteria. One group represents the stress the bacteria was exposed to, without having any ligand exposure and the red group shows the effect of the added ligand.



Figure 14: Control, no ligand or stress was added to the bacteria.


Figure15: Bases without ligand added


Figure 16: Bases with ligand added

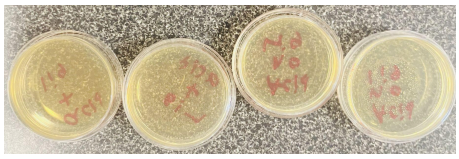

Figure 17: Acids with and without ligand added


Figure 18: Heat without ligand added

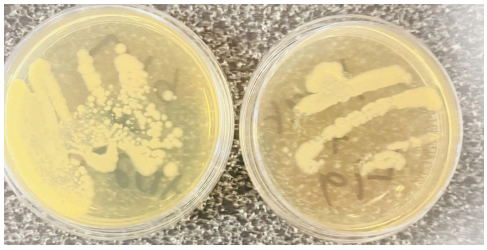
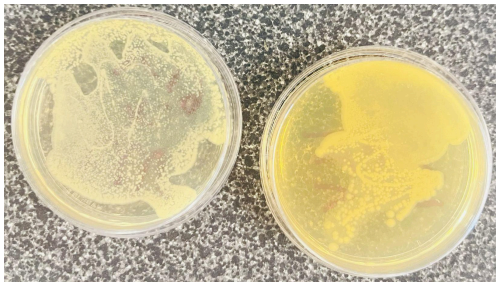

Figure 19: Heat and lig Added


Figure 20: UV without Ligand added


Figure 21: UV with ligand added

Conclusion/Error analysis/limits/future of drug discovery

CONCLUSION

The goal of this study was to find a ligand/small molecule through docking that reliably and specifically binds to a GLUT1DS affected GLUT1, or a similar protein, like XylE. The secondary goal was to find out if glycycoumarin could have an observed positive effect on bacterial growth under conditions that broadly disrupted the function of XylE.

In terms of docking and in-silico results, glycycoumarin was a potential candidate for research. It was cheaper to obtain and it was an isomer of one of the molecules with the highest affinity. As a result it was chosen. But because of the lack of measurable results the null hypothesis was neither proven nor rejected. As a result the effect of glycycoumarin on the XylE and its representativeness of the GLUT1 protein can not be verifiably proven by these results.

However there were some trials or groups that had observed differences. The acid group plate with glycycoumarin in trial 1 had more bacterial growth than the untreated acid-stressed plates. But because of the limited number of plates in the acid group (n=3) statistical testing cannot be done nor can significance be established.

In the second attempt a majority of the groups under stress had an overall increase in the percent coverage. Though these results are weak because they can not be statistically tested due to a small number of groups being tested. However these results are only observations and do not support that glycycoumarin can positively affect the function of XylE or if they have any relevance to GLUT1. So further investigation must be done in order to determine if glycycoumarin can even interact with GLUT1 favorably and if glycocoumarin can have a positive effect on a GLUT1DS affected version of GLUT1.

The results were also partially unexpected. The stressed only groups had more growth in bacteria than the control groups. This was unexpected because the stresses were meant to cause the XylE protein to have disrupted cellular and protein stability, potentially reducing XylE function along with the surrounding

parts of the bacteria; this was expected to cause a smaller amount of bacterial growth. But this was contradicted in the study, the stressed plates had more growth than the control plates. This could be explained with the inconsistent streaking burning the plating phase or possible adaptation of the bacteria during the growth period.

In conclusion, these results do not suggest that glycycoumarin can positively affect a degraded version of XylE. The experiments as a whole also do not establish if the potential effects of glycycoumarin on XylE would be present in GLUT1. However there were observed associations between increased bacterial coverage and the presence of glycycoumarin, specifically in dishes with sufficient nutrients with xylose as the only form of carbon. Further investigation must be done in order to see if glycycoumarin can correct the structure of a mutated GLUT1 and restore its function. Experiments that could do so would involve directly observing how GLUT1 interacts with glycycoumarin, through precise simulations that model how the protein and ligand changes its shape after it interacts with glycycoumarin; an experiment that has an organism with a specific mutated version of GLUT1 (mimicking the mutations that GLUT1DS has) which then measures how much glucose it can uptake before and after exposure to glycycoumarin would also help validate the effects of glycycoumarin and whether or not it has an effect on the function of a mutated GLUT1. Experiments that more directly explore the interaction between glycycoumarin and GLUT1 without needing to use XylE as a representative of GLUT1 that can directly measure the function of GLUT1 are recommended.

Error Analysis (Attempt 1):

The study had a variety of problems. The mistakes primarily reside in the in-vitro portion of the lab. The nutrient broth the E coli was subcultured in was left in the incubator after inoculation for 5 days, this meant that there be a reduced amount of bacteria in the broth [59], due to this depletion the E Coli ended up in stationary phase where growth is minimal and eventually after this phase there would be eventual bacterial death. Because of how long the nutrient broth was kept in the incubator there is a chance that the amount of E Coli picked up by the loop when inoculation is minimal. Another error lies in the xylose mixed in with the acid/base, for both trials the concentration of xylose could have been insufficient for the bacteria to live off of, because of this the bacteria levels in all of the dishes would be extremely low or unable to be viewed. The next problem lies in the inoculation, during inoculation the loop was not allowed to fully cooldown and as a result when it was dipped into the broth it might have killed the bacteria that were attached to it, so when plated it may have caused a smaller amount of bacteria to be deposited or even no bacteria.

For the acids and bases, the PH could have been too high or too low to allow the bacteria to grow. And because of the stress of both acidity and a lack of proper nutrition inside of the agar the E Coli could have died from the stress. The stressors of heat and UV exposure could also have caused unwanted side effects. Because of the fact that the heat that the bacteria was exposed to was prolonged and only impeded by a thin piece of plastic the E Coli could have been harmed to a greater than needed extent. This principal also applies to the UV, since it was directed to the bacteria at 254 nm this means that it is very effective at killing bacteria, and because of this the 30 seconds that the bacteria was exposed too might have caused

an overly harmful amount of exposure as there was only a small amount of bacteria present, so a small amount of UV would have been enough.

After analysis of the different factors, there is a strong possibility that the lack of growth was caused by improper preparation of the agar/xylose solution, and with inoculation. The control was exposed to nothing but still yielded no bacteria, this means that the bacteria weren't able to survive on the agar. The only process that was common between all of the plates was the agar and the inoculation, and as a result, there is a chance that one or both of these factors influenced the lack of growth. The inoculation is less likely as in trial 2 the inoculation was done very precisely while giving ample time for the loop to cool down and to pick up bacteria.

2nd Attempt errors:

In this trial the agar was replaced with a more nutritionally sufficient agar. With beef broth and peptone which provided nitrogen and phosphorus that allowed the bacteria to grow. But in this trial there were problems as well. The glycycoumarin was ~1 month old and as a result there is a possibility that the compound had broken down. Therefore when it was added to the bacteria groups there would be a chance that the effect it had was not due to the glycycoumarin itself. This meant that there is a possibility that the slight increase in bacteria growth could be due to a broken down version of the ligand. This added more uncertainty into the interpretation of the second attempt. Furthermore when inoculating the bacteria the stresses that were added, were done so in a large quantity. And because of how much has been added, the bacteria were unable to attach onto the surface of the agar, this was the reason why the basic and acidic environment were unable to have any bacterial growth. So, in future experiments lower levels of acid or base additions would be beneficial while still keeping the intended effect.

Furthermore there was an overgrowth of bacteria, this is known as partially confluent growth, within the 24 hour growth period causing a lack of discrete colonies to be found. This was due to allowing the bacteria to grow too much, and for too long. If the bacteria grew for too long there would be too many bacteria causing confluent growth. This could have been solved by plating the bacteria at a later time and returning to observe and collect data earlier. Or rather than streaking, the bacteria could have been plated by pipetting the nutrient broth infused bacteria onto the plate. This would allow the bacteria to be more spread out, and by doing so there is more space between the bacteria allowing the colonies to grow and remain disconnected allowing for more discrete colonies to be present. The final error was the low number of experimental groups, because of this small sample size there aren't many statistical tests to be run which caused the study to be statistically impaired.

Limitations:

The study was very limited in its ability to be extrapolated to humans. While XylE is a very strong structural homolog to GLUT1 [4] it is still varied in multiple ways. It is a symporter that relies on proton movement to fuel transport unlike the GLUT1 protein which relies on a chemical gradient to allow facilitated diffusion to occur. This means that while basic mechanisms are similar the proteins differ in

small yet functionally different ways. Furthermore, the stressors that were applied to the E Coli in hopes to simulate the deficiency that GLUT1 experiences were not very precise. The UV was more likely to affect the DNA of bacteria rather than the protein, the Heat was more likely to cause entire proteome degradation and the acids and bases were at PH that would have caused either: full proteome degradation, or be too weak to cause any actual effect. Finally because E Coli is an entirely different branch of cell type, there were many more ways that the study could have been inaccurate or unable to apply to humans. The prokaryotes could exhibit adaptation and become resistant to the provided stresses, possibly rendering the small molecule's purpose null. And finally the way that the ligand interacts with E Coli could be completely different than in how the ligand could interact with the human blood brain barrier due to environmental differences. The study was heavily limited by a lack of processing power, this lowered the scope of the docking process and thus decreased the specificity of the small molecules. There were also limitations in the lab environment, timing of the experiment was greatly affected due to shipping of materials and the arrival of the weekend. Because of a lack of experience and human error the lab experiment was not the most optimized. The prior mentioned errors also were a result of the mentioned limitations of the lab.

Potential applications in the future:

While this study was inconclusive it is also important to understand the underlying concepts and how they are being applied to the process of drug discovery and research as a whole. My research was an attempt to bring this process to the high school, a way to make this accessible. To find a way to optimize the process of drug discovery and participate and learn about ways that it could be emulated. The identification of candidates in this study was a less high-through put screening method than those of the industry. Rather than doing molecular dynamics simulations, and simulating the environment that the drugs might exist in, my docking simulation was less precise. Rather than reproducing exact mutations, I used blunt stressors in order to accomplish the desired effect.

But at its core the study emulated the principles of the industry of drug research, including the screening procedures and expression models albeit in a smaller and less precise scale. The scope and rigor were limited but the core principles I emulated are still being followed and refined for the past few years [60]. This experiment was inconclusive, and lacking in rigor. It was limited by the unavoidable risks of working in a school laboratory, and it was limited by the inexperience of the researcher, but it can also serve as a guide in miniature. A small-scale representation, limited by safety and financial constraints, of the experimental approaches in drug discovery and how they can be improved in the future.

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