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RESEARCH ARTICLE

Carbonic Anhydrase IX Inhibitors: Finding Potential Therapeutic Cancer Agents Through Virtual Screening

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Cancer is one of the leading causes of death and affects over seven million people each year (American Cancer Society, 2011). Carbonic anhydrase IX (CAIX) is an enzyme that increases the survival of cancer cells and renders treatments ineffective by regulating tumor pH. The purpose of the project was to discover and test novel inhibitors of CAIX that can potentially block tumor growth. A total of 86,228 compounds were virtually screened. The DNA sequence coding for the human carbonic anhydrase IX protein was inserted into a bacterial expression vector and expressed and purified via nickel affinity tag. Spectrophotometric enzyme assays measured the effectiveness of the compounds in vitro. Virtual screening results demonstrated that metoprolol, pentamidine isethionate, and Chembridge compounds #7653639 and #7633756 have predicted drug-likeness and binding strength to the CAIX active site through favorable interactions of Van der Waals forces and hydrogen bonds. In the enzyme assays, the mean velocities decreased as the tested concentrations of the compounds increased from 0.5 μM to 100 μM. Overall, the selected compounds showed promise. Successful inhibition of CAIX in vitro affirms the importance of virtual screening and may contribute to the discovery of novel therapeutic cancer agents.

INTRODUCTION

About 85% of all cancers are carcinomas, which are tumors of the epithelial tissue (Woolf, 2011), including some of the most common and the most fatal types of cancer. Carbonic anhydrase IX (CAIX, PDB ID: 3IAI) is an enzyme expressed in carcinomas (Alterio, 2009). It is part of the carbonic anhydrase (CA) family. These enzymes catalyze the reversible reaction in which a proton and bicarbonate are produced from carbon dioxide and water. Unlike other enzymes of the CA family, CAIX is expressed in many forms of cancer, but not in most normal tissues. CAIX is cell density-dependent and has shown to be activated in tumors under stressful conditions including hypoxia, the latter implying deprivation of adequate oxygen supply from tissues (Robertson, 2004). In addition, CAIX activity is associated with cellular reproduction and transformation, increased rates of metastasis, and poor prognosis in aggressive carcinomas (Chia, 2001).

Virtual screening was used in order to identify potential CAIX inhibitors; virtual screening is the process of utilizing molecular docking software to analyze biological structures in order to discover novel drugs (Soichet, 2004). In order to bring a new drug to the market, up to $800 million and over 10 years of research can be spent (Geldenhuys, 2006). Although only recently developed, virtual screening can accelerate the introduction of new drugs to market while reducing the cost of development by up to 50% (Geldenhuys, 2006). GOLD, the docking program used in the study, scans numerous conformations of compounds in a library. The program returns a score that accounts for factors of binding affinity, which in turn may indicate inhibition in an enzyme-ligand complex. Factors include the number of hydrogen bonds and the extent of van der Waals forces (Cummings, 2005).

It is hypothesized in this study that virtual screening methods along with standard molecular biology techniques will yield specific inhibitors of CAIX that may improve cancer therapy. The four main objectives of this project were 1) to predict potential CAIX inhibitors by virtually screening many different compounds, 2) to clone the CAIX gene into an expression vector with ligation independent cloning, 3) to express and purify the CAIX protein, and 4) to test the potency of discovered inhibitors through in vitro enzyme assays.

CAIX increases the survival of cancer cells by regulating tumor pH (Chiche, 2009). CAIX's negative effects are multifold. The activation of CAIX in the first stages of hypoxia allows the tumor cells to survive in increasingly hypoxic conditions. CAIX lowers tumor extracellular pH by producing protons. For normal cells, acidification of the micro-environment can be lethal, stopping vital intracellular processes such as cellular respiration. The CAIX of tumor cells prevents cancer cell death by producing HCO_3^-, which alkalizes the intracellular pH. Therefore, CAIX gives tumor cells an advantage in survival against normal cells (Figure 1). The extracellular acidification leads to chemo-resistance with slightly basic anti-cancer drugs (Švastová, 2004). In addition, radiation treatment is rendered less effective due to the lack of oxygen because free radicals that cause cellular tumor damage cannot be produced (Chia, 2009). Therefore, tumor cells aided by CAIX have further resistance to radiation treatment. Overall, the negative attributes of CAIX's functionality in cancer make CAIX an attractive target for inhibition.

METHODS AND MATERIALS

Cloning

Ligation independent cloning was used to insert the CAIX coding

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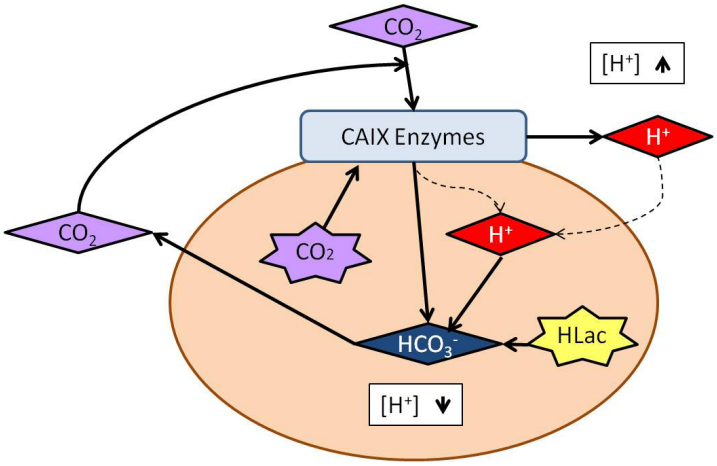

Figure 1. Reaction catalyzed by CAIX. In a tumor cell, CO_2 is generated from aerobic respiration or is present from the general environment. CAIX can turn CO_2 and water into $HCO3^-$ and H^+. When the extracellular pH becomes extremely acidic from the H^+, the $HCO3^-$ produced by CAIX regulates interior pH. Another source of acidic intracellular pH is Lactic Acid (HLac), which is produced from anaerobic respiration and/or aerobic glycolysis. It can be removed by intracellular titration with $HCO3^-$, further balancing the pH (Švastová, 2004).

DNA sequence into the pNIC28-Bsa4 expression vector designed by Gileadi et al. (2010) and obtained from the Arizona State University Plasmid Repository (Figure 2). Primers were designed to exclude parts of the CA9 sequence in polymerase chain reaction (PCR) that code for large hydrophobic regions. Hydrophobic residues cause difficulties in protein purification because of their low solubility and tendency to cause protein aggregation (Condron et al., 2008). The complete truncated amino acid sequence is the same as that on PDB used by Alterio et al (2009). PCR conditions followed EMD Millipore's standard *Thermococcus kodakaraensis* polymerase (KOD1), according to the manufacturer's instructions, with the exception of a 10 second annealing step at 59.6°C rather than at 55°C (EMD Milipore, 2013).

Gel electrophoresis and extraction were used in order to isolate the desired PCR products to be inserted into the expression plasmid. The PCR samples were electrophoresed through 0.9% agarose gels containing ethidium bromide in 1xTAE buffer. Gels were viewed using a UV trans-illuminator. The bands of 771 base pairs (size of PCR insert) were manually cut out. A Gel Extraction kit (Sigma-Aldrich; St. Louis, MO) and a PCR Clean Up kit (Sigma-Aldrich; St. Louis, MO) purified samples using included chemicals and micro-centrifuge columns.

Eco311 restriction enzymes aligned the pNIC28-Bsa4 plasmid. T4 DNA polymerase created complimentary ends on the PCR insert and the linearized pNIC-Bsa4. Once the insert and vector annealed, the hybrid plasmid was transformed into NEB5α E. coli cells (New England Biolabs; Ipswich, MA), which were then plated on a lysogeny broth (LB) agar with kanamycin antibiotic. Plasmids were isolated from E. coli using a Miniprep Kit (Qiagen; Valencia, CA). Plasmids with sequencing primers were sent to the University of Texas (UT) at Austin's Core Facilities and sequenced with the UT Core's standard Sanger DNA sequencing service.

Protein expression and purification

Positive clones were transformed into BL21 (DE3) competent cells. Colonies were inoculated into LB media overnight. After 16 hours, culture samples were adjusted to have a final OD600 (optical density at 600 nm wavelength) of 0.1. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 500 μM in order to induce CAIX expression. The expression took place overnight at room temperature (~20°C) rather than the standard 4 hours at 37°C. Cells were harvested by centrifuge and lysed by sonicator. Clarified liquid supernatant contained the CAIX protein ready for purification.

Ni-NTA agarose (Qiagen; Valencia, CA) was used to purify CAIX using a standard protocol for 6xHis-tagged proteins from E. coli (Qiagen, 2013). Fast protein liquid chromatography (FPLC) on a Superdex G75 column (General Electric Healthcare; Pittsburg, PA) purified the sample even further. CAIX protein concentrated using a Vivaspin concentrator (General Electric Healthcare; Pittsburg, PA) was stored in 20% glycerol at -20°C. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under a standard protocol (Caprette, 2012) for confirming the presence of the protein.

Virtual Screening

The carbonic anhydrase IX protein structure was obtained from the literature (Alterio, 2009) and viewed on the PyMol program (Schrödinger; Portland, OR). The protein was prepared for docking using the GOLD program (Jones et al., 1995). The active site cavity was measured as 7.5 angstroms around its central point. Two thousand eight hundred and nineteen hydrogen atoms were added. Four hundred and thirty excess water atoms and three excess ligands were deleted. This was done to optimize the virtual screening process. A script file was made for each run to distribute the docking jobs on multiple processors using the Sun Grid Engine (SGE). The job was run using an edited script command through the secure shell to the remote UT TI-3D computer cluster. In total, 85,000 compounds from seven libraries were screened (Table 1).

The top 1% of the total library size was saved as high scoring ligands after several rounds of screening. These compounds were analyzed further with Pymoland Lipinski's Rule of 5 (Lipinski, 2004). Lipinski's Rule of 5 is composed of four criteria related to drug absorption, distribution, metabolic effect, and excretion in the human body (Lipinski, 2004). Compounds selected in the final list of top 10 potential CAIX inhibitors (Table 2) for further analysis and testing had the highest binding scores, the strongest predicted interactions in the CAIX active site, and at least three out of the four requirements in Lipinski's Rules of 5 passed. Then the top four compounds to be analyzed in the enzyme assays were chosen on the criteria of both binding scores directly reported by GOLD program and statistical analysis.

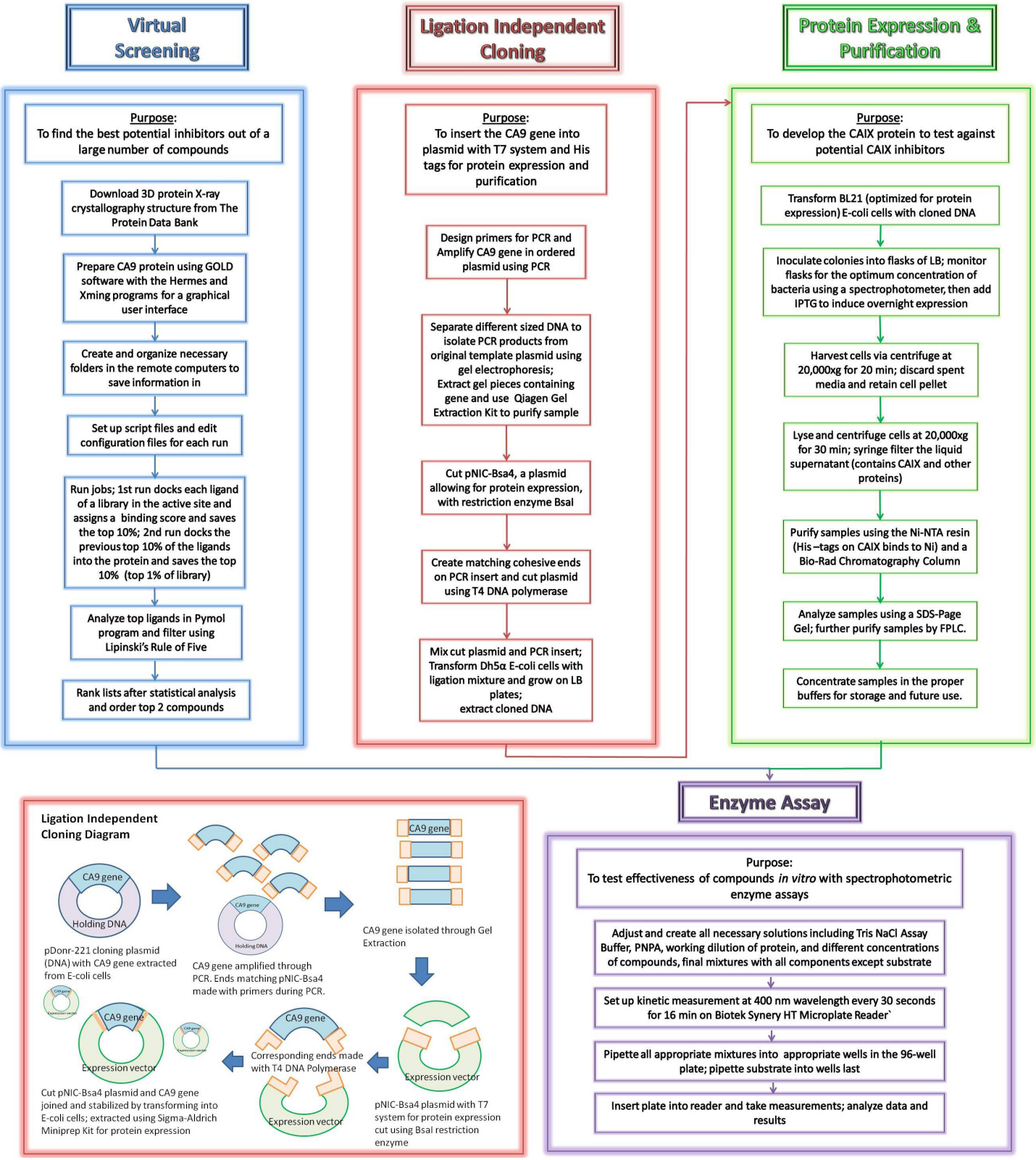

Figure 2. An overview of the methods used in this study.

Compound Library	Library Size
Chembridge Diversity 3D	49,797
Chembridge's MW Set	30,000
Chembridge's CB-Kin Library	4,000
Sigma's LOPAC Library	1280
NIH Library	445
Maybridge's HF9 Plates 5_9 Library	400
Chembridge's CB 306 Library	306

Table 1. Virtual screening libraries containing a total of 86,228 compounds. Some libraries contain a diverse set of compounds while others are focused on a certain property or target. The NIH and Sigma libraries contain compounds that have been through clinical trials.

Enzyme Assays

In order to determine the potency of the chosen compounds in vitro, spectrophotometric enzyme assays were carried out using 4-nitrophenyl acetate (PNPA) as the substrate.

PNPA (Sigma-Aldrich; St. Louis, MO) was used because of its structural similarity to HCO_3^-. In our study, the surrogate is the reactant although this reverse reaction catalyzed by CAIX does not take place in the human body in appreciable amounts because it is not nearly as abundant in a cell as CO_2. However, it is difficult to quantify the CO_2, H_2O, H^+, and HCO_3^- of the natural reaction. P-nitrophenol (PNP) and acetic acid are the products of the reaction when PNPA is the reactant. P-nitrophenol has an OD that is easily read by a spectrophotometer at 400 nm (Sies, 2005). Optical density at a 400 nm over time, or mean velocity, shows the rate at which CAIX catalyzes the reaction and makes PNP. Thus, a smaller mean velocity indicates an inhibited enzyme because less PNP is being made.

Assay buffer with a pH of 7.5 consisted of 25 mM tris and 150 mM NaCl. Compounds were diluted in dimethyl sulfoxide (DMSO) (Sigma-Aldrich; St. Louis, MO). Wells in the 96-well plates had 100 ng of enzyme in buffer, water, a final concentration of 1mM 4-nitrophenyl acetate (PNPA) and the chosen compound and concentration to be tested. The Biotek Synergy HT Microplate Reader (BioTek; Winooski, VT) measured OD of each sample every 33 seconds for a total of 20 minutes.

RESULTS

Agarose gel electrophoresis showed successful PCR amplification of the 771 base pair CA9 gene. Cutting of pNIC-Bsa4 by Eco3111 restriction enzyme was also confirmed (Figure 3). This demonstrates that the expression vector was successfully linearized which is necessary for subsequent cloning processes. The Basic Local Alignment Search Tool (Altschul, 1990) comparison between the target sequence and the sequence of the sample showed the CAIX coding DNA sequence was correctly inserted into pNIC-Bsa4 expression vector (Figure 3). This cloned hybrid CAIX-expression plasmid allows for CAIX protein expression.

Compound Identifier	Library Name	Binding Score	*z*-score within Library	# of Lipinski's Rules Violations
Metoprolol	Sigma's LOPAC Library	91.78	0.94	0
Pentamidine Isethionate	Sigma's LOPAC Library	87.47	0.14	1
7653639	ChemBridge-diversity3D	85.15	3.23	0
7633756	ChemBridge-diversity3D	84.51	3.05	0
7784819	ChemBridge-diversity3D	83.17	2.65	0
7628135	ChemBridge-diversity3D	83.13	2.64	0
7750322	ChemBridge-diversity3D	83.12	2.64	0
6399774	ChemBridge-diversity3D	82.95	2.59	0
6444968	ChemBridge-diversity3D	82.62	2.49	0
7955780	CB-kin	79.35	2.90	0

Table 2. The top 10 potential inhibitors of CAIX as chosen based on the results of virtual screening. The binding score is the GOLD fitness score. A higher z-score shows that the predicted binding strength of the particular molecule is higher than the other molecules of the same library.

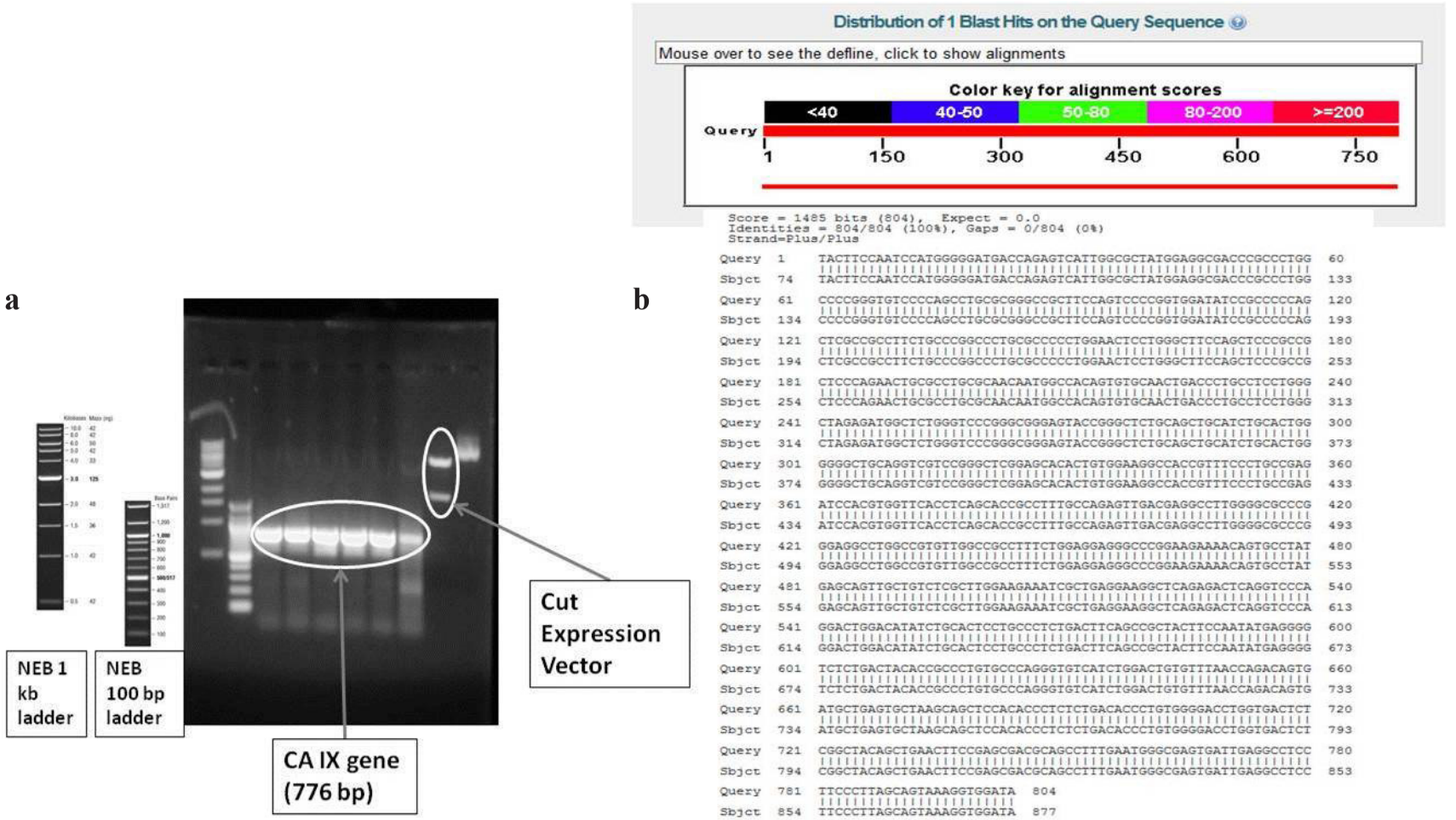

Figure 3. a) Agarose gel electrophoresis image showing successful PCR amplification of the CA9 gene in lanes 3-8. Comparison to 100 kb ladder in lane 2 confirms the DNA is the correct size of CDS at 774 bp. Lane 9 shows the cut pNIC-Bsa4 vector while lane 10 shows the uncut pNIC-Bsa4 control at 7.3 kb. The 1 kb ladder in lane 1 confirms the size of the cut vector pieces. **b) Ligation independent cloning results.** Basic Local Alignment Search Tool (BLAST) (Altschul, 1990) was used to compare sequencing facility results to the desired sequence of the CAIX gene in the expression vector. The BLAST image above demonstrates a 100% match between the query (desired sequence) to the subject, the sample that was cloned.

No-Compound Mean Velocities vs. Compound Mean Velocities				
	p-values			
	Metoprolol	Pentamidine	#7653639	#7633756
0.5 µM	0.1127	0.0342	0.0017	0.0247
1 µM	0.0426	0.0064	0.00028	0.0083
10 µM	0.0062	0.00047	0.00007	0.00007
100 µM	0.0302	< 0.00005	0.00026	< 0.00001

Table 3. Two-sample t-tests were used to determine if the mean velocities of control samples were significantly different from non-control samples. A *p*-value less than the chosen alpha level of .05 indicates statistical significance in order to reject the null hypothesis. Fifteen out of 16 sample comparisons reject the null hypothesis. This demonstrates evidence that the mean velocities of samples with compounds are significantly less than the mean velocities of the control samples.

After expression and purification, SDS-PAGE showed a protein band of approximately 28 kilodaltons. Because the CAIX protein has a size of 28 kilodaltons, it is concluded that the protein present in the sample is CAIX.

A box plot (Figure 4) compared the binding scores of the top compounds to those of other compounds. Chembridge #7653639, Chembridge #7633756, metoprolol, and pentamidine isethionate all had high binding scores and *z*-scores, which reflect the differences in binding scores of chosen compounds to those of other compounds in the same library. The binding scores ranged from 84.51 to 91.78 and result from similarly high Van der Waals and hydrogen bond sub-scores. All four passed the Lipinski's Rule of 5 Test for drug-likeness and were used in the enzyme assays.

As the concentration of the compounds increased from 0.5 µM to 100 µM, the average mean velocities decreased. The average mean velocity of the enzyme without any inhibitors was 2.184 mOD/min. At 100 µM, the top two inhibitors, Chembridge compound #7653639 and #7633756, had an average mean velocity of 1.519 mOD/min and 1.565 mOD/min, respectively (Figures 5, 6). The lower mean velocities indicate that the reaction catalyzed by CAIX progresses at a slower rate. Thus, the compounds can be concluded to result in CAIX's inhibition.

Two-sample t-tests with an alpha level of 0.05 were used to determine if the mean velocities of control wells were significantly different from experimental wells (Table 3). Fifteen out of 16 sample comparison (control vs. 0.5 µM concentration of metoprolol) returned *p*-values below the alpha level. This shows that the mean velocities of the control sample were significantly higher than those of the samples containing the compounds and are not likely due to random error.

DISCUSSION AND CONCLUSIONS

The hypothesis was supported by this study; specific inhibitors of CAIX, which may improve cancer therapy, were discovered by the successful completion of the 4 research objectives. The research objectives included the cloning of the CAIX gene, the expression and purification of the CAIX protein, the virtual screening of CAIX's X-ray crystallography structure, and finally, the testing of four chemical inhibitors to CAIX. The standard molecular biology methods used in this study, along with the less common methods such as ligation independent cloning rather than T/A cloning and overnight protein expression rather than four-hour expression, were shown to be effective in obtaining sufficient amounts of CAIX protein for use in enzyme assays. As compared to traditional methods, virtual screening yielded results of predicted inhibitors in a manner saving both time and resources.

All four compounds show evidence of inhibiting the CAIX protein when tested in vitro. The top two inhibiting compounds in the enzyme assay were Chembridge compound #7653639 and #7633756. The higher effectiveness of the two Chembridge compounds in enzyme inhibition would result from the greater affinity of these two compounds have to the CAIX active site. Chembridge #7653639 and #7633756 have molecular weights of 409 and 466, respectively (Chembridge, 2010). On the other hand, metoprolol and pentamidine isethionate have molecular weights of 685 and 593, respectively (Sigma-Aldrich, 2013). The results showing decreased effectiveness of the metoprolol and pentamidine isethionate as inhibitors may thus indicate a disadvantage in using larger compounds because they do not fit as well in the CAIX active site. The decreased inhibition may also be caused by a number of other factors such as greater number of hydrogen bonds or Van der Waals forces. Greater understanding of the causes for the compounds' effectiveness may be further elucidated with additional tests such as x-ray crystallography.

Although metoprolol and pentamidine isethionate have lower inhibition results, they offer a great case for drug repurposing because they are already on the market for different diseases. Pentamidine isethionate is currently used as an anti-infective agent (Western, 1970) and was tested in rats to see its effect on anti-tumor necrosis factors (Chen et al., 1994). CAIX inhibition by pentamidine isethionate may be very promising because it could target cancer in two separate ways. Metoprolol is commonly used drug for hypertension and heart disease (Benfield, 1986). Because these compounds already passed clinical trials, the road to becoming an official, generally used drug for cancer may be significantly shortened.

Inhibitors of carbonic anhydrase (CA) have previously been found in other studies. However, these drugs target the CA family as a whole instead of CAIX specifically. Many members of the family actually have beneficial effects for humans; thus, these known inhibitors have high toxicity and a number of side effects (Supuran, 2010). This research presented here is novel in that it concerns the inhibition of specifically CAIX, a protein that is only activated in cancer cells. Future studies with the four compounds would need to be carried out to determine their effect upon the CA family as a whole to further determine specificity.

While a variety of research has been done on CA using high-throughput screening and other trial and error based methods (Miyazaki, 2008), studies analyzing inhibitors via virtual drug screen-

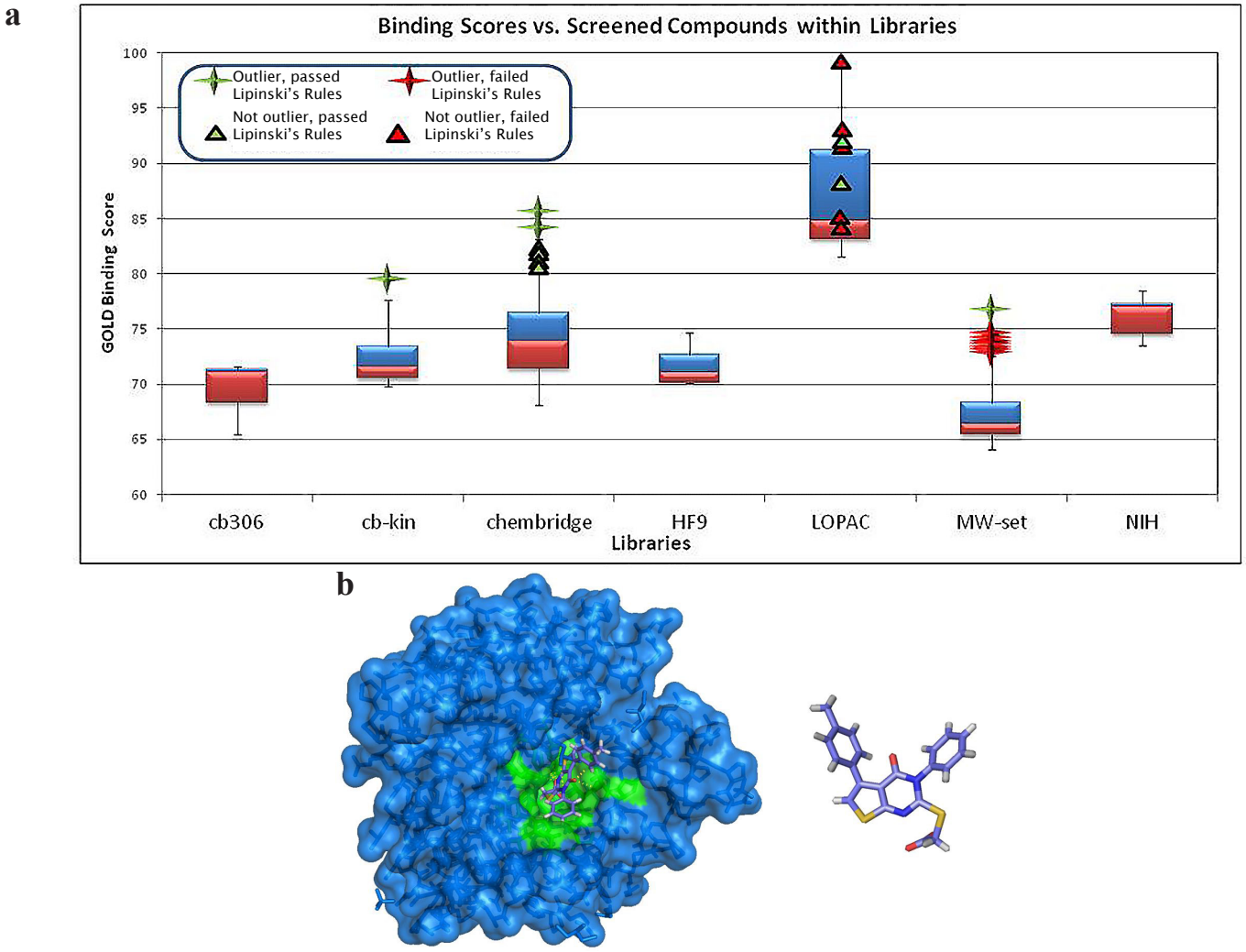

Figure 4: a) Box plot used to compare the binding score distribution between libraries. There were outliers (compounds with a score greater than 1.5 * inner quartile range (IQR) above the third quartile) in 3 libraries showing specific ligands with scores outside of their libraries' normal distribution. The top two chosen ligands that were examined are both outliers of Chembridge library. The other compounds chosen in the top 10 compounds list also show promise because of their distinct qualities and high binding scores. **b) CAIX structure (PDB #: 3IAI) with ChemBridge Compound #7653639 (Gold fitness score of 85.15).** The CAIX protein (blue) is shown using the PyMol program (Schrödinger; Portland, OR) with its active site (green), polar contacts (yellow), and compound (colored by element: carbon-light blue, hydrogen-white, nitrogen-dark blue, oxygen-red, sulfur-orange).

ing are limited. Traditional virtual drug screening is measured entirely by binding score (Soichet, 2004). This research chose to use a statistical process less commonly used (Sastry, 2013) to measure inhibition potential. A ligand's *z*-score within its own library was added to the screening process so that the integrity of the ligand was not solely based on a single binding score. By scoring significantly above other compounds, a ligand shows greater inhibition potential. Using *z*-scores helped the researchers to find ligands, such as the chosen Chembridge compounds, with low relative binding scores but with significant drug potential.

Additional studies will further enhance our understanding of the compounds' potential. A larger number of trials may be conducted to decrease the likelihood of random errors, which may arise from spectrophotometer error or sample composition variation from potentially insufficient mixing. Virtual screening may be done on more compound libraries to find additional potential inhibitors of CAIX. Also, the remaining compounds in Table 2 may be tested using enzyme assays. CAIX, and its prospective inhibitors may be tested in cell-based assays using animal and human cancer cells in a hypoxic environment. If further studies are successful, CAIX inhibitors in this study have the potential to drastically improve both the treatment and the prognosis of cancer patients all over the world.

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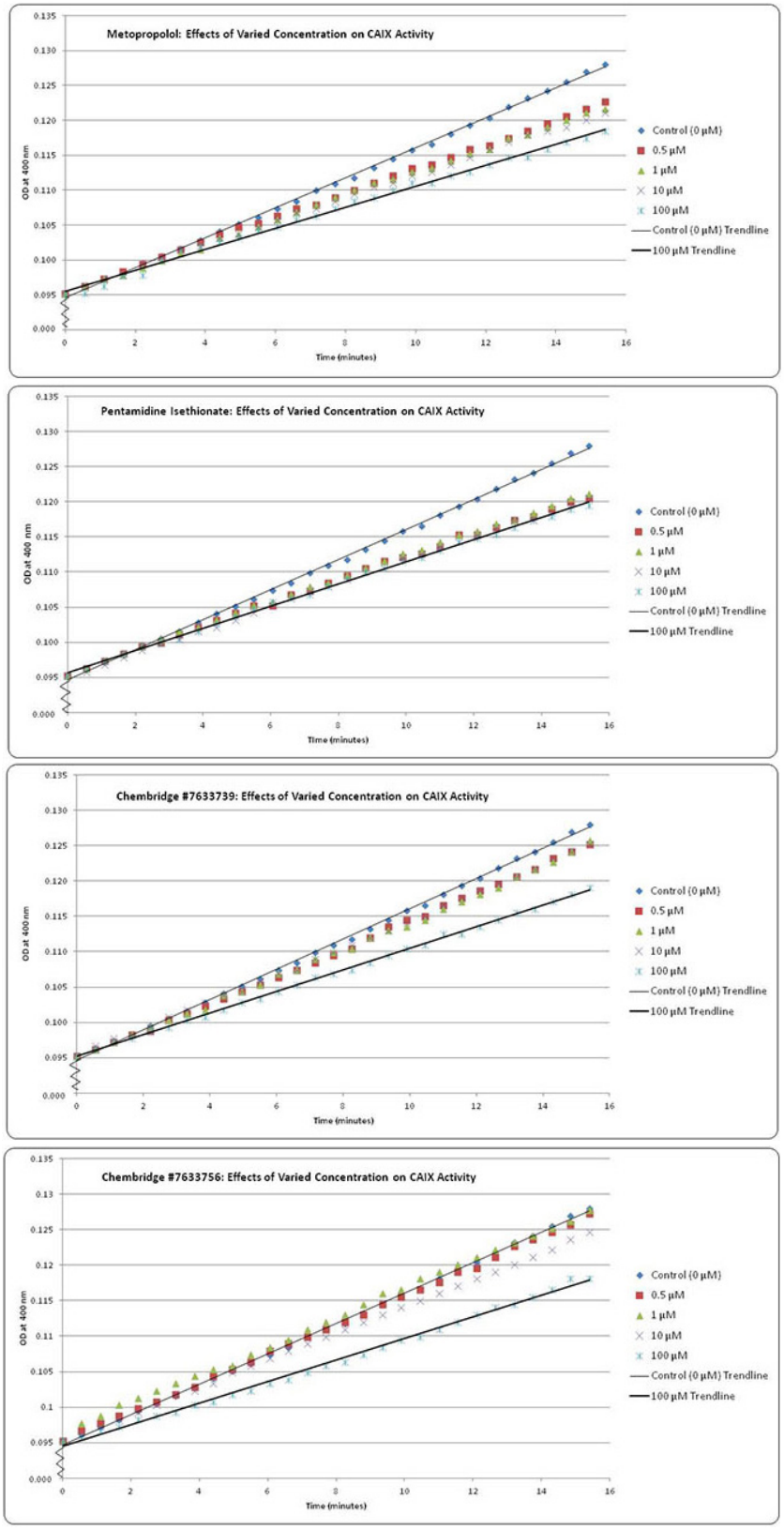

Figure 5: Different concentrations of each compound were tested against CAIX activity to hydrolyze PNPA to PNP and acetate. The concentrations tested were 0.5, 1, 10 and 100 µM. For each concentration, five trials were averaged. Optical Density at 400 nm (OD) over time (mean velocity/slope) represents the enzymatic activity. Thus, a lower slope indicates an inhibited enzyme. From 1 µM to 100 µM, the mean velocity decreased. The mean velocity of the control sample was 2.184 mOD (400 nm)/min. The mean velocities (at 100 µM compound concentration) were metoprolol - 1.789 mOD(400 nm)/min, pentamidine isethionate - 1.938 mOD(400 nm)/min, Chembridge #7653639 - 1.519 mOD(400 nm)/min, and Chembridge #7633756 - 1.565 mOD(400 nm)/min.

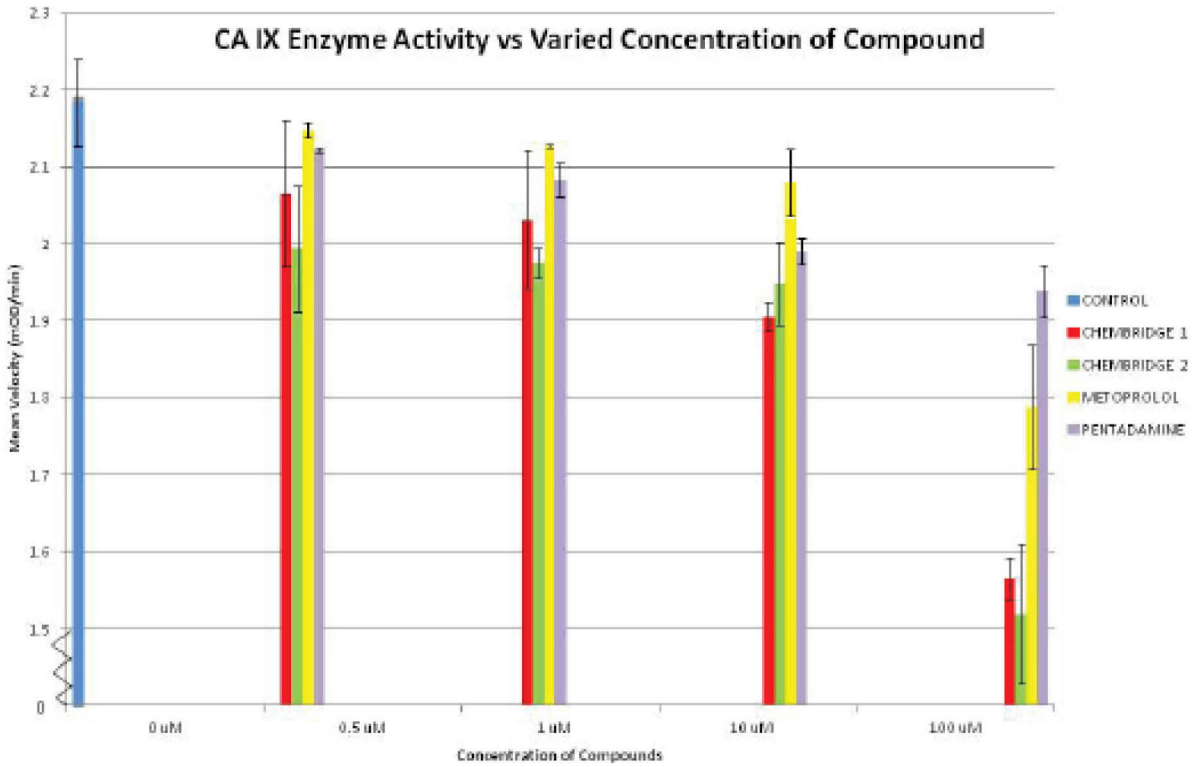

Figure 6: Bar graph representation of the enzymatic activity (mean velocity – also, the slope of each line in the Figure 5 graphs) of the CAIX enzyme under different concentrations of inhibitory compounds. The error bars indicate 1 standard deviation plus or minus of the average mean velocity.

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