A Comparative Study of Drug Affinities Determined by Thermofluor and Kinetic Analysis

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Abstract

Determining the binding affinity and potency in vitro is one of the significant steps that can give a clue for a new candidate drug during the drug discovery process. Thermofluor is a method used in measuring binding affinity (Kd) of protein-ligands interaction through determining the change in thermal denaturation temperature of protein using real time PCR (RT-PCR). Kinetic analysis assay is used to screen a library of compounds to calculate their potencies (IC50) and inhibition constants (Ki) and it can be performed by spectrophotometer technique. In this study, we used bovine carbonic anhydrase II (BCA II) enzyme, and four of its inhibitors as a model to compare drug affinities, which were determined either by Fluorescence Thermal Shift Assay (FTSA) using Sypro Orange dye or kinetic assay using 4-Nitrophenyl acetate as a substrate to measure the nonphysiologically esterase activity of CA. The inhibitors studied were Methazolamide, Brinzolamide, Dorzolamide HCl and Mafenide HCl. The Kd values were determined to be 5.4±0.085 µM, 1.2±0.44 µM, 2.08±0.63 µM, and IC50 values were 0.148±0.024 µM 0.129±0.015 µM 0.092±0.01 µM 1.715±0.16 µM whereas the Ki values were 4±0.55 nM, 3.5±0.5 nM, 2.5±0.5 nM and 46.5±6.5 nM for Methazolamide, Brinzolamide, Dorzolamide HCl and Mafenide HCl, respectively. The potencies (IC50) of the inhibitors were 10-50 fold lower than that of the Kd values. In addition, Kd values were higher compared to Ki values. Therefore, kinetic analysis is a more sensitive technique and requires a lower amount of the enzyme to measure drug affinity than FTSA.
MONTCLAIR STATE UNIVERSITY

A COMPARATIVE STUDY OF DRUG AFFINITIES DETERMINED BY THERMOFLUOR AND KINETIC ANALYSIS

By

Oruba Shatnawi

A Master's Thesis Submitted to the Faculty of

Montclair State University

In Partial Fulfillment of the Requirements

For the Degree of

Master of Science

January 2020

College of Science and Mathematics

Department of Chemistry and Biochemistry

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Montclair, NJ

2020
Acknowledgements

I would like to express my deep gratitude to my supervisor, Dr. Siekierka for his invaluable advice, continuous encouragement and direction.

I’m particularly thankful to my committees, Dr. Goody and Dr. Rotella for their support and encouragement throughout my study.

I would like to thank Tamara Kriess, Tyler Eck, Brittany Hart and Thomas Candela for their technical instruction, patient troubleshooting assistance and support.

I’m grateful to my husband, Salman for his inspiration, encouragement, moral help and patience while I completed my thesis. Without his support nothing could be done.

I dedicate my work to my parents, this research work would not have come to light without their blessing, to my daughters, Layan, Kenda and Dariya. To my Family and friends
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Background: Literature Review

Drug discovery

The drug discovery process takes about 12-15 years, and more than 1 billion dollars to come up with any new candidate drug (Fig. 1). It may take many years to build up a body of supporting evidence before selecting a target for a costly drug discovery program. Many challenges are present in drug discovery and pharmaceutical industry, such as target identification, hit to lead optimization and evaluation of drug candidates (Hughes, Rees, Kalindjian, & Philpott, 2011).

The hit to lead to process is a valuable technique for distinguishing drug candidates. Determining the binding affinity and potency in vitro, is one of the hit to lead optimization steps that can give a clue for the candidate drug (Wang, Dong, & Sheng, 2019). Creating of methods for rapid screening of inhibitors of specific enzyme from compound library is one of the main challenges in drug discovery, especially for new targets where the goal is to study and recognize their potential inhibitors (Lo et al., 2004).
Universal and valid drug discovery assay technique are desired in order to get ahead with the latest developments in combinatorial chemistry and genomics-based target production (Pantoliano et al., 2001). Enzymatic assays (e.g. Fluorescence or absorbance spectroscopy) are commonly used in measuring the potencies of compounds in laboratories. While binding assay such as Fluorescence Thermal shift Assay (FTSA) is used to measure the compound affinity. Many compounds that are assumed to be potential drugs in treating human diseases, are firstly known through studying their binding properties like affinity and potency (FDA, 2003).

**Enzymatic assay (kinetic analysis):**

Enzymatic assays play critical roles in determining the reaction rate, binding affinity, catalytic constant, and inhibition rate as well as screening library of compounds. (FDA, 2003).
One of the major assays is used to estimate $V_{max}$ (the maximum velocity of the reaction) and $K_M$ (Michaelis-Menten constant), which is the substrate concentration at half of $V_{max}$ (Eq. 1) (Choi, Rempala, & Kim, 2017). Where the rate of the reaction is measured in time course by monitoring the absorbance of the product release or the substrate consumption over period of time (Boeckx, Hertog, Geeraerd, & Nicolai, 2017).

$$v = \frac{V_{max}[S]}{K_m+[S]}$$  \hspace{1cm} \text{Eq. 1}

Where $[S]$ is the substrate constant; $V_{max}$ is the maximal velocity and $K_m$ is the Michaelis constant which is the substrate concentration of $V_{max}/2$

**IC$_{50}$ Assay:**

Dose response relationship is displayed by fitting the response against the log of the inhibitor concentration through the application of the equation of Four-Parameter Logistic Function (Eq. 2), (Krohn & Link, 2003).

$$y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{\log(IC_{50}) - \log(x) \times \text{HillSlope}}$$  \hspace{1cm} \text{Eq. 2}

Where $y$ is response, $x$: log of inhibitor concentration, Top and Bottom: plateaus in same unit as $y$, same log unit as $x$, Hillslope: slop factor or hill slop (unitless).

High throughput screening assays are mainly used to examine enzyme activity and inhibitor efficacy. A total inhibitor concentration that decreases enzyme activity by 50% ($IC_{50}$) is the most used pharmacokinetic measure of a drug's efficacy and potency. It is a quantitative measure that indicates the concentration of a drug or substance that is required to inhibit a specific biological activity by a half (Aykul & Martinez-Hackert, 2016).
**Inhibition constant $K_i$:**

Inhibition constant ($K_i$) is a guide of how potent an inhibitor is, and it is the concentration of the inhibitor desired to produce half maximum inhibition. For competitive inhibitors it can be calculated by Cheng-Prusoff equation (Eq. 3) (Lazareno & Birdsall, 1993).

\[
K_i = \frac{IC50}{1+[S]/Km}
\]  
Eq.3

**Binding assays:**

Binding affinity is the strength of the binding interaction between protein and its ligands (like inhibitors or activators). It is generally estimated by the binding affinity constant also known as equilibrium dissociation constant ($K_d$). The smaller the $K_d$ value, the greater the binding affinity of the ligand for its target (Pollard, 2010).

There are multiple methods available for detection of dissociation constant ($K_d$) value of protein-ligand interactions. Such as, Surface Plasmon Resonance (SPR) which is an approach that requires transfer of electromagnetic energy to electrons in a thin layer of metal (e.g. Gold) in contact with a solution.

Fluorescence Polarization (FP) is another method used; it measures the change in the rate of the rotation of a fluorophore which can be detected by alteration in polarization. This method depends on the binding of a fluorescent ligand to a protein which causes the ligand to rotate more slowly. Isothermal Titration Calorimetry (ITC): This method quantifies the heat produced from a chemical reaction in solution. Because almost all enzyme-ligand reactions release or consume heat, and no fluorescent labels of the enzyme or ligand are required. ITC is the only known method
capable to immediately measure the enthalpy, $\Delta H$, of a ligand binding to a protein (Mittermaier & Meneses, 2013).

Thermofluor also known as Thermal shift assay (TSA) or Differential Scanning Fluorimetry (DSF), measures the change in thermal denaturation temperature of a protein under different conditions (e.g., pH, salts, additives, drugs or mutations). And this method requires only RT-PCR machine, data can easily be analyzed as many protocols are available to quantify the data from TSA to rapidly demonstrate $K_d$ values and predict the binding affinity of protein-ligand interaction (Mittermaier & Meneses, 2013).

FTSA (Fluorescence Thermal Shift Assay) monitors thermal unfolding of proteins in the presence of a fluorescent dye which is highly fluorescent in non-polar environment, such as the hydrophobic sites on unfolded proteins, compared to aqueous solution where the fluorescence is quenched (Fig. 2). Usually, TSA can be applied for variety of enzymes where it is run by a real-time PCR instrument (Niesen, Berglund, & Vedadi, 2007).

This assay is used for detection of protein-ligand interactions. After the ligand binds to the folded protein, it will stabilize it. As a result of that, it will increase the melting temperature ($T_m$), which is needed to unfold the protein. In consequence, this will result in shifts in unfolding curves and $T_m$ to give ($\Delta T_m$), and analyzing these data gives the value of the dissociation constant ($K_d$) which determines the binding affinity of the ligand (Bai, Roder, Dickson, & Karanicolas, 2019).
Figure 2: The two-state transition curve. In the first state, the fluorescence intensity increases upon enzyme unfolding, where the hydrophobic region becomes exposed to the dye allowing it to bind with the enzyme. After reaching the plateau, in the second state, the fluorescence intensity begins to decrease as the denatured enzyme-dye complex starts to aggregate (Bruce, Cardew, Freitag-Pohl, & Pohl, 2019).

**Carbonic Anhydrase (CA) Overview:**

Carbonic Anhydrase is an enzyme which requires zinc ion to function as a Lewis acid in the reaction mechanism at the active site, where it can be coordinated to three histidine and a hydroxide ion to catalyze the reversible hydration of carbon dioxide (CO₂) and dehydration of bicarbonate ion (HCO₃⁻) (Eq. 4) (Iqbal, Nisar-Ur-Rahman, & Iqbal, 2014; Iyer, Barrese, Parakh, Parker, & Tripp, 2006).

\[
\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+ \quad \text{(Eq. 4)}
\]
CAs are present in plant and animal tissues. There are several classes of CAs; α, β, γ, δ and ζ. α-class is found in bacteria and mammals, and about 16 different mammalian isozymes such as CA I, CA II, CA III, CA IV ...etc. were described (Taslimi et al., 2016). CAs I and II are abundantly found in red blood cells of mammals where CA II is one of the most known isozymes in α-class that was discovered in 1933 by Meldrum and Roughton (Meldrum & Roughton, 1933). Bovine carbonic anhydrase II (BCA II) is similar to the human one (Iqbal et al., 2014) and it is a cytosolic, single-chain, approximately 30 KDa enzyme. In addition, CAs are found in mammals, and can be divided in four subgroups according to their localization; cytosolic, mitochondrial, membrane bound with extracellular domains or secreted (Krishnamurthy et al., 2008a).

Carbonic anhydrase enzyme is involved in different physiological processes, such as pH homeostasis, calcification, respiration, vision, gas exchange and bone resorption (Krishnamurthy et al., 2008a). These functions make it a target for many drugs that function as CA inhibitors. CA anhydrase inhibitors are used in many conditions such as; glaucoma, cancer, epilepsy, obesity, heart failure and intercranial hypertension. Examples include, sulfonamides which are the most common inhibitors class of CA (Iqbal et al., 2014). Additionally, methazolamide and brinzolamide which help in reduction of elevated intraocular pressure in patients with open-angle glaucoma or ocular hypertension, dorzolamide is used in the treatment of acute or chronic vascular hypertension and mafenide used as antibacterial agent (Brunton, L.B., Lazo, J.S., & Parker, 2005).

CA is an excellent model for studying enzyme kinetics for small molecule ligands due to several reasons. These include the fact that it does not contain disulfide bonds, it is monomeric, stable, it can be easily purified, it has known amino
acid sequences, and the mechanism of its catalytic activity is known. Additionally, the mechanism of inhibition of it is well understood and it is easy to evaluate the binding of ligands by different high throughput assays (Krishnamurthy et al., 2008a).

The assays that study the CA activity can be generally subdivided into main groups. One method includes CO$_2$ consumption detection, where carbonic dioxide and carbonate act as substrates for estimation of CA activity. Also, stopped-flow assay which estimates the CA activity by monitoring the pH changes of dehydration of HCO$_3^-$ to CO$_2$ or the reverse reaction (Krishnamurthy et al., 2008a). In 1965, Pocker and Stone established another type of assay to measure the CA activity, and a spectrophotometric technique for verifying the esterase activity of CA by catalyzing the non-phycological hydrolysis of esters like hydrolysis of 4-Nitrophenyl acetate (4-NPA). The product (nitrophenolate) ionizes to give yellow nitrophenolate anion which can be detected at 400 nm by using UV-spectrophotometer (y. pocker, 1965).

**Can we use FTSA to study the binding affinity?**

Generally, Thermofluor (FTSA) is easy, fast, and inexpensive. It does not require creating new assays with each new target and can be used also for any unknown compound method to measure the binding affinity of the compounds other than the known enzymatic assays, these advantages could be an improvement of drug discovery field (Matulis, Kranz, Salemme, & Todd, 2005).

Recently, FTSA method is used in studying the binding affinity $K_d$ of the hit compounds as qualitative measurement, and there are many approaches and protocols
applied in order to concede this method for quantitative measurement. Therefore, enzymatic assay has been conducted for a variety of enzymes with different ligands to determine IC$_{50}$ values and compared them with the binding affinity constant ($K_d$) values that is obtained from thermal shift assay.

Lo et al., (2004) compared the binding affinities measured by FTSA and ITC with the IC$_{50}$ values, which were measured by enzymatic assay, using β-site amyloid precursor protein-cleaving enzyme 1 (BACE1). They concluded that $K_d$ values from FTSA are correlated to the $K_d$s that were obtained from ITC and IC$_{50}$ from enzymatic assay. In addition, they suggested that candidate hits can be determined based on $\Delta T_m$ values, if FTSA can be run at appropriate compound concentrations in connection to their dissociation constants.

Bai et al., (2019) proposed and established new approach for analyzing FTSA data using experimental data for maltose binding protein (MBP) and maltose, and for two carbonic anhydrase isoforms with four of their inhibitors. Enzymatic assay was applied to determine the inhibition constant ($K_i$) to obtain an independent measure of the interaction between the enzyme and each of the inhibitors. They concluded that there is a correlation between the binding constant and the inhibition constant, especially for potency range between uM to mM range.

Smirnovienė, Smirnovas, & Matulis, (2017) studied the importance of inhibition and binding assays using carbonic anhydrase by applying enzymatic stopped-flow CO$_2$ assay and FTSA, respectively. $K_d$ of picomolar was successfully measured by FTSA, which is promising in drug discovery world. As a result, a correlation between IC$_{50}$ and $K_d$ is observed. Moreover, they suggested that the combination between the two methods can give a higher quality and precise data.
In this study, carbonic anhydrase enzyme was used as a model with four of its known competitive inhibitors (methazolamide, brinzolamide, dorzolamide HCl and mafenide HCL) to perform kinetic assays and FTSA using uncomplicated and straightforward protocol (Iyer et al., 2006; Laubach, A. E., Wang, E., & Anderson, 2015). Then comparison was made between the potency (IC\textsubscript{50}), inhibition constant (\(K_i\)) and binding affinity (\(K_d\)) to demonstrate if FTSA is suitable and practical for screening of inhibitors and identifying hit compound. In addition, optimization of the FTSA technique was done to build up an effective and easily use method to determine drug affinity.

**Materials and Methods**

**Carbonic Anhydrase concentration determination:**

Stock solutions of each of methazolamide (Sigma-Aldrich, St. Louis, MO), brinzolamide (Selleck Chemicals, Houston, TX) and mafenide HCl (Selleck Chemicals, Houston, TX) were prepared in 100% DMSO, and Dorzolamide HCl (Selleck Chemicals, Houston, TX) was prepared in distilled water to make 10 mM solution. 2 mg/ml of bovine CAII (Sigma-Aldrich, St. Louis, MO) was prepared using buffer (Tris-HCl, pH 8.2). 20X solution of SYPRO Orange dye (Invitrogen\textsuperscript{TM} Molecular Probes\textsuperscript{TM}, 5,000X Concentrate in DMSO) were prepared in (Tris-HCl, pH 8.2).

To monitor the enzyme unfolding a total of 20 \(\mu\)L of mixture contains 2 \(\mu\)l of SYPRO Orange dye (2X final concentration), 2 \(\mu\)l of Bovine CAII (0.0008-0.1) mg/ml, and 16 \(\mu\)l of Tris-HCl buffer were mixed on 96-well PCR plate. A18 \(\mu\)L of
buffer and 2 µL of Sypro Orange dye was used as control. Then the assay plate covered with a sheet of optically clear adhesive. Main measurements were carried out in duplicate. Then fluorescence was measured from 25°C for 2 minutes then 1°C/min to 95°C (excitation, 450-490 nm; detection, 560-580 nm.) Then, FTSAs were performed on a StepOnePlus Real-Time PCR System (ThermoFisher, Waltham, Massachusetts, USA.). Then data was fitted using Excel 2013 (Microsoft, Redmond, WA) program.

Initially, Sypro Orange dye was compared with GloMelt™ dye (Sybr Green dye manufactured by Biotium Inc. Fremont, CA). A total of 20 µl mixture was used in the assay with different concentrations of the enzyme (0.0008-0.1) mg/ml, with either 2 µl of 2X of Sypro Orange or 2X of Sybr Green, and 16 µl Buffer. 2 µl of dye and 18 µl of buffer were added together as a control. Then fluorescence was measured from 25°C for 2 minutes then 1°C/min to 95°C (excitation, 450-490 nm; detection, 560-580 nm.) by RT-PCR.

The signal of Sypro Orange was found to be stronger (Fig. 3). A possible explanation for this finding is the difference in the sensitivity of RT-PCR machine. In addition, Sypro Orange has many advantages compare to Sybr Green dye; it is inexpensive, easy to prepare and can be stored at room temperature.
Figure 3: Comparison of fluorescence signal with Sypro Orange dye and Sybr Green dye at 0.1 mg/ml BCA II concentration.

**Determination of kd of the inhibitors**

FTSA was run using a total of 20 µl mixture, which contained 2 µl of Sypro Orange dye (2X final concentration), 12 µl of Tris-HCl buffer, 2 µl of BCA II (0.1 mg/ml in assay) and 4 µL of different concentrations (0.23 – 100 µM) of either methazolamide, brinzolamide or dorzolamide HCl, and (0.46-200 µM) of Mafenide HCL were mixed in 96-well plate. As a positive control, 2 µl of Carbonic anhydrase, 2 µL of the dye and 16 µl of buffer were added together. 1% DMSO was used as a negative control. Then the assay plate covered with a sheet of optically clear adhesive and fluorescence was measured from 25°C for 2 minutes and then 1°C/min to 95°C (excitation, 450-490 nm; detection, 560-580 nm.). FTSAs were performed on a StepOnePlus Real-Time PCR System. Data evaluation and melting temperature $T_m$
determination were performed using the GraphPad Prism 8 software (GraphPad software, Inc) and applied Boltzmann Sigmoidal curve equation (Eq. 5).

\[
Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + \exp\left(\frac{X - \text{Slope}}{\text{Tm}}\right)}
\]  
Eq. 5

Where \(Y\): fluorescence intensity; \(X\): temperature; \(\text{Bottom}\): baseline fluorescence at low temperature; \(\text{Top}\): maximal fluorescence at the top; \(\text{Slope}\): the steepness of the curve; and \(\text{Tm}\): melting temperature of the enzyme.

Then the data was analyzed by GraphPad Prism 8 software using the single site ligand binding equation (Eq. 6) (Vivoli, Novak, Littlechild, & Harmer, 2014):

\[
Y = B + ((\text{T} - B) \times (1 - ((P - Kd) - X + \sqrt{((P+X+Kd)^2) - (4+P+X)}) )/2 \times P)
\]  
Eq. 6

Where \(P\): protein concentration. \(Kd\): dissociation constant (has the same unit as \(P\)). \(T\): melting temperature at high inhibitor concentration; \(B\): melting temperatures of no inhibitor concentration.

**Enzymatic assay:**

**Determination of the Enzyme concentration**

The catalytic activity of BCA II was monitored by the hydrolysis of the nonphysiologically ester (4-NPA) (Sigma-Aldrich, St. Louis, MO). The products of the hydrolysis reaction are acetate and nitrophenolate, which ionizes to give a bright yellow anion that is detected by measuring its absorbance at 400 nm with a Synergy™ 2 spectrophotometer (Biotek instruments Winooski, VT) using 96-well plates. For initial screens, 200 µl of assay solution containing 10 µl of (0.0008-0.1) mg/ml of
BCA II, 10 µl of 4-NPA and 180 µl of Tris-HCl buffer were dispensed into 96-well plates which were incubated for about 20-30 minutes at room temperature. As a control 10 µl of the enzyme and 190 µl of the buffer were mixed. Then, the absorbance was read at 400 nm. The data were plotted on Excel to choose the enzyme concentration that is suitable to use in the IC50 detection.

Esterase activity Assay

Calculation of Vmax and Km:

The assay was performed by adding 10 ul of different substrate concentrations of 4-NPA (0.00195-0.25) mM. In addition, 10 ul of 6 ng/ul BCA (II) and 180 ul of Tris-HCl buffer were mixed in 96-well plate. Then the absorbance was read at 400 nm using the spectrophotometer. Michaelis Menten equation (Eq. 1) was applied and the curve was plotted by GraphPad Prism 8. To find out Km value and Vmax (ξ = 21,000 M-1cm-1) (Krishnamurthy et al., 2008b).

Calculation of Ki:

To determine the inhibition constants (Ki) values for each one of the inhibitors, Cheng-Prusoff equation (Eq. 3) was applied.

Calculation of IC50:

The assays were performed by adding 10 µl of each methazolamide, brinzolamide or dorzolamide HCl (0.039-5) µM final concentration or 10 µl of mafenide HCl (0.078-10 μM), 10 ul of 6 ng/ul of the enzyme, 10 µl of 4-NPA substrate (0.25 nM final concentration) and 170 µl of the buffer. Less than 1% DMSO

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was used as a control. The normalized data were then fit to the 4-parameter nonlinear sigmoidal dose-response model using GraphPad Prism version 8 software to obtain the IC$_{50}$ and Hill slope (Eq. 2) (Iyer et al., 2006).

**Results**

The thermal unfolding of different concentrations of BCA II was monitored by RT-PCR thermocycler which generated curves that showed a two-state transition (Fig. 2). In the first state, the fluorescence intensity increases upon enzyme unfolding, where the hydrophobic region becomes exposed to the SYPRO Orange dye allowing it to bind with the enzyme. After reaching the plateau, in the second state, the fluorescence intensity begins to decrease as the denatured enzyme-dye complex starts to aggregate. Comparing the fluorescence intensity of different concentrations of BCA II showed that the 3.33 µM concentration of the enzyme gives the highest read of the fluorescence intensity (Fig. 4), so it was chosen to be used in the FTSA experiment to measure the binding affinity ($K_d$) of the inhibitors. Determination of protein stability and its melting temperature were obtained by FTSA, followed by data analysis using GraphPad Prism 8®, the average time was about 3 hours.
Figure 4: Thermal unfolding of different concentrations of BCA II (3.33 µM, 1.67 µM, 0.83 µM and 0.42 µM) monitored by 2X SYPRO Orange each in triplicate. Normalization was done by using Boltzmann equation.

The Melting temperatures $T_m$ (the midpoint of the first state of fluorescence transition) were calculated using (Eq.3). As the inhibitor binds at the hydrophobic region of the unfolding enzyme, the melting temperature $T_m$ increases. The calculated $T_m$ of the enzyme was higher in the presence of the inhibitor and positively correlated to the inhibitor concentration (Figures 5A, 6A, and 7A) show the shifts in melting temperature curves of methazolamide, brinzolamide and dorzolamide HCl, respectively.

The stability of the enzyme increases as the inhibitor concentration is increased. Binding affinity constants ($K_d$s) were calculated using (Eq. 4) for the four inhibitors and they were 5.4±0.085 µM, 1.2±0.44 µM, 2.08±0.63 µM for methazolamide, brinzolamide and dorzolamide, respectively. (Figures 5B, 6B and 7B) show $\Delta T_m$ values which are significant and increased with the inhibitor concentration.
$\Delta T_m$ is large for the higher affinity inhibitor and decreased when the affinity decreases. $K_d$ value of mafenide HCl was hard to detect and it was difficult to observe the $T_m$ shifts where $\Delta T_m$ was less than 2 °C. This could be due to the low solubility of mafenide HCl at high concentration (Figure 8).
Figure 5 (A) Thermal shift curves of unfolding transition of 3.33 µM CA II in the presence of 100 µM, 44.5 µM, 19.8 µM, 8.8 µM and 0 µM of Methazolamide. Data fit to Boltzmann equation gave midpoint $T_m$ of 71°C, 70°C, 69.5°C, 68.5°C and 65°C, respectively, ($R^2 \geq 0.9$). (B) $\Delta T_m$ s are ranging between (0 – 6) °C. n = 3 independent experiments.
Figure 6(A) Thermal shift curves of unfolding transition of 3.33 µM CA II in the presence of 100 µM, 44.5 µM, 19.8 µM, 8.8 µM and 0 µM of Brinzolamide. Data fit to Boltzmann equation gave midpoint $T_m$ of 77˚C, 76.7˚C, 75.6˚C, 74˚C and 64˚C, respectively, ($R^2 \geq 0.9$). (B) $\Delta T_m$ s are ranging between $0 – 13.45$ ˚C. n = 3 independent experiments.
Figure 7: (A) Thermal shift curves of unfolding transition of 3.33 µM CA II in the presence of 100 µM, 44.5 µM, 19.8 µM, 8.8 µM and 0 µM of Dorzolamide HCl. Data fit to Boltzmann equation gave midpoint $T_m$ of 76˚C, 74˚C, 73˚C, 72˚C and 64˚C, respectively, ($R^2 \geq 0.9$). (B) $\Delta T_m$ s are ranging between (0 – 12.6) °C. n = 3 independent experiments.
Figure 8: Thermal shift curves of unfolding transition of 3.33 µM BCA II in the presence of 200 µM, 133 µM, 12 µM and 0 µM of Mafenide HCl. Data fit to Boltzmann equation gave midpoint $T_m$ of 66.8°C, 66.7°C, 66°C and 65°C, respectively, ($R^2 \geq 0.9$). n = 3 independent experiments.
Vmax and Km results:

The $K_m$ value determined from the initial velocity data was found to be $10 \pm 3 \, \mu M$, which is an average of three trials and the error is the standard deviation of the three trials.

Figure 9: Plot of Michaelis-Menten equation. Velocity vs N-NPA concentration was fitted to the Michaelis-Menten equation (Eqn. 1) to determine the $K_m$ value for CA. The fit for CA II-catalyzed reaction was done using the program GraphPad Prsim ® version 8. The reactions were performed in triplicate and the average of the three $K_m$ values was $10 \pm 3 \, \mu M$. 4-NPA concentrations (0.00195-0.25) mM and 6 ng/µl of BCA II.

$K_i$ values were calculated using Cheng-Prusoff equation for competitive inhibitors (methazolamide, brinzolamide, dorzolamide HCl and mafenide HCl), where $K_m = 10\pm3 \, \mu M$ and substrate concentration was 0.25 mM (Laubach, A. E., Wang, E., & Anderson, 2015; Iyer et al., 2006):

$$K_i = \frac{IC_{50}}{1+[S]/K_m}$$
**Ki values** were 4±0.55 nM, 3.5±0.5 nM, 2.5±0.5 nM and 46.5±6.5 nM for methazolamide, brinzolamide, dorzolamide HCl and mafenide HCl, respectively. The reactions were performed in triplicate n=3.

**IC₅₀ results**

The results of the half maximal inhibitory concentration (IC₅₀) for methazolamide, brinzolamide, dorzolamide HCl and mafenide HCl were 0.148±0.024 µM, 0.129±0.015 µM, 0.092±0.01 µM and 1.715±0.16 µM, respectively (Fig.10).

![Graphs showing IC₅₀ values for different compounds](image)

Figure 10: IC₅₀ values were determined by fitting a dose-response (four parameters) curve of the inhibition (%) to the data, using the GraphPad Prism program. (R² ≥ 0.9) and n = 3 independent experiments.
The affinity constants ($K_d$) were about 10-50-fold higher than the potencies and they were not close in the magnitudes to those of $K_i$ values (Table 1).

Table 2: Values of $K_d$, $IC_{50}$ and $K_i$

<table>
<thead>
<tr>
<th>Inhibitor Name</th>
<th>$K_d$ (µM)</th>
<th>$IC_{50}$ (µM)</th>
<th>$K_i$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FTSA</td>
<td>Enzymatic assay</td>
<td>Enzymatic assay</td>
<td></td>
</tr>
<tr>
<td>Methazolamide</td>
<td>5.4±0.085</td>
<td>0.148±0.024</td>
<td>0.004±0.00055</td>
</tr>
<tr>
<td>Brinzolamide</td>
<td>1.2±0.44</td>
<td>0.129±0.015</td>
<td>0.0035±0.0005</td>
</tr>
<tr>
<td>Dorzolamide HCl</td>
<td>2.08±0.63</td>
<td>0.092±0.01</td>
<td>0.0025±0.0005</td>
</tr>
<tr>
<td>Mafenide HCl</td>
<td>ND</td>
<td>1.715±0.016</td>
<td>0.0465±0.0065</td>
</tr>
</tbody>
</table>

Statistical Analysis

For binding assays and enzymatic assays, sample standard deviation for each condition was calculated using the STDEV.S function in Microsoft Excel and Error ranges for fitted $IC_{50}$, $K_i$ and $K_d$ values were defined as 95% confidence intervals.

Discussion

The primary purpose of this study was to examine the ability of applying FTSA in determination of hit to lead compound in research study by comparing it with two of the commonly used traditional kinetic analysis methods by measuring the affinities of four known inhibitors of CA.
FTSA technique has number of advantages such as: it is easy to use, inexpensive, the RT-PCR machine used to run the assay is readily available in most of the laboratories and industries, it doesn’t depend on previous knowledge of the value of the affinity or potency to modify the conditions of the reaction, and it can be used as a primary detection method for the qualitative measurement of the binding affinity of unknown compounds which is one of the initial steps in the drug discovery process.

The findings of this study for (methazolamide, brinzolamide and dorzolamide HCl and mafenide HCl) indicate that the Kd values obtained by FTSA were about 10-50 fold higher than that of the IC$_{50}$ values that were obtained from the enzymatic assay. In addition, Kd values were higher compared to Ki values.

A commonly cited explanation for the difference of these measured values is that the reaction was conducted under different temperatures. FTSA uses a high temperature that can probably reach about $77 \, ^\circ C$ $T_m$ compared to the room temperature ($25^\circ C$) that is used when running the IC$_{50}$ assay (Redhead, Satchell, McCarthy, Pollack, & Unitt, 2017).

The need for a high concentration of the enzyme in this approach to get the proper fluorescence intensity is required to observe the melting temperature shifts between different concentrations of the inhibitor. Thereby making it hard to measure the precise Kd value for a potent drug (Vivoli et al., 2014).

Some reports in the literature concluded that there is a correlation between Kd values and IC$_{50}$ values, which were measured by FTSA and
traditional enzymatic assay, respectively (Bai et al., 2019; Lo et al., 2004; Smirnovienė et al., 2017).

The discrepancy between the values of the $K_d$ using the approach of FTAS which is used in this study as well as the values that were published using other approaches of FTSA could be due to consideration of the enthalpy values ($\Delta H$) of the reaction in their FTSA approach, which can help measuring low $K_d$s values. Especially, for high potent inhibitors (Smirnovienė et al., 2017).

Another finding in this study is that it was difficult to observe the shift in $T_m$ for mafenide HCl (low potency drug) that could be explained by low solubility at high concentration. For some ligands such concentration may be too high due to limited solubility. If the solubility is below the expected $K_d$, then the determination is impracticable and hard to obtain. (Cimmperman & Matulis, 2011) (Matulis et al., 2005).

Limitations of the FTSA include that some ligands may interfere with the dye or interact covalently with the unfolded enzyme. Enzyme folding could be reversible and can cause aggregation at higher temperature (Bai et al., 2019). The binding constant calculated by FTSA is measured at high temperatures and not in the physiological temperature, and this point should be taken in consideration when FTSA is compare with enzymatic assay. However, estimation of lower binding constants $K_d$ is applicable if the Van’t Hoff enthalpy ($\Delta H^\Theta$) of binding is known (Matulis et al., 2005). To determine thermal shift of weakly binding compounds, their concentration should be
more than the expected $K_d$. This could be an issue because they may have limited solubility at high concentration. As a result, it creates a hard observation of the thermal shift (Cimperman & Matulis, 2011).

Despite these limitations, FTSA has several advantages like its simplicity in use for identification of candidate hits, also its general applicability to large different target proteins. The IC$_{50}$ values do not recognize the mechanism of action. Consequently, various unrelated compounds may exhibit the same activity due to interactions at different sites. Moreover, FTSA can identify mechanistic information such as stoichiometry and differentiate covalent and noncovalent interactions. (Matulis et al., 2005)

**Conclusion**

The findings of this study which compared drug affinities determined by enzymatic analysis and FTSA showed that the potencies (IC$_{50}$) of the potent drugs were 10-50 folds lower than that of the $K_d$ values. In addition, $K_d$ values were higher compared to $K_i$ values. The higher values of $K_d$ could be explained by the different temperature conditions used in each assay and the need of high concentration of the enzyme, which make obtaining $K_d$ lower than a half of this concentration hard. In addition, it was difficult to distinguish the $T_m$ shifts of Mafenide HCl ($\mu$M potent drug) a possible factor could be its low solubility at high concentration.

Summing up the results, it can be concluded that kinetic analysis is more sensitive technique, doable and requires lower amount of the enzyme to measure drug affinity than FTSA. More experiments will be needed to verify whether various modifications of the FTSA approach (like using different buffers or salts) can predict more precise measurements of the binding affinity.


https://doi.org/10.1016/j.apsb.2019.05.004