L-Leucine, A Potential Drug to Inhibit Cancer Metastasis

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Abstract:

The human placental isoenzyme is a thermo-stable version of alkaline phosphatase. The high levels of ALP have been associated with tumor growth and cell migration due to epithelial to mesenchymal transformation. We hypothesize that L-leucine could be an inhibitor to inhibit ALP enzymatic activities by using colorimetric enzymatic assay. Our research found that L-leucine can significantly inhibit the enzymatic activity of ALP in a dose-dependent manner, which could be potentially use as a treatment to reduce cancer metastasis.

Introduction:

Alkaline phosphatase (ALP) is a membrane-bound, glycosylated, dimeric metalloenzyme found ubiquitously in the human body [1]. The human placental isoenzyme is a thermo-stable version of ALP encoded as ALPP gene on chromosome 2 [2]. During pregnancy, the level of ALP gradually increases reaching its peak in the third trimester, yet an unproportionally increase of ALP can signify abnormal pregnancy [3]. In addition, high levels of ALP have been associated with serinoma and ovarian cancer [2]. Recent research proves that ALP is associated with tumor growth. If ALP is effectively repressed in tumor cells, epithelial to mesenchymal transformation and cell migration can be significantly reduced such that metastasis becomes limited [4]. Certain human ALP can be inhibited by specific L-amino acids [5]. In fact, the inhibition of the placental isoenzyme by L-phenylalanine was one of the principal criteria used in identification of "Regan isoenzyme" in cancer patients [6]. Inspired by that, we will determine the effect of the addition of L-leucine, in a dose-dependent manner, on the kinetics of the placental ALP. The effect of the proposed non-competitive inhibitor will be assessed in an endeavor to be used as substituent to current pharmaceutical drugs such as Levimasole which limit metastasis.

Method:

Preparation of Sample

Placental alkaline phosphatase extract were resuspended to make the final concentration 1U . The unit of enzyme activity is defined as that amount of enzyme which will hydrolyze 1 µmol of substrate per min under standard assay conditions [4].

Enzymatic Activity Assay

After determining the unlimiting placental alkaline phosphatase concentration from enzyme saturation curve by using 0.5U - 3U of placental alkaline phosphatase, 1.5U placental alkaline phosphatase samples were mixed with various concentrations of L-Leucine(0.045mM, 0.09mM and 0.18mM) along with various concentration of NPP(0.056mM, 0.112mM, 0.56mM, 0.84mM and 1.12mM) in diethanolamine pH 9.8. P-nitrophenyl phosphate (NPP) was used as a substrate since the product inorganic phosphate and p-nitrophenol upon the hydrolysis by alkaline phosphatase. The latter product is a yellow colored molecule that absorbs at 410nm. The absorbance at 410nm was recorded at one second interval for 3 minutes.

Results:

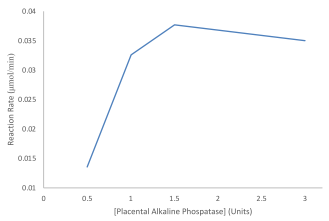

Figure 1: Enzyme Saturation Curve. Various concentrations of placental ALP was reacted with 11.2mM NPP enzymatic activity assay to determine the rate of catalysis as a function of enzyme concentration.

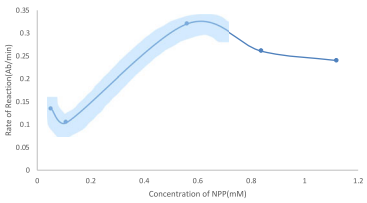

Figure 2: Michaelis-Menten Plot Various concentrations of the substrate, nitrophenyl phosphate (NPP) were added to 1.5 U/ul of ALP in triplicates at a temperature of 37 C to determine Michaelis Constant (Km) and the maximum velocity (V max)

Figure 3.1

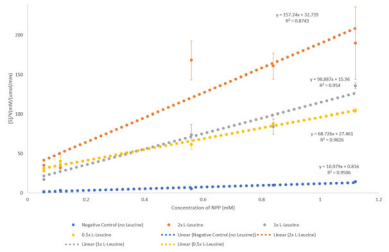

Figure 3.2

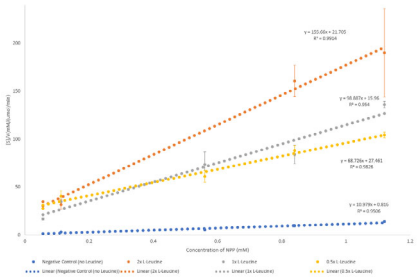

Figure 3: Hanes-Woolf Plot for Placental Alkaline Phosphatase with various concentration of L-Leucine.
1.5U of placental ALP was used to react with various concentration of NPP (1.12mM, 0.84mM, 0.56mM, 0.112mM and 0.056mM) in the presence of various concentration of L-Leucine inhibitor(0.18mM, 0.09mM and 0.045mM

Table 1.1

Substrate	Km [a] (mM)	Vmax [b] (µmol/min)	R^2 of Hanes-Woolf Plot
NPP only	0.09	0.093	0.95
0.5X L-Leucine + NPP	0.40	0.015	0.99
1 X L-Leucine + NPP	0.16	0.010	0.95
2 X L-Leucine + NPP	0.21	0.006	0.87

Table 1.2

Substrate	Km [a] (mM)	Vmax [b] (µmol/min)	R^2 of Hanes-Woolf Plot
NPP only	0.090	0.093	0.95
0.5X L-Leucine + NPP	0.40	0.015	0.99
1 X L-Leucine + NPP	0.16	0.010	0.95
2 X L-Leucine + NPP	0.17	0.0066	**0.98**

Table 1 - Effect of the non-competitive inhibitor, L-Leucine, on ALP reaction rate; Km and Vmax Calculated Based on Hanes Woolf Plot.

a. In Hanes-Woolf plot, Km is calculated by using the equation [S]v = [S]Vmax +KmVmax, where Km is -x intercept.

- Sample calculation, for the Hanes-Woolf Plot with 0.09mM L-Leucine, the equation is y = 98.887x + 15.96. When y = 0, the x intercept is -15.9698.887 = -0.16mM, therefore, the Km = 0.16mM.

b. In Hanes-Woolf plot, Vmax is calculated by using the equation [S]v = [S]Vmax +KmVmax, where Vmax is Km y-intercept.

- Sample calculation, for the Hanes-Woolf Plot with 0.09mM L-Leucine, the equation is y = 98.887x + 15.96.
  When x = 0, the y intercept is 15.96mM/(abs/min), therefore, the Vmax = 0.1615.96 =0.01 µmol/min.

Discussion and Conclusion:

The enzyme saturation curve plotted in Figure 1 displays that at an [ALP] of 1.5 U, the enzyme is no longer limiting to the rate of reaction therefore the effect of a change in the substrate concentration on the rate of the reaction can be assessed. While a drop in the rate of the reaction is observed at an [ALP] of 3 U, it is suggested that thus happened due to the sudden increment change initially 0.5 U (from 0.5 U to 1.5 U) then the increment was increased to 1.5 U to reach 3 U of [ALP]; if the increments remained constant a plateau would have been observed to further highlight that after 1.5 U of ALP, the rate is no longer affected by the enzyme's concentration.

This experiment held the enzyme concentration to be constant at 1.5 U/ul, the temperature to be at the 1.0 M diethanolamine buffer with 0.5mM MgCl2 pH = 9.8 at 37 C, and the pH at 8.0.

To determine the Michalis Constant (Km), the substrate concentration needed to reach half o the maximum velocity, the concentration of the substrate (NPP) was varied while measuring the rate of reaction by measuring the absorbance of the yellow NPP catalysis product, p-nitrophenol at 410 nm. Nevertheless, because the collected data does not obey the linear model of Michaelis-Menten and a Sigmoid curve (s-curve) was observed as shown in Figure 2, a Hanes-Woolf Plot was used to linearize the data and extrapolate that Km = 0.0896 mM and Vmax = 0.0925 µmol/min. Yet, the formation of an s-curve in Michaelis-Menten Plot, Figure 2, indicates that the placental ALP is an allosteric enzyme; ALP has multiple active sites that exhibit a cooperative property such that the binding of one active site affects the affinity of other active sites on the enzyme [1].

Figure 3 along with the calculations displayed in Table 1 show that as the concentration of L-leucine increases from 0.5 x to 2x, the Vmax ***significantly*** decreases by 44.0 % from 0.015 µmol/min to 0.0066 µmol/min respectively. While a noncompetitive inhibitor has constant Km values and a changing Vmax, Table 1 shows that the addition of L-Leucine caused a change in both. Therefore, L-leucine can not be considered as a non-competitive inhibitor, but it is a mixed inhibitor. In this case, the changing Vmax and Km show that L-leucine a affects the affinity of the substrate to the active site as it alters the geometry of the active site and thus affects catalysis and binding [7].

Figure 3 and table 1 have been put in duplicates to display the difference in the calculated Km and Vmax with the inclusion or absence of a suspected outlier. In Figure 3.1 and Table 1.1, the presence of the rate (S/V) at NPP concentration of 0.56mM, decreases the R^2 value of the trendline for 2X l-leucine from 0.9914 to 0.873 as shown in Figure 3.2, to 0.8743. This affects the interpretation of the Vmax trend where when this point in present the Km value changes from 0.21 (Table 1.1) to 0.17 (Table 1.2). To make the extrapolation of Vmax and Km more representative, the higher R^2 value is used to interpret the observed trend. Likewise, the Km obtained for 0.5x L-Leucine from both Table 1.1 and Table 1.2, are suspected to be outliers because the Km value calculated in the addition of 0 (NPP-control), 1x, and 2x of L-leucine almost remains consistent, slightly ranging from 0.1-0.2 mmol So, considering the 0.40 mM Km of the 0.5x L-leucine to be an outlier, Km is shown to remain constant. With that, Table 1.2 displays that the calculated Vmax ***significantly*** decreases by 44.0% from 0.015 µmol/min to 0.0066 µmol/min while Km remains almost constant. The constant Km and decreasing Vmax highlights that the affinity of the substrate to the active site remains unchanged (Km) while the efficiency of the catalysis is decreasing (Vmax). Hence, the obtained data confirms that L-leucine behaves as a non-competitive inhibitor and effectively decreases the placental ALP rate of reaction.

In conclusion, the data collected confirm that Placental ALP is an allosteric enzyme with multiple binding site that affect one another cooperatively, and the addition of L-leucine is effectively inhibiting ALP's catalytic activity in a noncompetitive manner. Thus, since the inhibition of ALP with L-leucine significantly decrease the rate of catalysis, and the L-form is safe to be consumed by humans, and has already been used as a dietary supplement to regulate metabolism and protein synthesis, if prescribed in the correct doses and designed to have a location specific, timed and gentle release, L-Leucine can be used as a substituent to Levamisole as L-leucine is a more naturally derived supplement with high efficacy in limiting ALP catalysis and thus the endothelial to mesenchymal transformation contributing to tumor metastasis.

Acknowledgement

We would like to thank Professor Annette Vincent for her supervision and expert advice throughout this entire project.

We are also grateful to Maya Kemaldean, Bernadette Bernales,Maheen Javed for their indispensable contribution

Reference:

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