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In Vitro Evaluation of Inhibitory Effect of Artemisinin and Dihydroartemisinin on Calcineurin Enzyme

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ABSTRACT

Background and Objective: Artimisinin (an anti-malaria drug) is extracted from *Artemisia annua* and its water soluble derivative is dihydroartimisinin. Previous *in vivo* and *in vitro* studies showed that it has an inhibitory effect on T cells. It is also useful for immunosuppression of immune system.

Materials and Methods: The stable state kinetic for comparison of inhibitory effect of artimisinin, dihydroartimisinin and cyclosporine A on calcineurin activation was used in this study. First, the best inhibitory concentration of artimisinin, dihydroartimisinin and cyclosporine A was calculated. Afterwards, the k_m and V_{max} of them in the presence of substrate, paranitrophenylphosphate (P-NPP) were measured. The K_I was calculated in the presence of cyclosporine A, artemisinin and dihydroartemisinin. In this study, we used distilled water instead of sample in blank and cyclosporinA as a positive control group.

Results: The V_{max} in the control group was 81.97 M/min and in the presence of cyclosporine A and artemisinin or dihydroartemisinin were 81.97 M/min and 66.225 M/min, respectively. The K_m in the absence of inhibitors was 1.886 M and in the presence of cyclosporine A and artemisinin or dihydroartemisinin was 2.819 M and 1.736, respectively. Also, K_I in the presence of artimisinin and dihydroartimisinin was 4.219×10^{-5} M and in the presence of cyclosporine A was 2.021×10^{-5}M.

Conclusion: This study indicates that the inhibitory power of artimisinin and dihydroartimisinin is almost equal and they inhibited calcineurin competitively, while inhibitory effect of cyclosporine A is non-competitive.

1. Introduction

Calcineurin, PP-2B, is a serine/ threonine protein phosphatase (1) that is expressed in the mammalian tissues including T lymphocytes and is especially abundant in the brain. It is a key enzyme of the rate-limiting step in the activation of T lymphocytes and an important regulator of nuclear factor of activated T cells (NFAT), which activates the transcription of genes for cytokines such as interleukin-2 and interferon. Therefore, inhibitors of calcineurin inhibit the activation of T lymphocytes and have been widely used to prevent allograft rejection after solid organ transplantation (2). Artemisinin is originally isolated from *Artemisia annua* (sweet wormwood) (3) and has a history of utilization for more than 2000 years in traditional medicine. In 1976, Chinese scientists reported the anti-malarial activity of artemisinin (3). Researchers showed that artemisinin can suppress the delayed type hypersensitivity (DTH) and has inhibitory effect on the activity of calmodulin. So, both *in vivo* (DTH response) and *in vitro* studies indicate the activity of artemisinin as an immunosuppressive agent (4-6).

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So far, the inhibitory effect of artemisinin and its derivatives on calcineurin enzyme has not been investigated and such studies can be useful in the design of effective derivatives of these drugs that have had the better absorption, distribution, biotransformation and excretion. In this study, we determined the appropriate dose of substrate and inhibitors, then calculated constant values of kinetics (K_m and V_{max}) in the presence and absence of inhibitors.

2. Materials and Methods

para-nitrophenolphosphate (P-NPP), cyclosporine A, calcineurin, artemisinin, dihydroartemisinin and other chemicals were obtained from Sigma (USA).
Spectrophotometer model was DU 800 UV/visible (BEKMAN).

2.1. Enzyme assay

Phosphatase activity of calcineurin was assessed by measuring the para-nitrophenols (P-NPs) released from para-nitrophenolphosphate (P-NPP) as a substrate by a modification of the method of Pallen and Wang (7). Briefly, an hour before the experiment begins, water bath was pre-heated to 37 °C. Then, the activation of calcineurin was occurred at 37 ^{o}C for 30 min in 1.5 ml phasphatase buffer containing 50 mM Tris-HCL (pH 7.4), 100 mM $CaCl_2$, 5 mg/ml BSA and 1 mg/ml calmodulin was prepared at 20 mM Tris–HCL(pH 7.4) and 40 mM $NiCL_2$ was prepared in distilled water (as the calcineurin assay buffer) and 10 mU/ml calcineurin prepared in enzyme solution, then 1.5 ml P-NPP (2 mg/ml) was added and after 20 min, the absorbance was read at 405 nm (Spectrophotometer model was DU 800 UV/visible, BEKMAN). To end the reaction, 1 ml NaOH (1 N) was added to each sample.

2.2. Determination of the optimum concentration of the inhibitors

All stages of the experiment were similar to the enzyme assay except that 1.5 ml calcineurin (10 mU/ml) was pre-incubated with 75 µl of different concentrations of inhibitors (10^{-1} to 10^{-10} mM was prepared in phahsphatase buffer) for 20 min and 75 µl P-NPP (1.5 mg/ml) was added to start the reaction. At the end, the minimum OD_{405nm} was selected as the best concentration of inhibitors.

2.3. Preparation of standard curve

For preparation of standard curve, we added 0, 66, 134, 200, 264, and 330 µl of P-NPP (1mM) into 6 tubes in duplicate to generate 0, 66, 134, 200, 264, and 330 nM/tube P-NPP standard, then the volume was brought to 2 ml with calcineurin assay buffer, afterwards 167 µl of calcineurin enzyme was added to each tube and mixed and incubated for 60 min at 37 °C and at final, all reaction were stopped by 1 ml NaOH (1 N) into each tube and OD was measured at 405 nm.

2.4. Evaluation of the mode of calcineurin inhibition

The inhibition mode of the inhibitors was determined using the optimum concentration of the inhibitors. Briefly, 75 µl of inhibitors equal to 10^{-4} mM (artemisinin and dihydroartemisinin were dissolved in acetonitril) were pre-incubated with 1.5 ml of calcineurin solution for 20 min at 37 ^{o}C in one set of tubes. In another set of tubes, calcineurin was pre-incubated with 75 µl buffer phasphatase (pH 7.4). Then, 75 µl of P-NPP at increasing concentrations (0.5-3.5 mg/ml) was added to both sets of reaction mixture to start the reaction. The reaction was incubated for 20 min at 37 ^{o}C and 1.5 ml NaOH (1N) was added to stop the reaction. The reduction in the amount of released P-NPs was determined spectrophotometrically using a para-nitrophenol standard curve and converted to reaction velocities. A double reciprocal plot (Lineweaver-Burk plot) (1/V versus 1/(S), V is reaction velocity and (S) is substrate concentration) was determined by analysis of the double reciprocal using Michaelis-Menten kinetics.

2.5. Statistical analysis

Statistical analysis was performed using SPSS version 17. The data were analyzed by one way analysis of variance (ANOVA). All the results were expressed as mean ±SE for triplicate determinations.

3. Results

The result of the determination of the optimum concentration of the inhibitors is presented in Fig 1. In this regard, the minimum OD_{405nm} for artemisinin and dihydroartemisinin solution is at concentration of 10^{-4} mM and minimum OD_{405nm} for cyclosporine A solution is about 10^{-5} mM, so this result reveals that the best concentration for all of the inhibitors is 10^{-4} mM. There are also significant difference between artemisinin / dihydroartemisinin and cyclosporine A ($p < 0.05$), while it was not significant between artemisinin and dihydroartemisinin ($p > 0.05$).

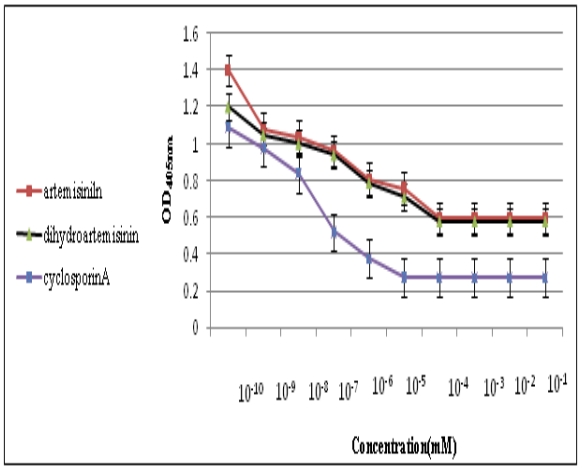

Figure 1: Determination of the optimum concentration of the inhibitors ($p > 0.05$) between artemisinin and dihydroartemisinin and between artemisinin/dihydroartemsinin and cyclosporine A ($p<0.05$).

As results of standard curve in figure 2 show, in this range, there is a linear relationship between OD_{405nm} and their substrate concentrations.

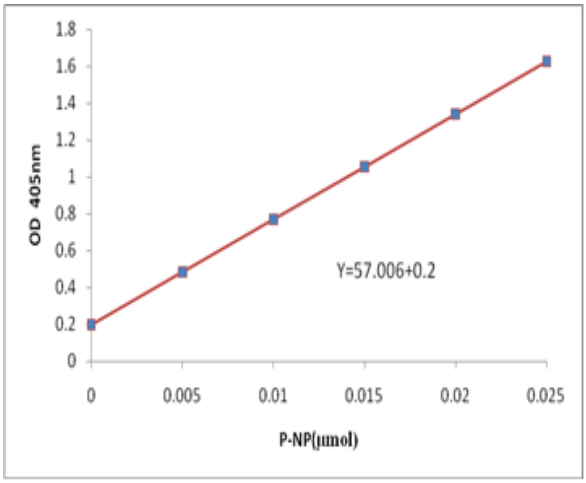

Figure 2: Standard curve of P-NP (all concentrations were prepared in duplicate)

According to results in Table 1, Km, Vmax and KI for artemisinin and dihydroartemisinin are 1.736, 66.225 and 4.219 $\times 10^{-5}$, respectively and these kinetic constants for cyclosporine A are 2.819, 81.967 and 2.021 $\times 10^{-5}$, respectively and Km and Vmax for P-NPP are 1.886 and 81.967, respectively.

Table1: Kinetic constants of artemisinin, dihydroartemisinin and cyclosporine A

kinetic constants	Substrate and Inhibitors		
	Cyclosporine A	P-NPP	Artemisinin Dihydroartemisinin
k_m(M)	2.819	1.886	1.736
v_{max} (M/min)	81.967	81.967	66.225
k_I(M)	2.021×10^{-5}	----------	4.219×10^{-5}

In order to determine the mode of inhibition of this enzyme by artemisinin, dihydroartemisinin and cyclosporine A, lineweaver-Burke plot was made. This plot showed that V_{maX} for artemisinin, dihydroartemisinin and P-NPP is almost the same and K_m for artemisinin and dihydroartemisinin inhibition has increased (Figure 3) and K_m for cyclosporine A inhibition and P-NPP is almost the same, but V_{max} fpr cyclosporine A has decreased (Figure 4).

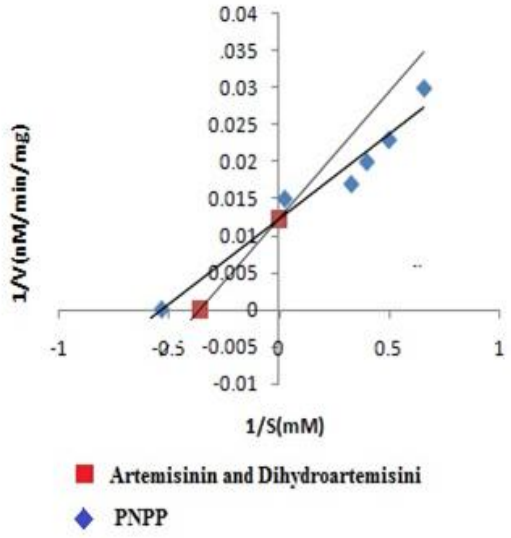

Figure 3: Mode of inhibition of calcineurin by artemisinin and dihyroartemisinin

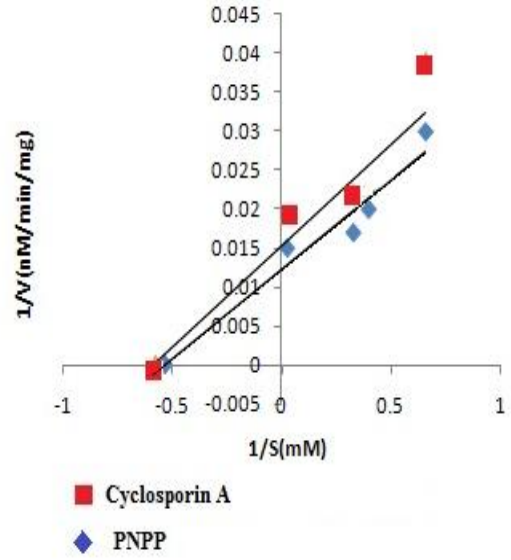

Figure 4: Mode of inhibition of calcineurin by cyclosporine A

4. Discussion

Calcineurin (CaN), a serine/threonine phosphatase enzyme, is expressed in immune cells, muscle cells and neurons and plays significant roles in regulating immunological responses in lymphocytes cells (8). Immunosuppressive drugs cyclosporine A (CsA) and FK506 is used in patients of organ transplantation as inhibitors of CaN (9). These inhibitors finally inhibit the T-cell activation (10). These drugs have different side effects including nephrotoxicity, hyperkalemia, hypomagnesaemia, hyperlipidemia, hypertension, gingival hyperplasia and hypertrichosis in addition to being diabetogenic (11, 12). We used P-NPP as a substrate because of its colored product that is readable at 405 nm and cyclosporine A is used as a standard inhibitor. Artemisinin and dihydroartemisinin was dissolved in acetonitril because of their hydrophobic property. Because the OD_{405nm} has direct correlation with the amount of NPP produced, the lowest OD_{405nm} mean is minimum production of NPP and that mean is the lowest concentration of inhibitors that has had maximum inhibitory effect, so we chose the concentration of 10^{-4} mM of inhibitors. The V_{max} in the absence of inhibitors (81.97 M/min) is equal to the V_{max} in the presence of artemisinin or dihydroartemisinin (81.97 M/min) and is higher than the V_{max} in the presence of cyclosporine A (66.225 M/min) which indicated that probably cyclosporine A non-competitavely inhibits the calcineurin.

The K_m in the absence of inhibitors (1.886 M) was less than the K_m in the presence of artemisinin or dihydroartemisinin (2.819 M) and is approximately equal with it in the presence of cyclosporine A (1.736 M) which probably indicated that a higher substrate concentration is needed for rectifying artemisinin or dihydroartemisinin effect and these inhibitors competitively inhibit the enzyme. The K_I in the presence of cyclosporine A (2.021×10^{-5} M) is one half of K_I in presence of artemisinin or dihydroartemisinin (4.219×10^{-5} M) which indicated that used concentrations of artemisinin and dihydroartemisinin are two times of cyclosporine A.

Taken together, it was found out that artemisinin and dihydrartemisinin have inhibitory effect on calcineurin and their inhibitory effects are the same and inhibit the enzyme competitively. For inhibition of calcineurin, artemisinin and dihydrartemisinin should be used at concentrations two times of cyclosporine A.

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