Effect of dihydropyrimidine derivatives on kinetic parameters of E. carotovora L-asparaginase

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Abstract- Purified L-asparaginase in combination with other anticancer drugs like pyrimidine derivatives is administered usually in the body to treat ALL. In the present study, L-asparaginase was purified from Erwinia carotovora up to 247.6 fold and its catalytic properties were studied in the presence of eight different dihydropyrimidine (DHP) derivatives, out of eight derivatives only two viz 4- (2'-hydroxy phenyl) -6- methyl -2- thioxo)-1 -N - benzilydene - 1, 4 - dihydropyrimidine - 5 - carboxylic acid ethyl ester (P₁) and 4 -(2'-hydroxy-5'-chlorophenyl)-5-acetyl-6-methyl-2 pyrimidinone (P₂) were found to be activators of L-asparaginase. Their catalytic effect was assayed at optimum pH 8.6 and at temperature 35°C in the absence and presence of derivatives P₁ and P₂ (20-40 µM) at 0.02-0.1 mM concentration of asparagine. It was found that derivatives below the concentration 5 µg/ml have no effect on the activity. Derivative P₁ is found to be a strong activator of the asparaginase activity that was reflected by an increase in the Vₘₐₓ (1.75 fold by P₁ and 2.80 fold by P₂ respectively) and decrease in the Kₘ (0.91 fold by P₁ and 0.81 fold by P₂ respectively). The activation of asparaginase is explained by suppressing the cooperativity for the substrate, producing hyperbolic kinetics with Km of 0.080 mM and by 3 fold increase in the Vₘₐₓ of the enzyme. The activation by derivative P₁ and P₂ were additive, at optimal or suboptimal concentrations of both activators (up to 30 µg/ml). The DHP derivatives were further analyzed for quantitative SAR study (QSAR) by using PASS, online software to determine their Pa value. Toxicity and drug relevant properties were analyzed by PALLAS software in terms of their molecular weight and log p values. The results showed both the derivatives P₁ and P₂ are positive modulators of asparaginase activity and may support the development of novel combination therapy for the treatment of Leukemia and solid blood tumors.

Key words: Erwinia carotovora, L- asparaginase, dihydropyrimidine derivatives, activation, cooperativity

Introduction
L-asparaginase (L-asparagine amino hydrolase, E.C.3.5.1.1) catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonia [1-5]. L-asparaginase is an essential amino acid for the growth of tumor cells, while the growth of normal cells is not dependant on its requirement. The presence of L-asparaginase deprives the tumor cells from an important growth factor asparagine and therefore they fail to survive. Cleavage of external asparagine may block protein synthesis in the leukemic cells and thereby causes apoptosis of tumor cells [6-9]. Thus L-asparaginase has been a clinically acceptable anti tumor agent for the effective treatment of acute lymphoblastic leukemia (ALL) and lymph sarcoma [10, 11]. However, it has been reported that clinical employment of bacterial asparaginase is accompanied by the development of side effects. Besides minor side effects such as an allergic reaction and vomiting, L-asparaginase therapy of ALL has some serious side effects. Hypersensitivity reactions to chemotherapeutic antineoplastic agents such as L-asparaginase are most common [12], with many other unusual complications of neutropenia [13], tubular and glomerular dysfunction [14], drug induced pancreatitis [15], acute hepatic dysfunctions [16], cerebral thrombotic complications [17], growth hormone deficiency [18] and deficiency of antithrombin [19]. Therefore, the treatment protocols of ALL and lymph sarcoma do not employ only L-asparaginase as a single agent; instead, it is always a part of multiple agent regimens and combined with drugs having definitive immunosuppressive effects [20]. An immunosuppressive agent prevents the immune system from reaction with appearance of foreign protein (asparaginase) in patients’ blood circulation [21]. Although the asparaginase has been found in number of organisms like serum of guinea pig and rodents, chicken liver, yeast, molds, plants and number of bacteria, not all of these enzymes are clinically active [22]. L-asparaginase from E. coli and E. chrysanthemii has been used as
chemotherapeutics in ALL for the past three decades [23, 24]. However recent discoveries have indicated that the L-asparaginase from E. carotovora is more efficient and exhibits fewer side effects as compared to L-asparaginase obtained from E. coli and E. chrysanthemii [25]. Pyrimidine molecules, which comprises an integral part of nucleic acids now a days are of special importance in medicinal chemistry and received considerable attention because of their diversified activities such as potential antihypertensive agents [26] antiviral [27], antitumor [28], antibacterial agents [29] and immunosuppressive agents [30]. The broad spectrum biological activities of DHP derivatives are depends upon the stereochemistry between the aryl group and DHP ring [31]. It is proposed that L-asparaginase in chemotherapy regimen should have high affinity towards the substrate asparaginase and selectively deprives the tumor cells [32].

The diversified activities of DHP derivatives specially antitumor and antileukemic properties of asparaginase prompted us to synthesize different pyrimidine derivatives, to extract and purify E. carotovora L-asparaginase and to study its catalytic activity in absence and presence of 4- (2'-hydroxy phenyl)-6-methyl-2-thioxo)-1 -N-benzilydene - 1, 4 - dihydropyrimidine - 5 -carboxylic acid ethyl ester (P1) and 4-(2'-hydroxy-5'- chlorophenyl)-5-acetyl-6-methyl-2-pyrimidinone (P2), the dihydropyrimidine derivatives.

**Materials and methods**

**Materials**

All the chemicals were purchased from Hi Media Laboratories (Mumbai, India). All other reagents used were of analytical grade and used without further purification unless stated otherwise.

**Enzyme production and partial purification**

Asparaginase (E.C.3.5.1.1) was obtained from E. carotovora (M.T.C.C.1428) by fermentation [33] and purified according to method of Kawaga et.al. [34]. Protein was measured by the Bradford dye binding method using bovine serum albumin as the standard [35].

**Molecular weight determination**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 3mm slab gel of 6 % acryl amide in a tris borate buffer pH 7.2 containing 0.1 % SDS. The gels were stained with 0.025 % coomassie brilliant blue R-250 and destained by 1.0 % acetic acid solution. phosphorylase b (97.3kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), soya bean trypsin inhibitor (20 kDa), and lysozyme (14.3 kDa) were used as molecular weight markers [36].

**Chemistry of P1 and P2 dihydropyrimidine derivatives**

The synthesis of dihydropyrimidine derivatives P1 and P2 have already been reported elsewhere [37, 38]. A mixture of Urea / thiourea / thiourea derivatives (0.1 M), acetyl acetone/ Ethyl acetoacetate (0.1 M) and substituted aromatic aldehyde (0.1 M) in methanol (25 ml) and catalytic amount of piperidine was added to the mixture and refluxed for 3h. The contents were kept for refrigeration for overnight. The solid separated out was filtered and recrystallized from proper solvent.

Melting points of the dihydropyrimidine derivatives P1 and P2 were recorded on an Electro thermal digital melting point apparatus and were uncorrected. IR spectra were obtained on a Nicolet FTIR 500 Spectrophotometer using KB pellets.1H NMR (400 MHZ) spectra were recorded on Bruker Avance Spectrometer in DMSO d6. Chemical shifts were recorded in δ ppm with reference to TMS as an internal standard.

**Protein Measurements and L-asparaginase assay**

A 0.1 ml sample of purified enzyme solution, 0.9 ml of 0.1M sodium borate buffer (pH 8.5) and 1 ml of 0.04 M L-asparagine solutions were combined and incubated for 10 min at 37 ºC. The reaction was terminated by the addition of 0.5 ml of 15% tri chloro acetic acid. After centrifugation, 0.2 ml supernatant was diluted to 8 ml with distilled water, treated with 1.0 ml of Nessler’s reagent and 1.0 ml of 2.0 M NaOH. The color reaction was allowed to proceed for 15 min before the absorbance at 500 nm was determined. The absorbance was then compared to a standard curve prepared from solutions of ammonium sulfate as the ammonia source [39]. One international unit of L-asparaginase is the amount of enzyme which liberates 1µmole of ammonia in 1min at 37 ºC [40]. The protein content of the enzyme was made on whole cell suspension or crude enzyme preparations from the calibration curve of bovine serum albumin by the standard method [41].

**Determination of kinetic parameters**

The K_m and V_max values were determined in the presence and absence of DHP derivatives (20-40 µg/ml) at 0.10-0.50 mM asparagine
concentration. The $K_m$ and $V_{max}$ values were obtained from Lineweaver double reciprocal plot [42].

**Reaction Mechanism of L-asparaginase**

The precise mechanism of L-asparaginase is still unknown although the hydrolysis (figure 1) is known to proceed via a beta acyl intermediate [43].

**Proposed Mechanism of interaction of DHP derivative $P_1$ and $P_2$ with asparaginase**

Both the derivatives interacts with the reactive functional groups of enzymes, DHP derivative $P_1$ with -COOC$_2$H$_5$ (ethyl ester) (scheme 1) and $P_2$ with -COCH$_3$ (acetyl) group (scheme 2) may be coupled covalently to a reactive group of asparaginase especially lysine residues or the N terminal of amino group in proteins under conditions of mild pH and temperature to yield the imine derivative of $P_1$ and $P_2$ (Scheme 3 and 4).

**Drug prediction Analysis**

All the computational work was performed using the BioMed Cache, molecular designing and modeling tool developed for Microsoft operating system developed for Microsoft operating system. Pharmacological activities of DHP derivatives were developed by QSAR study using PASS (Prediction of Activity Spectrum for Substance) online software. It is used to determine $Pa$ values. Osiris Property explorer an organic chemistry portal was used for evaluation of toxicity and drug relevant properties of DHP derivatives. While PALLAS, a computer tool was used for studying molecular weight, total Hydrogen Bond Donor count (HBD) and Hydrogen bond Acceptor count (HBA). The Log P values were calculated and studied according to the Lipinski rule of 5 in the form of Lipinski score.

**Results and discussions**

The synthesized DHP derivatives were characterized by IR and NMR spectral analysis. 4-(2'-Hydroxy phenyl) - 6 -methyl - 2-thioxo-1-N - benzylidene - 1, 4 -dihydropyrimidine - 5 -carboxylic acid ethyl ester ($P_1$) M .P. 217ºC, yield 85 %. (Scheme 1) IR (KBr, $V_{max}$ , cm$^{-1}$) : 3340, 3210, 1730,1595,1480,1118,2172,1080. 1H NMR (DMSO-d$_6$): 9.67 (s, 1H, NH, D$_2$O exchangeable) 7.45 - 7.90 ( m, 9H, Ar - H), 9.30 (s,1H, N= C-H), 2.15 (s, 3H, CH$_3$), 4.10 (q, 2H, -COOCH$_2$CH$_3$), 3.95 (t, 3H, -COOH$_2$ CH$_3$), 5.15 (s, 1H,4-C-H), 10.50 (s, 1H, OH, D$_2$O exchangeable).

4-(2'- Hydroxy -5'- chlorophenyl) - 5 - acetyl - 6 -methyl - 2- pyrimidinone ($P_2$) M.P = 204ºC, yield 80% (Scheme 2) IR (KBr, $V_{max}$ cm$^{-1}$) 3360, 3206, 3065, 1685, 1665, 1495, 1090. 1H NMR (DMSO-d$_6$): 9.67 (s, 1H, NH, D$_2$O exchangeable), 10.50 (s, 1H, OH, D$_2$O exchangeable), 1.98 (s, 3H, CH$_3$), 3.48 (s, 3H, -COCH$_3$), 7.10-7.62 (m, 3H, Ar-H), 4.92 (s, 1H, 4-CH). The partial purification of the asparaginase crude extract was obtained by ammonium sulfate (70%) precipitation. The total protein was decreased from 1.41 mg to 0.29 mg in the ammonium sulfate precipitation step. The specific activity was increased to 0.751 U/mg and 9.83 U/mg after the S-sepharose gel filtration and DEAE cellulose column chromatography respectively (Table 2). SDS-PAGE showed one band of enzyme with electrophoretic mobility of 9.8 cm. By using different standard proteins of known molecular weights it was found that the apparent molecular weight of Erwinia carotovora (M.T.C.C.1428) asparaginase was 36.5 kDa (Figure 2). The possible effect of all the eight DHP derivatives on asparaginase activity was initially assayed at fixed concentrations (0.085 mM) of the substrate and at various concentrations (1-30 µg/ml) of DHP derivatives. Out of eight only two DHP derivatives were able to activate rate of asparagine hydrolysis by the enzyme (unpublished data for $P_3$-$P_8$). The maximal activation of asparaginase by $P_1$ was about 114.8 fold with an EC$_{50}$ of 10 µg/ml and by $P_2$ was about 142 fold with an EC$_{50}$ of 12 µg/ml (Figure 3, Table 4). This activation by DHP derivatives was completely reversible by dilution, did not affect the native molecular asparaginase structure that was confirmed by SDS -PAGE. Results of the kinetic parameters obtained for both the in the absence and in the presence DHP derivatives shown (Table 3 and Figure 4-6). The activation by DHP derivatives is revealed by an increase in the $V_{max}$ (1.75 and 2.8 fold by $P_1$ and $P_2$ derivatives respectively) and decrease in the apparent $K_m$ (from 0.087 mM in the control to 0.080 mM (Figure 3) and 0.071 mM (Figure 4) in the presence of $P_1$ and $P_2$ derivatives respectively). With the purified enzyme obtained by ourselves was of two components one co-operative with lower $S_{0.5}$ [0.045 mM; $h$ (Hill coefficient) = 2.28 Figure 7a] and $V_{max}$ (2.84 I.U.) a second non cooperative component with higher $K_m$ (0.080 mM) and $V_{max}$ (14.28 I.U.) In the presence of both the derivatives the saturation curve of asparaginase became hyperbolic ($h = 1,$
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Figure 7 b, c) with an apparent $K_m$ of 0.080 mM, 0.071 mM and $V_{max}$ 25 IU and 40 IU respectively. The probable mechanism of activation of asparaginase in presence of derivative $P_1$ and $P_2$ may be due to the fact that the derivatives may bind to the site distant from the binding site especially with lysine residues or N terminal amino acid residues to form imine derivatives of asparaginase. Thereby it contributes to the proper orientation of the substrate and influence the rate of catalysis and substrate affinity. It has been reported that none of the lysine residues is located in the interior region of protein involved in substrate binding and catalysis [44].

Drug Prediction Analysis has shown that derivatives $P_1$ and $P_2$ provide important physiological properties for Asparaginase activation. The results suggested that activity of DHP derivatives depends upon the molecular weight and polarity of DHP derivatives. $P_1$ with $P_a$ values of 0.42 and $P_2$ with 0.38 were found to be nearer to the standard $P_a$ value 0.5. The $P_1$ MW (391) was found to be more effective than $P_2$ MW (279.5). The Log P values of $P_1$ (4.458) and $P_2$ (5.246) gives important information about its hydrophobicity and hydrophilicity and found to be effective as values are nearer to the standard.

Conclusions
The present investigation on the catalytic activity of L-asparaginase was prompted by our interest in the reactivity and hypersensitivity of L-asparaginase during therapeutic use. In the present study, the results clearly shows that derivatives $P_1$ and $P_2$ behave like positive modulators of homotetrameric Erwinia asparaginase activity [45], acting through binding site that is different from the substrate binding site. Derivative $P_1$ and $P_2$ have no structural similarity to the true substrate of the enzyme and its activation is reversible, indicating that the effect may be due to a conformational change of the enzyme in presence of derivatives. Activation of L-asparaginase by the derivative $P_1$ and $P_2$ is additive and this data reveals that two derivatives may interacting with two different sites of L-asparaginase. The ($P_2$) pyrimidinone derivative having chloro group was less effective in modulating the activity of L-asparaginase as compared to the thioxo ($P_1$) pyrimidine derivative. The structural changes of derivatives also affect catalytic activity of asparaginase and affinity for the substrate. The asparaginase injections has wide medicinal applications in treating Leukemia and other blood system tumors, as high catalytic activity of asparaginase in presence of derivative $P_1$ and $P_2$ would deplete the concentration of exogenous asparagine that is the prerequisite for growing tumor cells. The Asparaginase injections consist of reduced glutathione, water and supplementary materials [46]. In this aspect it would be feasible on biotechnological ground to provide it as supplementary material like other compounds. Such preparation process has the features of high stability and has well maintained structure and activity of asparaginase. In our laboratory studies we also found the potent inhibitory activity of $P_1$ and $P_2$ derivative against the asparagine synthetase (unpublished data). The reported data opens the questions of possible role of DHP derivatives in combination chemotherapy protocols for ALL.

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References
Table 1- Activity and purity of L-asparaginase from *E. carotovora* obtained at different steps of Purification

<table>
<thead>
<tr>
<th>sample</th>
<th>Volume (ml)</th>
<th>Activity (U/ml)</th>
<th>Total activity (units)</th>
<th>Protein concentration (mg/ml)</th>
<th>Specific activity (U/mg)</th>
<th>Fold purification (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>100</td>
<td>0.05</td>
<td>5.65</td>
<td>1.41</td>
<td>0.04</td>
<td>----</td>
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<tr>
<td>S-sepharose column</td>
<td>30</td>
<td>0.63</td>
<td>19.1</td>
<td>0.85</td>
<td>0.75</td>
<td>18.9</td>
</tr>
<tr>
<td>CM cellulose</td>
<td>30</td>
<td>1.07</td>
<td>32.3</td>
<td>0.78</td>
<td>1.3</td>
<td>34.7</td>
</tr>
<tr>
<td>Ammonium sulfate precipitation</td>
<td>25</td>
<td>1.84</td>
<td>46.1</td>
<td>0.54</td>
<td>3.4</td>
<td>85.9</td>
</tr>
<tr>
<td>DEAE cellulose</td>
<td>15</td>
<td>2.85</td>
<td>42.7</td>
<td>0.29</td>
<td>9.8</td>
<td>247.6</td>
</tr>
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</table>

Table 2- Summary of the kinetic parameters of asparaginase in the absence and in the presence of DHP derivatives P₁ and P₂

<table>
<thead>
<tr>
<th>Addition</th>
<th>Kₘ (mM)</th>
<th>Vₘₐₓ (IU)</th>
<th>S₀.₅</th>
<th>Hill coefficient (h)</th>
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<tr>
<td>None</td>
<td>0.087, 0.16₀ᵃ</td>
<td>14.28, 7.2₀ᵃ</td>
<td>2.84₀ᵇ</td>
<td>0.045₀ᵇ</td>
</tr>
<tr>
<td>P₁</td>
<td>0.071</td>
<td>25.0</td>
<td>0.045</td>
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<tr>
<td>P₂</td>
<td>0.080</td>
<td>40.0</td>
<td>0.045</td>
<td>944-1</td>
</tr>
</tbody>
</table>

Kinetic parameters (mean ± SD) are derived from the corresponding double-reciprocal plots by linear least squares fitting from three different experiments. ᵃVₘₐₓ and Kₘ values of second noncooperative component of the saturation curve for asparaginase activity. ᵇVₘₐₓ, S₀.₅ and h of the first component of the saturation curve for asparaginase activity.

Table 3- Additive effect of DHP derivatives P₁ and P₂ on the *Erwinia carotovora* asparaginase

<table>
<thead>
<tr>
<th>Additions (µg/ml)</th>
<th>P₁ (µg/ml)</th>
<th>P₂ (µg/ml)</th>
<th>asparagine (0.40mM)</th>
</tr>
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<tr>
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<td>----</td>
<td>----</td>
<td>1</td>
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<tr>
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<td>----</td>
<td>36.3</td>
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<tr>
<td>10</td>
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<td>----</td>
<td>62.6</td>
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<td>110</td>
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<td>114</td>
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<td>----</td>
<td>10</td>
<td>----</td>
<td>97.7</td>
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</tr>
<tr>
<td>50</td>
<td>50</td>
<td>----</td>
<td>68.6</td>
</tr>
</tbody>
</table>

Enzyme activity determined at a fixed concentration of 0.40mM for asparagine. The activity in the absence of both derivatives was taken as 1. The values presented (mean ± SD from three different experiments) are the quotient of (activity observed at different concentration of Pyrimidine derivative) / (activity obtained in its absence).
Scheme 1: 4-(2'-hydroxy phenyl) - 6 -methyl - 2- thioxo-1- N - benzilydene - 1,4 dihydropyrimidine - 5 - carboxylic acid ethyl ester (P₁)

Scheme 2: 4-(2'-hydroxy -5'- chlorophenyl) - 5 - acetyl -6 - methyl - 2- pyrimidinone (P2)

Scheme 3. Possible mechanism of P₁ imine derivative formation
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Scheme 4: Possible mechanism P2 imine derivative formation

**Figure 1.** Reaction mechanism of L-asparaginase

**Figure 2.** SDS PAGE of the purified L-asparaginase from the *E. carotovora*
Lane 1 molecular weight marker proteins, Lane 2 purified asparaginase, Lane 3 purified asparaginase in presence of derivatives P1 and P2, Lane 4 crude preparation
Figure 3. Effect of different amount of Pyrimidine derivative on the L-asparaginase catalytic activity
The activity observed at asparagine concentration of 0.085 mM in the absence of pyrimidine derivative. The values presented (mean ± SD from three different experiments) are ((activity observed at different concentration of pyrimidine derivative)/(activity obtained in its absence)). The half maximal dose for activation, Kai was obtained from double reciprocal plots of the results presented.

Figure 4. Lineweaver Burk plot of activation of Erwinia asparaginase by pyrimidine derivative P1 with asparagines concentration (0.085 mM), enzyme (3.4 U/mg) in sodium borate buffer(100mM, pH 8.5) to a total volume of 3.05 ml.
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Figure 5. Lineweaver Burk plot of activation of Erwinia asparaginase by pyrimidine derivative P2 with asparagines concentration (0.085 mM), enzyme (3.4 U/mg) in sodium borate buffer (100mM, pH 8.5) to a total volume of 3.05 ml.

Figure 6. Lineweaver Burk plot of activation of Erwinia asparaginase by pyrimidine derivative P1 and P2 with asparagines concentration (0.085 mM), enzyme (3.4 U/mg) in sodium borate buffer (100mM, pH 8.5) to a total volume of 3.05 ml.

Figure 7 a, b and c. Hill plot of asparaginase catalytic activity in presence and absence of dihydropyrimidine derivatives with asparagine concentration (0.085 mM), enzyme (3.4 U/mg) in sodium borate buffer (100mM, pH 8.5) to a total volume of 3.05 ml.