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# Biocatalytic synthesis of valaciclovir using commercial enzymes

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## ARTICLE INFO

## ABSTRACT

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Proof-of-concept has been demonstrated for the biocatalytic transformation of aciclovir into valaciclovir, attaining high conversions from a solid-to-solid biotransformation in L-valine methyl ester using various formulations of Subtilisin Carlsberg activated for use in organic solvent.

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Valaciclovir **1**, the active ingredient of Valtrex<sup>®</sup>, is a water soluble L-valine ester prodrug of the HIV reverse transcriptase inhibitor aciclovir **2**. It can be hydrolysed to **2** and L-valine **3** in vivo by the action of enzymes, one of which is the 'so-called' human biphenyl hydrolase-related protein (hBph-rp) that has been cloned by Puente and López-Otin and expressed in *E. coli* (Scheme 1).<sup>1</sup>

Valaciclovir is currently produced commercially by a two-step process using 1,3-dicyclohexylcarbodiimide (DCC) coupling of *N*benzyloxycarbonyl-L-valine (CBz-L-valine) and aciclovir followed by hydrogenolysis with a palladium catalyst to remove the protecting group (Scheme 2). Alternative chemical methods for preparing valaciclovir have been reported but, like the original method of manufacture, all require the use of an amino acid moiety containing a nitrogen protecting group which has to be removed in a subsequent step.<sup>2</sup>

The work of Puente and López-Otin led us to consider the possibility of synthesising valaciclovir by reverse hydrolysis using hydrolase enzymes (Scheme 2, dashed arrow).<sup>1</sup> An atom-efficient, chemoselective method of preparing amino acid esters that does not require protection of the amino acid nitrogen atom might provide significant production cost benefits for manufacture through the use of fewer chemical transformations and reducing the costs of starting materials.

The use of mammalian enzymes in active pharmaceutical ingredient (API) production, particularly in the final chemistry stage, is undesirable due to concerns over the risk of infection by transmissible spongiform encephalopathies (TSEs).<sup>3</sup> Therefore, we decided to screen commercial hydrolases and a selection of non-commercial hydrolases prepared from microbial sources, for activity using the human enzyme hBph-rp as a positive control.

To identify non-mammalian commercial and non-commercial enzymes that might be capable of synthesising valaciclovir, we decided to screen for valaciclovir hydrolysis activity as this can be performed under mild aqueous conditions. Although the preferred enzyme for hydrolysis will not necessarily be preferred in the reverse reaction, particularly in the case of lipases where the water nucleophile appears to be introduced through a separate tunnel,<sup>4</sup> this screening approach reduces the potential to miss a hit through the use of inappropriate conditions in the more difficult synthesis reaction.

Using a hydrolysis procedure adapted from the work of Kim et al.,<sup>5</sup> 62 immobilised and free commercial enzyme preparations were screened and 19 were competent catalysts for the hydrolysis of valaciclovir to aciclovir. When the same enzyme from different suppliers was tested, no qualitative difference was observed. Of the microbial strains that were tested based on our selection criteria,<sup>6</sup> 5/6 yeast strains, 7/14 Gram-negative bacterial strains, 0/5 Gram-positive bacterial strains and 1/6 filamentous fungi strains gave extracts displaying valaciclovir hydrolysis activity.

Given the large number of commercial enzymes that were found to display valaciclovir hydrolysis activity, subsequent synthetic studies focused on these, and in particular Subtilisin A (Subtilisin protease from *Bacillus licheniformis*, also known as Subtilisin Carlsberg) and ChiroCLEC-BL (a cross-linked form of the same enzyme), which displayed the highest activity of all the enzymes tested including hBph-rp.

We anticipated that the synthesis of valaciclovir by reverse hydrolysis would be highly challenging to realise biocatalytically because of the poor solubility of L-valine and aciclovir in both organic and aqueous solvents. This is further hampered by the zwitterionic nature of L-valine which reduces the concentration of the protonated acid form necessary for acyl enzyme formation at pH values that are considered to be compatible with enzyme activity. The possibility that the favoured amino acid form might be generated within the enzyme active site, along with precedent for the enzymatic esterification of amino acids with sugars,<sup>7</sup> led us to pursue this option. However, all attempts to esterify L-valine with aciclovir failed to yield the desired product with the enzymes tested.<sup>8</sup>



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Scheme 1. Reagents: hBph-rp, pH 7.4 phosphate buffer.



Scheme 2. Reagents: (i) CBz-L-valine, DCC, DMAP, DMF; (ii) H<sub>2</sub>, 5% Pd/C, DMF, 0.5 M HCl<sub>ag</sub>; (iii) desired enzyme catalysed esterification, L-valine.

Given the high polarity of the starting materials and product, we postulated that in an organic solvent they might bind tightly to the protein resulting in inhibition. However, this appeared unlikely to be product related given that Ke and Klibanov have demonstrated the propanolysis of racemic methyl phenylalaninate in organic solvent in the presence of CLEC enzymes.<sup>9</sup> In fact, we found that the transesterification of valaciclovir with methanol could also be effected under these conditions by a variety of enzymes of which Subtilisin A and ChiroCLEC-BL were again preferred, both giving very similar activity. If L-valine were inhibiting the esterification reaction, then spiking experiments should inhibit the transesterification, but we found this not to be the case, and so presumably the esterification reaction is limited by the rate of acyl enzyme formation.

Following the successful transesterification of valaciclovir with methanol, we instead turned our attention to the reverse of this reaction as a potential biocatalytic route to valaciclovir (Scheme 3). Irreversible acyl donors such as *p*-nitrophenyl or vinyl esters that have been used to selectively prepare amino acid esters of other nucleoside analogues such as nelarabine,<sup>10</sup> ribavarin<sup>11</sup> and lobucavir<sup>12</sup> were not considered as they require preparation from expensive N-protected amino acids. We instead chose the hydrochloride salt of L-valine methyl ester **5** as an acyl donor for this non-selective transformation as it can be easily produced in a single step from the amino acid, would give direct access to product as the desired hydrochloride salt and provide transient salt protection of the nitrogen atom.<sup>13</sup>

Disappointingly, attempts to transesterify L-valine methyl ester hydrochloride with aciclovir in the presence of 4 Å molecular sieves and various enzymes failed to afford any valaciclovir. Testing the free amine of **5** instead, enantiopure valaciclovir was finally produced in the presence of ChiroCLEC-BL with best rates observed



Scheme 3. Desired enzyme catalysed transesterification.



**Figure 1.** Screening results for the transesterification of L-valine methyl ester (free amine) with aciclovir using ChiroCLEC-BL or Subtilisin A at room temperature in the presence of 4 Å molecular sieves.

in neat methyl L-valinate or with *tert*-butanol cosolvent (Fig. 1). Surprisingly, Subtilisin A was unable to produce valaciclovir under these conditions even though it had displayed similar activity to ChiroCLEC-BL in the reverse reaction. Given that all enzymes require a minimum quantity of water for activity,<sup>14</sup> we suspected that the discrepancy in activity between Subtilisin A and ChiroC-LEC-BL in the forward and reverse transesterification reactions might have arisen as a result of different catalyst hydration levels in the different solvents used and that the CLEC enzyme might have contained sufficient water before solvent addition.

Unfortunately ChiroCLEC-BL is no longer commercially available and so it was important to obtain an alternative formulation of Subtilisin Carlsberg that gives a comparable activity before reaction optimisation. The activation of lyophilised enzymes in organic solvent has been studied extensively,<sup>15</sup> but we found that by using the simple procedure reported by Chen et al.,<sup>16</sup> Subtilisin A, precipitated from aqueous solution with *tert*-butanol, gave comparable wt/wt activity to the CLEC enzyme following filtration and an acetone wash. In fact, the cheap, commercial formulation Alcalase 2.5 L that is, available in bulk quantities, could be treated in a similar manner to give a catalyst of comparable activity and both could be cross-linked with glutaraldehyde in organic solvent to produce an insoluble version of the enzyme without loss of activity. Alternatively the enzyme could be precipitated in situ by the addition of Alcalase 2.5 L directly to the reaction mixture. However, Alcalase 2.5 L contains a significant quantity of 1,2-propanediol (30%) and conversions were inferior using this technique, presumably due to competition of the diol nucleophile with aciclovir.

With a suitable first generation catalyst in hand we next turned our attention to improving the productivity of the transesterification reaction. We found that increasing the reaction temperature had a significant positive impact on reaction rate and was optimum at 50 °C. Water content was critical to maintain activity with optimum rates observed at 1-3% v/v of water. Further increases in water content resulted in reduced product formation. Under identical conditions, untreated Subtilisin A gave no product, illustrating that hydration of the dehydrated catalyst in organic solvent was not sufficient for reactivation as previously observed by Halling et al.<sup>17</sup> Presumably this is because the protein is rigid in organic solvent, preventing conformational change.<sup>18</sup>

The reaction is a solid-to-solid transformation where both starting material and product have similarly poor solubility (<1 mg/ml in *tert*-butanol or L-valine methyl ester) and so the substrate solution concentration remains constant throughout the majority of the reaction resulting in a linear rate until high conversions. Attempts to improve the reaction rate by the use of a range of ionic liquids to enhance substrate solubility were unsuccessful.

Under optimum conditions, yields of up to 89% were achieved at 20 mg/ml concentration using either precipitated or precipitated



**Figure 2.** Gram scale synthesis of valaciclovir in the presence of precipitated Alcalase at 50 °C, with and without applied vacuum (250 mbar).

and cross-linked catalyst with no observable racemisation.<sup>19</sup> On a gram scale, by replacing molecular sieves with reduced pressure (250 mbar) as a driving force, preliminary experiments gave conversions of up to 70% from a 20 volume process (Fig. 2). However, the mixtures were observed to become more viscous over the time course of the reactions, even where a constant volume was maintained by periodic addition of fresh valine methyl ester. In fact, aciclovir/valaciclovir mixtures, isolated from laboratory scale reaction by filtration, acidification and precipitation were found to contain a significant quantity of polymeric valine.

Treatment of neat L-valine methyl ester with precipitated Alcalase (30 mg in 1 ml of L-valine methyl ester) at 50 °C gave an emulsion after three days whereas in a parallel experiment, in the absence of catalyst, no solid was observed to form over the same period.

Proof-of-concept has been demonstrated for the biocatalytic transformation of aciclovir into valaciclovir, attaining high conversions from a solid-to-solid biotransformation in L-valine methyl ester using various forms of Subtilisin Carlsberg activated for use in organic solvent. Subtilisin Carlsberg ultimately proved to be unsuitable for scale-up, displaying insufficient activity towards the hydrochloride salt of L-valine methyl ester that might have allowed direct access to valaciclovir as the desired hydrochloride salt and inadequate selectivity towards the more active free base, resulting in polymer formation. In order to realise a practical process for the biocatalytic production of valaciclovir it would, therefore, be necessary to identify an alternative enzyme or Subtilisin Carlsberg variant that accepts L-valine methyl ester hydrochloride as an acyl donor or that accepts the free amine of L-valine methyl ester as an acyl donor but not an acceptor. In the latter case, the rate of valaciclovir synthesis might be expected to improve in the absence of competing peptide formation. An improved enzyme might also allow access to more efficient processes for the preparation of a variety of amino acid ester prodrugs of nucleosides<sup>20</sup> and other drugs<sup>21</sup> that have recently attracted considerable interest.

We have also demonstrated that Subtilisin Carlsberg and other hydrolases can transesterify L-valine ester hydrochloride salt (valaciclovir). Transient protection of nitrogen should prevent competing polypeptide formation and given that L-valine represents a particularly hindered amino acid, it is likely that a range of amino acid ester salts could be used as acylating agents for the resolution of racemic alcohols. Amino acid ester based acylating agents would be cheap and produce a basic product, that is, readily separable from the neutral starting alcohol, therefore, avoiding the need for chromatographic separation. This would provide a complementary approach to the use of succinic anhydride and a single step alternative to the use of the *N*-Boc protected amino acid vinyl esters recently reported by Moody and coworkers.<sup>22</sup>

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   In a typical experiment, water (40 µl) was added to the catalyst (40 mg) and acyclovir (40 mg) in L-valine methyl ester (2 ml). After brief shaking 4 Å molecular sieves were added and the resultant mixture stirred at 50 °C. 25 µl samples of the reaction mixture were taken at regular intervals, diluted with 10% TFA in H<sub>2</sub>O (1 ml) and passed through a 4 µm PTFE syringe filter. Analysis was performed by HPLC, injecting 5 µl of diluted sample onto a Spherisorb phenyl (250 × 4.6 mm, 5 µm) column and eluting with 20:80:0.1 MeOH/H<sub>2</sub>O/TFA at a flow rate of 1 ml/min, with the UV detector set at 254 nm. Valaciclovir enantiopurity was determined by HPLC using a Crownpak CR (+) column and eluting with H<sub>2</sub>O/MeOH/90% aqueous HClO<sub>4</sub> (950:50:5) as the mobile phase at a flow rate of 0.75 ml/min, with the UV detector set at 254 nm.
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