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# 9-(2-Hydroxypropyl)adenine: A Novel Fraudulent Substrate of HSV1-Thymidine Kinase. An Interdisciplinary Study

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## Introduction

Herpes Simplex virus type 1 thymidine kinase (HSV1 TK) phosphorylates thymidine (dT) to thymidine monophosphate (dTMP) playing a key role in reactivation from the latency and replication of herpes simplex viruses. Acyclovir (ACV) and gancyclovir (GCV) are today the only therapeutic compounds to interfere with a severe HSV infection. Those molecules act as fraudulent substrates blocking virus proliferation by dead end complexes with the viral DNA after being activated by the HSV-specific TK. Furthermore, HSV1 TK was more recently used as a suicide enzyme in gene therapy of cancer (1,2) and AIDS (3) in combination with ACV. The molecular basis of the selective therapy, that uses HSV1 TK as target, is the difference in substrate specificity between the human cellular and the herpesviral TK isoenzymes. Because of the important therapeutic implications, HSV is not only linked to viral infection but also with other diseases such as Kaposi's Sarcoma (4) and Alzheimer disease (5), and the increase of resistance towards ACV and GCV, intensive efforts have been directed towards the search of new compounds with general antiviral activity (6). In this study we report the results of a first cycle of structure-based drug-design aiming the development of new compounds for antiviral and gene therapy.

## Results and Discussion

The result of the evaluation of the DOCK parameter using dT and the emptied X-ray structure (7) (Fig 1) is shown in figure 2. The orientation of dT in the HSV1-TK binding site, found by DOCK, corresponds almost exactly with the crystallographic determined one.

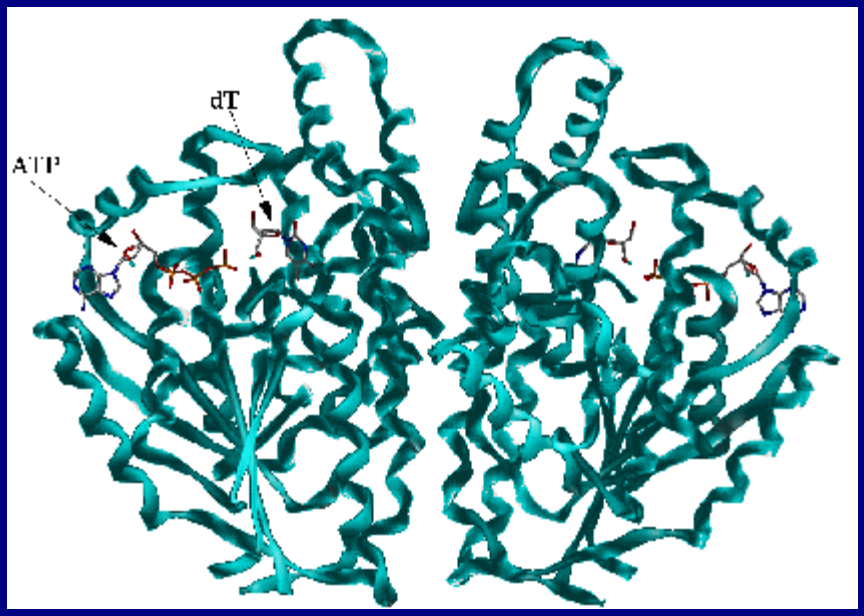


Figure 1

Plot of the X-ray structure of the symmetric HSV1-TK homo-dimer with bound ATP and thymidine (dT) showing the binding site of substrate and cosubstrate. ATP and dT are displayed as color coded capped stick. The backbone is indicated by a shaded ribbon (cyan).

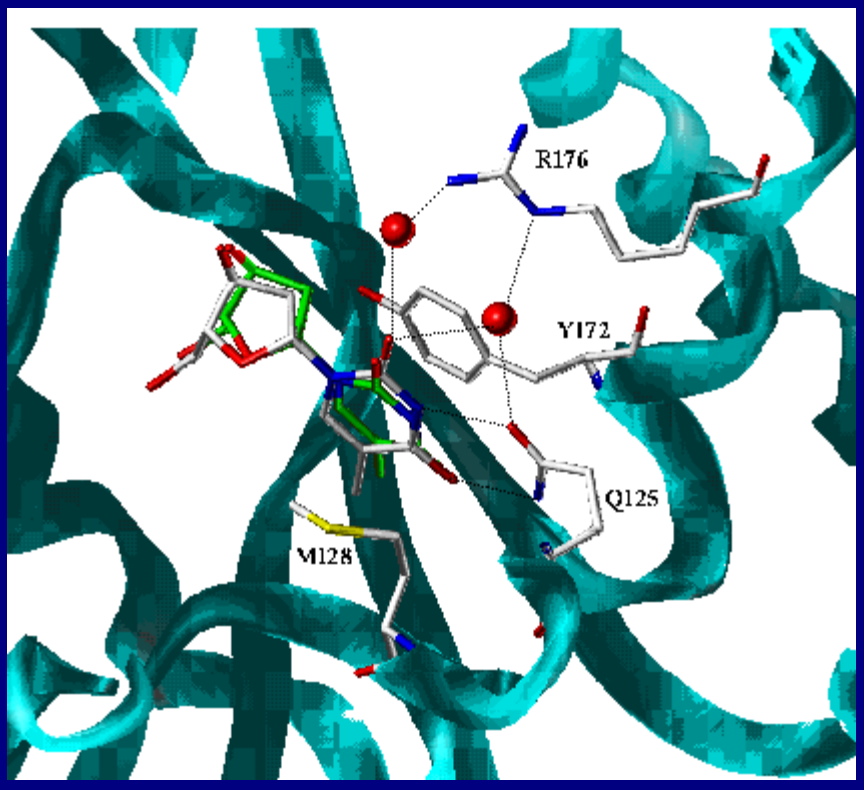


Figure 2

Zoom into the thymidine binding site indicating the crystallograpically determined mode of binding of Thymidine. dT and the amino acids that are directly involved in substrate binding, are displayed as color coded capped sticks (C atoms: white). Water molecules are shown as spheres. The hydrogen bond network is given as dashed lines. The most favorable binding orientation of dT determined using DOCK (DOCK score=170) is presented as color coded capped sticks (C atoms: green).

By means of the evaluated protocol a series of adenine derivatives (6) has been evaluated as putative ligands for HSV1-TK. The resulting most favorable binding orientation of one of them, compound 9-(2-Hydroxypropyl)adenine (9-HPA), is reported in figure 3. The overall orientation of the adenine base and the orientation of the OH group of the hydroxypropyl moiety towards the reaction center let us hypothesize that compound 9-HPA may be a fraudulent substrate of HSV1-TK. This hypothesis has been submitted to various experimental verifications whose result are reported here.

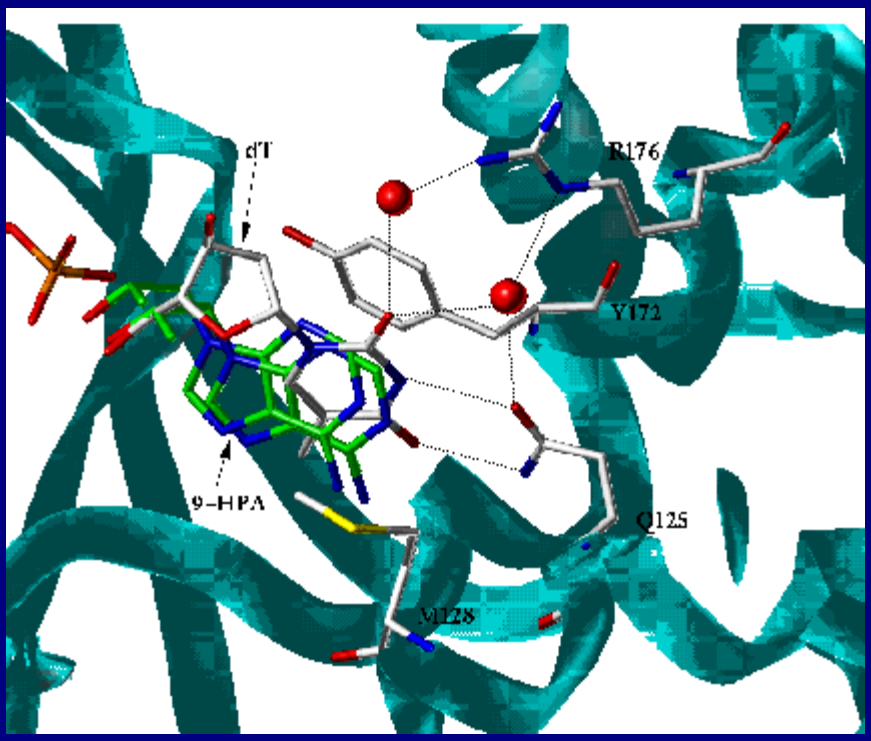


Figure 3

Most favorable binding orientations of 9-HPA [color coded capped sticks (C atoms: green)] in the thymidine binding site, found by DOCK (DOCK score=136-142), compared to the crystallographically determined mode of binding of dT. The g-phosphate of ATP, indicating the location of the reaction center, dT and the amino acids that are directly involved in substrate binding, are displayed as color coded capped sticks (C atoms: white). Water molecules are shown as spheres. The hydrogen bond network is given as dashed lines.

Figure 4 shows the results of the UV-measurement that monitors the ADP formation during the phosphorylation reaction catalyzed by HSV1-TK. The used concentration of TK were 3, 4, 5 and 7 ug/reaction. The measurements show a clear increase in velocity of ADP formation depending upon the amount of TK. The observed ADP formation is a specific signal of the TK catalyzed phosphorylation reaction between 9-HPA and ATP. Thus, these results indicate that 9-HPA is binding in the thymidine binding site of TK and acts as a substrate.

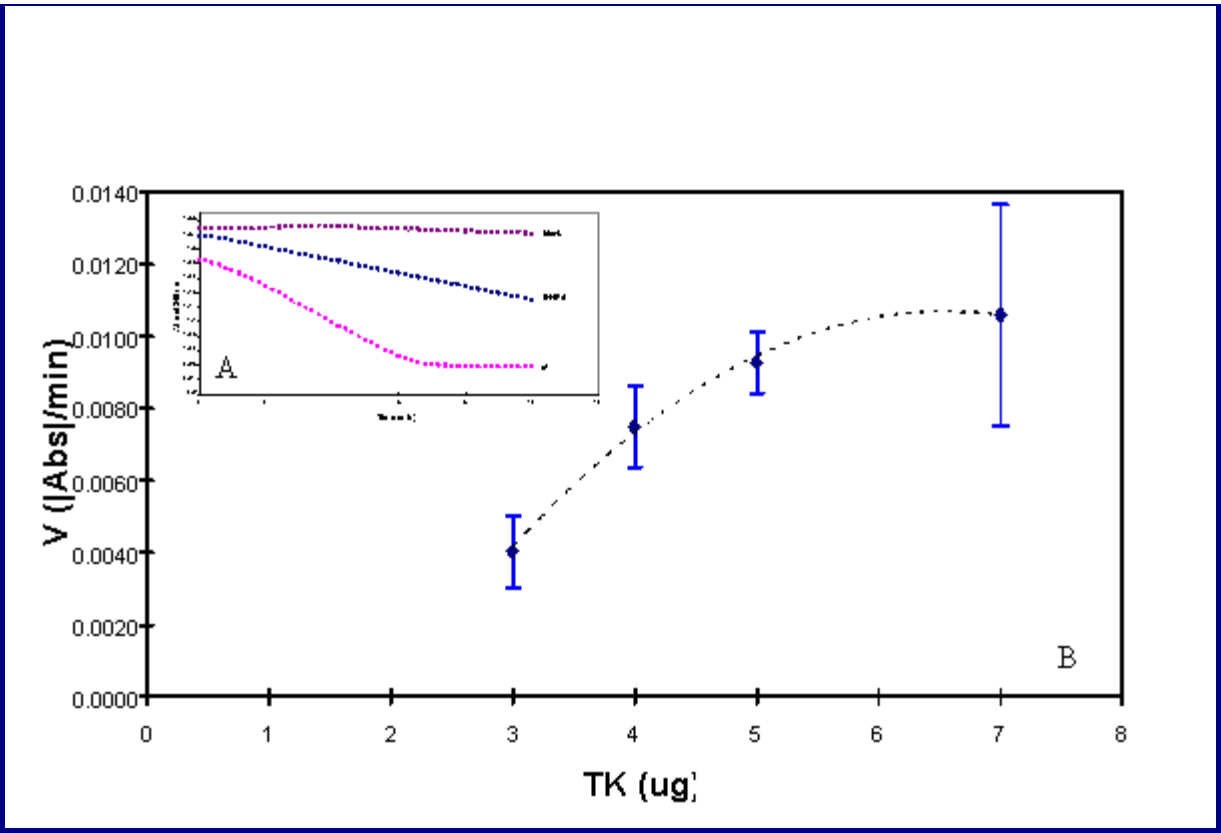


Figure 4

A) Plot of typical experimental values obtained with the coupled enzyme assay using UV-spectrophotometry. The blank shows the decrease of the absorbance in absence of substrate. The dT and 9-HPA curve illustrate the typical decrease of the absorbance in presence of dT (as reference) and of 9-HPA respectively. B) Plot of the ADP formation velocity in dependence of the amount of HSV1-TK when 9-HPA is presented as substrate at a concentration of 4 mM. The presented values are mean values of 5 independent measurements and take into account the spontaneous hydrolysis of ATP to ADP. The relative standard deviation is given as vertical bars.

HPLC was used as a second independent method to monitor ADP formation during phosphorylation reaction. Figure 5A shows the quality of the chromatographic separation for the different experiments. The ratio "Area of ADP/Area of ATP" was used for the evaluation of the results. The area under the peak of ATP served as normalization factor. The mean values of the calculated ratio as well as the relative standard deviation of the four different experiments are reported in Figure 5B.

In all the experiments, formation of ADP could be depicted but the amount of released ADP, during the "9-HPAex" and the "dTex" experiments, clearly differs from this of the blanks (Fig. 5A, 5B).

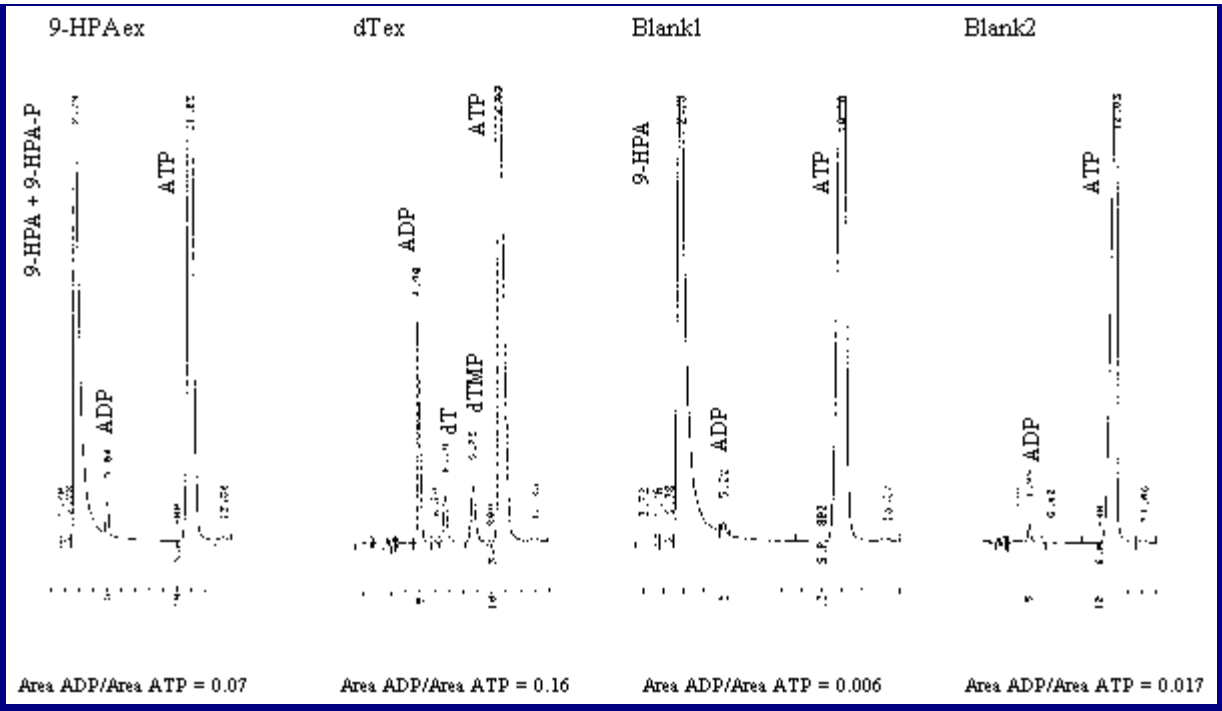


Figure 5 A

HPLC chromatograms used for monitoring ADP formation under following experimental conditions: (9-HPAex): 5mM ATP + 6mM 9-HPA+ 5mM Mg2+ + 1 ug TK; (dTex): 5mM ATP + 2 mM dT + 5mM Mg2+ + 1 ug TK; (Blank 1): 5mM ATP + 6mM 9-HPA + 5mM Mg2+ (no TK); (Blank 2): 5mM ATP + 1 ug TK + 5mM Mg2+ (no 9-HPA) Samples were incubated during 1 hour at 37 oC. All the samples were diluted 1:15 and the injection volume for the HPLC was 20 ml. No signal for 9-HPA-P could be depicted by the chromatographic method. From the experience of using this chromatographic system with the adenine series (adenosine, adenosine-monophosphate) we can extrapolate that the 9-HPA-P signal is overlapping with the 9-HPA one.

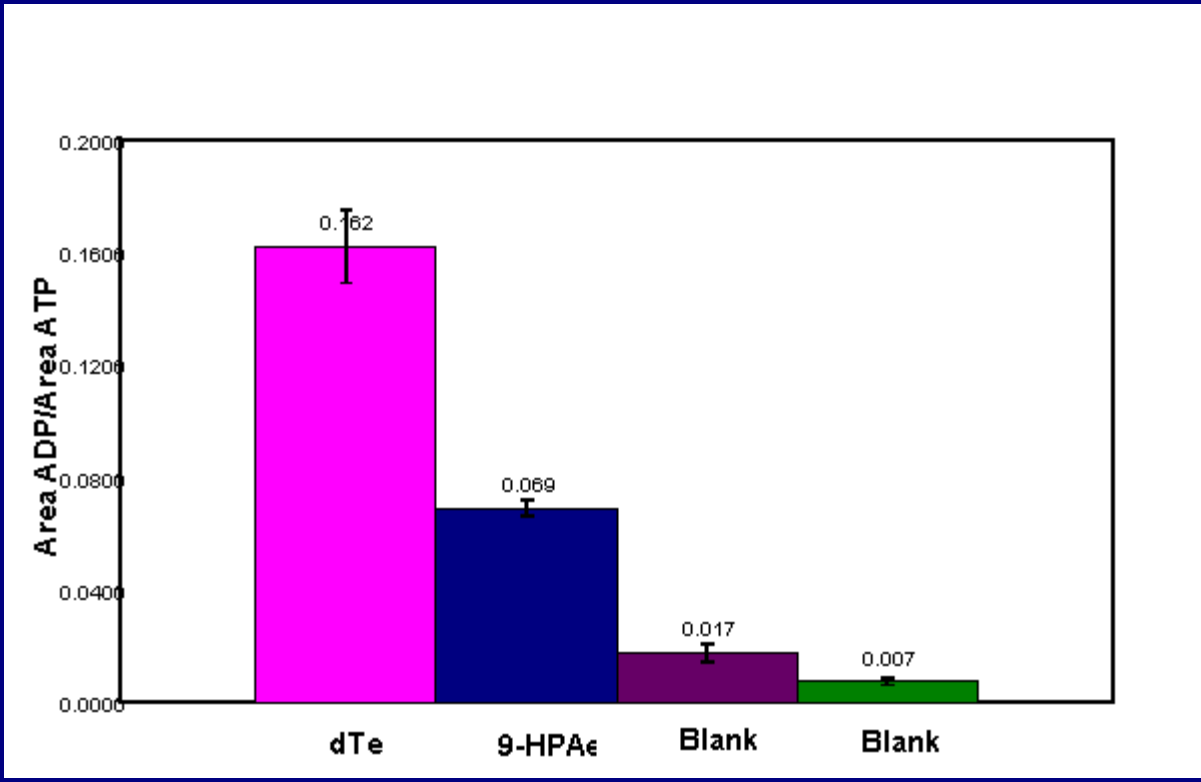


Figure 5B

Histogram of the ratio between area of ADP and area of ATP resulting from the different experiments. Blank 1 and Blank 2

experiments were performed to monitor spontaneous (reaction independent) hydrolysis of ATP to ADP by the experimental conditions. The "dTex" experiment serves as reference. The relative standard deviation is given as vertical bars. The experiments have been performed in triplicate.

The HPLC results show that the increase of the ADP signal is due to the specific phosphorylation of dT to dTMP and of 9-HPA to 9-HPA-monophosphate (9-HPA-P) catalyzed by TK. They confirm that 9-HPA is a substrate of HSV1-TK. They also indicate that the affinity of 9-HPA to TK is less than this of dT (Km 0.2uM) and therefore, a higher Km value has to be expected.

Figure 6 reports the results of the kinetic measurements. These were performed using radioactive labeled dT and 9-HPA as an "inhibitor". Vi (initial velocity) was measured by monitoring the increase of radioactively labeled dTMP in absence and presence of 9-HPA. The affinity of 9-HPA was calculated as Ki. The slope of the regression curves in the reciprocal plot (Fig. 6B) increases with the increase of the concentration of 9-HPA. The fact that the intersection of the curves is not optimal (Fig. 6B), (for a competitive inhibitor all curve should point on the y-axis) is probably due to the low affinity of compound 9-HPA, that causes an increased scattering of the measured values. The Ki value for 9-HPA has been calculated using the slope replot (Fig. 6), as the intersection of the curves is not optimal. These data show that 9-HPA is binding in the thymidine binding site of HSV1-TK and competes with dT. The calculated Ki for 9-HPA is 5.3 mM +/- 0.28 mM (n=3).

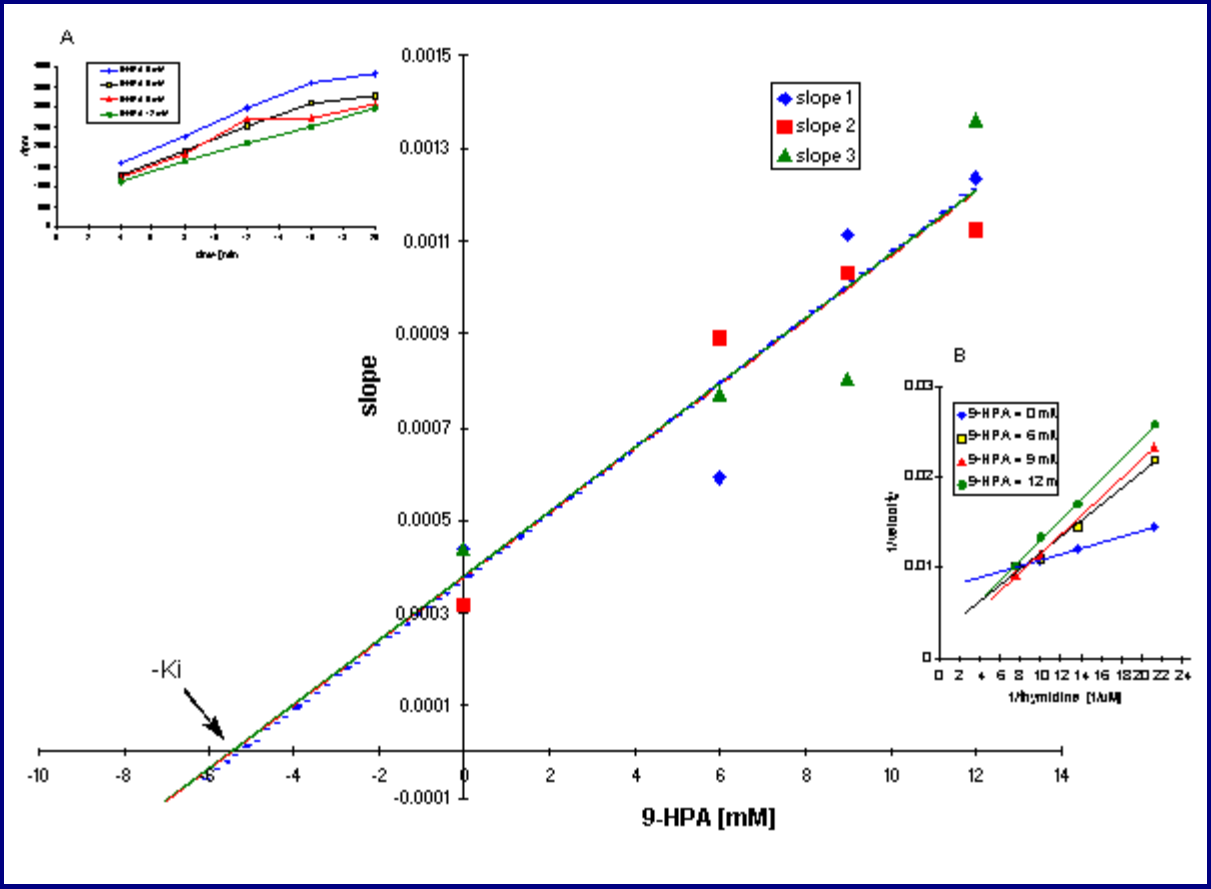


Figure 6

Reciprocal plot of the slope as function of the 9-HPA concentration for the determination of Ki value of 9-HPA. A) Example of a time profile of the formation of radioactivelly labelled dTMP in absence and in presence of 9-HPA by a dT concentration of 0.075 uM. B) Typical Lineweaver-Burk plot of product inhibition by 9-HPA.

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# Summary and Conclusion

- The results of the docking, suggested that 9-(2-hydroxypropyl)adenine (9-HPA) could possibly be a fraudulent substrate of TK.
- The kinetic and analytic experiments showed that:

- 9-HPA competes with the natural substrate dT

- 9-HPA binds to HSV1 TK and has a Ki value of 5.3 mM

- 9-HPA is phosphorylated by HSV1 TK

Our prediction has been confirmed and we conclude that 9-(2-hydroxypropyl)adenine is indeed a novel fraudulent substrate of HSV1 and may be a useful lead compound for structure-based drug design of more potent therapeutic compounds.

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# Material and Methods

- *Molecular Modelling*

. A protocol for DOCK V3.0 has been evaluated and validated using the information from the x-ray structure of HSV1-TK in complex with dT and ATP. Shape and electrostatic served as scoring function.

. Conformational search performed for the ligands (InsightII, Biosym).

. The PM3-Hamiltonian of MOPAC has been applied for minimization and calculation of the electrostatic potential charges (ESP).

. DelPhi used to calculate the required electrostatic potential map for the enzyme.

. Putative ligands were evaluated using the previously evaluated DOCK protocol.

. Visualization of the results by means of the program SYBYL V.6.3 (Tripos Associates, St. Louis).

- *Indirect monitoring of the phosphorylation of 9-HPA by determining the formation of ADP*

*UV-spectrophotometry*: The amount of ADP released was determined by monitoring the oxidation of NADH to NAD+ with UV spectrophotometry at 340 nm using a cascade of two enzymatic reactions, namely pyruvate kinase and lactate dehydrogenase. Several concentrations of HSV1-TK and correspondent blanks were measured.

*High performance liquid chromatography*: The amount of ADP released by the phosphorylation reaction has been monitored using ion-pair chromatography (Column: RP-18; Solvent: $NaH_2PO_4$ 0.2M, Tetrabutylammonium-hydrogensulfate 25mM, 3% MeOH; Flow: 1.1 ml/min; Detection: UV 254 nm)

- *Kinetic measurement of product inhibition by 9-HPA*

Kinetic studies, measuring the conversion of labeled thymidine to thymidine monophospate, were performed in presence and absence of 9-HPA, using the DEAE-cellulose method (8).

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## Comments

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