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Effect of alkyl groups on the cellular hydrolysis of stavudine phosphoramidates

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Abstract—We examined the effect of cellular metabolism of three alkyl-substituted amino acid ester phosphoramidate derivatives of stavudine in different cell lines. Marked cell-to-cell differences were found in both the rate of hydrolysis and chiral selectivity. This selectivity implies that different enzymes may be involved in the metabolism of these compounds depending on the cell type involved. Notably, both the methyl and ethyl substituted derivatives underwent hydrolysis in presence of various cell lines, whereas the *tert*-butyl substituted compound was resistant to hydrolysis implying that steric hindrance associated with this group along with electron density may play a key role in the hydrolysis profile of these compounds. Additionally we found this mimicked the hydrolysis profiles obtained for bacterial enzymes. Furthermore, our results suggest that the site of attack of the cellular enzymes is confined to the ester side chain of the molecule. This result is also consistent with our earlier observation using bacterial enzymes as well as using 'D' isomers.

1. Introduction

Stampidine is a novel phosphoramidate derivative of stavudine that is being developed as a potential anti-HIV agent.[1–5] Stampidine was 100 times more active than stavudine and twice as active as zidovudine against nine clinical HIV-1 isolates of non-B envelope subtypes (A, C, F, and G) originating from South America, Asia, and sub-Saharan Africa.[1] Stampidine was effective against genotypically and phenotypically nucleoside analog reverse transcriptase inhibitor (NRTI)-resistant HIV-1 isolates as well as non-nucleoside inhibitor (NNRTI)-resistant HIV-1 isolates at subnanomolar to low nanomolar concentrations.[1] Orally or intraperitoneally administered stampidine exhibited significant and dose-dependent in vivo anti-HIV activity against an NRTI-resistant clinical HIV-1 isolate in severe combined immunodeficient (SCID) mice reconstituted with peripheral blood (PBL) mononuclear cells from seronegative human donors.[4] In the feline immunodeficiency virus (FIV)-infected domestic cat model for AIDS, orally administered stampidine showed a dose-dependent anti-retroviral effect.[5] Stampidine therapy was not associated with any clinical or laboratory evidence of significant toxicity at individual dose levels as high as 500 mg/kg or at cumulative dose levels as high as 8.4 g/kg. Stampidine exhibited favorable pharmacokinetic behavior in mice, rats, dogs, and cats following oral administration.[6,7] The documented in vitro potency of stampidine against primary clinical HIV-1 isolates with genotypic and/or phenotypic NRTI- or NNRTI-resistance as well as non-B envelope subtypes together with its in vivo antiretroviral activity in HIV-infected Hu-PBL SCID mice and FIV-infected cats warrants its further development as a new anti-HIV drug.

Hydrolysis of stampidine results in generation of its active metabolite ala-d4T-monophosphate. A better understanding of the key determinants of the hydrolytic activation of stampidine may provide the basis for modifications in the structure that may improve its potency. The generation of the active metabolite of stampidine was originally proposed to require an esterase-mediated hydrolysis of the carbomethoxy group associated with the alanine side chain of stampidine.[8–28] Additionally, we hypothesized that in various tissue microenvironments the metabolism of stampidine may also occur

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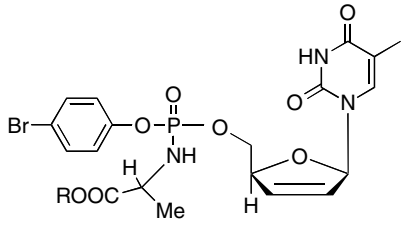
through the action of hydrolytic enzymes other than esterase. Our results provided additional evidence that stampidine as well as other halogen-substituted phosphoramidate derivatives of stavudine can also be metabolized by lipase.[29] Additionally, our earlier studies utilizing molecular modeling and a comparison of the hydrolysis rate constants revealed a chiral preference of the lipase active site for the putative *S*-stereoisomer of these compounds.[29] Our model indicates that the lipase-mediated formation of the cyclic intermediate is a key step in metabolism of stampidine and relies on the initial configuration of the stereoisomers. In a subsequent study, we demonstrated that proteases too can mediate the conversion of phosphoramidate derivatives into the active metabolite.[30] We have also examined the hydrolysis of stampidine using several different cell lines to further show that esterases are not the only enzymes responsible in the metabolism of stampidine.[31] In addition, we explored the chiral selectivity of each of the cell lines for hydrolyzing each of the stereoisomers of stampidine. These studies provided evidence that enzymes other than esterase are likely involved in the hydrolysis as esterase exhibited minimal chiral selectivity.[29] We continued our studies and examined the metabolism of various alkyl group substituted phosphoramidate derivatives of stavudine using bacterial enzymes. Notably, each of the bacterial enzymes hydrolyzed these compounds with a chiral preference.[32] We also found that the *tert*-butyl substituted compound was not hydrolyzed by any of the bacterial enzymes unlike methyl or ethyl substituted compounds. Based on this result we proposed that the resistance shown by *tert*-butyl group substituted phosphoramidate derivative is due to its inability to enter the enzyme pocket caused by steric hindrance.[32] In the present study, we examined the metabolism of these alkyl substituted compounds toward various cell lines to establish whether such a trend is shown by these phosphoramidate compounds.

2. Results and discussion

Synthesis of various ester substituted phosphoramidate derivatives of stavudine was achieved following the Scheme 1.[10–15] A one-pot synthetic procedure was adopted for this purpose and is briefly described here as a general method for the synthesis of all the compounds shown in Table 1.

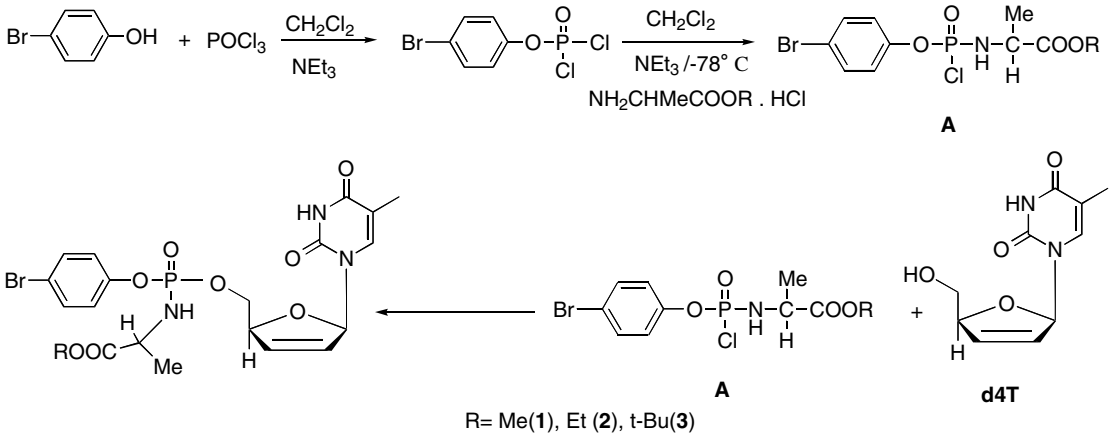
We used five different cell lines to examine the intracellular metabolism of various alkyl substituted amino acid ester phosphoramidates of d4T. The cells used in the present study comprise of the following: Jurkat (T-cell line), Cos-7 (monkey kidney cell line), RAW264

Table 1. Chiral selectivity of phosphorus stereoisomers of phosphoramidate derivatives of stampidine with different cell treatment



R	Cells used	Fastest hydrolyzing isomer	Rate (h)
Me (**1**)	RAW264 (macrophages)	'*R*'	4.03
Et (**2**)	RAW264 (macrophages)	'*R*'	4.63 ± 1.07
t-Bu (**3**)	RAW264 (macrophages)	'*R*'	1.19 ± 0.03
Me	Cos-7 (monkey kidney cells)	'*R*'	1.46 ± 0.2
Et	Cos-7 (monkey kidney cells)	'*R*'	3.52 ± 1.40
t-Bu	Cos-7 (monkey kidney cells)	'*R*'	0.20 ± 0.14
Me	Jurkat cells	'*S*'	0.61 ± 0.11
Et	Jurkat cells	'*S*'	1.46 ± 0.25
t-Bu	Jurkat cells	—	0.0[a]
Me	LL2—lung cells	'*S*'	0.56 ± 0.22
Et	LL2—lung cells	'*S*'	3.04 ± 0.98
t-Bu	LL2—lung cells	—	0.09 ± 0.05
Me	CEM cells	'*S*'	1.41 ± 0.75
Et	CEM cells	'*S*'	2.07 ± 0.63
t-Bu	CEM cells	—	0.04

Tentative stereochemistry assignment based on previous studies.[29]
[a] Not hydrolyzed even after prolonged incubation.



Scheme 1.

(macrophage-like cell line), CEM (T-leukemia cell line), and LL2 (mouse lung cancer cell line). Cells in phosphate buffer were treated separately with alkyl substituted amino acid ester phosphoramidate derivatives of d4T and incubated at 37 °C. The incubated samples were then centrifuged and the supernatants were collected at various time intervals and analyzed by HPLC. A guard column was used in order to avoid any protein or particulate from entering the HPLC analytical column. In 15 min, treatment of RAW264 cells with methyl ester substituted phosphoramidate derivative of d4T (**1**) resulted in the formation of d4T and *p*-bromophenol. By 30 min, we observed an increase in the amount of d4T and *p*-bromophenol; however, one of the isomers was still present in intact form (Fig. 1). Treatment of ethyl substituted phosphoramidate derivative of d4T (**2**) with RAW264 macrophage-like cell line resulted in the formation of d4T and *p*-bromophenol in 15 min. Similar to the results obtained for **1**, we observed that one of the isomer was preferentially hydrolyzed compared to the other (Fig. 2). Interestingly, the second isomer of both compounds was hydrolyzed by RAW264 cells. In the past we have tentatively assigned the stereochemistry at the phosphorus center of methyl substituted phosphoramidate derivative as '*R*' based on molecular modeling studies.[29] Based on the above criteria, we propose that the ethyl substituted phosphoramidate derivatives' second peak may be assigned a tentative stereochemistry as '*R*'. Figure 3 shows the HPLC chromatogram obtained for *tert*-butyl substituted phosphoramidate derivative of d4T (**3**) treated with RAW264 cell line at various time intervals. Even after 45 min we observed that both the peaks (A and B) and the hydrolysis was comparatively slower. At 90 min peak B in the chromatogram was hydrolyzed preferentially although the rate of hydrolysis was slow.

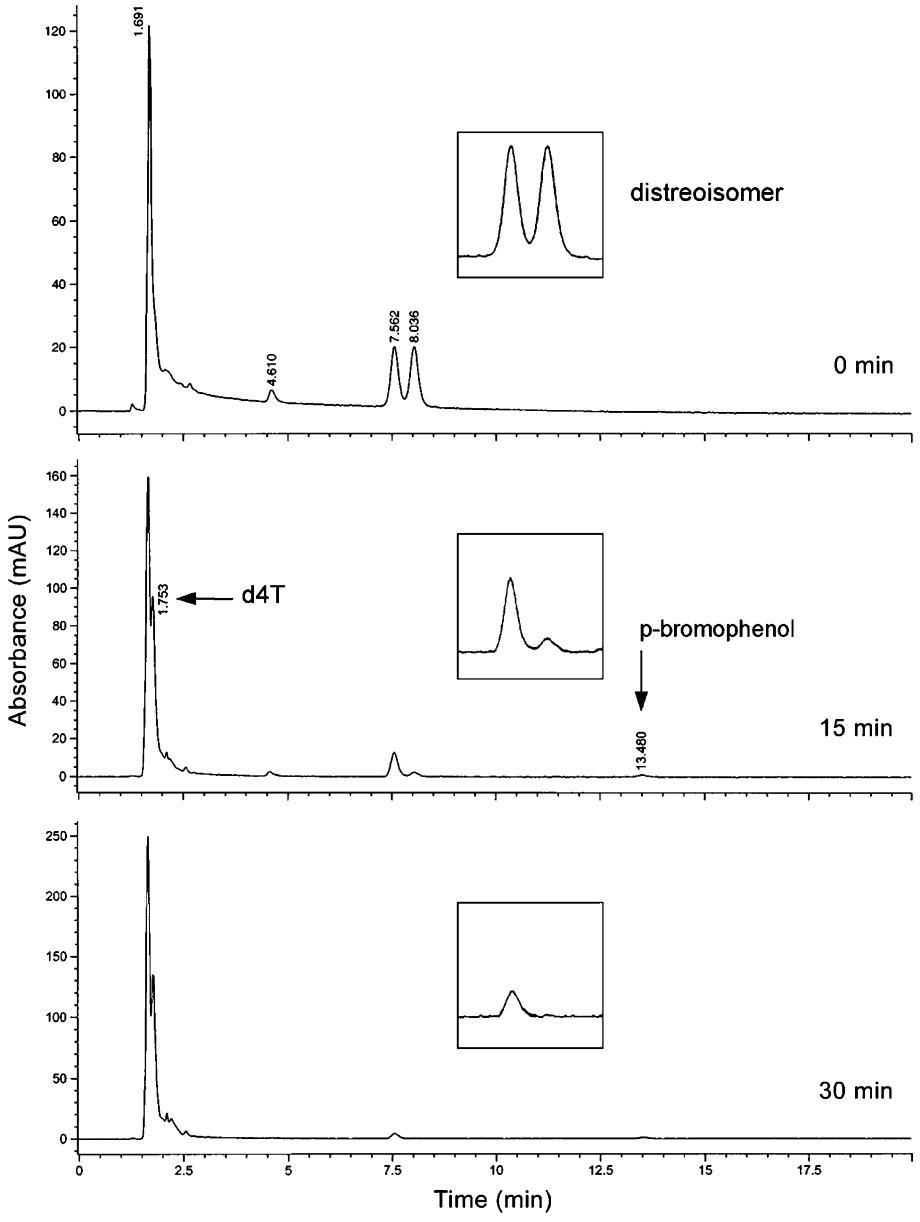


Figure 1. HPLC profile of supernatant from 30×10^6 RAW264 cells treated with **1** at various time intervals.

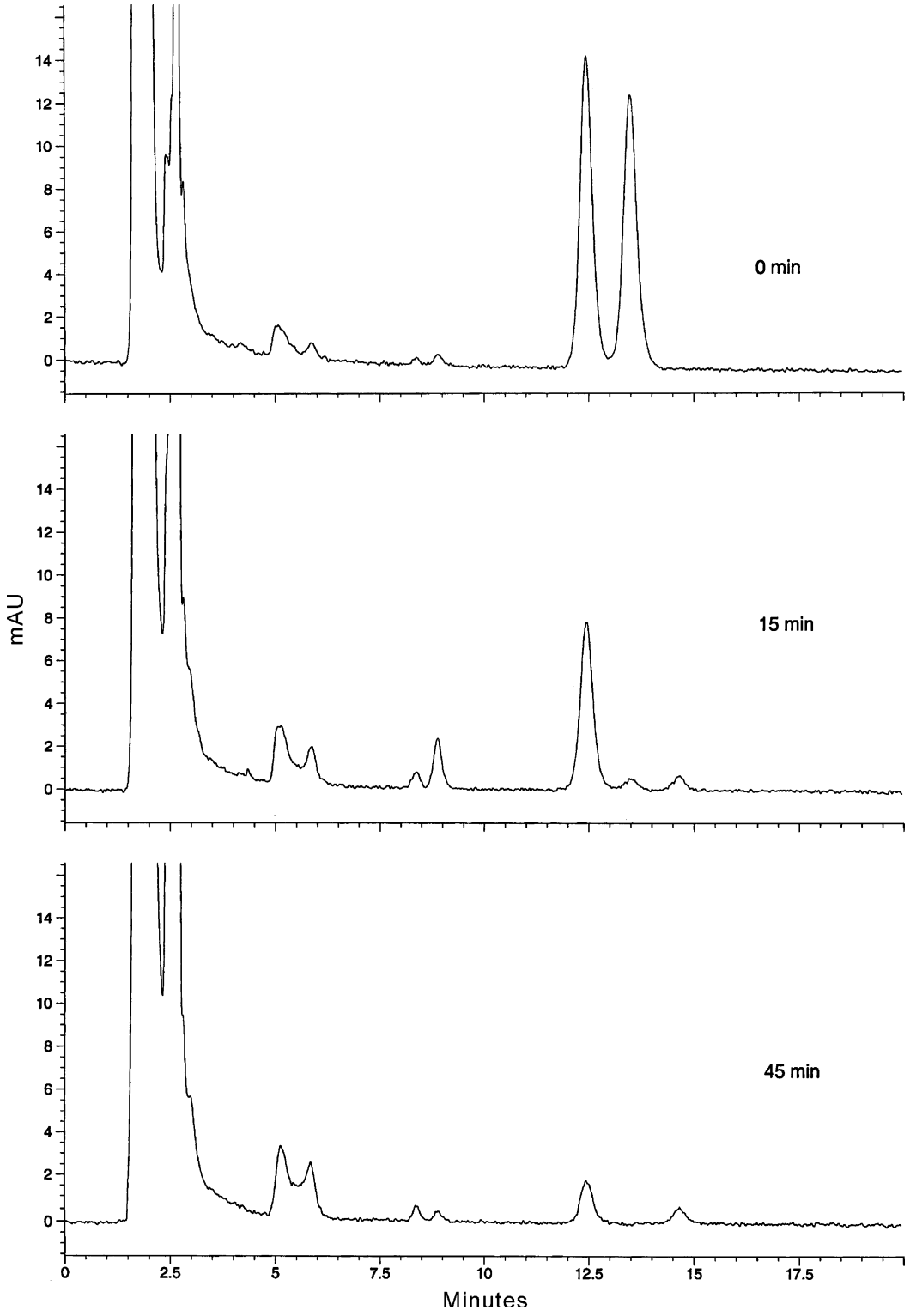


Figure 2. HPLC profile of supernatant from 30×10^6 RAW264 cells treated with **2** at various time intervals.

Both *p*-bromophenol and d4T were formed as evidenced from the small peak at 13.8 and 1.9 min. Similar results were observed with Cos-7 cells wherein the second peak (B) was hydrolyzed when **1** or **2** was used (Fig. 4). Furthermore, one of the isomers was hydrolyzed preferentially, implying that similar enzymes in RAW264 and Cos-7 cell lines may be responsible for the chiral selectivity of hydrolysis. Figure 5 shows the HPLC profile of **3** treated with Cos-7 cell line at various time intervals. This compound was found to be resistant to hydrolysis even after prolonged periods of time. For example, even after 4.5 h we found that very little (if any) hydrolysis was taking place. This result further illustrates that *tert*-butyl compound was not recognized by the enzymes present in the cell line, consistent with the result obtained with the RAW264 cell line. Once again we

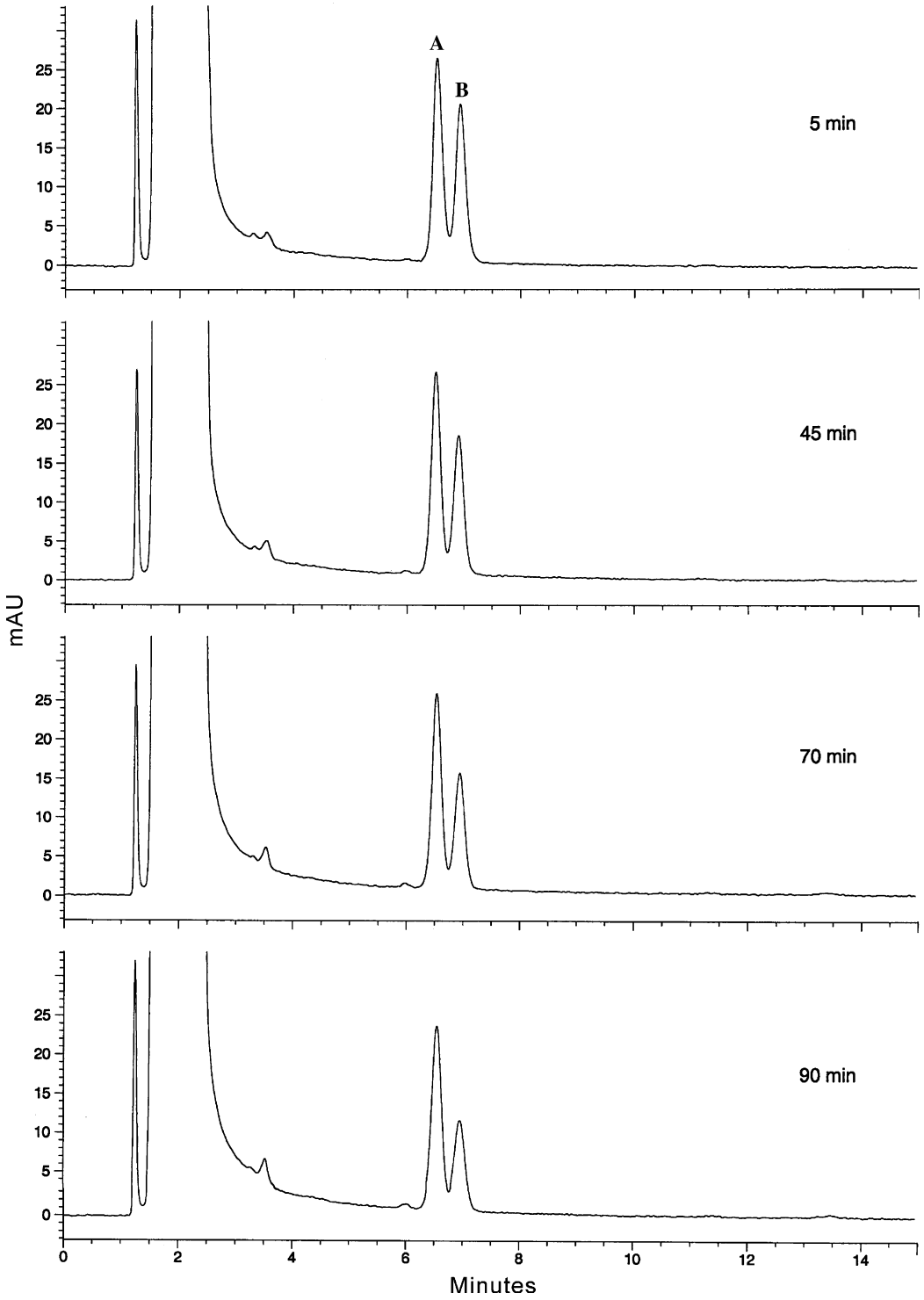


Figure 3. HPLC profile of supernatant from 30×10^6 RAW264 cells treated with **3** at various time intervals.

postulate that *tert*-butyl group does not enter the enzyme pocket due to steric factors associated with its bulky structure. Alternative explanations for this observation include electron density related differences in hydrolytic rates.

For the next set of experiments, we used Jurkat cells. Treatment of Jurkat cells with methyl substituted phosphoramidate of stavudine (**1**) resulted in the intracellular hydrolysis of this compound within 30 min. One isomer underwent hydrolysis faster than the other. Additionally, it was evident that this isomer was different from the isomer undergoing hydrolysis in RAW264 and Cos-7 cells. Thus, two different types of enzymes each preferentially recognizing one of the isomers may be operative in different cell types. A similar result was obtained when **2** was incubated with Jurkat cells as can be seen from Figure 6.

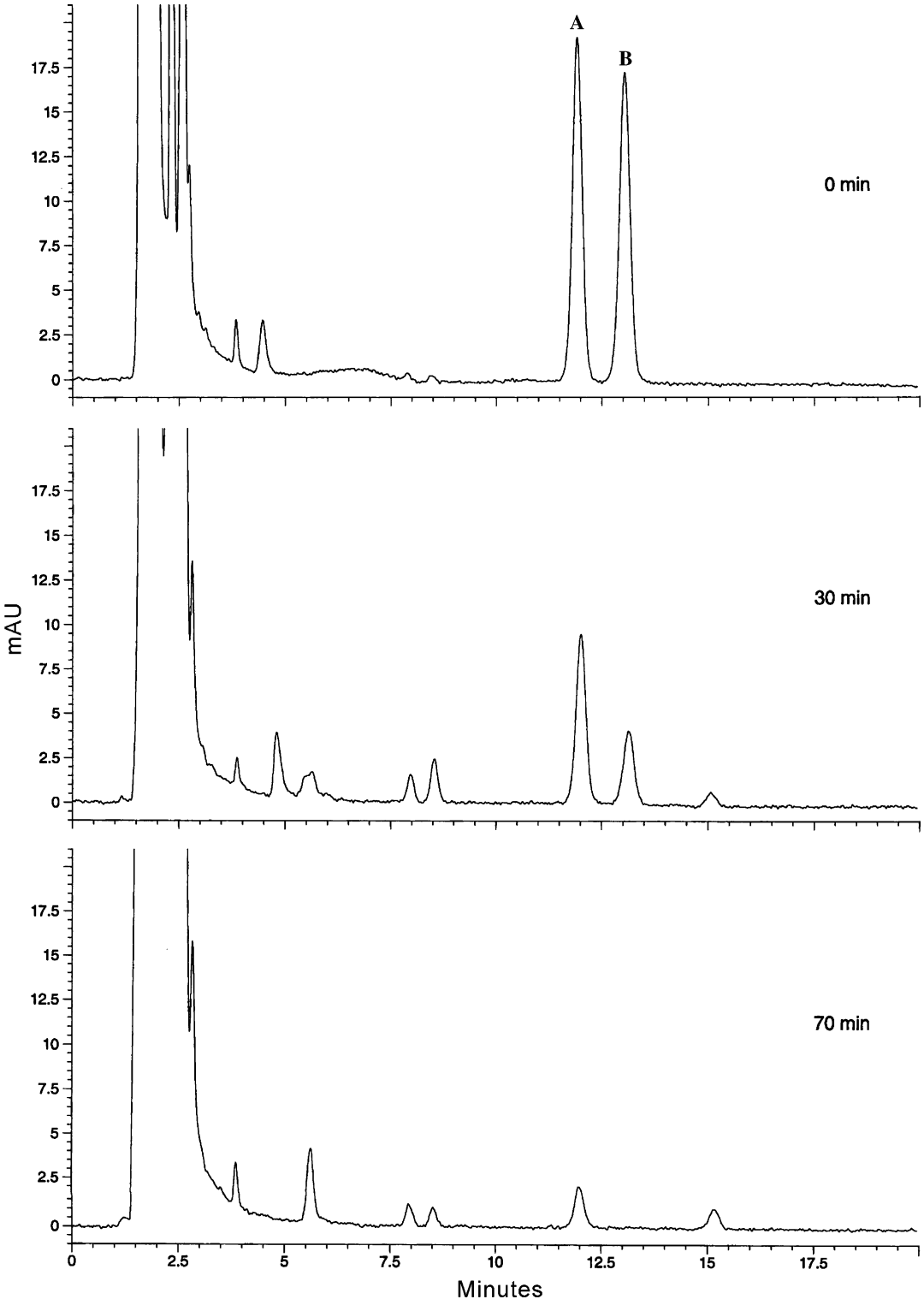


Figure 4. HPLC profile of supernatant from 29.3×10^6 Cos-7 cells treated with **2** at various time intervals.

Within 30 min of incubation we could observe formation of *p*-bromophenol. On the other hand, incubation of **3** with Jurkat cells did not hydrolyze the compound even after 1.5 h once again proving that the *tert*-butyl group in the structure of the ester side chain of the molecule is resistant to hydrolysis by enzymes present in the cells.

We also incubated **1** with CEM cells and monitored the reaction using HPLC. The hydrolysis of **1** in CEM cells mimicked its hydrolysis profile in Jurkat cells. A similar result was obtained with **2** treated with CEM cells. Once again the *tert*-butyl substituted compound **3** was resistant to hydrolysis when incubated with CEM cells. (Fig. 7). Even after prolonged period of time (150 min)

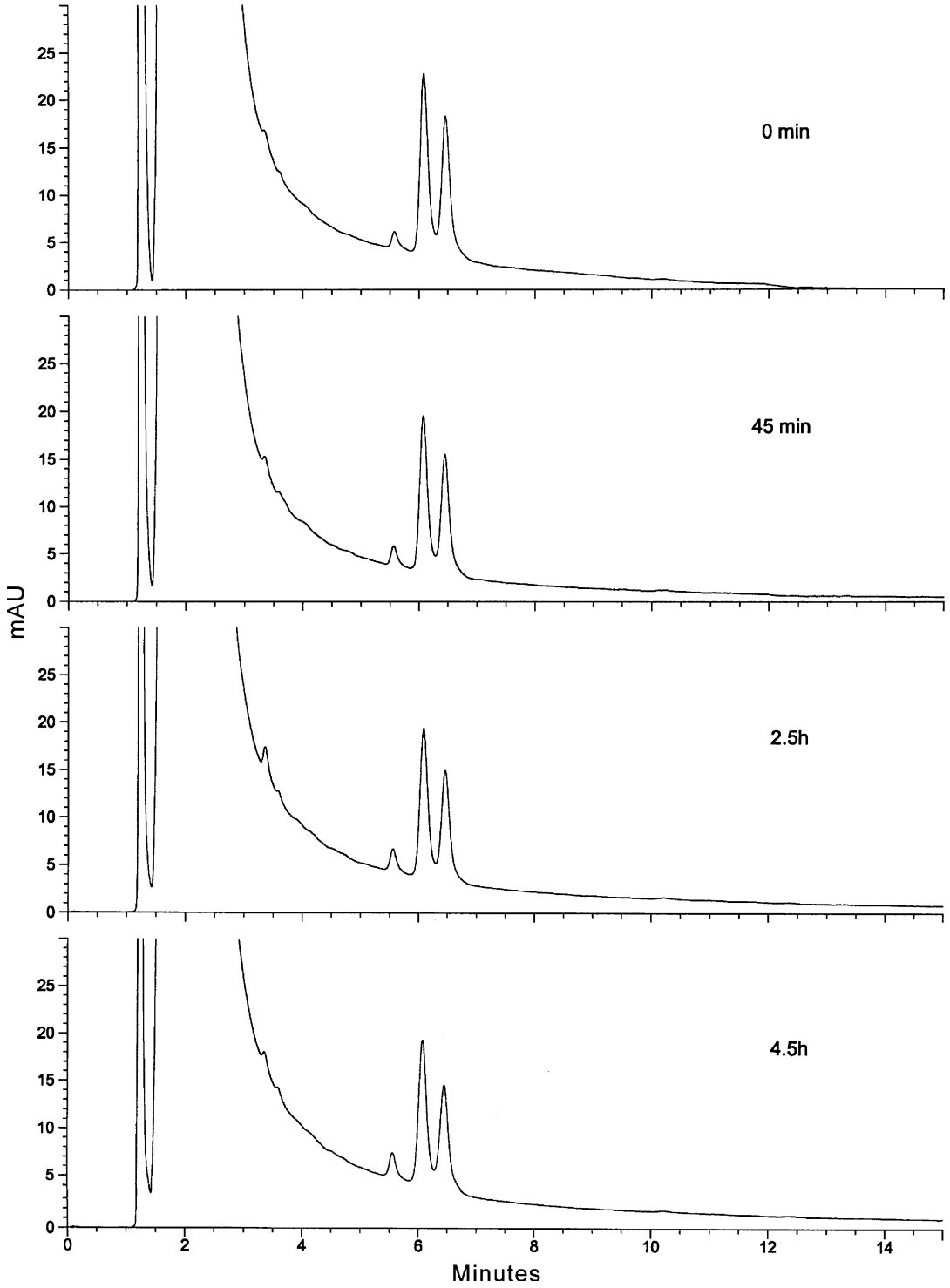


Figure 5. HPLC profile of supernatant from 29.3×10^6Cos-7 cells treated with **3** at various time intervals.

3 only showed a very slow hydrolysis. This is consistent with the results observed for other cell lines incubated with compound **3**.

In the final experiment, we examined the hydrolysis profile of all three compounds in LL2 lung cancer cells. Compounds **1** and **2** showed considerable hydrolysis with time as can be seen from Figures 8 and 9, respectively. One of the isomers was hydrolyzed preferentially than the other, a trend similar to that observed with Jurkat cells. Similarly, treatment of **3** with LL2 cell line indicated resistance to hydrolysis a trend identical to that observed for other cell lines.

From these examples, it is evident that enzymatic hydrolysis is an essential step for the metabolism of phosphoramidate derivatives of d4T. Furthermore,

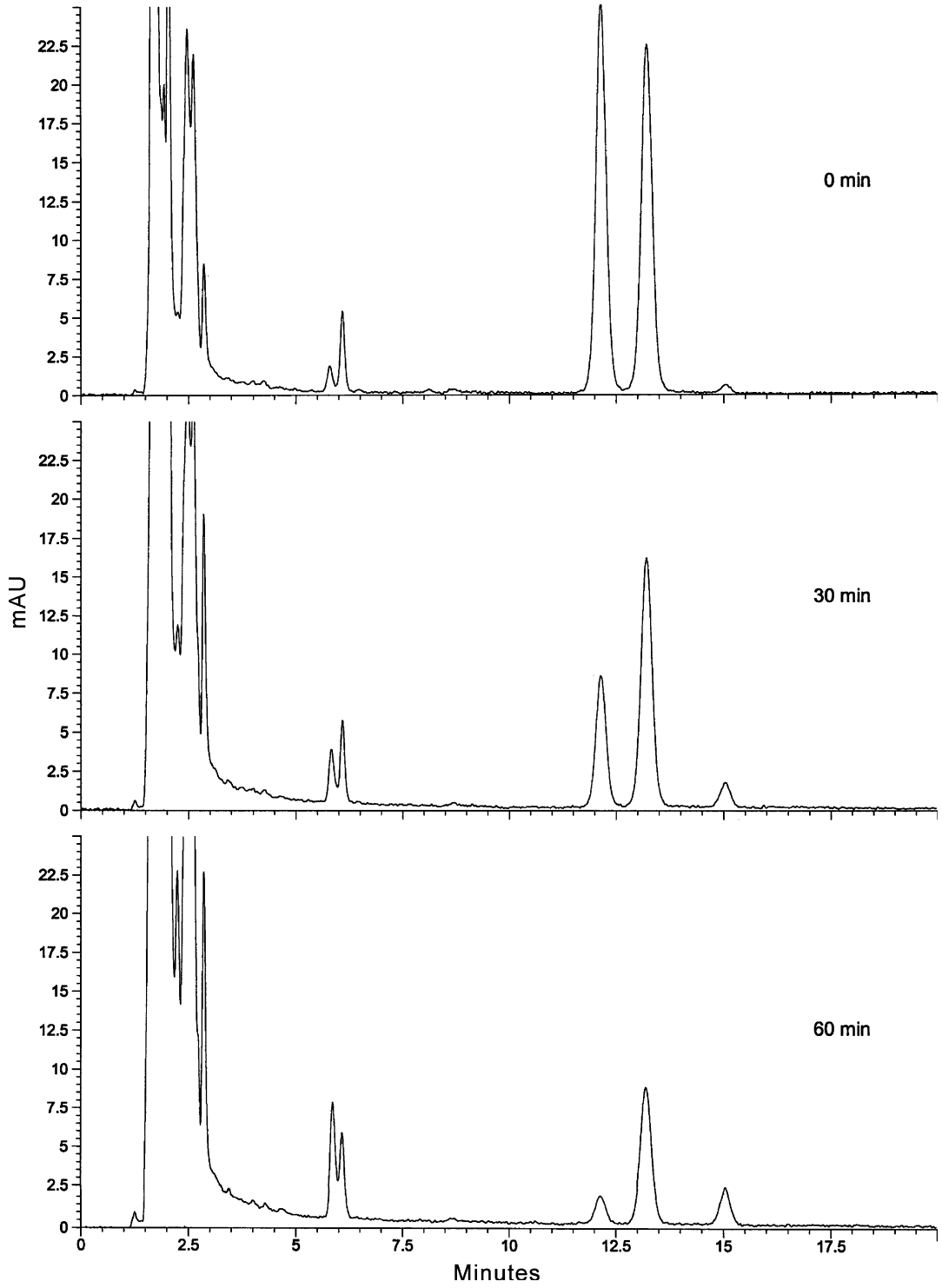


Figure 6. HPLC profile of supernatant from 29.5×10^6 Jurkat cells treated with **2** at various time intervals.

we also suggest that different enzymes may be playing a role in metabolizing each of the compounds depending on the cell line used. Additionally we propose that certain ester side chains such as methyl or ethyl are preferred and bulkier substitutions such as *tert*-butyl group case poor recognition of the derivative by the hydrolyzing enzymes occurs perhaps due to the inability of this side group to enter the enzyme pocket for hydrolysis.

3. Comparison between bacterial enzymes and cells

It has been postulated that phosphoramidate derivatives of d4T are activated by an enzymatic hydrolysis mediated by esterases.[10–28] In the past we have established that apart from esterases, other enzymes can also hydrolyze the ester side chain of the phosphoramidate derivatives.[29,33] We have also demonstrated that enzyme inhibitors can slow down the rate of hydrolysis.[33]

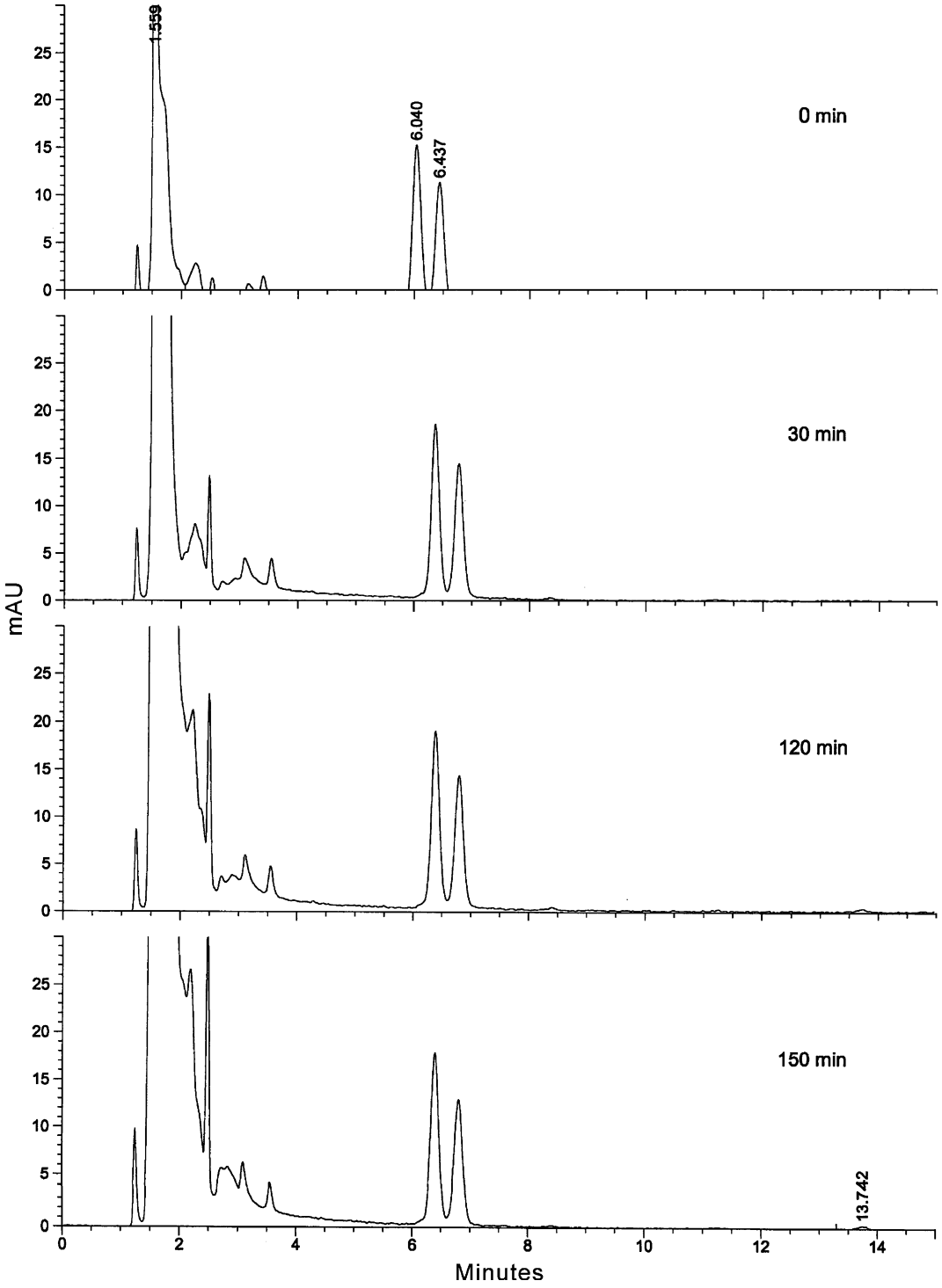


Figure 7. HPLC profile of supernatant from 30×10^6 CEM cells treated with **3** at various time intervals.

Furthermore, we and others[23–26] discovered that enzyme attack takes place at the ester side chain of the molecule (not at the phosphorus center).[34]

After establishing that the ester side chain of the molecule is the site of attack, we also examined the hydrolysis profile of phosphoramidate derivatives with various ester side groups in the presence of bacterial enzymes, including esterase, lipase, and protease. Incubation of all the derivatives with the different ester substitutions with esterase showed that methyl and ethyl substituted compounds undergo facile hydrolysis. The *tert*-butyl substituted compound underwent extremely slow hydrolysis. In general, the *tert*-butyl substituted compound was resistant to hydrolysis by bacterial enzymes. We now show that both methyl and ethyl compounds undergo hydrolysis by various cell lines, whereas *tert*-butyl substituted compound **3** does not.

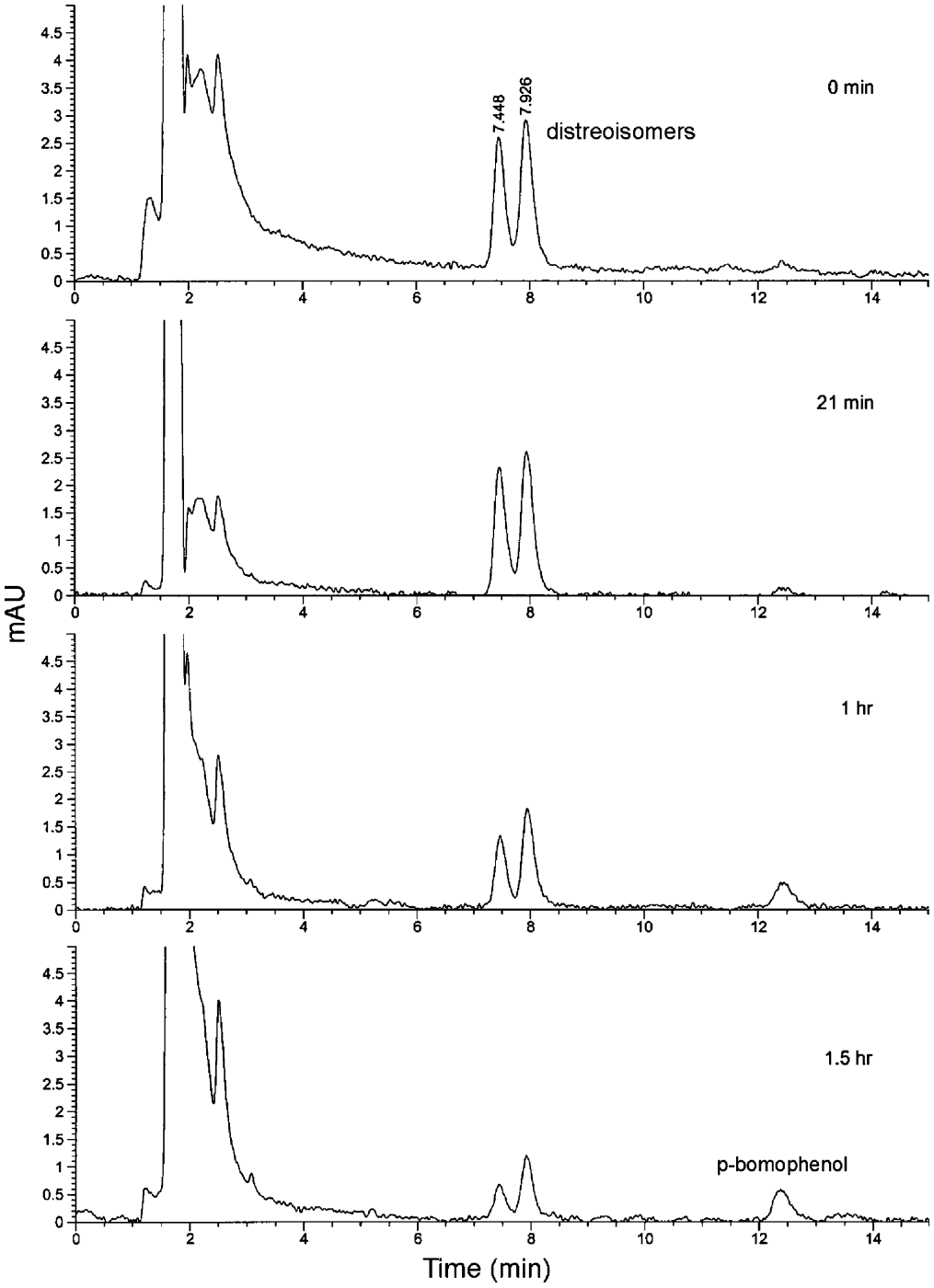


Figure 8. HPLC profile of extracts from 33×10^6 LL2 mouse lung cells treated with **1** at various time intervals.

4. Comparison of rate of hydrolysis

Table 1 shows the rate of hydrolysis of all the three compounds in presence of each cell line used in the present study. The rates indicate a first order rate constant for both the isomers. Although multiple steps may be involved in the hydrolysis during treatment with cells, for simplicity and comparative purposes, we assumed that the rate of formation of products follows a first order kinetics, especially in the early kinetic cycle.

The fastest rate of hydrolysis for most of the compounds was observed in RAW264 cells and the slowest rate of hydrolysis was observed with Jurkat cells. The rate of hydrolysis of methyl-substituted compound **1** in RAW264 cells was approximately three times faster than its rate of hydrolysis in Cos-7 cells and seven times faster than its rate of hydrolysis in Jurkat cells. Similarly the rate of hydrolysis for compound **1** was four times faster in RAW264 cells than in LL2 cells and approximately four times faster than in CEM cells.

The ethyl substituted compound **2** showed an almost identical trend when treated with similar cells. For example, the fastest rate of hydrolysis was observed

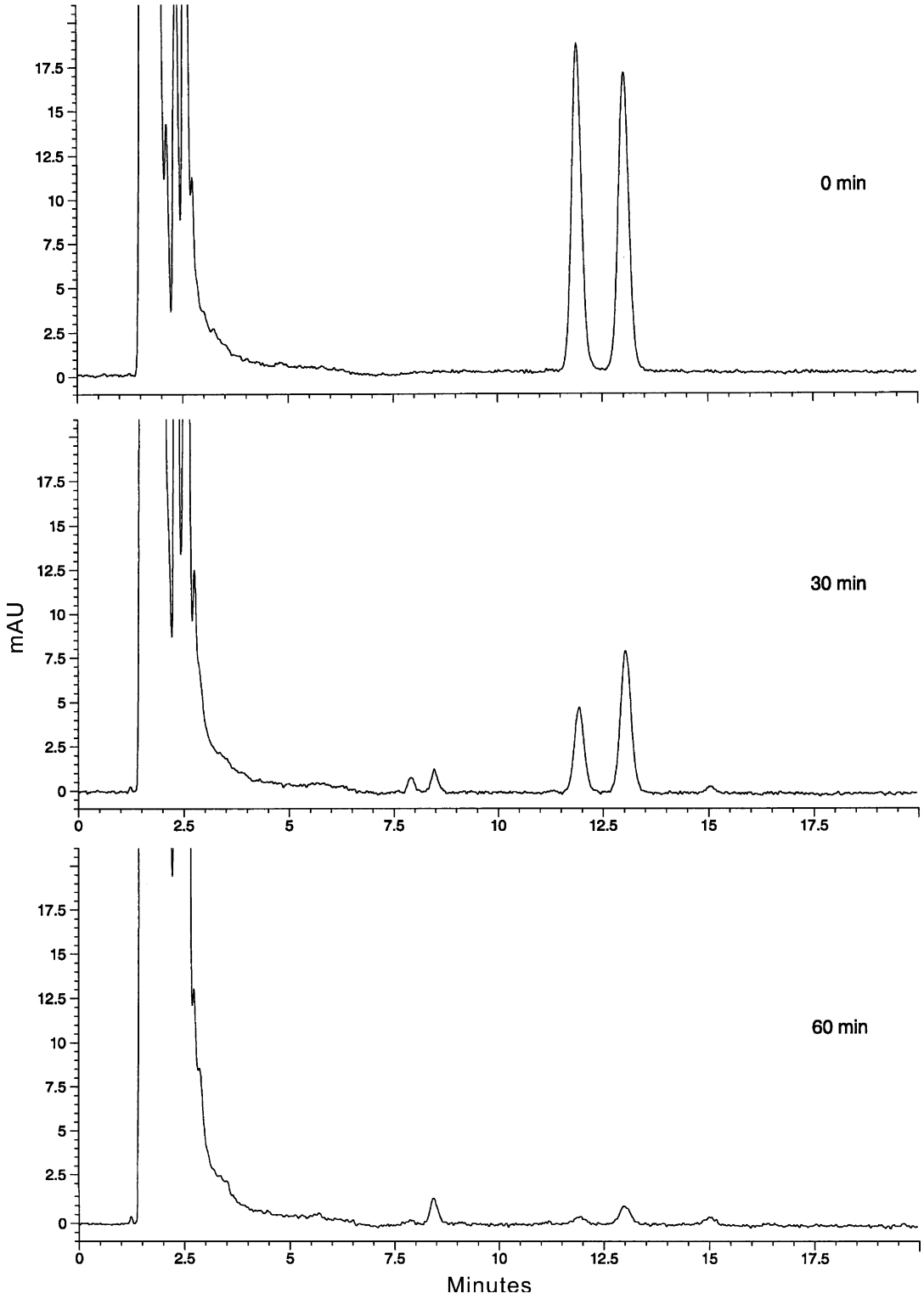


Figure 9. HPLC profile of supernatant from 33×10^6 LL2 mouse lung cells treated with **2** at various time intervals.

in RAW264 cells followed by Cos-7 cells and LL2 lung cells. The rate of hydrolysis for **2** was slightly faster than its rate of hydrolysis in Cos-7 cells (4.62 vs 3.52). The ethyl substituted compound **2** showed a threefold faster hydrolysis rate in RAW264 cells as compared to Jurkat cells. Contrary to the results obtained for methyl substituted compound **1**, the ethyl substituted compound **2** showed only a twofold higher rate of hydrolysis in RAW264 cells as compared to CEM cells. However, the trend was similar to that obtained for methyl substituted compound. This is expected due to the fact that methyl and ethyl substitutions should not change drastically the structural features of the phosphoramidates.

When we examined the rate of hydrolysis of *tert*-butyl substituted compound (**3**), we observed that the highest rate of hydrolysis was seen in RAW264 and Cos-7 cells (1.19 and 0.20). The rate of hydrolysis of this compound in LL2 as well as CEM cells was very low (0.09 and 0.04) and interestingly the compound did not undergo hydrolysis in the presence of Jurkat cells. In general, the rate of hydrolysis of this compound in all the cell lines was found to be very slow demonstrating the fact that the *tert*-butyl substituted compound is very resistant to enzyme-mediated hydrolysis. This may be rationalized due to a steric hindrance as well as variation in electron density caused by bulky *tert*-butyl group hampering its ability to enter the enzyme's active pocket.

It is also possible that the same protein is present in the cells but in different quantities which may be responsible for the observed results. In order to find out whether this factor influences the rate of hydrolysis and the stereochemistry, we quantified the protein contents of few cells. The experimental protocol for such a study is described in Section 5. Table 3 shows the soluble protein content obtained for two cell lines. It is evident from the table values that there is no significant variation in the soluble protein content between these two cell lines. Hence, we conclude that the amount of protein content does not affect the results of our present study.

Figure 10A shows the rate of hydrolysis of alkyl-substituted phosphoramidates treated with various cells under identical experimental conditions. It is evident that **2** shows invariably the highest rate of hydrolysis followed by methyl and *tert*-butyl substituted compounds. In our earlier studies using bacterial enzymes, we also observed that ethyl substituted compound showed highest rate of hydrolysis (Table 2), implying that ethyl group is preferred in the case of phosphoramidate derivatives (Fig. 10B). Additionally, we observe an identical trend between bacterial enzymes and cell treated systems implying perhaps that similar enzymes may be responsible for the hydrolysis of these phosphoramidate derivatives. Furthermore, it also supports our earlier proposal that the site of attack of the enzyme is the ester side chain of the molecule.

Depending on the cell line utilized we observed varying degrees of chiral selectivity in the hydrolysis of phosphoramidate isomers (Table 1). Notably, both RAW264

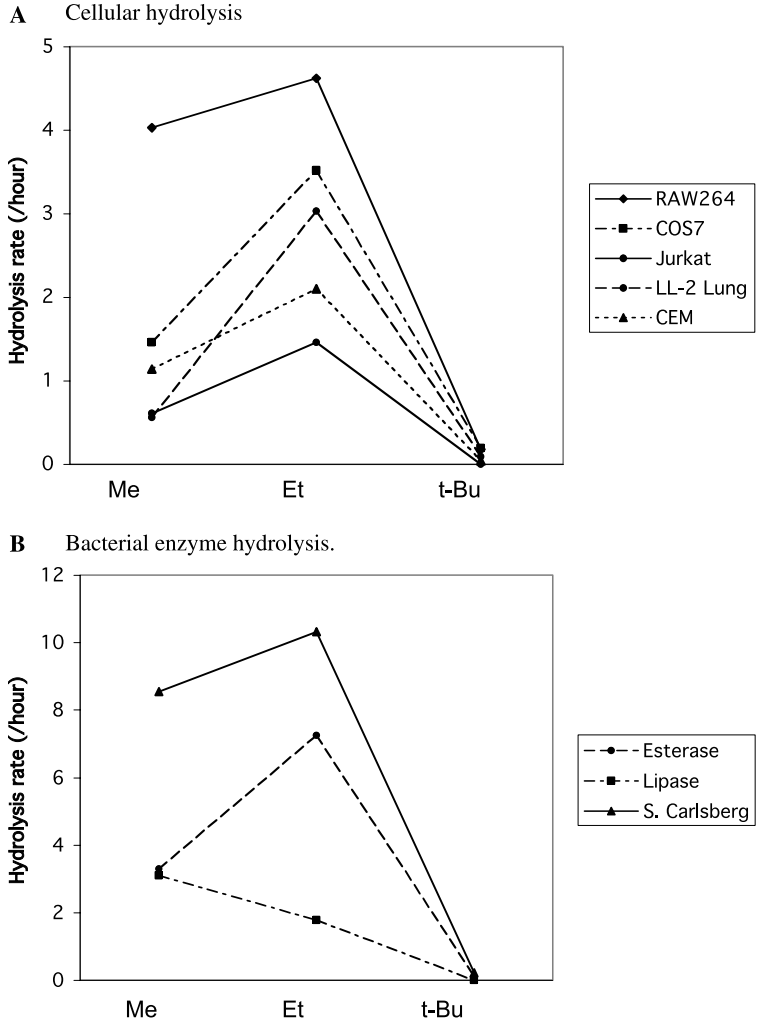
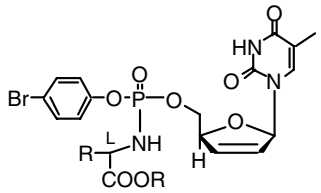


Figure 10. Cellular and bacterial enzymatic hydrolysis of various alkyl substituted phosphoramidate derivatives.

Table 2. Rate of hydrolysis of various substituted esters of *p*-bromophenyl alaninylphosphoramidate derivatives of d4T



Compound	Ester Group®	Alkali	Esterase	Lipase	S. Carlsberg
1	Me	2.07 ± 0.33	3.3 ± 0.5	3.1 ± 0.5	8.55 ± 0.7
2	Et	4.58 ± 0.52	7.25 ± 0.4	1.78 ± 0.1	10.32 ± 1.38
3	*t*-Bu	1.18 ± 0.13	0.11 ± 0.05	0.0[a]	0.22 ± 0.1

Rates are expressed per hour and for three independent trials.
[a] Did not hydrolyze the compound even after 45 min at room temperature.

and Cos-7 cells showed a preference for the '*R*' isomer of the phosphoramidate derivative in all the three compounds, while '*S*' isomer was preferred for other cell lines. In summary, the results presented herein support our notion that a change in the alkyl group in the ester side chain of phosphoramidate derivative of d4T causes significant alteration in the rate of hydrolysis. This provides additional evidence that the site of attack is the ester side chain of the molecule.

5. Experimental

Compounds were prepared following literature reported procedures.[10–28] The purity and structure of the compounds were confirmed using standard analytical techniques. For the hydrolysis experiments the compounds were first dissolved in methanol (3–4 mg/3 ml) and 100 μl of methanolic solution was transferred into a vial to which 900 μl water was added and the contents shaken to ensure thorough mixing. This diluted solution (100 μl) was used for the studies.

5.1. Enzymes

Bacterial enzymes used in the present study were obtained from Biocatalytic Inc and were used as such without further purification. The experimental procedure followed for bacterial enzyme studies has been reported previously.[29]

5.2. Cellular studies

The cells were propagated in RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate, 90%; fetal bovine serum, 10%, at 37 °C in a humidified atmosphere containing 5% CO_2. The cell line was obtained from the Cell Biology Laboratory of the Parker Hughes Cancer Center. To obtain these cells, 30 million frozen cells were plated in a T-150 flask with 20 ml of medium and incubated at 37 °C for 48 h. The cells were collected in a 50 ml conical tube and centrifuged at 1000 rpm for 5 min, the supernatant decanted off, and the cells were re-suspended in 90 ml of medium and grown further in three T-150 flasks with 30 ml each at 37 °C for another 48 h to give 120 million cells. The cells were collected by centrifugation at 1000 rpm for 5 min, subsequently washed thrice in 1XPBS, and then re-suspended in 2 ml of 1XPBS. No significant differences existed in the protein content of the different T-cell lines (Table 3).

Table 3. Soluble protein content of T-cell lines (mg/30×10^6 cells)

Cells	Trial 1	Trial 2	Trial 3	Average
CEM	1.36	1.57	1.50	1.48
Jurkat	1.22	2.02	1.39	1.54

Table 4. Number of cells used in each experiment (10^6)[35,36]

Cell type	1	2	3	4	5	Average (±SD)
LL2	35	35	35	30	30	33 ± 3
CEM	30	30	30	30	30	30 ± 0
Jurkat	30	30	30	28	—	30 ± 1
RAW264	30	30	30	30	30	30 ± 0
Cos-7	30	30	30	27	—	29 ± 2

For the HPLC measurements, 0.5 ml of the suspended cell mixture containing cells (Table 4) in PBS buffer was taken and treated with compounds and incubated at 37 °C. At various time intervals, an aliquot of the supernatant was drawn and assayed using HPLC. Authentic samples were used to identify the products during cell-mediated hydrolysis of various experimental compounds. A lichrospher-RP-18 column was used (4.0×250 mm) for this purpose and the eluent was a mixture consisting of 65% water containing 0.1% TEA and TFA and 35% of acetonitrile. The flow rate was maintained at 1 ml/min and the column was maintained at room temperature throughout the analysis. The rate of reaction was calculated using a first order kinetic equation.

5.3. Protein content determination

For the determination of soluble protein content of the cells, a bicinchoninic acid assay (BCA) procedure (Sigma procedure # TPRO-562) was used. In brief, standard solution of human albumin (Sigma, A 9511) in water was prepared and was treated with Cu-BCA to

form the chelate and the absorbance was measured using a UV–vis spectrometer following at 562 nm. Seven to eight standard solutions were made and a calibration curve was obtained. Thirty million cells were taken in a centrifuge tube and were lysed using 1 ml of lysing buffer containing a mixture of ammonium chloride, EDTA, and sodium bicarbonate. The resulting mixture was centrifuged (9× 1000 rpm) for 5 min and 100 μl of the supernatant was drawn and mixed with 2 ml Cu-BCA solution (1:50 ratio). The above mixture was incubated at 37 °C for 30 min and the absorbance was measured at 562 nm. From the absorbance values, the soluble protein content of the extract was obtained. The above experiment was repeated three times using each time a fresh batch of the cells.

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