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# Synthesis and anticancer activity of 5′-chloromethylphosphonates of 3′-azido-3′-deoxythymidine (AZT)

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ABSTRACT

A series of novel *N*-alkyl 5′-chloromethylphosphonates of 3′-azido-3′-deoxythymidine (**6**–**15**) was synthesized by means of phosphonylation of 3′-azido-3′-deoxythymidine (**4**) with *P*-chloromethylphosphonic ditriazolide (**3**) followed by a reaction with the appropriate amine. The synthesized phosphonamidates **6**–**15** were evaluated for their cytotoxic activity in two human cancer cell lines: oral (KB) and breast (MCF-7) using the sulforhodamine B (SRB) assay. The highest activity in KB human cancer cells was displayed by phosphonamidate **8** ($IC_{50}$ = 5.8 μg/mL), however, this compound was less potent than the parent AZT ($IC_{50}$ = 3.1 μg/mL). Phosphonamidate **10** showed only moderate activity ($IC_{50}$ = 12.1 μg/mL) whereas the other phosphonamidates proved inactive. Similarly, the highest activity in MCF-7 human cancer cells was displayed by phosphonamidate **8** ($IC_{50}$ = 3.7 μg/mL) but it proved somewhat less active than AZT ($IC_{50}$ = 2.6 μg/mL). Some activity was also displayed by phosphonamidate **10** ($IC_{50}$ = 12.8 μg/mL) but the other phosphonamidates were found inactive. Hydrolysis studies indicate that the synthesized phosphonamidates are likely to act as prodrugs of the parent nucleoside (AZT). Transport measurements showed that the most active phosphonamidates (**8** and **10**) were able to permeate across the intestinal epithelium in vitro. The apparent permeability coefficients determined in Caco-2 cell monolayers indicated that these compounds could be moderately absorbed in humans.



## 1. Introduction

Several nucleoside analogues have found important use as antiviral[1] and anticancer[2–4] therapeutics. Notably, 3′-azido-3′-deoxythymidine (AZT, zidovudine) has been developed as an anti-HIV drug for the treatment of acquired immunodeficiency syndrome (AIDS).[5] On the other hand, there are some examples of application of AZT as an antitumor agent in combination with either cisplatin, methotrexate or 5-fluorouracil in the therapy of advanced colon cancer.[6] In addition, Wagner reported the potent inhibitory activity of AZT in cultured human breast cancer cells.[7] It is assumed that the mechanism of anticancer action of AZT involves its intracellular conversion to the 5′-triphosphate (via the corresponding mono- and diphosphate). The 5′-triphosphate of AZT acts as a competitive inhibitor of DNA polymerases and a chain terminator of the nascent DNA strand due to the lack of a 3′-hydroxyl group.[6] The development of AZT prodrugs which can easily penetrate the lipid-rich cell membranes and then undergo hydrolysis to the 5′-monophosphate or its analogues has therefore become an increasingly important area of research.[8–12] The 5′-monophosphate of AZT itself can not be employed as a prodrug since it is negatively charged at physiological pH and consequently too polar to cross the cell membranes.[9] Moreover, the blood and cell surface phosphohydrolases effectively convert nucleoside 5′-phosphates to the parent nucleosides.[9]

A number of AZT monophosphate prodrugs was synthesized and evaluated for their antiviral and anticancer activity.[13] Meier developed the so-called *cyclo*Sal-pronucleotide approach using the bifunctional salicylic alcohol to protect the phosphate group.[14] McGuigan found that some alkyloxy phosphoramidates,[15,16] diaryl phosphates[17] and aryloxy phosphoramidate diesters[18,19] of AZT were effective pronucleotides[12] whereas amino acid phosphoramidate monoesters[20,21] of AZT were investigated by Wagner.[9,11] Périgaud elaborated *S*-acyl-2-thioethyl (SATE) phosphotriesters[22,23] and phosphoramidate diesters[24] of AZT as useful pronucleotides.[25]

It should be noted that there were also several attempts to synthesize phosphonate prodrugs of AZT. Thus, Rosowsky published the synthesis and antiretroviral activity of 5′-phosphonoformates of AZT having the carboxyl group esterified with long chain alcohols.[26] Other 5′-phosphonoformate esters of AZT incorporating *S*-(pivaloyl)thioethyl (*t*-butyl-SATE) group were described by

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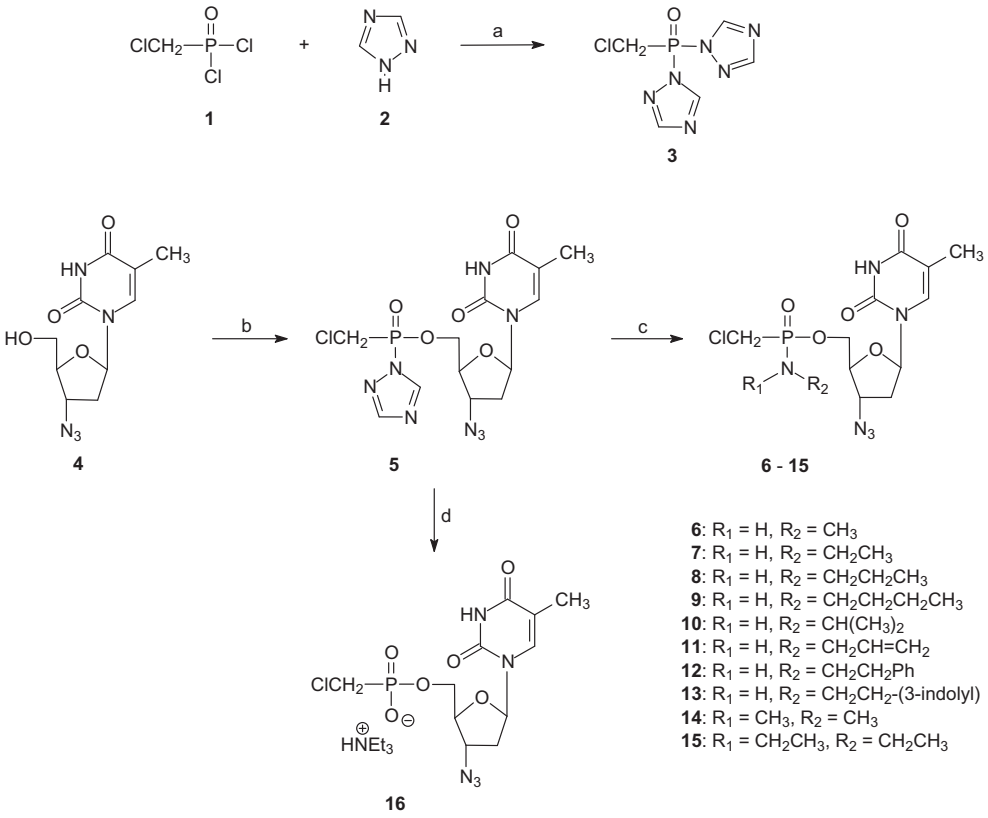


**Scheme 1.** Synthesis of the 5′-chloromethylphosphonates of 3′-azido-3′-deoxythymidine. Reagents and conditions: (a) $NEt_3$, $CH_3CN$, rt; (b) **3**, pyridine, rt; (c) $R_1$–NH–$R_2$, rt; (d) $H_2O$, pyridine, $NEt_3$, rt.

Meier.[27] Shirokova reported the synthesis and anti-HIV activity of two series of 5′-phosphonoformate and 5′-phosphonoacetate derivatives of AZT.[28] Recently, Kraszewski examined dinucleoside phosphonate–phosphates[29] and 5′-α-hydroxyphosphonates[30] of AZT as potential anti-HIV agents.

The aim of our study was to synthesize novel phosphonate prodrugs of AZT with potential anticancer or antiviral properties. In this paper, we report the synthesis of *N*-alkyl 5′-chloromethylphosphonates of 3′-azido-3′-deoxythymidine (AZT) (**6**–**15**) and evaluation of their cytotoxic activity in two human cancer cell lines: oral (KB) and breast (MCF-7). Moreover, chemical stability and permeability of the most active compounds across intestinal barrier in vitro was investigated.

## 2. Results and discussion

### 2.1. Chemistry

A series of novel phosphonamidates **6**–**15** was synthesized by phosphonylation of 3′-azido-3′-deoxythymidine (**4**) with *P*-chloromethylphosphonic ditriazolide (**3**) according to the procedure outlined in Scheme 1. *P*-Chloromethylphosphonic ditriazolide (**3**) was prepared by reaction of *P*-chloromethylphosphonic dichloride (**1**) with 1,2,4-triazole (**2**) in the presence of triethylamine in acetonitrile. Reaction of compound **3** with 3′-azido-3′-deoxythymidine (**4**) in the presence of pyridine afforded the key intermediate **5**, which, in situ, was treated with the appropriate amine (or amine hydrochloride in the presence of triethylamine) to give desired products **6**–**15** in 70–85% yield. Treatment of intermediate **5** with water, in the presence of triethylamine and pyridine, afforded *P*-chloromethylphosphonate **16**.

$^{31}P$ NMR spectra of products **6**–**15** revealed the presence of two diastereoisomers due to a chiral center being formed at the phosphorus atom. There were two close signals, in the ratio of approximately 1:1, in each $^{31}P$ NMR spectrum. Also thin layer chromatography of compounds **6**–**15** was consistent with the presence of two diastereoisomers showing two overlapping spots but we were unable to resolve them by silica gel column chromatography. However, it was possible to resolve the two diastereoisomers by HPLC on reversed-phase column (see Experimental section, compound **8** and **10**).

It is worth mentioning that the use of *P*-chloromethylphosphonic dichloride (**1**), rather than its triazolide counterpart **3**, resulted in the formation of a considerable amounts of the symmetrical (5′–5′) dinucleoside phosphonate. Application of 2- and 4-chlorophenyl phosphoroditriazolides[31–33] for the phosphorylation and methylphosphonic ditriazolide[34–36] for the phosphonylation of 5′-protected nucleosides has been reported in the phosphotriester synthesis of oligonucleotides and provided an inspiration for the development of our method.

It should be noted that *P*-chloromethyl group remains intact throughout the synthesis of phosphonamidates **6**–**15**. Apparently, nucleophilic substitution of the chlorine atom at the *P*-chloromethyl group by 1,2,4-triazole anion or alkyl amines does not take place during the course of the reaction. However, our attempts to perform nucleophilic substitution reaction on phosphonamidate **6** with azide or iodide anion (using sodium azide or iodide in DMF at 100 °C) were unsuccessful. Below 90 °C the reaction did not proceed but at higher temperatures it resulted in the mixture of products due to attack of the nucleophiles on the 5′-carbon atom of **6**, which leads to the cleavage of the ester bond. Reactivity of the *P*-chloromethyl group towards nucleophilic substitution is

enhanced in phosphonate esters bearing electron-withdrawing groups (e.g., trifluoroethyl).[37,38]

### 2.2. In vitro cytotoxicity

The synthesized phosphonamidates **6**–**15** were tested for their cytotoxic activity in two human cancer cell lines: oral (KB) and breast (MCF-7) employing sulforhodamine B (SRB) assay.[39] The highest activity in KB human cancer cells was displayed by phosphonamidate **8** ($IC_{50}$ = 5.8 μg/mL), however, it was less potent than parent AZT ($IC_{50}$ = 3.12 μg/mL). Phosphonamidate **10** showed only moderate activity ($IC_{50}$ = 12.1 μg/mL) whereas the other phosphonamidates proved inactive. Likewise, the highest activity in MCF-7 human cancer cells was displayed by phosphonamidate **8** ($IC_{50}$ = 3.7 μg/mL) which was somewhat less active than AZT ($IC_{50}$ = 2.6 μg/mL). Some activity was also shown by phosphonamidate **10** ($IC_{50}$ = 12.8 μg/mL) whereas the other phosphonamidates were inactive. The findings show that the phosphonamidate **8** with *N*-*n*-propyl substituent was the most potent compound in both the cancer cell lines (KB and MCF-7) and its activity is comparable with that of the parent AZT. Also phosphonamidate **10** with the *N*-*i*-propyl substituent exhibited moderate activity in both the cell lines. However phosphonamidate **9** with a longer *N*-*n*-butyl chain substituent was 13-fold less potent than **8** in KB cancer cells and 22-fold less potent than **8** in MCF-7 cancer cells. It is possible several reasons for this. The phosphonamidate **9** could become so hydrophobic that it is poorly soluble in aqueous phase. Alternatively, it could be caught by fat depots and never reach the intended site. Finally, hydrophobic compounds are often more susceptible to metabolism and subsequent elimination.[40]

Partition coefficient (log*P*) values of the compounds **6**–**15** were calculated[41] to determine a possible correlation between the cytotoxicity data and lipophilicity (Table 1). All of the AZT phosphonamidates were more lipophilic than AZT (log*P* = 0.06)[30], with log*P* values ranging from 0.11 to 2.37. The most active compounds **8** and **10** showed moderate values of log*P*, 1.08 and 1.02 respectively. However, linear regression analysis did not reveal any correlation between log*P* values and the cytotoxicity data.

Before we propose a mechanism of action of the target compounds some explanation for their design rationale is needed. We chose phosphonate compounds with relatively stable P–C bond and nonionic character, to ensure sufficient lipophilicity and hydrolytic stability. Some of them can be further phosphorylated to triphosphate analogues. For example, anti-HIV drug tenofovir (a phosphonate) is phosphorylated by cellular kinases to its active metabolite—tenofovir diphosphate.[43] Since *N*-alkyl *P*-methylphosphonates of AZT did not exhibit any significant cytotoxic activity (unpublished results) we turned our attention to the *P*-chloromethylphosphonates. The *P*-chloromethyl group is more polar then the *P*-methyl and to some extent can mimic the hydroxyl on a phosphorus atom.[44] Moreover, we anticipated that the *P*-chloromethylphosphonate could be converted to the *P*-hydroxymetylphosphonate and then oxidized to the *P*-carboxylphosphonate which after decarboxylation is transformed to the phosphate via the transient H-phosphonate.[26,28] From phosphonamide moiety we expected more stability than phosphonodiester could give to the structure of the target compound. Furthermore, lipophilicity of the phosphonamidates can be modulated by changing the nature of *N*-alkyl substituents.

Although the biochemical mode of action of the AZT phosphonamidates requires more detailed studies, we think that these compounds, due to their nonionic character, can penetrate the cell membranes as indicated by encouraging values of their partition coefficients (Table 1). Once inside the cells, the phosphonamidates could be hydrolyzed or metabolized to AZT either directly or via 5′-chloromethylphosphonate **16**. Finally, AZT after enzymatic phosphorylation to its 5′-triphosphate, can act as an inhibitor of DNA polymerases.

**Table 1**
In vitro cytotoxic activity of the synthesized compounds **6**–**15** in two human cancer cell lines: oral (KB) and breast (MCF-7)

| Compound | Cytotoxicity ($IC_{50}$, μg/mL)[a] ± SD[b] | | Log*P*[c] |
|---|---|---|---|
| | KB | MCF-7 | |
| **6** | 52.0 ± 1.7 | 63.2 ± 1.4 | 0.11 |
| **7** | >100 | >100 | 0.60 |
| **8** | 5.8 ± 0.8 | 3.7 ± 0.7 | 1.09 |
| **9** | 77.0 ± 3.5 | 82.0 ± 3.1 | 1.58 |
| **10** | 12.1 ± 1.9 | 12.8 ± 1.6 | 1.02 |
| **11** | 47.0 ± 4.1 | 34.0 ± 3.1 | 0.96 |
| **12** | >100 | >100 | 2.31 |
| **13** | 80.0 ± 4.9 | >100 | 2.37 |
| **14** | 65.0 ± 1.8 | 22.8 ± 1.2 | 0.66 |
| **15** | 85.0 ± 1.7 | 67.0 ± 2.5 | 1.64 |
| Zidovudine (AZT) | 3.12 ± 0.26 | 2.60 ± 0.25 | 0.06[30] |
| Cytarabine (standard) | 0.99 ± 0.02 | 0.93 ± 0.06 | −2.32[41] |

[a] $IC_{50}$ is the compound concentration required to inhibit cell growth by 50%.
[b] SD (standard deviation) of three independent experiments.
[c] Log*P* (logarithm of partition coefficient) was calculated using 'log $P_{Knowwin}$' method.[42]

### 2.3. Chemical stability

Chemical stability of the most active compounds **8** and **10** was studied in the phosphate buffer (pH 7.2, 7.4 and 7.8) at 37 °C and the concentration of the compound under study of 0.5 mM (Table 2). The composition of the reaction mixtures was analyzed by HPLC on reversed-phase column. It was established that the hydrolysis of both compounds **8** and **10** at pH 7.2 was very slow and after 7 h only a small fraction of each compound (6% and 4% respectively) underwent hydrolysis with formation of AZT as the only product. At pH 7.4 the hydrolysis was somewhat faster, 17% and 13% of compound **8** and **10**, respectively was hydrolyzed after 7 h. This time, AZT was detected as a major product and also a small amount of phosphonate **16** was formed. A further increase of the pH to 7.8 resulted in about 50% hydrolysis of compound **8** after 7 h; AZT was detected as a major product and phosphonate **16** as a minor one. The amount of phosphonate **16** did not exceed 11% of the total amount of products. The hydrolysis of compound **10** at pH 7.8 was slower than that of **8** and only 36% of **10** reacted after 7 h. Authentic samples of AZT and phosphonate **16** were used to identify the products.

More detailed stability studies of compound **8** and **10** at pH 7.8 are presented in Fig. 1.

The hydrolysis studies suggest that, the synthesized phosphonamidates act as depot forms of AZT rather than 5′-phosphonate prodrugs of AZT.[28]

### 2.4. Caco-2 permeability assay

In view of their cytotoxic activity and chemical stability, potential oral absorption of phosphonamidates **8** and **10** was also evaluated. In order to determine their permeability across the intestinal barrier we used the Caco-2 cell culture system. The expected good

**Table 2**
Hydrolysis of compounds **8** and **10** in phosphate buffer (pH 7.2, 7.4 and 7.8) at 37 °C after 7 h

| pH | Amount of the compound consumed (%) | |
|---|---|---|
| | **8** | **10** |
| 7.2 | 6 ± 1 | 4 ± 1 |
| 7.4 | 17 ± 3 | 13 ± 2 |
| 7.8 | 50 ± 5 | 36 ± 4 |

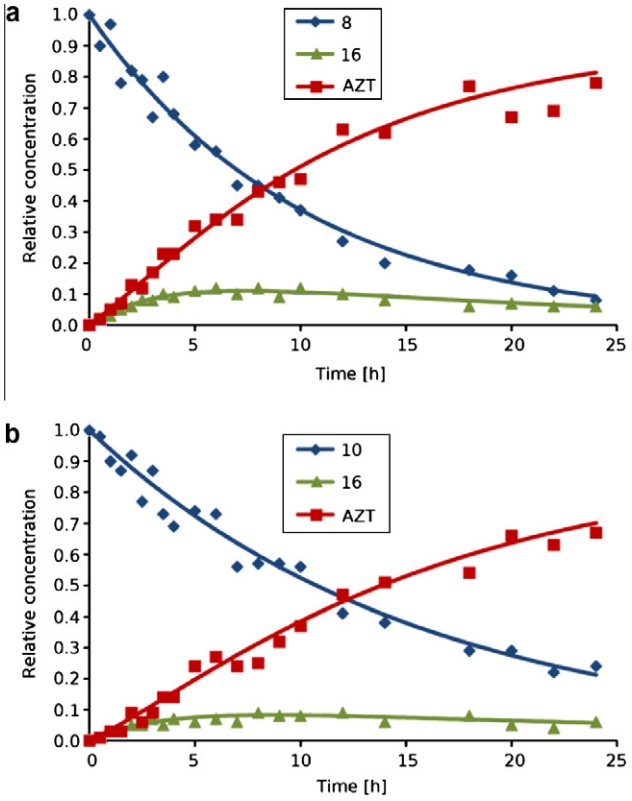


**Fig. 1.** Hydrolysis of compound **8** (a) and **10** (b) at 0.5 mM initial concentration in phosphate buffer (pH 7.8) at 37 °C.

correlation between permeability in vitro and oral drug absorption in the human was based on previous studies in this cell line.[45,46]

Prior to carrying out a transport study, the cytotoxicity and the effect of compounds tested on Caco-2 monolayer integrity and intestinal barrier properties was examined. No cytotoxicity of substances at 100 μM concentration in transporting medium after a 2 h exposure was observed. The transepithelial electrical resistance (TEER) measurement indicated high integrity of Caco-2 cell monolayer in both cultures, before and after transport experiments (data not shown).

The transport of phosphonamidates **8** and **10** was evaluated at a target concentration of 100 μM and was initiated by adding the test solution to the apical (A) compartment for absorptive transport or basolateral (B) compartment for exsorptive transport determination. The solution from acceptor compartment was sampled at 15 min intervals and the concentration of a transported compound was analyzed. The results indicated that phosphonamidates **8** and **10** were able to cross the Caco-2 monolayer in both absorptive and secretory direction and no significant difference in their permeability was observed. The cumulative fraction

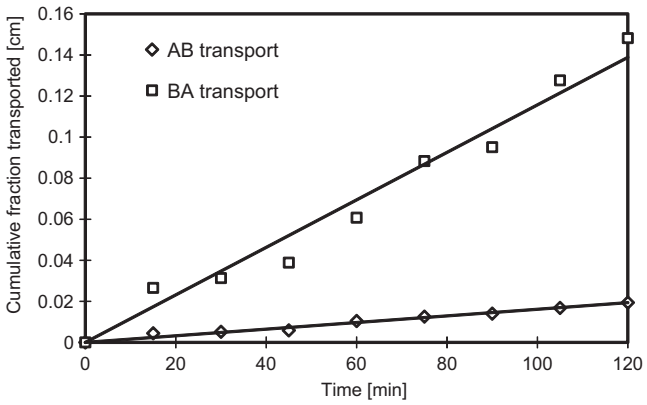


**Fig. 2.** Cumulative fraction of phosphonamidate **8** in bidirectional AB (absorptive) and BA (exsorptive) transport across Caco-2 cell monolayer.

transported (CTF) in the receiver compartment increased linearly with time, independently on transport direction (Fig. 2). The time-course experiments showed that the CFT of compounds **8** and **10** was significantly higher in the secretory direction (BA transport) than in the absorptive one (AB transport) (Fig. 2).

It was reflected in the apparent permeability coefficient ($P_{app}$) values determined for compounds transported in AB ($P_{app,AB}$) and BA direction ($P_{app,BA}$). As shown in Table 3, the $P_{app,BA}$ values were approximately fivefold higher than the $P_{app,AB}$ values suggesting that secretory transport rather than absorptive transport of phosphonamidates **8** and **10** is predominant in the Caco-2 system. These results indicate that phosphonamidates tested, similarly to the parent AZT, may be a substrate for efflux carrier modulating its absorptive permeability. The current data show that AZT efflux in the intestine is mainly mediated by the ATP-binding cassette (ABC) family of proteins, suggesting the intervention of P-glycoprotein (P-gp), multi-drug resistance proteins (MRP-2, MRP-4) and breast cancer-related protein (BCRPs) in these phenomena.[47–51]

In spite of the impact of efflux mechanism in phosphonamidates **8** and **10** absorption, the apical-to-basolateral permeability coefficients (3.22 and 3.36 × $10^{-6}$ cm/s, respectively) determined in the Caco-2 cell assay, suggest relatively high oral availability. The drug substances with $P_{app}$ values ranging from 1 to 10 × $10^{-6}$ cm/s can be classified as moderately (20–70%) absorbed compounds.[46] In vitro permeability of phosphonamidates **8** and **10** is comparable to permeability of the parent AZT reported in the literature. The $P_{app}$ values of AZT determined in the Caco-2 cell assay are usually in the range of 2.85 to 8.5 × $10^{-6}$ cm/s[52–55] and confirm moderate oral bioavailability of this drug (63 ± 10%).[56] A better prediction of phosphonamidates (**8** and **10**) absorption in humans could be obtained by using a P-gp inhibitor in bidirectional permeability studies. The inclusion of a P-gp inhibitor would probably result in increased absorptive and decreased secretory transport of the compounds tested.

**Table 3**
The apparent permeability coefficients of phosphonamidates **8** and **10** and the parent nucleoside (zidovudine, AZT)

| Compound | $P_{app}$ (×$10^{-6}$ cm/s)[a] ± SD[b] | | Log $P_{app}$[a] | | Log $tP_{app}$[c] |
|---|---|---|---|---|---|
| | A–B | B–A | A–B | B–A | |
| **8** | 3.22 ± 0.76 | 16.10 ± 0.33 | −5.49 | −4.79 | −5.53 |
| **10** | 3.36 ± 0.64 | 18.00 ± 0.47 | −5.47 | −4,75 | −5.52 |
| Zidovudine (AZT) | 2.85 ± 0.07[54] | | −5.55[54] | | −5.16 |
| | 4.0 ± 0.2[52] | | −5.40[52] | | |
| | 6.93 ± 0.17[53] | | −5.16[53] | | |
| | 8.5 ± 0.2[55] | | −5.07[55] | | |

[a] $P_{app}$ (apparent permeability coefficient) was determined using method described by Tavelin et al.[58], A – apical side, B – basolateral side.
[b] SD (standard deviation) of three independent experiments.
[c] $tP_{app}$ (theoretical value of $P_{app}$) calculated by method described by Guangli and Yiyu.[57]

It is noteworthy that the $P_{app,AB}$ values determined experimentally for both the compounds in the Caco-2 system were comparable to theoretically calculated ones ($tP_{app}$) using the method described by Guangli and Yiyu.[57] The log $P_{app}$ values, experimental as well as calculated, are presented in Table 3.

## 3. Conclusion

In summary, we have developed an efficient method for the synthesis of *N*-alkyl 5′-chloromethylphosphonates of 3′-azido-3′-deoxythymidine employing *P*-chloromethylphosphonic ditriazolide as phosphonylating agent. *P*-Chloromethylphosphonic ditriazolide was more selective than its dichloro counterpart and its use did not result in the formation of symmetrical (5–5′)dinucleoside phosphonates. It was established that the *P*-chloromethyl group in phosphonamidates **6**–**15** was chemically stable and did not undergo reactions with nucleophiles, such as amines and azide anion, at room temperature. The obtained compounds **6**–**15** were examined for their cytotoxic activity in two human cancer cell lines: oral (KB) and breast (MCF-7). The highest activity in KB human cancer cells was displayed by phosphonamidate **8** with the *N*-*n*-propyl substituent ($IC_{50}$ = 5.8 μg/mL) but it was less potent than the parent AZT ($IC_{50}$ = 3.1 μg/mL). Similarly, the highest activity in MCF-7 human cancer cells was displayed by phosphonamidate **8** ($IC_{50}$ = 3.7 μg/mL) which was somewhat less potent than AZT ($IC_{50}$ = 2.6 μg/mL). In view of our hydrolysis studies, the synthesized phosphonamidates are most probably the prodrug forms of the parent AZT. The results of transport studies suggest that phosphonamidates **8** and **10** with $P_{app}$ values of 3.22 and 3.36 × $10^{-6}$ cm/s, respectively, can be considered as moderately absorbed in humans.

## 4. Experimental section

### 4.1. Chemistry

NMR spectra were recorded on a Varian-Gemini 300 MHz spectrometer. Chemical shifts ($\delta$) are reported in ppm relative to the tetramethylsilane (TMS) peak. For $^{31}$P NMR spectra 85% phosphoric(V) acid in $D_2O$ was used as an external standard (coaxial inner tube). Mass spectra were measured on a Waters Micromass ZQ electrospray (ES) mass spectrometer. Elemental analyses were performed on EL III elemental analyzer (Elementar Analysensysteme GmbH, Germany). Thin layer chromatography (TLC) was performed on silica gel 60 $F_{254}$ precoated (0.2 mm) plates and vacuum flash column chromatography on silica gel 60 H (5–40 μm) purchased from Merck. High performance liquid chromatography (HPLC) was performed on a Waters chromatograph equipped with a Waters 996 UV-Vis photodiode array detector. Analytical HPLC was carried out on Waters XTerra $RP_{18}$ reversed-phase column (4.6 × 150 mm, 5 μm) using three eluting systems: (a) water–methanol (70:30), (b) 25 mM ammonium formate (pH 7.0)–methanol (80:20) and (c) phosphate buffer (20 mM $Na_2HPO_4$, pH was adjusted to 7.1 with $H_3PO_4$)–acetonitrile (80:20). Water contained 5% methanol to prevent bacterial growth. The flow rate was 1 mL/min and detection at 266 nm. Chemical reagents were purchased from Sigma-Aldrich. 3′-Azido-3′-deoxythymidine was prepared based on the literature procedure.[59]

#### 4.1.1. General procedure for the preparation of compounds 6–15

To a solution of *P*-chloromethylphosphonic dichloride (**1**) (153 mg, 0.914 mmol) in acetonitrile (2 mL) was added 1,2,4-triazole (**2**) (164 mg, 2.374 mmol) followed by triethylamine (189 mg, 1.865 mmol) and the reactants were stirred for 30 min at room temperature. Then to the mixture 3′-azido-3′-deoxythymidine (**4**) (100 mg, 0.374 mmol) and pyridine (2.30 mL) were added. The reaction mixture was stirred at room temperature for a further 1 h and the appropriate amine (4.57 mmol) or amine hydrochloride (4.57 mmol) and triethylamine (694 mg, 6.86 mmol) was added. After 1 h, the reaction mixture was evaporated under reduced pressure. To the residue was added saturated aqueous sodium bicarbonate (10 mL) and the mixture was extracted with chloroform (3 × 10 mL). The combined chloroform extracts were washed with water (10 mL), dried over anhydrous magnesium sulfate, filtered and evaporated to dryness. The residue was purified by silica gel column chromatography using chloroform – methanol (50:1, v/v) as eluent to give pure products **6**–**15** (yield 70–85%).

**4.1.1.1. 3′-Azido-3′-deoxythymidine 5′-(*N*-methyl-*P*-chloromethylphosphonate) (6).** $^1$H NMR ($CDCl_3$): $\delta$ 1.84 (d, 3H, $J$ = 1.0 Hz, 5-$CH_3$); 2.13–2.32 (m, 2H, H-2″, H-2′); 2.64–2.72 (m, 3H, N–$CH_3$); 3.27–3.41 (m, 3H, P–$CH_2$–Cl, NH–C); 3.47–3.58 (m, 1H, H-4′); 4.20–4.35 (m, 3H, H-5′, H-5″, H-3′); 6.24 (t, 1H, $J$ = 6.1 Hz, H-1′); 7,43 (d, 1H, $J$ = 1.1 Hz, H-6); 9.51 (s, 1H, 3-NH). $^{13}$C NMR ($CDCl_3$): $\delta$ 12.55 (5-$CH_3$); 27.06 (N–$CH_3$); 35.28 (d, $J_{P-C}$ = 155 Hz, P–$CH_2$–Cl); 37.11 (C-2′); 60.15 (C-3′); 63.07 (C-5′); 82.41 (C-1′); 85.47 (C-4′); 111.22 (C-5); 135.80 (C-6); 150.50 (C-2); 166.40 (C-4). $^{31}$P NMR ($CDCl_3$): $\delta$ 25.28; 25.34. MS-ESI $m/z$: 393; 395 $[M+H]^+$; 415; 417 $[M+Na]^+$; 391; 393 $[M-H]^-$; 427; 429; 431 $[M+Cl]^-$. Anal. Calcd for $C_{12}H_{18}ClN_6O_5P$: C, 36.70; H, 4.62; N, 21.40. Found: C, 36.72; H, 4.61; N, 21.42.

**4.1.1.2. 3′-Azido-3′-deoxythymidine 5′-(*N*-ethyl-*P*-chloromethylphosphonate) (7).** $^1$H NMR ($CDCl_3$): $\delta$ 1.20 (t, 3H, $J$ = 7.0 Hz, N-C-$CH_3$); 1.93 (d, 3H, $J$ = 3.0 Hz, 5-$CH_3$); 2.33–2.42 (m, 2H, H-2′, H-2″); 3.06–3.25 (m, 3H, N-$CH_2$-C, NH-Et); 3.60–3.71 (m, 2H, P-$CH_2$-Cl); 4.04–4.08 (m, 1H, H-4′); 4.21–4.40 (m, 2H, H-5′, H-5″); 4.42–4.51 (m, 1H, H-3′); 6.14 and 6.21 (t, 1H, $J$ = 6.5 Hz, H-1′); 7.38 (s, 1H, H-6); 9.54 (s, 1H, 3-NH). $^{13}$C NMR ($CDCl_3$): $\delta$ 12.48 (5-$CH_3$); 17.74 (N-C-$CH_3$); 35.18 (d, $J_{P-C}$ = 155 Hz, P–$CH_2$–Cl); 36.27 (N–$CH_2$–C); 37.24 (C-2′); 60.53 (C-3′); 63.10 (d, $^2J_{P-C}$ = 6.0 Hz, C-5′); 82.30 (C-1′); 85.44 (C-4′); 111.54 (C-5); 135.64 (C-6); 150.30 (C-2); 163.86 (C-4). $^{31}$P NMR ($CDCl_3$): $\delta$ 24.75; 24.87. MS-ESI $m/z$: 407; 409 $[M+H]^+$; 429; 431 $[M+Na]^+$; 445; 447 $[M+K]^+$; 405; 407 $[M-H]^-$; 441; 443; 445 $[M+Cl]^-$. Anal. Calcd for $C_{13}H_{20}ClN_6O_5P$: C, 38.39; H, 4.96; N, 20.66. Found: C, 38.43; H, 4.97; N, 20.64.

**4.1.1.3. 3′-Azido-3′-deoxythymidine 5′-(*N*-*n*-propyl-*P*-chloromethylphosphonate) (8).** $^1$H NMR ($CDCl_3$): $\delta$ 0.94 (t, 3H, $J$ = 7.3 Hz, N–C–C–$CH_3$); 1.55 (sextet, 2H, $J$ = 7.3 Hz, N–C–$CH_2$–C); 1.93 (d, 3H, $J$ = 1.1 Hz, 5-$CH_3$); 2.33–2.47 (m, 2H, H-2′, H-2″); 2.95–3.05 (m, 2H, N–$CH_2$–C–C); 3.28–3.36 (m, 1H, NH–Pr); 3.57–3.68 (m, 2H, P–$CH_2$–Cl); 4.02–4.10 (m, 1H, H-4′); 4.17–4.51 (m, 3H, H-5′, H-5″, H-3′); 6.14 and 6.21 (t, 1H, $J$ = 6.6 Hz, H-1′); 7.32 and 7.38 (d, 1H, $J$ = 1.1 Hz, H-6); 9.75 (s, 1H, 3-NH). $^{13}$C NMR ($CDCl_3$): $\delta$ 11.01 (N–C–C–$CH_3$); 12.45 (5-$CH_3$); 25.29 (N–C–$CH_2$–C); 35.06 (d, $J_{P-C}$ = 142 Hz, P–$CH_2$–Cl); 37.21 (C-2′); 43.08 (N–$CH_2$–C–C); 60.53 (C-3′); 63.61 (C-5′); 82.29 (C-1′); 85.43 (C-4′); 111.52 (C-5); 135.59 (C-6); 150.34 (C-2); 163.88 (C-4). $^{31}$P NMR ($CDCl_3$): $\delta$ 25.06; 25.23. MS-ESI $m/z$: 421; 423 $[M+H]^+$; 443; 445 $[M+Na]^+$; 459; 461 $[M+K]^+$; 419; 421 $[M-H]^-$; 455; 457; 459 $[M+Cl]^-$. Anal. Calcd for $C_{14}H_{22}ClN_6O_5P$: C, 39.96; H, 5.27; N, 19.97. Found: C, 39.98; H, 5.28; N, 19.96. HPLC: eluent (a), retention time ($t_R$) of 15.6 and 17.2 min in the ratio 1:1; eluent (c), retention time ($t_R$) of 13.9 and 15.4 min in the ratio 1:1.

**4.1.1.4. 3′-Azido-3′-deoxythymidine 5′-(*N*-*n*-butyl-*P*-chloromethylphosphonate) (9).** $^1$H NMR ($CDCl_3$): $\delta$ 0.92 (t, 3H, $J$ = 7.2 Hz, N–C–C–C–$CH_3$); 1.35 (sextet, 2H, $J$ = 7.4 Hz, N–C–C–$CH_2$–C); 1.43–1.56 (m, 2H, N–C–$CH_2$–C–C); 1.93 (d, 3H, $J$ = 1.4 Hz, 5-$CH_3$); 2.21–2.49 (m, 2H, H-2′, H-2″); 2.97–3.08 (m, 2H, N–$CH_2$–C–C–C);

3.30–3.40 (m, 1H, NH–C–C–C–C); 3.57–3.72 (m, 2H, P–$CH_2$–Cl); 4.02–4.17 (m, 1H, H-4′); 4.18–4.27 (m, 1H, H-3′); 4.32–4.51 (m, 2H, H-5′, H-5″); 6.14 and 6.21 (t, 1H, $J$ = 6.8 Hz, H-1′); 7.32 and 7.38 (d, 1H, $J$ = 1.4 Hz, H-6); 9.87 (s, 1H, 3-NH). $^{13}$C NMR ($CDCl_3$): $\delta$ 7.19 (5-$CH_3$); 8.36 (N–C–C–C–$CH_3$); 14.43 (N–C–C–$CH_2$–C); 28.91 (N–C–$CH_2$–C–C); 29.80 (d, $J_{P\text{-}C}$ = 144 Hz, P-$CH_2$-Cl); 31.96 (C-2′); 35.81 (N–$CH_2$–C–C–C); 55.29 (C-3′); 58.22 (d, $^2J_{P\text{-}C}$ = 6.0 Hz, C-5′); 77.03 (C-1′); 80.13 (C-4′); 106.18 (C-5); 130.22 (C-6); 144.98 (C-2); 158.49 (C-4). $^{31}$P NMR ($CDCl_3$): $\delta$ 24.85; 25.03. MS-ESI $m/z$: 435; 437 $[M+H]^+$; 457; 459 $[M+Na]^+$; 433; 435 $[M-H]^-$; 469; 471; 473 $[M+Cl]^-$. Anal. Calcd for $C_{15}H_{24}ClN_6O_5P$: C, 41.43; H, 5.56; N, 19.33. Found: C, 41.40; H, 5.57; N, 19.35.

**4.1.1.5. 3′-Azido-3′-deoxythymidine 5′-(*N*-*i*-propyl-*P*-chloromethylphosphonate) (10).** $^1$H NMR ($CDCl_3$): $\delta$ 1.23 (d, 6H, $J$ = 6.0 Hz, N-C($CH_3$)$_2$); 1.94 (d, 3H, $J$ = 1.4 Hz, 5-$CH_3$); 2.29–2.45 (m, 2H, H-2″, H-2′); 2.95–3.06 (m, 1H, NH-*i*Pr); 3.47–3.71 (m, 3H, P-$CH_2$-Cl, N-CH); 4.02–4.17 (m, 1H, H-4′); 4.19–4.40 (m, 2H, H-5′, H-5″); 4.45–4.50 (m, 1H, H-3′); 6.14 and 6.21 (t, 1H, $J$ = 6.7 Hz, H-1′); 7.31 and 7.38 (d, 1H, $J$ = 1.1 Hz, H-6); 9.41 (s, 1H, 3-NH). $^{13}$C NMR ($CDCl_3$): $\delta$ 12.53 (5-$CH_3$); 25.71, 25.91 (N–C($CH_3$)$_2$); 35.56 (d, $J_{P\text{-}C}$ = 142 Hz, P–$CH_2$–Cl); 37.27 (C-2′); 44.00 (N–CH); 60.54 (C-3′); 63.56 (C-5′); 82.33 (C-1′); 85.43 (C-4′); 111.53 (C-5); 135.50 (C-6); 150.16 (C-2); 163.59 (C-4). $^{31}$P NMR ($CDCl_3$): $\delta$ 23.34; 23.52. MS-ESI $m/z$: 421; 423 $[M+H]^+$; 443; 445 $[M+Na]^+$; 459; 461 $[M+K]^+$; 455; 457; 459 $[M-Cl]^-$. Anal. Calcd for $C_{14}H_{22}ClN_6O_5P$: C, 39.96; H, 5.27; N, 19.97. Found: C, 39.99; H, 5.26; N, 19.98. HPLC: eluent (a), retention time ($t_R$) of 14.5 and 16.1 min in the ratio 1:1.

**4.1.1.6. 3′-Azido-3′-deoxythymidine 5′-(*N*-allyl-*P*-chloromethylphosphonate) (11).** $^1$H NMR ($CDCl_3$): $\delta$ 1.93 (d, 3H, $J$ = 1.1 Hz, 5-$CH_3$); 2.26–2.49 (m, 2H, H-2′, H-2″); 3.53–3.72 (m, 5H, N-–$CH_2$–C=C, P–$CH_2$–Cl, H-4′); 4.02–4.18 (m, 1H, NH–C–C=C); 4.19–4.41 (m, 2H, H-5′, H-5″); 4.44–4.50 (m, 1H, H-3′); 5.14–5.30 (m, 2H, N–C–C=$CH_2$); 5.82–5.94 (m, 1H, N–C–CH=C); 6.13 and 6.19 (t, 1H, $J$ = 6.7 Hz, H-1′); 7.30 and 7.36 (d, 1H, $J$ = 1.1 Hz, H-6); 9.81 (s, 1H, 3-NH). $^{13}$C NMR ($CDCl_3$): $\delta$ 12.53 (5-$CH_3$); 35.25 (d, $J_{P\text{-}C}$ = 143 Hz, P–$CH_2$–Cl); 43.58 (C-2′); 50.59 (N–$CH_2$–C=C); 60.55 (C-3′); 63.75 (d, $J_{P\text{-}C}$ = 6,3 Hz, C-5′); 82.21 (C-1′); 85.43 (C-4′); 111.45 (C-5); 115.94 (N–C–C=$CH_2$); 135.36 (N–C–CH=C); 135.53 (C-6); 150.23 (C-2); 163.80 (C-4). $^{31}$P NMR ($CDCl_3$): $\delta$ 24.70; 24.86. MS-ESI $m/z$: 419; 421 $[M+H]^+$; 441; 443 $[M+Na]^+$; 417; 419 $[M-H]^-$; 453, 455; 457 $[M+Cl]^-$. Anal. Calcd for $C_{14}H_{20}ClN_6O_5P$: C, 40.15; H, 4.81; N, 20.07. Found: C, 40.11; H, 4.80; N, 20.05.

**4.1.1.7. 3′-Azido-3′-deoxythymidine 5′-(*N*-phenethyl-*P*-chloromethylphosphonate) (12).** $^1$H NMR ($CDCl_3$): $\delta$ 1.90 (d, 3H, $J$ = 1.0 Hz, 5-$CH_3$); 2.24–2.45 (m, 1H, H-2′, H-2″); 2.82 (t, 2H, $J$ = 6.2 Hz, N–C–$CH_2$–Ph); 3.21–3.56 (m, 6H, H-4′, NH–C–C–Ph, N–$CH_2$–C–Ph, P–$CH_2$–Cl); 3.94–4.07 (m, 2H, H-5′, H-5″); 4.19–4.31 (m, 1H, H-3′); 6.11 and 6.18 (t, 1H, $J$ = 6.6 Hz, H-1′); 7.19–7.34 (m, 6H, H-6, Ph); 9.68 (s, 1H, 3-NH). $^{13}$C NMR ($CDCl_3$): $\delta$ 12.52 (5-$CH_3$); 35.17 (d, $J_{P\text{-}C}$ = 143 Hz, P–$CH_2$–Cl); 37.27 (C-2′); 38.25 (N–C–$CH_2$–Ph); 42.63 (N–$CH_2$–C–Ph); 60.56 (C-3′); 63.57 (d, $^2J_{P\text{-}C}$ = 5.8 Hz, C-5′); 82.25 (C-1′); 85.39 (C-4′); 111.43 (C-5); 126.71 (Ph); 128.61 (Ph); 128.81 (Ph); 135.42 (C-6); 138.06 (Ph); 150.13 (C-2); 163.61 (C-4). $^{31}$P NMR ($CDCl_3$): $\delta$ 24.40; 24.62. MS-ESI $m/z$: 483; 485 $[M+H]^+$; 505; 507 $[M+Na]^+$; 481; 483 $[M-H]^-$; 517; 519; 521 $[M+Cl]^-$. Anal. Calcd for $C_{19}H_{24}ClN_6O_5P$: C, 47.26; H, 5.01; N, 17.40. Found: C, 47.22; H, 5.00; N, 17.39.

**4.1.1.8. 3′-Azido-3′-deoxythymidine 5′-[*N*-2-(1*H*-indol-3-yl) ethyl-P-chloromethylphosphonate] (13).** $^1$H NMR (DMSO-$d_6$): $\delta$ 1.77 (s, 3H, 5-$CH_3$); 2.26–2.45 (m, 2H, H-2′, H-2″); 2.76–2.88 (m, 2H, N–C–$CH_2$–ind); 3.09–3.20 (m, 1H, NH–C–C–ind); 3.26–3.53 (m, 5H, P–$CH_2$–Cl, N–$CH_2$–C–ind, H-4′); 3.65–3.78 (q, 1H, $J$ = 12.5 Hz, H-3′); 3.98–4.17 (m, 2H, H-5′, H-5″); 6.13 (t, 1H, $J$ = 6.7 Hz, H-1′); 6.94–7.56 (m, 6H, H-6, ind); 10.83 (s, 1H, NH–ind); 11.38 (s, 1H, 3-NH). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 12.10 (5-$CH_3$); 27.13 (d, $J_{P\text{-}C}$ = 142 Hz, P–$CH_2$–Cl); 34.32 (N–C–$CH_2$–ind); 35.73 (C-2′); 41.29 (N–$CH_2$–C–ind); 60.13 (C-3′); 63.11 (d, $^2J_{P\text{-}C}$ = 5.5 Hz, C-5′); 81.73 (d, $^3J_{P\text{-}C}$ = 6.7 Hz, C-4′); 83.51 (C-1′); 109.93 (ind); 111.37 (C-5); 112.03 (ind); 118.20 (ind); 120.86 (ind); 122.60 (ind); 122.86 (ind); 127.12 (ind); 135.78 (ind); 136.19 (C-6); 150.37 (C-2); 163.66 (C-4). $^{31}$P NMR (DMSO-$d_6$): $\delta$ 25.38; 25.58. MS-ESI $m/z$: 522; 524 $[M+H]^+$; 544; 546 $[M+Na]^+$; 560; 562 $[M+K]^+$; 520; 522 $[M-H]^-$; 556; 558; 560 $[M+Cl]^-$. Anal. Calcd for $C_{21}H_{25}ClN_7O_5P$: C, 48.33; H, 4.83; N, 18.79. Found: C, 48.35; H, 4.82; N, 18.77.

**4.1.1.9. 3′-Azido-3′-deoxythymidine 5′-(*N*,*N*-dimethyl-*P*-chloromethylphosphonate) (14).** $^1$H NMR ($CDCl_3$): $\delta$ 1.93 (d, 3H, $J$ = 1.1 Hz, 5-$CH_3$); 2.92–2.49 (m, 2H, H-2′, H-2″); 2.82 (d, 6H, $J$ = 3.7 Hz, N($CH_3$)$_2$); 3,63 and 3,64 (dd, 2H, $J$ = 4.8 Hz, $J$ = 9.5 Hz, P-$CH_2$-Cl); 4.03–4.17 (m, 2H, H-5′, H-5″); 4.30–4.41 (m, 2H, H-4′, H-3′); 6.15 and 6.23 (t, 1H, $J$ = 6.6 Hz, H-1′); 7.32 and 7.40 (d, 1H, $J$ = 1.1 Hz, H-6); 9.40 (s, 1H, 3-NH). $^{13}$C NMR ($CDCl_3$): $\delta$ 12.50 (5-$CH_3$); 34.10 (d, $J_{P\text{-}C}$ = 144 Hz, P–$CH_2$–Cl); 36.56 (N–$CH_3$); 37.33 (C-2′); 60.57 (C-3′); 63.34 (d, $^2J_{P\text{-}C}$ = 6.5 Hz, C-5′); 82.27 (d, $^3J_{P\text{-}C}$ = 6.4 Hz, C-4′); 85.29 (C-1′); 111.49 (C-5); 135.49 (C-6); 150.22 (C-2); 163.75 (C-4). $^{31}$P NMR ($CDCl_3$): $\delta$ 25.82; 25.89. MS-ESI $m/z$: 429; 431 $[M+Na]^+$; 445; 447 $[M+K]^+$; 405; 407 $[M-H]^-$; 441; 443; 445 $[M+Cl]^-$. Anal. Calcd for $C_{13}H_{20}ClN_6O_5P$: C, 38.39; H, 4.96; N, 20.66. Found: C, 38.43; H, 4.95; N, 20.65.

**4.1.1.10. 3′-Azido-3′-deoxythymidine 5′-(*N*,*N*-diethyl-*P*-chloromethylphosphonate) (15).** $^1$H NMR ($CDCl_3$): $\delta$ 1.04 (t, 6H, $J$ = 7.0 Hz, N(C-$CH_3$)$_2$); 1.86 (d, 3H, $J$ = 1.1 Hz, 5-$CH_3$); 2.12–2.40 (m, 2H, H-2′, H-2″); 3.24–3.41 (m, 6H, N($CH_2$–C)$_2$, P–$CH_2$–Cl); 3.81–4.11 (m, 3H, H-4′, H-5′, H-5″); 4.16–4.26 (m, 1H, H-3′); 6.14 and 6.22 (t, 1H, $J$ = 6.6 Hz, H-1′); 7.37 (d, 1H, $J$ = 0.9 Hz, H-6); 10.25 (s, 1H, 3-NH). $^{13}$C NMR ($CDCl_3$): $\delta$ 13.10 (5-$CH_3$); 13.55 (N(C–$CH_3$)$_2$); 33.46 (d, $J_{P\text{-}C}$ = 144 Hz, P–$CH_2$–Cl); 37.21 (C-2′); 37.97 (N($CH_2$–C)$_2$); 60.24 (C-3′); 63.08 (C-5′); 82.55 (d, $^3J_{P\text{-}C}$ = 6.6 Hz, C-4′); 85.11 (C-1′); 111.20 (C-5); 135.48 (C-6); 150.30 (C-2); 163.71 (C-4). $^{31}$P NMR ($CDCl_3$): $\delta$ 25.64; 25.72. MS-ESI $m/z$: 457; 459 $[M+Na]^+$; 433; 435 $[M-H]^-$; 469; 471; 473 $[M+Cl]^-$. Anal. Calcd for $C_{15}H_{24}ClN_6O_5P$: C, 41.43; H, 5.56; N, 19.33. Found: C, 41.41; H, 5.57; N, 19.34.

### 4.1.2. Procedure for the preparation of compound 16

To a solution of *P*-chloromethylphosphonic dichloride (**1**) (153 mg, 0.914 mmol) in acetonitrile (2 mL) was added 1,2,4-triazole (**2**) (164 mg, 2.374 mmol) followed by triethylamine (189 mg, 1.865 mmol) and the reactants were stirred for 30 min at room temperature. Then to the mixture 3′-azido-3′-deoxythymidine (**4**) (100 mg, 0.374 mmol) and pyridine (2.30 mL) were added. The reaction mixture was stirred at room temperature for a further 1 h and a mixture of water (0.112 mL), triethylamine (240 mg, 2.372 mmol) and pyridine (0.748 mL) was added. After 20 min, the reaction mixture was evaporated under reduced pressure. To the residue was added saturated aqueous sodium bicarbonate (20 mL) and the mixture was extracted with chloroform (3 × 10 mL). The combined chloroform extracts were washed with water (10 mL), dried over anhydrous magnesium sulfate, filtered and evaporated to dryness. The residue was dissolved in chloroform (1 mL) and added dropwise to stirred *n*-hexane (80 mL). The resulting colorless precipitate was collected by filtration and dried in vacuo over phosphorus pentoxide to afford product **16** (128 mg, yield 71%).

**4.1.2.1. 3′-Azido-3′-deoxythymidine 5′-(*P*-chloromethylphosphonate) triethylammonium salt (16).** $^{1}$H NMR (DMSO-$d_6$): $\delta$ 1.16 (t, 9H, $J$ = 7.1 Hz, N(C–$CH_3$)$_3$); 1.91 (d, 3H, $J$ = 1.0 Hz, 5-$CH_3$); 2.03–2.22 (m, 2H, H-2″, H-2′); 2.94 (q, 6H, $J$ = 7.1 Hz, N($CH_2$–C)$_3$); 3.28–3.41 (m, 2H, P–$CH_2$–Cl); 3.47–3.58 (m, 1H, H-4′); 4.20–4.35 (m, 2H, H-5′, H-5″); 4.48 (m,1H, H-3′); 6.14 (t, 1H, $J$ = 6.6 Hz, H-1′); 7.56 (d, 1H, $J$ = 1.0 Hz, H-6); 11.21 (bs, 1H, 3-NH). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 8.55 (N(C–$CH_3$)$_3$); 12.08 (5-$CH_3$); 36.19 (N($CH_2$–C)$_3$); 39.28 (d, $J_{P\text{-}C}$ = 155 Hz, P–$CH_2$–Cl); 44.96 (C-2′); 60.85 (C-3′); 63.37 (C-5′); 83.01 (C-1′); 84.47 (C-4′); 109.22 (C-5); 136.16 (C-6); 150.54 (C-2); 163.40 (C-4). $^{31}$P NMR (DMSO-$d_6$): δ8.95. MS-ESI (negative mode) $m/z$: 378; 380. HPLC: eluent (a), retention time ($t_R$) of 7.6 min.

### 4.1.3. Chemical stability studies

Hydrolysis of the compounds **8** and **10** was carried out in the 0.066 M phosphate buffer at pH 7.2, 7.4 and 7.8 at 37 °C and the concentration of the compound under study of 0.5 mM. The aliquots were taken out after certain intervals and frozen. The composition of the reaction mixtures was analyzed by HPLC under the conditions described above.

## 4.2. In vitro cytotoxicity

### 4.2.1. Cell cultures

Human cancer cells KB (*carcinoma nasopharynx*) were cultured in RPMI 1640 medium and human cancer cells MCF-7 (breast cancer cell line) were cultured in DMEM medium. Each medium was supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin solution. The cell lines were kept in the incubator at 37 °C. The optimal plating density of cell lines was determined to be $5 \times 10^4$. Both the cell lines were obtained from The European Collection of Cell Cultures (ECACC) supplied by Sigma–Aldrich.

### 4.2.2. In vitro cytotoxicity assay

The protein-staining sulforhodamine B (SRB, Sigma–Aldrich) microculture colorimetric assay, developed by the National Cancer Institute (USA) for in vitro antitumor screening was used in this study, to estimate the cell number by providing a sensitive index of total cellular protein content, being linear to cell density.[39] The monolayer cell culture was trypsinized and the cell count was adjusted to $5 \times 10^4$ cells. To each well of the 96 well microtiter plate, 0.1 mL of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was washed out and 100 μL of six different compound concentrations (0.1, 0.2, 1, 2, 10 and 20 μM) were added to the cells in microtitre plates. The tested compounds were dissolved in DMSO (20 μM) and the content of DMSO did not exceed 0.1%; this concentration was found to be nontoxic to the cell lines. The cells were exposed to compounds for 72 h. After that, 25 μL of 50% trichloroacetic acid was added to the wells and the plates were incubated for 1 hour at 4 °C. The plates were then washed out with the distilled water to remove traces of medium and next dried by the air. The air-dried plates were stained with 100 μL SRB and kept for 30 min at room temperature. The unbound dye was removed by rapidly washing with 1% acetic acid and then air dried overnight. The optical density was read at 490 nm. All cytotoxicity experiments were performed three times. Cell survival was measured as the percentage absorbance compared to the control (non-treated cells). Zidovudine and cytarabine (Sigma–Aldrich) were used as the internal standards.

## 4.3. In vitro permeability assay

### 4.3.1. Cell culture

Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC) and supplied by Sigma-Aldrich. Cells were cultured in DMEM medium (Sigma), supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL), 1% non-essential amino acids 100X (NEAA, Sigma) and 50 mg/L gentamycin (Gibco BRL). For transport experiments, Caco-2 cells were plated at a density of $4.4 \times 10^5$ cells/cm$^2$ on polycarbonate membranes (Millicell, 12 mm diameter) with pore size of 0.4 μm (Millipore). Cells were maintained at 37 °C, in a humidified, 5% $CO_2$, 95% air atmosphere. Growth medium was replaced twice weekly. Cell confluence was monitored by transepithelial electrical resistance (TEER) measurements. TEER was determined employing the Millicell Electrical Resistance System (Millipore). Transport experiments were performed on monolayers, 21 days post-seeding.

### 4.3.2. Transport experiments

Solutions of compounds **8** and **10** at concentration of 100 μM were prepared in transport medium (Hank's balanced salt solution containing 10 mM HEPES buffer, pH 7.4). The solutions were filtered through a 0.22 μm membrane (Millex GP, Millipore) and pre-heated at 37 °C before permeability experiments.

Prior to the experiments, the monolayers were washed twice with transport medium and pre-incubated at 37 °C for 30 min. After the pre-incubation, TEER of the monolayers was measured to check cell monolayer integrity. Only monolayers with TEER values above 800 Ω cm$^2$ were used in experiments. Transport was initiated by adding transport medium to the acceptor side and the solution of the test compound to the donor side. The Caco-2 cell monolayers were placed in the incubator (37 °C) on the plate shaker. At 15 min intervals, the sample from acceptor compartment was taken and the concentration of compound transported was analyzed by HPLC. Each sample volume was replaced with fresh pre-heated (37 °C) transport medium. To quantify transport of compounds tested across the Caco-2 cell monolayer, the apparent permeability coefficient ($P_{app}$) was used. The $P_{app}$ values were determined as previously described.[58] Experimentally derived data are shown as mean ± SD ($n$ = 3).

## 4.4. Theoretical calculation of Caco-2 cell permeability

The Caco-2 prediction model, developed by Guangli and Yiyu,[57] based on descriptors generated by open source software Chemistry Development Kit (CDK) was used to compute Caco-2 apparent permeability for the studied compounds. In this model membrane permeability of compounds is determined by a number of hydrogen bond donors and three molecular surface area properties. The correlation coefficients of the experimental and predicted Caco-2 apparent permeability for their test set was as high as 0.85.

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