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Synthesis of 3′-azido-2′,3′-dideoxy-5-fluorouridine phosphoramidates and evaluation of their anticancer activity

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ABSTRACT

A series of novel 4-chlorophenyl *N*-alkyl phosphoramidates of 3′-azido-2′,3′-dideoxy-5-fluorouridine (**12**–**21**) were synthesized by means of phosphorylation of 3′-azido-2′,3′-dideoxy-5-fluorouridine (**4**) with 4-chlorophenyl phosphoroditriazolide (**10**) followed by a reaction with the appropriate amine. The synthesized phosphoramidates (**12**–**21**) were evaluated for their cytotoxic activity in three human cancer cell lines: cervical (HeLa), oral (KB) and breast (MCF-7) using the sulforhodamine B (SRB) assay. The highest activity in all the investigated cancer cells was displayed by phosphoramidate **13** with the *N*-ethyl substituent and its activity was much higher than that of the parent nucleoside. Also phosphoramidate **17** with the *N*-propargyl substituent exhibited good activity in all the used cell lines.

1. Introduction

A number of pyrimidine and purine analogues, especially nucleoside analogues, have found broad application as antiviral [1] and anticancer [2–4] therapeutics. In particular, 5-fluoropyrimidine analogues including 5-fluorouracil (FUra), 5-fluoro-2′-deoxyuridine (floxuridine, FdU) and 5′-deoxy-5-fluoro-N^4-pentyloxycarbonylcytidine (capecitabine, CAP) play an important role among anticancer drugs (Fig. 1) [5,6]. 3′-Azido-2′,3′-dideoxy-5-fluorouridine (AddFU), similarly like FdU, possesses 5-fluorouracil moiety but in sugar part instead of 3′-hydroxyl it has 3′-azido group and in this resembles 3′-azido-3′-deoxythymidine (AZT). AddFU exhibits not only anticancer activity in murine leukaemia (L1210) cells [7,8], but also moderate antiviral activity against HIV-1 virus [9] and some activity against herpes simplex virus (type 1 and 2) [8], vaccinia virus as well as vesicular stomatitis virus [8].

5-Fluoropyrimidine-based drugs (FUra, FdU and CAP) are mainly used in the treatment of colon, breast, gastrointestinal and ovary tumours [10,11]. The mechanism of anticancer action of 5-fluorouracil and 5-fluoro-2′-deoxyuridine primarily involves their intracellular conversion to 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP), which acts as an irreversible inhibitor of thymidylate synthase [3]. Other proposed modes of anticancer action of 5-fluorouracil include its transformation *via* 5-fluoro-2′-deoxyuridine to 5-fluoro-2′-deoxyuridine 5′-triphosphate (FdUTP), which is incorporated to DNA and transformation to 5-fluorouridine 5′-triphosphate (FUTP), which in turn is incorporated to RNA [3]. It has been shown that nucleic acids (DNA and RNA) having thymine and uracil residues, respectively, replaced with a 5-fluorouracil residue are less stable and their function is altered, which leads to inhibition of cell division [3]. Capecitabine easily penetrates the cell membrane and inside the cells, after three enzymatic transformations, releases 5-fluorouracil, which is further converted into FdUMP [11]. In fact, FdUMP – an irreversible inhibitor of thymidylate synthase is the common active metabolite of 5-fluorouracil, 5-fluoro-2′-deoxyuridine and capecitabine. Therefore, a considerable effort has been directed into the synthesis of prodrugs (pronucleotides) of FdUMP with a protected 5′-phosphate group [5,6]. These prodrugs are designed to easily penetrate the cell membrane and release FdUMP inside the cell as a result of chemical or enzymatic hydrolysis [12,13]. Furthermore, the liberated FdUMP would require only a second and third phosphorylation for the conversion to FdUTP, a next active metabolite, which can be incorporated to DNA. The FdUMP itself cannot be employed as a drug because it is negatively charged at

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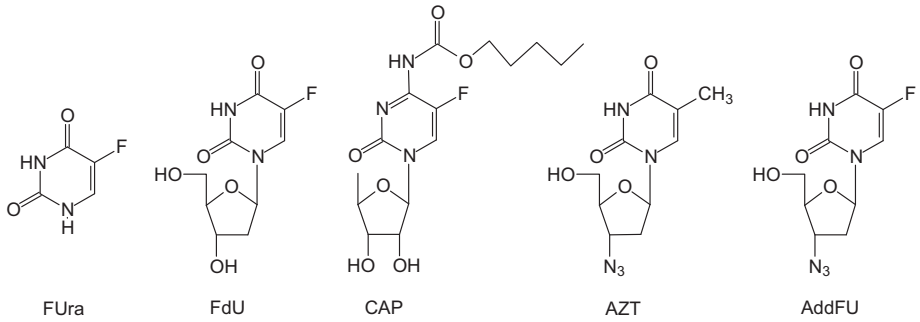


Fig. 1. 5-Fluoropyrimidine-based drugs and AZT.

physiological pH and consequently too polar to cross the cell membrane [12,14]. In addition, the blood and cell surface phosphohydrolases effectively convert nucleoside 5′-phosphates to the parent nucleosides [14]. Several FdUMP prodrugs were synthesized and evaluated for their anticancer activity. Jones published the synthesis of a series of cyclic phosphates of FdU and their cytotoxic activity against murine leukaemia L1210 cells [15]. Farquhar reported synthesis of 5′-(1,3,2-dioxaphosphorinan-2-yl) and 5′-[4-(pivaloyloxy)-1,3,2-dioxaphosphorinan-2-yl] derivatives of FdU [16,17]. Borch described synthesis of series of *N*-haloalkyl phosphoramidate diesters of FdU, which are able to release FdUMP intracellularly [18,19]. Meier reported synthesis of series of cyclosaligenyl FdUMP derivatives as prodrugs of FdUMP [20]. Recently, McGuigan and Balzarini reported synthesis and anticancer activity of aryl *N*-amino acid phosphoramidates of FdU [5,6]. Also recently, Jain and Kalman described synthesis of *N*-amino acid 3′,5′-cyclic-phosphoramidates of FdU [21].

On the other hand, AddFU bears a resemblance to AZT, which found an important use as anti-HIV agent [1,22]. There are also some examples of application of AZT as an anticancer agent in combination with either cisplatin, methotrexate or 5-fluorouracil in the therapy of advanced colon cancer [23]. Moreover, Wagner reported the potent inhibitory activity of AZT in cultured human breast cancer cells [24]. It is assumed that the mechanism of anticancer action of AZT involves its intracellular conversion to the 5′-triphosphate, which can act as a competitive inhibitor of DNA polymerases and a chain terminator of the growing DNA strand due to the lack of a 3′-hydroxyl group [23].

Encouraged by the above studies we have set out to develop novel phosphoramidate prodrugs of AddFU with potential anticancer properties. In this paper, we report the synthesis of 4-chlorophenyl *N*-alkyl phosphoramidate diesters of 3′-azido-2′,3′-dideoxy-5-fluorouridine (AddFU) (**12**–**21**) and evaluation of their cytotoxic activity in three human cancer cell lines: cervical (HeLa), oral (KB) and breast (MCF-7).

2. Results and discussion

2.1. Chemistry

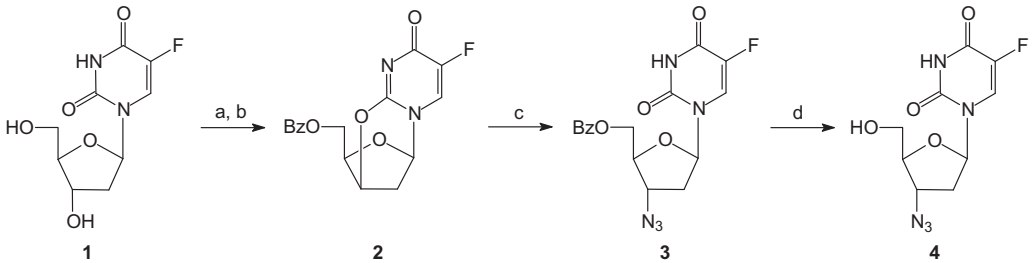
3′-Azido-2′,3′-dideoxy-5-fluorouridine (**4**) was synthesized by two different routes. In the first way, similar to the method of synthesis of 3′-azido-3′-deoxythymidine developed by Czernecki [25], 5-fluoro-2′-deoxyuridine (**1**) was converted into 2,3′-anhydro-5′-*O*-benzoyl-5-fluoro-2′-deoxyuridine (**2**) by a one-pot transformation involving two successive Mitsunobu reactions in a yield of 84% (Scheme 1).

Subsequent ring opening of the 2,3′-anhydro derivative **2** with lithium azide in DMF at 120 °C afforded 3′-azido-5′-*O*-benzoyl-5-fluoro-2′-deoxyuridine (**3**) in 21% yield. Attempts to improve the yield of the reaction by increasing the temperature did not give the expected results, because of the by-products formed. Compound **2** in comparison with 2,3′-anhydro-5′-*O*-benzoyl-thymidine [25] is more stable and less prone to nucleophilic substitution reaction with azide ion. However, the use of lithium azide in hexamethylphosphoramide (HMPA) in the presence of *p*-toluenesulfonic acid (PTSA), increased the yield of **3** to 65%. In the last step of the synthesis, 5′-*O*-benzoyl group was removed from compound **3** by treatment with methanolic ammonia to give **4** in 91% yield.

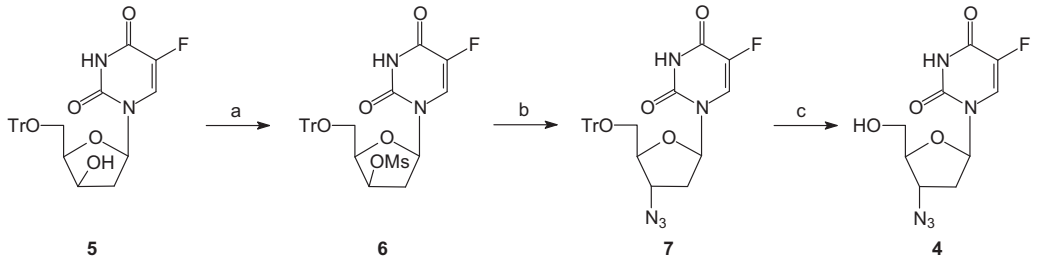
In the second synthetic route, 5-fluoro-2′-deoxyuridine (**1**) was transformed into 1-(5-*O*-trityl-2-deoxy-β-D-lyxofuranosyl)-5-fluoro uracil (**5**) by a previously published method [26]. Compound **5** was then converted to the 3′-*O*-mesyl derivative **6** by treatment with mesyl chloride in dry pyridine at room temperature (Scheme 2).

Subsequently, compound **6** was reacted with lithium azide in DMF at 90 °C to obtain **7**, which after detritylation with 0.5 M hydrochloric acid in methanol–tetrahydrofuran solution gave **4** in 68% yield.

Synthesis of 3′-azido-2′,3′-dideoxy-5-fluorouridine (**4**) by azidation of 1-(2-deoxy-3-*O*-mesyl-5-*O*-trityl-β-D-*erythro*-pentofuranosyl)-5-fluorouracil, described by Vanderhaeghe [8], led to the



Scheme 1. Synthesis of 3′-azido-2′,3′-dideoxy-5-fluorouridine *via* 2,3′-anhydro-5′-*O*-benzoyl-5-fluoro-2′-deoxyuridine. Reagents and conditions: (a) DIAD, PPh_3, $PhCO_2H$, DMF, rt; (b) DIAD, PPh_3, DMF, rt, yield 84%; (c) LiN_3, PTSA, HMPA, 120 °C, 3 h, yield 65%; (d) CH_3OH saturated with NH_3, rt, 12 h, yield 91%.



Scheme 2. Synthesis of 3′-azido-2′,3′-dideoxy-5-fluorouridine *via* 1-(5-*O*-trityl-2-deoxy-β-D-lyxofuranosyl)-5-fluorouracil. Reagents and conditions: (a) MsCl, pyridine, rt, 2 h; (b) LiN_3, DMF, 90 °C, 4 h; (c) 0.5 M HCl in CH_3OH–THF, rt, 3 h, yield 68%.

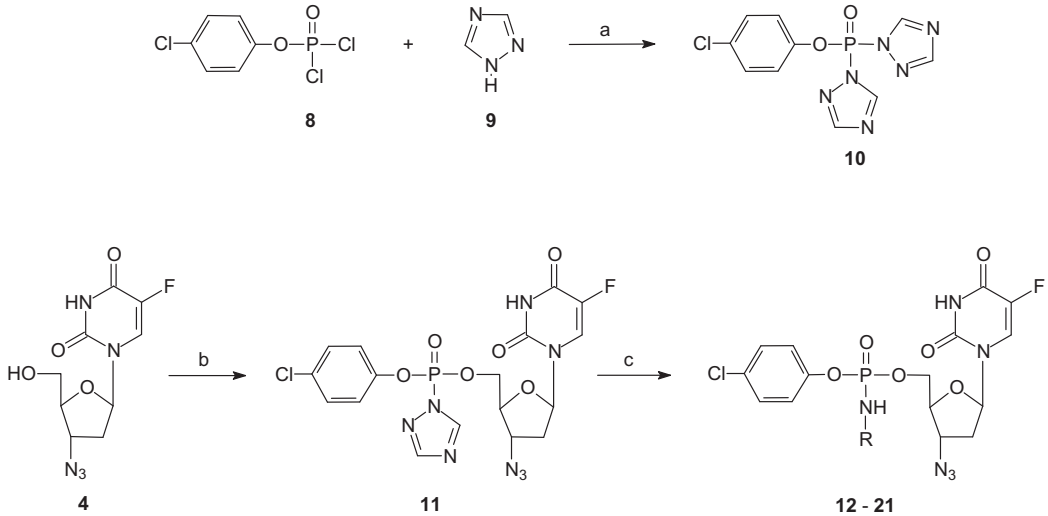
mixture of four compounds including 1-(3-azido-2,3-dideoxy-5-*O*-trityl-β-D-*erythro*-pentofuranosyl)-5-fluorouracil (**7**) and its *threo* isomer. Compound **7** was isolated and its detritylation gave the desired product **4**. 3′-Azido-2′,3′-dideoxy-5-fluorouridine can also be prepared by fluorination of 3′-azido-3′-deoxyuridine, but it requires the use of hazardous trifluoromethyl hypofluorite (CF_3OF) [7].

A series of novel 4-chlorophenyl *N*-alkyl phosphoramidate diesters of 3′-azido-2′,3′-dideoxy-5-fluorouridine (**12**–**21**) were synthesized by phosphorylation of 3′-azido-2′,3′-dideoxy-5-fluorouridine (**4**) with 4-chlorophenyl phosphoroditriazolide (**10**) according to the synthetic route shown in Scheme 3.

4-Chlorophenyl phosphoroditriazolide (**10**) was prepared by reaction of 4-chlorophenyl phosphorodichloridate (**8**) with 1,2,4-triazole (**9**) in the presence of triethylamine in acetonitrile. Reaction of compound **10** with 3′-azido-2′,3′-dideoxy-5-fluorouridine (**4**) in the presence of pyridine afforded reactive intermediate **11**, which was treated *in situ* with the appropriate amine (or amine hydrochloride in the presence of triethylamine) to give the desired products **12**–**21** in 67–86% yield.

^{31}P NMR spectra of products **12**–**21** revealed the presence of two diastereoisomers due to a chiral centre being formed at the phosphorus atom. There were two close signals, in the ratio of approximately 1:1, in each ^{31}P NMR spectrum. Thin layer chromatography of compounds **12**–**21** was also 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 a reversed-phase column (see experimental data for compounds **12** and **13**).

It is worth emphasizing that the use of 4-chlorophenyl phosphorodichloride (**8**), rather than its triazolide counterpart **10**, resulted in the formation of a considerable amounts of the symmetrical (5′-5′)dinucleoside phosphate. The application of 2- and 4-chlorophenyl phosphoroditriazolides [27–29] for the phosphorylation of 5′-protected nucleosides has been reported in the



12: R = CH_3
13: R = CH_2CH_3
14: R = CH_2CF_3
15: R = $CH_2CH_2CH_3$
16: R = $CH_2CH{=}CH_2$
17: R = $CH_2C{\equiv}CH$
18: R = $CH_2CH_2CH_2N_3$
19: R = $CH_2CH_2CH_2CH_3$
20: R = $CH_2CH_2CH_2CH_2N_3$
21: R = *(S)*-$CH(CH_3)CO_2CH_3$

Scheme 3. Synthesis of the 4-chlorophenyloxy *N*-alkyl phosphoramidates of 3′-azido-2′,3′-dideoxy-5-fluorouridine. Reagents and conditions: (a) NEt_3, CH_3CN, rt, 30 min; (b) **10**, pyridine, rt, 1 h; (c) R–NH_2, rt, 1 h.

phosphotriester synthesis of oligonucleotides and provided the inspiration for the development of our method.

It should be mentioned that 1-amino-3-azidopropane and its 1-amino-4-azidobutane homologue were synthesized from 1,3-dibromopropane and 1,4-dibromobutane, respectively, *via* the appropriate diazidoalkanes by a known method [30].

2.2. Biological activity

The synthesized phosphoramidates **12**–**21** were evaluated for their cytotoxic activity in three human cancer cell lines: cervical (HeLa), oral (KB) and breast (MCF-7) employing sulforhodamine B (SRB) assay [31]. The resulting cytotoxic activity data of the obtained phosphoramidates and reference compounds are presented in Table 1. The highest activity in HeLa cancer cells was displayed by phosphoramidate **13** (IC_{50} = 1.64 μM), and it was almost seven times more potent than the parent AddFU (IC_{50} = 11.06 μM). Phosphoramidate **17** exhibited also relatively high activity (IC_{50} = 4.21 μM) whereas phosphoramidates **12** and **16** were only somewhat more active then the parent nucleoside **4**. All the remaining phosphoramidates proved inactive in HeLa cancer cells. The highest activity in KB cancer cells was again demonstrated by phosphoramidate **13** (IC_{50} = 1.64 μM), which was almost five times more active than **4**. Significant activity, higher than that of AddFU, was also shown by phosphoramidate **17** (IC_{50} = 3.81 μM). Similarly, the highest activity in MCF-7 cancer cells was demonstrated by phosphoramidate **13** (IC_{50} = 1.88 μM), which was five times more active than the parent nucleoside. Moreover considerable activity, about two times higher than that of AddFU, was shown by phosphoramidates **12** and **17**. These findings clearly indicate that the phosphoramidate **13** with the *N*-ethyl substituent was the most potent compound in all investigated cancer cell lines (HeLa, KB and MCF-7) and its activity was consistently higher than that of the parent nucleoside (AddFU). Phosphoramidate **17** with the *N*-propargyl substituent was also found to have good activity in the three the cell lines whereas phosphoramidate **12** with the *N*-methyl substituent proved moderately active. However, phosphoramidates (**15** and **18**–**20**) with a longer *N*-alkyl chain substituents were less potent in the all cancer cells. There are several reasons to explain this. These phosphoramidates could become so hydrophobic that there are poorly soluble in aqueous phase. Alternatively, they could be caught by fat depots and never reach the intended site. Furthermore, hydrophobic compounds are often more susceptible to metabolism and subsequent elimination [32].

Partition coefficient (log *P*) values of the compounds **12**–**21** were calculated [34] to determine a possible correlation between the cytotoxicity data and lipophilicity (Table 1). All of the AddFU phosphoramidates were more lipophilic than AddFU (log *P* = −0.38), with log *P* values ranging from 1.68 to 3.16. The most active compounds **13** and **17** showed moderate values of log *P*, 2.05 and 1.84 respectively. However, linear regression analysis did not reveal any correlation between log *P* values and the cytotoxicity data.

Although the biochemical mode of action of the AddFU phosphoramidates requires more detailed studies, it can be envisaged 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 phosphoramidates could be hydrolyzed or metabolized to AddFU 5′-monophosphate, which is likely to be an inhibitor of thymidylate synthase. However, our phosphoramidates differ from those developed by McGuigan [5] because they lack the ester motif which starts the hydrolysis process to release the nucleoside analogue 5′-monophosphate. Therefore, our phosphoramidates have to be hydrolyzed by a different mechanism. It appears that the enzymatic hydrolysis of our phosphoramidates to FUra is unlikely because FUra shows lower activity than the most active phosphoramidates **13** and **17** (Table 1). Moreover, AddFU 5′-monophosphate after enzymatic phosphorylation to AddFU 5′-triphosphate (*via* the 5′-diphosphate), can act as a competitive inhibitor and of DNA polymerases and a chain terminator of the nascent DNA strand due to the lack of a 3′-hydroxyl group.

Table 1
In vitro cytotoxic activity of the synthesized compounds **12**–**21** in three human cancer cell lines: cervical (HeLa), oral (KB) and breast (MCF-7).

Compound	Cytotoxicity (IC_{50}, μM)[a] ± SD[b]			log *P*[c]
	HeLa	KB	MCF-7	
12	8.43 ± 0.84	8.64 ± 0.76	4.42 ± 0.95	1.68
13	1.64 ± 0.10	1.64 ± 0.18	1.88 ± 0.74	2.05
14	43.85 ± 0.22	40.53 ± 0.07	23.95 ± 0.13	2.60
15	25.85 ± 1.69	26.85 ± 0.12	27.25 ± 0.18	2.56
16	9.98 ± 0.74	8.59 ± 0.66	10.38 ± 0.40	2.32
17	4.21 ± 0.18	3.81 ± 0.12	5.41 ± 0.18	1.84
18	96.17 ± 0.59	99.29 ± 0.97	33.10 ± 0.09	2.65
19	32.89 ± 0.10	38.70 ± 0.17	41.79 ± 0.08	3.16
20	35.49 ± 0.23	46.61 ± 0.31	22.41 ± 0.23	2.92
21	38.40 ± 0.31	>100	38.40 ± 1.54	1.87
FUra	6.23 ± 0.46	4.84 ± 0.15	6.53 ± 0.82	−0.59
1 (FdU)	6.50 ± 0.24	8.69 ± 1.18	12.19 ± 1.34	−1.72
4 (AddFU)	11.06 ± 0.26	8.11 ± 0.33	10.32 ± 0.15	−0.38
Cytarabine (standard)	3.54 ± 0.16	4.07 ± 0.08	3.82 ± 0.25	−2.32 [33]

[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 [34].

3. Conclusion

In conclusion, we have developed an efficient method for the synthesis of 4-chlorophenyloxy *N*-alkyl phosphoramidates of 3′-azido-2′,3′-dideoxy-5-fluorouridine employing 4-chlorophenyl phosphoroditriazolide as a phosphorylating agent. 4-Chlorophenyl phosphoroditriazolide was more selective than its dichloro counterpart and its use did not result in the formation of symmetrical (5-5′)dinucleoside phosphates. The obtained compounds **12**–**21** were examined for their cytotoxic activity in three human cancer cell lines: HeLa (cervical), oral (KB) and breast (MCF-7). The highest activity in all the investigated human cancer cells was displayed by phosphoramidate **13** with the *N*-ethyl substituent and its activity was several times higher than that of the parent nucleoside (AddFU). Phosphoramidate **17** with the *N*-propargyl substituent exhibited also fairly high activity. Moreover, phosphoramidate **12** with the *N*-methyl substituent displayed moderate activity. However phosphoramidates (**15** and **18**–**20**) with a longer *N*-alkyl chain substituents were less potent in all the cell lines used.

4. Experimental protocols

4.1. Chemistry

NMR spectra were recorded on a Varian-Gemini 400 MHz spectrometer. Chemical shifts (δ) are reported in parts per million (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 XBridge C18 reversed-phase column (4.6 × 150 mm, 5 μm) using as an eluting system phosphate buffer (20 mM Na_2HPO_4, pH was adjusted to 7.1 with H_3PO_4)–methanol (40:60). The flow rate was 1 mL/min and detection at 266 nm. Chemical reagents were purchased from Sigma–Aldrich.

4.1.1. 2,3′-Anhydro-5′-O-benzoyl-5-fluoro-2′-deoxyuridine (**2**)

To a stirred solution of 5-fluoro-2′-deoxyuridine (**1**) (3.69 g, 15 mmol) and triphenylphosphine (5.90 g, 22.5 mmol) in DMF (30 mL) was added dropwise a solution of benzoic acid (2.75 g, 22.5 mmol) and diisopropyl azodicarboxylate (DIAD) (4.43 mL, 22.5 mmol) in DMF (7 mL). After 15 min second portion of DIAD (4.43 mL, 22.5 mmol) and triphenylphosphine (5.90 g, 22.5 mmol) in DMF (7 mL) was added dropwise and stirring was continued for 30 min. Then the mixture was poured into diethyl ether (370 mL) and the resulting suspension was chilled for 2 h. The white precipitate was filtered off and washed with diethyl ether; yield: 4.18 g (84%). Analytically pure sample of compound **2** was obtained by silica gel column chromatography using chloroform–methanol (60:1, v/v) as eluent. ^{1}H NMR (DMSO-d_6) δ: 2.55–2.69 (m, 1H, H-2′, H-2″), 3.17 (m, 1H, H-4′), 3.52 (m, 2H, H-5′, H-5″), 4.22 (m, 1H, H-3′), 5.08 (pseudo t, 1H, J = 6.1 z, H-1′), 6.84 (d, 1H, J = 3.5 Hz, H-6), 7.52–8.03 (m, 5H, Ph). ^{13}C NMR (DMSO-d_6) δ: 31.26, 59.38, 77.52, 85.42, 87.34, 125.59 (d, J_{C-F} = 36.8 Hz), 128.70, 129.13, 130.19, 133.46, 144.27 (d, J_{C-F} = 248.7 Hz), 151.70, 162.93 (d, J_{C-F} = 16.3 Hz), 166.84. ^{19}F NMR (DMSO-d_6) δ: −158.46 (d, 1F, J = 5.0 Hz). MS-ESI m/z: 333 $[M + H]^+$; 355 $[M + Na]^+$; 371 $[M + K]^+$; 331 $[M - H]^-$; 367, 369 $[M + Cl]^-$. Anal. Calcd for $C_{16}H_{13}FN_2O_5$: C, 57.83; H, 3.94; N, 8.43. Found: C, 57.89; H, 3.95; N, 8.45.

4.1.2. 3′-Azido-5′-benzoyl-2′,3′-dideoxy-5-fluorouridine (**3**)

A solution of **2** (3.99 g, 12 mmol), lithium azide (1.18 g, 24 mmol) and *p*-toluenesulfonic acid monohydrate (2.28 g, 12 mmol) in HMPA (130 mL) was heated for 3 h at 120 °C. After cooling, the mixture was poured into ice-water (1 L) and extracted with ethyl acetate (3 × 100 mL). The organic layer was washed with saturated aqueous sodium bicarbonate (30 mL), then with water (30 mL), dried over anhydrous magnesium sulphate, filtered and evaporated to dryness. The residue was purified by silica gel column chromatography using chloroform–methanol (100:1, v/v) as eluent to give pure **3** (yield: 2.93 g, 65%). ^{1}H NMR (DMSO-d_6) δ: 2.73–2.89 (m, 1H, H-2′, H-2″), 4.12–4.15 (m, 1H, H-4′), 4.46–4.69 (m, 2H, H-5′, H-5″), 4.78 (m, 1H, H-3′), 6.18 (pseudo t, 1H, J = 6.1 Hz, H-1′), 7.42 (d, 1H, J = 3.2 Hz, H-6), 7.49–8.05 (m, 5H, Ph), 11.38 (s, 1H, H-3). ^{13}C NMR (DMSO-d_6) δ: 31.26, 59.87, 63.52, 80.51, 83.42, 125.59 (d, J_{C-F} = 36.8 Hz), 128.75, 129.34, 130.24, 133.58, 144.28 (d, J_{C-F} = 248.7 Hz), 151.74, 162.94 (d, J_{C-F} = 16.3 Hz), 166.79. ^{19}F NMR (DMSO-d_6) δ: −158.46 (d, 1F, J = 5.0 Hz). MS-ESI m/z: 376 $[M + H]^+$; 398 $[M + Na]^+$; 414 $[M + K]^+$; 374 $[M - H]^-$; 410, 412 $[M + Cl]^-$. Anal. Calcd for $C_{16}H_{14}FN_5O_5$: C, 51.20; H, 3.76; N, 18.66. Found: C, 51.25; H, 3.77; N, 18.67.

4.1.3. 3′-Azido-2′,3′-dideoxy-5-fluorouridine (**4**)

Compound **3** (2.5 g) was suspended in methanol saturated with ammonia (200 mL) and the mixture was stirred at room temperature for 12 h. Then the mixture was evaporated to dryness. The residue was purified by silica gel column chromatography using chloroform–methanol (40:1, v/v) as eluent to obtain pure **4** (yield: 1.64 g, 91%). ^{1}H NMR (DMSO-d_6) δ: 2.31–2.45 (m, 1H, H-2′, H-2″), 3.69 (m, 1H, H-4′), 3.84 (m, 2H, H-5′, H-5″), 4.40 (m, 1H, H-3′), 6.06 (pseudo t, 1H, J = 6.1 Hz, H-1′), 8.20 (d, 1H, J = 6.8 Hz, H-6), 11.98 (s, 1H, H-3). ^{13}C NMR (DMSO-d_6) δ: 36.60, 59.55, 60.42, 84.14, 84.30, 124.60 (d, J_{C-F} = 34.3 Hz), 141.12 (d, J_{C-F} = 231.3 Hz), 149.03, 157.25 (d, J_{C-F} = 26.1 Hz). ^{19}F NMR (DMSO-d_6) δ: −166.82 (d, 1F, J = 7.2 Hz). MS-ESI m/z: 272 $[M + H]^+$; 294 $[M + Na]^+$; 310 $[M + K]^+$; 270 $[M - H]^-$; 306, 308 $[M + Cl]^-$. Anal. Calcd for $C_9H_{10}FN_5O_4$: C, 39.86; H, 3.72; N, 25.82. Found: C, 39.92; H, 3.73; N, 25.84.

4.1.4. 1-(2-Deoxy-3-O-mesyl-5-O-trityl-β-D-threo-pentofuranosyl)-5-fluorouracil (**6**)

To a stirred solution of compound **5** [26] (1.25 g, 2.21 mmol) in pyridine (25 mL) was added methanesulfonyl chloride (0.38 g, 3.32 mmol) and the stirring was continued at room temperature for 2 h. Then 5% aqueous solution of sodium bicarbonate (20 mL) was added to the reaction mixture and it was extracted with chloroform (3 × 30 mL). The organic layer was washed with water (20 mL), dried over anhydrous magnesium sulphate, filtered and evaporated to dryness. To the residue was added toluene (20 mL) and the mixture was evaporated to remove traces of pyridine. The crude compound **6** was purified by silica gel column chromatography using chloroform–methanol (180:1, v/v) as eluent to give pure **6** (yield: 1.25 g, 86%). ^{1}H NMR (DMSO-d_6) δ: 2.35 (s, 3H, SO_2CH_3), 2.41–2.51 (m, 2H, H-2′, H-2″), 3.65 (m, 1H, H-4′), 4.10–4.20 (m, 2H, H-5′, H-5″), 4.65 (m, 1H, H-3′), 6.14 (pseudo t, 1H, J = 6.2 Hz, H-1′), 7.19–7.474 (m, 15H, Ph), 8.00 (d, 1H, J = 6.8 Hz), 11.86 (s, 1H, H-3). ^{13}C NMR (DMSO-d_6) δ: 29.85, 36.61, 68.45, 68.83, 89.18, 91.61, 128.41 (d, J_{C-F} = 34.1 Hz), 132.71–134.84, 143.40 (d, J_{C-F} = 231.2 Hz), 150.57, 162.06 (d, J_{C-F} = 26.3 Hz). ^{19}F NMR (DMSO-d_6) δ: −167.58 (s, 1F). MS-ESI m/z: 567 $[M + H]^+$; 589 $[M + Na]^+$; 605 $[M + K]^+$; 565 $[M - H]^-$; 601, 603 $[M + Cl]^-$. Anal. Calcd for $C_{29}H_{27}FN_2O_7S$: C, 61.47; H, 4.80; N, 4.94. Found: C, 61.53; H, 4.81; N, 4.95.

4.1.5. 3′-Azido-2′,3′-dideoxy-5-fluoro-5′-tritylouridine (**7**)

To a solution of **6** (1 g, 1.95 mmol) in DMF was added lithium azide (0.14 g, 2.93 mmol) and the mixture was stirred at 90 °C for 3 h. Then the mixture was evaporated to dryness and the residue was purified by silica gel column chromatography using chloroform–methanol (180:1, v/v) as eluent to give pure **7** (yield: 0.79 g, 87%). ^{1}H NMR (DMSO-d_6) δ: 2.37–2.51 (m, 2H, H-2′, H-2″), 3.42 (m, 1H, H-4′), 3.86 (m, 2H, H-5′, H-5″), 4.56 (m, 1H, H-3′), 6.09 (pseudo t, 1H, J = 6.4 Hz, H-1′), 7.38–7.40 (m, 15H, Ph), 8.00 (d, 1H, J = 6.8 Hz, H-6), 11.92 (s, 1H, H-3). ^{13}C NMR (DMSO-d_6) δ: 35.95, 59.14, 62.74, 82.11, 83.98, 128.19 (d, J_{C-F} = 34.2 Hz), 134.52–138.42, 141.16 (d, J_{C-F} = 231.2 Hz), 148.93, 157.13 (d, J_{C-F} = 26.2 Hz). ^{19}F NMR (DMSO-d_6) δ: −166.53 (s, 1F). MS-ESI m/z: 514 $[M + H]^+$; 536 $[M + Na]^+$; 552 $[M + K]^+$; 512 $[M - H]^-$; 548, 550 $[M + Cl]^-$. Anal. Calcd for $C_{28}H_{24}FN_5O_4$: C, 65.49; H, 4.71; N, 13.64. Found: C, 65.55; H, 4.72; N, 13.65.

4.1.6. Procedure for the detritylation of **7**

To a solution of **7** (0.7 g, 1.36 mmol) in THF (4 mL) was added 0.5 M HCl in methanol (8 mL) and the mixture was stirred at room temperature for 3 h. Then the mixture was neutralized using 5% aqueous solution of sodium bicarbonate, filtered and evaporated to dryness. The residue was purified by silica gel column chromatography using chloroform–methanol (40:1, v/v) as eluent to give pure **4** (yield: 0.25 g, 68%).

4.1.7. General procedure for the synthesis of target compounds **12**–**21**

To a solution of 4-chlorophenyl phosphorodichloridate (**8**) (224 mg, 0.914 mmol) in acetonitrile (2 mL) was added 1,2,4-triazole (**9**) (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-2′,3′-dideoxy-

5-fluorouridine (**4**) (100 mg, 0.369 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) was added. In the case of synthesis of compounds **12**–**14** amine hydrochloride (4.57 mmol) and triethylamine (694 mg, 6.86 mmol) were added. When compound **21** was synthesized L-alanine methyl ester hydrochloride (4.57 mmol) and triethylamine (694 mg, 6.86 mmol) were 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 sulphate, filtered and evaporated to dryness. The residue was purified by silica gel column chromatography using as an eluent the mixture chloroform–methanol (from 100:1 to 40:1, v/v) to afford products **12**–**21** (yield 67–86%).

*4.1.7.1. 3′-Azido-2′,3′-dideoxy-5-fluorouridine 5′-O-(4-chlorophenyl N-methylphosphate) (**12**).* ^{1}H NMR (DMSO-d_6) δ: 2.32–2.48 (m, 2H, H-2′, H-2″), 2.47–2.49 (m, 3H, N–CH_3), 4.04 (m, 1H, H-4′), 4.16–4.29 (m, 2H, H-5′, H-5″), 4.47 (m, 1H, H-3′), 5.46 (m, 1H, NH–C), 6.10 (pseudo t, 1H, J = 6.0 Hz, H-1′), 7.23 (d, 2H, J = 8.9 Hz, 4-ClPh), 7.44 (d, 2H, J = 8.9 Hz, 4-ClPh), 7.96, 7.97 (d, 1H, J = 6.8 Hz, H-6), 11.92 (br s, 1H, 3-NH). ^{13}C NMR (DMSO-d_6) δ: 26.42, 35.88, 59.81, 65.13, 81.63, 84.37, 124.74 (d, J_{C-F} = 34.2 Hz), 128.69, 129.71, 139.00, 140.12 (d, J_{C-F} = 231.30 Hz), 148.87, 149.49, 157.02 (d, J_{C-F} = 26.20 Hz). ^{19}F NMR (DMSO-d_6) δ: −166.28 (d, J = 14.4 Hz, 1F). ^{31}P NMR (DMSO-d_6) δ: 6.20, 6.41. MS-ESI m/z: 475, 477 $[M + H]^+$; 497, 499 $[M + Na]^+$; 513, 515 $[M + K]^+$; 473, 475 $[M - H]^-$; 509, 511, 513 $[M + Cl]^-$. Anal. Calcd for $C_{16}H_{17}ClFN_6O_6P$: C, 40.48; H, 3.61; N, 17.70. Found: C, 40.53; H, 3.62; N, 17.71. HPLC: retention time (t_R) of 4.83 and 5.36 min in the ratio 1:1.

*4.1.7.2. 3′-Azido-2′,3′-dideoxy-5-fluorouridine 5′-O-(4-chlorophenyl N-ethylphosphate) (**13**).* ^{1}H NMR (DMSO-d_6) δ: 1.02 (t, 3H, J = 6.7 Hz, N–C–CH_3), 2.29–2.49 (m, 2H, H-2′, H-2″), 2.75–2.98 (m, 2H, N–CH_2–C), 3.97–4.08 (m, 1H, H-4′), 4.14–4.32 (m, 2H, H-5′, H-5″), 4.41–4.52 (m, 1H, H-3′), 5.57 (m, 1H, NH–C–C), 6.11 (pseudo t, J = 8.4 Hz, 1H, H-1′), 7.20, 7.24 (d, 2H, J = 12.0 Hz, 4-ClPh), 7.39, 7.44 (d, 2H, J = 12.0 Hz, 4-ClPh), 7.96, 7.97 (d, 1H, J = 9.2 Hz, H-6), 11.92 (br s, 1H, 3-NH). ^{13}C NMR (DMSO-d_6) δ: 22.06, 22.32, 40.52, 45.54, 64.73, 86.84, 89.44, 127.09, 129.89 (d, J_{C-F} = 34.2 Hz), 134.71, 143.74, 145.26 (d, J_{C-F} = 231.40 Hz), 146.65, 154.16, 162.16 (d, J_{C-F} = 26.21 Hz). ^{19}F NMR (DMSO-d_6) δ: −166.29 (t, 1F, J = 11.2 Hz). ^{31}P NMR (DMSO-d_6) δ: 6.37; 6.49. MS-ESI m/z: 489, 491 $[M + H]^+$; 511, 513 $[M + Na]^+$; 527, 529 $[M + K]^+$; 487, 489 $[M - H]^-$; 523, 525, 527 $[M + Cl]^-$. Anal. Calcd for $C_{17}H_{19}ClFN_6O_6P$: C, 41.77; H, 3.92; N, 17.19. Found: C, 41.80; H, 3.93; N, 17.20. HPLC: retention time (t_R) of 5.17 and 5.66 min in the ratio 1:1.

*4.1.7.3. 3′-Azido-2′,3′-dideoxy-5-fluorouridine 5′-O-[4-chlorophenyl N-(2,2,2-trifluoroethyl)phosphate] (**14**).* ^{1}H NMR (DMSO-d_6) δ: 2.30–2.49 (m, 2H, H-2′, H-2″), 3.58–3.70 (m, 2H, N–CH_2), 4.02 (m, 1H, H-4′), 4.17–4.31 (m, 2H, H-5′, H-5″), 4.45 (m, 1H, H-3′), 5.61 (m, 1H, NH-C-CF_3), 6.10 (pseudo t, 1H, J = 6.4 Hz, H-1′), 7.19, 7.23 (d, 2H, J = 8.8 Hz, 4-ClPh), 7.39, 7.44 (d, 2H, J = 8.8 Hz, 4-ClPh), 7.93, 7.94 (d, 1H, J = 6.8 Hz, H-6), 11.86 (br s, 1H, 3-NH). ^{13}C NMR (DMSO-d_6) δ: 35.55, 42.69, 60.47, 66.11, 81.36, 84.34, 122.07, 122.57, 124.85 (m), 129.60, 139.07, 140.09 (d, J_{C-F} = 231.40 Hz), 148.98, 149.19, 156.98 (d, J_{C-F} = 26.20 Hz). ^{19}F NMR (DMSO-d_6) δ: −166.25 (m, 1F), −71.81 (t, 3F, J = 13.2 Hz). ^{31}P NMR (DMSO-d_6) δ: 5.44; 5.62. MS-ESI m/z: 543, 545 $[M + H]^+$; 565, 567 $[M + Na]^+$; 581, 583 $[M + K]^+$; 541, 543 $[M - H]^-$; 577, 579, 581 $[M + Cl]^-$. Anal. Calcd for $C_{17}H_{16}ClF_4N_6O_6P$: C, 37.62; H, 2.97; N, 15.48. Found: C, 37.65; H, 2.98; N, 15.50.

*4.1.7.4. 3′-Azido-2′,3′-dideoxy-5-fluorouridine 5′-O-(4-chlorophenyl N-propylphosphate) (**15**).* ^{1}H NMR (DMSO-d_6) δ: 0.82, 0.90 (t, 3H, J = 10.0 Hz, N–C–C–CH_3), 1.41, 1.56 (s, 2H, J = 10 Hz, N–C–CH_2–C), 2.25–2.51 (m, 2H, H-2′, H-2″), 2.70–2.89 (m, 2H, N–CH_2–C–C), 4.06 (m, 1H, H-4′), 4.14–4.33 (m, 2H, H-5′, H-5″), 4.40–4.54 (m, 1H, H-3′), 5.61 (m, 1H, NH–C–C–C), 6.12 (t, 1H, J = 8.0 Hz, H-1′), 7.26 (d, 2H, J = 11.2 Hz, 4-ClPh), 7.46 (d, 2H, J = 11.2 Hz, 4-ClPh), 7.98, 8.00 (d, 1H, J = 9.2 Hz, H-6), 11.96 (br s, 1H, 3-NH). ^{13}C NMR (DMSO-d_6) δ: 11.01, 24.31, 35.69, 42.65, 59.63, 64.97, 81.56, 84.93, 121.79, 124.72 (d, J_{C-F} = 34.30 Hz), 129.50, 138.59, 140.16 (d, J_{C-F} = 231.40 Hz), 148.87, 149.61, 157.95 (d, J_{C-F} = 26.20 Hz). ^{19}F NMR (DMSO-d_6) δ: −166.29 (dd, 1F, J = 14.4, 7.2 Hz). ^{31}P NMR (DMSO-d_6) δ: 6.53; 6.54. MS-ESI m/z: 503, 505 $[M + H]^+$; 525, 527 $[M + Na]^+$; 541, 543 $[M + K]^+$; 501, 503 $[M - H]^-$; 537, 539, 541 $[M + Cl]^-$. Anal. Calcd for $C_{18}H_{21}ClFN_6O_6P$: C, 43.00; H, 4.21; N, 16.71. Found: C, 43.07; H, 4.22; N, 16.74.

*4.1.7.5. 3′-Azido-2′,3′-dideoxy-5-fluorouridine 5′-O-(4-chlorophenyl N-allylphosphate) (**16**).* ^{1}H NMR (DMSO-d_6) δ: 2.27–2.46 (m, 2H, H-2′, H-2″), 3.62, 3.69 (dd, 2H, J = 3.6 Hz, 12.0 Hz, N–CH_2–C=C), 3.85 (m, 1H, H-4′), 4.11–4.31 (m, 2H, H-5′, H-5″), 4.36–4.53 (m, 3H, H-3′, N–C–C=CH_2), 5.31–5.64 (m, 2H, NH–C–C=C, C–CH=C), 6.06, 6.11 (pseudo t, 1H, J = 6.0 Hz, H-1′), 7.24 (d, 2H, J = 8.8 Hz, 4-ClPh), 7.44 (d, 2H, J = 8.8, 4-ClPh), 87.97, 7.99 (d, 1H, J = 6.8, H-6), 11.93 (br s, 1H, 3-NH). ^{13}C NMR (DMSO-d_6) δ: 36.64, 40.01, 60.60, 66.54, 80.73, 84.35, 121.62, 122.65, 124.80 (d, J_{C-F} = 34.70 Hz), 129.62, 138.86, 139.15, 140.11 (d, J_{C-F} = 231.20 Hz), 148.84, 149.50, 157.09 (d, J_{C-F} = 26.20 Hz). ^{19}F NMR (DMSO-d_6) δ: −166.29 (m, 1F). ^{31}P NMR (DMSO-d_6) δ: 7.40; 7.53. MS-ESI m/z: 501, 503 $[M + H]^+$; 523, 525 $[M + Na]^+$; 539, 541 $[M + K]^+$; 499, 501 $[M - H]^-$; 535, 537, 539 $[M + Cl]^-$. Anal. Calcd for $C_{18}H_{19}ClFN_6O_6P$: C, 43.17; H, 3.82; N, 16.78. Found: C, 43.21; H, 3.83; N, 16.80.

*4.1.7.6. 3′-Azido-2′,3′-dideoxy-5-fluorouridine 5′-O-(4-chlorophenyl N-propargylphosphate) (**17**).* ^{1}H NMR (DMSO-d_6) δ: 2.09 (s, 1H, N–C–C–CH), 2.28–2.48 (m, 2H, H-2′, H-2″), 3.63–3.72 (m, 2H, N–CH_2–C–C), 4.04 (m, 1H, H-4′), 4.18–4.34 (m, 2H, H-5′, H-5″), 4.47 (m, 1H, H-3′), 5.32 (m, 1H, NH–C–C–C), 6.09 (pseudo t, 1H, J = 6.4 Hz, H-1′), 7.18, 7.24 (d, 2H, J = 8.8 Hz, 4-ClPh), 7.38, 7.42 (d, 2H, J = 8.8 Hz, 4-ClPh), 7.94 (d, 1H, J = 6.8 Hz, H-6), 11.78 (br s, 1H, 3-NH). ^{13}C NMR (DMSO-d_6) δ: 30.19, 35.83, 59.71, 59.79, 65.51, 73.70, 82.01, 84.35, 121.98, 124.62 (d, J_{C-F} = 34.20 Hz), 129.54, 138.58, 140.11 (d, J_{C-F} = 231.50 Hz), 149.07, 149.38, 157.09 (d, J_{C-F} = 26.20 Hz). ^{19}F NMR (DMSO-d_6) δ: −166.08 (d, 1F, J = 8.8 Hz). ^{31}P NMR (DMSO-d_6) δ: 5.51; 5.70. MS-ESI m/z: 499, 501 $[M + H]^+$; 521, 523 $[M + Na]^+$; 537, 539 $[M + K]^+$; 497, 499 $[M - H]^-$; 533, 535, 537 $[M + Cl]^-$. Anal. Calcd for $C_{18}H_{17}ClFN_6O_6P$: C, 43.34; H, 3.44; N, 16.85. Found: C, 43.40; H, 3.45; N, 16.87.

*4.1.7.7. 3′-Azido-2′,3′-dideoxy-5-fluorouridine 5′-O-[4-chlorophenyl N-(3-azidopropyl)phosphate] (**18**).* ^{1}H NMR (DMSO-d_6) δ: 1.64 (m, 2H, N–C–CH_2–C–N_3), 2.26–2.50 (m, 2H, H-2′, H-2″), 2.83–2.98 (m, 2H, N–CH_2–C–C–N_3), 3.33, 3.38 (t, 2H, J = 6.8 Hz, N–C–C–CH_2–N_3), 4.04 (m, 1H, H-4′), 4.18–4.31 (m, 2H, H-5′, H-5″), 4.39–4.51 (m, 1H, H-3′), 5.70 (m, 1H, NH–C–C–C–N_3), 6.12 (pseudo t, 1H, J = 6.4 Hz, H-1′), 7.21, 7.24 (d, 2H, J = 8.8 Hz, 4-ClPh), 7.39, 7.44 (d, 2H, J = 8.8 Hz, 4-ClPh), 7.96, 7.98 (d, 2H, J = 5.6 Hz, H-6), 11.94 (br s, 1H, 3-NH). ^{13}C NMR (DMSO-d_6) δ: 30.43, 38.01, 39.81, 48.21, 59.72, 65.01, 81.91, 84.36, 122.22, 124.74 (d, J_{C-F} = 34.20 Hz), 129.58, 139.03, 140.15 (d, J_{C-F} = 231.50 Hz), 149.00, 149.50, 157.02 (d, J_{C-F} = 26.20 Hz). ^{19}F NMR (DMSO-d_6) δ: −166.21 (t, 1F, J = 6.00 Hz). ^{31}P NMR (DMSO-d_6) δ: 6.34; 6.49. MS-ESI m/z: 544, 545 $[M + H]^+$; 566, 568 $[M + Na]^+$; 584, 586 $[M + K]^+$; 542, 544 $[M - H]^-$; 578, 580, 582 $[M + Cl]^-$. Anal. Calcd for $C_{18}H_{20}ClFN_9O_6P$: C, 39.75; H, 3.71; N, 23.18. Found: C, 39.81; H, 3.72; N, 23.20.

*4.1.7.8. 3′-Azido-2′,3′-dideoxy-5-fluorouridine 5′-O-(4-chlorophenyl N-butylphosphate) (**19**).* ^{1}H NMR (DMSO-d_6) δ: 0.81 (t, 3H, J = 7.2 Hz, N–C–C–C–CH_3), 1.22 (sextet, 2H, J = 7.2 Hz, N–C–C–CH_2–C), 1.33 (m, 2H, N–C–CH_2–C–C), 2.31–2.49 (m, 2H, H-2′, H-2″), 2.77–2.87 (m, 2H, N–CH_2–C–C–C), 3.99–4.06 (m, 1H, H-4′), 4.14–4.28 (m, 2H, H-5′, H-5″), 4.42–4.50 (m, 1H, H-3′), 5.57 (m, 1H, NH–C–C–C–C), 6.11 (pseudo t, 1H, J = 6.4 Hz, H-1′), 7.23 (d, 2H, J = 8.8 Hz, 4-ClPh), 7.44 (d, 2H, J = 8.8 Hz, 4-ClPh), 7.95 (m, 1H, H-6), 11.89 (br s, 1H, 3-NH). ^{13}C NMR (DMSO-d_6) δ: 13.56, 19.20, 33.15, 35.73, 40.53, 60.10, 65.03, 81.71, 84.27, 121.90, 124.73 (d, J_{C-F} = 34.20 Hz), 129.53, 138.95, 140.07 (d, J_{C-F} = 231.50 Hz), 149.04, 149.63, 157.04 (d, J = 26.2 Hz). ^{19}F NMR (DMSO-d_6) δ: −166.20 (m, 1F). ^{31}P NMR (DMSO-d_6) δ: 6.54; 6.66. MS-ESI m/z: 518, 520 $[M + H]^+$; 540, 542 $[M + Na]^+$; 556, 558 $[M + K]^+$; 516, 518 $[M - H]^-$; 552, 554, 556 $[M + Cl]^-$. Anal. Calcd for $C_{19}H_{23}ClFN_6O_6P$: C, 44.15; H, 4.49; N, 16.26. Found: C, 44.20; H, 4.50; N, 16.28.

*4.1.7.9. 3′-Azido-2′,3′-dideoxy-5-fluorouridine 5′-O-[4-chlorophenyl N-(4-azidobutyl)phosphate] (**20**).* ^{1}H NMR (DMSO-d_6) δ: 1.40–1.60 (m, 4H, N–C–CH_2–CH_2–C–N_3), 2.33–2.53 (m, 2H, H-2′, H-2″), 2.82–2.97 (m, 2H, N–CH_2–C–C–C–N_3), 3.31 (t, 2H, J = 6.6 Hz, N–C–C–C–CH_2–N_3), 4.07 (m, 1H, H-4′), 4.21–4.34 (m, 2H, H-5′, H-5″), 4.44–4.54 (m, 1H, H-3′), 5.71 (m, 1H, NH–C–C–C–C–N_3), 6.14 (pseudo t, 1H, J = 8.0 Hz, H-1′), 7.29 (d, 2H, J = 12.0 Hz, 4-ClPh), 7.48 (d, 2H, J = 12.0 Hz, 4-ClPh), 7.99, 8.00 (d, 1H, J = 9.6 Hz, H-6), 11.94 (br s, 1H, 3-NH). ^{13}C NMR (DMSO-d_6) δ: 23.34, 25.80, 38.40, 40.33, 50.25, 59.72, 65.04, 81.76, 84.62, 121.81, 124.59 (d, J_{C-F} = 34.40 Hz), 129.52, 138.64, 141.07 (d, J_{C-F} = 231.40 Hz), 149.52, 149.63, 157.00 (d, J_{C-F} = 26.10 Hz). ^{19}F NMR (DMSO-d_6) δ: −166.21 (m, 1F). ^{31}P NMR (DMSO-d_6) δ: 6.45; 6.59. MS-ESI m/z: 558, 560 $[M + H]^+$; 580, 582 $[M + Na]^+$; 596, 598 $[M + K]^+$; 556, 558 $[M - H]^-$; 592, 594, 596 $[M + Cl]^-$. Anal. Calcd for $C_{19}H_{22}ClFN_9O_6P$: C, 40.91; H, 3.97; N, 22.60. Found: C, 40.97; H, 3.98; N, 22.63.

*4.1.7.10. 3′-Azido-2′,3′-dideoxy-5-fluorouridine 5′-O-[4-chlorophenyl N-(methoxy-(S)-alaninyl)phosphate] (**21**).* ^{1}H NMR (DMSO-d_6) δ: 1.19–1.34 (m, 3H, N–C(CH_3)CO_2C), 2.33–2.53 (m, 2H, H-2′, H-2″), 3.62–3.64 (s, 3H, N–C(C)CO_2CH_3), 3.81–3.99 (m, 1H, N–CH(C)CO_2C), 4.01–4.11 (m, 1H, H-4′), 4.17–4.35 (m, 2H, H-5′, H-5″), 4.50 (m, 1H, H-3′), 5.42 (m, 1H, NH–C(C)CO_2C), 6.14 (m, 1H, H-1′), 7.23, 7.27 (d, 2H, J = 9.2 Hz, 4-ClPh), 7.43, 7.47 (d, 2H, J = 9.2 Hz, 4-ClPh), 7.97, 7.98 (d, 1H, J = 6.8 Hz, H-6), 10.49 (br s, 1H, 3-NH). ^{13}C NMR (DMSO-d_6) δ: 19.69, 35.68, 49.34, 51.92, 59.73, 65.97, 81.35, 84.26, 121.99, 124.72 (d, J_{C-F} = 34.30 Hz), 129.50, 138.79, 140.12 (d, J_{C-F} = 231.50 Hz), 148.93, 149.43, 157.02 (d, J_{C-F} = 26.20 Hz), 173.18. ^{19}F NMR (DMSO-d_6) δ: −166.15 (t, 1F, J = 5.4 Hz). ^{31}P NMR (DMSO-d_6) δ: 4.52; 4.54. MS-ESI m/z: 547, 549 $[M + H]^+$; 569, 571 $[M + Na]^+$; 585, 587 $[M + K]^+$; 545, 547 $[M - H]^-$; 581, 583, 585 $[M + Cl]^-$. Anal. Calcd for $C_{19}H_{21}ClFN_6O_8P$: C, 41.73; H, 3.87; N, 15.37. Found: C, 41.83; H, 3.88; N, 15.39.

4.2. Biological evaluation

4.2.1. Cell cultures

Human cancer cells HeLa (cervical cancer cell line) and 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% foetal 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 5×10^4. All 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 antitumour 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 [31]. The monolayer cell culture was trypsinized and the cell count was adjusted to 5×10^4 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 (containing 10% of water) (100 μL) 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 h 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). Cytarabine (Sigma–Aldrich) was used as the internal standard.

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