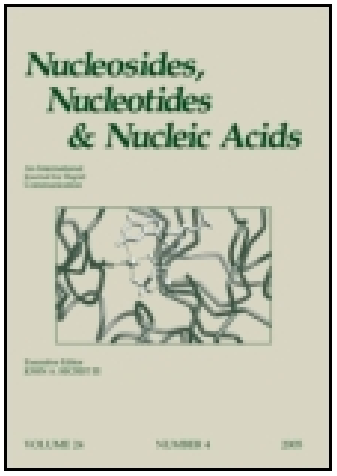

Nucleosides, Nucleotides and Nucleic Acids

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Synthesis of Novel 2′,3′-Didehydro-2′,3′-dideoxyinosine Phosphoramidate Prodrugs and Evaluation of their Anticancer Activity

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SYNTHESIS OF NOVEL 2′,3′-DIDEHYDRO-2′,3′-DIDEOXYINOSINE PHOSPHORAMIDATE PRODRUGS AND EVALUATION OF THEIR ANTICANCER ACTIVITY

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□ *An efficient synthesis of 4-chlorophenyl N-alkyl phosphoramidates of 2′,3′-didehydro-2′,3′-dideoxyinosine employing 4-chlorophenyl phosphoroditetrazolide as a phosphorylating agent is reported. Improved method for the synthesis of 2′,3′-didehydro-2′,3′-dideoxyinosine starting from inosine is also described. The synthesized phosphoramidates* ***11–18*** *were examined for their cytotoxic activity in three human cancer cell lines: cervical (HeLa), oral (KB), and breast (MCF-7) employing sulforhodamine B assay. The highest activity in all investigated cancer cell lines was displayed by phosphoramidate* ***13*** *with N-n-propyl substituent.*

Keywords Phosphorylation; phosphoramidate; 2′,3′-didehydro-2′,3′-dideoxyinosine; prodrug; anticancer activity

INTRODUCTION

Many nucleoside analogs have found important use as antiviral[1] and anticancer[2] therapeutics. Particularly, 2′,3′-dideoxyinosine (ddI, didanosine) has been developed as an anti-HIV drug for the treatment of acquired immunodeficiency syndrome.[3] Significant interest also concerned synthesis of 2′,3′-didehydro-2′,3′-dideoxyinosine (d4I) due to its potential biological activity.[4,5] Mechanism of antiviral or anticancer action of 2′,3′-dideoxynucleosides (ddN) primarily involves their intracellular conversion to 2′,3′-dideoxynucleoside 5′-triphosphates (ddNTPs) *via* 5′-mono- and

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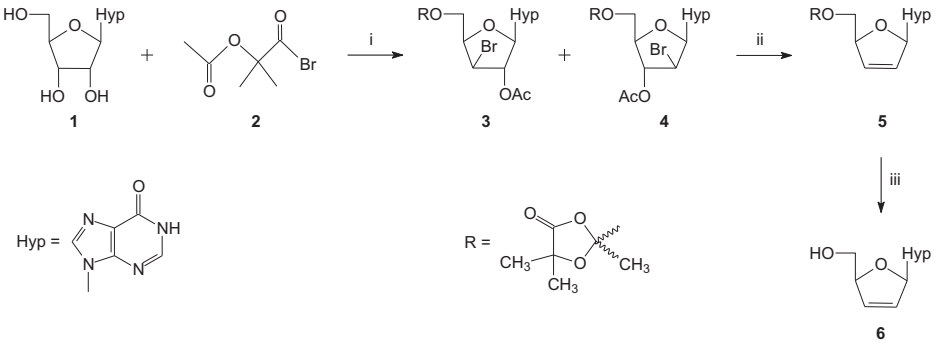
5′-diphosphates. The ddNTPs act as competitive inhibitors of DNA polymerases and chain terminators of a growing DNA strand due to the lack of a 3′-hydroxyl group.[6] Since, the first step of phosphorylation is usually the slowest one, attempts are made to synthesize prodrugs (pronucleotides) of 2′,3′-dideoxynucleoside 5′-monophosphates (ddNMPs) with a protected 5′-phosphate group.[7] These prodrugs are designed to easily cross the cell membrane and release ddNMPs inside the cell as a result of chemical or enzymatic hydrolysis.[6,8] Thus, ddNMPs liberated inside the cell require only the second and third phosphorylation steps for conversion to the ddNTPs. Of the current pronucleotide strategies, the phosphoramidate diester approach appears to be the most viable.[9]

The aim of our study was to synthesize novel phosphoramidate prodrugs of 2′,3′-didehydro-2′,3′-dideoxyinosine with potential antiviral or anticancer properties. In this paper, we report a method for the synthesis of 4-chlorophenyl *N*-alkyl phosphoramidate diesters of 2′,3′-didehydro-2′,3′-dideoxyinosine (**11–18**) and evaluation of their anticancer activity. Improved method for the synthesis of 2′,3′-didehydro-2′,3′-dideoxyinosine starting from inosine is also presented.

RESULTS AND DISCUSSION

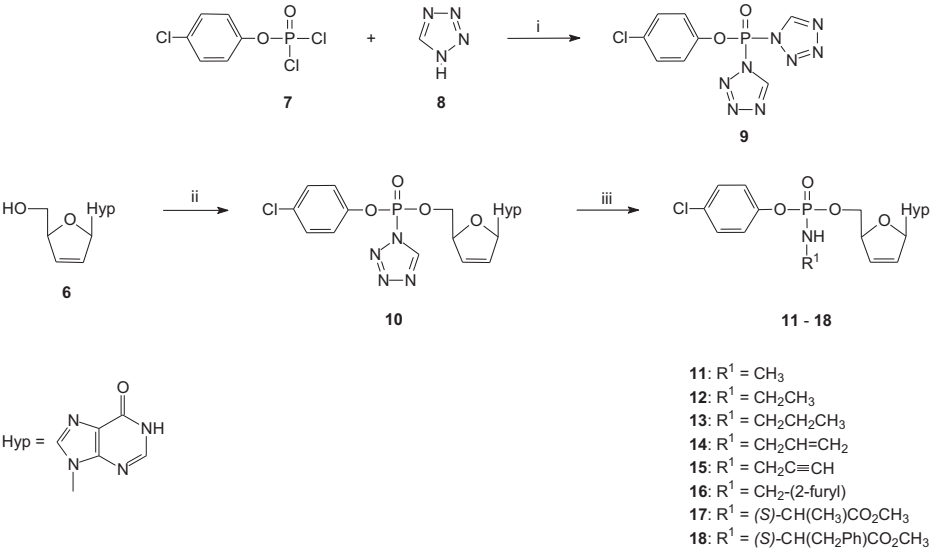
Chemistry

2′,3′-Didehydro-2′,3′-dideoxyinosine was synthesized from inosine according to the procedure shown in Scheme 1 adopting the methods described by Bhat[10] and Robins[5,11] with some modifications. In the first step of the synthesis, inosine (**1**) was reacted with 2-acetoxyisobutyryl bromide (**2**) in acetonitrile at room temperature to give the mixture of two isomeric inosine bromoacetates **3** and **4** in 72% yield. The amount of the 2-acetoxyisobutyryl bromide could be reduced to 2.5 equivalents. It was found that precooling of the reaction mixture to 5°C was not necessary as indicated in the original procedure.[10] Moreover, it was established that the addition of water to the acetonitrile was not essential.[5,11] Attempts to use nitromethane instead of acetonitrile did not improve the reaction yield.[4] Treatment of the mixture of bromoacetates **3** and **4** with activated zinc in THF effected reductive elimination to provide 5′-*O*-(2,5,5-trimethyl-1,3-dioxalan-4-on-2yl)-2′,3′-didehydro-2′,3′-dideoxyinosine (**5**) in 65% yield. The use of zinc-copper couple instead of the zinc did not increase the reaction yield appreciably. When reductive elimination of the bromo acetates **3** and **4** was carried out in DMF[5,11] or pyridine[4] the product **5** was obtained with a lower yield. In the last step of the synthesis, 5′-*O*-(2,5,5-trimethyl-1,3-dioxalan-4-on-2yl) group was removed from compound **5** by treatment with methanolic ammonia to furnish **6** in 89% yield.



SCHEME 1 Synthesis of 2′,3′-didehydro-2′,3′-dideoxyinosine (**6**) from inosine (**1**). Reagents and conditions: (i) CH_3CN, r.t., 3 hours; (ii) Zn, AcOH, THF, r.t., 5 hours; and (iii) NH_3/CH_3OH, r.t., overnight.

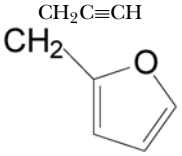
A series of novel 4-chlorophenyl *N*-alkyl phosphoramidate diesters of 2′,3′-didehydro-2′,3′-dideoxyinosine (**11–18**) were synthesized by phosphorylation of 2′,3′-didehydro-2′,3′-dideoxyinosine (**6**) with 4-chlorophenyl phosphoroditetrazolide (**9**) according to the synthetic route outlined in Scheme 2.



SCHEME 2 Synthesis of 4-chlorophenyl *N*-alkyl phosphoramidate diesters of 2′,3′-didehydro-2′,3′-dideoxyinosine (**11–18**). Reagents and conditions: (i) NEt_3, CH_3CN, r.t., 30 minutes; (ii) **9**, pyridine, r.t., 1 hour; and (iii) R-NH_2, r.t., 1 hour.

4-Chlorophenyl phosphoroditetrazolide (**9**) was obtained by reaction of 4-chlorophenyl phosphorodichloridate (**7**) with 1*H*-tetrazole (**8**) in the presence of triethylamine in acetonitrile. Reaction of compound **9** with 2′,3′-didehydro-2′,3′-dideoxyinosine (**6**) in the presence of pyridine afforded reactive intermediate **10**, which was treated in situ with the appropriate

TABLE 1 Preparation of 4-chlorophenyl *N*-alkyl phosphoramidates of 2′,3′-didehydro-2′,3′-dideoxyinosine (**11–18**) by phosphorylation of 2′,3′-didehydro-2′,3′-dideoxyinosine (**6**) with 4-chlorophenyl phosphoroditetrazolide (**9**)

Product	R^1	Yield[a] (%)
11	CH_3	68
12	CH_2CH_3	70
13	$CH_2CH_2CH_3$	87
14	$CH_2CH{=}CH_2$	85
15	$CH_2C{\equiv}CH$	83
16		86
17	(S)-CH($CH_3CO_2CH_3$)	67
18	(S)-CH(CH_2Ph_2)CO_2CH_3)	66

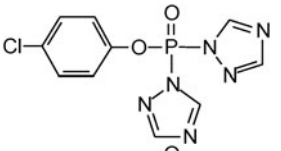
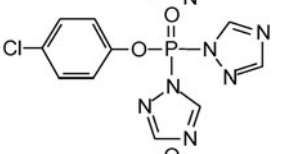
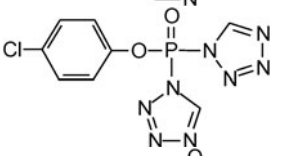
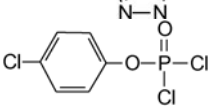
[a]Yield of isolated product.

amine (or amine hydrochloride in the presence of triethylamine) to give the desired products **11–18** in 66–87% yield (Table 1).

It should be noted that the use of 4-chlorophenyl phosphorodichloride (**7**), rather than its tetrazolide counterpart **9**, resulted in the formation of a considerable amounts of the symmetrical (5′-5′)dinucleoside phosphate. We also tried to employ 4-chlorophenyl phosphoroditriazolide[12] (obtained from **7** and 1*H*-1,2,4-triazole) as the phosphorylating reagent of **6** but the reaction yield was lower (Table 1, Table 2 entry 1). The addition of 1-methylimidazole as the nucleophilic catalyst[13] only slightly increased the yield of phosphorylation reaction (Table 2, entry 2). The application of 2- and 4-chlorophenyl phosphoroditriazolides[14–16] for the phosphorylation of 5′-protected nucleosides has been reported in the phosphotriester synthesis of oligonucleotides and provided the stimulus for the development of our method. We anticipate that developed by us method of the synthesis of 2′,3′-didehydro-2′,3′-dideoxyinosine phosphoramidates will also apply to the other 2′,3′-dideoxynucleosides, e.g., 2′,3′-dideoxyinosine (ddI), 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T), or 3′-azido-3′-deoxythymidine (AZT).

^{31}P NMR spectra of products **11–18** 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. Thin-layer chromatography (TLC) of compounds **11–18** 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 compound **13**).

TABLE 2 Optimization of the reaction conditions for the preparation of 4-chlorophenyl *N*-*n*-propyl phosphoramidate of 2′,3′-didehydro-2′,3′-dideoxyinosine (**13**)

Entry	Phosphorylating reagent	Extra nucleophilic catalyst	Yield[a] (%) of **13**
1		Not added	74
2		1-Methylimidazole	76
3		Not added	87
4		Not added	26

[a]Yield of isolated product.

Anticancer Activity

The synthesized phosphoramidates **11–18** 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.[17] The resulting cytotoxic activity data of the obtained phosphoramidates and reference

TABLE 3 In vitro cytotoxic activity of the phosphoramidates **11–18** and the parent nucleoside **6** in three human cancer cell lines: cervical (HeLa), oral (KB), and breast (MCF-7)

Compound	Cytotoxicity (IC_{50}, μM)[a] ± SD[b]			log Pc
	HeLa	KB	MCF-7	
11	72.5 ± 0.8	71.1 ± 0.7	78.4 ± 0.9	1.09
12	55.1 ± 0.5	54.6 ± 0.6	64.3 ± 0.7	1.47
13	46.7 ± 0.3	45.1 ± 0.2	53.4 ± 0.4	1.97
14	71.2 ± 0.8	73.4 ± 0.9	81.2 ± 0.8	1.74
15	60.1 ± 0.7	62.5 ± 0.6	71.3 ± 0.7	1.25
16	>100	>100	>100	1.44
17	96.7 ± 0.6	95.9 ± 0.7	99.1 ± 0.9	1.29
18	>100	>100	>100	2.75
6	>100	>100	>100	−0.97
Cytarabine (standard)	3.5 ± 0.1	4.1 ± 0.1	3.8 ± 0.1	−2.32 [18]

[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.[19]

compounds are presented in Table 3. The phosphoramidates **11–15** and **17** showed moderate cytotoxic activity. Among them the highest activity, in all investigated cancer cell lines, was displayed by phosphoramidate **13** with *N*-*n*-propyl substituent. Phosphoramidate **12** and **15** exhibited also relatively high activity whereas phosphoramidates **11**, **14**, and **17** were somewhat less potent. On the other hand, the phosphoramidates **16** and **18** as well as the parent nucleoside **6** proved inactive in three the cell lines.

Partition coefficient (log P) values of the compounds **11–18** were calculated[19] to determine a possible correlation between the cytotoxicity data and lipophilicity (Table 3). All of the d4I phosphoramidates were more lipophilic than d4I itself (log P = −0.97), with log P values ranging from 1.09 to 2.75. The most active compounds **12** and **13** showed moderate values of log P, 1.47 and 1.97, respectively. However, linear regression analysis did not reveal any correlation between log P values and the cytotoxicity data.

CONCLUSIONS

In summary, we have developed an efficient method for the synthesis of 4-chlorophenyl *N*-alkyl phosphoramidates of 2′,3′-didehydro-2′,3′-dideoxyinosine employing 4-chlorophenyl phosphoroditetrazolide as a phosphorylating agent. 4-Chlorophenyl phosphoroditetrazolide was more effective than its di(1,2,4-triazolo) counterpart. The obtained phosphoramidates **11–18** were examined for their cytotoxic activity in three human cancer cell line: cervical (HeLa), oral (KB), and breast (MCF-7). The highest activity in all the investigated cancer cell lines was displayed by phosphoramidate **13** with *N*-*n*-propyl substituent. Improved method for the synthesis of 2′,3′-didehydro-2′,3′-dideoxyinosine starting from inosine was also elaborated.

EXPERIMENTAL SECTION

Chemistry (General Methods)

^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on a Varian-Gemini 400 MHz spectrometer. Chemical shifts (δ) 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). 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.

2′,3′-Didehydro-2′,3′-dideoxyinosine (6)

To a stirred suspension of inosine (2.00 g, 7.46 mmol) in dry acetonitrile (15 mL) was added dropwise 2-acetoxyisobutyryl bromide (3.89 g, 18.61 mmol, 2.5 equiv) and stirring was continued at room temperature for 3 hours. Then the mixture was poured into saturated aqueous solution of sodium bicarbonate (50 mL) and extracted with ethyl acetate (4 × 15 mL). The combined ethyl acetate extracts were washed with water (10 mL), dried over anhydrous magnesium sulfate, filtered and evaporated under vacuum to dryness; yield of **3** and **4**: 2.69 g (72%). Next, the mixture of **3** and **4** (2.69 g, 5.37 mmol) was dissolved in THF (55 mL) and zinc dust (2.69 g) was added followed by acetic acid (0.25 mL). The reaction mixture was stirred at room temperature for 5 hours. After that, the mixture was filtered and the residue was washed with THF (15 mL). The combined filtrates were concentrated to about 20 mL and poured into 5% aqueous solution of EDTA trisodium salt (100 mL). The resulting solution was extracted with ethyl acetate (6 × 20 mL). The combined ethyl acetate extracts were washed 5% aqueous solution of sodium bicarbonate (20 mL) and with water (10 mL), dried over anhydrous magnesium sulfate, filtered and evaporated under vacuum to dryness; yield of **5**: 1.26 g (65%). Afterward, compound **5** (1.26 g, 3.48 mmol) was suspended in methanol saturated with ammonia (50 mL) and the mixture was stirred at room temperature for 12 hours. Then the mixture was evaporated under vacuum to dryness and the residue was crystallized from methanol; yield of **6**: 0.73 g (89%).

^{1}H NMR (400 MHz, DMSO-d_6): δ 3.62–3.58 (m, 2H, H-5′, H-5″), 4.89 (m, 1H, H-4′), 4.92 (s, 1H, OH), 5.88–6.15 (m, 1H, H-3′), 6.36–6.49 (m, 1H, H-2′), 6.91 (dd, J = 6.2 Hz, J = 3.8 Hz, 1H, H-1′), 8.09 (s, 1H, H-8), 8.12 (s, 1H, H-2), 12.33 (s, 1H, H-1); ^{13}C NMR (100 MHz, DMSO-d_6): δ 62.63, 87.99, 88.26, 124.00, 125.23, 134.68, 138.51, 145.97, 147.98, 156.65; MS (ESI+): *m/z* 235 $[M + H]^+$, 257 $[M + Na]^+$; Anal. Calcd for $C_{10}H_{10}N_4O_3$: C, 51.28; H, 4.30; N, 23.92. Found: C, 51.33; H, 4.31; N, 23.93.

Synthesis of 2′,3′-didehydro-2′,3′-dideoxyinosine phosphoramidates (11–18)

General Procedure

To a solution of 4-chlorophenyl phosphorodichloridate (**7**) (260 mg, 1.06 mmol) in acetonitrile (2 mL) was added 1*H*-tetrazole (**8**) (193 mg,

2.75 mmol) followed by triethylamine (220 mg, 2.17 mmol) and the reactants were stirred for 30 min at room temperature. Alternatively, instead of 1*H*-tetrazole other additives were used, as demonstrated for the synthesis of compound **13** (see Table 2), they include: 1*H*-1,2,4-triazole (190 mg, 2.75 mmol) (entry 1), mixture of 1*H*-1,2,4-triazole (190 mg, 2.75 mmol) and 1-methylimidazole (35 mg, 0.43 mmol) (entry 2). Then to the mixture 2′,3′-didehydro-2′,3′-dideoxyinosine (**6**) (100 mg, 0.43 mmol) and pyridine (2.7 mL) were added. The reaction mixture was stirred at room temperature for a further 1 hour and the appropriate amine (2.15 mmol) was added. In the case of synthesis of compounds **11** and **12** amine hydrochloride (2.15 mmol) and triethylamine (327 mg, 3.23 mmol) were added. When compounds **17** and **18** were synthesized L-alanine methyl ester hydrochloride or L-phenylalanine methyl ester hydrochloride (2.15 mmol) and triethylamine (327 mg, 3.23 mmol) were added. After 1 h, the reaction mixture was evaporated under reduced pressure. To the residue was added saturated aqueous sodium bicarbonate (15 mL) and the mixture was extracted with chloroform (5 × 20 mL). The combined chloroform extracts were washed with water (15 mL), dried over anhydrous magnesium sulfate, 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 **11–18** (yield 66–87%).

2′,3′-Didehydro-2′,3′-dideoxyinosine 5′-O-(4-chlorophenyl N-methylphosphate) (11)

^{1}H NMR (400 MHz, DMSO-d$_6$): δ 2.60–2.86 (m, 3H, CH$_3$), 4.15–4.31 (m, 2H, H-5′, H-5″), 5.08 (m, 1H, H-4′), 5.46 (m, 1H, P-NH), 6.08 (m, 1H, H-3′), 6.54 (m, 1H, H-1′), 7.08–7.20 (m, 1H, H-2′), 7.28, 7.30 (d, J = 9.0 Hz, 2H, 4-ClPh), 7.46, 7.49 (d, J = 9.0 Hz, 2H, 4-ClPh), 8.09 (s, 1H, H-8), 8.30 (s, 1H, H-2), 12.24 (s, 1H, NH-1); ^{13}C NMR (100 MHz, DMSO-d$_6$): δ 25.74, 64.06, 86.11, 88.24, 121.21, 124.58, 126.06, 129.51, 130.10, 133.83, 138.57, 146.01, 148.95, 149.31, 169.61; ^{31}P NMR (160 MHz, DMSO-d$_6$): δ 5.11, 5.38; MS (ESI+): *m/z* 438, 440 [M + H]$^+$, 460, 462 [M + Na]$^+$; Anal. Calcd. for $C_{17}H_{17}ClN_5O_5P$: C, 46.64; H, 3.91; N, 16.00. Found: C, 46.67; H, 3.92; N, 16.01.

2′,3′-Didehydro-2′,3′-dideoxyinosine 5′-O-(4-chlorophenyl N-ethylphosphate) (12)

^{1}H NMR (400 MHz, DMSO-d$_6$): δ 1.09 (t, J = 7.2 Hz, 3H, CH$_3$), 2.61–2.86 (m, 2H, N-CH$_2$), 4.15–4.37 (m, 2H, H-5′, H-5″), 5.22 (m, 1H, H-4′), 5.68 (m, 1H, P-NH), 6.12 (m, 1H, H-3′), 6.72 (m, 1H, H-1′), 7.09–7.18 (m, 1H, H-2′), 7.29, 7.34 (d, J = 8.3 Hz, 2H, 4-ClPh), 7.51, 7.56 (d, J = 8.3 Hz, 2H, 4-ClPh), 8.12 (s, 1H, H-8), 8.31 (s, 1H, H-2), 12.94 (s, 1H, NH-1); ^{13}C NMR (100 MHz, DMSO-d$_6$): δ 15.28, 35.60, 68.24, 85.49, 88.74, 122.38, 124.81, 126.18, 129.03,

130.90, 133.88, 138.94, 145.17, 147.89, 149.31, 167.84; ^{31}P NMR (160 MHz, DMSO-d_6): δ 5.42, 5.61; MS (ESI+): *m/z* 452, 454 $[M + H]^+$, 474, 476 $[M + Na]^+$; Anal. Calcd. for $C_{18}H_{19}ClN_5O_5P$: C, 47.85; H, 4.24; N, 15.50. Found: C, 47.88; H, 4.25; N, 15.51.

2′,3′-Didehydro-2′,3′-dideoxyinosine 5′-O-(4-chlorophenyl N-n-propylphosphate) (13)

^{1}H NMR (400 MHz, DMSO-d_6): δ 0.82, 0.90 (t, J = 10.2 Hz, 3H, CH_3), 1.39, 1.48 (s, J = 9.8 Hz, 2H, N—C—CH_2), 2.88–2.96 (m, 2H, N-CH_2), 4.10–4.28 (m, 2H, H-5′, H-5″), 5.01 (m, 1H, H-4′), 5.43–5.58 (m, 1H, P-NH), 6.01 (m, 1H, H-3′), 6.49 (m, 1H, H-1′), 7.12–7.14 (m, 1H, H-2′), 7.26, 7.28 (d, J = 8.8 Hz, 2H, 4-ClPh), 7.44, 7.48 (d, J = 8.8 Hz, 2H, 4-ClPh), 8.07 (s, 1H, H-8), 8.24 (s, 1H, H-2), 13.04 (s, 1H, NH-1); ^{13}C NMR (100 MHz, DMSO-d_6): δ 10.98, 24.82, 43.32, 66.26, 85.81, 88.31, 121.35, 124.40, 126.05, 129.54, 129.90, 133.46, 138.55, 145.68, 148.65, 149.19, 168.50; ^{31}P NMR (160 MHz, DMSO-d_6): δ 4.89, 5.18; MS (ESI+): *m/z* 466, 468 $[M + H]^+$, 488, 490 $[M + Na]^+$; Anal. Calcd. for $C_{19}H_{21}ClN_5O_5P$: C, 48.99; H, 4.54; N, 15.03. Found: C, 49.05; H, 4.55; N, 15.04; HPLC: retention time (t_R) of 5.17 and 5.66 min in the ratio 1: 1.

2′,3′-Didehydro-2′,3′-dideoxyinosine 5′-O-(4-chlorophenyl N-allylphosphate) (14)

^{1}H NMR (400 MHz, DMSO-d_6): δ 3.23–3.37 (m, 2H, N-CH_2), 4.02–4.24 (m, 3H, H-4′, H-5′, H-5″), 5.01–5.19 (m, 2H, N—C—C=CH_2), 5.70–5.82 (m, 2H, P-NH, N-C-CH =C), 6.18–6.27 (m, 1H, H-3′), 6.45–6.54 (m, 1H, H-1′), 6.78–6.89 (m, 1H, H-2′), 7.17–7.26 (m, 2H, 4-ClPh), 7.44–7.56 (m, 2H, 4-ClPh), 7.98 (s, 1H, H-8), 8.11 (s, 1H, H-2), 12.42 (s, 1H, NH-1); ^{13}C NMR (100 MHz, DMSO-d_6): δ 38.06, 67.39, 85.39, 92.60, 115.07, 121.95, 122.04, 126.09, 129.48, 131.60, 133.22, 136.43, 138.22, 146.04, 148.06, 149.62, 166.99; ^{31}P NMR (160 MHz, DMSO-d_6): δ 5.55, 5.59; MS (ESI+): *m/z* 464, 466 $[M + H]^+$, 486, 488 $[M + Na]^+$; Anal. Calcd. for $C_{19}H_{19}ClN_5O_5P$: C, 49.20; H, 4.13; N, 15.10. Found: C, 49.24; H, 4.14; N, 15.12.

2′,3′-Didehydro-2′,3′-dideoxyinosine 5′-O-(4-chlorophenyl N-propargylphosphate) (15)

^{1}H NMR (400 MHz, DMSO-d_6): δ 2.09 (s, 1H, N-C-C-CH), 3.62–3.78 (m, 2H, N-CH_2-C-C), 4.35 (m, 2H, H-5′, H-5″), 5.17 (m, 1H, H-4′), 6.13–6.22 (m, 1H, H-3′), 6.42–6.50 (m, 1H, H-2′), 6.78–6.89 (m, 1H, H-1′), 7.02–7.26 (m, 2H, 4-ClPh), 7.48–7.62 (m, 2H, 4-ClPh), 8.07 (s, 1H, H-8), 8.22 (s, 1H, H-2), 12.83 (s, 1H, NH-1); ^{13}C NMR (DMSO-d_6) δ 29.67, 71.82, 71.89, 80.60, 85.56, 88.46, 121.37, 121.44, 126.16, 129.54, 129.68, 133.37, 138.75, 145.70, 148.59, 148.94, 158.45; ^{31}P NMR (DMSO-d_6) δ 4.24, 4.58; MS (ESI+): *m/z*

462, 464 $[M + H]^+$, 484, 486 $[M + Na]^+$; Anal. Calcd. for $C_{19}H_{17}ClN_5O_5P$: C, 49.42; H, 3.71; N, 15.17. Found: C, 49.46; H, 3.72; N, 15.18.

2′,3′-Didehydro-2′,3′-dideoxyinosine 5′-O-[4-chlorophenyl N-methyl(2-furyl)phosphate] (16)

^{1}H NMR (400 MHz, DMSO-d_6) δ 3.64 (m, 2H, N-CH_2-2-furyl), 3.82–3.96 (m, 2H, H-5′, H-5″), 4.36 (m, 1H, H-4′), 6.11–6.34 (m, 2H, H-3′, H-2′), 6.43–6.58 (m, 1H, furyl), 6.81 (m, 1H, furyl) 7.02 (m, 1H, H-1′), 7.25–7.40 (m, 2H, 4-ClPh) 7.50–7.68 (m, 2H, 4-ClPh), 7.96 (s, 1H, H-8), 8.18 (s, 1H, H-2), 12.79 (s, 1H, NH-1); ^{13}C NMR (DMSO-d_6) δ 29.70, 66.03, 85.70, 88.41, 107.22, 110.30, 116.80, 121.36, 126.05, 129.51, 130.08, 133.62, 138.78, 142.06, 145.70, 148.66, 148.96, 149.12, 158.48; ^{31}P NMR (DMSO-d_6) δ 4.19, 4.64; MS (ESI+): *m/z* 504, 506 $[M + H]^+$, 526, 528 $[M + Na]^+$; Anal. Calcd. for $C_{21}H_{19}ClN_5O_6P$: C, 50.06; H, 3.80; N, 13.90. Found: C, 50.11; H, 3.81; N, 13.91.

2′,3′-Didehydro-2′,3′-dideoxyinosine 5′-O-[4-chlorophenyl N-(methoxy-(S)-alaninyl) phosphate] (17)

^{1}H NMR (400 MHz, DMSO-d_6) δ 1.25 (m, 3H, CCH_3), 3.78 (s, 3H, OCH_3), 3.94 (m, 2H, H-5′, H-5″), 4.18–4.28 (m, 1H, N-CH), 4.36–4.54 (m, 1H, H-4′), 6.11–6.28 (m, 1H, H-3′), 6.84 (m, 1H, H-2′) 7.08 (m, 1H, H-1′), 7.25–7.31 (m, 2H, 4-ClPh), 7.62–7.81 (m, 2H, 4-ClPh), 7.98 (s, 1H, H-8), 8.21 (s, 1H, H-2), 12.81 (s, 1H, NH-1); ^{13}C NMR (DMSO-d_6) δ 45.80, 52.59, 68.12, 85.61, 85.62, 88.57, 121.20, 121.51, 126.20, 129.65, 130.87, 133.38, 138.50, 145.31, 146.96, 148.95, 158.12, 167.76; ^{31}P NMR (DMSO-d_6) δ 3.18, 3.12; MS (ESI+): *m/z* 510, 512 $[M + H]^+$, 532, 534 $[M + Na]^+$; Anal. Calcd. for $C_{20}H_{21}ClN_5O_7P$: C, 47.12; H, 4.15; N, 13.74. Found: C, 47.17; H, 4.16; N, 13.75.

2′,3′-Didehydro-2′,3′-dideoxyinosine 5′-O-[4-chlorophenyl N-(methoxy-(S)-phenylalaninyl) phosphate] (18)

^{1}H NMR (400 MHz, DMSO-d_6) δ 2.98–3.08 (m, 2H, CH_2Ph), 3.69 (s, 3H, OCH_3), 3.89 (m, 2H, H-5′, H-5″), 4.31–4.40 (m, 1H, H-4′), 4.68 (m, 1H, N-CH) 6.18–6.38 (m, 1H, H-3′), 6.42–6.54 (m, 1H, H-2′), 6.89 (m, 1H, H-1′), 7.05–7.50 (m, 9H, 4-ClPh, C-Ph), 8.02 (s, 1H, H-8), 8.24 (s, 1H, H-2), 12.89 (s, 1H, NH-1); ^{13}C NMR (DMSO-d_6) δ 38.17, 52.17, 53.28, 70.15, 84.98, 89.45, 116.18, 122.32, 127.15, 128.85, 129.05, 129.39, 130.07, 131.87, 133.81, 135.05, 137.77, 147.81, 149.11, 151.28, 158.65, 172.18; ^{31}P NMR (DMSO-d_6) δ 5.21, 5.38; MS (ESI+): *m/z* 586, 588 $[M + H]^+$, 608, 610 $[M + Na]^+$; Anal. Calcd. for $C_{26}H_{25}ClN_5O_7P$: C, 53.30; H, 4.30; N, 11.95. Found: C, 53.35; H, 4.31; N, 11.96.

BIOLOGICAL EVALUATION

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% 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 5×10^4. All the cell lines were obtained from The European Collection of Cell Cultures (ECACC) supplied by Sigma-Aldrich.

In vitro Cytotoxicity Assay

The protein-staining 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.[17] 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 hours, 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 hours. 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 minutes 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 (nontreated cells). Cytarabine (Sigma-Aldrich) was used as the internal standard.

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