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SYNTHESIS AND CHARACTERIZATION OF NOVEL PHOSPHORYLATED DERIVATES OF ZIDOVUDINE: ANTICANCER ACTIVITY AGAINST HUMAN BREAST CANCER CELL LINES (MCF7)

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Abstract
A series of novel zidovudine derivatives **4a-c** were synthesized in two step process with high yields. In the first step AZT was reacted with substituted phenyl phosphorodichloridate in the presence of triethylamine (TEA) in dry tetrahydrofuran (THF) /pyridine to obtain the mono chloride intermediates **4a-c**. They were further reacted with ethylene glycol to afford the title compounds **4a-c**. The title compounds were screened for their anticancer activity against human breast cancer MCF7 cells. Among the three compounds 4b shows potential and 4a, 4b were exhibits good anticancer activity.

Key Words: Zudovudine, Human breast cancer cells, Anticancer activity

Introduction

Nucleoside analogs are used in anti-cancer, anti-(retro) viral and immunosuppressive therapies. The nucleosides are phosphorylated to nucleotide mono-, di-, and triphosphates intracellularly. The triphosphate form inhibits human and viral polymerases and reverse transcriptase's and is incorporated into nucleic acids. Although the mono- and diphosphates are not the main active metabolites, they possess pharmacological activity as well (Jansen et al., 2010; Parker and Cheng, 1990; Peter et al., 1996). To date, six nucleoside reverse transcriptase inhibitors abacavir (ABC), didanosine (ddI), lamivudine (3TC), stavudine (d4T), zalcitabine (ddC) and zidovudine (AZT) have been approved by the U.S. Food and Drug Administration (FDA) (Barry et al., 1996; Kewn et al., 2000; Boyle et al., 2005; Satheesh Krishna et al., 2010).

Nucleoside 5'-triphosphates are a class of very important compounds in biological systems. Naturally occurring deoxyribo- and ribonucleoside triphosphates are the basic building blocks for enzymatic synthesis of DNA and RNA *in vivo*. Modified nucleoside triphosphates have received much attention in search for potential therapeutic and diagnostic agents and in the study of numerous biochemical and pharmacological processes (Boyle et al., 2005; Koteswara Rao et al., 2010a; 2011; 2012; Babu et al., 2012; 2014). Nucleoside

analogues (e.g., lamivudine, ribavirin, stavudine, zidovudine, etc.) are the prodrugs which are to be phosphorylated to their active 5′-triphosphate (TP) form in order to compete with their natural deoxyribonucleoside triphosphate (dNTP) counterpart for incorporation into the synthesized DNA. In the search for more effective antiviral and antitumor agents, various modifications of nucleosides have been proposed. Lamivudine, ribavirin, stavudine and zidovudine are nucleoside analogs that have demonstrated efficacy in treating viral diseases (Burgess and Cook, 2000; Sarafianos et al., 1999; Reddy et al.,2011).

Breast cancer is the most common and deadly malignancy in women around the world. It accounts for 60% of cancer death rates in women. It is believed that, estrogens have played vital role in breast cancer development and growth and particularly estrogen stimulated growth in tumor cells need estrogen receptors (ERs). Although many drugs are available in the market for combating this pylorious disease, however they doesn't work properly in view of their limitations including low solubility, high cytotoxicity and ADMET abnormalities. Therefore it is an imperative to treat this, safe and durable anticancer molecules.

Earlier studies have suggested that phosphorylated drugs showed potential anticancer (Rao et al., 2010), antiviral, antioxidant (Reddy et al., 2011; Rao et al., 2010b; 23. Alahari et al., 2010) and antifungal (Sanapalli et al., 2008), antialzheimers (Valasani et al., 2013a, b; 2014a,b; c; Vangavaragu et al., 2014) and antimalarial properties. Therefore the present study was aimed to investigate the synthesis of novel phosphorylated anologues of Zudovudine and evaluated for their anticancer activity against human breast cancer MCF7 cells.

RESUSLTS AND DISCUSSION

The synthetic route (**Scheme - 1**) involves the addition of a solution of substituted -phenyl phosphorodichloridate (**2**) in dry tetrahydrofuran (THF) to the stirred solution of zidovudine (AZT) in THF and pyridine at -10 ºC in the presence of TEA over a period of 15 min. The reaction mixture was further stirred at 0ºC and continued stirring for 7 h. After completion of the reaction, it was filtered to remove triethylamine hydrochloride. The filtrate containing the intermediate (**3a-c**) was used for next step. In the next step, mono chloride (**3a-c**) and TEA were added at -40 °C with stirring to a solution of various nucleosides in pyridine and slowly raised to 40-50 °C and stirred for 38 h to get title compounds (**4a-c**). The Structures of synthesized dinucleotide analogues are presented in **Figure 1**.

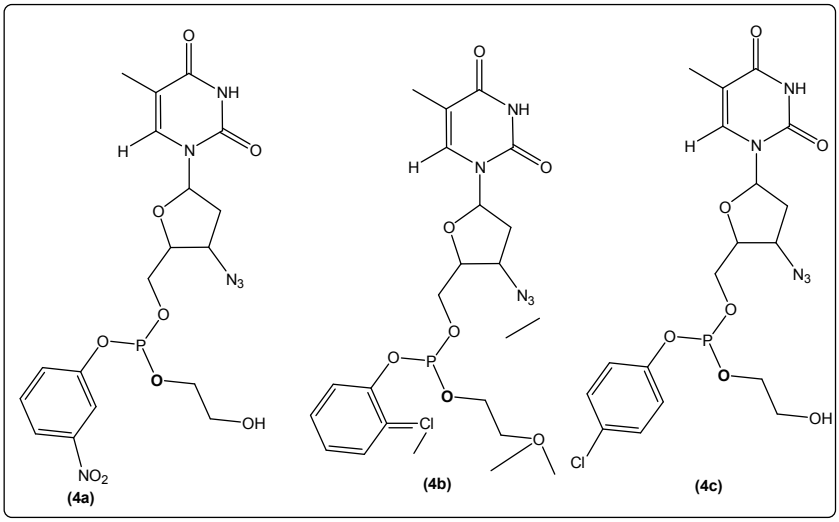
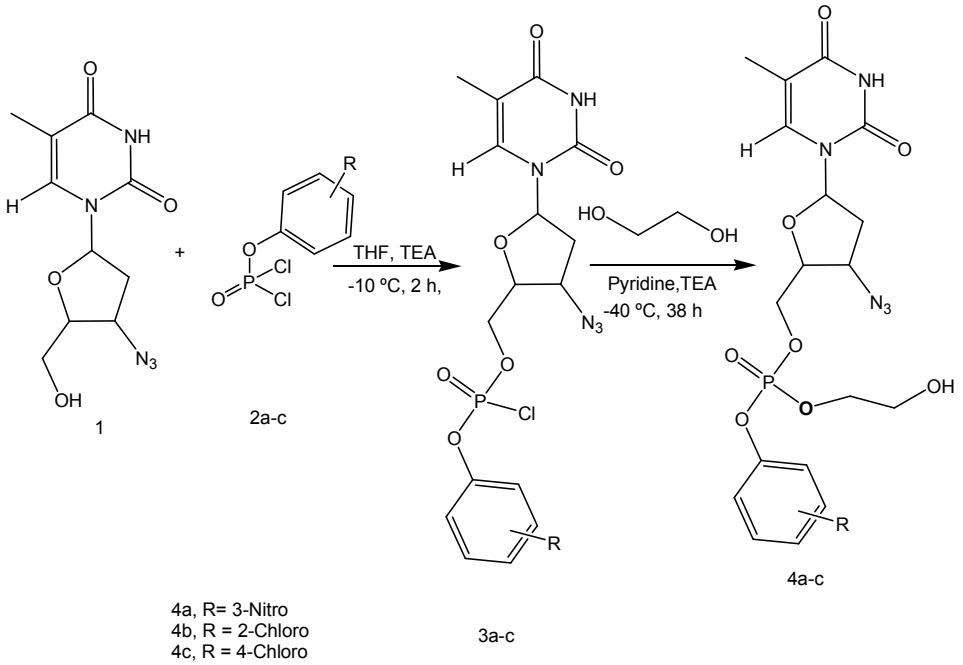


Figure 1: Structures of phosphorylated derivatives of AZT.

The chemical structures of all the title compounds **4a-c** were characterized by IR, ^{1}H, ^{13}C, ^{31}P NMR and HRMS studies and their data are presented in the experimental section. Characteristic IR stretching absorptions were observed in the regions 1212–1231 cm^{-1}, 3375–3385 cm^{-1} and 3412–3416 cm^{-1} for P=O (Deval et al., 2002), N–H (Ray et al.,2002), O–H (Kiran et al., 2008) respectively. In the ^{1}H NMR spectra of title compounds, the chemical shifts of aromatic hydrogens of the phenyl ring appeared as doublets in the region δ 7.41–8.22 (Kiran et al., 2008) and -$CONH_2$ protons appeared as singlets in the region δ 7.55-7.59 (Chitra et al., 2010). The NH protons in thymine ring were observed as a singlet in the region δ 8.52-11.34. The protons in triazole ring were observed as a singlet in the region δ 8.52-9.92 (Zatorski et al., 1996; Moriyama et al., 2008). ^{13}C NMR chemical shifts for title compounds were observed in their expected regions. ^{31}P NMR signals were observed in the region δ 4.23 to 22.52 (Miao et al., 2002; Ahmadibeni et al., 2006).



Scheme 1: Synthetic route of targeted molecules

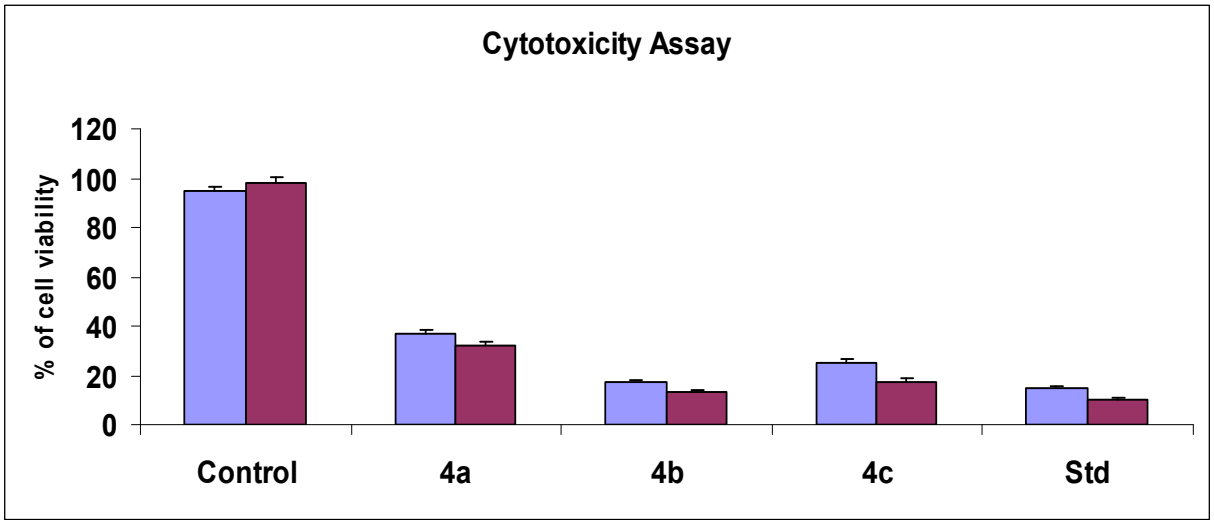


Fig.1. Cytotoxicity of test compounds against MCF-7 breast cancer cells by Trypan blue assay. Cytotoxicity of test compounds against MCF-7 cells by Trypan blue assay at 10 µg/mL (Blue color) and 100µg/mL (brick red color). C= Control (DMSO), S= Standard (Doxorubicin), values are expressed as mean ± SD for % cell viability. P<0.001.

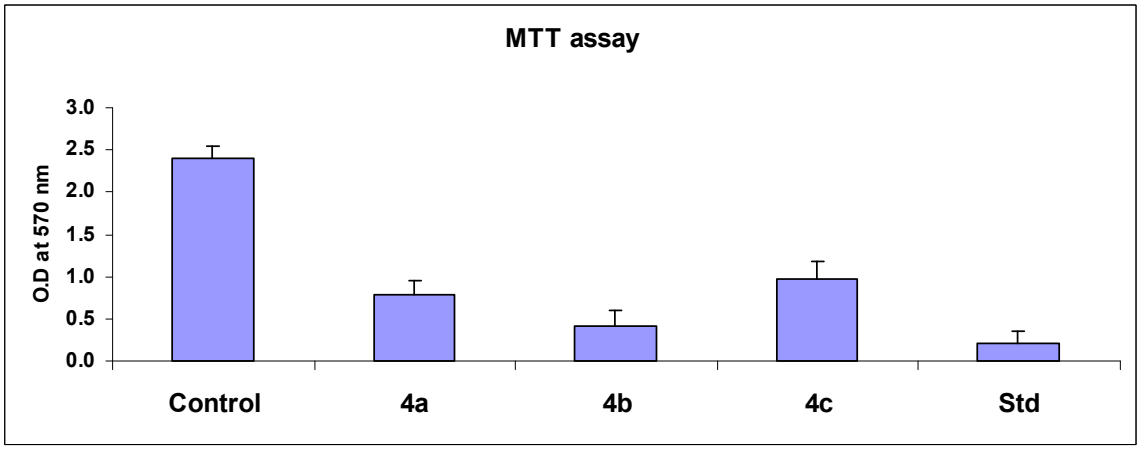


Fig.2. Anti prolifirative activity of test compounds against MCF7 human breast cancer cells by MTT assay. C (Control); Compounds (4a-c); Standard (Doxorubicin).

Antiprolifirative activity of newly synthesized derivatives of Zudovudine (4a-c) against human breast cancer cells (MCF7) was examined by Trypan blue and MTT assays. All the synthesized compounds were tested for their anticancer activity at 10 and 100 µg/Ml. Amongst the three compounds, 4b and 4a shown potential anticancer activity when compared to compound 4c, expected reason is compound 4a and b having amino group in that aromatic ring. The antiprolifirative activities were shown in Figures 1 and 2 compared with positive standards like Doxorubicin.These results pave the way for future design and development of adeno carcinoma breast cancer compounds and also more potent drugs for cancer. The compounds 4b and 4a could be considered as lead molecules which are to be screened for their efficacy in animal model.

MATERIALS AND METHODS

Chemicals purchased from Sigma-Aldrich, Merck and Lancaster, were used without further purification. All solvents used for spectroscopy and other physical studies were reagent grade and were further purified by literature methods. Melting points were determined using a calibrated thermometer by Guna Digital Melting Point apparatus. Infrared spectra (IR) were recorded as potassium bromide (KBr) discs on a Nicolet 380 FT-IR spectrophotometer. Absorptions are reported in wave numbers (cm-1). ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded in DMSO-d_6 on a Bruker AMX 400 MHz spectrometer operating at 400 MHz for ^{1}H, 100 MHz for ^{13}C and 161.9 MHz for ^{31}P NMR. The ^{1}H and ^{13}C chemical shits were expressed in parts per million (ppm) with reference to tetramethylsilane (TMS) and ^{31}P chemical shifts to 85 % H_3PO_4. HRMS, GCMS, LCMS, APCI mass spectra were recorded on a Jeol SX 102 DA/600 Mass spectrometer. Elemental analyses were performed by Central Drug Research Institute, Lucknow, India. The following abbreviations were used while presenting the NMR data s = singlet, d = doublet, t = triplet and m = multiplet.

SYNTHESIS

General procedure

To the stirred solution of substituted-phenyl phosphorodichloridate (137.8 mg, 1.00 mmol) in dry tetrahydrofuran (20 mL) and pyridine (20 mL), was added a solution of zidovudine (228.8 mg, 1.00 mmol) in THF (20 mL) at -10 ºC in the presence of TEA (101.2 mg, 1.00 mmol) over a period of 15 min. The reaction mixture was further stirred at 0 ºC and continued stirring for 7 h, progress of the reaction was monitored by TLC, (ethylacetate: hexane 1:1). After completion of the reaction, it was filtered to remove triethylamine hydrochloride. The filtrate containing the intermediates **3a-c** was used for next step. To a solution of ethylene glycol in pyridine (20 mL), and TEA were added at -10 °C with stirring and slowly raised to 40-50 °C and stirred for 38 h to get title compounds. After completion of the reaction, triethylamine hydrochloride was removed by filtration and the solvent was removed in a rota-evaporator to obtain crude product. It was purified by column chromatography on silica gel using acetone: methanol (9:1) as eluent to afford the title compounds **4a-c**. The synthetic protocol for all the compounds **4a-c** is depicted in **Scheme 1** and their spectral data are given below.

(3-Azido-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-2-yl)methyl (2-hydroxyethyl) (3-nitrophenyl) phosphite (4a)

Yield: 62 %; IR (KBr): 3393 (-NH), 1683 (C=O) cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.31 (s, 1H), 7.51-7.54 (m, 2H), 7.31-7.37 (m, 2H), 6.05 (d, 1H, J = 7.6 Hz), 4.46-4.48 (m, 3H), 4.21 (s, 1H), 3.94-4.05 (m, 4H), 3.76-3.80 (m, 4H), 2.40 (s, 3H). ^{13}C NMR (400 MHz, DMSO-d_6): δ 165.7, 163.4, 156.2, 148.5, 143.6, 135.1, 132.1, 110.8, 95.8, 85.1, 73.5, 67.5,

64.2, 59.2, 38.7, 13.1. ^{31}P (161.9 MHz, DMSO-d_6): δ 21.26. HRMS: MF: $C_{18}H_{21}N_6O_9P$: $[MH]^+$: found 496.1108, calcd 496.1114.

(3-Azido-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-2-yl)methyl (2-chlorophenyl) (2-hydroxyethyl) phosphite (4b).

Yield: 67 %; IR (KBr): 3386 (-NH), 1685 (C=O) cm^{-1}; ^{1}H-NMR (500 MHz, MeOD): δ 10.14 (s, 1H), 7.32 (d, 2H, J = 9 Hz), 6.80-6.85 (m, 2H), 6.32-6.34 (m, 1H), 5.89-5.92 (m, 2H), 4.45-4.47(m, 1H), 4.05-3.95 (m, 5H), 3.78-3.86 (m, 4H), 2.40 (s, 3H).^{13}C NMR (400 MHz, DMSO-d_6): δ (ppm) 164.2, 156.5, 152.8, 141.5, 138.6, 133.4, 126.4, 126.3, 121.9, 111.2, 98.6, 94.7, 88.2, 86.6, 71.2, 66.5, 59.1, 38.4, 13.8. ^{31}P (161.9 MHz, DMSO-d_6): δ 7.52. HRMS: MF: $C_{18}H_{21}ClN_5O_7P$ $[MH]^+$: found 485.0867, calcd : 485.0872.

(3-Azido-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-2-yl)methyl (4-chlorophenyl) (2-hydroxyethyl) phosphite (4c).

Yield: 70 %; IR (KBr): 3350-3420 (-OH, -NH_2), 1685 (C=O), 1231(P=O) cm^{-1}; ^{1}H-NMR (400 MHz, MeOD): δ 9.92 (s, 1H), 6.8-6.82 (m, 2H), 6.53-6.56(m, 2H), 4.61-4.63(m, 3H), 4.46-4.48(m, 4H), 4.34-4.37(m, 2H), 3.78-3.86 (m, 4H), 2.40 (s, 3H).. ^{13}C NMR (500 MHz, DMSO-d_6): δ (ppm) 163.4, 156.2, 152.1, 148.5, 143.6, 141.5, 135.1, 126.6, 121.4, 111.9, 101.4, 95.8, 73.5, 72.2, 64.2, 59.2, 38.7, 13.1. ^{31}P (161.9 MHz, DMSO-d_6): δ 11.28. HRMS: MF: $C_{18}H_{21}ClN_5O_7P$: $[MH]^+$: found 485.0867, calcd: 485.0874.

Pharmacology

Cell lines and culturing: The MCF-7 human breast adenocarcinoma cells were routinely maintained as monolayer in DMEM medium supplemented with 2 mM/L glutamine (Himedia), 10% fetal bovine serum (FBS, Himedia) and 10 µg/mL ciprofloxacin in humidified incubator (Binder) containing 5% CO_2, 95% air at 37 ºC.

Trypan blue assay : The MCF-7 cells were seeded at 2 x 10^5 cells per well in 6-well tissue culture plates in DMEM media supplemented with 10% FBS, incubated for 24 h at 37 º C in CO_2 incubator for cell viability assay by Trypan blue dye exclusion (Kumi-Diaka et al., 1999). Later the medium was replaced with fresh medium containing various concentrations of synthesized compounds and 0.1% of DMSO (served as solvent control), incubated for 24-48 h at 37 ºC. Doxorubicin, a potent inhibitor of MCF-7 cell lines was used as Standard control to evaluate the potency of the test compounds. To assess the cell viability after incubation aliquots from both floating and trypsinized adherent cells were mixed with a 0.4% trypan blue solution in 1:1 (v/v) and loaded on to a haemocytometer. The cells were observed and counted under light microscope, live cells are clear and the dead cells are blue in color. The concentration of the compound that inhibited cell growth by 50% (IC_{50}) was determined from cell survival plots. The obtained data were presented as mean ± SE (n=3) percentage of viable cell per sample was calculated as follows.

Viable cells (%) = [(Total cells- Dead cells) / Total cells] ×100%

MTT assay; Cell proliferation was assayed by MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay as described by Mosmann et al, (1983). The exponentially growing MDAMB-231 cells were collected and re suspended in fresh culture medium with 10% FBS. Before the treatment with synthesized compounds and the standard (Celecoxib), cells were washed with PBS and fresh medium was added and incubated in 96-well plates in the presence or absence of various concentrations of synthesized compounds for 24 h in a final volume of 100 lL. At the end of the treatment, 20 lL of MTT (5 mg/mL in PBS) was added to each well and incubated for an additional 4 h at 37 ————————————C. The purple-blue formazan precipitate was dissolved in 100 lL of DMSO and the optical density was measured at 570 nm on Bio-Rad, micro titer plate reader. An increase in number of living cells results in an increase in

the total metabolic activity in the sample. This increase directly correlates to the amount of purple formazan crystals formed. Data are expressed as mean ± SD, (n = 3)

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