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ORIGINAL RESEARCH

Phosphorylated ganciclovir derivatives: design, synthesis and in vitro and in vivo immunomodulatory activity

Kuruva Chandra Sekhar · Devineni Subba Rao · Kalla Chandra Mouli · Tartte Vijaya · Chamarthi Naga Raju

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Abstract Synthesis of a series of novel phosphorylated ganciclovir derivatives with bioactive amines/aminoacid esters as substituents was accomplished. These compounds were structurally characterized by IR, NMR (^{1}H, ^{13}C, ^{31}P), mass spectra and CHN analysis. The compounds (**5a–m**) have been evaluated for its in vitro and in vivo immunomodulatory activities. The amino acid ester-substituted ganciclovir derivatives, especially the **5g** and **5i**, increased the intracellular killing activity of the stimulated neutrophils. The in vivo experiment results show that the administration of compounds **5g** and **5i** (8 mg/kg body weight) to diet-induced immune impaired obese rats ameliorated the significant increase in immune cell counts (lymphocytes, neutrophils and monocytes). In addition, TNF-α, IL-6 and IL-1 secretions were considerably restored to normal by the compounds **5g** and **5i** with regulation in the release of C-reactive protein suggesting their potentiating effect on immune system dysfunction. These in vitro and in vivo results suggest that the phosphorylated ganciclovir derivatives **5g** and **5i** are strong immunomodulators.

Keywords Phosphorylated ganciclovir derivatives · In vivo immunomodulatory activity · In vitro nitroblue tetrazolium test · Immune cell counts · Immune modulating biofactors

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Introduction

Ganciclovir is a guanine containing acyclic nucleoside prodrug (Cytovene, RxList) that is activated by phosphorylation (Nichols and Boeckh, 2000). This undergoes triphosphorylation to become active, with the initial monophosphorylation catalyzed by UL97-encoded kinase and subsequently by cellular kinases (Raymund, 2011). Ganciclovir triphosphate inhibits viral deoxyribonucleic acid (DNA) synthesis through competitive incorporation during viral DNA synthesis, thereby leading to DNA chain termination (Karen, 2006). It has long been used as antiviral medication with the success in treating cytomegalovirus, human herpes virus infections, even it is also effective against pneumonia, encephalitis and gastrointestinal diseases (Meyers, 1991; Jeffery *et al.,* 2011).

Viral infections such as herpes, cytomegali and human immunodeficiency viruses have substantial impact on host immune responses (Chereshnev *et al*., 2013; Brennan, 2001; Lorenzo *et al.,* 2013). Following infection, viruses infiltrate the cell and produce immediate-early antigens that regulate DNA production. During the ensuing, the virus also produces late antigens that direct nucleocapsid protein production. It also causes up-regulation of (interleukin) IL-2, the master regulatory molecules of immune response and down regulation of major histocompatibility complex (MHC)-1 molecules on the surface of infected cells to evade host immune recognition (Brennan, 2001). The initial promise of antiviral agents has been offset by the development of antiviral resistance in specific populations, such as immune compromised hosts (McHutchison *et al*., 1998).

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In addition, for many viruses effective antivirals are not available. Augmenting the host's natural immune response to viruses by the supplementation of immune modulating molecules is, however, an alternative approach in the antiviral therapy (Dockrell and Kinghorn, 2001). Hence, it is imperative for the treatment of active viral diseases with a combination of immunomodulation and antiviral therapy (Blair and Emily, 2008).

The previous findings in our laboratory on biological evaluation of phosphorylated drugs displayed broad spectrum of potential pharmacological activities (Rao *et al.*, 2010; Rao *et al.*, 2011). Considering the above, the primary effort in the present study, therefore, is to synthesize the phosphorylated derivatives of ganciclovir and further to screen them for the in vitro and in vivo immune modulating potentials.

Materials and methods

Chemistry

Chemicals were procured from Sigma-Aldrich and Merck and used as such without further purification. All solvents used for spectroscopic and other physical studies were reagent grade and were further purified by standard methods (Armarego and Perrin, 1997). Melting points (mp) were determined using a calibrated thermometer by Guna Digital Melting Point apparatus and expressed in degree centigrade (°C) and are uncorrected. Infrared spectra (IR) were obtained on a Perkin-Elmer Model 281-B spectrophotometer. Samples were analyzed as potassium bromide (KBr) disks. Absorptions were reported in wave numbers (cm^{-1}). ^{1}H and ^{13}C NMR spectra were recorded as solutions 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 shifts were expressed in parts per million (ppm) with reference to tetramethylsilane (TMS) and ^{31}P chemical shifts to 85 % H_3PO_4. LCMS mass spectra were recorded on a Jeol SX 102 DA/600 mass spectrometer.

*Synthesis of 2-amino-9-[(2-[(2-aminoethyl)amino]methyl-2-oxo-1,3,2λ^5-dioxaphosphinan-5-yl)oxy]methyl-6,9-dihydro-1H-6-purinone (**5a**)*

2-Amino-9-((1,3-dihydroxypropan-2-yloxy)methyl)-1H-purin-6(9H)-one [ganciclovir (**1**)] (0.001 mol) was dissolved in the mixture of solvents tetrahydrofuran (THF) (10 mL) and pyridine (5 mL). To this stirred solution of Ganciclovir, chloromethylphosphonic dichloride (**2**) (0.001 mol) in 5 mL of THF was added dropwise over a period of 15 min at −10 °C in the presence of 1,1,3,3-tetramethylguanidine (TMG) (0.002 mol) as a base. After completion of the addition, the reaction mixture was stirred for 2 h at 0 °C to offord an intermediate, 2-amino-9-([2-(chloromethyl)-2-oxo-1,3,2λ^5-dioxaphosphinan-5-yl]oxymethyl)-6,9-dihydro-1*H*-6-purinone (**3**). The reaction progress was monitored by thin layer chromatography (TLC) using methanol:ethyl acetate (1:1). After completion of the reaction, it was filtered to remove TMG·HCl. Ethane-1,2-diamine (**4a**) (0.001 mol) in dry THF (10 mL) was added dropwise to the filtrate (**3**) at −10 °C and stirring was continued for 4 h at 50 °C. The progress of the reaction was monitored by TLC using methanol:ethyl acetate (1:1). After completion of the reaction, it was filtered to remove TMG·HCl, and the filtrate was concentrated in a rotaevaporator to obtain crude product. The residue was dissolved in chloroform (10 mL) and washed with 1 M hydrochloric acid solution (2 × 15 mL), saturated sodium bicarbonate (2 × 10 mL), and then water (3 × 15 mL). Then the organic phase was dried over $MgSO_4$ and distilled under vacuum, and the residue was purified by column chromatography on silica gel by eluting with 5 % methanol in chloroform to afford pure product of 2-amino-9-[(2-[(2-aminoethyl)amino]methyl-2-oxo-1,3,2λ^5-dioxaphosphinan-5-yl)oxy]methyl-6,9-dihydro-1*H*-6-purinone with high yield, 79 %.

The same experimental procedure was adopted for the preparation of the remaining title compounds **5b–l** (Scheme 1) using various amines (**4b–f**) and amino acid esters (**4g–l**).

Spectroscopic analysis

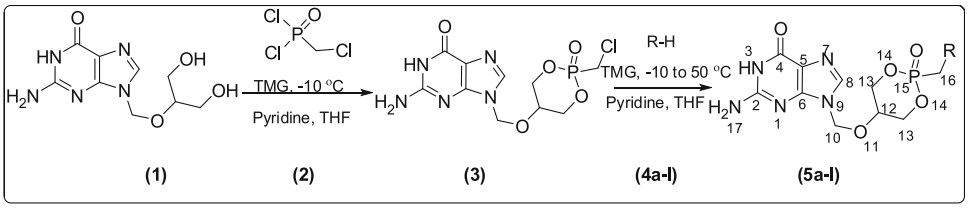
*2-Amino-9-[(2-[(2-aminoethyl)amino]methyl-2-oxo-1,3,2λ^5-dioxaphosphinan-5-yl)oxy]methyl-6,9-dihydro-1H-6-purinone (**5a**)*

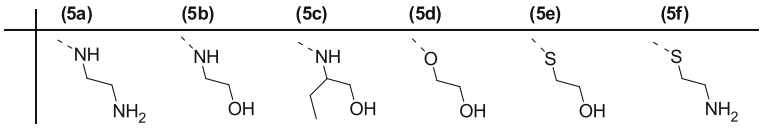
Yield 79 %; white solid, mp 235–237 °C; IR (KBr): $\bar{\nu}$ 3,196, 3,137, 3,097, 3,057, 1,250, 1,021, 1,025, 728 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.65 (br, s, 1H, H-3, NH), 7.79 (s, 1H, H-8), 6.50 (s, 2H, H-17, NH_2), 5.42 (s, 2H, H-10), 4.48–2.47 (14H, H-12 to H-16 and H-1′-4′); ^{13}C NMR (100 MHz, DMSO-d_6): δ 157.3 (C-4), 154.2 (C-2), 151.6 (C-6), 137.7 (C-8), 116.8 (C-5), 73.7 (C-13), 72.0 (C-12), 69.6 (C-10), 50.1 (C-2′), 43.4 (C-16), 40.5 (C-3′); ^{31}P NMR(161.9 MHz, DMSO-d_6): δ −10.9; LC MS (%): *m/z* 374.3 (65 %), 254.4 (100 %); Anal. Calcd. for $C_{12}H_{20}N_7O_5P$: C 38.61; H 5.40; N 26.26; Found: C 38.51; H 5.28; N 26.19.

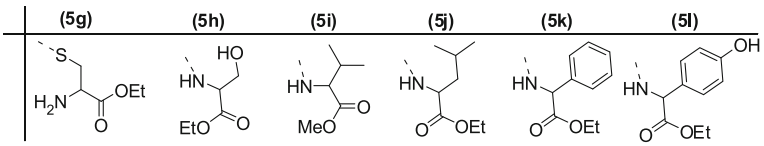
*2-Amino-9-[(2-[(2-hydroxyethyl)amino]methyl-2-oxo-1,3,2λ^5-dioxaphosphinan-5-yl)oxy]methyl-6,9-dihydro-1H-6-purinone (**5b**)*

Yield 75 %; white solid, mp 229–231 °C; IR (KBr): $\bar{\nu}$ 3,436, 3,155, 3,075, 3,023, 1,245, 1,018, 1,022, 771 cm^{-1};

Scheme 1 Synthesis of phosphorylated derivatives of ganciclovir







^{1}H NMR (400 MHz, DMSO-d_6): δ 10.63 (br, s, 1H, H-3, NH), 7.72 (s, 1H, H-8), 6.55 (s, 2H, H-17, NH_2), 5.49 (s, 2H, H-10), 4.45-2.91 (13H, H-12 to H-16 and H-1′-4′); ^{13}C NMR (100 MHz, DMSO-d_6): δ 157.4 (C-4), 154.6 (C-2), 151.2 (C-6), 137.5 (C-8), 116.8 (C-5), 73.9 (C-13), 72.6 (C-12), 69.4 (C-10), 59.4 (C-3′), 52.9 (C-2′), 43.9 (C-16); ^{31}P NMR(161.9 MHz, DMSO-d_6): δ −11.0; LC MS (%): *m/z* 375.1 (72 %), 254.4 (100 %); Anal. Calcd. for $C_{12}H_{19}N_6O_6P$: C 38.51; H 5.12; N 22.45; Found: C 38.42; H 5.04; N 22.36.

*2-Amino-9-([2-([1-(hydroxymethyl)propyl]aminomethyl)-2-oxo-1,3,2λ^5-dioxaphosphinan-5-yl]oxymethyl)-6,9-dihydro-1H-6-purinone (**5c**)*

Yield 68 %; white solid, mp 243–245 °C; IR (KBr): $\bar{\nu}$ 3,445, 3,148, 3,082, 3,049, 1,257, 1,016, 1,024, 742 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.63 (br, s, 1H, H-3, NH), 7.76 (s, 1H, H-8), 6.58 (s, 2H, H-17, NH_2), 5.49 (s, 2H, H-10), 4.49–1.02 (17H, H-12 to H-16 and H-1′-6′); ^{13}C NMR (100 MHz, DMSO-d_6): δ 157.4 (C-4), 154.9 (C-2), 151.7 (C-6), 137.5 (C-8), 116.2 (C-5), 73.7 (C-13), 72.4 (C-12), 69.8 (C-10), 60.7 (C-3′), 60.4 (C-2′), 42.3 (C-16), 24.3 (C-5′), 11.5 (C-6′); ^{31}P NMR(161.9 MHz, DMSO-d_6): δ −18.7; LC MS (%): *m/z* 403.7 (90 %), 254.4 (100 %); Anal. Calcd. for $C_{14}H_{23}N_6O_6P$: C 41.79; H 5.76; N 20.89; Found: C 41.71; H 5.64; N 20.77.

*2-Amino-9-[(2-[(2-hydroxyethoxy)methyl]-2-oxo-1,3,2λ^5-dioxaphosphinan-5-yloxy)methyl]-6,9-dihydro-1H-6-purinone (**5d**)*

Yield 82 %; white solid, mp 268–270 °C; IR (KBr): $\bar{\nu}$ 3,467, 3,104, 3,042, 1,256, 1,013, 1,019, 768 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.66 (br, s, 1H, H-3, NH), 7.72 (s, 1H, H-8), 6.54 (s, 2H, H-17, NH_2), 5.46 (s, 2H, H-10), 4.44–3.11 (12H, H-12 to H-16 and H-2′-4′); ^{13}C NMR (100 MHz, DMSO-d_6): δ 157.7 (C-4), 154.6 (C-2), 151.4 (C-6), 137.8 (C-8), 116.5 (C-5), 73.3 (C-13), 72.7 (C-12), 70.7 (C-2′), 69.1 (C-10), 63.9 (C-16), 62.4 (C-3′); ^{31}P NMR(161.9 MHz, DMSO-d_6): δ −14.4; LC MS (%): *m/z* 376.4 (68 %), 254.4 (100 %); Anal. Calcd. for $C_{12}H_{18}N_5O_7P$: C 38.41; H 4.83; N 18.66; Found: C 38.32; H 4.73; N 18.57.

*2-Amino-9-[(2-[(2-hydroxyethyl)sulfanyl]methyl-2-oxo-1,3,2λ^5-dioxaphosphinan-5-yl)oxy]methyl-6,9-dihydro-1H-6-purinone (**5e**)*

Yield 80 %; white solid, mp 246-248 °C; IR (KBr): $\bar{\nu}$ 3,420, 3,091, 3,045, 1,248, 1,014, 1,028, 752 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.62 (br, s, 1H, H-3, NH), 7.74 (s, 1H, H-8), 6.54 (s, 2H, H-17, NH_2), 5.44 (s, 2H, H-10), 4.49–2.39 (12H, H-12 to H-16 and H-2′-4′); ^{13}C NMR (100 MHz, DMSO-d_6): δ 157.5 (C-4), 154.3 (C-2), 151.7 (C-6), 137.4 (C-8), 116.9 (C-5), 73.2 (C-13), 72.9 (C-12), 69.7 (C-10), 57.6 (C-3′), 38.6 (C-2′), 32.9 (C-16); ^{31}P NMR(161.9 MHz, DMSO-d_6): δ −13.6; LC MS (%): *m/z* 392.3 (82 %), 254.4 (100 %); Anal. Calcd. for $C_{12}H_{18}N_5O_6PS$:C 36.83; H 4.64; N 17.90; Found: C 36.71; H 4.50; N 17.74.

*2-Amino-9-[(2-[(2-aminoethyl)sulfanyl]methyl-2-oxo-1,3,2λ^5-dioxaphosphinan-5-yl)oxy]methyl-6,9-dihydro-1H-6-purinone (**5f**)*

Yield 73 %; white solid, mp 251–253 °C; IR (KBr): $\bar{\nu}$ 3,218, 3,063, 3,027, 1,232, 1,017, 1,021, 772 cm^{-1}; ^{1}H NMR

(400 MHz, DMSO-d_6): δ 10.61 (br, s, 1H, H-3, NH), 7.70 (s, 1H, H-8), 6.57 (s, 2H, H-17, NH_2), 5.49 (s, 2H, H-10), 4.43–2.32 (13H, H-12 to H-16 and H-2′-4′); ^{13}C NMR (100 MHz, DMSO-d_6): δ 157.2 (C-4), 154.7 (C-2), 151.4 (C-6), 137.5 (C-8), 116.7 (C-5), 73.4 (C-13), 72.3 (C-12), 69.7 (C-10), 41.4 (C-3′), 39.2 (C-2′), 32.4 (C-16); ^{31}P NMR(161.9 MHz, DMSO-d_6): δ −15.7; LC MS (%): *m/z* 391.5 (75 %), 254.4 (100 %); Anal. Calcd. for $C_{12}H_{19}N_6O_5PS$: C 36.92; H 4.91; N 21.53; Found:C 36.84; H 4.86; N 21.40.

*Ethyl 2-amino-3-[(5-[(2-amino-6-oxo-6,9-dihydro-1H-9-purinyl) me thoxy]-2-oxo-1,3,2λ^5-dioxabphosphinan-2-ylmethyl) sulfanyl] propanoate (**5g**)*

Yield 65 %; white solid, mp 296–298 °C; IR (KBr): $\bar{v}$ 3,162, 3,106, 3,068, 1,245, 1,023, 1,029, 765 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.32 (br, s, 1H, H-3, NH), 8.66 (s, 2H, H-8′, NH_2), 7.68 (s, 1H, H-8), 6.43 (s, 2H, H-17, NH_2), 5.49 (s, 2H, H-10), 4.48-1.32 (15H, H-12 to H-16 & H-2′-7′); ^{13}C NMR (100 MHz, DMSO-d_6): δ 168.3 (C-4′), 157.5 (C-4), 154.4 (C-2), 151.9 (C-6), 137.2 (C-8), 116.1 (C-5), 73.6 (C-13), 72.3 (C-12), 69.7 (C-10), 62.1 (C-6′), 54.4 (C-3′), 36.5 (C-2′), 31.9 (C-16), 15.8 (C-7′); ^{31}P NMR(161.9 MHz, DMSO-d_6): δ −23.3; LC MS (%): *m/z* 463.6 (88 %), 254.4 (100 %); Anal. Calcd. for $C_{15}H_{23}N_6O_7PS$: C 38.96; H 5.01; N 18.17; Found: C 38.87; H 4.93; N 18.11.

*Ethyl 2-[(5-[(2-amino-6-oxo-6,9-dihydro-1H-9-purinyl)methoxy]-2-oxo-1,3,2λ^5-dioxaphosphinan-2-ylmethyl)amino]-3-hydroxypropanoate (**5h**)*

Yield 70 %; white solid, mp 232–234 °C; IR (KBr): $\bar{v}$ 3,445, 3,155, 3,092, 3,076, 1,252, 1,028, 1,021, 772 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.37 (br, s, 1H, H-3, NH), 7.62 (s, 1H, H-8), 6.41 (s, 2H, H-17, NH_2), 5.47 (s, 2H, H-10), 4.48–1.35 (17H, H-12 to H-16 and H-1′-8′); ^{13}C NMR (100 MHz, DMSO-d_6): δ 166.7 (C-3′), 157.2 (C-4), 154.8 (C-2), 151.4 (C-6), 137.4 (C-8), 116.4 (C-5), 74.5 (C-2′), 73.8 (C-13), 72.1 (C-12), 69.9 (C-10), 61.1 (C-5′), 60.9 (C-7′), 41.8 (C-16), 15.4 (C-6′); ^{31}P NMR(161.9 MHz, DMSO-d_6): δ −20.6; LC MS (%): *m/z* 447.13 (79 %), 254.4 (100 %); Anal. Calcd. for $C_{15}H_{23}N_6O_8P$: C 40.36; H 5.19; N 18.83; Found: C 40.28; H 5.13; N 18.77.

*Methyl 2-[(5-[(2-amino-6-oxo-6,9-dihydro-1H-9-purinyl)methoxy]-2-oxo-1,3,2λ^5-dioxaphosphinan-2-ylmethyl)amino]-3-methylbutanoate (**5i**)*

Yield 63 %; white solid, mp 264–267 °C; IR (KBr): $\bar{v}$ 3,144, 3,118, 3,063, 1,248, 1,036, 1,018, 775 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.42 (br, s, 1H, H-3, NH), 7.66 (s, 1H, H-8), 6.45 (s, 2H, H-17, NH_2), 5.51 (s, 2H, H-10), 4.52-1.02 (19H, H-12 to H-16 and H-1′-7′); ^{13}C NMR (100 MHz, DMSO-d_6): δ 170.7 (C-3′), 157.5 (C-4), 154.2 (C-2), 151.7 (C-6), 137.4 (C-8), 116.7 (C-5), 73.7 (C-13), 72.9 (C-12), 69.1 (C-10), 62.4 (C-2′), 51.3 (C-5′), 41.4 (C-16), 33.4 (C-6′), 20.2 (C-7′); ^{31}P NMR(161.9 MHz, DMSO-d_6): δ -19.8; LC MS (%): *m/z* 445.2 (73 %), 254.4 (100 %); Anal. Calcd. for $C_{16}H_{25}N_6O_7P$: C 43.24; H 5.67; N 18.91; Found: C 43.20; H 5.59; N 18.82.

*Ethyl 2-[(5-[(2-amino-6-oxo-6,9-dihydro-1H-9-purinyl)methoxy]-2-oxo-1,3,2λ^5-dioxaphosphinan-2-ylmethyl)amino]-4-methylpentanoate (**5j**)*

Yield 58 %; white solid, mp 275–277 °C; IR (KBr): $\bar{v}$ 3,134, 3,112, 3,065, 1,253, 1,027, 1,023, 761 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.49 (br, s, 1H, H-3, NH), 7.63 (s, 1H, H-8), 6.44 (s, 2H, H-17, NH_2), 5.53 (s, 2H, H-10), 4.52–1.02 (23H, H-12 to H-16 and H-1′-9′); ^{13}C NMR (100 MHz, DMSO-d_6): δ 165.2 (C-3′), 157.3 (C-4), 154.8 (C-2), 151.3 (C-6), 137.8 (C-8), 116.1 (C-5), 73.4 (C-13), 72.8 (C-12), 69.7 (C-10), 58.3 (C-5′), 59.8 (C-2′), 39.4 (C-7′), 24.4 (C-8′), 23.2 (C-9′), 41.1 (C-16), 15.7 (C-6′); ^{31}P NMR(161.9 MHz, DMSO-d_6): δ −18.9; LC MS (%): *m/z* 473.4 (58 %), 254.4 (100 %); Anal. Calcd. for $C_{18}H_{29}N_6O_7P$: C 45.76; H 6.19; N 17.79; Found: C 45.67; H 6.11; N 17.72.

*Ethyl 2-[(5-[(2-amino-6-oxo-6,9-dihydro-1H-9-purinyl)methoxy]-2-oxo-1,3,2λ^5-dioxaphosphinan-2-ylmethyl)amino]-2-phenylacetate (**5k**)*

Yield 67 %; white solid, mp 241–243 °C; IR (KBr): $\bar{v}$ 3,157, 3,125, 3,078, 1,246, 1,049, 1,023, 757 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.62 (br, s, 1H, H-3, NH), 7.70 (s, 1H, H-8), 7.05-7.21 (m, 5H, Ar'-H), 6.48 (s, 2H, H-17, NH_2), 5.50 (s, 2H, H-10), 4.84 (s, NH, H-1′), 4.52–1.31 (13H, H-12 to H-16 and H-1′-6′); ^{13}C NMR (100 MHz, DMSO-d_6): δ 168.7 (C-3′), 157.2 (C-4), 154.9 (C-2), 151.3 (C-6), 137.2 (C-8), 132.2 (C-Ph), 127.4 (C-Ph), 126.9 (C-Ph), 125.4 (C-Ph), 116.6 (C-5), 73.1 (C-13), 72.8 (C-12), 69.7 (C-10), 64.8 (C-2′), 59.2 (C-5′), 40.4 (C-16), 15.5 (C-6′); ^{31}P NMR(161.9 MHz, DMSO-d_6): δ −24.7; LC MS (%): *m/z* 493.5 (88 %), 254.4 (100 %); Anal. Calcd. for $C_{20}H_{25}N_6O_7P$: C 48.78; H 5.12; N 17.07; Found: C 48.70; H 5.05; N 17.00.

*Ethyl 2-[(5-[(2-amino-6-oxo-6,9-dihydro-1H-9-purinyl) methoxy]-2-oxo-1,3, 2λ^5dioxaphosphinan-2-ylmethyl) amino] 2-(4-hydroxy phenyl) acetate (**5l**)*

Yield 72 %; white solid, mp 282–284 °C; IR (KBr): $\bar{v}$ 3,585, 3,168, 3,115, 3,063, 1,283, 1,037, 1,026, 745 cm^{-1}; NMR (400 MHz, DMSO-d_6): δ 10.37 (br, s, 1H, H-3, NH), 7.74 (s, 1H, H-8), 6.82-7.08 (m, 4H, Ar'-H), 6.58 ^{1}H (s, 2H,

H-17, NH_2), 5.62 (s, 1H, Ph-OH), 5.45 (s, 2H, H-10), 4.65-1.38 (14H, H-12 to H-16 and H-1′-3′); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.2 (C-3′), 157.4 (C-4), 156.4 (C-Ph-OH), 154.6 (C-2), 151.2 (C-6), 137.5 (C-8), 127.5 (C-Ph), 128.1 (C-Ph), 115.2 (C-Ph), 116.8 (C-5), 73.9 (C-13), 72.6 (C-12), 69.4 (C-10), 64.2 (C-2′), 59.7 (C-5′), 40.4 (C-16), 15.1 (C-6′); ^{31}P NMR(161.9 MHz, DMSO-d_6): δ -21.4; LC MS (%): *m/z* 509.9 (76 %), 254.4 (100 %); Anal. Calcd. for $C_{20}H_{25}N_6O_8P$: C 47.25; H 4.96; N 16.53; Found: C 47.15; H 4.84; N 16.44.

In vitro immunomodulatory activity (Nitroblue tetrazolium test)

Neutrophils were isolated from venous blood of healthy volunteers. The heparinized blood (5 mL containing 100 units of heparin) was added to 1 mL of 4.5 % dextran B in physiological saline. The mixture was gently shaken and allowed to stand for 60 min at room temperature to sediment erythrocytes. Neutrophils were isolated by Ficoll-Hypaque density gradient centrifugation according to Ferrante and Thong (Ferrante and Thong, 1980). After the removal of the residual erythrocytes by hypotonic lysis, the neutrophils were washed with Hank's balanced salt (HBS) solution. The cells were suspended at a final concentration of 5×10^6 neutrophils/mL in HBS solution for NBT reduction test (Basaran *et al.*, 1997). The viability of neutrophils was tested by trypan blue exclusion and was greater than 90 %. A suspension of neutrophils (5×10^6/mL) was prepared in 0.5 mL of phosphate buffered saline (PBS) solution in different tubes; 0.1 mL of PBS solution (control) and 0.1 mL of endotoxin-activated plasma (standard) were added to 1st tube and 2nd tubes, respectively, 0.1 mL of different concentrations (10, 20, 40, 100 and 1,000 μg/mL) of the test samples (**5a**–**m**) was added to the other tubes. 0.2 mL of freshly prepared 0.15 % nitroblue tetrazolium (NBT) solution was added to each tube and incubated at 37 °C for 20 min. The tubes were centrifuged at 400×*g* for 3–4 min to discard the supernatant. The cells were resuspended in a small volume of PBS solution. A thin film was made with the drop on the slide, dried, fixed by heating, counter stained with dilute carbol fuchsin for 15 s. The slide was washed under tap water, dried and focused under 100× oil immersion objectives. Two hundred neutrophils were counted for the % of NBT positive cells containing blue granules/lumps (Wilkinson, 1981).

In vivo immunomodulatory activity (high-fat diet-induced obese rats with altered immunity)

Experimental design

Male Wistar strain Albino rats weighing 110–130 g were purchased from Sri Venkateswara Animal Agency at Bangalore, India. Animals were housed at five rats per cage in an air-conditioned room at 23 ± 1 °C, 55–60 % relative humidity, and a 12 h light/dark cycle (07:00 lights on, 19:00 lights off), and were given a laboratory regular rodent diet for 1 week. The high-fat diet (HFD) with energy density 466.78 kcal/100 g purchased primarily from National Institute of Nutrition (NIN), Hyderabad, India, was used to induce obesity with altered immunity. The caloric density of the normal diet (ND) was 292 kcal/100 g and obtained from Hindustan Lever Limited, Bombay. All of animal experiments were carried out according to the guidelines of the Sri Venkateswara University's Institutional Animal Care and Use Committee (No./02(i)/a/CPCSCA/IAEC/SVU/TV). For treatments with compounds **5a**–**m**, after acclimatization for 1 week, rats were randomly divided into fifteen groups ($n = 5$ per group), and respectively fed a normal diet (ND) + vehicle, a high-fat diet (HFD) + vehicle, HFD + **5m** (ganciclovir): 8 mg/kg bodyweight (b.w), HFD + **5a**: 8 mg/kg b.w, HFD + **5b**: 8 mg/kg b.w, HFD + **5c**: 8 mg/kg b.w, HFD + **5d**: 8 mg/kg b.w, HFD + **5e**: 8 mg/kg b.w, HFD + **5f**: 8 mg/kg b.w, HFD + **5g**: 8 mg/kg b.w, HFD + **5h**: 8 mg/kg b.w, HFD + **5i**: 8 mg/kg b.w, HFD + **5j**: 8 mg/kg b.w, HFD + **5k**: 8 mg/kg b.w and HFD + **5l**: 8 mg/kg b.w. The doses of compounds were determined by preliminary tests using concentrations of 50, 100, and 200 mg/kg as per Organization for Economic Co-operation and Development (OECD)-423 guidelines. The lethal dose (LD50) was determined as 80 mg/kg b.w. Hence, the experimental dose (1/10) 8 mg/kg b.w was selected for the immunomodulatory effect. Compounds were dissolved in distilled (2 mg/mL) water for oral administration at the desired doses in a volume of 5 mL/kg daily at 8:00 h. All groups were treated for 8 weeks. At the end of the treatment period, blood was collected through retro-orbital bleeding under chloroform anesthesia. The serum samples were prepared by centrifugation of the collected blood samples (2,500 rpm for 15 min), then stored at −80 °C for biochemical determinations. Total counts of immune cells viz. lymphocytes, neutrophils, monocytes were determined using hematology analyser. Levels of cytokines such as TNF-α, IL-6, IL-1 and C-reactive protein (CRP) in serum samples were determined by specific enzyme-linked immunosorbent assay (ELISA) techniques according to the manufacturer's instructions (Genex Bio, New Delhi, India).

The concentration of cytokines was determined spectrophotometrically at 450 nm. A standard curve using cytokines standards was also constructed. The cytokine concentrations for unknown samples were calculated according to standard curve.

Statistical analysis

All data are presented as mean ± S.D. Differences between groups were analyzed using the one-way analysis

of variance (ANOVA) with Dunnett's *t* test. A value of $P < 0.01$ was considered statistically in significant using GraphPad InStat 3 statistical analytical software.

Results and discussion

Chemistry

Chloromethylphosphonic dichloride (**2**) in THF/Pyridine was reacted at −10 °C with ganciclovir (**1** or **5m**) in the presence of 1,1,3,3-tetramethylguanidine (TMG) as a base. After completion of the addition, the reaction mixture was stirred for 2 h at 0 °C to obtain an intermediate, 2-amino-9-([2-(chloromethyl)-2-oxo-1, 3, $2\lambda^5$-dioxa phosphinan-5-yl]oxymethyl)-6,9-dihydro-1*H*-6-purinone (**3**). It was filtered to remove TMG.HCl. Various amines (**4b–f**) and amino acid esters (**4g–l**) were reacted with (**3**) at −10 to 0 °C and further stirring was continued for 4–7 h by raising the temperature to 35–50 °C. The progress of the reaction was monitored by TLC using methanol:ethyl acetate (1:1) as eluent. After completion of the reaction, it was filtered to remove TMG·HCl, and the filtrate was concentrated in a rota-evaporator to get crude product. It was dissolved in chloroform (10 mL) and washed with 1 M hydrochloric acid (2 × 15 mL), saturated sodium bicarbonate solution (2 × 10 mL), and then water (3 × 15 mL). Then the organic phase was dried ($MgSO_4$) and distilled under vacuum, and the residue was purified by column chromatography on silica gel eluting with 5 % methanol:chloroform to obtain the title compounds (**5a–l**).

The resulted compounds were characterized by their spectral analysis. The IR spectra of **5a–l** showed the expected absorption bands at 3,218–3,027 and 1,283–1,232 cm^{-1} for the NH and P=O stretching vibrations, respectively (Thomas, 1974). Aliphatic OH bands were observed in between 3,445 and 3,425 for the compounds **5b**, **5c**, **5d**, **5e**, **5h** and **5l**. ^1H NMR signals and proton counting of the title compounds were observed in their expected region. All ^{13}C signals of phosphorylated ganciclovir derivatives were observed in their expected regions (Prasadaraju *et al.*, 2009). ^{31}P NMR signals appeared in the range of −24.7 to −10.9 ppm as expected for the P=O group of the title compounds.

In vitro immunomodulatory activity

To investigate the potential immunomodulating activity of the synthesized compounds, the phosphorylated derivatives of ganciclovir were subjected to in vitro nitroblue tetrazolium (NBT) test and in vivo activity on high-fat diet-induced immune impaired obese rats.

The NBT dye reduction test gives information about the phagocytic and intracellular killing functions of neutrophils

Table 1 Effect of the phosphorylated ganciclovir derivatives on NBT-positive neutrophils (%)

Test sample	NBT-positive neutrophils (%)				
	10 μg/mL	20 μg/mL	40 μg/mL	100 μg/mL	1,000 μg/mL
NC	18.41 ± 0.03	19.36 ± 0.15	24.59 ± 0.04	25.42 ± 0.16	27.57 ± 0.08
PC	39.67 ± 0.17*	49.57 ± 0.25*	51.34 ± 0.36*	54.14 ± 0.48*	58.68 ± 0.11*
5m	39.43 ± 0.18	53.21 ± 0.3	55.89 ± 0.25	58.23 ± 0.17	60.11 ± 0.22
5a	40.12 ± 0.31	42.51 ± 0.11	45.35 ± 0.12	47.30 ± 0.11	50.46 ± 0.43
5b	33.38 ± 0.16	45.98 ± 0.53	48.54 ± 0.16	50.57 ± 0.18	55.25 ± 0.31
5c	31.37 ± 0.21	43.15 ± 0.27	46.76 ± 0.12	53.98 ± 0.15	54.32 ± 0.14
5d	34.31 ± 0.20	46.44 ± 0.31	49.85 ± 0.33	51.23 ± 0.17	53.02 ± 0.20
5e	32.25 ± 0.26	43.73 ± 0.11	47.93 ± 0.14	52.15 ± 0.28	55.35 ± 0.16
5f	33.77 ± 0.13	45.64 ± 0.32	49.32 ± 0.12	50.36 ± 0.11	57.91 ± 0.21
5g	45.03 ± 0.24#	56.34 ± 0.15#	58.04 ± 0.32#	60.16 ± 0.24#	66.82 ± 0.17#
5h	39.80 ± 0.06	39.92 ± 0.19	48.11 ± 0.50	53.09 ± 0.15	56.02 ± 0.24
5i	41.18 ± 0.17#	55.09 ± 0.26#	60.83 ± 0.22#	62.45 ± 0.15#	65.14 ± 0.46#
5j	39.17 ± 0.12	40.92 ± 0.11	49.31 ± 0.21	51.44 ± 0.23	58.03 ± 0.15
5k	40.62 ± 0.13	43.11 ± 0.23	46.63 ± 0.12	52.91 ± 0.27	60.32 ± 0.10#
5l	39.58 ± 0.21	42.46 ± 0.14	49.49 ± 0.13	52.31 ± 015	62.25 ± 0.08#

Values are mean ± SD. Statistical analysis was by ANOVA followed by Dunnett's *t* test

5m, ganciclovir; *PBS (NC)* phosphate buffer saline (normal control), *EAP (PC)* endotoxin-activated plasma (positive control)

* $P < 0.01$ as compared to normal control

# $P < 0.01$ as compared to endotoxin-activated plasma

which are necessary for normal microbicidal activity. The dye is taken into neutrophils by phagocytosis and then stimulation of the hexose monophosphate-shunt pathway (HMP) of glucose oxidation and concomitant changes in oxidative metabolism lead to the reduction of the dye (Akbay *et al.*, 2002).

The ganciclovir and all its synthesized phosphorylated derivatives at the concentrations of 10, 20, 40, 100 and 1,000 μg/mL increased the intracellular killing activity of stimulated neutrophils assayed by in vitro NBT reduction test. The results are shown in Table 1. Among them, compounds **5g** and **5i** showed significant ($P < 0.01$) activity as compared to the standard ganciclovir (**5m**) and endotoxin-activated plasma indicating their possible immunomodulatory effect. **5k** and **5l** also showed significant activity at high dose (1,000 μg/mL) compared with the positive control group, but did not show a significant effect with ganciclovir (**5m**). A number of derivatives of guanosine and analogs thereof have been studied over the years for their ability to stimulate the immune system in mouse and human models. The structural requirements for guanosine analogs to be immunomodulatory are such that the pattern of purine ring substitution is absolutely critical. The consequences of immune stimulation by guanosine analogs include both humoral and cellular immune responses (Jongdae *et al.,* 2003). Our results reported in the present study shed light on these events.

In vivo immunomodulatory activity

The immunomodulatory study of ganciclovir and its phosphorylated derivatives was also performed using the high-fat diet-induced immune impaired obese animal model. Obesity is associated with an impaired immune response (Alexia *et al.,* 2007). Diet-induced obesity is a more physiologically relevant model of human obesity (Ikejima *et al.,* 2005). The marked body weights of rats reflecting obesity by high-fat diet is considerable and is accompanied by increased immune cells viz., total lymphocytes, neutrophils, monocytes were evident (Sonia and Ranjit Chandra, 2001). Furthermore, several lines of evidence have supported a link between adipose tissue and immunocompetent cells. This interaction is illustrated in obesity, where excess adiposity and impaired immune

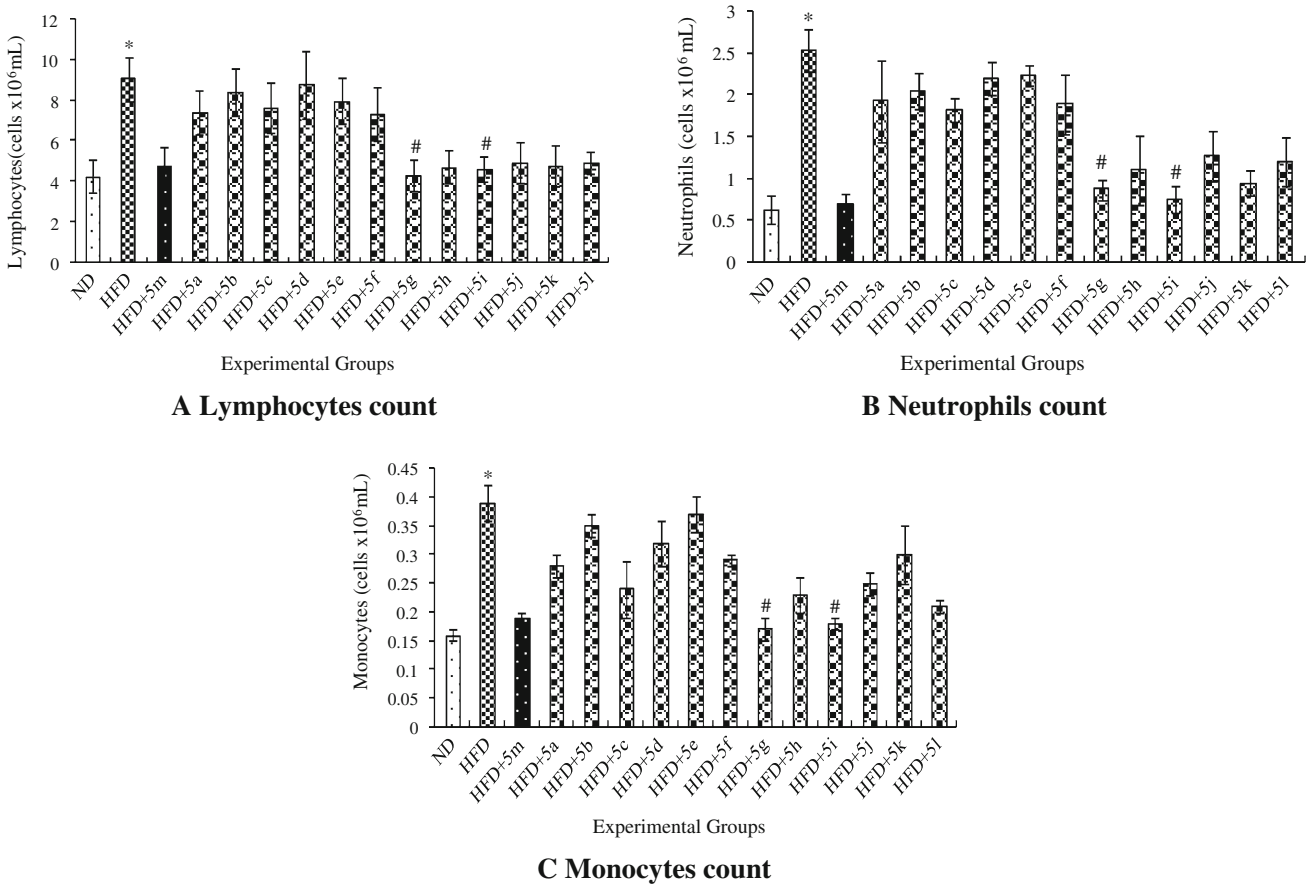


Fig. 1 The levels of total immune cell counts in impaired immune obese rats by the treatment of phosphorylated derivatives of ganciclovir. Values are mean ± SD; $^{*}P < 0.01$ as compared to normal diet-ND (control group), $^{\#}P < 0.01$ compared to high-fat diet-HFD (obese group)

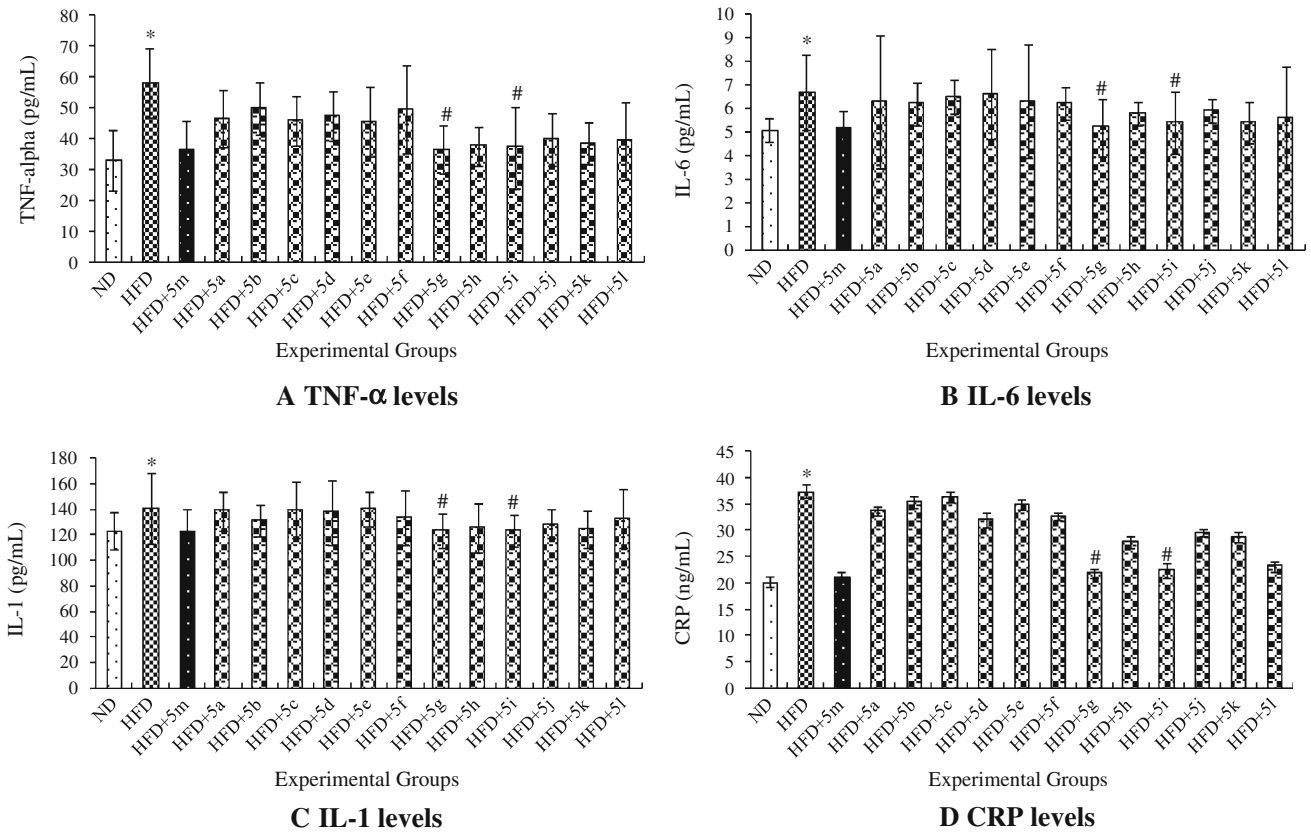


Fig. 2 The levels of biofactors in immune impaired obese rats upon the treatment with phosphorylated derivatives of ganciclovir. Values are mean ± SD; $^{*}P < 0.01$ as compared to normal diet-ND (control group), $^{\#}P < 0.01$ as compared to high-fat diet-HFD (obese group)

function have been described in both humans and genetically obese rodents (Marti *et al.,* 2001). The impact of obesity on immune cells has been evaluated with results suggesting that being overweight or obese is associated with higher immune cell levels (Womack *et al.,* 2007). In the present study, oral administration of compounds **5g** and **5i** (8 mg/kg b.w) significantly ameliorated the counts of total lymphocytes, neutrophils and monocytes (Fig. 1a–c) induced by high-fat diet in immune impaired rats when compared normal controls.

The development of high-fat diet-induced obesity is associated with an altered leptin production (Lin *et al.,* 2000). Leptin is mainly secreted by the adipose tissue and has a significant immune modulatory role (Laharrague *et al.*, 1998; Lord *et al.*, 1998), demonstrating that mouse lymphocytes express the leptin receptor, and that leptin modulates in these cells for increased cytokines (TNF-α, IL-6 and IL-1) production. Considering the aforementioned issues, it seemed plausible to assume that the increased lymphocyte count reported here with the high-fat diet fed immune impaired rats would affect the cytokine production.

In this study, we also investigated the effects of Ganciclovir and its synthetic derivatives on immune-related biofactors (cytokines) in obese rats. Data presented in Fig. 2a–c) show the effects of ganciclovir and its derivatives on TNF-α, IL-6 and IL-1 levels in the experimental animals. Cytokines (TNF-α, IL-6 and IL-1) secretion was not affected significantly by the compounds (**5a**–**5f**), while the compounds (**5g** and **5i**) exhibited significantly considerable levels (36.5 ± 7.5, 5.2 ± 1.2, 122.9 ± 13.5 and 37.1 ± 13.2, 5.4 ± 1.3, 123.6 ± 12.4 pg/mL respectively) when compared to the rats with dysfunctioned immune system.

Impaired immunity in high-fat diet-induced obese rats is associated with increased production of the cytokines including IL-6 (Santos-Alvarez *et al.,* 1999). Second, IL-6 induces up-regulation of CRP (Shamsuzzaman *et al.,* 2004). Thus, we prompted to investigate the possibility of the compounds **5a**–**m** which could be potent immunomodulators acting via CRP. In view of the above, in this article, we present evidence that upon the treatment with compounds **5g** and **5i** to immune impaired obese rats, the levels of CRP (21.9 ± 0.51 and 22.3 ± 1.37 ng/mL) were significantly restored to normal as compared to high-fat diet alone fed rats with immune dysfunction (Fig. 1d). However, CRP production was not affected significantly in

other immune functions altered rats with the phosphorylated derivatives of ganciclovir (**5a–5f**, **5h**, **5j–5l**) suggesting their immune modulating potential.

Conclusion

In conclusion, the data corroborate that the ganciclovir and its phosphorylated derivatives (GPDs) can alter the intracellular killing activity of the stimulated neutrophils indicating their involvement in immune response. In addition, the in vivo experiment indicated that the GPDs might be cytokine modulators that regulate the TNF-α, IL-6 and IL-1 secretion levels, further IL-6 production also induces up-regulation in CRP (biofactors for impaired immunity in obesity) levels. Furthermore, the GPDs seem to augment the immune cell proliferation, the useful indicator of an immune response to treatment. When compared with the title compounds, amino acid ester-substituted ganciclovir derivatives presented the potent pharmcological activities. These in vitro and in vivo results suggest that the GPDs might be strong immunomodulators. Investigations of synthetic compounds and their analogs or derivatives that can modulate the immune system are gaining attention in the research areas of immunopharmacology. Together, targeting the immune response with these GPDs is highly effective in mobilizing or restoring immunity, supporting the potential therapeutic use of this novel immunomodulators as adjuvants in the treatment of immune mediated and viral diseases.

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