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Original article

Phenyl phosphoramidate derivatives of stavudine as anti-HIV agents with potent and selective in-vitro antiviral activity against Adenovirus

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Abstract

Adenoviruses are responsible for a broad range of clinical diseases that may be associated with high mortality, including pneumonia, hepatitis, encephalitis, hemorrhagic cystitis, nephritis, and gastroenteritis in immunocompromised patients, including HIV-infected individuals. Here we report the identification of halo-substituted stavudine phenyl phosphoramidate derivatives as a new class of dual-function anti-HIV agents with potent and selective anti-adenovirus (ADV) activity. We examined the investigational stavudine phenyl phosphoramidate derivative stampidine and 12 structurally similar stavudine derivatives for anti-ADV activity. All 13 derivatives of stavudine, including stampidine, were substantially more potent than stavudine and inhibited ADV-induced plaque formation at nanomolar IC_{50} values. Compounds with halo substitutions in the phenyl ring as well as the unsubstituted compound **607** were more potent than compounds with methoxy, methyl, or cyano substitutions. Compound **113** (stampidine) with a 4-Br substitution and compound **609** with a 4-Cl substitution were identified as the most potent lead anti-ADV agents. Compound **113/**Stampidine inhibited ADV-induced plaque formation in skin fibroblasts in a concentration-dependent fashion with a mean (±S.E.M.) IC_{50} value of 17 ± 2 nM without any evidence of cytotoxicity even at 100 μM. Similarly, compound **609** inhibited ADV-induced plaque formation with an IC_{50} value of 27 ± 3 nM. We next sought to determine if the lead compounds **113** and **609** can also inhibit other viruses. Both compounds exhibited potent anti-HIV activity at nanomolar concentrations. However, neither compound exhibited any antiviral activity against non-HIV viruses, including Cytomegalovirus (CMV), Type I or Type II herpes simplex viruses (HSV-1, HSV-2), enterovirus ECHO 30, or respiratory syncytial virus (RSV) (IC_{50} > 100 μM). The remarkable anti-ADV potency of the lead compounds stampidine and compound **609** warrants the further development of these promising new antiviral agents for possible clinical use in ADV infected patients.

Keywords: HIV; Phosphoramidates; Stampidine; Adenovirus

1. Introduction

Adenovirus (Ad) infection results in significant morbidity and mortality in both immunocompetent and immunosuppressed hosts. Adenoviruses are responsible for a broad range of clinical diseases that may be associated with high mortality, including pneumonia, hepatitis, encephalitis, hemorrhagic cystitis, nephritis, and gastroenteritis in immunocompromised patients, including HIV-infected individuals [1–8]. Adenovirus colitis is a common cause of diarrhea in HIV-infected patients and it may facilitate enteric infection with Cytomegalovirus (CMV) [9]. Gastrointestinal adenovirus excretion occurs at an advanced stage of HIV disease, and is associated with a poor prognosis [10]. In a recent study, Sabin et al. [10] examined HIV-infected patients in a very advanced stage of disease and those presenting with diarrhea for whom the survival of HIV-infected patients with adenovirus-positive diarrhea was 1 year. And this remained significant after accounting for differences in CD4 counts between the groups, suggesting that adenoviruses may contribute to mortality in this population [10].

Several nucleoside analogs, including 2′,3′-dideoxynucleoside 5′-triphosphates and 3′-fluoro-2′-deoxy-

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thymidine (FTdR), have been discovered as having antiviral activity against adenovirus by inhibiting the adenovirus DNA polymerase-mediated DNA replication in adenovirus-infected cells [11–14]. Most nucleoside analogs exhibit broad-spectrum antiviral activity [15,16], for example, (*S*)-1-(3-Hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC, Cidofovir, Vistide), an acyclic nucleoside phosphonate with broad-spectrum activity against a wide variety of DNA viruses including herpesviruses [Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), varicella-zoster virus (VZV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), human herpesvirus type 6 (HHV-6) and equine and bovine herpesviruses], papovaviruses [human polyoma virus and human papilloma virus (HPV)], adeno-, irido-, hepadna-, and poxviruses [16] is currently being pursued in the topical and/or systemic (intravenous) treatment of various infections due to CMV, HSV, VZV, EBV, HPV, polyoma-, adeno- and poxviruses. However, none of the currently available anti-HIV agents were reported to have anti-ADV activity and anti-ADV agents such as ribavarin cannot be administered long-term in HIV-infected patients because of the associated side effects such as severe hematologic toxicity [16,17]. Therefore, there is an urgent need for selective anti-ADV agents with more favorable safety profiles than the available nucleoside analogs as well as dual-function anti-HIV agents with anti-ADV activity.

Stavudine (STV)/d4T is a pyrimidine nucleoside analogue used in the treatment of human immunodeficiency virus (HIV) infection. It inhibits viral reverse transcriptase as do zidovudine (ZDV)/AZT, didanosine (ddI), zalcitabine (ddC) and lamivudine (3TC), which comprise the family of NRTI (18). The 5′-triphosphates of these NRTI, which are generated intracellularly by the action of nucleoside and nucleotide kinases, are potent inhibitors of HIV-1 RT [18]. The rate-limiting step for the generation of the bioactive STV metabolite STV-triphosphate is the conversion of STV to its monophosphate derivative [18]. In an attempt to overcome the dependence of stavudine on intracellular nucleoside kinase activation, we prepared stampidine (STAMP)/HI-113, STV-5′-[*p*-bromophenyl methoxyalaninyl phosphate], a novel aryl phosphate derivative of stavudine [19]. The presence of a single para-bromine group in the phenyl moiety of stampidine contributes to its ability to undergo rapid hydrolysis yielding the key active metabolite alaninyl-STV-monophosphate (ala-STV-MP) [20]. STAMP has been shown to inhibit the replication of HIV-1 strain HTLV-IIIB, HIV-2, as well as the ZDV-resistant laboratory HIV-1 strain RT-MDR in human peripheral blood mononuclear cells at nanomolar concentrations [20]. In preliminary studies, we found that STAMP is substantially more potent than STV in inhibiting the replication of the laboratory HIV-1 strain HTLVIIIB in thymidine kinase-deficient T-cells [19–20]. STAMP was very well tolerated in BALB/c, CD-1 as well as CB17-SCID mice without any detectable acute or subacute toxicity at single intraperitoneal or oral bolus dose levels as high as 500 mg/kg [21–23]. Notably, daily administration of STAMP intraperitoneally or orally for up to eight consecutive weeks was not associated with any detectable toxicity at cumulative dose levels as high as 6.4 g/kg [21–23]. STAMP was 100-fold more potent than stavudine and twofold more potent than zidovudine against nine clinical HIV-1 isolates of non-B envelope subtype [21–23]. STAMP inhibited the in vitro replication of each one of 20 genotypically and phenotypically NRTI-resistant and 6 NNRTI-resistant HIV-1 isolates at subnanomolar to low nanomolar concentrations [21]. Orally administered STAMP exhibited significant and dose-dependent in vivo anti-HIV activity against the NRTI-resistant clinical HIV-1 isolate BR/92/019 in Hu-PBL-SCID mice [22,23]. A single 50 mg/kg or 100 mg/kg oral bolus dose of STAMP (a) yielded therapeutic concentrations of STAMP and its metabolite ala-STV-MP >4-logs higher than their respective IC_{50} values in 6 FIV-infected domestic cats without any adverse reactions, and (b) exhibited potent antiretroviral activity as evidenced by a ≥1-log decrease of the FIV load of circulating peripheral blood mononuclear cells [24]. A 4-week treatment course with STAMP administered in gelatin capsules twice daily showed a dose-dependent antiretroviral effect in chronically FIV-infected cats. The documented in vitro potency of STAMP against primary clinical HIV-1 isolates with genotypic and/or phenotypic NRTI- or NNRTI-resistance as well as non-B envelope subtype together with its in vivo antiretroviral activity in HIV-infected Hu-PBL SCID mice as well as FIV-infected cats prompted us to further develop this agent for clinical use.

The purpose of the present study was to examine stampidine and 12 structurally similar stavudine derivatives for anti-ADV activity. All 13 derivatives of stavudine, including stampidine, were substantially more potent than stavudine and inhibited ADV-induced plaque formation at nanomolar IC_{50} values. Compounds with halo substitutions in the phenyl ring as well as the unsubstituted compound **607** were more potent than compounds with methoxy, methyl, or cyano substitutions. Compound **113** (stampidine) with a 4-Br substitution and compound **609** with a 4-Cl substitution were identified as the most potent lead anti-ADV agents. Both compounds inhibited ADV-induced plaque formation in skin fibroblasts at nanomolar concentrations. We next sought to determine if the lead compounds **113** and **609** can also inhibit other viruses. Both compounds exhibited potent anti-HIV activity. However, neither compound exhibited any antiviral activity against non-HIV viruses, including Type I or Type II herpes simplex viruses (HSV-1, HSV-2), enterovirus ECHO 30, or respiratory syncytial virus (RSV). These results establish halo-substituted stavudine phenyl phosphoramidate derivatives as a new class of dual-function anti-HIV agents with potent and selective anti-ADV activity. The remarkable anti-ADV potency of the lead compounds stampidine and compound **609** warrants the further development of these promising new antiviral agents for possible clinical use in ADV infected patients.

2. Chemistry

2.1. Chemicals

All chemicals were purchased from Aldrich (Milwaukee, WI). All syntheses were performed under a nitrogen atmosphere. ^{1}H, ^{13}C, ^{19}F and ^{31}P NMR were obtained on a Varian Mercury 300 instrument at ambient temperature in $CDCl_3$ or DMSO-d_6;. Chemical shifts are reported as δ values in parts per million downfield from tetramethylsilane ($\delta = 0$ ppm) as internal standard. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad peak. FT-IR spectra were recorded on a Nicolet Protege 460 spectrometer. MALDI-TOF mass spectra were obtained by using a Finnigan MAT 95 system. UV spectra were recorded by using a Beckmann UV-VIS spectrophotometer (Model 3DU 74000) with a cell path length of 1 cm. HPLC purification was achieved by using a reverse-phase Lichrospher column (250 × 4 mm, Hewlett-Packard, RP-18, Cat # 79925) and an isocratic flow (1 ml/min) consisting of water (70%) and acetonitrile (30%). Melting points were determined using a Melt John's apparatus and are uncorrected. Column chromatography was performed using silica gel obtained from Baker Company.

2.2. Anti-viral drugs

The synthetic procedures for preparation of stampidine, d4T-5′[para-bromophenyl methoxyalanininyl phosphate], have been previously described in detail [19]. Zidovudine was obtained from Toronto Research Chemicals Inc, Missassagua, Ontario, Canada. The physicochemical properties of the synthesized compounds were as follows:

2.3. Physical constants

2.3.1. 2′,3′-didehydro-3′-deoxy thymidine :(d4T)

Yield: 57%; mp. 165–166 °C; UV (MeOH) λ_{max} 204, 257 nm: IR (KBr): 3463, 3159, 3033, 1691, 1469, 1116, 1093 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 11.29 (br s, 1H), 7.63 (s, 1H), 6.80 (d, 1H, $J = 1.2$ Hz); 6.38 (d, 1H, $J = 5.9$ Hz), 5.90 (dd, 1H, $J = 1.1$, 4.7 Hz), 5.01(m, 1H), 4.76(s, 1H), 3.60 (dd, 2H, $J = 4.8$, 3.6 Hz), 1.71 (d, 3H, $J = 1.2$ Hz), ^{13}C NMR(DMSO-d_6) δ 164.4, 151.3, 137.2, 135.4, 126.4, 109.3, 89.2, 87.6, 62.4, 12.2; mass calculated: 224, found: 225(M + 1); HPLC retention time (t_R): 8.7 min.

2.3.2. 5′-[4-bromophenyl methoxylaninylphosphate]-2′,3′-didehydro-3′-deoxy thymidine (**113**)

Yield: 83%; UV (MeOH) λ_{max}: 209, 218 and 266 nm; IR (Neat): 3203, 3070, 2954, 2887, 2248, 1743, 1693, 1485, 1221, 1153, 1038, 912, 835, 733 cm^{-1}; ^{1}H NMR($CDCl_3$) δ 9.60–9.58 (br s, 1H), 7.45–7.42 (m, 2H), 7.30–7.09 (m, 4H), 6.37–6.27 (m, 1H), 5.93–5.88 (m, 1H), 5.04–5.01 (br m, 1H), 4.35–4.33 (m, 2H), 4.27–3.98 (m, 2H), 3.71–3.70 (s, 3H), 1.85–1.81 (s, 3H), 1.37–1.31 (m, 3H); ^{13}C NMR($CDCl_3$) δ 173.7, 163.8, 150.8, 149.7–149.6, 135.6–135.4, 133.1–132.5, 127.4–127.3, 121.9–121.7, 118.0, 111.2–111.1, 89.7–89.4, 84.4–84.3, 67.8–66.4, 52.5, 50.0–49.9, 20.7, and 12.3; ^{31}P NMR($CDCl_3$) δ 3.41, 2.78; MALDI-TOF mass calculated (M + Na) 567.2, found 567.1; HPLC t_R: 12.04 and 12.72 min.

2.3.3. 5′-[4-methoxy phenyl methoxylaninylphosphate]-2′,3′-didehydro-3′-deoxy thymidine (**598**)

Yield: 25%; UV (MeOH) λ_{max}: 223, 229 and 270 nm; IR (Neat): 3223, 3072, 2999, 2953, 2837, 1743, 1693, 1506,1443, 1207, 1153, 1111, 1034, 937, 837 and 756 cm^{-1}; ^{1}H NMR($CDCl_3$) δ 9.40 (br s, 1H), 7.30–7.00 (m, 5H), 6.83–6.81 (m, 1H), 6.37–6.27 (m, 1H), 5.91–5.86 (m, 1H), 5.00 (br m, 1H), 4.40–4.30 (m, 2H), 4.20–4.10 (m, 2H), 3.95–3.93 (s, 3H), 3.82–3.80 (s, 3H), 1.85–1.81 (s, 3H) and 1.39–1.29 (m, 3H); ^{13}C NMR($CDCl_3$) δ 174.0, 163.9, 156.6, 150.8, 143.5, 135.8–135.5, 133.3–132.9, 127.4–127.2, 121.2–120.9, 114.5, 111.2, 89.7–89.4, 84.5, 66.9–66.3, 55.5, 52.5, 50.6–49.9, 20.9, and 12.3; ^{31}P NMR($CDCl_3$) δ 3.82, 3.20; MALDI-TOF mass calculated (M + Na) 518.2, found 518.2; HPLC t_R: 5.83 and 6.26 min.

2.3.4. 5′-[3-dimethylaminophenyl methoxylaninylphosphate]-2′,3′-didehydro-3′-deoxy thymidine (**599**)

Yield: 18%, mp. 61–62 °C, UV (MeOH) λ_{max}: 203, 206, 211 and 258 nm; IR: 3448, 3050, 2952, 1691, 1506, 1450, 1247, 1143 cm^{-1}, ^{1}H NMR($CDCl_3$) δ 9.93 (s,1H), 7.27 (m, 1H), 7.04 (m, 1H), 6.97(m, 1H), 6.44(q, 3H), 6.24(m, 1H), 5.81 (t, 1H), 4.94 (t, 1H), 4.24(s, 2H), 4.03(m, 1H), 3.92(m, 1H), 3.64 ^{1} (d, 3H), 2.86(s, 6H), 1.77 ^{1} (d, 3H), 1.28 ^{1} (t, 3H); ^{13}C NMR($CDCl_3$) δ 173.7(d), 163.9(d), 151.3(t), 150.8(t), 135.5(d), 132.9(d), 129.5(d), 126.9(d), 111.0(d), 108.8(d), 107.2(q), 103.7(q), 89.3(d), 84.4(q), 66.7(d), 66.1(d), 52.3(d), 49.9(d), 40.2, 20.7(t), 12.2; ^{31}P NMR($CDCl_3$) δ 3.32, 2.70; HPLC t_R: 3.36 min.

2.3.5. 5′-[2,6-dimethoxyphenyl methoxyalaninyl phosphate]-2′,3′-didehydro-3′-deoxy thymidine (**600**)

Yield: 23%, mp. 51–53 °C, UV (MeOH) λ_{max}: 210 and 267 nm; IR: 3432, 3072, 2950, 1691, 1483, 1261, 1112, 931 cm^{-1}, ^{1}H NMR($CDCl_3$) δ 9.78 (s, 1H), 6.95(m, 3H), 6.48(t, 2H), 6.29(m, 1H), 5.81 (m, 1H), 4.36 (m, 3H), 4.02(m, 2H), 3.74(m, 6H), 3.63 ^{1} (t, 3H), 1.74 ^{1} (d, 3H), 1.29 ^{1} (m, 3H); ^{13}C NMR($CDCl_3$) δ 173.7(q), 163.9(d), 151.7(t), 150.8(t), 135.7(d), 133.1(d), 128.4(d), 126.8(d),125.0(d), 110.9(d), 104.8(t), 89.2(d), 84.6(d), 66.8(t), 55.8(d), 52.2(t), 49.7(d), 49.4(d), 21.0(d), 11.8(d); ^{31}P NMR($CDCl_3$) δ 4.97, 4.28; HPLC t_R: 6.55 min.

2.3.6. 5′-[4-cyanophenyl methoxyalaninyl phosphate]-2′,3′-didehydro-3′-deoxy thymidine(**601**)

Yield: 20%, mp. 62–63 °C, UV (MeOH) λ_{max}: 207, 213, 233, and 267 nm; IR: 3214, 3070, 2954, 2229, 1691, 1502,

1467, 1245, 1035, 925 cm^{-1}, ^1H NMR($CDCl_3$) δ 9.92 (s, 1H), 7.60 (m, 2H), 7.28(m, 2H), 7.16(m, 1H), 6.96 (m, 1H), 6.28(m, 1H), 5.86 (t, 1H), 4.99(m, 1H), 4.32 (m, 3H), 3.92(m, 1H), 3.65 ^{1} (m, 3H), 1.75 ^{1} (m, 3H), 1.29 ^{1} (m, 3H); ^{13}C NMR($CDCl_3$) δ 173.5(t), 163.7(d), 153.4(q), 150.7, 135.3(d), 133.7(d), 132.6(d), 127.2(d), 121.0(q), 117.9, 111.0(d), 108.6(d), 89.5(d), 84.2(d), 67.3(t), 52.5(d), 50.0(d), 20.6(t), 12.3(d); ^{31}P NMR($CDCl_3$) δ 4.15, 3.62; HPLC t_R: 5.02 min.

*2.3.7. 5′-[3-bromophenyl methoxyalaninyl phosphate]-2′,3′-didehydro-3′-deoxy thymidine(**602**)*

Yield: 15%, mp. 47–48 °C, UV (MeOH) λ_{max}: 208, 213, and 267 nm; IR: 3432, 3070, 2954, 1685, 1473, 1247, 941 cm^{-1}, ^1H NMR($CDCl_3$) δ 9.65 (s,1H), 7.34–7.11(m, 5H), 6.97 (m, 1H), 6.26(m, 1H), 5.87 (t, 1H), 4.98(m, 1H), 4.26 (m, 3H), 3.93(m, 1H), 3.67 ^{1} (m, 3H), 1.76 ^{1} (m, 3H), 1.32 ^{1} (t, 3H); ^{13}C NMR($CDCl_3$) δ 173.5(d), 163.8(d), 150.6(d), 135.4(d), 132.8 (d), 130.6(d), 128.0, 127.3(d), 123.3(q),122.3(d), 118.8(q), 111.1(d), 89.5(d), 84.4(q), 67.2(d), 52.6, 50.0(d), 20.7(t), 12.3(d); ^{31}P NMR($CDCl_3$) δ 3.36, 2.74; HPLC t_R: 10.3, 10.7 min.

*2.3.8. 5′-[4-bromo-2-chlorophenyl methoxyalaninyl phosphate]-2′,3′-didehydro-3′-deoxy thymidine(**603**)*

Yield: 18%, mp. 51–52 °C, UV (MeOH) λ_{max}: 215, and 267 nm; IR: 3415, 3222, 3072, 2952, 1691, 1475, 1245, 1085, 1035, 925 cm^{-1}, ^1H NMR($CDCl_3$) δ 9.52(s, 1H), 7.52(s, 1H), 7.32 (m, 2H), 7.22(m, 1H), 6.99 (m, 1H), 6.29(m, 1H), 5.90 (m, 1H), 5.00 (m, 1H), 4.33(m, 2H), 4.19(m, 1H), 4.01 (m, 1H), 3.67 (s, 3H), 1.79 ^{1} (m, 3H), 1.31 ^{1} (m, 3H); ^{13}C NMR($CDCl_3$) δ 173.5(q), 163.8(d), 150.8(d), 145.5(t), 135.3 (d), 132.8 (d), 130.9(d), 127.3(d), 126.2(d), 122.7(d), 117.8(d), 113.3(d), 89.6(d), 84.3(d), 67.5(d), 67.1(d), 52.6, 50.1, 20.8(t), 12.3(d); ^{31}P NMR($CDCl_3$) δ 3.11, 2.54; HPLC t_R: 18.6, 20.6 min.

*2.3.9. 5′-[4-flurophenyl methoxyalaninyl phosphate]-2′,3′-didehydro-3′-deoxy thymidine(**604**)*

Yield: 46%, mp. 42–44 °C, UV (MeOH) λ_{max}: 210 and 266 nm; IR: 3423, 3245, 3072, 2954, 1691, 1504, 1247, 1089, 1037, 939 cm^{-1}, ^1H NMR($CDCl_3$) δ 10.08(bs, 1H), 7.16(m, 1H), 7.08(m, 2H), 6.91(m, 3H), 6.20 (m, 1H), 5.79(t, 1H), 4.92(m, 1H), 4.42(t, 1H), 4.22(m, 2H), 3.85 (m, 1H), 3.58 ^{1} (m, 3H), 1.70 ^{1} (m, 3H), 1.22 ^{1} (m, 3H); ^{13}C NMR($CDCl_3$) δ 173.5(q), 163.8(d), 160.7, 157.5, 150.7(d), 145.7(q), 135.3(d), 132.7(d), 126.9(d), 121.3(t), 115.8(q), 110.8(d), 89.2(d), 84.2(d), 66.8(t), 52.2, 49.8(d), 20.4(d), 12.1(d); ^{31}P NMR($CDCl_3$) δ 3.80(d), 3.22(d); ^{19}F NMR($CDCl_3$) δ -42.8(t); HPLC t_R: 6.3, 6.6 min.

*2.3.10. 5′-[2-bromophenyl methoxyalaninyl phosphate]-2′,3′-didehydro-3′-deoxy thymidine(**605**)*

Yield: 20%, mp. 45–46 °C, UV (MeOH) λ_{max}: 207 and 267 nm; IR: 3432, 3072, 2954, 1685, 1475, 1245, 1089, 933 cm^{-1}, ^1H NMR($CDCl_3$) δ 9.55 (s, 1H), 7.47 (m, 2H), 7.24(m, 2H), 6.99(m, 2H), 6.29(m, 1H), 5.88(t, 1H), 5.00 (m, 1H), 4.35(m, 2H), 4.02 (t, 2H), 3.66 (s, 3H), 1.80 ^{1} (m, 3H), 1.30 ^{1} (m, 3H); ^{13}C NMR($CDCl_3$) δ 173. 6(t), 163.8(d), 150.8(d), 147.3(t), 135. 4(d), 133.0(t), 128.5(d), 127.2(d), 126.1(d), 121.3(q), 114.4(d), 111.3(d), 89.6(d), 84.3(d), 67.2(q), 52.5, 50.1(d), 29.6, 20.8(t), 12.4; ^{31}P NMR($CDCl_3$) δ 2.98, 2.37; HPLC t_R: 8.4, 9.2 min.

*2.3.11. 5′-[2-chlorophenyl methoxyalaninyl phosphate]-2′,3′-didehydro-3′-deoxy thymidine(**606**)*

Yield: 47%; mp. 43–45 °C, UV (MeOH) λ_{max}: 214, 215, 219 and 267 nm; IR: 3209, 3070, 2952, 1691, 1481, 1245, 1035, 931 cm^{-1}, ^1H NMR($CDCl_3$) δ 9.80(s, 1H), 7.39(t, 1H), 7.29(m, 1H), 7.20(m, 1H), 7.13(t, 1H), 7.01(t, 1H), 6.92(d, 1H), 6.24(m, 1H), 5.81(m, 1H), 4.94(m, 1H), 4.28(m, 3H), 3.96(m, 1H), 3.59 ^{1} (m, 3H), 1.72 ^{1} (m, 3H), 1.25 ^{1} (m, 3H); ^{13}C NMR($CDCl_3$) δ 173.5(t), 163.8(d), 150.8(d), 145.9(d), 135. 3(d), 132.7(d), 130.0, 127.5(d), 127.0(d), 124.8(q), 121.2(q), 111.0(d), 89.3(d), 84.3(d), 66.9(d), 52.3, 49.8(d), 20.5(t), 12.1(d); ^{31}P NMR($CDCl_3$) δ 3.23, 2.67; HPLC t_R: 7.6, 8.3 min.

*2.3.12. 5′-[phenyl methoxylaninylphosphate]-2′,3′-didehydro-3′-deoxy thymidine: (**607**)*

Yield: 46%; UV (MeOH) λ_{max}: 211 and 264 nm; IR (Neat): 3222, 2985, 2954, 1743, 1693, 1593, 1491, 1456, 1213, 1153, 1039, 931, 769 cm^{-1}; ^1H NMR ($CDCl_3$) δ 9.30 (br s, 1H), 7.30–7.10 (m, 6H), 6.85–6.82 (m, 1H), 6.36–6.26 (m, 1H), 5.91–5.85 (m, 1H), 5.00 (br m, 1H), 4.19–3.68 (m, 4H), 3.72, 3.71 (s, 3H), 1.83, 1.80 (d, 3H), 1.38–1.25 (m, 3H); ^{13}C NMR($CDCl_3$) δ 173.9, 163.7, 150.7, 149.7, 135.7–135.4, 133.2–132.9, 129.6–129.4, 127.3–127.2, 125.0–124.4, 120.0, 111.1, 89.6–89.4, 84.5–84.4, 66.9–66.3, 52.5–52.3, 50.0–49.6, 20.9 and 12.3; ^{31}P NMR($CDCl_3$) δ 2.66, 3.20; MALDI-TOF mass calculated (M + Na) 488.0, found 487.9; HPLC t_R: 5.54, 5.85 min.

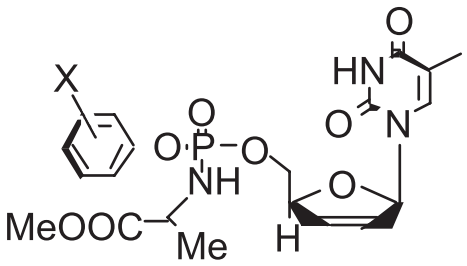
*2.3.13. 5′-[2,5-dichlorophenyl methoxyalaninyl phosphate]-2′,3′-didehydro-3′-deoxy thymidine(**608**)*

Yield: 30%, mp. 42–44 °C, UV (MeOH) λ_{max}: 211, 216, 220 and 268 nm; IR: 3423, 3205, 3072, 2954, 1691, 1475, 1245, 1093, 946 cm^{-1}, ^1H NMR($CDCl_3$) δ 9.43(s, 1H), 7.45(m, 1H), 7.25(m, 2H), 7.04(m, 1H), 6.99(q, 1H), 6.32(m, 1H), 5.88(m, 1H), 4.99(m, 1H), 4.32(m, 3H), 4.00(m, 1H), 3.67(s, 3H), 1.77 ^{1} (m, 3H), 1.33 ^{1} (t, 3H); ^{13}C NMR($CDCl_3$) δ 173.5(d), 163.8(d), 150.8(d), 146.4(d), 136.3, 132.7(t), 130.7(d), 127.4, 125.8, 123.7(d), 121.7(q), 111.2(d), 89.6(d), 84.3(t), 67.1(d), 52.6, 50.1, 29.6, 20.7(t), 12.3(d); ^{31}P NMR($CDCl_3$) δ 3.24, 2.60; HPLC t_R: 13.2 min.

*2.3.14. 5′-[4-chlorophenyl methoxyalaninyl phosphate]-2′,3′-didehydro-3′-deoxy thymidine(**609**)*

Yield: 40%, mp. 42–44 °C, UV (MeOH) λ_{max}: 202, 204, 212, 219 and 267 nm; IR: 3423, 3214, 3068, 2952, 1691, 1488, 1247, 1089, 929 cm^{-1}, ^1H NMR($CDCl_3$) δ 9.48 (d,

Table 1
Antiviral activity of arylphosphoramidate derivatives of stavudine



Compound number	X	HIV-1 isolates			Adeno	CMV
		$HTLVIII_B$	A17	RTMDR		
113	4-Br	1 ± 0	36 ± 21	21 ± 12	22 ± 9	>100
598	4-Ome	4 ± 2	121 ± 70	1014 ± 586	555 ± 115	>100
599	3-$N(Me)_2$	25 ± 16	70 ± 49	273 ± 158	230 ± 20	>100
600	2,6-OMe	6 ± 3	137 ± 79	1104 ± 637	290 ± 120	>100
602	3-Br	2 ± 2	42 ± 24	493 ± 285	235 ± 145	>100
603	4-Br, 2-Cl	1 ± 1	31 ± 18	234 ± 135	80 ± 10	>100
604	4-F	1 ± 0	91 ± 52	218 ± 126	100 ± 40	>100
605	2-Br	2 ± 0.7	42 ± 24	523 ± 302	39 ± 22	>100
606	2-Cl	3 ± 1	34 ± 20	187 ± 108	28 ± 12	>100
607	H	2 ± 0.6	36 ± 21	534 ± 308	57 ± 23	>100
608	2,5-diCl	1 ± 0	37 ± 22	22 ± 15	33 ± 17	>100
609	4-Cl	1 ± 0.3	79 ± 46	62 ± 43	27 ± 3	>100
d4T	–	18 ± 2	–	–	1230 ± 300	–
AZT	–	4 ± 1	55	68.0	–	–

All compounds synthesized showed satisfactory analytical data conforming their structures.
* IC_{50} values in nanomolar.

1H), 7.25(d, 3H), 7.12(t, 1H), 7.00(m, 1H), 6.30(m, 1H), 5.89(t, 1H), 5.01(m, 1H), 4.29(m, 3H), 4.05(t, 1H), 3.90(m, 1H), 3.69 [1] (d, 3H), 1.80 [1] (d,3 H), 1.32 [1] (d, 3H); ^{13}C NMR($CDCl_3$) δ 173.7(q), 163.7(d), 150.7, 148.6(q), 135.5(d), 132.9(d), 130.3(d), 129.5(d), 127.3(d), 121.4(q), 111.2(d), 89.5(d), 84.4(d), 67.2(d), 66.5(d), 52.6,50.1(d), 20.9(t), 12.4(d); ^{31}P NMR($CDCl_3$) δ 3.57, 2.82; HPLC t_R: 7.6, 8.3 min.

3. Results and discussion

Halo-substituted phenyl phosphoramidate derivatives of stavudine are potent anti-retroviral agents which inhibit the human immunodeficiency virus HIV-1 at nanomolar concentrations [22], but very little is known about their activity against other viruses. Table 1 shows the antiviral activity profiles of arylphosphoramidate derivatives of stavudine against various viral strains including a few mutated HIV strains such as $HTLVIII_B$, A17, and RTMDR. All the phosphoramidate derivatives showed potent antiviral activity against HTLVIIIB at nanomolar concentrations as compared to parent stavudine (18 nM). In particular compounds with electron withdrawing groups such as chloro or bromo and di-halo substituted derivatives were found to be amongst the most active compounds. In contrast, electron donating substituents such as OMe, $N(Me)_2$, and 2,6-dimethoxy compound showed 5–10-fold decrease in activity. In addition, the position of the substituents on the phenyl ring did not alter the activity profiles as evident from the table values.

We examined the antiviral activity profile of these derivatives against the NNRTI -resistant A17 (Y-181C) strain [26,27]. Among the phosphoramidate derivatives, those with electron withdrawing substituents showed nanomolar to low micromolar activity. The reduced activity shown by the cyano-substituted derivative may have been due to its fast rate of decomposition in aqueous media. In general compounds with electron donating substituents showed a 3–5-fold decrease in activity.

The multi drug resistant strain RTMDR carries many mutations, that are associated with both NRTI resistance (M41L, L74V, T215Y) as well as NNRTI resistance (V106N). Compounds **113, 608** and **609** were found to be the most potent among the derivatives studied.

We next examined the antiviral activity of stavudine and 13 phenyl phosphoramidate derivatives of stavudine against human adenovirus (ADV strain Type 5). Stavudine inhibited the cytopathic effects of ADV with an IC_{50} value of 12.3 ± μM. All 13 derivatives of stavudine were substantially more potent than stavudine and inhibited ADV-induced plaque formation with nanomolar IC_{50} values. Compounds with halo substitutions in the phenyl ring as well as the unsubstituted compound **607** were more potent than compounds with methoxy, methyl, or cyano substitutions. Compound **113** (stampidine) with a 4-Br substitution and com-

[1] Indicates the peaks corresponding to two isomers.

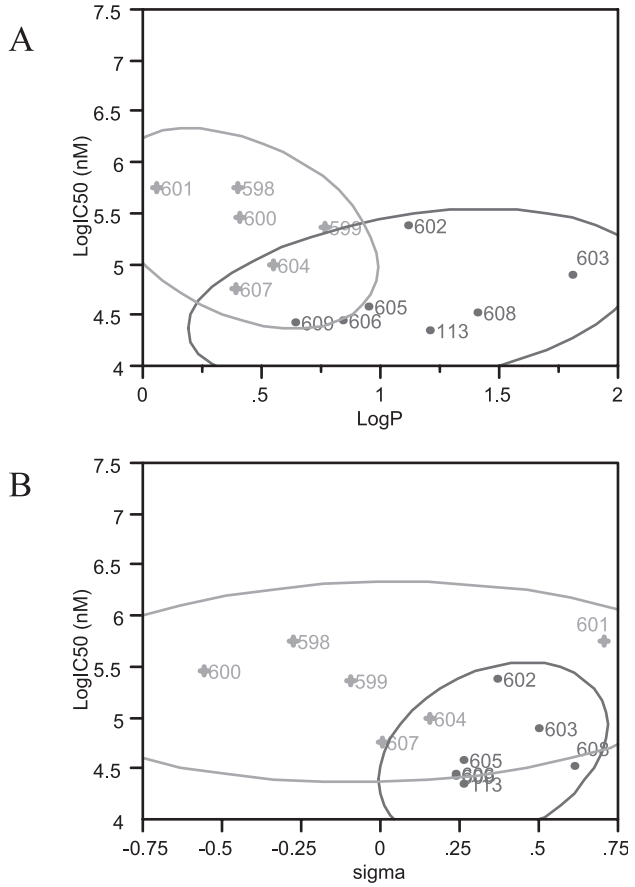


Fig. 1. Cluster analysis of sigma and log *P* vs. biological activity for Adenovirus. Each compound (compound number shown) represents a three-dimensional data point (sigma value, log *P*, log (IC_{50}) value). Wards distance clustering algorithm was used to cluster the compounds into two groups. Membership to the cluster groups is shown by the bivariate 95% confidence ellipses (red, green lines). Log *P* values show greater separation of the group membership than sigma values indicated by the degree of overlap.

pound **609** with a 4-Cl substitution were identified as the most potent lead anti-ADV agents. Compound **113/**Stampidine inhibited ADV-induced plaque formation in skin fibroblasts in a concentration-dependent fashion with a mean (±S.E.M.) IC_{50} value of 0.022 ± 0.009 μM without any evidence of cytotoxicity even at 100 μM. Similarly, compound **609** inhibited ADV-induced plaque formation with an IC_{50} value of 0.027 ± 0.003 μM (Table 1).

Fig. 1 shows the cluster analysis used for analyzing the structure–activity relationship of substituted phosphoramidate derivatives of stavudine against the adenovirus strain. The log IC_{50} vlaues (activity) for the adenovirus strain was plotted against Hammett sigma and partition coefficient. Bivariate plot of log *P* (Fig. 1A) vs. activity shows a greater separation between the two clusters than for Hammett sigma (Fig. 2B). Compounds with higher log *P* values tend to be more active (Fig. 1A; **605, 606, 113, 608, 602, 603**). Two compounds with electron withdrawing capability (Fig. 1B; **608, 113**) showed greater activity and lay outside the 95% confidence ellipse that represented low activity and low Hammett sigma values (green ellipse).

We next examined if the potency shown by these compounds was predicted by liphophilicity, electron donating capability or alkaline hydrolysis rates. Table 2 shows the physicochemical properties of phosphoramidate analogs of stavudine including Hammett sigma, partition coefficients, solubility in water and alkaline hydrolysis rate respectively. These values were used in correlation analysis against the IC_{50} values for each virus strain (Table 3). From the Hammett sigma values, we observed that electron withdrawing groups in general showed potent activity in comparison to electron donating substituents on the aryl moiety of these phosphoramidate derivatives against HTLV III_B. Indeed, a significant correlation was found between Hammett sigma and activity against $HTLVIII_B$ ($P = 0.012$). There was also a significant correlation between the hydrolysis rate of these phosphoramidate derivatives and activity against $HTLVIII_B$ ($P = 0.049$).

In case of viral strain A17, we observed statistically significant correlation of both Hammett sigma and hydrolysis rate against the antiviral activity with *P*-values of 0.029 and 0.036, respectively. Examination of RTMDR viral strain showed that only Hammett sigma had statistically significant correlation with a *P* value of 0.036. However, the hydrolysis rate did not show statistically significant correlation with RTMDR antiviral activity. Focusing on the activity profiles for ADV viral strain we found that there were no statistically significant correlations between antiviral activity and Hammett sigma or hydrolysis rate with these phosphoramidate derivatives (Table 3). A 3D plot of IC_{50} vs. log (hydrolysis rate) and Hammett sigma against A17 activity was performed. The statistics of multiple regression fit of Hammett sigma and Hydrolysis rate to log IC_{50} values for inhibition of A17 replication were as follows: $R^2 = 0.61$, $F_{2,9} = 7.13$, $P = 0.014$; log (IC_{50}) = 0.021 × log (hydrolysis) – 0.579 × Hammett sigma + 1.857.

Notably, both hydrolysis rate and Hammett sigma values can predict the antiviral activity of the phosphoramidate derivatives for A17 virus ($P = 0.014$). In case of ADV we found that there was a clear trend between electron withdrawing groups and the antiviral activity with borderline significance (Table 3).

Examination of the liphophilicity values for these derivatives demonstrated that there were no correlations between log *P* values and IC_{50} values for all the viral strains studied except RTMDR ($P = 0.039$). Thus, the liphophilicity plays a minor role in predicting the antiviral activity of these phosphoramidate derivatives.

In the above discussion, we have been interested in chemical hydrolysis rate of these phosphoramidate derivatives in alkaline medium principally to understand whether any correlation can be drawn between physicochemical properties and antiviral activity. However, one must be cognizant of the fact that there could be marked differences between cellular enzymatic hydrolysis rates compared to chemical hydrolysis rate. Due to the complexity in measuring hydrolysis rate in cellular system, we opted to study the rate in alkaline solu-

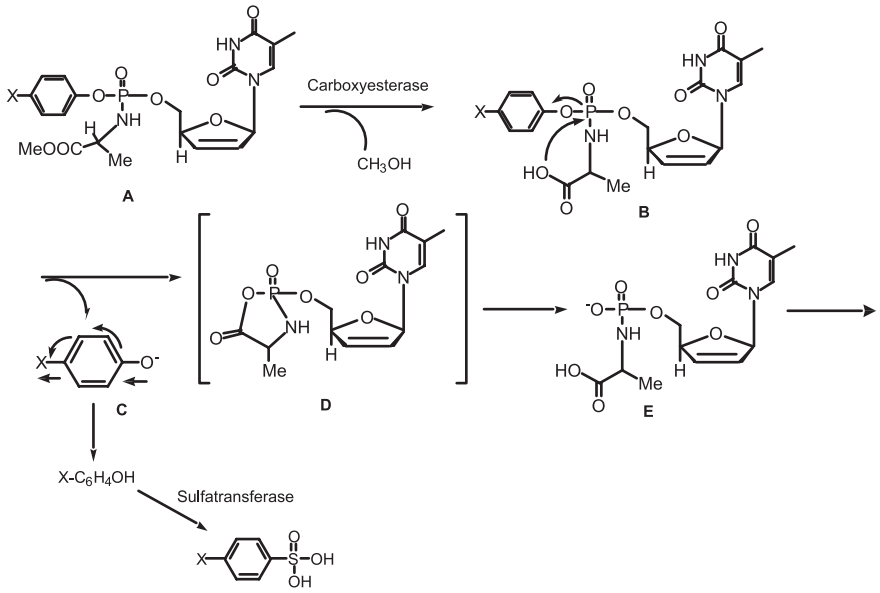
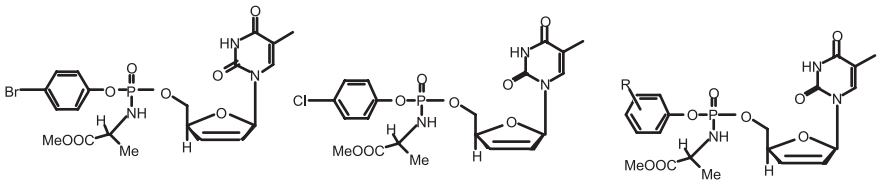


Fig. 2. Literature proposed metabolic pathway of phosphoramidate derivatives of stavudine [28] [In the first step, compound B is generated by carboxyesterase by hydrolysis of the methylester. The subsequent step involves an intramolecular cyclization at the phosphorous center with simultaneous elimination of the phenoxy group to form the cyclic intermediate (D). In the presence of water, this intermediate is converted into the active metabolite (E). The generated phenolic moiety (C) is converted into benzene sulfonic acid by sulfatransferase enzyme.].

tions for simplicity. Based on the literature pathway [28] shown in Fig. 2, we anticipate that electron withdrawing groups at the phenyl moiety will tend to enhance the rate of hydrolysis of these phosphoramidate derivatives to subsequently yield alanine substituted stavudine monophosphate. In the course of our pharmacokinetic investigations of phosphoramidate derivatives of stavudine, we discovered that a new enzyme was involved in the process as depicted in Fig. 2. The sulfur analog thus obtained in our pharmacokinetic studies was identified using LC/Mass as well as by independent synthesis of an authentic sample [29]. The above observation tends to point out that there may be additional enzymes to be involved in the cellular processes. Accordingly, we recognize that hydrolysis rate at which the phenyl moiety leaves the

Table 2
Physicochemical properties and hydrolysis rate of phosphoramidate derivatives of Stavudine (d4T)



Compound number	Substituents (R)	σ-value	Oct/water (log P)	Solubility (mg/ml)	Hydrolysis rate (min^{-1})
113	4-Br	0.26	1.21	3.0	0.0210
598	4-Ome	–0.28	0.39	11.9	0.0102
599	3-NMe_2	–0.10	0.76	18.0	0.0058
600	2,6-Ome	–0.56	0.40	43.6	0.0029
601	4-CN	0.70	0.05 *	4.0	0.1199
602	3-Br	0.37	1.12	5.7	0.0338
603	4-Br, 2-Cl	0.50	1.81	1.8	0.1500
604	4-F	0.15	0.54	7.5	0.0117
605	2-Br	0.26	0.95	4.2	0.0336
606	2-Cl	0.24	0.84	7.3	0.0370
607	H	0.00	0.38	44.7	0.0082
608	2,5-diCl	0.61	1.41	3.7	0.1840
609	4-Cl	0.24	0.64	1.4	0.0216
Stavudine			–0.59	83.0	

* The value from 4-CN substituent was not included in regression analysis clear separation was not obtained between octanol and water.

Table 3
Regression analysis of biological activity with physiochemical properties

		Virus strain (regression of $1C_{50}$ vs. parameter)			
Parameter	Statistic	HTLV IIIB	A17	RTMDR	ADV
Hammett sigma	Spearman Rho	–0.7	–0.63	–0.61	–0.51
	P-value	**0.012**	**0.029**	**0.036**	0.091
Hydrolysis rate	Spearman Rho	–0.58	–0.61	–0.57	–0.47
	P-value	**0.049**	**0.036**	0.055	0.125
Log (*P*)	Spearman Rho	–0.49	–0.57	–0.60	–0.41
	P-value	0.106	0.055	**0.039**	0.183

Inhibition of viral replication (IC_{50}) for each viral strain was correlated with each of the three physiochemical parameters; Hammett Sigma, Hydrolysis Rate and Partition Coefficient (log *P*), using non-parametric regression analysis (Spearman Rank). The Spearman Rho value was computed on ranked data to measure the strength and direction of the correlation. Significant correlations ($P < 0.05$) are highlighted in bold. Since one test out of 20 is expected to yield a positive, we calculated the false discovery rate to be 10%.

phosphoramidate derivatives may be a key step in the cellular process.

We next sought to determine if the lead compounds **113** and **609** can also inhibit other viruses. Both compounds exhibited potent anti-HIV activity but neither compound exhibited any antiviral activity against Type I or Type II herpes simplex viruses (HSV-1, HSV-2), enterovirus ECHO 30, CMV or respiratory syncytial virus (RSV) ($IC_{50} > 100$ μM) (Table 4).

In summary, we have established that phosphoramidate derivatives of stavudine with various substituents attached on the phenyl ring influences the antiviral activity towards various viral strains. In particular we have demonstrated that chemical hydrolysis rate as well as Hammett sigma values can be used to predict the antiviral activity of these types of nucleoside analogs.

4. Conclusions

The results establish halo-substituted stavudine phenyl phosphoramidate derivatives as a new class of dual-function anti-HIV agents with potent and selective anti-ADV activity. Unlike other nucleoside analogs with anti-ADV activity, such as ribavarin or HPMC, stampidine was selective active only against adenovirus, which suggests a higher susceptibility of adenovirus DNA polymerase to this novel nucleoside analog. A selective antiviral activity is not unprecedented for nucleoside antiviral drugs. For example, a number of novel 5-substituted 2′deoxypyrimidine nucleosides exhibited antiviral activity against herpes simplex virus type 1 and type 2 strains V3 but not against adenovirus [17]. However, stampidine and compound **609** are the first nucleoside analogs to be identified as dual-function anti-HIV agents with selective anti-ADV activity. The remarkable anti-ADV potency of the lead compounds stampidine and compound **609** warrants the further development of these promising new antiviral agents for possible clinical use in ADV infected patients.

5. Experimental protocols

5.1. Partition coefficients

The octanol/water partition coefficient was determined by the shake flask method. The phosphoramidate analogs were added to 2 ml of water and 2 ml of octanol in a glass vial. The mixture was shaken for 4 h at room temperature. The two phases were carefully separated and filtered through a Millipore filter and analyzed by HPLC. The partition coefficient was calculated using the ratio of the area under the curve for octanol and water, respectively.

5.2. Viruses

The HIV-1 strains used in this study were HTLV-IIIB, A17 (Y181C), and RTMDR (M41L, L74V, T215Y, V106N) [25]. The non-HIV viruses included in the present study were the

Table 4
Antiviral activity profiles of compound **113**(stampidine) and compound **609**

Virus	IC_{50}, (μM)		
	Compound **113** (Stampidine)	Compound **609**	STV
HIV-1, HTLV-IIIB	0.001 ± 0.000	0.001 ± 0.000	0.023 ± 0.008
CMV, Strain AD169	>100	>100	>100
HSV-1, Strain HF	>100	>100	>100
HSV-2, Strain G	>100	>100	>100
Adenovirus, Type 5	0.022 ± 0.009	0.027 ± 0.003	12.3 ± 3
Enterovirus, ECHO 30	>100	>100	>100
RSV, Strain Long	>100	>100	>100

Plaque assays were used to examine the activity of stampidine and compound **609** against non-HIV viruses. The skin fibroblast cell line SF (ATCC CRL-2097) was used as a target for AD169, Adenovirus Type 5, and ECHO 30, VERO cell line (ATCC CCL-81) was used as a target for HF and G, and HEP-2 cell line (ATCC CCL-23) was used as target for the RSV strain Long. Results are expressed as the average IC_{50} values in μM. Stavudine (STV) was included as a control.

gancyclovir-sensitive Cytomegalovirus (CMV) strain AD169 (ATCC VR-538), acyclovir-sensitive Herpes Simplex Virus (HSV) Type I strain HF (ATCC VR-260), acyclovir-sensitive HSV Type II strain G (ATCC VR-734), Adenovirus strain Type 5, Strain Adenoid 75 (ATCC VR-5), Enterovirus strain, ECHO 30, Strain Bastianni (ATCC VR-322), and Respiratory Syncytial Virus (RSV), Strain Long (ATCC VR-26).

5.3. In vitro assays of anti-HIV-1 activity

Normal human peripheral blood mononuclear cells (PBMNC) from HIV-negative donors were cultured 72 h in RPMI 1640 supplemented with 20% (v/v) heat –inactivated fetal bovine serum (FBS), 3% interleukin-2, 2 mM L-glutamine, 25 mM HEPES, 2 g/l $NaHCO_3$, 50 μg/ml gentamicin, and 4 μg/ml phytohemagglutinin prior to exposure to HIV-1 at a multiplicity of infection (MOI) of 0.1 during a 1 h adsorption period at 37 °C in a humidified 5% CO_2 atmosphere. Subsequently, cells were cultured in 96-well microtiter plates (100 μl/well; 2×10^6 cells/ml) in the presence of various concentrations of compounds **113** or **609** and aliquots of culture supernatants were removed from the wells on the 7th day after infection for p24 antigen assays, as previously described [25]. The applied p24 enzyme immunoassay (EIA) was the unmodified kinetic assay commercially available from Coulter Corporation/Immunotech, Inc. (Westbrooke, ME), which utilizes a murine mAb to HIV core protein coated onto microwell strips to which the antigen present in the test culture supernatant samples binds. Percent viral inhibition was calculated by comparing the p24 values from untreated infected cells (i.e., virus controls).

5.4. Plaque formation assays

Plaque assays were used to examine the activity of the compounds against non-HIV viruses. The skin fibroblast cell line SF (ATCC CRL-2097) was used as a target for AD169, Adenovirus Type 5, and ECHO 30, VERO cell line (ATCC CCL-81) was used as a target for HF and G, and HEP-2 cell line (ATCC CCL-23) was used as a target for the RSV strain Long. These cell lines were cultured at 1×10^5 cells/well in 24-well (all but ECHO 30 infected SF) or 6-well (ECHO 30 infected SF) tissue culture plates with 0.9% methylcellulose or 0.4% SeaPlaque agarose semisolid support. Minimum Essential Medium (MEM) with Earle's salts (Gibco), L-glutamine, non-essential amino acids, 2% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, and 0.05% gentamicin served as the culture medium. The incubation times were 3 days for HF, G, ECHO 30, and Long and 7 days for AD169 and Adenovirus Type 5. Plaque counting was performed with a 20× dissecting microscope for AD169, Type 5, ECHO 30, and RSV Long, and with a light box for HF and G. The fixative agent was crystal violet for all viruses except for the CMV strain AD169 for which methylene blue was used. Percent inhibition of plaque formation was calculated by comparing the plaque numbers from the test substance-treated infected cells with the plaque numbers from untreated infected cells (i.e., virus controls). The IC_{50} values were determined using the Statview statistics program (SAS Institute, Inc.).

5.5. Statistical analysis

Each drug was tested at 6–7 different concentrations ranging from 0.0001 μM to 100 μM. Each assay was set up in triplicate wells and repeated 1–3 times. An IC_{50} value was calculated from each set of triplicate wells using nonlinear regression modeling of the linearized form of an exponential equation ($\ln y = \ln b_0 + b_1 x$; where y = % inhibition and x = drug concentration and the IC_{50} was calculated from the fitted parameters b_0 and b_1; Statview). Hydrolysis rate constants were determined by fitting single exponential decay equations to the disappearance of the compound in alkali conditions (all R^2 values >0.85). Log transformed hydrolysis rate constant, Hammett sigma and log P were correlated to inhibition constants for each virus type using non-parametric Spearman Rank tests. Multivariate relationships between Inhibition constant, log P and Hammett sigma for adenovirus were investigated using Hierarchical Cluster Analysis and the separation of clusters were shown using bivariate plots. Multiple regression model was used to fit Hammet Sigma and Hydrolysis rate to Inhibition constant for each virus type and significant relationships were shown using a 3D plot. All calculations were performed using JMP Software (SAS Institute Inc., Cary, NC). P-values less than 0.05 were deemed significant. No corrections for multiple comparisons were performed.

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