

Short communication

Cellular pharmacology of 9-(β -D-1,3-dioxolan-4-yl)guanine and its lack of drug interactions with zidovudine in primary human lymphocytes

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Amdoxovir, currently in Phase II clinical trials, is rapidly converted to 9-(β -D-1,3-dioxolan-4-yl)guanine (DXG) by adenosine deaminase *in vitro* and in humans. The cellular pharmacology of DXG in primary human lymphocytes, including dose–response relationships, intracellular half-life of DXG triphosphate (DXG-TP), and combination studies were determined. DXG produced high levels of DXG-TP with a long half-life (16 h) in activated human peripheral blood mononuclear cells. Since zidovudine (ZDV) and DXG select for

different resistance mutations, co-formulation of the these two drugs is an attractive proposition. A combination study between DXG and ZDV showed no reduction of DXG-TP or ZDV-TP. Taken together, these results suggest that an appropriately designed DXG prodrug could be given once a day and that co-formulation with ZDV might be a possibility.

Keywords: cellular pharmacology, DXG, nucleoside analogues, NRTI

The emergence of resistant HIV strains during therapy has made it a major challenge to develop drugs that delay, prevent or attenuate the onset of resistance. Therefore, several nucleoside reverse transcriptase inhibitors (NRTIs) are under development as second line therapies for individuals infected by viruses with common mutations such as M184V and thymidine analogue mutations (TAMs). Although a number of NRTIs in development are pyrimidines, such as Racivir, Dextelucitabine (2',3'-dideoxy-2',3'-dideoxy-5-fluorocytidine, reverset, D-D4FC, DFC, RVT), AVX-754 (SPD-754, (-)dOTC, (-)-2'-deoxy-3'-oxa-4'-thiocytidine) and β -D-dioxolane-thymine (DOT), only a few are purines. Amdoxovir (AMDX, (-)- β -D-2,6-diaminopurine dioxolane, DAPD) is a purine nucleoside in Phase II clinical trials for the treatment of HIV-1 infections (<http://clinicaltrials.gov/show/NCT00432016>).

DAPD was developed as a nucleoside analogue prodrug that is deaminated by adenosine deaminase to the 2'-deoxyguanosine analogue, 9-(β -D-1,3-dioxolan-4-yl)guanine (DXG), to circumvent the limited aqueous solubility and oral bioavailability of DXG. Activation of DXG requires intracellular phosphorylation to the triphosphate DXG-TP, which is a potent and selective

inhibitor of HIV-1, HIV-2 and hepatitis B virus (HBV) in human cell lines. The antiretroviral spectrum of DXG-TP includes potent activity against wild-type and drug-resistant forms of HIV-1 reverse transcriptase (RT), including RT enzymes containing M184V/I, TAMs (specifically M41L, D67N, K70R, L210W, T215Y/F and K219Q/E/N) and the 69SS double-insert mutations, and against HBV in human cell lines *in vitro* (Chin *et al.*, 2001, Seignerres *et al.*, 2002, Ying *et al.*, 2000). Resistance in HIV-1 develops slowly *in vitro*, and is associated with mutations at K65R or L74V (Bazmi *et al.*, 2000).

To date, close to 200 subjects have safely received DAPD in seven Phase I and II studies (www.rfspharma.com). DAPD has an excellent safety profile in subjects receiving treatment for up to 96 weeks and is very effective at decreasing viral load in HIV-infected individuals, including those with extensive NRTI mutations. A Phase I/II pharmacokinetic/pharmacodynamic study in HIV-infected individuals to identify a potential co-formulation of DAPD with 3'-azido-3'-deoxythymidine (zidovudine; ZDV) has been completed and suggests that ZDV enhances the activity of DAPD (Murphy R, Zala C, Ochoa C, Tharnish P, Mathew J,

Fromentin E, Asif G, Hurwitz SJ, Kivel NM & Schinazi RF [2008] Pharmacokinetics and potent anti-HIV-1 activity of amdoxovir plus zidovudine in a randomized double-blind placebo-controlled study. *15th Conference on Retroviruses and Opportunistic Infections*. Boston, MA, USA, 3–6 February 2008. Abstract J126).

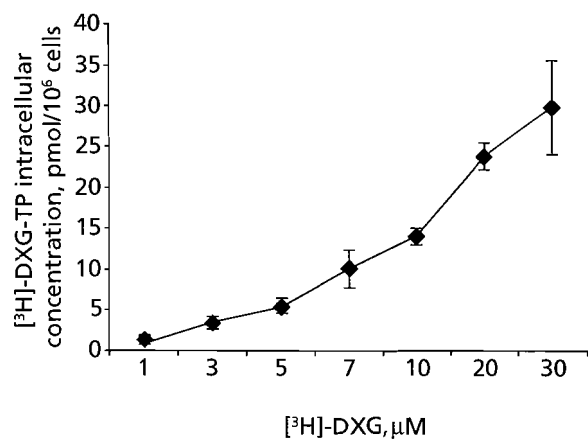
Although extensive pharmacology studies have been conducted in humans, *in vitro* cellular pharmacology including assessments of dose–response relationships, accurate determination of the intracellular half-life of DXG-TP, and combination studies have yet to be published.

To achieve a better understanding of DXG phosphorylation, phytohaemagglutinin-stimulated primary human peripheral blood mononuclear cells (PBMCs) (1×10^6 cells/per time point) were incubated in the presence of different concentrations of [^3H]-DXG (1, 3, 5, 7, 10, 20 and 30 μM) for 4 h. To determine the cellular half-life of DXG-TP, [^3H]-DXG (30 μM) was incubated in PBMCs for 4 h at 37°C in a 5% CO_2 atmosphere. The cells were then washed three times with drug-free medium to remove extracellular DXG and re-incubated in drug-free cell culture medium for specific time periods (0, 1, 2, 4, 8, 12, 24 and 48 h). Combination studies with ZDV were also performed in which radiolabelled-DXG (10 μM) was co-incubated with ZDV (0.1, 1 and 10 μM) or [^{14}C]-ZDV (10 μM) was co-incubated with DXG (1, 10 and 100 μM) for 2 h in PBMCs. All studies were conducted in triplicate.

Cells were then processed to remove extracellular DXG: at selected times cells were centrifuged for 10 min at $350 \times g$ at 4°C; the pellets were resuspended and washed two to three times with cold phosphate-buffered saline (PBS); viable cells were counted using Vi-cell XR counter (Beckman Coulter, Fullerton, CA, USA; viability >98%). The intracellular metabolites of DXG were then extracted by incubation for 2 h at -20°C with 60% methanol/water (1 ml), and the extracts collected and centrifuged at 14,000 rpm (Eppendorf Centrifuge Model 5415C, Hamburg, Germany) for 5 min, before being dried under a gentle filtered air flow and stored at -20°C until they were assayed. The residues were resuspended in 100 μl of water and aliquots were injected into a high-pressure liquid chromatography (HPLC) system.

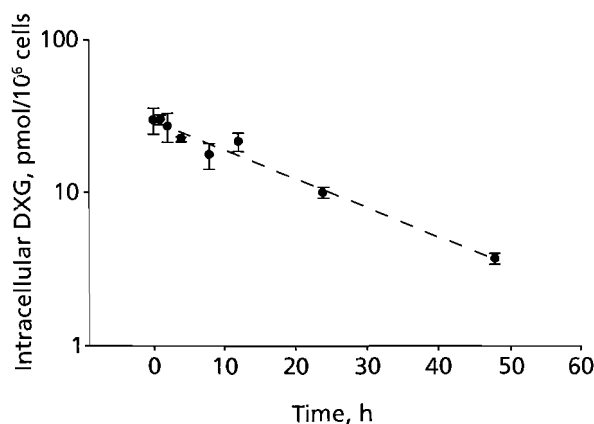
Separation of DXG metabolites was performed by ion-pairing reverse phase HPLC on a Columbus 5 μm C_{18} column (250 \times 4.6 mm; Phenomenex, Torrance, CA, USA) using a Varian Pro Star HPLC model 210 with manual injection (Walnut Creek, CA, USA). The mobile phase consisted of buffer A (25 mM ammonium acetate with 5 mM tetrabutylammonium phosphate [TBAP]; pH 7.0) and buffer B (methanol). Elution was performed using a multistage linear gradient of buffer B from 10% to 50%. The limit of detection was ~ 0.01 pmol/ 10^6 cells. Radioactivity was quantified using a 2500 TR liquid scintillation analyzer (PerkinElmer, Life and Analytical Sciences, Wellesley, MA, USA). DXG-TP was identified based on an authentic standard.

Figure 1. DXG-TP levels in PHA-stimulated PBMCs after incubation with different concentrations of DXG for 4 h



[^3H]-DXG (250–1,000 dpm/pmol) phosphorylation in phytohaemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMCs) for 4 h. DXG, 9-(β -D-1,3-dioxolan-4-yl)guanine; TP, triphosphate.

Figure 2. Decay study of DXG-TP in PHA-stimulated PBMCs



The half-life ($t_{1/2}$) of DXG-TP in phytohaemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) after 4 h incubation with [^3H]-DXG (30 μM , 250 dpm/pmol). The average half-life derived from three experiments was 16.04 ± 0.61 h. DXG, 9-(β -D-1,3-dioxolan-4-yl)guanine; TP, triphosphate.

The quantification of ZDV-TP was performed using a sensitive and specific liquid chromatography tandem mass spectrometry (LC-MS/MS) method. Dried extracts were reconstituted in ultra-pure water (100 μ l) containing lamivudine-triphosphate (3TC-TP; 100 nM) as internal standard (IS) and filtered (0.22 μ m nylon centrifuge tube) at 16,000g for 5 min to remove insoluble particulates; 45 μ l were injected on the column. The separation was accomplished using a Dionex Packing Ultimate 3000 modular LC system (Dionex, Sunnyvale, CA, USA) consisting of a quaternary pump, vacuum degasser, thermostated autosampler and column compartment. A weak anion exchange chromatography was performed on a Biobasic AX, 1 \times 100 mm, 5 μ m column; 20% acetonitrile was maintained during the entire run (25 min). The initial mobile phase consisted of 20 mM ammonium acetate. A pH gradient was accomplished in 4 min using 2 mM ammonium phosphate adjusted to pH 11 with ammonium hydroxide. The flow rate, 100 μ l/min, was increased to 200 μ l/min at the end of the run to remove any late-eluting impurities. The autosampler temperature was maintained at 4°C and the column temperature was maintained at 20°C. The retention times were 6.41 min for ZDV-TP and 3TC-TP (IS).

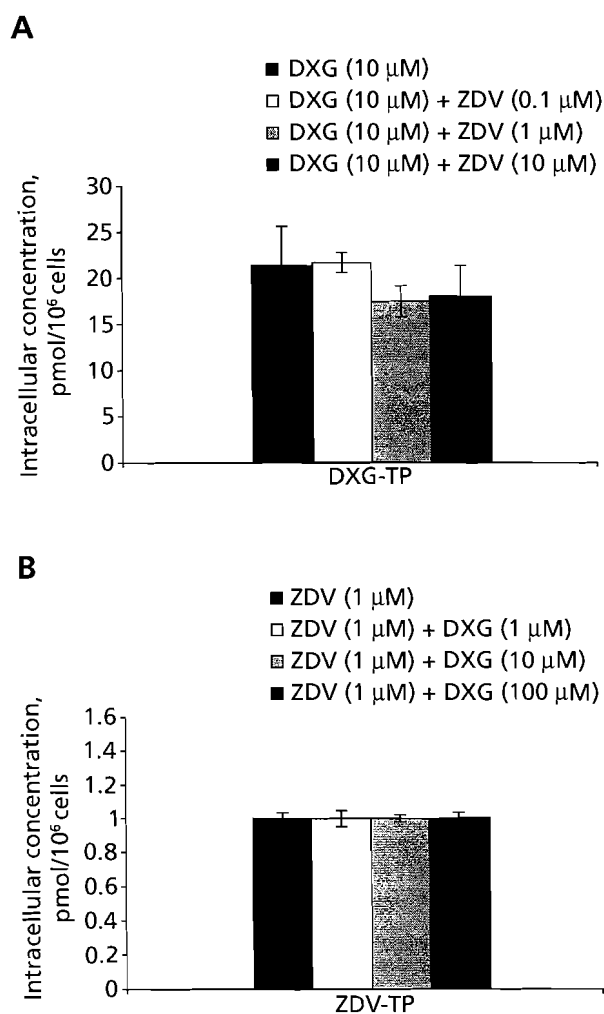
A TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Electron Corp., Waltham, MA, USA) was used for detection. The mass spectrometer was operated with a spray voltage of 3.0 kV, sheath gas at 50 (arbitrary units), ion sweep gas at 0.3 (arbitrary units), auxiliary gas at 0 (arbitrary units), and a capillary temperature of 350°C. The collision cell pressure was maintained at 1.9 mTorr. Two positive ion selected reactions were monitored for ZDV-TP (m/z 508 \rightarrow m/z 81; collision energy 20 V) and for 3TC-TP (m/z 470 \rightarrow m/z 112; collision energy 27 V). Thermo Xcalibur software was used to control both the HPLC and the mass spectrometer and to perform data analysis. Calibration curves were generated using ZDV-TP standard serially diluted in blank PBMCs suspensions ranging from 100 fmol/10⁶ cells to 10,000 fmol/10⁶ cells. The limit of quantification was 100 fmol/10⁶ cells; r^2 values were ≥ 0.999 .

DXG was phosphorylated in primary human PBMCs and the major metabolite was DXG-TP. DXG-TP reached concentrations up to 14 pmol/10⁶ cells after incubation with 10 μ M DXG, and the ratio of DXG to DXG-TP was ~1:3 (Figure 1). The cellular accumulation of DXG-TP was linear and was not saturated at concentrations =30 μ M (Figure 1).

The intracellular half-life ($t_{1/2}$) of DXG-TP was measured in PBMCs after 4 h incubation with 30 μ M DXG (Figure 2). DXG-TP reached a concentration of 29.8 \pm 5.7 pmol/10⁶ cells and declined with a half-life of 16.0 \pm 0.6 h. This relatively long half-life suggests that prodrugs delivering DXG are candidates for once daily dosing.

ZDV is an attractive drug for co-formulation with DXG, as these drugs are activated by different phosphorylation pathways and select for different resistance mutations. Furthermore, previous reports suggest that the mutation K65R in HIV-1 reverse transcriptase, which confers cross-resistance to DXG and DAPD, can revert ZDV-resistant virus to ZDV sensitivity (Gu *et al.*, 1999, Mewshaw *et al.*, 2002). Previous *in vitro* studies have also suggested that DXG could act synergistically with ZDV,

Figure 3. A competition study between DXG and ZDV to measure potential drug–drug interactions at the phosphorylation level



Nucleoside triphosphate concentrations in the competition study between: (A) [³H]-DXG (10 μ M, 500 dpm/pmol) with different concentrations of ZDV (0.1, 1 and 10 μ M) and (B) ZDV (1 μ M) with different concentrations of DXG (1, 10 and 100 μ M) in human peripheral blood mononuclear cells at 2 h. DXG, 9-(β -D-1,3-dioxolan-4-yl) guanine; TP, triphosphate, ZDV, zidovudine.

lamivudine and nevirapine (Gu *et al.*, 1999). On the basis of these studies, we conducted a combination study with DXG and ZDV to measure potential drug–drug interactions at the phosphorylation level. When DXG (10 μM) was combined with different ZDV concentrations (up to 10 μM), no significant effect was noted on DXG-TP levels (Figure 3). Similar results were observed when DXG (concentrations up to 100 μM) were co-incubated with ZDV (1 μM). No reduction in ZDV-TP was observed in primary human PBMCs (Figure 3). These results were expected given that these nucleoside analogues do not share kinases in their phosphorylation pathways.

In summary, human PBMCs accumulated high levels of DXG-TP, which declined. The combination study between DXG and ZDV demonstrated no reduction in DXG-TP or ZDV-TP formation. On the basis of these preclinical studies, the results of a recently completed Phase I/II study on the usefulness of DAPD with ZDV (<http://clinicaltrials.gov/show/NCT00432016>; Murphy R, Zala C, Ochoa C, Tharnish P, Mathew J, Fromentin E, Asif G, Hurwitz SJ, Kivel NM & Schinazi RF [2008] Pharmacokinetics and potent anti-HIV-1 activity of amdoxovir plus zidovudine in a randomized double-blind placebo-controlled study. *15th Conference on Retroviruses and Opportunistic Infections*. Boston, MA, USA, 3–6 February 2008. Abstract J126), a multinational Phase IIb study involving at least 60 HIV-1-infected individuals is planned using a fixed-dose combination of DAPD with ZDV, as add-on therapy in treatment-experienced HIV-1-infected persons.

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