

1                   **Antiviral effect of Cidofovir on Parvovirus B19 Replication**

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6   Running Title: Parvovirus B19 Inhibition by Cidofovir

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1 **Abstract**

2 Parvovirus B19 (B19V) is a human ssDNA virus responsible for a wide range of clinical  
3 manifestations, still lacking for a specific antiviral therapy. The identification of compounds active  
4 against B19V may add therapeutic options to the treatment of B19V infections, that now entirely  
5 relies on symptomatic treatments. In the search for compounds possibly inhibiting B19V  
6 replication, a particular focus was raised to cidofovir, an acyclic nucleoside phosphonate broadly  
7 active against dsDNA viruses. The present study was aimed at evaluating the effect of cidofovir  
8 against B19V in two model systems, the UT7/EpoS1 cell line and erythroid progenitor cells (EPC),  
9 generated from peripheral blood mononuclear cells. Experiments were carried out at different  
10 multiplicity of infections and cidofovir concentrations (0-500  $\mu$ M) during a course of infection. The  
11 effects of cidofovir on B19V replication were assessed by qPCR assays while influence of cidofovir  
12 on host cells was measured by cell proliferation and viability assays. Our findings demonstrated  
13 that cidofovir has a relevant inhibiting activity on B19V replication within infected UT7/EpoS1, and  
14 that the effect on B19V DNA amounts is dose-dependent allowing for the determination of EC<sub>50</sub>  
15 and EC<sub>90</sub> values (7.45 – 41.27  $\mu$ M, and 84.73 – 360.7  $\mu$ M, respectively). In EPCs, that constitute a  
16 cellular population close to the natural target cells in bone marrow, the inhibitory effect was  
17 demonstrated to a lesser extent, however provoking a significant reduction on B19V DNA amounts  
18 at 500  $\mu$ M (68.2 – 92.8 %). To test infectivity of virus released from EPCs cultured in the presence  
19 of cidofovir, cell culture supernatants were used as inoculum for a further course of infection in  
20 UT7/EpoS1 cells, indicating a significant reduction in viral infectivity at 500  $\mu$ M cidofovir,. Since the  
21 drug did not interfere with the overall cellular DNA synthesis and metabolic activity, the observed  
22 effect of cidofovir could be likely related to a specific inhibition of B19V replication.

23

24 **Keywords:**

25 Parvovirus B19; cidofovir; replication inhibition; quantitative PCR;

## 1. Introduction

Parvovirus B19 (B19V) is a human pathogenic virus in the Parvoviridae family (King et al., 2011). The pathogenetic potential of B19V is ample (Gallinella, 2013; Young and Brown, 2004). The virus shows a marked tropism for erythroid progenitor cells in the bone marrow, where productive infection induces apoptosis and a block in erythropoiesis that can be manifested as a transient or persistent erythroid aplasia (Chisaka et al., 2003). Anaemia as a consequence of the block in erythropoiesis usually becomes clinically relevant when an underlining condition is present, such as expanded erythropoiesis compensating for haematological disorders, or in case of inadequacy of the specific antiviral immune response (Brown, 2000). The virus has additional capability to infect other cellular types, such as endothelial, stromal or synovial cells, although with dissimilar degrees of expression, normally resulting in the mere persistence of virus in tissues (Bonvicini et al., 2010; Manning et al., 2007; Norja et al., 2006) but sporadically also leading to productive viral replication. In these cases, consequences on infected cells can vary from the unapparent to the overtly cytotoxic by mechanisms not limited to induction of apoptosis (Bonvicini et al., 2012; Duechting et al., 2008; Lu et al., 2006). The common clinical manifestations of infection are erythema infectiosum (fifth disease), mainly in children, or arthropathies, mainly affecting adults, but the virus has been implicated in a growing spectrum of other different pathologies, among them myocarditis (Modrow, 2006) and rheumatic (Kerr, 2000) or autoimmune (Lehmann et al., 2003) diseases. The virus can be transmitted to the foetus, where its tropism for erythroid progenitor cells can lead to consequences such as foetal death and/or hydrops foetalis (Bonvicini et al., 2009; Bonvicini et al., 2011; Enders et al., 2004).

No specific antiviral therapy has been developed or evaluated for B19V. Currently, treatment can only be symptomatic or supportive, in particular blood transfusions are required to overcome acute or chronic anaemia, while intravenous immunoglobulins (IVIG) are considered the only available option to neutralise infectious virus in case of incompetence of the immune system (Crabol et al., 2012; Mouthon and Lortholary, 2003). However, IVIG are seldom able to clear infection unless a specific antiviral immune response becomes effective.

1 A motivation for the development of a specific antiviral therapy would then be for treatment of  
2 haematological consequences of acute or chronic infections, especially in immunodeficient  
3 patients, or to reduce the inflammatory aspects of infections, or possibly for prophylaxis in selected  
4 cases. The incomplete characterization of the viral proteins and related molecular mechanisms,  
5 and the unavailability of systems to produce large amounts of virus in *in vitro* cell cultures,  
6 presently hampers both the rationale design of specifically targeted drugs as well the high  
7 throughput screening of available chemical libraries.

8 An approach that could be explored was the investigation of known antiviral compounds for a  
9 possible activity against B19V. In particular, the acyclic nucleoside phosphonate cidofovir (CDV)  
10 has shown activity against all five families of human, not retro-transcribing dsDNA viruses (De  
11 Clercq, 2007; De Clercq and Holy, 2005). The present study was aimed to investigate its potential  
12 to inhibit B19V, a ssDNA virus, depending for its replication on cellular DNA polymerase activity.  
13 Two cellular systems were suitable for this study, the human myeloblastoid cell line UT7/EpoS1  
14 that presents a restricted pattern of permissiveness, allowing viral replication but only a limited  
15 yield of infectious virus (Wong and Brown, 2006), and *in vitro* derived erythroid progenitor cells,  
16 whose permissiveness depends on cell differentiation and that constitute a cellular population  
17 similar to the primary target cells in the bone marrow (Filippone et al., 2010; Wong et al., 2008).  
18 The activity of virus in infected cells, and a possible role of CDV, have been investigated by  
19 quantitative PCR assays for the determination of variation of viral nucleic acids in the presence of  
20 different concentrations of CDV within a time course of infection. Results indicate that CDV can  
21 interfere with the viral replication, although the degree of inhibition is different in the two cellular  
22 environments.

## 1 **2. Materials and Methods**

### 2 **2.1. Cells**

3 UT7/EpoS1 cells were cultured in IMDM (Cambrex), supplemented with 10% FCS (Cambrex) and  
4 2 U/mL rhu erythropoietin (NeoRecormon, Roche), at 37°C and 5% CO<sub>2</sub>. Cells were kept in culture  
5 at densities between 2x10<sup>5</sup>-1x10<sup>6</sup> cells/mL, and used for infection experiments when at a density of  
6 3x10<sup>5</sup> cells/mL (Wong and Brown, 2006).

7 Erythroid progenitor cells (EPCs) were generated *in vitro* from peripheral blood mononuclear cells  
8 (PBMC), via culture in a medium containing erythropoietic growth factors (Filippone et al., 2010).  
9 PBMC, obtained from volunteer staff members, were isolated by Ficoll-Paque centrifugation, then  
10 cultured as described. The cells were maintained at 37°C in 5% CO<sub>2</sub>, after 4±1 days of culture  
11 were split and then maintained at a density of 0.5/1.0x10<sup>6</sup> cells/mL until day 9±1, when used for  
12 infection experiments.

### 13 **2.2. Flow Cytometry analysis**

14 EPCs were characterized using flow cytometry (FACSCalibur, Becton Dickinson). Aliquots of 5 x  
15 10<sup>5</sup> EPCs were stained with antibodies specific for erythroid differentiation markers (CD36, CD71,  
16 CD235α) and known B19V receptors (globoside, α<sub>5</sub>β<sub>1</sub> integrin). CD36 and CD71 expression was  
17 evaluated by phycoerythrin (PE)-labelled monoclonal antibodies (BD Biosciences). CD235a, α<sub>5</sub>β<sub>1</sub>  
18 integrin and globoside expression was evaluated by monoclonal mouse anti-CD235a (BD  
19 Biosciences), monoclonal mouse anti-α<sub>5</sub>β<sub>1</sub> (Immunological Sciences) and polyclonal rabbit anti-  
20 globoside (Matreya), followed by anti-mouse-Alexa 488 (Life Technologies) or anti-rabbit- FITC  
21 (DakoCytomation) antibody, respectively. Data were analysed using the Cell Quest Pro Software  
22 (Becton Dickinson).

### 23 **2.3. Infection**

24 A B19V viremic serum sample, identified in our laboratory in the course of routine diagnostic  
25 analysis and available for research purposes according to Italian privacy law, was used as source  
26 of virus for the infection experiments. The viremic serum contained 10<sup>12</sup> B19V (genotype 1)

1 genome copies (geq)/mL, as determined by quantitative PCR analysis (Bonvicini et al., 2013a),  
2 and resulted negative by routine diagnostic NAT assays to other viruses, including HIV, HBV, HCV,  
3 HSV, VZV, EBV, CMV, HHV8, AdV, BKV.

4 For infection of both UT7/EpoS1 and EPCs,  $5 \times 10^5$  cells were incubated in 50  $\mu$ L volume of PBS, in  
5 the presence of 5  $\mu$ L of viremic serum, diluted in PBS in order to obtain different multiplicities of  
6 infection (moi, expressed as geq/cell), from  $10^4$  geq/cell to  $10^1$  geq/cell. Following adsorption for 2  
7 h at 37°C, the inoculum virus was washed and the cells were incubated at 37°C in the respective  
8 complete medium at an initial density of  $5 \times 10^5$  cells/mL.

#### 9 **2.4. Cidofovir**

10 Cidofovir (CDV), [(S)-1-[3-hydroxy-2-(phosphonylmethoxy)propyl]cytosine], was purchased from  
11 Sigma at a purity degree >98%. The drug was dissolved in H<sub>2</sub>O and stored at -20°C as a 5 mg/mL  
12 stock solution.

#### 13 **2.5. Cell viability and proliferation assays**

14 The effects of CDV on viability and proliferation of UT7/EpoS1 and EPCs were monitored,  
15 respectively, by the alamarBlue assay, a redox indicator dye that yields a fluorescent signal in  
16 response to metabolic activity (Life Technologies), and by evaluation of 5-bromo-2'-deoxyuridine  
17 (BrdU) incorporation into newly synthesised DNA (Cell proliferation ELISA BrdU Assay, Roche  
18 Diagnostics), according to manufacturers' instructions. For experiments, cells were seeded at the  
19 density of  $5 \times 10^4$  cells/well in a 96-well culture microplate, and volumes of 100  $\mu$ L of CDV freshly  
20 diluted in medium at the different concentrations (0.1 – 500  $\mu$ M) were added, either in the absence  
21 or presence of 10  $\mu$ M of BrdU. Cells were then cultured for 72h, for UT7/EpoS1 cells, or 24h, for  
22 EPCs. Cells grown in absence of both chemicals were also included as control samples.

#### 23 **2.6. Nucleic acids purification**

24 Equal amounts of cell cultures, corresponding to  $2 \times 10^5$  cells, were collected at the appropriate time  
25 points following infection. Collected cells were processed by using the EZ1 viral nucleic acid kit on  
26 a EZ1 platform (Qiagen), following the manufacturer's instructions, in order to obtain a total nucleic  
27 acid fraction, containing both viral DNA and RNA in elution volumes of 120  $\mu$ L.

## 1 **2.7. Quantitative real-time PCR**

2 Standard targets for quantification of viral nucleic acids were obtained from plasmid pHR0, that  
3 contains an insert corresponding to the complete internal region of B19 virus genome (nt. 345-  
4 5245). Primers for the amplification of viral targets were the pair R2210-R2355, located in the  
5 central exon of B19V genome, suitable for amplification of both viral DNA and the whole set of viral  
6 transcripts; pair R1882-R2033, suitable for the amplification of the mRNAs coding for NS protein;  
7 pair R4883-R5014, suitable for the amplification of the mRNAs coding for capsid VP1 and VP2  
8 proteins. For control and normalisation with respect to the number of cells, a target sequence in the  
9 region of genomic DNA coding for 18S rRNA (rDNA) was amplified by using the primers 18Sfor  
10 (CGGACAGGATTGACAGATTG) and 18Srev (TGCCAGAGTCTCGTTCGTTA) (Bonvicini et al.,  
11 2006; Bonvicini et al., 2008). All oligonucleotides were obtained from MWG Biotech.

12 For quantitative analysis of viral DNA, an aliquot of the eluted nucleic acids, corresponding to 0.1  
13 volumes of experimental samples, was directly amplified. For quantitative analysis of viral RNA, a  
14 parallel aliquot of experimental samples was first treated with Turbo DNAfree reagent (Ambion)  
15 and then amplified. Quantitative real-time PCR and RT-PCR were carried out by using the  
16 RotorGene 3000 system (Corbett Research) and SybrGreen detection of amplification products.  
17 Amplification reactions were performed by using QuantiTect PCR SybrGreen PCR Kit or  
18 QuantiTect SybrGreen RT-PCR Kit (Qiagen), including 10 pmol of each specific primer pair, as  
19 described. Quantitation of viral DNA and of total viral RNA was obtained by the absolute  
20 quantitation algorithm, converting quantification cycle (C<sub>q</sub>) values to geq number using external  
21 calibration curves obtained from respective standard targets (Bustin et al., 2009).

## 22 **2.8. Immunofluorescence assay**

23 Aliquots of cell culture samples, each corresponding to 0.5-1.0x10<sup>5</sup> cells, were centrifuged at 4000  
24 g for 5 min. Pelleted cells were washed with PBS, spotted onto glass slides and fixed with 1:1  
25 acetone:methanol for 10 min at -20°C. Slides were then incubated at room temperature for 1h with  
26 a monoclonal mouse antibody against VP1 and VP2 proteins (MAB8293, Chemicon), diluted 1:200  
27 in PBS/BSA 1%. After washing in PBS, slides were incubated for 1h with AlexaFluor488 anti-

1 mouse secondary antibodies (Life Technologies), diluted 1:20 in PBS/BSA 1%. After further  
2 washing, slides were stained with Evans blue, washed, mounted and viewed on a fluorescence  
3 microscopy with a fluorescein isothiocyanate filter set.

#### 4 **2.9. Statistical analysis**

5 Experiments were carried out in triplicate series, and for each quantitative determination triplicate  
6 values were obtained. Statistical analysis was carried out by using the programme GraphPad Prism  
7 version 5.00 for Windows (GraphPad Software, San Diego California, USA). Linear regression  
8 analysis was carried out to correlate input moi values and calculated amounts of viral nucleic acids.  
9 One-way ANOVA (analysis of variance) followed by the Dunnett' Multiple comparison test was used  
10 to compare data obtained in different experimental conditions. EC<sub>50</sub> and EC<sub>90</sub> values for the different  
11 moi were determined by non-linear regression curve on percentage inhibition of viral activity for each  
12 different concentration of CDV.

### 1 3. Results

#### 2 3.1. UT7/EpoS1 cells, B19 virus and cidofovir

3 UT7/EpoS1 cells were infected with B19V, at different moi in the range of  $10^4$  -  $10^1$  geq/cell, in the  
4 presence of different concentrations of CDV, and the effects on viral replication were assessed by  
5 qPCR analysis. Following incubation to allow penetration of virus (2 hpi), infected cells were  
6 divided and cultured in the absence, as a control, and in the presence of different concentrations of  
7 CDV (0.1 – 500  $\mu$ M) for a course of infection (72 hpi). Equivalent cell fractions were collected at 2  
8 hpi and at 72 hpi for the different conditions. Quantitative determination of viral nucleic acids, to  
9 determine viral activity, and of cellular rDNA for normalisation purposes, was carried out by qPCR.  
10 For the rDNA target, the coefficient of variation of quantification cycle values for all different  
11 samples was minimal ( $\leq 3\%$ ), so this parameter was considered invariant and the normalisation by  
12 rDNA not required for the following quantitative determination of viral targets.

13 In Figure 1A, a time course of infection in UT7/EpoS1 cells shows that the highest increase in DNA  
14 amount occurs at 72 hpi. Then, in Figure 2A, calculated amounts of viral DNA are plotted as a  
15 function of the moi, at 2 hpi and at 72 hpi for the different concentrations of CDV added to infected  
16 cell cultures. Linear regression analysis showed a correlation with the moi used, for the different  
17 experimental samples, in the whole test range. In particular, the correlation evident at 2 hpi  
18 indicated that virus attachment and penetration was driven by concentration of virus, while the  
19 extent of replicative activity of the viral genome resulted in a variation in the amount of viral DNA  
20 from 2 hpi to 72 hpi. The slopes for the 2 hpi and 72 hpi sample series are statistically equivalent,  
21 thus indicating a constant relative biological activity of B19V within the UT7/EpoS1 cells, while the  
22 statistically significant difference in elevations might be attributed to the inhibitory effects of CDV on  
23 viral DNA replication. In the presence of CDV, progressively lower increases of viral DNA were  
24 detected in the 0.1 – 100  $\mu$ M range, while at 500  $\mu$ M the amount of viral DNA at 72 hpi was lower  
25 than what detected at 2 hpi suggesting an effective inhibition of viral DNA replication.

26 The effect of CDV on B19V replication, as a dose-dependent reduction in the amount of viral DNA  
27 detected at 72 hpi, could be expressed and quantified in two possible ways. First, for any given  
28 moi, inhibition of replicative activity could be directly determined by quantification of viral DNA at 72

1 hpi for the different concentrations, compared to what present in absence of drug (Figure 3A).  
2 Then, given the parallelism between regression curves, activity of virus in infected cells could also  
3 be determined by interpolation of quantitative values obtained for the different experimental  
4 conditions on the reference curve obtained in the absence of drug, yielding an experimental  
5 infectious titre as the equivalent to a nominal moi (Figure 4A). Both methods produced concordant  
6 results, indicating a progressive inhibition of viral replicative activity in dependence of CDV  
7 concentration. By expressing this dose dependent relationship between CDV concentration and  
8 replicative activity as percentage inhibition of replication, non-linear regression curve allowed  
9 determining  $EC_{50}$  and  $EC_{90}$  values for CDV for all the different moi tested (Figure 5 and Table 1).  
10 The amount of total viral RNA synthesised in infected cells was determined at 72 hpi. The  
11 presence of CDV led to a significant reduction in the amount of viral RNA only for high moi and the  
12 highest concentration tested (Figure 6A). The relative abundance of mRNAs coding for NS protein  
13 was in the range of 1-3% of total viral RNA, in accordance with previous results, irrespective to the  
14 different concentrations of CDV. The relative abundance of mRNAs coding for capsid VP1 and  
15 VP2 proteins was in the range of 15-22%, also in accordance with previous results, for the  
16 concentrations of CDV in the range 0-100  $\mu$ M, but was reduced to 5-8% for CDV at 500  $\mu$ M, an  
17 effect possibly linked to a shift in the expression profile of B19V due to the marked inhibition of its  
18 replicative activity (Bonvicini et al., 2006; Bonvicini et al., 2008).  
19 The effects of CDV on viral activity were also investigated by immunofluorescence detection of  
20 viral capsid proteins. At the moi of  $10^4$  and in the absence of CDV, 5.0% of cells were positive to  
21 the detection of viral capsid proteins. This value was reduced to 3.9% at 50  $\mu$ M, 0.5% at 100  $\mu$ M,  
22 while at 500  $\mu$ M no positive cells could be detected. At lower multiplicities, due both to the  
23 restrictive pattern of infection in UT7/EpoS1 cells and the low sensitivity of the method, the small  
24 number of positive cells did not allow any reliable quantitative evaluation of inhibitory activity of  
25 CDV.  
26 The possible cytotoxic or cytostatic effects of CDV on UT7/EpoS1 cells were investigated by  
27 alamarBlue assay, to assess cell viability, and BrdU incorporation assay, to assess variations in

1 cell proliferation rate. Results show that CDV added to cell cultures did not alter cell viability or  
2 proliferation to a statistically significant extent (Figure 7A, B).

### 3 **3.2. EPC cells, B19 virus and cidofovir**

4 EPCs were generated *in vitro* from PBMC, via culture in a medium containing erythropoietic growth  
5 factors. Following  $9 \pm 1$  days of culture, the cellular population showed a wide distribution of  
6 erythroid differentiation markers (65-75 % CD36, 70-80 % CD71, 65-75 % CD235a) and known  
7 viral receptors (60-65 % globoside and 55-65 %  $\alpha_5\beta_1$  integrin). At this differentiation stage, when  
8 highly permissive for viral replication (Filippone et al., 2010; Wong et al., 2008), EPCs were  
9 infected with B19V, at different moi in the range of  $10^4 - 10^1$  geq/cell, according to the same  
10 experimental scheme followed for UT7/EpoS1 cells except that the time course of infection was  
11 limited to 24 hpi, a time showing the highest increase in the DNA amount and considered optimal  
12 for assessing the viral activity in a single round of infection (Figure 1B).

13 In Figure 2B, calculated amounts of viral DNA are plotted as a function of the moi, at 2 hpi and at  
14 24 hpi for the different concentrations of CDV added to infected cell cultures. As for UT7/EpoS1  
15 cells, also for EPCs the amount of viral DNA within infected cells showed a correlation with the moi  
16 used, both at 2 hpi and at 24 hpi, for the different experimental series, in the whole test range. The  
17 slopes for the 2 hpi and 24 hpi sample series, at different concentrations of CDV, are statistically  
18 equivalent, also indicating a constant relative biological activity of B19V within the EPCs. However,  
19 a statistically significant difference in elevations that could be attributed to the inhibitory effects of  
20 CDV on viral DNA replication was evident only for the 500  $\mu\text{M}$  concentration. The effect of 500  $\mu\text{M}$   
21 CDV in inhibiting B19V DNA replication was also evident in Southern Blot Analysis (Figure 8),  
22 when comparing the amount of DNA at 24 hpi, and when determining an interpolated experimental  
23 infectious titre (Figures 3B and 4B, reported as percentage inhibition of activity in Table 1). Even at  
24 this highest concentration of CDV, the reduction in the amount of viral RNA at 24 hpi was not  
25 significant (Figure 6B). The relative abundance of mRNAs coding for NS protein was in the range  
26 of 1-3% of total viral RNA, and that of mRNAs coding for capsid VP1 and VP2 proteins was in the  
27 range of 35-45%, values comparable to what previously determined for bone marrow mononuclear  
28 cells (Bonvicini et al., 2006; Bonvicini et al., 2008), without any significant correlation with the

1 concentration of CDV. By immunofluorescence, viral capsid proteins were detected in a fraction of  
2 cells not statistically different from controls (10.8 compared to 14.0 % for moi of  $10^4$ , 1.1 compared  
3 to 1.8 % for moi of  $10^3$ ). Finally, CDV added to cell cultures did not alter EPCs viability or  
4 proliferation to a statistically significant extent (Figure 7C, D).

### 5 **3.3. B19V in EPCs, cidofovir and infectivity**

6 To test yield and infectivity of virus released from EPCs, the cell culture supernatants harvested at  
7 24 hpi from EPCs infected with B19V at the moi of  $10^4$ , and cultured in presence of different  
8 concentrations of CDV, were used for infection of UT7/EpoS1. For this purpose, UT7/EpoS1 cells  
9 were incubated for 2 hours in the presence of 50  $\mu$ L of the different supernatants, washed, then  
10 incubated for an additional time course of 72 hours. Quantitative analysis was carried out on cell  
11 culture supernatants, and on UT7/EpoS1 cell fractions harvested at 2 hpi and 72 hpi, for evaluation  
12 of B19V DNA content.

13 The amount of viral DNA present in supernatants derived from EPCs was proportional to what  
14 detected in the corresponding cellular fractions (Figure 9 left, compare with Figure 3B). The  
15 amount of B19V DNA present within UT7/EpoS1 cells at 2 hpi was then proportional to what  
16 present in the supernatant (Figure 9 centre), reflecting the release of infectious viral particles from  
17 infected EPCs. In these, a reduced amount of viral DNA was observed in the 500  $\mu$ M CDV  
18 samples. The increase in the amount of viral DNA within UT7/EpoS1 cells from 2 hpi to 72 hpi as a  
19 measure of relative viral infectivity was significantly lower for virus obtained from EPCs cultured in  
20 the presence of 500  $\mu$ M CDV (Figure 9 right). The overall reduction in virus yield and infectivity  
21 was 92.1% when comparing the 500  $\mu$ M sample to control in the absence of CDV. This result  
22 arises as the additive effect of the inhibition of replication within EPCs, with a lower production of  
23 infectious virions (68-70% reduction measured in supernatants and 2 hpi samples), and a reduced  
24 activity of viral DNA within UT7/EpoS1 cells (75% reduction measured in the 72 hpi samples).

#### 1 4. Discussion

2 The present study was aimed at investigating the potential of the broad spectrum antiviral drug  
3 CDV to inhibit parvovirus B19, a ssDNA virus responsible for a wide spectrum of clinical  
4 manifestations, and presently lacking both of a causative therapy and of a vaccine to prevent the  
5 infection. In our experiments, we could show that the antiviral activity of the CDV is also effective  
6 against this human pathogenic virus. This constitutes the first evidence of activity of an antiviral  
7 compound against B19V, and in the same time expands the antiviral range of CDV to encompass  
8 also a ssDNA virus in addition to the families of dsDNA viruses (De Clercq, 2007; De Clercq and  
9 Holy, 2005).

10 Due to the characteristics of B19V and its restricted tropism, only two cellular systems were aptly  
11 used, namely the UT7/EpoS1 cell line and *in vitro* differentiated EPCs. UT7/EpoS1 is the most  
12 permissive and commonly used cellular line when investigating B19V (Wong and Brown, 2006). In  
13 these, most of the cells possess the receptors for the virus and can be infected, but the intracellular  
14 environment is restrictive as active replication is shown to occur only in a subset of cells (Bonvicini  
15 et al., 2013b), possibly depending on still uncharacterised S-phase factors. At the population level,  
16 the degree of replication of viral DNA shows relative increases comparable to fully permissive  
17 systems, while major impairment occurs at the level of capsid protein production and yield of  
18 infectious virus (Gallinella et al., 2000). EPCs, on the other hand, represent a cellular system more  
19 closely resembling the natural target cells within bone marrow environment. Circulating EPCs can  
20 be cultivated from peripheral blood and induced to differentiate along the erythroid lineage,  
21 progressively acquiring sensitivity to viral infection and full permissiveness to viral replication  
22 (Filippone et al., 2010; Wong et al., 2008).

23 In the evaluation of a possible antiviral activity against B19V, the use of qPCR assays for  
24 determining the dynamics of viral nucleic acids production can offer several advantages linked to a  
25 highly accurate evaluation of replication activity within infected cells. In both cellular systems, a  
26 direct relationship was present between multiplicity of infection and detected amount of viral  
27 nucleic acids, such as viral DNA measured at 2 hpi (adsorbed/internalised virus), at 24/72 hpi  
28 (replicative activity), or RNA at 72 hpi (transcription). This relationship could be approximated by a

1 linear regression in the moi range of  $10^4$ - $10^1$  viral genomes per cell, and this allowed to calculate,  
2 for every infected cell sample and CDV concentration, the effect on the amount of viral nucleic  
3 acids produced (Y-axis shift), as well as the reduction in infectivity (X-axis shift) compared to a  
4 control reference titration in the absence of drug. Finally, these data could be expressed as  
5 percentage inhibition and allowed calculating standard  $EC_{50}$  or  $EC_{90}$  values.

6 An inhibitory effect of CDV was evident in UT7/EpoS1 cells as a regular dose-dependent inhibition  
7 of viral replicative activity, for all the moi tested, up to the point where 500  $\mu$ M was successful in  
8 reducing the amount of viral DNA present at 72 hpi to levels lower than input virus. This effect was  
9 less evident in peripheral blood derived EPCs, where only the the 500  $\mu$ M concentration showed a  
10 significant inhibition of viral replicative activity. This difference in the two cellular systems may be  
11 ascribed to a different permeability of cells to the compound, or to different mechanisms of  
12 interference with the normal metabolism of nucleotides. The antiviral activity of CDV is due to its  
13 incorporation in synthesised DNA that may hamper its template activity, or may induce chain  
14 termination (Magee and Evans, 2012). A different degree of incorporation in viral DNA, or a  
15 different activity of DNA repair mechanisms, can contribute to the diverse activity of CDV in normal  
16 cells like EPCs compared to a myeloblastoid cell line like UT7/EpoS1 (De Schutter et al., 2013).

17 CDV did not exert any significant cytotoxic or cytostatic effect in these cells, implying a basis for  
18 the selectivity of action against the virus that deserves further investigation. Southern blot analysis  
19 of viral DNA showed a reduction in the amount of viral ssDNA and dsDNA forms, rather than to  
20 presence of incomplete, terminated replicative intermediates or products. Finally, in the EPCs  
21 system where infectious viral particles were produced and released in the supernatant, the overall  
22 reduction in infectivity induced by 500  $\mu$ M CDV was also evident following a second cycle infection.

23 The  $EC_{50}$  and  $EC_{90}$  values determined in UT7/EpoS1 cells were close to those obtained for dsDNA  
24 viruses, having their own DNA polymerases (Williams-Aziz et al., 2005) or exploiting the cellular  
25 enzymes (Bernhoff et al., 2008). The demonstration of some efficacy of CDV against B19V should  
26 be considered of interest, and bears some promise to the development of antiviral drugs directed  
27 against a human pathogenic virus. B19V is highly dependent on cellular machinery and offers few  
28 virus specific targets, but the capacity of a broad spectrum antiviral compound to interfere with its

1 biological activity indicated the presence of pathways susceptible to potential inhibition in a very  
2 complex and as yet not fully characterised virus-cell interaction network.

3 The development and availability of a compound active against B19V may add therapeutic options  
4 to the treatment of infections, that now relies entirely on symptomatic or supportive rather than  
5 causative treatments. Inhibition of the replicative activity of the virus and reduction in the yield of  
6 infectious virus should be relevant when the pathology induced by the virus is mainly linked to an  
7 active replication and proapoptotic effect. Transient or persistent red cell aplasia may benefit from  
8 a reduction in the viral replication rate, while in the case of incompetence of the immune system to  
9 control the infection, a pharmacological inhibition of viral replication may be considered of high  
10 impact, given the substantial inefficacy and costs of IVIG infusion.

11 Further progress in our knowledge of the biological characteristics of B19V and of its interaction  
12 with different cellular environments will possibly lead to a better understanding of the viral targets  
13 suitable for a specific antiviral approach, but in the meantime some progress might also be  
14 obtained investigating the activity and mechanism of action of known compounds, such as CDV or  
15 related lipid conjugates (Rinaldo et al., 2010; Williams-Aziz et al., 2005), that are characterised by  
16 a broad spectrum antiviral activity.

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1 **Figure captions**

2  
3 Figure 1. Time course of B19V infection in UT7/EpoS1 cells and EPCs.

4 Amount of B19V DNA, determined by qPCR, in infected UT7/EpoS1 cells (A) or EPCs (B). Cells  
5 infected at the different moi have been collected at the indicated time points between 2 hpi and at  
6 72 hpi following culture in the absence of CDV. Log of B19V DNA target copies (geq/20000 cells)  
7 is plotted as a function of the time points. Values are mean of three independent experiments and  
8 bars indicate the standard error of means.

9  
10 Figure 2. B19V DNA as a function of moi.

11 Amount of B19V DNA, determined by qPCR, in infected UT7/EpoS1 cells (A) or EPCs (B). Cells  
12 infected at the different moi have been collected at 2 hpi and at 72 (A) or 24 (B) hpi following  
13 culture in the presence of the different concentrations of CDV. Log of B19V DNA target copies  
14 (geq/20000 cells) is plotted as a function of the Log of moi. Values are mean of three independent  
15 experiments and bars indicate the standard error of means. Lines are linear regression analysis for  
16 the respective experimental series. The slopes of the lines are not statistically different (for  
17 UT7/EpoS1,  $p=0.31$ ; for EPCs,  $p=0.19$ ), while elevations are ( $p<0.0001$ ).

18  
19 Figure 3. Analysis of variance in the amount of B19V DNA as a function of CDV concentration.  
20 Amount of B19V DNA, determined by qPCR, in UT7/EpoS1 cells (A) or EPCs (B), infected at  
21 different moi and cultivated for 72 (A) or 24 (B) hpi in the presence of the different concentrations  
22 of CDV. Values are mean of three independent experiments and bars indicate the standard error of  
23 means. Analysis of variances among Log of B19V DNA target copies (geq/20000 cells) within a  
24 given moi indicate differences in B19V replicative activity compared to control sample (0  $\mu$ M). \* p  
25 value 0.01 – 0.05, \*\* p value 0.001 – 0.01, \*\*\* p value < 0.001.

26  
27 Figure 4. Analysis of variance in infectivity of B19V as a function of CDV concentration.

1 Variation in infectivity for B19V in UT7/EpoS1 cells (A) or EPCs (B), following a time course of  
2 infection, determined by interpolation of quantitative values obtained for the different experimental  
3 conditions on the reference curve obtained in the absence of CDV. Values are mean of three  
4 independent experiments and bars indicate the standard error of means. The experimental  
5 infectious titre is equivalent to a nominal moi in the absence of CDV. \* p value 0.01 – 0.05, \*\* p  
6 value 0.001 – 0.01, \*\*\* p value < 0.001.

7  
8 Figure 5. Normalised response to CDV in B19V infected UT7/EpoS1 cells.

9 The plot shows the normalised response curve (% inhibition) as a function of CDV concentration  
10 for the different moi of B19V in UT7/EpoS1 cells. EC<sub>50</sub> and EC<sub>90</sub> values derived from this plot are  
11 reported in Table 1.

12  
13 Figure 6. Analysis of variance in the amount of B19V RNA as a function of CDV concentration.  
14 Amount of B19V total RNA, determined by qPCR, in UT7/EpoS1 cells (A) or EPCs (B), infected at  
15 different moi and cultivated in the presence of the different concentrations of CDV. Values are  
16 mean of three independent experiments and bars indicate the standard error of means. Analysis of  
17 variances among Log of B19V RNA target copies (geq/20000 cells) within a given moi indicate a  
18 significant reduction in transcriptional activity only for high moi and 500 µM CDV compared to  
19 control (0 µM), only for UT7/EpoS1 cells \*\* p value 0.001 – 0.01.

20  
21 Figure 7. Effects of CDV on viability and proliferation of UT7/EpoS1 cells (A, B) and EPCs (C, D).  
22 Results of alamarBlue assay (A, C) as an indicator of cell metabolic activity are expressed as  
23 relative fluorescence intensity units (RFU). Results of BrdU incorporation as an indicator of cell  
24 proliferation for are expressed as optical density (OD) values.

25  
26 Figure 8. Molecular forms of B19V DNA in infected EPCs.  
27 Southern Blot analysis of B19V DNA obtained from EPCs infected at the moi of 10<sup>4</sup> geq/cell, in the  
28 absence or presence of 500 µM CDV, following a time course of infection. After EcoRI cleavage,

1 standard agarose gel electrophoresis and alkaline downward blotting on Nylon membrane,  
2 hybridization was carried out by using a full-length digoxigenin-labelled DNA probe, recognised by  
3 anti-digoxigenin Fab, alkaline phosphatase conjugated, and NBT/BCIP colorimetric detection  
4 (Roche). Typical viral ssDNA and dsDNA forms are evident and the intensity of bands correlate  
5 with the amount of DNA determined by qPCR.

6

7 Figure 9. Infectivity of B19V released from CDV-treated EPCs.

8 Cell culture supernatants harvested at 24 hpi from EPCs infected with B19V at the moi of  $10^4$ , and  
9 cultured in presence of different concentrations of CDV, were used for infection of UT7/EpoS1 cells  
10 to test infectivity of virus released from cells. Values are mean of three independent experiments  
11 and bars indicate the standard error of means. Log amount of B19V DNA detected in aliquots of  
12 the supernatant (5  $\mu$ L volume), at 2 hpi and at 72 hpi (20000 cells) is shown for the different  
13 concentrations of CDV. \*\*\* p value < 0.001.

1 **Table 1**

2

	moi (geq/cell)			
	10 <sup>1</sup>	10 <sup>2</sup>	10 <sup>3</sup>	10 <sup>4</sup>
UT7/EpoS1				
EC <sub>50</sub> [μM]	41.27	29.65	7.45	34.35
EC <sub>90</sub> [μM]	89.42	84.73	85.45	360.70
EPCs				
% inhibition	92.78	71.29	83.89	68.21

3

4 For UT7/EpoS1 cells, EC<sub>50</sub> and EC<sub>90</sub> [μM] values for the different moi were obtained by non-linear  
5 regression analysis from the percentage inhibition values obtained for each different concentration  
6 of CDV. For EPCs, only percentage inhibition values for the CDV concentration of 500 μM relative  
7 to control could be calculated.

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