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Hydroxyurea inhibits parvovirus B19 replication in erythroid progenitor cells

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ABSTRACT

Parvovirus B19 (B19V) infection is restricted to erythroid progenitor cells (EPCs) of the human bone marrow, leading to transient arrest of erythropoiesis and severe complications mainly in subjects with underlying hematological disorders or with immune system deficits. Currently, there are no specific antiviral drugs for B19V treatment, but identification of compounds inhibiting B19V replication can be pursued by a drug repositioning strategy. In this frame, the present study investigates the activity of hydroxyurea (HU), the only disease-modifying therapy approved for sickle cell disease (SCD), towards B19V replication in the two relevant cellular systems, the UT7/EpoS1 cell line and EPCs. Results demonstrate that HU inhibits B19V replication with EC_{50} values of 96.2 μM and 147.1 μM in UT7/EpoS1 and EPCs, respectively, providing experimental evidence of the antiviral activity of HU towards B19V replication, and confirming the efficacy of a drug discovery process by drug repositioning strategy. The antiviral activity occurs *in vitro* at concentrations lower than those affecting cellular DNA replication and viability, and at levels measured in plasma samples of SCD patients undergoing HU therapy. HU might determine a dual beneficial effect on SCD patients, not only for the treatment of the disease but also towards a virus responsible for severe complications.

1. Introduction

Parvovirus B19 (B19V) is a human virus in the Parvoviridae family with an ample pathogenic potential [1–4]. Common clinical manifestations are erythema infectiosum in children, arthropathies mainly in adults, however the virus has been associated to many other different pathologies and inflammatory processes affecting diverse tissues and organs. A main characteristic of B19V is its selective tropism for erythroid progenitor cells (EPCs) in the bone marrow, or the equivalent during fetal development. As a consequence, B19V infection can result in chronic pure red blood cell aplasia mostly in individuals with compromised immune system or transient aplastic crisis (TAC) in patients under conditions of hematopoietic stress in which there is increased red cell destruction worsening anemia [5,6]. This complication is particularly common in young children with sickle cell disease (SCD), and less frequent, but present, in adults [7–9]. In these subjects, red blood cell (RBC) transfusions are adequate to treat a transient aplastic episode reducing the risk of circulatory collapse from severe anemia, or other B19V-related events (marrow necrosis, acute splenic sequestration, acute chest syndrome, fat embolism) [10–12]. RBC transfusions are also indicated to overcome chronic anemia and, in case of fetal infection, intrauterine transfusions are a therapeutic option in the treatment of fetal anemia and non-immune hydrops associated to B19V [13]. Chronic anemia due to persistent infection is responsive to intravenous immunoglobulin (IVIG) administrations, but high dosage and repeated courses may be necessary, and symptoms can recur when IVIG treatment is interrupted [14,15]. Currently, there are no recognized antiviral drugs as an alternative to the supportive treatments however in certain B19V-infected patients a specific therapy would provide a substantial benefit preventing bone marrow failure or inflammatory aspects of the infection.

The absence of the complete characterization of B19V proteins and related cellular machinery interactions makes difficult a rational drug design strategy. The NS protein, with its replicative activities and effects on cellular environment, and the phospholipase domain in the VP1u region may constitute relevant therapeutical targets [3,4], however these proteins have not been characterized to the availability of crystallographic structures, and entries in UniprotKB database only refer to predicted domains [16], thus preventing accurate target-based molecular modeling. Also, the interactions of viral proteins with cellular counterparts

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are only partially described and understood [3,4]. Thus, efforts in the search for compounds inhibiting B19V replication can be aptly directed towards a drug repositioning approach. anti-B19V drug discovery can take advantage of the availability of two cellular systems, the myeloblastoid cell line UT7/EpoS1 and the human primary EPCs able to support B19V replication [17], thus suitable to test antiviral drugs. On the other hand, animal *in vivo* models, although possible, are not easily amenable to experimental use for this purpose [18,19].

In this frame, the anti-B19V activity of cidofovir has been demonstrated, widening the antiviral spectrum of the acyclic nucleoside phosphonate [20,21]. The drug exerts a potent antiviral effect in infected myeloblastoid cell line UT7/EpoS1 and a relevant inhibitory activity in primary human EPCs when extendedly exposed to high doses [22,23], thus limiting its potential use *in vivo*.

This evidence encourages the search for other anti-B19V candidates and, herein, the repositioning opportunity of hydroxyurea (HU) in the treatment of B19V infection was evaluated. HU is an S-phase inhibitor of DNA synthesis targeting cellular ribonucleotide reductase (RR) enzyme [24], thus leading to the inhibition of cell proliferation, including the hematopoietic cell compartment that is involved in the pathogenesis of B19V infection. The potential use of HU as antiviral agent has been previously explored both *in vitro* and *in vivo* in combined therapy with deoxynucleoside analogs with the assumption that HU depletes the intracellular deoxyribonucleotide pools required for viral replication, enhancing deoxynucleoside analogs incorporation [25–28]. Finally, the drug is the only disease-modifying therapy approved by FDA for SCD in adults [29,30], and its "off-label" use is increasing in the pediatric SCD population [31–35], in which B19V is cause of severe complications.

Given all these observations, the present study investigates the possible inhibitory effects of HU against B19V replication in the two relevant model cellular systems, UT7/EpoS1 cells and in EPCs, by means of quantitative evaluation of viral nucleic acids synthetized within a time course of infection. In addition, a complete characterization of the cellular effects of HU in the selected experimental systems was carried out including viability, proliferation, and cytotoxicity, together with the immunophenotypic characterization of erythroid-associated markers and cell cycle analysis by flow cytometry in the EPCs.

2. Materials and methods

2.1. Drug

Hydroxyurea (HU) was purchased from Sigma-Aldrich (St. Louis, MO, USA) at a purity degree >98%, solubilized in water and filter sterilized. Aliquots were stored at −20°C and used in the experiments in the range 0.1–50 mM.

2.2. Cells

UT7/EpoS1 cells were cultured in Iscove modified Dulbecco medium (IMDM) (Sigma-Aldrich, St. Louis, MO, USA), containing 10% fetal bovine serum (Carlo Erba Reagents, Milan, Italy) and 2 U/mL rhu erythropoietin (NeoRecormon, Roche S.p.A, Monza, Italy), at 37°C and 5% CO_2. Cells were used for experiments 24 h after a fresh medium exchange, when at a density of 3–5 × 10^5 cells/mL.

For the generation of EPCs from peripheral blood mononuclear cells (PBMC), a previously established protocol was adopted [17]. PBMC were obtained from leukocyte-enriched buffy coats of anonymous blood donor, available for institutional research purposes from the Immunohematology and Transfusion Service (S. Orsola-Malpighi University Hospital, Bologna, Italy, authorization 0070755/1980/2014, issued by Head of Service). Following isolation by Ficoll-Paque centrifugation (GE Healthcare Bio-Science AB, Uppsala, Sweden), PBMC were cultured in IMDM supplemented with 20% serum substitute BIT 9500 (StemCell Technologies, Vancouver, Canada), and enriched with 900 ng/mL ferrous sulphate, 90 ng/mL ferric nitrate, 1 μM hydrocortisone, 3 U/mL rhu erythropoietin, 5 ng/mL rhu IL-3 and 20 ng/mL rhu stem cell factor (SCF) (Life Technologies, Carlsband, CA, USA). Cells were maintained at 37°C in 5% CO_2 and kept at 10^6 cells/mL until experiments.

2.3. Cell proliferation and cell viability assays

Cell proliferation measurements were assessed in UT7/EpoS1 cells and EPCs, at 8 ± 2 days from isolation, by evaluating the 5-bromo-2′-deoxyuridine (BrdU) incorporation into the newly synthetized genomic DNA in a 48 h-period (Cell proliferation ELISA BrdU Assay, Roche Diagnostics, Indianapolis, IN, USA). For analysis, cells at a density of 5 × 10^4 cells/100 μL were seeded in 96-well plate and grown in regular medium or supplemented with different concentrations of HU (0.1–50 mM). At 24 h, 10 μM of BrdU were added to cells, the cells were harvested at 48 h and the amount of labeled DNA in the cell population was measured according to manufacturer's instructions. Data were expressed as mean percentage values of treated cells relative to untreated controls.

The assessment of cellular viability and metabolic function was carried out through the measurement of mitochondrial dehydrogenase enzyme activity by the WST-8 based assay (Cell Counting Kit-8, CCK-8, Dojindo Molecular Technologies, Rockville, MD, USA). For these experiments, UT7/EpoS1 cells and EPCs were seeded at the density of 5 × 10^4 cells/100 μL in a 96-well plate, and cultured for 48 h with the different concentrations of HU (0.1–50 mM). After addition of the CCK-8 solution, the amount of the formazan dye generated by dehydrogenases in living cells was measured according to manufacturer's instructions. Data were expressed as mean percentage values of treated cells relative to untreated controls.

The assessment of potential cytotoxic properties of HU on the investigated cellular systems was carried out by measuring the lactate dehydrogenase enzyme (LDH) released in the cell culture medium through damaged plasma membranes (Cytotoxicity LDH Assay kit-WST, Dojindo Molecular Technologies, Rockville, MD, USA). For the assay, cells were cultured at a density of 5 × 10^4 cells/100 μL in a 96-well plate with different concentrations of HU (0.1–50 mM), and, following 48 h of incubation, the cell-free supernatants (100 μL) were recovered from each well, and the LDH activity was determined following manufacturer's instructions. HU cytotoxicity was expressed as percentage values relative to the 100% lysis controls, included in the test, and untreated controls.

2.4. B19V infection

UT7/EpoS1 cells and EPCs, at 8 ± 2 days from isolation, were infected with B19V at a multiplicity of infection (moi) of 10^4 viral genomes/cell. The inoculum virus consisted in a B19V viremic serum sample, containing 10^{12} B19V (genotype 1) genome copies (geq)/mL, as determined by qPCR assay [36], available for research purposes according to the Italian privacy law. The viremic serum resulted negative to B19V specific IgM and IgG antibodies by a commercial chemiluminescence immunoassay (Liaison XL, Diasorin S.p.A, Italy) and to other viruses (HIV, HBV, HCV, HSV, VZV, EBV, CMV, HHV8, AdV, BKV) by nucleic acid testing (NAT) assays.