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Original article

Foscarnet reduces FGF2-induced proliferation of human umbilical vein endothelial cells and has antineoplastic activity against human anaplastic thyroid carcinoma cells

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ABSTRACT

In contrast to many reports dealing with inhibitors of growth factor receptors like VEGF or FGFR, only few reports of low molecular weight inhibitors, which are directed against growth factors itself, are known. Here, foscarnet, an antiviral drug which inhibits several viral DNA polymerases by mimic pyrophosphate of nucleotides, was identified to interact with fibroblast growth factor 2 and stabilize the growth factor against tryptic digestion similar like the non-nitrogen containing bisphosphonates clodronate and etidronate that we have reported just recently as inhibitors of FGF-induced cell proliferation. Foscarnet competes with ATP against the binding on fibroblast growth factor 2 at the heparin/ATP-binding domain. This indicates binding of foscarnet at the heparin-binding domain of FGF2. This interaction of foscarnet with fibroblast growth factor 2 reduces FGF2-induced proliferation of human umbilical vein endothelial cells and intracellular signaling via ERK1/2 kinases in this cell line. Additionally, foscarnet reduces in a dose-dependent manner proliferation of CAL-62 cells that belong to anaplastic thyroid carcinoma, a rare but lethal type of thyroid cancer that expresses FGF2.

1. Introduction

Low molecular weight inhibitors (e.g. sunitinib or sorafenib) of growth factor receptors like VEGFRs or FGFRs are known and used in clinic for several cancer species as an antiangiogenic therapy option [1]. In contrast, to our knowledge low molecular weight inhibitors of fibroblast growth factor 2 (FGF2) itself are not under clinical investigation. Recently we demonstrated that some non-nitrogen-containing bisphosphonates like clodronate and etidronate interact with the growth factor FGF2 and reduce FGF2-induced proliferation of endothelial cells [2]. This inhibitory activity of the bisphosphonates (BPs) probably based on binding to FGF2 and subsequent change of the secondary structure of the growth factor. Interestingly, the phosphonate groups of the BPs are important for the interaction with FGF2. These phosphonate groups probably bind to positively changed amino acids located in the heparin-binding domain (HBD) of FGF2 [2].

First hints that there is an interaction of bisphosphonates with FGF2 based on growth factor stabilizing experiments. ATP as well as clodronate and etidronate are able to stabilize and protect FGF2 from tryptic digestion [2,3].

Foscarnet (Fig. 1) is a broad spectrum antiviral mimicking the pyrophosphate group of nucleotides that is the leaving group from nucleotide transfer reactions [4]. Foscarnet is used for the treatment of cytomegalovirus (CMV) disease as well as herpex simplex, varicella zoster and HIV [5–8]. The first crystal structure of foscarnet bound to CMV-DNA polymerase is presented just recently [4].

Here, we report the first time interaction of foscarnet with FGF2. This interaction was investigated with a focus on the influence on FGF2-induced proliferation of endothelial cells as well as on influence on proliferation of anaplastic thyroid cancer cells (CAL-62). Anaplastic thyroid cancer is a rare but rapidly lethal carcinoma [9] with no therapeutic options for cure or prolonged survival [10].

2. Materials and methods

2.1. Material

Foscarnet was obtained from Sigma-Aldrich (Taufkirchen, Germany). All antibodies were purchased from SantaCruz Biotechnology (Santa Cruz, USA). Fibroblast growth factor 2 (FGF2) as well as variant form FGF2(K134A) were expressed and purified as described before [11]. Human umbilical vein endothelial cells (HUVECs) and endothelial growth medium were purchased from Promocell (Heidelberg, Germany). Human thyroid anaplastic

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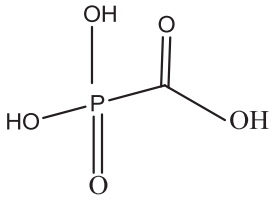

Foscarnet

Fig. 1. Structure of foscarnet.

carcinoma cell line CAL-62 was obtained from German Collection of Microorganisms and Cell Cultures (DMSZ, Braunschweig, Germany).

2.2. Proteolytic cleavage of FGF2

Protection of FGF2 and FGF2(K134A) against proteolytic digestion by trypsin was anlysed by incubating FGF2 (2 μg; final concentration: 5 μM) in the presence or absence of foscarnet (25 μM). FGF2 was preincubated with foscarnet in a volume of 20 μL in 25 mM Tris-HCl (pH 7.5) at 37 °C for 15 min. Then trypsin (50 ng) was added and incubation was continued for an additional 2 h. Trypsin activity was stopped by addition of sample buffer and heating at 95 °C for 5 min. The products of protease cleavage were analysed by SDS-PAGE with 15% minigels and proteins were visualized by silver staining.

2.3. Immunoblot analysis

Cell lysates of HUVECs were produced by sonication and by using phosphatase inhibitor cocktail 3 (Sigma-Aldrich, Taufkirchen, Germany) together with 1 mM Na-vanadate and 5 mM NaF. Proteins (30 μg) were electroblotted onto nitrocellulose membranes and incubated overnight with mouse α-tubulin antibody (1:10,000; all antibodies: SantaCruz Biotechnology, USA), ERK1/2 (1:400) and p-ERK1/2 (1:400). Secondary antibody (1:2500) was added at room temperature for 1 h.

2.4. Cell proliferation assay

HUVECs and CAL-62 proliferation were assayed with the Cell Titer 96AQ Assay kit according to the manufacturer's instruction (Promega, Mannheim, Germany). In brief, HUVECs were plated on 96-well-plates at 8×10^3 cells/well in 100 μL endothelial growth medium (EGM) with supplements (Promocell, Heidelberg, Germany) for 24 h. Then the medium was replaced by endothelial basal medium (EBM) containing bovine serum albumin (0.1% (w/v)) instead of supplements. After 1 h of incubation, medium was replaced by EBM containing FGF2 (20 ng/mL), preincubated for 15 min at 37 °C with or without foscarnet.

CAL-62 cells were plated on 96-weel-plates at 8×10^3 cells/well in 100 μL DMEM (PAA, Pasching, Austria) for 24 h. Then the medium was replaced by fresh DMEM with or without foscarnet. After 48 h of incubation, 20 μL of CellTiter 96AQ solution was added to the cells and incubated for additional 2–4 h. The quantity of formazan product as measured by the absorbance at 492 nm is directly proportional to the number of living cells. The assay was performed in duplicate ($n = 10$).

2.5. CD measurement

Far-UV spectra (195–250) were recorded at FGF2 concentration of 25 μM with a Jasco-J600 spectropolarimeter at room temperature in a 0.1-cm cell with four accumulations. Measurements were performed in 25 mM Tris-HCl (pH 7.5), and all spectra were corrected for buffer and foscarnet background. The results are expressed as ellipticities related to the mean residue weight of amino acids as described before [12].

2.6. Labeling of FGF2 with [γ-^{32}P]ATP

FGF2 was radiolabeled as described before [11]. Briefly, FGF2 (2 μg) was incubated in a volume of 15 μL with 6 μCi [γ-^{32}P]ATP. In some samples foscarnet was added. After incubation for 15 min at 37 °C, reactions were stopped by adding SDS-PAGE sample buffer. Radiolabeled FGF2 was separated by SDS-PAGE with 15% minigels. The gels were dried and finally phosphorylation was detected by autoradiography or phosphoimaging using Fujifilm BAS-1800 II imager (Fuji, Japan).

2.7. Statistics

Differences between experimental groups were evaluated by the analysis of variance (ANOVA) followed by Scheffe's test for multiple comparisons.

3. Results

3.1. Foscarnet stabilizes FGF2 against proteolytic digestion

ATP binds to FGF2 at the heparin-binding domain (HBD) of the growth factor [11,13]. Interaction of some bisphosphonates with FGF2 is probably located at this binding domain [2]. Binding of bisphosphonates on FGF2 was detected indirectly by the ability to stabilize the growth factor against proteolytic digestion [3]. The phosphate/phosphonate groups of the ligands are important for this interaction. Furthermore, interaction of clodronate with FGF2 reduces FGF2-induced proliferation of HUVECs. Here we tested foscarnet (phosphonoformic acid), an antiviral drug and a structural mimic of pyrophosphate. Interestingly, pyrophosphate was identified as a moderately active stabilizer of FGF2 just recently [12].

Foscarnet is also able to stabilize FGF2 against tryptic digestion; it can protect FGF2 (5 μM) partially against trypsin degradation at concentrations of 25 μM and, more distinct, at 100 μM (Fig. 2). As a control we demonstrated that foscarnet itself is not able to inhibit trypsin activity at the used concentration of 100 μM (data not shown).

3.2. Heparin/ATP-binding domain of FGF2 is involved in foscarnet-binding

Interestingly, no stabilizing effect of foscarnet could be obtained when using the variant form FGF2(K134A) (Fig. 3). FGF2(K134A) is rapidly degraded by trypsin independently of present foscarnet. This FGF2-variant form possesses an amino acid replacement located at the HBD leading to a drastically reduced heparin and ATP-binding capacity of the growth factor [11,14].

Furthermore, ATP and foscarnet compete against the same binding domain as demonstrated in Fig. 4. Increasing foscarnet concentrations lead to a reduced ability of FGF2 to bind radioactive labeled ATP. For example, addition of 100 μM foscarnet to FGF2 reduces the ATP-binding capacity of the growth factor significantly.

3.3. Moderate change of secondary structure of FGF2 after foscarnet-binding

We collected CD spectra of FGF2 alone and FGF2 preincubated with foscarnet to investigate putative conformational changes of