Nuclear Medicine and Biology 43 (2016) 27–34

Contents lists available at ScienceDirect

Nuclear Medicine and Biology

journal homepage: www.elsevier.com/locate/nucmedbio

In vivo monitoring of the anti-angiogenic therapeutic effect of the pan-deacetylase inhibitor panobinostat by small animal PET in a mouse model of gastrointestinal cancers

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ARTICLE INFO

Article history:
Received 19 June 2015
Received in revised form 29 September 2015
Accepted 16 October 2015

Keywords:
Histone deacetylase inhibitor
Panobinostat
Colon cancer
Hepatocellular carcinoma
Positron emission tomography
RGD peptide

ABSTRACT

Introduction: Deacetylase inhibitors have recently been established as a novel therapeutic approach to solid and hematologic cancers and have also been demonstrated to possess anti-angiogenic properties. Although these compounds show a good efficacy in vitro and in vivo, no data on monitoring and predicting treatment response are currently available. We therefore investigated the effect of the pan-deacetylase inhibitor panobinostat (LBH589) on gastrointestinal cancer models and the suitability of 2-[^{18}F]FGlc-RGD as a specific agent for imaging integrin $\alpha_v\beta_3$ expression during tumor angiogenesis using small animal positron emission tomography (PET).
Methods: The effect of panobinostat on cell viability in vitro was assessed with a label-free impedance based real-time analysis. Nude mice bearing HT29 and HepG2 tumors were treated with daily i.p. injections of 10 mg/kg panobinostat for 4 weeks. During this time, tumor size was determined with a calliper and mice were repeatedly subjected to PET imaging. Tumor tissues were analyzed immunohistochemically with a focus on proliferation and endothelial cell markers (Ki-67, Meca-32) and by Western blot applying specific markers of apoptosis.
Results: In vitro, panobinostat inhibited the proliferation of HepG2 and HT29 cells. Contrary to the situation in HepG2 tumors in vivo, where panobinostat treatment is known to reduce proliferation and vascularization resulting in a decreased tumor growth, HT29 tumors did not show any effect on these parameters. We demonstrated by Western blotting, that panobinostat induced apoptosis in HT29 tumors in vivo. Longitudinal PET imaging studies in HepG2 tumor-bearing mice using 2-[^{18}F]FGlc-RGD demonstrated that the standard uptake value (SUV_{max}) in HepG2 tumors was significantly decreased by 39% at day 7 after treatment. The comparative PET study using HT29 tumor-bearing animals did not reveal any response of the tumors to panobinostat treatment.
Conclusions: Small-animal PET imaging using 2-[^{18}F]FGlc-RGD was successfully applied to the non-invasive monitoring of the HepG2-tumor response to panobinostat in nude mice early after begin of treatment. Thus, PET imaging of angiogenesis using 2-[^{18}F]FGlc-RGD could be a valuable tool to monitor panobinostat therapy in further preclinical studies.
Advances in knowledge and implications for patient care: When successfully translated to the clinical surrounding, PET imaging of angiogenesis could therefore facilitate therapy planning and monitoring of therapy success with panobinostat in hepatocellular carcinoma.

1. Introduction

Colorectal cancer is one of the most common malignancies with high incidence and mortality. About 800,000 new cases are diagnosed each year and approximately half a million patients have been estimated to die by colorectal cancer in the United States [1–3].

Hepatocellular carcinoma (HCC) is the most common primary tumor of the liver and represents the 2nd and 5th most common cause of cancer related death in men and women, respectively, worldwide [2,4]. Most cases occur in pre-existing liver cirrhosis [5], particularly due to chronic viral hepatitis, alcohol abuse or hemochromatosis.

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http://dx.doi.org/10.1016/j.nucmedbio.2015.10.003
0969-8051/

Inhibitors of histone deacetylases (HDAC) have been established as potent novel anticancer therapies in hematologic and solid tumors [6–8]. Panobinostat (LBH589) is a pan-deacetylase inhibitor showing strong pro-apoptotic and anti-proliferative effects in various tumor entities [9,10]. Moreover, in the subcutaneous HepG2 xenograft model of HCC, it has been shown that daily i.p. injections of 10 mg/kg panobinostat lead to a significant growth delay mediated by reduced angiogenesis in tumor xenografts, as shown by endothelial cell staining with the Meca-32 antibody indicating the significantly decreased microvessel density after panobinostat treatment [11]. In addition, we previously demonstrated that panobinostat leads to a dose-dependent down-regulation of VEGF and CTGF in HepG2 and Hep3B cells in vitro and in vivo [12].

Treatment opportunities and monitoring options, including positron emission tomography (PET) methodology [13], have to be further improved for their wider availability. Especially the context between tumor proliferation and neoangiogenesis including the influence of $\alpha_v\beta_3$-integrins has been investigated very rarely [14–16].

In the field of molecular imaging, PET has emerged as an imaging modality with superior sensitivity for the predictive in-vivo imaging of therapy success. Among the key players of molecular targets that are regulated by angiogenesis, the $\alpha_v\beta_3$-integrin can be addressed by radiolabeled RGD-containing peptides. Various radiolabeled cyclic RGD peptides have been described so far [17,18]; among these, the glycopeptide [^{18}F]Galacto-RGD [19] is the most extensively validated substance that has been evaluated in clinical studies [20,21]. Following the concept of 'click chemistry' introduced by Sharpless et al. [22], we have successfully developed a glycosylated RGD peptide (2-[^{18}F]FGlc-RGD) for PET imaging of integrin $\alpha_v\beta_3$ expression as a marker of tumor angiogenesis in vivo [23].

In this work, we aim at studying the effect of the pan-deacetylase inhibitor panobinostat (LBH589) in tumor xenograft models and the suitability of non-invasive small-animal PET imaging with 2-[^{18}F]FGlc-RGD to assess anti-angiogenic effects as a prognostic marker for tumor response.

2. Materials and methods

2.1. Cell culture

HT29 colon adenocarcinoma and HepG2 hepatocellular carcinoma cell lines were grown as monolayers in RPMI-1640 and Dulbecco's modified Eagle's medium (DMEM), respectively, supplemented with 10% fetal calf serum and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were routinely tested for contamination with mycoplasma and tests were always negative. All cell culture medium components were obtained from Biochrom AG, Berlin, Germany. Panobinostat was kindly provided by Novartis Pharma GmbH, Basel, Switzerland and processed as described previously [11].

2.2. Impedance-based real-time cell viability analysis

Cell viability, IC_{50} and cell doubling times were determined by continuous label-free measurement using the xCELLigence RTCA system (Roche Molecular Diagnostics, Mannheim, Germany) as described previously [24]. In brief, 10,000 cells/well were seeded to 96-well E-Plates. After 24 h cells were treated with different concentrations of panobinostat (0.1–10 μM). Cell index, an arbitrary unit defined as (Rn-Rb)/15Ω, with Rn being the impedance of the well containing cells and Rb being the background impedance of the well with medium only, was continuously measured every 5 min for 72 h. Results were normalized to the time point of treatment and expressed as mean of three wells per treatment group. The doubling time was calculated with the formula "cell index = A * 2^(t/cell index doubling time)". IC_{50} values were calculated from a sigmoidal dose-response curve according to the formula "bottom + (top − bottom)/(1 + 10^(x − log IC_{50}))".

2.3. Analysis of cell proliferation

Proliferation rate was validated by counting the number of viable cells in a Neubauer chamber after staining with Trypan blue at the indicated time points and concentrations of panobinostat. Results for this assay were then correlated with xCELLigence data and analyzed statistically.

2.4. Xenograft models

All animal experiments were performed in compliance with the protocols approved by the local Animal Protection Authorities (Regierung Mittelfranken, Germany, no. 54-2532.1-15/08). Cells were harvested and resuspended in sterile physiologic NaCl solution. 5×10^6 cells were injected subcutaneously into the flank of 6–8 week old male athymic nude mice (Harlan Winkelmann GmbH, Germany). Nine animals were used for each treatment group. Animals were kept in a light- and temperature-controlled environment and provided with food and water ad libitum. Tumor size was determined three times a week by measurement using a calliper square. When subcutaneous tumors reached a diameter of 7 mm, mice were treated with daily intraperitoneal injections of 10 mg/kg panobinostat for up to four weeks as described previously [11]. Untreated animals served as a control group. During the treatment period, mice were repeatedly subjected to PET imaging. At the end of the treatment period, mice were sacrificed and tumor specimens were snap-frozen in liquid nitrogen or fixed in phosphate-buffered formalin solution for further analyses.

2.5. Quantitative real time RT-PCR

Isolation of total RNA and synthesis of cDNA was performed as described previously [25]. Quantitative real-time PCR was performed on a CFX96 system (BioRad) using QuantiTect primers obtained from Qiagen (Hilden, Germany) as described. Results were normalized to GAPDH content for each sample.

2.6. Immunohistochemistry

Formalin-fixed tumor specimens were embedded in paraffin. 5 μm sections were cut and processed as described previously [11]. Routine H&E staining was performed to assess basic histomorphology. Proliferation rate was determined after staining with an anti-Ki-67 antibody (Dako, Glostrup, Denmark) and determining of positively stained nuclei in four representative high power fields (HPF) per section in relation to all nuclei in the section counterstained with hematoxylin as described. The Ki-67 labeling index is defined as percentage of Ki-67 positive nuclei per HPF. Endothelial staining was performed on snap frozen sections using the anti-MECA-32 antibody produced in a rat hybridoma cell line [11,26].

2.7. Western blot analysis of procaspase-3, caspase-3 and PARP cleavage

Western blot analysis was performed as described previously [27]. Briefly, the lysates of cultured HT29 or HepG2 cells were extracted by resuspending the cell pellet in appropriate volume (250–450 μl) of lysis buffer (4 M urea, 0.5% SDS, 62.5 mM Tris, protease inhibitor, 1 mM PMSF, pH = 6.8). After incubation on ice for 60 min, the lysate was resuspended by vortexing and the proteins were further solubilized by 10–15 cycles of ultrasonic pulses. Protein lysates of xenograft HT29 and HepG2 tissue were generated by resuspending 50 mg of the respective tissue in 500 μl lysis buffer. After 15 s of homogenization (level 6; Ultra-turax homogenisator, IKA) the lysates were incubated on ice for 30 min. Afterwards, the protein lysate was separated from the cell