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Flubendazole induces mitotic catastrophe and apoptosis in melanoma cells

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ABSTRACT

Flubendazole (FLU) is a widely used anthelmintic drug belonging to benzimidazole group. Recently, several studies have been published demonstrating its potential to inhibit growth of various tumor cells including those derived from colorectal cancer, breast cancer or leukemia via several mechanisms. In the present study we have investigated cytotoxic effects of FLU on malignant melanoma using A-375, BOWES and RPMI-7951 cell lines representing diverse melanoma molecular types. In all three cell lines, FLU inhibited cell growth and proliferation and disrupted microtubule structure and function which was accompanied by dramatic changes in cellular morphology. In addition, FLU-treated cells accumulated at the G2/M phase of cell cycle and displayed the features of mitotic catastrophe characterized by formation of giant cells with multiple nuclei, abnormal spindles and subsequent apoptotic demise. Although this endpoint was observed in all treated melanoma lines, our analyses showed different activated biochemical signaling in particular cells, thus suggesting a promising treatment potential of FLU in malignant melanoma warranting its further testing.

1. Introduction

Metastatic melanoma is an aggressive form of skin cancer, with a high mortality rate. Although melanoma represents < 5% of all diagnosed skin cancers, the World Health Organization has indicated that its incidence is increasing faster than any other type of malignancy, mainly due to the general population's increasing exposure to ultraviolet light (Daud et al., 2017). The extensive danger of melanoma is based not only on the increasing incidence but also on its tendency to fast metastasizing and on extensive development of drug resistance - the biggest reason of treatment failure. While the prognosis of melanoma is promising with early diagnosis, upon its systemic spread where the effective therapy is still missing survival rates are significantly lower – the main reason for ongoing searches for new treatment options.

One of the promising strategies in the development of novel antioneoplastic medicines is drug repositioning (or repurposing). Drug repositioning is the process of searching for new indications of existing drugs (Shim and Liu, 2014). Repositioned drugs have the advantage of decreased development costs and decreased time to market compared to traditional discovery candidates, mainly due to the availability of previously collected pharmacokinetic, toxicology, and safety data (Padhy and Gupta, 2011). Among the potential pharmacological candidates for repurposing is also a group of benzimidazoles whose several members have already shown antitumor activities in various preclinical models (Michaelis et al., 2015; Nygren et al., 2013).

Flubendazole (FLU) is a derivative benzimidazole which has been extensively evaluated in humans and animals for the treatment of intestinal parasites as well as for the treatment of systemic worm infections (Spagnuolo et al., 2010). The mechanism of action of benzimidazole compounds in helmints can be explained by their increased affinity to tubulin, which leads to the inhibition of tubulin polymerization and results in the interference with microtubule-mediated transport in helminth tissues. Accordingly, benzimidazoles were found to interact with mammalian tubulin too, although their affinity is weaker in comparison to the helminth tubulin.

Cytotoxicity of various benzimidazoles including FLU has been proved in a number of in vitro and in vivo tumor models (Canova et al., 2017). FLU first showed its anti-tumor activity in leukemia and myeloma cells where at low and pharmacologically feasible concentrations it induced mitotic catastrophe and cell death and delayed tumor growth in vivo (Spagnuolo et al., 2010). Moreover, in recently published studies, FLU inhibited proliferation of several breast cancer cell lines (Hou et al., 2015), colon cancer cell lines (Kralova et al., 2016) and neuroblastoma cells (Michaelis et al., 2015). Despite the fact that currently no information exists about FLU effects in melanoma, reports on cytotoxic activity of mebendazole, a related benzimidazole compound, in

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melanoma cells as well as in xenographts appear promising (Doudican et al., 2008; Doudican et al., 2013) and suggest a wider scientific potential of other benzimidazole compounds including FLU in this type of malignancy.

Based on these facts, we have investigated the effect of FLU on three melanoma cell lines A-375, BOWES and RPMI-7951 representing diverse molecular types of this malignancy. Here we report that FLU inhibited the proliferation of all three employed cell lines with a similar IC50 equalling to 0.25 μM (RPMI-7951), 0.90 μM (BOWES) and 0.96 μM (A-375) at 72 h of exposure. FLU-dependent antiproliferative activity was associated with accumulation of cells at the G2/M phase of cell cycle and their extensive morphological changes including the specific alterations of the microtubular network. Thus treated cells finally underwent mitotic catastrophe characterized by formation of giant cells with multiple nuclei, abnormal spindles and caspase-dependent apoptosis. Although this endpoint was observed in all treated melanoma lines, our analyses showed different activated biochemical signaling in particular cells, thus suggesting a promising treatment potential of FLU in malignant melanoma warranting its further testing.

2. Materials and methods

2.1. Chemicals

Flubendazole was purchased from Janssen Pharmaceutica (Prague, Czech Republic), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, dithiotreitol (DTT), dimethylsulfoxide (DMSO), 4′,6-diamidino-2-phenylindole (DAPI), Triton-X, propidium iodide (PI), Dulbecco's Modified Eagle's Medium (DMEM), sodium dodecyl sulfate (SDS), bicinchoninic acid (BCA) kit for protein determination were purchased from Sigma-Aldrich (Prague, Czech Republic). Eagle's Minimum Essential Medium (EMEM) was supplied by LGC Standards (Lomianki, Poland), fetal bovine serum from Invitrogen (Carlsbad, CA, USA) and bovine serum albumin (BSA) from Fluka (Prague, Czech Republic). WST-1 was purchased from Roche Diagnostics (Manheim, Germany). Polyvinylidene difluoride (PVDF) membrane was obtained from BIO-RAD Laboratories (Prague, Czech Republic). All other chemicals were of highest analytical grade.

2.2. Cell culture and treatment conditions

Human melanoma cell lines were purchased from ATCC. A-375 (ATCC® CRL1619™) are adherent, epithelial cells hypotriploid with a modal number of 62 chromosomes whose *BRAF* and *CDKN2A* genes are mutated. BOWES (ATCC® CRL9607™) are adherent, epithelial, heteroploid cells with wild-type *BRAF*. RPMI-7951 (ATCC® HTB66™) are adherent, epithelial-like cells derived from metastasis to the lymph node, hyperdiploid (47–66 chromosomes) with mutant *BRAF*, *TP53*, *CDKN2A* and *PTEN*.

A-378 and BOWES were maintained in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin. RPMI-7951 was cultivated in EMEM medium with 10% FBS and 1% penicillin/streptomycin. Cells were maintained upon standard conditions (37 °C, 5% CO_2 in an incubator) and passaged twice a week upon 90% cells confluence using 0.05% EDTA/trypsin. Only mycoplasma-free cells were used for experiments.

Stock solutions of FLU were prepared in DMSO and stored in aliquots at 6 °C. Cells were treated with medium containing 1 μM FLU, the final concentration of DMSO in the medium was 0.1%. Control samples were treated with medium containing 0.1% DMSO.

2.3. Cytotoxicity assay

2.3.1. The WST assay

The cells were cultured in 96-well plates and treated with FLU in concentration range 0.01 μM–10 μM. After 24-, 48- and 72-hours exposure, the medium was removed and the cells were washed twice with 150 μl PBS. Then, 100 μl of culture medium containing WST-1 (0.3 mg/ml) was added to each well. Absorbance of the samples was measured immediately at 450 nm/650 nm wavelength (Tecan Infinite M200; Tecan, Salzburg, Austria). The samples were then placed in the incubator and the absorbance was measured again after 2 h of incubation. Each sample was assayed in 6 parallels and three independent experiments were performed. The viabilities of treated cells were expressed as percentage of untreated controls (100%).

2.3.2. Test of proliferation using the xCELLigence system

The system measures electrical impedance across interdigitated microelectrodes integrated at the bottom of tissue culture E-plates. The impedance measurement provides quantitative information about the biological status of the cells, including cell number, viability and morphology.

Ninety microliter of culture medium was added into each well and plates were inserted in the device for background measurement. Then, 100 μl of cell suspension (containing 1500 cells) was added in duplicate to the appropriate wells. The growing impedance (corresponding to cell proliferation) was measured every 30 min for 24 h. After 24 h, 10 μl of treatment medium was added into each well, so that final concentration of FLU was 1 μM. Plates were inserted back into the device and impedance was measured every hour for 72 h. Each sample was assayed in duplicate and three independent experiments were performed.

2.4. Time-lapse videomicroscopy

A-375, BOWES and RPMI-7951 cell lines were seeded into plastic tissue-culture dishes with glass bottom and left for 24 h in an incubator with 5% CO_2 at 37 °C. Next, the growth medium was replaced with a medium containing flubendazole. The tissue-culture dishes were transferred into a time-lapse imaging system BioStation IM (Nikon, Prague, Czech Republic) combining an incubator, a motorized microscope and a cooled CCD camera. Recording was carried out in a multipoint and multichannel manner employing various time-lapse modes and upon small as well as high magnifications to allow global as well as detailed view of changes in behavior of treated cell populations. Recorded sequences were subsequently semi automatically analyzed with the software NIS Elements AR 3.20 (Nikon, Prague, Czech Republic).

2.5. Cell cycle distribution analysis

2.5.1. Flow cytometry

Cells were cultured in 75 cm^2 culture flasks in 20 ml of culture medium for 12 and 24 h. Then the culture medium was replaced by fresh medium containing 1 μM of FLU or 0.1% DMSO as a control. After 12 or 24 h of treatment, the cells were trypsinized, collected by centrifugation, washed with PBS and fixed with ice-cold 70% ethanol while gently vortexing. Fixed samples were stored overnight in 4 °C. Then they were washed with PBS and incubated with 0.5 ml of the Vindelov solution (1.2 g/l TRIS, 0.6 g/l NyCl, 0.01 g/l RNase and 0.05 g/l propidium iodide) for 50 min at 37 °C. The samples were analyzed at the FC500 Cytomics Flow cytometer (Beckman Coulter,Hialeah, FL, USA) with PI fluorescence detected in FL3 channel. Cell cycle distributions in control and treated samples were analyzed with MultiCycle AV for Windows (Phoenix Flow Systems, San Diego, USA) and three independent experiments were performed.

2.5.2. Percentage of cells in S-phase by EdU labeling

EdU (5-ethynyl-2′-deoxyuridine) is incorporated into DNA during active DNA synthesis. The cells were cultured in 96-well plates and treated with FLU in concentration 1 μM. After 24 hours exposure, the cells were labeled with EdU (Click-iT® EdU Flow Cytometry Assay Kit,