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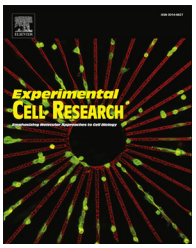

BH3 mimetics reduce adhesion and migration of hepatoblastoma and hepatocellular carcinoma cells

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ABSTRACT

Advanced stages of tumour and development of metastases are the two major problems in treating liver tumours such as hepatoblastoma (HB) and hepatocellular carcinoma (HCC), in paediatric patients. Modulation of apoptosis in HB cells enhances the sensitivity of these cells towards various drugs and has been discussed to enforce treatment. We analysed the effect of apoptosis modulators, BH3 mimetics, on mechanisms of dissemination such as adhesion or migration of HB and HCC cells.

BH3 mimetics such as ABT-737 and obatoclax can reduce cell migration in a scratch assay as well as adhesion of HB and HCC cells to matrigel. Immunofluorescence staining of *F*-actin demonstrated that development of lamellipodia, which are important for migration, decreased. BH3 mimetics increase the level of activated caspases 3 and 7 in HUH6 cells. This results in the degradation of GTPase Cdc42, which can be determined by western blot analysis. A pan-caspase inhibitor can block the migration and degradation of Rho-GTPase. In summary, our study showed that BH3 mimetics not only enhance drug sensitivity but also may prevent metastasis by inhibiting HB and HCC cell motility.

Introduction

Hepatoblastoma (HB) is the most common liver tumour in paediatric patients. Its annual incidence is very constant. In western countries, approximately 2.5 new diagnoses are made per 1 million children who are younger than 15 years [1]. Surgically complete tumour resection represents a realistic chance of cure for these patients. However, only 50–70% of all tumours can be resected at diagnosis [2]. Event-free survival (EFS) has improved in the last decade. For standard-risk patients, optimised chemotherapy increases the chance of tumour resection and complete cure to 95% [3]. Patients with recurrence of HB who exhibit lung metastases at the time of diagnosis are considered to be at high risk [5]. EFS of high-risk HB patients is less than 69% [4]. Therefore, more attention should be given to developing new treatment regimens to overcome multidrug resistance and dissemination during treatment.

Drug resistance, which develops in 80% of high-risk HB patients during chemotherapy [6], and metastasis are closely linked to apoptosis [7]. Tumour cells, including HB cells, evade the cellular homeostasis by expressing high levels of anti-apoptotic proteins such as BCL-2 [8,9]. Many anti-apoptotic proteins block cytochrome c release from the mitochondria by sequestering pro-apoptotic BH3-only proteins such as tBid, Bad and Bim [10,11]. Silencing experiments with small BH3 mimetic molecules have shown that these molecules enhance the anti-tumour activity of different drugs in in vitro and xenograft models of HB [9,12,13]. At present, different drugs have been developed using combinatorial chemistry that compete with the BH3 domain. Some of these drugs such as ABT-263, an oral

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counterpart of ABT-737 and obatoclax have been used in clinical trials on different malignancies [13,14]. The BH3 mimetic ABT-737 was developed by binding the BH3 domain of anti-apoptotic proteins such BCL-2, BCL-XL and BCL-W [13]. As a single agent, ABT-737 induces apoptosis in small-cell lung carcinoma (SCLC) and several lymphoid malignancies. However, it is less effective against most solid tumours [13,15]. Obatoclax works in combination with MCL-1, an anti-apoptotic molecule; however, its binding affinity to other anti-apoptotic proteins is lower than that of ABT-737 [10,13].

Bcl-2 proteins are also involved in the metastasis of tumour cells, as determined by the overexpression of Bcl-2 and inhibition of Bim [16–19]. Different components of the cytoskeleton such as actin, gelsolin, lamin and plectin are targets of caspases during early apoptotic events [20–24]. Moreover, key enzymes that organise the *F*-actin network with GTPase activity are the direct substrates of caspase 3 and 7 and play an important role in linking apoptosis to cell motility [25,26]. Blocking of BCl-2 proteins also affects other homeostasis pathways involving autophagy. BH3 mimetics disrupt the interaction of anti-apoptotic proteins BCL-2 and BCL-XL with Beclin-1. Subsequently, the vacant Beclin-1 triggers the autophagy pathway [27,28].

Modulation of apoptotic state in HB and HCC cells using BH3 mimetics enhances their susceptibility to different drugs [9]. In this study, we investigated BH3 mimetics not only as modulators of apoptosis but also as modulators of adhesion and migration in HB and HCC cells, which may help in reducing metastasis.

Methods

Drugs

BH3 mimetic ABT-737 (Abbott, Abbott Park, IL) and obatoclax (Selleck Chemicals, Houston, TX) were dissolved in DMSO. The final concentration in the cell culture was between 0.03 and 1 μM. DMSO concentration in cultures did not exceed 0.1%. The pan-caspase inhibitor Z-VAD(OMe)-FMK (zVAD, Bachem AG, Bubendorf, Switzerland) was used at concentrations of 25 μM.

Cells and culture conditions

In this study the HB cell line HUH6 and the HCC cell line HUH7 (both from Japanese Collection of Research Bioresources (JCRB)) and the paediatric HCC cell line HC-AFW1 were used [29]. Tumour cells were cultured as a monolayer in Dulbecco's MEM medium (Biochrom, Berlin, Germany) supplemented with 10% foetal calf bovine, 1% glutamine and 1% penicillin/streptomycin (all additives from Biochrom, Berlin, Germany). HUH6 cells were grown at 37 °C in a humidified atmosphere containing 5% carbon dioxide. To remove the adherent cells from the dishes, trypsin-EDTA (PAA Laboratories, Pasching, Austria) was used.

Cell migration assay

Tumour cells were treated with 0.03, 0.1 and 0.3 μM BH3-mimetics for 24 h. Cells were transferred into double chamber culture inserts (Ibidi GmbH, Munich, Germany) at density of 4×10^4 per chamber and cultured for further 24 h in the absence of BH3 mimetics. The culture chamber was removed and the distance between the cell layers was documented and measured at five different regions after 0, 4, 6 and 24 h (Axio-Vison, Carl-Zeiss Vision GmbH, Aalen, Germany). The distance of migration was calculated as a difference to the starting point of migration experiment. Each concentration of BH3 mimetics was tested in triplicates.

Apoptosis assay via flow cytometry

HUH6 cells were treated with 0.3 μM obatoclax and 1 μM ABT-737 for 24 h or 48 h. HUH6 cells were harvested by Trypsin-EDTA after the treatment and stained with Annexin-V-FLUOS Staining Kit (Roche, Mannheim, Germany) to detect apoptotic cells.

Senescence

HUH6 cells were seeded at a density of $5 \times 10^5/cm^2$ and treated 24 h later with 0.3 μM obatoclax or 1 μM ABT-737. The next day senescence was detected in cultures using the acid beta galactosidase staining kit (Cell signalling, Danvers, MA). Senescent cells were detectable through the blue staining of X-gal reagent. Blue cells were counted automatically by ImageJ (NIH, USA) using pictures of confluent cultures.

Immunofluorescence staining

HUH6 cells were cultured on Poly-L-lysine (Sigma Aldrich GmbH, Steinheim, Germany) coated glass cover slips as described for cell migration assays. Cells were treated with 0.3 μM obatoclax previously and during the migration for 24 h. HB cells were fixed with 3.5% paraformaldehyde for 10 min, washed with PBS twice and permeabilized with 0.1% Triton X/PBS for 5 min at room temperature. The tumour cells were stained with FITC-Phalloidin (1:200, Alexis Biochemicals, Lauen, Switzerland) to detect *F*-actin by fluorescence microscopy (Axioscope 40 and Axiovision, Carl Zeiss, Jena, Germany).

Western blot analysis

The Western Blot analysis and chemoluminescent detection were performed as described previously [30]. CDC42 protein was detected with the monoclonal antibody ab41429 (Abcam, Cambridge, United Kingdom). As a loading control GAPDH was revealed by polyclonal antibody (Santa Cruz, CA, USA).

Cell adhesion assay

Tumour cells were treated with obatoclax and ABT-737 with indicated concentrations for 24 h in duplicates. A suspension of 5×10^4 cells/ml of these cells was given to the 10% matrigel (Becton Dickinson GmbH, Heidelberg, Germany) coated glass plates in culture chambers. Non-adherent cells were washed away 30 minutes later. Adherent cells were fixed with 3.5% paraformaldehyde and stained with DAPI (1 μg/ml, Sigma, Munich, Germany). Nuclei of adherent cells in three regions of 2.4 mm for each treatment were counted automatically on fluorescent micrographs by Image J (NIH, USA).