Cancer Letters 404 (2017) 79–88

Contents lists available at ScienceDirect

Cancer Letters

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

Original Article

Sensitivity towards the GRP78 inhibitor KP1339/IT-139 is characterized by apoptosis induction via caspase 8 upon disruption of ER homeostasis

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ARTICLE INFO

Article history:
Received 12 May 2017
Received in revised form
5 July 2017
Accepted 7 July 2017

Keywords:
Sodium *trans*-[tetrachlorobis(1H-indazole) ruthenate(III)]
Ruthenium
Extrinsic pathway of programmed cell death
GRP78
Caspase 8

ABSTRACT

The ruthenium drug and GRP78 inhibitor KP1339/IT-139 has already demonstrated promising anticancer activity in a phase I clinical trial. This study aimed to identify mechanisms underlying increased sensitivity to KP1339 treatment. Based on a screen utilizing 23 cell lines, a small panel was selected to compare KP1339-sensitive and low-responsive models. KP1339 sensitivity was neither based on differences in ruthenium accumulation, nor sensitivity to oxidative stress or constituents of KP1339 (ruthenium chloride and indazole). Subsequently, the biochemical response to KP1339 was analyzed using whole genome expression arrays indicating that, while sensitive cell lines were characterized by "response to chemical stimuli" and "regulation of cell death", low-responsive cells preferentially activated pathways controlling cell cycle, DNA repair, and metabolism. Cell culture experiments confirmed that, while low-responsive cells executed cell cycle arrest in G2 phase, pronounced apoptosis induction via activation of caspase 8 was found in sensitive cells. Cell death induction is based on a unique disruption of the ER homeostasis by depletion of key cellular chaperones including GRP78 in combination with enhanced KP1339-mediated protein damage.

Introduction

Due to the major limitations (adverse effects and rapid resistance development) of systemic therapy for late stage patients, still high interest in the development of more effective and better tolerated drugs exists [1,2]. One of them is KP1339, which has already proven distinct anticancer activity, while being extraordinarily well tolerated [3]. Thus, in a clinical phase I study of KP1339,

Abbreviations: 4-PBA, 4-phenylbutyric acid; CHOP, C/EBP-homologous protein; CI, combination index; CFTR, cystic fibrosis transmembrane conductance regulator; DAPI, 4′,6-diamidine-2′-phenylindole dihydrochloride; DCF-DA, 2′,7′−dichlorofluorescin diacetate; EPR, enhanced permeability and retention; ER homeostasis, endoplasmatic reticulum homeostasis; Ero1α, endoplasmic reticulum oxidoreductase 1 alpha; FACS, fluorescence-activated cell sorting; FBS, fetal bovine calf serum; GO, gene ontology; GOrilla, gene ontology enrichment analysis and visualization tools; GRP78, 78 kDa glucose-regulated protein; GSH, glutathione; logFC, logarithmic fold change; ICP-MS, inductively coupled plasma mass spectroscopy; Ire1α, inositol-requiring enzyme 1; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide; KP1339, sodium *trans*-[tetrachlorobis(1H-indazole)ruthenate(III)]; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; NSCLC, non-small cell lung cancer; PARP, poly(ADP-ribosyl)polymerase; PBS, phosphate-buffered saline; PERK, protein kinase-like endoplasmic reticulum kinase; ROS, reactive oxygen species; RDC, ruthenium-derived compounds; STR, short-tandem-repeat; TMAH, tetramethylammoniumhydroxide; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

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http://dx.doi.org/10.1016/j.canlet.2017.07.009
0304-3835/

ten cases of disease stabilization out of 38 evaluable patients were observed [3] especially for tumor entities known for very limited treatment options (e.g. NSCLC, metastasized neuroendocrine tumor, or colon cancer). One patient with a carcinoid tumor achieved a durable partial response and remained on treatment for 100 weeks. Noteworthy, dose-limiting effects (nausea, creatinine levels $\leq$ grade 2) were comparably mild [3].

The exceptional good tolerability of KP1339 is based on its dual prodrug nature: Tumor accumulation via albumin binding followed by tumor-specific activation by reduction [4–7]. This rapid binding also supports passive tumor targeting by the "enhanced permeability and retention" (EPR) effect, which describes the accumulation of larger particles but also proteins such as albumin in the intercellular space of malignant tissue due to the combination of chaotic blood vessel formation with a lack of lymph drainage [8,9]. Importantly, malignant cells frequently take up albumin by endocytosis/macropinocytosis as source of amino acids to meet the requirements of their often upregulated metabolism [10]. By this mechanism, cancer cells accumulate distinctly higher intracellular levels of protein-bound drugs compared to non-malignant tissue [9]. After uptake of albumin-bound KP1339, we propose that the second prodrug mechanism takes place: activation by reduction inside the malignant cells, which results in tumor-specific release of the reactive Ru(II) species as also seen with the indazolium salt KP1019 [11,12]. With regard to the exact mode of action, KP1339 has been recently identified as potent inhibitor of GRP78 activation [13], an ER stress-sensing chaperone [14].

For further preclinical studies, it is essential to acquire information about the intracellular mechanism of action preferably in combination with biomarkers to indicate the response of the malignant cells to treatment. As in the phase I clinical trial diverse tumors responded favorably to KP1339 treatment, the aim of this study was to identify and further characterize KP1339-sensitive cancer cell models to allow the future definition of potential markers indicative for response to KP1339 treatment.

Material and methods

Chemicals

KP1339 (Suppl. Fig. 1) was prepared as described previously [15] and dissolved in dimethyl sulfoxide (dimethyl sulfoxide concentrations in all experiments were below 1%). Tetramethylammoniumhydroxide (TMAH) was purchased from Merck (Darmstadt, Germany), TRAIL from Life Technologies (Carlsbad, California, USA), Z-VAD-FMK from Enzo Life Sciences (Lausen, Switzerland), Z-IETD-FMK (THP medical products, Vienna, Austria) and Indazole from Polivalent-95 (Chisinau, Republic of Moldova). All other substances were purchased from Sigma-Aldrich (St. Louis, MO). All solutions were freshly prepared before usage.

Cell culture

Detailed information as well as culture and MTT seeding conditions for cell lines used are provided in Supplementary Table 1. All cultures were grown under standard cell culture conditions, regularly checked for *Mycoplasma* contamination and their authentication verified either by short-tandem-repeat (STR) profiling or array comparative genomic hybridization. Fetal bovine calf serum (FBS) was purchased from PAA (Linz, Austria).

Cytotoxicity assays

Cells were plated in 96-well plates (number of used cells is given in Supplementary Table 1) and allowed to recover for 24 h before drug treatment for 72 h. Cell survival was determined by MTT assay following the manufacturer's recommendations (EZ4U, Biomedica, Vienna, Austria). Cytotoxicity was calculated using the Graph Pad Prism software (La Jolla, USA) (using a point-to-point function) and expressed as IC_{50} values calculated from full dose-response curves (drug concentrations resulting in 50% reduction of viable cells compared to untreated control cells cultured in parallel). Synergism is expressed by combination index (CI) according to Chou and Talalay [16] using CalcuSyn software (Biosoft, Ferguson, MO, USA).

Western blot analyses

Sample collection, protein separation and Western blotting were performed as described [17]. The antibodies used including source and dilutions are given in Supplementary Table 2.

Cell cycle analysis

After seeding (1×10^5 cells/well in 6-well plates) with 24 h recovery, cells were treated for 24 h and (after fixation in 70% ethanol) prepared for staining with 5 μg/ml propidium iodide as previously published [18]. Fluorescence intensity was measured by flow cytometry using a FACS Calibur (Becton Dickinson, Palo Alto, CA) and quantified by ModeFit software (Becton Dickinson and Company, New York, USA).

Preparation of DAPI-stained nuclei

Briefly, 1×10^5 cells were seeded in 6-well plates, allowed to recover for 24 h and treated for another 24 h. Subsequently, cells were collected and cytospins prepared using a cytocentrifuge (Cytospin 4, Thermo Scientific, Waltham, USA) at 400 rpm for 5 min. After fixation with ethanol/acetone (−20 °C, 1:1) cells were stained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI). For each slide, 200 to 300 nuclei were scored as normal, mitotic or apoptotic.

Time-lapse microscopy analyses

1×10^5 cells were seeded in a cell culture dish (3.5 cm diameter), allowed to recover for 24 h, and treated with 100 μM KP1339. Filming started immediately after treatment (a humidified incubation chamber ensured stable cell culture conditions throughout the experiment (37 °C, 5% CO_2)). Photomicrographs (20× magnification) were taken with an OLYMPUS IMT-2 inverted microscope connected to a DSLR camera (Canon 100D) every minute for 72 h.

Inductively coupled plasma mass spectroscopy (ICP-MS)

To determine cellular accumulation of KP1339, cells were incubated with 100 μM KP1339 for 3 h at 37 °C, washed twice with PBS, then lysed in 500 μl of TMAH at room temperature for 5 min. The resulting lysates were dissolved in 25 ml 0.6 M HNO_3. Ruthenium concentrations were determined using an Elan 6100 (PerkinElmerSciex Instruments, Boston, MA).

Total-RNA isolation and whole genome gene expression array

All details on RNA preparation and array analysis are given in the Supporting Information.

Results

Cytotoxicity screen for KP1339 sensitivity

In order to identify cell models with enhanced sensitivity towards KP1339, a broad panel of cell lines (n = 23) was screened (Fig. 1a). In general, responsiveness to KP1339 was not associated with a specific tumor entity, p53- or k-ras mutation status. Thus, most of the tested cell lines had IC_{50} values around 100 μM. However, five cell lines were characterized by enhanced KP1339 sensitivity ($IC_{50} \leq 50$ μM): Capan1, Capan2, HCC3, and HCT116 as well as its p53-deleted subline HCT116/p53ko. For subsequent studies, a panel of five models was chosen: sensitive Capan1 and HCT116 vs. low-responsive CCL13, SW480 and KB-3-1. First, we tested whether the sensitivity towards KP1339 was based on its constituents (ruthenium, indazole) or increased drug accumulation in sensitive cell lines. Table 1 clearly demonstrates that sensitivity to KP1339 is not linked to enhanced responsiveness to ruthenium or indazole treatment. In addition, no significant difference in drug accumulation was detected (Table 1). Also, altered sensitivity to disturbance of redox homeostasis did not offer an explanation, as neither sensitivity to H_2O_2 (Table 1) nor cellular GSH levels (Suppl. Fig. 2a) correlated with KP1339 responsiveness. In accordance, also no correlation of KP1339 sensitivity with the levels of drug-induced intracellular reactive oxygen species induction was found (Suppl. Fig. 2b).