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Original Article

The Hsp70 inhibiting peptide aptamer A17 potentiates radiosensitization of tumor cells by Hsp90 inhibition

Daniela Schilling [a, b, *], Carmen Garrido [c, d], Stephanie E. Combs [a, b], Gabriele Multhoff [a, b]

[a] Department of Radiation Oncology, Klinikum rechts der Isar, Technische Universität München, Munich, Germany
[b] Institute of Innovative Radiotherapy (iRT), Department of Radiation Sciences (DRS), Helmholtz Zentrum München, Munich, Germany
[c] INSERM UMR 866, Team Labeled LNCC, Faculty of Medicine, Dijon, France
[d] Anticancer Center Georges François Leclerc (CGFL), Dijon, France

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ABSTRACT

The inhibition of heat shock protein 90 (Hsp90) is a promising strategy to increase the radiosensitivity of tumor cells. However, Hsp90 inhibition induces the expression of Hsp70 which is a prominent cytoprotective protein. Therefore, dual targeting of Hsp70 and Hsp90 might be beneficial to increase the radiosensitivity of tumor cells. Hsp70 inhibiting peptide aptamers have been shown to increase the sensitivity of tumor cells to apoptosis induced by different anticancer drugs. Herein, we studied the radiosensitizing activity of the Hsp70 inhibiting peptide aptamer A17 in combination with the Hsp90 inhibitor NVP-AUY922. Whereas A17 significantly increased apoptosis induction by NVP-AUY922 it did not significantly affect the radiosensitivity of human lung and breast cancer cells. However, Hsp70 inhibition by the aptamer A17 potentiated the radiosensitizing effects of the Hsp90 inhibitor NVP-AUY922. Mechanistically we speculate that an increased number of DNA double strand breaks and an enhanced G_2/M arrest might be responsible for the increased radiosensitization in A17 expressing tumor cells. Therefore, the simultaneous inhibition of Hsp90 and Hsp70 combined with radiotherapy might provide a promising anti-cancer strategy.

Introduction

The molecular chaperone heat shock protein 70 (Hsp70, HspA1A) is well known to execute multiple cytoprotective functions. Hsp70 plays a major role in the correct folding of newly synthesized proteins, the transport of proteins across membranes and in the assembly of multiprotein complexes. In normal cells, the Hsp70 expression under physiological conditions is low but strongly induced upon stress (e.g. heat shock). In contrast to normal cells, tumor cells constitutively express high amounts of Hsp70. Hsp70 overexpression inhibits apoptosis, increases tumorigenicity, mediates resistance against radio- and chemo-therapy and is associated with poor prognosis [14]. Accordingly, silencing or inhibition of Hsp70 has been shown to sensitize tumor cells against chemotherapeutic drugs and radiation [10]. Recently, Rerole et al. demonstrated that Hsp70-targeting peptide aptamers such as A17 inhibited Hsp70, thereby increasing the sensitivity of tumor cells against various anticancer drugs like cisplatin, 5-FU or etoposide [16]. Furthermore, they showed a strong tumor regression in vivo by a treatment of mice with the peptide aptamer A17 in combination with cisplatin or 5-FU [16].

Inhibition of Hsp70 in combination with Hsp90 inhibition has been considered as an attractive anticancer strategy. Hsp90 inhibition causes the degradation of multiple oncogenic client proteins thereby downregulating simultaneously several signaling pathways which are involved in tumor cell survival and radioresistance [3,9]. Hsp90 inhibitors have been demonstrated to increase the radiosensitivity of tumor cells but in parallel they strongly enhance the expression of cytoprotective Hsp70 [18]. Therefore, dual targeting of Hsp90 and Hsp70 is an emerging concept to render tumor cells more susceptible to radiation therapy. Actually, reducing Hsp70 expression by inhibiting heat shock factor 1 (HSF1), the transcription factor responsible for Hsp70 induction, clearly increased the radiosensitizing effect of the Hsp90 inhibitor NVP-AUY922 [20]. However, this approach was not specific for Hsp70

Abbreviations: Hsp, heat shock protein; HSF1, heat shock factor 1; SER, sensitizing enhancement ratio.

* Corresponding author. Department of Radiation Oncology, Klinikum rechts der Isar, Technische Universität München, Ismaninger Str. 22, 81675 München, Germany. Fax: +498941404477.
E-mail address: daniela.schilling@tum.de (D. Schilling).

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and impaired tumor cell killing by natural killer (NK) cells [21]. Therefore, herein we investigated whether the specific inhibition of Hsp70 by a peptide aptamer alone or in combination with the Hsp90 inhibitor NVP-AUY922 can enhance the radiosensitivity of the radioresistant tumor cell lines H1339 and T47D.

Material and methods

Reagents and treatment

10 mM stock solution of NVP-AUY922 (Novartis) was prepared in 100% DMSO. Dilutions were performed in PBS. A vehicle control with the respective amount of DMSO diluted in PBS (maximal 0.001%) was used in all experiments. If not indicated otherwise, cells were incubated for 24 h with NVP-AUY922.

Cells and cell culture

The human lung (H1339) and breast (T47D) cancer cell lines were cultured as described previously [20]. The authenticity of the tumor cell lines was tested by the DSMZ (German Collection of Microorganisms and Cell Cultures). Cells were routinely checked and determined as negative for mycoplasma contamination.

Transfection

H1339 and T47D cells were transfected either with a control plasmid or with a plasmid bearing the A17 aptamer [16] by using attractene transfection reagent (Qiagen). The selection for plasmid bearing cells was performed with 400 μg/ml G418.

Western blot analysis

Cells were lysed as described previously [17]. On immunoblots, proteins were detected with antibodies against Hsp70 (ADI-SPA-810, Enzo Life Sciences), myc-tag (#2276, cell signaling) and β-actin (A5316, Sigma–Aldrich).

Proliferation assay

Cells were seeded into 96-well plates. 24 h after seeding, various concentrations of NVP-AUY922 were added. As a control, 0.001% DMSO was added. The proliferation was measured using the colorimetric alamarBlue assay, according to the manufacturer's instructions (Biosource, Camarillo, CA, USA). Briefly, alamarBlue was added and 4 h after incubation at 37 °C, the absorption at 570 nm and 630 nm (reference wavelength) was measured using an absorbance microplate reader (ELx808; BioTek). The proliferation of untreated cells incubated with DMSO was set to 100% in each experiment.

Apoptosis assays

Annexin V: Cells were washed with Annexin V-binding buffer and incubated with Annexin V-FITC (Roche) for 15 min at room temperature. After a washing step propidium iodide (PI) was added for 1 min and cells were analyzed on a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA).

Active caspase 3: Cells were washed, fixed, permeabilized, and stained with FITC-conjugated mouse monoclonal anti-active-caspase-3 mAb according to the manufacturer's instructions (BD, # 550480) and analyzed on a FACSCalibur™ flow cytometer (BD Biosciences).

Clonogenic assay and irradiation

To measure the radiosensitivity, clonogenic assays were performed as described previously [18]. The cells were seeded in 12-well plates, one day later treated with NVP-AUY922 and 24 h later irradiated using the RS225A irradiation device (Gulmay Medical Ltd) at a dose rate of 1 Gy/min (70 keV). After irradiation the medium was exchanged by drug-free medium. On day 9 (H1339) or 16 (T47D) after seeding, colonies were fixed, stained and counted. Survival curves were fitted to the linear quadratic model and α and β values were calculated on the basis of the equation $\ln SF = -\alpha \times D - \beta \times D^2$ using Sigmaplot (Systat Software Inc).

Cell cycle analysis

To analyze the cell cycle distribution, cells were fixed, stained with propidium iodide in the presence of RNase and analyzed on a FACSCalibur flow cytometer (BD Biosciences) as described previously [19]. The cell cycle distribution was calculated using Modfit software (Verity software house Inc).

γH2AX staining

Cells were fixed in 70% ethanol, washed, permeabilized with 0.15% Triton X-100 and stained with anti-phospho-Histone H2A.X (Ser139), FITC conjugate antibody. After washing cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences). Relative mean fluorescence intensity (MFI) values were calculated since the basal fluorescence intensities varied in different antibody lots.

Statistics

Statistical analysis was performed using SPSS 18.0.2 software (IBM). Normal distribution was proven by the Shapiro–Wilk test and the Student's t-test was used to evaluate significant differences ($^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$). All experiments were independently performed at least 3 times.

Results

Hsp70 targeting peptide aptamer A17 sensitizes tumor cells to Hsp90 inhibition

The two radioresistant cancer cell lines H1339 and T47D were chosen as a tumor model [20]. The cell lines were transfected either with a control plasmid or a plasmid bearing the Hsp70 targeting aptamer A17 fused to myc-tag which have been previously described [16]. Successful transfection of both cell lines with A17 is demonstrated by detection of myc-tag (Fig. 1). As expected, the expression levels of Hsp70 remained unaltered after transfection with the Hsp70 inhibiting aptamer A17.

The aptamers alone did not influence viability or cell growth compared to control cells (data not shown). Only in combination with the Hsp90 inhibitor NVP-AUY922 the proliferative capacity of A17 harboring H1339 and T47D cells was found to be more severely reduced in a concentration-dependent manner compared to control cells (Fig. 2A).

In line with the reduced proliferation, expression of A17 significantly increased apoptosis induced by NVP-AUY922 treatment in H1339 and T47D cells, as demonstrated by Caspase-3 (Fig. 2B) and Annexin V staining (Fig. 2C). These data indicate that the Hsp70 inhibitor, peptide aptamer A17, sensitizes tumor cells to Hsp90 inhibition.

A17 increases radiosensitization by NVP-AUY922

A comparison of the intrinsic radiosensitivity of the control and A17 bearing tumor cell lines revealed that the aptamer per se did not significantly affect the radiosensitivity of H1339 and T47D cells (Fig. 3). However, T47D cells harboring the aptamer A17 revealed a slightly higher radiosensitivity – indicated by a lower D_{50} value – than T47D control cells (Table 1). When control tumor cells were treated with low concentrations of NVP-AUY922 (2, 5, and 10 nM) before irradiation, the radiosensitivity was slightly but not significantly increased (Fig. 3). In contrast, NVP-AUY922 significantly enhanced the radiosensitivity of tumor cells expressing the Hsp70

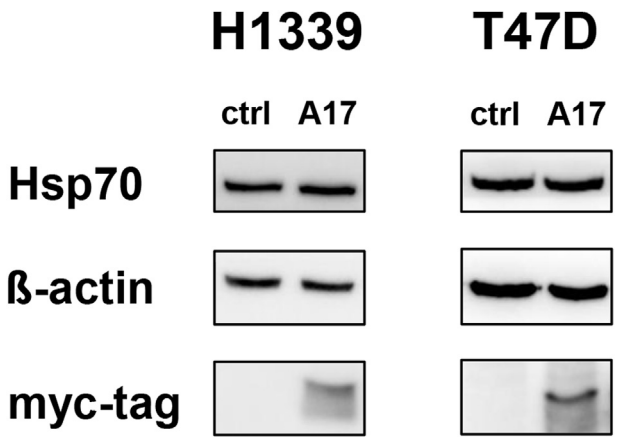

Fig. 1. Representative immunoblot showing the successful transfection of H1339 and T47D tumor cells with the Hsp70 targeting aptamer A17. Expression of myc-tag and Hsp70 in H1339 and T47D tumor cells transfected with control (ctrl) or A17 expression plasmid.