Relevance of subcellular localization of extracellular signal-regulated kinase 1/2 (ERK1/2) for cisplatin resistance

Shahana Dilruba 1, Martin Michaelis 2, Jindrich Cinatl jr. 3, and Ganna V. Kalayda 1

1 Institute of Pharmacy, Department of Clinical Pharmacy, University of Bonn, Germany, 2 Centre for Molecular Processing and School of Biosciences, University of Kent, Canterbury, UK, and 3 Institute of Medical Virology, Clinics of the Goethe University, Frankfurt/Main, Germany

Introduction

Cisplatin is a widely used anticancer drug. Despite a significant rate of initial response, cisplatin treatment often results in the development of chemo resistance leading to therapeutic failure [1]. The resistance mechanisms underlying resistance acquisition to cisplatin are not completely understood. Cisplatin has been described as interacting with DNA to form DNA adducts, primarily intrastrand crosslinks, activating a number of signal transduction pathways and culminating in the initiation of apoptosis [2]. Extracellular signal-regulated kinase 1/2 (ERK1/2) is activated in many cell lines in response to cisplatin treatment [3, 4]. Upon activation, ERK1/2 is supposed to translocate into the nucleus and induce the expression of survival genes. Studies conducted so far were focused on total ERK1/2 activation and not on the cellular distribution of this protein. We hypothesized that translocation of activated ERK1/2 (p-ERK1/2) to the nucleus may be responsible for p-ERK1/2-mediated cisplatin resistance, and that alterations in the localization of ERK1/2 may enhance the cytotoxicity caused by cisplatin. In this project, we investigated the effect of p-ERK1/2 translocation into the nucleus on cisplatin resistance in ovarian cancer cells.

Materials and methods

Cell culture

The human ovarian carcinoma cell lines A2780 and the cisplatin-resistant variant A2780cis were obtained from the European Collection of Cell Cultures (ECACC). EFO27 cells were obtained from the DSMZ (Braunschweig, Germany). The corresponding cisplatin-resistant sub-line EFO27 CDDP 2000 was derived from the Resistant Cancer Cell Line (RCCL) collection (www.kent.ac.uk/stms/cmp/RCCL/RCCLaboute.html). The A2780 cell lines were cultured in RPMI-1640 (PAN Biotech, Aidenbach, Germany) supplemented with 10% fetal calf serum, 0.6 mM L-glutamine, 100 I.E./mL penicillin and 0.1 mg/mL streptomycin at 37 °C in a humidified chamber with 5% CO2. The EFO27 cell lines were cultured in IMDM supplemented with 10% fetal calf serum, 0.6 mM L-glutamine, 100 I.E./mL penicillin, and 0.1 mg/mL streptomycin. The medium of EFO27 CDDP 2000 cells was additionally supplemented with 2 µg/mL cisplatin.

Determination of cell viability (determination of EC50 values)

Effects of cisplatin on cell viability were evaluated by an MTT-based assay. Nine dilutions of cisplatin (50 nM, 0.1, 1, 3, 6, 10, 33, 67, and 100 µM) were added to the cells in triplicate (100 µL/well). After 72 hours of incubation, 20 µL of a 5 mg/mL MTT solution in phosphate-buffered saline (PBS) were added to each well, and the cells were incubated at 37 °C for 60 minutes. Subsequently, medium was discarded, and 100 µL of di-
Figure 1. A: Detection of ERK1/2 activation (p-ERK1/2) by Western blot in response to 60 µM cisplatin treatment for 1 hour in cisplatin-sensitive A2780 and EFO27 and their resistant variants A2780cis and EFO27′CDDP<sub>2000</sub>, respectively. GAPDH served as loading control. B: Immunofluorescence staining of p-ERK1/2 after cisplatin treatment in A2780 and EFO27 cell lines as well as their corresponding cisplatin-resistant variants. p-ERK1/2 was detected with Alexa Fluor 488 conjugated secondary antibody (green), nuclei were stained with DAPI (blue). Scale bar, 50 µm.
methyl sulfoxide was added to each well, yielding purple solutions. The optical density was measured at 590 nm using a Multiskan™ microplate reader (Thermo Scientific, Waltham, MA, USA). The results were analysed and the pEC_{50} (pEC_{50} = -\log EC_{50}, EC_{50} is the drug concentration that produces 50% of the maximum possible response) values were determined using the GraphPad Prism™ analysis software package (sigmoidal dose response, variable slope).

**Western blot analysis**

Cellular extracts (20 µg of total protein) were resolved using 10% SDS-PAGE under denatured reducing conditions and transferred to PVDF membrane. The membranes were blocked with 5% nonfat milk, washed, and incubated with the following primary antibodies: anti-p-ERK1/2 at 1 : 1000 (Cell Signaling Technology Europe B.V., Leiden, The Netherlands), GAPDH at 1 : 20,000 (GeneTex Inc., Biozol Diagnostica Vertrieb GmbH, Eching, Germany). Horseradish peroxidase-conjugated secondary antibody (1 : 5,000, Southern Biotech, Birmingham, AL, USA) was used to detect the bound primary antibody. Immune complexes were visualized after incubation with Pierce™ ECL Western Blotting Substrate (Thermo Scientific, Life Technologies GmbH, Darmstadt) on a ChemiDoc™ XRS+ System (Bio-Rad Laboratories GmbH, Munich, Germany).

**Immunofluorescence staining**

Immunofluorescence staining was performed to detect nuclear translocation of p-ERK1/2 in response to 100 µM cisplatin treatment for 1 hour. The control cells were not treated with cisplatin, and a staining control was kept where the primary antibody was omitted to exclude the probability of unspecific binding of the secondary antibody. Cells were grown for 1 day on Labtek chamber slides at 20,000 cells/well, after which the medium was removed, and the cells were washed three times with ice-cold PBS (5 minutes per wash) and fixed in 4% paraformaldehyde for 20 minutes at room temperature. After another 3-minute washes in PBS, cells were permeabilized in 0.5% Triton-X in PBS for 10 minutes at room temperature, washed again with PBS, and blocked in 5% chicken serum (Life Technologies) for 1 h at room temperature. Cells were then incubated overnight at room temperature with the polyclonal rabbit anti-p-ERK1/2 primary antibody diluted 1 : 200 (Santa Cruz Biotechnology, Heidelberg, Germany). The next day, the cells were washed three times with PBS and incubated for 45 minutes with chicken antirabbit Alexa 488 conjugated secondary antibody (Life Technologies, Darmstadt, Germany) at a 1 : 250 dilution. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) for 5 minutes, washed three times with PBS and mounted for visualization. Cells were visualized with a confocal laser-scanning microscope (Nikon A1, Nikon Engineering Co., Ltd., Kanagawa, Japan).

**Statistics**

Results were investigated for statistically significant differences using the Student’s t test. p-values < 0.05 were considered to be significant.

**Results and discussion**

**ERK1/2 activation in response to cisplatin treatment**

As shown in Figure 1A, A2780 and A2780cis cells showed the activation of ERK1/2 following exposure to 60 µM cisplatin for 1 hour. In EFO27 and EFO27CD-Dp\textsuperscript{2000} cells, ERK1/2 activation in response to cisplatin treatment was also observed, but in this cell line pair, ERK1/2 is already activated in untreated cells (Figure 1A).

**Immunofluorescence staining to detect p-ERK1/2 translocation into the nucleus in response to cisplatin treatment**

After treatment of EFO27 and EFO27CDDp\textsuperscript{2000} cells with 100 µM cisplatin for 1 hour, translocation of p-ERK1/2 into
the nucleus was observed as demonstrated by colocalization of p-ERK1/2 (green) and the nuclear marker DAPI (blue) (Figure 1B). No clear nuclear accumulation of p-ERK1/2 was observed in A2780 and A2780cis cells (Figure 1B).

Inhibition of p-ERK1/2 signaling decreases cisplatin cytotoxicity in the A2780 cell line pair but has an opposite effect in the EFO27 cell line pair. The cisplatin pEC\textsubscript{50} values were 5.63 ± 0.21 for A2780, 4.91 ± 0.11 for A2780cis, 5.00 ± 0.05 for EFO27 and 4.41 ± 0.04 for EFO27\textsuperscript{CDDP\textsuperscript{2000}} cells (mean ± SEM, n = 4 – 6). This corresponds to the EC\textsubscript{50} values of 2.34 µM for A2780, 12.3 µM for A2780cis, 10 µM for EFO27, and 38.9 µM for EFO27\textsuperscript{CDDP\textsuperscript{2000}} cells, clearly showing decreased cisplatin sensitivity of cisplatin-resistant variants.

To investigate the role of ERK1/2 activation in cancer cell drug response, ERK1/2 activation was inhibited using U0126, a pharmacological inhibitor of MEK1/2, the upstream kinase that phosphorylates ERK1/2. The effect of U0126 on cisplatin cytotoxicity was studied. As U0126 was diluted in medium from a DMSO stock solution (to a final concentration of 0.2% DMSO), an additional control was kept by treating cells only with 0.2% DMSO (Figure 2). Sensitive cells were treated with 10 µM cisplatin, and resistant cells were treated with 30 µM cisplatin for 72 hours. U0126 was added at a final concentration of 20 µM. Figure 2 shows the effect of cisplatin, U0126, and the combination of both on the viability of cells determined using an MTT assay. The results reveal that the inhibition of ERK1/2 activation leads to an increase in cell survival in A2780 and A2780cis cells (Figure 2A, B). This suggests that the cisplatin-induced ERK1/2 activation contributes to the anticancer effects of cisplatin in A2780 and A2780cis cells.

Differing effects were found in the EFO27 cell lines (Figure 2C, D). Inhibition of ERK1/2 activation by U0126 enhanced cisplatin cytotoxicity in sensitive EFO27 cells. It should be noted, however, that cell viability after treatment with both cisplatin and U0126 was similar to cell viability after treatment with U0126 alone. This implies...
no substantial influence of the inhibition of ERK1/2 activation on cisplatin cytotoxicity in the EFO27 cell line. In EFO27/CDDP2000 cells, U0126 had no effect on the cell sensitivity to cisplatin either. Given high basal activation of ERK1/2 in EFO27 cell lines, it may be possible that U0126 did not substantially inhibit ERK1/2 activation at the concentration used. Taking into account p-ERK1/2 translocation into the nucleus upon cisplatin exposure, our data indicate that ERK1/2 activation may instead account for survival response in the EFO27 cell line pair.

Conclusions

Our results suggest that the activation of ERK1/2 may contribute to the anticancer effects exerted by cisplatin in A2780 cells, whereas it may be involved in survival mechanisms in EFO27 cells. In response to cisplatin, activated ERK1/2 (p-ERK1/2) translocates into the nucleus in EFO27 cells but not in A2780 cells. In future experiments, the localization of the activated ERK1/2 will be manipulated to further investigate the effect of p-ERK1/2 localization on cisplatin resistance in these ovarian cancer cells.

Acknowledgment

This project was supported by the Federal Ministry of Education and Research (BMBF) and the Bonn International Graduate School for Drug Sciences (BIGS DrugS), the Hilfe für krebskranke Kinder Frankfurt e.V., the Frankfurter Stiftung für krebskranke Kinder, the Royal Society, and the Kent Cancer Trust.

References


