Targeting z-Crystallin by aspirin restores the sensitivity to cisplatin in resistant A2780 ovarian cancer cells

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Short Report

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Abstract

Purpose

Ovarian cancer is the deadliest gynaecologic malignancies worldwide. Platinum based chemotherapy is the mainstay treatment for ovarian cancer; however, frequent recurrence and chemoresistance onset in patients with advanced diseases remain a therapeutic challenge. Although mechanisms underlying the development of chemoresistance are still ambiguous, the B-cell lymphoma-2 (Bcl-2) family is closely associated with chemoresistance in ovarian cancer. We previously disclosed that Zeta-Crystallin (CryZ) is a post-transcriptional regulator of \textit{Bcl-2} gene expression, by binding to \textit{bcl-2} mRNA and increasing its half-life. Here, we investigated the role of CryZ as a novel therapeutic target in ovarian carcinoma by modulating the protein activity with acetylsalicylic acid (ASA) to restore chemosensitivity.

Methods

Inhibition of CryZ binding activity to \textit{Bcl-2} and \textit{Bcl-xl} mRNA targets by ASA was evaluated in A375 cells. Cytotoxicity assays were conducted in A2780S and A2780R ovarian cancer cells to evaluate if CryZ binding activity inhibition and \textit{CryZ} silencing were able to reverse cisplatin resistance.

Results

ASA inhibits the binding of CryZ to \textit{Bcl-2} and \textit{Bcl-xl} mRNAs. Furthermore, ASA-treatment or \textit{CryZ} silencing are able to increase and restore the chemosensitivity in both sensitive and resistant A2780 ovarian cancer cell lines, respectively.

Conclusion

In this research article we demonstrated that the pharmacological or genetic inhibition of CryZ restores the sensitivity to cisplatin in a model of sensitive or resistant ovarian cancer cells. These findings suggest a new gene-targeted chemotherapeutic approach to restore the cytotoxicity in drug-resistant ovarian cancers and increase the sensitivity in non-resistant cells.

Background

Ovarian cancer is the deadliest gynaecological tumour. Inherent resistance or acquired resistance is the main cause of treatment failure. Even today, the mechanisms responsible for resistance in ovarian cancer treatment are poorly understood [1]. Regarding acquired resistance, the deregulation of anti-apoptotic genes \textit{Bcl-2} and \textit{Bcl-xl} has been well established [2, 3]. Expression of both genes is regulated by post-transcriptional mechanisms, including the interactions between stabilizing or destabilizing RNA-binding proteins (RBP) and their mRNAs [4]. In the last few years, several aspects of the post-transcriptional control of \textit{Bcl-2} have been characterized, and it has been demonstrated the existence of a complex regulative network controlled by the interaction of an adenylate-uridylate-rich element (ARE) in the 3'UTR region of \textit{Bcl-2} mRNA, with different ARE-binding proteins (AUBPs) [5, 6]. Among the \textit{Bcl–2} AUBPs we
identified Zeta-Crystallin (CryZ), demonstrating its role in increasing Bcl-2 mRNA stability in human leukaemia cell lines [7]. CryZ is also endowed with enzymatic activity, particularly acting as NADPH dependent quinone reductase. In the effort to identify pharmacological inhibitors of CryZ, the group of Bazzi MD found that aspirin-like analgesics are potent inhibitors of its enzymatic activity [8, 9]. Paracetamol (acetaminophen), ibuprofen (2-(4-isobutyl phenyl)-propionic acid), salicylic acid and aspirin (ASA) inhibit the enzyme to varying degrees; the latter being the most potent CryZ inhibitors. The inhibition of CryZ enzymatic activity by ASA occurs through the interaction with a specific binding site, closed to that of NADPH [10, 11], therefore it appears to be a non-competitive inhibitor with respect to NADPH, interacting with both the free form of CryZ as well as the CryZ/NADPH complex.

The most relevant data on the antitumor role of ASA and other non-steroidal anti-inflammatory drugs (NSAIDs) derive from epidemiologic studies, in a large panel of tumour types [12]. Furthermore, a synergistic effect of classical chemotherapeutic agents and ASA on cancer growth and apoptosis has been observed in a human myeloma xenograft model [13]. Also, a reduction in incidence of ovarian cancer has been observed in patients who take ASA regularly [10]. In preclinical ovarian cancer models, ASA treatment determined a significant reduction in Bcl-2 protein level with concomitant increase in caspase 3, revealing its antitumor and antiangiogenic effects [14]. Bcl-2 and Bcl-xl play major roles in the pathobiology and chemoresistance of ovarian cancer, and their inhibitions were useful for treatment [15].

In the present research study, we demonstrated that ASA inhibits the binding of CryZ to the mRNAs of the anti-apoptotic Bcl-2 and Bcl-xl genes. In addition, we revealed that modulation of CryZ activity using ASA or its downregulation by specific siRNA increases sensitivity in both sensitive or drug-resistant human ovarian cancer cells in response to chemotherapeutic drugs.

**Material and Methods**

**Cell Culture**

The human A2780S and A2780R ovarian cancer cell lines and the A375 melanoma cell line were purchased from ATCC (ATCC Manassas, USA). A2780S and A2780R cells or A375 cells were maintained in RPMI-1640 or DMEM medium, respectively, supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 U/mL) (Euroclone, Milan, Italy) at 37°C in a humidified incubator with 5% CO2. Cells were periodically screened for mycoplasma contamination using PCR.

**RNA-Amply-Seq analysis**

Total RNA was isolated from A2780S and A2780R cells by means of NucleoSpin Total RNA kit according to the manufacturer’s protocol (Macherey-Nagel, PA, USA). RNA was quantified by Qubit™ 3.0 Fluorometer (Invitrogen, MA, USA). After quantification, quality of RNA was evaluated using the Agilent RNA 6000 Nano LabChip® kit with the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). RNA-Sequencing (RNA-Seq) analysis was performed on an Ion Proton System for NGS (Life Technologies, Carlsbad, USA). Briefly, for each cell line, an experimental triplicate was analysed. 10 ng of total RNA was reverse
transcribed using the Ion AmpliSeq Transcriptome Human Gene Expression kit (Life Technologies, Carlsbad, USA), according to manufacturer’s instruction. The cDNA was amplified using Ion AmpliSeq Transcriptome Human Gene Expression core panel (Life Technologies, Carlsbad, USA). The analysis method is described in Mini et al. [16]; the resulting transcriptome dataset obtained has not been published.

**Western blot assay**

For Western blot analysis, cells were lysed in cold radioimmunoprecipitation assay (RIPA) lysis buffer (1% NP-40, 150 mM NaCl, 5 mM EDTA, 0.25% NaDOC, 50 mM Tris-HCl pH 7.5, 0.1% SDS) supplemented with protease and phosphatase inhibitors (Merck Life Science, Milan, Italy). Total proteins extracted from the cells were quantified with Bradford reagent (Merck Life Science, Milan, Italy). Aliquots of total protein (30 µg) were electrophoresed on 8–12% (v/v) SDS-PAGE gel (Thermo Fisher Scientific, Milan, Italy), transferred onto nitrocellulose membranes (Bio-Rad, CA, USA) and probed overnight at 4°C with primary antibodies against CryZ [7], Bcl-2 (clone 100, Thermo Fisher Scientific), Bcl-xl (SC-8392, Santa Cruz Biotechnologies) and beta-Actin (bACT, Thermo Fisher Scientific). Bound primary antibodies were detected using goat anti-mouse or goat anti-rabbit IgG Alexa Fluor 680 or 800 secondary antibodies (LI-COR® Bioscience, Lincoln, NE, USA). Membranes were visualized at the Odyssey Infrared Imaging System (LI-COR® Bioscience, Lincoln, NE, USA Euroclone, Milan, Italy) and densitometric analysis was performed by Odyssey Image studio software (LI-COR® Bioscience, Lincoln, NE, USA Euroclone, Milan, Italy) Quantity One software (Bio-Rad, CA, USA).

**Silencing of CryZ**

The A2780S and A2780R were transfected with 100 nM specific siRNA (IDT, Leuven, Belgium) against human *CryZ* by means of jetPRIME® (Polyplus, Illkirch, France) according to the instruction manual.

**In vitro transcription**

*Renilla luciferase* RNA was *in vitro* transcribed to be used as internal RT-qPCR control. A PCR product containing the ORF of Renilla luciferase was obtained from pGL4.71 plasmid, inserting the T7 RNA polymerase consensus by means of PCR reaction. This product was used as a template in the *in vitro* transcription reaction using MEGAscript T7 Transcription kit (Ambion, USA). Obtained RNA was purified using the RNAeasy kit (Qiagen, USA) and quantified by Qubit™ 3.0 Fluorometer.

**RNA Immunoprecipitation Assay**

RNA protein immunoprecipitation (RIP) was performed based on [17]. In brief, A375 cells were treated with ASA 1 mM (the concentration previously demonstrated to inhibit CryZ enzymatic activity in cell free biochemical assays [7]) for 24h, then cells were lysed in non-denaturing lysis buffer (20 mM Tris-HCl pH 8.0, 137 mM NaCl, 1% Nonidet P-40, 2 mM EDTA) in presence of protease inhibitors cocktail (Merck, USA) and 100 U/ml RNasein ribonuclease inhibitor (Promega, USA). 1 mg of protein extracts were incubated with 15 µl of Dynabeads protein G (ThermoFisher, USA), which were pre-washed with wash buffer (100
mM Tris-HCl pH 7.4, 2 mM EDTA, 150 mM NaCl, 1% Triton X100, 0.2 mM sodium orthovanadate), and with 1 ug of mouse anti-CryZ antibody (2A9DD1, Abcam, USA) or mouse IgG (Santa Cruz, USA) for 18h at 4°C. Supernatants were then collected, as internal protein control samples, and beads were washed four times using wash buffer. After washing, 30% of beads were used to elute protein for subsequent Western Blotting analysis of immunoprecipitation, and the remaining 70% of beads were subjected to RNA extraction. To this aim, beads were incubated for 15 min at 55°C in 100 µl of NT2 buffer (50 mM Tris-HCl pH 7.4, 1 mM MgCl₂, 150 mM NaCl, 0.05% Nonidet P-40) supplemented with 0.5 mg/ml of proteinase K and 0.1% SDS. 1 ng of in vitro transcribed Renilla luciferase RNA (as internal control) was added, and total RNA was extracted using RNAqueous total RNA Isolation kit (ThermoFisher, USA). Equal volumes of RNA were retrotranscribed using iScript kit (Biorad). Quantification of Bcl-2, Bcl-xl, beta-2 microglobulin and Renilla luciferase levels was performed through real-time PCR with specific primers (listed in Table 1) carried out using SsoAdvancedTM universal SYBR® Green Supermix (Bio-Rad, USA) in a CFX96 Real Time Detection System instrument (Bio-Rad, USA).

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>CryZ</td>
<td>CCCGTGGAGACACATACTCGCTCTG</td>
<td>GTAAACAGTGTGGTCTGCTGCAAGAGC</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>GAGGATTGTGGCTTTTGGAGTTTCG</td>
<td>GAAATCAAACAGAGGCGATGCTGG</td>
</tr>
<tr>
<td>Bcl-xl</td>
<td>CAGGCCGAGTGTGTTGAACCTGCGG</td>
<td>CATCTCCTTGTCTACGCTTCCACG</td>
</tr>
<tr>
<td>Beta-2 Microglobulin</td>
<td>AGTATGCCTGCCGTGTAAC</td>
<td>GCGGCATCTTCAAAACCTCCA</td>
</tr>
<tr>
<td>Renilla luciferase</td>
<td>GTCGAGACCATGCTCCCAAGCA</td>
<td>TTGCGGACATCTGGAGACGT</td>
</tr>
</tbody>
</table>

### Cytotoxicity and drug resistance reversal assays

The growth inhibitory effects of ASA and CryZ-siRNA in combination with CDDP were evaluated in A2780S and A2780R after 72 hours treatment by means of the sulforhodamine B (SRB) assay. Briefly, exponentially growing cells were seeded in 96-well plates in complete RPMI 1640 at a plating density of 4x10³ cells/well. After 24 hours, cells were exposed to ASA at 1 and 2.5 µM (concentration doses comparable to those in vivo [18], and previously shown not affecting A2780 cell viability [11]) with an increase ranging of concentrations from 1 nM to 100 µM of CDDP or CryZ-siRNA 100nM plus CDDP at the same concentration described above. Each concentration was tested in triplicate. In all cases, after 48-hour of drugs or siRNA-drug exposure, cells were fixed with 10% trichloroacetic acid and stained with 0.4% SRB in 1% acetic acid. The SRB fixed to the cells was dissolved in 10 mM Tris-HCl and the absorbance was read at 540 nm on Victor X5 (Perkin Elmer). IC50 values were calculated using GraphPad Prism v7 software (GraphPad Prism La Jolla, CA, USA). The IC50 drug concentration resulting in a 50% reduction in the net protein content (as measured by SRB staining) in drug treated cells as compared to
untreated control cells was determined. All the reported IC50 values represent the mean of at least three independent experiments.

**Statistical analysis**

$t$-Tests were used for pairwise comparisons. $P$ values $< 0.05$ were considered statistically significant. All data were analyzed using GraphPad Prism v7 software (GraphPad Prism La Jolla, CA, USA).

**Results**

**Bcl-2, Bcl-xl and CryZ expression levels are higher in resistant-compared to sensitive-A2780 cells.**

We analysed the expression levels of *Bcl-2, Bcl-xl* and *CryZ* genes in the A2780 sensitive and A2780 resistant cells (A2780S and A2780R, respectively) at both RNA and protein levels. As shown in Fig. 1A, analysis of transcriptomic data reveals that the A2780R cells show higher expression levels of the three genes compared to the sensitive cells. In agreement with mRNA expression levels, Western Blotting analysis revealed that while in sensitive cells the Bcl-2 protein is apparently absent, its levels result dramatically increased in resistant cells (Fig. 1B). In addition, Bcl-xl and CryZ protein levels are higher in A2780R compared to A2780S cells (Fig. 1B).

**ASA impairs the binding of CryZ to Bcl-2 and Bcl-xl mRNAs.**

As shown in the Fig. 1C-D, RNA-immunoprecipitation experiments carried out on the A375 cells using the anti-CryZ antibody or unrelated IgG as control demonstrate that treatment with 1 mM ASA for 24 hours drastically decreased the amount of *Bcl-2* and *Bcl-xL* mRNAs bound by CryZ, but not *b2-microglobulin* used as negative control. This data demonstrates that the presence of ASA impairs the binding of CryZ to these mRNAs, probably by interfering with the binding domain of CryZ to the AU-rich regions. Based on the evidence that CryZ is a stabilizing post-transcriptional regulator [7], this result suggests an increase in destabilization of the two mRNAs following ASA treatment, which could affect drug sensitivity.

**ASA restores the chemosensitivity to CDDP chemotherapeutics in A2780R cells.**

Table 2 shows how treatment with ASA (1 and 2.5 µM) together with CDDP at different doses for 72 hours in A2780S and A2780R cells significantly decreases the apoptotic threshold, increasing sensitivity to CDDP. To highlight the fact that CryZ may be at the basis of the reversion of the resistant phenotype, we performed cytotoxicity experiments following CryZ silencing, treating A2780S and A2780R cells with CDDP at different doses for 48 hours. As reported in Table 1, CryZ silencing in the A2780R and A2780S cells determined an induction of drug sensitivity, highlighting that CryZ has a major role in drug resistance. Furthermore, these experiments highlight that the inhibition of CryZ binding on the *Bcl-2* and *Bcl-xl* mRNAs by ASA has a fundamental relevance in increasing sensitivity in the A2780S cell line and in reversing resistance in the A2780R cell line.
Table 2  
**Cytotoxicity (IC50)**

<table>
<thead>
<tr>
<th>IC50 µM</th>
<th>A2780S</th>
<th>A2780R</th>
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<tbody>
<tr>
<td>CDDP</td>
<td>2.55</td>
<td>11.16</td>
</tr>
<tr>
<td>CDDP + 1µM ASA</td>
<td>1.41</td>
<td>9.31</td>
</tr>
<tr>
<td>CDDP + 2.5µM ASA</td>
<td>1.31</td>
<td>6.32</td>
</tr>
<tr>
<td>siRNA CryZ + CDDP</td>
<td>1.57</td>
<td>7.34</td>
</tr>
</tbody>
</table>

**Discussion**

The onset of resistance to chemotherapy drugs is the main determinant of the failure of anticancer chemotherapy in tumours initially sensitive to the drugs [19]. Human cell lines resistant to anticancer drugs represent an *in vitro* useful model for studying the mechanisms of action of drugs and the development of tumor resistance. Here, we show that ASA inhibits the binding of CryZ to the mRNAs of Bcl-2 and Bcl-xl, two important anti-apoptotic genes and that it is able to revert the drug resistance in ovarian cancer cells. We previously demonstrated that CryZ is a stabilizing RBP of Bcl-2 [7], while this is the first demonstration that CryZ is a new RBP of Bcl-xl. The mRNAs of both antiapoptotic genes are physiologically able to bind AUBPs by AU-rich sequence element in the 3’-UTR of their mRNAs, thus modulating the recruitment of the degradation machinery constituted by the exosome complex. Therefore, we revealed that CryZ could act as Bcl-2 and Bcl-xl AUBP in A2780 ovarian cancer cells. Treatment with ASA drastically reduces the ability of CryZ to bind to the target mRNAs, thus it could decrease their stability and ultimately lead to a reversal of the resistant phenotype. This is the first demonstration in ovarian cancer cell lines that chemoresistance can be reverted by the modulation of the post-transcriptional mRNA regulatory pathway. We are conscious of the limits of this brief communication in that ASA has a number of other effects that can affect drug sensitivity. However, the fact that CryZ silencing mimics the ASA effect at a similar extent suggests that inhibition of CryZ mRNA binding by means of ASA may play a major role. Further studies are needed to finely address mechanistic issues.

**Declarations**

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**Conflict of interest.** Authors have no financial interests to declare.

**Acknowledgements.** Not applicable

**Research involving human participants and/or animals.** Not applicable

**Data availability.** Not applicable
References


**Figures**

**Figure 1**

A) Transcriptomic analysis of *Bcl-2*, *Bcl-xl* and *CryZ* genes in A2780S and A2780R; data represent mean ± SD of at three independent experiments (*p < 0.05). B) Western Blotting analysis of Bcl-2, Bcl-xl and CryZ...
in A2780S and A2780R; the image is representative of three different experiments. bACT was used as housekeeping control. C) Immunoprecipitation assay of CryZ in extracts of A375 cells treated with ASA or with the DMSO alone; bACT was used as control. D) Evaluation of $Bcl-2$, $Bcl-xl$ and $b2M$ mRNA expression in immunoprecipitated CryZ-RNA complexes by RNA-immunoprecipitation assay (*p < 0.05).