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ORIGINAL ARTICLE

Alpha-1-Antitrypsin Antagonizes Cisplatin-Induced Cytotoxicity in Prostate Cancer (PC3) and Melanoma Cancer (A375) Cell Lines

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Abstract Increased circulating alpha-1-antitrypsin (AAT) correlates with cancer stage/aggressiveness, but its role in cancer biology is unclear. We revealed antagonistic effect of AAT to cisplatin-induced cytotoxicity in prostate (PC3) and melanoma (A375) cancer cell lines. Moreover, AAT abrogated cytotoxicity of MEK inhibitor U0126 in PC3 cell line. Weaker antagonistic effect of AAT on cytotoxicity of PI3/Akt and NF-kB inhibitors was also observed. In addition, cisplatin increased AAT gene expression in transfected PC3 cells. However, AAT derived from transfected PC3 cells did not antagonize cisplatin-induced cytotoxicity. In conclusion, these results suggest possible association between high circulating AAT and cisplatin resistance.

Keywords Alpha-1-antitrypsin · Cisplatin · Prostate cancer · Melanoma cancer

Introduction

Alpha-1-antitrypsin (AAT), an acute phase, 52 kDa glycoprotein is archetypal serine protease inhibitor (also known as SERPINA1). As the most abundant anti-protease, its main physiological role is the protection of lower respiratory tract by inhibition of neutrophil elastase and other endogenous serine proteases [1]. AAT is synthesized predominantly by hepatocytes, and normal plasma level in healthy subjects is 20–53 μM (1.5–3.5 mg/mL). During inflammation plasma level of AAT increases fourfold [2]. Extrahepatic sites of AAT synthesis include macrophages, monocytes, neutrophils, intestinal and lung epithelial cells [3–6]. Aside from anti-protease protection, local production of AAT by pulmonary cells plays important role in anti-inflammatory response to inflammatory stimulus [7].

Growing evidence supports multi-functional role of AAT. Thus, AAT has been shown to exert cyto-protective effect on vascular smooth muscle cells in serum deprived conditions, as well as in renal and hepatic ischemia/reperfusion models of apoptosis [8, 9]. AAT inhibits apoptosis in alveolar and bronchial epithelial cells [10, 11]. Also, AAT exerts direct anti-apoptotic effect on alveolar epithelial cells, regardless of its anti-inflammatory or anti-protease activity, by inhibiting activity of caspase 3 both in vivo and in cell free system [12]. Furthermore, direct inhibition of caspases 3, 7, and 6 has been recently also reported, indicating a specific inhibitory effect of AAT on executioner caspases [13].

Role of AAT in cancerogenesis and metastasis has been recognized, but insufficiently clarified. It was established that AAT expression status in tumour cells may represent a prognostic biomarker of tumour growth and progression [14, 15]. Recently, AAT was identified as a protein required for cancer cell migration and invasion of lung adenocarcinoma cells [16]. Moreover, C-terminal fragment of AAT was revealed to enhance invasiveness in pancreas adenocarcinoma cells and breast tumour cells [17, 18]. The increase plasma level of AAT was recognized as potential diagnostic and prognostic tumour marker [19–22].

Given the previously described anti-apoptotic role of AAT and its elevated expression in malignancy, we tested whether

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AAT affects the cisplatin-induced cytotoxicity in following human cancer cell lines - prostate PC3 and melanoma A375. Using small molecule-inhibitors of signalling pathway we showed an anti-apoptotic effect of AAT in the both cancer cell lines and this effect is most likely exerted via mitogen activated protein kinase (MAPK) signalling cascade. This further implies that elevated AAT can reduce efficiency of cisplatin therapy. These results might indicate that the serum level of AAT could be useful for monitoring response to treatment with cisplatin and other blockers of MEK1/2, PI3K/Akt pathways as well as NF-κB. Further clinical studies need to be conducted to evaluate prognostic characteristics of AAT as tumour marker.

Materials and Methods

Materials

Fetal calf serum (FCS), RPMI-1640, phosphate-buffered saline (PBS), dimethyl sulfoxide, cisplatin and propidium iodide (PI) were obtained from Sigma (St. Louis, MO). Annexin V-FITC (AnnV) was from Biotium (Hayward, CA). Commercial plasma derived AAT Prolastin® (Talecris Biotherapeutics, Inc., USA) was a kind gift from Beocompass, Belgrade, Serbia. SB202190 (MAPK p38 inhibitor), SP600125 (c-jun N-terminal kinase inhibitor), MG132 (NF-kappaB inhibitor) and rapamycin (mTOR inhibitor) were obtained from Sigma (St. Louis, MO) while U0126 (mitogen-activated protein/extracellular signal-regulated kinases 1 and 2 (MEK1/2) inhibitor), PD98059 (MEK1/2 inhibitor) and LY294002 (PI3K/Akt inhibitor), were obtained from Cell Signaling Technology (Danvers, MA).

Cell Lines and Transfection

Human melanoma A375 and androgen independent prostate PC3 cells were a kind gift from Prof. Ferdinando Nicoletti (Department of Biomedical Sciences, University of Catania, Italy). Cells were routinely maintained in HEPES-buffered RPMI-1640 medium supplemented with 10 % FCS, 2 mM L-glutamine, 0.01 % sodium pyruvate, and antibiotics at 37 °C in a humidified atmosphere with 5 % CO_2. For treatment with AAT cells were maintained in medium supplemented with 1 % FCS 2 h before and during the treatment. After standard trypsinization, cells were cultivated at 1×10^4 / well in 96-well plates for viability measurement and 2.5×10^5 / well in 6-well plates for flow cytometry and immunoblot analysis.

PC3 cells were transfected with 4 μg of plasmid (empty vector pcDNA3, pcDNA3-M AAT and pcDNA3-Z AAT) using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. PC3 cells were selected 24 h post-transfection by treatment with 250 μg/mL G418 (Gibco).

Determination of Cell Viability by Crystal Violet Assay

The viability of adherent viable cells was detected by crystal violet (CV) assay [23]. In brief, cells were exposed to different doses of AAT (0.04–40 μM) alone or in combination with cisplatin (3–30 μM) for 24 h and then fixed with 4 % PFA 10 min at room temperature (RT). Cells were stained with 1 % crystal violet solution for 15 min at RT, washed in tap H_2O, dried and the dye was dissolved in 33 % acidic acid. The absorbance of dissolved dye was measured at 540 nm with the reference wavelength at 670 nm. Results are presented as percentage of the control value that was arbitrarily set to 100 %.

Examination of Combined Effect of Cisplatin and AAT Using Isobologram Analysis

Analysis was done from dose-response curves of PC3 and A375 cell viability treated with AAT alone, with cisplatin alone or with their combination for 24 h. Combinations getting 20–50 % of cytotoxicity were presented as the concentration of single agent alone that produced that amount of toxicity. FCI < 1 is considered synergistic, FCI > 1 is considered antagonistic.

Detection of Apoptosis Using AnnexinV-FITC/PI Staining

PC3 cells were treated with 15 μM cisplatin and 100 μM U0126, alone or in combination with 20 μM AAT for 24 h, then trypsinized and stained with AnnV-FITC/PI (Biotium, Hayward, CA) following manufacturer's instruction. Cells were analysed with FACS Calibur flow cytometer (BD, Heidelberg, Germany) using Cell Quest Pro software (BD).

Caspase Detection

For determination of caspase activity Apostat staining was used. Cells were treated as previously with 15 μM of cisplatin concomitantly with 20 μM of AAT. At the end of cultivation cells were detached and stained with Apostat (R&D) for 30 min at 37 °C. Finally, cells were washed in PBS, resuspended and analysed with FACS Calibur flow cytometer (BD, Heidelberg, Germany) using Cell Quest Pro software.

Acridine Orange Staining of Autophagic Vesicles

For detection of the acidic autophagic vesicles, hallmark of autophagy, supravital acridine orange staining was applied. Briefly, cells were exposed to the same treatment with Cisplatin and/or prolastin as mentioned above. At the end of treatment, cells were trypsynized, subsequently washed and stained with 1 μM of Acridin orange (Labo-Moderna, Paris,

France) 15 min at 37 °C. Finally, cells were washed with PBS and analysed with FACS Calibur flow cytometer.

Analysis of AAT Expression in Transfected PC3 Cells Using Quantitative Real Time PCR

Total RNA from PC3 cell line, stably transfected with pcDNA3–AAT constructs and treated with cisplatin, was isolated using RNeasy Plus Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription reactions were performed using High Capacity cDNA Reverse Transcription (Life Technologies). Quantitative real-time PCR (qRT-PCR) was performed in 10 μL reactions containing 5 μL Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies), 1 μl template cDNA and 0.4 μl (10 μM) of each of following primers: 5′-ATGCTGCCCAGAAGACAGATA-3′ and 5′-CTGAAGGCGAACTCAGCCA-3′ amplifying a 91-bp AAT fragment [11], and 5′- AGGAAGGAAGGCTGGAAGAG-3′ and 5′-GGACTTCGAGCAAGAG-3′ amplifying a 138-bp β-actin fragment (endogenous control). Amplification was performed using the ABI PRISM 7500 (Applied Biosystems) and PCR cycling parameters used were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All PCR reactions were performed in triplicates. Results were analysed with the software provided with the ABI Prism7500 Real Time PCR system.

Analysis of ERK 1/2 Signalling Using Western Immunoblot

PC3 cells were treated with either cisplatin, AAT or their combination for 4 h and cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.5); 120 mM NaCl; 0.5 % NP40; 1 x protease inhibitors (Complete, EDTA free, Roche). Cell lysates were subjected to SDS PAGE followed by transfer onto PVDF membrane (Millipore) and western blotting (WB). Membranes were probed with anti-ERK1/2, anti-p-ERK1/2 (Cell Signaling) and HRP-conjugated anti-rabbit antibody (Invitrogen). Antibody complexes were detected by enhanced chemiluminescence, using ECL SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Waltham, MA, USA).

Statistics

The results are presented as means ± SD of triplicate observations from one representative of at least three repeated experiments. The significance of the differences between various treatments was assessed by analysis of variance (ANOVA), followed by a Student-Newman-Keuls test. A p value less than 0.05 was considered significant.

Results

AAT Antagonized the Activity of Cisplatin on Cancer Cell Lines PC3 and A375

We evaluated the influence of AAT on the efficacy of cisplatin-induced apoptosis in androgen-independent prostate PC3 cells and human melanoma A375 cells, both sensitive to cisplatin. Cells were exposed to AAT for 2 h before the addition of cisplatin. After the incubation for 24 h cell viability was estimated by CV assay. Type of interaction (synergism, antagonism or additivity) between tested compounds was evaluated by isobologram and results are presented in Fig. 1. While both agents decreased the viability of PC3 and A375 cells in dose dependent manner, isobologram curves clearly indicated antagonistic action between cisplatin and AAT in combined treatment. Obtained data revealed that presence of AAT in tumour microenvironment could antagonize the cisplatin therapy. For further analysis we chose PC3 cell lines.

AAT Neutralized Cisplatin-Induced Apoptosis

Cisplatin is commonly used cytostatic whose basic mechanism of action is induction of apoptosis. To determine the mechanism which is responsible for observed cyto-protective activity of AAT in cisplatin-treated cells, PC3 cells were incubated with 15 μM cisplatin and 20 μM AAT, alone and in combination, for 24 h when the Ann/PI double staining was performed. For cell treatment we chose physiologically relevant concentration of AAT. The aim was to simulate early stage of acute phase, considering that increased level of AAT correlates with advanced stages of cancer. Flow cytometry analysis showed remarkable increase in percentage of early apoptotic cells marked as $Ann^{+}PI^{-}$ upon the cisplatin, but not when AAT was applied in mentioned dose (Fig. 2.). However, the percentage of apoptotic cells was significantly reduced in AAT-cisplatin combined treatment suggesting cyto-protective role of AAT in the presence of cisplatin.

Induced apoptosis by cisplatin exposure was followed with slightly elevated caspase activity (7.9 % in cisplatin treated vs 2.9 % in control). On the other hand, AAT neither alone nor concomitantly to cisplatin diminished their activity (7.3 % in AAT treated vs. 8 % in AAT+ cisplatin) indicating that neutralization of apoptosis was not a result of caspase inhibition. Having in mind that autophagy can oppose the apoptosis, we next explore the presence of autophagic vesicles, as the hallmark of autophagic process, in the cytoplasm of cells in the same experimental setting. While increased red fluorescence was detected in 3.7 % of untreated cells, AAT alone triggered autophagy in 11.6 % of cells. Exposure to cisplatin led to development of acidic, autophagic formation in 21.8 %. The percentage of cells with intensified autophagic process was further observed in concomitant treatment of AAT and

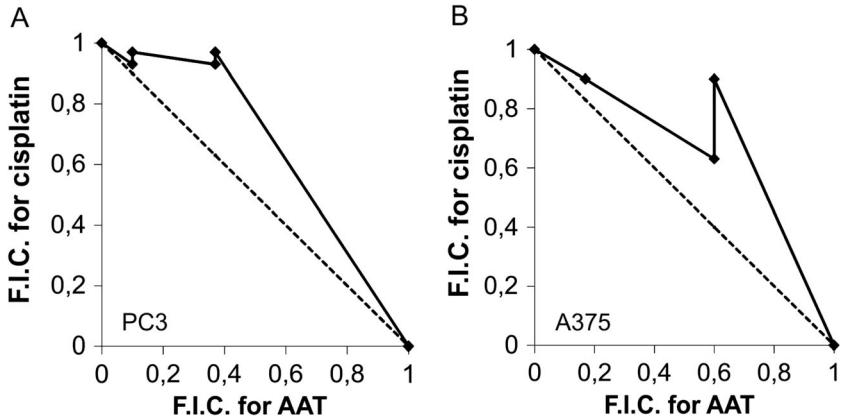

Fig. 1 AAT antagonizes cisplatin-induced cytotoxicity. **a** PC3 and **b** A375 cells were treated with various concentrations of cisplatin (3.5–30 μM) in the presence of AAT (0.04–40 μM). After 24 h, cell viability was determined by CV assay, and isobologram curves calculated from a representative of three independent experiments are presented. Fraction inhibitory concentration (F.I.C.): concentration of each agent in combination/concentration of each agent alone, F.I.C. < 1 is considered synergistic; F.I.C. > 1 is considered antagonistic

cisplatin (32.9 %) raising the possibility that autophagy is responsible for cytoprotective role of AAT.

AAT Neutralized Apoptosis Triggered by Small Molecule Inhibitor U0126

It is well documented that changes in signalling pathways which define cell decision to proliferate, differentiate or die correlate with malignant transformation. Therefore, small molecule inhibitors created to block different pathways were found to be efficient in preclinical and clinical trials [24]. We further aimed to determine the influence of AAT on the effectiveness of molecular targeted therapy by identification of molecular mechanisms that are responsible for anti-apoptotic effect of AAT on PC3 cells. For this purpose PC3 cells were treated with different inhibitors in concentrations selected as cytotoxic, and with combined treatment of AAT and inhibitors.

After 24 h of incubation cell viability was determined by CV assay. Following inhibitors - SB202190, LY294002, SP600125 and MG132 were applied in a dose of 50 μM, PD98059 and

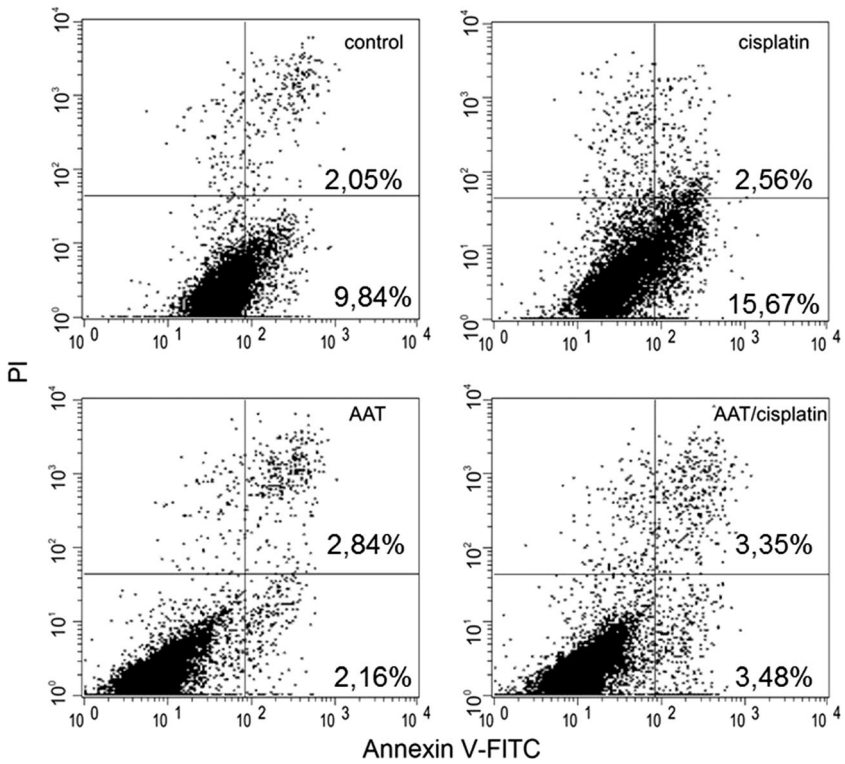

Fig. 2 AAT neutralized cisplatin-induced apoptosis in PC3 cells. Cells were incubated with 15 μM cisplatin, 20 μM AAT, alone or in combination for 24 h, stained by AnnV/PI and analyzed by FACS. Representative FACS plots are presented

U0126 at 100 μM while rapamycin was used at 10 μM. Inhibitors were applied alone and in combination with 20 μM AAT. Treatment of PC3 cells with U0126, MG132, LY294002 and rapamycin significantly reduced cell viability (Fig. 3a). Presence of AAT was abrogated the cytotoxicity of blockers of MEK1/2, PI3K/Akt pathways as well as NF-κB. The major effect was observed in the case of U0126, and this inhibitor was selected for further analysis. Interestingly, in PC3 cell line, AAT exerted very similar cyto-protective capacity against U0126 and cisplatin (Fig. 3b) indicating MEK/ERK signalling pathway as target for AAT. Therefore, ERK protein expression was analysed upon the 2 and 4 h of treatment with cisplatin with or without AAT. According to the western blot analysis, AAT induces ERK phosphorylation in combination with cisplatin, but not on its own (Fig. 3c).

AAT Originating from Transfected PC3 Cells Did not Affect Cisplatin-Induced Apoptosis

In addition, we aimed to test whether endogenous expression of AAT by PC3 cells exerts the same effect on cisplatin-induced cytotoxicity, as the AAT from cellular microenvironment. Also, we explored whether AAT polymorphism affects its cisplatin-antagonistic properties. For this purpose, we have selected a functionally normal, wild type AAT variant (M), and clinically significant mutation associated with human AAT deficiency (Z variant).

Stable transfectants were established by selecting cells with G418 for 2 weeks and AAT expression was analysed by qRT-PCR. As already established, PC3 cells were shown to express AAT [25, 26] and stable transfection increased AAT expression more than 100 times fold (Fig. 4b). Treatment with cisplatin led to increased AAT gene expression in transfected PC3 cells (Fig. 4a). Also, there was an up-regulation of the AAT expression in cells transfected with empty vectors, suggesting that cisplatin regulates AAT expression from both genomic AAT promoter and vector CMV promoter.

In contrast to effects of exogenous AAT, endogenous expression of AAT did not change the effectiveness of cisplatin in PC3 cells. This might be explained by the fact that even though transfection significantly increased levels of AAT mRNA, this expression was insufficient to elicit response. In detail, evaluation of apoptosis through double staining of Ann/PI showed the same percentage of early and late apoptotic cells after the treatment

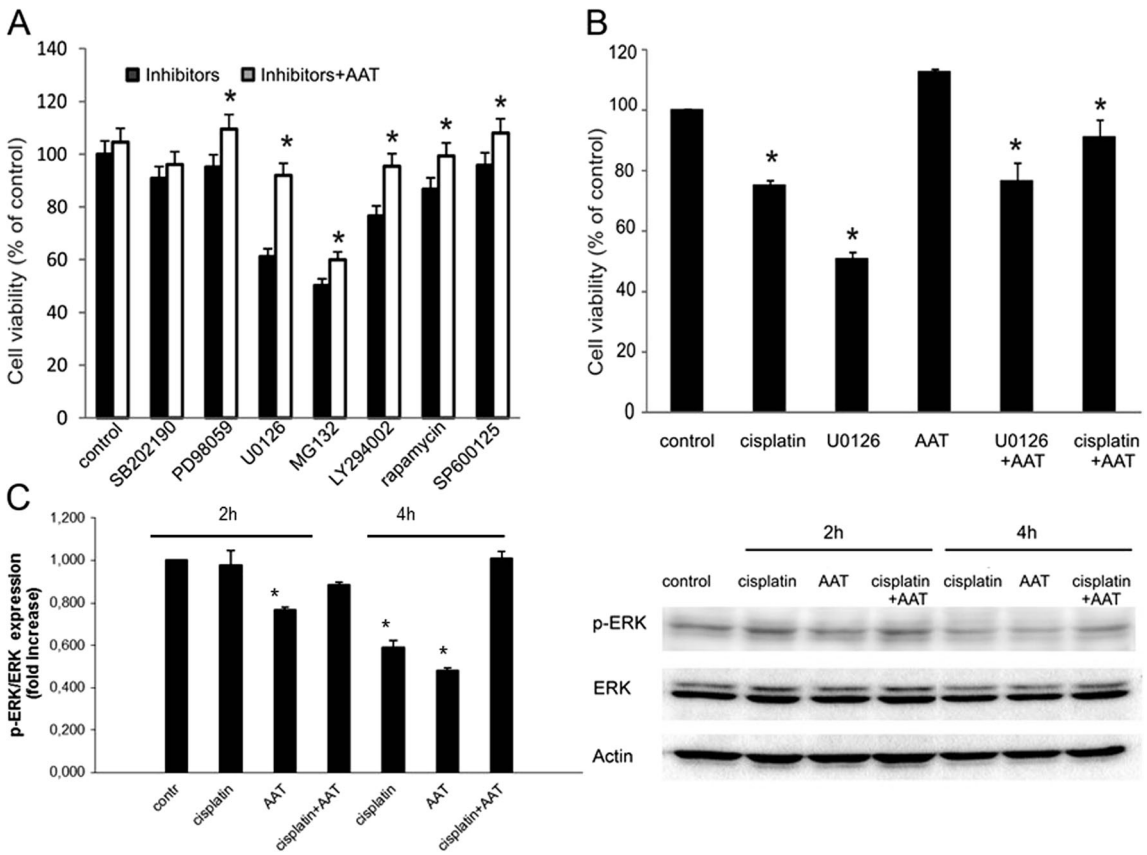

Fig. 3 Interaction of AAT with inhibitors of different signalling pathways. **a** and **b** PC3 cells were treated with SB202190, LY294002, SP600125, MG132, PD98059, U0126, rapamycin or cisplatin, alone or in the presence of 20 μM AAT and viability was determined after 24 h by CV test. The data are presented as mean ± SD from a representative of three independent experiments. *$p < 0.05$, refers to adequate controls. **c** ERK1/2 expression was analysed by WB after 2 h and 4 h of incubation with cisplatin and AAT, alone or in combination. Densitometric analysis was presented as mean ± SD from two experiments with similar results

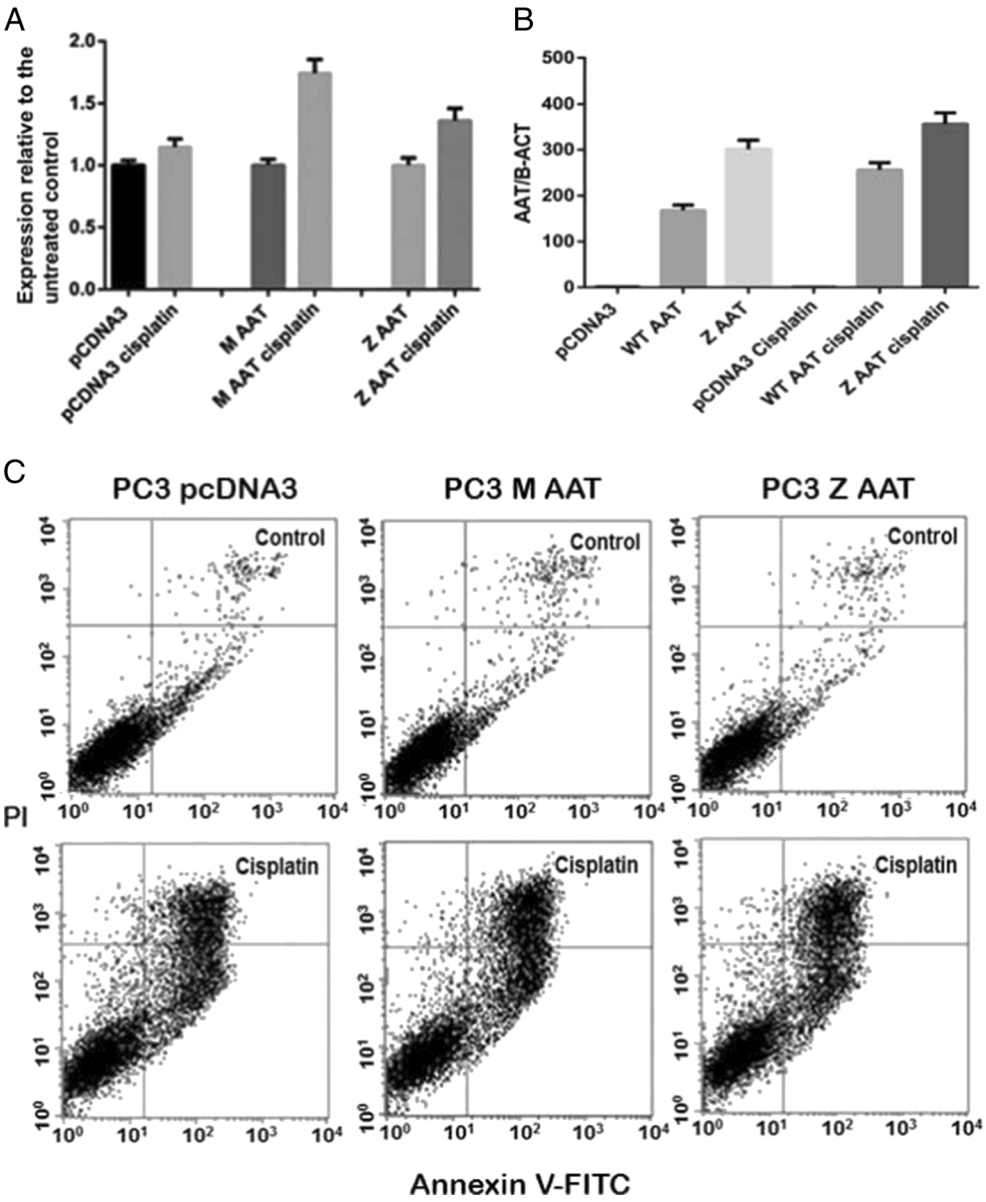

Fig. 4 Endogenous expression of AAT in PC3 cells did not affect the cisplatin activity. PC3 cells were stably transfected with M AAT, Z AAT or empty vector (pcDNA3). **a** Expression of AAT in transfected cells treated with 15 μM cisplatin for 24 h was analysed by qRT-PCR and shown relative to the expression in the untreated transfected cells. **b** Expression of AAT shown relative to beta actin. **c** Transfected cells were exposed to 15 μM cisplatin for 24 h and then stained by AnnV/PI

with cisplatin in cells transfected with empty vector, M or Z AAT (Fig. 4c).

Discussion

Other than anti-protease, alpha-1-antitrypsin exerts various physiological functions, such as anti-inflammatory, pro-survival and anti-apoptotic. However, despite the well documented clinical data regarding positive correlation between AAT plasma levels and cancer stage and progression [27–30], its role in cancer biology is still not clarified. Moreover, it has been found that different molecular forms of AAT can exert diverse effects on tumour cell viability of human breast cancer pancreatic cancer, and melanoma cell lines, from suppression to induction their activity in vitro [18, 31, 32]. Clinically is very important to determine whether this acute-phase protein has an impact on biological activity of malignant cells and consequently on the cancer aggressiveness and treatment outcome.

Cisplatin is one of the most potent anti-tumour agents widely used in protocols for the treatment of many solid tumours [33]. It was reported that more than 80 % of internalized cisplatin was found in non-DNA fractions indicating that its interaction with other biomolecules could also contribute to its biological effect [34]. Identification of factors and pathways involved in cisplatin activity could improve understanding of mechanisms involved in chemoresistance. Having in mind previously mentioned, this study opens the question of whether AAT could influence chemotherapy efficacy in the case of general or molecular targeted therapy.

Our study revealed that AAT present in cell microenvironment antagonized cytotoxic activity of cisplatin in prostate (PC3) and melanoma (A375) cancer cell lines. Isobologram curves clearly indicated antagonistic action between cisplatin and AAT in the combined treatment in both cell lines. For the first time we revealed that cytoprotective activity of AAT was achieved through inhibition of apoptosis, but is not a result of caspase activation. Importantly, inhibited apoptosis in combined treatment was accompanied with intensified autophagic process suggesting its relevance for the observed cytoprotection. Similarly, we recently published the same phenomenon on colon cancer cell line HCT116, resistant to cisplatin [35]. Precisely, cultivation of cells in serum deprived conditions promoted autophagy with an aim to provide the energy and therefore support survival. Addition of inhibitor of MEK1/2, UO126, induced apoptosis and abolished autophagy. However, in the presence of AAT autophagic process was preserved and as a consequence, apoptosis was abrogated. This effect could be tightly connected with Bcl-2/Beclin 1 dissociation enabling the formation of autophagosomes [36, 37]. For Bcl-2 dissociation its phosphorylation is necessary. Despite the fact that major regulator of Bcl-2 phosphorylation is JNK [38], cytotoxic activity of inhibitor created to shut down JNK, SP600125 was not compromised by AAT addition. Moreover, we found that extracellular AAT abrogated cytotoxicity of MEK inhibitor U0126 in PC3 cell line, and in lesser extent cytotoxicity of inhibitors of PI3/Akt pathway and NF-κB. These data indicated the relevance of ERK1/2 activity in autophagy-apoptosis interplay and AAT role in cytoprotection. Our results could be related to the finding that elevation of circulatory AAT is associated with resistance to cisplatin in the experimental Lewis lung carcinoma model [39]. Taken that physiologically-relevant concentration of AAT antagonized cisplatin-induced cytotoxicity, it is reasonable to assume that increased circulatory level of AAT can lead to cisplatin resistance. ERK activation by AAT has been previously reported in fibroblasts where AAT stimulated fibroblast proliferation and pro-collagen production [40]. In light of these results, it could be assumed that pro-survival effect that AAT has shown in serum-free conditions could also be linked to the activation of ERK by AAT. However, data presented in this study revealed that in our experimental conditions AAT and cisplatin alone decreased ERK phosphorylation. Combined treatment oppositely strongly potentiated ERK phosphorylation. This effect subsequently could be associated with Bcl-2 dissociation from complex Bcl-2/Beclin 1 [36, 37] and sustained autophagy as a consequence.

The precise mechanism of antagonistic action of AAT to cisplatin-induced cytotoxicity remains to be investigated. In this regard it should be mentioned that selective and direct modification of active site of AAT on Met^{358} by cisplatin which disables its anti-protease activity has been described [41]. Although AAT-cisplatin interaction should be considered, it is highly unlikely that this is the mechanism of action in this case, since AAT also antagonized cytotoxicity of small molecule inhibitors such as U0126.

According to our knowledge, for the first time we revealed that cisplatin increased AAT gene expression in transfected PC3 cells. Influence of anticancer therapy on elevation of AAT gene expression could be clinically significant considering that was shown association between elevated expression of AAT gene in different cancer tissues and worse prognosis, for instance in HLA-positive cervical carcinoma, in colorectal cancer and in gastric cancer [42–44].

In contrast to AAT in cell microenvironment, we showed that the AAT originating from the PC3 cancer cells did not impair effectiveness of cisplatin. Chemically modified structure of AAT derived from different types of cancer cells have been well documented [45–47]. In this regard, it should be considered that the chemically modified AAT from cancer cells exerts different biological activity in relation to the AAT synthesized in non-malignant cells present in the cancer cell microenvironment.

The main result of this study emphasizes that under certain circumstances presence of AAT in cancer microenvironment may affect therapeutic efficacy of cisplatin. These results suggest that apoptotic-based chemotherapy could be ineffective due to synergistic effect of increased AAT as an acute phase protein and cisplatin-induced AAT gene expression.

Considering that the acute phase response of the host immune system can be activated by the presence of the cancer, primary resistance to cisplatin can be ascribed to presence of the increased AAT level in the tumour microenvironment. In addition, secondary resistance to cisplatin may be a consequence of inflammation caused by chemotherapy-induced cell necrosis in response to the treatment [48].

Since the advanced stages of cancer are often accompanied by significantly high levels of AAT, results of our study could eventually be translated towards the identification of non-responders to inhibitors of Ras-Raf MEK-ERK signalling pathway.

Clinical studies have recognised plasma level of AAT as a predictive biomarker of cisplatin-based combined chemo-radiotherapy [49], and for early detection of recurrence and/or widespread metastasis caused by ineffective therapy [50].

In conclusion, given that potential mechanism involves MEK-ERK signalling pathway, high level of serum AAT could be evaluated as a predictive biomarker for response to chemotherapy based upon inhibition of Raf-Ras MEK-ERK signalling cascade.

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Compliance with Ethical Standards

Conflict of Interest The authors have declared no conflicts of interest.

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