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# Ailanthone increases oxidative stress in CDDP-resistant ovarian and bladder cancer cells by inhibiting of Nrf2 and YAP expression through a post-translational mechanism

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## Abstract

Chemoresistance represents one of the main obstacles in treating several types of cancer, including bladder and ovarian cancers, and it is characterized by an increase of cellular antioxidant potential. Nrf2 and YAP proteins play an important role in increasing chemoresistance and in inducing antioxidant enzymes. It has been reported that Ailanthone (Aila), a compound extracted from the Ailanthus Altissima, has an anticancer activity toward several cancer cell lines, including chemoresistant cell lines. We have examined the effect of Aila on proliferation, migration and expression of Nrf2 and YAP proteins in A2780 (CDDP-sensitive) and A2780/CP70 (CDDP-resistant) ovarian cancer cells. Furthermore, to clarify the mechanism of Aila action we extended our studies to sensitive and CDDP-resistant 253J-BV bladder cancer cells, which have been used in a previous study on the effect of Aila. Results demonstrated that Aila exerted an inhibitory effect on growth and colony formation of sensitive and CDDP-resistant ovarian cancer cells and reduced oriented cell migration with higher effectiveness in CDDP resistant cells. Moreover, Aila strongly reduced Nrf2 and YAP protein expression and reduced the expression of the Nrf2 target GSTA4, and the YAP/TEAD target survivin. In CDDP-resistant ovarian and bladder cancer cells the intracellular oxidative stress level was lower with respect to the sensitive cells. Moreover, Aila treatment further reduced the superoxide anion content of CDDP-resistant cells in correlation with the reduction of Nrf2 and YAP proteins. However, Aila treatment increased Nrf2 and YAP mRNA expression in all cancer cell lines. The inhibition of proteolysis by MG132, a proteasoma inhibitor, restored Nrf2 and YAP protein expressions, suggesting that the Aila effect was at post-translational level. In accordance with this observation, we found an increase of the Nrf2 inhibitor Keap1, a reduction of p62/SQSTM1, a Nrf2 target which leads Keap1 protein to the autophagic degradation, and a reduction of P-YAP. Moreover, UCHL1 deubiquitinase expression, which was increased in bladder and ovarian resistant cells, was down-regulated by Aila treatment.
In conclusion we demonstrated that Aila can reduce proliferation and migration of cancer cells through a mechanism involving a post translational reduction of Nrf2 and YAP proteins which, in turn, entailed an increase of oxidative stress particularly in the chemoresistant lines.

## Keywords

CDDP-Resistance; Ovarian cancer cells; Bladder cancer cells; Ailanthone; Oxidative stress level; Superoxide anion; Nrf2; Yap; Keap1; p62/SQSTM1; Protein degradation; UCHL1

## 1. Introduction

Chemoresistance represents one of the main obstacles in treating several types of cancer, including bladder and ovarian cancers. Although an increase of oxidative stress is common in tumor cells, in chemoresistant cells a great increase of the transcription of antioxidant genes contributes to the resistance towards chemotherapeutic pro-oxidant drugs. This adaptive response is mediated by induction of Nrf2 (NF-E2-related factor 2) transcription factor, the main regulator of the antioxidant potential of the cells [1]. In physiological conditions, Nrf2 is bound to the Keap1 inhibitor (Kelch-like ECH-associated protein 1), which drives Nrf2 to proteasomal degradation [2]. In response to an increase of oxidative stress, the cysteine residues of Keap1 become oxidized, resulting in a conformational change of the Keap1–Nrf2 complex which leads to the release of Nrf2 [2]. When released from Keap1, Nrf2 moves to nuclei, and, after dimerization with Maf proteins, binds with antioxidant response element (ARE)/electrophile response element (EpRE) and activates genes involved in antioxidant action [2]. It has been proposed that Nrf2 is an important agent for maintaining drug resistance in cancer cells [3]. In particular, Nrf2 has been demonstrated to be involved in CDDP resistance in ovarian cancer and its knockdown sensitized ovarian cancer cells to CDDP treatment [4]. Moreover, in a previous paper we demonstrated a crosstalk between Nrf2 and YAP proteins, which contributes to maintaining the antioxidant potential and chemoresistance in bladder cancer cells [5]. YAP is a downstream component of the Hippo tumor-suppressor pathway [6]. Hippo pathway phosphorylates YAP on Ser127 leading to its cytoplasm sequestration or on Ser 381 leading to its ubiquitination and degradation [6]. Conversely, unphosphorylated YAP can translocate into the nucleus, and, through the binding to the TEADs transcription factors, trigger the expression of diverse genes involved in cell survival, proliferation and migration [7]. Moreover, YAP, through the binding to FoxO1 can stimulate the transcription of antioxidant genes such as MnSOD and catalase [8]. Importantly, several reports indicated that YAP activation induces cancer stem cell proliferation, chemoresistance, and metastasis and its inhibition increases sensitivity toward chemotherapeutic treatment [9]. In ovarian cancer cells YAP was required for maintaining the expression of specific genes that may be involved in chemoresistance [10] and the inhibition of the YAP pathway enhances CDDP sensitivity in human ovarian adenocarcinoma cells [11].

Ailanthone (Aila) is a natural compound extracted from the tree Ailanthus altissima. This compound, in traditional Chinese medicine, has been employed to treat several diseases [12]. In the recent years, several reports demonstrated that Aila was able to inhibit the proliferation of different cancer cell lines [[13], [14], [15], [16], [17], [18], [19], [20], [21]]. Most of the time, this inhibition is accompanied by cell cycle arrest in a specific cell cycle phase [[14], [15], [16], [17]] and apoptosis [14,15,17]. Inhibition of migration and induction of autophagia have been also observed after Aila treatments [16,18]. It has been shown that Aila is able to affect gene expression or protein activity of a number of targets, such as specific cell cycle proteins [15,16], apoptotic gene regulators (i.e., bax and bcl-2) [14,15,17], as well as to inhibit signalling pathways, such as Pi3K/AKT/mTOR and Ras/Raf/MEK/ERK/mTOR [15,18]. Moreover, it has been shown the ability of Aila to modulate specific miRNAs [16,18]. Little is known on the ability of Aila to overcome chemoresistance. To this regard, it has been demonstrated that Aila, through the binding with the co-chaperone p23 protein, sensitized castration-resistant prostate cancer cells to the treatment [15,19], and that it can reverse multidrug resistance by inhibiting the P-glycoprotein-mediated efflux in K562 cells resistant to doxorubicin [20]. Recently, we demonstrated that Aila down-regulated Nrf2, YAP and c-Myc expression and inhibited proliferation and migration of CDDP- resistant bladder cancer cells [21] but, until now, no data has been provided about the effect of Aila in ovarian cancer cells. Ovarian cancer is one of the most serious gynecological malignancies. The first-line treatment is represented from surgery and platinum-based chemotherapy. The main limitation of the clinical usefulness of platinum (CDDP or carboplatin) is the high incidence of chemoresistance [22]. In the present paper we analyzed the effect of Aila on the expression of Nrf2 and YAP proteins and the respective target genes in sensitive and CDDP-resistant ovarian cancer cells. Moreover, to verify whether the Alia action was common to cancer cells from

diverse origins, we analyzed the mechanism of Aila activity in ovarian and bladder cancer cells, both sensitive and CDDP-resistant.

## 2. Material and method

### 2.1. Cell lines and culture conditions

253J B–V and 253J B–V CDDP-resistant cells (253J B–V C-r) were obtained as previously reported [5]. A2780 and A2780/CP70 cells were purchased from the American Type Culture Collection (ATCC, USA). All the cell lines were cultured in RPMI 1640 medium, supplemented with fetal bovine serum (FBS (10%), streptomycin (100 µg/ml), and penicillin (100 units/ml), and maintained in a 5% CO2, 37 °C humidified incubator. For cell treatments, Aila (Baoji Herbest, Bio-Tech Co., China) or CDDP (Merck Life Science S.r.l., Milan, Italy) were used.

### 2.2. MTT assay and colony-forming assay

A2780 and A2780/CP70 were seeded (1500 cells/well) in a 96-well plate with 200 µl of serum-supplemented medium. After cell treatment with Aila or CDDP, the viability was assessed by MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Merck Life Science S.r.l) assay, as previously reported [21].

To analyze colonies formation, A2780 and A2780/CP70 were seeded (500 cells/well) in a 6-well plate. After 24h, cells were treated with CDDP or Aila, further cultured for 10 days and subsequently fixed and stained, as previously described [21]. The count of colonies was performed by the function “Analyze Particles” of the ImageJ software (https://imagej-nih-gov.bibliopass.unito.it/ij/National Institutes of Health, USA; version 1.52).

### 2.3. Measurement of the cell's redox status

Cells were seeded (200,000 cells/well) in a 6-well plate in 2 ml of serum-supplemented RPMI medium. The intracellular oxidative stress level was evaluated by incubating cells with dichlorodihydrofluorescein diacetate (DCF-DA, Invitrogen, Carlsbad, CA, USA), while dihydroethidium (DHE, Merck Life Science S.r.l.) was used to specifically detect ROS, in particular the superoxide anions. The amount of the respective fluorescent products (2,7-dichlorodihydrofluorescein -DCF-, and 2-hydroxyethidium) were measured by FACScan cytometer (Becton Dickinson Accuri) [23].

### 2.4. Cell invasion assay

A2780 and A2780/CP70 were plated (2000 cells) onto the apical side of 50 µg/mL. Matrigel-coated filters of a Boyden chamber (BD Biosciences, San Jose, CA) in serum-free medium with or without Aila, while medium with 20% FCS was used as chemoattractant in the basolateral chamber. The invasion assay was then performed as previously described [21]. Data are shown as percentages of the inhibition of treated cells in comparison with the rate of migration measured on untreated cells [24]. Results are expressed as percent of control values: 65 ± 4 cells per microscope field in A2780 cells, and 72 ± 4 in A2780/CP70 cells.

### 2.5. Western blot analysis

Western blot analysis in A2780, A2780/CP70, 253J B–V, and 253J B–V C-r cells were performed as already reported [5]. We have used the following antibodies: from Santa Cruz Biotechnology, Inc.: β-actin (sc-47778), YAP (sc-15407), Nrf2 (sc-722), Keap1 (sc-33569), UCHL1 (C-4, sc-271,639), survivin (D-8, sc-17779); from Cell Signaling Technology, Inc.: GAPDH (14C10, 2118), pYAP (4911); from Merck Life Science S.r.l: GSTA4 (SAB 1401164-100UG); from Immunological Sciences (Rome, Italy): p62/SQSTM1 (AB-81677). To detect the bands, membranes were incubated with Clarity Western ECL Substrate (Bio-Rad Laboratories S.r.l. Segrate, Milan, Italy, #1705061), and the chemiluminescent signal was captured by autoradiography film (Santa Cruz Biotechnology, sc-201697), or visualized by using a Bio-Rad visualizer (Bio-Rad Molecular

imager ChemiDoc XRS+). The signal intensities relative to the protein products were quantified by densitometric scanning using the ImageJ software.

### 2.6. Gene expression analysis by quantitative real-time polymerase chain reaction (qRT-PCR)

After RNA isolation from A2780, A2780/CP70, 253J B–V, and 253J B–V C-r cells by using the TRIzol™ Reagent (Thermo Fisher Scientific), equal amount of RNA (1 µg) was incubated with 1 U DNaseI (Invitrogen, Monza, Italy), followed by retro-transcription (Superscript III RT kit, Invitrogen, Monza, Italy). Gene expression levels of Nrf2 and YAP, and Abelson (Abl) gene as housekeeping control, were measured by quantitative real-time PCR (qRT-PCR) in an iCycler (Bio-Rad Laboratories S.r.l.), as previously described [5]. Variations of gene expression were calculated using the ΔΔCt method [25].

### 2.7. MG132 treatment

MG132, or carbobenzoxy-Leu-Leu-leucinal (Merck Life Science S.r.l), is a peptide aldehyde inhibitor of the 26S proteasome complex. To evaluate the Aila effect on Nrf2 and YAP expressions after proteasome inhibition, A2780, A2780/CP70, 253J B–V, and 253J B–V C-r cells (200,000 cells/well) were seeded in a 6-well plates, and treated with 0.5 µg/ml Aila in the presence or absence of 5 µM MG132 for 24 h. At the end of the treatment, cells were harvested, washed with PBS, and frozen at −80 °C until protein extraction.

### 2.8. Statistical analysis

Differences between experimental groups were evaluated by one-way ANOVA followed by the Bonferroni post-test (GraphPad InStat software (San Diego, CA, USA). We considered statistically significant values of $p \leq 0.05$.

## 3. Results

### 3.1. Aila inhibited viability and proliferation of both CDPP-sensitive and CDDP-resistant ovarian cancer cells

The sensitivity or the resistance of ovarian cancer cells to CDDP was tested by exposing cells to increasing concentration of CDDP. MTT analysis confirmed the CDDP resistance of A2780/CP70 cells (S1 supplementary data). To evaluate the effect of Aila in ovarian cancer cell lines, we exposed these cells to increasing concentrations of Aila and evaluated cell growth through MTT and colony forming assays. Aila was more effective than CDDP in inhibiting the growth of both sensitive and resistant cells (Fig. 1A). Colony forming assays, performed in A2780 and in A2780/CP70 cells (Fig. 1B and C, D), also confirmed the Aila ability in inhibiting cell growth, and, in particular, the higher effectiveness of Aila, with respect to CDDP, was most pronounced in A2780/CP70 cells.

### 3.2. Aila inhibited invasion of both CDPP-sensitive and CDDP-resistant ovarian cancer cells

The invasion assay, performed by using Aila concentrations unable to affect cell growth (Fig. 2A), demonstrated that Aila, at concentrations of 0.01 and 0.05 µg/ml, significantly affected cell invasion (Fig. 2B). Furthermore, the effect exerted by 0.05 µg/ml of Aila was higher in A2780/CP70 than in A2780 cells (about twice).

### 3.3. Aila reduced Nrf2, YAP protein expression and activity in both CDPP-sensitive and CDDP-resistant ovarian cancer cells

A reduction of Nrf2 protein expression in A2780 and A2780/CP70 cells, treated with increasing Aila concentrations, was observed 24 and 48 h after the treatment (Fig. 3A and B). The down-regulation of Nrf2 activity was confirmed by the reduction of the expression of the Nrf2 target GSTA4 (Fig. 3C and D). To evaluate whether the reduction of Nrf2 expression was accompanied by a reduction of YAP expression, YAP protein expression was tested in A2780 and A2780/CP70 cells, 24 and 48h after Aila treatment. Results showed that Aila inhibited YAP protein expression in A2780 and A2780/CP70 cells, starting from the Aila

concentration of 0.1–0.5 μg/ml (Fig. 4A and B). The inhibition of YAP/TEAD transcriptional activity was confirmed by the simultaneous reduction of the survivin expression, a YAP/TEAD target gene. (Fig. 4C and D).

### 3.4. Aila increased the intracellular oxidative stress level in ovarian and bladder cancer cells

Since both Nrf2 and YAP proteins are involved in the regulation of cellular oxidative levels, we analyzed the effect of Aila treatment in the ovarian cancer cells and in 253J-BV (sensitive) and 253J-BV-Cr (CDDP-resistant) bladder cancer cells, in which the inhibitory effect of Aila treatment on Nrf2 and YAP protein expression was previously reported [21]. In both CDDP-resistant ovarian and bladder cancer cells the levels of Nrf2 and YAP proteins are higher than in respective sensitive clones (Fig. 5A and B). According to these observations, we found a lower content of intracellular oxidative stress level (Fig. 5C and D) and superoxide anion (Fig. 5E and F) in resistant lines, with respect to the sensitive ones. Moreover, the Aila treatment increased the superoxide anion content in CDDP resistant cells. The increase of superoxide anion was evident also in sensitive cells but at lower level compared with CDDP-resistant cells (Fig. 6).

### 3.5. Aila reduced Nrf2, YAP protein expression through a post-translational mechanism

To verify whether the inhibitory effect of Aila on Nrf2 and YAP protein expression could depend on the reduction of their transcription or, otherwise, could depend on the protein degradation, we analyzed Nrf2 and YAP mRNA in both bladder and ovarian cancer cells, sensitive and resistant to CDDP. In both sensitive and CDDP-resistant ovarian and bladder cancer cells we observed an increase of Nrf2 (Fig. 7A and B) and YAP (Fig. 7C and D) mRNA expression 24 and 48 h after Aila treatments. These results demonstrated that the reduction of Nrf2 and YAP protein amounts, in both bladder and ovarian cancer cells, did not depend on the reduction of transcription but it was at the post-translational level.

Since both Nrf2 and YAP proteins are degraded through the proteasomal pathway, we incubated ovarian and bladder cancer cells, of control or treated with Aila, with MG132, a well known proteasomal inhibitor [26]. Results demonstrated that a 24 h incubation with MG132 restored the amounts of Nrf2 and YAP proteins similar to the control, in both ovarian (Fig. 8) and bladder cancer cells (Fig. 9), thus confirming that the reduction of Nrf2 and YAP proteins, after Aila treatment, could depend on the increase of protein degradation.

Degradation of Nrf2 is mediated by the binding with Keap1, which induces ubiquitination and proteasomal degradation of Nrf2 [2]. In Aila-treated cells the reduction of Nrf2 expression could be accompanied by a reduction of p62/SQSTM1 (sequestosome 1) protein expression, which has been shown to be a Nrf2 target [27]. Moreover, p62/SQSTM1 protein was also able to bind Keap1 and lead it to autophagic degradation [27], thus, a reduction of this protein could contribute to increasing the level of Keap1 expression, and, of consequence, an increase of Nrf2 protein degradation. According to our hypothesis, Keap1 expression was found up-regulated and p62/SQSTM1down-regulated in ovarian (Fig. 10A and B) and bladder (Fig. 10C and D) Aila-treated cells.

It has been reported that phosphorylation of YAP on S381 creates a binding site for a complex able to recognize and ubiquitinate YAP, leading it to proteasomal degradation [28]. The analysis of pYAP expression was performed at 6 h from the Aila treatment (we chose a time before the reduction of total YAP protein expression occurred) and demonstrated that the YAP phosphorylation on S381 was increased in all the cell lines tested (Fig. 11).

### 3.6. Aila inhibited UCHL1 expression in ovarian and bladder cancer cells

The ubiquitination of target proteins is a reversible process. The activity of ubiquitin ligases is counteracted by deubiquitinating enzymes (DUBs), which catalyze the removal of covalently attached ubiquitin moieties. Among the DUBs enzymes, the Ubiquitin C-terminal hydrolases (UCHs) have gained particular attention, due to the role played in the progression of diverse tumors [29]. We found that UCHL1 expression was

significantly increased in both ovarian and bladder CDDP-resistant cells (Fig. 12A, C) and its expression was down-regulated by Aila treatment (Fig. 12D and E), suggesting that the inhibition of its expression could be involved in the Aila-induced Nrf2 and YAP protein degradation.

## 4. Discussion

Our results demonstrated that Aila reduced proliferation and migration of ovarian cancer cells. In particular Aila inhibited the growth of A2780/CP70 ovarian cancer cells which did not respond to CDDP treatment and reduced cell migration in these cells with a higher effectiveness than in A2780 cells. Analogously with that observed in bladder cancer cells [21], these actions could be related to the inhibition of Nrf2 and YAP protein expression, since both proteins play an important role in reducing proliferation and invasion of cancer cells [1,7]. The Aila-dependent reduction of the expression of GSTA4, p62/SQSTM1 and survivin, which are Nrf2 and YAP/TEAD targets, respectively, demonstrated that Aila was also able to inhibit Nrf2 and YAP/TEAD transcriptional activity.

Beyond cell proliferation and chemoresistance, Nrf2 and YAP proteins have been also involved in the regulation of oxidative stress, through the induction of antioxidant enzymes, such as glutathione S-transferases (GSTs) and heme oxygenase 1, which are activated by Nrf2 [2] and catalase and MnSOD, which are activated by YAP/FoxO1 pathway [8]. Moreover, Nrf2 and YAP proteins are linked through a cross-talk, which reduced YAP expression when Nrf2 was inhibited and vice versa [5]. We demonstrated that the amount of the intracellular oxidative stress level was lower in CDDP-resistant cells than in sensitive ones, in accordance with the higher expression of Nrf2 and YAP proteins in CDDP-resistant cells. Aila treatment greatly increased superoxide anion level by decreasing Nrf2 and YAP protein expression, in particular in CDDP-resistant lines, in which these two proteins were expressed at high level.

Until now the mechanism of Aila action in inhibiting Nrf2 and YAP protein expression in ovarian and bladder cancer cells has not been investigated. Our results demonstrated that Aila treatment, increased Nrf2 and YAP proteasomal degradation. Indeed, the mRNA level of Nrf2 and YAP was increased after Aila treatment, demonstrating that the reduction of Nrf2 and YAP proteins did not depend on the reduction of transcription. The observation that the reduction of the protein expression was reversed by the use of the proteasome inhibitor MG132, demonstrated that the decrease of Nrf2 and YAP protein levels depended on the increase of proteasomal degradation. We postulated that the increase of Nrf2 and YAP mRNA in Aila-treated cells probably depended on a compensatory attempt of the cells, that was not sufficient to balance the increase of degradation.

The Nrf2 protein degradation was linked to the binding with the Keap1 inhibitor [30]. Thus, the increase of Keap1 expression sustained the increase of Nrf2 degradation. The Keap1 role in several malignances has been widely investigated [31]. It has been demonstrated that the inactivation of Keap1 was paralleled by Nrf2 over-expression, which, in turn, enhanced the antioxidant capacity of cancer cells and decreased their sensitivity to chemotherapeutics [32]. In our cellular models, the increase of Keap 1 expression in Aila-treated cells, could depend on the reduction of p62/SQSTM1 protein. Indeed, p62/SQSTM1 is a receptor for ubiquitinated proteins that are degraded by autophagy [33] and can bind Keap1 to drive it toward the autophagic degradation [34]. Moreover, an increase of p62/SQSTM1 protein was also related to a poor prognosis of several types of cancers, including ovarian cancer [35,36]. We demonstrated that Aila treatment increased Keap1 expression and decreased the expression of p62/SQSTM1 protein. Analogously, Aila treatment induced an increase of YAP phosphorylation on Ser 318, which has been demonstrated to be critical for YAP ubiquitination [6].

The ubiquitination is a reversible process that is regulated by ubiquitinating enzymes and DUBs [37]. DUBs are isopeptidases that cleave polyubiquitin chains and remove ubiquitin from target proteins, thus

protecting protein from degradation [38]. Among the 100 DUBs identified, UCHs have gained attention thanks to their role in malignances. UCHL1 is the most studied UCH member, its expression has been found to be increased or decreased in relation of the tumor type and, also in the same tumors, conflicting results are reported [29]. However, UCHL1 was found to be related to the antioxidant potential of the cells, since it increased GSH in radio-resistant cancer cells, and to metastatization in breast and lung cancers [29, 38]. These data prompted us to investigate its expression in sensitive and CDDP-resistant bladder and ovarian cancer cells. Results obtained demonstrated that UCHL1 expression was increased in chemo-resistant bladder and ovarian cancer cells, compared with the corresponding sensitive counterpart, thus suggesting a role of UCHL1 in CDDP-resistance. The treatment with Aila reduced UCHL1 expression, suggesting that this reduction could be in relation, not only with the increased degradation of Nrf2 and YAP proteins, but could also contribute to the increase of oxidative stress.

The Aila effect on the protein degradation was previously reported [19]. He and collaborators found that Aila increased the ubiquitine-proteasome degradation of the androgen receptor (AR) through the binding to the co-chaperone protein p23. However, the effect of Aila could depend on the tissue type and the tissue-specific protein expression. We cannot exclude that, in ovarian and bladder cancer cells, Aila could affect other mechanisms as well as the apoptosis induction, as described in melanoma [17], or in human hepatoma cell line [15]. However, we must consider that the reduction of Nrf2 and YAP expression can affect several pathways involved in chemoresistance, including cell cycle arrest and apoptosis [39,40].

In conclusion, the ability of Aila to reduce the proliferation and invasion of cancer cells, resistant to conventional treatment, may represent an important tool in the therapy of CDDP-resistant ovarian and baldder cancer cells. The clarification of the mechanism of action of this compound may stimulate further investigations, leading to a clinical use of Aila.

## Declaration of competing interest

The authors declare no conflict of interest.

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**Fig. 1.** Viability and cell proliferation in A2780 and A2780/CP70 cells. A: MTT assay 72 h from the treatment with the indicated concentrations of Aila or CDDP. Results are expressed as percent of control, and are the mean ± SD of six separate experiments. **p ≤ 0.01vs control cells. ##p ≤ 0.01 vs. sensitive A2780. B: Colony forming assay. Number of colonies are expressed as percent of control number in cells treated with Aila or CDDP, and results are the mean ± SD of four separate experiments. **p ≤ 0.01 and *p ≤ 0.05 vs. control. ##p ≤ 0.01 vs. sensitive A2780 cells. C, D: Images of representative colony forming assays.

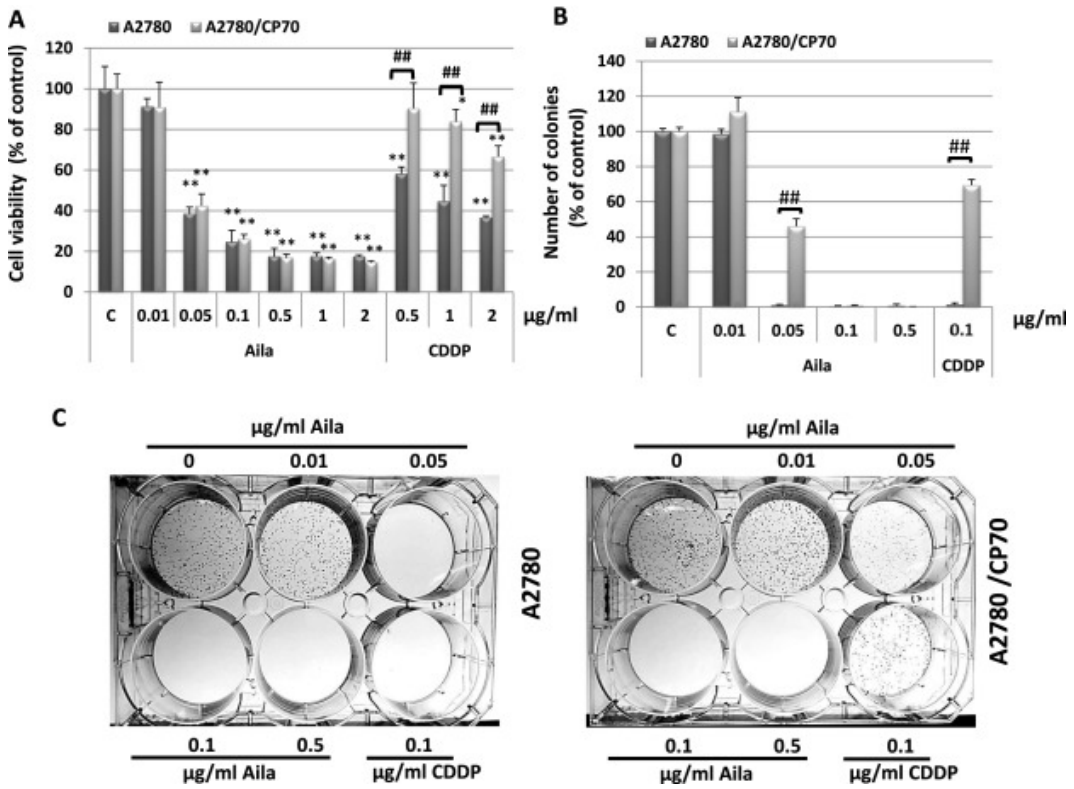



**Fig. 2**. Invasion assay of A2780 and A2780/CP70 cells treated with Aila at concentrations unable to affect cell growth. A: MTT assay at 24 h from the treatment. Results are expressed as percent of control, and are the mean ± SD of four separate experiments. B: invasion assay at 24 h. Data are expressed as percentages of inhibition versus the control. Data are mean ± SD of five independent experiments.**p ≤ 0.01 and *p ≤ 0.05 vs. control; ##p ≤ 0.01 vs. sensitive A2780 cells.

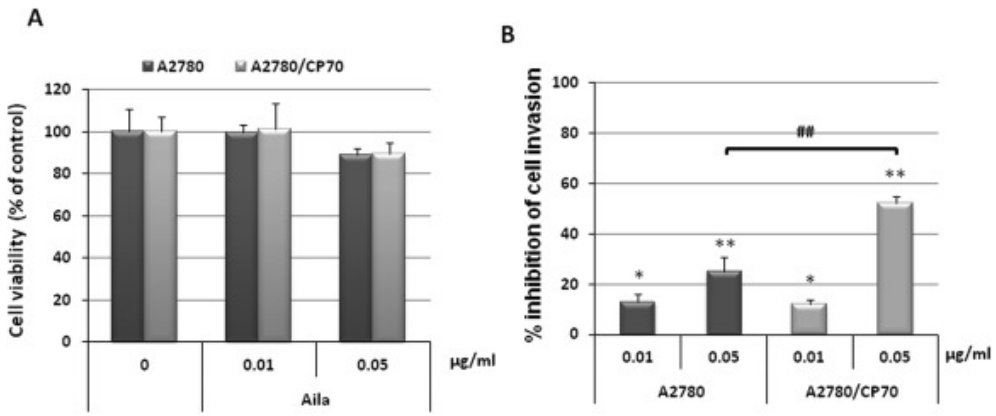


**Fig. 3**. Western blot analysis of Nrf2 (A) and GSTA4 (C) expressions in A2780 and A2780/CP70 cells, untreated (0) or treated with Aila at the indicated concentrations, and harvested after 24 or 48 h. B and D: densitometric scanning of Nrf2 and GSTA4 expression normalized using the β-actin signal. Data are the mean ± SD from three independent experiments. **p ≤ 0.01 and *p ≤ 0.05 vs. control.

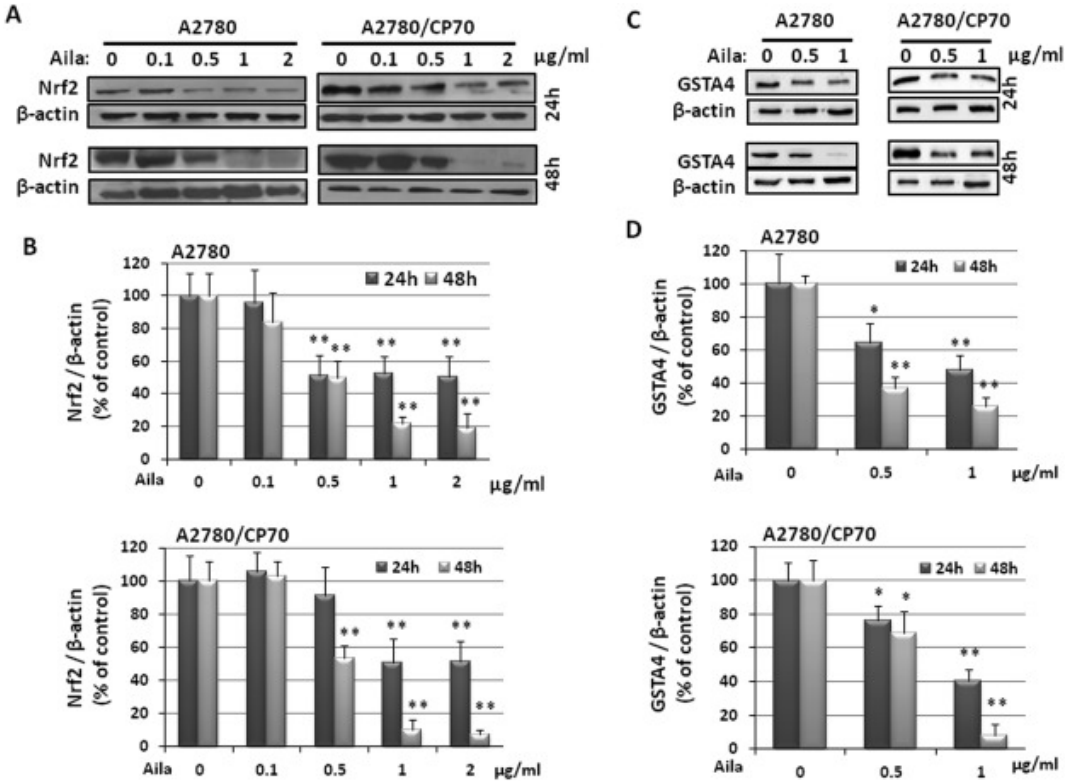


**Fig. 4**. Western blot analysis of YAP (A) and Survivin (C) expression in A2780 and A2780/CP70 cells, untreated (0) or treated with Aila at the indicated concentrations, and harvested after 24 or 48 h. B and D: densitometric scanning of YAP and Survivin expression normalized using the β-actin signal. Data are the mean ± SD from three independent experiments. **p ≤ 0.01 vs. control.

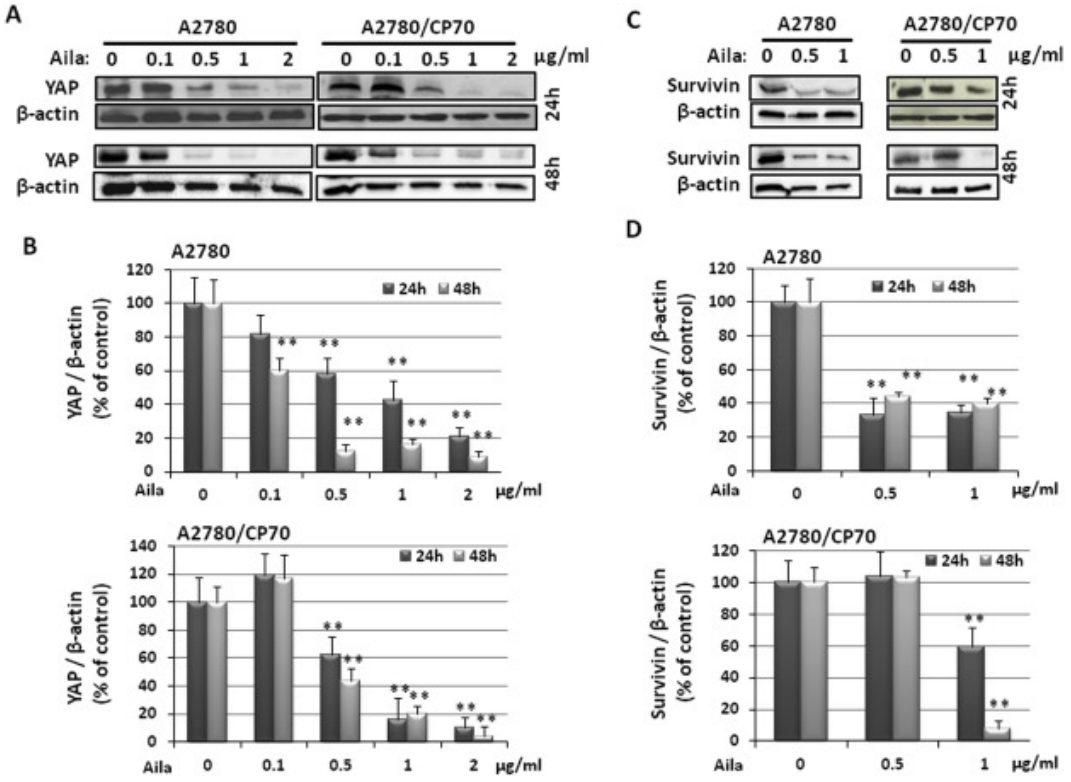


**Fig. 5**. YAP and Nrf2 protein expression, intracellular oxidative stress level, and superoxide anion production in untreated cells. Western blot analysis of YAP and Nrf2 and protein expression in A2780 and A2780/CP70 cells (panel A) and in 253J B–V and 253J B–V C-r cells (Panel B). To the right: quantitative densitometry of protein products normalized to the GAPDH signal. Data are indicated as means ± SD from three independent experiments. **p ≤ 0.01 vs. sensitive cells. Intracellular oxidative stress level in A2780 and A2780/CP70 cells (panel C) and in 253J B–V and 253J B–V C-r cells (Panel D). Representative histogram from flow cytometric analysis using DCF-DA in untreated cells are shown. To the right: bar graph showing median fluorescence intensity (MFI) values, expressed as means ± SD. Superoxide anion production in A2780 and A2780/CP70 cells (panel E) and in 253J B–V and 253J B–V C-r cells (Panel F). Representative histograms from flow cytometric analysis using DHE in untreated cells are shown. To the right: bar graphs showing the median fluorescence intensity (MFI) values, presented as means ± SD. **p ≤ 0.01 sensitive vs. chemoresistan cells.

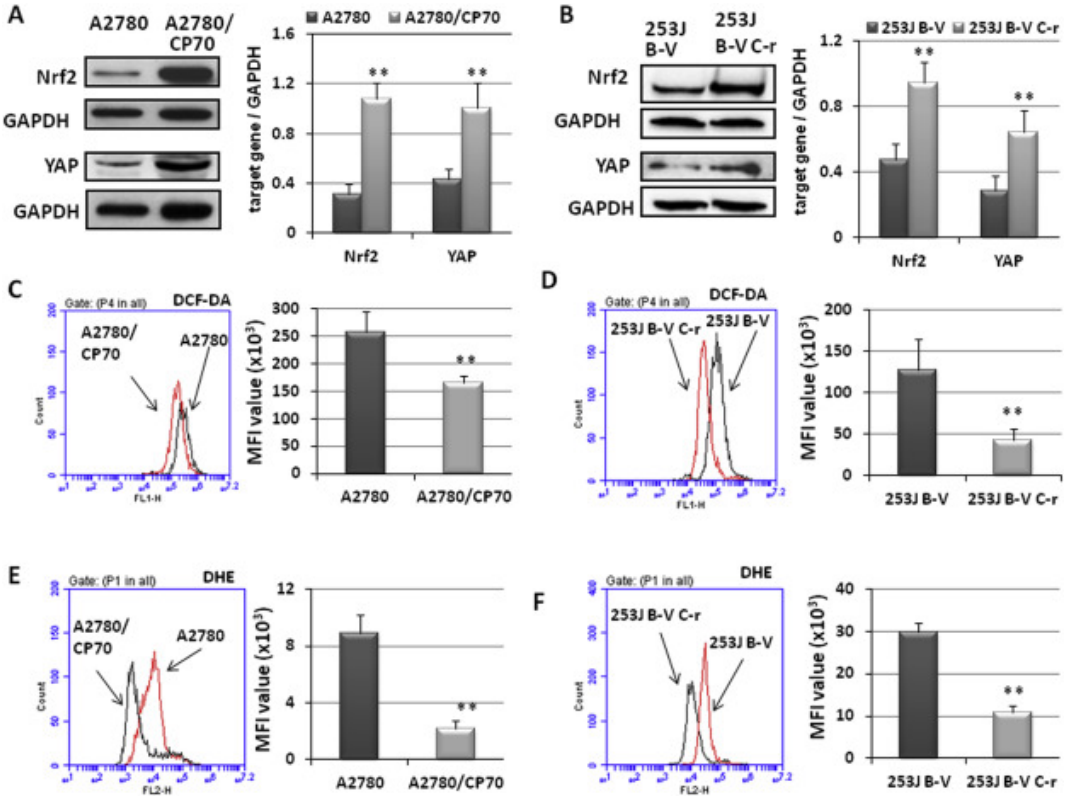


**Fig. 6**. Superoxide anion production after Aila treatments. Panel A: bar graph showing the relative median fluorescence intensity (MFI) values, presented as means ± SD, in A2780 and A2780/CP70 cells untreated (0) or treated with Aila at the indicated concentrations, and harvested after 24 or 48 h. Panel B: bar graph showing the relative median fluorescence intensity (MFI) values, presented as means ± SD, in 253J B–V and 253J B–V C-r cells untreated (0) or treated with Aila at the indicated concentrations, and harvested after 24 or 48 h, *p ≤ 0.05 and **p ≤ 0.01 vs. respective control.

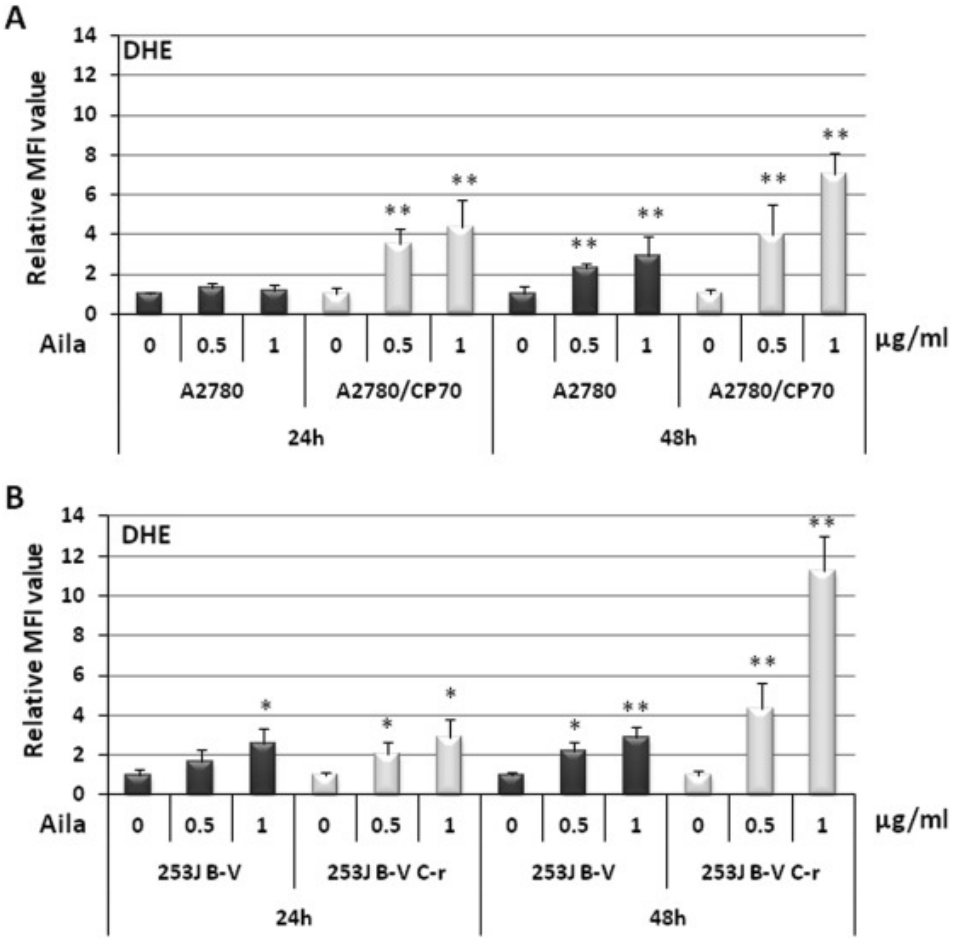


**Fig. 7**. Nrf2 (A) and YAP (C) mRNA expression in A2780 and A2780/CP70 ovarian cancer cells treated with Aila at the dose and time indicated. Nrf2 (B) and YAP (D) mRNA expression in 253J B–V and 253J B–V C-r bladder cancer cells treated with Aila at the dose and time indicated. mRNA expression was evaluated by qRT-PCR. Abelson (Abl) gene was utilized as housekeeping control. All analyses were carried out in triplicate; results showing a discrepancy greater than one cycle threshold in one of the wells were excluded. The results were analyzed using the ΔΔCt method. *p ≤ 0.05 and **p ≤ 0.01 vs. respective control.

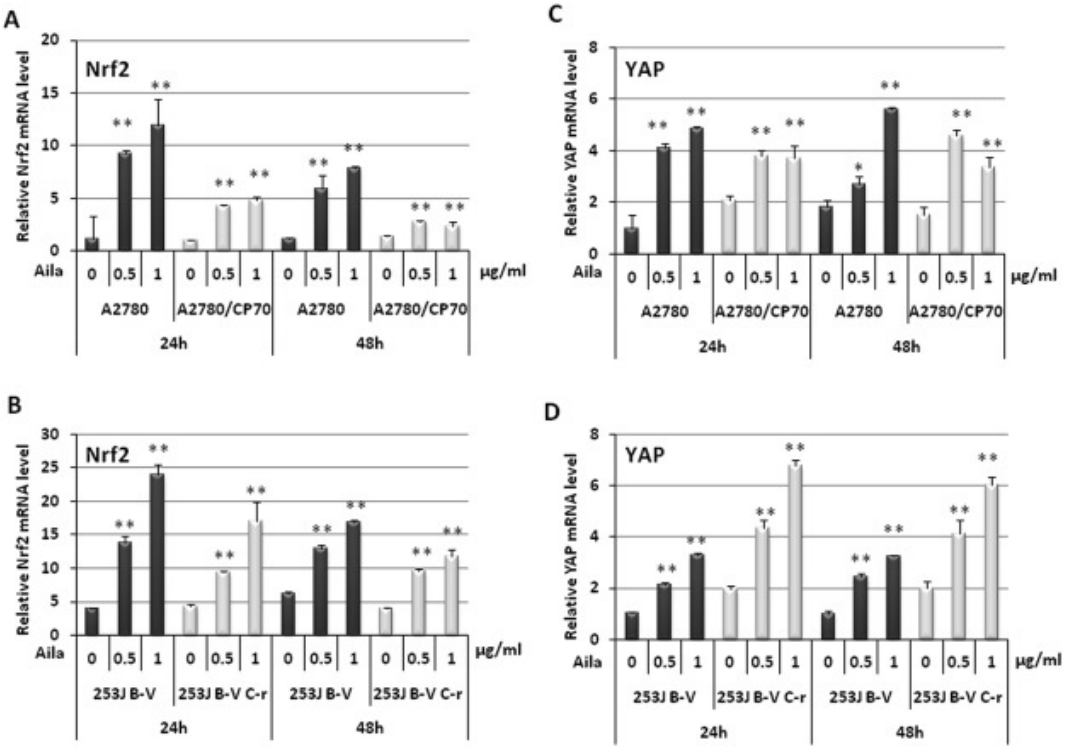


**Fig. 8**. WB analysis of Nrf2 (A) and YAP (C) expression in A2780 and A2780/CP70 ovarian cancer cells untreated (C) or treated with 5 µM MG132 (MG); with 0.5 µg/ml Aila, or 5 µM MG132 + 0.5 µg/ml Aila (MG + Aila), collected 24 h after the treatment. B and D: densitometric scanning of Nrf2 and YAP expression normalized using the β-actin signal. Data are the mean ± SD from three independent experiments. **p ≤ 0.01 vs. control, ##p ≤ 0.01 MG + Aila vs. Aila.

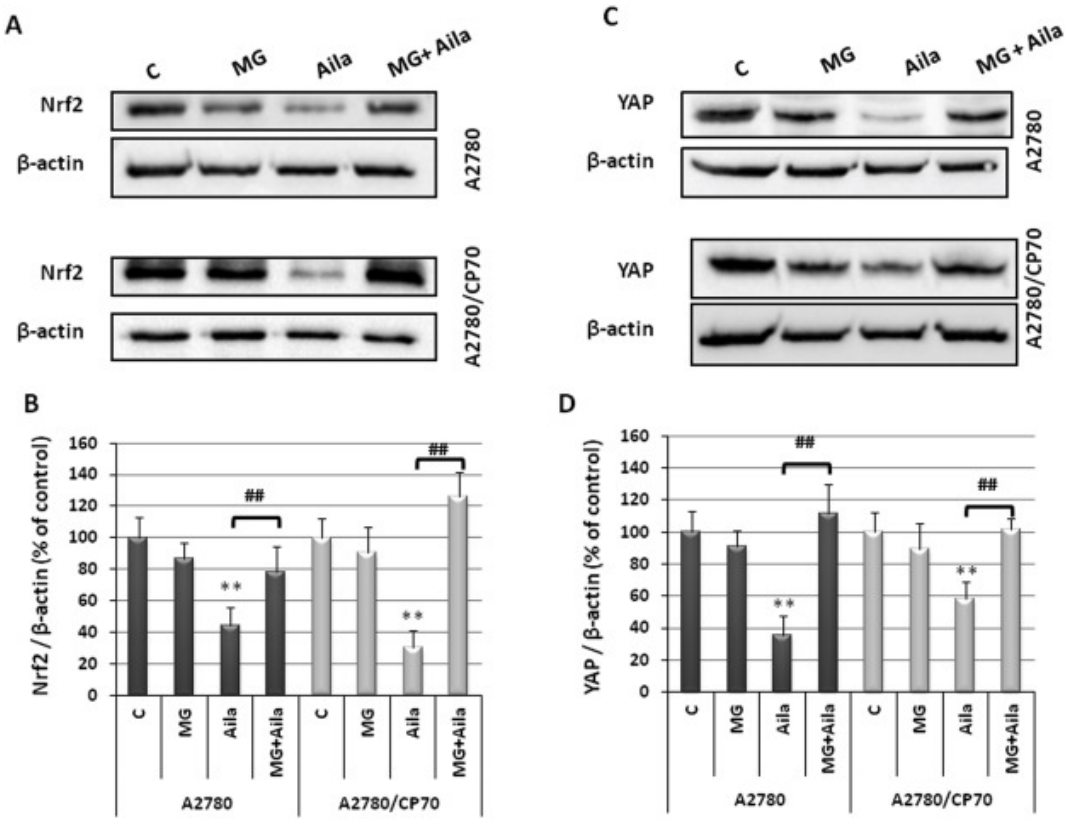


**Fig. 9**. WB analysis of Nrf2 (A) and YAP (C) expression in 253J B–V and 253J B–V C-r bladder cancer cells, untreated (C) or treated with 5 µM MG132 (MG); with 0.5 µg/ml Aila, or 5 µM MG132 + 0.5 µg/ml Aila (MG + Aila), or, collected 24 h after the treatment. B and D: densitometric scanning of YAP and Survivin expression normalized using the β-actin signal. Data are the mean ± SD from three independent experiments. **p ≤ 0.01 vs. control, ##p ≤ 0.01 MG + Aila vs. Aila.

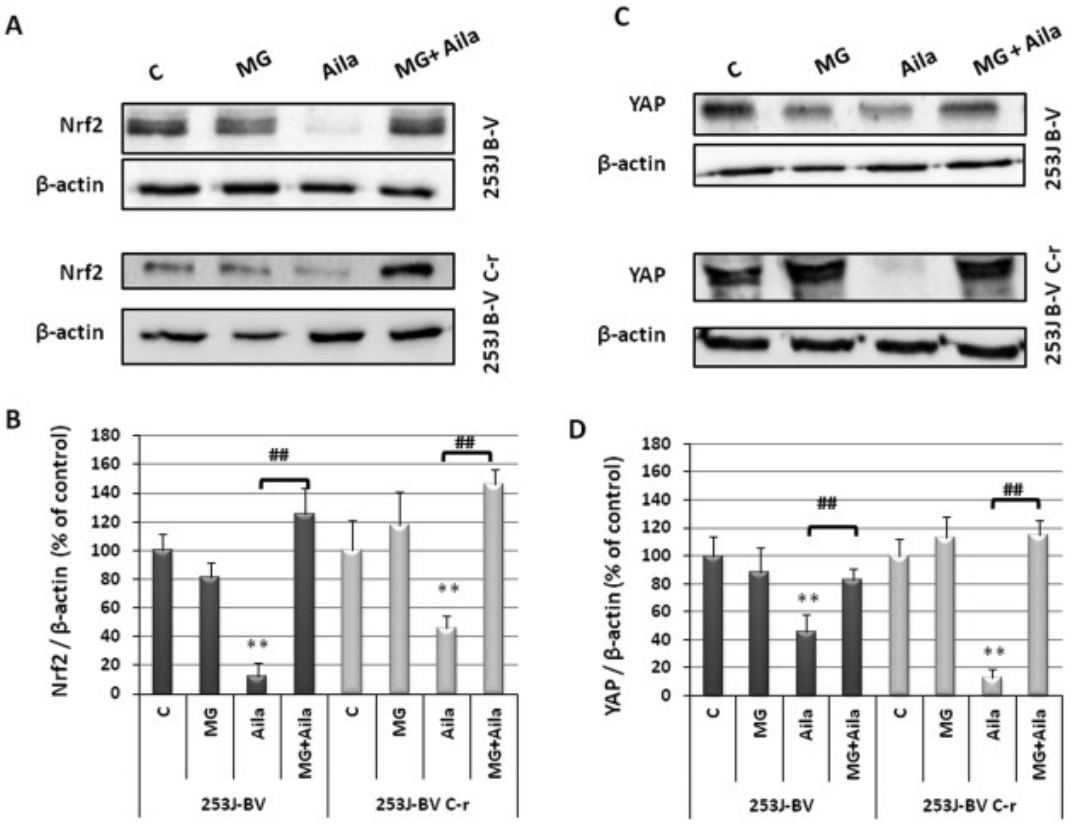


**Fig. 10**. Western blot analysis of Keap1 and p62 expression in A2780 and A2780/CP70 cells (A), and 253J B–V and 253J B–V C-r bladder cancer cells (C), untreated (0) or treated with Aila at the indicated concentrations, and harvested after 24 h. B and D: densitometric scanning of Keap1 and p62 expression normalized using the β-actin signal. Data are the mean ± SD from three independent experiments. *p ≤ 0.05 and **p ≤ 0.01 vs. control.

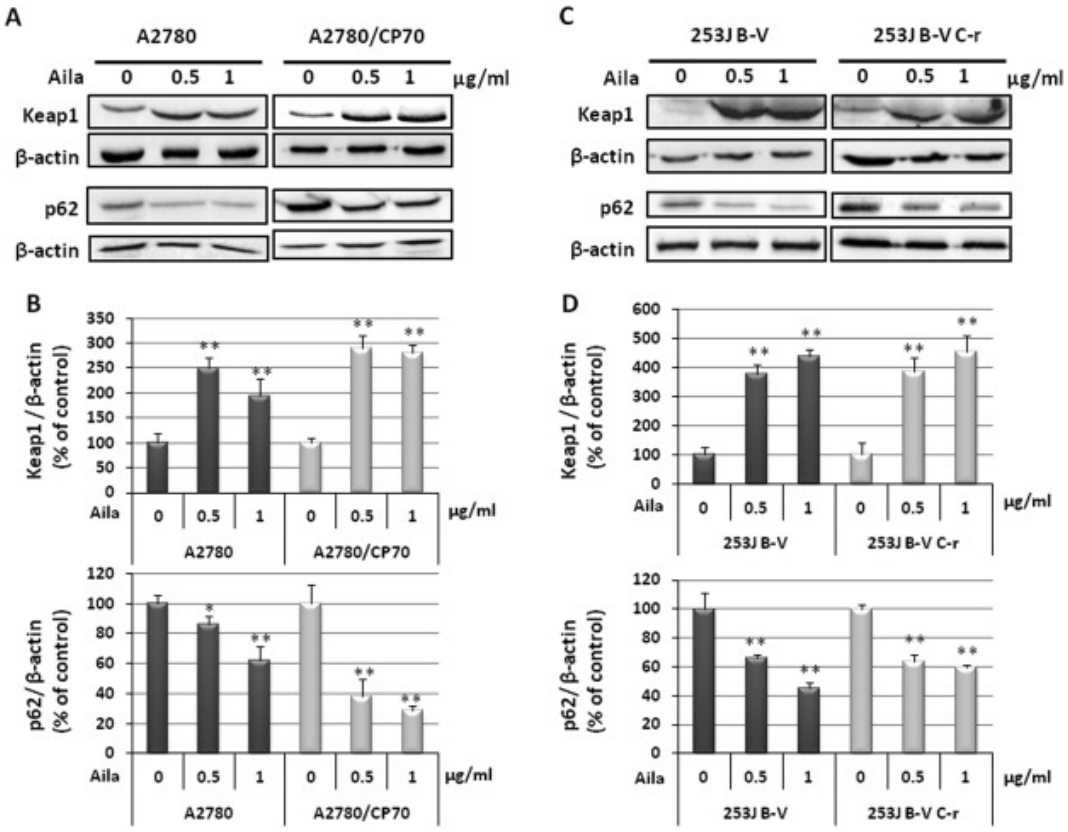


**Fig. 11**. Western blot analysis of pYAP expression in A2780 and A2780/CP70 cells (A) and 253J B–V and 253J B–V C-r bladder cancer cells (C) untreated (0) or treated with Aila at the indicated concentrations, and harvested after 24 h. B and D: densitometric scanning of pYAP expression normalized using the β-actin signal. Data are the mean ± SD from three independent experiments. **p ≤ 0.01 vs. control.

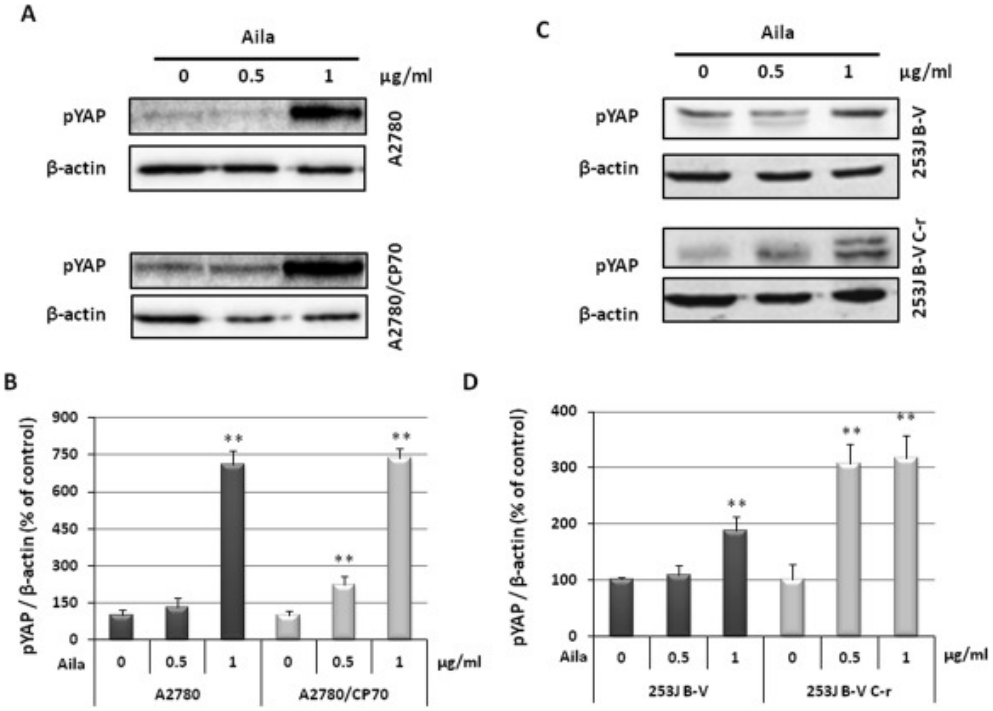
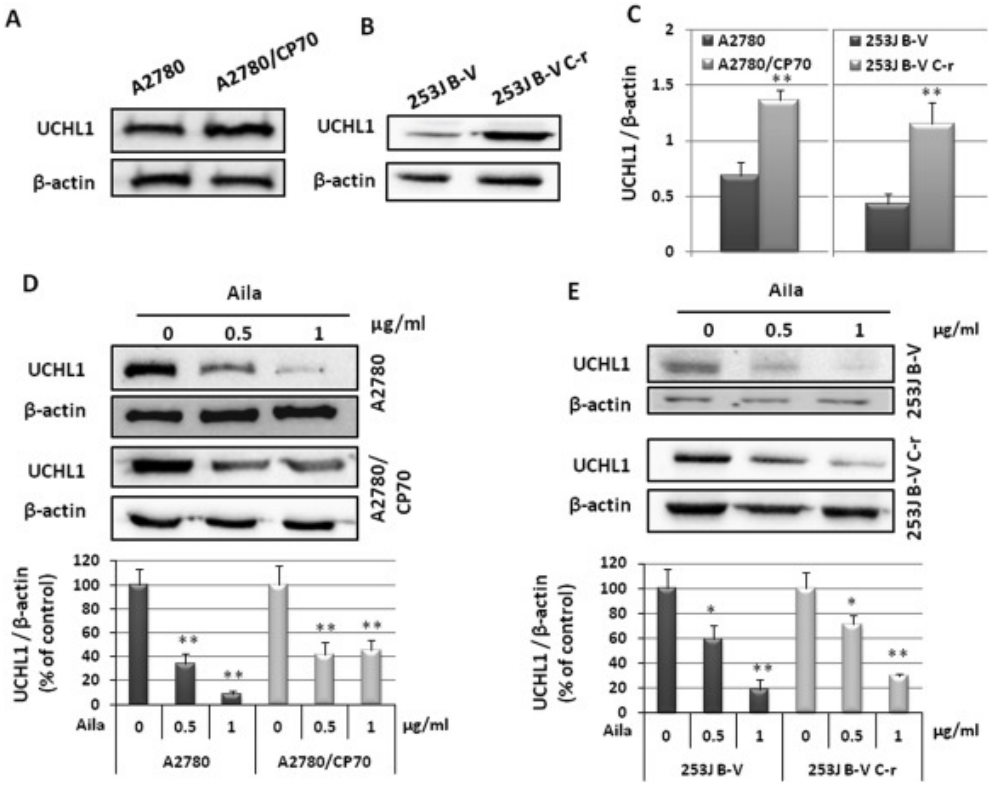


**Fig. 12**. Western blot analysis of UCHL1 basal expression in A2780 and A2780/CP70 cells (A) and 253J B–V and 253J B–V C-r bladder cancer cells (B). C: densitometric scanning of UCHL1 expression normalized using the β-actin signal. **p ≤ 0.01 vs. respective sensitive clone. Western blot analysis of expression in A2780 and A2780/CP70 cells (D) and 253J B–V and 253J B–V C-r bladder cancer cells (E), untreated (0) or treated with Aila at the indicated concentrations, and harvested after 24 h. Below: densitometric analysis. Data are the mean ± SD from three independent experiments. *p ≤ 0.05 and **p ≤ 0.01 vs. control.



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