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ORIGINAL ARTICLE

ASNA1, an ATPase targeting tail-anchored proteins, regulates melanoma cell growth and sensitivity to cisplatin and arsenite

O. Hemmingsson · Y. Zhang · M. Still · P. Naredi

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Abstract
Purpose ASNA1 is homologous to *E. coli* ArsA, a well characterized ATPase involved in efflux of arsenite and antimonite. Cells resistant to arsenite and antimonite are cross-resistant to the chemotherapeutic drug cisplatin. ASNA1 is also an essential ATPase for the insertion of tail-anchored proteins into ER membranes and a novel regulator of insulin secretion. The aim of this study was to determine if altered ASNA1 levels influenced growth and sensitivity to arsenite and cisplatin in human melanoma cells.
Methods Cultured melanoma T289 cells were transfected with plasmids containing sense or antisense *ASNA1*. Cells were exposed to cisplatin, arsenite and zinc. Cell growth and chemosensitivity were evaluated by the MTT assay and apoptosis by a TUNEL assay.
Results ASNA1 expression was necessary for growth. T289 clones with decreased ASNA1 expression exhibited $51 \pm 5\%$ longer doubling times than wildtype T289 ($P = 0.0091$). After exposure to cisplatin, ASNA1 down-regulated cells displayed a significant increase in apoptosis. The cisplatin IC_{50} in ASNA1 underexpressing cells was $41.7 \pm 1.8\%$ compared to wildtype ($P = 0.00097$) and the arsenite IC_{50} was $59.9 \pm 3.2\%$ of wildtype IC_{50} ($P = 0.0067$).
Conclusions Reduced ASNA1 expression is associated with significant inhibition of cell growth, increased apoptosis and increased sensitivity to cisplatin and arsenite.

Keywords ASNA1 · Cisplatin · Arsenite · Melanoma · Drug resistance

O. Hemmingsson (✉) · Y. Zhang · M. Still · P. Naredi
Department of Surgical and Perioperative Sciences,
Umeå University Hospital, 90185 Umeå, Sweden
e-mail: oskar.hemmingsson@surgery.umu.se

Introduction

Cisplatin and other platinum-based chemotherapeutic drugs are widely used in the clinic against solid tumours. However, their efficiency is limited by resistance caused by mechanisms that reduce drug accumulation, increase DNA repair and inhibit apoptosis [24]. These mechanisms are not fully understood and thus there are no efficient way to circumvent resistance and treatment failure. Human tumour cells display the RASP phenotype consisting of cross-resistance between arsenite (As), antimonite (Sb) and cisplatin (Pt). This is associated with impaired accumulation of arsenic and cisplatin, suggesting a common resistant mechanism [17, 18].

The human ATPase ASNA1 is evolutionary conserved and homologous to *E. coli* arsA [11]. *ArsA* encodes a cytosolic oxyanion-dependent ATPase [19] that binds to the transmembrane channel protein ArsB to form an efflux pump for arsenite and antimonite [27]. There is no known homologue to ArsB in eukaryotes but disruption of ASNA-1 in *C. elegans* results in increased sensitivity to arsenite and antimonite [28] suggesting a conserved role in metalloid resistance. The yeast ASNA1 homologue Arr4 has recently been described in several membrane associated functions. Arr4 acts in Golgi-to-ER traffic [22] and disruption of Arr4 results in increased sensitivity to arsenite [23]. Sharing homology with evolutionary conserved proteins associated with arsenite and antimonite detoxification, ASNA1 is a candidate to also confer cisplatin resistance.

ASNA1 is expressed in human tumour cells and in several normal tissues [13]. The protein has ATPase activity stimulated by arsenite [12] and is detected in the cytoplasm, the perinuclear region and the nucleolus [14]. ASNA1 was recently described as a positive regulator of human insulin secretion and proven mandatory for the growth in *C. elegans*

[8]. In mammalian cells, ASNA1 is a targeting factor for posttranslational insertion of tail-anchored proteins into the endoplasmatic reticulum [25]. As Bcl-2 and SNAREs are examples of tail-anchored proteins [6] ASNA1 could also be a regulator of apoptosis and vesicular efflux.

The aim of this study was to explore the influence of altered ASNA1 levels on growth and on cellular sensitivity to arsenite and cisplatin. Reduced *ASNA1* expression is associated with increased sensitivity to arsenite and cisplatin. Consistent with recent observations in *C. elegans,* ASNA1 downregulation significantly inhibited cellular growth.

Materials and methods

Cell culture

Human melanoma T289 cells [26] and the cisplatin-resistant subline T289/DDP [15] were grown in RPMI 1640 (Invitrogen, Paisley, UK) supplemented with 10% FBS and 1× HITES (10 nM hydrocortison, 5 µg/ml insulin, 5 µg/ml transferrin, 10 nM beta-estradiol and 5 ng/ml selenite, all from Sigma, Stockholm, Sweden), termed complete medium. The medium for transfected cells containing a neomycin resistance gene was supplemented with 0.3 mg/ml G418 (Invitrogen). The medium for siRNA-transfected cells was supplemented with 1% penicillin streptomycin, PEST (Invitrogen). Cells were grown in monolayer culture at 37°C in humidified air with 5% CO_2.

Plasmid constructs

The human *ASNA1* cDNA (GenBank AF047469) was obtained by PCR using primers flanking the entire 1047 bp coding sequence (5′-CGAGGAAATGGCGGCAG-3′ and 3′-GTAAAGTGTGAGTGGAAGGT-5′) with a human liver cDNA as template. The PCR product was separated in a 1% low melt agarose gel and the product purified in a Qiaquick spin column (Kebo lab, Spånga, Sweden). The cDNA was inserted into the *Eco* R1 site of the mammalian expression vector pTarget (SDS, Falkenberg, Sweden) in either sense or antisense direction. An empty vector was also ligated. The pTarget vectors contained a CMV-promotor and a G418-resistance gene. The vectors were transformed into JM109 cells and the plasmids were prepared by the Qiagen 500 plasmid prep (Kebo lab) according to the instructions from the manufacturer. The plasmids were cut by plasmid-specific BamHI- and *ASNA1*-specific ClaI and separeted in a 1% agarose gel to identify clones producing plasmids with sense or antisense *ASNA1*. The pTarget vectors were sequenced to confirm the orientation of the *ASNA1* cDNA.

Plasmid transfection

T289 cells were transfected with the pTarget-*ASNA1*-sense, pTarget-*ASNA1*-antisense and pTarget-empty vector plasmids using lipofectamine reagent (Invitrogen) according to the instructions from the supplier. Briefly, one µg plasmid DNA and 4 µl lipofectamine were mixed in 0.8 ml serum-free medium. Cells were incubated for 5 h at 37°C with the DNA-lipofectamine mixture and for additional 19 h after adding RPMI 1640 supplemented with 20% FBS. After the transfection, cells were diluted in complete medium supplemented with 1 mg/ml G418 and seeded for clonal expansion. After 3–4 weeks, independent cell clones were randomly isolated and plated separately.

siRNA constructs and transfection

siRNA oligos of 21 nucleotides complementary to *ASNA1* were obtained at Dharmacon siDesign centre (Dharmacon, Dallas, TX, USA). An *ASNA1* complementary oligo was selected (targets CCAGAUGGAGGACCUGUAU starting at base pair 912 of the coding sequence, genebank AF047469). A control-oligo (id siRNA#2, Dharmacon) was also used. T289 cells in complete medium supplemented with 1% penicillin streptomycin, PEST, grew to 70% confluency and were then transfected with siRNA oligos using opti-mem (Invitrogen) according to the instructions from the manufacturer.

Antibodies

Polyclonal rabbit and chicken anti-ASNA1 antibodies were generated by immunizing animals with a recombinant ASNA1-GST fusion protein at Agrisera, Vännäs, Sweden. The specificity of the immunized serum against ASNA1 was confirmed by ELISA and compared to preserum. Both 6xHis-ASNA1 and ASNA1-GST fusion proteins were detected on ELISA. The antibody showed the same immunohistochemical staining pattern as a previously used monoclonal anti-ASNA1 antibody, 5G8 [14], but with less background. Immunoblotting of total cell lysates detected two bands on western blot, a stronger 41 kDa band and a weaker 37 kDa band. Western blot on recombinant ASNA1 detected only the 41 kDa band. Immunoprecipitation on total cell lysates also displayed ASNA1 on the 41 kDa level. Transfection of T289 cells with a plasmid construct containing the full-length ASNA1 resulted in overexpression of the 41 kDa band.

Immunoblotting

Exponentially growing cells were lysed in ice-cold buffer containing 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 nM

EDTA, 1% Triton X-100, 5 mM DTT, 0.1 mM PMSF and 1% protease inhibitor cocktail (Sigma). The supernatant was collected after spinning in 14,000 rpm for 20 min at 4°C. The total protein concentration of the lysates was determined using the DC protein assay (Biorad, Sundbyberg, Sweden). A volume of 30 µg total cellular protein was separated in 10% SDS-PAGE gels and electrotransferred to immobilon-p membranes (Millipore, Sundbyberg, Sweden). After blocking in 5% fat-free milk in 1× TBS, the membranes were incubated with a primary rabbit anti-ASNA1 antibody (1:2,000; 1 h at room temperature). After washing, the membranes were incubated with a secondary horseradish peroxidase-conjugated donkey anti-rabbit antibody (Amersham Pharmacia Biotech, Uppsala, Sweden). The antibodies were visualized by the chemiluminescent ECL-kit (Amersham Pharmacia Biotech). To confirm equal loading, membranes were stripped for 30 min at 50°C in a buffer containing 100 mM beta mercaptoetanol, 2% SDS and 0.0625 M Tris (pH 6.7), and then incubated with a monoclonal mouse anti-PCNA antibody (Dakopatts AB, Älvsjö, Sweden) or a monoclonal anti-beta-actin antibody (Sigma), both detected as described above. Quantity One 4.6.5.094 (Bio-Rad Laboratories Inc) was used to quantify detected bands on western blots. Loading density of each band representing ASNA1 expression was adjusted for PCNA expression.

Immunofluorescence and immunocytochemistry staining

T289 cells were cultured in complete medium on chamber slides at overnight. The cells were fixed in 3% paraformaldehyde for 10 min at room temperature, washed in PBS and then blocked for 1.5 h in blocking solution containing PBS supplemented with 3% BSA and 0.25% Triton X-100. The primary polyclonal rabbit anti-ASNA1 antibody was added to the slide chamber in blocking solution for 1 h, followed by washing and 45 min incubation in dark with secondary goat anti-rabbit fluorescent IgG and goat anti-rabbit IgG-peroxidase (Immunkemi, Järfälla, Sweden). The slides were mounted with vectashield (Immunkemi). The positive staining was examined under light and fluorescent microscope at 20× and 100× (Zeiss Axiophot). T289 wildtype and transfected cells were photographed with Dage-MTI DC330E in parallell experiments to compare morphology.

Cell growth assay

T289 cells in suspension were stained by trypan blue and counted in a Bürkel chamber. 1,000 cells were seeded in 200 µl complete medium on 96-well plates. The plates were incubated at 37°C in humidified air with 5% CO_2 for 24, 48, 72 and 96 h. Anchorage-dependent cell growth was determined daily in six wells by the MTT colorimetric growth assay [10, 16]. Exactly 10 µl of 5 mg/ml MTT (Sigma) was added to each well and incubated at 37°C in humidified air with 5% CO_2 for 4 h. After removal of the medium, the dye crystals were dissolved in acidified isopropanol. The optical density was measured on an ELISA plate reader at 540 nm. Each experiment was performed at least three times.

Cell death assay

The apoptotic fraction was determined by the in situ cell death detection kit (Roche Diagnostics Scandinavia, Bromma, Sweden). In brief, cells were seeded on 2-well culture slides and grown 24 h at 37°C. To evaluate apoptosis after cisplatin treatment, samples were exposed to 100 µg/ml cisplatin (Platinol®, Bristol Myers Squibb AB, Bromma, Sweden) for 2 h prior to the TUNEL reaction. After fixation with 3% paraformaldehyde during 1 h, the slides were incubated 10 min in blocking solution containing methanol and 0.3% H_2O_2. To allow permeabilisation, the cells were incubated with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. Exactly 100 µl of the TUNEL reaction mixture was added and incubated 60 min in a humidified chamber at 37°C. The positive control was treated with 100 µl 0.5 mg/ml DNase 1 prior to the TUNEL reaction. The slides were then incubated with 100 µl converter-POD 60 min in a humidified chamber at 37°C followed by the incubation for 10 min in 200 µl DAB-substrate. The slides were mounted under glass coverslip and evaluated under light microscope.

Chemosensitivity assay

To assess chemosensitivity in pTarget *ASNA1* plasmid transfected cells, 1,500 cells were seeded in 200 µl complete medium in each well on 96-well plates. T289 wildtype cells and T289 cells transfected with empty pTarget vector were used as controls. After 24 h in a CO_2 chamber, the cells were subjected to either 0–15 µM sodium arsenite (Sigma) for 48 h or 0–500 µM zinc chloride (Göteborgs termometerfabrik, Gothenburg, Sweden) for 72 h or 0–6 µg/ml cisplatin for 72 h. To assess chemosensitivity in siRNA-transfected cells, 9,000 cells were seeded directly after transfection in 180 µl complete medium in each well on 96-well plates. After incubation for 48 h the cells were exposed to 0–100 µg/ml cisplatin for 1 h, washed in PBS and incubated in complete medium for additional 48 h. T289 wildtype and T289 transfected with control siRNA were used as controls. Each concentration was added to triplicate wells. Each experiment was performed at least three times.

Cell number in each well was determined by the MTT assay as described above. The concentration needed to

reduce the cellular population to 50% (IC_{50}) was determined.

Statistical analysis

Results were expressed as mean ± SEM. The doubling times and the IC_{50} values were calculated from the linear regression of the logarithmically transformed slope illustrating cell number versus time and cell number versus concentration. Three representative series were selected for each experiment. The relative doubling time, apoptotic fraction or drug sensitivity for each cell clone was determined by relating to T289 wildtype or T289 empty vector. Statistical analysis was carried out by two-sided student's *t* test. A P value < 0.05 was set as level of statistical significance. SPSS 11.0.2 for Mac OS X was used for statistical calculations.

Results

Expression of ASNA1

ASNA1 expression in different cell lines grown in cell culture was determined by western blot. The human melanoma cell line T289 had relatively high levels of ASNA1 compared to other cell lines used in our laboratory. The cisplatin-resistant subline T289/DDP displayed increased levels of ASNA1 compared to parental T289 (Fig. 1a).

To investigate the phenotype in cells with altered ASNA1 levels we transfected T289 melanoma cells with either *ASNA1* sense or *ASNA1* antisense constructs. An empty vector construct was used as a control. ASNA1 expression in empty vector-transfected cells was 105 ± 6% of wildtype expression on western blot ($P = 0.51$) (Fig. 1d). After transfection with the sense construct, 19 independent clones were picked and all grew well during clonal expansion. Out of these, three clones displayed a constitutive overexpression of ASNA1 compared to T289 wildtype and T289 empty vector control. Two ASNA1 overexpressing clones, s10 and s15, were chosen for further studies. Quantification of western blots revealed that the ASNA1 overexpression in these clones were 186 ± 5% and 188 ± 9% compared to wildtype ($P = 0.0037$ and $P = 0.011$, respectively) (Fig. 1c). After transfection with the antisense construct, 32 independent clones were isolated and observed during expansion. Four clones stopped growing and were discarded. Eight clones grew slowly compared to wildtype. On western blot, six out of the 28 clones displayed decreased ASNA1 levels compared to T289 wildtype and T289 empty vector control. All of these belonged to the group of slowly growing clones. No surviving clone expressed less than 40% of parental T289 ASNA1 expression. Two clones with constitutive downregulated ASNA1 levels, asD4 and asE2, were chosen for additional characterization. The level of ASNA1 in clone asD4 and asE2 was reduced to 41 ± 8% and 45 ± 5% compared to that in T289 wildtype-cells ($P = 0.018$ and $P = 0.0066$, respectively) (Fig. 1d).

The effect of reduced ASNA1 expression was also evaluated by transient transfection of T289 cells with a siRNA directed to the *ASNA1* mRNA. Four *ASNA1*-specific oligonucleotides were constructed and all were found to downregulate the ASNA1 expression by approximately 50% when introduced into T289 cells. Downregulation of ASNA1 levels was detected from day 1 to day 5 after siRNA transfection. One oligonucleotide was chosen for further studies. Transfection of control siRNA into T289 resulted in no change of ASNA1 expression on western blot compared to T289 wildtype (Fig. 1b).

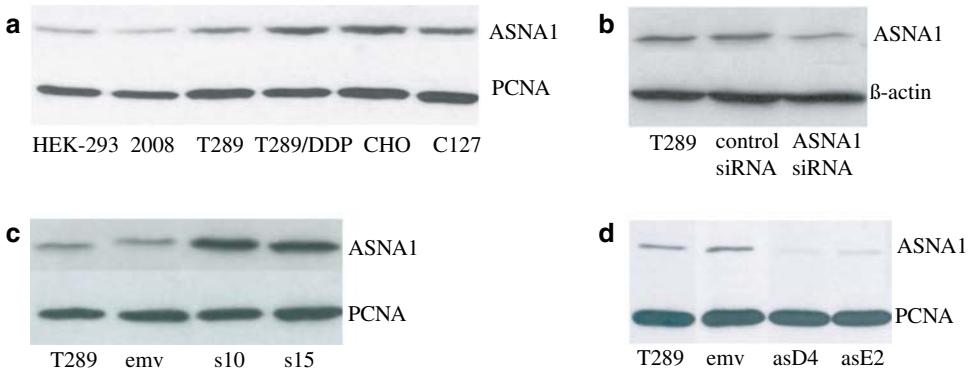

Fig. 1 Western blots displaying ASNA1 expression; ASNA1 was detected at 41 kDa level by a polyclonal rabbit anti-ASNA1 antibody. PCNA or beta-actin expression was used as loading control. **a** ASNA1 expression in different cell lines: human embryonic kidney (HEK-293), human ovarian carcinoma (2008), human melanoma (T289), cisplatin resistant subline of T289 (T289/DDP), chinese hamster ovary (CHO) and mouse fibroblast (C127), **b** ASNA1 expression in *ASNA1* siRNA treated cells compared to control siRNA treated cells and T289 wildtype, **c** T289 wildtype and sublines transfected with empty vector (emv) or a plasmid containing sense *ASNA1* (s10 and s15) and **d** T289 wildtype and sublines transfected with empty vector (emv) or a plasmid containing antisense *ASNA1* (asD4 and asE2)

Morphology of ASNA1 antisense transfected cells

Immunocytochemical and immunofluorescent staining of cells with altered ASNA1 expression revealed changed morphology in the ASNA1 deficient clones. These cells were larger and had a weak nuclear staining compared to wildtype (Fig. 2). The general staining intensity correlated with the ASNA1 levels detected on western blot as described above. ASNA1 overexpressing clones had similar morphology as wildtype.

Retarded growth rate in ASNA1 antisense downregulated cells

Anchorage dependent growth in T289 cells with altered ASNA1 expression was evaluated by the MTT-assay. The doubling times of the clones with increased ASNA1-expression, s10 and s15, was similar to that of the T289 wildtype (37.3 ± 2.9 h) and T289 empty vector control cells (Table 1). However asD4 and asE2 in which ASNA1 was downregulated grew significantly slower, as was observed also during the initial clonal expansion. ASNA1 deficient asD4 cells displayed 51 ± 5% longer doubling time than wildtype ($P = 0.0091$) (Table 1).

Table 1 Doubling times in T289 cells and the plasmid transfected sublines

Cell	Doubling time (h)	Doubling time (quotient)
T289	37.3 ± 2.9	1
emv	39.6 ± 3.1	1.02 ± 0.05
asD4	59.6 ± 6.8	1.51 ± 0.05**†
asE2	47.5 ± 4.1	1.26 ± 0.06*
s10	35.6 ± 3.1	1.02 ± 0.03
s15	33.4 ± 2.2	0.96 ± 0.02

T289, wildtype is compared to empty vector transfected clone (emv); *ASNA1*, antisense transfected clones (asD4 and asE2); *ASNA1*, sense transfected clones (s10 and s15). Data are expressed as mean ± SEM

* $P < 0.05$ compared to T289

** $P < 0.01$ compared to T289

† $P < 0.05$ compared to emv

Increased chemosensitivity in ASNA1 antisense downregulated cells

To test the hypothesis that ASNA1 is involved in cellular detoxification of arsenite and cisplatin, we compared chemosensitivity in cells with altered ASNA1 levels in two

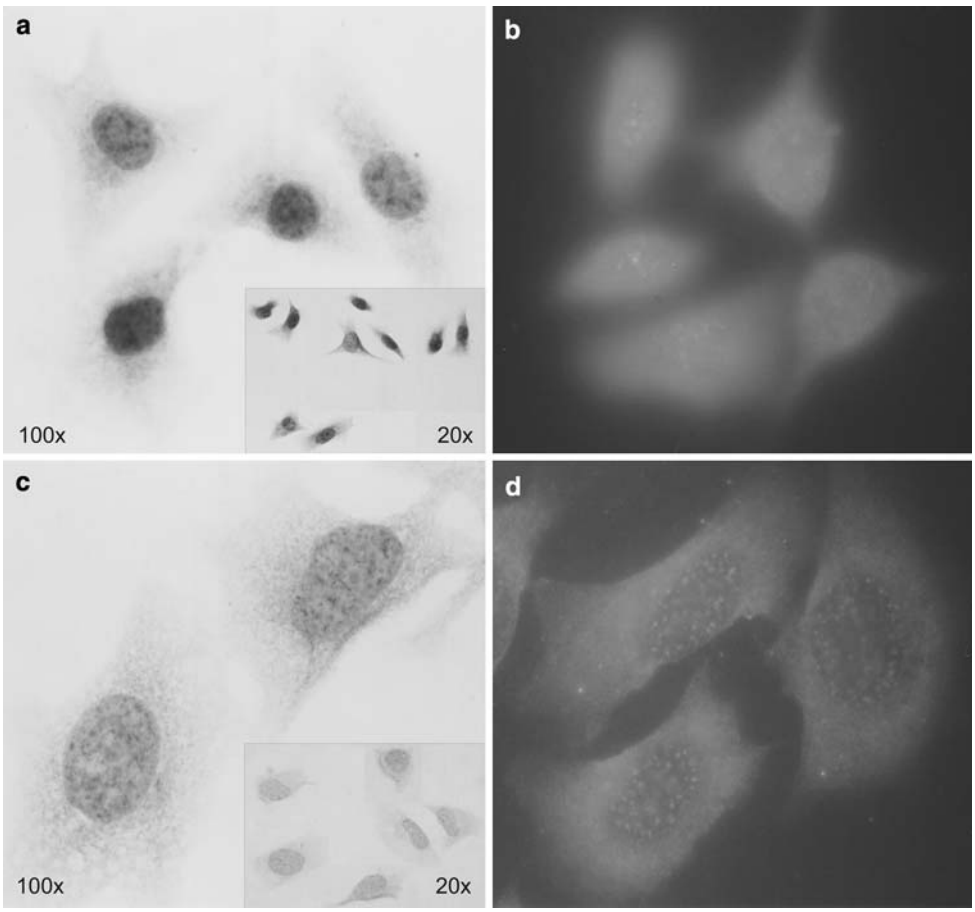

Fig. 2 Human melanoma T289 wildtype (**a**, **b**) is compared to ASNA1 antisense transfected subline asD4 (**c**, **d**). Immunocytochemical staining of T289 cells at 100× and 20× Zeiss axiophot (**a**, **c**). Immunofluorescent staining of T289 cells at 100× Zeiss axiophot (**b**, **d**)

different settings. We compared T289 wildtype and plasmid-transfected T289 cells in a continuous 48 h exposure to sodium arsenite or 72 h exposure to cisplatin or zinc chloride. The viability after exposure was determined by the MTT assay. The IC_{50} values for all examined metal salts were similar in the ASNA1 overexpressing, wildtype and empty vector transfected T289 cells; however, the antisense ASNA1 downregulated cells were significantly more sensitive to sodium arsenite and cisplatin. While the sodium arsenite IC_{50} for the T289 wildtype cells was 11.1 ± 1.0 μM, that for the antisense ASNA1 clone asD4 was 6.4 ± 0.3 μM ($P = 0.020$). The IC_{50} for cisplatin in the wildtype T289 cells was 2.2 ± 0.1 μg/ml compared to 1.1 ± 0.05 μg/ml for the asD4 cells ($P = 0.0058$). There was no difference in sensitivity to zinc chloride between ASNA1 deficient and wildtype T289 cells (Table 2; Fig. 3).

Chemosensitivity to cisplatin was also evaluated for T289 cells transiently transfected with an *ASNA1* siRNA. Due to the transient downregulating effect of siRNA, we exposed the cells to high cisplatin concentrations during 1 h. Transient suppression of ASNA1 expression significantly increased sensitivity to cisplatin. The IC_{50} for cisplatin in the ASNA1 siRNA treated cells was 23.8 ± 2.7 μg/ml compared to 37.3 ± 5.3 μg/ml in parental T289 ($P = 0.0046$) and 31.9 ± 4.2 μg/ml in the siRNA control T289 cells ($P = 0.012$).

Increased apoptosis in ASNA1 antisense downregulated cells

A TUNEL assay was conducted to determine whether the *ASNA1* underexpressing clones with a slower growth rate also displayed increased apoptosis. Spontaneous apoptotic cells were counted after 24 h on a culture slide. T289 wildtype cells demonstrated $6.3 \pm 0.7\%$ apoptosis. T289 empty vector control and ASNA1 overproducing s10 and s15 showed similar numbers, while ASNA1 antisense cells asD4 and asE2 exhibited increased apoptosis ($15.7 \pm 0.9\%$ $P = 0.010$ and $17.0 \pm 1.2\%$ $P = 0.073$, respectively). After exposure to 100 μg/ml cisplatin, a moderate increase in apoptosis was observed in T289 wildtype cells ($9.7 \pm 1.2\%$ $P = 0.12$), empty vector control and the ASNA1 overexpressing clones. A significant increase in apoptosis was observed in the ASNA1 downregulated clones asD4 and asE2 ($38.7 \pm 2.6\%$ $P = 0.00048$ and $40.7 \pm 0.9\%$ $P = 0.018$, respectively) (Fig. 4).

Discussion

Emergence of cisplatin-resistance during cancer treatment is a major clinical problem. We have previously reported cross-resistance between arsenite, antimonite and cisplatin, termed the RASP phenotype. The phenotype is associated with decreased accumulation of arsenite and the cisplatin analogue [3H]DEP in resistant cells, suggesting that these metal ions share a common resistance mechanism [18]. Here we show that ASNA1, a conserved ATPase involved in nematode growth [8] and resistance against arsenite and antimonite [28] also controls growth and sensitivity to cisplatin and arsenite in human tumour cells.

This is the first study describing a phenotype in human cells with altered ASNA1 levels. Plasmid transfections were used to establish clones in which ASNA1 expression was elevated or reduced. We report a significantly increased sensitivity to cisplatin and arsenite in T289 melanoma cells in which ASNA1 levels have been downregulated. Cisplatin induced apoptosis was enhanced in ASNA1

Table 2 Chemosensitivity in T289 cells and sublines transfected with empty vector (emv), *ASNA1* antisense (asD4 and asE2) or *ASNA1* sense (s10 and s15)

Cell	Sodium arsenite IC50		Cisplatin IC50		Zinc chloride IC50	
	(μM)	Quotient	(μg/ml)	Quotient	(mM)	Quotient
T289	11.1 ± 1.0	1	2.19 ± 0.09	1	0.19 ± 0.02	1
emv	10.6 ± 0.6	0.97 ± 0.04	1.85 ± 0.13	0.85 ± 0.06	0.21 ± 0.01	1.12 ± 0.09
asD4	6.4 ± 0.3	0.59 ± 0.06*††	1.13 ± 0.05	0.52 ± 0.04**†	0.21 ± 0.03	1.12 ± 0.02*
asE2	6.7 ± 0.9	0.60 ± 0.03**†	0.91 ± 0.04	0.42 ± 0.02**†	0.21 ± 0.00	1.16 ± 0.16
s10	11.6 ± 0.6	1.06 ± 0.04	2.05 ± 0.05	0.93 ± 0.06	0.19 ± 0.01	1.02 ± 0.08
s15	11.7 ± 0.2	1.06 ± 0.03	2.01 ± 0.07	0.92 ± 0.06	0.20 ± 0.01	1.08 ± 0.16

IC_{50} values after 48 h exposure to sodium arsenite or 72 h exposure to cisplatin or zinc chloride. Data represent mean ± SEM from three representative series

* $P < 0.05$ compared to T289

** $P < 0.01$ compared to T289

† $P < 0.05$ compared to emv

†† $P < 0.01$ compared to emv

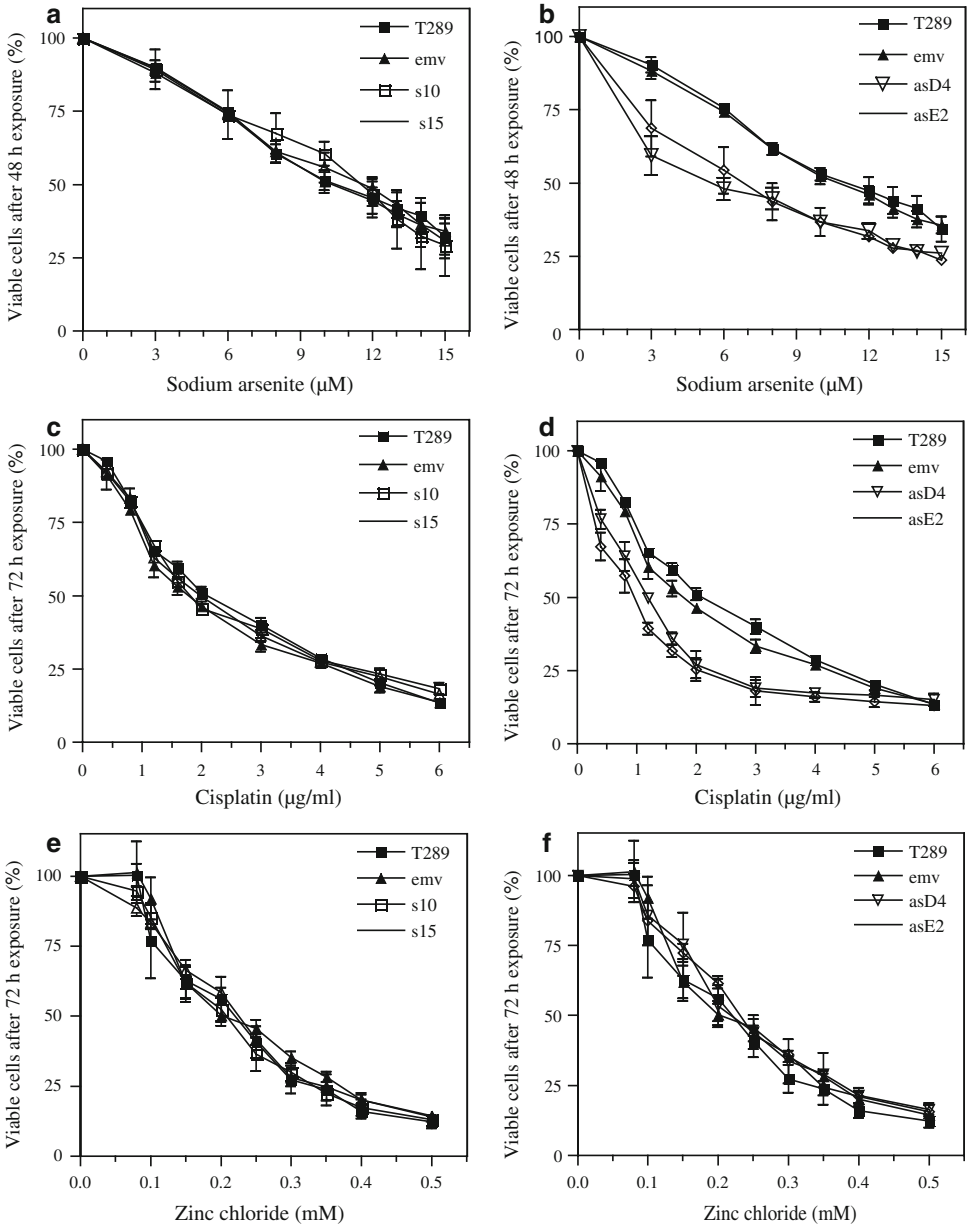

Fig. 3 Chemosensitivity in human melanoma T289 cells and plasmid transfected sublines. Empty vector transfected cells (emv) were used as control. *ASNA1* sense transfectants (s10 and s15) are shown in **a**, **c** and **e**. *ASNA1* downregulated antisense transfectants (asD4 and asE2) are shown in **b**, **d** and **f**. Cells were exposed 48 h to sodium arsenite or 72 h to cisplatin or zinc chloride. Each concentration was added to triplicate wells. Viability was determined by the MTT assay. Each data point represents mean ± SEM from three independent experiments

downregulated cells. The twofold increased cisplatin sensitivity of ASNA1 deficient cells might be regarded as modest but clinically significant resistance to cisplatin in vivo is associated with only 1.5–3.0-fold changes in sensitivity to cisplatin when measured in vitro [2, 3].

In this study, overexpression of ASNA1 did not generate any change in metal salt sensitivity. We found that in the subline T289/DDP selected for acquired resistance to cisplatin [15], ASNA1 is overexpressed. T289/DDP is also cross-resistant to arsenite [14]. One possible explanation is that, in a selection process that extends over several passages, resistance is caused by different mechanisms. One mechanism could be overexpression of several proteins in the hypothetical ars-transporter. Transfection and overexpression of ASNA1 would not necessarily lead to increased resistance unless other components of the transporter are also upregulated.

In early prokaryotic life, cells were forced to survive in a metal-polluted enviroment and thereby evolved resistance systems against toxic metals. ASNA1 is the human homologue of the well-characterized ArsA ATPase in *E. coli* [11]. ArsA is the catalytic subunit of the ars-transporter responsible for pumping arsenate, arsenite and antimonite out of the cell. ArsA and ASNA1 have several characteristics in common. Their sequence homology was preserved during evolution and other homologues have been identified in a wide variety of species [5]. They are both arsenite-stimulated ATPases and they contain a distinct NTP-binding motif which places them within the same superfamily of ATPases [9, 11, 12].

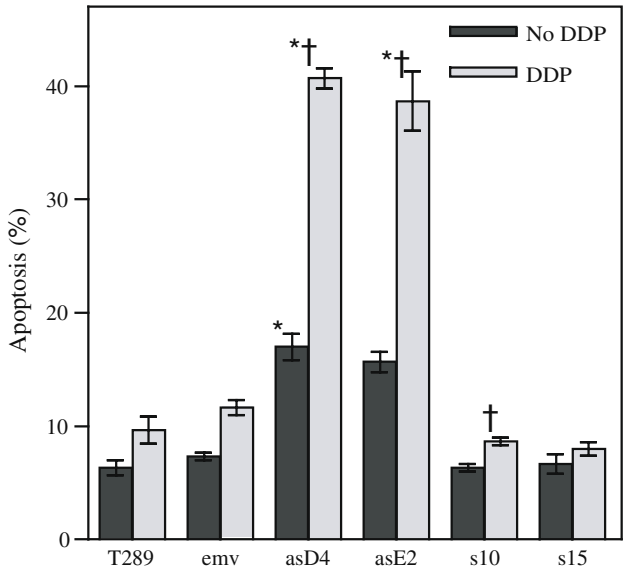

Fig. 4 TUNEL-assay; apoptosis in human melanoma T289 cells and plasmid transfected sublines, empty vector (emv), *ASNA1* antisense (asD4 and asE2) and *ASNA1* sense (s10 and s15). *Bars* represent mean apoptosis ± SEM after 24 h on a culture slide (*filled*) and after 2 h exposure to 100 μg/ml cisplatin (DDP) (*grey*). Significant increase in apoptosis is indicated by *asterisik* ($P < 0.05$ compared to T289 and emv) and *dagger* ($P < 0.05$ compared to cisplatin untreated cells)

Three observations support the hypothesis that ASNA1 is a key protein in a mechanism that mediates the RASP-phenotype and that downregulation of ASNA1 results in a specific phenotype rather than in generally defective cells. First, we did not observe any correlation between ASNA1 expression and sensitivity to zinc chloride. Secondly, a *C. elegans* asna-1 mutant strain is sensitive to arsenite- and antimonite but shows wildtype resistance against Pb(II), Cu(II), Al(III), Cr(VI), and Zn(II) [28]. Thirdly, arsenite stimulate the ATPase activity of human and *C. elegans* ASNA-1, while metal ions unrelated to the RASP-pheotype do not [12, 28].

E.coli ArsA binds to transmembrane ArsB to export arsenite and antimonite. There is no known homologue to ArsB in eukaryotic cells and ASNA1 is described in several cellular pathways. ASNA-1 in yeast, Arr4, regulates intracellular membranes in Golgi-to-ER transport and sporulation [4]. ASNA1 was recently shown to be a targeting factor for posttranslational insertion of tail-anchored proteins into the endoplasmatic reticulum (ER) of bovine cells [25]. The tail-anchored SNARE proteins mediate intracellular membrane fusion events [7] and an ASNA1-SNARE complex could be involved in the reported vesicular efflux of platinum [20, 21]. A second class of tail-anchored proteins involved in cisplatin resistance is the Bcl-2 family [6, 24]. The current view on the interactions of the three Bcl-2 protein families and apoptosis [1] is in agreement with our findings and ASNA1 could then have a key role in regulation of apoptosis by binding to either Bcl-2 or BAX. Furthermore, we have recently demonstrated that ASNA1 promotes insulin secretion in *C. elegans* and mammals [8]. Thus, ASNA1 seems to be involved in disparate functions in the eukaryotic cell even if the ASNA1 dependent chemoresistance presented here appears to be substrate-specific.

During selection of plasmid transfected antisense clones it became obvious that some clones grow extremely slowly. After clonal expansion and analysis of ASNA1 expression, we found that all clones with reduced levels of ASNA1 grew slowly. Neither the mock transfected nor ASNA1 overexpressing clones grew slowly. The clones with the lowest ASNA1 expression grew too slowly to be useful in chemosensitivity assays. ASNA1 expression in the *ASNA1* antisense transfected clones we managed to passage was never below 40% of that in the wildtype cells. Hence, a basal level of ASNA1 is needed for cell growth. This is supported by our observations in *C. elegans* where ASNA-1 mutant worms arrest in the first larval stage but continue larval development when expressing human ASNA1 [8]. A TUNEL assay showed that at least part of the growth defect among the ASNA1 downregulated melanoma cells was due to increased apoptosis. The difference in apoptosis compared to wildtype was enhanced after cisplatin treatment.

In conclusion, downregulation of ASNA1 increases the cellular susceptibility to cisplatin and arsenite and results in retarded growth rate and increased apoptosis. One possible mechanism is the recent finding that ASNA1 is a targeting factor for posttranslational insertion of tail-anchored proteins into intracellular membranes.

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