ASNA-1 activity modulates sensitivity to cisplatin

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ABSTRACT

Cancer can be cured by platinum based chemotherapy but resistance is a major cause of treatment failure. Here we present the nematode *Caenorhabditis elegans* as a model to study interactions between the platinum drug cisplatin and signaling pathways in vivo. Null mutations in a single gene, *asna-1*, makes worms hypersensitive to cisplatin. The metalloregulated ATPase ASNA-1 promotes insulin secretion and membrane insertion of tail-anchored proteins. Using structural data from ASNA-1 homologs, we identify specific ASNA-1 mutants that are sensitive to cisplatin while still able to promote insulin signaling. Mutational analysis reveals that hypersensitivity of ASNA-1 mutants to cisplatin remains in absence of CEP-1/p53 or apoptosis. Human ASNA1 can substitute for the worm gene, indicating a conserved function. Cisplatin sensitivity is not affected by decreased insulin signaling in wild type nematodes or restored insulin signaling in *asna-1* mutants. These findings provide a functional insight into ASNA-1, demonstrate that *C. elegans* can be used to characterize cisplatin resistance mechanisms and propose that rationally designed drugs against ASNA-1 can sensitize cancer cells to cisplatin.
INTRODUCTION

Mammalian responses to metals have implications in both cancer chemotherapy and environmental toxicology. Use of the platinum compound cisplatin has improved the outcome for cancer patients for more than three decades. Recent development of oxaliplatin for treatment of colon cancer and arsenic trioxide for acute myeloid leukemia have increased the interest in metal complexes (1). Cisplatin interacts with DNA, resulting in DNA adducts that inhibit both replication and transcription (2). It induces apoptosis or necrosis in tumor cells while dose-limiting side effects also occur in non-dividing cells like neurons and nephrons (3). Although cisplatin can cure cancer, most tumors eventually develop a multifactorial resistance and some are intrinsically resistant. Addressing the causes of this treatment failure remains a longstanding and relevant question. A combination of cisplatin and a targeted drug for increased tumor sensitivity would improve outcome for cancer patients.

Our work with cultured human cells suggests that ASNA1 is a potential drug target to circumvent cisplatin resistance. Cisplatin resistant tumor cell lines overexpress ASNA1 (4, 5) while tumor cells with downregulated ASNA1 expression display increased apoptosis and increased sensitivity to cisplatin and arsenite (5, 6). This is consistent with the previous observations that cisplatin resistant cells are cross resistant to arsenite and antimonite (7, 8) and that ASNA1 is a well conserved homologue to a subunit of a bacterial efflux pump for metalloids (9-11).
ASNA1 has ATPase activity which is stimulated by arsenite (12) and the protein is detected in the cytoplasm, the perinuclear region and the nucleolus (13). Recently, ASNA1 gained much interest as the first identified factor for membrane insertion of tail-anchored proteins, which are involved in diverse important functions like vesicular transport (SNAREs) and apoptosis (Bcl-2) (14-18). ASNA1 could mediate cisplatin resistance through these pathways since vesicular efflux of cisplatin has been reported (19) and antiapoptotic Bcl-2 is up-regulated in cisplatin resistant cells (20). While the role for ASNA-1 in insertion of tail-anchored proteins is intensively studied in yeast, less is known about the functions of ASNA1 in metazoans in vivo. Reduced expression of ASNA1 in cell lines results in retarded growth (5) and knock-out of ASNA-1 in mouse causes embryonic lethality (21). Therefore, it has not been possible to study phenotypes in complete absence of ASNA1. Here, we developed a model to study cisplatin in the nematode Caenorhabditis elegans where genetic asna-1 null mutants can be used. C. elegans is a well established organism to study individual signaling pathways in response to drug exposure (22). Human and C. elegans ASNA-1 share 54% amino acid sequence identity and a conserved ATPase domain. C. elegans asna-1 null mutants have greatly decreased insulin/IGF signaling activity that can be rescued by expression of the human ASNA1 gene, indicating conservation of function (23).

Here we ask whether asna-1 mutants are sensitive to cisplatin and if this would be secondary to apoptosis, retarded growth or lack of ASNA-1 promoted insulin signaling.
METHODS

C. elegans techniques

Handling of C. elegans strains was as described (24). Experiments were carried out at 20°C unless stated otherwise. N2 is the wild type parent for all strains in this study. Injection RNAi and feeding RNAi was done as described (25). For injection RNAi of asna-1 or zk637.4, the full-length cDNA clone was amplified by PCR using primers with T7 promoter sequences at the 5’ ends. The amplified DNA was used as substrate for in vitro synthesis of dsRNA and its subsequent clean up using a kit (Ambion) following manufacturer’s suggestions. asna-1 (sv42) was isolated from a deletion library (23). asna-1(ok938), zk637.3 (gk367), cep-1(gk138), egl-1(n1084n3082), daf-2(e1370) and daf-7(e1372) were obtained from the C. elegans Genetics Center.

Plasmids and transgenic strains

pVB202GK: P_{asna-1}::human ASNA1

pVB222GK: P_{asna-1}::asna-1::GFP, GFP fused to the last codon of asna-1

pVB275GK: P_{elt-2}::asna-1

pVB277GK: P_{daf-28}::asna-1

pVB308GK: P_{osm-6}::asna-1

The plasmids listed above and the transgenic strains generated using these plasmids have been described previously (23). The worm strain bearing the transgene containing pVB222GK was integrated into the genome. In all other cases the transgenes were maintained as extrachromosomal.
arrays. The *osm-6* promoter is expressed only in head neurons (26) and the neuronal wild type expression of *asna-1* is in a subset of these head neurons (23). The *elt-2* promoter is exclusively expressed in the intestine (27) and the *daf-28* promoter is expressed in the intestine and in head neurons (28).

pVB402GK: Same as pVB222GK except for a 3bp deletion for the His-164 codon.

pVB464GK: Same as pVB222GK except for change of codons 285 and 288 from cysteine to serine.

pVB402GK and pVB464GK were expressed in worms on extrachromosomal arrays generated by co-injecting 50 µg/mL of each plasmid along with pCC:GFP at 50 ug/mL (which is expressed in coelomocytes) to follow the inheritance of the transgenes,

pVB507GK: full length *ced-3* cDNA cloned into feeding RNAi plasmid L4440 as a NheI/SacI fragment.

Antibodies and western blot

Worms were lysed in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and loading dye with DTT. Worms were centrifuged at 13600 rpm 1 min, suspended in lysis buffer and boiled for 5 min. Lysates were separated in a 10 % SDS gel and transferred to immobilon-p filters (Millipore) prior to ASNA-1 detection by a rabbit anti-worm ASNA-1 polyclonal antibody (23). Loading control was performed by coomassie (Serva) staining of the immobilon-p filter or by stripping of the filter in 1M Glycine-HCl (pH 2.5) for 10min and 1M Tris (pH 7.5) for 10 min followed by detection of
α-tubulin by a mouse anti tubulin antibody. Loading density was quantified by the Quantity One® 4.5.6 software.

Chemosensitivity assays

*asna-1 (RNAi)* animals arrest as L1 larvae and to obtain the same condition for wild type strain N2, worms hatched in the absence of food. L1 arrested larvae were transferred to NGM plates containing 0-250 µg/ml zinc chloride (Göteborgs termometerfabrik); 0-200 µg/ml cadmium chloride (Sigma); 0-50 mg/ml potassium antimonyl tartrate (Sigma); 0-300 µg/ml sodium arsenite (Sigma) or 0-500 µg/ml cisplatin (Platinol® Bristol Myers Squibb). Larvae were incubated 24±1 h at 20°C on all metal containing plates except on cadmium chloride where they were kept 72±1 h due to delayed toxicity. To test the effect of metal salts on adult worms, they were maintained on MYOB agar (275 mg Tris-Cl (Sigma), 120 mg Tris-OH (Sigma), 1.55 g Bacto-peptone (Becton Dickinson), 800 µl cholesterol 5 mg/ml (Sigma), 1 g NaCl, 10 g agar (Merck) and 250 ml H2O gave 2x MYOB medium) with 0-500 µg/ml cisplatin, 0-600 µg/ml sodium arsenite, 0-1600 µg/ml zinc chloride or 0-1500 µg/ml copper sulphate. The adult animals were fed with OP50 strain of *E. coli* transferred from NGM-plates. Wild type (N2) and worms heterozygous for an *asna-1* mutation (*ok938* and *sv42*) were used as controls on cisplatin agar. Young adult worms were incubated 24±1 h at 20°C on metal containing plates and death was determined by absence of touch-provoked movement when probed with a platinum wire. To test chemosensitivity in solution, young adult worms were exposed for 24 h to 0-500 µg/ml...
cisplatin in S-medium. They were then transferred to an agar plate where death was determined by absence of touch-provoked movement.

Chemotaxis assay

One side of a MYOB plate without cisplatin was cut out and MYOB-medium containing 300 µg/ml cisplatin was poured in that area. Age synchronous young adult worms were put in the middle of the plate and the ratio of worms on each side of the plate was determined after 1 h.

Pharyngeal pumping rate

Worms were exposed to control medium or medium containing 300 µg/ml cisplatin on plates for 2 h. The pharyngeal pumping rate of the worms lying in the plates was measured using a dissection light microscope.

Site specific mutagenesis of asna-1

Multiple amino acid sequence alignments were done using the ClustalW Service (http://www.ebi.ac.uk/clustalw/). The protein source and GenBank Accession numbers of the aligned sequences are H. sapiens (AAC03551), C. elegans (P30632) and S. cerevisiae (NP_010183). Deletion of the ASNA-1 His164 codon and the substitution of serines for Cys285 and Cys288 were performed using mutagenic primers. These were designed for both strands to amplify the gene in two parts using outside primers corresponding to the beginning of the asna-1 promoter and the last codon of asna-1. Both outside primers were designed to have Sph1 sites. The two obtained fragments were mixed and subjected to a
second round of PCR using the outside primers. The resulting 1.9kb PCR fragment was digested with Sph1 and cloned into the GFP vector pPD95.77 which had been linearized with Sph1. This resulted in asna-1:gfp fusion genes in which gfp was fused to the last codon of asna-1. Both constructs were fully sequenced.

daf-2 experiment

The daf-2 (e1370) mutant is a temperature sensitive strong loss-of function mutant (29). Mutants grow reproductively at 15°C and do not enter the dauer state. Mutants grown throughout their life at 25°C become dauer larvae and never reach adulthood because of loss of gene activity. To obtain daf-2 (e1370) mutant adults with greatly reduced gene activity, worms have to be shifted from 15°C to 25°C after the L2 stage when commitment to the dauer state is no longer possible. Age synchronous N2 and daf-2 (e1370) mutants were grown at 15°C (the non-dauer permissive temperature) to late L2. Worms were then shifted to 25°C to reduce or eliminate daf-2 gene function in daf-2 (e1370) mutants. asna-1 (ok938) mutants were grown at 20°C. One day after the L4 stage, young adults were transferred to cisplatin plates at 25°C. Death was scored after 24 h incubation on cisplatin containing MYOB plates.

daf-7 dauer escape assay

The strong temperature sensitive daf-7 (e1372) mutant (30) was used to reduce DAF-7 activity. The mutants grow reproductively (non-dauer) at 15°C but become 100% dauer larvae at 25°C. daf-7 (e1372) mutants carrying pVB402GK or pVB464GK expressing ASNA-1^{AH164} and ASNA-1^{C285SC288S} respectively were grown at 25°C. Dauer escape was
evaluated after 96 h by determining the number of adult (non-dauer) worms on the plates. This ability of transgene bearing daf-7 mutants grown at 25°C to leave the dauer state and progress to adulthood is termed dauer escape. No dauer escape was seen among daf-7 (e1372) control animals lacking a transgene, confirming that the temperature of the incubator was nonpermissive for daf-7 dauer exit.

Microscopy
The worms were anesthetized by mounting them in a drop of M9 containing 1mM levamisol on a microscope slide containing a 2% agar pad. Worms were examined by microscopy within 10 minutes after mounting. Cisplatin influence on tissue morphology was determined using a 100x objective in a Leica DMRP microscope, and photographed with Deltapix DP450 software. Body volume was measured at 5x (Leica DMRP and Deltapix DP450 software) by SnapMeasue in Adobe illustrator CS3 13.0.2. Body volume was calculated as the cylindrical volume between the pharynx and the rectum.

Statistical analysis
LC$_{50}$ values were determined using a binary logistic regression model. Chi-square tests were performed to calculate level of statistical significance when comparing survival at a single metal salt concentration. A Mann-Whitney test was performed to compare ASNA-1 density levels on western blots, pharyngeal pumping rates and body volumes. A level of significance was set at 0.05. SPSS 18.0 for Mac OSX was used for statistical analysis.
RESULTS

To determine the effect of ASNA-1 on chemoresistance, *asna-1(RNAi)* animals (which arrest as L1 larvae (23)) were tested along with arrested wild type larvae of the same age. We observed a significant increase in sensitivity to cisplatin, sodium arsenite and antimony potassium tartrate in *asna-1 (RNAi)* larvae compared to wild type larvae (Table 1). No difference was observed in sensitivity to cadmium chloride or zinc chloride. In agreement, cisplatin resistant tumor cells are cross resistant to arsenite and antimonite (7, 8) and ASNA1 underexpressing mammalian cells are hypersensitive to the same metal salts (5, 6). This emphasizes the similarity in the resistance pattern between worms, cell lines and resistant tumor cells.

Next, we tested chemoresistance in two *asna-1* mutants, *ok938* (11, 23) and *sv42* (23), which bypass the L1 arrest and grow to become sterile thin adults due to deposit of *asna-1* gene product from the mother that is heterozygous for the *asna-1* mutation. Adult wild type worms and *asna-1* heterozygotes were intrinsically resistant to the maximum possible cisplatin concentration in agar (500µg/ml), (Figure S1). By contrast, both *asna-1(ok938)* and *asna-1(sv42)* mutants were cisplatin sensitive and died within 24 hours, displaying a LC50 of 251 µg/ml and 235 µg/ml respectively (Figure 1A and S2), (p<0.001 compared to wild type for both observations). The *sv42* deletion affects both *asna-1* and the upstream genes *zk637.4* and *zk637.3. zk637.4(rnaI)* worms and *zk637.3 (gk367)* mutant worms were not sensitive to cisplatin (Figure S3). Further, since worms bearing a deletion of *asna-1* alone in the *asna-1 (ok938)* strain were as hypersensitive to cisplatin as the *asna-1 (sv42)*
mutants, we conclude that inactivation of *asna-1* alone is sufficient to cause cisplatin hypersensitivity. This is confirmed by a rescue experiment described below. Consistent with a previous report (11), *asna-1* mutants were also hypersensitive to arsenite with a LC$_{50}$ of 111 µg/ml compared to 279 µg/ml in wild type, p<0.001 (Figure 1B). Cisplatin shares resistance pattern and plasma membrane transporters with copper (31-33). The LC$_{50}$ for copper in *asna-1* mutants (500 µg/ml) was significantly lower than that of wild-type animals (849 µg/ml), p<0.001 (Figure 1C). As with the larval *asna-1(rnai)* experiment, there was no difference in sensitivity to zinc chloride between the mutants and the controls (Figure 1D). This indicates that *asna-1* mutants display a substrate specific phenotype related to cisplatin, arsenite and copper rather than general metal sensitivity. *C. elegans asna-1* mutants expressing either wild type *asna-1* or human ASNA1 on transgenes were both rescued for the cisplatin hypersensitivity phenotype (Figure 1E). Thus, human ASNA1 can substitute for the worm homolog and likely mediates cisplatin resistance by conserved mechanisms. Cisplatin resistant human tumor cells generated by serial exposure to cisplatin overexpress ASNA1 (5, 6). In *C. elegans*, we observed an increase in ASNA-1 protein levels after 48 h exposure to 100 or 200 µg/ml cisplatin in agar plates (Fig 1F). A 2.8±0.35 fold increase in the steady state levels of ASNA-1 protein was detected in worms exposed to 200µg/ml cisplatin (p<0.05).

We asked whether *asna-1* mutants display increased chemosensitivity because of an inability to sense and avoid chemicals. To test this we first determined the pharyngeal pumping rate after two hours on agar with or without cisplatin. *asna-1* mutants on cisplatin agar had decreased pharyngeal pumping rate compared to wild type (Table S1), excluding...
the possibility that increased oral intake of cisplatin is the reason for hypersensitivity.

Secondly, worms were put on plates with cisplatin agar on one side and control agar on the other. The relative number of worms on each side was determined after one hour. Avoidance to cisplatin was observed neither in wild type nor in asna-1 mutants (Table S2). Finally, when worms were exposed to cisplatin in solution where avoidance is impossible, asna-1 mutants were again more sensitive to cisplatin than wild type (Figure S4).

Cisplatin can induce apoptosis and necrosis in tumor cells (3) and apoptosis is increased by downregulation of ASNA1 in a human cell line (5). To test whether apoptosis is involved in the cisplatin sensitivity of C. elegans asna-1 mutants, we blocked the apoptosis signaling pathway and evaluated resistance. We created a double mutant between asna-1 and cep-1/p53 and a double mutant between asna-1 and egl-1/BH-3only (which lacks all somatic apoptosis) (34). Thirdly, we exposed asna-1 mutants to ced-3/caspase RNAi that was effective because germline apoptosis was eliminated. asna-1 mutants did not become more resistant when apoptosis was blocked by depleting cep-1, egl-1 or ced-3 activity (Figure 2, A to C). asna-1;cep-1 double mutants were slightly more sensitive to cisplatin compared to asna-1 single mutants (p<0.05) but there was no significant difference between cep-1 and wild type (p=0.121) (Figure 2A). While observing worms exposed to cisplatin, both wild type and asna-1 mutants showed signs of necrosis, most obviously in the head region (Figure S5). We conclude that apoptosis is not involved in the increased cisplatin sensitivity seen in adult asna-1 mutant worms.
The prokaryotic ASNA-1 homologue ArsA contains three metal binding residues (Cys113, Cys172 and His148) (35) that activate the ArsA ATPase after binding of arsenite or antimonite. We deleted the asna-1 codon for His164 that corresponds to His148 in ArsA. By western blot analysis of several transgenic lines expressing ASNA-1ΔH164:GFP we identified a line in which the ASNA-1ΔH164:GFP expression level was equal to levels of wild type ASNA-1 (Figure 3A). The asna-1ΔH164:gfpr transgene rescued the growth phenotype of asna-1 mutants as much as the wild type transgene (Figure 3D and S6), showing that ASNA-1ΔH164:GFP is functional. However, these worms were still cisplatin sensitive (Figure 3B), identifying His164 as a target in ASNA-1 to increase cisplatin sensitivity.

The ASNA-1 homologue in yeast (Get3) functions as a homodimer and His172 in Get3 (corresponding to ASNA-1 His164) is the transition site for alteration between two dimer conformations important for targeting of tail-anchored proteins (17). The Get3 homodimer is linked by Cys285 and Cys288 from each half of the dimer and when these residues are mutated, Get3 is nonfunctional and unable to dimerize (17, 36). His172, Cys285 and Cys288 are conserved between yeast, C. elegans and humans (FigS7). We changed ASNA-1 Cys285 and Cys288 to serines and selected a transgenic strain in which the ASNA-1C285S C288S:GFP expression level was similar to that of wild type ASNA-1 (Figure 3A). ASNA-1C285S C288S is functional since its expression rescues the growth phenotype of asna-1 mutants (Figure 3D and S6). However, worms expressing ASNA-1C285S C288S were sensitive to cisplatin (Figure 3C), identifying ASNA-1 Cys285 and Cys288 as another possible target for metal drug sensitivity.
Rescue of the asna-1 growth phenotype by asna-1\(^{ΔH164}\) and asna-1\(^{C285S\,C288S}\) (Figure 3D and S6) indicates that insulin signaling is restored by these transgenes. To confirm this finding we performed the dauer escape assay. Worms remain in the dauer stage permanently at 25°C if they are mutant for daf-7/TGFβ. A compensatory increase in insulin pathway activity by overexpression of some insulins or wild-type ASNA-1 allows daf-7 dauers to exit the dauer stage and become adults (23). Using this assay we observed that 23% of daf-7(e1372) dauers expressing the asna-1\(^{C285S\,C288S}\) transgene (n=103) and 26% of daf-7 dauers expressing the asna-1\(^{ΔH164}\) transgene (n=105) exited the dauer state at 25°C. Hence, these transgenes expressing mutant forms of asna-1 are capable of enhancing insulin signaling. This shows that the His164, Cys285 and Cys288 residues are essential for cisplatin resistance but not for insulin signaling and that it should be possible to design drugs that target ASNA-1 promoted cisplatin resistance without interfering with insulin signaling.

In C. elegans, mutants in both the insulin/IGF receptor homologue daf-2 and asna-1 have greatly decreased insulin signaling (23). To further test whether ASNA-1 dependent chemoresistance is a consequence of the influence of ASNA-1 on insulin signaling we exposed daf-2 (e1370) mutants to cisplatin. While asna-1 mutants died on cisplatin plates, no death was scored in the daf-2 mutants (Figure 4A), suggesting that reducing insulin signaling by itself has no effect on cisplatin sensitivity. These results are consistent with our data in table 1 where starved wild type larval controls, which lack insulin signaling (37), were more resistant than asna-1 (RNAi) treated larvae, which also lack insulin signaling.
In addition, *daf-2* mutants are resistant to copper (38) while *asna-1* mutants are hypersensitive (Figure 1C). Thus depletion of ASNA-1 affects pathways other than insulin signaling to cause metal salt hypersensitivity.

As another test of separability between the insulin and cisplatin phenotype of ASNA-1 we expressed *asna-1* under tissue specific promoters in *asna-1* mutants. *asna-1* expression under an intestinal (*pelt-2*) or an insulin (*p_{daf-28}* promoter rescued the resistance in a significant manner (Figure 4B). The insulin promoter (*p_{daf-28}* and the *asna-1* promoter drives *asna-1* expression in head neurons and in the intestine. The intestine is considered the most important organ for detoxification in worms (39). Consistant with this notion, *asna-1* expression only in head neurons (under the *posm-6* promoter) did not rescue the sensitivity of *asna-1* mutants (Figure 4C). However, *asna-1* expression in head neurons is sufficient to rescue insulin signaling in *asna-1* mutants (23). These observations reinforce the notion that the insulin function of ASNA1 is distinct from its cisplatin related function.
DISCUSSION

Platinum based chemotherapy is widely used and increasingly important to treat cancer. Despite three decades of clinical use and intense research, there is still a need for greater knowledge about cellular responses to platinum compounds. Cisplatin is generally considered to be toxic through its interaction with DNA but it also induces cell death through ER stress, independently of DNA damage (40). A more detailed understanding of the mode of action by cisplatin would increase the possibility to circumvent cisplatin resistance. Furthermore, recent clinical studies have highlighted that a targeted drug against EGFR has a detrimental effect on patient survival when given together with platinum based drugs in patients with KRAS mutated tumors (41). This underscores the importance of finding useful models to study interactions between genetic factors, targeted drugs and platinum based chemotherapy. This work presents a model for such studies.

We have identified ASNA-1 as a target to reduce cisplatin resistance and characterized this in vivo using genetic techniques in the nematode *C. elegans*. Molecular pathways related to human diseases are highly conserved between *C. elegans* and humans. The existing mutation libraries and in vivo markers for cell damage provide useful tools to further study cisplatin in *C. elegans*. This model can also be used for chemical drug screens and functional genomic screens in vivo.

Recent efforts to describe the structure of the ASNA-1 homologue in yeast (Get3) have resulted in different models for ASNA-1 targeting of tail-anchored proteins (TA-proteins).
In the nematode, it has been possible to use these data to study the function of metazoan ASNA-1 \textit{in vivo}. While several reports identify a pair of cysteins in ASNA-1 important for ASNA-1 dimerization and targeting of TA-proteins, we show that mutated ASNA-1 lacking these cysteins is capable of promoting insulin signaling but cannot rescue the cisplatin hypersensitivity phenotype. A drug blocking these residues of ASNA-1 could sensitize tumors to cisplatin while preserving other functions of ASNA-1. This illustrates the translational possibilities between \textit{C. elegans} and cancer medicine and underscores the importance of functional studies \textit{in vivo} in addition to structure biology.

Downregulation of mammalian ASNA1 results in retarded growth, increased cisplatin sensitivity, increased apoptosis and decreased insulin signaling (5, 23). Studying ASNA-1 in \textit{C. elegans} made it possible to separate these phenotypes. Here we show that asna-1 mutant cisplatin hypersensitivity is seen even when insulin signaling and the growth phenotype is rescued and in absence of apoptosis. Cisplatin resistant tumor cells are often resistant to apoptosis signaling (2) but according to these results, they could be resensitized to cisplatin if the chemotherapy was combined with a targeted therapy against ASNA1.

Cisplatin resistant cells are cross-resistant to arsenite, antimonite and copper (7, 8, 33). ASNA-1 mutants are hypersensitive to all these substrates but not to cadmium or zinc.

Several reports show that the copper transporters CTR1, ATP7A and ATP7B can mediate cisplatin influx and efflux (31, 32). ASNA-1 mutant hypersensitivity to copper indicate that ASNA-1 could regulate copper transporters to promote copper and cisplatin resistance. We speculate that the conserved metalloregulated ATPase ASNA1 regulates diverse tail-
anchored proteins in response to toxic metals in metazoans. Cisplatin resistance is multifactorial and ASNA1 could be a unique target to interfere with several resistance factors.

In conclusion, we demonstrate the validity of *C. elegans* as a model to identify and characterize cisplatin response mechanisms and that a targeted therapy against ASNA1 could resensitize cisplatin resistant cells and improve outcome for cancer patients.
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REFERENCES


### Table 1

<table>
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<th>Metal salt</th>
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Table 1. Chemosensitivity in *asna-1(rnai)* worms. Worms were injected with *asna-1* dsRNA and the progeny arrested in the first larval stage L1 due to absence of ASNA-1 promoted insulin signaling. Embryos were hatched on agar plates without food to also induce L1 arrest in the wild type strain *N2*. Larvae were exposed to various concentrations of cisplatin, arsenite, antimonite or zinc for 24 h, or to cadmium chloride for 72 h. Death was determined by absence of touch provoked movement. A binary logistic regression model was used to calculate LC$_{50}$ and p-values. *** indicates p<0.001. Data are collected from three experiments.
FIGURE LEGENDS

Figure 1. Chemosensitivity of asna-1 mutants and induction of ASNA-1 expression by cisplatin. Young adult worms were exposed to metal salts and death was determined by absence of touch provoked movement after 24 h. Data are presented as mean survival (%) ± SEM from at least three experiments. Black squares represent wild type (N2) and open circles represent asna-1 (ok938) mutants (A-D). (A) asna-1 mutants (n=164) compared to wild type (n=233) after exposure to cisplatin. (B) asna-1 mutants (n=310) compared to wild type (n=581) after exposure to sodium arsenite. (C) asna-1 mutants (n=164) compared to wild type (n=149) after exposure to copper sulphate. (D) asna-1 mutants (n=278) compared to wild type (n=250) after exposure to zinc chloride. (E) Survival after 24 h on 300 µg/ml cisplatin. asna-1 (sv42) mutants (n= 25) are compared to asna-1 mutants carrying a transgene expressing asna-1 under the control of its own promotor (n=48) (*** p<0.001). Next to it, asna-1 (ok938) mutants (n=82) are compared to asna-1 mutants carrying a transgene expressing human asna1 under the control of the asna-1 promotor (n=48) (*** p<0.001). (F) Western blot of lysates of wild type worms (N2) after 48 h exposure of 0, 100 or 200 µg/ml cisplatin. Loading control by α-tubulin.
Figure 2. Blockage of apoptosis has no effect on cisplatin sensitivity. Young adult worms were exposed to control agar (black bars) or agar containing 300 µg/ml cisplatin (grey bars). Bars represent mean survival ± SEM after 24 h exposure. Data are collected from at least three experiments. The level of statistical significance is indicated for comparisons discussed in the text. n.s. indicates no significance. (A) On cisplatin, asna-1;cep-1/p53 (n=41) is not more resistant than asna-1 alone (n=46). Rather, asna-1;cep-1 is more sensitive (* p<0.05) but no significant difference is observed between cep-1 and wild type. (B) On cisplatin, asna-1;egl-1/BH3 (n=112) worms are not more resistant than asna-1 alone (n=186). (C) Wild type worms (N2) and asna-1(ok938) mutants were exposed to feeding RNAi to eliminate ced-3/caspase function. Control RNAi (L4440) was performed in parallel. On cisplatin agar, asna-1;ced-3(rnai) worms (n=76) are not more resistant than asna-1 (n=32).
Figure 3. Structure-function analysis of ASNA-1. (A) Western blot comparing expression of wild type ASNA-1 and ASNA-1ΔH164:GFP or ASNA-1C285S, C288S:GFP in worms heterozygous for the asna-1 mutation (ok938). Coomassie staining as loading control. (B) Worms expressing ASNA-1ΔH164:GFP in the asna-1 mutant background (n=51) fail to rescue the cisplatin sensitivity seen in asna-1(ok938) mutants (n=57) and are more sensitive than N2 (n=54) after 24 h on 300 µg/ml cisplatin (*** p<0.001 by a chi square test). Error bars represent SEM. (C) Worms expressing ASNA-1C285S C288S:GFP in the asna-1 mutant background (n=38) fail to rescue the cisplatin sensitivity seen in asna-1(ok938) mutants (n=61) and are more sensitive than N2 (n=80) on 300 µg/ml cisplatin (*** p<0.001 by a chi square test). Error bars represent SEM. (D) Transgenic expression of ASNA-1:GFP (D4), ASNA-1ΔH164:GFP (D5) or ASNA-1C285S, C288S:GFP (D6) results in a partial rescue of the growth phenotype observed in asna-1 (ok938) mutant (D3). 5x magnification in Leica DMRP, scale bars indicate 100 µm.
Figure 4. ASNA-1 confers cisplatin resistance independent of insulin signaling. Worms were exposed to 300 µg/ml cisplatin for 24 h. Bars represent mean survival ± SEM. Each experiment was performed three times and a chi square test was used to calculate statistical significance. The level of statistical significance is indicated for comparisons discussed in the text. (A) *daf-2/insulin IGF receptor (e1370)* mutants (n=162) were more resistant than both *asna-1(ok938)* mutants (n=78) and N2 (n=130) on cisplatin agar (**p<0.001 for both observations). (B) *asna-1* was expressed under an intestinal (*Pelt-2*) or an insulin (*Pdaf-28*) specific promotor in an *asna-1(sv42)* mutant background. Worms expressing *Pelt2:asna-1* (n=24) or *Pdaf-28:asna-1* (n=37) were resistant to cisplatin compared to *asna-1(sv42)* mutants (n=32) (**p<0.001 and *p<0.05 respectively). (C) *asna-1* expression under a head neuronal (*Posm-6*) specific promotor in an *asna-1(sv42)* mutant background did not rescue the cisplatin hypersensitivity phenotype. *asna-1* mutants expressing *Posm-6:asna-1* (n=56) were cisplatin sensitive as compared to both N2 (n=70) (**p<0.001) and *asna-1* mutants (n=84) (**p<0.01).
Figure 1.

(A) Cisplatin (µg/ml) vs. survival (%)
(B) Sodium arsenite (µg/ml) vs. survival (%)
(C) Copper sulphate (µg/ml) vs. survival (%)
(D) Zinc chloride (µg/ml) vs. survival (%)
(E) Graph showing survival (%) with different treatments.
(F) Western blot analysis of ASNA-1 and α-tubulin with cisplatin concentration.
Figure 2.
Figure 3.

A

B

C

D

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Figure 4.
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ASNA-1 activity modulates sensitivity to cisplatin
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