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 mutation 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 homologues, 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 suggest that rationally designed drugs against ASNA-1 can sensitize cancer cells to cisplatin. Cancer Res; 70(24); 10321–8. ©2010 AACR.

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 3 decades. Recent development of oxaliplatin for treatment of colon cancer and arsenic trioxide for acute myeloid leukemia has 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 whereas 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) whereas 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 of a subunit of a bacterial efflux pump for metalloids (9–11).

ASNA1 has ATPase activity that 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 (TA-proteins), which are involved in diverse important functions like vesicular transport (SNAREs) and apoptosis (Bcl-2; refs. 14–18). ASNA1 could mediate cisplatin resistance through these pathways because vesicular efflux of cisplatin has been reported (19) and antiapoptotic Bcl-2 is upregulated in cisplatin-resistant cells (20). Although the role for ASNA1 in insertion of TA-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 knockout of ASNA1 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 in which 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 ASNA1 share 54% amino acid sequence identity and a conserved ATPase domain. C. elegans asna-1 null mutants have greatly decreased insulin/insulin like growth factor 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 whether this would be due 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 were done as described (25). For injection RNAi of asna-1 or zkd37.4, 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 cleanup using a kit (Ambion) following manufacturer’s suggestions. asna-1 (sv42) was isolated from a deletion library (23). asna-1 (ok938), zkd37.3 (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<sub>asna-1</sub> human/ASNA1
pVB222GK: P<sub>asna-1</sub>:asna-1::GFP, GFP fused to the last codon of asna-1
pVB275GK: P<sub>elt-2</sub>:asna-1
pVB277GK: P<sub>daf-28</sub>:asna-1
pVB308GK: P<sub>omn-6</sub>:asna-1

The plasmids listed earlier in the text 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 His164 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 coinjecting 50 μg/mL of each plasmid along with pCC3/GFP at 50 μg/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 Nhe1/Sac1 fragment.

Antibodies and Western blot

Worms were lysed in 10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, and loading dye with DTT. Worms were centrifuged at 13,600 rpm for 1 minute, suspended in lysis buffer, and boiled for 5 minutes. 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 1 mol/L of Glycine-HCl (pH 2.5) for 10 minutes and 1mol/L of Tris (pH 7.5) for 10 minutes 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 nematode growth medium (NGM) plates containing 0 to 250 μg/mL of zinc chloride (Göteborgs termometerfabrik); 0 to 200 μg/mL of cadmium chloride (Sigma); 0 to 50 mg/mL of potassium antimonyl tartrate (Sigma); 0 to 300 μg/mL of sodium arsenite (Sigma) or 0 to 500 μg/mL of cisplatin (Platino Bristol Myers Squibb). Larvae were incubated for 24 ± 1 hours at 20 °C on all metal-containing plates except on cadmium chloride, where they were kept for 72 ± 1 hours 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 H<sub>2</sub>O gave 2x MYOB medium] with 0 to 500 μg/mL of cisplatin, 0 to 600 μg/mL of sodium arsenite, 0 to 1600 μg/mL of zinc chloride, or 0 to 1500 μg/mL of copper sulfate. The adult animals were fed with OP50 strain of Escherichia 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 for 24 ± 1 hours at 20 °C on metal-containing plates, and death was determined by absence of touch-provoked movement when they were probed with a platinum wire. To test chemosensitivity in solution, young adult worms were exposed for 24 hours to 0 to 500 μg/mL of cisplatin in S-medium. They were then transferred to an agar plate on which death was determined by absence of touch-provoked movement.

Chemoattract 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 hour.

Pharyngeal pumping rate

Worms were exposed to control medium or medium containing 300 μg/mL cisplatin on plates for 2 hours. 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 Homo sapiens (AAC03551), C. elegans...
(P30632), and Saccharomyces 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 2 parts using outside primers corresponding to the beginning of the asna-1 promotor and the last codon of asna-1. Both outside primers were designed to have Sph1 sites. The 2 obtained fragments were mixed and subjected to a second round of PCR using the outside primers. The resulting 1.9 kb PCR fragment was digested with Sph1 and cloned into the GFP vector pPD95.77 that 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 reproducitively 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-hour 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 reproducitively (non-dauer) at 15°C but become 100% dauer larvae at 25°C. daf-7(e1372) mutants carrying pVB402GK or pVB464GK expressing ASNA-1H164 and ASNA-1C285/C288S, respectively, were grown at 25°C. Dauer escape was evaluated after 96 hours 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 1 mmol/L of 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 ×100 objective in a Leica DMRP microscope, and photographed with Deltapix DP450 software. Body volume was measured at ×5 (Leica DMRP and Deltapix DP450 software) by SnapMeasure in Adobe Illustrator CS3 13.0.2. Body volume was calculated as the cylindrical volume between the pharynx and the rectum.

Statistical analysis
LC50 values were determined using a binary logistic regression model. The χ² test was used 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; ref. 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 with wild-type larvae (Table 1). No

<table>
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<th>Metal salt</th>
<th>Wild type (mg/mL)</th>
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<th>asna-1(rnai) (mg/mL)</th>
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<tr>
<td>Cisplatin</td>
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<td>237</td>
<td>0.20±*</td>
<td>187</td>
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<tr>
<td>Sodium arsenite</td>
<td>0.31</td>
<td>175</td>
<td>0.067±</td>
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<td>Antimony potassium tartrate</td>
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<td>104</td>
<td>10.7±</td>
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<td>116</td>
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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 induce L1 arrest in the wild-type strain N2. Larvae were exposed to various concentrations of cisplatin, arsenite, antimonite, and zinc for 24 hours, or to cadmium chloride for 72 hours. Death was determined by absence of touch-evoked movement. A binary logistic regression model was used to calculate LC50 and P values. Data are collected from 3 experiments.

*P < 0.001.
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 among worms, cell lines, and resistant tumor cells.

Next, we tested chemoresistance in 2 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 (500 μg/mL; Supplementary Fig. 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 (Fig. 1A; Supplementary Fig. S2; P < 0.001 compared with wild type for both observations). The sv42 deletion affects both asna-1 and the upstream genes zk637.4 and zk637.3. zk637.4(sv42) mutants alone are not sensitive to cisplatin (Supplementary Fig. S3). Further, because 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 later in this article. Consistent with a previous report (11), asna-1 mutants were also hypersensitive to arsenite with a LC50 of 111 μg/mL compared with 279 μg/mL in wild type (P<0.001; Fig. 1B). Cisplatin shares resistance pattern and plasma membrane transporters with copper (31–33). The LC50 for copper in asna-1 mutants (500 μg/mL) was significantly lower than that of wild-type animals (849 μg/mL), P < 0.001 (Fig. 1C). As with the larval asna-1(rnaI) experiment, there was no difference in sensitivity to zinc chloride between the mutants and the controls (Fig. 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 (Fig. 1E). Thus, human ASNA1 can substitute for the worm homologue 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 ASNA1 protein levels after 48-hour exposure to 100 or 200 μg/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 2 hours on agar with or without cisplatin. asna-1 mutants on cisplatin agar had decreased pharyngeal pumping rate compared with wild type (Supplementary

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**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 hours. Data are presented as mean survival (%) ± SEM from at least 3 experiments. Black squares represent wild type (N2) and open circles represent asna-1(ok938) mutants (A–D). A, asna-1 mutants (n = 164) compared with wild type (n = 233) after exposure to cisplatin. B, asna-1 mutants (n = 310) compared with wild type (n = 281) after exposure to zinc chloride. E, survival after 24 hours on 300 μg/mL cisplatin. asna-1(sv42) mutants (n = 25) are compared with 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 with 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-hour exposure of 0, 100, or 200 μg/mL cisplatin. Loading control by α-tubulin.
n.s.

activate the ArsA ATPase after binding of arsenite or anti-binding residues (Cys113, Cys172, and His148; ref. 35) that
worms.

necrosis, most obviously in the head region (Supplementary
0.121; Fig. 2A). Observation of worms exposed to cisplatin
cep-1
no significant difference between
cep-1
and wild type.

cep-1
double mutants were slightly more sensitive to cisplatin
depleting
asna-1
because germline apoptosis was eliminated.
asna-1

cep-1
cep-1/caspase RNAi

asna-1;egl-1/BH3

asna-1;egl-1
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asna-1

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asna-1

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(ok338) 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(mal) worms (n = 76) are not more resistant than asna-1 (n = 32).

Table S1), excluding the possibility that increased oral intake of cisplatin is the reason for hypersensitivity. Second, worms were put on plates with cisplatin agar on 1 side and control agar on the other. The relative number of worms on each side was determined after 1 hour. Avoidance of cisplatin was observed neither in wild-type nor in asna-1 mutants (Supplementary 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 (Supplementary Fig. 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-3-only (which lacks all somatic apoptosis; ref. 34). Third, 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 (Fig. 2A–C). The asna-1; cep-1 double mutants were slightly more sensitive to cisplatin compared with asna-1 single mutants (P < 0.05) but there was no significant difference between cep-1 and wild type (P = 0.121; Fig. 2A). Observation of worms exposed to cisplatin showed that both wild-type and asna-1 mutants had signs of necrosis, most obviously in the head region (Supplementary Fig. 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 3 metal-binding residues (Cys113, Cys172, and His148; ref. 35) that activate the ArsA ATPase after binding of arsenite or anti-monite. We deleted the asna-1 codon for His164 that corresponds to His148 in ArsA. By Western blot analysis of several transgenic lines expressing ASNA-1<sup>H164F</sup> GFP, we identified a line in which the ASNA-1<sup>H164F</sup>GFP expression level was equal to levels of wild-type ASNA-1 (Fig. 3A). The ASNA-1<sup>H164F</sup>GFP transgene rescued the growth phenotype of asna-1 mutants as much as the wild-type transgene (Fig. 3D and Supplementary Fig. S6), showing that ASNA-1<sup>H164F</sup>GFP is functional. However, these worms were still cisplatin sensitive (Fig. 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 2 dimer conformations important for targeting of TA-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 (Supplementary Fig. S7). We changed ASNA-1 Cys285 and Cys288 to serines and selected a transgenic strain in which the ASNA-1<sup>C285S C288S</sup>GFP expression level was similar to that of wild-type ASNA-1 (Fig. 3A). ASNA-1<sup>C285S C288S</sup> is functional because its expression rescues the growth phenotype of asna-1 mutants (Fig. 3D; Supplementary Fig. S6). However, worms expressing ASNA-1<sup>C285S C288S</sup> were sensitive to cisplatin (Fig. 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<sup>H164F</sup> and asna-1<sup>C285S C288S</sup> (Fig. 3D; Supplementary Fig. 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

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 (gray bars). Bars represent mean survival ± SEM after 24-hour exposure. Data are collected from at least 3 experiments. The level of statistical significance is indicated for comparisons discussed in the text. (n.s., not significant). 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.

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are mutant for \textit{daf-7}/TGFβ. A compensatory increase in insulin pathway activity by overexpression of some insulins or wild-type \textit{ASNA-1} allows \textit{daf-7} dauers to exit the dauer stage and become adults (23). Using this assay we observed that 23\% of \textit{daf-7}(e1372) dauers expressing the \textit{asna-1}C285S C288S transgene \((n = 103)\) exited the dauer stage at 25°C. Hence, these transgenes expressing mutant forms of \textit{asna-1} are capable of enhancing insulin signaling. This shows that the H164, 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 \textit{ASNA-1}–promoted cisplatin resistance without interfering with insulin signaling.

In \textit{C. elegans}, mutants in both the insulin/insulin-like growth factor receptor homologue \textit{daf-2} and \textit{asna-1} have greatly decreased insulin signaling (23). To further test whether \textit{ASNA-1}–dependent chemoresistance is a consequence of the influence of \textit{ASNA-1} on insulin signaling, we exposed \textit{daf-2}(e1370) mutants to cisplatin. Although \textit{asna-1} mutants died on cisplatin plates, no death was scored in the \textit{daf-2} mutants (Fig. 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 to cisplatin than \textit{asna-1}(null)–treated larvae, which also lack insulin signaling (23). In addition, \textit{daf-2} mutants are resistant to copper (38) whereas \textit{asna-1} mutants are hypersensitive (Fig. 1C). Thus, depletion of \textit{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 \textit{ASNA-1}, we expressed \textit{asna-1} under tissue-specific promoters in \textit{asna-1} mutants. \textit{asna-1} expression under an intestinal (\textit{p}_{\text{cut}}) or an insulin (\textit{p}_{\text{daf-2}}) promoter rescued the resistance in a significant manner (Fig. 4B). The insulin promoter (\textit{p}_{\text{cut}}) and the \textit{asna-1} promoter drive \textit{asna-1} expression in head neurons and in the intestine. The intestine is considered the most important organ for detoxification in worms (39). Consistent with this notion, \textit{asna-1} expression only in head neurons (under the \textit{p}_{\text{daf-2}} promoter) did not rescue the sensitivity of \textit{asna-1} mutants (Fig. 4C).

However, \textit{asna-1} expression in head neurons is sufficient to rescue the insulin signaling defect in \textit{asna-1} mutants (23). These observations reinforce the notion that the insulin function of \textit{ASNA-1} is distinct from its cisplatin-related function.

**Discussion**

Platinum-based chemotherapy is widely used and is increasingly important in the treatment of cancer. Despite 3 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 endoplasmic reticulum 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 among genetic factors, targeted drugs, and platinum-based chemotherapy. This work presents a model for such studies.

We have identified \textit{ASNA-1} as a target to reduce cisplatin resistance and characterized this \textit{in vivo} using genetic
ASNA-1 and Cisplatin Sensitivity

Figure 4. ASNA-1 confers cisplatin resistance independent of insulin signaling. Worms were exposed to 300 μg/mL cisplatin for 24 hours. Bars represent mean survival ± SEM. Each experiment was performed 3 times and a χ² test was used to calculate statistical significance. The level of statistical significance is indicated for comparisons discussed in the text. A, daf-2/insulin/insulin-like growth factor 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 (Pdaf-2) or an insulin (Psm-6)-specific promotor in an asna-1(sv42) mutant background. Worms expressing Psm-6:asna-1 (n = 24) or Pdaf-2:asna-1 (n = 37) were resistant to cisplatin compared with asna-1(sv42) mutants (n = 32). ***, P < 0.001; *, P < 0.05. C, asna-1 expression under a head neuronal (Pposm-6)-specific promotor in an asna-1(sv42) mutant background did not rescue the cisplatin hypersensitivity phenotype. asna-1 mutants expressing Pposm-6:asna-1 (n = 56) were cisplatin sensitive as compared with both N2 (n = 70; ***, P < 0.001) and asna-1 mutants (n = 84; **, P < 0.01).

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 TA-proteins. In the nematode, it has been possible to use these data to study the function of metazoan ASNA-1 in vivo. Although 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 cysteines 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 C. elegans and cancer medicine and underscores the importance of functional studies 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 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 are rescued and also 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 indicates that ASNA-1 could regulate copper transporters to promote copper and cisplatin resistance. We speculate that the conserved metal-regulated ATPase ASNA1 regulates diverse TA-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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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