Published OnlineFirst October 21, 2010; DOI: 10.1158/0008-5472.CAN-10-1548

Cancer Research

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* ASNA-1 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).

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Note: Supplementary material for this article is available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-10-1548

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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 *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 cleanup 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 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 pCC: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 (Platinol 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_2O 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.

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 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*

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(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* promoter 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 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-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 reproductively (non-dauer) at 15°C but become 100% dauer larvae at 25°C. *daf-7*(*e1372*) mutants carrying pVB402GK or pVB464GK expressing ASNA-$1^{\Delta H164}$ and ASNA-$1^{C285S\ C288S}$, 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

LC_{50} values were determined using a binary logistic regression model. The χ^2 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 1. Chemosensitivity in *asna-1(rnai)* worms

Metal salt	Wild type		*asna-1(rnai)*	
	LC_{50} (mg/mL)	*n*	LC_{50} (mg/mL)	*n*
Cisplatin	0.38	237	0.20[a]	187
Sodium arsenite	0.31	175	0.067[a]	132
Antimony potassium tartrate	38.9	104	10.7[a]	87
Zinc chloride	0.22	196	0.21	197
Cadmium chloride	0.057	116	0.052	110

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-provoked movement. A binary logistic regression model was used to calculate LC_{50} and *P* values. Data are collected from 3 experiments.

[a] $P < 0.001$.

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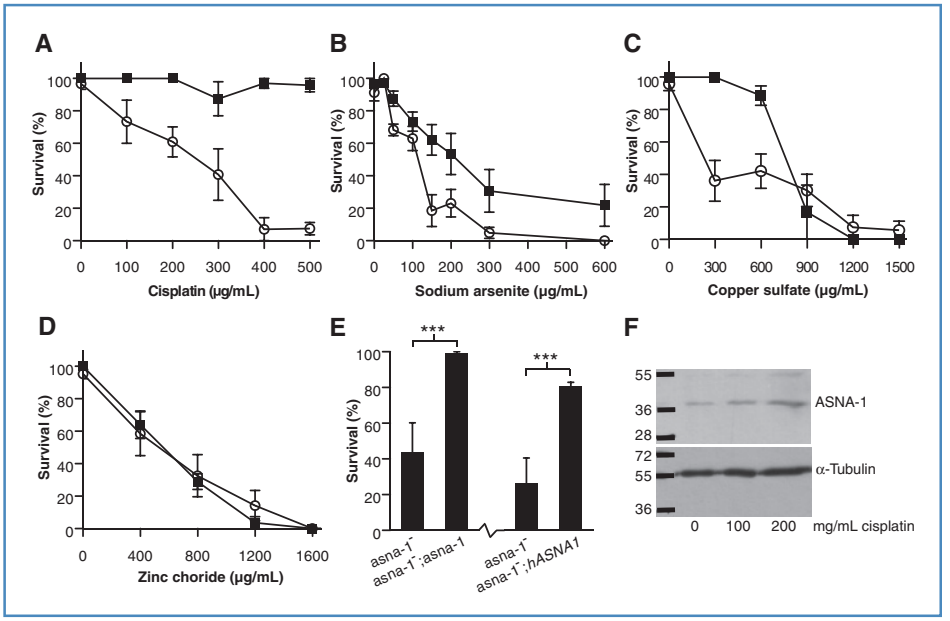

Figure 1. Chemosensitivity of *asna-1* mutants and induction of ASNA-1 expression by cisplatin. Young adult worms were exposed to metal salts and death was determined by absence of touch-provoked movement after 24 hours. Data are presented as mean survival (%) ± SEM from at least 3 experiments. Black 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 ($n = 581$) after exposure to sodium arsenite. C, *asna-1* mutants ($n = 164$) compared with wild type ($n = 149$) after exposure to copper sulfate. D, *asna-1* mutants ($n = 278$) compared with wild type ($n = 250$) 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.

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 LC_{50} 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(rnai)* worms and *zk637.3*(*gk367*) mutant worms were 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 LC_{50} 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 LC_{50} for copper in *asna-1* mutants (500 μg/mL) was significantly lower than that of wild-type animals (849 μg/mL), $P < 0.001$ (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 ASNA-1 protein levels after 48-hour 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 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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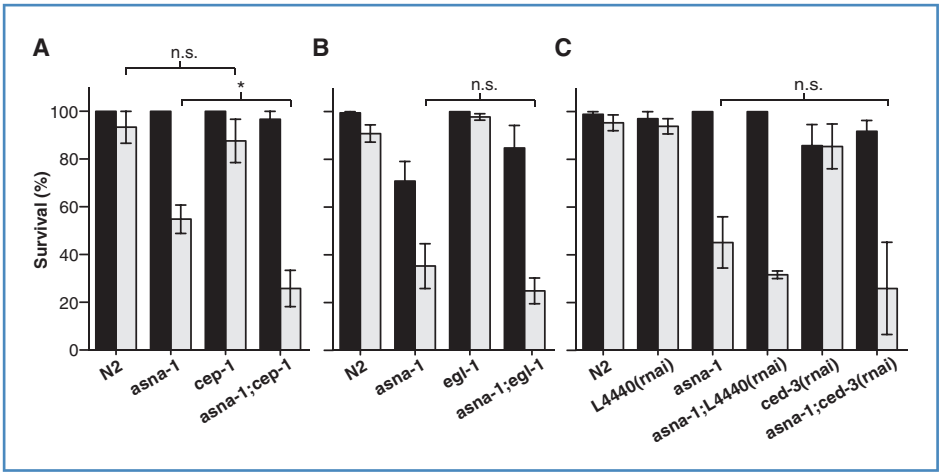

Figure 2. Blockage of apoptosis has no effect on cisplatin sensitivity. Young adult worms were exposed to control agar (black bars) or agar containing 300 μg/mL cisplatin (gray bars). Bars represent mean survival ± SEM after 24-hour exposure. Data are collected from at least 3 experiments. The level 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. 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* ($n = 32$).

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 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-$1^{\Delta H164}$:GFP, we identified a line in which the ASNA-$1^{\Delta H164}$:GFP expression level was equal to levels of wild-type ASNA-1 (Fig. 3A). The *asna-*$1^{\Delta H164}$*: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-$1^{\Delta H164}$: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-$1^{C285S\ C288S}$:GFP expression level was similar to that of wild-type ASNA-1 (Fig. 3A). ASNA-$1^{C285S\ C288S}$ is functional because its expression rescues the growth phenotype of *asna-1* mutants (Fig. 3D; Supplementary Fig. S6). However, worms expressing ASNA-$1^{C285S\ C288S}$ 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-*$1^{\Delta H164}$ and *asna-*$1^{C285S\ C288S}$ (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

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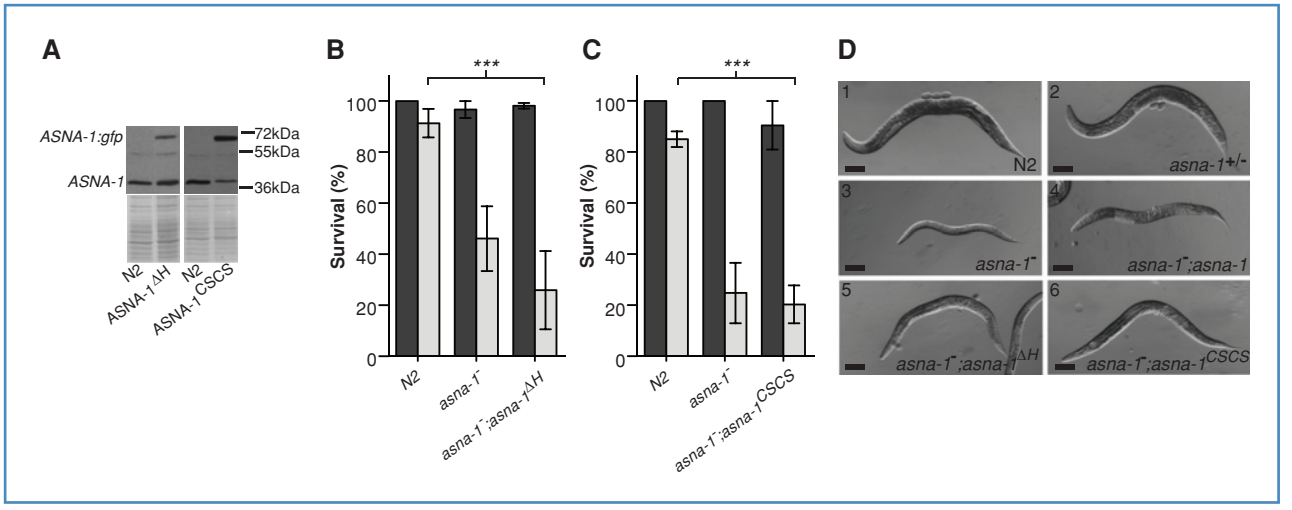

Figure 3. Structure-function analysis of ASNA-1. A, Western blot comparing expression of wild-type ASNA-1 and ASNA-1$^{\Delta H164}$:GFP or ASNA-1$^{C285S,\ C288S}$:GFP in worms heterozygous for the *asna-1(ok938)* mutation. Coomassie staining as loading control. B, worms expressing ASNA-1$^{\Delta H164}$:GFP 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 hours on 300 μg/mL cisplatin (***, $P < 0.001$ by a χ^2 test). Error bars represent SEM. C, worms expressing ASNA-1$^{C285S\ C288S}$: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 χ^2 test). Error bars represent SEM. D, transgenic expression of ASNA-1:GFP (D4), ASNA-1$^{\Delta H164}$:GFP (D5) or ASNA-1$^{C285S,\ C288S}$:GFP (D6) results in a partial rescue of the growth phenotype observed in *asna-1(ok938)* mutant (D3). Magnification in Leica DMRP, ×5; scale bars indicate 100 μm.

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*$^{\Delta H164}$ 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/insulin-like growth factor 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. Although *asna-1* mutants died on cisplatin plates, no death was scored in the *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 Table1, where starved wild-type larval controls, which lack insulin signaling (37), were more resistant to cisplatin than *asna-1(rnai)*–treated larvae, which also lack insulin signaling (23). In addition, *daf-2* mutants are resistant to copper (38) whereas *asna-1* mutants are hypersensitive (Fig. 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 (p_{elt-2}) or an insulin (p_{daf-28}) promoter rescued the resistance in a significant manner (Fig. 4B). The insulin promoter (p_{daf-28}) and the *asna-1* promoter drive *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, *asna-1* expression only in head neurons (under the p_{osm-6} promoter) did not rescue the sensitivity of *asna-1* mutants (Fig. 4C). However, *asna-1* expression in head neurons is sufficient to rescue the insulin signaling defect in *asna-1* mutants (23). These observations reinforce the notion that the insulin function of 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 ASNA-1 as a target to reduce cisplatin resistance and characterized this *in vivo* using genetic

Published OnlineFirst October 21, 2010; DOI: 10.1158/0008-5472.CAN-10-1548

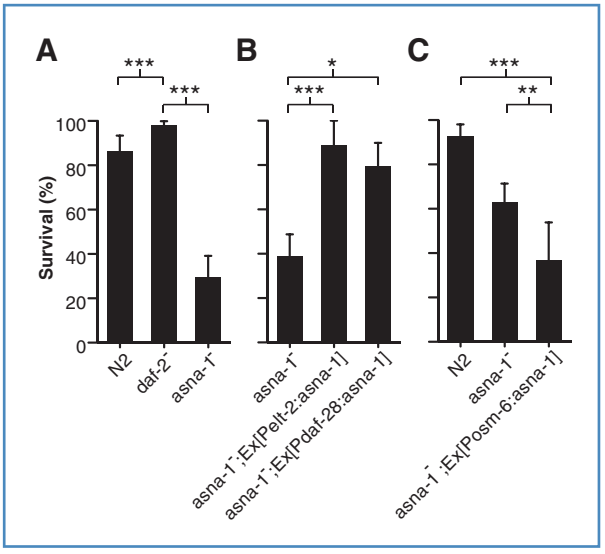

Figure 4. ASNA-1 confers cisplatin resistance independent of insulin signaling. Worms were exposed to 300 μg/mL cisplatin for 24 hours. Bars represent mean survival ± SEM. Each experiment was performed 3 times and a χ^2 test was used to calculate statistical significance. The level of statistical significance 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 (P_{elt-2}) or an insulin (P_{daf-28})-specific promotor in an *asna-1(sv42)* mutant background. Worms expressing P_{elt2}:*asna-1* ($n = 24$) or P_{daf-28}:*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 (P_{osm-6})-specific promotor in an *asna-1(sv42)* mutant background did not rescue the cisplatin hypersensitivity phenotype. *asna-1* mutants expressing P_{osm-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 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 *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 metalloregulated 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.

Acknowledgments

The authors are grateful to Eva-Maj Hägglöf, Agneta Rönnlund, Jyothsna Chitturi, and Hanna Wolf-Watz for technical assistance. The authors thank Hans Stenlund for statistical support. Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR).

Grant Support

This work was supported by the Swedish Cancer Society (CAN2007/901; P. Naredi), the Swedish Research Council (K2008-68X-20803-01-3; P. Naredi), the Cancer Research Foundation in Northern Sweden (P. Naredi, O.Hemmingsson), JC Kempe Memorial Foundation Scolarship Fund (O.Hemmingsson), and Västerbottens County Council (ALF-means and Spjutspetsmedel; P. Naredi).

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Received 04/29/2010; revised 09/22/2010; accepted 10/16/2010; published OnlineFirst 10/21/2010.

References

1. Hambley TW. Chemistry. Metal-based therapeutics. Science 2007;318:1392–3.

2. Kelland L. The resurgence of platinum-based cancer chemotherapy. Nat Rev Cancer 2007;7:573–84.

Published OnlineFirst October 21, 2010; DOI: 10.1158/0008-5472.CAN-10-1548

3. Wang D, Lippard SJ. Cellular processing of platinum anticancer drugs. Nat Rev Drug Discov 2005;4:307–20.
4. Kurdi-Haidar B, Heath D, Naredi P, Varki N, Howell SB. Immunohistochemical analysis of the distribution of the human ATPase (hASNA-I) in normal tissues and its overexpression in breast adenomas and carcinomas. J Histochem Cytochem 1998;46:1243–8.
5. Hemmingsson O, Zhang Y, Still M, Naredi P. ASNA1, an ATPase targeting tail-anchored proteins, regulates melanoma cell growth and sensitivity to cisplatin and arsenite. Cancer Chemother Pharmacol 2009;63:491–9.
6. Hemmingsson O, Nojd M, Kao G, Naredi P. Increased sensitivity to platinating agents and arsenite in human ovarian cancer by down-regulation of ASNA1. Oncol Rep 2009;22:869–75.
7. Naredi P, Heath DD, Enns RE, Howell SB. Cross-resistance between cisplatin and antimony in a human ovarian carcinoma cell line. Cancer Res 1994;54:6464–8.
8. Naredi P, Heath DD, Enns RE, Howell SB. Cross-resistance between cisplatin, antimony potassium tartrate, and arsenite in human tumor cells. J Clin Invest 1995;95:1193–8.
9. Kurdi-Haidar B, Aebi S, Heath D, Enns RE, Naredi P, Hom DK, et al. Isolation of the ATP-binding human homolog of the arsA component of the bacterial arsenite transporter. Genomics 1996;36:486–91.
10. Shen J, Hsu CM, Kang BK, Rosen BP, Bhattacharjee H. The Saccharomyces cerevisiae Arr4p is involved in metal and heat tolerance. Biometals 2003;16:369–78.
11. Tseng YY, Yu CW, Liao VH. Caenorhabditis elegans expresses a functional ArsA. Febs J 2007;274:2566–72.
12. Kurdi-Haidar B, Heath D, Aebi S, Howell SB. Biochemical characterization of the human arsenite-stimulated ATPase (hASNA-I). J Biol Chem 1998;273:22173–6.
13. Kurdi-Haidar B, Hom DK, Flittner DE, Heath D, Fink L, Naredi P, et al. Dual cytoplasmic and nuclear distribution of the novel arsenite-stimulated human ATPase (hASNA-I). J Cell Biochem 1998;71:1–10.
14. Stefanovic S, Hegde RS. Identification of a targeting factor for post-translational membrane protein insertion into the ER. Cell 2007;128:1147–59.
15. Schuldiner M, Metz J, Schmid V, Denic V, Rakwalska M, Schmitt HD, et al. The GET complex mediates insertion of tail-anchored proteins into the ER membrane. Cell 2008;134:634–45.
16. Rabu C, Schmid V, Schwappach B, High S. Biogenesis of tail-anchored proteins: the beginning for the end? J Cell Sci 2009; 122:3605–12.
17. Mateja A, Szlachcic A, Downing ME, Dobosz M, Mariappan M, Hegde RS, et al. The structural basis of tail-anchored membrane protein recognition by Get3. Nature 2009;461:361–6.
18. Bozkurt G, Stjepanovic G, Vilardi F, Amlacher S, Wild K, Bange G, et al. Structural insights into tail-anchored protein binding and membrane insertion by Get3. Proc Natl Acad Sci USA 2009;106:21131–6.
19. Safaei R, Katano K, Larson BJ, Samimi G, Holzer AK, Naerdemann W, et al. Intracellular localization and trafficking of fluorescein-labeled cisplatin in human ovarian carcinoma cells. Clin Cancer Res 2005;11:756–67.
20. Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene 2003;22:7265–79.
21. Mukhopadhyay R, Ho YS, Swiatek PJ, Rosen BP, Bhattacharjee H. Targeted disruption of the mouse Asna1 gene results in embryonic lethality. FEBS Lett 2006;580:3889–94.
22. Kaletta T, Hengartner MO. Finding function in novel targets: C. elegans as a model organism. Nat Rev Drug Discov 2006;5:387–98.
23. Kao G, Nordenson C, Still M, Ronnlund A, Tuck S, Naredi P. ASNA-1 Positively Regulates Insulin Secretion in C. elegans and Mammalian Cells. Cell 2007;128:577–87.
24. Brenner S. The genetics of Caenorhabditis elegans. Genetics 1974;77:71–94.
25. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 1998;391:806–11.
26. Collet J, Spike CA, Lundquist EA, Shaw JE, Herman RK. Analysis of osm-6, a gene that affects sensory cilium structure and sensory neuron function in Caenorhabditis elegans. Genetics 1998;148: 187–200.
27. Fukushige T, Hawkins MG, McGhee JD. The GATA-factor elt-2 is essential for formation of the Caenorhabditis elegans intestine. Dev Biol 1998;198:286–302.
28. Li W, Kennedy SG, Ruvkun G. daf-28 encodes a C. elegans insulin superfamily member that is regulated by environmental cues and acts in the DAF-2 signaling pathway. Genes Dev 2003;17:844–58.
29. Gems D, Sutton AJ, Sundermeyer ML, Albert PS, King KV, Edgley ML, et al. Two pleiotropic classes of daf-2 mutation affect larval arrest, adult behavior, reproduction and longevity in Caenorhabditis elegans. Genetics 1998;150:129–55.
30. Ren P, Lim CS, Johnsen R, Albert PS, Pilgrim D, Riddle DL. Control of C. elegans larval development by neuronal expression of a TGF-beta homolog. Science 1996;274:1389–91.
31. Holzer AK, Samimi G, Katano K, Naerdemann W, Lin X, Safaei R, et al. The copper influx transporter human copper transport protein 1 regulates the uptake of cisplatin in human ovarian carcinoma cells. Mol Pharmacol 2004;66:817–23.
32. Samimi G, Safaei R, Katano K, Holzer AK, Rochdi M, Tomioka M, et al. Increased expression of the copper efflux transporter ATP7A mediates resistance to cisplatin, carboplatin, and oxaliplatin in ovarian cancer cells. Clin Cancer Res 2004;10:4661–9.
33. Safaei R, Katano K, Samimi G, Naerdemann W, Stevenson JL, Rochdi M, et al. Cross-resistance to cisplatin in cells with acquired resistance to copper. Cancer Chemother Pharmacol 2004;53:239–46.
34. Conradt B, Horvitz HR. The C. elegans protein EGL-1 is required for programmed cell death and interacts with the Bcl-2-like protein CED-9. Cell 1998;93:519–29.
35. Zhou T, Radaev S, Rosen BP, Gatti DL. Structure of the ArsA ATPase: the catalytic subunit of a heavy metal resistance pump. EMBO J 2000;19:4838–45.
36. Metz J, Wachter A, Schmidt B, Bujnicki JM, Schwappach B. The yeast Arr4p ATPase binds the chloride transporter Gef1p when copper is available in the cytosol. J Biol Chem 2006;281:410–7.
37. Baugh LR, Sternberg PW. DAF-16/FOXO regulates transcription of cki-1/Cip/Kip and repression of lin-4 during C. elegans L1 arrest. Curr Biol 2006;16:780–5.
38. Barsyte D, Lovejoy DA, Lithgow GJ. Longevity and heavy metal resistance in daf-2 and age-1 long-lived mutants of Caenorhabditis elegans. Faseb J 2001;15:627–34.
39. McGhee JD. The C. elegans intestine. WormBook 2007:1–36.
40. Mandic A, Hansson J, Linder S, Shoshan MC. Cisplatin induces endoplasmic reticulum stress and nucleus-independent apoptotic signaling. J Biol Chem 2003;278:9100–6.
41. Punt CJ, Tol J. More is less – combining targeted therapies in metastatic colorectal cancer. Nat Rev Clin Oncol 2009;6:731–3.

Published OnlineFirst October 21, 2010; DOI: 10.1158/0008-5472.CAN-10-1548

Cancer Research
The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

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Cancer Res 2010;70:10321-10328. Published OnlineFirst October 21, 2010.