ASNA1 and cisplatin resistance
- studies in C. elegans and in human tumor cells

Oskar Hemmingsson
To Eda, Agnes and Lovisa

"What is true for E. coli is true for an elephant"

Jacques Monod
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Abstract

Platinum based chemotherapy is widely used to treat cancer. Cisplatin (diamminedichloroplatinum) combination treatments provide cure for metastatic testicular cancer and prolong survival for patients suffering from ovarian, head and neck, bladder and non small cell lung cancer. Tumors that initially respond to treatment may eventually acquire resistance, resulting in treatment failure. Cisplatin resistant cells are crossresistant to arsenite and antimonite and these metalloids are exported from bacteria by the ars-operon.

In this thesis, we describe the human ArsA homolog, ASNA1, as a protein involved in a novel resistance mechanism to cisplatin, arsenite and antimonite. ASNA1 was downregulated by antisense and siRNA techniques in human melanoma and ovarian carcinoma cell lines. These cells displayed increased sensitivity to arsenite and the platinum based drugs cisplatin, carboplatin and oxaliplatin. In both melanoma and ovarian carcinoma, cisplatin resistant cells overexpressed ASNA1.

Blockage of ASNA1 resulted in increased apoptosis and retarded growth, complicating further characterization of ASNA1 in human cell lines. ASNA1 also promotes insulin signaling and mediates membrane insertion of tail-anchored proteins. To explore different aspects of ASNA1 function with respect to cisplatin resistance, we used the model organism C. elegans.

In the nematode C. elegans, asna-1 (rnai) treated larvae were hypersensitive to cisplatin, arsenite and antimonite. Adult asna-1 mutant worms were cisplatin sensitive and this hypersensitivity was seen even when apoptosis was blocked. Expression of human ASNA1 rescued the cisplatin hypersensitivity in asna-1 mutants, showing conservation of function. Transgene expression of mutated forms of asna-1 separated the cisplatin hypersensitivity phenotype from the insulin signaling phenotype of asna-1 mutants. Three ASNA-1 residues, His164, Cys285 and Cys288 were identified as essential for ASNA-1 promoted cisplatin resistance but not for insulin signaling. Finally, studies of the C. elegans germline revealed increased numbers of apoptotic cells in asna-1 mutants.

In conclusion, C. elegans is a suitable model organism to identify and characterize cisplatin response mechanisms. A targeted therapy against ASNA1 could sensitize cisplatin resistant cells and improve outcome for cancer patients.
List of original papers

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### Abbreviations

- **5-FU**: 5-fluorouracil  
- **ATP**: Adenosine triphosphate  
- **cDNA**: Complementary DNA  
- **CED**: Cell death abnormal  
- **Cys**: Cysteine  
- **Da**: Dalton  
- **DAF**: Dauer formation abnormal  
- **DAPI**: 4',6-diamidino-2-phenylindole  
- **DDP**: Cisplatin (cisdiamminedichloroplatinum)  
- **DNA**: Deoxyribonucleic acid  
- **dsRNA**: Double stranded RNA  
- **ER**: Endoplasmic reticulum  
- **FBS**: Fetal bovine serum  
- **FDA**: Food and drug administration  
- **Get**: Guided entry of tail-anchored proteins or golgi to ER traffic  
- **GFP**: Green fluorescent protein  
- **GSH**: Glutathione  
- **GSTπ**: Glutathione-S transferase π  
- **His**: Histidine
<table>
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<tr>
<td>IC₅₀</td>
<td>The concentration of a drug that is required for 50% inhibition</td>
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<td>IGF</td>
<td>Insulin-like growth factor</td>
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<tr>
<td>LC₅₀</td>
<td>Median lethal concentration or lethal concentration 50%</td>
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<td>MMR</td>
<td>Mismatch repair</td>
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<tr>
<td>MTT</td>
<td>(3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide)</td>
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<tr>
<td>NER</td>
<td>Nucleotide excision repair</td>
</tr>
<tr>
<td>RASP</td>
<td>Resistance to arsenite, antimonite and platinum</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>SRP</td>
<td>Signal recognition particle</td>
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<tr>
<td>TA</td>
<td>Tail-anchored</td>
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<tr>
<td>TGFβ</td>
<td>Transforming (or tumor) growth factor beta</td>
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<td>TMD</td>
<td>Transmembrane domain</td>
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Introduction

Insight into the complexity of human biology is facilitated by knowledge about our evolutionary history. Model organisms share conserved cellular pathways with humans and provide a basic map for biological connections and a toolbox to test hypotheses. This thesis is built on the concept that we share cellular resistance mechanisms with our biological ancestors and that these mechanisms are important in cancer treatment. The ATPase ASNA1 is related to a highly conserved bacterial resistance factor for metalloids and we ask if ASNA1 promotes cisplatin resistance in human tumor cells. Our hypothesis is tested both in the nematode Caenorhabditis elegans and in human cell lines. Conservation of fundamental biological processes is a prerequisite for this work. We characterize ASNA1 as a highly conserved resistance factor and as a target to increase the therapeutic effect of platinum based chemotherapy.

Cancer is a devastating disease. Platinum based chemotherapy is a cornerstone in oncology treatments and there is an urge for increased knowledge about cellular resistance mechanisms and platinum interactions with signaling pathways. We present C. elegans as a novel model system for research on platinum based drugs and use this model to characterize ASNA1.

C. elegans as a model organism

C. elegans represents an excellent model system for genetic, biochemical and functional studies in vivo. The 1 mm long nematode (Fig. 1) is easy and inexpensive to grow on agar plates or in liquid culture. It exists in two sexes; hermaphrodites that give rise to genetically identical progeny and males that can be used in crosses to transfer mutant genes. The life cycle of C. elegans is 3.5 days at 20°C and involves an embryonic stage, four larval stages, and an adult stage in which the worms are sexually mature (1).

C. elegans worms are readily amenable to forward and reverse genetic analysis. Gene knockdown by RNAi is possible through injection of dsRNA or through feeding of worms with bacteria expressing the target RNA (2, 3). In addition, collections of strains carrying mutant alleles simplify phenotypic analysis of specific mutations. The C. elegans genome has been sequenced and about sixty percent of human genes have a homolog in the worm and a third have a direct ortholog (4). The cell lineage is mapped and transparency
allows studies of individual cells and expression of GFP markers for analysis in vivo.

Figure 1. *C. elegans* adult hermaphrodite. The transparent nematode is easily studied using light microscopy. The pharynx (P) in the head consists of an anterior and a posterior bulb connected by isthmus. The intestine (I) goes from the end of the pharynx in the head to the anus in the tail region. The vulva (V) is an anatomical landmark on the ventral body wall and distinguishes adults from larvae. Dorsal to the vulva is the uterus (U), containing embryos. The gonad consists of two arms, (anterior and posterior). Only one of the gonad arms normally appears in a focal plane while the other is hidden by the intestine. The proximal gonad contains oocytes (O) that become fertilized by sperm in the spermatheca before entry into the uterus. The gonad turns to the dorsal side of the worm at the gonad loop region (G) and continues to the distal tip where germline development is initiated.

**Drug discovery in *C. elegans***

*C. elegans* provides a platform for both drug screening processes and genetic studies of molecular pathways targeted by specific drugs (5). For example, *C. elegans* models have been applied to understand the pharmacodynamic mechanisms behind volatile anesthetics (6), anthelmintic drugs (7), statins (8), anticonvulsant and antidepressive drugs (5). High throughput screens in *C. elegans* have aimed at finding new targets for treatment of Parkinson’s disease, obesity and type 2 diabetes (5, 9).

For cancer research, studies in *C. elegans* have provided understanding in apoptosis and RAS signaling pathways (10). Studies in *C. elegans* was the first to reveal critical components in apoptosis like CED-3 (caspases) and CED-4 (Apaf-1) (11, 12). The potential to use *C. elegans* as a tool for cancer drug discovery has so far not been fully exploited but is illustrated by a few reports about the mode of action of farnesyltransferase inhibitors (13) and the metabolism of 5-fluorouracil (14). This study provides a model to study platinum based chemotherapy in *C. elegans*.

**Apoptosis in *C. elegans* germ cells**

Adult *C. elegans* animals contain postmitotic somatic cells and a two armed gonad that contains proliferating germ cells. Germline development starts in the distal end of the gonad arm where germ cells are in mitosis (Fig. 2). More
proximally, germ cells enter meiosis in the transition zone where DAPI-stained nuclei appear with a characteristic crescent as chromosomes cluster on one side of the nucleus. Germ cell apoptosis occurs in the gonad loop region where developing cells are in the pachytene stage of meiotic prophase (15). Germ cells share a common cytoplasm and are engulfed by the surrounding somatic sheath cells after apoptosis (16, 17). Apoptotic corpses can be observed in vivo using Nomarski microscopy and engulfment can be visualized by sheath cell expression of engulfment specific genes fused to GFP.

Figure 2. Germline development in C. elegans hermaphrodites. The germline from one gonad arm is shown. Pictures show DAPI-stained nuclei (100x) in the corresponding part of the germline. Germ cell development starts at the distal end of the gonad and is regulated by the distal tip cell. Meiosis starts in the transition zone. Apoptotic cells are observed closer to the gonad loop region where cells are in the pachytene stage of meiosis. Germ cells share a common cytoplasm but differentiate into individual cellularized oocytes in the proximal gonad. The lower part of the figure is reproduced with permission from (15).

Pathways for programmed cell death are highly conserved between the C. elegans and humans. In the germline, apoptosis occurs both physiologically and as a consequence of DNA damage. The latter is dependent on the p53 homolog CEP-1 that transcriptionally upregulates the BH3-only homologs EGL-1 and CED-13. Binding of EGL-1 to the Bcl-2 homolog CED-9 induces a conformational change in CED-9, making it unable to sequester the Apaf-1 homolog CED-4. When CED-4 is released from CED-9, it associates in a tetramer and activates the caspase homolog CED-3, resulting in apoptosis.
(Fig. 3) (11, 15). This simplified model for apoptosis is very similar to what occurs in mammalian cells after DNA-damage.

Under normal conditions, more than 50% of the germ cells undergo apoptosis. This physiological apoptosis is EGL-1 independent and is triggered by an unknown mechanism. However, it requires CED-3 and CED-4 and is inhibited by CED-9 (11).

Figure 3. Regulation of apoptosis in C. elegans. The core apoptotic machinery is highly conserved between C. elegans and mammals. Apoptosis is triggered by the caspase homolog CED-3 after activation of proCED-3 by the CED-4 tetramer apoptosome. CED-4, the worm homolog of Apoptotic protease activating factor Apaf-1, is sequestered as a dimer by the Bcl-2 homolog CED-9. When EGL-1 binds to CED-9, CED-4 is released and can activate CED-3. EGL-1, the homolog to BH-3 only, is transcriptionally upregulated by the p53 homolog CEP-1 after genotoxic stress in the germline. It also regulates developmental cell death in the soma but not in the germline.

**Developmental apoptosis in the C. elegans soma**

Apoptosis occurs in somatic cells during embryonic and larval development. A C. elegans hermaphrodite generates 1090 cells during development and out of these, exactly 131 cells undergo apoptosis. 113 cell deaths occur in the embryo (between 250 and 450 minutes after fertilization) and neuronal apoptosis is observed in the larval stage L2. No apoptotic cell death occurs in somatic cells after the L2 stage (11). The developmental cues that activate apoptosis are unknown for most of the 131 cell deaths. However, somatic apoptotic cell death, unlike physiological germ cell death, is dependent on EGL-1 binding to CED-9 (11, 18).
**Insulin/IGF signaling in C. elegans**

In adverse conditions or if exposed to a pheromone, the worm can enter an alternative stage, termed the dauer stage (Fig. 4) (19). *C. elegans* amphid sensory neurons and posterior intestinal cells promote growth in the non-dauer life cycle by secreting the insulin-like peptide DAF-28 that is genetically upstream (and the likely ligand) of the insulin/IGF1 receptor homolog DAF-2 (20). Insulin signaling controls dauer entry by regulating the subcellular localization of the forkhead family transcription factor DAF-16. In animals with reduced insulin signaling, DAF-16::GFP is observed in the nuclei and in growing animals, DAF-16 is cytoplasmic (21-23). In parallel with insulin signaling, the TGFß/DAF-7 pathway also controls dauer entry (19). Mutations in the insulin signaling pathway and the TGFß pathway show synergistic effects (24) and overexpression of insulin-like peptides can compensate for mutations in the TGFß pathway (25).

![Figure 4. The life cycle of C. elegans. Embryonic development continues 10-12 h (at 20°C) ex utero before hatching. Larvae arrest in the first larval stage (L1) under starvation conditions but resume growth when food becomes available. The normal life cycle takes 3.5 days at 20°C. Each larval stage is separated by a molt which involves exchange of the exoskeleton. From the middle of the L1 stage, larvae can decide to enter an arrested state termed dauer. They can remain in this state for months or continue growth to the L4 stage.](image)

Studies of the influence of ASNA-1 on insulin signaling illustrates how the nematode can be exploited for functional analysis of a protein in the context of a defined signaling pathway. It was shown that insulin producing cells in wild type and *asna-1(rnai)* worms express similar levels of DAF-28::GFP but *asna-1(rnai)* animals secrete less DAF-28::GFP into the pseudoceolomic...
fluid. In addition, DAF-16::GFP is nuclear in asna-1(rnai) worms, as in worms with reduced insulin signaling. Overexpression of asna-1 bypasses the requirement for TGFβ signaling in dauer development, showing that ASNA-1 stimulates the insulin signaling pathway that acts in parallel with TGFβ signaling. ASNA-1 in worms acts non-autonomously to promote growth since expression in head neurons is sufficient to rescue the growth phenotype of asna-1 mutants. This further reinforces the notion that ASNA-1 promotes growth via a hormone like mechanism. Human ASNA1 rescues the phenotype of ASNA-1 deficient worms and ASNA1 influence on insulin secretion was confirmed in a mammalian system (25). This shows that ASNA-1 promotes insulin signaling and that ASNA-1 function is conserved from worms to humans.

**Cancer treatment**

Cancer is the second leading cause of death in the western world. One in four deaths in the United States is due to cancer (26) and the global incidence in cancer is expected to rise (27). The challenge to treat a disease that originates from the host tissue is met by three major treatment modalities. First, surgical removal of solid tumors and adjacent tissues provide the basis for cancer treatment. Second, radiation therapies aim at damaging the cancer cell DNA that is more susceptible than DNA in normal cells. Third, pharmacological treatment has gained importance in cancer therapy since the 1960s when studies showed that chemotherapy could cure leukemia patients (28). Since then, combinations of these three modalities are standard clinical practice. This study focuses on an important class of chemotherapy drugs, the platinum based drugs.

The term chemotherapy was coined by Paul Ehrlich in the beginning of the 1900s and defined as the use of chemicals to treat a disease. He also developed a treatment for syphilis based on arsenicals in 1908 (28). Ninety years later, a study showed that arsenic trioxide can be used to treat acute promyelocytic leukemia (29). This delay illustrates the challenge to develop a drug that is both potentially therapeutic and toxic. Over the years, a combination of studies in experimental model systems and clinical trials has resulted in chemotherapy regimens that provide cure for leukemias, lymphomas and solid tumors.
The platinum based chemotherapeutic drugs have been exceptionally successful in treatments of a wide variety of cancer diseases. Cisplatin was the first platinum drug and revolutionized cancer chemotherapy when it was approved in 1978. Testicular cancer was previously fatal but treatment with cisplatin, bleomycin and etoposide provide cure for 96% of the patients (26, 30).

In recent years, research on cancer pharmacology has focused on three perspectives. First, mapping of tumor cell resistance against chemotherapy has aimed at development of more efficient drugs. Second, treatment adjustment after characterization of the tumor has provided an individualized treatment regimen. Third, development of targeted drugs that hit a defined molecular pathway in the tumor cell has evolved as a result of increased knowledge in molecular biology. Model systems are required to understand the tumor biology in response to chemotherapy alone or in combination with targeted drugs. This study describes ASNA1 as a novel cisplatin resistance mechanism and C. elegans as a new model to study interactions between platinum-based drugs and individual signaling pathways.

**Cisplatin**

Platinum based chemotherapy has been a clinical success during three decades. Rosenberg et al. discovered cisplatin (cis-diammine-dichloro-platinum) by serendipity when they found that platinum electrodes induced filamentous growth of *Escherichia coli* (31). Later, they also reported that cisplatin suppresses tumor growth (32) and it was approved for cancer treatment by the US Food and Drug Administration (FDA) in 1978 (33).

**Clinical use**

Patients suffering from testicular and ovarian cancer have been treated with cisplatin since the FDA approval 1978 (33). With cisplatin regimens, the overall cure rate for testicular cancer surpass 90% (34). Carboplatin has replaced cisplatin in ovarian cancer treatment because it causes less nephrotoxicity. Cisplatin and carboplatin are also used against head and neck, bladder and non small cell lung cancer (34). Oxaliplatin is given in combination with 5-fluorouracil and leucovorin to patients with metastatic colorectal cancer. Platinum based chemotherapy is often included in treatment regimens with novel targeted drugs. For example, bevacizumab that targets vascular endothelial growth factor (VEGF) significantly improves
survival for patients suffering from non-small-cell lung cancer when used in combination with carboplatin and paclitaxel (35).

Mode of action

Cisplatin is a neutral square planar 300.1 Da molecule containing a platinum atom surrounded by two chloride ions and two amino groups in a cis-position (Fig. 5). Cisplatin becomes activated in the cytoplasm as chloride groups are replaced by water (33). This aquation process is facilitated by the low chloride concentration in the cytoplasm (36). Cisplatin binds primarily to purine bases in DNA and forms DNA-protein and DNA-DNA adducts where intrastrand adducts are believed to be the most toxic. DNA distortion by the adducts results in binding of damage recognition proteins which hinder both replication and transcription (36), inhibit nucleotide excision repair (37) and promote apoptosis (36). These damage recognition proteins include the nonhistone chromosomal high-mobility group 1 and 2 (HMG1 and HMG2), the mismatch repair complex (MMR), the human upstream binding factor (UBF) and the TATA-box binding protein (TBP).

Apoptosis is considered the principal mode of cell death after cisplatin treatment. However, necrosis is also observed in tissues exposed to cisplatin and most prominent at high concentrations (38). It is estimated that only a small fraction of intracellular cisplatin reacts with DNA while the rest is bound to nucleophilic sites at proteins, RNA, membrane phospholipids or thiol-containing molecules. If these cisplatin targets interfere with apoptosis signaling or the energy supply of the cell, necrosis might occur (39). It is also shown that cisplatin can induce apoptosis through ER stress independent of DNA damage (40).

Different platinum based drugs

Three platinum antitumor drugs are available in the clinic; cisplatin, carboplatin and oxaliplatin (Fig. 5). They form the same type of adducts at the same sites on the DNA (37). Carboplatin and cisplatin are crossresistant and share the same range of clinical activity (34). Oxaliplatin adducts are recognized differently by damage recognition proteins. The mismatch repair complex (MMR) binds more specifically to cisplatin and carboplatin adducts and defects in MMR results in resistance to these drugs but not to oxaliplatin (37). Consequently, oxaliplatin is toxic to some tumors with acquired
resistance to cisplatin (41). In addition, oxaliplatin is the only platinum drug used against colorectal cancer (33).

Satraplatin is an orally active version of carboplatin and has shown promising results against prostate cancer but has not yet gained FDA approval (33). Picoplatin is designed to avoid inactivation by thiol-containing proteins and has shown antitumor activity in a phase II study on ovarian cancer (42).

![Platinum based drugs](image)

Figure 5. Platinum based drugs. The three clinically approved platinum based drugs; cisplatin, carboplatin and oxaliplatin.

**Side effects of platinum based drugs**

Gastrointestinal toxicity is common for all platinum drugs. Cisplatin neurotoxicity can result in both ototoxicity and peripheral neuropathy. Furthermore, nephrotoxicity limits the dose interval for cisplatin even if it partially can be treated medically with prehydration, mannitol and diuretics (43). Cisplatin enters proximal tubule cells through the OCT2 transporter and induces apoptosis and necrosis (34, 44). When cisplatin is activated in the cytoplasm by aquation at the sites of the chloride ions, the chloride leaving group might induce nephrotoxicity. Carboplatin was developed to leave a less toxic group. In addition, neither carboplatin nor oxaliplatin interacts with OTC2 (43). Carboplatin is also less nephrotoxic than cisplatin but as potent as cisplatin against ovarian cancer (33). However, carboplatin can induce myelosuppression with thrombocytopenia. The main dose-limiting side effect of oxaliplatin is neurotoxicity with sensory peripheral neuropathy (33).
Cisplatin resistance

The main clinical obstacle with platinum based chemotherapy is resistance. Some tumors are intrinsically resistant to cisplatin and some tumors that are originally sensitive to platinum can eventually acquire resistance, resulting in treatment failure. This is most apparent for ovarian cancer where the initial clinical complete remission rate of 75% declines to a 5-year survival of 45% (26) and an even worse overall survival (45). Cisplatin resistance is multifactorial. Understanding of resistance mechanisms is required to develop more efficient drugs or a targeted therapy that can hit on the Achilles heel of the resistant tumor cell. Cisplatin resistance can be due to inactivation of the drug, increased DNA-repair, decreased apoptosis signaling or decreased drug accumulation.

Inactivation

In the cytoplasm, cisplatin becomes highly reactive and may be inactivated by nucleophilic proteins. Resistant tumor cells contain elevated levels of glutathione (GSH) which inactivate cisplatin by GSH-S transferase π (GSTπ) catalyzed thiol conjugation (36). High levels of GSTπ are associated with a twofold reduction in survival in head and neck cancer patients (46). Canfosfamide is a prodrug that is activated by GSTπ, thus theoretically more active in resistant cells (33). A phase I-II study on non-small cell lung cancer recently showed promising results when canfosfamide was given together with carboplatin as first line therapy. This illustrates the principle of adding a drug to specifically treat resistant cells (47). Metallothioneins contain cysteins that bind to cisplatin but while some report a 5-fold increase in metallothionein levels in resistant cells, others have not observed any differences, emphasizing the multifactorial nature of resistance (36).

Damage recognition and DNA repair

An enhanced DNA repair system attenuates the apoptotic process following cisplatin exposure. The major platinum adduct repair system is the nucleotide excision repair (NER) and the NER proteins ERCC1 and XPA are overexpressed in resistant tumors (36). Knock-down of ERCC1 by siRNA enhances cisplatin sensitivity and ERCC1 mRNA levels correlate to clinical resistance in ovarian cancer (33). Testicular cancer cells have a low capacity to repair DNA and very low expression of ERCC1 and XPA. This is an
explanation for the beneficial cisplatin response in testicular cancer (33). The mismatch repair complex (MMR) does not repair cisplatin adducts but instead activates apoptosis signals. MMR proteins are downregulated in resistant cells. The MMR complex recognizes cisplatin and carboplatin but not oxaliplatin adducts explaining the different resistance patterns between these drugs (37). Damage recognition protein HMG1 shields DNA from repair and is overexpressed in resistant cells (36).

Apoptosis

Once cisplatin-DNA adducts are recognized by damage recognition proteins, several signaling pathways are activated to promote cell cycle arrest, DNA repair or apoptosis. The intricate signaling network that decides whether the cell should survive or die can be affected by the genetic background and new mutations in the tumor DNA. Overexpression of HER-2/neu is associated with inhibition of apoptosis and cisplatin resistance, possibly by HER2 inhibition of Bad through the ERK and PI3K/Akt pathways (36). Furthermore, p53 activation is critical for cisplatin-induced apoptosis. p53 mutations are observed in 50% of all cancers but platinum sensitive seminomatous germ cell tumors predominantly carry wild type p53 (36). p53 mutations can disrupt cell cycle G1 arrest where cells are most sensitive to cisplatin. Mutations in exon 4-9 of p53 prevents DNA binding and Bax activation. Bax is further inhibited by overexpression of Bcl-2 in cisplatin resistant cells (48).

Drug accumulation

Reduced drug accumulation in resistant cells is a significant mechanism of cisplatin resistance. Reductions of the order of 20-70% have been reported from a variety of resistant cell lines (49). Several in vitro studies have also shown that accumulation of carboplatin and oxaliplatin determines cellular sensitivity (44). Autopsy samples have shown a correlation between platinum concentration in the tumor and clinical response (50). Cisplatin accumulation occurs by several mechanisms including both passive diffusion and facilitated transport. The neutral species of the drug might cross the plasma membrane by passive diffusion while charged aquated species needs a transport mechanism to cross the lipid bilayer.
Passive diffusion was previously considered as the main influx mechanism since uptake was linear, non-saturable and independent of a temperature shift from 37°C to 4°C (44). Later studies indicate that energy dependent transporters mediate uptake since ATPase inhibitors and metabolic inhibitors reduce accumulation (51, 52). Since accumulation is non-saturable, it is assumed that several transporters can mediate cisplatin influx (44). Cisplatin is too big for most gated channels but aquaporin 9 (AQP9) has a sufficient pore size and its expression is decreased in platinum resistant cells (44). AQP9 is also involved in arsenite transport (53) and is thereby another candidate for mediating the RASP-phenotype (crossResistance to As, Sb and Pt).

The standard theory explaining cisplatin nephrotoxicity is OCT2 mediated cisplatin influx in tubular cells. OCT2 belongs to the large group of solute carriers (SLC). Another member of this family is the copper transporter CTR1 (44). Deletion of the conserved transmembrane protein CTR1 in yeast results in cisplatin and copper resistance associated with decreased uptake (54). CTR1 also mediates cisplatin uptake in ovarian cancer cells (55, 56). Cisplatin exposure results in rapid internalization and degradation of CTR1 and other uptake processes must be responsible for subsequent accumulation of cisplatin (57). Oxaliplatin is less dependent on CTR1 than cisplatin and carboplatin (44).

Cisplatin and copper shares crossresistance patterns based on both decreased influx and increased efflux and the copper export pumps ATP7A and ATP7B are strongly expressed in resistant cells (58). ATP7A/B contains a metal binding motif that is capable of coordinating platinum (44). Increased expression of ATP7A after platinum treatment is associated with poorer survival in ovarian cancer patients (59). Similarly, ATP7B positive ovarian cancers are more resistant to cisplatin than ATP7B negative tumors (60), resulting in inferior median survival (33 compared to 66 months).

Transfected cells with increased ATP7B expression accumulate low levels of cisplatin while expression of ATP7A results in increased accumulation (61). ATP7A transfectants are still cisplatin resistant and the intracellular platinum is found in the vesicular fraction, indicating that ATP7A compartmentalize platinum for inactivation. Consistent with this notion, platinum co-localizes with ATP7A in vesicles belonging to golgi, lysosomal and secretory pathway compartments (62). Cisplatin resistant cells release more protein as exosomes. Exosomes from resistant cells also contained increased levels of cisplatin, ATP7A and ATP7B (63).
**The RASP phenotype**

Ovarian cancer cells acquire cisplatin resistance when they are selected in cisplatin medium over several passages. These cells show crossresistance to antimony potassium tartrate. Similarly, an antimony potassium tartrate selected sub-line is crossresistant to cisplatin (64). Considering that the bacterial *ars* efflux system for antimony also mediates arsenite transport, the resistant cell lines were tested for arsenite resistance. Cells are resistant to arsenite if they are selected for resistance against either cisplatin or antimony. Resistant cells accumulate less platinum and arsenite, indicating that a transport mechanism mediates this cross resistance. This phenotype is termed the RASP-phenotype (Resistance to *As*, *Sb* and *Pt*) (65) and led to the hypothesis that conserved efflux systems for arsenite and antimonite also could mediate resistance to cisplatin. Here, we develop this hypothesis by asking if ASNA1, the human homolog of the catalyzing *ars* subunit ArsA, mediates cisplatin resistance.

**Arsenic in medicine**

Arsenic has several roles in medicine, it serves as a drug for leukemia and infections diseases but is also known as a carcinogen, a poison and an environmental toxin (66). Exposure to the toxic metalliod is endemic in some parts of the world (67). Arsenic in ground water wells in Latin America and Asia is a severe public health problem. Chronic exposure to arsenic results in peripheral gangrene (“blackfoot disease”), cardiovascular disease and diabetes. Arsenic exposure is also associated with skin- bladder- and lung cancer (68). Organic or inorganic arsenic exists in a pentavalent (*AsV*) or a more toxic trivalent form (*AsIII*). Arsenic targets sulfhydryl groups in proteins and induces apoptosis, mainly through induction of reactive oxygen species (ROS) by interferrance with the thioredoxin system (69).

Despite its reputation as a poison, arsenic has been used as a drug by Greek and Chinese healers for more than two milleniums. In 1910, Paul Ehrlich introduced salvarsan, an organic arsenic compound, to treat syphilis and tuberculosis (66). Another organic arsenical, melarsoprol, is used to treat trypanosomiasis, even tough its use is limited by severe toxicity (66, 70). It was suggested to use *As₂O₃* against acute promyelocytic leukemia (APL) based on positive results of traditional medicine in China (66). Soignet et al. later reported complete remission in 11 of 12 studied patients who were treated with *As₂O₃* against relapse of APL (29). *As₂O₃* is now standard treatment for relapse of APL (71). The APL-specific t(15;17) translocation
results in fusion of the promyelocytic leukemia gene (PML) with the retinoic acid receptor alpha (RARα). PML-RARα fusion protein homodimers block differentiation by inhibition of retinoic acid (RA) functions. RA is the first line treatment for APL and the first example of an oncogene targeted therapy. Both RA and As₂O₃ treatment results in degradation of PML-RARα but while RA binds to RARα, As₂O₃ targets the PML part (66). Thus, As₂O₃ provides a second line targeted therapy for APL. In addition, PML-RARα induces NADPH activation and ROS generation, making APL cells more sensitive to arsenic induced oxidatice stress (69).

**ASNA1**

Given the fact that cisplatin resistant cells are crossresistant to arsenite and antimonite (64, 65) and that mechanisms that confer metalloid resistance are evolutionary conserved (72), possible cisplatin resistance mechanisms could be found in metalloid resistance systems in bacteria. ASNA1 is the human homolog to the bacterial arsenite stimulated ATPase ArsA (73). Human ASNA1 and arsA share 27% homology and a conserved ATPase domain, the GKGVVGKT Walker A motif (73, 74). They belong to a specific family of ATPases (SIMIBI for Signal recognition particle, MinD and BioD), originally GTPases that have acquired ATPase activity during evolution (75). This thesis tests the hypothesis that ASNA1 promotes resistance to cisplatin and arsenite in human cancer cells.

**ASNA1 is a conserved protein mediating metalloid resistance**

To survive in metal polluted environments, bacteria developed resistance systems early during evolution (72). The arsRDABC-operon is well characterized in *E. coli* and promotes resistance to the trivalent metalloids arsenite and antimonite (Fig. 6). The ASNA1 homolog ArsA is an ATPase driving metalloid efflux through the transmembrane protein ArsB (76). Bacterial ArsA consists of two homologous halves, has two nucleotide binding domains and one metalloid binding domain (77). Binding of arsenite or antimonite to ArsA stimulates ATPase activity (78). ArsR is a regulatory protein while ArsC converts arsenate (V) to arsenite (III) (72). ArsD is a metallochaperone, providing arsenite to ArsA (79). arsA have known homologs in higher organisms while arsB only is found in bacteria. Still, the
arsA-homologs get3/arr4 in \textit{S. cerevisiae} (80) and \textit{asna-1} in \textit{C. elegans} (81) are involved in metalloid resistance. Get3 mutants are hypersensitive to As, Co, Cr, Cu and heat. This is reversed by expression of Get3 but not of ATPase deficient Get3. Get3-GFP is seen diffusely in the cytoplasm but under stress conditions, GFP-punctae appear, indicating membrane association (80). Furthermore, two reports suggest that copper regulates Get3 activity. First, Get3 associates with a transport protein (Gef1) when copper is available (82). Second, Get3 activates G-protein signaling by inducing nucleotide exchange after direct binding to a G-alpha subunit. Get3 binding to G alpha is also dependent on copper (83).

![Diagram of the bacterial Ars efflux system for arsenite and antimonite.](image)

The metalloid binding site of ArsA (Cys113, Cys172 and His148) (77) is only partially conserved in \textit{C. elegans} ASNA-1 where ArsA His148 corresponds to ASNA-1 His164. Nevertheless, the ATPase activity of \textit{C. elegans} MBP-ASNA-1 fusion protein is stimulated by both arsenite and antimonite and \textit{asna-1} mutants are hypersensitive to these substrates (81). Human GST-ASNA1 fusion protein is weakly stimulated by arsenite (84).

Sequence comparison of archeal, bacterial and eukaryotic homologs of ASNA1 indicate that eukaryotes may have inherited ASNA1 from an archeal ancestor since we share hydrophobic residues that are absent in bacterial ArsA (85). These residues are important for binding of ASNA1 to tail-anchored proteins. While the bacterial ASNA1 homolog ArsA binds to
transmembrane ArsB for metalloid efflux, eukaryotic ASNA1 might induce a more complex response by regulating tail-anchored proteins. For example, targeting of tail-anchored Bcl-2 or SNAREs could regulate apoptosis and vesicular efflux of cisplatin.

**ASNA1 expression**

ASNA1 RNA is detected in human tissue samples from heart, brain, pancreas, lung, liver, skeletal muscle, kidney and placenta (73). Immunohistochemistry analysis by a monoclonal anti-ASNA1 antibody revealed staining in heart, pancreas, liver, spleen, stomach, kidney, adrenal gland, and skeletal muscle (86). No staining was detected in normal tissues from breast, cerebellum, thyroid, uterine epithelium, testis, lung or large or small intestine but breast carcinomas stained positively for ASNA1. A polyclonal rabbit anti-ASNA1 antibody from our lab was used to detect ASNA1 expression in human brain, breast, testis, kidney, skin, stomach, lung and liver (Naredi et al. unpublished). Use of both the monoclonal and the polyclonal antibody showed a cell specific staining pattern. For example, the beta cells of the islets of Langerhans in pancreas are positive for ASNA1 while the alpha cells are negative (25). Human ASNA1 is detected in the cytoplasm, in the nucleolus and in the perinuclear region (87).

**Influence of ASNA1 on insulin secretion**

*S. cerevisiae* ASNA1 homologue Get3 is implicated in vesicular transport and get3 mutant strains display defects in secretion. Furthermore, Get3 localization changes from golgi to ER upon increases in nutrient levels (88). In humans, ASNA1 is highly expressed in beta cells and correspondingly, ASNA-1 is expressed in insulin producing cells in *C. elegans* (25). This led to the hypothesis that ASNA1 could influence insulin secretion. As described above, extensive studies in *C. elegans* have shown that ASNA-1 promotes insulin signaling. In mammals, downregulation or overexpression of ASNA1 in mouse insulinaoma cells results in decreased or increased insulin secretion respectively. Taken together, these results show that ASNA1 positively regulates insulin secretion (25).
**ASNA1 is a targeting factor for tail-anchored proteins**

Tail-anchored (TA) proteins are bound to cellular membranes by a single transmembrane domain (TMD) at the C-terminus. The N-terminus is cytosolic and carries out a variety of essential functions such as vesicular transport, protein translocation and apoptosis. A bioinformatic screen has identified 325 human TA-proteins and among these are the SNARE-proteins and the Bcl-2 family (89). TA-proteins must be targeted post-translationally since the C-terminal membrane sequence is hidden in the ribosome during translation. Thus, they cannot follow the co-translational SRP-dependent membrane insertion pathway.

Biochemical analysis in rabbit reticulocyte lysates identified mammalian ASNA1 as a chaperone mediating membrane insertion of TA-proteins (Fig. 7) (90). ASNA1 mediated insertion was dependent on ATP hydrolysis, TMD-binding and a proteinaceous receptor at the ER (90, 91). Parallel studies in yeast showed that Get3 interacts with the TMD of several TA-proteins and that Get1 and Get2 are the ER receptors for Get3 (Fig. 7) (92, 93). Subsequently, Get4 and Get5 were identified as interacting partners to Get3 in the cytoplasm (94) and in association with the ribosome (95). It is suggested that Get4/5 associate with Sgt2 and guide TA-proteins to Get3 (96).

![Figure 7: A model for Get3/ASNA1 targeting of tail-anchored proteins](image)

ASNA1 and the yeast homolog Get3 mediate insertion of tail-anchored (TA) proteins into the ER. Homology between yeast and mammals are indicated in the same color. TA-proteins are guided from the ribosome to Get3/ASNA1 by a Get4/TRC35-Get5/Ubl4A complex that associates with Sgt2 in yeast and with Bat3 in mammals. Dimers of Get3/ASNA1 exist in an open conformation in the cytosol but close as they bind to Get4/TRC35 and a TA-protein. While Get1 and Get2 are the ER receptors for Get3 in yeast, it is not known how ASNA1 binds to the ER in mammals. The WRB-protein is the human homolog to Get1.
In mammalian cells there is so far no known receptor for ASNA1 in the ER and yet no evidence for an association between Sgt2-homologs and ASNA1. Instead, immunodepletion of Bat3 from rabbit reticulocyte lysate results in loss of ASNA1 dependent TA-protein membrane insertion (97). Bat3 promotes TA-protein binding to ASNA1 and forms a complex with the mammalian homologs of Get4 and Get5 (TRC35 and Ubl4A) (98).

Three TA-protein insertion pathways are described during the last few years. These are a post-translational SRP-dependent route, a Hsc70/Hsp40 dependent route and an ASNA1 mediated pathway (99). Depletion of the SRP-receptor or small molecule inhibition of Hsp40/Hsc70 has no effect on TA-protein membrane insertion (99) but loss of the ASNA1 homolog Get3 in yeast results in misinsertion of ER-proteins into mitochondria, suggesting that ASNA1/Get3 is indispensable for correct TA-protein localization (93).

**Structure of the yeast ASNA1 homolog Get3**

Several recent studies on Get3 have led to proposals for structural models for TA-protein insertion (99-104). The common five findings in these studies are as follows. First, Get3 exists as a homodimer. Second, a zinc ion between the monomers is coordinated by Cys residues that are essential for Get3 function (Fig. 8). Third, each monomer contains an ATPase domain and an alpha helical domain rich in Met-residues that bind TA-proteins. Hydrophobic TA-protein transmembrane domains are hidden in the cleft between the monomers. Fourth, a conformational change in the alpha-helical domain affect the ATPase domain, suggesting a functional link between these regions. Finally, they suggest that transition between a closed and an open dimer conformation is dependent of the state of the nucleotide bound to Get3.
Figure 8. Dimerization of *S. cerevisiae* Get3. (A) The molecular surface of monomer A is shown in silver and the drawing of monomer B is shown in gold. The dimer interface is located at the bottom of the picture. A zinc ion is shown in a blue sphere and two magnesium ions at the nucleotide-binding sites are shown in red. (B) The intermolecular zinc-finger motif. A zinc ion is bound by C285 and C288 from each monomer. C285, C288 and alpha helix 12 are labeled. The zinc ion is shown in blue. The figure is reproduced with permission from (103).
METHODS

Cell culture

Cells were grown in monolayer culture at 37°C in humidified air with 5% CO₂. Human melanoma T289 cells (105) and the cisplatin-resistant subline T289/DDP (106) were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1x HITES (10 nM hydrocortisone, 5 µg/ml insulin, 5 µg/ml transferrin, 10 nM beta-estradiol and 5 ng/ml selenite). The human ovarian cancer cell line 2008 (107) and the cisplatin resistant subline 2008/A (65) were grown in RPMI 1640 supplemented with 5% FBS. The medium for transfected cells containing a plasmid bearing a neomycin resistance gene was supplemented with 0.3 mg/ml G418. The medium for siRNA-transfected cells was supplemented with 1% penicillin streptomycin, PEST.

Plasmid construct and transfection of human cells

To obtain cells with altered ASNA1 expression, we made a plasmid construct for transfection of cells. The human ASNA1 cDNA was obtained by PCR using primers flanking the entire 1047 bp coding sequence with human liver cDNA as template. The PCR-product was separated in a 1% low melt agarose gel and the product purified in a spin column. The cDNA was inserted into the Eco R1 site of the mammalian expression vector pTarget in either sense or antisense direction and an empty vector was also ligated. The vectors contained a CMV-promoter and a G418 resistance gene. They were transformed into JM109 cells and the plasmids were prepared by a kit. The plasmids were cut by plasmid backbone specific BamHI and ASNA1 specific ClaI and separated through a 1% agarose gel to identify clones containing plasmids with sense or antisense ASNA1. The vectors were sequenced to confirm the orientation of the ASNA1 cDNA. T289 and 2008 cells were transfected with the plasmids using lipofectamine reagent. Cells were incubated in serum-free medium containing plasmid DNA and lipofectamine for 4-5 h at 37°C and then medium supplemented with FBS was added. The next day the medium was supplemented with 1 mg/ml G418 to select transfected cells and cells were seeded for clonal expansion. After 2-4 weeks, independent cell clones were randomly isolated and plated separately.
ASNA1 expression in the selected clones was determined by western blot analysis.

**siRNA constructs and transfection**

ASNA1 specific siRNA oligos of 21 nucleotides were obtained at Dharmacon siDesign center. An oligo was selected which targets 19 nucleotides starting at base pair 912 of the ASNA1 coding sequence (Genbank AF047469). A control-oligo was also used. T289 cells in medium supplemented with 1% penicillin streptomycin grew to 70% confluency in 6-well plates. The cells were incubated 4 h with oligofectamin, opti-mem and siRNA before addition of medium with FBS. Cells were transferred to 96-well plates for chemosensitivity assays and to a 6-well plate for western blot.

**Antibodies**

Polyclonal anti-ASNA1 antibodies were generated by immunizing rabbits with a recombinant ASNA1-GST fusion protein at Agrisera, Vännäs, Sweden. The specificity of the immune serum against ASNA1 was confirmed by ELISA and compared to pre serum. Both 6xHis-ASNA1 and ASNA1-GST fusion proteins were detected on ELISA. Immunoblotting of total cell lysates detected a stronger 41 kDa band and a weaker 37 kDa band. Western blot on recombinant ASNA1 detected only the 41 kDa band. Immunoprecipitation on total cell lysates also displayed ASNA1 on the 41 kDa level. Transfection of T289 cells with a plasmid construct containing the full-length ASNA1 resulted in overexpression of the 41 kDa band (Fig. 9). Polyclonal antibodies against *C. elegans* ASNA-1 was generated in parallel (25).

**Immunoblotting**

Exponentially growing cells were lysed in ice-cold buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton, 5 mM DTT, 0.1 mM PMSF and 1% protease inhibitor cocktail. The supernatant was collected after spinning in 14,000 rpm 20 min at 4°C. The total protein concentration of the lysates was determined using the DC Protein Assay (Biorad). C. *elegans* worms were lysed in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton and loading dye with DTT. Worm lysates were centrifuged
at 13600 rpm 1 min and boiled for 5 min. Lysate proteins were separated in 10% SDS-PAGE gels and electrotransferred to immobilon-p membranes. After blocking in 5% fat-free milk in 1x TBS, the membranes were incubated with a primary polyclonal rabbit anti-ASNA1 or a polyclonal rabbit anti-worm ASNA-1 antibody (1:2000, 1 h at room temperature). After washing, the membranes were incubated with a secondary horseradish peroxidase-conjugated donkey anti-rabbit antibody (Amersham Pharmacia Biotech). The antibodies were visualized by the chemiluminescent ECL-kit (Amersham Pharmacia Biotech). To confirm equal loading, membranes were stripped and then incubated with an anti-PCNA antibody, an anti-beta-actin antibody, or an anti-tubulin antibody, all detected as described above. Software Quantity One 4.6.5 was used to quantify detected bands on western blots.

Figure 9. Western blot demonstrating the 41 kDa ASNA1 protein. T289 cells and sublines transfected with an empty vector (emv) or plasmids containing sense-ASNA1 (S10 and S15) display the 41 kDa ASNA1. A weaker band at 37 kDa is not upregulated in S10 and S15. PCNA is used as a loading control.

Native gel electrophoresis

Worms were lysed by mechanical treatment in 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, and Protease inhibitor cocktail (Roche). Lysates were separated in a 7.5 % acrylamide gel under non denaturing conditions and then transferred to immobilon-p filters (Millipore). Detection was done as described above.

Cell growth and chemosensitivity assays

The MTT assay was used for cell growth and chemosensitivity assays (108, 109). Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
bromide) is reduced to purple formazan by living cells. Cells were counted in a Bürkel chamber and seeded on 96-well plates. Each sample was tested in triplicate wells. To determine a value representing cell number, cells were incubated with MTT (Sigma) for 4 h. After removal of the medium, the formazan dye crystals were dissolved in acidified isopropanol. The optical density was measured on an Elisa plate reader. Each experiment was performed at least three times.

To validate the method and obtain a relation between cell number and optical density, ovarian cancer 2008 cells in suspension were stained by trypan blue and counted in a Bürkel chamber. Cells were seeded on 96-well plates at concentrations ranging from 4000 to 128000 cells per well. Cells were allowed to anchor for 4 h prior to incubation with MTT. A linear regression for cell number versus optical density results in $R^2=0.997$ (Fig 10).

![Figure 10. MTT-values corresponds to number of cells. The optical density of dissolved formazan crystals after MTT exposure was directly proportional to the number of cells.](image)

To determine growth, cells were grown for 24, 48, 72 or 96 h prior to the MTT colorimetric growth assay. To assess chemosensitivity, cells were exposed to either sodium arsenite, zinc chloride, copper (II) sulphate, cisplatin, carboplatin or oxaliplatin. After 72 h incubation, cell number in each well was determined by the MTT assay. Arsenite toxicity was measured after 48 h. The concentration needed to reduce cellular population by 50% (IC$_{50}$) was determined.

To assess chemosensitivity in siRNA-transfected cells, cells were seeded on 96-well plates directly after transfection. After incubation for 48 h the cells
were exposed to cisplatin for 1 h. They were then washed in PBS and incubated in normal medium for additional 48 h before the MTT-assay.

**Cell death assay**

Apoptosis was detected by a TUNEL based in situ cell death detection kit for light microscopy (Roche). Cells were seeded on 2-well culture slides and grown 24 h. To evaluate apoptosis after cisplatin treatment, cells were exposed to 100 µg/ml cisplatin for 2 h prior to the TUNEL reaction. The positive control was treated with 0.5 mg/ml DNase. For fixation, slides were incubated 1 h with 3% paraformaldehyde and then 10 min in methanol and 0.3% H₂O₂. A 2 min incubation on ice with 0.1% triton and 0.1% sodium citrate preceded addition of the TUNEL reaction mixture and incubation 60 min at 37°C. The slides were then incubated 60 min with converter-POD (peroxidase conjugated antifluorescein antibody) followed by 10 min in DAB, a peroxidase substrate. The slides were mounted under glass coverslip and evaluated under light microscope.

**C. elegans strains and techniques**

Handling of *C. elegans* strains, injection RNAi and feeding RNAi was done as previously described (1, 3). Experiments were carried out at 20°C unless stated otherwise. N2 is the wild type parent for all strains in this study.

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). A *rnai* feeding construct for *ced-4* was obtained from a *C. elegans rnai* library. The full length *ced-3* cDNA was cloned into feeding RNAi plasmid L4440 as a Nhe1/Sac1 fragment (pVB507GK).

Mutant strains used in this study include *asna-1* (sv42) that was isolated from a deletion library (25). *asna-1(ok938), zk637.3 (gk367), cep-1(gk138), egl-1(n1084n3082), daf-2(e1370), daf-7(e1372), ced-9(n1653ts), ced-9(n1950gf), op1s110(Plim-::yfp-act-5) and bcl539(Plim-ced-1::gfp) were obtained from the *C. elegans* Genetics Center.

Transgenic strains expressing extrachromosomal human *ASNA1* or *asna-1*
under the *elt-2* the *daf-28* or the *osm-6* promoter have been described previously (25). A plasmid expressing *asna-1::gfp* was integrated into the genome (25).

Using mutagenic primers, we generated plasmids expressing *asna-1::gfp* carrying a deletion of the His164 codon (pVB402GK) or a change of codons 285 and 288 from cysteine to serine (pVB464GK). In both cases mutagenic primers were designed for both strands and were used 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 fragments thus obtained 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 to verify that the desired change was the only one in the gene. These were expressed extrachromosomally by coinjecting 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.

**C. elegans 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 agar plates containing 0-250 µg/ml zinc chloride; 0-200 µg/ml cadmium chloride; 0-50 mg/ml potassium antimonyl tartrate; 0-300 µg/ml sodium arsenite or 0-500 µg/ml cisplatin. 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. Higher concentrations of metal salts are required to test the effect of metal salts on adult worms. To avoid metal salt precipitates, adult worms were tested on MYOB agar. 2x MYOB medium (275 mg Tris-Cl, 120 mg Tris-OH, 1.55 g Bacto-petunone, 800 µl cholesterol 5 mg/ml, 1 g NaCl, 10 g agar and 250 ml H2O) was autoclaved. 6 ml 2x MYOB agar and 6 ml test solution was stirred in a petri dish on a heating block to obtain agar containing 0-500 µg/ml cisplatin, 0-600 µg/ml sodium arsenite, 0-1600 µg/ml zinc chloride or 0-1500 µg/ml copper sulphate. Adult animals were fed with OP50 strain of *E. coli*. Since bacteria do not grow on cisplatin plates, they were transferred from a NGM plate. 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 to 0-500 µg/ml cisplatin for 24 h in S-medium. They were then transferred to an agar plate where death was determined by absence of touch-provoked movement.

**C. elegans daf-2 and daf-7 experiments**

The *daf-2 (e1370)* mutant is a temperature sensitive loss-of-function mutant (110). Mutants grow reproductively at 15°C. 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 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. The strong temperature sensitive *daf-7 (e1372)* mutant (111) was used to reduce DAF-7 activity. The mutants grow reproductively at 15°C but become 100% dauer larvae at 25°C. *daf-7 (e1372)* mutants carrying extrachromosomal arrays expressing ASNA-1ΔH164 or ASNA-1C285S C288S were grown at 25°C. Dauer escape was evaluated after 96 h by determining the number of adult (non-dauer) worms on the plates.

**C. elegans microscopy**

For DAPI-staining, worms or dissected gonads were fixed in -20°C acetone and then incubated in 0.1 µg/ml DAPI in M9. For Syto-12 staining, worms were incubated with 50 µM Syto-12 in dH₂O with bacteria for 3-4 h at room temperature and then transferred to NGM agar plates to destain for 1 h. Worms were anesthetized by mounting them in M9 and levamisole. Worms were examined by microscopy within 10 minutes after mounting. Cisplatin influence on tissue morphology and embryonic apoptosis was determined using a 100x objective in a Leica DMRP microscope, and photographed with Deltapix DP450 software. Body volume was measured at 5x magnification (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**

To compare chemosensitivity between cell clones, the slope illustrating cell number versus concentration was created. The slope was transformed logarithmically and we calculated the linear regression. From the linear regression formula, the concentration needed to inhibit growth to 50% was determined (IC$_{50}$). To compare cell growth, doubling times were calculated from the linear regression of the logarithmically transformed slope illustrating cell number versus time. Three representative series were selected for each experiment. The relative IC$_{50}$-value or doubling time value for each cell clone was determined by relating to wild type or empty vector transfected cell clones. Results were expressed as mean±SEM. Statistical analysis was carried out by two-sided student’s t-test.

To compare nematode chemosensitivity, we determined the concentration that was lethal to 50% of the population (LC$_{50}$). LC$_{50}$-values and levels of statistical significances were determined using a binary logistic regression model. Each worm was coded in three columns representing concentration of test substance, worm category and status (dead or alive, 0 or 1). LC$_{50}$ is counted from:

$$\ln(p/(1-p)) = @ + \beta_1 x + \beta_2 D$$

When LC$_{50}$=x, p=0.5. That gives $\ln(p/(1-p))=\ln(1)=0$

$$0 = @ + \beta_1 x + \beta_2 D$$

$$x = (@ + \beta_2 D)/(-\beta_1)$$

D is worm category (1 or 0). The variables in the equation gives @, $\beta_1$ and $\beta_2$.

Chi-square tests calculated 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.
AIMS

Overall aim:

Discover a target to treat cisplatin resistant tumors and ultimately improve the possibility for patients to be cured of their cancer disease.

Specific aims:

1. Explore the influence of altered ASNA1 levels on human tumor cell growth and on sensitivity to arsenite and cisplatin.

2. Determine if lowered ASNA1 levels results in general sensitivity to platinum based drugs in a second human tumor cell line.

3. Develop a C. elegans model to study responses to cisplatin in vivo in different genetic backgrounds.

4. Determine whether C. elegans asna-1 mutants are sensitive to cisplatin and if this is secondary to apoptosis, retarded growth or lack of ASNA-1 promoted insulin signaling.

5. Determine if the process of apoptosis is abnormal in C. elegans asna-1 mutants.
RESULTS

Expression of ASNA1 in cisplatin resistant cells

ASNA1 expression was determined using western blots. The human melanoma cell line T289 had relatively high levels of ASNA1 compared to other cell lines used in our laboratory. The cisplatin-resistant subline T289/DDP displayed increased levels of ASNA1 compared to parental T289 (Fig 11A), suggesting that ASNA1 is upregulated in resistant cells. Cisplatin is frequently used against ovarian cancer and the ovarian carcinoma cell line 2008 is well established for cisplatin research. A subline of 2008, termed 2008/A, was previously obtained by selection in cisplatin. 2008/A is 17-fold more resistant to cisplatin compared to wild type and also resistant to antimonite and arsenite (65). The ASNA1 expression was measured to evaluate if ASNA1 is involved in the RASP-phenotype (Resistance to As, Sb and Pt) of 2008/A. These cells displayed 177±10% ASNA1 expression compared to wild type (p<0.05) (Fig. 11B), indicating that upregulation of ASNA1 could be a part of a resistance mechanism.

Generation of cell lines with altered ASNA1 expression

To investigate the phenotype in cells with altered ASNA1 levels we transfected T289 melanoma cells and 2008 ovarian carcinoma cells with either ASNA1 sense or ASNA1 antisense constructs. An empty vector construct was used as a control. ASNA1 expression in empty vector transfected T289 cells was 105 ± 6% of wild type expression, as determined by western blot. The ASNA1 sense construct aimed at increasing ASNA1 expression. After transfection of T289 with the sense construct, three out of 19 clones displayed a constitutive overexpression of ASNA1 compared to wild type T289. Two clones, s10 and s15, were chosen for further studies. Quantification of bands on western blots revealed that the ASNA1 overexpression in these clones were 186 ± 5% and 188 ± 9% compared to wild type (p<0.005 and p<0.05 respectively) (Fig. 11A). After transfection of ovarian cancer cell line 2008, two out of 24 clones, s18 and s23, exhibited obvious overexpression of ASNA1. S18 had the highest ASNA1 expression, 300 ± 6% of wild type (p<0.001) (Fig. 11B).
The ASNA1 antisense construct was set up to establish clones with decreased ASNA1 expression. After transfection of T289 cells, 32 independent clones were isolated. Four clones stopped growing and could not be used for further analysis. Eight clones grew slowly compared to wild type. Six clones displayed decreased ASNA1 protein levels on western blot. All of these belonged to the group of slowly growing clones. No surviving clone expressed less than 40% of parental T289 ASNA1 protein levels. Two clones, asD4 and asE2, with constitutive low levels of ASNA1 were chosen for further studies. These clones expressed 41 ± 8% and 45 ± 5% of wild type ASNA1 protein levels (p<0.05 and p<0.01 respectively) (Fig. 11A). When ASNA1 was examined by immunocytochemical and immunofluorescent staining, the decreased staining intensity of cells transfected with antisense ASNA1 correlated with the decreased ASNA1 levels detected by western blot. ASNA1 deficient cells were also larger compared to wild type.

![Figure 11. Cellular levels of ASNA1 determined by western blot.](image)

(A) Malignant melanoma cell line T289, the cisplatin resistant subline T289/DDP and T289 cells transfected with empty vector (emv), sense-ASNA1 (s10 and s15) and antisense-ASNA1 (asD4 and asE2). PCNA was used as a loading control. (B) Ovarian carcinoma cell line 2008, the cisplatin resistant subline 2008/A and 2008 cells transfected with empty vector (emv), sense-ASNA1 (s18) and antisense-ASNA1 (as4). T289 cells transfected with control siRNA (#siRNA) and ASNA1 specific siRNA. β-actin was used as a loading control.

After ASNA1 antisense transfection of 2008 cells, 32 clones were randomly isolated. Two clones died during clonal expansion and six clones grew slowly. Three clones, as4, as9 and as28, had decreased levels of ASNA1 protein. All three belonged to the slowly growing clones. As9 showed the lowest level of ASNA1 expression but grew too slowly to be compared to other clones with regard to chemosensitivity. As4 expressed 42 ± 4% of wild type ASNA1 protein levels (p<0.005) (Fig. 11B) while as28 displayed 71 ± 5% of wild type levels (p<0.05).
The ASNA1 expression was also reduced by transient transfection of T289 cells with a siRNA directed to the ASNA1 mRNA. Four ASNA1 specific oligonucleotides were constructed and all downregulated the ASNA1-expression by approximately 50%. SiRNA mediated downregulation of ASNA1 was detected from day 1 to day 5 after transfection. One oligonucleotide was chosen for further studies. Transfection of control siRNA into T289 did not result in altered ASNA1 protein levels on western blot (Fig. 11B).

**Retarded growth in ASNA1 downregulated cells.**

The ASNA1 downregulated cells grew slowly during clonal expansion. We wished to quantify this growth defect by determining the doubling time. For this, we applied the colorimetric MTT-assay. The doubling times of the T289 and 2008 clones with increased ASNA1-expression was similar to that of wild type and empty vector control cells. However, T289 asD4 and asE2 in which ASNA1 was downregulated grew significantly slower, as was observed also during the initial clonal expansion. ASNA1 deficient asD4 cells displayed 51±5% longer doubling time than wild type (p<0.01). Correspondingly, the doubling time for ovarian cancer 2008 antisense clone as4 was 40±5.5% longer than parental 2008 (p<0.05). The 2008 subline as28 with only a moderate downregulation of ASNA1 also grew slowly but to a less extent, displaying 28±2.3% longer doubling times compared to wild type (p<0.01). Finally, the 2008 subline with the lowest ASNA1 expression (as9) grew too slowly to be used in experiments. This suggests that ASNA1 is necessary for growth and that the level of downregulation corresponds to the severity of the growth defect.

**Increased chemosensitivity in ASNA1 downregulated cells.**

To test the hypothesis that ASNA1 is involved in cellular detoxification of arsenite and cisplatin, we compared chemosensitivity in human tumor cells with altered ASNA1 levels. The viability was determined by the MTT assay after a continuous 48 h exposure to sodium arsenite or 72 h exposure to cisplatin, carboplatin, oxaliplatin, copper sulphate or zinc chloride. For all examined metal salts, the concentrations needed to inhibit growth by 50% (IC50) were similar in ASNA1 overexpressing cells, empty vector transfected cells and wild type cells. Thus, ASNA1 overexpression alone is not sufficient to cause resistance although cisplatin resistant cells overexpress ASNA1.
Even though ASNA1 is not sufficient to cause resistance, it is necessary for resistance. The ASNA1 downregulated T289 cells were significantly more sensitive to sodium arsenite and cisplatin (Fig 12A-B). While the IC\textsubscript{50} for sodium arsenite in non-transfected T289 cells was 11.1 ± 1.0 µM, that for the antisense ASNA1 clone asD4 was 6.4 ± 0.3 µM (p<0.05). The IC\textsubscript{50} for cisplatin in wild type T289 cells was 2.2 ± 0.1 µg/ml compared to 1.1 ± 0.05 µg/ml for the asD4 cells (p<0.01). The second ASNA1 deficient T289 clone asE2 was also significantly hypersensitive to cisplatin and arsenite.

These results were confirmed in the other cell line, the ovarian carcinoma 2008. We observed increased sensitivity in ASNA1 deficient 2008 clone as4 where IC\textsubscript{50} for arsenite was 72 ± 6% of the wild type value (p<0.05) and the IC\textsubscript{50} for cisplatin was 63 ± 3% of parental 2008 (p<0.01). The ASNA1 downregulated as4 cell line was also hypersensitive to the second and third generation platinum drugs carboplatin and oxaliplatin (Fig. 12C), displaying IC\textsubscript{50}-values reaching 45 ± 5% and 60 ± 3% of wild type IC\textsubscript{50} to the respective drug (p<0.01 for both observations). Thus, downregulation of ASNA1 results in increased sensitivity to all three chemotherapeutic platinum compounds and arsenite. The ASNA1 antisense clone as28 exhibited only a moderate decrease in ASNA1 expression. Nevertheless we observed a statistically significant increase in sensitivity to arsenite, cisplatin and carboplatin in as28 compared to parental 2008 or empty vector transfected cells.

Chemosensitivity to cisplatin was also evaluated after transient siRNA mediated knockdown of ASNA1 in T289 cells. We exposed cells to high concentrations of cisplatin during 1 hour and let them grow for 48 h without cisplatin before test of viability by the MTT assay. Transient ASNA1 suppression significantly increased the cisplatin sensitivity. The IC\textsubscript{50} for cisplatin was 24 ± 3 µg/ml in the ASNA1 siRNA treated cells compared to 37 ± 5 µg/ml in parental T289 (p<0.005) and 32 ± 4 µg/ml in the siRNA control T289 cells (p<0.05). This supports that our findings in cells transfected with antisense ASNA1 are ASNA1 specific and not due to off-target effects.

There was no difference in sensitivity to zinc chloride or copper sulphate between ASNA1 deficient and wild type cells, indicating that downregulation of ASNA1 results in a substrate specific phenotype rather than generally sensitive cells.
Figure 12. Chemosensitivity in cells with altered expression of ASNA1. Error bars represent SEM. Wild type (T289 or 2008) is compared to empty vector transfected cells (emv), ASNA1 underexpressing antisense clones (as) and ASNA1 overexpressing sense clones (s). (A) Cisplatin sensitivity after 72h exposure. (B) Arsenite sensitivity after 48h exposure. (C) Sensitivity to carboplatin and oxaliplatin after 72h exposure.
Increased apoptosis in ASNA1 downregulated cells.

Clones underexpressing ASNA1 displayed increased chemosensitivity and retarded growth. A TUNEL-assay was conducted to determine whether these cells underwent apoptosis more frequently than wild type cells. 6.3 ± 0.7% of T289 wild type cells demonstrated apoptosis after 24 h on a culture slide. Empty vector control and ASNA1 overproducing s10 and s15 cell lines showed similar extent of apoptosis while ASNA1 underexpressing clones (asD4 and asE2) exhibited increased apoptosis (15.7 ± 0.9%, p<0.01 and 17.0 ± 1.2%, p=0.073 respectively). After exposure to 100 µg/ml cisplatin, a moderate increase in apoptosis was observed in T289 wild type cells (9.7 ± 1.2%, p=0.12 compared to non treated cells), empty vector control and the ASNA1 overexpressing clones. A significantly increased apoptosis fraction was observed in the ASNA1 downregulated clones asD4 and asE2 (38.7 ± 2.6% and 40.7 ± 0.9%, p<0.05 compared to non treated cells). ASNA1 downregulated cell lines exposed to cisplatin also displayed significantly more apoptosis than cisplatin treated wild type cells (p<0.05). In conclusion, ASNA1 deficient cells are more prone to undergo apoptosis, especially after cisplatin treatment, and this could at least in part explain the retarded population growth and the increased chemosensitivity in these cells.

ASNA1 mediated cisplatin resistance is conserved from worms to humans

Knock-down of ASNA1 in human cell lines results in retarded growth, increased apoptosis, increased chemosensitivity (112, 113) and decreased insulin secretion (25). The severe growth phenotype of ASNA1 downregulated human tumor cells complicates further studies in cell cultures. ASNA1 cannot be knocked-down under 40% of wild type levels of ASNA1, thus we cannot establish cell lines to study phenotypes in complete absence of ASNA1. To understand if ASNA1 mediated cisplatin resistance is a consequence of the other aspects of ASNA1 function, we wished to determine whether the modulation of the cisplatin phenotype by ASNA1 is conserved in another model system. In the nematode C. elegans, animals can be studied in complete absence of the ASNA1 homolog ASNA-1 in vivo.

asna-1(RNAi) treated animals arrest in the first larval stage L1 due to lack of ASNA-1 promoted insulin signaling (25). These larvae were tested for chemoresistance along with arrested wild type larvae of the same age. L1 larvae were obtained by putting embryos on plates without food. In agreement with the RASP-phenotype seen in human cell lines (64, 65)
(resistance to As, Sb, Pt), ASNA-1 depleted larvae were hypersensitive to cisplatin, sodium arsenite and antimony potassium tartrate when compared to wild type larvae (Table 1). No difference was observed in sensitivity to cadmium chloride or zinc chloride, showing that the ASNA-1 dependent resistance is substrate specific and similar between nematodes and humans.

Table 1. Chemosensitivity in asna-1(rnai) worms. L1 larvae on plates without food were exposed to various concentrations of cisplatin, arsenite, antimonite or zinc for 24 h, or to cadmium chloride for 72 h. Data are collected from three experiments. LC50 represents the lethal concentration for 50% of the population. *** indicates p<0.001.

<table>
<thead>
<tr>
<th>Metal salt</th>
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<tr>
<td></td>
<td>LC50 (mg/ml)</td>
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<tr>
<td>Cisplatin</td>
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<tr>
<td>Sodium arsenite</td>
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<td>175</td>
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<tr>
<td>Antimony potassium tartrate</td>
<td>38.9</td>
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<td>Zinc chloride</td>
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<td>Cadmium chloride</td>
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Two asna-1 mutant alleles are available, ok938 (25, 81) and sv42 (25). asna-1 mutants are bred from heterozygous mothers since homozygous animals are sterile. The heterozygous mother deposits asna-1 gene product to the progeny and asna-1 mutants can thereby bypass the L1 arrest seen in asna-1 (rnai) animals and grow to become thin sterile adult worms. This allowed us to study chemoresistance in adult worms on plates with food. Wild type worms and asna-1 heterozygotes were intrinsically resistant to 500µg/ml cisplatin, which is the highest possible cisplatin concentration in agar. By contrast, young adult asna-1(ok938) and asna-1(sv42) mutants were cisplatin sensitive (Fig. 13A-B). The concentration of cisplatin that was lethal to 50% of the population (LC50) was 251 µg/ml and 235 µg/ml respectively (p<0.001 compared to wild type for both observations). Consistent with a previous report (81) and experiments in cell lines and C. elegans larvae, asna-1 mutants were also hypersensitive to arsenite (Fig. 13D) with a LC50 of 111 µg/ml compared to 279 µg/ml in wild type (p<0.001). The LC50 of copper in asna-1 mutants (500 µg/ml) was significantly lower than that of wild-type animals (849 µg/ml, p<0.001) (Fig. 13F). This is of particular interest since cisplatin shares resistance pattern and plasma membrane transporters with copper (56, 61, 114).
Figure 13. Chemosensitivity in adult C. elegans nematodes after 24h exposure. Wild type nematodes (black boxes) are compared to asna-1 mutants (open circles). Error bars represent SEM. (A) asna-1 (ok938) mutant sensitivity to cisplatin. (B) asna-1 (sx42) mutant sensitivity to cisplatin. (C) asna-1 (ok938) mutant sensitivity to cisplatin in solution. (D) asna-1 (ok938) mutant sensitivity to sodium arsenite. (E) asna-1 (ok938) mutant sensitivity to zinc chloride. (F) asna-1 (ok938) mutant sensitivity to copper sulphate.

To test if asna-1 mutants display increased chemosensitivity because of an inability to sense and avoid chemicals we determined the pharyngeal pumping rate, avoidance behavior and sensitivity to cisplatin in solution. asna-1 mutants on cisplatin agar had decreased pharyngeal pumping rate compared to wild type, excluding the possibility that increased oral intake of cisplatin is the reason for hypersensitivity. Avoidance behavior was
scored on plates with cisplatin agar on one side and control agar on the other. Avoidance to cisplatin was observed neither in wild type nor in \textit{asna-1} mutants after one hour. Finally, when worms were exposed to cisplatin in solution where avoidance is impossible, \textit{asna-1} mutants were still more sensitive to cisplatin ($LC_{50}$ 229 µg/ml) than wild type ($LC_{50}$ >500 µg/ml, \( p<0.001 \)) (Fig 13C). The exposure of \textit{asna-1} mutants to cisplatin in agar or in solution results in similar $LC_{50}$ values, suggesting that these two methods can substitute one another.

There was no difference in sensitivity to zinc chloride between the mutants and the controls (Fig. 13E) and this further emphasizes that \textit{asna-1} mutants display a substrate specific phenotype rather than general metal sensitivity.

We have shown that cisplatin resistant human tumor cells overexpress ASNA1. They are generated by serial exposure to cisplatin. To determine if ASNA-1 expression is induced by cisplatin in \textit{C. elegans}, we exposed worms to 100 or 200 µg/ml cisplatin in agar plates for 48 h. Worms exposed to 200µg/ml cisplatin displayed a 2.8±0.35 fold increase in ASNA-1 protein expression by western blot analysis (\( p<0.05 \)) (Fig 14A).

![Figure 14](image)

**Figure 14.** (A) Levels of ASNA1 in worm lysates after 48h cisplatin exposure is determined by western blotting. \( \alpha \)-tubulin was used as a loading control. (B) Mean survival ± SEM after 24h exposure to 300µg/ml cisplatin. \textit{asna-1} mutants are compared to \textit{asna-1} mutants expressing either \textit{C. elegans} ASNA-1 or human ASNA1.

To determine whether \textit{asna-1} mutants are hypersensitive to cisplatin due to lack of ASNA-1 and not due to non-specific mutations, we expressed \textit{asna-1} under its own promoter in \textit{asna-1} mutants. This construct fully rescued the \textit{asna-1} mutant cisplatin phenotype (Fig. 14B). Furthermore, to determine whether human ASNA1 protects cells from cisplatin toxicity by the same mechanism as worm ASNA-1, we expressed human ASNA1 under the \textit{asna-
promoter in worms. This construct also resulted in significant rescue of the \textit{asna-1} mutant resistance (Fig. 14B). This shows that ASNA1 mediates resistance by a conserved mechanism and validates \textit{C. elegans} as a model to study ASNA-1 and cisplatin resistance.

\textbf{asna-1 mutants are cisplatin sensitive in absence of apoptosis}

As noted, downregulation of ASNA1 results in increased apoptosis in two human cell lines. Cisplatin induces apoptosis in tumor cells (34) but cisplatin resistant cells often carry mutations in p53 or upregulate Bcl-2, resulting in a higher threshold for apoptosis (36). To test if the \textit{asna-1} mutant cisplatin phenotype is a consequence of ASNA-1 influence on apoptosis, we blocked the apoptosis signaling pathway in \textit{C. elegans} and evaluated resistance. We created a double mutant between \textit{asna-1} and \textit{cep-1/p53} which is involved in DNA-damage induced apoptosis both in humans and nematodes (115). \textit{asna-1;cep-1} double mutants were not more resistant than \textit{asna-1} alone (Fig. 15A), as would have been expected if \textit{asna-1} mutant hypersensitivity was dependent on \textit{cep-1}. Instead, the double mutants were slightly more sensitive to cisplatin (p<0.05), although \textit{cep-1} mutants were not more sensitive than wild type. This suggests that blockage of ASNA1 in human tumor cells might increase cisplatin sensitivity even in cells that are cisplatin resistant due to mutations in p53.

Downstream of CEP-1, the BH-3-only homolog EGL-1 promotes apoptosis by blocking CED-9/Bcl-2 (18). \textit{egl-1} mutants lack all somatic apoptosis (116). An \textit{egl-1;asna-1} double mutant was not more resistant than \textit{asna-1} (Fig. 15B), again demonstrating that apoptosis is not required for \textit{asna-1} mutant chemosensitivity. Finally, the caspase homolog CED-3 (117, 118) was downregulated by \textit{rna{i}}. This eliminated germline apoptosis, showing that \textit{rna{i}} was effective. \textit{asna-1} mutants did not become more resistant when apoptosis was blocked by \textit{ced-3 (rnai)} (Fig. 15C).

Both wild type and \textit{asna-1} mutants showed signs of necrosis, most obviously in the head region (Fig. 15D). We conclude that apoptosis is not involved in the increased cisplatin sensitivity seen in adult \textit{asna-1} mutant worms.
Figure 15. (A-C) 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. (A) Chemosensitivity of worms carrying mutations in asna-1 and the p53-homolog cep-1. (B) Chemosensitivity of worms carrying mutations in asna-1 and the BH3-only homolog egl-1 (C) Chemosensitivity of asna-1 mutants exposed to ced-3 (rnai) or control (rnai) (L4440). CED-3 is the worm homolog of caspases. (D) Vacuoles as a sign of necrosis in N2 and an asna-1 mutant worm after 4h exposure to 300µg/ml cisplatin.

**C. elegans asna-1 mutants display increased apoptosis**

*C. elegans asna-1* mutants are hypersensitive to cisplatin in absence of apoptosis while tumor cells display increased levels of cisplatin induced apoptosis when ASNA1 levels are reduced. If ASNA-1 only mediated a transport mechanism for cisplatin, ASNA-1 deficient cisplatin treated cells could undergo necrosis or apoptosis depending on their genetic background. Alternatively, ASNA-1 could promote cisplatin resistance by affecting several pathways, possibly by regulating diverse tail-anchored proteins. The *C. elegans* germline was used as a model to study ASNA-1 influence on apoptosis.

Oocytes that are homozygous for the *asna-1* mutation will still contain some ASNA-1 from heterozygous germ cells because the cells in the gonads share a common cytoplasm. *asna-1* mutants can escape from the L1 arrest due to
this phenomenon and this indicates that ASNA-1 is expressed in *C. elegans* germ cells.

![Figure 16](image)

**Figure 16.** The germline of a *C. elegans* hermaphrodite. An adult worm is stained with DAPI to visualize cell nuclei. (A) The anterior gonad arm showed at 20x magnification. The scale bar indicates 100 µm. Germ cell proliferation starts at the distal tip (DT) of the gonad. Cell death can be seen where cells are in the pachytene stage of meiosis (P). At the gonad loop (GL), the germline turns 180° and oocyte development continues in the proximal gonad on the ventral side of the worm towards the vulva (V). (B) The distal part of the gonad is shown at 100x magnification. The scale bar indicates 25 µm. Cells are in mitosis at the most distal part to the right and in the transition zone of meiosis to the left. (C) Germ cells in the pachytene stage of meiosis are shown at 100x magnification. The scale bar indicates 25 µm.

To study apoptosis in the germline, we first characterized the germline in *asna-1* mutants. Worms were stained with DAPI to visualize the germ cell nuclei. Each wild type gonad contains a distal zone with proliferating mitotic cells, a transition zone and a proximal zone with germ cells in meiosis (Fig. 16). DAPI-stained sperm are seen as dots in the spermatheca. In *asna-1* mutants, the germline is underproliferated and contains few cells in mitosis (Fig. 17) and no sperm. Somatic expression of *asna-1* in *asna-1* mutants rescues ASNA-1 promoted insulin signaling (25), *asna-1* mutant growth defects and ASNA-1 dependent metalloid resistance (119). However, somatic expression of *asna-1* fails to rescue the germline phenotype of *asna-1(ok938)*
mutants (Fig. 17), showing that germ cell expression of asna-1 is required for germline homeostasis.

Figure 17. Germ cell nuclei from adult C. elegans hermaphrodites are stained with DAPI and observed at 100x magnification. Scale bars indicate 25 µm. (A) The distal part of the germline in wild type with cells in mitosis. (B) The distal part of the germline in an asna-1 (ok938) mutant. Cells in the pachytene stage of meiosis are seen. (C) The distal part of the germline in an asna-1 (ok938) mutant with somatic expression of asna-1. (D) Wild type germ cells close to the gonad loop region are in the pachytene stage of meiosis. (E) asna-1 (ok938) mutant germ cells close to the gonad loop region. (F) Germ cells close to the gonad loop region in asna-1 (ok938) mutants with somatic expression of asna-1.

Germline proliferation is controlled by the somatic distal tip cell and the Notch signaling pathway (120, 121). In addition, insulin signaling stimulates germline proliferation in C. elegans larvae (122). Since ASNA-1 promotes insulin signaling, this could explain the underproliferated germline. Two observations suggest that other aspects of ASNA-1 mediate the germline phenotype of asna-1 mutants. First, somatic expression of asna-1 rescues insulin signaling but not the germline phenotype. Second, worms mutant for the insulin receptor homolog daf-2 display reduced larval germline proliferation while no defects are seen in adults (122). In wild type worms, the number of germ cells increases between the last larval stage L4 and adulthood while half of the germ cell population is lost during the same period in asna-1 mutants. asna-1 mutants with somatic expression of asna-1 and a rescued insulin signaling pathway also lose germ cells after the last larval stage.
To examine if loss of *asna-1* mutant germ cells is due to increased apoptosis we expressed *ced-1:gfp* (*bcIs39*) in *asna-1* (*sv42*) mutants. CED-1 is expressed in somatic sheath cells that surround the germline in the gonad (123). CED-1:GFP is visualized during an early phase of engulfment of apoptotic corpses in the germline. *asna-1* (*sv42*) mutants expressed 5.0±0.28 (n=20) germ cells corpses per gonad compared to 2.2±0.20 (n=20) in wild type (p<0.001) (Fig. 18). Expression of CED-1:GFP was specific for apoptosis since the expression disappeared in a *ced-3(rnai)* background.

**Figure 18.** Germ cell apoptosis is visualized by CED-1:GFP. Gonads from young adult worms are observed at 100x magnification. (A) Wild type worm carrying a transgene (*bcIs39*) expressing *ced-1:gfp*. (B) *asna-1* (*sv42*) mutant expressing *ced-1:gfp*.

*actin:yfp* (*opIs110*) is another marker for engulfment (124) and was crossed into *asna-1* (*sv42*) mutants. *asna-1* (*sv42*) mutants were positive for 5.6±0.27 (n=45) corpses compared to 1.6±0.15 (n=20) in *sv42/ht2g* that are heterozygous for the *asna-1* mutation (p<0.001). In a *ced-3(rnai)* background, *asna-1* mutant actin:YFP expression decreased to 0.58±0.12 (n=55) corpses per gonad (p<0.001).

SYTO-12 stains corpses after engulfment (124). *asna-1(ok938)* mutants are negative for SYTO-12, indicating that the engulfment process is defective in *asna-1* mutants. An engulfment defect can not explain the underproliferative and sterile phenotypes of *asna-1* mutants (124). In addition, when inhibiting apoptosis in *asna-1* mutants by *ced-3(rnai)*, these worms display an increased density of germ cell nuclei. Thus, blockage of ASNA1 results in increased chemosensitivity both in absence of apoptosis and by altering the threshold for apoptosis.
**ASNA-1 is not required for CED-9 function**

We speculated that ASNA-1 might interact with the tail-anchored Bcl-2 homolog CED-9 to inhibit apoptosis and this hypothesis was tested in *C. elegans*. First, we checked for phenotype enhancement of a temperature sensitive (ts) *ced-9* allele. At 25°C, *ced-9* (*n1653ts*) mutants are sterile and they have a germline phenotype similar to *asna-1* with increased apoptosis and few cells in mitosis but the corpses are positive for SYTO-12. At 15°C and 20°C, *ced-9* (*n1653ts*) are healthy but after feeding *rnai* against *asna-1*, their progeny become sterile, slowly growing and lack oocytes. These phenotypes were not seen in wild type worms exposed to feeding *asna-1(rnai)*. Thus, *asna-1(rnai)* enhances the *ced-9(ts)* phenotype at lower temperatures.

To explore if Ced-9/Bcl-2 requires ASNA-1 to inhibit apoptosis, we evaluated somatic apoptosis in a *ced-9* gain of function (gf) allele. Age synchronous embryos at the age of 280-340 min after fertilization were examined by Nomarski microscopy and the apoptotic corpses were counted. In wild type, a medium of 8.9 corpses (n=12) was counted per embryo (Fig. 19). *ced-9* (*n1950gf*) embryos (treated with control *rnai*) displayed a medium of 0.29 cells in apoptosis (n=17). Neither feeding *rnai* or injection *rnai* against *asna-1* could increase the number of apoptotic corpses in *ced-9* (*n1950gf*) embryos. Thus, ASNA-1 is not required for CED-9 function.

![Figure 19. A *C. elegans* embryo at the comma stage is shown at 100x magnification. The scale bar indicates 25 µm. Arrows indicate apoptotic corpses.](image-url)
Identification of essential ASNA-1 residues for chemoresistance

The prokaryotic ASNA-1 homologue ArsA contains three metal binding residues (77). One of these (His148) is conserved in eukaryotic ASNA-1 and corresponds to C. elegans ASNA-1 residue His164 (Fig. 20). We deleted the asna-1 codon for His164 and expressed ASNA-1^{His164}:GFP in transgenic animals by extrachromosomal arrays. We identified a line in which the ASNA-1^{His164}:GFP expression level was equal to wild type levels of ASNA-1. ASNA-1^{His164}:GFP is functional since it rescues the growth phenotype of asna-1 mutants as much as the wild type transgene (Fig. 21D-E). However, these worms were still cisplatin sensitive (Fig. 21A), identifying His164 as a target in ASNA-1 to increase cisplatin sensitivity.

Figure 20. Comparison of amino acid sequences of human ASNA1, C. elegans ASNA-1 and S. cerevisiae Get3. The dashes indicate the gaps introduced to maximize sequence alignment. (*) indicates conserved residues. (c) and (l) indicate high and low similarity respectively. C. elegans His164, Cys285 and Cys288 are boxed. The walker A motif is indicated by (w) and encodes the binding site for ATP. The signal transduction domains switch 1 and switch 2 are indicated by (1) and (2) respectively. Hydrophobic residues involved in tail-anchor binding in yeast are indicated by (H).
The yeast ASNA-1 homolog Get3 forms a homodimer that is linked by Cys285 and Cys288. These residues are essential for Get3 dimerization and function (82, 101). We changed ASNA-1 Cys285 and Cys288 to serines and selected a transgenic strain with expression of ASNA-1\textsuperscript{C285S C288S}:GFP equal to wild type expression of ASNA-1. In contrast to yeast Get3 without these cysteines, ASNA-1\textsuperscript{C285S C288S}:GFP is functional since it rescues the growth phenotype of asna-1 mutants (Fig. 21D-E).

Figure 21. Identification of essential residues in ASNA-1. (A) asna-1 mutants expressing asna-1 carrying a deletion in the codon for His\textsuperscript{164} (asna-1\textsuperscript{ΔH}) are sensitive to 24h exposure of 300µg/ml cisplatin. (B) asna-1 mutants expressing asna-1 without Cys285 and Cys288 (asna-1\textsuperscript{CSCS}) are sensitive to 24h exposure of 300µg/ml cisplatin. (C) A western blot of a native gel of worm lysates show two bands for ASNA-1 and two bands for ASNA-1:GFP. asna-1 mutants expressing asna-1\textsuperscript{CSCS}:gfp also display two ASNA-1 bands on the native blot (lane 4). (D) asna-1 mutant growth defect (D3) is compared to wild type (D1), asna-1 heterozygotes (D2) and asna-1 mutants expressing asna-1:gfp (D4). Expression of asna-1\textsuperscript{ΔH}:gfp (D5) or asna-1\textsuperscript{CSCS}:gfp (D6) results in rescue of the growth defect in asna-1 mutants. Pictures from microscopy at 5x magnification and scale bars indicate 100 µm. (E) Standardized body volume ± SEM relative to the medium of wild type (N2). The body volume of asna-1 mutants is 19% of wild type. asna-1 mutants expressing ASNA-1, ASNA-1\textsuperscript{ΔH}, or ASNA-1\textsuperscript{C285S C288S} have a significantly larger body volume measuring 37%, 37% and 49% of wild type volume respectively.

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Western blot of native proteins revealed two bands when ASNA-1C285S C288S-GFP was probed with an anti-ASNA-1 antibody (Fig. 21C), presumably marking a monomer and dimer of ASNA-1C285S C288S-GFP. This suggests that ASNA-1 forms a more stable homodimer than Get3, independent of Cys285 and Cys288. However, asna-1 mutants expressing ASNA-1C285S C288S were still sensitive to cisplatin (Fig. 21B). This identifies ASNA-1 Cys285 and Cys288 as another possible target for increased metal drug sensitivity.

Separable roles for ASNA-1 in chemoresistance and insulin signaling

Expression of asna-1ΔH164 and asna-1C285S C288S rescued the asna-1 mutant growth phenotype (Fig. 21D-E). This indicated that these transgenes are able to restore insulin signaling. To confirm this, we performed the dauer escape assay. Worms remain in the dauer stage permanently if they lack daf-7/TGFβ activity. An increased activity in the insulin pathway can compensate for blockage of the daf-7 signaling pathway and results in exit of the daf-7 mutant from the dauer stage. Overexpression of some insulins or wild-type ASNA-1 allows daf-7 dauer to exit the dauer stage and become adults (25). To test if ASNA-1C285S C288S or ASNA-1ΔH164 promote insulin signaling, we expressed these proteins in the daf-7(e1372) mutant background. We observed that 23 % of daf-7(e1372) dauer expressing the asna-1C285S C288S transgene (n=103) and 26 % of daf-7 dauer expressing the asna-1ΔH164 transgene (n=105) exited the dauer state. Hence, these transgenes are capable of enhancing insulin signaling while they are unable to promote cisplatin resistance.

C. elegans animals that are mutant for either the insulin/IGF receptor homologue daf-2 (125) or asna-1 have greatly decreased insulin signaling (25). If the cisplatin hypersensitivity of asna-1 mutants was due to decreased insulin signaling, daf-2 (e1370) mutants would also be sensitive. However, no death was scored among the daf-2 mutants exposed to cisplatin (Fig. 22A). These results are consistent with our chemosensitivity data from starved larvae which lack insulin signaling (126). Also here, wild type larvae were more resistant than asna-1 (RNAi) treated larvae. Finally, daf-2 mutants are resistant to copper (127) while we have shown that asna-1 mutants are hypersensitive.

To understand in which tissues ASNA-1 is required to promote chemoresistance, we expressed asna-1 under tissue specific promoters in asna-1 mutants. This also became another test for separability between the
insulin and cisplatin phenotype of ASNA-1. The asna-1 promoter drives 
asna-1 expression in head neurons and in the intestine and the intestine is 
considered the most important organ for detoxification in worms (128). 
asna-1 expression under an intestinal (Pelt-2) promoter rescued the resistance 
in a significant manner (Fig. 22B). This means that intestinal expression of 
ASNA-1 is sufficient for resistance. When asna-1 expression was limited to 
head neurons (under the Posm-6 promoter), animals were cisplatin sensitive 
(Fig. 22C). In contrast, asna-1 expression in head neurons is sufficient to 
rescue insulin signaling in asna-1 mutants (25), again separating ASNA-1 
promoted chemoresistance from insulin signaling. In conclusion, blockage of 
insulin signaling does not result in increased cisplatin sensitivity and 
restoration of insulin signaling in asna-1 mutants does not rescue resistance. 
This shows that the chemoresistance phenotype of ASNA-1 is separable from 
the insulin phenotype.

Figure 22. 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. (A) daf-2/insulin IGF receptor (e1370) 
mutants were more resistant than both asna-1 mutants and N2 on cisplatin agar. (B) asna-1 was expressed 
under an intestinal (Pelt-2) or an insulin (Pdaf-28) specific promoter in an asna-1 mutant background. Worms 
expressing Pelt2:asna-1 or Pdaf-28:asna-1 were more resistant to cisplatin compared to asna-1(sv42) mutants. 
(C) asna-1 expression under a head neuronal (Posm-6) specific promoter in an asna-1 mutant background did 
ot rescue the cisplatin hypersensitivity phenotype.
DISCUSSION

Perspectives on platinum based drugs and ASNA1

After three decades in the clinic, platinum based drugs still gain importance in the treatment of cancer patients. Oxaliplatin treatment for colorectal cancer, the third most common cancer disease in both men and women, has revived the interest in platinum based drugs. Cisplatin, carboplatin and oxaliplatin are continuously tested in new regimens against cancer and new platinum based substances are in the pipeline for clinical approval (33).

Cisplatin regimens provide cure for patients suffering from metastatic testicular cancer (34). To obtain the same beneficial response in other tumor diseases, we must circumvent resistance. The multifactorial nature of cisplatin resistance (36) challenges us to target an essential resistance mechanism to resensitize cancer cells. In this study, we describe ASNA1 as a novel resistance factor for platinum based drugs and arsenite.

Several observations suggest that ASNA1 is an important resistance factor. First, ASNA1 is highly conserved during evolution, showing that ASNA1 has been important for survival (73). The biological history of ASNA1 goes back to archaean bacteria (85) and ASNA1 could have gained several roles in cellular protection against metalloids during the long presence in evolution. Second, ASNA1 promotes membrane insertion of tail-anchored proteins which are involved in diverse important cellular functions (90). This indicates that ASNA1 regulates cellular homeostasis on multiple levels. Third, our results indicate that ASNA1 deficiency both results in increased apoptosis per se and in increased cisplatin sensitivity in absence of apoptosis. This suggests that ASNA1 acts in at least two pathways to protect cells against cisplatin. Translated to the clinical situation, this implies that blockage of ASNA1 would sensitize cancer cells to cisplatin, regardless whether they carry or acquire mutations in the apoptosis signaling pathway or not.

Downregulation of ASNA1 results in cisplatin hypersensitivity that was of the same magnitude in T289 melanoma and 2008 ovarian carcinoma cells. This hypersensitivity, is measured as a 2-fold decrease in IC50 and previous reports state that clinically significant resistance to cisplatin in vivo is associated with only 1.5-3.0-fold changes in sensitivity to cisplatin when measured in vitro (52, 129). asna-1 mutant nematodes were hypersensitive
to copper and the ASNA-1 homolog in yeast is regulated by copper (82, 83). However, no copper sensitivity phenotype was detected in human cells with altered levels of ASNA1. Cells selected or transfected for copper resistance are reported to exhibit larger changes in cisplatin sensitivity than copper sensitivity (58), indicating that it can be difficult to detect a minor change in copper sensitivity.

Cisplatin resistant cells overexpress ASNA1 and cisplatin induces ASNA-1 expression in C. elegans. However, overexpression of ASNA1 alone is not sufficient to cause resistance. This suggests that ASNA1 acts in association with other proteins to promote resistance. To gain resistance, several subunits in this stipulated resistance mechanism must be upregulated. Since downregulation of ASNA1 results in increased sensitivity, ASNA1 is necessary for resistance and constitutes an essential subunit in the mechanism where it is working.

C. elegans as a model system for cisplatin research

Cisplatin has a broad cellular toxicity that affects multiple pathways in the cell (36). Model systems are required to study how cisplatin affects the tumor cell. We present Caenorhabditis elegans as a multicellular model organism to study platinum based drugs. To explore the function of a drug, C. elegans can fill the gap between in vitro studies in cells and in vivo experiments in mammalian model organisms (130, 131). Experiments in C. elegans allowed us to separate different functions of ASNA1. Downregulation of ASNA1 in human tumor cells affects growth, chemoresistance, insulin signaling and apoptosis (25). Our experiments in C. elegans demonstrate that the chemoresistance phenotype is separate from the other aspects of ASNA1. In C. elegans, it was possible to study phenotypes in complete absence of ASNA-1 in vivo. This is much more complicated in mammalian systems since ASNA-1 knock-out is lethal to mouse embryos (132) and ASNA1 knockdown results in retarded growth in human cell lines.

Gene expression is readily up- or downregulated in C. elegans, making this an excellent model system to study signaling pathways and phenotypes in a specific genetic background (5). On the basis of our studies, we believe that C. elegans can facilitate prediction of tumor responses to cisplatin in different genetic backgrounds. In addition, platinum based drugs are often combined with a targeted drug that blocks a specific pathway and C. elegans can help us identify which pathways to block to increase the tumor response in the clinic. Clinical studies have shown 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 (133). This underscores the importance of a useful model to study interactions between genetic factors, targeted drugs and platinum based chemotherapy.

In this study, we use nematodes that are either adult or arrested in the first larval stage. When these worms are exposed to cisplatin, the somatic cells are postmitotic. This allowed us to separate the growth phenotype from the chemoresistance phenotype of ASNA1. The *C. elegans* germline consist of proliferating cells that can undergo apoptosis either physiologically or as a consequence of DNA damage (15). However, the ASNA1 germline phenotype was distinct from wild type, complicating studies of cisplatin sensitivity of *asna-1* mutant germ cells. Instead, we used the germline to start deciphering ASNA1 involvement in apoptosis signaling. *C. elegans* is an established model organism to study apoptosis (16). The transparent nematode allows studies of individual cells *in vivo*. Expression of GFP linked to proteins expressed during apoptosis ameliorates identification and quantification of apoptosis. The *C. elegans* germline could be a useful model to study the genetics behind cisplatin induced apoptosis.

Cisplatin is neurotoxic and nephrotoxic and is thereby affecting postmitotic cells also in human patients (43). Ototoxicity and peripheral neuropathy are common side effects to cisplatin treatment (34). We studied pharyngeal pumping rates to determine whether *asna-1* mutant worms ingested more than wild type on cisplatin agar. Cisplatin treated *asna-1* mutant worms displayed a lower pump rate than wild type and at a level seen when neuronal signaling is abolished (David Raizen, personal communication). This suggests that *asna-1* is required for neuronal protection during cisplatin treatment and that the pharyngeal pumping assay (131) can be used to study the neurotoxic effects of cisplatin.

*C. elegans* is a suitable platform to test if ASNA-1 targeting of tail-anchored proteins is necessary for chemoresistance. If TA-protein binding is required for ASNA-1 promoted resistance, knock-down of TA-proteins in *C. elegans* can reveal pathways mediating this resistance. To further explore cisplatin function and resistance in the *C. elegans* model, several screening processes are available (130).
ASNA1, a conserved resistance mechanism

The hypothesis behind this study is built on two observations. First, cisplatin resistant tumors are crossresistant to arsenite and antimonite and this is associated with decreased accumulation (the RASP-phenotype) (64, 65). Second, ASNA1 is the human homolog to the ATPase ArsA which mediates efflux of arsenite and antimonite in bacteria (73). Thus, we asked whether ASNA1 promotes resistance to cisplatin and if ASNA1 knockdown results in increased sensitivity. We show, both from a functional and a structural perspective, that information about ASNA1 homologs can be used to characterize the protein. Our results from human cells and C. elegans demonstrate that ASNA1 activity is essential for resistance to platinum based drugs and to the ArsA substrates arsenite and antimonite. This is consistent with the idea that ASNA1 is part of a conserved mechanism mediating the RASP-phenotype. Bacterial ArsA-mediated efflux of metalloids is dependent of ArsB, a transmembrane protein (76). Eukaryotic cells have no known ArsB-homolog and even though eukaryotic ArsA-homologs promotes metalloid resistance (80, 81), it has not been tested if they act by stimulating metalloid efflux.

During this study, we have learned that ASNA1 promotes membrane integration of tail-anchored proteins (90), a diverse group of proteins involved in several essential functions in the cell. A recent report compared the amino acid sequences of archael, bacterial and eukaryotic homologs of ASNA1 and found that residues involved in TA-protein binding are found in archael but not in bacterial ancestors to ASNA1 (85). This indicates that we might have inherited ASNA1 from archa where ArsA both stimulates arsenite efflux through ArsB and has the structural capacity to bind TA-proteins.

ASNA1 and the yeast homolog GET3 are involved in resistance to arsenite and antimonite (80, 81). GET3 activity is regulated by copper (82, 83) and ASNA-1 is stimulated by arsenite (81). This is interesting since both copper and arsenite share resistance patterns with cisplatin (64, 65, 114). How ASNA-1 and GET3 are regulated by metalloids remains to be elucidated since arsenite binding cystein residues in ArsA are absent in eukaryotic homologs (77, 78). Nevertheless, we have shown that worms expressing ASNA-1 without a conserved arsenite-binding histidine residue are hypersensitive to cisplatin. This illustrates how structural data from ASNA1 homologs can be used to identify targets to specifically block one function of ASNA1. It also demonstrates the benefit of C. elegans as model for functional studies in vivo. Applying the same principle, we showed that a pair of ASNA-1 cystein residues is required for cisplatin resistance. These residues, Cys285 and
Cys288, corresponds to cysteins involved in dimerization of the yeast homolog GET3 (101). Cys285 and Cys288 from each half of the homodimer coordinate a zinc ion. Copper and cisplatin binds to CXXC-motifs (134) (like Cys285, Cys288) and it is tempting to speculate that ASNA-1 function is mimicked by metalloid interaction with this site.

The ultimate evidence for conservation of ASNA1 function is rescue of asna-1 mutant cisplatin resistance by expression of human ASNA1. This finding validates *C. elegans* as a model to study ASNA1 and shows that ASNA1 promotes cisplatin resistance by a conserved mechanism.

**Possible ASNA1 interacting partners for cisplatin resistance**

ASNA1 acts in multiple cellular pathways (25, 90). This enables a stipulated ASNA1 targeting drug to increase cisplatin sensitivity by several means and raises hope that ASNA1 could be the essential target to overcome multifactorial cisplatin resistance. However, ASNA1 is required for growth and ASNA-1 knock-out mice die as embryos (132). To block ASNA1 dependent resistance without severe side effects, we must understand how ASNA1 promotes resistance.

Studies of the yeast homolog Get3 suggests that ASNA1 binding of TA-proteins is dependent on certain hydrophobic residues in ASNA1 (96). *C. elegans* animals expressing ASNA-1 without these residues could be studied to evaluate if the cisplatin phenotype of ASNA-1 is secondary to targeting of tail-anchored proteins.

Cisplatin toxicity in asna-1 mutant worms is independent of cisplatin effects on DNA replication because somatic cells are postmitotic in the adult nematode. Cisplatin also inhibits transcription and induces oxidative and ER stress in the cell (40, 135). Several TA-proteins are involved in oxidative and ER stress (89). For example, ASNA1 regulates ER stress by integration of the tail-anchored stress-associated endoplasmatic reticulum protein 1 (SERP1) into the ER (91).

Determination of cisplatin accumulation in ASNA1 deficient cells will explain if ASNA1, as the bacterial homolog ArsA, promotes resistance by efflux of toxic metals. Studying cisplatin accumulation in eukaryotic cells, it is important to also study the subcellular localization of platinum. Overexpression of the copper efflux pump ATP7A results in cisplatin resistance and increased total cell accumulation because cisplatin is
sequestered in the vesicular fraction (61). It is also shown that cisplatin is exported from the cell through vesicles (62). Stimulation of vesicular efflux could possibly be mediated by ASNA1 through membrane insertion of tail-anchored SNARE-proteins.

**Concluding remarks and future perspectives**

ASNA1 is a conserved cisplatin resistance factor and targeting of ASNA1 or its interacting partners could resensitize cisplatin resistant tumors. To reach the ultimate goal to improve cancer treatment, the essential ASNA1 interacting partners should be identified and this will require further studies in model organisms. To go from bench to bedside, drug targets in ASNA1 dependent pathways have to be found.

The hypothesis behind this study is built on observations in tumor cells and bacteria. We have tested our hypothesis in model systems and our conclusion is based on empirical data. A possible short-track to find drug targets to increase cisplatin sensitivity is to perform a screen. High throughput screening for genes or small molecules that increase cisplatin sensitivity is facilitated by our establishment of *C. elegans* as a model for cisplatin research (130). Nevertheless, validation of hits in a screening must be performed. In this study, we show how *C. elegans* can be used to characterize a target to increase cisplatin sensitivity.

Metal based treatments will be continuously important in cancer treatment (136). Cisplatin was found in studies of bacteria (31, 32) and arsenite treatment for acute promyelocytic leukemia originates from Chinese traditional medicine (66). Both cisplatin and arsenite were developed as cancer drugs by researchers who observed biological phenomena and translated that to established cancer medicine by testing of scientific hypothesis. To find the next generation of cancer drugs we must use our model systems and an open mind to translate our observations to improved cancer medicine.
CONCLUSIONS

1. Downregulation of ASNA1 inhibits growth of human tumor cell lines and results in increased sensitivity to cisplatin, carboplatin, oxaliplatin and arsenite.

2. ASNA1 is upregulated in cisplatin resistant cells and in cisplatin treated nematodes but overexpression of ASNA1 is not sufficient to cause resistance.

3. *C. elegans* is a suitable model organism to characterize cisplatin resistance mechanisms in relation to signaling pathways and specific genetic backgrounds.

4. ASNA1 is a conserved cisplatin resistance factor and *C. elegans asna-1* mutants are hypersensitive to cisplatin independent of ASNA-1 influence on growth, apoptosis or insulin signaling.

5. Apoptosis is increased in ASNA1 deficient human tumor cells *in vitro* and *C. elegans asna-1* mutant germlines contain an increased number of apoptotic cells *in vivo*. 


I det första delarbetet visar vi att ett minskat ASNA1 uttryck i tumörceller från malignt melanom leder till en ökad känslighet för cisplatin och arsenik. Dessa celler genomgår oftare programmerad celldöd (apoptos) jämfört med celler med ett normalt ASNA1 uttryck. Vi visar också att cisplatinbehandlade celler som blivit motståndskraftiga mot cisplatin uttrycker mer ASNA1 än obehandlade celler.

I det andra delarbetet beskriver vi att ett minskat ASNA1 uttryck ger en ökad cisplatinlönsamhet i tumörceller från äggstockscancer. Cisplatin baseras på platina och det finns två andra platina-cellgifter, carboplatin och oxaliplatin. Vi visar att ASNA1 även reglerar sammansättningen för dessa cellgifter.

När vi minskar ASNA1 uttrycket minskar också tumörcellernas förmåga att föröka sig genom celldelning. Sedan tidigare vet vi att ASNA1 också reglerar insöndring av insulin. För att studera om celler med minskat ASNA1 uttryck är cisplatinlönsamma på grund av tillväxthämning, insulinpåverkan eller apoptos, utvecklade vi en metod för att studera cisplatin i C. elegans.
C. elegans är en 1 mm lång genomskinlig rundmask som enkelt kan undersökas i mikroskop. C. elegans arvsmassa är helt kartlagd och ungefär 60 procent av människans gener har en besläktad gen i masken. Den stora fördelen med C. elegans är att det är relativt enkelt att förändra uttrycket av gener. Det gör att flera viktiga signalvägar som styr till exempel apoptos, först beskrevs i C. elegans.

I det tredje delarbetet visar vi att maskar som saknar ASNA-1 är känsliga för cisplatin medan vanliga maskar istället är mycket motståndskraftiga. Skillnaden kvarstår i larver som slutat växa vilket visar att denna känslighet inte har med tillväxt att göra. När signalvägar för apoptos slås ut kvarstår också skillnaden i cisplatinkänslighet. Det talar för att blockering av ASNA1 kan vara effektivt även i tumörCELLER som har mutationer i gener som styr apoptos. När vi uttrycker humant ASNA1 i maskar utan eget ASNA-1 så blir de motståndskraftiga mot cisplatin. Det visar att människans ASNA1 fungerar på samma sätt som maskens ASNA-1. Vi identifierar också tre aminosyror i ASNA-1 som krävs för cisplatinresistens men inte för insöndring av insulin. Slutligen visar vi att ASNA-1 måste uttryckas i maskens tarm för att ge cisplatinresistens medan det räcker med uttryck i nervceller för att ASNA-1 ska reglera insulin. Därmed kan vi skilja ASNA-1:s funktion avseende cisplatinresistens från dess funktioner avseende insulin, tillväxt och apoptos.

Trots att apoptos inte krävs för att maskar utan ASNA1 ska vara mer känsliga för cisplatin så har vi sett att celler med låga nivåer av ASNA1 ofta genomgår apoptos. ASNA1 styr hur en stor grupp av proteiner (tail-anchored proteins) binder till membran i cellen. I denna grupp finns Bcl-2 som hämmar apoptos. I delarbete fyra studerar vi apoptos i C. elegans förplantningsorgan, gonaden. Maskar utan ASNA-1 har ett ökat antal celler i apoptos i gonaden. Våra data talar dock emot att ASNA-1 skulle reglera funktionen av proteinet CED-9 som är maskens motsvarighet till Bcl-2.

Vår slutsats är att studier i C. elegans ger oss mycket information om hur individuella gener och signalvägar påverkar känsligheten för cisplatin. När cisplatin kombineras med nya målinriktade mediciner är det viktigt att kunna förutse effekten av dessa och här kan studier i C. elegans spela en viktig roll. ASNA1 bidrar till cisplatinresistens och blockering av ASNA1 ger en ökad känslighet för platina baserade cellgifter som cisplatin, vilket i förlängningen kan förbättra prognosen vid behandling av cancer.
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