Stannin, a protein that localizes to the mitochondria, sensitizes NIH-3T3 cells to trimethyltin and dimethyltin toxicity

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Running Title: Selective Toxicity of Organotins

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ABSTRACT

Stannin (Snn) is a highly conserved, 88-amino acid protein which may mediate the selective toxicity of organotins. Snn is localized in tissues with known sensitivity to trimethyltin (TMT), including the central nervous system, immune system, spleen, kidney and lung. Cells in culture that do not express Snn show considerable resistance to TMT toxicity. *In vitro*, Snn peptide can bind TMT in a 1:1 ratio, and can de-alkylate TMT to dimethyltin (DMT). We now show that transfection with Snn sensitized TMT-resistant NIH-3T3 mouse fibroblasts to both TMT and DMT cytotoxicity. Triple label confocal microscopy of Snn-transfected cells and Percoll gradient purification of mitochondria showed Snn localized to the mitochondria and other membrane structures. The mitochondrial localization of Snn coupled with its ability to bind and de-alkylate organotin compounds indicate a possible mechanism by which selective alkyltin toxicity may be mediated.
INTRODUCTION

Alkyltins have been used as heat stabilizers in polyvinylchloride tubing, curing agents for rubber production, disinfectants in hospitals, and as biocides (Aschner and Aschner, 1992; Snoeij et al., 1987). Toxicity from accidental exposure to alkyltins has lead to considerable research characterizing their selective toxicity in mammals (Boyer, 1989; Ross et al., 1981; Snoeij et al., 1987).

Trimethyltin (TMT) is a potent toxicant that selectively kills cells in the central nervous system (CNS), immune system, spleen, lung and kidney (Brown et al., 1979; Philbert et al., 2000; Snoeij et al., 1985). The ‘TMT Syndrome’ had been used to characterize the phenotype observed following intoxication in rodents; abnormalities following intoxication include spontaneous seizures, self-mutilation, vocalizations, whole body tremors, and hyper-reactivity to touch (Dyer et al., 1982). Human exposure results in pathology closely resembling that of other mammals (Feldman et al., 1993; Ross et al., 1981). TMT intoxication resulted in phenotypic changes indicative of apoptotic cell death, which include chromatin condensation, nuclear fragmentation, mitochondrial dysfunction, reactive oxygen species (ROS) production, membrane blebbing, and caspase activation (Brown et al., 1979; Geloso et al., 2002; Jenkins and Barone, 2004; LeBel et al., 1990; Stine et al., 1988). Within the CNS, TMT selectively destroyed neurons located in the neocortex, amygdala, and olfactory tubercle, but its most striking effects were in the hippocampal formation (Balaban et al., 1988; Brown et al., 1979; Thompson et al., 1996).

Subtractive hybridization of TMT-treated versus untreated control rat brain was used to isolate the stannin (Snn) gene product. (Krady et al., 1990). In situ hybridization of adult rat brain for Snn mRNA demonstrated that Snn was expressed in the hippocampal formation, neocortex, and other limbic structures, correlating with areas sensitive to TMT (Dejneka et al.,...
Northern blots of rat tissue mRNA revealed that Snn was expressed in spleen, brain, kidney, and lung, but was below detection levels in liver, heart, skeletal muscle or testes (Dejneka et al., 1997). Examination of TMT toxicity in cultured cells showed that cells expressing Snn were considerably more sensitive to TMT (Thompson et al., 1996). Conversely, pre-incubation with antisense oligonucleotides directed against Snn mRNA protected primary neurons in culture from TMT toxicity (Thompson et al., 1996).

Molecular characterization of Snn predicted an 88 amino acid protein expressed only in vertebrates (Toggas et al., 1992). Furthermore, this protein exhibits a high level of conservation with rat, mouse and human Snn differing by only two amino acids in the carboxy-terminus. Lower vertebrates such as frog (*Xenopus Laevis*) and Zebrafish (*Danis Rerio*) share 89% and 91% similarity with the human protein sequence, respectively. Structural analysis using several algorithms predicts an amino-terminal transmembrane domain followed by a hydrophilic loop (Dejneka et al., 1997). The carboxy terminal domain appears to be a membrane-associated random coil (Veglia, et al, 2003).

Direct association of Snn with alkyltins has been shown using a peptide corresponding to amino acids 29-37 of Snn (Buck et al., 2003). These nine amino acids located within the predicted cytoplasmic loop, contain two cysteines (amino acids 32 and 34), forming a vicinal thiol. Vicinal thiols are implicated in heavy metal binding, suggesting a possible binding domain for TMT. Electrospray ionization mass spectrometry of the Snn-peptide in the presence of tri-substituted alkyltins showed that Snn bound to, and de-alkylated TMT to dimethyltin (DMT). Snn remained in association with DMT in a [1:1] ratio after the methyl group was removed (Buck et al., 2003).
Although TMT toxicity has been studied in depth, molecular characterization of DMT toxicity and its intracellular targets are not as well characterized. DMT is used as a heat stabilizer for polyvinylchloride tubing and may contaminate drinking water supplies (Braman and Tompkins, 1979; Sadiki and Williams, 1996). In rats, DMT can be absorbed through the digestive tract and passed through the placenta to the pup where it accumulates in the fetal blood and brain (Noland et al., 1983). DMT is less toxic \textit{in vitro} and \textit{in vivo} when compared to tri-substituted organotins (Komulainen and Bondy, 1987; Mushak et al., 1982); this reduced toxicity may be due to its lower hydrophobicity, and inability to readily cross the plasma membrane. TMT exposure can result in mitochondrial dysfunction and increased reactive oxygen species, though its specific target(s) remain unclear (LeBel et al., 1990; Stine et al., 1988).

We now demonstrate that Snn is a mitochondrial target for TMT and DMT toxicity. Subcellular localization of Snn in mitochondria and other organelles indicates a possible common link to alkyltin toxicity. Furthermore, we show that Snn transfection into NIH-3T3 fibroblasts sensitized these cells to the toxic effects of TMT and to a lesser extent, DMT.
MATERIALS AND METHODS

Materials. The empty vector pcDNA3.1, T4 DNA ligase, Xho-I and HinDIII endonucleases were obtained from Invitrogen/GibcoBRL (Carlsbad, CA). Subcloning efficiency DH5-α E. Coli were purchased from Stratagene (La Jolla, CA). Cell culture reagents were obtained from Invitrogen/GibcoBRL; including 0.25% Trypsin, EDTA, and antibiotic/antimitotic mixture and high glucose Dulbecco’s Modified Eagle medium (DMEM) containing sodium pyruvate and pyridoxine hydrochloride. Lipofectamine2000 and Opti-MEM I containing HEPES buffer, sodium bicarbonate and L-glutamine were also purchased from Invitrogen/GibcoBRL. Trimethyltin chloride and dimethyltin dichloride were a gift from Dr. James O’Callahan, NIOSH/CDC (Morgantown, WV).

Antibodies. Mouse anti-FLAG (M2 monoclonal) and rabbit anti-FLAG (polyclonal) were purchased from Sigma (St. Louis, MO). Rabbit anti-active caspase-3 and rabbit anti-caspase-9 were purchased from Cell Signaling Technology (Beverly, MA). Mouse anti-α-spectrin was obtained from Chemicon (Temecula, CA), and rabbit anti-Elk-1, goat anti-GRP-75, goat anti-GRP-78, HRP-conjugated goat anti-mouse, HRP-conjugated goat anti-rabbit, and HRP-conjugated rabbit anti-goat were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). FITC-conjugated donkey anti-goat and Texas Red-conjugated donkey anti-rabbit were purchased from Jackson Immunoresearch Laboratories (West Grove, PA).

Subcloning. The Snn open reading frame, with the addition of the amino-terminal FLAG-tag epitope, was subcloned into pcDNA3.1 using the full Snn cDNA sequence in pBluescript (Dejneka et al., 1998) as a template. PCR primers were constructed containing the FLAG-tag epitope sequence added to Snn’s amino terminal (N-terminal primer: GGG CTC GAG ATG GAC TAC TCT ATT ATG GAC TAC AAG GAC TAC AAG GAC GAC GAC GAC AAG ATG TCT ATT ATG GAC...
CAC AGC; C-terminal primer: GGG AAG CTT TCA GCC ATG CAC TTC CGG G). The pcDNA3.1 vector and the Snn-FLAG insert were digested using Xho1 and HinDIII restriction endonucleases. Snn-FLAG was ligated into pcDNA3.1 using T4 DNA ligase, and subsequently transformed into DH5α E-coli. Transformed DH5α were plated onto agar plates using ampicillin (50 µg/mL) as a selection marker. Plates were incubated at 37°C for 12 hours. Colonies were transferred to LB media containing ampicillin at 50 µg/mL. Plasmid DNA for Snn-FLAG-pcDNA3.1 was extracted (plasmid miniprep kit, QIAGEN, Valencia, CA), and positive clones were sequence verified. GFP-pcDNA3.1 was constructed with a carboxy terminal FLAG-tag using the same method and restriction sites (N-terminal primer: GGG AAG CTT ACC ATG GTG AGC AAG GGC G; C-terminal primer: GGG CTC GAG TCA CTT GTC GTC GTC GTC CTT GTA GTC CTT GTA CAG CTC GTC CAT GC).

Cell culture. NIH-3T3 mouse fibroblasts (American Type Tissue Collection, Rockville, MD) were cultured in high glucose DMEM with 10% heat-inactivated Cool Calf-2 Serum (Sigma), with antibiotic/antimitotic mixture. For subculturing, cells were plated into 75mm² poly-D-lysine-coated flasks (Corning, Corning, NY), and maintained at 37°C with 5% CO₂.

Transient Transfection. NIH-3T3 cells were plated at 3.0x10⁵ cells per 25 mm² flask and allowed to grow for 24 hours at 37°C. Six µL of Lipofectamine2000 and 4.8µg of either: Snn-FLAG-pcDNA3.1, GFP-FLAG-pcDNA3.1, or vehicle was incubated with 200 µL of OptiMEM for 20 minutes at 24°C. This mixture was then added to the cells and incubated for a total of 24 hours. Average transfection efficiency was measured to be 25-30%, calculated by the expression of GFP after 24 hours. Pictures were taken using the Nikon Eclipse TE2000-S microscope and a FITC filter at 100x magnification. The number of FITC-fluorescent cells was counted as a function of the total cells within the observed field.
Immunofluorescent laser-scanning confocal microscopy. Cells were transfected with Snn-FLAG-pcDNA3.1 and allowed to incubate at 37°C for 24 hours. Cells were washed three times with phosphate buffered saline (PBS). Cells were fixed with 4.0% paraformaldehyde for 5 minutes, washed with PBS 3 times, then permeabilized with 0.1% Triton-X100 in PBS for 2 minutes. The rabbit polyclonal anti-FLAG, and goat polyclonal anti-GRP-75 antibodies were then added (1:200 dilution), and incubated at 24°C for 1 hour. Cells were washed three times with PBS and then incubated for 20 minutes with normal donkey serum (1:50). The Texas Red-conjugated donkey anti-rabbit and FITC-conjugated donkey anti-goat antibodies were added (1:250 dilution) and incubated at room temperature for one hour. Nuclear staining using 4’,6-diamidino-2-phenylindole, dihydrochloride (DAPI) was performed following immunocytochemistry. Cells were washed with PBS 3 times, and incubated with 3.0 µM DAPI stain for 5 minutes. Cells were rinsed 3x with PBS and mounted with Gel-Mount mounting media (Fisher, Pittsburgh, PA). Immunofluorescence was visualized using the Leica DMI RE2 laser-scanning confocal microscope in the Penn State Confocal Microscopy Facility. Triple emission micrographs were taken; DAPI, FITC and Texas Red excitation was accomplished using 405 nm, 488 nm and 514 nm lasers, and emission was measured at 420-480 nm, 500-580 nm, and 610-670 nm, respectively. All pictures were taken using a 10x eyepiece, 63x objective, and either 2.5x or 4x fine focus zoom to achieve 1600x and 2500x magnification, respectively.

Subcellular fractionation by differential centrifugation. NIH-3T3 cells were plated at 1.5x10^6 cells per 75 mm² flask and incubated at 37°C for 24 hours. Cells were then transfected using methods described above. A total of 14.4µg of DNA and 18.0 µL of Lipofectamine2000 were used per 75 mm² flask. After a 24 hour incubation, cells were trypsinized and resuspended in 500 µL homogenization buffer (20 mM Hapes-NaOH, pH 7.2, 0.25 M sucrose, 1.0 mM MgCl₂, 1.0
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mM EDTA, and mammalian protease inhibitor cocktail [1:50; Sigma]) Cells were incubated for 5 minutes on ice and sonicated three times for 5 seconds each. Cells were centrifuged at 1000 rpm (3000g) in an Eppendorf tabletop microfuge; the pellet was labeled (P1). The supernatant was transferred to a new tube and centrifuged at 20,000 rpm (~33,000g) using a TLA100.3 rotor in a Beckman Ultracentrifuge; this pellet was labeled (P2). The supernatant was transferred to a new tube and centrifuged at 55,000 rpm (~100,000g), using same rotor and ultracentrifuge; this pellet was labeled (P3) and the supernatant labeled (S3). Pellets were then resuspended in lysis buffer containing: 20 mM Tris (pH 7.5), 150 mM NaCl, 1.0 mM EDTA, 1.0 mM EGTA, 1.0% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na3VO4, and [1:50] mammalian protease inhibitor cocktail. Ten µg of total protein was used for Western blot analysis.

**Percoll gradient purification of mitochondria.** NIH-3T3 cells were plated at 1.5x10^6 cells per 75 mm² flask and incubated at 37°C for 24 hours. Cells were transfected as described above. Cells were then incubated for 24 hours, trypsinized and resuspended in 500 µL homogenization buffer (10 mM Hepes-NaOH, pH 7.5, 0.25 M sucrose, 1.0 mM dithiothrietol, and [1:50] mammalian protease inhibitor cocktail). Cells were incubated on ice for 10 minutes and homogenized by 30 strokes with a Teflon Dounce homogenizer. The mixture was then centrifuged for 10 minutes at 1000 rpm (3000g) in an Eppendorf tabletop microfuge. The supernatant was transferred to a new tube and centrifuged for 10 minutes at 14,000 rpm (27,000g). The (P2) pellet was then resuspended in 500 µL of mitochondrial isolation buffer (0.25 M mannitol, 25 mM Hepes-NaOH, pH 7.5, 0.5 mM EGTA, and [1:50] mammalian protease inhibitor cocktail). This mixture was loaded on top of 7 mL of mitochondrial isolation buffer containing 30% Percoll (Sigma). The mixture was then centrifuged for 30 minutes at
27,000 rpm (95,000g; 4°C) using an SW-40Ti rotor in a Beckman Ultracentrifuge. Fractions taken include (1) the top 1.5 mL of Percoll gradient, (2) the dense band approximately 40% through the Percoll gradient, and (3) the membrane collected at the bottom of the Percoll gradient (resuspended in 100 µL of lysis buffer). Twenty-five µL of total lysate was used for Western blot analysis.

**SDS-PAGE and Western blot analysis.** Following transient transfection, cells were trypsinized (0.25% trypsin; GibcoBRL) and resuspended in lysis buffer (described above). The BCA protein assay was performed to obtain protein concentrations. An equal amount of protein was added to Nu-PAGE LDS sample buffer containing laurel dodecyl sulfate (LDS) and β-mercaptoethanol. Samples were denatured at 37°C for 10 minutes. Proteins were separated on Nu-PAGE precast polyacrylamide gels (SDS-PAGE; Invitrogen) and transferred to nitrocellulose membrane (BioRAD, Hercules, CA). Nitrocellulose membranes were incubated at room temperature for 15 minutes in blocking solution containing tris-buffered saline (TBS), 0.1% polyoxyethylene (20) sorbitan monolaurate (Tween-20, EM Science, Gibbstown, NJ) and 5% non-fat dry milk. Membranes were incubated with primary antibody (1:1000 dilution in blocking solution) overnight at 4°C, followed by a 15-minute wash in TBS with 0.1% Tween-20 (TBS-T). Membranes were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000 dilution) at room temperature for one hour in blocking solution, then washed in TBS-T for one hour. Membranes were visualized using enhanced chemiluminescence reagent (ECL; Amersham Pharmacia, Piscataway, NJ), and exposed to ECL film (Amersham Pharmacia), and developed using an X-ray developer.

**Cytotoxicity Measurement.** Cells were plated into 12-well plates at a concentration of 3×10^4 cells per well and incubated at 37°C for 24 hours. One µL of Lipofectamine2000 and either 1.0
µg of GFP-FLAG-pcDNA3.1, Snn-FLAG-pcDNA3.1, or vehicle were incubated at 24°C for 20 minutes in 50 µL OptiMEM. This mixture was added to the cells and allowed to incubate at 37°C for 20 hours. TMT (either 0.2 or 1.0 µM), or DMT (either 1.0 or 5.0 µM) was then added. Cells were incubated at 37°C for 24 hours. Media was aspirated and cells were trypsinized and resuspended in 1.0 mL of media. Cells (100 µL) were added to 10 µL of 0.4% trypan blue. Viable and non-viable cells were then counted using a hemocytometer.
RESULTS

**Snn-FLAG expression in NIH-3T3 mouse fibroblasts.** Snn was engineered with an N-terminal FLAG epitope and subcloned into the pcDNA3.1 vector. The FLAG-tag antibodies were then used to detect Snn-FLAG expression. Green fluorescent protein (GFP) was subcloned into the pcDNA3.1 vector with the addition of the FLAG-tag. The GFP-FLAG-pcDNA3.1 construct was used as an expression control on Western blot to verify relative levels Snn expression in NIH-3T3 cells (Figure 1A). Given the transfection efficiencies of 25-30%, the extent of sensitization in these experiments may be masked, in part, by the presence of non-transfected cells. Laser-scanning confocal microscopy of Snn-FLAG-pcDNA3.1 and GFP-FLAG-pcDNA3.1 transfected NIH-3T3 cells indicate the relative levels of expression of both constructs (Figure 1B).

**Snn-FLAG localizes to the mitochondria.** We initially performed subcellular fractionation using differential centrifugation to determine the organelle(s) to which Snn localizes. Characterization of the content of each of the fractions was based on protocol described by (Graham, 1982). Western blots were performed to analyze the presence of Snn in the different fractions. Only a minimal amount of Snn-FLAG immunoreactivity was found in the P1 fraction (nuclei and heavy mitochondria), indicating that Snn is most likely not found in the nucleus or nuclear membrane (Figure 2). Furthermore, only a small amount of Snn-FLAG immunoreactivity was found in the cytosolic (S3) fraction, which may represent Snn in membrane that was not removed from the cytosol during the centrifugation process. Presence of Snn in the S3 fraction may also represent protein being trafficked to some membrane structure. A majority of Snn was found in the P2 (mitochondria, endoplasmic reticulum, peroxisomes) and P3 fractions (total membranes), at all timepoints tested (20 and 24 hour timepoints shown).
Differences between the amount of Snn in the P2 or P3 fractions over a range of timepoints was tested. No significant changes were observed in the amount of Snn present in the P2 or P3 fractions at any timepoint tested (data not shown).

To determine if Snn specifically localized to the mitochondria, further purification of the P2 pellet was performed. First, to maintain a higher level of mitochondrial integrity, acquisition of the P2 pellet was modified through the use of differentially-prepared homogenization and Percoll gradient buffers, and plasma membrane disruption through the use of a Dounce homogenizer. Through this method, approximately 75% of cells were disrupted, as verified through trypan blue inclusion. After the separation of the P2 fraction, a self-resolving Percoll gradient was used to separate intact mitochondria from endoplasmic reticulum and peroxisomes based on organelle density. Western blot examination of three of the Percoll fractions revealed the presence of Snn in the second fraction, indicated by anti-FLAG immunoreactivity (Figure 3A). The presence of mitochondria in the second fraction was confirmed by immunoreactivity of glucose regulatory protein-75 (GRP-75), also known as mitochondrial-specific heat shock protein-70 (HSP-70).

Verification of mitochondrial purity in the second Percoll gradient fraction was accomplished by screening for the presence of protein markers for other organelles that may contaminate the mitochondrial fraction (Figure 3B). Minimal contamination was noted from nucleus (anti-Elk-1), endoplasmic reticulum (anti-GRP-78), or cytoskeleton (anti-α-spectrin). This indicates that Snn-FLAG immunoreactivity in the second Percoll gradient fraction represented the presence of Snn in mitochondria. Quantitation of the presence of each organelle marker was performed. The average pixel density found in total cell lysate was compared to the pixel density observed in the purified mitochondria (Figure 3C).
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Immunofluorescent laser scanning confocal microscopy was performed to visualize the co-localization of Snn and the mitochondrial protein GRP-75 (Figure 4). Snn-FLAG-pcDNA3.1 transfected cells can be visualized adjacent to non-transfected cells. All cells within the selected fields show immunofluorescence for GRP-75 (green fluorophore) and positive DAPI staining (blue fluorophore). The Snn-FLAG-pcDNA3.1 transfected cells show immunofluorescence for Snn (red fluorophore). The co-localization of Snn and GRP-75 is indicated by the presence of yellow overlay. The presence of a pool of Snn that does not co-localize with GRP-75 most likely represents Snn that localizes to other intracellular compartments. This is consistent with the presence of Snn in several fractions in the differential centrifugation experiments.

**Stannin over-expression sensitizes NIH-3T3 cells to TMT and DMT toxicity.** NIH-3T3 fibroblasts are resistant to TMT-induced toxicity; furthermore, they express very low levels of Snn, compared to other immortalized cell lines (Thompson et al., 1996). Thus, NIH-3T3 cells were chosen as a model to test conferrence of TMT sensitivity by Snn over-expression. After treatment with 0.2 µM TMT for 24 hours, significantly increased trypan blue inclusion was noted in Snn-FLAG-pcDNA3.1 transfected cells, compared to GFP-FLAG-pcDNA3.1 transfected and non-transfected control cells (Figure 5). No differences between groups were revealed in the 1.0 µM TMT-treated conditions.

Recent research has shown that Snn may directly dealkylate TMT and retain association with the subsequent product, DMT (Buck et al., 2003). This led us to examine Snn’s possible role in mediating DMT toxicity. Initial screening of high and low Snn expressing cells (HEK-293 and NIH-3T3 cells, respectively) revealed that NIH-3T3 cells exhibited resistance to DMT-induced cytotoxicity, as compared to HEK-293 cells (data not shown). Transfection of NIH-3T3 cells with Snn-FLAG-pcDNA3.1 conveyed significant sensitization to cytotoxicity at both 1.0
and 5.0 µM of DMT (Figure 6). Conversely, transfection of GFP-FLAG-pcDNA3.1 did not sensitize cells to DMT-induced toxicity, when compared with non-transfected controls. This indicates that Snn over-expression can confer increased sensitivity to DMT-induced, as well as to TMT-induced cytotoxicity.

**Snn overexpression does not increase caspase activation following TMT and DMT treatment.** TMT administration elicits cellular responses that are indicative of both necrotic and apoptotic cell death. To determine if the increased cytotoxicity was mediated by caspases, we analyzed the cellular content of activated caspase-9 and -3 following TMT and DMT treatment. NIH-3T3 fibroblasts were transfected with Snn-FLAG-pcDNA3.1, GFP-FLAG-pcDNA3.1, or no DNA and treated with TMT (0.2 µM) or DMT (1.0 µM or 5.0 µM). The 1.0 µM treatment of TMT elicited cytotoxicity in all transfection conditions; therefore, only the 0.2 µM concentration was used to analyze differential caspase activation. Western blot analysis of GFP and non-transfection conditions following TMT treatment revealed a small amount of active caspase-9 immunoreactivity (Figure 7a). Conversely no active caspase-9 immunoreactivity was found in the Snn-transfection condition following TMT treatment. Western blot analysis for active caspase-3 revealed that all TMT treatments elicited caspase-3 activation under all transfection conditions. Furthermore, no differences were noted in active caspase-3 immunoreactivity between Snn and control conditions, +/- TMT treatment. Western blot analysis of DMT-treated cells revealed that the 1.0 and 5.0 µM treatments did not result in significant activation of caspase-3 or caspase-9 in any of the transfection conditions. This is indicated by the lack of immunoreactivity for active caspase-3 and active (p39) caspase-9 (Figure 7b). These data indicate that Snn overexpression in NIH-3T3 cells does not pre-activate caspase cascades.
Furthermore, caspase cascades are not differentially activated following TMT and DMT treatment in Snn transfected cells compared to control conditions.
DISCUSSION

Our results demonstrate that a significant portion of Snn localizes to mitochondria following transfection of NIH-3T3 fibroblasts. Because our model system employs transient transfection and expression of Snn is under the control of a strong cytomegaloviral (CMV) promoter, the levels of Snn may exceed those normally encountered. It is likely that Snn is also present in endoplasmic reticulum. Transcript/Translation summaries from Sanger Institute protein analyses indicate that Snn may also contain a secretion signal (Snn; www.ensembl.org). Thus, Snn may localize to endoplasmic reticulum and Golgi apparatus during processing. This is consistent with the current differential centrifugation data showing Snn localized to multiple compartments. These results show that isolated and purified mitochondria contained Snn within that compartment.

The mechanism by which Snn is targeted to the mitochondrial membrane is unclear. Sequence analysis of Snn does not reveal the existence of a known mitochondrial localization signal; however, this is not uncommon. Tail-anchored proteins are known to localize to membrane surfaces that face the cytosol, including the outer mitochondrial membrane, though they do not contain a traditional mitochondrial localization signal (Borgese et al., 2003; Habib et al., 2003). Recent evidence has also shown that proteins may translocate to the mitochondria following alterations in their phosphorylation status (Bjur and Jope, 2003; Robin et al., 2003). Analysis of Snn’s amino acid sequence reveals multiple residues that have high probabilities of phosphorylation (data not shown), which may provide a possible mechanism for mitochondrial targeting.

The high level of sequence conservation of Snn across vertebrates, and the temporal and spatial patterns of expression suggest an important function for this protein. However, no
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homologies have been found for Snn, nor does Snn contain any domains that may provide insight into a possible intracellular role. For these reasons, research has concentrated on Snn’s role in mediation of organotin toxicity. Snn transfection sensitized cells that normally show resistance to TMT and DMT-induced cytotoxicity. Furthermore, we have shown that DMT is only toxic to NIH-3T3 cells when they over-express Snn. It is possible that Snn is a common factor required for organotin toxicity, with TMT as the most potent toxicant.

Comparative toxicity studies following TMT administration in vivo and in vitro show that DMT is less toxic than TMT (Komulainen and Bondy, 1987; Mushak et al., 1982). Our results indicated that DMT did not elicit cytotoxicity when administered to NIH-3T3 cells, or NIH-3T3 cells transfected with GFP. NIH-3T3 cells that were transfected with Snn, however, exhibited marked sensitivity to DMT. This indicates that DMT may interact with Snn to elicit cytotoxicity. Although Snn-overexpression conferred DMT sensitivity to NIH-3T3 cells, TMT treatment produced a more potent cytotoxicity. This may indicate that the interaction of Snn with TMT and subsequent dealkylation of TMT to DMT plays an integral role in the molecular basis of TMT-induced cytotoxicity. Oligonucleotide-mediated knockdown of Snn expression in vitro protected primary neurons in culture from TMT-induced cytotoxicity (Thompson et al., 1996). Together, this evidence indicates that inhibition of the TMT-Snn interaction may block TMT-induced cell death.

Morphological and molecular evidence following TMT exposure has indicated that TMT treatment can result in apoptotic and necrotic cell death, alluding to multiple mechanisms of toxicity (Brown et al., 1979; Bruccoleri et al., 1998; Geloso et al., 2002; Gunasekar et al., 2001; Jenkins and Barone, 2004). TMT treatment of PC-12 cells in vitro results in caspase-3 and -9 activation (Jenkins and Barone, 2004). Here we show corroborating increases in caspase-3 and -9 activation.
activation following TMT treatment of NIH-3T3 cells. Snn overexpression did not pre-activate caspase cascades, nor did it amplify caspase activity following TMT treatment. DMT-treated cells exhibited a total lack of caspase activation under all conditions. This suggests that caspase activation is not a common phenomenon following treatment with organotins.

Significant increases in trypan blue inclusion occurred only in Snn-overexpressing cells treated with TMT and DMT. TMT-elicited increase in caspase activity and cytotoxicity are likely to occur via separate mechanisms in cells transfected with Snn. Furthermore, TMT-induced caspase-9 activation was attenuated by overexpression of Snn; this may indicate that Snn overexpression drives a form of cell death that is not dependent upon mitochondrial-mediated activation of caspase-9.

Recent reports indicated that p38/MAPK inhibitors can effectively block TMT-induced cytotoxicity in vitro (Jenkins and Barone, 2004), providing another possible mechanism by which Snn may mediate organotin-induced cytotoxicity. These experiments, as well as the current data, examined organotin-induced cytotoxicity in vitro. The molecular mechanisms seen in transfected cells in vitro may not reflect the toxicological effects observed following TMT administration in vivo. Additional research is required to elucidate the molecular basis of TMT-induced cytotoxicity using both in vivo and in vitro models.

In conclusion, these data show that over-expression of Snn can confer sensitivity to two organotins. Moreover, given the mitochondrial localization and the putative role of Snn as an organotin-binding protein, we suggest a possible mechanism whereby TMT and DMT can bind to and disrupt Snn (Figure 8). The Snn/organotin interaction leads to cellular toxicity that may be mediated by multiple mechanisms, including JAK/STAT, MAPK, caspase-independent
apoptotic, or necrotic pathways. Further work will be needed in order to determine the normal function for this highly conserved protein.
REFERENCES


FOOTNOTES

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FIGURE LEGENDS

**Figure 1**: Stannin expression in NIH-3T3 cells. Cells were transfected with either GFP-FLAG-pcDNA3.1, Snn-FLAG-pcDNA3.1 or with vehicle control. After a 24 hour incubation, cells were harvested and used for Western blots or confocal microscopy analysis for anti-FLAG immunoreactivity. (A) Western blot analysis for anti-FLAG immunoreactivity. Note that immunoreactivity is observed for both Snn and GFP using the anti-FLAG antibody; 20 µg of total protein was loaded in each lane. Lanes: 1. Non-transfection; 2. GFP-FLAG-pcDNA3.1 transfection; 3. Snn-FLAG-pcDNA3.1 transfection. (B) Laser scanning immunofluorescent confocal micrographs of anti-FLAG immunoreactivity. GFP exhibited diffuse staining equally distributed throughout the cytosol, whereas Snn exhibited punctate intracellular staining.

**Figure 2**: Subcellular localization of stannin by differential centrifugation fractionation. NIH-3T3 Cells were transfected with Snn-FLAG-pcDNA3.1, incubated for 24 hours, and subcellular fractions were collected. Twenty µg of total protein was used in Western blots. The denotation corresponds to these fractions: (P1) = 3000g fraction, (P2) = 22,000g fraction, (P3) = 100,000g fraction, and (S3) = cytosol. Snn-FLAG immunoreactivity was detected in the (P2), and (P3) fractions, with some present in the (S3) fraction. The (P1) fraction was devoid of Snn-FLAG immunoreactivity.

**Figure 3**: Percoll gradient purification of mitochondria. Cells were transfected with Snn-FLAG-pcDNA3.1, incubated for 24 hours and then the (P2) fraction was isolated. Next, organelles within the (P2) fraction were density-separated using a self-forming Percoll gradient. (A) Western blots analyzed for the mitochondrial marker anti-GRP-75 (HSP-70), and anti-FLAG.
Lanes: (L) NIH-3T3 cell lysate; (1) top fraction from Percoll gradient; (2) dense band that had migrated 40% through the Percoll gradient, corresponding to mitochondria; (3) dense membrane collected at bottom of Percoll gradient. Note that immunoreactivity for the mitochondrial marker GRP-75 and Snn-FLAG was present only in the cell lysate and mitochondrial lanes. (B) Corresponds to Western blots verifying the purity of the Percoll purified mitochondria. Lanes: Controls for total cell lysate from non-transfected cells (lanes 1 and 2), and Snn-FLAG transected cells (lanes 3 and 4); Percoll purified mitochondria from non-transfected cells (lanes 5 and 6), and Snn-FLAG transected cells (lanes 7 and 8). Organelle markers are GRP-75 for mitochondria, GRP-78 for endoplasmic reticulum, α-spectrin for cytoskeleton, and Elk-1 for nucleus. (C) Quantitation of immunoreactivity in Western blots of organelle markers. Four lanes representing total cell lysate and mitochondrial samples were grouped and the average taken. Grey bars represent the average pixel value for total lysate conditions, and the black bars represent the average pixel value for the mitochondrial samples, ± S. E. M.

Figure 4: Triple-label laser scanning confocal microscopy of Snn-FLAG transfected NIH-3T3 cells. Cells were transfected with Snn-FLAG-pcDNA3.1, incubated for 24 hours, then treated with anti-FLAG antibodies, anti-GRP-75 antibodies, and DAPI stain. Blue fluorophore (UV emission) corresponds to DAPI stained nuclei. Green fluorophore (FITC emission) corresponds to GRP-75 immunoreactivity. Note that all cells are stained with DAPI and show GRP-75 immunoreactivity. Red fluorophore (Texas red emission) corresponds to Snn-FLAG immunoreactivity. Arrows in each panel indicate cells expressing Snn-FLAG. Micrographs were taken at 2500x magnification.
**Figure 5**: TMT-induced cytotoxicity in Snn-FLAG-pcDNA3.1 transfected NIH-3T3 cells. Cells were transfected with Snn-FLAG-pcDNA3.1, incubated for 20 hours, and then treated with either 0.2 µM, 1.0 µM DMT or no TMT, and incubated for 24 hours. Cells were harvested and the number of non-viable cells quantitated using the trypan blue inclusion method. Denotation refers to: NT = non-transfection; GFP = GFP-FLAG-pcDNA3.1 transfection; SNN = Snn-FLAG-pcDNA3.1 transfection. After treatment with 0.2 µM TMT, the Snn-overexpressing cells exhibited significantly increased cytotoxicity, as compared to GFP-transfected or non-transfected controls (Student’s t-test; p < 0.01). After treatment with 1.0 µM TMT, cytotoxicity was increased in all groups, as compared with untreated controls (Student’s t-test, p < 0.01). Data are shown as percent of control; mean values ± S.E.M.

**Figure 6**: DMT-induced cytotoxicity in Snn-FLAG-pcDNA3.1 transfected NIH-3T3 cells. Cells were transfected with Snn-FLAG-pcDNA3.1, incubated for 20 hours, and then treated with either 1.0 µM, 5.0 µM DMT or no DMT, and incubated for 24 hours. Cells were harvested and the number of non-viable cells quantitated using the trypan blue inclusion method. Denotation refers to: NT = non-transfection; GFP = GFP-FLAG-pcDNA3.1 transfection; SNN = Snn-FLAG-pcDNA3.1 transfection. At both concentrations of DMT, Snn over-expression significantly increased the number of cells included with trypan blue, as compared to GFP-transfected and non-transfected controls (Student’s t-test, p < 0.01). Data are shown as percent of control; mean values ± S.E.M.

**Figure 7**: Caspase-9 and -3 activity following TMT and DMT treatment. Cells were transfected with Snn-FLAG-pcDNA3.1, GFP-FLAG-pcDNA3.1, or no DNA and incubated for 20 hours.
TMT (0.2 µM) or DMT (1.0 µM, 5.0 µM) was then added and incubated for 24 hours. Cells were lysed and 50 µg of total protein analyzed on Western blots. Panel (A) shows Western blots of TMT-treated cells; immunoreactivity for activated caspase-3 was visible in all transfection conditions when treated with 0.2 µM TMT. Pro-caspase-9 immunoreactivity was visible in all conditions. A small amount of activated (p39) caspase-9 immunoreactivity was present in non-transfected and GFP-transfected cells treated with 0.2 µM TMT. No immunoreactivity for activated (p39) caspase-9 was present in Snn-transfected cells +/- TMT. Panel (B) shows Western blots of DMT-treated cells. Immunoreactivity for activated caspase-3 was not visible in any condition. Immunoreactivity for pro-caspase-9 was visible in all conditions; however, activated (p39) caspase-9 immunoreactivity was absent in all conditions.

**Figure 8:** Model of proposed role for interaction between stannin and methyltins. Shown is a model of the possible localization of Snn in the outer mitochondrial membrane. In this orientation, the vicinal di-thiol (residues 32 and 34) provides a possible binding domain for organotins. Shown is the proposed interaction of Snn with TMT and its subsequent de-alkylation. The localization of Snn to mitochondria provides a mechanistic link to the mitochondrial toxicity observed following TMT exposure. The direct interaction of stannin with TMT and DMT is based on data from Buck et al., (2003).
Figure 2

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P1

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P3

S3
Figure 3a

(A) Fractions

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Figure 3b

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GRP 75
GRP78
α-spectrin
ELK-1
Figure 5

cells included with trypan blue expressed as % of control

[TMT]

- 0 μM TMT
- 0.2 μM TMT
- 1.0 μM TMT

Legend:
- NT
- GFP
- SNN
Figure 7a

(A) 0.2 μM TMT models for caspase-9 and p-39. The graph shows a comparison between No-trans, GFP, and SNN conditions.

- Pro-caspase-9 levels decrease in the presence of 0.2 μM TMT.
- p-39 levels remain relatively constant.
- Caspase-3 levels are significantly reduced in all conditions.

- Graphs illustrate the effect of 0.2 μM TMT on caspase-9, p-39, and caspase-3.