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Suppression of Cytosolic NADPH Pool by Thionicotinamide Increases Oxidative Stress and Synergizes with Chemotherapy

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Abbreviations

6-AN, 6-amino nicotinamide; DLBCL, Diffuse Large B-Cell Lymphoma; G6PD, glucose-6-phosphate dehydrogenase; NADK, NAD⁺ Kinase; NADS, thionicotinamide adenine dinucleotide ; NADPS, thionicotinamide adenine dinucleotide phosphate ; ROS, reactive oxygen species ; ThioNa, thionicotinamide.

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Abstract NAD⁺ kinase (NADK) is the only known cytosolic enzyme that converts NAD⁺ to NADP⁺, which is subsequently reduced to NADPH. The demand for NADPH in cancer cells is elevated as reducing equivalents are required for the high levels of nucleotide, protein and fatty acid synthesis found in proliferating cells, as well as for neutralizing high levels of reactive oxygen species (ROS). The aim of this study was to determine whether inhibition of NADK activity is a valid anti-cancer strategy alone and in combination with chemotherapeutic drugs known to induce ROS. *In vitro* and *in vivo* inhibition of NADK with either shRNA or thionicotinamide inhibited proliferation. Thionicotinamide enhanced ROS produced by several chemotherapeutic drugs and produced synergistic cell kill. NADK inhibitors alone or in combination with drugs that increase ROS-mediated stress may represent an efficacious antitumor combination and should be explored further.

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Introduction

Cancer cells have three basic needs for proliferation: ATP for a source of energy, nutrients for macromolecular synthesis, and NADPH for the synthesis of nucleic acids and lipids and the maintenance of redox status in cells (Vander Heiden, 2011). To meet these enhanced needs, cancer cells have an altered metabolism, e.g., aerobic glycolysis rather than oxidative phosphorylation (the Warburg effect), thereby generating high levels of ROS (reactive oxygen species) as compared to normal cells (Vander Heiden *et al.*, 2009). In order to survive the increase in ROS, cancer cells control oxidative damage primarily through the activities of glutathione reductase and thioredoxin reductase, both of which require NADPH to function as a reducing agent (Estrela *et al.*, 2006; Lu and Holmgren, 2014). Therefore, down regulation of NADPH production is predicted to have a selective and two-pronged negative effect on tumor survival: inhibition of critical biosynthetic pathways and reduction in the ability of cancer cells to handle ROS.

The inhibition of NADK in cancer cells may represent a novel treatment strategy (Hsieh *et al.*, 2013). Cytosolic NADK is an enzyme responsible for generating NADP, which is then rapidly converted to NADPH by reductases. Together, NAD and NADP are involved in a variety of cellular pathways including metabolism, energy production, protein modification and reactive oxygen species detoxification (Ying, 2008). NADP/H is the core of biosynthetic pathways for lipids, amino acids and nucleotides as substrates or cofactors. The ability of cancer cells to rapidly proliferate requires these pathways to be functioning at high efficiencies; a lack of synthetic precursors can lead to a halt in cell growth and eventual death (Cairns *et al.*, 2011).

In this paper, we identified and validated a novel anti-cancer approach: down regulation of NADPH levels through the inhibition of NADK and glucose-6-phosphate dehydrogenase

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(G6PD) using thionicotinamide (ThioNa). Treatment of cancer cells with thionicotinamide lowered NADPH pools, compromised biosynthetic capabilities and inhibited cell growth. As a result of the decrease in NADPH levels, proliferating tumor cells, already stressed by high levels of ROS, were unable to protect a further increase in ROS generated by chemotherapeutic drugs, and consequently underwent apoptosis.

Materials and Methods

Cell culture

C85 human colon cancer cells (Longo *et al.*, 2001) and RL human Diffuse Large B-Cell Lymphoma (DLBCL) cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum in a 37°C incubator with 5% CO₂.

Cytotoxicity assay

Five thousand C85 cells per well were plated in 96-well plates in RPMI 1640 media (Gibco) supplemented with 10% FBS (Invitrogen). After overnight culture, spent media was removed and fresh media containing drug was added and plates were incubated for 96 hours. The Cell Titer 96 Aqueous One Solution (Promega) assay was used to assess cell viability at the end of the experiment according to manufacturers' protocol. Data was analyzed using the GraphPad Prism 4 software package (GraphPad Software Inc.).

Western blotting

Cells treated as appropriate were scraped into a micro centrifuge tube. After brief centrifugation, cell pellets were lysed in RIPA buffer containing a commercial protease inhibitor mix (Roche) and phosphatase inhibitor (50mM sodium fluoride and 10mM sodium orthovanadate). After quantification by Bradford protein assay (Bio-Rad Laboratories), proteins were resolved by 10%

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SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories). After blocking the membrane with 5% nonfat dry milk prepared in Tris buffered saline + 0.1% tween-20, the membrane was incubated with the desired primary antibody according to manufacturer's directions at 4°C overnight. The membrane was washed in Tris buffered saline + 0.1% tween-20 and incubated for two hours at room temperature with the appropriate peroxidase-conjugated secondary antibody. Bands were visualized using an enhanced chemiluminescence kit (Pierce). Anti-dihydrofolate reductase, anti-cleaved caspase-3 (Asp175) and anti-PARP was purchased from Cell Signaling Technology, anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and anti-phospho-H2A.X (Ser139) was purchased from Millipore and anti-NAD⁺ kinase was purchased from Abnova. Anti-mouse secondary was purchased from Santa Cruz Biotechnologies. Band intensity quantification was performed using ImageJ (NIH) with at least three replicates.

shRNA knockdown

C85 cells were transfected with a GIPZ NADK shRNA plasmid (GE Life Sciences, clone V3LHS_411242) according to manufactures' protocol. After two days, cells were cultured in 4µg/mL puromycin to select for cells expressing shRNA for two weeks. After knockdown of NADK was confirmed by western blot, cells were maintained in 2µg/mL puromycin.

Drug synergy study

5,000 cells/well were plated in 96-well plates. The following day, cells were treated with the appropriate drug/drug combination and incubated for 96 hours. An MTS assay (Promega) was performed to assess cell viability. The data was analyzed for synergy using Calcsyn software

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and the Chou-Talalay method (Chou and Talalay, 1984). $CI < 1$ =synergy; $CI=1$, additive; $CI > 1$ = antagonism.

Colony formation assay

250 cells/well were plated in 6 well plates and treated as indicated. Plates were cultured for 10-14 days and then fixed with 0.1% Crystal Violet stain in methanol. Colonies were counted and analyzed using ImageJ.

NADK enzymatic assay

This coupled assay measured the formation of NADP by conversion to NADPH by an excess of glucose 6- phosphate dehydrogenase. Reactions were carried out in 50mM Tris HCl, 5mM MgCl₂, 5mM glucose-6-phosphate, 50mM ATP and 18mM NAD⁺, 0.05μg of human G6PD and 0.5μg of human NADK. NADS or NADPS was added to a concentration of 500uM and reactions were incubated at room temperature for 30 minutes. An absorbance spectrum from 500 to 300nm was read using a Beckman spectrophotometer. All reagents were sourced from Sigma-Aldrich.

Human glucose-6-phosphate dehydrogenase (G6PD) inhibition assay

Reactions were carried out in 50mM Tris-HCl, 5mM MgCl₂, 5mM glucose-6-phosphate, and 0.05μg of human G6PD with varying amounts of NADP⁺ or NADPS. Reaction rate was monitored at 340nm using a Beckman spectrophotometer.

Reactive oxygen species detection

30,000 cells/well were plated in glass-bottom black-walled 96-well plates. The next day, cells were treated with the appropriate drug or drug combination and incubated for 24 hours. After

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treatment, cells were assayed for ROS production using a DCFDA ROS kit (Abcam) according to the manufactures directions.

NADP/NADPH quantification

3×10^6 cells were plated in 10cm dishes and incubated overnight. Cells were then treated as described. After treatment, control and treated cells were washed quickly with 5mL PBS twice. Any residual PBS in the plate was completely removed and 0.3mL PBS was added and the cells were scraped and transferred into 1.5mL microcentrifuge tube.

For the quantitation of NADPH and NADH, the samples were extracted by adding 0.6mL 0.4M KOH (Litt *et al.*, 1989). The samples were vortexed 30 seconds, and sonicated on ice for 20 seconds for three times. The suspension was centrifuged at 14,000g for 5min at 4°C and heated at 60°C for 30min. The samples were stored at -80°C until HPLC analysis. Total protein in the sample was determined by Bradford protein assay method following the manual protocol (BioRad). Quantitation of reduced pyridine nucleotides (NADPH/NADH) was carried out using a liquid chromatographic system (Hitachi, Tokyo, Japan) equipped with an L-7100 pump, L-7200 autosampler, and L-7480 fluorescence detector with excitation and emission wavelengths set at 340nm and 460 nm respectively. The separations were performed using a Luna PFP (2) column (5 μ m, 250mm \times 4.6mm, Phenomenex CA, USA) at 30°C. The extraction samples were injected into the system and eluted using mobile phase KH₂PO₄, (0.1M, pH 6) and methanol (95:5, v/v) at flow rate of 1.0 mL/min. NADPH and NADH in the samples were quantitated using a standard calibration curve. The amount of NADPH and NADH in the cells was expressed as nanomoles per milligram protein.

For the quantitation of NADP, the samples were extracted in 0.1mL of 1N HCl on ice for 15min. After centrifugation at 14,000g for 5min at 4°C, the acid extracts were adjusted to pH (~7.4)

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using 0.2M tris base and reduced to NADPH, using NADP cycling buffer (0.165 M Tris-HCl (pH 8.0) containing 16.5mM MgCl₂, 8.3mM glucose-6-phosphate and 8.3 units/mL glucose-6-phosphate dehydrogenase(Ogasawara *et al.*, 2009). Then the samples were incubated for 5 min at 37°C and heated at 60°C for 30 min. The samples were cooled and transferred to glass vials and a 50µL sample was injected into the HPLC system and analyzed using the same analytical HPLC method described above for NADPH and NADH. The calibration standards were prepared using NADP as substrate in NADP cycling system.

³H 4,5-Leucine incorporation to measure the rate of protein synthesis

8x10⁵ C85 cells/well were seeded in 6-well plates and cultured overnight. The next day, media was removed and replaced with media containing 2µCi/ml ³H 4,5-leucine for 2 hours. Cells were harvested with perchloric acid and precipitated proteins were assayed for ³H 4,5-leucine incorporation using a scintillation counter.

Measurement of lipid biosynthesis: Oil Red O assay

60,000 cells/well were seeded in 6-well plates and cultured overnight. The following day, spent media was removed and media-containing drug was added and plates were incubated for 48 hours. Low concentrations of drug were used to reduce experimental error to do high levels of cell death. For staining, media was removed and wells were washed with PBS and fixed with 10% formalin for 1 hour. After formalin was removed, wells were washed in ddH₂O and 60% isopropyl alcohol and left to dry completely. 1mL of Oil Red O solution was added to each well for 10 minutes. Stain was removed and washed with ddH₂O until washes became clear. Plates were air-dried and 1mL of 100% ethanol was used to elute Oil Red O from the stained cells. Elutions were collected and absorbance at 500nm was recorded using a Beckman

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spectrophotometer. Cell counts of identically treated replicates were used to calculate absorbance per cell value.

Human xenograft studies in immunosuppressed mice

C85 xenograft: NOD/SCID gamma male mice (a gift from Dr. Sharon Pine), 20-25 g, were inoculated s.c with 1×10^6 C85 cells or 1×10^6 C85 cells expressing shRNA directed against NADK. Animals were dosed with 100mg/kg thionicotinamide on days 3, 5, 7 and 9 post-xenograft. Animals were monitored tumor size and weight and signs of toxicity three times weekly. Tumor volume was determined using calipers and was calculated with the following equation: $\text{Volume} = (\text{width})^2 \times (\text{length}/2)$. There were at least eight animals in each cohort.

RL xenograft: NOD/SCID gamma male mice (a gift from Dr. Sharon Pine), 20-25 g, were inoculated s.c with 2.5×10^6 RL cells. When animals exhibited xenografts measuring $\sim 200 \text{mm}^3$ (Day 1), animals were dosed with 100mg/kg thionicotinamide on days 1, 3, 5, 7 and 9. Animals were monitored tumor size and weight and signs of toxicity three times weekly. Tumor volume determined using calipers and was calculated with the following equation: $\text{Volume} = (\text{width})^2 \times (\text{length}/2)$. There were at least eight animals in each cohort.

Results

We initially compared knockdown of NADK using shRNA (Supplemental Figure 1) with thionicotinamide in C85 cells, and found that inhibition of NADK by either method led to marked inhibition of colony growth (Figure 1C). This experiment, together with our previous study showing that NADK inhibition lowered NADPH levels (Hsieh *et al.*, 2013), established NADK as a valid target for drug development. Thionicotinamide is the active moiety of two

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previously identified NADK inhibitors, NADS and NADPS (Figure 1A). Treatment of C85 cancer cells with thionicotinamide results in an identical loss of dihydrofolate reductase levels, a G1/S block (Hsieh *et al.*, 2013) and similar toxicity profiles as NADS and NADPS; i.e., thionicotinamide is a prodrug, and is converted intracellularly to NADPS (Figure 1B and 1C).

Previous studies have shown that NADS and NADPS can be synthesized from thionicotinamide using porcine liver powder (Stein *et al.*, 1963). Using an enzymatic assay, NADS can be phosphorylated to NADPS by NADK. The addition of recombinant human glucose-6-phosphate dehydrogenase (hG6PD) to the reaction allows NADPS to be reduced to NADPSH (Figure 2A).

The finding that NADPS is a substrate for hG6PD led us to investigate the ability of NADPS to inhibit G6PD activity (Figure 2B and 2C). A K_i value of $\sim 1\mu\text{M}$ was found for NADPS, as compared to a K_m of $7.1\mu\text{M}$ for NADP (Wang and Engel, 2009). Therefore, thionicotinamide, by conversion to NADPS acts not only as an inhibitor of NADK, but also inhibits G6PD, thus both activities may contribute to its anti-cancer effects.

As previously noted, the level of nicotinamide in the media used for culturing cells has a large effect on the toxicity of NADS and NADPS (Hsieh *et al.*, 2013). To investigate whether nicotinamide levels affect thionicotinamide toxicity, we performed a colony formation assay, varying the levels of nicotinamide ($0\mu\text{M}$, $8.2\mu\text{M}$ and $32.8\mu\text{M}$) to assess the effect on cells treated with thionicotinamide or cells with knocked down NADK (Figure 3A). Control cells were largely indifferent to nicotinamide levels, as were knockdown cells. However, in cells treated with thionicotinamide, there was a direct relationship between high nicotinamide concentration and lower toxicity in both colony size and number (Figure 3B and 3C).

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The mechanism(s) by which exogenous nicotinamide dilutes the effect of thionicotinamide is not clear, possibilities are that nicotinamide may prevent thionicotinamide uptake or its incorporation into NAD (Figure 3D). Given these predicted mechanisms of action, exogenous nicotinamide addition would not be expected to affect the growth of normal cells as there are a variety of *de novo* NAD⁺ pathways (Chiarugi *et al.*, 2012) or those with a knockdown of NADK, as observed. NADK is still required for the conversion of NAD⁺ to NADP⁺; knockdown of NADK would still result in effectively lower NADP⁺ despite increased levels of NAD generated by nicotinamide.

The administration of thionicotinamide, a NADK inhibitor and a G6PD inhibitor, on cellular levels of NADP⁺ and NADPH should be significant (Icard and Lincet, 2012). To elucidate the effects of thionicotinamide, we monitored changes in cellular pools of NADP and NADPH via HPLC in C85 colon cancer cells. As expected, NADP and NADPH levels were reduced by 60-70% after 24 hours of exposure to 100µM thionicotinamide (Figure 4A and 4B).

Both the oxidized form (NADP) and reduced form (NADPH) are critical to macromolecular biosynthetic pathways (Patra and Hay, 2014). To determine if thionicotinamide inhibited lipid synthesis, we examined the level of fatty acids in cells treated with thionicotinamide using Oil Red O staining (Sikkeland *et al.*, 2014). Thionicotinamide had a significant effect on fatty acid levels in C85 cells (Figure 4C). Likewise, protein synthesis rates, as measured by ³H 4,5-leucine incorporation were also depressed in thionicotinamide treated C85 cells (Figure 4D). These results demonstrate the adverse effect of reduction of cellular levels of NADP and NADPH on cancer both lipid and protein synthesis.

A substantial requirement of NADPH in the cell is for the defense against reactive oxygen species (ROS) (Pollak *et al.*, 2007). High levels of ROS can damage proteins and DNA

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and cause cell death if left unchecked, and tumor cells with elevated levels of ROS require active management of ROS levels. Treatment with thionicotinamide caused a modest increase in steady state ROS levels 24 hours after exposure as detected using DCFDA staining (Figure 5A). In the presence of an oxidative stressor such as H₂O₂, ROS levels are significantly increased when C85 cells were treated with thionicotinamide, demonstrating a loss of protection against oxidative stressors (Figure 5B).

To further explore the ability of thionicotinamide to potentiate oxidative stressors we treated C85 cells with menadione, a vitamin K analog and known generator of ROS (Beck *et al.*, 2011), and examined ROS levels. Cells treated with a combination thionicotinamide and menadione exhibited significantly higher levels of ROS in a dose dependent manner (Figure 5C). Similarly, C85 cells exhibiting a knockdown of NADK contained higher levels of ROS when treated with menadione (Figure 5C). Loss of NADK and/or G6PD activity results in a decreased capacity to neutralize ROS, as NADP/NADPH pool size is reduced and NADP⁺ synthesis and reduction are inhibited.

As some commonly used chemotherapy drugs are known to induce ROS (Sinha *et al.*, 1989; Maehara *et al.*, 2004; Chintala *et al.*, 2010), we investigated whether the combination of thionicotinamide with these drugs would result in synergistic cell death. We found that gemcitabine, docetaxel and irinotecan all increased ROS levels and exhibited synergistic cell kill at ED75 and ED90 when combined with thionicotinamide as analyzed by the Chou–Talalay method (Figure 5D) (Chou and Talalay, 1984). A concomitant increase in ROS levels was observed when thionicotinamide was combined with chemotherapy, possibly explaining the synergy observed (Figure 5D).

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To determine if the decreased ability of cells to alleviate high ROS levels after treatment with thionicotinamide led to increased activity by chemotherapy, we investigated the level of double strand DNA breaks in cells treated with irinotecan, with and without thionicotinamide present. Using γ -H2AX levels as a marker for double strand DNA breaks (Petitprez *et al.*, 2013), we found that cells treated with thionicotinamide and irinotecan contained a higher level of γ -H2AX at a range of concentrations than cells treated with irinotecan alone (Figure 6A). The increased toxicity of this combination was confirmed by examining cleaved caspase-3 and PARP, indicating that cells are undergoing apoptosis when treated with both irinotecan and thionicotinamide (Figure 6B).

Our previous studies demonstrated that lowering NADPH levels by knockdown of NADK or by treatment with thionicotinamide, an inhibitor of both NADK and G6PD, caused decreased tumor cell growth *in vitro*. Therefore, it was important to demonstrate that thionicotinamide, as a lead compound for development of more potent inhibitors of NADK, had anti-tumor activity *in vivo*, and with less toxicity than 6-aminonicotinamide, (6-AN) a potent inhibitor of G6PD (Köhler *et al.*, 1970). 6-AN was not developed further as an anti-cancer drug because of severe neurotoxicity seen in early clinical trials (HERTER *et al.*, 1961). To find a safe dose for *in vivo* studies, we first performed a limited toxicity study in NOD- SCID gamma mice, and determined that the LD₅₀ was approximately 800 mg/kg administered every other day for two days; at this dose level, 3 of 8 mice died (Supplemental Figure 2). Importantly, unlike mouse toxicity studies with 6-AN (Dietrich *et al.*, 1958), there was no evidence of neurotoxicity.

For xenograft studies, we generated tumor cells with stable knockdown of NADK and compared effects on tumor growth with thionicotinamide treatment to determine if tumor regression would result without toxicity. As we previously observed that C85 cells produce

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rapidly proliferating xenografts (Longo *et al.*, 2001), dosing of thionicotinamide was performed as soon as tumors were palpable. The reduction of NADK levels in the stable knockdown cells drastically slowed tumor proliferation as compared to untreated C85 tumors (Figure 7A). Thionicotinamide dosing at 100 mg/kg every other day for four cycles also provided a marked reduction of tumor growth (Figure 7A, inset), however, once dosing was halted, the effect was largely lost (Figure 7A). Thionicotinamide was well tolerated, with no reduction of weight observed, and importantly, with no evidence of neurotoxicity (Supplemental Figure 3).

In a second series of *in vivo* experiments, we tested the effect of thionicotinamide treatment on RL, a Diffuse Large B-Cell Lymphoma (DLBCL) xenografts to determine the spectrum of thionicotinamide activity (Figure 7B). When tumors were approximately 200mm³ in size, treatment was initiated using a 100mg/kg dose of thionicotinamide every other day for five cycles. The treated cohort demonstrated moderate tumor regression for the duration of treatment and exhibited a prolonged decrease in growth rate post-treatment.

Discussion

The lowering NADPH levels by inhibition of NADK or G6PD has recently been recognized as a target for cancer drug development (Pandolfi *et al.*, 1995; Kirsch *et al.*, 2009; Petrelli *et al.*, 2011). While a few inhibitors of NADK have been described, they have lacked potency, and have not advanced to preclinical or clinical evaluation (Petrelli *et al.*, 2009). 6-Aminonicotinamide, an inhibitor of G6PD, had demonstrated antitumor effects, but there was clear evidence of neurotoxicity in animals, and also in patients. Further clinical evaluation was stopped because neurotoxicity limited dose escalation. The cause of this side effect is not known, though is theorized to be due to the death of glial cells by 6-AN (Kim and Wenger, 1973; Penkowa *et al.*, 2003). Importantly, in contrast to what was observed with 6-aminonicotinamide,

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in our xenograft experiments, thionicotinamide did not cause neurotoxicity in mice, suggesting that other inhibitors of NADK and or G6PD may not induce this deleterious side effect. A potentially significant source of toxicity may result from effects on highly proliferative immune cells. The strain of mice used to assay for thionicotinamide toxicity, NOD/SCID, lack this component of the immune system and possible negative effects remain unknown. Further testing is required to understand the full toxicological profile of thionicotinamide and NADK inhibition. Selectivity of NADK inhibition in cancer cells vs normal slowly proliferating tissues would result as most are not actively dividing, generate less ROS, and require less robust anabolic pathways (Vander Heiden, 2011).

In highly proliferative cells, aberrant metabolism and protein expression leads to increased rates of ROS production (El Sayed *et al.*, 2013). Cancer cells attempt to counteract the accumulation of ROS by increasing production of NADPH and glutathione (GSH), the most abundant antioxidant (Estrela *et al.*, 2006). NADP⁺ dependent malic enzyme and isocitrate dehydrogenase (IDH) 1 and 2, as well as G6PD also generate NADPH to help provide cancer cells protection against excessive ROS (Ying, 2008, p. 200). Due to the similar molecular structure of NADS and NADPS to NAD⁺ and NADP⁺, the inhibition of the malic and IDH enzymes is possible and we cannot rule out their role in thionicotinamide toxicity. However, the significant loss of NADP/H levels in the cell is expected to lower the activity of many NADP/H utilizing enzymes, likely making direct inhibition through thionicotinamide compounds a secondary effect.

Though we focus on the effects of inhibiting cytosolic NADK in this manuscript, it is important to consider the newly discovered and characterized mitochondrial NADK (mNADK) (Ohashi *et al.*, 2012; Zhang, 2015). A previous study (Zhang, 2015) found mNADK had lower

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activity compared to NADK and was in fact found to have lower expression in liver tumor samples, in contrast to overexpression of NADK in a variety of cancer types (unpublished). Having established the importance of cytosolic NADK in cancer, regardless of mitochondrial NADK, we expect that compounds selectively targeting cytosolic NADK would be preferable as they would spare off target mitochondrial effects in patients while displaying anti-tumor activity. Future efforts to develop specific inhibitors of NADK should consider the possible role mNADK may play in cancer.

The identification and study of new drivers of cancer metabolism have led to insights that can be exploited therapeutically (Pelicano *et al.*, 2004; Teicher *et al.*, 2012). This study is the first to explore the suppression of NADPH metabolism through the dual inhibition of NADK and G6PDH. The lowering of NADPH pools result in decreased biosynthesis of macromolecules vital to cancer cell growth, and the effects of this are seen *in vitro* and *in vivo* through thionicotinamide treatment or knockdown of NADK. These results support further investigation of the disruption of NADPH metabolism by targeting NADK, including an analysis of the clinical significance of NADK, development of a new generation of potent and selective NADK inhibitors and determination of cancer phenotypes particularly amenable to NADK inhibition.

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Authorship Contributions

Participated in research design: Tedeschi, Abali, Kerrigan, Scotto, Bertino

Conducted experiments: Tedeschi, Lin, Gounder, Kerrigan

Contributed new reagents or analytic tools: Lin, Gounder

Performed data analysis: Tedeschi, Bertino, Lin

Wrote or contributed to the writing of the manuscript: Tedeschi, Lin, Gounder, Kerrigan, Abali,
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Footnotes

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Figure Legends

Figure 1: Thionicotinamide is a prodrug of NADS and NADPS. A, all three compounds result in the destabilization of dihydrofolate reductase (DHFR), an indication of NADK inhibition. Methotrexate (MTX) causes a stabilization of DHFR and results in an increase of detectable protein. B, these compounds have similar toxicity profiles in C85 colorectal cancer cells. C, NADK shRNA knockdown and ThioNa toxicity result in similar colony growth in C85 cells.

Figure 2: NADPS is both a substrate and inhibitor of human G6PD. A, NADPS, derived from NADS phosphorylated by NADK in this reaction, can be reduced by G6PD to NADPSH, which absorbs at 405nm. B, NADPS inhibits NADP reduction. C, Using a Dixon plot, the K_i of NADPS for human G6P

D is $1\mu\text{M}$, as opposed to the NADP K_m of $7.1\mu\text{M}$ (Wang and Engel, 2009).

Figure 3: Exogenous nicotinamide in culture media can abrogate ThioNa toxicity. A, ThioNa toxicity is inversely correlated with nicotinamide levels. Untreated C85 cells and C85 cells stably knocking down NADK are unaffected. B, average colony increases in ThioNa treated cells as nicotinamide levels increase. C, average colony number increases as nicotinamide levels increase. D, the proposed intracellular biosynthetic pathway from ThioNa to NADPSH.

Figure 4: Treatment with ThioNa reduces cellular pools of NADP/NADPH and inhibits biosynthetic pathways. A, NADP and B, NADPH cellular pools decrease in C85 cells with treatment of $100\mu\text{M}$ ThioNa. C, the protein synthesis rate, measured by ^3H 4,5-leucine

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incorporation, is reduced with treatment of ThioNa. D, neutral fatty acid levels in cells treated with ThioNa are reduced, as measured by Oil Red O staining.

Figure 5: ThioNa causes a rise in cellular ROS levels and synergizes with chemotherapy. A, treatment of C85 cells with 100 μ M of ThioNa, NADS or NADPS cause an increase in steady-state ROS levels. B, C85 cells under oxidative stress from 1mM hydrogen peroxide (H₂O₂) are more sensitive when treated with 100 μ M ThioNa. C, C85 cells treated with thionicotinamide or containing a knockdown of NADK are more sensitive to menadione, a generator of ROS. D, ThioNa synergizes with ROS-inducing chemotherapy gemcitabine, docetaxel and irinotecan. CI values (Chou and Talalay, 1984) and ROS levels after 24 hours of treatment are described. * indicates a p-value ≤ 0.05 when compared to untreated cells.

Figure 6: The combination of ThioNa and irinotecan result in DNA damage and induction of apoptosis. A, an increase in γ -H2AX, an indication of DNA double strand breaks, is markedly increased in C85 cells treated with thionicotinamide and irinotecan. B, the presence of cleaved caspase 3 and PARP in C85 cells treated with ThioNa and irinotecan is indicative of apoptosis.

Figure 7: NADK inhibition is effective in xenograft models of colon cancer and lymphoma. A, stable knockdown of NADK in C85 cells caused slow growth in xenografts. NOD/SCID mice bearing C85 xenografts treated with 100mg/kg ThioNa displayed inhibited tumor growth for the duration of treatment (inset) with little low general toxicity. B, moderate tumor regression was observed in a second xenograft study using the DLBCL cell line RL using a dose of 100mg/kg ThioNa. Arrows indicate treatment.

Figure 1

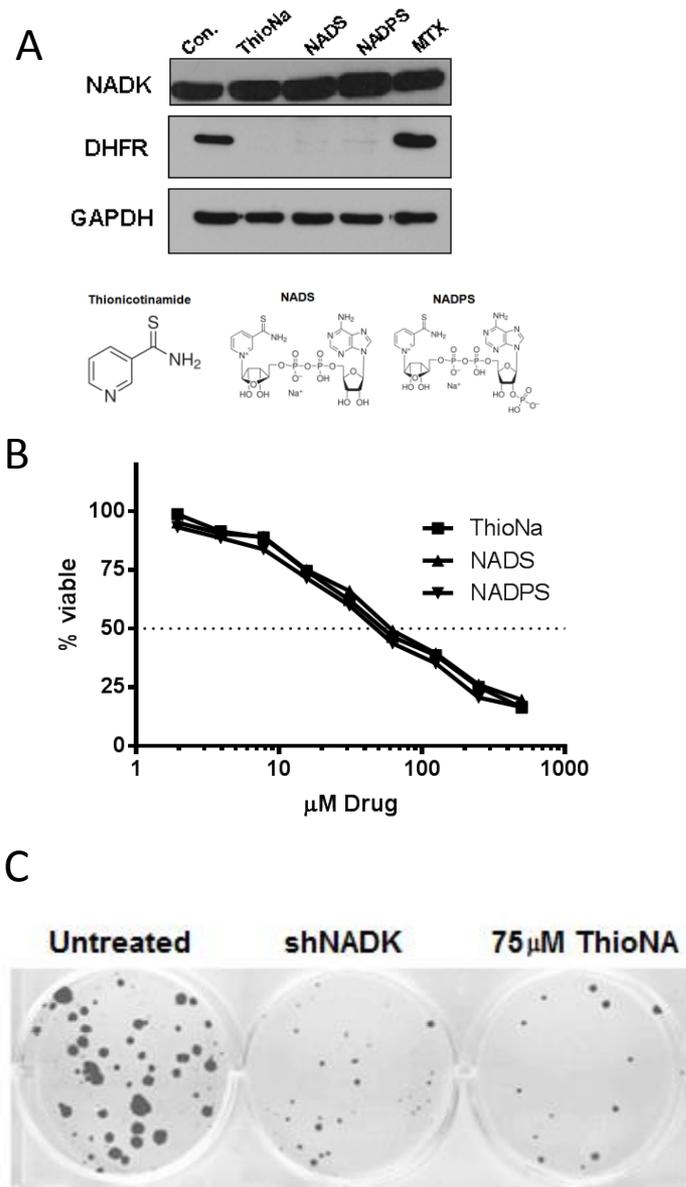


Figure 2

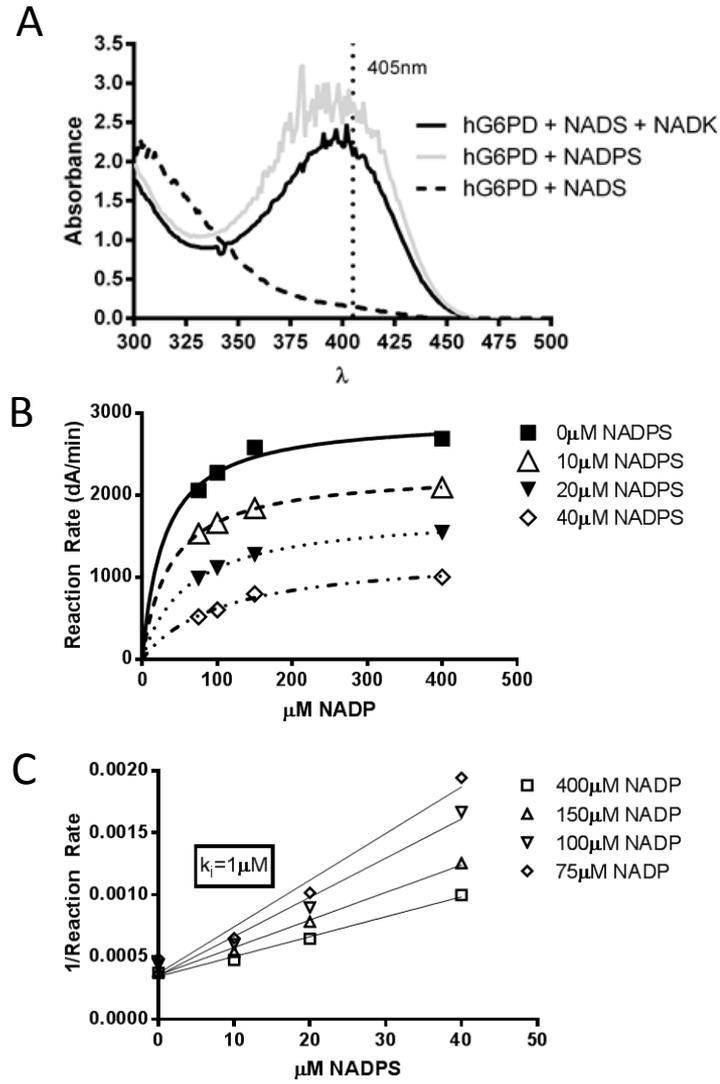
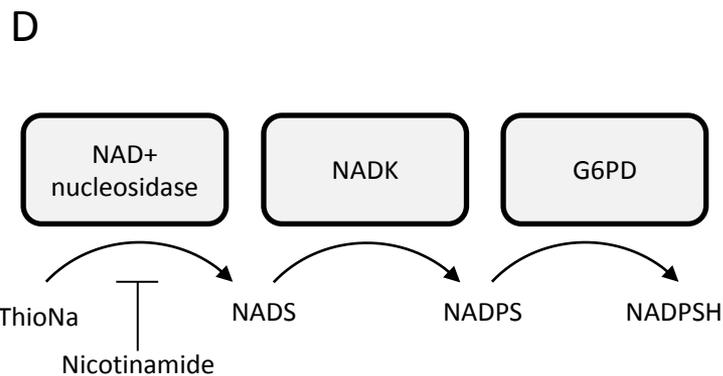
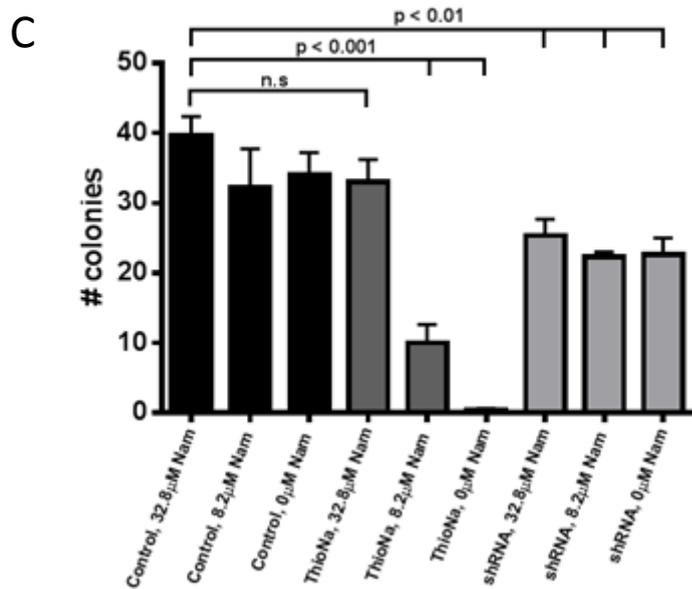
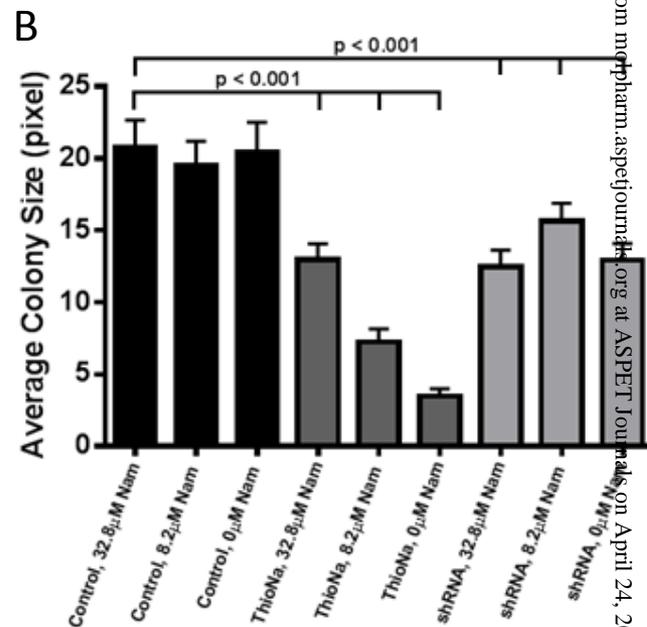
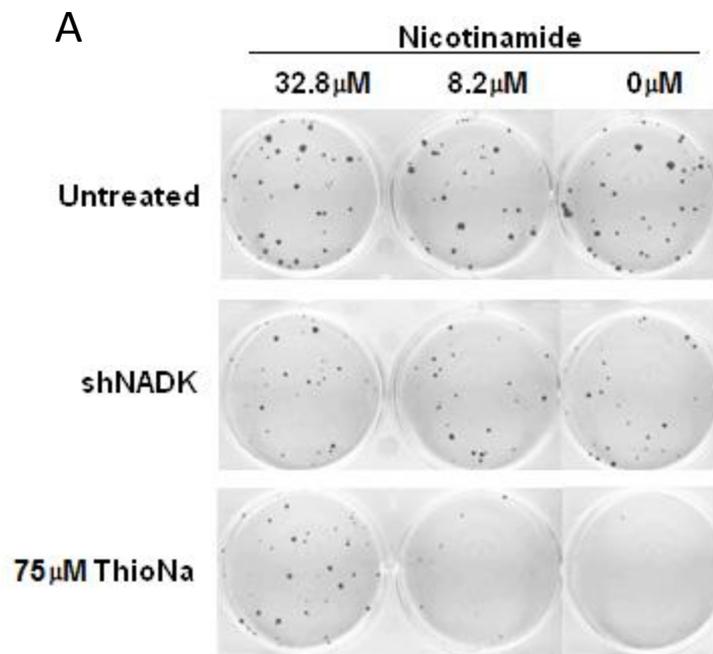


Figure 3



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Figure 4

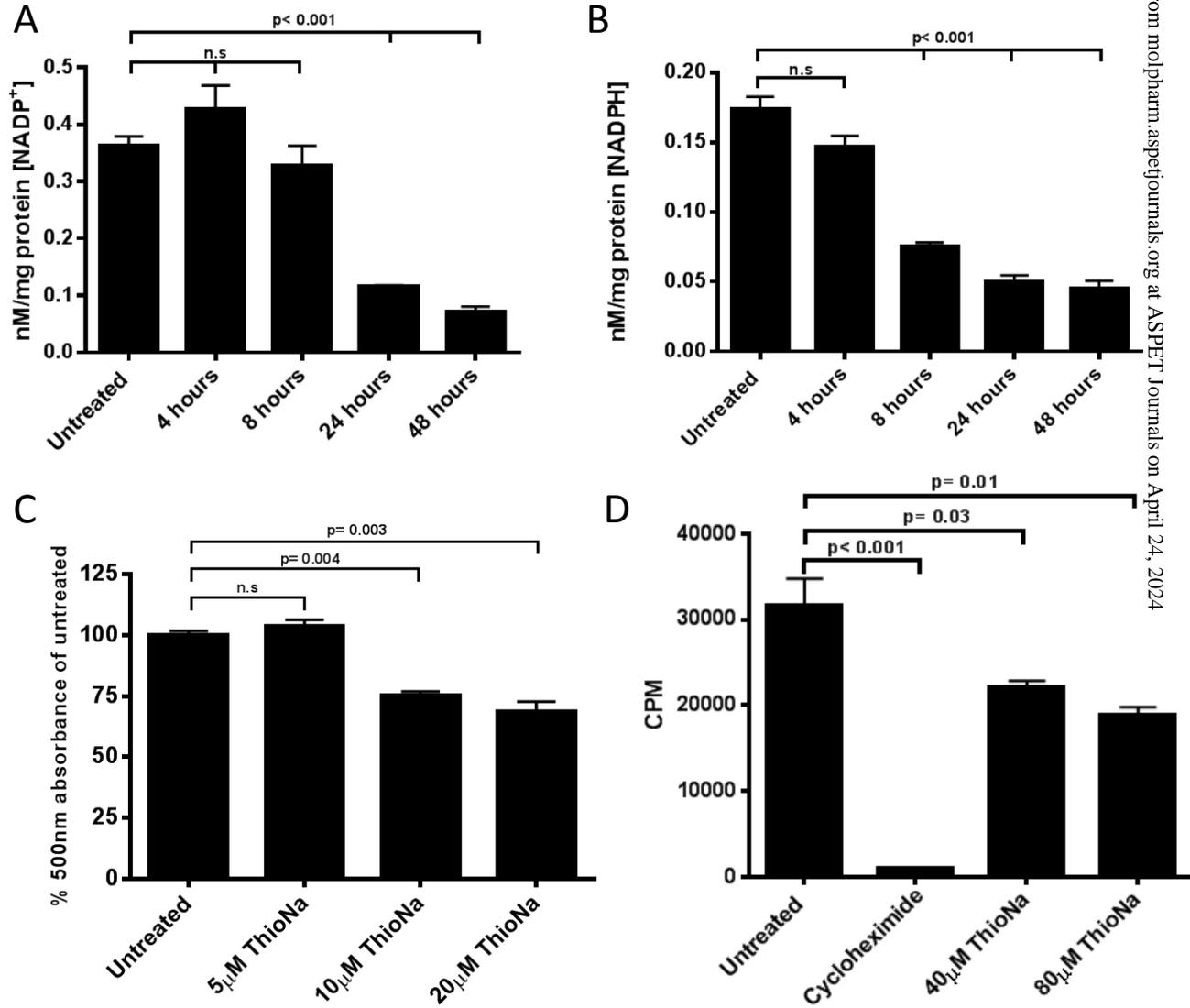
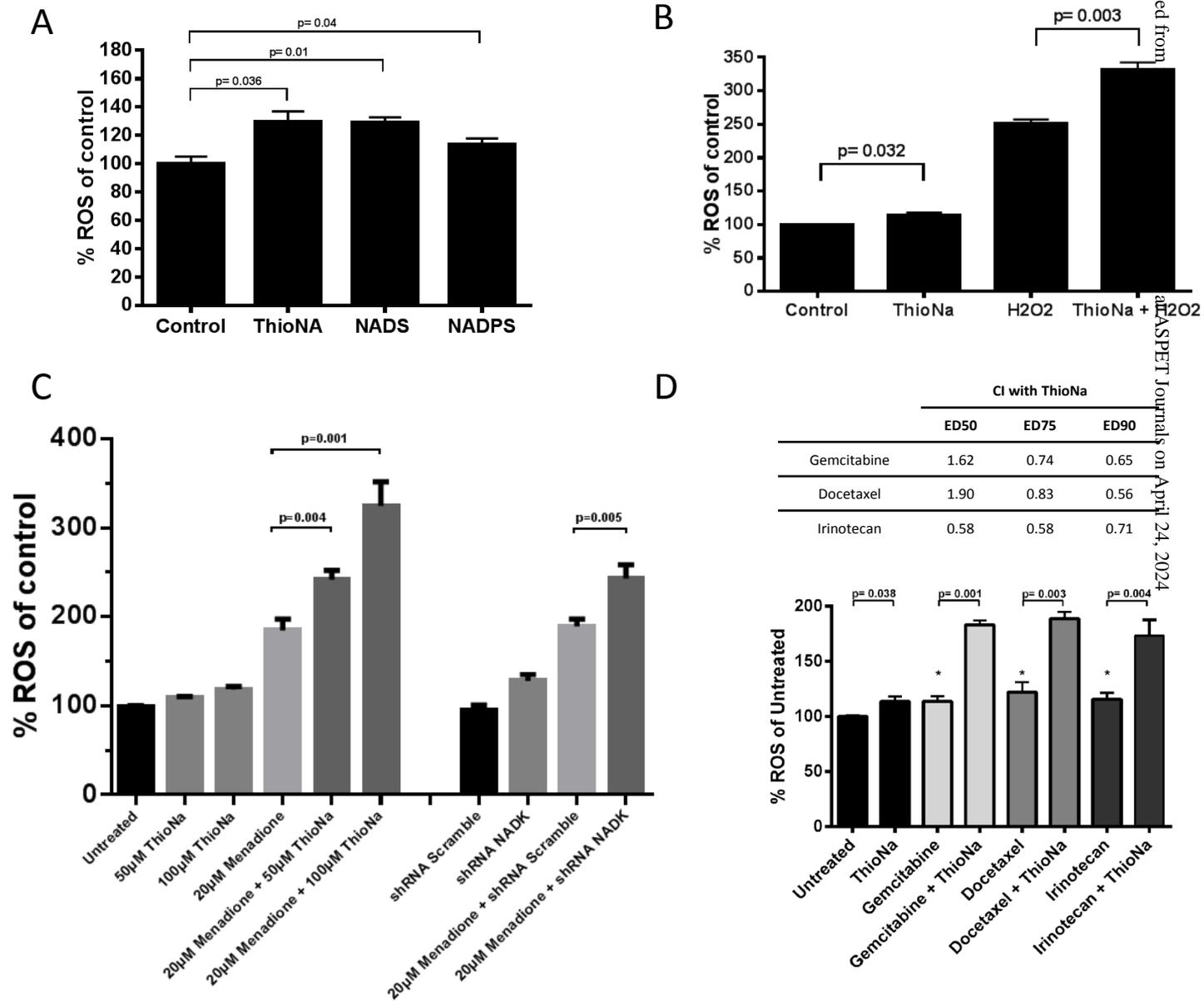


Figure 5



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Figure 6

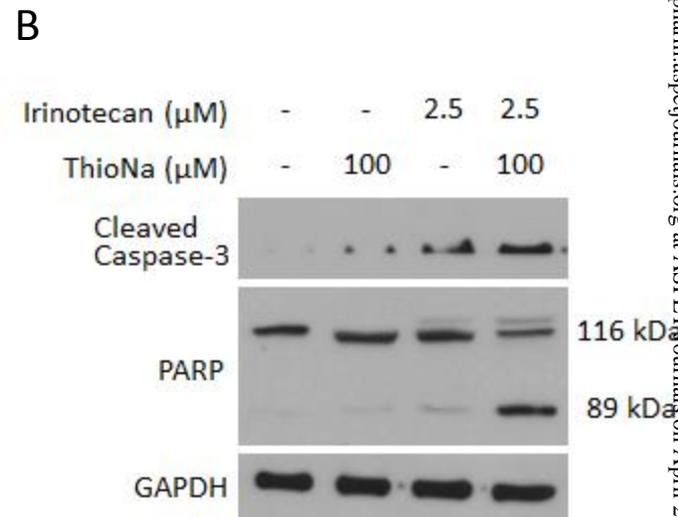
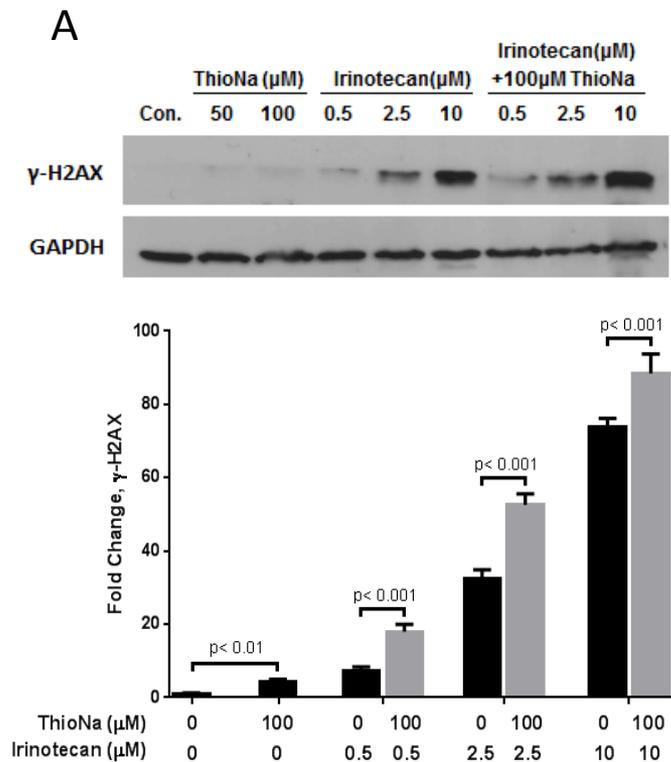
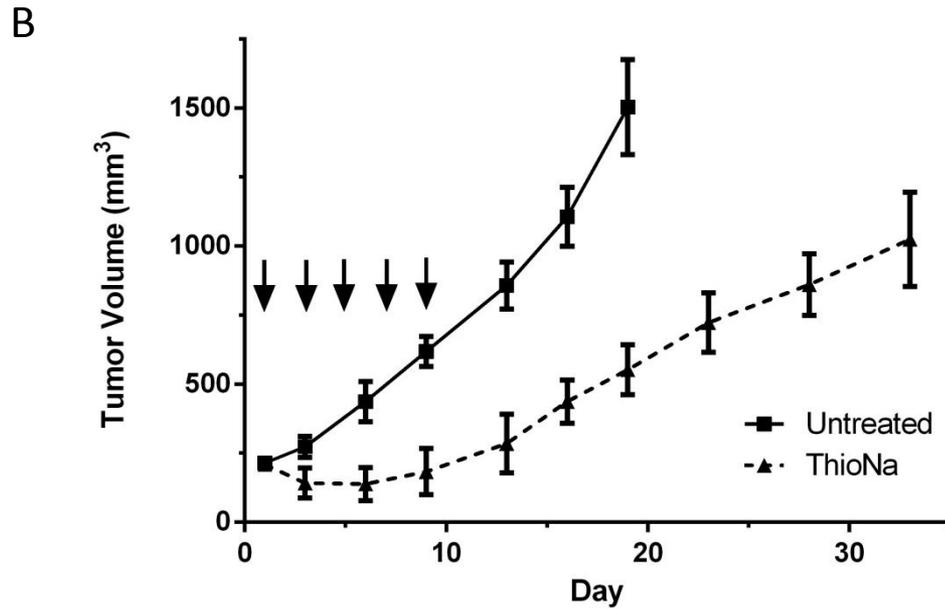
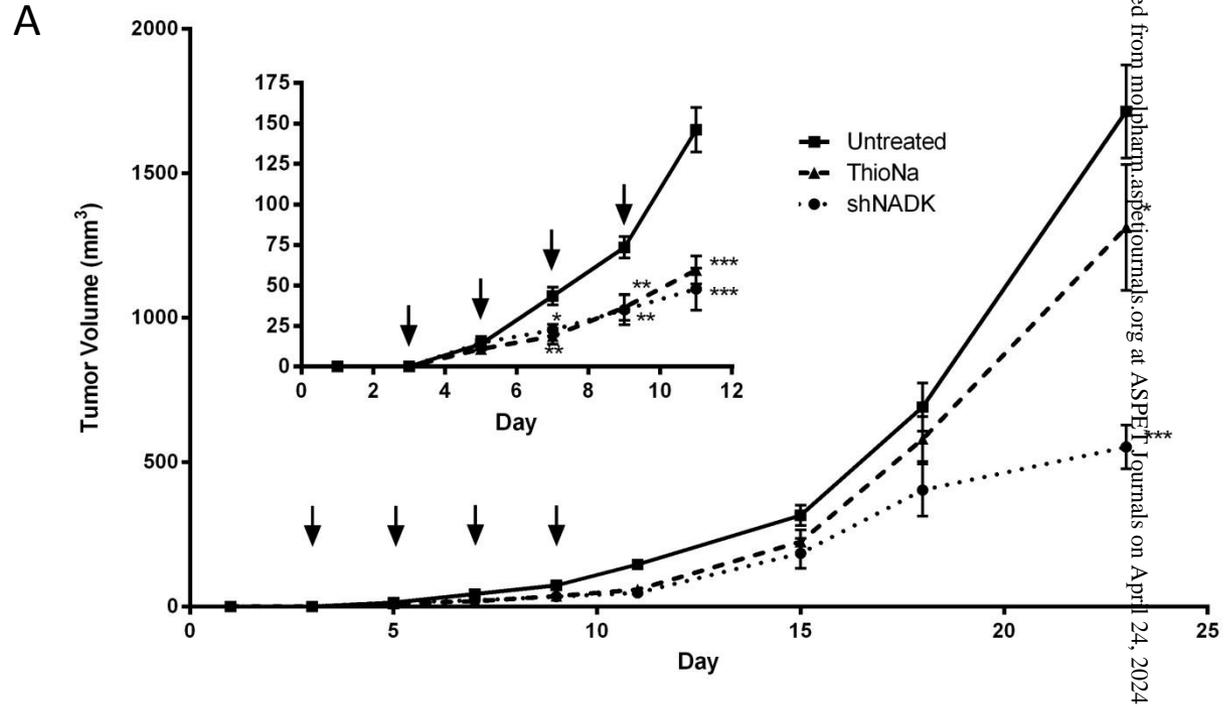


Figure 7

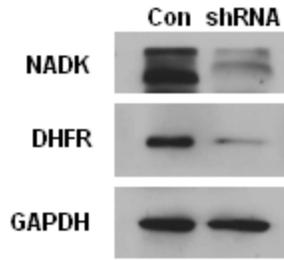


Supplemental Data for Molecular Pharmacology MOL#96727

Suppression of Cytosolic NADPH Pool by Thionicotinamide Increases Oxidative Stress and Synergizes with Chemotherapy

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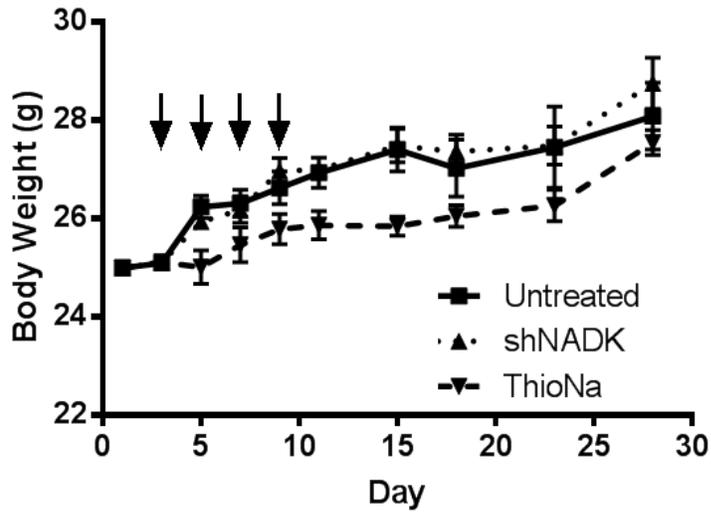


Supplemental Figure 1: Western blot of C85 cells transfected with shRNA directed against NADK. Note the loss of DHFR levels, an indication of a loss of NADK activity.

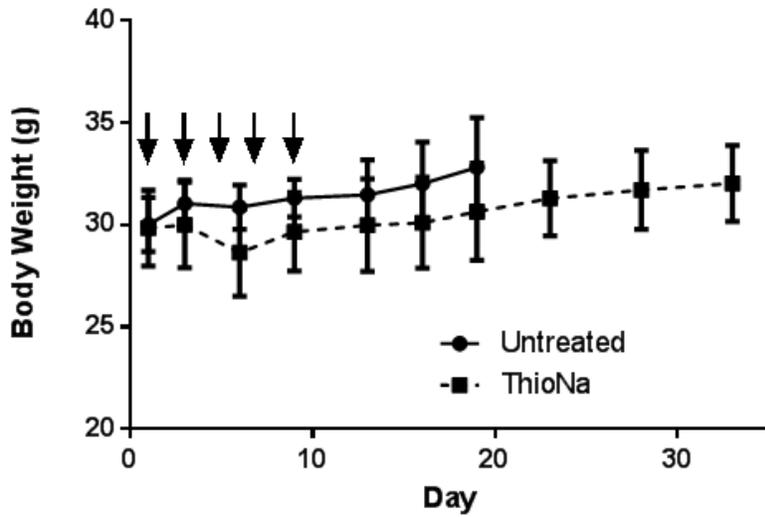
Dose	Mice Dead	Total Mice
50 mg/kg	0	8
100 mg/kg	0	8
400 mg/kg	0	7
800 mg/kg	3	8

Supplemental Figure 2: Female NOD- SCID mice were administered varying doses of ThioNa every other day for two days (a total of two doses). ThioNa LD₅₀ was determined to be approximately 800 mg/kg using this schedule.

A



B



Supplemental Figure 3: Thionicotinamide is well tolerated *in vivo*. A, body weight from mice bearing C85 xenografts. B, body weight from mice bearing RL xenografts. Arrows indicate treatment with 100mg/kg thionicotinamide.