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

Philip M. Tedeschi, HongXia Lin, Murugesan Gounder, John E. Kerrigan, Emine Ercikan Abali, Kathleen Scotto, and Joseph R. Bertino

Departments of Pharmacology and Medicine, Rutgers Cancer Institute of New Jersey (P.M.T., H.L., M.G., J.E.K., K.S., J.R.B.), and Department of Biochemistry (E.E.A.), Rutgers University, New Brunswick, New Jersey

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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). We determined whether inhibition of NADK activity is a valid anticancer strategy alone and in combination with

chemotherapeutic drugs known to induce ROS. In vitro and in vivo inhibition of NADK with either small-hairpin RNA or thionicotinamide inhibited proliferation. Thionicotinamide enhanced the 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.

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, such as aerobic glycolysis rather than oxidative phosphorylation (the Warburg effect), thereby generating high levels of reactive oxygen species (ROS) as compared with normal cells (Vander Heiden et al., 2009). 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, downregulation 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 NAD⁺ kinase (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 ROS 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).

We identified and validated a novel anticancer approach: downregulation of NADPH levels through the inhibition of NADK and glucose-6-phosphate dehydrogenase (G6PD) using thionicotinamide. 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 themselves from 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 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum in a 37°C incubator with 5% CO₂.

Cytotoxicity Assay. We plated 5000 C85 cells per well in 96-well plates in RPMI 1640 medium (GIBCO/Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Invitrogen/Life Technologies, Carlsbad, CA). After overnight culture, the spent medium was removed, and fresh medium containing the drug was

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ABBREVIATIONS: 6-AN, 6-aminonicotinamide; G6PD, glucose-6-phosphate dehydrogenase; HPLC, high-pressure liquid chromatography; mNADK, mitochondrial NAD⁺ kinase; NADK, NAD⁺ kinase; NADS, thionicotinamide adenine dinucleotide; NADPS, thionicotinamide adenine dinucleotide phosphate; PBS, phosphate-buffered saline; ROS, reactive oxygen species; shRNA, small-hairpin RNA.

added; the plates then were incubated for 96 hours. The Cell Titer 96 Aqueous One Solution (Promega, Madison, WI) assay was used to assess cell viability at the end of the experiment according to the manufacturer's protocol. Data were analyzed using the GraphPad Prism 4 software package (GraphPad Software, San Diego, CA).

Western Blotting. The cells that had been treated as appropriate were scraped into a microcentrifuge tube. After brief centrifugation, cell pellets were lysed in radioimmunoprecipitation assay buffer containing a commercial protease inhibitor mix (Roche Applied Science, Indianapolis, IN) and phosphatase inhibitor (50 mM sodium fluoride and 10 mM sodium orthovanadate). After quantification by Bradford protein assay (Bio-Rad Laboratories, Hercules, CA), the proteins were resolved by 10% 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 the manufacturer's directions at 4°C overnight. The membrane was washed in Tris-buffered saline + 0.1% Tween-20 and incubated for 2 hours at room temperature with the appropriate peroxidase-conjugated secondary antibody. The bands were visualized using an enhanced chemiluminescence kit (Pierce Biotechnology, Rockford, IL).

Anti-dihydrofolate reductase, anti-cleaved caspase-3 (Asp175), and anti-poly(ADP-ribose) polymerase were purchased from Cell Signaling Technology (Beverly, MA). Anti-glyceraldehyde 3-phosphate dehydrogenase and anti-phospho-H2A.X (Ser139) were purchased from Millipore (Millipore Bioscience Research Reagents, Temecula, CA), and anti-NAD⁺ kinase was purchased from Abnova (Taipei, Taiwan). Anti-mouse secondary was purchased from Santa Cruz Biotechnology (Dallas, TX). The band intensity quantification was performed using ImageJ (<http://imagej.nih.gov/ij/>) with at least three replicates.

Small-Hairpin RNA Knockdown. C85 cells were transfected with a GIPZ NADK small-hairpin RNA (shRNA) plasmid (clone V3LHS_411242; GE Healthcare Bio-Sciences, Pittsburgh, PA) according to the manufacturer's protocol. After 2 days, the cells were cultured in 4 μ g/ml of puromycin for 2 weeks to select for cells expressing shRNA. After knockdown of NADK had been confirmed by Western blot analysis, the cells were maintained in 2 μ g/ml of puromycin.

Drug Synergy Study. We plated 5000 cells/well in 96-well plates. The next day, the cells were treated with the appropriate drug-drug combination and incubated for 96 hours. A methanethiosulfonate assay (Promega) was performed to assess cell viability. The data were analyzed for synergy using CalcuSyn software (Biosoft, Cambridge, UK) and the Chou-Talalay method (Chou and Talalay, 1984), where CI < 1 = synergy; CI = 1, additive; CI > 1 = antagonism.

Colony Formation Assay. We plated 250 cells/well in six-well plates and treated them as indicated. The plates were cultured for 10 to 14 days and then fixed with 0.1% Crystal Violet stain in methanol. The colonies were counted and analyzed using ImageJ.

NADK Enzymatic Assay. The NADK enzymatic coupled assay measured the formation of NADP by conversion to NADPH by means of an excess of glucose 6-phosphate dehydrogenase. The reactions were performed in 50 mM Tris HCl, 5 mM MgCl₂, 5 mM glucose-6-phosphate, 50 mM ATP, 18 mM NAD⁺, 0.05 μ g of human G6PD, and 0.5 μ g of human NADK. We added thionicotinamide adenine dinucleotide (NADS) or thionicotinamide adenine dinucleotide phosphate (NADPS) to a concentration of 500 μ M, and the reactions were incubated at room temperature for 30 minutes. An absorbance spectrum from 500 to 300 nm was read using a Beckman spectrophotometer (Beckman Coulter, Brea, CA). All reagents were sourced from Sigma-Aldrich (St. Louis, MO).

G6PD Inhibition Assay. Reactions were performed in 50 mM Tris-HCl, 5 mM MgCl₂, 5 mM glucose-6-phosphate, and 0.05 μ g of human G6PD with varying amounts of NADP⁺ or NADPS. The reaction rate was monitored at 340 nm using a Beckman spectrophotometer.

ROS Detection. We plated 30,000 cells/well in glass-bottom black-walled 96-well plates. The next day, the cells were treated with

the appropriate drug or drug combination and were incubated for 24 hours. After treatment, cells were assayed for ROS production using a 2',7'-dichlorofluorescein diacetate ROS kit (Abcam, Cambridge, MA) according to the manufacturer's directions.

NADP/NADPH Quantification. We plated 3×10^6 cells in 10-cm dishes and incubated them overnight. The cells were then treated as described. After treatment, the control and treated cells were washed quickly with 5 ml of phosphate-buffered saline (PBS) twice. Any residual PBS in the plate was completely removed, 0.3 ml PBS was added, then the cells were scraped and transferred into 1.5-ml microcentrifuge tube.

For the quantitation of NADPH and NADH, the samples were extracted by adding 0.6 ml of 0.4 M KOH (Litt et al., 1989). The samples were vortexed 30 seconds, and sonicated 3 times on ice for 20 seconds. The suspension was centrifuged at 14,000g for 5 minutes at 4°C and heated at 60°C for 30 minutes. The samples were stored at -80°C until the high-pressure liquid chromatography (HPLC) analysis. The total protein in the sample was determined by Bradford protein assay method following the protocol manual (Bio-Rad Laboratories).

Quantitation of reduced pyridine nucleotides (NADPH/NADH) was performed 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 340 and 460 nm, respectively. The separations were performed using a Luna PFP (2) column (5 μ m, 250 $\times$ 4.6 mm; Phenomenex, Torrance, CA) at 30°C. The extraction samples were injected into the system and eluted using mobile phase KH₂PO₄ (0.1 M, pH 6.0) 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 of protein.

For the quantitation of NADP, the samples were extracted in 0.1 ml of 1 N HCl on ice for 15 minutes. After centrifugation at 14,000g for 5 minutes at 4°C, the acid extracts were adjusted to pH ~7.4 using 0.2 M Tris base, and reduced to NADPH using NADP cycling buffer (0.165 M Tris-HCl (pH 8.0) containing 16.5 mM MgCl₂, 8.3 mM glucose-6-phosphate, and 8.3 units/ml G6PD (Ogasawara et al., 2009)). Then the samples were incubated for 5 minutes at 37°C and heated at 60°C for 30 minutes. The samples were cooled and transferred to glass vials, and a 50- μ l sample was injected into the HPLC system and analyzed using the same analytic HPLC method described earlier for NADPH and NADH. The calibration standards were prepared using NADP as substrate in the NADP cycling system.

[³H]4,5-Leucine Incorporation to Measure the Rate of Protein Synthesis. We seeded 8×10^5 C85 cells/well in six-well plates and cultured them overnight. The next day, the medium was removed and replaced with medium containing 2 μ Ci/ml [³H]4,5-leucine for 2 hours. The cells were harvested with perchloric acid, and the precipitated proteins were assayed for [³H]4,5-leucine incorporation using a scintillation counter.

Measurement of Lipid Biosynthesis: Oil Red O Assay. We seeded 60,000 cells/well in six-well plates and cultured them overnight. The next day, the spent medium was removed and medium containing the drug was added. The plates were then incubated for 48 hours. Low concentrations of thionicotinamide were used to reduce experimental error due to high levels of cell death. For staining, the medium was removed, and the wells were washed with PBS and fixed with 10% formalin for 1 hour. After the formalin had been removed, the wells were washed in ddH₂O and 60% isopropyl alcohol and were left to dry completely. We added 1 ml of Oil Red O solution to each well for 10 minutes. The stain was removed, and the plates were washed with ddH₂O until the rinses became clear. The plates were air dried, and 1 ml of 100% ethanol was used to elute Oil Red O from the stained cells. Elutions were collected, and the absorbance at 500 nm was recorded using a Beckman spectrophotometer. Cell counts of identically treated replicates were used to calculate the absorbance per cell value.

Human Xenograft Studies in Immunosuppressed Mice. C85 xenograft: NOD/SCID γ male mice (a gift from Dr. Sharon Pine), 20–25 g,

were inoculated subcutaneously with 1×10^6 C85 cells or 1×10^6 C85 cells expressing shRNA directed against NADK. The animals were dosed with 100 mg/kg thionicotinamide on days 3, 5, 7, and 9 after xenografting. The animals were monitored tumor size and weight and signs of toxicity 3 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 γ male mice (a gift from Dr. Sharon Pine), 20–25 g, were inoculated subcutaneously with 2.5×10^6 RL cells. When the animals exhibited xenografts measuring $\sim 200 \text{ mm}^3$ (day 1), the animals were dosed with 100 mg/kg thionicotinamide on days 1, 3, 5, 7, and 9. Animals were monitored tumor size and weight and signs of toxicity 3 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 Fig. 1) with thionicotinamide in C85 cells, and found that inhibition of NADK by either method led to marked inhibition of colony growth (Fig. 1C). This experiment, together with our previous study showing that NADK inhibition lowered NADPH levels (Hsieh et al., 2013),

Fig. 1. Thionicotinamide (ThioNa) 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 thionicotinamide toxicity result in similar colony growth in C85 cells. Con., control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Fig. 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 405 nm. (B) NADPS inhibits NADP reduction. (C) Using a Dixon plot, the K_i of NADPS for human G6PD is $1 \mu\text{M}$, as opposed to the NADP K_m of $7.1 \mu\text{M}$ (Wang and Engel, 2009).

established NADK as a valid target for drug development. Thionicotinamide is the active moiety of two previously identified NADK inhibitors, NADS and NADPS (Fig. 1A). Treatment of C85 cancer cells with thionicotinamide resulted in an identical loss of dihydrofolate levels, a G1/S block (Hsieh et al., 2013), and similar toxicity profiles as NADS and NADPS; that is, thionicotinamide is a prodrug and is converted intracellularly to NADPS (Fig. 1, B and C).

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 G6PD to the reaction allows NADPS to be reduced to NADPSH (Fig. 2A).

The finding that NADPS is a substrate for recombinant human G6PD led us to investigate the ability of NADPS to inhibit G6PD activity (Fig. 2, B and C). A K_i value of $\sim 1 \mu\text{M}$ was found for NADPS, as compared with a K_m of $7.1 \mu\text{M}$ for NADP (Wang and Engel, 2009). Therefore, thionicotinamide,

Fig. 3. Exogenous nicotinamide in culture media can abrogate thionicotinamide (ThioNa) toxicity. (A) Thionicotinamide toxicity is inversely correlated with nicotinamide levels. Untreated C85 cells and C85 cells stably knocking down NADK are unaffected. Representative wells are shown for each condition. (B) Average colony increases in thionicotinamide-treated cells as nicotinamide levels increase. (C) Average colony number increases as nicotinamide levels increase. (D) The proposed intracellular biosynthetic pathway from thionicotinamide to NADPSH. Nam, nicotinamide; n.s., not significant.

by conversion to NADPS, acts not only as an inhibitor of NADK but also as an inhibitor for G6PD, thus both activities may contribute to its anticancer effects.

As previously noted, the level of nicotinamide in the medium 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, 8.2, and 32.8 μ M) to assess the effect on cells treated with thionicotinamide or cells with knocked down NADK (Fig. 3A). The control cells were largely indifferent to nicotinamide levels, as were the 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 (Fig. 3, B and C).

Fig. 4. Treatment with thionicotinamide (ThioNa) reduces cellular pools of NADP/NADPH and inhibits biosynthetic pathways. (A) NADP and (B) NADPH cellular pools decrease in C85 cells with 100 μ M thionicotinamide treatment. (C) The protein synthesis rate, measured by [³H]4,5-leucine incorporation, is reduced with thionicotinamide treatment. (D) Neutral fatty acid levels in cells treated with thionicotinamide are reduced, as measured by Oil Red O staining. CPM, counts per minute; n.s., not significant.

The mechanism(s) by which exogenous nicotinamide dilutes the effect of thionicotinamide is not clear; the possibilities include that nicotinamide may prevent thionicotinamide uptake or its incorporation into NAD (Fig. 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 effects of 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 100 μ M thionicotinamide (Fig. 4, A and B).

Both the oxidized form (NADP) and reduced form (NADPH) are critical to macromolecular biosynthetic pathways (Patra

and Hay, 2014). To determine whether thionicotinamide inhibited lipid synthesis, we examined the level of fatty acids in the 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 (Fig. 4C). Likewise, protein synthesis rates, as measured by [³H]4,5-leucine incorporation, were also depressed in thionicotinamide-treated C85 cells (Fig. 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 ROS (Pollak et al., 2007). High levels of ROS can damage proteins and DNA 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 2',7'-dichlorofluorescein diacetate staining (Fig. 5A). In the presence of an oxidative stressor such as H₂O₂, the ROS levels were significantly increased when C85 cells were treated with thionicotinamide, demonstrating a loss of protection against oxidative stressors (Fig. 5B).

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

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), then examined the ROS levels. Cells treated with a combination thionicotinamide and menadione exhibited significantly higher levels of ROS in a dose-dependent manner (Fig. 5C). Similarly, C85 cells exhibiting a knockdown of NADK contained higher levels of ROS when treated with menadione (Fig. 5C). Loss of NADK and/or G6PD activity results in a decreased capacity to neutralize ROS, as the 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 (Fig. 5D) (Chou and Talalay, 1984). A concomitant increase in ROS levels was observed when thionicotinamide was combined with chemotherapy, possibly explaining the synergy observed (Fig. 5D).

To determine whether 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 when compared with the cells treated with irinotecan alone (Fig. 6A). The increased toxicity of this combination was confirmed by examining cleaved caspase-3 and poly(ADP-ribose) polymerase, indicating

that cells are undergoing apoptosis when treated with both irinotecan and thionicotinamide (Fig. 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 antitumor activity in vivo and with less toxicity than 6-aminonicotinamide (6-AN), a potent inhibitor of G6PD (Köhler et al., 1970) that was not developed further as an anticancer drug because of the 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 γ mice, and determined that the LD₅₀ was approximately 800 mg/kg administered every other day for 2 days; at this dose level, 3 of 8 mice died (Supplemental Fig. 2). Importantly, unlike the mouse toxicity studies with 6-AN (Dietrich et al., 1958), there was no evidence of neurotoxicity.

For the xenograft studies, we generated tumor cells with stable knockdown of NADK and compared the effects on tumor growth with thionicotinamide treatment to determine whether tumor regression would result without toxicity. We had previously observed that C85 cells produce rapidly proliferating xenografts (Longo et al., 2001); dosing of thionicotinamide was performed as soon as the tumors were palpable. The reduction of NADK levels in the stable knockdown cells drastically slowed tumor proliferation as compared with untreated C85 tumors (Fig. 7A). Thionicotinamide dosing at 100 mg/kg every other day for four cycles also provided a marked reduction of tumor growth (Fig. 7A, inset); however, once dosing was halted, the effect was largely lost (Fig. 7A). Thionicotinamide was well tolerated, with no reduction of weight observed, and importantly with no evidence of neurotoxicity (Supplemental Fig. 3).

Fig. 6. Combination of thionicotinamide (ThioNa) and irinotecan results 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 poly(ADP-ribose) polymerase (PARP) in C85 cells treated with thionicotinamide and irinotecan is indicative of apoptosis. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Fig. 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 100 mg/kg thionicotinamide (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 diffuse large B-cell lymphoma cell line RL using a dose of 100 mg/kg of thionicotinamide. Arrows indicate treatment. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

In a second series of *in vivo* experiments, we tested the effect of thionicotinamide treatment on RL, forming diffuse large B-cell lymphoma xenografts, to determine the spectrum of thionicotinamide activity (Fig. 7B). When the tumors were approximately 200 mm³ in size, treatment was initiated using a 100 mg/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 after 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). Although 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-AN, 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 the neurotoxicity limited dose escalation. The cause of this side effect is not known, though it 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-AN, 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 NOD/SCID strain of mice used to assay for thionicotinamide toxicity lack this component of the immune system, so the possible negative effects remain unknown. Further testing is required to understand the full toxicologic profile of thionicotinamide and NADK inhibition. Selectivity of NADK inhibition in cancer cells versus normal, slowly proliferating tissues would result because 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, the most abundant antioxidant (Estrela et al., 2006). NADP⁺-dependent malic enzyme and isocitrate dehydrogenase 1 and 2 as well as G6PD also generate NADPH to

help provide cancer cells with 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 isocitrate dehydrogenase enzymes is possible, so 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 using enzymes, likely making direct inhibition through thionicotinamide compounds a secondary effect.

Though we focused on the effects of inhibiting cytosolic NADK in this study, it is important to consider the newly discovered and characterized mitochondrial NADK (mNADK) (Ohashi et al., 2012; Zhang, 2015). In a previous study, Zhang (2015) found mNADK had lower activity compared with NADK and fact had lower expression in liver tumor samples, in contrast with the overexpression of NADK in a variety of cancer types (unpublished data). Having established the importance of cytosolic NADK in cancer, regardless of mNADK, we expect that compounds selectively targeting cytosolic NADK would be preferable as they would spare off-target mitochondrial effects in patients while displaying antitumor 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). Our study is the first to explore the suppression of NADPH metabolism through the dual inhibition of NADK and G6PDH. The lowering of NADPH pools results 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 the cancer phenotypes particularly amenable to NADK inhibition.

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, Scotto, Bertino.

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Address correspondence to: Dr. Joseph R. Bertino, Rutgers Cancer Institute of New Jersey, 195 Little Albany Street, Room 3033, New Brunswick, NJ 08903. E-mail: bertinoj@cinj.rutgers.edu.