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ORIGINAL ARTICLE

Nicotinamide mononucleotide adenylyltransferase2 overexpression enhances colorectal cancer cell-kill by Tiazofurin

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Colorectal cancer cells exhibit limited cytotoxicity towards Tiazofurin, a pro-drug metabolized by cytosolic nicotinamide mononucleotide adenylyltransferase2 (NMNAT2) to thiazole-4-carboxamide adenine dinucleotide, a potent inhibitor of inosine 5′-monophosphate dehydrogenase required for cellular guanylate synthesis. We tested the hypothesis that colorectal cancer cells that exhibit low levels of NMNAT2 and are refractory to Tiazofurin can be rendered sensitive to Tiazofurin by overexpressing NMNAT2. Transfection of hNMNAT2 resulted in a six- and threefold cytoplasmic overexpression in Caco2 and HT29 cell lines correlating with Tiazofurin-induced enhanced cell-kill. Folate receptors expressed on the cell surface of 30–50% colorectal carcinomas were exploited for cellular targeting with Tiazofurin encapsulated in folate-tethered nanoparticles. Our results indicated that in wild-type colorectal cancer cells, free Tiazofurin-induced EC_{50} cell-kill was 1500–2000 μM, which was reduced to 66–156 μM in hNMNAT2-overexpressed cells treated with Tiazofurin encapsulated in non-targeted nanoparticles. This efficacy was improved threefold by encapsulating Tiazofurin in folate-tethered nanoparticles to obtain an EC_{50} cell-kill of 22–59 μM, an equivalent of 100–300 mg m^{-2} (one-tenth of the approved dose of Tiazofurin in humans), which will result in minimal toxicity leading to cancer cell-kill. This proof-of-principle study suggests that resistance of colorectal cancer cell-kill to Tiazofurin can be overcome by sequentially overexpressing hNMNAT2 and then facilitating the uptake of Tiazofurin by folate-tethered nanoparticles, which enter cells via folate receptors.

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INTRODUCTION

Colorectal cancer is the second most common cause of death from cancer in men and women in the United States with an estimated 103 170 new cases and 40 290 deaths in 2012 (http://www.cancer.gov/cancertopics/types/commoncancers). It is notable that 40–50% of patients who undergo potentially curative surgery alone eventually relapses and die of metastatic disease.[1] Combination chemotherapy for metastatic colon cancer includes 5-fluorouracil, leucovorin, oxaliplatin and anti-vascular endothelial growth factor monoclonal antibody as first-line treatment, which result in a median survival of 10–15 months.[2–4] As conventional therapy is relatively non-specific, with cytotoxicity affecting both tumor and normal cells, there is a need to explore selective targeting of colorectal cancer that spares normal cells.

Nicotinamide adenine dinucleotide (NAD^+) is a high-energy molecule that can be reduced to form NAD dehydrogenase (NADH) in various cellular processes. The NAD^+/NADH ratio has an important role in the intracellular redox state and is a good index of the metabolic state of the cell. NAD^+ is synthesized by the action of nicotinamide mononucleotide adenylyltransferase (NMNAT), which utilizes NMN and ATP as substrates. Although NAD^+ can be formed either by conserved *de novo* or salvage pathways, both pathways are mediated by NMNAT, the crucial rate-limiting step to catalyze the final product NAD^+. Of three human NMNAT isoforms, NMNAT1 is found in nucleus;[5,6] NMNAT2 is present in cytoplasm in Golgi bodies;[7–9] and NMNAT3 is in mitochondria.[8,10]

Tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide) (TR) is a nucleoside pro-drug, which is phosphorylated to tiazofurin 5′-monophosphate (TRMP) and then converted by the action of NMNAT, to its active metabolite, thiazole-4-carboxamide adenine dinucleotide (TAD), an analog of NAD^+. TAD inhibits inosine 5′-monophosphate dehydrogenase (IMPDH), which is a rate-limiting enzyme in the synthesis of guanylates, including GTP and dGTP (Figure 1) that results in cancer cell death.[11,12] The sensitivity to Tiazofurin is related to cellular NMNAT2 activity and Tiazofurin-resistant cell lines exhibit decreased NMNAT activity.[13,14]

Tiazofurin induced a specific reduction in GTP that correlated with response in patients in Phase I/II clinical trials with chronic myeloid leukemia in blast crisis.[15,16] This led to its approval as an orphan drug for the treatment of patients with chronic myelogenous leukemia in blast crisis.[17]

Although an earlier Phase II study with Tiazofurin in 21 patients with colorectal cancer revealed no significant antitumor activity,[18] there was insufficient information to explain the lack of activity based on the levels of NMNAT, effect on IMPDH or GTP levels within target cells. We subsequently determined that Tiazofurin had to be administered over longer periods (1-h infusion for 10 days) to effectively inhibit IMPDH thereby reduce GTP levels sufficient to induce an antitumor effect.[19]

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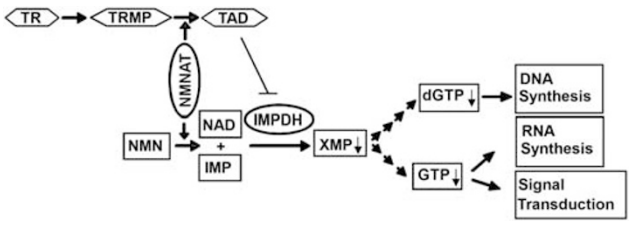

Figure 1. Schematic representation of Tiazofurin activation to TAD and inhibition of guanylate synthesis. TR is activated through the intermediate TRMP to form TAD, an analogue of NAD by NMNAT. TAD competes for the NAD site on IMPDH and inhibits guanylate synthesis.

When compared with leukemic cell lines, colorectal cancer cell lines required higher doses of Tiazofurin to effect cell-kill *in vitro* and to exhibit antitumor activity in mice transplanted with the colorectal cancer HT29 cells.[20] Subsequent preclinical studies with Tiazofurin demonstrated significant antitumor activity with reduced dose-dependent micro-invasiveness and metastasis formation in a liver metastasis model of human colorectal carcinoma in athymic mice.[21]

Our earlier studies with murine tumors that were intrinsically sensitive or resistant to Tiazofurin suggested that tumors which exhibited high NMNAT activity had the capacity to avidly metabolize Tiazofurin to TAD.[13,22] This supported the concept that tumors which exhibited high NMNAT activity would generate high TAD levels that would result in concurrent decrease in cellular GTP concentration and thereby confer much greater sensitivity to Tiazofurin.[22] Therefore, we hypothesized that increasing cellular levels of NMNAT2 by transfection with NMNAT2 into colorectal cancer cells, which were initially less sensitive to Tiazofurin because of low levels of NMNAT2, could subsequently be rendered more sensitive to this pro-drug.

A related issue is that the combination of specific gene and drug delivery to target cells *in vivo* remains a challenge. Tumor-specific delivery of therapeutic agents to cancer cells can be achieved by exploiting folate receptors, which bind bio-degradable, biologically inert, bi-layer lipid nanoparticles that are linked to the ligand folic acid.[23–26] Therefore, to increase the specificity of uptake of Tiazofurin into colorectal cancer, we took advantage of the fact that 30–50% of these cells express cell surface folate receptors.[27–29] So we also tested the hypothesis that specific uptake of the pro-drug Tiazofurin encapsulated in folate-tethered nanoparticles via folate receptors would allow for greater metabolism of Tiazofurin within cancer cells that overexpress NMNAT2.

MATERIALS AND METHODS

Cell culture

Human colorectal cancer cell lines Caco2, HCT15, HCT116, HT29 and HCC2998 were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD. These cell lines were maintained in 5% CO_2 at 37 °C in minimum essential media containing 2.3 μM folic acid (Life Technologies, Grand Island, NY) supplemented with 1% penicillin, streptomycin, amphotericin B (Invitrogen, Carlsbad, CA), and 10% fetal bovine serum (FBS) for all cell lines except Caco2 which required 20% FBS for optimal growth. Caco2 and HT29 cell lines were adapted to grow in a low-folate (LF) media by stepwise reduction of the folate concentration in the medium and were stably adapted to grow in folate-free minimum essential media supplemented with 20% and 10% FBS, respectively. The complete LF medium with 20% FBS for Caco2 cell line contained 27.2 nM 5-methyltetrahydrofolate and the LF medium with 10% FBS for HT29 cell line contained 13.6 nM 5-methyltetrahydrofolate.[30] These cell lines were cultured in LF medium for at least 12 weeks before they were used in the experiments to promote full expression of folate receptors,[31] and logarithmically growing cells were utilized for all studies.

Establishment of stably transfected cell lines

The *hNMNAT2* gene amplification was performed using Accuprime pfx DNA polymerase (Invitrogen) with the primers 5′-GGGGTACCCGATGACC GAGACCACCAAGAC-3′ and 5′-GCTCTAGAATCGATGCTAGCCGGAGCC GGAGGCATTG-3′ from EST246487 clone (American Type Tissue Collection, Manassas, VA).[9] The PCR product was digested by *Kpn*I and *Xba*I and cloned into the vector pTracerEFV5/HisA (Invitrogen). The correct plasmid pTracer-hNMNAT2 constructs were confirmed by restriction enzyme analysis and sequencing.

Colorectal cancer cell lines were transfected with either pTracer-hNMNAT2 or the control plasmid pTracerEFV5/HisA using the lipofectamine reagent (Invitrogen). Zeocin resistant and green fluorescence protein (GFP) expressing cell clones were further analyzed by fluorescence-activated cell sorting (FACS) (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Cytoplasmic, mitochondrial and nuclear protein extraction

Nuclear and cytoplasmic fractions of colorectal cancer cell lines were separated and purified using the Pierce protein separation kit (Thermo Scientific, Rockford, IL). Mitochondrial protein was prepared as described.[32] Protein concentration was determined using the Pierce Bicinchoninic acid protein assay kit.

Generation of hNMNAT2-silenced Caco2 cell lines to demonstrate the consequences of hNMNAT2 overexpression

Colorectal cancer Caco2 cell line-overexpressing hNMNAT2 were transfected with short hairpin (shRNA) control plasmid and shRNA NMNAT2 (sc-62693, Santa Cruz biotechnology, Santa Cruz, CA) for 8 h and puromycin-resistant cell clones were selected. Silencing of hNMNAT2 was confirmed by western blot analyses.

Silencing of hNMNAT1 or hNMNAT3 in wild-type and hNMNAT2-overexpressed human colorectal cancer cell lines

Initial studies were carried out to establish optimal conditions (for cell density, transfection reagent and small interference RNA (siRNA) concentrations) for transfection using siRNA (hNMNAT1 (s34981) or hNMNAT3 (s51394), Invitrogen) in Caco2 and HT29 cell lines as per manufacturer's instructions. Incubations were conducted at 37 °C for 24–168 h to determine silencing of respective genes.

Quantitative real-time PCR for assessing hNMNAT3 expression

Total RNA was extracted and purified from the cell lines after transfecting with siRNA hNMNAT3 using GenElute mRNA miniprep kit (Sigma-Aldrich, St Louis, MO) according to the manufacture's instruction. Single-stranded cDNA was synthesized from total RNA using the affinity script QPCR cDNA Synthesis kit (Agilent Technologies, Santa Clara, CA) and real-time PCR for each target was performed with Brilliant II SYBR Green QPCR master mix (Agilent technologies) using the Agilent Mx3005P QPCR system. (Primer sets for NMNAT3: 5′-ATGAAGAGCCGAATACCTGTG-3′ and 5′-CTAGCTTGTCTTGCCCTCAG-3′; and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 5′-TGATGACATCAAGAAGGTGGTGAAG-3′ and 5′-TCCTTGGAGGCCATGTGGGCCAT-3′). All the PCR reactions were performed in duplicates with at least three independent studies with following thermocycling conditions: 94 °C for 10 min followed by 40 cycles of 94 °C for 10 s, 60 °C for 20 s and 72 °C for 20 s. Each mRNA level was normalized with the internal GAPDH control. Quantification of the transcripts was made by the ΔΔCt method.

Analysis of NMNAT2 activity

NMNAT2 enzyme activity in the cytoplasmic samples was analyzed by a discontinuous assay system using alcohol dehydrogenase, and NADH formed was monitored at 340 nm as described.[9] Enzyme rate calculations were based on NADH extinction coefficient of 6220 $M^{-1} cm^{-1}$. One unit of enzyme activity was defined as the capacity to form 1 μmol of NADH per min.

Determination of IMPDH activity and NAD^+ concentration

Colorectal cancer cells were extracted, IMPDH activity and NAD concentrations were assayed as described.[9,14]

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Western blot analyses

Cell extracts (30 μg protein) were separated on 12% polyacrylamide gels and transferred to nitrocellulose membrane and probed with primary anti-hNMNAT2 antibody,[9] anti-IMPDH2 monoclonal antibody, anti-GAPDH antibody or anti-MTC 02 antibody (mitochondrial-specific marker), and bound antibody was probed with secondary anti-mouse or anti-rabbit horseradish peroxidase antibody (Antibody Solutions, Palo Alto, CA) and detected by enhanced chemiluminescent western blotting detection system (Molecular Imager, Gel-Doc XR$^+$, Bio-Rad Laboratories, Hercules, CA).

Determination of GTP and TAD concentrations in colorectal cancer cell lines

For the determination of GTP and TAD, colorectal cancer cell lines Caco2 and HT29 (3×10^6 cells) were treated with concentrations of Tiazofurin (Figure 6) or with saline for 4 h and cell extracts were prepared as detailed earlier.[33] A 10 μl aliquot of the neutralized extract was injected into Phenomenex Gemini 250 × 4.6 mm, 5 μm C18 110Å column (Torrance, CA) maintained at 29 °C, pre-equilibrated with buffer A (0.1 M potassium phosphate, pH 6.0) and then subjected to a stepwise gradient of buffer A with 20% methanol, pH 6.0 over 36 min and analyzed by Shimadzu high-performance liquid chromatography (Canby, OR). Under these conditions, GTP and TAD eluted at 10 and 22.5 min, respectively, separated from other nucleotides.

Preparation of nanoparticles

Folate-tethered nanoparticles and non-targeted nanoparticles containing either Tiazofurin or calcein of the following compositions were used in the study: folate-tethered nanoparticles were composed of distearoylphosphatidylcholine/cholesterol/polyethyleneglycol-folate-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (56:40:0.1 v/v); and non-targeted nanoparticles were composed of distearoylphosphatidylcholine/cholesterol/polyethyleneglycol-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (56:40:0.1 v/v). All lipids were obtained from Avanti Polar Lipids, Alabaster, AL. Briefly, 100 mg lipid mixture was dissolved in 3 ml chloroform and dried to a thin film in a round-bottom flask on a rotary evaporator under reduced pressure for 1 h. The dried lipid mixture was then rehydrated with 1 ml Tiazofurin (1.0 M in phosphate buffered saline, pH 7.4) or calcein (1.0 mM in phosphate buffered saline, pH 7.4) by constantly rotating the round-bottom flask at 60 °C with intermittent vortexing. The resulting suspension of multilamellar vesicles was then subjected to 10 cycles of freezing and thawing, sonicated for 10 min using an ultrasonic cleaner (EMC model 250, Hickory, NC) and extruded 10 times each, through 400-, 200- and 100-nm pore size polycarbonate membranes using a stainless steel extruder (Northern Lipid, Vancouver, British Columbia, Canada) circulated with 60 °C water. The resulting nanoparticles encapsulating either Tiazofurin or calcein were then separated from the free-drug on a Sepharose CL-4B column (10 × 1.5 cm) that was pre-equilibrated with phosphate buffered saline. The opaque nanoparticle fractions of 2 ml eluted in the void volume and the concentration of Tiazofurin was determined in a 50-μl aliquot. The samples were centrifuged at 18 000 *g* for 5 min to pellet nanoparticles and an aliquot of the supernatant was used to measure free Tiazofurin. To quantify entrapped Tiazofurin within the pellet, 0.5 ml 1% aqueous sodium dodecyl sulfate in water was added to solubilize nanoparticles, and the mixture was brought up to 1 ml with water and transferred to a quartz cuvette for reading absorbance at 238 nm in a spectrophotometer (Molecular Devices, Specra Max 250, Sunnyvale, CA). To measure the calcein concentration in nanoparticles, calcein fluorescence was resolved by excitation at 495 nm and emission at 515 nm using a fluorescence spectrophotometer (FluroMax-2, ISA Instruments, Edison, NJ).[24] The size distribution of the various nanoparticles preparations was determined by light scattering, and the median size of all nanoparticles preparations was 127 ± 36 nm in diameter. All nanoparticle containing samples were stored at 4 °C and used within 2 weeks of preparation. During this period, no significant leakage of calcein or Tiazofurin was detected by gel filtration, and <1% change in concentration was noted (data not shown).

FACS analysis of calcein encapsulated in folate-tethered and non-targeted nanoparticles

To analyze calcein uptake in nanoparticles, 0.2×10^6 cells growing in normal or LF medium were cultured and folate-targeted or non-targeted nanoparticles containing calcein (1–100 μM calcein) in 0.5 ml folic acid-free minimum essential media medium containing 10 or 20% FBS was added. To block the entry of calcein, cells were incubated with 1 mM folic acid (pH adjusted to 7.0) for 20 min at 37 °C before the addition of nanoparticles containing calcein, and cells were then incubated for 2 h at 37 °C on a shaking platform. The medium from the plates was aspirated, washed and immediately analyzed on a FACS.

Cytotoxicity assay

A dye-based cell proliferation assay kit (Promega, Madison, WI) was used to determine the cytotoxicity exhibited by wild-type and hNMNAT2-overexpressed cell lines towards Tiazofurin. Colorectal cancer cells (1500 cells/0.1 ml) were dispensed into 96-well tissue culture plates. In one set of experiments, cells were treated 24 h later with serial dilutions of free Tiazofurin and incubated for 72 h at 37 °C. In second set of experiments, cells were treated 24 h later with free Tiazofurin or with nanoparticles containing Tiazofurin for 2 h at 37 °C on a shaking platform, cells were aspirated to remove medium containing Tiazofurin and then fresh media was added and cells incubated for 72 h at 37 °C. After 72 h incubation, 20 μl 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium reagent was added to each well, mixed and further incubated for 2 h at 37 °C and the extent of color that developed in each reaction was read at 490 nm using a microplate reader (Spectra MAX 250, Molecular Devices). Cell-kill data were analyzed using the Graphpad Prism 4 software (GraphPad Software, San Diego, CA). Effective cell-kill concentration [EC_{50}] of Tiazofurin was defined as the concentration of the drug required to reduce cell proliferation by 50%.

Statistical analysis

All experiments were conducted at least three times with each data point carried out in triplicate. The observed results in the two different groups were compared using Student's *t*-tests for grouped data. Significance in all tests was set at a 95% or greater confidence level ($P < 0.05$).

RESULTS

Relationship of basal hNMNAT2 expression and Tiazofurin cell-kill in colorectal cancer cell lines

Western blot analysis for hNMNAT2 expression revealed highest signals in HCT15, moderate expression in HT29 and lower signals in Caco2 and HCC2998 cell lines when normalized against GAPDH (Figure 2a). By contrast effective cell-kill concentrations [EC_{50}] values after exposure to Tiazofurin in HCT15, HCT116, HT29, HCC2998 and Caco2 cell lines were 63.9, 72.6, 124.7, 274.3 and 450.0 μM, respectively (Figure 2b). There was a good correlation between hNMNAT2 expression and the effectiveness of Tiazofurin-induced cell-kill. Thus, HCT15 cell line that exhibited highest cell-kill with least Tiazofurin concentration also showed the highest level of hNMNAT2 expression; whereas Caco2 showed exactly the opposite. Indeed, the ratio of hNMNAT2 expression to Tiazofurin cell-kill in five colorectal cancer cell lines showed a significant inverse correlation with an R^2 value of 0.86 (Figure 2c).

Transfection with hNMNAT2 and its influence in colorectal cancer cell lines

Overexpression of hNMNAT2 was achieved in colorectal cancer cell lines Caco2 and HT29 using pTracer-hNMNAT2 mediated by lipofectamine. FACS analyses of GFP expression (indicated by an arrow) in Caco2 and HT29 clones confirmed overexpression of hNMNAT2 protein (Figures 2d and g). The Western blot analyses of FACS-positive cells revealed an approximate six- and threefold overexpression of hNMNAT2 in Caco2 and HT29 hNMNAT2-overexpressed cell lines, respectively, compared with their wild-type controls (Figures 2e and h). However, hNMNAT2 overexpression did not alter the expression levels of hNMNAT1 or hNMNAT3 in comparison with their wild-type counterparts in their regions of localization (nucleus and mitochondria) (Figures 2f and i). Therefore, these hNMNAT2-overexpressed cell lines of Caco2 and HT29 were used for further experiments.

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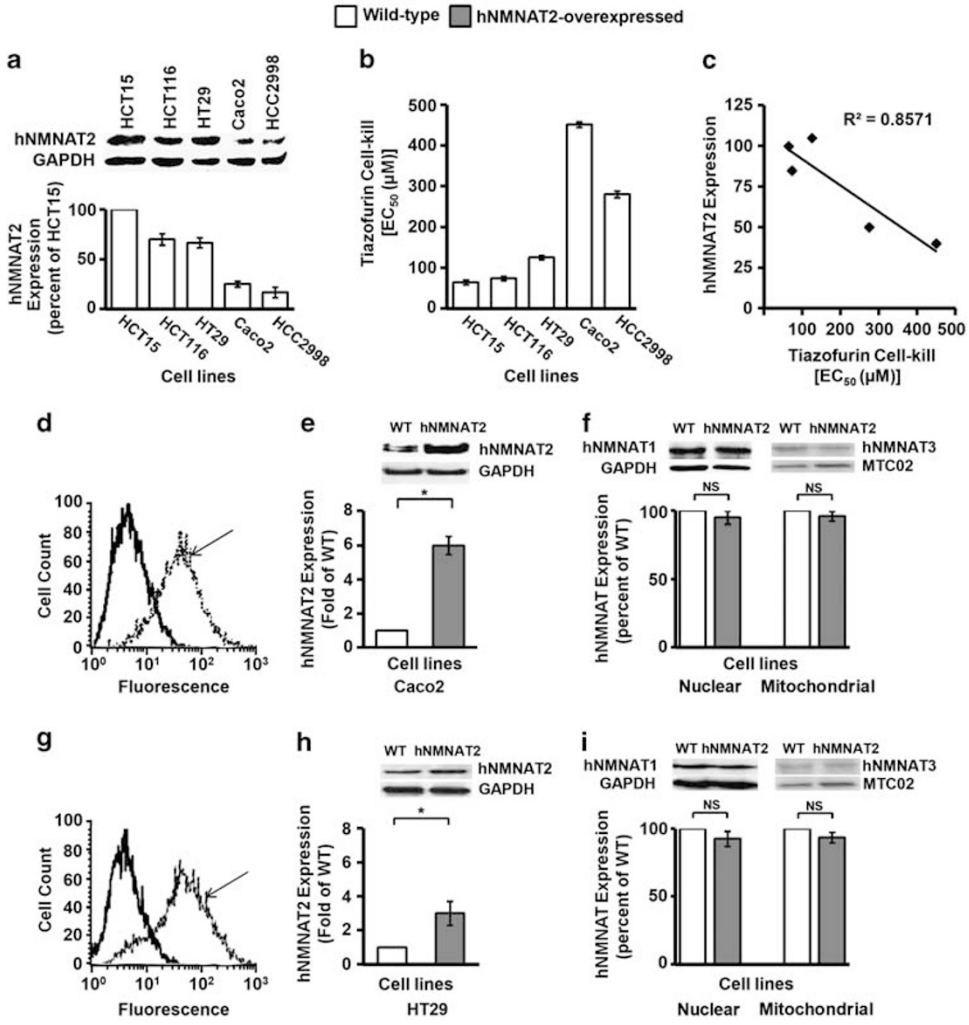

Figure 2. Relationship of hNMNAT2 expression with Tiazofurin cytotoxicity in human colorectal cancer cell lines. (**a**) Western blots analyses showing hNMNAT2 expression in colorectal cancer cell lines and the bar graph specifies pooled densitometric scanned data from three independent experiments of hNMNAT2 expression normalized by GAPDH expression shown as a percent of HCT15. (**b**) Cytotoxicity of colorectal cancer cell lines to Tiazofurin. (**c**) Regression analysis of relationship between hNMNAT2 expression and cytotoxicity to Tiazofurin. (**d**, **g**) FACS analysis of GFP fluorescence in wild-type and hNMNAT2-overexpressed Caco2 and HT29 cell lines (—wild-type; ----- hNMNAT2-overexpressed), arrow indicates fluorescence peak specific to GFP expression. (**e**, **h**) Western blot analyses of hNMNAT2 expression in wild-type (WT) and hNMNAT2-overexpressed colorectal cancer cell lines and pooled densitometric scanned data from three independent experiments. (**f**, **i**) Western blot analyses of hNMNAT1 and hNMNAT3 in WT and hNMNAT2-overexpressed colorectal cancer cell lines, Caco2 and HT29 and pooled densitometric scanned data from three independent experiments. Unless otherwise specified, the results are presented as the mean ± s.d. (error bars) from three independent sets of experiments ($n = 3$) with each data point performed in triplicate. Symbols, Asterisk denotes statistical significance ($P < 0.05$) and NS denotes no significance.

Both biosynthetic and salvage pathways for NAD^+ synthesis depend on NMNAT activity and perturbations in NAD^+ levels can affect cellular metabolism and survival. Hence we compared NAD^+ pools in wild-type and hNMNAT2-overexpressed Caco2 cell lines using continuously coupled NMNAT and alcohol dehydrogenase assay (Figure 3). The results demonstrated that there was no significant change in NAD^+ levels between wild-type and hNMNAT2-overexpressed cell lines (Figure 3a). As NAD^+ is one of the substrates for IMPDH activity, wherein *IMPDH1* is a cellular constitutive gene and *IMPDH2* is an inducible gene which is the target for Tiazofurin action,[34] we determined if IMPDH2 expression was altered in hNMNAT2-overexpressed cell line. The results demonstrated that there was no significant difference in the protein expression levels of IMPDH2 between wild-type and hNMNAT2-overexpressed cell lines (Figure 3b).

To establish the relationship of hNMNAT2 protein overexpression (determined by western blot analysis) with NMNAT enzyme activity, wild-type and hNMNAT2-overexpressed Caco2 cell lines were subjected to cytoplasmic extraction and NMNAT enzyme activity was determined. The results showed a 2–3-fold increased enzyme activity (Figure 3c), which correlated to some extent with hNMNAT2 protein overexpression determined by western blot analysis (Figure 2e).

Relationship of IMPDH2 inhibition with cell-kill by Tiazofurin

We next tested the hypothesis that TAD inhibits IMPDH, resulting in colorectal cancer cell-kill. Therefore, to delineate the effect of Tiazofurin treatment on IMPDH activity, the wild-type and hNMNAT2-overexpressed Caco2 cell lines were treated with sub-lethal concentrations of Tiazofurin for 4 h and IMPDH activity was determined. As the concentrations of Tiazofurin selected in this study were sub-lethal to the wild-type Caco2 cell line, there was no reduction in IMPDH activity (Figure 3d); however, the

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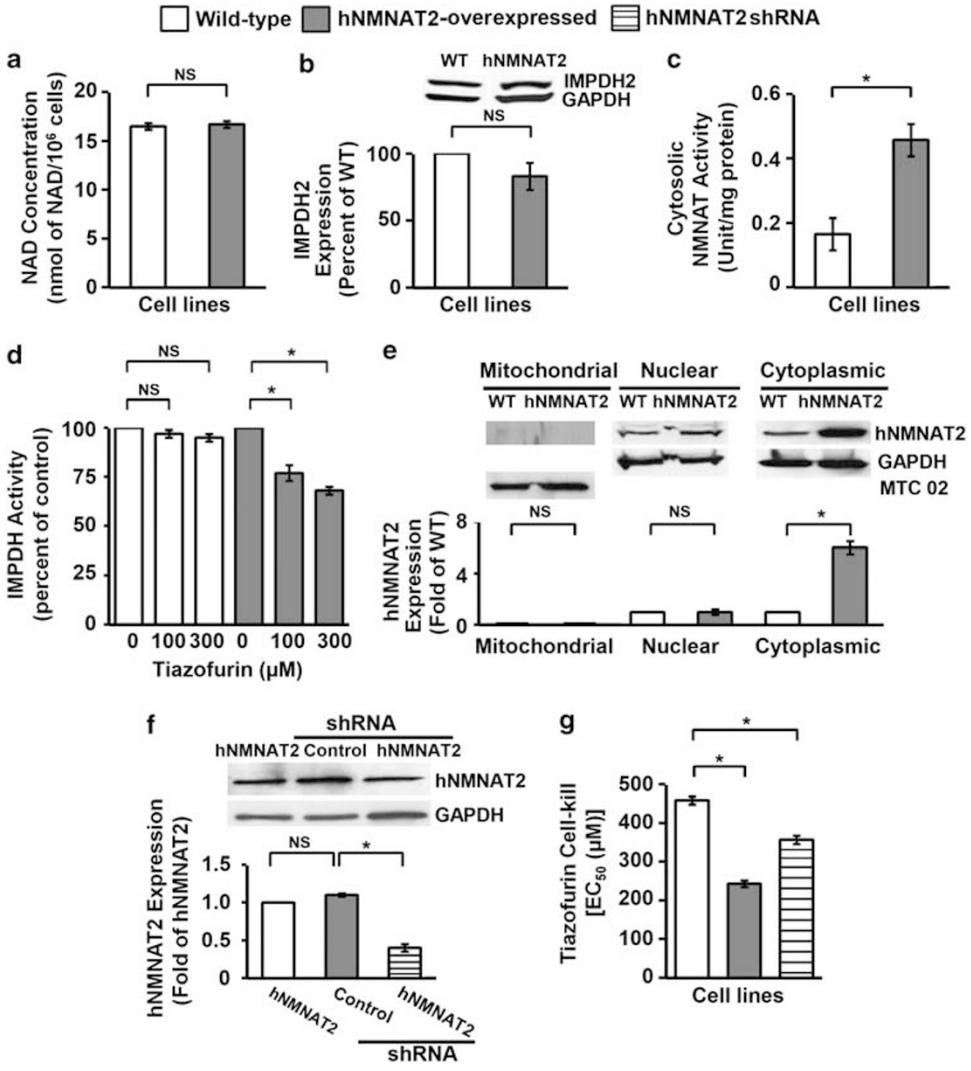

Figure 3. Consequence of hNMNAT2 overexpression in Caco2 cell lines. (**a**) Effect of hNMNAT2 overexpression on NAD concentration. Cells were processed and NAD levels were determined in the cytoplasmic extracts. **(b)** Western blot analyses of IMPDH2 expression in wild-type (WT) and hNMNAT2-overexpressed cell line. The bar graph of IMPDH2 expression was normalized to GAPDH and is shown as a percent of wild-type expression. (**c**) Cytosolic NMNAT activity in wild-type and hNMNAT2-overexpressed cell line. (**d**) The influence of Tiazofurin treatment on IMPDH enzyme activity in wild-type and hNMNAT2-overexpressed cell lines. Basal IMPDH activity in saline treated wild-type and hNMNAT2-overexpressed cell lines was 1.85 μM of XMP synthesized per mg protein per min. (**e**) Mitochondrial, nuclear and cytoplasmic expression of hNMNAT2 in wild-type (WT) and hNMNAT2-overexpressed Caco2 cell lines. (**f**) Silencing of hNMNAT2 expression in hNMNAT2-overexpressed cell line. Expression of hNMNAT2 in overexpressed cell line was silenced by shRNA hNMNAT2 and its expression was determined by Western blot analysis. (**g**) Cytotoxicity towards Tiazofurin in wild-type, hNMNAT2-overexpressed and hNMNAT2-silenced cell lines. Unless otherwise specified, the results are presented as the mean ± s.d. (*error bars*) from three independent sets of experiments ($n = 3$) with each data point performed in triplicate. Symbols, Asterisk denotes statistical significance ($P < 0.05$) and NS denotes no significance.

hNMNAT2-overexpressed cell line exhibited a significant inhibition of IMPDH activity (Figure 3d), suggesting a close relationship of IMPDH inhibition to Tiazofurin cell-kill. As shown in Table 1, EC_{50} of Tiazofurin was reduced by about 200% in hNMNAT2-overexpressed Caco2 and HT29 cell lines. These studies demonstrate that cytotoxic action of Tiazofurin was in good agreement with IMPDH inhibitory activity in the hNMNAT2-overexpressed cell line.

Cellular localization of hNMNAT2 overexpression

To confirm the overexpression of hNMNAT2 in colorectal cancer cells was confined to the cytoplasm, the wild-type and hNMNAT2-overexpressed Caco2 cell lines were subjected to differential extraction procedures to separate mitochondrial, nuclear and cytoplasmic fractions of the proteins. These fractions were subsequently tested for expression of hNMNAT2 by western blot and the intensity of the bands were normalized with respective housekeeping genes and compared using densitometric analysis. The results demonstrated a 2–3-fold higher expression of hNMNAT2 only in the cytoplasm of hNMNAT2-overexpressed Caco2 cell line with no increase of hNMNAT2 in other cellular compartments (Figure 3e).

Effect of silencing various hNMNAT isoforms on cell-kill by Tiazofurin

The hNMNAT2-overexpressing Caco2 cell line was silenced (~70%) by shRNA hNMNAT2 transfection as confirmed by western blot analyses (Figure 3f) it was further examined to

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Table 1. Effect of Tiazofurin on cell-kill in wild-type and hNMNAT2-overexpressed cell lines

Cell line	*Cytotoxicity (EC_{50} (μM))*		*Percent change (compared with wild-type)*
	Wild-type	*hNMNAT2 overexpressed*	
Caco2	450 ± 10	225 ± 6*	200
HT29	130 ± 8	80 ± 3*	163

Experimental details are provided in the Methods section.
*Denotes statistical significance ($P < 0.05$).

determine the influence of Tiazofurin treatment. Wild-type Caco2 cell line exhibited an EC_{50} of 450 μM, whereas hNMNAT2-overexpressed cell line showed a two-fold reduction in EC_{50} value (225 μM). However, silencing of hNMNAT2-overexpressed isoform with shRNA resulted in a partial reversal of cell-kill by Tiazofurin with an EC_{50} value of 380 μM, indicating that enhanced sensitivity to Tiazofurin was related to hNMNAT2 overexpression (Figure 3g).

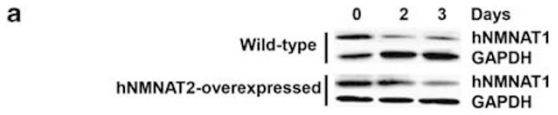
Next, we investigated whether the overall cell-kill with Tiazofurin was specifically related to hNMNAT2. To confirm this, we first silenced hNMNAT1 expression with siRNA hNMNAT1 in both wild-type and hNMNAT2-overexpressed Caco2 cell lines. The results showed that despite siRNA hNMNAT1 treatment for 3 days, which resulted in 80% reduction in hNMNAT1 expression (Figure 4a), there was no change in cell-kill by Tiazofurin in either wild-type or hNMNAT2-overexpressed Caco2 cell lines (Figure 4b). Likewise, in wild-type and hNMNAT2-overexpressed HT29 cell lines, an 80–85% reduction in hNMNAT1 expression (Figure 4c) had no significant effect in Tiazofurin-induced cell-kill (Figure 4d).

We then examined the outcome of silencing hNMNAT3 on Tiazofurin-induced cell-kill in these colorectal cancer cell lines. To determine the influence of silencing hNMNAT3 expression, we had to depend on mRNA expression rather than protein expression (because of technical difficulties in isolating and analyzing mitochondrial hNMNAT3 protein from very small numbers of siRNA-treated cells). Treatment with siRNA hNMNAT3 for 7 days did not affect the cell proliferation in either Caco2 or HT29 cell lines. However, although treatment of wild-type (Figure 5a) and hNMNAT2-overexpressed (Figure 5b) Caco2 cell lines with siRNA hNMNAT3 for 5 days resulted in about 80–90% reduction in mRNA expression of hNMNAT3, respectively, there was no change in the dose of Tiazofurin required to produce 50% cell-kill (Figure 5c). Parallel studies to silence hNMNAT3 expression in wild-type (Figure 5d) and hNMNAT2-overexpressed (Figure 5e) HT29 cell lines for 5 days with siRNA hNMNAT3, which resulted in about 80% decrease in mRNA expression of hNMNAT3 in both wild-type and hNMNAT2-overexpressed HT29 cell lines, respectively, also did not influence cell-kill by Tiazofurin (Figure 5f).

Taken together, our data demonstrated that overexpression of hNMNAT2 did not perturb expression of hNMNAT1 or hNMNAT3 isoforms. In addition, silencing of the endogenous expression of hNMNAT1 or hNMNAT3 did not alter Tiazofurin cell-kill in either wild-type or hNMNAT2-overexpressed colorectal cancer cell lines, demonstrating that overexpression of hNMNAT2 isoform that is localized in the cytoplasm resulted in intracellular activation of Tiazofurin to TAD in these cell lines and thereby inhibiting IMPDH that in turn results in reduced colorectal cancer cell proliferation.

Effect of Tiazofurin treatment on GTP concentration in wild-type and hNMNAT2-overexpressed colorectal cancer cell lines

Next, we sought to verify that overexpression of hNMNAT2 leads to greater activation of Tiazofurin to its active form, TAD, and results in a more profound inhibition of IMPDH which in turn,



b

Caco2 cell line	Control [EC_{50} (μM)]	siRNA hNMNAT1 [EC_{50} (μM)]	Reduction in protein expression (%)
Wild-type	450 ± 10	445 ± 10	80 ± 12*
hNMNAT2-overexpressed	225 ± 6	228 ± 8	80 ± 15*

d

HT29 cell line	Control [EC_{50} (μM)]	siRNA hNMNAT1 [EC_{50} (μM)]	Reduction in protein expression (%)
Wild-type	130 ± 8	127 ± 9	85 ± 14*
hNMNAT2-overexpressed	80 ± 5	85 ± 6	80 ± 10*

Figure 4. Influence of siRNA hNMNAT1 transfection on its expression and cell-kill by Tiazofurin in colorectal cancer cell lines. (**a**, **c**) Western blot analyses of hNMNAT1 expression in wild-type and hNMNAT2-overexpressed cell lines on siRNA hNMNAT1 transfection as a function of treatment (days). (**b**, **d**). Influence of 3-day treatment with siRNA hNMNAT1 on Tiazofurin cell-kill in colorectal cancer cell lines. The results represent the mean ± s.d. from three independent sets of experiments with each data point performed in triplicate. Unless otherwise specified, the results are presented as the mean ± s.d. (error bars) from three independent sets of experiments ($n = 3$) with each data point performed in triplicate. Symbols, Asterisk denotes statistical significance ($P < 0.05$).

leads to a reduction in GTP. Wild-type and hNMNAT2-overexpressed Caco2 and HT29 cell lines were incubated with either saline (control) or 50–400 μM Tiazofurin for 4 h. Caco2 cell lines exhibited 1.5-fold higher basal GTP level when compared with HT29 cell lines (Figure 6a versus c). As shown in Figure 6a, Tiazofurin treatment (100–400 μM) in the wild-type Caco2 cell line resulted in 27–42% decrease in GTP levels, whereas treatment with the same concentrations of Tiazofurin in the hNMNAT2-overexpressed Caco2 cell line resulted in higher reduction (33–62%) in GTP pools. Likewise, there was a 28–46% decrease in GTP pools following treatment of the wild-type HT29 cell line with Tiazofurin (50–150 μM), (Figure 6c), while similar treatment of Tiazofurin in the hNMNAT2-overexpressed HT29 cell line resulted in greater reduction (34–65%) in GTP levels (Figure 6c). Thus, in both cell lines, a Tiazofurin-induced reduction in GTP concentration correlated with higher levels of TAD in hNMNAT2-overexpressed cell lines (Figures 6b and d). In general, however, HT29 cell lines exhibited twofold higher levels of TAD compared with Caco2 cell lines, which was inversely related to the basal GTP concentration in these cells.

Cellular uptake of folate-tethered nanoparticles containing calcein or Tiazofurin via folate receptors in colorectal cancer cell lines

In all the previous experiments, free Tiazofurin was continuously incubated with cells for 72 h to induce cell-kill. In the following experiments to provide relevance to Tiazofurin administration in humans, Tiazofurin was incubated with cells only for 2 h. Therefore, we sought to determine whether folate receptor targeting through the use of Tiazofurin that was encapsulated in folate-tethered nanoparticles improved the efficiency of delivery

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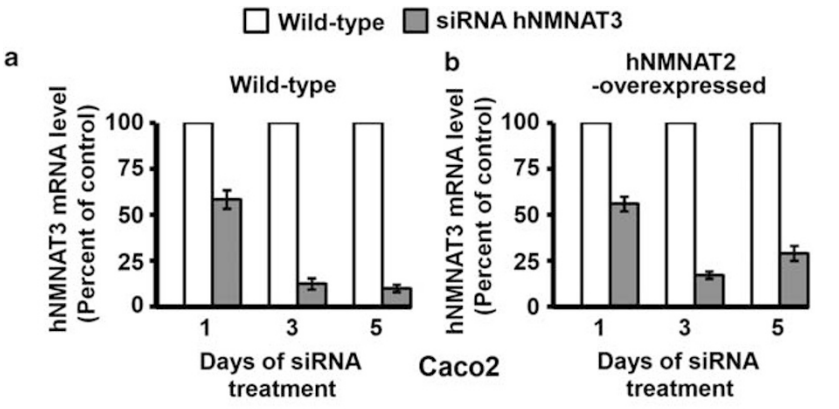
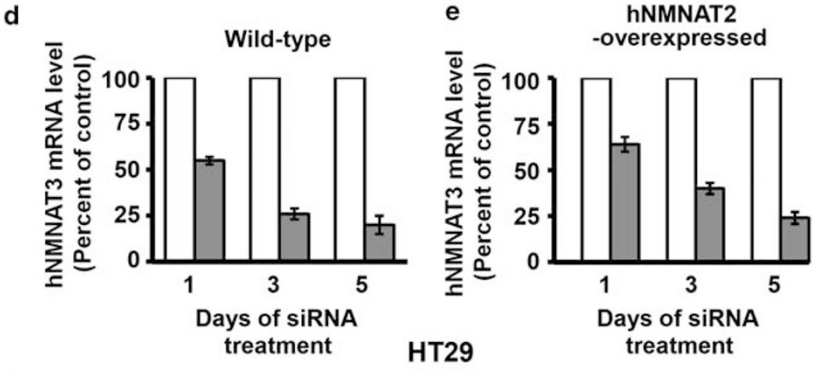


c

Caco2 cell line	Control [EC_{50} (μM)]	siRNA hNMNAT3 [EC_{50} (μM)]	Reduction in mRNA expression (%)
Wild-type	450 ± 10	458 ± 8	90 ± 3*
hNMNAT2-overexpressed	225 ± 6	228 ± 5	76 ± 6*



f

HT29 cell line	Control [EC_{50} (μM)]	siRNA hNMNAT3 [EC_{50} (μM)]	Reduction in mRNA expression (%)
Wild-type	130 ± 8	137 ± 5	79 ± 8*
hNMNAT2-overexpressed	80 ± 5	75 ± 6	75 ± 7*

Figure 5. Effect of siRNA hNMNAT3 transfection on mRNA expression and its influence on cell-kill by Tiazofurin in colorectal cancer cell lines. Quantitative real-time PCR determination of hNMNAT3 mRNA levels in Caco2 (**a**, **b**) and HT29 (**d**, **e**) cell lines following transfection as a function of treatment (days). (**c**, **f**) Influence of 5 days treatment with siRNA hNMNAT3 on Tiazofurin cell-kill in colorectal cancer cell lines. The results represent the mean ± s.d. from three independent sets of experiments with each data point performed in triplicate. Unless otherwise specified, the results are presented as the mean ± s.d. (error bars) from three independent sets of experiments ($n = 3$) with each data point performed in triplicate. Symbols, Asterisk denotes statistical significance ($P < 0.05$).

of Tiazofurin into colorectal cancer cells when compared with non-targeted nanoparticles containing Tiazofurin and free Tiazofurin. FACS analysis indicated a threefold and a twofold higher uptake of calcein encapsulated in folate-tethered nanoparticles by Caco2 and HT29 cell lines, respectively, when compared with calcein in non-targeted nanoparticles (results not shown).[24] The addition of folic acid before incubation with calcein encapsulated in folate-tethered nanoparticles profoundly decreased the mean uptake of calcein fluorescence by over 90%, suggesting that the specificity of calcein uptake was mediated via folate receptors (results not shown). With the wild-type Caco2 cell line, folate-tethered nanoparticles containing Tiazofurin exhibited an EC_{50} (cell-kill value) of 216 μM when compared with non-targeted nanoparticles, which showed EC_{50} of 410 μM and free Tiazofurin exhibited an EC_{50} of 2000 μM, indicating a nine-fold advantage in cell-kill when Tiazofurin was encapsulated in folate-tethered nanoparticles. By contrast, in the hNMNAT2-overexpressed Caco2 cell line an EC_{50} of 59 μM was obtained when Tiazofurin was encapsulated in folate-tethered nanoparticles (Table 2); this 29-fold reduction in EC_{50} was therefore attributable to the *hNMNAT2* gene therapy. Similarly with HT29 cell lines, the wild-type HT29 cell line exhibited EC_{50} of 81 μM when Tiazofurin was encapsulated in non-targeted nanoparticles, when compared with an EC_{50} value of 50 μM for Tiazofurin encapsulated in folate-tethered nanoparticles, which was a 30-fold reduction compared with free Tiazofurin administration. Moreover, hNMNAT2-overexpressed HT29 cell line exhibited

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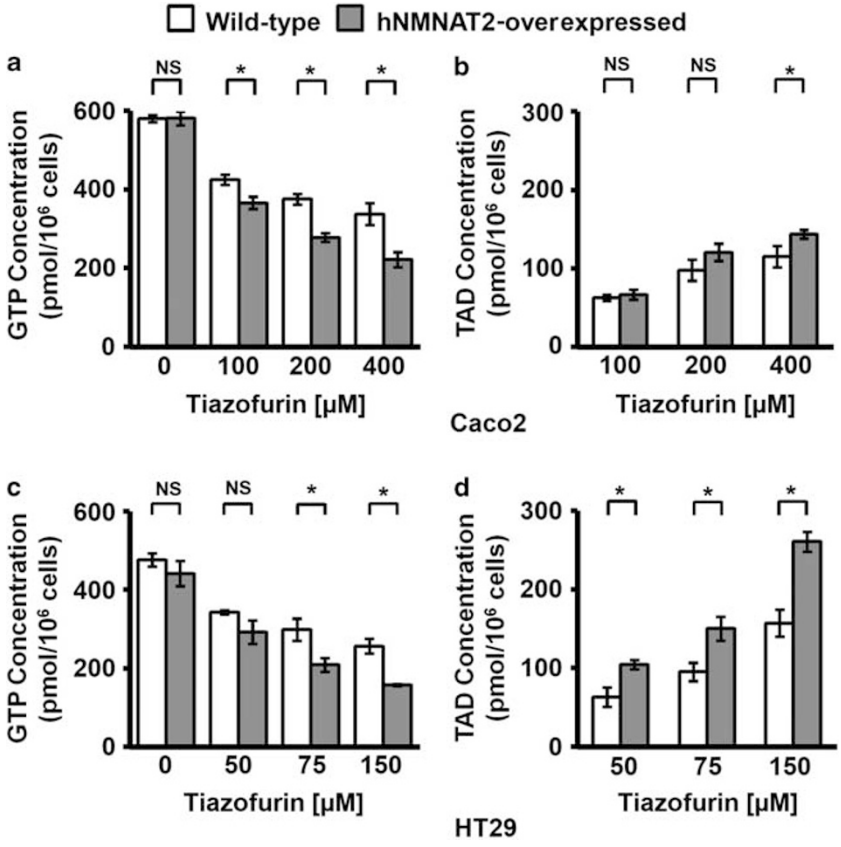

Figure 6. Effect of Tiazofurin treatment on GTP concentrations in colorectal cancer cell lines (**a**, **c**). Influence of Tiazofurin on TAD levels in wild-type and hNMNAT2-overexpressed Caco2 (**b**) and HT29 (**d**) cell lines. Unless otherwise specified, the results are presented as the mean ± s.d. (error bars) from three independent sets of experiments ($n = 3$) with each data point performed in triplicate. Symbols, Asterisk denotes statistical significance ($P < 0.05$) and NS denotes no significance.

Table 2. Mode of Tiazofurin delivery on colorectal cancer cell-kill

Tiazofurin delivery to cells	*Wild-type*		*hNMNAT2 overexpressed*	
	Tiazofurin cell-kill EC_{50} (μM)	*Fold change*[a]	*Tiazofurin cell-kill EC_{50} (μM)*	*Fold change*[a]
Caco2				
Free	2027 ± 240	1	1716 ± 375	1
Non-targeted	410 ± 9*	5	156 ± 5*	11
Targeted	216 ± 6*	9	59 ± 3*	29
HT29				
Free	1507 ± 430	1	1690 ± 516	1
Non-targeted	81 ± 3*	19	66 ± 7*	24
Targeted	50 ± 4*	30	22 ± 3*	73

Experimental details are provided in the Methods section.
*Denotes statistical significance ($P < 0.05$).
[a]Fold change compared with Free Tiazofurin cell-kill.

an EC_{50} of 22 μM for Tiazofurin when it was encapsulated in folate-tethered nanoparticles (Table 2). Thus, in hNMNAT2-overexpressed HT29 cell line, the concentration of Tiazofurin was further decreased by three-fold when encapsulated in folate-tethered nanoparticles bringing the total to 73-fold reduction (Table 2).

DISCUSSION

The dose of Tiazofurin approved for treatment of patients with chronic myelogenous leukemia in blast crisis is 2200 mg m^{-2} given as a 1-h infusion, which resulted in a peak plasma Tiazofurin concentration of 441 μM.[15,16,19] However, colorectal cancer cells are less responsive to Tiazofurin than chronic myelogenous leukemia cells as indicated by their differences in cytotoxic response.[14] As the use of an even higher Tiazofurin starting dose could induce increased drug side-effects and could interfere with treatment,[19] we investigated alternative approaches to improve the therapeutic index using Tiazofurin in colorectal cancer cell lines. Although TAD has been synthesized[35] and can potentially be encapsulated in nanoparticles for delivery into cancer cells, our earlier studies demonstrated that the activity of TADase, the enzyme which degrades TAD to Tiazofurin 5′-monophosphate and AMP, was high in colorectal cancer cell.[22,36] Therefore, induced overexpression of NMNAT and efficient delivery of the pro-drug Tiazofurin into cells to increase the concentration of TAD within the cytoplasm of colorectal cancer cells was a better logical approach.

As colorectal cancer cell lines express lower levels of hNMNAT2 compared with leukemia, hepatoma, neuroblastoma and other cancer cell lines,[14,22,37] we hypothesized that transfection-mediated

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upregulation of human NMNAT2 in colorectal cancer cell lines would make them more susceptible to Tiazofurin at lower concentrations. Our observation of minimal difference in EC_{50} cell-kill observed between wild-type colorectal cancer cell lines and their corresponding overexpressed hNMNAT2 cell lines might relate to short time of 2 h Tiazofurin exposure that may not be enough for the pro-drug activation. We compared colorectal cancer cell-kill by targeted delivery of Tiazofurin in relation to calculated doses of Tiazofurin administered to cancer patients.[19] These calculations indicated that about 6795–9000 mg m^{-2} dose would be required to provide EC_{50} (1500–2000 μM) equivalent response in colorectal cancer when free Tiazofurin is utilized. However, if Tiazofurin is encapsulated in non-targeted nanoparticles this dose can be reduced to 411–2060 mg m^{-2} (based on EC_{50} of 81–410 μM), which is less than currently administered dose of Tiazofurin in patients. It is likely that increased colorectal cancer cell sensitivity to Tiazofurin in liposomal nanoparticles may be related to longer circulation time of the drug similar to that seen with liposomal doxorubicin.[38] Encapsulating Tiazofurin in folate-tethered nanoparticles[39], further reduced the dose equivalent by about two-fold by encapsulating Tiazofurin in folate-tethered nanoparticles to EC_{50} of 22–59 μM in colorectal cancer cells, this is equivalent to a calculated Tiazofurin dose of 100–300 mg m^{-2} and about one-tenth of the current dose. Such doses have minimal toxicity in Phase I/II studies[15,16,19] and will significantly improve the therapeutic index of this preparation of Tiazofurin.

When we examined the functional impact of hNMNAT2 overexpression in cancer cells on several downstream parameters, we determined that overexpressed hNMNAT2 was confined to cytoplasm and did not perturb NAD^+ levels. This was similar to an earlier observation that overexpressed NMNAT1 in yeast did not alter NAD^+ under steady-state conditions.[40] Tiazofurin treatment of hNMNAT2-overexpressed cancer cells also led to an expected decrease in hIMPDH2 activity (without reducing IMPDH2 protein levels[34]) and this led to a concurrently lower GTP concentration. Silencing of hNMNAT2 overexpression with shRNA hNMNAT2 led to a partial reversal of cell-kill by Tiazofurin. Moreover, silencing the expression of hNMNAT1 or hNMNAT3 isoforms with siRNA did not alter Tiazofurin-induced cell-kill, suggesting that the hNMNAT2 isoform was able to convert the pro-drug to its active form. This was confirmed by measured TAD concentrations within cells.

A factor that may have influenced the effectiveness of Tiazofurin as a cytotoxic agent was the base-line levels of GTP in cells. Thus, the basal GTP concentration was about 1.5-fold higher in Caco2 cell lines than in HT29 cell lines. As a result, a higher concentration of Tiazofurin was required to achieve a similar cell-kill in Caco2 cells when compared with HT29 cells. This protective effect afforded by higher basal levels of intracellular GTP can potentially explain the susceptibility of various tissues to excess cytotoxicity of Tiazofurin. These data raise the possibility that the side-effects of Tiazofurin may be predicted based on the basal GTP concentration (that is, more GTP leads to less side-effect). Conversely, an increase in hNMNAT2 levels could also allow for more pro-drug conversion to its active form leading to more cytotoxicity and side-effects. Such studies are under investigation in our laboratory.

Our approach with the induced overexpression of hNMNAT2 requires additional refinement to ensure selectivity in expression of the gene in colon cancer cells *in vivo*. Others have employed specific promoters such as carcinoembryonic antigen to ensure selectivity of expression in target tissues/cells.[41] Other gene-directed enzyme pro-drug therapy systems have been successful in providing cancer cell-kill. For example, Herpes simplex virus thymidine kinase with ganciclovir therapy was shown to be effective in breast cancer cells[42] and *E. coli* cytosine deaminase with 5-fluorocytosine[43] treatment was cytotoxic to human lung, pancreatic and cervical cancer cell lines. We have earlier shown a relationship between transduction of folate receptor-α gene using adeno-associated virus2 into human cervical cancer HeLa cells, which resulted in activation of thymidine kinase leading to increased sensitivity of cancer cells to 3′-azido-3′-deoxythymidine.[44] This is in line with activation of ganciclovir by Herpes simplex virus thymidine kinase resulting in increased cytotoxicity to breast cancer cells.[42]

In summary, we have described relationship of hNMNAT2 expression with sensitivity to Tiazofurin in colorectal cancer cells. The increase in TAD synthesis led to a decrease in hIMPDH2 activity with concurrent lower GTP concentration and reduced EC_{50} value. Increased delivery of Tiazofurin through the targeting folate receptors on cancer cell surfaces through the use of encapsulated Tiazofurin markedly reduced the EC_{50} by about 30- to 73-fold. The overexpression of folate receptors can be manipulated to target folate receptors for treatment of tumors that express folate receptors, thereby avoiding uptake by most healthy tissues that express few if any of folate receptors.[45] Consequently, these studies demonstrate the feasibility of developing a targeted (folate receptor), gene-directed (*hNMNAT2*) enzyme pro-drug (Tiazofurin) therapy for colorectal cancer and elucidates methods to improve therapeutic efficacy of Tiazofurin in colorectal cancer.

ABBREVIATIONS

FR, folate receptors-α; IMPDH, inosine 5′-monophosphate dehydrogenase; NMNAT, nicotinamide 5′-mononucleotide adenylyltransferase; TAD, thiazole-4-carboxamide adenine dinucleotide; TRMP, tiazofurin 5′-monophosphate; TR, Tiazofurin (2-ß-D-ribofuranosylthiazole-4-carboxamide).

CONFLICT OF INTEREST

A provisional patent application based on this work was filed by the technology transfer program of the United States Department of Veteran Affairs, Washington, DC for Drs Hiremagalur N Jayaram, Asok C Antony and Praveen Kusumanchi. The remaining authors, Drs Yonghua Zhang, Mehul B Jani, Nagesh H Jayaram, Rehana A Khan and Yingsheng Tang declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

HNJ and ACA contributed to conception and design of the research, acquision of data, analysis and interpretation of data, drafting of the manuscript. PK, YZ, MBJ, NHJ, RAK and YT contributed to the acquisition of data, analysis and interpretation of data and final approval of the manuscript.

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