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RESEARCH ARTICLE

Preclinical models of nicotinamide phosphoribosyltransferase inhibitor-mediated hematotoxicity and mitigation by co-treatment with nicotinic acid

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Abstract

Nicotinamide adenine dinucleotide (NAD) is an essential co-factor in glycolysis and is a key molecule involved in maintaining cellular energy metabolism. Nicotinamide phosphoribosyltransferase (NAMPT) catalyzes the rate-limiting step of an important salvage pathway in which nicotinamide is recycled into NAD. NAMPT is up-regulated in many types of cancer and NAMPT inhibitors (NAMPTi) have potential therapeutic benefit in cancer by impairing tumor metabolism. Clinical trials with NAMPTi APO-866 and GMX-1778, however, failed to reach projected efficacious exposures due to dose-limiting thrombocytopenia. We evaluated preclinical models for thrombocytopenia that could be used in candidate drug selection and risk mitigation strategies for NAMPTi-related toxicity. Rats treated with a suite of structurally diverse and potent NAMPTi at maximum tolerated doses had decreased reticulocyte and lymphocyte counts, but no thrombocytopenia. We therefore evaluated and qualified a human colony forming unit-megakaryocyte (CFU-MK) as *in vitro* predictive model of NAMPTi-induced MK toxicity and thrombocytopenia. We further demonstrate that the MK toxicity is on-target based on the evidence that nicotinic acid (NA), which is converted to NAD via a NAMPT-independent pathway, can mitigate NAMPTi toxicity to human CFU-MK *in vitro* and was also protective for the hematotoxicity in rats *in vivo*. Finally, assessment of CFU-MK and human platelet bioenergetics and function show that NAMPTi was toxic to MK and not platelets, which is consistent with the clinically observed time-course of thrombocytopenia.

Keywords

Colony forming unit-megakaryocyte, hematotoxicity, platelet

History

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Introduction

Nicotinamide phosphoribosyltransferase (NAMPT) is a key enzyme involved with cellular energy metabolism through nicotinamide adenine dinucleotide regeneration. Cells require the constant replenishment of NAD for survival due to the constitutive activity of NAD degradative enzymes including poly and mono(ADP-ribose) polymerases, CD38 ecotenzymes and the sirtuins (Houtkooper et al., 2010). *De novo* synthesis of NAD from tryptophan is insufficient to keep pace with NAD degradation and utilization; therefore, salvage pathways catalyzed by NAMPT and nicotinic acid phosphoribosyltransferase domain containing 1 (NAPRT1) are crucial for NAD regeneration (Houtkooper et al., 2010). Cells that are starved of NAD deplete their ATP supply, and die due to autophagy or apoptosis (Montecucco et al., 2013; Pittelli et al., 2010).

Nicotinamide phosphoribosyltransferase inhibition is a potentially promising pharmacologic target to inhibit tumor growth for several reasons. Tumor cells have greatly increased glycolytic activity and NAD requirement compared to healthy cells (Vander Heiden et al., 2009), tumors are dependent on NAD salvage pathways due to the absence of *de novo* synthesis of NAD (Xiao et al., 2013), and NAMPT expression is up-regulated in tumors (Bi & Che, 2010). Accordingly, several small molecule NAMPT inhibitors (NAMPTi) have been evaluated as anticancer agents (Konieczna et al., 2013; Olesen et al., 2010; Vander Heiden et al., 2009).

Clinical trials with two structurally diverse NAMPTi, APO-866 (also known as FK-866) and GMX-1778 (also known as CHS-828), failed to demonstrate sustained efficacy in cancer patients due to dose-limiting, Grade 3-4 thrombocytopenia with platelet count nadirs occurring between days 10 and 15 of dosing (Holen et al., 2008;

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Hovstadius et al., 2002). Patients also had a generally mild Grade 2-3 lymphopenia and anemia (Holen et al., 2008; Hovstadius et al., 2002; von Heideman et al., 2010). These hematological toxicities are considered to be the major class liability of NAMPTi in the clinic. Preclinical evidence of NAMPTi- thrombocytopenia is limited to one report of GMX-1778 at lethal doses causing an acute reduction in platelet counts of B6D2F1 mice (Olesen et al., 2010). The relevance of this observation to clinical thrombocytopenia is not clear, given the preclinical dose used (above maximum tolerated dose, MTD). It has also not been established whether NAMPTi have direct effects on circulating platelets or primarily suppress platelet production by toxicity to bone marrow megakaryocytes (MK), although the delayed timing of thrombocytopenia observed clinically post-dose is most consistent with an effect on MK (Kenney & Stack, 2009). The identification of the cellular target of toxicity is important when selecting an appropriate model to investigate strategies of risk mitigation during preclinical drug development prior to first in human trials.

Our objective in this work was to establish a translatable preclinical model of NAMPTi-induced thrombocytopenia to inform decision-making for early risk mitigation and candidate selection. By using a structurally diverse suite of NAMPTi, including compounds associated with clinical thrombocytopenia, we qualified the human CFU-MK assay as an *in vitro* toxicity model and showed that preclinical species (mouse, rat, monkey) did not adequately model the thrombocytopenia. Furthermore, we demonstrated that the mechanism of toxicity was on-target based on the similarity of the hematotoxicity across chemically distinct NAMPTi, and the abrogation of NAMPTi toxicity on CFU-MK *in vitro* and hematotoxicity in rats *in vivo* by nicotinic acid (NA) co-treatment. We also determined that NAMPTi was toxic to MK and not platelets, which is consistent with the clinically observed time-course of thrombocytopenia.

Materials and methods

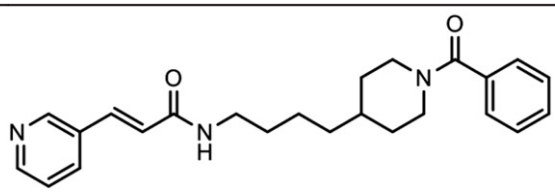
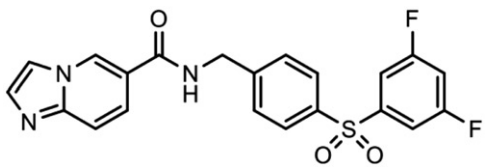
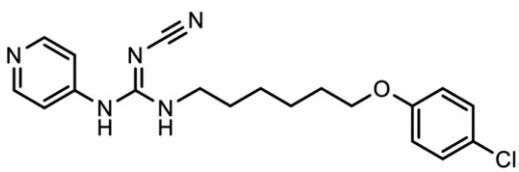
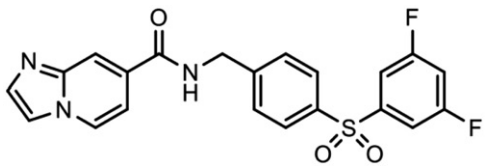
Synthesis and structures of NAMPTi reagents

The syntheses, chemical structures and cellular potencies associated with the NAMPTi studied in this work are summarized in Table 1. GNE-643 is a close structural isomer of GNE-617 but has reduced potency in cell culture experiments of NAMPT activity (Oh et al., 2014). Thus, GNE-643 served as a key reference compound for many of the assessments described in this work to distinguish toxicity related directly to NAMPT inhibition versus off-target effects. Studies were performed with all four NAMPTi unless otherwise noted.

Rat toxicity studies

In separate studies of up to 15 days duration, Sprague-Dawley (Crl:CD) rats (Charles River Labs, Hollister, CA), typically five males per group, were administered vehicle (60%

Table 1. Chemical structures and cellular potencies of different NAMPTi evaluated for hematopoietic toxicity *in vitro* and *in vivo*.

NAMPTi		Cellular IC_{50} (nM)*	Cell potency reference	Synthesis reference
APO-866		1	Zheng et al. (2013b)	Galli et al. (2008)
GNE-617		2	Zheng et al. (2013b)	Zheng et al. (2013b)
GMX-1778		5	Zheng et al. (2013b)	Schou et al. (1997)
GNE-643		360	Oh et al. (2014)	Oh et al. (2014)

*A2780 cancer cell proliferation inhibition.

PEG400, 10% EtOH and 30% sterile water) or the following NAMPTi: GNE-617 or GMX-1778 up to 30 mg/kg/day once daily by oral gavage (PO); GMX-1778 at 100/mg/kg/day PO, or GNE-643 at 60 mg/kg/day twice daily by PO; or APO-866 at 60 mg/kg/day twice daily intraperitoneally all at a dose volume of 5 mL/kg with the exception of GNE-643 that had a dose volume of 10 mL/kg. In one study, NA at 120 mg/kg/day (formulated as a solution in water at a dose volume of 7 mL/kg) twice daily by oral gavage (equivalent to a pharmacologically relevant human dose) was administered as a single agent or co-administered with GNE-617 at 30 mg/kg/day once daily, for 7 days. Clinical pathology and anatomic pathology evaluation were performed 24 h following the final dose. Blood was collected from the retro-orbital plexus under isoflurane anesthesia for routine clinical pathology evaluation on a Cobas Integra 400 (Roche Diagnostics, Indianapolis, IN). Blood samples for hematology analysis were analyzed on a Sysmex XT 2000iV (Sysmex America, Inc, Mundelein, IL). Systemic drug concentrations were measured in separate toxicokinetic groups during the dosing period. The systemic exposures were equivalent or greater than those that demonstrated efficacy in mouse tumor xenograft models (data not shown). Toxicokinetic profiles are provided by Zabka et al. (2015). Dose-limiting toxicity and lethality (not related to hematotoxicity) was generally observed at the highest dose level tested among the NAMPTi. The exception was the less potent structural analog (GNE-643), which did not have dose limiting toxicities in the 6-day rat study (Misner et al., personal communication; Zabka et al., 2015;).

All studies were conducted according to a written study protocol and approved by an Institutional Animal Care and Use Committee in compliance with national regulations on animal welfare standards.

NAMPT and NAPRT1 mRNA analysis

Megakaryocytes derived after 14-day *in vitro* differentiation of human primary CD34 + hematopoietic stem cells, or bone marrow mononuclear cells, were analyzed for NAMPT and NAPRT1 mRNA expression. Total RNA was extracted from frozen cell pellets using the miRNeasy mini Kit (217004, Qiagen, Valencia, CA). Samples were processed according to manufacturer's instructions. All extractions were performed with an on-column RNase-free DNA digestion (79254, Qiagen, Germantown, MD). Total RNA was quantified by Nanodrop (Thermo Scientific, Wilmington, DE). mRNAs were converted to cDNA with reagents from bulk version of High Capacity cDNA Reverse Transcription Kit (4368814, Life Technologies, Carlsbad, CA) or the High Capacity RNA-to-cDNA Kit (4387406, Life Technologies). Reverse transcription (RT) reactions were performed in a total volume of 50 µL, with a standardized RNA input across samples. Reactions were performed in a thermal cycler according to manufacturer's instructions. qPCR was performed in 384-well format on the ViiA 7 (Life Technologies) according to standard protocols. Briefly, 2X Taqman Universal Master Mix II (4440040, Life Technologies) was mixed with a 20X Taqman assay (Life Technologies) and a cDNA input standardized across sample types, in a total volume of 20 µL. mRNA was quantified by a 40-cycle qPCR reaction. Samples were quantified with Human NAMPT (Hs00237184_m1), Human NAPRT1 (Hs00376971_g1) and Human GAPDH (Hs02758991_g1). All assays were performed in triplicate with concurrent appropriate blanks.

The quality of amplification curves was confirmed by inspection. Further analysis was performed using Spotfire (TIBCO Software 5, Somerville, MA) to normalize sample input by the endogenous reference, GAPDH. Data were expressed as the normalized dCt.

CFU-MK assays

Clonogenic megakaryocyte (CFU-MK) progenitors , from human and cynomolgus monkey donor marrow were assessed in a collagen-based system (StemCell Technologies, Vancouver, BC, Canada) containing recombinant human (rh)IL-3 (10 ng/mL), rhIL-6 (10 ng/mL) and rhTpo (50 ng/mL). Mouse CFU-MK were assessed in a collagen-based system (StemCell Technologies) containing recombinant murine (rm) IL-3 (10 ng/mL), rhIL-6 (10 ng/mL) and rhTpo (50 ng/mL). Various concentrations of NAMPTi (1000, 100, 30, 10, 3, 1 and 0.1 nM) were added to these media formulations for human, monkey and mouse samples. The NAMPTi APO-866, GMX-1778 and GNE-617 (0–100 nM) were combined with NA (1000, 100 and 10 nM) in human samples. Human bone marrow ($n = 3$) (ReachBio, Seattle, WA) or cynomolgus monkey bone marrow mononuclear cells ($n = 2$) (AllCells, Alameda, CA) were added at concentrations of $7–10 \times 10^4$ per culture, in triplicate and incubated for 14 days. The gel was extracted from the culture dish, placed on glass slides, fixed in methanol/acetone (Fisher Scientific, Waltham, MA), and stained using an anti-human CD41a antibody with an alkaline phosphatase detection system (StemCell Technologies). For mouse CFU-MK, bone marrow cells from the femur of 7–8 week old Balb/C mice were added at concentrations ranging from 8 to 22×10^4 per culture, in triplicate cultures (each replicate was from a different donor) and incubated for 7 days. The gel was extracted from the culture dish, placed on glass slides, fixed in acetone and then stained for acetylcholinesterase and CFU-MK were enumerated.

We were unsuccessful in our attempts to develop a rat CFU-MK assay. Human and mouse formulations were tested, substituting rat cytokines for the human cytokines where available (i.e. IL-6 and IL-3). However, the colony number was exceptionally low despite cell concentrations greater than the number used for mouse or human CFU-MK assays. The same rat BM cells supported myeloid CFU-GM (not part of the current study), which suggested the cells were viable and functional. We are aware of only one report of successful development of a rat CFU-MK assay, published after our studies were completed (Evstatiev et al., 2014).

MK cytotoxicity assay

To assess cytotoxicity of NAMPTi on MK at various stages of differentiation, human MK were differentiated from CD34 + hematopoietic stem cells (HSCs) in liquid culture. HSC from three separate donors were isolated from bone marrow mononuclear cells (BM-MNCs, from AllCells) using the CD34 MicroBead Kit, an MS Column, and a MiniMACS

Separator (Miltenyi Biotec Inc., Auburn, CA). The isolated cells were cultured in ultra-low attachment flasks (Cat # CLS3814-24EA, Sigma, St. Louis, MO). HSCs were expanded for 5 days at 37 °C/5% CO_2/85% RH in StemSpan serum-free medium (Cat # 09650, StemCell Technologies) supplemented with SCF, IL3, IL6 and Flt3 (Peprotech, Rocky Hill, NJ). On the 5th day, cells were re-suspended in MK differentiation medium (SCF, TPO, IL-3,IL-6,IL-9 and IL-1b prepared at Genentech Inc., South San Francisco, CA) with added NAMPTi (0.3, 1, 3, 10, 30, 100 nM) and NA (1000 nM) or vehicle and transferred to a low attachment 6-well plate (Sigma, Cat # CLS3471-24EA) at a density of 750 000 cells/well and incubated for 14 days to obtain mature MKs. Aliquots of the MK cultures at days 3, 6, 9 and 14 of treatment were transferred to a 96-well black/clear bottom plate (Perkin Elmer, Akron, OH). An equal volume of CellTiter-Glo luminescence Cell viability reagent (Promega, Cat # G7570) was added to the plate. Plates were shaken for 2 min at 750 rpm and incubated at room temperature in the dark for 10 min. The luminescence signal was measured on a PerkinElmer Envision instrument.

Platelet function testing

Platelet-rich plasma (PRP) was isolated by low speed centrifugation (200*g* for 20 min) from acid citrate dextrose-anticoagulated whole blood collected from healthy human donors. For light transmission aggregometry (LTA), PRP was treated with NAMPTi (10, 20, 30 and 50 μM) then stimulated with 30 μM Trap6 platelet agonist (Anaspec, Fremont, CA) to determine if NAMPTi could affect agonist-induced aggregation, or treated with 50 μM NAMPi alone to determine if the inhibitors were proaggregatory. Aggregation was measured on an aggregometer (Chrono-Log 700; Chrono-Log Corporation, Havertown, PA). For the 50 μM NAMPTi treatment, platelet aggregometry was performed both immediately on addition of the NAMPTi and after 1 h of pre-incubation with NAMPTi.

Platelet activation markers were measured by flow cytometry (BD LSR Fortessa cell analyzer, San Jose, CA) on PRP stimulated with a Trap6 agonist, immediately after an addition of 50 μM NAMPTi or following a 1 h pre-incubation with the NAMPTi. Activation of CD61 expressing platelets was assessed by measuring the expression of PAC and CD62p (BD Biosciences, San Jose, CA) by flow cytometry.

Platelet bioenergetics

Platelet-rich plasma was re-suspended in unbuffered Dulbecco's Modified Eagle Medium to a final concentration of 30×10^6 cells/150 μL and treated with 50 μM NAMPTi, or 30 mM of Trap6 platelet agonist as the positive control and 2 μM oligomycin to inhibit ATP synthetase as the negative control. The Seahorse XF96 analyzer (Seahorse Bioscience, North Billerica, MA) was used to measure mitochondrial respiration by oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of platelets after the addition of compounds.

Statistical analysis

Two-tailed Student's *t*-test performed in CFU-MK assays showed significance at $p < 0.01$ and $p < 0.001$. For rat hematology parameters, independent Student's *t*-tests and one-way ANOVA with Dunnett's multiple comparison *post hoc* testing was performed with significance shown at $p < 0.01$ and $p < 0.001$.

Results

Characterization of the hematologic toxicity of NAMPTi in the rat

In acute rat toxicity studies of up to 15 days dosing, administration of potent NAMPTi, APO-866, GMX-1778, GNE-617 and GNE-643 were associated with moderately-markedly decreased circulating lymphocyte and/or reticulocyte counts (Figure 1). However, there was no reduction in platelet counts despite exposures to APO-866 and GMX-1778 that exceeded clinical exposures (Free *C*max 6.73 μM or 0.115 μM for APO-866 or GMX-1778, respectively). As suppression of bone marrow platelet production may take time to impact the circulating platelet pool, we also measured platelet counts at one and two weeks recovery from GNE-617 dosed for 5 days at the maximum tolerated dose and duration. Platelet counts were also not reduced in the non-dosing recovery period (data not shown).

Hematopoietic tissue changes were characterized microscopically by decreased lymphocyte cellularity in lymph nodes, thymus and spleen, decreased extramedullary hematopoiesis in the spleen, and decreased cellularity in the bone marrow due to decreases in lymphoid and erythroid lineages (Figure 2). At non-tolerated doses of NAMPTi due to cardiac insufficiency (Misner et al., personal communication), megakaryocytes were relatively spared despite a marked decrease in bone marrow cellularity (Figure 2). These findings demonstrate that NAMPTi have a different hematotoxicity profile in the rat compared to humans, specifically, rats do not exhibit thrombocytopenia. Conversely, rats do manifest the lymphoid and erythroid toxicity observed in humans. The reason for this lineage-specific hematopoietic toxicity profile in rat versus human is not understood.

Human CFU-MK are a model of NAMPTi induced MK toxicity

Given that administration of NAMPTi was not associated with decreased platelet counts in rats, the rat was not a preclinical model of clinical NAMPTi-related thrombocytopenia. Therefore, we first investigated whether *in vitro* human CFU-MK assays were a suitable model system. Clonogenic assays performed in a semi-solid media measure cytotoxic effects on megakaryocyte progenitors (CFU-MK) by enumerating the number of mature MK colonies generated from hematopoietic stem cells (Pessina et al., 2009).

We confirmed the NAMPTi drug target and NAPRT1 salvage pathway expression in human MKs cultured from bone marrow progenitors. Both were expressed in the majority of donors, however, levels of NAPRT1 showed considerable inter-individual variability (Figure 3).

Treatment with potent NAMPTi APO-866, GMX-1778 and GNE-617 was associated with profound decreases in human CFU-MK (Figure 4 and Table 2). GNE-643, the less potent inhibitor, showed a much-reduced effect on CFU-MK

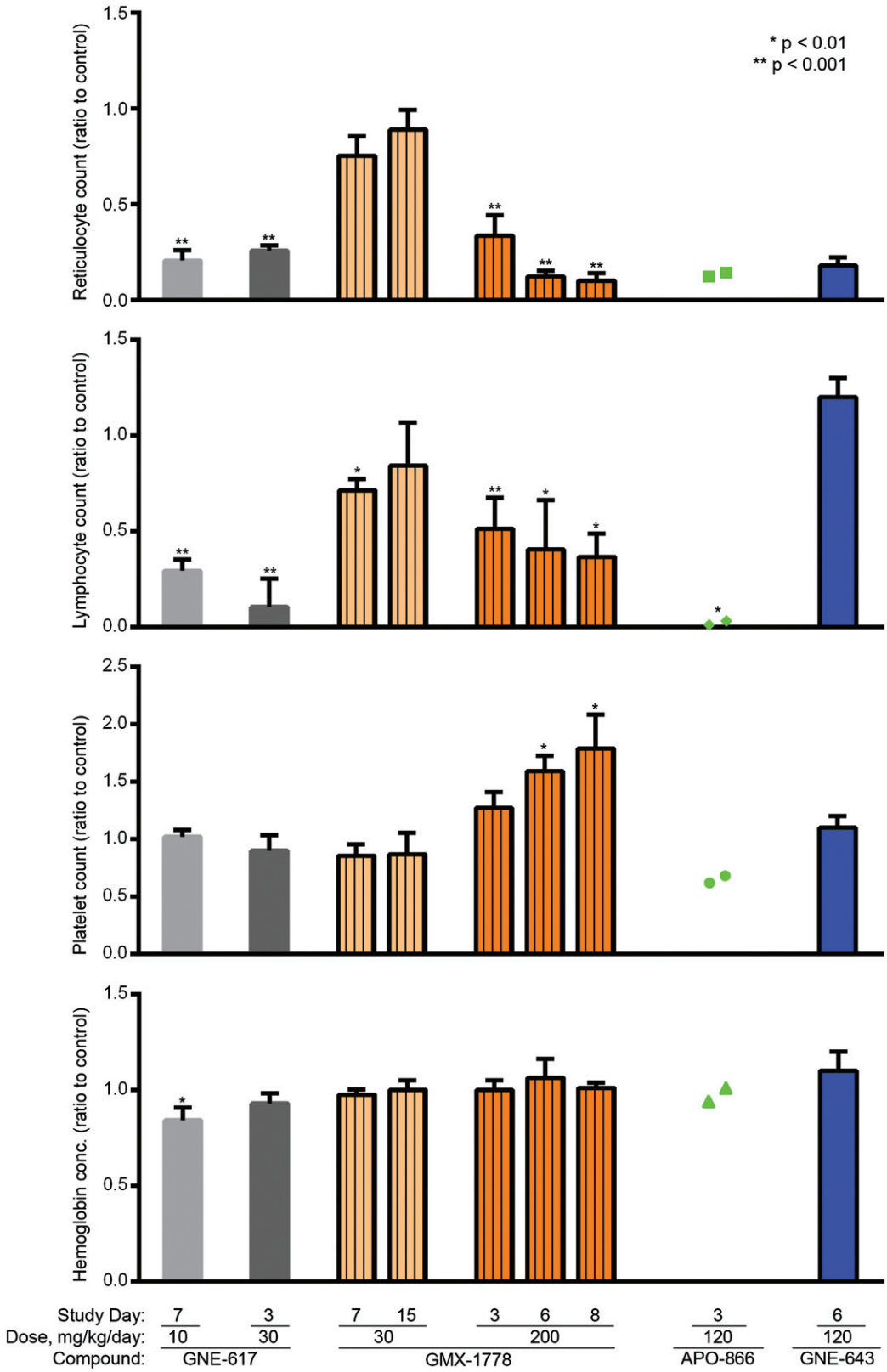


Figure 1. Hematologic effects of NAMPTi in rats. Rats were treated once or twice daily with the noted doses of various NAMPTi for up to 15 days, followed by hematology evaluation 24 h after the last dose. Hematology data are presented relative to vehicle control groups with mean and SD excepting APO-866 with data points displayed for the two surviving rats. Reticulocyte and lymphocyte counts were generally reduced by treatment with various NAMPTi (*$p < 0.01$, **$p < 0.001$); however, there was no consistent reduction in platelets.

in the same experiments. This finding was consistent with an effect on proliferation and/or viability of MK progenitors.

We evaluated mouse and monkey CFU-MK to inform species selection to assess NAMPTi-related thrombocytopenia. We were unsuccessful in our attempts to develop a Rat CFU-MK assay. Mouse CFU-MKs were generally less sensitive to NAMPTi toxicity compared to monkey and human CFU-MKs and monkey was an order of

magnitude less sensitive than human CFU-MK for two of the four NAMPTi (Table 2). These data confirmed that human CFU-MK was the most suitable model system for assessing the NAMPTi-related thrombocytopenia *in vitro* and to explore strategies to mitigate this toxicity in a clinically relevant manner.

To determine whether cell death contributed to a decrease in CFU-MK, we performed a biochemical assessment of cytotoxicity in a liquid cell culture system that differentiated primary CD34 + human stem cells to MK. Potent NAMPTi at concentrations up to 100 nM were associated with dose-dependent decreases in cellular ATP levels to <10% control consistent with cell death (Figure 5A and B). The less potent NAMPTi analog GNE-643 was not associated with any discernible decreases in cellular ATP in human MK cultures. Cytotoxicity was demonstrated beginning at day 3 of the 14-day period of MK differentiation and maturation (the first time-point analyzed) with the maximum effect beginning from day 6 (Figure 5 and Supplementary Figure 1). The cytotoxicity of MK lineage cells tracked with NAMPTi anti-proliferative potency in the human A2780 cancer cell line (Table 1). Together these investigations show a remarkable decrease in the clonogenicity and viability of MK progenitors in response to NAMPTi consistent with a reduced capacity for platelet production and support an on-target mechanism of toxicity.

NA co-treatment protects MK progenitors from NAMPTi toxicity

The cytoprotective effect of NA, a substrate for the NAPRT1 salvage pathway, was evaluated in the human CFU-MK assay with potent NAMPTi APO-866, GMX-1778 and GNE-617 (Figure 6 and Table 3). Increasing concentration of NA co-treatment (from 10 nM to 1000 nM) resulted in a

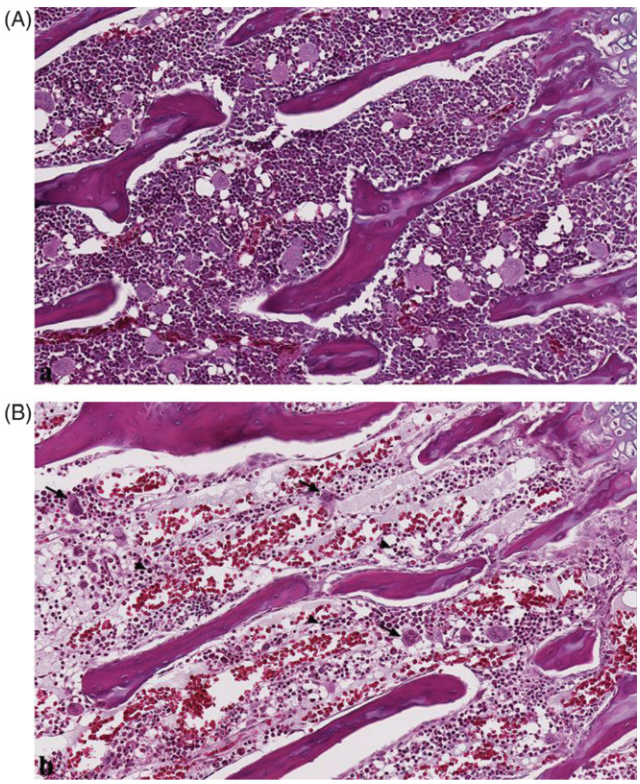


Figure 2. Representative images (H&E, 20×) from rat of (a) normal bone marrow and (b) NAMPTi-induced bone marrow toxicity following administration of APO-866 (120 mg/kg/day IP) for three days, demonstrating a marked decrease in cellularity (score 4 out of 5) with the remaining cells comprised predominantly of megakaryocytes (arrow) and granulocytes (arrowhead).

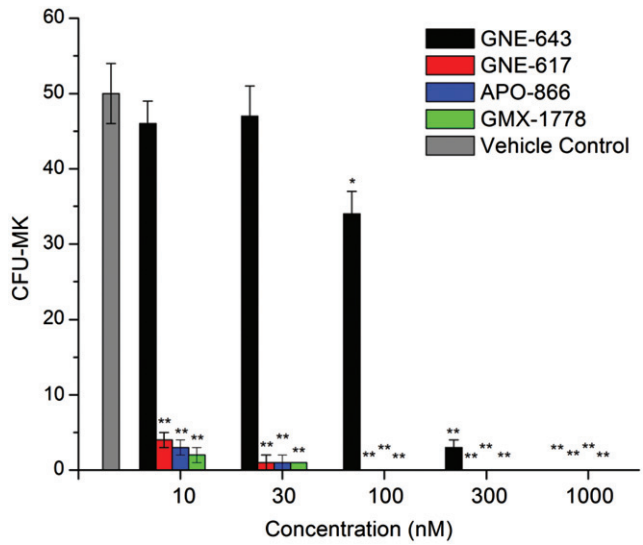


Figure 4. Potent NAMPTi reduce human CFU-MK. Cultured human CFU-MK were treated with NAMPTi over a 14 days. Relative to vehicle control, potent NAMPTi APO-866, GMX-1778 and GNE-617 all exhibited marked, dose-responsive inhibition of MK colony formation, ($*p < 0.01$, $**p < 0.001$; IC_{50} values < 10 nM). In contrast, the less potent NAMPTi GNE-643 was considerably less toxic ($IC_{50} = 136$ nM).

Figure 3. Inter-individual variation in the expression of NAMPT and NAPRT1 in human MKs. Expression of NAMPT and NAPRT1 were measured in MKs derived from human primary CD34 + hematopoietic stem cells in liquid culture (MK1-6), and human bone marrow mononuclear cells in CFC assays (MK-7). Gene expression was normalized to GADPH and expressed as delta CT (mean and SD of triplicate reactions). NAMPT was consistently detected in all human MK donors, however, NAPRT1 was variably expressed.

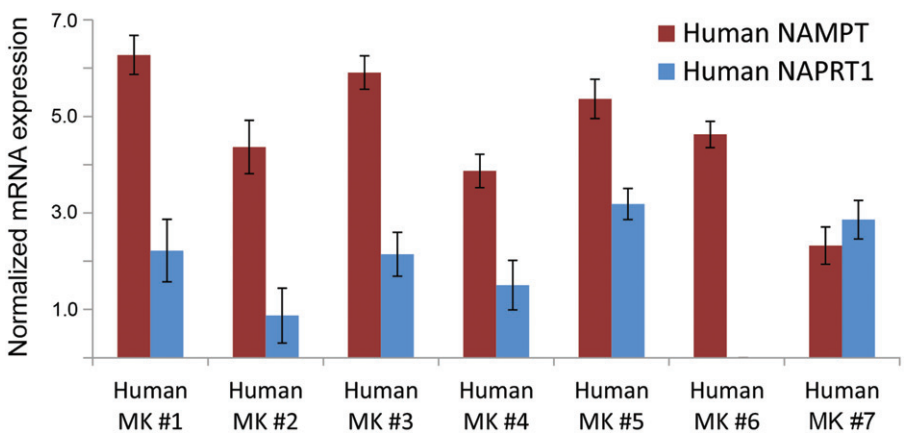


Table 2. Comparison of NAMPTi toxicity in CFU-MK of different species.

NAMPTi	Mouse $IC_{50} \pm SE$ (nM)	NHP $IC_{50} \pm SE$ (nM)	Human $IC_{50} \pm SE$ (nM)
APO-866	≤10	≤10	≤10
GMX-1778	290 ± 40	53 ± 28	<10
GNE-617	91 ± 2	<10	<10
GNE-643	16 700 ± 1800	3500 ± 1100	136 ± 8

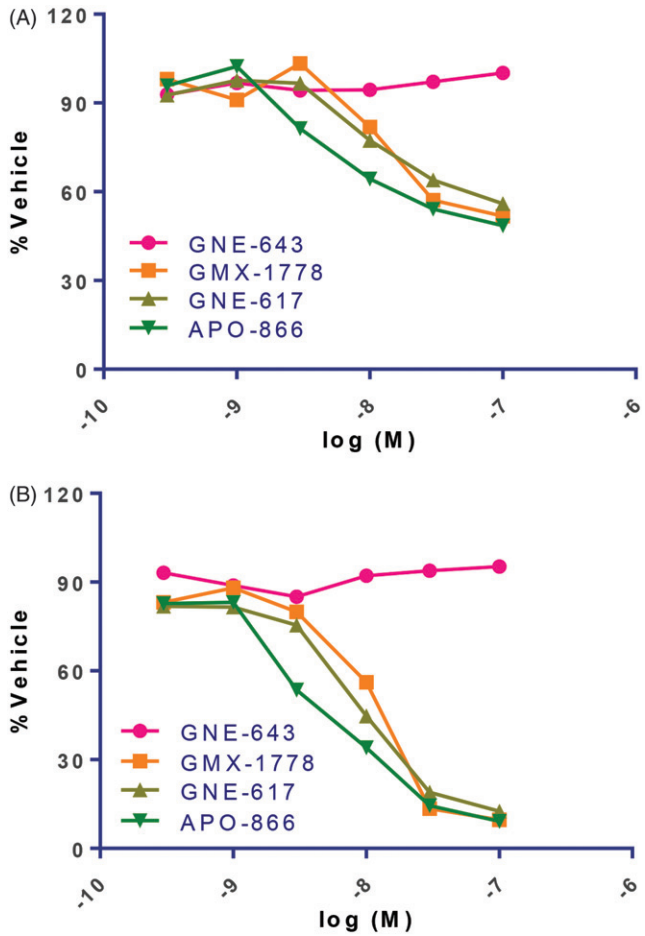


Figure 5. Potent NAMPTi reduce the cellular viability of MK progenitors. Human CD34 + cells were differentiated to MK *in vitro* in the presence of different concentrations (0.3 to 100 nM) of NAMPTi. Cell viability was measured by ATP levels, with representative data from (A) Day 3, and (B) Day 6, of the differentiation. Potent NAMPTi GNE-617, APO-866, GMX-1778 caused a dose-responsive decrease in ATP levels that was maximal at Day 6. In contrast, the less potent NAMPTi GNE-643 did not decrease ATP levels.

dose-dependent increase in IC_{50} values of NAMPTi toxicity to MK progenitors ($IC_{50} > 100$ nM).

In a time course study, in liquid culture of differentiated CD34 + cells co-treated with GNE-617, 1000 nM NA fully mitigated NAMPTi-induced toxicity in human MKs with a maximum effect beginning from day 6 of the 14-day differentiation and maturation period (Figure 7A and B, and Supplementary Figure 2). Thus, NA provided a dose-dependent protective effect for NAMPTi toxicity on MKs, demonstrating that the MK toxicity was on-target and supporting NA co-treatment as a potential risk mitigation strategy.

NA can partially mitigate NAMPTi-induced rat hematotoxicity *in vivo*

Rats were a suitable preclinical model for NAMPTi toxicity to lymphoid and erythroid lineages; therefore, we investigated whether administration of NA could also protect against these hematotoxicities *in vivo*. Co-treatment of NA with GNE-617 for 7 days increased the maximum tolerated dose of GNE-617 and mitigated the NAMPTi-induced effects on reticulocyte count (Figure 8A and B). Microscopic evaluation of the bone marrow showed a mild decrease in cellularity and partial mitigation of NAMPTi-induced bone marrow toxicity (data not shown). These findings support the principle that NA administered *in vivo* can mitigate NAMPTi toxicity to sensitive hematopoietic cell lineages and confirm the *in vitro* to *in vivo* translatability of this risk mitigation strategy for hematotoxicity.

NAMPTi has no direct toxicity on platelet function or bioenergetics

It had not been established whether NAMPTi toxicity was confined to MK or if a direct effect on platelets contributed to clinical thrombocytopenia. We therefore evaluated the direct effects of NAMPTi on platelet function and metabolism using platelets isolated from healthy human donors. Platelet function was assessed by light transmission aggregometry and expression of activation markers by flow cytometry. Concentrations of NAMPTi up to 50 μM had no direct proaggregatory effect on platelets, nor an additive effect on agonist-induced platelet aggregation (Supplementary Figure 3 and data not shown). We also assessed direct effects of NAMPTi on platelet activation using the expression of platelet activation markers CD62P and activated GPIIBIIA (i.e. PAC-1 binding) as indicators. NAMPTi did not induce platelet activation as measured by PAC1-binding or CD62P expression (data not shown).

The effect of NAMPTi on human platelet mitochondrial respiration was measured *in vitro*. Concentrations of up to 50 μM of NAMPTi (GNE-643, APO-866, GNE-617, GMX-1778) did not cause a significant reduction in the oxygen consumption rate or increase in extracellular acidification rate when compared to media alone (Supplementary Figure 4A and B, respectively). These data demonstrate that NAMPTi did not directly affect platelet bioenergetics or function; therefore, the thrombocytopenia observed in human patients treated with NAMPTi was unlikely to be caused by direct toxicity to platelets in circulation.

Discussion

Small molecule NAMPT inhibitors cause dose-limiting thrombocytopenia in the clinic that did not allow exploration of potentially efficacious doses or an evaluation of the therapeutic index. In this study, we qualified a CFU-MK model of NAMPTi-induced thrombocytopenia with a suite of structurally diverse NAMPTi and demonstrate that the CFU-MK is translatable to clinical risk assessment. By using chemically distinct NAMPTi and applying NA co-treatment in the CFU-MK assay we confirm the NAMPTi effects were on-target and that NA could protect against toxicity to MK

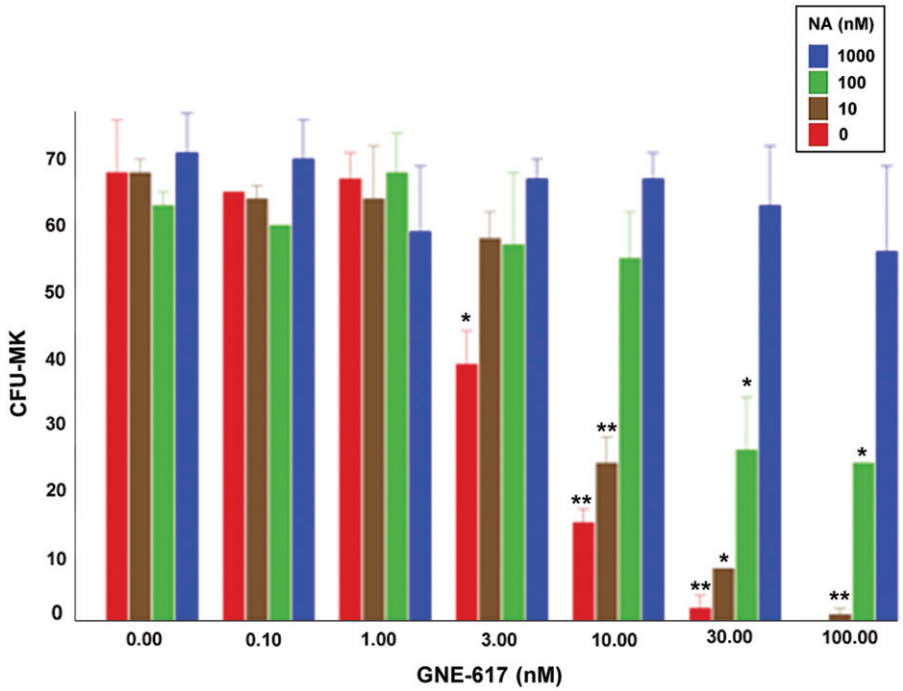


Figure 6. Nicotinic acid mitigates GNE-617 toxicity on human CFU-MK progenitors. Human CFU-MK were assessed in a CFC assay with different concentrations of GNE-617 (0–100 nM) in the presence of various concentrations of NA (0–1.0 μM). NA was not toxic to human CFU-MK. NA showed a dose-dependent mitigation of GNE-617 toxicity, with complete protection by the top dose of 1 μM NA (*$p < 0.01$, **$p < 0.001$).

Table 3. Mitigation of NAMPTi toxicity on human CFU-MK by NA co-treatment.

NA co-treatment	APO-866 IC_{50} ± SE (nM)	GMX-1778 IC_{50} ± SE (nM)	GNE-617 IC_{50} ± SE (nM)
0	1.9 ± 0.2	2.4 ± 0.2	5.8 ± 1.2
10 nM	2.2 ± 0.4	3.2 ± 0.05	8.8 ± 0.6
100 nM	57 ± 21	29 ± 11	25.1 ± 6.8
1000 nM	>100	>100	>100

cells. Furthermore, we demonstrate that the mechanism of toxicity is through direct effects on megakaryocytes and not platelets. The relative resistance of mouse CFU-MK and absence of an effect on platelet count in rat toxicity studies to NAMPTi, reveal that rodents underpredict clinical thrombocytopenia.

Administration of NAMPTi to rats was not associated with decreases in circulating platelets or MK depletion by routine hematology and microscopic evaluation of hematopoietic tissue in studies in rats of up to 15 days duration at maximal tolerated doses (Figure 1) with systemic drug concentrations that exceeded that associated with thrombocytopenia in clinical studies (Holen et al., 2008; Hovstadius et al., 2002; Lindhagen et al., 2004). In our *in vivo* preclinical studies, rats had selective hematotoxicity with depletion of tissue lymphoid and erythroid populations and corresponding decreases in circulating lymphocyte and reticulocyte count, similar to mice treated with tolerated doses of APO-866 for 4 days (Nahimana et al., 2009) and patients (Holen et al., 2008; Hovstadius et al., 2002; von Heideman et al., 2010). Collectively, these data indicate that NAMPTi primarily target lymphoid and erythroid populations in rodents and thus model these clinical toxicities. However, rodents are not a suitable nonclinical model to recapitulate clinical thrombocytopenia associated with NAMPTi treatment.

We therefore established a tractable *in vitro* preclinical model of clinical thrombocytopenia to explore strategies to characterize and manage this toxicity. The model was qualified using structurally diverse NAMPTi, including molecules (APO-866 and GMX-1778) that were associated with thrombocytopenia in clinical trials. The predictivity of the colony forming cell assay as an *in vitro* model of clinically-relevant neutropenia is well established for the CFU-GM assay (Pessina et al., 2003). The CFU-MK assay has also shown utility as a predictive model of drug-induced thrombocytopenia (Pessina et al., 2009) and thus a potentially useful system for applying to NAMPTi toxicity. We first confirmed that human MKs expressed the NAMPT target (Figure 3). NAMPTi that had potent anti-tumor activity (Table 1) were also toxic to CFU-MK, with an IC_{50} of less than 10 nM (Table 2). The relatively inactive GNE-643 molecule (Table 1) was orders of magnitude less potent on CFU-MK consistent with on-target toxicity (Table 2). APO-866 and GMX-1778 IC_{50} in this system (<10 nM) corresponded to similar exposures in clinical testing that were associated with thrombocytopenia (steady state concentration of 14 nM APO-866 and a free *C*-max < 5 nM GMX-1778) (Holen et al., 2008; Hovstadius et al., 2002; Lindhagen et al., 2004). Using a comparable human colony forming cell assay system to explore toxicity to progenitors of different hematopoietic cell lineages, others have shown that the same concentrations of APO-866 that caused profound reduction of CFU-MK in our study had no effect on CFU-granulocyte/macrophage progenitors (Nahimana et al., 2009). Furthermore, APO-866 was not toxic to isolated human CD34+CD38- hematopoietic stem cells (Nahimana et al., 2009). These data indicate that NAMPTi MK toxicity in clonogenic assays is specific to MK lineage and not due to general effects on stem cells, pluripotent progenitor cells or proliferating cells in this system. It is not understood why the MK lineage are particularly sensitive to NAMPT inhibition.

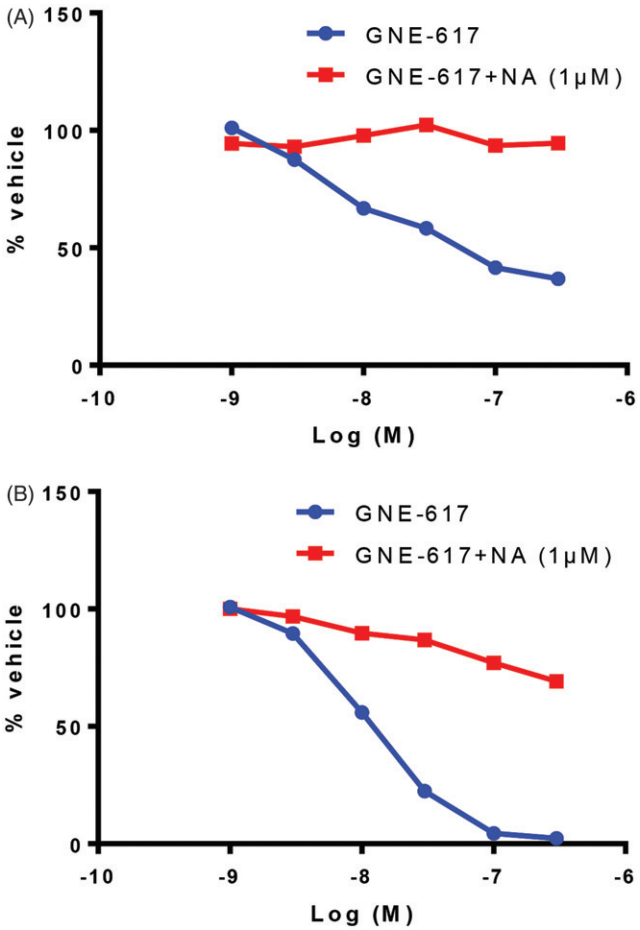


Figure 7. Nicotinic acid mitigates GNE-617 toxicity at various stages of MK cell differentiation and maturity. Human CD34 + cells were differentiated to MK *in vitro* in the presence of different concentrations (0.3 to 100 nM) of NAMPTi GNE-617 in the absence or presence of 1 μM NA. Cell viability was measured by ATP levels, with representative data from (A) Day 3, and (B) Day 6, of the differentiation. In the absence of NA, GNE-617 caused a dose-responsive decrease in MK precursor viability. In the presence of 1 μM NA, this toxicity was maximally prevented from Day 3.

We also excluded that NAMPTi were directly toxic to platelets by showing no effect on the bioenergetics, activation or aggregation potential of platelets (Supplementary Figure 3, 4A and B). The CFU-MK assay is accordingly a valid *in vitro* model for evaluating NAMPTi-related thrombocytopenia and confirms the mechanism of toxicity that is consistent with the observed clinical time-course of thrombocytopenia.

We found that the predominant hematological toxicity of NAMPTi in the rodent is suppression of erythroid and lymphoid lineages (Figures 1 and 2) in contrast to the thrombocytopenia in cancer patients (von Heideman et al., 2010). This differential species sensitivity to NAMPTi toxicity in blood cells has previously been noted between rat and human lymphocytes, where GMX-1778 (CHS-828) had a greater inhibition of primary lymphocytes of humans compared to rat (Lindhagen et al., 2004). In contrast, the kinetics of cell death occurs more rapidly with APO-866 treatment of mouse primary lymphocytes (with less reduction in cellular NAD), compared to human lymphocytes (Pittelli et al., 2010). Our data also demonstrate a species-specific sensitivity of CFU-MK to NAMPTi with human most sensitive followed by monkey and mouse (Table 2). We could not directly compare rat to the other species, as we were unsuccessful in our effort to develop a rat CFU-MK CFC assay. The relative toxicity of various NAMPTi on human and monkey CFU-MK, but not mouse CFU-MK, generally tracked with cellular-based assessment of NAMPTi potency (Table 1). We also observed discordance between the rank molecular potency in mouse CFU-MK compared to human CFU-MK, with a considerably lower IC_{50} of APO-866 compared to GNE-417 and GMX-1778 in mouse MK progenitors. Species differences in the potency of NAMPTi in cell-based assays, at variance with ranking in species-specific enzymatic assays, has been previously described and suggests potential differences in pharmacologic activity of individual NAMPTi across preclinical species used in regulated drug development (Zheng et al.,. 2013a,b). Collectively, these findings may explain the lack of

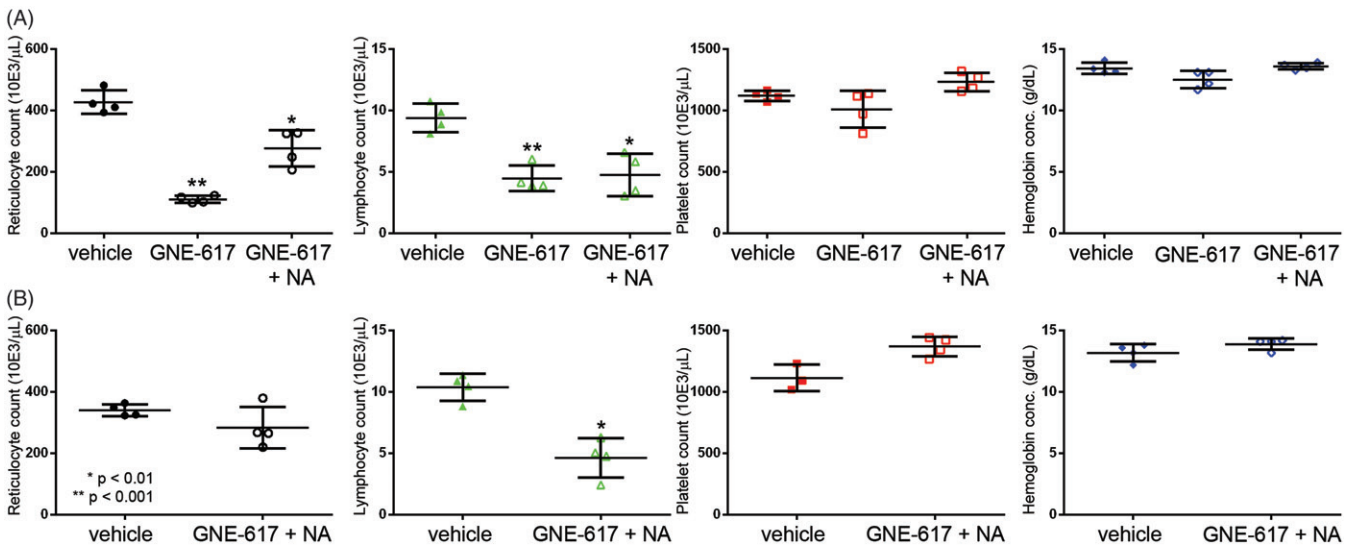


Figure 8. Protection of NAMPTi-erythroid toxicity in rats by NA. Rats were given 120 mg/kg/day NA twice daily by oral gavage and co-administered 30 mg/kg/day GNE-617 once daily for 7 days followed by hematology evaluation on (A) Day 3, or (B) Day 8. NA extended the tolerability of NAMPTi from two daily doses to at least seven daily doses and mitigated hematologic toxicity to reticulocytes.

thrombocytopenia in the rat and uncertainty in prediction of clinical toxicity from preclinical safety studies with different NAMPTi.

This work supports the utility of the CFU-MK assay in species selection for preclinical risk assessment when there is a target- or class-related risk of thrombocytopenia that is due to toxic effects on megakaryocytopoiesis. The assay could also be used for screening lead molecules for MK toxicity. Using the CFU-MK assay, we show that the mouse is not a suitable toxicity species for assessing thrombocytopenia for NAMPTi and the monkey was less sensitive to the effects on CFU-MK for two of four NAMPTi. From these findings, we contend that the human CFU-MK assay is the most clinically translatable preclinical model for thrombocytopenia induced by NAMPTi. This conclusion is consistent with the published *in vivo* data for APO-866 in patients (Holen et al., 2008); in mice, where thrombocytopenia was noted only at exposures of APO-866 above the MTD (Nahimana et al., 2009; Olesen et al., 2010); and in monkeys where thrombocytopenia was not reported (Holen et al., 2008). A limitation of our work is the lack of *in vitro* to *in vivo* correlative data for rat and the monkey. We were unsuccessful in multiple attempts to develop a rat CFU-MK assay, using conditions that worked for monkey, human and mouse assays, and rat bone marrow cells that supported other hematopoietic progenitor assays. Due to retinal and cardiac toxicities observed in the rat (Misner et al., personal communication; Zabka et al., 2015), we ceased development of NAMPTi and did not perform large animal studies.

Co-treatment of NA protected CFU-MK from NAMPTi-induced toxicity in our model of NAMPTi-thrombocytopenia. We confirmed that MKs express both NAMPT and NAPRT1 (Figure 3) and found that 1000 nM of NA, when co-administered concentrations up to 100 nM NAMPTi, completely abrogated toxicity in the CFU-MK assay (Table 3, Figure 6). NA protection was associated with the normalized ATP levels of MK precursors consistent with replenishment of cellular NAD levels via the NAPRT1-mediated salvage pathway (Figure 7). Despite the wide inter-individual variability in NAPRT1 expression of MK derived from human bone marrow (Figure 3), these levels of NAPRT1 are adequate to support MK survival in the presence of supplementary NA.

In rat toxicity studies, 120 mg/kg/day NA was well-tolerated and co-treatment increased the maximum tolerated dose of GNE-617 (Figure 8), reproducing previous findings in mice for NA co-treatment with APO-866 (Beauparlant et al., 2009; Olesen et al., 2010). Due to the lack of an animal model of NAMPTi-induced thrombocytopenia, we were unable to definitively demonstrate NA rescue of NAMPTi thrombocytopenia *in vivo*. In a different model, supra-physiologic supplementation of nicotinamide (a substrate of NAMPT) in healthy rodents, failed to augment megakaryocytopoiesis in mice despite beneficial findings when applied to CFU-MK *in vitro* (Konieczna et al., 2013). However, our rat studies demonstrate the protective effect of NA for NAMPTi hematotoxicity *in vivo* (Figure 8) and suggest this approach would be feasible to test in the clinic.

Conclusion

In conclusion, the work presented provides a translatable *in vitro* cell-based model for NAMPTi-induced thrombocytopenia in the clinic and provides evidence for direct effects on MK as the mechanism of toxicity. The CFU-MK assay provides a useful resource in conjunction with traditional *in vivo* models for decision-making during candidate selection.

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Declaration of interest

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Supplementary material available online
Supplementary Figures 1, 2, 3 and 4.