On-Target Effect of FK866, a Nicotinamide Phosphoribosyl Transferase Inhibitor, by Apoptosis-Mediated Death in Chronic Lymphocytic Leukemia Cells

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Abstract

Purpose: Chronic lymphocytic leukemia (CLL) remains incurable despite advances in therapy. In this study, we characterize the effect of nicotinamide phosphoribosyltransferase (NAMPT) inhibition by FK866 in primary CLL cells from patients with various clinical prognostic markers.

Experimental Design: CLL cells were treated with FK866 to assess viability by Annexin V/PI staining. Functional analysis of FK866 included time- and concentration-dependent evaluation of cellular NAD, ATP, mitochondrial membrane potential (MMP), reactive oxygen species (ROS), and apoptotic signaling. Chemosensitization potential by FK866 to fludarabine was also assessed. Prognostic markers were correlated with drug response.

Results: FK866 induced CLL cell death by depleting cellular NAD content by day 1, followed by a drop in ATP on day 2. We observed loss of MMP, ROS increase, and induction of apoptotic signaling at day 3. On-target activity of FK866 was confirmed by NAD-mediated rescue of NAD and ATP loss, apoptotic signaling, and viability. The response to FK866 was independent of most prognostic markers. Higher doses were required with short lymphocyte doubling time and positive CD38 status, whereas CLL cells resistant to fludarabine in vitro and from patients with del17p13.1 were equally sensitive to FK866. FK866 did not upregulate the p53-target p21, nor did the p53 activator Nutlin improve FK866-mediated cell death. Furthermore, fludarabine and FK866 were synergistic at clinically relevant concentrations.

Conclusions: NAMPT inhibition by FK866 may be a potential treatment for CLL, including patients with del17p13.1 or other high-risk features. FK866 may complement standard agents to enhance their efficacy and/or allow dose reduction for improved tolerability.

Introduction

Chronic lymphocytic leukemia (CLL) is an incurable disease characterized by the abnormal accumulation of mature CD19/CD5-positive monoclonal B lymphocytes in peripheral blood (PB), lymph nodes, spleen, and bone marrow. Patient outcomes are very heterogeneous, ranging from stable disease and good long-term prognosis to rapidly progressing, aggressive disease and death despite therapeutic interventions (1). Several biomarkers, such as unmuted status of the immunoglobulin heavy chain variable region (IgVh), presence of zeta-associated protein tyrosine kinase 70 (Zap-70), presence of the surface marker CD38, or some cytogenetic aberrancies are associated with disease progression, aggressiveness, and poor treatment response (2, 3). The worst prognosis, involving rapid progression of disease, multidrug resistance, and early relapse, is associated with a deletion in 17p13.1 (del17p13.1) that is linked to reduced p53 function (4). There is, thus, a requirement for new therapeutic approaches to treat these high-risk patients.

Although altered cellular metabolism was recognized as a characteristic of cancer cells by Otto Warburg almost a century ago and was popularized in the 1950s (5), only recently have actual functional links been established between oncogenic pathways and cellular metabolism (6). As a result, “altered metabolism” has now been recognized as one of the “hallmarks of cancer” (7). Deregulated metabolic processes have, thus, become an attractive target for cancer therapy (8).

A critical factor in cellular metabolism is nicotinamide adenine dinucleotide (NAD), a coenzyme in adenosine triphosphate (ATP)–synthesizing mitochondrial electron transport chain reactions.
**Translational Relevance**

Chronic lymphocytic leukemia (CLL) remains incurable with available treatment options. This study describes targeting nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme in salvage generation of NAD, by FK866 as a potential treatment approach in CLL. FK866 induced apoptosis in CLL cells independent of most prognostic markers and showed equal efficacy in CLL cells with fludarabine resistance in vitro and those harboring a deletion in 17p13.1. Cells from patients with positive CD38 status and short lymphocyte doubling time required higher doses of FK866. We found no evidence of p53 involvement in FK866 action. Furthermore, fludarabine and FK866 were synergistic at clinically relevant concentrations. As FK866 is described to be well tolerated, translation to clinical practice is promising. This study highlights NAMPT inhibition as a novel approach and an alternate strategy for CLL treatment that can be used in combination with standard agents with the potential to alter outcomes for all patients, including those with high-risk features.

transport chain reactions. NAD consumption is also linked to intracellular signaling reactions (9–13) some of which have been associated with cancer physiology (14–16). Although NAD can be generated de novo from tryptophan precursors, the major route of NAD biosynthesis in lymphocytes is through a nicotinamide-salvage process, which is dependent on the rate-limiting enzyme, nicotinamide phosphoribosyltransferase (NAMPT; refs. 15, 17). NAMPT has been demonstrated to be overexpressed in several cancers, suggesting a critical role in tumor physiology (18–21).

FK866 (N-[4-(1-benzoyl-4-piperidinyl)butyl]-3-(3-pyridinyl)2-ethylpropenamide) was initially identified as a non-competitive inhibitor of NAMPT (22), but was later shown to compete with nicotinamide to bind to the catalytic pocket (23, 24). FK866 reduces intracellular NAD content, resulting in selective cancer cell death in solid tumors (22, 25–27), and emerging evidence suggests that it is also active in blood cancers (28–33). FK866 has been demonstrated to be effective in blood cancer cell lines, where it leads to ATP reduction and delayed induction of cell death. Whereas some studies demonstrated the induction of apoptosis with FK866 (22, 32), others showed caspase-independent autophagic cell death (25, 28–30). FK866 was safe and well tolerated in a phase I study that included 24 patients with various advanced solid tumors that were refractory to standard treatments. Thrombocytopenia was the dose-limiting toxicity in this study, suggesting that this drug is a good candidate to transition into the clinic (34).

Although FK866 is known to cause cell death in a variety of models of hematologic malignancies, the mechanism of cell death is debated in the literature and unknown for CLL cells. In addition, response to FK866 with clinical parameters has not been previously described. In this study, we investigate the effect of NAMPT inhibition by FK866 on cellular energy content, cell viability, and the impact on apoptotic signaling in primary CLL cells from patients with different prognostic markers, including those with high-risk features.

These studies show that NAMPT inhibition by FK866 is a novel and potentially important treatment for CLL, alone or in combination with other drugs, and may particularly be of interest for patients with high-risk features, including del17p13.1.

**Materials and Methods**

**Patients**

PB from consented CLL patients or donors without CLL were used. Patients with CLL had a confirmed diagnosis according to standard criteria (1). Patient characteristics were provided by the Manitoba Blood and Marrow Tumor Bank and are listed in Supplementary Table S1. One patient was classified as small lymphocytic leukemia (SLL) and was included in our CLL cohort. The study was performed according to the World Medical Association’s Declaration of Helsinki (6th version, Seoul, South Korea, 2008) and authorized by the human research ethics board at the University of Manitoba (approval number HS15746).

**Primary cells**

PB mononuclear cells (PBMC) from the blood of patients with CLL or donors without CLL were extracted by Ficoll density-gradient centrifugation. For lower-count CLL samples (lymphocytes $\leq 40 \times 10^6/\mu$L) and B-cell purification of control donors, the B Cell Enrichment Antibody Cocktail RosetteSep (STEMCELL Technologies) was used. Freshly isolated cells were used for all experiments at $4 \times 10^6$ cells/mL. All experiments were carried out in RPMI-1640 containing 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) at 37°C, 5% CO2 in a humidified atmosphere.

**Reagents**

For immunoblotting, anti-vinuculin antibody was from Abcam (ab18058). Antibodies directed against pan-actin (#4968), caspase-3 (#66565 and #6662), caspase-9 (#9502), Mcl1 (#4572), XIAP (#2042), PARP (#9542), p21 (WAF/Cip1; #2947), cytochrome C (#11940), COX IV (#4850), pAMPK (#2535), Erk1/2 (#4695), pErk1/2 (#9106), and pmTOR (#2971) were from Cell Signaling Technology. Anti-NAMPT/PBEF (#130058) was from Santa Cruz Biotechnology. Vinculin and pan-actin served as loading control. Secondary horseradish peroxidase–labeled antibodies were purchased from Bio-Rad. FK866, the p53-activator Nutlin-3, and etoposide were purchased from Sigma and dissolved in DMSO to a stock concentration of 10 mmol/L and dissolved in DMSO to a stock concentration of 10 mmol/L for FK866 and Nutlin-3 and 20 mmol/L for etoposide, respectively. Fludarabine was obtained from Enzo Life Sciences and dissolved in DMSO to a stock concentration of 35 mmol/L. All drug stocks were stored at $-30^\circ$C. β-NAD sodium salt was purchased from Sigma and freshly dissolved in water to a stock concentration of 10 mmol/L.
before use. The superoxide indicator dihydroethidium (DHE) was obtained from Molecular Probes as a 5 mmol/L solution stabilized with DMSO and stored at −30°C with protection from light. Tetramethylrhodamine methyl ester (TMRM) was obtained from Molecular Probes, dissolved in DMSO at 500 mmol/L, stored at 4°C, and protected from light.

ATP, NAD, and caspase-3/7 assays

The commercially available kits CellTiter-Glo Luminescent Cell Viability Assay (#G7571), NAD/NADH Glo Assay (#G9071), and Caspase-3/7 Glo Assay (#G8091) were purchased from Promega and used to assess cellular ATP levels, NAD levels, and caspase-3/7 activity, respectively. Luminescent read-outs were performed on the SPECTRAmax GEMINI XS luminometer (Molecular Devices).

Annexin V/PI, reactive oxygen species, and mitochondrial membrane potential assays

The Annexin V-FITC Apoptosis Detection Kit (# 556574; BD Biosciences) was used following the manufacturer’s instructions. Annexin V/PI double-negative cells were
FK866 reduces cellular NAD and ATP content in a time- and concentration-dependent manner. CLL cells were treated with vehicle control and FK866, as indicated. ATP and NAD content was assessed daily for 4 days (n = 6). ATP and NAD content was normalized to vehicle control and displayed as relative luminescent units. NAD content significantly decreased in a concentration-dependent manner as early as day 1 in concentrations equal or greater to 10 nmol/L compared with control (top). Although unchanged at day 1, at day 2, ATP content decreased significantly at doses 10 nmol/L or greater with further decrease at days 3 and 4 (bottom). Paired Student t test was used to assess statistical significance. Ctrl, vehicle control. **, P < 0.01; ****, P < 0.0001.

considered viable. Viable cells were normalized to a vehicle-treated control.

To assess levels of reactive oxygen species (ROS) or the mitochondrial membrane potential (MMP), cells were incubated with DHE at a final concentration of 2.5 μmol/L (ROS) or TMRM at a final concentration of 25 nmol/L (MMP) for 20 minutes at room temperature protected from light. Fluorescence was assessed using the BD LSRII flow cytometer and FACSDiva software. Analysis was done using FlowJo 8 Software.

Immunoblotting

Cells were lysed using radioimmunoprecipitation assay (RIPA) cell-lysis buffer containing a protease inhibitor (cComplete Protease Inhibitor Cocktail; Roche) and a phosphatase inhibitor (PhosStop Phosphatase Inhibitor Cocktail; Roche). For separation of the mitochondrial fraction, the Mitochondria Isolation Kit for Cultured Cells (Pierce) was used following the manufacturer’s instructions. Lysates were separated on 4% to 12% Bis/Tris precast gels (Invitrogen). Proteins were blotted onto nitrocellulose membrane by means of wet transfer. Primary antibody incubation was carried out in Tris-buffered saline (TBS), 0.05% Tween-20 (TBS-T), including 5% bovine serum albumin (BSA) or 5% nonfat milk at 4°C overnight. Incubation with secondary, horseradish peroxidase (HRP)-labeled antibodies was carried out for 1 hour at room temperature in TBS-T, either containing 5% BSA or 5% nonfat milk, matching primary antibody solution. Signals were visualized using an enhanced chemiluminescence reagent (PerkinElmer) on an automated developer (Kodak). Densitometric analysis was carried out using ImageJ software (imagej.net).

Rescue experiments

CLL cells were incubated with culture medium supplemented with freshly dissolved exogenous (ex) NAD to a final concentration of 100 μmol/L for 18 hours before the addition of FK866 at 25 nmol/L. Controls included FK866-treated cells without NAD supplementation and vehicle (DMSO)-treated cells with or without NAD supplementation. The NAD/NADH Glo Assay did not detect NAD at concentrations up to 150 μmol/L in the absence of cells (data not shown). Cells were collected for immunoblotting after 2 days and ATP content, NAD content, and Annexin V/PI-based viability were assessed following 3 days of FK866 treatment.

CompuSyn analysis

Drug combination analysis, according to the Chou–Talay Combination Index model (35, 36), was performed using CompuSyn 1.0 (CombioSyn, Inc.). Measure of synergism is given as combination indices (CI), where CI < 1, = 1, and >1 indicate synergism, additivity, and antagonism, respectively. Comparison of drug efficacy, alone and in combination, is given as dose reduction indices (DRI), which represent fold decrease in the drug concentration required to achieve a given level of efficacy.

Data management and statistics

Data management and analysis was performed using Microsoft Excel 2010 and GraphPad Prism 6. Data are presented as dose–response curves, bar charts, or scatter plots generated in GraphPad Prism 6. Dot plots from flow cytometric analyses were generated in FlowJo 8. Statistical analysis was performed using GraphPad Prism 6. The associations between patients’ clinical parameters and their sensitivity to FK866 were calculated by the Fisher exact test of independence, and logistic regression models were built to estimate odds ratios (OR) in univariable models using SAS9.3. AP value ≥ 0.05 was considered not significant (ns), whereas P values <0.05, <0.01, <0.001, and <0.0001 were considered ***, ***, and ****, respectively.

Results

The NAMPT inhibitor FK866 causes dose-dependent and selective loss of viability in primary CLL cells

Immunoblot analysis demonstrated upregulation of NAMPT protein levels in primary CLL cells compared with PBMCs and purified B cells from non-CLL controls (Fig. 1A). Densitometry on 21 CLL and four control PBMC samples.

Figure 2. FK866 reduces cellular NAD and ATP content in a time- and concentration-dependent manner. Cells were treated with vehicle control and FK866, as indicated. ATP and NAD content was assessed daily for 4 days (n = 6). ATP and NAD content was normalized to vehicle control and displayed as relative luminescent units. NAD content significantly decreased in a concentration-dependent manner as early as day 1 in concentrations equal or greater to 10 nmol/L compared with control (top). Although unchanged at day 1, at day 2, ATP content decreased significantly at doses 10 nmol/L or greater with further decrease at days 3 and 4 (bottom). Paired Student t test was used to assess statistical significance. Ctrl, vehicle control. **, P < 0.01; ****, P < 0.0001.
showed a 3-fold higher normalized densitometric value in CLL cells compared with control PBMCs and 1.5-fold higher normalized densitometric value compared with control B cells (1.15/C6 0.07 for CLL cells, 0.37/C6 0.05 for control PBMCs, 0.70/C6 0.07 for control B cells; Supplementary Fig. S1). As FK866 is not directly cytotoxic, but requires catabolic reactions to deplete cellular energy stores (22), CLL cells and control PBMCs were incubated for 4 days with doses of FK866 in concentrations ranging from 1 nmol/L to 1,000 nmol/L for CLL cells and 0.2 nmol/L to 2,000 nmol/L for control PBMCs. Effects on cell viability were measured by Annexin V/PI staining. A significantly lower lethal dose, which killed 50% of cells (LD_{50}) was obtained for FK866 in CLL cells (mean LD_{50} 7.3 nmol/L; range, 0.7–56.9 nmol/L) compared with control PBMCs (mean LD_{50} 270.7 nmol/L; range, 224.6–312.2 nmol/L; Fig. 1B). The individual responses are visualized in a heat map, highlighting the increased sensitivity of CLL cells to FK866 treatment as compared with the control PBMCs (Fig. 1C). A dose range surrounding the determined LD_{50} value confirmed a significant drop in viability between 5 nmol/L and 10 nmol/L of FK866 (Fig. 1D). This is illustrated by representative dot plots (Fig. 1E). Together, these results indicate that FK866 selectively induces cell death in primary CLL cells.

**FK866 reduces cellular NAD and ATP content in a time- and concentration-dependent manner**

FK866 inhibits NAMPT, the rate-limiting enzyme for the generation of NAD, which is an essential component of the mitochondrial electron transport chain to generate ATP. Primary CLL cells were treated with 1, 10, 25, 50, and 100 nmol/L of FK866, and cellular NAD and ATP levels were measured daily for 4 days (Fig. 2). The level of NAD had
significantly fallen with 10 nmol/L FK866 by day 1 and this was followed by a further decline in NAD at day 2 (Fig. 2, top). Although ATP levels were stable at day 1, they significantly dropped in a concentration-dependent manner at day 2 (Fig. 2, bottom).

Positive CD38 status and short lymphocyte doubling time predict response to FK866

CLL samples (n = 39) demonstrated variable cell death response to FK866 with 
LD50 values ranging from 0.7 to 56.9 nmol/L. We went on to determine if the variable response to FK866 could be attributable to clinical prognostic markers. No statistically significant associations were found between response to FK866 based on LD50 and age, gender, Rai stage, white blood cell counts (WBC), B2-microglobulin (B2M), mutational status of the immunoglobulin variable heavy chain (IgVh), or positive Zap-70 status. In contrast, positive CD38 status and shorter lymphocyte doubling time (LDT ≤ 12 months), both markers of increased cellular proliferation, showed significantly

Figure 4. FK866 is independent of in vitro response to fludarabine (Fluda) and does not upregulate p21. A, CLL cells from unselected patients were treated with vehicle control at concentrations ranging from 1 to 1,000 nmol/L FK866 or 5 μmol/L fludarabine for 4 days. LD50 values for FK866 were derived from dose–response curves. Sensitivity to fludarabine is demonstrated as FDC. FK866 sensitivity (LD50) and fludarabine sensitivity (FDC) were plotted against each other. The basis of Pearson correlation coefficient r, there was no significant linear dependence between these two variables (r = 0.2365; 95% confidence interval, −0.559 to 0.1424). B, CLL cells were treated with FK866 for 18 hours before addition of fludarabine at constant concentration ratios of 1 (FK866) to 500 (fludarabine) for 3 days (n = 5). Compusyn analysis was performed to address drug synergy. DRI curve is displayed. The presence of FK866 allowed significant dose reduction of fludarabine at all concentrations tested. C, FISH results were available for 17 samples. Dose–response curves demonstrate comparable response to FK866 in CLL cells with del17p13.1 (n = 5) or wt17p13.1 (n = 12). D, CLL cells were treated with vehicle control (Ctrl), 5 nmol/L FK866, 10 μmol/L etoposide (Eto), or 10 μmol/L fludarabine for 4 days with or without addition of 0.5 μmol/L Nutlin (n = 7). Viability was assessed by Annexin V/PI staining. Although Nutlin significantly improved etoposide- and fludarabine-induced viability reduction, FK866-induced viability reduction remained unaffected. * P < 0.05; ** P < 0.001. E, CLL cells were treated as described for D for 2 days and subjected to immunoblotting. While in the absence of Nutlin, etoposide and fludarabine induced p21; FK866 did not upregulate p21 in either wt17p13.1 or del17p13.1 cells. As expected, in del17p13.1 cells, etoposide and fludarabine only slightly upregulated p21 (left). Although Nutlin stabilized etoposide and fludarabine induced p21, it did not lead to p21 induction in FK866-treated cells from a wt17p13.1 sample (right). Background-corrected densitometric values were normalized to control and are displayed below the blot (n = 5 for wt17p13.1; n = 1 for del17p13.1).
higher FK866 LD$_{50}$ values (Fig. 3; Supplementary Table S2). Statistical findings were valid with two different cutoffs for LD$_{50}$ (8 and 14 nmol/L).

Another high-risk group is of relapsed patients. We grouped our cohort into treatment-naive patients and patients who had failed at least one line of treatment (Supplementary Table S1). The in vitro response to FK866 was significantly lower (higher LD$_{50}$) in the relapsed subgroup (25.5 ± 6.0 nmol/L) compared with the treatment-naive subgroup (8.4 ± 2.1 nmol/L; Supplementary Fig. S2 and Supplementary Table S2). However, there was a statistically significant association between CD38 and treatment status by Fisher exact test ($P = 0.0233$). When multivariable regression analysis was performed to adjust for CD38, no significant association between treatment status and sensitivity to FK866 was found (data not shown).

#### FK866 is independent of in vitro response to fludarabine and does not upregulate p21

As FK866 effectively induced cell death in CLL cells with several adverse prognostic markers, we went on to evaluate the impact of FK866-mediated cell death on CLL cells with in vitro resistance to fludarabine and samples known to harbor del17p13.1.

To determine whether FK866 had a mechanism of action different from other antitumor agents such as fludarabine, we evaluated the correlation between in vitro response to fludarabine based on fraction of dead cells (FDC) and FK866 based on LD$_{50}$ in unsampled samples (Fig. 4A). The correlation coefficient $r$ suggested an inverse correlation based on increased FDC values with increased sensitivity and decreased LD$_{50}$ values with increased sensitivity with no statistical significance in our cohort ($r = -0.2365$). Therefore, CLL cells with low in vitro sensitivity to fludarabine were equally sensitive to FK866 as fludarabine sensitive CLL cells. Although fludarabine resistance indicates high-risk disease, these results demonstrate that FK866 and fludarabine have different modes of cell kill and that FK866 has potential clinical activity for drug-resistant patients.

Fludarabine has been described to downregulate NAD. This is believed to be the result of activation of PARP, the major consumer of NAD in the cell, at late stages of apoptosis (37). We addressed the question of potential synergy between FK866 and fludarabine based on their concentration-dependent manner at day 2. The increase observed loss of NAD and ATP at days 1 and 2, respectively (Fig. 2), there was no change in MMP after 1 day of treatment with Nutlin, suggesting a mode of action independent of p53 (Fig. 4D). To further support this, we addressed the impact of FK866, fludarabine, and etoposide on the immediate p53 downstream target protein p21 by immunoblotting. Although at this time and concentration, FK866 significantly reduced NAD as well as ATP contents (Fig. 2), no upregulation of p21 was observed (Fig. 4E). In CLL cells with wt17p13.1, incubation with etoposide (10 µmol/L) or fludarabine (10 µmol/L) induced upregulation of p21, whereas only limited p21 upregulation in CLL cells from a patient with a del17p13.1 was observed (Fig. 4E left). Furthermore, the presence of Nutlin did not result in p21 induction upon FK866 treatment, whereas etoposide- and fludarabine-mediated induction of p21 was enhanced by Nutlin in a wt17p13.1 sample (Fig. 4E right) as supported by normalized densitometric values below the blot. This suggests that FK866-mediated cell death acts independent of p53.

#### FK866 treatment induces mitochondrial dysfunction and activates apoptotic signaling

MMP is crucial for maintaining electron transport chain function to generate ATP. NAD is a central component in this process and its downregulation by NAMPT inhibition by FK866 would be expected to compromise this process. We determined the effect of FK866 on the MMP in a time- and concentration-dependent manner. Although we observed loss of NAD and ATP at days 1 and 2, respectively (Fig. 2), there was no change in MMP after 1 day of FK866 treatment, but a concentration-dependent reduction was detected at day 2, and on day 3 the MMP was significantly reduced (Fig. 5A). To further assess the impact on mitochondrial damage, we assessed cytochrome C (Cyt C). Cyt C levels from mitochondrial extracts decreased in a time- and concentration-dependent manner with increasing doses of FK866 (Fig. 5B). Overall reduction of Cyt C in the absence of FK866 at day 3 may be attributed to spontaneous apoptosis. Similarly, an indicator of defective electron transport in the mitochondria is the generation of ROS. In agreement with the MMP, ROS levels were stable at day 1 and increased in a concentration-dependent manner at day 2. The increase in ROS was most pronounced at day 3 (Fig 5C).
FK866 induces mitochondrial dysfunction and activates apoptotic signaling. CLL cells were treated for 1, 2, or 3 days with vehicle control, 1, 10, 25, and 50 nmol/L of FK866 for flow cytometric analysis or vehicle control, 1, 10, and 50 nmol/L for immunoblot analysis and caspase-3/7 assay. A, the MMP was assessed by TMRM staining. The bar graph demonstrates a time- and concentration-dependent loss of the MMP starting at day 2, which became statistically significant on day 3 at 25 and 50 nmol/L of FK866 (n = 3). (Continued on the following page.)
timing of MMP breakdown and ROS generation were comparable with viability reduction, as assessed by Annexin V/PI staining (Fig. 5D). The effect of FK866 on Annexin V staining (Figs. 1E and 6B) in CLL cells suggests that this agent induces cell death via apoptosis. To confirm this, CLL cells were treated with FK866 for 1, 2, and 3 days. A concentration-dependent cleavage of PARP and caspase-3, along with down-regulation of the procaspases 9 and 3 was visible at day 2. Similar effects were seen on the anti-apoptotic proteins XIAP and Mcl1. These changes became more pronounced by day 3. The significant time- and concentration-dependent activation of effector caspases 3/7 by FK866 was further confirmed by a luminescent assay (Fig. 5E). In contrast, using the lowest dose of FK866 (1 nmol/L), no apoptotic signaling was observed using any time points and read-outs (Fig. 5). However, this concentration of FK866 increased the levels of Mcl1, XIAP (Fig. 5E), and ATP (Fig. 2) with activation of AMPK, mTOR, and Erk1/2 at early time points (Supplementary Fig. S3). These findings suggest a potential initial autophagic response as a result of the energy crisis induced by low-dose FK866.

Exogenous NAD rescues the impact of FK866 on cellular energy content, viability, and apoptotic signaling

To further evaluate the on-target effect of FK866, we incubated CLL cells with exogenous NAD (exNAD, 100 μmol/L) for 18 hours before a 3-day exposure to 25 nmol/L FK866. ExNAD almost doubled the intracellular content of NAD (compare control vs. exNAD, Fig. 6A, left), suggesting it to cross the plasma membrane as demonstrated before in other cell types (38, 39). Addition of exNAD rescued the effect of FK866 on intracellular NAD as well as ATP content (Fig. 6A, left and right, respectively), confirming FK866 action through downregulation of NAD. In addition, exNAD protected the cells from the effects of FK866 on cell death (Fig. 6B), caspase-3 cleavage, and the downregulation of Mcl1 (Fig. 6C).

Discussion

An unexplored area in CLL therapy is targeting deregulated metabolism. NAMPT is a metabolic enzyme, essential for the salvage generation of NAD. Preclinical studies have shown that FK866, a NAMPT inhibitor, is effective in killing leukemic cell lines and has the ability to decrease cancer cell survival in xenograft models of leukemia, myeloma, and lymphoma (28, 30, 31, 40). In the present study, we exploited NAD-salvage generation as a clinically relevant target in CLL by assessing the cytotoxicity of FK866 against CLL cells in vitro, and how this activity correlates with markers of high-risk, aggressive, and drug-resistant disease.

Previous studies report that hematopoietic progenitors are less sensitive to FK866 than tumor cells (30, 31, 41). In the present study, we demonstrated that primary CLL cells overexpress NAMPT in comparison with control PBMCs and non-CLL B lymphocytes. CLL cells were more sensitive to FK866 with a LD₅₀ of 7.3 nmol/L, well below doses achieved in a phase II clinical trial (34).

We confirmed the on-target activity of FK866 by demonstrating that the addition of NAD rescued CLL cells from the phenotypic effects of FK866 (Fig. 6). However, heterogeneous NAMPT protein levels between patients (Fig. S2) did not predict sensitivity to FK866 (data not shown). Our data parallel previously published data in multiple myeloma cell lines and primary cells with no correlation between NAMPT levels and cytotoxic response to FK866 (28).

The timing of NAD and ATP loss along with cell death observed with FK866 in primary CLL cells was comparable with that described in other cancers (22, 25, 42). Whether the NAD and subsequent ATP reduction by FK866 treatment leads to the induction of autophagy or apoptosis as a mechanism of cell death has been debated in the literature (22, 25, 28–30, 32). This suggests that neither is solely responsible for the cell death seen with NAD depletion and that the mode of death may depend on cell context. In our study, we clearly demonstrated the induction of apoptosis (Fig. 1E; Fig. 5E, Fig. 6B) and caspase activation (Fig. 5E and F) in primary CLL cells at doses that induced cell death. However, we did observe indication for induction of autophagy at early doses and/or early time points (Fig. 2; Fig. 5E; Supplementary Fig. S3). These results suggest that an autophagic response to low doses of FK866, and/or at early time points may counteract the initial cellular energy crisis caused by a slight depletion in NAD. However, when the balance is tipped to a point of no return, apoptosis occurs. A later pro-death effect through autophagy is also possible.

Although the cytotoxic response to FK866 varied between samples with the LD₅₀ values ranging from 0.7 to 56.9 nmol/L, all CLL samples were highly sensitive to FK866 with no statistical differences in response based on common clinical prognostic features, including age, gender, Rai stage, white blood cell counts, β2M, mutational status of IgVh,
and ZAP-70 (Fig. 3; Supplementary Table S2), suggesting that FK866 was equally effective across a variety of markers. LDT and CD38 positivity were found to predict decreased sensitivity to FK866 (Fig. 3; Supplementary Table S2). This may reflect the functional link of the enzymatic activity of CD38 as a NADase (43). Therefore, the presence of CD38 may result in a higher turnover of NAD through salvaging of nicotinamide, suggesting either increased total levels or activity status of NAMPT, both of which scenarios would explain the need for higher FK866 doses. In addition, in CLL, a positive CD38 status is a negative prognostic marker associated with poor response to chemotherapy (44). This

Figure 6. exNAD rescues the impact of FK866 on cellular energy content, viability, and apoptotic signaling. CLL cells were cultured in media supplemented with 100 μmol/L exNAD for 18 hours. Samples were then treated with 25 nmol/L FK866 for 2 days for immunoblot analysis and for 3 days for analysis of cellular energy content and viability. Further experimental setups included FK866 treatment alone, exNAD supplementation alone, and vehicle-treated control (Ctrl). A, exNAD nearly doubled intracellular NAD, whereas FK866 treatment almost completely abolished NAD content (left) and ATP content (right) after 3 days. exNAD significantly protected CLL cells from FK866-induced NAD (n = 3, left) and ATP loss (n = 11, right). B, exNAD rescued FK866-induced viability reduction based on Annexin V-FITC/PI negativity as demonstrated by bar chart (n = 6, top left) and representative dot blot (bottom). The paired Student t test was used to assess statistical significance. C, cleavage of caspase-3 and downregulation of Mcl1, which were induced by FK866 treatment, were also rescued by exNAD supplementation. Vinculin served as a loading control. Representative immunoblots are depicted (n = 3). *, p < 0.05; ***, p < 0.0001.
is attributed to an increased proliferation status as reflected by an increased LDT (45–48), and would suggest that higher levels of FK866 are required to sufficiently shut down cellular energy generation. In line with this, increased LD50 values observed in our relapsed patient subgroup were codependent on CD38. It has to be considered, that the CD38 status is variable and dependent on time and topographic localization of the CLL cell within the body (49, 49, 50). Therefore, further studies examining the role of CD38 in response of CLL cells to NAMPT inhibition are required.

Although the sample size is limited, we demonstrated that FK866 is equally effective in high-risk patients frequently associated with the presence of del17p13.1 (Fig. 4C; refs. 4, 51). FK866 did neither upregulate p21, a direct downstream target of intact p53 (Fig. 4E), nor did the p53 activator Nutlin improve FK866-induced cell death (Fig. 4D), suggesting that FK866 acts independent of this pathway in CLL. It is believed that resistant clones emerge despite standard treatments with relapsed patients having drug-resistant disease with an increased likelihood of being del17p13.1 (51). Thus, NAMPT inhibition by FK866 with standard therapy might prolong remissions and decrease the emergence of resistant clones. Mechanistically, fludarabine can also act through a p53-independent mechanism, which activates PARP to deplete NAD, thus causing a severe energy crisis potentiating cell death (37). The results of our drug combination studies demonstrated that FK866 could enhance the antitumor activity of fludarabine at clinically achievable concentrations for both drugs (Fig. 4B). Further studies examining the mechanism of this drug combination and alternative agents are required to explore the full potential of NAMPT inhibition in CLL.

Overall, in this study, we identified NAMPT inhibition by FK866 as a potential targeted treatment in CLL in the upfront and in high-risk patients, including those with a del17p13.1. Moreover, initial clinical studies have indicated that this is a well-tolerated drug currently in clinical trials with ease of translation to the clinic. Thus, inhibition of NAD metabolism by FK866 may be a novel and important approach, either alone or in combination with existing agents, for the treatment of CLL.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: I. Gehrke, S.B. Gibson, V. Banerji


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): I. Gehrke, E.D.J. Bouchard, A.G. Poeppl, J.B. Johnston, V. Banerji

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): I. Gehrke, E.D.J. Bouchard, S. Beiggi, A.G. Poeppl, V. Banerji

Writing, review, and/or revision of the manuscript: I. Gehrke, E.D.J. Bouchard, J.B. Johnston, S.B. Gibson, V. Banerji

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.G. Poeppl, V. Banerji

Study supervision: V. Banerji

Acknowledgments

The authors thank Donna Hewitt, Erin Streu, Jean Guevara, Mandy Squires, and the Manitoba Blood and Marrow Tumor Bank for providing samples and clinical patient characteristics. The authors also thank Dr. Sadi AlMuftaki for carrying out FISH analysis and Michelle Queen for IgYh analysis. The authors acknowledge and thank all patients who donated their blood for this study.

Grant Support

This work was supported in part by operating funds awarded to V. Banerji by the CancerCare Manitoba Foundation, the Manitoba Medical Service Foundation, and the University of Manitoba Research Grant Program. E.D.J. Bouchard is supported by the Thorlakson Foundation, and I. Gehrke is supported by a joint fellowship from the Manitoba Health and Research Council and the CancerCare Manitoba Foundation.

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Received March 12, 2014; revised June 12, 2014; accepted July 2, 2014; published OnlineFirst August 29, 2014.

References


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*Clin Cancer Res* Published OnlineFirst August 29, 2014.

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