Reciprocal Potentiation of the Antitumoral Activities of FK866, an Inhibitor of Nicotinamide Phosphoribosyltransferase, and Etoposide or Cisplatin in Neuroblastoma Cells

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ABSTRACT

NAD is an essential coenzyme involved in numerous metabolic pathways. Its principal role is in redox reactions, and as such it is not heavily "consumed" by cells. Yet a number of signaling pathways that bring about its consumption have recently emerged. This has brought about the hypothesis that the enzymes that lead to its biosynthesis may be targets for anticancer therapy. In particular, inhibition of the enzyme nicotinamide phosphoribosyl transferase has been shown to be an effective treatment in a number of preclinical studies, and two lead molecules [N-[4-(1-benzoyl-4-piperidinyl)butyl]-3-(3-pyridinyl)-2E-propenamide (FK866) and (E)-1-[6-(4-chlorophenoxy)hexyl]-2-cyano-3-(pyridin-4-yl)guanidine (CHS 828)] have now entered preclinical trials. Yet, the full potential of these drugs is still unclear. In the present study we have investigated the role of FK866 in neuroblastoma cell lines. We now confirm that FK866 alone in neuroblastoma cells induces autophagy, and its effects are potentiated by chloroquine and antagonized by 3-methyladenine or by down-regulating autophagy-related protein 7. Autophagy, in this model, seems to be crucial for FK866-induced cell death. On the other hand, a striking potentiation of the effects of cisplatin and etoposide is given by cotreatment of cells with ineffective concentrations of FK866 (1 nM). The effect of etoposide on DNA damage is potentiated by FK866 treatment, whereas the effect of FK866 on cytosolic NAD depletion is potentiated by etoposide. Even more strikingly, cotreatment with etoposide/cisplatin and FK866 unmasks an effect on mitochondrial NAD depletion.

Introduction

NAD is an essential coenzyme involved in numerous metabolic pathways, and as such it is not heavily "consumed" by cells. Yet, it has been extensively demonstrated that a number of signaling pathways bring about its consumption (Berger et al., 2004; Koch-Nolte, et al., 2009). For example, NAD is a substrate for poly(ADP ribosyl)ation reactions, mono(ADP-ribosyl)ation reactions, and acetylation reactions and is the precursor for a number of molecules involved in calcium signaling (e.g. ADP-ribose, cyclic ADP-ribose, nicotinic acid adenine dinucleotide phosphate). Furthermore, the possibility that NAD (or precursors and metabolites) can also exist in cells to reuse nicotinamide released by NAD metabolism is unclear. In the present study we have investigated the role of FK866 in neuroblastoma cell lines. We now confirm that FK866 alone in neuroblastoma cells induces autophagy, and its effects are potentiated by chloroquine and antagonized by 3-methyladenine or by down-regulating autophagy-related protein 7. Autophagy, in this model, seems to be crucial for FK866-induced cell death. On the other hand, a striking potentiation of the effects of cisplatin and etoposide is given by cotreatment of cells with ineffective concentrations of FK866 (1 nM). The effect of etoposide on DNA damage is potentiated by FK866 treatment, whereas the effect of FK866 on cytosolic NAD depletion is potentiated by etoposide. Even more strikingly, cotreatment with etoposide/cisplatin and FK866 unmasks an effect on mitochondrial NAD depletion.

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olizing enzymes. This pathway, known as the “NAD salvage pathway” is schematically composed of two enzymes: nicotinamide phosphoribosyl transferase (NAMPT; also known as visfatin or PBEF1), which forms nicotinamide mononucleotide from nicotinamide and phosphoribosyl-pyrophosphate, and nicotinamide mononucleotide adenyllyl transferase, which leads to NAD from nicotinamide mononucleotide and ATP.

The link between NAMPT and cancer therapy is rapidly strengthening (Imai, 2009; Galli et al., 2010). First, NAMPT has been shown to be up-regulated in a number of solid tumors (Bi and Che, 2010). Second, NAMPT has been shown to be involved in angiogenesis by inducing vascular endothelial growth factor and matrix metalloproteinase 2/9 expression (Drevs et al., 2003; Kim et al., 2007). Third, an important role in tumorigenesis or tumor treatment has been postulated or proven for a number of NAD-using enzymes (Haigis and Sinclair, 2010; Rouleau et al., 2010; e.g. PARP, sirtuins). Fourth, and most important, two inhibitors of NAMPT [N-[4-(1-benzoyl-4-piperidinyl)butyl]-3-(3-pyridinyl)-2-EP-propenamide (FK866) and (E)-1-[6-(4-chlorophenoxy)hexyl]-2-cyano-3-(pyridin-4-yl)guanidine (CHS 828)] have been shown to have potent antitumoral activity in vitro and in vivo (Hasmann and Schemainda, 2003; Olesen et al., 2008). Indeed, both drugs have entered clinical trials for a number of malignancies and are now in phase I/II (www.clinicaltrials.gov). These reports have also brought a surge of interest in the development of novel NAMPT inhibitors, and it is now emerging that these agents may also be useful in inflammatory and autoimmune diseases (Busso et al., 2008; Bruzzone et al., 2009).

Initial observations suggested that FK866 induced cellular NAD depletion followed by ATP depletion, and this brought about apoptotic cell death (Hasmann and Schemainda, 2003). Yet, more recently, our group observed that, in neuroblastoma cells, NAD depletion by FK866 leads to autophagy (Billington et al., 2008a,b). A number of reports have confirmed, in different cell types, both modes of cell death (Bruzzone et al., 2009; Cea et al., 2009; Hsu et al., 2009; Nahimana et al., 2009).

Given the possible influence of NAD-depleting agents on downstream effectors such as PARPs and sirtuins, it could be postulated that these drugs could synergize with other chemotherapeutic agents. Indeed, it has been reported that FK866 potentiates the actions of lactate dehydrogenase inhibitors, as well as those of etoposide and cisplatin. As such, the combination of FK866 and etoposide is given by cotreatment of cells with ineffective concentrations of FK866 (1 nM). Cell death by cotreatment is via apoptosis and necrosis. The effect of etoposide on DNA damage is potentiated by FK866 treatment, whereas the effect of FK866 on cytosolic NAD depletion is potentiated by etoposide.

Materials and Methods

Cell Culture. SH-SY5Y cells were cultured as described previously (Billington et al., 2008b). SK-N-BE and GI-ME-N cells were cultured in RPMI medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate/ nonessential amino acids, 2 mM glutamine, 10 units/ml penicillin, and 100 μg/ml streptomycin. HEp2 cells were cultured in minimal essential medium (Sigma-Aldrich) supplemented with 10% FBS, 2 mM glutamine, 10 units/ml penicillin, and 100 μg/ml streptomycin. IMR-32 cells were cultured in minimal essential medium (Sigma-Aldrich) supplemented with 10% FBS, 2 mM glutamine, 10 units/ml penicillin, and 100 μg/ml streptomycin. Cerebellar granules were prepared from postnatal day 7 rat cerebellum as described previously (D’Mello et al., 1993) and cultured in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) supplemented with 10% FBS, 2 mM glutamine, 10 units/ml penicillin, 100 μg/ml streptomycin, 1% sodium pyruvate, and 25 mM KCl. Cells were maintained in a humidified incubator supplied with 5% CO2/95% air at 37°C and were subcultured as needed by detaching the cells with 0.25% trypsin and 5 mM EDTA. For immunoblot analysis, cells were cultured onto 60-mm plates. After the indicated treatments, cells lysates were prepared, and protein concentration was determined using the Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Thirty micrograms of total protein from cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting using anti-LC3B (Sigma-Aldrich), anti-antioxidant (Calbiochem, San Diego, CA), or anti-α-tubulin (Sigma-Aldrich) to monitor for equal loading and/or purity of fractions.

Viability Assays. Viability of cells was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay as described elsewhere (Colombano et al., 2010). Apoptotic Nuclei Staining. For analysis of nuclear integrity, neuroblastoma cells were plated on 12-mm glass coverslips and treated the next day. Hoechst 33342 dye (Sigma-Aldrich) was used for nuclear staining of living and apoptotic cells (fragmented nuclei). In brief, the dye (0.8 μg/ml) was added to cultured cells for 30 min followed by fixing in 4% paraformaldehyde for 10 min at room temperature. Cells were washed twice with PBS and mounted onto cover slips to be visualized with a UV filter (360/395 nm). Lactate Dehydrogenase Assay. Neuroblastoma cells were cultured in 96-well plates, and LDH release was determined using the LDH cytotoxicity assay kit after 48 h of treatment (Roche Diagnostics, Indianapolis, IN).

Annexin V/Propidium Iodide Staining. SH-SY5Y cells were cultured in 60-mm plates. After the indicated treatment cells were detached with trypsin/EDTA and incubated 30 min with annexin V-APC (1 mg/ml) and propidium iodide (PI; 1 μg/ml). After the incubation time, cells were analyzed using the FACScalibur cell analyzer (BD Biosciences, San Jose, CA).

GFP-LC3B Translocation. Exponentially growing cells were transfected with EGFP-LC3B (cloned in the pLEGF plasmid from Clontech, Mountain View, CA) by liposome transfection (Lipo- fectamine 2000; Invitrogen, Carlsbad, CA) and selected with puromycin for approximately 3 weeks. After this time, cells stably expressed the EGFP-LC3B fusion protein and translocation was analyzed using a TCS-S2P laser-scanning microscope (Leica, Wet-

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Generation of a GFP\(^\text{+/ATG7}\) Stable Cell Line. A pGITZ vector encoding siRNAs specific for ATG7 or a scrambled siRNA (Open Biosystems, Huntsville, AL) were used. For lentiviral infection of SH-SY5Y cells, we transiently transfected human embryonic kidney 293T cells and, 48 h later, collected the infectious supernatant and used it to infect (over 48 h) SH-SY5Y cells. After this, supernatant was removed and cells were cultured in the presence of \((2R,3S,4R,5R,6S,5\text{-amino}-6\text{-[(1R,2S,3S,4R,6S)-4,8-diamo-3\text{-[(2R,3R,4R,5S)-3,5-dihydroxy-5-methyl-4-methyldinoanoxan-2-y]oxy-2-hydroxy)cyclohexyl]oxy-2,4,6-tri-}
\text{hydroxyethyl} \text{oxane-3,4-diol (G418) (1 mg/ml) for clonal separation and sorted for GFP expression. GFP}\(^\text{+}\) cells were kept in culture or lysed in Laemmli buffer for Western blot analysis. When indicated, GFP\(^\text{+}\) cells were resorted to obtain a brighter GFP\(^\text{+}\) population.

NAD(P) Cycling Assay. Total cellular NAD(P) was measured as described previously (Billington et al., 2008b).

ATP Assay. Neuroblastoma cells were cultured in 96-well plates and after the indicated treatment ATP levels were determined using the ATP bioluminescent somatic cell assay kit (Sigma-Aldrich).

Preparation of Subcellular Fractions. SH-SY5Y and SK-N-BE cells were grown in 75-cm plates. After treatment (24 h), cells were lysed in buffer A \((20 \text{ mM HEPES, pH 7.6, } 10 \text{ mM KCl, } 1.5 \text{ mM MgCl}_2, 0.1 \text{ mM EDTA, } 1 \text{ mM dithiothreitol (DTT), } 250 \text{ mM sucrose, } 10 \text{ mg of aprotinin per ml, } 10 \text{ mg of leupeptin per ml, } 10 \text{ mM benzamidine, } 1 \text{ mM phenylmethylsulfonyl fluoride (PMSF), } 0.2 \text{ mM sodium orthovanadate and } 1 \text{ mM NaF})\) and homogenized (60 strokes) with a syringe with a 27-\text{g} needle. The absence of intact cells per ml, 10 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.2 mM sodium orthovanadate, 1 mM NaF). Protein kept on ice. The pellet containing mitochondria were washed and supernatants, containing the cytosolic fractions, were collected and before confocal analysis.

For NAD level determination, nuclear, cytosolic, and mitochondrial fractions were diluted with an equal volume of HClO\(_4\) (2 M) and kept on ice, and NAD levels were quantified using the protocol described previously (Billington et al., 2008b).

\(\gamma\)-H2AX Immunofluorescence. HeLa cells were grown on 12-mm glass coverslips. At the end of experiments, cells were fixed in 4% paraformaldehyde. Fixed cells were permeabilized by a 7-min treatment with 0.5% Triton X-100 in bovine serum albumin, blocked with gelatin and bovine serum albumin in PBS for 1 h, and immunoprobed with the primary antibody for another 1 h at room temperature. Incubation with secondary antibodies (Alexa Fluor 546 goat anti-mouse; Invitrogen) was performed for 30 min at room temperature. Nuclei were stained with 0.2 \text{ mM To-PRO (Molecular Probes)} for 10 min. Images were acquired with a Leica TCS-SP2 Laser-scanning microscope.

Micro-Gel Electrophoresis (Comet) Assay. HeLa cells were cultured in 24-well plates (100,000 cells/well). After the indicated time, cells were centrifuged, washed with calcium- and magnesium-free PBS, detached with trypsin/EDTA, and resuspended in 500 \text{ µl of PBS}. In a 2-ml tube, 50 \text{ µl of the cell suspension (10,000 cells)} and 500 \text{ µl of melted LMAgarose were mixed, and 75 µl was pipetted onto a prewarmed Comet slide. The slides were placed flat in the dark at 4°C for 10 min to allow the mixture to solidify and then immersed in prechilled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma base, 1% Triton X-100, 10% dimethyl sulfoxide, pH 10.0) at 4°C for 2 h. Slides were removed from the lysis solution, tapped, and immersed in alkali solution for 30 min at room temperature in the dark. Slides were electrophoresed at low voltage (300 mA, 25 V, 4°C) for 20 min. Slides were washed three times in a neutralization solution (0.4 M Tris-HCl, pH 7.5) and air-dried for 20 min. Slides were stained with SYBR Green stain (Invitrogen) and allowed to air dry at room temperature for 30 min. SYBR Green-stained Comet slides were viewed with a fluorescence microscope and analyzed using Comet Assay Analysis System software (Comet Assay 4; Perceptive Instruments, Suffolk, UK).

Immunohistochemistry of Neuroblastoma Slices. Formalin-fixed, paraffin-embedded tissue blocks of histologically proven neuroblastomas were retrieved from the archives of the Ospedale Maggiore della Carità pathology unit (Novara, Italy), and 3-\text{µm sections were obtained. After deparaffinization and heat-induced antigen retrieval in EDTA solution, mouse monoclonal anti-Nampt antibody (Enzo Life Sciences, Inc., Farmingdale, NY) was used at a 1:1000 concentration in Ventana antibody diluent (Ventana Medical Systems, Tucson, AZ) and incubated for 60 min. The Dako Envision anti-mouse-rabbit secondary antibody (Dako North America, Inc., Carpenteria, CA) was used for 30 min. The 3,3'\text{-diaminobenzenide-based staining system was used (Dako North America, Inc.). Slides}

Fig. 1. NAMPT is present in neuroblastoma tumors and its inhibition leads to cell death in vitro. A, representative immunohistochemical staining of a neuroblastoma tumor. Four separate tumors showed similar staining. B, concentration-response curves of FK866 in neuroblastoma cell lines and cerebellar granule cells. Values are mean ± S.E.M. of 8 to 16 replicates from two to four separate experiments.

A

B

viability (% of control)

100

10

0.1

0.01

0.001

1

0.1

10

100

[FK866] nM

SH-SY5Y

SK-N-BE

HME-N

- BNF-22

+ cerebellar granules

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were then counterstained with ematoxylin, dehydrated, and processed according to routine laboratory protocols.

Results

NAMPT Is Present in Neuroblastoma Samples and Is a Possible Pharmacological Target. To investigate whether NAMPT is present in neuroblastoma tumors, we investigated its presence in four samples retrieved from the pathology archive of the hospital. In all tumors, NAMPT staining was evident (Fig. 1A for an example). Staining was dishomogenous between Ospedale Maggiore della Carità and appeared both cytoplasmic and nuclear.

We next performed concentration-response curves on four cell lines originally obtained from neuroblastoma tumors. These cell lines exhibit different pathological features and genetic alterations (Thiele, 1998). In all four cell lines, FK866 triggered cell death with IC$_{50}$ values below 10 nM (Fig. 1B). The approximate IC$_{50}$ values were SH-SY5Y, 3.4 ± 1.2 nM; SK-N-BE, 5.6 ± 3.1 nM; GI-ME-N, 3.4 ± 1.2 nM; IMR-32, 2.6 ± 0.9 nM. As a control, we also performed cytotoxicity assays on primary cerebellar granule cells neurons, where FK866 was unable to induce substantial cell death at concentrations up to 1 μM (Fig. 1B).

FK866 Induces Autophagy in SH-SY5Y Cells. We then decided to evaluate whether autophagy was responsible for these effects. IMR-32 cells are difficult to grow and do not adhere well to glass coverslips; therefore, we decided to concentrate on the other three cell lines. To characterize the effect of FK866 in neuroblastoma cells, we generated a stable SH-SY5Y cell line overexpressing the chimeric protein EGF-P-LC3. In this cell line, treatment with FK866 (10 nM) for 32 h yielded the formation of autophagic vacuoles, as observed in confocal microscopy by colocalization of EGF-P-LC3 with Lysotracker (Fig. 2A) as described previously (Billington et al., 2008b). This effect was slow, because it was not evident after 24 h of treatment. To confirm that this effect could not be attributed to overexpression, we also prepared cell lysates from control SH-SY5Y cells grown in the presence or absence of FK866 (10 nM) to investigate the conversion of LC3-I to LC3-II, the phosphatidylethanolamine conjugate enriched in autophagosomes (Mizushima and Yoshimori, 2007). Indeed, LC3-II was abundant in FK866-treated cells (Fig. 2B). Similar data were obtained with another compound that we have previously synthesized that also targets NAMPT (C. Traveli and A. A. Genazzani, unpublished work and Colombano et al., 2010). Furthermore, experiments performed on SK-N-BE cells yielded identical results, i.e., LC3-II conversion in the presence of FK866 (10 nM, Fig. 2B). In GI-ME-N cells, LC3-II levels were also high under basal conditions; therefore, it was difficult to establish unequivocally whether an increase occurred upon FK866 incubation (data not shown).

LC3 immunoblotting is controversial, because an increase in the LC3-II lower band may represent both an increase in autophagy or a decreased autophagic flux (Klionsky et al., 2008). To discriminate between these two hypotheses, we incubated SH-SY5Y or SK-N-BE cells in the presence of chloroquine (5 μM), which should increase LC3-II in the presence of increased autophagy. Indeed, this was the case, confirming bona fide autophagy (Fig. 2B).

Although a number of chemotherapeutic agents (e.g., tamoxifen, temozolomide, arsenic trioxide) are known to induce autophagy, the contribution of this phenomenon on tumor treatment is still debated (Kondo et al., 2005). In a simplistic view, autophagy can be considered both as a means to kill tumoral cells or, more likely, a means for cells to resist treatment. To investigate what contribution autophagy might have on FK866-induced cell death in SH-SY5Y cells, we decided to generate a SH-SY5Y cell line with reduced levels of ATG7, which is essential for vesicle elongation and therefore for autophagy to proceed. We used a lentiviral construct encoding for GFP and for a siRNA against ATG7, thereby generating ATG7-GFP$^+$ cells. We were surprised to find that FK866 was significantly less cytotoxic in ATG7$^-$ cells (Fig. 2C); whereas the IC$_{50}$ value for FK866 in control SH-SY5Y cells was 3.5 ± 0.4 nM, it shifted to 12.6 ± 0.4 nM in ATG7-silenced cells. Use of a scrambled siRNA had no effect (4.9 ± 0.26 nM). It is well known that this molecular approach does not completely ablate expression of the target protein, and indeed we were still able to observe ATG7 protein, albeit at reduced levels, in ATG7$^-$ cells (Fig. 2C). Therefore, to further validate our result, we sorted cells for intensity of the GFP signal, which should inversely correlate with residual ATG7 activity. In accord with the data above, the brightest GFP$^+$ cells were more resistant to FK866 compared with the general ATG7-silenced cell population (IC$_{50}$ of 17.9 ± 1.7 nM). It should also be noted that this macroautophagy can proceed in an ATG7-independent fashion (Nishida et al., 2009); therefore, this may also have a contribution.

We then used chloroquine, which inhibits autophagy-some-lysosome fusion. As shown in Fig. 2D, chloroquine strongly potentiated the cytotoxic nature of FK866 by shifting the IC$_{50}$ left-ward by approximately 10-fold after 24-h treatment. Furthermore, FK866-induced cell death was anticipated in the presence of chloroquine, because it was already evident after 24 h (Fig. 2E). Similar data were obtained on SK-N-BE cells (Fig. 2E) and GI-ME-N cells (see supporting information).

Last, we incubated cells in the presence of 3-MA, an inhibitor of class III phosphatidylinositol 3-kinase that partici-
pates in vesicle nucleation, an early step of autophagy. 3-MA alone did not have a significant effect on cell viability in SH-SY5Y or SK-N-BE cells, but was able to protect cells from FK-866-induced cell death. This effect was particularly evident at 48 h, whereas it was partly ablated after 72 h (Fig. 2F). A protective effect at 48 h was also evident in GI-ME-N cells (see supporting information).

**FK866 Potentiates the Cytotoxic Effect of Cisplatin/Etoposide.** The data presented above show that autophagy is an important mediator of FK866-induced cell death. Given this observation and the controversy among the link between this phenomenon and antitumoral treatments, we reasoned that the potential of FK866 might be better used in combination therapy. Indeed, NAD depletion antagonizes, alongside basic metabolism, other pathways (e.g., PARPs, sirtuins) that might play an important role in mediating the actions of other drugs. To this extent, we performed concentration-response curves of combretastatin and paclitaxel, two agents acting on tubulin, and cisplatin and etoposide in the presence or absence of 1 nM FK866, which per se is devoid of any activity on cell viability (92 ± 1.3% compared with control) to investigate possible synergisms. Cytotoxic responses to combretastatin and paclitaxel were not affected by cotreatment for 48 h with FK866 (although these cells are sensitive to these agents). On the contrary, a striking potentiation was observed with etoposide, an inhibitor of topoisomerase II, and cisplatin, a cross-linker of DNA. For SH-SY5Y cells, these drugs alone displayed IC\(_{50}\) for cytotoxicity of 670 ± 100 and 790 ± 140 nM, respectively, and these were reduced to 1.0 ± 2.0 and 2.1 ± 0.2 nM in the presence of 1 nM FK866 (Fig. 3A). This is a striking 300- to 600-fold shift in the concentration-response curve. For SK-N-BE cells, these agents alone displayed IC\(_{50}\) for cytotoxicity of 3.0 ± 1.4 and 1.7 ± 0.7 μM, respectively, and these were reduced to 24 ± 3.6 and 73 ± 14 nM in the presence of 1 nM FK866 (Fig. 3B). For GI-ME-N cells, these drugs alone displayed IC\(_{50}\) for cytotoxicity of 38 ± 17 and 7.5 ± 1.2 μM, respectively, and these were reduced to 276 ± 92 and 255 ± 170 nM in the presence of 1 nM FK866 (Fig. 3C). Similar results were obtained in HeLa cells, where etoposide shifted its IC\(_{50}\) from 610 ± 98 to 12 ± 2.9 nM and cisplatin shifted its IC\(_{50}\) from 500 ± 86 to 8.3 ± 1.7 nM (Fig. 3D).

We then set out to establish whether this potentiation could confer further specificity to treatments. We incubated primary glial cells or cerebellar granule neurons with 1 nM FK866 and 100 nM etoposide or cisplatin. Neither combination treatment yielded significant cell death. Such treatment would have led to approximately 70% cell death in SH-SY5Y cells, 60% cell death in SK-N-BE, 30% cell death in GI-ME-N cells, and 70% cell death in HeLa cells. FK866 alone yielded a cytotoxic effect in glial cells at higher concentrations, and a concentration-response curve confirmed this finding.

**The Cytotoxic Effect of FK866 and Cisplatin/Etoposide Is Mediated Mainly by Necrotic Cell Death.** We then decided to explore the mode of cell death. We therefore investigated autophagic cell death, necrosis, and apoptosis. As shown in Fig. 4A, in SH-SY5Y cells the combination of etoposide + FK866 was unable to potentiate further the LC3-II formation induced by either agent alone, suggesting that autophagy might not be involved. Further evidence for this comes from the absence of effect of 3-methyladenine on the cytotoxicity induced by the drug combination in SH-SY5Y cells (Fig. 4B). We then analyzed LDH release from cell cultures treated for 48 h with the drug combination. As shown in Fig. 4C for SH-SY5Y cells, FK866 or etoposide alone did not display a significant effect, whereas the combination yielded a significant release of this intracellular enzyme. Similar effects on LDH release were obtained in SK-N-BE and GI-ME-N cells, suggesting that the mode of cell death is likely to be necrotic (see supporting information). FK866 at a higher concentration (10 nM) was also unable to increase LDH release substantially at this time point, probably because of its capacity to induce autophagy and induce a tardive cell death.

Whether apoptosis was also involved was assessed by determination of nuclear fragmentation using Hoechst staining in SH-SY5Y cells (Fig. 4, C and D; 40 h after treatment). In this assay, nuclear fragmentation was evident, providing evidence for a contribution from apoptotic cell death. Nonetheless, fluorescence-activated cell sorting analysis (Fig. 4, E and F; 40 h after treatment) showed that most cells were propidium iodide-positive and annexin V-negative, suggesting that necrosis was prominent.

**Cisplatin/Etoposide Potentiates NAD and ATP Depletion Induced by FK866.** We then asked whether the two hallmarks of FK866 action, global NAD depletion followed by ATP depletion (Hasmann and Schemainda, 2003), could also contribute to the actions of the combination treatment. As can be observed in Fig. 5, 100 nM etoposide or 100 nM cisplatin alone were unable to reduce NAD levels in SH-SY5Y cells. Likewise, in SK-N-BE cells 100 nM etoposide or 300 nM cisplatin (a concentration that yields similar cell death to 100 nM in SH-SY5Y cells) were unable to elicit any effect (Fig. 5). FK866 at a concentration of 1 nM reduced NAD levels by a maximum of approximately 20% after 24 h. As a comparison, FK866 at 10 nM reduced NAD levels by approximately 80% after 24 h. The combination of FK866 and etoposide/cisplatin induced a drastic drop of NAD levels in both SH-SY5Y and SK-N-BE cells. We were surprised to find that the combination yielded a significant drop in NAD levels after 6 h (see Supplemental Material). A similar pattern was observed when following ATP levels over time (Fig. 5). Indeed, the ATP drop induced by the combination was significantly greater than the one induced by 10 nM FK866 alone.

NAD levels are compartmentalized in cells, although debate persists about the subcellular distribution of NAD-synthesizing and -metabolizing enzymes. We therefore treated cells with compounds alone or in combination for 24 h and proceeded to fraction the different subcellular compartments (Fig. 6). In SH-SY5Y cells, treatment with 10 nM FK866 significantly affected the cytosolic pool (44 ± 4.7% of control), mildly affected the nuclear pool (74 ± 2.6% of control), and to a lesser extent affected the mitochondrial NAD (86 ± 5.3% of control). We have recently become aware that, in parallel, another laboratory has performed similar experiments in HeLa cells, also showing that FK866 affects the cytosolic pool without significantly altering the mitochondrial pool (Pittelli et al., 2010). Low concentrations of either FK866, etoposide, or cisplatin alone were unable to alter NAD content in any of the three compartments. On the contrary, the joint application had an effect on both the cytosolic and the nuclear pool, albeit at a similar or lower extent compared with 10 nM FK866. We were surprised to find that with this treatment the mitochondrial content was affected to a higher extent.
compared with FK866 (61.6 ± 4.4%). A similar effect on the mitochondrial pool was also observed in SK-N-BE cells cotreated with FK866 and etoposide (see Supplemental Material).

This finding warranted investigation of the possible convergence of FK866 and etoposide/cisplatin on mitochondria. To investigate whether this convergence could be direct, we used isolated mouse liver mitochondria and investigated Ca²⁺-retaining capacity and the mitochondrial respiratory chain in the acute setting after pretreatment of 2 h. In brief, these parameters were investigated in the presence of 10 nM FK866, 1 nM FK866, 100 nM etoposide, or the combination of the latter two. None of the parameters investigated were altered by the treatments (see Supplemental Material). It has been suggested that NAMPT may be present in mitochondria (Yang et al., 2007). Yet, a recent study by Pittelli et

Fig. 3. FK866 potentiates the cytotoxic effect of cisplatin and etoposide in neuroblastoma cells. A to D, FK866 was used at a concentration of 1 nM, which yielded no cytotoxicity after 48 h of treatment. E, effect of FK866, etoposide, or cisplatin in cerebellar granule cells or cultured astrocytes after 48 h of treatment. Values are mean ± S.E.M. of at least eight determinations from 2 separate experimental days.
Fig. 4. The combination of FK866 and etoposide induces necrosis and apoptosis. A, Western blot of endogenous LC3 processing. Experiment is representative of at least three separate experiments on separate cultures. B, viability of cells treated with FK866 (1 nM) and/or etoposide (100 nM). C, % of LDH release and DNA fragmentation. D, Hoechst staining at 32h and 40h. E, Flow cytometry analysis. F, Annexin V-APC and PI staining.
al. (2010) was not able to replicate these results. Furthermore, immunoblotting experiments of isolated mouse liver mitochondria failed to evidence a specific band corresponding to NAMPT (data not shown). These data, taken together, would therefore make it unlikely that the mitochondria is a direct relevant target of FK866, although it does not exclude that different cellular pathways converge on mitochondria.

**FK866 Potentiates the DNA Damage Induced by Cisplatin/Etoposide in HeLa Cells.** Because both cisplatin and etoposide share the capacity to induce DNA damage and NAD depletion should antagonize the reparative PARP activity, we turned our attention to a possible synergism at this level. Because HeLa cells are one of the best-studied systems for DNA damage mechanisms, and synergism was also apparent in this cell type, we opted to investigate this model (Fig. 7).

First, we investigated the amount of DNA damage in HeLa cells treated with the single agents or the combination by following the recruitment of γH2Ax to DNA damage foci in immunocytochemistry. In control or in FK866 (1 nM)-treated cells, specific γH2Ax nuclear staining was present in a minority of cells (after 32 h treatment, 14 and 16% of total cells, respectively). Most of these cells displayed fewer than 10 foci per cell. On the contrary, treatment of cells with 100 nM etoposide induced an increase in the number of positive cells (59% of cells after 24 h and 67% after 32 h). The amount of γH2Ax-positive cells was not substantially modified by cotreatment with FK866 (1 nM; 67% after 24 h and 69% after 32 h). A more detailed analysis, however, revealed a difference between etoposide-treated and etoposide + FK866-treated cells. As shown in Fig. 7C, in cells treated only with etoposide (100 nM), the number of foci per cell was limited (cells with more than 20 foci were approximately 25%). Such a number was substantially increased by cotreatment with FK866 at both 24 and 32 h. These data suggest that FK866 is able to potentiate the effect on DNA damage induced by low concentrations of etoposide. Nonetheless, it should be noticed that performing γH2Ax immunostaining with concentrations of etoposide that induce similar cell death alone (e.g., 10 μM) yields substantially different patterns of staining. In brief, treatment for just 1 h with 10 μM etoposide is sufficient to yield more than 30 foci in virtually all cells (data not shown). Such a qualitative observation suggests that synergism occurs by a plurality of mechanisms.

To confirm a converging action of FK866 and etoposide on DNA damage, we investigated this directly by using the Comet assay. This single-cell approach allows for direct vi-
sualization of DNA damage by performing an electrophoresis run and staining DNA with SYBR Green. Control and FK866-treated cells did not display measurable DNA damage. Etoposide induced a modest DNA fragmentation that could be quantified (Fig. 7D) by an increased fluorescence of the tail (percentage of tail DNA), an increase in the tail length (not shown), and an increase in tail moment (the product of the tail length × the fractional amount of DNA in the tail). All of these indices of DNA damage were significantly increased by FK866 treatment, supporting the conclusion that the DNA damage induced by etoposide is increased in the presence of FK866.

Discussion

In the present article, we provide three separate conclusions: 1) NAD-depleting agents are effective as cytotoxic agents in neuroblastoma cell lines; 2) the use of autophagy-interfering agents modulates this effect; and 3) FK866 and cisplatin/etoposide exhibit a strong synergic effect in neuroblastoma cells that might have important therapeutic repercussions.

We now show that FK866 alone at concentrations below 100 nM is effective in inducing cell death in four distinct neuroblastoma cell lines of different origin, histological type, and genetic alterations (SH-SY5Y, SK-N-BE, GI-ME-N, and IMR-32). Likewise, we had previously shown that SNB-19 and SNB-75, two other cell lines, were sensitive to NAD-depleting agents (Colombano et al., 2010). These data, together with the immunohistochemical data showing the presence of NAMPT in these tumors, suggests that these agents might have a role in neuroblastoma therapy. Indeed, CHS 828, a drug that shares a similar mechanism of action, has been shown to lead to tumor regression and metastatic spread in a orthotopic neuroblastoma mouse model (Fuchs et al., 2009). In our hands, FK866 showed selectivity for the tumoral cells compared with primary neurons, but it is unlikely that this selectivity will be maintained in all cell types, because NAD is a universal energy currency. For these reasons, we felt that NAD depletion might have a higher value in combination treatments.

Because FK866 induces autophagy in these cell lines, we investigated whether modulating this mode of cell death might affect the actions of the drug. Indeed, 3-MA and ATG7 silencing reduced the extent of FK866-induced cell death. This is supported by a recent article describing the ability of 3-methyladenine to prevent FK866-induced cell death in human leukemia cells (Zoppoli et al., 2010). Conversely, chloroquine was able to potentiate the actions of FK866. Although these data may seem contradictory, the most likely explanation is that autophagy needs to be triggered for FK866 to execute its action on cell death. Yet, if autophagy is triggered and then blocked downstream of elongation (i.e., downstream of ATG7) but before autophagosome-lysosome fusion (i.e., the site of chloroquine action), then cell death is more pronounced. These data strengthen the notion that completion of autophagy is protective. It should be noted that the combination of anticancer agents + chloroquine has already been postulated (e.g., temozolomide and chloroquine in glioblastoma or...
tamoxifen and chloroquine in breast cancer) and therefore our observation might also have translational implications (www.clinicaltrials.gov; Kondo et al., 2005). Although the effect of chloroquine is most likely caused by its effect on autophagy, we cannot exclude that it might be related to its effect on DNA, because it is a known intercalating agent (Pivonková et al., 2010). Obviously, FK866 induces a concentration-dependent NAD decrease, and it is likely that at higher concentrations (and therefore lower NAD levels) other modes of cell death might take over.

Alongside autophagy-modulating agents, it could be postulated that lowering NAD levels might potentiate the effect of other chemotherapeutic agents. Indeed, a number of enzymes involved in DNA repair (PARP, sirtuins) as well as mitochondria highly depend on NAD levels and are affected by chemotherapy. We now show that low, per se ineffective concentrations of FK866 are able to potentiate the effect of cisplatin and etoposide (the two principal DNA-damaging agents used in the therapy of neuroblastoma; Maris et al., 2007). Although chemopotentiating effects of FK866 with other agents have been shown previously in other cell types (Pogrebniak et al., 2006), the effect reported here is significantly more pronounced. Indeed, maximal effects are obtained at concentrations of both compounds that alone are completely inefficacious. Although we concentrated on etoposide and cisplatin because these represent the drugs of choice for neuroblastoma therapy, these data are in accord with data obtained with methylmethanesulfonate (Yang et al., 2007), TRAIL in leukemia and temozolomide, and a base excision repair inhibitor in glioblastoma (Goellner et al., 2011). How these drugs synergize is yet unknown. Our data suggest that inhibition of NAMPT increases DNA damage induced by cisplatin or etoposide, and, conversely, that NAD and ATP depletion induced by FK866 is potentiated by DNA-damaging agents. In this respect, it has been recently reported that TRAIL + FK866 synergize in inducing a decrease in NAD and ATP levels in leukemia cells (Zoppoli et al., 2010). A possible link between these two phenomena is given by PARP activation, which participates in DNA repair processes (Heitz et al., 2010) and is a NAD-consumsing enzyme.

In conclusion, our study reports a striking synergism between cisplatin/etoposide and FK866 in neuroblastoma, which deserves to be followed with further work in preclinical in vivo models and, hopefully, in humans. It should be noted that neuroblastoma is known to be relatively chemoresistant; therefore, our in vitro data are particularly encouraging. In addition, both etoposide and FK866 are moderately selective for cancer cells, and their selectivity is based on high proliferation rates. It could be envisaged that, by using both drugs...
at lower concentrations, their selectivity could synergize as well.

Authorship Contributions

Participated in research design: Travelli, Boldorini, Di Lisa, Canonic, and Genazzani.

Conducted experiments: Travelli, Drago, Maldi, and Kaludercic.

Contributed new reagents or analytic tools: Galli and Tron.

Performed data analysis: Travelli, Kaludercic, and Genazzani.

Wrote or contributed to the writing of the manuscript: Travelli and Genazzani.

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References


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