

Anticancer agent CHS-828 inhibits cellular synthesis of NAD

Uffe Høgh Olesen^{a,b,*}, Mette Knak Christensen^b, Fredrik Björkling^b, Marja Jäättelä^c,
Peter Buhl Jensen^{b,d}, Maxwell Sehested^{a,b}, Søren Jensby Nielsen^{a,b}

^a Experimental Pathology Unit, National University Hospital, Biocentre, Building 2, 3rd Floor, Ole Maaloes Vej 5, 2200 Copenhagen, Denmark

^b TopoTarget AIS, Symbion Science Park, Fruebjergvej 3, 2100 Copenhagen, Denmark

^c Apoptosis Department, Centre for Genotoxic Stress, Institute for Cancer Biology, Danish Cancer Society, 2100 Copenhagen, Denmark

^d Department of Oncology, Finsen Centre, National University Hospital, 2100 Copenhagen, Denmark

Received 3 January 2008

Available online 15 January 2008

Abstract

Malignant cells display increased demands for energy production and DNA repair. Nicotinamide adenine dinucleotide (NAD) is required for both processes and is also continuously degraded by cellular enzymes. Nicotinamide phosphoribosyltransferase (Nampt) is a crucial factor in the resynthesis of NAD, and thus in cancer cell survival. Here, we establish the cytotoxic mechanism of action of the small molecule inhibitor CHS-828 to result from impaired synthesis of NAD. Initially, we detected cross-resistance in cells between CHS-828 and a known inhibitor of Nampt, FK866, a compound of a structurally different class. We then showed that nicotinamide protects against CHS-828-mediated cytotoxicity. Finally, we observed that treatment with CHS-828 depletes cellular NAD levels in sensitive cancer cells. In conclusion, these results strongly suggest that, like FK866, CHS-828 kills cancer cells by depleting NAD.

© 2008 Elsevier Inc. All rights reserved.

Keywords: NAD; FK866; CHS-828; Nampt; PBEF; Visfatin; Metabolism; Cancer

NAD is a crucial cofactor of the oxidative respiratory chain, as well as a degradable substrate for several important enzymes including poly(ADP-ribose) polymerases (PARPs) and sirtuins [1], that are involved in genomic stability, apoptosis, aging, stress resistance, and metabolism [2–5]. In the absence of NAD resynthesis, these processes lead to NAD depletion and cell death through apoptosis or necrosis [6–8]. The main pathway for NAD resynthesis in mammals (the salvage pathway) uses nicotinamide as a substrate. The first, rate-limiting step is catalyzed by nicotinamide phosphoribosyltransferase (Nampt), also known as pre-B cell colony-enhancing factor 1 (PBEF) or visfatin

[9]. Blocking this enzyme will, as a consequence of the NAD turnover, lead to a gradual reduction of the amount of cofactor available.

Since most cancer cells have continuous PARP activation through DNA damage and genomic instability [6,10–12] and have higher energy consumption demands relative to non-transformed cells [13], they are expected to be more vulnerable to the inhibition of NAD synthesis than non-transformed cells [14]. This hypothesis has led to the development of an NAD synthesis inhibitor for the clinical treatment of cancer. FK866, a specific inhibitor of Nampt [14], has recently successfully completed a phase I study [15] and is currently in several phase I/II and II trials for the treatment of various cancers. It is believed to compete with nicotinamide [16] and thereby disrupt the synthesis of NAD, and it is so far the only published small molecule inhibitor demonstrated to interfere with Nampt function.

CHS-828 is a pyridyl cyanoguanidine compound originally identified in a screen for antihypertensive compounds [17]. It was shown to be a potent inhibitor of cell growth in

Abbreviations: NAD, nicotinamide adenine dinucleotide; Nampt, nicotinamide phosphoribosyltransferase; PARP, poly(ADP-ribose) polymerase; PBEF, pre-B cell colony-enhancing factor 1.

* Corresponding author. Address: Experimental Pathology Unit, National University Hospital, Biocentre, Building 2, 3rd Floor, Ole Maaloes Vej 5, 2200 Copenhagen, Denmark.

E-mail address: uho@topotarget.com (U.H. Olesen).

a broad range of tumor cell lines, allowing the progression of CHS-828 into a phase I study in oncology [18]. The mechanism of action of this small molecule inhibitor has since its initial publication in 1999 remained unsolved but recently it has been hypothesized to involve the inhibition of NF- κ B [19,20].

In this paper, we show a number of mechanistic similarities between the structurally different compounds FK866 and CHS-828. Together, our results strongly indicate that CHS-828 exerts its cytotoxic effect through inhibition of NAD synthesis.

Materials and methods

Cell lines and cell culture. Cell culture media and reagents were purchased from Invitrogen unless otherwise stated. Human small cell lung carcinoma OC-NYH (NYH) cells have been described (as GLC-2) [21]. All cells were maintained in RPMI 1640 with GlutaMax supplemented with 10% FBS (Perbio, Thermo Fischer Scientific) and 100 U/ml penicillin–streptomycin. Resistance towards CHS-828 was reached by culturing NYH cells with stepwise increasing concentrations of drug over a prolonged period of time until a satisfying level of resistance was obtained as previously described for other chemotherapeutics [22]. This resulted in the cell line NYH/CHS. The cells retained full resistance after 30 passages without CHS-828.

Drugs and reagents. All chemical reagents were purchased from Sigma–Aldrich, as were PS-1145, BMS-345541, and MG132. QNZ was obtained from Calbiochem (Merck). Our laboratory synthesized CHS-828 as described previously [23]. FK866 was supplied by TopoTarget A/S (Copenhagen, Denmark).

Cell culture assays. Cells were plated in 96-well plates (5000 cells/well for 48/72 h treatment, 1000 cells/well for 6 days treatment) 24 h before use and then incubated with drug for the time indicated. NYH/CHS was passaged without drug before use in these assays. The CyQuant assay (Invitrogen) was performed according to the manufacturer's recommendations except that due to the low adherence of NYH cells the plates were centrifuged for 5 min at 300g before the medium was removed. An incubation period of 45 min was used for the CyQuant reagent and fluorescence was measured at 535 nm. Data were analysed with Prism (GraphPad). For ATP/proliferation measurements, the CellTiterGlo assay (Promega) was performed according to the manufacturer's instructions. To determine the ATP levels, the results were correlated to cell number by normalizing to CyQuant data, and analysis was performed using Prism. Clonogenic assay was carried out using an assay with three week continuous drug exposure as previously described [24]. Counting of colonies was carried out in triplicate. Analysis was performed using Prism.

Western blot analysis. Cells were lysed in a buffer containing 20 mM NaCl, 25 mM MOPS, 2 mM EDTA, 2 mM Sodium Orthovanadate, 0.1% NP-40, 10% glycerol and 1% Ettan™ protease inhibitor mix (Amersham) using sonication. Protein concentrations were determined by Bio-Rad Protein Assay (Bio-Rad) according to the manufacturer's instructions. Proteins were separated by SDS–PAGE and blotted to a nitrocellulose membrane using the NuPAGE® Novex BisTris (XCell SureLock™) system (Invitrogen). Namp1/PBEF antibodies were obtained from Abcam and used at a dilution of 1:500 followed by anti-rabbit HRP-conjugated antibody (Amersham) at a dilution of 1:5000. HRP-conjugated GAPDH goat-antibody was purchased from SantaCruz, and used at a dilution of 1:2000. Detection of HRP-conjugated antibodies was performed with ECL Plus Blotting Reagent (Amersham) using a ChemiDoc XRS/Quantity One documentation system (Bio-Rad).

Quantification of NAD⁺/NADH synthesis. Logarithmically growing cells in Ø 6-cm culture dishes were treated with/without 10 nM CHS-828 or 1 nM FK866 for 24 h. After harvesting, 10⁶ cells were washed with cold PBS. EnzyChrom™ NAD⁺/NADH assay kit (BioAssay Systems, California) was used to determine the levels of NAD⁺ and NADH using the

manufacturer's guidelines with all steps performed at 4 °C, except for the final enzymatic process, which was performed at room temperature. Fluorescence data obtained was based on $\Delta(650\text{ nm}/550\text{ nm})$. A standard curve was made in accordance with the kit, and the data was fitted using Microsoft Excel. Protein concentrations in lysates were determined as described above, and NAD⁺ and NADH levels were normalized. Final data preparation and presentation was performed using Prism.

Results

The NYH/CHS cell line displays cross-resistance towards FK866

To investigate the potential of using FK866 to treat drug resistant cancers, FK866 cytotoxicity has been investigated in a number of drug resistant cell lines including a number of common multi drug resistant cell lines, none of which showed any significant cross-resistance to FK866 (our unpublished data). Following up on this we examined the activity of FK866 in the CHS-828 resistant small cell lung carcinoma cell line NYH/CHS. To our surprise, in a clonogenic assay, which examines long-term survival of cells, NYH/CHS was resistant to a comparable degree to both CHS-828 and FK866 as shown in Fig. 1A (178- and 115-fold, respectively). This cross-resistance was also seen in a short-term cell proliferation assay (CyQuant) based on quantification of DNA (data not shown). The cross-resistance was unexpected given the structural differences between CHS-828 and FK866 (Fig. 1B). In comparison, a screen of NYH/CHS sensitivity to other putative anti-cancer drugs revealed no cross-resistance to several inhibitors of NF- κ B (PS-1145, BMS-345541, and QNZ) or to an inhibitor of the proteasome (MG132) as shown in Fig. 1C. Similar lack of cross-resistance was observed for doxorubicin, camptothecin, vincristine, staurosporine, taxol, fluorouracil, 5-azacytidine, and belinostat (data not shown). We conclude that the NYH/CHS cell line is selectively cross-resistant to FK866, suggesting that CHS-828 may function by a mechanism related to the modulation of NAD synthesis similarly to FK866.

Early ATP loss is correlated with cell death for both CHS-828 and FK866

A distinct feature of FK866-induced cell death is that it is preceded by a drop in cellular ATP concentrations occurring between 24 and 48 h of treatment. The lowered ATP level is due to the depletion of NAD interfering with the oxidative energy production. Likewise, a drop in cellular ATP levels after 24 h of treatment has been reported after treatment with CHS-828, with cytotoxicity occurring much later (75% at 72 h) [25]. We examined whether this ATP loss occurs similarly following treatment with both drugs and whether it correlates with their IC₅₀ values for cell death in NYH cells. The IC₅₀ values obtained for reduction of ATP after 48 h correlated well with the induction of cell death after 6 days for both CHS-828 and FK866 in NYH cells (Figs. 2 and 3). No such decrease in cellular ATP con-

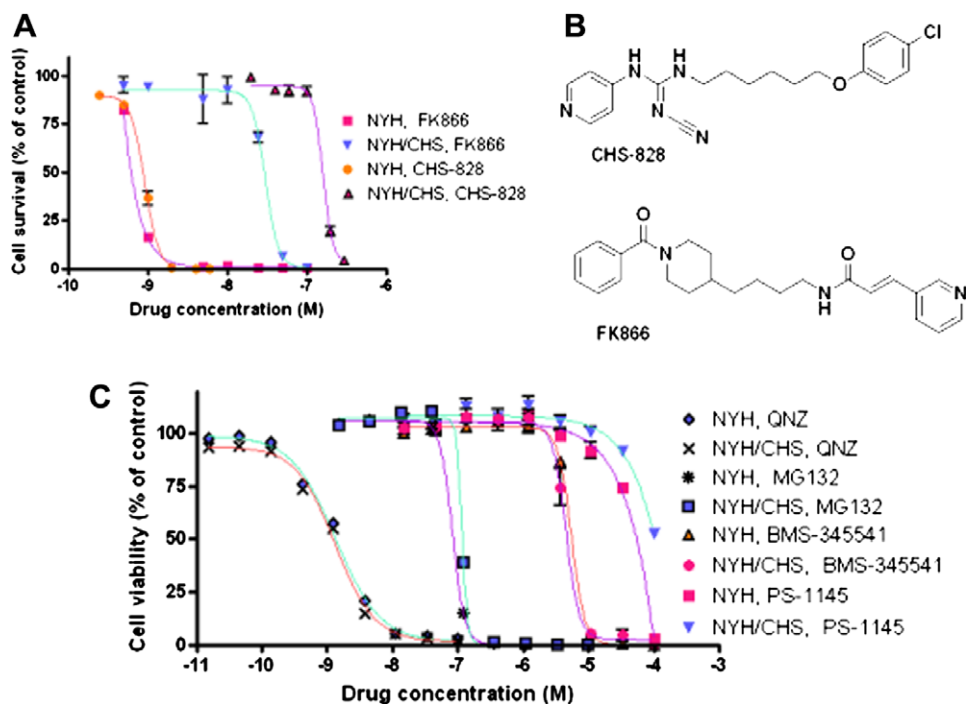


Fig. 1. (A) Cell survival for NYH and NYH/CHS cells measured by clonogenic assay. (B) Chemical structures of CHS-828 and FK866. (C) A screen for cross-resistance with NYH and NYH/CHS for the indicated inhibitors of NF- κ B measured by CellTiterGlo cell proliferation assay for a standard 3 days treatment. (Error bars indicate SEM, x-axes are logarithmic).

centrations was seen for other compounds tested, including doxorubicin and taxol (data not shown).

High concentrations of nicotinamide protect NYH cells from CHS-828

Although FK866 inhibits Nampt directly, it is possible to negate this inhibition by supplying high concentrations of nicotinamide [14]. It is believed that the high amount of substrate competes directly with FK866 for binding to Nampt, leading to sufficient NAD production through the NAD salvage pathway (Fig. 3C) for the cells to survive. To test whether CHS-828 could have a mechanism of action similar to FK866, we investigated the protective effect of nicotinamide when treating cells with CHS-828.

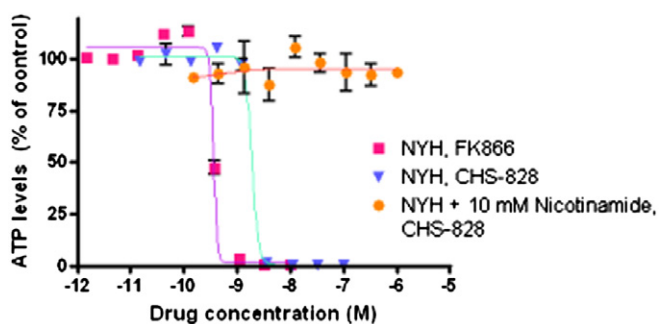


Fig. 2. ATP levels after 48 h treatment with FK866 and CHS-828 (with/without 10 mM nicotinamide) normalized to cell density. (Error bars indicate SEM, the x-axis is logarithmic).

As can be seen from Fig. 3A, a slight protective effect was seen at 0.1 and 1 mM nicotinamide and this effect increased to more than 1000-fold at 10 mM for CHS-828. This correlated well with what we found (Fig. 3B) and what has previously been documented for FK866 [14] where protection is partial at 1 mM nicotinamide and is complete at 10 mM. Nicotinic acid did not rescue NYH or NYH/CHS cells from cell death upon treatment with CHS-828 or FK866 (data not presented). This is presumably a consequence of the nicotinic acid synthesis pathway (Fig. 3C) being inactive in NYH cells similar to what has been found for HEPG2 cells [14], where nicotinamide but not nicotinic acid protects from cell death from FK866. Also, nicotinamide protected the cells against the early drop in ATP caused by CHS-828 (Fig. 2). The protective effect of nicotinamide strongly indicates a crucial involvement of NAD synthesis in the cytotoxic effect of CHS-828.

CHS-828 reduces NAD^+ and NADH levels in cells

To further characterize CHS-828 and the mechanism of CHS-828 resistance in NYH/CHS cells we investigated the NAD levels of untreated and CHS-828-treated NYH and NYH/CHS cells. As can be observed in Fig. 4A, treatment of NYH cells with 10 nM CHS-828 for 24 h lead to a dramatic drop in NAD levels. This is consistent with the NAD reduction in cells treated with FK866 (Fig. 4A). In comparison, CHS-828 and FK866 induced little or no reduction of NAD^+ /NADH levels in NYH/CHS cells at concentrations

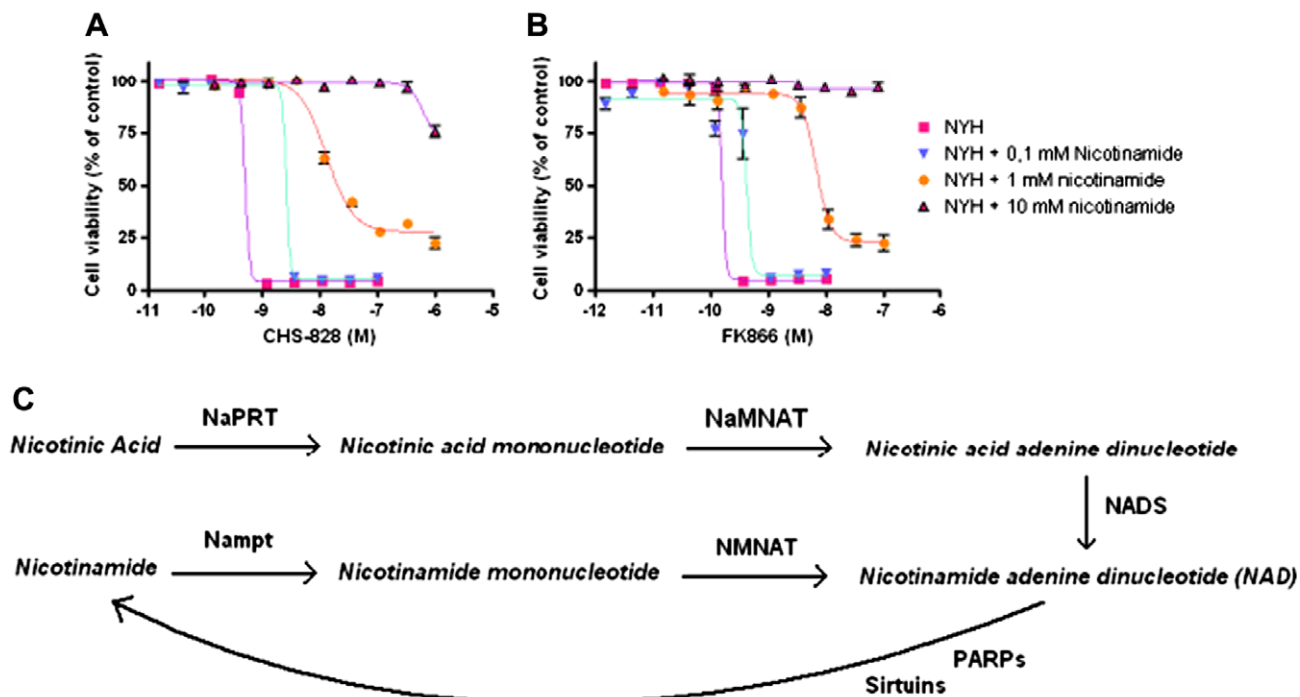


Fig. 3. Effect of nicotinamide on the cytotoxicity of CHS-828 (A) and FK866 (B) as measured by CyQuant after 6 days of treatment. (Error bars indicate SEM, x-axes are logarithmic.) (C) The mammalian NAD salvage pathways. Abbreviations used: NaPRT, nicotinic acid phosphoribosyltransferase; Nampt, nicotinamide phosphoribosyltransferase; NaMNAT, nicotinic acid mononucleotide adenylyltransferase; NMNAT, nicotinamide mononucleotide adenylyltransferase; NADS, NAD synthase; PARPs, poly(ADP-ribose) polymerases; Sirtuins, sirtuin 1–7.

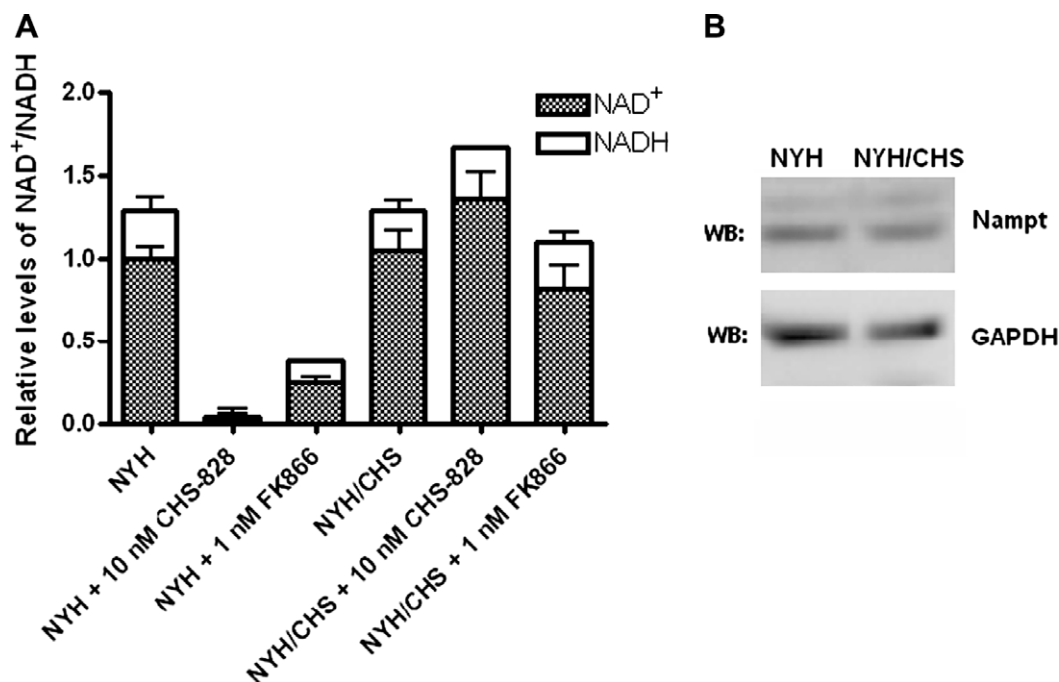


Fig. 4. (A) Cellular levels of NAD⁺ and NADH (24 h treatment) relative to the level of NAD⁺ in untreated NYH cells. (Error bars indicate SEM.) (B) Western blot analysis of Nampt and GAPDH protein expression.

sufficient to kill NYH cells. We examined whether this resistance seen for NYH/CHS was due to an increased expression of the Nampt gene but as Fig. 4B shows, the protein level of Nampt was similar for both cell lines.

Discussion

Originally thought as an investigation of the efficacy of the metabolic cell death pathway induced by FK866 in cell

lines resistant to other chemotherapeutics, this study lead to novel indications of the mechanism of action for the structurally distinct CHS-828 (Fig. 1B). CHS-828 has previously been hypothesized to function through NF- κ B inhibition [20], but the exact mechanism remained unclear. We originally found no common resistance mechanism for NYH/CHS cells, and so far increased resistance compared to NYH has not been observed for any type of compound including several substances known to inhibit NF- κ B directly or indirectly. This indicates a specific resistance mechanism for CHS-828. For FK866, we observe cross-resistance comparable to CHS-828 in NYH/CHS cells. This gives a strong indication that CHS-828 might be modulating the NAD metabolism. This corresponds well with CHS-828 inducing an early increase in glycolytic flux, decrease of ATP and shutdown of DNA and protein synthesis [25–28]. We have found that the drop in ATP levels after 48 h is similar for both FK866 and CHS-828, and that it occurs at drug concentrations able to kill NYH cells upon prolonged drug exposure.

A strong indication of CHS-828 having a mechanism of action closely related to the function of Nampt and thus the cellular NAD levels is the fact that high concentrations of nicotinamide are able to completely block the cytotoxic action of CHS-828. The levels of nicotinamide needed correspond well with the published data for FK866 [14]. Since nicotinamide can act as an antidote the hypothesis that CHS-828 exerts its effect through the synthesis of NAD is strengthened. Furthermore, the impact of treatment on the cellular NAD⁺/NADH levels is of similar magnitude for both CHS-828 and FK866 in NYH cells, whereas NAD⁺/NADH levels remain unchanged in NYH/CHS cells at the same drug concentrations.

Very recently, additional support for CHS-828 functioning by NAD depletion has been presented by Roulston et al. [29]. These authors have performed an extensive metabolic screen on CHS-828 where they found NAD⁺ to be significantly downregulated. They have established markedly decreased NAD⁺ levels as early as 6 h after initiation of treatment. They found nicotinic acid to protect HeLa cells from the toxic effect of CHS-828 but due to the content of nicotinic acid in their preparations of nicotinamide they could not discern a separate protective effect of high concentrations of nicotinamide. Most interestingly, they have performed *in vitro* studies of the inhibitory effect of CHS-828 on Nampt and found that the enzyme is inhibited by CHS-828 with an apparent K_i of 2.6 nM. Although resulting from a different initial approach, the results from Roulston et al. support our own hypothesis that CHS-828 acts as an inhibitor of Nampt.

Previously published results on CHS-828 are in accordance with inhibition of NAD synthesis as its primary mechanism of action. Thus, CHS-828 only induces death in cancer cells after prolonged periods of treatment, and this effect is preceded by a sharp drop in cellular ATP levels [25,28]. CHS-828 has been described as inducing an atypical form of cell death [30], sometimes mimicking apoptosis

and sometimes resembling necrosis [31–33]. One study also points out that pretreatment with CHS-828 inhibits induction of apoptosis by etoposide and instead leads to slow necrotic cell death [31]. This protection against apoptosis and the differing results on cell death could be explained by the fact that varying cell lines have been examined and differing basal levels of NAD and depletion rates could be crucial in determining whether enough ATP is present to execute apoptosis, or whether the cells will succumb to necrosis. Taken together, those results are consistent with CHS-828 controlling the cell death pathway depending on the level of ATP available.

We have previously found no significant difference between NYH and NYH/CHS cell lines with regard to plating efficiency, growth rate, drug uptake, and expression of major drug transporters (unpublished results). Similarly, examination of other CHS-828 resistant cell lines yielded no clue to the origin of the resistance [34]. NYH/CHS clearly displays resistance against reduction in NAD levels by FK866 and CHS-828, and we determined that this difference was not simply due to a changed expression of Nampt. The resistance could be due to metabolic adaptation or a mutation of Nampt in NYH/CHS but further research is required to fully delineate the mechanism of resistance in NYH/CHS cells.

In conclusion, our findings indicate that CHS-828 acts as an inhibitor of NAD synthesis, likely by direct inhibition of Nampt. Although this mechanism of CHS-828 needs closer inspection, it would seem that we have discovered a new chemical class of Nampt inhibitors.

Acknowledgment

The authors thank Sanne Christansen, Annette Nielsen, and Mette Frandsen for technical support.

References

- [1] R.A. Frye, Characterization of five human cDNAs with homology to the yeast SIR2 gene: Sir2-like proteins (sirtuins) metabolize NAD and may have protein ADP-ribosyltransferase activity, *Biochem. Biophys. Res. Commun.* 260 (1999) 273–279.
- [2] J.C. Ame, C. Spelshauer, G. de Murcia, The PARP superfamily, *Bioessays* 26 (2004) 882–893.
- [3] D. D'Amours, S. Desnoyers, I. D'Silva, G.G. Poirier, Poly(ADP-ribose)ylation reactions in the regulation of nuclear functions, *Biochem. J.* 342 (Pt 2) (1999) 249–268.
- [4] M.C. Haigis, L.P. Guarente, Mammalian sirtuins—emerging roles in physiology, aging, and calorie restriction, *Genes Dev.* 20 (2006) 2913–2921.
- [5] L.R. Saunders, E. Verdin, Sirtuins: critical regulators at the crossroads between cancer and aging, *Oncogene* 26 (2007) 5489–5504.
- [6] S. Beneke, J. Diefenbach, A. Burkle, Poly(ADP-ribose)ylation inhibitors: promising drug candidates for a wide variety of pathophysiologic conditions, *Int. J. Cancer* 111 (2004) 813–818.
- [7] S.W. Yu, H. Wang, M.F. Poitras, C. Coombs, W.J. Bowers, H.J. Federoff, G.G. Poirier, T.M. Dawson, V.L. Dawson, Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor, *Science* 297 (2002) 259–263.

- [8] S.J. van Wijk, G.J. Hageman, Poly(ADP-ribose) polymerase-1 mediated caspase-independent cell death after ischemia/reperfusion, *Free Radic. Biol. Med.* 39 (2005) 81–90.
- [9] J.R. Revollo, A.A. Grimm, S. Imai, The regulation of nicotinamide adenine dinucleotide biosynthesis by Nampt/PBEF/visfatin in mammals, *Curr. Opin. Gastroenterol.* 23 (2007) 164–170.
- [10] G.J. Hageman, R.H. Stierum, Niacin, poly(ADP-ribose) polymerase-1 and genomic stability, *Mutat. Res.* 475 (2001) 45–56.
- [11] C. Lengauer, K.W. Kinzler, B. Vogelstein, Genetic instabilities in human cancers, *Nature* 396 (1998) 643–649.
- [12] F. Nomura, M. Yaguchi, A. Togawa, M. Miyazaki, K. Isobe, M. Miyake, M. Noda, T. Nakai, Enhancement of poly-adenosine diphosphate-ribosylation in human hepatocellular carcinoma, *J. Gastroenterol. Hepatol.* 15 (2000) 529–535.
- [13] P.L. Pedersen, The cancer cell's "power plants" as promising therapeutic targets: an overview, *J. Bioenerg. Biomembr.* 39 (2007) 1–12.
- [14] M. Hasmann, I. Schemainda, FK866, a highly specific noncompetitive inhibitor of nicotinamide phosphoribosyltransferase, represents a novel mechanism for induction of tumor cell apoptosis, *Cancer Res.* 63 (2003) 7436–7442.
- [15] K. Holen, L.B. Saltz, E. Hollywood, K. Burk, A.R. Hanauske, The pharmacokinetics, toxicities, and biologic effects of FK866, a nicotinamide adenine dinucleotide biosynthesis inhibitor, *Invest. New Drugs* (2007).
- [16] J.A. Khan, X. Tao, L. Tong, Molecular basis for the inhibition of human NMPRTase, a novel target for anticancer agents, *Nat. Struct. Mol. Biol.* 13 (2006) 582–588.
- [17] P.J. Hjarnaa, E. Jonsson, S. Latini, S. Dhar, R. Larsson, E. Bramm, T. Skov, L. Binderup, CHS 828, a novel pyridyl cyanoguanidine with potent antitumor activity in vitro and in vivo, *Cancer Res.* 59 (1999) 5751–5757.
- [18] A. Ravaud, T. Cerny, C. Terret, J. Wanders, B.N. Bui, D. Hess, J.P. Droz, P. Fumoleau, C. Twelves, Phase I study and pharmacokinetic of CHS-828, a guanidino-containing compound, administered orally as a single dose every 3 weeks in solid tumours: an EORTC study, *Eur. J. Cancer.* 41 (2005) 702–707.
- [19] S.B. Hassan, H. Lovborg, E. Lindhagen, M.O. Karlsson, R. Larsson, CHS 828 kill tumour cells by inhibiting the nuclear factor-kappaB translocation but unlikely through down-regulation of proteasome, *Anticancer Res.* 26 (2006) 4431–4436.
- [20] L.S. Olsen, P.J. Hjarnaa, S. Latini, P.K. Holm, R. Larsson, E. Bramm, L. Binderup, M.W. Madsen, Anticancer agent CHS 828 suppresses nuclear factor-kappa B activity in cancer cells through downregulation of IKK activity, *Int. J. Cancer* 111 (2004) 198–205.
- [21] L. de Leij, P.E. Postmus, C.H. Buys, J.D. Elema, F. Ramaekers, S. Poppema, M. Brouwer, A.Y. van der Veen, G. Mesander, T.H. The, Characterization of three new variant type cell lines derived from small cell carcinoma of the lung, *Cancer Res.* 45 (1985) 6024–6033.
- [22] P.B. Jensen, B.S. Sorensen, M. Sehested, E.J. Demant, E. Kjeldsen, E. Friche, H.H. Hansen, Different modes of anthracycline interaction with topoisomerase II. Separate structures critical for DNA-cleavage, and for overcoming topoisomerase II-related drug resistance, *Biochem. Pharmacol.* 45 (1993) 2025–2035.
- [23] C. Schou, E.R. Ottosen, H.J. Petersen, F. Björkling, S. Latini, P.V. Hjarnaa, E. Bramm, L. Binderup, Novel cyanoguanidines with potent oral antitumour activity, *Bioorg. Med. Chem. Lett.* 7 (1997) 3095–3100.
- [24] P.B. Jensen, I.J. Christensen, M. Sehested, H.H. Hansen, L. Vindelov, Differential cytotoxicity of 19 anticancer agents in wild type and etoposide resistant small cell lung cancer cell lines, *Br. J. Cancer.* 67 (1993) 311–320.
- [25] S. Ekelund, R. Larsson, P. Nygren, Metabolic effects of the cytotoxic guanidino-containing drug CHS 828 in human U-937 lymphoma cells, *Anticancer Res.* 22 (2002) 2269–2274.
- [26] S. Ekelund, G. Liminga, F. Björkling, E. Ottosen, C. Schou, L. Binderup, R. Larsson, Early stimulation of acidification rate by novel cytotoxic pyridyl cyanoguanidines in human tumor cells: comparison with *m*-iodobenzylguanidine, *Biochem. Pharmacol.* 60 (2000) 839–849.
- [27] S. Ekelund, P. Nygren, R. Larsson, Guanidino-containing drugs in cancer chemotherapy: biochemical and clinical pharmacology, *Biochem. Pharmacol.* 61 (2001) 1183–1193.
- [28] P. Martinsson, G. Liminga, S. Dhar, M. de la Torre, A. Lukinius, E. Jonsson, S. Bashir Hassan, L. Binderup, J. Kristensen, R. Larsson, Temporal effects of the novel antitumour pyridyl cyanoguanidine (CHS 828) on human lymphoma cells, *Eur. J. Cancer* 37 (2001) 260–267.
- [29] A. Roulston, C. Bernier, H. Chan, M.O. Gratton, A. Jang, E. Koch, M. Lavoie, D. Paquette, M. Mitchell, A. Berger, L. Belee, X. Billot, G. Shore, P. Beauparlant, in: *Molecular Targets and Cancer Therapeutics*, AACR, San Francisco, California, 2007, p. A81.
- [30] P. Martinsson, M. de la Torre, L. Binderup, P. Nygren, R. Larsson, Cell death with atypical features induced by the novel antitumoral drug CHS 828, in human U-937 GTB cells, *Eur. J. Pharmacol.* 417 (2001) 181–187.
- [31] P. Martinsson, S. Ekelund, P. Nygren, R. Larsson, The combination of the antitumoural pyridyl cyanoguanidine CHS 828 and etoposide in vitro—from cytotoxic synergy to complete inhibition of apoptosis, *Br. J. Pharmacol.* 137 (2002) 568–573.
- [32] H. Lovborg, P. Martinsson, J. Gullbo, S. Ekelund, P. Nygren, R. Larsson, Modulation of pyridyl cyanoguanidine (CHS 828) induced cytotoxicity by 3-aminobenzamide in U-937 GTB cells, *Biochem. Pharmacol.* 63 (2002) 1491–1498.
- [33] C.M. Hansen, D. Hansen, P.K. Holm, R. Larsson, L. Binderup, Cyanoguanidine CHS 828 induces programmed cell death with apoptotic features in human breast cancer cells in vitro, *Anticancer Res.* 20 (2000) 4211–4220.
- [34] J. Gullbo, H. Lovborg, S. Dhar, A. Lukinius, F. Oberg, K. Nilsson, F. Björkling, L. Binderup, P. Nygren, R. Larsson, Development and characterization of two human tumor sublines expressing high-grade resistance to the cyanoguanidine CHS 828, *Anticancer Drugs* 15 (2004) 45–54.