HKI 46F08, a novel potent histone deacetylase inhibitor, exhibits antitumoral activity against embryonic childhood cancer cells

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Embryonic childhood cancer such as neuroblastoma and medulloblastoma are still a therapeutic challenge requiring novel treatment approaches. Here, we investigated the antitumoral effects of HKI 46F08, a novel trifluoromethyl ketone histone deacetylase (HDAC) inhibitor with a nonhydroxamic acid type structure. HKI 46F08 inhibits in-vitro HDAC activity in cell-free assays with a half maximal inhibitory concentration of 0.6 μmol/l and intracellular HDAC activity with a half maximal inhibitory concentration of 1.8 μmol/l. The compound reduces viability of both cultured neuroblastoma and medulloblastoma cells with an EC50 of 0.1–4 μmol/l. HKI 46F08 efficiently arrests tumor cell proliferation, represses clonogenic growth and induces differentiation and apoptosis in both MYCN-amplified and nonamplified neuroblastoma cells. In summary, we identified HKI 48F08 as a structural novel, potent HDAC inhibitor with strong antitumoral activity against embryonic childhood cancer cells in the low micromolar range. ***Anti-Cancer Drugs*** **19:849–857 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Embryonic childhood cancers are characterized by an undifferentiated histology resembling immature embryonic tissue with a clinical manifestation shortly after birth or within the first years of life. Despite recent progress using multimodal treatment protocols, advanced stage disease remains a therapeutic challenge. For example, neuroblastoma is a highly malignant tumor derived from precursor cells of the neural crest [1,2]. Advanced stage disease or *MYCN* oncogene-amplified tumors have a dismal prognosis despite intense multimodal therapy including high-dose chemotherapy with autologous stem cell rescue [3]. Therefore, novel treatment options are urgently warranted. We have previously shown that pharmacological inhibition of histone deacetylases (HDACs) may be a promising novel strategy for neuroblastoma treatment leading to inhibition of cell proliferation, clonogenic growth, induction of apoptosis and differentiation, and activation of retinoblastoma tumor suppression networks [4,5].

HDAC inhibitors are a novel class of compounds with promising antitumoral activities [6]. Exposure of cancer cells to HDAC inhibitors results in inhibition of cell proliferation, induction of apoptosis, induction of differentiation, inhibition of invasion and migration, inhibition of clonogenic growth and also in antiangiogenic effects in a variety of in-vitro and animal cancer models [6,7]. HDAC inhibitors change transcription of a number of genes. One of the key genes, however, having a central function in mediating HDAC inhibitor effects on cells seems to be p21Waf1/Cip1 [8]. On the basis of their chemical structure, HDAC inhibitors are grouped into different categories: short-chain fatty acids, hydroxamic acids, electrophilic ketones, cyclic tetrapeptides, and benzamides [6]. A few compounds are now being tested in phase I and II clinical trials [6,7] and recently, the hydroxamic acid SAHA has been approved by the FDA for the treatment of cutaneous T-cell lymphoma in adults [9]. Many HDAC inhibitors such as the short-chain fatty acid derivatives, however, require millimolar concentrations or, as the case with trichostatin A, are considered to be too toxic and are thus not suitable for in-vivo applications. Therefore, further development and identification of novel HDAC-inhibiting compounds are required.

We have recently screened a natural compound library using a novel fluorescence-based HDAC activity assay [10]. This screen yielded several novel compounds with

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HDAC inhibitory activity. Of these, HKI 46F08 appeared particularly interesting because of its novel structure and potent HDAC-inhibitory activity.

Here, we show that HKI 46F08 inhibits cellular HDAC activity at low micromolar concentrations, induces histone and tubulin hyperacetylation and displays antitumoral effects against several neuroblastoma and medulloblastoma cell lines.

Methods

Cell culture

Human neuroblastoma cell lines SH-EP and Kelly were cultured in RPMI 1640, WAC2 in RPMI 1640 supplemented with L-glutamine, BE(2)-C cells in Dulbecco's modified Eagle's medium. Human medulloblastoma cell lines were cultured in EMEM (Daoy) and in improved MEM (D458 and D487), respectively. All media were supplemented with 10% (v/v) fetal calf serum and cells were cultured in a humidified atmosphere with 5% CO_2. Neuroblastoma cell lines WAC2 and SH-EP cells were kindly provided by Manfred Schwab [11]. BE(2)-C and Kelly neuroblastoma cells were purchased from ECACC (Wiltshire, UK) or DSMZ (Braunschweig, Germany), respectively. Medulloblastoma cell line Daoy was purchased from ATCC (Manassas, Virginia, USA), and D458 and D487 cells were provided by Darrel D. Bigner, Duke University Medical Center [12,13]. Cell numbers were determined using an automated cell counter (Coulter). Compounds were dissolved in dimethylsulfoxide (DMSO) as indicated. The maximum final concentration in culture medium was 0.1% v/v DMSO and was therefore applied in all control experiments. At this concentration, no unspecific or toxic effects of DMSO were observed in the applied cell culture models.

Histone deacetylase activity assay

For determination of half maximal inhibitory concentration (IC_{50}) values, HDAC preparations from rat liver extracts (Calbiochem, Darmstadt, Germany) were incubated with increasing concentrations of HKI 46F08 in the presence of an acetylated fluorogenic substrate using a fluorescence-based HDAC assay as described previously [14]. Release of fluorescent 7-amino-4-methylcoumarin which correlates with deacetylating HDAC enzyme activity was measured in a 96-well plate reader. For assessment of inhibition of endogenous HDAC activity in neuroblastoma cells, the HDAC activity assay was modified as follows: cells were precultured overnight in a 96-well plate at 20 000 cells/well followed by adding the fluorogenic substrate and respective HDAC inhibitor for 3 h [14]. Cells were lysed by adding lysis buffer [50 mmol/l Tris/HCl, pH 8.0, 137 mmol/l NaCl, 2.7 mmol/l KCl, 1 mmol/l $MgCl_2$ with 1% (v/v) NP40] with 10 mg/ml trypsin. Read-out of the fluorescence signal intensity was performed in a 96-well plate reader. In control experiments, direct interference of HKI 46F08 with fluorescence signal was ruled out.

Western blot analysis

Detection of acetylated histone H4 proteins was performed by western blot analysis as we have described elsewhere [15] using an anti-histone H4-acetyl antibody from Upstate (Billerica, Massachusetts, USA). Acetylated tubulin proteins were detected using an anti-acetyl-tubulin antibody from Sigma (Munic, Germany). Levels of p21WAF1/Cip1 were probed using anti-p21WAF1/Cip1 antibody from Upstate. Equal loading of proteins was documented by reprobing the membranes with an anti-β-actin antibody from Sigma.

Quantitative reverse transcription-PCR

Quantification of mRNA expression was done by real time reverse transcription-PCR using SYBR green dye (Invitrogen, Carlsbad, California, USA) as described previously. Primer sequences used in this study were published previously [5].

Soft agar assay

Clonogenic growth of BE(2)-C neuroblastoma cells was determined using a soft agar assay system as described previously [5]. Clones were counted after culturing for 14 days in the presence of HDAC inhibitors as indicated in the figures.

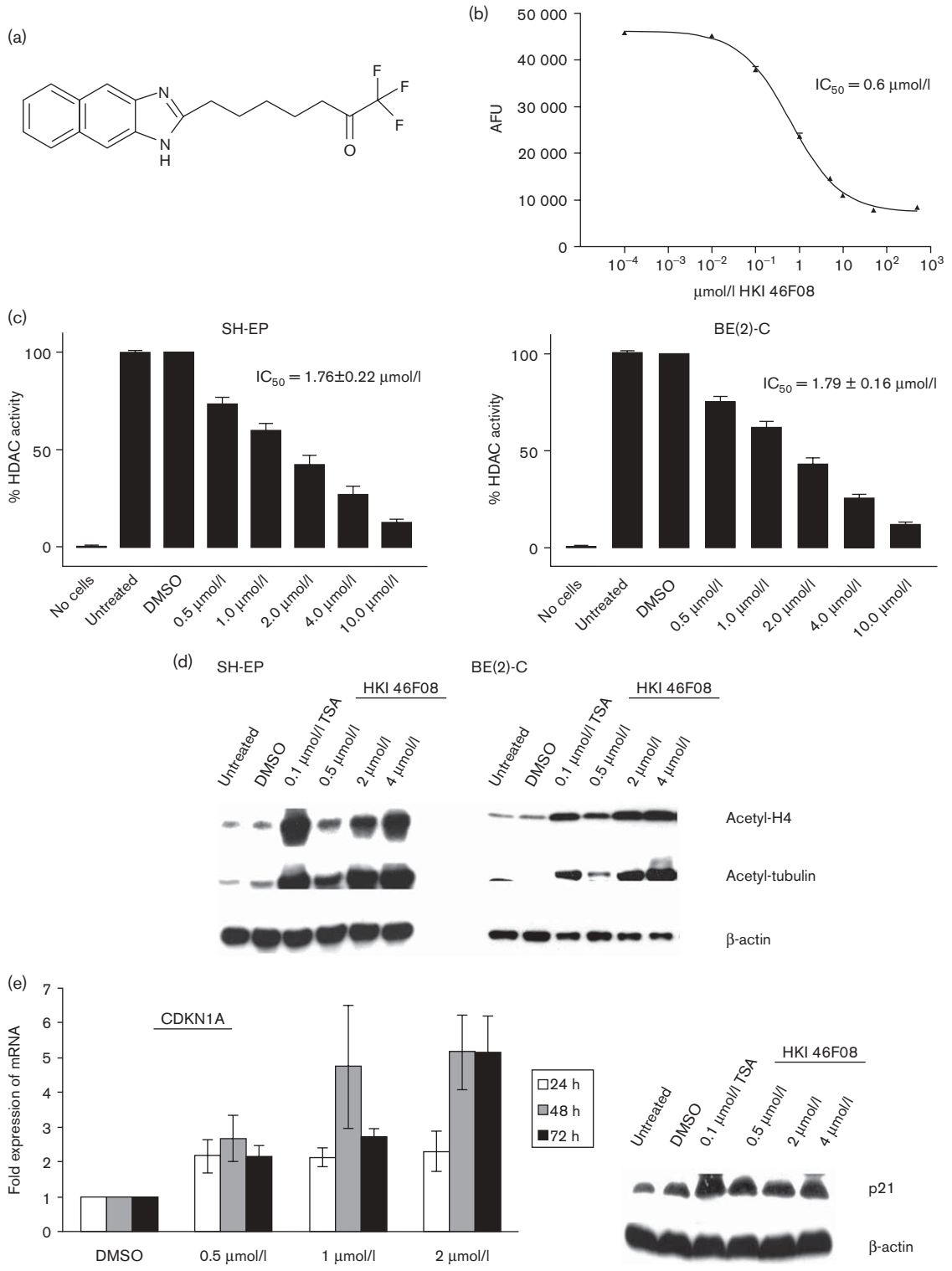
Determination of apoptosis and viability of cells

Cells were treated for 24 h with HDAC inhibitors and caspase 3-like activity was quantified in lysates using the caspase-3/CPP32 fluorometric assay kit from BioVision (Mountain View, California, USA) as described in the

HKI 46F08 inhibits histone deacetylase (HDAC) activity. (a) Chemical structure depicting the trifluoromethyl ketone with a nonhydroxamic acid type structure. (b) In-vitro inhibition of HDAC activity from rat liver preparation. Shown are arbitrary fluorescence intensities (AFU). (c) Intracellular inhibition of endogenous HDAC activity in SH-EP and BE(2)-C neuroblastoma cells. Values are expressed relative to endogenous HDAC activity of dimethylsulfoxide (DMSO) solvent-treated cells. Experiments were repeated at least three times and mean and standard errors were calculated as indicated. (d) Western blot analysis of histone H4 and tubulin acetylation after treatment of SH-EP and BE(2)-C neuroblastoma cells with HKI 46F08. Trichostatin A (TSA), a pan-HDAC inhibitor served as a positive control, β-actin as a protein loading control. (e) Induction of p21WAF1/Cip1 (CDKN1A) gene expression by HKI 46F08 in SH-EP neuroblastoma cells. Left panel: p21WAF1/Cip1 mRNA levels determined by quantitative reverse transcription-PCR. Induction of mRNA was statistically significant ($P<0.01$) with 1 and 2 μmol/l HKI 46F08 at all time points investigated. Right panel: Western blot analysis of p21WAF1/Cip1 protein levels after 24 h treatment of cells with HKI 46F08. Experiments were repeated three times and similar results were obtained. TSA served as a positive control, β-actin as a protein loading control. DMSO: solvent-treated control cells were cultured in the presence of 0.1‰ v/v DMSO corresponding to the maximum DMSO concentration applied with the compounds.

manufacturer's manual. Cell viability was determined after treatment for 48 h with HDAC inhibitor using the colorimetric WST-1 assay kit from Roche (Mannheim, Germany) as described in the manufacturer's manual. Metabolic activity was assessed with the tetrazolium derivative WST-1, which is reduced to a soluble colored formazan product by cellular mitochondrial dehydrogenases. The metabolic activity measured corresponds

Fig. 1



to the number of living cells and is used as a screening assay for cell viability [16].

Statistical methods

IC_{50} values were calculated with GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, USA) using non-linear regression curve fit. Statistical differences of mRNA expression data were determined using two-sided Student's *t*-test.

Results

HKI 46F08 is a potent histone deacetylase inhibitor *in vitro* and in tumor cells

We have recently screened a natural compound library using a fluorescence-based HDAC activity assay. Of the 3719 compounds screened, HKI 46F08, a trifluoromethyl ketone compound with a nonhydroxamic acid type structure, was identified as a novel HDAC inhibitor [10]. The chemical structure of the compound is depicted in Fig. 1a. Determination of in-vitro IC_{50} using HDAC activity from rat liver extracts in cell-free assays revealed an IC_{50} of 0.6 µmol/l (Fig. 1b). Next, we tested the ability of this compound to inhibit endogenous HDAC activity in cultured MYCN oncogene single copy (SH-EP) and amplified [BE(2)-C] neuroblastoma cells. HKI 46F08 inhibited HDAC enzymatic activity in both cultured neuroblastoma cell lines with an IC_{50} of 1.8 µmol/l (Fig. 1c). Furthermore, the compound induced hyperacetylation of histone H4 and tubulin proteins in a concentration-dependent manner indicative of targeting class I and II HDACs (Fig. 1d). As expression of the p21Waf1/Cip1 (CDKN1A, Cip/Waf1) gene has been found as a marker for HDAC inhibition in a variety of cultured cell models [8,17–22], we investigated the expression of this gene after exposure of cells to HKI 46F08. The compound induced CDKN1A mRNA expression significantly ($P < 0.01$) with 1 and 2 µmol/l HKI 46F08) (Fig. 1e). p21Waf1/Cip1 protein expression was confirmed by western blot analysis (Fig. 1e). Thus, HKI 46F08 is a potent inhibitor of HDAC activity in neuroblastoma cells with an IC_{50} of approximately 1.8 µmol/l which is in good correlation with the IC_{50} of the compound of 0.6 µmol/l determined *in vitro*, indicating efficient cellular uptake.

Inhibition of cell viability and proliferation of neuroblastoma and medulloblastoma cells by HKI 46F08

We next investigated the ability of HKI 46F08 to inhibit cell viability and proliferation of several neuroblastoma and medulloblastoma cell lines. As amplification of the MYCN oncogene is known to play a major role in tumor biology and clinical outcome of neuroblastoma [2,23], we used MYCN single copy cells (SH-EP), MYCN stably transfected cells (WAC2), and MYCN-amplified cells [BE(2)-C, Kelly] in these experiments. HKI 46F08 reduced cell viability in all neuroblastoma (Fig. 2a) and medulloblastoma (Fig. 2b) cell lines investigated in a concentration-dependent manner. The EC50 of cell viability was in the range of 0.1–4 µmol/l. We then exemplarily determined the effect of the compound on cell proliferation in SH-EP cells. Again, the compound inhibited cell proliferation in a concentration-dependent manner with a complete growth arrest at 2 and 4 µmol/l (Fig. 2c). For comparison, we included the HDAC inhibitor valproic acid in the viability and growth curve experiments, which is already used in clinical cancer trials (Fig. 2a and b).

Thus, HKI 46F08 inhibits cell viability and proliferation of neuroblastoma and medulloblastoma cells in the low micromolar range.

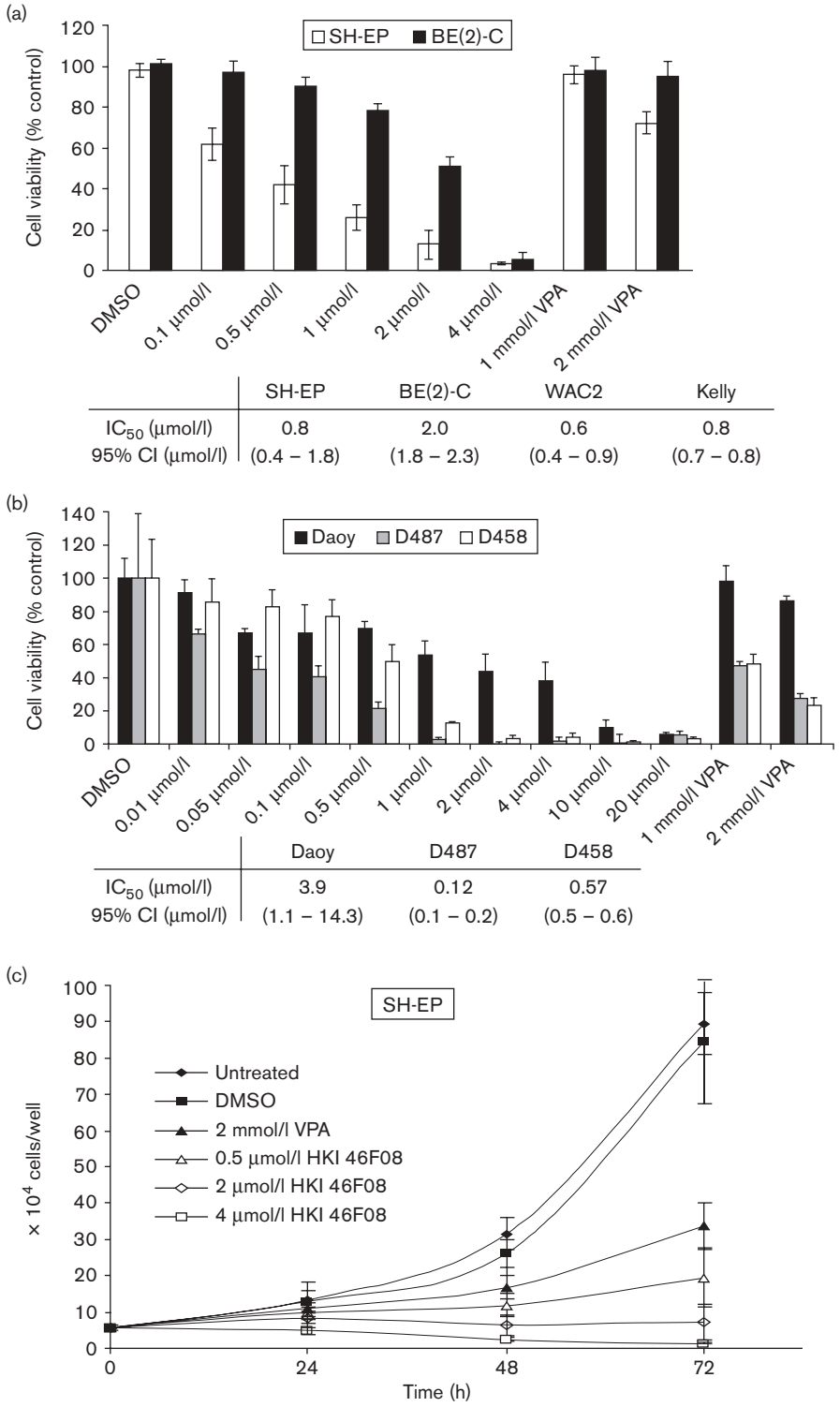
HKI 46F08 significantly inhibits clonogenic growth

Clonogenic growth of tumor cells in soft agar correlates well with tumorigenicity in mouse models, aggressive tumor biology *in vivo* and is often used as an assay for malignant transformation. For example, BE(2)-C MYCN oncogene-amplified neuroblastoma cells are highly tumorigenic in mice and show significant clonogenic growth in soft agar, whereas SH-EP MYCN single copy neuroblastoma cells neither form tumors in mice nor exhibit clonogenic growth in soft agar [5,24]. We therefore examined the influence of HKI 46F08 on clonogenic growth of BE(2)-C neuroblastoma cells in soft agar assays. At a concentration of 0.5 µmol/l, the compound already showed significant inhibition of clonogenic growth which was completely abolished at 2 µmol/l (Fig. 3 upper and lower panel). Again, the concentration required to efficiently inhibit clonogenic growth corresponds to the IC_{50} of the compound on *in vitro* and intracellular inhibition of HDAC enzymatic activity.

Induction of differentiation and apoptosis by HKI 46F08

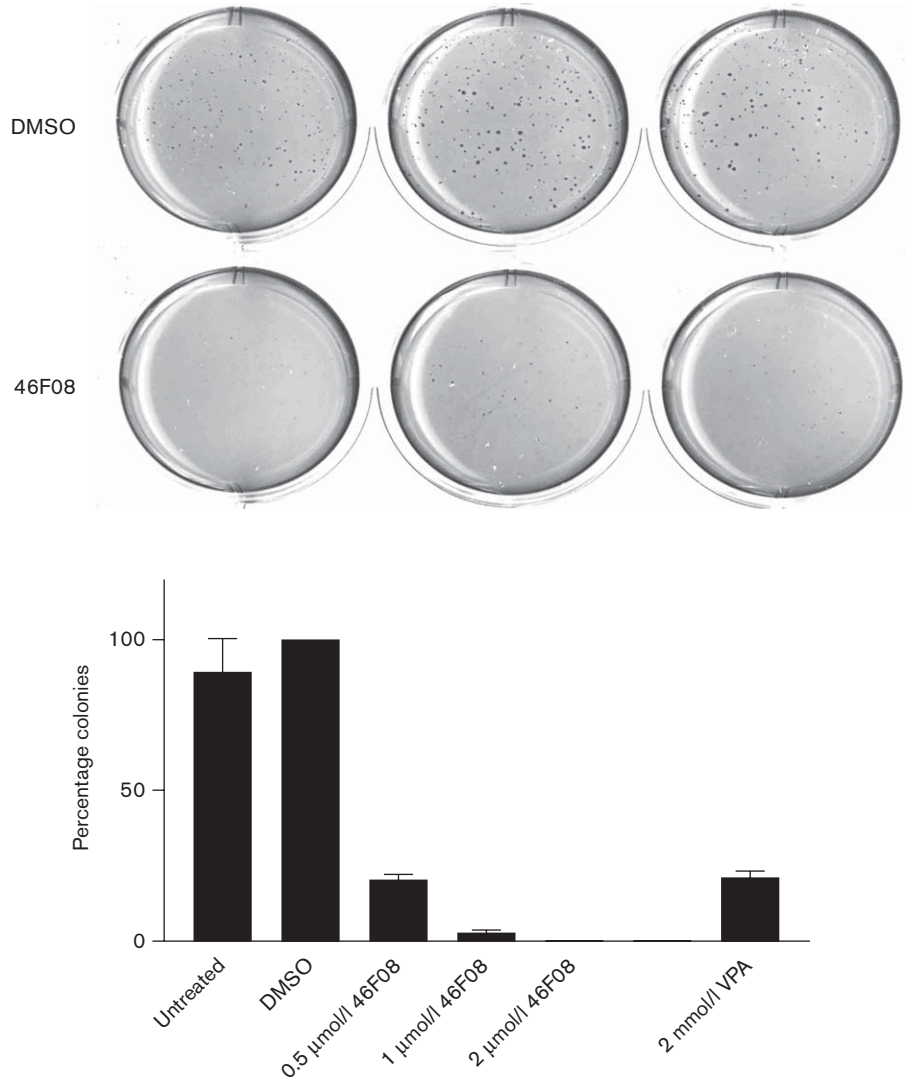
We observed morphological changes resembling neurite-like extensions after treatment of neuroblastoma cells with HKI 46F08 within the first 24 h (Fig. 4a, upper panel). Thereafter, cells became rounded and detached from the culture flask, suggestive of apoptosis (Fig. 4a, lower panel). We therefore investigated both cellular programs exemplarily in SH-EP neuroblastoma cells in response to HKI 46F08 treatment. The expression of a panel of marker genes of neuronal and neuroendocrine differentiation by quantitativ reverse transcription-PCR revealed induction of neurofilament, synapsin, synaptophysin, and MAP2 after 24 h (Fig. 4b). As a marker for apoptosis, we determined caspase-3-like activity, which was increased before the observed microscopic changes, indicative of induction of apoptosis by HKI 46F08 (Fig. 4c). Additionally, investigation of MYCN oncogene expression in the MYCN-amplified neuroblastoma cell line BE(2)-C by HKI 46F08 did not reveal changes of expression at the mRNA level (data not shown).

Fig. 2



HKI 46F08 inhibits cell viability and proliferation of neuroblastoma and medulloblastoma cells. (a) Determination of cell viability by WST-1 assay of SH-EP and BE(2)-C neuroblastoma cells after treatment with HKI 46F08 for 48 h. Values are expressed relative to untreated control cells. For comparison, results of treatment with 1 and 2 mmol/l valproic acid (VPA) are shown (inserted tables). IC_{50} of cell viability of SH-EP, BE(2)-C, WAC2 and Kelly neuroblastoma cells are shown. Confidence Intervals (CI) are depicted in parenthesis. (b) Determination of cell viability by WST-1 assay of Daoy, D487, D458 medulloblastoma cells. Values are expressed relative to untreated cells (c) Determination of absolute cell numbers following treatment of SH-EP neuroblastoma cells with HKI 46F08. Results for 2 mmol/l VPA treatment are shown for comparison. Experiments were repeated at least three times and mean and standard errors were calculated as indicated. Dimethylsulfoxide (DMSO): solvent-treated control cells were cultured in the presence of 0.1‰ v/v DMSO corresponding to the maximum DMSO concentration applied with the compounds.

Fig. 3



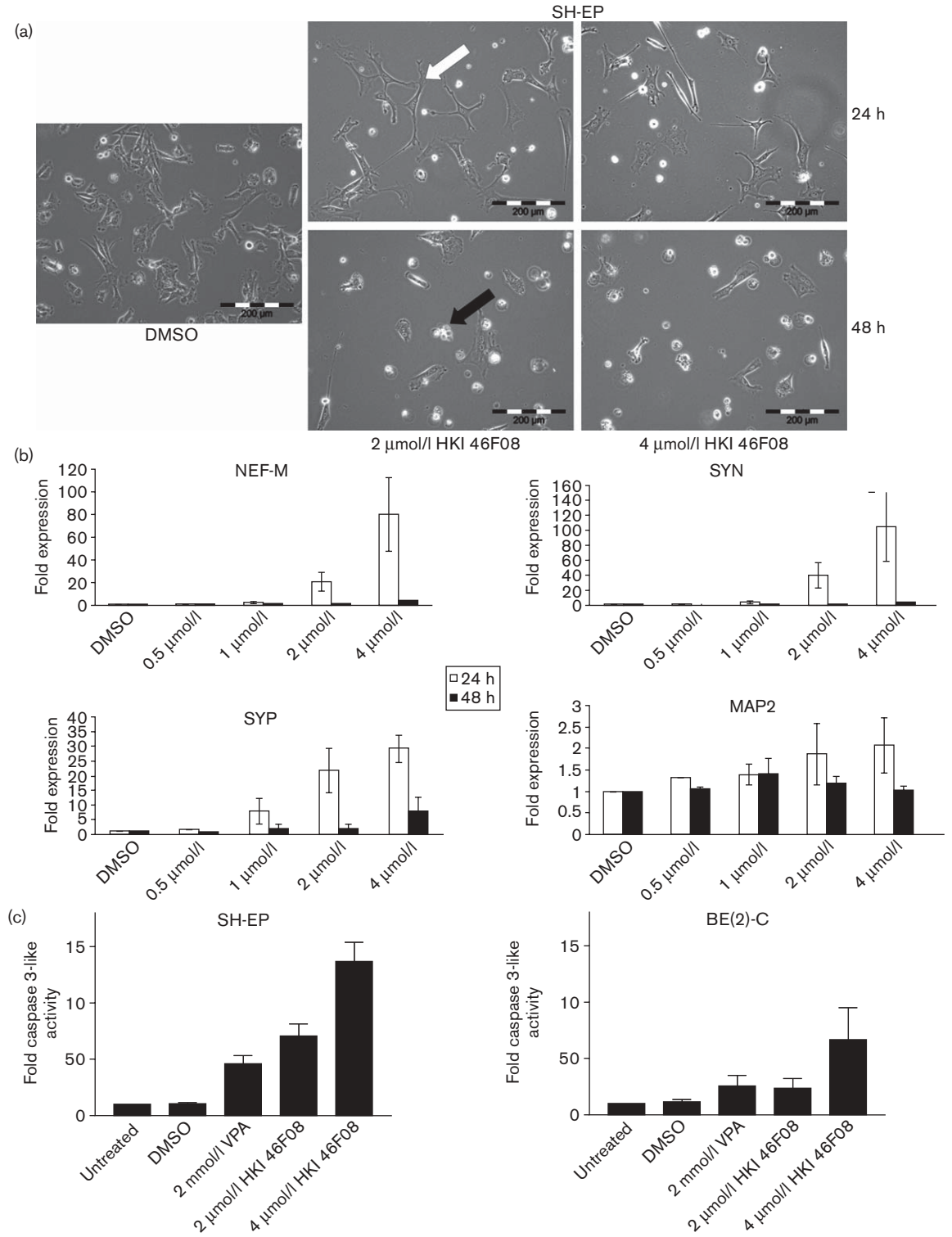
HKI 46F08 inhibits clonogenic growth of neuroblastoma cells. BE(2)-C neuroblastoma cells are able to form colonies in soft agar which is significantly inhibited by HKI 46F08. Upper panel: photograph of a typical six-well soft agar plate run in triplicates. Dimethylsulfoxide (DMSO): solvent-treated control cells form numerous colonies which can be strongly inhibited with 0.5 µmol/l HKI 46F08. Lower panel: quantification of colony numbers by counting of visible colonies. Values are expressed relative to DMSO-treated control cells. Experiments were repeated at least three times and mean and standard errors were calculated as indicated. DMSO: solvent-treated control cells were cultured in the presence of 0.1‰ v/v DMSO corresponding to the maximum DMSO concentration applied with the compounds.

Discussion

We have characterized the antitumoral potency of a novel HDAC inhibitor, HKI 46F08, which was identified by screening a compound library [10]. In this screening campaign, the validation assays already revealed some hints that this novel compound might be effective against neuroblastoma cells [10]. Here, we show that HKI 46F08 indeed proved to be effective against several pediatric embryonal cancer cell lines derived from neuroblastoma and medulloblastoma. HKI 46F08 inhibits HDAC activity in a cell-free in-vitro assay as well as intracellular HDAC activity in cultured neuroblastoma cells with an IC_{50} of about 1–2 µmol/l. It seems that the compound is a pan-HDAC inhibitor because of induction of histone and tubulin hyperacetylations, which are surrogate parameters for inhibition of class I and class II HDACs, respectively. The compound is a novel trifluoromethyl ketone displaying a nonhydroxamic acid type structure and therefore will serve as a novel lead compound for further optimization.

We found that HKI 46F08 is very effective against MYCN single copy as well as amplified neuroblastoma cells based on the following observations: (i) it inhibits

Fig. 4



HKI 46F08 induces differentiation and apoptosis in SH-EP neuroblastoma cells. (a) Morphological changes of cells after 24 and 48 h of HKI 46F08 treatment. Note outgrowth of neurite-like structures after 24 h (white arrow) and appearance of rounded, detached cells after 48 h (black arrow). (b) mRNA expression of neuronal differentiation markers by quantitative reverse transcription-PCR after 24 h (white bars) and 48 h (black bars). MAP2, microtubulin-associated protein 2; NEF-M, neurofilament, medium chain; SYN, synapsin; SYP, synaptophysin. Experiments were repeated at least three times and mean and standard errors were calculated as indicated. (c) Induction of caspase 3-like activity in SH-EP and BE(2)-C neuroblastoma cells after treatment with HKI 46F08. DMSO: solvent-treated control cells were cultured in the presence of 0.1‰ v/v DMSO corresponding to the maximum DMSO concentration applied with the compounds.

cell viability and cell proliferation at low micromolar concentrations, (ii) it strongly reduces clonogenic growth of cells in soft agar, and (iii) the compound induces neuronal differentiation and apoptosis. Similar reduction of cell viability was observed in medulloblastoma cells. HKI46F08 induced differentiation followed by apoptosis in the neuroblastoma cell lines investigated. We speculate that induction of expression of differentiation marker mRNA is only transient during first 24 h of treatment, which is then followed by activation of an apoptotic program which overruns the differentiation program with subsequent degradation of mRNAs [25].

One of the key target genes induced by HDAC inhibitors is the cyclin-dependent kinase inhibitor 1A (CDKN1A, p21WAF1/Cip1) [8]. HKI 46F08 also significantly induced expression of this gene time and concentration dependent at the mRNA and protein level. p21WAF1/Cip1 induction seems to be independent of the genetic background and type of HDAC inhibitor used. For example, the cyclic tetrapeptide HC toxin similarly induces p21WAF1/Cip1 expression in MYCN-amplified BE(2)-C neuroblastoma cells [5] as we have observed with HKI 46F08 in MYCN single copy neuroblastoma cell in this study. Of note, the HKI46F08 compound does not affect viability of normal skin fibroblasts in 10-fold higher concentrations [10].

We have previously shown that the tetracyclic peptide HDAC inhibitor Helminthosporium carbonum toxin is very active against neuroblastoma cells [4,5] indicating that HDACs are promising targets for potential neuroblastoma therapy. This is further supported by an earlier study demonstrating activity of HDAC-inhibitors against pediatric embryonal tumor cell lines *in vitro*, in a xenograft model and in a child with glioblastoma multiforme [26–28]. Several HDAC inhibitors are currently being investigated in clinical trials in adults [7] and recently, the first compound of this class was approved by the FDA for the treatment of cutaneous lymphoma [29].

In summary, we have identified a novel HDAC inhibitor, HKI 46F08, as a compound with promising activity against several pediatric embryonal cancer cell lines requiring further toxicity and efficacy evaluation in animal models.

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