Inhibition of NF-κB activation by the histone deacetylase inhibitor 4-Me₂N-BAVAH induces an early G₁ cell cycle arrest in primary hepatocytes

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Abstract. *Objective*: Benzoylaminoalkanohydroxamic acids, including 5-(4-dimethylaminobenzoyl)aminovaleric acid hydroxamide (4-Me₂N-BAVAH), are structural analogues of Trichostatin A, a naturally occurring histone deacetylase inhibitor (HDACi). 4-Me₂N-BAVAH has been shown to induce histone hyperacetylation and to inhibit proliferation in Friend erythroleukaemia cells *in vitro*. However, the molecular mechanisms have remained unidentified. *Materials and Methods*: In this study, we evaluated the effects of 4-Me₂N-BAVAH on proliferation in non-malignant cells, namely epidermal growth factor-stimulated primary rat hepatocytes. *Results and Conclusion*: We have found that 4-Me₂N-BAVAH inhibits HDAC activity at non-cytotoxic concentrations and prevents cells from responding to the mitogenic stimuli of epidermal growth factor. This results in an early G₁ cell cycle arrest that is independent of p21 activity, but instead can be attributed to inhibition of cyclin D1 transcription through a mechanism involving inhibition of nuclear factor-kappaB activation. In addition, 4-Me₂N-BAVAH delays the onset of spontaneous apoptosis in primary rat hepatocyte cultures as evidenced by down-regulation of the pro-apoptotic proteins Bid and Bax, and inhibition of caspase-3 activation.

INTRODUCTION

Temporal changes in chromatin structure play an important role in eukaryotic gene transcription by affecting the affinity of histone proteins for DNA and other chromatin-associated proteins (Workman & Kingston 1998). In this context, acetylation of N-terminal lysine residues in nucleosome-associated histones has been well studied. It is a reversible process catalysed by histone acetyltransferases and histone deacetylases (HDACs). A balanced histone acetylation status is essential for correct progress of cell proliferation, apoptosis and differentiation

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Table 1. *In vitro* HDAC inhibition potency, metabolic stability and biological effects of TSA and 4-Me$_2$N-BAVAH

	TSA	4-Me$_2$N-BAVAH	Reference
HDAC inhibition potency (IC$_{50}$)	11 ± 2 nM	1920 ± 405 nM	Elaut *et al.* 2007
Metabolic stability in hepatocyte suspensions	30 min	3 h	Elaut *et al.* 2004
Effects on differentiation in cultured hepatocytes	↑ albumin secretion	↑↑ albumin secretion	Papeleu *et al.* 2003 and this paper
	↑ CYP1A, 2B1, 3A2	↑↑ CYP1A, 2B1, 3A2	Henkens *et al.* 2007
	↑ Cx32, Cx43	↑↑ Cx32, Cx43	Vinken *et al.* 2006
	↓ Cx26	↓↓ Cx26	Vinken *et al.* 2006

4-Me$_2$N-BAVAH, 5-(4-dimethylaminobenzoyl)aminovaleric acid hydroxamide; HDAC, histone deacetylase; TSA, Trichostatin A.

(Workman & Kingston 1998; Kouzarides 1999). Indeed, improper HDAC recruitment or activity often results in uncontrolled cell proliferation and tumour development (Marks *et al.* 2001). This explains the current popularity for development of HDAC inhibitors (HDACi) as targeted anticancer drugs. *In vitro* experiments have revealed that these compounds effectively induce cell cycle arrest and differentiation in a wide variety of neoplastic cell lines (Marks *et al.* 2001; Yoshida *et al.* 2001; Vanhaecke *et al.* 2004; Marks & Jiang 2005; Papeleu *et al.* 2005) some of which are presently being evaluated in clinical trials (Piekarz & Bates 2004; Marks & Jiang 2005). However, *in vitro* and *in vivo* efficacy of HDACi, in particular hydroxamic acid-based compounds like Trichostatin A (TSA), are limited due to rapid metabolic inactivation of the functional group (Elaut *et al.* 2002; Elaut *et al.* 2004; Sanderson *et al.* 2004). 5-(4-Dimethylaminobenzoyl)-aminovaleric acid hydroxamide (4-Me$_2$N-BAVAH), a structural analogue of TSA, appears to be more stable in rat hepatocyte suspensions (Table 1; Elaut *et al.* 2004) and has previously been shown to inhibit HDAC activity and proliferation in Friend erythroleukaemia cells (Jung *et al.* 1999). The molecular mechanisms, however, remain unidentified.

The majority of studies dealing with the elucidation of molecular mechanisms associated with HDACi-induced inhibition of cell proliferation have been performed in tumour-derived cell lines. In general, cancer cell proliferation is suppressed due to the stimulating effect of HDACi on expression of growth-inhibitory genes, including *p21* (Marks *et al.* 2001; Yoshida *et al.* 2001; Vanhaecke *et al.* 2004). Cancer cells, however, exhibit severely disturbed regulation of the cell cycle due to mutations in tumour-suppressor genes or overexpression of oncogenes. Primary cultured hepatocytes, stimulated with appropriate mitogens, including epidermal growth factor (EGF), have proven to be a valuable alternative for studying cell cycle-related signal transduction (Papeleu *et al.* 2004). Although cell division rarely occurs in the adult healthy liver, hepatocytes quickly respond to injury by initiating an adaptive proliferative process (Koniaris *et al.* 2003). One of the best-characterized *in vivo* models in this respect is 70% partial hepatectomy (Koniaris *et al.* 2003). An analogous proliferative response occurs during the two-step collagenase perfusion used to isolate hepatocytes from the liver (Loyer *et al.* 1996). Responsiveness to growth factors is ensured in a pre-replicative priming phase and is essential for cells to pass the G$_1$ restriction point and subsequent progression through the cell cycle (Koniaris *et al.* 2003; Mitchell & Gilgenkrantz 2003). EGF is a known *in vivo* and *in vitro* hepatocyte mitogen and its role in the control of hepatocyte proliferation has been studied extensively. Upon binding to its receptor, EGF activates a number of signalling pathways that ultimately converge on expression of cyclin D1, a pivotal player in G$_1$ phase progression (Talarmin *et al.* 1999; Rescan *et al.* 2001; Coutant

et al. 2002). Induction of this D-type cyclin appears to be a rate-limiting step in hepatocyte proliferation (Loyer *et al.* 1996; Albrecht & Hansen 1999; Talarmin *et al.* 1999; Rescan *et al.* 2001; Coutant *et al.* 2002; Nelsen *et al.* 2003). In the present study, we investigated the effects of the TSA-like hydroxamic acid-containing HDACi 4-Me$_2$N-BAVAH on EGF-induced proliferation in primary rat hepatocytes.

MATERIALS AND METHODS

Reagents

Minimal essential medium, medium 199 and crude collagenase type I came from Sigma-Aldrich (Bornem, Belgium). [methyl-^{3}H]-thymidine (25 Ci/mmol) and [α-^{32}P]-dCTP (3000 Ci/mmol) came from Amersham Pharmacia Biotech (Buckinghamshire, UK). Recombinant human EGF was from Promega (Leiden, The Netherlands). 4-Me$_2$N-BAVAH (purity $\geq$ 96%) was synthesized as previously described (Jung *et al.* 1999) and stock solutions of 100 mM were prepared in ethanol. Final ethanol concentrations in the media did not exceed 0.05% (v/v) (used as solvent control). All other reagents were readily commercially available and of molecular biology grade.

Cell isolation and culture

Procedures for housing rats and isolation and culture of primary rat hepatocytes were approved by the local ethical committee for animal experiments of the Vrije Universiteit Brussel, Brussels, Belgium. Hepatocytes were isolated from male outbred Sprague-Dawley rats (200–300 g, Charles River Laboratories, Brussels, Belgium) (Papeleu *et al.* 2006). They were kept under controlled environmental conditions (a 12-h light : dark cycle) and were fed a standard diet (Animalabo A04, water *ad libitum*). Cells (viability > 90%; assessed by trypan blue exclusion) were plated at a density of 0.4×10^5 cells/cm^2 in a mixture of 75% minimal essential medium and 25% medium 199 supplemented with bovine serum albumin (1 mg/mL), bovine insulin (5 µg/mL), 10% foetal bovine serum and per millilitre: 7.3 IU benzyl penicillin, 50 µg streptomycin sulfate, 50 µg kanamycin monosulfate and 10 µg sodium ampicillin (Papeleu *et al.* 2004). Four hours after plating, fresh serum-free medium containing 0.5 µg/mL hydrocortisone sodium hemisuccinate and 50 ng/mL human recombinant EGF was added to the cells. Exposure to the HDACi 4-Me$_2$N-BAVAH began at time of plating unless indicated otherwise. Media were renewed daily.

Fluorescence-activated cell sorting

Ploidy of hepatocyte nuclei was measured by fluorescence-activated cell sorting (FACS) analysis. Cells were washed twice with ice-cold phosphate-buffered saline and subsequently were incubated in a hypotonic fluorochrome solution (50 µg/mL propidium iodide in 0.1% sodium citrate and 0.1% Triton X-100) for 1 h at room temperature in the dark. Propidium iodide fluorescence of individual cells was analysed on a FACStar[Plus] (Becton Dickinson Biosciences, San José, CA, USA).

Western blotting

At the indicated time points, cultured hepatocytes were harvested in ice-cold phosphate-buffered saline. For total cell protein extraction, cell pellets were prepared as described (Loyer *et al.* 1996) and histones were prepared according to Cousens *et al.* (1979). Total protein (25 or 50 µg/lane) or histone (20 µg/lane) were resolved on sodium dodecyl sulfate-polyacrylamide gel

electrophoresis, blotted on to nitrocellulose membranes (Amersham Pharmacia Biotech) and visualized with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) as recommended by the manufacturer.

Antibodies used were rabbit polyclonal antiphospho-p44/42 MAP kinase (Thr202/Tyr204) (Cell Signalling Technologies, Beverly, MA, USA), anticdk1 and anti-IκBα (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anticaspase-3 (Calbiochem-Novabiochem, San Diego, CA, USA) and anti-acetylated histone H4 (CamproScientific, Amsterdam, The Netherlands); goat polyclonal antip21 and anti-Bax were from Santa Cruz Biotechnology and anti-Bid was from R & D Systems, Abingdon, Oxfordshire, UK. Mouse monoclonal antibodies used were anticyclin D1 (Neomarkers, Fremont, CA, USA), anti-GAPDH (Abcam, Cambridge, UK) and antipan-ERK (Transduction Laboratories, Lexington, KY, USA).

DNA synthesis

Cells were cultured in triplicate and were incubated with [methyl-^{3}H]-thymidine (25 Ci/mmol; 2 μCi/mL) for 24 h prior harvesting. Incorporation of the radiolabel was measured by liquid scintillation counting (Wallac 1410; counting efficiency: 67.5%) after overnight precipitation of the DNA in 15% trichloroacetic acid (Loyer *et al.* 1996).

Northern blotting

Total RNA was extracted with the simian virus total RNA isolation system (Promega, Leiden, The Netherlands). Northern blotting and visualization of RNA by autoradiography was performed as described previously (Loyer *et al.* 1996). cDNA probes used were the purified 1.3-kb *Eco*RI mouse *cyclin D1* insert from the pcBZ plasmid (Dr Sherr, Memphis, TN, USA) and the purified 0.75-kb *Eco*RI–*Hin*dIII rat *waf1* insert from the pcRW0.8–pBluescript SK plasmid (Dr Ilyin, Rennes, France).

Luciferase reporter gene assay for nuclear factor-kappaB activity

Nuclear factor-kappaB (NF-κB) activation was analysed by measuring the expression of an NF-κB-dependent luciferase reporter gene that was transfected by adenoviral gene transfer as described previously (El Bakkouri *et al.* 2005; Wullaert *et al.* 2005). Primary hepatocytes were seeded in triplicate in 24-well plates at a density of 0.4×10^5 cells/cm² and were infected the day after with a replication-deficient adenovirus at a total multiplicity of infection of 75. The adenovirus mixture comprised 50% adenovirus expressing an NF-κB-dependent luciferase reporter gene (AdNF-κBluc) (gift from Dr B. McGray, University of Iowa College of Medicine, Iowa City, IA, USA; Sanlioglu *et al.* 2001) and 50% adenovirus expressing a β-galactosidase gene under a constitutive active cytomegalovirus promoter (AdLacZ). To test the effect of the inhibitor of κBα (IκBα) super-repressor (IκBα^{sr}), the adenovirus mixture was comprised of 25% AdNF-κBLuc, 25% AdLacZ and either 50% of control adenovirus without transgene (AdRR5) or 50% virus expressing the IκBα^{sr} transgene (AdIκBα^{sr}) (gift from Dr R. Hay, University of St. Andrews, St. Andrews, Scotland, UK; Grempler *et al.* 2004). One day after infection, cells were either untreated or were stimulated with EGF for 6 h. Recombinant adenoviruses were generated in HEK293 cells as previously described (Wullaert *et al.* 2005). NF-κB promoter activity was assessed by measuring luciferase (Luc) activity in cell extracts (De Valck *et al.* 1996). β-galactosidase (Gal) activity was measured using the Galactostar reporter gene assay system (Applied Biosystems, Foster City, CA, USA). Luc values were normalized for Gal values to correct for differences in infection efficiency and were plotted as fold NF-κB activation (relative to the basal level of Luc in the absence of EGF, after normalization with Gal).

Cell viability

Lactate dehydrogenase (LDH) leakage was measured using the Merckotest (VWR International, Leuven, Belgium) and was calculated (in percentage) by the ratio: [$100 \times$ LDH activity in supernatant]/[LDH activity in (supernatant + cells)].

Albumin secretion

Medium samples were collected at indicated time points and were analysed for their albumin content by ELISA (Dunn *et al.* 1991).

Identification and separation of 4-Me$_2$N-BAVAH metabolites

Hepatocyte monolayer cultures were incubated with 50 µM 4-Me$_2$N-BAVAH for 20 h. Medium samples were collected every 20 min during the first hour and every hour during the next 6 h of culture; final samples were taken 8 and 20 h after administration of the HDACi. Separation and identification of 4-Me$_2$N-BAVAH and its acid and amide metabolites were performed as previously described (Elaut *et al.* 2004).

Statistical analysis

Results were expressed as the mean ± SD. Statistical analyses were performed using a Student's *t*-test with the significance level being set at 0.05.

RESULTS

4-Me$_2$N-BAVAH inhibits EGF-induced cell proliferation in primary hepatocytes

To determine whether the selected doses of HDACi 4-Me$_2$N-BAVAH effectively block HDAC activity, primary hepatocytes were cultured for 48 h either in the absence (0 µM) or presence of 10, 25 and 50 µM 4-Me$_2$N-BAVAH. Figure 1 shows that 4-Me$_2$N-BAVAH enhanced histone H4 acetylation in a concentration-dependent way. Ten and twenty-five micromolars of the HDACi
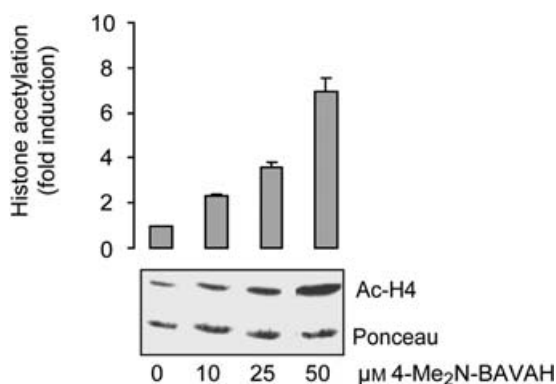

Figure 1. Effect of 4-Me$_2$N-BAVAH on histone H4 acetylation. Primary rat hepatocytes were cultured in EGF-containing medium (50 ng/mL, added 4 h after plating) either in the absence (0 µM) or presence of 10, 25 or 50 µM 4-Me$_2$N-BAVAH (added from time plating during 48 h). After 48 h of culture, nuclear histones were extracted and histone H4 acetylation levels were evaluated by Western blotting, using a polyclonal antibody, specifically directed against hyperacetylated forms of histone H4. A representative blot of three independent experiments is shown. Ponceau S staining is shown as a control for sample loading and transfer efficiency. Quantification of Western blots by densitometry is from three independent experiments (means ± SD are shown), control values were arbitrarily set at 1.

induced a slight to moderate increase in histone H4 acetylation (2.35 ± 0.05- and 3.56 ± 0.23-fold induction, respectively), whereas a 7-fold (6.91 ± 0.63) increase in acetylation levels was found upon exposure to 50 μM 4-Me$_2$N-BAVAH.

Next, we evaluated whether 4-Me$_2$N-BAVAH could effectively inhibit EGF-induced hepatocyte proliferation. Thus, the same concentrations of HDACi were added to the culture media from time of plating, for 3 consecutive days. DNA synthesis was measured by means of ^{3}H-thymidine incorporation every 24 h (Fig. 2a). In addition, induction of cyclin D1, a rate-limiting step in EGF-induced hepatocyte proliferation, was evaluated by Western blot analysis (Fig. 2b). Exposure of EGF-stimulated hepatocytes to increasing concentrations of 4-Me$_2$N-BAVAH revealed a clear dose-response relationship. Inhibition of DNA synthesis and cyclin D1 protein accumulation in the presence of 50 μM 4-Me$_2$N-BAVAH correlated well with the observation that a significantly higher proportion of cells had a G$_0$/G$_1$ DNA content ($P < 0.001$, Student's *t*-test) and a significantly lower number remained in G$_2$/M ($P < 0.05$, Student's *t*-test) (Fig. 2c). LDH leakage into the culture medium was not increased in hepatocytes that were exposed to 50 μM 4-Me$_2$N-BAVAH, indicating that cytotoxicity did not contribute to inhibition of cell proliferation (Fig. 2d). Albumin secretion, reflecting overall cell functionality, was even significantly increased upon continuous exposure of the cells to 4-Me$_2$N-BAVAH (Fig. 2e).

4-Me$_2$N-BAVAH induces an early G$_1$ cell cycle arrest by inhibiting cyclin D1 transcription

Figure 3a illustrates that 4-Me$_2$N-BAVAH had less or no inhibitory effect on DNA synthesis when added either in mid-G$_1$ (corresponding to 24 h of culture) or after the restriction point (corresponding to 48 h of culture). In these conditions, cells were able to pass the restriction point, as evidenced by the presence of cyclin D1, and to subsequently proceed through S phase as evidenced by the presence of cdk1, an S/G$_2$/M marker (Fig. 3b). These data suggest that 4-Me$_2$N-BAVAH interferes with signals that trigger early cell cycle events. Cells that have progressed already into the mid- and mid-late-G$_1$ phase were no longer sensitive towards the antiproliferating activity of 4-Me$_2$N-BAVAH.

As shown in Fig. 2b, 4-Me$_2$N-BAVAH inhibits cyclin D1 protein expression in EGF-stimulated hepatocytes in a dose-dependent manner. Cyclin D1 is a key mediator of mitogenic signalling in primary hepatocytes. In these cells, cyclin D1 is only expressed following mitogenic stimulation and is indicative for passage of the restriction point, located in mid-late-G$_1$ (Loyer *et al.* 1996). Its absence in the presence of 50 μM 4-Me$_2$N-BAVAH thus indicates that cells were blocked somewhere in the G$_1$ phase prior to the restriction point. Reversion experiments were performed to more precisely define where in G$_1$ cells were arrested. For this purpose, primary proliferating hepatocytes were exposed to 50 μM 4-Me$_2$N-BAVAH for 24 h from time of plating. Thereafter, cells were cultured in medium deprived of 4-Me$_2$N-BAVAH for 3 consecutive days. DNA replication was analysed at the indicated time points. Hepatocytes in these reversion experiments started to incorporate ^{3}H-thymidine with a delay of 24–48 h (Fig. 3c), which corresponds with the lag period for initiation of DNA synthesis in EGF-stimulated control cultures. A concomitant delay in cyclin D1 protein expression was observed (Fig. 3d). Next, Northern blots were performed to investigate whether down-regulation of cyclin D1 occurred at the pre-translational level. These experiments revealed that 4-Me$_2$N-BAVAH suppressed cyclin D1 expression at the RNA level in EGF-stimulated rat primary hepatocytes (Fig. 3e).

4-Me$_2$N-BAVAH down-regulates cyclin D1 RNA expression through inhibition of NF-κB activation

Different EGF-activated pathways, including the extracellular signal-regulated kinase (ERK) (Rescan *et al.* 2001; Coutant *et al.* 2002) and the p70S6 kinase (Nelsen *et al.* 2003) pathways,
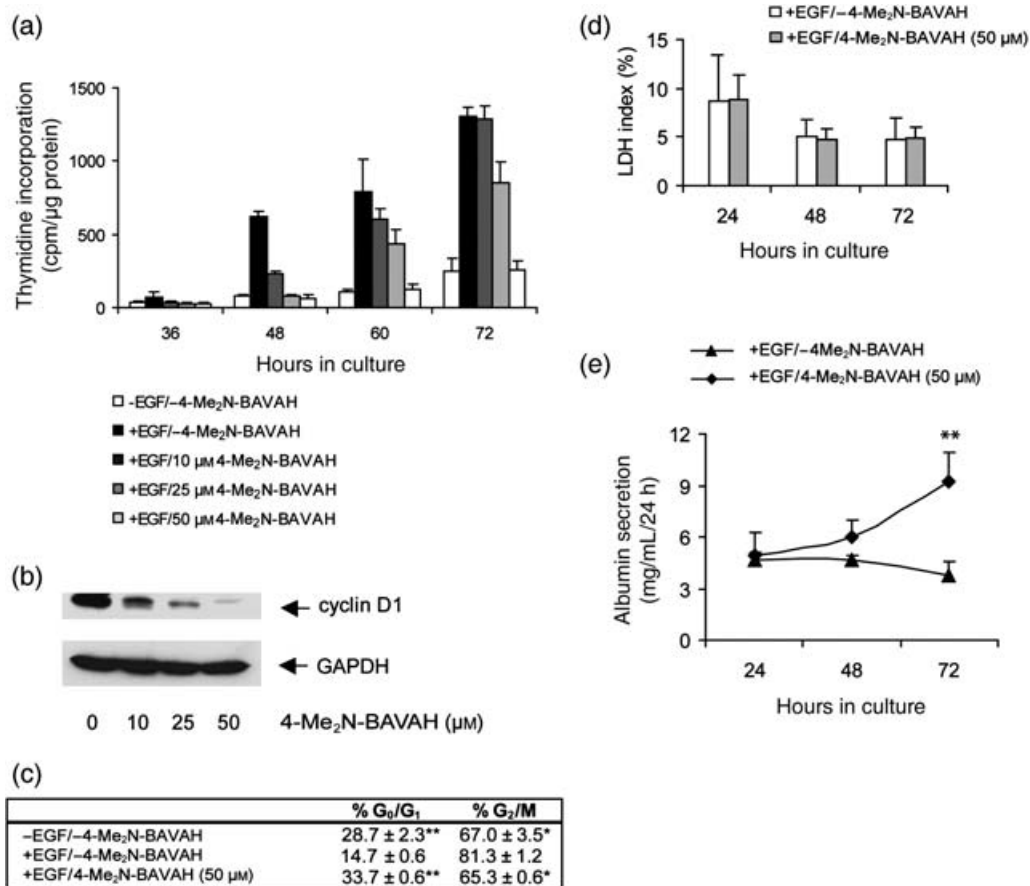
Figure 2. (a) 4-Me$_2$N-BAVAH dose-dependently inhibits EGF-induced DNA synthesis in primary hepatocytes. Hepatocytes were cultured in triplicate as indicated. ^{3}H-thymidine incorporation was measured at the indicated time points. Data shown are the means ± SD of triplicate cultures from one experiment and are representative for four other experiments. Cells cultured in the absence of EGF serve as a negative control for hepatocyte proliferation whereas cells stimulated with EGF (50 ng/mL) 4 h after plating serve as a positive control for hepatocyte proliferation. Treatment with increasing concentrations of the HDACi started from time of plating. **(b) 4-Me$_2$N-BAVAH dose-dependently inhibits cyclin D1 protein accumulation.** Hepatocytes were cultured in the presence of EGF alone (added 4 h after plating) and treated or not with 10, 25 or 50 μM 4-Me$_2$N-BAVAH from time of plating until 72 h of culture. Cells were then harvested and analysed for cyclin D1 protein expression (50 μg of total cellular proteins were loaded on SDS-PAGE) ($n = 3$). The membranes were re-probed with anti-GAPDH antibody to verify sample loading. **(c) Effect of 4-Me$_2$N-BAVAH on cell cycle distribution of primary hepatocytes.** Primary hepatocytes were cultured as indicated and their cell cycle distribution was evaluated at 72 h of culture by FACS analysis as described under Materials and Methods ($n = 3$). The relative high percentage of G$_2$/M hepatocytes in the non-proliferating conditions (– EGF/–4-Me$_2$N-BAVAH and +EGF/50 μM 4-Me$_2$N-BAVAH) can be explained by the fact that mononuclear tetraploid hepatocytes (1 × 4n G$_2$/M) could not be discriminated from binuclear diploid hepatocytes (2 × 2n G$_0$/G$_1$). *$P < 0.05$ or **$P < 0.001$ for significant differences from EGF-stimulated untreated control cultures according to a Student's *t*-test. **(d) Effect of 4-Me$_2$N-BAVAH on hepatocyte viability.** LDH leakage data are expressed as LDH index (%) and means ± SD are given ($n = 5$). **(e) Effect of 4-Me$_2$N-BAVAH on albumin secretion.** Medium samples were taken at the indicated time points and analysed for their albumin content by ELISA. Data shown are the means ± SD of five experiments, **$P < 0.01$.
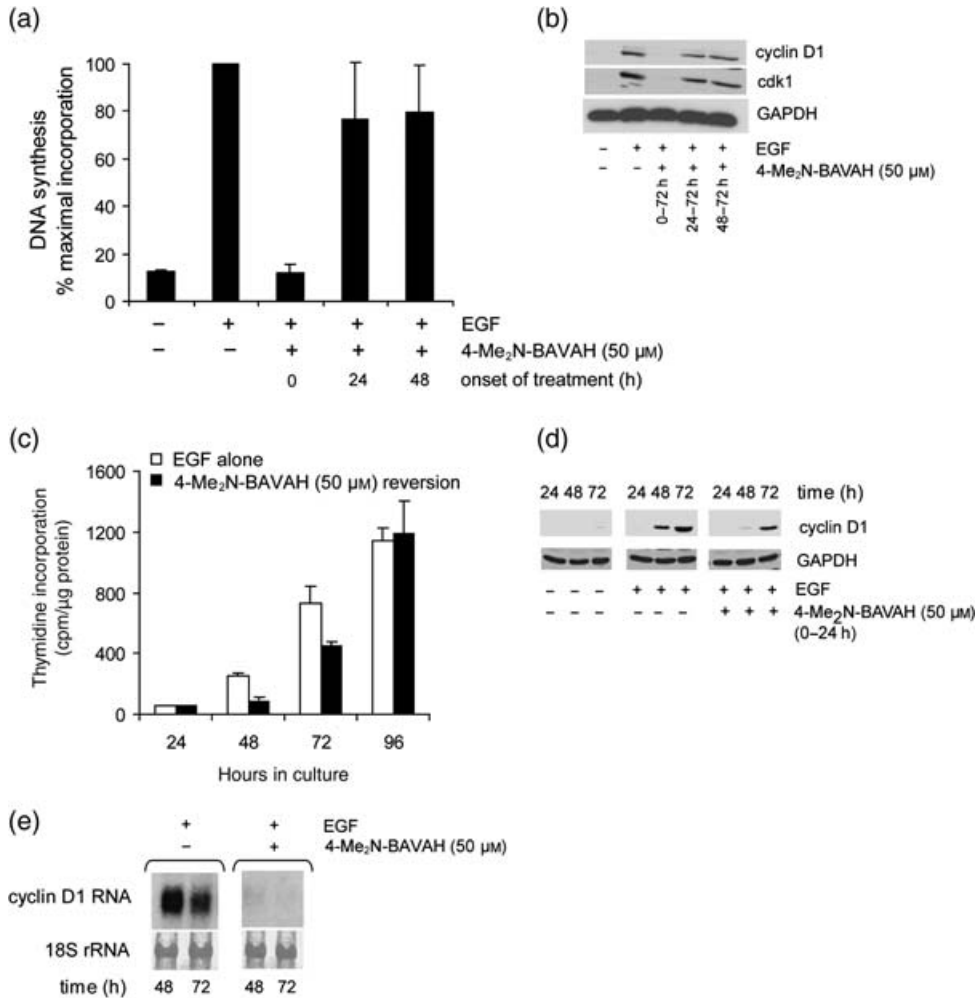
Figure 3. 4-Me$_2$N-BAVAH induces an early G$_1$ cell cycle arrest by inhibiting cyclin D1 transcription. (a) Inhibition of DNA synthesis depends on time of onset of treatment. Freshly isolated hepatocytes were cultured either in the absence of EGF or stimulated with EGF 4 h after plating. Exposure to 50 μM 4-Me$_2$N-BAVAH started at 0, 24 or 48 h after plating and was maintained until 72 h after plating. DNA synthesis was measured at 72 h of culture by means of ^{3}H-thymidine incorporation. Results are expressed as the percentage of maximal incorporation observed in hepatocytes cultured in the presence of EGF alone. The values obtained in this condition were arbitrarily set at 100% ($n = 3$). (b) Freshly isolated hepatocytes were cultured as described under (a). At 72 h of culture, cells were harvested and total cell protein extracts were prepared. 25 μg of proteins were loaded on gel and analysed for their cyclin D1 and cdk1 protein expression ($n = 3$). GAPDH was used as a loading control. (c) Reversion experiments. Freshly isolated hepatocytes were cultured in triplicate either in the presence of EGF alone or in the presence of both EGF and 4-Me$_2$N-BAVAH (50 μM) for 24 h. Thereafter, cells were cultured for an additional 48 h in the presence of EGF alone. ^{3}H-thymidine incorporation was measured at the indicated time points. Data shown are the means ± SD of triplicate measurements of three independent experiments (except for values measured at 96 h where $n = 2$). (d) Hepatocytes were cultured in the presence of 50 μM 4-Me$_2$N-BAVAH for 24 h. Thereafter, cells were cultured for an additional 48 h in the presence of EGF alone. Cyclin D1 expression was analysed by Western blotting at 24, 48 and 72 h of culture (25 μg of proteins/lane, $n = 3$). GAPDH was used as loading control. (e) Cyclin D1 RNA expression was analysed by Northern blotting. Hepatocytes were cultured in the presence of EGF (50 ng/mL; added 4 h after plating) and either exposed or not to 4-Me$_2$N-BAVAH (50 μM). At 48 and 72 h of culture, cells were harvested and RNA was extracted. 10 ng of total RNA was loaded on a 1.1% (w/v) agarose gel. 18S rRNAs dyed by methylene blue staining was used as a loading control ($n = 3$).
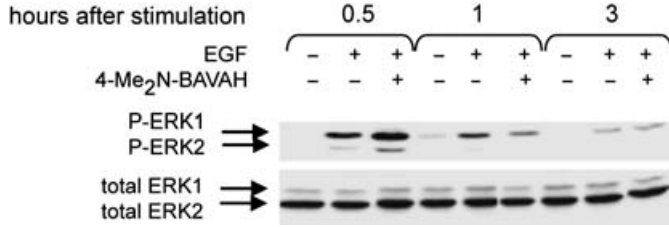
Figure 4. Effect of 4-Me$_2$N-BAVAH on ERK1/2 phosphorylation. As signal transduction kinases are often only transiently induced after growth factor stimulation, hepatocytes were first synchronized in the G$_1$ phase by culturing them in the absence of EGF for 48 h. Hepatocytes were subsequently either stimulated or not with EGF (50 ng/mL) and exposed to 50 μm 4-Me$_2$N-BAVAH where indicated. The level of ERK1/2 phosphorylation was analysed by Western blotting (50 μg proteins/lane) 0.5, 1 and 3 h later. Total ERK staining serves as a loading control ($n = 3$).

mediate the expression of cyclin D1 and can as such trigger early cell cycle events. These kinases are often only transiently induced shortly after growth factor stimulation (Nelsen *et al.* 2003). Therefore, in order to investigate the potential role of both pathways in the HDACi-induced cyclin D1 down-regulation, hepatocytes were first synchronized in the G$_1$ phase by culturing them in the absence of EGF for 48 h. They were subsequently stimulated with EGF and either exposed or not exposed to 4-Me$_2$N-BAVAH (50 μm). Activation of both pathways was analysed 0.5, 1 and 3 h after growth factor stimulation. No substantial effects on the phosphorylation of the ribosomal subunit S6 were observed, at least not at residues Ser235/236 (data not shown). Because S6 phosphorylation by the p70S6 kinase plays a role in the translation of cyclin D1 (Chou & Blenis 1995), these findings confirm our observation that 4-Me$_2$N-BAVAH inhibits cyclin D1 expression at the RNA level (Fig. 3e). Shortly after stimulation of synchronized primary hepatocytes with EGF, rapid and transient phosphorylation of ERK1/2 occurred (Fig. 4). 4-Me$_2$N-BAVAH did not inhibit EGF-mediated ERK activation. Instead, the HDACi slightly enhanced ERK1/2 phosphorylation levels during the initial 30 min, which decreased rapidly thereafter to control levels (Fig. 4). These data indicate that inhibition of the ERK pathway most likely is not involved in the inhibition of cyclin D1 transcription by 4-Me$_2$N-BAVAH.

Previous reports have shown that *cyclin D1* gene expression can be activated by NF-κB in transformed cells and in synchronized mouse embryo fibroblasts (reviewed in Joyce *et al.* 2001). To verify whether induction of *cyclin D1* by EGF in primary rat hepatocytes was NF-κB-dependent, cells were infected with adenovirus encoding the dominant negative, *IκBα* super-repressor gene (AdIκBα^{sr}) (Fig. 5a). IκBα^{sr} is a mutated IκBα protein in which Ser32 and 36 are replaced with Ala. Consequently, IκBα^{sr} can no longer be phosphorylated and therefore is not degraded by the proteasome. As such, IκBα^{sr} behaves as a constitutively active inhibitor of NF-κB activation in different cell types, both *in vitro* and *in vivo* (Roff *et al.* 1996; El Bakkouri *et al.* 2005; Wullaert *et al.* 2005). IκBα^{sr} has also been shown to delay and reduce *cyclin D1* expression in mouse embryo fibroblasts, HeLa cells and C2C12 myoblasts (Joyce *et al.* 2001). Concomitantly, we show here that AdIκBα^{sr}-infected primary hepatocytes have significantly reduced EGF-induced cyclin D1 protein levels (Fig. 5a; $P < 0.05$). As this observation is similar to the effect of 4-Me$_2$N-BAVAH on cyclin D1 expression, it was investigated whether this HDACi could inhibit EGF-induced NF-κB activation. Using a luciferase reporter gene assay, we could show that, like adenoviral infection with IκBα^{sr}, exposure to the HDACi 4-Me$_2$N-BAVAH (50 μm) substantially inhibited EGF-induced NF-κB activation in primary hepatocytes (Fig. 5b).
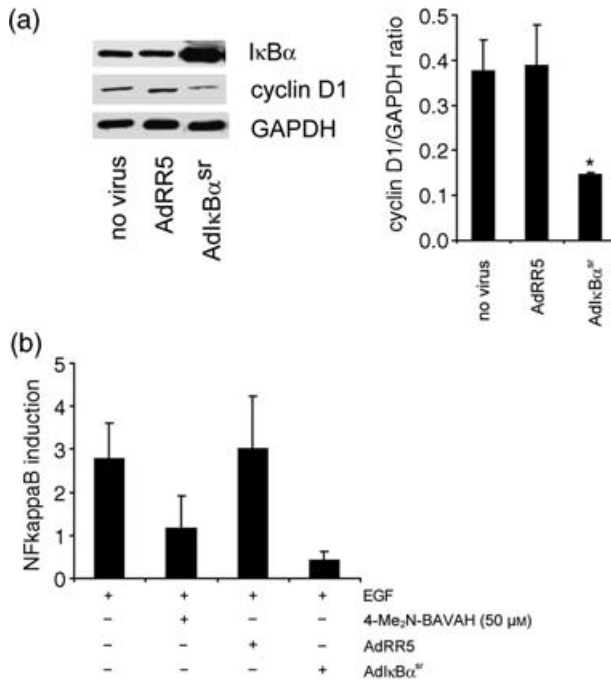
Figure 5. 4-Me$_2$N-BAVAH inhibits EGF-mediated NF-κB activation in primary hepatocytes. (a) Effect of AdIκBα^{sr} on cyclin D1 expression in primary rat hepatocytes. Primary rat hepatocytes were either not infected or infected with AdRR5 or AdIκBα^{sr}. The day after, hepatocytes were stimulated with EGF for 6 h. Cell extracts were prepared and analysed for IκBα and cyclin D1 expression by Western blot (15 μg proteins/lane). Cyclin D1 protein expression was quantified by densitometry. Data are expressed as the ratio cyclin D1/GAPDH. GAPDH was used as a control for transfer efficiency and equal sample loading, *$P < 0.05$. (b) Primary rat hepatocytes were infected with AdNF-κBluc and AdLacZ alone, or in combination with either AdRR5 or AdIκBα^{sr}. 24 h after infection, hepatocytes were stimulated with EGF (50 ng/mL), with or without the HDACi 4-Me$_2$N-BAVAH (50 μM), for 6 h. Relative (Luc/Gal) NF-κB activity was assessed by measuring Luc (luciferase) and Gal (β-galactosidase) activities, and plotted as fold NF-κB activation (relative to the basal level of Luc in the absence of EGF, after normalization with Gal). Values shown are the mean ± SD of triplicate measurements.

4-Me$_2$N-BAVAH-induced G$_1$ cell cycle arrest is not mediated by p21

Induction of proliferation arrest by up-regulating the p21 cdk inhibitor protein is a main trait of HDACi in tumour cells (Sambucetti *et al.* 1999; Chiba *et al.* 2004; Vanhaecke *et al.* 2004). As previously described (Albrecht & Hansen 1999; Nelsen *et al.* 2001), p21 expression is induced upon EGF stimulation and tightly correlates with the expression of cyclin D1 in these cells during proliferation (Fig. 6a). In primary hepatocytes, however, EGF-induced p21 expression, was not further enhanced upon exposure to 4-Me$_2$N-BAVAH; instead, p21 protein expression was prevented (Fig. 6a).

We investigated whether p21 might be only transiently induced due to either instability of the HDACi in the culture medium or to rapid biotransformation of the HDACi by the hepatocytes; both leading to low levels of HDACi in the culture medium. As induction of p21 by EGF occurs between 24 and 48 h of culture, cells were provided with fresh EGF-containing medium supplemented or not with 4-Me$_2$N-BAVAH at 24 h and p21 RNA and protein levels were monitored at short intervals (0.5, 1, 6 and 24 h) within this time frame. In untreated, control, cultures, p21 RNA and protein became detectable 0.5 h after medium renewal. Peak levels were reached 24 h
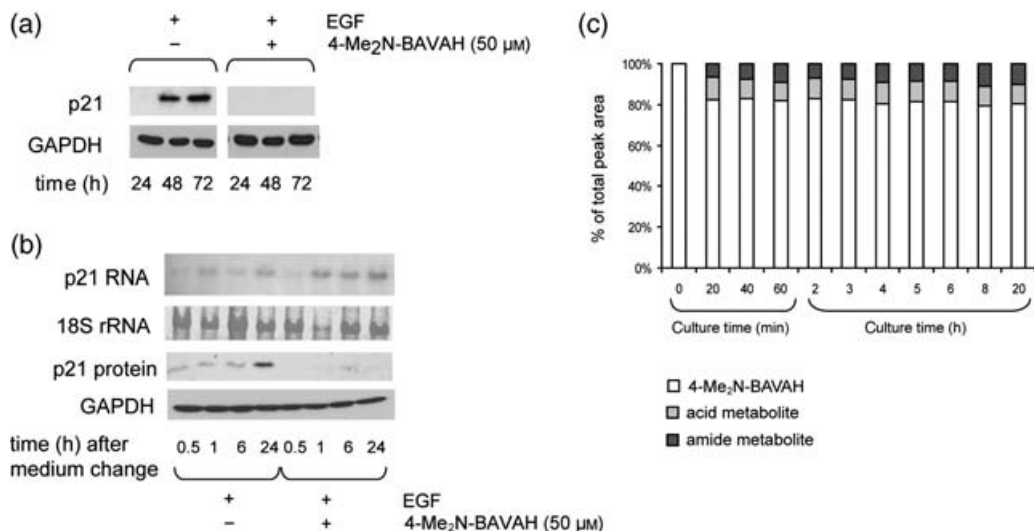
Figure 6. 4-Me$_2$N-BAVAH-induced cell cycle arrest occurs in the absence of p21 protein induction. (a) Hepatocytes were cultured in the presence of EGF (50 ng/mL; added 4 h after plating) and either treated or not with 50 μM 4-Me$_2$N-BAVAH from time of plating. Cells were harvested at the indicated time points. p21 protein expression was analysed by Western blotting after SDS-PAGE of 50 μg of total cellular proteins (*n* = 3). Blots were reprobed with anti-GAPDH antibody to verify equal sample loading and transfer efficiency. (b) Hepatocytes were cultured in the presence of EGF (50 ng/mL; added 4 h after plating) and either treated or not with 50 μM 4-Me$_2$N-BAVAH from time of plating. At 24 h of culture, cells were provided with fresh medium of the same composition. Cells were then harvested at 0.5, 1, 6 and 24 h post medium renewal, corresponding to 24.5, 25, 30 and 48 h of culture. For analysis of p21 RNA expression, 10 ng of total RNA was loaded on a 1.1% (w/v) agarose gel. 18S rRNAs dyed by methylene blue staining was used as a loading control. p21 protein expression (25 μg proteins/lane) was evaluated by Western blotting. Membranes were re-probed with anti-GAPDH antibody to verify transfer efficiency and equal sample loading (*n* = 3). (c) Metabolic stability of 4-Me$_2$N-BAVAH in rat hepatocyte cultures. The initial concentration of 4-Me$_2$N-BAVAH was 50 μM. Aliquots were taken from the culture medium immediately after addition of the HDACi (0 min), and every 20 min during the first hour of culture. Thereafter, aliquots were taken every hour during a period of 6 h; final aliquots were taken at 8 and 20 h of culture. 4-Me$_2$N-BAVAH and its major phase I metabolites were quantified by high-pressure liquid chromatography combined with electrospray ionization mass spectrometry/ultraviolet detection. Results are expressed as percentage of the total peak area in the samples taken at the indicated time points.

later, corresponding to 48 h of culture. In the presence of the HDACi, a strong decrease in p21 protein, but not RNA expression was seen (Fig. 6b). In addition, it could be shown that no extensive or rapid biotransformation of 4-Me$_2$N-BAVAH into inactive metabolites occurred (Fig. 6c).

4-Me$_2$N-BAVAH delays the onset of spontaneous apoptosis in primary hepatocytes

In Fig. 5b, we show that the HDACi 4-Me$_2$N-BAVAH inhibits EGF-mediated NF-κB activation. Inhibition of NF-κB activity has previously been reported to enhance the pro-apoptotic activity of HDACi (Mayo *et al.* 2003). As spontaneous apoptosis is a well-known pernicious event in primary hepatocyte cultures (Baily-Maitre *et al.* 2002), we wished to determine whether 4-Me$_2$N-BAVAH, through its action on NF-κB activity, facilitated this spontaneous cell death or not. Data reported in Fig. 7 show that, in proliferating primary hepatocytes, 4-Me$_2$N-BAVAH decreased the expression of the pro-apoptotic proteins Bid and Bax. In addition, cleavage of pro-caspase-3 into the smaller active p18 fragment was reduced from 24 h of culture onwards (Fig. 7b).
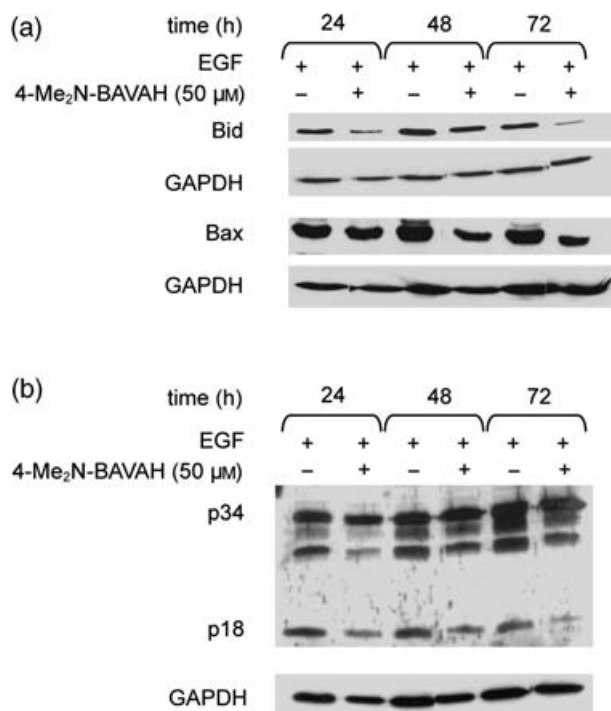
Figure 7. 4-Me₂N-BAVAH delays the onset of spontaneous apoptosis in primary rat hepatocytes. Hepatocytes were cultured in the presence of EGF alone (50 ng/mL; added 4 h after plating) and exposed or not to 50 μM 4-Me₂N-BAVAH (from time of plating). Cells were harvested at the indicated time points and analysed for (a) Bid and Bax protein expression (50 μg proteins/lane) and (b) caspase-3 activation (50 μg proteins/lane) ($n = 3$).

DISCUSSION

4-Me₂N-BAVAH is an amide-based hydroxamic acid, containing HDACi with structural similarity to TSA. Despite its higher metabolic stability (Elaut *et al.* 2004, Table 1), 4-Me₂N-BAVAH appeared less potent than TSA in HDAC inhibition assays (Table 1). However, given the complex role and regulation of HDACs in gene transcription, it is clear that the selection of a suitable compound should not be based solely on IC_{50} values (reflecting enzyme inhibition activity) but also on its biological activity and efficacy. Some clear advantages of 4-Me₂N-BAVAH over TSA with respect to metabolic stability and biological activity are summarized in Table 1. Jung *et al.* (1999) have previously reported the antiproliferative activity of 4-Me₂N-BAVAH in a malignant cell line, yet no attention was paid to the underlying molecular mechanisms. In this study, we have investigated the effects of 4-Me₂N-BAVAH on EGF-induced proliferation in rat primary hepatocytes. The latter is widely recognized as a valuable alternative to cell lines for studying cell cycle-related signal transduction (Papeleu *et al.* 2004).

Our data reveal that primary hepatocytes exposed to non-cytotoxic concentrations of 4-Me₂N-BAVAH become irresponsive to the mitogenic activity of EGF. This is evidenced by the inhibition of cyclin D1 gene transcription, and corresponds with cell cycle arrest in early G_1 phase of the cell cycle. Cyclin D1 plays a pivotal role in the regulation of hepatocyte proliferation

by growth factors, including EGF (Loyer *et al.* 1996; Nelsen *et al.* 2001). Transfection of hepatocytes with cyclin D1 was found to promote their proliferation *in vivo* as well as *in vitro* (Albrecht & Hansen 1999; Nelsen *et al.* 2001). One of the key players in transcriptional regulation of cyclin D1 is the MEK/ERK pathway. Talarmin *et al.* (1999), for instance, described that ERK activation preceded the induction of cyclin D1 mRNA expression after partial hepatectomy. Yet, 4-Me$_2$N-BAVAH had no inhibitory effect on ERK1/2 phosphorylation, excluding a role for this pathway in the transcriptional down-regulation of cyclin D1 by the HDACi.

Another link between EGF stimulation and G$_1$-to-S-phase progression is provided by NF-κB activation. In unstimulated cells, NF-κB is predominantly located in the cytoplasm through interaction with IκB inhibitory proteins, such as IκBα (Moynagh 2005). Upon activation by pro-inflammatory cytokines, including tumour necrosis factor and IL-1, IκBα is phosphorylated and subsequently degraded by the proteasome (Joyce *et al.* 2001; Moynagh 2005). This allows rapid and transient translocation of NF-κB to the nucleus, where it can activate transcription of a wide variety of genes that are involved in cell proliferation, including cyclin D1 (Joyce *et al.* 2001). We provide evidence that activation of *cyclin D1* gene transcription by EGF in rat primary hepatocytes is NF-κB-dependent. Indeed, hepatocytes infected with adenovirus expressing IκBα[sr] showed reduced expression of NF-κB-dependent luciferase reporter gene upon EGF stimulation, and concomitant down-regulation of EGF-induced *cyclin D1* expression. Exposure of EGF-stimulated hepatocytes to 4-Me$_2$N-BAVAH inhibited NF-κB activation in a manner similar to AdIκBα[sr], arguing that HDACi inhibits *cyclin D1* gene transcription through a mechanism involving NF-κB inhibition. Although in most cases, HDACi exposure has been associated with activation of NF-κB (Ashburner *et al.* 2001; Mayo *et al.* 2003), we show that, as in colonic epithelial cells (Yin *et al.* 2001), the opposite occurs in proliferating rat primary hepatocytes.

In contrast to cancer cell lines, in which HDACi induces reversible cell cycle arrest by up-regulating expression of cyclin-dependent kinase inhibitor p21 (Sambucetti *et al.* 1999; Lagger *et al.* 2003), 4-Me$_2$N-BAVAH-induced G$_1$ cell cycle arrest was not associated with p21 protein accumulation. Although this could be due to impaired translation and/or enhanced proteolysis, the fact that none of the functions of p21 (such as assembly of cyclin D1/cdk4 complexes and inhibition of their activity) are required might also in part explain this finding. In addition, we show that lack of p21 induction is not due to metabolic instability of 4-Me$_2$N-BAVAH in primary hepatocyte cultures. HDACi-mediated p21-independent cell cycle arrests have also been described in other non-tumourous cell systems, including p21 null mouse fibroblasts (Wharton *et al.* 2000) and normal human dermal fibroblasts (Glaser *et al.* 2003). This could thus represent a general mechanism of action of HDACi in non-malignant cells, which might contribute to their tumour-selective apoptotic features (Papeleu *et al.* 2005). Indeed, primary rat hepatocytes exposed to 4-Me$_2$N-BAVAH show delayed onset of spontaneous apoptosis, which could be a result of decreased p21 protein levels. In primary hepatocytes and models of liver regeneration, for example, forced expression of p21 has been shown to favour apoptosis (Kobayashi & Tsukamoto 2001; Papakyriakou *et al.* 2002; Qiao *et al.* 2002), whereas significant reduction in apoptosis has been observed in p21–/– primary mouse hepatocytes (Sheahan *et al.* 2004).

In conclusion, 4-Me$_2$N-BAVAH induces early G$_1$ cell cycle arrest in EGF-stimulated primary rat hepatocytes by inhibiting NF-κB activation and subsequent cyclin D1 transcription. In contrast to frequently observed tumour cells, HDACi-induced cell cycle arrest in primary rat hepatocytes occurs independently of p21 induction, which could represent a general mechanism of action of HDACi in non-malignant cells.

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