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Characterisation of the *in vitro* activity of the depsipeptide histone deacetylase inhibitor spiruchostatin A

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ABSTRACT

We recently completed the total synthesis of spiruchostatin A, a depsipeptide natural product with close structural similarities to FK228, a histone deacetylase (HDAC) inhibitor (HDI) currently being evaluated in clinical trials for cancer. Here we report a detailed characterisation of the *in vitro* activity of spiruchostatin A. Spiruchostatin A was a potent (sub-nM) inhibitor of class I HDAC activity *in vitro* and acted as a prodrug, requiring reduction for activity. Spiruchostatin A was a potent (low nM) inhibitor of the growth of various cancer cell lines. Spiruchostatin A-induced acetylation of specific lysine residues within histones H3 and H4, and increased the expression of $p21^{cip1/waf1}$, but did not induce acetylation of α-tubulin. Spiruchostatin A also induced cell cycle arrest, differentiation and cell death in MCF7 breast cancer cells. Like FK228, spiruchostatin A was both an inducer and substrate of the ABCB1 drug efflux pump. Whereas spiruchostatin A and FK228-induced protracted histone acetylation, hydroxamate HDI-induced short-lived histone acetylation. Using a subset of HDI-target genes identified by microarray analysis, we demonstrated that these differences in kinetics of histone acetylation between HDI correlated with differences in the kinetics of induction or repression of specific target genes. Our results demonstrate that spiruchostatin A is a potent inhibitor of class I HDACs and anti-cancer agent. Differences in the kinetics of action of HDI may be important for the clinical application of these compounds.

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1. Introduction

There is a great deal of interest in HDIs as potential new therapeutic agents for cancer [1,2]. HDACs catalyse deacetylation of the ε-amino group of lysine amino acid residues in the N-terminal tails of histones, as well as many other proteins, and thereby counter the activity of histone acetyltransferases. Histone acetylation plays a major role in controlling gene expression, by modulating chromatin packaging in combination with other post-translational modifications (such as methylation and phosphorylation) creating a ''histone code'' which is read by transcriptional regulators that recognise these marks [1,2].

There are 18 HDACs, divided into three major families [1–3]. Class I and II enzymes are zinc dependent, whereas class III enzymes (sirtuins) require NAD+ for activity. Class I (HDACs 1, 2, 3, 8 and 11) and class II enzymes (HDACs 4, 5, 6, 7, 9 and 10) possess distinct functional roles. For example, whereas class I enzymes are widely expressed and localised in the nucleus, class II enzymes have more restricted expression and shuttle between the cytoplasm and nucleus. Class I HDACs are considered to play a predominant role in maintaining the proliferation and survival of cancer cells [4–6], whereas HDAC5 and HDAC9, for example, are involved in preventing hypertrophy in the heart [7] and HDAC6 can act to deacetylate α-tubulin [8].

HDIs interfere with HDAC activity resulting in hyperacetylation of histones and other proteins. HDI induce growth arrest, cell death and differentiation in various sensitive cancer cells, with comparatively little effect on normal cells [1,3,9–11]. The molecular mechanisms that contribute to the activity of HDI in cancer cells and their relative sparing of normal cells are not fully understood. Surprisingly, HDI have been shown to modulate the expression of only a relatively small subset of genes, approximately 3–7% of the genome [1,2,12,13]. HDACs are important for the action of oncoproteins activated in various cancers, such as Bcl-6 or the PML-RARα fusion protein, and for silencing of growth inhibitory genes, such as $p21^{cip1/waf1}$ and $p16^{ink4a}$ [1–3,8,9,11]. Although most attention has focused on effects mediated by acetylation of histones on transcription, effects on DNA repair and replication, and histone-independent effects may also be important. For example, the function of key cancer-related proteins such as p53, E2F, pRb, STAT3, NF-κB and α-tubulin are all modulated by acetylation and therefore by HDI [1–3,14].

HDI are rapidly being developed as new therapeutic agents for cancer [1–3]. The two most advanced compounds are SAHA (also known as vorinostat; Zolinza, Merck), which has been approved for the treatment of recurrent cutaneous T-cell lymphoma and FK228 (Romidepsin, Gloucester Pharmaceuticals) which is in clinical trials. SAHA is an orally available synthetic hydroxamate whereas FK228 is a natural bicyclic depsipeptide, produced by fermentation of *Chromobacterium violaceum* (Fig. 1). Trichostatin A (TSA) is a naturally occurring hydroxamate that is a potent HDI [15], but is considered too toxic for clinical development (Fig. 1). Other HDI are relatively well tolerated. Frequent toxicities include fatigue, nausea, vomiting and thrombocytopenia [16–18]. Modest single agent activity has been seen in early phase trials to date and combination use with both conventional cytotoxics and molecularly targeted therapies is being evaluated in the clinic [19].

All HDI in clinical development inhibit class I/II zinc dependent HDACs with varying degrees of selectivity. The basis for activity has been revealed by structural studies [20]. In general, HDI comprise three key structural elements, a ''functional group'' which chelates the Zn atom in the base of a deep cleft comprising the active site of these enzymes, a short ''spacer'' which mimics the length of the side chain of the lysine residue and a ''cap'' structure which makes additional stabilising contacts with the enzyme around the rim of the active site [20]. The active site of class I/II HDACs is relatively well conserved between individual enzymes whereas amino acids beyond the rim of the active site are more variable. In TSA, the hydroxamate group chelates the Zn atom and the relatively simple cap largely interacts with well-conserved residues close to the active site. Simple hydroxamates therefore show relatively little selectivity towards classes I and II enzymes [21,22]. FK228, which acts as a prodrug, undergoes intracellular reduction of the disulphide bond releasing a free –

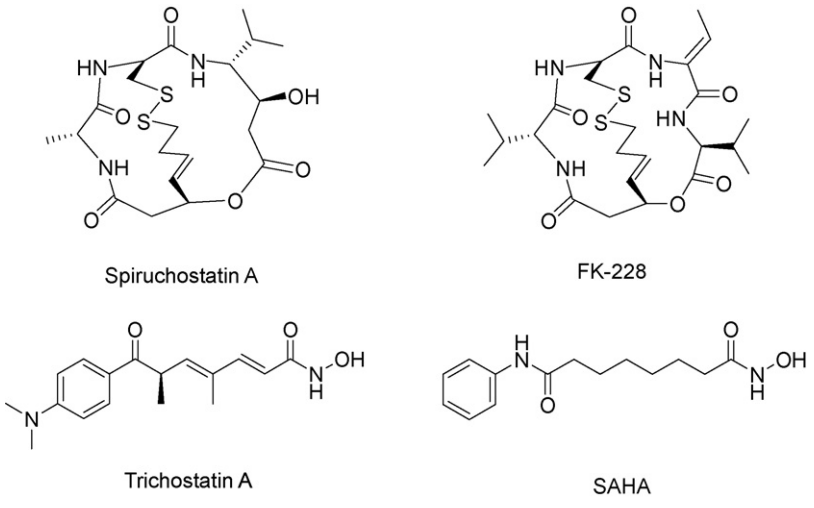

Fig. 1 – Chemical structures of HDI.

SH group which is thought to chelate its active site Zn atom [21]. The larger cyclic tetrapeptide cap structure is thought to make additional contacts compared to the simple cap structures of TSA and SAHA, and this may explain the observed selectivity of FK228 towards class I enzymes [21].

Spiruchostatin A (Fig. 1) is a natural bicyclic depsipeptide from the organism *Pseudomonas* sp. with structural similarities to FK228 and was identified in a screen for activators of transforming growth factor beta-mediated signalling [23]. Both compounds share the intramolecular disulfide bond that, in FK228, is reduced intracellularly to provide the zinc-binding group. We recently completed the total synthesis of spiruchostatin A and used initial cell-based assays to confirm its activity as an HDI [24]. Here we have performed a more detailed characterisation of the *in vitro* HDAC inhibitory activity of spiruchostatin A and describe differences in kinetics of histone acetylation and gene expression profiles compared to hydroxamate pan-HDIs such as TSA and SAHA.

2. Materials and methods

2.1. Compounds and cell culture

Spiruchostatin A and FK228 were prepared as previously described [24,25]. TSA, and all other chemicals unless stated, were obtained from Sigma, Poole, UK. SAHA was from Alexis Biochemicals, Nottingham, UK. All compounds were prepared as stock solutions in dimethyl sulfoxide (DMSO) and stored at −80 °C. BT474 cells were a kind gift of Dr. J.P. Blaydes, University of Southampton, UK. Normal human dermal fibroblasts (NHDF) were from Cambrex (Wokingham, UK). All other cell lines were from ECACC, Salisbury, UK. Cancer cell lines were maintained in DMEM (MCF7, BT474), RPMI1640 (A2780) or McCoy's 5A (HT29, MES-SA, MES-SA/Dx5) media with 100 μg/ml Penicillin G, 100 μg/ml Streptomycin and 292 μg/ml L-glutamine (all from Invitrogen, Paisley, UK) with 10% (v/v) foetal calf serum (PAA laboratories, Pasching, Austria) at 37 °C in 10% (v/v) CO_2. NHDF were maintained in Fibroblast Basal Medium and 10% (v/v) FCS and 0.5 μg/ml hydrocortisone (all from Cambrex) at 37 °C in 5% (v/v) CO_2. Cells were washed five times in medium lacking compound to remove drug in ''wash-out'' experiments.

2.2. In vitro HDAC assays

Inhibition of HDAC1 and HDAC6 activity was performed as previously described [21]. For reduction, spiruchostatin A was incubated overnight with dithiothreitol (DTT).

2.3. Cell growth inhibition assays

Cells (1000/well in a 96-well plate) were incubated for 6 days in the presence of drug, prior to analysis of cell number using the CyQuant reagent (Molecular Probes, OR, USA), according to the manufacturer's instructions. IC_{50} values were derived from multiple data points each performed in duplicate, and non-linear regression was performed using GraphPad Prism Software (GraphPad Software, San Diego, CA, US).

2.4. Immunoblotting

The following antibodies were used for immunoblotting; mouse monoclonal anti-α-tubulin (TAT1, Cancer Research UK Research Services, London, UK), mouse monoclonal anti-acetylated α-tubulin (6-11B-1, Sigma, Poole, UK), mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) (PC10, Cancer Research UK Research Services, London, UK), mouse monoclonal anti-$p21^{cip1/waf1}$ (SX118, Pharmingen, Oxford, UK), rabbit polyclonal anti-acetylated histone H3K9, histone H3K14 and histone H4K8 (all Upstate Cell Signalling Solutions, Dundee, UK). Immunoblotting for α-tubulin and acetylated α-tubulin, $p21^{cip1/waf1}$ was performed as previously described using detergent lysates [26]. Immunoblotting for acetylated histones was performed using whole cell lysates, prepared by resuspending washed cell pellets in SDS-PAGE sample loading buffer, sonication and heating at 95 °C for 3 min. Immunoblotting for PCNA expression was performed as a loading control in all experiments.

2.5. Cell cycle analysis

Cells plated at a density of 3×10^5 per 35 mm well, were treated with spiruchostatin A or DMSO. Analysis of cell cycle phase and sub-G1 cell fraction using flow cytometry and staining with propidium iodide was performed as previously described [27].

2.6. Differentiation analysis

Cells plated on to microscope cover slips at a density of 3×10^5 per 35 mm well, were treated with spiruchostatin A or DMSO. Differentiation analysis by Nile Red staining according to the methods of Greenspan et al. [28] was performed as previously described [27].

2.7. ABCB1 analysis

Cells were plated at a density of 3×10^5 per 35 mm well and treated with HDI or DMSO as a solvent control for 16 h. Cells were collected by trypinisation, washed and resuspended in PBS. Cells were incubated with anti-ABCB1 antibody (MRK16, Kamiya Biomedical Company, Seattle, US) or mouse IgG2Ak isotype control antibody (BD Pharmingen, Oxford, UK) on ice in the dark for 45 min. Cells were collected by centrifugation, washed twice in PBS, incubated with FITC-conjugated goat F(ab') anti-mouse IgG (R&D systems, Abingdon, UK) on ice in the dark for 30 min. Cells were collected by centrifugation, washed twice in PBS and resuspended in 500 μl PBS. Cells were then analysed by flow cytometry on a FACScaliber flow cytometer (BD Biosciences, Oxford, UK) using CellQuest software.

2.8. Gene expression microarray analysis

To perform the microarray experiment, 10^7 MCF7 cells were incubated with 200 nM of TSA, or the equivalent volume of DMSO, for 6 h. For each condition (i.e., TSA or DMSO), two separate samples of cells were treated, and each cell sample was used to prepare a probe that was hybridised to a single

array. Cells were collected by trypsinisation and centrifugation and total cellular RNA was prepared using TRIzol Reagent (Invitrogen, Paisley, UK), according to the manufacturer's instructions. cRNA was synthesized from total RNA using the CodeLink™ Expression Assay Reagent Kit (GE Healthcare, Amersham, UK) according to the manufacturer's instructions. Probes were prepared by *in vitro* transcription, labelling and fragmentation of the DNA and were hybridised to GE Healthcare CodeLink™ Uniset 20 K human gene BioArrays (GE Healthcare, Amersham, UK) containing 20,289 probe sets, corresponding to 408 controls and 19,881 discovery genes. The arrays were washed according to the manufacturer's instructions and results visualised with a GenePix™ 4100A microarray scanner (Molecular Devices, Wokingham, UK). The default Codelink™ per-array normalisation was used, where array are adjusted to have a common median expression value. There were a number of reporters with common sequence/ID. To cater for these we averaged over the duplicates whenever they existed. Microarray data was analysed using *R* (www.r-project.org) and Bioconductor (www.bioconductor.org). To select the significantly differentially expressed genes, an empirical Bayes' *t*-test between the two groups was used, as implemented in Bioconductor's 'limma' package. We filtered on genes that had a *p*-value of less than 0.001.

2.9. *Quantitative reverse transcription polymerase chain reaction (Q-RT-PCR) assays*

Total RNA was prepared using TRIzol (Invitrogen, Paisley, UK) according to the manufacturer's instructions. First strand cDNA was prepared using oligo(dT)15 primer (Promega, Southampton, UK) and murine Moloney leukaemia virus reverse transcriptase (Promega, Southampton, UK). Real-time Q-RT-PCR analysis was performed using Applied Biosystems Assays-on-demand probes sets according to the manufacturer's instructions (Applied Biosystems, Warrington, UK). Expression assays used for this study were Hs00170014 (CTGF), Hs00191243 (RAB33A), Hs00202033 (SIRT4), Hs00248508 (RGL1), Hs00178563 (IRS1), Hs00191466 (ICB-1), Hs00270951 (FOXC2), Hs00189461 (BPTF) and Hs99999905 (GAPDH). All reactions were performed in triplicate using the ABI PRISM 7900 Sequence detection System (Applied Biosystems, Warrington, UK) according to the following thermal cycle protocol: 94 °C 10 min, followed by 40 cycles of 94 °C for 15 s and 60 °C for 1 min. Control reactions with no cDNA added were run on each plate for each Taqman gene Expression Assay used and no amplification was detected in any control reaction. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA expression was used to calculate relative expression levels for all genes analysed.

3. Results

3.1. *Inhibition of in vitro HDAC activity by spiruchostatin A*

We previously completed the total synthesis of spiruchostatin A and demonstrated that it induced acetylation of histone H4 and activated the $p21^{waf1/cip}$ promoter in MCF7 breast cancer cells, consistent with HDI activity [24]. To further characterise spiruchostatin A we first analysed its effects on HDAC activity using *in vitro* assays. FK228 is relatively selective for class I HDACs and acts as a prodrug, requiring reduction of the disulphide bond for activity. Unreduced FK228 is inactive in *in vitro* assays but is active when added to cells since the compound is reduced by the intracellular environment following uptake [21]. We therefore compared the activity of the oxidised and reduced forms of spiruchostatin A against both HDAC1 (class I) and HDAC6 (class II). As a control we used TSA, a potent inhibitor of class I and II HDACs [29]. Reduced spiruchostatin A was a potent (sub-nM) inhibitor of HDAC1, but showed markedly reduced activity against HDAC6 (Table 1). By contrast, TSA was approximately equally effective versus HDAC1 and HDAC6. Reduced spiruchostatin A was therefore a more potent HDI than TSA and was ~600-fold more effective versus HDAC1 than HDAC6. Unreduced spiruchostatin A was essentially inactive against both HDAC1 and HDAC6 confirming the requirement for reduction for activity.

Table 1 – Inhibition of HDAC1 and 6 in *in vitro* assays

	HDAC1	HDAC6
Reduced spiruchostatin A	0.62 ± 0.05[a]	360 ± 40
TSA	15.3 ± 1.6	61.2 ± 12.7
Unreduced spiruchostatin A	>100,000	>100,000

[a] IC_{50} (nM) for enzyme inhibition (mean ± S.D.).

3.2. *Activity of spiruchostatin A in cell-based assays*

FK228 is amongst the most potent HDI reported and we therefore determined the effects of spiruchostatin A on the growth of a small panel of cancer cell lines, including breast (MCF7 and BT474), ovarian (A2780) and colon (HT29) carcinomas (Fig. 2A and Table 2). Spiruchostatin A was a potent (i.e., low nM) inhibitor of the growth of all cell lines. We also compared the activity of various HDI in MCF7 breast cancer cells and fibroblasts (NHDF), a well-established model system for comparison of effects in malignant and normal cells [11]. The depsipeptide HDIs (spiruchostatin A and FK228) were the most potent, whereas the hydroxamates SAHA, and TSA were markedly less active. By comparison to MCF7 cells, NHDF were less sensitive to each of these four HDIs with respect to growth inhibition, consistent with effects previously reported for SAHA [11]. Although this difference in sensitivity was modest, there was close consistency across these four inhibitors of a

Table 2 – Growth inhibition by spiruchostatin A in human cancer cell lines

Cell line	IC_{50} (nM)[a]
MCF7	5.7 ± 0.7
BT474	6.7 ± 1.4
A2780	6.3 ± 2.4
HT29	1.2 ± 0.7

[a] Data are mean ± S.D. derived from a minimum of two separate experiments.

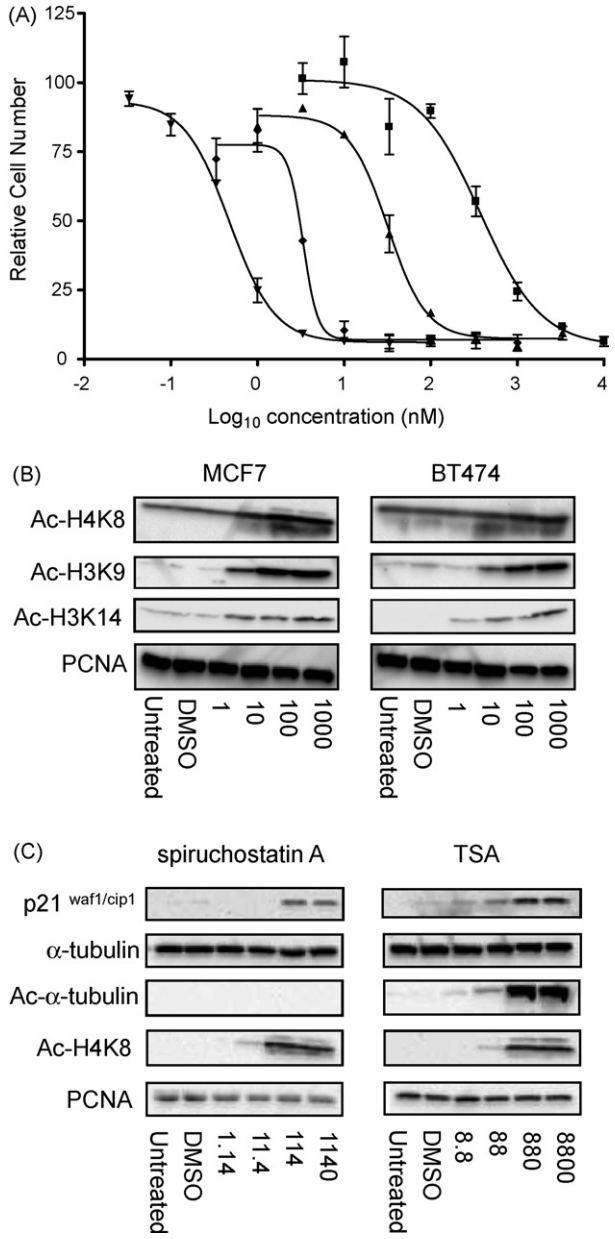

Fig. 2 – Effects of spiruchostatin A on cancer cell growth and protein acetylation and expression. (A) Representative growth inhibition experiments. MCF7 cells were incubated with the indicated concentrations of compounds. After 6 days, relative cell numbers were determined using the CyQuant reagent. Error bars are derived from duplicate determinations. The absorbance value obtained from untreated cells was used to set the 100 value for relative cell number. FK228 (▼); spiruchostatin A (◆); TSA (▲); SAHA (■); DMSO, as a solvent control, had no effect on cell growth at any concentration tested (up to the equivalent of the highest dose of each test compound). (B) Effect of spiruchostatin A on histone acetylation. MCF7 and BT474 breast cancer cells were incubated with the indicated concentrations of spiruchostatin A (nM), DMSO (solvent control) or left untreated. Cells were collected 24 h later and analysed by immunoblotting using the indicated antibodies. Experiments shown are representative of a minimum of two separate experiments. (C) Effect of spiruchostatin A and TSA on α-tubulin acetylation and $p21^{cip1/waf1}$ expression. MCF7 cells were incubated with the indicated concentrations (nM) of spiruchostatin A or TSA, DMSO (solvent control) or left untreated. The concentrations of HDI used were equivalent to 0.2×, 2×, 20× and 200× IC_{50} for growth inhibition in MCF7 cells in 6-day assays (Table 3). Cells were collected 24 h later and analysed by immunoblotting using the indicated antibodies. Experiments shown are representative of a minimum of two separate experiments.

Table 3 – Growth inhibition by HDI in MCF7 breast cancer and normal human dermal fibroblast cell lines

	Spiruchostatin A	FK228	TSA	SAHA
MCF7	5.7 ± 0.7[a]	0.8 ± 0.2	44 ± 7	500 ± 42
NHDF	25 ± 7.7	4.5 ± 0.8	165 ± 35	2000 ± 50

[a] Data are mean IC_{50} (nM) ± S.D. derived from a minimum of two separate experiments. NHDF, normal human dermal fibroblasts.

four- to sixfold lower potency against NHDF compared to MCF7 cells. Spiruchostatin A is therefore a potent growth inhibitory molecule, with activity in a range of cancer cell lines and which shows diminished potency of effect for fibroblasts comparable to other HDI.

We next analysed the effects of spiruchostatin A on protein acetylation and expression. In both MCF7 and BT474 breast cancer cells, incubation with spiruchostatin A resulted in a dose dependent increase in the acetylation of specific lysine residues in histones H3 and H4 (Fig. 2B). Histone acetylation was increased at concentrations of 10 nM (close to the IC_{50} value for growth inhibition in these cells) although higher concentrations (100 nM) were required for maximal effects. We also analysed the effect of spiruchostatin A on α-tubulin, a specific substrate for HDAC6 (Fig. 2C). In these experiments we compared the activity of spiruchostatin A with TSA. We considered it important in such comparisons between compounds to compare 'equipotent' doses to allow for the variation in potency which we have demonstrated. We therefore used doses in this, and in subsequent comparative experiments in this work, based on multiples of the respective IC_{50} value for growth inhibition in MCF7 cells at 6 days (Table 3). Thus in this comparison, compounds were analysed at multiples of 0.2–200-fold of their respective IC_{50} values. Consistent with the *in vitro* data (Table 1), spiruchostatin A did not promote acetylation of α-tubulin, even at concentration ~200-fold higher than that required for growth inhibition, whereas TSA caused marked accumulation of acetylated α-tubulin. Both spiruchostatin A and TSA induced expression of the cyclin dependent kinase inhibitor $p21^{cip1/waf1}$, a well-established and key gene expression target of HDIs (Fig. 2C).

HDI cause cell cycle arrest, cell death and/or differentiation in cancer [1,3,9–11]. We therefore analysed the effects of spiruchostatin A on DNA content in MCF7 breast cancer cells (Fig. 3A). In both time course and dose response experiments spiruchostatin A caused a pronounced accumulation of cells in the G_2/M phase (Fig. 3B and C). There

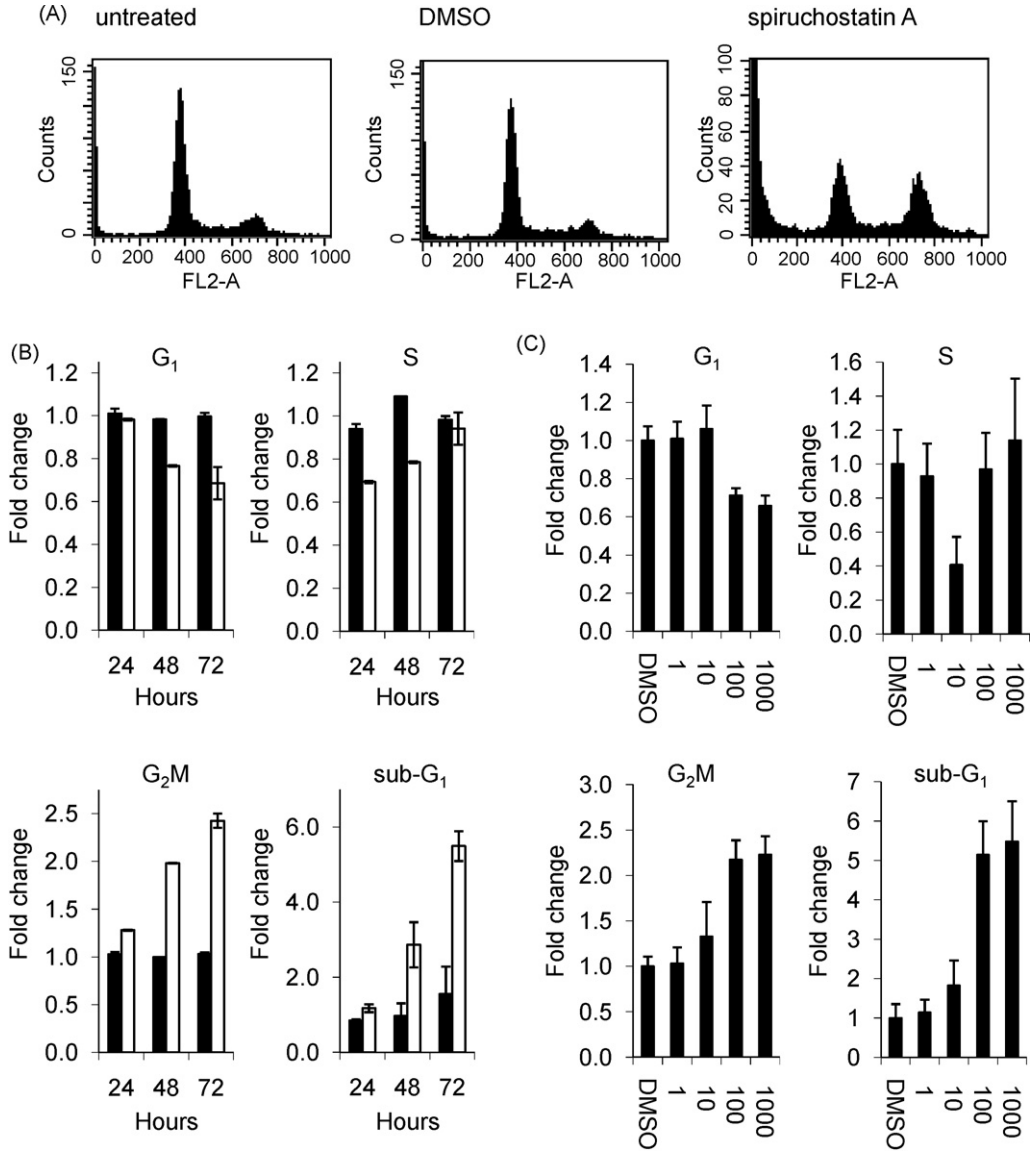

Fig. 3 – Effects of spiruchostatin A on the cell cycle (A). Representative cell cycle profiles of MCF7 cells treated for 72 h with DMSO (solvent control), spiruchostatin A (70 nM) (i.e., at ~10× the IC_{50} value for growth inhibition in the 6-day MCF7 assay, Table 3), or untreated control cells. Cell cycle profiles in response to spiruchostatin A were assessed in MCF7 cells treated (B) at 70 nM for the indicated durations (DMSO closed bars; spiruchostatin A open bars) or (C) for 48 h at the indicated concentrations (nM). Cells were collected and cell cycle distribution analysed by flow cytometry. Results show mean cell number derived from two separate experiments (±S.E.M.) relative to untreated cells which have been normalised to one.

was a reduction in the proportion of cells entering S-phase at early time points and lower concentrations indicating an additional block within the G_1-phase under these conditions. There was also evidence for induction of cell death, demonstrated by an increase in the proportion of cells with sub-G_1 DNA content. Since G_2/M phase cell cycle arrest is often associated with differentiation, we analysed the accumulation of cytoplasmic milk fat droplets as a marker of differentiation in breast cancer cells [10]. Incubation with spiruchostatin A for 24 h caused a significant ($p = 0.019$; Student's *t*-test) increase in the number of cytoplasmic lipid droplets (Fig. 4).

3.3. *Spiruchostatin A is an inducer and substrate for the ABCB1 drug efflux pump*

Drug efflux pumps are important determinants of resistance to drug therapy in malignant cells. HDI induce the expression of ABCB1 (MDR and pgp1) and FK228 (but not SAHA) is an efficient substrate for ABCB1-mediated drug efflux [30–32]. We therefore analysed the interactions between spiruchostatin A and ABCB1. Q-RT-PCR analysis demonstrated that spiruchostatin A strongly induced ABCB1 RNA expression in MCF7 cells (Fig. 5A). Other HDI tested also induced ABCB1 RNA. Cell surface expression of ABCB1 protein was also increased in

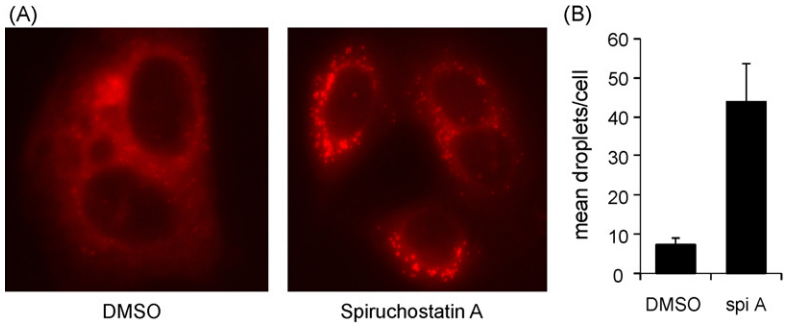

Fig. 4 – Effects of spiruchostatin A on lipid droplets. MCF7 cells were treated with spiruchostatin A (10 nM) for 24 h or DMSO as a control. Cells were stained using Nile Red to detect lipid droplets. (A) Representative images of DMSO or spiruchostatin A-treated cells (40× magnification). (B) The mean lipid droplet count per cell was determined in three separate experiments. The graph shows the mean of the counts per cells from three separate experiments (±S.D.).

spiruchostatin A or FK228-treated cells, although not as strongly as ABCB1 RNA (Fig. 5B).

To determine whether spiruchostatin A is a substrate for ABCB1-mediated efflux we compared activity in the MES-SA cell line, derived from a uterine sarcoma, and MES-SA/Dx5 cells a multi drug resistant subline of MES-SA with high expression of ABCB1 [33]. Whereas spiruchostatin A was a potent inhibitor of the growth of MES-SA cells, MES-SA/Dx5

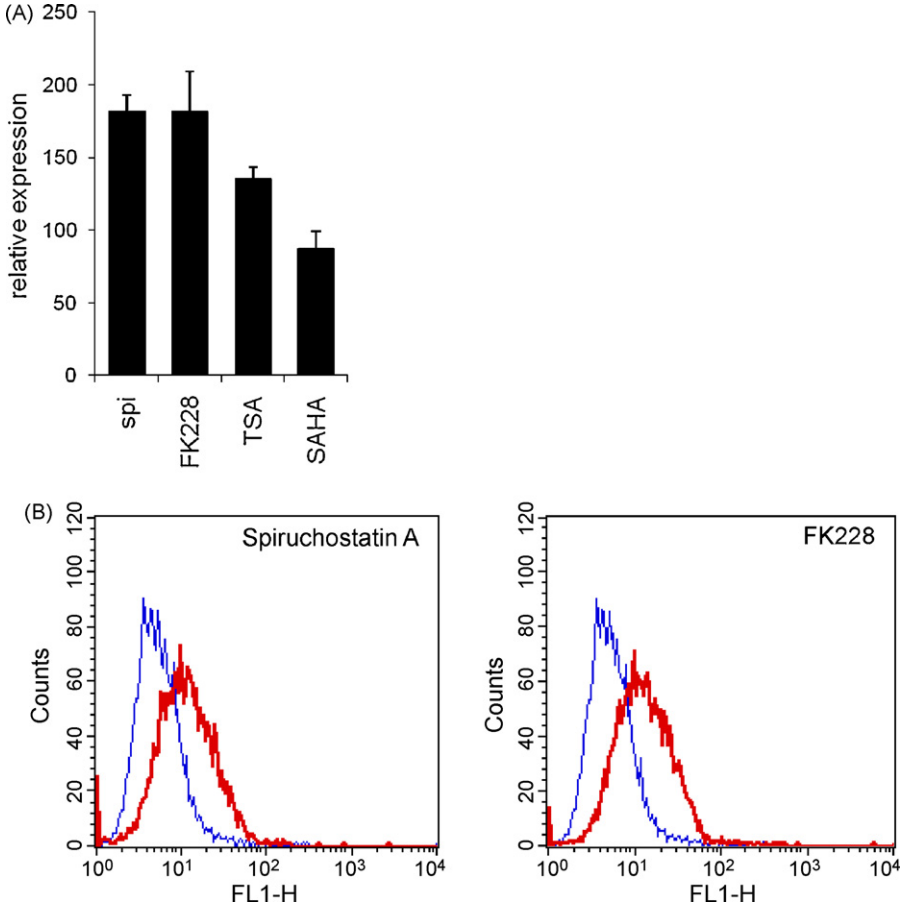

Fig. 5 – Regulation of ABCB1 by spiruchostatin A and other HDI. (A) MCF7 cells were treated with HDI at equipotent concentrations (for growth inhibition in a 6-day assay, Table 3; spiruchostatin A, 360 nM; FK228, 50 nM; TSA, 2.8 μM; SAHA, 32 μM) or an equivalent amount of DMSO as a solvent control. After 16 h, RNA was isolated the expression of *ABCB1* and *GAPDH* RNA were analysed by Q-RT-PCR. The graph shows the expression of ABCB1 normalised to GAPDH (mean of duplicate determinations ± S.D.) relative to DMSO-treated cells (set to 1). Experiment is representative of three. (B) MCF7 cells were treated with spiruchostatin A (45 nM) or FK228 (6.5 nM) (blue) or an equivalent amount of DMSO (red) as a solvent control. After 16 h, cell surface expression of ABCB1 was analysed by flow cytometry. Experiment is representative of two.

Table 4 – Effect of ABCB1 over expression on growth inhibition by HDI

	MES-SA	MES-SA/Dx5
Spiruchostatin A	3.1 ± 0.7[a]	440 ± 136
FK228	0.9 ± 0.1	547 ± 274
TSA	29 ± 8	51 ± 18
SAHA	406 ± 237	884 ± 29

[a] Data are mean IC_{50} (nM) ± S.D. derived from a minimum of two separate experiments.

cells were resistant with an approximately 140-fold higher IC_{50} value (Table 4). Similar results were obtained for FK228 where an approximately 600-fold comparative resistance in the ABCB1 over expressing cell line was seen, whereas the hydroxamate HDI TSA and SAHA were approximately equally effective in the two cell lines. Therefore, like FK228, spiruchostatin A is both an inducer and substrate for ABCB1.

3.4. *Kinetics of histone acetylation induced by spiruchostatin A*

In initial experiments we demonstrated that spiruchostatin A-induced protracted histone acetylation for up to 72 h, whereas the effect of TSA was limited to 24 h [34], suggesting that the kinetics of histone acetylation induced by different classes of HDI were distinct. To investigate this further we performed a series of time course and wash-out experiments in MCF7 cells using spiruchostatin A, FK228, TSA and SAHA (Fig. 6). All inhibitors were used at equipotent concentrations (i.e., ~2× IC_{50} for growth inhibition, Table 3). Cells were exposed to the inhibitors for a total of 2, 16 or 72 h prior to analysis of histone acetylation. In addition, drug was removed after 2 or 16 h exposure and the remaining histone acetylation analysed at 72 h. In this way, we could monitor the onset of action and its decay after washout of drug. The times points were selected to most clearly demonstrate differences between depsipeptide and hydroxamate HDI based on pilot experiments. Although

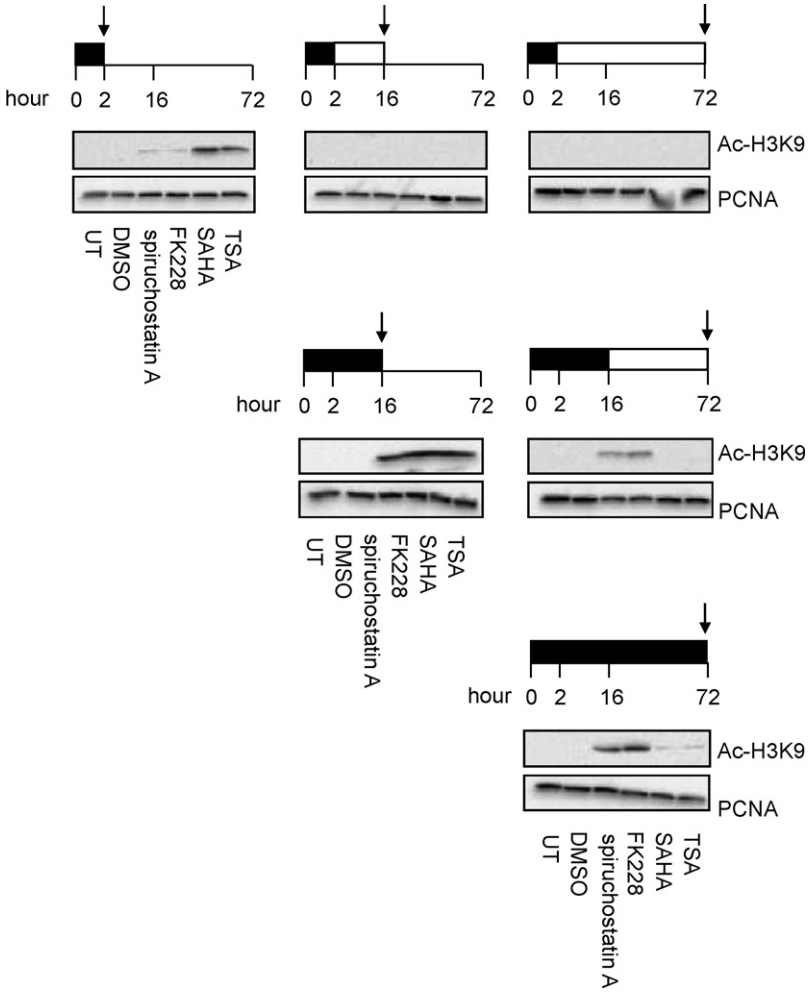

Fig. 6 – Kinetics of histone acetylation in breast cancer cells treated with depsipeptide and hydroxamate HDIs. MCF7 cells were incubated with the indicated HDIs at approximately equipotent concentrations. Concentrations used were spiruchostatin A 15 nM, FK228 1.5 nM, TSA 80 nM, SAHA 1000 nM (each equivalent to ~2× IC_{50} for 6-day growth inhibition; Table 3). Cells were exposed to compounds for 2, 16 or 72 h as indicated (black bars), washed thoroughly, and incubated further in growth medium without drug supplementation as indicated (open bars) before harvesting at the indicated time points (arrows). Cells were then analysed by immunoblotting using the indicated antibodies. Experiment representative of two separate experiments.

the induction of acetylation by FK228 or spiruchostatin A was relatively delayed (requiring greater than 2 h of drug exposure), depsipeptide-induced acetylation was protracted and was detectable after 72 h continuous exposure. In addition, histone acetylated also persisted following drug washout as it remained detectable at 72 h even if drug was removed as early as 16 h. By comparison, SAHA or TSA-induced early histone acetylation at 2 h which only persisted to 16 h, and was not detectable at 72 h, even after continuous exposure. SAHA or TSA-induced acetylation was also rapidly lost after drug wash out (within 30 min—data not shown). Thus, the kinetics of action of depsipeptide and hydroxamate HDI are distinct, at least at the level of global histone acetylation.

3.5. *Effects of HDI on gene expression*

A key downstream effect of HDI is modulation of gene expression and we determined whether differences in the induction of global histone acetylation by HDI impacted on the kinetics of modulation of specific gene expression in MCF7 cells. Primary modulation of gene expression by HDI will cause a cascade of secondary and tertiary events and it was clearly important to focus on early events in these studies. Although a number of microarray studies have identified HDI target genes in breast and other cancer cells, many of these have used relatively high concentrations of inhibitors and/or protracted incubation times (e.g. 24 h [12]). We therefore performed gene expression microarray analysis to identify a set of early TSA-responsive genes. We identified a total of 87 genes regulated in MCF7 cells treated with TSA for 6 h, representing 0.44% of the total number of genes analysed (Supplementary Table 1). Of these, 46 (53%) were up-regulated and 41 (47%) were down-regulated. Gene ontology analysis demonstrated that the genes were involved in diverse functions, including apoptosis, RNA processing, signalling and transcription (Supplementary Table 2). Genes involved in transcriptional control were particularly over-represented in the subset of regulated genes. We selected 8 genes involved in diverse functions for further analysis (CTGF, RAB33A, FOXC2, SIRT4, ICB-1 (C1orf38), RGL1, IRS1 and BPTF) and confirmed their regulation using Q-RT-PCR. There was a high correlation when comparing the fold change for these genes measured by Q-RT-PCR and the gene expression microarray ($R^2 = 0.812$).

To investigate the kinetics of regulation of these genes, we analysed their expression in MCF7 cells that had been treated with spiruchostatin A or TSA at equipotent doses (at approximately 4× IC_{50} for growth inhibition, Table 3) for 3 or 24 h. These time points were selected since TSA induces histone acetylation by 3 h, but spiruchostatin A does not, and both drugs induce histone acetylation at 24 h (Fig. 6). All of the genes were regulated by TSA at 3 h, and most maintained differential expression at 24 h (Fig. 7). By contrast, spiruchostatin A did not alter expression of any of these genes at 3 h exposure. However, each gene was found to undergo induction or repression by 24 h exposure. Therefore, TSA and spiruchostatin A control the expression of a common set of genes in MCF7 cells, despite differences in selectivity for class I/II enzymes but with a differing kinetic profile which mirrors differences in kinetics of histone acetylation.

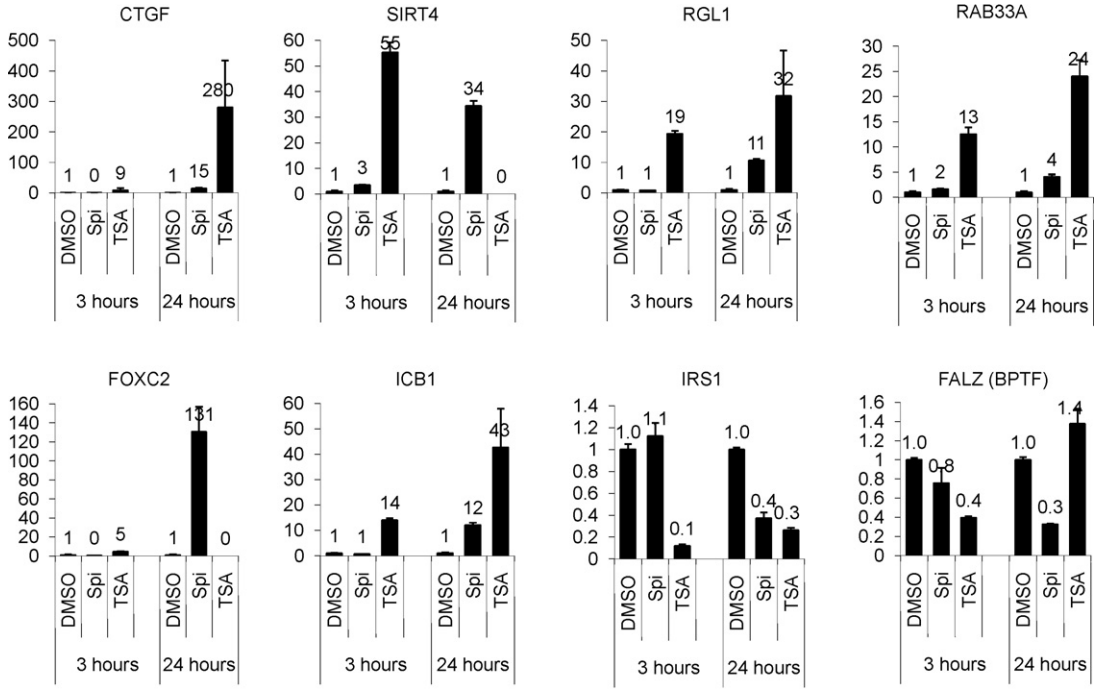

Fig. 7 – Effect of spiruchostatin A and TSA on kinetics of transcription in breast cancer cells. MCF7 cells were incubated with approximately equipotent concentrations (approx. 4× IC_{50} for growth inhibition in a 6-day assay; Table 3) of TSA (200 nM) or spiruchostatin A (spi; 20 nM), or DMSO as a control. RNA was prepared after 3 and 24 h, and expression of the indicated genes analysed by Q-RT-PCR. The expression of each gene was normalised to levels of glyceraldehyde-3-phosphate dehydrogenase RNA, and the relative expression of DMSO-treated cells was set at 1.0 for each time point. The fold induction relative to DMSO-treated cells is shown. Data are mean ± S.D. of triplicate determinations.

4. Discussion

We have analysed the activity of spiruchostatin A, a natural product originally isolated from a *Pseudomonas* extract and with structural similarities to FK228, an HDI in clinical trials for cancer. It is not clear why *Pseudomonas* sp. produces significant levels of HDI as potent as spiruchostatin A. It is possible that bacteria might exploit HDI to modulate cell growth control, by controlling the acetylation of non-histone proteins. However, the FK228 biosynthetic gene cluster in *C. violaceum* no. 968 contains two potential resistance genes which may prevent the accumulation of excess intracellular FK228 [35]. This perhaps suggests that certain bacteria utilise cyclic depsipeptides to inhibit the growth of other competing microorganisms in the environment. For example, HDI might inhibit the growth of bacteria by modulating acetylation on non-histone proteins, as well as fungi via histone-dependent mechanisms.

Spiruchostatin A shared many characteristics with FK228. Both compounds are potent (low/sub-nanomolar) HDIs, with selectivity towards class I HDACs and require reduction for activity, indicating that they act as prodrugs. Relative selectivity towards class I enzymes is likely to stem from the complex ''cap'' structure, comprising the peptide-like ring of these compounds. Additional contacts around and beyond the ''rim'' of the active site may contribute to enhanced potency and also selectivity, since variable amino acids between individual HDAC enzymes lie beyond the active site [20]. α-Tubulin acetylation was not seen with spiruchostatin A consistent with *in vitro* selectivity of depsipeptides towards HDAC1 versus HDAC6. Analysis of HDAC selectivity was restricted to a single representative of class I and II enzymes, and like all HDI, the selectivity profile of spiruchostatin A towards all class I and II enzymes is therefore incompletely described. The inability of depsipeptide compounds to inhibit HDAC6, and probably other class II enzymes does not appear to limit their activity as anti-cancer agents, as measured by *in vitro* effects on cell proliferation, differentiation and survival, and regulation of gene expression. These findings support the rationale for exploration of depsipeptide structure function relationships, through the generation of unnatural analogues, to better understand issues relating to HDAC selectivity and subsequent biological effect.

Characterisation of spiruchostatin A demonstrated a profile of biological effects including growth inhibition, cell cycle arrest, induction of apoptosis, induction of $p21^{waf1/cip1}$, and differentiation consistent with that seen with other HDIs. Comparative sparing of effects was demonstrated for growth inhibition using the model of fibroblasts compared to malignant cells, comparable in magnitude across the inhibitors studied, and consistent with previous results for SAHA [11]. With respect to cell cycle arrest, our findings are consistent with those of others, that either G1 or G2M arrest may be seen with HDIs, depending on cell line model system, treatment duration and dose used [9–11]. Our results and others suggest that the more selective depsipeptide HDIs retain a very similar profile of anti-cancer activities to more promiscuous hydroxamates in cell line model systems, consistent with the idea that class I enzymes are the key targets for HDI in malignant cells [4–6].

Looking to differences between agents, greater potency was seen across a panel of human solid tumour cell lines for FK228 and spiruchostatin A compared to hydroxamate and benzamide HDI. Of note, however, the depsipeptide HDI were relatively inactive in a cell model of multi drug resistance resulting from increased ABCB1 (pgp and MDR1) expression. Whilst ABCB1 induction appears to be a feature of all HDI studied to date, the depsipeptides appear also to be efficient substrates. This might be relevant to clinical application of HDIs but may also be a feature of depsipeptide compounds that would be amenable to alteration through structural modification of depsipeptide analogues.

In terms of differences between FK228 and spiruchostatin A, we have so far not detected clear differences between these two structurally similar compounds in this cell line-based study. Thus 'on target' effects (both therapeutic and possibly toxic) in clinical application of these agents would be predicted to be broadly similar but remains speculative at this stage. The potential to optimise drug like properties such as reduction of MDR1-mediated efflux exists through generation of further analogues of these agents. Other toxicities such as cardiac effects seen with FK228 might also be modified.

We also discovered striking differences with respect to the kinetics of global histone acetylation induced by depsipeptide and hydroxamate HDI. The hydroxamates induced a rapid but transient histone acetylation with swift loss of histone acetylation following removal of drug from the culture medium. By comparison the effects of depsipeptides were delayed but prolonged, and maintained following drug washout. The mechanisms for these kinetic effects remain to be fully elucidated, but many rate-limiting steps in the action of depsipeptides can be envisioned. For example, these larger compounds might be expected to accumulate within and exit from cells more slowly than lower molecular weight hydroxamates. It is also possible that efficient uptake of compounds may require active transporters, and these may be saturable or take time to be induced. Finally reduction of the bicyclic depsipeptide HDI may be a rate-limiting step. Future studies will investigate directly the kinetics of uptake, reduction and metabolism of depsipeptide HDI and the impact of structural modification, however, these will be demanding since analysis may have to be performed under non-oxidising conditions.

It was important to determine whether these differences in global histone acetylation correlated with differences in the kinetics of gene regulation, which is dependent on local chromatin modulation, and/or acetylation of specific transcription factors. Of the eight genes that we have studied in detail, all were regulated rapidly by TSA (i.e., within 3 h). In contrast, although the same 8 genes were each induced or repressed at 24 h in spiruchostatin A-treated cells, they required longer exposure to drug with none undergoing modification at 3 h. Therefore differences in kinetics of global histone acetylation that we have detected do appear to mirror effects on transcriptional regulation (both induction and repression) of HDI early responsive target genes. Regardless of this kinetic difference, the selectivity of spiruchostatin A for class I HDACs did not alter which genes underwent transcriptional regulation, at least of the limited set of eight target genes that we have assessed here.

We identified a total of 87 genes which appear to respond relatively rapidly to HDAC inhibition by TSA in breast cancer cells. It will be important to determine in future studies to what extent these changes in RNA are associated with alterations in protein expression, and to investigate the functional significance of these targets. This should include analysis of other cancer cell types and normal cells to start to better understand the therapeutic window offered by these drugs. Since we selected a relatively low concentration of TSA and an early time point for study, it is perhaps not surprising that only a small proportion of the genes identified in our microarray study have previously been shown to be regulated by HDI. Examples include RGL1, SNAI2, SILV, ABCB1 (MDR1), NFIC, SIRT4, DYRK3, GATA3, CTGF, FALZ (BPTF), SOX9 and SERPINI1 [30,36–41]. Notably, we did not identify any of the 13 ''core'' HDI responsive genes identified in breast cancer cells by Glaser et al. [12]. Within our microarray experiment, this included $p21^{waf1/cip1}$ which is considered as a ''core'' HDI target gene and important for the growth inhibitory effects of these compounds. We did however find protein expression levels to be up regulated at higher equipotent doses at 24 h spiruchostatin A and TSA (and FK228 and SAHA, data not shown). The study of Glaser et al. used a concentration of TSA up to 100 times the concentration required for growth inhibition and studied gene expression changes at 24 h. Therefore $p21^{waf1/cip1}$ is regulated relatively late by HDI, supporting the idea that our TSA early response gene set identifies relatively early changes in gene expression more closely associated with the primary effects of these drugs, compared to previous studies. It is undoubtedly also the case that differences in microarray platforms and methods of analysis are likely to contribute to this variation, as well as the precise cell type studied. Better characterisation of early (perhaps more direct) transcriptional modification in response to HDI, compared to late (perhaps secondary) effects will be of value in elucidation of the pleiotropic actions of these drugs.

Genes differentially regulated by TSA in our microarray experiment were involved in a diverse array of functions, including development (SERPINI1), metabolism (DHRS2), RNA-splicing (RNPC2), structure (MAP1A) and transport (SLC16A10) (Supplementary Table 1). Analysis of GO terms demonstrated that DNA-binding transcription factors were particularly abundant amongst TSA regulated genes. For example, SNAI2 (SLUG), a zinc-finger transcription factor was up-regulated by TSA. SNAI2 is a transcriptional repressor that represses BRCA2, cytokeratins, metallothioneins and E-cadherin transcription in breast carcinoma and is a determinant in tumour cell invasiveness [42–45]. In contrast to SNAI2, most of the transcription factors were down-regulated after TSA treatment. For example, GATA3 is a transcriptional activator, but also a direct target for acetylation [46]. Low expression of GATA3 is associated with invasive breast cancers [47]. Similarly, down-regulation of PgR may be relevant to breast cancer progression. Another striking feature of the TSA effect identified was a relative preponderance for genes linked to chromatin remodeling, suggesting activation of rapid feedback loops in HDI-treated cells including increased expression of SIRT4 and DYRK3 and decreased expression of SUV420H1, BANP/SMAR1 and ARID2. SIRT4 is a class III HDAC, which regulates epigenetic silencing by deacetylating histones in a NAD-dependent manner [48]; BANP/SMAR1 is a tumour suppressor protein that represses cyclin D1 by recruiting the HDAC1/SIN3 complex and deacetylating chromatin [49]; DYRK3 is a protein kinase that phosphorylates histone H2B [50]; SUV420H1 is a histone methyltransferase [51]; and ARID2 is a subunit of a SWI/SNF family chromatin remodelling complex that mediates transcriptional activation by nuclear receptors [52]. Many regulated genes were also involved in signalling. For example members of the Rassignalling pathway RGL1, RASGRP1, RAB33A and RASA4 [53], the tyrosine kinase FYN that has been implicated in the regulation of cell growth [54], and IRS-1 a down-stream signaling protein for insulin and insulin-like growth factor 1 receptor [55]. Our findings may have clinical relevance. Firstly, the ability of depsipeptides to promote durable acetylation responses might contribute to their efficacy but also to the patterns of toxicity. This may be important for dosing regimens used in clinical trials of HDI. The optimal duration of HDAC blockade, and whether this should be continuous or intermittent, remains to be defined. Benefits may be gained in the trade off between efficacy and toxicity by exploring this in clinical development. Secondly, there may also be implications for scheduling in combinations with other anticancer agents. Scheduling in cell-based assay systems has been shown to be important to optimally exploit synergism in some combinations with chemotherapy [56–58]. Thirdly, the variable kinetics of histone acetylation and gene expression profiles, which we have shown, may be important for interpretation of translational endpoints and biomarker profiles in clinical trials of HDIs.

Finally, our synthetic route to production of spiruchostatin A provides the basis on which to generate a series of FK228/spiruchostatin A analogues to select compounds which exploit benefits either with reduced toxicity or altered selectivity profiles. This holds potential value both as a scientific tool for dissection of HDI function, particularly surrounding the issue of HDAC selectivity, and for clinical development in various cancer indications and other diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2008.06.004.

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