A novel small molecule hydroxamate preferentially inhibits HDAC6 activity and tumour growth

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Background: This study investigates whether a histone deacetylase subtype 6 (HDAC6) inhibitor could be used in the treatment of solid tumours.

Methods: We evaluated the effect of a novel inhibitor, C1A, on HDAC6 biochemical activity and cell growth. We further examined potential of early noninvasive imaging of cell proliferation by [18F]fluorothymidine positron emission tomography ([18F]FLT-PET) to detect therapy response.

Results: C1A induced sustained acetylation of HDAC6 substrates, α-tubulin and HSP90, compared with current clinically approved HDAC inhibitor SAHA. C1A induced apoptosis and inhibited proliferation of a panel of human tumour cell lines from different origins in the low micromolar range. Systemic administration of the drug inhibited the growth of colon tumours in vivo by 78%. The drug showed restricted activity on gene expression with <0.065% of genes modulated during 24 h of treatment. C1A treatment reduced tumour [18F]FLT uptake by 1.7-fold at 48 h, suggesting that molecular imaging could provide value in future studies of this compound.

Conclusion: C1A preferentially inhibits HDAC6 and modulates HDAC6 downstream targets leading to growth inhibition of a diverse set of cancer cell lines. This property together with the favourable pharmacokinetics and efficacy in vivo makes it a candidate for further pre-clinical and clinical development.

Histone deacetylase (HDAC) enzymes that affect the acetylation status of histones and various cellular proteins have been recognised as therapeutic targets in central nervous system disorders and cancer (Xu et al, 2007; Kazantsev and Thompson, 2008; Haberland et al, 2009; Marks and Xu, 2009). Two inhibitors, vorinostat (SAHA) and romidepsin, have been approved by the FDA for treatment of cutaneous T cell lymphoma (Duvic and Vu, 2007; Piekarz et al, 2009). There are 18 HDACs, which are classified structurally into class I (HDAC1, HDAC2, HDAC3, HDAC8), class IIa (HDAC4, HDAC5, HDAC7, HDAC9), class IIb (HDAC6, HDAC10), class III (sirtuins; SIRT1-7) and class IV (HDAC11) groups. Class I HDACs are mostly nuclear, ubiquitously expressed and display enzymatic activity towards histone substrates. Class II members shuttle between the nucleus and the cytoplasm, target primarily non-histone substrates and their expression is tissue specific. In parallel with the explosion of HDAC research, the number of HDAC inhibitor (HDACI) drug candidates has grown exponentially in the last decade. Most HDACIs reported to date inhibit multiple HDAC isoforms, particularly HDAC1 and HDAC3, and although they have desirable antiproliferative effects, a number of these also elicit profound side effects, including bone marrow suppression, weight loss, fatigue and cardiac arrhythmias (Bruserud et al, 2007; Marsoni et al, 2008). The discovery of HDACIs with different isoform profiles or indeed isoform selective inhibition is important to elucidate the mechanism of action of specific HDAC enzymes,
and may hold greater promise than their non-selective counterparts, by minimising toxicity.

HDAC6 has recently emerged as an attractive target for the treatment of cancer (Boyault et al, 2007; Lee et al, 2008; Kawada et al, 2009; Rivieccio et al, 2009). HDAC6 was shown to be the deacetylase for a diverse set of substrates involved in tumourigenesis, such as HSP90, α-tubulin, cortactin and peroxiredoxins, but unlike other HDACs, inhibition of HDAC6 is believed not to be associated with severe toxicity; HDAC6 knockout in mice does not lead to embryonic lethality (Hubbert et al, 2002; Haggerty et al, 2003; Bali et al, 2005; Zhang et al, 2007; Parmigiani et al, 2008; Witt et al, 2009; Kaluza et al, 2011). To date, HDAC6 selective inhibitors (tubacin, tubastatin A) have only contributed to validate HDAC6 as a target, but their unfavourable pharmacokinetic (PK) profiles have prevented them from further pre-clinical and clinical development, making them only good research tools (Haggerty et al, 2003; Butler et al, 2010).

Here we report the development of a novel HDAC6 inhibitor, C1A. We show that C1A induces cell death and significant growth inhibition in a panel of cancer cells, and because of its favourable PK profile, is efficacious in solid tumours in vivo.

**MATERIALS AND METHODS**

HDAC deacetylase activity. HDAC enzyme inhibition was assessed as described elsewhere, using recombinant enzymes and 7-amino-4-methylcoumarin (AMC)-labelled substrates from p53 residues 379–382: the activities of HDACs 1, 2, 3, 6 and 10 were assessed using the substrate RHKKAc; HDAC8 activity was assayed using a specific substrate RHKAcKAc (Schultz et al, 2004; Butler et al, 2010). Activities of the Class IIA HDACs (HDAC4, 5, 7, 9) were assessed using a Class IIA-specific substrate (R, Acetyl-Lys(trifluoroacetyl)-AMC; Lahm et al, 2007).

Growth inhibitory assay. Drug concentrations that inhibited 50% of cell growth (GI50) were determined using a sulphorhodamine B (SRB) Growth inhibitory assay. Cells were cultured for 24 h and subsequently treated with different compounds: C1A (synthesised in-house), SAHA (R, Acetyl-Lys-acetyllysine; Cayman Chemicals, Ann Arbor, MI, USA), and C1 was prepared in 10% DMSO, 10% Solutol (Solutol HS15; BASF) and 10% Tween 20 in PBS, and administrated at 20 mg kg \(^{-1}\) intraperitoneally (i.p.) to female BALB/c mice (Harlan, Bicester, UK).

Cell cycle analysis. Cells were treated with C1A or SAHA for 24 h. Collected cells were fixed with ethanol and stained with propidium iodide in PBS for 3 h. Cell cycle distributions were determined using flow cytometry (FACS canto, Becton Dickinson, Oxford, UK) and analysed using the flowJo software (TreeStar Inc, Ashland, OR, USA). In each analysis, 10 000 events were recorded.

Caspase-3/7 assay. Caspase-3/7 activity was determined using Promega’s caspase-3/7 assay according to the manufacturer’s instructions (Promega, Madison, WI, USA). In brief, cells were incubated for 1 h with Caspase-Glo reagent, and the enzymatic activity of caspase-3/7 was measured using a TopCount NXT microplate luminescence counter (PerkinElmer, Waltham, MA, USA) and normalised to protein content.

**PK profiling.** C1A was prepared in 10% DMSO, 10% solutol HS15 (BASF) and 10% Tween 20 in PBS, and administrated at 20 mg kg \(^{-1}\) intraperitoneally (i.p.) to female BALB/c mice (Harlan, Bicester, UK). Full details are described in Supplementary Information. All animal experiments were done by licensed investigators in accordance with the United Kingdom Home Office Guidance on the Operation of the Animal (Scientific Procedures) Act 1986 (HMSO, London, UK, 1990) and within guidelines set out by the United Kingdom National Cancer Research Institute Committee on Welfare of Animals in Cancer Research (Workman et al, 2010).

**Tumour xenographs.** HCT-116 cells (5 × 10\(^6\)) were injected subcutaneously in 100 μl volumes into the flanks of female nu/nu-BALB/c athymic nude mice (Harlan). Tumour measurements were performed daily and volumes were calculated using the formula (length (mm)) × (width (mm)) × (depth (mm)) × π/6. When tumours reached a volume of 50–100 mm\(^3\), treatment with different compounds was initiated. Throughout the 14-day treatment period, animal weights and tumour volumes were determined daily.

**Ki-67 immunostaining.** Tumours treated with C1A or vehicle were excised after imaging, fixed in formalin, embedded in paraffin and cut into 5.0 μm sections, and tumour proliferation was determined as previously described (Leighton et al, 2006a,b, 2008; Nguyen et al, 2009). The normalised uptake value at 60 min post injection (NUV\(_{60}\)) was used for comparisons (Nguyen et al, 2009).

**[\(^{18}\)F]Fluorothymidine positron emission tomography imaging.** HCT-116 cells (5 × 10\(^6\)) cells were injected on the back of female nu/nu-BALB/c mice. At 24 or 48 h post treatment with C1A given at 40 mg kg \(^{-1}\) QD, the animals were scanned on a dedicated small animal PET scanner (Siemens Inveon PET module) following a bolus intravenous injection of ~3.7 MBq of [\(^{18}\)F]fluorothymidine ([\(^{18}\)F]FLT) as previously described (Leighton et al, 2006a,b, 2008; Nguyen et al, 2009). The normalised uptake value at 60 min post injection (NUV\(_{60}\)) was used for comparisons (Nguyen et al, 2009).

**Statistical analyses.** Statistical analyses were done using GraphPad prism software. Differences between groups were analysed by two tailed Student’s t-tests. Differences in tumour growth in nude mice were analysed by repeated measures two-way ANOVA. P-values < 0.05 using a 95% confidence intervals were considered significant. Data are reported as mean ± s.e.m. of at least three independent experiments unless otherwise stated. *P < 0.05, **P < 0.005, ***P < 0.0001, NS, not significant.

**Additional methods.** Additional methodology is described in Supplementary Information—Materials and Methods.
C1A is an effective HDAC6 inhibitor. C1A was developed based on the structure of naturally occurring pan HDACIs trichostatin A and SAHA. Although maintaining the hydroxamate part of the molecule, responsible for the binding to the Zn$^{2+}$ pocket of HDAC in general, the aromatic nitrogen mustard moiety, originally introduced to enhance duration of drug action, was found to enhance preferential binding to HDAC6 (Figure 1A; Finnin et al, 1999). A molecular modelling approach was used to illustrate the preferential affinity of C1A to HDAC6 catalytic domain (cd) II (Figure 1B and C) relative to HDAC1cd (Figure 1D and E). In many aspects, the binding mode for the two isoforms, HDAC1 and HDAC6, is very similar. Both are bound to the Zn$^{2+}$ ion in the bottom of the pocket in a bidentate fashion. There are hydrophobic interactions between the conjugated carbon chain of C1A and the side chains of Phe150 and Phe205 in HDAC1cd and the side chains of Phe620 and Phe680 in HDAC6cdII. However, there are important differences as can be seen by comparing the water accessible surfaces. For HDAC1cd, the aniline mustard binds to the surface of the protein at an unfavourable solvent-exposed area. The HDAC6cdII pocket is much wider and can much better accommodate the inhibitor, and the aniline mustard moiety binds to a well-defined hydrophobic groove, which contributes to the observed isoform selectivity.

C1A inhibited class I/II HDACs, and sirtuins, with highest affinity for HDAC6 (IC$_{50}$ = 479 nM) versus HDAC1 (IC$_{50}$ = 14 μM; Figure 1F and Supplementary Table S1). HDAC8 was also inhibited by C1A at submicromolar concentrations (Supplementary Table S1). Initial reports suggesting that HDAC8 drug inhibition had little effect in solid tumour-derived cell lines (e.g., HCT-116) triggered our attention towards HDAC6 inhibition and its application in our experimental models of cancer (Balasubramanian et al, 2008).

C1A treatment affects known HDAC6 substrates. We evaluated the effect of C1A on HDAC6 substrates. In human colon cancer HCT-116 cells, we observed an increase in the acetylation of α-tubulin and HSP90, known substrates of HDAC6, as early as 4 h after 1 μM C1A treatment (Figure 2A and B). The effect of C1A on acetylation of α-tubulin was drug concentration-dependent at 4 and 24 h (Figure 2B and C). Treatment with the clinically licensed broad-spectrum inhibitor, SAHA, also increased acetylation of α-tubulin. Unlike C1A, however, SAHA increased acetylation of histone H3, a biomarker for inhibition of class I HDACs (Figure 2A and C). When treated continuously for 4 h (10 μM) and drug was subsequently removed by washing to mimic intermittent dosing in vivo, acetylation of α-tubulin was maintained for 3 h after washout of C1A but not with SAHA (Figure 2D). These results show that C1A is associated with a sustained effect on HDAC6 response that would allow the drug to be dosed less frequently.

The loss of chaperone activity of HSP90 is a functional consequence of its acetylation (Scroggins et al, 2007). CDK4 is a recognised client protein of the HSP90 chaperone and is degraded upon HSP90 inhibition (Banerji et al, 2005). Both C1A and SAHA were associated with a decline of CDK4 expression, consistent with HSP90 inhibition (Figure 2E). As a control, treatment of cells with the HSP90 inhibitor, 17-AAG, also decreased CDK4 expression in these cells (Figure 2E). Treatment with positive control tubastatin A, a HDAC6 inhibitor tool compound, was also associated with a decline of CDK4, concomitant with a drug concentration-dependent increase of the acetylated form of α-tubulin (Supplementary Figure 1).

Similarly, HCT-116 cells transfected with HDAC6 shRNA showed increased acetylation of α-tubulin concomitant with a decrease of CDK4 (Supplementary Figure S2).

C1A does not promote non-specific DNA alkylation. We wondered if the presence of a nitrogen mustard moiety in C1A promoted non-specific DNA alkylation. As DNA alkylating agents will generally be more active in cell lines deficient in the DNA repair machinery, we tested the potency of the drug in cell lines deficient in DNA repair proteins. We showed that, in contrast to the DNA alkylating agent chlorambucil, the growth inhibitory potency of C1A was not affected by DNA repair defects (Figure 2F), suggesting that the nitrogen mustard moiety in C1A does not induce non-specific DNA alkylation.

HDAC6 inhibition is associated with antiproliferative activity and apoptosis. C1A inhibited the growth of a panel of 17 human...
C1A is a selective inhibitor of HDAC6 associated with a sustained effect. (A) Inhibition of HDAC6 substrates by C1A (1 μM; 4 h) relative to SAHA in HCT-116 cells. (B) Drug concentration-dependent acetylation of α-tubulin at 4 h, and (C) at 24 h relative to acetyl histone H3 expression. (D) Effect of 10 μM C1A or SAHA on acetylation of α-tubulin in HCT-116 cells following 4 h drug incubation and washout (cells collected 3 h after washout). (E) Cognate effect of C1A and SAHA and C1A on HSP90 client protein CDK4 following 24 h treatment. The HSP90 inhibitor, 17-AAG, was used as a positive control. (F) Effect of C1A on the growth of DNA repair-deficient and repair-competent cells relative to chlorambucil. UV23 and UV96 cell lines are deficient in XPB and ERCC1, respectively; proteins involved in the nucleotide excision repair pathway. Irs1 and Irs1SF are deficient in XRCC2 and XRCC3, respectively; proteins involved in the homologous recombination repair pathway. Xrs5 (XRCC5–) is deficient in UV23 and UV96 cell lines are deficient in XPB and ERCC1, respectively; proteins involved in the nucleotide excision repair pathway. Irs1 and Irs1SF are deficient in XRCC2 and XRCC3, respectively; proteins involved in the homologous recombination repair pathway. Xrs5 is deficient in XRCC5; protein involved in the non-homologous end-joining pathway. Growth curves in parental repair-competent cell lines AA8 (WT) and CHO-K1 (WT) are also presented.

cancer cell lines, including cell lines derived from 8 different histological types of solid tumours and one type of B-cell malignancy, with a mean growth inhibitory effect (GI_{50}) of 3.1 ± 2.2 μM following 72 h continuous exposure (Table 1). When the cells were treated for 6 h, washed and allowed to grow for an additional 72 h in drug-free growth medium, the growth inhibitory effect of C1A was more than 300-fold greater than for SAHA (Figure 3A). Under washout conditions, the latter did not show any antiproliferative effect at the concentrations tested. The cellular effect of C1A was further characterised in HCT-116 cells to investigate the mechanism of growth inhibition. Flow cytometry studies showed that treatment of cells with C1A for 24 h increased the sub-G1 population in a drug concentration-related manner (from 4% in untreated cells to 64% at the highest concentration tested, i.e. 10 μM; Figure 3B and C), suggesting an apoptotic mechanism. The characteristic increased sub-G1 fraction was also demonstrated in A2780 human ovarian cancer cell line but not in the caspase-3-deficient human breast cancer cell line, MCF7 (Supplementary Figure S3; Janicke, 2009). In contrast, SAHA increased the fraction of cells arrested in G2/M (from 30–41%) in the HCT-116 cells. SAHA also induced a sub-G1 population, but unlike C1A this effect reached a plateau at 22% from a low drug concentration. We confirmed the drug-induced increase in sub-G1 as occurring via apoptosis by measuring caspase-3/7 activity: both C1A and SAHA induced a drug concentration-related increase in caspase-3/7 activity (Figure 3D); increased caspase-3/7 activity was further confirmed in HCT-116 cells by flow cytometry (FLICA positive/SYTOX red negative; Supplementary Figure S4). Drug concentration-dependent increase in caspase-3/7 activity was also seen in these cells with tubastatin A (Supplementary Figure S5).
Caspe-3/7 activation by C1A also occurred in A2780 cell line but not in the caspase-3-deficient MCF7 breast cancer cell line (Supplementary Figure S3). Proliferation of cells transfected with HDAC6 shRNA was inhibited by 24% after 3 days of seeding, but unlike drug treatment no difference in caspase-3/7 activity was detectable (Supplementary Figure S6). Flow cytometry studies revealed that, cells transfected with HDAC6 shRNA had an increased sub-G1 population (from 1.2% for shRNA-scramble to 21.8% for shRNA HDAC6), suggesting that the peak of caspase-3/7 may have occurred at an earlier time point.

The cell cycle inhibition caused by SAHA was accompanied by increases observed in plasma concentration (Figure 4B). Maximum drug concentration was rapidly achieved (30 min) when given i.p. Notably, drug levels equivalent to two-fold GI50 in HCT-116 cells could be achieved within 24 h, suggesting that the peak of caspase-3/7 may have occurred at an earlier time point.

Table 1. Growth inhibitory effect of C1A compared with SAHA in a panel of cancer cell lines (mean ± s.d.)

| Cell type  | Cell line | C1A (µM) | SAHA (µM) |
|------------|-----------|----------|-----------|
| Breast     | MCF7      | 3.3 ± 0.15 | 3.5 ± 0.6 |
|            | MDA-MB-435| 7.7 ± 0.2  | 0.96 ± 0.05 |
|            | T47D      | 4.5 ± 0.03 | 1.2 ± 0.2 |
| Colon      | HCT-116   | 3.3 ± 1.6  | 0.48 ± 0.1 |
| Endometrial| Ishikawa  | 5.6 ± 0.03 | 0.67 ± 0.05 |
| Epidermal  | A431      | 4.1 ± 0.6  | 1.1 ± 0.1 |
| Lung       | AS49      | 5.8 ± 2.2  | 1.8 ± 0.6 |
| Myeloma    | ARH-77    | 3.1 ± 0.55 | 0.48 ± 0.24 |
|            | KMS-11    | 0.48 ± 0.24 | 0.27 ± 0.03 |
| Neuroblastoma| SH-SYSY  | 0.28 ± 0.01 | 0.92 ± 0.3 |
|            | Kelly     | 0.18 ± 0.01 | 0.4 ± 0.12 |
|            | S-K-NAS   | 3.3 ± 1.5  | 0.49 ± 0.02 |
|            | BE2C      | 0.66 ± 0.19 | 0.63 ± 0.08 |
| Ovarian    | A2780     | 0.96 ± 0.44 | 0.82 ± 0.1 |
|            | IGROV1    | 1.1 ± 0.2  | 1.2 ± 0.2 |
| Prostate   | LNCAP     | 3.7 ± 1.5  | 1.7 ± 1.2 |
|            | DU-145    | 4.6 ± 0.6  | 0.54 ± 0.01 |

Caspase-3/7 activity of C1A combined with its favourable PK features favoured efficacy in the human breast xenograft models. Different schedules and dose levels of C1A were tested in the HCT-116 xenograft model and antitumour activity was found to be dose-related. Doubling times were 4.4 ± 1.1, 8.3 ± 1.0, 7.6 ± 1.2 and 9.5 ± 1.5 days for vehicle control mice and mice treated with C1A at 40 mg kg⁻¹ q.o.d., 20 mg kg⁻¹ q.d. and 20 mg kg⁻¹ b.i.d., respectively; Figure 4C). C1A treatment was associated with a tumour growth delay (TGD2x) of 4.0 ± 0.8, 3.8 ± 1.2 and 5.7 ± 1.4 days and a tumour growth inhibition (TGI) of 57, 69 and 78% compared with vehicle at 40 mg kg⁻¹ q.o.d., 20 mg kg⁻¹ q.d. and 20 mg kg⁻¹ b.i.d., respectively; there was no general toxicity in any of the treated cohorts as determined by changes in body weight (Figure 4D).

In vivo antitumour activity is associated with molecular and imaging biomarker changes. Biochemical target modulation in vivo was determined by measuring levels of acetylated α-tubulin in HCT-116 tumours. Following a single dose of C1A at 40 mg kg⁻¹, acetylated α-tubulin could be detected at 6h and sustained up to 24 h (Figure 5A). No modulation of acetylation of histone H3 was detectable, confirming a lack of inhibitory effect on
class I HDACs. Furthermore, tumour uptake of the proliferation imaging biomarker, $[^{18}]$FLT (Shields et al, 1998; Leyton et al, 2006a), decreased after C1A treatment (Figure 5B). Radioactivity in tumour regions normalised to that of the heart of the same animal was used quantitatively to compare the effects of C1A (Leyton et al, 2006a). There was a 1.7-fold decrease in tumour $[^{18}]$FLT uptake (1.7-fold; $P<0.0001$) (Figure 5C). We further evaluated if C1A induced apoptosis in vivo. Activated caspase-3 expression and subsequent fragmented DNA staining (TUNEL) increased 6- ( $P=0.0082$) and 7-fold ( $P<0.0001$), respectively, together with a decrease of DNA content ( $P=0.023$; Figure 5D).

Figure 5. Molecular and imaging biomarkers for monitoring the response to C1A. Nude mice bearing HCT-116 tumour xenografts were injected with either vehicle or C1A at 40 mg kg$^{-1}$ per day. (A) Expression of acetylated $\alpha$-tubulin and H3 over time following a single injection of C1A at 40 mg kg$^{-1}$. (B) Computed tomography (CT; top) and corresponding $[^{18}]$FLT-PET-CT (bottom). For visualisation, cumulative images up to 60 min are displayed. The tumour is indicated by an arrow. Quantitative $[^{18}]$FLT uptake at 24 and 48 h was expressed as radiotracer uptake in tumours normalised to that of heart at 60 min (NUV$_{60; n=4}$). (C) Ki-67 immuno-stained tumour sections following treatment for 2 days. Proliferative cells have brown staining. Ki-67 labelling index was calculated from 10 random fields of view per slice (2 slices per tumour, and 3 tumours per group). The Ki-67-positive cells were expressed as a percentage of total cells. (D) Tumour active caspase-3 and TUNEL immunohistochemistry analysis. Tumour tissues were removed after PET imaging (following treatment for 2 days) and stained for active (cleaved) caspase-3 (Δcaspase-3) and DNA fragmentation (TUNEL assay). Representative images of histological tumour sections are shown. Staining intensities were expressed as per cent staining per field using 10 random fields of view per slice (2 slices per tumour and 3 tumours per group). (E) Differential gene expression of HCT-116 tumours 24 h following a single injection of C1A at 40 mg kg$^{-1}$ and compared with vehicle-treated (n=3).

Gene expression signature following C1A treatment in vivo. Although HDAC6 has been described as being predominantly cytoplasmic, it has also been suggested to act as a nuclear cofactor (Palijan et al, 2009). To assess if the mechanism of response to C1A in vivo was related at least in part to transcriptional activation, directly or indirectly via its substrates, a gene array was performed in the HCT-116 xenograft model. Of the 20 000 genes tested, only 13 were deregulated (0.065%) at 24 h following a single injection of C1A (40 mg kg$^{-1}$) in keeping with the class II (HDAC6) effect of C1A (i.e., minor effect in the regulation of genes; Figure 5E) in contrast to SAHA (LaBonte et al, 2009). The following genes were upregulated: BST2, FHL1, PTPRG, ED44, NBPF8-11/14-16, XAF1, PTB, PIPlK1, BAX, SRDS5A, NBPF8-11/14-16, XAF1, PTB, PIPlK1, BAX, SRDS5A, RAD23B, IPO7, XAF1, and NBPF8-11/14-16.
NBPF(8-11, 14-16), XAF1, PIP5KL1, BAX, SRD5A1, DHCR24, TAPBP, PTRRG, IPO7 and RAD23B; HIST2H2AC was down-regulated. Among those, the pro-apoptotic factors XAF1 (gene encoding XIAP-associated factor 1, an antagonist of X-linked inhibitor of apoptosis protein) and BAX (gene encoding Bcl-2-associated X protein) were upregulated, which could explain in part the antitumour activity. Of note is the upregulation of RAD23B that was recently proposed as a predictive marker of response to HDACIs generally (Fotheringham et al, 2009). To confirm changes in protein expression, western blot analysis was performed on the excised tumours. Levels of XAF1, PIP5KL1 and RAD23B were indeed higher following treatment with C1A (Supplementary Figure S8).

**DISCUSSION**

Cancer remains an unmet medical need with one in three people affected by the disease. New therapies with improved efficacy and reduced side effect profile are needed. We have shown that a potent small molecule inhibitor of HDAC, in particular HDAC6, inhibits proliferation and induces cell death in tumour cells lines from diverse histological background.

There is an increasing interest in HDAC6 as a therapeutic target. Although HDAC6 inhibitors including tubacin and tubastatin A (Haggarty et al, 2003; Butler et al, 2010) have been reported, the lack of inhibitors with favourable PK profile has stilled progress within the field. More recently, a new HDAC6 inhibitor ACY-1215 was reported as an inhibitor of multiple myeloma cell growth when combined with bortezomib (Santo et al, 2012). The chemical structure of ACY-1215 is not available for comparison and the effect of ACY-1215 on solid tumours was not evaluated in that study. We report the mechanism of action and growth inhibitory properties of a new HDACI, C1A in a solid tumour model. HCT-116 xenograft model was chosen because it represented the highest HDAC6 expression, has intermediate sensitivity to C1A (thus, data are not biased to high sensitivity tumours only) and it has been used extensively as a model for other HDACIs, including genomic and imaging studies (Leyton et al, 2006a; Witter et al, 2008; LaBonte et al, 2009). Furthermore, there is a need to develop therapies for colorectal cancer, which accounts for 10–15% of all cancers and is the second leading cause of cancer deaths in western countries. Traditionally used for nearly 50 years, 5-fluouracil (5-FU) has led to a small benefit in survival (Buyse et al, 1988). More recently, combination regimens comprising of 5-FU leuvorin irinotecan, cetuximab or oxiloplatin have been used of innovative clinical trials of novel agents. In this regard, C1A treatment reduced tumour [18F]FLT uptake by 1.7-fold at 48 h, suggesting that molecular imaging could provide value in future studies of this compound. [18F]FLT-PET uptake was previously shown to be altered only at later time points with broad-spectrum HDACI LAQ-824 (Leyton et al, 2006a). The imaging output was consistent with the observed reduction in Ki-67 immunostaining in excised tumour samples.

In keeping with the preferential HDAC6 activity, the drug showed restricted activity on gene expression in vivo with <0.065% of genes modulated during 24 h of treatment. Notably, however, although HDAC6 modulation was seen in vivo, we cannot wholly attribute the changes in gene expression seen after C1A treatment to an HDAC6 mechanism, as we cannot completely rule out accumulation of C1A into the tumour cells to levels high enough to also inhibit other HDAC classes. The lack of an increase in histone H3 acetylation together with the absence of typical gene expression profile characteristic of class I HDAC inhibition, for instance activation of CDKNIA that encodes p21WAF1/CIP1, suggest that the observed gene expression profile was not due to inhibition of class I HDAC. As per the in vitro studies, the observation that C1A induced pro-apoptotic genes including XAF1 and BAX requires further investigation.

HADC6 inhibitor with antitumour activity
In conclusion, C1A preferentially inhibits HDAC6 and modulates HDAC6 downstream targets, leading to growth inhibition of a diverse set of cancer cell lines. This property together with the favourable PKs and efficacy in vivo makes it a candidate for further pre-clinical and clinical development. Acetyl-

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