

Efficacy of MS-275, a selective inhibitor of class I histone deacetylases, in human colon cancer models

TOMKE UTE BRACKER¹, ANETTE SOMMER², IDUNA FICHTNER³,
HORTENSIA FAUS¹, BERNARD HAENDLER¹ and HOLGER HESS-STUMPP¹

¹Global Drug Discovery, Therapeutic Research Group Oncology, and ²Global Drug Discovery, Target Discovery, Bayer Schering Pharma AG, 178 Müllerstr., 13353 Berlin; ³Max-Delbrück Center for Molecular Medicine, 10 Robert-Rössle-Str., 13125 Berlin-Buch, Germany

Received April 1, 2009; Accepted June 11, 2009

DOI: 10.3892/ijo_00000406

Abstract. N-(2-aminophenyl)-4-[N-(pyridine-3-yl-methoxy-carbonyl) aminomethyl] benzamide (MS-275) is a second generation histone deacetylase (HDAC) inhibitor with significant anti-tumor efficacy currently in clinical development. We investigated the effect of MS-275 treatment on various colon cancer cell lines, as well as on mouse xenograft models derived from human colorectal cancer. MS-275 exerted strong anti-proliferative effects in five cell lines and increased the acetylation of histones 3 and 4. *In vivo* testing of the compound in eight different models of human colon cancer derived from primary colorectal cancers or from established cell lines revealed that five models were responders, two non-responders and one an anti-responder. Gene expression profiles were determined in order to identify genes and pathways differentially regulated upon MS-275 treatment in responder versus non-responder models. Principle component analysis revealed a correlation of the anti-tumor efficacy with the sub-clustering of the MS-275 treatment groups in 7 out of 8 models. Although the overall gene expression pattern was rather unique for each individual model, 129 genes were significantly up- and 58 genes significantly down-regulated in at least 2 out of 5 responder models in response to MS-275 treatment. We identified potential biomarkers for response to MS-275, such as PRA1, MYADM and PALM2-AKAP2 which were up-regulated in all responder models and down-regulated or unchanged in all non-responder models. Our results provide a starting point for the development of clinically relevant biomarkers for predicting a response to MS-275 and the understanding of the mode of action of this HDAC inhibitor.

Introduction

In recent years it has become clear that deregulation of epigenetic processes plays a major role in tumorigenesis (1-5). The term epigenetics relates to changes in gene expression elicited by modifications of histone marks such as acetylation or methylation as well as of DNA methylation, all of which do not alter the DNA sequence (6-11). These modifications may be transmitted over generations or be of more transient nature.

Local modification of histone acetylation is an important factor regulating the access of transcription factors to DNA (5,7,10). This is controlled by histone acetyltransferases and histone deacetylases (HDACs), and plays an important role for normal cellular functions including regulation of cell cycle progression, differentiation and apoptosis (12-14). The abnormal activity of these key modulators of chromatin structure may be associated with the development of a malignant phenotype. Indeed, overexpression of HDACs as well as aberrant recruitment to promoters leading to condensation of chromatin and to silencing of tumor suppressor genes has been reported in several cancer types (12-15).

The inhibition of HDAC activity results in the hyperacetylation of the tails of histone 3 (H3) and histone 4 (H4). This facilitates the relaxation of the chromatin structure and allows the re-expression of silenced genes, including p21 (16-21). In addition, several genes are repressed following HDAC inhibition and, intriguingly, the number of down-regulated genes may be as high as that of up-regulated ones (22). Generally, pro-apoptotic and anti-proliferative genes show increased expression after HDAC inhibitor treatment whereas anti-apoptotic and pro-growth genes are down-regulated (23-25). Furthermore, direct acetylation and regulation of transcription factors such as p53, STAT1 or steroid receptors leading to modifications in gene expression patterns also account for some of the effects of HDAC inhibitors (26,27). A third mechanism of action not directly linked to gene regulation has been evidenced. It involves the direct acetylation of non-transcriptional targets such as HSP90, tubulin or Ku70 (26).

HDAC inhibitors represent a structurally diverse group of compounds. Most, like suberoylanilide hydroxamic acid

Correspondence to: Dr Bernard Haendler, TRG Oncology, Bayer Schering Pharma AG, 178 Müllerstr., D-13353 Berlin, Germany
E-mail: bernard.haendler@bayerhealthcare.com

Key words: colon cancer, chromatin, gene expression, histone deacetylase inhibitor, biomarker

(SAHA) and trichostatin A (TSA) inhibit the activity of many if not all HDACs (28-34). N-(2-aminophenyl)-4-[N-(pyridine-3-yl-methoxy-carbonyl) aminomethyl] benzamide (MS-275) has a much more restricted profile (28,35-37). Several studies indicate that MS-275 is a selective inhibitor of HDAC1 and HDAC2, with IC_{50} values in the sub-micromolar range (28,31,32). The compound shows significant anti-tumor efficacy *in vitro* and *in vivo* and is currently in phase I/II clinical trials (35).

In the current study, we investigated the efficacy of MS-275 in human colon cancer models *in vitro* and *in vivo*. A large number of colorectal cancers show, in addition to genetic alterations such as p53 and β -catenin mutations, epigenetic abnormalities such as changes in DNA methylation and in histone modification pattern (38). Also, overexpression of HDAC2 has frequently been observed in colon cancers (39). We first determined the effects of MS-275 on the proliferation of colon cancer cell lines and analyzed pharmacodynamic markers. We then treated tumors grown as xenografts from eight different human colon cancer models with MS-275 and determined gene expression profiles. Since not all models showed a significant tumor inhibition upon treatment, the comparison of these expression profiles may represent the basis to learn more about the mode of action of MS-275 and to find markers that may help to distinguish between responders and non-responders to treatment.

Material and methods

Cell culture and reagents. Human colon carcinoma cell lines Colo205, HCT116, HT29, LoVo and SW480 were purchased from the American Type Culture Collection (Bethesda, MD, USA) or the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and cultured according to the guidelines. For immunoblot analysis, cells were treated with 0.3 or 3 μ M MS-275 (Bayer Schering Pharma AG, Berlin, Germany) for 24, 48 or 72 h. Cells were rinsed with PBS and centrifuged at 1200 x g for 5 min. Total protein extracts were prepared using the M-PER reagent (Perbio, Bonn, Germany) and protein concentrations were determined with the BCA-Assay (Perbio).

SDS-PAGE and immunoblotting. For SDS-polyacrylamide gel electrophoresis (PAGE), total protein extracts were prepared in sample buffer (4 x LDS sample buffer, 10 x reducing agent), heated at 95°C for 5 min and loaded onto 4-12% NuPAGE Bis-Tris gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (all accessories and buffers for PAGE were purchased from Invitrogen, San Diego, CA, USA) and blocked in 5% skimmed milk in TBS containing 0.1% Tween-20 (TBST) for 30 min. The following primary antibodies were diluted in 5% skimmed milk in TBST: anti-p21 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-acetylated histone 3 and histone 4 (1:2000 and 1:20000, respectively; Upstate, Chemicon, Hampshire, UK); anti-HDAC2 (1:2000; Abcam, Cambridge, UK); anti-GAPDH (1:5000; Zytomed Systems, Berlin, Germany), and incubated overnight at 4°C. Membranes were washed three times with TBST and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit

antibodies (1:10000 in 5% skimmed milk in TBST, Dianova, Hamburg, Germany) for 45 min at room temperature. Before chemiluminescence detection (ECL plus, GE Healthcare, Munich, Germany) membranes were washed three times with TBST.

Proliferation assay. After trypsinization, cells were seeded in 96-well plates at 10,000-30,000 cells per well, depending on growth properties, and allowed to adhere overnight. MS-275, dissolved in DMSO and diluted in medium, was added at different concentrations ranging from 0.001 to 10 μ M. After 72 h the medium was replaced with 30% Alamar Blue solution (diluted in medium; Invitrogen) and the cells were incubated for 4 h at 37°C (80% humidity, 95/5% air/CO₂). Inhibition of proliferation was detected colorimetrically by measuring the excitation at 590 nm and plotting it against MS-275 concentration. The IC_{50} values were calculated using these graphs.

Gene expression knock-down experiments. For small interfering RNA (siRNA) silencing of HDAC2, a predesigned siRNA pool and the non-targeting siCONTROL siRNA pool was used (Dharmacon, Perbio). HCT116 cells were seeded at a concentration of 1000 cells per well in a 96-well plate and transfected with 20 nM siRNA using the HiPerFect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. Twenty-four hours after transfection, different concentrations of MS-275, ranging from 0.001 to 10 μ M, were added to the cells. Proliferation was assessed with the Alamar Blue assay after 72 h of MS-275 treatment. For validation of HDAC2 knock-down efficiency and viability of the cells, 10⁵ cells were seeded in a 24-well plate and transfected with 20 nM siRNA. RNA and proteins were isolated from the same well using the AllPrep RNA/Protein Kit (Qiagen) according to the standard protocol. HDAC2 knock-down efficiency was measured by quantitative real-time polymerase chain reaction (PCR) and immunoblotting.

Animal experiments. For tumor xenograft experiments, 6-week-old female nude mice (NMRI-nu/nu) were utilized. HCT116 cells (1.5x10⁶), HT29 cells (1x10⁶) and LoVo cells (1.5x10⁶) were mixed with matrigel (BD Biosciences, Heidelberg, Germany) and injected subcutaneously into one flank. Human colon carcinoma tissue was obtained from primary tumors (Co5854, Co5776 and Co5676) or liver metastases of colon cancer (Co5841 and Co6044). Tumor fragments (2-3 mm³) were transplanted onto the flanks of anaesthetized nude mice. Primary xenograft models were established at the Max-Delbrück-Center for Molecular Medicine, Berlin, Germany (40). After the tumors became palpable, MS-275 treatment was initiated. The mice received a daily oral dose of 0, 10 or 40 mg/kg MS-275. Irinotecan (Campto[®], Pfizer, Karlsruhe, Germany) was used as a positive control substance and given intraperitoneally at a daily dose of 15 mg/kg. The diameter of the tumors was measured twice weekly with a caliper and tumor volumes were calculated by the formula: (width² x length) x 0.5. The median values of treated versus control (T/C) values of the groups were used to evaluate the therapeutic efficacy.

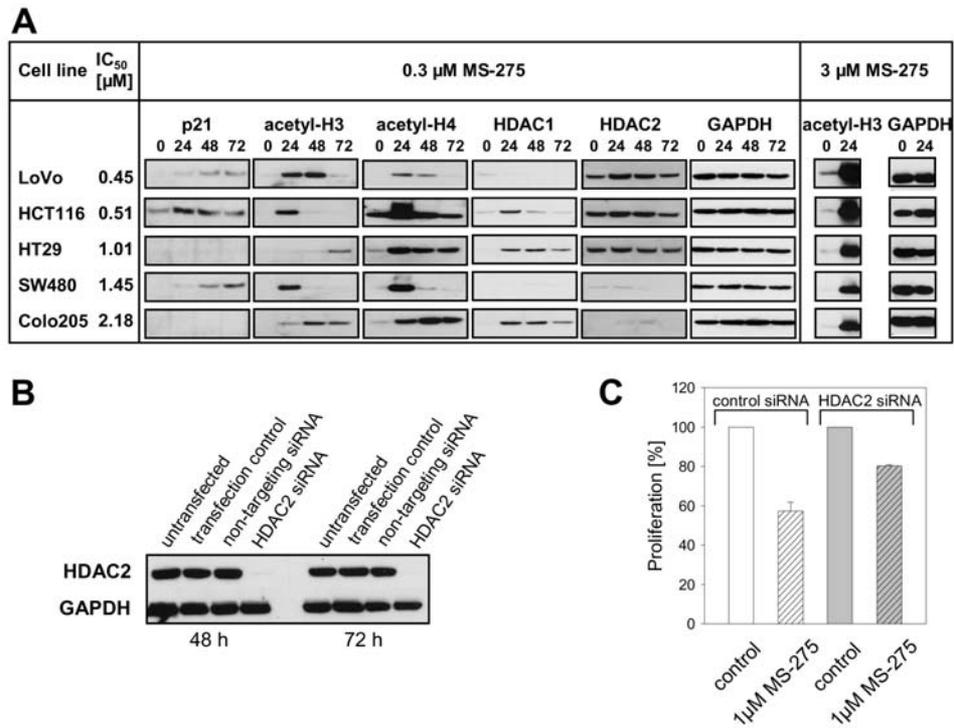


Figure 1. Analysis of MS-275 pharmacodynamic markers in colon cancer cell lines. (A) Immunoblotting analysis of p21 expression, of H3 and H4 acetylation, and of HDAC1 and HDAC2 levels after MS-275 treatment for the indicated timepoints (in hours). GAPDH levels were determined as controls. (B) Evaluation of HDAC2 protein down-regulation after siRNA treatment of HCT-116 cells for the indicated timepoints. (C) Impact of HDAC2 levels on the anti-proliferative effects of MS-275 on HCT116 cells.

RNA extraction. Tumor tissue was taken from sacrificed animals, snap-frozen and stored at -80°C until use. Total RNA was extracted using the RNeasy mini kit (Qiagen) according to the manufacturer's recommendations including a DNase I (Qiagen) step to digest genomic DNA. The quality of total RNA was checked for integrity using the RNA LabChips on the Agilent Bioanalyzer 2100 (Agilent Technologies Inc., Palo Alto, CA, USA) and the concentration was determined on a Nanodrop spectrophotometer (Peqlab, Erlangen, Germany).

In vitro RNA transcription and hybridization to Affymetrix GeneChips. The One-Cycle Eukaryotic Target Labeling Kit (Affymetrix Inc., Santa Clara, CA, USA) was used according to the manufacturer's instructions. Briefly, $2\ \mu\text{g}$ of high quality total RNA were reverse-transcribed using a T7 tagged oligo-dT primer for the first-strand cDNA synthesis reaction. After RNase H-mediated second-strand cDNA synthesis, the double-stranded cDNA was purified and served as template for the subsequent *in vitro* transcription reaction which generates biotin-labeled complementary RNA (cRNA). The biotinylated cRNA was then cleaned up, fragmented and hybridized to GeneChip HGU133Plus2.0 expression arrays (Affymetrix), which contain 54675 probe sets. The GeneChips were washed and stained with streptavidin-phycoerythrin on a GeneChip Fluidics Station 450 (Affymetrix). After washing, the arrays were scanned on an Affymetrix Gene-Chip 3000 scanner with autoloader and barcode reader. A total of 169 HGU133Plus2.0 arrays were processed.

Data analysis. The quality of the hybridized arrays was analyzed with the Expressionist Pro 4.0 Refiner software

(Genedata, Basel, CH). The following analyses were performed, based on raw intensities of individual oligonucleotide features (probes): the experiments were grouped according to similarity and potential outlier experiments were removed (or selected for re-hybridization and/or re-fragmentation), the quality of a particular experiment was compared with a virtual reference experiment, which was computed as average of all feature intensities of all arrays in that group. Moreover, defects on the arrays were masked. Here, for each array, the spatial signal distribution was compared with the reference experiment of the experiment group it belonged to. Regions with sharp boundaries which had consistently higher or lower feature intensities compared to the reference experiment were flagged as defects and excluded from further analysis. In addition, a signal correction (distortion and gradient) was performed, the control gene statistics were calculated, and an overall classification of the quality of the experiments was provided.

The probe intensities on each array were summarized with the MAS5.0 summarization algorithm and the refined and summarized data were loaded into the CoBi database (Genedata). The analysis of the probeset-specific signal intensities was performed with the Expressionist Pro 4.0 Analyst software (Genedata). The data set was median normalized. Pathway analyses were performed with the GeneGo Metacore database and software tools.

Quantitative real-time RT-PCR. Aliquots of the total RNA from all tumors belonging to one treatment group were pooled and reverse-transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) containing $3\ \mu\text{g}$ RNA, $5\ \text{ng}/\mu\text{l}$ random hexamer primer and

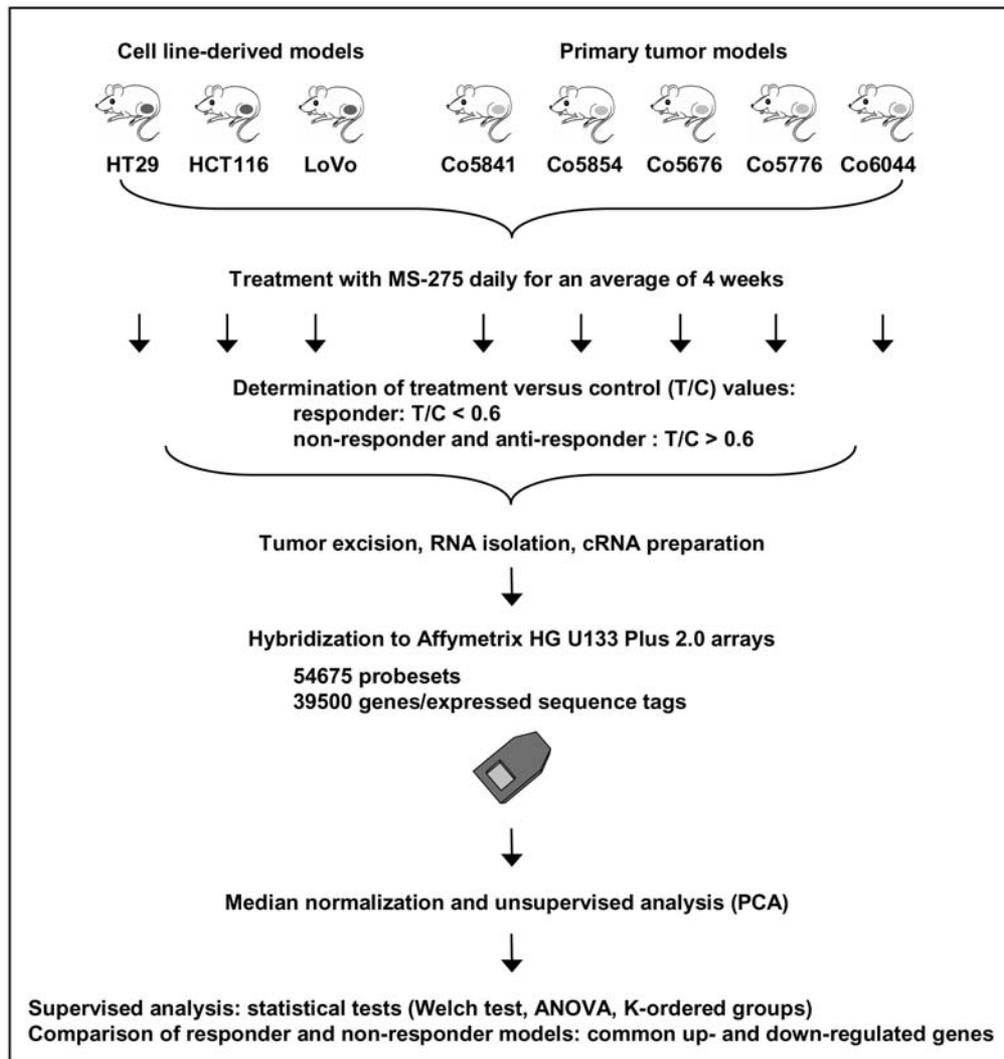


Figure 2. Experimental workflow for analysis of human colon cancer xenograft models. Eight different models were used for treatment with MS-275. The mice (N=8 per treatment group) were given vehicle control, 10 or 40 mg/kg of MS-275. RNA from individual tumors was isolated, cRNA was synthesized and hybridized to Affymetrix HGU133Plus2.0 arrays. The normalized dataset was subjected to unsupervised and supervised analyses with the Expressionist Pro 4.0 Analyst software.

1 mM deoxynucleotide triphosphate mix in a final volume of 20 μ l. After an initial denaturation step at 65°C for 5 min, the reverse transcriptase reaction was carried out at 25°C for 10 min, 50°C for 50 min and 85°C for 5 min. Real-time PCR was carried out using predesigned TaqMan assays and TaqMan Fast Universal Master mix on an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems Applera Deutschland GmbH, Darmstadt, Germany). Three genes were selected for quantitative real-time PCR analysis: prenylated rab acceptor 1 (PRA1, assay ID Hs00197506), myeloid-associated differentiation marker (MYADM, assay ID Hs00414763_m1) and paralemmin 2-A kinase anchor protein 2 (PALM2-AKAP2, assay ID HS00364914_m1). The levels of 18S rRNA transcripts (assay reference 4319413E) were determined as control. The real-time PCR reaction was performed in 96-well plates and contained 100 ng cDNA, 12.5 μ l 2 x TaqMan Fast Universal Master mix, 1.25 μ l TaqMan Assay Mix and 1.25 μ l 18S rRNA primers/probe mix in a 25 μ l reaction mix. After an initial denaturation step at 95°C for 20 sec, 40 cycles consisting of denaturation at

95°C for 3 sec and annealing at 60°C for 30 sec were carried out. Triplicate values were determined for each sample.

The relative expression of each gene was determined on the basis of the comparative threshold cycle method ($\Delta\Delta$ CT method). A standard curve was generated with 18S rRNA in every real-time PCR run. Each CT value was normalized for the CT value of the 18S rRNA gene (Δ CT). Then, the normalized gene expression value of the calibrator (i.e., Δ CT of the vehicle control group) was subtracted from the normalized target gene expression (i.e., Δ CT of the MS-275 treatment groups) to obtain the $\Delta\Delta$ CT value and the fold change of the gene in the treatment groups relative to the untreated control tumors was calculated.

Results

In vitro efficacy of MS-275. We assessed the anti-proliferative effects of MS-275 on the five established colon cancer cell lines LoVo, HCT116, HT29, SW480 and Colo205 by determining cell numbers after a 3-day treatment. MS-275

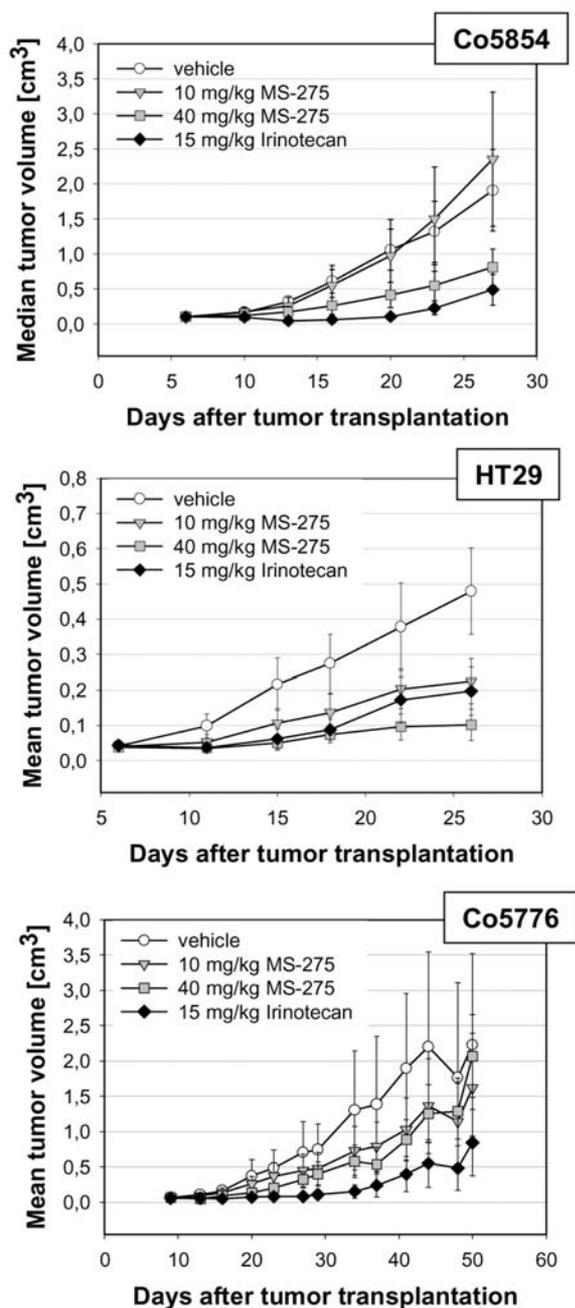


Figure 3. Efficacy of MS-275 on tumor growth in the responder models Co5854 and HT29, and in the non-responder model Co5776. Tumor volume was determined at the indicated timepoints. Irinotecan treatment was used as comparison.

inhibited all cell lines with IC_{50} values varying from 0.45 to 2.18 μ M (Fig. 1A). The treatment led to arrest in the G_0/G_1 phase and apoptosis (data not shown). We then tested the induction of the cyclin-dependent kinase inhibitor p21 which is induced upon treatment by several HDAC inhibitors and reflects the anti-proliferative effects of this compound class. We found that at a sub- IC_{50} concentration (0.3 μ M), MS-275 induced p21 expression in LoVo, HCT116 and SW480 cells, whereas no such effect was detectable in HT29 and Colo205 cells (Fig. 1A). A 10-fold higher concentration of MS-275 induced p21 expression in Colo205, but not in HT29 cells (not shown). We then examined changes in histone acetylation.

Stimulation of H3 acetylation was observed after 24-48 h in all cell lines, except in HT29 cells where it was observed only after 72 h of treatment. The amount of acetylated H3 was further increased when a higher MS-275 concentration (3 μ M) was used. An increase in H4 hyperacetylation was observed in all cell lines after 24 h of MS-275 treatment, albeit to different extents.

HDAC1 and HDAC2 are the main targets of MS-275 (28,35-37). We therefore used immunoblotting to look at their expression levels in the different colon cancer cell lines (Fig. 1A). For HDAC1, very low basal levels were found. An induction was initially observed upon MS-275 treatment in HCT116, HT29 and Colo205 cells, followed by a decrease after 72 h. LoVo and SW480 cells showed almost no expression of HDAC1. Thus, the HDAC1 protein levels did not correlate with the anti-proliferative activity of MS-275. Concerning HDAC2, we found that LoVo, HCT116 and HT29 cells strongly expressed it. SW480 and Colo205 had no detectable levels which were very slightly stimulated after MS-275 treatment. Thus, a higher HDAC2 expression level correlated with a high potency of MS-275.

In order to determine whether this finding had a functional relevance for MS-275 efficacy, we performed RNAi knock-down studies and subsequent proliferation assays in HCT116 cells. The HDAC2 mRNA level was dramatically reduced after siRNA transfection (not shown) and HDAC2 protein was no longer detectable, as assessed by immunoblotting (Fig. 1B). The down-regulation of HDAC2 was specific, as no reduction of HDAC1 mRNA or protein levels was seen (not shown). HDAC2 knock-down reduced the efficacy of MS-275 in proliferation assays. Only 20% growth inhibition was observed 72 h after 1 μ M MS-275 treatment in HCT-116 cells transfected with HDAC2-specific siRNAs compared to more than 40% in the control transfected cells (Fig. 1C). The respective IC_{50} values were 1.25 and 0.89 μ M. Thus, knock-down of HDAC2 partly abrogated the anti-proliferative effect of MS-275, suggesting that this isoform was the relevant molecular target.

In vivo efficacy of MS-275. In order to better evaluate the therapeutic efficacy of MS-275 in colon cancer, different primary tumor models (Co5854, Co5676 and Co5776), two metastasis models (Co5841 and Co6044), as well as three xenograft models derived from the cell lines that responded best to MS-275 treatment (HCT116, HT29 and LoVo) were tested (Fig. 2). Tumor-bearing mice were treated with 0, 10 or 40 mg/kg MS-275 once daily, and the tumor volumes were measured twice weekly. The growth curves observed for three tumors (Co5854, HT29 and Co5776) after MS-275 treatment are depicted, in comparison to irinotecan treatment (Fig. 3). The anti-tumor efficacy of MS-275 given daily at a 40 mg/kg concentration to treat the different colon cancer models is shown in Table I. The growth of 5 out of 8 models was significantly inhibited, as the tumor volume ratio between treated and control mice (T/C) was below 0.6 (i.e., 40% tumor growth inhibition). Two models were classified as non-responders with T/C values of 0.7 and 1.22 (Co5841 and Co5776, respectively). The LoVo model grew faster than the control following MS-275 treatment (T/C value of 2.04) leading to its classification as an anti-responder. Thus, this

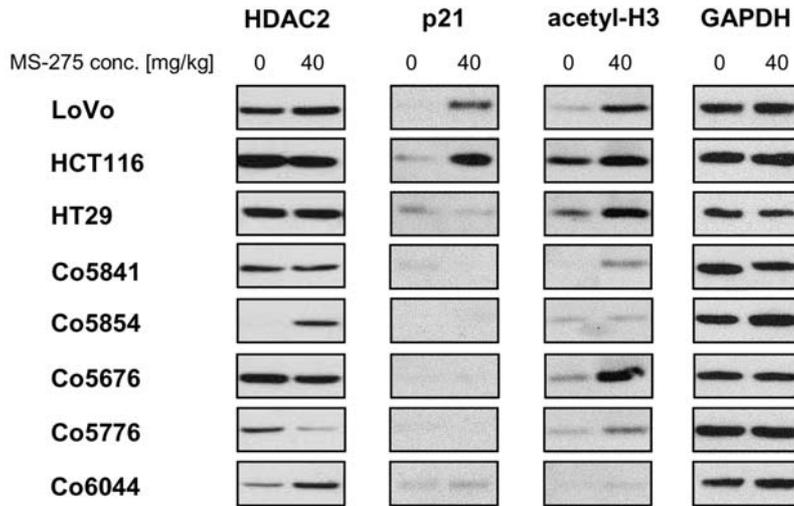


Figure 4. Analysis of pharmacodynamic markers *in vivo*. Tumors were treated with 40 mg/kg MS-275 and analyzed by immunoblotting for their levels of HDAC2, p21 and acetylated H3 at the end of the study. GAPDH levels were determined as controls.

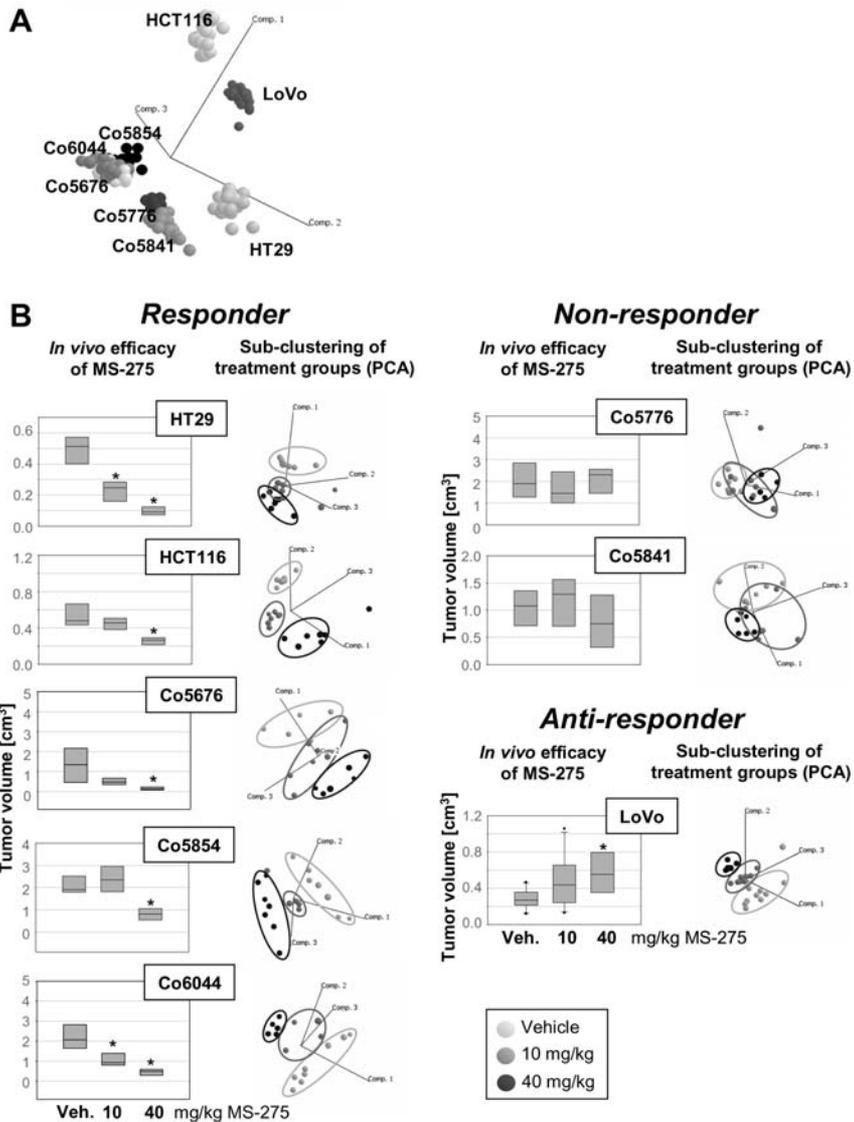


Figure 5. PCA of all models and correlation between MS-275 efficacy and clustering. (A) PCA clustering of all models. (B) PCA of each individual model indicates sub-clustering for treatment groups and correlation to response to MS-275 for responder and anti-responder models. The box plots show the tumor volume at the end of the experiment. The asterisk indicates a statistically significant change in tumor volume of treated animals compared to untreated ones ($p < 0.05$).

Table I. *In vivo* anti-tumor efficacy of MS-275 in experimental human colon carcinoma models.^a

Model	Origin	Duration of experiment (days)	Median volume of control tumors (cm ³)	Median volume of MS-275 treated tumors (cm ³)	T/C	Classification
HT29	Cell line	26	0.51	0.09	0.18	Responder
HCT116	Cell line	21	0.48	0.26	0.54	Responder
LoVo	Cell line	58	0.27	0.55	2.04	Anti-responder
Co5676	Tumor	49	1.35	0.12	0.09	Responder
Co5776	Tumor	50	1.90	2.31	1.22	Non-responder
Co5841 ^b	Tumor	46	1.08	0.76	0.70	Non-responder
Co5854	Tumor	27	1.91	0.82	0.43	Responder
Co6044 ^b	Tumor	29	2.07	0.50	0.24	Responder

^aTumor models were transplanted into nude mice and treated daily with 40 mg/kg MS-275. ^bTumors originating from secondary liver metastatic deposits.

study revealed that some models were responders and others non-responders or even anti-responders to MS-275 treatment.

Immunoblotting experiments were then performed to determine the effects of MS-275 on HDAC2 and p21 protein levels, and on H3 acetylation (Fig. 4). HDAC2 expression was detected in most untreated tumors (with the exception of Co5854) and in all treated tumors. Both up- and down-regulation were observed following MS-275 treatment. Concerning p21, strong re-expression was seen only in treated LoVo and HCT116 cells tumors. Hardly any signal was detected in the other tumor models. The induction of p21 did not correlate with the response to MS-275 as LoVo was an anti-responder and HCT116 a responder model. Concerning H3 acetylation, an increase was observed in most cases after MS-275 treatment, with the exception of Co5854 and Co6044.

Gene expression profiling - unsupervised analysis. RNA isolated from the xenograft models was subjected to global gene expression profiling in order to identify genes that were regulated by MS-275 and genes selectively regulated in responder versus non-responder tumor models (Fig. 2). Total RNA from each individual tumor was isolated, the corresponding cRNA synthesized and hybridized to a total of 169 Affymetrix HGU133Plus2.0 GeneChips. After median normalization the individual arrays were subjected to principle component analysis (PCA) to remove outliers at the expression level. Co-clustering of samples derived from each established cell line or from each colorectal cancer was visible, regardless whether the mice were treated with MS-275 or not (Fig. 5A). The three primary tumor responder models Co5841, Co5676 and Co6044 showed similar gene expression patterns in the PCA and differed from the two primary non-responder models Co5841 and Co5776. Consequently, among primary tumors, the PCA distinguished between responders and non-responders (Fig. 5A). Altogether these results indicate that the overall gene expression patterns differed more among the models than did the expression changes induced by MS-275 treatment for each individual model.

Next, we asked whether response to MS-275 correlated with a sub-clustering of treatment groups and therefore

performed PCA on each individual model (Fig. 5B). For all responder models, we observed a sub-clustering that distinguished the non-treated from the two MS-275-treated tumor groups at the gene expression level. Conversely, such a clustering was not observed in the two primary tumor models that did not respond to MS-275. The situation was different for the anti-responder model LoVo for which a dose-dependent increase in tumor growth was seen. Here, sub-clustering of the non-treated and of each treated group was observed.

Gene expression profiling - supervised analysis. Next, supervised analyses were performed to determine if MS-275 treatment led to uniform alterations of gene expression patterns. The following comparisons were performed for each model: 10 mg MS-275 versus control, 40 mg MS-275 versus control, 10 and 40 mg MS-275 versus control, and 10 versus 40 mg MS-275. For statistical analyses, we chose a Welch-test and selected regulated genes with a fold change >5 and a p-value <10⁻⁴. In addition, analysis of variation (ANOVA; p<10⁻⁵) and K-ordered groups tests (p<10⁻²) were carried out. The most significantly regulated genes in presence of 40 mg/kg MS-275 as identified with all three statistical tests are listed in Table II. This analysis showed that genes were regulated more significantly in cell line models than in primary models. Also, more genes were up- than down-regulated in responder models whereas in non-responder models, most genes were down-regulated. When looking at the most significantly regulated genes in each individual model, we observed rather heterogeneous changes. The responder models HT29 and Co5854 showed a strong up-regulation of genes involved in cell adhesion and cell-cell communication [for instance LGALS1 (18.8-fold) and NRCAM (90-fold)], whereas the non-responder models Co5776 and Co5841 showed a down-regulation of genes involved in cell adhesion [CLDN4 (1.6-fold) and TSPAN5 (2-fold)]. In HT29 cells, CEACAM6 and CEACAM7 were up-regulated 4- and 17.7-fold, respectively. MS-275 treatment of the HCT116 and Co5676 models led to down-regulation of cell cycle-promoting genes like TNFSF4 (20-fold), BTC

Table II. Most significantly regulated genes in colon carcinoma xenograft models in response to 40 mg/kg MS-275.^a

Model	Up-regulated			Down-regulated		
	Gene symbol	Probeset ID	Fold change	Gene symbol	Probeset ID	Fold change
HT29	LGALS1	201105_at	18.83	AKAP7	205771_s_at	0.05
	CEACAM7	206199_at	17.66	LGALS2	208450_at	0.10
	PTPRO	208121_s_at	16.30	MUC5AC	214385_s_at	0.17
HCT116	FAM49A	209683_at	34.51	TNFSF4	207426_s_at	0.05
	NGFRAP1L1	229963_at	18.44	KLRC1	206785_s_at	0.10
	FLJ35767	241367_at	10.32	KLRC3	207723_s_at	0.13
Co 5676	HLA-DQB1	212998_x_at	13.20	PAPSS2	203058_s_at	0.43
	RNF182	230720_at	8.14	KPNA2	211762_s_at	0.53
	BAMBI	203304_at	6.23	HIGD1A	221896_s_at	0.53
Co 5854	NRCAM	204105_s_at	89.52	AQP3	39248_at	0.26
	TNNT1	213201_s_at	35.30	MATN2	202350_s_at	0.30
	SCG5	203889_at	20.01	ST6GALNAC1	227725_at	0.35
Co 6044	TNNT1	213201_s_at	21.59	TCN1	205513_at	0.08
	TUBB2B	214023_x_at	14.25	KRT6B	213680_at	0.17
	NES	218678_at	9.25	CDKN1C	213348_at	0.20
LoVo	NES	218678_at	13.16	FOXL1	243409_at	0.20
	NSBP1	221606_s_at	12.20	MLPH	218211_s_at	0.20
	SCNN1A	203453_at	11.98	HHIP	237466_s_at	0.21
Co 5776	NUPL1	204435_at	1.58	SLC16A1	1557918_s_at	0.24
	RAP1GDS1	209444_at	1.52	P4HB	200654_at	0.57
	ORC4L	203351_s_at	1.51	CLDN4	201428_at	0.64
Co 5841	PSMB4	228204_at	1.78	GSN	214040_s_at	0.21
	DSG3	235075_at	1.55	RNF144	204040_at	0.47
	IQGAP3	229538_s_at	1.52	NETO2	218888_s_at	0.48

^aGenes were selected by the overlap of three different statistical tests [Welch-test ($p < 10^{-4}$), ANOVA ($p < 10^{-5}$) and K-ordered groups ($p < 10^{-2}$)], respectively.

(4.4-fold) and KPNA2 (1.9-fold). In the non- and anti-responder models Co5776, Co5841 and LoVo, genes involved in the regulation of transcription were up-regulated [NSBP1 (12.2-fold), GTF2H1 (1.4-fold) and EZH2 (1.4-fold)] or down-regulated [FOXL1 (5-fold) and GLIS3 (3.1-fold)]. Interestingly, gelsolin (GSN) was down-regulated 4.8-fold in the Co5841 model.

As we did not observe a strong overlap of regulated genes between the five responder models, we used less stringent criteria. We called for a significant change in the expression level in at least two of the three statistical tests. With this new setting we identified 116 up- and 43 down-regulated genes after MS-275 treatment that exhibited a dose-dependent regulation in at least 4 out of 5 responder models. Interestingly, 34 of these genes showed up-regulation in all responders and down-regulation or no change of regulation in at least 2 out of 3 non-responders, whereas 13 genes exhibited down-regulation in all responders and up-regulation or no change in at least two out of three non-responders (Table III). These genes may serve as biomarkers to predict the response to MS-275.

Confirmation of microarray data by quantitative real-time RT-PCR. The three most significantly regulated genes showing up-regulation in most responder models but not in non-responder models were those coding for PRA1, MYADM and PALM2-AKAP2 (Fig. 6A). Quantitative real-time PCR was performed to confirm these findings. Total RNA was extracted from each treatment group for each model. The normalized gene expression levels in treated tumors relative to the expression in untreated tumors are shown in Fig. 6B. The quantitative real-time PCR analysis confirmed the up-regulation of these three genes in at least four of the five responder models and the down-regulation or no change in at least 2 of the 3 non-responder models. However, in the LoVo model, the absence of up-regulation of PRA1 and MYADM could not be confirmed by quantitative real-time RT-PCR.

Pathway analysis. To gain a deeper insight into the mechanisms of action of MS-275, we performed a pathway analysis using the GeneGo Metacore database and software (Fig. 7). Genes regulated by MS-275 treatment as compared

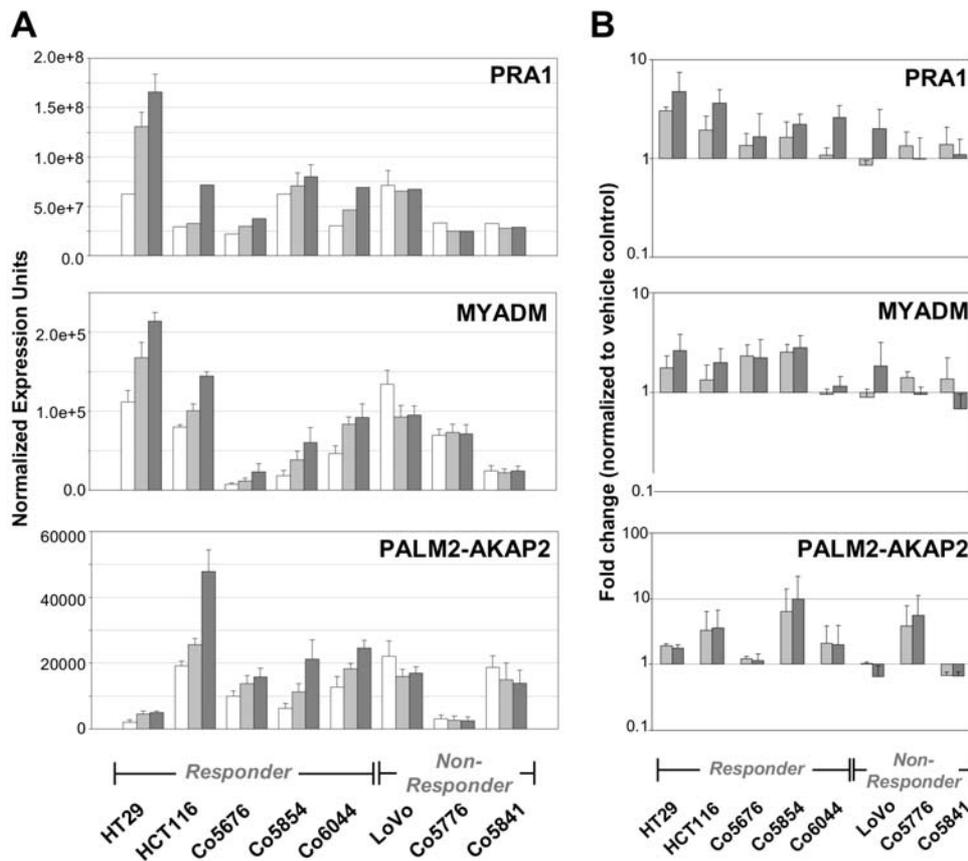


Figure 6. Examples of genes regulated upon MS-275 treatment. Genes were selected by the overlap of 2 out of 3 statistical tests [Welch-test ($p < 10^{-4}$), ANOVA ($p < 10^{-5}$) and K-ordered groups tests ($p < 10^{-2}$)]. (A) Normalized expression of three selected genes showing an up-regulation in responder but not in non-responder or anti-responder models. Normalized expression levels for control (white columns), 10 mg/kg MS-275 (light grey columns) and 40 mg/kg MS-275 (dark grey columns) are shown. (B) Confirmation of expression changes of selected genes by quantitative real-time PCR of pooled RNA from each tumor and treatment group. Fold changes normalized to vehicle control are shown in light grey columns (10 mg/kg MS-275) and dark grey columns (40 mg/kg MS-275). Mean values \pm SD of three independent real-time PCR runs are shown.

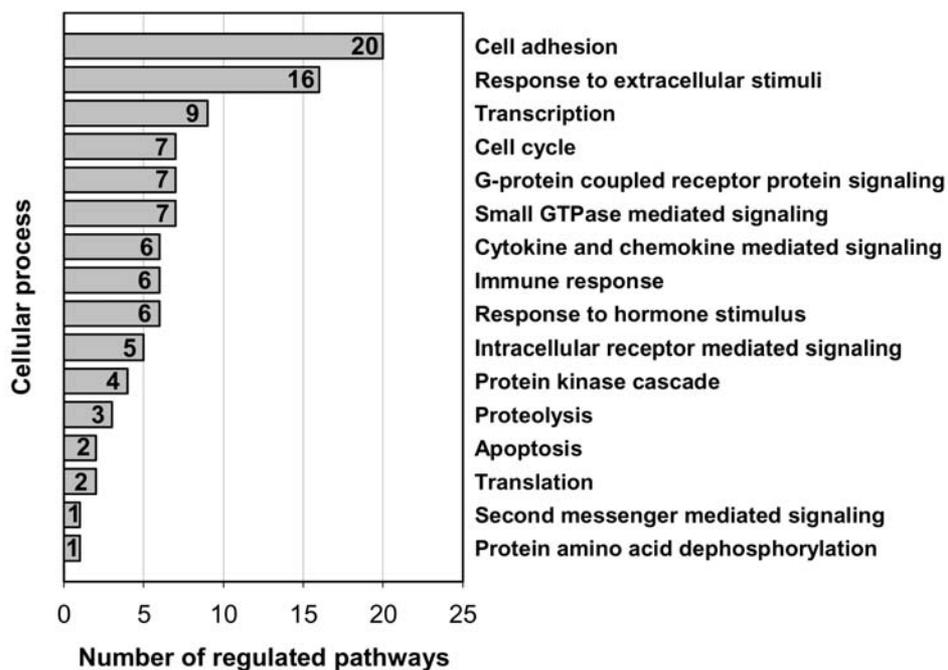


Figure 7. The pathways most significantly regulated after MS-275 treatment (40 mg/kg) were categorized using the GeneGO Metacore database according to their involvement in cellular processes. One pathway can be a member of more than one process.

Table III. Oppositely regulated genes in responder and non-responder models following treatment with 40 mg/kg MS-275.^a

A, Log2 ratio vehicle control versus 40 mg/kg MS-275											
Gene symbol	Probeset ID	Responders					Non-responders			Up in responders	Down or no change in non-responders
		HT29	HCT116	Co5676	Co5854	Co6044	LoVo	Co5776	Co5841		
PALM2-											
AKAP2	202760_s_at	2.4	2.5	1.6	3.4	1.9	0.8	0.8	0.7	5/5	3/3
PRA1	203136_at	2.7	2.4	1.7	1.3	2.3	0.9	0.8	0.9	5/5	3/3
OAT	201599_at	1.3	1.4	1.2	1.2	2.6	0.9	1.0	0.8	5/5	3/3
PRSS23	202458_at	1.0	2.4	2.0	1.6	1.3	0.4	0.8	0.8	5/5	3/3
C20orf3	206656_s_at	1.4	1.8	1.5	1.3	1.3	0.9	0.9	1.0	5/5	3/3
MYADM	225673_at	1.9	1.8	3.2	3.3	2.0	0.7	1.0	1.0	5/5	3/3
SH3KBP1	223082_at	1.8	1.2	1.6	1.1	1.5	0.8	0.9	1.0	5/5	3/3
DKFZP											
564O0823	225809_at	3.4	2.3	1.7	1.9	2.1	1.4	0.7	0.9	5/5	2/3
LITAF	200706_s_at	1.5	1.9	1.4	1.3	1.6	1.1	0.9	0.9	5/5	2/3
NSMCE1	224666_at	1.4	1.1	1.2	1.6	1.7	0.9	1.1	1.0	5/5	2/3

B, Log2 ratio vehicle control versus 40 mg/kg MS-275

B, Log2 ratio vehicle control versus 40 mg/kg MS-275											
Gene symbol	Probeset ID	Responders					Non-responders			Down in responders	Up or no change in non-responders
		HT29	HCT116	Co5676	Co5854	Co6044	LoVo	Co5776	Co5841		
BAZ1A	217986_s_at	0.6	0.6	0.8	0.9	1.0	1.2	1.1	1.1	5/5	3/3
LACTB2	222714_s_at	0.5	0.9	0.5	0.8	0.6	1.8	1.0	1.0	5/5	3/3
FLJ21345 fis	234219_at	0.7	0.9	0.4	1.0	0.4	1.5	1.0	1.0	5/5	3/3
BID	211725_s_at	0.6	0.8	0.6	0.6	0.6	0.8	1.1	1.1	5/5	2/3
C6orf51	225083_at	0.5	0.4	0.8	0.6	0.5	0.9	1.2	1.0	5/5	2/3
HMG2	208668_x_at	0.8	0.6	0.6	0.8	0.9	0.9	1.1	1.1	5/5	2/3
HSPC268	226780_s_at	0.6	0.6	0.5	0.5	0.7	0.9	1.0	1.1	5/5	2/3
LOC644246	230388_s_at	0.5	0.9	0.7	0.7	0.6	0.8	1.2	1.2	5/5	2/3
ADI1	217761_at	0.4	0.5	0.6	0.6	0.7	0.4	1.0	1.2	5/5	2/3
TRMT5	221952_x_at	0.7	0.5	0.5	0.8	0.8	0.9	1.1	1.1	5/5	2/3

^aThe genes most significantly up-regulated in responder models and down- or not regulated in non-responder models are shown in (A). The genes most significantly down-regulated in responder models and up- or not regulated in non-responder models are shown in (B). The ratio refers to the comparison of the 40 mg/kg and the vehicle control group. Genes were selected by the overlap of two of three different statistical tests (either Welch-test, $p < 10^{-4}$ or ANOVA, $p < 10^{-5}$ and K-ordered groups, $p < 10^{-2}$).

to the corresponding control groups were submitted to pathway analysis. Affected pathways were sorted according to the total number of affected genes and the significance of the regulation (p-value). The 100 most significantly regulated pathways were categorized according to the cellular processes they affected ($p < 0.017$). This revealed that the most perturbed cellular processes following MS-275 treatment were cell adhesion, response to extracellular stimuli and transcription.

Discussion

This study was devised to examine the anti-tumor effects of MS-275 in *in vitro* and *in vivo* models of human colorectal cancer. MS-275 is a potent HDAC inhibitor with selectivity for the class I family members (28,35-37). Treatment of five colon cancer cell lines with MS-275 at a sub IC_{50} concentration revealed that efficacy did not correlate with changes in previously described pharmacodynamic markers such as p21 expression or H3 and H4 acetylation. However, the expression

of HDAC2 in the cell lines correlated best with MS-275 efficacy *in vitro*. In line with this, HDAC2 knock-down resulted in a partial abrogation of the anti-proliferative potency of the compound. These results are consistent with previously published ones reporting that loss of HDAC2 expression and activity in sporadic cancers rendered them resistant to the HDAC inhibitor TSA (41). Transfection of wild-type HDAC2 into the HDAC2-deficient colon cell line RKO restored TSA sensitivity (41). We found that the five colon cancer cell lines, as well as the eight xenograft models expressed full-length HDAC2. This isoform is therefore likely to be responsible for at least some of the anti-proliferative effects we observed. On the other hand, the fact that nearly complete elimination of HDAC2 did not entirely obliterate the anti-proliferative effect of the compound strongly suggests that additional cellular targets might be involved in the anti-tumor effects of MS-275.

Predicting the efficacy of MS-275 *in vitro* can therefore not solely be based upon examination of a single marker.

Only the combined examination of the above-mentioned markers, and probably of even more, will allow the prediction of the efficacy of MS-275. Strong p21 induction as well as HDAC2 expression combined with strong and early H3 and H4 hyperacetylation, as seen in HCT116 cells, correlated with good response. The same holds true for the interpretation of these markers *in vivo*. We did not observe that HDAC inhibition led to H3 and H4 hyperacetylation and p21 induction of expression, the claimed hallmarks of HDAC inhibition, in all tested *in vivo* models of colon cancer. These changes can thus not be ascribed to every model of human cancer and every HDAC inhibitor. Our systematic analysis of different xenograft models as well as the *in vitro* analysis of five different colon cancer cell lines showed that there was a need for new, more sensitive and reliable biomarkers to predict the response and sensitivity of tumors to the treatment with MS-275.

We therefore performed microarray analyses of eight colon cancer xenograft models to learn more about the mode of action of MS-275 and to find markers that may help to distinguish between MS-275 responders and non-responders. The present study revealed a rather non-uniform response towards MS-275 treatment, which reflected the molecular heterogeneity of the xenograft models used. We did not see a correlation between the different types of xenograft models, either derived from established cell lines or colorectal cancer, and the response towards MS-275. We identified two responder cell lines (HT29 and HCT116) and one anti-responder (LoVo), two responder primary models (Co5854 and Co5676) and one non-responder (Co5776), as well as one metastasis model responder (Co6044) and one non-responder (Co5841). The gene expression profiles of these models were rather heterogeneous and the PCA revealed more differences among individual models than among treatment groups.

With the help of stringent statistical tests, we identified a number of potential biomarkers for MS-275. These markers were up-regulated dose-dependently upon MS-275 treatment in the five responder models and showed no induction in the three non-responder models. PRA1 is a ubiquitous protein which binds to prenylated Rab GTPases (42). Its overexpression impairs TCF/ β -catenin signaling, which plays a prominent role in colon cancer, possibly by limiting nuclear translocation of β -catenin (43). This was linked to ERK1/2 dephosphorylation. Re-expression of PRA1 upon MS-275 treatment may therefore lead to inhibition of the TCF/ β -catenin pathway by blockade of the nuclear translocation and thus prevention of the transcriptional stimulation of genes involved in tumor formation and proliferation. MYADM is associated with the differentiation of hematopoietic and acute promyelocytic leukemia cells (44,45). All-trans retinoic acid treatment induces expression of this differentiation marker in NB4 cells. The up-regulation of this gene upon MS-275 treatment may reflect the potential of the compound to induce differentiation in tumor cells and could serve as a differentiation and response biomarker for MS-275. Little is known about PALM2-AKAP2 (46). The corresponding mRNA is a naturally occurring cotranscribed product of the adjacent PALM2 and AKAP2 genes but the significance of this co-transcribed mRNA and the function of its putative protein product have not yet been determined.

A comparison of expression profiles of different HDAC inhibitors revealed a very small overlap of regulated genes,

pointing out the importance of specific studies for each HDAC inhibitor when it comes to evaluating and validating stratification biomarkers. We studied the effects of MS-275 in eight different systems and found the overlap between regulated genes to be rather small, suggesting that each model carried individual patterns of epigenetic marks that contribute to their differential gene expression and response to treatment.

Pathway analysis revealed that MS-275 strongly affected cell adhesion pathways. Among the 100 most significantly affected pathways, 20 were involved in the regulation of cell-cell adhesion, communication, motility, or tumor invasion. Several cell adhesion genes were up-regulated in MS-275 responder models but down-regulated in non-responder models. In the HT29 and HCT116 models many integrins and tetraspanins, such as β 1-integrin, CD9 or CD81, were up-regulated, whereas these genes were down-regulated in Co5841, Co5776 and LoVo. Besides that, E-cadherin, an important suppressor of epithelial tumor cell invasiveness and metastasis which is epigenetically silenced through promoter methylation in many carcinomas (47), was down-regulated in the non-responder models Co5841 and Co5776 upon MS-275 treatment. This implies that MS-275 did not induce the re-expression of E-cadherin, but rather promoted the hypermethylated status of the primary non-responder models. E-Cadherin as well as claudin 4 (CLDN4), which were down-regulated in Co5776, are decreased in diffuse type and poorly differentiated tumors (47,48). Dysfunction of these proteins may therefore play a role in disruption of cell-cell adhesion. In conclusion, MS-275 might increase the adhesive properties of tumor cells in responder models thus preventing the metastatic spread and the immune escape of the malignant cells, whereas in non-responder models most cell adhesion molecules were down-regulated.

Taken together, the different *in vitro* and *in vivo* effects of MS-275 as well as the expression changes induced in responder and non-responder models revealed the pleiotropic effects of this compound on different cellular pathways affecting tumors and their microenvironment.

Acknowledgements

The excellent contributions of our technical assistants Nicole Kelm, Bianca Timpner, Karola Henschel and Anja Klinner are gratefully acknowledged. We also thank all other internal and external colleagues who supported this project. This work was supported by EPITRON, an Integrated Project funded by the European Union under the 6th Framework Programme (LSHC-CT-2005-518417).

References

1. Oki Y and Issa JP: Review: recent clinical trials in epigenetic therapy. *Rev Recent Clin Trials* 1: 169-182, 2006.
2. Baylin SB and Ohm JE: Epigenetic gene silencing in cancer - a mechanism for early oncogenic pathway addiction? *Nat Rev Cancer* 6: 107-116, 2006.
3. Kubicek S, Schotta G, Lachner M, Sengupta R, Kohlmaier A, Perez-Burgos L, Linderson Y, Martens JH, O'Sullivan RJ, Fodor BD, Yonezawa M, Peters AH and Jenuwein T: The role of histone modifications in epigenetic transitions during normal and perturbed development. *Ernst Schering Res Found Workshop* 57: 1-27, 2006.
4. Esteller M: Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat Rev Genet* 8: 286-298, 2007.

5. Wang GG, Allis CD and Chi P: Chromatin remodeling and cancer, Part I: Covalent histone modifications. *Trends Mol Med* 13: 363-372, 2007.
6. Goldberg AD, Allis CD and Bernstein E: Epigenetics: a landscape takes shape. *Cell* 128: 635-638, 2007.
7. Kouzarides T: Chromatin modifications and their function. *Cell* 128: 693-705, 2007.
8. Esteller M: Epigenetic gene silencing in cancer: the DNA hypermethylome. *Hum Mol Genet* 16 (Spec No 1): R50-R59, 2007.
9. Agger K, Christensen J, Cloos PA and Helin K: The emerging functions of histone demethylases. *Curr Opin Genet Dev* 18: 159-168, 2008.
10. Yang XJ and Seto E: Lysine acetylation: codified crosstalk with other posttranslational modifications. *Mol Cell* 31: 449-461, 2008.
11. Abreu PA, Dellamora-Ortiz G, Leao-Ferreira LR, Gouveia M, Braggio E, Zalberg I, Santos DO, Bourguinhon S, Cabral LM, Rodrigues CR and Castro HC: DNA methylation: a promising target for the twenty-first century. *Expert Opin Ther Targets* 12: 1035-1047, 2008.
12. Glozak MA and Seto E: Histone deacetylases and cancer. *Oncogene* 26: 5420-5432, 2007.
13. Mottet D and Castronovo V: Histone deacetylases: target enzymes for cancer therapy. *Clin Exp Metastasis* 25: 183-189, 2008.
14. Johnstone RW: Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. *Nat Rev Drug Discov* 1: 287-299, 2002.
15. Pan LN, Lu J and Huang B: HDAC inhibitors: a potential new category of anti-tumor agents. *Cell Mol Immunol* 4: 337-343, 2007.
16. Wang LG, Liu XM, Fang Y, Dai W, Chiao FB, Puccio GM, Feng J, Liu D and Chiao JW: De-repression of the p21 promoter in prostate cancer cells by an isothiocyanate via inhibition of HDACs and c-Myc. *Int J Oncol* 33: 375-380, 2008.
17. Bruserud O, Stapnes C, Ersvaer E, Gjertsen BT and Rynningen A: Histone deacetylase inhibitors in cancer treatment: a review of the clinical toxicity and the modulation of gene expression in cancer cell. *Curr Pharm Biotechnol* 8: 388-400, 2007.
18. Perez-Balado C, Nebbioso A, Rodriguez-Grana P, Minichiello A, Miceli M, Altucci L and de Lera AR: Bispyridinium diones: histone deacetylase inhibitors with selective activities. *J Med Chem* 50: 2497-2505, 2007.
19. Huang BH, Laban M, Leung CH, Lee L, Lee CK, Salto-Tellez M, Raju GC and Hooi SC: Inhibition of histone deacetylase 2 increases apoptosis and p21Cip1/WAF1 expression, independent of histone deacetylase 1. *Cell Death Differ* 12: 395-404, 2005.
20. Ocker M and Schneider-Stock R: Histone deacetylase inhibitors: signalling towards p21cip1/waf1. *Int J Biochem Cell Biol* 39: 1367-1374, 2007.
21. Hoshino I, Matsubara H, Akutsu Y, Nishimori T, Yoneyama Y, Murakami K, Komatsu A, Sakata H, Matsushita K and Ochiai T: Gene expression profiling induced by histone deacetylase inhibitor, FK228, in human esophageal squamous cancer cells. *Oncol Rep* 18: 585-592, 2007.
22. Glaser KB, Staver MJ, Waring JF, Stender J, Ulrich RG and Davidsen SK: Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines. *Mol Cancer Ther* 2: 151-163, 2003.
23. Chiba T, Yokosuka O, Fukai K, Kojima H, Tada M, Arai M, Imazeki F and Saisho H: Cell growth inhibition and gene expression induced by the histone deacetylase inhibitor, trichostatin A, on human hepatoma cells. *Oncology* 66: 481-491, 2004.
24. Milutinovic S, D'Alessio AC, Detich N and Szyf M: Valproate induces widespread epigenetic reprogramming which involves demethylation of specific genes. *Carcinogenesis* 28: 560-571, 2007.
25. Tavares TS, Nanus D, Yang XJ and Gudas LJ: Gene microarray analysis of human renal cell carcinoma: the effects of HDAC inhibition and retinoid treatment. *Cancer Biol Ther* 7: 2008.
26. Glozak MA, Sengupta N, Zhang X and Seto E: Acetylation and deacetylation of non-histone proteins. *Gene* 363: 15-23, 2005.
27. Faus H and Haendler B: Post-translational modifications of steroid receptors. *Biomed Pharmacother* 60: 520-528, 2006.
28. Hu E, Dul E, Sung CM, Chen Z, Kirkpatrick R, Zhang GF, Johanson K, Liu R, Lago A, Hofmann G, Macarron R, De los Frailes M, Perez P, Krawiec J, Winkler J and Jaye M: Identification of novel isoform-selective inhibitors within class I histone deacetylases. *J Pharmacol Exp Ther* 307: 720-728, 2003.
29. Inoue S, Mai A, Dyer MJ and Cohen GM: Inhibition of histone deacetylase class I but not class II is critical for the sensitization of leukemic cells to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. *Cancer Res* 66: 6785-6792, 2006.
30. Moradei O, Vaisburg A and Martell RE: Histone deacetylase inhibitors in cancer therapy: new compounds and clinical update of benzamide-type agents. *Curr Top Med Chem* 8: 841-858, 2008.
31. Beckers T, Burkhardt C, Wieland H, Gimmnich P, Ciossek T, Maier T and Sanders K: Distinct pharmacological properties of second generation HDAC inhibitors with the benzamide or hydroxamate head group. *Int J Cancer* 121: 1138-1148, 2007.
32. Khan N, Jeffers M, Kumar S, Hackett C, Boldog F, Khramtsov N, Qian X, Mills E, Berghs SC, Carey N, Finn PW, Collins LS, Tumber A, Ritchie JW, Jensen PB, Lichenstein HS and Sehested M: Determination of the class and isoform selectivity of small-molecule histone deacetylase inhibitors. *Biochem J* 409: 581-589, 2008.
33. Xu WS, Parmigiani RB and Marks PA: Histone deacetylase inhibitors: molecular mechanisms of action. *Oncogene* 26: 5541-5552, 2007.
34. Bolden JE, Peart MJ and Johnstone RW: Anticancer activities of histone deacetylase inhibitors. *Nat Rev Drug Discov* 5: 769-784, 2006.
35. Hess-Stumpp H, Bracker TU, Henderson D and Politz O: MS-275, a potent orally available inhibitor of histone deacetylases-the development of an anticancer agent. *Int J Biochem Cell Biol* 39: 1388-1405, 2007.
36. Rosato RR, Almenara JA and Grant S: The histone deacetylase inhibitor MS-275 promotes differentiation or apoptosis in human leukemia cells through a process regulated by generation of reactive oxygen species and induction of p21CIP1/WAF1. *Cancer Res* 63: 3637-3645, 2003.
37. Saito A, Yamashita T, Mariko Y, Nosaka Y, Tsuchiya K, Ando T, Suzuki T, Tsuruo T and Nakanishi O: A synthetic inhibitor of histone deacetylase, MS-27-275, with marked in vivo antitumor activity against human tumors. *Proc Natl Acad Sci USA* 96: 4592-4597, 1999.
38. Kondo Y and Issa JP: Epigenetic changes in colorectal cancer. *Cancer Metastasis Rev* 23: 29-39, 2004.
39. Zhu P, Martin E, Mengwasser J, Schlag P, Janssen KP and Gottlicher M: Induction of HDAC2 expression upon loss of APC in colorectal tumorigenesis. *Cancer Cell* 5: 455-463, 2004.
40. Fichtner I, Slisow W, Gill J, Becker M, Elbe B, Hillebrand T and Bibby M: Anticancer drug response and expression of molecular markers in early-passage xenotransplanted colon carcinomas. *Eur J Cancer* 40: 298-307, 2004.
41. Ropero S, Fraga MF, Ballestar E, Hamelin R, Yamamoto H, Boix-Chornet M, Caballero R, Alaminos M, Setien F, Paz MF, Herranz M, Palacios J, Arango D, Orntoft TF, Aaltonen LA, Schwartz S, Jr and Esteller M: A truncating mutation of HDAC2 in human cancers confers resistance to histone deacetylase inhibition. *Nat Genet* 38: 566-569, 2006.
42. Gougeon PY, Prosser DC, Da-Silva LF and Ngsee JK: Disruption of Golgi morphology and trafficking in cells expressing mutant prenylated rab acceptor-1. *J Biol Chem* 277: 36408-36414, 2002.
43. Kim JT, Cho MY, Choi SC, Kim JW, Chae SK, Yoon DY, Kim JW and Lim JS: Prenylated Rab acceptor 1 (PRA1) inhibits TCF/beta-catenin signaling by binding to beta-catenin. *Biochem Biophys Res Commun* 349: 200-208, 2006.
44. Cui W, Yu L, He H, Chu Y, Gao J, Wan B, Tang L and Zhao S: Cloning of human myeloid-associated differentiation marker (MYADM) gene whose expression was up-regulated in NB4 cells induced by all-trans retinoic acid. *Mol Biol Rep* 28: 123-138, 2001.
45. Wang Q, Li N, Wang X, Shen J, Hong X, Yu H, Zhang Y, Wan T, Zhang L, Wang J and Cao X: Membrane protein hMYADM preferentially expressed in myeloid cells is up-regulated during differentiation of stem cells and myeloid leukemia cells. *Life Sci* 80: 420-429, 2007.
46. Hu B, Copeland NG, Gilbert DJ, Jenkins NA and Kilimann MW: The paralemmin protein family: identification of paralemmin-2, an isoform differentially spliced to AKAP2/AKAP-KL, and of palmdelphin, a more distant cytosolic relative. *Biochem Biophys Res Commun* 285: 1369-1376, 2001.
47. Reinhold WC, Reimers MA, Maunakea AK, Kim S, Lababidi S, Scherf U, Shankavaram UT, Ziegler MS, Stewart C, Kouros-Mehr H, Cui H, Dolginow D, Scudiero DA, Pommier YG, Munroe DJ, Feinberg AP and Weinstein JN: Detailed DNA methylation profiles of the E-cadherin promoter in the NCI-60 cancer cells. *Mol Cancer Ther* 6: 391-403, 2007.
48. Michl P, Barth C, Buchholz M, Lerch MM, Rolke M, Holzmann KH, Menke A, Fensterer H, Giehl K, Lohr M, Leder G, Iwamura T, Adler G and Gress TM: Claudin-4 expression decreases invasiveness and metastatic potential of pancreatic cancer. *Cancer Res* 63: 6265-6271, 2003.