

Differential response of p53 and p21 on HDAC inhibitor-mediated apoptosis in HCT116 colon cancer cells *in vitro* and *in vivo*

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Abstract. We investigated the effect of a novel histone deacetylase inhibitor, A-423378.0, on the colon carcinoma cell line HCT116 and genetically modified derivatives lacking either p21^{cip1/waf1} or p53. HCT116 cell lines were incubated with A-423378.0 at different concentrations for 3-120 h. Cell viability, proliferation and apoptosis rates were determined and verified by Western blot, detection of mitochondrial membrane potential breakdown $\Delta\Psi_m$, activation of caspases-3, -8 and cytokeratin 18 cleavage. A subcutaneous xenograft model was established in NMRI mice with daily intraperitoneal injections of 10 mg/kg for 14 days. All three HCT116 cell lines responded to A-423378.0 treatment in a dose- and time-dependent manner via induction of apoptosis as measured by breakdown of $\Delta\Psi_m$ and BrdU incorporation. We identified that A-423378.0 induced the expression of TRAIL and TRAIL receptor, especially TRAIL-R2/hDR5, which was up-regulated in HCT116 cells after treatment with A-423378.0. *In vivo*, a growth inhibitory effect was observed with HDAC-I treatment, which was paralleled by

a down-regulation of PCNA and a concomitant induction of apoptosis. Treatment of wild-type or knock-out HCT116 cells with A-423378.0 exerts potent anti-proliferative and pro-apoptotic effects *in vitro* and *in vivo*. A-423378.0 was able to induce apoptosis in both p21^{WAF1} and p53 deficient tumour cells, which appeared to be mediated by the intrinsic cell death pathway. Interestingly, the effects of A-423378.0 on the extrinsic cell death pathway through activation of TRAIL and its signalling pathway indicate that A-423378.0 may be a potent new therapeutic compound for the treatment of advanced colorectal cancer.

Introduction

Colorectal cancer is one of the leading causes of cancer-related deaths worldwide. Colorectal cancer represents 9.4% of all incidents of cancer in men and 10.1% in women and whilst only surgical resection is still the preferred method of treatment capable of curing colon cancer, adjuvant therapy continues to play an important role in preventing the recurrence and metastasis of colon carcinoma (1).

The transition from normal colonic mucosa to adenomatous polyp to adenocarcinoma is a gradual process involving genetic (e.g. mutations, amplifications, deletions) and epigenetic (e.g. DNA methylation, histone modifications) events (2,3).

Epigenetic events are a key driving force in gene expression without changes in the DNA coding sequence and occur throughout all stages of tumorigenesis. A major example of epigenetic transcriptional control is the modification of histones (4). Histones are basic proteins that form complexes with DNA called nucleosomes, resulting in compact structure of chromatin. Basic amino acids of the histones can be modified post-transcriptionally with ubiquitin or methyl-, acetyl- and phosphate groups. Acetylation of lysine residues of the histones weakens their ability to bind to DNA and induces a change of DNA conformation which is essential for binding of transcription factors to the promoter

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Abbreviations: HDAC, histone deacetylase; HDAC-I, histone deacetylase inhibitor; HF, human foreskin fibroblasts; TRAIL, tumour necrosis factor-related apoptosis inducing ligand

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regions of regulatory genes (e.g. cell cycle) (5,6). Transcriptionally activated genes are associated with hyperacetylated chromatin, whereas transcriptionally silenced genes are associated with hypoacetylated chromatin (7). This is a reversible process which is highly regulated by two groups of enzymes, histone acetyl transferases (HAT) and histone deacetylases (HDAC) (6,8).

Inhibition of HDACs has recently been established as a new and promising means of cancer therapy (6,9). This tumour growth inhibition by HDAC inhibitors (HDAC-I) has been shown to involve the re-expression of the cyclin-dependent kinase (CDK) inhibitor p21^{WAF1}, which is a key target of the tumour suppressor gene p53 (10-12). p21^{WAF1} overproduction is central for stress-induced, p53-mediated G1 and sustained G2 cell cycle arrests (13,14), yet, p21^{WAF1} expression can be driven by other factors that do not rely on p53, which itself is frequently mutated in a variety of different tumour cells (15).

In response to DNA damage and other stressors, p53 is activated by several post-transcriptional modifications, which in turn activates downstream targets to induce cell cycle arrest and/or apoptosis (16,17). p53 mediated induction of apoptosis can be regulated by the intrinsic cell death pathway that relies on mitochondrial membrane depolarization as well as by the extrinsic cell death pathway that relies on activation of death receptors, primarily those of the tumour necrosis factor super-family of receptors. One such death receptor pathway that has generated great interest in cancer therapy in recent years is the tumour necrosis factor-related apoptosis inducing ligand (TRAIL) (also known as Apo2L) cell death pathway. TRAIL has been shown to induce apoptosis in a wide variety of transformed cells with little or no toxicity in normal (non-transformed) cells (18,19).

The primary aim of our study was to investigate the effect of a novel non-hydroxamic acid HDAC-I, A-423378.0 (compound 6) (20), on cell viability, proliferation and apoptosis of different HCT116 colon carcinoma cell lines. Both, the tumour suppressor p53 as well as the cell cycle regulator p21^{WAF1} play critical roles in cell proliferation and survival (13,14,21), and are commonly mutated or epigenetically inactivated in progressive colorectal cancers (22,23). As has been shown previously, HDAC-I therapy (24) could influence or restore the functions of these genes, we studied the novel HDAC-I A-423378.0 (20) on wild-type as well as p53 and p21^{WAF1} deficient HCT116 human colorectal carcinoma cell lines *in vitro* and *in vivo* using a xenograft model. In an attempt to understand the mechanisms of action of A-423378.0-induced cell death, we identified that members of the TRAIL signalling pathway and TRAIL itself was being regulated by the actions of A-423378.0 in the investigated HCT116 cell lines. We therefore suggest that A-423378.0 may be a potent new therapeutic compound for the treatment of advanced colorectal cancer.

Materials and methods

Cell culture. HCT116^{wt}, HCT116^{p21-/-} and HCT116^{p53-/-} cells (provided by Professor B. Vogelstein, Johns Hopkins University, Baltimore, MD) were cultured in RPMI-1640 medium (Biochrom, Berlin, Germany) containing 10% foetal

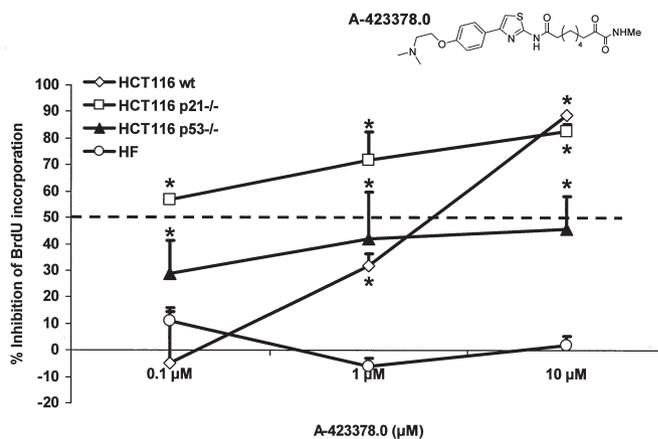


Figure 1. Structure of A-423378.0 and dose response curve (DRC) on inhibition of BrdU incorporation of A-423378.0 on different HCT116 and HF cells after 24-h incubation. BrdU incorporation was used as a marker of cell proliferation. Dotted line represents 50% inhibition of cell proliferation (IC₅₀). Values represent percent inhibition of BrdU incorporation compared to untreated controls (= 0%) and are mean ± SD of three independent experiments. *P<0.05.

bovine serum (FBS; Biochrom), penicillin (10⁷ U/l) and streptomycin (10 mg/l) in humidified conditions of 37°C and 5% CO₂. Primary human foreskin fibroblasts (HF) cultured in Dulbecco's modified Eagle's medium (DMEM, Biochrom) with the same supplements served as non-malignant controls. The use of primary normal colonic epithelial cells was prohibitive as they are unstable under normal cell culture conditions. The HDAC-I A-423378.0 (Fig. 1) was a gift of Abbott Laboratories, Abbott Park, IL, USA. A-423378.0 is a non-selective inhibitor of the class I and class II HDAC enzymes, does not apparently inhibit the tubulin deacetylase activity (TDAC) of HDAC 6, has IC₅₀ values in the 2-4 nM range and showed anti-proliferative activity in many cell types (20).

BrdU-incorporation ELISA. Cells (5x10³) per well were seeded into 96-well microtiter plates (Becton-Dickinson, Heidelberg, Germany) and incubated in complete growth medium in the presence or absence of A-423378.0 for 24 h. DNA-synthesis was assessed using bromodeoxyuridine (BrdU) incorporation (Cell Proliferation ELISA, BrdU Colorimetric, Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions and as described previously (11).

Analysis of apoptosis using fluorescence activated cell sorting (FACS). FACS analysis was employed for the quantification of apoptosis in HCT116 cells and their genetically modified variants treated with the HDAC-I A-423378.0 as indicated. Briefly, 5x10⁴ cells per well were seeded in 6-well tissue culture plates (Becton-Dickinson) and incubated with different concentrations of the HDAC-I A-423378.0 as described above for 3-72 h. Culture supernatants were collected and cells washed twice in PBS, trypsinized and lysed in a hypotonic solution containing 0.1% sodium citrate, 0.1% Triton X-100 and 50 µg/ml propidium iodide (Sigma, Deisenhofen, Germany). Analysis of labelled nuclei was performed on

FACSCalibur fluorescence-activated cell sorter (FACS) using CellQuest software (Becton-Dickinson). The percentage of apoptotic cells was determined by measuring the fraction of nuclei with a sub-diploid DNA content. Ten thousand events were collected for each sample analyzed. The antibody against TRAIL-R2/TNFRSF10B/hDR5 (R&D Systems, Minneapolis, MN, USA) was used at 100 ng/ml in apoptosis stimulating experiments.

Analysis of mitochondrial membrane potential $\Delta\Psi_m$. Mitochondrial injury was measured by JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbo-cyanine iodide) staining (Sanova Pharma GmbH, Vienna, Austria). Briefly, cells were adjusted to a density of 0.2×10^6 /ml, trypsinized and washed in PBS. The cells were resuspended in 1 ml of complete medium, and stained with $5 \mu\text{g/ml}$ JC-1 for 15 min in humidified conditions of 37°C and 5% CO_2 in total darkness. The cells were then washed twice in PBS and resuspended in 0.5 ml of PBS. Analysis was assessed by FACS scan after 24 to 72-h incubation time as described (25).

Immunohistological assessment of apoptosis. Cleavage of cytokeratin 18 by activated caspases-3 and -7 reveals a neo-epitope that is specifically recognized by the M30 antibody (CytoDeath, Roche Molecular Biochemicals). Cells were stained according to the manufacturer's instructions after 12-72 h of incubation with A-423378.0. Analysis was performed on a Zeiss Axioplan fluorescence microscope (Carl Zeiss, Göttingen, Germany) with OpenLab software (Improvision, Heidelberg, Germany).

Assessment of caspase activity. The Caspase Colorimetric Assay (R&D Systems) was used to determine the enzymatic activity of caspase-3 and -8. The assay was performed according to the manufacturer's instructions after 24 h of incubation with increasing concentrations of A-423378.0. Caspase activation leads to cleavage of the provided colorimetric substrates conjugated to *p*-nitroaniline (DEVD-pNA for caspase-3 or IETD-pNA for caspase-8) and was measured photometrically at 405 nm using a Tecan Genios photometer (Tecan Deutschland GmbH, Crailsheim, Germany).

Xenograft model of colorectal cancer. HCT116 cell lines were harvested and resuspended in sterile physiologic NaCl solution. Cells (5×10^6) were injected subcutaneously into the flank of 4 to 6-week-old male NMRI mice (Harlan Winkelmann GmbH, Germany). Eight animals were used for each treatment group. Animals were kept in a light- and temperature-controlled environment and provided with food and water *ad libitum*. Tumour size was determined daily by measurement using a calliper square. When subcutaneous tumours reached a diameter of 7 mm, daily intraperitoneal treatment over 14 days was started using the HDAC-I A-423378.0 (10 mg/kg) or physiologic saline solution for the control population. Animals were sacrificed by cervical dislocation and tumour samples collected. Tumour samples were fixed in 10% phosphate-buffered formalin or snap-frozen in liquid nitrogen. The appropriate ethical approval was sought and granted before any experiments on animals

had begun by the Regional Government of Lower Franconia, Würzburg, Germany (#54-2531.31-9/06). All procedures were in compliance with UKCCCR guidelines for the Welfare of Animals in Experimental Neoplasia.

TUNEL assay and PCNA immunohistochemistry. Tumour tissue was fixed with 10% phosphate-buffered formalin and embedded in paraffin. Sections ($5 \mu\text{m}$) were cut and stored at room temperature until use. Routine histology (hematoxylin-eosin staining) was performed in order to evaluate basic histomorphological features of the specimens. For PCNA immunohistochemistry, sections were dewaxed, rehydrated and processed by microwave heating in citrate buffer (pH 6.0). Specimens were incubated with a primary monoclonal mouse anti-PCNA antibody (1:200, Novocastra, Newcastle upon Tyne, UK) overnight at 4°C and visualized using streptavidin-biotin-complex (Biogenex, San Ramon, CA) or Dako Envision polymer (DakoCytomation Co., Hamburg, Germany) and developed using 3-hydroxy-2-naphthylacide-2,4-dimethylanilide as substrate. Nuclei were counterstained with hematoxylin. Replacement of primary antibodies by non-immune mouse or rabbit serum (BioGenex) or Tris-buffered saline (pH 7.2) served as negative controls. For determination of apoptosis, TUNEL staining was performed on formalin-fixed and paraffin-embedded tissue sections using the In-situ Cell Death detection Kit (Roche) according to the manufacturer's instructions and as described previously (26). Sections were permeabilized with proteinase K (30 min, 37°C) and peroxidase blocked in methanol containing 0.3% H_2O_2 . Fluorescent nucleotides mixed with terminal deoxynucleotide transferase were added for 60 min at 37°C followed by incubation with converter POD (peroxidase conjugated anti-fluoresceine antibody) for 30 min at 37°C . Slides were developed using diaminobenzidine substrate (DAB) for 10 min and counterstained using methylene green (7.5%) for 7 min at room temperature.

Slides were digitized and analyzed with the Ce2001 Cell Explorer software (BioSciTec, Frankfurt, Germany). Quantification (extensity) and semi-quantification (intensity and distribution) were performed for 4 independent high power fields in each slide, using electronic filtering for respective signals.

Protein extraction and Western blot analysis. Total protein was extracted from cultured cells by adding 2X sample buffer (10 mM sodium chloride, 0.5% Nonidet NP40 (Amresco, Solon, OH, USA), 20 mM Tris-HCl pH 7.4, 5 mM magnesium chloride, $10 \mu\text{g/ml}$ complete protease inhibitor cocktail (Roche Diagnostics), 1 mM phenylmethylsulfonylfluoride). DNA was sheared by pipetting up and down for 3 min at room temperature. Samples were boiled at 95°C for 15 min, centrifuged at 13,000 rpm for 10 sec and then subjected to 14% SDS-PAGE (Invitrogen, Carlsbad, USA). After blocking overnight at 4°C in a buffer containing PBS, 0.1% Tween-20 and 5% low fat milk powder, nitrocellulose membranes were incubated for 90 min either with primary antibodies [TRAIL (Chemicon, Temecula, CA, USA) 1:500; p21 (BD PharMingen, Rockville, MD, USA, Clone SX118) 1:500; BCL-2 (BD Transduction Lab, Rockville, MD, USA, Clone 7) 1:200; Bax (Santa Cruz Biotechnology, Santa

Table I. Primer sequences for quantitative real-time PCR.

Primer	Sense (5'-3')	Antisense (5'-3')	bp
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	225
TRAIL/hApo2L	TGTGTGGCTGTAACCTTACGTGTACTTT	GGGCTGTTCATACTCTCTTCGTCAT	139
TRAIL-R1/hDR4	GCCTGTAACCGGTGCACAGA	CATGGGAGGCAAGCAAACAA	70
TRAIL-R2/hDR5	CCTGCTGGATGCCTTGGA	CCAGAGCTCAACAAGTGGTCCT	74
TRAIL-R3/hDcR1	TGGCCCCACAGCAACAGA	CACGGGTTACAGGCTCCAGTAT	90
TRAIL-R4/hDcR2	CTTACCTCAAAGGCATCTGTCTCA	CATTGTCTCCGCCCA	110

bp, size of PCR product.

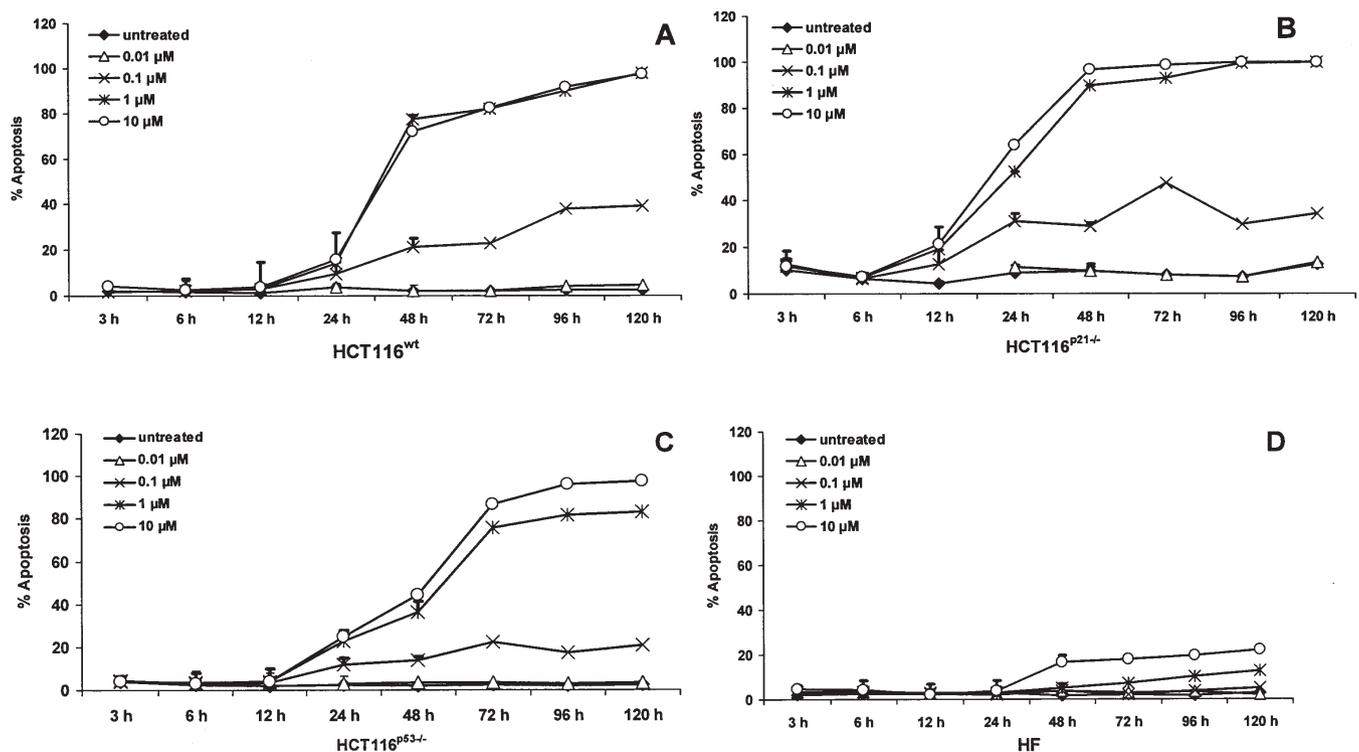


Figure 2. Induction of apoptosis is time- and dose-dependent. Shown are apoptosis rates measured by flow cytometric analysis of sub-diploid nuclei stained with propidium iodide after incubation with A-423378.0 at 0.01-10 μ M over a time course of 3-120 h. Values are mean \pm SD of three independent experiments. (A) HCT116^{wt}; (B) HCT116^{p21-/-}; (C) HCT116^{p53-/-}; (D) HF.

Cruz, CA, USA, N-20) 1:250]; BID (BD PharMingen 1:1,000). All primary anti-TRAIL-receptor antibodies (goat anti-human IgG) were obtained from R&D Systems): TRAIL-R1/TNFRSF10A/hDR4, TRAIL-R2/TNFRSF10B/hDR5, TRAIL-R3/TNFRSF10C/hDcR1 and TRAIL-R4/TNFRSF10D/hDcR2 at 1:200. Membranes were washed three times for 10 min in a buffer containing PBS and 0.1% Tween-20 and were incubated with a peroxidase coupled secondary antibody (1:1,000, Sigma) for 1 h at room temperature. Reactive bands were detected with the ECL chemiluminescence reagent (Amersham Pharmacia Biotech, Freiburg, Germany) and analyzed using GelScan 5 software (BioSciTec). Signals were standardized to β -actin (1:5,000, Sigma) content.

Quantitative real-time PCR. For quantitative real-time PCR, total RNA was prepared from 0.25×10^6 cells from each cell line treated with 1-10 μ M A-423378.0 for 12 h as described previously using the RNeasy Kit in combination with DNase treatment (Qiagen, Hilden, Germany), (27). cDNA synthesis was performed using Superscript II RNase H-reverse transcriptase (Invitrogen) with dT15 (TIB-Biomol, Berlin, Germany) and random hexamer primers (Promega, Heidelberg, Germany).

Primers for quantitative real-time PCR amplification of TRAIL and the TRAIL-receptors were obtained from MWG Biotech AG (Ebersberg, Germany). Sequences were chosen according to the literature and are given in Table I (28). PCR was carried out with the LightCycler FastStart DNA Master

SYBR Green I kit (Roche) according to the manufacturer's instructions and as described previously. After an initial denaturation at 95°C for 10 min, 40 cycles with 95°C for 10 sec, 60°C for 5 sec and 72°C for 10 sec were performed. Measurements were standardized to GAPDH.

Statistical analysis. Statistical analysis was performed using Microsoft Excel 2003. Significance was calculated using the t-test for paired samples. $P < 0.05$ was regarded as significant.

Results

A-423378.0 treatment decreases cell proliferation in HCT116 colon carcinoma cells. In order to assess the effects of A-423378.0 (Fig. 1) on the proliferation of the colorectal cell line HCT116 and its variants, proliferation assays were established on cells treated with various concentrations of A-423378.0 ranging from 0.1 μM to 10 μM for 24 h. Generally, the HCT116 colon carcinoma cells demonstrated a significant reduction of proliferation which was dose-dependent compared to untreated controls. Specifically, there was a reduction in proliferation of the HCT116^{wt} of 89% compared to untreated controls at a concentration of 10 μM of A-423378.0 and a reduction of proliferation of 32% at 1 μM ($\text{IC}_{50} = 2.1 \mu\text{M}$). There was no reduction of proliferation at the lowest concentration of 0.1 μM in this cell line (Fig. 1). Interestingly, a similar observation was seen in the HCT116^{p21-/-} treated with A-423378.0. In this cell line, there was an 83% reduction of proliferation at 10 μM , a 72% reduction at 1 μM and a 57% reduction at 0.1 μM ($\text{IC}_{50} \sim 0.04 \mu\text{M}$). Furthermore, and similar to the previous two cell lines, we also observed a reduction of proliferation in the HCT116^{p53-/-} which demonstrated a 46% reduction at 10 μM , a 42% reduction at 1 μM and a 27% reduction at 0.1 μM of A-423378.0 ($\text{IC}_{50} > 10 \mu\text{M}$). There was no significant reduction in proliferation of HF cells when treated with the HDAC-I A-423378.0 ($\text{IC}_{50} > 10 \mu\text{M}$).

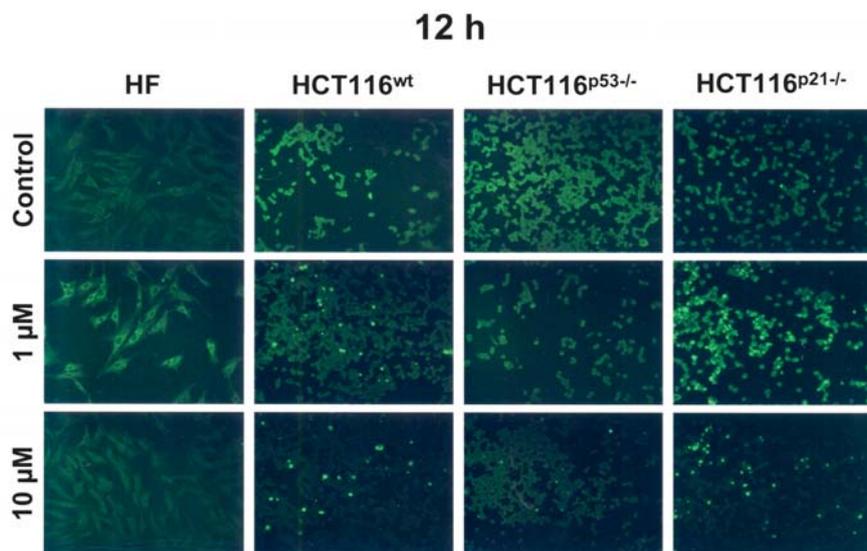
A-423378.0 treatment induces apoptosis of HCT116^{wt}, HCT116^{p21-/-} and HCT116^{p53-/-} in a dose- and time-dependent manner. From our studies we discovered that treatment of the colorectal cell lines with the HDAC-I A-423378.0 induced significant levels of apoptosis at concentrations ranging from 0.1 to 10 μM . Therefore, all future experiments that involved treatment using A-423378.0 were done in the concentration range described above. We observed an increased induction of apoptosis at 12-h post-treatment with A-423378.0 at a concentration of 1.0 μM in all three cell lines investigated, which peaked between 48 to 72-h post-treatment. The highest level of apoptosis observed was 89.7% in HCT116^{p21-/-}, 81.8% in HCT116^{wt} and 75.8% in HCT116^{p53-/-} (Fig. 2A-C) compared with only a 12.6% induction of apoptosis in our normal HF cells (Fig. 2D), consistent with the effects observed for cell proliferation. We confirmed an apoptotic phenotype by performing immunofluorescence staining for cytokeratin 18 cleavage fragments in cell lines treated with A-423378.0. The data are summarized in Fig. 3A and B. Interestingly, we observed significantly fewer cleavage fragments in HCT116^{p53-/-} cells compared to HCT116^{wt} and HCT116^{p21-/-} cells.

Treatment with A-423378.0 disrupts the mitochondrial transmembrane potential ($\Delta\Psi_m$). The technique of JC-1 staining was developed with the intent to detect $\Delta\Psi_m$ in intact, viable cells. For this purpose JC-1 acts as a marker of mitochondrial activity. We employed this technique to determine the mode of action of A-423378.0 in the HCT116 cell types. Compared to untreated controls, there was a reduction in red fluorescence by 54.8% in HCT116^{p21-/-}, 6.7% in HCT116^{wt} and 21.9% in HCT116^{p53-/-} when treated with 1.0 μM A-423378.0 for 24 h (Fig. 3C). Furthermore, after 48 h of treatment with A-423378.0, there was a further decrease in red fluorescence and thus a reduction of $\Delta\Psi_m$ of 74.9% in the HCT116^{p21-/-}, 73.9% in the HCT116^{wt} and 10.6% in the HCT116^{p53-/-} cells compared to untreated controls was observed (data not shown). In the HCT116^{p53-/-}, $\Delta\Psi_m$ was reduced to similar levels as the HCT116^{wt} and the HCT116^{p21-/-} but only after 72 h (data not shown). There was no significant decrease in $\Delta\Psi_m$ in HF cells after treatment with A-423378.0 for 24 h (Fig. 3C) and 48 h (data not shown). Furthermore, regression analysis comparing the induction of apoptosis as demonstrated by flow cytometry and JC-1 staining for mitochondrial viability demonstrated a very strong correlation in all cell types ($R^2 = 0.873$ for HCT116^{p53-/-}, 0.893 for HCT116^{p21-/-} and 0.930 for HCT116^{wt}, respectively) (data not shown).

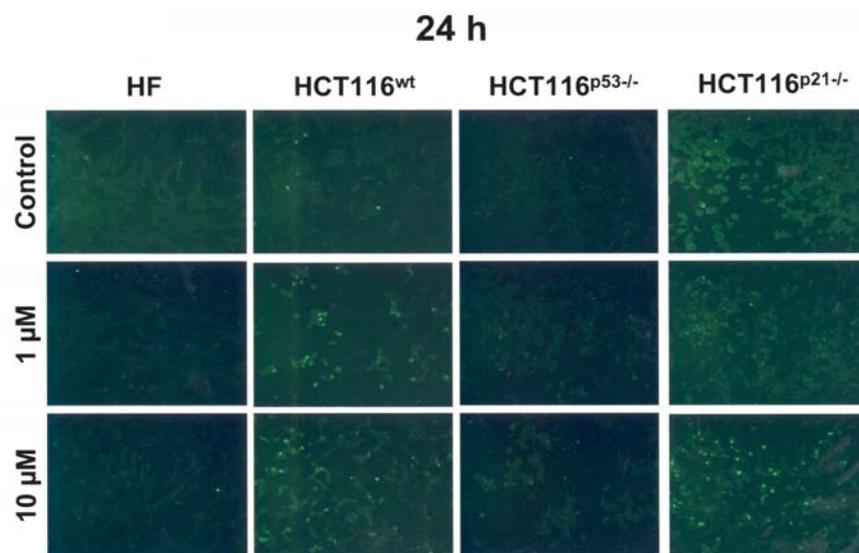
A-423378.0 reduces tumour size in vivo. To investigate the *in vivo* effects of A-423378.0 we generated a xenograft model of colorectal cancer using the HCT116 cell lines in 4-6-week old male NMRI mice. Tumours were allowed to develop subcutaneously and treatment began when tumour size reached a mass of 7 mm. Animals were treated by daily intraperitoneal injections with 10 mg/kg A-423378.0 for 14 days. At the end of 14 days animals were sacrificed and tissue samples collected. The data were normalized against saline treated animals and given the value of 1.0 at the start (day 1) of treatment. Tumour size in the treatment groups was expressed relative to control animals (i.e. relative to 1.0). After 14 days of treatment with A-423378.0 we observed a relative tumour mass of 1.2 in HCT116^{p21-/-} xenografts when compared to saline treated animals which demonstrated a relative tumour size of 1.3. A-423378.0 treated HCT116^{p53-/-} xenografts demonstrated a relative tumour size of 1.5 compared to 2.0 in untreated xenografts ($P < 0.05$), while HCT116^{wt} presented with a relative tumour size of 1.6 in those animals that received A-423378.0 and 2.1 in untreated xenografts ($P < 0.05$). It should be noted that the HCT116^{p21-/-} xenografts had a slower growth rate than the HCT116^{wt} and HCT116^{p53-/-} xenografts in the control group (Fig. 4A). Overall, treatment with A-423378.0 caused a minimum relative decrease in tumour size of 50% in all HCT116 xenografts. During the duration of the experiment animal weight remained stable in all treatment groups, and no adverse drug reactions were noticed. All animals survived until the treatment endpoint of 14 days.

A-423378.0 reduces tumour size in vivo by a reduction of proliferation and an induction of apoptosis. Similar to the data described in our *in vitro* experiments above, we demonstrated that the reduction in relative tumour size from our

A



B



C

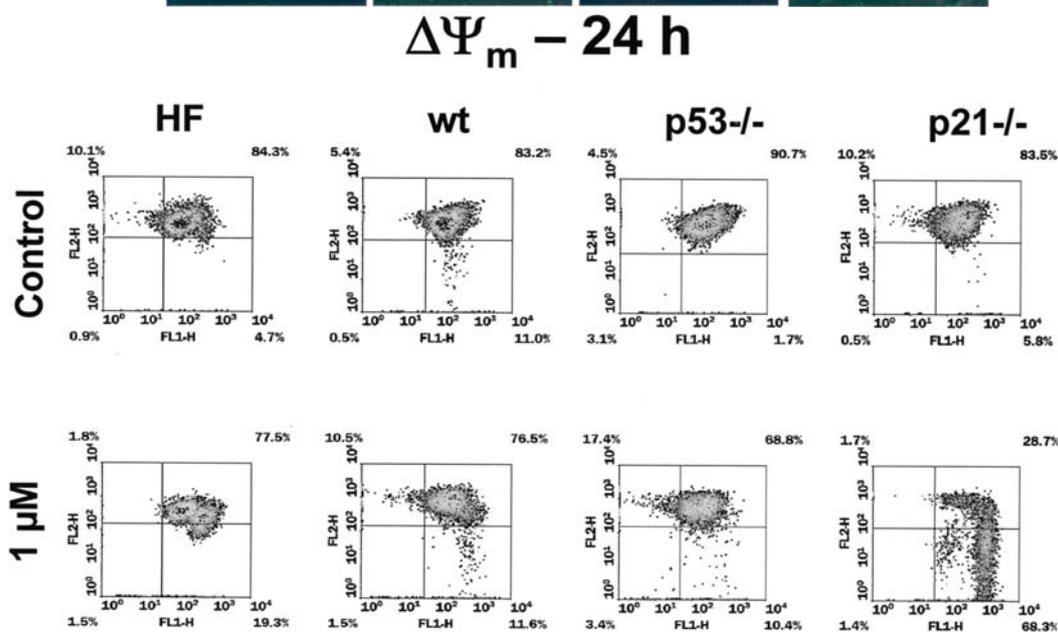


Figure 3. Verification of apoptosis in HCT116 and HF cell lines. Immunofluorescence staining for cytokeratin 18 cleavage fragments showing apoptotic bodies in cells after treatment with A-423378.0 for 12 h (A) and 24 h (B). Apoptotic cells show intense green fluorescence, while non-apoptotic cells exhibit only background fluorescence. (C) FACS analysis of mitochondrial transmembrane potential after JC-1 staining and treatment with 1 μ M A-423378.0. Shown are representative density plots of green vs. red fluorescence depicting all acquired events. Red fluorescence, intact mitochondrial potential; green fluorescence, breakdown of mitochondrial potential. Analysis of the percentage of events acquired in the upper right quadrant provided results for cells with intact $\Delta\Psi_m$.

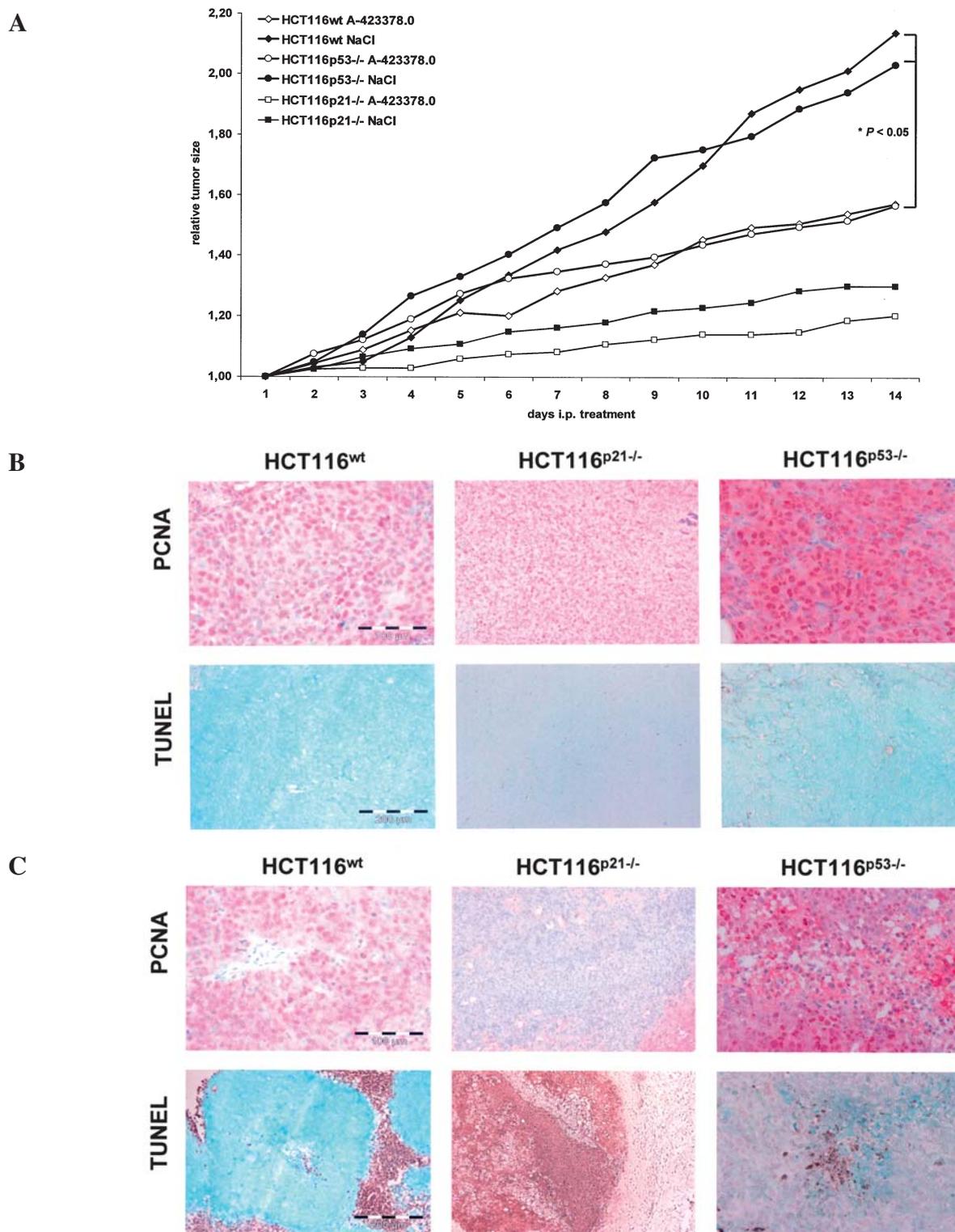


Figure 4. Results of *in vivo* application of A-423378.0. (A) Relative tumour size in the xenograft model (n/group = 8). Daily intraperitoneal (i.p.) treatment with 10 mg/kg A-423378.0 reduced the tumour size significantly compared to a control group with a daily i.p. injection of NaCl. At day 14, the endpoint of treatment, the treated tumours show a significant difference in relative tumour size compared to the control. * $P < 0.05$. Representative examples of TUNEL staining and PCNA immunohistochemistry in different HCT116 xenografts after daily i.p. injections of (B) NaCl or (C) 10 mg/kg bodyweight A-423378.0. PCNA positive cells show an intense red nuclear signal, while negative signals exhibit nuclei counterstained with hematoxylin. TUNEL positivity is determined by strong dark-brown staining. Magnification is x400 for PCNA stainings of HCT116^{wt} and HCT116^{p53-/-}, all other images were obtained with a x200 magnification.

xenograft model of colorectal cancer was due to a combination of a reduction in cell proliferation and an induction of apoptosis. We employed the use of TUNEL

staining for the detection of apoptotic cells and staining for PCNA for proliferation. We noted a 3-fold increase in apoptotic cells in the HCT116^{wt} treated with A-423378.0, a

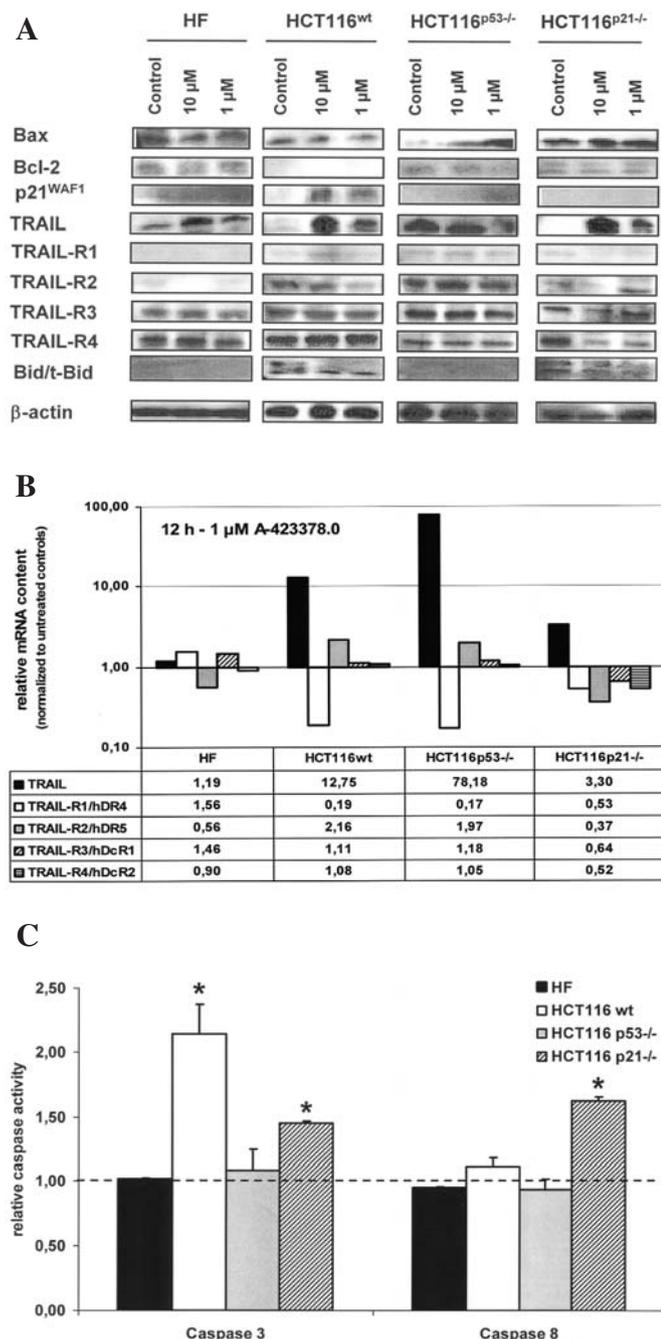


Figure 5. Expression and activity of apoptosis related molecules. (A) Representative Western blots from cells treated with 10 or 1 μ M A-423378.0 for 12 h. Specimens were probed with antibodies against bax, bcl-2, p21^{WAF1}, TRAIL, the different TRAIL receptors, bid/t-bid and β -actin as an internal standard. (B) mRNA levels of TRAIL and its receptors after 12-h incubation with 1 μ M A-423378.0. Results are normalized to GAPDH and expressed relative to untreated controls (set at 1.0). Results shown are mean values of three independent mRNA preparations pooled for PCR analysis. (C) Relative activity of caspase-3 and -8 after 24-h incubation with 1 μ M A-423378.0. Activity levels for untreated controls were set at 1.0 (dotted line). Results are mean \pm SD of three independent experiments. * P <0.05.

1.8-fold increase in apoptotic cells in the HCT116^{p21^{-/-}}, and a 6-fold increase in apoptotic cells in the HCT116^{p53^{-/-}}. Analysis of proliferative cells revealed that there was a reduction in proliferation of 26.1% in the HCT116^{wt}, a reduction in proliferation of 31.0% in the HCT116^{p21^{-/-}}, and

a reduction of 7.3% in the HCT116^{p53^{-/-}} xenografts after treatment with A-423378.0 when compared to control (Fig. 4B-C). These data suggest that A-423378.0 exerts its effects via reducing proliferation and inducing apoptosis.

A-423378.0 induces apoptosis in a Bax-dependent manner and leads to an up-regulation of TRAIL-expression in p53 activated cells. A-423378.0 treatment induced an up-regulation of pro-apoptotic molecules such as Bax with a concomitant constancy or partially reduction in the expression of anti-apoptotic molecules such as Bcl-2. Fig. 5A demonstrates an increase in the expression of pro-apoptotic Bax especially in HCT116^{p53^{-/-}} and HCT116^{p21^{-/-}} cell lines. Importantly, there were no significant changes in Bax or Bcl-2 in the normal human foreskin fibroblast cell line (Fig. 5A). The enhanced increase in expression of Bax with a concomitant constancy or partially decrease in the expression of Bcl-2 is strongly suggestive of a pro-apoptotic phenotype as the Bax/Bcl-2 ratio is skewed in favour of the pro-apoptotic Bax.

p21^{WAF1} was also strongly induced only in the HCT116^{wt} after 12 h of treatment with A-423378.0. There was no change in p21^{WAF1} expression in the other cell lines tested.

There are a number of different compounds that regulate the expression of TRAIL and its cognate receptors. Importantly, it has recently been shown that HDAC-I not only regulate TRAIL receptors, but TRAIL itself and are responsible for overcoming TRAIL-resistance in tumours (29). We therefore assessed whether A-423378.0 was indeed able to regulate the TRAIL intermediates. We demonstrated that there was no TRAIL expression in untreated HCT116^{wt} and HCT116^{p21^{-/-}}. However, upon A-423378.0 stimulation (with either 1 or 10 μ M), there was a marked induction of TRAIL protein (>6-fold) after 12 h. This was notably different in the HCT116^{p53^{-/-}} cell line which already displayed quite a strong expression of TRAIL and hence no further induction of TRAIL was noted. Further, normal human foreskin fibroblast demonstrated a weak expression of TRAIL that was only moderately increased with A-423378.0 stimulation.

To investigate the connection between the extrinsic and the intrinsic apoptosis cascade, the expression of bid and the caspase-8-dependent cleavage to t-bid was analysed by Western blotting. HCT116^{wt} and HCT116^{p21^{-/-}} showed an expression of bid and treatment with A-423378.0 produced the pro-apoptotic fragment t-bid. Bid and t-bid were not detected in HCT116^{p53^{-/-}} and HF. As these cell lines showed an increased resistance to A-423378.0, expression and cleavage of bid may be involved in the apoptotic response to this HDAC-I.

A-423378.0 leads to increased expression of TRAIL and TRAIL-R2/DR5 mRNA. The molecular mechanisms of TRAIL sensitization are not very well understood and there is much discussion as to the actual role that the TRAIL-receptors perform. However, it is implied, that the mechanism of TRAIL sensitization may involve the up-regulation of death receptors or activation of intracellular signalling pathways of TRAIL. We therefore investigated the

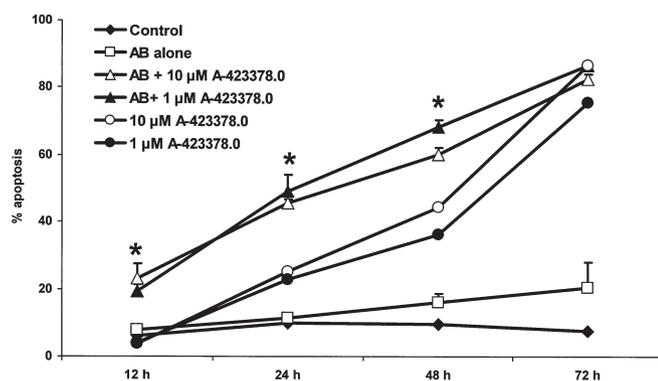


Figure 6. Stimulation of TRAIL-R2/hDR5 with an activating antibody accelerates the apoptotic response of HCT116^{p53-/-} cells to A-423378.0. Cells were incubated with the antibody at 100 ng/ml alone or in combination with 1 or 10 μ M A-423378.0. Apoptosis was determined as sub-diploid events by flow cytometry after propidium iodide staining. Shown are mean \pm SD of three independent experiments. *P<0.05 vs. treatment with A-423378.0.

role of these receptors in our system. We identified that the pro-apoptotic death receptors TRAIL-R1/hDR4 and TRAIL-R2/hDR5 were not expressed in untreated or stimulated normal human foreskin fibroblasts, while the anti-apoptotic decoy receptors TRAIL-R3/hDcR1 and TRAIL-R4/hDcR2 were. When we compared the HCT116 cell lines, we identified that TRAIL-R1 was only weakly detectable, while there was a strong induction of TRAIL-R2. This observation seems to correlate with previous reports by Butler *et al* (29) in other systems and different HDAC-inhibitors and may indicate to a novel yet general mechanism of action for HDAC-inhibitors. We noted that the TRAIL decoy receptors were expressed at low levels in all cell types, except for HCT116^{p21-/-} cells, where we noted a down-regulation of both decoy receptors after 12-h treatment with A-423378.0 (Fig. 5A).

Specifically, and concordant with protein levels, the expression of TRAIL mRNA remained relatively stable in HF cells after 12-h incubation with 1 μ M A-423378.0. In the tumour cell lines, TRAIL mRNA significantly increased to 12.7-fold of untreated controls in HCT116^{wt}, 78.2-fold in HCT116^{p53-/-} and 3.3-fold in HCT116^{p21-/-} (Fig. 5B).

A-423378.0 treatment lead to a pronounced down-regulation of TRAIL-R1/hDR4 mRNA in the tumour cells (Fig. 5B), while it was slightly increased in HF. In contrast, mRNA of the death receptor TRAIL-R2/hDR5 was doubled after 12-h incubation with 1 μ M A-423378.0 in HCT116^{wt} and HCT116^{p53-/-}, while it was diminished in HCT116^{p21-/-} and HF. Expression of the decoy receptors TRAIL-R3/hDcR1 and TRAIL-R4/hDcR2 was stable in HCT116^{wt} and HCT116^{p53-/-}, but was diminished in HCT116^{p21-/-}. Yet, in this cell line, the overall changes in expression of pro-apoptotic molecules (i.e. especially TRAIL as well as TRAIL-R1 and TRAIL-R2) and of anti-apoptotic molecules (i.e. TRAIL-R3 and TRAIL-R4) was still in favour of a pro-apoptotic outcome. In HF, mRNA levels increased to ~1.5-fold of untreated controls for TRAIL-R3 or remained stable for TRAIL-R4. Similar results were obtained for incubations with 10 μ M A-423378.0 in all cell lines and all PCR experiments (data not shown).

A-423378.0 activates the extrinsic and intrinsic caspase cascade. In order to understand whether the up-regulation of TRAIL and its pro-apoptotic receptors after incubation with A-423378.0 activates caspase cascades, we used a colorimetric substrate cleavage assay specific for activated caspase-3 or caspase-8, respectively. While no change in caspase activity was observed for non-responsive HF control cells, a strong induction of caspase-3 (>2.4-fold) was observed in HCT116^{wt} after 24-h incubation with 1 μ M A-423378.0. In this cell line, no additional activation of caspase-8 was observed. In contrast, HCT116^{p21-/-} cells showed an increase in both, caspase-3 and caspase-8 activity (1.4- and 1.6-fold, respectively), indicating that both pathways are activated here and corroborating the increased sensitivity of this cell line to A-423378.0 treatment. The more resistant HCT116^{p53-/-} cells did not show an increase in caspase activities, indicating a p53-dependent but caspase-independent way of cell death, e.g. the formation of reactive oxygen species.

Stimulation of TRAIL-R2/hDR5 accelerates the apoptotic response of HCT116^{p53-/-}. HCT116^{p53-/-} demonstrated the greatest resistance against A-423378.0 treatment in all experiments but showed a strong expression of TRAIL and the pro-apoptotic death receptor TRAIL-R2/hDR5 on protein and mRNA level (Fig. 5A and B) after treatment with A-423378.0. We therefore incubated this cell line with an activating antibody against hDR5 alone or in combination with A-423378.0 as described (Fig. 6). Stimulation of TRAIL-R2/hDR5 alone lead to a slow increase in apoptosis, reaching 20.5% after 72 h (P<0.05 vs. untreated control). Combining the antibody treatment with A-423378.0 increased the number of sub-diploid events in flow cytometry to 23.2 and 19.4% (10 and 1 μ M A-423378.0, respectively) already after 12 h, compared to 3.75 and 4% for A-423378.0 alone. This acceleration of the apoptotic response was similarly observed after 24 and 48 h (Fig. 6).

Discussion

Using different molecular techniques we demonstrated that treatment with the novel HDAC inhibitor A-423378.0 decreases cell proliferation and induces apoptosis in HCT116 wild-type cells as well as in genetically modified variants lacking p21^{WAF1} or p53 *in vitro* and in a xenograft model *in vivo* in a dose- and time-dependent manner with little or no effect in non-transformed human foreskin fibroblasts. Compared to the wild-type HCT116 cells with intact p53 and p21^{WAF1}, there was no reduction in overall apoptosis rate in the knock-out variants but rather an accelerated increase of apoptosis in the p21^{WAF1} deficient cells.

Overall, the induction of apoptosis by A-423378.0 seems to be independent of p53 and p21^{WAF1} status according to recent literature (30,31). A previous study highlighted that loss of p21^{WAF1} protein expression induces a post-transcriptional increase of p53 levels resulting in a modification of the balance between the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax in favor of Bax (32). p21^{WAF1} expression can be provoked through factors other than p53, e.g. extracellular signal regulated

kinase (ERK) pathways, causing a p53-independent p21^{WAF1} expression by HDAC-I treatment which was not observed in our experimental setting (33-36). Furthermore, other HDAC-I like SAHA have been shown to stimulate p21^{WAF1} via binding to two sp1 sites located at -82 and -69 relative to the transcription start site, i.e. a direct transcriptional activation independent of p53 (31,37). Interestingly, results of studies performed on HCT116^{wt}, HCT116^{p21^{-/-}} and HCT116^{p53^{-/-}} cells indicate that apoptosis can be induced independently of the cellular status of p53 or p21^{WAF1} under chemotherapeutic treatment with e.g. camptothecin or n-butyrate (30,38). In the absence of p53, chemotherapeutic-induced apoptosis decreases, while G2-M checkpoint activation increased, which is reversed in the absence of p21^{WAF1} (39). This corroborates our observations on HCT116^{wt}, HCT116^{p21^{-/-}} and HCT116^{p53^{-/-}} cells *in vitro* and on their cognate xenografts *in vivo*, where we demonstrated the possibility of a p21^{WAF1} and p53 independent induction of apoptosis by HDAC-I treatment.

In order to better understand the mechanisms of action of A-423378.0 we identified that members of the TRAIL signalling pathway and indeed TRAIL itself was being induced by the actions of A-423378.0. TRAIL can interact with any one of 5 receptors, two of which act as death receptors and are termed TRAIL-R1/hDR4 and TRAIL-R2/hDR5. The other three, bind TRAIL and act as decoy receptors termed TRAIL-R3/DcR1, TRAIL-R2/DcR2 and osteoprotegerin (OPG). Work from some groups has clearly determined a reliance of TRAIL on either p21 or p53 status, however, this is by no means an absolute argument as there is ample evidence for both TRAIL-R1 and TRAIL-R2 regulation in a p53- and p21-independent manner (19,40,41). We demonstrated a strong induction of TRAIL by HDAC-I-treatment in colorectal cancer cells with an activated p53 gene, as was the case with the human foreskin fibroblasts and the HCT116^{wt} or HCT116^{p21^{-/-}} cells: TRAIL-R1 is only weakly expressed, while the pro-apoptotic receptor TRAIL-R2 expression is enhanced in responsive tumour cells, independent of their p53 or p21 status. Although a slight downregulation has been demonstrated on mRNA levels in HCT116^{p21^{-/-}} cells, the overall ratio of pro- and anti-apoptotic TRAIL receptors is still in favour of apoptosis, as here also a suppression of the two decoy receptors has been found. The antibody-mediated stimulation of TRAIL-R2 accelerated the apoptotic response of HCT116^{p53^{-/-}} cells to A-423378.0, indicating that the use of pro-apoptotic receptor agonists could further facilitate the effect of HDAC-I in p53-deficient cells.

The mechanisms of differential sensitivity of different tumour types or between tumours of the same type to TRAIL are not well understood. There appear to be multiple mechanisms, including increased expression of the decoy receptors for TRAIL, and the over-expression of intracellular inhibitory proteins such as FLIP or intracellular inhibitors of apoptosis molecules (IAPs). We, through our data and the work of others have not been able to demonstrate a consistent correlation between TRAIL receptor expression and sensitivity to TRAIL-induced apoptosis. The data here demonstrate an association between HDAC inhibition and the TRAIL signalling system. Future pathway-inhibiting

experiments are aimed at investigating the role of A-423378.0 in sensitizing colon carcinoma cells to TRAIL-induced apoptosis.

Work from other groups has demonstrated that TRAIL was able to induce apoptosis in colon carcinoma cells, e.g. HCT116 independently of the p53 tumour suppressor gene, which is inactivated in more than half of human cancers (42-45). These observations could strengthen and explain why we saw a high level of TRAIL expression in the p53 deficient HCT116^{p53^{-/-}} and human foreskin fibroblasts where the p53 gene is not activated. We conclude that A-423378.0 could also induce apoptosis via an up-regulation of TRAIL-R2/hDR5 mRNA and an enhanced expression of the cognate apoptosis inducing ligand TRAIL, independent of the p53 and p21 status.

The observations we described were confirmed by several studies that demonstrated HDAC-inhibitor mediated apoptosis via the mitochondrial-mediated cell death pathway in cancer cells (25). Our data suggest that apoptosis was induced via the mitochondrial pathway as shown by the disruption of $\Delta\Psi_m$. HCT116^{p21^{-/-}} had the fastest reduction of the transmembrane potential followed by HCT116^{wt} cells. In HCT116^{p53^{-/-}} cells, reduction of $\Delta\Psi_m$ was delayed, possibly reflecting the central role of p53 in mitochondria-regulated apoptosis (46).

The mitochondria-mediated pathway of apoptosis induction seems to be sufficient in HCT116^{wt} cells after treatment with A-423378.0 as we observed a strong induction of caspase-3 activity here, but no change in caspase-8. As caspase-8 was only activated in HCT116^{p21^{-/-}} cells, this finding could explain the accelerated pro-apoptotic response in these cells as both pathways, the TRAIL mediated extrinsic and the mitochondria-mediated intrinsic pathway, may be activated concomitantly here. Similarly, the delayed response of the HCT116^{p53^{-/-}} cells, which did not show a significant level of caspase activation may hint at a slower, caspase-independent cell death pathway, e.g. the formation of reactive oxygen species that has been previously linked with HDAC-I treatment. This view is further corroborated by the expression of bid and cleavage to t-bid in HCT116^{wt} and HCT116^{p21^{-/-}} cell lines only, which were most sensitive to A-423378.0 treatment. These results confirm previous findings of caspase-independent induction of apoptosis in pancreatic cancer cells by the HDAC-I Trichostatin A or SAHA where a similar pattern of caspase-3 and caspase-8 levels was observed (47,48).

In summary, HDAC-I-mediated apoptosis is possible in p21^{WAF1} or p53 deficient tumour cells with HCT116^{p21^{-/-}} and HCT116^{wt} cells appearing to be more sensitive to A-423378.0 stimulation than HCT116^{p53^{-/-}}. This could reflect a central role for the p53 suppressor gene in apoptosis-induction, where it activates the p21^{WAF1} gene (13,14,21,49). The difference observed between the cell lines was not seen *in vivo*, where all three HCT116 xenografts presented a similar growth inhibition by A-423378.0 treatment. This could be explained by various factors including drug metabolism or the interference between the tumour and stromal layers, which can possibly be able to affect the expression of growth or survival factor receptors (e.g. integrins) and their downstream signalling *in vivo* (50). Furthermore, the

TRAIL-receptor expression may vary between our *in vitro* and our *in vivo* models, where in addition to the human system, the differing murine TRAIL system (with 1 death receptor and 3 TRAIL-like decoy receptors) may influence the effect of A-423378.0 on HCT116 colon cancer xenografts (51). Overall and in concordance with the cell culture experiments, a growth inhibition as evidenced by reduced PCNA staining and increased apoptosis as shown by TUNEL staining was also observed in the xenograft model.

In conclusion, HDAC-inhibitors like A-423378.0 may contribute to the adjunct therapy of advanced stages of colorectal cancer, where escape mutations through chromosomal instability of p21^{WAF1} and p53 exist (52). The involvement of TRAIL-mediated apoptosis pathways by A-423378.0 and the known prolongation of S-phase with sensitization to conventional chemotherapeutic drugs, make this HDAC inhibitor a promising new agent in the treatment of advanced tumours.

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