

# The histone-deacetylase inhibitor MS-275 and the CDK-inhibitor CYC-202 promote anti-tumor effects in hepatoma cell lines

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**Abstract.** Effective therapies for advanced stages of hepatocellular carcinoma (HCC) have yet to be developed. We investigated how far a combination of the HDAC inhibitor MS-275 and the CDK inhibitor CYC-202 synergizes to inhibit proliferation and promotes apoptosis of hepatoma cells *in vitro*. Human hepatoma cell lines Hep3B and HepG2 as well as primary human foreskin fibroblasts as non-malignant controls were cultured under standardized conditions and incubated with increasing concentrations of CYC-202 and MS-275 as single agents and in combination. After 24 to 72 h, apoptosis was analyzed by flow cytometry (propidium iodide, JC-1) and by immunocytochemistry for cytokeratin 18 fragmentation. DNA synthesis was assessed using bromodeoxyuridine incorporation. Protein was separated for Western blotting against p21, bax and bcl-2 and fluorimetric activity assays against caspase 3 and 8. The results showed that the combination of CYC-202 and MS-275 leads to better pro-apoptotic effects than the employment of single substances. Apoptosis was induced via the mitochondrial pathway as evidenced by a shift in the bax/bcl-2 ratio and breakdown of mitochondrial transmembrane potentials. Caspase assays revealed a strong induction of caspase 3 but not of the extrinsic initiator caspase 8. In conclusion, combination therapy with the biomodulators MS-275 and CYC-202 is a promising treatment option for HCC.

## Introduction

Hepatocellular carcinoma (HCC) belongs to the most common malignancies worldwide with an estimated incidence of 560,000 cases (1) and ~1 million cancer-related deaths per year (2). Most cases occur in pre-existing liver cirrhosis (3,4), particularly due to chronic viral hepatitis, alcohol abuse or hemochromatosis. Overall 5-year survival remains poor (5) as only a limited number of patients are eligible for curative treatment options such as surgical resection or orthotopic liver transplantation. Patients at advanced stages need palliative treatment including transarterial chemoembolization (TACE), percutaneous ethanol injection (PEI), cryosurgery, hormonal therapy, radiation therapy and systemic chemotherapy (2). Advanced HCC is known to be highly chemotherapy-resistant. Therefore, the development of an effective chemotherapeutic approach is urgently needed and pro-apoptotic biomodulators may be promising agents in the treatment of HCC.

Histone deacetylases (HDAC) play an important role in gene and protein expression regulation and are new targets for anti-cancer drug development (6-8). Histone-deacetylase inhibitors (HDACi) are able to induce gene expression, differentiation, apoptosis and growth arrest (7,8). A direct consequence of HDAC inhibition is the increase of chromatin histone acetylation, which leads to a relaxation of the chromatin structure (7). This change permits the modulation of a selected gene and protein expression, e.g. the cell cycle inhibitor p21<sup>cip1/waf1</sup> (9). MS-275, a synthetic benzamide, induces chromatin hyperacetylation and inhibits HDAC enzyme activity (10). It is structurally dissimilar from other HDAC inhibitors such as SAHA or trichostatin A (TSA) and has inhibitory activities against solid malignancies as well as against haematological tumors *in vitro* and *in vivo* (10-13). As mentioned above (9) the HDAC-inhibitory activity of MS-275 increases the expression of the cyclin-dependent kinase inhibitor (CDKi) p21<sup>cip1/waf1</sup> and leads to accumulation of cells in the G<sub>1</sub> phase (14). The up-regulation of p21<sup>cip1/waf1</sup> is accompanied by the down-regulation of cyclin D1 and consequently growth arrest in G<sub>1</sub> (15).

Seliciclib (CYC-202) is the R-enantiomer of roscovitine (16). It binds to the ATP-binding pocket of cyclin-dependent

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kinases and is a potent Cdk2-cyclin E inhibitor (17). It also inhibits Cdk7, Cdk9, Cdk5 and other target molecules such as MAPK (18,19). It was recently demonstrated that this compound potentiates apoptotic induction by modulating the p53 response (20). In a phase I trial, CYC-202 led to disease stabilisation in patients with advanced solid malignancies (21).

We investigated whether and how far the combination of MS-275 and CYC-202 influences proliferation and apoptosis of hepatoma cells, as the two compounds interfere with the cell cycle progression by direct or indirect inhibition of cyclin-dependent kinases and may therefore have synergistic effects.

## Materials and methods

**Reagents and cell culture.** Human hepatoma cells HepG2 (p53wt) and Hep3B ( $\Delta$ p53) as well as human foreskin fibroblasts (HF) were cultured on six-well tissue culture or 96-well plates (Becton-Dickinson, Mannheim, Germany) in RPMI-1640 (Biochrom, Berlin, Germany) or Dulbecco's modified Eagle's medium (DMEM) (Biochrom) containing 10% fetal bovine serum (FBS, Gibco BRL, Karlsruhe, Germany), penicillin ( $10^7$  U/l, Biochrom) and streptomycin (10 mg/l, Biochrom) under standardised conditions (37°C, 5% CO<sub>2</sub>). The hepatoma cell lines were obtained from ATCC, Rockville, Maryland, USA. HF cells were used as non-malignant controls.

CYC-202 (r-roscovitine) was a gift from Cyclacel Ltd. (Dundee, Scotland, UK) and MS-275 from Schering Pharma (Berlin, Germany). For the experiments, the substances were dissolved in dimethyl sulfoxide (DMSO, 0.5%, Sigma, Deisenhofen, Germany). Further dilutions to concentrations of  $10^{-4}$  to  $10^{-7}$  M were performed using complete cell culture medium.

**Flow cytometry of apoptosis.** Cells were starved for 24 h in medium containing 0.125% FBS to achieve cell cycle synchronization and then washed twice with phosphate-buffered saline (PBS, Biochrom), treated with trypsin EDTA (0.05% trypsin and 0.02% EDTA, Biochrom), seeded at a density of  $0.5 \times 10^6$  per well and incubated for 24, 48 or 72 h in the presence of CYC-202 or MS-275 alone or in combination. For quantification of apoptosis, cells were washed twice with PBS, trypsinized and lysed in a hypotonic solution containing 0.1% sodium citrate, 0.1% Triton X-100 and 50  $\mu$ g/ml propidium iodide (Sigma). An analysis of labelled nuclei was performed on a FACSCalibur fluorescence-activated cell sorter (FACS) using CellQuest software (both from Becton-Dickinson, Heidelberg, Germany). 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 analysed.

**Determination of DNA synthesis.** DNA synthesis as a marker for cell proliferation was measured by bromodeoxyuridine (BrdU) incorporation using the cell proliferation ELISA (Roche Molecular Biochemicals, Mannheim, Germany), which was based on the incorporation of BrdU into newly synthesized DNA and antibody-mediated detection of BrdU in DNA, as previously described (22).

**Analysis of mitochondrial membrane potential  $\Delta\Psi_m$ .** Mitochondrial injury was assessed by JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarboyanine iodide) staining (Sanova Pharma GmbH, Vienna, Austria) as previously described (23). Briefly, cells were adjusted to a density of  $0.2 \times 10^6$ /ml, trypsinized, washed in PBS, resuspended in 1 ml medium, and stained with 5  $\mu$ g/ml JC-1 for 15 min at 37°C in an atmosphere containing 5% CO<sub>2</sub> in the dark. Cells were then washed twice in PBS and resuspended in 0.5 ml PBS. An analysis was performed by FACScan and the mitochondrial function was assessed as JC-1 green (uncoupled mitochondria, detector FL-1) vs. red (intact mitochondria, detector FL-2) fluorescence. For the quadrant analysis, 10,000 events were collected and gated for cell viability according to the FSC-SSC plot.

**Immunohistological assessment of apoptosis.** Cleavage of cytokeratin 18 by activated caspase 3 and 7 reveals a neo-epitope that is specifically recognized by the M30 antibody (CytoDeath, Roche Molecular Biochemicals, Mannheim, Germany) as previously described (23). Cells were stained according to the manufacturer's instructions after 24 h of incubation with MS-275 and CYC-202. An analysis was performed on a Zeiss Axioplan fluorescence microscope (Carl Zeiss, Göttingen, Germany) with OpenLab software (Improvision, Heidelberg, Germany).

**Western blotting of proteins involved in apoptosis.** Trypsinized and washed cells were lysed by adding 100  $\mu$ l 2X sample buffer (2 mM NEM, 2 mM PMSF, 4% SDS, 4% DTT, 20% glycerol, 0.01% bromophenol blue, 2 M urea, 0.01 M Na-EDTA and 0.15 M Tris-HCl) to  $10^6$  cells. 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 min and then subjected to 14% SDS-PAGE (precast gels; Invitrogen, Karlsruhe, Germany). After blocking overnight at 4°C in a buffer containing PBS, 0.1% Tween-20 and 4% low-fat milk powder, nitrocellulose membranes were incubated for 90 min with polyclonal rabbit anti-human bcl-2 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal rabbit anti-human bax (1:500, Santa Cruz Biotechnology), polyclonal mouse anti-human p21 (1:500, BD Biosciences, San Jose, CA, USA) or  $\beta$ -actin (1:5000, Sigma) antibodies. Membranes were washed twice for 10 min in a buffer containing PBS, 0.1% Tween-20 and 4% low-fat milk powder and incubated with an anti-rabbit or anti-mouse IgG combined with peroxidase (1:1000, Sigma) for 1 h at room temperature. Reactive bands were detected with the ECL chemiluminescence reagent (Amersham Pharmacia-Biotech, Freiburg, Germany) using a Fluor-Chem 8900 digital image analyser (AlphaInnotech, San Leandro, CA). A densitometric analysis was performed using Gelscan 5.01 (BioSciTec, Frankfurt, Germany). Values were normalized to  $\beta$ -actin levels and are shown as changes relative to untreated cells.

**Assessment of caspase 3 and 8 activity.** Cells ( $5 \times 10^4$ ) were treated with the indicated concentrations of CYC-202 and MS-275 alone or in combination for the indicated time. Total protein was isolated as previously described and measured by BCA assay (Pierce, Perbio). Caspase 3 and 8 activities were

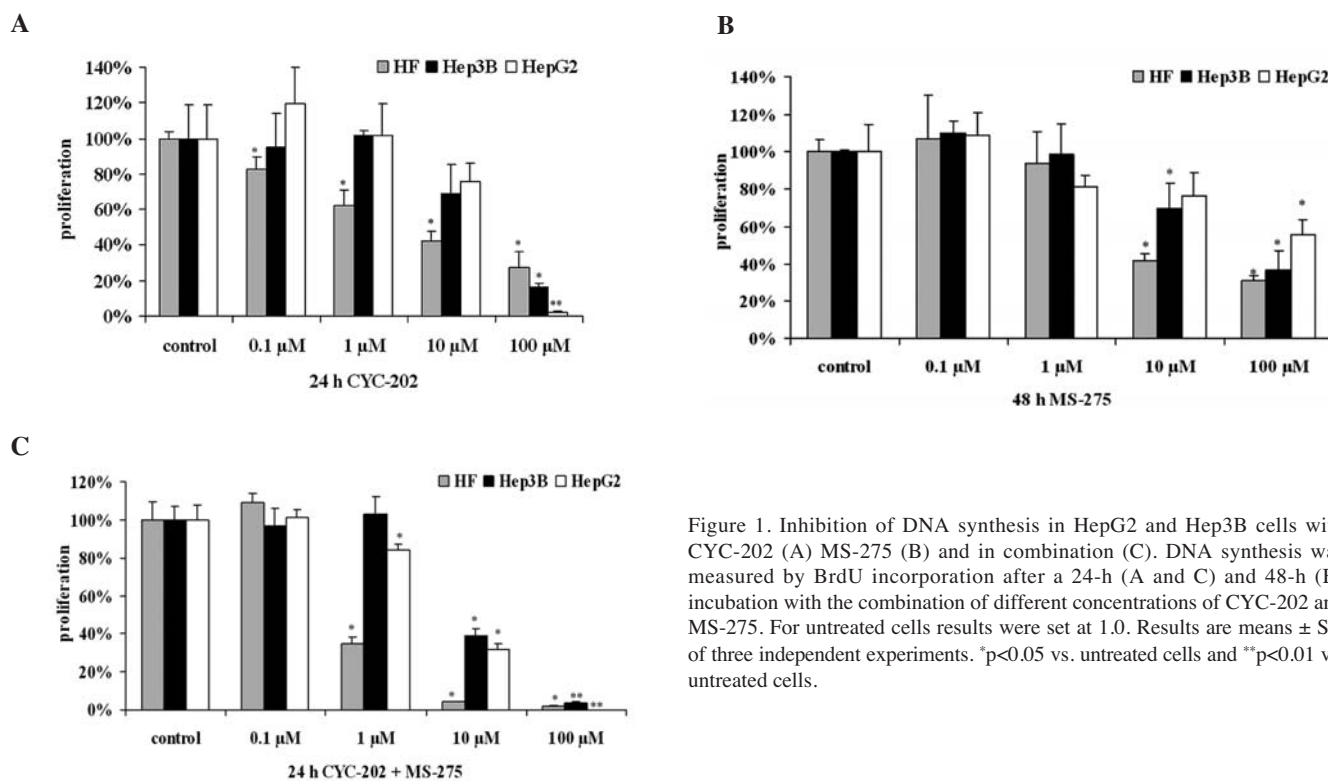


Figure 1. Inhibition of DNA synthesis in HepG2 and Hep3B cells with CYC-202 (A) MS-275 (B) and in combination (C). DNA synthesis was measured by BrdU incorporation after a 24-h (A and C) and 48-h (B) incubation with the combination of different concentrations of CYC-202 and MS-275. For untreated cells results were set at 1.0. Results are means  $\pm$  SD of three independent experiments. \* $p < 0.05$  vs. untreated cells and \*\* $p < 0.01$  vs. untreated cells.

determined from 5  $\mu$ g of total protein by the Caspase-Glo 3/7 and 8 assay, respectively (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luminescence was measured using a luminometer (Genios, Tecan Germany GmbH, Crailsheim, Germany) and expressed as RLU. Results were normalised to untreated controls set at 1.0.

**Statistical analysis.** Statistical analysis was performed using Excel for Windows. Significant differences were calculated using the t-test for paired samples.  $P \leq 0.05$  was regarded as significant and  $P \leq 0.01$  as highly significant.

## Results

**Combination of MS-275 and CYC-202 inhibits cell cycle progression and increases suppression of hepatoma cell DNA synthesis.** BrdU incorporation, which correlates with DNA synthesis and proliferation, was determined in experiments using combinations of MS-275 and CYC-202 after testing the single substances at different concentrations (0.1 to 100  $\mu$ M) and different times after 24 and 48 h (Fig. 1A and B).

Relative to untreated controls (=100%) incubation for 24 h with CYC-202 (10  $\mu$ M) reduced proliferation to 69% (Hep3B) ( $p > 0.05$ ) and 75% (HepG2) ( $p > 0.05$ ), respectively, (Fig. 1A). A further increase of CYC-202 to a concentration of 100  $\mu$ M showed a reduction of the proliferation rate to 16% (Hep3B) ( $p \leq 0.05$ ) and 2% (HepG2) ( $p \leq 0.01$ ), respectively. Concentrations  $< 10$   $\mu$ M did not show a clear decrease of the proliferation rate. Forty-eight hour incubation with CYC-202 at a concentration of 1  $\mu$ M led to a proliferation rate of 86% in Hep3B cells ( $p \leq 0.05$ ). In HepG2 cells no further efficacy (87%) or significance ( $p > 0.05$ ) was observed. Concentrations  $> 1$   $\mu$ M were similar to the results after 24 h incubation

(data not shown). Therefore, we did not continue incubation for 72 h.

After 24 h incubation with MS-275, cell proliferation decreased dose-dependently in Hep3B cells compared to controls: 77% at 1  $\mu$ M, 75% at 10  $\mu$ M and 52% at 100  $\mu$ M. After 48 h a clear effect was only observed at concentrations of 10  $\mu$ M or higher. Thus, the proliferation of Hep3B was reduced to 70% at 10  $\mu$ M and to 37% at 100  $\mu$ M. HepG2 cells were less sensitive for MS-275 and needed a concentration of at least 10  $\mu$ M to reduce the proliferation rate to 78% after 24 h ( $p > 0.05$ ). A concentration of 100  $\mu$ M resulted in a proliferation rate of 56% ( $p > 0.05$ ; data not shown). An incubation time of 48 h showed a proliferation rate of 81% at 1  $\mu$ M ( $p > 0.05$ ; Fig. 1B). Further increase of the concentration of MS-275 after 48 h had the same effect as after 24 h.

The combination of CYC-202 and MS-275 showed no inhibition at a concentration of 0.1 and 1  $\mu$ M after 24 h in the investigated cell lines (Fig. 1C). A surprising effect was observed at a concentration of 10 and 100  $\mu$ M as cell proliferation was reduced to 39% ( $p \leq 0.05$ ) and 4% ( $p \leq 0.01$ ), respectively, in Hep3B and to 32 and 0% in HepG2 ( $p < 0.05$ ). HepG2 cells also showed a reduced proliferation of 84% at 1  $\mu$ M ( $p < 0.05$ ). After 48 h a low inhibition of cell proliferation occurred at a concentration of 0.1  $\mu$ M to 95% ( $p > 0.05$ ; data not shown). A further increase of concentration inhibited cell proliferation at 1  $\mu$ M to 88%, 10  $\mu$ M to 41% and 100  $\mu$ M to 14% ( $p > 0.05$ ) in Hep3B and to 56, 7 and 0%, respectively, in HepG2. Thus, the combination treatment significantly decreased BrdU incorporation as compared to the two single agents with a greater sensitivity in HepG2 cells.

**Combination of MS-275 and CYC-202 induces apoptosis in hepatoma cells.** The effect of different MS-275 and CYC-202

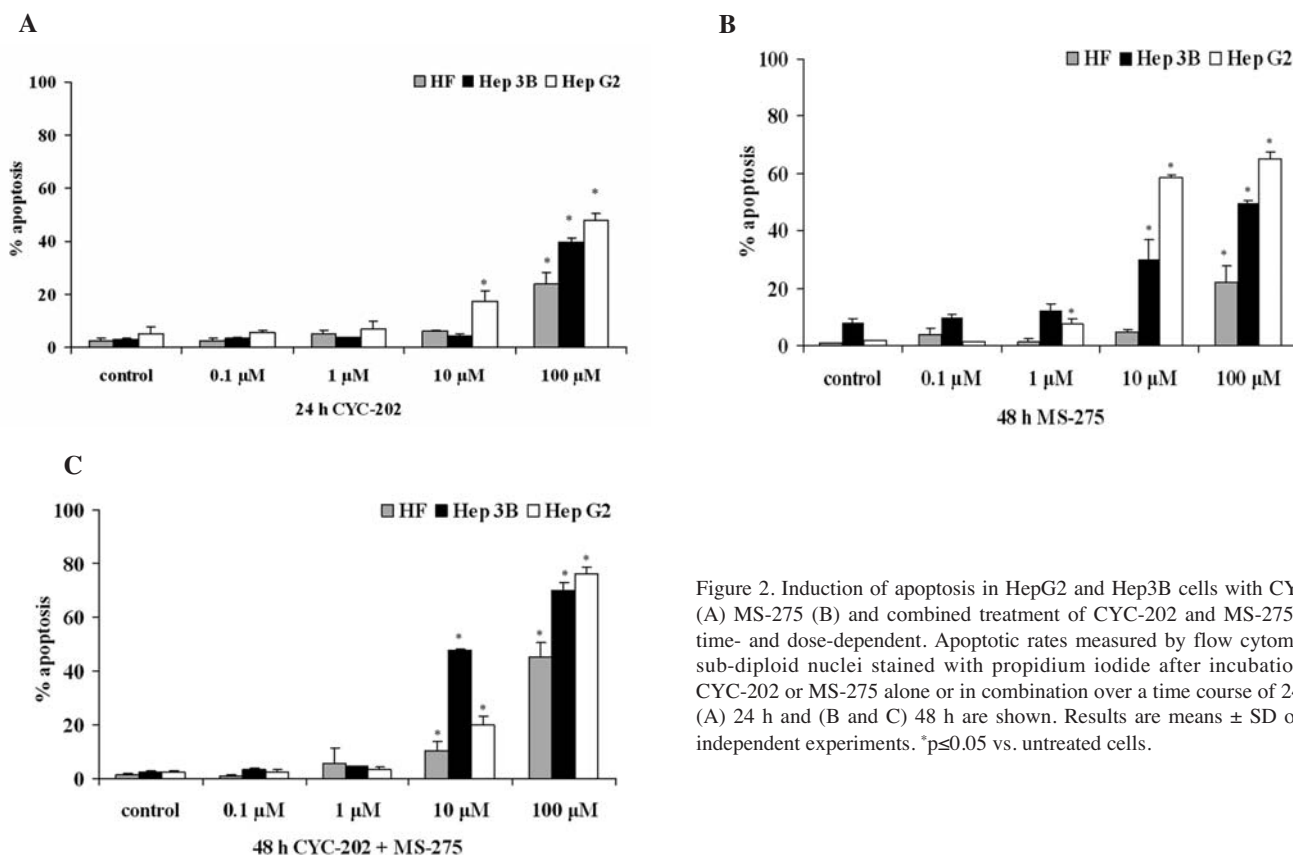


Figure 2. Induction of apoptosis in HepG2 and Hep3B cells with CYC-202 (A) MS-275 (B) and combined treatment of CYC-202 and MS-275 (C) is time- and dose-dependent. Apoptotic rates measured by flow cytometry of sub-diploid nuclei stained with propidium iodide after incubation with CYC-202 or MS-275 alone or in combination over a time course of 24-72 h. (A) 24 h and (B and C) 48 h are shown. Results are means  $\pm$  SD of three independent experiments. \* $p \leq 0.05$  vs. untreated cells.

concentrations alone (0.1 to 100  $\mu$ M) or in combination with Hep3B and HepG2 cell apoptosis was determined by flow cytometry (propidium iodide, FACS) after 24, 48 and 72 h. Significant effects of CYC-202 in Hep3B after 24 h were only observed at a concentration of 100  $\mu$ M by reaching ~40% apoptosis. Lower concentrations remained ineffective (Fig. 2A). Apoptosis increased to 6.5 and 16% after 48 and 72 h, respectively, at 10  $\mu$ M CYC-202, while 100  $\mu$ M increased apoptosis to 68 and 77%, respectively (data not shown). HepG2 cells were again more sensitive to CYC-202 than Hep3B cells. Thus, 10  $\mu$ M or 100  $\mu$ M CYC-202 induced ~20 and 48% apoptosis, respectively, after 24 h. Prolonged incubation for 48 and 72 h led to an increased rate of apoptosis from 60 to 80% at 100  $\mu$ M (data not shown).

Treatment of Hep3B cells with MS-275 showed a continual increase of apoptosis, which was time- and dose-dependent (Fig. 2B). The incubation with a concentration of 10  $\mu$ M led to an apoptotic rate of 18% after 24 h, 30% after 48 h and 62% after 72 h (data not shown). MS-275 (100  $\mu$ M) induced an apoptotic rate of 20% after 24 h, 49% after 48 h and 77% after 72 h. Treatment of Hep2G cells was also time- and dose-dependent. Therefore, at 10  $\mu$ M apoptosis increased to 10% after 24 h and to 59% after 48 h (Fig. 2B). A further increase of MS-275 to 100  $\mu$ M led to an apoptotic rate of 65% after 48 h (Fig. 2B), which did not increase after 72 h.

A combination of the two substances, CYC-202 and MS-275, increased the apoptotic cell rate of Hep3B cells in a time- and dose-dependent manner. After a 24 h incubation with 10  $\mu$ M, the two substances induced an apoptotic rate of 20% and treatment with a combination of 100  $\mu$ M increased apoptosis to 33% (data not shown). A further increase of 47%

apoptosis at 10  $\mu$ M and 70% at 100  $\mu$ M occurred after 48 h treatment (Fig. 2C). No further increase was observed after 72 h incubation in Hep3B (data not shown). Similar results were obtained for HepG2. After 24 h we noted an apoptotic rate of 5% (10  $\mu$ M) and 54% (100  $\mu$ M), respectively (data not shown). A further incubation of 48 h increased apoptosis to 20% (10  $\mu$ M) and 76% (100  $\mu$ M) (Fig. 2C). After 72 h, no further increase was observed at these concentrations, but 1 and 10  $\mu$ M also led to an increase in sub-G<sub>1</sub>-events (data not shown).

*The combination of MS-275 and CYC-202 induces a high activation of p21<sup>cip1/waf1</sup>.* In Western blotting, expression of the proapoptotic bax in HF cells was not detectable after incubation with the single agents MS-275, CYC-202 or their combination (all at 100 or 10  $\mu$ M) at different times at 24 or 48 h (Fig. 3). In contrast, an expression of bax was noted in HepG2 cells after 24 h. A high expression of the anti-apoptotic bcl-2 was detectable in HF cells, especially after 48 h as a sign of protection against apoptosis (Fig. 3). These results confirm the results of flow cytometry (Fig. 2), where apoptosis in HF cells is lower than in HepG2. Of note was the high expression of p21<sup>cip1/waf1</sup> in HepG2 cells mainly treated with MS-275 alone or in combination with CYC-202 after 24 and 48 h, which confirms the p53-dependent control of p21<sup>cip1/waf1</sup>. In Hep3B cells no expression of p21<sup>cip1/waf1</sup> was observed (data not shown).

*Loss of  $\Delta\Psi_m$  parallels induction of apoptosis.* Flow cytometry of the mitochondrial membrane potential by JC-1 staining revealed that in Hep3B cells, ~88% of untreated control cells



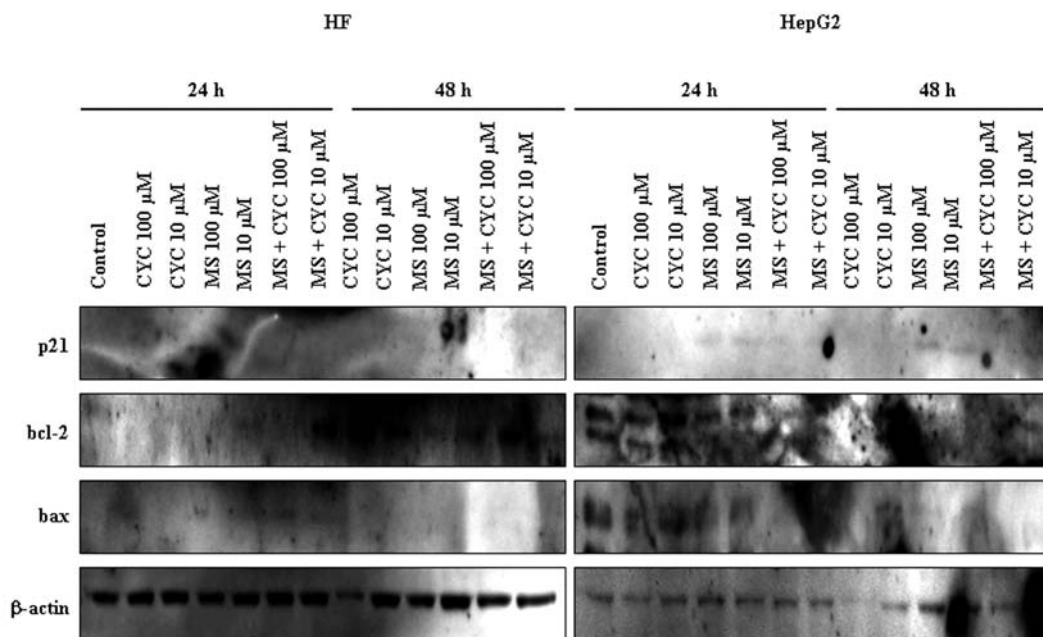


Figure 3. Western blotting of HepG2 and HF treated with different concentrations of CYC-202 and MS-275 alone or in combination for 24 and 48 h. Samples were probed with antibodies against bax, bcl-2, p21<sup>cip1/waf1</sup> and β-actin as an internal standard to show the equal loading of lanes.

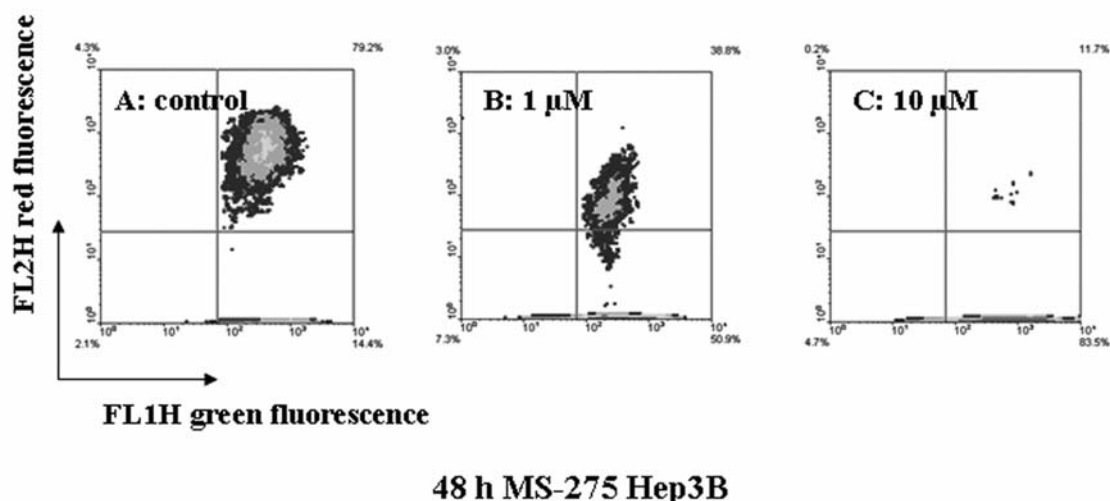


Figure 4. JC-1 staining of the mitochondrial potential  $\Delta\psi_m$ . Representative examples of Hep3B cells treated with MS-275 for 48 h are shown. (A) Untreated control, (B) 1  $\mu$ M and (C) 10  $\mu$ M. A quadrant analysis of 10,000 acquired events, gated for cell viability, was performed to determine the percentage of cells stained positively for monomeric (green fluorescence detector FL1-H, x-axis) as well as dimeric JC-1 (red fluorescence detector FL2-H, y-axis).

contained intact mitochondria, while CYC-202 reduced this level time- and dose-dependently by 85% (100  $\mu$ M) and 17% (1  $\mu$ M) after 24 h; and 40% (10  $\mu$ M) and 7% (1  $\mu$ M) after 48 h (data not shown). MS-275 reduced the mitochondrial membrane potential time- and dose-dependently to 86% (100  $\mu$ M) and 61% (10  $\mu$ M) after 24 h (data not shown), and 62% (100  $\mu$ M) and 6% (10  $\mu$ M) after 48 h (Fig. 4). These values correlated significantly with the apoptotic induction as determined by propidium iodide staining with a Spearman's correlation coefficient ( $R^2$ ) of 0.9 and were confirmed in HepG2 cells (data not shown).

*Increased cleavage of cytokeratin 18 by MS-275 and CYC-202.* Verification of apoptosis with immunofluorescence staining of cytokeratin 18 cleavage fragments showed a marked increase in positively stained cells with morphological signs of apoptosis after treatment with MS-275 and CYC-202 alone (Fig. 5) and in combination (data not shown). In the untreated controls only background fluorescence was detected.

*MS-275 and CYC induce activity of caspase 3 but not of caspase 8.* A high caspase 3 activity was detected in HepG2 and Hep3B cells treated with the single agents alone or in

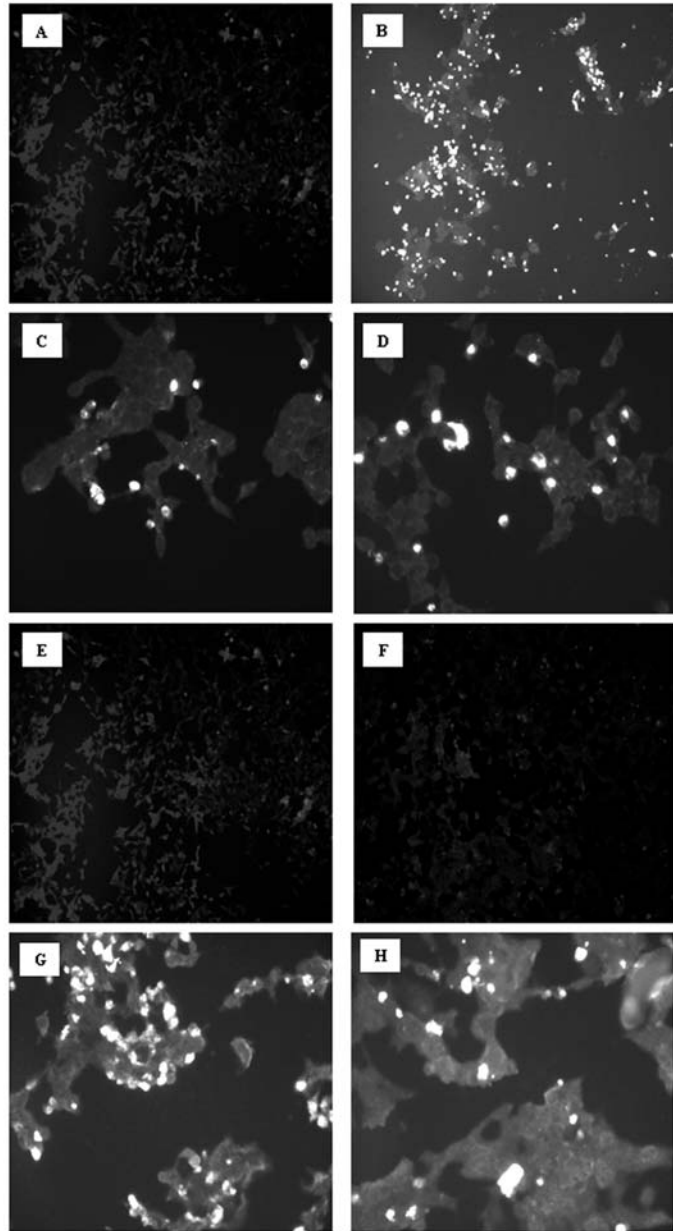


Figure 5. Immunofluorescence staining of apoptotic cytochrome c cleavage. Representative examples of HepG2 cells treated with MS-275 and CYC-202 for 24 and 48 h (x10). Apoptotic cells exhibit bright fluorescence, while vital cells show only background fluorescence. (A) Untreated control, 24 h (x10); (B) 100  $\mu$ M CYC-202, 48 h (x10); (C) 10  $\mu$ M CYC-202, 24 h (x40); (D) 10  $\mu$ M CYC-202, 48 h (x40); (E) untreated control, 48 h (x10); (F) 10  $\mu$ M MS-275, 48 h (x10); (G) 100  $\mu$ M MS-275, 48 h (x40) and (H) 10  $\mu$ M MS-275, 48 h (x40).

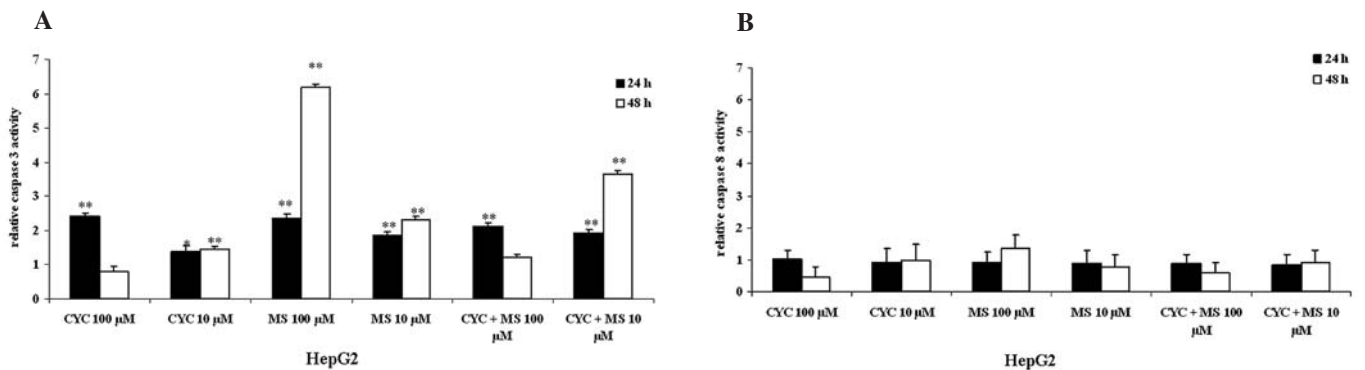


Figure 6. Assessment of caspase 3 (A) and 8 activity (B) in HepG2 cells after treatment with CYC-202 and MS-275 for 24 and 48 h by a luminometric substrate cleavage assay. Activity of caspase 3 and of 8 was set at 1.0 for untreated controls. Values for treated cells are expressed in relation to untreated control cells. Means  $\pm$  SD of three independent experiments are shown. \* $p \leq 0.05$  vs. untreated cells and \*\* $p \leq 0.01$  vs. untreated cells.

combination after 24 and 48 h (Fig. 6A), proving apoptotic cell death. Activity of the initiator caspase of the extrinsic apoptotic pathway, caspase 8, was noted in neither HepG2 (Fig. 6B), nor in Hep3B cells (data not shown), thereby confirming JC-1 and Western blotting results showing mitochondrial cell death.

## Discussion

Hepatocellular carcinoma (HCC) is one of the most common and progressive malignancies worldwide. The prognosis for patients with HCC is poor because HCC is chemotherapy-resistant and there is no effective treatment for this advanced and metastatic disease (1,2,5).

Several therapeutic agents have been evaluated and we have shown that histone-deacetylase inhibitors, such as trichostatin A (TSA) or SAHA, had potent anti-proliferative and pro-apoptotic effects in hepatoma cell lines *in vitro* alone (22) or in combination with chemotherapeutic substances (24), which are ineffective without SAHA, but led to mitochondrial apoptotic induction in SAHA-sensitized hepatoma cells. The histone-deacetylase inhibitor MS-275 is structurally dissimilar to SAHA or TSA (10) and specifically inhibits class I HDAC isoforms (25).

In our *in vitro* experiments we showed that the combination of the histone-deacetylase inhibitor MS-275 and the CDK inhibitor CYC-202 in human hepatoma cell lines Hep3B and HepG2 had anti-proliferative (Fig. 1C) and pro-apoptotic effects (Fig. 2C). Furthermore, a combination of these agents had an additive anti-tumoral efficacy (Fig. 1), confirming the results described above (22,24).

MS-275 induced the expression of the endogenous CDKi p21<sup>cip1/waf1</sup> in a p53-dependent manner in HepG2 but not in Hep3B cells as was observed previously for other HDACi (9,23,26). This observation explains the additive effects of the combination of CYC-202 and MS-275, while MS-275 increases the expression of CDKi p21<sup>cip1/waf1</sup> and leads to an accumulation of cells in the G<sub>1</sub> phase (14,27), CYC-202 functions as a competitive antagonist and is a potent inhibitor of the Cdk2-cyclin E complex as well as of Cdk7, Cdk9 and Cdk5 (18). Although we observed a pronounced growth arrest in the combination of MS-275 and CYC-202 in non-malignant fibroblasts, this cell line did not respond to a significant induction of apoptosis, indicating that the pro-apoptotic effects of this therapy are independent of the cell cycle blocking effects and that this treatment may exert tolerable toxicities *in vivo*. The up-regulation of p21<sup>cip1/waf1</sup> has also been shown to lead to the suppression of bcl-2 in human hepatoma cells (28) and therefore shift the bax/bcl-2 ratio towards pro-apoptosis as was demonstrated here.

In our study, MS-275, as well as the combination with CYC-202, induces apoptosis via the intrinsic mitochondrial pathway as shown by JC-1 staining, down-regulation of bcl-2 and no activation of caspase 8 which confirms previous findings for other HDACi by us and others (22,23,27,29-32). Apart from a direct transcriptional regulation of proliferation- and survival-regulating genes by HDACi, activation of the intrinsic apoptotic-inducing pathway can be triggered by a HDACi-mediated generation of reactive oxygen species (ROS) that leads to DNA double-strand breaks (33-35).

Interestingly, roscovitine has been shown to enhance the generation of ROS, and amplify HDACi effects (36).

HDACi have been shown to mediate their effects via p53-dependent and -independent pathways (23). Activation of the p53-response (e.g. by generation of ROS) has recently been demonstrated for roscovitine (20,37,38) which explains the greater sensitivity of p53 wild-type HepG2 cells compared to p53-deficient Hep3B cells in our experiments.

In addition to combinations of an HDACi with conventional cytotoxic agents, e.g. 5-FU or irinotecan (24), agents that specifically target tumor-specific molecular alterations have been investigated. However, combinations on pro-apoptotic receptor agonists, e.g. TRAIL (23,31,39), or small molecule receptor tyrosine kinase inhibitors, such as sorafenib (27) or imatinib (40) are emphasized.

In conclusion, CYC-202 and MS-275 inhibit cell proliferation and growth, induce apoptosis and influence the expression and activity of apoptotic- and proliferation-related proteins. In contrast to monotherapy, a combination of CYC-202 and MS-275 is highly effective in yielding proliferation and enhancing apoptosis in human hepatoma cells *in vitro*. The combination of CDK-inhibitor CYC-202 and the histone deacetylase-inhibitor MS-275 acts synergistically. Our results *in vitro* can serve as the basis for further *in vivo* experiments and clinical trials.

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