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Mutual amplification of apoptosis by statin-induced mitochondrial stress and doxorubicin toxicity in human rhabdomyosarcoma cells

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1 Besides their cholesterol-lowering effect, 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors (statins) show antiproliferative behaviour, which has been suggested as a promising anticancer strategy. However, the signalling cascades leading to statin-induced death of cancer cells are poorly characterized.

2 Here we show that statins activate the mitochondrial pathway of apoptosis in rhabdomyosarcoma RD cells *via* translocation of Bax from the cytosol to mitochondria. The prototypical representative of statins, simvastatin, induced consecutive activation of caspase 9 and 3 in a concentration-dependent manner.

3 The permeability transition pore inhibitor bongkrekic acid was capable of completely preventing simvastatin-induced caspase 9 and 3 activity, corroborating the mitochondrial pathway of apoptosis as the sole mechanism of statin action. Alternative pathways *via* death receptors, that is, caspase 8 or calpain activation, were not triggered by simvastatin.

4 Simvastatin-treated RD cells could be completely rescued from apoptosis by the co-application of mevalonic acid, indicating that deprivation of cholesterol precursors is essential for statin-induced apoptosis.

5 However, pretreatment with subthreshold concentrations of simvastatin was sufficient to augment doxorubicin toxicity *via* the mitochondrial apoptotic machinery. Moreover, the presence of doxorubicin increased the potency of simvastatin to trigger caspase activation.

6 Taken together, these data highlight the therapeutic anticancer potential of statins and their additivity and mutual sensitization, in combination with doxorubicin in human rhabdomyosarcoma cells.

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Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; PBS, phosphate-buffered saline

Introduction

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, commonly referred to as statins, are widely used in hypercholesterolaemia and preventing cardiovascular events (S4 group, 1994; 2000). Statins inhibit the rate-limiting step in the mevalonate pathway and by this prevent endogenous *de novo* synthesis of cholesterol (Goldstein & Brown, 1990). Although the therapeutic effect of statins has been attributed to HMG-CoA reductase inhibition, recently, reports that show additional pleiotropic properties like prevention of bone mass loss (Mundy *et al*., 1999), antiproliferative (Munro *et al*., 1994) and anti-inflammatory effects (Weitz-Schmidt, 2002) have accumulated, which may help additionally to explain the reduction of atherosclerotic plaques and cardiovascular complications (Bellosta *et al*., 2000; Vaughan, 2003).

Statins have been faced with concerns regarding their long-term safety. In particular, experiments with rodents and epidemiological studies in humans suggested potential carcinogenicity of statins or low cholesterol levels (Kritchevsky & Kritchevsky, 1992; Newman & Hulley, 1996). In contrast, long-term studies on cardiovascular diseases treated with statins reveal no evidence for a risk of cancer due to low serum cholesterol levels or the drugs *per se* (S4 group, 2000; Bjerre & Lelorier, 2001). Furthermore, a nested case–control study demonstrated that cancer was less often diagnosed under statin treatment (28%) compared to patients receiving bile acid-binding resins (Blais *et al*., 2000). In the line of the latter findings, phase I and II trials are currently under investigation to evaluate statin's role and efficacy in various human tumour types (Larner *et al*., 1998; Kawata *et al*., 2001; Kim *et al*., 2001).

Although preclinical trials successively confirm an anticancer efficacy of statins, the molecular mechanisms leading to cell death are poorly investigated (Chan *et al*., 2003). Nevertheless, *in vitro* data show that statins are capable of arresting cancer cells in the G_1/S phase transition *via* a mevalonate-dependent mechanism (Jakobisiak *et al*., 1991; Keyomarsi *et al*., 1991). Most importantly, statins are capable of triggering apoptosis in a tumour-specific manner (Rao *et al*., 1998; Fritz *et al*., 2003; Li *et al*., 2003). Cell lines deriving from breast and prostate cancer are insensitive to statin-induced apoptosis (Dimitroulakos *et al*., 2001). In contrast, tumour

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cells deriving from leukaemia, squamous carcinoma, lung tumours, the central nervous system and pancreas cancers are sensitive to statins (Hawk *et al*., 1996; Kikuchi *et al*., 1997; Clutterbuck *et al*., 1998; Dimitroulakos *et al*., 2001). Nevertheless, a tumour-specific delineation of the statin-induced apoptotic pathway is ill defined and therefore a prerequisite for rational therapeutic administration.

As the overall outcome in paediatric rhabdomyosarcoma is still a therapeutic challenge, we have chosen human rhabdomyosarcoma RD cells to investigate statin sensitivity. Simvastatin and lovastatin induce apoptosis in these cells *via* the mitochondrial pathway strictly dependent on cholesterol precursors. Bax translocation independent of caspase 8 or calpain plays a crucial role in simvastatin-induced apoptosis. Consecutive activation of caspase 9 and 3 by simvastatin was sensitized by the anthracyclin doxorubicin in a concentration-dependent manner. This synergistic induction of cell death by the combination of simvastatin and doxorubicin highlights the therapeutic potential of statins as novel anticancer drugs.

Methods

Reagents

The detergent 3((3-cholamidopropyl)-dimethylammonio)-1-propane sulphonate (CHAPS), DL-mevalonic acid, bongkrekic acid and protease-inhibitors (pefablock, leupeptin and aprotinin) were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Goat anti-rabbit Cy3-tagged antibody and horseradish peroxidase-conjugated antibodies were purchased from Amersham Pharmacia Biotech (Little Chalfront, Buckinghamshire, U.K.); MitoTracker®- and Alexa-488-conjugated goat anti-mouse antibody from Molecular Probes (Eugene, OR, U.S.A.). An antibody against p53 (Ab-6) was from Calbiochem (San Diego, CA, U.S.A.), against Bax (N-20) from Santa-Cruz (Santa-Cruz, CA, U.S.A.) and against actin (AC-40) from Sigma Chemical Co. (St Louis, MO, U.S.A.). An antibody against cleaved caspase 3 was purchased from Cell Signalling Technology (Beverly, MA, U.S.A.). Simvastatin and lovastatin were provided by Merck Research Laboratories (Rahway N.J., U.S.A.) and alternatively purchased from Calbiochem (San Diego, CA, U.S.A.).

Cell culture

All experiments were carried out with cultured human rhabdomyosarcoma cells (RD cells) of the spindle-cell type obtained from ATCC (Manassas, VA, U.S.A.). The RD cells were kept in growth medium (Dulbecco's modified Eagle's medium (DMEM), 10% foetal calf serum (FCS), 50 U ml^{-1} penicillin G and 50 μg ml^{-1} streptomycin) at 37°C under 5% CO_2 for 2–5 days prior to experiments.

Caspase assays

RD cells were incubated with various concentrations of statins and/or other drugs for the indicated times and concentrations. Thereafter, the cells were washed with phosphate-buffered saline (PBS) and exposed to ice-cold caspase-lysis buffer (25 mM HEPES (pH 7.4), 5 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2$, 5 mM DTT), which was supplemented with protease inhibitors immediately before application (1.4 μg ml^{-1} aprotinin; 10 μg ml^{-1} leupeptin; 100 μM pefablock). Membranes and microsomal fractions were separated by centrifugation at 26.000 × *g* at 4°C for 20 min. The supernatant was collected and the pellet resuspended in 400 μl caspase lysis buffer. Finally, the samples were stored at −80°C. The substrates for caspase 3 (Ac-Asp-Glu-Val-Asp-AFC (Ac-DEVD-AFC)), caspase 8 (Ac-Leu-Glu-Thr-Asp-AFC (Ac-LETD-AFC)) and caspase 9 (Ac-Leu-Glu-His-Asp-AFC (Ac-LEHD-AFC)) were obtained from Alexis Biochemicals (Carlsbad, CA, U.S.A.). Aliquots of the supernatant (10–50 μg) were incubated with reaction buffer (25 mM HEPES (pH 7.4), 10% sucrose, 1.4 mM CHAPS and 5 mM DTT) and 50 μM 7-amino-4-trifluoromethylcoumarin (AFC)-conjugated substrate at 37°C for 90 min in the dark. The change in fluorescence was measured at an excitation wavelength of 405 nm and an emission wavelength of 535 nm by a fluorescence plate reader, VICTOR-2 (Perkin-Elmer, Wellesley, MA, U.S.A.). As a negative control, the AFC-conjugated substrates were diluted in lysis buffer and reaction buffer in the absence of protein.

Calpain assay

A calpain assay kit was obtained from Calbiochem (San Diego, CA, U.S.A.). Purified calpain was used as a positive control. As a negative control, the reaction was carried out in the presence of a calpain inhibitor (Z-LLY-FMK). The reaction was started by the addition of a fluorescent calpain substrate (Ac-LLY-AFC). After 1 h at 37°C in the dark, the generated fluorescence was measured similar to caspase assays.

Western blot analysis

The pellets and supernatants obtained for the caspase assays were also used for Western blot analysis. The proteins were resolved on an SDS–polyacrylamide gel and transferred to a nitrocellulose membrane (pore size 0.45 μM, Schleicher & Schuell; Dassel, Germany). For immunodetection, the membrane was blocked and exposed to antibodies against Bax or actin. Appropriate secondary antibodies conjugated to horseradish peroxidase enabled detection by enhanced chemiluminescence detection system from Pierce (Rockford, IL, U.S.A.).

Immunohistochemistry and confocal fluorescence microscopy

RD cells treated as indicated in the figure legends were washed with PBS and fixed with 4% paraformaldehyde (PFA) for 30 min. The cells were permeabilized for 5 min with 0.1% Triton X-100 and 0.1% citrate in PBS and blocked with 2% BSA in PBS for 60 min. The slides were exposed to antibodies against Bax, p53, cleaved caspase 3 or mitochondria (Alexa 488-conjungated Mito-Tracker®) diluted 1 : 200 for 1 h at 37°C. The cells were incubated for an additional hour with the appropriate secondary antibodies, Cy3-conjugated (diluted 1 : 500) or Alexa-488-conjugated (diluted 1 : 500). The slides were dried, mounted and stored at 4°C. As negative control, cells were treated under identical conditions without applying a primary antibody. Images were collected using a confocal microscope from Zeiss (Axiovert 100), equipped with an argon laser system (LSM 410; Jena, Germany). The digitized pictures

were stored and prepared for presentation off-line using MetaMorph® software (West Chester, PA, U.S.A.).

Miscellaneous procedures

Experiments were at least repeated twice and presented as mean ± s.d., if not otherwise stated. The concentration–response curves were subjected to nonlinear least-squares regression to the Hill equation using the Sigmaplot software (Jandl, Erkrath, Germany). Statistical analysis was carried out by Student's *t*-test, or multiple comparison was performed by ANOVA and *post hoc* Scheffe test. A value of $P<0.05$ was considered statistically significant.

Results

Morphological effects of simvastatin and lovastatin on human RD cells

Human RD cells undergo dramatic morphological changes after exposure to simvastatin for 24 h (Figure 1a–f). Typically, the cells were shrunken and the ratio of the nuclear/cytosolic volume increased. The overall shape of the cells turns into a spherical form with condensed nuclei and finally cells detach from the surface. Mevalonic acid was capable of rescuing the cells from simvastatin-induced morphological alterations which are typical for cells undergoing apoptosis (Figure 1c and f).

If the conjecture holds true that 30 μM simvastatin induces apoptosis or cell-cycle arrest already after 24 h, one would expect that the number of viable cells should decrease within this time scale. The two members of the statin family, simvastatin (Figure 1g) and lovastatin (Figure 1h), reduced the number of viable cells in a time- and concentration-dependent manner. Already at therapeutical concentrations between 0.1 and 1 μM, the statins were capable of reducing the cell number by 10–40% within 48 h. The reduction in cell number was more pronounced with lovastatin, especially at a concentration of 1 μM (Figure 1h). This is surprising and unclear at the moment because the potency to inhibit the HMG-CoA reductase is comparable for simvastatin and lovastatin (Corsini *et al*., 1999). Nevertheless, the reduction in cell number was reversed by mevalonic acid (data not shown).

Simvastatin-induced caspase activation

In order to confirm apoptosis, we measured the activation of caspases. The effector caspase 3 plays a central role in

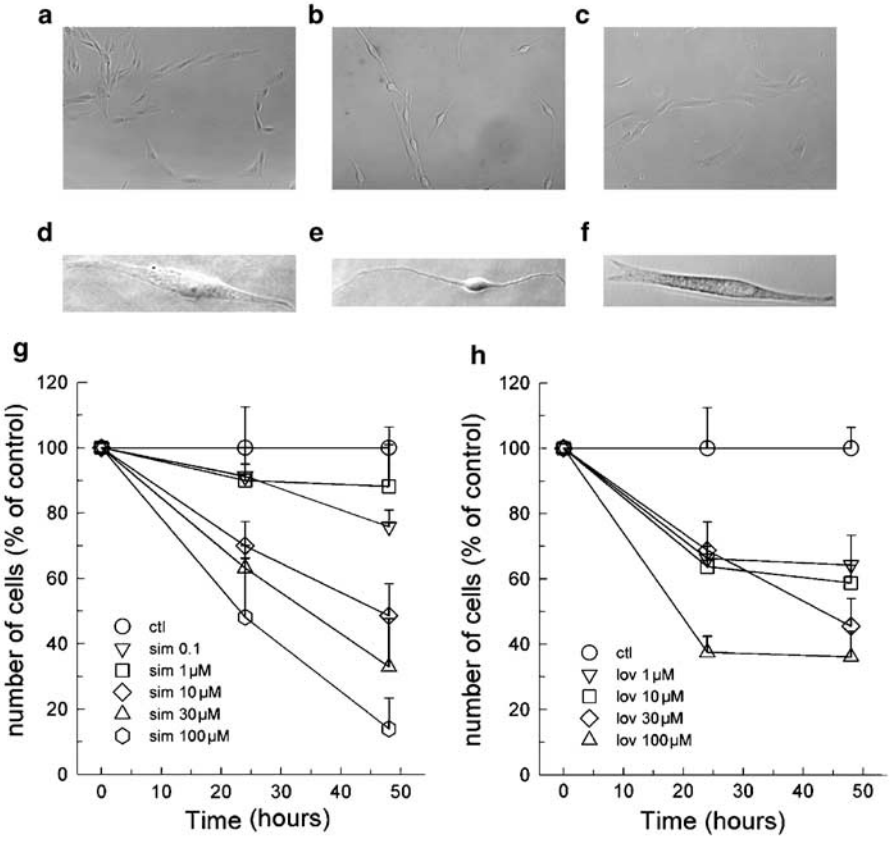
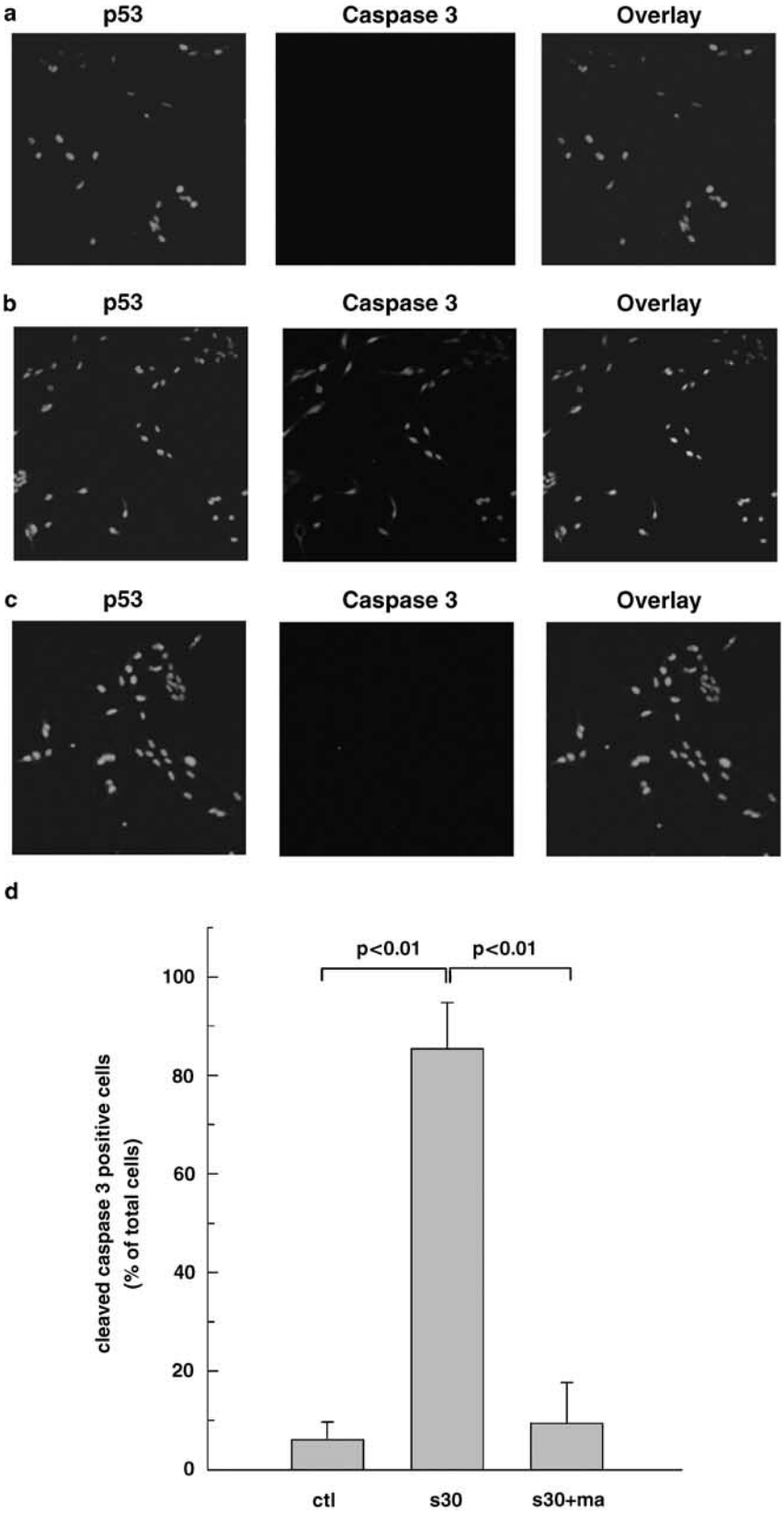


Figure 1 Statin-induced reduction in viable RD cells. RD cells were kept in growth medium in the absence (a, d) and presence (b, e) of 30 μM simvastatin or the combination of 30 μM simvastatin with 1 mM mevalonic acid (c, f) for 24 h. Overviews (a–c; × 20) and magnifications of representative cells (d–f; × 63) were photographed under a confocal laser microscope. The kinetics of simvastatin- (G; Sim) and lovastatin- (H; Lov) induced reduction of viable RD cells were observed over 48 h. Trypanblue-stained cells were counted and subtracted from the total number of attached RD cells. The data represent the mean ± s.e.m., of three pictures taken under the confocal laser microscope.

apoptosis as it translocates from the cytosol into the nucleus upon activation (Ferri & Kroemer, 2001). While under control conditions cleaved caspase 3 was hardly detectable (Figure 2a), upon simvastatin administration the fraction of activated caspase 3 increased significantly in the nuclei (Figure 2b and d, $P<0.01$). The increment of cleaved caspase 3-positive cells was



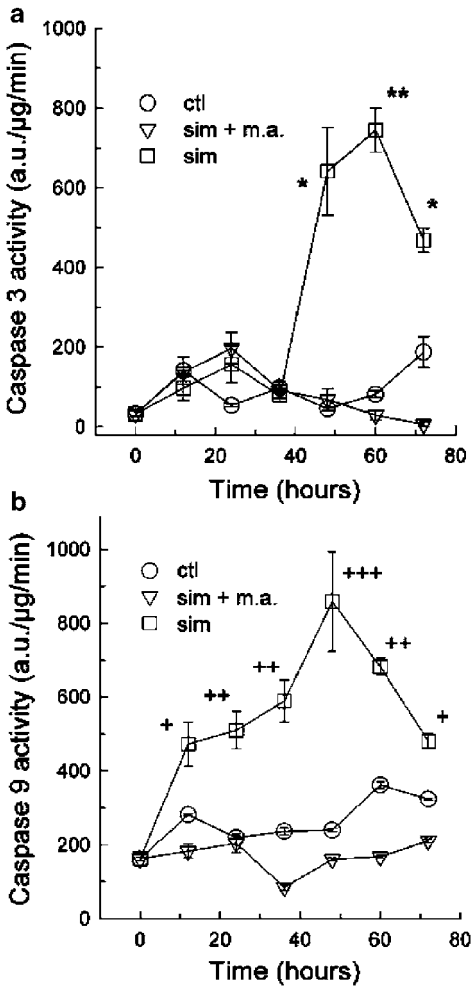


Figure 3 Time course of the simvastatin-induced caspase 3 and 9 activation. RD cells were incubated in the absence (ctl) and presence of 10 μM simvastatin (sim) or 10 μM simvastain plus 1 mM mevalonic acid (sim + m.a.). The cytosolic (50 μg) fraction of the cells was used to cleave specific fluorescent substrates for caspase 3 (a) and 9 (b). The data are given as mean values $\pm$ s.e.m. ($n = 8$–16). The asterisks and crosses denote statistical significance between control and simvastatin treatment ($^{*}P < 0.005$; $^{**}P < 0.001$; $^{+}P < 0.01$; $^{++}P < 0.005$, $^{+++}P < 0.001$).

approximately 70%, which is in good agreement with a 70% reduction of viable cells by 30 μM simvastatin after 48 h (Figure 1g). The co-application of mevalonic acid prevented simvastatin-induced caspase 3 cleavage (Figure 2c).

The kinetic analysis of caspase activity facilitated the discrimination between different onsets of simvastatin-induced caspase activation. The very downstream caspase 3 showed a strong activation peak between 48 and 60 h upon simvastatin exposure (Figure 3a). Moreover, the caspase 3 activation was again completely abrogated by the co-application of mevalonic acid, confirming the immunostainings described in Figure 2. Since two signalling pathways, a death-receptor-mediated and mitochondrial stress-induced cascade, lead to caspase 3 activation, we have also measured the activity of caspase 8 and 9 (Ferri & Kroemer, 2001; Igney & Krammer, 2002). Caspase 9 activity was significantly elevated in RD cells after 12 h of exposure to 10 μM simvastatin (Figure 3b). Again, the co-administration of mevalonic acid could completely prevent caspase 9 activation by simvastatin. Of utmost importance is the fact that the kinetics of simvastatin-induced caspase 9 activation precede that of caspase 3. Conversely, caspase 8 was not triggered by simvastatin (data not shown). Even in cells exposed to 30 μM simvastatin for 1 day, caspase 8 activity equals that measured in untreated RD cells (960.7 $\pm$ 19.8 *versus* 1168.3 $\pm$ 27.5 a.u. μg min^{-1}, respectively; mean $\pm$ s.e.m., $n = 4$).

Calpain is not activated in RD cells by statins

As we have already excluded a death receptor-mediated activation of caspase 8, possibly activation of the Ca^{2+}-activated cysteine protease, calpain, may participate in statin-induced apoptosis (Wang, 2000; Hajnoczky *et al*., 2003). Even high concentrations of simvastatin or lovastatin were not capable of triggering specific cleavage of the fluorescent calpain substrate and did not exceed the basal level determined by the negative control (Figure 4). So far, these observations strongly favour the interpretation that simvastatin and

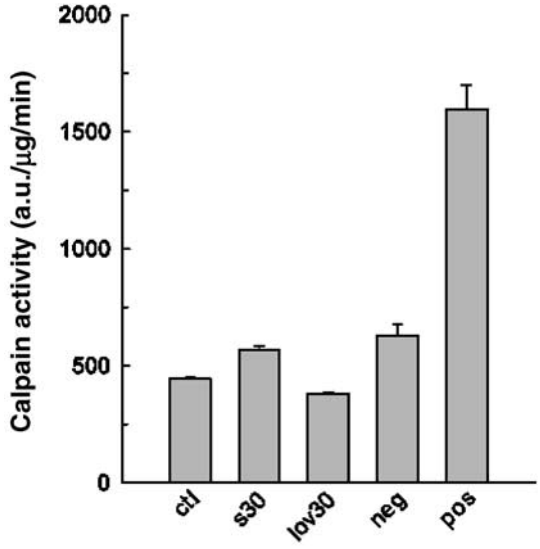


Figure 4 Statins did not induce calpain activation. RD cells were incubated for 24 h in the absence (ctl) and presence of 30 μM simvastatin (s30) or 30 μM lovastatin (lov30), and the cytosolic fraction of such treated cells was used to measure calpain activity. Background activity was obtained in the presence of a calpain inhibitor (neg) and purified calpain was used as a positive control (pos). The bars represent mean $\pm$ s.e.m. ($n = 3$) obtained from experiments carried out in duplicates.

Figure 2 Simvastatin-induced caspase 3 activation is abrogated by 1 mM mevalonic acid. RD cells were incubated for 24 h in the absence (a) and presence of 30 μM simvastatin (b) or 30 μM simvastatin supplemented with 1 mM mevalonic acid (c). Immunostaining of p53 (green) and cleaved caspase 3 (red) was visualized in RD cells with antibodies directed against the respective proteins and using Alexa 488- and Cy3-fluorophore-tagged secondary antibodies. Overlays were obtained by superimposing the pictures taken from p53 and cleaved caspase 3 immunostainings. The quantification of RD cells positive for cleaved caspase 3 is given in panel d in the absence (ctl) or presence of 30 μM simvastatin (s30) or 30 μM simvastatin and 1 mM mevalonic acid (s30 + m.a.). The data represent the mean $\pm$ s.e.m. obtained from three experiments.

lovastatin trigger the mitochondrial pathway of apoptosis in a caspase 8- and calpain-independent manner.

Concentration-dependent activation of caspase 9 and 3 by statins

The survival of RD cells exposed to simvastatin or lovastatin was strictly dependent on the concentration of statins (Figure 1g and h). In the case that apoptosis *via* the mitochondrial pathway is responsible for this observation, one would expect a concentration-dependent activation of caspase 9 and 3 by statins. This assumption is corroborated by the data depicted in Figure 5. The concentration–response curves were fitted to the Hill equation and gave EC_{50} values of $12.5 \pm 4.9\,\mu$M for simvastatin and $14.4 \pm 2.1\,\mu$M for lovastatin-activated caspase 9 (Figure 5a). Equal potency was retained by simvastatin to induce caspase 3 activity with an EC_{50} of $11.1 \pm 3.2\,\mu$M (Figure 5b).

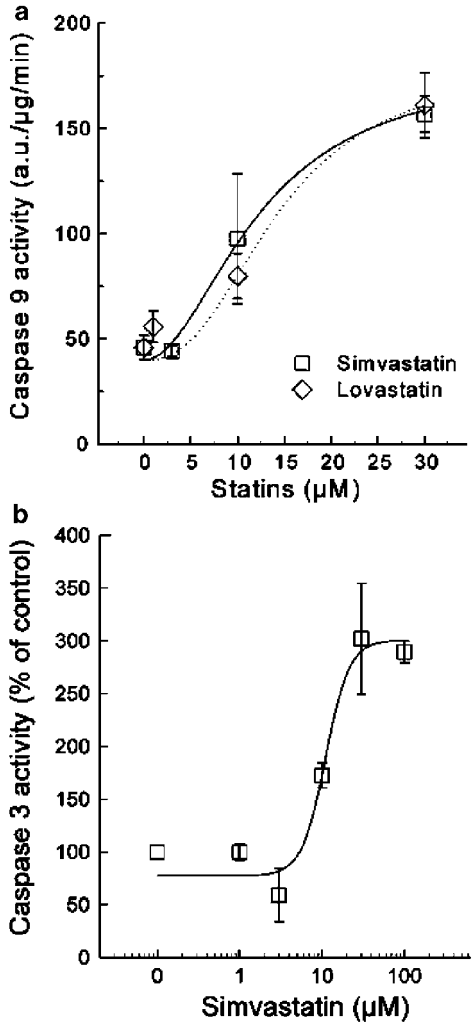


Figure 5 Concentration-dependent activation of caspase 9 and 3 by simvastatin and lovastatin. For concentration–response curves, RD cells were incubated for 24 h in the absence and presence of simvastatin or lovastatin. Aliquots of the cytosolic fraction of such treated RD cells (50 μg) were used to measure caspase 9 (a) and 3 (b) activity. The symbols represent the mean $\pm$ s.e.m. obtained from six to 11 experiments carried out in duplicates.

Simvastatin induces translocation of Bax from the cytosol into mitochondria

So far, these data indicate an activation of the mitochondrial pathway of apoptosis. Bax is functionally linked to mitochondrial swelling and formation of the mitochondrial permeability transition pore complex, and is therefore an essential upstream signal (Ferri & Kroemer, 2001; Debatin *et al*., 2002). Typically, Bax is arrested in the cytosol and upon an apoptotic stimulus it translocates into the outer mitochondrial membrane (Ferri & Kroemer, 2001). Using the Western blot analysis, in untreated RD cells Bax is mainly detectable in the cytosolic fraction. Conversely, in cells treated with simvastatin, the amount of Bax is reduced in the cytosolic fraction, but gives a strong response in the microsomal fraction (Figure 6a and b). In order to corroborate this observation, immunostainings were used as a second experimental approach (Figure 6c and d). Bax is condensed to the perinuclear space and is superimposable with the mitochondrial localization. Under control conditions, Bax is homogenously distributed over the cytosol and covers regions of the cells that were negative for mitochondrial staining (Figure 6c). The simvastatin-induced translocation of Bax was again abrogated by 1 mM mevalonic acid (data not shown).

We have also used a pharmacological approach to confirm activation of the mitochondrial pathway of apoptosis by application of simvastatin. Bongkrekic acid is a potent inhibitor of the adenine nucleotide transporter, which is an essential part of the mitochondrial permeability transition pore complex (Klingenberg, 1980; Debatin *et al*., 2002). While bongkrekic acid *per se* had no effect on caspase 3 and 9 activity, it was capable of completely preventing caspase activation induced by 30 μM simvastatin (Figure 7).

Synergistic efficacy of doxorubicin and simvastatin to induce caspase-specific apoptosis

The anthracycline doxorubicin is widely used to treat various types of cancer, especially soft tissue sarcoma in children (Hortobagyi, 1997; Koscielniak *et al*., 2002). Doxorubicin is known to induce apoptosis in a dose-dependent mechanism in many cell lines including rhabdomyosarcoma cells (Tomek *et al*., 2003). In our case, doxorubicin was capable of increasing the potency of simvastatin to trigger caspase 9 and 3 activity. The EC_{50} values for simvastatin-induced caspase 9 activation were progressively shifted from $12.4 \pm 4.9\,\mu$M in the absence of doxorubicin to $6.8 \pm 1.1\,\mu$M and $4.0 \pm 1.3\,\mu$M ($P < 0.01$) in the presence of 0.1 μM and 1 μM doxorubicin, respectively (mean $\pm$ s.d., $n = 4$). Similar results were obtained for caspase 3 (data not shown). If doxorubicin is capable of sensitizing RD cells for simvastatin, conversely, simvastatin should facilitate doxorubicin-caused apoptotic stimuli. Thus, a strong activation of caspase 9 or 3 by 30 μM simvastatin was further enhanced significantly by increasing concentrations of doxorubicin (Figure 8a and b). In order to mimic a therapeutic situation, RD cells were treated with a subthreshold concentration of 1 μM simvastatin. Although not significant, it is clear that caspase 9 and 3 were activated within 48 h in a consecutive manner (Figure 8c). Interestingly, in RD cells pretreated with 1 μM simvastatin, the doxorubicin toxicity was significantly augmented compared to RD cells without simvastatin pretreatment (Figure 8d). Taken together, these results confirm

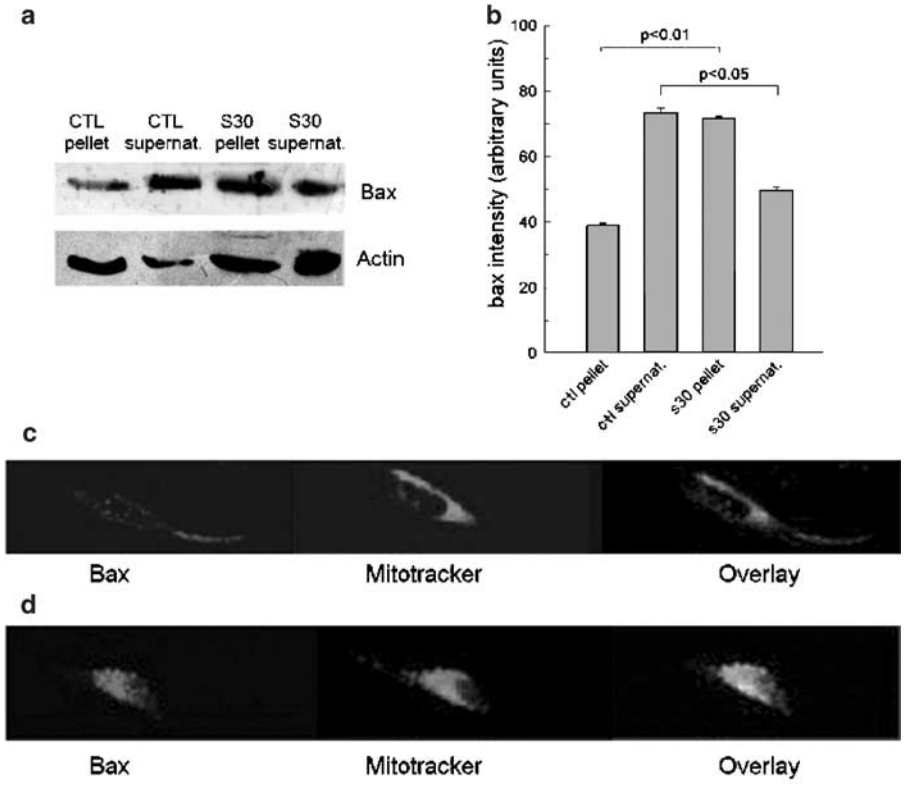


Figure 6 Simvastatin triggers the translocation of Bax from the cytosol into the microsomal fraction of RD cells. RD cells were incubated for 24 h in the absence (CTL) and presence of 30 μM simvastatin (s30). The cells were harvested and cytosolic (supernat.) and microsomal (pellet) fractions were obtained by centrifugation. The protein was subjected to Western blot analysis using antibodies to Bax and actin (a). The intensity of the Bax protein band was recorded densitometrically with the Scion® software program and normalized to that of the actin band (b). Statistical significance was calculated using Student's *t*-test. The bars represent the mean values $\pm$ s.e.m. obtained from five measurements. Immunostaining of mitochondria and Bax in untreated (c) and 30 μM simvastatin (d)-treated RD cells using Mitotracker® (green) and a specific antibody against Bax (red). The distribution of Bax was visualized by a Cy3-conjugated secondary antibody. The Overlay was generated by superimposing the images produced by Cy3 and Mitotracker®. The pictures depict a single plane obtained by confocal laser scan microscopy.

that simvastatin and doxorubicin synergistically enhance the mitochondrial pathway of apoptosis in human RD cells.

Discussion

The rhabdomyosarcoma is highly malignant and is the most common soft tissue sarcoma in childhood (McDowell, 2003). Although the overall survival of childhood rhabdomyosarcoma has improved during the recent decades, poor treatment response and drug toxicity are still major clinical problems. A possibly new approach in the treatment of tumours is the use of HMG-CoA reductase inhibitors, commonly referred to as the statins (Wong *et al*., 2002; Chan *et al*., 2003). Statins are introduced to treat hypercholesterolaemia and are well tolerated, besides occasionally occurring skeletal muscle side effects (Corsini *et al*., 1999). At the moment, phase I and phase II studies on adjuvant tumour therapy with statins are available; however, little is known about the molecular events leading to apoptosis of cancer cells due to statin exposure, especially when combined with other chemotherapeutic agents.

We have assessed the molecular mechanisms leading to apoptosis by the prototypical statin simvastatin in human rhabdomyosarcoma RD cells. Caspase 9 and 3 were activated in a time- and concentration-dependent manner (Figures 3 and 5). Both activations are fully reversible with mevalonic acid and are therefore attributable to the inhibition of the HMG-CoA reductase. Simvastatin and lovastatin are equipotent to inhibit HMG-CoA reductase (Corsini *et al*., 1995). At 1 μM, lovastatin already gave a strong signal to reduce viable cells (Figure 1h) compared to simvastatin (Figure 1g). The reason for this discrepancy is unclear at the moment. Nevertheless, the potency to trigger caspase 9 activity was nearly identical for simvastatin and lovastatin (Figure 5). This observation is an indirect hint that the apoptotic stimulus is raised from a common target, that is, depletion of a cholesterol precursor. The main apoptotic features evoked by simvastatin were abrogated by mevalonic acid. Thus, depletion of a cholesterol precursor is essential to explain statin effects rather than cholesterol depletion *per se*. This assumption is based on the fact that cholesterol is present in the cell culture medium in concentrations of 0.2–0.4% (suppliers information), which is sufficient to prevent cholesterol depletion in RD cells.

Statins arrest the growth of cancer cells at the G1/S phase boundary of the cell cycle in a p53-independent manner (Jakobisiak *et al*., 1991; Keyomarsi *et al*., 1991; Rao *et al*., 1998). At the molecular level, cyclin-dependent kinase 2 was found to be downregulated, and conversely $p21^{cip1}$ and $p27^{kip1}$ were upregulated (Rao *et al*., 1998). As statins are engaged in cell cycle arrest, this does not suffice to trigger apoptosis (Rao

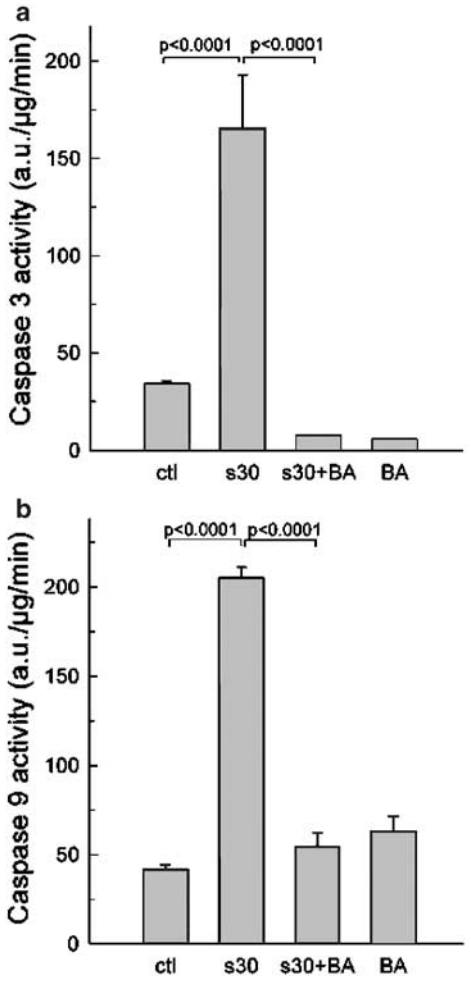


Figure 7 Bongkrekic acid abrogated simvastatin-induced caspase 3 and 9 activation. Caspase 3 (a) and 9 (b) activity was measured in the cytosolic fraction of RD cells incubated for 24 h in the absence (ctl) and presence of 30 μM simvastatin (s30), 30 μM simvastatin plus 4 μM bongkrekic acid (s30 + BA) or 4 μM bongkrekic acid (BA). The data represent mean values ± s.e.m. The level of statistical significance was calculated from multiple comparison analysis with ANOVA and *post hoc* Scheffe's test ($n = 8$–24).

et al., 1998; Dimitroulakos *et al*., 2001). A concentration of 1 μM simvastatin was enough to initiate a consecutive activation of caspase 9 and 3 within 48 h (Figure 8c). Moreover, the increment in cleaved caspase 3-positive cells (Figure 2d) is comparable to the reduction of viable RD cells by 30 μM simvastatin within 48 h (Figure 1g). These observations lend support to the contention that apoptosis in RD cells is responsible for cell number reduction rather than cell cycle arrest.

As the central mechanism of simvastatin-induced apoptosis, we could elucidate a translocation of the proapoptotic Bcl-2 family member Bax into mitochondria (Figure 6). The activation and oligomerization of Bax can be induced by truncated Bid, which in turn is a substrate of caspase 8 or calpain (Luo *et al*., 1998; Mandic *et al*., 2002). In RD cells, both proteases are not activated by simvastatin, indicating that alternative activation mechanisms for Bax translocation may exist (Figure 4). This observation is confirmed by the findings of Wei *et al*. (2001), who could demonstrate that Bid−/− cells are still sensitive for many apoptotic agents, like staurosporine, etoposide and ultraviolet light. Endogenous Bax was elucidated as an important determinant of chemosensitivity in paediatric tumour cell lines, including rhabdomyosarcoma cells, independent of Bcl-2 expression and p53 status (McPake *et al*., 1998). The authors claim that Bax may therefore represent a useful prognostic indicator for effectiveness of chemotherapeutics like doxorubicin, actinomycin D or topotecan.

By the combination of simvastatin with doxorubicin, our results suggest that statins increase the vulnerability of RD cells for apoptosis, as we observe additive effects on caspase 9 and 3 activation in a concentration-dependent manner (Figure 8). On the one hand, a high concentration of doxorubicin (1 μM) decreased the EC_{50} for simvastatin to trigger caspase 9 activity from 12.4 ± 4.9 to 4.0 ± 1.3 μM ($P < 0.01$). Conversely, caspase activity maximally stimulated by 30 μM simvastatin was further enhanced by increasing concentrations of doxorubicin (Figure 8a and b). Moreover, preincubation of RD cells with 1 μM simvastatin, a concentration usually used within conventional cholesterol-lowering therapies (Corsini *et al*., 1999), was sufficient to significantly augment the toxicity of doxorubicin (Figure 8d). Thus, the coadministration of statins with other chemotherapeutic agents may facilitate an apoptotic response in cancer cells.

This assumption is corroborated by various preclinical studies and clinical trials (Bjerre & Lelorier, 2001; Wong *et al*., 2002; Chan *et al*., 2003). *In vitro* studies have shown that statins amplify the cytotoxic effect of not only chemotherapy but also radiation. Lovastatin sensitized HeLa cells to gamma radiation-induced cytotoxic effects independent of p53/p21Waf1- and NF-κB-related mechanisms (Fritz *et al*., 2003). Similar results were obtained in primary human acute myeloid leukaemia cells which responded to radiochemotherapy more sensitive in the presence of statins (Li *et al*., 2003). In three colon cancer cell lines, 10–30 μM lovastatin augmented sulindac-induced apoptosis and the authors conclude that the combinatory application of the drugs may improve chemopreventive effects of sulindac (Agarwal *et al*., 1999). Clinical studies were conducted on various tumour types including solid tumours of the prostate and central nervous system, anaplastic astrocytoma and glioblastoma multiforme, advanced gastric cancer and advanced hepatocellular cancer (Thibault *et al*., 1996; Larner *et al*., 1998; Kawata *et al*., 2001; Kim *et al*., 2001). Only in advanced hepatocellular cancers pravastatin treatment resulted in a significant increase in median survival of 18 months compared to 9 months in the control (Kawata *et al*., 2001). Of utmost importance is the fact that in this study pravastatin was combined with standard therapeutics, that is, 5-fluorouracil. In all other studies mentioned above, statins were not combined with other anticancer drugs (Thibault *et al*., 1996; Larner *et al*., 1998; Kim *et al*., 2001). The dosage of statins also varied very much, between pulsed high dosage for 1 week or continuous application at lower dosages. Besides dosage and scheduling, an alternative explanation was that statin-insensitive tumours were included into the phase I and phase II studies. For the first time, Dimitroulakos *et al*. (2001) investigated systematically 80 human cancer cell lines for their sensitivity to lovastatin. Interestingly, those tumour cells which were sensitive to the antiproliferative effects of retinoids were found most sensitive toward lovastatin-induced apoptosis. Rhabdomyosarcoma cells, including RD cells, were found to be highly susceptible toward lovastatin treatment (Dimitroulakos *et al*., 2001).

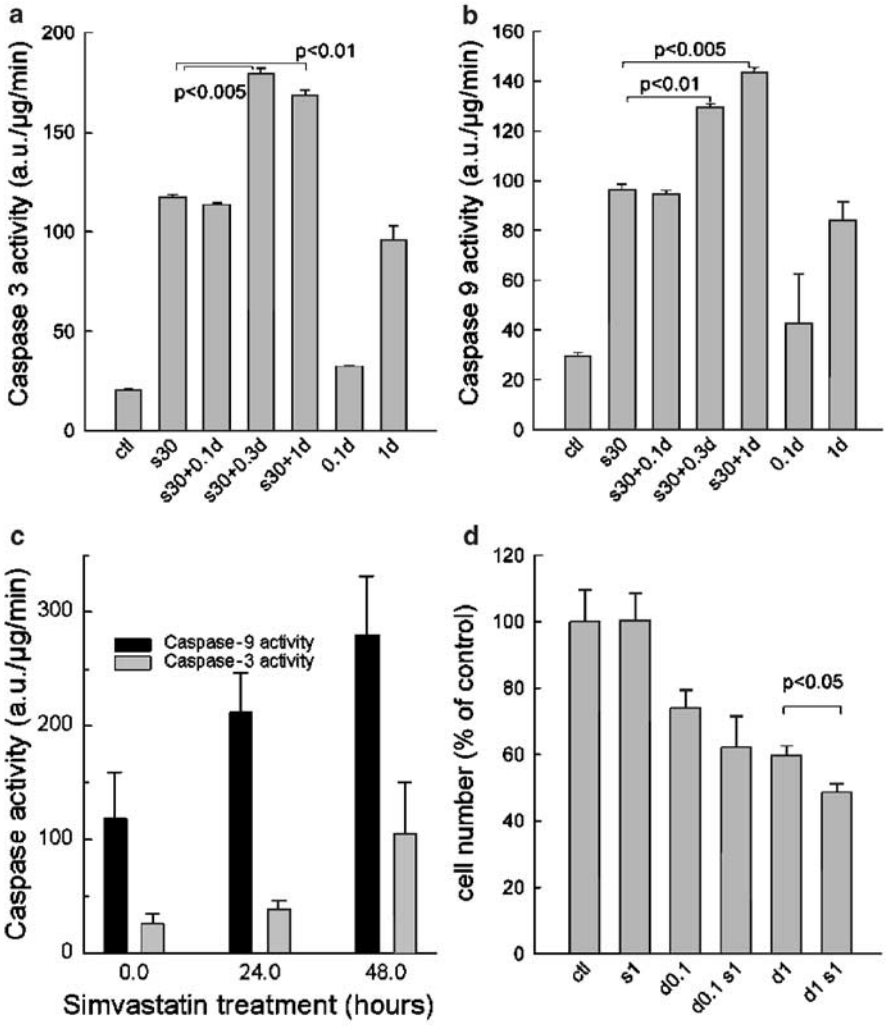


Figure 8 Additivity of simvastatin- and doxorubicin-induced apoptotic efficacy. Caspase 3 (a) and 9 (b) activity was determined in the cytosolic fraction of RD cells in the absence (ctl) and presence of 30 μM simvastatin (s30), 30 μM simvastatin plus 0.1, 0.3 or 1 μM doxorubicin (s30 + 0.1d, s30 + 0.3d or s30 + 1d) and 0.1 or 1 μM doxorubicin (0.1d, 1d) alone. The bars represent the mean ± s.e.m. The indicated statistical significance was calculated from multiple comparison analysis with ANOVA and *post hoc* Scheffe's test ($n = 2$–4). (c) Time-dependent caspase activation in the presence of 1 μM simvastatin in RD cells. The bars represent the mean ± s.e.m. ($n = 3$). (d) Reduction of viable RD cells preincubated with 1 μM simvastatin for 24 h and thereafter incubated for additional 24 h in the presence of 1 μM simvastatin (s1), 1 μM simvastatin plus 0.1 or 1 μM doxorubicin (d0.1s1, d1s1). Untreated RD cells (ctl) were compared with cells treated with 0.1 or 1 μM doxorubicin (d0.1, d1) for 24 h. Similar to Figure 1g and h, the number of viable cells was counted from photographs taken under the microscope. Statistical significance was obtained using Student's *t*-test. Five pictures (approximately 200 cells per picture) were counted for each experimental condition. The bars represent mean ± s.d.

In the present study, we could confirm this observation and extend it by delineation of the signalling cascade triggering simvastatin and lovastatin-induced apoptosis. Furthermore, the combination of doxorubicin and simvastatin sensitized RD cells for apoptosis by enhancing caspase 9 and 3 activity, synergistically. Thus, the strategy to combine statins with chemotherapeutic agents may result in a more complete cell death in tumour cells, leading to new effective anticancer therapy.

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