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ORIGINAL PAPER

Regulation of apoptosis in human melanoma and neuroblastoma cells by statins, sodium arsenite and TRAIL: a role of combined treatment versus monotherapy

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Abstract Treatment of melanoma cells by sodium arsenite or statins (simvastatin and lovastatin) dramatically modified activities of the main cell signaling pathways resulting in the induction of heme oxygenase-1 (HO-1) and in a downregulation of cyclooxygenase-2 (COX-2) protein levels. Through heme degradation and the production of carbon monoxide and biliverdin, HO-1 plays a protective role in different scenario of oxidative stress followed by mitochondrial apoptosis. Both sodium arsenite and statins could be efficient inducers of apoptosis in some melanoma cell lines, but often exhibited only modest proapoptotic activity in others, due to numerous protective mechanisms. We demonstrated in the present study that treatment by sodium arsenite or statins with an additional inhibition of HO-1 expression (or activation) caused a substantial upregulation of apoptosis in melanoma cells. Sodium arsenite- or statin-induced apoptosis was independent of BRAF status (wild type versus V600E) in melanoma lines. Monotreatment required high doses of statins (20–40 μM) for effective induction of apoptosis. As an alternative approach, pretreatment of melanoma cells with statin at decreased doses (5–20 μM) dramatically enhanced TRAIL-induced apoptosis, due to suppression of the NF-κB and STAT3-transcriptional targets (including COX-2) and downregulation of cFLIP-L (a caspase-8 inhibitor) protein levels. Furthermore, combined treatment with sodium arsenite and TRAIL or simvastatin and TRAIL efficiently induced apoptotic commitment in human neuroblastoma cells. In summary, our findings on enhancing effects of combined treatment of cancer cells using statin and TRAIL provide the rationale for further preclinical evaluation.

Keywords Apoptosis · TRAIL · Statin · Sodium arsenite · Melanoma · Neuroblastoma

Abbreviations

COX-2	Cyclooxygenase-2
DR4	Death receptor-4
DR5	Death receptor-5
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorter
HO-1	Heme oxygenase-1
IETD	*N*-acetyl-Ile-Glu-Thr-Asp-CHO (aldehyde)
IL1β	Interleukin1β
IL6	Interleukin-6
IκB	Inhibitor of NF-κB
IKK	Inhibitor of nuclear factor kappa B kinase
LEHD	*N*-acetyl-Leu-Glu-His-Asp-CHO (aldehyde)
MAPK	Mitogen-activated protein kinase
MFI	Medium fluorescence intensity
NF-κB	Nuclear factor kappa B
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PARP-1	Poly (ADP-ribose) polymerase-1
PTGS2	Prostaglandin-endoperoxide synthase 2
ROS	Reactive oxygen species
TNFα	Tumor necrosis factor alpha
TRAIL	TNF-related apoptosis inducing ligand
TRAIL-R	TRAIL-Receptor
zVAD	Carbobenzoxy-valyl-alanyl-aspartyl-[*O*-methyl]-fluoromethylketone

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Introduction

Regulation of the optimal levels of reactive oxygen species (ROS) and suppression of oxidative stress in living cells requires precise function of numerous protective mechanisms, including induction of gene expression of several antioxidant enzymes, such as catalase, superoxide dismutase-2 (SOD-2), glutathione peroxidase, glutathione reductase and heme oxygenase-1 (HO-1). Inducible HO-1, as well as constitutive HO-2, catalyzes the first rate-limiting step of heme degradation producing carbon monoxide (CO) and biliverdin, which is further converted to bilirubin that possesses strong anti-oxidant activity [1, 2]. Furthermore, CO inhibits enzymatic activities of numerous hemoproteins resulting in downregulation of intracellular respiration and ROS production. The ability of HO-1 to catabolize free heme prevents induction of the mitochondrial apoptotic pathway that could operate as the final resolution to maintain homeostasis at the whole organism level via programmed death of particular cell populations [3–5]. Since inflammation is often linked with high levels of ROS production, the induction of HO-1 expression and enzymatic activity is also involved in the protective anti-inflammatory response [2, 6]. On the other hand, cancer development and progression (including melanoma) is linked with hypoxia, suppression of mitochondrial respiration, increased production of ROS, which are inducers of genomic instability, and establishing a general pro-inflammatory phenotype that is maintained in cancer cells through gene expression of the proinflammatory cytokines, such as IL6, IL1β and TNFα and the corresponding receptors [7]. Paradoxically, suppression of the pro-inflammatory response of cancer cells could substantially block tumor development [8] and sensitize cancer cells to death receptor-mediated apoptosis [9].

In our previous investigations, we used sodium arsenite treatment (1–5 μM) for induction of apoptosis in human melanoma cells [10, 11]. Similar treatment was successfully used for therapy of acute promyelocytic leukemia (APL) and multiple myeloma (MM) [12, 13]. However, the mitochondrial apoptotic pathway was induced only at relatively low levels by clinically proved concentrations (2–5 μM) of sodium arsenite in most melanoma lines and required additional proapoptotic sensitization through specific suppression of the cell survival pathways, such as MEK-ERK, PI3K-AKT or IKK-NF-κB [10, 11].

In recent years, effects of statins (popular drugs that are widely used to down-regulate cholesterol production) on mitochondrial function, ROS production and HO-1 induction have been extensively investigated [14, 15]. In the present study, we further investigated a role of HO-1 suppression in the substantial upregulation of sodium arsenite- or statin-induced cell death in human melanoma cells. We found greater sensitization and killing of melanoma cells through combined treatment of cancer cells by the exogenous TRAIL and statins. Such dramatic sensitization of TRAIL-Receptor (TRAIL-R) mediated apoptosis by sodium arsenite [16] or by statins (the current study) offers a new modality for a possible therapy of melanoma, incident number of which progressively increased in USA and worldwide during the last 50 years [17].

In spite of the remarkable progress in the investigation of carcinogenesis and treatment of melanoma based on the application of specific inhibitors of permanently active mutated BRAF (V600E) [18–20], there is a critical necessity for alternative treatment of melanoma cells carrying wt BRAF (that represents 40–60% of melanoma cases) [21] and for overcoming resistance to specific inhibitors of BRAF (V600E) that could occur after several months of a successful treatment.

Results

A role of HO-1 expression in protection of MEF against sodium arsenite-induced apoptosis

To make a general assessment for a role of HO-1 inducible expression in anti-apoptotic protection we used *HO1*(*HMOX1*) Null mouse embryonic fibroblasts (MEF) (Fig. 1a), which were previously established at Solomon Snyder's laboratory [22]. Using nuclear DNA staining by PI and flow cytometry, we determined substantial differences in apoptotic response of *HO1*-deficient versus normal MEF 24 h after dose-dependent (2.5–20 μM) sodium arsenite exposure. While 5 μM sodium arsenite induced only a 2-fold increase in apoptosis in *HO1*-deficient cells, 10 μM sodium arsenite killed a vast majority of these cells (Fig. 1b). LEHD-fmk, a specific inhibitor of caspase-9, substantially suppressed levels of apoptosis induced by 10 μM arsenite, highlighting activation of the caspase-9 dependent mitochondrial apoptotic pathway, while the effect of IETD-fmk, a caspase-8 and caspase-6 inhibitor, on apoptosis was relatively modest. Finally, pan-caspase inhibitor zVAD-fmk (50 μM) substantially blocked apoptosis in both wt and *HO1*-deficient MEF induced by 10 μM sodium arsenite (Fig. 1c). Taken together, these results indicated involvement of the mitochondrial pathway and its substantial acceleration in *HO1*-deficient MEF following sodium arsenite treatment. Although the role of the mitochondrial pathway in mediation of arsenite-induced apoptosis is well known [10, 23], establishing a protective effect of HO-1 expression against arsenite-induced apoptosis identified a new potential target for

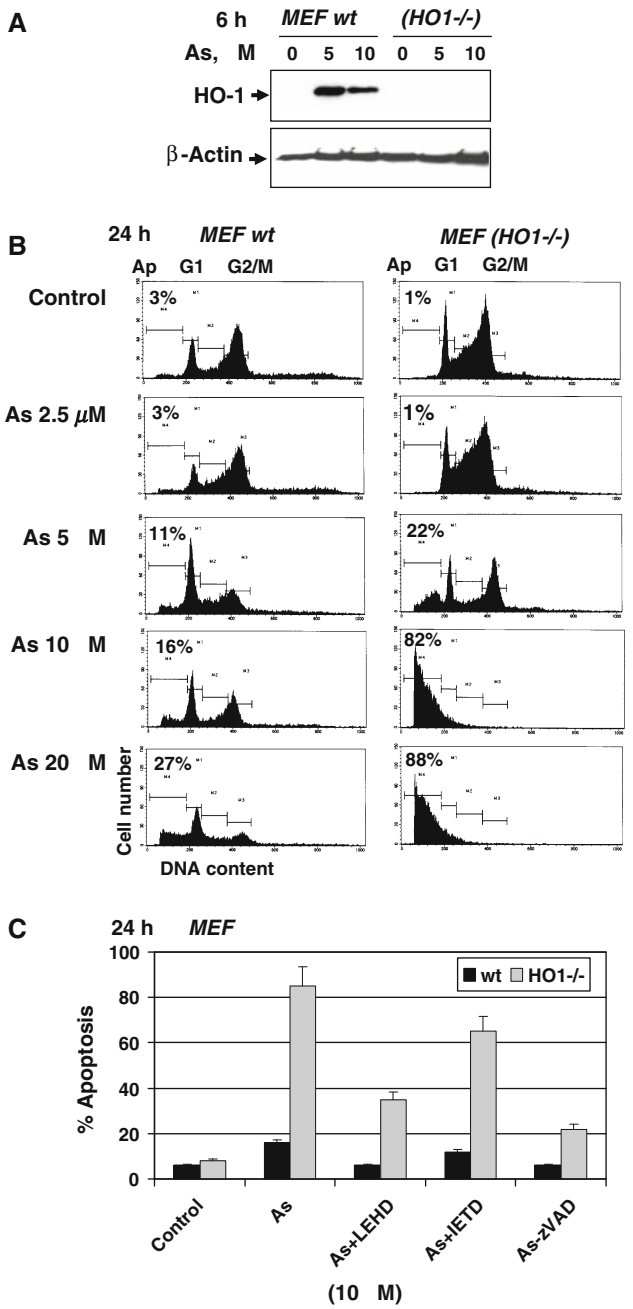

Fig. 1 *HO1* Null mouse embryonic fibroblasts (MEF) are very sensitive to sodium arsenite induced death. **a** Western blot analysis of HO-1 induction by sodium arsenite in MEF. **b**. Cell cycle and apoptosis analysis of wt and *HO1* Null MEF, which were treated with increased doses of sodium arsenite (2.5–20 μM), was performed using PI staining of DNA and the flow cytometry. Apoptotic (pre-G1) levels are indicated. **c** Down-regulation of arsenite-induced apoptosis (10 μM) by specific caspase inhibitors, LEHD (a caspase-9 inhibitor), IETD (a caspase-8 inhibitor), zVAD (a general caspase inhibitor) used at dose 50 μM

regulation of apoptosis not only in normal, but also in cancer cells that are often characterized with highly inducible HO-1 activation upon exposure to different forms of stress.

Upregulation of sodium arsenite-induced apoptosis in melanoma cells

Based on these results, we decided to upregulate sodium arsenite-induced apoptosis in melanoma cells [10] using additional suppression of HO-1. Sodium arsenite treatment at low doses (2.5–5 μM), which was used successfully in the clinic for treatment of APL [24], induced high protein levels of HO-1, while down-regulating protein expression of cyclooxygenase-2 (COX-2/PTGS2), one of the critical proinflammatory enzymes, in WM793, FEMX and LOX human melanoma lines (Fig. 2a). However, caspase-3 driven cleavage of PARP-1, a characteristic feature of apoptotic commitment, was observed in WM793 and FEMX, but not in LOX cells (Fig. 2a) resulting in pronounced apoptosis for WM793 and FEMX cells, but only G2/M arrest for resistant LOX cells 24 h after treatment (Fig. 2b). This certainly indicated that HO-1 activation was not the only critical factor that determined a resistance to sodium arsenite. Beside HO-1 induction, multiple cell regulatory mechanisms, including the basal AKT and NF-κB activities, could determine differential response of melanoma lines to sodium arsenite treatment [9–11]. Additional analysis of normal human melanocytes, normal fibroblasts IMR-90 and several melanoma lines confirmed our previous observations [10] and demonstrated relatively low apoptotic levels in normal cells, as well as in LOX and WM9 melanoma cells, while pronounced apoptosis in WM793 and FEMX melanoma cells induced by 5–10 μM sodium arsenite treatment. Apoptosis could be partially blocked by zVAD-fmk (50 μM), a universal caspase inhibitor (Fig. 2c). Interestingly, arsenite-induced apoptosis was independent on BRAF mutation status: both WM793 [BRAF (V600E)] cells and FEMX (BRAF wt) cells were sensitive to sodium arsenite treatment (Fig. 2c).

Since sodium arsenite induced HO-1 transcription and translation [25], we used a HO1-RNAi construct for stable transfection and suppression of HO-1 expression in WM793 melanoma cells. Control WM793 cells were transfected with the empty vector pSR-GFP/Neo (Fig. 3a). We observed that HO1-RNAi substantially suppressed arsenite-induced HO-1 induction in transfected cells (Fig. 3a). Sodium arsenite-induced PARP-1 cleavage (an indication of caspase-3 activation) was detected in WM793 cells with suppressed HO-1 expression 6 h after treatment. Levels of PARP-1 cleavage were notably increased in these cells, compared to the control cells 12 h after treatment (Fig. 3a). This was followed by a significant upregulation of apoptotic levels for *HO1* deficient cells that were determined as % cells positively stained by Annexin-V-PE (red) among GFP-positive permanently transfected WM793 cells (Fig. 3b).

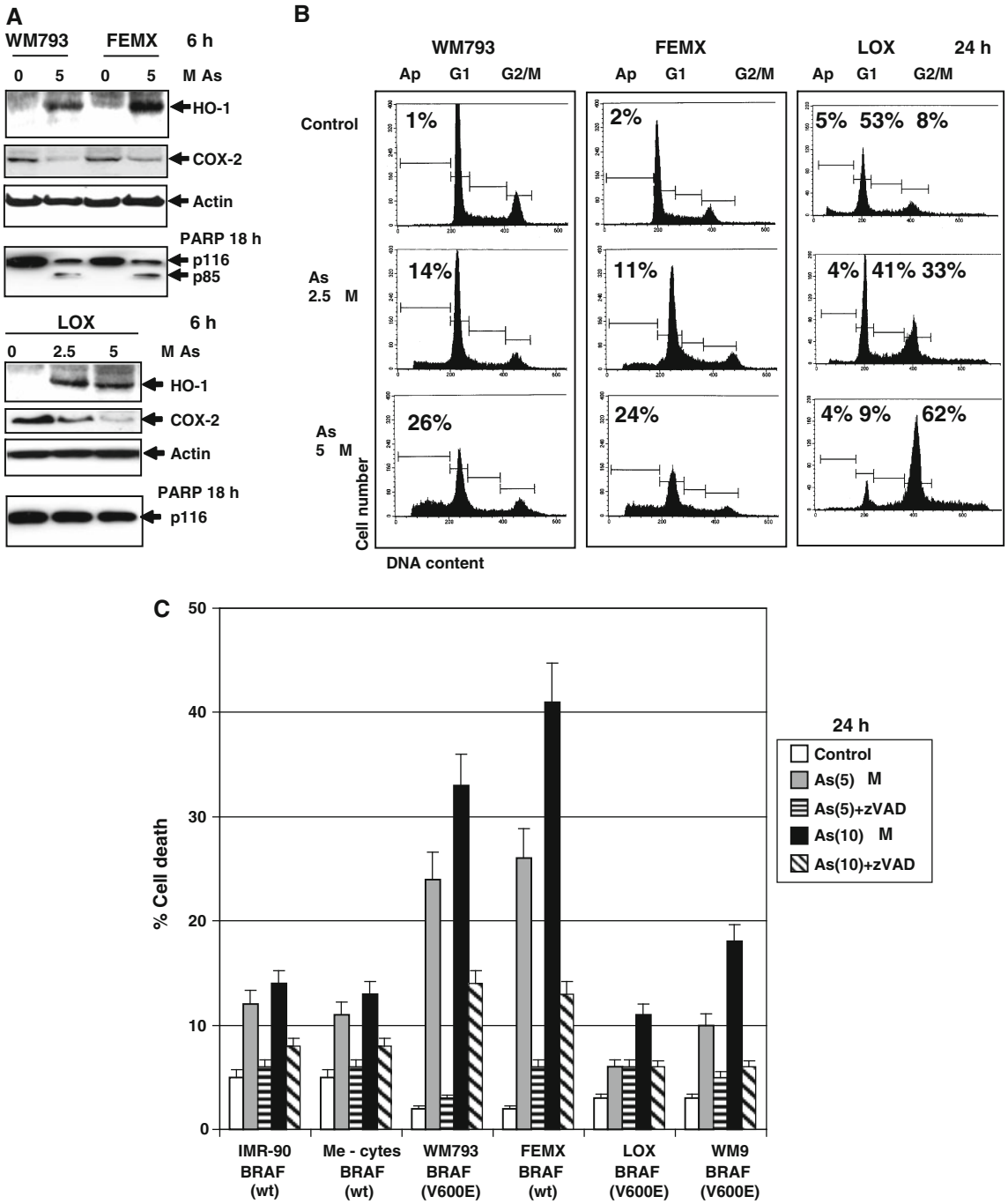

Fig. 2 Induction of HO-1 protein expression and apoptotic commitment in human melanoma cell lines after sodium arsenite treatment. **a** Western blot analysis of protein levels of HO-1 and COX-2 6 h after sodium arsenite (5 μM) treatment and PARP-1 cleavage 18 h after sodium arsenite treatment of indicated melanoma lines. Actin was used as a protein loading control. **b** Cell cycle and apoptosis analysis 24 h after sodium arsenite treatment using PI staining of DNA and the flow cytometry. Apoptosis (pre-G1) levels are indicated for WM793, FEMX and LOX melanoma cells. G1 and G2/M levels are indicated for LOX cells. Results of a typical experiment are shown. **c** Cell death levels of normal human fibroblasts IMR-90, human melanocytes and melanoma cell lines were determined 24 h after sodium arsenite (5–10 μM) treatment in the presence or absence of a caspase inhibitor zVAD (50 μM) using PI staining of DNA and the flow cytometry. BRAF status (wt or V600E) of melanoma lines is indicated. *Error bars* represent mean ± SD for four independent experiments (Student's t test, $P < 0.05$)

As an alternative approach to increase apoptotic response, we used an inhibitor of enzymatic activity of HO-1, Zn(II) containing Protoporphyrin IX [PPIX(Zn)]. Sodium arsenite (5 μM), in combination with PPIX(Zn) at a dose of 20 μM, additionally increased (from 23 to 45%) apoptotic levels of WM793 cells 24 h after treatment

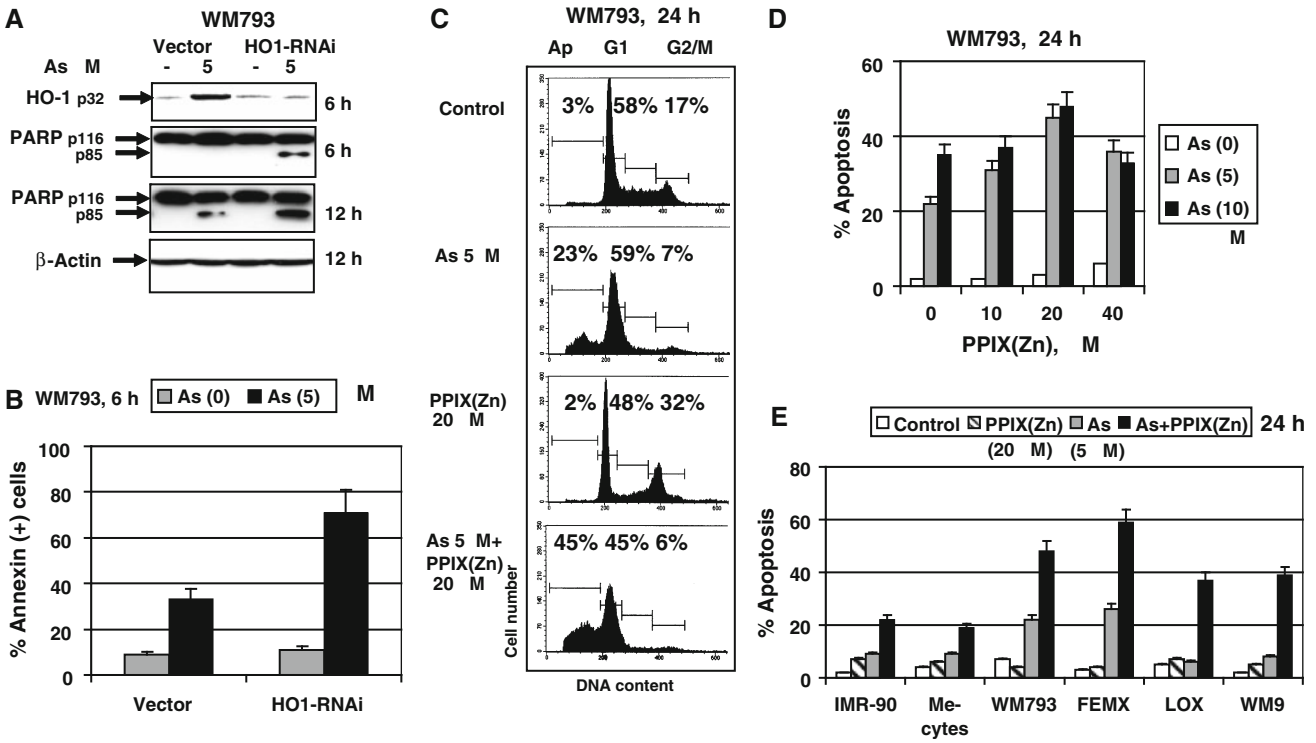

Fig. 3 Inhibition of HO-1 expression by specific RNAi or chemical inhibition of HO-1 enzymatic activity by Zn(II) containing Protoporphyrin IX [PPIX(Zn)] dramatically increased sodium arsenite-induced apoptosis in WM793 melanoma cells. **a** WM793 cells were permanently transfected either with the empty vector pSR-GFP/Neo (Oligoengine) or with the specific HO1-RNAi construct (Oligoengine) based on this vector. Western blot analysis was performed for determination of HO-1 protein levels in the control and *HO1* deficient melanoma cells 6 h after sodium arsenite (As, 5 μM) treatment. PARP-1 protein levels were determined 6 and 12 h after treatment. Beta-Actin was used as a loading control. **b** The early apoptotic levels induced by sodium arsenite treatment. Annexin-V-PE (red) staining of WM793 cells permanently transfected with the empty vector or with HO1-RNAi (green) before and 6 h after sodium arsenite (5 μM) treatment were determined using the flow cytometry. **c**, **d** Cell cycle–apoptosis analysis of WM793 cells 24 h after treatment with sodium arsenite (5–10 μM) in the presence or absence of increased doses of PPIX(Zn) (10–40 μM). A typical experiment is shown in (**c**). **e** Effects of PPIX(Zn) at the dose of 20 μM on up-regulation of apoptotic levels in human melanocytes and melanoma lines. Apoptotic levels were determined using PI staining of DNA and the flow cytometry. *Error bars* represent mean ± SD for four independent experiments (Student's *t* test, $P < 0.05$)

(Fig. 3c). The combination of 5 μM sodium arsenite and 20 μM PPIX(Zn) was most efficient for apoptotic induction in several melanoma lines, including resistant LOX, while relatively mild effects were observed in normal fibroblasts and melanocytes (Fig. 3d, e). Hence, results of genetic and enzymatic inhibition of HO-1 demonstrated substantial upregulation of arsenite-induced apoptosis in human melanoma cells. There was a narrow window of concentrations of PPIX(Zn) (10–20 μM), when apoptotic response in melanoma cells induced by 5 μM sodium arsenite in the presence of PPIX(Zn) was substantially higher, than in normal cells. An additional increase in a concentration of PPIX(Zn) was quite toxic for normal cells and caused massive cell death by necrosis. Taken together, these results indicated that HO-1 activation was necessary, but not sufficient for protection of melanoma cells against apoptosis induced by sodium arsenite treatment. Suppression of HO-1, however, was linked with substantial upregulation of arsenic-induced apoptotic levels in human melanoma cell lines.

Sodium arsenite treatment further sensitizes melanoma cells to TRAIL-induced apoptosis: a probable role for COX-2 downregulation

A vast majority of cancer cell lines exhibited low sensitivity to treatment by TRAIL alone and required an additional sensitizer in combination with TRAIL [26]. We previously demonstrated such a role for sodium arsenite as a sensitizer for TRAIL-induced death via suppression of cFLIP expression and additional upregulation of surface expression of TRAIL-R2/DR5 in several human melanoma lines, including WM793, WM9 and LU1205 [16]. As expected, A375 human metastatic melanoma cells (TRAIL-R2/DR5-positive) demonstrated a similar response to combined treatment with TRAIL and sodium arsenite (Fig. 4a). Inhibition of HO-1 enzymatic activity by PPIX(Zn) did not notably affect apoptosis induced by TRAIL, but decreased levels of (TRAIL + sodium arsenite)-induced apoptosis in melanoma cells (Fig. 4b), demonstrating a probable role of HO-1 in promoting activity of

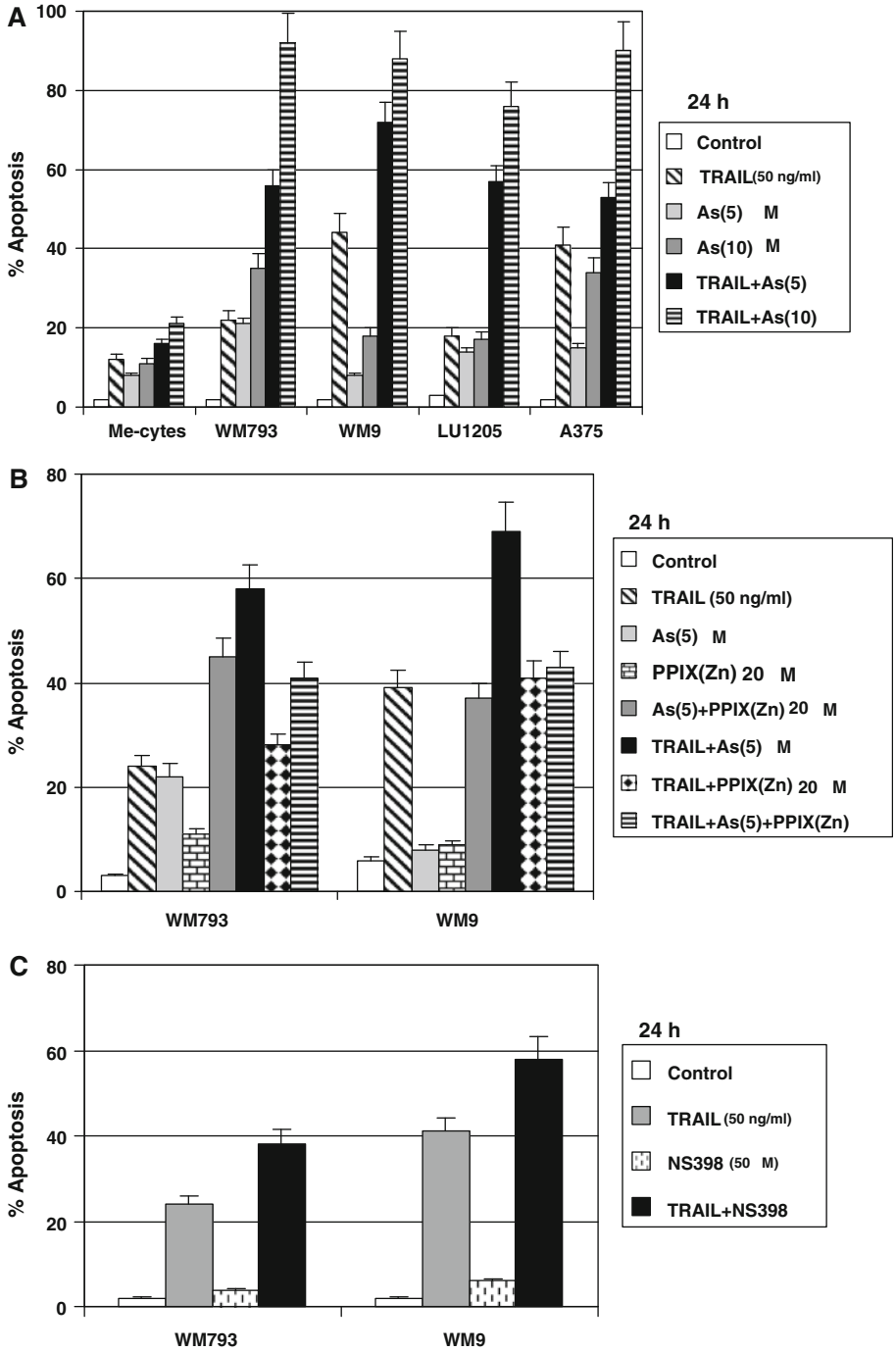

Fig. 4 Sensitization of human melanoma cells to TRAIL-induced apoptosis by sodium arsenite treatment. **a** Human melanocytes (me-cytes) and melanoma lines, WM793, WM9 and LU1205 and A375 were treated by TRAIL (50 ng/ml), sodium arsenite (As) (5–10 μM) alone or in combination. Apoptotic levels were determined using PI staining of DNA and the flow cytometry. **b** Effects of PPIX(Zn) (20 μM) on sodium arsenite- or TRAIL-induced apoptosis in WM793 and WM9 cells 24 h after treatment. **c** Effect of NS390 (50 μM), a COX-2 enzymatic inhibitor, on TRAIL-induced apoptosis in WM793 and WM9 cells

the death receptor-mediated apoptotic pathway. Sodium arsenite/HO-1 was found to cause downregulation of anti-apoptotic COX-2 protein levels (Fig. 2a). Furthermore, HO-1 activation has been observed to inhibit enzymatic activities of numerous hemoproteins, including COX-2, through CO binding to the heme [5]. These data suggested that COX-2 suppression mediated by sodium arsenite and HO-1 might be additional factor for upregulation of TRAIL-induced apoptosis. Indeed, an alternative inhibition of COX-2 enzymatic activity by NS398 (50 μM) also upregulated levels of TRAIL-induced apoptosis in WM9 and WM793 melanoma cells (Fig. 4c).

Statins as inducers of HO-1 expression and cell death of cancer cells

To extend our observations regarding a regulatory role of HO-1 for apoptotic signaling, we used statins as inducers of HO-1 expression in melanoma cells. Statins are widely prescribed drugs for decreasing cholesterol levels, because

they inhibit 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase), the rate-limiting enzyme, which controls production of mevalonate, an intermediate of the biosynthesis of cholesterol [27]. Furthermore, mevalonate is a precursor of several lipid isoprenoid intermediates, such as geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP), which are critically important for posttranslational modification and proper function of small GTP-binding proteins, including Rho, Rac and Ras that play pivotal role in cell signaling in normal and cancer cells [28]. Furthermore, via control of cholesterol biosynthesis and cell signaling, statins demonstrated a strong anti-inflammatory potential that also included the induction of HO-1 expression in different cell systems [14, 29].

Indeed, our data demonstrated that simvastatin at the dose 10–40 μM strongly upregulated HO-1 expression in WM793 and WM9 melanoma cells (Fig. 5a). Furthermore, numerous effects of simvastatin treatment on signaling proteins were observed in both melanoma lines with certain quantitative differences: a dose-dependent inhibition of ERK1/2 phosphorylation and activation; the absence of inhibitory effects of simvastatin for phospho-AKT in WM793 cells with high AKT activity and suppression of AKT activity by high dose of simvastatin (40 μM) in WM9 cells with low AKT activity; suppression of the basal STAT3 Tyr phosphorylation in WM9 cells by 40 μM simvastatin. Furthermore, a dramatic downregulation of NF-κB p65 protein levels, which was followed by COX-2

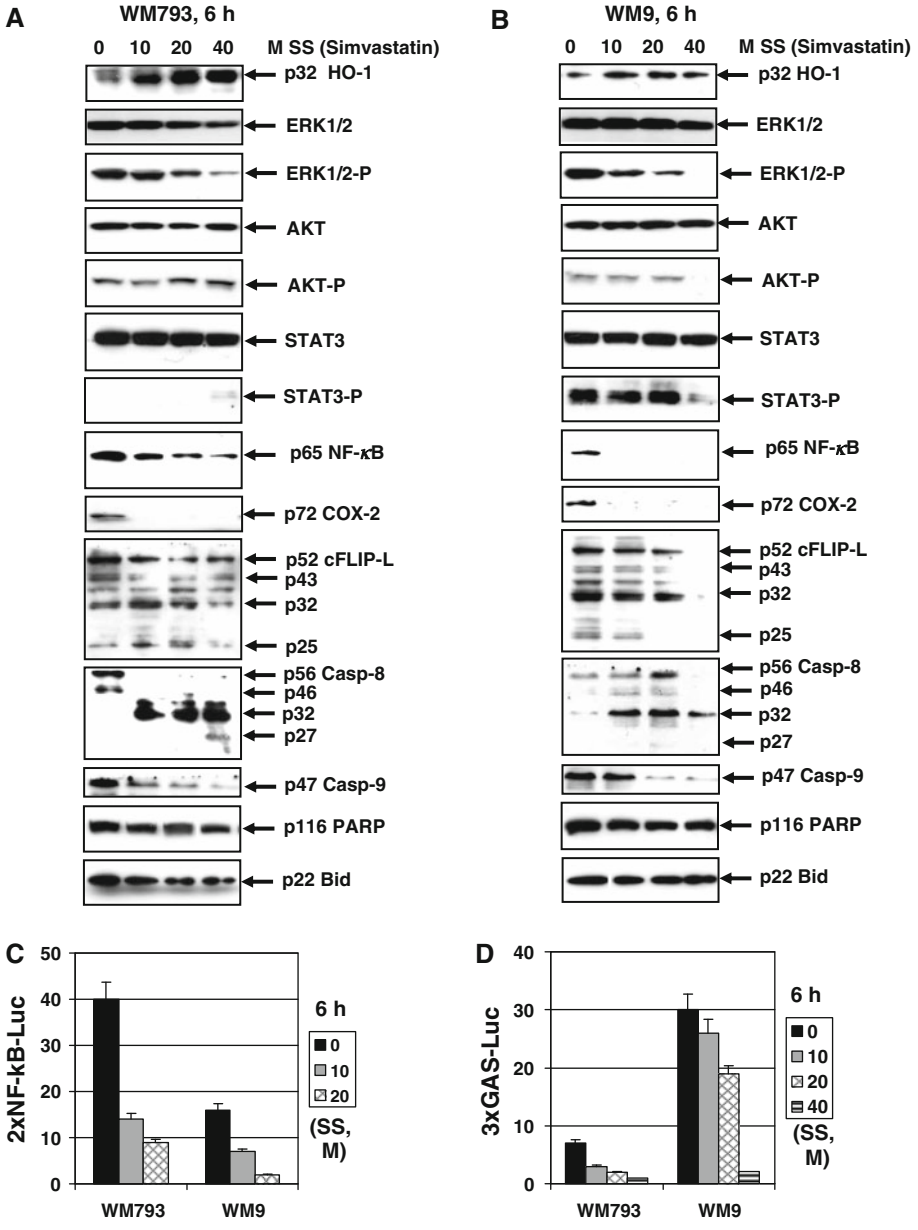

Fig. 5 Dose-dependent effects of simvastatin (SS) on cell signaling pathways in WM793 and WM9 melanoma cells. **a**, **b** Western blot analysis of indicated proteins in WM793 and WM9 melanoma cells. **c**, **d** Effect of simvastatin (SS) on NF-κB-dependent luciferase reporter activity and GAS/STAT-dependent luciferase activity (normalized to β-gal activity) in transiently transfected melanoma cells. Control cells (0 μM SS) were treated only with DMSO (at final concentration 0.1%) that was used for dissolving simvastatin

downregulation, was observed in both melanoma lines as a result of simvastatin treatment (Fig. 5a, b). Simultaneously, cleavage and activation of pro-caspase-8 and pro-caspase-9 was detected, which was also accompanied by a permanent downregulation of cFLIP-L levels (a caspase-8 inhibitor) and changes in the levels of its cleaved products 6 h after statin treatment (Fig. 5a, b). At this time point, however, only a mild decrease of the p116 subunit of PARP-1 (a caspase-3 target) was detected. A pronounced cleavage of Bid was also not observed (Fig. 5a, b). A role of the activation of the caspase-8-mediated pathway during statin-induced apoptosis is not completely understood. However, the basal production and secretion of TNFα [10] and surface expression of the endogenous FasL [30] and TRAIL [16] in melanoma cells potentially could be responsible for the subsequent activation of the death receptor signaling pathway under conditions where down-regulation of cFLIP-L and COX-2 anti-apoptotic activities were induced by statin.

Determination of NF-κB-dependent and GAS/STAT-dependent luciferase activity further demonstrated statin-dependent suppression of the general NF-κB transacting activity in both WM793 and WM9 cells. STAT-dependent luciferase activity was also suppressed in WM9 cells and was almost undetectable in the control and statin-treated WM793 cells (Fig. 5c, d). In summary, these results demonstrate a rapid sensitization of statin-treated cells to apoptosis through both the endogenous and exogenous pathways due to numerous changes in apoptosis-related proteins.

Indeed, simvastatin treatment at low doses (2.5 μM) resulted in an increased ratio of G1/G2 and the subsequent dose-dependent induction of apoptosis in WM793 and WM9 melanoma cell populations (Fig. 6a–c). As expected, a 50-fold excess of mevalonate, a downstream product of the reaction catalyzed by HMG-CoA reductase, almost completely suppressed simvastatin-induced apoptosis (Fig. 6b), highlighting the specificity of simvastatin, as an inhibitor of HMG-CoA reductase activity, for apoptotic signaling. Interestingly, GGPP at significantly lower doses (10 μM) also substantially blocked apoptosis in melanoma lines (Fig. 6c), indicating a critical role of geranylated small G-proteins in cell survival and protection against apoptosis in melanoma cells.

Induction of apoptosis by atorvastatin in melanoma cells was relatively low, compared to simvastatin (Fig. 6d). On the other hand, the HO-1 inhibitor, 20 μM PPIX(Zn), further upregulated levels of apoptosis induced by 20–40 μM atorvastatin and simvastatin in WM793 and WM9 melanoma cells (Fig. 6d). In contrast, zVAD-fmk (50 μM), a universal caspase inhibitor, substantially decreased levels of simvastatin-induced apoptosis in melanoma cells (Fig. 6d). Using control and *HO1* deficient WM793 cells (see Fig. 2b) we also observed upregulation of apoptotic levels in *HO1* deficient cells for a range of simvastatin concentrations (10–40 μM) (Fig. 6e). In summary, these results demonstrate pronounced statin-induced apoptosis (at high doses of statin) in WM793 and WM9 cells that could be further increased by suppression of HO-1 expression/activation. These data identified HO-1 expression, which operated in concert with other defensive mechanisms, as an important factor protecting against statin-induced apoptosis. Interestingly, simvastatin at relatively low concentration (5 μM) notably upregulated arsenite induced apoptosis, possibly via inhibition of ERK activation. U0126, a specific inhibitor of MEK-ERK pathway, demonstrated a similar effect (Supplementary Fig. 1).

Melanoma cells produce and secrete a variety of cytokines, including NF-κB-dependent IL1β, TNFα and IL6, which are known as major proinflammatory cytokines and regulators of cell proliferation and metabolism. These cytokines could be potential targets of negative regulation by statins, because statin treatment resulted in a down-regulation of a general NF-κB-dependent transcription (see Fig. 5c). Next we determined the effects of simvastatin on production and secretion of proinflammatory cytokines in cell growth media of melanoma lines. We observed pronounced negative effects of simvastatin treatment on secretion of TNFα and IL1β that were produced at very low pg/ml concentration (Fig. 7a, b). In contrast, a relative downregulation of IL6 secretion (produced at much higher doses, ng/ml) by simvastatin into the WM9 cell media was slight, but significant. A notable down-regulation of IL6 secretion was not observed for WM793 cells after simvastatin treatment (Fig. 7c). The absence of downregulation of IL6 secretion by simvastatin, but its effect on down-regulation of IL1β was previously reported [31]. This indicated that simvastatin treatment of cancer cells did not affect, in some circumstances, well known pro-inflammatory targets, such as IL6. As expected, the exogenous IL6 added at high concentration (100 ng/ml) to the cell media downregulated apoptotic levels induced by simvastatin in WM9, but not in WM793 melanoma cells (Fig. 7d).

We performed analysis of cell death induction by statins in normal human fibroblasts IMR-90, human melanocytes and several melanoma lines. Simvastatin at doses of 10–40 μM induced different levels of apoptosis in cancer cell lines, in the range between 10 and 70% 24 h after treatment: high levels of apoptosis were observed for WM793, FEMX and WM9 melanoma lines, while a slight response, which was similar to the response of normal cells, was detected for WM35 and HHMSX melanoma lines; a modest response was measured in WM164 and WM852 melanoma lines (Fig. 7d). While lovastatin produced a similar induction of apoptosis, apoptotic effect of atorvastatin (10–40 μM) was not well pronounced in human melanomas (Supplementary Fig. 2). We would like to highlight that WM793 and WM9 cells contain mutated *BRAF* (V600E), while FEMX and WM852 cells have

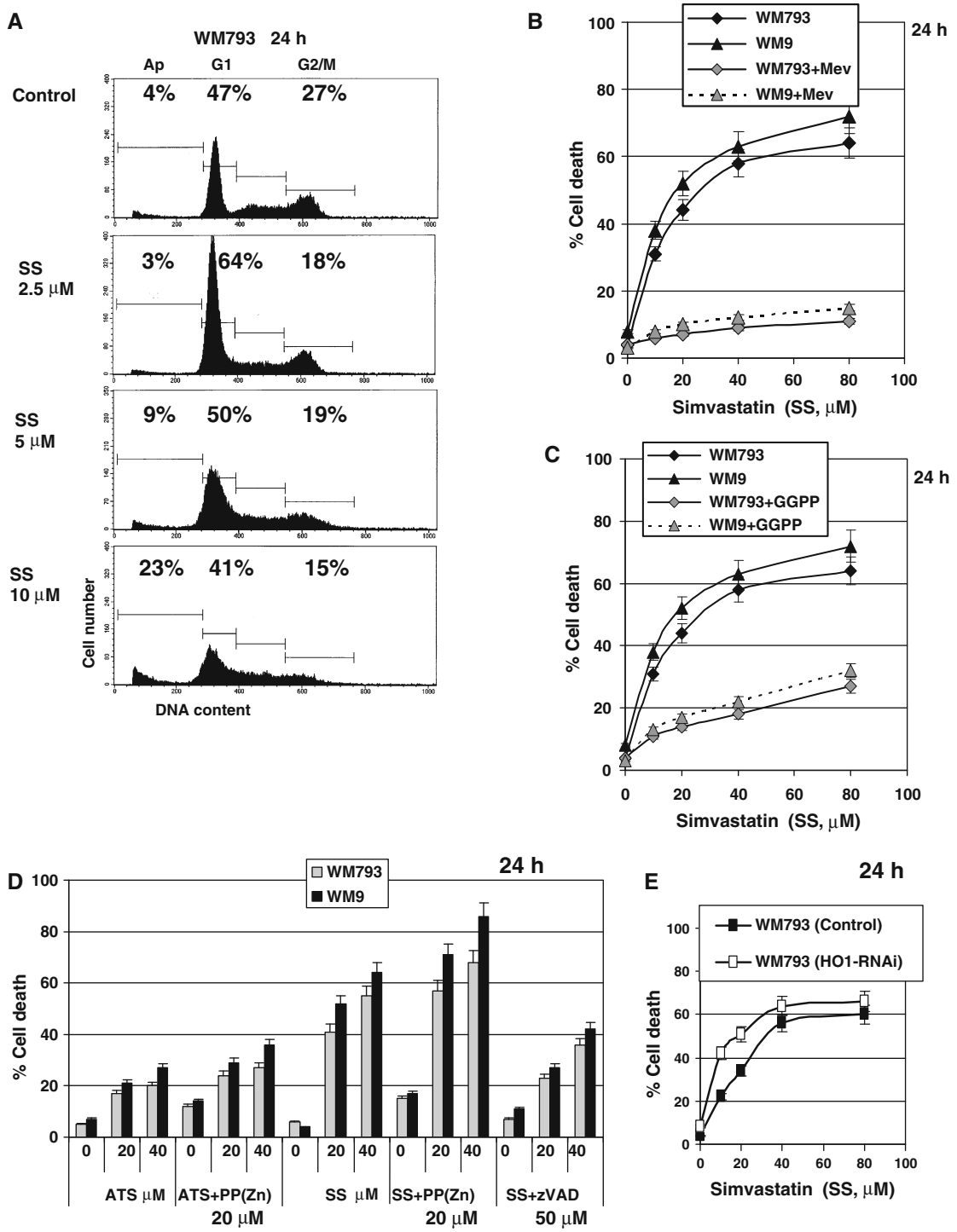

Fig. 6 Statins-induced apoptosis in WM793 and WM9 melanoma cells. **a** Cell cycle-apoptosis analysis of simvastatin (SS) treatment on WM793 melanoma cells. Percentage of cells at distinct phases of cell cycle and apoptotic (pre-G1) levels were determined using PI staining of DNA and the flow cytometry. Control cells (0) were treated only with DMSO (at final concentration 0.1%) that was used for dissolving simvastatin. **b** Suppression of simvastatin-induced apoptosis by 50-fold excess of mevalonate (Mev) that was added 15 min after simvastatin (SS) into the cell culture of WM793 and WM9 cells. **c** Effect geranylgeranyl pyrophosphate (GGPP) (10 μM) on simvastatin-induced apoptosis in WM793 and WM9 cells. **d** Effects of HO-1 inhibitor PPIX(Zn) on statin-induced apoptosis. Apoptosis was initiated by atorvastatin (ATS), simvastatin (SS) (0–40 μM) alone either in combination with HO-1 inhibitor PPIX(Zn), 50 μM, or in combination with caspase inhibitor zVAD, 50 μM. **e** Apoptosis levels in the control WM793-neo cells or in WM793 cells permanently transfected with HO-1 RNAi that suppressed HO-1 expression. Apoptotic levels were determined using PI staining of DNA and the flow cytometry. *Error bars* represent mean ± SD for four independent experiments (Student's *t* test, $P < 0.05$)

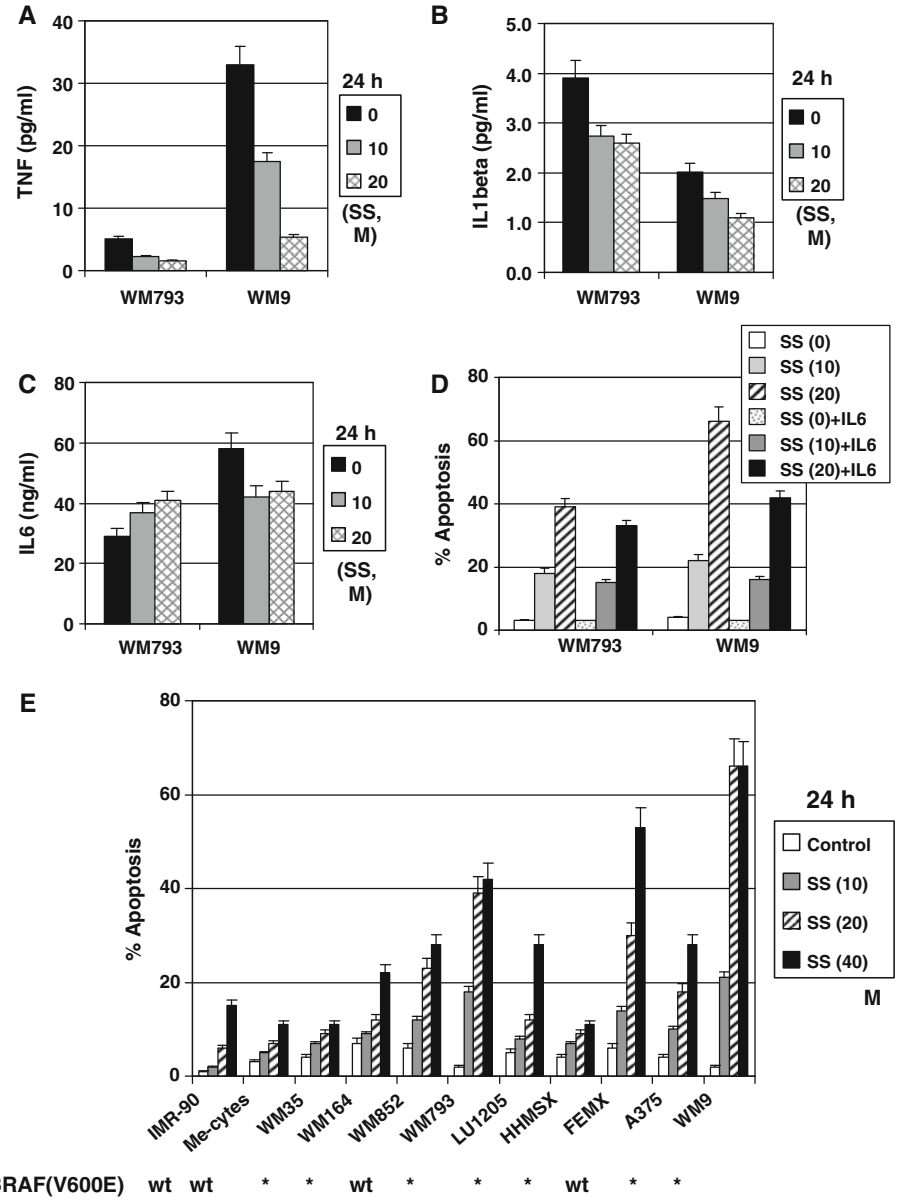

Fig. 7 Dose-dependent effects of simvastatin on apoptosis levels in normal and cancer cells. **a–c** IL1β, TNFα and IL6 levels (normalized for 100,000 cells) in culture medium of melanoma WM793 and WM9 cells were determined 24 h after simvastatin (SS) treatment by ELISA. **d** Exogenous IL6 (100 ng/ml) down-regulated simvastatin (SS)-induced apoptosis in WM9 cells. **e** Doze-dependent effect of simvastatin (SS) on induction of apoptosis in IMR-90 normal human fibroblasts, melanocytes and human melanoma lines was determined using PI staining of DNA and the flow cytometry. BRAF status (wt or V600E) of melanoma lines is indicated. Control cells (SS, 0 μM) in all lines were treated only with DMSO (at final concentration 0.1%)

normal *BRAF* [32]. Our results indicated that melanoma cells with normal *BRAF* could also be treated and killed by simvastatin at relatively high concentrations.

Statin doses routinely used for decreasing cholesterol levels in human patients are 1–2 μM, while specific apoptotic effects for some melanoma cell lines were well pronounced only at doses 20–40 μM. Next we used statins at lower doses (5–20 μM) in a combination with TRAIL for an additional upregulation of TRAIL-induced apoptosis in cancer cells.

Statin treatment sensitizes melanoma cells to TRAIL-induced apoptosis

Melanoma lines used in the present study exhibited different levels of TRAIL-R2/DR5 surface expression, with well pronounced expression in WM793, LU1205, WM9, LOX and A375 cells; surface expression of TRAIL-R1/DR4 was not well-defined [33, 34]. Based on similarity between sodium arsenite and statins in the regulation of the key signaling pathways and levels of apoptosis-related proteins (such as cFLIP-L), we determined effects of combined TRAIL and simvastatin treatment on apoptosis in human melanoma lines. TRAIL alone (50 ng/ml) and simvastatin alone (5–20 μM) could induce apoptosis at different levels in distinct melanoma lines. The combination of TRAIL and simvastatin produced increased caspase-3-mediated cleavage of PARP-1 by 4 h after treatment of WM793 cells, when effect of the individual inducers was not detectable (Fig. 8a). Both TRAIL and simvastatin alone induced a modest PARP cleavage only 16 h after treatment, while effect of a combined treatment

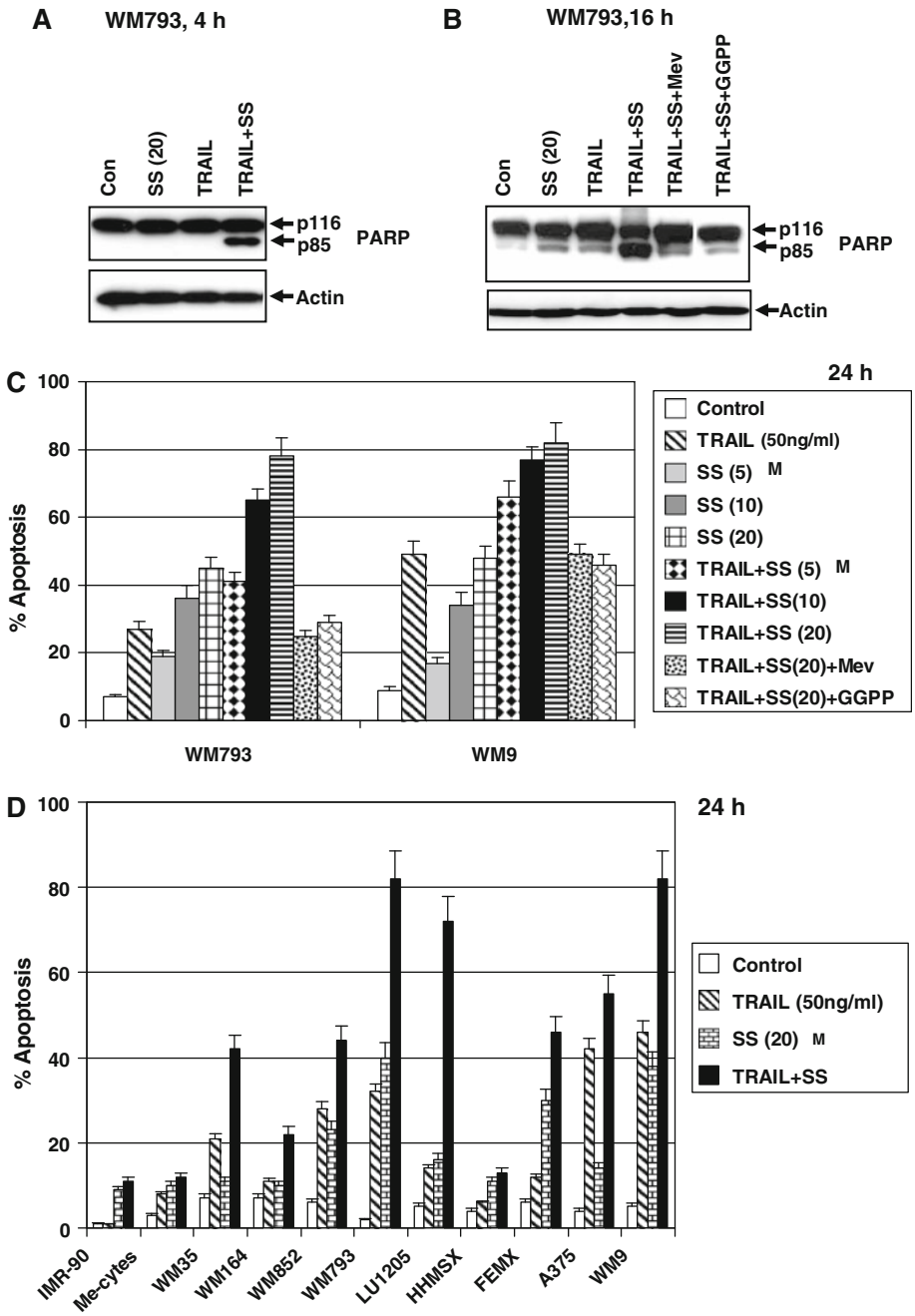

Fig. 8 Sensitization of human melanoma cells to TRAIL-induced apoptosis by simvastatin treatment. **a**, **b** Western blot analysis of PARP-1 cleavage in WM793 melanoma cells after TRAIL (50 ng/ml) and simvastatin (SS, 20 μM) treatment in the presence or the absence of mevalonate (Mev, 1 mM) or geranylgeranyl pyrophosphate (GGPP, 10 μM). **c**, **d** Effect of co-stimulation by simvastatin (5–20 μM) on TRAIL-induced apoptosis in normal (IMR-90 and melanocytes) and indicated cancer cell lines. Apoptotic levels (pre-G1) were determined using PI staining of DNA and the flow cytometry. *Error bars* represent mean ± SD for four independent experiments (Student's *t* test, $P < 0.05$)

was really strong at this time point (Fig. 8b). The maximal dose-dependent effect of simvastatin on TRAIL-induced apoptosis was occurred with 20 μM in WM793 and WM9 cells, although it was evident at 5 μM (Fig. 8c). Upregulation of TRAIL-induced apoptosis by simvastatin, as well as PARP-1 cleavage, was diminished in the presence of 50-fold excess of mevalonate (Fig. 8b, c), highlighting a role of the inhibition of the mevalonate pathway for upregulation of apoptosis. GGPP (10 μM) was an efficient suppressor of TRAIL and simvastatin induced PARP-1 cleavage and apoptosis (Fig. 8b, c), indicating a critical role of the remote downstream products of the mevalonate biosynthetic pathway (that are vitally important for post-translational modification of the Rac/Rho family members) in the anti-apoptotic protection.

We further used a combination of TRAIL (50 ng/ml) and simvastatin (20 μM) for upregulation of apoptosis in additional melanoma lines (Fig. 8d). Normal fibroblasts and melanocytes, as well as WM164 and HHMSX melanoma cells, exhibited low levels of apoptosis after a combined treatment, due to low surface expression of TRAIL-R2/DR5. In contrast, WM793, LU1205, A375 and

WM9 melanoma cells demonstrated enhanced apoptosis; medium apoptotic induction was observed in WM35, FEMX and WM852 cells (Fig. 8d). Additive effects of combined treatment with TRAIL and simvastatin on upregulation of apoptosis were likely dependent on several parameters such as the level of TRAIL-R surface expression, efficiency of the death-inducing signaling complex (DISC) formation [35], suppression of anti-apoptotic proteins, cFLIP-L and COX-2, and changes in NF-κB- and STAT3-dependent anti-apoptotic gene expression. Our results highlighted the usefulness of combined treatment for apoptotic induction in cell culture and could serve as a basis for subsequent in vivo experiments.

Sodium arsenite or statin treatment sensitizes neuroblastoma cells to TRAIL-induced apoptosis

Due to common origin from the embryonic neural crest, there is a certain similarity in gene expression, regulation of cell signaling pathways and apoptosis in melanocytes and neurons [36] and probably in melanomas and neuroblastomas. Sodium arsenite was a powerful inducer of HO-1 expression in human neuroblastoma HTB-11 cells (Fig. 9a) similarly to the levels observed in melanoma cells (see Fig. 2a). Sodium arsenite treatment also resulted in PARP-1 116 kb down-regulation/cleavage (Fig. 9a) and in induction of apoptosis in HTB-11 cells (Fig. 9b). Inhibition of HO-1 enzymatic activity by PPIX(Zn) increased arsenite-induced apoptosis in both A375 melanoma and HTB-11 neuroblastoma cells (Fig. 9c). Furthermore, HTB-11 cells demonstrated a pronounced response to combined treatment by TRAIL and sodium arsenite (Fig. 9b, c). PPIX(Zn) did not notably affect apoptosis induced by TRAIL, but decreased levels of (TRAIL + sodium arsenite)-induced apoptosis in melanoma and neuroblastoma cells (Fig. 9b, c).

HTB-11 neuroblastoma cells were relatively resistant to statin treatment at a dose range of 1–20 μM. However, a combination of TRAIL (50 ng/ml) and simvastatin (20 μM) or TRAIL and atorvastatin (20 μM) demonstrated efficient for upregulation of apoptosis in HTB-11 neuroblastoma cells, compared to A375 melanoma cells (Fig. 9d). In contrast to A375 cells, HTB-11 cells produced and secreted high levels of the proinflammatory IL6. Simvastatin treatment did not decreased IL6 levels in the cell growth media, as we previously observed for WM793 cells (see Fig. 7c), indicating that anti-inflammatory effects of simvastatin were limited.

Hence, results obtained demonstrated a significant upregulation of TRAIL-mediated apoptosis by combined treatment with simvastatin not only in melanoma, but also in neuroblastoma cells.

Discussion

Melanoma is the fifth most common cancer in men and the seventh most common cancer in women. It is estimated that 68,130 people in the United States were diagnosed in 2009, and 8,700 died because of it (Melanoma Cancer Overview by American Cancer Society, November 2010; www.cancer.org). In spite of substantial progress in understanding the molecular mechanisms of melanoma carcinogenesis and extensive development of new treatment modalities during the last 5 years, induction of apoptotic commitment in metastatic melanomas and overcoming resistance to new targeting treatment [for example a resistance to specific BRAF (V600E) inhibitors] remain significant problems [18, 37–39]. Our general expectation was that combined treatment based on activation of the TRAIL/TRAIL-R apoptotic pathway, which was quite specific for cancer cells, together with metabolic inhibitors could be potentially beneficial approaches for cancer therapy. In the present study, we have been focused first on suppression of inducible HO-1 activity that could result in further upregulation of ROS-induced apoptosis in human melanomas. Surprisingly, sodium arsenite and statins could affect many common targets, exhibiting anti-inflammatory effects through suppression of the IKK-NF-κB pathway [40], its critical transcriptional target COX-2 [41] and a dramatic induction of HO-1 activity. Stress-induced HO-1 activation is often a result of mitochondrial targeting followed by release of heme-containing cytochrome *c*, which should be degraded: holoenzyme through the proteasome system [42] and heme by HO-1. Furthermore, HO-1 could be further involved in the maintenance of the anti-inflammatory response by suppression of COX-2 enzymatic activity and prostaglandin-E2 production. An alternative pathway that operates in parallel, a cytochrome *c* interaction with the apoptosome, results in the activation of the caspase-9-dependent apoptotic pathway that ultimately determines a life-or-death balance [43]. Even though our results demonstrated enhanced upregulation of the mitochondrial apoptotic pathway by HO-1 suppression, a challenging problem that remains is the absence of suitable physiological inhibitors of HO-1, because Zn containing PPIX (widely used in cell culture experiments) is cytotoxic for in vivo treatment.

Our observations and published data also demonstrated that monotreatment with statin induced pronounced apoptotic death in some melanoma cell lines only at relatively high doses (5–20 μM) [44]. Animal experiments with the B16F10 mouse melanoma model exhibited a suppression of tumor growth by treatment with 1.6 μM simvastatin [45]. However, a regular statin treatment of patients for the control of cholesterol levels appears does not display a pronounced anti-melanoma protection [46, 47].

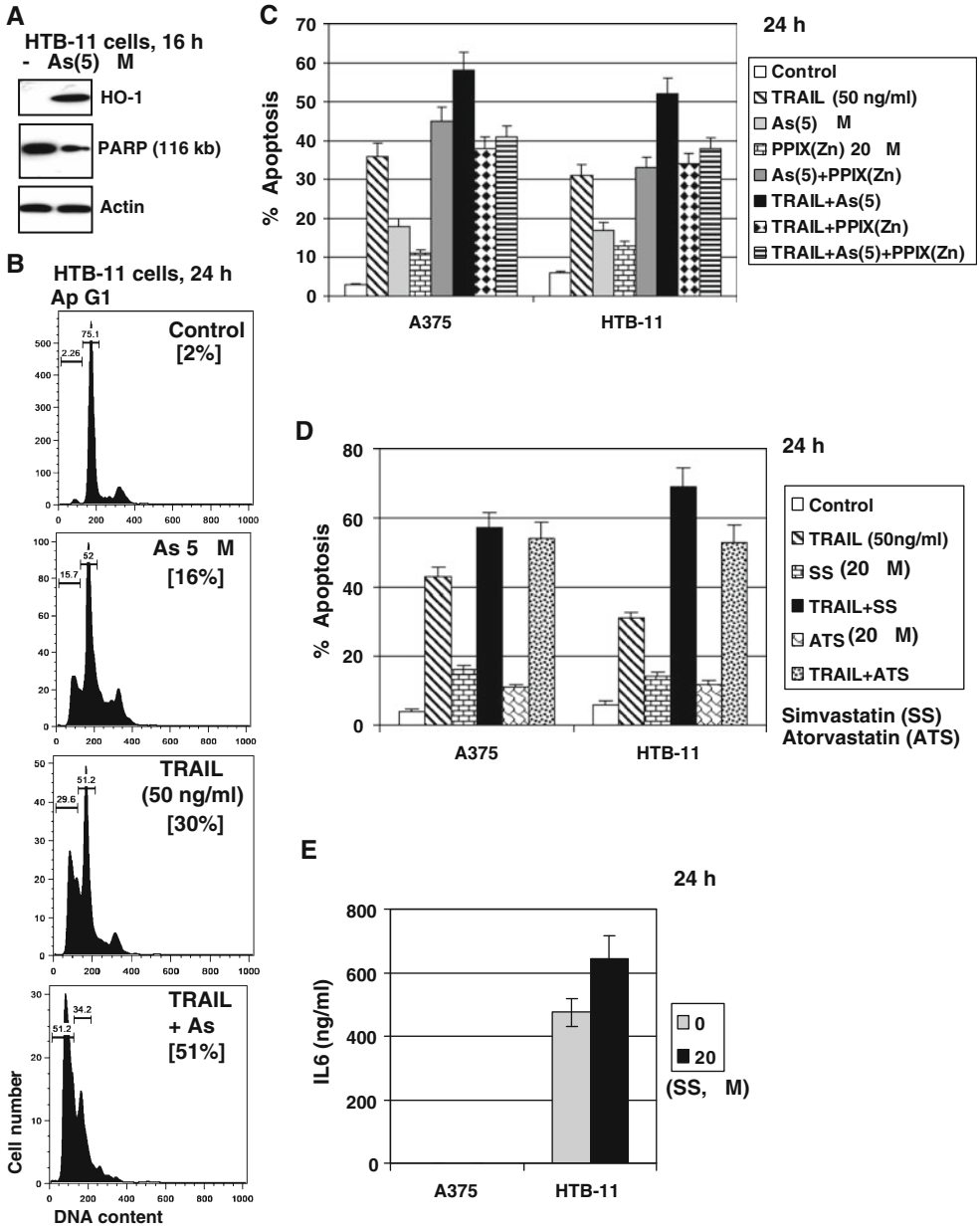

Fig. 9 Sensitization of HTB-11 human neuroblastoma cells to TRAIL-induced apoptosis by sodium arsenite or simvastatin treatment. **a** Western blot analysis of HO-1 induction and PARP-1 levels in HTB-11 cells 16 h after sodium arsenite exposure. **b** A combined treatment of sodium arsenite and TRAIL induced high levels of apoptosis in HTB-11 neuroblastoma and cells. Apoptotic levels are indicated. **C.** Effects of PPIX(Zn), 20 μM on sodium arsenite-induced apoptosis and (TRAIL + sodium arsenite)-induced apoptosis in HTB-11 neuroblastoma and A375 melanoma cells. **d** A combined treatment of statin and TRAIL significantly increased TRAIL-mediated apoptosis in HTB-11 and A375 cells. Apoptotic levels (pre-G1) were determined using PI staining of DNA and the flow cytometry. *Error bars* represent mean ± SD for four independent experiments (Student's *t* test, $P < 0.05$). **e** IL6 levels (normalized for 100,000 cells) in culture medium of melanoma A375 and neuroblastoma HTB-11 cells were determined 24 h after simvastatin (20 μM) treatment using ELISA

Furthermore, multiple side effects of statin treatment for normal cells linked with mitochondrial destruction, apoptosis and the subsequent myopathy were reported [48]. Therefore, solid evidence does not exist that statin monotherapy would be efficient for in vivo treatment.

An alternative approach was an upregulation of TRAIL-induced apoptosis in melanoma cells with additional sensitization of cancer cells by sodium arsenite [16] or statins (in the present study). Specific inhibitors of IKK-NF-κB [49] or JAK2-STAT3 [9], which exhibited a strong

antiinflammatory response, specific inhibitors of pro-inflammatory regulators, such as COX-2 inhibitors, and more general inhibitors of the inflammatory response, like resveratrol [33, 50], curcumin [51] and several others, could be efficient sensitizers of cancer cells to TRAIL-induced apoptosis. Statins with certain antiinflammatory activities [29, 41] might play a similar role in upregulation of death-receptor-mediated apoptosis in melanoma and neuroblastoma cells. Costimulatory effects of statins on TRAIL-induced apoptosis in glioblastomas [52] and on FasL-mediated apoptosis of smooth muscle cells were previously observed [53]. Interestingly, that through inhibition of COX-2 activity, HO-1 alone had a proapoptotic role in TRAIL-mediated apoptosis in melanoma cells. That was in contrast to the antiapoptotic role of HO-1 in the mitochondria-dependent apoptosis induced by sodium arsenite or statins at higher doses. The proapoptotic effect of HO-1 was very similar with NS398-mediated suppression of COX-2 activity, which also sensitized melanoma cells to TRAIL-mediated apoptosis (see Fig. 4e). The analogous effects of COX-2 inhibitors on TRAIL-induced apoptosis in different cancer models were previously described [54].

Even though proapoptotic effects of statins for melanoma cells have been previously reported in several publications [44, 55, 56], sensitization of melanoma cells to TRAIL-induced apoptosis by statins, which was investigated in our study, potentially could be a new modality for melanoma therapy. It was especially important, that combined treatment of statin and TRAIL was also quite effective for killing BRAF wt melanoma cell lines, such as WM852 and FEMX (see Fig. 8), where mutation specific (BRAF V600E) inhibitors did not work.

Furthermore, numerous data are available about proapoptotic effects of arsenic [57], TRAIL alone and TRAIL in different combinations for induction of apoptosis in human neuroblastoma cells [50]. Combined treatment of neuroblastoma cells with TRAIL and sodium arsenite or TRAIL and simvastatin that was very efficient in our in vitro study (see Fig. 9) might also serve as a potential treatment modality for induction of high levels of apoptosis in this type of tumor. In summary, our results further highlighted significance of combined targeting of cell signaling pathways for efficient and specific elimination of cancer cells.

Materials and methods

Materials

Sodium arsenite, simvastatin, atorvastatin, mevalonic acid (lithium salt) and GGPP were obtained from Sigma-Aldrich (St. Louis, MO, USA). Human *Killer*-TRAIL was purchased from Axxora (San Diego, CA, USA). Lovastatin was purchased from Calbiochem/EMD Chemicals (San Diego, CA, USA). COX-2 inhibitor NS398 was obtained from Cayman Chemical (Ann Arbor, MI, USA). Human recombinant IL6 was obtained from R&D Systems (Minneapolis, MN, USA). Zn(II) containing Protoporphyrin IX, an inhibitor of HO-1, was purchased from Frontier Scientific (Logan, UT, USA).

Cell culture

The primary human embryonic lung fibroblasts IMR-90 (Coriell Cell Repository, Camden, NY, USA) were maintained in medium supplemented with 15% fetal bovine serum, vitamins, non-essential amino acids and antibiotics. Normal human melanocytes were obtained from the Department of Dermatology, Yale University (New Haven, CT, USA) and maintained in TICVA medium. Human melanoma cell lines LU1205 (also known as 1205lu), WM9, WM35, WM164, WM793, WM852 [58], FEMX, LOX, HHMSX [59] and A375 and human neuroblastoma cell line HTB-11 (SK-N-SH) were maintained in DMEM medium supplemented with 10% fetal bovine serum, L-glutamine and antibiotics. Cells were grown at 37°C with 5% CO_2. A375 and HTB-11 cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA).

Transfection and luciferase assay

The NF-κB luciferase reporter containing two κB binding sites, and STAT-Luc reporter containing three repeats of GAS sites from the Ly6A/E promoter were used to determine NF-κB and STAT transactivation, respectively. Transient transfection of different reporter constructs (1 μg) together with pCMV-β-galactosidase (0.25 μg) into 5×10^5 cells was performed using Lipofectamine (Life Technologies-Invitrogen, Carlsbad, CA, USA). Proteins were prepared for β-Gal and luciferase analysis 16 h after transfection. Luciferase activity was determined using the Luciferase assay system (Promega, Madison, WI, USA) and was normalized based on β-galactosidase levels.

Apoptosis studies

Surface levels of TRAIL-R1/DR4 and TRAIL-R2/DR5 were determined by staining with the PE-labeled mAbs from eBioscience (San Diego, CA, USA). A FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) combined with the CellQuest program was used to perform flow cytometric analysis.

For induction of apoptosis, cells were exposed to sodium arsenite (1–20 μM) or statin (1–80 μM) alone or in the presence of protoporphyrin IX (ZnII) at a dose 10–40 μM. Cells were also exposed to soluble TRAIL (50 ng/ml) alone or in a combination with sodium arsenite (5 μM) or simvastatin (5–20 μM). Apoptosis was then assessed by PI staining and quantifying the percentage of hypodiploid nuclei (pre-G1) using FACS analysis.

Western blot analysis

Total cell lysates (50 μg protein) were resolved on SDS-PAGE, and processed according to standard protocols. The monoclonal antibodies used for Western blotting included: anti-β-Actin (Sigma, St. Louis, MO, USA); anti-FLIP (NF6) (Axxora, San Diego, CA, USA); anti-caspase-8, anti-caspase-9, anti-caspase-3 (Cell Signaling, Danvers, MA, USA); anti-COX-2 (Cayman Chemical, Ann Arbor, MI, USA). The polyclonal antibodies used included anti-phospho-p44/p42 MAP kinase (Thr202/Tyr204) and anti-p44/p42 MAP kinase; anti-phospho-AKT (Ser473) and anti-AKT; anti-p65 NF-κB, anti-STAT3, anti-phospho-STAT3 (Tyr705); anti-PARP-1; anti-Bid (Cell Signaling, Danvers, MA, USA); anti-HO-1 (Enzo Life Sciences, Plymouth Meeting, PA, USA). The secondary antibodies were conjugated to horseradish peroxidase; signals were detected using the ECL system (Thermo Scientific, Rockford, IL, USA).

ELISA

Antibody pairs used in sandwich ELISA for this study were all commercially available. Kits to detect IL6, TNFα and IL1β were from Life Technologies/Invitrogen (Carlsbad, CA, USA).

RNAi targeting of HO-1 mRNA

The empty vector pSR-GFP/Neo was obtained from Oligoengine (Seattle, WA, USA). RNAi of 19 nucleotides (AGATTGCCCAGAAAGCCCT), designed to target human HO-1 mRNA within nucleotides 526–544 were expressed using pSR-GFP/Neo (HO-1-RNAi) plasmid construct, which also produced a marker GFP protein. Human melanoma cells WM793 have been used for HO-1 targeting. Melanoma cells were transfected with indicated expression vectors using Lipofectamine (Life Technologies/Invitrogen, Carlsbad, CA).

For detection of apoptotic levels after treatment with sodium arsenite or statin, GFP-positive stably transfected cells were stained by PE-labeled Annexin-V (red) (BD-Pharmingen, San Diego, CA, USA). A FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) combined with the CellQuest program was used to perform flow cytometric analysis of double-stained cells.

Statistical analysis

Data from 3 to 4 independent experiments were calculated as means and standard deviations. Comparisons of results between treated and control groups were made by the Students' t tests. A P value of 0.05 or less between groups was considered significant.

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Conflicts of interest No potential conflicts of interest were disclosed.

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