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Agonists of an ecdysone-inducible mammalian expression system inhibit Fas Ligand- and TRAIL-induced apoptosis in the human colon carcinoma cell line RKO

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Abstract

The ecdysone-inducible mammalian expression system is frequently used for inducible transgene expression *in vitro* and *in vivo*. Here, we describe a strong antiapoptotic effect of ecdysone analogs in the human colon carcinoma cell line RKO, which is in contrast to published data that ecdysteroids do not influence mammalian cell physiology. Inhibition of Fas ligand- and TNF-related apoptosis-inducing ligand-induced apoptosis by muristerone A occurs at the level of caspase-8 activation and is neutralized by phosphatidylinositol-3-kinase/Akt, protein kinase C and mitogen-activated protein kinase inhibitors. Microarray, Northern and Western blot analysis revealed that incubation of RKO cells with muristerone A leads to changes in gene expression levels, including an upregulation of *bcl-x$_L$* mRNA and protein levels. Our data imply that ecdysteroids and ecdysone mimics can induce and/or repress gene transcription in RKO and other mammalian cells, thereby influencing the apoptotic behavior. Therefore, the ecdysone-inducible mammalian expression system may not be suitable for the analysis of apoptosis-related genes.
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Abbreviations: hFasL, human Fas ligand; TRAIL, TNF-related apoptosis-inducing ligand; Casp-8, 3, 9, caspase-8, 3, 9; PI3K, phosphatidylinositol-3-kinase; PKB, protein kinase B; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; MAPKK/MKK, mitogen-activated protein kinase kinase; RXR, retinoid X receptor; CHX, cycloheximide; PI, propidium iodide; DISC, death-inducing signaling complex; FLIP S/L, FLICE-like inhibitory protein (short/long); Cyt *c*, cytochrome *c*; ERK1/2, extracellular signal-regulated kinase 1/2; PI(4,5)P2, phosphatidylinositol-4,5-bisphosphate; PI(3,4,5)P3, phosphatidylinositol-3,4,5-triphosphate; PMA, phorbol 12-myristate 13-acetate; NF-κB, nuclear factor-κB; DYRK, dual-specificity tyrosine-phosphorylation-regulated protein kinase; MNB, minibrain gene; MMP, mitochondrial membrane potential; PDK, phosphoinositide-dependent kinase; PH domain, Plekstrin homology domain; ILK, integrin-linked kinase; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; S.D., standard deviation; S.E.M., standard error of the mean; EtOH, ethanol

Introduction

Ecdysone is an insect steroid hormone belonging to a larger family of ecdysteroids, which trigger metamorphosis in insects, for example *Drosophila melanogaster*.[1,2] Recently, an ecdysone-regulated gene switch has been developed as an inducible expression system for mammalian cells.[3,4] A chimeric protein composed of the VP16 activation domain fused to an ecdysone receptor with altered DNA-binding specificity (VgEcR) heterodimerizes with the retinoid X receptor (RXR) and attaches to a unique synthetic response element not recognized by natural nuclear hormone receptors. Binding of specific inducers to the receptor dimer leads to its activation and consequently to the transcriptional upregulation of any gene of interest located downstream of the synthetic response element recognized by the artificial VgEcR/RXR transcription factor. Different ecdysone analogs can be used to induce the system, among them the herbal steroids muristerone A and ponasterone A, and other nonsteroidal ecdysone mimics including [*N*-(3-methoxy-2-ethylbenzoyl)-*N'*-(3,5-dimethylbenzoyl)-*N'*-tert-butyl hydrazine], which is commercially available under the name GS™-E.[4]

The low basal activity of the ecdysone-inducible mammalian expression system, in combination with a high induction rate, makes it an attractive alternative for tightly regulated expression of genes in cultured cells and transgenic mice.[3] In addition, the known inducers of the system possess a lipophilic nature useful for efficient penetration into all tissues, short half-lives, which allow precise and potent inductions, and favorable pharmacokinetics that prevent storage and expedite clearance. As an additional advantage, ecdysteroids are neither toxic nor teratogenic, and they should not affect mammalian physiology,[3] although a recent publication reported effects of muristerone A and ponasterone A on cytokine signaling in mammalian cells.[5]

We used an established system of ecdysone-inducible p27 overexpression in the human colon carcinoma cell line RKO[6] to analyze the functional relationship between death receptor-mediated apoptosis and the cell cycle. During the course of

these experiments, we noticed that the substances used to induce p27 expression (namely muristerone A, ponasterone A and GS™-E) exhibited a strong antiapoptotic effect independent of the ecdysone-inducible system. We then set out to analyze the antiapoptotic influence of these ecdysone analogs on mammalian cells in more detail.

Results

Muristerone A protects from death receptor-mediated apoptosis in the human colon carcinoma cell line RKO

Since we planned to apply the commercially available ecdysone-inducible expression system in human RKO colon carcinoma cells to analyze the cell cycle dependency of CD95/Fas-induced apoptosis, we tested whether the broadly used inducer of the system – muristerone A[4] – has an effect on Fas-mediated apoptosis. In a first experiment, we incubated wild-type RKO cells (without any components of the ecdysone-inducible system) for 48 h with muristerone A at a concentration of 3 μM, a treatment that on its own had no toxic effect (Figure 1a, left half, black bar). At 24 h after addition of muristerone A, we applied soluble recombinant human Fas ligand (hFasL) (10 ng/ml, together with 0.1 μg/ml cycloheximide (CHX) plus 1 μg/ml anti-FLAG antibody for FasL crosslinking) to the muristerone A-containing medium for an additional 24 h. This resulted in $6 \pm 1\%$ of cells appearing apoptotic with fragmented DNA (Figure 1a, right half, black bar), detectable in the sub-G1 area of the cell cycle profile of fixed cells containing propidium iodide (PI)-stained DNA. Under the same killing conditions, 36% of RKO cells not treated with muristerone A underwent hFasL-mediated apoptosis (Figure 1a, right half, white bar). Apparently, muristerone A significantly inhibits FasL-induced cell death in RKO cells.
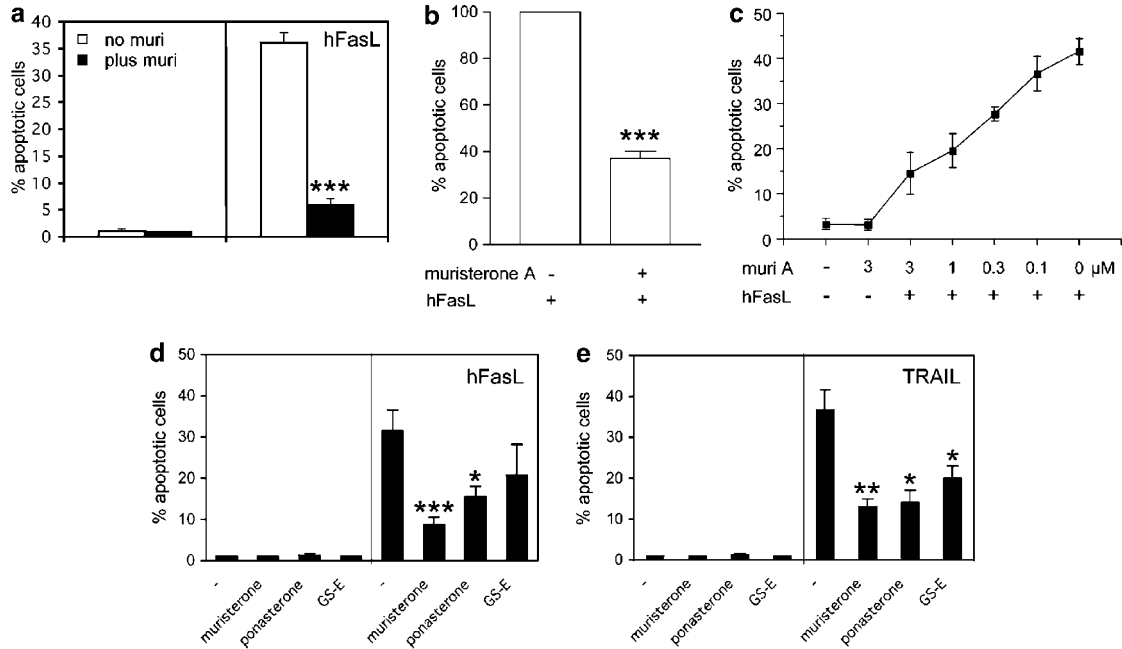

Figure 1 Muristerone A, ponasterone A and GS™-E inhibit FasL- and TRAIL-induced apoptosis in the human colon carcinoma cell line RKO. (**a**) A 24 h of incubation with soluble recombinant hFasL (10 ng/ml) resulted in $36 \pm 2\%$ apoptotic cell death, as measured by PI staining of EtOH-fixed cells. When treated with 3 μM muristerone A 24 h before addition of FasL, only $6 \pm 1\%$ of cells were killed (right half). The graph shows the average of four independent experiments. White bars represent RKO cells without muristerone A treatment, whereas black bars correspond to RKO cells treated with muristerone A. The bars in the left half of the figure depict control incubations without FasL. Error bars: Standard deviation (S.D.). The difference between percentages of FasL-killed cells treated with or without muristerone A is statistically highly significant (*t*-test $P < 0.0001$, indicated with three stars). (**b**) In 18 independent experiments, only 37% cells were killed after incubation with 3 μM muristerone A for 24 h and 10 ng/ml FasL for 4 h, as compared to cells without muristerone A pretreatment (set as 100% in this presentation). This difference in FasL cell killing is highly significant ($P < 0.0001$). Error bars: Standard error of the mean (S.E.M.). (**c**) The protective effect of muristerone A against treatment with hFasL (10 ng/ml for 24 h) is concentration dependent. The average of three independent experiments is shown; error bars indicate standard deviations. (**d**) Other ecdysone homologs and mimics (ponasterone A (3 μM) and the synthetic GS™-E (9 μM)) also inhibit hFasL-induced apoptosis in RKO cells. Cells were preincubated for 24 h with ecdysone analogs and then treated with FasL (24 h, 10 ng/ml). While RKO control cells in the absence of further substances responded to FasL killing with $32 \pm 5\%$ cell death, only $9 \pm 2\%$ of cells were killed in the presence of muristerone A, $16 \pm 3\%$ with ponasterone A and $21 \pm 8\%$ after GS™-E treatment. The figure represents the average of four independent experiments. The left half of the figure shows that incubation with ecdysone analogs alone without FasL was not toxic for the cells. Error bars: S.D. *t*-Test values are for the difference between FasL and FasL plus muristerone A ($P < 0.0001$, highly significant), between FasL and FasL plus ponasterone A ($P = 0.0157$, significant) and between FasL and FasL plus GS-E ($P = 0.0733$, not significant). (**e**) Ecdysone analogs also inhibit TRAIL-induced apoptosis in RKO cells. Incubation with TRAIL (10 ng/ml His-tagged killer TRAIL) resulted in $37 \pm 5\%$ apoptotic cells in control cell cultures. Treatment of the cells with muristerone A, ponasterone A or GS™-E before addition of TRAIL ligand decreased the extent of apoptosis. With muristerone A, only $13 \pm 2\%$ of cells were killed, with ponasterone A, $14 \pm 3\%$ and with GS™-E, $20 \pm 3\%$. The figure represents the average of three independent experiments. In the left half, control incubations without TRAIL are shown. Error bars: S.D. *P*-values are for the difference between TRAIL and TRAIL plus muristerone A ($P = 0.0085$, very significant), between TRAIL and TRAIL plus ponasterone A ($P = 0.0108$, significant) and between TRAIL and TRAIL plus GS-E ($P = 0.0246$, significant)

Figure 1b represents the summary of 18 independent experiments. Since absolute numbers (in percent) of cells killed by FasL treatment varied between individual experiments, the amount of dead cells in the absence of muristerone A was set to 100%, and the fraction of dead cells after FasL incubation in muristerone A-treated cells was determined. In the presence of 3 μM muristerone A, very constantly only 37% of cells died after addition of FasL, as compared to cells without muristerone A.

We went on to show that the protective effect of muristerone A is dose dependent (Figure 1c). With decreasing concentrations of muristerone A applied to the medium, the proportion of dead RKO cells after hFasL treatment increased.

Two other substances are frequently used instead of muristerone A to induce the ecdysone receptor gene switch in mammalian cells: ponasterone A (another ecdysone homolog[4]) and the synthetic nonsteroidal ecdysone mimic GS™-E (Invitrogen). Interestingly, both substances were also able to inhibit hFasL-induced apoptosis in RKO cells (Figure 1d, right half). Neither of them showed any toxic effects at the concentrations employed (Figure 1d, left half). It is worth noting that, among the three substances tested, the ability to inhibit apoptosis correlated with the potency of target gene induction from the ecdysone-inducible system. Muristerone A exhibited the strongest apoptosis-reducing capacity (Figure 1b and d) and similarly activated the ecdysone-inducible system very potently (data not shown), while GS™-E displayed the weakest activity in both assays.

In the next experiment, we tested whether the VgEcR/RXR inducers also inhibit mammalian apoptosis initiated by other death ligands. The recombinant soluble form of TNF-related apoptosis-inducing ligand (TRAIL) induces apoptosis in cell lines from a broad spectrum of human cancers, whereas most normal human cell types are insensitive to the ligand.[7] We found that muristerone A, ponasterone A and GS™-E were not only able to decrease FasL-induced apoptosis, but also RKO cell death initiated by TRAIL (Figure 1e, right half).

We then tested whether cell lines other than RKO would be influenced by muristerone A in their apoptotic behavior. Different other human colon carcinoma cell lines (HCT116, CaCo2, DLD1) displayed no altered cell death behavior after muristerone A treatment. However, we detected a significant inhibition of FasL- and TRAIL-induced apoptosis by muristerone A in SW620 (human colon carcinoma) and HC11 (nontransformed mouse epithelial) cells (see Supplementary Figure 1a and b), although the extent of protection by muristerone A was clearly smaller as observed with RKO cells. We also found two cell lines – A20 (mouse B-cell lymphoma) and HeLa (human cervix adenocarcinoma) cells – which were more (and not less) sensitive toward FasL killing in the presence of muristerone A (Supplementary Figure 1c and d).

In summary, our data illustrate that muristerone A and analogs such as ponasterone A and GS™-E inhibit death receptor-mediated apoptosis in RKO cells induced by hFasL and TRAIL, demonstrating that unexpectedly and in contrast to published data[3,4,8] these compounds profoundly influence mammalian cell physiology.

Muristerone A inhibits Fas-mediated apoptosis at the level of caspase-8 activation

Upon ligand binding, death receptors activate the extrinsic apoptosis pathway, which in so-called type II cells like RKO is amplified by the intrinsic or mitochondrial apoptosis pathway.[9,10] Death receptor-induced apoptosis can be inhibited at different steps of the cell death program, and we tried to find out at which level muristerone A interferes with the death receptor-mediated signaling cascade. First, we quantified Fas receptor expression at the cell surface. As demonstrated in Figure 2a, treatment of RKO cells with muristerone A did not influence Fas receptor cell surface expression.

Following Fas receptor activation and formation of the intracellular Fas receptor complex (DISC for 'death-inducing signaling complex'[11]), caspase-8 (Casp-8) becomes autocatalytically activated in this complex by 'induced proximity'.[12] We therefore analyzed Casp-8 activity upon stimulation with soluble hFasL in muristerone A-treated and untreated RKO cells (Figure 2b). Incubation of the cells with muristerone A for 24 h led to decreased Casp-8 activation after addition of hFasL. Importantly, treatment of the cells with muristerone A alone (without Fas activation) led to decreased basal Casp-8 activity, suggesting a general Casp-8 inhibition by muristerone A in RKO cells. The overall moderate Casp-8 activity generated after Fas activation in RKO cells confirms the classification as type II cells, suggested by the fact that these cells are only killed by recombinant hFasL upon CHX sensitization (leading to the inhibition of protein translation[10]). The inhibition of Casp-8 activation by muristerone A is supported by Western blot analysis, which reveals decreased cleavage of pro-Casp-8 after FasL treatment in the presence of muristerone A (see Supplementary Figure 2). Since RKO cells do not express detectable levels of FLIP$_L$ or FLIPs (FLICE-like inhibitory protein (short/long); data not shown), these inhibitory proteins can be excluded as a cause for decreased Casp-8 activity.[13]

Upon triggering of the death receptor pathway, RKO cells release cytochrome *c* (Cyt *c*) into the cytosol.[14] This release can be measured by flow cytometry with the help of specific antibody binding to Cyt *c* after permeabilization of the cells (Figure 2c). A 4 h of RKO cell incubation with hFasL led to a decrease in mitochondrial-localized Cyt *c* in 38% of muristerone A-free cells, whereas mitochondrial Cyt *c* was released in only 13% of muristerone A pretreated cells following addition of hFasL.

To further investigate mitochondrial events upon death ligand treatment, we studied the mitochondrial membrane depolarization that occurs in cells undergoing apoptosis.[14,15] For this experiment, we used JC-1 (5,5′,6,6′-tetrachloro-1,1′, 3,3′-tetraethylbenzimidazolylcarbocyanine iodide) as a specific mitochondrial membrane potential (MMP)-dependent probe (Figure 2d). hFasL stimulation of RKO cells results in MMP depolarization, which can be measured by FACS analysis as an increased population of cells with detectable JC-1 monomer fluorescence in FL-1 (525 nm). Pretreatment of the cells with muristerone A prevented hFasL-induced MMP depolarization.

Once Cyt *c* is released into the cytosol, it elicits the assembly of a large protein complex, the apoptosome, which
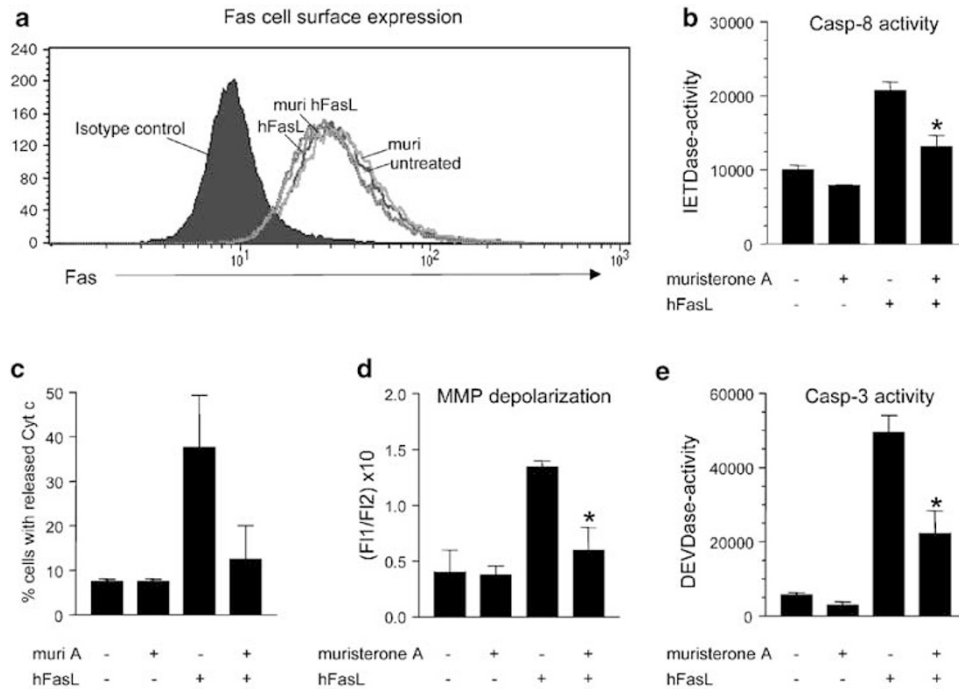
Figure 2 Inhibition of death receptor-mediated apoptosis by muristerone A occurs at the level of Casp-8 activation. (**a**) Fluorescence-activated cell sorter (FACS) analysis of Fas receptor expression levels at the cell surface with an FITC-coupled anti-hFas antibody revealed no significant differences between untreated and muristerone A-treated (3 μM, 24 h) RKO cells in the presence or absence of hFasL (20 ng/ml for 4 h). The experiment was repeated twice with similar results. (**b**) Quantification of Casp-8 activity with an AFC-labeled Casp-8-specific IETD peptide showed decreased Casp-8 activation 4 h after addition of FasL (10 ng/ml) in RKO cells treated for 24 h with 3 μM muristerone A (13 163 relative units (RU)), compared to FasL-treated cells without muristerone A (20 706 RU). The first two bars indicate constitutive Casp-8 activity in nonapoptotic RKO cells in the absence of FasL, with and without muristerone A. The figure shows the average of two independent experiments, each performed in triplicate. Error bars: S.E.M. The difference in the Casp-8 activity between FasL and FasL plus muristerone A-treated cells is statistically significant ($P = 0.0143$). (**c**) Muristerone A prevents Cyt *c* release in response to 4 h of incubation with 10 ng/ml hFasL. Only 13% of muristerone A-treated RKO cells released mitochondrial Cyt *c* upon addition of FasL, compared to 38% of cells in the absence of muristerone A. This result was obtained by FACS analysis of fixed RKO cells after intracellular staining with a specific antibody recognizing Cyt *c*. The graph represents the average of two independent experiments. Error bars: S.E.M. In both experiments, only one-third of the muristerone A-treated cells released Cyt *c* after incubation with FasL, and this difference is statistically significant ($P = 0.0335$). However, because of divergent absolute values obtained for cells treated with FasL only, the differences between the average percent numbers for FasL and FasL plus muristerone A are statistically not significant. (**d**) Measurement of MMP with the fluorescent MMP-dependent dye JC-1 showed that there was no MMP depolarization in cells treated with muristerone A (3 μM, 24 h) before hFasL (20 ng/ml, 4 h) incubation, while MMP breakdown was observed in apoptotic cells without muristerone A protection. The figure represents the average of two independent experiments. Error bars: S.E.M. The difference in cell proportions with depolarized MMP between FasL- and FasL plus muristerone A-treated samples is significant ($P = 0.0358$). (**e**) Casp-3 activity was significantly decreased in hFasL-treated RKO cells (10 ng/ml, 4 h) after 24 h of preincubation with 3 μM muristerone A (22 000 RU) compared to RKO cells treated with hFasL in the absence of muristerone A (49 000 RU). The figure displays the average of two independent experiments, each performed in triplicate. Error bars: S.E.M. The difference in the Casp-3 activity between FasL- and FasL plus muristerone A-treated cells is statistically significant ($P = 0.0210$)

contains activated Casp-9 and facilitates further activation of effector Casp-3.[16] Indeed, triggering of the Fas-mediated death pathway in RKO cells resulted in large amounts of activated Casp-3 (Figure 2e). As expected, RKO cells pretreated with muristerone A prior to Fas stimulation displayed much less Casp-3 activity, and muristerone A even decreased basal activation of Casp-3 without Fas ligation. This finding was confirmed by Western blot analysis, since generation of the cleaved large and small Casp-3 subunits after FasL treatment was diminished in the presence of muristerone A (see Supplementary Figure 2).

Our data suggest that the protection from death receptor-induced apoptosis conferred by muristerone A (and other inducers of the ecdysone system) in RKO cells occurs at the level of Casp-8 activation within the intracellular DISC complex. This finding is supported by our observation that cleavage of the BH3-only Bcl-2 family member Bid (which is a

direct Casp-8 target[17,18]) after addition of hFasL is inhibited in muristerone A-treated RKO cells (data not shown).

Downregulation of the PI3K/Akt signaling pathway and PKC and MAPK inhibitors restore apoptosis sensitivity in muristerone A-treated cells

Next, we wanted to investigate the molecular mechanism responsible for the antiapoptotic effect of muristerone A in mammalian cells. An increasing number of published experiments suggest that activation of the phosphatidylinositol-3-kinase (PI3K) pathway inhibits apoptosis not only at the mitochondrial level by phosphorylation and sequestration of the proapoptotic BH3-only Bcl-2 family member Bad (see Downward[19] and Vivanco and Sawyers[20] and references therein), but also at the level of Casp-8 activation at the DISC, perhaps by Casp-8 phosphorylation.[21–25]

To test whether PI3K signaling is responsible for muristerone A-mediated impairment of cell death, we used the specific PI3K inhibitor LY294002, which is a competitive and reversible inhibitor of the PI3K–ATP-binding site.[26] As illustrated in Figure 3a, LY294002 displayed a small toxic effect when used alone at a concentration of $25 \mu M$ (6.7% dead cells *versus* 1.8% untreated cells), and it enhanced hFasL-induced apoptosis (35.5% dead cells *versus* 27.2% cells treated with hFasL alone). When muristerone A-treated RKO cells were pretreated with LY294002 before incubation with hFasL, 20.5% of the cells were found to be apoptotic, compared to 9.8% after hFasL treatment in the presence of muristerone A, but without LY294002. In summary, the apoptotic response of RKO cells to hFasL treatment, which was decreased by the addition of muristerone A from 27.2% dead cells to 9.8%, was restored to 20.5% in the further presence of LY294002.

To confirm the importance of PI3K signaling for the antiapoptotic effect of muristerone A, we made use of another PI3K inhibitor, wortmannin, which irreversibly inhibits PI3K by binding to its catalytic subunit.[26] In contrast to LY294002, this chemical demonstrated no toxic activity in RKO cells. However, like LY294002, wortmannin partially resensitized RKO cells toward hFasL-induced apoptosis in the presence of muristerone A (Figure 3b).

One (indirect) target of activated PI3K is the serine/threonine kinase Akt/protein kinase B (PKB), which, upon activation, directly phosphorylates Bad and other apoptosis-relevant proteins (see Downward[19] and Vivanco and Sawyers[20] and references therein). To analyze the involvement of activated Akt/PKB in the PI3K-dependent inhibition of apoptosis by muristerone A, we used a specific Akt/PKB inhibitor from Calbiochem ([1L-6-hydroxymethyl-chiro-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate]). As can be seen in Figure 3c, this substance only slightly increased the number of dead cells when applied on its own, but impressively restored the ability of muristerone A-treated RKO cells to undergo Fas-induced apoptosis.

When the same experiment was repeated with Go6976 (a specific inhibitor of another serine/threonine kinase family, the calcium-dependent protein kinase C (PKC) isoforms[27]) instead of a PI3K or Akt/PKB inhibitor, apoptosis sensitivity upon hFasL treatment was again re-established to great extent in muristerone A-protected RKO cells (Figure 3d). PKC has been reported to inhibit apoptosis at the level of Casp-8 activation within the Fas receptor DISC,[28] a finding that is compatible with the observed PKC-dependent decrease of FasL-induced Casp-8 activation in muristerone A-treated RKO cells. In addition, PKC influences apoptosis by regulating the amount of Bcl-x_L,[29,30] a member of the antiapoptotic Bcl-2 subfamily, whose expression level in muristerone A-treated cells was also investigated in the course of this study (see below).

Mitogen-activated protein kinases (MAPK) have well-established important roles in apoptosis regulation.[31] One mechanism how activated p42/44 MAP kinases (extracellular signal-regulated kinase 1/2 (ERK1/2)) suppress apoptosis is by upregulating Bcl-x_L,[32–34] and in mammary epithelial cells a significant decrease in Bcl-x_L protein has been reported by using pharmacological inhibitors of both the PI3K/Akt and the

p42/44 MAPK pathways.[35] We used a specific inhibitor of the activation of mitogen-activated protein kinase kinase (MAPKK), PD 98,059,[36] to investigate the role of p42/44 MAP kinases in muristerone A-induced inhibition of RKO cell apoptosis. Figure 3e shows that inhibition of the p42/p44 MAPK pathway by PD 98,059 once again reintroduces FasL sensitivity in muristerone A-treated cells.

To test whether muristerone A treatment activates the pathways blocked by the above-mentioned inhibitors, and whether these inhibitors indeed prevented activation of their target pathways at the concentrations used in this study, we performed Western blot analysis with lysates prepared from RKO cells incubated with the different substances (Figure 4). PI3K activation and subsequent generation of phosphatidyl-inositol-3,4,5-trisphosphate (PI(3,4,5)P3) is a prerequisite for Akt phosphorylation at T308 and S473, by which Akt kinase is activated to fulfill cell survival functions.[26] Incubation of RKO cells with muristerone A leads to elevated Akt S473 phosphorylation, while application of the PI3K inhibitor Ly 294002 prevents Akt phosphorylation (see Figure 4a). A similar inhibitory result is obtained with the specific Akt inhibitor that was used in Figure 3c.

To prove the efficiency of the inhibitor of PKCα and PKCβ isoenzymes, Go6976, at the concentration used in Figure 3d, we performed Western blot analysis with an antibody recognizing phosphorylated PKC substrates.[37] As a positive control, we treated RKO cells with the PKC activator phorbol 12-myristate 13-acetate (PMA), and detected a strong increase in PKC substrate phosphorylation. On the contrary, incubation of the cells with Go6976 under different conditions always resulted in declined phosphorylation of PKC substrates as compared to cells that were treated similarly but without Go6976 (Figure 4b). However, the sensitivity of this assay did not allow us to determine whether muristerone A application directly stimulates PKC activity.

Finally, we could show that incubation of RKO cells with muristerone A leads to activation of the p42/p44 MAPK pathway, as demonstrated by increased phosphorylation of both MAP kinases. Usage of the MAPK inhibitor PD 98,059 at the concentration used in Figure 3e resulted in decreased p42/44 MAPK phosphorylation and activation (Figure 4c).

Taken together, we could demonstrate direct activation of the PI3K/Akt and the p42/44 MAPK pathways by muristerone A, and our results with inhibitors for the different signaling pathways suggest that muristerone A-mediated protection against apoptosis in RKO cells is positively influenced by the PI3K/Akt, the PKC and the p42/44 MAPK pathways. Interestingly, activation of all these three signaling cascades is known to result in Bcl-x_L upregulation (see below).

Muristerone A treatment leads to upregulation of Bcl-x_L in RKO cells

Akt regulates cellular survival through phosphorylation of different downstream substrates that directly or indirectly control the apoptotic machinery. One of the pathways activated by Akt is the nuclear factor-κB (NF-κB) pathway,[38,39] which leads to transcriptional upregulation of prosurvival genes, including the antiapoptotic Bcl-2 family
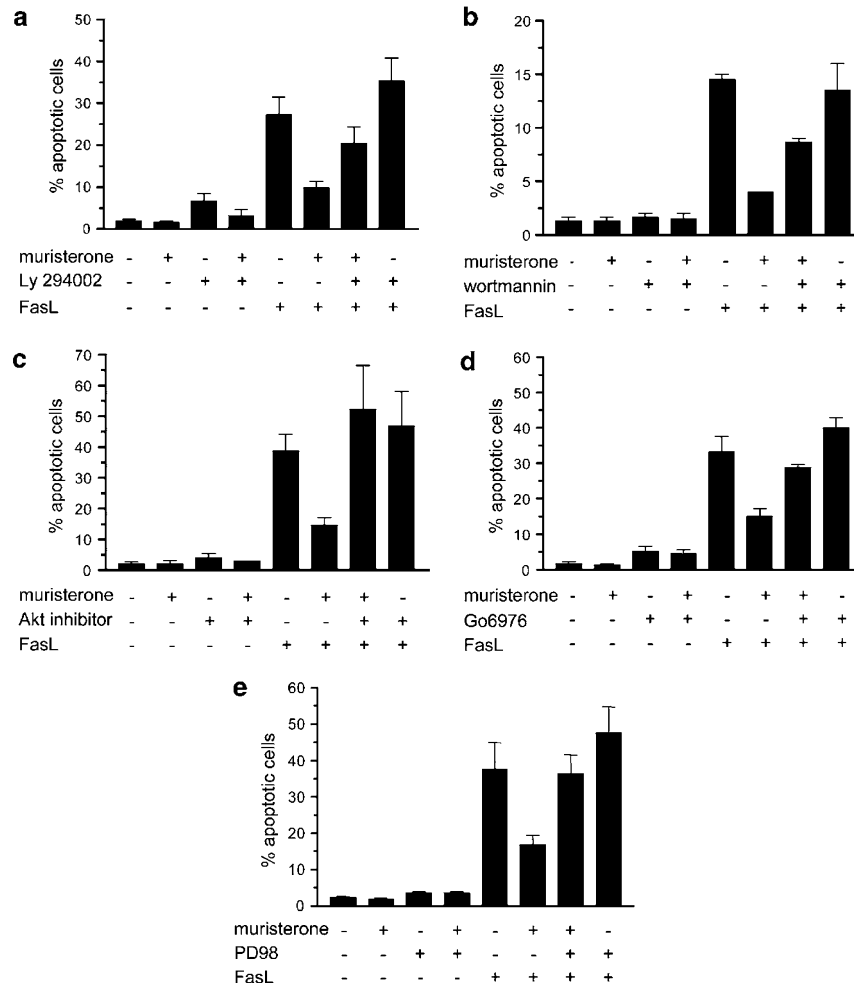
Figure 3 Inhibition of apoptosis by muristerone A depends on PI3K/Akt, PKC and p42/44 MAPK signaling. (**a**) The PI3K inhibitor LY294002 inhibits muristerone A-mediated protection from hFasL-induced apoptosis in RKO cells. At a concentration of 25 μM, LY294002 alone was slightly toxic (6.7 $\pm$ 1.8% dead cells as determined by FACS analysis after PI staining of EtOH-fixed cells) and enhanced hFasL-induced apoptosis from 27.2 $\pm$ 4.3% without LY294002 up to 35.5 $\pm$ 5.4%. Simultaneous treatment of cells with muristerone A and LY294002 resulted in 20.5 $\pm$ 4.0% apoptotic cells in response to incubation with hFasL, while only 9.8 $\pm$ 1.6% of cells died by FasL killing when protected by muristerone A (without coincubation with LY294002). This figure represents the average of seven independent experiments. Error bars: S.E.M. The difference in the proportion of dead cells between FasL and FasL plus muristerone A is very significant ($P=0.0025$), the difference between FasL plus muristerone A and FasL plus muristerone A plus LY294002 is significant ($P=0.0297$) and the difference between FasL and FasL plus muristerone A plus LY294002 is not significant ($P=0.2722$). (**b**) The PI3K inhibitor wortmannin (200 μM) inhibits muristerone A-mediated protection from hFasL-induced apoptosis. At a concentration of 200 μM, wortmannin displayed no toxic effect when added to RKO cells. The graph shows the average of three independent experiments. Error bars: S.E.M. The difference in percent dead cells between FasL and FasL plus muristerone A is very significant ($P=0.0023$), the difference between FasL plus muristerone A and FasL plus muristerone A plus wortmannin is very significant ($P=0.0017$) and the difference between FasL and FasL plus muristerone A plus wortmannin is very significant ($P=0.0020$). (**c**) Like LY 294002 and wortmannin, a specific Akt/PKB inhibitor ([1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate]; 25 μM) abolishes apoptosis inhibition by muristerone A in RKO cells. The Akt inhibitor showed slight proapoptotic activity (4%) when applied on its own. The average of four independent experiments is shown. Error bars: S.E.M. The difference in the amount of dead cells between FasL and FasL plus muristerone A is very significant ($P=0.0076$), the difference between FasL plus muristerone A and FasL plus muristerone A plus Akt inhibitor is significant ($P=0.0429$) and the difference between FasL and FasL plus muristerone A plus Akt inhibitor is not significant ($P=0.4170$). (**d**) Calcium-dependent PKC isoforms are involved in the antiapoptotic muristerone A effect. The PKC inhibitor Go6976 abolished muristerone A protection from hFasL in RKO cells. At the chosen concentration of 5 μM, Go6976 induced 5.2 $\pm$ 1.5% cell death when applied on its own. The figure represents the average of three independent experiments. Error bars: S.E.M. The difference in the percentage of dead cells between FasL and FasL plus muristerone A is significant ($P=0.0209$), the difference between FasL plus muristerone A and FasL plus muristerone A plus Go6976 is very significant ($P=0.0034$) and the difference between FasL and FasL plus muristerone A plus Go6976 is not significant ($P=0.3878$). (**e**) The MAPK pathway inhibitor PD 98,059 (2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one) prevents inhibition of apoptosis by muristerone A in RKO cells. PD 98,059 was used at a concentration of 10 μM. Four independent experiments were performed. Error bars: S.E.M. The difference in the proportion of dead cells between FasL and FasL plus muristerone A is significant ($P=0.0382$), the difference between FasL plus muristerone A and FasL plus muristerone A plus PD 98,059 is significant ($P=0.0133$) and the difference between FasL and FasL plus muristerone A plus Go6976 is not significant ($P=0.9056$). All inhibitors were added to the medium 1 h before incubation of the cells with 3 μM muristerone A for another 24 h. At 1 h before addition of recombinant FasL, the inhibitors were again supplemented. After 4 h of incubation with FasL, cells were harvested for quantification of apoptosis

member Bcl-x$_L$ (see Barkett and Gilmore[40] and references therein). The idea that the antiapoptotic activity of muristerone A may depend on gene induction is supported by the observation that muristerone A needs to be added to RKO cells several hours before the induction of apoptosis to be effective in suppressing cell killing; simultaneous incubation of
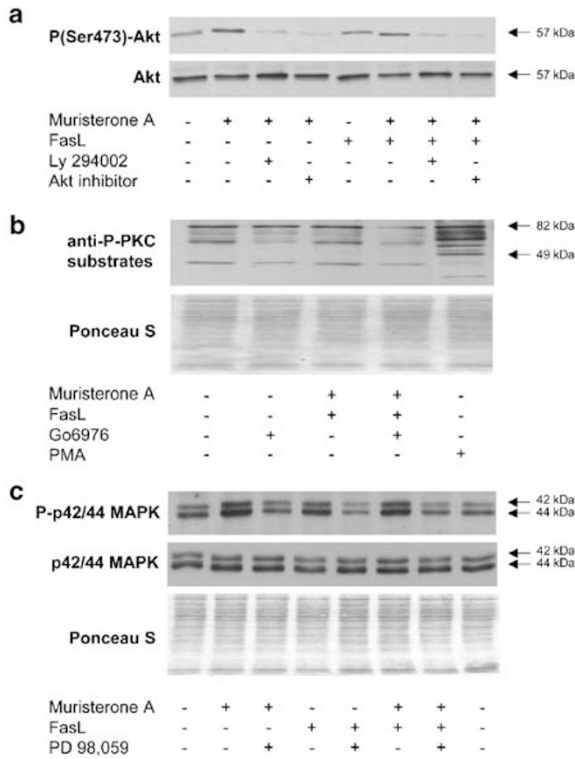
Figure 4 PI3K/Akt, PKC and p42/p44 MAPK inhibitors lead to decreased activity of their targets in RKO cells, while muristerone A enhances Akt and p42/p44 MAPK phosphorylation. (a) Western blot analysis of RKO protein lysates with a phospho-specific anti-Akt antibody after treatment of cells with inhibitors of the PI3K/Akt pathway, FasL and muristerone A. Phosphorylation of Akt Ser473 is increased by muristerone A treatment and decreased upon addition of the PI3K inhibitor Ly 294002 or incubation with the specific Akt inhibitor described in Figure 3. The Western blot performed with an antibody recognizing equal amounts of total Akt protein (second panel) is shown as a control. (b) Go6976, an inhibitor of the calcium-dependent PKCα/β isoforms, decreases phosphorylation of PKC substrates when applied to RKO cells under different conditions. Western blot analysis was performed with an antibody recognizing phosphorylated substrates of the PKC kinase.[37] Ponceau S staining of the membrane proves equal loading of protein lysates. Whether muristerone A treatment of cells stimulates phosphorylation of PKC targets could not be determined with the given sensitivity of the assay. (c) Western blot analysis of RKO protein lysates with a phospho-specific anti-p42/44 MAPK antibody. Cells were treated with the MAPK pathway inhibitor PD 98,059, FasL and muristerone A. While muristerone A increases phosphorylation of p42/44 MAPK proteins, incubation with PD 98,059 prevents MAPK phosphorylation. Equal loading of protein lysates is demonstrated by incubation of a membrane with identical amounts of the same protein lysates with an antibody recognizing total p42/p44 MAPK proteins, and by Ponceau S staining of the membrane. The exact inhibitor, FasL and muristerone A concentrations and treatment conditions used for the cells before lysate preparation are identical to the ones described in Figure 3

the cells with muristerone A and the proapoptotic hFasL leads to a similar amount of cell death as in cells cultured with hFasL alone (data not shown).

Furthermore, activation of PKC and the p42/44 MAPK pathway have both been linked to increased amounts of Bcl-x_L[29,30,32–34] (see above). We therefore analyzed Bcl-x_L protein levels in RKO cells after incubation with muristerone A in the presence or absence of hFasL. Treatment of RKO cells with muristerone A for 24 h led to the upregulation of the Bcl-x_L protein, as determined by Western blot analysis (Figure 5). When recombinant hFasL was added to RKO
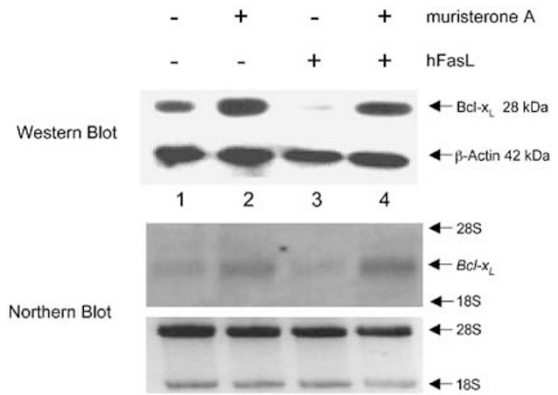

Figure 5 Muristerone A increases Bcl-x_L mRNA and protein expression. Analysis of Bcl-x_L protein levels by Western blot demonstrated that incubation of RKO cells with 3 μM muristerone A for 24 h upregulates the amount of Bcl-x_L protein (lane 2 compared with lane 1). Induction of apoptosis by recombinant hFasL (10 ng/ml) led to a dramatic decrease in Bcl-x_L levels in RKO cells (lane 3), presumably due to the previously described cleavage by activated Casp-3.[41,42] When the cells were preincubated with muristerone A before addition of FasL, Bcl-x_L protein levels were stabilized and did not vanish (lane 4). The control incubation of the membrane with anti-actin antibody proves equal protein loading. The lower part of the figure shows bcl-x_L mRNA levels in RKO cells, as determined by Northern blot analysis. Cells were incubated for 24 h with 3 μM muristerone A and/or with 20 ng/ml FasL for 4 h. Treatment of cells with muristerone A increased bcl-x_L mRNA levels, a result that confirmed the data obtained by gene expression profiling with Affymetrix oligonucleotide arrays (see Table 1). 18S and 28S rRNAs are displayed as loading controls. Both Western blot and Northern blot experiments were repeated twice with similar results

cells to induce apoptosis, Bcl-x_L levels massively decreased, presumably as a result of degradation by Casp-3.[41,42] The resulting C-terminal Bcl-x_L fragment has been reported to exert proapoptotic activity. Interestingly, muristerone A treatment of the cells prior to the addition of hFasL completely reversed the induced reduction in Bcl-x_L protein and stabilized Bcl-x_L levels, most likely as a result of the observed muristerone A-mediated Bcl-x_L upregulation. We also noticed an inhibition of UV-induced apoptosis in RKO cells upon muristerone A treatment (data not shown), an observation that is well explained by the elevated Bcl-x_L levels.[43,44]

We then analyzed bcl-x_L mRNA levels in RKO cells upon muristerone A treatment to find out whether the increase in Bcl-x_L protein is due to transcriptional upregulation of the gene. As shown in Figure 5, addition of muristerone A results in enhanced bcl-x_L mRNA amounts in the absence or presence of recombinant hFasL. As a conclusion, muristerone A stimulates bcl-x_L mRNA transcription, leading to elevated Bcl-x_L protein levels.

In addition to its established mitochondria-related anti-apoptotic activity, Bcl-x_L has been implicated in the inhibition of Casp-8 activation after treatment of cells with the death ligand TRAIL.[45] Such data are consistent with our observation that muristerone A induces upregulation of Bcl-x_L and prevents Casp-8 activation after apoptotic death receptor triggering.

Muristerone A influences gene expression in mammalian carcinoma cells

Mammalian steroid hormones bind to nuclear hormone receptors and thereby activate gene transcription.[46] Since muristerone A protects human RKO cells from apoptosis only

Table 1 Differentially expressed genes in RKO cells treated with muristerone A and hFasL

	Fold expression		
hFasL *versus* untreated	muri+hFasL *versus* untreated	Muri *versus* untreated	GeneBank no. and description
	1.2	1.4	L07597: ribosomal protein S6 kinase 1 (RPS6KA1)
	1.4	1.7	M69043: MAD-3 mRNA encoding IκB-like activity
	1.4	1.4	M93425: protein tyrosine phosphatase (PTP-PEST)
−1.4	1.3	1.3	Z23115: bcl-x$_L$
	3.3	2.4	D87116: MAP kinase kinase 3b
	3.1	2.4	L36719: MAP kinase kinase 3 (MKK3)
	7.9	1.1	Endothelial cell growth factor 1
−1.6		1.4	Cluster Incl. M34309: epidermal growth factor receptor (HER3)
	1.7	1.3	M92287: cyclin D3 (CCND3)
	−9.4	−9.1	U10906: cyclin-dependent kinase inhibitor p27kip1
−1.6			U01062: type 3 inositol 1.4.5-trisphosphate receptor (ITPR3)
−1.4			Cluster Incl. U01062:type 3 inositol 1.4.5-trisphosphate receptor (ITPR3)
	1.4	1.5	M35416: Human GTP-binding protein (RALB)
	1.4	1.4	Cluster Incl. M29893:low molecular mass GTP-binding protein (ral)
	1.7	1.6	Cluster Incl. AF030234: splicing factor Sip1
	1.3	1.5	Cluster Incl. M36341:ADP-ribosylation factor 4 (ARF4)
	−1.5	−1.4	Cluster Incl. Z35491:novel glucocorticoid receptor-associated protein
−1.4	1.4	2.3	Cluster Incl. M14648: cell adhesion protein (vitronectin) receptor alpha subunit
−2.1			X52773: retinoic acid receptor-like protein
1.7			Cluster Incl. U13021: positive regulator of programmed cell death ICH-1L (Ich-1)
−1.5			Cluster Incl. AB019036: geranylgeranyl pyrophosphate synthase
−6.3	−3.2	−1.3	Cluster Incl. M27396: asparagine synthetase
	1.4	2.5	Cluster Incl. AF032108: integrin alpha-7
	−14.0	−4.4	Cluster Incl. U58496: mnb protein kinase homolog hp86 (DYRK)
	−7.0	−12.9	U52373: Human serine/threonine kinase MNB (mnb) mRNA
	−1.3	−1.3	Cluster Incl. S68134: CREM (cyclic AMP-responsive element modulator beta isoform)
	1.2	1.3	Cluster Incl. AF056490: cAMP-specific phosphodiesterase 8A (PDE8A)
	1.7	1.3	Cluster Incl. AF035119: deleted in liver cancer-1 (DLC-1)
	4.4	6.2	Cluster Incl. AJ001189: oligophrenin 1
	1.3	1.7	Cluster Incl. U68723: checkpoint suppressor 1
5.1			M29037: 17 beta-hydroxysteroid dehydrogenase (17BHSDI)
	1.5	1.5	Cluster Incl. M69013: guanine nucleotide-binding regulatory protein (G-y-alpha)
−1.2	1.5	1.6	Cluster Incl. X71973: phospholipid hydroperoxide glutathione peroxidase (GPx-4)

Total RNA was isolated from cells that were either left untreated, incubated for 48 h with 3 μM muristerone A ('muri') or treated for 24 h with hFasL (50 ng/ml plus 1 μg/ml anti-Flag antibody, no cycloheximide) with ('muri+hFasL') or without ('hFasL') muristerone A addition 24 h previously. After cRNA synthesis, an oligonucleotide microarray analysis was performed with Affymetrix HG-U95A DNA chips. Gene expression profiles derived from all three conditions were compared pairwise using Affymetrix analysis software

after several hours of preincubation, it is reasonable to assume that this and other ecdysone analogs influence mammalian gene regulation. We therefore analyzed the gene expression profile of untreated and muristerone A-treated RKO cells (the latter with or without incubation with hFasL for 24 h) using the Affymetrix oligonucleotide array HG-U95A. Some of the results are presented in Table 1.

Incubation of RKO cells with muristerone A did indeed influence gene expression. Table 1 shows a selection of differentially expressed genes. Notably, *bcl-x$_L$* mRNA was upregulated upon muristerone A treatment, which is consistent with our finding that Bcl-x$_L$ RNA and protein levels were elevated in RKO cells incubated with the hormone.

Only six genes were up- or downregulated more than two-fold in both comparisons of untreated with muristerone A-treated cells and untreated with muristerone A plus hFasL-treated cells. They encode the following gene products: MAPKK3b, MKK3, dual-specificity tyrosine-phosphorylation-regulated protein kinase (DYRK), minibrain gene (MNB), oligophrenin and p27. *p27* mRNA is decreased by a factor of 9 in the presence of muristerone A. However, downregulation of this cell cycle inhibitor is not responsible for the anti-apoptotic muristerone A activity since we used a muristerone

A-inducible expression system to overexpress p27 protein in RKO cells and found that upregulation of p27 (rather than downregulation by muristerone A) did not abolish the protective influence of muristerone A upon hFasL treatment (Supplementary Figure 3; here we also show that over-expression of another cell cycle inhibitor, p21, did not influence apoptosis inhibition by muristerone A). Whether any of the other genes listed in Table 1 are involved in the inhibition of apoptosis by ecdysone analogs remains to be established. It is worth noting that, in addition to *p27*, several other cell cycle regulators are differentially expressed (e.g. *cyclin D3*, which is strongly upregulated at the protein level after addition of muristerone A, and the destruction of which upon FasL treatment is inhibited by muristerone A; data not shown), suggesting that treatment of mammalian cells with ecdysone may influence both apoptosis and proliferation.

Discussion

The ecdysone-inducible expression system has been widely used to express transgenes in mammalian cells in an inducible manner.[3,4,8] Ecdysone homologs like muristerone A and ponasterone A are added to the culture medium to

activate an artificial dimeric transcription factor belonging to the nuclear hormone receptor class. These ecdysone homologs have been reported to have no effect on mammalian cell physiology and to be inert in mammalian cell culture.[3] Here, we show that functional ecdysone homologs and mimics (muristerone A, ponasterone A, GS™-E) protect human colon carcinoma cells from hFasL- and TRAIL-induced cell death in a dose-dependent manner. Their ability to inhibit apoptosis in mammalian cells correlated with the potency of induction of gene expression under the control of the artificial DNA response element to which the VgEcR/RXR receptor dimer binds. While death receptor cell surface levels were not influenced, cell death was inhibited by muristerone A at the level of Casp-8 activation, which consequently led to inhibition of downstream events, such as MMP breakdown, Cyt c release and Casp-3 activation.[14] Interference with the PI3K/Akt survival pathway ablated the protective muristerone A effect, as did inhibitors of the calcium-dependent PKC isoforms and of the p42/44 MAPK pathways. Muristerone A treatment of human RKO cells also led to an upregulation of the antiapoptotic Bcl-2 family member $bcl-x_L$, which compensated for the Casp-3-mediated Bcl-x_L cleavage and degradation normally observed during apoptotic cell death.[41,42] This increase in Bcl-x_L levels in RKO cells after muristerone A treatment correlated with raised protection from UV-induced apoptosis. Affymetrix oligonucleotide microarray analysis revealed further changes in gene expression in RKO cells upon incubation with muristerone A. In particular, $bcl-x_L$ and certain cell cycle-related genes ($p27$, $cyclin D3$) were deregulated, suggesting that ecdysone homologs and mimics influence the apoptotic and proliferative behavior of mammalian cells. However, we can exclude that the antiapoptotic effect of muristerone A on RKO cells depends on cell cycle progression, since the same protective effect was observed in cells that were arrested in the G1 cell cycle phase by overexpression of CDK inhibitors p27 or p21.

Our data identify two levels at which muristerone A interferes with the apoptotic program: inhibition of Casp-8 activation at the intracellular death receptor domain (after Casp-8 recruitment into the DISC[11]) and Bcl-x_L upregulation. Both events may follow after activation of the same survival pathway, the PI3K/Akt signaling pathway,[26] which is directly stimulated by muristerone A, as judged by increased Akt-Ser473 phosphorylation. Indeed, we did not observe any protection from apoptosis by muristerone A after blockage of the PI3K/Akt pathway, either at the level of the PI3K itself (LY294002, wortmannin) or at the level of the Akt kinase (Akt inhibitor). Inhibition of Casp-8 activation by PI3K has been reported earlier, although the exact molecular mechanism remains unclear.[21–25] We did not see any changes in the expression level of the FLIP protein, a catalytically inactive Casp-8 homolog that exists in two isoforms (FLIP long and short) and that, after recruitment into the DISC, is able to inhibit Casp-8 activation.[13] However, some data indicate that, in certain situations, FLIP long may rather increase the amount of activated Casp-8[47]). PI3K indirectly activates Akt by phosphorylating phosphatidylinositol-4,5 bisphosphonate (PI(4,5)P2) to PI(3,4,5)P3.[26] Akt translocates to the cell membrane and interacts with PI(3,4,5)P3 via its plekstrin homology (PH) domain, which becomes phosphorylated at

two residues (Thr308 and Ser473) by phosphoinositide-dependent kinase (PDK) 1, PDK2 and integrin-linked kinase (ILK). Phosphorylated Akt behaves as an active kinase, and a well-known Akt phosphorylation target involved in apoptosis regulation is the proapoptotic BH3 domain-only protein Bad (see Downward[19] and Vivanco and Sawyers[20] and references therein). Once phosphorylated by Akt, Bad binds to 14-3-3 and becomes sequestered into the cytosol, where it no longer interacts with and inactivates antiapoptotic Bcl-2 family members. Akt can also phosphorylate and activate IκB kinase α, which, in turn, phosphorylates IκB, targeting it for degradation.[38,39] This leads to the nuclear translocation and activation of NF-κB and transcription of NF-κB-dependent prosurvival genes, including $bcl-x_L$ (see Barkett and Gilmore[40] and references therein).

Two other pathways are well known to result in transcriptional $bcl-x_L$ upregulation, the p42/44 MAPK pathway and the PKC signaling cascade.[29,30,32–34] We could show that muristerone A treatment increases phosphorylation of p42/44 MAPK, and inhibitors of the p42/44 MAPK and PKC pathways abolished protection by muristerone A, as the inhibitors of the PI3K pathway did. It is possible that activation of one of these pathways stimulates one or both of the other signaling cascades; however, we did not observe changes in Akt phosphorylation level when the PKC inhibitor had been applied, and $vice versa$ (data not shown), arguing for an independent activation of at least these two pathways by muristerone A.

Alternatively, the amount of $bcl-x_L$ mRNA may increase directly after nuclear hormone receptors become activated by ecdysone mimics. Transcriptional upregulation of $bcl-x_L$ has been reported for glucocorticoids[48,49] and estrogen.[50]

Elevated Bcl-x_L protein amounts may also account for the observed inhibition of Casp-8 activation in muristerone A-treated cells. Overexpression of Bcl-x_L in the human acute myelogenous leukemia cell line HL-60 suppresses TRAIL-induced apoptosis by abrogating caspase activation, including Casp-8.[45] Several publications suggest a 'feedback loop' of Casp-3 activating Casp-8 downstream of mitochondrial damage, which by itself is inhibited by Bcl-x_L.[51,52] In addition, inactivation of Casp-8 on mitochondria of Bcl-x_L-expressing MCF7 cells has been published as another regulatory mechanism for Bcl-x_L to prevent apoptosis.

Muristerone A displayed its antiapoptotic activity in the RKO cells only after several hours of incubation before the toxic stimulus, which argues that inhibition of apoptosis depends on gene induction and/or gene repression. This hypothesis is further supported by the changes in mRNA levels identified by microarray analysis. The most likely explanation is a direct activation of mammalian steroid hormone receptors by the ecdysone mimics. Interestingly, in addition to its role as an activator of estrogen receptor (ER)-mediated transcription, estrogen has been implicated in nongenomic regulation of intracellular signaling pathways through protein–protein interactions.[53] Cell membrane-localized ER has been demonstrated to interact with the regulatory subunit of PI3K and thereby to increase PI3K and Akt activity.[54,55] Such direct PI3K activation by a muristerone A-activated steroid hormone receptor is supported by the fact that we did not observe any

transcriptional changes in the components of the PI3K/Akt pathway upon muristerone A treatment.

Our current model of apoptosis inhibition in mammalian cells by ecdysone homologs and mimics involves activation of mammalian steroid hormone receptors by these substances, followed by direct activation of PI3K/Akt, PKC and p42/44 MAPK activity, leading to transcriptional upregulation of bcl-x_L (in the case of PI3K/Akt via IκB phosphorylation and NF-κB activation).

We were not the first to notice an influence of ecdysone homologs on PI3K activity. An earlier study reported that muristerone A and ponasterone A potentiate the IL-3-dependent activation of the PI3K/Akt pathway in the pro-B-cell line Ba/F3, which requires IL-3 for growth.[5] The authors noted a muristerone A-induced phosphorylation of Akt and of the Akt target protein Bad; however, this muristerone A-dependent phosphorylation was not detected in the absence of IL-3. The question of whether the IL-3-dependent increase in Bad phosphorylation leads to enhanced survival in muristerone A-treated Ba/F3 cells was not addressed.

Our own studies revealed an antiapoptotic influence of muristerone A on further mammalian cell types other than RKO. Interestingly, we also found cell lines that were more sensitive toward apoptotic stimuli when incubated with muristerone A. Such different outcomes of muristerone A treatment are reminiscent of mammalian steroid hormones, which in different cell types either inhibit or induce apoptosis (for review see Pushkala and Gupta[56]). This similarity further supports the notion that ecdysone homologs may activate mammalian steroid hormone receptors.

In the light of our results, experimental apoptosis studies using the ecdysone-inducible system have to be re-evaluated to exclude the possibility that antiapoptotic effects observed in such studies were mediated by muristerone A and other ecdysone mimics rather than by the induced gene of interest.

Materials and Methods

Cell lines and reagents

The human colon carcinoma cell line RKO (ATCC no. CRL-2577, propagation in Dulbecco's modified Eagle's medium (DMEM), 10% FCS) and the ecdysone-inducible p27- and p21-RKO cell lines (DMEM, 10% FCS, 200 μg/ml zeocin, 500 μg/ml G418) were obtained from Dr. Mathias Schmidt (Altana Pharma AG, Germany). SW620 is a human colorectal adenocarcinoma cell line (ATCC no. CCL-227), HeLa is a human cervix adenocarcinoma cell line (ATCC no. CCL-2), A20 is a mouse B-cell lymphoma line (ATCC no. TIB-208), and nontransformed human HC11 breast epithelial cells were kindly provided by B Groner (Georg-Speyer-Haus, Frankfurt, Germany). Muristerone A, ponasterone A and GS™-E were purchased from Invitrogen, PI, LY294002, Go6976, PD 98,059, digitonin, saponin and Triazol from Sigma, the Akt inhibitor ([1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate]), PMA and wortmannin from Calbiochem, JC-1 from Molecular Probes and Casp-3- and Casp-8-specific fluorogenic substrates from BioVision.

Apoptosis induction

To induce Fas-mediated apoptosis, cells were incubated with the indicated concentrations (usually 10 ng/ml) of recombinant soluble hFasL (Alexis),

together with 1 μg/ml M2 anti-FLAG antibody (Sigma) for FasL crosslinking and 100 ng/ml CHX (Sigma). To induce TRAIL-mediated apoptosis, cells were incubated with 10 ng/ml recombinant soluble TRAIL (Killer-TRAIL, His-tagged, Alexis) and CHX.

Apoptosis detection and cell cycle analysis

For cell cycle analysis and apoptosis quantification by flow cytometry, cells were collected, fixed with ice-cold 70% ethanol (EtOH) and kept at 4°C overnight. The next day, cells were washed with 38 mM sodium citrate (pH 7.5) and stained with PI (12.5 μg/ml)/DNase-free RNase A (200 μg/ml; Roche) in sodium citrate buffer, incubated at 37°C for 20 min and then measured with a FACS (FACSCalibur, Becton Dickinson). Cell Quest Pro software (Becton Dickinson) was used for the analysis of results.

Flow cytometric analysis of mitochondrial membrane potential ($\Delta\Psi_m$)

To measure $\Delta\Psi_m$, hFasL-treated or untreated RKO cells were incubated with the dye JC-1 (10 μg/ml; Molecular Probes) for 20 min at 37°C. Immediately after incubation, samples were cooled down to 4°C and analyzed with a FACSCalibur (Becton Dickinson). Cells with depolarized mitochondrial membranes appeared in FL-1 (JC-1 monomers), whereas living cells with intact MMP were detected in FL-2 (JC-1 aggregates).

Cell surface receptor expression levels

A total of 2×10^5 cells were washed with ice-cold phosphate-buffered saline (PBS), incubated for 30 min on ice with saturating amounts of primary antibody (anti-Fas-FITC, clone DX2, PharMingen), washed with PBS and analyzed with a FACSCalibur (Becton Dickinson) and the CellQuest Pro software. The isotype negative control was performed with an anti-mIgG-FITC antibody (PharMingen).

Caspase activity

Casp-3 and Casp-8 activities were measured in cell lysates as follows: cells were stimulated for 4 h with 10 ng/ml hFasL, 1 μg/ml M2 anti-Flag antibody and 100 ng/ml CHX. Lysate triplicates from 1.5×10^6 cells each were incubated in lysate buffer for 10 min on ice and then in reaction buffer (plus 1 mM DTT) containing AFC-labeled Casp-3-specific peptide DEVD or Casp-8-specific peptide IETD for 1 h at 37°C. Caspase activity was determined fluorometrically using a fluorescence plate reader with a 405 nm excitation filter and a 505 nm emission filter. Blank values (substrate in buffer without cells) were subtracted from values obtained with cell lysates (relative units (RU) = sample value−blank value).

Cytochrome c release (FACS)

Cells were harvested, washed with cold PBS, pelleted and permeabilized (50 ng/ml digitonin in PBS for 5 min at room temperature), so that Cyt c released from mitochondria in apoptotic cells could be washed away. Upon fixation with 2% formaldehyde, cells were incubated for 1 h at room temperature with blocking buffer (0.05% saponin, 3% bovine serum albumin (BSA) in PBS), followed by incubation overnight at 4°C with primary anti-Cyt c antibody (6H2.B4; Pharmingen). After incubation with secondary FITC-labeled anti-mouse antibody for 40 min on ice, the percentage of nonapoptotic cells containing mitochondrial Cyt c was quantified (FACSCalibur, Becton Dickinson).

Western blot analysis

Cells were lysed in 10 mM KCl, 1.5 mM MgCl$_2$, 10 mM Tris-HCl (pH 7.4), 0.5% SDS and 100 nM PMSF plus protease inhibitors (Roche), and protein contents were quantified with Biorad protein quantification solution. For analysis of phosphorylated proteins, the lysis buffer contained 1 mM Na-orthovanadate and 150 nM ocadaic acid, and lysates were prepared in boiling lysis buffer to inhibit phosphatases. Lysates were fractionated by SDS-PAGE analysis using 12.5% polyacrylamide gels. Proteins were then electroblotted onto Immobilon-P membranes (Millipore) and blocked later in 1% nonfat dried milk, 1% BSA and 0.05% Tween 20 in PBS. To detect phosphorylated proteins, TBST buffer (2.5 mM Tris-HCl (pH 8.1), 0.09% NaCl, 0.1% Tween) was used instead for blocking and antibody incubation. The following antibodies were applied: anti-Bcl-x$_L$ (2H12; Pharmingen), monoclonal anti-Casp-8 antiserum (clone C15; kindly provided by K Schulze-Osthoff, University of Duesseldorf, Germany), anti-Casp-3 (H-277; Santa Cruz), anti-Akt antibody (#9272; Cell Signaling), anti-phospho-Akt (Ser473) antibody (#9271; Cell Signaling), anti-phospho-(Ser) PKC substrate antibody (#2261; Cell Signaling), anti-p44/42 MAP kinase antibody (#9102; Cell Signaling), anti-phospho-p44/42 MAPK (Thr202/Tyr204) E10 antibody (#9106; Calbiochem) and anti-β-actin (C11; Santa Cruz). Ponceau S staining solution to visualize protein loading on the membrane was from Fluka. Specific proteins were detected with horseradish peroxidase-labeled secondary antibodies (Amersham) and ECL blotting substrate (Amersham).

Northern blot analysis

A total of 4×10^5 RKO cells were plated subconfluently for each treatment condition. The next day, cells were either treated or not with 3 μM muristerone A. After 24 h, recombinant human FasL (20 ng/ml) was added to some cultures, and 4 h later RNA was isolated using the RNeasy Mini kit from Quiagen. The protective effect of muristerone A against FasL killing had been tested before with an aliquot of the cells by FACS analysis of PI-stained fixed cells. Northern blot analysis was performed according to standard protocols. The RNA was blotted from a paraformaldehyde-containing agarose gel onto a Hybond N$^+$ membrane (Amersham), which was hybridized with the ^{32}P-labeled full-length human *bcl-x$_L$* coding sequence as a probe in ULTRAhyb™ hybridization solution from Ambion at 50°C overnight.

Isolation of total RNA and oligonucleotide microarray analysis

For microarray analyses, we used the Affymetrix GeneChip platform, employing a standard protocol for sample preparation and microarray hybridization that has been described in detail previously. Briefly, 2×10^6 cells were homogenized with Triazol (Sigma). Upon phenol–chloroform extraction, RNA was pelleted with isopropanol, washed with EtOH, dried and then redissolved in H$_2$O. Further analysis with HG-U95A chips (Affymetrix, Santa Clara, CA, USA) was performed in Professor Tarik Möröy's laboratory (Institut für Zellbiologie (Tumorforschung), University Hospital Essen, Germany). The total RNA was converted into double-stranded cDNA using an oligodeoxythymidine primer containing the T7 RNA polymerase-binding site (5′-GCATTAGCGGCCGCGAAATTAATAC GACTCACTATAGGGAGA-(dT)$_{24}$V-3′; MWG Biotech, Munich, Germany) for first-strand synthesis. After generation of double-stranded cDNA from the first-strand cDNA, biotinylated cRNA was synthesized by *in vitro* transcription using the BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, New York, NY, USA). Labeled cRNA was purified on

RNeasy columns (Qiagen, Hilden, Germany), fragmented and hybridized to Affymetrix HG-U95A microarrays. The arrays were washed and stained according to the manufacturer's recommendations and finally scanned in a GeneArray scanner 2500 (Agilent, Palo Alto, CA, USA).

Array images were processed to determine signals and detection calls (present, absent, marginal) for each probe set using the Affymetrix Microarray Suite 5.0 software (MAS 5.0; statistical algorithm). Scaling across all probe sets of a particular array to give an average intensity of 1000 was performed to compensate for variations in the amount and quality of the cRNA samples and in other experimental variables of nonbiological origin.

Pairwise comparisons of treated *versus* untreated samples were carried out with MAS 5.0, which calculates the significance (change *P*-value) of each change in gene expression using a Wilcoxon ranking test. To limit the number of false positives, we restricted further target identification to those probe sets that received at least one present detection call in the uninduced/induced sample pair. Probe sets exhibiting a change in *P*-value < 0.001 or > 0.999 were identified by filtering, using the Affymetrix Data Mining Tool 3.0, and only those passing these cutoffs in both independent RNA samples were considered as targets.

Statistical analysis

t-Test was performed using GraphPad Prism version 3.0a for Macintosh, GraphPad Software, San Diego, CA, USA. *P*-values smaller than 0.05 were considered as statistically significant (***$P < 0.001$; **$0.001 < P < 0.01$; *$0.01 < P < 0.05$).

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd).