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ORIGINAL PAPER

The plant alkaloid and anti-leukemia drug homoharringtonine sensitizes resistant human colorectal carcinoma cells to TRAIL-induced apoptosis via multiple mechanisms

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Abstract TNF-related apoptosis-inducing ligand (TRAIL) is a pro-apoptotic ligand from the TNF-alpha family that is under consideration, along with agonistic anti-TRAIL receptor antibodies, as a potential anti-tumor agent. However, most primary human tumors are resistant to monotherapy with TRAIL apoptogens, and thus the potential applicability of TRAIL in anti-tumor therapy ultimately depends on its rational combination with drugs targeting these resistances. In our high-throughput screening for novel agents/drugs that could sensitize TRAIL-resistant colorectal cancer cells to TRAIL-induced apoptosis, we found homoharringtonine (HHT), a cephalotaxus alkaloid and tested anti-leukemia drug, to be a very effective, low nanomolar enhancer of TRAIL-mediated apoptosis/growth suppression of these resistant cells. Co-treatment of TRAIL-resistant RKO or HT-29 cells with HHT and TRAIL led to the effective induction of apoptosis and the complete elimination of the treated cells. HHT suppressed the expression of the anti-apoptotic proteins Mcl-1 and cFLIP and enhanced the TRAIL-triggered activation of JNK and p38 kinases. The shRNA-mediated down-regulation of cFLIP or Mcl-1 in HT-29 or RKO cells variably enhanced their TRAIL-induced apoptosis but it did not markedly sensitize them to TRAIL-mediated growth suppression. However, with the notable exception of RKO/sh cFLIP cells, the downregulation of cFLIP or Mcl-1 significantly lowered the effective concentration of HHT in HHT + TRAIL co-treatment. Combined HHT + TRAIL therapy also led to the strong suppression of HT-29 tumors implanted into immunodeficient mice. Thus, HHT represents a very efficient enhancer of TRAIL-induced apoptosis with potential application in TRAIL-based, anti-cancer combination therapy.

Keywords Harringtonine · Apoptosis · Death receptor · cFLIP · Mcl-1

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Introduction

New natural and rationally designed drugs are being developed and tested in the never-ending battle against cancer [1–5]. Among these agents is the pro-apoptotic ligand TNF-related apoptosis-inducing ligand (TRAIL), which can, along with agonistic anti-TRAIL receptor antibodies, selectively induce the apoptosis of cancer cells. These TRAIL-related apoptogens are currently being evaluated in phase I/II clinical trials (recently reviewed in [6–8]). TRAIL binding to its pro-apoptotic receptors TRAIL-R1/DR4 or TRAIL-R2/DR5 leads to the rapid formation of the intracellular multiprotein death-inducing signaling complex (DISC), which, in addition to the ligand/receptor cluster, is

composed of the adapter protein FADD, the initiator procaspases-8/-10 and the caspases-8/-10 antagonist cFLIP [9, 10]. In addition to these core DISC components, other proteins such as PEA-15/PED, DDX-3, TRADD, DJ-1 or MADD can, in a cell- and conditionally-specific manner, associate with the TRAIL receptosome and negatively affect either DISC formation or caspase-8 processing [11–15].

Though TRAIL induces the apoptosis of a number of cultured cancer cells [16, 17], many primary cancer cells are resistant to TRAIL, either intrinsically and/or through anti-apoptotic signals from the tumor microenvironment. Among these protective signals are the activation of the PI3K/Akt pathway, the increased expression of anti-apoptotic proteins such as cFLIP, Mcl-1, Bcl-XL or XIAP and the suppressed expression/modification of pro-apoptotic molecules (caspases, Bid, pro-apoptotic TRAIL receptors) [8, 18]. Moreover, the TRAIL-induced activation of NF-κB signaling can enhance the survival and proliferation of these resistant cancer cells [19]. For these reasons, "smart" therapy combining TRAIL apoptogens with current tested anti-tumor drugs seems to be a preferred choice. Indeed, many drugs such as DNA damaging agents, histone deacetylase or proteasome inhibitors, Bcl-2 and IAP antagonists, natural products as well as therapeutic antibodies can synergize with TRAIL, effectively eliminating resistant cancer cells of various origin [20–22]. The sensitizing mechanisms, though not fully understood, include the increased expression of TRAIL receptors or initiator/effector caspases, the production of reactive oxygen species (ROS), the downregulation of anti-apoptotic proteins (cFLIP, Mcl-1), the suppression of anti-apoptotic signaling (NF-κB or PI3K inhibitors) and the stabilization of pro-apoptotic proteins such as active caspases or tBid (recently reviewed in [20]).

Currently, many natural products such as wogonin, nimbolide and others have emerged as potent sensitizers of resistant cancer cells to TRAIL-induced apoptosis [23–25]. Using a high-throughput screening (HTS) approach, we uncovered homoharringtonine (HHT), an alkaloid originally isolated from Cephalotaxus harringtonia, and found it to be one of the most potent sensitizers of resistant colorectal cancer cells to TRAIL-induced apoptosis. HHT has been used in traditional Chinese medicine for the treatment of diverse malignancies. Recently, its semisynthetic variant Omacetaxine mepesuccinate (marketed under the name Omapro) has been considered as an alternative therapeutic option in the treatment of imatinib-resistant chronic myeloid leukemias [26, 27]. HHT can inhibit proteosynthesis, which in affected cells leads to the proteolytic degradation of short-lived anti-apoptotic proteins such as Mcl-1, cFLIP, survivin and others [28]. HHT also suppresses the growth of non-leukemia cancer cells [29, 30], and in our model of TRAIL-resistant RKO or HT-29 colorectal cancer cells, HHT treatment led to the downregulation of Mcl-1 and cFLIP expression. However, the shRNA-mediated downregulation of either Mcl-1 or cFLIP expression in these cells did not fully sensitize them to TRAIL-induced growth suppression. HHT also very robustly cooperated with TRAIL in the growth suppression of HT-29 tumors in immunodeficient mice. Thus, HHT in combination with TRAIL or with agonistic anti-TRAIL receptor antibodies could represent an interesting option in anticancer therapy.

Materials and methods

Cell cultures and chemicals

The human colon carcinoma cell lines RKO and HT-29 and normal colon epithelial cells CCD 841 were purchased from ATCC. RKO and HT-29 cells were maintained in DMEM + 10 % FCS at 37 °C and 5 % CO_2. CCD 841 cells were cultivated in collagen-coated dishes in the recommended medium (ATCC, CRL-1790) supplemented with 10 % FCS. All chemicals used were purchased from Sigma-Aldrich unless stated otherwise.

High-throughput screening

HT-29 cells were plated in 384-well plates (Corning) at a density of 2,500 cells/25 µl/well using a Multidrop Combi dispenser (Thermo Scientific) and cultivated overnight. Then, library compounds were added using pintool (V&P Scientific) coupled to a JANUS Automated Workstation (PerkinElmer) to a final concentration of 1 µM in the absence or in combination with 100 ng/ml of recombinant TRAIL (Killer TRAIL, ENZO LS). The compound library included the Library of Pharmacologically Active Compounds (LOPAC1280, Sigma-Aldrich), the Prestwick Chemical Library (Illkirch, France) and the NIH Clinical Trial Collection (NIH, USA). In total 2,448 various unique compounds were used in the course of HTS screening. The cells were cultivated for 48 h, and then their viability was determined by the CellTiter-Blue assay (Promega) and an EnVision microplate reader (PerkinElmer).

Analysis of cell proliferation/survival and activation of effector caspases

The long-term survival of treated cells was determined by the WST-1 (Roche) and CellTiter-Blue (Promega) assays using a 96- or 384-well format, respectively, according to the manufacturers' recommendations. The real-time kinetic xCELLigence-based cell survival assay was performed according to the manufacturer's recommendations. Briefly,

3,000–5,000 cells were plated in triplicate into individual wells of an E-plate (Roche), placed into the monitoring unit in a CO_2 incubator and monitored for growth. When the cells reached approximately 40–50 % confluency, HHT and/or TRAIL were added and the on-line monitoring continued for an additional 60–70 h. The activity of the effector caspases-3/7 was quantified by the Caspase-Glo 3/7 assay (Promega) in 384-well plates, according to the manufacturer's instructions.

DISC precipitation

Cells grown to 80 % confluence in 140 mm cell culture dishes were pretreated with 50 nM HHT for 3 h, then the medium was replaced with ice-cold medium and the cells were incubated on ice for 10 min. Where applicable, biotin-labeled TRAIL (bio-TRAIL) was added to a final concentration of 1 μg/ml for 20 min. After this pre-incubation, the cold medium containing bio-TRAIL was replaced with warm medium at 37 °C (without bio-TRAIL), and the cells were incubated at 37 °C for 0–40 min. At selected time points, the culture dishes were placed on ice, and the cultivation medium was replaced with 20 ml of ice-cold phosphate-buffered saline (PBS). After washing with ice-cold PBS, the cells were scraped and centrifuged (800×*g*, 4 °C, 5 min). The cell pellets were lysed in ice-cold lysis buffer (1 % NP-40, 20 mM Tris–Cl pH 7.5, 150 mM NaCl, 10 mM NaF, 10 mM EDTA, 1 mM Na_3VO_4, 10 % glycerol) supplemented with protease inhibitors (complete protease inhibitor cocktail, Roche) on ice for 30 min, and the lysates were then centrifuged (15,000×*g*, 4 °C, 30 min). The cleared lysates were diluted to the same protein concentration (usually 3 mg/ml) and incubated with streptavidin agarose beads (Thermo Scientific) at 4 °C for 2 h. After five washes with 10 volumes of the lysis buffer, the DISC precipitates were directly eluted with 2× SDS sample buffer (95 °C, 5 min) and analyzed by Western blotting.

Cell lysis and western blotting

Cells were washed with ice-cold PBS, scraped, centrifuged, and the cell pellets lysed in ice-cold lysis buffer (1 % Triton X-100, 25 mM Tris–HCl pH 7.4, 150 mM NaCl, 10 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4) supplemented with protease inhibitors (Complete, Roche) on ice for 30 min. The lysates were centrifuged (15,000×*g*, 4 °C, 30 min), and the amount of total protein in the supernatants was quantified by the BCA assay (Pierce). The supernatants were diluted 1:1 with 2× SDS sample buffer, heated to 95 °C for 5 min and analyzed by Western blotting (usually 50 μg of total protein was loaded per well). The antibodies against following proteins were used: Bcl-XL (2764), Bid (2002), caspase-9 (9502), FADD (2782), IκBα (8414), P-IκBα (2859), NF-κB/p65 (4764), P-NF-κB/p65 (3033), SAPK/JNK (9252), P-SAPK/JNK Thr183/Tyr185 (9251), p-38 MAPK (9212) and P-p38MAPK (9211) from Cell Signaling; Mcl-1 (559027) and Caspase-8 (559932) from BD Biosciences; β-actin (SC-1615) and Bak (SC-832) from Santa Cruz Biotechnology; cFLIP (ALX-804-428) and Bim (ALX-804-351) from Enzo Life Science; caspase-10 (M059-3) from MBL and DR5 (SAB3500427) from Sigma-Aldrich.

Flow cytometry analysis of apoptosis and receptor expression

For the Annexin V-FITC assay of apoptotic cells, the cell cultures (typically 1.5×10^5 cells in each well of a 24-well plate) were harvested by trypsinization, washed once with PBS and once with ice-cold Annexin binding buffer. Then, Annexin V-FITC (Apronex Biotechnologies) was added to a final concentration of 2 μg/ml and the samples incubated on ice for 20 min. After adding Hoechst 33258 to a final concentration of 0.5 μg/ml, the samples were analyzed by flow cytometry on a LSRII (BD Biosciences).

For flow cytometry analysis of death receptor expression, cells were harvested by trypsinization and incubated in a blocking buffer (PBS + 0.2 % gelatin and 0.1 % sodium azide—PBS-GA) containing 20 % heat-inactivated human serum for 10 min. Then, the samples were incubated on ice for 30 min with antibodies against DR4, DR5 (10-403, 11-461, EXBIO) or FAS/CD95 (DX2, R&D System Inc.) diluted in PBS-GA to a final concentration of 2–5 μg/ml. The cells were then washed twice with ice-cold PBS-GA and incubated with phycoerythrin-conjugated goat anti-mouse IgG1 (Southern Biotech, diluted in PBS-GA to 4 μg/ml) on ice for 30 min. After two final washes, the cells were resuspended in PBS-GA containing 0.5 μg/ml Hoechst 33258, and the cell surface expression of receptors on living cells (Hoechst 33258-negative) was analyzed by flow cytometry on a LSRII.

Preparation of RKO and HT-29 cells with the downregulated expression of cFLIP or Mcl-1 and RKO cells with transiently suppressed expression of p53

HEK293T cells were transfected with the lentivirus packaging plasmids pMD2G, psPAX (Addgene) and pLKO.1 carrying shRNA against cFLIP (4 shRNA), Mcl-1 (5 shRNA) (all from Open Biosystems/Hermo Scientific). Two days later the lentiviral particles were purified from the supernatant using PEG/it Virus Precipitation Solution (SBI, LV810A-1). RKO and HT-29 cells were transduced at MOI 5 and selected in medium containing puromycin (3 μg/ml) for 4 days. Cells displaying the effective (80–90 %) downregulation of cFLIP (TRCN0000007229—shFLIP1, TRCN0000007230—shFLIP2) or Mcl-1 (TRCN0000005515—shMcl-1-2,

TRCN0000005517—shMcl-1-4, TRCN0000005518—shMcl-1-5) expression were used for further experiments. For Mcl-1, the effective downregulation of its expression was achieved by co-transduction of the target cells with shMcl-1-5+2 (=shMcl-1 5/2) or shMcl-1-5+4 (=shMcl-1 5/4) expressing lentiviruses.

Expression of p53 in RKO cells was downregulated by their Lipofectamine RNAiMax-mediated transfection with p53 On-TARGET siGENOME (Thermo Scientific/Dharmacon) according to the manufacture's recommendations. Two days post-transfection, the p53 downregulation was confirmed by Western blotting and cells were used within the following day for experiments.

Subcutaneous xenograft studies

Female immunodeficient NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mice (Jackson Laboratory) were maintained in individually ventilated cages. HT-29 cells were harvested, suspended in PBS, and injected (10^7 cells/mouse) subcutaneously into the left dorsal flank of 8–12-week-old mice. When tumors reached 800–1,000 mm^3, the animals were randomized into four groups and treated. The control group was injected with vehicle (PBS) intraperitoneally. HHT (1.3 or 2.6 mg/kg; 26 or 52 μg/mouse) and TRAIL (25 mg/kg; 500 μg/mouse) were administered intraperitoneally in a volume of 300 μl. The animals were treated with these agents and their combination on days 1, 3 and 5; when the tumors in the control group reached 2.5–3 cm in diameter, the animals were sacrificed and the tumors weighed.

Statistical analysis

Data are presented as mean ± SD (standard deviation) for the given numer of independent experiments. Student's unpaired *t* test or one-way ANOVA for multiple comparisons were used to determine significant differences between the experimental groups. Values of $*p < 0.05$ were considered significant, $**p < 0.01$ very significant and $***p < 0.001$ extremely significant.

Results

Homoharringtonine enhances the TRAIL-induced apoptosis and growth suppression of TRAIL-resistant colorectal cancer cells but not normal colon epithelial cells

TRAIL-induced apoptosis/growth suppression of primary tumors is rather inefficient. The new emerging concept based on TRAIL-related apoptogens encompasses tailored selection of TRAIL-induced apoptosis enhancing agents combined with molecular targeting (e.g., to EGFR via anti-EGFRcs Fv-TRAIL fusion protein) [31, 32]. We were interested in identifying novel TRAIL sensitizers, and thus we screened several chemical libraries for compounds that would significantly enhance TRAIL-mediated growth suppression of resistant colon carcinoma cells (HT-29). Compounds that showed to be at least two times more efficient in decreasing the viability of HT-29 cells in the presence of TRAIL were considered as primary hits. These hits were validated in dose–response for their ability to enhance TRAIL-mediated apoptosis (Suppl. Fig. 1). Among the positive hits were previously identified sensitizers such as camptothecin and thapsigargin [33, 34] Other validated hits were emetine and its analog cephaeline, the inhibitors of protein synthesis anisomycin, the alcohol dehydrogenase inhibitor disulfiram and HHT. HHT was by far the most potent TRAIL sensitizer and the only compound showing activity at low nanomolar concentrations.

Homoharringtonine consistently and strongly sensitized not only HT-29, but also other TRAIL-resistant carcinoma cell lines such as RKO and SW620 to TRAIL-induced apoptosis (Fig. 1a and data not shown). In contrast, the normal colon epithelial cell line CCD 841 and normal human fibroblasts remained resistant to combined HHT + TRAIL treatment (Fig. 1a and data not shown). HHT also synergized with TRAIL in enhancing the TRAIL-induced activation of effector caspases (Fig. 1b, c). For RKO cells the saturation in caspase-3/7 activity was reached at 35 ng/ml and for HT-29 at 76 ng/ml of recombinant TRAIL (LC50 = 7 and 15 ng/ml, respectively) with a constant 100 nM HHT (Fig. 1b). Vice versa, titrating HHT under a constant LC50 concentration of TRAIL revealed that the saturating concentration of HHT required for the activation of effector caspases is 50–60 nM with a LC at 15 nM (Fig. 1c). More efficient activation of caspases is necessary but might not be sufficient for the reliable elimination of cancer cells. Thus, by various approaches, we analyzed the long-term survival of resistant cancer cells treated with HHT, TRAIL and their combination. Both xCELLigence- and WST-1-based survival assays confirmed that only the combined effect of 50 nM HHT and TRAIL could lead to the effective long-term elimination of cancer cells (Fig. 2 and Suppl. Fig. 2a, c). In agreement with the Annexin-FITC staining, combined HHT + TRAIL treatment of normal colon epithelial cells with sub-lethal and lethal combinations of HHT + TRAIL did not markedly change their viability (Fig. 2 and Suppl. Fig. 2b).

Homoharringtonine modifies TRAIL-induced proximal signaling in colorectal cancer cells and the expression of some apoptosis-regulating proteins

For the efficient induction of TRAIL-induced apoptosis, the essential requirements are (a) the presence and

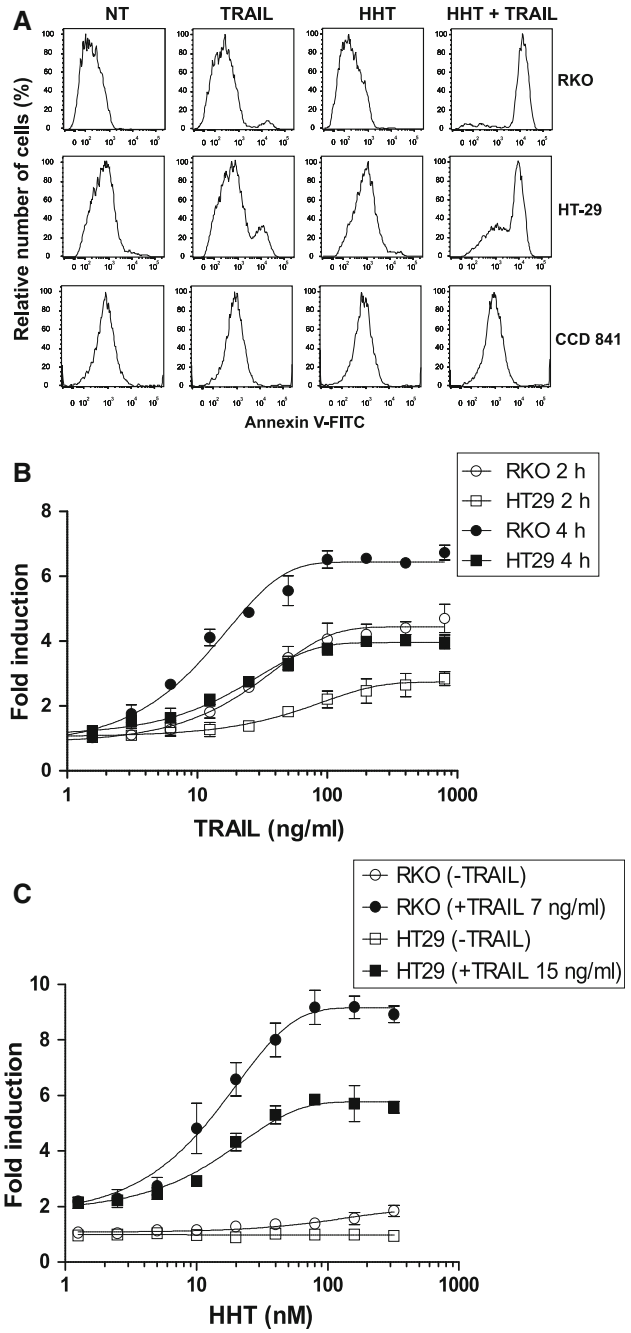

Fig. 1 Homoharringtonine enhances TRAIL-induced pro-apoptotic signaling in resistant colorectal cancer cells. HT-29 or RKO cells were treated with TRAIL, HHT or their combination and assayed for the manifestation of apoptosis by Annexin V-FITC staining and the activity of caspase-3. **a** Cells were incubated with 50 nM HHT, 20 ng/ml TRAIL or their combination for 5 h, stained with Annexin-V-FITC and analyzed by flow cytometry. **b** The concentration-dependent activation of caspase-3 in cells co-treated with 100 nM HHT and TRAIL for 2 and 4 h was quantified by the Caspase 3/7 GLO assay. **c** Titration of HHT at a fixed concentration of TRAIL (7 ng/ml for RKO and 15 ng/ml for HT-29 cells) quantified by the caspase 3/7 GLO assay. The experiments were run in triplicate in 384-well plates and were repeated three times. Means and standard deviations are shown

accessibility of pro-apoptotic TRAIL receptors and (b) the efficient formation of the DISC platform leading to rapid self-processing and the activation of caspase-8. However, short or long-term treatment (4 or 15 h) of RKO or HT-29 cells with 50 nM HHT did not significantly affect the cell surface expression of TRAIL receptors (we even noticed a small but reproducible decrease in TRAIL-R2/DR5 expression in RKO cells; Suppl. Fig. 3 and Fig. 3—lower staining of DR5 in the cell lysates).

Next, we aimed to assess the efficacy of DISC formation and the processing of the initiator caspases. In RKO cells, TRAIL engagement led to the rapid formation of the DISC and the processing of both initiator caspases-8 and -10 (Fig. 3a, left side of the left panel). In RKO cells pre-treated with HHT for 3 h, TRAIL addition resulted in a lesser quantity of DISC proteins in the precipitates (Fig. 3a, right side of the left panel); however, the processing of the initiator caspases and cFLIP cleavage in the DISC were not significantly affected. A decrease of both DR5 and also cFLIP levels (both L and S forms) in HHT-pretreated RKO cells was also observable in the cell-free lysates used for DISC precipitation in response to TRAIL addition (Fig. 3a, right panel). Similarly in HT-29 cells we also observed a drop in cFLIP levels in precipitated DISC complexes and no observable differences in the efficacy of activation of the initiator caspases (data not shown).

In addition to the activation of caspases, TRAIL-induced proximal signaling also feeds into MAP kinase and NF-κB pathways. TRAIL-treatment of RKO cells resulted in the strong but transient activation of NF-κB signaling (phosphorylation of IκB and p65/RelA, transient degradation of IκB, Fig. 3b). IκB phosphorylation and degradation were evident at 30 and 60 min post-TRAIL treatment respectively, also in cells co-treated with TRAIL + HHT. However, after co-treatment, in contrast to TRAIL-only-treated cells, there was no recovery of IκB expression at 2 h post-treatment (p.t.), and we even observed the stronger phosphorylation of p65/RelA at this time point. HHT alone did not have any effect on canonical NF-κB signaling (Fig. 3b, upper panels). Treatment with either HHT or TRAIL alone also activated the JNK signaling pathway and induced JNK1/2 phosphorylation as early as 30–60 min p.t. (Fig. 3b, middle panels). Interestingly, there was a strong synergism in JNK1/2 phosphorylation in RKO cells co-treated with HHT + TRAIL. Similarly, the TRAIL-induced phosphorylation of p38 MAP kinase was significantly enhanced at 60 min post-treatment in cells co-treated with TRAIL + HHT (Fig. 3b, lower panels). Published reports have connected the increased activation of JNK and p38 kinases with the apoptosis-sensitizing effects of HHT [35, 36], and these authors used MAP kinase inhibitors for the suppression of HHT-mediated apoptosis. However, neither the JNK inhibitor SP600125 nor the p38 inhibitor SB202190 had any effect

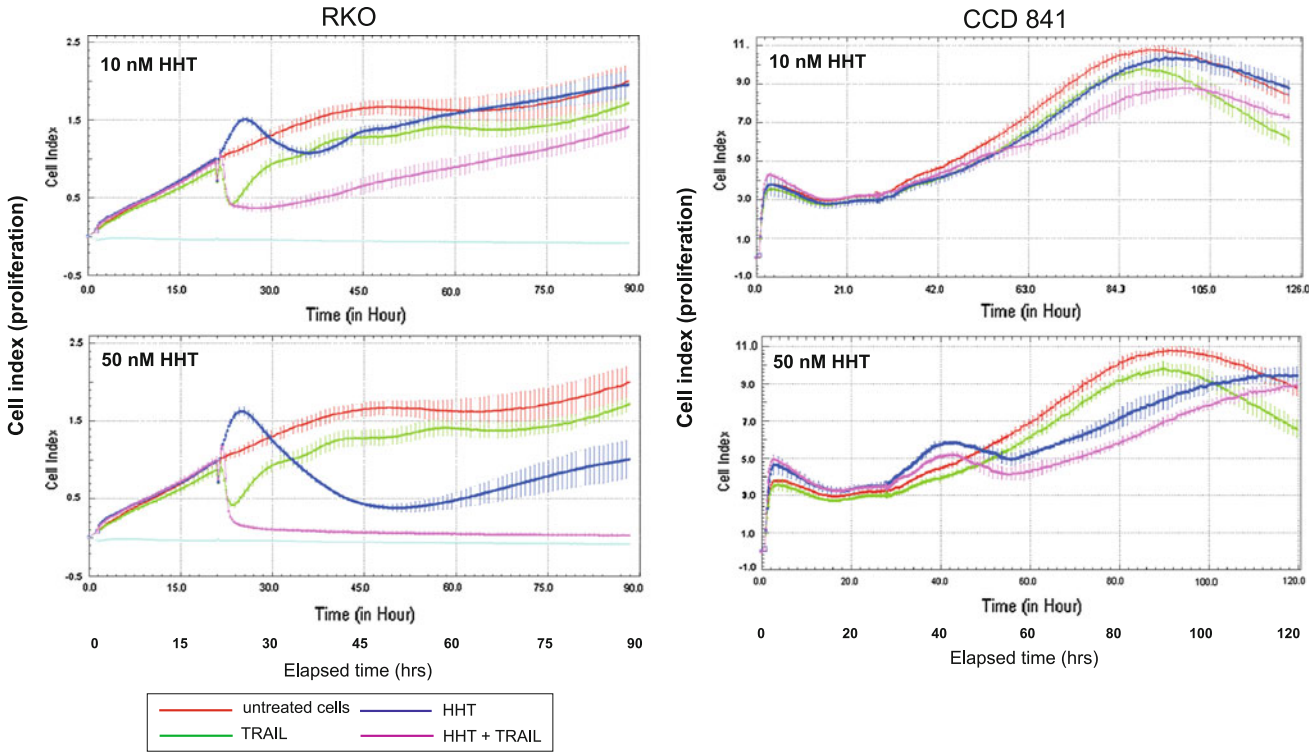

Fig. 2 HHT collaborates with TRAIL in the growth suppression of resistant cancer cells. 5,000 cfu of cancer RKO or normal CCD 841 cells were seeded in triplicate into a 96-well E-plate and treated with 100 ng/ml TRAIL, 10 or 50 nM HHT or their combination in the xCELLigence RTCA analyzer (Roche). The cell index representing cell proliferation was measured every 15 min. The data represent one out of four experiments with similar outcomes

(positive or negative) on the HHT-induced sensitization of either RKO or HT-29 cells to TRAIL-induced apoptosis (data not shown), indicating none or minor role of these kinases in the HHT-mediated sensitization of these cells to TRAIL. In contrast to MAP/stress kinases inhibitors blocking NF-κB signaling with IKKα inhibitors Bay 11-7082 surprisingly resulted in blunting the HHT-mediated sensitization of RKO cells to TRAIL-induced apoptosis (Suppl. Fig. 4 and not shown). Especially at higher 20 μM concentration that effectively suppressed IκB phosphorylation, Bay 11-7082 reproducibly attenuated TRAIL-triggered externalization of phosphatidyl choline (Suppl. Fig. 4a) and activation of caspases (Suppl. Fig. 4b). However, this concentration was also significantly toxic for RKO cells as documented by over 25 % dead, Hoechst-positive cells after their 5 h treatment with Bay 11-7082 alone or in combination with HHT/TRAIL (Suppl. Fig. 4A, right panel). Likely also for Bay's toxic side effects, long-term survival of Bay 11-7082/HHT/TRAIL-treated cells was very low and similarly as for HHT + TRAIL-treated cells virtually no cells survived this combined treatment.

The significant down-regulation of cFLIP expression in HHT + TRAIL-treated cells (see Fig. 3a) as well as published data on HHT as a translation inhibitor [37] prompted us to assess the effect of HHT on the stability of apoptosis-regulating proteins. Western blots of RKO or HT-29 lysates of cells treated with 50 nM HHT for up to 6 h documented a significant, time-dependent decrease of both cFLIP-L and, even more strongly, of cFLIP-S levels starting between 1 and 2 h p.t. We did not see any effect of HHT on the cellular levels of Bak, Bax, Bcl-XL, Bim or XIAP (Fig. 4a and data not shown), but the stability of the short-lived protein Mcl-1 was strongly affected by HHT treatment and its levels dramatically dropped within 2–4 h post-treatment (Fig. 4a). Kinetics of caspase processing and cleavage of some of their substrate was then analyzed in HHT + TRAIL-treated RKO cells. The cells were either treated with 50 nM HHT and 100 ng/ml TRAIL alone for up to 4 h or they were co-treated for the same time periods. In contrast to HHT- or TRAIL-only treated cells, cells co-treated with the combination of HHT + TRAIL efficiently within the first 2 h processed both initiator caspases- and-10. After the second hour of the treatment also caspase-8 substrate tBid and mitochondrial-activated caspase-9 were fully or strongly processed/activated only in HHT + TRAIL-treated cells (Fig. 4b). In addition the combined treatment even accelerated HHT-mediated downregulation of cFLIP-S or Mcl-1.

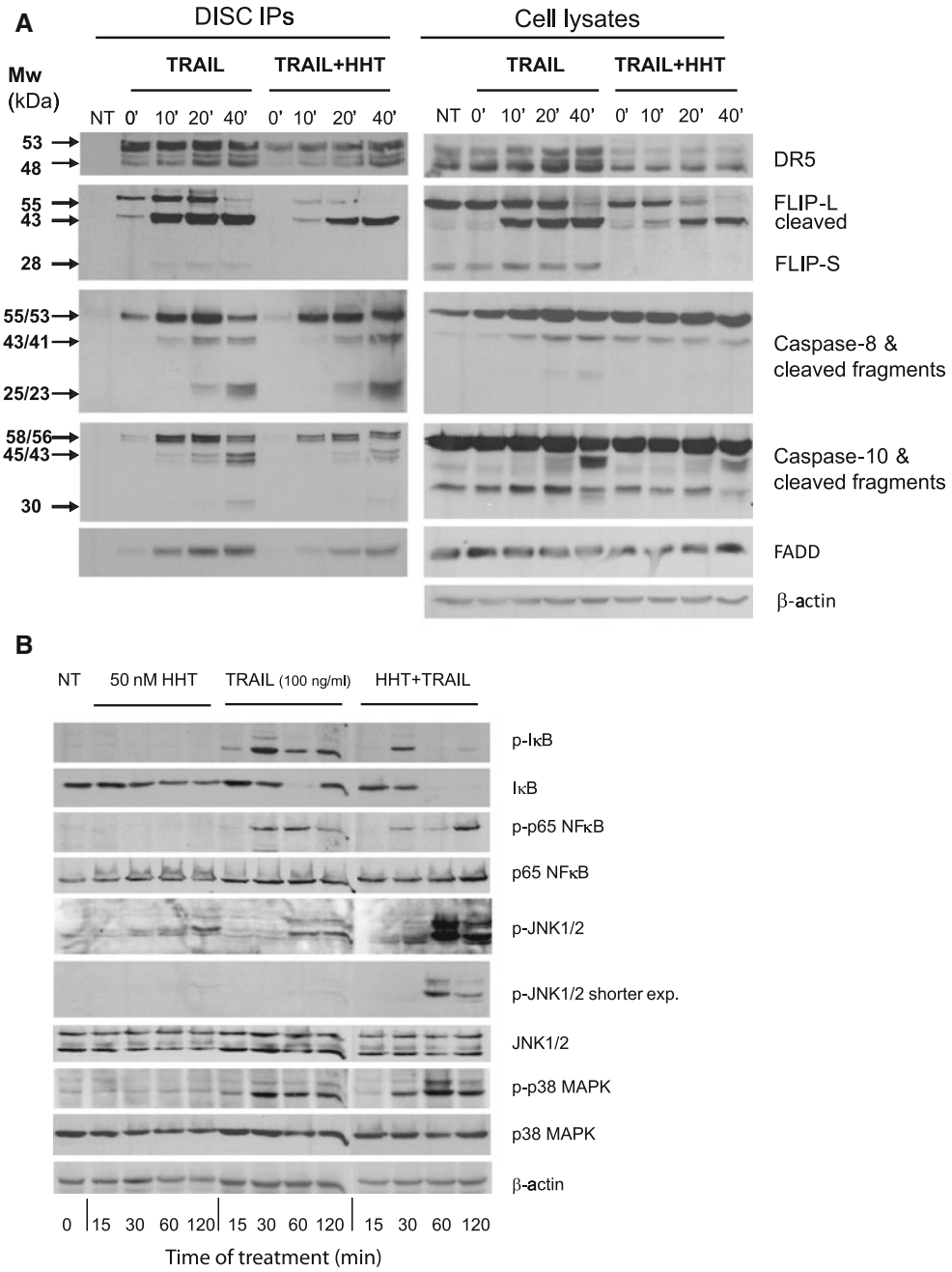

Fig. 3 Homoharringtonine affects TRAIL-induced proximal signaling in RKO cells. **a** Precipitation of TRAIL receptor DISC from Biot-TRAIL- and Biot-TRAIL + HHT-treated RKO cells (Biot-TRAIL was used at a concentration of 1 μg/ml and HHT at 50 nM). The precipitated proteins and cell lysates were analyzed by Western blotting using the indicated antibodies. **b** RKO cells were treated with HHT, TRAIL or their combination for up to 120 min, and the expression and phosphorylation status of the proteins shown were determined by Western blotting. The data represent typical blots from two (**a**) or three (**b**) independent experiments

Downregulation of cFLIP or Mcl-1 enhances TRAIL-induced apoptosis but is not sufficient for TRAIL-mediated growth suppression of resistant cells

As HHT treatment of TRAIL-resistant cells led to a time-dependent downregulation of cFLIP and Mcl-1, two major inhibitors of TRAIL-induced apoptosis, we aimed to assess the individual contribution of these proteins to the TRAIL-resistant phenotype of RKO and HT-29 cells. Using two different shRNA-expressing lentiviruses for each gene, we established both RKO and HT-29 cells with the downregulated expression of either cFLIP or Mcl-1. In both cell lines we achieved at least an 80–90 % decrease in the expression of either cFLIP or Mcl-1 (Fig. 5a). The downregulation of Mcl-1 enhanced TRAIL-induced pro-apoptotic signaling in both cell lines (Fig. 5b and Suppl. Fig. 5a, b, right graphs). However, to our surprise downregulating cFLIP augmented TRAIL-induced apoptosis only in HT-29 cells, but it had no or even an inhibitory effect on the TRAIL-induced apoptosis of RKO cells. Similarly, using a sub-optimal, 25 nM concentration of HHT enhanced the TRAIL-induced apoptosis of both RKO and HT-29 cells with the downregulated expression of Mcl-1 and of HT-29 cells with the downregulated expression

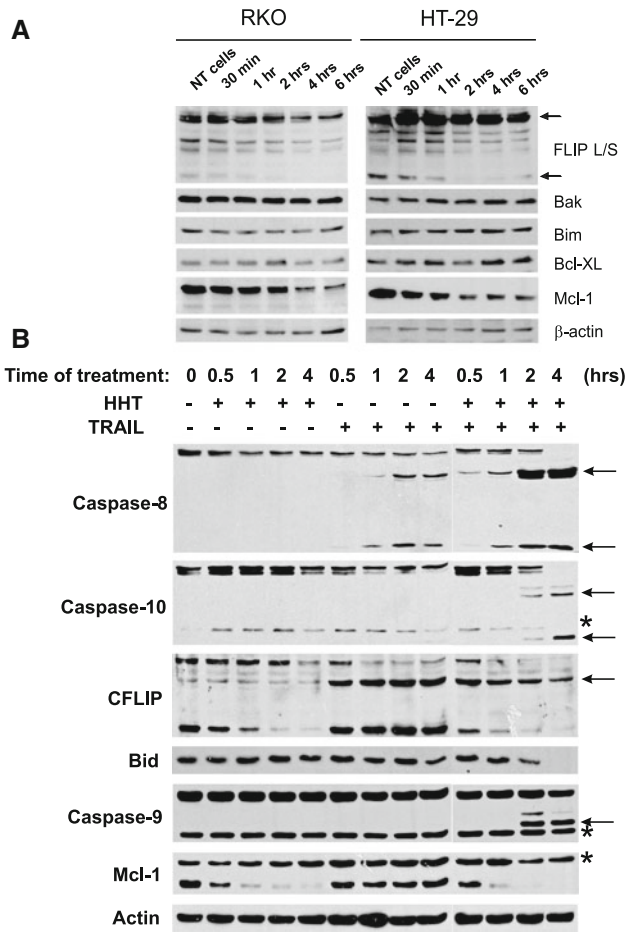

Fig. 4 Homoharringtonine suppresses the expression of some anti-apoptotic proteins and enhances processing of caspases in TRAIL-resistant cancer cells. **a** RKO or HT-29 cells were treated with 50 nM HHT for up to 6 h, and the expression of selected apoptosis-regulating proteins was analyzed by Western blotting. Representative Western blots from three independent experiments are shown. *Arrows* point to cFLIP-L and cFLIP-S, respectively. **b** RKO cells were treated with HHT (50 nM), TRAIL (100 ng/ml) and their combination in time-dependent manner and the expression/processing of selected, TRAIL-mediated apoptosis-related caspases and proteins was analyzed by Western blotting. *Arrows* indicate processed caspases and cleaved cFLIP-L, and *asterisk* then non-specifically stained proteins

of FLIP, but such treatment led to much lower pro-apoptotic responses in RKO cells with the downregulated expression of cFLIP (Fig. 5b and Suppl. Fig. 5).

The results of viability/proliferation assays of these cells with suppressed cFLIP or Mcl-1 expression followed a quite similar pattern. Both CellTiter-Blue and xCELLigence viability tests showed that despite the significant increase of apoptosis in TRAIL-treated cells with the downregulated expression of Mcl-1 or cFLIP (only for HT-29), knocking-down either of these anti-apoptotic proteins had only a marginal effect on the long-term survival of TRAIL-treated cells (Suppl. Fig. 5, left panels and Suppl. Fig. 6). However, especially the suppressed expression of Mcl-1 markedly boosted the HHT-mediated sensitization of both RKO and HT-29 cells to TRAIL-induced apoptosis, as documented by more than a twofold lower viability in comparison to control cells in the CellTiter-Blue assay (Suppl. Fig. 5) and virtually no survival of treated cells in the xCELLigence test (compare none and shMcl-1; Suppl. Fig. 6). Strangely, RKO cells with the downregulated expression of cFLIP virtually became resistant to the sensitizing effect of HHT and TRAIL + HHT (Suppl. Fig. 5, left panels and Suppl. Fig. 6). These cells basically reflected just the anti-proliferative effects of HHT itself.

RKO cells in contrast to HT-29 contain wt p53 and thus it might be possible that p53 presence/activity could make RKO cells even more vulnerable to the combined effect of HHT + TRAIL. But this premise was not fulfilled, as RKO cells with by siGENOME downregulated expression of p53 responded to either TRAIL alone or TRAIL + HHT with no difference in the efficacy or kinetics of their apoptosis (data not shown).

The combined action of homoharringtonine and TRAIL efficiently suppressed the growth of TRAIL-resistant cancer cells in vivo

To validate our in vitro findings documenting the very strong sensitizing impact of HHT on the TRAIL-induced apoptosis/growth suppression of resistant cancer cells, we used an in vivo model with HT-29 cells subcutaneously xenotransplanted into immunodeficient NOD/SCID mice. A cohort of 36 mice was injected with 10^7 cells, and when the resulting tumors reached approximately 1 cm^3, the mice were divided into six groups and treated three times (every second day) with intraperitoneal injections of TRAIL (25 mg/kg), HHT (1.3 mg/kg) or a combination of TRAIL with HHT. Within 18 days of tumor growth, the tumor mass in untreated mice increased approximately 3.5-fold; both individual treatments had only a marginal inhibitory effect on tumor growth, but the tumors in animals co-treated with HHT + TRAIL ceased growing, and the animals did not suffer any adverse effects (Fig. 6). All mice in the HHT + TRAIL-treated cohort survived the treatment until their sacrifice at the end of the experiment.

Discussion

Primary tumors only weakly react to mono-therapy with TRAIL apoptogens, and even binary or ternary combinations of TRAIL with sensitizing agents were only partly effective in a number of phase I/II trials. Thus, if TRAIL apoptogens are still to be considered as potential anti-tumor drugs, tailored combination treatment that would overcome

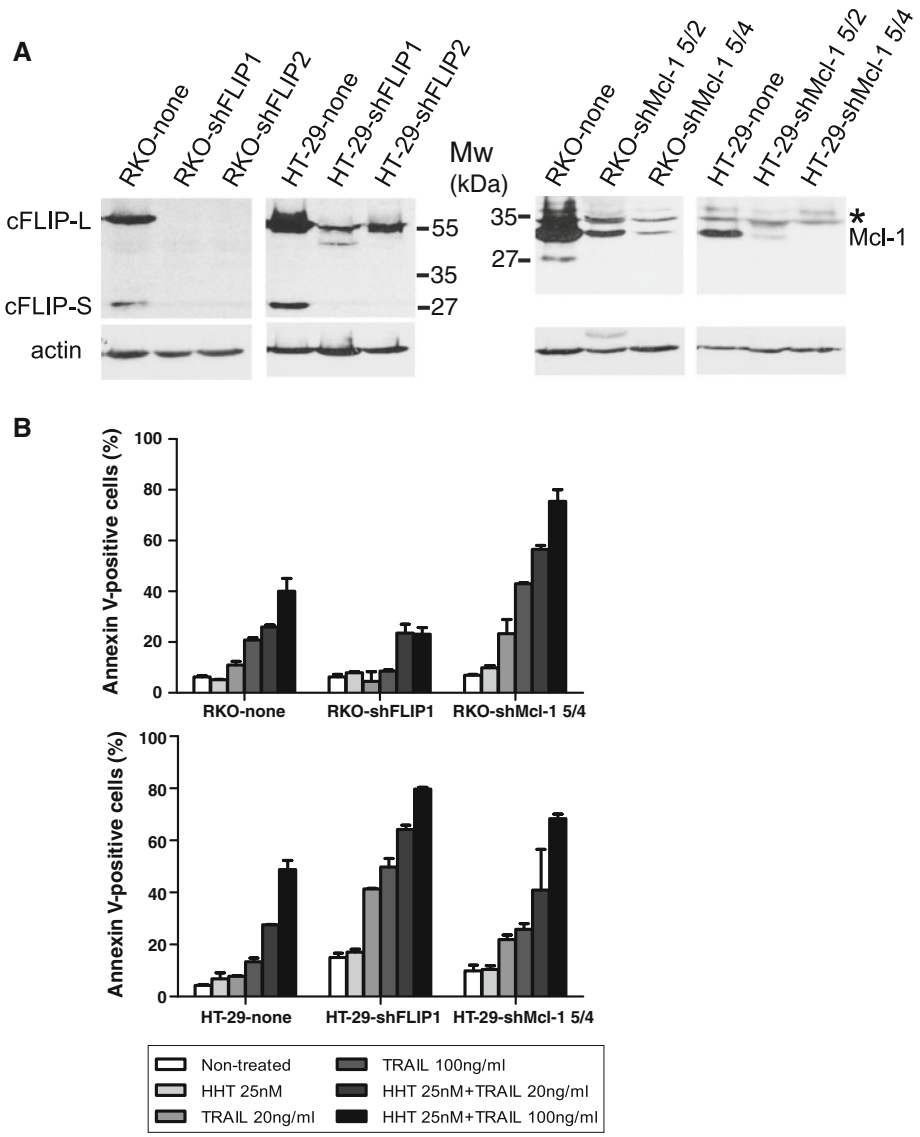

Fig. 5 Downregulation of Mcl-1 or cFLIP predominantly leads to the increased sensitivity of resistant cancer cells to TRAIL-induced apoptosis. RKO or HT-29 cells were transduced with cFLIP- or Mcl-1-expressing lentiviruses and analyzed for their sensitivity to TRAIL- or HHT + TRAIL-induced apoptosis. **a** Western blots of mixed populations of RKO and HT-29 cells stably transduced with the pLKO1 lentivirus (none) and two different cFLIP or Mcl-1 shRNAs. *Asterisk* at Mcl-1 signal indicates non-specific staining. **b** The activation of TRAIL-induced apoptosis in these cells treated for 5 h with the indicated reagents was analyzed by Annexin-V-FITC staining and flow cytometry. The data represent means and standard deviations of three independent experiments from shFLIP and shMcl-1 expressing RKO cells

the acquired resistance of cancer cells and have few, or better, no side effects would be an optimal therapeutic option [31].

Here we show that HHT, an inhibitor of translation and a tested anti-leukemia drug, efficiently sensitizes resistant cancer cells to their TRAIL-mediated elimination both in vitro and in vivo and, within the therapeutic window, is not harmful to normal cells or the organism. In agreement with the published data we also found that in our model of TRAIL-resistant colorectal cancer cell lines, HHT caused, in a time- and concentration-dependent manner, a fairly rapid (within 2–4 h) drop in the cellular levels of two important regulators of TRAIL-induced apoptosis—the anti-apoptotic proteins Mcl-1 and cFLIP [38, 39]. Also as expected, the shRNA-mediated downregulation of either of these proteins significantly enhanced TRAIL-induced apoptosis but surprisingly had only a marginal effect on TRAIL-mediated growth suppression. However, shRNA treatment lowered the effective sensitizing concentration of HHT by at least two-fold.

In contrast to HT-29 cells, downregulation in RKO cells provided quite ambiguous results. While the downregulation of Mcl-1 strongly boosted both TRAIL-induced apoptosis and HHT-mediated sensitization, cells with downregulated cFLIP expression became more resistant not only to TRAIL, but also to their HHT-mediated sensitization to TRAIL-induced apoptosis. This was quite an unexpected finding as the downregulation of cFLIP in various cancer cell lines has generally been connected with the strong enhancement of TRAIL- or FasL-induced apoptosis [40–42]. However, cFLIP-L, in sharp contrast to cFLIP-S or -R, is likely not just a competitive inhibitor of caspase-8 activation, but also, due to its high affinity for caspase-8, either a platform in a cytoplasmic caspase-8-activating complex

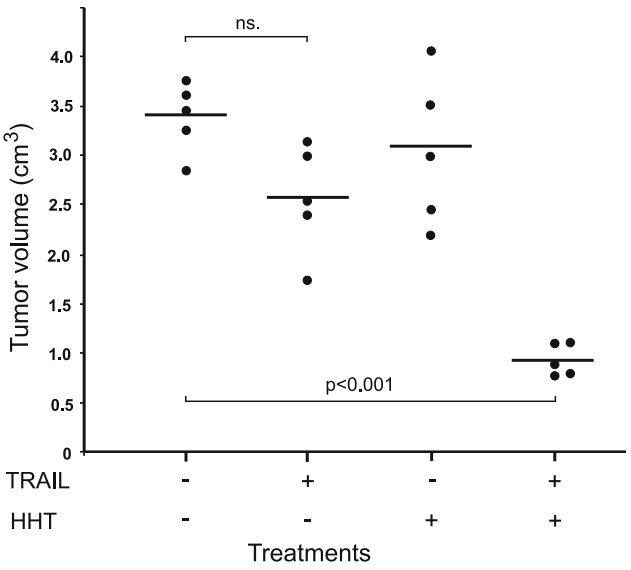

Fig. 6 Homoharringtonine assists TRAIL in suppressing the growth of colon cancer cells in immunodeficient mice. HT-29 cells (10^6 cfu) were subcutaneously injected into a cohort of NOD/SCID mice, and when the tumors reached approximately 1 cm^3 in size, these mice (at least five per group) received three intraperitoneal injections (every second day) of TRAIL (25 mg/kg), HHT (1.3 mg/kg) or their combination (25 mg/kg TRAIL plus 1.3 mg/kg HHT). At day 10 post-treatment the mice were sacrificed, and the size of their tumors was determined. The average volumes and standard deviations are shown with *marked* statistical significance

[43] or even an enhancer of caspase-8 processing in the DISC at low (sub) stochiometric concentrations [44, 45]. RKO cells express significantly less cFLIP than HT-29 cells, and thus shRNA-mediated downregulation basically eliminated cFLIP expression in these cells. This apparently made RKO cells more resistant even to the combined effects of HHT + TRAIL, indicating a positive role for low levels cFLIP-L in the fine tuning of TRAIL-induced apoptotic signaling in these cells. This finding might be in apparent contradiction with the HHT-mediated downregulation of cFLIP in RKO cells. However, in HHT-treated RKO cells the more unstable cFLIP-S is rapidly downregulated, but the decrease in cFLIP-L levels is only modest, and cFLIP-L is still detectable in the DISC precipitates (and lysates) of HHT + TRAIL-treated RKO cells.

Intracellular levels of Mcl-1 were many times shown as important decision nodes for various apoptotic stimuli including e.g. BH3 analog ABT-737 [46], and Mcl-1 expression alone or together with cFLIP was targeted and suppressed by a number of existing and novel drugs such as roscovitine, quercetin or aspirin plus sorafenib, enhancing thus (not exclusively) TRAIL-induced apoptosis of cancer cells [47–49]. Multikinase inhibitor sorafenib or ectopic expression of cMyc blocked TRAIL-induced, NF-κB-mediated expression of Mcl-1 and thus sensitized TRAIL-resistant cells to TRAIL-induced apoptosis [50]. In contrast to this generally accepted anti-apoptotic role of NF-κB signaling we found out that blocking NF-κB signaling by BAY 11-7082, a well-known inhibitor of IKKα also attenuated sensitizing effect of HHT and suppressed TRAIL-induced apoptosis of RKO cell. This unexpected finding is however supported by recent publication from Simone Fulda's group. In this communication the authors for the first time document that in glioblastoma cell lines, blocking NF-κB signaling pathway by overexpression of dominant-negative non-phosphorylated IκB also suppressed TRAIL-induced apoptosis likely via for yet unknown reasons less efficient caspase-8 processing in DISC [51].

Being a potent inhibitor of translation, HHT could be potentially harmful to normal cells in combination with TRAIL, similarly as another anti-cancer drug and proteasome inhibitor, bortezomib (Velcade) [52]. On the other hand, bortezomib has proven to be a very efficient enhancer of the TRAIL-induced apoptosis of therapy-resistant glioblastoma or melanoma cells and Lexatumumab-resistant BJAB cells [53–55] and within the therapeutic window also safe to normal cells. Similarly, combining HHT with TRAIL was not only effective in vitro, but also suppressed the growth of xenotransplanted TRAIL-resistant HT-29 cells in immunodeficient mice, thus proving its efficacy and safety within the therapeutic window. As an already tested drug in cancer treatment, HHT could, after thorough testing, represent a potentially interesting option for sensitizing TRAIL-resistant cancer cells to apoptosis.

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Conflict of interest The authors declare that they have no conflict of interest.

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