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RESEARCH PAPER

Methyl jasmonate down-regulates survivin expression and sensitizes colon carcinoma cells towards TRAIL-induced cytotoxicity

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This study is dedicated to the late Professor Eliezer Flescher, whose research pursued the discovery of new cancer therapies, with the hope that it follows his spirit.

Keywords
colorectal cancer; methyl jasmonate; TRAIL; survivin; apoptosis; sensitization

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BACKGROUND AND PURPOSE

Methyl jasmonate (MJ) is a plant stress hormone with selective cytotoxic anti-cancer activities. The TNF-related apoptosis-inducing ligand (TRAIL) death pathway is an attractive target for cancer therapy. Although TRAIL receptors are specifically expressed in primary cancer cells and cancer cell lines, many types of cancer cells remain resistant to TRAIL-induced cytotoxicity. Here we have assessed a possible synergy between MJ and TRAIL cytotoxicity in colorectal cancer (CRC) cell lines.

EXPERIMENTAL APPROACH

CRC cell lines were pre-incubated with sub-cytotoxic concentrations of MJ followed by TRAIL administration. Cell death was determined by XTT assay and microscopy. Cytochrome *c* release, caspase cleavage, TRAIL-associated factors, X-linked inhibitor of apoptosis (XIAP) and survivin protein levels were detected by immunoblotting. Survivin transcription was examined by RT-PCR.

KEY RESULTS

Pre-treatment with MJ resulted in increased TRAIL-induced apoptotic cell death, increased cytochrome *c* release and caspase cleavage. TNFRSF10A, TNFRSF10B, TNFRSF10D, Fas-associated death domain and cellular FLICE-like inhibitory protein remained unchanged during MJ-induced TRAIL sensitization, whereas MJ induced a significant decrease in survivin protein levels. Overexpression of survivin prevented MJ-induced TRAIL cytotoxicity, implying a role for survivin in MJ-induced TRAIL sensitization. MJ decreased survivin mRNA indicating that MJ may affect survivin transcription. In a β-catenin/transcription factor (TCF)-dependent luciferase activity assay, MJ decreased TCF-dependent transcriptional activity.

CONCLUSION AND IMPLICATIONS

MJ, at sub-cytotoxic levels, sensitized CRC cells to TRAIL-induced apoptosis. Thus, combinations of MJ and TRAIL, both selective anti-cancer agents, have potential as novel treatments for CRC.

Abbreviations

cFLIP, cellular FLICE-like inhibitory protein; CRC, colorectal cancer; FADD, Fas-associated death domain; IAP, inhibitors of apoptosis; MJ, methyl jasmonate; PARP, poly ADP ribose polymerase; TRAIL, TNF-related apoptosis-inducing ligand

Introduction

Colorectal cancer (CRC) is one of the most common cancer types in the developed world and ranks second place as a cause of death from malignancy (Midgley and Kerr, 1999; Rougier and Mitry, 2003). The common adjuvant treatment to CRC consists of pharmacological treatment regimens based on protocols involving 5-fluorouracil and folinic acid as well as new cytotoxic agents such as oxaliplatin and irinotecan (Midgley and Kerr, 2000; Rougier and Mitry, 2003). However, resistance to chemotherapy remains a major problem in the treatment of CRC. Therefore, development of new treatment strategies for CRC patients is urgently needed.

Tumour necrosis factor-α-related apoptosis-inducing ligand (TRAIL, also called Apo2L) and its receptors are a subgroup of the TNF superfamily capable of mediating specific cancer cell death (LeBlanc and Ashkenazi, 2003; Falschlehner *et al.*, 2007). TRAIL can bind two cell surface death receptors, TNFRSF10A (TRAIL-R1/DR4) and TNFRSF10B (TRAIL-R2/DR5) that contain functional intracellular death domains (DD) and transmit death signals into the cell (LeBlanc and Ashkenazi, 2003; Falschlehner *et al.*, 2007). On binding TRAIL, the TRAIL receptors undergo trimerization and recruit Fas-associated death domain (FADD) adaptor protein to their DD (Kischkel *et al.*, 2000; Sprick *et al.*, 2000). FADD in turn interacts with pro-caspase 8/FLICE to form a complex at the receptor level called the death-inducing signalling complex (DISC) (Kischkel *et al.*, 2000; Sprick *et al.*, 2000). As the DISC is formed, it induces the activation of caspase 8, which in turn activates downstream effector caspases (extrinsic apoptotic pathway) or cleaves the BH3-only protein (Bid) which translocates to the mitochondria to activate the intrinsic apoptotic pathway (Luo *et al.*, 1998). In addition, TRAIL can bind to two decoy receptors, TNFRSF10C (TRAIL-R3/DcR1) and TNFRSF10D (TRAIL-R4/DcR2), that lack functional DD and thus cannot transmit death signals (LeBlanc and Ashkenazi, 2003; Falschlehner *et al.*, 2007). Moreover, TRAIL-induced death signalling is negatively regulated by the cellular FLICE-like inhibitory protein (cFLIP, also known as CFLAR), an inhibitor of caspase 8, which interacts with FADD, thus antagonizing TRAIL-induced apoptosis (Roth and Reed, 2004).

TRAIL receptors are highly expressed in primary tumours and various cancer cell lines including CRC cells (Van Geelen *et al.*, 2004). TRAIL cytotoxicity is very specific towards cancer cells while sparing most normal cells. Therefore, TRAIL-induced cell death is a very attractive potential target for cancer therapy (Koschny *et al.*, 2007; Johnstone *et al.*, 2008). Nevertheless, many tumours and cancer cells display an intrinsic resistance towards TRAIL-induced apoptosis (Van Geelen *et al.*, 2004). Possible mechanisms of TRAIL resistance include a high expression of cFLIP (Ricci *et al.*, 2004), Bax deficiency (LeBlanc *et al.*, 2002) and high expression of inhibitors of apoptosis (IAP) family proteins, such as X-linked IAP (XIAP) and survivin (Chawla-Sarkar *et al.*, 2004; Ricci *et al.*, 2004; Van Geelen *et al.*, 2004). One approach to overcome TRAIL resistance is to combine the administration of TRAIL with that of a second drug capable of sensitizing cells towards TRAIL-induced apoptosis, by a variety of mechanisms such as up-regulation of TRAIL receptors (Kim *et al.*, 2008), down-regulation of cFLIP (Hernandez *et al.*, 2001; Palacios *et al.*, 2006), down-regulation of XIAP and survivin (Schultze *et al.*, 2006; Mori *et al.*, 2007) and combinations of these mechanisms.

Methyl jasmonate (MJ) is a plant stress hormone found in our laboratory to exhibit selective activities towards primary cancer cells and various types of cancer cell lines (Fingrut and Flescher, 2002; Rotem *et al.*, 2005). MJ is a fatty acid-derived cyclopentanone with a molecular weight of 224 and it is very hydrophobic and is not charged. MJ induces cell death primarily by triggering mitochondrial perturbation, by interacting with mitochondrial-membrane bound hexokinase, inducing its detachment from the mitochondria (Goldin *et al.*, 2008). As both MJ and TRAIL cytotoxicity are very selective towards cancer cells, we examined the possible effects of combined treatment with MJ and TRAIL, on CRC cells. Here we show that pre-incubation with MJ, at sub-cytotoxic concentrations, sensitized CRC cells to TRAIL-induced cytotoxicity and enhanced TRAIL-dependent caspase cleavage. MJ-induced TRAIL sensitization was probably not mediated by up-regulation of TRAIL receptors, or down-regulation of cFLIP, but involved down-regulation of survivin protein levels via a decrease of its mRNA levels. Therefore, we propose that the combination of MJ and TRAIL holds great potential for the treatment of cancer.

Methods

Cell culture

A range of CRC cell lines, SW480, HT29, LS180 (ATCC, Manassas, VA, USA), and HCT116 (kindly provided by Prof. Bert Vogelstein, Johns Hopkins University School of Medicine, Baltimore, MD, USA), were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 1 mM L-glutamine, 100 U·mL^{-1} penicillin, 100 μg·mL^{-1} streptomycin (all from Biological Industries, Beit-Haemek, Israel). Cells were maintained in a humidified chamber of 95% air 5% CO_2 at 37°C.

Cytotoxicity assay

Cells (5×10^3 per well) were plated into 96-well microtiter plates and were allowed to adhere before treatment. The next day, cells were pre-incubated with MJ for 24 h before treatment with TRAIL for another 8 h (HCT116 and SW480 cells) or 24 h (HT29 and LS180 cells). Cell proliferation was determined using the XTT kit (Biological Industries). Optical density was read at 490 nm with a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA). The optical density is directly proportional to the number of living cells in culture. Cytotoxicity of each treatment was calculated as percentage of control untreated cells.

Phase microscope and DAPI analysis

For phase microscopy analysis, SW480 cells were grown in 6-well plates, allowed to adhere before being treated the next day with 0.5 mM MJ for 24 h followed by treatment with 200 ng·mL^{-1} TRAIL for another 8 h. The cells were then observed directly under an Olympus IX50 inverted microscope (Olympus America Inc., Center Valley, PA, USA) at ×400 magnification. For nuclear fragmentation DAPI analysis,

SW480 cells grown on cover slips in 6-well plates were allowed to adhere and treated the next day with 0.5 mM MJ for 24 h followed by another 4 h with 100 ng·mL^{-1} TRAIL. Then, the cover slips were fixed with formaldehyde and stained with DAPI. Apoptotic nuclei were observed under an upright Olympus BX40 fluorescence microscope at ×400 magnification. In both phase and DAPI fluorescence microscopy analysis, for each treatment, several images from different fields were taken.

Cytochrome c release assay

SW480 cells were grown in 10 cm plates and treated with 0.5 mM MJ for 24 h followed by treatment with TRAIL (100 ng·mL^{-1}) for 4 h. Cells were harvested and washed twice with PBS and were incubated for 15 min on ice in homogenizing buffer (20 mM HEPES pH 7.5, 250 mM sucrose, 1 mM EGTA, 1 mM EDTA, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF and protease inhibitors). The cells were then lysed by 25 strokes with Dounce homogenizer followed by centrifugation (800× *g*, 4°C for 5 min). The supernatants were collected and centrifuged (15 000× *g*, 4°C for 15 min). The resultant supernatants, designated as cytoplasmic fractions, were immediately boiled in sample buffer. Equal amounts of protein of each sample was separated on 12% SDS-PAGE and cytochrome *c* was detected by Western blot.

Preparation of cell lysates and Western blot analysis

Cells were seeded in 6 cm plates and allowed to adhere. After treatment with the relevant agents, the cells were washed twice with PBS and lysed in 100 µL radioimmunoprecipitation assay buffer (25 mM Tris, pH 7.4, 150 mM KCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) for 5 min on ice. The cells were then scraped into Eppendorf tubes and cell lysates were centrifuged at 17 500× *g* for 15 min at 4°C. Sample buffer was added followed by boiling for 4 min. Similar amounts of proteins (30 µg) from each treatment were loaded, separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk for 30 min, followed by overnight incubation at 4°C with the different primary antibodies. The blots were washed, incubated with the relevant horseradish peroxidase-conjugated secondary antibody for 30 min, and developed with electrochemiluminescence (Biological Industries).

Flow cytometry analysis of cell surface death receptors

SW480 and HCT116 cells were analysed for the surface expression of TNFRSF10A, TNFRSF10B TRAIL receptors and TNFRSF10C, TNFRSF10D TRAIL decoy receptors, by indirect staining with primary mouse anti-human TNFRSF10A, TNFRSF10B (eBioscience Inc., San Diego, CA, USA), and TNFRSF10C, TNFRSF10D (R&D Systems Inc., Minneapolis, MN, USA) antibodies, followed by FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) as follows: 5×10^5 cells were incubated with the relevant primary antibody (5 µg antibody in 50 µL PBS supplemented with 5% FBS) on ice for 30 min. After incubation, cells were washed with PBS supplemented with 5% FBS twice and reacted with FITC-conjugated goat anti-mouse IgG secondary antibody on ice for 30 min. As a negative control, cells were incubated with the secondary antibody alone at the same conditions. Following washing with PBS supplemented with 5% FBS, the expression of these receptors was analysed by FACS (Becton Dickinson and Co., Franklin Lakes, NJ, USA).

Survivin stable transfection

HCT116 cells were co-transfected with pCDNA3 plasmid encoded for HA-tagged human survivin (kindly provided by Prof. Yoel Kloog, Department of Neurobiology, Faculty of Life Science, Tel-Aviv University) and an empty pSuper vector containing a puromycin resistance codon sequence, in a 9:1 ratio (survivin : pSuper). Cells were grown under puromycin (3 µg·mL^{-1}) to produce separate colonies. Positive colonies expressing HA-survivin were chosen for further experiments.

RT-PCR analysis

RNA was isolated from each treatment using a total RNA purification kit (QIAGEN, Valencia, CA, USA), followed by TURBO™ DNase treatment (Ambion, Austin, TX, USA). Samples of RNA (2µg) were used to synthesize cDNA using Affinity Script™ Reverse Transcriptase (Stratagene, Cedar Creek, TX, USA), 25 nmol dNTPs mix (LAROVA, Teltow, Germany) and 500 ng oligo dT primer (Promega Corporation, Madison, WI, USA) at total reaction volume of 20 µL. PCR was performed employing Taq Master Mix (LAROVA) with 50 ng cDNA template and 1 µM of each primer. The following primer sequences were used: survivin-forward 5′-3′-CTTTC TCAAGGACCACCGCATC, survivin-reverse 5′-3′ CAATCCAT GGCAGCCAGCTGC, and GAPDH-forward 5′-3′-GAAGGT GAAGGTCGGAGTC, GAPDH-reverse 5′-3′ GAAGATGGTGA TGGGATTTC, resulting in a 392 base pairs PCR product for survivin and a 226 base pairs PCR product for GAPDH. PCR conditions were as follows: an initial denaturation step at 94°C for 2 min followed by 29 cycles for survivin or 25 cycles for GAPDH of denaturation for 30 s, annealing for 30 s at 60°C, extension at 72°C for 1 min and a final extension step at 72°C for 3 min. PCR products were separated on 1% agarose gel and visualized by ethidium bromide.

Luciferase reporter gene assays

To assay for transcription factor (TCF)-mediated transcription, SW480, HCT116 and HEK 293 cells were seeded at 1×10^5 cells per well in a 24-well plate and allowed to adhere. Cells were transfected the next day with a total of 1.1 µg of the following plasmids: pGL3-OT (pTOPFLASH) or pGL3-OF (pFOPFLASH) luciferase reporter constructs containing three copies of either a wild-type or mutated TCF binding element, respectively, together with pCMV-Renilla as internal control. Twenty-four hours after transfection, SW480 and HCT116 cells were incubated with the indicated concentration of MJ, and HEK 293 cells were treated with 30 mM LiCl with or without MJ for additional 24 h. Cells were then harvested, lysed and their luciferase activity was measured using a Dual-Luciferase Reporter Assay System Kit (Promega). To assay for NF-κB-mediated transcription, HCT116 cells were transfected with a total of 1.1 µg NF-κB-responsive luciferase plasmid; this encodes the firefly luciferase reporter gene under the control of a minimal CMV promoter and tandem repeats of

the NF-κB transcriptional response element, together with the pCMV-Renilla plasmid. Twenty-four hours later, the cells were treated with MJ and after another 24 h, the cells were harvested and subjected to luciferase activity measurement as above.

Statistical analysis

The Bliss additivism model (Goldoni and Johansson, 2007) was employed to calculate the expected cytotoxic effect of combining MJ and TRAIL with the following formula: $E_{bliss} = E_{MJ} + E_{TRAIL} - E_{MJ} \times E_{TRAIL}$, where E_{MJ} and E_{TRAIL} are the percentage cytotoxicity obtained by MJ and TRAIL alone, respectively, at specific concentrations. E_{bliss} is the percentage cytotoxicity that would be expected if the combination of the two drugs was exactly additive. Therefore, if the experimentally measured percentage cytotoxicity is greater than E_{bliss}, the combination is considered to be synergistic. For survivin overexpression experiments and luciferase reporter gene assay, results are presented as mean ± SD of *n*-independent experiments. Statistical significance was assessed using the two-tailed Student's *t*-test. *P*-value < 0.05 was considered statistically significant.

Materials

Methyl jasmonate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human TRAIL/Apo2L was purchased from PeproTech Inc. (Rocky Hill, NJ, USA). XIAP and caspase 8 mouse monoclonal antibodies were from MBL Co. (Nagoya, Japan). Mouse monoclonal cytochrome *c* (clone 6H2) and FADD mouse monoclonal antibodies were from BD Biosciences (San Jose, CA, USA). Survivin mouse monoclonal antibody, Bid and procaspase 9 rabbit polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Caspase 9, caspase 3, cleaved caspase 3 and PARP rabbit polyclonal antibodies were from Cell Signaling Technology, Inc. (Danvers, MA, USA). cFLIP mouse monoclonal antibody (NF6) was from ALEXIS® Biochemicals (Lausen, Switzerland). Mouse monoclonal TNFRSF10A and TNFRSF10B antibodies were from eBioscience Inc. Mouse monoclonal TNFRSF10C and TNFRSF10D antibodies were from R&D Systems Inc. Actin mouse monoclonal antibody (clone C4) was from MP Biomedicals (Solon, OH, USA). Rabbit and mouse horseradish peroxidase-conjugated and rabbit FITC-conjugated antibodies were from Jackson ImmunoResearch Laboratories, Inc. Drugs and molecular target nomenclature in this manuscript follow Alexander *et al.*, (2009).

Results

MJ sensitizes CRC cells towards TRAIL-induced cytotoxicity

We evaluated the cytotoxic effect of combined MJ and TRAIL treatment in various CRC cell lines by employing MJ at concentrations inducing low cytotoxicity (0.25–1 mM) with a range of 10–200 ng·mL^{-1} TRAIL. As determined by XTT, HCT116 and SW480 cells exhibited relative sensitivity to TRAIL, as reflected by a relatively short time (8 h) needed for TRAIL-induced cytotoxicity (Figure 1A). In contrast to HCT116 and SW480 cells, HT29 and LS180 CRC cell lines were more resistant to TRAIL, exhibiting substantial TRAIL-induced cytotoxicity only after 24 h (Figure 1B), whereas incubation for 8 h with TRAIL did not induce significant cytotoxicity in these cells (data not shown). In both SW480 and HCT116 cells, pre-incubation with MJ for 24 h followed by treatment with TRAIL for another 8 h, resulted in a significant dose-dependent cell toxicity increase in comparison to each agent given alone (Figure 1A). Moreover, the relatively TRAIL-resistant HT29 and LS180 cells also exhibited increased sensitivity towards the combination of TRAIL and MJ, resulting in significant dose-dependent synergistic cell death (Figure 1B). Similar results were obtained in DLD1 cells, and p53-deficient HCT116 cells exhibited also similar sensitivity towards the combined treatment as their parental cells (data not shown). The latter finding and the fact that p53 is mutated in SW480 and HT29 cells (Hasegawa *et al.*, 1995) excludes the possibility that p53 is involved in the cell death process induced by MJ and TRAIL. Co-administration of MJ and TRAIL in SW480 cells for 8 h did not result in increased cytotoxicity (Figure 1C), suggesting that MJ pre-incubation before treatment with TRAIL is needed to induce certain molecular event(s) that facilitate(s) the enhanced TRAIL-induced cell death.

MJ augments TRAIL-induced morphological characteristics of cell death and increases cytochrome c release to the cytoplasm

SW480 cells that were pre-incubated with 0.5 mM MJ for 24 h followed by TRAIL treatment for an additional 8 h were analysed by phase microscopy. Massive cell damage could be observed as an increased incidence of loosely rounded up cells in the MJ and TRAIL combined treatment compared to non-treated cells, or to cells treated by MJ or TRAIL alone (Figure 2A). Similar results were seen in HCT116 and HT29 cells (data not shown). Next, we examined SW480 cell nuclei by DAPI staining upon treatment with MJ, TRAIL or their combination. As demonstrated in Figure 2B, there is a substantial increase of apoptotic nuclear pattern staining, as illustrated by condensed and fragment-shaped nuclei, in cells pre-treated with MJ and subsequently treated with TRAIL, compared to those treated with TRAIL alone. Moreover, an enhanced TRAIL-induced cytochrome *c* release into the cytoplasm, a key feature of activation of the intrinsic pathway of apoptosis, was seen in SW480 cells pre-incubated with 0.5 mM MJ (Figure 2C).

Pre-incubation with MJ enhances TRAIL-induced caspase cleavage

We next explored the possible molecular mechanism(s) involved in the synergistic cell death demonstrated above. First, we studied death signal transmission. Upon TRAIL binding, TRAIL receptors recruit adaptor proteins forming DISC at the cell membrane, resulting in recruitment and activation of caspase 8 (Kischkel *et al.*, 2000; Sprick *et al.*, 2000). Active caspase 8 directly activates effector caspases ('extrinsic' apoptotic pathway), and may also activate the 'intrinsic' apoptotic pathway by cleavage of Bid. The truncated Bid is translocated to the mitochondria where it mediates mitochondrial membrane permeabilization and cytochrome *c* release. Cytochrome *c* in turn facilitates the

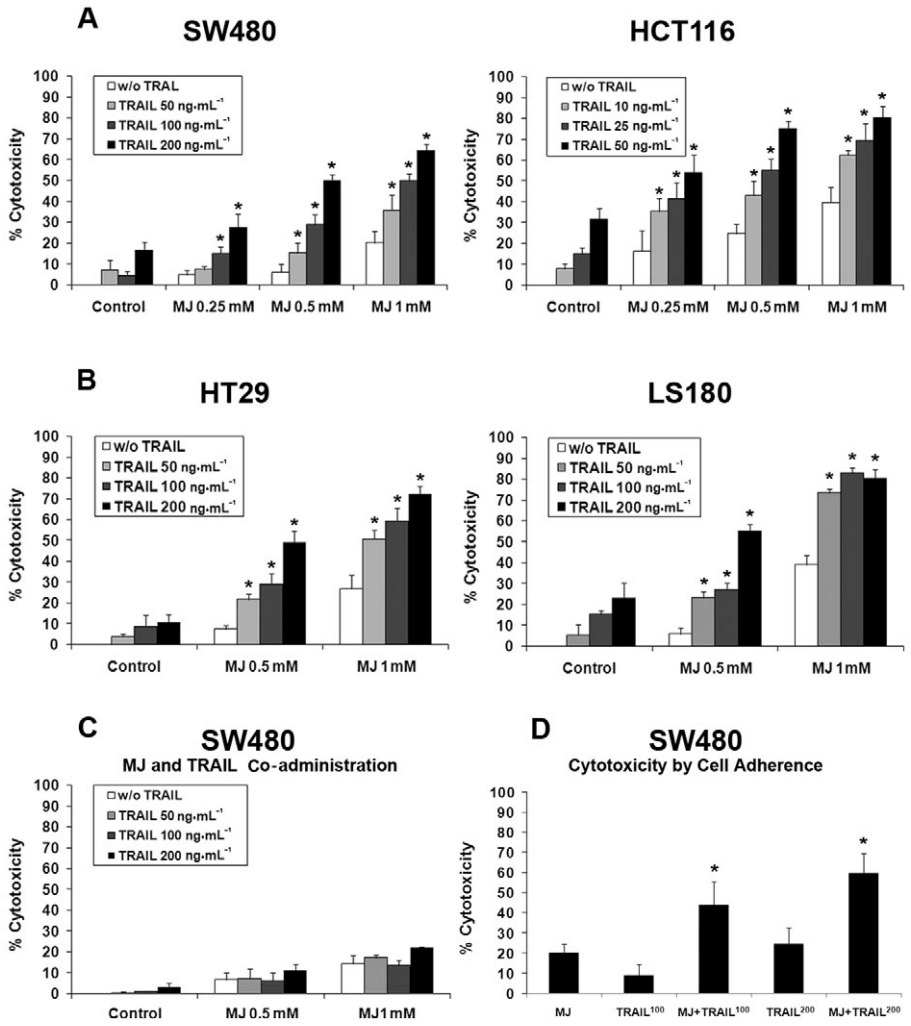

Figure 1

Pre-incubation of MJ augments TRAIL-induced cytotoxicity in various CRC cell lines. SW480 and HCT116 cells were incubated with concentrations of MJ inducing low cytotoxicity, for 24 h followed by treatment with TRAIL for another 8 h (A); HT29 and LS180 cells were incubated with MJ for 24 h followed by TRAIL for another 24 h (B), and SW480 cells were co-administered with MJ and TRAIL for 8 h (C). Cell toxicity was then evaluated by XTT. Bars labelled w/o TRAIL, refer to cells treated with MJ alone at the indicated concentrations. (D) SW480 cells were grown and treated as in (A) except that cytotoxicity was evaluated by loss of cell adherence measured by protein concentration determination of adherent cells using a Bio-Rad, Lowry-based, protein assay kit. $TRAIL^{100}$ and $TRAIL^{200}$ refer to TRAIL concentrations in $ng{\cdot}mL^{-1}$; MJ was used at 0.5 mM. In all of the panels, the data represent percentage cell toxicity of control untreated cells (the control cells thus are calculated to have zero toxicity) and are shown as mean ± SD for a minimum of three experiments. Asterisks indicate that the experimentally measured combined cytotoxic effect of MJ and TRAIL is greater than the expected additive effect as calculated using the Bliss additivism model, as described in *Methods*.

formation of the apoptosome that comprises the adaptor Apaf-1 and caspase 9, and the latter directly cleaves and activates the effector caspase, caspase 3 (Luo *et al.*, 1998; Falschlehner *et al.*, 2007). Therefore, we measured the cleavage of caspase 8, Bid, caspase 9 and subsequently that of the executive caspase 3 and its substrate poly ADP ribose polymerase (PARP), by means of Western blot. In both SW480 and HCT116 cells there was an enhanced caspase 8 cleavage in MJ and TRAIL combined treatment compared to TRAIL administered alone, as demonstrated by the decrease in procaspase 8 protein levels and also by the appearance of p43- and p18-cleaved caspase 8 isoforms in HCT116 cells (Figure S1). No caspase 8 cleavage was observed when 0.5 mM MJ was given alone, a concentration that facilitated the enhanced TRAIL-induced cell death as demonstrated in Figure 1. In a similar manner, increased Bid and caspase 9 cleavage was detected in cells treated with the MJ and TRAIL combination (Figure 3) suggesting that MJ pre-treatment affects TRAIL-induced intrinsic apoptotic pathway activation as well. As with caspase 8 activation, no Bid or caspase 9 cleavage was seen in cells treated with MJ alone. Finally, both caspase 3 effector caspase and its substrate PARP cleavage were enhanced in cells receiving the combined treatment of MJ and TRAIL (Figure 3).

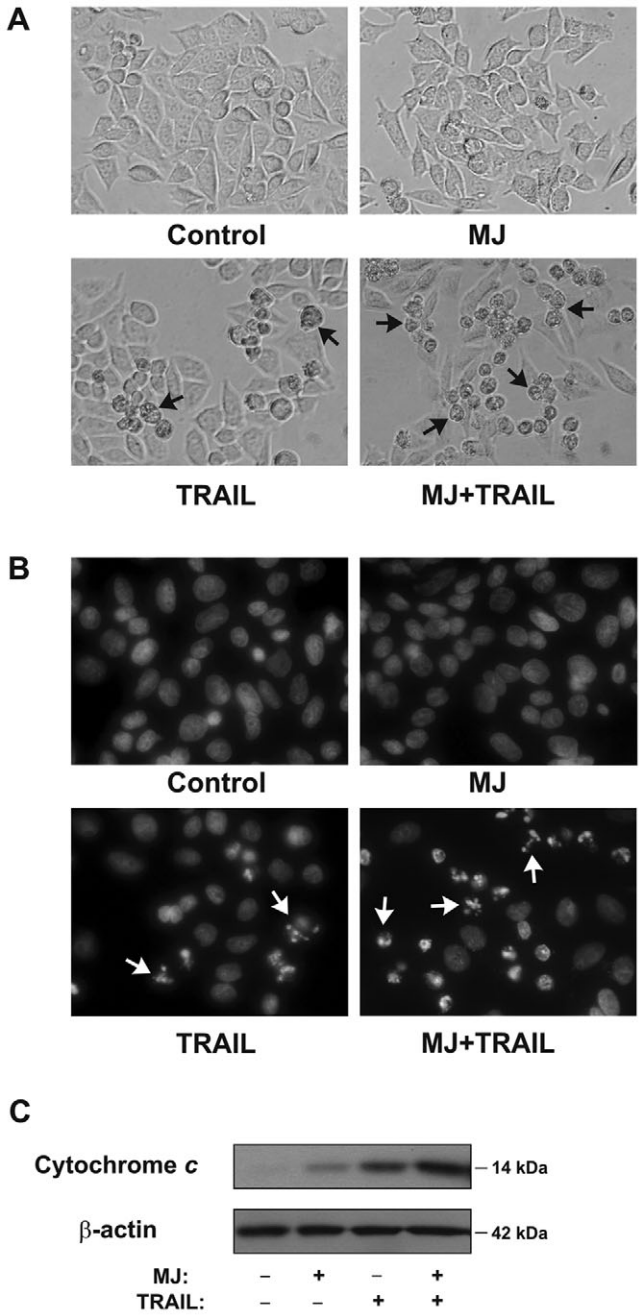

Figure 2

Combined MJ and TRAIL treatment enhances apoptotic cell death. (A) Microphotograph (phase microscopy) of SW480 cells treated with 0.5 mM MJ for 24 h followed by 200 ng·mL^{-1} TRAIL administration for another 8 h. Damaged loose, rounded up cells are indicated with black arrows; x400 magnification (B). SW480 cells grown on cover slips were pre-incubated with 0.5 mM MJ for 24 h followed by another 4 h with 100 ng·mL^{-1} TRAIL. Cells were fixed with formaldehyde and stained with DAPI. Apoptotic, fragment-shaped nuclei are indicated by white arrows; x400 magnification (C). Enhanced TRAIL-induced cytochrome *c* release in cells pre-incubated with MJ. SW480 cells were pre-incubated for 24 h with 0.5 mM MJ followed by treatment with TRAIL (100 ng·mL^{-1}) for additional 4 h. Cells were then harvested and cytochrome *c* release was measured as described in Methods.

TNFRSF10A, TNFRSF10B, TNFRSF10D and cFLIP are not involved in MJ sensitization towards TRAIL-induced cell death

Because MJ pre-incubation sensitized cells towards TRAIL-induced cytotoxicity (Figures 1 and 2) and facilitated an augmented TRAIL-dependent caspase activity (Figure 3), we examined the molecular factors that may play a role in this process. First, we examined the cell surface protein expression of the TRAIL receptors, TNFRSF10A and TNFRSF10B, as their up-regulation has been shown to be involved in a number of different cases of drug-dependent TRAIL sensitization. To this end, we incubated SW480 and HCT116 cells with MJ for 24 h and analysed the expression of TNFRSF10A and TNFRSF10B by FACS. We found that TNFRSF10A cell surface protein levels were not changed upon MJ administration in both SW480 and HCT116 cells (Figure 4A, upper panel). As for TNFRSF10B cell surface protein levels, those did not change in SW480 cells upon MJ treatment, whereas in HCT116 cells, TNFRSF10B protein levels were up-regulated (Figure 4A, middle panel). Based on these results, we concluded that TNFRSF10A is not involved in MJ sensitization towards TRAIL-induced cytotoxicity. Moreover, although that TNFRSF10B cell surface expression levels were increased in HCT116 cells, the different pattern seen in SW480 and the fact that in HCT116 cells, MJ given at 0.5 mM, which is sufficient to sensitize HCT116 cells to TRAIL, resulted in only a very small increase in cell surface levels of TNFRSF10B, suggest that TNFRSF10B is probably not responsible for MJ-induced TRAIL sensitization even in HCT116 cells.

To examine whether down-regulation by MJ of the TRAIL decoy receptors TNFRSF10C and TNFRSF10D may be involved in TRAIL sensitization, we analysed their cell surface expression by FACS as well. Both SW480 and HCT116 cells did not express TNFRSF10C (data not shown). Interestingly, while TNFRSF10D cell surface levels were unchanged upon MJ administration to SW480 cells, its levels were slightly increased in HCT116 cells treated with MJ (Figure 4A, lower panel). These results exclude the possibility that MJ induces TRAIL sensitization by down-regulation of decoy receptors. We next analysed, by Western blot, levels of FADD adaptor protein and cFLIP protein after MJ treatment. FADD adaptor protein expression levels did not change in SW480 cells and in HCT116 cells FADD expression was even decreased, upon MJ administration (Figure 4B). Moreover, while in many circumstances, cFLIP down-regulation is a common mechanism of drug-induced TRAIL sensitization, in our system cFLIP long isoform (cFLIP$_L$) protein levels were not altered upon MJ administration, at concentrations displaying synergism with TRAIL, in both SW480 and HCT116 cells (Figure 4B), indicating that cFLIP was not involved in TRAIL sensitization induced by MJ.

MJ induces a decrease in survivin protein and mRNA levels

Survivin and XIAP are both members of the IAP protein family known to play important roles in apoptosis regulation, and their down-regulation was reported to be involved in several cases of drug-induced TRAIL sensitization (Schultze *et al.*, 2006; Mori *et al.*, 2007). Moreover, down-regulation of survivin or XIAP by means of RNA interference was shown to

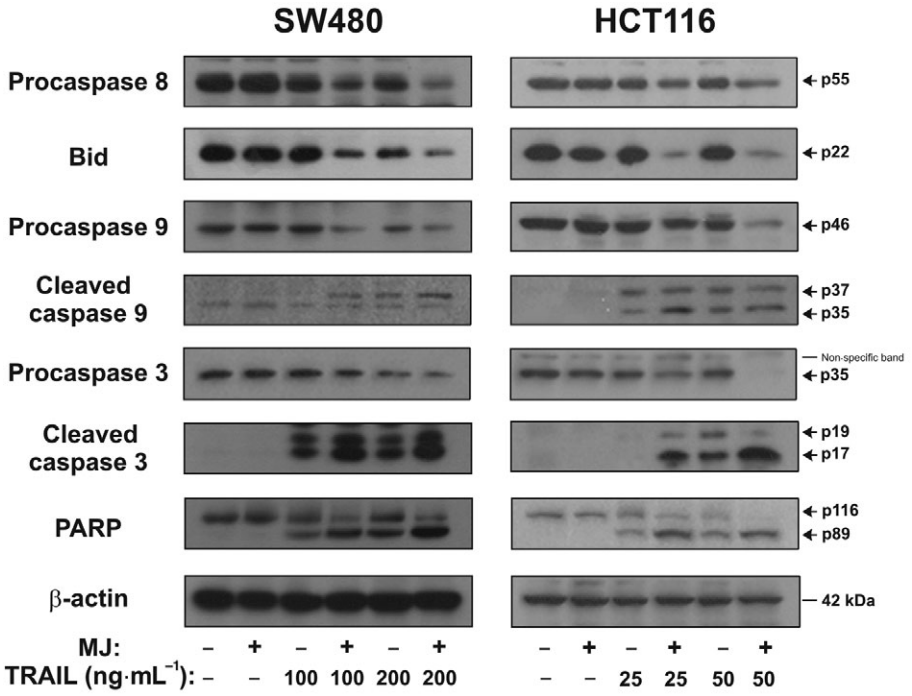

Figure 3

MJ enhanced TRAIL-dependent cleavage of caspases and Bid. SW480 (left panels) and HCT116 cells (right panels) were pre-incubated for 24 h with 0.5 mM MJ followed by TRAIL treatment for another 4 h at the indicated concentrations. Cells were then harvested and analysed by Western blot for the different caspases, PARP and Bid proteins as indicated in the Figure.

sensitize cancer cell lines towards TRAIL-induced cytotoxicity (Chawla-Sarkar *et al.*, 2004; Yamaguchi *et al.*, 2005). Therefore, we measured survivin and XIAP protein expression levels after MJ treatment. While XIAP protein levels were not modified by treatment with MJ for 24 h, in both SW480 and HCT116 cell lines, survivin protein levels were decreased significantly in a dose-dependent manner (Figure 5A). The decrease in survivin protein expression was substantial even at low MJ concentrations that are capable of TRAIL-induced cytotoxicity augmentation (Figure 5A and see Figure 1), implying that survivin may play an important role in MJ-induced TRAIL sensitization. Similar results were obtained in HT29 and LS180 cells (Figure S2). Examination of the time course of survivin expression revealed that its protein levels were decreased to a large extent, only upon 24 h of MJ treatment (Figure 5B), supporting our assumption that MJ pre-incubation for a sufficient period is needed to sensitize the cells towards TRAIL-induced cytotoxicity (Figure 1A,C). Moreover, HCT116 cells stably transfected with human survivin were significantly less sensitive towards MJ-induced TRAIL sensitization (Figure 5C). Survivin overexpression inhibited the cytotoxicity induced by TRAIL administered alone, and also that of MJ administered alone (Table S1), but had no significant effect on spontaneous cell death (data not shown). Next, we analysed survivin mRNA levels after treatment with MJ for 24 h by RT-PCR, and found that survivin mRNA levels were decreased in a similar manner to that of its protein levels, in both SW480 and HCT116 cells (Figure 6A). These results imply that MJ may affect survivin transcription or survivin mRNA stability. Interestingly, in a gene array experiment examining MJ-treated MDA-MB-231 breast carcinoma cells, MJ caused a significant decrease of survivin mRNA levels in comparison to non-treated cells (C. Horrix and M.R. Berger, pers. comm.).

MJ suppresses β-catenin/TCF-dependent luciferase activity

Survivin is known to be involved in CRC carcinogenesis and is highly expressed in CRC cells (Kawasaki *et al.*, 1998; Kim *et al.*, 2003). We thus explored the mechanism by which MJ induced survivin mRNA down-regulation. Several factors are known to regulate survivin transcription, that is, NF-κB, Sp1 and the Wnt/TCF signalling pathway (Zhang *et al.*, 2006). Mutations in the tumour suppressor gene APC, a negative regulator of the Wnt pathway, are an important initiating genetic alteration in the development of CRC (Zhang *et al.*, 2001; Kim *et al.*, 2003). Moreover, in CRC cells the Wnt pathway plays an important role in survivin transcriptional regulation (Zhang *et al.*, 2001). Therefore, we examined whether MJ administration affects the Wnt/TCF pathway by employing a TCF-luciferase reporter gene assay (Gazit *et al.*, 1999) and found that MJ induced a significant reduction of TCF-dependent luciferase activity (Figure 6B), indicating that the MJ-induced decrease in survivin mRNA levels is likely to be specifically affected by TCF-dependent transcriptional activity. Next, we treated HEK 293 cells (which, in contrast to CRC cell lines, have normal, non-mutated Wnt/TCF signalling components) with LiCl, a known activator of Wnt signalling (Klein and Melton, 1996), together with MJ and tested TCF-dependent luciferase activity. Indeed, MJ substantially

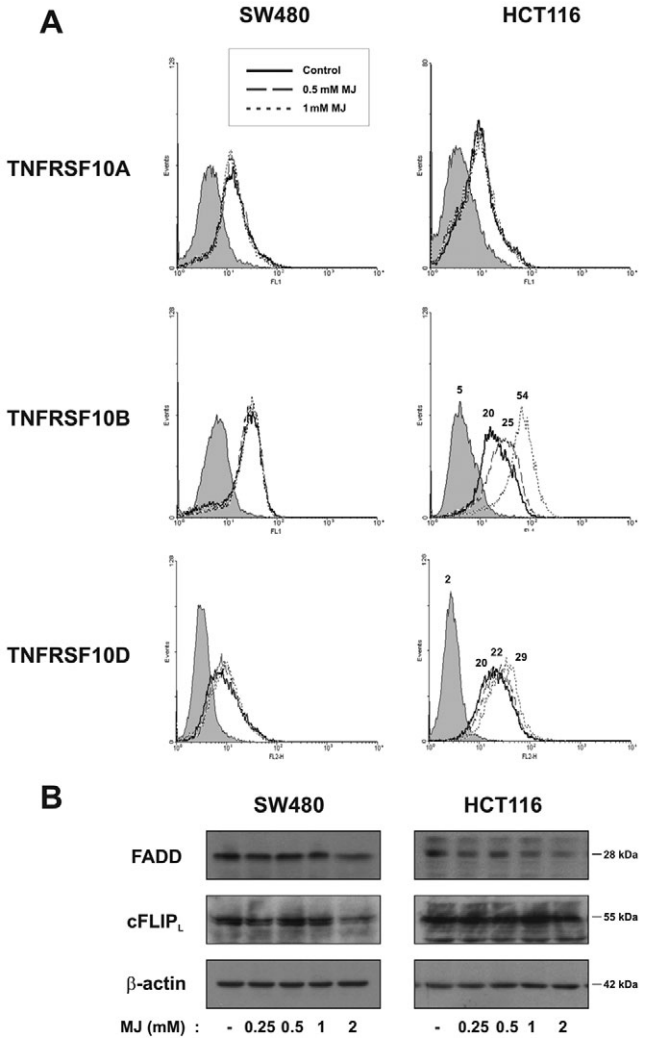

Figure 4

Effct of MJ on death receptors and death receptors-associated proteins. (A) SW480 and HCT116 cells were treated for 24 h with MJ and were analysed for cell surface expression of TNFRSF10A, TNFRSF10B and TNFRSF10D by FACS. Gray filled histograms: control cells incubated with secondary antibody alone. Open histograms: cells treated with antibodies for death receptors, under control (no MJ) or with MJ (0.5 or 1 mM). Numbers, representing geometrical mean values, are shown for HCT116 TNFRSF10B and TNFRSF10D, where a change in their expression levels is seen. (B) SW480 (left panels) and HCT116 cells (right panels) were incubated with the indicated MJ concentrations for 24 h and the protein levels of FADD and cFLIP$_L$ were then analysed by Western blot.

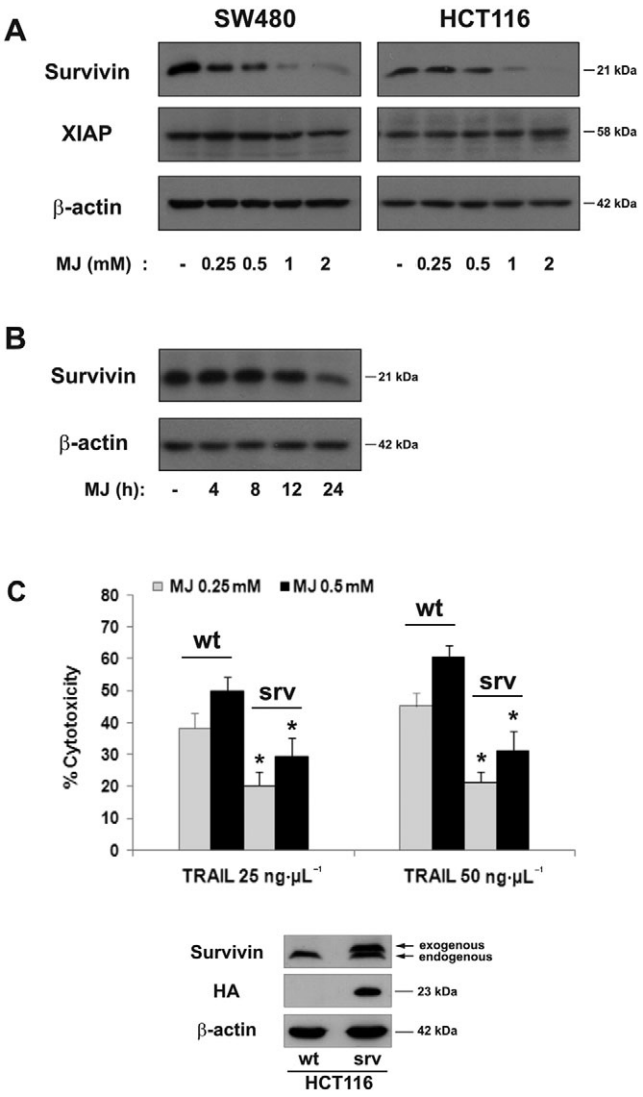

Figure 5

MJ decreased survivin but not XIAP protein levels. (A) SW480 (left panels) and HCT116 cells (right panels) were treated for 24 h with different MJ concentrations. Cells were then lysed and analysed for survivin and XIAP protein levels by Western blot. (B) SW480 cells were treated with MJ (1 mM) for the different times indicated in the Figure. Cells were then analysed by Western blot for survivin protein levels. (C) HCT116 cells stably expressing survivin (srv) and parental wt-HCT116 cells (wt) were treated with MJ for 24 h followed by TRAIL for another 8 h at the indicated concentrations. XTT assay was then performed. *$P < 0.05$, srv-HCT116 cell cytotoxicity was significantly lower than in wt-HCT116 cells; two-tailed Student's *t*-test. Cell lysates from parental and survivin stably transfected HCT116 cells were examined for survivin expression by Western blot using anti-survivin and anti-HA antibodies.

decreased LiCl-induced TCF-dependent luciferase activity (Figure 6C), further indicating the ability of MJ to affect TCF-dependent transcription. To evaluate whether the effects of MJ on survivin down-regulation was specific to Wnt/TCF, we transfected HCT116 cells with a NF-κB-dependent luciferase construct and treated them with MJ. MJ did not have an effect on the activity of NF-κB luciferase (Figure 6D). All together, these results demonstrate that MJ affects the Wnt/TCF pathway, and this may explain its ability to decrease survivin mRNA levels, by inhibition of transcription.

Discussion and conclusions

Cytotoxicity induced by TRAIL has emerged in recent years as a promising modality in cancer therapy, as TRAIL and its receptors are expressed preferentially in cancer cells. Many preclinical studies, and even several clinical trials, have been

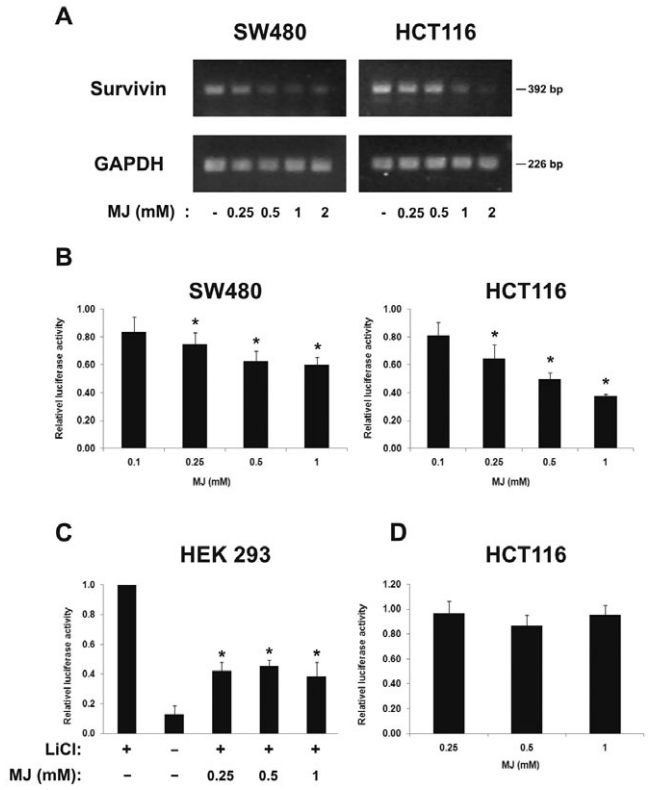

Figure 6

MJ suppresses survivin transcription. (A) MJ decreased survivin mRNA levels. SW480 (left panels) and HCT116 cells (right panels) were incubated for 24 h with the indicated MJ concentrations. Cells were then harvested and RNA was isolated. To detect survivin mRNA levels, an equal RNA amount of each preparation was evaluated by RT-PCR with specific primers designed for human survivin. Samples were then analysed by agarose gel electrophoresis. (B) MJ affects TCF-dependent luciferase activity. SW480 and HCT116 cells were transfected with the pTOPFLASH/pFOPFLASH reporter and the pCMV-Renilla plasmids. Twenty-four hours later, cells were incubated with the indicated concentrations of MJ. Forty-eight hours post transfection cells were harvested and subjected to luciferase activity measurement. (C) MJ affects TCF-dependent luciferase activity in HEK 293 cells induced by LiCl. HEK 293 cells were transfected with pTOPFLASH/pFOPFLASH reporter and the pCMV-Renilla plasmids and treated 24 h later with 30 mM LiCl, with or without MJ, for another 24 h. Cells were then harvested and subjected to luciferase activity measurement. (D) MJ does not affect NF-κB-dependent luciferase activity. HCT116 cells were transfected with NF-κB reporter and the pCMV-Renilla plasmids. Twenty-four hours later the cells were treated with MJ and after another 24 h, the cells were harvested and subjected to luciferase activity measurement. The data in B–D illustrate relative luciferase activity compared to control untreated cells (B,D) or relative luciferase activity compared to LiCl treated cells (C) and are shown as mean ± SD of a minimum of three independent experiments. *$P < 0.05$, significantly less than (B) untreated cells, or (C) LiCl-treated cells; two-tailed Student's *t*-test.

performed (Ashkenazi and Herbst, 2008; Johnstone *et al.*, 2008). However, one major obstacle facing TRAIL treatment is the fact that many cancer cells exhibit innate TRAIL resistance. Consequently, a common approach in TRAIL treatment is administration of recombinant TRAIL or agonistic anti-TRAIL receptor monoclonal antibodies, together with anti-cancer agents capable of TRAIL sensitization. Some of these drugs are anti-cancer drugs that are already in the clinic, while others include inhibitors of NF-κB, histone deacetylases, PI3K pathway, proteasome and IAP (Ashkenazi and Herbst, 2008; Johnstone *et al.*, 2008). While some of these combinations seem to be efficient *in vivo*, the majority of these treatments are not (Johnstone *et al.*, 2008), and some of these combinations are toxic (Koschny *et al.*, 2007).

We have previously demonstrated that MJ is capable of inducing cancer cell death in a selective manner via mitochondrial perturbation (Rotem *et al.*, 2005), by a direct interaction and detachment of mitochondrial bound hexokinase (Goldin *et al.*, 2008) which is overexpressed in cancer cells, and could explain the selectivity of MJ towards cancer cells. Here, we show that pre-incubation with MJ, at concentrations inducing low cytotoxicity, followed by TRAIL administration, resulted in a significant enhanced TRAIL-induced apoptosis in CRC cell lines, thereby illustrating an efficient combination of two highly selective anti-cancer agents. Thus, we assume that MJ has other characteristics in addition to its direct mitochondrial-cytotoxic effect. MJ-induced TRAIL sensitization was demonstrated in this study in several ways, that is, XTT assay, increased DAPI-stained nuclear fragments, increased TRAIL-induced cytochrome *c* release to the cytoplasm and increased TRAIL-induced caspase cleavage. We were able to induce TRAIL sensitization by MJ in cancer cell lines other than CRC cells as well, including breast and leukaemic cell lines (Z. Raviv and E. Flescher, pers. comm.), indicating that combinations of MJ and TRAIL may be applicable to the treatment of other types of cancer besides CRC.

Although in many cases, cFLIP down-regulation is one of the factors playing an important role in drug-induced TRAIL sensitization, this is most probably not the case in our system, as cFLIP protein levels were not changed by MJ treatment. Moreover, TNFRSF10A expression on the cell surface was unchanged upon MJ administration in both SW480 and HCT116 cells, and that of TNFRSF10B was not altered by MJ in SW480 cells. Thus, despite the fact that in HCT116 cells TNFRSF10B levels were up-regulated, we conclude that the possibility of TRAIL receptor up-regulation being a key mechanism underlying the enhancement by MJ of TRAIL-induced cell death and caspase activity is highly unlikely, especially due to the fact that 0.5 mM MJ induced a slight increase of TNFRSF10B levels (Figure 4A), whereas it induced significant TRAIL sensitization in these cells (Figure 1A). Moreover, the finding that the cell surface expression of the TRAIL decoy receptor, TNFRSF10D, was slightly up-regulated upon MJ administration in HCT116 cells is not in favour of the idea of the deregulation of TRAIL receptors after MJ treatment, being responsible for TRAIL sensitization by MJ. MJ was involved with the intrinsic apoptotic pathway as it induced cytochrome *c* release. However, it is most likely that the effect of MJ on TRAIL-induced cytochrome *c* release was a result of the enhanced caspase 8 and subsequent Bid cleavage, that was induced by TRAIL, rather than MJ activation of the intrinsic apoptotic pathway, given that MJ induced a moderate cytochrome *c* release, and that caspase 9 cleavage, a downstream event of cytochrome *c* release, was not induced by treatment with MJ alone.

Inhibitors of apoptosis are important modulators of cell death exerting their regulatory effects in part by caspase inhibition, and their down-regulation is of great importance in cancer therapy (LaCasse *et al*., 2008). We have shown here that, in SW480 and HCT116 CRC cells, MJ affected survivin but not XIAP protein expression and that survivin down-regulation by MJ was carried out via down-regulation of its mRNA levels. It is well known that knockdown of survivin by means of siRNA sensitizes cancer cells towards TRAIL-induced apoptosis, as demonstrated for human hepatocellular carcinomas (Yamaguchi *et al*., 2005), melanoma cancer cells (Chawla-Sarkar *et al*., 2004), and most relevant to our study, SW480 cells (Goda *et al*., 2008). The removal of caspase inhibition by down-regulation of survivin results in enhanced TRAIL-induced caspase cleavage. Survivin is known to inhibit effector caspases but the study of Goda *et al*. reinforces the possibility that survivin may affect caspase 8 cleavage as well, as siRNA directed against survivin augmented TRAIL-induced caspase 8 cleavage in SW480 cells (Goda *et al*., 2008). The results of Goda *et al*. support previous findings suggesting that caspase 3 can modulate the complete activation of caspase 8 (Pirnia *et al*., 2002). Accordingly, we conclude that survivin down-regulation, as demonstrated in our study could enhance TRAIL-induced processing of the initiator caspase 8, besides that of the effector caspase 3. siRNA directed against survivin was able to sensitize hematopoietic cancer cells also to cytotoxicity induced by the Bcr-abl tyrosine kinase inhibitor STI571 (Wang *et al*., 2005), supporting the notion that down-regulation of survivin is an important target in cancer therapy. Moreover, down-regulation of survivin is involved in several cases of drug-induced TRAIL sensitization (Schultze *et al*., 2006; Yoo and Lee, 2007; Goda *et al*., 2008). We have demonstrated in this study that overexpression of survivin in HCT116 cells significantly protected against MJ-induced TRAIL sensitization. Therefore, we conclude that survivin down-regulation is a key factor in MJ-induced TRAIL sensitization in our system. Overexpression of survivin did not cause a complete suppression of the effects of MJ on TRAIL-induced cytotoxicity. Therefore, an additional mechanism for MJ-induced TRAIL sensitization should be considered. It might appear that the MJ dose-dependent down-regulation of survivin (Figures 5 and 6) did not correlate with the sensitization to TRAIL-induced apoptosis that pre-treatment of MJ conferred (Figure 1), particularly in HCT116 cells at the lower doses of MJ. Indeed, TNFRSFB up-regulation could explain this discrepancy. However, one should take into consideration that survivin is also down-regulated in HT29 and LS180 cells and that TNFRSFB up-regulation occurs only in HCT116 cells. Not only that, as discussed above, the cell surface expression of TNFRSFD TRAIL decoy receptors was also up-regulated in HCT116 cells. Thus, the evidence in our hands does not allow us to state conclusively that an increase in TNFRSFB can resolve the apparent discrepancy. A possible alternative explanation to this inconsistency might be that HCT116 cells are inherently more sensitive to TRAIL (Ozoren and El-Deiry, 2002), thus even minor down-regulation in survivin levels could have a marked effect on TRAIL sensitization. In addition, as mentioned above, there could be other factor/s, yet to be discovered, involved in MJ-induced TRAIL sensitization, besides down-regulation of survivin.

Interestingly, high cytotoxic MJ concentrations affect survivin expression in human neuroblastoma cell lines (Tong *et al*., 2008). Here we show that lower MJ concentrations, which by themselves were only marginally toxic, were able to down-regulate survivin in CRC cells. Also, we have shown earlier that MJ, given at sub-lethal concentrations, was associated with anti-metastatic activities of melanoma cancer cells (Reischer *et al*., 2007), and others have shown that a low dose of MJ was very effective at inducing the myelomonocytic differentiation of human myeloid leukaemia HL-60 cells (Ishii *et al*., 2004). Taken together, these findings support the notion that in addition to its cytotoxic activity, MJ has non-cytotoxic effects, depending on the concentration administered, and both characteristics can be beneficial for cancer therapy.

Survivin is highly expressed in CRC (Kim *et al*., 2003) and negatively correlates with survival (Kawasaki *et al*., 1998). As up-regulation of survivin is frequently found in CRC, it is very likely that high levels of survivin contribute to TRAIL resistance. CRC is thought to originate from the expansion of colonic crypt cells as a result of aberrant gene expression caused by transcriptional activity of the Wnt/TCF pathway (Polakis, 2000). This pathway plays an important role in survivin transcriptional regulation in CRC cells (Zhang *et al*., 2001). In normal cells, wild-type APC represses survivin expression, thus limiting cell proliferation (Zhang *et al*., 2001). Mutations in APC and β-catenin are common in CRC and other types of cancer (Li *et al*., 2002). As a result of these mutations, β-catenin is accumulated and the β-catenin–TCF complex becomes transcriptionally active for downstream targets, including survivin and other proliferative genes and oncogenes, resulting in tumourigenesis (Li *et al*., 2002). We showed here that MJ treatment affected TCF-luciferase reporter gene-dependent activity, indicating that MJ-induced decrease of survivin mRNA levels may be affected, via the β-catenin/TCF pathway. Moreover, given that MJ is capable of affecting the β-catenin/TCF pathway and the crucial role of this pathway in the onset of colon cancer, we speculate that MJ could be used as a potential chemopreventive agent in the battle against cancer.

In summary, we propose that combinations of MJ and TRAIL would have several therapeutic advantages: (i) drug combinations facilitate decreasing the dosage of each drug given alone; (ii) MJ and TRAIL are very selective towards cancerous cells, sparing normal cells; and (iii) evading the barrier of TRAIL resistance of several cancers by MJ-induced TRAIL sensitization. Our study is strictly *in vitro*, thus further *in vivo* studies need to be carried out in order to further evaluate the efficiency of this type of combined therapy.

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Science and Technology to MRB and EF, and by The Israel Cancer Association through the Estate of the late Alexander Smidoda to EF.

Sadly, we note that Professor Flascher died during the review of this paper.

Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 MJ induces enhanced TRAIL-dependent caspases 8 cleavage. HCT116 cells were pre-incubated for 24 h with 0.5 mM MJ followed by TRAIL administration for another 4 h. Cells were then harvested and analysed by Western blot for caspases 8. p55 indicates the procaspase 8 isoform. p43 and p18 indicate the caspase 8 cleaved isoforms.
Figure S2 MJ induces decrease of survivin protein levels. HT29 and LS180 cells were treated for 24 h with the different MJ concentrations. Cells were then lysed and analysed for survivin protein levels by Western blot.
Table S1 HCT116 cells stably expressing survivin (srv) and parental wt-HCT116 cells (wt) were treated with MJ (0.25 or 0.5 mM) for 24 h followed by TRAIL administration (25 or 50 $ng{\cdot}mL^{-1}$) for another 8 h. Cells were then subjected to XTT cell viability assay. Results are percentage cytotoxicity of control non-treated cells and are mean ± SD of at least three independent experiments

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