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The Synthetic Cannabinoid WIN 55,212-2 Sensitizes Hepatocellular Carcinoma Cells to Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL)-Induced Apoptosis by Activating p8/CCAAT/Enhancer Binding Protein Homologous Protein (CHOP)/Death Receptor 5 (DR5) Axis

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ABSTRACT

In this article, we demonstrate that the synthetic cannabinoid *R*-(+)-(2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrol[1,2,3-de]-1,4-benzoxazin-6-yl)-(1-naphthalenyl) methanone mesylate (WIN 55,212-2) sensitizes human hepatocellular carcinoma (HCC) cells to apoptosis mediated by tumor necrosis-related apoptosis inducing ligand (TRAIL). The apoptotic mechanism induced by treatment with WIN/TRAIL combination involved the loss of the mitochondrial transmembrane potential and led to the activation of caspases. In HCC cells, WIN treatment induced the up-regulation of TRAIL death receptor DR5, an effect that seemed to be related to the increase in the level of p8 and CHOP, two factors implicated in cellular stress response and apoptosis. This relationship was suggested by the observation that the down-regulation of p8 or CHOP by specific small interfering RNAs attenuated both WIN-mediated DR5 up-regulation and the cytotoxicity induced by WIN/TRAIL cotreatment. Moreover, WIN induced a significant decrease in the levels of some survival factors (survivin, c-inhibitor of apoptosis protein 2, and Bcl-2) and in particular in that of the active phosphorylated form of AKT. This event seemed to be dependent on the transcription factor peroxisome proliferator-activated receptor-γ whose level significantly increased after WIN treatment. Therefore, both the induction of DR5 via p8 and CHOP and the down-regulation of survival factors seem to be crucial for the marked synergistic effects induced by the two drugs in HCC cells. Taken together, the results reported in this article indicate that WIN/TRAIL combination could represent a novel important tool for the treatment of HCC.

The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor family, is a potent apoptosis-inducing cytokine. TRAIL seems to specifically kill a wide variety of cancer cells in culture and xenografted tumors while sparing most normal cells (Ray and Almasan, 2003; Falschlehner et al., 2007). TRAIL-induced apoptosis is associated with the interaction of this ligand with two closely related membrane receptors, DR4 (TRAIL-R1) and DR5 (TRAIL-R2), whereas two other TRAIL receptors, DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4), are not involved in apoptotic signal, serving as decoy proteins (Sheridan et al., 1997). The binding of TRAIL to DR4 and DR5 receptors results in the interaction with the adaptor molecule Fas-associated death domain leading to the recruitment and

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ABBREVIATIONS: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; HCC, hepatocellular carcinoma; DR, death receptor; CB, cannabinoid receptor; CHOP, CCAAT/enhancer binding protein homologous protein; IAP, inhibitor of apoptosis protein; PPARγ, peroxisome proliferator activated receptor-γ; MTT, 3-[4,5-dimethylthiazolyl-2] 2,5-diphenyl-tetrazolium bromide assay; $DiOC_6$, 3–3-dihexyloxacarbocyanine; z-VAD-fmk, benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; WIN 55,212-2, WIN, *R*-(+)-(2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrol[1,2,3-de]-1,4-benzoxazin-6-yl)-(1-naphthalenyl) methanone mesylate; siRNA, small interfering RNA; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; RT-PCR, reverse transcriptase-polymerase chain reaction; bp, base pair; PCR, polymerase chain reaction; siCHOP, small interfering RNAs against CCAAT/enhancer binding protein homologous protein; siScr, scrambled small interfering RNA; sip8, p8 small interfering RNA; CI, combination index; XIAP, X-linked inhibitor of apoptosis protein; ROS, reactive oxygen species; GW9662, 2-chloro-5-nitrobenzanilide.

cleavage of the initiator caspase-8 and the consequent activation of executioner protease cascade (Kischkel et al., 2000).

It has been demonstrated that many malignant cells are resistant to TRAIL signaling, and this is frequently correlated to the loss of TRAIL receptors on cell surface (Zhang and Fang, 2005). Thus, it is important to find therapeutic agents capable of sensitizing resistant cancer cells to TRAIL-induced apoptosis. There are many studies demonstrating that the combination of different anticancer agents with TRAIL induces additive or synergistic tumor cell death (Griffith and Kemp, 2003; Elrod and Sun, 2008; Shamimi-Noori et al., 2008; Siegelin et al., 2009). It has been demonstrated that the efficacy of histone deacetylase inhibitors in sensitizing hepatocellular carcinoma (HCC) cells to TRAIL induced apoptosis (Carlisi et al., 2009).

Cannabinoids, originally derived from the plant *Cannabis sativa*, and their endogenous and synthetic counterparts elicit a wide range of central and peripheral effects, mediated mostly through cannabinoid receptors CB1 and CB2, which differ in tissue distribution, physiological role, and signaling mechanisms (Herkenham et al., 1991; van der Stelt and Di Marzo 2005). Current interest in cannabinoids is focused on their involvement in the regulation of cell death and survival. The antiproliferative effects of these compounds, observed more than 30 years ago, have been reported in various cancer cells, including breast and prostate cancer, PC12 pheochromocytoma, and malignant gliomas (Guzmán, 2003; Velasco et al., 2004; Sarfaraz et al., 2005). The action of cannabinoids in tumor cells is related to the decrease in cell viability, proliferation, adhesion, and migration, as well as the modulation of angiogenesis and metastasis (Guzmán, 2003). Although these results encourage the use of cannabinoids as anticancer agents, the use is often limited by their psychoactive effects; thus, in recent years, synthetic CB receptor agonists with activities similar to those exerted by natural cannabinoids but without side effects have been developed. Among these, WIN 55,212-2 (WIN), a synthetic CB1/CB2 receptor agonist, seems to be particularly promising. It has been reported that WIN induces apoptosis in some tumor cell lines, such as human prostate cancer cells and mantle cell lymphoma (Gustafsson et al., 2006; Sarfaraz et al., 2006). Our previous studies demonstrated that WIN is very effective in inducing apoptosis in HepG2 cells through a mechanism involving the reduction in the levels of some survival factors (survivin, phospho-AKT, 72-kDa heat shock protein, and Bcl-2) and the activation of proapoptotic ones (Bax, Bcl-X_S, and t-Bid) (Giuliano et al., 2009).

The aim of the present study was to investigate whether WIN is capable of sensitizing TRAIL-resistant HCC cells to TRAIL-induced apoptosis and to underlie the mechanism by which the combined treatment achieves killing of these cells. Data reported show that the combined treatment with subtoxic doses of WIN and TRAIL dramatically induces apoptosis in three different HCC cell lines. This event seems to be a consequence of two different effects: 1) the WIN-induced DR5 up-regulation mediated by p8 and CHOP, two critical mediators of cell death; and 2) the down-regulation of both phospho-AKT and some survival factors of IAP family. Thus, treatment with WIN/TRAIL combination may synergistically stimulate and accelerate death receptor-triggered apoptotic pathway, making this new drug combination promising for clinical outcome.

Materials and Methods

Reagents. WIN 55,212-2 and GW9662 were purchased from Sigma-Aldrich (St. Louis, MO). Soluble human recombinant TRAIL/APO2L was obtained from PeproTech (EC Ltd., London, UK). Benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) from Promega (Madison, WI). Stock solutions were prepared in dimethyl sulfoxide and opportunely diluted in culture medium. The final concentration of dimethyl sulfoxide never exceeded 0.04%.

Cell Cultures. Human HCC HepG2 cells were grown in RPMI 1640 medium, supplemented with 1.0 mM pyruvic acid; Hep3B and SK-Hep1 cells were cultured in Dulbecco's modified Eagle's medium high glucose. Nonessential amino acid solution (1.0 mM) was added only in Hep3B culture medium. All of the media were also supplemented with 2.0 mM L-glutamine, 10% (v/v) heat-inactivated fetal calf serum, and antibiotic antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B; Sigma-Aldrich). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. For the experiments, cells were seeded at 60 to 70% confluence, unless otherwise indicated, and allowed to adhere overnight. Then, cells were kept in serum-free medium at least for 6 h before treatments. Control cells were cultured in the presence of vehicle alone.

3-[4,5-Dimethylthiazolyl-2] 2,5-diphenyl-tetrazolium Bromide Cell Viability Assay. The effects on cell viability were determined by 3-[4,5-dimethylthiazolyl-2] 2,5-diphenyl-tetrazolium bromide assay (MTT; Sigma-Aldrich) as reported previously (Giuliano et al., 2009). The absorbance at 570 nm (test wavelength) and at 630 nm (reference wavelength) was measured using an enzyme-linked immunosorbent assay microplate reader (Dynex Technologies, Chantilly, VA). Cell survival was estimated as a percentage of the control value.

Hoechst Staining. The morphological apoptotic changes were analyzed by Hoechst 33258 staining. Cells seeded in 96-well plates were fixed with methanol/acetic acid (3:1) for 10 min at room temperature, washed in phosphate-buffered saline (PBS), and stained for 10 min in PBS containing 40% paraformaldehyde and 10 μg/ml Hoechst 33258. Morphological evaluations of nuclear condensation and fragmentation were performed immediately after staining by means of fluorescent microscope equipped with an automatic photomicrograph system (Leica, Wetzlar, Germany).

Flow Cytometric Analysis of Annexin V-FITC/Propidium Iodide-Stained Cells. Apoptotic cells were quantified by measuring the externalized phosphatidylserine residues by using Annexin V-FITC/propidium iodide kit (BD Biosciences, San Jose, CA) following the manufacturer's instructions. After treatment, cells were collected, washed with ice-cold PBS, and suspended in a binding buffer at a concentration of 10^6 cells/ml. Then, cells were incubated for 15 min with FITC-conjugated Annexin V and propidium iodide and analyzed by Epics XL flow cytometer using Expo32 software (both from Beckman Coulter, Fullerton, CA). Annexin V-positive/propidium iodide-negative cells (bottom right quadrant) were considered to be early apoptotic, whereas the bottom left quadrant contains the vital (double-negative) cell population.

Measurement of Mitochondrial Transmembrane Potential. Mitochondrial transmembrane potential ($\Delta\psi$m) dissipation was measured by using 3,3-dihexyloxacarbocyanine ($DiOC_6$), a lipophilic fluorochrome that exclusively emits within the spectrum of green light and accumulates in the mitochondrial matrix under the influence of $\Delta\psi$m. After treatment with the drugs, HCC cells were harvested by trypsinization, incubated with 40 nM $DiOC_6$ for 20 min at 37°C, washed with PBS, and analyzed by flow cytometry using excitation and emission setting of 488 and 525 nm, respectively. The percentage of cells showing a lower fluorescence, reflecting the loss of $\Delta\psi$m, was determined by comparison with untreated controls using Expo32 software. Carbonyl cyanide *m*-chlorophenylhydrazone (50 μM), a protonophore that completely de-energizes mitochondria by

dissipating the transmembrane potential, was used as a positive control for maximum $\Delta\psi m$ disruption (data not shown).

Western Blotting Analysis. After treatment with the compounds, protein extracts were prepared by washing the cells in PBS and incubating for 20 min in ice-cold lysis buffer supplemented with protease inhibitor cocktail, as reported previously (Giuliano et al., 2009). After sonication three times for 10 s and evaluation of protein concentration, equal amounts of protein samples (60 μg/lane) were subjected to SDS-polyacrylamide gel electrophoresis and then electrotransferred to a nitrocellulose membrane for the detection with specific antibodies. The blots were developed using the alkaline phosphatase colorimetric system. Bands were quantified by densitometric analysis with the use of Quantity One Image Software (Bio-Rad Laboratories, Hercules, CA). The correct protein loading was verified by means of both red Ponceau staining and immunoblotting for β-actin. All of the antibodies used were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA), except for anti-caspase-8, -9, and -3 (Cell Signaling Technology, Danvers, MA), anti-caspase-6 (Sigma-Aldrich), and anti-DR5 (ProSci, Poway, CA).

RT-PCR Analysis. Total RNA was isolated from cells using RNeasy Mini kit (QIAGEN, Valencia, CA) with a DNase digestion step using the RQ1 RNase free DNase (Promega). cDNA was subsequently obtained by means of the GeneAmp Kit for reverse transcriptase-polymerase chain reaction (PerkinElmer Life and Analytical Sciences, Waltham, MA) as described previously (Drago-Ferrante et al., 2008). The following sense and antisense primers, respectively, were used to amplify the human *p8* gene (5′-GAAGAGAGGCAGGGAAGACA-3′ and 5′-CTGCCGTGCGTGTCTATTTA-3′; 571-bp product), human *CHOP* gene (5′-GGCAGCTGAGTCATTGCC-3′ and 5′-GCAGATTCACCATTCGGTCA-3′; 496-bp product), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene (5′-TGACATCAAGAAGGTGGTGA-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′; 200-bp product). PCR reactions were performed using the following parameters: 95°C for 5 min, 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min, followed by a final extension step at 72°C for 5 min. The number of cycles (26–28 cycles for p8, 30 cycles for CHOP, and 22–25 cycles for GAPDH) was adjusted to allow detection in the linear range. Samples without reverse transcriptase, RNA, or Taq polymerase were used as internal controls for each RT-PCR assay. PCR products were electrophoresed in 1% agarose gel and visualized by ultraviolet transillumination.

For real-time PCR analysis, cDNA samples were amplified using IQ SYBR Green Supermix (Bio-Rad Laboratories) according to the manufacturer's instructions. The following primers were used: *DR5* gene: sense, 5′-GCACTCACTGGAATGACCTC-3′; antisense, 5′-GCCTTCTTCGCACTGACAC-3′ (annealing temperature = 55°C); and *GAPDH* gene: sense, 5′-TGACATCAAGAAGGTGGTGA-3′; antisense, 5′-TCCACCACCCTGTTGCTGTA-3′ (annealing temperature = 55°C). All reactions were performed in triplicate. For each PCR, we checked the linear range of a standard curve of serial dilutions. The relative quantification of DR5 gene expression was evaluated after normalization with the GAPDH gene as endogenous control. Data processing and statistical analysis were performed by using IQ5 Cycler software (Bio-Rad Laboratories).

Small Interfering RNAs. Two different small interfering RNAs (siRNAs) against CHOP (siCHOP) (D-004819-01-0005 and D-004819-02-0005) and scrambled siRNA (siScr), as a negative nonsilencing control, were purchased from Dharmacon RNA Technologies (Chicago, IL). p8 siRNA (sip8) [sense, 5′-GGAGGACCCAGGACAGGAUd(TT)-3′] was obtained from Eurogentec (Seraing, Belgium). Cells (2×10^5) were plated in six-well plates and cultured in antibiotic- and fetal calf serum-free RPMI 1640 medium for 24 h before transfection. Then, cells were transfected with 100 nM siCHOP, sip8, or siScr using Metafectene Pro (Biontex Laboratories GmbH, Martinsried/Planegg, Germany) (6 μl) in a final volume of 1 ml of serum-free RPMI. Six hours after transfection, the medium was replaced with fresh RPMI, and cells were treated with WIN and TRAIL alone or in combination as indicated. Data reported in Fig. 6 were obtained by using siRNA duplexes D-004819-02-0005, which results were more efficacious than D-004819-01-0005 in CHOP silencing.

Statistical Analysis and Evaluation of Synergy. Cell viability data were expressed as the mean ± S.E. and evaluated by Student's *t* test. Differences were considered significant when the *p* values were less than 0.05.

To quantify the effects of drug combination and to determine eventual synergistic actions, the median-effect method, originally described by Chou and Talalay (1984), was used. Cells were treated with different doses of WIN and TRAIL alone or in combination at fixed molar ratios for 24 h. The relative survival was assessed, and the concentrations that reduced cell viability of 25, 50, and 75% (IC_{25}, IC_{50}, and IC_{75}, respectively) were established. Then, combination indices (CIs) were calculated for different dose-effect levels based on parameters derived from median-effect plots. A CI value significantly less than 1 indicates synergy, a CI not significantly different from 1 indicates addition, and a CI significantly more than 1 indicates antagonism. Synergy is defined as a combination of two agents that has a greater therapeutic effect than would be expected by the simple addition of the individual effects of each drug.

Results

The Synthetic Cannabinoid WIN Sensitizes HCC Cells to TRAIL-Induced Apoptosis. Our previous results demonstrated that hepatoma HepG2 cells are resistant to TRAIL (Carlisi et al., 2009), whereas they are sensitive to apoptotic effects induced by the synthetic cannabinoid WIN (Giuliano et al., 2009). In the present research, we investigated whether WIN could sensitize HCC cells to TRAIL-mediated apoptosis also inducing synergistic effects. For the experiments, we used HepG2, Hep3B, and SK-Hep1 cells, three different human HCC lines. Cells were treated for various times with different doses of WIN and TRAIL used alone or in combination. As reported in Fig. 1A, after treatment with TRAIL (20 or 50 ng/ml) or WIN (2–10 μM) for 24 h, cell viability was only slightly decreased. Instead, WIN/TRAIL combined treatments resulted in a marked reduction in cell viability, which was observed under conditions of a fixed WIN concentration and varied TRAIL concentrations or vice versa. The effect was similar for the three cell lines; however, because HepG2 cells represent a widely used experimental model of human hepatoma, most of the experiments were performed with this cell line. Time-dependent experiments indicated that the combination of WIN (5 μM) and TRAIL (50 ng/ml) clearly reduced HepG2 cell viability already at 8 h of treatment (−38%) and after 24 h, cell viability diminished by approximately 70% (Fig. 1B). A similar time course was observed in Hep3B cells, whereas in SK-Hep1 cells, the cytotoxic effects appeared earlier in SK-Hep1 cells than in HepG2 and Hep3B cells (data not shown).

The above reported results suggested that the combination of the two compounds produces synergistic effects. The median effect method of Chou and Talalay (1984) was applied to evaluate the magnitude of the combined effects. At this purpose, increasing doses of WIN and/or TRAIL were used to determine the amount of the drugs that induces IC_{25}, IC_{50}, and IC_{75}, respectively. Then, the CIs were calculated for each level of cytotoxicity. In all three examined cell lines, great synergistic effects (CI < 1) were found already when low doses of WIN and TRAIL were used (Fig. 1C). Because the

combination of 5 μM WIN/20 ng/ml TRAIL significantly reduced HepG2, Hep3B, and SK-Hep1 cell viability, this combination was chosen for the subsequent experiments.

The study of cell morphology by means of light microscopy evidenced that WIN/TRAIL combined treatment for 24 h induced the appearance of the typical features of apoptosis in all three HCC cell lines; cells became rounded, detached, and floated in the medium (Fig. 1D). Hoechst 33258 fluorescence assay confirmed that the compounds did not induce appreciable modifications when used alone, whereas when used in combination, it caused chromatin condensation and fragmentation in the bulk of cells. Similar effects were observed when we evaluated the externalization of phosphatidylserine on the plasma membrane, a typical marker of early apoptosis, by means of Annexin V/propidium iodide cytofluorimetric assay. When the two drugs were added together, already at 8 h of treatment, a high percentage of cells (approximately 40%) were Annexin V-positive/propidium iodide-negative.

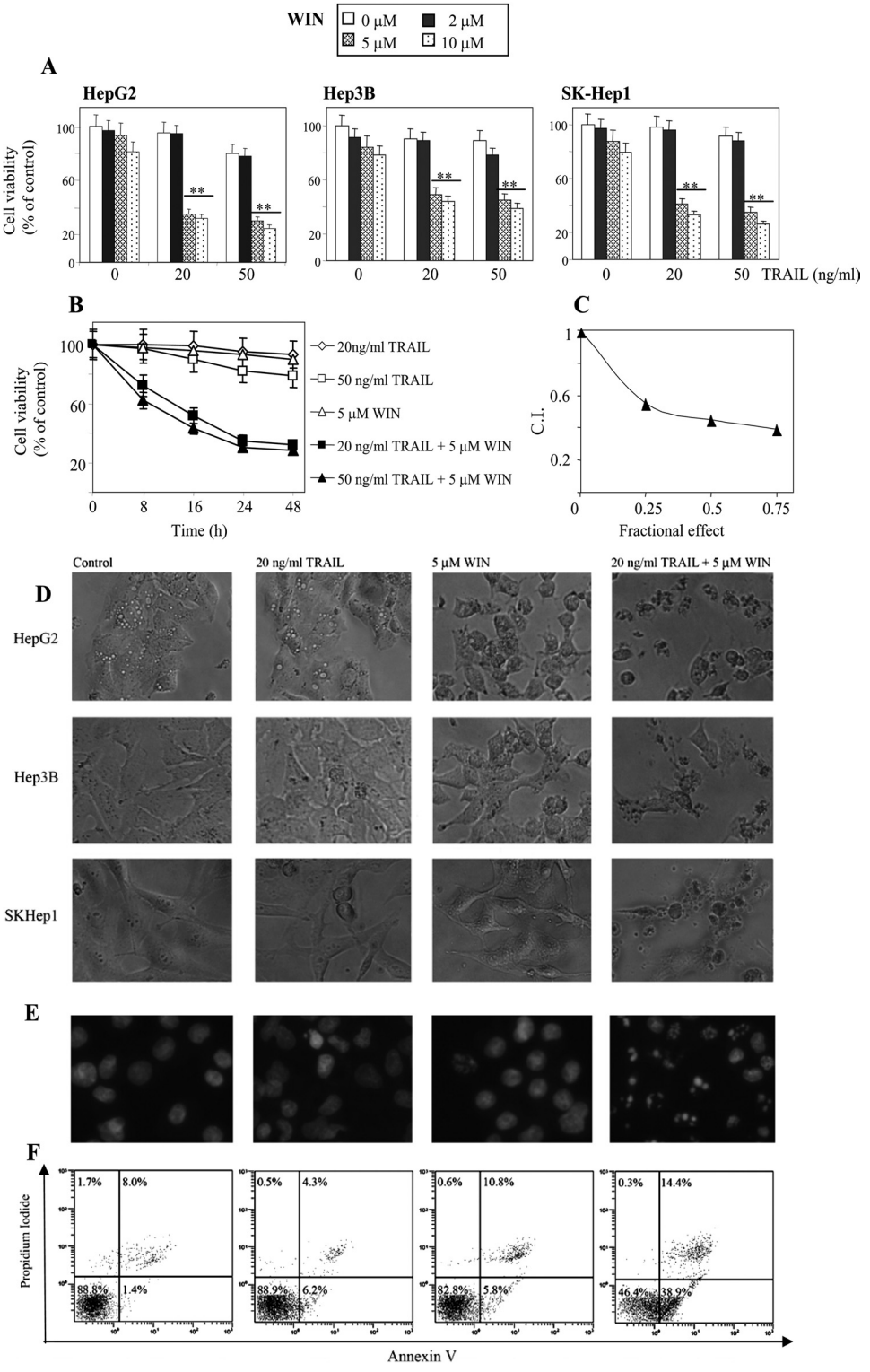


Fig. 1. WIN sensitizes HCC cells to TRAIL-induced apoptotic cell death. A, the effect of WIN and/or TRAIL on HepG2, Hep3B, and SK-Hep1 cell viability. Cells were treated with WIN and/or TRAIL for 24 h at the indicated concentrations. B, time-dependent effect of WIN and/or TRAIL on HepG2 cell viability. Cell viability was estimated by MTT assay as reported under *Materials and Methods* and expressed as the percentage of control value. Data are the means ± S.E. of four independent experiments involving triplicate assays. **, $p < 0.01$ versus control untreated cells. C, synergistic effects exerted on HepG2 cells by WIN/TRAIL combinations. HepG2 cells were treated for 24 h with the two compounds at concentrations, which changed in a fixed ratio. At the end of treatment, cytotoxic effects were measured, and data were used to calculate the CI at the different fractional effects as reported under *Materials and Methods*. D, morphological changes of HepG2, Hep3B, and SK-Hep1 cells observed under light microscopy. E, Hoechst 33258 fluorescence assay to detect typical DNA condensation in HepG2 cells. Cells were treated for 24 h with the drugs used at the indicated concentrations. F, flow cytometric analysis of Annexin V/propidium iodide-labeled HepG2 cells treated for 8 h, as indicated. Then, Annexin V-FITC and propidium iodide were added as described in the manufacturer's instructions. A flow cytometric analysis was performed to quantify the percentage of live and early apoptotic cells. In D, E, and F, representative data from at least three different experiments with similar results are shown.

Figure 1, E and F, shows the results obtained in HepG2 cells as representative of the effects induced by the drugs in the three cell lines.

Cotreatment of HepG2 Cells with WIN and TRAIL Causes Dissipation of the Mitochondrial Transmembrane Potential and Activation of Caspases. Next, we analyzed whether apoptosis induced by the WIN/TRAIL combination was associated with mitochondrial depolarization and activation of caspases. By using $DiOC_6$, a mitochondria-specific and voltage-dependent dye, we evaluated the loss of $\Delta\psi m$. The results indicated that treatment of HepG2 cells for 24 h with WIN or TRAIL alone induced only a slight dissipation of $\Delta\psi m$, whereas when the cells were treated with the combination of the two drugs, approximately 45% of cells resulted as depolarized (Fig. 2A).

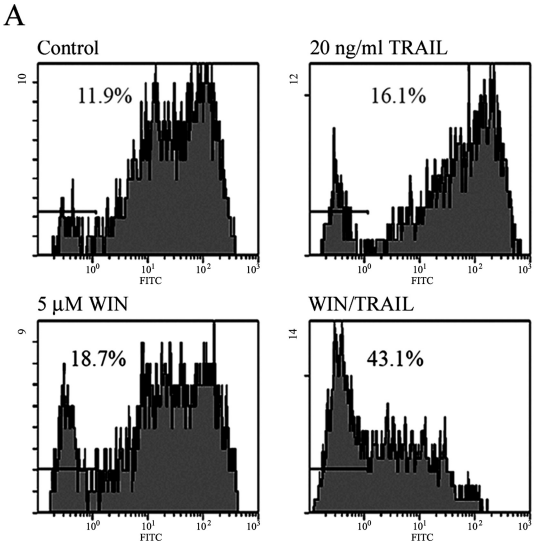
Western blotting analysis showed that treatment of HepG2 cells for 24 h with WIN/TRAIL combination led to the activation of caspase-8 and caspase-9, resulting in a marked decrease in the respective procaspase forms and a concomitant appearance of cleavage products. WIN/TRAIL treatment also induced the activation of executioner caspase-6 and caspase-3, as demonstrated by the reduction of the bands related to the inactive forms (Fig. 2B). No significant effect on the levels of the examined caspases was observed when the cells were treated with the two compounds used separately.

Treatment with WIN/TRAIL Combination Induces the Up-Regulation of PPARγ and Down-Regulation of Some Survival Factors. It has been reported that PPARγ, a member of the nuclear receptor family, which has been shown to possess antineoplastic activity in many cancer cells (Krishnan et al., 2007), can mediate antiproliferative action of cannabinoids (O'Sullivan, 2007), including WIN, as demonstrated previously by us in HepG2 cells (Giuliano et al., 2009). As shown in Fig. 3A, 5 μM WIN significantly enhanced the level of PPARγ in HepG2 cells. The effect, which was already evident at 8 h of treatment, reached the maximum at 24 h. Treatment with 20 ng/ml TRAIL did not modify the level of the transcription factor when used alone, whatever the incubation time, whereas when TRAIL was added to WIN, we observed at 8 h of treatment a further increase in PPARγ level. Instead, at 24 h, combined treatment induced a decrease in the band corresponding to PPARγ and the appearance of another band at lower molecular weight, presumably a degradation form of the protein. Because z-VAD-fmk, the pan inhibitor of caspases, counteracted the appearance of this band, we suppose that PPARγ degradation was a caspase-dependent event occurring in the late phase of apoptosis (Fig. 3A). In addition, in Hep3B and SK-Hep1 cells, WIN also induced an increase in PPARγ level, although the effect was less evident and appeared differently in time.

It is well known that in many cancer cells, apoptosis can be inhibited by the expression of high levels of survival factors, including IAP and Bcl-2 family members, and the down-regulation of these proteins is a key event to trigger cell death (LaCasse et al., 2008; Kang and Reynolds, 2009). Thus, we evaluated the effect of WIN and TRAIL, used alone or in combination, on the level of survivin, c-IAP2, XIAP, and Bcl-2. WIN caused a clear reduction in the level of survivin in all three cell lines, whereas c-IAP2 was clearly reduced in HepG2 and SK-Hep1 cells but not in Hep3B, and the level of XIAP and Bcl-2 decreased only in Hep3B and HepG2 cells, respectively. The effects appeared after 8 h of treatment in concomitance with PPARγ increase, reaching the maximum at 24 h (Fig. 3B). The levels of these survival factors were not modified in the presence of TRAIL alone, whereas the intensity of the corresponding bands further decreased after treatment with WIN/TRAIL combination.

AKT is another important survival factor, vital to the growth and survival of cancer cells. For this reason, drugs that down-regulated the AKT pathway can be promising for cancer therapy (Engelman, 2009). Here, we demonstrate that in HepG2 and Hep3B cells, the level of the phosphorylated active form of AKT significantly decreased after treatment with WIN for 24 h. The addition of TRAIL potentiated the effect of WIN, and in this condition, the band corresponding to phospho-AKT almost disappeared. In SK-Hep1 cells, the effect was observed only in cotreated cells (Fig. 3B).

We demonstrated previously in WIN-treated cells the relationship between the increase in the level of PPARγ and the down-regulation of survival factors by using GW9662, an irreversible antagonist of PPARγ (Giuliano et al., 2009). Here, we observed that the same antagonist, administered at a dose that did not exert any cytotoxicity, also counteracted



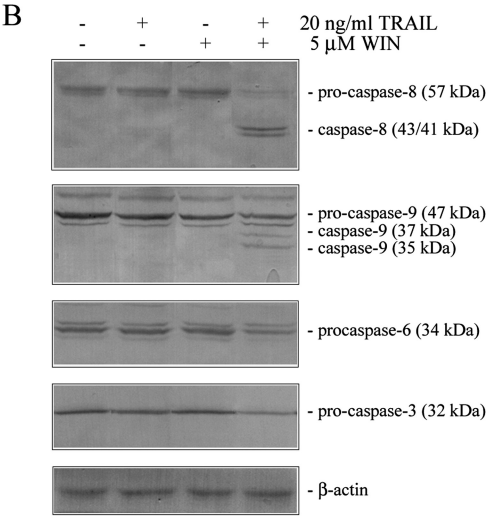


Fig. 2. WIN/TRAIL combined treatment induces mitochondrial transmembrane potential dissipation and activation of caspases. A, evaluation of ΔΨm dissipation in HepG2 cells treated for 24 h with 5 μM WIN and 20 ng/ml TRAIL used alone or in combination. ΔΨm was quantified by flow cytometry as reported under *Materials and Methods*. B, activation of caspase activities in HepG2 cells treated with WIN and/or TRAIL for 24 h. Cell lysates were analyzed by Western blotting using specific antibodies against the different caspase activities as reported under *Materials and Methods*. Actin blots were included to show equal protein loading for all of the samples. The results are representative of four independent experiments with similar results.

the effects on survival factors levels in HepG2 and Hep3B cells. It is noteworthy that GW9662 was ineffective in SK-Hep1 cells, suggesting that in these cells the down-regulation of these proteins is a PPARγ-independent event (Fig. 3B, lane 5). It is interesting that GW9662 also attenuated by approximately 30% the cytotoxic effect induced by WIN/TRAIL combination on HepG2 and Hep3B cells, whereas it did not modify SK-Hep1 cell viability. Figure 3C reports the effect induced by GW9662 in HepG2 cells.

WIN Induces DR5 Up-Regulation Sensitizing HCC Cells to TRAIL Action. It is well known that TRAIL resistance is often associated with the loss of the specific DR4 and DR5 death receptors or the up-regulation of the decoy DcR1 and DcR2 receptors (Sheridan et al., 1997). Because the expression of these proteins can be modulated by a number of compounds, we were interested in determining their levels in 5 μM WIN-treated HCC cells. Figure 4A shows that at 24 h of treatment, WIN induced an increase in the levels of DR4 and, in particular, in that of DR5, whereas the levels of DcR1 and DcR2 were only slightly decreased after treatment (data not shown).

Our experiments demonstrated that in HepG2, Hep3B, and SK-Hep1 cells, WIN-induced DR5 increase was progressive with time; the effect was clearly evident at 8 h of treatment, reaching the maximum at 16 and 24 h (Fig. 4B). DR5 increase was a consequence of the transcriptional activation induced by WIN treatment. In fact, real-time quantitative RT-PCR for DR5 mRNA expression, performed in HCC cells, showed that 5 μM WIN increased the level of DR5 transcript, reaching approximately 2-fold the control value already at 8 h of treatment (Fig. 4C). The effects on both DR5 mRNA and protein levels were specifically induced by the cannabinoid; in fact, the addition of TRAIL did not modify WIN effects (Fig. 4, C and D, top).

It has been documented that in cancer cells, the down-regulation of c-FLIP, an antiapoptotic factor related to death receptors, can contribute to the enhancement of TRAIL-induced apoptosis (Park et al., 2009). Western blotting analysis showed that WIN

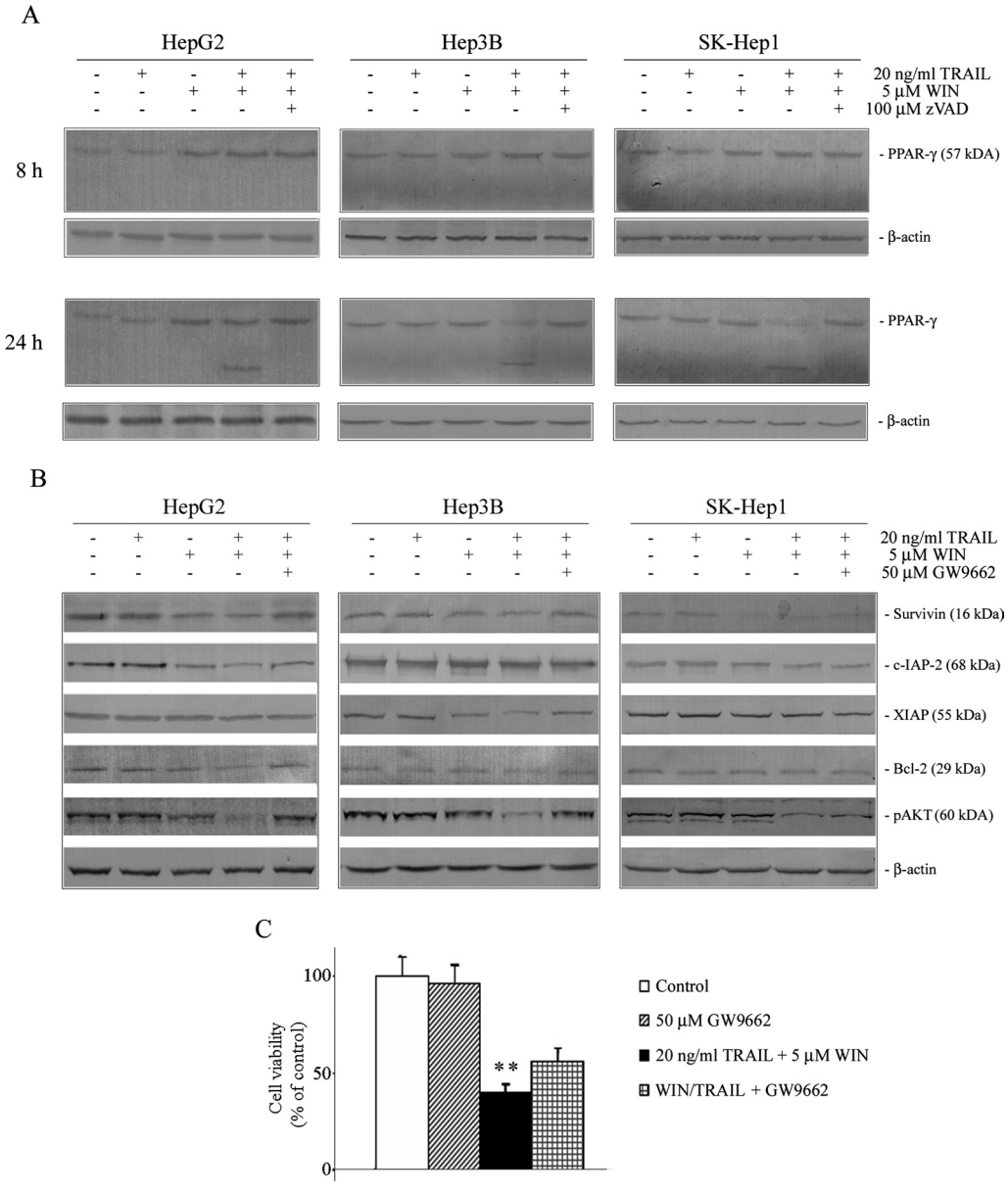


Fig. 3. WIN-sensitized TRAIL-mediated apoptosis is accompanied by up-regulation of PPARγ and down-regulation of survival factors. A, effects induced by WIN or TRAIL, used alone or in combination, on PPARγ level in HCC cells treated for 8 and 24 h in the presence of absence of z-VAD-fmk. B, effects induced by the compounds on the level of the survival factors survivin, c-IAP2, XIAP, Bcl-2, and phospho-AKT. HCC cells were treated for 24 h with the two compounds used alone or in combination in the presence or absence of the PPARγ inhibitor GW9662 as indicated. Cell lysates were prepared as reported under *Materials and Methods*, resolved by SDS-polyacrylamide gel electrophoresis and Western blotting. Actin blots were included to show equal protein loading for all of the samples. The results are representative of four independent experiments with similar results. C, effect induced by 50 μM GW9662 on viability of HepG2 cells treated for 24 h with WIN/TRAIL combination. Cell viability was estimated by MTT assay, as reported under *Materials and Methods*, and expressed as the percentage of control cells. Data were the means ± S.E. of four independent experiments involving triplicate assays. **, $p < 0.01$ versus control untreated cells.

induced a marked down-regulation of both the long and short isoforms of the protein. In addition, in this case, the effect obtained in WIN-treated cells was not modified by TRAIL (Fig. 4D, bottom). The addition of GW9662 partially counteracted WIN effect on c-FLIP level, indicating that this event is almost in part dependent on PPARγ activation.

WIN-Induced DR5 Up-Regulation Is a CHOP-Dependent Event. Next, we were interested in investigating the underlying mechanism by which WIN induces DR5 up-regulation. It has been demonstrated that in hepatic stellate cells, DR5 up-regulation is regulated by PPARγ (Wang et al., 2009). The addition of PPARγ inhibitor GW9662 to WIN/TRAIL-treated cells failed to counteract WIN-induced increase in both mRNA and protein DR5 levels (Fig. 4, C and D), indicating that, in our experimental model, DR5 increase is a PPARγ-independent event.

Another upstream activator of DR5 in certain types of cancer is CHOP (also known as GADD153), a transcription factor of CCAAT/enhancer binding protein homologous protein family, which is strictly correlated with endoplasmic reticulum stress and participates in endoplasmic reticulum-mediated apoptosis (Chen et al., 2007). Results reported in Fig. 5A show that in HCC cells, CHOP level was significantly increased after WIN treatment in a time-dependent manner. The event was clearly evident at 8 h in accordance with the cannabinoid-induced DR5 increase. CHOP up-regulation occurred at the transcription level, as demonstrated by semiquantitative RT-PCR analysis (Fig. 5B). The effects on CHOP

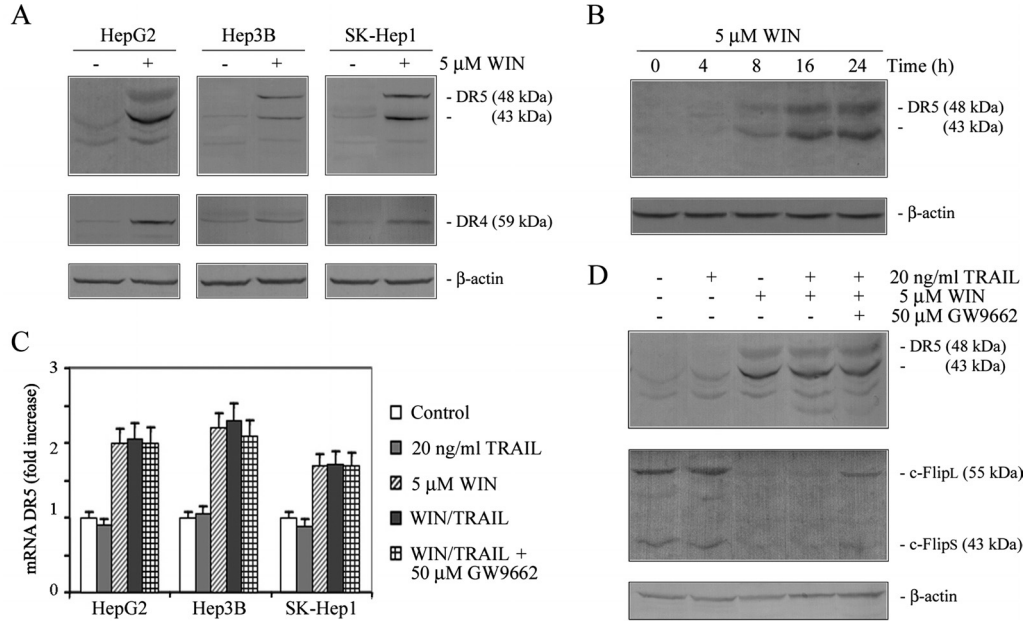


Fig. 4. WIN induces up-regulation of both mRNA and protein expression levels of DR5 death receptor. A, evaluation of DR5 and DR4 TRAIL receptors in HCC cells treated with WIN for 24 h. B, time-dependent effect on the level of DR5 induced by WIN treatment in HepG2 cells. C, real-time RT-PCR to evaluate the effect of the compounds on the level of DR5 transcript in HCC cells treated for 8 h in the presence or absence of 50 μM GW9662. Real-time RT-PCR was performed as reported under *Materials and Methods*. D, effects of WIN/TRAIL combined treatment on the level of DR5 and c-FLIP measured in HepG2 cells incubated for 24 h with the compounds in the presence or absence of 50 μM GW9662. Western blotting analysis was performed as reported under *Materials and Methods*. Actin blots were included to show equal protein loading for all of the samples. The results are representative of four independent experiments with similar results.

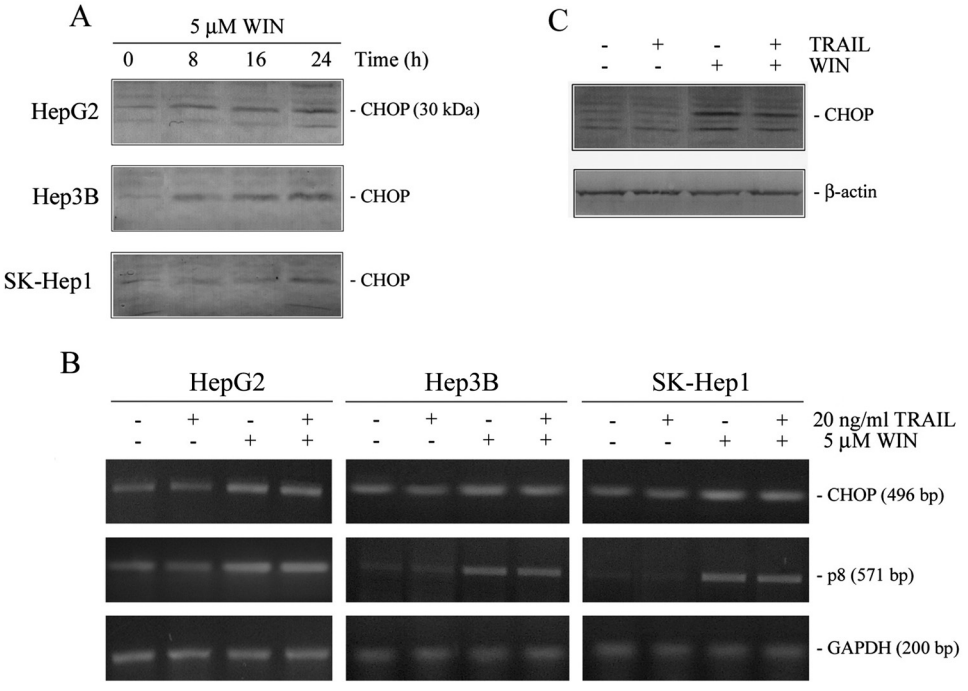


Fig. 5. The apoptotic effects of WIN/TRAIL combination are dependent on up-regulation of CHOP and p8. A, time-dependent increase in the level of CHOP induced by 5 μM WIN in HCC cells. B, CHOP and p8 are transcriptionally induced by WIN treatment. HCC cells were treated for 8 h with WIN and/or TRAIL at the indicated concentrations. The expression of CHOP and p8 transcripts was determined by semiquantitative RT-PCR. GAPDH mRNA levels were evaluated as an internal control. Results are representative of at least three separate experiments. C, effects induced by WIN and/or TRAIL in HepG2 cells after 24 h of treatment. CHOP expression level was analyzed by Western blotting analysis as reported under *Materials and Methods*. Actin blots were included to show equal protein loading for all the samples.

expression were induced by the cannabinoid alone; in fact, also in this case, the addition of TRAIL did not modify WIN effects on CHOP mRNA and protein levels (Fig. 5, B and C). Because it has been described that up-regulation of CHOP and DR5 can be dependent on ROS generation (Lee et al., 2009), we examined the effects induced by the addition of *N*-acetylcysteine or glutathione on both the viability of WIN/TRAIL-treated cells and the levels of DR5 and CHOP. The results seemed to exclude the involvement of oxidative stress; in fact, the examined parameters were unmodified after the addition of the antioxidants (data not shown).

There has been identified recently a correlation between CHOP and p8 (also designated as candidate of metastasis-1 or nuclear protein-1), a stress-regulated protein that is implicated in a number of functions, including the induction of apoptosis in tumor cells (Carracedo et al., 2006; Chowdhury et al., 2009). Because p8 is an essential mediator of cannabinoid antitumor action in gliomas (Carracedo et al., 2006), we tested the involvement of this factor in the antiproliferative effect of WIN in HCC cells. Semiquantitative RT-PCR demonstrated that p8 mRNA levels increased after 8 h of WIN treatment in all three cell lines. p8 up-regulation seemed to be more evident in Hep3B and SK-Hep1 cells because in these cells, the basal level of p8 mRNA was almost undetectable. The increase was also observed in WIN/TRAIL-treated cells, whereas it was not observed in the presence of TRAIL alone (Fig. 5B).

To clarify whether p8 and CHOP proteins are critical for WIN-mediated DR5 up-regulation, we down-regulated the expression of these factors by siCHOP or sip8 mRNAs and examined the effect on DR5 activation and cell death in response to WIN and TRAIL, used alone or in combination. In initial experiments, CHOP expression was evaluated in siCHOP-transfected WIN-treated HepG2 cells. Figure 6A shows that the transfection with siCHOP reduced WIN-dependent CHOP up-regulation compared with treated cells transfected with siScr. Likewise, siCHOP also clearly abrogated WIN-induced DR5 increase, both at mRNA and protein levels, confirming that CHOP up-regulation is required for this event (Fig. 6, A and B).

To evaluate the existence of a p8/CHOP/DR5 axis, we also studied the effect of WIN after silencing of p8 expression. After confirming the reduction in the level of p8 transcript in the presence of the specific siRNA (Fig. 6C), we studied the levels of CHOP and DR5 in sip8-transfected cells after WIN treatment. As shown in Fig. 6D, the levels of both of these factors were significantly decreased in WIN-treated transfected cells. It is important to note that we found that in siCHOP- or sip8-transfected HepG2 cells, WIN/TRAIL combined treatment exerted a more minor cytotoxic effect than that observed in cells transfected with scrambled siRNA (Fig. 6E).

Discussion

The aim of this study was to investigate the effect of cannabinoids in modulating TRAIL sensitivity and in activating apoptosis in TRAIL-resistant HCC cells. Data presented in this article demonstrate for the first time a strong synergistic interaction between WIN, a synthetic ligand of cannabinoid receptors, and TRAIL in HCC cell lines. Treatment with a combination of subtoxic doses of the two drugs effectively reduced the viability of HepG2, Hep3B, and SK-Hep1 cells, three HCC cell lines characterized by a different origin and tumorigenic degree. Cell death observed after treatment with WIN/TRAIL combination was associated with the activation of an apoptotic pathway, which involved the dissipation of transmembrane mitochondrial potential and the activation of caspase activities.

Our article clearly indicates that many events induced by WIN can be responsible for sensitization of HCC cells to TRAIL-induced apoptosis. The results provided evidence that

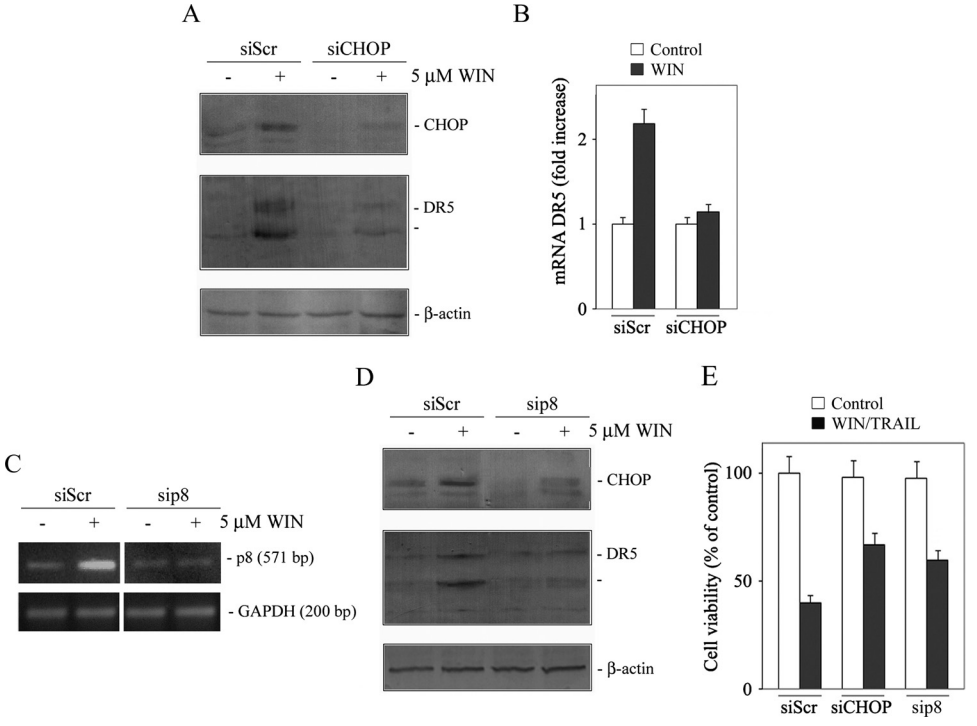


Fig. 6. RNA interfering against CHOP or p8 affected DR5 expression and HepG2 cell viability. A, Western blotting analysis of CHOP and DR5 expression levels and real-time RT-PCR (B) for DR5 mRNA in siCHOP transfected cells. C, semiquantitative RT-PCR of p8 transcripts and Western blotting analysis (D) of the levels of CHOP and DR5 in sip8-transfected cells. HepG2 cells were transfected for 6 h with siCHOP, sip8, or siScr. At the end, cells were treated with 5 μM WIN for another 8 h for evaluation of p8 mRNA or 16 h for CHOP and DR5 protein expression levels. The results are representative of three independent experiments with similar results. E, knockdown of CHOP or p8 expression attenuated cytotoxic effect of WIN/TRAIL combined treatment. siCHOP- or sip8-transfected HepG2 cells were treated with 5 μM WIN/20 ng/ml TRAIL for 24 h. Cell viability was estimated by MTT assay as reported under *Materials and Methods* and expressed as the percentage of control value. Data were the means ± S.E. of three independent experiments involving triplicate assays.

treatment with WIN potently and rapidly up-regulated TRAIL receptor DR5 at both mRNA and protein expression levels, an event that is involved in the sensitization to TRAIL-induced apoptosis by different compounds. DR5 up-regulation was accompanied by the activation of caspase-8 and decrease in c-FLIP level, an antiapoptotic protein that operates as an endogenous antagonist of caspase-8.

To ascertain the molecular mechanism through which WIN enhanced DR5 expression, at first we hypothesized an involvement of PPARγ. This hypothesis was suggested by the observation that PPARγ, a factor that is precociously increased by WIN treatment, has been shown to enhance DR5 expression in hepatic stellate cells (Wang et al., 2009). However, in line with other authors (Zou et al., 2007; Kim et al., 2008a), data we obtained by using the specific PPARγ inhibitor GW9662 demonstrated that in HCC cells, the increase in DR5 level was a PPARγ-independent event.

CHOP is a key transcription factor involved in apoptosis induced by many events, such as reticulum stress, DNA damage, nutrient starvation, or anticancer drugs (Oyadomari and Mori, 2004). A relationship has been demonstrated between the up-regulation of CHOP and the increase of DR5. Such an event seemed to be responsible for the reactivation of TRAIL signaling in tumor cells (Yoshida et al., 2005). Moreover, Carracedo et al. (2006) showed that in glioma cells, as well as in many other tumor cell lines, CHOP up-regulation can be also mediated by tetra-hydrocannabinol. In line with these observations, our data indicate that WIN treatment markedly augmented mRNA and protein levels of CHOP already after 8 h of treatment in concomitance with the increase in the level of DR5. This effect was also observed in WIN/TRAIL cotreated cells. The role of CHOP/DR5 axis in apoptosis induced by combined treatment was also suggested by the observation that knockdown of CHOP induced by siRNA significantly down-regulated DR5 level and concomitantly reduced cell death induced by WIN/TRAIL. Therefore, we conclude that CHOP is a key player in this mechanism.

CHOP up-regulation was often related to ROS increase, as demonstrated in human renal cancer cells treated with withaferin A (Lee et al., 2009). However, in other cases, as shown in glioma cells treated with arsenic trioxide (Kim et al., 2008b), CHOP up-regulation was a ROS-independent event. In accordance with these studies, our results seemed to exclude the involvement of oxidative stress as CHOP inducer in HCC cells treated with WIN, because the addition of *N*-acetylcysteine and reduced glutathione, which are two powerful antioxidants, did not modify the effects of WIN on apoptosis and on the levels of both CHOP and DR5.

To individuate the mechanism responsible for the enhancement in the expression of CHOP, we evaluated the possible involvement of p8, an endoplasmic reticulum stress-regulated protein, which has been recently demonstrated to mediate cannabinoid action in tumor cells (Carracedo et al., 2006). Experimental evidence reported in the present study make plausible the hypothesis that p8 increase can be responsible for CHOP up-regulation. In fact, p8 transcript was markedly increased after treatment of HCC cells for 8 h with WIN, and after p8 silencing, the WIN-dependent up-regulation of both CHOP and DR5 was significantly reduced. However, at present, we do not know the mechanism by which WIN triggers p8 activity; it is possible that ceramide is also involved in these cells, as demonstrated in glioma cells treated with tetra-hydrocannabinol (Carracedo et al., 2006). Studies are in progress to evaluate the level of de novo synthesized ceramide in HCC cells treated with WIN.

Another mechanism by which WIN can sensitize HCC cells to WIN/TRAIL-mediated apoptosis seems to be related to the decrease in the level of survival factors. In many experimental models, the down-regulation of these proteins represents a way to address apoptosis by anticancer drugs. Here, we demonstrate that in HCC cells, WIN induced a clear reduction in the level of phosphorylated active form of AKT, which can be considered a crucial factor for the growth and survival of human cancer cells. In addition, some members of the IAP family and Bcl-2 were down-regulated after WIN treatment. These events seem to be mediated by PPARγ, whose levels were early up-regulated by WIN treatment. In HepG2 and Hep3B cells, the use of the specific PPARγ antagonist (GW9662) counteracted the decrease in survival factors and in concomitance partially counteracted the WIN/TRAIL effect on cell viability, indicating that the down-regulation of survival factors in these cells is a PPARγ-dependent event. The addition of TRAIL slightly potentiated the effects of WIN on PPARγ and survival factor levels at 8 h of treatment. This event could be related to β-catenin, whose levels are very high in hepatoma cells. In fact, it has been demonstrated recently that this factor is a negative regulator of PPARγ gene expression (Almeida et al., 2009) and that, in resistant cancer cells, TRAIL- and troglitazone-induced apoptosis is preceded by a caspase-dependent cleavage of β-catenin (Senthivinayagam et al., 2009). Thus, it is plausible to hypothesize that also in hepatoma cells, the further increase in PPARγ level could be dependent on the cleavage of this protein induced by WIN/TRAIL combined treatment.

Although treatment with WIN/TRAIL combination was effective in all three hepatoma cell lines, the comparative study showed some differences. In particular, in SK-Hep1 cells the expression of the survival factors was differently influenced by treatment and seemed to be independent of WIN-induced PPARγ up-regulation. We have undertaken a research to investigate the causes of the observed differences.

In conclusion, data reported in this article indicate that in apoptosis induced by WIN/TRAIL combination, two different mechanisms are involved: on the one hand, the WIN-mediated early activation of p8 and CHOP causes up-regulation of TRAIL receptor DR5, thus sensitizing resistant HCC cells to TRAIL; on the other hand, WIN treatment through enhancement of

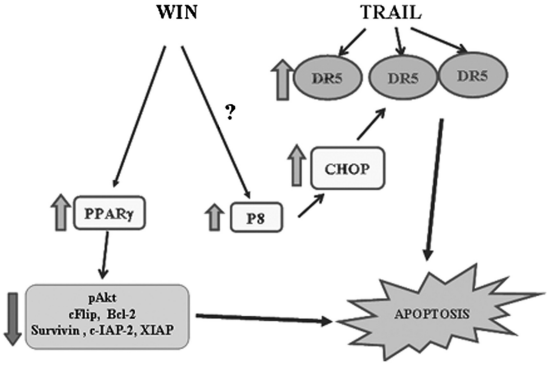


Fig. 7. Schematic representation of the proposed mechanism of WIN/TRAIL-induced apoptosis on HCC cells.

PPARγ leads to the down-regulation of survival factors further contributing to cell death (Fig. 7). Although other studies will be required to support an eventual clinical application of WIN/TRAIL cotreatment, this novel drug combination may represent, in our opinion, a promising anticancer strategy.

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