Cannabinoids Induce Apoptosis of Pancreatic Tumor Cells via Endoplasmic Reticulum Stress–Related Genes

Arkaitz Carracedo,[1] Meritxell Gironella,[2] Mar Lorente,[1] Stephane Garcia,[2] Manuel Guzmán,[1] Guillermo Velasco,[1] and Juan L. Iovanna[2]

[1]Department of Biochemistry and Molecular Biology I, School of Biology, Complutense University, Madrid, Spain and [2]U624 Institut National de la Sante et de la Recherche Medicale, Marseille, France

Abstract

Pancreatic adenocarcinomas are among the most malignant forms of cancer and, therefore, it is of especial interest to set new strategies aimed at improving the prognostic of this deadly disease. The present study was undertaken to investigate the action of cannabinoids, a new family of potential antitumoral agents, in pancreatic cancer. We show that cannabinoid receptors are expressed in human pancreatic tumor cell lines and tumor biopsies at much higher levels than in normal pancreatic tissue. Studies conducted with MiaPaCa2 and Panc1 cell lines showed that cannabinoid administration (*a*) induced apoptosis, (*b*) increased ceramide levels, and (*c*) up-regulated mRNA levels of the stress protein p8. These effects were prevented by blockade of the CB_2 cannabinoid receptor or by pharmacologic inhibition of ceramide synthesis *de novo*. Knockdown experiments using selective small interfering RNAs showed the involvement of p8 via its downstream endoplasmic reticulum stress–related targets activating transcription factor 4 (ATF-4) and TRB3 in Δ^9-tetrahydrocannabinol–induced apoptosis. Cannabinoids also reduced the growth of tumor cells in two animal models of pancreatic cancer. In addition, cannabinoid treatment inhibited the spreading of pancreatic tumor cells. Moreover, cannabinoid administration selectively increased apoptosis and TRB3 expression in pancreatic tumor cells but not in normal tissue. In conclusion, results presented here show that cannabinoids lead to apoptosis of pancreatic tumor cells via a CB_2 receptor and *de novo* synthesized ceramide-dependent up-regulation of p8 and the endoplasmic reticulum stress–related genes *ATF-4* and *TRB3*. These findings may contribute to set the basis for a new therapeutic approach for the treatment of pancreatic cancer. (Cancer Res 2006; 66(13): 6748-55)

Introduction

Pancreatic cancer is one of the most malignant and aggressive forms of cancer (1). With an incidence of 10/10,000 for men and 7/10,000 for women, it represents the fourth most common death-causing cancer in the United States (2) and the fifth in the Western world overall (3). About 95% of pancreatic cancers cases are ductal adenocarcinomas. The anatomic localization of the pancreas and the nonspecific nature of the symptoms result in a complex and delayed diagnosis. Therefore, at the time of detection, 85% of patients show metastasic infiltrations in proximal lymphatic nodes, liver, or lungs, and only 15% to 20% of the tumors are typically found resectable (1). In addition, <20% of the operated patients survive up to 5 years. Treatment of unresectable tumors is currently based on administration of fluorouracil chemoradiation for locally advanced tumors and gemcitabine chemotherapy for metastatic disease (1). However, despite maximal optimization of these therapies, the median survival for the affected patients remains ~1 year. It is therefore of especial interest to set new therapeutic strategies aimed at improving the prognostic of this deadly disease.

The hemp plant *Cannabis sativa* produces ~70 unique compounds known as cannabinoids, of which Δ^9-tetrahydrocannabinol (THC) is the most important owing to its high potency and abundance in cannabis (4). THC exerts a wide variety of biological effects by mimicking endogenous substances, the endocannabinoids anandamide (5) and 2-arachidonoylglycerol (6), which bind to and activate specific cannabinoid receptors. Thus far, two cannabinoid-specific $G_{i/o}$ protein-coupled receptors have been cloned and characterized from mammalian tissues (7): The CB_1 receptor is particularly abundant in discrete areas of the brain but is also expressed in peripheral nerve terminals and various extraneural sites. In contrast, the CB_2 receptor was initially described to be present in the immune system (8) although, recently, it has been shown that expression of this receptor also occurs in cells from other origins (9–11).

One of the most exciting areas of research in the cannabinoid field is the study of the potential application of cannabinoids as antitumoral agents (12). Thus, cannabinoid administration has been shown to curb the growth of several models of tumor xenografts in rats and mice (12). This antitumoral action of cannabinoids relies, at least in part, on the ability of these compounds to directly affect the viability, via induction of apoptosis or cell cycle arrest, of a wide spectrum of tumor cells in culture (12). In addition, cannabinoid treatment inhibits tumor angiogenesis (13–15). Both CB_1 (9, 11, 16, 17) and CB_2

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M. Gironella and M. Lorente, as well as J.L. Iovanna and G. Velasco, contributed equally to this work. A. Carracedo did experiments of cell viability, Western blot, RNA isolation from cultured cells, real-time quantitative PCR, transfections with siRNA, quantification of ceramide levels, and experiments with s.c. and intrapancreatic tumor xenografts and contributed to experiment design, data analysis, and discussion. M. Gironella participated in the experiments with tumor xenografts, immunofluorescence, and RNA isolation from human tumor samples and participated in data analysis and discussion. M. Lorente did RNA isolation from tumor samples, real-time quantitative PCR analysis, transfections with siRNAs, TUNEL staining of tumor samples, and immunofluorescence experiments and participated in data analysis and discussion. S. Garcia processed tumor samples. M. Guzmán participated in experimental design, data analysis, and discussion, as well as critical reading of the manuscript. G. Velasco coordinated the general experimental design, data analysis, and discussion and wrote the manuscript. J.L. Iovanna participated in general experimental design, data analysis, and discussion, as well as critical reading of the manuscript.

Requests for reprints: Guillermo Velasco, Department of Biochemistry and Molecular Biology I, School of Biology, Complutense University, c/ José Antonio Novais s/n, 28040 Madrid, Spain. Phone: 34-91-394-4668; Fax: 34-91-394-4672; E-mail: gvd@bbm1.ucm.es.

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receptors (11, 18, 19) have been shown to mediate the growth-inhibiting action of THC and related cannabinoids on tumor cells (12). The present study was therefore undertaken to investigate (*a*) the antitumoral action of cannabinoids in pancreatic cancer and (*b*) the molecular mechanisms involved in that effect.

Materials and Methods

Reagents. THC was a kind gift from Dr. Javier Fernández-Ruiz (Complutense University, Madrid, Spain) and SR141716 and SR144528 were a kind gift from Sanofi Synthelabo (Montpellier, France). WIN 55,212-2 was from Sigma Chemical Co. (St. Louis, MO), myriocin (ISP-1) was from Biomol (Plymouth Meeting, PA), and C_2-ceramide was from Alexis (San Diego, CA).

Cell culture and viability. MiaPaCa2 and Panc1 were cultured in DMEM containing 10% heat-inactivated fetal bovine serum (FBS), 5 units/mL penicillin, and 5 mg/mL streptomycin. Capan2 and BxPc3 were cultured in RPMI 1640 containing 10% heat-inactivated FBS, 2 mmol/L glutamine, 5 units/mL penicillin, and 5 mg/mL streptomycin. Cells were transferred to a serum-free medium (except Capan2 and BxPc3, which were transferred to a medium containing 0.1% serum) 18 hours before the different treatments. THC, WIN 55,212-2, SR141716, and SR144528 stock solutions were prepared in DMSO. Control incubations contained the same amount of DMSO. No significant effect of DMSO was observed in any of the parameters determined throughout this study at the final concentration used (0.1-0.2%, v/v). Cell viability was determined using the CellTiter96 Aqueous One Solution Reagent (MTS, Promega, Madison, WI) according to the instructions of the manufacturer.

Transfections. Seventy-five percent confluent MiaPaCa2 cells were transfected with the different small interfering RNAs (siRNA) using the X-tremeGENE siRNA Transfection Reagent (Roche, Basel, Switzerland) according to the instructions of the manufacturer. Twenty-four hours after transfection, cells were trypsinized and seeded at a density of 5,000/cm^2. Cells were transferred to a serum-free medium 18 hours before the different treatments. Transfection efficiency was monitored using a control fluorescent siRNA (Qiagen, Hilden, Germany).

RNA interference. Double-stranded RNA duplexes corresponding to human p8 (5′-GGAGGACCCAGGACAGGAU), human activating transcription factor 4 (ATF-4; 5′-GCCUAGGUCUCUUAGAUGA), human TRB3 (5′-UCAUCUAAGAGAACCUAGGC), and a nontargeted control (5′-UUCUCCGAACGUGUCACGU) were purchased from Eurogentec (Liege, Belgium).

Caspase 3/7 activity. Caspase 3/7 (DVEDase activity) was determined according to the instructions of the manufacturer using a luminogenic substrate (Caspase Glo, Promega). Luminiscence was determined in a Microplate Fluorescence Reader FLUOstar Optima (BMG Labtech, Offernburg, Germany). One unit of caspase activity is defined as the amount of active enzyme necessary to produce an increase in the lumiscence of 1 arbitrary unit after 1 hour.

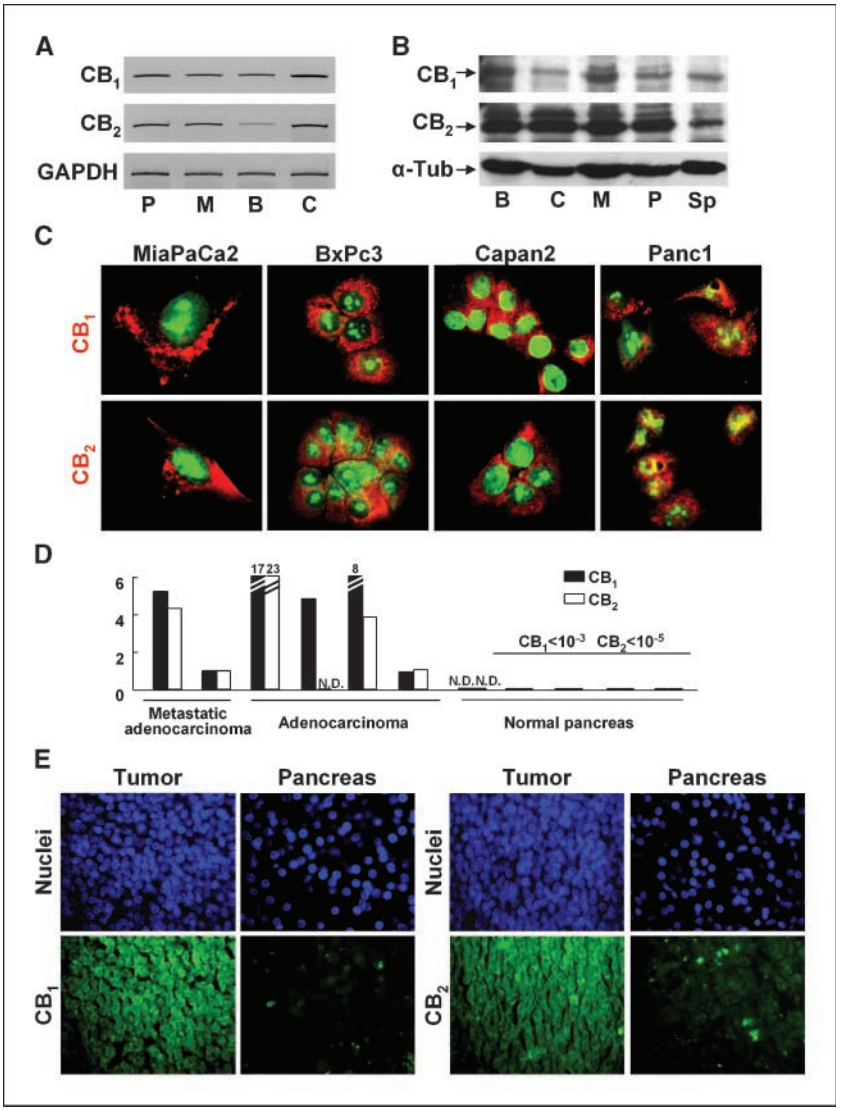

Figure 1. Pancreatic tumor cells express cannabinoid receptors. *A,* total RNA was isolated from the corresponding cell lines and reverse transcription-PCR (RT-PCR) using selective primers for human CB_1, CB_2, or GAPDH was done. Representative of two experiments. *P,* Panc1; *M,* MiaPaCa2; *B,* BxPc3; *C,* Capan2. *B,* cell lysates were obtained from the corresponding cell lines and CB_1, CB_2, and α-tubulin protein levels were determined by Western blot. Spleen (*Sp*) was used as a positive control for CB_1 and CB_2 expression. Representative of two experiments. *C,* representative (63×) images of CB receptor–stained pancreatic tumor cells. Cannabinoid receptors are stained in red; nuclei are stained in green. *D,* total RNA was isolated from pancreatic biopsies diagnosed as metastasic adenocarcinoma, adenocarcinoma, and normal pancreas, and CB_1 and CB_2 mRNA levels were determined by real-time quantitative PCR. The plot represents gene expression (in fold number) of each individual sample relative to one of the tumor samples. mRNA levels in normal pancreas samples were much lower [<1,000-fold (CB_1 receptor) or <100,000-fold (CB_2 receptor)] than in the tumor of reference. *N.D.,* nondetectable. *E,* representative (63×) images of CB receptor immunostaining obtained from three human pancreatic tumor biopsies and three samples of normal human pancreas. Cannabinoid receptors are stained in green; nuclei are stained in blue.

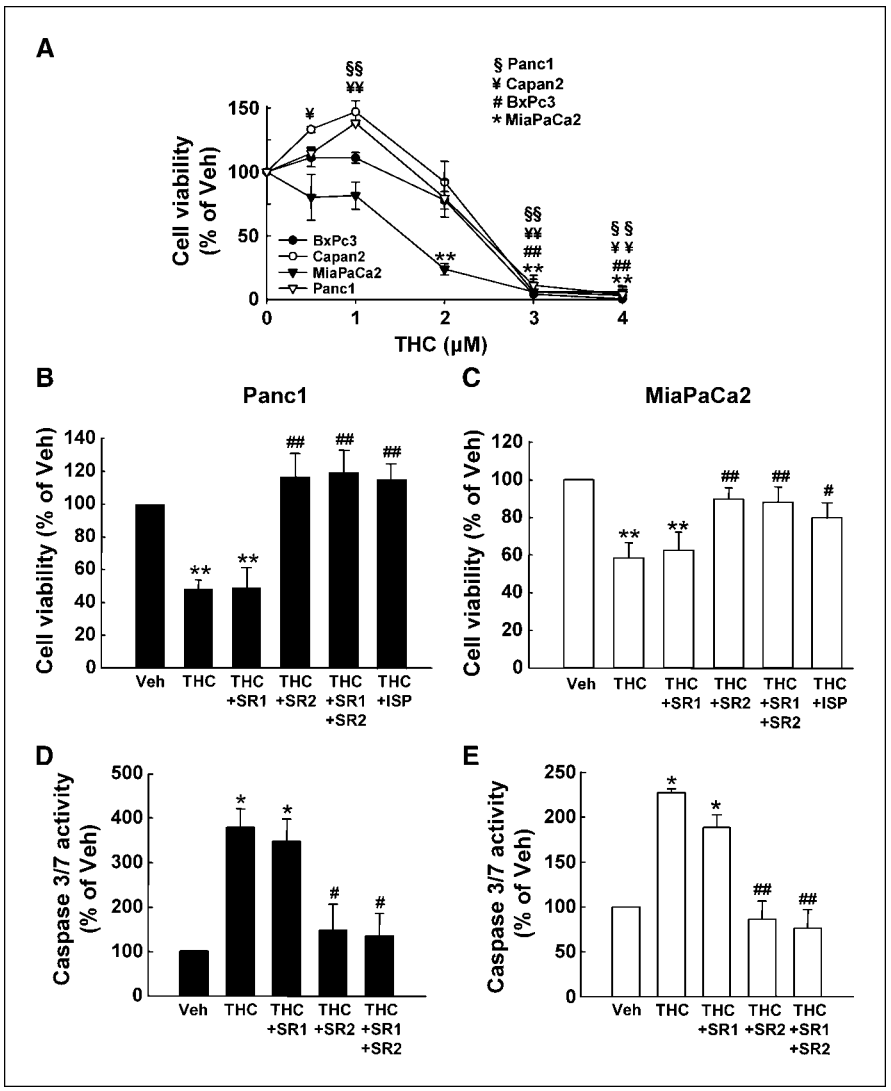

Figure 2. THC induces apoptosis of pancreatic tumor cells. *A,* cells were incubated for 66 hours with the indicated doses of THC and cell viability was determined. Results correspond to five different experiments and are expressed as the percentage of viability relative to each corresponding vehicle-treated cells. ¥, $P < 0.05$; **, ##, §§, ¥¥, $P < 0.01$, significantly different from vehicle-treated cells. *B* to *E,* cells were preincubated with vehicle (*Veh*), 1 μmol/L SR141716 (*SR1*), 1 μmol/L SR144528 (*SR2*), 1 μmol/L SR141716 plus 1 μmol/L SR144528, or 1 μmol/L ISP-1 (*ISP*; *B* and *C*) for 20 minutes, further treated with 2.75 μmol/L THC (*Panc1*, *B* and *D*) or 2.0 μmol/L THC (*MiaPaCa2, C* and *E*) for 66 hours (*B* and *C*) or 24 hours (*D* and *E*), and cell viability (*B* and *C*; $n = 5$) or DVEDase (*caspase 3/7, D* and *E*; $n = 3$) activity was determined. Results are expressed as percentage of vehicle-treated cells. *, $P < 0.05$; **, $P < 0.01$, significantly different from vehicle-treated cells. #, $P < 0.05$; ##, $P < 0.01$, significantly different from THC-treated cells.

Ceramide levels. Ceramide levels were determined as previously described (20). Briefly, after incubation of the cells in the different conditions, lipids were extracted, saponified, and incubated with *E. coli* diacylglycerol kinase in the presence of [γ-^{32}P]ATP. Finally, ceramide 1-phosphate was resolved by TLC.

Reverse transcription-PCR analysis. RNA was isolated using the RNeasy Protect kit (Qiagen) including a DNase digestion step using the RNase-free DNase kit (Qiagen). cDNA was subsequently obtained using the first-strand cDNA synthesis kit (Roche). The following sense and antisense primers, respectively, were used to amplify human CB_1 (CGTGGGCAGCCTGTTCCTCA and CATGCGGGCTTGGTCTGG; 408-bp product), human CB_2 (CGCCGGAAGCCCTCATACC and CCTCATTCGGGCCATTCCTG; 522-bp product), human p8 (GAAGAGAGGCAGGGAAGACA and CTGCCGTGCGTGTCTATTTA; 571-bp product), human TRB3 (GCCACTGCCTCCCGTCTTG and GCTGCCTTGCCCGAGTATGA; 538-bp product), human ATF-4 (AGTCGGGTTTGGGGGCTGAAG and TGGGGAAAGGGGAAGAGGTTGTAA; 436-bp product), and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GGGAAGCTCACTGGCATGGCCTTCC and CATGTGGGCCATGAGGTCCACCAC; 322-bp product). PCR reactions were done using the following parameters: 95°C for 5 minutes, 94°C for 30 seconds, 57°C (CB_1, CB_2, p8, ATF-4, and GAPDH) or 52°C (TRB3) for 30 seconds, and 72°C for 1 minute followed by a final extension step of 72°C for 5 minutes. The number of cycles (25-26 cycles for ATF-4, 26-28 cycles for p8, 28-29 cycles for TRB3, 22-25 cycles for GAPDH, and 36-38 cycles for CB_1 and CB_2) was adjusted to allow detection in the linear range. Finally, PCR products were separated on 1.5% agarose gels.

Real-time quantitative PCR. cDNA was obtained with Transcriptor (Roche). TaqMan probes were obtained from Applied Biosystems (Foster City, CA). Amplifications were run in a 7900 Real-time PCR System (Applied Biosystems). Each value was adjusted by using 18S RNA levels as reference.

Human tumor samples. Pancreatic tumor samples were obtained from "Hopital Nord," Marseille, France.

S.c. xenografts. Tumors were induced by s.c. injection of 20×10^6 MiaPaCa2 cells in PBS supplemented with 0.1% glucose in immunodeficient nude mice. When tumors reached an average size of 250 mm^3, animals were assigned randomly to various groups and injected peritumorally for 15 days with THC (15 mg/kg/d), JWH (1.5 mg/kg/d), or vehicle in 100 μL of PBS supplemented with 5 mg/mL defatted and dialyzed bovine serum albumin (BSA). Tumors were measured with external caliper and volume was calculated as $(4\pi/3) \times (\text{width}/2)^2 \times (\text{length}/2)$.

Intrapancreatic tumors. Immunodeficient mice were anesthetized, operated to allow access to the abdominal cavity, and 10×10^6 MiaPaCa2 cells were injected directly in the pancreas. Animals were allowed to recover from the operation for 10 hours and assigned randomly to control or treatment groups. Vehicle or WIN 55,212-2 (in 150 μL of PBS supplemented with 5 mg/mL defatted and dialyzed BSA) was given daily for 14 days in a single i.p. injection (1.5 mg/kg for 2 days, 2.25 mg/kg for 2 additional days, and 3.0 mg/kg for 10 additional days).

Western blot. Western blot analysis was done as described (20). Anti-CB_1 and anti-CB_2 receptor antibodies (Affinity Bioreagents, Golden, CO) were used.

Immunostainning and terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling. Pancreatic tumor cell lines were fixed in 4% buffered paraformaldehyde. Samples from human biopsies and tumor xenografts were dissected and fixed in 10% buffered formalin and then paraffin embedded. After deparaffinization, H&E-safran staining was done (samples from tumor xenografts). For the rest of experiments, samples were blocked to avoid nonspecific binding. Terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) was done using the *in situ* cell death detection kit (Roche). For CB receptors and TRB3 immunodetection, slides were incubated with a rabbit anti-human/mouse CB_1 receptor antibody (1:500; Affinity Bioreagents), a rabbit anti-human/mouse CB_2 receptor antibody (1:500; Affinity Bioreagents), and a rabbit anti-TRB3 antibody (1:500; Abcam, Cambridge, United Kingdom). Slides were further incubated in the dark (1 hour, room temperature) with a secondary antirabbit antibody-Alexa Fluor 594 (1:500; cell lines and tumor xenografts) or an antirabbit antibody-Alexa Fluor 488 (1:500; samples from human biopsies; Molecular Probes, Leyden, the Netherlands). Finally, Yoyo-1 (Molecular Probes; 1:5,000; cell lines and tumor xenografts) or Hoechst 33342 (Sigma Chemical; 10 μg/mL; human biopsies) were added to stain nuclei. Fluorescence images were acquired using a Leica Axiovert S100 TV microscope (Carl Zeiss, Oberkochen, Germany). Pixel quantification was determined with Metamorph-Offline software (Universal Imaging, Downingtown, PA).

Statistics. Unless otherwise specified, results shown represent mean ± SD Statistical analysis was done by ANOVA with a post hoc analysis by the Student-Neuman-Keuls test, except for data in Fig. 5*E* and *F*, which were analyzed by a χ^2 test.

Results

Activation of the CB_2 cannabinoid receptor induces apoptosis of pancreatic tumor cells *in vitro*. We investigated the effect of cannabinoids on pancreatic tumor cells. First, we determined the expression of cannabinoid receptors in four different human pancreatic tumor cell lines as well as in biopsies of human pancreatic tumors. CB_1 and CB_2 cannabinoid receptor mRNA (Fig. 1*A*) and protein (Fig. 1*B* and *C*) were expressed in Panc1, MiaPaCa2, Capan2, and BxPc3 cell lines. In addition, mRNA

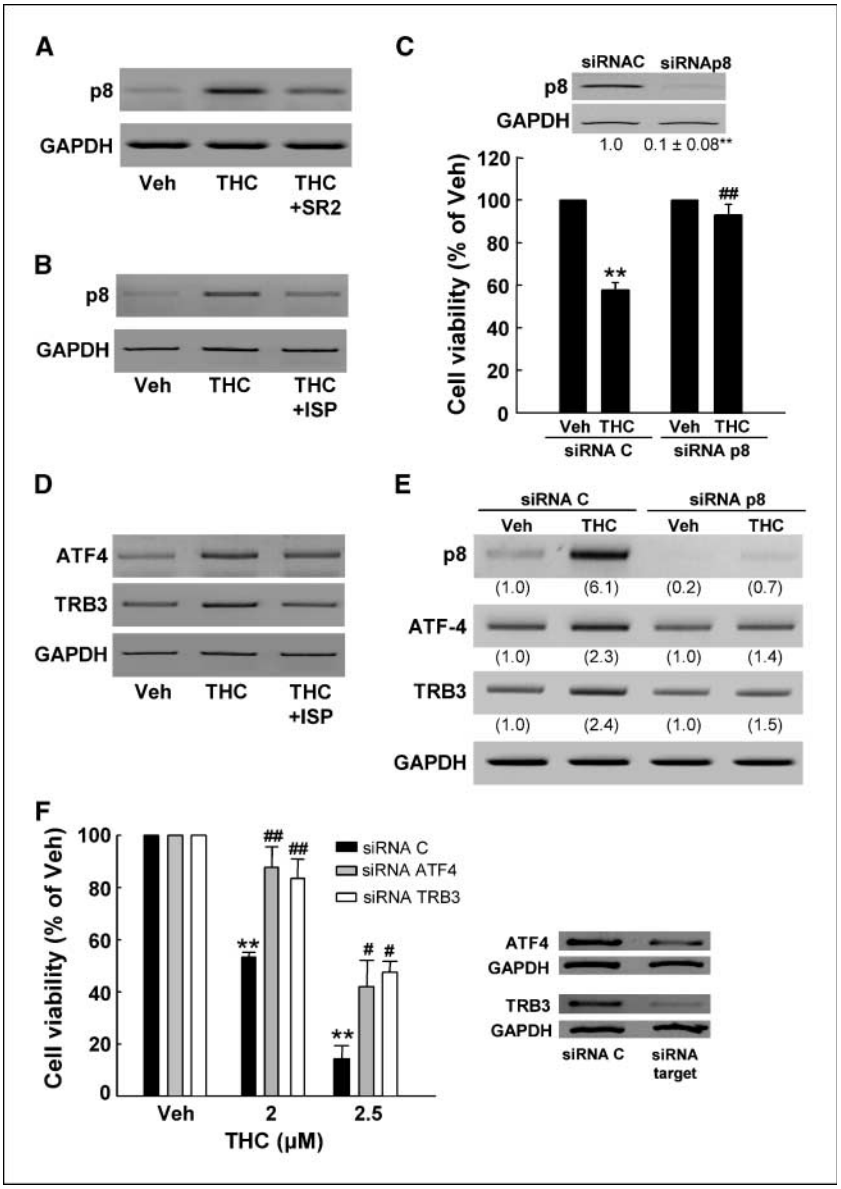

Figure 3. p8, ATF-4, and TRB3 are involved in THC-induced apoptosis of pancreatic tumor cells. *A* and *B*, MiaPaCa2 cells were preincubated with vehicle, 1 μmol/L SR144528 (*A*), or 1 μmol/L ISP-1 (*B*) for 20 minutes and further treated with 2.0 μmol/L THC. After 18 hours, total RNA was isolated and RT-PCR using selective primers for human p8 or GAPDH was done. Representative of three experiments. *C*, MiaPaCa2 cells were transfected with control (*siRNA C*) or p8-selective (*siRNA p8*) siRNA, treated with vehicle or 2.0 μmol/L THC for 66 hours, and cell viability was determined. Results correspond to seven different experiments and are expressed as the percentage of cell viability relative to the corresponding vehicle-treated cells. *Top*, a control of p8 knockdown after 18 hours of treatment. Values below the panel correspond to real-time quantitative PCR analysis of p8 mRNA levels in control siRNA– and p8-selective siRNA–transfected cells; *columns*, mean fold decrease relative to control siRNA–transfected cells; *bars*, SD. **, $P < 0.01$, significantly different from vehicle-treated and control siRNA–transfected cells. ##, $P < 0.01$, significantly different from THC-treated control siRNA–transfected cells. *D*, MiaPaCa2 cells were preincubated with vehicle or 1 μmol/L ISP-1 for 20 minutes and further treated with 2.0 μmol/L THC. After 18 hours, total RNA was isolated and RT-PCR using selective primers for human ATF-4, TRB3, or GAPDH was done. *A* and *B*, representative of three experiments. *E*, MiaPaCa2 cells transfected with control siRNA or p8-selective siRNA were treated with vehicle or 2.0 μmol/L THC for 18 hours, total RNA was isolated, and RT-PCR using selective primers for p8, ATF-4, TRB3, and GAPDH was done. Representative of three experiments. Values of gene induction as determined by real-time quantitative PCR (expressed as fold increase relative to control siRNA–transfected vehicle-treated cells in that experiment) are shown in parentheses. *F*, MiaPaCa2 cells transfected with control, ATF-4-selective, and TRB3-selective siRNAs were treated with vehicle or the indicated doses of THC for 66 hours and cell viability was determined. Results correspond to five different experiments and are expressed as the percentage of cell viability relative to the corresponding vehicle-treated cells. *Right*, a control of ATF-4 and TRB3 knockdown after 18 hours of treatment. **, $P < 0.01$, significantly different from vehicle-treated control siRNA–transfected cells. #, $P < 0.05$; ##, $P < 0.01$, significantly different from THC-treated control siRNA–transfected cells at each corresponding dose.

for cannabinoid receptors was expressed in several human pancreatic tumor biopsies, whereas in samples obtained from normal pancreatic tissue, mRNA levels for these receptors were very low or could not be detected (Fig. 1*D*). This difference between tumor and nontransformed pancreatic tissue was further confirmed by immunofluorescence analysis of CB receptors both in human biopsies from pancreatic cancer (Fig. 1*E*) and in pancreatic tumors generated in mice (Supplementary Fig. S1; see below). In both cases, expression of cannabinoid receptors was detected clearly in the tumor nodules but hardly in the surrounding pancreatic tissue.

Next, we tested the effect of THC on cell viability. Incubation with THC led to a dose-dependent decrease in cell viability in the four lines tested (Fig. 2*A*). Because cells exhibited a different sensitivity to THC treatment, we chose MiaPaCa2 as the most sensitive line and Panc1 as a less sensitive line to confirm the involvement of cannabinoid receptors in THC antiproliferative action. Incubation with the CB_2-selective antagonist SR144528, but not with the CB_1-selective antagonist SR141716, prevented THC-induced loss of cell viability in both lines (Fig. 2*B* and *C*). Likewise, in MiaPaca2 and Panc1 cells, THC led to caspase-3 activation, a characteristic of apoptotic cell death, and preincubation with SR144528 abrogated this effect (Fig. 2*D* and *E*).

Because *de novo* synthesized ceramide has been implicated in CB receptor–mediated apoptosis of glioma cells (19, 20), we tested the involvement of this pathway in our model. As shown in Supplementary Fig. S2*A*, incubation with THC led to ceramide accumulation in MiaPaca2 and Panc1 cell lines, and preincubation with SR144528 or ISP-1, a selective inhibitor of serine palmitoyltransferase (the enzyme that catalyzes the rate-limiting step of ceramide biosynthesis; ref. 21), prevented this accumulation. In addition, ISP-1 prevented the THC-induced (*a*) decrease of cell viability (Fig. 2*B* and *C*) and (*b*) activation of caspase 3 (Supplementary Fig. S2*B* and *C*), indicating that *de novo* synthesized ceramide is involved in THC-induced apoptosis of pancreatic tumor cells.

The stress-regulated protein p8 is involved in THC-induced apoptosis of pancreatic tumor cells. p8 (also designated as candidate of metastasis 1) is a stress-regulated protein related to the architectural factor HMG-I/Y (22). This protein has been implicated in a number of functions including the induction of apoptosis of pancreatic tumor cells (23). In addition, it has been shown that ceramide treatment leads to p8 up-regulation (24) and we have very recently identified this protein as an essential mediator of cannabinoid antitumoral action in gliomas (25). We therefore tested the involvement of this protein in the antiproliferative effect of THC in our cells. p8 mRNA levels increased after THC treatment of MiaPaca2 cells, and incubation with SR144528 (Fig. 3*A*) or ISP-1 (Fig. 3*B*) prevented this effect. Moreover, knockdown of p8 mRNA using a selective siRNA prevented THC-induced apoptosis of MiaPaCa2 cells (Fig. 3*C*), confirming the implication of this gene in the response to THC in our model.

Our next step was to identify genes downstream of p8 that could participate in the antitumoral effect of THC. By comparing the mRNA expression profiles of p8-deficient fibroblasts and fibroblasts with enforced p8 expression, several p8-dependent genes have been implicated in apoptotic signaling (25). Among these genes, we selected the endoplasmic reticulum (ER) stress–related proteins ATF-4 (26, 27) and TRB3 (28) as potential mediators of p8-dependent effects in our cells.

Incubation with THC led to a parallel increase in p8, ATF-4, and TRB3 mRNA levels, which was prevented by incubation with ISP-1 (Fig. 3*D*). In addition, knockdown of p8 mRNA prevented ATF-4 and TRB3 up-regulation (Fig. 3*E*). Moreover, knockdown of ATF-4 or TRB3 mRNA also prevented THC-induced apoptosis (Fig. 3*F*). Taken together, these observations support that challenge with THC triggers a ceramide- and p8-controlled apoptotic response in pancreatic tumor cells, which involves up-regulation of these genes.

Antitumoral effect of cannabinoid in pancreatic cancer models *in vivo*. To evaluate the antiproliferative effect of cannabinoids on pancreatic tumors *in vivo*, we first generated tumor xenografts by s.c. injection of MiaPaCa2 cells in immunodeficient mice. As shown in Fig. 4, peritumoral treatment with THC or the CB_2-selective (and therefore psychoactivity devoid; ref. 19) cannabinoid agonist JWH-133 reduced notably the growth of the established pancreatic tumors.

Next, we generated tumors by intrapancreatic injection of MiaPaCa2 cells to investigate the antitumoral effect of cannabinoids in a model that resembles more closely the niche of pancreatic cancer spreading. The synthetic cannabinoid agonist WIN 55,212-2 was selected for i.p. administration owing to its better bioavailability than THC and other classic cannabinoids. Experiments were done to confirm that this compound, which exhibits affinity for both cannabinoid receptor types (7), induces apoptosis of pancreatic cancer cells via the same endoplasmic reticulum stress–related proapoptotic pathway as THC (Supplementary Fig. S3). Administration of WIN 55,212-2 not only remarkably reduced the growth of intrapancreatic tumors (Fig. 5*A*) but also significantly decreased the extension of the tumor cells to proximal (Fig. 5*B*) and distal (Fig. 5*C*) organs, indicating that cannabinoid treatment affects the growth and spreading of pancreatic tumor cells.

To investigate the mechanisms involved in this cannabinoid action *in vivo*, we analyzed the tissue samples obtained in these experiments. Treatment with WIN 55,212-2 increased the number of apoptotic cells in tumor nodules (Fig. 6*A*) but not in normal

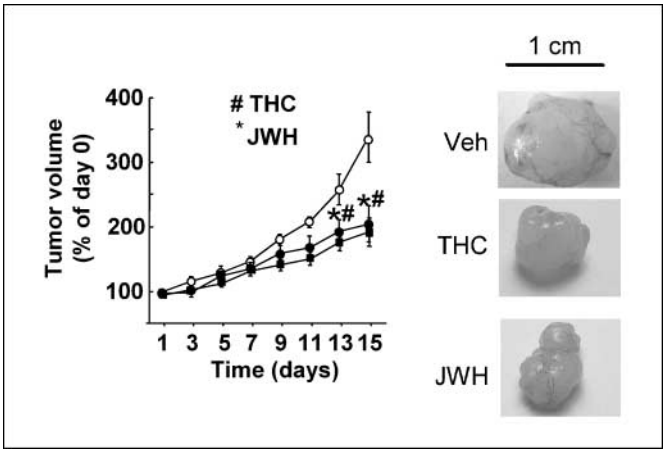

Figure 4. Cannabinoid treatment reduces the growth of pancreatic tumor cells *in vivo*. S.c. tumors were generated as described in Materials and Methods. When tumors reached the desired size (day 0), animals were treated with either vehicle (○), THC (15 mg/kg/d; ●), or JWH-133 (1.5 mg/kg/d; ■) for 15 days ($n = 6$ for each experimental group). Tumor volume was measured at the indicated times. Results are expressed as the percentage of tumor volume growth relative to day 0. *, #, $P < 0.05$, significantly different from vehicle-treated tumors at the corresponding day of treatment. Photographs of a representative vehicle-, THC-, and JWH-treated tumor.

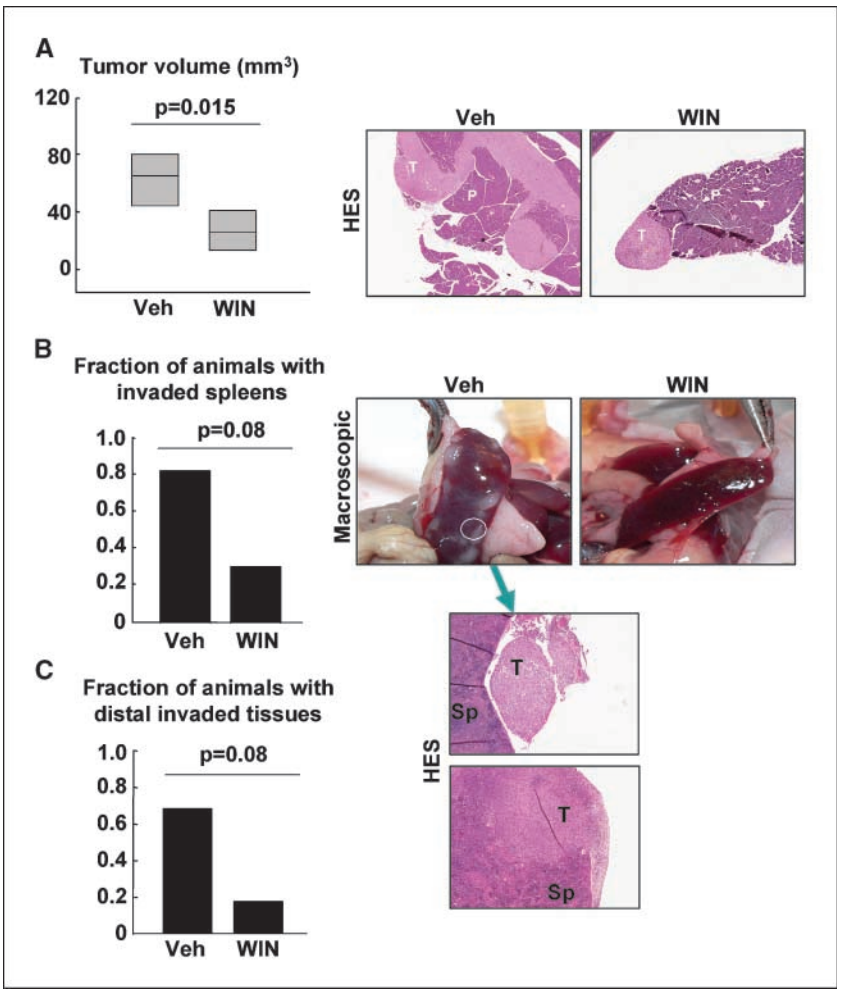

Figure 5. Cannabinoid treatment reduces the growth and the spreading of pancreatic tumor cells *in vivo*. Intrapancreatic tumors were generated as described in Materials and Methods. Animals were treated with either vehicle or WIN 55,212-2 (1.5 mg/kg for 2 days, 2.25 mg/kg for 2 additional days, and 3.0 mg/kg for 10 additional days; $n = 6$ for each experimental group). The day after the 14-day treatment, animals were sacrificed and tumor size was measured (*A*) and the presence of tumor nodules in spleen (*B*) and distal organs (liver, diaphragm, intestine, and stomach; *C*) was analyzed. Results are expressed as the average tumor size for each experimental group (*A*) and the fraction of animals with invaded (at least one positive nodule) spleen (*B*) or distal organs (*C*). *A, right,* images of H&E-safran–stained pancreas from vehicle-treated and WIN 55,212-2-treated animals. *T,* tumor tissue; *P,* normal pancreatic tissue. *B, right,* photographs of representative spleens from vehicle-treated and WIN 55,212-2-treated animals. *C, right,* H&E-safran staining of vehicle-treated spleens showing two tumor nodules [one attached (*top*) and the other invading (*bottom*) the organ]. *Sp,* normal splenic tissue.

pancreatic tissue (Fig. 6*B*) or in the spleen (Supplementary Fig. S4*A*). In contrast, cannabinoid administration did not significantly modify the percentage of proliferating (proliferating cell nuclear antigen–positive) cells in the tumors (data not shown). Next, we analyzed the expression of the proapoptotic protein TRB3 because this protein is downstream of p8 and ATF-4 (Fig. 5; refs. 28, 29) and has been proposed to be responsible for the execution of endoplasmic reticulum stress–induced apoptosis (28, 29). WIN 55,212-2 administration increased the expression of TRB3 in tumor nodules (Fig. 6*C*) but not in normal pancreatic tissue (Supplementary Fig. S4*B*) or in the spleen (Supplementary Fig. S4*C*). Taken together, these results indicate that cannabinoid administration selectively induces apoptosis in pancreatic tumor cells *in vivo*.

Discussion

Despite many years of intensive research, pancreatic cancer remains as the fourth leading cause of cancer death in the United States (1, 2) and the fifth in the Western world overall (3). Current approved therapies based on the administration of fluorouracil chemoradiation for locally advanced tumors and gemcitabine chemotherapy for metastatic disease have only slightly increased the median survival of affected patients (1). In the present report, we show that cannabinoids induce apoptosis of pancreatic tumor cell lines *in vitro* and exert a remarkable growth-inhibiting effect in models of pancreatic cancer *in vivo*. The latter effect is evident under various experimental settings (cells inoculated at different sites and cannabinoids administered by different routes). Moreover, our results also show that cannabinoids exert a strong inhibitory effect on the spreading of pancreatic tumor cells not only to adjacent locations such as spleen but also to distal tissues such as liver, diaphragm, stomach, and intestine, thus suggesting that these agents may also decrease the propagation of pancreatic tumor cells.

Although the pancreatic tumor biopsies and cell lines analyzed expressed both CB_1 and CB_2 cannabinoid receptors, our findings indicate that the CB_2 receptor is the one that plays a major role in the proapoptotic effect of cannabinoids in these cells. Previous observations had shown that the CB_2 receptor is involved in the antitumoral effect of cannabinoids in gliomas (9, 19), skin carcinomas (11), lymphomas (18), and prostate carcinomas (30), which may be clinically relevant as CB_2-selective activation is not linked to the typical marijuana-like psychoactive effects of CB_1 activation (7). However, the molecular mechanisms involved in those CB_2-mediated actions are only partially understood. Results presented here, together with data obtained in rat glioma and human astrocytoma cells (25), show that p8 and ATF-4 up-regulation mediates cannabinoid-induced apoptosis via induction of the proapoptotic protein TRB3 (29, 31). Of interest, it has been recently shown that ATF-4 regulates TRB3 expression to induce apoptosis of human transformed cells (29). This pathway is triggered by endoplasmic reticulum stress (29), and our results support that it is also involved in cannabinoid-induced apoptosis of

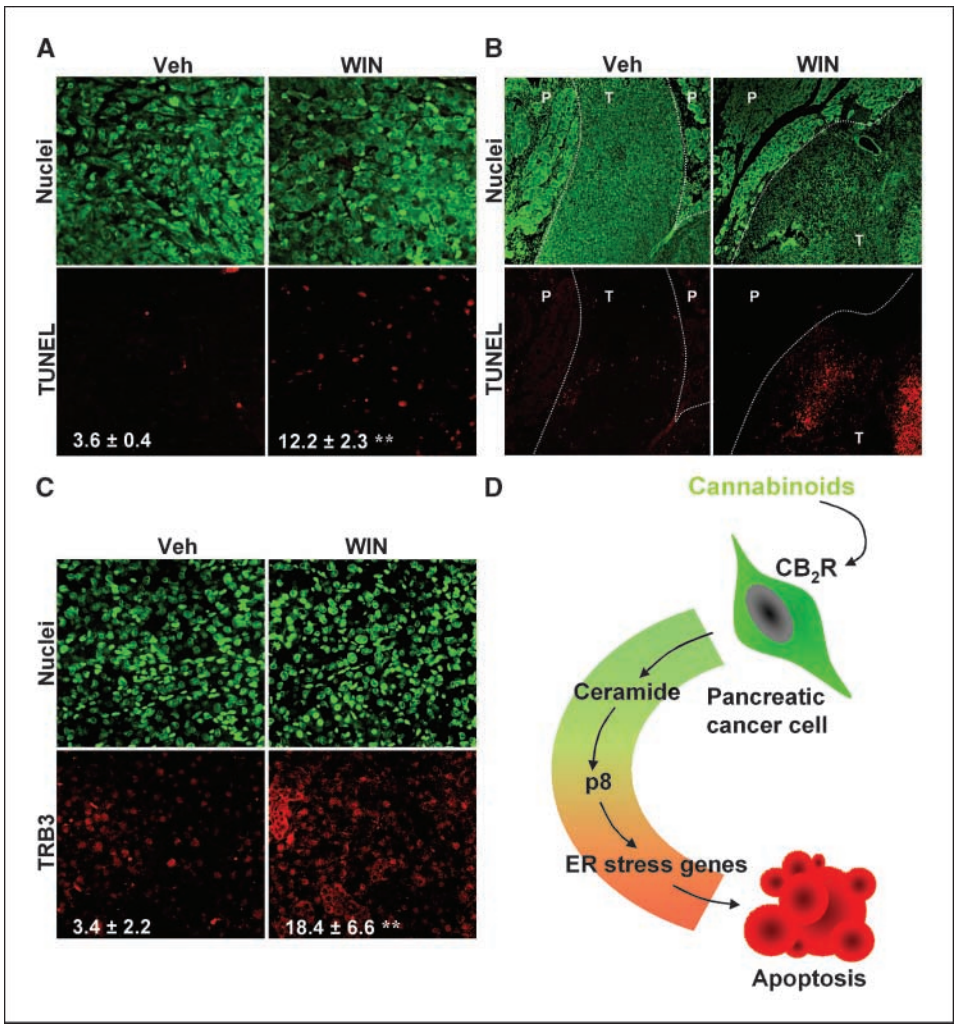

Figure 6. Cannabinoid treatment increases TRB3 expression and apoptosis in pancreatic tumors but not in normal pancreatic tissue. Intrapancreatic tumors were generated as described in Materials and Methods. Animals were treated with either vehicle or WIN 55,212-2 (1.5 mg/kg for 2 days, 2.25 mg/kg for 2 additional days, and 3.0 mg/kg for 10 additional days; $n = 6$ for each experimental group). The day after the 14-day treatment, animals were sacrificed, pancreatic tissues containing tumors were fixed, and sections were prepared. *A,* representative images (×40) of TUNEL-stained pancreatic tumors. Values in the lower left corner of the bottom images correspond to 12 sections of three dissected tumors for each condition and are expressed as the percentage of TUNEL-positive cells relative to the total number of cells in each section. *B,* representative images (×20) of TUNEL-stained pancreas. *T,* tumor tissue; *P,* normal pancreatic tissue. *C,* representative images (×40) of TRB3-stained pancreatic tumors. Values in the lower left corner of the bottom images correspond to 18 sections of three dissected tumors for each condition and are expressed as the percentage of TRB3-stained area relative to the total area in each section. *D,* schematic representation of the proposed mechanism of cannabinoid-induced apoptosis on pancreatic tumor cells.

human pancreatic tumor cells *in vitro* and *in vivo*. Of potential interest for future cannabinoid-based therapies, cannabinoid treatment does not seem to activate this pathway in normal pancreas or spleen, suggesting that these agents may activate the endoplasmic reticulum stress proapoptotic pathway selectively in tumor cells.

On the other hand, our data also implicate *de novo* synthesized ceramide in the proapoptotic effect of THC. Many chemotherapeutic agents have been shown to mediate their antiproliferative effects via regulation of ceramide production (32). In line with these observations, we had previously shown that this proapoptotic sphingolipid participates in the antitumoral action of cannabinoids in glioma cells (9, 13, 20). Here we show that *de novo* synthesized ceramide is involved in THC-induced up-regulation of p8, ATF-4, and TRB3 in pancreatic tumor cells. Interestingly, p8 had been shown to be regulated by ceramide (24, 25) and we have also observed that pharmacologic inhibition of ceramide synthesis *de novo* decreases basal p8 expression (data not shown). Thus, our findings indicate that the mechanism of cannabinoid-induced apoptosis of human pancreatic tumor cells involves a CB_2 receptor–dependent accumulation of *de novo* synthesized ceramide that leads to p8, ATF-4, and TRB3 up-regulation (Fig. 6*D*).

One of the major problems that face cancer therapies is the resistance to chemotherapy. It is worth noting that low doses of THC (but not of WIN 55,212-2) slightly induced proliferation of Panc1 and Capan2, but not of MiaPaca2 and BxPc3 cells, an effect that does not depend on cannabinoid receptors (data not shown). A similar growth-promoting effect of cannabinoids at submicromolar concentrations has previously been described by us (33) and others (34) for several transformed cells. Interestingly, our observations suggest that this behavior may be related with a lower sensitivity to THC proapoptotic action, as higher doses of this compound (although still within the low micromolar range) are necessary to induce CB receptor–dependent apoptosis of the human pancreatic (present study) and astrocytoma[3] cell lines that exhibit this proliferative response. Thus, the potential development of future cannabinoid-based therapies for this or other types of tumors should ideally evaluate the sensitivity of each particular tumor to these compounds. In our laboratories, we are currently investigating the gene expression profile associated with a higher sensitivity/resistance of tumor cells to cannabinoid treatment to improve the selectivity and efficiency of a potential cannabinoid-based therapy.

In conclusion, results presented here show that cannabinoids exert a remarkable antitumoral effect on pancreatic cancer cells

[3] Authors' unpublished observations.

in vitro and *in vivo* due to their ability to selectively induce apoptosis of these cells via activation of the p8-ATF-4-TRB3 proapoptotic pathway. These findings may help to set the basis for a new therapeutic approach for the treatment of this deadly disease.

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References

1. Li D, Xie K, Wolff R, Abbruzzese JL. Pancreatic cancer. Lancet 2004;363:1049–57.
2. Edwards BK, Brown ML, Wingo PA, et al. Annual report to the nation on the status of cancer, 1975–2002, featuring population-based trends in cancer treatment. J Natl Cancer Inst 2005;97:1407–27.
3. Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ. Cancer statistics. CA Cancer J Clin 2003;53: 5–26.
4. Gaoni Y, Mechoulam R. Isolation, structure and partial synthesis of an active constituent of hashish. J Am Chem Soc 1964;86:1646–7.
5. Devane WA, Hanus L, Breuer A, et al. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 1992;258:1946–9.
6. Mechoulam R, Ben-Shabat S, Hanus L, et al. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. Biochem Pharmacol 1995;50:83–90.
7. Howlett AC, Barth F, Bonner TI, et al. International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. Pharmacol Rev 2002;54:161–202.
8. Munro S, Thomas KL, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. Nature 1993;365:61–5.
9. Galve-Roperh I, Sanchez C, Cortes ML, Gómez del Pulgar T, Izquierdo M, Guzman M. Anti-tumoral action of cannabinoids: involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation. Nat Med 2000;6:313–9.
10. Benito C, Nunez E, Tolon RM, et al. Cannabinoid CB2 receptors and fatty acid amide hydrolase are selectively overexpressed in neuritic plaque-associated glia in Alzheimer's disease brains. J Neurosci 2003;23: 11136–41.
11. Casanova ML, Blazquez C, Martinez-Palacio J, et al. Inhibition of skin tumor growth and angiogenesis *in vivo* by activation of cannabinoid receptors. J Clin Invest 2003;111:43–50.
12. Guzman M. Cannabinoids: potential anticancer agents. Nat Rev Cancer 2003;3:745–55.
13. Blazquez C, Gonzalez-Feria L, Alvarez L, Haro A, Casanova ML, Guzman M. Cannabinoids inhibit the vascular endothelial growth factor pathway in gliomas. Cancer Res 2004;64:5617–23.
14. Blazquez C, Casanova ML, Planas A, et al. Inhibition of tumor angiogenesis by cannabinoids. FASEB J 2003; 17:529–31.
15. Portella G, Laezza C, Laccetti P, De Petrocellis L, Di Marzo V, Bifulco M. Inhibitory effects of cannabinoid CB1 receptor stimulation on tumor growth and metastatic spreading: actions on signals involved in angiogenesis and metastasis. FASEB J 2003;17:1771–3.
16. Melck D, De Petrocellis L, Orlando P, et al. Suppression of nerve growth factor Trk receptors and prolactin receptors by endocannabinoids leads to inhibition of human breast and prostate cancer cell proliferation. Endocrinology 2000;141:118–26.
17. De Petrocellis L, Melck D, Palmisano A, et al. The endogenous cannabinoid anandamide inhibits human breast cancer cell proliferation. Proc Natl Acad Sci U S A 1998;95:8375–80.
18. McKallip RJ, Lombard C, Fisher M, et al. Targeting CB2 cannabinoid receptors as a novel therapy to treat malignant lymphoblastic disease. Blood 2002;100: 627–34.
19. Sanchez C, de Ceballos ML, del Pulgar TG, et al. Inhibition of glioma growth *in vivo* by selective activation of the CB(2) cannabinoid receptor. Cancer Res 2001;61:5784–9.
20. Gomez del Pulgar T, Velasco G, Sanchez C, Haro A, Guzman M. *De novo*-synthesized ceramide is involved in cannabinoid-induced apoptosis. Biochem J 2002;363: 183–8.
21. Hanada K. Serine palmitoyltransferase, a key enzyme of sphingolipid metabolism. Biochim Biophys Acta 2003; 1632:16–30.
22. Encinar JA, Mallo GV, Mizyrycki C, et al. Human p8 is a HMG-I/Y-like protein with DNA binding activity enhanced by phosphorylation. J Biol Chem 2001;276: 2742–51.
23. Malicet C, Lesavre N, Vasseur S, Iovanna JL. p8 inhibits the growth of human pancreatic cancer cells and its expression is induced through pathways involved in growth inhibition and repressed by factors promoting cell growth. Mol Cancer 2003;2:37.
24. Vasseur S, Vidal Mallo G, Fiedler F, et al. Cloning and expression of the human p8, a nuclear protein with mitogenic activity. Eur J Biochem 1999; 259:670–5.
25. Carracedo A, Lorente M, Egia A, et al. The stress-regulated protein p8 mediates cannabinoid-induced apoptosis of tumor cells. Cancer Cell 2006;9:301–12.
26. Ma Y, Brewer JW, Diehl JA, Hendershot LM. Two distinct stress signaling pathways converge upon the CHOP promoter during the mammalian unfolded protein response. J Mol Biol 2002;318:1351–65.
27. Back SH, Schroder M, Lee K, Zhang K, Kaufman RJ. ER stress signaling by regulated splicing: IRE1/HAC1/ XBP1. Methods 2005;35:395–416.
28. Ord D, Ord T. Characterization of human NIPK (TRB3, SKIP3) gene activation in stressful conditions. Biochem Biophys Res Commun 2005;330:210–8.
29. Ohoka N, Yoshii S, Hattori T, Onozaki K, Hayashi H. TRB3, a novel ER stress-inducible gene, is induced via ATF4-CHOP pathway and is involved in cell death. EMBO J 2005;24:1243–55.
30. Sarfaraz S, Afaq F, Adhami VM, Mukhtar H. Cannabinoid receptor as a novel target for the treatment of prostate cancer. Cancer Res 2005;65: 1635–41.
31. Mayumi-Matsuda K, Kojima S, Suzuki H, Sakata T. Identification of a novel kinase-like gene induced during neuronal cell death. Biochem Biophys Res Commun 1999;258:260–4.
32. Ogretmen B, Hannun YA. Biologically active sphingolipids in cancer pathogenesis and treatment. Nat Rev Cancer 2004;4:604–16.
33. Galve-Roperh I, Rueda D, Gomez del Pulgar T, Velasco G, Guzman M. Mechanism of extracellular signal-regulated kinase activation by the CB(1) cannabinoid receptor. Mol Pharmacol 2002;62:1385–92.
34. Hart S, Fischer OM, Ullrich A. Cannabinoids induce cancer cell proliferation via tumor necrosis factor α-converting enzyme (TACE/ADAM17)-mediated transactivation of the epidermal growth factor receptor. Cancer Res 2004;64:1943–50.

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