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**FARMACOGENÉTICA
DE LOS AINEs**

Personalizar el
Tratamiento



**PERSONALIZED
MEDICINE OF
DEMENTIA**

Pharmacogenomics of
Alzheimer's Disease



**EFFECTS OF FR-91
ON HUMAN TUMOR
CELL LINES**

Effects of FR-91 on human tumor cell lines

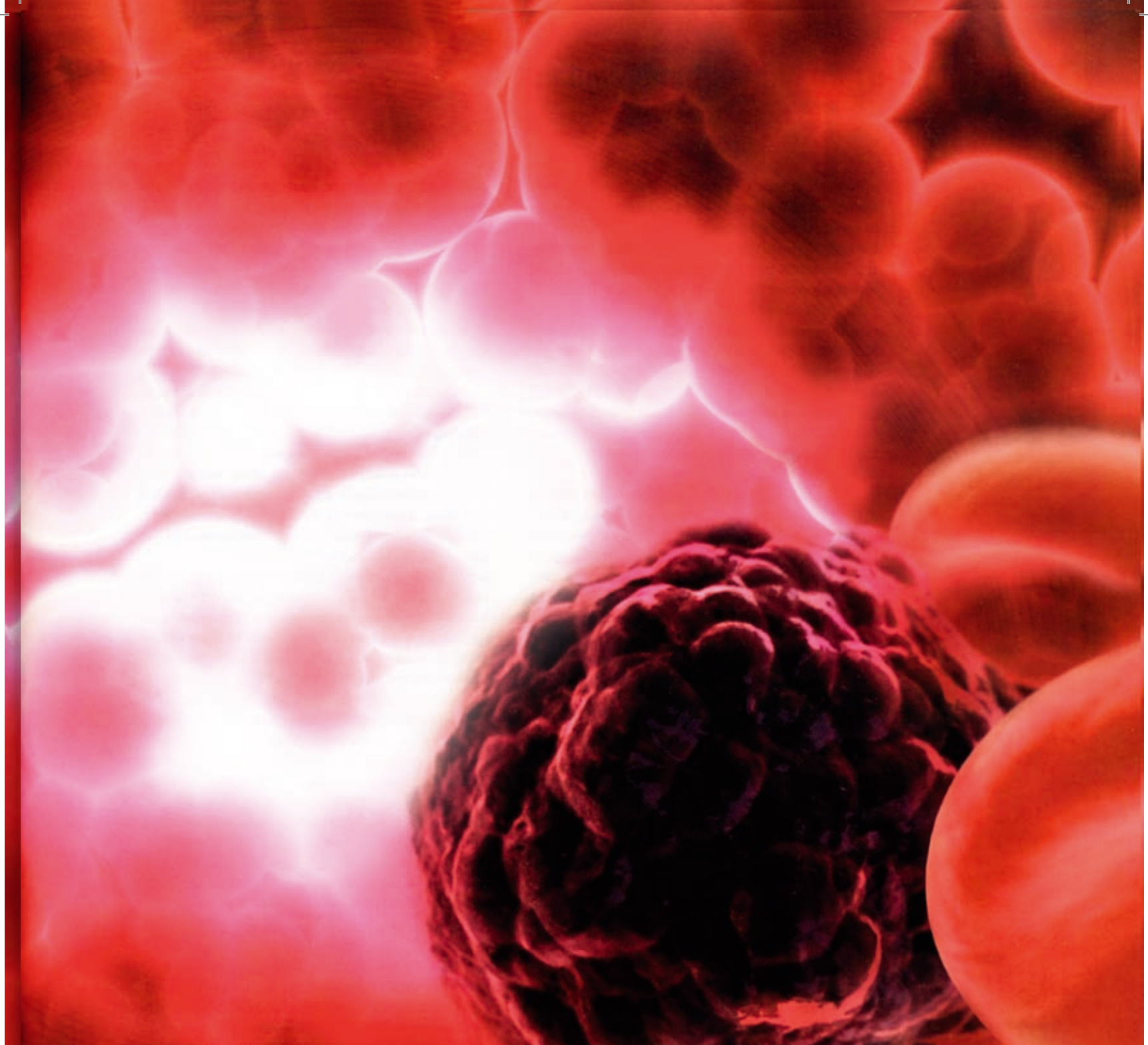
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APOPTOSIS has been an intensive research area, which involves the study of compounds that trigger or inhibit this mechanism of death. Being an important process implicated in many pathological diseases, including cancer, a number of new natural compounds has been investigated in the attempt to inhibit or trigger this fundamental cellular process making apoptosis amenable to new biopharmaceutical interventions. The aim of the study was to investigate the antitumor effect of FR-91, a standardized lysate of microbial cells belonging to the *Bacillus* genus, against SW872 (human adipose cells), SW982 (human synovial sarcoma

line), HL-60 (promyelocytic cells), HS 274.T (breast adenocarcinoma), HS 313.T (lymphoma), H2126 (lung adenocarcinoma), TOV 21G (epithelial ovarian cancer), WM 115 (melanoma), and HS 281T (breast adenocarcinoma) human tumor cell lines. We used the MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) assay to study the growth inhibition activity of FR-91 and apoptosis. We report the potential apoptogenic activity after 24 hours incubation with 10, 25 and 50 $\mu\text{l/ml}$ of FR-91. Apoptosis, determined by DNA cellular fragmentation, was observed in HL-60, HS 313T, SW872, SW982 and TOV-21G cell lines treated with



25 and 50 $\mu\text{l/ml}$ of FR-91. The highest level of growth inhibition, cytotoxicity assay, was observed in SW872 (55, 78, 87%), SW982 (50, 70, 87%) and TOV-21G (42, 66, 85%), with respect to untreated cells, while the results of the expression of genes associated with apoptosis indicated a down-regulation of Bcl-2 in all cell lines. Taken together these results suggest that FR-91 contains small peptides which can contribute to the induction of apoptosis. Further investigations will be required to clarify the nature of these bioactive constituents and the in vivo effects of FR-91 on tumor-induced in experimental animal models.

Introduction

Dysregulated proliferation appears to be a hallmark of increased susceptibility to neoplasia. Cancer prevention is generally associated with inhibition, reversion, or retardation of cellular hyperproliferation. Both epidemiological and experimental studies have shown that a variety of food and food components, by exhibiting several biological properties that are able to modulate mammalian immune system, could be used for the prevention of many degenerative diseases^{1,2,3}, and would be more economical and less painful, representing a new rational >

Effects of FR-91 on human tumor cell lines

approach for cancer control. It is well known that dietary flavonoids and isoflavonoids behave as general cell growth inhibitors. One of their biological properties in plants, in fact, is providing resistance to fungal or bacterial growth, and although most flavonoids and isoflavonoids appear nontoxic to humans and animals, they have demonstrated to inhibit proliferation in many kinds of cultured human cancer cell lines^{4,5,6,7}. Antiproliferative effects of quercetin⁸, taxifolin⁹, nobiletin¹⁰, and tangretin¹¹ at 2-8 µg/ml for 3-7 day on squamous cell carcinoma, meningioma, colon carcinoma, leukaemia, and lung carcinoma tumor cell lines have been reported.

Studies on mice, rats, and chickens have shown that many animals carry latent oncogenic viruses that usually remain harmless but occasionally become activated to cause the development of tumors or leukaemia¹². The activation process can be triggered by several external or internal factors, such as radiation, certain hormones, or chemicals. However, the activation of the latent tumor-inducing viruses can be substantially delayed, or even prevented, by avoiding or reducing exposure to tumor-inducing factors, and also by the use of natural products¹³.

The bacteria that reside in the intestinal tract generally have a symbiotic relationship with their host. Beneficial bacteria produce natural antibiotics¹⁴ to keep pathogenic bugs in check (preventing diarrhea and infections) and produce some B vitamins in the small intestine where they can be utilized. Beneficial bacteria help a) with food digestion^{15,16,17} by providing extra enzymes, such as lactase, in the small intestine; b) strengthen the immune system^{18,19} right in the gut where much of the interaction between the outside world and the body goes on; and c) prevent food allergies^{20,21,22}. In addition, they can help to prevent cancer at various stages of development²³. These good bacteria can improve mineral absorption,

maximizing food utilization. However, the balance of beneficial and potentially pathogenic bacteria in the gut is dependent on the diet.

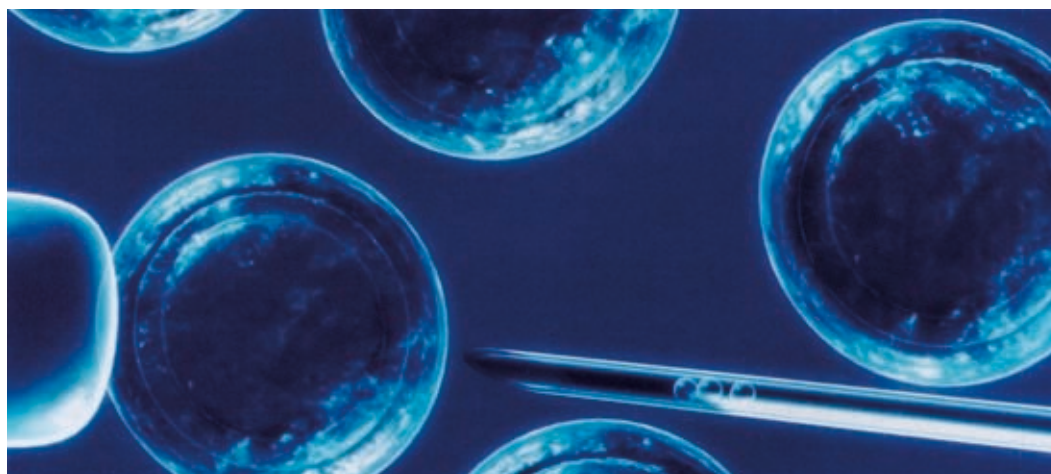
Studies in rats have shown that probiotics can inhibit the formation of aberrant crypt foci, thought to be a pre-cancerous lesion in the colon. Some of the best results were obtained with a probiotic strain consumed with inulin, a type of fructooligosaccharide. Total aberrant crypt foci, chemically induced, were reduced 74% by the treatment of rats with inulin and *B. longum*, but only 29 and 21% by *B. longum* and inulin alone, respectively²⁴. There was a synergistic effect in using both products together. Similar synergy was seen in rats with azoxymethane-induced colon cancer in another study. Rats fed Raffilose, a mixture of inulin and oligofructose, or Raffilose with *Lactobacilli rhamnosus* (LGG) and *Bifidobacterium lactis* (Bb12) had a significantly lower number of tumors compared to the control group²⁵. A probiotic mixture, without any prebiotic, given to rats fed azoxymethane reduced colon tumors compared to the control (50% vs 90%), and also reduced the number of tumors per tumor-bearing rat²⁶.

In the present study, different concentrations of FR-91, a standardized lysate of microbial cells belonging to the Bacillus genus, have been used to investigate potential antitumor activity against SW872, SW982, HL-60, HS 274.T, HS 313.T, H2126, TOV 21G, WM 115, and HS 281T human tumor cell lines.

Materials and methods

Cell lines and cell cultures

Human adipose cells (SW872) and human synovial sarcoma line (SW 982) were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Cells were maintained



in L-15 (Leibovitz-15 medium with L-glutamine [PAA, The Cell Culture Company, Pasching, Austria]), supplemented with 10% heat-inactivated fetal bovine serum (FBS, PAA) and sodium bicarbonate [1.5 g/liter; Gibco] and incubated at 37°C and 5% CO₂. HL-60 (promyelocytic cells) cell line was obtained from the American Type Culture Collection. Cells were grown in RPMI 1640 medium supplemented with 10% heat inactivated FBS, 100 IU penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. HS 274.T (breast adenocarcinoma) cell line was obtained from the American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated FBS, 100 IU penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. HS 313.T (lymphoma) cell line was obtained from the American Type Culture Collection. Cells were grown in DMEM supplemented with 10% heat inactivated FBS, 100 IU penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. H2126 hypotriploid cell line from metastatic site, pleural effusion adenocarcinoma, was obtained from the American Type Culture Collection. Cells were grown in DMEM/F12 supplemented with 5% heat inactivated FBS, 100 IU penicillin, 100 µg/ml streptomycin, G5 supplement, and 2 mM L-glutamine. TOV 21G epithelial ovarian cancer cell line was obtained from the American Type Culture Collection. Cells were grown in DMEM supplemented with 10% heat inactivated FBS, 100 IU penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. WM 115 (melanoma) cell line was obtained from the American Type Culture Collection. Cells were grown in Minimal essential Medium Eagle (MEM), supplemented with 10% heat inactivated FBS, 100 IU penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. HS 281T (breast adenocarcinoma) cell line was obtained from the American Type Culture Collection. Cells were grown in DMEM supplemented with 10% heat inactivated FBS, 100 IU penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. Cell stocks were maintained in liquid nitrogen.

Prior to use in experimental assays, the cells growing in monolayer were released from the culture flask with 0.25% trypsin (PAA), washed twice with fresh medium, and seeded onto 96-well microculture flat plates. Viable cell counts were confirmed prior to each experiment using trypan exclusion.

Chemicals

HEPES-buffered RPMI 1640 medium, DMEM, DMEM/F12, L-15, MEM, FBS, trypsin, G5 supplement, and CryoMaxx S were obtained from PAA. Glutamine and antibiotics (penicillin and streptomycin) were purchased from Sigma-Aldrich (Madrid, Spain).

MTT reduction assay

Cell proliferation assay was determined using MTT assay. Cell lines (1 x 10⁵ cells/ml) were incubated in 96 well plates with different doses of FR-91 (10, 25 and 50 µl/w) for 24 hours. Ten µl of MTT (10 mg/ml) was added to each well and incubated further at 37°C for 4 hours. After incubation, MTT-formazan precipitate was dissolved in 100 µl of DMSO and absorbance was recorded at 570 nm in an automatic plate reader (BioRAD instrument). Data are presented as percentage of cytotoxicity of treated versus untreated cells.

DNA ladder assay

DNA ladder assay was carried out as per standard method. This method prevents the contamination of entire genomic DNA with fragmented DNA. Briefly, after treatment with FR-91, cells were harvested, washed twice with cold PBS and lysed for 30 min at 4°C in lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.2% Triton X-100) using zirconium beads and automatic cell lyser. After centrifugation at 15,000 x g for 20 min, the supernatants was treated with protease inhibitor cocktail and 0.5% SDS for 1 hour at 37°C. DNA was extracted twice with phenol and precipitated with 150 mM NaCl and two volumes of ethanol at -20°C. DNA precipitate was washed twice with cold 70% ethanol, dissolved in TE buffer and treated for 1 hour with Rnase at 37°C. Finally, DNA precipitates were stained with propidium iodide, electrophoresed on 2% agarose gel and visualized in an automatic gel documentation system (BioRAD system).



The balance of beneficial and potentially pathogenic bacteria in the gut is dependent on the diet

Quantitative estimation of DNA fragmentation using an enzyme-linked immunosorbent assay (ELISA)

After trypsin treatment, cells collected by brief centrifugation were plated to a density of 110⁴ cells per well on flat-bottomed 96-well plates (Costar) and incubated overnight in complete medium at 37°C under a 5% CO₂ atmosphere. The next day, the culture medium was exchanged for 100 µl of complete medium with or without the indicated concentration of 10, 25 and 50 µl >>

Effects of FR-91 on human tumor cell lines

Table 1

Oligonucleotides used in the RT-PCR

p53	sense	5' -AAAACCTTACCAAGGCAACTA- 3'
	antisense	5' -TGAAATATTCTCCATCGAGT- 3'
p21	sense	5' -CATGTCCGATCCTGGTGATG- 3'
	antisense	5' -AGTGCAAGACAGCGACAAGG- 3'
Bcl-2	sense	5' -TGCACCTGACGCCCTTCAC- 3'
	antisense	5' -AGACAGCCAGGAGAAATCAAACAG- 3'
Bax	sense	5' -ACCAAGAAGCTGAGCGAGTGTC- 3'
	antisense	5' -ACAAAGATGGTCACGGTCTGCC- 3'

of FR-91 and incubated for an additional 24 h. In some experiments, cells were preincubated with z-VAD-fmk (Promega) or the adenosine kinase inhibitor 5'-iodotubercidin (IT; Sigma) prior to incubation with FR-91. Thereafter, DNA fragmentation was quantitatively estimated in the remaining attached cells using an ELISA (Cell Detection ELISA^{PLUS}, Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's protocol. Absorbance at 405 nm (reference at 492 nm) was measured in each well, and the means \pm SD were plotted as a function of the concentration of the indicated reagent. All control incubations contained the maximal concentration of DMSO, which was typically <0.1%; DMSO concentrations of up to 1.25% did not induce significant DNA fragmentation (data not shown). Each independent experiment was carried out in triplicate using all the different tumor cell lines.

RNA isolation and RT-PCR

Total cellular RNA was isolated by lysis in a guanidinium isothiocyanate buffer followed by single step phenol-chloroform-isoamyl alcohol extraction²⁷. Briefly, cells were harvested and lysed in a solution containing 4M guanidinium isothiocyanate, 25mM sodium citrate (pH 7.0), 0.5% sodium sarkosine and 0.1M β -mercaptoethanol. Sequentially, 1/10 volume of 2M sodium acetate (pH 4.9), one volume of phenol and 1/5 volume of chloroform-isoamyl alcohol (49:1, v:v) were added to the homogenate. After vigorous shaking for 30 seconds, the solution was centrifuged at 10,000 x g for 15 minutes at 4°C. RNA in the aqueous phase was precipitated by the addition of 0.5 ml isopropanol. One μ g of total RNA was reverse-transcribed into cDNA by incubating with 200 units of reverse transcriptase in 20 μ l of reaction buffer containing 0.25 μ g of random primers and 0.8 mM dNTPs at 42°C for one hour. Two μ l of the cDNA was used for the PCR reaction as templates. The PCR was performed in buffer containing 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM

dNTPs, 1 μ M of each primer and 5 units Taq DNA polymerase for 30 cycles of denaturing at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min. The resulting PCR products were analyzed by 1.5% agarose gel electrophoresis. Sequences for the specific primers used in the PCR were summarized in Table 1.

Statistical analysis

All statistical tests were performed with the use of SPSS (version 11.0; SPSS Inc, Chicago) and a *P* value <0.05 indicated statistical significance. Data were analyzed by using a two-factor repeated-measures analysis of variance (ANOVA) followed by a post hoc analysis where relevant (one-factor repeated-measures ANOVA, followed by Tukey's tests for a significant effect of dose and paired *t* tests for a significant effect of FR-91 treatment).

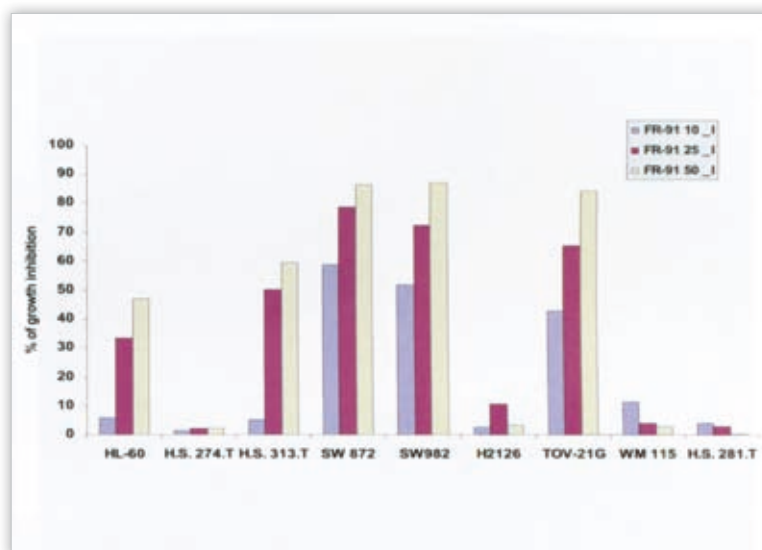
Results

FR-91 growth inhibition in tumor cell lines

To examine the possible antineoplastic effect of FR-91 in tumor cells, we first determined its effects on cell growth by the MTT assay, which measures the metabolically live cells based on their mitochondrial dehydrogenase activity. As shown in Fig 1, FR-91 caused growth inhibition in a dose-dependent manner. The cytotoxicity was not restricted to a specific cell line, since five different cell lines were sensible to the effects of FR-91. The highest level of growth inhibition was observed in SW872 (55%, 78%, 87%), SW982 (50%, 70%, 87%) and TOV-21G (42%, 66%, 85%), while HL-60 (6%, 33%, 48%) and HS 313.T (5%, 50%, 60%) showed a slight, but significant, cytotoxic effect with respect to untreated cells. At the lowest concentration of FR-91 (10 μ l/ml) a significant difference between controls and treated cells was observed in SW 872, SW 982, and TOV-21G cell lines. No cytotoxic effects were observed with HS 274.T, H2126, WM 115, and HS281.T tumor cell lines. To determine if the antiproliferative effect was reversible, SW872, SW982, and TOV-21G cells were treated with 25 μ l/ml FR-91 or culture medium for 48 hours. The medium-treated and FR-91-treated cells were then incubated in complete medium for 0, 6, 24, 48, and 72 hours followed by trypsinization and counting of the cells. After 48 hours of treatment with FR-91, the cell number had decreased by 50% in all cell lines. Once FR-91 was removed, the cell number did not increase but rather showed a small further decrease, whereas the cell number in the absence of treatment, as expected, increased with time (Fig 2). These data indicate that the FR-91 effect was not reversible.

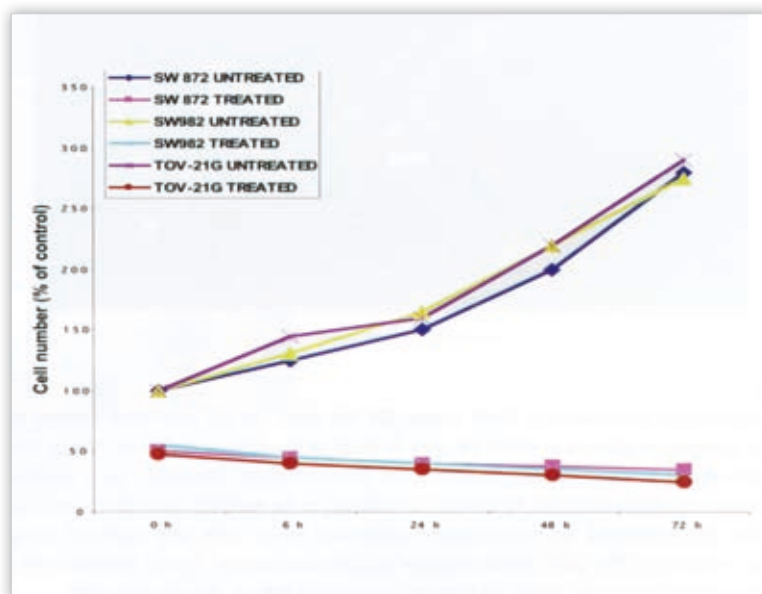
FR-91 induction of apoptosis in tumor cell lines

Next, we tested whether the administration of FR-91 induced any cytotoxic effects on SW872, SW982, HL-60, HS 274.T, HS 313.T, H2126, TOV 21G, WM 115, and HS 281T human tumor cell lines. When cells were treated with 10, 25, and 50 $\mu\text{l/ml}$ of FR-91 for 24 hours and then examined morphologically by light microscopy, a portion of the cells exhibited condensation (arrow-head) and cleavage (arrow) of the nuclei, findings that are typical of apoptosis (Fig. 3). No such images were observed in control, untreated cells. The following results, expressed as absorbances (570 nm), clearly show that the treatment with FR-91 indeed induced apoptosis in HL-60 (FR-91 25 $\mu\text{l/ml}$: 0.96, $p < 0.001$ vs negative, 0.12, and positive, 1.47, controls); FR-91 50 $\mu\text{l/ml}$: 1.1, $p < 0.001$ vs negative, 0.18, and positive, 1.72, controls); HS 313T (FR-91 25 $\mu\text{l/ml}$: 0.77, $p < 0.001$ vs negative, 0.18, and positive, 1.72, controls); FR-91 50 $\mu\text{l/ml}$: 0.68, $p < 0.001$ vs negative, 0.18, and positive, 1.72, controls), SW872 (FR-91 10 $\mu\text{l/ml}$: 0.58, $p < 0.001$ vs negative, 0.17, and positive, 1.64, controls); FR-91 25 $\mu\text{l/ml}$: 0.76, $p < 0.001$ vs negative, 0.17, and positive, 1.64, controls); FR-91 50 $\mu\text{l/ml}$: 1.55, $p < 0.001$ vs negative, 0.17, and positive, 1.64, controls), SW982 (FR-91 10 $\mu\text{l/ml}$: 0.77, $p < 0.002$ vs negative, 0.24, and positive, 1.8, controls); FR-91 25 $\mu\text{l/ml}$: 0.99, $p < 0.001$ vs negative, 0.17, and positive, 1.64, controls); FR-91 50 $\mu\text{l/ml}$: 1.48, $p < 0.001$ vs negative, 0.17, and positive, 1.64, controls), and TOV-21G (FR-91 25 $\mu\text{l/ml}$: 0.58, $p < 0.001$ vs negative, 0.17, and positive, 1.8, controls); FR-91 50 $\mu\text{l/ml}$: 1.6, $p < 0.001$ vs negative, 0.17, and positive, 1.8, controls), cell lines (Fig. 4). DNA prepared from HL-60, HS 313T, SW872, SW982 and TOV-21G cells treated with FR-91 for 24 hours showed oligonucleosomal ladder fragmentation on agarose gel electrophoresis (data not shown). No signs of apoptosis were observed on HS 274.T (FR-91 10 $\mu\text{l/ml}$: 0.22, $p = 0.28$ vs negative, 0.2, and positive, 1.65, controls); FR-91 25 $\mu\text{l/ml}$: 0.24, $p = 0.08$ vs negative, 0.22, and positive, 1.65, controls); FR-91 50 $\mu\text{l/ml}$: 0.2, $p = 0.33$ vs negative, 0.22, and positive, 1.65, controls), H2126 (FR-91 10 $\mu\text{l/ml}$: 0.18, $p = 0.32$ vs negative, 0.2, and positive, 1.75, controls); FR-91 25 $\mu\text{l/ml}$: 0.23, $p = 0.78$ vs negative, 0.2, and positive, 1.75, controls); FR-91 50 $\mu\text{l/ml}$: 0.22, $p = 0.52$ vs negative, 0.2, and positive, 1.75, controls), WM 115 (FR-91 10 $\mu\text{l/ml}$: 0.23, $p = 0.25$ vs negative, 0.14, and positive, 1.69, controls); FR-91 25 $\mu\text{l/ml}$: 0.22, $p = 0.27$ vs negative, 0.14, and positive, 1.69, controls); FR-91 50 $\mu\text{l/ml}$: 0.24, $p = 0.07$ vs negative, 0.14, and positive, 1.69, controls), and HS 281T (FR-91 10 $\mu\text{l/ml}$: 0.2, $p = 0.25$ vs negative, 0.24, and positive, 1.81, controls); FR-91 25 $\mu\text{l/ml}$: 0.22, $p = 0.27$ vs negative, 0.24, and positive, 1.81, controls); FR-91 50 $\mu\text{l/ml}$: 0.24, $p = 0.07$ vs negative, 0.24, and positive, 1.81, controls) (Fig. 5).



▲ *Figure 1*

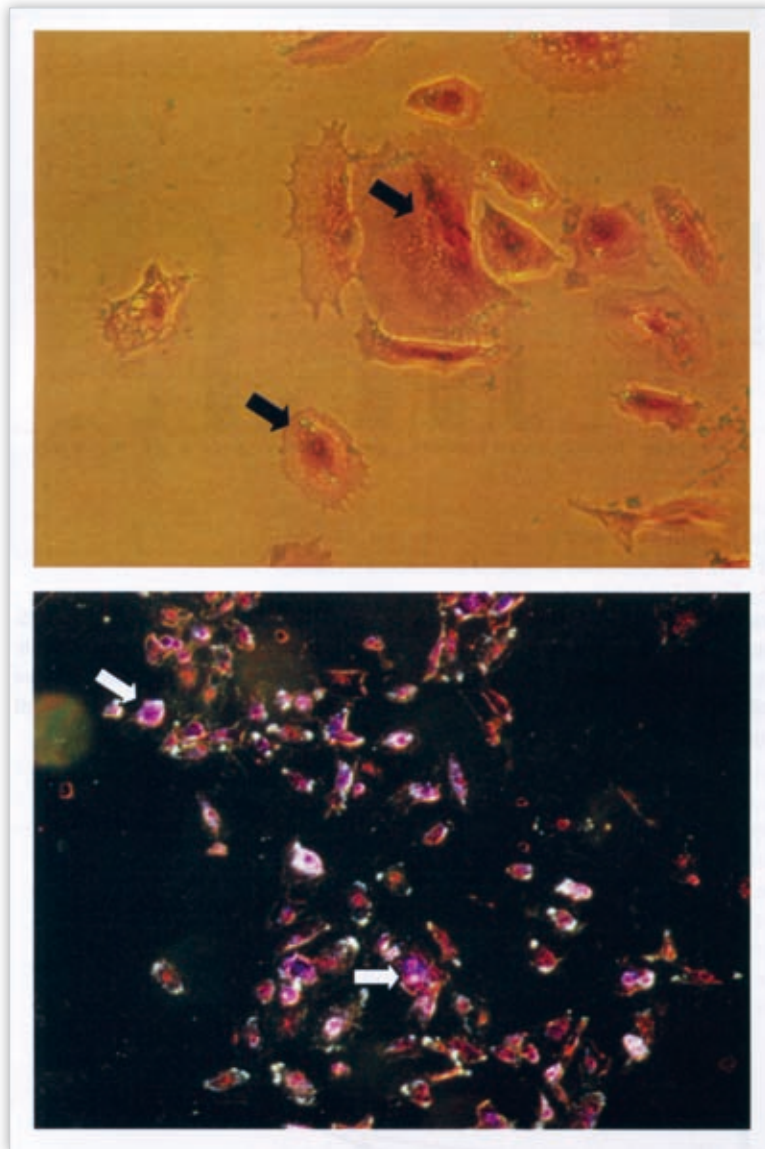
Growth inhibition of SW872, SW982, HL-60, HS 274.T, HS 313.T, H2126, TOV 21G, WM 115, and HS 281T human tumor cell lines. Cells were plated on 96 well plates and exposed to three 10, 25 and 50 $\mu\text{l/ml}$ of FR-91. The growth inhibition is expressed as percentage from cells exposed to culture medium. The mean shown was calculated from five independent experiments performed by triplicate.



▲ *Figure 2*

Antiproliferative effects of FR-91 in SW 872, SW 982, and TOV-21G cells. Cells were treated for 48 hours with 25 $\mu\text{l/ml}$ of FR-91 or complete medium. The medium was replaced with only medium, and after various times (0-72 hours) the cells were trypsinized and counted. Results are expressed relative to control (48 hours incubation with medium). All values are mean of triplicate cultures in three independent experiments.

Effects of FR-91 on human tumor cell lines



▲ **Figure 3**
Morphological examination of FR-91 treated SW 982 cells. SW 982 cells were cultured in the presence or absence of either 25 $\mu\text{l/ml}$ of FR-91 and 1 $\mu\text{l/ml}$ of apoptosis inducer mix (Actinomycin D, Camptothecin, Cycloheximide, Dexamethasone, Etoposide), as a positive control of apoptosis induction, for 24 hours as indicated in the materials and methods section. After Giemsa-staining, the morphological appearance of the cells was examined using light microscopy. The black arrows indicate nuclear condensation. Typical apoptotic cells, characterized by cleaved nuclei, are indicated by the white arrows. Magnification 400x.

Down-regulation of Bcl-2 gene expression in FR-91-treated tumor cell lines

To further investigate the molecular mechanisms responsible for the FR-91 induced apoptosis in HL-60, HS 313T, SW872, SW982 and TOV-21G cell lines, the gene expression of some apoptosis-related genes such as p53, p21, Bax and Bcl-2 was analyzed by RT-PCR. In *Fig. 6*, FR-91 treatment caused the down-regulation of Bcl-2 gene expression, while other genes were not affected, which therefore resulted in the relative increase of Bax/Bcl-2 ratio.

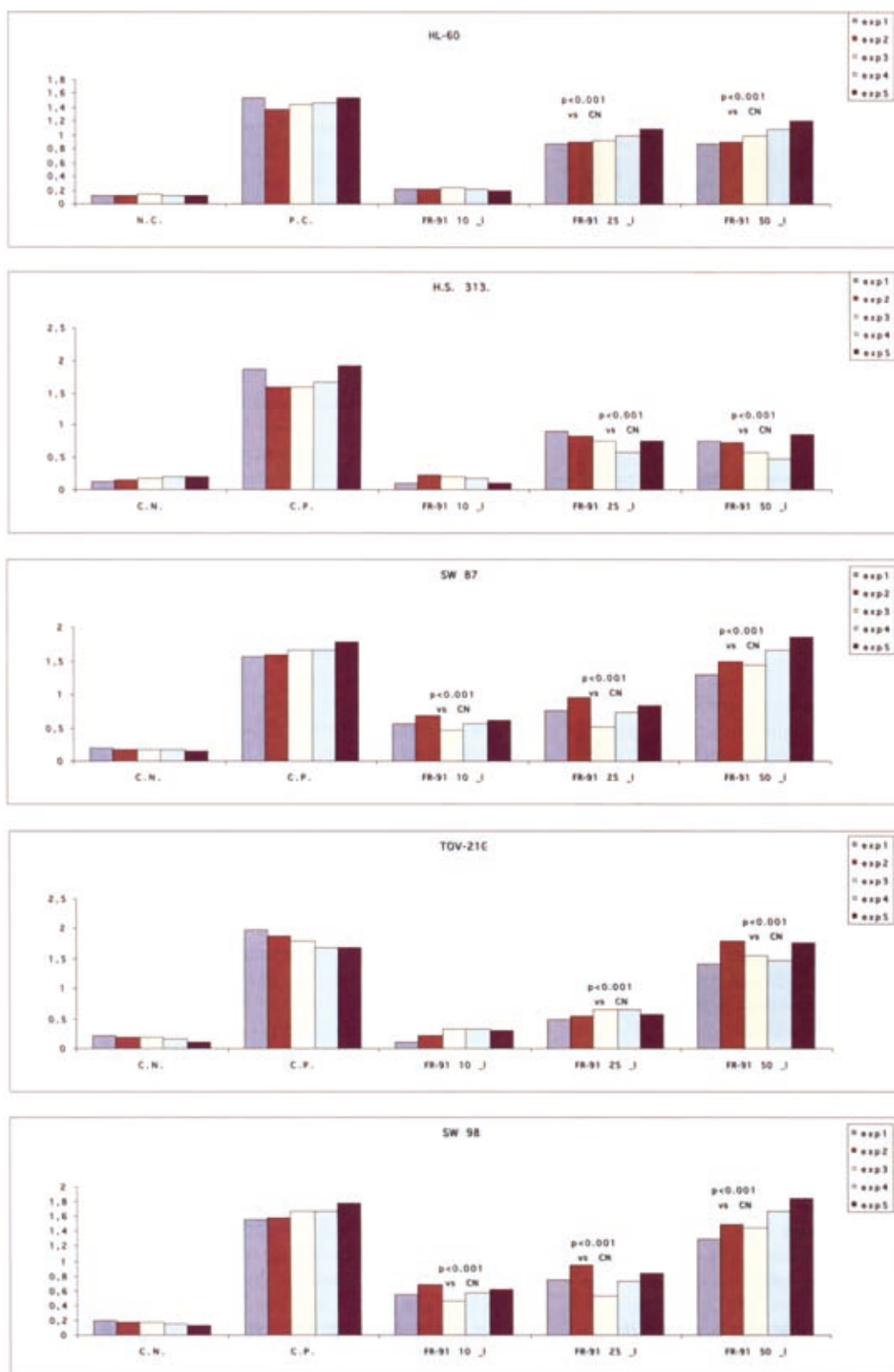
Discussion

In the attempt of understanding and treating diseases, natural components have been discovered, by trial and error, and used for thousands of years by a significant fraction of the population in many countries or regions of the world. It is estimated that approximately 25% of the drugs prescribed worldwide at present derive from plants and 60% of antitumor/anti-infectious drugs already on the market or under clinical investigations are of natural origin, and extracts from plants such as Taxol²⁸, curcumin²⁹, phenolic acids³⁰ and flavonoids³¹ are reported to inhibit tumor growth in many types of cancer. Tumor growth is generally associated with marked changes in hematopoiesis and immune response, myelosuppression and anemia. The immune system has several potential means to limit or even prevent tumor growth. These include: **a)** specific T cell-mediated immunity against tumor associated transplantation antigens (TATAs) on tumor cells; **b)** production of antibodies against TATAs and/or other antigenic structures associated with the tumor cells; **c)** natural cell-mediated immunity against tumors, which consists mainly of natural killer (NK) cells and activated macrophages; and **d)** natural anti-tumor antibodies.

Although there is some evidence for substantial anti-tumor effects of specifically induced or natural antibodies, particularly if they are cytotoxic in the presence of complement or in combination with lymphocytes or macrophages that express receptors for the Fc portion of immunoglobulins (and hence can mediate antibody-dependent cell-mediated cytotoxicity (ADCC)), most attention and experimental data have been focused on cell-mediated immunity as the basis for possible resistance of the host against progressive growth of tumor. There has been much interest and controversy for many years over the possible role of each of these immunological mechanisms in prevention of the initial development of detectable tumors.

Considerations along these lines are encompassed in the hypothesis of immunological surveillance against tumors. An updated version of this hypothesis, which includes the potential involvement of NK cells and other aspects of natural immunity as well as specific T-cell mediated immunity has been reported^{32,33,34}.

The most compelling support for this hypothesis has come from observations, in clinical situations as well as in experimental animal models, of substantially more frequent tumor development in immunodeficient individuals as compared to individuals with normally functioning immune



◀ **Figure 4**

Detection of DNA fragmentation in HL-60, HS 313T, SW872, SW982 and TOV-21G cell lines after culturing 24 hours in the presence of the indicated concentrations of FR-91 and 1 μl/ml of apoptosis inducer mix (Actinomycin D, Camptothecin, Cycloheximide, Dexamethasone, Etoposide), as a positive control of apoptosis induction. Experiments were repeated five times and the results are expressed as optical density.

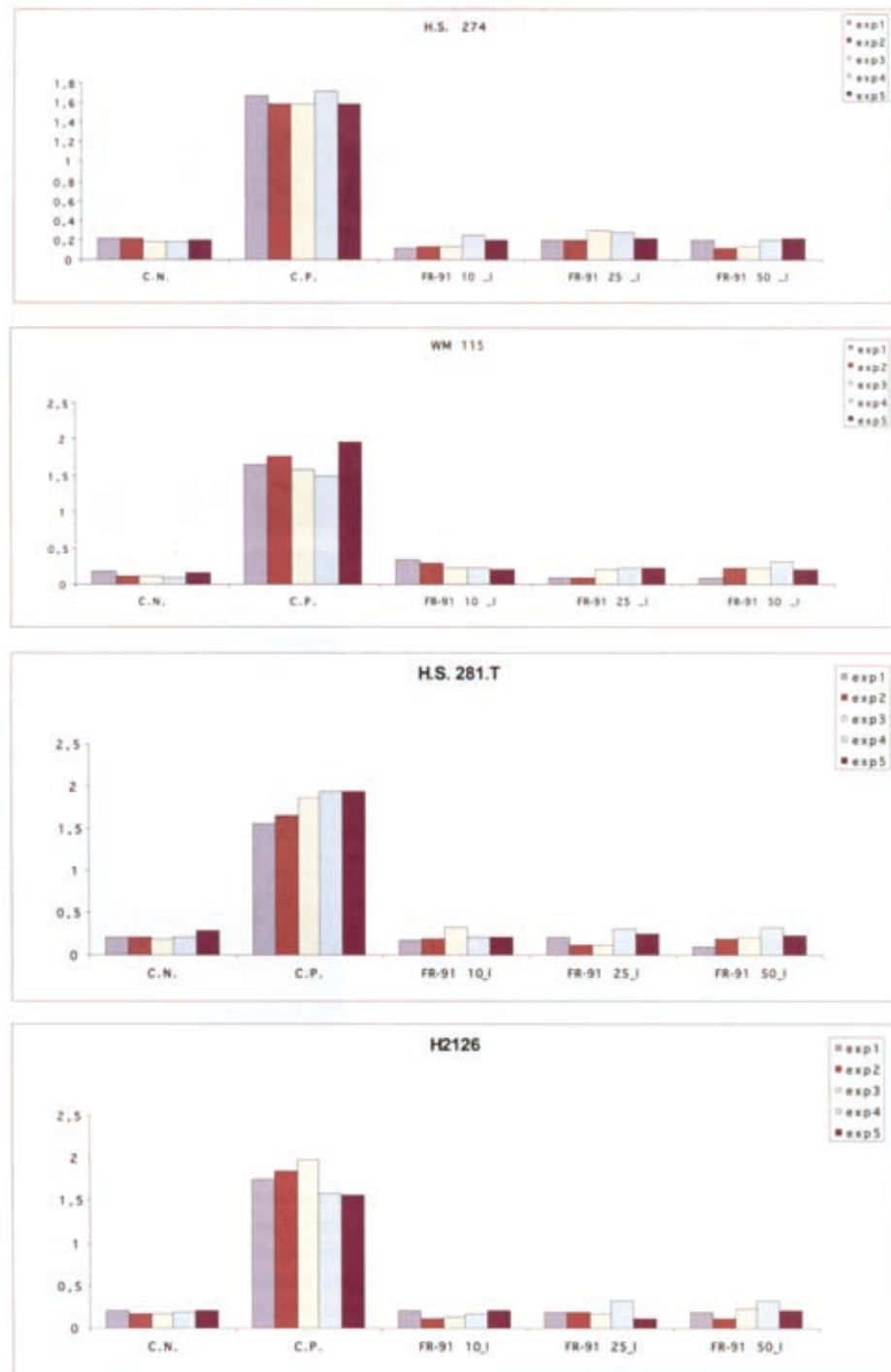
system. For example, the frequent development of lymphoproliferative disease in immunosuppressed organ transplant recipients, patients with infection by the human immunodeficiency virus (HIV), or in children with congenital immunodeficiencies

points strongly to a key role of the immune system in preventing this type of cancer^{35,36}. However, it has been very difficult to determine clearly which components of the immune system are most critical for effective immunological surveillance. >>

Effects of FR-91 on human tumor cell lines

Figure 5 ▶

Detection of DNA fragmentation in HS 274.T, H2126, WM 115, and HS 281T cell lines after culturing 24 hours in the presence of the indicated concentrations of FR-91 and 1 µl/ml of apoptosis inducer mix (Actinomycin D, Camptothecin, Cycloheximide, Dexamethasone, Etoposide), as a positive control of apoptosis induction. Experiments were repeated five times and the results are expressed as optical density.



It remains quite possible that several different arms of the immune system contribute to resistance against primary tumor development, with the appearance of overt malignant disease possibly representing failure of more than one line of defense.

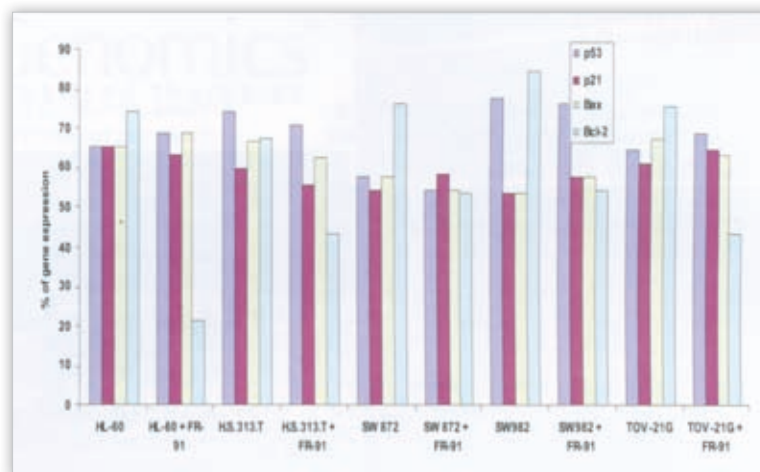
Although at least 120 chemical substances useful as antineoplastic drugs, and among them Paclitaxel (Taxol) isolated from three species of the genus *Taxus*, are still isolated from plants

throughout the world, research regarding the role of natural compounds in the treatment of different types of tumors still remains controversial. Epidemiological studies assessing dietary intake of natural compounds, although firmly based on biologically plausible hypotheses, have provided support for the association between reduced risk and bioactive intake, in most, but not all, studies published to date. Although the FR-91 extract has demonstrated significant *in vitro* and *in vivo* antineoplastic

activities against different tumor cell lines, the mechanism of this effect has not fully examined. In this study, FR-91 demonstrated selective cytotoxicity *in vitro* for human tumor cells tested (liposarcoma, sarcoma, promyelocitic, ovarian, and lymphoma cancer cell lines) when compared to untreated cells. Moreover, FR-91 showed the greatest cytotoxicity towards HS 313.T cells that are resistant to chemotherapy. This compound's ability to effectively kill several types of tumors without significant cytotoxicity to normal cells indicates that this compound may be a potentially chemotherapeutic agent. The FR-91 induced apoptosis occurred in a dose-dependent manner and was accompanied by the disruption of the mitochondrial transmembrane potential (data not shown) and the activation of caspase-3 and perhaps caspase-8.

The results of this study suggest that FR-91 inhibited proliferation of tumor cells by a mechanism that involves cytotoxicity. The predominant form of cell death is likely apoptosis since evidence of apoptotic cell death was seen initially. However, at longer times of incubations, necrotic cell death was also observed. It is likely that necrotic cell death occurred as a secondary event and is a phenomenon seen *in vitro* due to the lack of white cell phagocytosis. The possibility cannot be excluded, however, that FR-91 causes both forms of cell death and the incidence of these forms of death depends on the concentration that is used and the length of incubation.

Apoptosis is regulated and executed by different interplay of many genes responsive to various stimuli. There are two central pathways that lead to apoptosis: 1) positive induction by ligand binding to a plasma membrane receptor and 2) negative induction by loss of a suppressor activity. Positive-induction involves ligands related to TNF, while negative induction of apoptosis by loss of a suppressor activity involves the mitochondria. The study of apoptosis in cancer therapy is very important³⁷. It has been proved that occurrence of cancers is due to the loss of control of normal apoptosis and the disturbance of balance between cell apoptosis and cell proliferation³⁸. The apoptosis related genes (bcl-2 family) is divided in two categories: apoptotic repressor and apoptotic promoter. Bcl-2 is an important apoptotic repressor, while Bax is one of the most important apoptotic promoters. The protein it encodes can combine with Bcl-2 to form compounds which resist the action of repressing apoptosis. But it has a positive regulatory action³⁹. Recent studies indicate that the regulation of apoptosis by Bcl-2 and Bax is not only based on the level



▲ **Figure 6**

FR-91 induced the Bcl-2 down-regulation in HL-60, HS 313T, SW872, SW982 and TOV-21G cell lines. Cells were treated with 25µl of FR-91 as indicated for 24 hours and cells were harvested for RNA isolation and RT-PCR. Positive controls were treated with 1 µl/ml of apoptosis inducer mix (Actinomycin D, Camptothecin, Cycloheximide, Dexamethasone, Etoposide). Experiments were repeated three times and the results are expressed as % of gene expression compared to positive controls.

of either of the two regulatory proteins but also based on the ratio of them. If the ratio is high, the cells go to apoptosis⁴⁰. All cell lines used in the presented study have been induced by a variety of chemical reagents to undergo apoptosis through different pathways such as p53-dependent pathway or Bcl-2 family-related pathway.

To clarify the molecular mechanism of apoptosis mediated by different concentrations of FR-91, we examined the expression of genes including p53, p21, Bax and Bcl-2 family by RT-PCR. Results indicated that apoptosis occurred in HL-60, HS 313T, SW872, SW982 and TOV-21G cell lines treated with 25 µl/ml of FR-91 accompanied by the dose-dependent down-regulation of Bcl-2 gene expression, while others were not significantly changed (*Fig. 6*). In the present study, we demonstrated that the relative increase of apoptotic Bax/Bcl-2 ratio correlated well with FR-91-induced apoptosis in different human cell lines. It is possible that FR-91, through an appropriate signal, induces a conformational change in the Bax which moves to the mitochondrial membrane where it causes release of mitochondrial cytochrome c into the cytosol.

In conclusion, the use of FR-91 extracts exhibited an apoptosis-inducing effect in various human tumor cell lines. Although further studies must be performed to elucidate the mechanisms by which FR-91 induces apoptosis in tumor cell lines, the present data indicates that FR-91 might be a useful chemotherapeutic compound for patients with different types of tumors. ■



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