Effect of the Silybin-Phosphatidylcholine Complex (IdB 1016) on the Development of Mammary Tumors in HER-2/neu Transgenic Mice

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Abstract

Silybin, a main component of the milk thistle of *Silybum marianum*, has been reported to possess anticancer activity. We investigated the effects of IdB 1016, a complex of silybin with phosphatidylcholine, on the development of mammary tumors appearing spontaneously in HER-2/neu transgenic mice. The mechanisms involved in the antitumor effect of IdB 1016 were evaluated by studying the apoptosis, senescent-like growth arrest, intratumoral leukocyte infiltrate, and the expression of HER-2/neu and p53 in tumoral mammary glands from transgenic mice and in human breast SKBR3 tumor cells. The administration of IdB 1016 delayed the development of spontaneous mammary tumors, reduced the number and size of mammary tumor masses, and diminished lung metastasization in HER-2/neu transgenic mice. In tumoral mammary glands from IdB 1016–treated mice, a down-regulation of HER-2/neu gene expression was associated with an increased senescent-like growth arrest of tumor cells, and an increased infiltrate of neutrophils, CD4, and CD8 T cells. Both senescent-like growth arrest and apoptosis were significantly increased and were associated with a reduced $p185^{HER\text{-}2/neu}$ protein and an increased p53 mRNA in SKBR3 *in vitro* treated with IdB 1016 in comparison with control cells. The results show the antitumor effect of IdB 1016 in the development of spontaneous mammary tumors in HER-2/neu transgenic mice. The effect of IdB 1016 might be related to the down-regulation of HER-2/neu expression and the induction of senescent-like growth arrest and apoptosis through a p53-mediated pathway in tumor cells. [Cancer Res 2007;67(5):2022–9]

Introduction

There is an increasing interest in identifying potent preventive and therapeutic agents against breast cancer. Among the naturally occurring flavonoids, silymarin, a standardized extract of the milk thistle of *Silybum marianum*, has been found to exert high antitumor-promoting activity (1). Silybin, the main component of the silymarin complex, has been shown to have comparable antitumor effects and is as strong an antioxidant as silymarin (1). Most of the antitumor activity of silybin has been discovered through *in vitro* studies in tumor cell lines (2–5). In these studies, silybin was generally found to induce growth inhibition and apoptosis of a panel of human and murine tumor cell lines, and to potentiate the therapeutic potential of doxorubicin, cisplatin, and carboplatin. Fewer studies have been conducted on the antitumor effect exerted by *in vivo* supplementation with silybin or silymarin. Most data on the *in vivo* effects of these compounds have been drawn from studies done in mice treated with carcinogens or in nude mice bearing human xenografts (6–10); although informative, these data may not be entirely relevant to the development of most cancers in humans, in which the tumor is spontaneous and is initiated by the clonal expansion from a single cell *in vivo*.

The possible therapeutic application of silybin is hampered by its poor enteral absorption, which accounts for its very low bioavailability. IdB 1016 (silipide) is a complex of silybin with phosphatidylcholine. After oral administration, IdB 1016 gives plasma levels significantly higher than those found after the administration of silybin to rats (11, 12) or to humans (13). When tested in animal models, IdB 1016 was found to be very active in inhibiting the growth of human ovarian cancer xenografted into athymic mice and in potentiating the activity of suboptimal doses of cisplatin (14, 15). IdB 1016 has been shown to be very well tolerated and largely free of any adverse effects both in preclinical and in clinical studies.

The HER-2/neu proto-oncogene encodes a receptor-like transmembrane protein with a relative molecular mass of 185 kDa (p185) and homology to epidermal growth factor receptor (16). Overexpression of HER-2/neu is present in 15% to 40% of all human breast cancers and is associated with a poorer rate of survival and a higher risk for recurrent disease after primary therapy (17, 18). The causal relationship of this association is indicated by the evidence that transgenic mice that express activated neu develop multiple mammary tumors at an early age (19). Overexpression of HER-2/neu can lead to neoplastic transformation, as shown in transgenic mice, in which the activated neu transgene expression results in rapid conversion of the normal mammary epithelium to the malignant phenotype (20). Furthermore, overexpression rather than mutation of neu may be the primary mechanism contributing to breast cancer because examination of primary breast cancer biopsies has yet to reveal comparable activating mutations (21, 22).

For many years, the anticancer effect exerted by chemotherapeutic drugs has been related to the induction of apoptosis; more recently, it has been shown that an alternative pathway to cell death leading to permanent growth arrest in cancer cells may occur and may be involved in the anticancer effect of many substances. This phenomenon, called senescent-like growth arrest, is a cellular response that resembles replicative senescence occurring in normal cells and that may be crucial for protection against cancer development (23–25). A commonly used surrogate marker of senescence is the senescence-associated β-galactosidase

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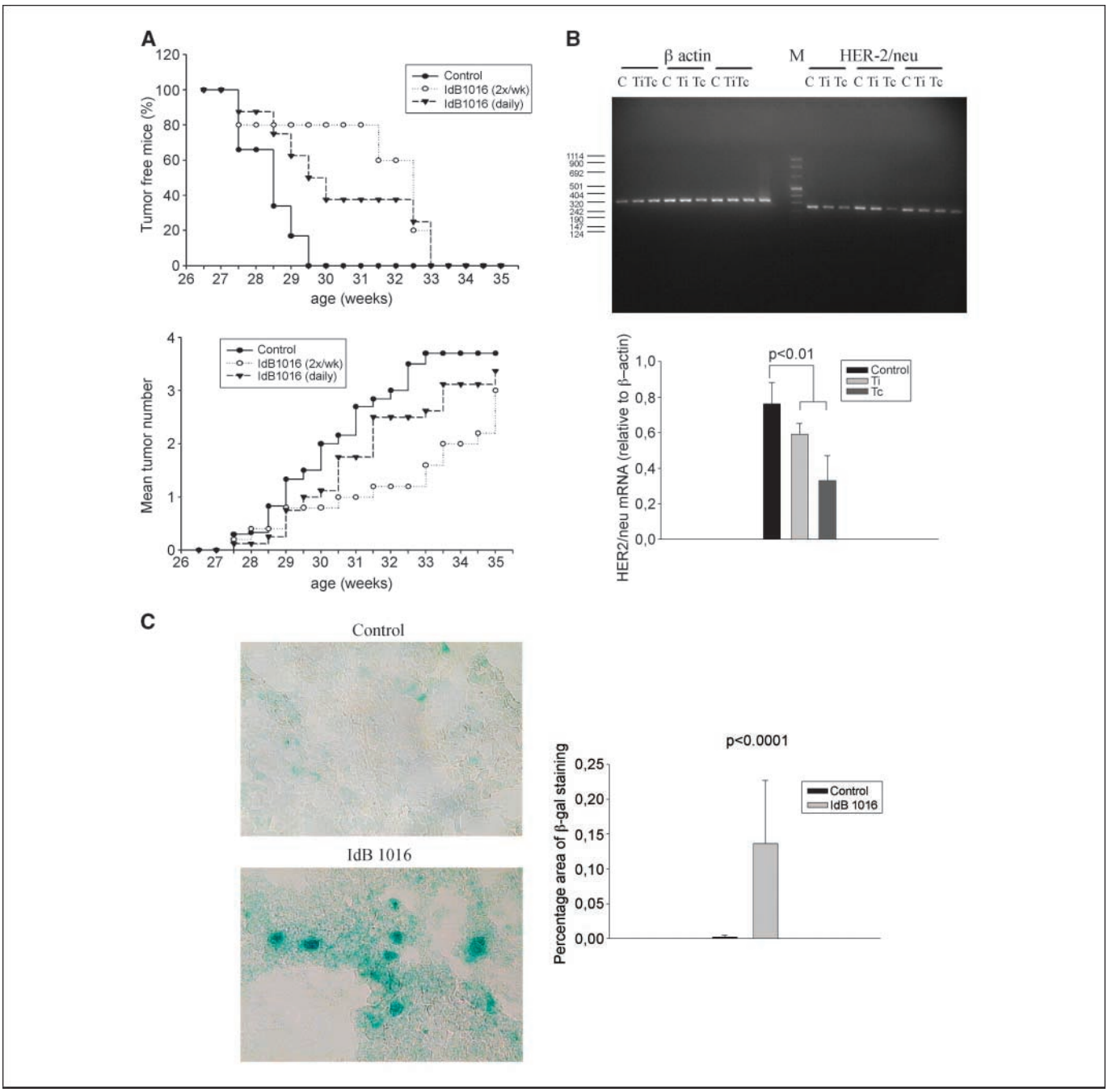

Figure 1. Effect of IdB 1016 administration on tumor incidence, HER-2/neu mRNA expression, and senescent-like growth arrest in tumoral mammary glands. *A,* HER-2/neu transgenic mice were supplemented daily or twice a week with IdB 1016 (450 mg/kg/d, equivalent to 414 μmol/L/kg of silybin) and analyzed for the kinetics of tumor incidence (percentage of tumor-free mice) and the tumor multiplicity (mean number of palpable mammary carcinomas per mouse). Difference in tumor incidence was significant between IdB 1016 (daily) versus control ($P < 0.05$) and IdB 1016 (twice a week) versus control ($P < 0.02$). Differences in tumor multiplicity were significant between IdB 1016 (daily) versus control from week 33 ($P < 0.05$), and IdB 1016 (twice a week) versus control from week 31 ($P < 0.02$). Data are from one of two experiments each done with 10 animals per group. *B,* mammary tumors from daily (*Tc*) or twice a week (*Ti*) IdB 1016–treated and control HER-2/neu transgenic mice were analyzed for HER-2/neu mRNA expression by reverse transcription-PCR. The RNA reverse-transcribed and amplified was normalized for β-actin expression in individual samples; *columns,* mean; *bars,* SD. Data are representative of one of three independent experiments involving three mice for each group. *C,* cryostat sections of tumoral mammary glands from control or IdB 1016 (twice a week)–treated mice were examined for the presence of senescent cells by SA-β-gal assay. A total of three tumors for each mice group were examined. For each tumor, two sections were analyzed. The evaluation of senescent cells relative to five fields at the microscope was quantified through image analysis; *columns,* mean percentage area of positive cells; *bars,* SD. Data are representative of one of two independent experiments.

(SA-β-gal). The SA-β-gal is a reagent required for identifying senescent cells using a staining procedure. The assay is based on a histochemical stain for β-gal activity at pH 6. Under these conditions, β-gal activity is easily detectable in senescent cells, but is undetectable in presenescent cells (26). Like other damage responses of normal cells, such as quiescence and apoptosis, senescent-like terminal proliferation arrest involves the function of wild-type p53 (27, 28).

The aim of the present article is to investigate the effect of IdB 1016 on the development of mammary tumors appearing spontaneously in HER-2/neu transgenic mice at an early age. The mechanisms involved in the antitumoral action were analyzed studying the effects determined by IdB 1016 on the expression of mRNA for HER-2/neu and p53, on the levels of $p185^{HER-2/neu}$ protein, on the induction of senescent-like growth arrest or apoptosis in tumor cells, and on the intratumoral leukocyte infiltrate.

Materials and Methods

Animals. FVB/N transgenic female mice for the activated rat neu oncogene were obtained from Charles River (Hollister, CA) and were maintained under specific pathogen–free conditions under a standard light/dark regimen (12 h light/12 h darkness) in our animal facilities. Mice were housed in plastic non–galvanized cages and fed with standard pellet food (Nossan, Italy) and tap water *ad libitum*. Procedures and facilities followed the requirements of Commission Directive 86/609/EEC concerning the protection of animals used for experimental and other scientific purposes. Italian legislation is defined in D.L. no. 116 of January 27, 1992.

Tumor cell lines. SKBR3 is a human breast adenocarcinoma cell line overexpressing human HER-2/neu. Confluent monolayers (5×10^5 cells/cm^2) of the tumor cell lines growing in their complete medium [DMEM plus 10% fetal bovine serum; all from Invitrogen S.R.L., san Giuliano Milanese (MI), Italy] were treated with a 0.25% solution of trypsin in PBS and used for *in vitro* experiments.

Experimental design. Female FVB/N HER-2/neu mice were analyzed in two different experiments. IdB 1016 (silipide) is a complex of silybin (2-[2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-1,4-benzodioxin-6-yl]-2,3-dihyidro-3,5,7-tri-hydroxy-4h-1-benzopyran-4-one) with phosphatidylcholine obtained by precipitation with n-hexane of an acetonic solution of silybin and phosphatidylcholine in a 1:1 molar ratio. In contrast to silybin, this complex is a light yellow powder freely soluble in aromatic hydrocarbons, ethers, and chlorinated solvents. IdB 1016 (supplied by INDENA, Milan, Italy) was suspended in 0.5% carboxymethylcellulose in sterile water at a concentration of 45 mg/mL, and was administered at a dose of 450 mg/kg, equivalent to 414 μmol/L/kg silybin, through oral gavage. Mice, at the age of 24 weeks, were randomly divided into three groups which received (*a*) IdB 1016 daily for 1 month, (*b*) IdB 1016 twice a week for 1 month for a total of eight administrations, and (*c*) carboxymethylcellulose (0.5%) in sterile water (control group). Twice a week, all mice were palpated for the detection of mammary tumors. The neoplastic masses were measured with calipers in the two perpendicular diameters. Progressively growing masses of >3 mm in mean diameter were regarded as tumors. In each group of mice, the number of lung metastases present at the end of the treatment was evaluated using China ink. HER-2/neu expression was evaluated in tumor masses of 4 to 5 mm of diameter. In *in vitro* experiments, SKBR3 cells, after overnight serum deprivation, were treated with IdB 1016 at doses ranging from 7.9 to 126.5 μg/mL (corresponding to 2.25-44 μmol/L silybin) for the times indicated. In selected experiments, SKBR3 tumor cells were treated with silybin (supplied by INDENA) at the concentrations of 25, 50, or 100 μmol/L.

Quantification of apoptosis by flow cytometry. Apoptosis was measured through (*a*) detection of mitochondrial permeability transition event (early apoptosis), and (*b*) subdiploid DNA peak analysis after staining with propidium iodide (late apoptosis). For evaluation of the change in the mitochondrial membrane potential, cells were stained with JC-1 according to the protocol provided in the MitoPT Kit (Alexis Italia, Vinci-Biochem, Fi, Italy). For subdiploid DNA evaluation, cells were washed twice in PBS after incubation and resuspended in 0.75 mL of hypotonic fluorochrome solution containing 50 μg/mL of propidium iodide (Calbiochem, La Jolla, CA) in 0.1% sodium citrate plus 0.1% Triton X-100 (Sigma-Aldrich S.R.L., Milano, Italy) in polypropylene tubes (Nunc, VWR International S.R.L., Milano, Italy). The tubes were placed at 4°C in the dark overnight before the flow-cytometric analysis (29). The propidium iodide fluorescence of individual nuclei was determined using a XL flow cytometer (Coulter, Hialeah, FL) and the percentage of apoptotic nuclei was determined on the basis of the number of subdiploid DNA peak in the DNA fluorescence histogram.

RNA extraction and reverse transcription-PCR. The expression of mRNA for HER-2/neu was evaluated in mammary tumor masses from IdB 1016–treated or untreated mice by reverse transcription-PCR. After homogenization of tissue samples, RNA was extracted using TRI-REAGENT according to the manufacturer's instructions (Sigma-Aldrich). After treatment with DNase (Invitrogen, San Diego, CA), RNA concentrations were determined by spectrophotometer (Scientific Instruments UV1601 Shimadzu, Columbia, MD). cDNA was synthesized from 0.1 μg of RNA incubating RNA with deoxynucleotide triphosphate (0.5 mmol/L), Oligo dT (12.5 ng/μL), First Strand buffer (1×), Moloney murine leukemia virus reverse transcriptase (10 units/μL), RNase inhibitor (1units/μL), and DTT (0.01 mmol/L) all from Invitrogen S.R.L., in a final volume of 20 μL. The samples were incubated at 37°C for 1 h and 95°C for 10 min; subsequently, cDNA was frozen at −20°C until use. PCR was done incubating 5 μL of cDNA with a reaction mixture containing: PCR buffer (1×), $MgCl_2$ (1.5 mmol/L), deoxynucleotide triphosphate (200 μmol/L), specific forward and reverse primers (0.8 μmol/L of each), Taq DNA

Table 1. Effect of supplementation with IdB 1016 on lung metastasizing and on leukocyte reactive infiltrate in HER-2/neu transgenic female mice

	Control	IdB 1016 (twice a week)	IdB 1016 (daily)	*P*
Lung metastases				
Mean no. of metastases	3.33	2.00	3.33	Not significant
Mice with metastases (%)	83	60*	67	0.01
Mean size of metastases (mm)	2.1	1*	1	0.01
Maximum size of metastases (mm)	4	1	1	Not significant
Leukocyte infiltrate †				
Neutrophil	1.2 ± 0.5	4.8 ± 1.8		0.008
Macrophage	48.3 ± 5.0	39.5 ± 21.4		Not significant
CD4+	4.4 ± 0.7	8.5 ± 0.7		0.0008
CD8+	2.1 ± 0.6	7.2 ± 0.7		0.0001

*Difference with control mice was significant ($P < 0.01$).
†Cell counts were determined at ×400 in 0.180 mm^2/field. At least five fields/tissue were evaluated. Values are expressed as the mean ± SD.

polymerase (1 unit/μL) in a total volume of 50 μL (Promega Italia, Milano, Italy). The samples were incubated in a GeneAmp PCR System 9700 (Perkin-Elmer S.p.A., Monza (MI), Italy) for a total of 35 cycles for HER-2/neu, and 30 cycles for β-actin. Each cycle consisted of 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C for HER-2/neu; 1 min at 94°C, 2 min at 63°C, and 1 min at 72°C for β-actin.

The primers for rat HER-2/neu, human p53, and human or mouse β-actin were purchased from Invitrogen S.R.L. using DNA published cDNA sequences. The rat HER-2/neu fragment of 220 bp was defined by the forward primer, 5′-CTGGAGGACGTGCCGCTTGTA; and the reverse primer, 5′-ATAGCTCCACACATCACTCTG. The human p53 fragment of 214 bp was defined by the forward primer, 5′-GGCCCCTCCTCAGCATCTTAT; and the reverse primer, 5′-GTCTTCCAGTGTGATGATGGTG. Mouse β-actin fragment of 265 bp by the forward primer, 5′-TGGAATCCTGTGGCATCCATGAAAC; and the reverse primer, 5′-TAAAACGCAGCTCAGTAACAGTCCG; human β-actin fragment of 77 bp by the forward primer, 5′-GCGAGAAGATGACCCAGATC; and the reverse primer, 5′-GGATAGCACAGCCTGGATAG. The PCR products and a molecular weight standard [DNA molecular weight marker VIII, Roche S.p.A., Monza (MI), Italy] were visualized after electrophoresis in a 1.5% agarose gel containing 1 μg/μL ethidium bromide. Densitometric analysis was done using the GelDoc 2000 (Bio-Rad Laboratories, Italy).

Flow cytometric measurement of $p185^{HER\text{-}2/neu}$. Subconfluent cultures of SKBR3 tumor cells were starved overnight removing serum and were treated with 31.6, 63.2, or 126.5 μg/mL of IdB 1016 for 24 h. To evaluate $p185^{HER\text{-}2/neu}$ protein through flow cytometry, tumor cells were washed with a solution of PBS, 5% FCS, and 0.04% NaN_3. SKBR3 tumor cells were incubated with a FITC-labeled anti-$p185^{HER\text{-}2/neu}$ monoclonal antibody (Ab-3; Oncogene Science, Cambridge, MA) for 30 min at 4°C. The cell pellet was washed with PBS with 0.1% NaN_3 and resuspended in Isoton II (Coulter). FITC-labeled mouse IgG (PharMingen, San Diego, CA) was used as isotype control. Cell fluorescence was analyzed through a XL flow cytometer (Coulter).

SA-β-gal staining and image analysis. Visualization of SA-β-gal for the detection of senescent cells was done using the Senescent Cells staining kit (Sigma-Aldrich) according to the manufacturer's instructions. Relative numbers of stained cells were determined in independent countings and analyzed statistically. The digital images of *in vivo* and *in vitro* samples obtained from Leica Imaging Systems Ltd. (Cambridge, United Kingdom) were imported to Image J program (version 1.24o, NIH) and converted to 8-bit grayscale images. Image analysis was then used to determine the percentage area of β-gal staining. Quantitative studies of β-gal–positive cells were done by evaluating four randomly chosen fields in each sample. The area of β-gal–positive cells was measured after thresholding to determine the absorbance that corresponded to specific staining of positive cells. Subsequently, the percentage area of β-gal–positive cells was calculated by dividing the area of the reaction product by the total area of the measured field.

Morphologic analysis. Groups of three mice bearing a 3 × 3 mm mammary tumor were killed for each group. Tumor tissues were embedded in Tissue-Tek optimum cutting temperature compound (Miles, Inc., Elkhart, IN) frozen in liquid nitrogen and preserved at −80°C until sectioning. For immunohistochemistry, 4-μm cryostat sections were fixed in acetone (at −20°C) for 5 to 10 min and washed twice with PBS. Endogenous peroxidase was quenched with 0.1% hydrogen peroxide in methanol for 5 min. The slides were washed in PBS and incubated in normal blocking serum for 20 min to prevent nonspecific binding; they were washed again and immunostained with purified rat anti-mouse monoclonal antibody to CD8a (Ly-2), CD4 (L3T4), MAC-3, and Ly-6G (Gr-1; PharMingen) for 30 min at room temperature. Antibody binding was localized using an anti-rat biotinylated secondary antibody (Vector Laboratories, Burlingame, CA), StreptABComplex conjugated with alkaline phosphatase (Dako Italia, Milan, Italy), and Fuchsin Substrate-Chromogen System (Dako). Staining with isotype-matched rat IgG was done as a negative control. Slides were counterstained with hematoxylin. Quantitative studies of the immunohistochemically stained reactions were done by evaluating five randomly chosen fields in each sample. Individual cells were counted under a ×400 microscopic field (×40 objective and ×10 ocular lens: 0.180 mm^2/field).

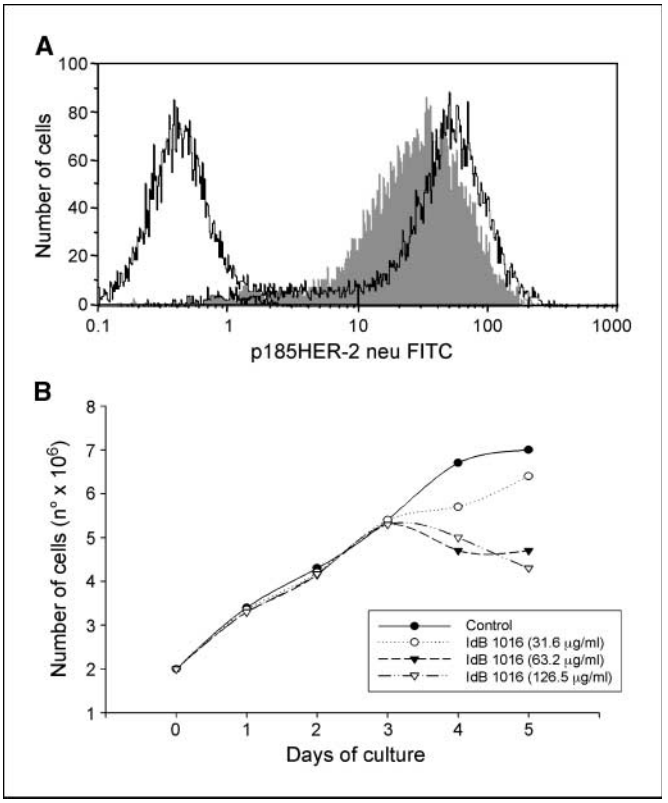

Figure 2. Effect of *in vitro* IdB 1016 treatment on the growth and protein expression of $p185^{HER\text{-}2/neu}$ in the human SKBR3 tumor cell line. *A,* after overnight starvation, subconfluent cultures of SKBR3 cells were supplemented with 31.6, 63.2, or 126.5 μg/mL of IdB 1016 (corresponding to 11, 22, or 44 μmol/L of silybin) for 24 h and analyzed for $p185^{HER\text{-}2/neu}$ protein levels. *Shaded histograms,* 100 μmol/L IdB 1016–treated tumor cells; *continuous lines*, isotypic control (*left*) and untreated tumor cells (*right*). At least 10,000 events were counted. Data are representative of one of three independent experiments. *B,* SKBR3 tumor cells were treated with 31.6, 63.2, or 126.5 μg/mL of IdB 1016 and analyzed for cell growth. On day 0, SKBR3 were seeded in culture flasks in complete medium and were cultured for 24 h; after overnight incubation without serum to synchronize their cell cycle, cells were treated without or with the indicated amounts of IdB 1016 in complete medium. Differences between 63.2 and 126.5 μg/mL IdB 1016–treated and control tumor cells were statistically significant ($P < 0.05$).

Statistical analysis. Differences in tumor incidence were evaluated by the Mantel-Haenszel log-rank test; differences in tumor multiplicity were evaluated by Student's *t* test; differences in lung metastasization were evaluated by Fisher exact test, and differences in mRNA expression and in immune variables were evaluated by Student's *t* test.

Results

Effect of IdB 1016 administration on the kinetics of tumor growth and on lung metastasization. As shown in Fig. 1*A* (*top*), the first mammary tumor appeared in untreated mice at 27.5 weeks of age. The tumor incidence increased progressively with increasing age of mice, affecting 50% or 100% of tumor-bearing mice at 28.5 or 29.5 weeks of age, respectively. In HER-2/neu mice treated with both continuous or intermittent IdB 1016, the appearance of the first mammary tumor was not significantly delayed. However, a slower kinetic of tumor incidence was found in treated mice compared with control mice. At week 29.5, when 100% of control mice were bearing one or more tumor masses, 80% or

50% of IdB 1016–supplemented mice through intermittent or continuous administration, respectively, were still tumor-free. Fifty percent of the mice bore tumors at 29.5 or 32.5 weeks of age in following continuous ($P < 0.05$ versus control) or intermittent ($P < 0.02$ versus control) IdB 1016, respectively. All mice bore tumors at week 33. The kinetics of tumor development were similar in the two experiments done.

As shown in Fig. 1*A* (*bottom*), the mean number of mammary tumors progressively increased in control mice. A significant reduction of the mean tumor number was observed in IdB 1016–treated mice either through continuous or intermittent administration. Differences in tumor multiplicity were significant between IdB 1016 (daily) versus control from week 33 ($P < 0.05$) and IdB 1016 (twice a week) versus controls from week 31 ($P < 0.02$).

As shown in Table 1, IdB 1016 reduced the percentage of mice with metastasis and the mean size of lung metastasis in comparison with control mice. The difference was statistically significant only for intermittent IdB 1016 administration ($P < 0.01$).

Effect of *in vivo* IdB 1016 administration on leukocyte tumor infiltrate. We analyzed leukocyte infiltrate in the mammary tumor samples from control and IdB 1016–treated mice. As shown in Table 1, IdB 1016 administration was associated with an increased number of neutrophils ($P < 0.008$), CD4 ($P < 0.0008$), and CD8 ($P < 0.0001$) lymphocytes, in comparison with control mice.

Effect of *in vivo* IdB 1016 administration on HER-2/neu expression. To evaluate the effect of IdB 1016 on tumor cells, we studied mRNA expression for HER-2/neu on tumor cells following reverse transcription-PCR of total RNA extracted from mammary tumors of IdB 1016–treated and control mice. As shown in Fig. 1*B*, the expression of mRNA for HER-2/neu was reduced in mammary tumors from IdB 1016–treated mice compared with control mice [mean relative expressions versus β-actin were 0.76 ± 0.12, 0.59 ± 0.06, and 0.33 ± 0.14; mean ± SD, for samples from controls, IdB 1016-intermittently treated (Ti) and IdB 1016-continuously treated (Tc) mice; $P < 0.01$ versus controls].

Induction of a senescence-like phenotype by *in vivo* administration of IdB 1016. As shown in Fig. 1*C* (*left*), IdB 1016 treatment increased the number of cells with a senescence-like phenotype in mammary tumor samples, as evidenced by the augmented number of cells with enlarged and flattened morphology and with increased SA-β-gal staining. Image analysis processing showed a significantly increased percentage area of SA-β-gal staining in tumor masses from IdB 1016–treated in comparison with control mice (0.136 ± 0.09 versus 0.002 ± 0.003; $P < 0.0001$; Fig. 1*C, right*).

Effect of *in vitro* IdB 1016 treatment on p185$^{HER-2/neu}$ protein. We then analyzed the p185 protein on SKBR3 tumor cells after 24 h of incubation with IdB 1016. As shown in Fig. 2*A*, the expression of p185$^{HER-2/neu}$ was reduced on SKBR3 cells after treatment with 126.5 μg/mL of IdB 1016 (equivalent to 44 μmol/L of silybin, mean fluorescence values: 35.7 and 23.0 for control and IdB 1016–treated tumor cells, respectively). A similar reduction of p185$^{HER-2/neu}$ protein on tumor cells was observed using 31.6 or 63.2 μg/mL of IdB 1016 (equivalent to 11 or 22 μmol/L of silybin; data not shown). The 24-h incubation of SKBR3 with silybin reduced the p185$^{HER-2/neu}$ protein to a concentration of only 100 μmol/L (data not shown).

Induction of a senescence-like phenotype by *in vitro* treatment with IdB 1016. To evaluate the effect of IdB 1016 on tumor cell growth, 2×10^6 SKBR3 cells were seeded in culture flasks and cultured for 2 days, they were then starved overnight removing serum and, on day 3, IdB 1016 was added. As shown in Fig. 2*B*, untreated SKBR3 cells continued their progressive growth on days 4 and 5 of culture; in the presence of 31.6 μg/mL of IdB 1016 (equivalent to 11 μmol/L of silybin), SKBR3 tumor cells showed a slower kinetics of growth in comparison with control cultures; 63.2 and 126.5 μg/mL of IdB 1016 (equivalent to 22 and 44 μmol/L of silybin) induced a growth arrest in SKBR3 tumor cells. As shown in Fig. 3*A*, IdB 1016–treated SKBR3 tumor cells showed an increased number of cells with senescence-like

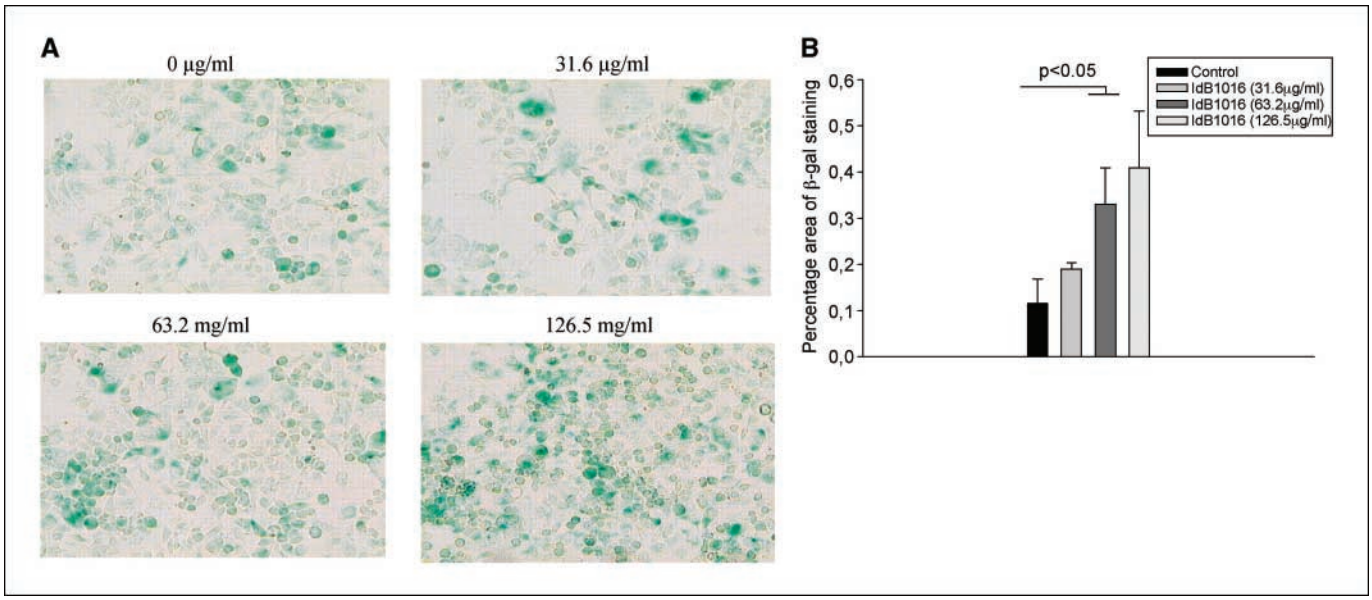

Figure 3. Effect of *in vitro* IdB 1016 treatment on senescent-like growth arrest in a human SKBR3 tumor cell line. *A,* SKBR3 tumor cells were cultured without or with 31.6, 63.2, or 126.5 μg/mL IdB 1016 (corresponding to 11, 22, or 44 μmol/L silybin) and were examined for the presence of senescent cells by SA-β-gal assay. *B,* the evaluation of senescent cells relative to five fields at the microscope was quantified through image analysis; *columns,* mean percentage area of positive cells; *bars,* SD. Data are representative of one of two independent experiments. Differences between 63.2 and 126.5 μg/mL IdB 1016–treated and control tumor cells were statistically significant ($P < 0.05$).

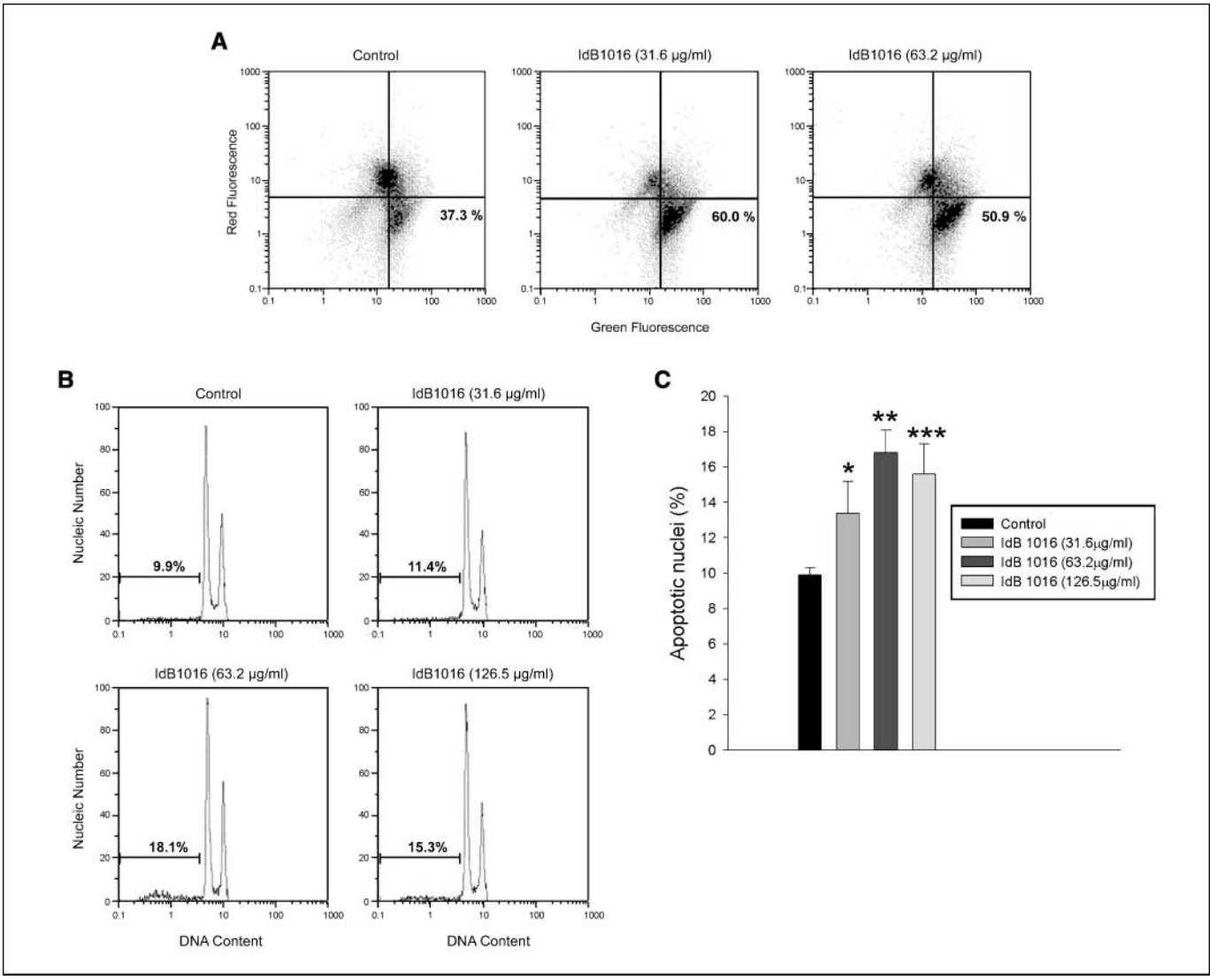

Figure 4. Effect of *in vitro* IdB 1016 treatment on apoptosis in a human SKBR3 tumor cell line. SKBR3 tumor cells were cultured in medium alone or supplemented with 31.6, 63.2, or 126.5 μg/mL IdB 1016 (corresponding to 11, 22, or 44 μmol/L silybin) for 24 h and analyzed for the number of apoptotic cells following mitochondrial permeability transition event (*A*) or propidium iodide staining (*B*). *Columns,* mean percentage of subdiploid nuclei number as assessed by propidium iodide staining; *bars,* SD (*C*). *, $P = 0.01$; **, $P = 0.0001$; ***, $P = 0.0007$, statistical significance versus controls. At least 10,000 events were counted. Results are representative of one of three independent experiments.

phenotype. As evidenced by image analysis processing, the increase in senescence-like cells was statistically significant in 63.2 and 126.5 μg/mL of IdB 1016 cultures in comparison with control cultures ($P < 0.05$).

Effect of *in vitro* IdB 1016 treatment on apoptosis of SKBR3 tumor cell line. To gain insight into the mechanism of action of IdB 1016, we assessed SKBR3 tumor cells cultured in the presence or absence of IdB 1016 for features of apoptotic cells. As shown in Fig. 4*A*, 24-h IdB 1016 treatment increased the percentage of early apoptosis in SKBR3 tumor cells, as evidenced by the higher number of cells with reduced mitochondrial membrane potential ($\Delta\Psi$). The staining with propidium iodide evidenced an increased number of subdiploid DNA peaks in 48-h IdB 1016–treated SKBR3 cells (Fig. 4*B* and *C*). No increase of apoptosis was observed in 24-h IdB 1016–treated cells by propidium iodide staining (data not shown). The incubation of SKBR3 with silybin induced apoptosis only at a concentration of 100 μmol/L (data not shown).

Effect of *in vitro* IdB 1016 treatment on p53 mRNA expression in the SKBR3 tumor cell line. To establish whether the effect of IdB 1016 on senescent-like growth arrest and on apoptosis was related to a modulation of p53 expression, we analyzed p53 mRNA expression in SKBR3 tumor cells after *in vitro* IdB 1016 treatment. As shown in Fig. 5*A*, the expression of p53 mRNA was increased in SKBR3 cells treated for 5 h with 126.5 μg/mL of IdB 1016 (equivalent to 44 μmol/L of silybin; relative expression of p53 versus β-actin was 0.20 ± 0.03, 0.55 ± 0.41, 0.28 ± 0.14, and 3.43 ± 0.89; mean ± SD; for samples from control, 31.6, 63.2, or 126.5 μg/mL IdB 1016; $P = 0.03$ for 126.5 μg/mL IdB 1016 versus control). After *in vitro* overnight incubation, p53 mRNA expression was increased in SKBR3 cells treated with low IdB 1016 doses (7.9 and 15.8 μg/mL), with a plateau at IdB 1016 concentrations of 31.6, 63.2, or 126.5 μg/mL (relative expression of p53 versus β-actin was 1.26 ± 0.11, 1.98 ± 0.26, 2.05 ± 0.49, 2.30 ± 0.62, 2.63 ± 0.40, and 2.65 ± 0.35; mean ± SD, for samples

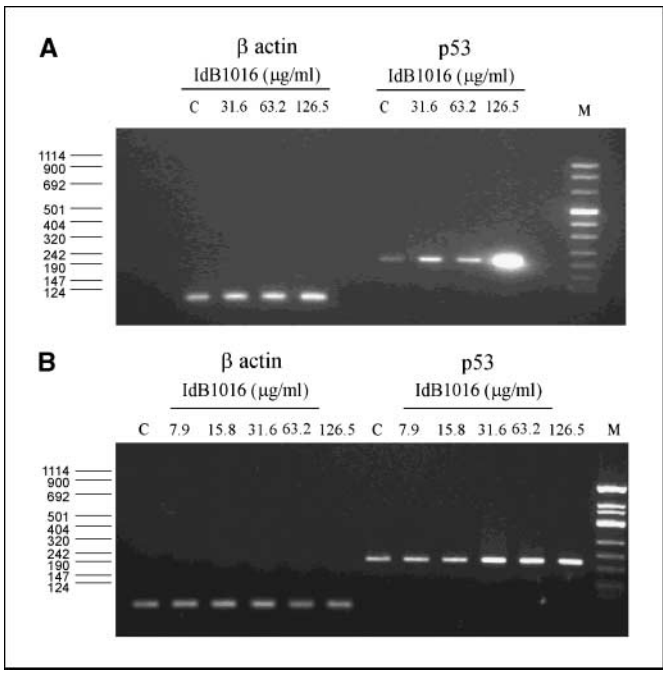

Figure 5. Effect of *in vitro* IdB 1016 on the expression of p53 mRNA in SKBR3 tumor cells. SKBR3 tumor cells were cultured for 5 h (*A*) without or with 31.6, 63.2, or 126.5 μg/mL IdB 1016 (corresponding to 11, 22, or 44 μmol/L silybin) or for 24 h (*B*) without or with 7.9, 15.8, 31.6, 63.2, or 126.5 μg/mL IdB 1016 (corresponding to 2.25, 5.5, 11, 22, or 44 μmol/L silybin) and were examined for p53 mRNA expression by reverse transcription-PCR. The RNA reverse-transcribed and amplified was normalized for β-actin expression in individual samples. Relative expressions versus β-actin were: (*A*) 0.20 ± 0.03, 0.55 ± 0.41, 0.28 ± 0.14, and 3.43 ± 0.89; mean ± SD, for samples from control, 31.6, 63.2, or 126.5 μg/mL IdB 1016 ($P = 0.03$ for 126.5 μg/mL IdB 1016 versus control); (*B*) 1.26 ± 0.11, 1.98 ± 0.26, 2.05 ± 0.49, 2.30 ± 0.62, 2.63 ± 0.40, and 2.65 ± 0.35; mean ± SD, for samples from control, 7.9, 15.8, 31.6, 63.2, or 126.5 μg/mL IdB 1016 (at least $P < 0.05$ versus control). Data shown are representative of one of three independent experiments.

from control, 7.9, 15.8, 31.6, 63.2, or 126.5 μg/mL IdB 1016; at least $P < 0.05$ versus control; Fig. 5*B*).

Discussion

The results reported in this article show for the first time the antitumor activity of IdB 1016, a complex of silybin and phosphatidylcholine, in a spontaneous breast cancer tumor model. The data shows that (*a*) IdB 1016 administration delays the development of spontaneous mammary tumors and reduces the number of mammary tumor masses and the number of lung metastases in HER-2/neu transgenic mice; (*b*) that IdB 1016 down-regulates HER-2/neu gene expression, induces p53 expression, and increases senescent-like growth arrest and apoptosis in tumoral mammary glands and in human breast SKBR3 tumor cell line.

HER-2/neu transgenic mice spontaneously develop tumors in their mammary glands at an early age (19, 30). We show that the administration of IdB 1016 before the appearance of palpable mammary tumors determines a significant slowing down of the kinetic of tumor development, a lower number of tumor masses, and a lower frequency and size of lung metastasis. Because in FVB/N transgenic mice, mammary tumors are histologically evident at 12 weeks of age (data not shown), and the IdB 1016 treatment was started in 24-week-old mice, our data suggests that the antitumoral effect of IdB 1016 in our experimental model may be considered therapeutic rather that chemopreventive. IdB 1016 is a silybin-phosphatidylcholine complex able to increase the bioavailability and therapeutic efficiency of silybin. After oral administration, IdB 1016 has been shown to give plasma levels significantly higher than those found after the administration of silybin or silymarin to rats (11, 12) or to humans (13). IdB 1016 has been shown to be very well tolerated and largely free of any adverse effect both in preclinical and in clinical studies. Our data on the antitumoral effect of IdB 1016 in HER-2/neu transgenic mice confirm and extend previous studies conducted in transplanted mouse models. In these studies, IdB 1016 administration was found to inhibit the growth of human ovarian cancer xenografts in nude mice and to potentiate the antitumor activity of cisplatin (14, 15).

The effectiveness of silybin as an anticancer agent has been previously reported to be related to its cytoprotective activity, antiproliferative effects on cancer cells, and to the induction of tumor cell apoptosis. In this study, we extend the actual knowledge on the mechanism of action of silybin, providing further potential mechanisms involved in its anticancer effect. In our experimental model, IdB 1016 was able to down-regulate HER-2/neu expression inducing apoptosis and senescent-like terminal proliferation arrest both *in vivo* and *in vitro*. IdB 1016 induced increased levels of apoptosis in SKBR3 tumor cells. However, levels of cell death were low and did not completely explain the effect of IdB 1016 on tumor development; furthermore, a significant increase of apoptosis was not found after examining mammary tumor masses from IdB 1016–treated versus control mice (data not shown). It has been suggested that senescent-like growth arrest may be a significant determinant of tumor response to anticancer agents in conditions in which the induction of cell death plays a minimal role in their antiproliferative effect (23, 25). Our data show that IdB 1016 induces senescent-like growth arrest both *in vivo* and *in vitro*. In fact, an increased number of cells positive for SA-β-gal were found in mammary tumors from IdB 1016–treated mice. Furthermore, *in vitro* IdB 1016 was able to slow down the kinetics of growth and to increase the number of SA-β-gal–positive cells in the SKBR3 tumor cell line. Both apoptosis and senescent-like growth arrest were associated with a down-regulation of HER-2/neu expression in IdB 1016–treated mice and in SKBR3 cultures. The relationship between reduced expression of HER-2/neu oncogene expression and induction of apoptosis was previously reported by us (30) and others (31) in other experimental models. To our knowledge, this is the first study showing a correlation between down-regulation of the HER-2/neu oncogene and the activation of senescent-like growth arrest.

Both apoptosis and accelerated senescence are regulated largely by p53. In cancer cells, although the senescent-like growth arrest does not seem to be dependent on functional p53, ectopic p53 expression has been shown to promote cellular senescence (32). Furthermore, introduction of wild-type p53 into a p53 null H299 lung cancer cell line was able to induce senescent-like growth arrest, with no evidence of apoptosis (33). Our data on the increased expression of p53 in IdB 1016–treated SKBR3 tumor cells agrees with the above findings showing the promoting activity of p53 in inducing senescent-like growth arrest.

In transgenic mice, the overexpression of HER-2/neu is under the control of the mouse mammary tumor virus promoter (20), which has been found to be hormone-dependent (34). Because silybin can bind to a purified steroid receptor and because the estrogenic effects of silymarin have been observed in

ovariectomized rats (35), we did experiments to exclude the potential effect of IdB 1016 on the promoter of HER-2/neu transgene. As we have reported in a study on another natural substance with estrogenic properties (30), the evidence that IdB 1016 *in vitro* modulates the HER-2/neu expression in SKBR3 tumor cells, in which HER-2/neu is not under the control of mouse mammary tumor virus promoter, clearly shows that IdB 1016 directly modulates the expression of HER-2/neu gene. In order to compare the effect of the phospholipid complex IdB 1016 with silybin, in selected experiments, we tested the activity of silybin on $p185^{HER-2/neu}$ protein and apoptosis. The data obtained, showing the ability of silybin to down-regulate $p185^{HER-2/neu}$ protein and to induce apoptosis in SKBR3 tumor cells only at the higher silybin concentration tested (100 μmol/L), confirm the lower bioavailability of silybin in comparison with IdB 1016.

The increased number of neutrophils, CD4, and CD8 lymphocytes found in mammary tumors from IdB 1016–treated in comparison with control animals suggests a local immune activation, even if the direct or indirect effect of IdB 1016 on immune cells remains to be further evaluated.

In conclusion, this study shows that IdB 1016 may exert important antitumor effects delaying the development and metastasizing capacity of tumors in mice transgenic for the HER-2/neu oncogene. The antitumor effect of IdB 1016 might be related to the down-regulation of HER-2/neu expression and the induction of senescent-like growth arrest and apoptosis through a p53-mediated pathway in HER-2/neu overexpressing mammary tumors and in tumor cells.

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