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Antiangiogenic and antitumor activities of berberine derivative NAX014 compound in a transgenic murine model of HER2/neu-positive mammary carcinoma

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Abstract

Berberine (BBR) is a natural isoquinoline alkaloid with proven antiangiogenic and anticancer activities. We recently demonstrated that BBR and its synthetic derivative 13-(4-chlorophenylethyl)berberine iodide, NAX014, exert antiproliferative activity against HER2-overexpressing breast cancer cells, inducing apoptosis, modulating the expression of cell cycle checkpoint molecules involved in cell senescence, and reducing both HER2 expression and phosphorylation on tumor cells. In this study, we examined the anticancer properties of BBR and NAX014 in a transgenic mouse model which spontaneously develops HER2-positive mammary tumors. Repeated intraperitoneal injections of a safety dose (2.5 mg/kg) of NAX014 delayed the development of tumors, reducing both the number and size of tumor masses. In vivo sidestream dark field videomicroscopy revealed a significant lower vessel density in mammary tumors from NAX014-treated mice in comparison with the control group. Immunohistochemical evaluation using CD34 antibody confirmed the reduced vessel density in NAX014 group. Statistically significant increase of senescence associated β-galactosidase and p16 expression, and reduced expression of heparanase were observed in tumors from NAX014-treated mice than in tumors from control animals. Finally, NAX014 treatment decreased the level of perforine and granzyme mRNA in mammary tumors. Berberine did not show any statistically significant modulation in comparison with control mice. The results of the present study indicate that NAX014 is more effective than BBR in exerting anticancer activity delaying the development of mammary tumors in mice transgenic for the HER-2/neu oncogene. The antitumor efficacy of NAX014 is mainly related to its effect on tumor vascular network and on induction of tumor cell senescence.

Introduction

Breast cancer is the most frequent cancer among women with an estimated 1.67 million new cancer cases diagnosed in 2012 (1). The HER2 receptor is over-expressed in 20–30% of invasive breast cancer and is associated with aggressive phenotype, lower survival rate and higher risk for recurrent disease after primary therapy.

Many bioactive plant-derived compounds have gained widespread attention for their potential role in preventing, inhibiting
and reversing the progression of cancer disease. Several in vitro and in vivo studies showed the antitumor properties of a large number of natural compounds versus a wide range of tumor types (2). We previously demonstrated the anticancer effect of resveratrol, silybin-derived complex and annatto-tocotrienols on the development and metastatization of HER2/neu-overexpressing breast cancer in a transgenic murine model (3–5).

The naturally occurring isoquinoline alkaloid berberine (BBR) is a promising phytochemical found in a wide variety of traditional herbs used in Traditional Chinese Medicine (6). BBR has shown a wide range of pharmacological effects, including both in vitro and in vivo anticancer properties (7).

In cell culture studies, BBR showed a proapoptotic activity (8,9) and was able to induce cell cycle arrest and a senescent-like phenotype (5,10). In vitro studies showed its antiangiogenic activity (11,12) and the ability to reduce the expression of heparanase (13), an endoglycosidase whose over-expression in tumor cells confers an invasive phenotype and induces an angiogenic response in vivo (14). The in vivo anticancer effect of BBR has only been investigated so far in murine models transplanted with tumor cells, while no data exist from spontaneous cancer models that more closely reproduce the human disease.

We recently showed that anticancer properties of BBR could be enhanced by the introduction of chemical modifications in appropriate positions of BBR skeleton. BBR-derived compounds NAX012 and NAX014, which have aromatic moieties bonded to the 13-position of parent alkaloid, exerted a higher cytotoxic effect than BBR in HER-2 overexpressing breast cancer cells by inducing both apoptosis and cellular senescence (5). This effect was associated with a greater reduction in HER-2 expression and phosphorylation as compared to BBR, suggesting a potential link between HER-2 downregulation and antitumor in vitro activity (5). Similarly, cell cycle arrest, apoptosis and autophagy were observed after treatment with BBR derivatives NAX012, NAX014 and NAX018 in human colon carcinoma cells (15).

The aim of this study was to investigate the acute and chronic dose-dependent toxicity of BBR and NAX014 compounds in Friend Virus B-type (FVB) mice and to evaluate the effect of the administration of a safety dose of BBR and NAX014 on the development of spontaneous mammary tumors in HER-2/neu transgenic mice. Furthermore, the induction of cellular senescence and apoptosis in tumors and the changes in tumor vascularization, immune infiltration, heparanase and HER-2/neu expression were examined to identify the mechanisms involved in the antitumoral action of BBR and/or NAX014.

Materials and methods

**Chemicals**

BBR and NAX014 (Figure 1A) were provided by Naxopharma. 13-[4-chlorophenyl(ethyl)]berberine iodide (NAX014) was prepared and characterized as previously reported (16) starting from commercial BBR chloride hydrate (ca. 17% H2O), which was purchased from Shanghai Trust & We, China. The purity (>97%) of the derivative NAX014 was assessed by HPLC on a Jasco system LC-2000 series (Jasco, Europe) with an Agilent Eclipse XDBC-C18 (4.6×150×3.5 mm) column (Agilent Technologies). The flow rate of the mobile phase (50% water, 50% acetonitrile plus 0.1% trifluoroacetic acid) was maintained at 1 ml/min and absorbance was measured at 235, 265, 340 and 420 nm. The structure was confirmed by HNMR ((200 MHz, DMSO-d6); 10.02 (s, 1H), 9.87 (s, 1H), 9.86 (s, 1H), 8.33 (d, 1H), 8.24 (d, 1H), 7.95 (d, 1H), 7.38 (d, 2H), 7.22 (d, 2H), 7.05 (s, 2H), 6.16 (s, 2H), 4.12 (s, 3H), 4.11 (s, 3H), 4.02 (m, 2H), 3.29 (t, 2H), 2.88 (m, 4H).

**Cell lines and animals**

Human breast adenocarcinoma SK-BR-3 cell line was obtained from American Type Culture Collection (Rockville, MD) through IZSLER Biobank (Brescia, Italy) (IZSLER Cell Bank code BT.1 C156). After resuscitation, cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin (all from Invitrogen, Milan, Italy), and frozen for future use. Before experiments, SK-BR-3 cells were characterized for cell morphology, growth rate and HER-2 expression. All experiments were performed within 3 months after cells thaw and mycoplasma contamination was monitored by Hoechst DNA staining. FVB female mice and FVB/N HER-2/neu transgenic female mice for the activated rat neu oncogene were obtained from Charles River (Hollister, CA) and maintained under specific pathogen-free conditions under a standard 12h light/12h dark regime in our animal facilities. Mice were housed in plastic nongalvanized cages and fed with standard pelleted food (Harlan Laboratories, Udine, 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. The experimental protocols were also approved by the Institutional Animal Care Committee of the Ministry of Health (Italy) and by the Animal Research Ethics Committee of ICRCCS-INRCA.

**Acute and subchronic toxicity and median lethal dose (LD50)**

Groups (n = 4) of female FVB mice (age 20 weeks, weight 25–28g) were intraperitoneally injected with BBR or NAX014 dissolved in DMSO at 10 mg/ml and diluted in sterile water for injection at doses of 2.5, 5, 10 and 20 mg/kg for two times/week. Control group (n = 7) received 10% DMSO (v/v) in sterile water. Body weights were measured twice a week. The animals were closely observed for any signs of toxicity during the first 5h, and the number of dead animals was recorded at 24, 48 and 72h. To observe the subchronic toxicity, the surviving animals were subjected to the same treatment, twice a week, for the following 4 weeks. The animals were inspected daily and weighed during the study period. At the end of the experiment, all animals were killed by carbon dioxide and subjected to terminal necropsy.

**Experimental design**

Female FVB/N mice were analyzed in three different experiments. In each experiment, 4-month-old mice were randomly divided into three groups (n = 10) which received the following treatments by intraperitoneal injection for two times a week, at intervals of 3–4 days: (i) 2.5 mg/kg BBR, (ii) 2.5 mg/kg NAX014 and (iii) 1.3% DMSO (v/v) in sterile water (control group). The two perpendicular diameters of the neoplastic masses were measured with caliper to calculate the tumor volume ([length × width]/2). Progressively growing masses of >3 mm in mean diameter were regarded as tumors. The mice were killed for ethical reasons when the largest diameter of tumors exceeded 10 mm. In vivo analyses and tissue collection were performed at this time point. Anesthesia was induced by inhalation of 4% isoflurane and maintained with 2% isoflurane, in a stream of oxygen, via facial mask. A midline incision of the skin was performed and the tumor masses were exposed, making every effort to avoid damage to the tumor vasculature. The tumor vasculature was visualized by means of side-stream dark field (SDF) videomicroscopy as described below. Thereafter, the tumor masses were removed and frozen in liquid nitrogen at −70°C for subsequent analyses.

**Reverse transcription, PCR and real-time PCR**

Total RNA was isolated from tumor tissues using RNeasy kit (Qiagen, Milan, Italy) according to the manufacturer’s instructions. The first-strand cDNA was synthesized starting from 1 µg of total RNA using RevertAid.
First Strand cDNA Synthesis Kit (Fermentas, Thermo Scientific). PCR for perforin, granzyme B and β-actin were performed as described previously (17). Relative quantification of mRNA expression was achieved using fluorescent dye SYBR green (Bio-Rad, Richmond, CA) during PCR amplification (IQ5 Real-time Detection System, Bio-Rad). The primers used were as follows: β-actin, 5′-TTGGTTTGGCTCCACAC-3′ and 5′-ACCAGCGAGCGATATGG-3′; p16, 5′-CTACCCCGATTTCAAGGTGAT-3′ and 5′-TTGAGCAGAAGAGCTGCTACGT-3′. Variation in gene expression in treated samples respect to the control was determined using the formula: \[ \Delta \Delta C_t = (C_{\text{treated sample}} - C_{\text{control sample}}) - (C_{\text{house-keeping gene}}) \].

Western blot analysis
Whole cell extracts were carried out as described previously (18). Tumor tissue samples were homogenized by gentleMACS Dissociator (Miltenyi Biotech). Immunoblotting was performed using HER-2, heparanase 2 (HPA2), p21 or β-Actin antibodies (Santa Cruz Biotechnology). Blots were visualized using a luminal-based detection system (GE Healthcare, Chicago, IL). For quantitative protein determination, densitometry measurements of western blot bands were performed using GS-800 Calibrated Densitometer (Bio-Rad).

SA-β-gal staining and image analysis
Senescence-associated SA-β-gal activity was detected and analyzed as described previously (4).

Detection of in situ cell death
For in situ staining of apoptotic cells, the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method was performed using a kit (Roche, Milan, Italy). Detection was done with dianinobenzidine substrate (Vector Laboratories, CA). Negative control without terminal transferase was included. Positive control was done treating tumor cells with 80U/ml DNaseI (Invitrogen) to induce DNA strand breaks. Sections were counterstained with hematoxylin. Apoptotic cells were quantified by evaluating five randomly chosen fields in each sample. Individual cells were counted under ×250 microscopic field.

Intravital assessment of tumor vascularization with SDF imaging
Sidestream dark field imaging technology (Microscan, Microvision Medical, Amsterdam, The Netherlands) was used to evaluate the tumor vascularization. The Microscan is a hand-held video microscope system enabling the real-time intravital observation of the blood flow in microvascular beds (19). Application of SDF videomicroscopy for in vivo evaluation of vascularization and perfusion of mammary tumors in HER-2/neu transgenic mice has been described elsewhere (20).

In each mouse, videos from the different tumor masses (2–3 sites per mouse, at least 10 s/site) were recorded with adequate focus, contrast and stability. The videos were stored as Audio Video Interleave files and analyzed offline using a dedicated computer software package (Automated Vascular Analysis Software, Microvision Medical BV, Amsterdam, The Netherlands). In each video, all visible vessels were identified and manually traced; the total vessel density (mm vessel/mm²) was automatically calculated as the total length of all the identified vessels divided by the total area of the image. Total vessel density values calculated on different sites were averaged, resulting in one total vessel density value per mouse.

Immunohistochemistry
Mammary tumor samples were frozen in liquid nitrogen and stored at −70°C. Six-micrometer-thick sections were air-dried overnight and then fixed in acetone (10 min). Sections were successively incubated overnight at 4°C with the monoclonal antibody anti CD34 (clone My10) (dilution 1:20, BD Biosciences, San Jose, CA). The reaction was revealed by the streptavidin–biotin peroxidase technique (Envision peroxidase kit, Dako Cytomation, Milan, Italy). After incubation with 0.05% 3,3′-diaminobenzidine (Sigma-Aldrich, Milan, Italy) in 0.05M Tris buffer, pH 7.6 with 0.01% hydrogen peroxide, sections were counterstained with Mayer’s hematoxylin (BioOptica, Milan, Italy), dehydrated in ethanol and coverslipped with Eukitt mounting medium (Electron Microscopy Sciences, PA, USA). Negative controls were performed by substituting the primary antibody with non-immune serum. All counts were performed blinded to the animal outcome. The density of microvessels (MVD) was defined counting the number of CD34 positive

Figure 1. Chemical structure of berberine chloride and NAX014 compounds (A). Survival curves of FVB mice injected with 2.5, 5, 10 and 20 mg/kg of BBR or NAX014 (B). Body weight changes (as a percent of the day 0 weight) in subchronic toxicity study (C).
small vessels in a 400× microscopic field (0.16 mm²/field). Any stained cell or cluster that was clearly separated from adjacent MVD was considered a single countable MVD. The average of the MVD in five fields was calculated and MVD was expressed as mean CD34 positive vessel count per mm².

Statistical analysis
All experiments were performed at least three times. The LD₅₀ was determined by the Spearman–Karber method (21). Differences in tumor incidence were evaluated by the Mantel–Haenszel log-rank test; differences in tumor volume and tumor multiplicity were evaluated by analysis of variance and Student–Newman–Keuls post hoc tests; differences in mRNA expression, CD34 immunostaining and immune parameters were evaluated by ANOVA and Bonferroni tests. For SDF imaging analysis, Kruskal–Wallis test was used to evaluate differences between groups, with Dunn’s multiple comparison post hoc test. Difference between means was considered significant at P < 0.05. Data analysis was performed with SigmaStat software version 1.03 (Jandel Scientific, Germany) and Systat 10 (SPSS) software.

Results
Acute and subchronic toxicity study
The LD₅₀ in FVB mice was determined to be 10.9 mg/kg for the BBR compound and 30.9 mg/kg for NAX014 (Figure 1B). No dead mouse was observed in the control group. In the subchronic toxicity study, no significant alterations on food consumption, body weight (Figure 1C) and mortality were observed over the 2-week study period. Nevertheless, at the end of the experiment, necropsy revealed macroscopic organ (spleen, liver, kidneys) damage (Table 1).

Prevention of mammary carcinogenesis
We examined the effect of BBR and NAX014 treatment on spontaneous HER-2/neu mammary carcinogenesis in transgenic mice. According to the results from the subchronic toxicity study, we choose to use the safety dose of 2.5 mg/kg bw for both compounds. This was administered by intraperitoneal injection twice a week, starting from the 16th week of age. The administration was well tolerated, with no signs of toxicity and no significant effect on body weight in any group (data not shown). As shown in Figure 2A, the first mammary tumor appeared in control mice at 20.5 weeks of age followed by NAX014 and BBR-treated mice at 22 and 23 weeks of age, respectively. The tumor incidence increased progressively with increasing age of mice, affecting 50% of control-DMSO and BBR-treated mice at 25 weeks of age and NAX014-treated mice at 26.5 weeks of age. Whereas animals from the control group were all tumor bearers at week 28, 10 or 30% of mice from BBR or NAX014 groups, respectively, were still tumor free at this week. All mice were tumor bearers at week 28.5 in the BBR group, whereas the appearance of tumors in all mice was delayed in NAX014 group until week 32.5 (Figure 2A). In mice treated with NAX014, but not in those which received BBR, the kinetics of appearance of mammary tumors was significantly different when compared with control group (P = 0.027). As shown in Figure 2B, a significant reduction of tumor volume was observed between 26 and 28.5 weeks of age in NAX014-treated mice compared with control group (P < 0.05 by Student–Newman–Keuls Method). The mean number of tumor masses also resulted lower, compared with control group, in NAX014-treated mice, although the differences between groups were not statistically significant (Figure 2C). No statistically significant difference between BBR-treated and control groups was observed in the kinetics of tumor volume and multiplicity (Figure 2B and C).

In vivo evaluation of vascularization of mammary tumors in HER-2/neu transgenic mice through SDF videomicroscopy
Twenty-six mice were evaluated in total (control, n = 7; BBR, n = 10; NAX014, n = 9). The tumor masses from control group showed

Table 1. Extent of tissue injury* in FVB mice induced by BBR and NAX014 compounds

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<th>Group</th>
<th>Dose (mg/kg)</th>
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*Gross pathology of liver, kidney and spleen was performed at the term of treatment: 4 weeks, 2 times/week administration. The severity of lesions was graded as −, negative; ±, mild; ++, severe.
Figure 2. Inhibition of mammary carcinogenesis in HER-2/neu transgenic mice treated with BBR or NAX014 compounds. FVB/N transgenic mice (n = 10 for each group) were intraperitoneally injected two times a week with 2.5 mg/kg of BBR, NAX014 or vehicle alone and analyzed for the kinetics of tumor incidence (A), tumor volume (B) and tumor multiplicity (C). The percentage of tumor-free mice was calculated as the cumulative number of mice with tumors and mice that were tumor-free. Statistical significance was assessed by Mantel–Haenszel log-rank test. (B) Mean tumor volume was calculated as the sum of tumor volumes/total number of tumors. Statistical analysis, assessed using a two-way analysis of variance (ANOVA) with a Student–Newman–Keuls post hoc test, is reported in the text. P < 0.05 was deemed a statistically significant difference. (C) Mean tumors number per mouse was calculated as the cumulative number of incident tumors/total number of FVB/N mice.
higher vessel density as compared to the BBR and NAX014 groups (total vessel density of 17.2 (13.2–21.2) mm/mm² versus 12.07 (10.7–13.4) mm/mm² for BBR and 9.9 (7.8–12.1) mm/mm² for NAX014) although a statistically significant difference was found only in the comparison between NAX014 and controls (P < 0.01) (Figure 3).

Evaluation of MVD density by CD34 immunostaining and granzyme B and perforin expression in mammary tumors in HER-2/neu transgenic mice

We quantified MVD in tumor masses from HER-2/neu transgenic mice through anti-CD34 immunostaining (Figure 4A). Four tumors per group were analyzed. Control tumors showed...
a MVD of 17.0 mean vessels per field (95% CI = 14.5–19.5) in the area of most intense neovascularization while the tumors from BBR-treated group displayed mean 15.8 vessels per field (95% CI = 11.0–20.6) and those from NAX014—11.3 (95% CI = 9.5–13.2). A statistically significant decrease in MVD compared with control group was observed for the NAX014 group ($P < 0.003$).

To evaluate the presence of cytotoxic lymphocytes in mammary tumors, tumor masses from control and treated mice were analyzed for the content of mRNA encoding granzyme B and perforins. Nine tumor masses were evaluated in total (control, $n = 3$; BBR, $n = 3$; NAX014, $n = 3$) through conventional PCR. Figure 4B shows the relative expression of both genes as the means of densitometric values normalized for $\beta$-actin expression in individual samples. Both mRNAs were found expressed in tumor masses from all groups of treatment. Levels of granzyme B expression were slightly reduced even if not significantly in BBR and in NAX014 compared with control, while perforin expression resulted significantly lower in NAX014-treated mice ($P < 0.05$) (Figure 4B).

Analysis of senescent-like phenotype and in situ apoptosis in tumor tissue from BBR and NAX014-supplemented mice

NAX014 and, to a lesser extent, BBR administration increased the number of cells with a senescent-like phenotype in mammary tumor samples, as evidenced by the increased SA-$\beta$-gal staining ($P < 0.05$ for NAX014 versus control, Figure 5A). In order to establish whether the effect of BBR and NAX014 supplementation on senescent-like growth arrest observed on tumor masses of treated mice could be related to a modulation of molecular cell-cycle checkpoint regulators, we measured the mRNA levels of p53, p21WAF1, p16INK4a and the expression of p27 protein in mammary tumor samples. As shown in Figure 5B NAX014 induced a significant dose-dependent upregulation of p16INK4a mRNA level (with 4.6-fold increase versus control group, $P < 0.05$) as defined by a change in the extent of expression at the reverse transcription-polymerase chain reaction analysis. In tumors from BBR-treated group, the p16INK4a mRNA level was 1.7-fold increased versus control group. No significant modulation in p53 and p21WAF1 expression was observed in the analyzed samples (data not shown). Western blot analysis showed a non-significant increase in the p27 expression in tumor tissues from both BBR- and NAX014-treated mice (Figure 5D).

The presence of apoptosis in mammary tumor masses was then evaluated in situ using TUNEL assay. No difference between groups of treatment versus control group was found regarding the number of apoptotic cells in tumor masses (Figure 5C). No evidence of apoptosis was present in negative control. Strong positivity was obtained in DNase-treated tumor samples (positive control).

Effect of BBR and NAX014 on HER-2 and heparanase protein expression

The effect of BBR and NAX014 on HER-2 and HPA2 expressions was evaluated in tumor tissues derived from treated mice by western blot analysis. As shown in Figure 5D, BBR and NAX014 administration tended to reduce HER-2 expression in tumor masses ($P > 0.05$ versus controls). While no significant
modulation in HPA2 expression was observed in tumors from BBR-treated mice in comparison to controls, a downregulation of HPA2 expression was detected in tumors from NAX014-treated mice ($P < 0.05$) (Figure 5D). The HPA2 downregulation was confirmed by in vitro treatment of human SK-BR-2 breast cancer cells with 50 $\mu$M NAX014 for 24 h (Figure 5E).

**Discussion**

A growing body of evidences shows that phytochemicals can interfere with tumor initiation and progression by triggering molecular pathways correlated with cell cycle arrest and/or cell death (22). In order to enhance the biological properties of natural molecules, in a number of studies structural analogs of natural products have been synthesized and analyzed for their chemopreventive and chemotherapeutic potential (23,24).

The isoquinoline alkaloid BBR, used in Traditional Chinese Medicine for thousands of years and currently available as a nutritional supplement, has been extensively investigated for its interesting pharmacological and biological activities (25). Antineoplastic properties of BBR have been investigated in both cellular and animal models and several mechanisms of action have been identified. Cell cycle arrest and apoptosis have been observed after BBR treatment of different types of human cancer cells (9,26,27). However, there are also various data describing the induction of senescence in cancer cells by BBR. Indeed, BBR was shown to induce senescence in human glioblastoma cells by downregulation of EGFR-MEK-ERK signaling pathway...
and number. The studies, conducted in animal models challenged the kinetics of tumor growth in comparison with the control group. Instead, the IP administration of 2.5 mg/kg suppressed the growth of nasopharyngeal carcinoma cells, abrogating STAT-3 activation (31). Also, BBR (5 and 10 mg/kg, twice per week for 4 weeks) resulted effective in reducing tumor weights and volumes in LNCap cell-bearing mice in a p53-dependent manner (32). In a mouse model of chemically induced colorectal carcinogenesis, BBR administration (40 mg/kg by oral gavage) suppressed colon epithelial proliferation and tumorigenesis through inhibition of mTOR and NF-κB activation (33). Our results do not completely agree with the studies reported above. Although BBR exerted some anticancer effects in our cancer model, they were generally not significant. We think that both the model and the conditions we used may account for the different results. In our experiments we used the safe dose of 2.5 mg/kg for 2 times/week, considering that the murine model requires a long-term administration (until 17 weeks), which is substantially longer than that used in the previous in vivo studies. Furthermore, the different characteristics of the transgenic murine models used (spontaneously developing tumors versus transplantation of parental tumors or exposure to carcinogens) must be taken into account. The overexpression of activated HER-2/neu in FVB/N transgenic mice is sufficient to efficiently transform mammary epithelial cells with consequent development of multiple HER-2/neu-positive tumor masses in 100% of mice (34). The ‘aggressiveness’ of this animal model and its closely correlation with human oncologic disease make it useful for screening novel anticancer drugs. On the other hand, murine models of cancer involving the challenge of mice with a bolus of tumor cells provide information that, while informative, may not be entirely relevant to cancer development in humans, where the tumor is initiated by the clonal expansion from a single cell in vivo.

Our results show that the mechanisms involved in the anticancer effect of NAX014 are related to both indirect effects on tumor environment and direct induction of cancer cellular senescence.

In our study, NAX014-mediated growth inhibition of mammary tumors was associated with a significant reduction in tumor vascularization. We applied SDF videomicroscopy to evaluate the in vivo vascular network of tumors (20). The lower vessel density in mammary tumors, which was confirmed through standard anti-CD31 immunostaining, clearly supports the antiangiogenic effect of NAX014 which is associated with a delayed tumor development. This effect was linked to the NAX014-dependent inhibition of heparanase which, in turn, has been reported to correlate with increased tumor vascularity. Furthermore, heparanase is an endoglycosidase which cleaves heparan sulfate and hence participates in degradation and remodeling of the extracellular matrix. Heparanase is preferentially expressed in human tumors and its over-expression in tumor cells confers an invasive phenotype in experimental animals. It seems then possible that the NAX014-dependent inhibition of heparanase could determine both the lower vessel density and the reduced invasiveness of tumors. The reduced tumor vascularization determined by NAX014 could also be related to the decreased intratumoral immune infiltration, as demonstrated by the lower granzyme and perforin detection. This evidence may represent a negative effect of NAX014, which deserves further investigations.

A growing number of scientific evidences attributes to BBR antiangiogenic properties. BBR-treated gastric cancer cells (SC-M1) under hypoxic condition resulted unable to induce in vitro HUVEC migration and tube formation (35). A decreased vascular endothelial growth factor expression was observed in both normoxic and hypoxic SC-M1 cultures (35). In C57BL/6 mice, BBR at 10 mg/kg showed significant reduction in tumor-specific capillary formation and in various proangiogenic factors and proinflammatory mediators involved in tumor angiogenesis (11). Furthermore, BBR has been shown to inhibit the metastasis and...
invasion of T24 bladder cancer cells by reducing the heparanase expression (13). The antiangiogenic effect of BBR in our experimental model has been only weakly detected and this could be again due to the low dosage of administration. On the other hand, the NAX014 compound showed potent antiangiogenic properties at the same dose of BBR treatment. Based on previous observation in cultured SK-BR-3 cells, NAX014 seems to act through the same molecular pathways affected by BBR (5).

The effectiveness of BBR and NAX014 as anticancer agents has been previously reported to be related to their antiproliferative effects on cancer cells and to the induction of tumor cell apoptosis (5,15). In the present in vivo study, we have found prosenescent effects induced by NAX014 on tumor cells. This was mainly demonstrated by the increased SA-β-gal staining and the 3–4 fold upregulation of p16 mRNA in mammary tumors, as well as by the increased p27 protein expression. Unexpectedly, no statistically significant changes were observed in apoptosis in in vivo NAX014-treated mice. We hypothesize that the NAX014 dose administered was sufficient to induce senescence but not apoptosis. Further studies are needed to confirm this possibility and find a way to increase NAX014 dose administration without inducing toxicity.

In conclusion, this study demonstrates that NAX014 may exert important anticancer effects by delaying the development of tumors in mice transgenic for the HER-2/neu oncogene. The antitumor effect of NAX014 may be related to the reduced tumor vascularity and inhibition of degradation and remodeling of the tumor extracellular matrix, and to the induction of cellular senescence in tumoral mammary glands.

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Conflict of Interest
P.L. is co-founder of Naxospharma. The described compound may have commercial value. The other authors declare no competing interest.

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