**Substance P Autocrine Signaling Contributes to Persistent HER2 Activation That Drives Malignant Progression and Drug Resistance in Breast Cancer**

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**Abstract**

ERBB receptor transmodulation by heterologous G-protein–coupled receptors (GPCR) generates functional diversity in signal transduction. Tachykinins are neuropeptides and proinflammatory cytokines that promote cell survival and cancer progression by activating several GPCRs. In this work, we found that the pain-associated tachykinin Substance P (SP) contributes to persistent transmodulation of the ERBB receptors, EGFR and HER2, in breast cancer, acting to enhance malignancy and therapeutic resistance. SP and its high-affinity receptor NK-1R were highly expressed in HER2 primary breast tumors (relative to the luminal and triple-negative subtypes) and were overall correlated with poor prognosis factors. In breast cancer cell lines and primary cultures derived from breast cancer samples, we found that SP could activate HER2. Conversely, RNA interference-mediated attenuation of NK-1R, or its chemical inhibition, or suppression of overall GPCR-mediated signaling, all strongly decreased steady-state expression of EGFR and HER2, establishing that their basal activity relied upon transdirectional activation by GPCR. Thus, SP exposure affected cellular responses to anti-ERBB therapies. Our work reveals an important oncogenic cooperation between NK-1R and HER2, thereby adding a novel link between inflammation and cancer progression that may be targetable by SP antagonists that have been clinically explored.

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**Introduction**

Inflammation is a hallmark of cancer (1). Chronic inflammation is closely related to tumor development and progression; it supplies the tumor with a milieu of proinflammatory cytokines that can modulate the inflammation-related tumor cell signaling (2, 3). Some inflammatory conditions increase the risk of the onset of cancer by enhancing the proliferation and oncogenic potential of adjacent epithelia. In cancers that are already established, persistent inflammation enhances tumor growth and progression by activating several mechanisms that support tumors, such as enhanced angiogenesis and stromal cell recruitment. Inflammatory mediators can also cause genetic instability (4, 5) and favor the fixation of epigenetic alterations (6).

Several proinflammatory cytokines, such as chemokines, induce signal transduction through different families of G-protein coupled receptors (GPCR). Activation of secondary messengers, such as PKC and adenylate cyclase, results in the diversification of G-protein signal transduction (7, 8). In some cancer cell types, the mitogenic stimulus activated by G-proteins is partly mediated by transmodulation of the EGF receptor (EGFR; refs. 9, 10). The members of the ERBB family, including EGFR, HER2, HER3, and HER4, are receptors with tyrosine kinase (RTK) activity, and signaling networks triggered by them play a pivotal role in tumor initiation and progression (11, 12). In particular, HER2 is overexpressed in approximately 25% of the patients with breast cancer and is associated with worse prognosis and lower survival rates (13, 14). The precise pathways interconnected with RTK to fine tune cellular responses are still unknown, but emerging evidence indicates that transmodulation by heterologous GPCRs may play an important role in them (7, 8). It is particularly noteworthy that the cross-talk between GPCRs and RTKs allows for RTK activation even in the absence of ligands (7).
Therefore, identifying new modulators of RTKs activity could be highly useful in the search for new therapeutic targets.

In this work, we have investigated the transmodulation of the ERBB system by the neuropeptide/proinflammatory mediator Substance P (SP), a pleiotropic molecule synthesized by the TACI gene. SP is related to processes of neurogenic inflammation, wound healing, hematopoiesis, microvasculature permeability, leukocyte trafficking, cell survival, and metastatic dissemination (15–19). The three classical members of the mammalian tachykinin family are SP and neurokinin A (NKA), both encoded by the TAC1 gene, and neurokinin B (NKB) encoded by the TAC3 gene. There is a third mammalian tachykinin gene (TAC4) that codifies for hemokinins and endokinins (15, 20, 21). These tachykinins exercise their biologic functions through their preferential binding to the tachykinin receptors NK-1R (TACR1), NK-2R (TACR2), and NK-3R (TACR3), respectively, although each tachykinin can bind to the three tachykinin receptors with different affinity (22). NK-1R, the high-affinity SP receptor, is a GPCR that is overexpressed in breast cancer (19). In this work, we identify a new aberrant signaling network in breast cancer based on the oncogenic cooperation between the tachykinergic system and HER2/EGFR signaling. The data presented here support the idea that the SP produced within a proinflammatory setting can enhance tumor malignancy by activating HER2 and EGFR. In addition, we showed that the network could be exploited for the development of new therapeutic strategies based on the dual inhibition of activation and transactivation mechanisms.

Materials and Methods

Cell lines

All human breast cancer cell lines were purchased from American Type Culture Collection and were validated by single locus short tandem repeat typing (Bio-Synthesis, Inc.) before its use.

Isolation of human primary breast cancer cells

Nineteen histologically confirmed breast cancer tumors were collected at the Hospital Clinic in Barcelona, Spain under the approval of the Institutional Review Board. Tumor tissue was processed to obtain single cells by digestion with a mixture of collagenase and hyaluronidase, and the cells were frozen in FBS with 10% dimethyl sulfoxide until further analysis.

Time-course studies

In cell lines. To determine the effects of SP treatment on HER2, EGFR, p42/44-MAPK, and Akt activation, cells were treated at the indicated times with SP 100 nmol/L, washed, and rapidly frozen until protein extraction.

In primary cultures. Tumor cells were thawed, filtered to ensure single-cell suspensions, and kept in Mammary Epithelial Basal Medium supplemented with growth factors for 3 hours at 37°C. Each single-cell suspension was split into 3 groups: control, SP 6 minutes, and SP 10 minutes. They were serum starved for 2 hours, and then treated with PBS or SP 100 nmol/L at 37°C. After treatment, the cells were washed, fixed in paraformaldehyde 4%, and resuspended in PBS with glycerol 10%. A cytospin slide centrifuge was used to concentrate and fix the samples on glass slides until further immunodetection of activated HER2.

In vivo experiments

Animal experiments were carried out in accordance with the regulations of our institution’s ethics commission, following the guidelines established by the regional authorities of Catalonia, Spain. The specific procedures are outlined in the Supplementary Materials and Methods online.

Western blot analysis

Briefly, cells or tumor pieces were lysed in ice-cold radioimmunoprecipitation assay buffer. Protein quantification and detection were conducted as described elsewhere, applying the primary antibodies at the dilutions indicated in the Supplementary Materials and Methods online.

Immunofluorescence

Cell lines and cytospin slides containing the cells from the primary cultures were immunostained with a combination of different antibodies as explained in the Supplementary Materials and Methods online. The slides were counterstained with 4’, 6-diamidino-2-phenylindole (DAPI; Roche Diagnostics) for 15 minutes and mounted with a coverslip using ProLong Gold antifade reagent (Invitrogen, Molecular Probes).

Fluorescence microscopy and image acquisition

Images were acquired using a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems Heidelberg GmbH). In the images of the primary cultures, the mean fluorescence (pHER2 staining; integrated density/area) of selected cells (those pan-CK positive) was quantified using a custom macro in the Fiji software (a distribution of ImageJ-Wayne Rasband, NIH) as explained in detail in the Supplementary Materials and Methods online.

Tumor subtype classification

Tumor subtype definitions in this study were as follows: luminal A [estrogen receptor (ER)⁺ and/or progesterone receptor (PR)⁺], HER2⁻, luminal B (ER⁺ and/or PR⁺, HER2⁺), HER2⁺ (ER⁺, PR⁺, HER2⁺), basal-like (ER⁻, PR⁻, HER2⁻, CK5/6⁻, and/or EGFR⁺), and unclassified (negative for all markers).

Immunohistochemistry procedure and evaluation

Immunohistochemistry for NK-1R, NK-2R, SP, COX2, or smooth muscle actin (SMA) was conducted following standard protocols as indicated in the Supplementary Materials and Methods online. For the semiquantitative analysis, an immunoreactive score (IRS) was derived by multiplying the percentage of positive cells (PPi) by the staining intensity (SI). The SI was determined as: 0 for no staining; 1+ for weak staining; 2+ for moderate staining; 3+ for strong staining, and 4+ for very strong staining. We classified the IRS into 4 groups: 0 (negative staining), 1 (staining score >0 and <100), 2 (staining score >100 and <200), and 3 (staining score ≥200).
Statistical analysis

Detailed statistic methods used to compare differences between groups can be found in the Supplementary Materials and Methods online.

Results

NK-1R and SP are highly expressed in human primary tumors of the HER2\(^{+}\) subtype, and circulating SP levels are increased in breast cancer patients

The expression levels of NK-1R, NK-2R, and SP were scored as both continuous and ordinal variables (for further details see Materials and Methods; Supplementary Tables S1 and S2), and both statistical analyses showed similar correlations with clinical characteristics. NK-1R, NK-2R, and SP were expressed by luminal cells from normal breast tissue (Supplementary Fig. S1), and overexpressed in tumors (Fig. 1A), in agreement with previous reports (19). In tumors, NK-1R, NK-2R, and SP expression was detected in 94%, 86%, and 52% of the 318 samples analyzed, respectively (Fig. 1A–C). With few exceptions, we did not find significant intratumor heterogeneity for the expression of these proteins; when present, they were expressed in the majority of the cells within each tumor. Expression of either NK-1R or SP was positively associated with tumor histopathologic characteristics related to poor prognosis. For example, NK-1R expression levels were associated with ER and PR negativity, and p53 overexpression. SP expression was associated with a high histologic grade, ER and PR negativity, and a high Ki67 proliferation index. In contrast, NK-2R expression was associated with low histologic grade, ER and PR positivity, and no p53 overexpression (Supplementary Tables S1 and S2). Interestingly, we found that NK-1R and SP expression were significantly higher in tumors of the HER2\(^{+}\) subtype, when the expression levels were considered as either continuous or ordinal variables (Fig. 1D–I), and the same positive association was found in tumors with HER2 gene amplification (Supplementary Tables S1 and S2). In contrast, NK-2R expression was lowest in this particular tumor subtype (Fig. 1E and H).

We next determined the SP concentration in the serum of 139 breast cancer patients and 92 healthy controls by ELISA.
The levels of SP were significantly higher in patients with breast cancer than in healthy controls, although we did not observe any association with a particular tumor subtype or any other clinicopathologic characteristic (Fig. 1J and Supplementary Table S3).

**Human tachykinins modulate EGFR and HER2 activation in breast cancer cells**

Considering that the higher expression of NK-1R and SP in HER2⁺ tumors could be the consequence of functional cooperation between the two signaling systems, we next explored the effects of SP on a panel of breast cancer cell lines with (MDA-MB-453, SK-BR-3, BT-474) and without (MDA-MB-231, MCF7) amplification or overexpression of the ERBB2 gene (23), and with overexpression of the EGFR gene (MDA-MB-468; Fig. 2A). We detected expression of the three main tachykinin receptors, TACR1, TACR2, and TACR3, and expression of TAC1 (which codifies for SP) by quantitative PCR (qPCR) in all the cell lines (Fig. 2B). We further confirmed the expression of NK-1R and SP by immunofluorescence in all the cell lines (Fig. 2C). Through time-course studies, we observed that SP quickly induced the phosphorylation of HER2 Tyr1248 (specific...
tyrosine residue that serves as docking site for the adaptor molecules that will activate the Ras-ERK pathways; refs. 24–26) within the first 6 to 10 minutes after stimulation in all the cell lines (Fig. 2D; the densitometric quantification is shown in Supplementary Fig. S2A). The activation of HER2 within this time frame was consistently observed in all the replicates conducted, although the exact time point of maximum activation varied. The kinetics of HER2 activation was dose dependent, showing faster activation with increasing concentrations of SP (Supplementary Fig. S3). SP treatment also induced the phosphorylation of EGFR Tyr1068 (ligand activated; ref. 27) and EGFR Tyr845 (Src activated; ref. 28), as well as the p42/44-MAPK and Akt pathways (Fig. 2D and Supplementary Fig. S2A).

By immunofluorescence, we also observed an increase in the levels of phospho-HER2 Y1248 in the cell membrane of MDA-MB-453 cells (HER2+), and an increase in the formation of endocytic vesicles containing activated EGFR in the MDA-MB-468 cell line (EGFR+; Supplementary Fig. S2B; ref. 29) after SP treatment.

The mitogen-activated protein kinase (MAPK) is a common downstream pathway triggered by both HER2 and NK-1R activation (7, 30, 31). Then, to confirm that the phosphorylation of HER2 caused by SP treatment was indeed activating HER2, we determined the capability of SP to activate the MAPK pathway in the presence of HER2 inhibitors. Using the MDA-MB-453 cell line, we observed that the inhibition of HER2 with either lapatinib or AG825 decreased the capability of SP to activate the p42/44-MAPK (Supplementary Fig. S4A and S4B), suggesting that the activation of MAPK by SP partially depends on the transactivation of HER2.

**SP transactivates HER2 in human primary tumor cells**

Because HER2 is a target for therapy in patients with breast cancer, we next studied the clinical relevance of SP-mediated HER2 activation. We determined the activation of phospho-HER2 Y1248 after SP treatment for 6 and 10 minutes in 1896 single cells obtained from 19 breast cancer primary cultures of different cancer subtypes that were classified by immunohistochemistry as having some degree of expression of HER2 (Supplementary Fig. S5 and Supplementary Table S4). We used multicolor immunofluorescence and confocal microscopy to analyze and quantify the levels of phospho-HER2 Y1248 in pan-cytokeratin-positive cells (to select only epithelial cells; Fig. 3A). The basal levels of phospho-HER2 Y1248 were higher in the control samples derived from HER2+ and luminal B...
tumors than in the luminal A or triple-negative tumors (Fig. 3B and C). Although overall we only observed a statistically significant increase in phosho-HER2 Y1248 by SP in cells derived from tumors of the HER2\(^+\) and triple-negative subtypes (Fig. 3D), there was some intergroup variation in the tumors of the luminal A subtype. In fact, in 7 out of 10 luminal A tumors, SP activated HER2, in agreement with the results obtained with breast cancer cell lines (Supplementary Fig. S6). We did not observe activation of HER2 in the cells derived from luminal B tumors at the time points tested. There could be several reasons for the lack of HER2 activation in this subgroup; for instance, due to low levels of NK-1R expression in luminal B compared with HER2\(^+\) tumors (Fig. 1D) or because of a lack of additional signaling mediators involved in the transactivation process with differential intracellular signaling.

**SP/NK-1R autocrine/paracrine signaling in breast cancer cells elicits a constitutive transactivation of HER2 and EGFRs**

Because NK-1R is the high-affinity receptor for SP, we next decided to investigate its contribution to SP-mediated HER2 transactivation. The MDA-MB-231 cell line was transfected with the pcDNA3.1(+)\(\)\(-\)TACR1 vector, enriched with NK-1R+ cells by fluorescence-activated cell sorting (Supplementary Fig. S7A), and the expression of NK-1R confirmed by qPCR (Supplementary Fig. S7B). Upon SP stimulation, the levels of pHER2 Y1248 and p-p42/44-MAPK were significantly increased in the cells transfected with pcDNA3.1(+)\(-\)TACR1 compared with mock cells (Fig. 4A and Supplementary Fig. S7C), indicating that NK-1R is involved in SP-induced HER2 transactivation. The inhibition of TACR1 by siRNA in the SK-BR-3 and BT-474 cell lines caused a significant decrease in

**Figure 4.** NK-1R signaling modulates EGFR and HER2 activity in breast cancer cells. Representative images of Western blot analyses corresponding to experiments showing: A, the contribution of NK-1R to the phosphorylation of HER2 and p42/44-MAPK in the MDA-MB-231 cell line transfected with pcDNA3.1(+)\(-\)TACR1 or the empty vector and treated with SP 100 nmol/L; B, the effects of TACR1 inhibition by siRNA on the steady state of pHER2 in different breast cancer cell lines. NK-1R expression is shown as a control for siRNA efficiency; C, the effects of L-733,060 20 \(\mu\)mol/L (SKBR3), 20 \(\mu\)mol/L (BT-474), 30 \(\mu\)mol/L (MDA-MB-453) on the phosphorylated and total levels of HER2, EGFR, p42/44-MAPK, and Akt in different breast cancer cell lines; D, the effects of the triple inhibition of NK-1R, NK-2R, and NK-3R with L-733,060 (20 \(\mu\)mol/L), MEN 10376 (30 \(\mu\)mol/L), and SB 218795 (20 \(\mu\)mol/L), respectively, on the phosphorylation of HER2, p42/44-MAPK, and Akt in different breast cancer cell lines; E, the effects of the G-protein inhibitor pertussis toxin (PTX) on the phosphorylated and total levels of HER2, p42/44-MAPK, and Akt in different breast cancer cell lines. The plots accompanying each panel show the densitometric quantification of the Western blot analyses relative to the expression of tubulin, which was used to ensure equal protein loading. All the Western blot analyses and the quantitative data are for a minimum of 3 replicates and are presented as mean ± SD and compared with t test (two tailed) as *, \(P < 0.05\); **, \(P < 0.01\); and ***, \(P < 0.001\).
these data suggest that the continuous presence of SP in a tumor oncogene addiction to NK-1R could imply in part on NK-1R-mediated effects on HER2 and EGFR basal activity. We also observed that signal transduction through phospho-p42/44-MAPK in BT-474 and/or phospho-Akt in SK-BR-3, BT-474, and MDA-MB-468 was also inhibited in cells treated with L-733,060 (Fig. 4C). Moreover, the blockade of NK-1R, NK-2R, and NK-3R in the SKBR3 cell line caused almost a complete inhibition of HER2 phosphorylation and a significant decrease in the Akt survival signaling pathway (Fig. 4D), thereby further confirming the tachykinergic system to be a modulator of the steady state (or basal activation) of HER2.

Besides the well-known role of several GPCR ligands as activators of EGFR and HER2 (7, 9, 32), their contribution to the basal activity of RTKs has not been intensely studied. For this reason, we next determined whether GPCR-mediated signaling modulates the activity of HER2 in breast cancer cells. We observed that the inhibition of pertussis toxin-sensitive G-proteins strongly decreased the pHER2 Y1248 in all the cell lines, and total HER2 in the SK-BR-3 and BT474 cells. In some cases, a decrease in p42/44-MAPK and Akt was also observed (Fig. 4E and Supplementary Fig. S7D). All together these data suggest that G-protein signaling in breast cancer cells directly modulates HER2 activation.

**Chronic overexposure to SP in vivo has mitogenic activity and enhances HER2 activation**

To further study the effects of overexposure to SP on tumor growth in vivo, we subcutaneously implanted mini osmotic pumps challenged to continuously deliver 500 nmol/L of SP for 28 days in severe combined immunodeficient mice (SCID) mice wearing MDA-MB-231 (HER2+) or MDA-MB-453–derived (HER2−) xenograft tumors. We aimed to determine whether long-term SP exposure, simulating a scenario of chronic inflammation, could favor the outgrowth of tumor cells with different properties and thus influence the progression of tumors with different phenotype: HER2− or HER2+.

Chronic SP delivery for 28 days did not affect the tumor growth or the final tumor weight in any of the tumor models (Fig. 5A and B). However, we observed a significant increase of total HER2 in the SP-treated tumors by Western blot analysis in both models (Fig. 5C) and by immunohistochemistry (Fig. 5D) in the MDA-MB-453. In the MDA-MB-231–derived xenografts, we also detected a weak expression of HER2 in the treated tumors, compared with the control tumors that were totally negative. The MAPK pathway was also highly expressed in the MDA-MB-231 SP-treated tumors. In addition, in both tumor models, there was a significant increase in the number of mitotic bodies (Supplementary Fig. S8A and S8B) probably due to the known mitogenic activity of SP (33, 34). These data suggest that the continuous presence of SP in a tumor, like in a situation of chronic inflammation, could enhance the expression of HER2 in tumor cells with different phenotypes.

**The NK-1R antagonist L-733,060 inhibits tumor growth in vivo in HER2- and EGFR-expressing tumors**

We next decided to study the effects of NK-1R inhibition on the in vivo growth of the breast cancer cell lines MDA-MB-453 (HER2+), MDA-MB-468 (EGFR+), and MDA-MB-231 (HER2− and EGFR−). The tumor growth was significantly decreased only in MDA-MB-453 and MDA-MB-468–derived xenograft tumors treated with L-733,060 (5 mg/kg three times weekly; Fig. 5E and F), but not in the MDA-MB-231 tumors (Fig. 5G), suggesting that the antitumor effects of NK-1R inhibition in vivo depend on the modulatory properties of NK-1R signaling on HER2 and EGFR activity. Final tumor weight was significantly reduced by L-733,060 only in the HER2+ model derived from MDA-MB-453, and slightly decreased in the MDA-MB-468 cells (Fig. 5H and I). On the other hand, we did not observe significant effect on the growth or final tumor weight of the MDA-MB-231–derived tumors (Fig. 5G and J). At the protein level, there were no significant differences in HER2 or EGFR, suggesting that NK-1R inhibition does not select for particular cell subpopulations, and the treatment with L-733,060 only decreased p42/44-MAPK and Akt in the MDA-MB-231–derived tumors (Fig. 5K–M). In addition, we noticed a slight decrease in the expression of the COX2 proinflammatory mediator as a consequence of NK-1R inhibition in all the tumor models, and a significant decrease in α-SMA–positive cells, suggesting that the blockade of NK-1R modulates both tumor and stromal properties (Supplementary Fig. S9A–S9C).

**Chronic SP treatment alters cellular responses to therapies targeting HER2 and EGFR**

Because the hyperactivation of ERBB signaling by proinflammatory components may influence cellular responses to targeted therapies, we next studied the effects of short or chronic exposure to SP on the cellular responses to HER2 and EGFR inhibitors in the MDA-MB-453 (HER2+) or MDA-MB-468 (EGFR+) cell lines. To develop a model of SP overexposure, we kept the different cell lines growing in the absence (control group) or presence of SP (100 nmol/L/day) for over 5 months. The cell lines chronically treated with SP showed increased levels of total and activated HER2 and EGFR, and in some cases a higher activation of the p42/44-MAPK and Akt pathways (Supplementary Fig. S10A–S10D).

Next, we studied the changes in IC50 values for the EGFR tyrosine kinase inhibitor AG1478, the HER2 tyrosine kinase inhibitor AG825, and the dual EGFR/HER2 tyrosine kinase inhibitor lapatinib upon acute (72 hours) or chronic (5 months) SP treatment. A short exposure to 100 or 500 nmol/L of SP had little or no effect on the IC50 value in any cell line, whereas only the response of the MDA-MB-453 cells to AG825 upon addition of 100 nmol/L SP and that of the MDA-MB-468 cells to AG1478 upon 500 nmol/L SP stimulation were significantly affected (Fig. 6A and B and Supplementary Table S5). However, chronic treatment with SP for up to 5 months induced a significant increase in the...
Figure 5. Modulation of tumor growth in vivo by SP/NK-1R. A, volume of MDA-MB-231 and MDA-MB-453 xenograft tumors treated with saline or SP for 27 days (mean ± SEM). B, final tumor weight. Sample size (n) is indicated. C, Western blot analysis of MDA-MB-231 and MDA-MB-453 xenografts, and box plots showing the densitometric quantification (right, tubulin was used to ensure equal protein loading) for the proteins indicated. D, representative images for the immunohistochemical detection of HER2. Scale bar, 50 μm. Volume of MDA-MB-453 (E), MDA-MB-468 (F), and MDA-MB-231 (G) xenograft tumors treated with the NK-1R inhibitor L-733,060 (mean ± SEM) and final tumor weight (H–J), respectively. Western blot analysis of MDA-MB-453 (K), MDA-MB-468 (L), and MDA-MB-231 (M) xenografts and box plots showing the densitometric quantification (right, tubulin was used to ensure equal protein loading) for the proteins indicated. The boxes show the 25th to 75th percentile, and the whiskers extend to the 5th and 95th percentiles. Yellow diamonds in each plot indicate the mean. Data were compared with t test (two tailed). Significant P values are indicated as *, P < 0.05 and **, P < 0.01.
viability of the MDA-MB-453 and MDA-MB-468 cell lines after being treated with AG1478, AG825, and lapatinib (Fig. 6A and B and Supplementary Table S5), as reflected by the differences in the IC_{50} values. These results indicate that chronic exposure to SP affects the response to EGFR and HER2-targeted therapies.

**Combination of the NK-1R antagonist L-733,060 with anti-HER2 therapies synergistically inhibits the proliferation of breast cancer cells**

We next used MTT assays and applied combination index (CI) equations (35) to determine whether the inhibition of NK-1R activity could enhance the effects of EGFR or HER2 inhibitors. Treatment with L-733,060 synergized with the cytotoxic effects of AG825 (CI = 0.5686) and lapatinib (CI = 0.653) in the MDA-MB-453 cell line and also with lapatinib in the MDA-MB-468 cells (CI = 0.4494), although the combination of L-733,060 with AG1478 (CI = 1.7642) in the MDA-MB-468 cell line indicated an antagonist effect at the doses used (Fig. 6C and Supplementary Table S5). These results strongly support the idea that the modulatory properties of NK-1R on ERBB receptors could be exploited to increase the effectiveness of targeted therapies for the treatment of breast cancer by concomitant inhibition of NK-1R and ERBB receptors.

**Discussion**

Although the oncogenic addiction to HER2 signaling is therapeutically exploited for breast cancer treatment, the disappointing clinical responses observed in a significant percentage of patients suggest that additional mechanisms regulating the HER2 pathway may be used to fine tune the cancer cell responses to anti-HER2 therapies. The work presented here highlights a previously unidentified role of SP/NK-1R autocrine/paracrine constitutive signaling as a modulator of the basal activation of HER2 and EGFR in breast cancer—a mechanism with several clinical implications.

The transmodulation of RTKs by SP and other neuropeptides and proinflammatory mediators (36, 37) can serve as a mechanism for RTK activation in a ligand-independent way (7). The fact that the steady states of HER2 and EGFR depend upon the activity of additional signaling pathways implies that these instigator pathways could be therapeutically used to downregulate the activation of RTKs. For example, our laboratory has previously shown that SP inhibition decreases EGFR and HER2 activity and induces apoptosis, and that SP blockade is effective in inducing cell death even in cell lines that are resistant to the EGFR/HER2 inhibitors lapatinib and trastuzumab (17). Likewise, in this work we have shown that the chemical inhibition of NK-1R, alone or in combination with the inhibition of NK-2R and NK-3R, or the silencing of TACR1 gene by siRNA strongly decreases HER2 activity in all the cell lines studied, as well as the NK-1R antagonist L-733,060 reduces tumor growth in SCID mice harboring HER2^+ or EGFR^+ tumor cells. Although we did not reach high levels of TACR1 inhibition to avoid the effects of massive cell death, even TACR1 gene silencing by 50% to 70% showed effects on the steady state of HER2, and given the diversity of GPCRs expression in cancer cells, it is probable that this finding may be applied to a huge variety of ligands. It should be noted that a vast number of receptors
involved in tumor progression, such as some cytokine and chemokine receptors, fall into the category of GPCRs, implying that steady G-protein activation is always present in cancer cells. In this context, we observed that the inhibition of G-protein-mediated signaling decreases HER2 activity and expression, supporting the idea that the dual blockade of activation and transactivation pathways could enhance the therapeutic response to particular targeted therapies. Our results also imply that the lack of target dependency in some anti-HER2 therapies could be due, in part, to NK-1R transactivation mechanisms in those tumors that express the NK-1R receptor. In fact, we observed that an overexposure to SP in vivo increases HER2 expression in HER2þ or HER2– xenograft tumors and that this could be a relevant finding to consider in relation to those tumors initially classified as HER2 negative; the constant signaling of SP in tumor cells could favor a long-term selection of cells with increased levels of HER2. Although in our model, we did not observe an increase in tumor volume despite HER2 increase, it is likely that in a longer period of time, this constant activation of HER2 would translate in increased tumor volume, as it can be expected from situations of chronic inflammation in humans. Furthermore, in vitro experiments show that chronic exposure to SP also increases HER2 and/or EGFR expression and activation in the cell lines studied. Moreover, NK-1R inhibition synergizes with anti-EGFR and anti-HER2 therapies thereby significantly reducing cell survival.

The modulation of RTK activation by NK-1R also suggests oncogenic cooperation between these two types of receptors during tumor progression. As we observed in human primary tumors, NK-1R and SP are highly expressed in those of the HER2þ subtype, and SP can activate HER2 in primary cultures derived from human tumors with and without HER2 overexpression. In addition to HER2 overexpression, our current results identify NK-1R and SP as two new markers associated with other clinicopathologic characteristics related to a poor prognosis such as p53 overexpression, ER negativity expression, high proliferation, and a high histologic grade (38, 39).

In addition, the relevance of SP signaling in breast cancer could be context dependent. For instance, the specificity of NK-1R signal transduction depends on the formation of scaffolding protein complexes after its activation (40). In fact, the coexpression of EGFR and/or HER2 in the same cancer cell could provide SP with additional signal transduction adaptors, thus diversifying its effects. In a similar way, the apoptotic effects of NK-1R inhibition could depend on its ability to block the oncogenic dependence on HER2 and EGFR, as we observed in vivo where only HER2þ or EGFRþ tumors were affected after NK-1R inhibition. Moreover, as we showed in this work, not all SP receptors are related to the activation of the same tumor-promoting mechanism; for example, NK-2R receptor showed a correlation with ER- and PR-positive tumors in contrast with NK-1R, which is associated with ER- and PR-negative tumors. Finally, SP activation of the MAPK pathway has been shown to be RTK dependent (40) or independent (41), suggesting a cell-type-specific modulation of SP signaling.

In conclusion, the results presented here imply another mechanism by which inflammation, via SP, could enhance tumor aggressiveness. Furthermore, the interplay between multiple signaling pathways, such as those controlled by the receptors NK-1R and HER2, implies that to achieve a complete therapeutic response with therapies designed to target RTK, both mechanisms (activation and transactivation) may be inhibited, as we have shown for NK-1R and HER2 in the present work.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
Transmodulation of HER2 by SP in Breast Cancer


Substance P Autocrine Signaling Contributes to Persistent HER2 Activation That Drives Malignant Progression and Drug Resistance in Breast Cancer


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