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Role of Cannabinoid Receptor CB₂ in HER2 Pro-oncogenic Signaling in Breast Cancer


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Abstract

Background: Pharmacological activation of cannabinoid receptors elicits antitumoral responses in different cancer models. However, the biological role of these receptors in tumor physio-pathology is still unknown.

Methods: We analyzed CB₂ cannabinoid receptor protein expression in two series of 166 and 483 breast tumor samples operated in the University Hospitals of Kiel, Tübingen, and Freiburg between 1997 and 2010 and CB₂ mRNA expression in previously published DNA microarray datasets. The role of CB₂ in oncogenesis was studied by generating a mouse line that expresses the human V-Erb-B2 Avian Erythroblastic Leukemia Viral Oncogene Homolog 2 (HER2) rat ortholog (neu) and lacks CB₂, and by a variety of biochemical and cell biology approaches in human breast cancer cells in culture and in vivo, upon modulation of CB₂ expression by si/shRNAs and overexpression plasmids. CB₂-HER2 molecular interaction was studied by colocalization, coimmunoprecipitation, and proximity ligation assays. Statistical tests were two-sided.

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Results: We show an association between elevated CB2 expression in HER2+ breast tumors and poor patient prognosis (decreased overall survival, hazard ratio [HR] = 0.29, 95% confidence interval [CI] = 0.09 to 0.71, P = .009) and higher probability to suffer local recurrence (HR = 0.09, 95% CI = 0.049 to 0.54, P = .003) and to develop distant metastases (HR = 0.33, 95% CI = 0.13 to 0.75, P = .009). We also demonstrate that genetic inactivation of CB2 impairs tumor generation and progression in MMTV-neu mice. Moreover, we show that HER2 upregulates CB2 expression by activating the transcription factor ELK1 via the ERK cascade and that an increased CB2 expression activates the HER2 pro-oncogenic signaling at the level of the tyrosine kinase c-SRC. Finally, we show HER2 and CB2 form heteromers in cancer cells.

Conclusions: Our findings reveal an unprecedented role of CB2 as a pivotal regulator of HER2 pro-oncogenic signaling in breast cancer, and they suggest that CB2 may be a biomarker with prognostic value in these tumors.

The classical and the most recent molecular classification of breast cancer recognizes a specific entity characterized by the overexpression of the tyrosine kinase receptor (TKR) human V-Erb-B2 Avian Erythroleukemia Viral Oncogene Homolog 2 (HER2) (1-4). Activation of TKRs turns on key signaling pathways involved in cell proliferation, development, differentiation, and angiogenesis, among other processes (5). HER2 gene amplification/protein overexpression is detected in 20% to 30% of primary breast cancers and is a predictor of poor prognosis and deficient response to chemotherapy (6).

The endocannabinoid system (ECS) is a cell communication system that participates in the control of different physiological functions such as pain perception, motor behavior, and food intake, just to mention a few (7,8). It consists of two cannabinoid-specific G protein–coupled receptors (GPCRs), CB1 and CB2, their endogenous ligands, and the enzymes that produce and metabolize these ligands (7,8). A large number of studies demonstrate that the pharmacological activation of the ECS by different strategies (eg, activation of cannabinoid receptors, inhibition of endocannabinoid degradation) leads to antitumoral responses (7-11). Additionally, it has been shown that the ECS is deregulated in a variety of cancers (7,12-14). Although strong evidence points to the cannabinoid receptor CB2, as a drug target for antitumoral therapy in several types of cancer (11,14,15), there is no information on its role in tumor generation and progression. Here we show an unprecedented pro-oncogenic role of the cannabinoid receptor CB2 in HER2+ breast cancer and unveil that this GPCR is a pivotal regulator of HER2 signaling.

Methods
Tissue Microarrays
PFA-fixed and paraffin-embedded blocks of tumor tissue from cases operated in the University Hospitals of Kiel, Tübingen, or Freiburg between 1997 and 2010 were used for tissue microarray (TMA) construction. All patients gave informed consent, and the study was authorized by the respective Hospital Ethics Committees. TMAs were generated by punching two 1 mm spots of each patient’s sample. This resulted in two series of 166 and 483 tumor samples. Complete histopathological information was available for all the patients. Additionally, for the 483-sample series (TMA #2), date and cause of death as well as date of local and/or distant relapse were also available.

Immunohistochemical Analysis
Tissue sections were subjected to a heat-induced antigen retrieval step prior to exposure to an anti-CB2 receptor or an anti-ERBB2 primary antibody (Supplementary Table 1, available online). Immunodetection was performed using the Envision method with DAB as the chromogen. For CB2 expression, cases were scored as 0 (no staining), 1 (weak staining), 2 (moderate staining), or 3 (high staining). ERBB2 staining was scored by one independent pathologist in each institution (University Hospitals of Kiel, Tübingen, or Freiburg) in accordance with HercepTest manufacturer’s guidelines.

Generation of MMTV-neu:CB2+/- Mice and Sample Collection
All procedures involving animals were performed with the approval of the Complutense University Animal Experimentation Committee according to the EU official regulations. Generation of the congenic strain MMTV-neu:CB2+/- was accomplished by mating MMTV-neu mice with CB2+/- mice (see the Supplementary Methods, available online). Females were palpated twice weekly for mammary gland nodules. As soon as tumors appeared, they were routinely measured with external caliper, and volume was calculated as (4π/3) x (width/2) x (length/2). Animals were sacrificed and mammary glands, breast tumors, and lungs were collected (see the Supplementary Methods, available online) at the following time points: 1) when the first tumor in each animal appeared, 2) 40 days after the appearance of the first tumor, and 3) 90 days after the appearance of the first tumor.

Statistical Analysis
The Pearson’s chi-squared test was used for statistical analysis of the human samples included in the TMAs. Kaplan-Meier survival curves were statistically compared by the log-rank test. Analysis of variance (ANOVA) with a post hoc analysis by the Student-Newman-Keuls’ test was routinely used for the rest of the analyses. Unless otherwise stated, data are expressed as mean ± SD. All statistical tests were two-sided, unless otherwise specified. A P value of less than .05 was considered statistically significant.

Additional methods are available in the Supplementary Methods (available online).

Results
Prognostic Relevance of Tumor CB2 Expression
In two small cohorts of human samples, we previously reported that CB2 mRNA expression is associated with higher histological grades and increased HER2 expression (16) and that the CB2 protein was present in the vast majority of HER2+ tumors (17). Here, we analyzed CB2 expression in a much larger series of tissue sections (649 breast human samples included in different tissue microarrays [TMAs]). CB2 expression was scored as 0 (no staining), 1 (weak staining), 2 (moderate staining), or 3 (high staining) (Figure 1A). We observed that nontumor
Conversely, CB2 was expressed by a very large fraction of human DNA microarray datasets (HR = 0.25, 95% CI = 0.07 to 0.71) than HER2+/low CB2 (scores 0 and 1) patients. Similar observations were made when CB2 mRNA levels were analyzed in tissue microarray (TMA) samples: scores 0, 1, 2, and 3 correspond to no, low, moderate, and high staining, respectively. Scale bar = 500 µm.  

**Figure 1.** Analysis of CB2 protein expression in human breast cancer samples. A) Representative images showing CB2 expression scoring according to intensity staining in tissue microarray (TMA) samples: scores 0, 1, 2, and 3 correspond to no, low, moderate, and high staining, respectively. Scale bar = 200 µm. B) Representative CB2 immunohistochemical staining in a human nontumor breast tissue sample included in the analyzed TMAs. Inset, CB2 staining (brown) in a macrophage is shown as a CB2 staining-positive control. Scale bar = 200 µm. C) Association between CB2 expression (as determined by staining scoring) and the molecular features of breast tumor samples included in the TMAs. The Pearson’s chi-squared test was used for statistical analysis. All statistical tests were two-sided.

Breast tissue expressed undetectable levels of CB2 (Figure 1B). Conversely, CB2 was expressed by a very large fraction of human breast adenocarcinomas (476 out of 629, ie, 75.6%). CB2 expression was highly associated to HER2+ tumors (P < .001) (Figure 1C), while no association between CB2 expression and hormone-sensitive (P = .66) or triple-negative tumors (P = .14) was detected (Figure 1C). Thus, 96.7% of the HER2+ samples scored positive for CB2 expression (Figure 1C). Moreover, 65.2% of them expressed elevated levels of CB2 (scores 2 and 3) (Figure 1C). Importantly, these HER2+/high CB2 patients had decreased overall survival (hazard ratio [HR] = 0.29, 95% confidence interval [CI] = 0.09 to 0.71, P = .009) (Figure 2A) and higher probability for suffering local recurrence (HR = 0.09, 95% CI = 0.0049 to 0.54, P = .003) (Figure 2B) and for developing distant metastases (HR = 0.33, 95% CI = 0.13 to 0.75, P = .009) (Figure 2C) than HER2+/low CB2 (scores 0 and 1) patients. Similar observations were made when CB2 mRNA levels were analyzed in public DNA microarray datasets (HR = 0.25, 95% CI = 0.07 to 1.05, P = .06 in Figure 2D; HR = 0.43, 95% CI = 0.18 to 0.94, P = .04 in Figure 2E; and HR = 0.52, 95% CI = 0.33 to 0.83, P = .007 in Figure 2F) (18–20). Of interest, this association was not observed in HER2- patients (Figures 2G-I; HR = 0.71, 95% CI = 0.30 to 1.56, P = .36 in G; HR = 0.83, 95% CI = 0.36 to 1.86, P = .64 in H; and HR = 0.48, 95% CI = 0.14 to 1.30, P = .14 in I). Together, these results show a strong association between CB2 expression and tumor aggressiveness in HER2+ breast cancer.

**Impact of CB2 Knock-out on Breast Tumor Generation and Progression**

We next analyzed whether there was a cause and effect link between elevated CB2 expression and increased aggressiveness in HER2+ tumors. First, we observed that CB2 expression in the noncancerous mammary glands of adult wild-type (WT) female mice was virtually undetectable (Supplementary Figure 1A and B, available online). Moreover, it remained very low and unchanged during adult mammary gland development (Supplementary Figure 1A and B, available online). These results suggest that CB2 may not play a major role in the physiology of the healthy adult mammary gland. Next, we analyzed breast tumor generation and progression in an animal model of HER2-driven breast cancer (the MMTV-neu mouse) in which CB2 expression was knocked out (Supplementary Figure 2, A and B, available online). MMTV-neu:CB2−/− mice (n = 42) showed a striking delay in tumor onset as compared with their WT littermates (n = 67) (P = .03) (Figure 3A). Upon early detection, tumor histological features were very similar in both groups (low-grade adenocarcinomas with no lymphatic invasion) (Supplementary Figure 2C, available online), the only apparent difference between them being their mitotic index (medium in WT animals and low in the CB2−/− population) (Supplementary Figure 2C, available online). Forty days after their appearance, 100.0% of the CB2 KO-derived tumors kept their original histological characteristics, while
Figure 2. Prognostic relevance of tumor CB2 expression. Kaplan-Meier curves for overall survival (A, D-G) and metastasis-free survival (C and I). Numbers below x-axes correspond to the number of patients at risk in each group at the indicated time points. Data plotted in panels (A–C) correspond to the 224 HER2+ samples included in TMA #2. Data plotted in panels (D and E) correspond to the 65 human V-Erb-B2 Avian Erythroblastic Leukemia Viral Oncogene Homolog 2 (HER2)+ samples included in tissue microarray (TMA) #2 (see Methods). Data plotted in panels (G–I) correspond to the 224 HER2- samples included in TMA #2. Data plotted in panels (D and E) were obtained from the microarray data sets published in ArrayExpress database (accession number E-TABM-158) (D) and GEO database (accession number GSE3143) (E). Data plotted in (F) were obtained from (20) through the Kaplan-Meier Plotter (www.kmplot.com). In (D–I), samples were ranked by CB2 mRNA expression, and the best cutoff was manually selected. In (F), the best cutoff was automatically selected by the software. Survival curves were statistically compared by the log-rank test. All statistical tests were two-sided.

40.0% of those derived from WT animals were solid carcinomas with necrotic areas (Supplementary Figure 2C, available online). At the final stage of the disease (90 days after tumor appearance), CB2+/– tumors had negligible changes in their histology (although 40.0% of them presented necrotic areas), but the WT group included 40.0% of solid carcinomas, 40.0% of tumors with necrotic areas, and 20.0% of tumors with evident signs of lymphatic invasion (Supplementary Figure 2C, available online), all of them signs of more aggressive tumors.

The lack of CB2 receptors also reduced the number of tumors generated per animal (P = .03) (Figure 3B) and slowed down tumor growth (P = .003) (Figure 3C). The delayed tumor onset and the decreased tumor multiplicity and growth associated with the lack of CB2 receptors were accompanied by reduced levels of cyclin D1 and increased levels of the CDK inhibitor p21 in the tumors (Figure 3, D and E), both of which are hallmarks of HER2-induced malignant transformation and progression (21–23). Additionally, the levels of the endocannabinoid anandamide were higher in CB2+/– tumors (Supplementary Figure 2D, available online), and the mRNA levels of the enzyme responsible for anandamide degradation (FAAH) were lower in CB2-deficient tumors than in CB2+/– tumors (Supplementary Figure 2E, available online).
Figure 3. Impact of CB2 knock-out on breast tumor generation and progression. A) Kaplan-Meier curves for tumor onset in MMTV-neu:CB2 wild-type (WT) and MMTV-neu:CB2 KO mice. Numbers below x-axes correspond to the number of mice at risk in each group at the indicated time points. Results were analyzed by the log-rank test. B) Number of tumors generated per animal 90 days after first tumor arousal. C) Tumor volume 70 days after tumor appearance. D) Western blot analysis of Cyclin D1 and p21 in tumors generated by the indicated mice. Three representative samples per experimental group are shown. E) Densitometric analysis of the levels of the indicated proteins (determined by Western blot; n = 7 for MMTV-neu:CB2 WT tumors, and n = 6 for MMTV-neu:CB2 KO tumors). Results are expressed in arbitrary units. F) Percentage of animals with lung metastases 90 days after tumor arousal. Lung tumor masses were classified as macrometastases when they were visible to the naked eye at dissection and as micrometastases when they were only detectable by hematoxylin and eosin staining. G) mRNA levels (as determined by real-time quantitative polymerase chain reaction) of Tenascin C, SPARC, and COX2 in tumors generated by the indicated mice. Results are expressed in arbitrary units (n = 16 for MMTV-neu:CB2 WT tumors and n = 16 for MMTV-neu:CB2 KO tumors). Except in A, data were analyzed by analysis of variance with a post hoc analysis by the Student-Newman-Keuls’ test. * P < .05; ** P < .01 vs MMTV-neu:CB2 WT mice. All statistical tests were two-sided.
Together, these observations demonstrate that HER2 promotes the CB2 promoter and that this interaction is enhanced upon CB2 deficiency (Figure 5A). Moreover, immunoprecipitation of HER2 in HEK cells produced the coprecipitation of CB2, and vice versa (Figure 5B). The HER2-CB2 molecular association in cancer cells was confirmed using the proximity ligation assay. Thus, ectopic overexpression of HER2 in MDA-MB-231 cells enhanced the levels of CB2 (Figure 5C), and this effect was accompanied by the appearance of fluorescent dots, i.e., HER2-CB2 heteromers (Figure 5D). This fluorescent signal was not evident either in cells that do not express HER2 (with very low levels of CB2) or in cells in which CB2 expression was knocked down by means of selective shRNA (Figure 5D). Importantly, the presence of HER2/CB2 heterodimers was detected in human breast cancer cells that endogenously overexpress HER2 (Figure 5E) and in HER2-positive human breast cancer tissue (Figure 5F). Again, the heteromer fluorescent signal was not evident either when CB2 was stably silenced in cells (Figure 5E) or in tumors that do not overexpress HER2 (Figure 5F). These results confirm that CB2 is upregulated by HER2 and support that HER2 and CB2 form heteromers in cancer cells.

Role of CB2 in the Control of the Pro-oncogenic Features of HER2+ Breast Cancer Cells

We next analyzed the biological consequences of the HER2-induced CB2 upregulation. HER2 overexpression in MDA-MB-231 cells (which effectively enhanced CB2 levels) (Figure 5C) increased cancer cell viability (Figure 6A) and stimulated properties of cancer cells intimately related to tumor progression, i.e., invasion (Figure 6B) and anchorage-independent growth (Figure 6C). These effects were prevented by CB2 knock-down (Figure 6, A-C). Likewise, genetic silencing of CB2 (Supplementary Figure 3D, available online) reduced cell viability (Figure 6D), cell invasion (Figure 6E), colony formation in soft agar (Figure 6F), and the expression of metastasis markers (Supplementary Figure 3E, available online) in cells endogenously overexpressing HER2, an effect that was not observed in HER2-negative cells. Moreover, the growth of MDA-MB-231 HER2-overexpressing orthotopic xenografts (Figure 6G) and the generation of lung metastases (Figures 6, H and I; Supplementary Figure 3F, available online) were statistically significantly impaired upon stable CB2 knock-down (Supplementary Figure 3G, available online). Similarly, tumors generated from HER2-amplified cells statistically significantly reduced their growth when CB2 was silenced (Figure 6H). Collectively, these data show that CB2 promotes pro-oncogenic responses in a HER2 context.

Potential Targets of CB2-HER2 Pro-oncogenic Signaling

Next, we wanted to identify CB2 targets responsible for its pro-tumoral activity. Upon modulation of CB2, and HER2 expression and by means of a phospho-kinase array, we detected substantial alterations in some members of the SRC family of receptor tyrosine kinases, which has been extensively related to cancer (34–36). Specifically, we found that the expression of phosphorylated LYN, LCK, YES, FGR, HCK, and FYN decreased upon CB2 knock-down (Supplementary Figure 4, A and B, available online). However, this effect was observed both in HER2+ and HER2- cells (Supplementary Figure 4, A and B, available online), which suggests that, although these may be relevant CB2 targets in breast cancer, they are not HER2+ context specific. Of interest, the phosphorylated form of another member of the SRC family (c-SRC), which has particular relevance in cancer development and progression (34), was specifically upregulated upon HER2 overexpression activated ELK1, an effect that was accompanied by the activation of ERK (Figure 4B). Of interest, incubation with the MEK inhibitor U0126 prevented the enhancement of p-ELK1 levels (Figure 4B). Moreover, pharmacological inhibition of MEK (Figure 4C) and genetic knock-down of ELK1 (Figure 4D; Supplementary Figure 3B, available online) blocked the increase in CB2 mRNA levels elicited by HER2 overexpression. Likewise, ELK1 knock-down (Supplementary Figure 3C, available online) decreased CB2 mRNA levels in breast cancer cells that endogenously overexpress HER2, an effect that was not observed in HER2-negative cells (Figure 4E). By chromatin immunoprecipitation assays, we confirmed that ELK1 physically interacts with the CB2 promoter and that this interaction is enhanced upon HER2 overexpression and is prevented by inhibition of the ERK cascade (Figure 4F). Moreover, ELK1 was able to activate the CB2 promoter. Thus, transfection of HEK293T cells with a luciferase reporter encoding the CB2 gene promoter (pGL3-CB2) together with a constitutively active ELK1-expressing plasmid resulted in an increased luciferase activity when compared with cells transfected with pGL3-CB2 only (Figure 4G). Point mutations in the CB2 promoter revealed that the putative ELK1-binding sites located at positions -71 and -89 are the ones responsible for ELK1-induced activation of CB2 expression (Figure 4G). Together, these observations demonstrate that HER2 promotes CB2 upregulation by activating the transcription factor ELK1 via ERK activation. Supporting the relevance of this observation, the analysis of 1453 human breast cancer samples from seven different public DNA microarrays (19,28–33) showed a strong association (P < .001) between ELK1 and CB2 mRNA expression (Figure 4, H and I), and the immunofluorescence analysis of HER2+ breast cancer biopsies revealed that CB2-positive cancer cells presented nuclear ELK1 immunoreactivity (Figure 4J).

Analysis of the Potential Molecular Interaction Between HER2 and CB2

We wanted to determine whether the HER2-CB2 functional crosstalk was the result of a molecular interaction between the receptors. Immunofluorescence analysis of human HER2+ breast cancer cells revealed that the two receptors colocalize
Figure 4. Mechanistic insight into the association between CB2 and human V-Erb-B2 Avian Erythroblastic Leukemia Viral Oncogene Homolog 2 (HER2) expression.

A) ERBB2/HER2 protein expression (upper panel) and CB2 mRNA expression (lower panel) in MDA-MB-231-HER2 (231-HER2) and MDA-MB-231 cells (231). B) Western blot analysis of the indicated proteins, in the presence or in the absence of the MEK inhibitor U0126 (5 µM). C-D) CB2 mRNA expression (in arbitrary units), in the presence/absence of U0126 (C), or after transfection with ELK1 siRNAs (siELK1) or with a nontargeted siRNA (siC) (D) (n = 3 independent experiments).

E) Effect of ELK1 knockdown on CB2 mRNA expression in different human breast cancer cells endogenously overexpressing (black bars) or not (gray bars) HER2. Results are expressed in arbitrary units vs mRNA expression in the corresponding cells transfected with a control siRNA (siC), which was set at 1 in all cases (white bar).

F) ChIP assay in cells treated with or without U0126. Immunoprecipitation was performed with an anti-ELK1 Ab (or a nonspecific rabbit IgG as control).

G) CB2 gene promoter activity as determined by a luciferase reporter (n = 3 independent experiments). Drawings (left) represent the CB2 promoter construct transfected in each case. A constitutively active ELK1-containing plasmid was always cotransfected with the CB2 promoter. Line 1, CB2 promoter empty vector. H and I) Correlation of CB2 and ELK1 expression (analyzed by the Pearson’s correlation test) in human breast cancer samples from seven public DNA microarrays ([19,24,28,30,31,33] in [H] and [31] in [I]). J) Immunofluorescence analysis of CB2 (green) and phospho-ELK1 (red) in a HER2-positive (upper panels) and a HER2-negative (lower panels) human breast cancer sample. Cell nuclei are stained in blue. Scale bar = 100 µm. Except in (H and I), data were analyzed by analysis of variance with a post hoc analysis by the Student-Newman-Keuls’ test. *P < .01 vs vehicle-treated (C) or siC-transfected (D) 231 cells; † P < .05 vs vehicle-treated (C) or siC-transfected (D) 231-HER2 cells; ‡ P < .01 vs WT (G). All statistical tests were two-sided.
overexpression and downregulated by CB2 knock-down in that high-HER2 context (Figure 7A; Supplementary Figure 4B, available online). Moreover, when CB2 expression was restored in MDA-MB-231-HER2 shCB2 cells, p-c-SRC levels were increased, an effect that was not evident in the MDA-MB-231 that do not overexpress HER2 (Figure 7B). Likewise, a decrease in p-c-SRC upon CB2 silencing was observed in a panel of five breast cancer cell lines that endogenously overexpress HER2 (Figure 7C).

Next, we tested whether c-SRC was responsible for CB2-driven oncogenesis. First, we observed that mouse NIH/3T3 embryonic
Figure 6. Role of CB2 in the control of the pro-oncogenic features of HER2+ breast cancer cells. A-F) Cell viability as determined by the MTT test (A and D), invasion in matrigel-coated Boyden chambers (B and E), and number of colonies generated in soft agar (C and F) of MDA-MB-231 (231) and MDA-MB-231-HER2 cells (231-HER2) (A and B) or of the indicated HER2-positive or HER2-negative cell lines (D-F) stably expressing a shRNA selectively targeting CB2 (shCB2) or a scrambled shRNA (shC). G and J) Evolution of tumor volume in mice orthotopically injected with either 231 or 231-HER2 cells stably expressing shCB2 or an shC (G), or with the indicated HER2-amplified cells stably expressing the same shRNAs (J). H) Evaluation of the number of lung metastases generated per animal by injection of lung-seeking MDA-MB-231-HER2 cells (231-HER2-LM) stably expressing shCB2 or an shC into the mouse lateral tail vein. I) Quantification of the lung bioluminescence signal in the two experimental groups. Data were analyzed by analysis of variance with a post hoc analysis by the Student-Newman-Keuls’ test (n ≥ 3 independent experiments, except in [G-J]). * P < .05 and ** P < .01 vs the corresponding shC cell line; † P < .01 vs shC-231-HER2 cells. All statistical tests were two-sided.
fibroblasts acquire clonogenic properties upon overexpression of either CB2 or HER2 (Figure 8, A and B). Moreover, the ability of these cells to form colonies in soft agar statistically significantly increased when the two receptors were simultaneously overexpressed (Figure 8, A and B). Disruption of c-SRC signaling by using a c-SRC-dominant-negative construct prevented the oncogenic phenotype induced by CB2 plus HER2 (Figure 8, A and B). Of interest, the HER2-mediated increased clonogenicity was prevented by blocking c-SRC signaling, the CB2-induced clonogenic response was not (Figure 8, A and B), which indicates that CB2 promotes c-SRC activation (and the subsequent clonogenic response) via HER2. We then performed colony formation experiments with human HER2-amplified breast cancer cells. Specifically, we overexpressed CB2 in five HER2+ cell lines and observed an increase in the levels of p-c-SRC (Figure 8C). As expected, this increase in activated c-SRC was accompanied by an enhanced clonogenicity (Figure 8D). Importantly, pharmacological inhibition of c-SRC with Saracatinib (a SRC family/Abi dual-kinase inhibitor) in CB2-overexpressing cells kept both clonogenicity (Figure 8D) and p-c-SRC expression (Figure 8C) at the same level as in pcDNA3-transfected cells treated with the inhibitor, which further suggests that CB2-driven oncogenesis is mediated by c-SRC activation. Finally, and in further support of a causal link between the CB2/HER2/c-SRC axis and pro-oncogenic events, we found a decreased c-SRC and AKT activation in tumors generated by CB2-deficient animals (which present a less aggressive phenotype [Figure 3]) with respect to their WT littermates (Figure 8, E and F), and the analysis of human tumor biopsies revealed that HER2+ breast cancer cells expressing activated c-SRC also expressed CB2 (Figure 8G).

Discussion

Here we demonstrate not only that the cannabinoid receptor CB2 exerts a remarkable pro-oncogenic function in HER2+ breast cancer but also that CB2 plays a pivotal role in HER2-mediated pro-oncogenic signaling (Figure 8H). It is widely accepted that GPCRs and TKRs control critical biological processes intimately related to oncogenesis and that the functional cross-talk between members of these two receptor superfamilies (eg, transactivation of TKRs by GPCR-mediated signaling) may have important consequences in the progression and resistance to TKR-targeted therapies of some types of cancer (37,38), including HER2+ breast cancer (39). In some cases, the functional cross-talk between GPCRs and TKRs might rely on a physical interaction between receptors. Regarding HER2 specifically, it has been reported that this receptor can form a complex with the β2-adrenergic receptor in the heart and brain, which is required for mitogen-activated protein kinase activation induced by multiple GPCR agonists in cardiac myocytes (40). Here we show for the first time that a TRK (HER2) forms heteromers with a GPCR (CB2) in cancer cells. These findings reveal an unprecedented mechanism of control of HER2 activity that involves cannabinoid receptor CB2, and they suggest that the simultaneous targeting of the two receptors (or common downstream effectors) may be a reasonable therapeutic strategy. Because dual-targeting approaches are showing positive results in preclinical and clinical contexts when the targets are different members of the ERBB family (mainly ERBB1 and ERBB2) or even different domains of the same receptor (neutralizing antibodies + tyrosine kinase inhibitors, for example) (41), it is tempting to speculate that the combination of anti-HER2 compounds with cannabinoids targeting CB2 may have synergistic antitumoral effects. Interestingly, there is evidence showing that, at least at the preclinical level, the combination of cannabinoids with other anticancer therapies results in improved responses when compared with the corresponding individual treatments (11,42-45). It would therefore be desirable that future clinical trials determine whether these preclinical findings can be extrapolated to the cancer patient.

Our findings also show that the nonreceptor tyrosine kinase c-SRC plays a pivotal role in CB2-induced HER2 pro-oncogenic signaling (Figure 7). c-SRC promotes cell proliferation, survival, migration, and angiogenesis (34), and its deregulation is associated with oncogenesis (35,36) and poor patient prognosis (46). Together, these features make c-SRC an excellent target for the clinical development of specific inhibitors (35,36,46,47). In HER2+ breast cancer in particular, c-SRC activation has been implicated in the generation of brain metastases (48), a condition that has no curative treatment, and in the development of trastuzumab resistance (49). Interestingly, treatment of brain metastasis–bearing mice with a combination of Lapatinib (which targets ERBB1 and ERBB2) and a c-SRC inhibitor slowed down the growth of the metastases (48), and treatment of trastuzumab-resistant cells/tumors with a c-SRC inhibitor restored trastuzumab response (49). These data suggest that blocking c-SRC may be an effective manner to treat two important remaining clinical challenges in HER2+ breast cancer: the management of highly metastatic tumors (especially those colonizing the central nervous system) and trastuzumab resistance (both innate and acquired). Nonetheless, additional experiments should be performed to analyze the involvement of CB2 in c-SRC-mediated trastuzumab resistance and generation of brain metastases.
The proposed role of CB2 in HER2-driven pro-oncogenic signaling is that HER2 enhances CB2 expression by activating the transcription factor ELK1 via ERK. Increased CB2 expression promotes HER2 pro-oncogenic signaling by activating the tyrosine kinase c-SRC.
Our results clearly reveal a pro-oncogenic role of CB2 in HER2+ breast cancer. However, it has been widely described that pharmacological activation of this particular receptor exerts antitumor effects in different models of breast (14,16,17,50,51) and many other types of cancer (11). Further experiments should be performed to get a deeper insight into the molecular details of this bimodal effect of CB2 receptor functionality. For example, it would be interesting to know whether different cannabinoid stimuli (ie, an endogenous tone vs an exogenous pharmacological activation) produce a different activation of CB2/HER2-mediated signaling in terms of intensity and/or specific pathways.

Finally, we have observed a strong association between higher CB2 protein expression in HER2+ breast tumors and lower CB2 receptor (CB2R) expression in HER2+ breast cell lines compared with the respective less aggressive (low grade) tumors. Recently, an association between CB2 expression and overall and disease-free survival of patients with squamous cell carcinoma of the head and neck has also been reported (55). All these observations may serve to exploit CB2 as a new prognostic marker in oncology (at least in certain types of tumors).

In conclusion, here we present solid insight into an unprecedented pro-oncogenic effect of the CB2-HER2 signaling axis. However, we acknowledge some limitations in our study. First, the cell and animal models used in this study are well validated on translational grounds, but they do not fully recapitulate the pathology found in the actual HER2+ breast cancer patient. In this respect, although we have been able to unravel a strong association between CB2 expression in human HER2+ tumor specimens and the prognosis of the donor patients, it would be desirable that this association be established in larger patient populations to further support the potential impact of our observations on therapeutic decision-making. This should be ideally accompanied by clinical studies aimed at evaluating the safety and efficacy of strategies targeting, for example, CB2 and SRC, in combination with widely accepted anti-HER2+ breast cancer chemotherapies and immunotherapies. Additionally, although our findings support the existence of HER2-CB2 heteromers, further studies should be performed to unveil the functional relevance of these complexes in human breast cancer and whether—and, if so—how they actually drive CB2/HER2-evoked signaling at different stages of tumor malignancy. Notwithstanding such limitations, our study provides the first proof of concept on the action of CB2, as a new key player in HER2+ breast cancer biology.

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**Notes**

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