Therapeutic targeting of HER2–CB2R heteromers in HER2-positive breast cancer

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Although human epidermal growth factor receptor 2 (HER2)-targeted therapies have dramatically improved the clinical outcome of HER2-positive breast cancer patients, innate and acquired resistance remains an important clinical challenge. New therapeutic approaches and diagnostic tools for identification, stratification, and treatment of patients at higher risk of resistance and recurrence are therefore warranted. Here, we unveil a mechanism controlling the oncogenic activity of HER2: heteromerization with the cannabinoid receptor CB2R. We show that HER2 physically interacts with CB2R in breast cancer cells, and that the expression of these heteromers correlates with poor patient prognosis. The cannabinoid Δ9-tetrahydrocannabinol (THC) disrupts HER2–CB2R complexes by selectively binding to CB2R, which leads to (i) the inactivation of HER2 through disruption of HER2-HER2 homomers, and (ii) the subsequent degradation of HER2 by the proteasome via the E3 ligase c-CBL. This in turn triggers antitumor responses in vitro and in vivo. Selective targeting of CB2R transmembrane region 5 mimicked THC effects. Together, these findings define HER2-CB2R heteromers as new potential targets for antitumor therapies and biomarkers with prognostic value in HER2-positive breast cancer.

Breast cancer is a very heterogeneous disease in terms of molecular markers, prognosis, and treatments. According to all subclassification methods, there is a specific subtype that is characterized by overexpression of human epidermal growth factor receptor 2 (HER2), which represents roughly 15 to 20% of all breast tumors (1, 2). HER2 belongs to the ERBB (HER) receptor tyrosine kinase family, which consists of four members: HER1 (epidermal growth factor receptor; EGFR), HER2, HER3, and HER4. HER2 promotes oncogenic signaling by modulating the expression and activity of proteins controlling cell proliferation, differentiation, death, migration, and angiogenesis. Activation of HER2 is achieved by ligand- or overexpression-induced dimerization with other members of the family, followed by trans-phosphorylation and autophosphorylation of the two constituents of the HER homo/heterodimer in their cytosolic kinase domains (3, 4). Overexpression of HER2 in some ways is a paradigm for the design of targeted therapies for the management of this subtype of tumors. Thus, trastuzumab, a recombinant humanized monoclonal anti-HER2 antibody, has significantly improved the outcome of these patients (1, 4, 5). Despite its efficacy in many HER2+ breast cancer cases, some patients do not respond to this treatment and others eventually progress. Identifying the molecular mechanisms underlying HER2 activation (i.e., dimerization, trans- and autophosphorylation) has allowed the design of additional tools to overcome resistance to trastuzumab and


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improve the treatment of these tumors. For example, pertuzumab, another anti-HER2 monoclonal antibody, was designed to specifically target the dimerization domain of HER2, and lapatinib, a tyrosine kinase inhibitor, to selectively inhibit the trans- and autophosphorylation of HER1 and HER2 (1, 4, 5). Simultaneous targeting of HER2 at different levels (i.e., combination of the aforementioned agents) is showing better clinical outcomes than anti-HER2 monotherapies, but some patients still present with either innate or acquired resistance (1, 5). Therefore, new/complementary therapeutic approaches are urgently needed to both identify and treat this patient population.

Cannabinoids, the active constituents of cannabis, produce antitumor responses in preclinical models of cancer, including HER2+ breast cancer (6–9). In most cases, the antitumor responses are produced by binding and activating cannabinoid receptors. CB1R and CB2R, the two cannabinoid receptors described so far, belong to the G protein-coupled receptor (GPCR) superfamily of membrane proteins. While CB1R, the main receptor responsible for the psychoactive effects of cannabis, is widely expressed throughout the body and especially abundant in the central nervous system, CB2R, in healthy individuals, is mainly restricted to elements of the immune system. However, increasing evidence shows that the expression of this receptor is augmented in many pathological states, including cancer (6–8). In fact, the preclinical research conducted so far in preclinical models of HER2+ breast cancer points to CB2R as the main target of cannabinoid antitumor action (10, 11).

Here, we aimed at getting a deeper insight into the mechanisms of HER2 activation/inactivation, to provide new potential targets for treatment of HER2+ tumors. Specifically, we studied the functional relevance of a recently described heteromer between HER2 and the cannabinoid receptor CB2. We have previously reported the presence of these complexes in HER2+ breast cancer tumors (12), but their role in HER2 function is as yet unknown. In this context, the main goal of this study was to determine the role of HER2–CB2R heteromers in HER2+ breast cancer pathology and, overall, whether these structures could be new targets for anticancer treatments.

Results
HER2–CB2R Heteromer Expression Correlates with Poor Patient Prognosis. We have previously described that CB2R promotes HER2 prooncogenic signaling, and that these two membrane receptors physically interact in HER2+ breast cancer cells and tissue (12). However, the functional relevance of these heteromers is completely unknown. To evaluate their role in breast cancer, we first analyzed the expression of these complexes in a series of 57 human HER2+ breast cancer biopsies obtained at the time of first diagnosis, before any treatment (13). Proximity ligation assays (PLAs) (Fig. 1D) showed that higher HER2–CB2R expression in tumors is associated with lower disease-free patient survival (Fig. 1B), as well as with higher spread to regional lymph nodes and Ki67 overexpression (SI Appendix, Table S1). To further validate these observations, we performed similar analyses in

![Figure 1](image-url)

Fig. 1. HER2–CB2R heteromer expression correlates with poor patient prognosis. Proximity ligation assays were performed in tissue microarrays and patient-derived xenografts. (Scale bars, 25 μm.) For the TMAs, samples were ranked based on HER2–CB2R heteromer expression (i.e., PLA signal), and the best cutoff was manually selected. (A) Representative confocal microscopy images of a low– and a high–heteromer-expressing sample in TMA 1. The red dotted signal corresponds to the heteromers, and the blue staining corresponds to cell nuclei. (B–D) Kaplan–Meier curves for disease-free survival [from samples included in TMA 1 (n = 57) (B) or TMA 2 (n = 39) (C)] and overall patient survival [from the HER2+ samples included in TMA 2 (n = 33) (D)]. Curves were statistically compared by the log-rank test (p < 0.05). (E and F, Upper) Representative images of HER2–CB2R heteromer expression in two pairs of PDXs, consisting of a PDX established from the patient’s primary tumor and a sample derived from a metastasis in the same patient [in the liver in one case (E), and in a lymph node in the other (F)]. (E and F, Lower) Quantification of HER2–CB2R heteromer expression in the PDX samples. Results are expressed as PLA ratio (number of red dots per cell), and error bars represent SEM (n = 7 technical replicates in primary tumor samples; n = 5 in metastatic samples). HR, hazard ratio.
an additional TMA containing 39 human high-grade HER2+ ductal breast cancer samples obtained before any treatment (TMA 2 in *Materials and Methods*). High HER2–CB-R heteromer expression was also associated with poor patient prognosis, specifically lower disease-free and overall patient survival (Fig. 1 C and D). Positive and negative controls for HER2–CB-R heteromer expression are shown in *SI Appendix*, Fig. S1 A–D. The separate analysis of either HER2 or CB-R by immunohistochemistry confirmed these issues. First is that increased heteromer expression is not just a consequence of individual receptor overexpression. Thus, similar HER2–CB-R heteromer levels were found in tumors with low, medium, or high HER2 expression (*SI Appendix*, Fig. S1E), as well as with no, low, medium, or high CB-R expression (*SI Appendix*, Fig. S1F). Second is that HER2–CB-R heteromer expression is a better prognostic marker than HER2 alone or CB-R alone. Thus, no association between HER2 expression and disease-free survival was found in TMA 1 (*SI Appendix*, Fig. S2A) for CB-R expression, although there seemed to be an association trend with disease-free survival, it did not reach statistical significance either (*SI Appendix*, Fig. S2B). In addition, we analyzed heteromer expression in two pairs of patient-derived xenografts (PDXs). Each pair consisted of one PDX generated from the patient’s primary tumor and another PDX generated from the corresponding metastasis (in the liver in one case, and in a lymph node in the other). Consistent with HER2–CB-R complexes correlating with poor patient prognosis, in both cases we observed significantly higher heteromer expression in the metastatic tissue with respect to the corresponding primary tumor (Fig. 1 E and F). Together, these results show that HER2–CB-R heteromers are specific receptor complexes present in HER2+ breast cancer tissue, and are associated with tumor recurrence and spreading.

Δ²-Tetrahydrocannabinol Disrupts HER2–CB-R Complexes and Impairs HER2–CB-R Heteromer Disruption by THC Induces HER2+ Breast Cancer Cell Viability. Since HER2–CB-R heteromer expression seems to be linked to tumorigenic processes in cancer cells (Fig. 1), we next studied whether these complexes could be targets for antitumor therapies. It has been previously described that CB-R activation in different models of HER2+ breast cancer leads to cancer cell death by apoptosis and inhibition of tumor growth, angiogenesis, and metastasis (10, 11). To determine if HER2–CB-R heteromers are involved in this cannabinoid antitumor action, we analyzed their expression in response to Δ²-tetrahydrocannabinol (THC; the main bioactive constituent of cannabis). We first used HEK293 cells transiently transfected with HER2 and CB-R as a model. In that system, we confirmed the formation of HER2–CB-R complexes by bioluminescence resonance energy transfer (BRET) (Fig. 2 A and B). The heteromer signal significantly decreased upon THC treatment (Fig. 2C). The cannabinoid-induced decrease in both HER2–CB-R and cell viability relied on CB-R activation, as indicated by the preventive effect of the CB-R-selective antagonist SR144528 (SR2; Fig. 2 C and E). In addition, and supporting the idea that HER2–CB-R heteromers are unique signaling structures, we observed that upon exposure to THC, CB-R coupling shifts to a different set of heterotrimeric G-proteins. Thus, in cells only expressing CB-R, THC induced the coupling of the receptor to Gα13, while it promoted the coupling to Gαi, when HER2 and CB-R were co-expressed (*SI Appendix*, Fig. S3).

To determine whether the effects observed in HEK293 cells also occur in more physiological settings, we ran a series of experiments in two different human HER2+ breast cancer cell lines (BT474 and HCC1954). THC decreased the viability of both cell lines in a concentration-dependent manner (Fig. 2F), an effect that was again prevented by SR2 (Fig. 2G). The interaction between HER2 and CB-R in these cells was then analyzed by communoprecipitation upon overexpression of an HA-tagged form of CB-R. THC treatment diminished the amount of CB-R that communoprecipitated with HER2 in both cell lines, which points to a cannabinoid-induced disruption of the heteromer (Fig. 2H). The decrease in HER2–CB-R complexes was not due to a reduction in the receptors’ expression, as they remained unchanged after a 4-h THC treatment (Fig. 2H). To further support the idea that THC disrupts HER2–CB-R heteromers, we performed PLAs in the two breast cancer cell lines in native conditions (i.e., under no overexpression of HER2 or CB-R). Data showed that THC decreases the amount of these complexes by activating CB-R (Fig. 2 I and J).

HER2–CB-R Heteromer Disruption by THC Hampers HER2 Activation. HER2 activation occurs upon dimerization with other members of the HER family, followed by trans- and autophosphorylation of the intracellular domains of each protomer (13). We analyzed whether disruption of the HER2–CB-R heteromer by THC had any effect on this activation process. First, and to determine which specific HER dimers may be affected, we analyzed HER2–CB-R disruption, we evaluated the expression of the four members of the HER family in the two HER2+ cell lines used in our studies. In addition to HER2, we found HER1 and HER3 overexpression in at least one of them compared with a luminal (MCF7) or a basal (MDA-MB-231) breast cancer cell line (Fig. 3A). We therefore studied the effect of THC on HER2–HER1, HER2–HER2, and HER2–HER3 heteromers in HCC1954 cells. Neither HER2–HER1 nor HER2–HER3 complexes were affected by cannabinoid treatment (Fig. 3 B and C). In contrast, THC significantly diminished the amount of HER2–CB-R complexes. HER2–CB-R reduction was prevented by SR2 (Fig. 3 D and E). HER2–HER2 homodimer reduction upon THC challenge, and involvement of CB-R in this effect, were further confirmed by BRET in HEK293 HER2–CB-R cells (Fig. 3F). As expected, THC produced no such action in HEK293 cells lacking CB2 receptors (Fig. 3G). In line with these observations, THC decreased the levels of HER2 phosphorylated at Tyr1248 (Fig. 3 H and I), one of the main autophosphorylation sites in this receptor. Taken together, these observations demonstrate that HER2–CB-R heteromer disruption by THC hampers HER2 activation by interfering with its homodimerization.

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increased the amount of c-CBL in BT474 and HCC1954 cells (Fig. 5 D and E). Moreover, THC augmented the extent of HER2 phosphorylation at Tyr1112, the residue that is specifically recognized by c-CBL and triggers HER2 polyubiquitination (16) (Fig. 5F). Involvement of c-CBL in HER2 degradation was further supported by genetic blockade. siRNA-driven targeting of this E3 ligase prevented THC-induced decrease of total HER2 levels in the two breast cancer cell lines tested (Fig. 5 G and H).

Collectively, these findings demonstrate that THC disrupts HER2–CB2R heteromers, blocks HER2 activation, and promotes its degradation through the proteasome system via c-CBL activation, which results in antitumor responses.

HER2–CB2R Heteromer Disruption by Targeting CB2R Transmembrane 5 Mimics THC Effects. To determine whether the effects described above were THC-specific or could also be produced by other tools that disrupt HER2–CB2R heteromers, we used two different experimental approaches aimed at blocking the physical interaction between HER2 and CB2R. First, and to determine which part of the cannabinoid receptor is involved in the interaction with HER2, we generated a series of truncated proteins containing the N-terminal domain of CB2R, followed by one of the seven transmembrane (TM) domains of the receptor and its C-terminal domain. All constructs contained an HA tag in the N-terminal domain (Fig. 6A). Coimmunoprecipitation assays in HEK293 cells cotransfected with HER2 and the different CB2R constructs showed a potential interaction between HER2 and TMs 1, 3, 4, and 5 of CB2R (Fig. 6B). To determine which of them was more plausible to participate in the physical interaction between the two receptors, we performed bimolecular fluorescence complementation (BiFC) assays in HEK293 cells (Fig. 6C). A fluorescent, proximity-evoked signal was observed when the HER2 fusion protein was cotransfected with the CB2R fusion protein and the constructs containing CB2R TMs 1, 2, 3, 6, and 7 (Fig. 6D). This signal was significantly reduced upon cotransfection with CB2R TMs 4 and 5 (Fig. 6D), which is indicative of heteromer disruption. Since TM5 has been previously described to be involved in interactions between GPCRs (17–19), we focused our studies on this specific transmembrane domain. A TM5-targeted peptide (CB2R TAT-TM5) was then used to prevent the association between CB2R and HER2. The use of this type of peptide has been widely reported in the literature, and it is broadly accepted as a tool for disrupting GPCR–GPCR interaction (20, 21). BiFC experiments confirmed that this tool selectively blocks the formation of HER2–CB2R heteromers (Fig. 6E). Thus, the fluorescent signal indicative of the presence of HER2–CB2R heteromers disappeared when cells were incubated with the CB2R TAT-TM5 peptide, and not when they were challenged with a D44R TAT-TM5–targeted peptide (used as negative control) (Fig. 6E). Similar data were obtained when PLA were carried out in native untransfected HER2+ breast cancer cells (Fig. 7A), that is, a significant decrease in the dotted fluorescent signal corresponding to the heteromers appeared upon CB2R TAT-TM5 treatment, which was not evident when the D44R TMS5 peptide was used (Fig. 7A and B). Of interest, and as observed for THC, disruption of HER2–CB2R heteromers by the CB2R TAT-TM5 peptide produced (i) HER2 inactivation, as demonstrated by a dramatic decrease in the formation of HER2–HER2 homodimers (Fig. 7 C and D) and in the levels of phosphorylated HER2 (Fig. 7 E and F); (ii) HER2 degradation, evidenced by a marked reduction in total HER2 protein levels (Fig. 7 E and F); and (iii) a concomitant decrease in the viability of HER2+ breast cancer cells (Fig. 7G) that was not observed in wild-type HEK293 cells, which do not express either HER2 or CB2R (Fig. 7G). Altogether, these results show that disruption of HER2–CB2R heteromers, either with THC or with other tools aimed at interfering with the physical interaction between CB2R TM5 and the transmembrane domain of HER2, dramatically impairs the viability of HER2+ breast cancer cells.
Discussion

Here, we describe a mechanism controlling the activity of HER2 that may constitute a new target for antitumor treatments. Specifically, we observed that HER2 physically interacts with a membrane receptor that does not belong to the HER family (cannabinoid receptor CB2-R), thus forming HER2-CB2-R heteromers, and that disrupting these complexes triggers the inactivation and degradation of HER2, promoting in turn antitumoral responses. The HER2-CB2-R heteromers described herein fulfill the three criteria required for demonstrating receptor heteromerization (20, 21): First, the heteromer component (HER2 and CB2-R) interaction is demonstrated by proximity-based techniques and communoprecipitation; second, HER2-CB2-R heteromers exhibit properties distinct from those of the protomers, as demonstrated by the coupling of CB2-R to different heterotrimeric G proteins depending on whether it is part of the heteromer or not; and third, heteromer disruption leads to a loss of heteromer-specific properties, as demonstrated by the fact that while HER2 is a necessary step for HER activation, and in fact some drugs cotransfected with HER2-Rluc, HER2-YFP, and a CB2-R untagged receptor (n = 3) (F), or an empty vector (n = 4) (G) (used as a negative control for THC activation). In C and E–G, results are expressed as percent vs. vehicle-treated cells, set as 100%, and graph bars represent SEM. (H) Expression of pH2R444 in BT474 and HCC1954 cells, as determined by Western blot, upon THC treatment at the indicated time point. Quantitation. Results are normalized vs. the corresponding total HER2 levels at each individual time point, and expressed as fold increase vs. time 0, set at 1 (n = 4 in BT474; n = 7 in HCC1954). Error bars represent SEM. Unpaired independent groups of two were analyzed by two-tailed Student’s t test. When multigroup comparison was required, data were analyzed by one-way ANOVA with Tukey’s post hoc test. *P < 0.05, **P < 0.01 vs. vehicle-treated cells; ***P < 0.01 vs. THC. n.s., not significant.

peutic targets for disorders such as addiction, pain, Parkinson’s disease, and schizophrenia (21–23). Heteromers between cytokine and adrenergic receptors have also been described, with implications in blood pressure regulation (21, 23), or between different GPCRs in distinct endocrine systems, which may constitute new targets for endocrine-related disorders (24). Cannabinoid receptors in particular have been long described as constituents of particular GPCR receptor heteromers. Thus, CB2-R physically interacts with CB1-R (25), serotonin (26), adenosine (22, 27), opioid (28), orexin (29), and angiotensin (30) receptors, and with the cannabinoid-related orphan receptor GPR55 (31). CB2-R, on the other hand, has been shown to form heteromers with GPR55 (32, 33) and CXCR4 (34, 35). Although several RTK–RTK heteromers and GPCR–GPCR heteromers have been previously described, there are very few examples of physical interaction between RTKs and GPCRs yet. Transactivation of RTKs by GPCRs and vice versa has been reported and in some cases physical interactions have been suggested, but no solid proof of the existence of such heteromers has been provided in most cases (36). Usually, colocalization, communoprecipitation, and pharmacological transmodulation of the protomers in nonnative cell systems are the only evidence suggesting the presence of the heteromer, but this is clearly insufficient. Colocalization does not provide enough subcellular resolution to establish close proximity, and even communoprecipitation cannot go too far apart to directly modulate one another. Transactivation is no doubt a very interesting pharmacological process, but demonstrating the existence of receptor heteromers has additional importance in terms of providing new druggable therapeutic targets. To the best of our knowledge, the best-characterized RTK–GPCR heteromer is that formed by HER2 and β2-adrenergic...
receptors (38) or serotonin 5-HT receptors (39), which play important roles in synaptic plasticity; and by EGFR and GPR54, which seem to promote breast cancer cell invasiveness (40). Here, we comprehensively describe the existence of heteromers between HER2 and CB2R and provide compelling evidence showing that their disruption promotes antitumoral responses both in vitro and in vivo, which may constitute a new strategy to treat HER2+ breast tumors. It is tempting to speculate that other HER2-overexpressing tumors such as gastric or gastroesophageal adenocarcinomas (41) may express similar CB2R–HER2 heteromers, and therefore respond in a similar way to treatments aimed at breaking up these complexes.

Our previous work had shown that, in the absence of exogeneously applied cannabinoids, CB2R plays a protumoral role in HER2+ contexts (12). In line with this study, here we report that heteromer expression correlates with poor patient prognosis. On the other hand, there is solid evidence that pharmacological activation of CB2R produces antitumoral responses in HER2+ preclinical settings (10, 11). Considering all these observations, we propose the following model for HER2–CB2R function in breast cancer (Fig. 8). Under no pharmacological treatment, HER2+ breast cancer cells express high levels of HER2, which up-regulate the expression of CB2R via the mechanisms described in ref. 12. HER2 and CB2R then form heteromers in the plasma membrane, thereby protecting HER2 from degradation and favoring its canonical oncogenic signaling, resulting in protumoral responses (Fig. 8A). When cells are exposed to THC (or to other tools that prevent HER2–CB2R interaction), the two receptors physically separate. In addition, HER2–CB2R disruption triggers inactivation of HER2 (by breaking HER2–HER2 homodimers) and increases its susceptibility to degradation. As a final consequence of HER2 degradation and CB2R activation, an antitumor response is produced (Fig. 8B). It is also conceivable that in cellular contexts of very high HER2 expression, the role of CB2R in HER2 signaling may be less relevant than in those with just high HER2 levels. The experiments we have conducted clearly show a direct impact of THC on the viability of cancer cells in culture and also in vivo. However, we cannot rule out the involvement of other cell types in the full antitumor response induced by THC. For example, immune cells and endothelial cells express CB2R as well, and it is reasonable to speculate that they may be affected by THC. In fact, it has been previously demonstrated, for example, that THC impairs tumor angiogenesis by blocking endothelial cell migration and blood vessel formation and elongation (reviewed in ref. 42). Of note, antitumor responses upon CB2R activation have also been described in non-HER2+ contexts. It would be interesting to analyze whether in those situations CB2R acts as a monomer in the plasma membrane or if it forms heteromers with other RTKs like HER1 (EGFR), which is overexpressed in many different types of tumors (41). In favor of the latter, Elbaz et al. (43) recently reported that CB2R impairs oncogenic EGFR/EGFR signaling in ER+ breast cancer cells. Although not proved, the authors suggested that EGFR and CB2R may be forming complexes, and that CB2R activation might disrupt them (43). In addition, and similar to what we observed here, pharmacological activation of CB2R induced the death of prostate cancer cells in culture, an effect that was accompanied by a significant down-regulation of EGFR (44), and coexpression of EGFR with CB2R was associated with poor patient prognosis in this type of cancer (45). These observations demonstrate a functional interaction between another cannabinoid receptor (CB1R) and another member of the HER family (HER1) that could be due to a mere transactivation process or to a physical interaction similar to that described here between CB2R and HER2.

In summary, our findings unveil a mechanism of regulation of HER2 activity, and support HER2–CB2R heteromers as new therapeutic targets for the management of HER2+ breast cancer. Although THC efficiently achieves heteromer disruption, our data set the basis for the design of new antitumor drugs aimed at breaking this interaction. In addition, it would be interesting to design an alternative method to detect and quantify these heteromers in human samples. Thus, anti-HER2–CB2R antibodies or similar tools would allow not only corroboration of the prognostic value described herein but also an easy transfer of this knowledge to clinical practice.
Materials and Methods

Cell-Viability Assays. Cells were seeded at a density of 5,000/cm² in 10% FBS-containing medium. Twenty-four hours later, they were serum-starved overnight and then treated with THC for 24 h. Cells were then fixed and stained with a crystal violet solution (0.1% crystal violet, 20% methanol in H₂O) for 20 min. After intensive washing with water, the stained cells were solubilized in methanol and absorbance was measured at 570 nm.

Cell Cultures and Transfections. Human breast adenocarcinoma cell lines HCC1954 (CRL-2338), BT474 (HTB-20), MCF7 (HTB-22), and MDA-MB-231 (HTB-26) and the human embryonic kidney cell line HEK293T (CRL-1573) were purchased from American Type Culture Collection. They were all authenticated by short tandem repeat profiling (Genomics Core Facility at “Alberto Sols” Biomedical Research Institute) and routinely tested for mycoplasma contamination. Cells were cultured in RPMI (HCC1954, BT474), MEM (MCF7), DMEM (MDA-MB-231, HEK293T) supplemented with 10% FBS and 1% penicillin/streptomycin, and BT474 and MCF7 cells with 10 μg/mL insulin as well. They were all maintained at 37 °C in an atmosphere of 5% CO₂.

For cell-culture experiments, THC (THC Pharm) was dissolved in DMSO. Unless otherwise indicated, the concentration used was 3 μM for HCC1954 cells and 4 μM for BT474 and HEK293T cells. The CB₂-receptor antagonist SR144528 (SR2) (Tocris Bioscience) and lactacystin (Calbiochem) were dissolved in DMSO and added to the cell cultures (1 μM) 1 h prior to THC.

Expression vectors were transiently transfected with FuGENE HD transfection reagent (Promega) in human breast cancer cells, and polyethyleneimine (PEI) (Sigma-Aldrich) in HEK293T cells. Transient genetic knockdown was done by selective siRNA transfection with DharmaFECT 1 transfection reagent (Dharmacon). Selective siRNAs to knock down human c-CBL were purchased from Thermo Fisher Scientific.

Western Blot Analysis. Cells and tumors were lysed in RIPA buffer supplemented with 1 mM sodium orthovanadate, 1 mM PMSF, 2 μg/mL aprotinin, and 2 μg/mL leupeptin (Sigma-Aldrich). Total lysates were resolved by SDS/PAGE and electrophoretically transferred to PVDF membranes. After blocking with 5% (wt/vol) nonfat dry milk in TBS-Tween, membranes were incubated with the following antibodies overnight at 4 °C: rabbit polyclonal anti-HER2 (C-18; Santa Cruz Biotechnology); mouse monoclonal anti-HER2 (44E7) and rabbit polyclonal anti–phospho-HER2 (Tyr1112) (Cell Signaling Technology); rabbit polyclonal anti-HER1 (06-847; EMD Millipore); rabbit polyclonal anti-HER3 (1B2E, 4754; Cell Signaling Technology); rabbit polyclonal anti-HER4-HER2 (AC-74; Sigma-Aldrich); mouse monoclonal anti-c-CBL (clone 17; BD Biosciences); mouse monoclonal anti-STUB1 (CHIP) (ab2917; Abcam); and mouse monoclonal anti-ubiquitin (P4D1; Santa Cruz Biotechnology). Secondary antibodies were chosen according to the species of origin of the primary antibodies, and detected by an enhanced chemiluminescence system (Bio-Rad). β-Actin was used as loading control. Densitometric analysis of the relative expression of the protein of interest vs. the corresponding control (β-actin or total HER2) was performed with ImageJ (NIH).

Commmunoprecipitation Assays. HCC1954 and BT474 cells were transiently transfected with pcDNA3.1-HA-cCB₂R (UMR cDNA Resource Center, University of Missouri, Rolla) or the corresponding empty vector (pcDNA3) (Invitrogen) with FuGENE HD transfection reagent (Promega). HEK293 cells were transiently cotransfected with pcDNA3-HA-cCB₂R, pcDNA3.1-HA-cCB₂R, or pcDNA3 containing the different CB₂R transmembrane constructs (see below) using PEI (Sigma-Aldrich). Forty-eight hours after transfection, cells were lysed in a buffer containing 10 mM Hepes (pH 7.5), 120 mM NaCl, 1 mM EDTA, 10 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 50 mM sodium fluoride, 0.5 mM sodium orthovanadate, and 0.3% CHAPS and supplemented with 1 mM benzamidine and 0.1 mM PMSF. Cell lysates (1 mg) were incubated with anti-HER2 antibody (C-18; Santa Cruz Biotechnology) for 20 min. After intensive washing with water, the stained cells were solubilized in methanol and absorbance was measured at 570 nm.

**Fig. 5.** HER2–CB₂R heteromer disruption by THC induces HER2 degradation via the c-CBL E3 ligase. Western blot-based analyses of the effect of different pharmacological and genetic tools on THC-induced HER2 degradation. (A and B) Effect of lactacystin (LAC, 1 μM) on BT474 cells (n = 4). (C–F) Effect of THC (4 h) on ubiquitinated HER2 (UB) (C), on c-CBL and CHIP levels (D and E), or on HER2 phosphorylation at Tyr1112 (F) in the indicated breast cancer cell lines. (G and H) HER2 protein expression after genetic silencing of c-CBL with selective siRNAs (siCBL). A nontargeted siRNA was used as a control (siC). The densitometric analyses of HER2 immunoblots were normalized to β-actin (n = 4 in B; n = 6 in E; n = 4 in H). Results are expressed as fold increase vs. vehicle-treated cells, set at 1, and graph bars represent SEM. Unpaired, independent groups of two were analyzed by two-tailed Student’s t test. When multigroup comparison was required, data were analyzed by one-way ANOVA with Tukey’s post hoc test. *P < 0.05, **P < 0.01 vs. vehicle-treated group; *P < 0.05, **P < 0.01 vs. THC-treated group.
CR transmembrane domain 5 is involved in HER2-CR heterodimerization. (A) Schematic representation of the HA-tagged truncated forms of CR used in this study. Each construct contains the HA tag, followed by the N-terminal domain of the receptor, one of its seven transmembrane domains, and the C-terminal end. (B) Each of the seven CR constructs (named HA-TMX, where X is the corresponding transmembrane domain) and a pcDNA3-HER2 plasmid was coexpressed in HEK293 cells. Immunoprecipitation of HER2 with an anti-HER2 antibody was followed by Western blot analysis with an anti-HA antibody. Full-length pcDNA3-HA-CR was also coexpressed with HER2 as a positive control of interaction. (C) Schematic representation of the bimolecular fluorescence complementation experiments between HER2-CFP and CR-R-YPF in the absence (Upper) or presence of the CR transmembrane constructs (Lower). (D) and (E) Complementation signal (i.e., fluorescence at 530 nm) of HEK293 cells transfected with CR-CFP, HER2-YPF, and the indicated CR TM constructs (n = 3) (D), or after 4 h of incubation with the indicated TAT-TM peptides (4 μM) (n = 3) (E). Results were analyzed by one-way ANOVA with Tukey’s post hoc test. Error bars represent SEM. **p < 0.01 vs. pcDNA3 (D) or vehicle-treated group (E).

Fig. 6. CR transmembrane domain 5 is involved in HER2-CR heterodimerization. (A) Schematic representation of the HA-tagged truncated forms of CR used in this study. Each construct contains the HA tag, followed by the N-terminal domain of the receptor, one of its seven transmembrane domains, and the C-terminal end. (B) Each of the seven CR constructs (named HA-TMX, where X is the corresponding transmembrane domain) and a pcDNA3-HER2 plasmid was coexpressed in HEK293 cells. Immunoprecipitation of HER2 with an anti-HER2 antibody was followed by Western blot analysis with an anti-HA antibody. Full-length pcDNA3-HA-CR was also coexpressed with HER2 as a positive control of interaction. (C) Schematic representation of the bimolecular fluorescence complementation experiments between HER2-CFP and CR-R-YPF in the absence (Upper) or presence of the CR transmembrane constructs (Lower). (D) and (E) Complementation signal (i.e., fluorescence at 530 nm) of HEK293 cells transfected with CR-CFP, HER2-YPF, and the indicated CR TM constructs (n = 3) (D), or after 4 h of incubation with the indicated TAT-TM peptides (4 μM) (n = 3) (E). Results were analyzed by one-way ANOVA with Tukey’s post hoc test. Error bars represent SEM. **p < 0.01 vs. pcDNA3 (D) or vehicle-treated group (E).

Real-Time Quantitative PCR. RNA was isolated with TRIzol reagent (Invitrogen), and cDNA was obtained with Transcriptor Reverse Transcriptase (Roche Applied Science). Real-time quantitative PCR assays were performed using FastStart Master Mix with Rox (Roche). The primers used for ERBB2 (HER2) were as follows: forward, 5′-CCCTGCACCTCCTGGATA-3′; reverse, 5′-CCAGAGGCGTAGA-3′. Each value was adjusted by using β-actin (forward, 5′-CCAAACCGGGAAAGATGA-3′; reverse, 5′-CCAGAGGCCGTA-CCAGATGAG-3′) and β-glucuronidase (forward, 5′-CCCTGCTCCATCTC-TATTC-3′; reverse, 5′-TCCCCACAGGGAGTGTGTAG-3′) levels as references.

In Situ Proximity Ligation Assays. For PLAs in the TMA and in sections of the patient-derived xenographs, samples were deparaffinized and submitted to heat-induced antigen retrieval in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0). TMA-, PDX-, and xenograft-derived slices were permeabilized with PBS containing 0.01% Triton X-100. For PLAs in cell cultures, cells were seeded on glass coverslips at 5,000/cm². After overnight serum starvation, cells were treated for 4 h with THC, TAT-TM peptides (4 μM), or the corresponding vehicle. They were then fixed in 4% paraformaldehyde and permeabilized with 0.05% Triton X-100. In Situ Proximity Ligation Assays. For PLAs in the TMA and in sections of the patient-derived xenographs, samples were deparaffinized and submitted to heat-induced antigen retrieval in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0). TMA-, PDX-, and xenograft-derived slices were permeabilized with PBS containing 0.01% Triton X-100. For PLAs in cell cultures, cells were seeded on glass coverslips at 5,000/cm². After overnight serum starvation, cells were treated for 4 h with THC, TAT-TM peptides (4 μM), or the corresponding vehicle. They were then fixed in 4% paraformaldehyde and permeabilized with 0.05% Triton X-100. Heteroreceptors were detected by using the Duolink In Situ PLA Detection Kit (Sigma-Aldrich) following the manufacturer’s instructions. For detection of HER2-CR heteromers, cells were incubated with equal amounts of a rabbit to PVDF membranes. Membranes were blotted with anti-HA antibody (Cell Signaling Technology).

Ubiquitination Assays. Cells were lysed after 4 h of THC or DMSO treatment using RIPA buffer supplemented with 1 mM sodium orthovanadate, 0.1 mM PMFS, and 20 mM N-ethylmaleimide. Cell lysates (1 mg) were immunoprecipitated with an anti-HER2 antibody (C-18; Santa Cruz Biotechnology) or preimmune IgG overnight at 4 °C on a rotating wheel. Cell lysates were then incubated with protein G-Sepharose and then washed in RIPA lysis buffer. Finally, immunoprecipitates were resuspended in sample buffer containing 2-mercaptoethanol. Samples were then resolved and electrophoretically transferred to PVDF membranes and blotted with mouse monoclonal anti-ubiquitin antibody (M1D; Santa Cruz Biotechnology).

Fig. 7. HER2-CR heteromer disruption by targeting CR TM5 mimics THC effects. (A–D) Effect of TM peptides on HER2-CR and HER2-CR heteromer expression as determined by PLA. (A and C) Representative PLA images in the indicated breast cancer cell lines, after treatment for 4 h with vehicle (DMSO), a TAT-TM peptide targeting CR TM5 (4 μM), or a TAT-TM peptide targeting dopamine receptor D44 (4 μM), used as a negative control. Dimer signal is in red, and cell nuclei are in blue. Scale bars, 25 μm. (B and D) Results (n = 7 technical replicates) are expressed as percent of PLA (red dots per cell) vs. vehicle-treated cells, set as 100%. (E) pHER2/HER2 and HER2 protein levels, as determined by Western blot, after treatment with vehicle, CR TM5, or D44R-TM5 peptides for 24 h in BT474 and HCC1954 cells. (F) Denitrosometric analysis of HER2 normalized to β-actin (n = 3). Results are represented as fold increase vs. vehicle-treated cells, set as 1. (G) Viability of HCC1954, BT474, and HER2 knockdown cells in response to the indicated treatments for 24 h. Data (n = 4) are represented as percent vs. vehicle-treated cells, set as 100%, and graph bars represent SEM. One-way ANOVA with Tukey’s post hoc test. **p < 0.01 vs. vehicle-treated cells.
anti-CB\(_2\)R antibody (101550; Cayman Chemical) directly linked to a plus PLA probe, and a rabbit anti-HER2 antibody (C-18; Santa Cruz Biotechnology) directly linked to a minus PLA probe. For detection of other HER2 heterodimers, cells were incubated with a mixture of equal amounts of a mouse anti-HER2 antibody (44E7; Cell Signaling Technology) and rabbit anti-HER1 antibody (06-847; EMD Millipore) for HER2–HER1 detection, or with a rabbit anti-HER3 antibody (182E; Cell Signaling Technology) for HER2–HER3 detection. A plus anti-rabbit PLA probe and a minus anti-mouse PLA probe were used. For negative controls, one of the primary antibodies was omitted. Ligation and amplification were done with In Situ Detection Reagent Red (Sigma-Aldrich), and slides were mounted in DAPI-containing mounting medium. Samples were analyzed with a Leica SP2 confocal microscope (Leica Microsystems) and processed with ImageJ software. Heteromer expression was calculated as the number of red fluorescent spots (indicating that receptors are within sufficient proximity) per total cells in the field. Representative images for each condition were prepared for figure presentation by applying brightness and contrast adjustments uniformly using Adobe Photoshop CS5.

**Fusion Proteins for BRET and BiFC Assays.** Sequences encoding amino acid residues 1 to 155 and 156 to 238 of YFP Venus hemitruncated proteins. The human cDNAs for HER2, cannabinoid (CB\(_2\)), dopamine (D44R), and Ghrelin (GHS-R1a) receptors, cloned into pcDNA3.1, were amplified without their stop codons using primers harboring EcoRI and BamHI sites to clone CB\(_2\)R and GHS-R1a; Xhol and EcoRI to clone D44R; or Nhel and Xhol to clone HER2. Amplified fragments were subcloned to be in-frame with restriction sites of pRLuc-N1 (PerkinElmer) or pEYPFP-N1 (enhanced yellow variant of GFP; Clontech) vectors, to generate plasmids that express proteins fused to RLuc or YFP on the C-terminal end (HER2–RLuc, D44R–RLuc, HER2–YFP, CB\(_2\)R–YFP, or GHS-R1a–YFP). For BiFC experiments, the cDNA for HER2, CB\(_2\)R, and D44R were also subcloned into pcDNA3.1-nVenus or pcDNA3.1-cYFP to generate a plasmid that expresses the receptor fused to the hemitruncated nYFP Venus or hemitruncated cYFP Venus on the C-terminal end of the receptor (HER2-nVenus, D44-nVenus, or CB\(_2\)R-cVenus).

**CB\(_2\)R Transmembrane Mutants.** A pcDNA3-HA-CB\(_2\)R plasmid was used as template for the generation of seven mutants containing an HA tag, followed by the N-terminal domain, one transmembrane domain, and the C-terminal domain of CB\(_2\)R. To assure the correct orientation of the resulting peptides, in constructs containing even-numbered transmembrane domains, the sequences corresponding to the transmembrane domains were reversed. The primers used to generate these constructs are shown in *SI Appendix, Table S2.*

**HIV TAT-TM Peptides.** Peptides containing the amino acid sequence of CB\(_2\)R and D4R transmembrane domains S were used as heteromer-disrupting agents. To allow intracellular delivery and the correct membrane orientation, they were fused (at the C-terminal domain) to the cell-penetrating HIV TAT peptide. Their resulting TAT-TM peptides were TMS-TAT-CB\(_2\)R: DYYSLWSLKLAFL-FSGIITTYGHLWGYGRKKRQRRRR and TMS-TAT-D4R: YVVYSSVYCFSLPFCPLMLLLWATGYGRKKRQRRRR. They were synthetized at the Peptide Synthesis Facility at University Pompeu Fabra.

**Bioluminescence Resonance Energy Transfer Assays.** HEK293 cells were transiently cotransfected with a constant amount of a CDNA encoding HER2 or D44R fused to RLuc protein (HER2–RLuc, D44R–RLuc) as donor, and with increasing amounts of a CDNA of the other receptor fused to YFP (CB\(_2\)R–YFP, HER2–YFP, GHS-R1a–YFP) as acceptor. For quantification of protein–YFP expression, fluorescence at 530 nm was analyzed in a FLUOstar Optima fluorimeter (BMG Labtech). Fluorescence of cells expressing the BRET donor only was subtracted from these measurements. BRET signal was analyzed 1 min after addition of the bioluminescent substrate coelenterazine H. The net BRET is defined as [(long-wavelength emission)/(short-wavelength emission)] – Cf, where Cf corresponds to [(long-wavelength emission)/(short-wavelength emission)] for the Rluc construct expressed alone in the same experiment. BRET is expressed as milli-RET units (mBU; net BRET × 1,000). In BRET curves, BRET was expressed as a function of the ratio between fluorescence and luminescence × 100 (YFP/RLuc). To calculate maximum BRET (BRET\(_\text{max}\)) from saturation curves, data were fitted using a nonlinear regression equation and assuming a single phase with GraphPad Prism software.

**Bimolecular Fluorescence Complementation Assays.** HEK293 cells cotransfected with HER2 fused to the YFP Venus N terminus (nYFP) and CB\(_2\)R fused to the YFP Venus C terminus (cYFP) were treated with vehicle, the CB\(_2\)R mutants, or the indicated TAT-TM peptides (4 μM) for 4 h at 37 °C. Fluorescence at 530 nm (which only appears after YFP complementation due to proximity of the two receptors fused to cYFP and nYFP hemiproteins) was quantified in a FLUOstar Optima fluorimeter (BMG Labtech). Protein fluorescence expression was determined as the fluorescence of the sample minus fluorescence of nontransfected cells. Cells expressing HER2-nYFP and nYFP or CB\(_2\)R-cYFP and cYFP showed similar fluorescence levels to nontransfected cells.

**Antibody-Capture [\(^{35}\)S]GTP\(_S\) Scintillation Proximity Assays.** Specific activation of different subtypes of G\(_i\) proteins by THC (5 μM) was determined as previously described (46). Briefly, cell-membrane homogenates from the four different cell lines [HEK293 cells transiently overexpressing HER2, CB\(_2\)R, both receptors (HER2–CB\(_2\)R) simultaneously, or the corresponding empty vector (pcDNA3)] were incubated in 96-well isoplates (PerkinElmer Life Sciences) in incubation buffer containing 0.4 nM [\(^{35}\)S]GTP\(_S\) (PerkinElmer) and 50 or 100 μM GDP for G\(_\alpha\)s, G\(_\alpha\)i2, G\(_\alpha\)i3, and G\(_\alpha\)12/13; Santa Cruz Biotechnologies) and PVT scintillation proximity assay beads coated with protein A (PerkinElmer) were used. Radioactivity was quantified on a Microbeta TriLux scintillation counter (PerkinElmer).

**Animals and Treatments.** All procedures involving animals were performed with the approval of the Complutense University Animal Experimentation Committee and Madrid Regional Government, according to European official regulations. For the generation of orthotopic tumors, 5 × 10\(^6\) HCC1954 cells were injected into the fourth right mammary fat pad of anesthetized (with 4% isoflurane) 6-wk-old SCID female mice (Envigo). Tumor volume was routinely measured with an external caliper, and when it reached an average volume of 200 mm\(^3\), animals were randomly assigned to the different experimental groups: THC (1.5 mg animal\(^{-1}\) dose\(^{-1}\)) or sesame oil as vehicle.

**Patient-Derived Xenografts.** Human breast tumors used to establish PDXs were from biopsies or surgical resections at Vall d’Hebron University Hospital, and were obtained following the institutional guidelines and approval of

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**Fig. 8.** Schematic drawing of the proposed mechanism of control of HER2 activity by CB\(_2\)R. (A) HER2 forms heteromers with CB\(_2\)R at the plasma membrane of HER2+ breast cancer cells, protecting it from degradation and favoring its prooncogenic signaling. (B) Disruption of HER2–CB\(_2\)R heteromers, either by THC or by specific tools targeting CB\(_2\)R transmembrane domain S, triggers inactivation of HER2 by inducing the separation of HER2–HER2 homodimers and increasing HER2 susceptibility to degradation by the E3 ligase c-CBL. HER2 degradation and CB\(_2\)R activation result in antitumor responses.
the institutional review boards at Vall d’Hebron University Hospital in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients who provided tissue. Fragments of patient samples were implanted into the mammary fat pad of NOD CB17-Prkdcscid (NOD/SCID) (S-NOD-55; F: Janvier) and maintained with 17β-estradiol (1 μM) (E8875-1G; Sigma) in the drinking water. Mice were maintained and treated in accordance with the institutional guidelines of the Vall d’Hebron University Hospital Care and Use Committee.

Tissue Microarrays. Two different tumor series, in a TMA format, were used in this study. TMA 1 consisted of 57 samples corresponding to newly diagnosed HER2+ breast cancer patients operated at 12 de Octubre University Hospital between 1999 and 2013, and prior to any treatment. TMA 2 was previously described in ref. 47, and contained 138 high-grade ductal breast cancer samples obtained before treatment at the Vall d’Hebron University Hospital, Virgen del Rocio Hospital (Seville, Spain), and MD Anderson Cancer Center (Madrid, Spain) between 2003 and 2014. Of them, 39 corresponded to HER2+ cases. In both cases, paraformaldehyde-fixed and paraffin-embedded blocks of tumor tissue were used to generate the corresponding TMAs by punching two 1-mm spots of each patient’s biopsy.

Statistics. Kaplan–Meier survival curves were statistically compared by the log-rank test. The best cutoff was manually selected for each TMA. In TMA 1, the PLR signal ranged from 1.3 to 16.0, and the cutoff was set at 8.0. In TMA 2, the PLR signal ranged from 1.5 to 6.0, and the cutoff was set at 4.0. Unpaired, independent groups of two were analyzed by two-tailed Student’s t-test. When multigroup comparison was required, data were analyzed by one-way ANOVA. Post hoc tests were performed. Tumor growth curves from vehicle- and THC-treated animals were statistically compared by two-way ANOVA. Significance level was below 0.05 in all cases. Results are shown as mean ± SEM, and the number of experiments is indicated in every case. All analyses were carried out using GraphPad software.

Data supporting the findings of this study are available within the paper and SI Appendix.

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