ErbB Receptor Signaling and Therapeutic Resistance to Aromatase Inhibitors

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Abstract We have investigated the effect of HER-2 overexpression on resistance to the aromatase inhibitor letrozole in MCF-7 breast cancer cells stably expressing cellular aromatase (MCF-7/CA). MCF-7/CA cells overexpressing HER-2 showed a >2-fold increase in estrogen receptor (ER)–mediated transcriptional reporter activity upon treatment with androstenedione compared with vector-only control MCF-7/CA cells. Cotreatment with letrozole did not abrogate androstenedione-induced transcription and cell proliferation in HER-2-overexpressing cells. Chromatin immunoprecipitation assays using cross-linked protein-DNA from MCF-7/CA/HER-2 cells indicated ligand-independent association of the ERα coactivators AIB-1 and CBP to the promoter region of the estrogen-responsive *pS2* gene. Upon treatment with androstenedione, there were increased associations of AIB1 and CBP with the pS2 promoter in the HER-2-overexpressing compared with control MCF-7/CA cells. These results suggest that ligand-independent recruitment of coactivator complexes to estrogen-responsive promoters as a result of HER-2 overexpression may play a role in the development of letrozole resistance.

The standard treatment for estrogen receptor–positive (ER$^+$) breast cancer includes several agents that antagonize the effects of estrogen (1). Tamoxifen, a selective ER modulator with ER-antagonistic and ER-agonistic activity, is still the agent of choice in the majority of postmenopausal women with ER$^+$ breast cancer. Second-line therapy typically has consisted until recently of aromatase inhibitors, such as letrozole, anastrozole, or exemestane, which block endogenous production of estrogen from testosterone and androstenedione. A number of recent studies have suggested superiority of aromatase inhibitors over tamoxifen, which currently is no longer considered the agent of choice in the majority of postmenopausal patient with hormone-dependent breast cancer. Mouridsen et al. (2) compared the efficacy of tamoxifen with letrozole in 900 patients with advanced ER$^+$ or progesterone receptor–positive (PR$^+$) breast cancer. Patients treated with letrozole had improved time to progression (median, 41 versus 26 weeks), overall response rate (30% versus 20%), and rate of clinical benefit (49% versus 38%). The Arimidex, Tamoxifen Alone, or in Combination Study compared the aromatase inhibitor anastrozole to tamoxifen as adjuvant therapy in >9,000 postmenopausal women with early, ER$^+$ cancer. Patients received either drug or a combination of the two. There were fewer recurrences and new primary breast cancers as well as a statistically better disease-free survival rate in the anastrozole arm compared with the other two arms (3).

These data fit several preclinical observations that support the therapeutic targeting of breast tumor cell aromatase. One study identified aromatase mRNA and protein in tumor cells of postmenopausal breast cancer patients. Treatment of histocultures from these specimens with testosterone increased cell proliferation (4), implying that intratumor aromatase has functional significance. ER$^+$ MCF-7 xenografts in ovariectomized nude mice exhibited increased tumor estradiol and cellular proliferation in response to androstenedione (5). In another study, MCF-7 xenografts transfected with the aromatase gene were inhibited by either aromatase inhibitors or the pure antiestrogen fulvestrant (ICI 182,780), whereas the dual ER agonist/antagonist tamoxifen had little effect (5, 6). These studies support both the importance of intratumor aromatase in the progression of breast cancer and the use of aromatase inhibitors as first-line therapy in patients with ER$^+$ mammary tumors.

Oncogene Signaling and Antiestrogen Resistance

ER$^+$ breast cancers typically progress from antiestrogen sensitive to being antiestrogen resistant. Several resistance mechanisms have been proposed. These include the rare loss of ER by tumors, the uncommon selection of cells with ER mutations, alterations in the intracellular pharmacology and/or binding of antiestrogens to breast cancer cells, development of

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ligand-independent ER-mediated transcription, and, generally more accepted, the perturbation of the interactions between ER and coactivators and corepressors of transcription (reviewed in ref. 7). Tamoxifen can repress or activate transcription of estrogen-target genes. Upon the emergence of resistance, the agonistic effects of selective ER modulators like tamoxifen can predominate, leading to breast tumor growth and/or endometrial cancer (8).

Several data suggest a causal association between overexpression or aberrant activity of HER-2/*neu* (erbB2) and antiestrogen resistance (9, 10). The HER-2 receptor is the product of the HER-2 proto-oncogene and a member of the epidermal growth factor (EGF) family of receptor tyrosine kinases, which also includes EGF receptor (EGFR; HER1 and erbB1), HER-3 (erbB3), and HER-4 (erbB4). Upon binding of ligand to either the EGFR, HER-3, or HER-4, the HER-2 receptor is recruited as the preferred partner of these ligand-bound receptors into a kinase-active, phosphorylated heterodimeric complex, which activates signaling pathways that lead to enhanced proliferation and survival of tumor cells (11). HER-2 overexpression in breast cancers is associated with a more metastatic behavior and poor patient prognosis (12). Patients with HER-2-overexpressing tumors exhibit lower response rates and/or shorter duration of response to antiestrogen therapy (13). Overexpression of the EGFR and/or its ligands has also been associated with antiestrogen resistance (14). These data suggest that the EGFR/HER-2 signaling network is a robust molecular therapeutic target in antiestrogen-resistant human breast carcinoma.

Overexpression of HER-2 in tamoxifen-sensitive MCF-7 breast cancer cells results in mitogen-activated protein kinase (MAPK) hyperactivity and resistance to antiestrogens (15–17). In addition, ER^+ breast cancer cells grown in estrogen-depleted conditions exhibit increased MAPK activity (18, 19). In turn, this activated MAPK makes cells more sensitive to the mitogenic effects of low concentrations of estrogen (18). MAPK promotes increases ER phosphorylation of Ser^{118} and increases ER association with coactivators but decreases association with corepressors, thus favoring hormone-induced gene transcription (20). Administration of the EGFR tyrosine kinase inhibitor gefitinib for 4 to 6 weeks to patients with $ER^+/EGFR^+$ primary breast cancers has recently been shown to inhibit Ser^{118} ERα phosphorylation, Tyr^{845} EGFR phosphorylation, and tumor size (21), strongly suggesting that the activated EGFR cross-talks with ER signaling and potentially regulates its function in hormone-dependent breast cancer. In turn, hormone-activated ER can increase transcription of EGFR ligands (22), thus establishing a positive feedback loop that amplifies the output of the cross-talk between polypeptide growth factor and steroid receptors. Because both aberrant EGFR and HER-2 can hyperactivate MAPK, overexpression of these receptors can enhance the agonistic effects of tamoxifen on ER-mediated transcription and therefore lead to antiestrogen resistance. In addition to facilitating the agonistic effects of tamoxifen, post-translational modifications of the ER and/or its coactivators by oncogene signals can also result in ER supersensitivity to low doses of endogenous estradiol and early escape from aromatase inhibitors (see below).

Elevated MAPK activity has been reported in ER^+ breast cancer cells subjected to chronic estrogen deprivation (18, 19). Whether acquired overexpression of EGFR and/or HER-2 mediates the MAPK hyperactivity observed as a result of estrogen deprivation is not clear. Thus, we have hypothesized that high EGFR/HER-2 and/or MAPK signaling may induce hyper-responsiveness to low levels of estradiol, permit adaptation of ER^+ breast tumor cells to estrogen deprivation, and eventually result in hormone independence and resistance to aromatase inhibitors. However, a study by Ellis et al. (23) suggests the opposite. In this study, postmenopausal women with ER^+, $EGFR^+$, and/or $HER\text{-}2^+$ tumors responded clinically to 4 months of therapy with letrozole but poorly to tamoxifen, suggesting that $EGFR^+$ and/or $HER\text{-}2^+$ tumors are highly hormone dependent. This result is intriguing in that EGFR and HER-2 can activate several other ER-independent transforming signaling pathways (11) that per se can contribute to tumor progression and that should not be inhibited by inhibition of aromatase. Considering that the assessment of clinical response in this study was done after a short period of 4 months, the possibility that EGFR and/or HER-2 can mediate early escape from aromatase inhibitor therapy and estrogen deprivation cannot be ruled out. Indeed, in patients treated for 12 weeks with neoadjuvant anastrozole in the IMPACT trial, the reduction of proliferation as measured by Ki67 immunohistochemistry seemed short-lived (24), suggestive of an early escape from the aromatase inhibitor as a function of oncogene overexpression. Therefore, we have tested the hypothesis that overexpression of EGFR/HER-2 will result in acquired resistance to aromatase inhibitors.

One therapeutic approach for the inhibition of EGFR and HER-2 has been the generation of ATP-competitive small molecules that bind to the ATP site in the receptors' catalytic domain (reviewed in ref. 14). Two promising tyrosine kinase inhibitors of the HER network are gefitinib (ZD1839) and erlotinib (OSI-774). Both have been shown to inhibit breast cancer cells that express EGFR and HER-2 *in vitro* and *in vivo* (25, 26). MCF-7 cells selected for resistance to the pure antiestrogen fulvestrant (ICI 182,780) exhibited increased dependence on EGFR/MAPK signaling. The resistant cells were extremely sensitive to gefitinib (27). In another study, tamoxifen-resistant MCF-7 cells exhibited markedly elevated levels of EGFR and HER-2 as well as activated MAPK compared with wild-type cells. Tamoxifen resistance did not develop if the selection was done in the presence of gefitinib (28). Finally, Shou et al. showed that gefitinib eliminated HER-2/ER cross-talk and restored the antitumor effect of tamoxifen *in vivo* in MCF-7 cells stably overexpressing HER-2 (17). These cumulative data suggest that activation of EGFR/HER-2 signaling is causal to acquired tamoxifen resistance.

HER-2 Overexpression and Resistance to Letrozole

We have set out to determine if forced expression of HER-2 results in resistance to letrozole in MCF-7 cells that have been stably transfected with an aromatase gene cDNA (MCF-7/CA cells; ref. 4). The MCF-7/CA cells were provided by Richard Santen (University of Virginia in Charlottesville). These cells form tumors in nude mice that are inhibited by letrozole treatment and can be maintained in culture in estrogen-depleted conditions in the presence of 10 nmol/L androstenedione (29). We stably transduced MCF-7/CA cells with pBabe-erbB2 (HER-2) or pBabe (control) retroviral vectors and selected in puromycin as described (30) and confirmed HER-2 expression by immunoblot (Fig. 1A). To

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test for antiestrogen resistance, we have examined luciferase expression in cells transiently transfected with a pGLB-MERE plasmid as described (15). This plasmid contains a double consensus estrogen response element into the *Hin*dIII site of pGLB. MCF-7/CA/vector and MCF-7/CA/HER-2 cells were plated in estrogen-depleted medium containing 25 nmol/L androstenedione. Intracellular aromatase converted androstenedione to estradiol and thus activated estrogen response element–induced transcription and luciferase expression. Interestingly, basal and androstenedione-induced ER reporter activity was higher in MCF-7/CA/HER-2 cells, and the ligand-induced activity was markedly less sensitive to 1 nmol/L letrozole compared with MCF-7/CA cells (Fig. 1B).

The ligand-independent ER reporter activity prompted us to next examine the components of the ER transcription complex in the well-characterized ER-responsive pS2 promoter by using chromatin immunoprecipitation. The reporter activity suggested that in the absence of androstenedione (or estradiol), the ER would be constitutively bound to transcriptional coactivators, such as AIB-1 and the histone acetylase CBP. Cells were treated with androstenedione ± letrozole or estradiol and cross-linked with formaldehyde. After precipitation of chromatin fragments with ERα, AIB-1, or CBP antibodies, DNA contained in immune complexes was amplified by PCR using specific primers for the promoter regions of the *pS2* gene. The pS2 PCR product was detectable in ERα, AIB-1, and CBP pull downs from untreated MCF-7/CA/HER-2 cells but not in MCF-7/CA controls. Treatment with androstenedione clearly induced the pS2 product in both cell types, but the induced levels were higher and unresponsive to letrozole in the HER-2-overexpressing cells (Fig. 2). Consistent with higher transcriptional activity in the presence of androstenedione (Fig. 1B), the estradiol-induced pS2 PCR primer product was more abundant in HER-2-overexpressing cells than in control cells.

MCF-7/CA/HER-2 cells showed increased proliferation in medium containing charcoal-stripped serum supplemented with androstenedione compared with MCF-7/CA/vector cells. Cotreatment of androstenedione with the aromatase inhibitor letrozole did not abrogate androstenedione-mediated cell proliferation in MCF-7/CA/HER-2 cells. MCF-7/CA/HER-2 cells were also resistant to letrozole-induced apoptosis in estrogen-depleted medium. Aromatase activity of both MCF-7/CA/HER-2 and MCF-7/CA/vector cells was reduced to similar basal levels by treatment with letrozole, suggesting that drug resistance cannot be explained by an intrinsic insensitivity of the aromatase enzyme itself to letrozole.[5] These results suggest that ligand-independent recruitment of coactivator complexes to estrogen-responsive promoters as a result of HER-2 overexpression may play a role in developing letrozole resistance.

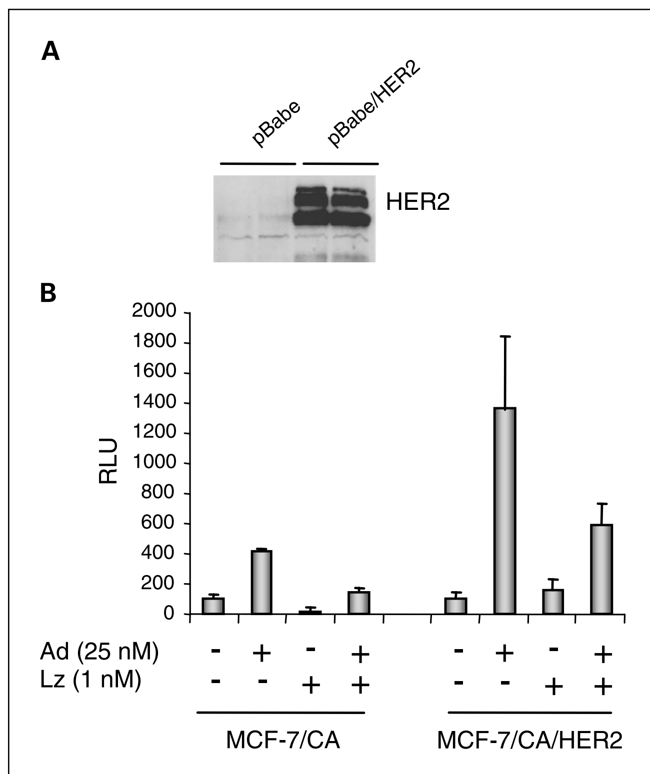

Fig. 1. HER-2 overexpression abrogates letrozole-mediated inhibition of androstenedione (*Ad*)–induced ER transcription. *A,* HER-2 immunoblot of MCF-7/CA cells stably transduced with pBabe-HER-2 or p-Babe vector. *B,* indicated cells were cotransfected in phenol red–free medium supplemented with 10% charcoal-stripped fetal calf serum with a pGLB-MERE plasmid and pCMV-R1 (*Renilla reniformis*) in triplicate wells and then stimulated for 24 hours with 25 nmol/L androstenedione (Sigma, St. Louis, MO) ± 1 nmol/L letrozole (provided by Dean Evans, Novartis, Basel, Switzerland). Firefly and *R. reniformis* luciferase activities were determined using the Dual Luciferase Assay System (Promega, Madison, WI), and the data were expressed as relative luciferase units (*RLU*) by using the firefly/*R. reniformis* luciferase activity ratio as described (35).

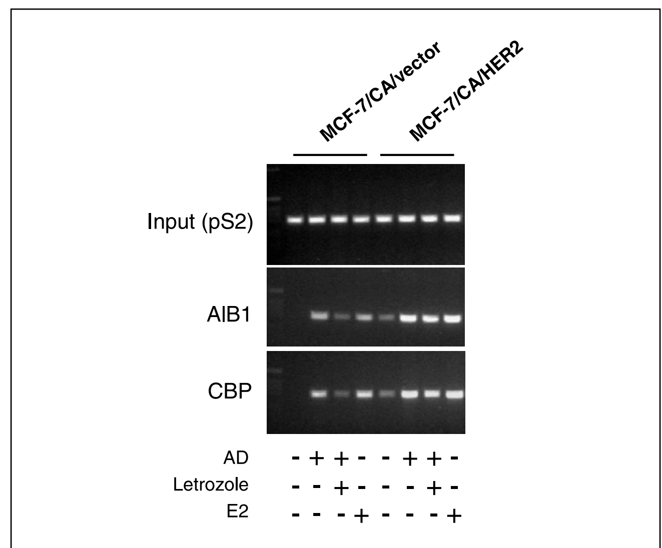

Fig. 2. ER is constitutively associated with coactivators of transcription in HER-2-overexpressing cells. Cells were incubated in phenol red–free medium supplemented with 10% charcoal-stripped FCS for 3 days and then serum starved for an additional 24 hours. Monolayers were then treated with androstenedione (*AD*; 25 nmol/L) ± letrozole (1 nmol/L) or estradiol (1 nmol/L) for 3 hours, washed with PBS, and cross-linked with 1% formaldehyde at 37°C for 10 minutes as described (36). Occupancy of the estradiol-responsive pS2 promoter by ERα or the transcriptional coactivators AIB-1 or CBP was assessed by chromatin immunoprecipitation as described by Shou et al. (17). Relevant pS2 promoter sequences were PCR amplified from chromatin-protein complexes precipitated with ERα, AIB-1, or CBP antibodies. *Input lane,* DNA that was PCR amplified from DNA-protein extracts before immunoprecipitation.

[5] All described in Shin et al., submitted for publication.

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Clinical Implications

These data suggest the use of antiestrogens in combination with EGFR/HER-2 signaling inhibitors in hormone-dependent breast cancers that also overexpress EGFR and/or HER-2. One potential difficulty of testing this approach is achieving rapid patient accrual to randomized studies, as only a minority of patients with ER^+ or PR^+ tumors also exhibit detectable levels of EGFR or HER-2 gene amplification (21, 31). Other data, however, would support the enrollment of ER^+/PR^+ tumors with low EGFR/HER-2 levels in these combination trials. For example, MCF-7 cells selected for resistance to the pure antiestrogen fulvestrant show increased EGFR and MAPK levels. This resistance is abrogated if the selection is done in the presence of the EGFR inhibitor gefitinib or MAPK inhibitors (27). Second, a recent report indicates that 3 of 26 (12%) tumors that relapse early while on adjuvant tamoxifen therapy exhibit high levels of HER-2 protein and/or gene amplification at the time of recurrence, suggesting that oncogene over-expression is acquired during antiestrogen escape (32). Furthermore, MCF-7 cells stably expressing aromatase that become resistant to letrozole overexpress P-MAPK, a major signal-transducing pathway regulated by erbB receptors. This resistance was reversed by gefitinib or MAPK kinase inhibitors (33). Finally, the *EGFR* gene contains a 96-bp intron fragment that is repressed by estradiol; therefore, estrogen depletion up-regulates EGFR transcription (34), providing a molecular explanation for some of the abovementioned studies. Taken together, these data provide a strong rationale for including patients with ER^+ breast cancer and low levels of EGFR and HER-2 into trials of antiestrogens and inhibitors of EGFR/HER-2 signaling.

Open Discussion

Dr. Steven Come: What does acquired resistance look like in terms of the extent of growth factor signaling in these models, compared to the clinic? Is it a matter of degree? If you have a patient whose tumor is 3+ HER-2/*neu* positive, that tumor is so driven by growth factors at that point that there's no turning back. The only setting where you possibly could have an impact is with the tumors that are clinically HER-2/*neu* negative but will up-regulate to some degree to escape. I'm surprised that gefitinib would be a choice to treat HER-2/*neu*–positive disease. I wouldn't think it would be very good in the clinic.

Dr. Carlos Arteaga: I would not choose gefitinib to block HER-2. To block the ErbB pathway in conjunction with an antiestrogen, I would choose the EGFR/HER-2 inhibitor lapatinib. Having said that, you saw the data from Charles Coombes in $ER^+/EGFR^+$ tumors [Lancet Oncol 2005;6:383–91]. He was using 250 mg/day of gefitinib, which is a dose that probably is not that great in blocking the wild-type EGF receptor, and he found a remarkable inhibition of phosphorylation of ER at serine 118.

Dr. Come: Right, but would it be a matter of degree? Where would you make your impact? You would assume that most patients who are resistant don't have clinically evident HER-2/*neu* overexpression. But it still might be up enough to escape from the antiestrogens.

Dr. Mitch Dowsett: I think Dr. Come's point is perhaps more relevant to EGFR than it is to HER-2, because most of the data on HER-2 indicate that some measurable degree of overexpression is needed. Coombes' data and your own work with erlotinib suggest that these agents are having an effect at lower levels of EGFR expression. The issue here is whether Coombes has a diagnostic that will tell us where the EGFR inhibitors are going to be effective. Or, in fact, is it that they're effective right across the board? We don't really know that from his data, because it's such an unusual diagnostic.

Dr. Kathleen Pritchard: It was surprising to see as much response as there was to gefitinib in that study, for a drug that's not supposed to work in breast cancer.

Dr. Stephen Johnston: He selected them. He screened about 350 cases to get 50 patients in the end of the study. He screened a lot of cases using two different antibodies.

Dr. Arteaga: Yes, 348 cases, and they found 114 that were positive for either or both EGFR antibodies.

Dr. Johnston: Both in your study and Coombes' study, where you see the biggest effects are in early breast cancer. The biomarker studies in advanced breast cancer that Jose Baselga did with gefitinib [J Clin Oncol 2005;23:5323–33] and the study with erlotinib are disappointing, in terms of the fact that they haven't seen effects on Ki67 in tumors. Again, these advanced breast cancer studies are unselected. I'm interested in what you said about phospho-MAPK, because if that was something that could select out responders, maybe that could be done in Coombes' study as well, because he did see a lot of changes in phospho-MAPK. So it could be a surrogate for upstream growth factor pathway activity.

Dr. Arteaga: What these studies suggest to me is that the EGFR pathway is a major input to MAPK *in vivo*. Jose Baselga's study, like ours, showed inhibition of phospho-MAPK. His study did not show changes in Ki67. It could be because his study was in a highly metastatic, heavily pretreated population, and/or because they used gefitinib and we used erlotinib. One possibility is that erlotinib is just a better blocker of EGFR than gefitinib at the maximum tolerated dose of 150 mg/day. It also blocks HER-2, at the steady-state concentrations of approximately 3 μM that can be achieved at the MTD. Of course, it is disappointing that these drugs don't have much single-agent activity, but the fact that one can see tumor reduction and inhibition of Ser^{118} ER phosphorylation in the Coombes study, suggests to me that if MAPK is a major mechanism of escape from antiestrogens, the combination of MAPK inhibitors or inhibitors of MAPK activation with hormonal therapies may be effective or, at a minimum, worth testing.

Dr. Myles Brown: So, in your MCF-7 model, you have EFGR but no HER-2. The model for the gefitinib experiment we want to do clinically would be tamoxifen-sensitive and aromatase-sensitive cells, expressing EGFR/HER-2, to look for synergy with EGFR inhibition. That's the proposal you're making, that at normal, inducible, non-amplified EGFR signaling, there's enough of a feedback loop that you want to block that pathway as well.

Dr. Arteaga: Correct, and I think we should do that. With these models, I would not expect to see much of a change in a short-term experiment. An experiment related to your question was done by Angela Brodie in mice [Cancer Res 2005;65:5380–9]. She selected MCF-7_{CA} tumors that had been stably transfected with the aromatase gene in the presence of letrozole. Once they escaped letrozole, the selected tumors overexpressed p-MAPK. One would have to do the same selection experiment

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in the presence of an EGFR/HER-2 inhibitor. But I am afraid the experiment would have to be done *in vivo*.

Dr. Dowsett: In terms of the clinical studies, we do have a study which is just completing its recruitment at the moment with 180 patients. It is anastrozole for 2 weeks, then randomization to gefitinib or not, with maintenance on anastrozole. About 90 patients will receive gefitinib, and these are actually unselected, other than for ER positivity. The primary endpoint is Ki67, and we will actually have the clinical responses fairly soon. There are some patients who don't show Ki67 suppression. What will gefitinib do to those? And there are other patients who show suppression but then something of a recovery later on. Will gefitinib prevent that recovery? Those are the thoughts that provoked the study design.

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