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Devious signals from NFκB driving breast cancer progression

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Citation of original article 1:
P. Bhatia, M. M. Sanders, M. F. Hansen. Expression of receptor activator of nuclear factor-kappaB is inversely correlated with metastatic phenotype in breast carcinoma. ***Clinical Cancer Research*** 2005; **11**(1): 162–5.

Abstract of the original article 1
During normal bone remodeling, the receptor activator of nuclear factor-kappaB (RANK) interacts with its ligand RANKL, which is present on pre-osteoclasts, resulting in bone resorption and initiation of new bone formation. When breast cancer metastasizes to bone, normal bone remodeling is disturbed by invasion of tumor cells, resulting in osteolytic lesions. We have studied the expression of both RANK and RANKL in 10 nonneoplastic breast samples, 58 infiltrating ductal carcinoma (IDC), and 43 breast cancer bony metastases (BTM). RANK seemed to be present in all samples tested. However, whereas RANKL expression was observed in 90% of nonneoplastic breast, RANKL expression was only observed in 62% of nonmetastatic IDC, 31% of metastatic IDC, and 2% of osteolytic BTM lesions. This decreased or absent expression of RANKL in the tumor cells may allow RANK, which is normally expressed as a receptor on the cell surface, to target RANKL present on the cell surface of normal osteoblasts and stromal cells of the bone. Stimulation of the normal osteoblasts and stromal cells by the tumor cells may then lead to secondary osteoclastogenesis, resulting in the osteolytic phenotype common to breast metastases.

Citation of original article 2:
R. B. Riggins, A. Zwart, R. Nehra, R. Clarke. The nuclear factor kappaB inhibitor parthenolide restores ICI 182,780 (Faslodex; fulvestrant)-induced apoptosis in antiestrogen-resistant breast cancer cells. ***Molecular Cancer Therapeutics*** 2005; **4**(1): 33–41.

Abstract of the original article 2
The molecular mechanisms underlying the acquisition of resistance to the antiestrogen Faslodex are poorly understood, although enhanced expression and activity of nuclear factor kappaB (NFkappaB) have been implicated as a critical element of this phenotype. The purpose of this study was to elucidate the mechanism by which NFkappaB up-regulation contributes to Faslodex resistance and to determine whether pharmacologic inhibition of NFkappaB by the small molecule parthenolide could restore Faslodex-mediated suppression of cell growth. Basal expression of multiple NFkappaB-related molecules in MCF7-derived LCC1 (antiestrogen-sensitive) and LCC9 (antiestrogen-resistant) breast cancer cells was determined, and cells were treated with Faslodex or parthenolide. The effect of these drugs either singly or in combination was assessed by cell proliferation, estrogen receptor (ER)-dependent transcriptional activation, cell cycle analysis, and apoptosis assays. Expression of the p65 NFkappaB subunit and the upstream NFkappaB regulator IkappaB kinase gamma/NFkappaB essential modulator were increased in the resistant MCF7/LCC9 cells ($P = 0.001$ and 0.04,

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respectively). Whereas MCF7/LCC9 cells were unresponsive to Faslodex alone, parthenolide effectively inhibited MCF7/LCC9 cell proliferation and the combination of Faslodex and parthenolide resulted in a 4-fold synergistic reduction in cell growth ($P = 0.03$). This corresponded to a restoration of Faslodex-induced apoptosis ($P = 0.001$), with no observable changes in ER-dependent transcription or cell cycle phase distribution.

Because parthenolide has shown safety in Phase I clinical trials, these findings have direct clinical relevance and provide support for the design of clinical studies combining antiestrogens and parthenolide in ER-positive breast cancer.

Review

The nuclear factor-κB (NFκB) complex encompasses a family of inducible transcription factors expressed in almost all cells and mediating a signaling pathway now recognized as lying 'at the crossroads of life and death' [1]. NFκB activation occurs in response to extracellular chemical stresses, cytokines, and growth factors; and directly regulates the expression of hundreds of genes whose cellular influences extend well beyond those of the immune system where its critical role was first recognized almost two decades ago [2]. The NFκB family consists of five mammalian members (p50/NFκB1, p52/NFκB2, p65/relA, c-rel, and relB), all share a conserved 300 amino acid N-terminal Rel homology domain (homologous to that encoded by the avian oncogene, v-Rel) responsible for their dimerization, nuclear translocation, DNA-binding, and association with IκB inhibitory proteins [3,4]. All exist as homo- or heterodimers (the most abundant form is generally thought to be the p50/p65 heterodimer), although in resting cells NFκB is cytoplasmically sequestered as a latent complex bound to one or more members of the IκB protein family (IκBα, IκBβ, IκBε, IκBγ, Bcl-3, and the precursor Rel proteins p100 and p105). Through different signaling intermediates, various NFκB stimuli (e.g. TNFα, CD40 ligand, IL-1, LPS, TRANCE/receptor activator of nuclear factor-κB ligand (RANKL), EGF, phorbol esters, peroxides, ionizing radiation) converge to activate IκB kinase (IKK), a heterotrimeric cytoplasmic complex with two catalytic kinase subunits (IKKα and IKKβ, either of which may be phosphorylated by upstream kinases) and a regulatory scaffolding component (IKKγ/NEMO). This activated IKK complex in turn phosphorylates IκB, leading to its proteasomal degradation and translocation of NFκB into the nucleus, where it binds promoter-specific κB consensus DNA elements to direct transcription of over 180 known NFκB target genes.

NFκB regulated genes are known to affect a wide variety of anti-apoptotic, proliferative, motility and invasion promoting cellular responses critical for normal organ development and homeostasis, including that of the mammary gland [5]. Thus, it is not surprising that within the past decade we have seen a rapid surge in the number of reports implicating NFκB dysregulation in various chronic inflammatory disorders and assorted hematopoietic and epithelial malignancies, and identifying this pathway as a promising target for new molecular therapies [6–10]. Among the experimental and medicinal strategies shown to inhibit constitutively active NFκB are drugs that target either upstream NFκB activating signals or downstream IκB degradative mechanisms [11], including the potent and specific antioxidant pyrrolidine dithiocarbamate [12], proteasome inhibitors like MG-132 and PS-341 (bortezomib/velcade) [7], and sesquiterpene lactones like parthenolide (PA), a specific IKK inhibitor and component of feverfew now being evaluated in cancer patients [13,14].

Two new breast cancer studies, Bhatia *et al.* [15] and Riggins *et al.* [16], implicate distinct but equally devious roles for NFκB dysregulation in the clinical progression of breast cancer. Both reports offer compelling findings that need further validation and mechanistic clarification. The immunohistochemical study of Bhatia *et al.* evaluated tumor expression of RANKL, which activates NFκB upon binding to the surface receptor, receptor activator of nuclear factor-kappaB (RANK). They hypothesized that differential expression of RANKL or RANK might identify cancer cells predisposed to metastasis, and in particular formation of bony metastases associated with osteolytic complications. RANK is also known as osteoclast differentiation and activation receptor for its essential role in bone remodeling, mediated by osteoclastic activation of NFκB, and bone resorption. RANKL, widely expressed on the surface of both osteoblasts and bone stroma, can bind to either cellular RANK or the soluble decoy receptor, osteoprotegerin (OPG), also known as osteoclastogenesis inhibitory factor because of its role in preserving bone density. For example, the bone mineral preserving activity of estrogen is mediated by stromal estrogen receptor (ER)-induction of OPG; and in an opposite manner, excess glucocorticoids can demineralize bone by inhibiting synthesis of OPG. Thus, local bone density and homeostasis are regulated by the competitive balance between OPG and RANK for binding to RANKL [17]. Comparing

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paraffin-embedded samples of nonneoplastic breast ($n = 10$, NNB), infiltrating ductal carcinomas ($n = 58$, IDC; 26 nonmetastatic and 32 metastatic), and breast cancer bony metastases ($n = 43$, breast cancer bony metastases (BTM)), Bhatia *et al.* observed no difference in the uniformly strong surface expression of RANK in all NNB, IDC, and BTM tissues analyzed. In contrast, they observed significantly decreased or absent surface expression of RANKL by BTM (only 2% positive) and IDC (31% positive for metastatic IDC, 62% positive for nonmetastatic IDC), relative to NNB (90% positive). Their findings that virtually all NNB samples express both RANK and RANKL are consistent with earlier observations that intact NFκB signaling is required for normal mammary organogenesis, that mice lacking RANK or RANKL fail to form lobuloalveolar structures during pregnancy, and that normal bone metabolism and mammary gland development are linked by NFκB responses to calcium demand [5]. Bhatia *et al.* speculate that decreased expression of RANKL by RANK-positive IDC allows for metastatic bone seeding by IDC and interaction with RANKL-positive stroma that induces osteolysis. While an attractive hypothesis, there is insufficient clinical information provided to confirm this proposal. Likewise, Bhatia *et al.* do not provide other relevant information, such as the ER status of their different study samples.

ER-positive IDC, while generally more indolent than ER-negative IDC, preferentially metastasizes to bone [18]; and this well recognized clinical fact raises an interesting issue not addressed by Bhatia *et al.* RANKL-negative IDC bony metastases would be expected to have activated NFκB (by interaction of their surface RANK with RANK-positive stroma), and this proliferation and survival signal would serve to augment the clinical invasiveness of these ER-positive metastatic cells. Until recently, however, ER-positive breast cancers were said to lack activated NFκB on the basis of limited breast cancer surveys and the known inhibitory effects of activated NFκB on all steroid receptors including ER [19]. A more extensive breast cancer evaluation has now reported important new observations about NFκB activation in ER-positive primary breast cancers [20]: (i) those destined for later metastatic relapse express significantly higher levels of NFκB as compared to primary tumors that do not relapse; (ii) those with higher NFκB levels appear clinically resistant to adjuvant endocrine therapy by the antiestrogen tamoxifen; and (iii) the antiestrogen resistance associated with higher NFκB levels may be reversed by anti-NFκB agents like bortezomib or parthenolide. This newly recognized and potentially important clinical role of NFκB activation in determining the antiestrogen responsiveness of ER-positive breast cancers is the focus of the preclinical study by Riggins *et al.* [16], who evaluated NFκB expression in an ER-positive human breast cancer cell line model (MCF7/LCC9) resistant to the pure antiestrogen, fulvestrant (ICI-182,780).

Several laboratories have transfected ER-positive, tamoxifen-sensitive MCF-7 cells to show that constitutively activated receptor tyrosine kinases like ErbB2 and EGFR, or downstream kinases like Raf-1, MEK1 or Akt, all reduce ER levels and tamoxifen sensitivity; and importantly, ER levels and tamoxifen sensitivity can be restored in these endocrine-resistant sublines using NFκB inhibiting doses of parthenolide [20–22]. In contrast to these studies, Riggins *et al.* employed two different MCF-7 sublines developed under long-term selection conditions: fulvestrant-resistant MCF7/LCC9 cells and fulvestrant-sensitive but estrogen-independent MCF7/LCC1 cells [16]. An earlier study by this same laboratory had shown that MCF7/LCC1 cells possessed increased NFκB activity relative to parental MCF-7 cells, and that the MCF7/LCC1 phenotype could be restored to an estrogen-dependent state by downregulation of NFκB [23]. Riggins *et al.* now report that fulvestrant-resistant MCF7/LCC9 cells exhibit significantly increased NFκB expression (and activating kinase, NEMO/IKKγ) relative to MCF7/LCC1 cells. Moreover, parthenolide can restore fulvestrant sensitivity to these MCF7/LCC9 cells and the combination parthenolide–fulvestrant produces synergistic inhibition of MCF7/LCC9 growth based on enhanced induction of apoptosis. While the restorative and antiestrogen potentiating effects of parthenolide are presumed due to parthenolide's IKK inhibitory activity as others have shown [20], Riggins *et al.* showed no change in intracellular IκBα levels and could not confirm under their experimental conditions that parthenolide actually inhibited NFκB activity. Thus, these investigators were forced to conclude that parthenolide might be interacting synergistically with fulvestrant in MCF7/LCC9 cells by 'other alternative mechanisms' [16].

Validating *in vivo* studies are clearly needed to support the general conclusions reported to date using these various NFκB-stimulated, ER-positive, and endocrine-resistant breast cancer cell line models [16,20–22]. Anticipating such *in vivo* validation, it is not too early to begin designing clinical trials in which antiestrogens like tamoxifen or fulvestrant are combined with anti-NFκB agents like parthenolide or bortezomib to treat patients with endocrine refractory ER-positive breast cancer. Future *in vivo* studies might also attempt to confirm another potential benefit predicted by the findings from Bhatia *et al.* [15], that anti-NFκB therapeutics can disrupt or prevent the devious interplay between bone and tumor cells that results in osteolytic metastases.

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