Targeting Ribonucleotide Reductase M2 and NF-κB Activation with Didox to Circumvent Tamoxifen Resistance in Breast Cancer

Khyati N. Shah, Elizabeth A. Wilson, Ritu Malla, Howard L. Elford, and Jesika S. Faridi

Abstract

Tamoxifen is widely used as an adjuvant therapy for patients with estrogen receptor (ERx)-positive tumors. However, the clinical benefit is often limited because of the emergence of drug resistance. In this study, overexpression of ribonucleotide reductase M2 (RRM2) in MCF-7 breast cancer cells resulted in a reduction in the effectiveness of tamoxifen, through downregulation of ERβ66 and upregulation of the 36-kDa variant of ER (ERα36). We identified that NF-κB, HIF1α, and MAPK/JNK are the major pathways that are affected by RRM2 overexpression and result in increased NF-κB activity and increased protein levels of EGFR, HER2, IKKα, Bcl-2, RelB, and p50. RRM2-overexpressing cells also exhibited higher migratory and invasive properties. Through time-lapse microscopy and protein profiling studies of tamoxifen-treated MCF-7 and T-47D cells, we have identified that RRM2, along with other key proteins, is altered during the emergence of acquired tamoxifen resistance. Inhibition of RRM2 using sRRM2 or the ribonucleotide reductase (RR) inhibitor didox not only eradicated and effectively prevented the emergence of tamoxifen-resistant populations but also led to the reversal of many of the proteins altered during the process of acquired tamoxifen resistance. Because didox also appears to be a potent inhibitor of NF-κB activation, combining didox with tamoxifen treatment cooperatively reverses ER-α alterations and inhibits NF-κB activity. Finally, inhibition of RRM2 by didox reversed tamoxifen-resistant in vivo tumor growth and decreased in vitro migratory and invasive properties, revealing a beneficial effect of combination therapy that includes RRM2 inhibition to delay or abrogate tamoxifen resistance. Mol Cancer Ther; 14(11): 2411–21. ©2015 AACR.

Introduction

ERx plays a fundamental role in the etiology and progression of human breast cancer (1). Many therapies have been designed to inhibit the tumor-promoting effects of ERx; however, tamoxifen has been the drug of choice for all stages of ER-positive breast cancer. As adjuvant therapy, tamoxifen reduces the risk of recurrence and improves overall survival (OS) in patients with early breast cancer (2). Tamoxifen arrests cells in G1 and decreases expression of several ER target genes (3). Apart from blocking classical ER action, tamoxifen also induces DNA damage and apoptosis in ER-positive cells (4). Despite these benefits, some tumors recur due to acquired tamoxifen resistance giving rise to a subpopulation of unresponsive cells (5). Several mechanisms of acquired tamoxifen resistance have been reported, including downregulation of ERx expression (6). Tamoxifen resistance has also been linked to cross-talk between ERx and signaling pathways involving EGF receptor (EGFR), HER2/ERBB2, or insulin-like growth factor receptor I (IGFRI; ref. 7).

ERα36 is the classical ER of 66 kDa and is often referred to as simply ‘ER.’ Cells that have high levels of ERα66 are often termed ER-positive, whereas those lacking ERα66 are called ER-negative. Clinical evidence suggests that approximately 40% of ERα66-positive breast cancers also express a 36-kDa variant of ERx (ERα36), and this subset of patients is less likely to benefit from tamoxifen treatment (8, 9). Alternatively, endocrine-resistant cells can develop a compensatory signaling pathway downstream of ER that results in hyperproliferation and increased cancer cell survival. In this type of resistance, ERα36 may promote downstream signaling, such as the PI3K pathway (10). We have previously shown that breast cancer cells overexpressing activated AKT exhibit tamoxifen-stimulated cell proliferation and enhanced cell motility. Moreover, we identified that RRM2 was a key contributor to AKT-induced tamoxifen resistance (11).

Tamoxifen chemotherapy initially arrests tumor growth, but upon acquiring resistance, DNA synthesis is reactivated. The first step in DNA synthesis is conversion of ribonucleotides to their corresponding deoxyribonucleotides, catalyzed by the enzyme ribonucleotide reductase (RR; ref. 12). This reaction is also the rate-limiting step in DNA synthesis and cell division, and its activity is closely correlated with tumor growth rate and cell division (12). Ribonucleotide reductase is composed of RRM1 and RRM2. Although the levels of the RRM1 protein do not change substantially during the cell cycle, there is an S-phase correlated increase in the RRM2 protein (13). The activity of

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ribonucleotide reductase and therefore DNA synthesis and cell proliferation are controlled by RRM2. In contrast, p53R2 (RRM2B) is involved in supplying dNTPs for DNA repair during mitochondrial DNA synthesis in the G1–G2 phase of the cell cycle (14). Overexpression of the RRM2 subunit has been associated with malignant transformation and confers gemcitabine resistance (15). RRM2 functions in coordination with the S-phase checkpoint to regulate DNA damage, replication stress, and genomic instability, including mutagenesis (16, 17). Because chronic incubation of tamoxifen induces DNA damage, cells may upregulate RRM2 in an attempt to repair the DNA damage and are thus able to survive as a resistant population.

Because of the DNA-damaging property of tamoxifen, combination therapies that enhance tamoxifen activity are of clinical benefit. RRM2 is an attractive target for combination therapy, as it is overexpressed in breast cancer and contributes to development of resistance (11). Didox (3,4-dihydroxybenzohydroxamic acid) is a strong inhibitor of ribonucleotide reductase that interferes with DNA synthesis and repair by blocking the production of deoxyribonucleotides and has demonstrated antitumor effects for decades (12, 18–20). Phase I/II clinical trial studies in patients with cancer showed minimal toxicity and determined that the maximum tolerated dose of didox is 6 g/m², yielding peak plasma levels of 425 µmol/L (21, 22). Didox can be tolerated by patients with cancer at high dosages without major side effects, making didox attractive for use in clinical applications. We previously reported that the combination of didox and tamoxifen significantly reduced cell proliferation in AKT-induced tamoxifen resistance (11).

Here, using gain and loss of RRM2, we show that RRM2 is sufficient to confer tamoxifen resistance in breast cancer cells. We demonstrate that RRM2 is associated with increased NF-κB activity, ERα alterations, HER2 and EGFR upregulation, and increases in antiapoptotic pathways. Finally, didox significantly inhibits tamoxifen-induced in vivo tumor growth. Our results show that didox works synergistically with tamoxifen for the treatment and prevention of resistant breast cancer cells. Our data provide a preclinical rationale for evaluating tamoxifen in combination with didox for breast cancer treatment.

Materials and Methods

Cell culture and treatment

MCF-7, T47D, HCC1428, BT483, ZR-75-30, ZR-75-1, SKBR3, BT20, BT549, HCC2157, MDA-MB-468, and MDA-MB-231 cells were purchased from ATCC between July and December 2012. BT20, BT549, HER2, Bcl-2, pAKT, pER (S167) PERK, total ERK, total βH2AX, IKKα/β siRNA, 48, 17B-estradiol (E2, Sigma), 1 µmol/L 4-hydroxytamoxifen (T, Sigma), 30 µmol/L D, or a combination of 1 µmol/L T + 30 µmol/L didox (T + D) for 24 hours.

Western blot analyses

Western blotting was performed as previously described (11, 23). Briefly, cells were disrupted in RIPA buffer (Sigma) or tumors were homogenized in lysis buffer (Cell Signaling) supplemented with aprotonin, leupeptin, and okadaic acid (Sigma).

Lysates were clarified by centrifugation, and equal protein (75 µg) was used for Western blotting. RRM1, RRM2, RRM2B (Sigma), p53, ERα66 (Santa Cruz), ERα36 (Alpha Diagnostics), GAPDH, EGFR, HER2, Bcl-2, pAKT, pER (S167) PERK, total ERK, total βH2AX, IKKα/β reporter kit, NF-κB reporter kit, pPAR, caspase-9 (Cell Signaling), and pγH2AX (Millipore) were used for immunoblotting with secondary antibodies conjugated with IRDye 800CW or 680RD (LI-COR Biosciences) and visualized with a LI-COR Odyssey Imager.

Meta-analysis of breast cancer datasets

RRM2 expression profiles and clinicopathologic data of patients with breast cancer were obtained from publicly available breast cancer microarray (25–29) and NCBIGEO (30–34) datasets. Data from tamoxifen-treated ER-positive patients were classified as tamoxifen-resistant if metastasis was indicated. Oncotarget was used for data collection and analyses. P < 0.05 was considered significant as determined by the two-tailed t test. A Kaplan–Meier plotter (http://kmplot.com/breast/) was used to determine HRs (at 95% confidence intervals) and P values to assess the relevance of RRM2 expression to relapse-free survival (RFS), OS, and distant metastasis-free survival (DMFS) in tamoxifen-treated ER-positive patients (35).

Generation of stable RRM2-overexpressing MCF-7 breast cancer cell lines

MCF-7 cells were transfected with pCMV6-RRM2-myc-DDK or vector (Origene) using Fugene HD (Roche) and grown under genetin selection after 48 hours. Clones overexpressing RRM2 were expanded to generate stable expressing clones MCF-7/R2.1, MCF-7/R2.3, and population MCF-7/R2.pool. The population of MCF-7/VC cells stably express vector. Stable cells were routinely tested and authenticated according to the ATCC guidelines.

Transcription factor reporter assays

MCF-7/R2.1 and MCF-7 VC cells were reverse transfected onto the Cignal Finder 10-Pathway Reporter Array or the Cignal NF-κB Luciferase Reporter using SureFECT according to the manufacturer’s protocol (Qiagen). All RNA samples were treated and authenticated according to the ATCC guidelines.

siRNA-mediated suppression of RRM2

siRNA oligos targeting RRM2 (siRRM2) were designed and synthesized at Genentech Inc. Nontargeting siRNA and Dharmafect-1 were purchased (Dharmacon). Cells were transfected with siRNA by reverse transfection according to the manufacturer’s directions. Transfection efficiencies were evaluated relative to nontargeting control by RT-qPCR, and the suppression of RRM2 expression was sustained through day 7 for all breast cancer cell lines tested.

Pathway-specific expression arrays

Total RNA was isolated using RNeasy according to the manufacturer’s protocol (Qiagen). All RNA samples were examined for their concentration, purity, and integrity. The human Breast Cancer PCR array and the ECM and Adhesion Molecule (SABioscience) was used to assess the expression of...
84 breast cancer genes according to the manufacturer’s instructions. Data shown represent the average of two replicates and were normalized using the previously validated housekeeping gene RPL13A levels (23).

**Establishment and treatment of acquired tamoxifen-resistant cells**

MCF-7 and T-47D were treated with 1 μmol/L tamoxifen and dose was increased every 10 days until 5 μmol/L. Once established, TamR-resistant cells were maintained in continuous culture with 1 μmol/L tamoxifen. For protein profiling, replicate plates were treated with tamoxifen in this manner and lysates were collected over 30 days. Control lysates were collected after 3 days of treatment with vehicle. TamR lysates were collected with continuous treatment with 1 μmol/L tamoxifen alone or combined with didox for 5 days.

**Cell proliferation and motility assays**

For proliferation, 1,000 cells per well were plated in phenol red-free, DMEM/F12 medium with 2% CSS. After 24 hours, treatment media were replenished on alternate days. On assay days, CellTiter-96 Aqueous One Solution (Promega) was added, incubated for 1 hour, and measured at 490 nm. For the colony formation assay, 3,000 cells per well were cultured in 5% FBS phenol red-free DMEM. The following day, the cells were treated and allowed to grow for 14 days. Colonies were stained with crystal violet and analyzed. A modified scratch assay was performed by plating 20,000 cells in wells containing inserts (BD/DI Marinetsried). After 24 hours, inserts were removed, and the percent open area was calculated after 20 additional hours. Cell migration and invasion experiments were carried out using the QCM 24-well Colorimetric Cell Migration Kit or the Invasion Assay Kit (Chemicon) according to manufacturer’s protocols. These experiments were conducted in triplicate and data is shown as the mean ± SEM.

**Synergy determinations**

The IC_{50} value of tamoxifen and didox was determined for MCF-7 VC, MCF-7/TamR, and RRM2-overexpressing MCF-7 cells (MCF-7/R2.1). On the basis of the IC_{50} value, fixed dose ratios were used to determine five different drug combinations (i.e., 2X, 1X, X, X/2, X/4, where X is IC_{50} of an individual drug). Synergistic, additive, or antagonistic effects were determined using the combination index (CI) method developed by Chou and Talalay. Synergy, additivity, and antagonism are indicated by CI values of <1, 1, and >1, respectively (24).

**Xenograft studies**

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of the Pacific (Stockton, CA). RRM2-overexpressing MCF-7 cells (4 × 10^7 per site) were subcutaneously injected into the flank of ncr nude female ovariectomized nude mice (6–15 tumors per group). Mice were implanted with estrogen [0.72 mg/pellet, Innovative Research of America (IRA)] and tamoxifen as indicated (5 mg/pellet, IRA). Beginning day 7 as indicated, daily didox (200 or 425 mg/kg/d) was injected intraperitoneally after compound was freshly dissolved in water and filtered. The in vivo tumor volume was approximated using the formula for an ellipsoid [(4/3)π(r_1^2r_2)], where r_1 < r_2. Tumor volumes and body weight were measured weekly. No significant differences in body weight between treatment groups was observed. At study termination, tumors were harvested and either snap-frozen in liquid nitrogen and stored at –80°C (for Western analysis) or fixed with 10% neutral-buffered formalin for immunohistochemical analyses. Tumors were processed for immunoblot or immunohistochemical (by UC Davis VMTH) analyses as previously described (11). Representative data are shown from three independent xenograft experiments.

**Tissue microarrays analyses**

Breast cancer tissue microarrays (TMA; Biochain) with 70 cancer and 5 normal breast tissue duplicated cases were immunohistochemically stained with anti-RRM2 antibody by University of Pittsburgh Medical Center Histology Lab or anti-ER-α36 (Alpha Diagnostics) by UC Davis VMTH. The specificity of each antibody was confirmed using an isotype nonimmune IgG control. Slides were scored using standard microscopy and evaluated independently by two investigators. Average staining intensity and percentage of positive cells were multiplied to generate H-scores of 0 to 300.

**Results**

**RRM2 expression inversely correlates with ER expression in breast cancer**

RRM2 expression is upregulated in cancer and is associated with increased tumor aggressiveness, poor prognosis, and chemoresistance (11, 14–16). Here, we demonstrate that RRM2 expression is significantly upregulated in ER-negative as compared with ER-positive breast cancer cells, as is a 36-kDa variant of ER (HER36; Fig. 1A). Interestingly, of the ER-positive cells, ZR-75-1 alone has high levels of RRM2 and ERα36. We have previously shown that ZR-75-1 cells exhibit tamoxifen-resistant cell proliferation and that inhibition of RRM2 using siRRM2 not only restores tamoxifen sensitivity but also represses the DNA repair enzymes that protect these cells from tamoxifen-induced apoptosis (11).

Meta-analysis of five different Oncomine datasets revealed that RRM2 is highly expressed in ER-negative tumors (Fig. 1B, Supplementary Table S1A; refs. 25–29). Using survival data from tamoxifen-treated ER-positive patients, RRM2 mRNA expression was determined to be significantly higher in patients who experienced tumor relapse (Fig. 1C, Supplementary Table S1B; refs. 30–34). Kaplan–Meier survival plots (KMplots) of tamoxifen-treated ER-positive patients indicate that increased RRM2 expression is strongly correlated with reduced RFS and OS (Fig. 1D and E; ref. 35).

**Stable overexpression of RRM2 reduces tamoxifen sensitivity in MCF-7 cells**

We previously reported that inhibition of RRM2 reverses tamoxifen resistance in breast cancer cells (11). To strengthen the association between RRM2 and tamoxifen resistance, we transiently overexpressed RRM2 in ER-positive, tamoxifen-sensitive MCF-7 and T-47D cells and confirmed RRM2 expression by Western blot analysis (Supplementary Fig. S1). Relative to vector control (VC) cells, RRM2-overexpressing MCF-7 and T-47D cells exhibit reduced tamoxifen sensitivity and higher IC_{50} values resulting in resistance ratios of 3.2 to 3.3. Stable RRM2-overexpressing (clones MCF-7/R2.1, MCF/R2.2, and pool population MCF-7/R2.Pool) and vector control (MCF-7 VC) MCF-7 cells were
RRM2 overexpression increases NF-κB activity and gene transcription in breast cancer cells

RRM2 overexpression specifically upregulates the NF-κB, HIF1α, and MAPK/JNK proliferation and differentiation pathways (Fig. 2A). Interestingly, didox significantly inhibited NF-κB (P < 0.0007), HIF1α (P < 0.01), and MAPK/c-Jun (P < 0.03) activity in MCF-7/R2.1 cells. We also observed that RRM2 overexpression induced gene expression of EGFR, ERBB2/HER2, IL6, Bcl-2, PARP, VEGFA, MMP9, and BIRC5 and repressed the expression of TP53, BAD, ESR1 (ERα), CDKN1A, and CDKN2A (Fig. 2B). Using protein analysis, we confirmed the downregulation of ERα66 and upregulation of ERα36 with RRM2 overexpression. Decreases in p33 and increases in EGFR, HER2, and Bcl-2 protein levels were also confirmed. Furthermore, increased expression of the NF-κB–related IKK-α, IKK-γ, IKK-δ, IKK-ε, p50, RelB, C-Rel, and other NF-κB–related proteins was found. The expression of these proteins was reversed by siRRM2 or didox treatment (Fig. 2C).

Overexpression of RRM2 inhibited the tamoxifen induction of apoptotic proteins and the S139 phosphorylation of the DNA damage sensor H2AX (ref. 36; Fig. 2D). Yet, didox treatment significantly restored the ability of tamoxifen to induce S139 H2AX and induce cell death (increased PARP and caspase-9 cleavage) in MCF-7/R2.1 cells.

Tamoxifen-resistant MCF-7 cells express increased RRM2, altered ER expression, and elevated NF-κB signaling

We previously identified that RRM2 is overexpressed in acquired tamoxifen-resistant breast cancer cells and that didox treatment resensitizes these cells to tamoxifen-induced cell death (11). Here, we have generated two models to capture the events that occur during the emergence of acquired tamoxifen resistance. MCF-7 and T-47D cells were cultured in the presence of tamoxifen or vehicle control. Although tamoxifen induced cell death (Fig. 3A), eventually cells adapted and MCF-7TamR and T-47DTamR cells that were no longer sensitive to tamoxifen treatment were generated (Fig. 3B). Using lysates collected during this resistance process, we performed protein profiling experiments and confirmed the upregulation of RRM2 and ERα36 and downregulation of ERα66 (Fig. 3C). ERα66 S167 phosphorylation, a marker of tamoxifen resistance, was also increased (5). We further examined the effect on growth factor receptor signaling and observed increased EGFR, HER2, pERK, pAKT but decreased p53 levels. During the emergence of tamoxifen resistance, we also detected increased IKKα, IKKγ, IKKε, and p-IKK-α/β (S176/180). There was an induction in the NF-κB proteins, p50, RelB, and increased phosphorylation of p65 at S536 indicating.

Figure 1.
RRM2 inversely correlates with ERα66 expression. A, Western blot analysis of RRM2 and ER levels in ER-positive and ER-negative cells. B, meta-analysis demonstrates higher RRM2 expression in ER-negative (dark box) than in ER-positive patients with cancer (white box). C, tamoxifen-resistant patients (dark box) have higher RRM2 than tamoxifen-sensitive patients (white box). Fold change and P values are given in Supplementary Table S1. K-Mplots demonstrate overexpression of RRM2 is predictive of (D) lower RFS (HR = 4.9e–5) and (E) lower OS (HR = 0.00025) in tamoxifen-treated ER-positive patients.

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increased NF-κB activity. When examining apoptotic proteins, we observed an initial spike (days 1–5) and then decrease (day 10) in cleaved PARP and caspase-9 leading to the increase in the anti-apoptotic factor Bcl-2 (Fig. 3D). Finally, the emergence of tamoxifen-resistant populations occurred around day 15, which coincides with the activation of pERK1/2 and pAKT (S473) kinases.

Combining tamoxifen and didox circumvents resistance via cooperatively reducing ER alterations and NF-κB signaling

To evaluate the efficacy of tamoxifen and didox (T + D) combination therapy on the emergence of tamoxifen resistance, time-lapsed images were captured and significant cell rounding (indicative of cell death) was observed as early as day 1. Treatment with T + D prevented the emergence of tamoxifen resistance in parental cells and successfully eradicated TamR lines (Fig. 4A and B). Didox reduced RRM2, ERα66, and S167 phosphorylation of ERα and simultaneously increased ERα66 levels (Fig. 4C and D). Moreover, combination therapy induced apoptosis as indicated by increased S139 phosphorylation of γ-H2AX, increased cleavage of PARP and caspase-9, reduced levels of the antiapoptotic Bcl-2, reactivating RRM2-overexpressing and TamR cells to tamoxifen-induced cell death (Supplementary Fig. S3). Furthermore, didox reduced the expression of EGFR, HER2, pERK, and pAKT in T-47DTamR (Fig. 4D) and all but pAKT in MCF-7/TamR cells (Fig. 4B).

Because there was an observed increase in NF-κB signaling in TamR cells, we sought to examine the effect of T + D in this pathway. Didox reduces expression of the NF-κB–related IkKs, p50, RelB, pIKK, p-1κBα, and the S336 phosphorylation of p65 (Fig. 4B and D). Using an NF-κB reporter assay, we found that didox alone, and more significantly, T + D downregulates NF-κB activity in MCF-7/TamR, MCF-7TamR, and T-47DTamR cells (Fig. 4E).

Didox cooperates with tamoxifen to reduce cell proliferation and tumor growth

To evaluate the combined effects of T + D, cellular viability was measured using the CI (24). Compared with single-agent treatments, the combination of T + D significantly reduced cell viability in MCF-7/TamR1 and MCF-7TamR cells (Supplementary Fig. S4). CI values of 0.75 and 0.47 indicate synergistic actions of 1.25 μmol/L tamoxifen and 37.5 μmol/L didox in MCF-7/TamR1 and MCF-7TamR (but not parental MCF-7) cells, respectively, on reducing cell proliferation. Cell viability and toxicity studies indicate that with tamoxifen treatment, MCF-7/TamR1 and MCF-7TamR cells exhibit higher viability, lower toxicity, and higher IC50 than parental MCF-7 cells (Supplementary Fig. S5).

To determine the in vivo efficacy tamoxifen and didox (T + D) combination therapy in RRM2-overexpressing breast tumors, MCF-7/TamR1 cells were subcutaneously injected in mice treated with tamoxifen as indicated. Daily didox (200 or 425 mg/kg/d) treatment was begun as indicated 7 days after cell injection. Tumor mice treated with tamoxifen exhibited significantly greater tumor growth compared with mice treated with the combination of T + D200 (P < 0.04) and T + D425 (P < 0.003; Fig. 5A). It appears that targeting RRM2 with didox effectively circumvents
tamoxifen-resistant breast tumor growth. Analysis of xenograft tumors reveal lower RRM2 levels, higher ERα66 levels, and lower ERα36 levels in the T + D group (Fig. 5B–D). Unlike parental MCF-7 xenografts that require estrogen, RRM2-overexpressing xenografts treated with estrogen alone (Control) did not grow (Fig. 5A). These findings support our hypothesis that upon RRM2 overexpression, there are ER alterations that allow for tamoxifen resistance, including the loss of estrogen agonist activity (via loss of ERα66) and the gain of tamoxifen agonist activity (via gain of ERα36).

Overexpression of RRM2 is associated with higher migratory and invasive phenotype

Using a modified scratch assay and cell migration and invasion colorimetric kits, we demonstrated that MCF-7/R2 cells are highly motile, migratory, and invasive; yet, treatment with didox alone is sufficient to reverse these phenotypes (Fig. 6A–C, Supplementary Fig. S6). Using an ECM and Adhesion Molecules PCR array, we observed that cell adhesion genes such as VCAM1, PECAM, FN1, NCAM1, and ECM molecules such as ITGB3, COL15A1, MMP2, MMP3, and MMP9 were altered upon RRM2 overexpression and reversed with didox treatment (Fig. 6D). Meta-analysis of patient data shows that RRM2 is highly expressed in grade 3 tumors (Fig. S6). Using an ECM and Adhesion Molecules PCR array, we observed that cell adhesion genes such as VCAM1, PECAM, FN1, NCAM1, and ECM molecules such as ITGB3, COL15A1, MMP2, MMP3, and MMP9 were altered upon RRM2 overexpression and reversed with didox treatment (Fig. 6D). Meta-analysis of patient data shows that RRM2 is highly expressed in grade 3 tumors (Fig. S6). Using an ECM and Adhesion Molecules PCR array, we observed that cell adhesion genes such as VCAM1, PECAM, FN1, NCAM1, and ECM molecules such as ITGB3, COL15A1, MMP2, MMP3, and MMP9 were altered upon RRM2 overexpression and reversed with didox treatment (Fig. 6D). Meta-analysis of patient data shows that RRM2 is highly expressed in grade 3 tumors (Fig. S6).

Discussion

Tamoxifen effectively blocks estrogen-stimulated tumor growth by inhibiting the activity of ERα66 in breast cancer cells. Overexpression of tyrosine kinase signaling pathways and subsequent downregulation of ERα66 after long-term tamoxifen treatment is responsible for the development of acquired tamoxifen resistance in ERα66-positive primary tumors (3, 5–8). As well, tamoxifen resistance can contribute to greater breast tumor aggressiveness (37). Although the mechanisms underlying acquired tamoxifen resistance are largely unknown, we have demonstrated that RRM2 is upregulated in AKT-induced tamoxifen-resistant breast cancer cells and that inhibition of RRM2 by siRNA significantly overturns this resistance (11). This current study shows that RRM2 overexpression alone is sufficient to promote tamoxifen resistance, is expressed during the emergence of de novo tamoxifen resistance (Fig. 3C), and downregulates ESR1 (ERα) gene and protein expression (Figs. 2B and 3C). Using in vitro breast cancer cells, patient datasets, TMAs, and tumor xenografts, we report for the first
time that there is an inverse correlation between RRM2 and ERα66 and a direct correlation with ERα36. By inhibiting RRM2 using didox, we show that the emergence of acquired tamoxifen resistance is circumvented, whereas the downregulation of ERα66 and upregulation of ERα36 are reversed.

Our data suggest that ERα66 downregulation in RRM2-overexpressing breast cancers may occur through increases in NF-κB activation and signaling. Transrepression of ER by NF-κB has been proposed as a mechanism by which ER-positive breast cancer cells lose ER expression and, hence, gives rise to a subpopulation of tumor cells that are resistant to endocrine therapy (38, 39). Furthermore, we provide evidence that increased NF-κB signaling leading to ERα alterations are mediated via RRM2 overexpression and can be reversed using didox through its ability to inhibit NF-κB activation (Fig. 4E). We have shown that the inhibition of RRM2 by didox was able to eradicate MCF-7/TamR and T-47D/TamR populations by day 15, supporting the use of didox to resensitize breast cancer cells to tamoxifen therapy. More importantly, didox prevented the emergence of tamoxifen resistance in two models, suggesting the therapeutic use of didox to also prevent the emergence of tamoxifen resistance (Fig. 4A and B).

Here, by overexpressing RRM2, we were able to reproduce the tamoxifen-resistant phenotype and have identified that NF-κB, HIF1α, and MAPK/JNK are the major pathways that are affected (Fig. 2A). These pathways have been shown to play a significant role in mammary carcinogenesis and have been implicated in tumor resistance. In particular, the NF-κB pathway has been shown to regulate cell proliferation, differentiation, and invasion (39). Similar to our study, the NF-κB pathway was reported to be increased by RRM2 and inhibited by didox, strengthening the findings that RRM2 is a key regulator of the NF-κB pathway (40, 41). Although didox exerts its activity by destabilizing ribonucleotide reductase through its free radical scavenging and iron chelating properties, didox has also been shown to have strongly inhibit NF-κB activation (42).

Because of the ability of tamoxifen to induce cell death by causing DNA damage, we sought to determine whether RRM2 plays a role in protecting breast cancer cells from tamoxifen-induced cell death (4). It is likely that cells having high RRM2...
tamoxifen may improve RFS and OS. RFS and OS in tamoxifen-treated patients (Fig. 1D and E), that didox can resensitize breast cancer cells to tamoxifen-induced tamoxifen and didox treatment (Fig. 2D). This is the established mechanisms of chemoresistance (44). These RRM2-and caspase-9 cleavage, and increased Bcl-2 levels, which are well-known contributors on the pathway to tamoxifen resistance (48). Our data demonstrate strong evidence that RRM2 is important in regulating ER expression or patients who have relapsed after long-term tamoxifen treatment. Similarly, RRM2 was recently suggested as a prognostic marker associated with poor survival and tamoxifen resistance, which supports our findings that RRM2 is an important contributor on the pathway to tamoxifen resistance (48).

In summary, our findings strongly complement the current knowledge regarding the molecular mechanisms underlying acquired tamoxifen resistance in breast cancer and also provide strong evidence that RRM2 is important in regulating ERα expression, hence responsiveness to tamoxifen. Our data demonstrate for the first time that overexpression of RRM2 in MCF-7 breast cancer cells leads to upregulation of EGFR and NF-kB signaling, downregulation of ERα66 expression, and upregulation of ERα36, contributing to the generation of acquired tamoxifen resistance. Overexpression of RRM2 also enhances the proliferative capacity and the migratory and invasive abilities of MCF-7 breast cancer cells. Furthermore, cotreatment of tamoxifen and didox resulted in reduced proliferation rates with decreased in vitro migratory and invasive properties. Most importantly, the combination treatment produced significant tumor inhibition, suggesting a critical role of RRM2 in maintaining a malignant phenotype in breast cancer. These findings indicate that RRM2 may be potentially used as a prognostic factor in patients with breast cancer undergoing tamoxifen therapy and can be considered a potential therapeutic target in tumors that have acquired resistance to tamoxifen. Further study of the molecular mechanisms by which RRM2 is activated during the development of acquired tamoxifen resistance in breast cancer will provide more detailed insights regarding the biologic function of RRM2. Finally,

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Figure 5.
RRM2 inhibition reverses tamoxifen-induced tumor growth and in vivo ER alterations. A, mice bearing MCF-7/R2.1 xenografts were treated with tamoxifen (T), tamoxifen and didox 200 mg/kg/d (T + D200), tamoxifen and didox 425 mg/kg/d (T + D425), vehicle (C), or didox 425 mg/kg/d (D); n = 6–15. Didox treatment was initiated at day 7 after cell injection when RRM2-overexpressing MCF-7 xenografts reach maximum tumor volumes (30 mm³) in the absence of tamoxifen. Mean tumor volume ± SEM is shown. **P < 0.003 (vs. T); *P < 0.04 (vs. T); and *P < 0.05 (vs. C). RRM2 and ERα levels were analyzed by (B) immunohistochemistry (original magnification, ×200; bar, 10 μm) and (C) Western blotting. D, protein expression from independent tumor samples is shown as the mean fold control ± SD (n = 3). **P < 0.003; *P < 0.05; *, P < 0.07, vs. C). Representative data are shown from 3 independent experiments.

(images and tables are not included in the text, but are expected to be present in the full document).
these data provide a rationale for the combination of tamoxifen and didox therapy to circumvent or prevent tamoxifen resistance in breast cancer.

Disclosure of Potential Conflicts of Interest

H.L. Elford has ownership interest (including patents) in Molecules for Health Inc. No potential conflicts of interest were disclosed by the other authors.

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Figure 6.
Overexpression of RRM2 is associated with higher migratory and invasive properties. Didox inhibits (A) cell motility, (B) cell migration, and (C) cell invasion in MCF-7/R2.1 cells. D, fold gene change is shown as significantly repressed (negative) or induced (positive) with or without didox in MCF-7/R2.1 cells. E, overexpression of RRM2 is correlated with higher tumor grade. F, KMplots demonstrate that higher RRM2 in tamoxifen-treated ER-positive patients is correlated with decreased DMFS ($p = 0.00021$). G, RRM2 and ERα36 levels increase with tumor grade on a TMA. HRM2 (H) and ERα36 (I) H-scores increase with tumor grade (ANOVA; $p < 0.0003$ and $p < 0.0023$, respectively).


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