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Targeting the mevalonate pathway to overcome acquired anti-HER2 treatment resistance in breast cancer

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Abstract

Despite effective strategies, resistance (R) in HER2+ breast cancer (BC) remains a challenge. While the Mevalonate pathway (MVA) is suggested to promote cell growth and survival, including in HER2+ models, its potential role in resistance to HER2-targeted therapy is unknown. Parental HER2+ BC cells and their Lapatinib (L) R and L + Trastuzumab (LT) R derivatives were used for this study. MVA activity was found to be increased in LR and LTR cells. Specific blockade of this pathway with lipophilic but not hydrophilic statins and with the N-bisphosphonate zoledronic acid led to apoptosis and substantial growth inhibition of R cells. Inhibition was rescued by mevalonate or the intermediate metabolites farnesyl pyrophosphate (FPP) or geranylgeranyl pyrophosphate (GGPP), but not cholesterol. Activated YAP/TAZ and mTORC1 signaling, and their downstream target gene product Survivin, were inhibited by MVA blockade, especially in the LR/LTR models. Overexpression of constitutively active YAP rescued Survivin and phosphorylated-S6 levels, despite blockade of the MVA. These results suggest that the MVA provides alternative signaling leading to cell survival and resistance by activating YAP/TAZ-mTORC1-Survivin signaling when HER2 is blocked, suggesting novel therapeutic targets. MVA inhibitors including lipophilic statins and N-bisphosphonates may circumvent resistance to anti-HER2 therapy warranting further clinical investigation.

Introduction

The human epidermal growth factor receptor 2 (HER2) is amplified and/or overexpressed in about 15% of breast cancers (BC) termed as HER2-positive (HER2+), where it is a dominant driver of tumor growth. Effective anti-HER2 treatment with the HER2 monoclonal antibody trastuzumab (T) combined with chemotherapy has dramatically improved patient outcome (1). Several studies have shown that anti-HER2 drug combinations, including the lapatinib (L)+T (LT) regimen, are even more effective by more completely blocking the HER receptor layer (2), and are associated with high rates of pathological complete response in neoadjuvant clinical trials (3, 4). However, despite the potency of these drug combinations in blocking the HER receptor family, resistance still remains a clinical challenge. Using a panel of HER2+ BC cell line derivatives made resistant to the L and LT regimens, we found that resistance to HER2-targeted therapy may arise from i) re-activation of the HER2 receptor by various mechanisms including mutations in the HER2 receptor itself; or, ii) activation of escape/bypass pathways such as β -integrin (5, 6) or ER (7) that circumvent anti-HER2 therapy.

The mevalonate pathway is a biosynthetic process regulated by the master transcription factor Sterol Response Element Binding Protein (SREBP), primarily by SREBP-1a and -2 (8). Cholesterol is the primary end product of this pathway, while isoprenoids, dolichols, sterols, heme A, and ubiquinones are the major intermediate products (Figure S1A). Isoprenoids, particularly farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), play vital roles in a variety of cell processes including cell proliferation, motility, and survival (9). Increasing evidence suggests the important role of the mevalonate pathway in tumor initiation and progression via direct and systemic effects on tumor cells and cells of the immune system (10–13). Upregulation of this pathway promotes mammary cell transformation, and high levels of HMG-CoA-Reductase (HMGCR) and other enzymes within this pathway have been shown to correlate with poor survival in BC (14). Similarly, exogenous mevalonate administration promotes tumor growth *in vivo* (12), while blocking this pathway promotes anti-tumor effects both *in vitro* and *in vivo* (15). ERBB2 dependent upregulation of HMGCR activity has been reported in a HER2+ BC cell model, supporting the enzyme's potential oncogenic role in this subtype of BC (16). Statins, the commonly used cholesterol-lowering drugs, block the mevalonate pathway by specific inhibition of HMGCR, the rate-limiting enzyme. N-bisphosphonates (including zoledronic acid), another well-known group of mevalonate pathway inhibitors, target the enzyme farnesyl diphosphate synthase (FDPS) and block the formation of the downstream metabolites FPP and GGPP (17). Both statins and bisphosphonates have direct anti-tumor effects *in vitro* and *in vivo* (15) (18). However, the potential role of the mevalonate pathway in driving resistance to anti-HER2 therapies, and the therapeutic potential of mevalonate pathway inhibitors in overcoming this resistance, have not been explored.

YAP (Yes-associated protein) and its paralog TAZ (Transcriptional Coactivator With PDZ-Binding Motif) function as proto-oncoproteins in a wide variety of cancers and are phosphorylated and inhibited by multiple kinases. YAP and TAZ function as transcriptional coactivators, mainly for the TEAD family of transcription factors, which mediate the oncogenic potential of YAP/TAZ by inducing target genes involved in survival and proliferation (19, 20). Phosphorylation of specific residues on YAP and TAZ results in cytoplasmic sequestration and proteasome-mediated protein degradation (21, 22). Additionally, YAP/TAZ activity is regulated by multiple metabolic pathways (23), including the mevalonate pathway, in various cancer cell models (24, 25).

mTOR (mechanistic target of rapamycin) is a key nutrient, energy and stress sensor protein, that exerts its actions by forming two different complexes (mTORC1 & 2), which can then activate kinases including the S6 kinase and AKT (26). mTOR has been reported to mediate L resistance in HER2+ BC (27), although the mechanism remains unclear. *BIRC5*, encoding Survivin, is a member of the inhibitor of apoptosis (*IAP*) gene family, which plays a role in cell survival and the negative regulation of apoptosis (28, 29). Survivin is overexpressed in many cancers, and is associated with chemotherapy resistance and higher tumor recurrence (30). Survivin expression is regulated by various mechanisms including activation of tyrosine kinase receptor signaling (29) and the transcription factor YAP (31).

In this study we sought to assess the role of the mevalonate pathway in mediating anti-HER2 therapy resistance in models wherein HER2 signaling remains inhibited. We uncover the

mechanism by which the mevalonate pathway mediates resistance to HER2-targeted treatments. Activation of the pathway provides an alternative proliferation and survival signal to R cells, at least partly by activation of the YAP/TAZ transcription factor complex and further downstream mediators Survivin and mTORC1. Lastly, we demonstrate the therapeutic potential of mevalonate pathway inhibitors in overcoming this resistance.

Materials and methods

Reagents and Assays are described in detail in the Supplementary methods (SM) section.

Cell cultures.

The AU565 and UACC812 BC cell lines were purchased from the ATCC (Manassas, VA). AU565, SKBR3, and UACC812 cells and their cognate LR (resistant to L), TR (resistant to T), and LTR (resistant to L+T) cells were established and cultured as described in the SM.

Western blot.

Cells were lysed using RIPA buffer or Cell Signaling Lysis buffer (Cell Signaling, MA), and immunoblotting was performed as described in the SM. Primary antibodies are listed in the Supplementary Table S1.

Q-RT-PCR assay:

Primer sequences used are listed in Supplementary Table S2.

Knockdown by siRNA.

Non-specific control siRNA was Ambion® Silencer Select Negative Control #2. Reverse transfection was performed using the Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific). Cell lysates were harvested 72 hours post transfection for protein expression analysis. For cell growth assays, cells were fixed 6 days after transfection. SiRNA sequences are listed in Supplementary Table S3

Transient transfection of YAP plasmids.

XtremeGENE HP transfection reagent (Roche) was used for transient transfection per manufacturer's instructions. The following three YAP plasmids were used: hYAP2 (wild type) and YAP (S127A) (Addgene plasmids #17793 and #19050 respectively) (32, 33), and YAP (S127/381A) (Addgene plasmid #27378) (21). The plasmid pFLAG-CMV2 was used as a control vector. For transfection experiments using LR and LTR cells, the L and LT containing media was replenished 16–18 hours after transfection,

Cell growth assay.

Cell growth was analyzed with methylene blue staining. Treatments with the various inhibitors alone or in combination in the presence or absence of the mevalonate pathway metabolites were performed for 6 days. Pre-treatments are indicated where applicable. For YAP/TAZ knock-down experiments and YAP overexpression experiments, cells were treated with L for 6 days, 18–48 hours after transfection. Data was normalized to the control

treatment within each individual cell derivative from 4–8 biological replicates for each treatment group.

Luciferase reporter assay.

Luciferase assays were performed with a well-established SREBP responsive pSRE-luc reporter (34) and a YAP/TAZ-responsive 8XGTIC-luc reporter (Addgene plasmid 34615) (24, 35) respectively. Data was normalized to control treatment within each individual cell derivative from 4 biological replicates for each treatment group.

Statistical analysis.

For the YAP/TAZ siRNA growth experiment, data analyses were performed using SAS9.4 software for both siRNA combinations (siYT-1, si-YT-2) separately. Cell growth was compared using a general linear model for nonspecific siRNA, siRNA against YAP/TAZ, and their interaction for the P/LR/LTR cell derivatives. For purposes of plotting, model-estimated group means and 95% confidence limits were used. All the other statistical analyses were performed using Graphpad Prism 6.05 software. One-way or two-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons correction was conducted to examine the statistical differences in cell growth with various treatments, in relative light units in luciferase assays, and in mean expression levels of proteins among the groups of interest in RPPA analysis. All the data in growth, apoptotic, and luciferase assays are presented as mean $\pm$ SD, except the drug response assays in which data are presented with individual values from four to eight biological replicates. In drug response assays, the drug dose values were log transformed before curve fitting. Curve fitting was performed with a nonlinear variable slope (four-parameter) regression model using the least squares fitting method. LogIC₅₀ (relative) differences were assessed by the extra sum-of-squares F test. Values of $p < 0.05$ were considered to be statistically significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns: non-significant.

Results

Resistant cells are dependent on the mevalonate pathway for cell survival and proliferation.

The mevalonate pathway is a complex biosynthetic process comprising multiple enzymatic reactions that produce cholesterol and intermediate isoprenoids (Suppl. Figure S1A). To investigate if the mevalonate pathway is involved in acquired anti-HER2 therapy resistance, we used two HER2-amplified (+) cell lines, SKBR3 and AU565, and the HER2+/Estrogen Receptor (ER)-positive cell line UACC812, all of which were initially L/LT sensitive, and their L/LT resistant (R) derivatives in which HER signaling remains substantially inhibited by the continued presence of L or LT (Suppl. Fig. S1B) (7). RNA Seq data (uploaded to GEO, accession number: GSE132055) from the untreated and treated parental (P) cell lines and their R derivatives revealed that the average expression of the key genes in the mevalonate pathway (Supplementary Table S4), as a surrogate for pathway activity, was dramatically suppressed by treatment of P cells. However, expression was restored or further upregulated relative to P cells in all three R models (Suppl. Fig. S1C). Overall, these analyses suggested that resistant cell growth is associated with restoration of mevalonate

pathway expression. Increased expression levels of the mevalonate pathway enzymes were associated with higher SREBP activity, demonstrated by an increase in SRE-responsive luciferase reporter activity in both LR and LTR cells (Suppl. Fig. S1D). Overall, these analyses suggested potential involvement of the mevalonate pathway in the R phenotype.

Next, to directly examine the mevalonate pathway's role in R cell proliferation and survival, the LR or LTR derivatives of SKBR3 and AU565 cells together with their P cells were treated with statins to block the mevalonate pathway. Interestingly, simvastatin, at doses previously reported to have no cytotoxic effect on the immortalized breast cell line MCF-10A (36), dramatically inhibited the growth of LR and LTR cells in a dose dependent manner, and induced cell death (Figure 1A). On the other hand, the inhibitory effect of simvastatin on the growth of P and TR cells, where the HER2 pathway remains active, was significantly less, suggesting the specificity of the mevalonate pathway in mediating R cell growth when HER2 remains inhibited. Another lipophilic statin, atorvastatin, demonstrated a similar growth inhibition of LR/LTR vs. P cells (Suppl. Fig. S2A). In contrast, the hydrophilic statin pravastatin had no effect on cell growth (Suppl. Fig. S2B), probably due to the absence of the hepatic tissue specific transporter required for entry of hydrophilic statins into cells (37, 38). The cell growth inhibitory effect of simvastatin at high and low dosages was significantly reversed (Figure 1B & Suppl. Fig. S2C) by exogenous supplementation with mevalonate, suggesting that the growth inhibition by simvastatin was via specific blockade of the mevalonate pathway and that the LR/LTR cells are highly dependent on this pathway for growth and survival. A selective growth inhibitory effect was also observed using the UACC812 LTR model (Suppl. Fig. S3A) in which HER2 signaling remains completely inhibited (7). Growth inhibition in this model was also rescued by exogenous mevalonate (Suppl. Fig. S3B). Simvastatin selectively induced apoptosis in the SKBR3, AU565, and UACC812 R cells, as seen by induced cleaved PARP (c-PARP) protein levels (Figure 1C & Suppl. Fig. S3C), which was reversed by exogenous mevalonate (Suppl. Fig. S3D). Apoptotic induction was also confirmed by increased annexin V staining (Suppl. Fig. S4). However, we have previously shown that resistance to potent L+T inhibition in HER2+/ER+ cell models including UACC812 LTR is mediated by the ER pathway (7) and, since the mevalonate pathway increases levels of 25/27-hydroxy-cholesterol that can activate ER as an alternative ligand under estrogen deprivation (39), we focused mainly on ER-negative cell models to avoid this confounding effect.

Importantly, the addition of downstream metabolites of the mevalonate pathway, FPP and GGPP, either alone or in combination rescued cell growth inhibition by simvastatin (Figure 1D & E). However, neither squalene, the immediate downstream metabolite of FPP, nor its end product cholesterol could rescue the cell growth inhibition conferred by simvastatin treatment (Suppl. Fig. S5A & B). Together, these data suggest that the L and LT resistance is dependent on the restored mevalonate pathway activity at least partly via its intermediary metabolites FPP and GGPP.

The mevalonate pathway signals through mTOR and YAP/TAZ to mediate resistance.

To identify the key downstream effectors of the mevalonate pathway that facilitate resistance to potent HER2 inhibition, the SKBR3 LTR cells treated with vehicle, simvastatin, or

simvastatin + mevalonate for 24h were subjected to reverse phase protein array (RPPA) (Suppl. Fig. S6A). We found that simvastatin treatment up-regulated the levels of phosphorylated (p) p38 MAPK, JNK, and c-JUN proteins, an effect that was blocked by simvastatin + mevalonate treatment, suggesting that these changes in levels of proteins are associated with mevalonate pathway alterations (*Cluster 1*, Suppl. Fig. S6B). Several prior studies have demonstrated that simvastatin-induced cell growth inhibition is mediated by p38 MAPK (40) and JNK (41, 42). However, p38 MAPK and JNK inhibitors did not reverse the simvastatin-induced cell growth inhibition in SKBR3 LR and LTR cells (Suppl. Fig. S6D), suggesting, either that the growth inhibitory effect of simvastatin is not mediated entirely by either p38 MAPK or JNK, or alternatively, that these proteins changes are related to cellular stress due to the simvastatin-mediated growth inhibition.

RPPA analysis also showed that levels of several other proteins including YAP and phosphorylated ribosomal protein S6 (p-S6_S235/236), a surrogate marker for mTORC1 activity, were downregulated by simvastatin treatment and restored by exogenous mevalonate (*Cluster 2*, Suppl. Fig. S6C). Western blot analysis of the R cells revealed that while p-AKT was suppressed in these cells to levels unmeasurable by western blotting, the levels of p-S6 and p-4EBP1 (p4EBP1_T37/46) (another marker of mTORC1 activity) were suppressed in P cells treated with L or LT, but increased in LR and LTR derivatives of both SKBR3 and AU565 cells (Suppl. Fig. S7A & B). Based on our findings that mTOR is activated in the R cell models and that simvastatin has an inhibitory effect on the levels of p-S6 in these cells, we assessed whether mTOR may be involved in downstream signaling by the mevalonate pathway in the R cells. Upon simvastatin treatment, p-S6 levels were decreased in the SKBR3 LR/LTR models, but not in the P cells, and this reduction was completely reversed by exogenous mevalonate (Figure 2A & Suppl. Fig. S6C), corroborating previous reports that mTOR can be activated in an AKT-independent manner in lapatinib resistant cells (27, 43). Inhibition of mTORC1 by everolimus inhibited cell growth (Suppl. Fig. S8), indicating that mTORC1 is critical for the R growth.

RPPA analysis further identified YAP as one of the key proteins downregulated upon mevalonate pathway blockade, and several recent studies (24, 25) have reported that in various cancers, including BC, the mevalonate pathway regulates YAP/TAZ activity. We then tested whether YAP and TAZ function as downstream effectors of the mevalonate pathway to mediate resistance to HER2-targeted therapy. To further validate the alterations in YAP protein and activity levels observed by RPPA analysis upon pharmacologic blockade of the mevalonate pathway (Suppl. Fig. S5), the SKBR3 P, LR, and LTR cells were treated with simvastatin ± mevalonate for 24hrs. Consistent with the RPPA results, simvastatin treatment dramatically increased the inactive form of p-YAP (S127 or S381) and reduced total YAP/TAZ protein levels, respectively, which were completely restored by simultaneous exogenous mevalonate treatment (Figure 2B). Of note, simvastatin inhibited YAP/TAZ levels and activity in both P and R cells, though growth was selectively inhibited in the R cells. These data further suggest that the mevalonate pathway at least partly regulates YAP/TAZ independent of the growth state of the cells. Inhibitory effects of simvastatin on YAP/TAZ and p-S6 were also reversed by FPP and GGPP (Suppl. Fig. S9). In agreement with the increased levels of the pYAP_S127 inactive form with simvastatin treatment, immunofluorescence staining showed that YAP protein was localized in the cytoplasm after

simvastatin treatment and its nuclear localization was rescued by exogenous mevalonate (Suppl. Fig. S10). Additionally, a YAP/TAZ-responsive and TEAD-dependent luciferase reporter (8XGTIIIC-luc vector) assay showed that YAP/TAZ activity was increased by about 3 and 10-fold in the SKBR3 LR and LTR cells, respectively, compared to the P cells (Figure 2C). YAP/TAZ transcriptional activity was markedly suppressed by simvastatin but restored by mevalonate (Figure 2D), and by GGPP plus cholesterol, but not by cholesterol alone (Suppl. Fig. S11). Taken together, these data indicate that YAP/TAZ signaling is elevated in the LR and LTR derivatives and is regulated by the mevalonate pathway.

To determine whether the increased YAP/TAZ signaling observed in R cells plays a role in cell survival and growth, we performed concurrent knockdown of YAP and TAZ proteins in the SKBR3 P and R cells using two independent sets (siYT-1, si-YT-2) of previously validated YAP and TAZ siRNAs (24, 35). As shown in Suppl. Fig. S12, YAP/TAZ knockdown inhibited the growth of both P and R cells, however, the degree of cell growth inhibition upon YAP/TAZ knockdown was significantly greater in the LR/LTR cells compared to P cells (Figure 2E), suggesting a greater dependence of the R cells on the mevalonate pathway dependent YAP/TAZ signaling.

mTORC1 is activated through YAP/TAZ in the R cells.

Having identified both mTORC1 and YAP/TAZ as downstream components of the mevalonate signaling pathway, we investigated their functional relationship. Concurrent knockdown of YAP and TAZ markedly and selectively inhibited the p-S6 levels in both LR and LTR but not in P cells (Figure 3A and> Suppl. Fig. S13A). Ectopic overexpression of constitutively active YAP (S127A, and the double mutant S127/381A) strikingly increased mTORC1 activity in the SKBR3 and AU565 R cells, as evidenced by elevated p-S6 levels while AKT remained inhibited (Figure 3B & Suppl. Fig. S13B & C), and also reversed the simvastatin-mediated reduction in p-S6 levels (Figure 3B & Suppl. Fig. S13B &> 13C). These results suggest that in the R cells, mTORC1 activity is largely dependent on YAP. In contrast, in the P cells overexpression of YAP did not increase p-S6 levels, but partially restored the levels that were reduced by L treatment, which also blocked AKT and ERK1/2 activity (Suppl. Fig. S13D). Interestingly, similar to simvastatin, an AKT inhibitor (AZD5363) also reduced p-S6 levels and induced c-PARP, and when combined together, showed an additive effect (Suppl. Fig. S12A & B). This suggests that despite partial activation of mTOR by the low residual AKT signaling in R cells, mTORC1 is also directly regulated by the mevalonate pathway. Overall, these data suggest that in the LR and LTR cells in which HER and downstream AKT signaling remain blocked, reactivation of mTORC1 is largely dependent on the mevalonate pathway via activation of YAP/TAZ. In agreement with this notion, inhibition of p-S6 and induction of c-PARP by simvastatin in the P cells was observed only in the presence of an AKT inhibitor.

The anti-apoptotic factor Survivin (*BIRC5*) is a downstream mediator of YAP/TAZ signaling.

Since YAP/TAZ acts as a transcription factor, we studied the expression levels of several key YAP target genes (*BIRC5*, *CTGF*, *CYR61*), which are known to promote tumor growth and/or survival (44), in the P and R derivatives of SKBR3 cells. The mRNA expression level of *BIRC5*, encoding Survivin, was downregulated by simvastatin treatment in R, but not in P

cells (Figure 4A), and this inhibition was rescued by exogenous mevalonate. The expression of the other YAP/TAZ target genes (i.e., *CTGF* and *CYR61*), however, was not inhibited by simvastatin, and hence these were excluded from further studies (Suppl. Fig. S15A & B). Corresponding to the mRNA expression, we also observed a decrease in Survivin protein levels following simvastatin treatment in the R models, but not in the P cells (Figure 4B, Suppl. Fig. S15 C & D). We next attempted to link the regulation of *BIRC5* expression to YAP/TAZ in the R cells and observed that *BIRC5* mRNA levels were downregulated upon concurrent YAP/TAZ knockdown (Figure 4C & Suppl. Fig. S15E). Transient overexpression of the constitutively active YAP mutants in both SKBR3 LTR cells (Figure 4D) and AU565 LTR cells (Suppl. Fig. S16A) increased Survivin levels, and reversed the simvastatin-mediated inhibition of Survivin expression, suggesting that in the LTR cells, Survivin expression is largely dependent on mevalonate pathway-mediated YAP/TAZ activation. In both SKBR3 and AU565 cells, we did not observe a decrease in *SLC7A5* (Leucine transporter) expression upon inhibition of the mevalonate pathway with simvastatin (Suppl. Fig S16 B & C), suggesting that the mechanism by which YAP/TAZ activates mTORC1 in HER2+ BC cells with acquired resistance to anti-HER2 therapy is different from that reported previously in hepatic epithelial cells (45).

To assess the importance of mTOR in regulating Survivin expression (43) in the R models, the SKBR3 P, LR, and LTR cells were treated with low levels of the mTORC1/2 inhibitor AZD2014 and the mTORC1 inhibitor everolimus. mTORC inhibition, especially by AZD2014, suppressed Survivin levels in SKBR3LR and LTR cells (Suppl. Fig. S16D), indicating that in addition to being directly regulated by YAP/TAZ, Survivin is also regulated by mTOR signaling in the R cell models.

Inhibition of the mevalonate pathway or its downstream effectors increases the sensitivity of naive cells to L.

Since the HER2 therapy R cells are highly dependent on the mevalonate pathway and its downstream targets for growth and survival, we hypothesized that blockade of the mevalonate pathway or its downstream mediators will further increase the sensitivity of P cells to L. Indeed, upon blockade of the mevalonate pathway by simvastatin, the L dose response curves of both SKBR3 and AU565 P cells showed a significant left shift (Figure 5A). Simvastatin treatment alone had minimal effects on p-HER2_Y1221/1222, p-AKT_S473, p-ERK1/2_T202/Y204, and p-S6 protein levels. Interestingly, in the presence of simvastatin, L treatment showed a greater reduction in p-S6 levels, with lesser effect on p-HER2_Y1221/1222, p-AKT_S473, and p-ERK1/2_T202/Y204 (Suppl. Figs S17A & B). While the p-S6 level was not affected by L alone at a low dose of 25nM, this dose in combination with simvastatin almost completely suppressed p-S6 levels, suggesting that mevalonate pathway blockade enhances the efficacy of L in abrogating mTORC1 signaling. To assess if YAP/TAZ signaling plays a similar key role in modulating the L response, we knocked down YAP/TAZ or overexpressed a constitutively active YAP (S127/381A) mutant in P (L/LT sensitive) cells in the presence of L. Interestingly, YAP/TAZ knockdown shifted the L dose response curve of these cells significantly to the left (Figure 5B), while over-expression of YAP (S127/381A) significantly reduced their sensitivity to L (Suppl. Figs. S18A, 18B, 18C) and the dual HER1/2 TKI afatinib (Suppl. Fig. S18D), suggesting that

YAP/TAZ signaling modulates the cellular response to both TKIs. To investigate whether the ability of simvastatin to enhance sensitivity of cells to L is due to enhanced inhibition of mTORC1, the L response was assessed $\pm$ the mTORC1 inhibitor everolimus in AU565 and SKBR3 P cells. As shown in Figure 5C, everolimus rendered both the AU565 and SKBR3-P cells more sensitive to L, suggesting that mTORC1 inhibition substantially enhances L efficacy.

The N-bisphosphonate zoledronic acid effectively inhibits R cell growth.

Finally, we investigated whether acquired HER2-targeted therapy resistance could also be overcome by zoledronic acid (ZA), a clinically important mevalonate pathway inhibitor that blocks the enzymatic activity of FDPS (Suppl. Fig. S1A). Like simvastatin, ZA greatly inhibited SKBR3, AU565 and UACC812 R cell growth, with only modest effects on P cells (Fig. 6A & Suppl. Fig. S19A). This growth inhibition by ZA was rescued by GGPP, a metabolite downstream of FDPS, but not by the upstream metabolite mevalonate, demonstrating that ZA's inhibitory effect on R growth is via mevalonate pathway blockade (Fig. 6B & Suppl. Fig. S19B). Furthermore, ZA induced a greater apoptotic response in the R vs P models, as seen by increased c-PARP which was again reversed by the addition of GGPP (Fig. 6C). Like simvastatin, ZA increased levels of the inhibitory p-YAP, and decreased levels of total TAZ, p-S6, and Survivin (Fig. 6D), which were rescued by GGPP. Together, these results demonstrate that ZA exploits a similar mechanism as simvastatin to suppress the growth and survival of cells R to anti-HER2 therapy.

Lastly, withdrawal of L and LT from the culture media of the resistant cells, led to re-activation of HER2 signaling and reduced sensitivity to both Simvastatin and zoledronic acid (Suppl. Fig, S20). This suggests that sustained inhibition of HER2 signaling is indeed required for the mevalonate pathway to act as an escape mechanism of resistance.

Discussion

Despite the advent of multiple therapies targeting HER2, resistance is still common and remains a challenge. Using models of HER2+ BC cell lines made resistant to HER2-targeted therapies, we observed that resistance is associated either with re-activation of HER2 signaling, or with alternative signaling pathways that promote cell survival and growth when HER2 signaling is blocked. Using the dual HER1/2 tyrosine kinase inhibitor lapatinib, alone or in combination with trastuzumab, we now report a novel mechanism, representative of the latter method, by which HER2+ BC cells escape sustained HER2 inhibition. The LR and LTR cells in which HER signaling is completely blocked have adopted the mevalonate pathway as an escape route to promote resistant cell growth. This resistance involves activation of and increased dependence on the mevalonate pathway-YAP/TAZ-mTORC1-Survivin signaling axis, identifying a metabolic vulnerability that can be exploited pharmacologically by inhibiting the mevalonate pathway to overcome resistance, as illustrated in the working model presented in Figure 6E.

Statins and the amino-(N)-bisphosphonate zoledronic acid, which are used frequently in patients to lower blood cholesterol and improve bone density, respectively, specifically block the mevalonate pathway. We observed that while lipophilic statins, which can penetrate the

cell membrane, were cytotoxic to the resistant cells, the hydrophilic statins were not, probably due to absence of the transporter that is required for entry of hydrophilic statins into the cells (37, 46). Importantly, in BC, the majority of studies including a recent meta-analysis suggest that lipophilic but not hydrophilic statins have great protective effects and significantly reduce cancer-specific and all-cause mortality in BC patients (47). Our findings also showed that zoledronic acid was as effective as simvastatin in killing the resistant cells. We demonstrated that inhibition of the mevalonate pathway by both drugs can overcome resistance to anti-HER2 therapy, by inhibiting key downstream survival signaling, when HER2 signaling remains blocked. This selective and specific action of mevalonate pathway inhibitors in circumventing resistant growth of HER2+ BC cells presents a novel therapeutic application of these inhibitors in BC. We also showed that concomitant treatment of parental cells with these mevalonate pathway inhibitors may enhance the anti-tumor efficacy of anti-HER2 inhibitors, thereby offering additional therapeutic intervention.

YAP has been shown to mediate resistance to targeted therapies in several reports. Indeed, amplification or overexpression of YAP enables pancreatic, colon, and lung cancers to bypass oncogenic KRAS addiction (48, 49). In this study, we demonstrated that knockdown of YAP/TAZ leads to significantly greater growth inhibition in LR/LTR cells than in the parental cells which have intact HER2 signaling. Conversely, hyperactivation of YAP/TAZ by over-expression of constitutively active YAP attenuated the sensitivity of HER2+ BC cells to anti-HER2 therapy, thus establishing the role of YAP/TAZ signaling as an escape mechanism that can drive resistance in HER2+ BC. While YAP/TAZ itself was previously reported to increase microenvironment rigidity, hence mediating lapatinib resistance (50), our study demonstrates its key intermediary role to convey resistance to potent HER2 inhibition or to negate the antitumor effects of HER2 targeted therapy. In line with previous reports about mevalonate pathway mediated activation of YAP/TAZ (24, 51), our data demonstrated the role of the isoprenoid metabolites FPP and GGPP in the mevalonate pathway signaling to YAP/TAZ. The observation that addition of FPP and GGPP rescues R cell growth and YAP/TAZ signaling inhibition by mevalonate pathway inhibitors, whereas squalene and cholesterol could not, confirmed that it is indeed the intermediate isoprenoids which mediate signaling to YAP/TAZ.

In this study, we found that re-activated mTORC1 signaling in anti-HER2 resistant cells, where HER2 remains inhibited is a potential downstream effector that is upregulated at least partly by the mevalonate pathway via activation of YAP/TAZ. By overexpressing constitutively active forms of YAP, we showed that YAP overcomes simvastatin-mediated inhibition of mTORC1 signaling, suggesting that mTORC1 signaling is directly regulated by the mevalonate pathway and YAP/TAZ. Although previous studies (27, 43) have shown that PI3K-independent activation of mTORC1 confers lapatinib resistance, the underlying mechanisms of activation remained unclear. In this regard, our findings present a novel mechanism for AKT independent activation of mTORC1 signaling via the mevalonate pathway-YAP/TAZ axis. Of note, though our data suggests that mTOR reactivation is largely dependent on the mevalonate pathway-YAP/TAZ signaling, we found that inhibition of the low residual levels of AKT activity, which could be detected only in the presence of an AKT inhibitor, further inhibited the mTOR activity. We surmise that this residual AKT activity may be attributed to the fact that mTOR is both an effector and activator of AKT signaling

(52), or to alternative upstream signaling from the mevalonate pathway or other cellular kinases. Nevertheless, our data suggests that the combination of a mevalonate pathway inhibitor and an AKT inhibitor may be even more effective in overcoming anti-HER2 therapy resistance.

While exploring the downstream survival effectors of mevalonate pathway signaling through YAP/TAZ, we also found that Survivin, an anti-apoptotic protein that is a direct gene target of YAP, is regulated by the mevalonate pathway in resistant cells. In addition to its regulation by the mevalonate pathway and YAP/TAZ, we also observed inhibition of Survivin levels by mTORC1 inhibitors. This observation is in agreement with a previous report showing mTOR-mediated inhibition of Survivin in a lapatinib resistant cell line (43), suggesting that Survivin may also be regulated by mTORC1. Together, these findings imply that Survivin may be a key signaling junction that is responsive to multiple upstream stimuli in the cells resistant to anti-HER2 therapy.

In summary, this study highlights the role of the mevalonate pathway as a novel mechanism of resistance to anti-HER2 therapies in HER2+ BC cells when HER2 signaling remains inhibited. We identified and linked new targets of the mevalonate pathway, especially the YAP/TAZ-mTORC1-Survivin axis that could be further therapeutically exploited to reverse resistance to HER2-targeted therapies. Our findings warrant additional research to directly assess the potential impact of statins and N-bisphosphonates on HER2+ BC recurrence, as well as prospective validation in clinical trials to evaluate the potential additive or synergistic effects of statins when used with anti-HER2 therapies. Further, while new antibodies and more potent drugs against HER2 are being developed in the clinic, it will be important to understand the activity status of the HER receptor layer in the residual disease. Our recently launched SIMPHONY trial ( ), for patients with metastatic HER2+ BC who progressed on dual anti-HER2 therapy, will evaluate whether the addition of simvastatin to a dual anti-HER2 regimen can re-sensitize the tumors to anti-HER2 therapy. If clinically validated, our findings may help develop a new well-tolerated treatment approach.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations List:

R	Resistance/ Resistant
BC	Breast Cancer
FPP	Farnesyl Pyrophosphate
GGPP	Geranyl Geranyl Pyrophosphate
L	Lapatinib
T	Trastuzumab
LR	Lapatinib Resistant
LTR	Trastuzumab Resistant

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IMPLICATIONS

The MVA was found to constitute an escape mechanism of survival and growth in HER2+ breast cancer models resistant to anti-HER2 therapies. MVA inhibitors such as simvastatin and zoledronic acid are potential therapeutic agents to re-sensitize the tumors that depend on the MVA to progress on anti-HER2 therapies.

Figure 1. LR or LTR cells are highly dependent on the MVA pathway for growth and survival. (A) Cell growth assay with simvastatin (Sim) treatment. Parental (P) SKBR3 or AU565 cells in parallel with their resistant (R) derivatives were treated with increasing doses of Sim. Statistical significance levels are indicated for comparisons between P vs LR and P vs LTR models of each cell line. (B) Cell growth assay with 5 μM Sim $\pm$ 250 μM mevalonate (MVA) treatment. (C) Apoptotic analysis by western blotting for cleaved PARP (cPARP). Cells were treated with 5 μM Sim $\pm$ 250 μM MVA for 48hrs (SKBR3 model) and 72hrs (AU565 model) respectively. (D, E) Growth assays for Sim treatments $\pm$ FPP or GGPP (10 μM) of SKBR3 (D) or AU565 (E) cell models. Values of $p < 0.05$ were considered to be statistically significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns: non-significant.

Figure 2. mTORC1 and YAP/TAZ are downstream effectors of the MVA pathway.

(A) Western blotting analysis of HER signaling and its downstream signaling including AKT/mTORC1 and ERK1/2 upon Sim treatment $\pm$ MVA repletion. SKBR3 LR/LTR cells in parallel with their P cells were treated with Sim $\pm$ MVA. (B) Western blotting of YAP/TAZ signaling. SKBR3 P and the LR/LTR derivatives were treated with Sim, MVA, or Sim + MVA. (C, D) YAP/TAZ luciferase reporter assay (8XGTIIC-luc) for the SKBR3 cell models. Statistical significance levels are indicated for comparison between P vs LR and P vs LTR. (C) SKBR3 cell lysates were harvested 24hrs after transfection. Data are presented as relative light units (RLU %) normalized to that of SKBR3P cells. (D) Cells treated with Sim $\pm$ MVA. The data are presented as in C. Values of $p < 0.05$ were considered to be statistically significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns: non-

significant. (**E**, **F**) Growth assay with YAP/TAZ knock-down. Cells were silenced with two combinations of different siRNA sequences in SKBR3 P, LR, and LTR cells. Data are normalized to the control siRNA group (siCtrl) within individual cell derivatives. **E**: siYT-1: siYAP-1+siTAZ-1; **F**: siYT-2: siYAP-2+siTAZ-2.

Figure 3. mTORC1 is reactivated by YAP/TAZ signaling, in R cells. **(A)** Signaling analysis with YAP/TAZ silenced. Combination knock-down of YAP and TAZ was performed in cells, and cell lysates were collected and assessed by western blotting for YAP/TAZ levels and HER2 signaling. **(B)** Analysis for signaling alterations with YAP ectopic expression in resistant cells under Sim treatments. After transfection with wild type (hYAP2) or constitutively active mutant YAP forms (YAP (S127A) and YAP (S127/381A)), SKBR3 LTR cells were treated with DMSO or 5 μ M Sim.

Figure 4.

Survivin is a downstream mediator of YAP/TAZ signaling in the resistant cells. **(A)** BIRC5 (Survivin) gene expression analysis by q-RT-PCR. SKBR3 P and LTR derivatives were treated with Sim, MVA, or Sim+MVA. Data were normalized to GAPDH and control treatments of individual cells derivatives. Values of $p < 0.05$ were considered to be statistically significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns: non-significant. **(B)** Western blotting of YAP/TAZ and Survivin protein levels. SKBR3 P and the LTR derivatives were treated with Sim, or Sim+MVA. **(C)** Signaling analysis with YAP/TAZ silenced. Combination knock-down of YAP and TAZ was performed in SKBR3 P, LR, and LTR cells, and cell lysates were subjected to western blot for YAP/TAZ, Survivin, and p-S6 protein levels. **(D)** Analysis for signaling alterations with YAP ectopic expression in resistant cells under Sim treatments. Forty-eight hours after transfection with wild type (hYAP2) or mutant YAPs (YAP (S127A) and YAP (S127/381A)), SKBR3LTR cells were treated with DMSO or Sim.

Figure 5.

Blockade of the MVA pathway and its downstream effectors YAP/TAZ and mTOR enhances lapatinib efficacy. **(A)** Lapatinib response assays under MVA pathway blockade. SKBR3 P or AU565 cells were treated with multiple doses of lapatinib $\pm$ Sim. Data were normalized to the mean of non-lapatinib treatments within DMSO or Sim group respectively. **(B)** Growth assays for lapatinib (Lap) response under YAP/TAZ silencing. The SKBR3 P cells were transfected with siCtrl or siYT-1 siRNAs, and were treated with multiple doses of lapatinib for 5 days. Data are normalized to non-lapatinib treatment within each siRNA. **(C)** Lapatinib response assays under mTORC1 suppression. SKBR3 or AU565 cells were treated with lapatinib $\pm$ 5nM everolimus. Data are presented as in **A**. p-values were indicated for comparisons between logIC50 values of control vs treated curves.

Figure 6.

MVA pathway blockade by zoledronic acid (ZA) is effective in overcoming L/LT resistance by a mechanism similar to Sim. (A) Cell growth assay with ZA treatment. SKBR3 or AU565 P cells in parallel with their LR, TR, and LTR derivatives were treated with 12.5, 25, and 50 μM ZA. Statistical significance levels are indicated for comparisons between P vs LR and P vs LTR models of each cell line. (B) Cell growth assay with 20 μM or 50 μM ZA (in SKBR3 and AU565 cell models, respectively) $\pm$ GGPP treatment. For A & B, values of $p < 0.05$ were considered to be statistically significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns: non-significant. (C) Western blotting for the apoptotic marker cleaved PARP (cPARP) and YAP/TAZ signaling. cPARP, pYAP127, pYAP397, and TAZ were probed after SKBR3 cells were treated with ZA $\pm$ GGPP for 48hrs. (D) Western blotting of pS6 and

Survivin levels. SKBR3 P and the LTR derivatives were treated with ZA, GGPP, or ZA + GGPP for 36hrs for P-S6 and Survivin levels. (E) Working model presenting the alternative signaling in LR or LTR cells where HER signaling remains inhibited by sustained L/LT treatment. P cells treated with L/LT concentrations that substantially inhibit HER signaling and the downstream AKT/mTORC1 and ERK1/2 pathways undergo growth inhibition or cell death. To evade the HER signaling suppression, cells adopt the mevalonate pathway as an alternative pathway to survive. The MVA pathway activates downstream YAP/TAZ-mTORC1 and YAP/TAZ-Survivin signaling through FPP/GGPP by unknown mechanisms. The MVA pathway inhibitors (Sim and ZA) block the MVA pathway signals and help to overcome resistance when HER2 signaling remains blocked due to presence of L or LT. The red X's represent blockade of the HER/AKT pathway and the MVA pathway