ARTICLE

Combination of ZEN-3694 with CDK4/6 inhibitors reverses acquired resistance to CDK4/6 inhibitors in ER-positive breast cancer

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CDK4/6 inhibitors significantly prolong progression-free survival in patients with advanced hormone receptor-positive (HR+) HER2-negative breast cancer. Despite recent successes, patients acquire resistance, necessitating the development of additional novel therapeutic strategies. Bromodomain and extra-terminal domain (BET) proteins are key epigenetic regulators that interact with acetylated lysine (AcLys) residues of histones or transcription factors. BET proteins are directly involved in modulating estrogen receptor (ER) signaling and the cell cycle. Therefore, BET inhibitors can potentially offer new strategies in the treatment of advanced ER+ breast cancer. ZEN-3694 is an orally bioavailable small molecule BET inhibitor currently being evaluated in Phase 1/2 clinical trials (NCT03901469). To assess a potential combination strategy in a CDK4/6i resistant breast cancer population, we investigated the mechanism of action of ZEN-3694 combined with CDK4/6 inhibitors in the ER+ cell lines resistant to palbociclib or abemaciclib. Here, we describe that the combination of ZEN-3694 with CDK4/6i potently inhibits proliferation and induces apoptosis in CDK4/6i resistant cell lines. The resistance to both palbociclib and abemaciclib was associated with the strong upregulation of CDK6 and CCND1 protein levels, which was reversed by the ZEN-3694 treatment. Furthermore, RNAseq data and pathway analysis elucidated the combinatorial effects of ZEN-3694 with CDK4/6 inhibitors through significant downregulation of multiple pathways involved in cell cycle regulation, cellular growth, proliferation, apoptosis, inflammation, and cellular immune response. Our data indicate that ZEN-3694 has therapeutic potential in combination with CDK4/6 inhibitors in patients with advanced ER+ breast resistant to CDK4/6 inhibitors.

INTRODUCTION

Estrogen receptor-positive breast cancer (ER+) is a prevalent disease with over two million global new cases diagnosed in 2020 [1]. About 70–80% of women diagnosed with breast cancer are ER+. The standard of care for ER+ breast cancer patients include selective estrogen receptor modulators (e.g., tamoxifen), estrogen receptor degraders (SERDs, e.g., fulvestrant), aromatase inhibitors, as well as a combination of endocrine therapy with inhibitors of cyclin-dependent kinases (CDK4/6 inhibitors, CDK4/6i). Three approved CDK4/6 inhibitors (palbociclib, ribociclib, and abemaciclib) have been shown to significantly improve progression-free survival and were granted FDA-breakthrough status in combination with endocrine therapy for the treatment of metastatic breast cancer patients [2–4]. Although improvements have been made in treating metastatic ER+ breast cancer, resistance still occurs over time, making it essential to elucidate the mechanisms of intrinsic and *de novo* resistance.

Several resistance mechanisms to CDK4/6 inhibitors have been described recently, including the upregulation of CDK6, CCNE1, and loss of function of Rb [3]. Additional mechanisms of resistance involve the loss of ER and upregulation of PI3K/mTOR/AKT as well as HER2 pathways [5, 6]. Kettner et al. [6] described resistance

mechanisms associated with the upregulation of IL-6 and STAT3 pathways coupled with the downregulation of DNA repair machinery, which prompted investigating a combination of a specific STAT3 with a PARP inhibitor.

As the unmet need in metastatic breast cancer patients developing CDK4/6i resistance remains high, new targeted therapies are currently being evaluated in the clinical setting, which includes PI3K, HDAC, and PARP inhibitors, in combination with endocrine as well as other therapies.

Recently, it has emerged that BET proteins play an important role in hormone receptor-positive (HR+) breast cancer. BET proteins (BRD2, BRD3, and BRD4) are epigenetic readers known to regulate transcription through binding to the acetylated lysine (AcLys) tails of histones and transcription factors. It has been described that BRD3 and BRD4 are involved in the regulation of the cell cycle and E2F-Rb pathway, as well as the regulation of ESR1 transcription and ER signaling [7, 8].

Moreover, it has been reported that BET proteins play an important role in endocrine-resistant breast cancer. Feng et al. previously described that the BET inhibitor JQ1 was efficacious in tamoxifen-resistant ER+ breast cancer models, suggesting that a combination treatment may be a meaningful treatment strategy in patients progressing on endocrine therapies [7, 8].

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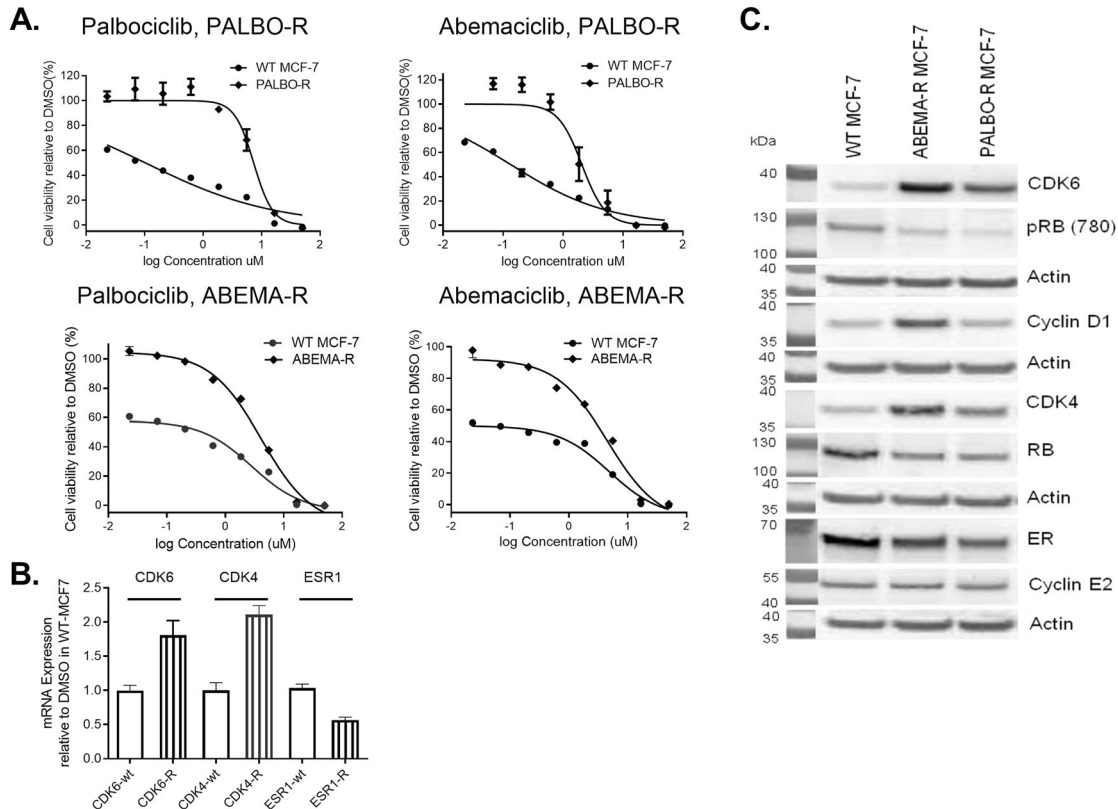
Fig. 1 Developing CDK4/6i-resistant cell lines. A Seven-day viability in WT and CDK4/6i resistant cells treated with palbociclib or abemaciclib. **B** Comparison of CDK4, CDK6, and ESR1 mRNA levels in PALBO-R MCF-7 cells in relation to WT at 6 h. **C** Comparison of ERα and G1-S phase cyclin protein levels in the CDK4/6i resistant vs. parental ER+ breast cancer cell lines.

ZEN-3694 is a novel bioavailable BET inhibitor currently undergoing Phase1/2 clinical trials in the metastatic castration-resistant prostate cancer (mCRPC, NCT02711956), as well as in patients with triple-negative breast cancer (TNBC, NCT03901469). As BET inhibitors can potentially offer a new strategy in reversing endocrine or CDK4/6i resistance, we elucidated the mechanisms of action of ZEN-3694 alone and in combination with CDK4/6 inhibitors in a panel of ER+ breast cancer cell lines resistant to palbociclib or abemaciclib. We investigated the mechanisms of the acquired CDK4/6i resistance to abemaciclib and palbociclib by RNAseq and Western blot analysis demonstrating that the altered pathways were similar but not identical.

Here, we report the mechanism of action of ZEN-3694 in combination with CDK4/6 inhibitors, demonstrating the reversal of CDK4/6i-resistance, which supports the potential use of this combination as a new strategy in patients developing resistance to CDK4/6 inhibitors in ER+ breast cancer.

RESULTS
Development of CDK4/6 inhibitor-resistant MCF-7 cell line variants
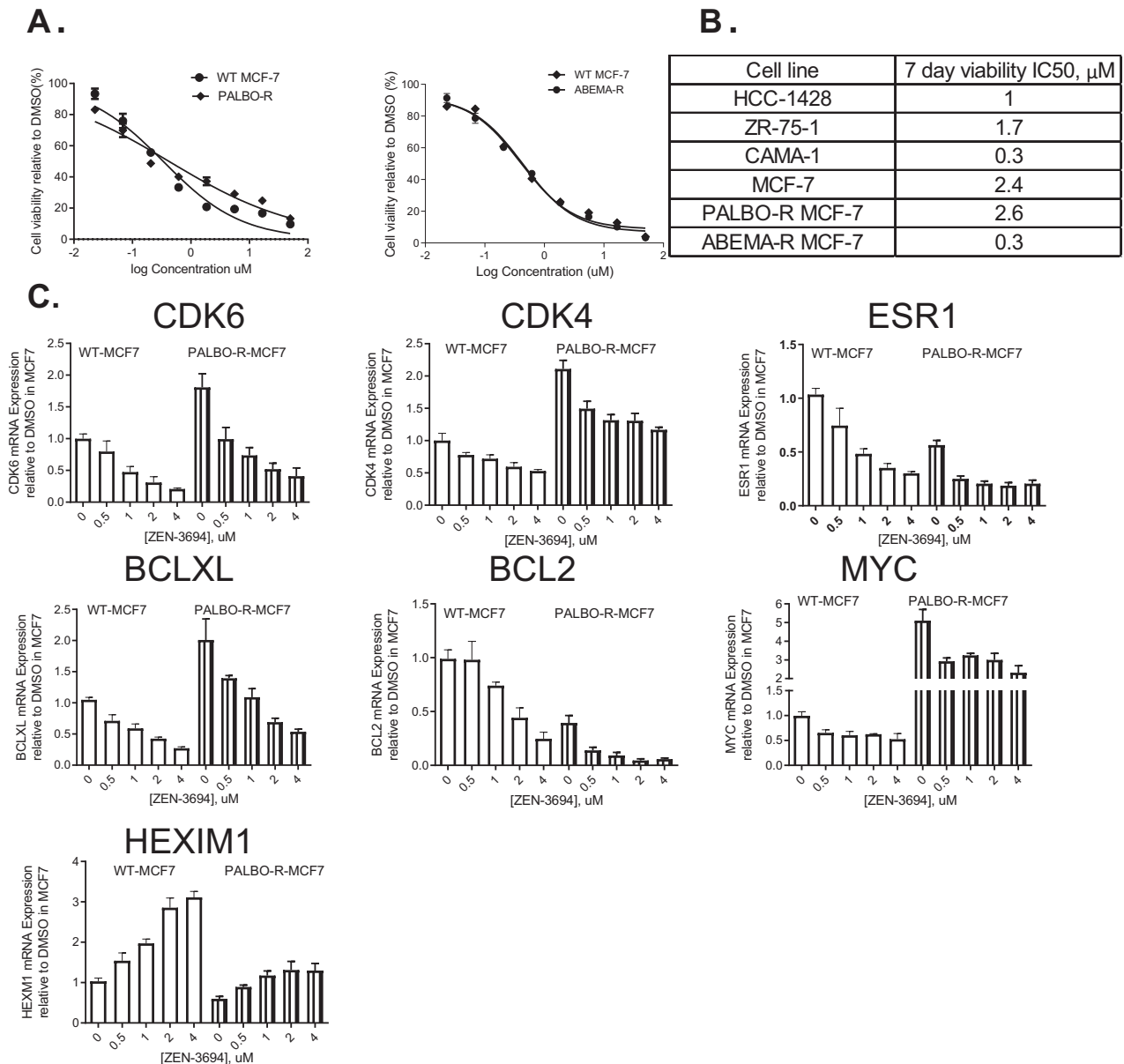To elucidate the mechanisms of resistance to CDK4/6 inhibitors and further investigate whether BET inhibition can reverse these effects, a panel of resistant MCF-7 variants was generated (Fig. 1). ER+ breast cancer cell line MCF-7 was continuously exposed to increasing concentrations of palbociclib or abemaciclib for several months. The developed resistance was confirmed by testing the potency of the CDK4/6 inhibitors in a viability assay. The obtained IC50 values of growth inhibition in response to CDK4/6i treatments were compared between the resistant and parental ER+ cell lines (Fig. 1A).

The resistant cell lines demonstrated a significant decrease in sensitivity towards corresponding CDK4/6 inhibitors. To understand whether the CDK4/6-phospho-Rb pathway is altered upon acquired resistance to CDK4/6i, we measured the mRNA levels of CDK6, CDK4, and ESR1 in palbociclib resistant MCF-7 (PALBO-R) vs. parental MCF-7 cell lines (Fig. 1B). The mRNA levels of both CDK4 and CDK6 were induced two-fold upon developing palbociclib resistance, while the mRNA levels of ESR1 were significantly reduced in PALBO-R in comparison to the wild type MCF-7 cell line.

These results are consistent with previous reports describing that resistance to CDK4/6 inhibitors is associated with the upregulation of CDK6 and CCNE1 [9, 10], as well as loss of total-Rb or phospho-Rb and downregulation of ER [7]. Therefore, we next determined whether the expression of select proteins involved in the regulation of the cell cycle has also been altered upon the acquired resistance. We performed Western blot analysis comparing the baseline levels of proteins of interest in the resistant cell lines in comparison to the parental cells and discovered that CDK6, CDK4, and cyclin D1 were significantly upregulated in PALBO-R and ABEMA-R-MCF-7 variants (Fig. 1C).

The acquired CDK4/6i resistance in these cells was also associated with reduced phospho-Rb and total Rb-protein levels, which is consistent with the mechanism of action of CDK4/6 inhibitors.

As described previously, CDK4/6 kinases regulate the E2F axis and thereby estrogen receptor transcription and signaling [8–10]. Similar mechanisms were reported by Yang et al., who showed a decrease of ERα protein levels in CDK4/6i resistant models with significant overexpression of either CDK6 or CCNE1 [9]. We then investigated whether the protein levels of estrogen receptors are affected in the resistant cell lines due to alterations in cell cycle

A.

B.

Cell line	7 day viability IC50, μM
HCC-1428	1
ZR-75-1	1.7
CAMA-1	0.3
MCF-7	2.4
PALBO-R MCF-7	2.6
ABEMA-R MCF-7	0.3

C.

Fig. 2 **ZEN-3694 inhibits proliferation of CDK4/6i sensitive and resistant ER+ breast cancer cell lines and inhibits markers of CDK4/6i-resistance. A** Effect of ZEN-3694 on the viability of resistant breast cancer cell lines resistant to CDK4/6 inhibitors. **B** Effect of ZEN-3694 on the viability of several ER+ breast cancer cell lines. **C** Effect of ZEN-3694 on mRNA transcripts in WT-MCF-7 and PALBO-R-MCF-7 at 6 h. ZEN-3694 upregulates HEXMI1, a known BET-dependent target engagement marker, in a concentration-dependent manner.

regulation and observed a decrease of ERα protein levels in CDK4/6i-resistant MCF-7, further suggesting deregulation of the cell cycle pathway upon developing acquired resistance (Fig. 1C).

ZEN-3694 inhibits proliferation of ER+ parental and CDK4/6i resistant breast cancer cell lines and inhibits markers of resistance

BET proteins are known to regulate ER transcription and play a major role in the maintenance of ER signaling [7, 8]. Feng et al. [8] have demonstrated that the combination of BET inhibitor (JQ1) and fulvestrant (SERD) was synergistic in inhibiting tumor growth of tamoxifen-resistant xenograft mouse models accompanied with a synergistic inhibitory effect on ERα protein levels. These results suggest that BETi treatments could improve the ability of hormonal therapy to inhibit ER signaling and thereby, the clinical progression of ER+ breast cancer.

Thus, we evaluated the effect of ZEN-3694 on the proliferation of ER+ breast cancer cell lines (Fig. 2). ZEN-3694 inhibited the proliferation of several ER+ cell lines at low micromolar concentrations and importantly had similar antiproliferative effects in wild type and CDK4/6i resistant cell lines, which indicates that ZEN-3694 can potentially alleviate the developed resistance to these agents (Fig. 2A, B).

As we demonstrated that ZEN-3694 can effectively inhibit proliferation in our CDK4/6i resistant cell lines, we next investigated whether it can also inhibit the mRNA expression of known markers contributing to the palbociclib resistance, such as CDK4, CDK6, ESR1, as well as such oncogenes as MYC, BCL2, BCLXL, and BET-dependent marker HEXIM1 (Fig. 2C). ZEN-3694 strongly inhibited ESR1 mRNA levels in the parental MCF-7 as well as resistant cell lines (Fig. 2C, Fig. S1). mRNA levels of MYC, CDK4, and CDK6 were concurrently upregulated with the acquired
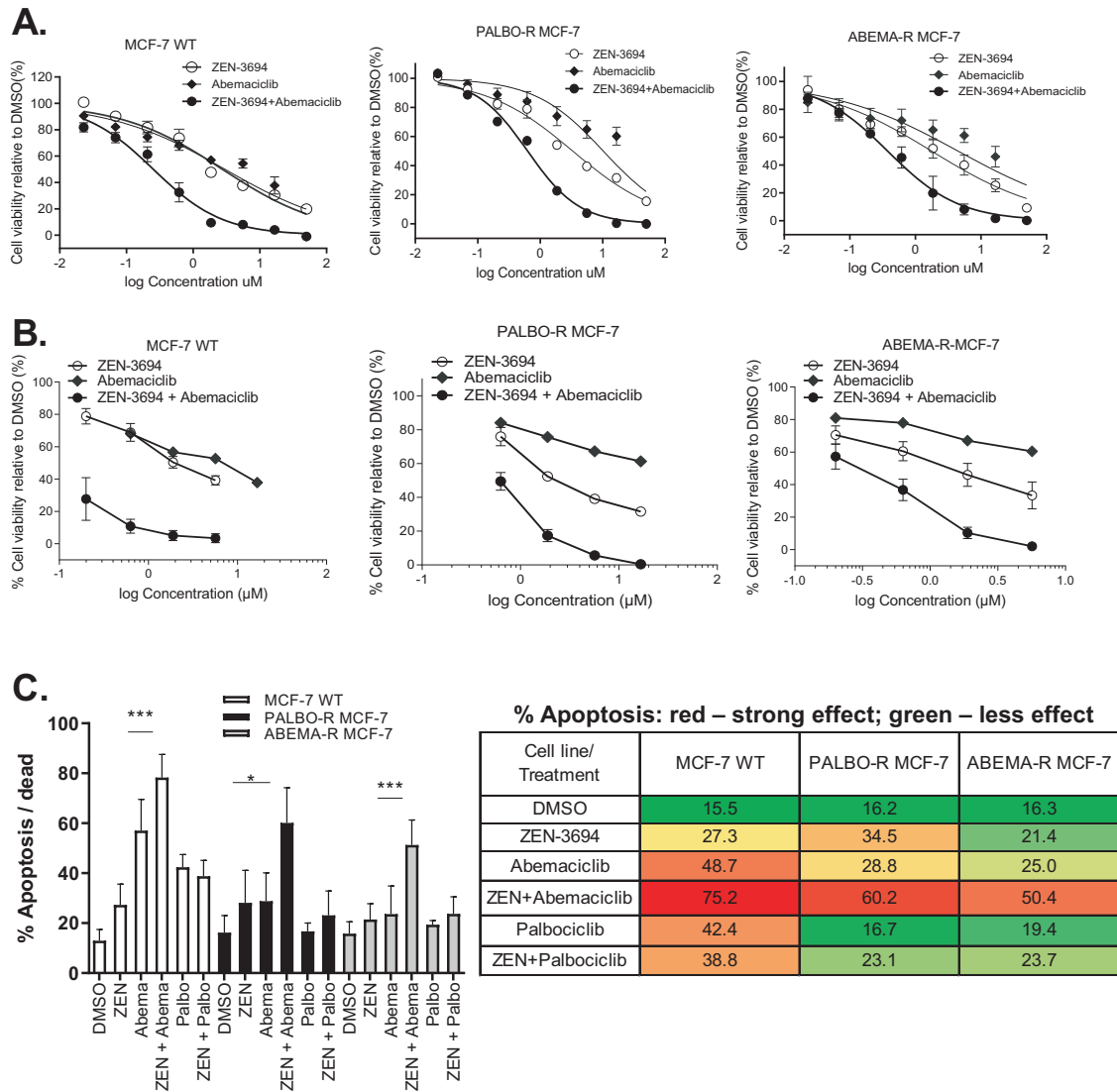
Fig. 3 The combination of ZEN-3694 and abemaciclib synergistically inhibits proliferation and induces apoptosis in WT and CDK4/6i-resistant ER+ breast cancer cell lines. A Combination of ZEN-3694 and abemaciclib synergistically inhibits proliferation in the parental and palbociclib or abemaciclib resistant MCF7 cell lines as shown in 8-point **A.** and four-point **B.** combination curves. **C** Combination of ZEN-3694 and abemaciclib synergistically induces apoptosis in WT and CDK4/6i-R breast cancer cells lines (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$). Apoptosis assay was measured by flow cytometry using FITC Annexin V Apoptosis Detection Kit. The heat map represents the average of apoptotic/dead cells measured in six independent experiments. The color-coding in the heat map goes as follows: red-strong apoptotic effect, yellow-medium, and green-less effect.

resistance, but inhibited by ZEN-3694, highlighting the possibility of ZEN-3694 to potentially reverse the developed resistance.
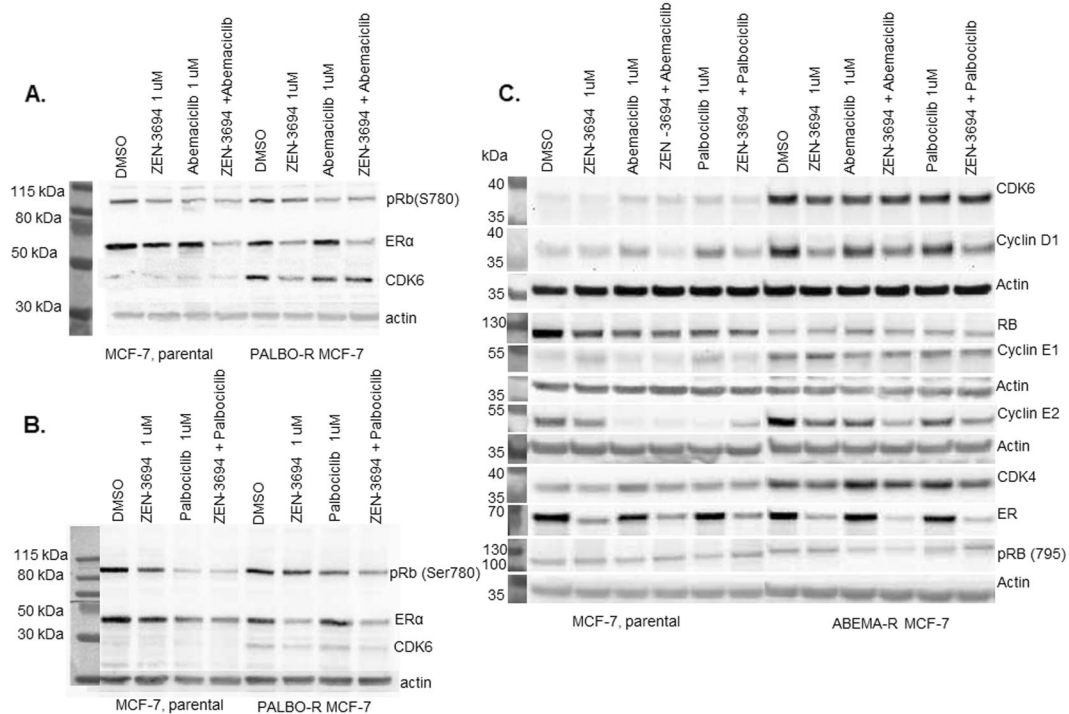
The combination of ZEN-3694 and abemaciclib synergistically inhibits proliferation and induces apoptosis in several WT and CDK4/6i-resistant ER+ breast cancer cell line variants

In order to elucidate whether combining CDK4/6 inhibitors with BET inhibition could provide an additional benefit for ER+ breast cancer treatment and whether BETi could potentially re-sensitize a resistant patient population to CDK4/6 inhibitors, we performed combination studies with ZEN-3694 and CDK4/6i in CDK4/6i sensitive and resistant cell lines (Fig. 3). Concurrent treatment with ZEN-3694 and abemaciclib in WT, PALBO-R, and ABEMA-R MCF-7 cell lines, demonstrated a significant effect on the inhibition of proliferation with the calculated CI values ranging between 0.07 and 0.36 (Fig. 3A, B).

In addition, we performed Annexin-V-FITC/PI flow cytometry with the extended panel of the ER+ CDK4/6 inhibitor-resistant

cell lines to assess whether the observed synergistic effects in ZEN-3694 and abemaciclib combination induced apoptosis (Fig. 3C).

Single-agent treatment with ZEN-3694 of parental, PALBO-R, and ABEMA-R MCF-7 cell lines induced apoptotic effects ranging from 21 to 35% (p values: ***<0.001; *<0.05). Palbociclib-induced apoptosis as a single agent in a parental MCF-7 cell line (42%, $p <$ 0.001) was decreased nearly twofold in PALBO-R MCF-7 (17% and 19%, $p < 0.001$). Abemaciclib treatments led to a higher level of apoptosis in the parental MCF-7 cell line (54%, $p < 0.001$) as compared to palbociclib, which was reduced to ~29% ($p < 0.001$) with the acquired palbociclib resistance and almost no effect in ABEMA-R-MCF-7. This is consistent with the reported differences in the mechanism of action for palbociclib vs. abemaciclib, indicating that abemaciclib has unique single-agent activity leading to the induction of apoptosis, while palbociclib or ribociclib are mostly known to induce cell cycle arrest [11]. This difference in the mechanism of action has contributed to the

Fig. 4 Effect of ZEN-3694, abemaciclib A, or palbociclib B and the combinations on cell cycle protein markers and ERα in WT, PALBO-R (**A**, **B**), and ABEMA-R MCF-7 (**C**).

approval of abemaciclib as a single agent treatment for patients with metastatic HR+ breast cancer.

Interestingly, the combination of ZEN-3694 with abemaciclib led to a significant induction of apoptosis in all resistant and parental cell lines, contributing to ~50–60% of apoptosis in CDK4/6i-resistant cell lines and up to an 80–90% increase in cell death in wild type cells. Importantly, such increased levels of apoptosis upon treatment with the ZEN-3694+abemaciclib combination was also observed in the abemaciclib-resistant cell lines, indicating that ZEN-3694 can potentially re-sensitize abemaciclib resistant patients to CDK4/6 inhibitors, thereby reversing acquired resistance. Interestingly, the combination of ZEN-3694 with palbociclib did not have significant effects in either the PALBO-R or ABEMA-R-MCF-7 cell lines.

As abemaciclib is the only CDK4/6 inhibitor approved as a single agent in patients with metastatic breast cancer, these results emphasize the potential importance and clinical relevance of a ZEN-3694 and abemaciclib combination in future clinical development.

ZEN-3694 downregulates drivers of CDK4/6i resistance
Next, we wanted to illustrate how ZEN-3694 alone or in combination with CDK4/6 inhibitors can reverse the acquired CDK4/6i resistance, by inhibiting CDK4, CDK6, or CCND1 protein levels upregulated in the resistant cell lines. The acquired CDK4/6i resistance resulted in the reduced phospho-Rb levels which suggest deregulation of this part of the cell cycle pathway (Fig. 4). We observed that ZEN-3694 significantly inhibited the upregulated CDK6 protein levels in both PALBO-R (Fig. 4A, B) and ABEMA-R MCF-7 cell lines (Fig. 4C). This is a significant finding, as Yang et al. [9] described that shRNA-mediated attenuation of elevated CDK6 levels to CDK6 levels close to the parental cell line re-sensitized the resistant cell line to CDK4/6 inhibitors.

In addition, ERα protein levels were inhibited by ZEN-3694 in wild-type and CDK4/6i resistant cell lines. These results indicate that ZEN-3694 alone and in combination with CDK4/6 inhibitors reverse the acquired resistance to CDK4/6i by downregulating some of the major drivers involved in the regulation of the cell cycle and ER pathways.
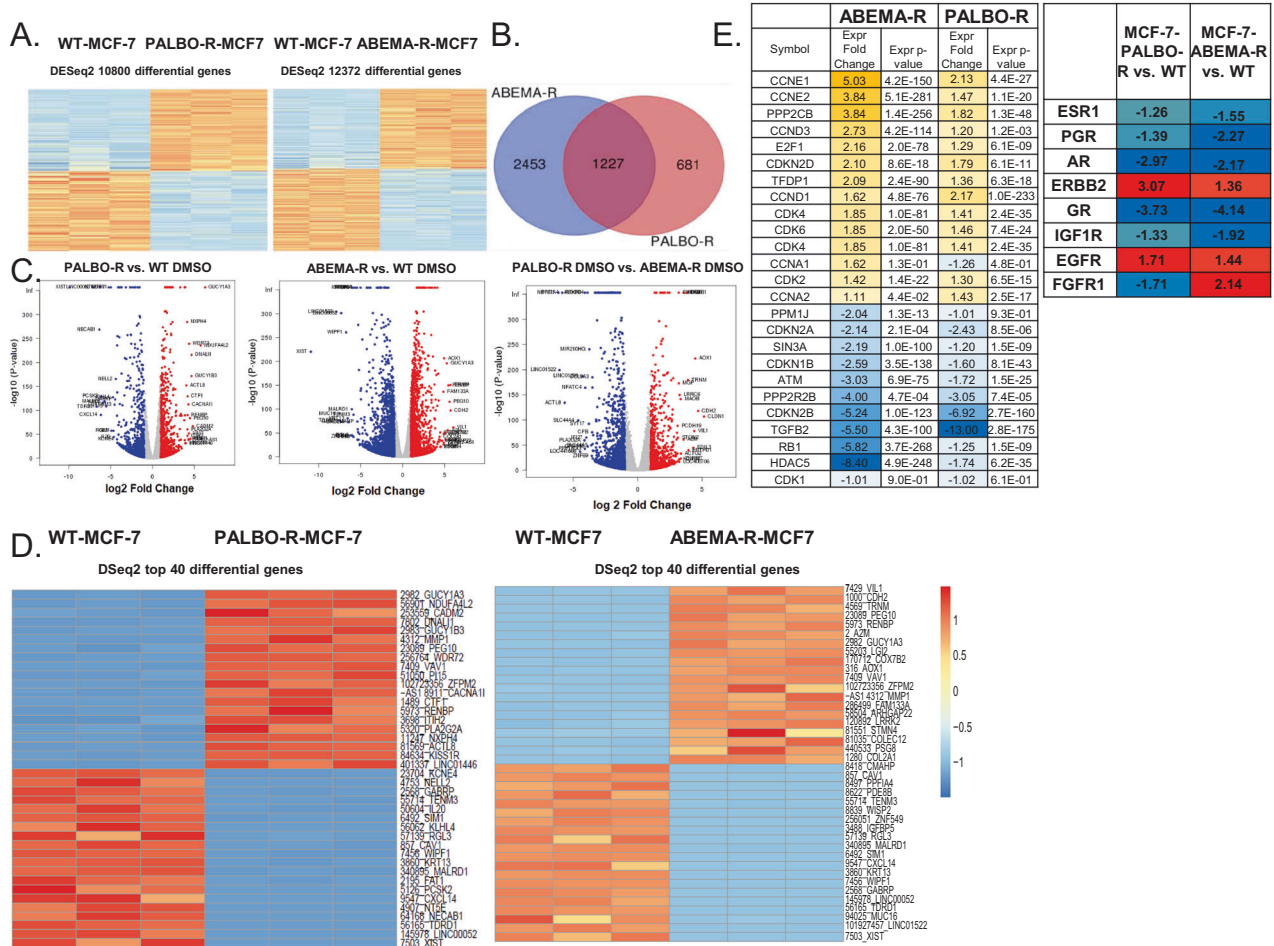
RNAseq analysis comparison of palbociclib and abemaciclib-resistant MCF-7 variants
In order to evaluate the mechanisms of palbociclib and abemaciclib resistance, we conducted RNAseq studies, comparing MCF-7-WT vs. palbociclib resistant and abemaciclib resistant cell lines (Fig. 5). RNA sequencing analysis highlighted major gene alterations in the PALBO-R and ABEMA-R MCF-7 variants in comparison to parental MCF-7 (Fig. 5A). Heatmaps of differentially expressed genes in PALBO-R-MCF-7 DMSO and ABEMA-R DMSO baselines vs. wild-type MCF-7 show that more than 10,000 differential genes were affected upon developing the resistance to both CDK4/6 inhibitors (Fig. 5A). 1,227 genes were shared between PALBO-R and ABEMA-R MCF-7 models, with ~680 and ~2400 gene changes strictly unique to PALBO-R or ABEMA-R-MCF-7 cell lines, respectively (Fig. 5B). Gene set enrichment analysis (GSEA) of the shared genes between the resistant cell lines revealed that the top ten pathways driving the resistance to CDK4/6 inhibitors involve estrogen receptor-related pathways as well as cell–cell signaling and signal transduction pathways.

Among the top 20 upregulated genes shared between PALBO-R and ABEMA-R resistant cell lines, GUCY1A3 and GUCY1B3 are known to play an important role in the eNOS signaling pathway, angiogenesis, and cancer progression [12] (Fig. 5C, D). VAV1 has been reported to be regulated by ER, and its overexpression is often associated with the increased levels of CCND1 and cell cycle progression [13, 14]. In addition, several mesenchymal markers like vimentin (VIM), fibronectin (FN1), and LCN2 were significantly elevated in the PALBO-R-MCF-7 cells, indicative of cells undergoing epithelial–mesenchymal transition (EMT) leading to the development of more aggressive breast cancer phenotypes. In both resistant cell lines, the following 6 genes were shared: VAV1, ZFPM2-AS1, MMP1, PEG10, GUCY1A3, and RENBP.

The tumor suppressor gene FAT1, a part of the Hippo signaling pathway, was among the most downregulated genes

A. WT-MCF-7 PALBO-R-MCF7   WT-MCF-7 ABEMA-R-MCF7

DESeq2 10800 differential genes   DESeq2 12372 differential genes

B.

ABEMA-R

2453   1227   681

PALBO-R

C.

PALBO-R vs. WT DMSO   ABEMA-R vs. WT DMSO   PALBO-R DMSO vs. ABEMA-R DMSO

E.

Symbol	ABEMA-R		PALBO-R	
	Expr Fold Change	Expr p-value	Expr Fold Change	Expr p-value
CCNE1	5.03	4.2E-150	2.13	4.4E-27
CCNE2	3.84	5.1E-281	1.47	1.1E-20
PPP2CB	3.84	1.4E-256	1.82	1.3E-48
CCND3	2.73	4.2E-114	1.20	1.2E-03
E2F1	2.16	2.0E-78	1.29	6.1E-09
CDKN2D	2.10	8.6E-18	1.79	6.1E-11
TFDP1	2.09	2.4E-90	1.36	6.3E-18
CCND1	1.62	4.8E-76	2.17	1.0E-233
CDK4	1.85	1.0E-81	1.41	2.4E-35
CDK6	1.85	2.0E-50	1.46	7.4E-24
CDK4	1.85	1.0E-81	1.41	2.4E-35
CCNA1	1.62	1.3E-01	-1.26	4.8E-01
CDK2	1.42	1.4E-22	1.30	6.5E-15
CCNA2	1.11	4.4E-02	1.43	2.5E-17
PPM1J	-2.04	1.3E-13	-1.01	9.3E-01
CDKN2A	-2.14	2.1E-04	-2.43	8.5E-06
SIN3A	-2.19	1.0E-100	-1.20	1.5E-09
CDKN1B	-2.59	3.5E-138	-1.60	8.1E-43
ATM	-3.03	6.9E-75	-1.72	1.5E-25
PPP2R2B	-4.00	4.7E-04	-3.05	7.4E-05
CDKN2B	-5.24	1.0E-123	-6.92	2.7E-160
TGFB2	-5.50	4.3E-100	-13.00	2.8E-175
RB1	-5.82	3.7E-268	-1.25	1.5E-09
HDAC5	-8.40	4.9E-248	-1.74	6.2E-35
CDK1	-1.01	9.0E-01	-1.02	6.1E-01

	MCF-7-PALBO-R vs. WT	MCF-7-ABEMA-R vs. WT
ESR1	-1.26	-1.55
PGR	-1.39	-2.27
AR	-2.97	-2.17
ERBB2	3.07	1.36
GR	-3.73	-4.14
IGF1R	-1.33	-1.92
EGFR	1.71	1.44
FGFR1	-1.71	2.14

D. WT-MCF-7   PALBO-R-MCF7   WT-MCF7   ABEMA-R-MCF7

DSeq2 top 40 differential genes   DSeq2 top 40 differential genes

Fig. 5 Comparison of palbociclib and abemaciclib-resistant cell lines using RNAseq analysis. A Heatmap **A.** and Venn diagram **B.** of differential genes in MCF-7 DMSO vs. PALBO-R or ABEMA-R DMSO. **C.** Volcano plot showing the gene differences/similarities between PALBO-R MCF-7 and ABEMA-R MCF-7 models (fold change ≥ 2 or ≤ -2-fold, $p \leq 0.01$). **D** Heatmaps of top 20 significant upregulated and 20 downregulated genes in PALBO-R and ABEMA-R cell lines are compared to MCF-7-WT. **E** Gene alterations involved in the cell cycle in PALBO-R and ABEMA-R-MCF-7 vs. WT. Receptor status in PALBO-R vs. ABEMA-R vs. WT MCF-7 ($p \leq 0.01$).

specific to the PALBO-R MCF-7 model. Downregulation of FAT1 has been previously linked to the promoting of CDK4/6i resistance through upregulation of CDK6 in breast cancer patients with poor prognoses [15].

As the cell cycle pathway is often affected upon developing resistance to the CDK4/6 inhibitors, we looked at whether the expression of the genes involved in the cell cycle regulation was also altered in the CDK4/6i resistance models. CCNE1/2, CCND1, and E2F1 were significantly upregulated in the ABEMA-R MCF-7 and to a lesser extent in PALBO-R MCF-7 cell lines (Fig. 5E). RB expression was significantly reduced in ABEMA-R MCF-7, but not in PALBO-R MCF-7.

In addition, we compared receptor status in PALBO-R vs. ABEMA-R cell lines as they are also known to be altered upon acquiring CDK4/6i resistance. GR, ER, and PR mRNA levels were significantly reduced in the resistant cell lines as compared to the parental MCF-7. The expression of EGFR and ERBB2 were elevated, consistent with Pancholi et al. describing the upregulation of several kinome players like EGFR as a tumor reprogramming mechanism in response to palbociclib resistance [16].

To elucidate the molecular mechanisms of CDK4/6i resistance further, we performed Ingenuity Pathway Analysis (IPA) of the RNAseq dataset, followed by comparative analysis of the canonical pathways significantly affected upon developing resistance to CDK4/6i (Table 1).

Our data suggest that resistance to palbociclib or abemaciclib occurred through a complex network of mechanisms involving the alteration of cell cycle regulation, intra-, and intercellular signaling, as well as immune response and growth factor signaling. The top pathways significantly upregulated upon developing palbociclib resistance included estrogen-mediated-S-phase entry, and cyclins and cell cycle regulation, immune response pathways (IL-8, IL-2, and PI3K signaling in B lymphocytes), and several related pathways involved in cell-cell adhesion such as ephrin receptor (Eph), Rac and integrin signaling by Rho GTPases, RhoA, and Gα12/13 signaling; all of these are known to play a significant role in cancer progression and invasion [15, 17].

The major pathways driving CDK4/6i resistance were similar between palbociclib-resistant vs. abemaciclib-resistant cell lines, but not identical. The shared pathways involved in CDK4/6i resistance included cell cycle regulation, estrogen-mediated-S-phase entry, IL-8, and integrin, Rac, and Tec kinase signaling. In addition, several alternative signaling pathways known to be drivers of cancer progression, invasion, and drug resistance were significantly elevated in both models, and including signaling by Rho family GTPases, VEGF, ErbB2/4, IGF-1, mTOR, and JNK signaling.

Some important differences between the mechanisms of acquired resistance to palbociclib vs. abemaciclib were observed. The significant upregulation of the eNOS, ephrin B, GNRH

Table 1. Comparison analysis of the pathways for PALBO-R and ABEMA-R MCF-7 cell lines vs. parental MCF-7.

Canonical pathways	PALBO-R MCF-7		ABEMA-R MCF-7	
	Z score	p Value	Z score	p Value
Ephrin receptor signaling	3.6	0.0	2.4	0.0
Signaling by Rho family GTPases	3.6	0.0	3.4	0.0
IL-8 signaling	3.5	0.0	3.0	0.0
G beta-gamma signaling	3.4	0.0	2.3	0.0
Rac signaling	3.3	0.0	3.1	0.1
Integrin signaling	3.3	0.0	3.7	0.1
Ovarian cancer signaling	3.3	0.0	3.9	0.0
Agrin interactions at the neuromuscular junction	3.3	0.0	2.4	0.0
B cell receptor signaling	3.3	0.0	2.8	0.0
Thrombin signaling	3.2	0.0	2.1	0.0
Cardiac hypertrophy signaling	3.1	0.0	0.2	0.0
Sphingosine-1-phosphate signaling	3.1	0.0	2.7	0.0
CCR3 signaling in eosinophils	3.0	0.0	1.8	0.0
PKCθ signaling in T lymphocytes	3.0	0.0	1.1	0.1
Synaptogenesis signaling pathway	2.9	0.0	0.1	0.0
RhoA signaling	2.9	0.0	2.7	0.1
Gα12/13 signaling	2.9	0.0	2.3	0.0
CXCR4 signaling	2.9	0.0	2.5	0.0
BMP signaling pathway	2.9	0.1	2.2	0.0
Antiproliferative role of somatostatin receptor 2	2.8	0.0	2.2	0.0
fMLP signaling in neutrophils	2.8	0.0	2.2	0.0
Cholesterol biosynthesis I	2.8	0.0	1.7	0.0
Cholesterol Biosynthesis III (via desmosterol)	2.8	0.0	1.7	0.0
nNOS signaling in neurons	2.8	0.0	1.1	0.0
Glioma signaling	2.8	0.0	2.2	0.0
Phospholipase C signaling	2.7	0.0	0.5	0.0
PI3K signaling in B lymphocytes	2.5	0.0	1.0	0.1
Estrogen-mediated S-phase entry	2.5	0.0	1.4	0.0
Adrenomedullin signaling pathway	2.5	0.0	1.3	0.0
VEGF signaling	2.4	0.0	3.0	0.1
PAK signaling	2.4	0.0	2.3	0.0
Glioblastoma multiforme signaling	2.4	0.0	1.8	0.0
Actin nucleation by ARP-WASP complex	2.4	0.0	3.7	0.0
GNRH signaling	2.4	0.0	0.7	0.0
CREB signaling in neurons	2.4	0.0	1.3	0.0
eNOS signaling	2.3	0.0	1.2	0.0
ErbB signaling	2.3	0.0	1.9	0.0
Non-small cell lung cancer signaling	2.2	0.0	2.4	0.0
Role of NFAT in cardiac hypertrophy	2.2	0.0	0.8	0.0
Ephrin B signaling	2.2	0.0	1.0	0.0
IGF-1 signaling	2.2	0.0	2.4	0.0
Role of NFAT in the regulation of the immune response	2.2	0.0	0.4	0.0
Huntington's disease signaling	2.2	0.0	1.6	0.0
Endocannabinoid developing neuron pathway	2.1	0.0	2.0	0.0
Renin-angiotensin signaling	2.1	0.0	1.4	0.0
Neuregulin signaling	2.1	0.0	1.6	0.0
Paxillin signaling	2.1	0.0	2.6	0.0
Gαq signaling	2.1	0.0	-0.2	0.0
Cholecystokinin/gastrin-mediated signaling	2.1	0.0	1.6	0.0
HGF signaling	2.1	0.0	1.1	0.0

Table 1 continued

Canonical pathways	PALBO-R MCF-7		ABEMA-R MCF-7	
	Z score	p Value	Z score	p Value
14-3-3-mediated signaling	2.1	0.0	1.8	0.0
Chemokine signaling	2.0	0.0	2.5	0.1
Opioid signaling pathway	2.0	0.0	1.4	0.0
HMGB1 signaling	2.0	0.0	1.3	0.1
IL-6 signaling	2.0	0.1	2.5	0.1
Apelin endothelial signaling pathway	2.0	0.0	2.0	0.0
Apelin cardiomyocyte signaling pathway	2.0	0.0	1.0	0.0
The NRF2-mediated oxidative stress response	2.0	0.0	1.0	0.0
SAPK/JNK signaling	2.0	0.0	0.2	0.0
Cyclins and cell cycle regulation	2.0	0.0	2.9	0.0
IL-3 signaling	2.0	0.0	1.7	0.0
VEGF family ligand–receptor interactions	2.0	0.0	1.3	0.0
Colorectal cancer metastasis signaling	1.9	0.0	2.5	0.0
Glioma invasiveness signaling	1.7	0.0	3.4	0.0
IL-2 signaling	1.7	0.0	1.9	0.0
STAT3 pathway	1.6	0.0	1.0	0.0
Cell cycle: G1/S checkpoint regulation	−2.7	0.0	−2.1	0.0
EIF2 signaling	−4.7	0.0	−1.8	0.0

signaling phospholipase C, and estrogen-mediated S-phase signaling, were only observed in the PALBO-R MCF-7 cell line. On the other hand, several pathways were considerably more affected in ABEMA-R-MCF-7 vs. PALB.O-R-MCF-7, including cell cycle regulation, VEGF, actin nucleation, and actin cytoskeleton signaling, IGF-1, IL-6, VEGF, actin nucleation by ARP–WASP complex, mTOR, and Gαq signaling.

RNAseq and IPA analysis of ZEN-3694, CDK4/6i and combination treatments of palbociclib and abemaciclib-resistant MCF-7 vs. parental cell lines

We then interrogated the mechanism of action of either ZEN-3694 alone or in combination with abemaciclib in CDK4/6i resistant vs. sensitive ER+ breast cancer models. RNAseq analysis revealed major gene alterations associated with these treatments. ZEN-3694 broadly regulated the gene expression of the parental and the resistant cell lines with more than 11,000 genes affected (Fig. 6). Abemaciclib or palbociclib single treatments led to significant gene alterations only in the parental MCF-7 cell line, with no significant gene changes observed in CDK4/6i resistant cell lines.

Next, the mechanisms of action of ZEN-3694 alone vs. in combination with CDK4/6 inhibitors were elucidated by performing IPA analysis of the RNAseq data and evaluating the key canonical pathways affected by these treatments. The majority of pathways upregulated in the PALBO-R MCF-7 cell line were strongly inhibited by ZEN-3694, including key pathways involved in tumorigenesis such as estrogen-mediated S-phase entry, STAT3, ILK, HMGB1, estrogen-dependent breast cancer signaling, as well as IL-7, IL-8, VEGF, and MAPK signaling (Fig. 6, Table S1).

Though ZEN-3694 alone had a significant impact on multiple pathways in the parental and the resistant cell lines, the effect of the ZEN-3694+abemaciclib combination was substantially more pronounced on pathways specifically involved in developing CDK4/6i resistance (Table 2, Table S2).

Notably, estrogen-dependent breast cancer signaling, IL-7 and IL-2 signaling, cyclins and cell cycle regulation, aryl hydrocarbon receptor, AMPK signaling, several key pathways involved in EMT and angiogenesis, known to play a key role in tumorigenesis of hormone-dependent ER+ breast cancer, were affected [17–21].

As we have described above, the resistance to abemaciclib or palbociclib occurs through a complex network of multiple mechanisms, including the deregulation of cell cycle or apoptosis signaling, the activation of several RTK pathways such as ErbBB, mTOR, VGEF, and STAT3, as well as an increase of several inflammation pathways such as IL-6, IL-8, and IL-2 all of which are strongly inhibited by ZEN-3694 in combination with abemaciclib. In addition, the ZEN-3694 + abemaciclib combination inhibits networks involved in cellular growth, proliferation, cellular development and intracellular signaling i.e., ILK, AMPK and PEDF, Ephrin Receptor, RhoGDI, signaling by Rho Family GTPases and others. The pathway analysis is shown above clearly demonstrates that the combination of ZEN-3694 and abemaciclib is a novel and effective therapeutic option in reversing CDK4/6i resistant breast cancer.

DISCUSSION

CDK4/6 inhibitors in combination with endocrine therapy were granted accelerated FDA approval and are now considered the gold standard for the treatment of metastatic ER-positive breast cancer. Despite these advancements, resistance to these therapies occurs over time and a more in-depth understanding of the mechanism of CDK4/6i resistance becomes imperative to drive the development of future clinically impactful targeted therapies. Several targeted combination therapies are being explored in clinical trials and include different PI3K, mTOR, AKT, and HDAC inhibitors, among others. For example, the PIK3CA inhibitor, alpeselib, in combination with fulvestrant has been recently approved for advanced ER+ breast cancer patients progressing on endocrine therapy or CDK4/6 inhibitors [22]. The AKT inhibitor, capivasertib, in a combination with fulvestrant has also shown some promising results in progression-free survival (10.3 vs. 4.8 months) in metastatic patients resistant to the standard of care therapies [23]. In addition, several clinical trials are currently
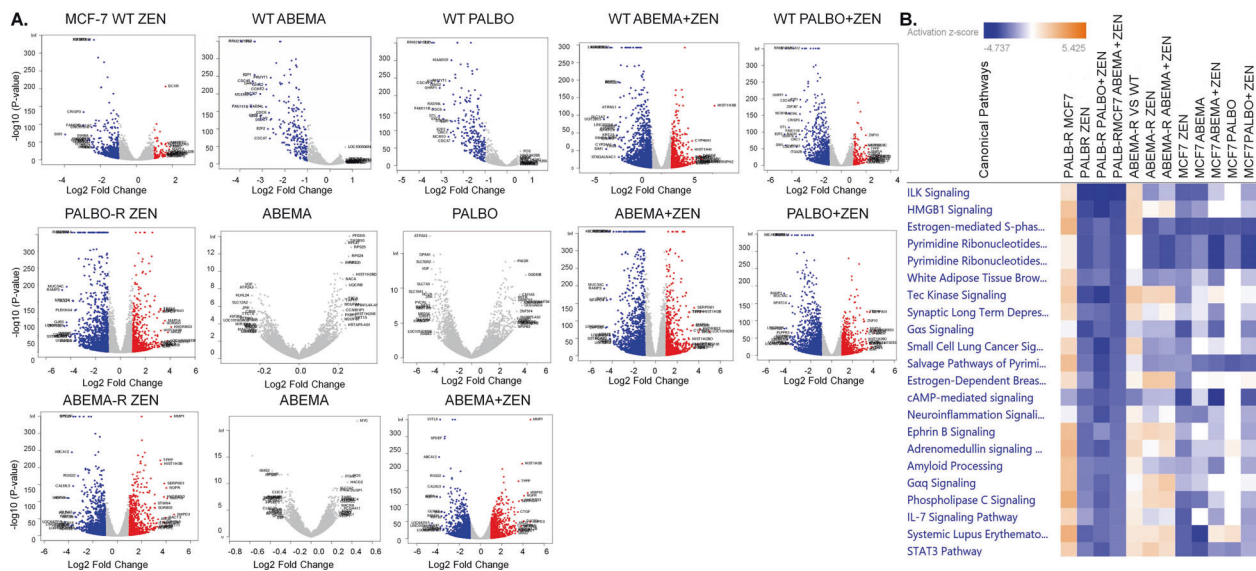
Fig. 6 RNAseq and IPA analysis of ZEN-3694, CDK4/6i and combination treatments in CDK4/6i-resistant vs. parental MCF7 cell lines. **A** Volcano plots of top gene changes with ZEN, abemaciclib, palbociclib, and combo treatments in WT-MCF-7, PALBO-R, and ABEMA-R cells. **B** Comparison analysis of all treatments of WT vs. CDK4/6i-R cells. Data were analyzed through the use of IPA® (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis).

ongoing evaluating triple combinations with CDK4/6 and PI3K pathway inhibitors together with endocrine therapy agents [3].

In this study, we have developed a panel of CDK4/6i resistant cell lines and explored the effects of BET inhibitor ZEN-3694 as a single agent and in combination with CDK4/6 inhibitors as a novel promising therapeutic strategy to combat the acquired resistance to CDK4/6i alone. We found that resistance to either palbociclib or abemaciclib was associated with the partial loss of ERα and the significant upregulation of CDK6 and CCND1, which is consistent with previous reports describing clinical resistance.

Based on our RNAseq studies and IPA analysis, we found that in addition to the upregulation of the cell cycle-related pathways, a network of mechanisms are implicated in developing CDK4/6 resistance which includes RTK pathways such as VEGF, ErbBB2/4, IGF-1, mTOR, and JNK signaling; inflammation-related pathways (IL-8, IL-2, PI3K signaling in B lymphocytes), and several pathways involved in cell-cell adhesion such as Ephrin receptor (Eph), Rac and Integrin signaling pathways, as well as signaling by Rho GTPases, RhoA, and Gα12/13.

Importantly, we demonstrate that ZEN-3694 treatments alone and to a greater extent in combination with abemaciclib leads to the reversal of the developed resistance through the down-regulation of key upregulated pathways, demonstrated by the significant inhibition of proliferation and synergistic increase of the apoptosis in our CDK4/6i sensitive and resistant ER+ breast cancer cell lines. Our results indicate that the ZEN-3694 + abemaciclib combination can effectively provide a new potential treatment option for treating patients with CDK4/6i-resistant breast cancer.

MATERIALS AND METHODS
Reagents
Abemaciclib and palbociclib were purchased from Selleck Chemicals (Houston, TX, USA). Cell cycle antibodies were obtained from Cell Signaling (Cell Cycle Regulation Antibody Sampler Kit #9932), ERα antibodies were purchased from Santa Cruz (sc-543, sc-8002). Tissue culture media and reagents were obtained from Thermofisher Scientific.

Establishment of the resistant cell lines
Breast cancer cell lines were obtained from ATCC. MCF-7, ZR-75-1, and HCC-1428 were maintained in 1640-RPMI media containing 10% FBS and penicillin/streptomycin. CAMA-1 cell line was maintained in DMEM-F12

supplemented 10% FBS/ penicillin/streptomycin/ plasmocin. CDK4/6i-resistant cell lines were obtained by continuous stepwise exposure to increasing concentrations of abemaciclib or palbociclib for 24 months until the resistance level was reached. The starting concentration of palbociclib was 0.1 µM, which was gradually increased to 10 µM and then further maintained. Abemaciclib-resistant cell line was also obtained by continuous exposure of increasing concentration of abemaciclib starting at 0.05 µM until 2 µM was reached and resistance was confirmed and maintained.

Cell viability
Breast cancer cell lines (MCF-7, Palbo-R-MCF-7, Abema-R MCF-7 cells) were plated at a density of 2500 or 7500 cells per well in 96 well flat bottom plates in 1640-RPMI media containing 10% FBS and penicillin/streptomycin, plasmocin and incubated for 24 h at 37 °C, 5% CO_2 after which media was replaced with 1640-RPMI containing 10% FBS with constant ratios of either ZEN-3694 or CDK4/6 inhibitors as single agents, or a combination of both drugs at four different concentrations (2X IC50, 1X IC50, 0.5X IC50, 0.25X IC50) in triplicates and incubated at 37 °C, 5% CO_2 for 7 days. The cells retreated as described above on the 3rd or 4th day. Triplicate wells were used for each concentration and wells containing only media with 0.1% DMSO were used as a control. To measure cell viability, 100 µl of a 1:100 dilution of GF-AFC substrate into the Assay Buffer (CellTiter Fluor Cell Viability Assay (Promega)) were added to each well and incubated at 37 °C, 5% CO_2 for an additional 30–90 min. Fluorescence at 380–400 nm Excitation/505 nm Emission was read in a fluorometer and the percentage of cell titer relative to DMSO-treated cells was calculated after correcting for background by subtracting the blank well's signal. IC50 values for single agents were calculated using the GraphPad Prism software. The presented data were obtained from multiple independent experiments are expressed as the means ± the standard deviations (SD). Quantification of synergy was done by calculating combination indices (CI) using the CalcuSyn software (Biosoft) based on the Chou-Talalay algorithm (Chou and Talalay, 1984), and averaging the CI values for the effective doses 50, 75, and 90. Statistical tests were performed using Prism 8 software (GraphPad, San Diego, CA, USA).

Flow cytometry
Breast cancer cell lines were seeded at 2.5×10^5 cells/2 mL in 6-well plates. The cells were treated after overnight adherence, retreated on the third or fourth day, then analyzed for apoptosis on the seventh day. Cells were stained with FITC Annexin V Apoptosis Detection Kit according to the manufacturer (BD Pharmingen) and immediately analyzed with a BD FACSCelesta flow cytometer. Data were analyzed and dot plots were made using FlowJo software. The reported data are ten independent repeats for

Table 2. Canonical pathways significantly affected by ZEN-3694 and abemaciclib combination.

Pathway category	Canonical pathways	PALBO-R ZEN-3694	PALBO-R MCF-7 ABEMA+ZEN-3694	PALBO-R MCF-7 DMSO
Cell cycle and cell cycle regulation, apoptosis	Estrogen-mediated S-phase entry	−2.89	−3.05	2.53
	Cyclins and cell cycle regulation	−1.46	−1.96	1.96
	Cell cycle regulation by BTG family proteins	−1.27	−1.67	1.27
	Estrogen-dependent breast cancer signaling	−1.79	−2.6	1.09
	Aryl hydrocarbon receptor signaling	−1.3	−1.85	1.48
	Role of CHK proteins in cell cycle checkpoint control	1.21	1.61	−0.28
Cellular growth, proliferation, and development	ILK signaling	−3.68	−3.97	1.05
	AMPK signaling	−1.31	−1.79	0.85
	Salvage pathways of pyrimidine ribonucleotides	−2.34	−2.65	2.2
	PEDF signaling	−1.35	−1.83	1.13
	GM-CSF signaling	−1.09	−1.8	2.4
	Pyridoxal 5′-phosphate salvage pathway	−1	−1.89	2.24
Cellular immune response/cytokine signaling	IL-7 signaling pathway	−1.57	−2.27	1.4
	IL-8 signaling	−1.89	−1.94	3.52
	IL-22 signaling	−1	−1.51	0.3
	IL-2 signaling	−0.94	−1.46	1.71
	Chemokine signaling	−1.62	−1.77	2.04
	Production of nitric oxide and reactive oxygen species in macrophages	−1.5	−1.75	0.13
	PI3K signaling in B Lymphocytes	−1.16	−1.73	2.53
Intracellular and second messenger signaling, cellular assembly and organization; cellular function and maintenance; cell morphology/cell-to-cell signaling	Gαq signaling	−2.19	−2.31	2.11
	Signaling by Rho family GTPases	−1.76	−2.02	3.6
	Phospholipase C signaling	−1.99	−2.29	2.73
	Dopamine-DARPP32 feedback in cAMP signaling	−1.54	−1.66	0.98
	Ephrin receptor signaling	−1.36	−1.6	3.64
	RhoGDI signaling	1.57	1.7	−1.29
Growth factor signaling/cell-to-cell signaling	Relaxin signaling	−1.22	−1.57	1.26
	GDNF family ligand–receptor interactions	−0.82	−1.51	1.04
Cell death and survival/cellular development	Glioblastoma multiforme signaling	−1.66	−2.18	2.41
	The antioxidant action of vitamin C	2.34	2.69	0
Cellular stress and injury	GP6 signaling pathway	−0.87	−2.02	0
	UVB-induced MAPK signaling	−0.89	−1.53	1.7
Cell morphology	Endothelin-1 signaling	−1.57	−1.84	1.08

WT-MCF7 and 8 independent repeats for ABEMA-R cell lines. *p* Values were calculated using a *t* test. Statistical tests were performed using Prism 8 software (GraphPad, San Diego, CA, USA).

Gene expression analysis
Breast cancer cells were trypsinized, counted, and plated in 96 well plates at a density of 50,000 cells per well in 100 μL of media containing RPMI-1640, 10% FBS, plasmocin, penicillin/streptomycin. After 24 h, the media was replaced with 100 μL of the media containing the serial dilutions of ZEN-3694 or DMSO at 0.1%. The cells were incubated for 6 or 24 h at 37 °C, with 5% CO_2. Immediately prior to harvesting the cells, 2× cell lysis solution was prepared from components of the mRNA Capture PLUS kit (Life

Technologies) according to the manufacturer's instructions. 60 μL of media was carefully removed and 45 μL of 2X Lysis buffer was added directly to each well and incubated for five min at room temperature to allow for complete cell lysis. The contents of the wells were mixed briefly, transferred to the mRNA Catcher PLUS plate, and incubated for 1 h at room temperature. Plates were washed three times with 100 μL of Wash Buffer (W15), eluted with 70 μL of Elution Buffer (E3) for 5 min at 68 °C in a polymerase chain reaction (PCR) machine, and immediately placed on ice. Real-time PCR reactions using the components of RNA UltraSense One-Step qRT-PCR System (Life Technologies, 11732927) were used along with Taqman primer probes and Cyclophilin A in a final volume of 8 μL per well master mix and 2 μL per well of RNA. One-step real-time PCR reactions were run on a ViiA™ 7 Real-Time PCR machine using standard conditions

and analyzed using Applied Biosystems software. Results were plotted as a percentage of the DMSO-treated control.

RNAseq

RNAseq samples were obtained according to the Active motif protocol.

Ingenuity Pathway Analysis (IPA) we evaluated the functional analysis of the driver genes–genes affected by somatic variants occurring in allele frequencies ≥25%—using the core analysis of IPA software (Qiagen, Hilden, Germany). We only considered the pathways with a score ≥20. Data were analyzed through the use of IPA (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis).

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AUTHOR CONTRIBUTIONS

RGP, CC, and OK conducted experiments, analyzed, and interpreted the data. OK and EvH conception and supervision of the study, wrote the paper. All authors corrected draft versions and approved the final version of the paper.

COMPETING INTERESTS

The authors are the employees and shareholders of Zenith Epigenetics.

ADDITIONAL INFORMATION

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