

20th of March 2019

Chuxia Deng, Ph. D.
Editor-in-Chief
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RE: Submission 32889s– Response Letter

Dear Dr. Deng,

Thank you for your email of March 1st, informing us of the reviewers' comments on our referenced manuscript. We have now modified the manuscript in accordance with the comments. We have included a marked-up copy of our manuscript file showing the changes we have made since the original submission. The changes are shown by using the "Track Changes" option in Microsoft Word. A clean copy of our revised manuscript that does not show our changes is also provided.

Reviewer 1:

- 1. The paper by Dr Bracht et colleagues is a very interesting research focusing on acquired resistance in EGFR-positive NSCLC. The authors investigated the role of combination of osimertinib plus pterostilbene a natural compound, that presents activity abrogating Src and STAT3 activation. The paper is extremely well written and argued and it is possible to consider it suitable for publication in the present***

Our reply: We highly appreciate the reviewer for his positive feedback on our work

Reviewer 2:

The authors examined treatment efficacy of osimertinib plus pterostilbene (a compound found in blueberries) in lung cancer cell lines with EGFR mutations. The authors found that pterostilbene monotherapy did not inhibit examined molecules (intracellular signaling molecules plus cMET) significantly, but the combination of osimertinib plus pterostilbene was synergistic. Although these phenomenon looks interesting, the mechanism for their observation is poorly understood, therefore the reviewer recommend additional experimtnets.

Major comments

- 1. The authors should perform the combination of EGFR siRNA inhibition plus pterostilbene to check the off-target effects of osimertinib.***

Our reply: We thank the reviewer for his suggestion, that indeed could shade more light on the mechanisms of action of osimertinib. However, we believe that this is out of the scope of the present manuscript. We and others have previously shown that osimertinib alone induces the activation of signaling nodes including Src, STAT3 or YAP1, as well as the expression of RTKs and non-RTKs (see Karachaliou et al EBioMedicine 2018; 29: 112-27, Chaib, et al J. Natl. Cancer Inst. 109 (9); and Ichihara et al Cancer Res. 77 (11), 2990–3000.). So, the scope of this manuscript was to see whether a natural compound can inverse this phenomena and subsequently prevent or delay resistance to osimertinib. Silencing of EGFR can mechanistically show whether our findings are related to the inhibition of the pathway in general or are drug related and we will explore in the future.

- 2. *The percentage of surviving cells after long time culture (~2 weeks or longer) should be determined for DMSO, osimertinib alone, pterostilbene alone, and the combination. The results of such experiment may suggest that the combination may have longer PFS in patients.***

Our reply: We fully agree with the comment of the reviewer. Considering that cell cultures or even in vivo models are never 100% representative of what may happen out in the clinic, we are running a single center proof of concept study with the combination of pterostilbene with EGFR inhibitors for EGFR-mutant patients and we expect to have soon some indicative findings.

- 3. *The authors should also test these combinations in vivo, since the mechanism of action is still unclear.***

Our reply: This is indeed a weakness of our study. We did not continue with the in vivo studies for the following reason:

- Although we show a synergism of pterostilbene with osimertinib in cell culture, our western blot experiments did not confirm this synergism at the protein level, compared to other combinations that we have tried (like TPCA1 and TPX-0005; see see Karachaliou et al EBioMedicine 2018; 29: 112-27, Chaib, et al J. Natl. Cancer Inst. 109 (9)). If any positive findings come out from our ongoing POC clinical study, we will go back and expand our experiments to this end.

- 4. *pMET should be tested by WB, if the authors want to say that the combination may work through MET. No data for AXL, although AXL is in the figure.***

Our reply: The reviewer again is very correct with his comment. For this reason we have now evaluated the phosphorylation of MET under different treatment conditions using western blot. We also added AXL and phosphorylated AXL in both western blots (Pterostilbene increasing doses, and combined Osimertinib and Pterostilbene treatment). We can see an upregulation of pMET with pterostilbene in both PC9 and H1975 cell lines. We have adapted figures 2 and 4 in the main manuscript.

Osimertinib and pterostilbene in EGFR-mutation-positive non-small cell lung cancer (NSCLC).

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ABSTRACT

Monotherapy with epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) still leads to incomplete responses in most EGFR-mutation positive non-small cell lung cancer (NSCLC) patients, often due to acquired resistance through activation of parallel compensatory pathways. We have previously shown that co-targeting EGFR, signal transducer and activator of transcription 3 (STAT3), and Src-yes-associated protein 1 (YAP1) was highly synergistic in vitro and in vivo. In the present study, we treated EGFR-mutation positive cell lines with the combination of osimertinib plus a natural compound, pterostilbene, which has been reported to abrogate Src and STAT3 activation.

Methods: Cell viability assays and immunoblotting were performed to reveal the mechanisms of action of pterostilbene, osimertinib and pterostilbene plus osimertinib in five EGFR-mutation positive NSCLC and one triple negative breast cancer (TNBC) cell lines.

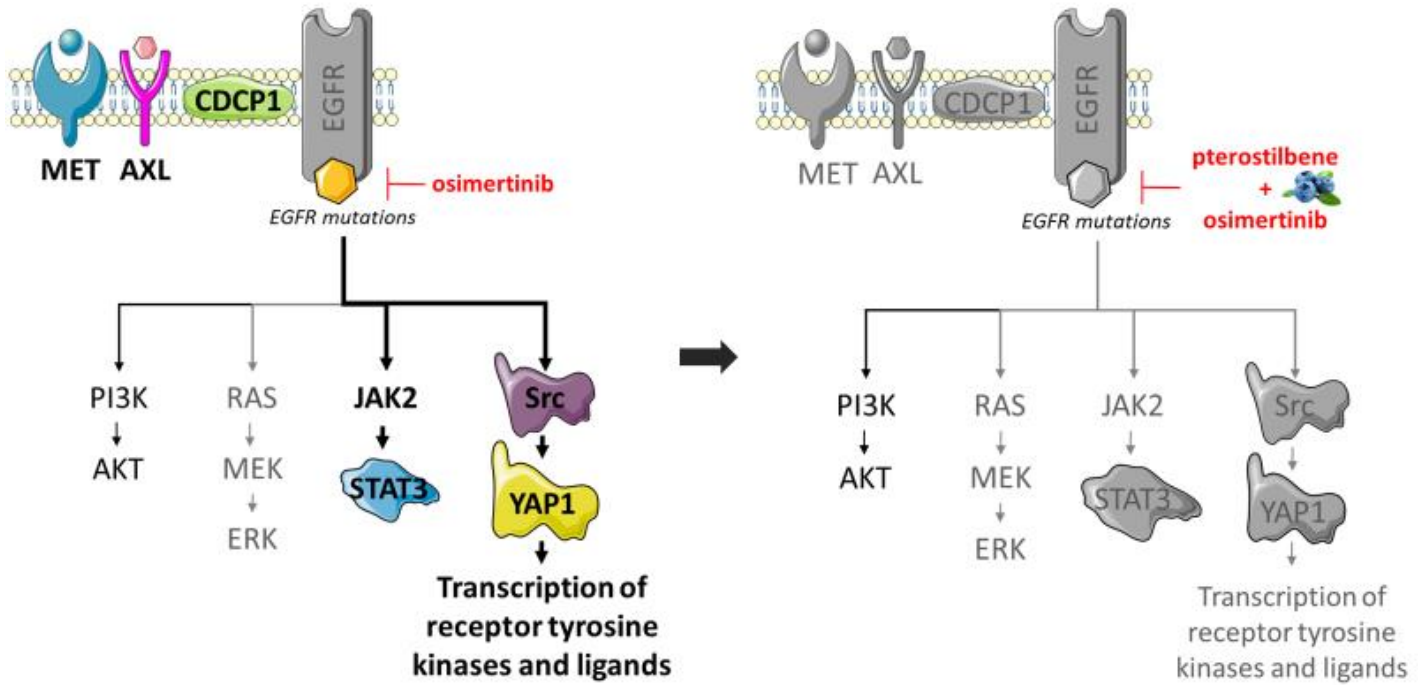
Results: Osimertinib plus pterostilbene yielded synergistic effects in all EGFR-mutation positive NSCLC cell lines investigated. Surprisingly, pterostilbene alone did not inhibit, nor downregulate Src phosphorylation in the EGFR-mutation positive NSCLC cell lines or the TNBC cell line, MDA-MB-231. However, the double combination of osimertinib plus pterostilbene reversed the osimertinib-induced STAT3, YAP1, and CUB domain-containing protein-1 (CDCP1) phosphorylation and slightly suppressed Src phosphorylation in PC9 and H1975 cells.

Conclusion: The results of this study indicate that pterostilbene may be used to abrogate the activated resistance pathways of single osimertinib treatment in EGFR-mutation positive NSCLC. Future studies should focus on in vivo translation and confirmation of these results.

Keywords: Pterostilbene, NSCLC, osimertinib, resistance.

GRAPHICAL ABSTRACT:

Pterostilbene to abrogate the activated resistance pathways of single osimertinib treatment



INTRODUCTION

Epidermal growth factor receptor (EGFR) mutations in non-small cell lung cancer (NSCLC) patients were discovered in 2004 [1]. To date, monotherapy with EGFR tyrosine kinase inhibitors (TKIs) still leads to incomplete responses in 95% of patients [2], often due to intrinsic or acquired resistance. Relevant signaling network- and crosstalk changes after EGFR blockade are underappreciated, including hyperactivation of signal transducer and activator of transcription 3 (STAT3) [3-7]. Ongoing and previously published research indicates that gefitinib, afatinib and osimertinib TKI treatments are unable to inhibit STAT3 activation, and lead to parallel compensatory activation of the Src-yes-associated protein 1(YAP1) signaling pathway [8-10]. We have previously shown that co-targeting EGFR, STAT3 and Src-YAP1 was highly synergistic in vitro and in vivo. We also found that several receptor tyrosine kinases (RTKs) and non-RTKs are upregulated at baseline or after treatment with gefitinib or osimertinib, limiting their therapeutic efficacy [8-11].

The genetic or pharmacologic inhibition of Src family kinases (SFKs) or YAP1 diminishes the phosphorylation of the RTK AXL and the transmembrane protein CUB domain-containing protein-1 (CDCP1) [10]. When overexpressed, both of these proteins are related to worse survival outcomes in patients treated with single EGFR TKIs. The combination of EGFR TKIs with a multikinase inhibitor, that inhibits janus kinase 2 (JAK2), Src and focal adhesion kinase (FAK), abrogates not only STAT3, but also YAP1 and SFKs activation and downregulates AXL and CDCP1 expression [10].

Pterostilbene (3,5-dimethoxy-4 β -hydroxy-trans-stilbene) is a stilbene of the family of phytoalexin compounds, found in blueberries and *Pterocarpus marsupium* (PM) heartwood. It is structurally similar to resveratrol, a compound found in red wine that has comparable antioxidant, anti-inflammatory, and anti-carcinogenic properties. Due to the presence of two methoxyl groups, pterostilbene has increased lipophilic and oral absorption and therefore increased bioavailability compared to resveratrol [12]. It has been shown that pterostilbene has apoptotic and anti-proliferative effects in solid tumors [13], including EGFR-mutation positive NSCLC [14]. In triple negative breast cancer (TNBC), pterostilbene abolished the activation of Src, FAK, paxillin and STAT3. Moreover, by altering mainly the Src-mediated signaling pathway

pterostilbene suppressed the metastatic potential of TNBC cells. It was also found to decrease the levels of mesenchymal markers, amongst which MET [15]. Pterostilbene also causes endoplasmic reticulum (ER) stress and consequently leads to apoptosis [14]. In addition, pterostilbene was shown to be safe in patients, even at high doses [16-18]. Henceforth, we posit that the combination of pterostilbene plus an EGFR TKI could substantially improve the outcome of single EGFR TKIs in EGFR-mutation positive NSCLC (**Figure 1**). In this study we explored whether pterostilbene inhibits compensatory osimertinib-induced signaling pathways, and if the combination can optimize the upfront therapy of EGFR-mutation positive NSCLC cells.

METHODS

Chemicals and reagents

Human lung adenocarcinoma PC-9 cells, harboring EGFR exon 19 deletion and H1975 cells, harboring EGFR exon 21 L858R mutation were provided by F. Hoffmann-La Roche Ltd. (Basel, Switzerland), and by Dr. Mayumi Ono, (Kyushu University, Fukuoka, Japan), respectively. EGFR exon 19 deletion positive HCC4006 and HCC827 cells were purchased from the American Type Culture Collection (ATCC). The H1975 cell line, harboring both EGFR exon 21 L858R and resistant T790M mutation as well as the TNBC MBA-MB-231 cell line were purchased from ATCC. All cell lines were maintained in RPMI (Roswell Park Memorial Institute medium) 1640 supplemented with 1% penicillin/streptomycin/glutamine (Gibco) and 10% fetal bovine serum (FBS; Gibco) in a 5% CO₂ 37°C cell culture incubator and routinely evaluated for mycoplasma contamination.

The primarily used pterostilbene compound (hereafter referred to as pterostilbene¹) was purchased from Amazon.com, Inc. (Catalog: B00BFUJ04Q), the second pterostilbene compound (hereafter referred to as pterostilbene²) was bought from Sigma Aldrich/Merck KGaA, (Darmstadt, Germany, Catalog: P1499-10MG) and osimertinib was bought from Selleck Chemicals (Houston, TX, U.S.). Drugs were prepared in dimethylsulfoxide (DMSO) at a concentration of 10-100 mM stock solutions and stored at -20°C. Further dilutions were made in culture medium to final concentration before use. All antibodies used in our study, including dilution and company catalog number can be found in **Table 1**.

Cell viability assay

Cells were seeded in 96-well plates at the following densities: 1×10^3 (PC9 and H1975) 1.5×10^3 (HCC4006, HCC827 and 11-18), and incubated for 24h, as previously described [8]. Cell viability was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma Aldrich, St Louis, MO, U.S). Cells were treated with serial dilutions of the drugs. For the half maximal inhibitory concentration (IC_{50}) determination, MTT viability assays were performed using pterostilbene doses ranging from 0-150 μ M. To determine the combined effect of pterostilbene and osimertinib, drug doses in viability assays were as follows: H1975 and HCC4006 cells were treated with osimertinib ranging from 0-90 nM, and pterostilbene ranging from 0-180 μ M, or with the combination of both. PC9 and HCC827 cells were treated with osimertinib ranging from 0-120 nM, and pterostilbene ranging from 0-120 μ M, or with the combination of both. 11-18 was treated with osimertinib ranging from 0-330 nM, and pterostilbene ranging from 0-120 μ M, or with the combination of both. After 72 h of treatment incubation, 0.5 mg/ml of MTT was added to the medium in the wells for 2 h at 37 °C and formazan crystals in viable cells were solubilized with 100 μ l DMSO and spectrophotometrically quantified using a microplate reader (Varioskan Flash; Thermo Fisher Scientific, Waltham, MA, U.S) at 565 nm of absorbance. Fractional survival was then calculated as percentage to control cells. Data of combined drug effects were subsequently analyzed by the Chou and Talalay method [19, 20]. Combination Index (CI) values <1 , $=1$ and >1 indicated synergism, additive effect and antagonism, respectively. All experiments were performed in biological triplicates.

Western blotting

For immunoblotting experiments, 1.5 million cells were seeded in T75 flasks (Starstedt, Newton, U.S). The next day, PC9, H1975 and MDA-MB-231 cells were either treated with increasing doses of pterostilbene (10, 20 and 50 μ M), or with 20 nM osimertinib, 10 μ M pterostilbene and a combination of both. Untreated cells received an equivalent dose of vehicle (DMSO). After 24 h cells were washed with cold PBS and re-suspended in ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris- hydrochloric acid in pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 150 mM sodium chloride, 1 mM ethylenediaminetetraacetic acid, 1 mM

sodium vanadate and 50 mM sodium fluoride) containing protease inhibitor mixture (Roche). Following cell lysis by sonication and centrifugation at 18620x g for 10 min at 4 °C, the resulting supernatant was collected as the total cell lysate. Briefly, the lysates containing 45 µg proteins were electrophoresed on 10% SDS-polyacrylamide gels (Life Technologies, Carlsbad, CA, U.S) and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad laboratories Inc., Hercules, CA, U.S). Membranes were blocked in Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE, U.S). All target proteins were immunoblotted with appropriate primary and horseradish peroxidase (HRP)-conjugated secondary antibodies. Chemiluminescent (HRP-conjugated) bands were detected in a ChemiDoc MP Imaging System (Bio-Rad laboratories Inc.). β -actin was used as an internal control to confirm equal gel loading. Experiments were performed in biological triplicates with similar results, and representative blots were shown.

RESULTS

Sensitivity of EGFR-mutation positive cells to pterostilbene and the combination of osimertinib plus pterostilbene.

To confirm the effects of pterostilbene on cell growth, cell viability assays were performed in 5 different EGFR-mutation positive NSCLC cell lines: PC9, H1975, HCC827, HCC4006 and 11-18. After establishing the half maximal inhibitory concentration (IC_{50}) of pterostilbene¹ in all cell lines (**Figure 2A**), it could be concluded that single pterostilbene¹ treatment was not able to induce anti-proliferative effects in these cell lines, with IC_{50} s ranging from 23.8 to 40.7 µM. However, when combining pterostilbene¹ with the third-generation TKI osimertinib (of which IC_{50} s were previously determined, and can also be found in **Figure 2A**), we obtained synergistic results in all EGFR-mutation positive NSCLC cell lines investigated, with a combination index (CI) ranging from 0.63 to 0.70 (**Figure 2B**). These results indicate a synergistic interaction between pterostilbene¹ and osimertinib in these cell lines.

Correlation between signaling nodes and cellular responses to pterostilbene or to the combination of osimertinib plus pterostilbene.

To determine which signaling nodes are being abrogated by pterostilbene¹ treatment, PC9 and H1975 cells were treated with different concentrations of pterostilbene¹ for 24h. Cell lysates were consequently used for immunoblotting and revealed different mechanisms of action of pterostilbene¹ on (non-)RTKs, as can be seen in **Figure 2C**. An increase of ERK, CDCP1, STAT3 and Src phosphorylation was observed upon different concentrations of pterostilbene¹ treatment. In contrast, both total and phosphorylated YAP1 were inhibited by pterostilbene¹ at higher concentrations. It can also be seen that phosphorylation of Src, YAP1, pCDCP1 and pAXL are correlated, indicating an interrelationship between these signaling nodes as previously described [10]. The increase in Src and MET phosphorylation compared to baseline after 24h treatment with 10 or 20 μ M pterostilbene¹ is opposite from previously published results, in which pterostilbene was reported to be a Src and MET inhibitor in TNBC [15].

To explore these contradictory results, the activity of pterostilbene¹ in the MBA-MB-231 TNBC cell line was determined. Cells were treated with 0 or 10 μ M pterostilbene¹ for 24 h, and Src activation was explored. Surprisingly, instead of inhibiting Src phosphorylation, pterostilbene¹ caused an induction of Src phosphorylation (**Figure 3A**). To confirm this, we purchased pterostilbene from a different supplier (pterostilbene²), and cell viability experiments were performed to compare the two drugs. In **Figure 3B** it can be seen that the two pterostilbene compounds have nearly identical anti-proliferative effects in two EGFR-mutation positive NSCLC cell lines. To verify these results in the TNBC cell line, MBA-MB-231 cells were treated with both pterostilbene compounds. The cell lysates were consequently used for immunoblotting, and again revealed similar mechanisms of action, as can be seen in **Figure 3C**. Both compounds clearly induce Src phosphorylation.

Although in our study pterostilbene was not able to inhibit Src phosphorylation, its effects on YAP1, Src, CDCP1 and AXL phosphorylation in EGFR-mutation positive cells led us to explore the combination of osimertinib plus pterostilbene¹. As we have previously reported, osimertinib alone attenuated ERK activation in both cell lines, but induced CDCP1 and Src activation in PC9 cells, and STAT3, YAP1 and AXL activation in both PC9 and H1975 cells (**Figure 4A**). In contrast, STAT3, YAP1 and

AXL phosphorylation are completely abrogated when treated with single pterostilbene¹. MET phosphorylation is slightly activated upon single pterostilbene¹ treatment in the PC9 cell line. While in both cell lines CDCP1 activation is inhibited compared to baseline levels, Src phosphorylation is not abrogated with single pterostilbene¹ treatment. Osimertinib plus pterostilbene¹ reversed the osimertinib-induced STAT3 and YAP1 phosphorylation, abolished CDCP1 and AXL activation and decreased Src phosphorylation. In the PC9 cell line, a restoration of MET phosphorylation levels to baseline was also observed with the double combination. The above results indicate that osimertinib treatment activates parallel compensatory pathways, which can be abrogated by combined osimertinib plus pterostilbene treatment.

DISCUSSION

EGFR-mutation-positive NSCLC cells co-express RTKs and non-RTKs, especially, Src, YES and FAK, which cannot be inhibited by single EGFR inhibitors, including the third-generation EGFR TKI osimertinib [8-10, 21-23]. EGFR, SFK and FAK concomitant inhibition enhances osimertinib activity and suppresses resistance [21-23]. We have confirmed our previous findings that the third generation TKI osimertinib instead of inhibiting, induces STAT3 and YAP1 (PC9 and H1975) and Src and CDCP1 phosphorylation (only PC9) in the PC9 and H1975 EGFR-mutation positive NSCLC cell lines [8-10].

Pterostilbene is a natural nutrient from blueberries that can be safely used in high doses [16-18]. Recent studies suggest that pterostilbene modulates the hallmarks of aging, like oxidative damage, inflammation, telomere attrition and cell senescence [24]. Interestingly, the inhibition of oxidative phosphorylation prevents the development of osimertinib resistance in EGFR-mutation positive NSCLC [25]. The anticancer potential of pterostilbene has been demonstrated in several tumor models, including lung cancer [14, 26] and TNBC [27]. In the present work, although we were not able to reconfirm the inhibitory effect of pterostilbene alone on Src and MET activation [15], or other downstream components (**Figure 2C**), the combination of osimertinib plus pterostilbene was synergistic in five-, and downregulated the activation of STAT3, Src-YAP1 and CDCP1 in two EGFR-mutation positive NSCLC cell lines (**Figure 4B**).

Pterostilbene was shown to modulate tumor-associated macrophages (TAMs), by modulating MUC1.²⁸ High MUC1 mRNA expression is correlated with poorer overall survival, and pterostilbene-based inhibition subverted the tumor-microenvironment to an anti-tumoral state by reducing stemness induction of TAMs. Therefore, MUC1 expression levels may predict which patients can benefit from pterostilbene treatment. Another important piece of information is that constitutively active STAT3 increases MUC1 expression levels on both the mRNA and protein level, explaining our synergistic in vitro results.²⁹ Therefore, further investigation in terms of feasibility and in vivo experiments should be carried out.

We are currently performing a proof of concept single-center clinical study, based on the model depicted in **Figure 4B**. This study will assess if pterostilbene is an economical but highly hopeful approach to increase the antitumoral activity of EGFR inhibitors in EGFR-mutation positive NSCLC patients, and should clarify whether this combined treatment strategy can be safely incorporated into routine clinical practice.

ABBREVIATIONS

ATCC: american type culture collection; CDCP1: cub domain-containing protein-1; CI: combination index; DMSO: dimethylsulfoxide; EGFR: epidermal growth factor receptor; ER: endoplasmic reticulum; FAK: focal adhesion kinase; FBS: fetal bovine serum; HRP: horseradish peroxidase; IC50: the half maximal inhibitory concentration; JAK2: janus kinase 2; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NSCLC: non-small cell lung cancer; PM: pterocarpus marsupium; PVDF: polyvinylidene difluoride; RIPA: radioimmunoprecipitation assay; RTKs: receptor tyrosine kinases; SDS: sodium dodecyl sulfate; SFKs: src family kinases; STAT3: signal transducer and activator of transcription 3; TAMs: tumor-associated macrophages; TKIs: tyrosine kinase inhibitors; TNBC: triple negative breast cancer; YAP1: src-yes-associated protein 1; 95%CI: 95% confidence interval.

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COMPETING INTERESTS

The authors have declared that no competing interest exists.

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TABLES

Table 1. Used antibodies, including dilutions and catalog numbers.

Western Blotting Primary Antibody	Dilution	Company and Catalog number
Rabbit anti-AXL	1:1000	Cell Signaling (#8661)
Rabbit anti-Phospho-AXL (Y779)	1:200	R&D Systems (#AF2228)
Mouse anti-Beta-actin	1:5000	Sigma Aldrich (#A5441)
Rabbit anti-CDCP1	1:1000	Cell Signaling (#4115)
Rabbit anti-Phospho-CDCP1 (Y707)	1:1000	Cell Signaling (#13111)
Rabbit anti-ERK1/2	1:1000	Cell Signaling (#9102)
Rabbit anti-Phospho-ERK1/2 (T202/Y204)	1:1000	Cell Signaling (#9101)
Rabbit anti-MET	1:1000	Cell Signaling (#8198)
Rabbit anti-Phospho-MET (Y1234/1235)	1:500	Cell Signaling (#3077)
Rabbit anti-Src	1:1000	Cell Signaling (#2109)
Rabbit anti-Phospho-Src Family (Y416)	1:1000	Cell Signaling (#6943)
Mouse anti-STAT3	1:1000	Cell Signaling (#9139)
Rabbit anti-Phospho-STAT3 (Y705)	1:1000	Cell Signaling (#9145)
Mouse anti-YAP1	1:1000	Cell Signaling (#12395)
Rabbit anti-Phospho-YAP1 (Y357)	1:1000	Abcam (#ab62751)
HRP-linked goat anti-rabbit (from donkey)	1:5000	GE Healthcare Life Sciences (NA934-1ML)
HRP-linked goat anti-mouse (from sheep)	1:5000	GE Healthcare Life Sciences (NA931-1ML)

FIGURES

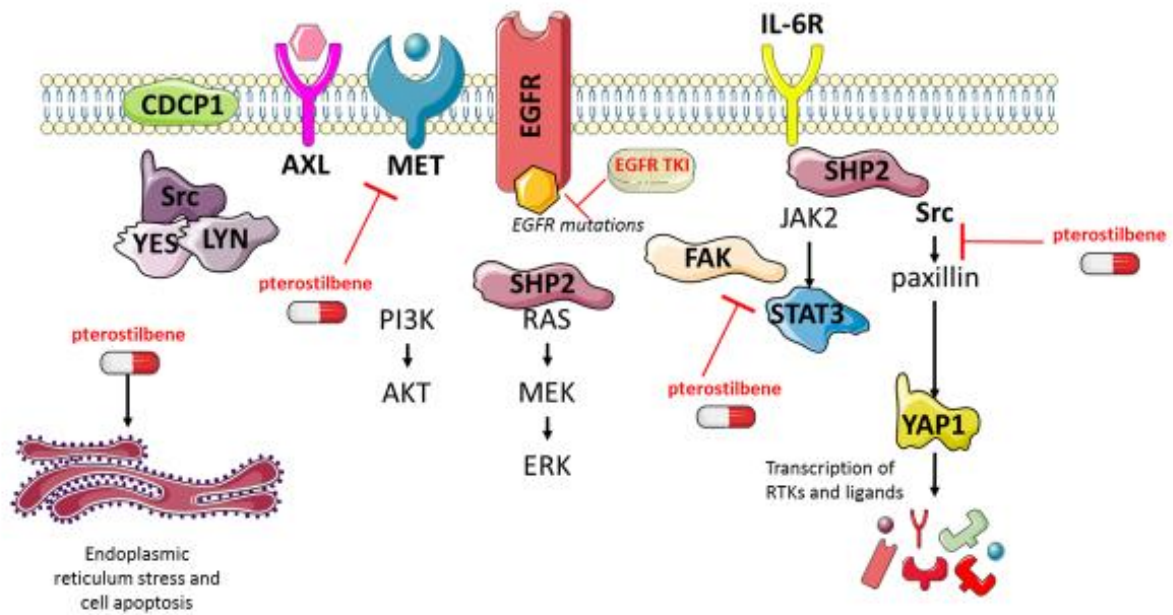


Figure 1. The effects of pterostilbene¹ and EGFR TKIs on RTKs and downstream components.

EGFR TKIs block signaling of the EGFR receptor and its downstream pathways. Previous research has shown that this process causes hyper-activation of compensatory pathways, such as STAT3 and Src-YAP1. Consequently, this leads to an up-regulation of RTKs and non-RTKs (e.g. MET, CDCP1), and therapy resistance. Pterostilbene has been shown to inhibit pathways involved in TKI resistance, such as STAT3 and Src-YAP1. Moreover, it was able to inhibit MET, and cause ER-stress and apoptosis. CDCP1: CUB domain-containing protein-1; EGFR: epidermal growth factor receptor; ER: endoplasmic reticulum; FAK: focal adhesion kinase; JAK2: janus kinase 2; RTKs: receptor tyrosine kinases; STAT3: signal transducer and activator of transcription 3; TKIs: tyrosine kinase inhibitors; YAP1: Src-yes-associated protein 1.

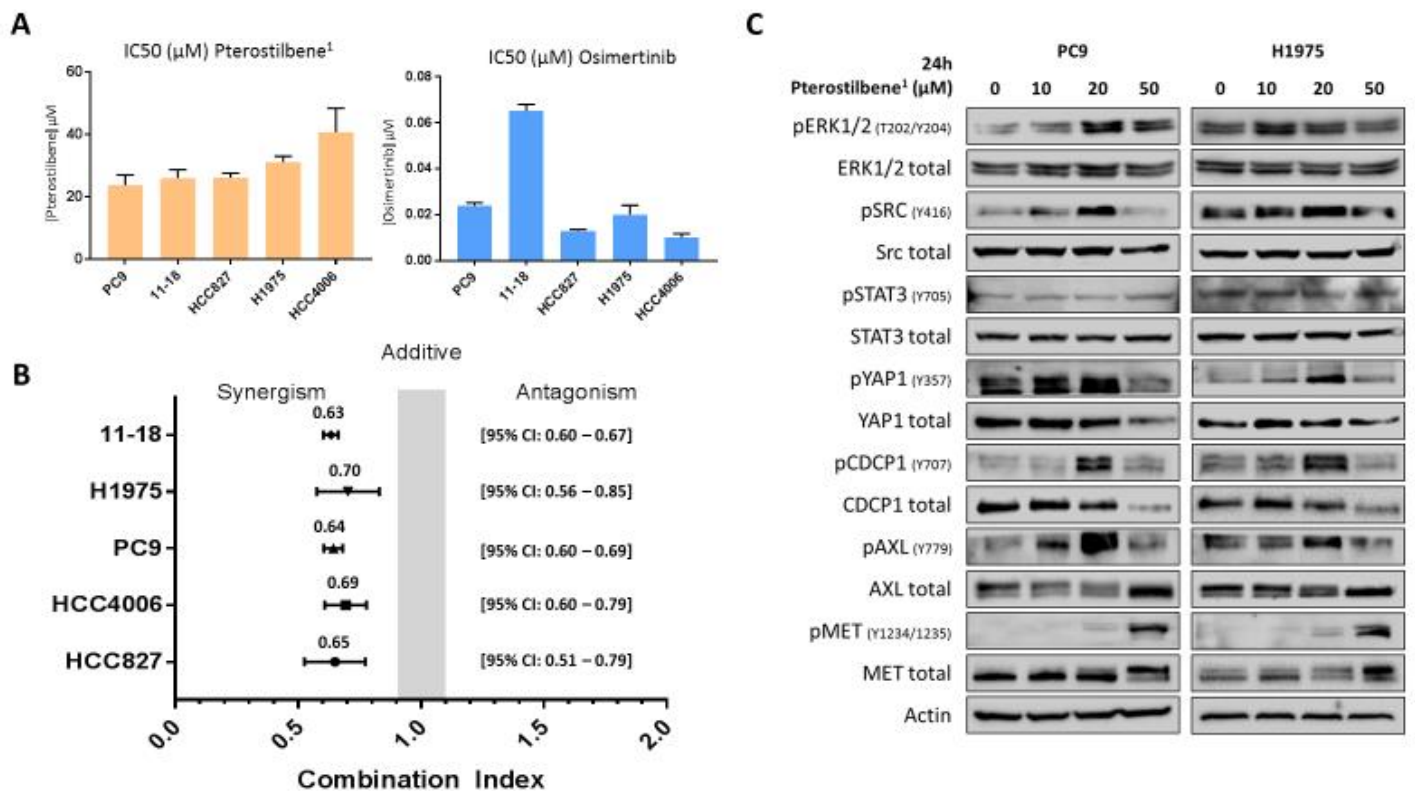


Figure 2. The effects of single and combined osimertinib and pterostilbene¹ treatment.

A: MTT cell viability assays were performed in 5 different EGFR-mutation positive NSCLC cell lines (PC9, H1975, 11-18, HCC4006 and HCC827), to determine the effects of pterostilbene¹ on cell viability, and to determine the IC₅₀ in each cell line. Results of previously performed experiments with osimertinib in the same cell lines are also shown. Experiments were performed in biological triplicates, and the average and standard deviations are shown. **B:** In all cell lines MTT cell viability assays were performed to explore the effect of combined osimertinib and pterostilbene¹ treatment. Combination indexes were calculated based on the Chou and Talalay method, and values <1, =1 and >1 indicate synergism, additive effect and antagonism, respectively. Experiments were performed in biological triplicates, and the averages and 95% CIs are shown. **C:** PC9 and H1975 cells were treated with different concentrations of pterostilbene¹ for 24 h. Untreated cells received an equivalent dose of vehicle (DMSO). Cell lysates were used for immunoblotting, and the effect of pterostilbene¹ treatment on downstream components was explored. Experiments were performed in biological triplicates with similar results, and a representative blot is shown. DMSO: dimethylsulfoxide; EGFR: epidermal growth factor receptor; IC₅₀: the half maximal inhibitory concentration; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NSCLC: non-small cell lung cancer; 95%CI: 95% confidence interval.

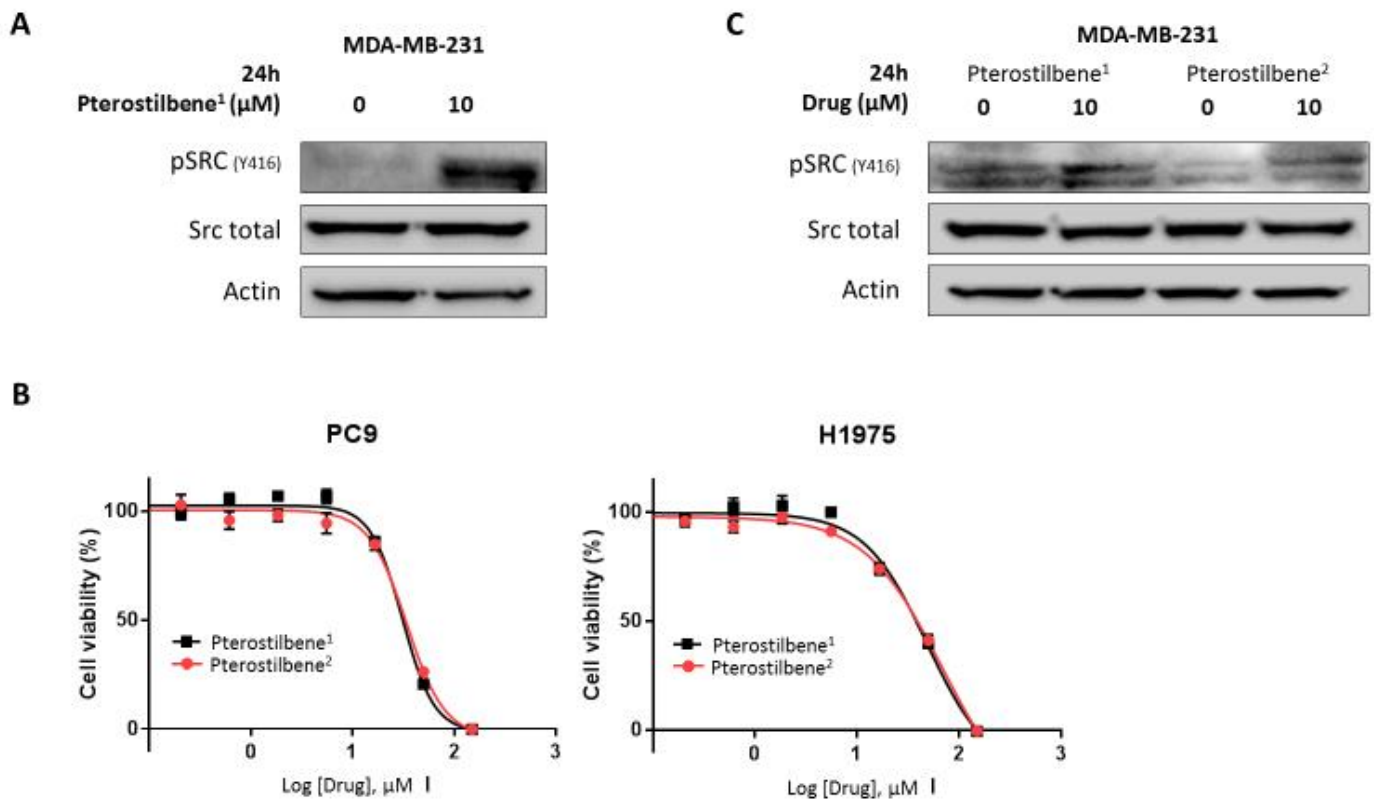


Figure 3. Unraveling the mechanisms of action of pterostilbene in TNBC and NSCLC cell lines.

A: The TNBC cell line MDA-MB-231 was treated with 10 μ M pterostilbene¹ for 24 h. Untreated cells received an equivalent dose of vehicle (DMSO). Cell lysates were used for immunoblotting, and the effect of pterostilbene¹ treatment on (phosphorylation of) Src was explored. Experiments were performed in biological triplicates with similar results, and a representative blot is shown. **B:** MTT cell viability assays were performed in 2 different EGFR-mutation positive NSCLC cell lines (PC9 and H1975), to compare the effects of the two different pterostilbene compounds (pterostilbene¹: Amazon; pterostilbene²: Sigma Aldrich) on cell viability. Experiments were performed in biological triplicates with similar results, and a representative plot is shown. **C:** To confirm a similar mechanism of action of the two pterostilbene compounds, MDA-MB-231 cells were treated with 10 μ M pterostilbene¹ or 10 μ M pterostilbene² for 24 h. Untreated cells received an equivalent dose of vehicle (DMSO). Cell lysates were used for immunoblotting, and the effect of pterostilbene treatment on (phosphorylation of) Src was explored. Experiments were performed in biological triplicates with similar results, and a representative blot is shown. DMSO: dimethylsulfoxide; EGFR: epidermal growth factor receptor; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NSCLC: non-small cell lung cancer; TNBC: triple negative breast cancer.

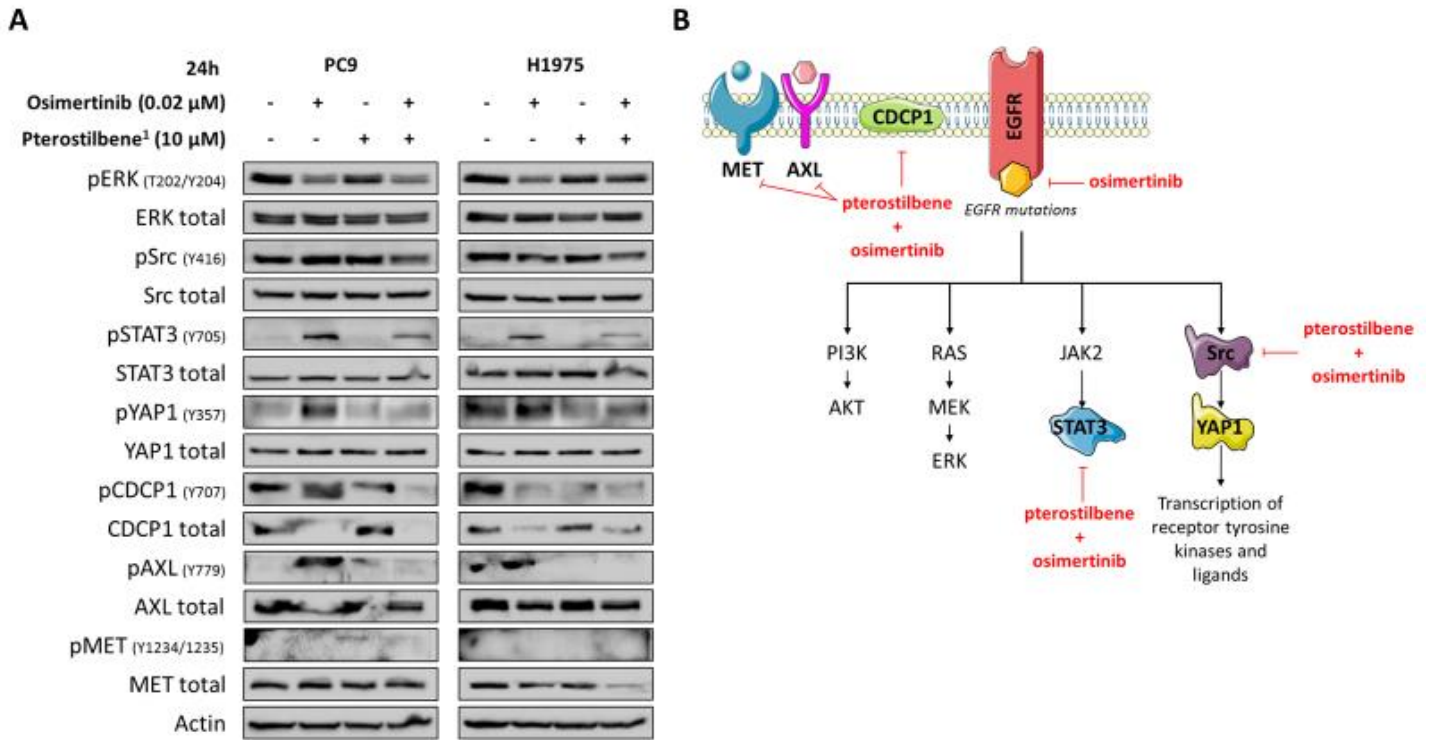


Figure 4. The effects of combined osimertinib and pterostilbene¹ treatment in NSCLC cell lines.

A: Two EGFR-mutation positive NSCLC cell lines (PC9 and H1975) were treated with single osimertinib (0.02 μ M), single pterostilbene¹ (10 μ M) or with the combination. Untreated cells received an equivalent dose of vehicle (DMSO). Cell lysates were used for immunoblotting and changes in RTKs and non-RTKs upon the different treatments were investigated in the two cell lines. Experiments were performed in biological triplicates with similar results, and a representative blot is shown. **B:** Our model: osimertinib blocks the signaling of the EGFR receptor and its downstream pathways. Our previous research has shown that this process causes hyperactivation of compensatory signaling nodes, including STAT3 and Src-YAP1. This leads to an up-regulation of RTKs and non-RTKs (e.g. MET, CDCP1), and ultimately to therapy resistance. The combination of pterostilbene with osimertinib abrogates the osimertinib-induced activation of STAT3, YAP1, CDCP1 and moderately abrogates Src activation. MET expression is also inhibited with the double combination in the H1975 cell line. CDCP1: CUB domain-containing protein-1; DMSO: dimethylsulfoxide; EGFR: epidermal growth factor receptor; NSCLC: non-small cell lung cancer; RTKs: receptor tyrosine kinases; STAT3: signal transducer and activator of transcription 3; YAP1: Src-yes-associated protein 1.