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, YAPI, 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.

**Key words:** Pterostilbene, NSCLC, osimertinib, therapy resistance

**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 (YAPI) 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'-hydroxy-trans-stilbene) is a stilbene of the family of phytoalexin compounds, found in 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 PC9 cells, harboring EGFR exon 19 deletion and 11-18 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 HCC406 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% fetal bovine serum (FBS; Gibco) and 10% penicillin/streptomycin/glutamine (Gibco) and 10% medium) 1640 supplemented with 1% fetal bovine serum (FBS; Gibco) in a 5% CO2 37°C cell culture incubator and routinely evaluated for mycoplasma contamination.

The primarily used pterostilibene compound (hereafter referred to as pterostilibene1) was purchased from Amazon.com, Inc. (Catalog: B00BFUJ04Q), the second pterostilibene compound (hereafter referred to as pterostilibene2) 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.

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

| Western Blotting Primary Antibody | Dilution | Company and Catalog number |
|-----------------------------------|----------|----------------------------|
| Rabbit anti-AXL (Y779)            | 1:1000   | Cell Signaling (#68661)    |
| 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 (#1115)     |
| 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 (#5307)    |
| Rabbit anti-Src                   | 1:1000   | Cell Signaling (#2109)     |
| Rabbit anti-Phospho-Src Family (Y146) | 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 | 1:5000   | GE Healthcare Life Sciences (NA934-1ML) |
| donkey)                           |          |                            |
| HRP-linked goat anti-mouse (from sheep) | 1:5000 | GE Healthcare Life Sciences (NA935-1ML) |

**Cell viability assay**

Cells were seeded in 96-well plates at the following densities: 1 x 10³ (PC9 and H1975) 1.5 x 10³ (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 (IC₅₀) determination, MTT viability assays were performed using pterostilibene doses ranging from 0-150 μM. To determine the combined effect of pterostilibene and osimertinib, drug doses in viability assays were as follows: H1975 and HCC4006 cells were treated with osimertinib ranging from 0-90 nM, and pterostilibene ranging from 0-180 μM, or with the combination of both. PC9 and HCC827 cells were treated with osimertinib ranging from 0-120 nM, and pterostilibene ranging from 0-120 μM, 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 μl 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 pterostilibene (10, 20 and 50 μM), or with 20 nM osimertinib, 10 μM pterostilibene 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 pterostilbene\(^1\) in all cell lines (Figure 2A), it could be concluded that single pterostilbene\(^1\) 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 pterostilbene\(^1\) with the third-generation TKI osimertinib (of which IC_{50}s were previously determined, and can 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 pterostilbene\(^1\) 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 pterostilbene\(^1\) treatment, PC9 and H1975 cells were treated with different concentrations of pterostilbene\(^1\) for 24h. Cell lysates were consequently used for immunoblotting and revealed different mechanisms of action of pterostilbene\(^1\) 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

Figure 2. The effects of single and combined osimertinib and pterostilbene\(^1\) 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 pterostilbene\(^1\) on cell viability, and to determine the IC_{50} 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 pterostilbene\(^1\) 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 with similar results, and a representative blot is shown. DMSO: dimethylsulfoxide; EGFR: epidermal growth factor receptor; IC_{50}: 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.
pterostilbene treatment. In contrast, both total and phosphorylated YAP1 were inhibited by pterostilbene 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 24 h treatment with 10 or 20 μM pterostilbene 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 pterostilbene in the MBA-MB-231 TNBC cell line was determined. Cells were treated with 0 or 10 μM pterostilbene for 24 h, and Src activation was explored. Surprisingly, instead of inhibiting Src phosphorylation, pterostilbene caused an induction of Src phosphorylation (Figure 3A). To confirm this, we purchased pterostilbene from a different supplier (pterostilbene3), 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 induced 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 pterostilbene. 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 pterostilbene. MET phosphorylation is slightly activated upon single pterostilbene 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 pterostilbene treatment. Osimertinib plus pterostilbene reversed the osimertinib-induced STAT3 and YAP1 phosphorylation, abolished CDCP1 and AXL activation.

![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 μM pterostilbene 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. B: MTT cell viability assays 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 μM pterostilbene or 10 μM pterostilbene 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.](http://www.ijbs.com)
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) in addition to 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. 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; SFRs: 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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