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α-TEA cooperates with chemotherapeutic agents to induce apoptosis of p53 mutant, triple negative human breast cancer cells via activating p73

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Abstract

Introduction: Successful treatment of p53 mutant, triple-negative breast cancers (TNBC) remains a daunting challenge. Doxorubicin (DOXO) and cisplatin (CDDP) are standard-of-care treatments for TNBC, but eventually fail due to acquired drug resistance and toxicity. Therefore, new treatments for overcoming drug resistance and toxicity in p53 mutant, TNBC are badly needed. Unlike p53, p73, a member of the p53 family, is usually not mutated in cancers and has been shown to regulate p53 mediated apoptotic signaling in p53 deficient cancers. Therefore, identification of anticancer agents that can activate p73 in p53-deficient cancers may provide a chemotherapeutic approach for treatment of p53 mutant cancers. Here we report on the reconstitution of the p53 tumor suppressor pathway in a p53-independent manner via p73 with combination treatments of α-TEA, a small bioactive lipid, plus DOXO or CDDP.

Methods: p53 mutant, TNBC cell lines, MDA-MB-231, BT-20 and MDA-MB-468 were used to evaluate the anticancer effect of chemotherapeutic drugs and α-TEA using Annexin V (FITC)/PI staining, western blot analyses, RT-PCR and siRNA knockdown techniques.

Results: Combination treatments of α-TEA plus DOXO or CDDP act cooperatively to induce apoptosis, caspase-8 and -9 cleavage, p73, phospho-c-Ab1 and phospho-JNK protein expression, and increase expression of p53 downstream mediators; namely, death receptor-5 (DR5), CD95/APO-1 (Fas), Bax and Noxa, as well as Yap nuclear translocation, plus reduce expression of Bcl-2. Knockdown of p73, c-Abl, JNK or Yap using siRNAs show that: (i) p73 plays a critical role in combination treatment enhanced apoptosis and the expression of pro- and anti-apoptotic mediators, and (ii) c-Abl, JNK and Yap are upstream mediators of p73 in combination treatment responses.

Conclusions: Data show that α-TEA in combination with DOXO or CDDP synergistically enhances apoptosis in TNBC via targeting p53 mediated genes in a p73 dependent manner and that p73 responses are downstream of c-Abl, JNK and Yap.

Introduction

Successful treatment of triple negative (estrogen receptor negative, progesterone receptor negative and Her-2 negative) breast cancers (TNBC) that are also p53 mutant remains elusive. Unfortunately, the anticancer efficacy of commonly used chemotherapeutic agents for TNBC, including doxorubicin (DOXO) and cisplatin (CDDP), are limited due to acquired drug resistance and toxicities [1, 2].

DOXO and CDDP are DNA damaging drugs that exert their anticancer actions via inhibition of cellular proliferation and induction of cell death by apoptosis [3, 4]. Tumor suppressor gene p53 plays a central role in the anticancer actions of DNA damaging agents. Loss of wild-type p53 functions leads to resistance to DNA damaging agents, such as DOXO and CDDP [5, 6]. Identification of anticancer agents that target p53 downstream genes via p53-independent mechanisms is of major clinical relevance, especially since p53 deficiency is a hallmark of many different cancer types.

p73 is a member of the p53 gene family [7]. Unlike p53 [8], p73 is rarely mutated or lost in cancers [9]. Although p53 deficient cancers are less responsive to chemotherapy, they are typically not completely drug resistant because other p53 family members such as p73, can replace p53 function in response to DNA damage [9-11]. Since p73 is usually not mutated in cancers and has been shown to regulate p53 target genes in p53 deficient cancers, identification of anti-cancer agents that can activate p73 in p53-deficient cancers will provide a chemotherapeutic approach for treatment of drug resistant p53 mutant cancers.

α-TEA (2,5,7,8-tetramethyl-2R-(4R,8R,12-trimethyltridecyl) chroman-6-yloxyacetic acid, called RRR-α-tocopherol ether-linked acetic acid analog or RRR-α-tocopheryloxyacetic acid, is a non-hydrolyzable ether analog of RRR-α-tocopherol [12]. α-TEA has been shown to be a potent pro-apoptotic agent both in *vitro* and *in vivo* in breast, prostate and ovarian cancer cells [12-20]. Recently,

α-TEA has been shown to delay tumor onset and inhibit the progression and metastatic spread in a clinically relevant model of spontaneous mammary cancer, further highlighting the translational potential of this anticancer agent [14]. Mechanisms involved in α-TEA induced apoptosis include: activation of JNK/c-Jun, p73/NOXA and Fas/DR5 and suppression of c-FLIP-L, survivin and phospho-Akt (pAkt), leading to death receptor mediated caspase-8 activation and mitochondria dependent apoptosis [15-20].

Data presented here show that α-TEA in combination with DOXO or CDDP significantly enhances apoptosis of p53 mutant, triple negative human breast cancer cells by targeting p73 mediated p53-dependent pro-apoptotic and anti-apoptotic genes via c-Abl, JNK and Yap signaling pathways.

Materials and methods

Chemicals

α-TEA was made in house as previously described [12]. DOXO and CDDP were purchased from Sigma (San Diego, CA). PI3K inhibitor (wortmannin) was purchased from Cell Signaling Technology (Beverly, MA).

Cell culture

p53 mutant, triple negative human breast cancer cell lines MDA-MB-231, BT-20 and MDA-MB-468 were purchased from the American Type Culture Collection (Manassas, VA). MDA-MB-231 and BT-20 cells were cultured in MEM media with 10% FBS and MDA-MB-468 cells were cultured in DMEM media with 10% FBS. All three p53 mutant TNBC cell lines (ER-, PR-, HER2 -/low) used in these studies were originally obtained from human samples so no isogenic counterparts expressing wildtype p53, estrogen receptor and progesterone receptor are available for use as controls. For experiments, FBS was reduced to 2% to better mimic low *in vivo* serum exposure and cells were allowed to attach overnight before treatments. α-TEA (40 mM) was dissolved in ethanol as stock solution. Concentrations of ethanol used in vehicle (VEH) treatments were 0.025-0.05% (v/v) to match ethanol content in the different final concentrations of α-TEA treatments. DOXO and CDDP were dissolved in H_2O.

Quantification of apoptosis

Apoptosis was quantified by Annexin V-FITC/PI assays following the manufacturer's instructions. Fluorescence was measured using Fluorescence Activated Cell Sorter (FACS) analyses with a FACSCalibur flow cytometer, and data were analyzed using CellQuest software (BD Biosciences, San

Jose, CA). Cells displaying phosphatidylserine on their surface (i.e. positive for annexin-V fluorescence) were considered to be apoptotic.

Nuclear and cytoplasmic fractionation

Cytoplasmic and nuclear fractions were prepared as previously described [21]. Briefly, whole cell lysates were centrifuged to obtain supernatant and pellet. The supernatant was centrifuged again and the resulting supernatant was used as the cytosolic fraction. The pellet was layered over a cushion of 1 ml sucrose buffer and centrifuged. The final pellet, lysed using RIPA buffer was used as the nuclear fraction.

Western blot analyses

Whole cell protein lysates were prepared and western blot analyses were conducted as described previously [22]. Proteins at 20-50 μg/lane were separated by SDS-PAGE and transferred to nitrocellulose (Optitran BA-S supported nitrocellulose, Schleicher and Schuell, Keene, NH). Antibodies to the following proteins were used: poly (ADP-ribose) polymerase (PARP), Fas, Bcl-2, Bax, total JNK and phospho-JNK (pJNK) (Santa Cruz Biotechnology, Santa Cruz, CA); p73 and NOXA (Imgenex, San-Diego, CA); pYap (Ser-127), Yap, p-cAbl (Tyr-245), c-Abl, pAkt (Ser-473), caspase-8, caspase-9, DR5 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Beverly, MA).

RT-PCR detection of Fas, DR5, Bax, Noxa and Bcl-2 mRNA expression

Total RNA was extracted using RNA isolation kit (Qiagen Inc. Valencia, CA). Semi-quantitative analyses were conducted to detect Fas, DR5, Bax, Noxa and Bcl-2 mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR) using the housekeeping gene β-actin as control. Total RNA was reverse transcribed to cDNA using Superscript RTase (250 U, Invitrogen, Carlsbad, CA) following the

manufacture's instructions. cDNA was used per PCR reaction with Taq PCR Master Mix Kit (Qiagen, Inc) plus 10 µM oligonucleotide primer pairs (Invitrogen). The primer sequences are presented in Table 1.

RNA interference

A scrambled RNA duplex purchased from Ambion (Austin, TX) that does not target any known mouse, rat or human gene was used as the nonspecific negative control for RNAi (referred to as control siRNA). Transfection of MDA-MB-231 cells with siRNA to p73, c-Abl, JNK, Yap or control (Ambion) was performed in 100 mm cell culture dishes at a density of 2 x 10^6 cells/dish using Lipofectamine 2000 (Invitrogen) and siRNA duplex, resulting in a final siRNA concentration of 30 nM following the company's instructions. After one day of exposure to transfection mixture, the cells were re-cultured in 100 mm dish at 2 x 10^6 cells/dish and incubated for one day followed by treatments.

Statistical Analysis

Apoptosis data were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey test for comparison of more than two treatments or a two-tailed student *t*-test for comparison between two treatments to determine statistical differences. Differences were considered statistical significant at $p < 0.05$.

Results

α-TEA, DOXO and CDDP induce apoptosis in p53 mutant, triple negative human breast cancer cells

The sensitivity of three p53 mutant, TNBC lines (MDA-MB-231, BT-20 and MDA-MB-468) to apoptosis induced by α-TEA, DOXO and CDDP was evaluated by determining EC_{50} values for apoptosis (Table 2). Data show that MDA-MB-468 cells exhibit the most sensitive phenotype and MDA-MB-231 cells exhibit the most resistant phenotype to apoptosis induced by DOXO and CDDP among the three cell lines. While the sensitivity of the three cell lines to α-TEA induced apoptosis is similar.

α-TEA cooperates with DOXO and CDDP to induce apoptosis of p53 mutant TNBC cells

Based on the EC_{50} values for apoptosis presented in Table 2, MDA-MB-231 and BT-20 cell lines, which are more resistant to DOXO and CDDP were chosen to study the combinational effects of α-TEA + DOXO or CDDP on apoptosis induction. Data showed that α-TEA at 10 and 20 μM significantly enhanced apoptosis in combination with DOXO and CDDP in MDA-MB-231 and BT-20 cells, respectively, in comparison to individual treatments (Figure 1a, b, c and d). Mean CI (combination index) for combination of α-TEA + DOXO was 0.41 $\pm$ 0.07 and 0.53 $\pm$ 0.05 for MDA-MB-231 and BT-20 cells, respectively (Table 3). Mean CI for combination of α-TEA + CDDP was 0.45 $\pm$ 0.10 and 0.75 $\pm$ 0.08 in MDA-MB-231 and BT-20 cells, respectively (Table 3). These data demonstrate that combinations of α-TEA + DOXO or CDDP synergistically induce apoptosis in both cell lines. Western blot analyses show that α-TEA at 20 μM cooperates with DOXO and CDDP to induce elevated levels of cleaved caspases-8, -9, and PARP in both cell lines (Figure 2a and b), indicating that apoptosis induced by these combinations involves both caspase-8 and caspase-9 activation.

p73 protein level is up-regulated by combinations of α-TEA + DOXO or CDDP and involved in combination induced apoptosis.

Since DOXO and CDDP as well as α-TEA have been shown to induce p73 up-regulation in breast cancer cells [11, 17, 23], the combination of α-TEA plus DOXO or CDDP was investigated for ability to cooperatively enhance p73 protein expression. Single treatments with DOXO, CDDP or α-TEA at sub-apoptotic levels for 24 hrs slightly increased p73 protein expression above control levels; whereas, combinations at the same levels markedly enhanced p73 protein expression in comparison with single treatments in both MDA-MB-231 and BT-20 cells (Figure 3a & b). siRNA to p73 significantly reduced the ability of combination treatments to induce apoptosis as determined by Annexin V (Figure 3c) and PARP analyses (Figure 3d) in MDA-MB-231 cells. Western blot data show that siRNA to p73 effectively silenced p73 protein expression (Figure 3d). These data indicate that p73 activation by combination treatments is critical for induction of cell death by apoptosis.

Combinations of α-TEA plus DOXO or CDDP up-regulate pro-apoptotic DR5, Fas, Bax, and Noxa mRNAs and proteins and down-regulate anti-apoptotic Bcl-2 mRNA and protein, all of which are mediated at least in part by p73

Published data show that p73 can regulate p53 dependent genes in p53 deficient cells [11]. To better understand the cellular events involved in p73 mediated apoptosis in combination treatments, mRNA and protein expression of p53 mediated pro-apoptotic mediators DR5, Fas, Bax, and Noxa, and anti-apoptotic mediator Bcl-2 were examined. Combinations of α-TEA + DOXO or CDDP enhanced DR5, Fas, Bax and Noxa mRNA (Figure 4a & b) and protein expression (Figure 4c & d) and decreased Bcl-2 mRNA (Figure 4a & b) and protein expression (Figure 4c & d) in MDA-MB-231 and BT-20 cells. siRNA knockdown of p73 was performed to determine if expression levels of these mediators were regulated by p73. siRNA to p73 in MDA-MB-231 cells effectively silenced p73 protein expression and blocked the ability of combinations to induce increased levels of DR5, Fas, Bax and Noxa

protein, as well as to decrease Bcl-2 protein levels (Figure 4e). These data suggest that combination treatments induce up-regulation of pro-apoptotic mediators and down-regulation of an anti-apoptotic mediator in a p73 dependent manner in p53 mutant, TNBC MDA-MB-231 and BT-20 cells. Recent studies in our lab show that DR5 pro-apoptotic signaling contributes to α-TEA-induced apoptosis [19-20]. To determine if DR5 contributes to combination treatment induced apoptosis, DR5 was functionally knocked-down with siRNA. Data indicate that silencing DR5 protein expression blocks combination induced apoptosis as determined by PARP cleavage (Figure 4f).

α-TEA cooperates with DOXO or CDDP to up-regulate pc-Abl and pJNK, which are upstream mediators of p73

Studies show that p73 can be up-regulated upon DNA damage via activation of c-Abl and JNK [23, 24]. To understand how p73 is activated by the combination treatments, phosphorylated levels of c-Abl and JNK2/1 were examined. Combinations of α-TEA plus DOXO or CDDP induced increased levels of pc-Abl (Tyr-245) and pJNK2/1 in both cell lines (Figure 5a and b). siRNA knockdown of c-Abl or JNK significantly reduced the ability of combination treatments to induce apoptosis in MDA-MB-231 cells as determined by Annexin V (Figure 5c) and PARP cleavage (Figure 5d). siRNA treatments blocked the ability of combination treatments to increase protein levels of p73 and block the ability of combination treatments to increase protein levels of DR5, Fas, Bax and Noxa, and to decrease level of Bcl-2 (Figure 5d). siRNA to c-Abl blocked the ability of combination treatments to induce increased levels of pJNK; whereas, siRNA to JNK had no effect on the ability of combination treatments to induce increased levels of pc-Abl (Tyr 245) (Figure 5d). These data show that activation of p73 is mediated by c-Abl and JNK in the combination treatments, and suggest that c-Abl, in part, regulates the phosphorylation status of JNK.

Yap is involved in combination induced apoptosis

Since Yap, a transcriptional co-activator Yes-associated protein, can interact with p73, resulting in enhanced p73 transcriptional activity [25] and stability [26-27], we determined if Yap contributes to combination induced apoptosis and increased p73 expression. siRNA knockdown of Yap significantly reduced the ability of combination treatments to induce apoptosis as measured by annexin V analyses (Figure 6a) and western blot analyses of PARP cleavage (Figure 6b). siRNA to Yap effectively reduced Yap protein levels and blocked combination treatment effects on p73 protein expression, as well as combination effects on DR5, Fas, Bax, Noxa and Bcl-2 protein expression (Figure 6b). These data show that Yap is a key player in combination treatment induced apoptosis mediated by p73.

Combination treatments induce Yap nuclear translocation, which is associated with suppression of phosphorylation of Akt and Yap.

Yap activity can be regulated by c-Abl via phosphorylation of Yap at Tyr-357, leading to its stabilization and higher affinity for p73 [28-29]. Furthermore, Yap can be negatively regulated by Akt [29-30]. Akt induces Yap phosphorylation at Ser-127, resulting in Yap cytosolic localization via promoting Yap binding with 14-3-3 resulting in inactivation of Yap [30]. Since α-TEA has been shown to decrease pAkt in prostate [15], ovarian [18], and breast cancer cells [data not shown] we examined the effect of combination treatments on Yap nuclear translocation, as well as pAkt and pYap expression. Combination treatments of MDA-MB-231 cells induced increased levels of Yap protein in the nuclear fraction and reduced levels of Yap protein in the cytoplasmic fraction. Histone 1 and GAPDH were used to evaluate purity of nuclear and cytoplasmic fractions, respectively, and served as lane load controls (Figure 7a). Furthermore, data show that DOXO and CDDP increased pAkt and pYap protein expression while α-TEA cooperated with DOXO or CDDP to suppress pAkt and pYap

in MDA-MB-231 (Figure 7b). These data suggest that Yap nuclear translocation may partially contribute to p73 mediated effects and that combination treatment down-regulation of pAkt correlates with decreased levels of pYap. To assess the role of Akt in DOXO and CDDP induced p73 protein expression, we examined the impact of PI3K/Akt inhibitor (wortmannin) on DOXO and CDDP induced p73 protein expression. Data show that wortmannin enhanced DOXO and CDDP induced up-regulation of p73 protein expression (Figure 7c), indicating a role for Akt in DOXO and CDDP increase in p73 expression. Data also show that wortmannin blocked DOXO and CDDP induced up-regulation of pAkt and pYap (Figure 7c), suggesting that suppression of pAkt enhances DOXO and CDDP induced p73 expression via down-regulation of pYap.

Discussion

p73 is an important target for treating p53 mutant cancers [10, 31-33]. The novel findings in this study are: (i) α-TEA, a potent anticancer analog of vitamin E, synergizes with DNA damaging agents DOXO and CDDP to induce apoptosis of human p53 mutant, triple negative human breast cancer MDA-MB-231 and BT-20 cells via targeting p73, (ii) combination treatments result in p73 dependent up-regulation of pro-apoptotic DR5, Fas, Bax and Noxa and down-regulation of anti-apoptotic mediator Bcl-2, all of which are p53 mediated apoptotic related genes, (iii) p73 and p73 mediated apoptotic events are regulated by c-Abl, JNK and Yap in combination treatments, and (iv) α-TEA downregulation of Akt partially contributes to p73 upregulation in combination treatments. Thus, our data, for the first time, identify α-TEA as a small bioactive anticancer agent that regulates p53 mediated genes via p53-independent mechanisms when combined with DNA damaging agents.

 As a transcription factor, p73 shares structural and functional similarities with p53 [9, 32, 33]. In cancer cells that express wild type p53, p73 has been reported to cooperate with p53 to induce

apoptosis [34]; whereas, in p53 mutant cancer cells p73 has been reported to induce apoptosis via activation of p53-inducible genes [11, 35]. Typically, p53 induces apoptosis via regulating apoptosis related genes; such as DR5, Fas, Bax, Noxa and Bcl-2 [36-37]. p73 is up-regulated in response to a subset of DNA-damaging agents, including DOXO, CDDP, camptothecin and etoposide [38]. Several p53 mediated apoptosis-related genes have been identified to be regulated by p73, such as Fas, Bax, Bim, Noxa and Puma [17, 39-41]. However, whether DR5 is a direct target of p73 is not well documented. It has been reported that DR5 is regulated by p73 in H1299 human non small lung cancer cells [42]. El-Deiry and co-workers used a high-throughput screen to identify small molecules that could activate p53 reporter activity, increase expression of p53 target genes such as p21(Waf1), DR5 and TRAIL and induce apoptosis in p53-deficient colon cancer cells [35]. Some of these compounds activated a p53 response by increasing p73 expression, and knockdown of p73 with siRNA reduced their ability to activate p53 reporter activity while other compounds acted in a p73-independent fashion [35]. In addition, they characterized a derivative of the plant alkaloid ellipticine as an anticancer agent which induces p73 and DR5 protein expression in a p53-deficient human colon carcinoma cell line [43]. However, neither of these studies showed direct evidence that p73 was regulating DR5 transcription. To the best of our knowledge, there is no direct evidence showing that p73 regulates DR5 transcription other than the lung cancer studies [42]. In addition, there is no evidence to indicate that p73 transcriptionally regulates Bcl-2. Thus, this study, for the first time, demonstrates that both DR5 and Bcl-2 are mediated at the transcriptional level by p73 in p53 mutant, TNBC MDA-MB-231 and BT-20 human breast cancer cells treated with α-TEA combined with DOXO or CDDP as determined by siRNA knockdown assays. Our previous data showed that DR5 is involved in α-TEA induced apoptosis since siRNA knockdown of DR5 blocked α-TEA induced apoptosis in MCF-7 and MDA-MB-231 human breast cancer cells [19-20]. Here, we demonstrated

that DR5 is necessary, at least in part, for apoptosis induced by α-TEA combination treatments with DOXO or CDDP.

Besides transcriptionally activating p53 mediated apoptotic genes, p73 has been reported to induce ER stress via transactivation of Scotin [44]. Since DR5 and Bcl-2 expression can be regulated by ER stress via CHOP [45-46] and since α-TEA has been shown to induce ER stress and CHOP expression [19] we cannot rule out the possibility that p73 regulates DR5 and Bcl-2 via ER stress in combination treatments. Further studies are needed to address this issue.

p73 is predominantly regulated at the post-translational level in response to DNA damaging agents. c-Abl and JNK are activated by DNA damaging agents and both are involved in p73 activation [23-24]. DOXO and CDDP have been shown to regulate p73 via c-Ab1 [23, 28-29]. c-Abl regulates p73 via different mechanisms; for examples, c-Abl can directly stabilize p73 via acetylation and phosphorylation of p73 [23, 47] and c-Abl can stabilize p73 and enhance p73 transcriptional activity via phosphorylation of Yap [28]. JNK has been reported to stabilize p73 via phosphorylation of p73 [24] and via JNK phosphorylation/activation of c-Jun [48]. In addition, JNK also activates p73 via enhancing c-Abl nuclear translocation [49]. In untreated cells, c-Abl is sequestered in the cytosol by 14-3-3 proteins. Upon exposure of cells to DNA damaging agents, JNK is activated and phosphorylates 14-3-3, resulting in the release of c-Abl into the nucleus, an event required for the induction of apoptosis in response to DNA-damaging agents [49]. Published [50] and present data show that c-Abl also regulates JNK via phosphorylation, suggesting cross-talk between c-Abl and JNK.

Yap (Yes-associated protein) is a transcriptional coactivator, which can interact with the p53 family member p73, resulting in an enhancement of p73's transcriptional activity [25-26] and stability [27]. A

potential mechanism of the p73 protein stabilization was recently suggested by Levy et al [27]. Namely, Yap competes with Itch, an E3 ubiquitin ligase involved in degradation of p73, for binding to p73 at the PPXY motif. Furthermore, Yap activity can be regulated by c-Abl via phosphorylation at Tyr-357, leading to a more stable form of Yap that exhibits a higher affinity to p73 [27]. Yap can be negatively regulated by Akt [30, 51]. Akt induces Yap phosphorylation at Ser-127, resulting in Yap cytosolic localization since phosphorylation of Yap at Ser-127 promotes Yap binding with 14-3-3 [30]. Thus, Yap activation can be regulated in a positive manner by c-Abl and in a negative manner by Akt. DNA damage can activate survival mediator Akt, resulting in reducing the anticancer efficacy of DNA damaging drugs. DOXO or CDDP induce activation of Akt in some cell lines [52-53]. Likewise, our data show that DOXO and CDDP induce elevated levels of pAkt not only in MDA-MB-231 (Figure 7), but also in MCF-7, MDA-MB-453 and BT-20 cells (data not shown). As expected, Akt inhibitors have been reported to enhance the anticancer effect of DOXO in MDA-MB-231 cells [54]. Data reported here show that Akt inhibitor wortmannin enhanced DOXO and CDDP mediated increases in p73 protein expression, which is associated with down-regulation of pAkt and pYap (Ser-127) in MDA-MB-231 cells. Taken together, these data suggest that Akt activation upon DNA damage may counteract p73 activation induced by JNK and c-Abl via inhibition of Yap nuclear translocation. Thus, our data suggest that Yap nuclear translocation plays an important role in p73 activation and that suppression of pAkt and its inhibitory phosphorylation of pYap contributes to enhanced Yap nuclear translocation in combination treatments.

How α-TEA induces p73 protein expression is not fully understood. Previously, we reported that JNK is involved in regulation of p73 in α-TEA-induced apoptosis of human breast cancer cells [19]. In this study, we found that α-TEA also induces increased levels of pc-Abl and Yap nuclear translocation, as well as suppresses pAkt and pYap, suggesting that c-Abl and Yap, as well as downregulation of

pAkt/pYap are also involved in α-TEA induced apoptosis. Noxa has been identified as a downstream mediator of p73 in α-TEA induced apoptosis [17]. However, whether other p53 mediated genes, such as Fas, DR5, Bax and Bcl-2 are regulated by 73 following α-TEA treatment has not been investigated. Since recent data show that ER stress mediated CHOP contributes to α-TEA induced up-regulation of DR5 and down-regulation of Bcl-2 [19] it will be important for future studies to address whether both CHOP and p73 contribute to DR5 upregulation and Bcl-2 downregulation in α-TEA induced apoptosis.

Mechanisms mediating the combined anticancer effects of α-TEA + DOXO or CDDP are diverse and not completely understood. These studies identified p73 as a key player in combination treatment induced apoptosis. In addition, data show that c-Abl, JNK and Yap play roles in combination treatment induced activation of p73. It is important to note that although both α-TEA and DNA damaging drugs DOXO or CDDP induce increased levels of pc-Ab1 and pJNK, only α-TEA and the combination of α-TEA plus DOXO or CDDP induces Yap nuclear translocation which is associated with inhibition of pAkt (Ser-473) and Akt phosphorylated pYap (Ser-127). Furthermore, a PI3K/Akt inhibitor was shown to enhance DOXO and CDDP up-regulation of p73, which was also associated with down-regulation of pAkt and pYap. Taken together, these data suggest that down-regulation of pAkt and pAkt mediated inactive form of Yap play important roles in p73 activation and apoptosis in combination treatments. Thus, α-TEA cooperates with DOXO or CDDP to induce p73 protein expression and apoptosis via not only activation of c-Abl and JNK, but also Yap, which may be regulated positively by c-Abl and negatively by Akt. Based on published reports and data presented here we proposed signaling events necessary for combination treatment induced apoptosis in p53 mutant, TNBC cells (Figure 8).

Conclusions

In summary, data demonstrated that α-TEA, a small bioactive lipid, cooperates with DNA damaging agents DOXO and CDDP to induce apoptosis in human breast cancer cells via targeting p53 inducible apoptotic related genes in a p73-dependent manner. These studies highlight the potential for activation of p73 as a promising target for treatment of p53 mutant, TNBC and identify α-TEA as an important candidate agent.

Abbreviations

α̃-TEA: RRR-α-tocopherol ether-linked acetic acid analog; CDDP: Cisplatin; DOXO: Doxorubicin; DR5: Death receptor 5; JNK: c-Jun N-terminal kinases; TNBC: Triple negative breast cancers; Yap: Yes associated protein.

Competing interests

US and international patents on α-TEA are held by the Research Development Foundation. Kimberly Kline, Bob G. Sanders and Weiping Yu are listed as inventors. No commercial applications or financial gain have been realized.

Authors' contributions

RT, WY, BGS and KK conceived and designed the study, analyzed the data, and drafted the manuscript. WY helped in cell culture study and RT performed all experiments.

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Figure legends

Figure 1. α-TEA cooperates with DOXO and CDDP to induce apoptosis of p53 mutant, triple negative human breast cancer cells. MDA-MB-231 and BT-20 breast cancer cells were treated with different concentrations of DOXO or CDDP alone or in combination with α-TEA for 24 hrs. FACS/Annexin V assays were used to determine the percentage of apoptotic cells **(a, b, c & d)**. Data are expressed as mean $\pm$ SD from three independent experiments. *$p<0.05$=significantly different from control and **$p<0.05$=significantly different from single treatments.

Figure 2. α-TEA cooperates with DOXO and CDDP to induce cleavage of caspase-8, -9 and PARP. MDA-MB-231 and BT-20 cells were treated with DOXO or CDDP alone or in combination

with α-TEA for 24 hrs. Western blot analyses were used to detect PARP, caspase-8, and caspase-9 cleavage (**a & b**). Data are representative of at least two independent experiments.

Figure 3. p73 is up-regulated by combination treatments and involved in combination induced apoptosis. MDA-MB-231 and BT-20 cells were treated with α-TEA, DOXO and CDDP alone or in combinations for 24 hrs. Western blot analyses were performed to detect protein levels of p73 with GAPDH serving as loading control (**a & b**). MDA-MB-231 cells were transfected with p73 siRNA or control siRNA for 2 days and treated with combinations of α-TEA + DOXO or α-TEA + CDDP for 24 hrs. FACS/Annexin V assays were used to determine the percentage of apoptotic cells (**c**). Western blot analyses were used to verify the knockdown efficiency of p73 siRNA and the effect of p73 siRNA on combination induced PARP cleavage (**d**). Data in a, b and d are representative of at least two independent experiments and the data in c are expressed as mean $\pm$ SD from three independent experiments. *$p<0.05$=significantly different from control siRNA determined by *t*-test.

Figure 4. α-TEA cooperates with DOXO or CDDP to up-regulate mRNA and protein of pro-apoptotic mediators DR5, Fas, Bax, and Noxa, and down-regulate anti-apoptotic Bcl-2 mRNA and protein, all of which are downstream targets of p73. MDA-MB-231 and BT-20 cells were treated with DOXO or CDDP alone or in combination with α-TEA for 24 hrs. mRNA levels of DR5, Fas, Bax, Noxa and Bcl-2 were determined by RT-PCR with β-actin serving as loading control (**a & b**). Protein levels of DR5, Fas, Bax, Noxa and Bcl-2 were determined by western blot analyses with GAPDH serving as loading control (**c & d**). The same treated samples as Figure 3d were used to detect the effect of siRNA to p73 on combination induced increase in protein levels of DR5, Fas, Bax, Noxa and decrease in Bcl-2 by western blot analyses with GAPDH as loading control (**e**). MDA-MB-231 cells were transfected with DR5 siRNA or control siRNA for 2 days and treated with combinations of

α-TEA + DOXO or α-TEA + CDDP for 24 hrs. Western blot analyses were used to determine the effect of siRNA to DR5 on combination induced PARP cleavage and to verify the knockdown efficiency of DR5 **(f)**. Data are representative of at least two independent experiments.

Figure 5. α-TEA cooperates with DOXO or CDDP to up-regulate pc-Abl and pJNK, which can serve as upstream mediators of p73. MDA-MB-231 and BT-20 cells were treated with DOXO or CDDP alone or in combination with α-TEA for 24 hrs. Protein levels of pc-Abl (Tyr-245), total c-Abl (tc-Abl), pJNK2/1, and total JNK2/1 (tJNK2/1) were determined by western blot **(a & b)**. MDA-MB-231 cells were transfected with c-Abl and JNK siRNAs, as well as control siRNA for 2 days and treated with combination of α-TEA + DOXO or CDDP for 24 hrs. Apoptosis was determined by Annexin V/FACS **(c)**. Western blot analyses were used to verify the knockdown efficiency of c-Abl and JNK siRNAs and the effect of c-Abl and JNK siRNAs on combination induced PARP cleavage, as well as p73 and p73 mediated DR5, Fas, Bax, Noxa and Bcl-2 **(d)**. Data in c are expressed as mean $\pm$ SD from three independent experiments and the data in a, b, and d are representative of at least two independent experiments. *p<0.05=significantly different from control siRNA determined by *t*-test.

Figure 6. Yap is involved in combination treatment induced apoptosis. MDA-MB-231 cells were transfected with Yap siRNA or control siRNA for 2 days and treated with combinations for 24 hrs. Apoptosis was determined by Annexin V/FACS **(a)**. Western blot analyses were used to verify the knock down efficiency of Yap siRNA and the effect of Yap siRNA on combination induced PARP cleavage, as well as p73 and p73 mediated DR5, Fas, Bax, Noxa and Bcl-2 **(b)**. Data in b are representative of at least two independent experiments. Data in a are expressed as mean $\pm$ SD from three independent experiments. *p<0.05=significantly different from control siRNA determined by *t*-test.

Figure 7. Combination treatments induce Yap nuclear translocation, which is associated with suppression of phosphorylation of Akt and Yap. Isolated cytosolic and nuclear fractions from MDA-MB-231 cells treated with α-TEA plus DOXO or CDDP were used to detect Yap tanslocation from cytosol to the nucleus by western blot analyses **(a)**. MDA-MB-231 cells were treated with DOXO or CDDP alone or in combination with α-TEA for 24 hrs. Protein levels of pAkt and pYap were determined by western blot analyses with total Akt and Yap serving as controls **(b)**. MDA-MB-231 cells were pre-treated with Akt inhibitor wortmannin at 1 μM or DMSO for 2 hrs followed by treatments with DOXO or CDDP for 24 hrs. Protein levels of p73, pAkt and pYap were determined by western blot analyses **(c)**. Data are representative of at least two independent experiments.

Figure 8. Proposed signaling pathways whereby the combination of α-TEA + DOXO or CDDP induces apoptosis in p53 mutant, triple negative MDA-MB-231 and BT-20 human breast cancer cells. p73 can be activated via multiple mechanisms and pathways including; (i) c-Abl and JNK can directly phosphorylate p73 to stabilize it, (ii) Yap in nucleus can bind with p73 to enhance its transcriptional activity and stability, and (iii) p73 can be transcriptionally regulated (not studied here). Yap can be regulated in a positive manner by c-Abl via phosphorylation enhancing its stability and transcriptional activity and in a negative manner by Akt via inhibiting translocation of Yap into the nucleus. c-Abl can directly phosphorylate JNK and JNK can enhance c-Abl nuclear translocation. Therefore, c-Abl, JNK and Yap play positive roles and Akt plays a negative role in p73 activation. Our data show that DNA damaging drugs DOXO and CDDP activated c-Abl and JNK, but also activated Akt, which can counteract c-Abl and JNK effects on activation of p73. Combination treatments not only act cooperatively to activate c-Abl and JNK, but also act cooperatively to inhibit pAkt and pYap (ser-127), leading to Yap nuclear translocation and p73 activation. In summary, combinations of α-TEA + DOXO or CDDP act cooperatively to up-regulate c-Abl/JNK, induce Yap nuclear translocation

and down-regulate pAkt/pYap, leading to activation of p73 and up-regulation of p73 mediated pro-apoptotic factors mediators, and down-regulation of Bcl-2, thereby restoring DOXO and CDDP chemotherapeutic potential in p53 mutant, triple-negative breast cancers.

Table 1. Primer sequences

Gene names	Forward	Reverse
Fas	5'-CAATGGGGATGAACCAGACTGC-3'	5'-GGCAAAAGAAGAAGACAAAGCC-3'
DR5	5'-GCCTCATGGACAATGAGATAAAGGTGGCT-3'	5'-CCAAATCTCAAAGTACGCACAAACGG-3'
Bax	5'-AGTAACATGGAGCTGCAGAGGATG-3'	5'-AGGAGGCTTGAGGAGTCTCACC-3'
Noxa	5'-CGTGTGTAGTTGGCATCTCC-3'	5'-AAGGAGTCCCCTCATGCAAG-3'
Bcl-2	5'-CCTGTGGATGACTGAGTACC-3'	5'-GAGACAGCCAGGAGAAATCA-3'
β-actin	5'-GGCGGCACCACCATGTACCCT-3'	5'- AGGGGCCGGACTCGTCATACT-3'

Table 2. EC_{50} values for apoptosis [a,b]

Cell lines	α-TEA (μM)	DOXO (μM)	CDDP (μM)
MDA-MB-231	41.7	46.5	70.8
BT-20	45.4	25.7	64.0
MDA-MB-468	35.4	8.5	40.7

[a]Cells were treated with different concentrations of α-TEA, DOXO, and CDDP for 24 hrs. Apoptosis was determined by Annexin V-FITC/PI staining/FACS as described in Material and Methods.

[b]The concentration that achieved 50% apoptosis (half maximal effective concentration; EC_{50}) was determined using commercially available software (Calcusyn; Biosoft, Manchester, United Kingdom).

Table 3. Combination Index (CI) of apoptosis[a]

Cell lines	α-TEA:drug[c]	CI[b] ED_{50}	ED_{75}	ED_{90}	Mean ± SD[d]	
DOXO						
MDA-MB-231	20:1	0.48	0.40	0.35	0.41 ± 0.07	Synergism [e]
BT-20	20:1	0.48	0.52	0.58	0.53 ± 0.05	Synergism
CDDP						

| MDA-MB-231 | 2:1 | 0.55 | 0.44 | 0.36 | 0.45 ± 0.10 | Synergism |
| BT-20 | 4:1 | 0.68 | 0.74 | 0.84 | 0.75 ± 0.08 | Synergism |

[a]MDA-MB-231 and BT-20 breast cancer cells were treated with different concentrations of α-TEA, DOXO and CDDP alone and in combinations for 24 hrs. Apoptosis was determined using Annexin V-FITC/PI staining /FACS assay as described in Material and Methods.

[b]For each combination treatment, a combination index (CI) was calculated using commercially available software (Calcusyn; Biosoft, Manchester, United Kingdom).

[c]The ratio for the concentrations that were used in combination treatments was determined from the data in Fig1.

[d]The mean $\pm$ SD is calculated from the CI values of ED_{50}, ED_{75} and ED_{90}.

[e]CI values <1.0 indicate synergism, CI values = 1.0 indicate additive effect and CI values >1.0 indicate antagonism.

	(a)	MDA-MB-231					
α-TEA (μM)	0	10	0	10	20	0	20
DOXO (μM)	0	0	0.5	0.5	0	1	1

	(b)	BT-20					
α-TEA (μM)	0	10	0	10	20	0	20
DOXO (μM)	0	0	0.5	0.5	0	1	1

	(c)	MDA-MB-231					
α-TEA (μM)	0	10	0	10	20	0	20
CDDP (μM)	0	0	0.5	0.5	0	1	1

	(d)	BT-20					
α-TEA (μM)	0	10	0	10	20	0	20
CDDP (μM)	0	0	0.5	0.5	0	1	1

(a)

α-TEA (20 μM)

	VEH		DOXO (0.5 μM)			VEH		CDDP (5 μM) (24 hrs)	
	−	+	−	+		−	+	−	+

Pro-caspase-8
ccaspase-8
Pro-caspase-9
ccaspase-9
Pro-PARP
cPARP
GAPDH

MDA-MB-231

(b)

α-TEA (20 μM)

	VEH		DOXO (0.5 μM)			VEH		CDDP (2.5 μM) (24 hrs)	
	−	+	−	+		−	+	−	+

Pro-caspase-8
ccaspase-8
Pro-caspase-9
ccaspase 9
Pro-PARP
cPARP
GAPDH

BT-20

Figure 2

(a) α-TEA (20 μM) — Control DOXO (0.5 μM) Control CDDP (5 μM) (24 hrs); p73; GADPH; MDA-MB 231

(b) α-TEA (20 μM) — Control DOXO (0.5 μM) Control CDDP (2.5 μM) (24 hrs); p73; GADPH; BT-20

(c) Control siRNA; p73 siRNA; Apoptosis (%); DOXO (0.5 μM); CDDP (5 μM); α-TEA (20 μM); MDA-MB 231

(d) Control siRNA; p73 siRNA (24 hrs); α-TEA (20 μM); DOXO (0.5 μM); CDDP (5 μM); Pro-PARP; cPARP; p73; GAPDH; MDA-MB 231

Figure 3

(a)

Control | DOXO (0.5 µM) | CDDP (5 µM) | (24 hrs)

α-TEA (20 µM): − + − + − +

DR5, Fas, Bax, Noxa, Bcl-2, β-actin

MDA-MB-231

(b)

Control | DOXO (0.5 µM) | CDDP (2.5 µM) | (24 hrs)

α-TEA (20 µM): − + − + − +

DR5, Fas, Bax, Noxa, Bcl-2, β-actin

BT-20

(c)

Control | DOXO (0.5 µM) | Control | CDDP (5 µM) | (24 hrs)

α-TEA (20 µM): − + − + − + − +

DR5, Fas, Bax, Noxa, Bcl-2, GAPDH

MDA-MB-231

(d)

Control | DOXO (0.5 µM) | Control | CDDP (2.5 µM) | (24 hrs)

α-TEA (20 µM): − + − + − + − +

DR5, Fas, Bax, Noxa, Bcl-2, GAPDH

BT-20

(e)

Control siRNA | p73 siRNA | (24 hrs)

α-TEA (20 µM): − + + − + +
DOXO (0.5 µM): − + − − + −
CDDP (5 µM): − − + − − +

p73, DR5, Fas, Bax, Noxa, Bcl-2, GAPDH

MDA-MB 231

(f)

Control | DOXO | DDDP

α-TEA (20 µM): − − + + + +
control siRNA: + − + − + −
DR5 siRNA: − + − + − +

Pro-PARP, cPARP, DR5, GAPDH

MDA-MB 231

Figure 4

(a)

α-TEA (20 µM)   Control   DOXO (0.5 µM)   Control   CDDP (5 µM)   (24 hrs)

pc-Abl (Tyr 245)
tc-Abl
pJNK (2/1)
tJNK (2/1)

MDA-MB 231

(b)

α-TEA (20 µM)   Control   DOXO (0.5 µM)   Control   CDDP (2.5 µM)   (24 hrs)

pc-Abl (Tyr 245)
tc-Abl
pJNK (2/1)
tJNK (2/1)

BT-20

(c)

Apoptosis (%)

Control siRNA
c-Abl siRNA
JNK siRNA

DOXO (0.5 µM)
CDDP (5 µM)
α-TEA (20 µM)

MDA-MB 231

(d)

Control siRNA   c-Abl siRNA   JNK siRNA   (24 hrs)

α-TEA (20 µM)
DOXO (0.5 µM)
CDDP (5 µM)

Pro-PARP
c PARP
pc-Ab1 (Tyr 245)
pJNK2/1
p73
DR5
Fas
Bax
Noxa
Bcl-2
GAPDH

MDA-MB 231

Figure 5

(a)

(b)

Figure 6

(a)

Nuclear fraction   **Cytoplasmic fraction**

| | VEH | DOXO (0.5 µM) | CDDP (5 µM) | | VEH | DOXO (0.5 µM) | CDDP (5 µM) | (24 hrs) |

α-TEA (20 µM): − + − + − +   − + − + − +

tYap

Histone 1

GAPDH

MDA-MB 231

(b)

| | Control | DOXO (0.5 µM) | Control | DOXO (0.5 µM) | (24 hrs) |

α-TEA (20 µM): − + − +   − + − +

pAkt (Ser 473)

tAkt

pYap (Ser 127)

tYap

MDA-MB 231

(c)

| | Control | DOXO (0.5 µM) | CDDP (5 µM) | (24 hrs) |

PI3K In (1 µM): − + − + − +

p73

pAkt (Ser 473)

pYap (Ser 127)

GAPDH

MDA-MB 231

Figure 7

α-TEA + DNA damage drugs

DNA damage drugs

pAkt

c-Abl → JNK

Yap

Nucleus

Yap

c-Abl

p73

Yap
p73
p300

p53 mediated genes
(DR5?, Fas, Bax, Noxa)

↓Bcl-2 ⊣

Apoptosis

Figure 8