

Synergistic effect of 17-allylamino-17-demethoxygeldanamycin with dehydroxymethylepoxyquinomicin on the human anaplastic thyroid carcinoma cell line KTC2

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Abstract: The use of targeted inhibitors has shown promise as an effective approach in cancer therapy. However, targeted therapies based only on one drug, such as 17-allylamino-17-demethoxygeldanamycin (17-AAG), have limited success, partly because cancer cells engage alternate pathways for survival and proliferation. In the present study, we evaluated whether dehydroxymethylepoxyquinomicin (DHMEQ), a nuclear factor κ B (NF- κ B) inhibitor, can enhance the antitumor activities of 17-AAG, a 90 kDa heat shock protein (Hsp90) inhibitor, in the anaplastic thyroid cancer cell line KTC2. We examined the effect of combined drug treatment vs single drug treatment on cell survival. Isobologram analysis was performed to distinguish the additive vs synergistic effects of the drug combination. Western blotting was performed to investigate apoptosis markers: caspase 3, poly(ADP-ribose) polymerase-1 (PARP-1), B-cell lymphoma-extra large (Bcl-XL), X-linked inhibitor of apoptosis (XIAP) and cellular inhibitor of apoptosis 2 (cIAP-2). Compared to monotherapy, the combined treatment enhanced growth-inhibitory effects in a synergistic manner and strongly potentiated apoptosis. These results demonstrate the first *in vitro* evidence that a combination of Hsp90 and NF- κ B inhibitors is a more effective modality for inhibiting cell proliferation and survival in anaplastic thyroid carcinoma cells than either agent alone, warranting further investigations.

Keywords: NF- κ B inhibitor; Hsp90 inhibitor; synergy; targeted inhibitor; combined treatment

INTRODUCTION

Anaplastic thyroid cancer (ATC) is a rare, undifferentiated thyroid cancer, with a high malignant potential and poor prognosis. The five-year survival rate from this type of cancer is less than 5%, with most patients dying within 3 to 6 months of diagnosis despite conventional multimodality treatment strategies, which include surgery, external beam radiotherapy and chemotherapy [1,2]. Treatment failure in ATC is often associated with resistance to chemotherapy drugs. Thus,

there is a pressing need for more effective therapeutic approaches, such as those including targeted inhibitors, specially when used in combination. Targeted inhibitors act on specific target molecules resulting in the inhibition of key signaling cascades in malignant tumor cells. Among the different classes of promising molecular targets for therapeutic purposes in ATC are heat shock proteins (HSP) and NF- κ B [2].

Hsp90 functions as a molecular chaperone by binding to different cellular proteins (protein kinases,

transcription factors, etc.), and supporting their proper folding, stability and function. Many of these client proteins are involved in cell proliferation, differentiation, invasion, metastasis and drug resistance and are thus frequently mutated or overexpressed in cancer. Intact Hsp90 chaperone function is what cancer cells are particularly dependent on, since inhibition of Hsp90 leads to ubiquitin-proteasome degradation of its client proteins. Moreover, enhanced Hsp90 affinity for mutated or functionally deregulated target proteins in cancer cells has been observed and documented. This explains why Hsp90, which is often overexpressed in cancer, plays a critical role in tumor cell growth and/or survival [3-5]. Hsp90 operates as an ATP-binding system. The benzoquinone ansamycin antibiotic geldanamycin and its less toxic analog with improved bioavailability, 17-AAG, were found to inhibit Hsp90 multi-chaperone complex formation by docking into the ATP-binding site in the N-terminal domain of this protein [6,7]. Although Hsp90 is expressed both in normal and tumor cells, it was determined that tumor cells harbor a finely-tuned Hsp90 fraction that exists in an altered conformation, with a 100-fold higher binding affinity for 17-AAG than Hsp90 derived from normal cells [8,9]. This, as well as the fact that Hsp90 regulates multiple proteins involved in different processes during cancer development and progression, makes it a unique anticancer target. Cytotoxic activities of 17-AAG were demonstrated in different thyroid cancer cell lines among others, including the anaplastic thyroid cancer cell lines ARO, FRO, ACT1, 8505C and CAL62 [10-13]. A number of phase I, II and III clinical trials of 17-AAG have been conducted so far, as summarized in [14]. Data from these clinical trials show that 17-AAG can be given safely at biologically active dosages, with mild toxicity. Although phase I and II clinical trials of 17-AAG as a single agent did not demonstrate considerable clinical efficiency, the combination of 17-AAG with other anticancer drugs showed more promise regarding the clinical benefit for cancer patients [14].

NF- κ B is a ubiquitously expressed proinflammatory transcription factor that regulates the expression of a number of genes involved in inflammation as well as in cell proliferation, apoptosis, invasion, angiogenesis and metastasis. NF- κ B is typically a heterodimer comprised of Rel family proteins p50 and p65. These dimers are usually located in the cytoplasm in an inactive form

as a result of association with inhibitory factors, I κ B proteins. A number of extracellular signals can lead to phosphorylation and degradation of I κ B, resulting in NF- κ B activation and translocation to the nucleus, where it binds to specific promoters and regulates target gene expression [15]. As an antiapoptotic factor, NF- κ B is one of the major reasons of resistance to chemotherapy, and it crucially affects the outcomes of cancer treatments [16]. ATC often exhibits constitutive and strong activation of NF- κ B, which is extensively correlated with resistance to therapy and the aggressiveness of ATC [17]. Furthermore, a recent study identified NF- κ B signaling pathways to be among the key pathways that regulate cancer stem cell properties in ATC [18]. An NF- κ B inhibitor – DHMEQ, a derivative of the antibiotic epoxyquinomicin C, has been found to inhibit tumor necrosis factor (TNF)-induced activation of NF- κ B by suppressing NF- κ B nuclear translocation, thus promoting apoptosis in a variety of cancer cells [19-22], including anaplastic thyroid carcinoma cells [23].

The aim of this study was to determine whether the combined treatment with 17-AAG and DHMEQ displays improved antitumor activity over either agent alone in the anaplastic thyroid carcinoma cell line KTC2. To our knowledge, this is the first time that the combined effect of these two agents is investigated in the anaplastic thyroid carcinoma cells.

MATERIALS AND METHODS

Reagents

Both 17-AAG and DHMEQ were dissolved in 99.9% dimethyl sulfoxide (DMSO) (Wako Chemicals, Osaka, Japan) at stock concentrations of 1 mM and 10 mg/mL, respectively, and stored at -20°C. Primary antibodies were obtained from the following sources: Bcl-XL polyclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA); XIAP polyclonal antibody, PARP-1 polyclonal antibody, and cleaved caspase 3 polyclonal antibody were all from Cell Signaling Technology (Danvers, MA, USA). The polyclonal antibody to cIAP-2 was from R&D Systems (Minneapolis, MN, USA). Secondary antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA).

Cell culture

The human anaplastic thyroid carcinoma cell line, KTC-2, derived from pleural effusion metastasis, with no TP53 mutation, was provided by the Department of Molecular Medicine, Medical School, Nagasaki University, Japan. Cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 5% fetal bovine serum (FBS) and 1% (w/v) penicillin/streptomycin in a 5% CO₂ humidified atmosphere at 37°C. Primary human thyroid cell culture was established as described [24] and maintained in a Dulbecco's Modified Eagle Medium (DMEM):F12 mixture (1:2) supplemented with 3% FBS and 1% (w/v) penicillin/streptomycin [all reagents were obtained from Sigma (St. Louis, MO, USA)].

Cell survival assay

Cell suspensions (100 µL, 3000 cells per well) were added to each well of 96-well plate flat-bottom microtiter plates (NalgeNunc International, Tokyo, Japan) in RPMI 1640 containing 5% FBS, and incubated for 24 h before treatment. Solutions, in increasing concentrations ranging from 1 nM to 1 µM of 17-AAG and with DHMEQ at a dose range of 0.1 µg/mL to 20 µg/mL, respectively, were added to each well in 11 µL medium. The control wells contained DMSO at a final concentration of 0.1%. After incubation, a water-soluble tetrazolium salt (WST)-based assay was performed as follows: 10 µL of the cell counting kit solution (CCK-8, Dojin, Osaka, Japan) were added to each well and incubated for 1 h at 37°C. Optical densities were measured at 450 nm in a microplate reader. Thus, the drug concentrations of 100 nM of 17-AAG and 2-10 µg/mL DHMEQ were determined as the most effective after 24, 48 and 72 h of treatment.

Analysis of the combined drug effects

Drug synergy was determined by the combination-index and isobologram analyses. Briefly, the combination-index (CI) is a quantitative representation of the degree of drug interaction calculated by the following equation $(CI) = (D)1/(D\chi)1 + (D)2/(D\chi)2$ using the CalcuSyn software (Biosoft, Ferguson, MO, USA) where (D χ)₁ and (D χ)₂ are the concentrations of each drug alone, which exert χ % effect while (D)₁ and (D)₂ are the

concentrations of drugs in combination, which elicit the same effect. The fraction affected (Fa) represents the fraction affected by a given drug concentration; the evolution of drug interactions can be assessed by the Fa-CI plot. On the basis of the dose-response curves obtained from the cell survival assay for KTC2 treated with the inhibitors, alone or in combination, the CI values were generated over the range of fraction affected (Fa) levels from 5%-95% growth inhibition. Synergism, additivity and antagonism are defined as CI<1, CI=1 and CI>1, respectively [25].

Preparation of cell extracts and Western blot

After the indicated treatment duration, the treated cells were washed twice in an ice-cold phosphate-buffered saline (PBS), collected in 1 mL of PBS and centrifuged at 1000 ×g for 3 min. The pellets were resuspended in 200 µL of lysis buffer (Cell Signaling Technology, Beverly, MA, USA) containing protease and phosphatase inhibitors and incubated for 15 min on ice. Lysates were then centrifuged for 15 min at 25000 ×g at 4°C, and the supernatants were stored at -80°C. Protein concentrations were determined with the bicinchoninic acid (BCA) assay kit (Sigma-Aldrich, St. Louis, MO, USA).

Western blotting analysis was performed according to standard procedures as described previously [26]. Proteins were resolved by the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA), and subjected to immunoblotting with appropriate primary and secondary antibodies. The antigen-antibody complexes were visualized using chemiluminescent reagents (Amersham, Arlington Height, IL, USA). Densitometry analysis was performed using ImageJ software [27].

RESULTS

Cytotoxic effects of 17-AAG and DHMEQ in anaplastic thyroid carcinoma cells

The combined treatment of 17-AAG and DHMEQ strongly enhanced the growth-inhibitory effect in KTC2 cells compared to each single drug treatment. This effect was already evident after 24 h of treatment, as shown in Fig. 1A. The inhibitory effect was increased

even more after 48 h of treatment (Fig. 1B) and by 72 h, no cells survived at any of the 4 indicated dose combinations (data not shown). In contrast, cultured primary thyrocytes showed substantially lower sensitivity to combined treatment, and no significant effect on primary cell survival was observed until the DHMEQ dose was increased to 10 µg/mL (Fig. 1C and 1D).

17-AAG and DHMEQ combined drug effect

The combination index method showed a synergistic antiproliferation effect of 17-AAG and DHMEQ in KTC2 cells, which was observed at all 4 dose combinations (Fig. 2).

Apoptotic changes in 17-AAG and DHMEQ treated thyroid cancer cells

Immunoblot analysis showed that the combined treatment triggered the cleavage of proapoptotic molecules,

caspase 3 and PARP-1, in KTC2 cells. Higher levels of cleaved PARP-1 and caspase 3 appeared after 48 h of combined treatment with 100 nM of 17-AAG and 2 µg/mL of DHMEQ, and this trend was also evident when higher doses (4, 8 and 10 µg/mL) of DHMEQ were used. Also, the combined treatment markedly reduced the expression of inhibitors of apoptosis Bcl-XL, XIAP and cIAP-2, when compared to the effect observed with either agent alone (Fig.3).

DISCUSSION

After promising preclinical studies of 17-AAG in various cancer cell lines and xenograft models [12,28-30], single agent clinical studies of this compound had limited success [14,29]. On the other hand, studies of 17-AAG in combination with other anticancer drugs showed more clinical benefit for cancer patients, despite drawbacks such as low water solubility and high hepatotoxicity, which could be addressed in the future by

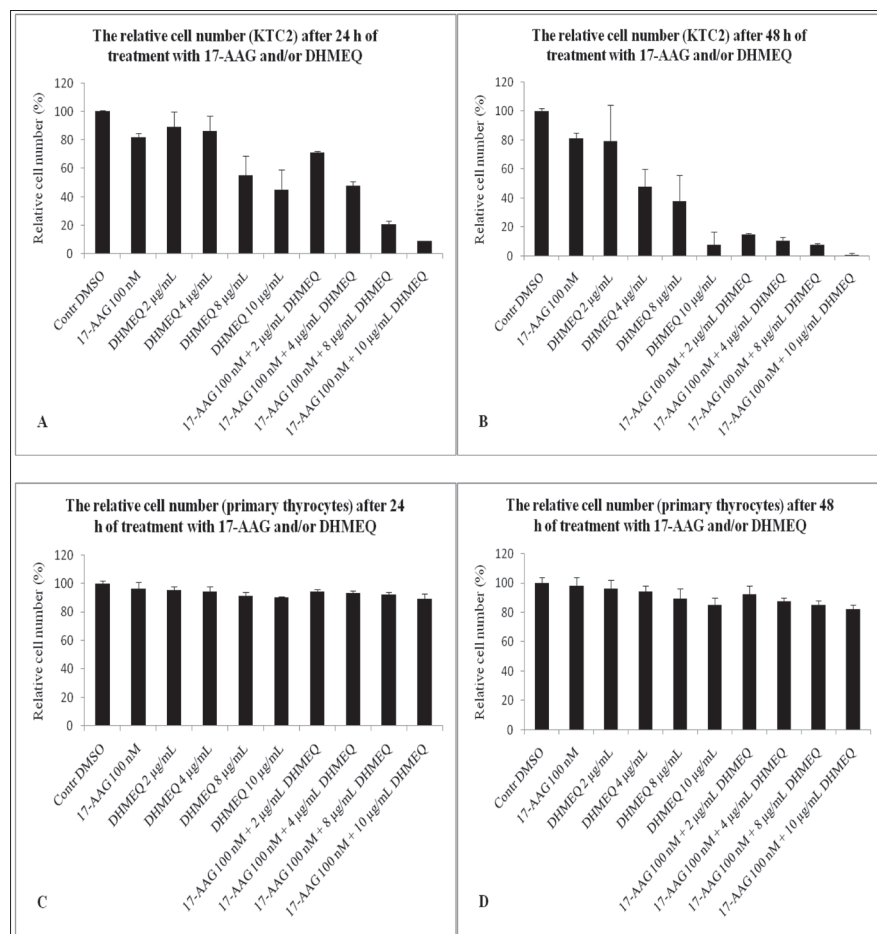


Fig. 1. Cell viability and synergy achieved by the combination of 17-AAG and DHMEQ in KTC2 cells and primary thyrocytes. Cells were treated with different concentrations of single or both drugs, and viabilities were determined by the WST-based assay as explained in the Materials and Methods. **A, B** – viability of KTC2 cells treated with the indicated concentrations of 17-AAG and DHMEQ for 24 h and 48 h, respectively; **C, D** – viability of cultured primary thyrocytes treated with the indicated concentrations of 17-AAG and DHMEQ for 24 h and 48 h, respectively. 17-AAG – 17-allyl-amino-17-demethoxygeldanamycin; DHMEQ – dehydroxymethylepoxyquinomicin; Bars, mean±SD from three replicates.

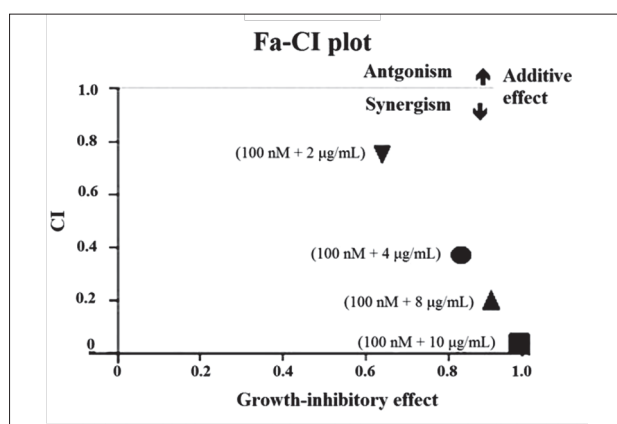


Fig. 2. Combination index analysis of 17-AAG/DHMEQ. The straight line (additivity line) is the locus of all dose pairs that should give the same effect. A dose pair below the line is synergistic, a dose pair on the line is additive, a dose pair above the line is antagonistic. 17-AAG was found to potently synergize with DHMEQ at 4 indicated dose pairs after 24, 48 and 72 h of treatment. The growth-inhibitory effect was measured by the WST-based assay. The figure represents the analysis after 24 h of treatment.

implementation of nanomaterials-based drug delivery carriers [14]. However, more studies in various types of malignant tumors are needed to assess the real clinical potential of this Hsp90 inhibitor, as well as to identify other agents capable of improving its anticancer effects. DHMEQ, an NF- κ B inhibitor, was shown to be a potent chemo- and immunosensitizing agent which, in combination with cytotoxic therapeutics resulted in significant reversal of resistance and tumor cell death in various tumors, including anaplastic thyroid carcinoma [20,31-36]. To our knowledge, no studies of the joint effect of these two compounds in cancer cells have been conducted so far.

In the present study, we analyzed the effects of the combined treatment of 17-AAG and DHMEQ in human anaplastic thyroid carcinoma cell line KTC2, as compared to the effect of either agent alone. Consistent with previous studies, both DHMEQ and 17-AAG

alone displayed a cytotoxic effect on thyroid cancer cells [10-12,23,36]. The combined treatment, however, already after the first 24 h strongly enhanced the growth-inhibitory effect compared with the single agent treatment. This effect was increased even more after 48 h, and by 72 h no cells survived. Moreover, we observed a synergistic antiproliferative effect of the combined treatment with all 4 dose combinations of the two agents. Furthermore, we established that the combined treatment of 17-AAG and DHMEQ potentiated apoptosis, this being probably one of the mechanisms for controlling tumor cell growth. Apoptosis induction was confirmed by cleavage of some of the characteristic indices of apoptosis, such as caspase 3 (the key executioner of apoptosis) and PARP-1 (the main cleavage target of caspase 3). Also, the combined treatment markedly reduced the expression of the antiapoptotic protein Bcl-xL, as well as the expression of cIAP-2 and XIAP, which are members of the inhibitors of apoptosis family (IAPs) and are known to be under transcriptional regulation of NF- κ B [37]. Consistent with previous studies, in our study the treatment

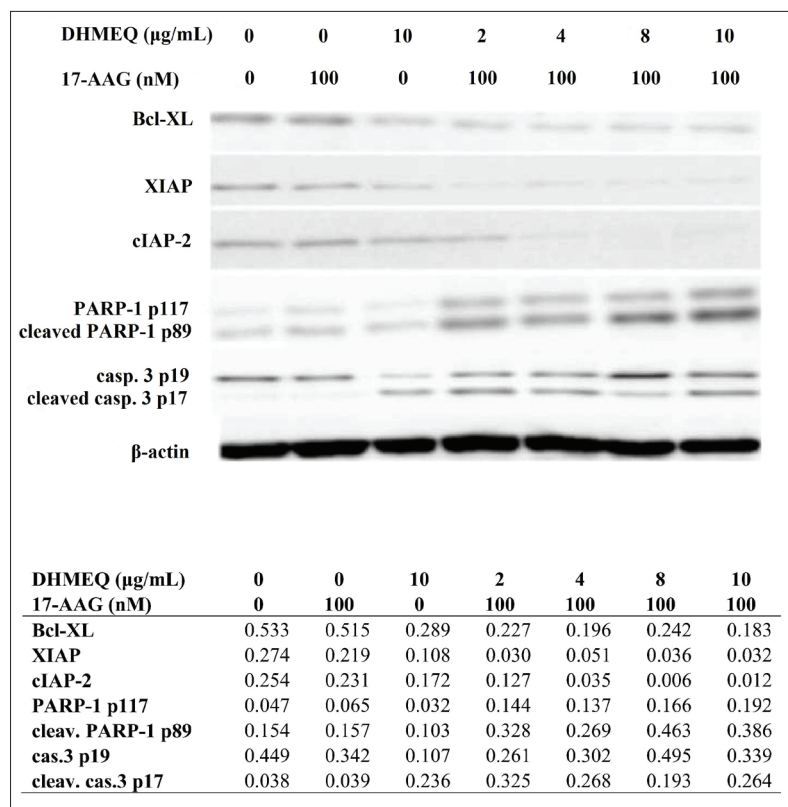


Fig. 3. Western blot analysis of the effect of the drugs on changes of pro- and anti-apoptotic factors. Cells were treated with the indicated concentrations of 17-AAG and DHMEQ for 48 h and whole-cell lysates were examined by Western blotting. β -actin was used as the loading control. The densitometry results are presented in the table below the Western blot. The expression levels of target proteins are normalized to β -actin.

with 17-AAG alone was not associated with caspase 3 cleavage nor significant changes of expression of Bcl-XL, XIAP and cIAP-2 [11,12] while DHMEQ treatment was most probably the result of NF- κ B inhibition [22, 23]. However, the changes in expression of the apoptotic markers were more prominent when the combined treatment of 17-AAG and DHMEQ was applied. The higher levels of cleaved PARP-1 and caspase 3 appeared after 48 h of combined treatment and were evident at all dose combinations. While some studies [22,23] have reported that the changes in expression level of Bcl-XL in ATC cells treated with DHMEQ were not as prominent as the suppression of IAPs, we showed that the combined treatment markedly reduced the expression level of this antiapoptotic protein, which was also accompanied by a significant reduction in the expression level of XIAP and cIAP-2. Taken together, these results are in agreement with other studies reporting that DHMEQ can act synergistically with different therapeutic agents in promoting apoptosis in various cancer cell lines [33,38-41]. In contrast to carcinoma cells, in primary thyrocytes the combination treatment was accompanied by more than 90% cell survival rate. This is in accordance with previous studies in which it was reported that cancer cells are significantly more sensitive to Hsp90 inhibition than non-transformed cells, as reviewed in [5,29], and studies indicating that normal thyroid epithelial cells, compared to cancer cells, show relative resistance to DHMEQ-induced apoptosis [23].

In light of the fact that dose-related toxicities were among the drawbacks for a more successful treatment with 17-AAG in clinical studies, another interesting finding of our study is that potent growth inhibition was achieved by using an overall lower dose of 17-AAG (when combined with various doses of DHMEQ) compared to doses used thus far [10-12,42,43]. This might indicate a potential strategy to decrease 17-AAG off-site toxicity in *in vivo* studies, justifying further investigations.

Finally, we would like to emphasize some of the limitations of the present study. The first is that the study included only one ATC cell line; it would be prudent to repeat the experiments on several other ATC cell lines. We also did not address the effect of 17-AAG and DHMEQ on the activity of their targets, Hsp90 and NF- κ B, respectively, in order to confirm their

interaction. Since quantitative Western blotting experiments using untested assumptions of proportionality for normalization can be misleading (as described in [44]), our results of the expression of apoptosis markers should be taken with caution when interpreting the results related to the effect of the drugs on apoptosis.

CONCLUSION

Herein we provide the first *in vitro* evidence that the combination of Hsp90 and NF- κ B inhibitors, 17-AAG and DHMEQ respectively, working in a synergistic manner, strongly inhibit cancer cell growth and potentiate apoptosis in human anaplastic thyroid carcinoma cells. Further *in vitro* and *in vivo* studies of the joint action of these two compounds are needed in order to clarify the underlying mechanisms, as well as to provide an estimate of their potential for translational applications in anaplastic thyroid carcinoma patients.

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