p53 independent radio-sensitization of human lymphoblastoid cell lines by Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin

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Abstract. Inhibition of heat shock protein 90 (Hsp90) is an attractive modality for cancer therapy. Recent studies presented that an Hsp90 inhibitor, 17AAG (17-allylamino-17-demethoxy-geldanamycin), enhanced tumor radio-sensitivity, while this was not observed in normal cells. One of the studies reported that the effect of this drug was only observed in tumor cells carrying the wild-type p53 gene, thus demonstrating p53-dependent tumor radio-sensitization by 17AAG. We have now tested the effects of 17AAG on two human lymphoblastoid cell lines from the same donor, TK6 cells with the wild-type p53 gene and WTK1 cells with the mutated p53 gene. The effects of 17AAG were tested at concentrations of 10 and 100 nM on various parameters, including growth inhibition of the cells, enhancement of radio-sensitivity by colony formation assay, apoptosis and chromosomal radio-sensitivity and abrogation of radiation induced G2/M checkpoint. When 100 nM 17AAG was applied, all of these parameters were enhanced in a similar fashion in both cell lines, indicating that the drug effect is p53-independent. Our results suggest that 17AAG is likely to be an effective sensitizer for radiotherapy, even on tumors with mutated p53.

Introduction

Heat shock protein 90 (Hsp90) is a molecular chaperone that plays a role in refolding and localization of proteins in cells and regulates signal transductions (1,2). Client proteins of Hsp90 include cdc2 and cdc25c which are involved in the G2/M check point (3). Cdc37 interacts with Hsp90 and is involved in regulation of Erk, Akt, mTOR and androgen-induced pathways (4). Hsp90 is over-expressed in many tumor types (2). Oncogenic client proteins of Hsp90 include EGFR, ErbB-2 (5), steroid hormone receptors (6), mutant p53 (7), Raf-1, Akt and HIF-1 (8). Therefore, modulation of Hsp90 offers a prospect of simultaneously inhibiting these multiple signaling pathways that have been implicated in the development of the malignant phenotype (9). Therefore, the inhibition of Hsp90 has recently been regarded as a promising strategy to treat various cancers (2,10).

17-Allylamino-17-demethoxy-geldanamycin (17AAG) is a derivative of geldanamycin, a benzoquinoid ansamycin compound and an inhibitor of the Hsp90 (5,11,12). Several studies reported that 17AAG has a significant anti-cancer property by inhibiting Hsp90 and the downstream signal pathways of client proteins (13,14). It was also reported that an inhibition of Hsp90 blocks the tumor motility and invasion *in vitro* and metastasis *in vivo* (8). Furthermore, 17AAG was also reported to function as a radio-sensitizer (3,13,14). 17AAG inhibits the PI3K-Akt pathway (13) which results in high apoptosis induction, and an inhibition of DSB repair was also reported in tumor cells but not in normal human cells (2,15). The Phase I clinical trial of 17AAG has been completed and the Phase II clinical trial is currently underway (16).

The drug effects often depend on the genetic background of particular tumor cells. The p53 tumor suppressor gene is the most commonly mutated in human cancers (17,18). Tumor cells with an altered p53 are generally more resistant to radiation than cells expressing wild-type p53. Using human squamous carcinoma cell lines, a recent study indicated that the radio-sensitization by 17AAG is dependent on the p53 status (19). We aimed at clarifying the effect of the p53 status on the radio-sensitization by 17AAG, and used human lympho-blastoid cell lines, TK6 with the wild-type p53 gene and WTK1 with the mutated p53 gene (20-22). We demonstrated that 17AAG-induced radio-sensitization is not dependent on p53 status in lymphoblastoid cells.

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Materials and methods

Cell lines and culture. Two human lymphoblastoid cell lines, TK6 with the wild-type p53 gene and WTK1 with the mutated p53 gene, were kindly supplied by Dr H.L. Liber, Colorado State University. They were grown in RPMI-1640 (Sigma, USA) supplemented with 10% heat-inactivated (56˚C for 30 min) fetal bovine serum, antibiotics and antimicotics (Gibco, Tokyo, Japan) in a humidified 5% CO_2 atmosphere at 37˚C. Cells were cultured in T25 flasks.

Irradiations and drug treatments. Exponentially growing cell cultures were irradiated at a dose of 1 Gy/min using a Pantac HF-320S type X-ray irradiator (Shimadzu, Kyoto, Japan). Irradiation of the cells in T25-flasks was carried out at room temperature. For the drug treatment, cells were pre-incubated in a medium containing 10 or 100 nM of 17AAG for 24 h prior to irradiation. The cells were irradiated in the same medium containing the drug and the medium was replaced the one without 17AAG after irradiation.

Cell proliferation and colony formation assay. The effects of 17AAG were tested on the growth of TK6 and WTK1 cells. The cells were cultivated in a medium containing either 10 or 100 nM of 17AAG and the cell number was counted every 12 h for 5 days using Coulter Counter.

As for the colony survival, the 17AAG-treated and irradiated cells were serially diluted and seeded onto 96-well plates. The cells were incubated without the drug at 37˚C for 10-14 days to allow for colonies to form. Colony survival was calculated as described previously (23) and expressed as a percentage normalized to that of untreated cells (as 100%).

Cell cycle distribution and analysis of apoptosis. 17AAG-treated and irradiated cells were monitored for cell cycle distribution. After 24 h of 17AAG pretreatment, cells were irradiated with 4 Gy of X-rays. Cells were incubated in a medium containing the drug for appropriate time. They were then fixed with 70% ethanol and analyzed for the distribution of DNA content and cell cycle phases using FACSCalibur (Becton-Dickinson).

TUNEL assay was used for detection of apoptotic cells with the use of the apoptosis detection kit (Molecular Probe, Tokyo, Japan). Briefly, log phase growing cells were treated with 17AAG for 24 h and then exposed to 4 Gy X-rays. The cells were further incubated in a medium containing the drug and monitored for apoptosis at 24 and 48 h after irradiation.

G2 chromosome assay. Chromosome radio-sensitivity was monitored by the G2 chromosomal assay using the method of Scott and co-worker (24-26). The drug-treated cells were exposed to 1 Gy X-rays, and incubated for another 30 min in the drug containing medium. Colcemid was then added to the culture and the cells were further incubated for 1 h to harvest mitotic cells. The cells were treated with 75 mM KCl for 20 min at 37˚C, fixed with methanol acetic acid solution and droped onto slides. Slides were stained by Giemsa solution for scoring the metaphase chromosomes that would have arisen in cells irradiated in mid to late G2.

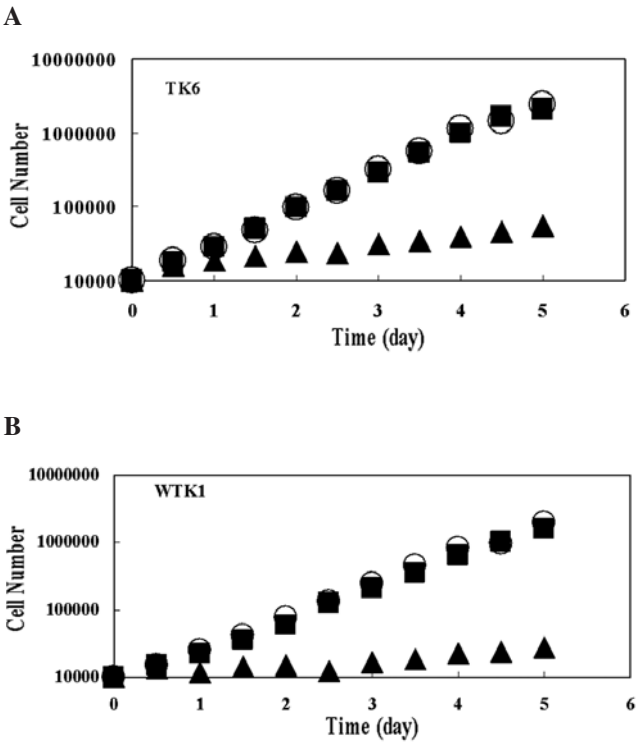

Figure 1. Suppression of cell growth by 17AAG. Cell growth was monitored for TK6 (A) and WTK1 (B) cell lines in the presence of 17AAG. ○, 0 nM; ■, 10 nM; ▲, 100 nM. Each data point represents average number of two separate experiments.

Results

The effects of 17AAG on the growth and the survival of TK6 and WTK1. We evaluated the drug toxicity by cell growth curves. Both TK6 and WTK1 cells double their number every 15 h in a fresh medium. The cell growth was not affected with 10 nM 17AAG in both TK6 and WTK1 cell lines. On the other hand, at a higher concentration of 100 nM, the cell growth was significantly reduced in both cell lines. The reduction was not dependent on p53 status although the effect was stronger in p53-mutated WTK1 (Fig. 1).

As for cell survival by colony formation, the 17AAG treatment sensitized both cell lines to radiation at 10 nM as well as at 100 nM (Fig. 2). Previous studies (20-22) indicated that p53-mutated WTK1 cells were more resistant to ionizing radiation than TK6 cells, which were indeed the case in our present study. TK6 cells lacked the shoulder portion of the survival curve and its D10 value was ~1.5 Gy. 17AAG enhanced radiation induced cell killing and the D10 value was decreased to ~1 Gy. On the other hand, WTK1 cells had the shoulder in the survival curve and D10 value was ~2.5 Gy. 17AAG-sensitized the cells but the shouldered nature of the survival curve was unaffected. As a consequence, the D10 value decreased to ~2 Gy. Enhancement of radio-sensitivity with 17AAG was clearly independent of p53 status.

Abrogation of the G2/M checkpoint and enhanced apoptosis by 17AAG. In order to understand the radio-sensitization by 17AAG, we investigated the cell cycle distribution of irradiated cells by FACScan. As is clear from the results of Fig. 3, the

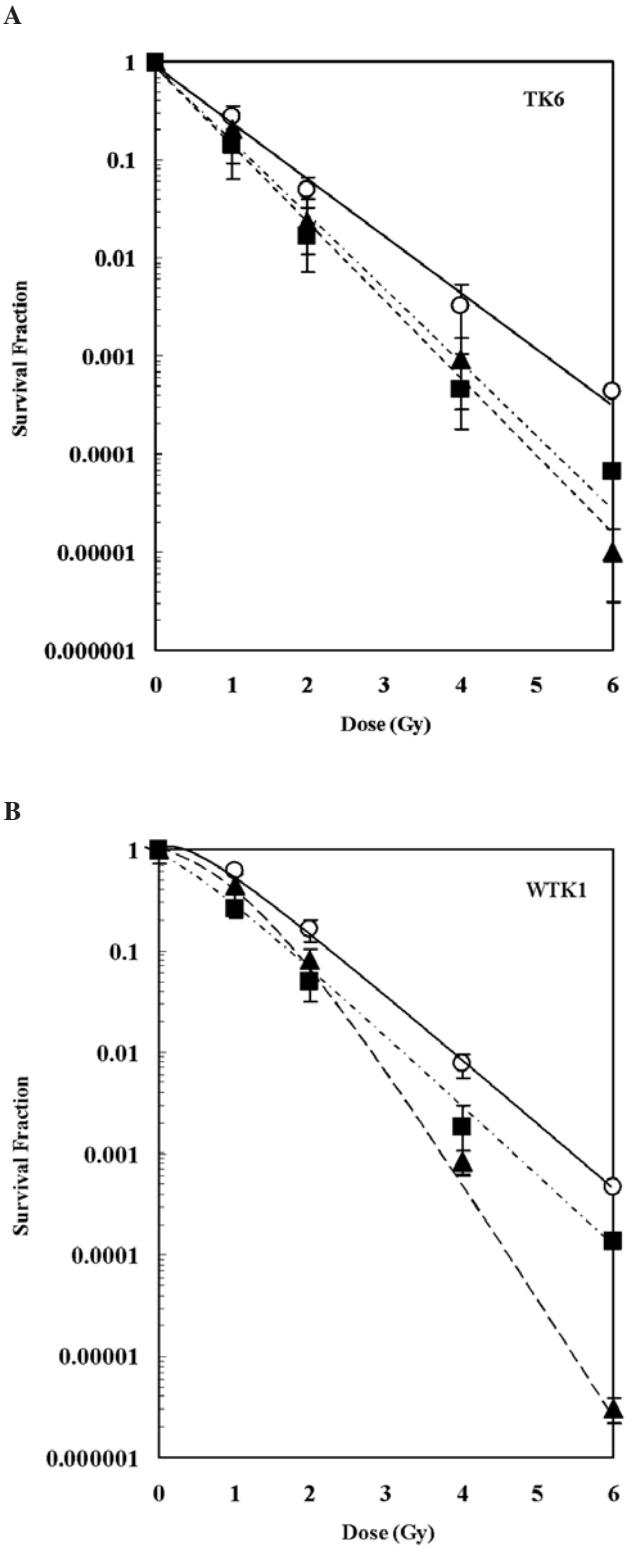

Figure 2. Clonogenic survival of irradiated cells and the effect of 17AAG. (A) TK6 (p53 wild-type) and (B) WTK1 (p53 mutated type) show the colony survival curves of X-irradiated cells treated with 17AAG. ○, 0 nM; ▲, 10 nM; ■, 100 nM.

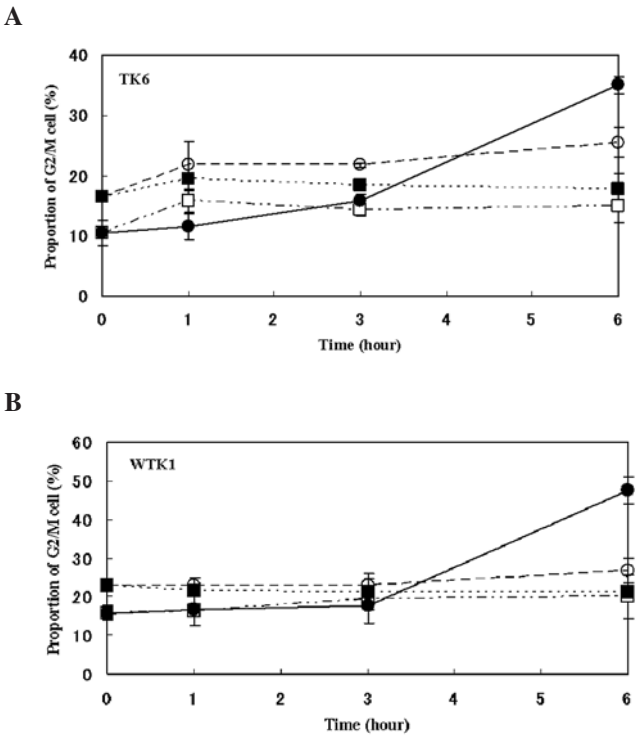

Figure 3. Abrogation of radiation-induced G2/M checkpoint by 17AAG. Accumulation of the cells in the G2/M phase of cell cycle was monitored for (A) TK6 cell and (B) WTK1. ●, X-ray only (4 Gy); ○, 17AAG (100 nM) and X-ray (4 Gy); ■, 17AAG only (100 nM); □, control.

G2/M check point was clearly functional in both cell lines and the fraction of cells in G2 phase increased after X-irradiation. However, when the cells were treated with 100 nM of 17AAG, this increase was not observed suggesting the abrogation of the G2/M checkpoint (Fig. 3). These results were consistent with a previous study (27). In addition, our present study indicates that this abrogation of the G2 checkpoint was independent of the p53 status of the cells.

Apoptosis is one of the basic modes of cell death (28) and lymphoblastoid cell lines are more prone to die by apoptosis than other cell types (29). The 24-h treatment with 17AAG itself was enough to induce apoptosis in both cell lines as measured by TUNEL assay (Fig. 4) and this is consistent with the previous studies (30,31). In addition, irradiation also induced apoptosis. When the two treatments, 17AAG pretreatment and irradiation, were combined, apoptotic cell death was greatly enhanced in TK6 cells (Fig. 4). Massive apoptotic cell death was observed in TK6 cells at 24 h after irradiation. In the case of WTK1, the apoptotic cell death by 17AAG and radiation were less pronounced than in TK6 cells. However, 17AAG definitely enhanced radiation induced apoptotic cell death in WTK1 cell line which was particularly pronounced at 48 h after irradiation. This suggests that the enhancement of apoptosis is not necessarily dependent on the p53 status in this lymphoblastoid cell system.

The 17AAG-mediated enhancement of chromosome radio-sensitivity. All the results above indicate that 17AAG enhances radio-sensitivity in both TK6 cells and in WTK1 cells. We have analyzed one more indicator of radio-sensitivity to further confirm the effects of 17AAG. The G2 chromosome radio-sensitivity is known as one of the most sensitive indicators and we used this to analyze the effects of 17AAG. The 24-h pretreatment of this drug slightly increased chromatid aberrations in both cell lines. In addition, the drug pre-treatment sensitized the cells to radiation induced G2

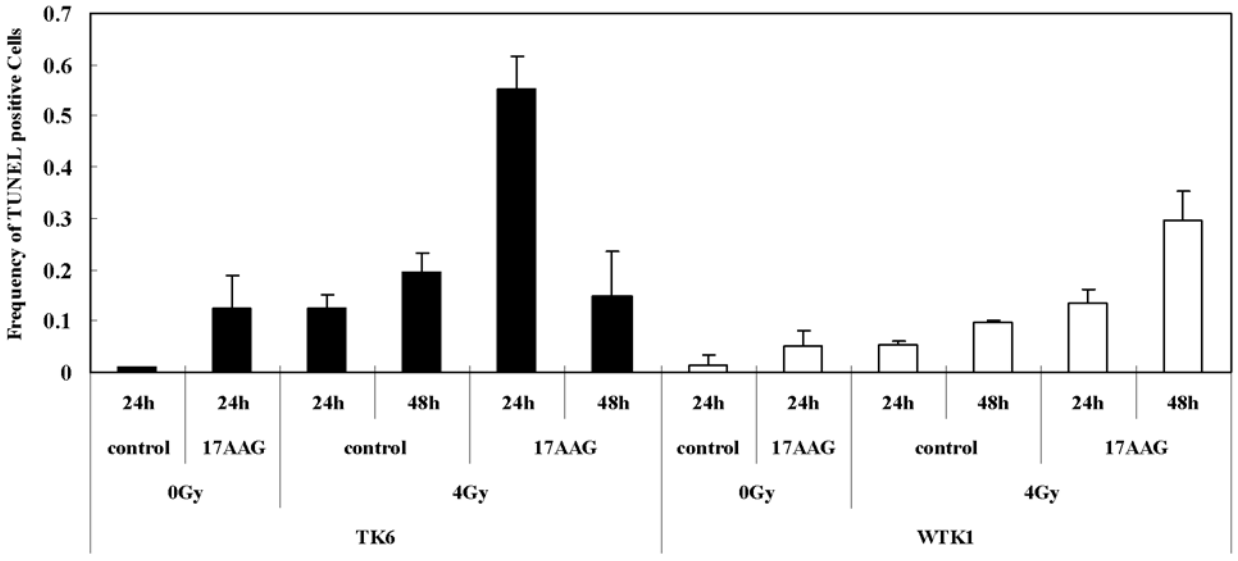

Figure 4. The frequency of apoptosis. Apoptosis was analyzed by the TUNEL assay for TK6 and WTK1 cells. The X-ray dose was 4 Gy and 17AAG 100 nM.

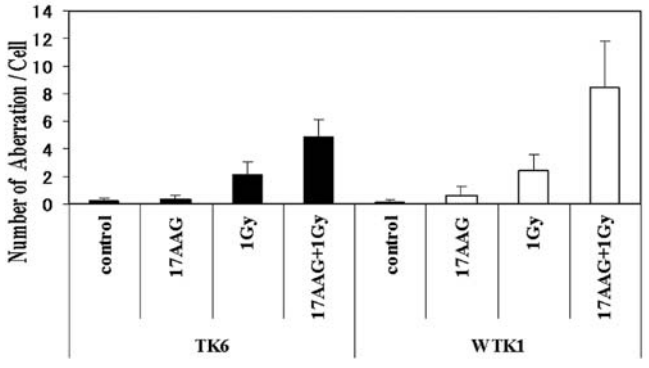

Figure 5. G2 chromosome analyses. G2 chromosome analyses were made on TK6 and WTK1 cells. The X-ray dose was 1 Gy and 17AAG 100 nM.

chromosome aberrations and this effect was more pronounced in WTK1 cells than in TK6 cells. Thus, the sensitization of radiation induced G2 chromosome aberrations by 17AAG was not dependent on the p53 status (Fig. 5).

Discussion

We demonstrated that 17AAG sensitized both TK6 cells and WTK1 cells to radiation. Radiation induced cell killing as assayed by colony formation was enhanced by the drug. 17AAG abrogated the radiation induced G2/M checkpoint which could well be one of the contributing factors of the radio-sensitization and this was consistent with previous studies (3,27). In addition, radiation-induced apoptosis and G2 chromosome aberrations were also enhanced by the drug in both cell lines.

The p53 tumor suppressor gene is the most commonly mutated gene in human cancer. The p53 tumor suppressor protein exhibits anti-proliferative effects such as cell cycle arrest and it also promotes apoptotic cell death (17,18). Also, it was reported that p53 mutations are often associated with decreased sensitivity to DNA damaging agents (17,19). Thereby, the p53 status plays a crucial role in cancer therapies including radio- and chemotherapy. Shintani *et al* reported that enhancement of radiation sensitivity by Hsp90 inhibitor such as 17AAG depended on the p53 status using oral squamous cell carcinoma OSCC cell lines (19). However, our present study using two human lymphoblastoid cell lines, TK6 with the wild-type p53 gene and WTK1 with the mutated p53 gene clearly demonstrated that all the parameters of radio-sensitivity was enhanced by 17AAG in both cell lines regardless of the p53 status. It is possible that the discrepancy between the previous study and ours might be due to the difference in cell lines used for the experiments. It is known that lymphoid cells are particularly sensitive to apoptosis. Indeed, the p53 mutated OSCC cell line used by Shintani *et al* did not show apoptosis after irradiation. However, our lymphoblastoid cell lines underwent apoptotic cell death even with the mutated p53 gene, although the degree of apoptosis was less pronounced in the p53 mutant cells than the wild-type cells.

Hsp90 has many client proteins which play important roles in cellular activities including signal transductions and cell cycle regulations (3,4,8,13,27). Some of the client proteins such as Akt and Bid are associated with cellular apoptosis (13,32,33). The client proteins associated with the induction of apoptosis include those of both p53-dependent and -independent pathways. In our study, we observed that 17AAG enhanced radiation-induced apoptosis in TK6 as well as WTK1 cells. These observations compelled us to conclude that 17AAG enhancement of apoptosis is p53-independent.

Other possible mechanisms of p53-independent radio-sensitization by 17AAG is the abrogation of the G2/M checkpoint (3,4,27). The G2/M checkpoint is not necessarily dependent on the function of p53 (34,35). The G2/M checkpoint suppresses the progression of radiation damaged cells to M phase (36,37). Abrogation of this by 17AAG forces the cells carrying unrepaired DNA damage to progress into M-phase and thus the DNA damage would be fixed and manifested as chromosome aberrations. Indeed, the results of Fig. 5 clearly indicated that 17AAG treatment of irradiated

cells increased the frequency of chromosome aberrations, a tell tale signature of unrepaired/misrepaired DNA damage. Chromosome aberrations hamper cell division and further growth even though cells may enter the next cell cycle phases and these may eventually result in reproductive or apoptotic cell death as shown here in Fig. 2 by clonogenic cell survival assay.

In summary, we have demonstrated that radio-sensitization by 17AAG is p53-independent in human lymphoblastoid cell lines. Thus, 17AAG could be used therapeutically in some of p53-mutated radio-resistant tumors.

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