Targeted therapy of human osteosarcoma with 17AAG or rapamycin: Characterization of induced apoptosis and inhibition of mTOR and Akt/MAPK/Wnt pathways

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Abstract. Osteosarcoma is highly resistant to current chemotherapy regimens. Novel therapeutic approaches, potentially involving targeting of specific survival pathways, are needed. We used 17-AAG to inhibit Hsp90 and rapamycin to inhibit mTOR, in the osteosarcoma cell lines, HOS and KHOS/NP. HOS and KHOS cells were treated for 24 and 48 h with 17-AAG or rapamycin and studied drug-induced apoptosis, cell cycle, mitochondrial membrane potential and levels of reduced glutathione (GSH), dephosphorylation of signal transduction proteins in the Akt/MAP kinase pathway and mTOR signaling. 17-AAG was a potent inducer of apoptosis, involving effective depletion of GSH and mitochondrial membrane (MM) depolarization, strong activation of caspase-8 and -9 and release of AIF from mitochondria to the cytosol. Furthermore, 17-AAG down-regulated pAkt, p44Erk, p-mTOR, p70S6, TSC1/2 and pGSK-3ß. Treatment with 17-AAG also caused down-regulation of cyclin D1, GADD45a, GADD34 and pCdc2 and upregulation of cyclin B1 and mitotic block. A decrease in Hsp90 and increase in Hsp70 and Hsp70 C-terminal fragments were also observed. Rapamycin was a less potent inducer of apoptosis, involving a small decrease in GSH and MM potential with no activation of caspases or release of AIF. Rapamycin strongly inhibited cell growth with an increase in G1 and a decrease in S-phase of the cell cycle concomitant with down-regulation of cyclin D1. Rapamycin also down-regulated the activity of p70S6, pAkt and p-mTOR, but had no effect on pGSK-3ß, p44Erk, pCdc2, TSC1/2 or Hsp70 or Hsp90. We conclude that Hsp90 inhibition merits further study in the therapy of osteosarcoma.

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Introduction

Sarcomas encompass a heterogeneous group of malignancies of mesenchymal origin, accounting for approximately 1% of adult malignancies, while soft tissue sarcomas comprise 7% and osseous sarcomas comprise 5% of childhood malignancies (1,2). Adult osteosarcoma is the second most frequent sarcoma with a low rate of response to current therapy due to inherent chemoresistance. Surgery, with or without radiation therapy represents the mainstay of therapy for early stage soft tissue sarcomas, while adjuvant chemotherapy is controversial. Pre- and post-operative chemotherapy play prominent roles in the treatment of bone sarcomas, such as anthracycline and/or ifosfamide-based chemotherapies which are the principal therapeutic agents in the adjuvant and metastatic soft tissue sarcomas settings. The response rates ranged between 15 and 65% (3-5). However, the majority of patients relapse and overall survival is dismal (6-8). Hence, there is a need to develop newer, novel therapeutic agents. Potential therapeutic approaches include targeting of angiogenesis, protein kinases, heat-shock protein 90 (Hsp90) and mammalian target of rapamycin (mTOR) signaling (9-13).

mTOR is key serine/threonine kinase acting downstream of the activation of Akt. Current data suggest that mTOR acts as a 'master switch' of cellular metabolism and key element in the growth and proliferation, particularly in the regulation of tumor cell proliferation and metastasis. mTOR is central to the regulation of apoptotic cell death involving p53, BAD, Bcl-2, p27 and c-Myc (14). Akt activates mTOR by activating the mTOR upstream regulator, tuberous sclerosis complex1/2 (TSC1/2), which in turn activates Rheb to phosphorylate mTOR complex 1 (mTORC-1). mTORC-1 then complexes with Raptor to phosphorylate p70S6 kinase for downstream activation of the S6 ribosomal protein to initiate protein synthesis. The mTORC1/Raptor complex also dissociates 4E-BP from eIF4-E resulting in activation of eIF4-E and overall protein synthesis (14,15). Hence, dysregulated mTOR activity is associated with several hamartoma syndromes, including tuberous sclerosis complex, and many types of cancer (16-20).

Several mTOR inhibitors have been studied in phase I/II clinical trials. Rapamycin (sirolimus), the rapamycin analogues

CCI-779 (temsirolimus) and AP23573 were recently studied in phase I trials in patients with solid tumors (21,22).

The heat-shock family of proteins, Hsp27, Hsp60, Hsp70, and Hsp90 are chaperone proteins and serve an important role in the regulation of cell stress and cell growth by affecting the turnover of client proteins to increase or decrease their intra-cellular activities through changes in their 3-D conformation and degradation (23). Hence, by interacting with a great variety of proteins, HSPs are capable of regulating oxidative stress, hypoxia, cell cycle, cell growth, cell death, angiogenesis and metastasis (23,24). In the apoptosis pathway, p53, BAD, caspase-3 and -9, Apaf-1, cytochrome c and AIF are the primary targets (23,24). Hsp90 is unique among chaperone proteins in its role as 'super chaperone' with strong ATPase activity and the capability to mobilize other chaperone proteins. Among the important targets of Hsp90 are the growth regulatory pathways of Akt, Bcr-abl; Raf/Ras pathway; MAP and Erk kinase pathways; VEGF and cyclin-dependent kinases (25,26). Hsp90 also facilitates the translocation of proteins such as p53 and mdm2 into the nucleus. In general, Hsp90 increases p53 stability and translocation to the nucleus and therefore it is considered a positive regulator of wt p53, whereas, stabilization of mutant p53 requires interaction of Hsp90 with the co-chaperone Hsp70 (27-29).

The Hsp90 inhibitor 17AAG is one of the most studied inhibitors of HSP90 (30-38). 17AAG destabilizes multiple tyrosine kinase receptors and other oncoproteins through Hsp90 inhibition, resulting in blocking of proliferation and induction of apoptosis.

In this study we tested the mechanism of action of rapamycin and 17AAG in osteosarcoma cell lines, HOS and KHOS/NP. HOS cells were derived from a female osteosarcoma patient and KHOS/NP cells were constructed by *in vitro* transfection of HOS cells with Kirsten murine sarcoma virus (Ki-MSV) (39). Unlike HOS cells, KHOS cells are capable of forming colonies in soft agar and form tumors in SCID mice.

We chose these cell lines for this osteosarcoma study because K-ras plays an important role in the biology of sarcoma and because these cell lines have been studied extensively before. The drugs used in this study, rapamycin and 17AAG are both promising target-specific drugs and could potentially be useful in the treatment of osteosarcoma patients.

We studied the effect of 17AAG and mTOR on apoptosis, mitochondrial membrane depolarization; the levels of reduced glutathione (GSH); and on proapoptotic and anti-apoptotic proteins. We also determined the effects of these drugs on cell cycle; Akt/MAPK and the mTOR pathways. These targets, as well as other targets were studied because of parallel changes observed by gene microarray profiling (Gazitt, unpublished data). Our results indicate that 17-AAG is a good inducer of apoptosis in both osteosarcoma cell lines whereas rapamycin is less effective in inducing cell death and primarily affects cell growth. Preliminary results from this work have been presented (Gazitt *et al*, 100th Annual meeting of the American Association for Cancer Research, abs. 710, 2007).

Materials and methods

Cell lines and cell culture. HOS and KHOS/NP sarcoma cell lines were obtained from ATCC. Cultures were performed in Eagle's MEM medium containing 10% fetal calf serum (ATCC). HOS cells were derived from a female osteosarcoma patient and KHOS/NP cells were constructed by *in vitro* transfection of HOS cells with Kirsten murine sarcoma virus (39). Unlike HOS cells, KHOS cells are capable of forming colonies in soft agar and form tumors in SCID mice.

Determination of cell viability and apoptosis. Cell viability was determined by the trypan blue dye exclusion. Five hundred cells were counted in duplicate samples. To induce apoptosis cells were treated with 17-AAG or rapamycin (Sigma-Aldrich Inc., Dallas, TX) at concentrations of 0-10 μM (rapamycin) or 0-1 μM of 17AAG (Sigma-Aldrich) for 24 and 48 h. For all assays adherent cells were detached by trypsin-EDTA and were combined with floating cells. Apoptosis was determined as before (40) by staining of exposed phosphatidylserine residues on apoptotic cells with Annexin V-FITC (BioVision, Palo Alto, CA). Stained cells were analyzed by flow cytometry (FACSAria, BDIS, San Jose, CA). Quantification of apoptosis was done by the Diva software. Twenty thousand cells were analyzed per sample.

Cell cycle analysis. The effect of 17AAG and rapamycin on cell cycle was determined by staining of cells with propidium iodide (PI) as previously described (40,41). Adherent cells were detached by trypsin-EDTA and combined with floating cells. Stained cells were analyzed by flow cytometry (FACSAria; BDIS) and cell cycle analysis was performed using the ModFit software (BDIS). Twenty thousand cells were analyzed per sample.

Determination of mitochondrial membrane depolarization. Tetramethylrhodamine ethyl ester (TMRE; Molecular Probes, Eugene, OR) was used to detect mitochondrial membrane depolarization as previously described (40,41). Briefly, cells ($2x10^5$/ml) were cultured and rapamycin or 17-AAG was added at various concentrations at the time-points indicated for each experiment. Adherent cells were detached by trypsin-EDTA and were combined with floating cells. For measurement of mitochondrial membrane depolarization, cells were harvested, washed and resuspended in FACS-buffer (PBS + 1% BSA) and TMRE (final concentration of 800 nM) was added and cells were further cultured for 20 min at 37˚C. Cells were then washed with FACS-buffer and the level of TMRE staining was determined by flow cytometry as previously described (40,41). Quantification of the extent of mitochondrial membrane depolarization was performed by measurement of the left shift of TMRE fluorescence in cells with depolarized mitochondria. Samples were run in the FACSAria flow cytometer and results were analyzed by the FACSDiva software. Twenty thousand cells were analyzed per sample.

Determination of intracellular reduced glutathione. Monochlorobimane (mBCl; Molecular Probes) was used to detect reduced glutathione (GSH). mBCl is a thiol-reactive, mem-

brane-permeable probe that fluoresces upon excitation at 405 nm. Although mBCl reacts with all free thiol (SH) groups, it preferentially forms adducts with the highly abundant cellular GSH in a reaction catalyzed by the enzyme glutathione-*S*-transferase (42). Assay was performed as described before (43). Briefly, cells ($5x10^5$/ml) were cultured with various concentrations of rapamycin or 17-AAG at the time-points indicated for each experiment. Adherent cells were detached by trypsin-EDTA and were combined with floating cells. For measurement of reduced GSH, cells were harvested, washed and resuspended in FACS-buffer (PBS + 1% BSA). mBCl (dissolved in DMSO) was added at a final concentration of 200 μM and cells were incubated at 37˚C for 40 min. Cells were then washed with FACS-buffer and mBCl fluorescence was determined by the FACSAria Flow Cytometer as above using the violet laser tuned to 407 nm. Decrease in the level of reduced glutathione was measured by the extent of the left shift of mBCl fluorescence peak. Results were quantified by FACSDiva software. Twenty thousand cells were analyzed per sample.

Determination of apoptotic proteins, cells cycle proteins and cell signaling proteins by Western immunoblotting. Cells were cultured as described above with rapamycin or 17-AAG for the time-points indicated. Adherent cells were detached by trypsin-EDTA and were combined with floating cells. Aliquots of $5\text{-}10x10^6$ cells were washed twice with PBS, total cellular protein was extracted and 50-100 μg of protein was resolved by SDS-PAGE. Gel electrophoresis, immunoblotting and quantification of protein bands were performed by densitometry (Bio-Rad) as previously described (43,44). Mouse monoclonal antibodies to Hsp70 (B6), Hsp90 (F8), Cdc2 (clone 17), cyclin D1 (A12), cyclin B1 (GNS1), GADD 45a (4-T27) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal anti-p-Cdc2, FLIP-s/l (G-11), GSK-3ß (0011A), p-GSK-3ß (ser9), PARP (250), TSC1/2, p-TSC1/2, and GADD 34 (S20) were also from Santa Cruz Biotechnology. Monoclonal antibodies to Akt1/2/3, p-Akt1/2/3 (ser 475), pro-caspase-9 (Asp 315), p44Erk; p-p44Erk, Akt, pAkt, mTOR, p-mTOR, p70S6, p-p70S6 were from Cell Signaling Technologies (Beverly, MA). Mouse monoclonal anti-active caspase-8 antibody was from NeoMarkers (Fremont, CA). Proteins were identified according to their migration on the blot using a ladder of biotinylated proteins (Bio-Rad). For loading controls, membranes were stripped and reprobed using mouse monoclonal anti-ß-actin antibody (Clone AC-15; Sigma, St. Louis, MO).

Determination of cytosolic AIF, Hsp70 and Hsp90 by Western immunoblotting. Cells were cultured with rapamycin or 17-AAG for up to 48 h. Adherent cells were detached by trypsin-EDTA and were combined with floating cells. Aliquots of $5\text{-}10x10^6$ cells were washed twice with PBS and the cytosolic fraction was prepared by resuspending the cell pellets in mitochondria/cytosol buffer (BioVision) and passing the cell suspension 10 times through a 22 gauge syringe needle. Cell homogenate was centrifuged at 10,000 x g for 10 min. at 4˚C to obtain the cytosolic fraction (40,43). For SDS-PAGE, 50 μg of protein was loaded onto each lane. Gel electrophoresis, immunoblotting and quantification of protein bands were performed by densitometry as previously described (40). Mouse monoclonal antibody to AIF (Clone E-1) was from Santa Cruz Biotechnology. Cytosolic ß-actin was used for loading control after stripping and reprobing as described above.

Results

Effect of 17AAG and rapamycin on apoptosis GSH, MMD and cell cycle in HOS and KHOS cells. We first tested 17-AAG and rapamycin for their potency to induce apoptosis as measured by Annexin V. HOS and KHOS cells were treated for 24 and 48 h with 0-1 μM of 17-AAG or 0-10 μM of rapamycin. The results obtained after 24 and 48 h of treatment were similar. The results obtained after 48 h are depicted in Fig. 1A. The results indicated that 17-AAG is a potent inducer of apoptosis in both sarcoma cell lines with an IC_{40} of 0.4 μM, at 48 h. At this concentration of 17AAG, the maximal mitochondrial membrane depolarization (MMD) was 40% and depletion of reduced glutathione (GSH) reached about 60% (Fig. 1A). The effect of 17AAG on cell viability was also tested. As can be noted in Fig. 1A the decrease in cell viability followed closely the results obtained for apoptosis. Similar results were obtained for HOS and KHOS cells. Only the results for HOS cells are shown.

Rapamycin-induced apoptosis was also dose-dependent. However, rapamycin was a weaker inducer of apoptosis in both sarcoma cell lines (IC_{25} of 4 μM, at 48 h), involving a 40% decrease in reduced glutathione and 30% of cells exhibiting mitochondrial membrane depolarization (Fig. 1B). The effect of 17AAG on cell viability was also tested. As can be noted in Fig. 1B the decrease in cell viability followed closely the results obtained for apoptosis. Similar results were obtained for HOS and KHOS cells and only the results for HOS cells at 48 h are shown.

Rapamycin was shown to affect cell growth, whereas 17AAG was reported to exert mitotic block, in other cell types (14,15). We therefore tested the effect of both drugs on cell cycle following 48 h of treatment. The results are depicted in Fig. 2. Treatment with 17AAG (0-0.5 μM) caused a dose-dependent increase in the percentage of cells in G2/M from 7.5 to 44.3% and from 6.2 to 52.7% for HOS and KHOS cells, respectively at maximal dose of 17AAG (Fig. 2). Arrows indicate G2/M block. In contrast to 17AAG, treatment with rapamycin at 0-5 μM (lower panel) resulted in blocking of cells in G1 (from 61.6 to 73%) with a marked (~90%) decrease in S-phase at maximal dose of rapamycin used. Left arrows indicate G1 block and right arrows indicate the position of S-phase, No effect on G2/M was observed with rapamycin. Similar results for rapamycin were observed in KHOS cells (data not shown).

17AAG and rapamycin induced down-regulation of GSK3, Akt/MAPK and mTOR. Previous studies have shown that 17AAG affects the Akt and MAP kinase pathway and rapamycin affects the mTOR pathway by a feedback loop mechanism (19). Our own studies of osteosarcoma cell lines using gene microarray profiling indicated changed in the expression of genes related to these pathways and the Wnt signaling pathway with a marked decrease in the expression

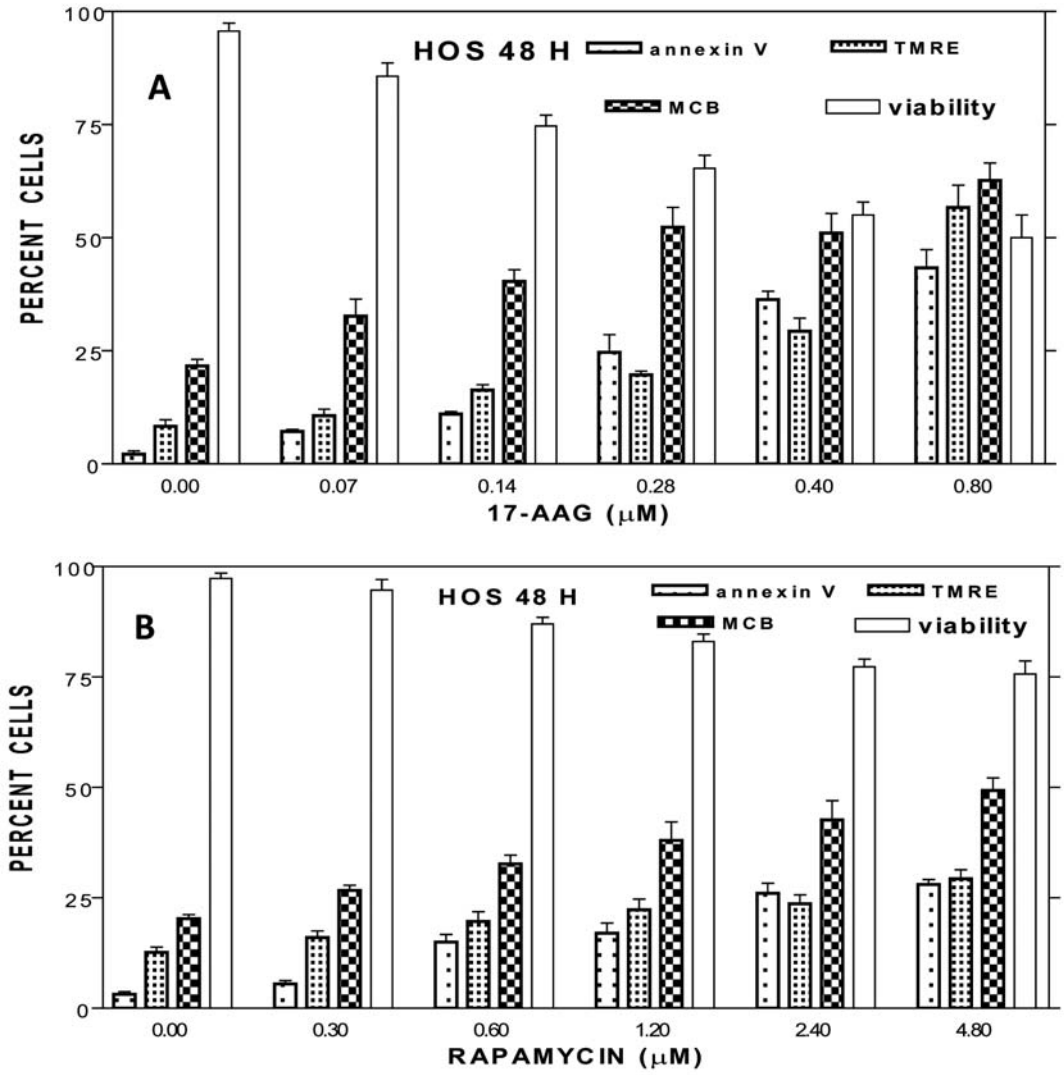

Figure 1. Effect of 17AAG and rapamycin on cell viability, apoptosis, GSH and MMD in HOS cells. Cells were treated for 24 and 48 h. Adherent cells were detached by trypsinization and were added to the floating cells. Apoptosis was determined by the extent of binding of Annexin V-FITC. Tetramethylrhodamine ethyl ester (TMRE) was used to detect mitochondrial membrane depolarization (MMD) and monochlorobimane (MCB) was used to detect the levels of reduced glutathione (GSH). Stained cells were analyzed by flow cytometry (FACSAria) and quantification was performed by the FACSDiva software. Bars represent the mean ± SD from at least 3 different experiments. Cell viability was determined by the trypan blue dye exclusion. At least 500 cells were counted.

of GSK3ß (Gazitt, unpublished data). We therefore tested the effect of 17AAG or rapamycin on these cell signaling proteins in HOS and KHOS cells following 24 and 48 h of treatment. The results are depicted in Figs. 3 and 4. Following treatment with 17AAG (A) we observed substantial (50- 80%) down-regulation of p-mTOR at 24 and 48 h in both cell lines, whereas rapamycin (R) had little effect on mTOR and no effect on p-mTOR (Fig. 3). Only slight inhibition of the down-stream protein, p70S6, was observed by either drug in HOS or KHOS cells with practically no effect on the level of p-p70S6 (Fig. 2). The glycogen synthase kinase-3ß (GSK-3ß) is the most important negative regulator of the Wnt/ß-catenin pathway in mammalian cells (45,46). Interestingly, both GSK-3ß and p-GSK-3 were markedly down-regulated (2-3-fold) by 17AAG in HOS and KHOS cells. No such effect was observed with rapamycin (Fig. 3).

We also tested the effect of 17AAG and rapamycin on upstream regulators of mTOR, TCS1/2 and pAkt and the p44 Erk of the MAPK pathway. The results are depicted in Fig. 4. Treatment with 17AAG (A) resulted in substantial down-regulation of TSC1/2 (upper band, arrow) and complete disappearance of the phosphorylated form of p44 ERK and about 50% decrease in pAkt after 24-48 h of treatment in both cell lines. The levels of p44ERK or Akt were not affected by 17AAG. In contrast to 17AAG, rapamycin (R) had no effect on TSC1/2, p-p44ERK, or pAkt with mild inhibition observed for Akt at 48 h. Similar effects were observed by each drug in both cell lines (Fig. 4).

Early effect of 17AAG and rapamycin on cell signaling and cell cycle regulatory proteins. To further investigate the kinetics of 17AAG and rapamycin-induced blocking of cell signaling and cell cycling, we examined early effects on the Akt, MAP/ERK kinase pathways, the mTOR signaling pathway and the cell cycle regulatory proteins, cyclin D1, cyclin B1, GADD45 and GADD34. HOS and KHOS cells

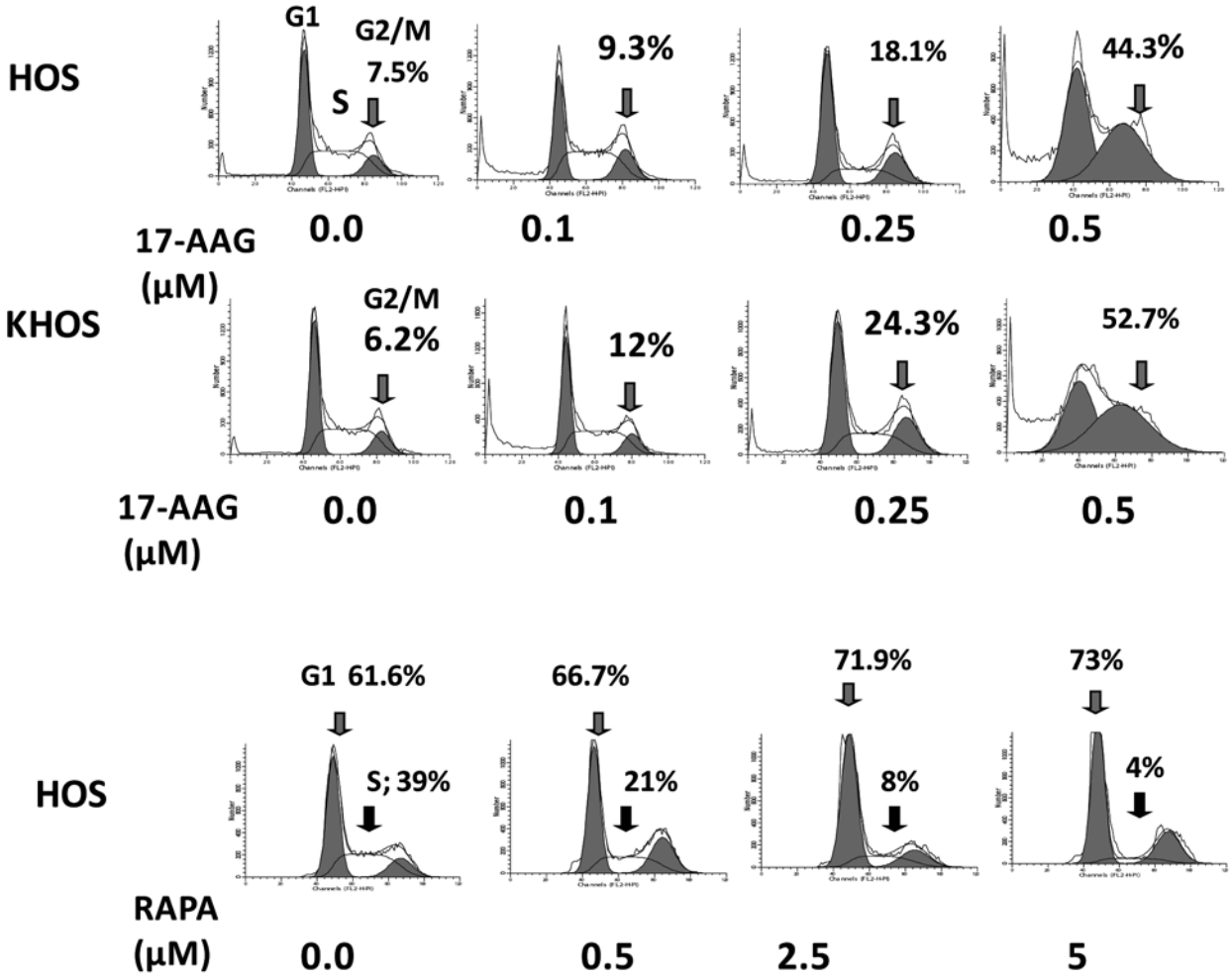

Figure 2. Effect of 17AAG and rapamycin on cell cycle in HOS and KHOS cells. Cells were treated for 48 h. Adherent cells were detached by trypsinization and were added to the floating cells. Cell cycle was determined by staining of cells with propidium iodide and results were analyzed by flow cytometry (FACSCalibur) and quantified by the ModFit software. The position of G2/M is marked in cells treated with 17AAG. The position of G1 and S-phase are marked in cells treated with rapamycin. Left red peak represents cells in G1 whereas red right peak represents cells in G2/M. The hatched area between the 2 peaks represents cells in S-phase of the cell cycle. Treatment with 17AAG resulted in blocking of cells in mitosis, whereas, treatment with rapamycin resulted in blocking of cells in G1 and a marked decrease in S-phase. HOS and KHOS cells responded in a similar way to rapamycin (results for KHOS cells are not shown).

were cultured without serum for 4 h with or without rapamycin (4 μM) or 17AAG (0.4 μM, T0). This was followed by adjusting the serum to 10% in order to stimulate growth signaling in these cultures. Serum-stimulated cultures with or without rapamycin (4 μM) or 17AAG (0.4 μM) were carried out. Cells were assayed at T0 (i.e. 4 h with no serum, with or without drugs) and at 1, 2, 4 and 24 h post-serum stimulation with or without drugs. Floating cells were combined with detached adherent cells. Cells were washed and total cellular protein was extracted and analyzed by Western immunoblotting for total Akt; p44Erk and TSC1/2 and their phosphorylated forms. The results for HOS cells are depicted in Fig. 5. A similar protocol was carried out for KHOS cells. Similar results were obtained for HOS and KHOS cells and only the results obtained with HOS cells are shown. As depicted in Fig. 5 treatment with 17AAG blocked phosphorylation of Akt, Erk and TSC1/2 at T0, before serum stimulation and at all other time-points tested. Interestingly, 17AAG down-regulated p-p44Erk, p-Akt, TSC1/2 and pTSC1/2, at all time-points tested. In contrast to 17AAG, rapamycin had no effect on Akt or Erk, TSC1/2, or their phosphorylated forms. A similar trend of results was observed for KHOS cells, although the effect was less robust (data not shown). These results suggest a very early effect of these drugs, before any measureable apoptotic event and further corroborate the results obtained for 24- and 48-h cultures depicted in Figs. 3 and 4. Furthermore, early down-regulation of p-p70S6, p-Akt and p-p44Erk were confirmed by flow cytometry with >50% decrease in the number of positive cells and a 2-3-fold decrease in mean fluorescence intensity (MFI) observed for p-p70S6 p-p44Erk and p-Akt after 3-6 h of treatment with 17AAG (data not shown).

Similar cultures and stimulation conditions to those described above for cell signaling were employed to determine early effects on cell cycle regulatory proteins. The results are depicted in Fig. 6 for HOS cells. 17AAG and rapamycin slightly down-regulated (2- and 4-h points) GADD34 with no effect on GADD45a. Down-regulation of cyclin D1 was observed by 17AAG and rapamycin, before serum stimulation (T0) and at 1, 2 and 24 h of serum stimulation. The levels of

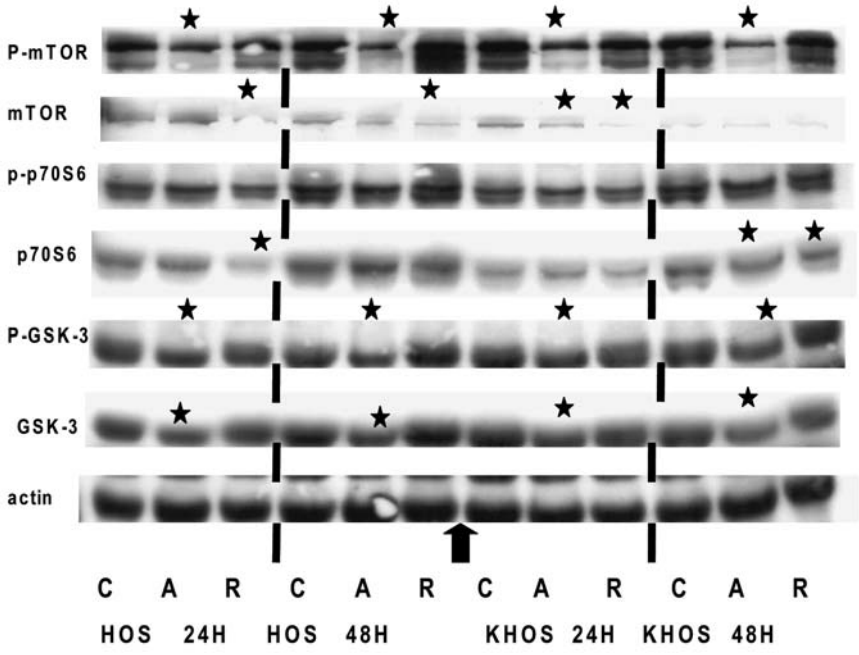

Figure 3. Effect of 17AAG and rapamycin on mTOR, p70S6 and GSK-3ß Cells were treated with 0.4 μM of 17-AAG or 5 μM of rapamycin. Adherent cells were detached by trypsinization and were added to the floating cells. Aliquots of 5-10x10^6 cells were washed with PBS and total cellular protein was extracted. 50-100 μg of protein was resolved by SDS-PAGE. Western immunoblotting was performed by standard procedures. Quantitation of protein band was done by densitometry. For loading controls, membranes were stripped and reprobed using mouse monoclonal anti-ß-actin antibody. Vertical arrows distinguish HOS from KHOS. Lines distinguish 24 h from 48 h of treatment. Asterisks represent significant and reproducible changes. Results shown are representative of at least 3 experiments. C refers to control; A refers to 17AAG and R refers to rapamycin. Similar effects of 17AAG and rapamycin was noted for HOS and KHOS cells.

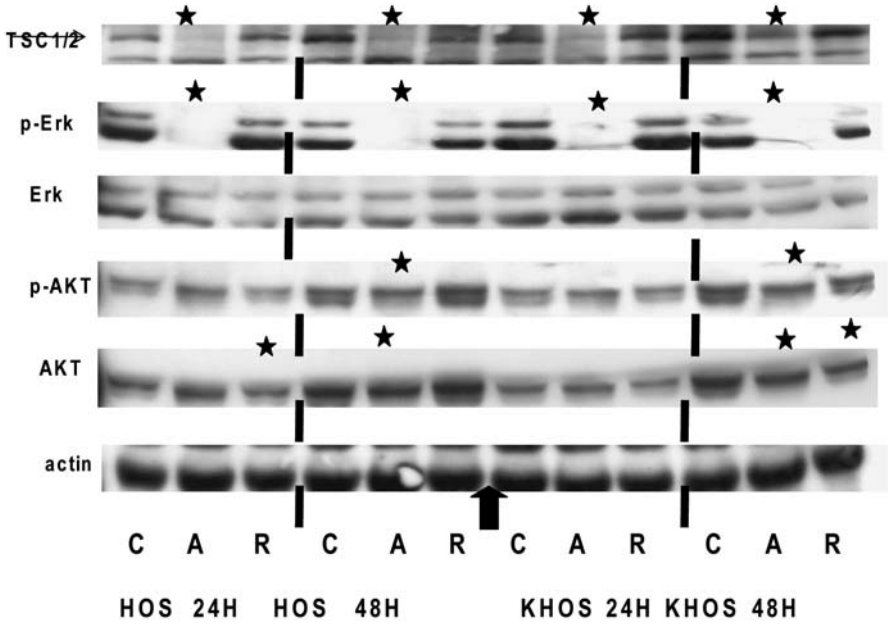

Figure 4. Effect of 17AAG and rapamycin on TSC1/2, Erk and Akt. Cells were treated with 0.4 μM of 17-AAG or 5 μM of rapamycin as described above. Adherent cells were detached by trypsinization and were added to the floating cells. Western immunoblotting was performed as described above (Fig. 3). Quantitation of protein band was done by densitometry. ß-actin was used for loading controls. Vertical arrows distinguish HOS from KHOS. Lines distinguish 24 h from 48 h of treatment. Asterisks represent significant and reproducible changes. Results shown are representative of at least 3 experiments. Horizontal arrow next to TSC1/2 band refers to the upper band of TSC1/2. C refers to control; A refers to 17AAG and R refers to rapamycin. Similar effects of 17AAG and rapamycin was noted for HOS and KHOS cells.

the mitotic cyclin, cyclin B1 were increased 24 h after stimulation by 17AAG (Fig. 6). Upregulation of cyclin B1 was confirmed also by flow cytometry with >50% increase in percent positive cells observed as early as 9 h after treatment with 17AAG. Similar results were obtained for KHOS cells (data not shown).

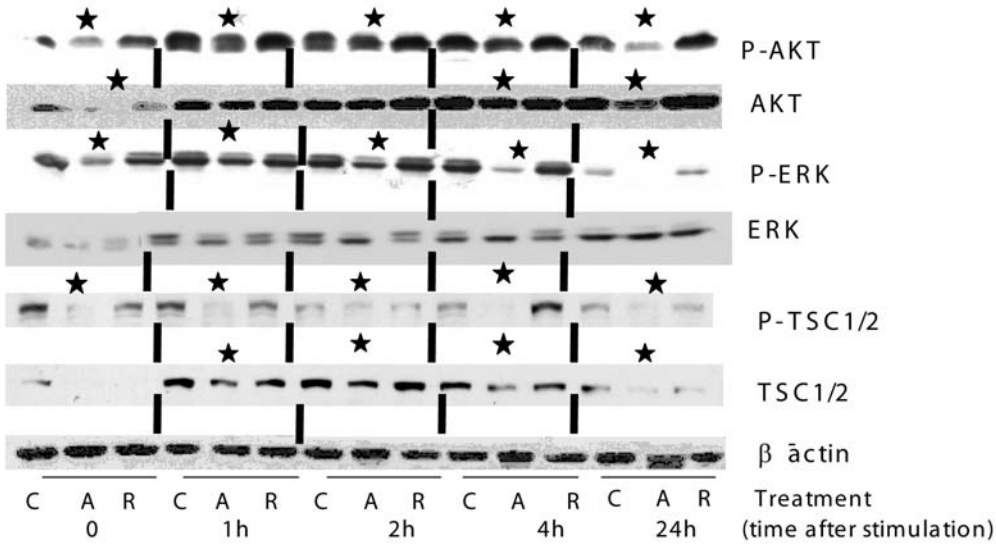

Figure 5. Early effects of 17AAG and rapamycin on cell signaling proteins in HOS cells. HOS cells were serum deprived for 4 h with or without 0.4 μM of 17-AAG or 5 μM of rapamycin and cell signaling was stimulated by addition of 10% serum with or without drugs for up to 24 h. Adherent cells were detached by trypsinization and were added to the floating cells. Cultures were assayed before serum stimulation (T0) and 1, 2, 4 and 24 h after stimulation as described in the text. Quantitation of protein band was done by densitometry. Lines distinguish different time-points of treatment. For loading controls, membranes were stripped and reprobed using mouse monoclonal anti-ß-actin antibody. Asterisks represent significant and reproducible changes. Results shown are representative of at least 3 experiments. C refers to control; A refers to 17AAG and R refers to rapamycin.

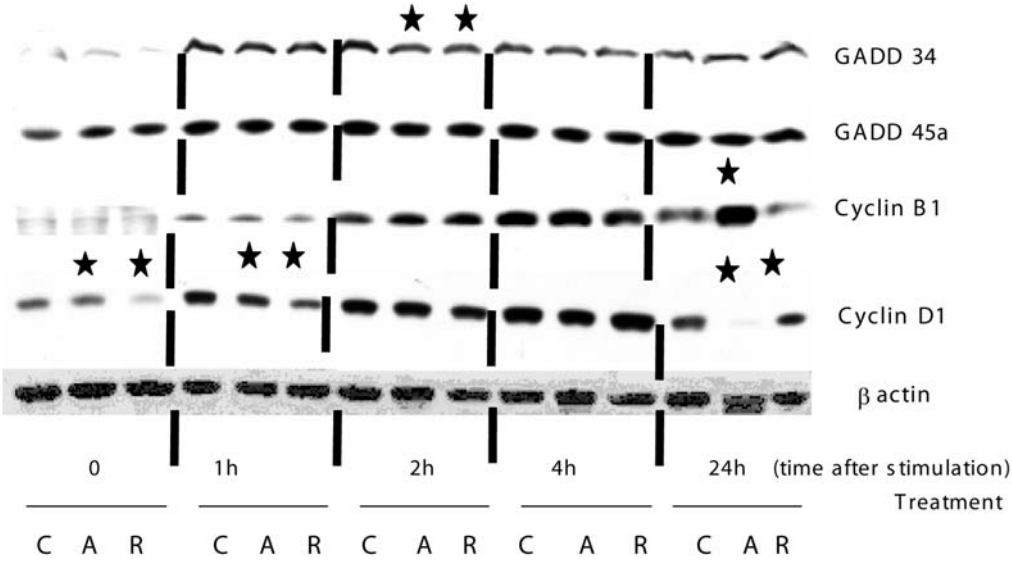

Figure 6. Early effects of 17AAG and rapamycin on cell cycle proteins in HOS cells. HOS cells were serum-deprived for 4 h with or without 0.4 μM of 17-AAG or 5 μM of rapamycin and cell growth was stimulated by addition of 10% serum as described above in the legends to Fig 5. For loading controls, membranes were stripped and reprobed using mouse monoclonal anti-ß-actin antibody. Quantitation of protein band was done by densitometry. Lines distinguish different time-points of treatment. Asterisks represent significant and reproducible changes. Results shown are representative of at least 3 experiments. C refers to control; A refers to 17AAG and R refers to rapamycin.

We then tested the long-term effect (24 and 48 h) of 17AAG and rapamycin on cell cycle regulatory proteins. The results are depicted in Fig. 7. Cyclin D1 levels were decreased (40-60%) by both drugs, whereas, cyclin B1 levels were increased (5-10-fold) by 17AAG only (Fig. 7 compare to Fig. 6 above). In addition, we observed a decrease (2-5-fold) in the levels of the mitotic cyclin kinase Cdc2 and p-Cdc2 at 24 and 48 h of treatment with 17AAG. No such effect was observed with rapamycin. These changes in cyclins and Cdc2 were compatible with the cell cycle changes measured by propidium iodide staining and flow cytometry depicted in Fig. 2.

Effect of 17AAG and rapamycin on heat-shock proteins in HOS and KHOS cells. Previous studies revealed that treatment with 17AAG resulted in a small decrease (20-30%) in Hsp90 and large increase (2-3-fold, 24 h, HOS cells) in the level of Hsp70 and its C-terminal degradation fragments of ~25 kDa (26). We observed similar effect of 17AAG in osteosarcoma cells. The results are depicted in Fig. 8. Following 24 and

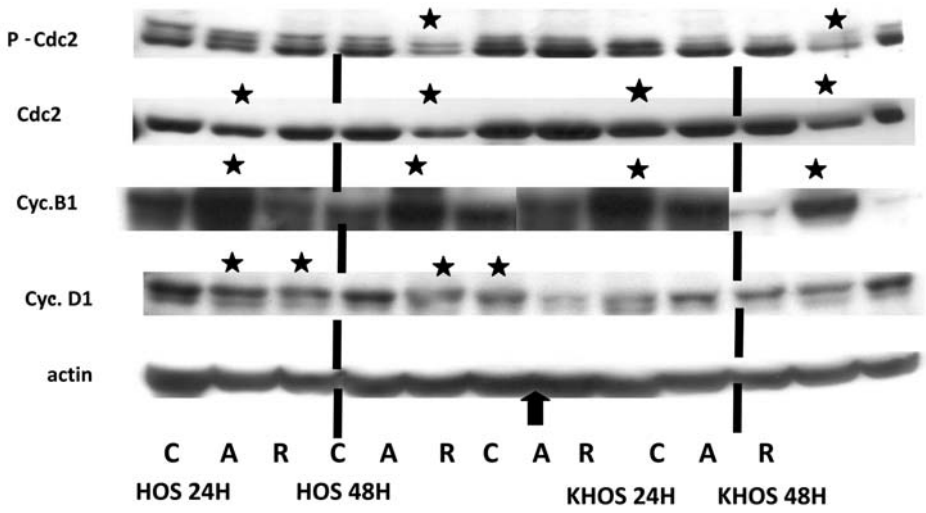

Figure 7. Effect of 17AAG and rapamycin on Cdc2, cyclin B1 and cyclin D1 in HOS and KHOS cells. Cells were cultured with 0.4 μM of 17-AAG or 5 μM of rapamycin for 24 or 48 h as described in Materials and methods. Adherent cells were detached by trypsinization and were added to the floating cells. For loading controls, membranes were stripped and reprobed using mouse monoclonal anti-ß-actin antibody. Quantitation of protein band was done by densitometry. Vertical arrows distinguish HOS from KHOS. Lines distinguish 24 from 48 h of treatment. Asterisks represent significant and reproducible changes. Results shown are representative of at least 3 experiments. C refers to control; A refers to 17AAG and R refers to rapamycin. Similar effects of 17AAG and rapamycin were noted for HOS and KHOS cells.

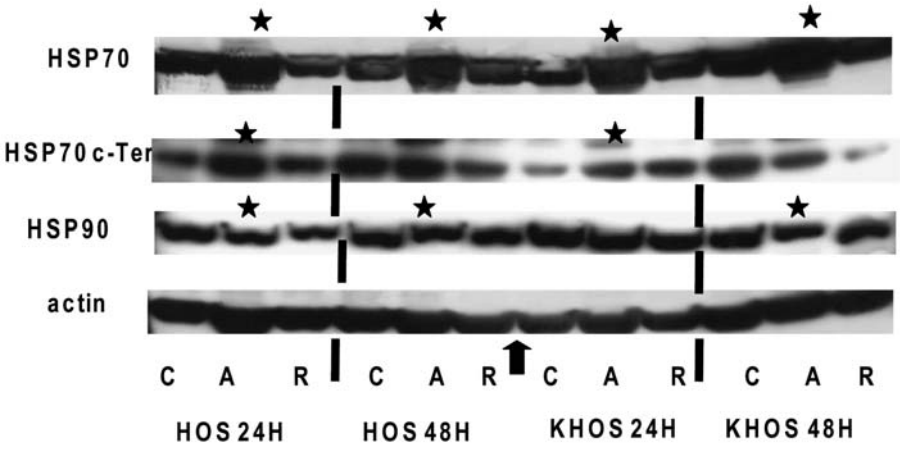

Figure 8. Effect of 17AAG and rapamycin on Hsp70 and Hsp90 in HOS and KHOS cells. Cells were cultured with 0.4 μM of 17-AAG or 5 μM of rapamycin as described in the text. Other legends are as in Fig. 3. For loading controls, membranes were stripped and reprobed using mouse monoclonal anti-ß-actin antibody. Quantitation of protein band was done by densitometry. Vertical arrows distinguish HOS from KHOS. Lines distinguish 24 from 48 h of treatment. Asterisks represent significant and reproducible changes. Results shown are representative of at least 3 experiments. C refers to control; A refers to 17AAG and R refers to rapamycin. An increase in cellular Hsp70 and its 30-kDa c-terminal fragment was noted in cells treated with 17AAG. Similar effects of 17AAG and rapamycin were noted for HOS and KHOS cells.

48 h of treatment with 17AAG or rapamycin we observed a substantial increase in the levels of Hsp70 and an increase (24-h point) in the C-terminal fragment of Hsp70, concomitant with a small decrease in Hsp90 at the 48-h point. No such changes were observed with rapamycin. No major differences were observed between HOS and KHOS in this respect. These results were further confirmed by proteomics (Gazitt *et al*, unpublished data).

Effect of 17AAG and rapamycin on proapoptotic proteins in HOS and KHOS cells. In order to elucidate the apoptotic pathway of 17AAG and rapamycin in HOS and KHOS cells we measured the levels and activation of cellular and cytosolic apoptotic proteins. Fig. 9 depicts the effect of 17AAG and rapamycin on caspase-8, -9, FLIP and PARP. We also determined the cytosolic levels of the apoptosis inducing factor, AIF. The results indicate that treatment with 17AAG results in an increase in the activation of caspase-9 as manifested by the decrease in the large peptide (upper band, horizontal arrow) of caspase-9. Treatment with 17AAG also resulted in a marked increase in the levels of activated caspase-8 in HOS and KHOS cells (Fig. 9). Rapamycin had no effect on these caspases. Most importantly, the level of cytosolic AIF was markedly increased (3-5-fold) by 17AAG, but not by rapamycin in both cell lines, at 24 and 48 h (Fig. 9).

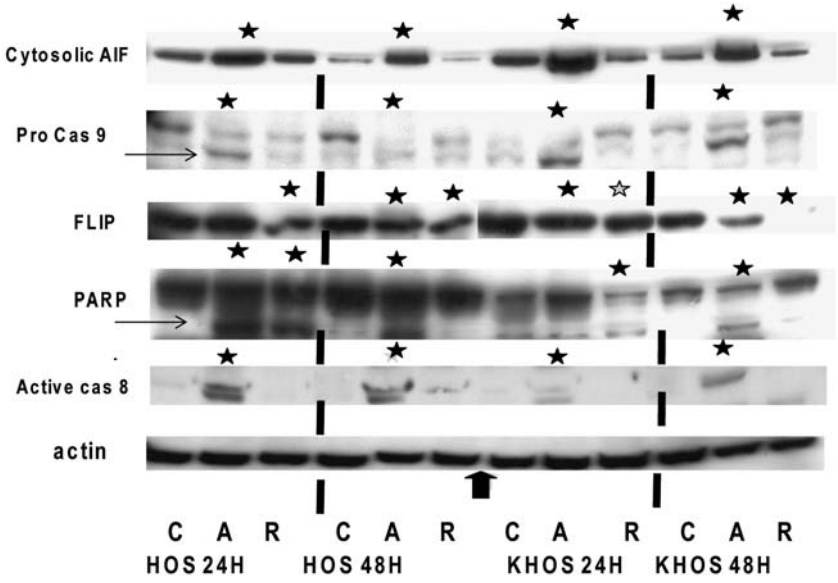

Figure 9. Effect of 17AAG and rapamycin on activation of caspases, PARP and FLIP/l and on cytosolic AIF and Hsp70 in HOS and KHOS cells. Cells were cultured with 0.4 μM of 17-AAG or 5 μM of rapamycin as described in the text. Other legends are as in Fig. 3. Cytosolic fraction was isolated as described in Materials and methods. For loading controls, membranes were stripped and reprobed using mouse monoclonal anti-ß-actin antibody. Quantitation of protein band was done by densitometry. Vertical arrows distinguish HOS from KHOS. Lines distinguish 24 from 48 h of treatment. Horizontal arrows indicate degradation peptides in caspase-9 and PARP lanes. Asterisks represent significant and reproducible changes. Results shown are representative of at least 3 experiments. C refers to control; A refers to 17AAG and R refers to rapamycin. An increase in cytosolic AIF and Hsp70 was noted in cells treated with 17AAG. Similar effects of 17AAG and rapamycin were noted for HOS and KHOS cells.

FLIP was decreased (5-10-fold) by both drugs at 24 and 48 h, especially in KHOS cells. Similarly, PARP was activated by both drugs with appearance of the 94-kDa degradation product (e.g., HOS 24 h; horizontal arrow).

These results, taken together, suggest that 17AAG activates the intrinsic and the extrinsic apoptotic pathways involving activation of caspase-8 and -9 and AIF in HOS and KHOS cells. In addition, 17AAG strongly deactivated the MAP/ERK/AKT/ß-catenin and mTOR pathways and blocked cells in mitosis concomitant with upregulation of the mitotic cyclin, cyclin B1 and down-regulation of the mitotic cyclin kinase, Cdc2. The effect of rapamycin, on the other hand, was predominantly on cell growth (G1 arrest) and depletion of S-phase, through blocking of mainly the mTOR pathway. These results explain the weaker apoptotic effect of rapamycin observed in these cell lines.

Discussion

Targeted therapy has become the mainstay of novel chemotherapy across all types of cancer. The most popular experimental and therapeutic targets involve angiogenesis; cancer survival pathways, such as the Akt/MAPK and mTOR pathways; apoptosis; and cell-cycle checkpoints. Many of these drugs are in various stages of preclinical and clinical development. However, scant information is known on the activity of this type of drugs in osteosarcoma, known for its drug-resistance. Hence, we undertook the task of detailed analysis of the mode of action of 2 promising target-specific drugs, 17AAG and rapamycin. Our results confirm results obtained previously by others for other solid tumors and report novel effects on targets not reported before for these drugs.

Mechanism of action of 17 AAG in osteosarcoma cells. Our results indicate that 17-AAG is a potent inducer of apoptosis in both sarcoma cell lines, involving early depletion of GSH, mitochondrial membrane depolarization and strong activation of caspase-8 and caspases-9 and activation of PARP. Among the new effects we observed down-regulation of FLIP and translocation of AIF from mitochondria to the cytosol. These combined observations suggest activation of multiple targets in the intrinsic and extrinsic apoptotic pathways as a result of blocking of Hsp90 as previously described for other cell types (23).

The most impressive effect of 17AAG was the very early and strong inhibition of the phosphorylated forms of p44Erk, GSK-3ß, Akt, mTOR and TSC1/2. Blocking of p44Erk and Akt by 17AAG has been reported before for other cell types (25). However, down-regulation of GSK-3ß pGSK-3ß by 17AAG have not been reported before. The reported effect of 17AAG on GSK-3ß and pGSK-3ß are important since ß-catenin and GSK-3ß play an important role in the etiology of osteosarcoma. Furthermore, GSK-3ß is an important client protein of Hsp90 and the Hsp90 chaperone activity is needed to stabilize the authophosphorylation and constitutive activation of pGSK-3ß (48). Thus, blocking of Hsp90 by 17AAG may have resulted in stabilization of ß-catenin and its many signaling pathways including cell cycle (e.g., cyclin D1, differentiation and apoptosis) (48,49). Down-regulation of GSK-3ß and cyclin D1 has been reported following knockdown (by SiRNA) of Cdc37, a co-chaperone of Hsp90 (49). We therefore suggest that at least part of the cell cycle and apoptotic effect observed with 17AAG resulted from blocking of GSK-3ß.

We also observed a profound effect of 17AAG on the cell cycle with a strong anti-mitotic activity, concurrent

with upregulation of the mitotic cyclin, cyclin B1 and down-regulation of the mitotic cyclin-dependent kinase, Cdc2 and its phosphorylated form. The observed mitotic block by 17AAG was confirmed by confocal imaging (Gazitt, unpublished data) and has been reported before for other cell types (25,49). 17AAG also down-regulated the G1 cyclin, cyclin D1, the DNA damage-related transcription factor GADD34 (50,51), but had no effect on the G1/S transcription factor, GADD45a (52). The observed decrease in cyclin D1 and GADD34 was confirmed independently in our lab using gene microarray analyses (Gazitt *et al*, 43rd Annual meeting of ASCO, abs. 10027, 2007). Based on these data and the low activity of the small responses obtained in phase I clinical trials in patients with solid tumors (53) we suggest that 17AAG could be used as a single drug for the treatment of osteosarcoma, but could be more effective if combined with other targeting drugs. In this respect, we are currently testing the combination of histone deacetylase inhibitors and 17AAG and preliminary results suggest synergy with 17AAG (Gazitt *et al*, unpublished data).

In addition, treatment with 17-AAG resulted in a marked increase in the levels of Hsp70 and Hsp70 degradation peptide known as the C-terminal fragment (~25 kDa). This finding is in agreement with the previously reported effect of blocking Hsp90 in other cell types (23,26). Hsp70 confers multiple antiapoptotic activities including blocking of the activation of caspase-9 and -3 and blocking of AIF and cytochrome c release to the cytosol and the ensuing apoptosis (26,47). Hence, the observed enhanced turnover of Hsp70 could have contributed to induced apoptosis. The observed increase in Hsp70 and its C-terminal fragment were confirmed independently by gene microarray profiling and proteomics using 2-D gels, mass spectrometry and direct sequencing (Gazitt *et al*, 100th Annual meeting of the American Association for Cancer Research, abs. 3702, 2007).

Mechanism of action of rapamycin in osteosarcoma cells. In our hands, the major effect of rapamycin in osteosarcoma cells was growth inhibition as manifested by down-regulation of cyclin D1, G1 arrest and in a marked decrease in the percent of cells in S-phase of the cell cycle. These effects of rapamycin have been described before for other cell types (14,15). On the other hand, rapamycin was less potent in the induction of apoptosis and treatment resulted in only a small decrease in GSH and mitochondrial damage, with no activation of caspases-8, -9, AIF, or in activation of PARP. Most importantly, rapamycin down-regulated Akt and mTOR but had no effect on p-mTOR, TSC1/2, GSK-3ß and p44Erk. A small inhibitory effect was observed for Akt and mTOR. It is noteworthy that in preliminary studies we observed no synergy or additivity between 17AAG and rapamycin when the 2 drugs were combined. In fact we observed a slight antagonistic effect. This could be the result of early blocking of cells in G1 which could result in a decrease in the potency of 17AAG which induces a mitotic block. Alternatively, lack of additivity or synergy could be the result of the previously documented activation of Akt as a result of blocking of mTOR.

Finally, it is noteworthy that osteosarcoma cell lines used in this study, HOS and KHOS were equally sensitive to both drugs despite the fact that KHOS cells are capable of forming colonies in soft agar and tumors in SCID mice (39).

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