Apoptosis of osteosarcoma cultures by the combination of the cyclin-dependent kinase inhibitor SCH727965 and a heat shock protein 90 inhibitor

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Osteosarcoma (OS) is an aggressive bone cancer typically observed in adolescents and young adults. Metastatic relapse accounts primarily for treatment failure, and obstacles to improving cure rates include a lack of efficacious agents. Our studies show apoptosis of OS cells prepared from localized and metastatic tumors by a novel drug combination: SCH727965 (SCH), a cyclin-dependent kinase inhibitor, and NVP-AUY922 (AUY) or other heat shock protein 90 inhibitor. SCH and AUY induced apoptosis when added simultaneously to cells and when AUY was added to and removed from cells before SCH addition. Sequential treatment was most effective when cells received AUY for ~12 h and when SCH was presented to cells immediately after AUY removal. The apoptotic protein Bax accumulated in mitochondria of cotreated cells but was primarily cytosolic in cells receiving either agent alone. Additional data show that SCH and AUY cooperatively induce the apoptosis of other sarcoma cell types but not of normal osteoblasts or fibroblasts, and that SCH and AUY individually inhibit cell cycle progression throughout the cell cycle. We suggest that the combination of SCH and AUY may be an effective new strategy for treatment of OS.

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Abbreviations: AAG, 17-allylamino-17-demethoxygeldanamycin; AUY, NVP-AUY922; BrdU, bromodeoxyuridine; CDK, cyclin-dependent kinase; DMAG, 17-(2-dimethylaminoethyl) amino-17-demethoxygeldanamycin; FACS, fluorescence-activated cell sorting; Hsp, heat shock protein; MAP, methotrexate, cisplatin, and doxorubicin; HSP990, NVP-HSP990; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; OS, osteosarcoma; SCH, SCH727965

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problematic due to toxicity, and AAG has poor aqueous solubility.\textsuperscript{16,17} The synthetic inhibitors HSP990 and AUY are currently in clinical trials.

In this study, we examined the effects of SCH and Hsp90 inhibitors on the survival of OS cell cultures prepared by us from resected OS tumors. Drugs were added to OS cells at the same time or at separate times, and their effects on the viability of normal osteoblasts was also determined. Our results suggest that the combination of SCH and Hsp90 inhibitor may be useful for treating OS.

Results

Apoptosis of OS cells by the combination of SCH and Hsp90 inhibitor. OS cell cultures were prepared from four resected tumors (designated OS1001, OS1002, OS1003, OS1004) as described in Materials and Methods. OS1001 was a localized tumor in a young adult. It showed excellent necrosis (>90\%) after MAP chemotherapy, and the patient remains disease-free more than 2 years from diagnosis. OS1003 and OS1004 were primary tumors in young adult patients with widespread and non-resectable metastatic disease at presentation. OS1003 and OS1004 received MAP chemotherapy in the neoadjuvant setting with 41\% and 90\% necrosis, respectively; a biopsy specimen of a metastasis of OS1003 had 88\% necrosis. The patients lived for 7 (OS1003) and 10 (OS1004) years from diagnosis after progressing through adjuvant MAP and additional chemotherapies. OS1002 was a previously treated, recurrent metastatic tumor without systemic therapy after recurrence; the patient has been lost to follow-up.

All four OS cultures expressed osteoblastic markers (osteopontin, osteocalcin and alkaline phosphatase) as determined by RT-PCR (Figure 1a). Normal human

![Cell lines and inhibitors. (a) RT-PCR was performed on cDNA derived from the indicated cell lines using probes for alkaline phosphatase, osteopontin, osteocalcin and GAPDH (loading control). NHOst cells are normal human osteoblasts and are included as a positive control. Control (lane 1) has no cDNA. (b) Structures of the inhibitors used in this study. (c) OS1002 cells received 10 nM SCH and the indicated inhibitors for 72 h. Percent apoptotic cells was determined by FACS analysis of annexin V-stained cells. Concentrations of inhibitors used were: MK2206 (1 \mu M); Gleevec (10 \mu M); etoposide (1 \mu M); doxorubicin (250 nM); dasatinib (100 nM); LY294002 (20 \mu M); SB203580 (10 \mu M); U0126 (10 \mu M); PD98059 (50 \mu M); AAG (1 \mu M); nicotinamide (100 nM); and nutlin (5 \mu M). Black bars denote cells receiving AAG, SCH or both.

Figure 1: Cell lines and inhibitors. (a) RT-PCR was performed on cDNA derived from the indicated cell lines using probes for alkaline phosphatase, osteopontin, osteocalcin and GAPDH (loading control). NHOst cells are normal human osteoblasts and are included as a positive control. Control (lane 1) has no cDNA. (b) Structures of the inhibitors used in this study. (c) OS1002 cells received 10 nM SCH and the indicated inhibitors for 72 h. Percent apoptotic cells was determined by FACS analysis of annexin V-stained cells. Concentrations of inhibitors used were: MK2206 (1 \mu M); Gleevec (10 \mu M); etoposide (1 \mu M); doxorubicin (250 nM); dasatinib (100 nM); LY294002 (20 \mu M); SB203580 (10 \mu M); U0126 (10 \mu M); PD98059 (50 \mu M); AAG (1 \mu M); nicotinamide (100 nM); and nutlin (5 \mu M). Black bars denote cells receiving AAG, SCH or both.
osteoblasts (NHOst) are shown as a positive control. The structures of SCH and of the Hsp90 inhibitors used in our study are diagramed in Figure 1b.

We previously showed that newly prepared OS cultures apoptose weakly when exposed to SCH alone for 72–96 h.4 To potentially improve this response, we cotreated OS1002 cells with 10 nM SCH and inhibitors of tyrosine kinases (dasatinib, Gleevec), mitogen-activated protein kinases (U0126, PD98059, SB203580), AKT signaling (MK2206, LY294002), class III histone deacetylases (nicotinamide), DNA synthesis (doxorubicin, etoposide), p53 (nutlin) or Hsp90 (AAG) for 72 h. Apoptosis was monitored by FACS (fluorescence-activated cell sorting) analysis of annexin V-stained cells; annexin V detects externalized phosphatidylserines, a marker of early apoptosis.18 None of the agents tested induced apoptosis in the absence of SCH, and only AAG increased responsiveness to SCH (Figure 1c). AAG by itself had little effect if any. Thus, Hsp90 inhibitors represent a means of sensitizing OS cells to SCH (or vice versa).

The apoptotic response of OS cells to SCH plus AAG was highly significant (Figure 2a) and was seen in all four OS cell lines (Figure 2b). Percentages of cells undergoing apoptosis were generally less than 10% for AAG, less than 20% for SCH, and more than 30% and as high as 50% for SCH plus AAG. Concentrations of AAG eliciting maximal responses in combination with SCH ranged from 0.1 μM to 5 μM (Figures 2b–e). DMAG (500 nM) also induced apoptosis when added to cells with SCH (Figure 1f). Some cotreated cells were both annexin V-positive and propidium iodide-positive, which is indicative of late apoptosis (‘secondary necrosis’) or of necrotic, non-apoptotic cell death (Supplementary Figures 1a–d).

Two additional Hsp90 inhibitors were tested: HSP990 and AUY. In combination with SCH, both inhibitors induced apoptosis of OS cells as effectively as did AAG and DMAG but at much lower concentrations (50 nM) (Figures 3a–c). As additional methods of monitoring apoptosis, we show caspase-3 activation (Figure 3d) and PARP (poly(ADP-ribose)
polymerase) cleavage (Figure 3e) in OS1002 cells cotreated with SCH and AUY. Caspases become active when cleaved and disrupt cell function to elicit cell death; PARP is a caspase-3 substrate.\(^{20}\) Bax, a member of the Bcl-2 family of apoptosis regulatory proteins, perforates the outer mitochondrial membrane to release cytochrome c, which indirectly activates caspase-3.\(^{21}\) Bax is cytosolic in healthy cells and translocates to mitochondria in response to apoptotic signals. We show that Bax accumulates in mitochondria of OS1003 cells cotreated with SCH and AUY as evidenced by its colocalization with the mitochondrial dye MitoTracker (Figure 3f). Colocalization was observed in 40.9% of cotreated cells as compared with 2.5%, 10.6% and 6.1% of control, SCH-treated and AUY-treated cells, respectively. Together, the data in Figures 2 and 3 show that SCH and Hsp90 inhibitors collaboratively induce robust apoptosis of OS cells.

Hsp90 inhibitors also boosted the apoptosis of OS cells cotreated with roscovitine (20 \(\mu\)M) or flavopiridol (200 nM) (Supplementary Figures 2a and b). These agents inactivate CDKs 1, 2 and 9, as does SCH; roscovitine also inactivates CDK7, and flavopiridol is a pan-CDK inhibitor (CDKs 1, 2, 4, 6, 7 and 9).\(^{22–25}\) Hsp90 inhibitors did not induce apoptosis when combined with PD0332991, which selectively targets CDK4 and CDK6 (Supplementary Figure 2c).\(^{26}\) This finding may be explained by redundant actions of PD0332991 and Hsp90 inhibitors: PD0332991 inactivates CDK4 and CDK6, and Hsp90 inhibitors downregulate CDK4 and CDK6.\(^{27,28}\)

To be useful in chemotherapy, drugs must selectively kill tumor cells while sparing normal cells. Cotreatment of normal human osteoblasts (hFOB1.19) or fibroblasts (WI38) with SCH and AUY did not appreciably increase the percentage of annexin V-positive cells (Figures 4a and b) or induce caspase

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**Figure 3** Caspase-3 activation and Bax translocation in OS cells cotreated with SCH and AUY. (a) OS1002 and OS1003 cells received 10 nm SCH, 100 nM HSP990 (990), or both for 48 h. Error bars indicate S.D. Asterisk indicates significant \(P\)-value. \(P\)-values for OS1002 cells are: SCH, 0.15; 990, 0.23; both, 0.007. \(P\)-values for OS1003 cells are: SCH, 0.14; 990, 0.01; both, 0.01. C: control. (b) OS1002 and OS1003 cells received 10 nm SCH and 100 nm AUY or both for 48 h. Error bars indicate S.D. Asterisk indicates significant \(P\)-value. \(P\)-values for OS1002 cells are: SCH, 0.008; AUY, 0.05; both, 0.001. \(P\)-values for OS1003 cells are: SCH, 0.22; AUY, 0.09; both, 0.0002. C: control. (c) OS cells received the indicated concentrations of inhibitors for 48 h. (a–c) Percent apoptotic cells was determined by FACS analysis of annexin V-stained cells. Black bars denote cells receiving SCH (d) OS1002 cells received 10 nm SCH and 50 or 100 nm AUY for 48 h. Cell extracts were western blotted with antibody to PARP or actin (loading control). (e) OS1002 cells received 10 nm SCH and 100 nm AUY for 24 h. Cell extracts were western blotted with antibody to caspase-3. C: control. (f) OS1003 cells received 10 nm SCH and 100 nm AUY for 48 h. Cells were co-stained with anti-Bax antibody (green), MitoTracker Red (red), and the nuclear marker DAPI (blue) as described in Materials and Methods. Magnification is \(\times\) 1890. Numbers to the right of the photographs indicate the percentage of cells with mitochondrial-localized Bax. C: control.
SCH induces apoptosis of OS cells previously exposed to AUY. We asked whether AUY and SCH induce apoptosis when added sequentially rather than simultaneously to cells. OS1002 cells received one inhibitor for 12 h, were refed with fresh medium containing the second inhibitor, and were harvested 36 h after refeeding. Cells did not apoptosis when exposed to SCH and then to AUY (Figure 5a). They did, however, apoptosis when the opposite was done: AUY followed by SCH was as effective as AUY and SCH together (≈27% apoptotic cells in both conditions). AUY was maximally effective when applied to cells for 12 h but only marginally effective when applied to cells for 6 h (Figure 5b). Effectiveness also depended on the timing of SCH addition relative to AUY removal. In the experiment shown in Figure 5c, OS1002 cells received AUY for 18 h and were incubated in fresh medium for 0, 1 or 4 h before addition of SCH; cells were harvested 48 h after SCH addition. When SCH was added to cells immediately after AUY removal, 30% of the cells apoptosed. Delaying SCH addition for 1 h reduced the percentage of apoptotic cells to 17%; delaying SCH addition for 4 h reduced it to 13%. Thus, sequential addition of AUY and SCH to OS cells is most effective when there is no gap between treatments.

Responsiveness of AUY-pretreated cells to SCH did not reflect the presence of residual AUY. AUY substantially reduced the phosphorylation (and thus the activity) of the Hsp90 client AKT; when AUY was withdrawn, phospho-AKT reaccumulated to near starting levels within 1 h (Figure 5d). Total amounts of AKT did not appreciably decline in cells receiving AUY for times up to 24 h; loss of AKT activity in the absence of changes in AKT abundance has also been observed in other systems. 29

**SCH and AUY inhibit cell cycle progression.** OS cells begin apoptosing within 12 h of addition of SCH plus AUY (Figure 6a). We performed a cell cycle analysis to determine whether cells are cycling or arrested at this time. First, we quantified DNA content by propidium iodide staining. OS1002 cells received SCH, AUY or both for 12, 15, 18 or 22 h. Cell cycle distributions were the same for all three treatments and at all time points (Figure 6b). On average, percentages of drug-treated cells in G0/G1, S and G2/M were 65%, 25% and 13%, respectively. In comparison, percentages of control cells in G0/G1, S and G2/M were 52%, 35% and 15%, respectively. The small decrease in percentage of cells in S phase and accompanying increase in percentage of cells in G0/G1 in drug-treated as compared with control cultures suggest that SCH and AUY, alone and together, modestly inhibit S phase entry. We note that cells were attached to the plates at all time points.

Second, as part of the same experiment, we monitored DNA synthesis by bromodeoxyuridine (BrdU) incorporation. Cells received BrdU 12 h after drug addition and were harvested 3, 6 and 10 h later (these time points correspond to the 15, 18 and 22 h time points in Figure 6b). The percentage of control cells incorporating BrdU increased progressively from 28% at 3 h to 45% at 10 h (Figure 6c). In contrast, the BrdU-labeled component of SCH-treated, AUY-treated, and co-treated populations remained constant. Whereas 22–29% of drug-treated cells were in S phase as detected by propidium iodide (Figure 6b), only 9–12% of the drug-treated cells were BrdU-labeled (Figure 6c). These data suggest that cells do not actively synthesize DNA in the presence of SCH, AUY or both. Cotreatment of cells with SCH and AUY for 12 h did not induce histone H2AX phosphorylation (Supplementary Figure 3), a marker of double-stranded DNA breakage, 30 and thus does not cause DNA damage.
Impaired S phase progression was not accompanied by a decrease in the percentage of G2/M cells (Figure 6b); this indicates that drug-treated cells do not exit G2/M. As further evidence, BrdU-positive cells accumulated in G0/G1 10 h after BrdU addition to control but not drug-treated cultures (Figure 6d). Collectively, our data suggest that SCH and AUY ‘freeze’ cells in multiple phases of the cell cycle. We conclude that OS cells cotreated with SCH and AUY are not cycling at the onset of apoptosis. Cell cycle arrest, however, is insufficient or unrelated to apoptosis: cell cycle arrest occurs in response to either SCH or AUY, whereas efficient apoptosis requires both SCH and AUY.

**Discussion**

Our studies show apoptosis of OS cultures by a novel drug combination: the CDK inhibitor SCH and an Hsp90 inhibitor. CDKs drive the cell cycle, which is often dysregulated in cancer, and ensure an ample supply of short-lived anti-apoptotic proteins. Hsp90 is essential for the proper functioning of numerous proteins and signal transduction pathways and is more susceptible to Hsp90 inhibitors in tumor cells than in normal cells. Thus, CDKs and Hsp90 are rational targets for drug intervention.

The OS cultures used in our experiments were derived from resected tumors and were passaged less than 20 times. Gillet et al. suggest that long-term culturing radically alters gene expression and distorts drug sensitivity: they found striking differences in the multi-drug resistance gene profiles of ovarian clinical samples versus established ovarian cancer cell lines. Thus, use of OS cultures minimizes potential culture-induced anomalies and increases the likelihood of obtaining information that will translate successfully to the clinic. We note that OS cultures are much less sensitive to SCH than are established OS cell lines such as U2OS and SaOs-2.4

We used four Hsp90 inhibitors in our experiments: AAG and DMAG, which are ansamycin derivatives, and HSP990 and AUY, which are synthetic small molecules classified as...
aminopyridine and resorcinol-containing, respectively. None induced OS apoptosis in the absence of SCH; all enhanced the weak apoptotic response elicited by SCH. HSP990 and AUY were especially potent, producing effects at concentrations of 25–50 nM. OS cells apoptosed when cotreated with SCH and an Hsp90 inhibitor regardless of whether their tumor of origin was localized or metastatic. Of particular note, OS1002 cells were derived from a tumor that recurred after chemotherapy. Combined application of SCH and Hsp90 inhibitor also induced the apoptosis of other sarcoma types but did not affect the survival of normal osteoblasts.

Interestingly, we show that AUY and SCH need not be present together to induce OS apoptosis: AUY can be added and removed before addition of SCH but not vice versa. This finding suggests that AUY elicits an event (or events) that renders OS cells responsive to SCH. For example, it may cause an anti-apoptotic protein to degrade or to lose its active conformation or may simply enervate cells by allowing buildup of toxic, unfolded protein aggregates. Sequential treatment was most effective when cells were primed with AUY for ~12 h and immediately exposed to SCH. Thus, the AUY-induced priming event is complete by 12 h and is short-lived in the absence of AUY.

As a reverse scenario, it is possible that SCH renders OS cells responsive to AUY. AUY may elicit both apoptotic and anti-apoptotic signals; negation of the anti-apoptotic signal by SCH would allow apoptosis to proceed. A potential anti-apoptotic signal is the induction of Hsp72. Hsp72 is a member of the Hsp70 family of molecular chaperones, and Hsp90 inhibitors upregulate its expression by activating the Hsf-1 transcription factor. Colon cancer cells depleted of Hsp72 (and a second Hsp70 isoform), apoptosed when exposed to AAG, whereas mock-depleted cells did not.

Figure 6  SCH and AUY inhibit cell cycle progression. (a) OS1002 cells received 20 nM SCH and 100 nM AUY for 12, 24 or 48 h. Percent apoptotic cells was determined by FACS analysis of annexin V-stained cells. (b) Cells received 20 nM SCH, 100 nM AUY, or both for 12, 15, 18 or 22 h. Cells were stained with propidium iodide, and cell cycle position was determined as described in Materials and Methods. (c) Cells received 20 nM SCH, 100 nM AUY, or both for 12 h. BrdU was added, and cells were harvested 3, 6 and 10 h after BrdU addition. Percent BrdU-labeled nuclei was determined as described in Materials and Methods. (d) Scatter plots for the 10 h time point in (c) are shown. The percentages of cells in S phase are indicated.
Similarly, Hsf-1-null transformed fibroblasts were more responsive to AAG than were their wild-type counterparts. Whether SCH prevents the upregulation of Hsp72 by Hsp90 inhibitors in OS cells remains to be determined.

Regardless of the mechanism, sequential application of SCH and AUY may allow flexibility when planning treatment schedules. Previous studies showed apoptosis of Rb-positive breast cancer cells sequentially exposed to AAG and taxol;
AAG was effective when added to cells less than 4 h before or after taxol.37

Our studies show that Bax is predominantly cytoplasmic in OS cells receiving SCH or AUY but accumulates in the mitochondria of cotreated cells. How SCH and AUY collaboratively trigger the mitochondrial translocation of Bax is unclear at present. Of interest is a recent study showing the interaction of Bax with the Hsp90 cochaperone p23 in the cytosol of healthy cells.38 Depletion or overexpression of p23 did not affect the subcellular location of Bax, thus suggesting that p23 controls functions of Bax unrelated to its location in the cell or that it modulates Bax location in concert with additional events.

In addition to inducing apoptosis, SCH and AUY also affected cell cycle progression. Alone or together, they blocked OS cells in G0/G1, S and G2/M. As robust apoptosis requires both inhibitors, cell cycle arrest is unrelated to or insufficient for the apoptosis of OS cells. Similar to our findings on OS cells, CDK inhibitors reduced the percentage of BrdU-positive S phase cells in myeloma, neuroblastoma, and colon, lung and breast carcinoma cultures; G2/M arrest, and in some instances G0/G1 arrest, was also observed.39–42 Hsp90 inhibitors arrest different tumor cell lines in different parts of the cell cycle: G0/G1 or G2/M, as shown by propidium iodide staining in several studies.38, 43–46 or G0/G1, G2 or M, as shown by Lyman et al.49 by combined use of a thymidine analog and cell cycle markers. As an exception, HCT116 colon carcinoma cells did not accumulate in either G0/G1 or G1/M in response to AAG.49 Thus, the cell cycle responses of tumor cells to Hsp90 inhibitors are variable and may reflect cellular genotype, at least in part.

Advances in surgical techniques and the advent of multi-agent chemotherapy have greatly improved the prognosis of OS patients. However, better treatments are still needed, particularly for patients with recurrent or metastatic disease. We suggest that SCH and AUY may be a promising strategy for treatment of OS and other types of sarcomas.

Materials and Methods

Cell culture. Fresh, finely minced sarcoma tissue was incubated with DNase I and collagenase D for 30 min at 37 °C, washed extensively and transferred to 10 cm2 culture flasks. Cells were inoculated in a 1:1 mixture of Ham’s F12 medium and Dulbecco’s Modified Eagle’s medium containing 10% fetal calf serum. After expansion of cultures, frozen stocks were prepared. Frozen stocks served as the source of the OS cultures used in experiments. OS cells were passaged less than 20 times. NHOst cultures were purchased from Lonza (Walkersville, MD, USA) and cultured in Osteoblast Growth Medium (OBM Bullet Kit, Lonza). NFOB1.19 cells (normal human fetal osteoblasts expressing temperature-sensitive SV40 large T antigen) were obtained from ATCC and cultured at the permissive temperature (37 °C). Percent BrdU-labeled cells and DNA content were plotted on the x and y axes, respectively, of the scatter plots.

Brdu staining. Cells received 30 µM Brdu for times indicated in the figure legends. Cells were removed from the plates and fixed in PBS and ethanol as described above for propidium iodide staining. Pelleted cells were resuspended in 2 N HCl and incubated for 30 min at 37 °C to denature DNA. Sodium borate (final concentration, 0.1 M) was added to neutralize the pH, and cells were washed thoroughly to remove any residual acid. Cells were incubated overnight at 4 °C in PBS containing 0.1% bovine serum albumin, 0.1% Tween-20 and a 1/20 dilution of mouse monoclonal anti-BrdU-FITC antibody (eBioscience, San Diego, CA, USA). After a further incubation at 4 °C for at least 4 h, cell cycle distribution was determined by FACS. DNA content (FL3, FL4 area) and relative amount of BrdU per cell (FL1, H-Anti-BrdU-FITC) are plotted on the x and y axes, respectively, of the scatter plots.

Statistical analysis. P-values were determined by a paired, two-tailed Student’s t-test of three or more samples. Treated groups were compared with untreated (control) groups. P-values < 0.05 are considered significant.

Conflict of Interest

The authors declare no conflict of interest.
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