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Preclinical Development

Molecular Cancer Therapeutics

The Cyclin-Dependent Kinase Inhibitor SCH 727965 (Dinaciclib) Induces the Apoptosis of Osteosarcoma Cells

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Abstract

Although rare, osteosarcoma is an aggressive cancer that often metastasizes to the lungs. Toward the goal of developing new treatment options for osteosarcoma, we show that the cyclin-dependent kinase (CDK) inhibitor SCH 727965 (SCH) induces the apoptosis of several osteosarcoma cell lines including those resistant to doxorubicin and dasatinib. Cell lines prepared in our laboratory from patients who had received adjuvant chemotherapy and explants derived from a human osteosarcoma xenograft in mice were also responsive to SCH. Apoptosis occurred at low nanomolar concentrations of SCH, as did CDK inhibition, and was p53-independent. SCH activated the mitochondrial pathway of apoptosis as evidenced by caspase-9 cleavage and accumulation of cytoplasmic cytochrome c. Amounts of the apoptotic proteins Bax and Bim increased in mitochondria, whereas amounts of the antiapoptotic proteins Mcl-1 and Bcl-x_L declined. Osteosarcoma cells apoptosed when codepleted of CDK1 and CDK2 but not when depleted of other CDK combinations. We suggest that SCH triggers the apoptosis of osteosarcoma cells by inactivating CDK1 and CDK2 and that SCH may be useful for treatment of drug-resistant osteosarcomas. SCH also induced the apoptosis of other sarcoma types but not of normal quiescent osteoblasts or fibroblasts. *Mol Cancer Ther; 10(6); 1018–27. ©2011 AACR.*

Introduction

Osteosarcoma is an aggressive bone cancer typically observed in children and adolescents. It occurs mostly in the long bones of the limbs and metastasizes primarily to the lungs (1). Treatment options include surgery, either amputation or limb salvage, and chemotherapy, most notably combinations of doxorubicin (Dox), methotrexate, and cisplatin (1, 2). The 10-year survival rate of patients with localized disease who receive chemotherapy is 60% to 70%. For patients with metastatic disease, it is less than 30%. Thus, the need for new treatments for patients unresponsive to current chemotherapy regimens is apparent.

Apoptosis is a program of events that results in cell death (3). It requires caspase (cysteine aspartyl protease) activity, and caspases become active when cleaved (4). Adaptor proteins facilitate the autocleavage of initiator caspases (e.g., caspase-8 and caspase-9), initiator caspases cleave effector caspases (e.g., caspase-3), and effector caspases disrupt cell function to elicit cell death. Two events initiate adaptor-mediated caspase cleavage: the binding of ligands to death receptors (the death receptor pathway) and the release of cytochrome c (Cyt c) from mitochondria (the mitochondrial pathway). Death receptors activate caspase-8, whereas Cyt c activates caspase-9; caspase-3 is common to both pathways.

Most chemotherapeutic agents induce apoptosis via the mitochondrial pathway (3). Regulators of this pathway include the Bcl-2 proteins and p53. There are 3 types of Bcl-2 proteins: antiapoptotic (e.g., Bcl-x_L and Mcl-1), single-domain apoptotic (termed as BH3-only), and multidomain apoptotic (e.g., Bax; ref. 5). When oligomerized, Bax perforates the outer mitochondrial membrane to release Cyt c. Antiapoptotic proteins block Bax oligomerization; BH3-only proteins such as Bim facilitate Bax oligomerization. p53 accumulates in cells exposed to chemotoxic drugs and promotes apoptosis by 2 mechanisms. It transactivates genes that encode apoptotic proteins, and it translocates to mitochondria where it interacts with Bcl-2 proteins (6). Many osteosarcomas exhibit p53 abnormalities, and mice expressing p53-null osteoblast progenitor cells develop osteosarcomas (7, 8).

Inhibitors of cyclin-dependent kinases (CDK) such as Roscovitine (Rosc, seliciclib) and Flavopiridol (Flav; alvocidib) induce the apoptosis of many tumor cell types. Our studies, for example, show apoptosis of melanoma and prostate carcinoma cells by Rosc (9, 10). CDKs are nuclear serine–threonine kinases; active CDK complexes contain both a cyclin and a CDK (11). Gene transcription requires the activity of CDK7 and CDK9. These CDKs phosphorylate the large subunit of RNA polymerase II (RNAP II) at

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distinct sites in its C terminus to facilitate promoter clearance (CDK7-cyclin H) and elongation of nascent transcripts (CDK9-cyclin T). Cell-cycle progression requires the activity of CDK4 and CDK6 (collectively referred to as CDK4/6), CDK2, and CDK1. CDK4/6 (with cyclin D1, D2, or D3) and CDK2 (with cyclin E) promote S-phase entry by phosphorylating and inactivating the retinoblastoma (Rb) protein; CDK2 (with cyclin A) and CDK1 (with cyclin A or cyclin B) propel cells through S phase and into mitosis, respectively. Whether CDK inhibitors induce apoptosis by repressing transcription or by perturbing the cell cycle (or both) is not clear.

SCH 727965 (SCH; dinaciclib) is a new CDK inhibitor developed by Paruch and colleagues (12). It inhibits the activity of CDK1, CDK2, and CDK9 *in vitro* with IC_{50} (inhibitory concentration) values of 1 to 4 nmol/L (13). In contrast, IC_{50} values for inhibition of CDK1 activity for Flav and Rosc are 12 to 200 and 400 nmol/L, respectively (13, 14). SCH does not inhibit the activity of non-CDKs such as Src family members and protein kinase C isoforms, even at concentrations of 10 mmol/L, and is more selective than Flav.

Here, we show that SCH induces the apoptosis of several osteosarcoma cell lines at low nanomolar concentrations and irrespective of p53 status. Our data suggest that SCH triggers apoptosis by inactivating CDK1 and CDK2 and via the mitochondrial pathway.

Materials and Methods

Cell culture

U2OS, MG63, and SaOs-2 cells were obtained from American Type Culture Collection (ATCC). LM7 cells were provided by Dr. E.S. Kleinerman (M. D. Anderson Cancer Center, Houston, TX). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 2 mmol/L L-glutamine, and 100 mg/mL penicillin/streptomycin. All are human osteosarcoma cell lines.

Normal human osteoblasts (NHOst) were purchased from Lonza and were cultured in Osteoblast Growth Medium (OGM BulletKit, Lonza, Catalog # 3207). Human fetal osteoblast (hFOB) 1.19 cells (NHOsts expressing temperature-sensitive SV40 large T antigen) were obtained from ATCC and were cultured at the permissive temperature (33.5°C) in a 1:1 mixture of Ham's F12/DMEM containing 0.3 mg/mL G418 and 10% fetal calf serum but lacking phenol red. WI-38 cells (normal human fibroblasts) were from our frozen stocks and were cultured in minimum essential medium containing 10% fetal calf serum. Commercially obtained cell lines were not authenicated by the authors.

Establishment of tumor-derived osteosarcoma cells

Fresh finely minced osteosarcoma tissue was incubated with DNase I and Collagenase D for 30 minutes at 37°C, washed extensively, and transferred to 10 cm^2 culture flasks. Cells were incubated in Ham's F12/DMEM containing 10% fetal calf serum. Three cell lines were established: OS8328, OS8521, and OS7770. OS8328 and OS8521 were resected after adjuvant chemotherapy; the percentage of necrotic cells was 40% to 50%. OS7770 was resected with no chemotherapy and with minimal necrosis. These cell lines were authenicated by monitoring the expression of osteoblast-specific markers by quantitative RT-PCR.

Preparation and assay of tumors *ex vivo*

Tumors were collected from patient-derived osteosarcoma xenografts by surgical resection of s.c. flank injections in SHO-severe combined immunodeficient mice (Charles River Laboratories). Tumors were processed into 0.1 to 0.3 mm fragments, and tumor fragments were resuspended in RPMI 1640 culture medium containing 10% fetal calf serum, 200 g/mL streptomycin, 200 g/mL penicillin, 25 mmol/L HEPES, and 0.1% $NaHCO_3$. Resuspended fragments were lightly seeded into culture dishes and treated with inhibitors as described in the text. After treatment, samples were collected by scraping and centrifuged at 500× g for 5 minutes at 4°C. Pellets were snap-frozen in a 95% ethanol and dry ice bath.

Annexin V binding

Cells were detached from plates with trypsin-EDTA and combined with floating cells. Cells were stained with Annexin V-FITC and PI/7-AAD (BD Pharmingen), and stained cells were analyzed by fluorescence-activated cell sorting (FACS), as described previously (15). Results shown in bar graphs were taken from the lower right quadrant of the plots and represent early apoptosis.

Western blotting

Cells were washed with ice-cold PBS, scraped into PBS, and collected by centrifugation. Pellets were resuspended in a lysis buffer containing 50 mmol/L HEPES, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 10% glycerol, 0.5% NP-40, 0.5% Tween 20, 1 mmol/L dithiothreitol, and protease inhibitor cocktail (Sigma) and vortexed for 20 minutes at 4°C; insoluble material was removed by centrifugation. Membrane, cytoplasmic, and mitochondrial fractions were prepared by a commercially available kit (Pierce). Proteins (50 μg) were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated sequentially in TBS containing 0.05% Tween-20 and 5% nonfat dry milk as follows: no addition, 1 hour at room temperature (blocking); primary antibody, overnight at 4°C; and secondary antibody (Amersham) diluted 1/1,000, 1 hour at room temperature. Bound secondary antibody was detected by West Pico and West Femto chemiluminescent substrates (Pierce).

Reagents

SCH was provided by Schering-Plough Corporation and Cancer Therapy Evaluation Program (CTEP); Flav was provided by CTEP. Rosc, Dox, and etoposide were purchased from EMD Bioscience. Antibodies were

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obtained from Cell Signaling (Rb, phospho-Rb, PARP, p53, caspase-3, -8, -9); BD Biosciences (Mcl-1, CDK4, $p27^{Kip1}$, phospho-$p27^{Kip1}$); Santa Cruz (CDK1, CDK2, CDK7, CDK9, Bcl-x_L); Bethyl Laboratories (phospho-RNAP II, RNAP II); Sigma (actin); Clontech (Cox4, Cyt c); and EMD Bioscience (Bax, Bim). Smartpool siRNAs for p53, CDK1, CDK2, and CDK9 were purchased from Dharmacon and introduced into cells using DharmaFECT.

Results

SCH abrogates the phosphorylation of CDK substrates in osteosarcoma cells

The structure of SCH is shown in Fig. 1A. To assess the effects of SCH on CDK activity *in vivo*, we monitored the site-specific phosphorylation of CDK substrates in 2 human osteosarcoma cell lines, SaOs-2 and U2OS. Cells received SCH at various concentrations and for various times, and cell extracts were Western blotted with the appropriate antibodies.

SCH attenuated the phosphorylation of RNAP II at serine 2 (Fig. 1 B–D) and the phosphorylation of the CDK inhibitor $p27^{Kip1}$ at threonine 187 (Fig. 1E). These sites are phosphorylated by CDK9 and CDK2, respectively (16, 17). Reductions in phosphorylation occurred at 12 to 40 nmol/L SCH and within 4 to 16 hours after SCH addition. SCH also reduced the phosphorylation of Rb at serine 807/811 (Fig. 1F). CDK1, CDK2, and CDK4/6 all phosphorylate Rb at this site (18–21), and we note that CDK1 also phosphorylates $p27^{Kip1}$ at threonine 187, at least *in vitro* (22, 23). SCH did not appreciably affect the phosphorylation of RNAP II at serine 5, the CDK7 site (Fig. 1D; ref. 16).

These findings suggest that SCH inhibits the activity of CDK2, CDK9, and perhaps CDK1 *in vivo*, as it does *in vitro*. Amounts of RNAP II, CDK9, CDK1, and Rb were unaffected by SCH, whereas amounts of CDK2 declined (Fig. 1B–F). Thus, reductions in CDK2 abundance may contribute to loss of CDK2 activity in osteosarcoma cells. Amounts of $p27^{Kip1}$ increased substantially (Fig. 1E), presumably due to stabilization: when phosphorylated at threonine 187, $p27^{Kip1}$ is ubiquitinated and degraded in the proteasome (24, 25). There was also a decrease in CDK7 abundance at 24 hours (Fig. 1D).

Inhibition of CDK4/6 activity *in vitro* requires 100-fold more SCH than does inhibition of CDK2 activity (IC_{50} values are 100 and 1 nmol/L, respectively). However, phosphorylation of Rb at the CDK4/6 site (serine 780; ref. 26) was significantly reduced in U2OS cells receiving 20 nmol/L SCH for 16 or 24 hours (Fig. 1F). Amounts of total Rb and CDK6 did not change, whereas amounts of CDK4 increased. We suggest that SCH indirectly inhibits CDK4/6 activity by increasing $p27^{Kip1}$ abundance (Fig. 1E) and reducing cyclin D abundance (data not shown).

SCH induces osteosarcoma apoptosis

Effects of SCH on the survival of 3 human osteosarcoma cell lines (SaOs-2, U2OS, and MG63) were determined.

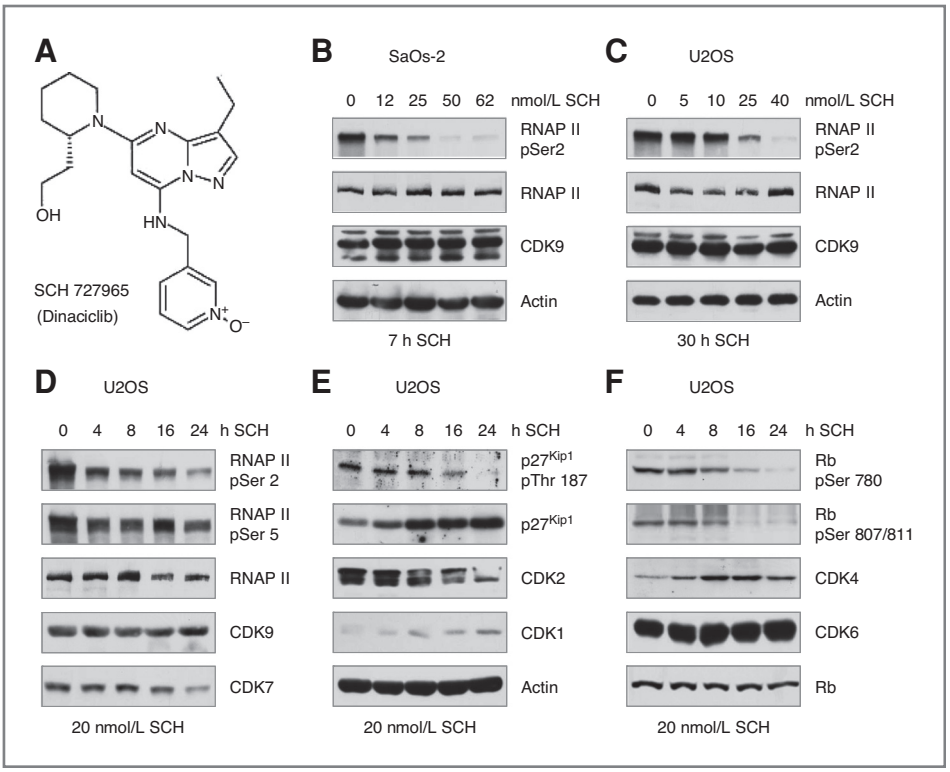

Figure 1. Effects of SCH on site-specific phosphorylation of CDK substrates in osteosarcoma cells. A, chemical structure of SCH. B to F, U2OS and SaOs-2 cells received the indicated amounts of SCH for the indicated times. Cell extracts were Western blotted with antibodies to the indicated proteins. D, E, and F are from the same experiment. Actin is shown as a loading control.

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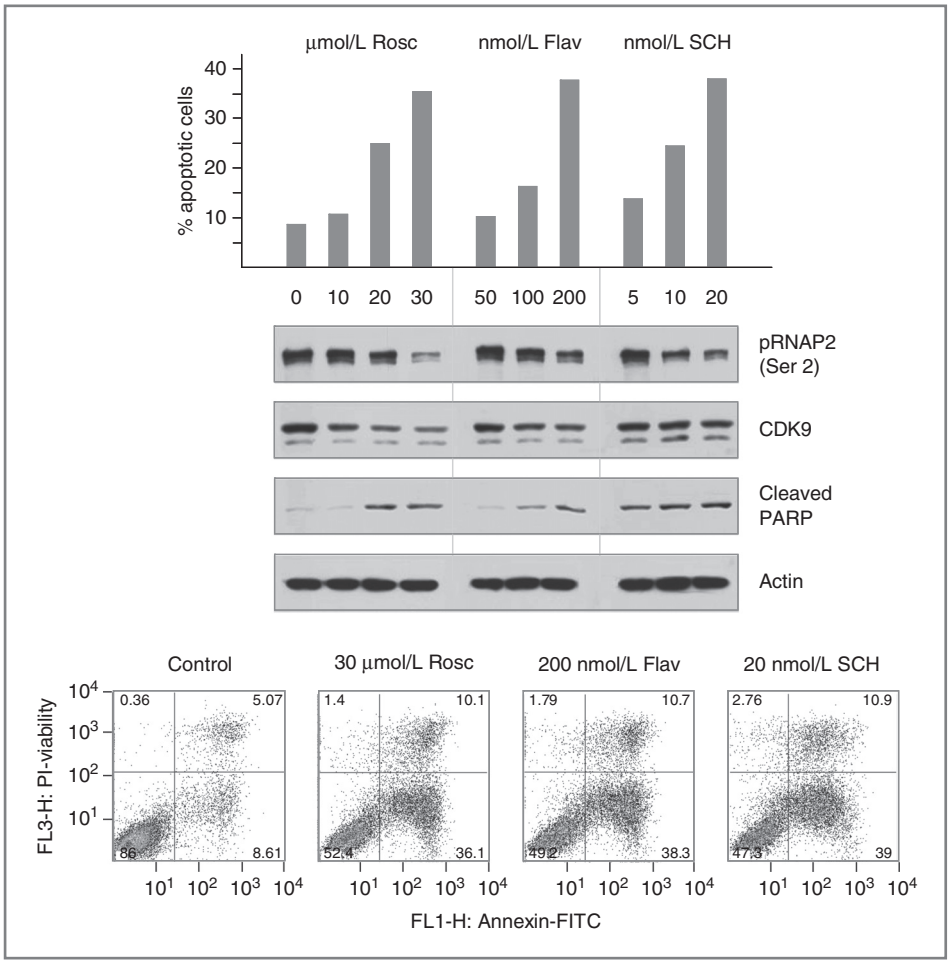

Figure 2. SCH-induced apoptosis of SaOs-2 cells: comparison with Rosc and Flav. SaOs-2 cells received the indicated amounts of Rosc, Flav, or SCH for 7 hours (Western blots) or 30 hours (bar graphs and plots). The percentage of apoptotic cells was determined by Annexin V binding (bar graph and plots). Protein abundance was determined by Western blotting of cell extracts with the indicated antibodies. Lower right quadrant of plots shows the number of cells in early apoptosis; these numbers were used in the bar graph. Upper right quadrant shows cells in late apoptosis or necrosis. pSer, phosphorylated serine.

Apoptosis was monitored by FACS analysis of Annexin V-stained cells and by cleavage of the caspase-3 substrate PARP. Annexin V detects phosphatidylserines on the outer surface of the cell; normally expressed on the inner leaflet of the plasma membrane, phosphatidylserines become externalized during early apoptosis (27).

In the first set of experiments, SaOs-2 cells received SCH or, for comparison, Rosc or Flav for 30 hours. Rosc inhibits the activity of CDK1, CDK2, CDK7, and CDK9, whereas Flav is a pan CDK inhibitor (28–31). All 3 inhibitors induced SaOs-2 apoptosis: amounts of cleaved PARP were similar, as were percentages of annexin-bound cells (approximately 35% at the highest concentrations tested; Fig. 2). However, SCH was effective at 1,000-fold and 10-fold lower concentrations than were Rosc and Flav, respectively. Concentrations of inhibitor that induced apoptosis were similar to those that attenuated RNAP II phosphorylation at serine 2 (CDK9 site).

In the second set of experiments, U2OS, MG63, and SaOs-2 cells received SCH at various concentrations for various times. All 3 cell lines apoptosed in response to SCH (Fig. 3A). MG63 cells were the least sensitive: onset of apoptosis was 8 to 16 hours after SCH addition, and the percentage of apoptotic cells was 18% at 48 hours. SaOs-2 cells were the most sensitive: onset of apoptosis was 4 hours after SCH addition, and the percentage of apoptotic cells was 40% at 16 hours. A similar percentage of U2OS cells apoptosed; however, a longer treatment time (48 hours) was required. The SCH dose dependence of apoptosis (10 to 40 nmol/L) mirrored that of CDK inhibition. Apoptosis did not correlate with p53 status: U2OS, MG63, and SaOs-2 cells express wild-type, mutant, and no p53, respectively (32, 33). Nor was p53 required: SCH induced the apoptosis of mock- and p53-depleted U2OS cells to a similar extent (Fig. 3B). p53 was depleted by RNA interference; Western blots show near elimination of p53 from cells receiving p53 siRNA.

Variants of SaOs-2 and U2OS also apoptosed when exposed to SCH. LM7 cells were derived from SaOs-2 cells by repeated i.v. recycling through the lungs of nude mice; unlike parental cells, they are highly metastatic (34). Approximately 30% of LM7 cells were annexin-positive after a 54-hour exposure to 20 nmol/L SCH (Fig. 3C). Dox-resistant U2OS cells were prepared in our laboratory by treating cells with gradually increasing concentrations of Dox over a 6-month period. Parental cells apoptosed when exposed to 400 nmol/L Dox for 48 hours, whereas resistant cells did not (Fig. 3D). However, both

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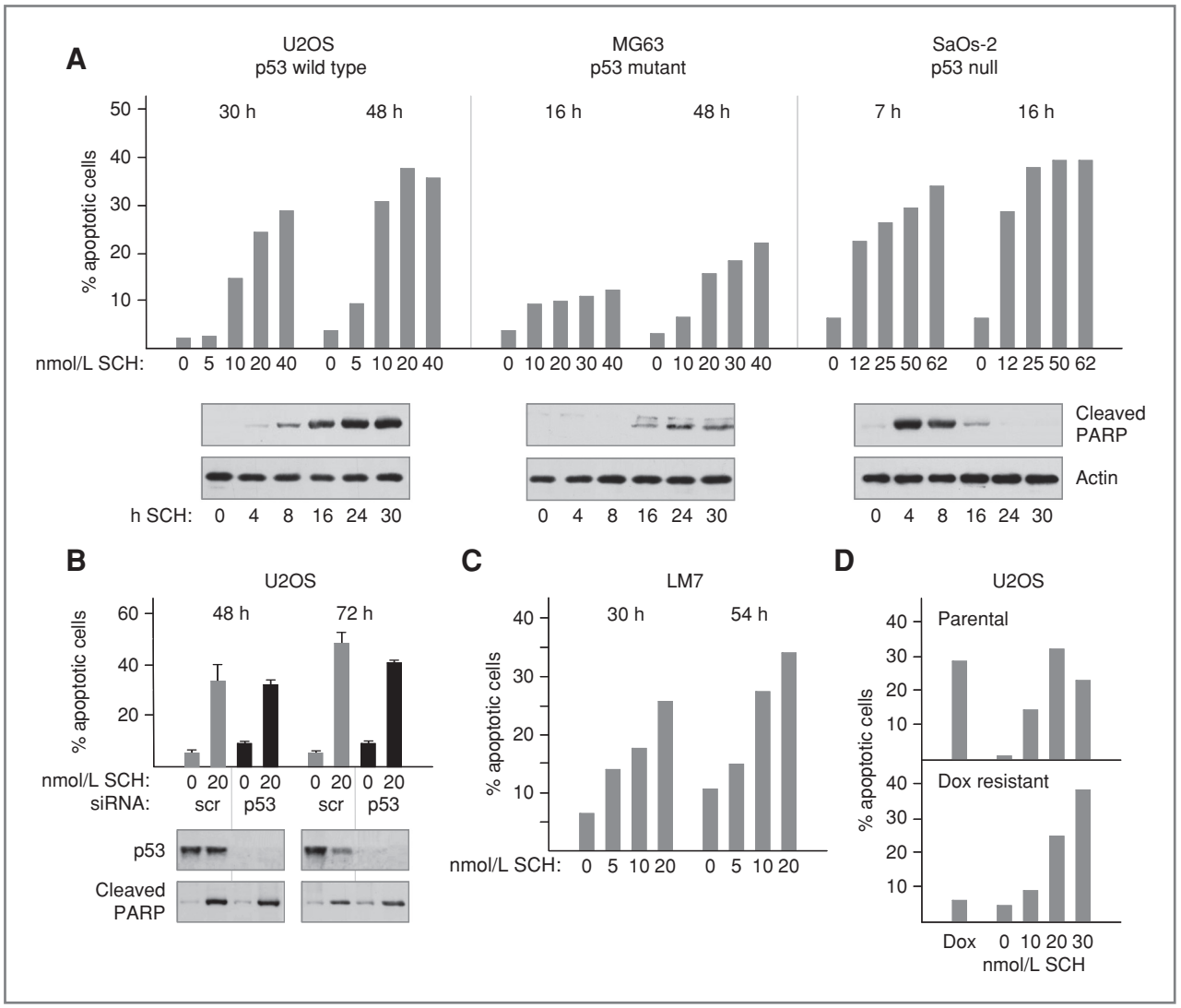

Figure 3. SCH-induced apoptosis of established osteosarcoma cell lines. A, cells received various concentrations of SCH for the indicated times (bar graphs) or 20 nmol/L SCH for the indicated times (Western blots). The 3 panels represent separate experiments. B, U2OS cells received scrambled siRNA (gray bars) or p53 siRNA (black bars) for 48 or 72 hours; SCH was added 24 hours before harvest. Western blots for p53 and cleaved PARP are from the same gel. C, LM7 cells received various concentrations of SCH for 30 or 54 hours. D, parental and Dox-resistant U2OS cells received 400 nmol/L Dox or the indicated amounts of SCH for 48 hours. A to D, the percentage of apoptotic cells was determined by Annexin V binding. B, error bars show SD.

populations were equally susceptible to SCH. When added with SCH, Dox did not appreciably enhance or inhibit U2OS or MG63 apoptosis (data not shown). A similar result was obtained for the combination of SCH and etoposide.

As a further test of SCH efficacy, we prepared cell lines from osteosarcoma tissue removed from patients who had (OS8328, OS8521) or had not (OS7770) received adjuvant chemotherapy. Patients responded poorly to chemotherapy on the basis of tumor necrosis (less than 90%; ref. 1). As determined by quantitative RT-PCR, all 3 cell lines expressed the osteoblastic markers osteopontin, osteocalcin, and alkaline phosphatase (data not shown). SCH induced apoptosis of OS770, OS8328, and OS8521 cells albeit to a small extent: 14% to 20% of cells were apoptotic 72 to 96 hours after SCH addition (Fig. 4A).

To more closely approximate tumors *in vivo*, tissue fragments of patient-derived xenografts grown in mice were placed in culture dishes and exposed to SCH or, for comparison, Rosc or Flav for 30 hours. All 3 inhibitors induced apoptosis of tumor fragments as assessed by PARP cleavage (Fig. 4B). As observed for cultured cells (see Fig. 2), SCH induced apoptosis at lower concentrations than did Rosc or Flav.

To be useful as a chemotherapeutic agents, a drug must selectively kill tumor cells while sparing normal cells. Quiescent nontransformed human osteoblasts (NHOst and hFOB1.19) were refractory to SCH: when exposed

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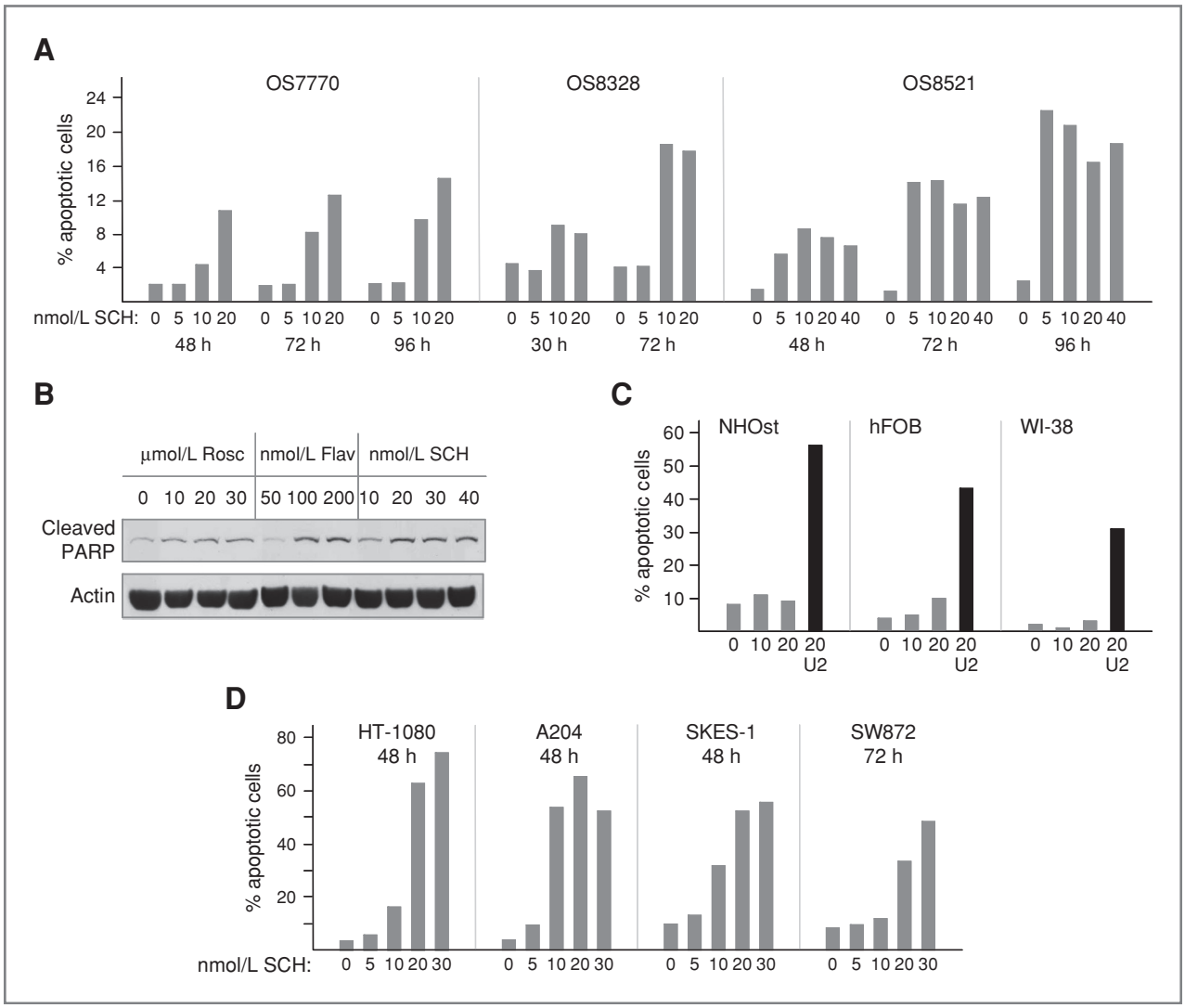

Figure 4. SCH-induced apoptosis of newly prepared osteosarcoma cell lines and osteosarcoma tumor fragments but not of normal osteoblasts or fibroblasts. A, Osteo7770, Osteo8328, and Osteo8521 cells received SCH at the indicated concentrations for the indicated times. The percentage of apoptotic cells was determined by Annexin V binding. B, fragments of osteosarcoma xenografts grown in mice were cultured in medium containing the indicated concentrations of Rosc, Flav, or SCH for 30 hours. Cell extracts were Western blotted with antibody to PARP or actin. C, density-arrested NHOst, hFOB1.19, and WI-38 cells (gray bars) received the indicated concentrations of SCH for 72 hours. U2OS cells (black bars, positive control) received 20 nmol/L SCH for 72 hours. The percentage of apoptotic cells were determined by Annexin V binding. The 3 panels represent separate experiments. D, cells received the indicated amounts of SCH for 48 or 72 hours. The percentage of apoptotic cells was determined by Annexin V binding.

to SCH for 72 hours, more than 90% of cells remained viable (Fig. 4C). WI-38 cells, which are nontransformed human fibroblasts, also survived a 72-hour exposure to SCH.

Other sarcoma types were sensitive to SCH: HT-1080 fibrosarcoma, A204 rhabdosarcoma, SKES-1 Ewing's sarcoma, and SW872 liposarcoma cells (Fig. 4D). The percentage of apoptotic cells for cells receiving SCH for 48 to 72 hours ranged from 44% (SW872) to 77% (HT1080). We note that not all sarcoma cell lines tested apoptosed in response to SCH: SKLMS leiomyosarcoma cells, for example, were refractory to SCH (data not shown).

Combined depletion of CDK1 and CDK2 induces apoptosis

How CDK inhibitors induce apoptosis is incompletely understood. They may do so by inactivating CDK7 and/or CDK9 and repressing transcription: this would deplete cells of short-lived survival proteins such as Mcl-1 (11, 35). Alternatively, inactivation of the cell-cycle CDKs and abrogation of cell-cycle progression may activate the DNA damage checkpoint or trigger mitotic catastrophe (36, 37). Inactivation of CDK1 and CDK2 also precludes downregulation of E2F-1 activity in S phase: when inappropriately activated, this transcription factor induces apoptosis (38, 39).

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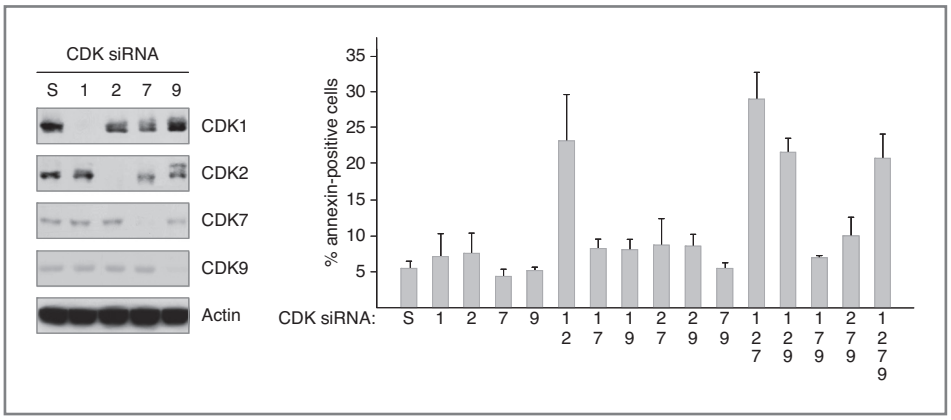

Figure 5. Apoptosis of osteosarcoma cells codepleted of CDK1 and CDK2. U2OS cells were transfected with siRNAs to CDK1, CDK2, CDK7, and CDK9 as indicated. Cells were harvested 72 hours after transfection. Cell extracts were immunoblotted with antibodies to the indicated proteins (Western blots). The percentage of apoptotic cells was determined by Annexin V binding (bar graphs). S, scrambled siRNA. Error bars show SD.

To identify the CDK(s) whose inactivation leads to osteosarcoma apoptosis, we transfected U2OS cells with siRNAs to CDK1, CDK2, CDK7, and CDK9, alone or in combination. Western blots confirmed depletion of these CDKs by their respective siRNAs (Fig. 5, Western blots). Cells depleted of a single CDK did not apoptose as monitored by annexin binding (Fig. 5, bar graph). Combined depletion of CDK1 and CDK2 induced apoptosis and was the only combination to do so. Cells codepleted of CDK1 and CDK2 apoptosed to a similar extent as did cells codepleted of CDK1, CDK2, and either or both CDK7 and CDK9. These findings suggest that SCH induces the apoptosis of osteosarcoma cells by inactivating CDK1 and CDK2.

SCH modulates the abundance of Bcl-2 proteins

SCH causes cleavage of PARP, which is a caspase-3 substrate (see Figs. 2, 3A and B, and 4B). In agreement with this finding, Western blots show cleaved (active) caspase-3 in extracts of SCH-treated but not control SaOs-2 cells (Fig. 6A). They also show cleaved caspase-9 but not cleaved caspase-8 in extracts of treated cells. Thus, SCH activates the mitochondrial but not the death receptor pathway of apoptosis. Inhibition of caspase-9 and caspase-3 with pharmacologic inhibitors rescued cells from SCH-induced apoptosis (Fig. 6B).

Consistent with mitochondrial damage, Cyt c accumulated in the cytoplasm of U2OS cells exposed to SCH for 24 hours (Fig. 6C). To approach mechanism, we monitored amounts of Bcl-2 proteins in mitochondrial extracts of U2OS cells treated with 20 nmol/L SCH for 24 hours. The apoptotic proteins Bax and Bim accumulated in mitochondria-enriched fractions of SCH-treated cells, whereas amounts of the antiapoptotic proteins Bcl-x_L and Mcl-1 decreased (Fig. 6D).

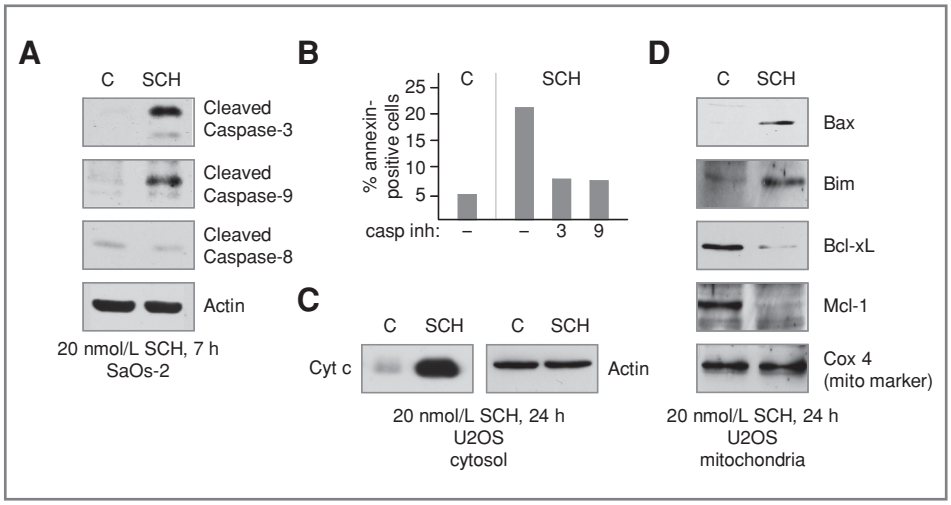

Figure 6. Activation of the mitochondrial pathway of apoptosis by SCH. A, SaOs-2 cells received 20 nmol/L SCH for 7 hours. Amounts of cleaved caspases were determined by Western blotting of whole cell extracts. B, SaOs-2 cells were pretreated with 50 mmol/L Z-DEVD-FMK (caspase-3 inhibitor) or Z-LEHD (caspase-9 inhibitor) for 1 hour. Cells then received 20 nmol/L SCH for 7 hours. The percentage of apoptotic cells was determined by Annexin V binding. C, U2OS cells received 20 nmol/L SHC for 24 hours. Cytosolic extracts were Western blotted with antibody to Cyt c or actin (cytoplasmic marker). D, U2OS cells received 20 nmol/L SCH for 24 hours. Mitochondrial fractions were Western blotted with antibodies to the indicated proteins.

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Discussion

Our studies show apoptosis of several osteosarcoma cell lines by the CDK inhibitor SCH. Most notable were those unresponsive to other drugs: U2OS cells selected for Dox resistance; MG63 cells, which do not respond to dasatinib as described in our previous publication (40); and 2 cell lines (OS8328 and OS8521) prepared in our laboratory from tumors of patients who had received adjuvant chemotherapy. Dox, which damages DNA, is part of a cocktail commonly used to treat osteosarcoma; dasatinib, which inhibits Src activity, is currently in clinical trials for osteosarcoma and other cancers (41). SCH also induced the apoptosis of metastatic SaOs-2 variants. These findings point to the potential value of SCH as a second-line osteosarcoma treatment. Parry and colleagues (13) showed apoptosis of a panel of tumor cell lines, including those expressing multidrug-resistance gene I, by SCH.

As a potential chemotherapeutic agent, SCH is attractive for 3 reasons. First, SCH selectively kills osteosarcoma cells: normal osteoblasts and fibroblasts remained viable when exposed to SCH for up to 72 hours at quiescence (which approximates their *in vivo* condition). Second, SCH kills osteosarcoma cells at low concentrations (10 to 40 nmol/L). As shown here and by others (36, 38), several CDK inhibitors (Rosc, Flav, BMI-1026, and AZ703) induce osteosarcoma apoptosis; however, concentrations of 100 nmol/L or greater were required. Third, SCH kills osteosarcoma cells in a p53-independent manner. We show that p53-null cells and p53-depleted cells respond to as SCH as efficiently as do cells expressing wild-type p53. The need for p53 function for CDK inhibitor–mediated apoptosis has been documented but is not universal and may be cell type-specific. Our previous study of Rosc, for example, showed preferential apoptosis of prostate cancer and melanoma cells expressing wild-type p53 (9, 10). Others reported p53-independent apoptosis of leukemia cells and lymphocytes by Rosc and Flav (42, 43). CDK-independent effects of SCH are also possible.

Similar to other CDK inhibitors (9, 44, 45), SCH activated the mitochondrial pathway of apoptosis in osteosarcoma cells: we show cleavage of caspase-9 but not caspase-8 and accumulation of cytosolic Cyt c in SCH-treated U2OS and SaOs-2 cells. Bax accumulated in mitochondria of SCH-treated cells and most likely contributes to Cyt c release: cytosolic Bax is monomeric and inactive, whereas mitochondrial Bax is usually oligomerized and active (i.e., capable of inserting itself into mitochondrial membranes; ref. 46). There was more Bim and less Mcl-1 and Bcl-x_L in mitochondria of treated than of untreated cells. Whether changes in the abundance of these proteins contribute to the apoptosis of SCH-treated cells is currently being investigated. Previous reports document Mcl-1 protein and mRNA downregulation in tumor cells treated with CDK inhibitors and suggest a link between $p18^{INK4C}$ expression, Mcl-1 expression, and resistance to Rosc-induced apoptosis (38, 47–51).

SCH attenuated the phosphorylation of CDK2-specific and CDK9-specific substrates in osteosarcoma cells. Whether it directly inhibits the activity of these CDKs *in vivo* as it does *in vitro* cannot be ascertained from our experiments but is likely. Inactivation of CDK1 by SCH *in vivo*, as *in vitro*, is also likely but was not formally established. U2OS cells apoptosed when codepleted of CDK1 and CDK2 but not when depleted of either alone. Knockdown of CDK7 and CDK9 did not affect osteosarcoma survival. Previous studies also show apoptosis of U2OS cells codepleted of CDK1 and CDK2 (52). We note that SCH also inhibits the activity of CDK5, which was not considered in our study. CDK5 is an atypical CDK: it does not require a cyclin partner for activity and is multifunctional (53).

We suggest that SCH initiates the apoptosis of osteosarcoma by inactivating CDK1 and CDK2. Apoptosis induced by codepletion of CDK1 and CDK2 was less than that induced by SCH. This may reflect inactivation of additional targets by SCH or the presence of residual amounts of CDK1 and CDK2 in depleted cells. CDK1 and CDK2 can substitute for each other; thus, the need to suppress both to elicit apoptosis is not surprising. In cells deficient for CDK2, for example, CDK1 bound cyclin E, whereas in cells deficient for CDK1, CDK2 bound cyclin B (52). The lack of effect of CDK7/CDK9 knockdown on U2OS survival suggests that SCH-induced apoptosis does not require turnover of labile proteins such as Mcl-1 or codepletion of CDK1 and CDK2 inhibits RNAP II activity or reduces Mcl-1 expression by a CDK7/CDK9-independent mechanism.

In summary, we suggest that SCH may be useful for treatment of osteosarcoma. It kills osteosarcoma cells, both in *in vitro* and *ex vivo* and including those resistant to other drugs, while sparing normal osteoblasts and fibroblasts. It does not require p53 for osteosarcoma apoptosis; as noted before, many osteosarcomas exhibit p53 abnormalities. It is effective at low concentrations and thus may be less toxic than other CDK inhibitors. Rosc, for example, was toxic at higher doses in a phase I clinical trial of malignant tumors refractory to conventional regimens (54). We also note that its effects are not limited to osteosarcoma: fibrosarcoma, rhabdosarcoma, Ewing's sarcoma, and liposarcoma cells also apoptosed in response to SCH.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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