Autophagy: A Novel Mechanism of Synergistic Cytotoxicity between Doxorubicin and Roscovitine in a Sarcoma Model

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Abstract

Doxorubicin is a genotoxic chemotherapy agent used in treatment of a wide variety of cancers. Significant clinical side effects, including cardiac toxicity and myelosuppression, severely limit the therapeutic index of this commonly used agent and methods which improve doxorubicin efficacy could benefit many patients. Because doxorubicin cytotoxicity is cell cycle specific, the cell cycle is a rational target to enhance its efficacy. We examined the direct, cyclin-dependent kinase inhibitor roscovitine as a means of enhancing doxorubicin cytotoxicity. This study showed synergistic cytotoxicity between doxorubicin and roscovitine in three sarcoma cell lines: SW-982 (synovial sarcoma), U2OS-LC3-GFP (osteosarcoma), and SK-LMS-1 (uterine leiomyosarcoma), but not the fibroblast cell line WI38. The combined treatment of doxorubicin and roscovitine was associated with a prolonged G_2-M cell cycle arrest in the three sarcoma cell lines. Using three different methods for detecting apoptosis, our results revealed that apoptotic cell death did not account for the synergistic cytotoxicity between doxorubicin and roscovitine. However, morphologic changes observed by light microscopy and increased cytoplasmic LC3-GFP puncta in U20S-LC3-GFP cells after the combined treatment suggested the induction of autophagy. Induction of autophagy was also shown in SW-982 and SK-LMS-1 cells treated with both doxorubicin and roscovitine by acridine orange staining. These results suggest a novel role of autophagy in the enhanced cytotoxicity by cell cycle inhibition after genotoxic injury in tumor cells. Further investigation of this enhanced cytotoxicity as a treatment strategy for sarcomas is warranted. [Cancer Res 2008; 68(19):7966–74]

Introduction

Sarcomas are a broad group of mesenchymal tumors that are notoriously chemoresistant. More than 50 histological types of sarcoma are described (1) with an overall 5-year survival for all stages of 50% to 60% (2–4). Only 20% of sarcomas respond to doxorubicin, which is the current standard of systemic therapy care for these tumors (5). Unfortunately, the clinical utility of doxorubicin, which is also used to treat a wide range of solid and nonsolid tumors, is limited by significant side effects, particularly irreversible cardiac toxicity (6). For these reasons, efforts to improve the efficacy of doxorubicin are essential and could benefit many patients suffering from a variety of cancers.

The mechanism of doxorubicin cytotoxicity involves "poisoning" the topoisomerase II enzyme, thereby interfering with the separation of daughter DNA strands and chromatin remodeling. In addition, doxorubicin intercalates into double-stranded DNA producing structural changes that interfere with DNA and RNA synthesis (6). Because it primarily damages double-stranded DNA, cells in the S phase of the cell cycle are more susceptible. Because of this cell cycle specificity, the cell cycle is a rational target for enhancing doxorubicin efficacy.

Cyclin-dependent kinases (Cdk) are essential cell cycle regulatory proteins, which ensure accurate and appropriate transition between cell cycle phases and ultimately cell division (7). In addition, Cdk function is an important determinant of the cellular response to DNA damage, including that caused by genotoxic chemotherapies, such as doxorubicin (8). To date, 13 different Cdks have been identified (Cdk1–13; ref. 9). The activity of individual Cdk types is restricted to specific phases of the cell cycle and dependent on binding with activating cyclin proteins (10). Because Cdks are involved in regulating many important aspects of the cell cycle, they provide an appealing target for cell cycle-directed anticancer therapy.

Currently, >100 direct and indirect Cdk inhibitors are available. One of the most studied, and apparently selective, direct Cdk inhibitors is the purine analogue, roscovitine (11). Roscovitine directly inhibits Cdks by occupying the ATP binding site of the catalytic subunit of the kinase protein and is relatively more specific for Cdk1 and Cdk2 than other Cdk inhibitors (12). It is a well-tolerated, oral agent, which has shown ability to arrest tumor growth as a single agent in phase I clinical trials (13) and is currently being tested in phase II clinical trials against non–small cell lung cancer and nasopharyngeal cancer. Other preclinical studies have shown enhanced cytotoxicity with a variety of chemotherapeutic agents when combined with roscovitine (14, 15). Because roscovitine can inhibit both Cdk1 and Cdk2, it has the potential to enhance the cytotoxicity of genotoxic chemotherapeutic agents by the combined effect of delayed cell cycle progression as well as inhibition of the DNA damage response pathway.

In the present study, we investigated the effect of the combination of doxorubicin with roscovitine in three different sarcoma cell lines and one immortalized fibroblast cell line. Our results show a nonapoptotic synergistic cytotoxicity in all three sarcoma cell lines, but not the fibroblasts, treated with both doxorubicin and roscovitine. This synergistic cell death was accompanied by both a prolonged G_2-M arrest and significant induction of autophagy.

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Materials and Methods

Cell culture. Fetal bovine serum (FBS) and cell culture medium were purchased from Hyclone Laboratories. All other chemicals were reagent grade. SW-982, SK-LMS-1, and WI38 (immortalized fibroblasts) were purchased from American Type Culture Collection and cultured in DMEM (SW-982, SK-LMS-1) or α-MEM (WI38) with 10% FBS. U20S cells stably transfected with LC3-GFP protein (U2OS-LC3-GFP) were kindly donated by Dr. Gordon B. Mills (M. D. Anderson Cancer Center, Houston, TX) and maintained in DMEM with 10% FBS. All cells were cultured and treated at 37°C in a humidified incubator containing 6.5% CO2. A 5-mmol/L stock solution of doxorubicin (Sigma) was made in sterile water and maintained at −20°C. A 10-mmol/L stock solution of roscovitine (synthesized and kindly provided by Drs. Herve Galons, Université René Descartes, Paris, France and Laurent Meijer, Protein Phosphorylation and Disease, Centre National de la Recherche Scientifique, Station Biologique, Roscoff, France) was made in DMSO and maintained at −20°C. Fresh drug was prepared for each experiment.

Cell cycle analysis. For DNA content analysis, harvested cells were centrifuged at 1,000 *g* for 5 min, washed with PBS, and fixed in 70% ethanol. Cells were then treated with RNase (10 μg/mL) for 30 min at 37°C, washed with PBS, resuspended, and stained in 1 mL of 69 μmol/L propidium iodide (PI) in 38 mmol/L sodium citrate for 30 min. The cell cycle phase distribution was determined by analytic DNA flow cytometry as described by Keyomarsi and colleagues (16). The percentage of cells in each phase of the cell cycle was analyzed using *Modfit* software (Verity Software House).

High-throughput clonogenic assay and combination index. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were modified for use as high-throughput clonogenic assays (HTCA) to determine cell viability. Cells were plated in 96-well plates at a density of 1.5 to 3 × 10^3 cells per well. After 24 h, cells were treated with drug-free medium, 0.001 to 0.02 μmol/L doxorubicin for 24 h, 5 to 25 μmol/L roscovitine for 48 h, or doxorubicin for 24 h then roscovitine for 48 h. Drug-free medium was replaced every 48 h after treatment. Six replicates were plated for each treatment group. Ten days after plating, 50 μL of 2.5 mg/mL MTT solution was added to each well and incubated for 4 h. The resultant blue formazan crystals were solubilized in 100 μL of buffer (0.04 N HCl and 1% SDS in isopropyl alcohol) for 1 h. Absorbance was read at 590 nm using a Wallac-1420 plate reader. Drug interactions were assessed using *CalcuSyn* software version 2.1 (Biosoft, Inc.) to determine the combination index of the combined treatment of doxorubicin and roscovitine.

Clonogenic assays. Cells (1 × 10^3) were plated onto 100-mm^2 dishes. After 24 h, cells were treated with fresh drug-free medium, 0.01 μmol/L doxorubicin (SK-LMS-1), 0.005 μmol/L doxorubicin (U2OS-LC3-GFP and SW-982) for 24 h, 20 μmol/L (SK-LMS-1) or 10 μmol/L (U2OS-LC3-GFP and SW-982) roscovitine for 48 h, or doxorubicin for 24 h then roscovitine for 48 h. Drug-free medium was applied at the end of each treatment. After 14 d, the cells were fixed and stained with crystal violet in 100% ethanol suspension. Plates were scored for the number of visible colonies of ≥2 mm.

Apoptosis assays. Both APC-conjugated Annexin V and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were used to determine the presence of apoptosis. Cells (2 × 10^5) were plated on 100-mm^2 plates. After 24 h, cells were treated with fresh drug-free medium, doxorubicin for 24 h, roscovitine for 48 h, or doxorubicin for 24 h then roscovitine for 48 h as described above. Cells were harvested and stained with Annexin V-APC and PI (Trevigen, Inc.) or by TUNEL using the APO-DIRECT kit (BD Biosciences PharMingen) according to the manufacturer's instructions (Trevigen, Inc.). Apoptosis was detected by flow cytometry.

Western blot analysis for apoptosis. For Western blot analysis, cells were homogenized by sonication and high-speed centrifugation. Cell lysate supernatant was assayed for total protein content and subjected to Western blot analysis as described by Rao and colleagues (17). Briefly, 25 μg of protein from each condition was electropheresed on a 10% SDS-polyacrylamide gel and transferred to Immunobilon P for 2 h. Membranes were washed at 4°C in Blotto [5% nonfat dry milk in 20 mmol/L Tris, 137 mmol/L NaCl, 0.25% Tween (pH 7.6)] overnight. After 6 washes in TBST [20 mmol/L Tris, 137 mmol/L NaCl, and 0.05% Tween (pH 7.6)], membranes were incubated in primary antibody (Parp; Cell Signaling) for 2 h (1 μg/mL in Blotto). Membranes were washed and incubated with goat anti-mouse horseradish peroxidase conjugate at a dilution of 1:5,000 for 1 h, washed, and developed with *Renaissance* chemiluminescence system as directed by the manufacturers (NEN Life Sciences Products).

Autophagy assays. U2OS-LC3-GFP cells (1 × 10^4) were plated on coverslips in 6-well plates. After 24 h, cells were treated with fresh drug-free medium, doxorubicin for 24 h, roscovitine for 48 h, or doxorubicin for 24 h then roscovitine for 48 h. The cells were fixed with 4% paraformaldehyde, stained with 4',6-diamidino-2-phenylindole, dilactate (Invitrogen), and observed with fluorescence microscopy (Leica DM 4000 B).

To detect autophagy in SK-LMS-1 and SW-982 cells, 1 × 10^4 cells were plated on coverslips in 6-well plates. After 24 h, cells were treated with fresh drug-free medium, doxorubicin for 24 h, roscovitine for 48 h, or doxorubicin for 24 h then roscovitine for 48 h. The cells were fixed with 4% paraformaldehyde for 15 min at room temperature then stained with acridine orange (AO; Polysciences, Inc.), 1 μg/mL in PBS at 37°C, in the dark, and observed immediately with fluorescence microscopy. To quantify the number of cell with acidic vesicles, cells were seeded into 6-well plates at a density of 4 × 10^4 cells per well and cultured overnight. The cells were stained with 1 μg/mL AO in DMEM at 37°C for 15 min. After incubation, the cells were washed with PBS and removed with trypsin-EDTA, resuspended, and analyzed by flow cytometry.

Statistical analysis. Each experiment was repeated at least thrice. The isobologram analysis and graphs were carried out using *Calcusyn* software (version 2.1; Biosoft, Inc.), which performs multiple drug dose-effect calculations using the median effects method described by T-C Chou and P. Talalay (18). Combination index (CI) values of <0.9 indicates synergy, CI of >0.9 and <1.2 indicate additivity, and CI of >1.2 indicate antagonism. Comparisons among groups were analyzed by two-sided *t* test. A difference of *P* value of ≤0.05 was considered to be statistically significant. All analyses were done with *SPSS* software, Version 12.0. The data represent the means of three or more samples with SE.

Results

Synergistic cytotoxicity between doxorubicin and roscovitine. High-throughput clonogenic assay (HTCA) was used to compare the cytotoxic effects of doxorubicin alone, roscovitine alone, and doxorubicin followed by roscovitine in SW-982, U2OS-LC3-GFP, SK-LMS-1 sarcoma, and WI38-immortalized fibroblast cell lines. A sequential drug treatment strategy (see Materials and Methods) was chosen based on previous reports in the literature demonstrating sequence–specific synergistic effects with administration of combination chemotherapy, and Cdk inhibitors (19, 20). As shown in a three-dimensional graph in Fig. 1*A*, dose-dependent increases in cell death were seen in all four cell lines when treated with doxorubicin alone (*X* axis). For SW-982, the percentage of cell death increased from 5% to 60% as cells were treated with increasing doxorubicin doses from 0.001 to 0.015 μmol/L (IC_{50}, ~0.0125 μmol/L). For U2OS-LC3-GFP, the percentage of cell death increased from 5% to 85% as cell were treated with increasing doxorubicin doses from 0.001 to 0.01 μmol/L (IC_{50}, between 0.005 and 0.0075 μmol/L). For SK-LMS-1, the percentage of cell death increased from 7% to 25% as cell were treated with increasing doxorubicin doses from 0.001 to 0.02 μmol/L (IC_{50}, not reached). For WI38, the percentage of cell death increased from 13% to 50% as cell were treated with increasing doxorubicin doses from 0.001 to 0.01 μmol/L (IC_{50}, 0.01 μmol/L). The percentage of cell death for all three sarcoma cell lines treated with roscovitine alone ranged between 5% and 30% as cells were treated with increasing roscovitine doses from 5 to 20 μmol/L (*Y* axis). For WI38, the percentage of cell death increased from 25% to 95% as cell were treated with increasing roscovitine doses from 5 to 25 μmol/L (IC_{50}, 10 μmol/L).

When the two drugs were combined, there was a significant increase in cell death (*Z* axis) in all three sarcoma cell lines compared with either doxorubicin or roscovitine alone in isobologram determinations. However, in the WI38 cells, the combination of doxorubicin and roscovitine did not result in such a synergistic cell death. CIs, as determined using the *CalcuSyn* software, showed synergistic cytotoxicity between doxorubicin and roscovitine in the three sarcoma cell lines but not the WI38 cells (Fig. 1*B*). For

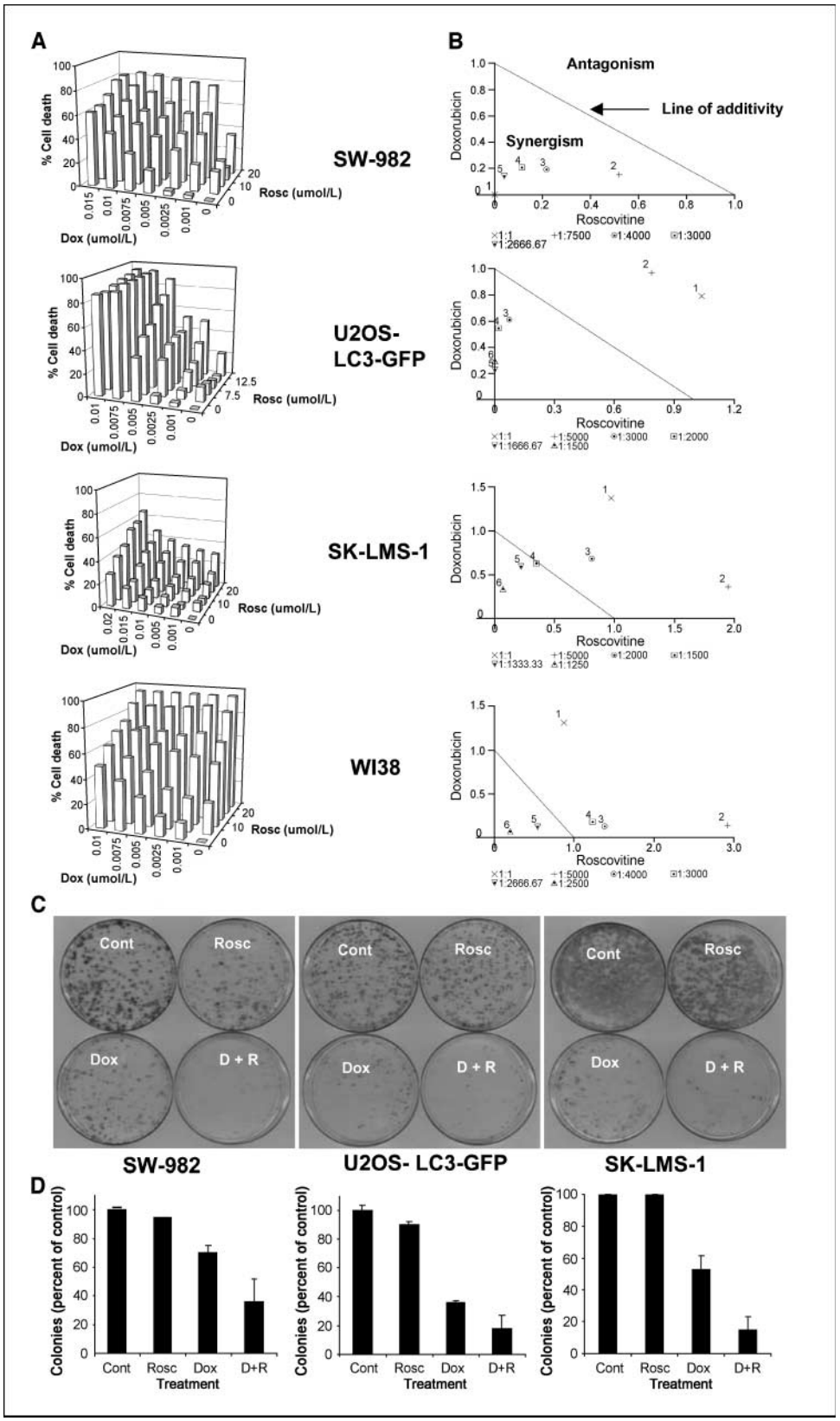

Figure 1. Synergistic effect of doxorubicin and roscovitine in sarcoma cells. *A,* HTCA was used to compare the cytotoxic effects of doxorubicin alone, roscovitine alone, and doxorubicin followed by roscovitine in SW-982, U2OS-LC3-GFP, SK-LMS-1, and WI38 cell lines [*X*-axis: doxorubicin (*Dox*) μmol/L; *Y*-axis: roscovitine (*Rosc*) μmol/L; *Z*-axis: percentage cell death]. *B,* isobologram analysis showed synergistic interactions in all three sarcoma cell lines. Isobologram analysis and graphs were obtained using *CalcuSyn* software, which performs drug dose-effect calculation using the median effect method described by Chou and Talalay (18). *C* and *D,* clonogenic assays also showed a synergistic effect between doxorubicin and roscovitine in the three cell lines (*Cont,* untreated; *D,* doxorubicin; *R,* roscovitine; *D + R,* doxorubicin plus roscovitine). Representative of three experiments.

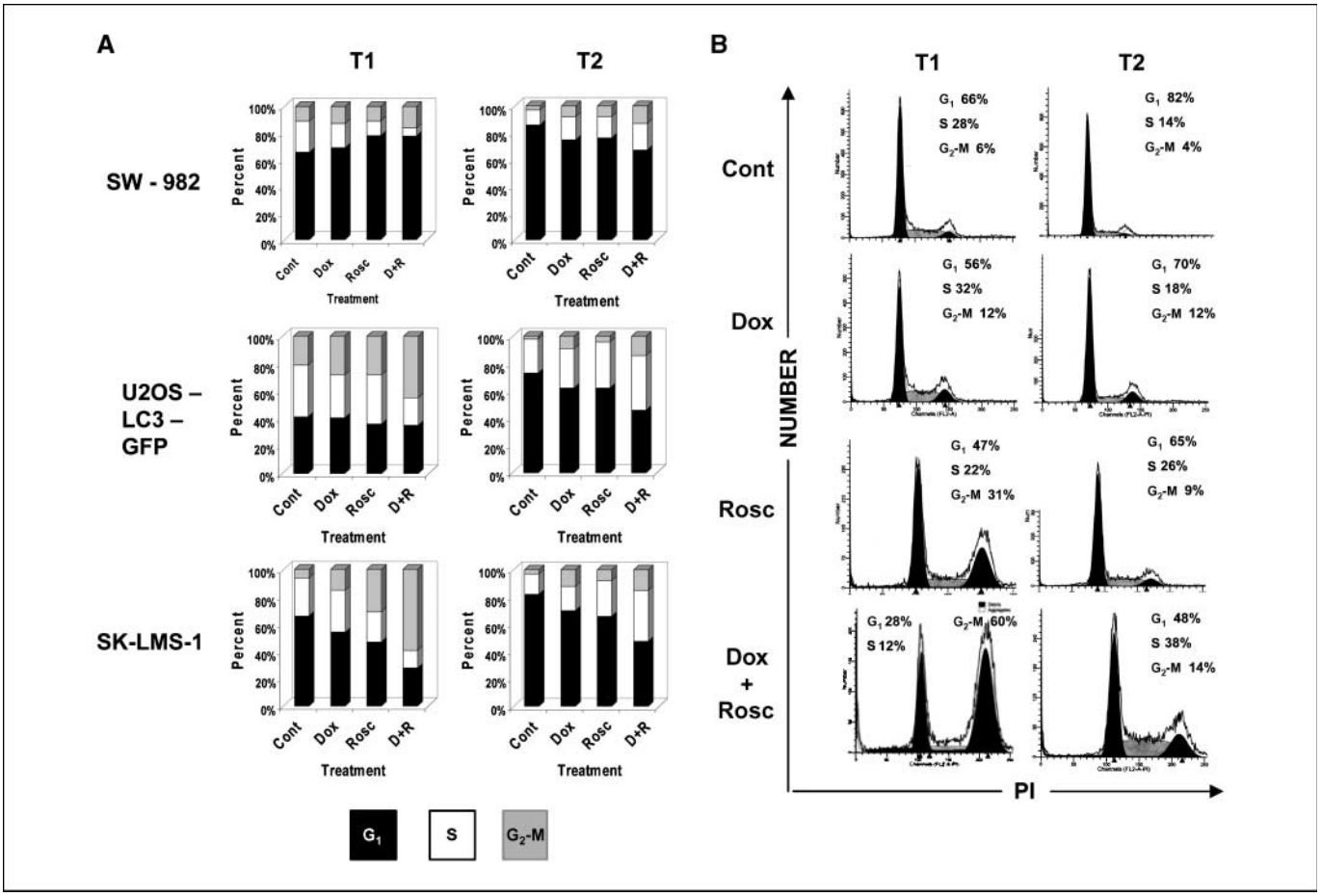

Figure 2. Effect of doxorubicin and roscovitine on cell cycle. *A,* SW-982 cells, U2OS-LC3-GFP cells, and SK-LMS-1 cells were treated with either 0.005 μmol/L (SW-982 and U20S-LC3-GFP) or 0.01 μmol/L (SK-LMS-1) doxorubicin for 24 h, 10 μmol/L (SW-982 and U20S-LC3-GFP) or 20 μmol/L (SK-LMS-1) roscovitine for 48 h, the combination of doxorubicin and roscovitine, or were untreated. At the completion of drug treatment (T1) and 72 to 96 h after the completion of drug treatment (T2), cells were harvested and the cell cycle distribution was determined using fluorescence-activated cell sorting (*B*). Cell cycle distribution histograms of SK-LMS-1. Representative of three experiments.

example, in SW-982 cells, treatment with either 0.005 μmol/L doxorubicin alone or 10 μmol/L roscovitine alone resulted in a cell death rate of only 15%. However, when treated first with 0.005 μmol/L doxorubicin then 10 μmol/L roscovitine, the percentage of cell death increased to 55% (CI, <0.9), suggesting synergism. Similarly, in U2OS-LC3-GFP, treatment with either 0.005 μmol/L doxorubicin alone or 10 μmol/L roscovitine alone resulted in a cell death of 30% and 7%, respectively. However, when treated first with 0.005 μmol/L doxorubicin then 10 μmol/L roscovitine, the percentage of cell death increased to 75% (CI<0.9). For SK-LMS-1, treatment with either 0.02 μmol/L doxorubicin alone or 20 μmol/L roscovitine alone resulted in a cell death of 25%. However, when treated first with 0.02 μmol/L doxorubicin then 20 μmol/L roscovitine, the percentage of cell death increased to 64% (CI, <0.9). Hence, in all three sarcoma cell lines, the combination of doxorubicin and roscovitine resulted in synergistic cell killings. On the other hand, for the WI38-immortalized fibroblast cells, the CIs were predominantly antagonistic (CI, >1.2) and not synergistic. This finding suggests that the increased cytotoxicity of the combined treatment is tumor cell specific.

The HTCA was complemented with conventional clonogenic assays in the sarcoma cell lines, and also showed a synergistic effect between doxorubicin and roscovitine in all three sarcoma cell lines (Fig. 1*C* and *D*). For SW-982 cells, there was a 10% decrease in the number of colonies formed after treatment with 10 μmol/L roscovitine alone, a 30% decrease after treatment with 0.005 μmol/L doxorubicin alone, and a 64% decrease in the number of colonies formed after treatment with doxorubicin (0.005 μmol/L) then roscovitine (10 μmol/L; $P \leq 0.006$). For U2OS-LC3-GFP cells, there was a 6% decrease in the number of colonies formed after treatment with 10 μmol/L roscovitine alone, a 64% decrease after treatment with 0.005 μmol/L doxorubicin alone, and a 82% decrease in the number of colonies formed after treatment with doxorubicin (0.005 μmol/L) then roscovitine (10 μmol/L; $P \leq 0.001$). For SK-LMS-1 cells, there was no significant change in the number of colonies formed after treatment with 20 μmol/L roscovitine alone. There was a 47% decrease after treatment with 0.01 μmol/L doxorubicin alone and a 85% decrease in the number of colonies formed after treatment with doxorubicin (0.01 μmol/L) then roscovitine (20 μmol/L; $P \leq 0.008$). Fig. 1*C* shows representative clonogenic plates from each experiment, and Fig. 1*D* shows quantization of the clonogenic assays under each condition.

Treatment with doxorubicin followed by roscovitine induces prolonged G_2 arrest. To determine the cell cycle effects of doxorubicin and roscovitine, both alone and in combination, DNA content was evaluated using PI staining followed by flow cytometry. Cell cycle distributions for the three sarcoma cell lines, both untreated and treated, are shown in Fig. 2*A*. After 24 hours of

treatment with doxorubicin alone, all 3 sarcoma cell lines showed an increase in the percentage of cells in G_2-M phase: 5% in SW-982, 6% in U20S-LC3-GFP, and 9% in SK-LMS-1 (Fig. 2*B*) compared with untreated cells. After 48 hours of treatment with roscovitine alone, there was an increase in the percentage of cells in G_1 by 13% for SW-982. U2OS-LC3-GFP and SK-LMS-1 showed a 7% and 25% increase in the percentage of cells in G_2-M phase compared with untreated cells, respectively. Combined treatment with doxorubicin for 24 hours followed by roscovitine for 48 hours resulted in an increase in the percentage of cells in G_2-M phase compared with untreated cells by 5% for SW-982, 24% for U2OS-LC3-GFP, and 45% for SK-LMS-1. Collectively, comparison of the changes in cell cycle distribution in response to each drug alone versus in combination suggests significant modulation of the cell cycle pathway by the combination of the two drugs. To assess the involvement of the cell cycle as the modulator of the observed synergistic response, we next performed cell cycle analysis at various time points: at the completion of drug treatment (T1) and 72 to 96 hours after the completion of drug treatment (T2).

As shown in Fig. 2*A*, untreated cells in all three sarcoma cell lines showed persistent cell division over the time course with increased percentage of cells residing in G_1 phase at the time of final analysis (T2), consistent with a contact-induced G_1 cell cycle arrest (compare control cells in T1 with T2). In addition, the percentage of cells in G_1 phase also increased in all three sarcoma cell lines treated with either doxorubicin or roscovitine alone, suggesting the persistence of cell division after drug treatment (compare doxorubicin alone cells in T1 with T2). In comparison, after combined treatment with doxorubicin and roscovitine, SW982 cells showed a smaller percentage of cells in G_1 compared with the initial time point (−19%) and a persistence of cells in G_2-M phase compared with untreated cells at the final time point (T2, 13% versus 3% of untreated cells). For U2OS-LC3-GFP cells, there was no significant change in the percentage of cells in G_1 compared with the initial time point (41% versus 46%, respectively) but a persistence of cells in G_2-M phase compared with untreated cells at the final time point (14% versus 2%). SK-LMS-1 cells showed an increase in the percentage of cells in G_1 compared with the combined treatment at the initial time point (19%) as well as a persistence of cells in the G_2-M phase (15% versus 3%) compared with untreated cells at the final time point (Fig. 2*B*). These findings translated into a 4-, 7-, and 5-fold increase in the percentage of SW982, U2OS-LC3-GFP, and SK-LMS-1 cells remaining in G_2-M phase, respectively, compared with untreated cells at the final time point with the combination treatment. These results suggest that treatment by roscovitine after doxorubicin not only inhibits the cell cycle but also the ability of the cell to recover from cytotoxic injury. All of these experiments were performed at least thrice in triplicate with similar results. Figure 2*B* is representative of a single experiment in the SK-LMS-1 cell line.

Synergistic cytotoxicity by doxorubicin and roscovitine is not due to increased apoptosis. We detected unique morphological changes by light microscopy of untreated and treated cells particularly in U2OS-LC3-GFP and SK-LMS-1 cells (Fig. 3*A*–*B*). As compared with untreated cells, cells treated with both doxorubicin and roscovitine became larger, displayed prominent fibrils, and were often multinucleated (see *arrows*). Based on the synergistic cytotoxicity observed with the combined drug treatment, it was clear that cell death was present but not through apoptosis. Three different methods were used to determine the degree of cell death due to apoptosis after treatment with either the single agents or the combination: Annexin V-APC staining, TUNEL staining, and expression of Parp (Fig. 4).

As shown in Fig. 4*A*, there was a significant increase in Annexin V-APC–positive SW-982 cells after treatment with either doxorubicin or roscovitine alone or in combination compared with untreated cells ($P \leq 0.004$). Roscovitine treatment alone lead to the greatest fold increase in Annexin V-APC–positive SW-982 cells (4-fold), and this was significant when compared with either doxorubicin treatment alone or in combination with roscovitine

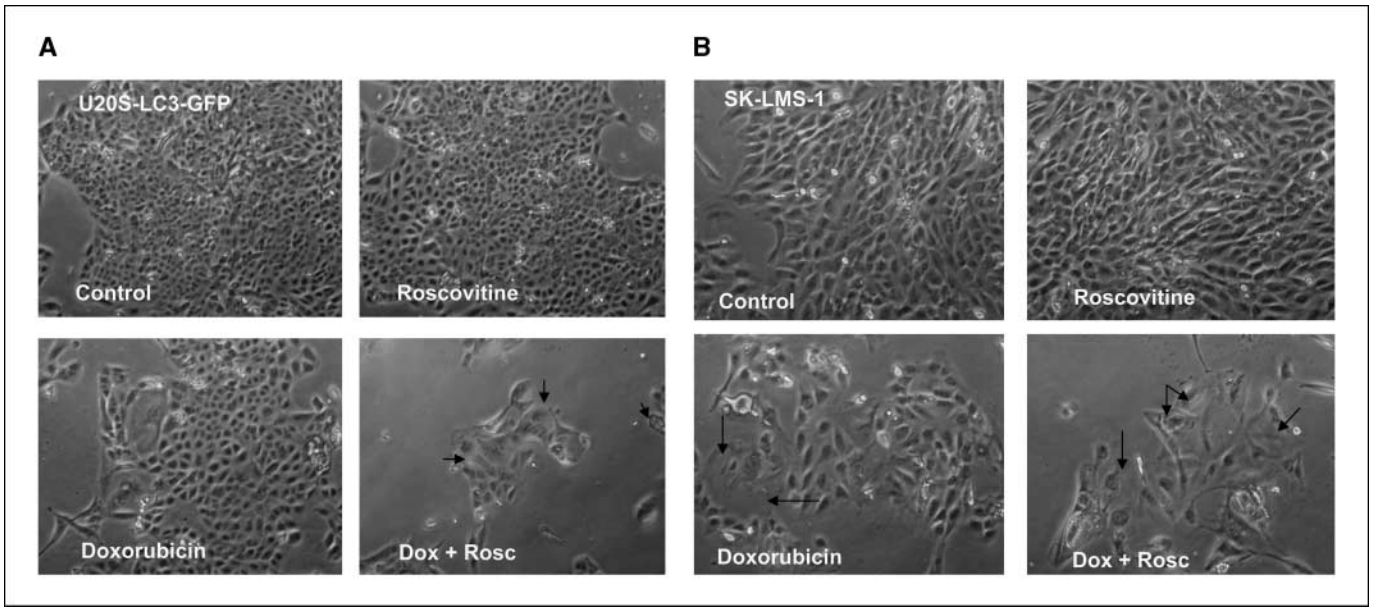

Figure 3. Morphologic changes observed by light microscopy. *A,* U2OS-LC3-GFP cells and (*B*) SK-LMS-1 cells were treated with either 0.005 μmol/L (U20S-LC3-GFP) or 0.01 μmol/L (SK-LMS-1) doxorubicin for 24 h, 10 μmol/L (U20S-LC3-GFP) or 20 μmol/L (SK-LMS-1) roscovitine for 48 h, the combination of doxorubicin and roscovitine, or left untreated. At 6 d, the cells were observed by light microscopy. *Arrows,* enlarged cells with prominent micofibrils and multinucleated cells seen in combination treatment.

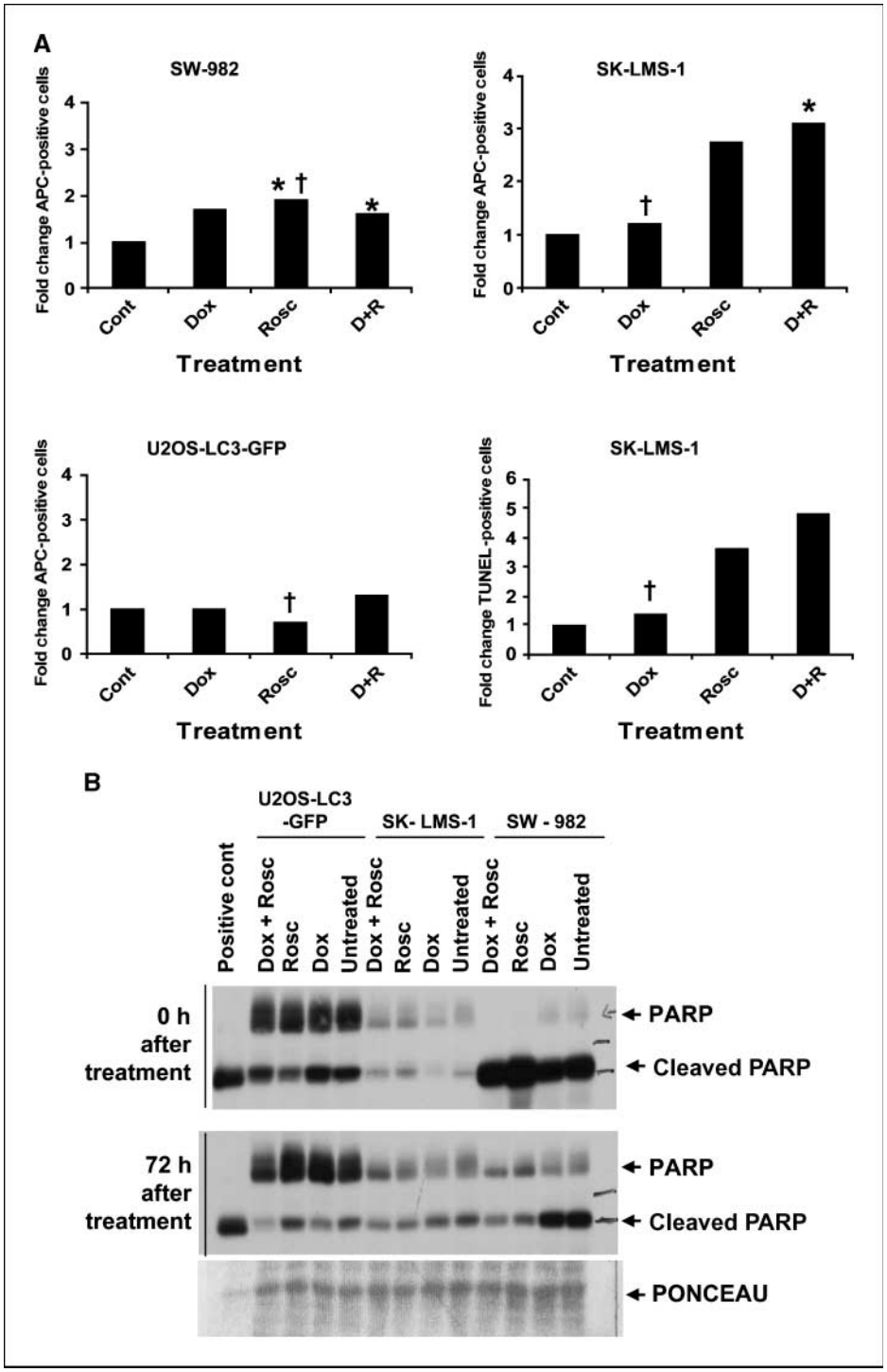

Figure 4. *A,* fluorescence-activated cell sorting–based apoptosis analyses. SW-982 (*top left*), U20S-LC3-GFP (*bottom left*), and SK-LMS-1 cells (*right*) were treated with either 0.005 μmol/L (SW-982 and U20S-LC3-GFP) or 0.01 μmol/L (SK-LMS-1) doxorubicin for 24 h, 10 μmol/L (SW-982 and U20S-LC3-GFP) or 20 μmol/L (SK-LMS-1) roscovitine for 48 h, the combination of doxorubicin and roscovitine, or were untreated. At 6 d, the cells were harvested and stained with either Annexin V-APC or TUNEL and subjected to fluorescence-activated cell sorting. *, significant difference compared with untreated cells ($P < 0.05$); †, significant difference compared with D + R ($P < 0.05$). Representative of three individual experiments. *B*, effect of combined treatment with doxorubicin and roscovitine on apoptosis. SW-982, U20S-LC3-GFP, and SK-LMS-1 cells were treated with either 0.005 μmol/L (SW-982 and U20S-LC3-GFP) or 0.01 μmol/L (SK-LMS-1) doxorubicin for 24 h, 10 μmol/L (SW-982 and U20S-LC3-GFP) or 20 μmol/L (SK-LMS-1) roscovitine for 48 h, the combination of doxorubicin and roscovitine, or left untreated. Western blot analysis using antibody specific for Parp (full-length and cleaved) was performed at 0 and 72 h after treatment. Positive cont, SW-982 cells treated with 4 μmol/L camptothecin for 12 h. Representative of three individual experiments.

($P \leq 0.021$). However, as shown previously, the combined treatment resulted in greater cell death than either doxorubicin or roscovitine alone, and because apoptosis was not significantly increased after the combination treatment, this finding supports the hypothesis of a nonapoptotic mechanism of synergistic cell death.

In comparison, there was no significant difference in Annexin V-APC–positive U2OS-LC3-GFP cells after treatment with either doxorubicin or roscovitine alone or in combination compared with untreated cells. For SK-LMS-1 cells, there was a significant increase in the Annexin V-APC–positive cells treated with the combination of doxorubicin and roscovitine compared with untreated cells and cells treated with doxorubicin alone ($P \leq 0.05$). This increase was not significantly different when compared with roscovitine alone. However, because the percentage of Annexin V-APC–positive cells was only 12% in cells treated with the combination of doxorubicin and roscovitine, these findings also support an alternative mechanism of synergistic cell death due to the combination treatment. Similar results were found using the TUNEL assay. SK-LMS-1 cells showed a slight increase in the TUNEL-positive cells treated with the combination of doxorubicin and roscovitine. This

difference was statistically significant when compared with doxorubicin alone (P = 0.05) but not roscovitine alone. However, similar to the Annexin-APC analysis, the percentage of TUNEL-positive cells was low (4%) in cells treated with the combination of doxorubicin and roscovitine again supporting an alternative mechanism of synergistic cell death due to the combination treatment.

Western blot analysis was also performed to determine whether the enhanced cell death was occurring through apoptosis. As shown in Fig. 4*B*, in all three sarcoma cell lines, the expression of the cleaved form of the apoptosis marker, Parp, decreased after treatment with the combination of doxorubicin and roscovitine compared with either doxorubicin or roscovitine alone. This finding further suggests that apoptosis is not the only mechanism of cell death due to the combined treatment of doxorubicin and roscovitine. Because the morphologic changes we observed with the drug combination were reminiscent of the homeostatic condition of autophagy, we explored whether an autophagy-mediated mechanism of cell death was associated with the synergistic cytotoxicity.

Synergistic cytotoxicity by doxorubicin and roscovitine is associated with autophagy. Microtubule-associated protein-1 light chain-3 (LC3), the homologue of the yeast *Apg8/Aut7p* gene, localizes on the autophagosomal membrane during autophagy (21). The LC3-GFP fused protein is used frequently to detect autophagy through the increased presence of GFP puncta within the cytoplasm (21). Figure 5*A* shows the difference in the presence of LC3-GFP punctuate structures in U2OS-LC3-GFP cells treated with doxorubicin alone, roscovitine alone, or with doxorubicin followed by roscovitine compared with untreated cells. The pictures clearly

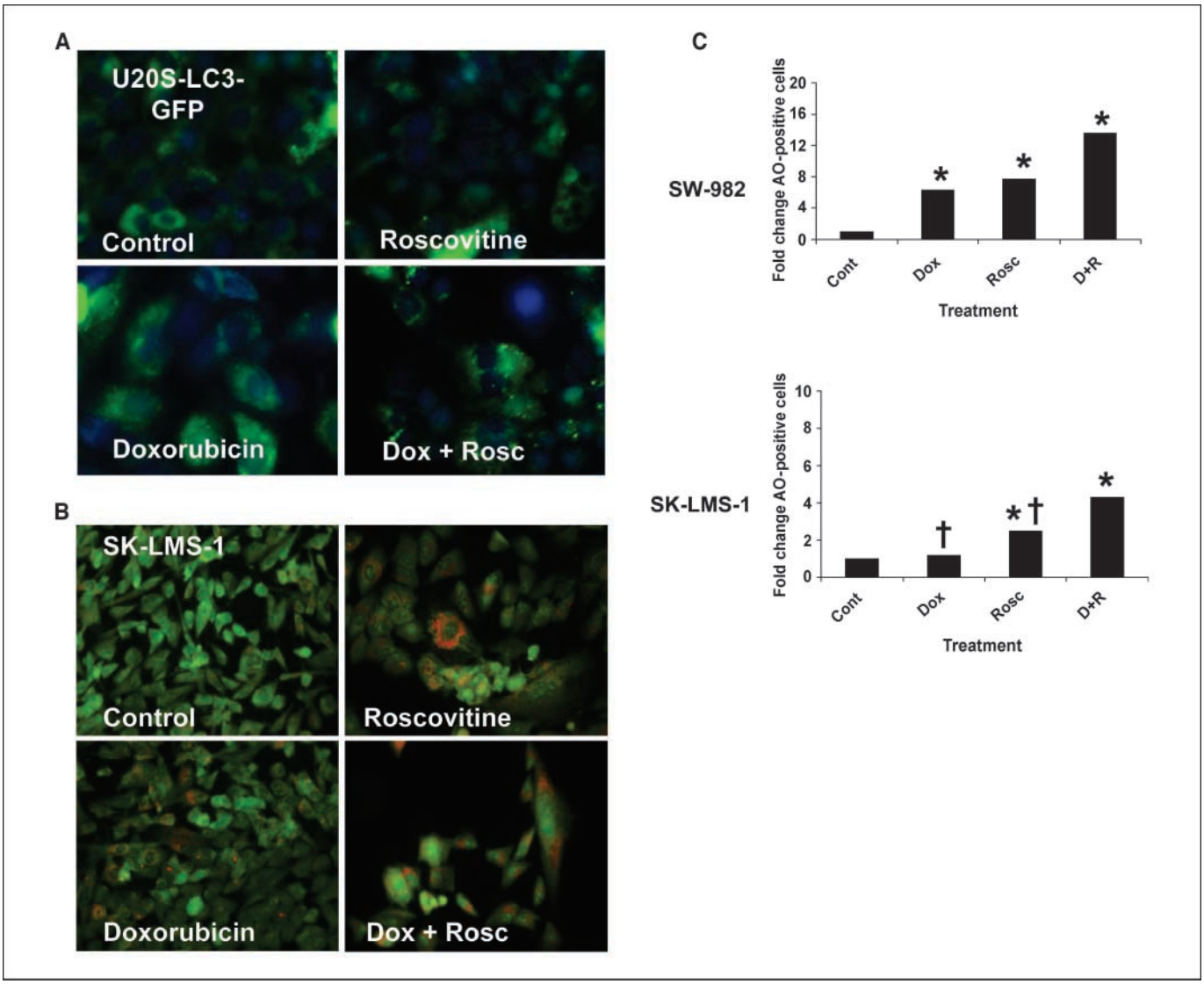

Figure 5. Induction of autophagy by combined treatment with doxorubicin and roscovitine. *A,* U20S-LC3-GFP cells were treated with either 0.005 μmol/L doxorubicin for 24 h, roscovitine for 48 h, the combination of doxorubicin and roscovitine, or left untreated. Cells were observed under fluorescence microscopy to detect the presence of LC3-GFP puncta, which are indicative of autophagy. Representative of three separate experiments. *B,* SK-LMS-1 cells were treated with either 0.01 μmol/L doxorubicin for 24 h, 20 μmol/L roscovitine for 48 h, the combination of doxorubicin and roscovitine, or left untreated. Seventy-two hours after treatment, cells were stained with AO and observed with fluorescence microscopy to detect the presence of AO puncta. *C,* SW-982 and SK-LMS-1 cells were treated with either 0.005 μmol/L (SW-982) or 0.01 μmol/L (SK-LMS-1) doxorubicin for 24 h, 10 μmol/L (SW-982) or 20 μmol/L (SK-LMS-1) for roscovitine 48 h, the combination of doxorubicin and roscovitine, or left untreated. Seventy-two hours after treatment, cells were stained with AO and quantified by fluorescence-activated cell sorting. *, significant difference compared with untreated cells (P < 0.05); †, significant difference compared with D + R (P < 0.05). Representative of three separate experiments.

show that treatment with either drug alone leads to increased autophagocytic LC3-GFP puncta, compared with untreated U2OS-LC3-GFP cells. Furthermore, cells treated with doxorubicin alone had more LC3-GFP puncta than cells treated with roscovitine alone. However, U2OS-LC3-GFP cells treated with both doxorubicin and roscovitine showed the most LC3-GFP puncta compared with cells treated with either drug alone. These findings suggested that autophagy may be associated with the mechanism of synergistic cytotoxicity of doxorubicin and roscovitine.

To determine whether autophagy was also present in SW-982 and SK-LMS-1, the cells were stained with AO. AO is concentrated in acidic vesicles such as the autpohagolysosome and has been used as a measure of autophagy (22). SK-LMS-1 and SW-982 cells treated with either doxorubicin or roscovitine developed significantly increased cytoplasmic AO puncta compared with untreated cells (Fig. 5*B*). Furthermore, SK-LMS-1 and SW-982 cells treated with both doxorubicin and roscovitine showed more AO puncta compared with cells treated with either doxorubicin or roscovitine alone. Quantization of AO staining by flow cytometry showed a significant increase in both the SW-982 and SK-LMS-1 cells treated with both doxorubicin and roscovitine compared with untreated cells and cell treated with either doxorubicin or roscovitine alone ($P \leq 0.003$ for all conditions; Fig. 5*C*). Taken together, these results suggested an autophagic mechanism of synergistic cytotoxicity due to the combined treatment of doxorubicin and roscovitine.

Discussion

Roscovitine synergistically increases doxorubicin cytotoxicity in sarcoma cells but not fibroblasts. Doxorubicin is a commonly used cytotoxic chemotherapy agent with a significant and use-limiting side effect profile. The ability to increase doxorubicin efficacy would positively effect many patients suffering from a variety of cancers. In this report, we show synergistic cytotoxicity between the Cdk inhibitor roscovitine and doxorubicin in three different sarcoma cell lines. However, immortalized human fibroblast cells were not responsive to such synergistic action, suggesting that the synergism observed is tumor specific. Previous studies have shown that Cdk inhibition mediates tumor cell–specific cell cycle arrest and cell death (23, 24). These effects occur through Cdk inhibition in both the early and late phases of the cell cycle. For example, within the G_1 phase, Cdk-mediated phosphorylation of Rb leads to E2F-1 transcription factor activation and up-regulation of the genes required for the transition into S phase (25). Cdk inhibition during G_1 can lead to G_1 arrest and in S phase can lead to inappropriately persistent E2F-1, resulting in both S-phase delay and apoptosis (26).

The G_2-M transition is also susceptible to Cdk inhibition. Cdk1 activation is essential for progression from the G_2 to M cell cycle phases, the progression of mitosis through metaphase and cell survival during the mitotic checkpoint (27, 28). Conditional knockdown of Cdk1 has been shown to result in extensive DNA rereplication and apoptosis (29). Inhibition of Cdk1 has also been shown to increase apoptosis after genotoxic stress, whereas prolonged Cdk1 inhibition alone can cause significant tumor cell–specific apoptosis (23, 24). Furthermore, Cdk1 inhibition during the spindle assembly checkpoint has been shown to cause tumor cell–specific cell cycle progression without cell division resulting in mitotic catastrophe (30). Because most conventional chemotherapy agents cause DNA damage and activate cell cycle checkpoints, it is logical that disruption of these checkpoints through a Cdk inhibitor, such as roscovitine, would increase their cytotoxic effects.

Roscovitine-enhanced doxorubicin cell death is associated with autophagy. We show here that apoptosis is not the only mechanism of synergistic cell death caused by the combination of doxorubicin and roscovitine. Instead, autophagy is significantly increased in the cells treated with the combination of doxorubicin and roscovitine. Autophagy is a membrane-trafficking process which, under normal conditions, degrades cytosolic proteins and organelles through engulfment into double-membraned vesicles (autophagosomes). Autophagosomes fuse with lysosomes to form autolysosomes and the contents are degraded (31). Autophagy is induced above basal levels by a wide variety of stimuli including nutrient deprivation and genotoxic stress and has a direct effect on cell viability. In this study, we have shown that autophagy can also be induced by the direct Cdk inhibitor, roscovitine, in the setting of previous cytotoxic treatment.

Cell cycle and autophagy. One of the more intriguing findings of this study is the induction of autophagy by the combination of doxorubicin and roscovitine in all three sarcoma cell lines despite different initial cell cycle effects of the individual drug treatments. Interestingly, the combined treatment with doxorubicin and roscovitine lead to a prolonged G_2-M arrest in all three sarcoma cell lines. Therefore, we postulate that this prolonged arrest, caused by the combined activation of the DNA damage checkpoint by doxorubicin followed by the inhibition of the Cdk1-cyclinB complex by roscovitine, may be a trigger for the induction of autophagy and ultimately cell death (Fig. 6).

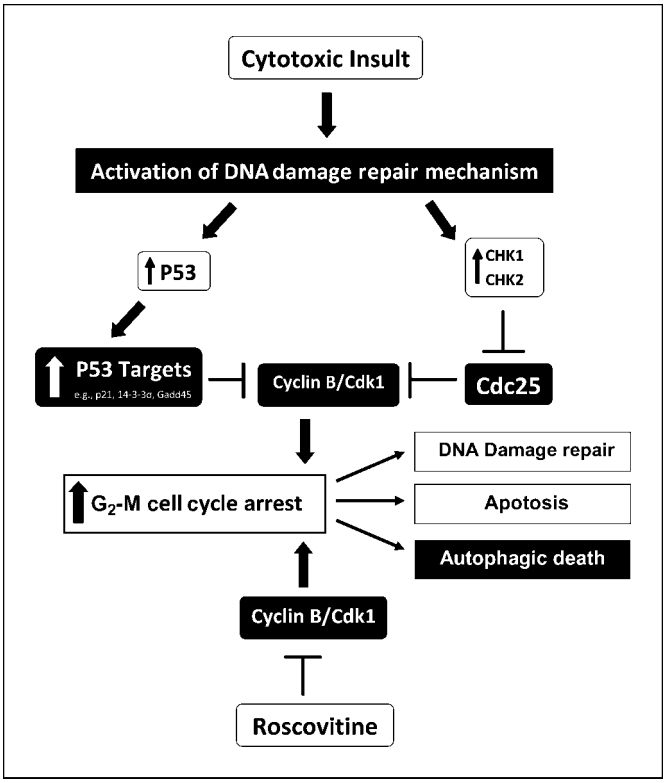

Figure 6. Proposed mechanism of autophagy-mediated synergistic cell death by genotoxic chemotherapy and cyclin-dependent kinase inhibition.

The relationship between cell cycle regulation and autophagy is not yet clearly defined. A variety of agents have shown induction of autophagy and autophagic cell death in association with a G_2 cell cycle arrest (32, 33). Based on our findings of the combination treatment of doxorubicin and roscovitine leading to synergistic cell death, induction of autophagy, and prolonged G_2 cell cycle arrest, we postulate the following model. DNA damage by doxorubicin (and potentially other genotoxic chemotherapy agents or treatments) activates the DNA-PK/ATM/ATR kinases, initiating two potential Cdk1-cyclin B inactivating cascades: (*a*) phosphorylation of Cdc25 by CHK1 and CHK2 and (*b*) (when present) activation of p53 and up-regulation of direct Cdk1-cyclin B inhibitors such as 14-3-3σ, GADD45, and p21. Injured cells undergo cell cycle arrest and autophagy to facilitate the repair of the damaged DNA. Once repaired, passage into mitosis occurs. However, pharmacological inhibition of the Cdk1-cyclin B complex by roscovitine also induces cell cycle arrest and autophagy, thereby preventing the cells from entering mitosis. Furthermore, due to the pivotal role of Cdk1-cyclin B in the G_2-M transition, the G_2-M DNA damage checkpoint may also be inhibited by roscovitine. In this manner, autophagy induced by a G_2 cell cycle arrest in the setting of genotoxic injury and Cdk inhibition, may be exploitable as a mechanism of cell death. Further investigation into the mechanism of synergy between cytotoxic agents and Cdk inhibition as well the role of the cell cycle in the initiation and outcome of autophagy (e.g., cell survival or cell death) will potentially provide new approaches to tumor-specific anticancer therapies.

Disclosure of Potential Conflicts of Interest

L. Meijer: coinventor on patent on roscovitine, licensed to Cyclacel. The other authors disclosed no potential conflicts of interest.

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