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Enhancement of Radiation Response by Roscovitine in Human Breast Carcinoma *in Vitro* and *in Vivo*

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ABSTRACT

Frequent deregulation of cyclin-dependent kinase (CDK) activation associated with loss of cell cycle control was found in most of human cancers. A recent development of a new class of antineoplasic agents targeting the cell cycle emerged as a small molecule CDK inhibitor, roscovitine, which presents potential antiproliferative and antitumoral effects in human tumors. Additional studies reported that roscovitine combined with cytotoxic agents can cooperate with DNA damage to activate p53 protein. However, little is known about the biological effect of roscovitine combined with ionizing radiation (IR) in human carcinoma, and no studies were reported thus far in p53 mutated carcinoma. In the breast cancer cell line MDA-MB 231, which lacks a functional p53 protein, we found a strong radiosensitization effect of roscovitine *in vitro* by clonogenic survival assay and *in vivo* in MDA-MB 231 xenograft model. Using Pulse Field Gel Electrophoresis, a strong impairment in DNA-double-strand break rejoining was observed after roscovitine and IR treatment as compared with IR alone. Cell cycle analysis showed a G_2 delay and no increase in radiation-induced apoptosis in the cells treated with IR or roscovitine and IR. On the other hand, we found a significant induction in micronuclei frequency after roscovitine and IR treatment as compared with IR alone. This effect was also observed in BALB murine cells in contrast to SCID murine cells, which are deficient in DNA-PKcs, suggesting a possible DNA-double-strand break repair defect in the nonhomologous end joining pathways. In MDA-MB 231 cells, the radiosensitization effect of roscovitine was associated with an inhibition of the DNA-dependent protein kinase activity caused by a marked decrease in Ku-DNA binding by using the electrophoretic mobility shift assay. In conclusion, we found a novel effect on DNA repair of the CDK inhibitor roscovitine, which acts as a radiosensitizer *in vitro* and *in vivo* in breast cancer cells lacking a functional p53.

INTRODUCTION

The CDKs[2] were recognized as key regulators of cell cycle progression through their association with regulatory subunits called cyclins (1). Deregulation of CDK activation or overexpression of cyclin, such as cyclin D and cyclin E, involved the G_1 phase and was frequently found in human cancers. Because uncontrolled cell growth is the hallmark of neoplasic cells, CDK inhibition appeared to be a potent target to cancer treatment. This generated a new category of compounds named small molecule CDK inhibitors, which can directly antagonize the action of CDKs (2). Among these, flavopiridol demonstrated interesting preclinical features, such as cell growth inhibition, induction of apoptosis, cell differentiation, and inhibition of angiogenic processes (2). In addition, this agent, now investigated in clinical trials, was found to act as an enhancer of chemotherapy induced apoptosis when combined with paclitaxel, doxorubicin, or etoposide (3). However, the relationship between the observed effects and CDK inhibition remains unclear.

Since flavopiridol, more potent and selective CDK inhibitors were synthesized. Roscovitine, an olomoucine-related purine, was found to be a potent inhibitor of the kinase activity of CDK1, CDK2, CDK5, and CDK7 (4, 5). This inhibitor was identified to bind specifically to the ATP-binding site of those CDKs in a competition mechanism. Concentrations of roscovitine at the micromolar levels were observed to exert strong inhibitory effects on the kinase activity of CDK1/cyclinB, CDK2/cyclinA, and CDK2/cyclinE complexes and prevented the cell cycle progression of mammalian cells at the G_1-S and G_2-M checkpoints. In addition, roscovitine was found to induce antiproliferative and antitumoral effects in human breast cancer cells (6), nucleolar fragmentation (7), and apoptosis in human cell lines (6, 8). Therefore, roscovitine is being considered as a potential anticancer agent. Recent studies reported that roscovitine can induce activation and stabilization of p53 by suppression of MDM2 expression (7, 9). Moreover, a synergistic activation of p53 was observed in p53 wild-type cancer cells when roscovitine was associated with DNA-damaging agents, such as camptothecin and IR (9, 10). However, the combined effect of roscovitine with IR to improve tumor control has never been obviously studied, and no studies were reported in p53 mutated carcinoma.

Our study has provided details on a mechanism involving roscovitine as radiosensitizer *in vivo* and *in vitro* in p53 mutated breast cancer MDA-MB231 cells. The observed radiosensitization effect was associated with a repair defect in the NHEJ pathway attributable to the inhibition of DNA-PK activity associated with a decrease in Ku-DNA binding.

MATERIALS AND METHODS

Cell Culture, Reagent, and Irradiation Conditions. The human breast carcinoma MDA-MB-231 (p53 mutated) cell line was purchased from the American Type Cell Culture and cultured according to its instructions. The mouse fibroblastic cell line SCID (DNA-PKcs deficient) and BALB were obtained from Dr. Mezzina (Genopole, France) cultured in RPMI 1640 (Sigma, Genopole, France), supplemented with 10% FCS, 0.1% L-glutamine, and 0.2% penicillin-streptomycin, and maintained in a 5% CO_2 humidified atmosphere at 37°C. For the *in vitro* experiments, Roscovitine (Alexis Coger, Co.) was dissolved in DMSO for a stock solution (14 mM) and kept at −20°C. γ-irradiation was delivered by ^{137}Cs source at a dose rate of 1.97 Gy/min.

Clonogenic Survival Assay. The cell lines were seeded in triplicate into T25 cm^2 flasks in a range of 200–800 cells/flask according to the condition tested. A single dose of irradiation and/or addition of the drug was done when cells were attached. Cells were cultured until 12 days in the incubator at 37°C in a 5% CO_2 humidified atmosphere. Colonies were fixed, stained with crystal violet, and counted. SF was estimated by the following formula: SF = number of colonies formed/number of cells seeded × plating efficiency of the control group.

Tumor Xenograft and Assessment of Antitumor Activity of the Combined Treatment. The *in vivo* experiments were carried out at the Institut Gustave Roussy under the Animal Care license n°C94-076-11 (Ministère de

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[2] The abbreviations used are: CDK, cyclin-dependent kinase; IR, ionizing radiation; SF, surviving fraction; T/C, treated *versus* control; DSB, double-strand break; FAR, fraction of activity released; PFGE, pulse field gel electrophoresis; NHEJ, nonhomologous end joining; EMSA, electrophoretic mobility shift assay; DNA-PK, DNA-dependent protein kinase.

l'Agriculture). Female athymic nude mice (6–8 weeks old) purchased from Janvier CERT (Le Genest St. Isle, France) were used. Tumor xenografts were obtained by s.c. injection of 3.10^6 cells in the right flank of nude mice. Xenografts were grown for 2 weeks to a mean tumor volume of 86 ± 17 mm^3. Roscovitine was first dissolved in absolute methanol (1 volume), and Tween 20 (10% volume for volume) was added. The roscovitine solution was thoroughly evaporated under N_2 and resolubilized in 0.9% NaCl (5 volume). Final roscovitine concentration was 1 mg/ml (2.8 mM). The vehicle (V) solution was composed of 2.5% Tween 20 in NaCl 0.9% and i.p. injected in mice of the control and irradiated (7.5 Gy) groups. The concomitant treatment was performed as follows: roscovitine was i.p. injected at the dose of 100 mg/kg, and within <20 min, a single dose of X-rays (7.5 Gy) was delivered locally (using a shielding device) on mice xenografts. Irradiation was carried out with 250 kV RT Phillips X-ray at a dose rate of 0.69 Gy/min (220 Kv, 20 mA, and 0.2 mm Cu filter). Mice were weighed, and the tumor size was measured twice a week with an electronic caliper. Individual mice follow-up was done over 30 days after the beginning of the treatment. The tumor volume was estimated from two-dimensional tumor measurements by the formula:

Tumor volume = length (mm) × width^2 (mm^2)/2.

In each group (six mice per group), the relative tumor volume was expressed as the Vt/Vo ratio (Vt as the mean tumor volume on a given day during the treatment and Vo as the mean tumor volume at the beginning of the treatment). Treatment efficacy was determined on the mean of two independent experiments according to the criteria from Langdon *et al.* (11). The percentage of T/C values was calculated from the mean of the relative tumor volume of T/C group at each day of the tumor measurement. The lowest T/C value within 4 weeks after treatment corresponded to the optimal T/C value. The optimal growth inhibition percentage is calculated as 100 minus the optimal T/C percentage value. The nonparametric Mann-Whitney *t* test (Statview software) was used to determine the statistical significance of the relative tumor volumes and comparisons among treatment groups.

Cell Cycle Analysis. For cell cycle analysis, cells were synchronized for 24 h in serum-free medium and then changed with a complete medium before 4 Gy irradiation and/or 5 μM roscovitine treatment. In brief, sham control and treated cells were harvested by trypsinization at the indicated time after treatment, washed with ice-cold PBS, fixed in 70% ethanol, and stored at 4°C. Before DNA analysis, DNA content was labeled with propidium iodide in the presence of the RNase (1 mg/ml). The presence of apoptotic cells was detected by the terminal deoxynucleotidyl transferase-mediated nick end labeling assay, according to the manufacturer's instructions (Apoptag *in situ* Apoptosis Detection Kit; Intergen). Flow cytometry analysis was performed on a FACScan, and data were analyzed by using Multi Cycle software (Becton Dickinson).

Micronuclei Assay. Cells (10^5) were seeded in duplicate into T25 cm^2 flasks and treated either by 4Gy irradiation dose and/or 5 μM roscovitine incubation for a culture time of 48 h. Cytochalasin B (Sigma) was added at a final concentration of 6 μg/ml for 24-h culturing before cell harvest. A sham control was done to determine the spontaneous micronuclei frequency. Cells were fixed into fixative solution (acetic acid:methanol, 1:3). Slides were made by dropping the cell suspension on clean object glasses with a drawn-out Pasteur pipette and stained in 4% Giemsa solution, rinsed with distilled water, and air dried. Micronuclei were scored in cells that have gone through one cell cycle after treatment, which led to the formation of binucleated cells. A total of 200 binucleated cells was scored per slide for the presence of micronuclei according to the criteria of Champion *et al.* (12).

Assessment of DNA-DSB Repair. Exponential growing cells (labeled with [^{3}H]thymidine) were treated with 5 μM roscovitine and/or irradiated on ice with 30 Gy and replaced in the incubator at 37°C for varying times. The cells were trypsinized on ice, washed with ice-cold PBS, and embedded in agarose plugs with the density of 2.10^5 cells/100 μl. The plugs were lysed with sarcosyl solution [1 mM EDTA (pH = 8.0), 10 mM Tris, 2% sarcosyl, and 1 mg/ml proteinase K] at 50°C for 38 h, then washed in TE buffer [10 mM Tris (pH = 8.0) and 1 mM EDTA], and incubated for 1 h at 37°C in TE buffer containing 0.1 mg/ml RNase. DNA plugs were washed with TE buffer before loading of the gel. The PFGE was performed in a CHEF-DR III (Bio-Rad, Hercules, CA) to allow separation of DNA fragments in the megabase size region. After the PFGE, separation of the gel was stained with ethidium bromide and photographed under UV light. The FAR is calculated from the activity in the lane relative to that in the lane plus the well. The results are expressed as the percentage of FAR remaining (FAR at the specified time/FAR at the initial time) and represented the mean of three independent experiments.

EMSA. Nuclear extracts of cells treated with 5 μM roscovitine and/or 4Gy irradiated were prepared for EMSA. Cells were scrapped and washed twice with ice-cold PBS by centrifugation. Cell pellets were resuspended in lysis buffer [1 M Tris (pH 8.0), 500 mM NaCl, 5 mM EDTA, 1 mM DTT, 50 mM NaF, 0.5 mM Na_3VO_4, and 0.1% NP40; protease inhibitor cocktail from Bio-Rad] for 10 min on ice and then sonicated briefly. After centrifugation (15,000 rpm) at 4°C for 15 min, the supernatant designed as the nuclear cell extracts was collected and stored at −80°C. The protein concentration was estimated using the Bradford assay (Bio-Rad). To prepare the double-strand DNA probe, two oligonucleotides (5′-GGG CCA AGA ATC TTA GCA GTT TCG GG-3′ and 5′-CCC GAA ACT GCT AAG ATT CTT GGC CC-3′) were end labeled with T4 polynucleotide kinase in the presence of [γ-^{32}P] ATP and subsequently annealed together. Ku-DNA binding reaction was performed on ice for 5 min with 20 μg of nuclear extract and 7.5 μl of binding buffer [20 mM Tris (pH 8), 2 mM EDTA, 20% glycerol, 0.4 M NaCl, and 0.25 μg/μl circular plasmid pBlue script II ks]. For supershift, 1 μg of monoclonal anti-Ku80 Ab-2 (Lab Vision Corp., Fremont, CA) was added to the binding mixture and incubated for an additional 10 min before gel electrophoresis. The samples were electrophoresed in 5% polyacrylamide gel for 3 h at 100 V. The gel was dried on Wathman 3M paper and exposed to phosphoimager intensifying screen overnight. After the scan of the gel, the band quantifications were done by imageQuaNT software.

Evaluation of DNA-PK Activity. DNA-PK activity was assayed by using a kit (Sigma TECT DNA-PK assay system; Promega) according to the manufacturer's protocol. Briefly, 20 μg of nuclear extract were incubated with a mix of DNA-PK, biotinylated peptide substrate, [γ-^{32}P]ATP, and either DNA-PK activation buffer or DNA-PK control buffer for 5 min at 30°C. Termination buffer was added, and 10 μl of each reaction sample were spotted onto a SAM^{2}TM biotin capture membrane. The SAM^{2}TM membrane squares were washed and dried before analysis by scintillation counting. The enzymatic activity of DNA-PK was expressed as the mean of two independent experiments at least.

RESULTS

***In Vitro* and *in Vivo* Radiosensitization Effect of Roscovitine in MDA-MB 231 Cells.** Clonogenic survival assays were performed to assess cellular sensitivity to the concomitant combination of roscovitine with IR. The maximum roscovitine concentration of 5 μM was tested corresponding to an IC_{15} estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay from a previous cytotoxicity study. As shown in Fig. 1*A*, we found a nonsignificant decrease of MDA-MB 231 clonogenic survival when 2.5 μM roscovitine was applied in combination with IR. At 5 μM roscovitine concentration, we have observed a marked enhancement of cellular radiation sensitivity. The clonogenic SF was decreased to 1.6-fold ($P = 0.003$) and 8-fold ($P = 0.015$) for 5 μM roscovitine combined, respectively, with 2 and 4 Gy irradiation doses.

The effect of combining roscovitine and IR was further studied *in vivo* against MDA-MB 231 tumor xenografts in athymic nude mice. Fig. 1*B* represents the relative tumor growth delay measured after roscovitine alone (100 mg/kg), IR alone (7.5Gy + vehicle), and concomitant regimen (100 mg/kg + 7.5Gy) in comparison with the sham control group (vehicle). No tumor growth delaying effect was observed with roscovitine alone with no difference in the tumor volume doubling time as compared with the control group (8 *versus* 7 days, respectively). In contrast, the combined treatment of roscovitine with IR significantly increased the antitumor effect of IR. Optimal growth inhibition of 73% was found on day 26 for the combined treatment group as compared with 54% for the irradiated group ($P = 0.02$, day 26, combined treatment *versus* IR). According to Langdon *et al.* (11), efficacy scoring was estimated as moderately active for IR alone and very active for the combined treatment, suggesting a possible synergistic effect of the combined treatment on

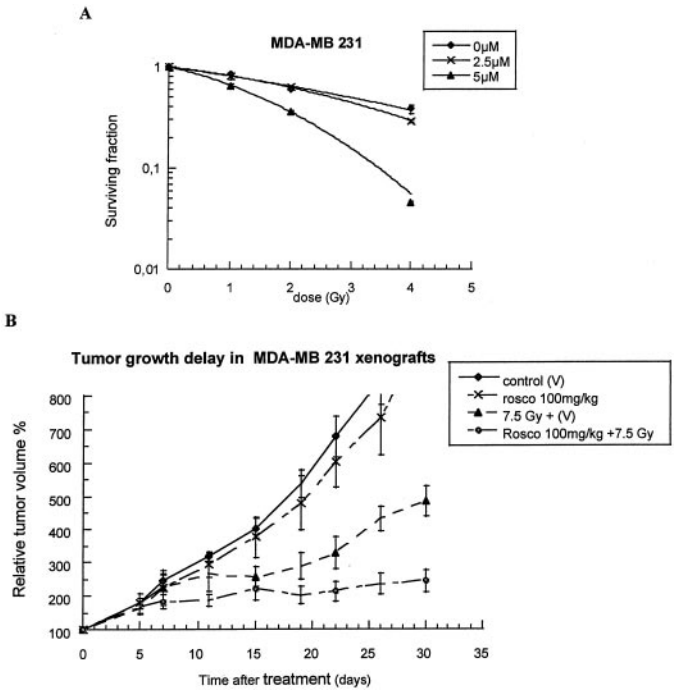

Fig. 1. *A*, clonogenic survival assays showing a radiosensitization effect of roscovitine in MDA-MB 231 (p53 mutated) cell line. After irradiation and/or roscovitine treatment and sham control treatment, cells were cultured ≤12 days until the colony formation. The results (in triplicate) of a representative experiment are shown; errors bars, SE. *B*, relative tumor growth delay measured after roscovitine alone (100 mg/kg), IR alone (7.5 Gy + vehicle), and concomitant regimen (100 mg/kg + 7.5 Gy) in comparison with the control group (vehicle) on MDA-MB 231 xenografts. Data represent the mean of two independent experiments (n = 6 mice/group in each experiment); error bars, SD.

MDA-MB 231 tumor xenografts. In addition, no significant decrease in mice body weight nor increase in normal tissue injury was observed in all treated groups during 30-day follow-up.

Potentialization of Cell Cycle Arrest with no Increased Radio-induced Apoptosis. To determine the effect of roscovitine and IR on the cell cycle distribution, we synchronized cells by 24-h starvation in serum-free medium before the different treatment. Cells were harvested at the indicated times and analyzed by flow cytometry (Fig. 2). Maximum G_2-M checkpoint arrest was found at 24 h in cells treated with roscovitine + 4 Gy resulting in 82% of the cell population blocked in G_2 phase and was associated with a pronounced decrease in DNA synthesis. This marked effect was sustained over 48 h after the combined treatment as compared with irradiation alone. No increase in sub-G_1 cell population was observed. The apoptosis level never exceeded 5% in all treated and sham control cells and was confirmed by the terminal deoxynucleotidyl transferase-mediated nick end labeling assay. In addition, Western blot analysis of Bcl2 protein has revealed no change in the expression level after either roscovitine, IR alone, or combination of roscovitine and IR treatment as compared with the untreated cells (data not shown). Growth curves were established to evaluate the effect of the combination of roscovitine and irradiation on cell proliferation in MDA-MB 231 cells. Roscovitine or irradiation treatment showed ~50% of cell growth inhibition as compared with the untreated cells. The combination of roscovitine and IR showed a marked antiproliferative activity starting 48 h after combined treatment of the cells, leading to 90% growth inhibition. This strong cytostatic effect was not correlated with a decrease in cell viability, because 80–95% of viable cells were found in all treated and control tumor cells over 4-day treatment (data not shown).

DNA-DSB Repair Defect Induced by the Combination of Roscovitine with IR. Furthermore, we investigated the radiosensitization effect of the combination of roscovitine and IR on DNA-DSB repair. Using PFGE, we studied the kinetics of DNA-DSB end joining after the combined treatment (5 μM roscovitine + 30 Gy) and 30 Gy alone in exponential growing MDA-MB 231 cells. As shown in Fig. 3*A*, rejoining of DNA-DSB followed biphasic kinetics. A significant difference in repair kinetics was observed in cells treated with 5 μM roscovitine + 30 Gy as compared with 30 Gy alone. One hour after roscovitine and IR treatment, a transient increase in the percentage of FAR remaining was observed, which could be associated with an increase in DNA-DSB level. Up to 6 h, the fast component of rejoining was characterized by a strong impairment of DNA-DSB rejoining in cells treated with the combined treatment as compared with 30 Gy alone. Moreover, this pronounced DSB repair defect was sustained up to 24 h, because cells treated with the combined treatment still present 59% of DSBs unrepaired as compared with 25% after a single dose of 30 Gy.

Therefore, we performed micronuclei assays in MDA-MB 231 cells treated 24 h with roscovitine, IR, and the combination roscovitine and IR. As shown in Fig. 3*B*, we found that roscovitine alone led to a 4.5-fold increase in micronuclei frequency as compared with basal level in the control cells. No significant difference in the induction of micronuclei was observed with the combination roscovitine + 2 Gy irradiation or 2 Gy alone in comparison with roscovitine alone (data not shown). Although, the micronuclei frequency was higher after 4 Gy irradiation alone. A maximum micronuclei frequency was found after roscovitine + 4Gy as compared with 4 Gy alone with 1.5-fold increased (P = 0.02). Thus, to further characterize which mechanism was implicated in this pronounced DSB repair defect, we examined the induction of micronuclei frequency in SCID (DNA-PKcs deficient) and BALB murine cells. In Fig. 3*B*, a 1.9-fold (P = 0.03) increase induction of micronuclei was found in BALB cells after roscovitine + 4 Gy treatment as compared with 4 Gy alone, whereas no significant difference was observed in SCID cells, suggesting the role of DNA-PK in this radiosensitization effect.

DNA-DSB Repair Defect Attributable to a Decrease in Ku-DNA Binding by Roscovitine Combined with IR. Next, we evaluated whether NHEJ repair pathway was involved in this misrejoining repair process in the MDA-MB 231 cell line. Using functional assay,

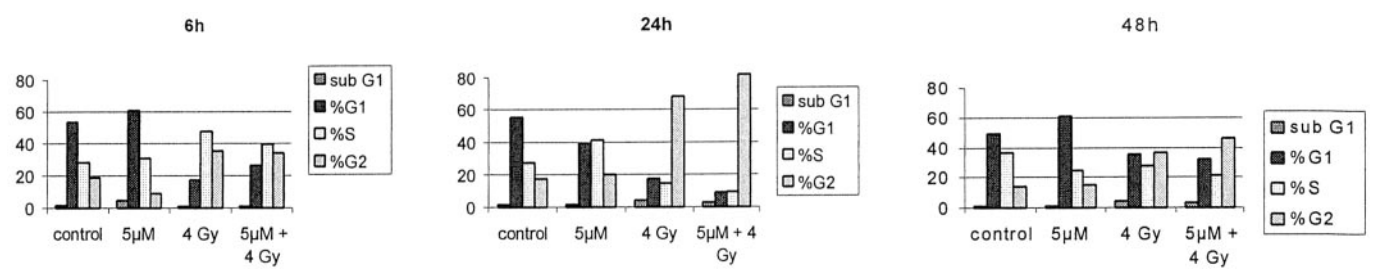

Fig. 2. Kinetic studies of cell cycle distribution after treatment in synchronized MDA-MB 231 cells. Flow cytometry analysis was performed at the indicated time on sham control samples and samples treated with 4 Gy, 5 μM roscovitine, and combination of 5 μM roscovitine + 4 Gy. A representative experiment from two independent experiments is shown.

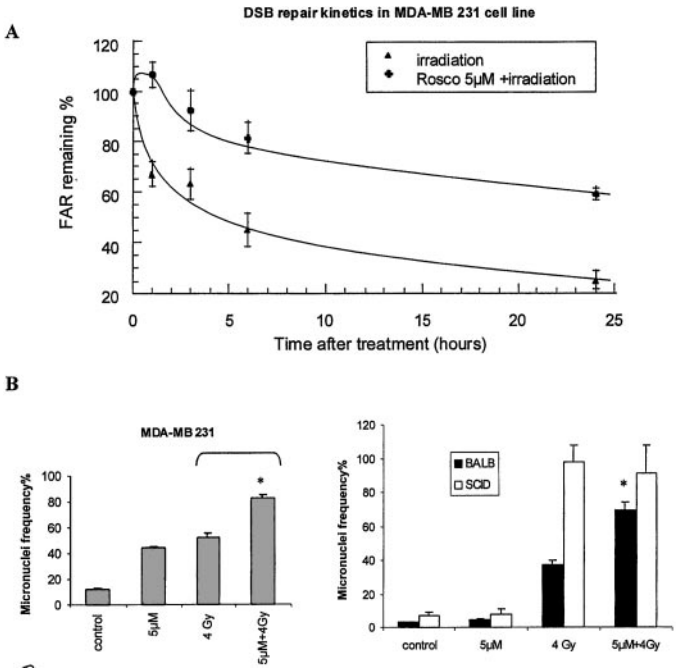

Fig. 3. *A*, DNA-DSB rejoining kinetics in exponential growing MDA-MB 231 cells after 5 μM roscovitine + 30 Gy and 30 Gy irradiation treatment, measured by conventional PFGE. The results are expressed as the percentage of FAR remaining (FAR at the specified time/FAR at the initial time) and represented the mean of three independent experiments; error bars, SD. *B*, micronuclei assay performed on MDA-MB 231 breast cancer cells and murine SCID (DNA-PKcs deficient) and BALB cells (used as control for DNA-PKcs). After 24-h treatment with 5 μM roscovitine and/or irradiation (4 Gy), cells were blocked by Cytochalasin B. Micronuclei were scored in cells that have gone through one cell cycle after treatment, which led to the formation of binucleated cells. Scoring was performed according to the criteria of Champion *et al.* (12). Data represent the mean of two independent experiments; error bars, SD. Statistical analysis by unpaired Student's *t* test: (4 Gy *versus* 5 μM + 4Gy), $*P < 0.05$.

roscovitine + 4 Gy showed a 0.7-fold decrease in the DNA-PK activity induced by 4 Gy alone (Fig. 4*A*). In addition, by using EMSA, we found a 3-fold ($P = 0.04$) decrease in Ku-DNA binding in cells treated with roscovitine + 4Gy as compared with 4 Gy alone. These results are consistent with DNA-PK activity for the same treatment conditions (Fig. 4*B*). Interestingly, Western blot analysis showed no differences in DNA-PKcs, Ku 70, or Ku 80 protein expressions after 24- and 48-h exposures with roscovitine and/or 4 Gy irradiation (data not shown). Therefore, we concluded that the radiosensitization effect of roscovitine was related to the inhibition of DNA-PK activity by a decrease in Ku-DNA binding in p53 mutated MDA-MB 231 cells.

DISCUSSION

Previous studies reported that roscovitine could sensitize cells to DNA-damaging agents, such as camptothecin and IR, by a synergistic activation of p53 (9, 10). Here, we observed for the first time that roscovitine was able to enhance the radiation response of p53 mutated breast cancer cells in *in vitro* and *in vivo* xenograft models. Thus, this addresses a new question on the mechanism involved in this radiosensitization effect.

At the dose as low as 5 μM, a potentialization in the inhibition of cell proliferation was found when roscovitine was added to IR. This cytostatic effect was correlated with an increase in cell cycle block at the G_2-M transition 24 h after the combined treatment and sustained until 48 h. Previous studies have reported that IR can induce G_2-M arrest in p53-deficient cells (13, 14). This is in agreement with our findings because we observed IR-induced G_2-M arrest in MDA-MB 231 cells, and this was reinforced by the combination with roscovitine. Using micronuclei assay, we found that roscovitine alone can generate DNA-DSB and cooperate synergistically with IR to enhance DNA damage. This finding was also observed by PFGE analysis, which revealed a transient increase in DNA-DSB induction at 1 h after roscovitine and IR treatment. In MDA-MB 231 cells, which lack a functional p53 protein, the G_2 checkpoint appears to be a critical determinant of cellular radiosensitivity. Recently, direct evidence that the DNA damage-induced G_2 delay is related to DNA repair activity was shown (15). The ability to repair DNA-DSB is fundamental to maintain genomic integrity. Indeed, DNA-DSB was considered as a lethal event if not repaired or misrepaired. Interestingly, we observed that the radiosensitization effect of roscovitine was not caused by an increase of apoptosis. A similar radiosensitization effect was reported in $p53^{-/-}$ cells treated by a new staurosporine analogue protein kinase C 412, which also presented an increase in the G_2 delay after the combined treatment (16). Therefore, it was interesting to investigate the radiosensitization effect of roscovitine on DNA repair pathways. The kinetic study of DNA-DSB rejoining in MDA-MB 231 showed a strong impairment of DSB repair when cells were treated with roscovitine and IR as compared with IR alone. Similar DNA rejoining kinetics were found in MO59J cells, which are deficient in DNA-PKcs (17). Using micronuclei assay, we demonstrated a marked DNA repair defect associated with roscovitine plus IR both in MDA-MB 231 and BALB cell lines. Although, no significant micronuclei induction was found in SCID cells (deficient for DNA-PKcs). This result provided some evidence that DNA-PK pathway could be involved in this radiosensitization effect.

On the other hand, we observed a significant micronuclei induction in murine BALB cells suggesting that normal cells can be sensitive to the combination of roscovitine and IR. However, we found no en-

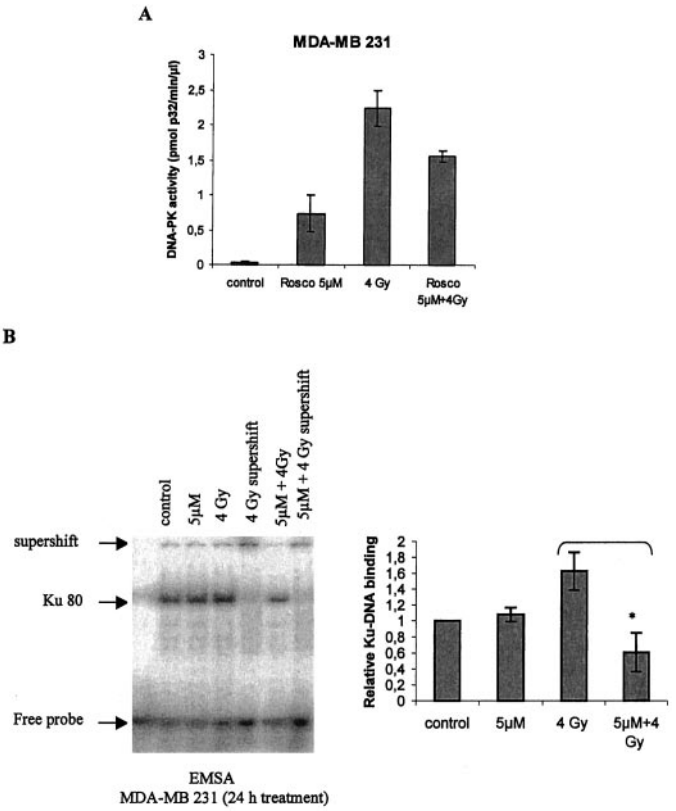

Fig. 4. DNA-PK activity (*A*) and Ku-DNA binding (*B*) after 24-h treatment in MDA-MB 231 cells. DNA-PK activity and the level of Ku-DNA binding by EMSA analysis were assessed on nuclear extracts. Results are the mean of three independent experiments; error bars, SD. Statistical analysis by unpaired Student's *t* test, $*P < 0.05$.

hancement of radiation response by roscovitine in normal human fibroblast and keratinocyte cells.[3] In addition, the *in vivo* experiment showed no increase in toxicity and normal tissue injury during the 30-day follow-up for the combined treatment as compared with IR alone.

The NHEJ pathway was found to be predominantly activated for repairing IR-induced DNA damage (18). After the introduction of DNA-DSB, the DNA-PK is activated by the recruitment of the catalytic subunit of DNA-PK (DNA-PKcs) through the binding of the Ku70/Ku80 heterodimeric regulatory component, which first recognized and stabilized DNA strand breaks (19). The NHEJ repair defect associated with roscovitine and IR was not attributed to a difference in either Ku70 or Ku80 or DNA-PKcs protein levels in the MDA-MB 231 cancer cells. On the other hand, Ku-DNA binding was markedly decreased in cells after 24-h treatment with roscovitine and IR as compared with IR alone. Furthermore, this was accompanied with a decrease in DNA-PK activity. Many studies have reported that cells lacking DNA-PK activity as a result of a mutation in any of the subunits are radiosensitive and deficient in DNA-DSB rejoining. Yet, Ku-deficient cells present a stronger radiosensitive phenotype than DNA-PKcs-deficient cells. Besides, DNA-PK activity is undetectable in Ku-deficient cell lines, indicating that DNA binding by Ku heterodimer is essential for its activation (19). Recently, it was found that the CDK inhibitor flavopiridol could bind to DNA and likely intercalate into duplex DNA with the same affinity of DNA-intercalating agents, such as doxorubicine (20). Hence, additional experiments are needed to investigate whether roscovitine might interact with DNA. Thus, the radiosensitization effect of roscovitine showing a decrease in Ku-DNA binding could be caused by a direct interaction with the DNA and/or indirect with Ku-DNA complex in a competitive mechanism.

Previous studies described roscovitine as a good candidate for modifying the IR response through p53-dependent mechanisms. In this study, for the first time, we bring up complementary evidence on the therapeutic potential both *in vitro* and *in vivo* of roscovitine combined with IR in cell lacking a functional p53. Beside its CDK inhibitor function, roscovitine can generate DNA-DSB and cooperate to enhance IR-induced DNA damages. Roscovitine is currently in clinical trials. Although our findings suggest that the combination of roscovitine with IR appears to be very promising, especially for breast cancer treatment, further investigation is needed to evaluate the therapeutic index before being tested in clinical trials.

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