Patupilone Acts as Radiosensitizing Agent in Multidrug-Resistant Cancer Cells *In vitro* and *In vivo*

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ABSTRACT

Interference with microtubule function is a promising antitumoral concept. Paclitaxel is a clinically validated tubulin-targeting agent; however, treatment with paclitaxel is often limited by taxane-related toxicities and is ineffective in tumors with multidrug-resistant cells. Patupilone (EPO906, epothilone B) is a novel non-taxane-related microtubule-stabilizing natural compound that retains full activity in multidrug-resistant tumors and is clinically less toxic than paclitaxel. Here we have investigated the effect of combined treatment with ionizing radiation and patupilone or paclitaxel in the P-glycoprotein-overexpressing, p53-mutated human colon adenocarcinoma cell line SW480 and in murine, genetically defined *E1A/ras*-transformed paclitaxel-sensitive embryo fibroblasts. Patupilone and paclitaxel alone and in combination with ionizing radiation reduced the proliferative activity of the *E1A/ras*-transformed cell line with similar potency in the sub and low nanomolar range. SW480 cells were only sensitive to patupilone, and combined treatment with low-dose patupilone (0.1 nmol/L) followed by clinically relevant doses of ionizing radiation (2 and 5 Gy) resulted in a supra-additive cytotoxic effect. Inhibition of the drug efflux protein P-glycoprotein with verapamil resensitized SW480 cells to treatment with low doses of paclitaxel alone and in combination with IR. In tumor xenografts derived from SW480 cells a minimal treatment regimen with patupilone and fractionated irradiation (1 × 2 mg/kg plus 4 × 3 Gy) resulted in an at least additive tumor response with extended tumor growth arrest. Analysis by flow cytometry *in vitro* revealed an apoptosis- and G_2-M-independent mode of radiosensitization by patupilone. Interestingly though, a transient accumulation of cells in S phase was observed on combined treatment. Overall, patupilone might be a promising alternative in paclitaxel-resistant, P-glycoprotein-overexpressing tumors for a combined treatment regimen using ionizing radiation and a microtubule inhibitor.

INTRODUCTION

Interference with microtubule function represents a clinically validated mechanistic concept for anticancer drugs. Microtubule-stabilizing agents suppress cancer cell growth by promoting accelerated assembly of excessively stable microtubules, which consequently leads to cell cycle arrest in G_2-M and eventual cell death (1–5). Although the exact mechanism of apoptosis-induction is not fully understood (4, 6–8), these agents are of high interest due to their capacity to induce apoptosis even in p53-mutated, otherwise chemo- and radioresistant cells (9). For a combined treatment modality with ionizing radiation (IR), the search for chemotherapeutic agents specifically arresting the cell cycle at G_2-M is tempting because G_2-M is the most radiosensitive cell cycle phase (10–13). Therefore, the combined use of microtubule-stabilizing agents such as taxanes in combination with IR might be clinically interesting to improve local tumor control (14–16).

Despite its clinical success for some tumor entities, treatment with taxanes (e.g., paclitaxel) is often limited by taxane-related toxicities, such as neutropenia, peripheral neuropathy, and alopecia (17–19), as well as development of multidrug resistance which is often associated with overexpression of P-glycoprotein. This has led to the search for novel microtubule-stabilizing agents lacking these limitations of taxanes (3, 20).

Epothilones, although structurally unrelated to the taxanes, exert strong microtubule-stabilizing effects (2). Patupilone (epothilone B, EPO906) and epothilone A are naturally occurring secondary metabolites that are produced by myxobacteria and were first described for their selective antifungal activity (21). These tubulin-polymerizing and microtubule-stabilizing agents are competitive inhibitors of paclitaxel-binding to tubulin, sharing with the taxanes a partially overlapping binding site (1, 22–25). Patupilone is a more potent microtubule stabilizer and antiproliferative agent than either demethylated epothilone B (epothilone A), the lactam analogue of epothilone B (aza-epothilone B, also known as BMS-247550), or paclitaxel (2, 26, 27). More importantly, patupilone is least sensitive to P-glycoprotein efflux pump–mediated multidrug resistance (1, 26–28).

Based on the close functional similarity to taxanes and the cellular response leading to an accumulation of the cell population in the radiosensitive G_2-M phase of the cell cycle, epothilones are presumed to sensitize tumor cells to ionizing radiation (2, 10–13). Here we profile the combined effect of

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the potent epothilone derivative patupilone with ionizing radiation against p53-deficient, paclitaxel-sensitive E1A/ras-transformed mouse embryo fibroblasts (MEF) and multidrug-resistant p53-mutant human colon adenocarcinoma tumor cells at low concentration both *in vitro* and *in vivo*. As the role of G_2-M cell cycle arrest in the radiosensitization mechanism, especially at low concentrations of these agents, is still controversial, we also closely analyzed cell cycle progression at low concentrations of patupilone and IR alone and in combination. We show here that patupilone can sensitize both paclitaxel-sensitive as well as paclitaxel-resistant cells to ionizing radiation, and propose an S-phase progression-related mechanism for radiosensitization.

MATERIALS AND METHODS

Paclitaxel and verapamil hydrochloride were purchased from Calbiochem (San Diego, CA). Patupilone (epothilone B, EPO906) was provided by Novartis Pharma (Basel, Switzerland).

Cell Cultures and Irradiation. p53-/- MEFs were derived from 13.5-day-old embryos and stably transfected with the two oncogenes *E1A* and *T24 H-ras* as described in ref. 29. These transformed MEFs were used at low passage numbers and cultured at 5% CO_2 atmosphere in DMEM containing 10% FCS and 10% bovine calf serum supplemented with penicillin and streptomycin. SW480 human colon adenocarcinoma cells were cultured in RPMI 1640 supplemented with 10% FCS, penicillin, and streptomycin. Irradiation of cell cultures was carried out at room temperature using a Pantak Therapax 300-kV X-ray unit at 0.7 Gy/min.

Cell Proliferation and Clonogenic Survival Assay. Tumor cell proliferation was assessed in 96-well plates with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide–like colorimetric Alamar Blue assay, which is based on detection of metabolic activity according to the protocol of the manufacturer (Biosource International, Camarillo, CA). Absorption was measured at 570 and 630 nm using a Tecan GENios spectrophotometer. To determine clonogenic survival, the number of single seeded cells was adjusted to obtain ~100 colonies per cell culture dish with a given treatment. After treatment with different regimens, cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and allowed to grow for 6 (*E1A/ras*-transformed MEFs) or 12 (SW480) days, respectively, before fixation in methanol/acetic acid (75%:25%) and staining with crystal violet. Only colonies with more than 50 cells/colony were counted. All proliferation and clonogenic assays were repeated as independent experiments at least twice. For combined treatment, cells were preincubated with patupilone, paclitaxel, or control solution 24 hours before irradiation. For *in vitro* experiments, patupilone and paclitaxel were dissolved in DMSO (1 mmol/L stock solution) and further diluted with media in the presence of 10% FCS. Verapamil, an inhibitor of the drug efflux protein P-glycoprotein, was dissolved in H_2O (1 mg/mL stock solution) and further diluted with FCS-containing media. For inhibition of this multidrug resistance–related efflux protein, cells were preincubated with verapamil 30 minutes before treatment with patupilone or paclitaxel.

Subcellular Fractionation and Immunoblotting. To obtain cytosolic and membranous cell constituents, subcellular fractionation of cell lysates was done essentially as described in ref. 30. Briefly, SW480 cells and transformed MEFs were cultivated in 10-cm dishes, washed twice with ice cold PBS, and then harvested by scraping the cells with 500 μL of precooled hypotonic lysis buffer (HEPES, pH 7.4, 25 mmol/L; β-glycerophosphate, 25 mmol/L; EDTA, pH 7.4, 2 mmol/L; leupeptin, 10 μg/mL; aprotinin, 10 μg/mL; Na_3VO_4, 1 mmol/L; phenylmethylsulfonyl fluoride, 1 mmol/L). The cell suspension was sonicated with 10 cycles (duty cycle, 50%; power output, 6) on ice using a tip sonicator and the cell lysate was centrifuged at 4°C for 15 minutes at 14′000 rpm. The supernatant (cytosolic fraction) was separated and the pellet was carefully washed with 1 mL of hypotonic lysis buffer and resuspended in 250 μL Triton lysis buffer (HEPES, pH 7.4, 25 mmol/L; β-glycerophosphate, 25 mmol/L; EDTA, pH 7.4, 2 mmol/L; leupeptin, 10 μg/mL; aprotinin, 10 μg/mL; Na_3VO_4, 1 mmol/L; phenylmethylsulfonyl fluoride, 1 mmol/L; Triton X-100, 1%). After a second sonication step, the sonicated lysate was centrifuged at 4°C for 15 minutes at 14′000 rpm, and the supernatant (clarified membrane fraction) was immediately frozen and stored at −70°C. The protein concentration was determined with the BioRad DC Protein assay (Bio-Rad Laboratories, Rheinach BL, Switzerland). Samples were separated by SDS-PAGE followed by Western blotting onto polyvinylidene difluoride membranes. Membranes were probed with the P-glycoprotein antibody anti-mdr (Ab-1, Calbiochem). Antibody detection was achieved by enhanced chemoluminescence (Amersham, Piscataway, NJ) according to the protocol of the manufacturer. All experiments were carried out independently at least thrice.

Cell Cycle Analysis. Cell cycle distribution was measured at distinct time points before (control) and after different treatment modalities. Bivariate distributions of 5-bromo-2′ deoxyuridine content (Sigma, Buchs SG, Switzerland) versus DNA content (propidium iodide) were analyzed according to the protocol of the manufacturers on the FITC-conjugated anti-5-bromo-2′ deoxyuridine antibody (Becton Dickinson Immunocytometry Systems, San Jose, CA). Analysis was done on an EPICS ELITE apparatus (Beckmann-Coulter). The data were analyzed using the MultiCycle (Phoenix Flow Systems, Inc., San Diego, CA) and WinMDI 2.8 software (Joseph Trotter, The Scripps Research Institute, La Jolla, CA). At least two independent experiments in duplicate were done for each set of data. Statistical analysis was done with the Fisher's *t* test.

Tumor Xenograft in Nude Mice and Administration of Patupilone and Irradiations. Human colon carcinoma cells (SW480) were injected subcutaneously (4×10^6 cells) on the back of 4- to 8-week-old athymic nude mice. Tumor volumes were determined from caliper measurements of tumor length (L) and width (l) according to the formula $(L \times l^2) / 2$. Tumors were allowed to expand to a volume of 200 $mm^3 \pm 10\%$ before start of treatment. Using a customized shielding device, mice were given a strictly locoregional radiotherapy of 4×3 Gy at 4 consecutive days using a Pantak Therapax 300-kV X-ray unit at 0.7 Gy/min. Patupilone (dissolved in 30% polyethylene glycol-300:70% saline) was applied intraveneously (at day 0 of the treatment) 24 hours before the first treatment with IR. Statistical Analysis of the *in vivo* data was done with the Mann-Whitney *U* test.

RESULTS

Antiproliferative Effect at Low Nanomolar Concentrations of Patupilone. The antiproliferative effect of patupilone was tested over 72 hours in genetically defined p53-deficient *E1A/ras*-transformed MEFs (Fig. 1*A*) and in the p53-mutated human colon adenocarcinoma cell line SW480 (Fig. 1*B*). Both cell lines were highly sensitive to patupilone; the SW480 tumor cells even responded in a subnanomolar range (IC_{50} ~ 0.2 nmol/L). A similar antiproliferative effect was observed in genetically identical but p53-wild-type *E1A/ras*-transformed MEFs (data not shown). Micromolar concentrations of epothilones are required for microtubule polymerization *in vitro*. However, epothilones also extensively accumulate inside cells and thus nanomolar concentrations of patupilone in the cellular media effectively translate into intracellular concentration levels sufficient for microtubule stabilization (2).

In parallel, the two cell lines were treated with increasing doses of paclitaxel. Whereas the *E1A/ras*-transformed cell line was sensitive to paclitaxel in the low nanomolar range (25-50 nmol/L, Fig. 1*C*), the SW480 tumor cells were resistant to paclitaxel up to 200 nmol/L (Fig. 1*D*), indicative for a paclitaxel-specific, but not epothilone-specific, treatment resistance for microtubule inhibitors in this cell line.

Antiproliferative and Clonogenic Cell Death–Inducing Effect by Combined Treatment with Patupilone and IR. Treatment of cells with microtubule inhibitors can induce a cell cycle arrest in the G_2-M phase (2). Based on the concept that microtubule inhibitors induce an accumulation of cells in this most radiosensitive phase of the cell cycle, the antiproliferative effect of patupilone in combination with ionizing radiation was analyzed in tumor cells, which were continuously pretreated for 24 hours with patupilone before irradiation.

Proliferation assays with increasing concentrations of patupilone and IR were done in both p53-/- *E1A/ras*-transformed MEFs (Fig. 2*A*) and SW480 cells (Fig. 2*B*). An at least additive antiproliferative effect was induced with doses that show less than 50% inhibition of proliferation over 72 hours when used alone. Likewise, treatment with IR in combination with paclitaxel induced an additive effect in both cell lines, but again high nanomolar concentrations of paclitaxel were required to achieve this effect in the human SW480 tumor cell line (Fig. 2*C* and *D*).

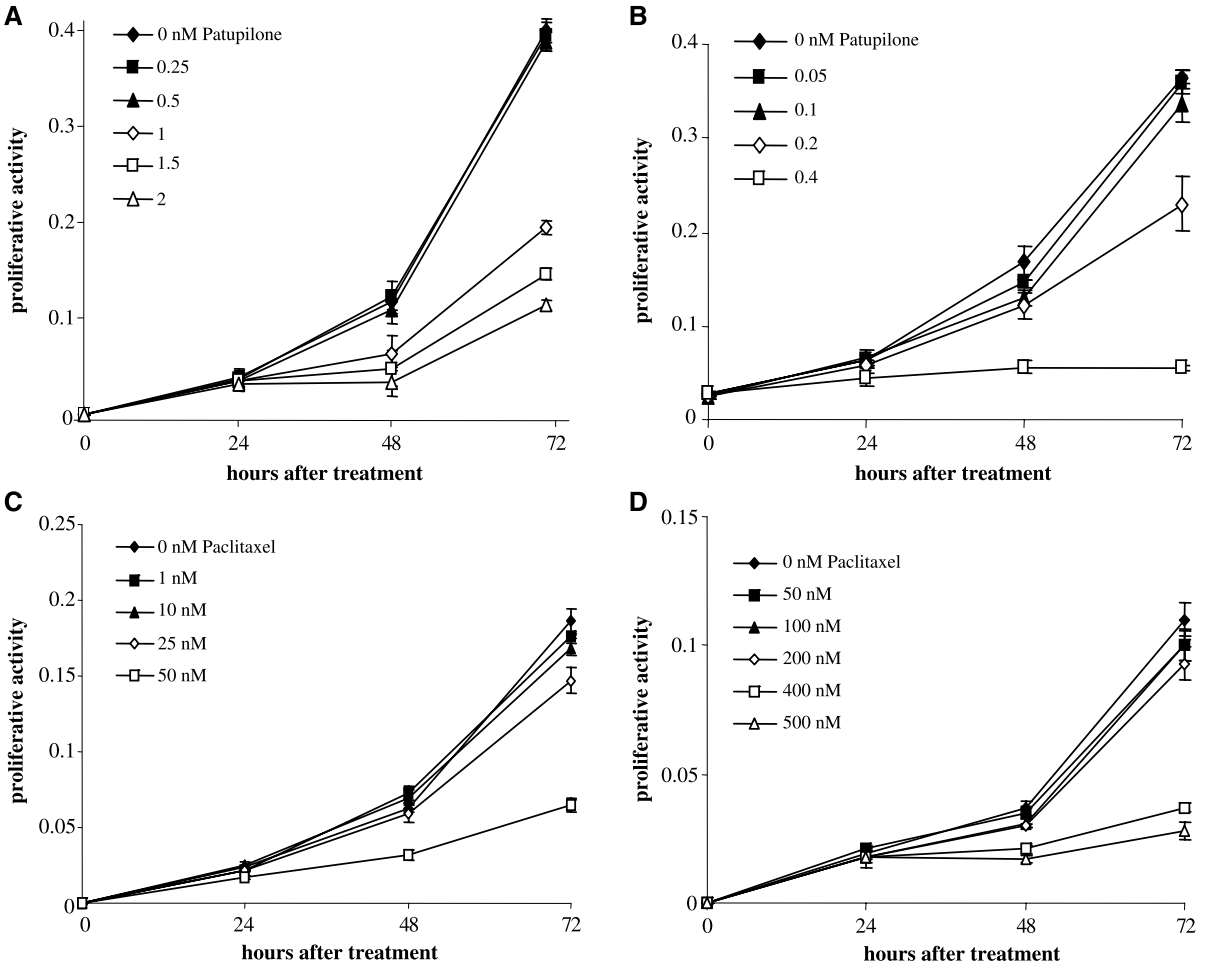

Fig. 1 Antiproliferative activity of patupilone and paclitaxel. p53-/- *E1A/ras*-transformed MEFs (*A*, *C*) and human colorectal p53-mutant SW480 cells (*B*, *D*) were treated with increasing doses of patupilone (*A*, *B*) or paclitaxel (*C*, *D*). The proliferative activity was determined at the indicated time points using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide–like Alamar Blue assay.

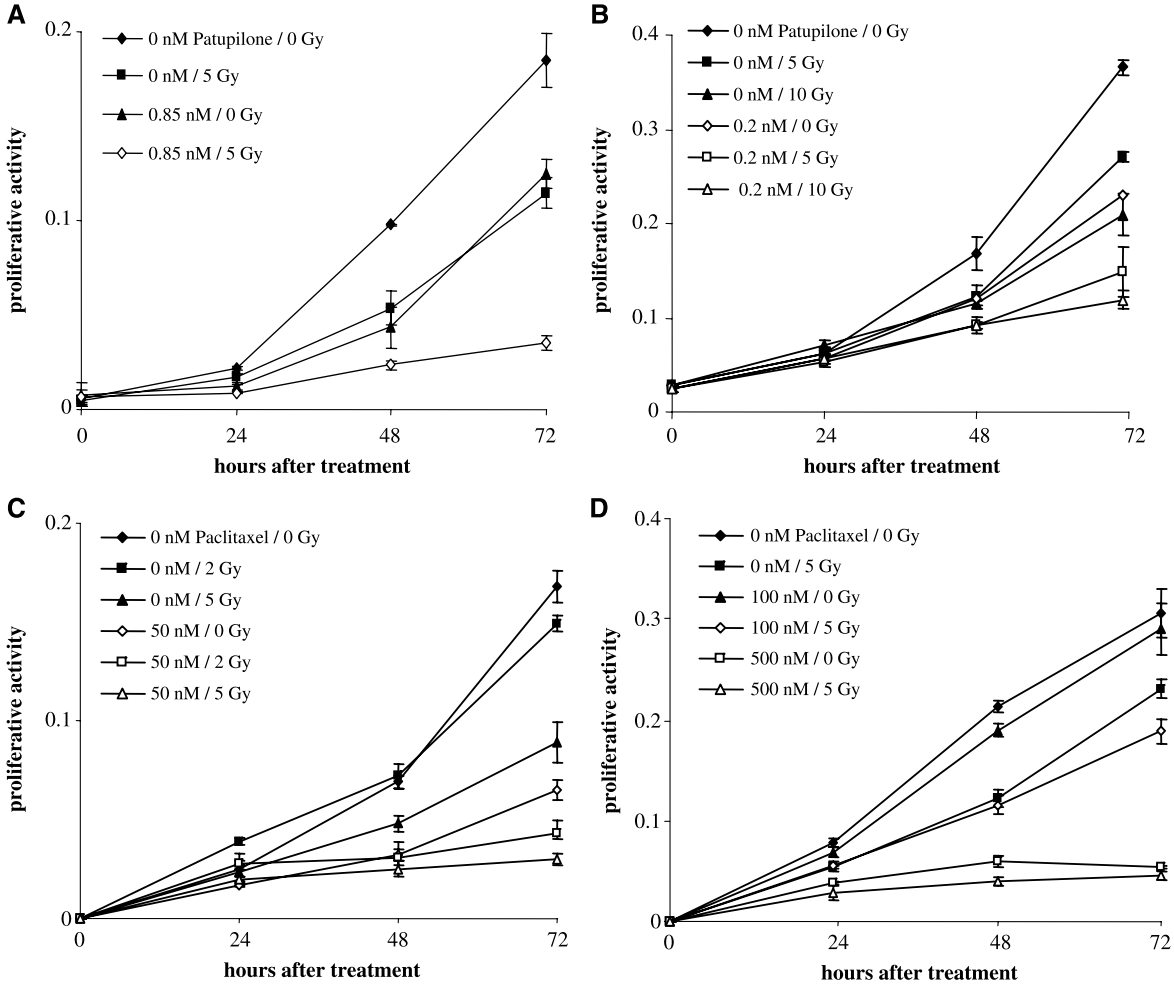

Fig. 2 Antiproliferative activity of patupilone or paclitaxel in combination with IR. p53-/- *E1A/ras*-transformed MEFs (*A*, *C*) and human colorectal p53-mutant SW480 cells (*B*, *D*) were treated with increasing doses of patupilone (*A*, *B*) or paclitaxel (*C*, *D*) in combination with IR. Both patupilone and paclitaxel were added 24 hours before irradiation. The proliferative activity was determined at the indicated time points using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide–like Alamar Blue assay.

Next, clonogenic survival assays were done with increasing concentrations of patupilone alone and in combination with irradiation (2 and 5 Gy) and the cellular response to IR and patupilone was compared with the radiosensitizing effect of paclitaxel. Clonogenic survival of MEFs and SW480 tumor cells was reduced on treatment with increasing concentrations of patupilone alone, but SW480 cells were more sensitive to patupilone than the p53-deficient transformed MEFs at all concentrations tested (Fig. 3*A* and *C*). Paclitaxel reduced clonogenic survival more drastically in the *E1A/ras*-transformed MEFs than in the SW480 cells and again in a dose range of 50 to 500 times higher than patupilone, as already observed in the proliferation assay (Fig. 3*B* and *D*). Preincubation of cells with patupilone at dose levels with only a minimal antiproliferative effect sensitized both tumor cell populations to IR and clonogenic survival was supra-additively reduced (Fig. 3*A* and *C*). A supra-additive effect of paclitaxel and IR was also observed against the p53-deficient transformed MEFs with increasing concentrations of paclitaxel (25-50 nmol/L, Fig. 3*B*). However, almost no additivity was observed on SW480 cells when IR was combined with paclitaxel at doses as high as 100 nmol/L paclitaxel (Fig. 3*D*). Overall these results show that whereas the oncogene-transformed MEFs are sensitive to both patupilone and paclitaxel, SW480 is a paclitaxel-refractory but patupilone-sensitive tumor cell line. Furthermore, patupilone has a supra-additive antiproliferative and clonogenic cell death–inducing effect when used in combination with IR.

The Multidrug Resistance–Related Efflux Pump P-Glycoprotein Is Overexpressed in the SW480 Cell Line and Renders SW480 Cells Paclitaxel-Resistant. Paclitaxel resistance in tumor cells is often due to overexpression of the multidrug resistance–related P-glycoprotein (as reviewed in ref. 31). Proliferation experiments were therefore done with SW480 cells, which were pretreated with the multidrug resistance modulator verapamil 30 minutes before paclitaxel treatment. Verapamil (5 μg/mL) resensitized SW480 cells to low doses of paclitaxel (10 nmol/L), indicative that enhanced P-glycoprotein activity is responsible for the paclitaxel-refractory effect in this cell line (Fig. 4*A*, compare with Fig. 1*D*). Verapamil alone did not have an antiproliferative effect at the applied concentration

and the antiproliferative effect of patupilone was mainly independent of verapamil. Western blotting indeed confirmed that P-glycoprotein was overexpressed in SW480 tumor cells in comparison with the paclitaxel-sensitive *E1A/ras*-transformed MEFs (Fig. 4*B*). The verapamil-dependent antiproliferative effect of paclitaxel in the SW480 cells was also tested in combination with increasing doses of IR (Fig. 4*C*). A strong supra-additive inhibitory effect on proliferative activity was determined on combined treatment with paclitaxel (10 nmol/L) and IR (2 and 5 Gy, respectively) but only in cells which were pretreated with the multidrug resistance modulator verapamil.

The Radiosensitizing Effect of Patupilone Is Not Mediated by G_2-M Phase Accumulation. Both microtubule inhibitors and IR can modulate cell cycle progression. The cooperative effect of their combined treatment has been linked to the G_2-M cell cycle arrest induced by microtubule inhibitors. We therefore analyzed cell cycle alterations at multiple time points in response to the different treatment modalities and especially with regard to the low concentration of patupilone (0.25 nmol/L) used for the combined treatment regimen. Pretreatment of SW480 cells with patupilone at this low concentration did not alter the G_2-M cell cycle distribution over 34 hours (Fig. 5*A*) or 38 hours (Fig. 5*B*), as determined by bivariate distribution analysis of DNA synthesis (5-bromo-2′-deoxyuridine incorporation) versus DNA content (propidium iodide amount). Extended accumulation of cells in the G_2-M phase was only observed after treatment with patupilone above 10 nmol/L (data not shown). On the other hand, IR alone induced a significant G_2-M arrest at the 10-hour time point ($P = 0.001$, control versus IR) and was again decreasing at the 14-hour time point after irradiation. Surprisingly, combined treatment of patupilone and IR induced a lower accumulation of cells in G_2-M at the 10-hour time point in comparison with the IR-induced G_2-M arrest (Fig. 5*A*, $P = 0.001$, IR versus combined) which was not further increased at the 14-hour time point and still below the IR-induced G_2-M arrest (Fig. 5*B*).

A loss of cells in G_1 paralleled these G_2-M-related cell cycle alterations (not shown). Close examination of the S-phase cell cycle distribution indicated a tendency that patupilone treatment induces a transient accumulation of cells in this phase (Fig. 5*C* and *D*), which was significantly enhanced in response to combined treatment with IR (Fig. 5*C*, $P = 0.002$, control versus combined; *D*, $P = 0.003$) but was minor in response to IR alone (Fig. 5*C*, $P = 0.008$, IR versus combined; *D*, $P = 0.005$). Thus, the combined treatment effect of patupilone and IR as reported here is not due to a microtubule inhibitor–induced accumulation of cells in the radiosensitive G_2-M phase but rather due to an S-phase-related process. Analysis of a subG_1-population did not reveal any significant changes, suggesting that apoptosis is not involved in these p53-mutated, radiation-resistant adenocarcinoma cells in response to these treatment modalities (data not shown).

Radiosensitizing Effect of Patupilone on Tumor Xenografts. Based on the at least additive effects of combined

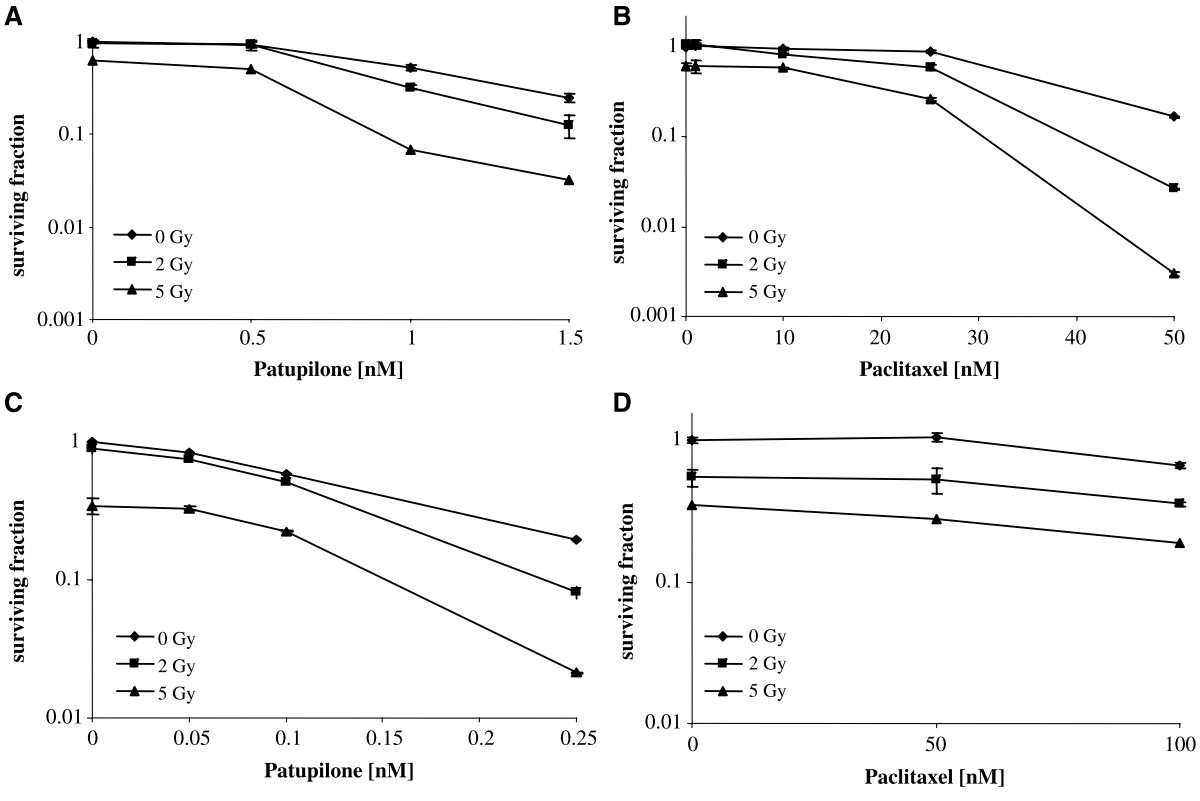

Fig. 3 Clonogenic survival of cells after treatment with either patupilone or paclitaxel in combination with IR. p53-/- *E1A/ras*-transformed MEFs (*A*, *B*) and SW480 cells (*C*, *D*) were treated with patupilone (*A*, *C*) or paclitaxel (*B*, *D*) alone and in combination with IR. Results of a representative experiment ($n = 2$).

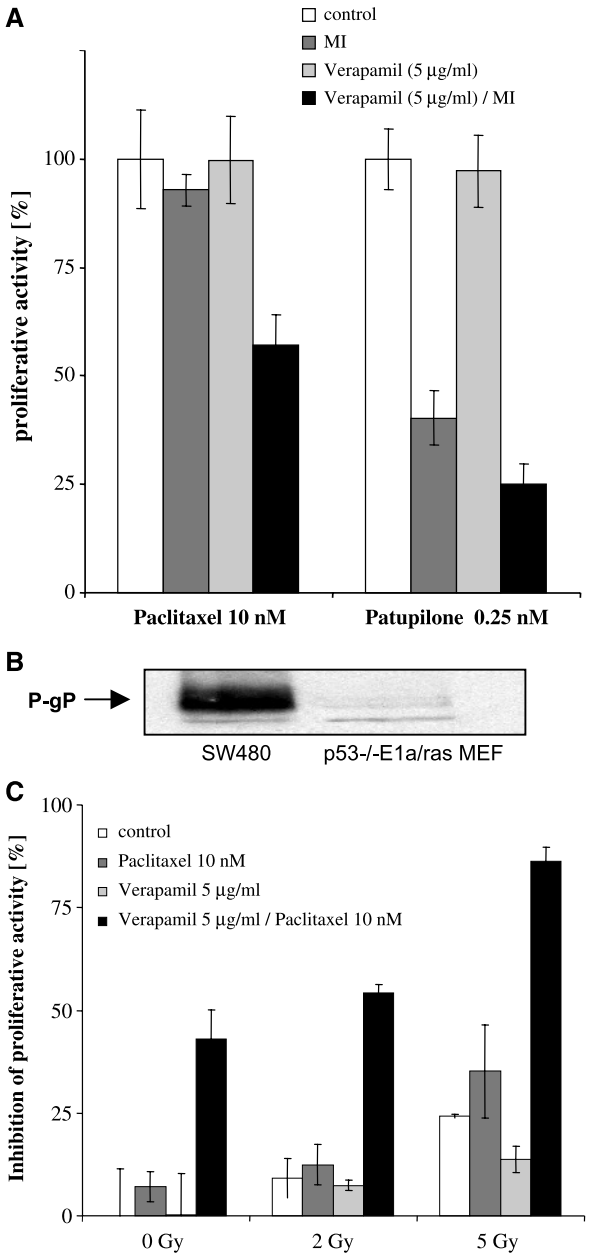

Fig. 4 Antiproliferative activity of paclitaxel after treatment with verapamil. *A,* SW480 cells were preincubated with verapamil (5 μg/mL) for 30 minutes and proliferative activity was determined 24 hours after treatment with the microtubule inhibitors (MI) paclitaxel or patupilone. *B,* P-glycoprotein expression was determined by Western blotting in the subcellular membrane fraction of SW480 cells and *E1A/ras*-transformed MEFs. *C,* SW480 cells were pretreated with verapamil as in *A* and irradiated 24 hours after paclitaxel treatment. Proliferative activity was determined 24 hours after treatment using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide–like Alamar Blue assay. The proliferative activity of untreated cells was set as 100%; inhibition of proliferative activity in untreated cells was set as 0%.

treatment *in vitro*, a combined treatment regimen with patupilone and IR was tested *in vivo* against paclitaxel-resistant tumors derived from human adenocarcinoma SW480 cells, s.c. injected into the back of nude mice. The solvent (polyethylene glycol-300/saline) for i.v. administration of patupilone, which is less toxic than the chremophore usually used for preclinical *in vivo* experiments with paclitaxel, was included as vehicle in control and IR-treated groups. Treatment was started when tumors reached a minimal size of 200 mm^3 ± 10% (day 12-17 after cell injection).

In vivo studies were done with locoregional application of IR using a shielding device and fractionated single doses of 3 Gy. This daily dose is applied when fractionated radiotherapy is used for the treatment of human malignancies. For practical reasons, only four fractions were chosen as treatment regimen, but the response to such a regimen has been previously found to be useful for treatment evaluation (32, 33). Figure 6 summarizes the effect of tumor treatment with patupilone alone (2 mg/kg once), IR alone (vehicle combined with 4 × 3 Gy), and in combination (2 mg/kg once combined with 4 × 3 Gy), in comparison with a vehicle alone–treated control group. Patupilone was applied 24 hours before the first of four fractions of irradiation applied on 4 consecutive days. Determination of treatment-related body weight changes only revealed a minor patupilone-dependent transient weight loss over 48 hours after patupilone application (<10%, data not shown) and no skin changes or tissue damage were observed in the co-irradiated healthy tissue area around the tumor during the follow-up period of tumor growth. Treatment with patupilone or IR alone resulted in a partial tumor growth suppression whereas combined treatment exerted a strong tumor growth control during treatment and the follow-up period (P = 0.0004, IR versus combined treatment). Overall these results show that patupilone might be a promising alternative in multidrug-resistant tumors for a combined treatment regimen using microtubule inhibitors and IR.

DISCUSSION

The search for novel microtubule inhibitors devoid of taxane-associated liabilites revealed multiple structurally distinct microtubule-stabilizing compounds, which are currently tested at the preclinical and clinical level as single agents or as part of a combined anticancer treatment modality (reviewed in ref. 34). Here we have examined the effect of the novel microtubule inhibitor patupilone alone and in combination with IR and determined at least additive antiproliferative and cytotoxic responses against p53-defective, radiation-resistant tumor cell lines. Importantly, patupilone at subnanomolar concentrations alone and in combination with IR retained a strong anticlonogenic effect against paclitaxel-resistant, P-glycoprotein-overexpressing human SW480 adenocarcinoma cells. Whereas treatment of SW480-derived xenografts in nude mice with patupilone or IR alone resulted in a partial tumor growth delay, a low-dose combined treatment regimen exerted a strong and significant tumor growth arrest.

Patupilone is currently tested in clinical phase I/II trials and already shows a broad antitumor activity. More importantly, patupilone is also able to induce responses in taxane-resistant cases (reviewed in ref. 34). Our own preclinical studies now show that patupilone is also able to sensitize taxane-resistant tumors to IR, and thus such a combined treatment modality qualifies for clinical trials in P-glycoprotein (*MDR1*)-overexpressing tumors. For example, our experiments were done with the human colon

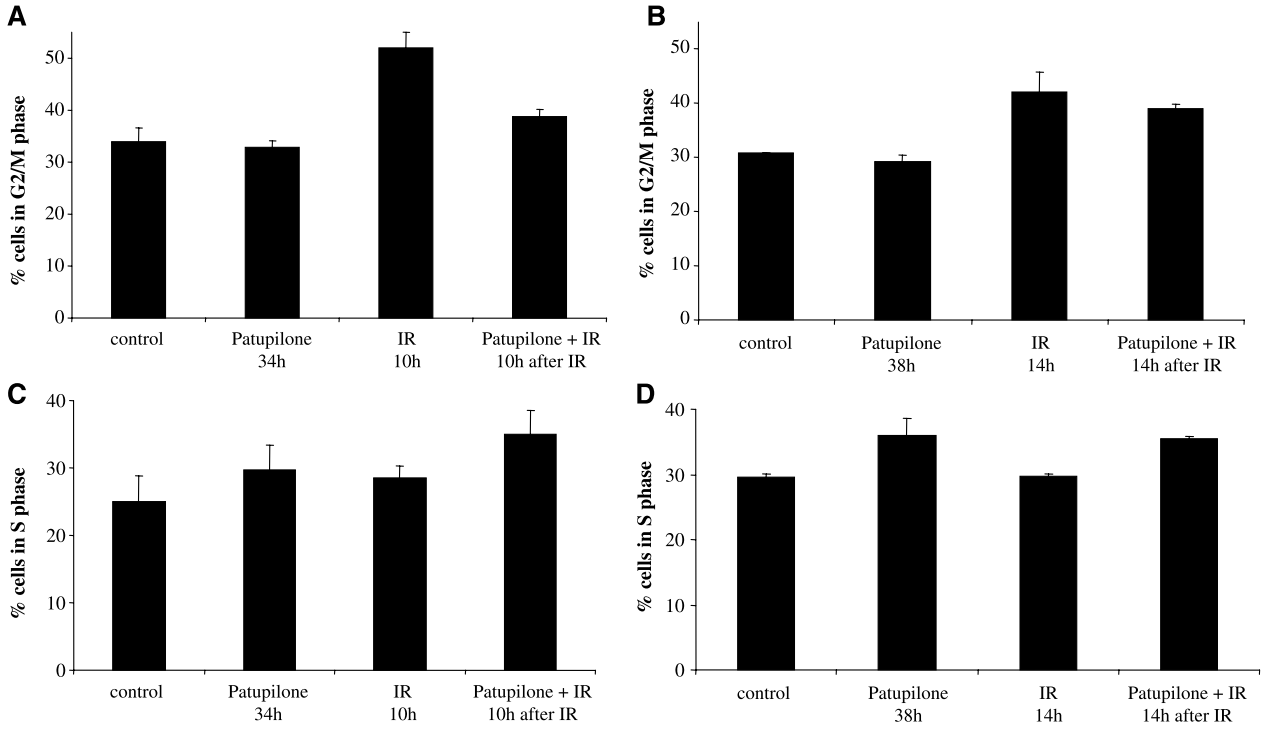

Fig. 5 Cell cycle distribution of SW480 cells after treatment with patupilone or IR, alone or in combination. SW480 cells were treated with patupilone (0.25 nmol/L), IR (5 Gy), alone or in combination, and cell cycle distribution was determined by bivariate flow cytometry. Columns, percentage of cells distributed in G_2-M phase (*A*, *B*) and S phase (*C*, *D*). Cells were preincubated with patupilone for 24 hours before irradiation and analyzed at the indicated time points. Data are representative of at least two independent experiments.

adenocarcinoma cell line SW480. A strong link exists between *MDR1* overexpression and tumorigenesis of colorectal cancer. Further, *MDR1* plays an important role in tumor progression in at least one proportion of colorectal cancer with high microsatellite instability (35, 36).

Microtubule inhibitors modulate microtubule stability and block the degradation and breakdown of the mitotic spindle apparatus which eventually leads to cell cycle arrest before completion of mitosis. Patupilone and paclitaxel induce this G_2-M arrest at high concentrations of 100 nmol/L and 1 μmol/L, respectively (2). Interestingly, in our experiments, we observed a radiosensitizing, supra-additive effect of patupilone with IR at concentrations of patupilone that did not lead to accumulation of cells into the radiosensitive G_2-M phase. As expected, treatment with IR alone resulted in an extended accumulation of SW480 cells in the G_2-M phase (37, 38). Detailed cell cycle analysis revealed that this effect was markedly reduced when cells were pretreated with a radiosensitizing concentration of 0.25 nmol/L patupilone. On the other hand, an enhanced accumulation of cells in S phase on combined treatment suggests that a decelerated S-phase transgression-related mechanism might be responsible for the supra-additive effect of patupilone in combination with IR. Thus, the radiosensitizing mechanism of microtubule inhibitors is not necessarily only related to a microtubule inhibitor–mediated G_2-M block as previously stated for paclitaxel (16). Interestingly, low concentrations of microtubule-stabilizing agents that are not associated with a G_2-M cell cycle block have been previously noted to cause cellular effects that may in part explain the radiosensitization reported here (e.g., low concentrations of epothilone B lead to aberrant mitosis and aneuploid cells in a manner that was associated with increased expression of stress genes and CD95; ref. 39).

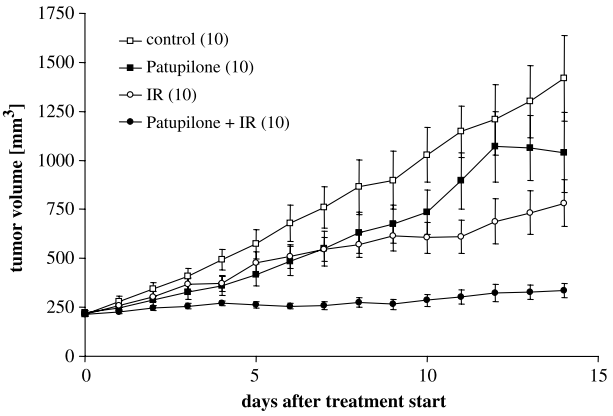

Fig. 6 The effect of patupilone and IR alone or in combination on the growth of SW480-derived xenografts in nude mice. Mice were treated with patupilone (2 mg/kg once) and IR (4 × 3 Gy, on 4 consecutive days), alone and in combination, with administration of patupilone or the vehicle 24 hours before the first fraction of IR. Points, mean (n = 10); bars, SE.

Multiple classes of agents exist that enhance IR-induced cell killing in S phase by different mechanisms. For example, 5-bromo-2′-deoxyuridine increases the amount of DNA damage and at the same time decreases the rate of DNA repair

(40). The clinically relevant nucleoside analogue gemcitabine (2′,2′-difluoro-2′-deoxycytidine) neither increases double-strand breaks nor decreases the rate of their repair (41, 42) but gemcitabine-mediated decrease in the dATP pool is relevant for S-phase-dependent radiosensitization (43, 44). Further genetic elements which activate an S-phase checkpoint, like enhanced expression of the transcription factor E2F-1, also enhance the cytotoxic effect of IR (45). Research on microtubules has mainly focused on its role during G_2-M and has strongly neglected other phases of the cell cycle thus far. Interestingly though, microtubule-associated proteins have been discovered which play a role in the cellular stress response to IR also in other stages of the cell cycle. For example, the microtubule-associated protein GTSE-1 is specifically expressed during S and G_2 phases of the cell cycle in response to DNA damage and controls stress-induced apoptosis (46). Eventually disturbance of microtubular integrity by microtubule inhibitors might affect the function of this DNA-damage response element or other microtubule-associated proteins. Own future studies will investigate the mechanism of low-dose patupilone radiosensitization during S phase and will in particular focus on the interference with the DNA repair machinery (47, 48).

To our knowledge, this is the first report investigating patupilone in combination with IR *in vitro* and *in vivo*. A previous report showed that the semisynthetic aza-epothilone derivative BMS-247550 is as potent as paclitaxel against human lung cancer cells *in vitro* and synergistically interacts with IR (49). The strong shift into the radiosensitive G_2-M cell cycle phase induced by BMS-247550 alone was proposed to be the mechanism for radiosensitization in the apoptosis-sensitive cell line evaluated. As outlined in this report, patupilone is much more potent than the taxane in both apoptosis- and radiation-resistant cell lines and reduces clonogenic survival in combination with IR independent of a G_2-M arrest *in vitro*. Although difficult to compare, combined treatment of IR with a single administration of patupilone *in vivo* seemed to result in a stronger tumor growth suppression than that reported for BMS-247550 (49).

Preclinical *in vivo* studies using patupilone as monotherapy (1×4 and 3×4 mg/kg) resulted in growth inhibition or tumor regression in several tumor models of lung, breast, colon, and prostate tumor xenografts including P-glycoprotein-overexpressing, paclitaxel-resistant KB-8511 tumor xenograft model (2). As determined in this report, combined treatment with a single, well-tolerated dose of patupilone followed by four fractionated administrations of IR exerted surprisingly strong tumor growth suppression in a colon adenocarcinoma model. Interestingly, patupilone is antiproliferative and induces apoptosis in microvascular and macrovascular endothelial cells also when applied in a low-dose treatment schedule (50). Especially in case of a single administration of patupilone the "metronomic" low-dose concentration remaining in the tumor circulation over time might still target the tumor vasculature. Thus, in parallel to its antitumor cell-directed effect, patupilone is also be considered antiangiogenic. Combined treatment of IR with inhibitors of angiogenesis is a promising strategy and has been tested with multiple antiangiogenic compounds (32, 51, 52), mainly directed against the vascular endothelial growth factor receptor–controlled endothelial cell signaling. Thus, IR as well as patupilone can target both the tumor tissue and the tumor vasculature and thus may cooperate on different tissue levels, which in turn may further enhance the overall antitumor effect of this combined modality.

Overall our *in vitro* and *in vivo* results suggest a multilayered synergistic response on the cellular and tumor tissue levels induced by IR and patupilone. The strong treatment response suggests the combination of patupilone and IR as a promising combined treatment modality especially in the treatment of multidrug-resistant paclitaxel-refractory tumor entities.

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