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Down-Regulation of DNA Topoisomerase IIα Leads to Prolonged Cell Cycle Transit in G_2 and Early M Phases and Increased Survival to Microtubule-Interacting Agents

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ABSTRACT

Microtubule binders are cell cycle-specific agents with preferential cytotoxicity toward mitotic cells. We have characterized vincristine-selected human leukemia cells to establish whether development of vincristine resistance was accompanied by changes in cell cycle kinetics and distribution. Our results indicate that vincristine resistance is accompanied by delayed G_2 transit and prolonged early mitosis in both the absence and the presence of the microtubule binder nocodazole. The altered G_2/M regulation is accompanied by resistance to short-term (12 h) but not continuous nocodazole exposure in agreement with the transient nature of the observed cell cycle alterations. Western blot analysis indicates that vincristine-selection is accompanied by down-regulation of topoisomerase IIα without detectable alterations of the other mitotic regulators studied, including Cdk1, p21, 14-3-3σ, and 14-3-3ϵ. This was associated with at least 7-fold less chromosome-associated topoisomerase IIα, decreased catalytic activity, and cross-resistance to topoisomerase II inhibitors. Characterization of isogenic cell lines expressing different levels of topoisomerase II proteins shows that cellular levels of topoisomerase IIα, but not the closely related topoisomerase IIβ, directly influence the cell cycle kinetics in G_2 and early mitosis as well as the resistance to nocodazole. These results underline the importance of topoisomerase IIα in late G_2 and early M phases and provide evidence for an as-yet-unsuspected interaction between topoisomerase II and microtubule-directed agents.

Microtubule interacting agents (also called tubulin binding agents or spindle poisons), such as vinca alkaloids and taxanes, comprise an important class of antineoplastic agents that act by interfering with tubulin dynamics during mitosis (for review, see Jordan and Wilson, 2004). Despite the efficacy of these agents toward both solid and hematological cancers, drug resistance to tubulin binding agents remains a serious clinical problem.

The different classes of microtubule-interacting agents share an important number of properties. They are natural products or semisynthetic compounds that bind to various sites on the β-tubulin subunit of the microtubules. Although tubulin binders act on microtubules throughout the entire cell cycle, cells are particularly vulnerable to this class of agents during the metaphase-to-anaphase transition, where a functional mitotic spindle is absolutely required for the proper positioning, separation, and migration of the mitotic chromosomes. Tubulin binders are able to induce at least three different types of cell cycle checkpoints: the prophase checkpoint (which delays chromosome condensation and metaphase entry), the mitotic spindle assembly/stability checkpoint (which delays the metaphase-to-anaphase transition), and a p53- and Rb-dependent G_1 tetraploidy checkpoint (which arrests polyploid cells that have exited mitosis without cytokinesis) (Trielli et al., 1996; Scolnick and Halazonetis, 2000; Meraldi et al., 2004).

One of the most widely reported mechanisms of resistance to tubulin binders is associated with overexpression of different ATP-binding cassette transporters such as P-glycoprotein/Mdr1 (ABCB1) and different MRP (ABCC) family mem-

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ABBREVIATIONS: ICRF-187, cardioxane; FITC, fluorescein isothiocyanate; DAPI, 4,6-diamidino-2-phenylindole; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ABC, ATP-binding cassette.

bers (for review, see Gottesman et al., 2002). Drug resistance has also been associated with microtubule-associated alterations, such as differential expression and/or mutations of β-tubulin isoforms or of microtubule-binding agents such as MAP4 (for reviews, see Drukman and Kavallaris, 2002; Orr et al., 2003). In contrast, the contribution of the different cell cycle checkpoints remains unclear, because loss of these checkpoints has been reported to provide either resistance (Wahl et al., 1996; Kasai et al., 2002) or increased sensitivity to microtubule inhibitors (Trielli et al., 1996; Scolnick and Halazonetis, 2000).

Gene expression analysis of human lung carcinoma cells showed that paclitaxel (Taxol) influenced the expression of several cell cycle regulators. In particular, after 24-h drug treatment, the cyclin-dependent kinase inhibitor $p21^{waf1/cip1/sdi1}$ (CDKN1A) was up-regulated, as was another Cdk1 modulator, 14-3-3σ. In contrast, Cdk1 (also known as cdc2 or $p34^{cdc2}$) and topoisomerase IIα were down-regulated (Chen et al., 2003). Similar findings were reported when the effect of paclitaxel was studied in vivo on ovarian carcinoma xenografts. It is interesting that topoisomerase IIα was consistently down-regulated, whereas p21 was up-regulated in the paclitaxel-responsive tumors (Bani et al., 2004). Proteome analysis of vinca alkaloid-sensitive and -resistant leukemia cells identified cell cycle regulators altered in both the response and in the resistance to vinca alkaloids (Verrills et al., 2003). These included 14-3-3ϵ, a protein reported to modulate the cellular effects of both Cdc25C phosphatase (Kumagai et al., 1998) and topoisomerase IIα (Kurz et al., 2000). It is noteworthy that most of these genes are known to play important roles in maintaining G_2 arrest in response to under-replicated or damaged DNA (Chan et al., 2000; Larsen et al., 2003). We speculated that the repression of proteins needed for the G_2-to-M transition could be a distinct mechanism of maintaining G_2 arrest, thereby preventing cells from entering mitosis where microtubule-interacting agents are most cytotoxic.

We report herein that down-regulation of topoisomerase IIα in both vincristine-resistant human leukemia cells and in untreated isogenic cell lines expressing different levels of topoisomerase II proteins is accompanied by delayed cell cycle progression during G_2 and early mitosis and increased resistance to the tubulin-interacting agent nocodazole. These results underline the importance of topoisomerase IIα in late G_2 and early M phases and provide evidence for an as-yet-unsuspected interaction between topoisomerase II and microtubule-directed agents.

Materials and Methods

Chemicals. Vincristine was obtained from Laboratoire Pierre Fabre (Castres, France) and nocodazole was purchased from Sigma (Saint-Quentin Fallavier, France). Symadex (compound C-1311) and amsacrine were synthesized by Dr. Marek Konieczny (Gdansk University of Technology, Gdansk, Poland). Mitoxantrone was a gift from Laboratoire Lederle (Rungis, France) and ICRF-187 (Cardioxane) was purchased from Chiron BV (Amsterdam, The Netherlands). [^{3}H]Thymidine (90 Ci/mmol), [^{14}C]thymidine (52 mCi/mmol), and [^{14}C]leucine (300 mCi/mmol) were obtained from Amersham Biosciences AB (Uppsala, Sweden).

DNA Substrates. Highly catenated kinetoplast DNA (form I) was purified from *Trypanosoma leishmania* (kindly provided by Prof. Jean-Pierre Dedet, Université de Montpellier I, Montpellier, France) and purified by cesium chloride/ethidium bromide density centrifugation.

Antibodies. Monoclonal antibodies directed toward topoisomerase IIα were obtained from Calbiochem (La Jolla, CA), monoclonal antibodies directed toward topoisomerase IIβ were purchased from BioTrend Chemikalien GmbH (Köln, Germany), whereas monoclonal mouse anti-Cdk1/Cdc2, polyclonal rabbit anti-p21, polyclonal goat anti-14-3-3ϵ, polyclonal goat anti-14-3-3σ, and polyclonal goat anti-actin antibodies were provided by Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies directed toward phosphotyrosine were obtained from Upstate Biotechnology (Lake Placid, NY). All secondary horseradish-conjugated antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA) and FITC-conjugated anti-mouse IgG antibodies were provided by Amersham Biosciences (Little Chalfont, Buckinghamshire, UK).

Cell Lines. Parental HL-60 cells and vincristine-selected multidrug-resistant HL-60 cells (HL60/Vinc) (McGrath et al., 1989) were a kind gift from Dr. Melvin Center (Kansas State University, Manhattan, KA). CEM and vincristine-selected CEM/VCR1000 cells (Kimmig et al., 1990) were generously provided by Dr. Johann Hofmann (University of Innsbruck, Innsbruck, Austria). For all experiments, HL-60/Vinc and CEM/VCR cells were reselected with 1 μM vincristine for 3 days followed by incubation in drug-free media for 1 to 4 weeks. This dose of vincristine is nontoxic to the resistant cell lines but lethal to the parental ones. All cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2/95% air atmosphere.

The DC-3F fibrosarcoma cell line, its 9-hydroxyellipticine-selected subline DC-3F/9-OH-E, and DC-3F/9-OH-E cells transfected with human topoisomerase IIα or human topoisomerase IIβ have been extensively characterized (Larsen and Jacquemin-Sablon, 1989; Dereuddre et al., 1997; Khelifa et al., 1999). The cells were maintained in minimal essential medium supplemented with 8% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin and grown at 37°C in a 5% CO_2/95% air atmosphere. All cells were screened routinely for *Mycoplasma* spp. by the DNA hybridization method (Gen-Probe, Inc., San Diego, CA).

Cell Cycle Analysis. All measurements were made using an epics Profile II flow cytometer (Beckman Coulter, Inc., Fullerton, CA) equipped with an argon laser to give 488 nm light. For cell cycle distribution studies, cells were fixed in 70% ethanol, rehydrated in PBS, and stained in PBS containing propidium iodide (20 μg/ml) and ribonuclease A (100 μg/ml) for 30 min at room temperature. Data from 10^4 cells were collected and analyzed by Multicycle software (Phoenix Flow Systems, San Diego, CA). Values are given as the mean of four independent experiments, each done in duplicate.

Mitotic Index. Exponentially growing nontreated cells (2×10^6) were collected, swelled in 75 mM KCl for 10 min at 4°C, and centrifuged for 5 min at 1000 rpm. Cells were fixed three times in 5 ml of freshly prepared Carnoy's fixative [methanol/acetic acid, 3:1 (v/v)] for 15 min at room temperature, spotted onto microscopic slides and stained with 0.1 μg/ml DAPI. At least 500 cells in 10 different microscopic fields were counted under 300× magnification using an epifluorescence microscope (BX60; Olympus, Tokyo, Japan) . Mitotic cells were photographed with a digital camera (DP50; Olympus). Values are given as the means of two independent experiments.

MPM-2 Staining. For indirect immunofluorescence staining, cells were fixed with 70% ethanol at −20°C overnight, rehydrated in ice-cold PBS for 10 min, and permeabilized in 0.25% Triton X-100/PBS for 5 min on ice. Cells were then washed with PBS and blocked in 1% BSA/PBS for 15 min at room temperature. After washing with PBS, cells were incubated for 1 h at room temperature with monoclonal mouse anti-MPM-2 (DakoCytomation Denmark A/S, Glostrup, Denmark) antibodies diluted at 1:100 in 0.5% BSA/PBS. Samples were washed with 0.2% Tween 20 and 0.5% BSA/PBS and incubated with anti-mouse IgG-FITC (Amersham Biosciences) for 1 h at room temperature, counterstained with 5 μg/ml propidium iodide and

analyzed by flow cytometry as described previously (Skladanowski and Larsen, 1997).

Quantitation of DNA Fragmentation by the Filter-Binding Assay. Cells were labeled with [^{14}C]thymidine (0.05 μCi/ml, 48 h) and chased in radiolabel-free medium for 6 h. After drug exposure, approximately 5×10^5 cells suspended in 5 ml of ice-cold PBS were applied onto Matricel filters (Gelman Science, Ann Arbor, MI) and washed twice with 5 ml of PBS. DNA fragmentation was determined by the filter binding assay.

Viability and Growth Inhibition. The viability of nocodazole-treated human leukemia cells was determined by the propidium iodide exclusion assay. In brief, propidium iodide was added directly to the medium (5 μg/ml final concentration) of drug-treated cells and the cell suspension was immediately analyzed by flow cytometry. Otherwise, cells were exposed to nocodazole for 12 h and postincubated in drug-free medium for an additional 60 h, and viability was determined by the MTT assay.

Exponentially growing leukemia cells were exposed to different drug concentrations for 1 h followed by postincubation for an additional 72 h in drug-free media. After postincubation, cells were counted with a Coulter counter (Beckman Coulter, Fullerton, CA), and the growth inhibition was determined as described earlier (Skladanowski et al., 1996). Values are given as the mean of three independent experiments, each done in duplicate.

Survival of the DC-3F parental and ellipticine-resistant fibrosarcoma cell lines was determined after 24-h drug exposure followed by a colony formation assay as described previously (Dereuddre et al., 1997). In brief, exponentially growing cells were plated into 60-mm Petri dishes (approximately 250 cells per dish) for 18 to 20 h at 37°C before the drug treatment. After 24-h drug exposure (each dose in triplicate), cells were washed with drug-free medium and postincubated for 5 to 7 days. Colonies were washed with 0.9% saline, fixed with ethanol, and stained with Giemsa stain. Colonies containing at least 50 cells were counted, and growth curves were plotted as survival fractions (number of colonies in drug-treated samples to controls) versus drug dose.

Western Blot Analysis. Exponentially growing cells (5×10^6 cells) were washed twice with PBS, resuspended in 200 μl of 0.9% saline with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A), and mixed with 200 μl of double-strength Laemmli buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromphenol blue, and 20% glycerol). Cell lysates were immediately denatured for 10 min at 95°C. Cell lysates (25 μl) were loaded onto 7.5% polyacrylamide-SDS gels, the proteins were separated by polyacrylamide gel-SDS electrophoresis and transferred to polyvinylidene difluoride filters in 0.192 M glycine, 0.025 M Tris, and 20% methanol for 4 h at 4°C. Membranes were saturated overnight at 4°C in TBS, pH 7.4, containing 5% nonfat milk and probed for 3 h with primary antibodies directed toward the topoisomerase IIα isoform (1:200 dilution) or the topoisomerase IIβ isoform (1:2000 dilution) at room temperature. After three 15-min washes in TBS/0.05% Tween 20, the blots were probed for 1 h at room temperature with a 1:10,000 dilution of peroxidase-conjugated secondary antibodies. After three washes in TBS/Tween 20, antibody binding was detected by enhanced chemiluminescence (Amersham Life Sciences, Amersham, UK) and quantitated by densitometric analysis using MicroImage Gel Pro software (Media Cybernetics, Silver Spring, MD). All experiments were done with at least three different cellular lysates. Equal loading of proteins was verified by probing dehybridized membranes for 1 h with polyclonal anti-actin antibodies (at 1:1000) followed by donkey anti-goat IgG (Jackson ImmunoResearch Labs) and detection by enhanced chemiluminescence.

Immunolocalization of DNA Topoisomerase IIα on Mitotic Chromosomes. The fraction of mitotic cells was enriched by nocodazole exposure (25 ng/ml) for 12 h. Approximately 5×10^6 cells were collected, swelled in 75 mM KCl for 10 min at 4°C, and centrifuged for 5 min at 1000 rpm. Cells were fixed three times in 5 ml of freshly prepared Carnoy's fixative [methanol/acetic acid, 3:1 (v/v)] for 15 min at room temperature, spotted onto microscopic slides, and air-dried. Chromosome spreads were then rehydrated in blocking solution (1% BSA in PBS) for 30 min at room temperature, and incubated for 1 h with anti-topoisomerase IIα-directed antibodies (Ab-1; Calbiochem, San Diego, CA) diluted in 0.5% BSA/PBS at 1:50. After three washes in PBS, chromosomes were incubated with anti-mouse FITC-conjugated antibodies (Amersham) diluted at 1:50. After three additional washes in PBS, chromosome preparations were air-dried and mounted in VectaShield solution (Vector Laboratories, Burlington, CA). All preparations were examined with an Axiovert 100M epifluorescence microscope equipped with appropriate filters using PlanApochromat 100× objective (Zeiss, Jena, Germany) and all data acquisition and image analysis were carried out by a LSM510 laser confocal scanning system (Zeiss).

Decatenation Assay. Nuclei were isolated from exponentially growing cells as described above, and the nuclei were extracted with 0.35 M NaCl for 30 min at 4°C (Lelievre et al., 1995). The nuclei were centrifuged at 11,000 rpm, and the supernatants were collected and frozen immediately after. Nuclear extracts were stored at −80°C and analyzed within 4 days. For decatenation assays, the reaction mixture contained 50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 100 mM KCl, 0.5 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM ATP, and 200 ng of kinetoplast DNA as described previously (Escargueil et al., 2001). The reaction was initiated by the addition of different concentrations of nuclear extract and was allowed to proceed at 37°C for the indicated times. Reactions were stopped by addition of 1% SDS, 0.5% bromphenol blue, and 30% glycerol. The samples were subjected to electrophoresis in 1.2% agarose gels at 5 V/cm for 6 h in Tris/borate/EDTA buffer, pH 8.3. Liberated minicircles were quantitated by densitometric scanning using a Chromoscan 3 densitometer (Joyce-Loebl and Co., Gateshead, UK). Decatenation assays were carried out with three different nuclear extract preparations.

Statistical Analysis. Comparisons of the cell cycle distribution between parental and drug-resistant cell lines were calculated by the Student's *t* test using the SigmaPlot program.

Results

Vincristine Resistance Is Accompanied by Altered Cell Cycle Distribution in G_2 and M. Cell cycle kinetics and distribution was determined for two different vincristine-resistant human leukemia cell lines, HL-60/Vinc and CEM/VCR1000, as well as for the corresponding parental cell lines. Both resistant cell lines are multidrug-resistant and have been reported to overexpress functional P-glycoprotein (McGrath et al., 1989; Kimmig et al., 1990). Comparison of untreated parental and resistant cell lines revealed no differences with respect to the cell doubling times, which corresponded to 23 h for HL-60 and HL-60/Vinc cells and 25 h for CEM and CEM/VCR1000 cells (data not shown). Likewise, the fraction of cells in the S phase of the cell cycle was comparable between parental and vincristine-resistant cells, with 44.9 ± 3.2% and 44.8 ± 3.8% for HL-60 and HL-60/Vinc, respectively, and 49.5 ± 4.7% and 46.7 ± 5.7% for CEM and CEM/VCR1000, respectively (Fig. 1A).

In contrast, the percentage of cells in the G_2/M phase of the cell cycle was enhanced in both vincristine-resistant cell lines with 9.0 ± 1.2% compared with 11.3 ± 1.2% for HL-60 and HL-60/Vinc, respectively, and 8.3 ± 0.7% versus 11.9 ± 1.2% for CEM and CEM/VCR1000, respectively. To establish whether the increased G_2/M fraction was due to an increased number of mitotic cells, the mitotic index (which principally represents metaphase cells but does not include prophase cells) was determined (Fig. 1B). We were surprised to find

that the mitotic indices of both resistant cell lines were only approximately half that observed for the sensitive parental cells; the mitotic index was 4.5 ± 1.4% for HL-60 cells compared with 2.2 ± 0.6% for HL-60/Vinc cells and 4.0 ± 0.8% for CEM cells versus 2.2 ± 0.9% for CEM/VCR1000 cells. These differences were statistically significant ($p < 0.01$). Because the cell doubling times were the same for parental and vincristine-selected cells, these results suggested that vincristine resistance was accompanied by an increased fraction of cells in G_2 and/or early mitosis.

Parental and Vincristine-Resistant Human Leukemia Cells Differ in the Temporal Response to Nocodazole. We next wished to establish whether the differences in cell cycle distribution observed for untreated parental and vincristine-resistant cells were maintained when the cells were treated with microtubule-interacting agents. The vincristine resistance of both HL-60/Vinc and CEM/VCR cells is associated with overexpression of P-glycoprotein/Mdr1 (ABCB1), a membrane-associated drug efflux pump able to transport most microtubule-interacting agents except for nocodazole. We therefore selected nocodazole for all following studies to exclude any influence of drug uptake and intracellular drug distribution on the results. As illustrated for HL-60 and HL-60/Vinc cells, continuous exposure to nocodazole (25 ng/ml) was accompanied by accumulation of more than 60% of the cell population in the G_2/M phase of the cell cycle (Fig. 2A). The fraction of parental HL-60 cells in G_2/M was maximal after 12-h drug exposure and then started to decrease. In comparison, the fraction of HL-60/Vinc cells with a G_2/M content remained elevated for an additional 8 h and started to decrease only after 20-h nocodazole exposure. To further confirm these findings, the fraction of MPM-2–positive cells was determined (Fig. 2*B*). The MPM-2 antibody specifically recognizes phosphoepitopes present in mitotic cells, particularly during the early phases of mitosis (Escargueil et al., 2000). In the presence of nocodazole, cellular levels of the MPM-2 epitope diminished after 9-h drug exposure in the parental HL-60 cells but were maintained for at least 16 h in the vincristine-resistant HL-60/Vinc cells. The differences in the fraction of MPM-2 positive cells between the two cell lines are statistically different ($p < 0.001$) by 12, 16, and 20 h.

Transient cell cycle arrest during G_2/M can be followed by either resumption of the cell cycle or by induction of apoptotic cell death. The presence of cells with apoptotic features suggested that the decrease of G_2/M cells was accompanied by apoptosis (results not shown). Apoptotic DNA fragmentation was assessed by a DNA filter binding assay (Fig. 2C). The results show that after 12-h nocodazole exposure, an increasing fraction of the total cellular DNA of the parental HL-60 cells is present as small fragments to reach 92.4 ± 4.6% of the total cellular DNA by 24 h. The fragmentation of cellular

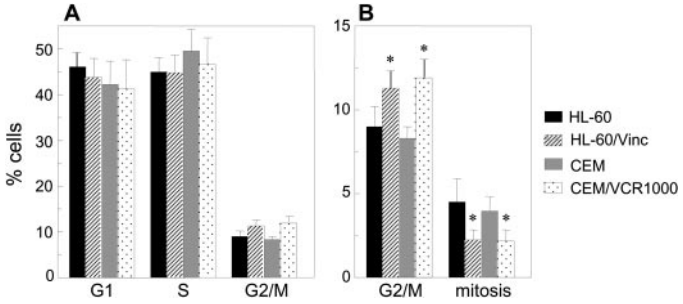

Fig. 1. Cell cycle distribution in parental and vincristine-resistant cells. A, the cell cycle distribution in parental and vincristine-resistant cells was determined by flow cytometry analysis. The values are means of at least three independent experiments (bars, S.D.). B, the G_2/M fraction of parental and vincristine-resistant cells was determined by flow cytometry as described in A, whereas the mitotic index was established after fixation as described under *Materials and Methods*. *, values obtained for parental and resistant cells are statistically significantly different ($p < 0.01$).

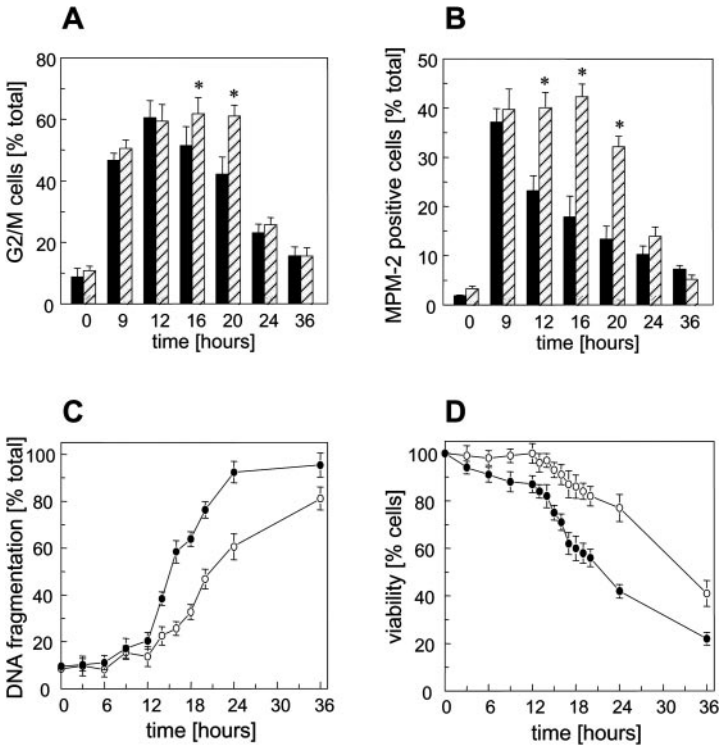

Fig. 2. Flow cytometry of parental and vincristine-resistant HL-60 cells in the presence of nocodazole. Parental (■, ●) and vincristine-resistant HL-60 cells (▨, ○) were incubated in the presence of nocodazole (25 ng/ml) for the indicated times. A, the G_2/M fraction of nocodazole-treated cells was determined by flow cytometry analysis. B, the fraction of MPM-2–positive cells was quantified by immunostaining with a MPM-2–directed antibody followed by flow cytometry. C, apoptotic DNA fragmentation was determined by a filter binding assay as described under *Materials and Methods*. D, the fraction of viable cells was determined by a propidium iodide exclusion assay. All values are means of at least two independent experiments, each done in duplicate (bars, S.D.). *, values obtained for parental and resistant cells are statistically significantly different ($p < 0.001$).

DNA was much slower for the vincristine-resistant HL-60/Vinc cells with 60.4 ± 5.6% DNA fragmentation by 24 h. For both cell lines, DNA fragmentation was followed by loss of membrane integrity and cell death as shown by a propidium iodide permeability assay (Fig. 2D). It is noteworthy that the occurrence of cell death was systematically delayed in the vincristine-resistant cells compared with the parental cells and was statistically significantly different ($p < 0.001$) for all time points from 12 h onward.

Prolonged G_2/M Transit in Vincristine-Resistant Cells Is Accompanied by Nocodazole Resistance. Transient inhibition of cell cycle progression in G_2/M is believed to provide additional time for repair of damaged DNA and other cellular lesions before chromosome separation and cell division. To determine whether the prolonged G_2/M transit in vincristine-resistant cells provided these cells with a survival advantage, parental and resistant cells were treated with different concentrations of nocodazole for 12 h, followed by postincubation in drug-free medium for 72 h and determination of cell viability by the MTT assay. The results (Fig. 3, A and B) show marked differences between parental and vincristine-resistant cells for all doses associated with at least 20% cell death. For example, at 5 μg/ml nocodazole, the viability of HL-60 cells was 16.4 ± 3.4% compared with 69.4 ± 2.7% for HL-60/Vinc cells, whereas the fraction of viable CEM cells was 9.8 ± 2.9% compared with 55.7 ± 3.2% for CEM/VCR cells. These differences are statistically significantly different ($p < 0.001$). In marked contrast, parental and vincristine-resistant cells were equally sensitive to continuous nocodazole exposure, with IC_{50} values of 9.6 ± 1.2 ng/ml for HL-60 compared with 9.4 ± 1.8 ng/ml for HL-60/Vinc and 21.9 ± 2.4 ng/ml for CEM compared with 20.5 ± 2.8 ng/ml for CEM/VCR. Therefore, the prolonged G_2 transit provided the vincristine-resistant cell lines with a time-dependent survival advantage in the presence of nocodazole in agreement with the transient nature of the observed cell cycle alterations.

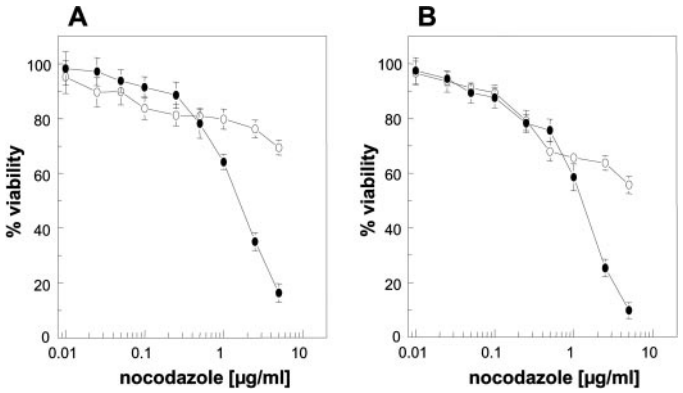

Fig. 3. Viability of nocodazole-treated parental and vincristine-resistant cells. Parental and vincristine-resistant cells were treated with the indicated concentrations of nocodazole for 12 h followed by postincubation in drug-free media for 72 h and determination of viable cells by the MTT assay. A, viability of HL-60 (●) and HL-60/Vinc cells (○). B, viability of CEM (●) and CEM/VCR cells (○). All values are means of at least two independent experiments, each done in duplicate (bars, S.D.).

Topoisomerase IIα Is Down-Regulated in Vincristine-Resistant Cells. To elucidate the molecular basis for the prolonged G_2 transit, the expression of important mitotic regulators was determined by Western blot analysis of parental and vincristine-resistant cells (Fig. 4). The proteins examined included $p34^{cdc2}$, the catalytic subunit of Cdk1, the cyclin kinase inhibitor $p21^{cip1/waf1/sdi1}$ and DNA topoisomerase IIα. In addition, we determined the expression of 14-3-3ε and 14-3-3σ, which are able to inhibit the activities of topoisomerase IIα and the Cdc25C protein phosphatase, respectively. The results show no clear differences for the expression of Cdk1, p21, or the two 14-3-3 proteins (Fig. 4). In addition, the relative distribution between the fast-migrating hypophosphorylated, catalytically active form of Cdk1 and the slow-migrating, catalytically inactive tyrosine-phosphorylated form of Cdk1 kinase was comparable for parental and vincristine-selected cell lines. We were surprised to find that topoisomerase IIα was down-regulated to approximately 33% of the initial levels in both vincristine-resistant cell lines. This was not the case for the closely related β isoform of topoisomerase II that has no known mitotic functions.

The Association of Topoisomerase IIα with Mitotic Chromosomes Is Decreased in Vincristine-Resistant Cells. We next wanted to establish whether the decreased expression of topoisomerase IIα in the vincristine-resistant

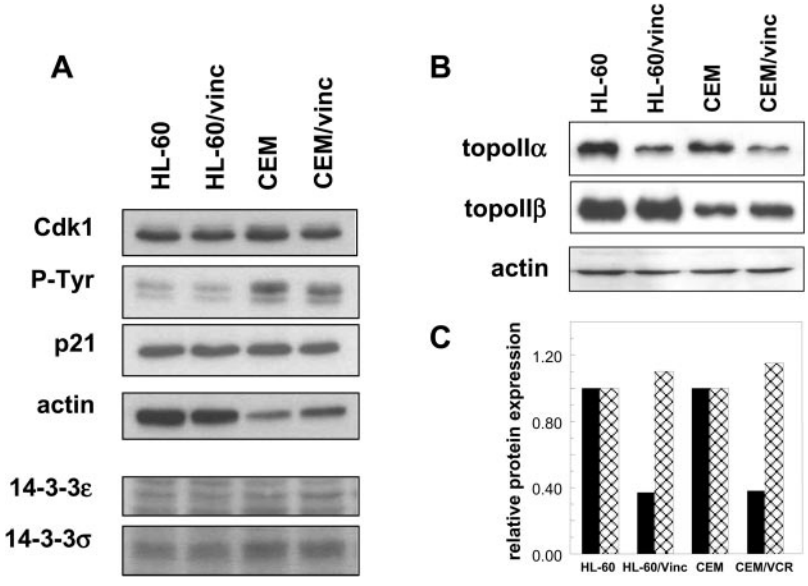

Fig. 4. Expression of Cdk1, phosphotyrosine, p21, 14-3-3ε, 14-3-3σ, topoisomerase IIα, and topoisomerase IIβ proteins in parental and vincristine-resistant cell lines. Cellular lysates were separated on PAGE-SDS gels and blotted onto polyvinylidene difluoride membranes. Immunoblotting was performed with the indicated antibodies (A and B). As an internal standard for equal loading, all membranes were rehybridized with antibodies directed against β actin. vinc, vincristine. C, differences in cellular levels of topoisomerase IIα and β as determined by densitometric analysis of the blots shown in B. VCR, vincristine.

cells influenced the association of the enzyme with mitotic chromosomes. Chromosome spreads of sensitive and vincristine-resistant cells were immunolabeled with a topoisomerase IIα-directed antibody and examined by confocal fluorescence microscopy (Fig. 5). Topoisomerase IIα localized to the chromosome arms of parental cells (Fig. 5A) with a clear concentration in centromeric regions apparent in certain images (Fig. 5C). The association of topoisomerase IIα with mitotic chromosomes of the vincristine-resistant cells was strongly reduced (Fig. 5B), although the overall labeling pattern appeared similar to that of the parental HL-60 cells. Scanning of fluorescence intensities of comparably sized chromosomes (Fig. 5, top, indicated with a line) on nonmodified images showed at least 7-fold difference in the levels of chromosome-associated topoisomerase IIα between sensitive and resistant cells (Fig. 5, bottom).

The Catalytic Activity of Topoisomerase II Is Reduced in Vincristine-Resistant Cells. The decatenation activities of nuclear extracts from parental and vincristine-resistant cells were determined to establish whether the different expression of topoisomerase IIα protein was accompanied by differences in the catalytic activity (Fig. 6). The amount of nuclear extracts needed to decatenate 50% of the kinetoplast DNA corresponded to 23 μg for the vincristine-resistant cells and 8 μg for the parental cells (Fig. 6A). The differences in catalytic activity were further confirmed by kinetic analysis that revealed that extracts from parental cells are 3- to 4-fold more active than extracts from vincristine-resistant cells (Fig. 6B). Thus, the down-regulation of topoisomerase IIα in vincristine-resistant cells was accompanied by decreased catalytic activity.

Vincristine-Resistant Cells Are Cross-Resistant to Topoisomerase Ii Inhibitors. The cytotoxic activity of different topoisomerase II inhibitors was determined to establish whether the reduced levels of topoisomerase IIα also influenced the sensitivity to topoisomerase II inhibitors. Because most topoisomerase II inhibitors are recognized by the P-glycoprotein, we selected topoisomerase II inhibitors that are either poor substrates for the P-glycoprotein (amsacrine, mitoxantrone) or, in the case of Symadex (C-1311), not recognized at all (Skladanowski et al., 1996). The results showed that vincristine-resistant cells were 4- to 20-fold cross-resistant to the tested topoisomerase II inhibitors compared with the parental cells (Table 1). It is interesting that the vincristine-resistant cells were also cross-resistant to ICRF-187, a topoisomerase II inhibitor that stabilizes noncovalent, rather than covalent complexes between DNA and topoisomerase II (Roca et al., 1994). Therefore, down-regulation of topoisomerase IIα in the vincristine-selected cells was accompanied by cross-resistance to different functional classes of topoisomerase II inhibitors.

Topoisomerase IIα Modulates the Cell Cycle Distribution in G_2 and M. Our studies revealed that vincristine-selection of human leukemia cells was accompanied by altered cell cycle progression in G_2 and M and by decreased expression of topoisomerase IIα. To establish whether cellular topoisomerase IIα levels have a direct influence on the cell cycle distribution, the cell cycle distribution was studied in isogenic cell lines expressing different levels of topoisomerase II protein. Previous results show that ellipticine selection of mammalian DC-3F cells was accompanied by different phenotypic changes including 4- to 5-fold down-regulation of topoisomerase IIα and complete loss of topoisomerase IIβ (Larsen and Jacquemin-Sablon, 1989; Khelifa et al., 1999). Transfection of the ellipticine-resistant cells with either topoisomerase IIα or IIβ was accompanied by expression of the corresponding proteins and partial restoration of the sensitivity to topoisomerase II inhibitors (Dereuddre et al., 1997; Khelifa et al., 1999). For comparison, we selected transfected clones that expressed topoisomerase IIα or IIβ at levels comparable with the parental DC-3F cells (Dereuddre et al., 1997; Khelifa et al., 1999).

The results showed that ellipticine resistance was accompanied by an increased fraction of cells in G_2/M (Fig. 7A) and a reduced mitotic index (Fig. 7B). Transfection of the DC-3F/9-OH cells with topoisomerase IIα was accompanied by a decreased G_2/M fraction and an increased mitotic index. In contrast, transfection with topoisomerase IIβ had only minor influence on the cell cycle distribution and the fraction of mitotic cells.

The mitotic index is principally a reflection of the number of metaphase cells and does not include prophase cells according to classic criteria. Prophase and metaphase cells can be distinguished by morphological features such as chromo-

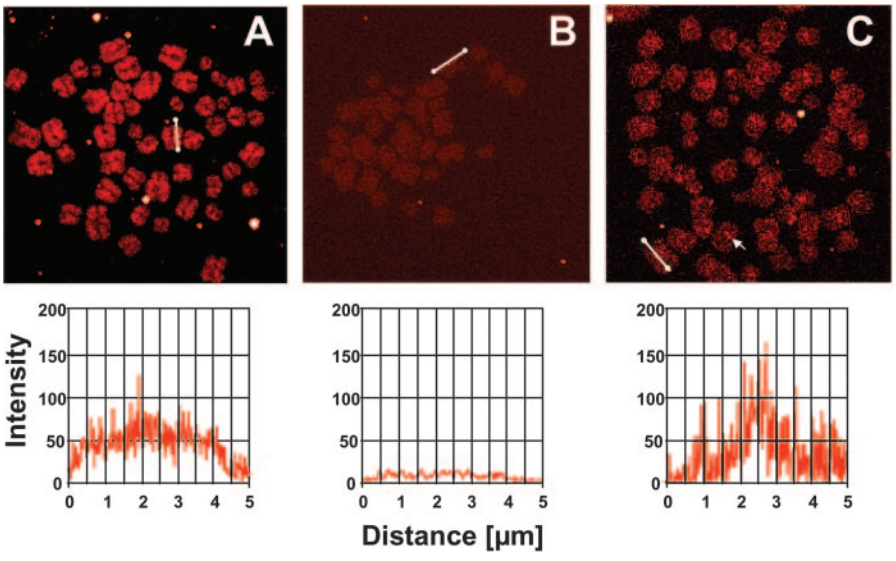

Fig. 5. The localization of topoisomerase IIα on mitotic chromosomes from parental and vincristine-resistant cells. Top, chromosomes from parental (A and C) and vincristine-resistant (B) HL-60 cells. Chromosome spreads were incubated with antibodies directed against topoisomerase IIα followed by FITC-labeled secondary antibodies and analyzed by confocal fluorescence microscopy. A and B represent total fluorescence signals, whereas C shows a section of the central part of the chromosomes. In B, the picture was processed by Adobe software to enhance the weak fluorescence signal and to permit visualization of the chromosomal topoisomerase IIα. Bottom, nonmodified images of comparable-sized chromosomes were scanned for fluorescence intensity (in pixels) along the lines indicated at the top.

some orientation, the degree of chromatin condensation, and the presence of a nucleolar remnant as illustrated in Fig. 8A. Determination of prophase and metaphase cells (Fig. 8B) showed a very modest fraction of prophase cells (0.32 ± 0.08%) in the parental cells compared with the fraction of metaphase cells (5.03 ± 0.7%). In contrast, the number of prophase cells (1.25 ± 0.3%) was much higher in the ellipticine-resistant cells, both in relative and absolute terms, because the fraction of metaphase cells was only 1.67 ± 0.26%. Transfection of the ellipticine-resistant cells with topoisomerase IIβ had marginal influence on the proportion of prophase and metaphase cells and corresponded to 1.32 ± 0.25% and 1.94 ± 0.3%, respectively. In marked contrast, transfection with topoisomerase IIα completely altered the proportion of prophase and metaphase cells to 0.26 ± 0.07% and 4.85 ± 0.6%, respectively. These values were comparable with those observed for the sensitive parental cells. Taken together, these experiments strongly suggest that the expression of topoisomerase IIα, but not topoisomerase IIβ, directly influences the cell cycle progression in G_2 and early mitosis.

To establish whether topoisomerase II protein levels directly influence the cellular sensitivity to microtubule interacting agents, the isogenic ellipticine-resistant cells were exposed to nocodazole for 24 h followed by postincubation in drug-free media and the cytotoxicity was determined by colony formation (Fig. 9). The results show that the expression of topoisomerase IIα plays an important role in the sensitivity to nocodazole with IC_{50} values corresponding to 0.09 μg/ml for the topoisomerase IIα transfectants compared with 0.30 μg/ml for the ellipticine-resistant parental cells. These changes are statistically significantly different ($p < 0.001$). In contrast, no significant differences were observed between the topoisomerase IIβ transfectants and the ellipticine-resistant parental cells.

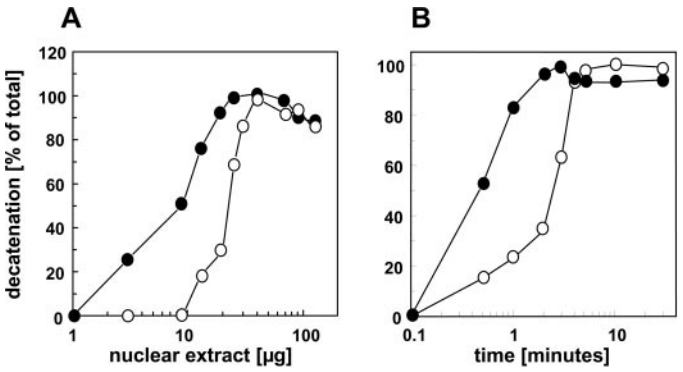

Fig. 6. Catalytic activity of topoisomerase II in nuclear extracts from parental and vincristine-resistant cells. Highly catenated kinetoplast DNA was incubated with nuclear extracts from HL-60 (●) or HL-60/Vinc (○) cells at 37°C, and the liberation of minicircles was determined by agarose gel electrophoresis. A, incubations were carried out with the indicated protein concentrations of nuclear extracts for 30 min. B, incubations were carried out with 50 μg of nuclear extracts for the indicated times. Data shown are typical of three independent experiments with different nuclear extracts.

TABLE 1

Growth inhibitory effects of different topoisomerase II inhibitors toward parental and vincristine-resistant HL-60 cells

Exponentially growing cells were exposed to different drug concentrations for 1 h, washed and resuspended in drug-free medium. After 72-h postincubation, cells were counted with a Coulter counter, and the growth inhibition was determined. Values are given as the mean of three independent experiments, each done in duplicate. The growth inhibitory effects of ICRF 187 were determined after 72-h continuous drug exposure. IC_{50} indicates the concentration inhibiting the growth of sensitive and resistant HL-60 cells by 50% compared to untreated control cells. -Fold resistance is the ratio between the IC_{50} values obtained for resistant (HL-60/Vinc) and sensitive cells (HL-60)

Compound	IC_{50}		-Fold Resistance
	HL-60	HL-60/Vinc	
	μM		
Amsacrine	0.9 ± 0.2	4.0 ± 0.6	4.4
Mitoxantrone	0.05 ± 0.01	1.0 ± 0.1	20.0
Symadex (C-1311)	0.5 ± 0.07	6.0 ± 0.9	12.0
ICRF-187	12.0 ± 2.1	24.0 ± 2.9	2.0

Discussion

Cell cycle arrest in the G_2 phase of the cell cycle can be triggered by a wide range of different stress stimuli known to influence DNA and nuclear functions. More recently, it has been reported that microtubule-interacting agents can also influence the expression of several important regulators of the G_2-to-M transition, such as Cdk1 kinase and topoisomerase IIα, which are down-regulated, and the cyclin-dependent kinase inhibitor p21 and 14-3-3 proteins that are up-regulated (Chen et al., 2003; Verrills et al., 2003; Bani et al., 2004). These findings suggest that tubulin interacting-agents might be able to influence the G_2-to-M progression, thereby retaining cells in a part of the cell cycle where they are less sensitive to the cytotoxic effects of such agents.

To explore this hypothesis, we characterized the cell cycle distribution in two independently selected human leukemia cell lines. The results showed no clear differences between vincristine-selected and parental cells with respect to the proportion of S phase cells or generation doubling times. In contrast, the G_2/M fraction was significantly enhanced in both resistant cell lines, whereas the fraction of metaphase cells was reduced. Therefore, vincristine selection resulted in enrichment of cells present in G_2 and/or early mitosis.

Western blot analysis was performed to compare cellular

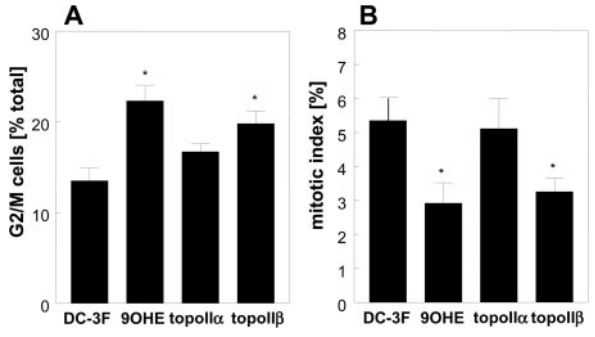

Fig. 7. Cell cycle distribution in parental and 9-hydroxyellipticine-resistant cells. Ellipticine selection of parental DC-3F cells is accompanied by 4- to 5-fold decreased expression of topoisomerase IIα and no detectable expression of topoisomerase IIβ. The topoisomerase II activity was restored by transfection of the ellipticine-resistant cells (9OHE) with either topoisomerase IIα (topoIIα) or IIβ (topoIIβ) as described previously in detail (Larsen and Jacquemin-Sablon, 1989; Larsen et al., 2003). A, the fraction of G_2/M cells in parental and ellipticine-resistant cell lines was determined by flow cytometry analysis. The values are means of at least three independent experiments (bars, S.D.). *, values obtained were significantly different ($p < 0.001$) for parental and ellipticine-resistant cells or significantly different ($p < 0.005$) in the case of parental and ellipticine resistant cells transfected with topoisomerase IIβ. B, the percentage of mitotic cells (mitotic index) was established after fixation of parental and ellipticine-resistant cells as described under *Materials and Methods*. The determination was done by counting 10 random fields with at least 500 cells in total. *, values obtained for resistant and parental cells were significantly different ($p < 0.001$).

levels of major G_2/M regulators in parental and vincristine-resistant cells. Because the same proteins often are implicated in both the response and the resistance to cytotoxic agents, we focused on genes previously shown to be influenced by exposure to paclitaxel, epothilone B, or vincristine (Chen et al., 2003; Verrills et al., 2003; Bani et al., 2004). We were surprised to find no detectable alterations for Cdk1, p21, 14-3-3σ, or 14-3-3ϵ. In clear contrast, topoisomerase IIα was down-regulated to approximately one-third the initial levels in both vincristine-resistant cell lines, accompanied by a 3- to 4-fold decrease in the catalytic activity and cross-resistance to topoisomerase II inhibitors.

Topoisomerase IIα is a dynamic molecule that can be either associated with DNA or present in the nucleoplasm (Tavormina et al., 2002). We therefore determined the association of topoisomerase IIα with mitotic chromosomes in parental and vincristine-resistant cells by immunoblotting followed by image analysis. The results showed that the association of topoisomerase IIα with mitotic chromosomes was reduced at least 7-fold in the vincristine-resistant cells compared with the sensitive parental cells. These findings suggested that the altered cell cycle progression of vincristine-selected cells might be directly related, at least in part, to the down-regulation of topoisomerase IIα.

Topoisomerase IIα is already known to play several essential roles during late G_2 and early mitosis (for reviews, see Larsen et al., 2003; Swedlow and Hirano, 2003). First, topoisomerase II is required for premitotic chromosome individualization, the process by which different chromosomes become untangled and converted into individual units (Gimenez-Abian et al., 2000). The protein also plays a direct role in early chromosome condensation by an as-yet poorly understood mechanism that seems to rely, at least in part, on protein-protein and DNA-protein interactions (Adachi et al., 1991). In addition, topoisomerase IIα is needed for recruitment of Cdk1 to the chromatin of mitotic chromosomes (Escargueil et al., 2001) and thus for mitotic phosphorylation of other chromatin-associated proteins such as the condensins. Finally, topoisomerase II is required for the separation of intertwined sister chromatids in prometaphase (Gimenez-Abian et al., 1995; Losada et al., 2002). A potential influence of topoisomerase IIα protein levels on the fraction of G_2 and prophase cells would therefore be consistent with the known biological functions of topoisomerase IIα during G_2 and early mitosis.

To further explore this hypothesis, we selected a panel of isogenic cell lines in which the topoisomerase II levels have been extensively characterized (Larsen and Jacquemin-Sablon, 1989; Dereuddre et al., 1997; Khelifa et al., 1999). Previous results showed that prolonged selection of parental DC-3F cells with the topoisomerase II inhibitor 9-hydroxyellipticine is accompanied by 4- to 5-fold decreased expression of the topoisomerase IIα protein and total loss of topoisomerase IIβ. We now report that the decreased topoisomerase II levels were accompanied by a significantly increased G_2/M fraction. To establish the individual roles of the two topoisomerase II isoforms, 9-hydroxyellipticine–resistant cells were transfected with either topoisomerase IIα or IIβ and two transfected clones selected where the catalytic activity had been restored to the same levels as in the sensitive parental cell line. It is interesting that transfection of the ellipticine-resistant cells with topoisomerase IIα resulted in a clear reduction of G_2 cells, whereas transfection with topoisomerase IIβ had no detectable influence on the G_2/M distribution. Morphological studies showed that the increased G_2/M fraction in cells with diminished topoisomerase IIα is a mixture of cells with classic G_2 morphology and of prophase cells. In contrast, the fraction of metaphase cells was diminished.

To further explore the influence of topoisomerase II on the sensitivity to microtubule-interacting agents, the different ellipticine-resistant cell lines were exposed to nocodazole for

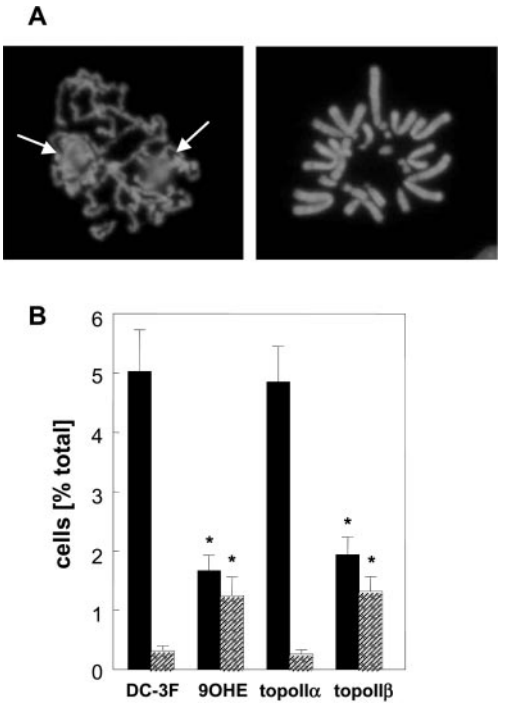

Fig. 8. The proportion of prophase and metaphase cells in parental (■) and ellipticine-resistant (▨) cells. A, the percentage of prophase and metaphase cells was determined according to morphological features (Leblond and El-Alfy, 1998). Left, typical prophase cell with undercondensed chromosomes. The nucleolar remnants are indicated with white arrows. Right, typical metaphase cell with fully condensed, aligned chromosomes. B, the percentage of prophase and metaphase cells was determined for parental and ellipticine-resistant cells. The values are means of two independent experiments (bars, S.D.). *, values obtained for parental and resistant cells were statistically significantly different ($p < 0.001$).

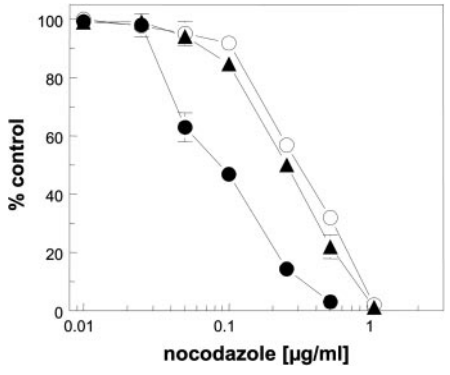

Fig. 9. Viability of nocodazole-treated DC-3F/9-OHE cells. Ellipticine-resistant cells (○) were transfected with either topoisomerase IIα (●) or IIβ (▲) and treated with the indicated concentrations of nocodazole for 24 h followed by colony formation in drug-free media. All values are means of at least two independent experiments, each done in duplicate. Error bars represent standard errors and are indicated when they exceed the symbol size.

24 h followed by postincubation in drug-free media. The results reveal that topoisomerase IIα, but not topoisomerase IIβ, directly influences the cellular sensitivity to microtubule-interacting agents.

Recent results have characterized the influence of small interfering RNA coding for topoisomerase IIα or IIβ on the cell cycle progression in untreated HeLa cells (Sakaguchi and Kikuchi, 2004). The results show that decreased topoisomerase IIα levels are associated with an increased G_2 fraction as well as an increased proportion of cells in the early phases of mitosis. In contrast, knockdown of topoisomerase IIβ had no apparent effect on the cell cycle. Thus, topoisomerase IIα was shown to directly influence G_2/early mitosis in two different genetic models (Sakaguchi and Kikuchi, 2004; this study). Furthermore, a similar phenomenon was observed in the current study for two clinically relevant models of acquired vincristine resistance. These results have important pharmacological implications because the topoisomerase IIα-mediated cell cycle modifications were accompanied by increased resistance to the microtubule-interacting agent nocodazole.

In the present studies, we consistently worked with resistant cell lines that had been reselected with vincristine at least 8 days before use. Therefore, the down-regulation of topoisomerase IIα would qualify as an adaptation process rather than a checkpoint mechanism. Although long-lasting, this is a dynamic response, because no differences in topoisomerase II levels and activities were observed when more than 4 weeks had passed because the selection process ended. Another major difference between our findings and classic G_2 and prophase checkpoints is that the modified cell cycle progression in G_2 and early M phases can be observed even for untreated cells.

It is interesting that down-regulation of topoisomerase IIα shows several similarities to the effects mediated by the prophase checkpoint protein Chfr. The Chfr ubiquitin ligase is activated upon mitotic stress and is able to modify key mitotic regulators, such as Plk1 and Aurora A, and to delay the activation of Cdk1/Cyclin B (Kang et al., 2002; Summers et al., 2005). Activation of Chfr is accompanied by prolonged chromosome condensation and a delay of approximately 6 h in metaphase entry (Scolnick and Halazonetis, 2000). It is noteworthy that transfection of Chfr into Chfr-deficient cells was accompanied by prolonged prophase, delayed metaphase entry, and resistance to both nocodazole and paclitaxel compared with the Chfr-deficient parental cells (Scolnick and Halazonetis, 2000). These results suggest that prophase prolongation and delayed metaphase entry are involved in both the checkpoint response and in the adaptation to microtubule poisons.

The down-regulation of topoisomerase IIα could be due to down-regulation of its promoter. The topoisomerase IIα promoter is regulated in a cell cycle-dependent manner, with maximal activity at the G_2/M interphase (Woessner et al., 1991), and contains five inverted CCAAT boxes (ICBs), two GC boxes, and an ATF site (Hochhauser et al., 1992). It has previously been reported that vinblastine is able to down-regulate the topoisomerase IIα promoter, at least in p53-proficient cells (Joshi et al., 2003). This is likely to also to be the case for p53-deficient cells, because microtubule inhibitors may lead to DNA damage as a result of abnormal mitosis, producing chromosomal breakage and other chromosomal damage (Chen et al., 2003).

In conclusion, we report here that down-regulation of topoisomerase IIα in vincristine-selected human leukemia cells and in isogenic cell lines expressing different levels of topoisomerase II proteins is accompanied by delayed cell cycle progression during G_2 and early mitosis and increased resistance to the tubulin-interacting agent nocodazole. Topoisomerase IIα is already known to be down-regulated after DNA damage and heat shock and is monitored by the G_2 catenation checkpoint. The observation that microtubule-interacting agents may also influence topoisomerase IIα expression makes it one of the most versatile proteins implicated in the control of the G_2-to-M transition.

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