Downregulation of DNA topoisomerase IIalpha leads to prolonged cell cycle transit in G2 and early M and increased survival to microtubule-interacting agents

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Abbreviations:
MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
ABC, ATP-binding cassette
Abstract

Microtubule binders are cell cycle-specific agents with preferential cytotoxicity toward mitotic cells. We have characterized vincristine-selected human leukemia cells to establish if development of vincristine resistance was accompanied by changes in cell cycle kinetics and distribution. Our results indicate that vincristine resistance is accompanied by delayed G2 transit and prolonged early mitosis both in the absence and presence of the microtubule binder nocodazole. The altered G2/M regulation is accompanied by resistance to short-term (12 h), but not to 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 downregulation of topoisomerase IIalpha without detectable alterations of the other mitotic regulators studied including CDK1, p21, 14-3-3sigma and 14-3-3epsilon. This was associated with at least 7-fold less chromosome-associated topoisomerase IIalpha, 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 IIalpha, but not the closely related topoisomerase IIbeta, directly influence the cell cycle kinetics in G2 and early mitosis as well as the resistance to nocodazole. These results underline the importance of topoisomerase IIalpha in late G2 and early M and provide evidence for a so far unsuspected interaction between topoisomerase II and microtubule-directed agents.
Introduction

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 semi-synthetic compounds that bind to various sites on the beta-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 that 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 G1 tetraploidy checkpoint that arrests polyploid cells that have exited mitosis without cytokinesis (Scolnick and Halazonetis, 2000; Trielli et al., 1996; Meraldi et al., 2004).

One of the most widely reported mechanisms of resistance to tubulin binders is associated with overexpression of different ABC transporters such as P-glycoprotein/Mdr1 (ABCB1) and different MRP (ABCC) family members (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 beta-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 since 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 taxol influenced the expression of several cell cycle regulators. In particular, after 24 h drug treatment, the cyclin-dependent kinase inhibitor p21<sup>waf1/cip1/sdi1</sup> (CDKN1A) was upregulated as was another CDK1 modulator, 14-3-3sigma. In contrast, CDK1 (also known as cdc2 or p34<sup>cdc2</sup>) and topoisomerase IIalpha were down-regulated (Chen et al., 2003). Similar findings were reported when the effect of taxol was studied in vivo on ovarian carcinoma xenografts. Interestingly, topoisomerase IIalpha was consistently downregulated whereas p21 was upregulated in the taxol-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 14-3-3epsilon, a protein reported to modulate the cellular effects of both Cdc25C phosphatase (Kumagai et al., 1998) and topoisomerase IIalpha (Kurz et al., 2000). It is notable, that most of these genes are known to play important roles in maintaining G2 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 G2 to M transition could be a distinct mechanism of maintaining G2 arrest and thus to prevent cells from entering mitosis where microtubule interacting agents are most cytotoxic.
We here report that downregulation of topoisomerase IIalpha 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 G2 and early mitosis and increased resistance to the tubulin-interacting agent nocodazole. These results underline the importance of topoisomerase IIalpha in late G2 and early M and provide evidence for a so far unsuspected interaction between topoisomerase II and microtubule-directed agents.

**Materials and Methods**

**Chemicals.** Vincristine was obtained from Laboratoire Pierre Fabre (Castres, France) while nocodazole was purchased from Sigma (Saint-Quentin Fallavier, France). Symadex (compound C-1311) and amsacrine were synthesized by Dr. Marek Konieczny at Gdansk University of Technology, Poland. Mitoxantrone was a gift from Laboratoire Lederle (Rungis, France) while ICRF-187 (Cardioxane) was purchased from Chiron BV (Amsterdam, The Netherlands). [3H]-thymidine (90 Ci/mmol), [14C]-thymidine (52 mCi/mmol) and [14C]-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 (Montpellier, France) and purified by cesium chloride/ethidium bromide density centrifugation.
Antibodies. Monoclonal antibodies directed toward topoisomerase IIalpha were obtained from Calbiochem (La Jolla, CA), monoclonal antibodies directed toward topoisomerase IIbeta were purchased from BioTrend Chemikalien GmbH (Köln, Germany), while monoclonal mouse anti-Cdc2, polyclonal rabbit anti-p21, polyclonal goat anti-14-3-3epsilon, polyclonal goat anti-14-3-3sigma 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 Labs (West Grove, PA) while FITC-conjugated anti-mouse IgG antibodies were provided by Amersham BioSciences (Amersham, 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 (Manhattan, KA). CEM and vincristine-selected CEM/VCR1000 cells (Kimmig et al., 1990) were generously provided by Dr. Johann Hoffman (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 at least one week but no more than four weeks. This dose of vincristine is non-toxic 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, penicillin (100 units/ml) and streptomycin (100 µg/ml) at 37 °C in a 5 % CO2/95 % air atmosphere.

The DC-3F fibrosarcoma cell line, its 9-hydroxyellipticine-selected subline DC-3F/9-0H-E and DC-3F/9-0H-E cells transfected with human topoisomerase IIalpha or human topoisomerase IIbeta have been extensively characterized previously (Larsen and Jacquemin-Sablon,
1989; Dereuddre et al., 1997; Khelifa et al., 1999). The cells were maintained in minimal essential medium (MEM) supplemented with 8% fetal calf serum, penicillin (100 units/ml) and streptomycin (100 µg/ml) and grown at 37 °C in a 5% CO2/95 % air atmosphere.

All cells were screened routinely for Mycoplasma by the DNA hybridization method (Gen-Probe, Inc., San Diego, CA).

**Cell cycle analysis.** All measurements were made using a Coulter EPICS Profile II flow cytometer (Coulter Electronics, Hialeah, FL) 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.** Two million exponentially growing non-treated 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 3 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 ten different microscopic fields were counted under 300x magnification using an Olympus BX60 epifluorescence microscope. Mitotic cells were photographed with Olympus DP50 digital camera. 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 (DAKO) antibodies diluted at 1:100 in 0.5 % BSA/PBS. Samples were washed with TBP buffer (0.2 % Tween 20/0.5 % BSA/PBS) and incubated with anti-mouse IgG-FITC (Amersham) for 1 h at room temperature, counterstained with 5 µg/ml propidium iodide and analyzed by flow cytometry as described (Skladanowski and Larsen, 1997).

**Quantitation of DNA fragmentation by the filter-binding assay.** Cells were labeled with [14C]-thymidine (0.05 µCi/ml, 48 h) and chased in radiolabel-free medium for 6 h. Following drug exposure, approximately 5×10^5 cells suspended in 5 ml ice-cold PBS were applied onto Matricel filters (Gelman Sciences) and washed twice with 5 ml 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. Briefly, 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. Alternatively, cells were exposed to nocodazole for 12 h, post-incubated in drug-free medium for an additional 60 h and the viability determined by the MTT assay.

Exponentially growing leukemia cells were exposed to different drug concentrations for 1 h followed by post-incubation for an additional 72 h in drug-free media. After postincubation, cells were counted with a
Coulter counter 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 earlier (Dereuddre et al., 1997). Briefly, exponentially growing cells were plated into 60-mm Petri dishes (about 250 cells per dish) for 18-20 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 post-incubated for 5-7 days. Colonies were washed with 0.9 % saline, fixed with ethanol and stained with Giemsa. Colonies containing at least 50 cells were counted and growth curves plotted as survival fractions (number of colonies in drug treated samples to controls) versus drug dose.

**Western blot analysis.** Exponentially growing cells (5 x 10^6 cells) were washed twice with PBS, resuspended in 200 µl 0.9 % saline with protease inhibitors (1 mM PMSF, 10 µg/ml each of aprotinin, leupeptin and pepstatin A, respectively) and mixed with 200 µl double strength Laemmlli buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4 % SDS, 0.2 % bromophenol blue and 20 % glycerol). Cell lysates were immediately denatured for 10 min at 95 °C. Twenty five µl of cell lysates were loaded onto 7.5 % polyacrylamide-SDS gels, the proteins were separated by PAGE-SDS electrophoresis and transferred to PVDF 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 % non-fat milk and probed for 3 h with primary antibodies directed toward the topoisomerase IIalpha isoform (1:200 dilution) or the topoisomerase IIbeta 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 MicrolImage Gel Pro software (Media Cybernetics). 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 IIalpha on mitotic chromosomes.** The fraction of mitotic cells was enriched by nocodazole exposure (25 ng/ml) for 12 h. About 5 x 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 RT, spotted onto microscopic slides and air dried. Chromosome spreads were then rehydrated in blocking solution (1 % BSA in PBS) for 30 min at RT and incubated for 1 h with anti-topoisomerase IIalpha-directed antibodies (Ab-1, Calbiochem) diluted in 0.5 % BSA/PBS at 1:50. Following 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 Labs, Burlingham, CA). All preparations were examined with an Axiovert 100M epifluorescence microscope equipped with appropriate filters using PlanApochromat 100x objective.
(Zeiss, 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 extracted with 0.35 M NaCl for 30 min at 4 °C (Lelievre et al., 1995). The nuclei were centrifuged at 11,000 rpm, the supernatants were collected and were 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 MgCl₂, 1 mM ATP, and 200 ng of kinetoplast DNA as previously described (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 % bromophenol 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 Joyce-Loebl Chromoscan 3 densitometer. 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 t-test using the SigmaPlot program.
Results

Vincristine-resistance is accompanied by an altered cell cycle distribution in G2 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 that corresponded to 23 h for HL-60 and HL-60/Vinc cells and to 25 h for CEM and CEM/VCR1000 cells (data not shown). Similarly, 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 G2/M phase of the cell cycle was enhanced in both vincristine-resistant cell lines with 9.0 % ± 1.2 compared to 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 if the increased G2/M fraction was due to an increased number of mitotic cells, the mitotic index (that principally represents metaphase cells but does not include prophase cells) was determined (Fig. 1B). Unexpectedly, the mitotic indices of both resistant cell lines were only about half of what was observed for the sensitive parental cells, with the mitotic index being 4.5 % ± 1.4 for HL-60 cells compared to 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). Since 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 G2 and/or early mitosis.

Parental and vincristine-resistant human leukemia cells differ in the temporal response to nocodazole. We next wished to establish if 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 G2/M phase of the cell cycle (Fig. 2A). The fraction of parental HL-60 cells in G2/M was maximal after 12 h drug exposure and then started to decrease. In comparison, the fraction of HL-60/Vinc cells with a G2/M content remained elevated for an additional 8 h and only started to decrease after 20 h nocodazole exposure. To further confirm these findings, the fraction of MPM-2 positive cells was determined (Fig. 2B). The MPM-2 antibody specifically recognizes phosphoepitopes present in mitotic cells, in particular 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 G2/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 G2/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 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). Importantly, the occurrence of cell death was systematically delayed in the vincristine-resistant cells compared with the parental cells, and was statistically different (p<0.001) for all time points from 12 h and onwards.

**Prolonged G2/M transit in vincristine-resistant cells is accompanied by nocodazole-resistance.** Transient inhibition of cell cycle progression in G2/M is believed to provide additional time for repair of damaged DNA and other cellular lesions prior to chromosome separation and cell division. To determine if the prolonged G2/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. 3A 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 to 69.4 % ± 2.7 for HL-60/Vinc cells whereas the fraction of viable CEM cells was 9.8 % ± 2.9 compared to 55.7 % ± 3.2 for CEM/VCR cells. These differences are statistically different with p<0.001. In marked contrast, parental and vincristine-resistant cells were equally sensitive to continuous nocodazole exposure with IC50 values of 9.6 ng/ml ±1.2 for HL-60 compared to 9.4 ng/ml ± 1.8 for HL-60/Vinc and 21.9 ng/ml ± 2.4 for CEM compared to 20.5 ng/ml ± 2.8 for CEM/VCR. Therefore, the prolonged G2 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.

**Topoisomerase IIalpha is down-regulated in vincristine-resistant cells.** To elucidate the molecular basis for the prolonged G2 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 p34cdc2, the catalytic subunit of CDK1, the cyclin kinase inhibitor p21cip1/waf1/sdi1 and DNA topoisomerase IIalpha. In addition, we determined the expression of 14-3-3epsilon and 14-3-3sigma, that are able to inhibit the activities of topoisomerase IIalpha 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 hypo-phosphorylated 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. Unexpectedly, topoisomerase IIalpha was downregulated to about...
one-third of the initial levels in both vincristine-resistant cell lines. This was not the case for the closely related beta isoform of topoisomerase II that has no known mitotic functions.

The association of topoisomerase IIalpha with mitotic chromosomes is decreased in vincristine-resistant cells. We next wanted to establish if the decreased expression of topoisomerase IIalpha in the vincristine-resistant cells influenced the association of the enzyme with mitotic chromosomes. Chromosome spreads of sensitive and vincristine-resistant cells were immunolabeled with a topoisomerase IIalpha-directed antibody and examined by confocal fluorescence microscopy (Fig. 5). Topoisomerase IIalpha 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 IIalpha 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 comparable-sized chromosomes (indicated with a line in Fig. 5, top panels) on non-modified images showed at least 7-fold difference in the levels of chromosome-associated topoisomerase IIalpha between sensitive and resistant cells (Fig. 5, bottom panels).

The catalytic activity of topoisomerase II is reduced in vincristine-resistant cells. The decatenation activities of nuclear extracts from parental and vincristine-resistant cells was determined to establish if the different expression of topoisomerase IIalpha 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 3-4 fold more active than extracts from vincristine-resistant cells (Fig. 6B). Thus, the downregulation of topoisomerase Ila alpha 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 in order to establish if the reduced levels of topoisomerase Ila alpha also influenced the sensitivity to topoisomerase II inhibitors. Since 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 to the parental cells (Table 1). Interestingly, the vincristine-resistant cells were also cross-resistant to ICRF-187, a topoisomerase II inhibitor that stabilizes non-covalent, rather than covalent complexes between DNA and topoisomerase II (Roca et al., 1994). Therefore, down-regulation of topoisomerase Ila alpha in the vincristine-selected cells was accompanied by cross-resistance to different functional classes of topoisomerase II inhibitors.

Topoisomerase Ila alpha modulates the cell cycle distribution in G2 and M. Our studies revealed that vincristine-selection of human leukemia cells was accompanied by altered cell cycle progression in G2
and M and by decreased expression of topoisomerase IIalpha. In order to establish if cellular topoisomerase IIalpha 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 4-5 fold downregulation of topoisomerase IIalpha and complete loss of topoisomerase IIbeta (Larsen and Jacquemin-Sablon, 1989; Khelifa et al., 1999). Transfection of the ellipticine-resistant cells with either topoisomerase IIalpha or topoisomerase IIbeta 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 IIalpha or beta 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 G2/M (Fig. 7A) and a reduced mitotic index (Fig. 7B). Transfection of the DC-3F/9-OH cells with topoisomerase IIalpha was accompanied by a decreased G2/M fraction and an increased mitotic index. In contrast, transfection with topoisomerase IIbeta 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 classical criteria. Prophase and metaphase cells can be distinguished by morphological features such as chromosome 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 to 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 since the fraction of metaphase cells was only 1.67 % ± 0.26. Transfection of the ellipticine-resistant cells with topoisomerase IIbeta 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 IIalpha 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 IIalpha, but not topoisomerase IIbeta, directly influence the cell cycle progression in G2 and early mitosis.

To establish if 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 IIalpha plays an important role in the sensitivity to nocodazole with IC_{50} values corresponding to 0.09 µg/ml for the topoisomerase IIalpha transfectants compared to 0.30 µg/ml for the ellipticine-resistant parental cells. These changes are statistically different (p<0.001). In contrast, no significant differences were observed between the topoisomerase IIbeta transfectants and the ellipticine-resistant parental cells.
Discussion

Cell cycle arrest in the G2 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 G2 to M transition such as CDK1 kinase and topoisomerase IIalpha that are downregulated, and the cyclin-dependent kinase inhibitor p21 and 14-3-3 proteins that are upregulated (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 G2 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 with regard to generation doubling times. In contrast, the G2/M fraction was significantly enhanced in both resistant cell lines while the fraction of metaphase cells was reduced. Therefore, vincristine-selection resulted in enrichment of cells present in G2 and/or early mitosis.

Western blot analysis was performed to compare cellular levels of major G2/M regulators in parental and vincristine-resistant cells. Since the same proteins often are implicated both in the response and in the resistance to cytotoxic agents, we focused on genes previously shown to be influenced by exposure to taxol, epothilone B or vincristine (Chen et al., 2003; Verrills et al., 2003; Bani et al., 2004). Unexpectedly, no detectable alterations were observed for CDK1, p21, 14-3-3sigma or 14-3-3epsilon. In clear contrast, topoisomerase IIalpha was downregulated.
to about one third of the initial levels in both vincristine-resistant cell lines which was accompanied by a 3-4 fold decrease in the catalytic activity and cross-resistance to topoisomerase II inhibitors.

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

Topoisomerase IIalpha is already known to play several essential roles during late G2 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 a so far poorly understood mechanism that, at least in part, seems to rely on protein-protein and DNA-protein interactions (Adachi et al., 1991). In addition, topoisomerase IIalpha 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 IIalpha protein levels on the fraction of G2 and prophase cells would
therefore be consistent with the known biological functions of topoisomerase IIalpha during G2 and early mitosis.

To further explore this hypothesis, we selected a panel of isogenic cell lines where 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 4-5 fold decreased expression of the topoisomerase IIalpha protein and total loss of topoisomerase IIbeta. We now report that the decreased topoisomerase II levels were accompanied by a significant increased G2/M fraction. To establish the individual roles of the two topoisomerase II isoforms, 9-hydroxyellipticine resistant cells were transfected with either topoisomerase IIalpha or topoisomerase IIbeta and two transfected clones selected where the catalytic activity had been restored to the same levels as in the sensitive parental cell line. Interestingly, transfection of the ellipticine-resistant cells with topoisomerase IIalpha resulted in a clear reduction of G2 cells while transfection with topoisomerase IIbeta had no detectable influence on the G2/M distribution. Morphological studies showed that the increased G2M fraction in cells with diminished topoisomerase IIalpha is a mixture of cells with classical G2 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 24 h followed by post-incubation in drug-free media. The results reveal that topoisomerase IIalpha, but not topoisomerase IIbeta, directly influence the cellular sensitivity to microtubule-interacting agents.
Recent results have characterized the influence of siRNA coding for topoisomerase IIalpha or topoisomerase IIbeta on the cell cycle progression in untreated HeLa cells (Sakaguchi and Kikuchi, 2004). The results show that decreased topoisomerase IIalpha levels are associated with an increased G2 fraction as well as an increased proportion of cells in the early phases of mitosis. In contrast, knockdown of topoisomerase IIbeta had no apparent effect on the cell cycle. Thus, topoisomerase IIalpha was shown to directly influence G2/early mitosis in two different genetic models (Sakaguchi and Kikuchi, 2004; this study). Furthermore, a similar phenomenon was observed for two clinically relevant models of acquired vincristine resistance (this study). These results have important pharmacological implications since the topoisomerase IIalpha-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. Therefore, the downregulation of topoisomerase IIalpha would qualify as an adaptation process rather than a checkpoint mechanism. Although long-lasting, this is a dynamic response, since no differences in topoisomerase II levels and activities were observed when more than 4 weeks had expired since the selection process ended. Another major difference between our findings and classical G2 and prophase checkpoints is that the modified cell cycle progression in G2 and early M can be observed even for untreated cells.

Interestingly, downregulation of topoisomerase IIalpha 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 approximately 6 hr delay in metaphase entry (Scolnick and Halazonetis, 2000). Importantly, transfection of Chfr into Chfr-deficient cells was accompanied by prolonged prophase, delayed metaphase entry and resistance to both nocodazole and taxol in comparison with the Chfr-deficient parental cells (Scolnick and Halazonetis, 2000). These results suggest, that prophase prolongation and delayed metaphase entry is involved in both the checkpoint response and in the adaptation to microtubule poisons.

The down-regulation of topoisomerase IIalpha could be due to downregulation of its promoter. The topoisomerase IIalpha promoter is regulated in a cell-cycle dependent manner, with maximal activity at the G2/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 downregulate the topoisomerase IIalpha promoter, at least in p53-proficient cells (Joshi et al., 2003). This is likely also to be the case for p53-deficient cells, since 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 here report that downregulation of topoisomerase IIalpha 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 G2 and early mitosis and increased resistance to the tubulin-interacting agent nocodazole. Topoisomerase IIalpha is already known to be downregulated following DNA damage and heat shock and is monitored by the G2 catenation checkpoint. The observation that topoisomerase IIalpha expression may also be influenced by microtubule-interacting agents
makes it one of the most versatile proteins implicated in the control of the G2 to M transition.

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References


Footnotes

A.S. and M.G.C. contributed equally to this work.

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Legends for Figures

**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, standard deviation). *B,* the G2/M fraction of parental and vincristine-resistant cells was determined by flow cytometry as described in *A,* while the mitotic index was established after fixation as described in experimental procedures. An asterisk indicates that the values obtained for parental and resistant cells are statistically different (p<0.01).

**Fig. 2.** Flow cytometry of parental and vincristine-resistant HL-60 cells in the presence of nocodazole. Parental (solid bars, ●) and vincristine-resistant HL-60 cells (hatched bars, ○) were incubated in the presence of nocodazole (25 ng/ml) for the indicated times. *A,* the G2/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 in experimental procedures. *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, standard deviation). An asterisk indicates that the values obtained for parental and resistant cells are statistically different (p<0.001).

**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, standard deviation).

**Fig. 4.** Expression of CDK1, phosphotyrosine, p21, 14-3-3epsilon, 14-3-3sigma, topoisomerase IIalpha and topoisomerase IIbeta 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, B). As an internal standard for equal loading, all membranes were rehybridized with antibodies directed against beta actin. C, differences in cellular levels of topoisomerase IIalpha and beta as determined by densitometric analysis of the blots shown in B.

**Fig. 5.** The localization of topoisomerase IIalpha on mitotic chromosomes from parental- and vincristine-resistant cells. Upper panels, chromosomes from parental (A,C) and vincristine-resistant (B) HL-60 cells. Chromosome spreads were incubated with antibodies directed against topoisomerase IIalpha followed by FITC-labeled secondary antibodies and analyzed by confocal fluorescence microscopy. Panels A and B represent total fluorescence signals while 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 II alpha. Lower panels, non-modified images of comparable-sized chromosomes were scanned for fluorescence intensity (in pixels) along the lines indicated in the upper panels.
**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 nuclear extracts for the indicated times. Data shown are typical of three independent experiments with different nuclear extracts.

**Fig. 7.** Cell cycle distribution in parental and 9-hydroxy ellipticine-resistant cells. Ellipticine-selection of parental DC-3F cells is accompanied by 4-5 fold decreased expression of topoisomerase IIalpha and no detectable expression of topoisomerase IIbeta. The topoisomerase II activity was restored by transfection of the ellipticine-resistant cells (9OHE) with either topoisomerase IIalpha (topollalpha) or topoisomerase IIbeta (topollbeta) as described in detail previously (Larsen and Jacquemin-Sablon, 1989; Larsen et al, 2003). **A**, the fraction of G2/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, standard deviation). An asterisk indicates that the values obtained were significant different (p<0.001) for parental and ellipticine-resistant cells or significant different (p<0.005) in the case of parental and ellipticine resistant cells transfected with topoisomerase IIbeta. **B**, the percentage of mitotic cells (mitotic index) was established after fixation of parental and ellipticine-resistant cells as described in experimental procedures. The determination was done by counting 10 random fields with at least 500 cells in total. An asterisk
indicates that the values obtained for resistant and parental cells were significantly different (p<0.001).

**Fig. 8.** The proportion of prophase and metaphase cells in parental (solid bars) and ellipticine-resistant (hatched bars) 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, standard deviation). An asterisk indicates that the values obtained for parental and resistant cells were statistically different (p<0.001).

**Fig. 9.** Viability of nocodazole-treated DC-3F/9-OHE cells. Ellipticine-resistant cells (O) were transfected with either topoisomerase IIalpha (●) or topoisomerase IIbeta (▲) 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.
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 post-incubation 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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>HL-60 [µM]</th>
<th>HL-60/Vinc [µM]</th>
<th>Fold resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amsacrine</td>
<td>0.9 ± 0.2</td>
<td>4.0 ± 0.6</td>
<td>4.4</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>0.05 ± 0.01</td>
<td>1.0 ± 0.1</td>
<td>20.0</td>
</tr>
<tr>
<td>Symadex (C-1311)</td>
<td>0.5 ± 0.07</td>
<td>6.0 ± 0.9</td>
<td>12.0</td>
</tr>
<tr>
<td>ICRF-187</td>
<td>12.0 ± 2.1</td>
<td>24.0 ± 2.9</td>
<td>2.0</td>
</tr>
</tbody>
</table>

**a** - IC$_{50}$ : concentration inhibiting the growth of sensitive and resistant HL-60 cells by 50 % compared to untreated control cells

**b** - ratio between the IC$_{50}$ values obtained for resistant (HL-60/Vinc) and sensitive cells (HL-60)