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Topoisomerase II and histone deacetylase inhibitors delay the G2/M transition by triggering the p38 MAPK checkpoint pathway

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When early prophase PtK_1 or Indian muntjac cells are exposed to topoisomerase II (topo II) inhibitors that induce little if any DNA damage, they are delayed from entering mitosis. We show that this delay is overridden by inhibiting the p38, but not the ATM, kinase. Treating early prophase cells with hyperosmotic medium or a histone deacetylase inhibitor similarly delays entry into mitosis, and this delay can also be prevented by inhibiting p38. Together, these results reveal that agents or stresses that induce global changes in chromatin topology during G2 delay entry into mitosis, independent of the ATM-mediated DNA damage checkpoint, by activating the p38 MAPK checkpoint. The presence of this pathway obviates the necessity of postulating the existence of multiple "chromatin modification" checkpoints during G2. Lastly, cells that enter mitosis in the presence of topo II inhibitors form metaphase spindles that are delayed in entering anaphase via the spindle assembly, and not the p38, checkpoint.

Introduction

The term "antephase" was coined in the mid 20th century to denote the period in late G2, "just before . . . prophase becomes visible," where cells delay in response to chemical and physical insults (Bullough and Johnson, 1951). However, in organisms containing large chromosomes, like rat kangaroos (PtK_1), newts, and Indian muntjacs, prophase can last for >1 h. Importantly, in these cells the commitment to mitosis does not occur until the nucleoli begin to fade, ~15 min before nuclear envelope breakdown (NEB; for review see Pines and Rieder, 2001). Before NEB, chromosome condensation can be arrested, and even reversed, by various treatments (for review see Mikhailov and Rieder, 2002). Thus, in cells like PtK_1 the end of G2, or antephase, can be expanded to encompass the early stages of chromosome condensation.

The arrest or reversal of chromosome condensation during prophase provides a unique visible cue that entry into mitosis has been delayed, and we have been using this feature to study how the G2/M transition is regulated. This assay has a numeric readout, the duration of prophase, and also a qualitative readout, the degree of chromatin condensation (a measure of CDK activity). In our initial studies we found that disassembling microtubules induces a 3–4-h delay in completing prophase (Rieder and Cole, 2000), a behavior that is likely mediated by a checkpoint involving the Chfr protein (Scolnick and Halazonetis, 2000; Chaturvedi et al., 2002; Matsusaka and Pines, 2004). Recently, we used this assay to explore how inhibiting topoisomerase II (topo II) and other enzymes involved in chromatin structure affect the G2/M transition. The results of these studies, which are described here, reveal that drugs which modify chromatin topology during late G2 delay entry into mitosis, independent of the ATM kinase, by activating the p38 MAPK checkpoint pathway.

Results

All topo II inhibitors delay the G2/M transition. To explore the mechanism behind this delay we treated antephase PtK_1 and Indian muntjac cells with various topo II inhibitors, and then followed their behavior by time-lapse video light microscopy. We used a topo II poison (adriamycin) known to

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Key words: mitosis; DNA; aclarubicin; merbarone; apicidin; ICRF-193

Abbreviations used in this paper: DSB, double-strand break; IMF, immunofluorescence; JNK, Jun-N-terminal; NEB, nuclear envelope breakdown; topo II, topoisomerase II.

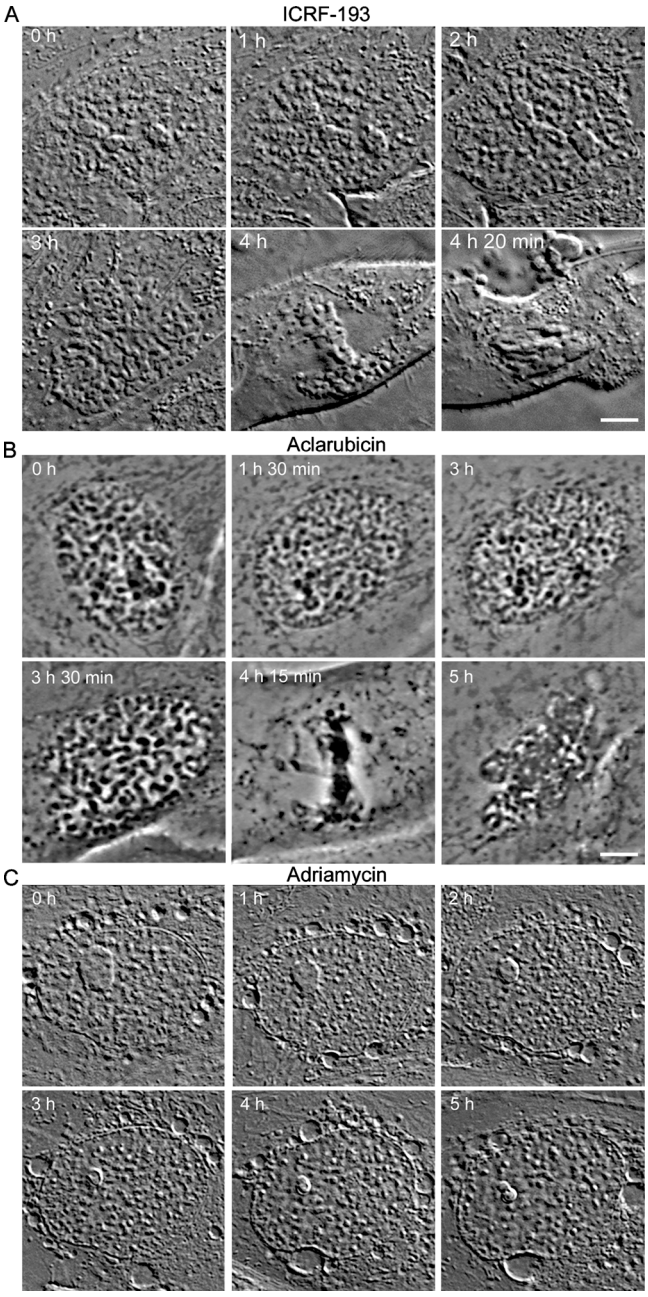


Figure 1. **Drugs that inhibit topo II delay progression through antephase/prophase.** Early to mid-prophase PtK_1 cells were located ~30 min after adding conditioned medium containing 4 μM ICRF-193 (A), 0.75–1 μM aclarubicin (B), or 10 μM adriamycin (C). They were then followed by time-lapse DIC (A and C) or phase-contrast (B) microscopy, to determine the duration of prophase and prometaphase/metaphase. Both ICRF-193 (A) and aclarubicin (B) significantly (4–5×) prolong the duration of prophase, which normally takes ~45 min at 37°C (see Tables I and II). Note that with both treatments the cells ultimately form normal looking metaphase spindles that entered an abnormal anaphase. By contrast, cells treated with adriamycin (C) remained in a prophase-like state until the recordings were terminated. Time in hours (h) and minutes (min), since the onset of recording, is in the upper left corner of each image. Bars, 10 μm.

produce double-strand breaks (DSBs), as well as catalytic inhibitors (ICRF-193, merbarone) that are not supposed to damage DNA. We also used aclarubicin which, by intercalating into DNA, inhibits decatenation (Perrin et al., 1998) by

Table I. **Duration of prophase in Ptk1 cells treated with topo II inhibitors, stress-inducting, and DNA-damaging agents**

Treatment	None	Caffeine (5–10 μM/4 h)	SB203580 (50 μM/1 h)
None	45 ± 3 (25)	44 ± 10 (9)	42 ± 9 (4)
Adriamycin (10 μM)	>600* (3)	>600*, $n = 5$	90 ± 53 (3)
ICRF-193 (4 μM)	224 ± 92 (18)	80 ± 37 (5)	51 ± 8 (5)
Merbarone (40 μM)	>600* (5)	>600* (2)	51 ± 12 (3)
Aclarubicin (0.75–1 μM)	163 ± 72 (18)	202 ± 49 (4)	55 ± 23 (7)
Anisomycin (5–7.5 ng/ml)	197 ± 80 (6)	>600* (4)	55 ± 20 (5)
50 laser pulses into nucleus	>600*	51 ± 7 (6)	>*120 (5)

The duration of prophase (min) in PtK_1 cells after disrupting chromatin structure in the presence of ATM/ATR (caffeine, 5–10 μM) or p38 (SB203580, 40–50 μM) kinase inhibitors. In the presence of SB203580 alone, PtK_1 cells enter mitosis and divide normally for up to 2 d (not depicted). Adriamycin, ICRF-193, merbarone, and aclarubicin all inhibit topo II. Anisomycin activates the p38 kinase, and laser irradiation selectively induces DSBs in the nucleus. The asterisk indicates that cells did not enter mitosis during the indicated time of observation.

preventing topo II from binding to chromatin (Kellner et al., 2002). Not unexpectedly all of these agents delayed progression through prophase (Table I). When exposed to 4 μM ICRF-193 or 1 μM aclarubicin the chromosomes in early to mid prophase cells decondensed, and then slowly recondensed, or they continued to slowly condense over a prolonged prophase period (Table I; Fig. 1, A and B). When these cells finally entered mitosis their chromosomes exhibited the typical nondecatenated phenotype (Gorbsky, 1994), i.e., they were less compacted than normal and the chromatids failed to separate during the ensuing anaphase (Fig. 1, A and B). Adriamycin "froze" cells in a prophase-like state for >10 h (Fig. 1 C), whereas merbarone arrested the cells in antephase after the chromosomes had decondensed (not depicted).

ICRF-193 and merbarone, but not aclarubicin, induce the formation of γ–H2AX complexes during antephase

To explore if catalytic inhibitors of topo II delay G2 in the absence of DSBs, we used the phosphorylation of histone H2AX on Ser^{139} (i.e., the formation of γ–H2AX complexes) as a sensitive visible assay for DSB formation (Rogakou et al., 1998; Caspari and Carr, 2002). We found that a 1-h treatment with 4 μM ICRF-193 or 40 μM merbarone produced multiple γ–H2AX foci in antephase cells (Fig. 2 A), although many fewer than adriamycin treatment. In contrast, treatment with aclarubicin did not produce γ–H2AX foci above that of the background (Fig. 2 A), even at concentrations that strip topo IIα from chromosomes in 40 min (Fig. 2 B). During interphase and mitosis chromatin bound topo IIα is in a rapid dynamic exchange with unbound topo II (Christensen et al., 2002; Tavormina et al., 2002). Thus, the induction of multiple γ–H2AX foci by ICRF-193 and merbarone imply (Caspari and Carr, 2002) that these drugs induce significant DSBs in vivo.

ATM is not involved in the antephase delay induced by catalytic inhibitors of topo II

During antephase adriamycin, ICRF-193 and merbarone activate the ATM kinase as evident from the fact that they in-

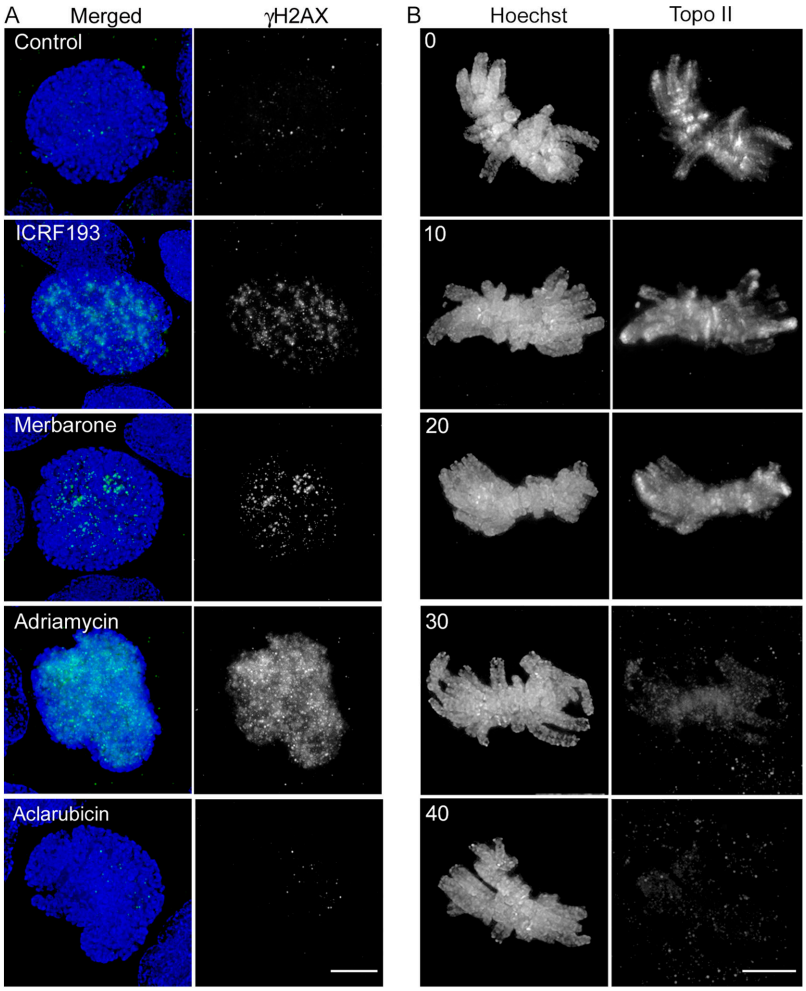
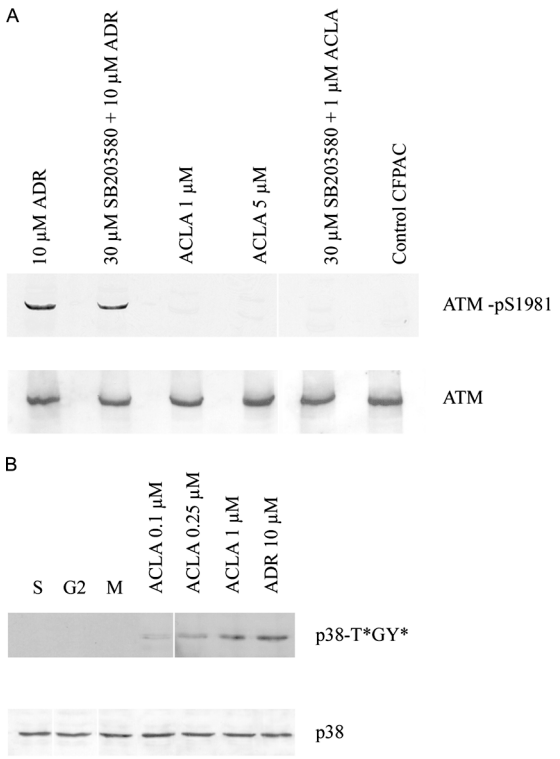


Figure 2. **Aclarubicin does not induce DSBs and depletes chromosomes of topo II.** (A) ICRF-193, merbarone, and adriamycin induce DSBs (γ–H2AX complexes) in prophase cells, whereas aclarubicin does not. CFPAC-1 cells were treated with DMSO (Control), 4 μM ICRF-193, 40 μM merbarone, 10 μM adriamycin, or 1 μM aclarubicin in conditioned media for 1 h. They were then fixed and stained as described previously for γ–H2AX complexes (Mikhailov et al., 2002). (Left) Hoechst 33342 and γ–H2AX antibody. (Right) γ–H2AX antibody channel. Note that all topo II inhibitors except aclarubicin induce formation of γ–H2AX complexes above that of the background. Bar, 10 μm. (B) PtK_1 cells were fixed 10, 20, 30, and 40 min after incubation in 1 μM aclarubicin, and stained for chromatin (left, Hoechst 33342) and the IMF localization of topo IIα (right). Note that aclarubicin depletes the chromosomes of topo IIα in 30–40 min. Bars, 10 μm.



duce γ–H2AX foci (Fig. 2 A), which requires ATM activity (Fernandez-Capetillo et al., 2002). Does aclarubicin, which does not induce γ–H2AX foci above that of background, similarly activate ATM? To answer this question we treated CFPAC-1 cultures for 1 h with adriamycin or aclarubicin before preparing whole cell extracts. We then stained Western blots of these extracts for total and activated (serine 1981-P; Bakkenist and Kastan, 2003) ATM. As predicted from our γ–H2AX studies, adriamycin activates the ATM kinase but aclarubicin does not, even at high concentrations (Fig. 3 A).

Figure 3. **ATM is activated by adriamycin but not aclarubicin, and both topo II inhibitors activate p38.** (A) CFPAC cells were grown for 4 h in the presence of DMSO (control) or SB203580. They were then exposed to different concentrations of aclarubicin or adriamycin. Whole cell lysates were separated on 6% acrylamide gels and blotted for activated (pS1981) and general ATM. Note that ATM is activated by adriamycin, but not aclarubicin, even at high concentrations. (B) Hela cells enriched in S, G2 and M-phase were collected after release from double thymidine block. Cells were exposed to different concentrations of aclarubicin (ACLA) and adriamycin (ADR) for 50 min during the G2-M transition. Whole cell lysates were then separated on a 12% acrylamide gel and blotted for activated (T*GY*) and general p38. Note that p38 is activated during G2/M by adriamycin and aclarubicin in a dose-dependent manner. White lines indicate that intervening lanes have been spliced out.

Next, we asked if aclarubicin causes cells lacking the ATM kinase to delay in late G2. For this experiment, we filmed fields of human GM16666A (−/− ATM) cell cultures in the presence or absence of 1 μM aclarubicin. From these records, we determined the percentage of cells in mitosis every hour, over a 7-h period, as well as the number that entered mitosis (underwent NEB) each hour. These data (Fig. 4) clearly reveal that aclarubicin rapidly delays the G2/M transition in cells lacking ATM.

Because aclarubicin delays the G2/M transition in the absence of ATM activation, the delay induced by this catalytic topo II inhibitor is not due to the DNA damage checkpoint. To determine if this is also true for other topo II inhibitors, we pretreated PtK_1 cultures for 4 h with 5–10 mM caffeine or wortmannin, potent inhibitors of the ATM kinase (Zhou et al., 2000), before adding topo II inhibitors. We found that caffeine or wortmannin did not prevent the antephase delay induced by adriamycin, merbarone, or aclarubicin (Table I). It did, however, shorten the ICRF-193 induced delay, which is consistent with reports that ICRF-193 delays cells in G2, at least in part via the ATM/ATR kinase (Deming et al., 2001). The inability of caffeine to override the adriamycin-induced antephase delay may be due to the extensive DNA damage caused by this poison (Fig. 2 A), which may trigger other pathways in addition to ATM. Regardless, because caffeine does not override the antephase delay induced by merbarone or aclarubicin, which compared with adriamycin produce significantly fewer DSBs (Fig. 2 A), the delay must be due to pathways not involving the ATM kinase.

Triggering p38 MAPK during antephase delays entry into mitosis

In addition to the ATM/DNA damage checkpoint, a caffeine-insensitive pathway appears to exist that delays cells in G2 in response to UV, IR, or γ-irradiation (Goldstone et al., 2001; Jha et al., 2002; Xu et al., 2002). In the case of γ and UV irradiation this arrest is mediated by the p38 MAPK (Bulavin et al., 2001; Dmitrieva et al., 2002; for review see Bulavin et al., 2002). This prompted us to ask if activating p38 during antephase, with concentrations of anisomycin (5–7 ng/ml) that do not affect protein synthesis (Bunyard et al., 2003), delays entry into mitosis. We found that anisomycin rapidly induced early to mid prophase PtK_1 cells to decondense their chromosomes and return to G_2 for >3 h (Table I). Osmotic stress, which is also a potent activator of p38 (Han et al., 1994; Dmitrieva et al., 2002), similarly induced early to mid prophase cells to decondense their chromosomes and delay in antephase (unpublished data). By binding to the ATP site on p38, the small molecule SB203580 potently and selectively inhibits the downstream activity of p38 without preventing its activating phosphorylation (Gum et al., 1998; Lisnock et al., 1998). Not unexpectedly, if p38 activity was prevented in PtK_1 with SB203580, before treating antephase cells with anisomycin (Table I) or hypertonic medium (not depicted), they entered prometaphase with near normal kinetics. Thus, activating p38 during antephase delays entry into mitosis, and this delay can be eliminated by inhibiting p38 with SB203580.

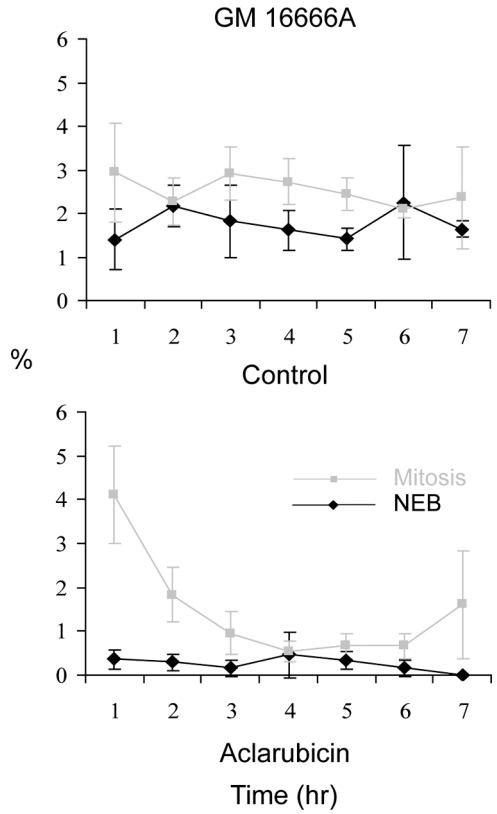


Figure 4. **Aclarubicin inhibits the G2/M transition in human cells lacking the ATM kinase.** Fields of human GM16666A cells, lacking a functional ATM (−/− ATM) kinase, were followed by video LM for 7 h before (top) and after exposure to 1 μM aclarubicin (bottom). The number of cells in mitosis at the end of each hour (gray squares), as well as those undergoing NEB during each hour (black squares), were determined for each field. Error bars represent SEM.

Topo II inhibitors activate the p38 pathway

To determine if topo II inhibitors activate p38 during G2/M we treated synchronized HeLa cells with adriamycin or aclarubicin. Western blots of whole cell extracts, immunostained for total and active p38 (T*GY*), confirm that p38 is not normally active in HeLa during S and G2/M (Fig. 3 B; for review see Deacon et al., 2003). However, it is clearly activated in a dose-dependent manner when G2/M cells are treated with adriamycin or aclarubicin (Fig. 3 B). Thus, inhibitors of topo II, including those that produce few if any DSBs, activate the p38 MAPK. P38 is highly conserved and antibodies against human p38 detected p38 in nonsynchronizable PtK_1 cells (unpublished data). We also found that agents known to stimulate (anisomycin) or inhibit (SB203580) p38 in human cells also work on marsupial cells.

Inhibiting p38 activity overrides the antephase delay caused by topo II inhibitors

We next incubated PtK_1 cultures in SB203580 before treating them with topo II inhibitors. We found that inhibiting p38 with SB203580 completely abolished the antephase delay seen after treating cells with ICRF-193, merbarone or aclarubicin, and it significantly reduced the delay after adria-

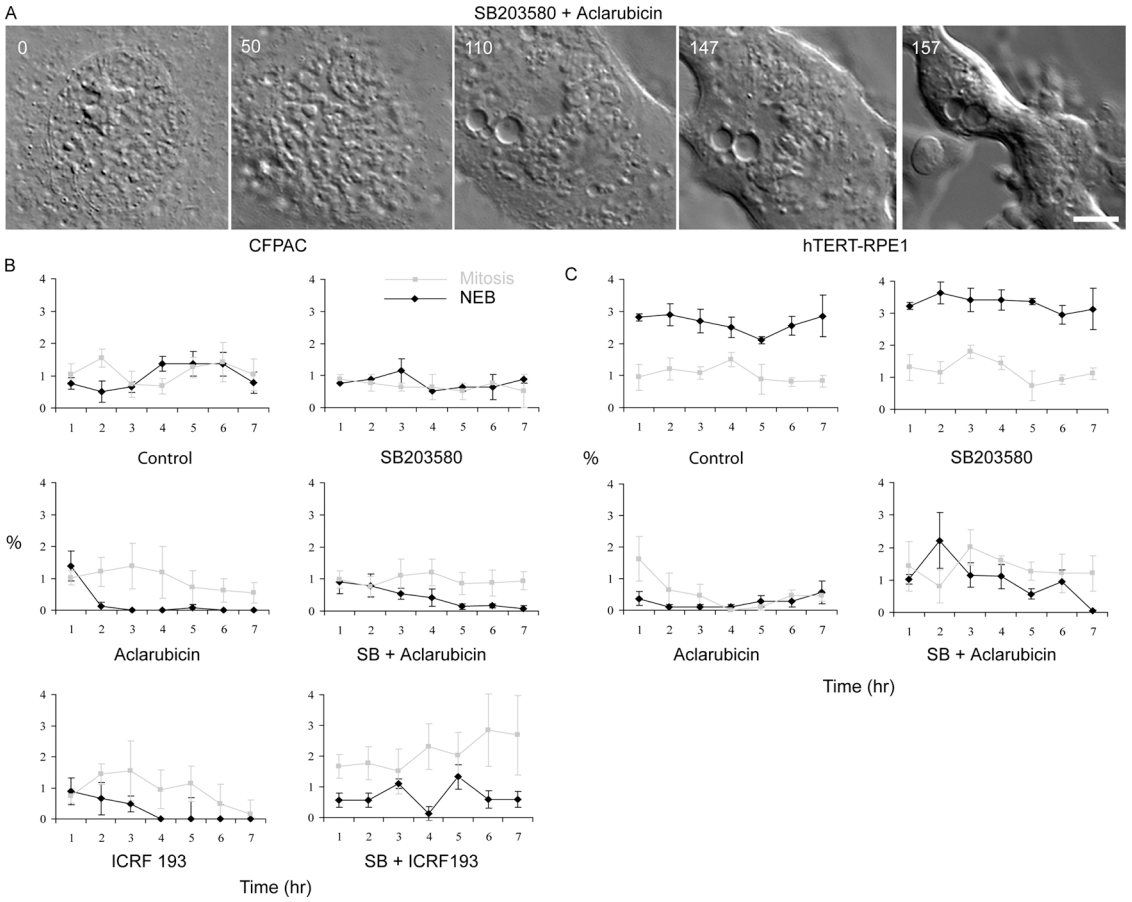


Figure 5. **Cells treated with topo II inhibitors in late G2 enter mitosis with normal kinetics when p38 is inhibited with SB203580.** (A) Sequential images of a SB203580 treated PtK_1 cell entering mitosis in the presence of 0.75 μM aclarubicin. Time in min is in top left corner of each frame. After NEB (50 min) this cell formed a metaphase spindle (110 min) which entered anaphase (147 min). In the presence of the p38 inhibitor, aclarubicin does not delay entry into mitosis although it does delay exit from mitosis. Bar, 10 μm. (B and C) CFPAC-1 (B) and hTERT-RPE1 (C) cells were followed for 7 h as described in Materials and methods. To distinguish cells blocked in mitosis from those entering mitosis from late G2, the percent of cells undergoing NEB (black line) and already in mitosis (gray line) were determined for each hour. Points are average data for every hour post-drug addition, error bars show standard error of the average. 30 μM SB203580 somewhat decreased the frequency of mitosis in CFPAC cells but it had no effect on hTERT-RPE1 cells.

mycin treatment (Table I; Fig. 5 A). During aclarubicin treatment the cells entered mitosis with little or no chromosome-bound topo II (Fig. 2 B). Pre-incubating PtK_1 cells with SB202474, an inactive analogue of SB203580, did not prevent the antephase delay (unpublished data). We then repeated these experiments with another potent p38 inhibitor, 2-(4-chlorophenyl)-4-(4-fluorophenyl)-5-pyridin-4-yl-1,2-dihydropyrazol-3-one (de Laszlo et al., 1998), and obtained the same results (unpublished data). Finally, the Jun-N-terminal (JNK) MAPK shares a high degree of structural and functional homology with p38. To determine if JNK is involved in the G2 delay induced by topo II inhibitors we inhibited this MAPK during prophase with 30 μM SP600125 (Bennett et al., 2001) and found that it did not prevent the antephase delay (unpublished data).

We then repeated the p38 inhibitor experiments on Indian muntjac (Table II; Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200405167/DC1), human CFPAC-1, and hTERT-RPE-1 cells (Fig. 5, B and C) and obtained similar results. The duration of visible prophase in CFPAC-1 and RPE-1 is ∼15 min, and by the time chromosome condensation is evident the cells are committed to mitosis. To determine how these cells respond to inhibiting topo II in late G2, in the presence or absence of active p38, we used video light microscopy to follow populations for 6–8 h after drug addition. When treated only with SB203580 the cells entered and completed prophase with normal kinetics for at least 7 h (Fig. 5, B and C). As with PtK_1 and Indian

Table II. **Duration of prophase in Indian muntjac cells treated with topo II inhibitors**

Treatment	None	SB203580 (50 μM/1 h)
None	55 ± 13 (16)	49 ± 16 (5)
Aclarubicin (0.75–1 μM)	300 ± 141 (3)	57 ± 4 (6)
ICRF-193 (4 μM)	384 ± 187 (3)	101 ± 35 (2)

Duration of prophase (min) in Indian muntjac cells treated with aclarubicin or ICRF-193 in the absence or presence of SB203580. Note that inhibiting p38 significantly reduces the duration of prophase in cells treated with topo II inhibitors. The data in A and B represent the average ± SD; the number of cells followed is shown in parentheses.

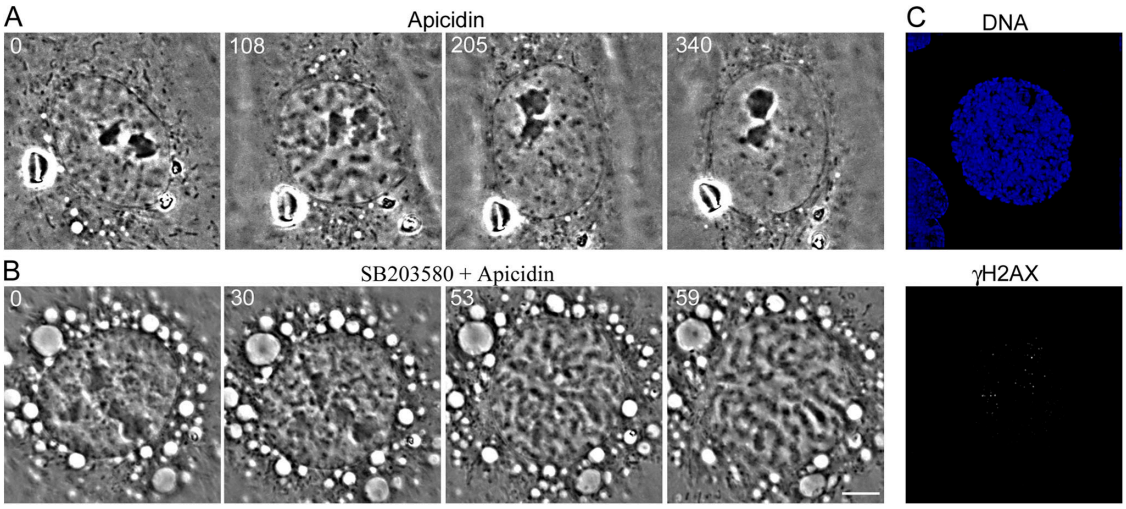


Figure 6. **Inhibiting histone deacetylase delays progression through antephase via a p38-dependent mechanism.** (A) PtK_1 cells treated in early prophase with apicidin either spend a prolonged period in prophase or, as illustrated here (top), decondense their chromosomes and return to antephase. (B) In contrast, when cells are exposed to the p38 inhibitor SB203580, and then apicidin, the duration of prophase is similar to nontreated controls (~50 min). (C) A 1-h treatment of antephase cells with 0.5 μM apicidin does not induce the formation of γ–H2AX foci (DSBs) above that of background. Time is in minutes indicated in top left corner of each frame. Bar, 10 μm.

muntjac cells, both ICRF-193 and aclarubicin rapidly inhibited entry into mitosis in CFPAC-1 (Fig. 5 B) and RPE-1 (Fig. 5 C) cultures. However, the inhibition could be largely overridden during the first several hours by first treating the cultures with SB203580. After 4–7 h in aclarubicin, CFPAC-1 and hTERT-RPE1 cells fail to enter mitosis even when p38 is inhibited. This is likely due to toxic effects arising, e.g., from the inability of late S or early G2 cells in aclarubicin-treated cultures to transcribe genes required for cell cycle progression.

Inhibiting histone deacetylase also delays the G2/M transition via p38

One interpretation of our results is that topo II inhibitors and osmotic shock impede the G_2/M transition because they induce abnormal chromatin topology which activates the p38 pathway. To explore whether other agents that globally disrupt chromatin structure delay cells in antephase via p38 we treated PtK_1 cells with apicidin, a potent histone deacetylase inhibitor (Witt et al., 2003). Because histone deacetylase is recruited to DNA by other proteins, inhibiting its activity during antephase with 0.5 μM apicidin should not, and does not (Fig. 6 C), induce γ–H2AX foci (DSBs) above background. However, others have shown that inhibiting histone deacetylase alters chromatin structure (Grunstein, 1997) and arrests cell cycle progression via an undefined checkpoint (Qiu et al., 2000). Not unexpectedly we found that inhibiting histone deacetylase during early prophase delayed entry into mitosis by either inducing the chromosomes to decondense ($n = 2$; Fig. 6 A), or by prolonging prophase (159 ± 87 min; $n = 3$). Importantly, this delay was eliminated when cells were pretreated with SB203580 (52 ± 11 min; $n = 3$; Fig. 6 B), but not caffeine (not depicted). For controls we treated early prophase cells with lumi-colcemid or cytochalasin D, which do not affect chromatin structure, and found that they entered mitosis with normal kinetics (not depicted; for review see Rieder and Cole, 2000).

Localized DSBs delay entry into mitosis via the ATM and not P38 checkpoint pathway

So far our data support the idea that global disruptions in chromatin topology delay cell cycle progression via the p38

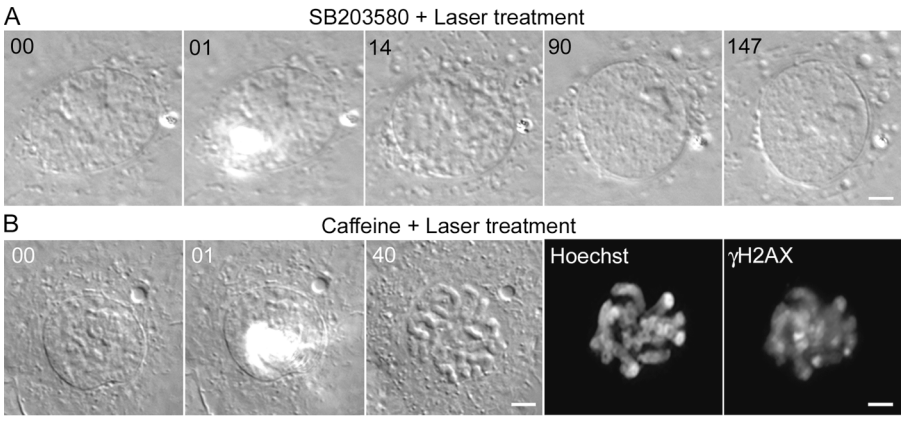


Figure 7. **Inhibiting p38 does not override the ATM/DNA damage checkpoint.** (A) When the nucleus of early prophase PtK_1 cells is irradiated with 50 pulses of laser light the chromosomes decondense and the cell returns to an interphase morphology. This response is not prevented by inhibiting p38 with SB203580 (A), but it is prevented when the ATM kinase is inhibited with caffeine (B). The cell in B was fixed after NEB and processed for the detection of both DNA (Hoechst) and DSBs (γ–H2Ax). Bars, 10 μm.

pathway independent of DSBs. This model predicts that inducing DSBs in just a few highly localized regions of the nucleus will not arrest antephase cells via the p38 pathway. To test this we "stitched" nuclei in antephase PtK_1 cells with 50 pulses of 546-nm laser light. This produces highly localized regions of γ–H2AX foci (unpublished data; for review see Rogakou et al., 1999) and delays antephase cells from entering mitosis (Rieder and Cole, 1998). When we repeated these experiments after inhibiting p38 with SB203580, the cells continued to decondense their chromosomes and were blocked in antephase (Table I; Fig. 7 A). However, if we pretreated cultures with 5–10 mM caffeine before stitching early prophase nuclei, the cells progressed into mitosis with normal kinetics even though they contained numerous DSBs (Table I; Fig. 7 B). This experiment reveals that SB203580 does not inhibit the ATM kinase. It also demonstrates that the localized disruption of chromatin does not activate p38, or that if it is activated under this condition it does not contribute to the cell cycle delay.

P38 activity is not required for progression through mitosis or for the spindle assembly checkpoint

Cells that enter mitosis in the presence of ICRF-193 form metaphase spindles that are delayed in entering anaphase (Mikhailov et al., 2002). Here, we report that aclarubicin-treated cells, driven into mitosis by inhibiting p38, also form spindles that are delayed in metaphase (Fig. 5 A). This was true for all cell types tested, including PtK_1 (Table III), Indian muntjac (Fig. S1 B), CFPAC (185 ± 54 min, $n = 4$ vs. 60 ± 7 min. $n = 9$), HeLa (160 ± 56 min, $n = 56$ vs. 46 ± 6 min, $n = 2$), and U2OS (238 ± 90 min, $n = 5$ vs. 43 ± 14 min, $n = 5$).

As reported by others (Deacon et al., 2003), we found that p38 is not activated as untreated HeLa cells transit from G2 into mitosis (Fig. 3 B). Therefore, it is not surprising that inhibiting p38 with SB203580 had no effect on the duration of mitosis in untreated PtK_1 cells (Table III). From our live cell studies it was also clear that the delay in mitosis caused by aclarubicin, or disrupting microtubule assembly with nocodazole, is not overridden by inhibiting p38 in PtK_1 (Table III) or Indian muntjac (Fig. S1 C). Under both conditions it is, however, rapidly eliminated when the cells are injected with a dominant negative Mad2 spindle assembly checkpoint component (see Mikhailov et al., 2002; unpublished data). Thus, p38 activation is not required for normal mitotic progression or for the spindle assembly checkpoint in PtK_1 or Indian muntjac cells.

Table III. **Duration of mitosis[a] in Ptk1 cells treated with nocodazole, anisomycin, or aclarubicin**

Treatment	None	SB203580
None	50 ± 2 (7)	57 ± 10 (8)
Nocodazole	136 ± 20 (13)	337 ± 107 (5)
Anisomycin (5–7.5 ng/ml)	46 ± 18 (6)	40 ± 12 (5)
Aclarubicin (1.5–3 μM)	128 ± 38 (10)	158 ± 54 (4)

The duration of mitosis (NEB to anaphase onset) in PtK_1 cells treated at 37°C with nocodazole, anisomycin, or aclarubicin, with or without prior treatment with the p38 inhibitor SB203580. The data represents the average ± SD; number of experiments is shown in parentheses.
[a]Nuclear envelope breakdown to anaphase onset.

Discussion

Topo II is the only enzyme that can cut and rejoin double-strand DNA, and it is used to relieve torsional stress caused e.g., during DNA replication, transcription and repair. The enzyme is particularly active during the G2 and M phases of the cell cycle where it is involved in many aspects of DNA metabolism and chromatin topology. Two features have made topo II a primary target for some of the most widely prescribed antibiotics and anticancer drugs currently in clinical use: it serves an indispensable function and it lacks biological redundancy (Froelich-Ammon and Osheroff, 1995; Kellner et al., 2002). Because deleting topo II is usually lethal, its function is studied primarily by disrupting its activity with "poisons" that stabilize the enzyme on cleaved DNA, or with "catalytic" inhibitors that bind the enzyme before it cuts DNA, or after it has been rejoined.

The synthesis of topo IIα starts in G1 and peaks in G2, whereas topo IIβ expression is continuous throughout the cell cycle (Kellner et al., 2002). As a result, poisons like ICRF-193 and merbarone, that preferentially bind to and inhibit topo IIα–catalyzed decatenation (Perrin et al., 1998), delay cells selectively in G2 (Deming et al., 2002). In contrast, by intercalating directly into chromatin aclarubicin prevents decatenation by both topo IIα and β (Perrin et al., 1998). As a result, this drug delays progression through all stages of the cell cycle including G2 (Teillaud et al., 1998).

The mechanism(s) by which topo II inhibitors delay cell cycle progression are only vaguely understood. Work with the catalytic inhibitor ICRF-193 suggested that this delay is mediated by a caffeine-sensitive pathway that monitors chromatin decatenation (Downes et al., 1994; Clifford et al., 2003). Subsequent work suggested that this "decatenation checkpoint" delays entry into mitosis, independent of the p53 pathway (Kaufmann et al., 2002), by using the ATR kinase and BRCA1 to inhibit the polo-like kinase (Deming et al., 2001, 2002; Kaufmann et al., 2002). The idea that a G2 "decatenation" checkpoint exists, distinct from the DNA damage checkpoint, is based largely on indirect observations and biochemical data that ICRF-193 does not induce DSBs. However, recent work (Huang et al., 2001; Wang and Eastmond, 2002), as well as our own data (Fig. 2), reveals that many of these drugs, including ICRF-193 and merbarone, do damage DNA in vivo. Furthermore, it is now clear that the ATR kinase implicated in the so called decatenation checkpoint has considerable overlap with the ATM kinase which arrests cells in response to DSBs (Durocher and Jackson, 2001). Finally, because sister chromatids do not become fully decatenated until the metaphase/anaphase transition, it is difficult to envision how a checkpoint monitoring the state of DNA catenation can delay the G2/M transition when cells normally enter mitosis with catenated chromatids.

Topo II and histone deacetylase inhibitors delay the G2/M transition by activating the p38 pathway

Our data reveal that, when applied to antephase (late G2) cells, topo II inhibitors delay entry into mitosis via the p38

MAPK, and not the ATM, pathway. Because this delay is triggered in minutes, by a route that functions well into prophase, it does not require activation of transcription factors (like p53) or new protein synthesis. The p38 MAPK pathway fulfills the criteria for a checkpoint control, at least during late G2: at this time it is normally not active and, when activated, it delays cell cycle progression via a route that shows a relief of dependence. Importantly, under many conditions this delay is transient and it is ultimately bypassed by an adaptation process, even when the problem cannot be fixed. This control provides a mechanism during the G2/M transition, as it appears to also do during the G1/S transition (Kyriakis and Avruch, 2001), for quickly delaying cell cycle progression in response to diverse stresses. In the absence of such a rapid response system, such stresses may well lead to chromosome segregation problems during mitosis independent of those generated by DNA damage. The p38 checkpoint pathway thus gives the cell time to recover, just before important transitional events, in cases where the insult is transient. If need be, it also allows other checkpoints that require transcription time to work.

What does the "topo II checkpoint" monitor if not chromatin decatenation? Topo II inhibitors either bind to chromatin (aclarubicin) or lock the enzyme on chromatin in an inactive form (adriamycin, ICRF-193, merbarone). One possibility is that as these drugs bind, they induce a global change in chromatin topology that delays the G2/M transition by activating the p38 pathway. This idea is supported by our data, and those of others, that osmotic shock and histone deacetylase inhibitors, which similarly induce global changes in chromatin topology, also delay the G2/M transition via the p38 pathway. It is also consistent with our finding that selectively damaging chromatin in just a few regions of the antephase nucleus delays entry into mitosis via the ATM and not p38 kinase pathway.

How could global changes in chromatin topology during antephase activate p38? One possibility is that regions of chromatin bind an unidentified factor that is released in response to abnormal chromatin topology. Once released this factor may interact with c-Abl and/or DNA-protein kinase (Kharbanda et al., 1997) to initiate a kinase cascade (Brancho et al., 2003) that activates p38. Active p38 can influence cell behavior by activating transcription factors or other kinases. Because the antephase response we describe is rapid, and occurs as chromosomes are condensing, it is not due to transcription factors like p53. Rather, the activation of p38 by abnormal chromatin topology likely initiates another kinase cascade, perhaps involving MNK1 (Fukunaga and Hunter, 1997), that produces the cell cycle delay. P38 can also directly interact with Cdc25B (Bulavin et al., 2001). The antephase checkpoint may work by ultimately blocking activation of cyclin A/CDK2 via Cdc25, which in response can occur independent of ATM/ATR (Goldstone et al., 2001; Mitra and Enders, 2004).

We find that the delay in entering mitosis induced in late G2 cells by topo II inhibitors is caffeine insensitive and does not involve the ATM kinase. Bakkenist and Kastan (2003) report that based primarily on immunofluorescence (IMF) data, osmotic stress, and histone deacetylase inhibitors induce a diffuse phosphorylation of ATM in the absence of DSBs. This suggested that ATM is activated globally by changes in chromatin structure, and then later accumulates at DSBs when present. Our results reveal that these same treatments delay the G2/M transition. However, we find that this delay is not overridden by inhibiting ATM with caffeine or wortmannin (or in −/− ATM cells), yet it is eliminated by preventing p38 kinase activity. We also find that topo II inhibitors which induce DSBs activate both ATM (as evidenced by γ–H2AX foci formation) and p38, but that inhibitors that do not induce DSBs (aclarubicin) do not activate ATM. Regardless, with the exception of adriamycin, which induces massive numbers of DSBs, all of these inhibitors delay entry into mitosis via the p38 and not ATM pathway. These results imply that, by itself, the global activation of ATM by changes in chromatin topology does not produce a late G2 delay independent of the p38 pathway.

P38 activity is not required for entry into mitosis or the spindle assembly checkpoint

The spindle assembly checkpoint delays anaphase when kinetochores are not stably associated with the spindle. Work on 3T3 and HeLa cell populations suggests that p38 is activated in response to spindle poisons (Deacon et al., 2003), and that this activity is required for the spindle assembly checkpoint (Takenaka et al., 1998). However, in situ studies conclude that p38 is normally active during mitosis, and that this activity is required to overcome this checkpoint (Campos et al., 2002). Cell sorting studies even suggest that inhibiting p38 does not influence the mitotic arrest or slippage of HeLa cells treated with nocodazole (Tsuiki et al., 2001).

As reported by others (Deacon et al., 2003) we found that p38 is not activated as untreated HeLa cells transit from G2 into mitosis (Fig. 3 B). We also found that inhibiting p38 does not influence the rate at which CFPAC-1 or hTERT-RPE1 cells enter mitosis (Fig. 5, B and C), or the duration of the mitotic delay induced in live PtK_1 or Indian muntjac cells by nocodazole or topo II inhibitors. This latter delay is, however, rapidly abrogated when cells are injected with a dominant negative construct of Mad2 (Mikhailov et al., 2002). From these observations we conclude that p38 activity is neither required for entry into mitosis, for normal mitotic progression, or for the spindle assembly checkpoint in PtK_1 or Indian muntjac cells.

Many of the chemical or physical insults that delay the G2/M transition also delay the metaphase/anaphase transition. With few exceptions, most of these globally perturb chromatin topology. Good examples here include chromatin damage caused by radiation (Mikhailov et al., 2002), and inhibitors of topo II (Illidge et al., 2000; Mikhailov et al., 2002) or histone deacetylase (Cimini et al., 2003). We propose that topo II and histone deacetylase inhibitors delay entry into and exit from mitosis because they bind to and induce structural changes in chromatin. During antephase these changes are detected by the p38 pathway. During mitosis they impede satisfaction of the spindle assembly checkpoint by deleteriously affecting kinetochore structure and thus their stable attachment to the spindle.

Finally, we found that cells arrested in G2 by drugs that prevent normal topo II function can be driven into a highly aberrant mitosis by simply overriding activation of the P38

MAPK. Many of these drugs are currently used as a primary or adjunct chemotherapy in cancer treatment (Froelich-Ammon and Osheroff, 1995; Kellner et al., 2002). One interesting avenue may therefore be to explore the clinical effects of combining topo II and p38 inhibitors.

Materials and methods

Cell culture

PtK_1, HeLa, U2OS, CFPAC-1, and hTERT-RPE1 were cultured on coverslips as detailed previously (Mikhailov et al., 2002). Indian muntjac cells were grown in DME supplemented with 10% FBS. GM1666A cells were grown on coverslips in DME supplemented with 10% FBS and 100 μg/ml hygromycin; 12 h before the experiment cells were placed in hygromycin-free media.

Coverslip cultures of PtK_1 and Indian muntjac cells were assembled into Rose chambers (Khodjakov and Rieder, 2004) at least 5 h before the start of each experiment, whereas those containing CFPAC, hTERT-RPE, and GM16666A cells at least 12 h before each experiment. Once assembled the Rose chambers were then incubated at 37°C.

Reagents

Adriamycin and caffeine were purchased from Sigma-Aldrich. Merbarone (5-(N-phenylcarboxamido)-2-thiobarbituric acid), aclarubicin (Aclacinomycin A), SB203580, SB 202474, JNK Inhibitor II (SP600125), apicidin, and 2-(4-chlorophenyl)-4-(4-fluorophenyl)-5-pyridin-4-yl-1,2-dihydropyrazol-3-one were purchased from Calbiochem. ICRF-193 was a gift from A. Creighton (St. Bartholomew's Hospital College, London, UK). In all instances, reagents were added to conditioned media before use.

Western blotting

Cells were washed in ice-cold PBS and scraped from the culture into cold buffer containing 20 mM Hepes, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 2 mM EDTA, 137.5 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM Na_3VO_4, 40 μM PMSF, 1% Triton X-100, and complete protease inhibitor cocktail (Roche Diagnostics Corp.). After 10 min on ice, the lysate was centrifuged at 14,000 rpm (4°C) for 10 min and the supernatant was used as whole cell extract. Equal amounts of protein were separated on reducing SDS-PAGE gels, immunoblotted and detected by ECL. To assay for general and active (T^{180}GY-phosphorylated) p38, we used antibodies from Cell Signaling Technology, Inc. and Promega Biosciences, Inc. General and S^{1981}-phosphorylated ATM was detected with antibodies from Cell Signaling ("5C2") and Rockland, Inc., correspondingly.

Immunochemical techniques

Rabbit antibody to human topo II was purchased from TopoGEN. Cells were fixed and stained for IMF, including γ–H2AX, as detailed previously (Mikhailov et al., 2002).

Laser irradiation and live cell video microscopy

Laser irradiation of early prophase nuclei was conducted with pulses of 532 nm light (Rieder and Cole, 1998). Nuclei were irradiated with 50 pulses as they were translated in a linear fashion through the fixed laser beam. Each pulse contained ~400 2 nJ of power as measured in the plane of the specimen.

All recordings were made on microscopes housed in a 37°C warm room, or inside custom built thermostatically regulated Plexiglas incubators.

For mitotic index studies coverslips of CFPAC-1 and hTERT-RPE1 cells were used at ~70% confluence. For long-term recordings, Rose chambers were mounted on the stage of Nikon Diaphot or TMS microscopes housed in a 37°C warm room. Fields of cells were time lapsed with a 20× phase contrast objective, and one image was acquired every 10 min for 6–10 h using Image Pro Plus (Media Cybernetics) or Scion Image (Scion Corp.). Sequential images were then assembled into movie stacks which were then visually analyzed, during each hour of recording, for: (a) the total number of cells within the field of view (usually 200–250); (b) the number of cells entering mitosis (i.e., undergoing NEB); and (c) the total number of mitotic cells.

Online supplemental material

One supplemental figure is included which illustrates that topo II inhibitors delay the G2/M transition in Indian muntjac cells via a p38-dependent mechanism. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200405167/DC1.

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