Cells Lacking DNA Topoisomerase IIβ are Resistant to Genistein

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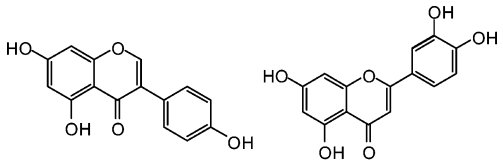
Evidence suggests that DNA topoisomerases (topos) may be involved in the anticancer and carcinogenic properties attributed to flavonoids. Using the cell-based assay TARDIS, the dietary flavonoids genistein (**1**) and luteolin (**2**) have been evaluated as topo I and topo II poisons and catalytic inhibitors in K562 leukemia cells. Both flavonoids induced topo II−DNA complexes, but they did not induce significant levels of topo I−DNA complexes. Genistein decreased the topo II−DNA complexes induced by the topo II poison etoposide, suggestive of a catalytic inhibition of topo II, and luteolin decreased the topo I−DNA complexes induced by the topo I poison camptothecin, indicative of a catalytic inhibition of topo I. Murine transgenic cells lacking topo IIβ were resistant to genistein-induced cell growth inhibition (XTT assays) and cytotoxicity (clonogenic assay). High levels of topo IIβ−DNA complexes were also observed in K562 cells exposed to genistein. These data suggest that topo IIβ has an important function in genistein-induced cell growth inhibition and cell death. The possible role of topoisomerases in the putative anticancer and carcinogenic properties of genistein and luteolin is discussed.

DNA topoisomerases (topos) are essential enzymes that govern DNA topology. During the normal catalytic cycle of these enzymes, transient enzyme-bridged DNA strand breaks are formed, which allow the enzyme to alter DNA topology; this allows cellular processes such as replication, transcription, recombination, and chromatin remodeling.[1,2] Topo I and topo II can be targeted by poisons and catalytic inhibitors. Topo I and topo II poisons (e.g., topotecan and etoposide, respectively) represent a group of clinically important anticancer drugs. These drugs stabilize the normally transient DNA breaks; then cellular processing converts these protein-bridged breaks into permanent strand breaks that trigger cell death.[3−5] Topo II poisons have been suggested to induce leukemia.[6−8] Catalytic inhibitors of topoisomerases, on the other hand, inhibit stages in the catalytic cycle of the enzymes, therefore preventing the formation of such DNA strand breaks and reducing the activity/toxicity of topo poisons. For instance, dexrazoxane can inhibit the catalytic activity of topo II and has clinical utility for the prevention of toxicity resulting from topo II poisons.[9]

Flavonoids are plant secondary metabolites widely distributed throughout the plant kingdom and are commonly present in plant-derived foods. Several dietary flavonoids have shown anticancer effects in vitro and in animal models of carcinogenesis, and some have entered clinical trials for the prevention or treatment of specific cancers.[10−13] Conversely, several studies have shown that flavonoids may be toxic and carcinogenic agents.[14−16]

DNA topoisomerases may play a role in the anticancer and carcinogenic effects shown by flavonoids. First, the inhibition of the catalytic activity of topo II has been suggested to be an important parameter for the selection of cancer chemopreventive agents, and some flavonoids are considered cancer chemopreventive agents able to inhibit the catalytic activity of topo II.[17] Second, topo I and topo II poisons are commonly used in cancer chemotherapy, and several flavonoids have been described as topo poisons.[18−24] Finally, topo poisons are known to produce DNA damage-induced toxicity,[6−8] and several flavonoids cause topo II-mediated DNA damage that may lead to infant leukemia.[14]

Several studies have revealed that specific flavonoids can inhibit or poison topo I and II. However, these are mostly in vitro studies and sometimes show contradictory results. For instance, Cho et al.[17] suggested that genistein was a catalytic inhibitor of topo I (relaxation assay), but Constantinou et al.[20] reported that it was not (relaxation assay). Flavonoids are abundant in our diet and are also frequent components of dietary supplements.[15] Therefore, it is important to clarify the effects of these dietary agents on topoisomerases in order to understand their potential anticancer effects and their possible toxicity.



Genistein (**1**) Luteolin (**2**)

In the present communication, the topo I and topo II poison and catalytic inhibition activity of the common dietary flavonoids genistein (**1**) and luteolin (**2**) has been evaluated using a cell-based immunofluorescence assay. The TARDIS assay (*t*rapped in *a*g*ar*ose *D*NA *i*mmunostaining) uses specific antibodies to DNA topo I or topo II to detect the protein covalently bound to the DNA in intact cells.[25,26] Murine transgenic cells lacking topo IIβ were then used to assess the importance of this enzyme as a cytotoxic target for these two flavonoids.

Results and Discussion

Genistein and luteolin were evaluated as topo I poisons in K562 leukemia cells using the TARDIS assay. Control or drug-treated K562 cells were embedded in agarose on microscope slides. The cells were then lysed to disrupt the cellular membranes and remove soluble proteins. After this, salt extraction was used to remove nuclear proteins and any noncovalently bound topo I from the DNA matrix. Drug-stabilized topo−DNA complexes remained and were detected by staining with isoform-specific antisera followed by an FITC-conjugated secondary antibody. Digital images of Hoechst (DNA) fluorescence and FITC immunofluorescence (drug-stabilized topo complexes) were captured, and levels of fluorescence were quantified. To obtain an appropriate concentration range, K562 cells

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Table 1. Evaluation of Genistein (**1**) and Luteolin (**2**) as Topo I and Topo II Poisons in K562 Leukemia Cells Using the TARDIS Assay[a]

flavonoid	IC_{50} (μM)		control	0.5 h	2 h	6 h	24 h	PC
genistein (**1**)	17.56 ± 1.3	topo I	0.40 ± 0.19	0.96 ± 0.16	0.44 ± 0.09	0.44 ± 0.07	0.18 ± 0.70	11.39 ± 3.79
		topo II	−0.09 ± 0.17	7.03 ± 4.11	20.40 ± 2.91	7.03 ± 3.07	12.62 ± 6.28	49.35 ± 6.28
luteolin (**2**)	14.65 ± 2.3	topo I	0.13 ± 0.25	0.49 ± 0.42	0.28 ± 0.10	0.51 ± 0.27	0.19 ± 0.29	6.72 ± 0.96
		topo II	0.39 ± 0.43	3.17 ± 1.70	4.08 ± 0.92	7.39 ± 1.68	5.05 ± 0.99	35.47 ± 3.97

[a] The IC_{50} growth inhibition activity in K562 cells was calculated using the XTT assay following a 5 day drug exposure. Both flavonoids were evaluated for topo I and topo II poison activity using the TARDIS assay. The flavonoids were used at the following concentrations (μM): genistein (175), luteolin (146); these concentrations are 10 times their IC_{50} value obtained in the XTT assay for a 5 day exposure. PC (positive control): the topo I poison camptothecin was tested at 10 μM for 1 h and the topo II poison etoposide at 10 μM for 2 h. Values show integrated green fluorescence (indicating topo poison activity) ± SEM and have been reduced for simplicity by 10^{-3}. All data are averaged from at least three independent experiments. The IC_{50} values (XTT assay, 5 day exposure) were also determined for camptothecin (0.04 ± 0.01 μM) and etoposide (0.32 ± 0.03 μM). Camptothecin and etoposide induced topo I− and I_I−DNA cleavable complexes, respectively, at concentrations 10 times their IC_{50} value: camptothecin (control: 0.19 ± 0.05, 2 h: 2.51 ± 1.03, 8 h: 1.58 ± 0.33, 24 h: 1.04 ± 0.31), etoposide (control: −0.03 ± 0.04, 2 h: 13.16 ± 4.27, 8 h: 20.87 ± 5.02, 24 h: 23.45 ± 5.35).

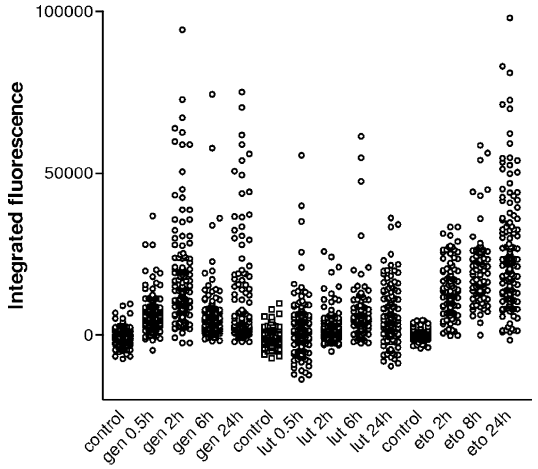

Figure 1. Evaluation of genistein (**1**) and luteolin (**2**) topo II poison activity in K-562 leukemia cells using the TARDIS assay. The plot illustrates representative individual experiments and shows the distribution of cleavable complexes in individual cells treated with 175 μM genistein, 146 μM luteolin, and 3 μM etoposide at different exposure times. These concentrations are 10 times their IC_{50} value obtained in the XTT assay for a 5 day exposure.

were treated for 5 days with the flavonoids, and their IC_{50} values were estimated using the XTT assay (Table 1). For the TARDIS assay, K562 cells were treated for 0.5, 2, 6, and 24 h with the flavonoids, and the levels of topo I−DNA complexes were evaluated using the TARDIS assay. Untreated cells or cells treated with the topo I poison camptothecin were used as negative and positive controls, respectively. Three independent experiments were carried out for each flavonoid for each exposure time. The results, shown in Table 1, indicate that none of the tested flavonoids induced significant levels of topo I−DNA cleavable complexes at any of the tested exposure times.

K562 cells were then treated for 0.5, 2, 6, and 24 h with genistein and luteolin, and the levels of topo II-DNA complexes were evaluated using the TARDIS assay. Untreated cells or cells treated with the topo II poison etoposide were used as negative and positive controls, respectively. The results, represented in Table 1 and Figure 1, show that both flavonoids induced topo II−DNA complexes in K562 leukemia cells. Genistein induced the highest levels of topo II−DNA complexes in cells treated for 2 h. Luteolin was approximately 2−3 times less active than genistein and induced the maximum levels of topo II−DNA complexes in cells treated for 6 h.

The topo II poison activity observed for genistein and luteolin in K562 cells is in agreement with previous research.[14,18−20] The lack of topo I poison activity of luteolin, however, disagrees with previous results.[22] The concentrations of flavonoids used in our experiments (10 × IC_{50}) are appropriate for this assay to detect drug-induced topo I− or topo II−DNA complexes, as standard topo I and topo II poisons such as camptothecin and etoposide can induce topo−DNA complexes at these concentrations. However, we incubated K562 cells for 2 h with luteolin at higher concentrations (30 × IC_{50}), and we did not observe topo I complexes (results not shown). The results showed that luteolin induced topo I−DNA complexes in vitro. In addition, using the cell-based SDS-K^+ precipitation assay, Chowdhury et al. observed that this flavonoid induced protein−DNA complexes in cells.[22] It is known that drugs that are topo poisons in vitro are not always topo poisons in cells. The SDS-K^+ precipitation assay is not specific for topoisomerases and does not differentiate between topo I−DNA complexes and topo II−DNA complexes. Since luteolin induces topo II complexes in cells (Table 1, Figure 1), it is possible that the topo−DNA complexes found by Chowdhury et al.[22] in cells were with topo II and not with topo I.

Table 2. Evaluation of Genistein (**1**) and Luteolin (**2**) as Catalytic Inhibitors of Topo I and Topo II in K562 Leukemia Cells Using the TARDIS Assay[a]

flavonoid	IC_{50} (μM)		IC_{50} + PC	$10IC_{50}$ + PC
genistein (**1**)	17.56 ± 1.3	topo I	112 ± 3	148 ± 5
		topo II	95 ± 3	67 ± 2
luteolin (**2**)	14.65 ± 2.3	topo I	99 ± 7	52 ± 8
		topo II	133 ± 19	85 ± 10

[a] K562 cells were treated with the positive controls (PC) camptothecin (topo I poison) and etoposide (topo II poison), alone or in combination with each flavonoid. Camptothecin was tested at 10 μM for 1 h and etoposide at 10 μM for 2 h. The flavonoids were tested for 24 h at the following concentrations (μM): genistein (17 and 175), luteolin (14 and 146); these concentrations are 1 and 10 times their IC_{50} value obtained in the XTT assay for a 5 day exposure. The levels of topo−DNA cleavable complexes induced by camptothecin and etoposide were normalized to 100%. The relative levels of topo−DNA cleavable complexes induced by camptothecin and etoposide in K562 cells pretreated with each flavonoid are shown in Table 2. Data were averaged from at least three independent experiments ± SEM.

Catalytic inhibitors of topoisomerases prevent the formation of topo−DNA complexes induced by topo poisons. Using the TARDIS assay, the ability of genistein and luteolin to decrease the topo I−DNA complexes induced by the topo I poison camptothecin and the topo II poison etoposide was evaluated (Table 2, Figure 2). The levels of topo−DNA complexes induced by camptothecin and etoposide in K562 cells were measured in the presence and absence of each flavonoid. Luteolin decreased the levels of topo I complexes induced by camptothecin, while genistein did not. Both flavonoids reduced the levels of topo II complexes induced by etoposide; the reductions produced by genistein were greater than those produced by luteolin.

Since genistein and luteolin showed topo II poison and/or inhibition activity, a topo II-deficient cell line (murine topo IIβ −/−) was used to evaluate the importance of this enzyme in the

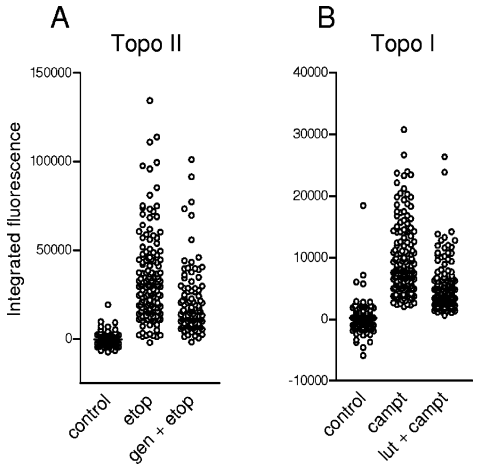

Figure 2. Evaluation of genistein (**1**) topo II catalytic activity and luteolin (**2**) topo I catalytic activity in K-562 leukemia cells using the TARDIS assay. (A) K562 cells were treated with the topo II poison etoposide (10 μM, 2 h) in the absence or presence of genistein (175 μM, 24 h). (B) K562 cells were treated with the topo I poison camptothecin (10 μM, 1 h) in the absence or presence of luteolin (146 μM, 24h). Plots show a representative individual experiment in which the distribution of cleavable complexes in individual cells can be observed.

growth inhibitory activity of these dietary agents.[27] Western blotting analysis confirmed that topo IIβ −/− cells did not contain any topo IIβ as previously reported.[27] Topo IIβ +/+ and topo IIβ −/− cells were treated for 4 h with each flavonoid at the concentrations shown in Figure 3. After treatment, cells were placed in drug-free medium for 68 h (to complete 3 days to observe effects on cell growth), and then cell viability was estimated using the XTT assay. Amsacrine (m-AMSA), a topo II poison with selectivity for the topo IIβ isoenzyme,[27] was used as a positive control (results not shown). Figure 3 shows that murine topo IIβ −/− cells treated with genistein at 100 and 300 μM were resistant to growth inhibition compared with murine topo IIβ +/+ cells ($p < 0.05$, paired, two-tailed *t*-test), while no significant differences were found for luteolin. To assess this effect further, clonogenic assays were carried out to examine the cytotoxicity of genistein on murine topo IIβ −/− and +/+ cells. Both cell lines were treated for 4 h with genistein at 10, 30, 100, and 300 μM. Figure 3C shows that genistein-induced cytotoxicity on topo IIβ −/− cells was markedly lower than in topo IIβ +/+ cells. For instance, the survival of topo IIβ +/+ cells exposed to 100 μM genistein was close to 0%, while the survival of topo IIβ −/− cells exposed to 100 μM genistein (or even 300 μM) was around 50%. There was a 6.4-fold difference in the IC_{50} for wild-type cells (47 μM) and for the knockout cells (300 μM). These results agree with a previous report indicating that two genistein-resistant cell lines derived from CCRF-CEM leukemia cells had a markedly reduced expression of topo IIβ, while topo IIα was unaffected.[28]

The resistance of cells lacking topo IIβ to genistein suggested that the β isoform of topo II was an important drug target for this flavonoid. The TARDIS assay was used with isoform-specific antisera to evaluate whether genistein induced topo IIβ complexes in individual cells. Figure 3D shows that genistein (175 μM, 2 h) induced higher levels of topo IIβ complexes than the positive control etoposide (10 μM, 2 h) in K562 cells. Genistein, under the same experimental conditions, also induced topo IIα complexes in K562 cells, but the levels of complexes were slightly lower than those induced by the positive control etoposide (control: 6 ± 170; genistein: 1355 ± 402; etoposide: 1599 ± 261). Collectively, our results suggest that genistein-induced cytotoxicity involves topo IIβ. However, other mechanisms (e.g., topo IIα poisoning) may contribute to genistein-induced cytotoxicity.

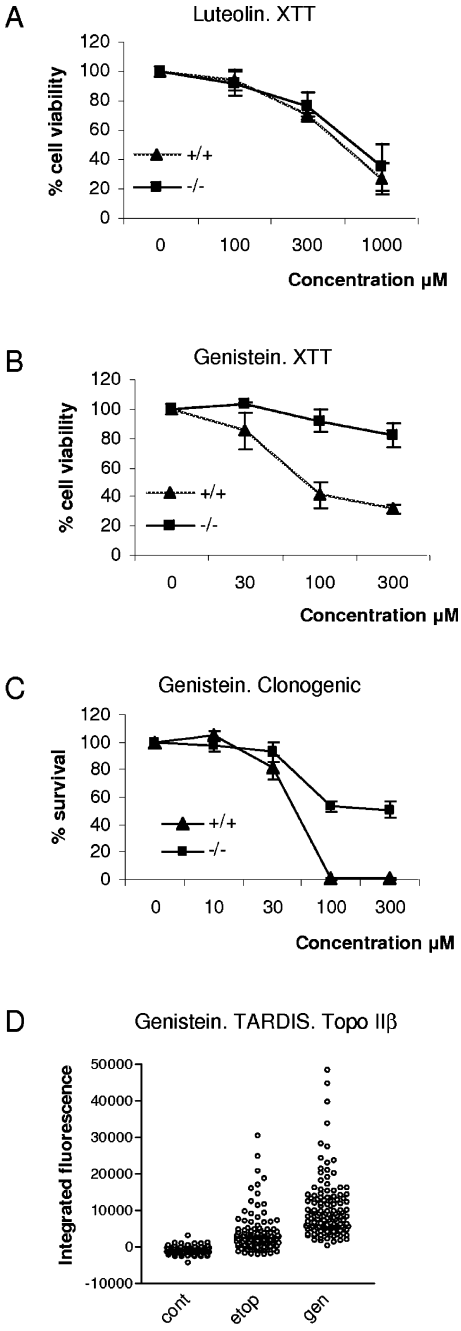

Figure 3. Evaluation of genistein activity on DNA topoisomerase IIβ. Topo IIβ +/+ and topo IIβ −/− cells were treated for 4 h with luteolin (A) and genistein (B) at different concentrations. After treatment and drug removal, cells were placed in drug-free medium to complete 3 days; then the percentage of cell viability in relation to untreated cells was estimated using the XTT assay. Three experiments were carried out, and data are expressed as mean ± SEM. There was a statistically significant difference (paired, two-tailed *t*-test) between the two cell lines for genistein 100 μM ($p = 0.004$) and 300 μM ($p = 0.02$). (C) Topo IIβ +/+ and topo IIβ −/− cells were placed in 9 cm plates and treated with genistein for 4 h. After drug removal, cells were cultured for 9 days and then cell survival was estimated using the clonogenic assay. Three experiments were carried out, and data are expressed as mean ± SEM. (D) Genistein induction of topo IIβ−DNA complexes in K562 (TARDIS assay). Three independent experiments showed that the levels of topo IIβ complexes were as follows: control 292 ± 38; etoposide (2 h, 10 μM) 3945 ± 711; genistein (2 h, 175 μM) 12 723 ± 4149. The plot shows a representative experiment in which the distribution of complexes in individual cells can be seen in a scattergram.

The present communication shows that the dietary flavonoids genistein and luteolin may have important in vivo effects on DNA topoisomerases; this may have relevance to several facets of cancer. First, topo II poisons are widely used in cancer chemotherapy, and genistein is shown here to behave as a topo II poison that induces DNA complexes with both topo IIα and topo IIβ. Interestingly, it has been reported that the levels of topo IIβ can be increased in human tumors compared with normal tissues[29,30] and that slow-growing tumors contain significant levels of this isoenzyme.[31] Genistein might therefore display anticancer effects in these types of tumors. Second, topo II inhibition has been suggested to be important in cancer chemoprevention,[17] and Table 2 and Figure 2A show that genistein behaves as a catalytic inhibitor of topo II in cells. This activity, however, occurs at relatively high concentrations. Considering the pharmacokinetics of dietary flavonoids in humans,[12] we suggest that it is unlikely that genistein or luteolin inhibits the catalytic activity of topo II and topo I when they are taken through the diet or supplements. Finally, topo II-mediated DNA damage may lead to toxic and carcinogenic effects,[6−8] and Table 1 and Figure 1 show that genistein and luteolin induce topo II complexes in cells. The in vivo plasma concentrations of genistein after supplementation has been reported to be 0.1−8 μM,[32] and the threshold for genistein induction of topo II−DNA-mediated clastogenicity has been suggested to be within this range.[33] This example suggests that some flavonoids may exert topo II-mediated toxic and carcinogenic effects when ingested at relatively high concentrations, such as those present in some dietary supplements. However, accumulating evidence suggests that consumption of flavonoid-containing foods is associated with a reduced cancer risk. This suggests that low concentrations of some flavonoids, achievable through a diet rich in plant-derived foods, may exert anticancer effects via topoisomerase-independent mechanisms, e.g., estogen receptor β mediated effects (30 nM).[34,35] In brief, evidence suggests that some flavonoids such as genistein or luteolin may produce anticancer effects at concentrations achievable through a diet rich in plant-derived foods (submicromolar) through topoisomerase-independent mechanisms (e.g., antioxidant, antiestrogenic). At higher concentrations (micromolar, noncytotoxic), these agents may induce topo II-mediated DNA damage that may produce carcinogenic effects. At high concentrations (micromolar, cytotoxic), these dietary agents may produce cancer chemotherapeutic effects by inducing topo II-mediated DNA damage.

Experimental Section

General Experimental Procedures. Human K562 leukemia cells were maintained as a suspension culture in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin (50 U/mL)/streptomycin (50 μg/mL). This cell line was maintained at 37° (5% CO_2). The murine topo IIβ −/− cell line and the wild-type topo IIβ +/+ cell line were grown as monolayers at 37 °C in a humidified atmosphere containing 5% CO_2.[27] These were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin. Cell culture reagents were obtained from Life Technologies. Genistein, luteolin, etoposide, camptothecin, and m-AMSA were purchased from Sigma. Stock solutions were prepared in DMSO (except for etoposide, which was in MeOH) and were stored at −20 °C.

XTT Assay. This is a colorimetric assay that allows the quantitative determination of cell viability. It is based on the capability of viable cells to transform the tetrazolium salt XTT into a formazan dye. Exponentially growing cells were seeded (2×10^3/well in 100 μL) into 96-well plates. Drugs were added to the plates 24 h later. Following the incubation period indicated in figure or table legends, cell viability was quantified using an XTT cell proliferation kit assay (Roche, Mannheim, Germany). After drug exposure, plates were incubated for 4 h with XTT before reading them on a Bio-Rad 550 plate reader at 450 nm. Cell viability was expressed as a percentage in relation to controls. All data were averaged from at least three independent experiments ± SEM.

Clonogenic Assay. The murine topo IIβ −/− cell line and the wild-type topo IIβ +/+ cell line were seeded (2.5×10^5/plate) into 9 cm plates. After 48 h, drug was added to exponentially growing cells at appropriate concentrations for 4 h. The clonogenic assay was carried out as described previously.[27]

Antibodies. Anti-topo II polyclonal antibodies were raised in rabbits. 18511 was raised to recombinant human topo IIα, 18513 to recombinant human topo IIβ C-terminal fragment and αCT to recombinant topo IIα C-terminal fragment. 18511 detected the α isoform specifically, and 18513 detected the β isoform specifically.[36] Western blots confirmed that αCT detected both isoforms of topo II (results not shown). This was the antibody used for all the experiments with topo II in which the isoenzyme is not specified. For topo I a polyclonal human antibody from Topogen (2012) was used. Antibodies were diluted in PBS containing 0.1% Tween 20 and 1% BSA. 18511 (topo IIα) and αCT (topo II α+β) were used at a 1:50 dilution, 18513 (topo IIβ) at 1:200, and 2012 (topo I) at 1:1000. For topo II (α, β, and α+β), the anti-rabbit FITC-conjugated second antibody (1262), from Sigma, was used at 1:200 dilution. For topo I, the goat anti-human FITC-conjugated second antibody (F5512), from Sigma, was used at 1:50 dilution.

Preparation of Slides. The slide preparation method is described in detail by Willmore et al.[25] Briefly, cells were seeded (3×10^4 cells/well) into six-well tissue culture plates. These were grown for ∼48 h, and drug was added to exponentially growing cells at appropriate concentrations. Microscope slides were precoated with agarose, and drug-treated or control (untreated) cells were immediately embedded in agarose and spread onto the slide. Slides were then placed in lysis buffer containing protease inhibitors for 30 min (after this stage slides could be stored at −20 °C in PBS containing 10% glycerol), followed by 30 min in 1 M NaCl plus protease inhibitors. Slides were then washed three times in PBS (5 min/wash) and exposed to primary antisera for 1 to 2 h. Slides were washed three times in PBS containing 0.1% Tween 20 (PBST) and subsequently exposed for 1 to 2 h to a secondary antibody (anti-rabbit fluorescein isothiocyanate (FITC)-conjugated secondary antibody, $F(ab')_2$ fragment; Sigma) diluted in PBST containing 1% w/v BSA. Slides were washed three times in PBST followed by an overnight wash in PBS containing protease inhibitors, at 4 °C.

Quantitation of Complexes. Slides were stained with Hoechst 33258 (10 μM in PBS; Sigma Chemical Co.) for 5 min, and cover slips were applied and secured. Images of blue (Hoechst-stained DNA) fluorescence and green (FITC-stained covalently bound topo II) immunofluorescence were then captured with an epifluorescence microscope attached to a cooled slow scan charge-coupled device camera. For each of eight randomly chosen fields of view, images of blue and green fluorescence were captured to give a total of ∼100 cells/dose for each antibody. Images were then analyzed to quantify the levels of Hoechst (blue) fluorescence and FITC (green) immunofluorescence with Imager 2 software (Astrocam, Cambridge, UK) based on Visilog 4 (Noesis, Paris, France). All images were corrected for stray light and camera background. Additionally, images were subjected to blue and green shade correction to compensate for variation in intensity of illumination and nonuniformities in light transmission.[25]

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