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SHORT COMMUNICATION

An Investigation into the Formation of *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide (DACA) and 6-[2-(dimethylamino)ethylamino]-3-hydroxy-7H-indeno[2,1-C]quinolin-7-one dihydrochloride (TAS-103) Stabilised DNA topoisomerase I and II Cleavable Complexes in Human Leukaemia Cells

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ABSTRACT. The antitumour agents DACA (XR5000; *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide) and TAS-103 (6-[2-(dimethylamino)ethylamino]-3-hydroxy-7*H*-indeno[2,1-*c*]quinolin-7-one dihydrochloride) have been shown to inhibit two essential nuclear enzymes *in vitro*, DNA topoisomerase I and DNA topoisomerase (topo) II. To examine whether DACA or TAS-103 stabilise topo I, topo IIα, and topo IIβ cleavable complexes in human leukaemia CCRF-CEM cells, the TARDIS assay (trapped in agarose DNA immunostaining) was used. This assay can reveal drug-stabilised topo–DNA complexes formed *in situ* in individual cells. The results showed that both DACA and TAS-103 can stabilise topo IIα cleavable complexes in these cells. Topo IIβ cleavable complexes were also formed, but only at high concentrations of DACA and TAS-103. The effect on topo I was less clear, with TAS-103 showing only low levels of cleavable complex formation and DACA having no detectable effect under these assay conditions. This is in contrast to the purified enzyme cleavable complex assay, where both DACA and TAS-103 poisoned topo I. Although both DACA and TAS-103 show a preference for topo IIα in whole cells using the TARDIS assay, the formation of low levels of topo I or topo IIβ cleavable complexes may still play a role in cell death. BIOCHEM PHARMACOL **60**;6:817–821, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. TARDIS assay; topoisomerase I; topoisomerase II; DACA; TAS-103; cleavable complexes

DNA topoisomerases are essential enzymes for DNA synthesis and cell division [1], and are known to be good targets for anticancer drugs. DNA topoisomerase (topo[‖]) poisons act by stabilising the normally transient cleavable complex stage of the catalytic cycle primarily by inhibiting the rate of DNA religation by the enzyme. Many topo poisons are in clinical use or in clinical trials. Two such drugs in phase II clinical evaluation are DACA and TAS-103. While most topo drugs target either topo I (e.g. CPT analogues) or topo II (α or β) (e.g. VP-16), DACA and TAS-103 have been demonstrated to be poisons of both topoisomerase I and II [2, 3], putting them in the same class of "joint inhibitors" as intoplicine and saintopin [4–6]. This dual targeting may have an advantage over poisons of topo I or II by simultaneously targeting these two enzymes which have different roles within the cell [1, 7] and by circumventing mechanisms of drug resistance due to alteration of a single target enzyme [8, 9].

In order to examine whether these joint inhibitors target both topo I and topo II in whole cells, studies with the recently published TARDIS assay [10, 11] were carried out. This method can directly measure topo I, topo IIα, and topo IIβ drug-stabilised cleavable complexes in individual cells. Briefly, cells are exposed to drug and then embedded in agarose on microscope slides. The cells are then lysed to remove the cell membrane and soluble proteins. Extraction with 1 M NaCl is used to remove non-covalently bound

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‖ *Abbreviations:* DACA, (XR5000; *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide); TAS-103, 6-[2-(dimethylamino)ethylamino]-3-hydroxy-7*H*-indeno[2,1-*c*]quinolin-7-one dihydrochloride; topo, DNA topoisomerase; FITC, fluorescein isothiocyanate; TARDIS, trapped in agarose DNA immunostaining; CPT, camptothecin; and VP-16, etoposide.

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nuclear proteins. This process leaves the DNA from each cell trapped in agarose along with any drug-stabilised cleavable complexes associated with the DNA. The covalently bound topo can then be detected *in situ* by immunofluorescence.

MATERIALS AND METHODS

Cell Culture

CCRF-CEM human acute lymphoblastic leukaemia cells were grown in RPMI-1640 supplemented with 3 mM L-glutamine, 10% foetal bovine serum, 50 U/mL of penicillin, and 40 μg/mL of streptomycin at 37° in a humidified atmosphere containing 5% CO_2. The cell line was tested for contamination with mycoplasma and found to be negative.

Drug Treatments

CPT (dissolved in DMSO) was purchased from ICN and VP-16 (dissolved in methanol) was purchased from Sigma. TAS-103 and DACA (both dissolved in DMSO) were supplied by Xenova. Exponentially growing cells ($\sim 5 \times 10^5$) were exposed to either of the four drugs for 2 hrs. Following drug exposure, cells were washed twice by centrifugation (400 × *g*, 3 min) in cold phosphate-buffered saline.

TARDIS Assay

The method for quantifying topo I and II cleavable complexes has been described previously [10–12]. For quantification of topo I cleavable complexes, slides were stained with a polyclonal antibody (2012) from TopoGen and a secondary FITC-conjugated goat anti-human immunoglobulin (Ig) G (Fab-specific) antibody from Sigma.

Cleavage of Labelled Plasmid DNA

Cleavage reactions were carried out as described previously [2]. Briefly, linearised ^{32}P-labelled pBR322 plasmid DNA was incubated with topoisomerase I (Life Technologies) for 10 min at 37° in the presence of varying concentrations of DACA, TAS-103, or CPT. Following SDS/proteinase K digestion, DNA was electrophoresed in 1% agarose and exposed to X-OMAT film to allow visualisation of cleavage events.

RESULTS AND DISCUSSION

CCRF-CEM cells were exposed to a range of concentrations of TAS-103 (0.1 to 10 μM) or DACA (0.1 to 100 μM) for 2 hr and then analysed using the TARDIS assay to detect drug-stabilised topo I, topo IIα, and topo IIβ cleavable complexes in individual cells. In addition, cells were exposed to CPT (100 μM) or VP-16 (100 μM) for 2 hr to act as controls for poisoning of topo I or topo IIα and β, respectively (data not shown). These cells were sensitive to DACA and TAS-103 with IC_{50} values of 474 and 5 nM, respectively, as determined by cytotoxicity assays looking at reduction in viable cell numbers relative to controls over a 5-day incubation period of constant exposure (data not shown).

Figure 1 shows scattergrams of CCRF-CEM cells exposed to DACA or TAS-103 in which each dot represents the integrated fluorescence level from a single cell. At 0.1 μM TAS-103 (Fig. 1a), levels of topo IIα FITC immunofluorescence were significantly higher than in untreated cells ($P < 0.0001$, Mann–Whitney Test) and further increases were seen at 1.0 and 10 μM TAS-103. As shown in Fig. 1b, there appears to be little change in topo IIβ FITC immunofluorescence levels at 0.1 and 1.0 μM; however, at 10 μM levels were significantly higher than in untreated cells ($P = 0.0055$). Low but significant levels of topo I FITC immunofluorescence were observed in cells exposed to all doses of TAS-103 (Fig. 1c). Similar results for TAS-103 were obtained in a replicate experiment (data not shown). Comparable levels of FITC immunofluorescence were produced by 0.1 μM TAS-103 and CPT in CCRF-CEM cells when exposed to these drugs for 2 hr. However, no significant increase in FITC immunofluorescence was seen with increasing concentrations of TAS-103, unlike CPT, which produced a dose-dependent increase in FITC immunofluorescence levels. This resulted in a 4.5-fold increase in FITC immunofluorescence levels observed with 10 μM CPT as compared to 10 μM TAS-103 [11].

DACA showed a dose-dependent increase in topo IIα FITC immunofluorescence levels (Fig. 1d), with 10 and 100 μM DACA giving significantly higher levels of cleavable complexes than the untreated cells ($P < 0.0001$). Only the highest concentration of 100 μM DACA showed any effect on topo IIβ FITC immunofluorescence ($P < 0.0001$) (Fig. 1e). No significant increase in topo I FITC immunofluorescence was detected at DACA concentrations up to 100 μM under these conditions (Fig. 1f).

Maximal responses seen with DACA and TAS-103 using the TARDIS assay were far lower than those seen with the controls, 100 μM VP-16 and 100 μM CPT (data not shown). This *in vivo* difference in the level of cleavable complex formation may be due to several factors, including the way in which the different molecules interact with the target. For example, both DACA and TAS-103 are strong intercalators and may inhibit topoisomerases primarily through their interaction with DNA [3, 13]. However, VP-16 is thought to have minimal interaction with DNA but still acts at the topoisomerase/DNA interface [14]. The optimum incubation period for maximum formation of cleavable complexes may also be different for the drugs tested.

The TARDIS assay is a specific and sensitive test for topoisomerase poisons and this preliminary study clearly shows that both DACA and TAS-103 can target topo IIα in whole cells. For both test compounds effects on topo IIβ were lower, with significance only being reached at higher

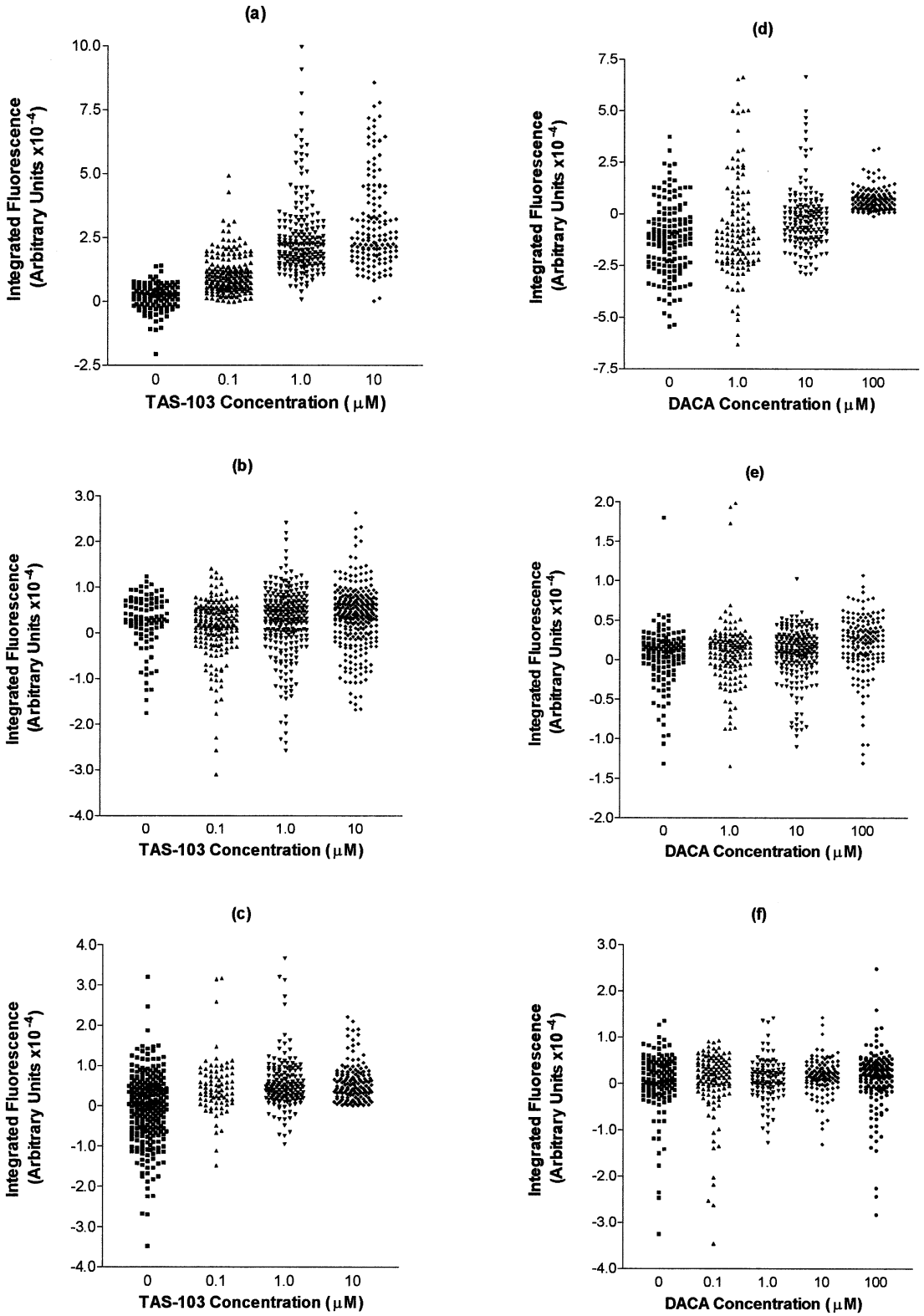

FIG. 1. Immunofluorescent quantification of TAS-103- and DACA-stabilised cleavable complexes in individual CCRF-CEM cells. CCRF-CEM cells were exposed to 0, 0.1, 1.0, and 10 μM TAS-103 for 2 hr or 0, (0.1 μM for topo I only), 1.0, 10, and 100 μM DACA for 2 hr. Slides were then prepared and stained with the appropriate primary antiserum and secondary FITC-conjugated antibody. (a), (b), and (c) represent scattergrams of topo IIα, topo IIβ, and topo I FITC immunofluorescence levels in cells exposed to TAS-103, respectively. (d), (e), and (f) represent scattergrams of topo IIα, topo IIβ, and topo I FITC immunofluorescence levels in cells exposed to DACA, respectively. The data shown are the results of one experiment.

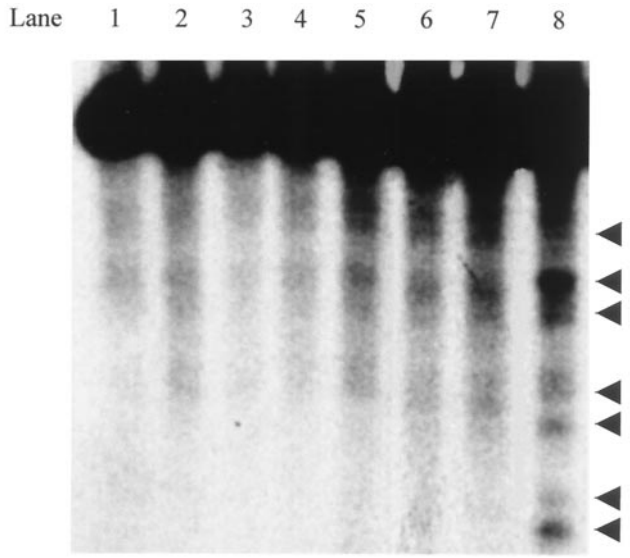

FIG. 2. Stimulation by DACA and TAS-103 of topo I-induced cleavage of ^{32}P-labelled linearised pBR322. Fifteen units of bovine thymus topo I (Life Technologies) and 4 × 10^6 cpm ^{32}P-labelled linearised pBR322 DNA were incubated in the presence or absence of the drugs DACA, TAS-103, or CPT. Lane 1 contains DNA alone; lane 2, DNA and topo I; lane 3, DNA, topo I, and DMSO (solvent control). Lanes 4 to 8 all contained DNA, topo I, and DMSO and lane 4, 0.1 μM TAS-103; lane 5, 0.3 μM TAS-103; lane 6, 0.1 μM DACA; lane 7, 0.3 μM DACA; lane 8, 1.0 μM camptothecin. Arrow heads indicate major cleavage points.

concentrations relative to those required for topo IIα. The effect on topo I was less clear. TAS-103 showed low (though significantly different) levels of topo I cleavable complex formation. With DACA, the topo I cleavable complexes were not above background under these conditions. These data are in agreement with that reported previously for TAS-103 using the caesium chloride/immunoblotting assay, which showed that at 3 μM TAS-103 both topo I- and topo II-stabilised cleavable complexes were formed [3].

DACA was recently tested in the transformed yeast strain JN394t2-4 (which is permeable to topo II poisons and carries a DNA repair mutation) with a plasmid expressing either topo IIα or topo IIβ [15]. Topo IIα transformants were found to be more sensitive (DACA IC_{50} = 50 μM) than topo IIβ transformants (DACA IC_{50} > 137 μM). This suggests that, although more potent against topo IIα than topo IIβ, DACA can still cause cytotoxicity through topo IIβ. No such data are available for topo I yeast transformants. Using a similar yeast genetic system, Byl *et al.* [16] have shown that TAS-103 does not kill yeast via topo I, but rather exerts its cytotoxic effects via topo II.

In a cell-free system using purified topo I, it was possible to detect cleavage of ^{32}P-labelled plasmid DNA in the presence of DACA or TAS-103 (Fig. 2). This confirms previous observations using similar cell-free assay systems that DACA and TAS-103 can target both topo I as well as topo II [16–18]. However, this dual targeting was not seen with DACA using the TARDIS assay. In addition, the cell-free system shows that DACA is more effective than TAS-103 in producing DNA cleavage with topo I (Fig. 2). This may be explained in part by the whole cell assay being a far more complex system relative to the purified enzyme assay. For example, both the DNA sequence and genomic localisation of topo poisons have been shown to vary [7]. Accessibility of these regions in CCRF-CEM whole cells may vary between drugs, resulting in the decreased ability of DACA to form topo I cleavable complexes as compared to TAS-103. In addition, in the cell-free system, the cleavage reaction was carried out for 10 min as compared to cleavable complex formation, which was measured after 2 hr using the TARDIS assay. It is possible that the topo I cleavable complex formation was not maximal with DACA after 2 hr and therefore was not detected. For example, the levels of CPT-stabilised cleavable complexes in K562 human leukaemia cells was significantly lower after 2 hr as compared to the levels detected after 1.5 hr of continuous drug exposure [11]. Recently, it has been shown that the reported inhibition of topo I-catalysed DNA relaxation by TAS-103 resulted from a drug-induced alteration in the apparent topology of the nucleic acid substrate [19].

Thus, it must be concluded that when the TARDIS assay is used, DACA and TAS-103 have a selectivity for topo IIα and are more effective in forming cleavable complexes with this enzyme than with topo IIβ or topo I. Nevertheless, this selectivity for topo IIα does not exclude the possibility that low levels of cleavable complexes formed with topo IIβ or topo I cleavable complexes may be involved in causing cell death.

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