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Mechanism of action of the dual topoisomerase-I and -II inhibitor TAS-103 and activity against (multi)drug resistant cells

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Abstract TAS-103 is a recently developed dual inhibitor of topoisomerase-I (topo-I) and topoisomerase-II (topo-II). TAS-103 has documented cytotoxicity in vitro and antitumor activity against a variety of mouse, rat, and human xenografts in vivo. *Purpose*: To determine TAS-103 activity against (multi)drug resistant cells in vitro and to delineate its mechanism of action. *Methods*: TAS-103 was evaluated for activity against three human multidrug-resistant cell lines representing resistance mediated by P-glycoprotein (Pgp)-, multidrug resistance protein (MRP), and lung resistance protein (LRP) as well as one camptothecin-resistant cell line associated with a mutated topo-I enzyme. Drug sensitivity following short (2 h), intermediate (6–8 h) and long term (24 h) exposures were compared. The mechanism of action was studied by evaluating inhibition of topoisomerase-I and -II specific DNA relaxation assays, drug-induced DNA/protein cross-link formation, and competitive DNA intercalation with ethidium bromide. *Results*: Increasing the exposure time only modestly potentiated TAS-103 cytotoxicity (3–5 fold) demonstrating a lack of strong exposure duration dependency. TAS-103 cytotoxicity was not affected by the presence of any of the drug resistance mechanisms studied. TAS-103 inhibits topo-I and -II activity in DNA relaxation assays, but in our assay system TAS-103 was found to have only a weak ability to induce DNA-protein crosslinks. DNA migration patterns in agarose gel electrophoresis indicate that TAS-103 can interact directly with DNA. Also its ability to displace ethidium bromide which has intercalated into the DNA provides an indication on the nature of drug-DNA interaction. *Conclusions*: TAS-103 cytotoxicity is not affected by the presence of Pgp, MRP, LRP or mutations in the CAM binding region of the topo-I enzyme and its growth-inhibitory effect appears to be weakly dependent on exposure duration. The presented evidence suggest that the inhibitory effects of TAS-103 on topo-I and -II may in part be related to its DNA binding rather than primarily through stabilization of topo-I or -II intermediates with DNA through specific binding to the enzymes.

Key words Topoisomerase-I · Topoisomerase-II · Drug resistance · Intercalation

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Introduction

DNA topoisomerases play a key role in controlling the topological structure of the DNA helix. There are two classes of DNA topoisomerases, class I and II, which differ in their functions and mechanisms of action [16, 26]. Class I topoisomerase (topo-I) enzymes act by making a transient break in one DNA strand allowing the DNA to 'swivel' and release torsional strain. Class II topoisomerase (topo-II) enzymes make transient breaks in both strands of the DNA allowing DNA strand passage which will result in 'unknotting' of the DNA. In case of a deficiency of topo-I, part of its function can be compensated for by topo-II enzyme [21].

The archetypal topo-I inhibitor camptothecin specifically inhibits the catalytic activity of the topo-I enzyme by stabilizing the DNA-topo-I complex formation. Once these stabilized complexes are encountered by moving DNA replication forks, through a cascade of events they result in lethal DNA double strand breaks [9].

Because of their potent antitumor activity in preclinical models topo-I-interactive drugs have been evaluated extensively. As a result many factors contributing

to the efficacy of this class of drugs have been recognized leading to the development of new agents with improved pharmacokinetic and pharmacodynamic properties compared to the camptothecin prototype. Currently, promising responses in traditionally poor responding tumors such as colorectal cancer are becoming available from clinical trials with Irinotecan (CPT-11) [18, 25].

One of the characterized mechanisms associated with resistance against topo-I interactive agents is a decreased level or activity of the target enzyme [13, 22]. Since the topo-I enzyme function is essential to very basic cell survival requirements such as DNA replication, transcription, and repair processes, commonly a decreased activity of topo-I is compensated for by topo-II [21, 22].

TAS-103 [6-[[2-(dimethylamino)ethyl]amino]-3-hydroxy-7H indeno [2.1-c]-quinolin-7-one dihydrochloride is a recently developed inhibitor of both topo-I and topo-II [23]. The dual targeting by this drug may be an important contributing factor in its high reported efficacy in vitro and in vivo and may additionally be useful for circumventing resistance mechanisms against topo-I-interactive agents associated with decreased topo-I activity.

In the present study the susceptibility of TAS-103 for resistance mechanisms associated with expression of P-glycoprotein, multidrug resistance protein, lung resistance protein (LRP) and mutated Topo-I was investigated. Additionally, exploratory studies were performed to reveal the mechanisms of action behind the dual target inhibition by this drug.

Materials and methods

Cell lines

Selection and culture conditions of all the resistant cell line models have been described previously. The human ovarian carcinoma cell lines A2780 and A2780-Dx5b [15] represent Pgp-mediated MDR with the A2780-Dx5b cell line displaying a 35-fold resistance to doxorubicin compared to its parental cell line. The human fibrosarcoma cell lines HT1080 vs HT1080/Dr4 [19, 20] represent MRP-mediated MDR with a 180-fold resistance to doxorubicin of the HT 1080/Dr4 cell line compared to the HT 1080 cell line. The human myeloma cell lines 8226 and 8226/MR20 [5] represent LRP-mediated MDR. The 8226/MR20 cell line is 100-fold resistant to doxorubicin. The human leukemia cell line CEM-C2 [4] contains a topo-I enzyme with a mutation in the binding region of camptothecin which confers a resistance of 1000-fold to camptothecin compared to its parental cell line.

Drug

TAS-103 (6-[[2-(dimethylamino)ethyl]amino]-3-hydroxy-7H indeno [2.1-c]-quinolin-7-one dihydrochloride) (Fig. 1) was provided by Taiho Pharmaceutical Co., LTD, Japan. Stock solutions of 10 mg/ml were prepared in sterile water and kept at 4 °C for up to 2 months without loss of activity.

Growth inhibition

Drug sensitivity was evaluated following short (2 h), intermediate (6 h), and long term (24 h) exposures. Cell survival of the A2780 and HT1080 cell lines was assessed by the sulphorhodamine B assay. Cells in exponential growth were seeded at a density of 1000 cells/well in 96-well microtitre plates (Falcon, Becton Dickinson Labware, Plymouth, U.K.) and allowed to attach overnight. The monolayers were exposed to TAS-103 24 h later. Following drug exposure, cells were washed, then incubated in drug-free medium. Evaluation of treatment was carried out four cell doubling times after drug treatment. Cells were then fixed with trichloro acetic acid, washed and stained with sulphorhodamine B. Absorbance was measured at 570 nm using a 96-well plate reader (EL340 BIO Kinetics Reader, BIO-TEK instruments Inc., Winooski, Vt).

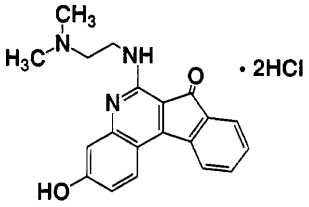

Fig. 1 Structure of TAS-103, 6-[[2-(dimethylamino)ethyl]amino]-3-hydroxy-7H indeno [2.1-c]-quinolin-7-one dihydrochloride

Drug sensitivity in 8226 and CEM cells was determined using the MTT assay. The cells were drug-treated as described above except that drug-treatment was performed in suspension culture at cell densities of 10^6 cells/ml. Cells were seeded at a density of 20,000 cells/well in 96-well plates (Falcon, Becton Dickinson Labware, Plymouth, U.K.) and incubated for 96 h.

All experiments were performed at least in quadruplicate and repeated at least three times. The drug concentrations that inhibited cell growth by 50% (IC_{50}) were obtained from analysis using curve fitting and modeling approaches as described previously [7].

Topo-I and -II catalytic assays

Inhibition of Topo-I and Topo-II catalytic activity was evaluated using a Topoisomerase-I assay kit and the Topoisomerase-II drug screening kit (TopoGEN, INC, Columbus, Ohio). Purified human topoisomerase-I (TopoGEN, Columbus, OH) was used as a source for topo-I in the topo-I assay kit. Assays were performed according to the manufacturer's instructions in the presence and absence of different concentrations of TAS-103. Reaction products were analyzed on a 1% agarose gel in the absence or presence of 0.5 μg/ml ethidium bromide as required by the manufacturer's instructions.

DNA-protein crosslink assay

DNA-protein crosslinks were analyzed following to a previously published method [27] with minor modifications. In brief, 10^6 cells prelabeled for 24 h with 0.025 μCi/ml [^{14}C]-thymidine were treated in suspension for 30 min with TAS-103 at various concentrations. After centrifugation at room temperature, cells were resuspended in 500 μl PBS (65 °C) and lysed by adding an equal volume of 5% SDS/20 m*M* EDTA/0.4 mg/ml salmon sperm DNA. Protein was precipitated by adding 16 μl of a 4 *M* KCl solution after which the pellet was washed three times in 10 m*M* Tris/HCl, 100 m*M* KCl, 1 m*M* EDTA and 0.1 mg/ml salmon sperm DNA. The final precipitate was hydrolysed in 1 ml of ddH_2O (70 °C) to which 90 μl of 12 N HCl had been added. Following a 1 h incubation at 70 °C, radioactivity was determined by liquid scintillation counting.

Flow cytometry

In order to determine the ability of TAS-103 to displace ethidium bromide, cells were incubated first for 30 min with a dose-range of TAS-103, washed and subsequently incubated with ethidium bromide (2.5 μg/ml) for 30 min. Quantitative fluorescence measurements were made using a FACSCAN (Becton Dickinson, San Jose,

Calif.) flow cytometer, using the 488 nm line of a 50 mW argon laser running at 15 mW output for excitation. Ethidium bromide emission was collected through a 650 nm longpass filter. Analysis was performed using the Win List software (Verity Software House, USA).

Results

Influence of several drug resistance mechanisms on TAS-103 growth inhibition

The A2780/Dx5b, HT1080/DR4 and 8226/MR20 cells which display a resistance level of 30-, 180-, and 100-fold, respectively against doxorubicin showed only a 2.25-, 0.25-, 0.91-resistance level against TAS-103. The CEM-C2 cells which have a mutated topo-I enzyme which makes it 1000-fold resistant to camptothecin only showed a 2.8-fold resistance level to TAS-103. The very minor differences observed in drug sensitivity to TAS-103 between the parental cell lines and their drug-resistant counterparts may have been accountable to minor variations in the topo-I and -II enzyme levels. It is obvious however, that the presence of Pgp, MRP, LRP, or a mutated topo-I, which confer high levels of resistance against doxorubicin or camptothecin did not confer high resistance levels against TAS-103. The lack of cross-resistance to TAS-103 in CEM-C2 cells may indicate either the presence of a compensation mechanism mediated by the topo-II enzyme or the binding of TAS-103 to a site different from that for camptothecin. Table 1 summarizes the IC_{50} data obtained following 2 h exposure to the drug.

Effect of exposure duration on TAS-103 cytotoxicity

The effect of exposure duration was evaluated in the A2780, A2780/Dx5b, CEM and CEM-C2 cell lines. For all four cell lines increasing the exposure duration from 2 to 24 h only minimally affected their sensitivity to TAS-103 with a range of differences of approximately 3 to 6 fold differences observed in the IC_{50} values between 2 h and 24 h exposures. Prolonging the drug exposure time affected the parental and resistant cell lines to the same extent. Table 2 summarizes the cytotoxicity data obtained for all four cell lines following the different exposure times.

Table 2 Drug exposure duration-dependent growth inhibition by TAS-103 against drug sensitive and drug resistant cell lines

Cell line	Exposure duration (h)	IC_{50} n*M*	95% Confidence interval	Relative resistance
A2780	2	14.15	10.77–17.52	1
	6	16.75	11.24–22.26	1
	24	4.94	4.62–5.29	1
A2780/Dx5b	2	31.78	28.41–35.18	2.25
	6	16.95	15.47–18.43	1.01
	24	8.17	7.63–8.71	1.65
CEM	2	55.18	48.02–62.31	1
	6	27.60	20.98–34.63	1
	24	10.63	6.62–14.61	1
CEM-C2	2	114.73	62.09–167.38	2.08
	6	67.40	−90.26–225.09	2.44
	24	19.63	11.54–27.72	1.85

In order to determine that the relatively minor contribution of drug exposure time was not related to instability of the drug in growth medium the following experiment was carried out. Two parallel cultures were set up. One was exposed to the drug for 2 h and the other was exposed for 2 h to culture medium plus drug removed from a 24 h exposure cell culture (equal cell number compared to the 2 h culture). The IC_{50} values for 2 h 'fresh' and 2 h 'old' medium were 14.76 n*M* and 24.61 nM, respectively. Thus, the potency of the drug after exposure to cell culture for 24 h was diminished by only 1.67-fold.

Inhibition of Topo-I catalytic activity by TAS-103

The ability of TAS-103 to inhibit topo-I catalytic activity in a cell-free system was compared with that of the standard topo-I inhibitor camptothecin. On an equimolar basis TAS-103 was found to be 10-fold more potent than camptothecin. Figure 2 shows an example of a direct comparison between TAS-103 and camptothecin with regard to cytotoxicity against a cell line (A2780 wt) following a short term (2 h) exposure. Figure 3 demonstrates that at concentrations < 2.46 μ*M* TAS-103 had no effect on the ability of topo-I to transform super coiled DNA into several topoisomer forms of relaxed DNA. However, at concentrations more than 2.46 μ*M*, TAS-103 inhibited the formation of DNA topoisomers

Table 1 Drug sensitivity of representative drug-resistant cell line models to TAS-103 following a 2-h drug exposure

Resistance mechanism	Cell line	IC_{50} n*M*	95% Confidence interval	Relative resistance
P-glycoprotein	A2780	14.15	10.77–17.52	1
	A2780/Dx5b	31.78	28.41–35.18	2.25
Multidrug resistance protein	HT1080	3623.95	2954.21–4293.68	1
	HT1080/Dr4	908.55	801.22–1015.73	0.25
Lung resistance protein	8226	2229.25	−46445.59–50904.09	1
	8226/MR20	2029.99	1637.62–2422.36	0.91
Mutated topo-I	CEM	55.18	48.02–62.31	1
	CEM-C2	114.73	62.09–167.38	2.08

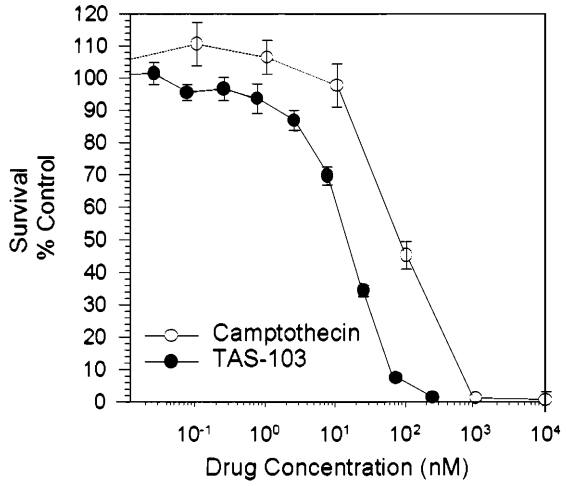

Fig. 2 Growth inhibitory effect of TAS-103 and camptothecin against A2780 wt cells following a 2 h drug exposure

but also affected migration of the DNA. In contrast, the effects of camptothecin were limited to inhibition of topoisomer formation at higher drug concentrations without affecting the DNA migration.

Inhibition of Topo-II catalytic activity by TAS-103

The inhibitory effects of TAS-103 on topo-II catalytic activity was compared with that of the standard topo-II inhibitor etoposide (VP16). Like VP16, TAS-103 at concentrations more than 2.46 μ*M* inhibited the formation of relaxed DNA from the supercoiled DNA substrate (Fig. 4). However, unlike VP16 the inhibition by TAS-103 does not coincide with the formation of linear DNA species as would be expected for agents

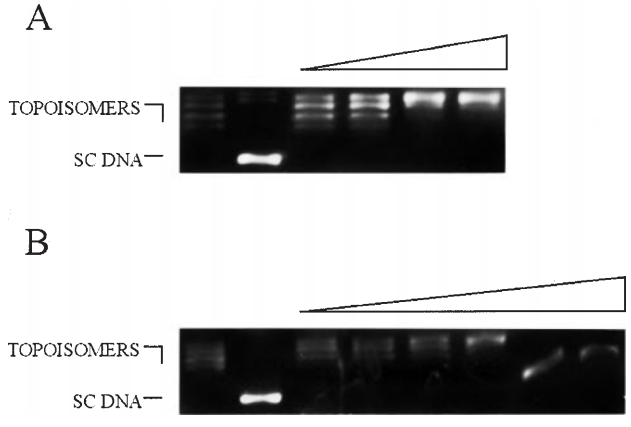

Fig. 3 Inhibitory effects of camptothecin (1a) and TAS-103 (1b) on the catalytic activity of topoisomerase I. The first two lanes represent a positive (topoisomers) and negative (super coiled DNA) control, the subsequent lanes represent the topo-I catalytic activity in the presence of increasing concentrations of each drug (*A*: Camptothecin 0.3 μ*M*, 3.0 μ*M*, 30 μ*M*, 300 μ*M*; B: TAS-103 24.6 n*M*, 246.0 n*M*, 2.46 μ*M*, 24.6 μ*M*, 246.0 μ*M*)

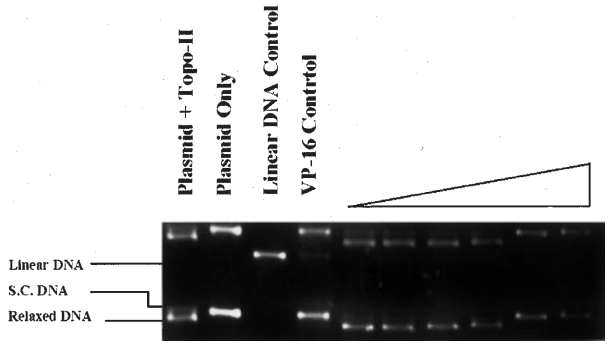

Fig. 4 Inhibitory effects of TAS-103 on the catalytic activity of topoisomerase II. The first four lanes represent controls; *lane 1*: plasmid + topo-II (positive control for topo-II activity), *lane 2*: plasmid only (negative control), *lane 3*: linear DNA (positive control), *lane 4*: topo-II activity in the presence of VP-16 (positive control for inhibitory action of a topo-II inhibitor). *Lanes 5–10*: topo-II activity in the presence of increasing concentrations of TAS-103 (2.46 n*M*, 24.6 n*M*, 246.0 n*M*, 2.46 μ*M*, 24.6 μ*M*, 246.0 μ*M*)

stabilizing the complex formation between topoisomerase-II and DNA (see lane 4; VP-16 control).

TAS-103 induced DNA-protein cross-link formation compared to camptothecin

The ability of TAS-103 to stabilize topo-I-DNA cross linking was evaluated using the A2780 cell line and was compared with that of camptothecin. As expected, camptothecin induced a dose-dependent increase in the amount of DNA-protein crosslinks as reflected by the increasing amounts of precipitable radioactivity following protein precipitation. It is well known that in order to demonstrate the DNA/protein crosslinks in this system, drug concentrations several magnitudes higher than the cytotoxic drug concentration range are required. In contrast to the effect of camptothecin, TAS-103 does not show significant DNA/protein cross-linking in this system. For comparison, the arrows in Figure 5 correspond with approximate IC_{90} values of each of the drugs following a 2 h drug exposure (Fig. 2). Thus whereas camptothecin induced DNA-protein crosslinking at concentrations less than 10-fold the IC_{90}, TAS-103 produces cross-linking, however, only at concentrations up to 1000-fold the IC_{90}.

Competitive DNA intercalation between TAS-103 and ethidium bromide

The ability of TAS-103 to interfere with the intercalating properties of ethidium bromide was studied in A2780 cells. Before incubation with ethidium bromide, cells were preincubated for 30 min with a dose range of TAS-103. Following the (30 min) incubation with ethidium bromide, cellular fluorescence was quantitatively

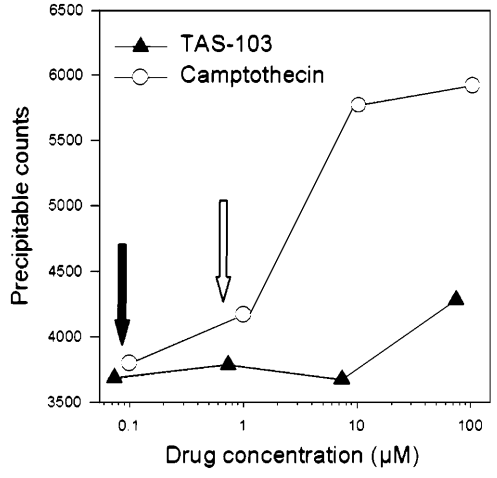

Fig. 5 Formation of DNA-protein crosslinks by camptothecin and TAS-103 as detected by SDS/KCl precipitation following 30 min drug exposure of A2780 cells. The arrows indicate equicytotoxic concentrations (IC_{90} for 2 h exposure)

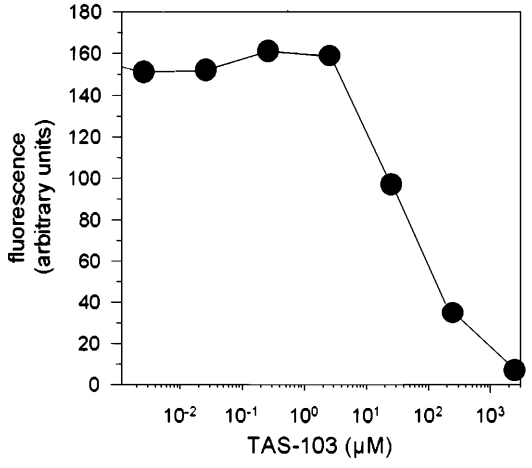

Fig. 6 Concentration dependent effect of TAS-103 pre-incubation on ethidium bromide intercalation in A2780 cells as determined by flow cytometry

assessed by flow cytometry. The data in Fig. 6 demonstrate that at concentrations more than 2.46 μ*M* TAS-103 reduces the ability of ethidium bromide to intercalate into the DNA of A2780 cells.

Discussion

One rationale for the combination of drugs in cancer chemotherapy is based on the idea that two or more drugs would be more successful than either one of the drugs alone with the premise that each drug attacks different intracellular targets. Because of their central role in DNA replication, transcription and repair processes, the topoisomerases are attractive targets for cancer chemotherapy especially since overexpression of these proteins has been demonstrated in several cancers although some cancers overexpress topo-I while others overexpress topo-II [6, 10, 11, 17, 24]. Topo-I expression is relatively stable throughout the cell cycle whereas topo-II expression varies with cell cycle progression (highest during G_2/M phase) [1, 8]. Because of its stable expression topo-I may be a more attractive target for chemotherapy than topo-II since potential cell cycle dependent mechanisms of drug escape may be avoided. One of the mechanisms associated with resistance against topo-I interactive drugs is a decreased activity of the topo-I [13, 22]. In some cases, decreased topo-I activity is compensated for by topo-II [21, 22]. The reciprocal relationship between the topoisomerase enzymes has initiated studies combining specific topo-I- and topo-II-interactive drugs but with reported results varying from antagonistic to additive and synergistic effects [3, 12, 14], the jury is still out on the best schedule of administration.

The quinoline derivative TAS-103 was developed as an anticancer agent targeting both topo-I and -II [23]. TAS-103 shows marked efficacy against a broad spectrum of human tumors in vitro and in vivo. In the present study the susceptibility of TAS-103 to several well-characterized drug resistance mechanisms was assessed. It was demonstrated that the presence of the MDR-associated proteins Pgp, MRP and LRP did not affect TAS-103 cytotoxicity. Monospecific topo-II inhibitors such as doxorubicin and VP-16 are common substrates for these resistance associated proteins, therefore, TAS-103 appears to have an advantage over a treatment approach combining a topo-I-specific inhibitor with a topo-II specific inhibitor. Notably, TAS-103 cytotoxicity appears relatively independent from drug exposure duration, this in contrast to many mono-specific topo-I inhibitors, the cytotoxicity of which generally increases with longer exposure times.

It is apparent from this study and others that TAS-103 indeed inhibits the catalytic activity of both topoisomerase-I and -II, but it appears to achieve this through mechanistically different ways than prototypical topo-I or topo-II drugs such as camptothecin and etoposide. Camptothecin causes a dose-dependent decrease in the DNA topoisomerase formation indicating inhibition of the topo-I catalytic cycle, which in the assay used results in formation of nicked-open circular DNA (Fig. 3a). The results of the topo-I assays for TAS-103 appears similar to that of camptothecin at lower concentrations, but at high concentrations more than 2.46 μ*M*, unlike camptothecin, TAS-103 alters the electrophoretic mobility of the DNA (Fig. 3b). In the topo-II assay, VP-16 inhibits relaxation of supercoiled DNA which is accompanied by the formation of linear DNA species which is used as a parameter for cleavable complex formation (Figure 4). In contrast, although TAS-103 like VP-16 inhibits the relaxation of supercoiled DNA this process is not accompanied by the formation of linear DNA species. This indicates that TAS-103 in this cell-free system did not stabilize the complex between topo-II and DNA.

The SDS/KCl precipitation data following camptothecin or TAS-103 exposure in a whole cell system indicate that TAS-103 inhibition of topo-I was not primarily related to stabilization of the complex between topo-I and DNA since protein-DNA complexes could not be detected in the assay used. These functions appear to contradict previously published data regarding TAS-103 that the formation of cleavable complexes between topo-I or topo-II and DNA can be demonstrated by means of immunoblotting following TAS-103 exposure of KB cells [23]. However, from the previously published data it is apparent that at equimolar concentrations (3 μM was used), TAS-103 produces lower DNA-protein crosslinks than camptothecin. Thus, if one would consider the immunoblotting assay as more sensitive than the SDS/KCl precipitation assay, this finding is consistent with the data in Fig. 5 in which camptothecin at 3 μM already has induced detectable DNA-protein crosslinks while TAS-103 has not. Unfortunately, the immunoblotting data have only been published for a single TAS-103 concentration (3 μM) so it is not possible to determine whether the immunoblot assay would detect a dose-dependent increase of DNA-protein crosslink formation following TAS-103 exposure.

The results presented here suggest that DNA binding of TAS-103 is a possible mechanism through which both topo-I and -II are inhibited and possibly trapped. The suggestion of DNA binding is provided not only by the results of the topo-I and -II catalytic assays but also by the competitive inhibition of ethidium bromide intercalation. It is possible that the previously used immunoblotting assay were able to detect trapped topo-I and -II at a detection threshold which is lower than that of the SDS/KCl protein precipitation assay. It should be noted however that the well-established DNA intercalator (and topo-II inhibitor) amsacrine does produce linear DNA species in the same topo-II assay as used in our experiments (TopoGen, manual for topoisomerase II drug screening kit) whereas TAS-103 does not. With the currently available data we cannot explain why this difference exists but a possible explanation could be that amsacrine actually binds the topo-II enzyme to the DNA while TAS-103 does not or to a lesser extent. The revealing of actual specific binding sites of TAS-103 to topo-I and topo-II would allow the determination whether the dual enzyme inhibition is a consequence of the drug-DNA interaction or of specific binding to the enzymes.

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