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ORIGINAL ARTICLE

The role of topoisomerases and RNA transcription in the action of the antitumour benzonaphthyridine derivative SN 28049

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Abstract
Purpose SN 28049 (N-[2-(dimethylamino)ethyl]-2,6-dimethyl-1-oxo-1,2-dihydrobenzo[b]-1,6-naphthyridine-4-carboxamide) is a DNA intercalating drug that binds selectively to GC-rich DNA and shows curative activity against the Colon 38 adenocarcinoma in mice. We wished to investigate the roles of topoisomerase (topo) I, topo II and RNA transcription in the action of SN 28049.
Methods We used clonogenic assays to study the cytotoxicity of SN 28049; RNA interference and enzyme assays to examine the role of topo I in SN 28049 action; ^{3}H uridine incorporation and reporter assays to study its effects on transcription; and RT-PCR to examine its ability to reduce endogenous h-TERT expression.
Results In clonogenic assays, SN 28049 showed a biphasic cytotoxic dose response curve in H460 cells typical of acridine derivatives such as N-[2-(dimethylamino) ethyl] acridine-4-carboxamide (DACA) although it was ~16-fold more potent. Down-regulation of topo IIα in HTETOP cells reduced the cytotoxicity of SN 28049, establishing its action as a topo IIα poison. Surprisingly, down-regulation of topo I in H460 cells by RNA interference sensitised them to the actions of SN 28049 and other topo II poisons. SN 28049 also inhibited topo I-mediated relaxation of supercoiled plasmid DNA. SN 28049 was also an inhibitor of transcription in HEK293 cells and was more potent at reducing luciferase expression from a GC-rich SP-1 binding promoter than from a non-GC-rich AP-1 binding promoter. The drug also reduced luciferase reporter gene expression driven by the SP-1-binding survivin promoter as well as reducing endogenous h-TERT expression in HEK293 cells whose promoter also contains SP-1 binding sites.
Conclusion We conclude that SN 28049 has a complex action that may involve poisoning of topo IIα, suppression of topo I and inhibition of gene transcription from promoters with SP-1 sites. These actions may contribute to the promising experimental solid tumour anticancer activity of SN 28049.

Keywords Topoisomerase · siRNA · Transcription · DACA · Topo poisons

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Introduction

Despite recent progress in the chemotherapy of cancer, success in advanced metastatic cancers is still very limited and drugs with novel actions are needed. Considerable attention has been paid to the development of small molecule drugs affecting signal transduction pathways [1] but relatively little attention has been paid to drugs that act by selective modification of gene transcription. Actinomycin D was one of the first DNA binding drugs to be used clinically and is known to bind very selectively to DNA rich in guanine-cytosine base pairs (GC-rich DNA), but suffered from the disadvantage of being highly susceptible to multidrug

resistance [2–4]. Mithramycin A and related analogues constituted a second class of antibiotic cytotoxins that bound by a novel mechanism to GC-rich DNA [5] but clinical trials demonstrated limited clinical activity, probably because of multiple cellular actions, poor pharmacological properties and susceptibility to multidrug resistance. N-[2-(dimethylamino)ethyl]acridine-4-carboxamide (DACA), a compound in which the acridine moiety provides DNA intercalation ability and the carboxamide side chain provides GC-selectivity, was first synthesised in this laboratory and has undergone phase I and II clinical trials [6–13], but low dose potency and an unusual form of toxicity prevented further development [14]. DACA had effects on both topoisomerase (topo) I and topo II [15, 16] but a variety of evidence suggested that the principal target was topo IIα [15, 17, 18].

A large number of compounds incorporating a DNA binding chromophore and a sequence-selective carboxamide side chain have been produced [19]. Of particular interest is a series of benzonaphthyridine derivatives, of which SN 28049 is the principal example (structure shown in Fig. 1). Several compounds in this series were able to induce complete regression of subcutaneous implants of the Colon 38 adenocarcinoma, a tumour which is relatively resistant to topo II-directed drugs such as amsacrine, doxorubicin and etoposide [20]. SN 28049 is ~50-fold more potent than DACA in vitro and 20-fold more dose-potent in vivo. Studies in this laboratory have shown that SN 28049 also binds more strongly to DNA and is more GC-selective than DACA (unpublished results). This report describes our investigation of the effect of SN 28049 on both topo activity and RNA synthesis. Modulation of topo IIα expression was carried out using the HTETOP cell line, in which the endogenous *TOPOIIα* gene is disrupted and transcription of an exogenous *TOPOIIα* cDNA is controlled by tetracycline transactivation [21, 22], while modulation of topo I expression was carried out using siRNA. The effect of this drug on gene expression was also carried out using reporter assays and RT-PCR. The results indicate that both DNA topo I and II and RNA polymerases are of potential relevance to its action.

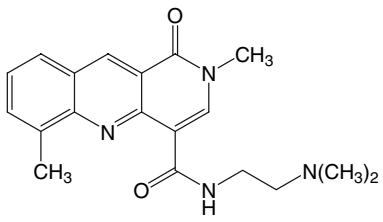

Fig. 1 Structure of SN 28049 (N-[2-(dimethylamino)ethyl]-2,6-dimethyl-1-oxo-1,2-dihydrobenzo[b]-1,6-naphthyridine-4-carboxamide)

Materials and methods

Materials

SN 28049 was synthesised in this laboratory and supplies for these studies were kindly provided by Mr. Graham Atwell. Amsacrine and DACA were also synthesised in this laboratory. Aclarubicin, camptothecin, penicillin and streptomycin were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and doxorubicin and etoposide were purchased as clinical vials (Farmitalia Carlo Erba, Clayton North, Australia and Bristol-Myers Squibb, Noble Park, VIC., Australia, respectively). Drugs were prepared as 2 mM stock solutions; SN 28049, DACA, aclarubicin and amsacrine in 50% v/v aqueous ethanol, and camptothecin in dimethyl sulphoxide. Drug solutions were stored at −20°C and clinical vials were stored at 4°C. Tetracycline was purchased from Calbiochem, EMD Biosciences Inc. (La Jolla, CA., USA) and prepared as a 1 mg/ml stock solution in PBS and stored at −20°C.

Cell lines

The H460 non-small-cell lung carcinoma cell line was purchased from the ATCC (Manassas, VA, USA). HTETOP cells have been described [21] and the HEK293 cells were a kind gift from R Burgeson (CBRC, MGH, Harvard Medical School, Charlestown, MA, USA). H460 cells were cultured in α-modified minimal essential medium supplemented with 5% fetal calf serum, while the HTETOP and HEK293 cells were cultured in D-modified minimal essential medium supplemented with 10% fetal calf serum. All cell lines were passaged weekly using trypsin (0.07% w/v) in citrate-buffered saline.

Clonogenicity assays

Assays were undertaken as described [23]. Briefly, H460 or HTETOP cells in exponential phase growth were exposed to increasing concentrations of cytotoxic agent for 1 h at 37°C, washed free of drug, and plated in triplicate (10^2 cells) in plastic 60-mm dishes. After 10 days of incubation at 37°C (in an atmosphere of 5% CO_2 in air), cultures were fixed and stained using methylene blue in 50% v/v aqueous ethanol. Colonies comprising 50 or more cells were counted. In the case of HTETOP cells, tetracycline (1 μg/ml) was added 24 h prior to treatment (where indicated) to reduce topo II expression. Cells were incubated with cytotoxic drugs (and tetracycline where indicated) for 1 h before being harvested, washed free of drug and tetracycline and assayed for clonogenic survival.

RNAi of topo I

A pool of four pre-designed siRNA molecules ('Smart-Pool') targeting human topo I and a single validated GAPDH siRNA were purchased from Dharmacon Inc. (Lafayette, CO, USA). A 5′-fluorescein labeled control siRNA was purchased from Qiagen GmbH (Hilden, Germany). Oligofectamine was purchased from Invitrogen (Carlsbad, CA, USA). H460 cells in logarithmic-phase growth were harvested with trypsin, and seeded (100,000 cells/well) into the wells of a 24-well plate and allowed to adhere overnight. SiRNA molecules were transfected at the indicated concentrations using Oligofectamine (1 μl per well; final volume 250 μl) in accordance with the manufacturer's instructions. All transfections were carried out in the absence of serum using OPTIMEM medium (Invitrogen, Carlsbad, CA, USA). Four hours post-transfection, 3× FBS supplemented α-MEM medium was added to each well and the cells were allowed to continue growth until harvesting the next day for western blotting analysis and IC_{50} assays.

Western blotting

Cells were recovered 24 h post-transfection by trypsin digestion, washed in phosphate-buffered saline (PBS) and immediately lysed in M-PER mammalian protein extraction reagent in accordance with the manufacturer's instructions (Pierce, Rockford, IL, USA), and a small aliquot was removed for protein concentration determination using the BCA assay (Sigma-Aldrich, Castle Hill, NSW, Australia). Samples were prepared for electrophoresis by adding NuPAGE LDS sample buffer (4×) and NuPAGE reducing agent (10×) and heating at 70°C for 10 min before loading (25 μg protein/ well) in the wells of a 10% pre-cast NuPAGE Novex Bis-Tris gel. All NuPAGE reagents were purchased from Invitrogen (Carlsberg, CA, USA). After electrophoresis, proteins were transferred to a nitrocellulose membrane and the membranes were blocked in PBS containing 0.5% Tween 20 (PBS-T) and 5% non-fat dried milk powder. For protein detection, primary and secondary antibodies were diluted 1:5,000 and 1:10,000, respectively, in PBS-T containing 5% milk powder and incubated overnight at 4°C for primary, and 1 h at room temperature for secondary antibodies. Mouse monoclonal anti-human topo I was purchased from BD Biosciences (San Jose, CA, USA), whereas rabbit polyclonal anti-human topo IIα and anti-GAPDH was purchased from Abcam (Cambridge, UK). Anti-mouse and rabbit HRP-conjugated secondary antibodies were purchased from Santa Cruz (San Diego, CA, USA). For detection, SuperSignal West Pico Chemiluminescent substrate was purchased from Pierce (Rockford, IL, USA), and used in accordance with the manufacturer's instructions.

IC_{50} growth inhibition assays

H460 and HTETOP cells with reduced topo I or II content were prepared and harvested as described and added to the wells of 96-well plates (2,500 cells/ well) containing cytotoxic drug that had already been serially diluted across the plate. The plates were then incubated for 4 days at 37°C. Growth inhibition was assessed by sulforhodamine B staining in three independent experiments. IC_{50}-values were determined from the regression line relating the percentage inhibition to logarithmic drug concentration.

[^{3}H]-Uridine incorporation

H460 cells (5×10^5 per well) were added to the wells of a 96-well plate and incubated overnight. Cytotoxic drugs were added, serially diluted across the plate and [5-^{3}H]-uridine (GE Healthcare, Little Chalfont, Buckinghamshire, UK) was added (0.04 μCi/ well; final volume 150 μl). For 1 h incubations the plates were placed in a waterbath (37°C) under CO_2 (5% in air) to maintain constant temperature during addition of drug, and then incubated for 1 h. For 24 h incubations, the plates were placed into a standard incubator (37°C in 5% CO_2 in air) for 24 h. At the end of the incubation the cells were aspirated onto glass fibre filters using a multiple automated harvester (LKB Wallac OY Beta Harvester, Skatron A/S, Norway). The filter mats were washed for 15 s with water to remove non-adherent material, dried, and the amount of [5-^{3}H]-uridine incorporated into the retained RNA was measured using a LKB Wallace Betaplate scintillation counter (Skatron A/S).

Luciferase reporter assays

The TransLucent AP-1 and SP-1 reporter vectors [pAP1(1)-Luc and pSp1-Luc, respectively] and the TransLucent SURVIVIN Gene Promoter Reporter Vector pSURVIVIN-Pluc were purchased from Panomics (Redwood City, CA, USA). HEK293 cells in log-phase growth were plated into the wells of a 24-well plate (1×10^5 cells/ well) 24 h prior to transfection. Cells were transfected with 0.2 μg reporter plasmid using FuGENE 6 (Roche Diagnostics, Mannheim, Germany). Fresh medium containing drug was added 4 h post-transfection and the cells were then incubated for a further 20 h at 37°C. Luciferase activity was measured using Promega's Luciferase Assay System (Madison, WI, USA) following extraction in Reportasol Extraction Buffer (Merck Biosciences, Darmstadt,

Germany). Reporter activity was normalised to protein content of the cellular extracts as measured by the BCA assay.

Topo I relaxation assay

A topo I drug screening kit was purchased from Topogen Inc. (Port Orange, Fl, USA) and calf thymus topo I was purchased from Invitrogen. Reactions were carried out in accordance with the manufacturer's instructions using 5 U of topo I and have been described [24]. Briefly, reactions were assembled on ice with supercoiled plasmid DNA (0.25 μg) being added to 1× relaxation reaction buffer followed by the addition of drug. Topo I was added last, samples were mixed and incubated for 30 min at 37°C before the reaction was terminated by the addition of prewarmed SDS (37°C; final concentration 1%) and additional proteinase K treatment (final concentration 50 μg/ml) for a further 30 min. Samples were loaded onto 1% agarose gels in TAE buffer [40 mM TRIS acetate buffer (pH 8.0) containing 1 mM EDTA] and electrophoresed at 1.4 V/cm for 14-16 h.

Results

Role of topo II in the action of SN 28049

The H460 human non-small cell lung carcinoma cell line was used to compare the cytotoxicities of SN 28049 and DACA in 1 h clonogenic survival assays (Fig. 2). SN 28049 showed a biphasic dose-response response curve similar to that of DACA, with reduced cytotoxicity at intermediate drug concentrations and higher cell killing at high concentrations. While the maximum degree of cell killing was similar for the two drugs, SN 28049 was more dose-potent with maximal cell killing occurring at 0.125 μM vs. 2 μM for DACA.

To investigate the specific role of topo IIα in the activity of SN 28049, we performed 1 h clonogenic assays in HTETOP human fibrosarcoma cells that were engineered to reduce topo IIα expression in the presence of tetracycline (Fig. 3). SN 28049 again showed a biphasic dose-response response curve similar to that for H460 cells. In cells not treated with tetracycline (topo IIα on) the maximum degree

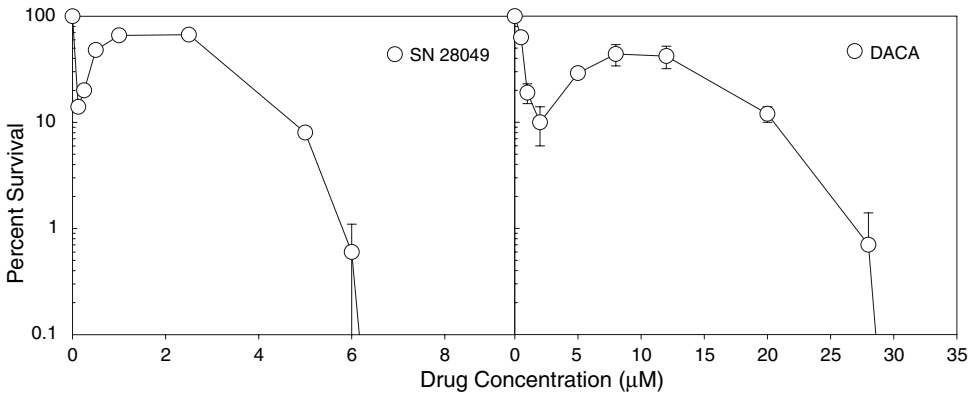

Fig. 2 Survival curves generated for H460 cells following a 1-h period of exposure to SN 28049 and DACA. Data are taken from single experiments plated in triplicate. To facilitate plotting, plates that did not contain colonies were arbitrarily scored at 0.001% survival

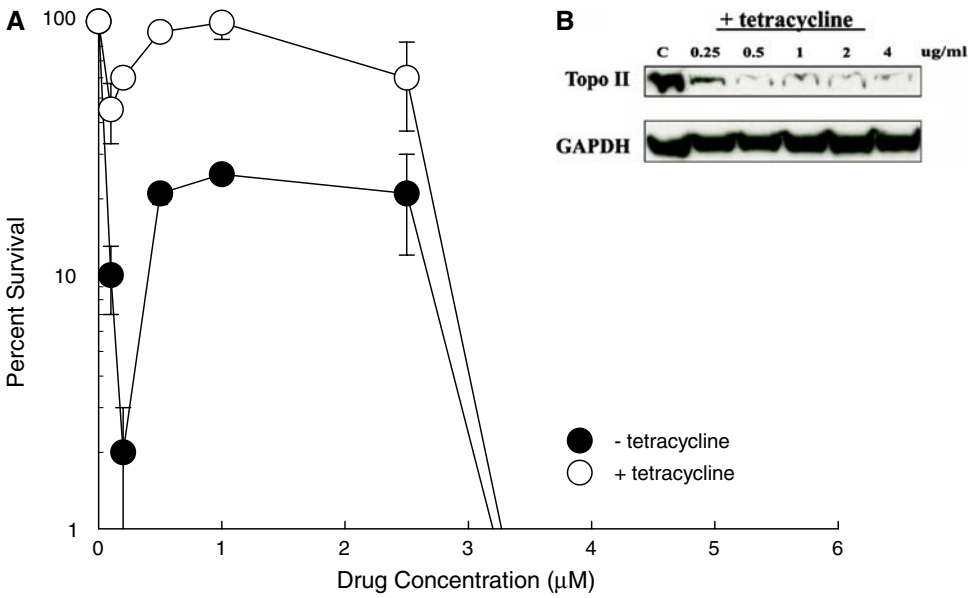

Fig. 3 Survival curves generated for HTETOP cells, with reduced topo IIα content, following a 1 h-period of exposure to SN 28049 (**a**). To reduce topo IIα content, HTETOP cells were exposed to tetracycline (1 μg/ml) 24 h prior to harvesting for the clonogenic assay. **b** Effects on topo IIα content in HTETOP cells following a 24 h exposure to tetracycline, as measured by western blotting

of cell killing (2% survival) occurred at 0.2 μM SN 28049. In tetracycline treated cells (reduced topo IIα) maximal cell killing was reduced to 60% of control. Reduction of topo IIα expression also reduced the effects of the topo II poisons amsacrine and doxorubicin (data not shown).

Growth inhibition assays were also carried out. The IC_{50}-value of SN 28049 for H460 cells was 8.8 nM, and the IC_{50}-values for HTETOP cells for a range of topo poisons are shown in Table 1. Topo II poisons were more than two-fold less active in the tetracycline treated cells, whereas camptothecin and aclarubicin, at resistance ratios of 1.2 and 1.3, respectively, were only slightly less active in the tetracycline treated cells. SN 28049 on the other hand was 1.9-fold less active.

Role of topo I in the action of SN 28049

To investigate the role of topo I in the action of SN 28049 we examined its ability to poison, or inhibit, purified calf thymus topo I-mediated relaxation of supercoiled plasmid DNA. The drug did not induce any detectable nicked open circles in this system suggesting that it did not poison topo I activity (Fig. 4b). However, it did inhibit relaxation activity of the enzyme at concentrations of 1 μM and above (Fig 4a, b).

In an alternative approach to determine whether SN 28049 could function as a topo I poison, we used RNA interference to reduce topo I expression in H460 cells and examined the effect on activity. We hypothesised that if SN 28049 was a topo I poison its activity would be reduced by this treatment. We used a pooled combination of four human topo I-targeting siRNA duplexes (Dharmacon's 'SmartPool') to transfect H460 cells and examined, by immunoblotting, the topo I content in the cells 24 h post-transfection. The results from a representative repeat experiment show that topo I expression was reduced in a dose-dependent manner with the highest reduction (88%) seen in cells transfected with 200 nM topo I-targeting siRNA (Fig. 5). We did not observed reduction in topo I levels in cells transfected with a positive siRNA control targeting GAPDH expression (50 nM; Fig. 5, Lane 3) or in cells transfected with a negative control siRNA (50 nM; Fig. 5, Lane 2). Moreover, in cells transfected with the topo I-targeting siRNA a reduction in the ratio of topo I to GAPDH expression was observed indicating the specificity of the effect. We then examined the consequences of reduced topo I expression (55% using 100 nM siRNA) on

Table 1 IC_{50}-values (nM) for HTETOP cells in the presence and absence of tetracycline

Drug	−Tetracycline	+Tetracycline	Ratio
SN 28049	6.7	13	1.9
DACA	70	170	2.4
Doxorubicin	9.9	25	2.5
Amsacrine	76	270	3.6
Camptothecin	16	19	1.2
Aclarubicin	2.4	3.0	1.3
Etoposide	44	370	8.6

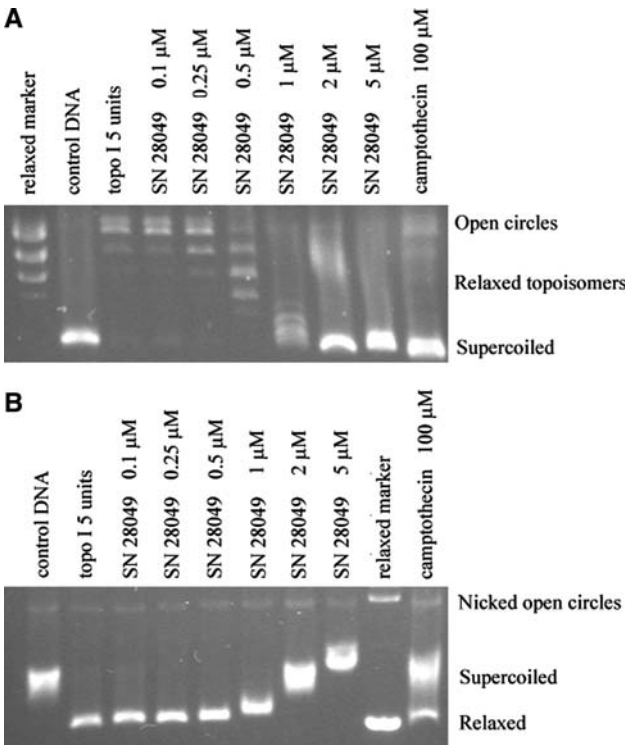

Fig. 4 Suppression of topo I-mediated relaxation of supercoiled plasmid DNA by SN 28049. At the end of the assay sample volumes were halved and analysed on separate 1% agarose gels containing ethidium bromide, 0.5 μg/ml (**a**), to detect nicked *open circles* or without ethidium bromide (**b**), to analyse relaxation activity

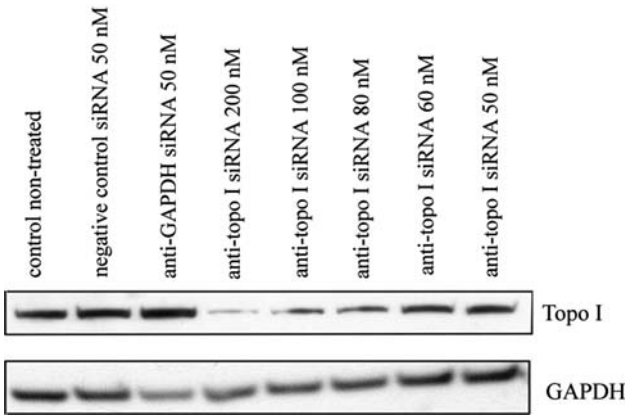

Fig. 5 Western blot analysis of H460 cells transfected with Dharmacon's 'SmartPool' of four siRNA molecules targeting human topo I, 24-h post-transfection. The image was scanned and band intensities measured. Percentage reductions in topo I levels with respect to control cells were 88% at 200 nM, 55% at 100 nM, 49% at 80 nM, 36% at 60 nM and 25% at 50 nM

the activity of a range of topo I and II-targeting drugs including SN 28049 by IC_{50} assays (Table 2). As expected, the topo I poison camptothecin was less active with an IC_{50}-value of 7.5 nM in the topo I reduced cells as compared to an IC_{50}-value of 3.6 nM in the negative-control siRNA transfected cells. The IC_{50}-values for SN 28049, the topo II poisons DACA, amsacrine, doxorubicin and etoposide, and the topo I/II inhibitor aclarubicin were all at least 2.1-fold lower in the topo I reduced cells as compared to the negative-control cells (Table 1). Repeated experiments gave similar results.

Effects on gene expression

We first examined the ability of SN 28049 to inhibit total RNA expression (predominantly ribosomal RNA expression) by examining its ability to inhibit ^{3}H-uridine incorporation in H460 cells. The results showed that SN 28049 was highly potent at inhibiting ^{3}H-uridine incorporation following a 1 h exposure to drug, inhibiting incorporation by 50% at 0.5 μM and by 100% at 10 μM (Fig. 6a). In contrast, following a 24 h exposure to drug (Fig. 6b), while SN 28049 still inhibited incorporation by 90% at 4 μM, at the highest concentration tested, amsacrine (20 μM), inhibited incorporation by only 70%.

We next examined the ability of SN 28049 to inhibit luciferase expression from reporter constructs containing defined transcription factor binding and promoter sequences in transfected HEK293 cells. SN 28049 was significantly more efficient ($P < 0.05$) at inhibiting luciferase expression from SP-1 (GC-rich) sequences than at inhibiting expression from non-GC-rich AP-1 sequences (Fig. 7a, c). Furthermore, the drug was also highly effective at inhibiting luciferase expression from the pSURVIVIN-Pluc construct that contains ~1 kb of the survivin promoter containing multiple SP-1 sites (Fig. 7b) [25]. At equimolar concentrations (50 nM) SN 28049 was more effective at inhibiting luciferase expression than etoposide, DACA and amsacrine (Fig. 7c). It was similar in potency however to doxorubicin and camptothecin.

An inhibitory effect was also observed against endogenous h-TERT expression, whose promoter also contains multiple SP-1 binding sites, as measured by RT-PCR (Fig. 8) [26]. Moreover, SN 28049 was more active in inhibiting h-TERT expression than amsacrine.

No inhibitory effect was seen against the housekeeper GAPDH gene, even when the cycle number of the PCR reaction was reduced from 30 to 24 cycles to be well within the linear amplification range (data not shown). The GAPDH promoter does not appear to contain SP-1 binding sites [27].

Table 2 IC_{50}-values (nM) of topo-directed anticancer drugs in H460 cells following RNA interference of topo I

Drug	Negative control siRNA	Topo I siRNA	Ratio[a]
Amsacrine	13	<3	>4.3
SN 28049	3.3	0.89	3.7
Doxorubicin	4	1.3	3.1
Etoposide	150	46	3.3
Aclarubicin	11	4.5	2.5
DACA	360	180	2.1
Camptothecin	3.6	7.5	0.5

H460 cells were transfected with 100 nM siRNA and harvested 24 h post-transfection for the assay

[a] IC_{50}-value for negative control siRNA treated H460 cells/IC_{50}-value for topo I-silenced H460 cells

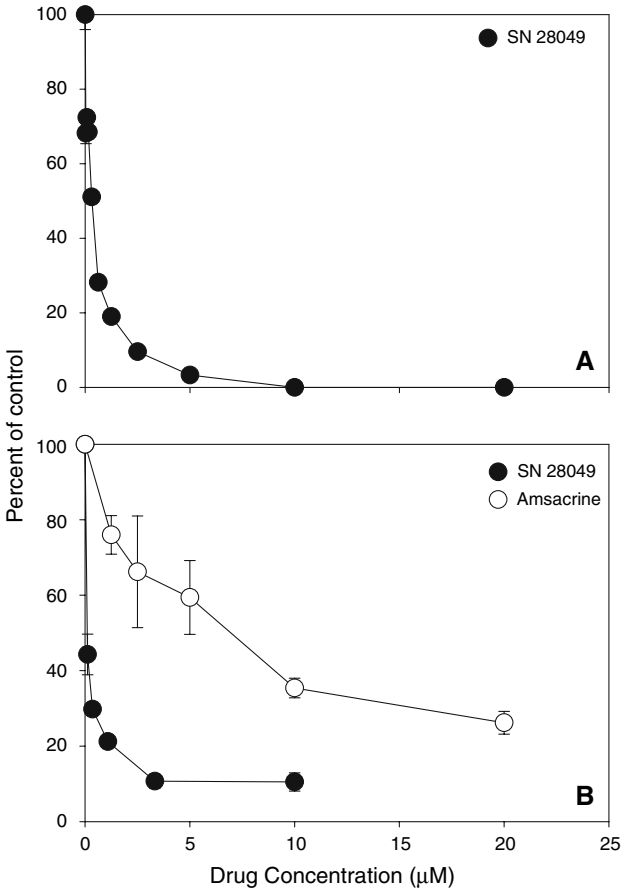

Fig. 6 Inhibition of ^{3}H-uridine incorporation in HEK293 cells by SN 28049 following a 1 h exposure to drug (**a**) and SN 28049 and amsacrine (**b**) following a 24-h exposure to drug

Discussion

In the present study, we investigated the mechanism of action of SN 28049, a GC-selective DNA binding drug with exceptionally high activity against the murine colon

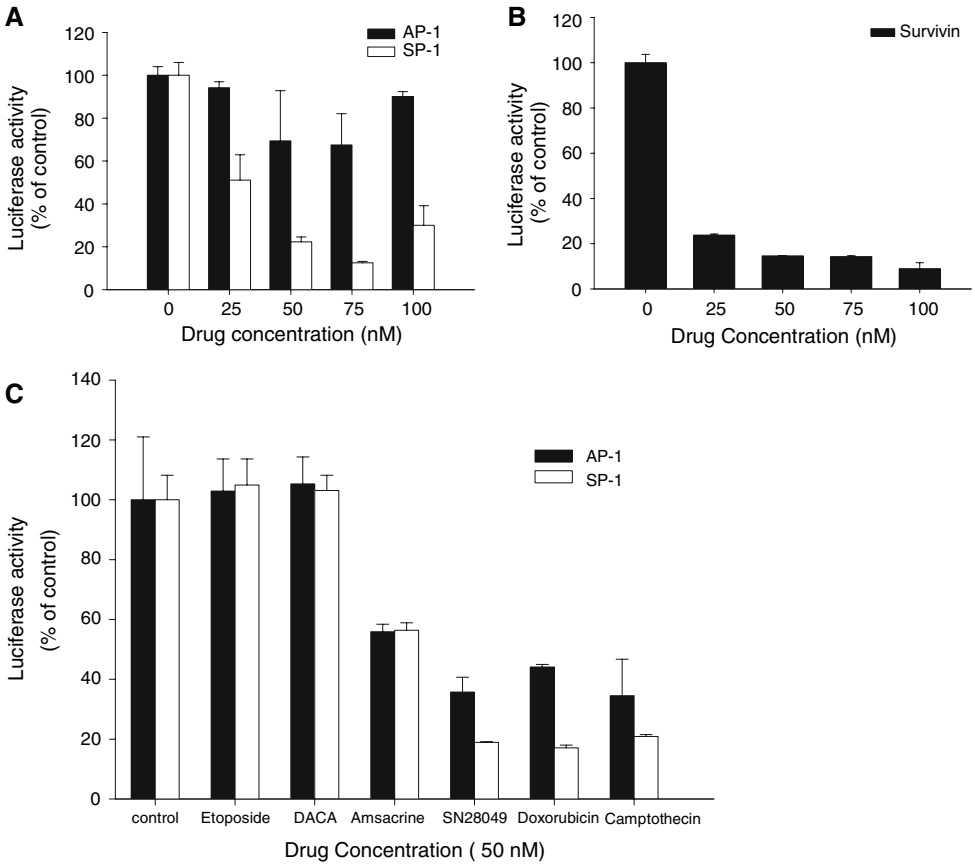

Fig. 7 Reductions in luciferase expression in HEK293 cells transfected with the GC-rich, SP-1 or non-GC-rich AP-1 promoter reporter in the presence of SN 28049 (**a**). Luciferase expression was significantly more reduced from the SP-1 construct than from the AP-1 construct $P < 0.05$. **b** Reductions in luciferase expression in HEK293 cells transfected with a reporter construct containing ~1 kb of the SP-1 regulated survivin promoter. **c** Effects of an equimolar exposure (50 nM) to topo-targeting drugs on SP-1 and AP-1 driven luciferase expression in HEK293 cells. Cells were exposed to drug for 20 h

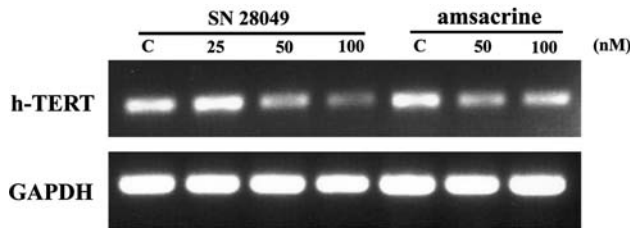

Fig. 8 RT-PCR analysis of the expression of endogenous h-TERT in HEK 293 cells following a 24 h exposure to SN 28049 and amsacrine

tumour Colon 38. Clonogenic survival curves for H460 lung cancer cells exposed to drug for 1 h had an unusual, biphasic shape (Fig. 2), with two phases of dose-dependent cytotoxicity separated by a phase of self-inhibition of cytotoxicity. The shape of the survival curve is very similar to that reported for the antitumour agent DACA [24] except that SN 28049 is ~16-fold more potent (Fig. 2). Previous studies with DACA showed that cell lines with reduced topo II activity failed to show the first phase of dose-dependent cytotoxicity, suggesting that this was topo II-dependent [17]. In this study, we used HTETOP cells which have been engineered such that topo IIα expression is reduced in the presence of tetracycline [21]. We have shown cytotoxicity in response to low concentrations of SN 28049 is strongly reduced when expression of topo IIα is reduced (Fig. 3), confirming and extending previous results. Cell proliferation assays also showed that addition of tetracycline to HTETOP cells decreased sensitivity to DACA, amsacrine, etoposide and doxorubicin, as well as to SN 28049 (Table 1), consistent with the hypothesis that all these drugs target topo IIα.

Previous studies with DACA and analogues with isolated topo enzymes have shown that drug concentrations that poison the activity of topo II (5–10 μM) also inhibit the relaxation action of topo I [24]. SN 28049 inhibits relaxation activity of purified topo I at 2 μM (Fig. 4) suggesting a similar action. More importantly, the data in Table 2 show that inhibition of topo I expression with siRNA sensitises cells to growth inhibition by SN 28049 and other topo II poisons. Yeast cells lacking topo I are hypersensitive to topo II poisons [28, 29] including DACA, possibly because of the requirement for topo II to compensate for the loss of topo I function. To the best of our knowledge this is the first demonstration of this phenomenon in human cells using RNAi. The results suggest a novel action for SN 28049 and DACA; namely that drug-induced suppression of topo I activity acts as a chemosensitiser for topo II-targeted cytotoxicity. This could help explain the in vivo high activity of SN 28049 and DACA against the Colon 38 murine adenocarcinoma in vivo [20, 30].

Our findings also demonstrate that SN 28049 has selective effects on transcription. Overall RNA expression, which probably comprises mainly ribosomal RNA, was inhibited by 50% at a drug concentration of ~0.5 μM at 1 h and 100 nM at 24 h, respectively, after adding drug to cultures (Fig. 6a, b). Expression of luciferase under the control of different promoters was measured under comparable conditions. Expression from a promoter containing non-GC-rich AP-1 sites was only slightly affected at 100 nM, whereas expression from a promoter containing GC-rich SP-1 sites, as well as that from the SP-1 containing survivin promoter, was inhibited at 25 nM by 50 and 80%, respectively. Expression of endogenous h-TERT which also contains SP-1 sites [26], was also inhibited by SN 28049 extending the luciferase results. Moreover, in related work conducted in our laboratory endogenous survivin gene expression was also found to be reduced in HCT116 cells following a 24 h exposure to 50 nM SN 28049, as measured by both RT-PCR and western blotting (C. Drummond et al., manuscript in preparation). Furthermore, at 50 nM, SN28049 strongly inhibited luciferase expression from both the SP-1 and AP-1 promoter constructs, as did camptothecin and doxorubicin, consistent with their known action as potent inhibitors of transcription. Etoposide and DACA, on the other hand, showed little effect at this concentration consistent with their generally weaker effects on transcription (D. J. Bridewell, unpublished). This finding further establishes SN28049 as a strong inhibitor of transcription with effects against GC-rich SP-1 sites.

Our work suggests the presence of novel activities associated with SN 28049 cytotoxicity. We have shown here that SN 28049 is not only a topo II poison and a suppressor of topo I activity, but also has selective effects on gene expression. Other compounds that have dual actions on topo I and II, such as aclarubicin, actinomycin D and the pyrazoloacridine NSC 366140, not only demonstrate strong antitumour activity but are also potent inhibitors of gene transcription [31–35]. The role that perturbations in transcription play in the cytotoxicity of topo targeting drugs is still unclear. Accumulation of p53 and the stimulation of p53-dependent apoptosis following inhibition of transcription has been reported [36–38] demonstrating that disruption of transcription can lead to cytotoxic events. Recently Derheimer et al. [38] have reported that p53 is strongly induced in human diploid fibroblasts when transcription is blocked, even in the absence of DNA damage, indicating the presence of a response pathway to transcriptional blockage. Compounds such as SN 28049 may be particularly efficient inducers of this pathway, culminating in cell death. Interestingly, work in our laboratory has shown that SN 28049 is a more potent inducer of p53 expression than either doxorubicin or etoposide at equitoxic concentrations despite the commonality of topo II targeting among these drugs (C. Drummond et al., manuscript in preparation).

The role of topo I in removing DNA supercoils that form in front of, and behind, advancing RNA polymerases is well known [39, 40]. Recently Koster et al. [41] reported that the accumulation of positive DNA supercoils following exposure to the topo I poison, topotecan, is associated with cytotoxicity [41]. As camptothecin is a potent inhibitor of transcription whereas amsacrine and etoposide (topo II poisons without topo I activity) are not [34, 42–45] disruption of topo I relaxation activity may be a key step in the inhibition of gene transcription. Consequently we hypothesise that topo II poisoning and the suppression of topo I relaxation activity by SN 28049 leads to the accumulation of DNA strand breaks, and possibly DNA supercoils stalling transcription, triggering both DNA damage and transcriptional blockage response pathways at the sites of bound drug. As SN 28049 is a GC-selective DNA binder, with strong activity against SP-1 sites, it is possible that cytotoxic DNA lesions will be localised to GC-rich sequences disrupting the expression of these genes.

Selective inhibition of gene expression may have a significant impact on tumour cell growth and survival [46]. For example, camptothecin exposure leads to selective reduction of expression of EGFR, HIF-1α and c-Myc [47–49], which are genes that strongly promote tumour cell growth and proliferation. Moreover, the bisphenazine XR5944 has been shown to selectively inhibit gene expression from oestrogen receptor binding sites, although not from SP-1 sites, suggesting that topo-targeting compounds that inhibit gene transcription may have unique gene inhibition signatures [50]. Interestingly SP-1 expression itself is highly elevated in many cancers where it appears to be involved in the promotion of tumourigenesis [51, 52]. Disruption of the expression of SP-1 regulated genes by SN 28049 may alter the balance between the expression of pro- and anti-apoptotic survival genes leading to apoptosis [46] and may in part explain the profound loss of viability seen in cells exposed to high concentrations of SN 28049 (Fig. 2). Although further work will be required to determine whether selective or general inhibition of gene expression does indeed form part of the overall mechanism of cytotoxicity of SN 28049 it may be possible to use SN 28049 therapeutically to target high expressing SP-1 cancers. Given that compounds such as actinomycin D, the anthracycline aclarubicin, SN28049, pyrozolacridine, that inhibit topo function and gene transcription all demonstrate strong antitumour activity suggests that mechanisms that disrupt topo function and inhibit gene transcription may be favourable for anticancer activity. These factors may help to explain why the novel DNA binding benzonaphthyridine derivative SN28049 has such high anticancer activity against the Colon 38 carcinoma.

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