Invest New Drugs (2011) 29:1102–1110
DOI 10.1007/s10637-010-9473-8

SHORT REPORT

# Action of SN 28049, a new DNA binding topoisomerase II-directed antitumour drug: comparison with doxorubicin and etoposide

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Received: 3 February 2010 / Accepted: 7 June 2010 / Published online: 22 June 2010


**Summary** *Aim*: We have examined the cellular action of SN 28049 (N-[2-(dimethylamino)ethyl]-2,6-dimethyl-1-oxo-1,2-dihydrobenzo[b]-1,6-naphthyridine-4-carboxamide), a DNA binding drug with curative activity against the Colon 38 transplantable murine carcinoma, on human tumour cells. Its action has been compared with that of two topoisomerase II-targetted drugs, etoposide and doxorubicin. *Methods*: The NZM3 melanoma and HCT116 colon carcinoma cell lines, each expressing wild-type p53, were cultured and responses were compared by flow cytometry, electrophoresis, microscopy, and growth of tumour xenografts. *Results*: Responses of NZM3 cells to all three drugs, as measured by histone H2AX γ-phosphorylation, induction of the p53 pathway and cell cycle arrest, were comparable and typical of those of topoisomerase II poisons. Xenografts of NZM3 cells responded to SN 28049 with a tumour growth delay of 16 days. In contrast, HCT116 cells had an attenuated DNA damage response to the drugs and SN 28049 had no *in vivo* activity, consistent with low topoisomerase II activity. However, SN 28049 inhibited HCT116 cell growth *in vitro* and activated the p53 pathway to induce a state with $G_2$/M-phase DNA content, low mitotic index and a high proportion of binucleate cells. Treated cells expressed cyclin E and the senescence marker β-galactosidase but showed low expression of cyclin B and survivin. In comparison, etoposide caused little p53 expression or cycle arrest, and doxorubicin had an intermediate effect. *Conclusion*: The action of SN 28049 in NZM3 cells is typical of a topoisomerase II poison, but the low topoisomerase IIα activity of HCT116 cells allowed the detection of a second antiproliferative action of SN 28049 in which cells undergo post-mitotic cycle arrest and induction of p53.

**Keywords** Topoisomerase · Chemotherapy · Tetraploid · Mitotic catastrophe · Colon · Melanoma

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## Introduction

Topoisomerase II expression is often elevated in tumour tissue and topoisomerase II poisons take advantage of such expression to induce DNA damage and cytotoxicity. Anthracyclines such as doxorubicin [1] and epipodophyllotoxins such as etoposide [2] are commonly used in the treatment of various forms of clinical cancer, while the DNA binding drug amsacrine (Fig. 1a), developed in this laboratory [3], is useful in second line treatment of acute leukaemia [4]. Subsequent studies in our laboratory have been concerned with the development of further DNA binding topoisomerase II poisons [5] with asulacrine (Fig. 1b) showing evidence of clinical activity in a Phase

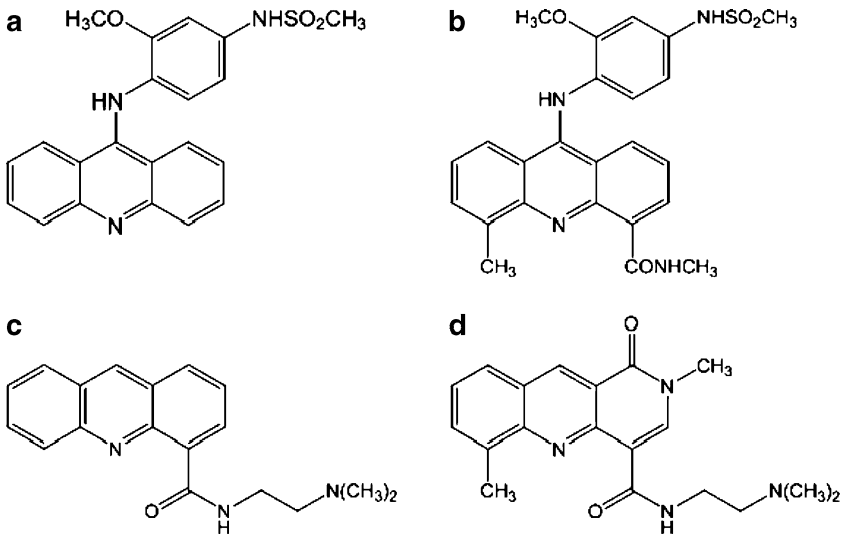


**Fig. 1** Chemical structures of amsacrine **a**, asulacrine **b**, DACA **c** and SN 28049 **d**

I/II clinical trial [6] and *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide dihydrochloride (DACA; Fig. 1c) progressing to Phase I trial [7]. DACA was superior to amsacrine, doxorubicin and etoposide against two murine models of carcinoma, Lewis lung and Colon 38 [8], but had low dose potency and clinical trials were limited by an unusual form of toxicity associated with a burning sensation near the infusion site [7]. Subsequent research in our laboratory has aimed to overcome this side effect by identifying compounds with increased activity and dose potency.

SN 28049 (Fig. 1d), a benzonaphthyridine derivative, not only has 20-fold higher dose potency than DACA in mice but is also the first DNA binding drug in our experience to show curative activity against the murine Colon 38 tumour [9, 10]. SN 28049 also has excellent pharmacological properties in that while its half-life in plasma and in normal tissues is approximately 3 h, its half-life in Colon 38 tumour tissue is 9 h [11]. It binds strongly to DNA with a strong selectivity for G-C rich DNA. The action of SN 28049 was studied in HTETOP human fibrosarcoma cells in which topoisomerase IIα could be down-regulated by addition of tetracycline. SN 28049 induced a 99% reduction of viability in HTETOP wild-type cells but only a 40% reduction when topoisomerase IIα was down-regulated [12], strongly suggesting that topoisomerase IIα is its major enzyme target. Moreover, Jurkat leukaemia cell lines that have developed resistance to doxorubicin or amsacrine [13] have a much reduced topoisomerase II content (Fig. 2) and are cross-resistant to SN 28049 (see Table 1). SN 28049 is currently a candidate drug for Phase I clinical trial.

In this report, we have further explored the action of SN 28049 in human tumour cells, comparing its effects on NZM3, a melanoma line developed in this laboratory [14], with those on HCT116, a colorectal cell line. We have also compared SN 28049 with doxorubicin and etoposide, two clinical agents that target topoisomerase II. The results demonstrate that while all three drugs induce responses in NZM3 cells that are typical of a topoisomerase poison, they do not do so in HCT116 cells. This cell line, which exhibits low topoisomerase II expression, has allowed characterisation of a second molecular action of SN 28049 which differs substantially from etoposide, with doxorubicin exhibiting intermediate properties.

## Materials and methods

### Materials

SN 28049 was synthesised at the Auckland Cancer Society Research Centre and stored as a 2 mM stock solution in 50%

**Table 1** $IC_{50}$ values for SN 28049, doxorubicin and etoposide in HCT116 and NZM3 cells (4-day assays). Comparison is made with previously published data [17] for Jurkat leukaemia cells and its two resistant sub-lines

| Cell line | SN 28049 | Doxorubicin | Etoposide |
|---|---|---|---|
| HCT116 | 8.4±0.5 nM | 10.5±1.0 nM | 210±30 nM |
| NZM3 | 24±0.5 nM | 29±1.0 nM | 1,500±1,000 nM |
| Jurkat | 6.7 nM | 7.0 nM | 160 nM |
| Jurkat/doxorubicin | 53 nM | 112 nM | 14,000 nM |
| Jurkat/amsacrine | 38 nM | 28 nM | 2,100 nM |

ethanol at –20°C. Camptothecin (Sigma Chemical Company) was reconstituted in dimethyl sulphoxide. Doxorubicin (DBL Pharmaceuticals) and etoposide (Bristol-Myers Squibb Pty Ltd) were obtained as clinical formulations and stored at 4°C. Drugs were diluted to working concentrations in culture medium immediately prior to use.

### Cell lines and antiproliferative assays

The HCT116 $p53^{+/+}$ colorectal carcinoma cell line was kindly provided by Dr B. Vogelstein [15]. The NZM3 melanoma line, which also expresses p53 [16], was developed in this laboratory [14]. Both lines were grown in α-modified minimal essential medium supplemented with 10% foetal bovine serum (FBS) and antibiotics. Insulin (10 μg/ml), transferrin (10 μg/ml) and selenite (10 ng/ml) were added to the growth medium for NZM3. Antiproliferative assays were carried out as previously described [17] and $IC_{50}$ values were defined as the drug concentration which reduced net growth at 4 days by 50%.

### Flow cytometric analysis

Cultures were set up at a density of $10^5$ cells/mL and grown overnight. Following drug exposure, cells were harvested and fixed in 70% ethanol at -20°C until analysis. Prior to analysis, samples were removed from ethanol and blocked overnight in phosphate buffered saline (PBS) containing 1% FBS. Where indicated, samples were stained with phospho-specific H2AX primary antibody (ser 139; Upstate) (1:500 in blocking buffer) and an Alexa Fluor 488 conjugated secondary antibody (Invitrogen; 1:400 in blocking buffer). Samples were stained for DNA content by addition of propidium iodide (20 μg/mL) and RNase (100 μg/mL). Fluorescence was measured by flow cytometry on a FACScan flow cytometer (Becton Dickinson) and analysed using Modfit LT software.

### Western blotting

All antibodies for western blotting were obtained from Santa Cruz Biotechnology Inc. and Cell Signalling Technology. Total cellular proteins were extracted (10 mM Tris-HCL pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 1% protease inhibitor cocktail), resolved on 10–12% acrylamide gels and transferred to nitrocellulose membrane. Membranes were probed with antibodies for p53 (sc-6243), $p21^{WAF1}$ (sc-397), survivin (sc-17779), cyclin E (sc-247), cyclin B (sc-245), FAS (sc-715), GAPDH (sc-20357) and Topoisomerase IIα (#4733). Following incubation with horseradish peroxidase conjugated secondary antibodies bands were detected by incubation with chemiluminescence substrate.

### Cell staining

Slides were prepared by cytospin centrifugation (5 min, 500 rpm, low acceleration) using 1-2 drops of cell suspension. When dry, the slides were dipped 5 times, for at least 1 second each time, in each staining solution of a Diff-Quik Staining Set (Dade Behring Laboratory Consumables). Slides were dried at room temperature. Coverslips were mounted onto the slides using Histomount. The slides were viewed at 40X magnification and representative images taken.

### Xenograft experiments

Rag1 immunodeficient mice were bred and housed under conditions of constant temperature and humidity with sterile bedding and food in this institution (Vernon Jansen Unit). All experiments were approved by the University of Auckland Animal Ethics Committee and conformed to the Guidelines for the Welfare of Animals in Experimental Neoplasia, as set out by the United Kingdom Coordinating Committee on Cancer Research. NZM3 and HCT116 cell lines were grown in culture, inoculated subcutaneously ($10^7$ cells/mouse) in one flank, and allowed to grow to a diameter of 4–6 mm before drug treatment. SN 28049 was dissolved in water and injected intraperitoneally Tumour size was measured twice weekly and tumour volumes were calculated as $0.52a^2b$, where $a$ and $b$ are the minor and major axes of the tumour, respectively. Tumour growth delay was determined as the difference in the number of days required for the control versus treated tumours to increase ten to times the initial volume.

## Results

### Relationship of topoisomerase IIα expression to drug inhibition

Cell extracts of the HCT116 line showed much lower expression of topoisomerase IIα than those of the NZM3 line (Fig. 2). Surprisingly, $IC_{50}$ values (concentrations for

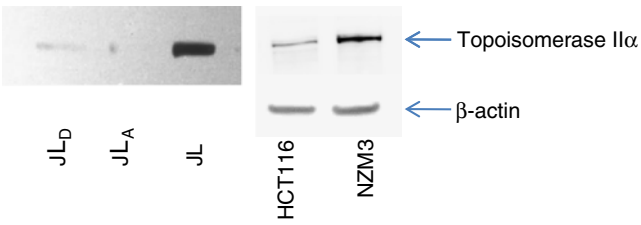


**Fig. 2** Identification of topoisomerase IIα (MW 107 K) in western blots of Jurkat/doxorubicin ($JL_D$), Jurkat/amsacrine ($JL_A$), Jurkat (JL), NZM3 and HCT116 cultures

50% growth inhibition), as determined for SN 28049, doxorubicin and etoposide, were lower for HCT116 cells than for NZM3 cells (Table 1).

## DNA damage responses

The ability of the three drugs to induce DNA damage was compared by measurement of γ-phosphorylation of histone H2AX [18]. Cells were cultured (6 h for NZM3; 5 h for HCT116) in the presence of a range of drug concentrations of SN 28049, doxorubicin or etoposide, then analysed for DNA content and histone γ-H2AX staining by two dimensional flow cytometry (representative results shown in Fig. 3). Clear evidence of DNA damage was obtained in NZM3 cells in response to all three drugs with large increases in relative fluorescence (Fig. 3j, l). In contrast, very little DNA damage was observed in HCT116 cells in response to SN 28049 at all concentrations tested (Fig. 3b), although an increase in the proportion of cells in the $G_2$/M population occurred over this time, indicating that cells were progressing through the cell division cycle. Some DNA damage was observed in HCT116 cells exposed to doxorubicin (Fig. 3d) and etoposide (Fig. 3f) with increases in relative fluorescence of approximately 2-fold, but the response to doxorubicin was less than the 5-fold increase observed in NZM3 cells. For comparison, HCT116 cells exposed for 3 h to the topoisomerase I-directed drug camptothecin (1 μM) showed an S-phase selective DNA damage response with an increase in relative fluorescence of almost 10-fold, consistent with its specificity for cells undergoing DNA replication (Fig. 3h).

## Response of the p53 pathway

Activation of the p53 pathway, which like γ-phosphorylation of histone H2AX is linked to DNA damage by the ATM enzyme complex [19], was compared. The concentration dependence of induced p53 expression was measured in

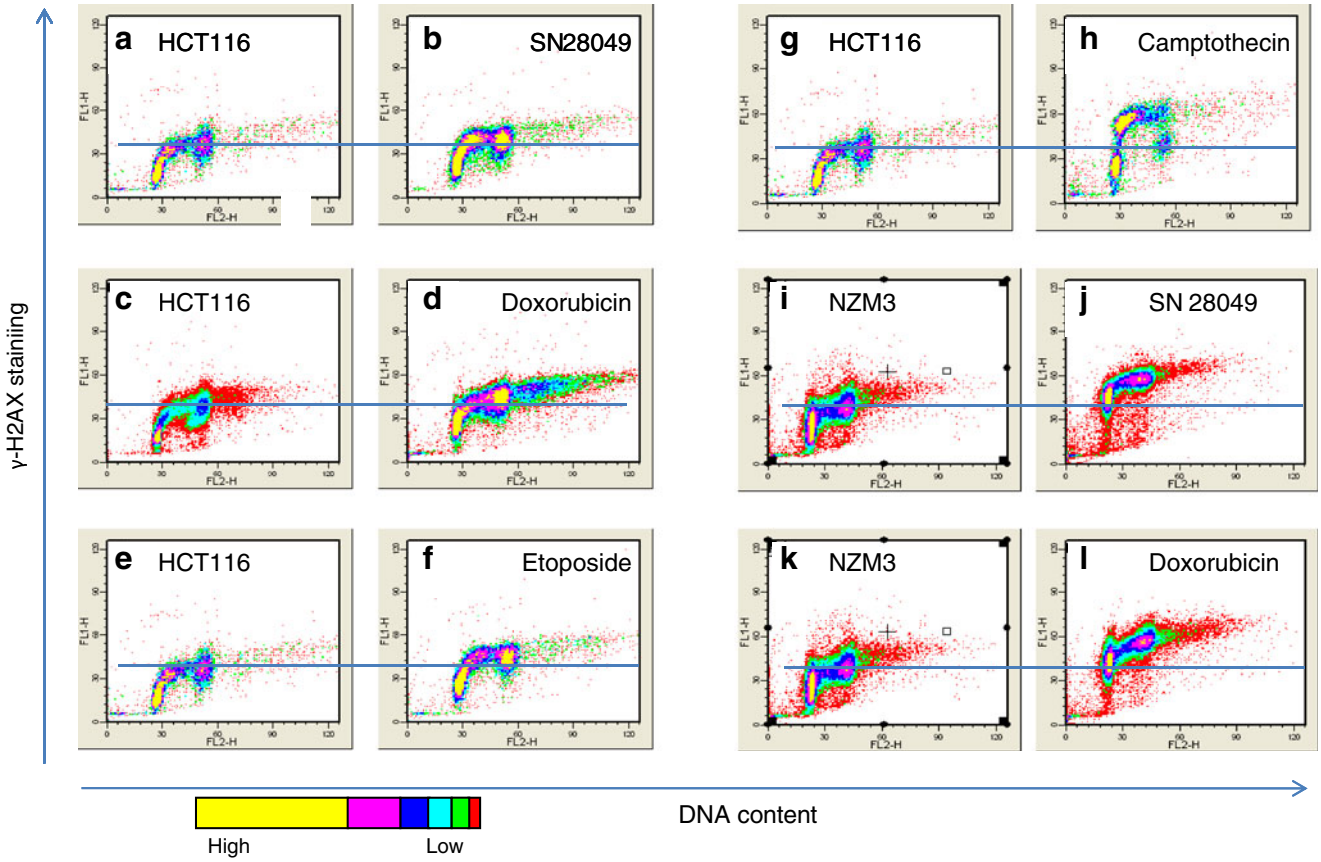


**Fig. 3** Examples of flow cytometry profiles of HCT116 cells exposed to SN 28049 (50 nM) for 5 h **b**, doxorubicin (50 nM) for 5 h **d**, etoposide (1,000 nM) for 5 h **f**, or to camptothecin for 3 h (1 μM; h) and of NZM3 cells exposed to SN 28049 (50 nM) for 6 h **j** or doxorubicin (50 nM) for 6 h **l**. Corresponding profiles for control cells are shown in **a**, **c**, **e**, **g**, **i** and **k**. Cells were analysed for γ-phosphorylation of H2AX by staining with fluorescent antibody (y-axis: logarithmic scale from 1 to 10,000) and for DNA content by staining with propidium iodide (x-axis: linear). The colours of the plot indicate of the relative density of cells in each region. The horizontal line on each graph is drawn through the position of the $G_2$-phase population of each control culture in order facilitate comparison of control and drug-treated cultures

NZM3 cells treated with SN 28049 and doxorubicin for 6 h. Increases were observed for concentrations of 50 nM and 100 nM, respectively, and responses for SN 28049 were larger than those for doxorubicin (Fig. 4b). Treatment of HCT116 cells with SN 28049 (25 nM) induced a time-dependent increase in p53 expression (Fig. 4a). The dependence on SN 28049 concentration was then compared with that of doxorubicin and etoposide following exposure of HCT116 cells for 5 h. SN 28049 induced a clear concentration-dependent increase in p53 expression, while etoposide caused little increase and doxorubicin showed an intermediate response (Fig. 4a). The expression of $p21^{WAF1}$ and FAS, which are transcription products of p53, was also measured in HCT116 cells. SN 28049 induced a large amount of FAS expression and a smaller amount of $p21^{WAF1}$, while etoposide induced small changes and doxorubicin showed intermediate effects (Fig. 5).

## Induction of cell cycle arrest

Since SN 28049 increased p53 expression in both cell lines, we tested whether this was associated with the induction of $G_1$-phase arrest. One method to measure this is to treat with drug in the presence of paclitaxel at a concentration (200 nM) that prevents cell division and to measure the ability of the drug to prevent existing $G_1$-phase cells to move to $G_2$/M phase by flow cytometry [16]. Cells were exposed to SN 28049 at concentrations up to 500 nM for 3 h, washed and resuspended in growth medium lacking SN 28049 but containing paclitaxel (200 nM) for a further 21 h. In the NZM3 line, paclitaxel exposure alone reduced the $G_1$-phase content from 66% to 37% and prior exposure to SN 28049 almost completely inhibited the loss of cells from $G_1$-phase. In the HCT116 line, paclitaxel exposure alone reduced the $G_1$-phase content from 37% to 1% (reflecting a shorter cell cycle time) and prior exposure to SN 28049 failed to inhibit loss of cells from $G_1$-phase (Fig. 6).

## Induction of tetraploid $G_1$-phase arrest in HCT116 cells

To investigate why SN 28049 induced p53 and p21 in HCT116 cells without inducing $G_1$-phase cycle arrest, cells were exposed to drug for 5 h, washed and grown in drug-free medium for a further 24 or 48 h, and analysed by flow cytometry. Cells exposed to SN 28049 at concentrations of 250 nM or higher were found to enter a state with a $G_2$/M-phase DNA content and to remain in this state for at least 48 h. Microscopic examination showed very few mitotic cells but an increased proportion of binucleate cells (Fig. 7). Cells also became positive for the senescence marker β-galactosidase (results not shown).

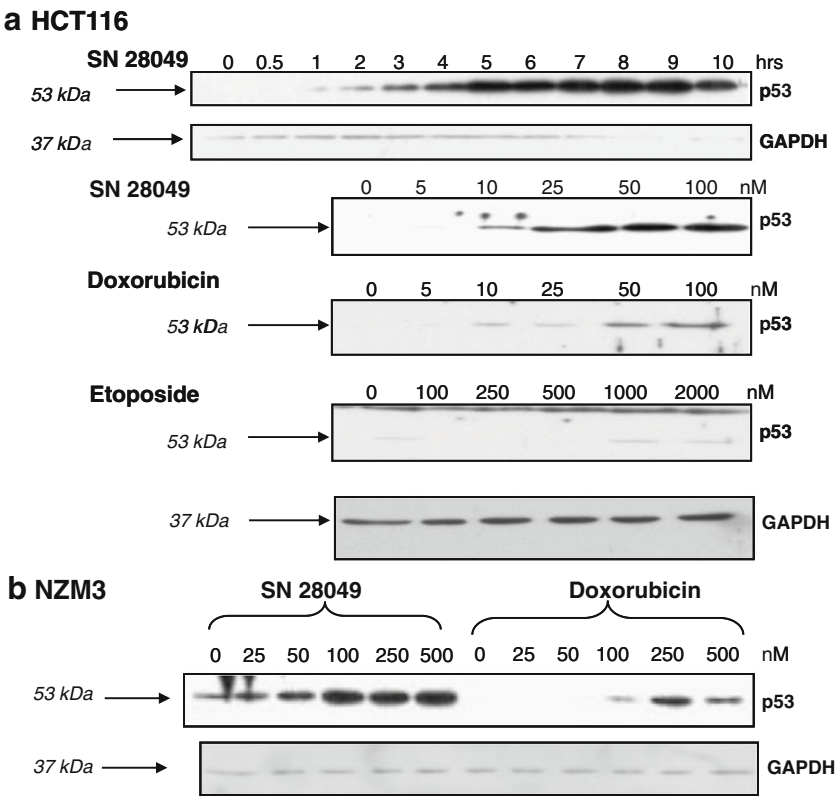


**Fig. 4** **a** Comparison of p53 responses of HCT116 cells exposed to SN 28049 (25 nM) at different times, and to SN 28049, doxorubicin or etoposide at the indicated concentrations for 5 h. **b** Comparison of p53 responses of NZM3 cells exposed to SN 28049 and doxorubicin at the indicated concentrations for 6 h. Relative protein loading is shown by GAPDH expression

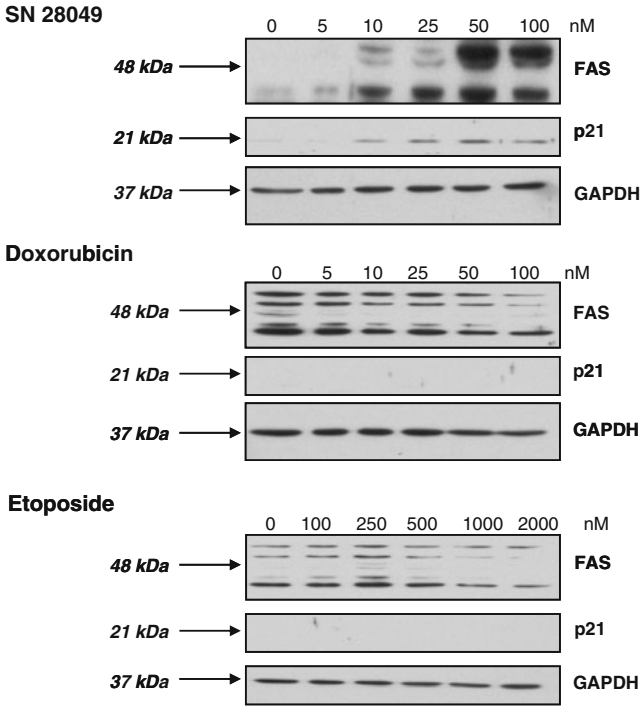


**Fig. 5** Induction of p21 and FAS in response to exposure to SN 28049, doxorubicin or etoposide for 5 h. Relative protein loading is shown by GAPDH expression

HCT116 cells were also treated with SN 28049 for 5 h, washed and grown in drug-free medium for a further 24, 48 or 72 h, and analysed for expression of p53, cyclin B (a $G_2$-phase cyclin), survivin and cyclin E (a $G_1$-phase cyclin). A concentration-dependent increase in expression of cyclin E, as well as a concentration-dependent decrease in expression of cyclin B and of survivin, occurred (Fig. 8). The effects of SN 28049 were also compared with those of doxorubicin or etoposide following a 5-hour drug exposure and subsequent growth in drug-free medium. A concentration-dependent increase in expression of cyclin E, as well as a concentration-dependent decrease in expression of cyclin B, occurred following exposure to doxorubicin, but not following exposure to etoposide (Fig. 8). Flow cytometric analysis of cells grown for 48 h in drug-free medium showed that most cells treated with doxorubicin (250 or 1,000 nM) continued to cycle and were present as an octaploid population. In contrast, cells treated with etoposide and grown for 48 h in drug-free medium were mainly diploid and showed a similar profile to that of untreated cells. Examination after exposure to doxorubicin followed by 48 h in drug-free medium revealed an increased frequency of binucleate cells and decreased frequency of mitotic cells, although the effects were not as marked as with SN 28049.

*In vivo* responses to SN 28049

To determine whether the *in vivo* response matched the *in vitro* results, NZM3 melanoma xenografts were treated with SN 28049 (Fig. 9). The tumour diameters at the commencement of treatment were approximately 5 mm and the xenografts responded with a 16 day growth delay to SN 28049, as compared to 6 days for etoposide. A similar experiment with HCT116 tumour xenografts treated with SN 28049 showed no growth delay (not shown).

## Discussion

SN 28049 is a DNA binding drug whose dominant action has been shown to be as a poison of topoisomerase IIα [12]. Its effects on NZM3 cells are similar to that of the clinical drug doxorubicin, and in fact SN 28049 has similar potency to this drug. Cellular responses, which include the

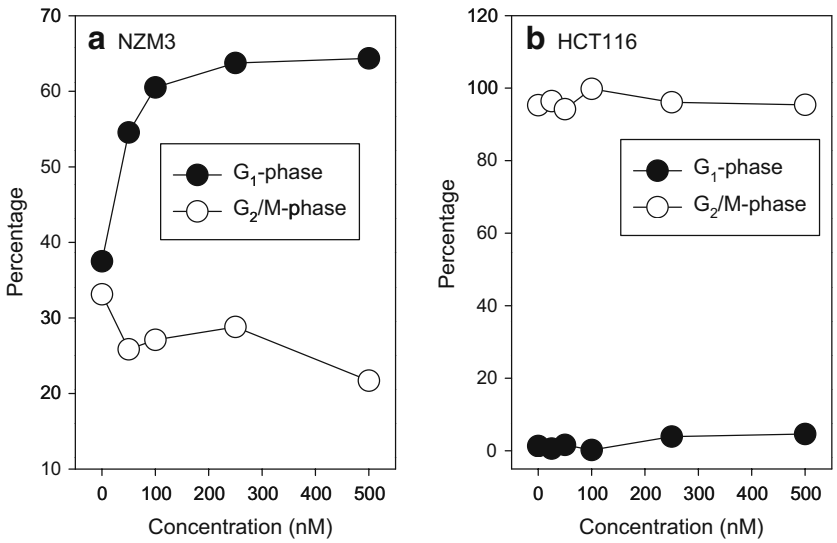


**Fig. 6** Ability of SN 28049 to induce $G_1$-phase arrest in NZM3 cells **a** and HCT116 cells **b**. Cells were exposed to the indicated concentrations of SN 28049 for 3 h and then incubated in the absence of SN 28049 but the presence of 200 nM paclitaxel for 21 h. Percentages of $G_1$-phase cells (●) and G2/M phase cells (○) were determined by flow cytometry

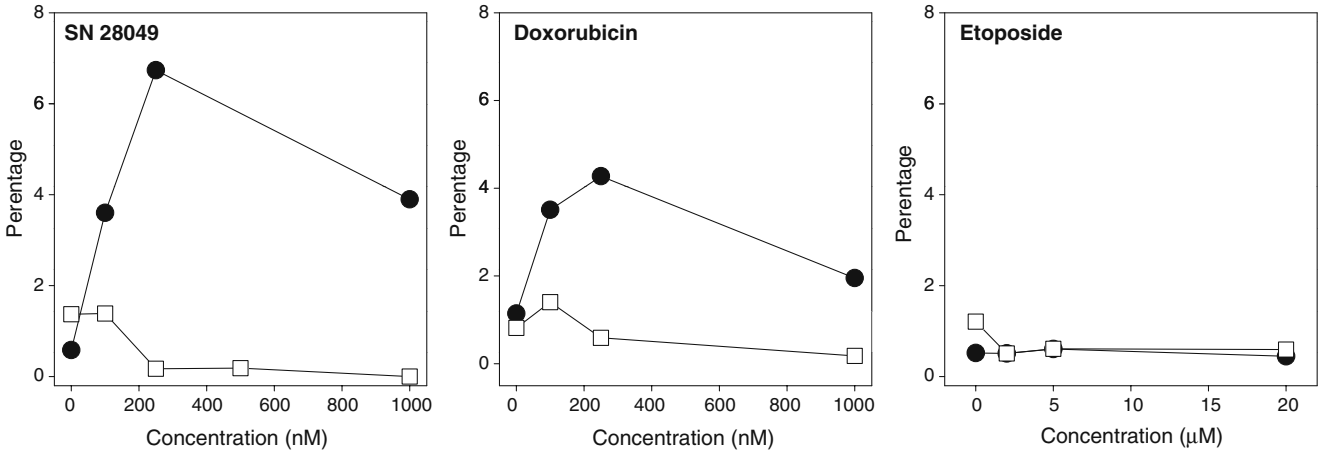


**Fig. 7** Effect of transient (5 h) exposure of HCT116 cells to SN 28049, doxorubicin or etoposide followed by 48 h of culture in the absence of drug on mitotic index (□) and on the incidence of binucleate or multinucleate cells (●)

induction of DNA damage (Fig. 3), accumulation of p53 (Fig. 4) and $G_1$-phase cell cycle arrest (Fig. 6), are typical of a topoisomerase II poison. SN 28049 thus behaves very much like a potent analogue of DACA, which is structurally similar and has also been shown to act as a poison of topoisomerase IIα [20].

In the HCT116 colon tumour line, DNA damage responses to all three drugs tested (SN 28049, doxorubicin and etoposide) were much smaller than those in the NZM3 cell line as judged by γ-H2AX phosphorylation (Fig. 3). However, the responses to doxorubicin and etoposide appeared to be slightly greater than that to SN 28049. The lower topoisomerase IIα content in the HCT116 line (Fig. 2) could provide a reason for the reduced DNA damage responses in comparison to NZM3. A frameshift mutation in exon 17 of the topoisomerase IIα gene, leading to a change in electrophoretic mobility of the protein, has been reported for this line [21], although there was little

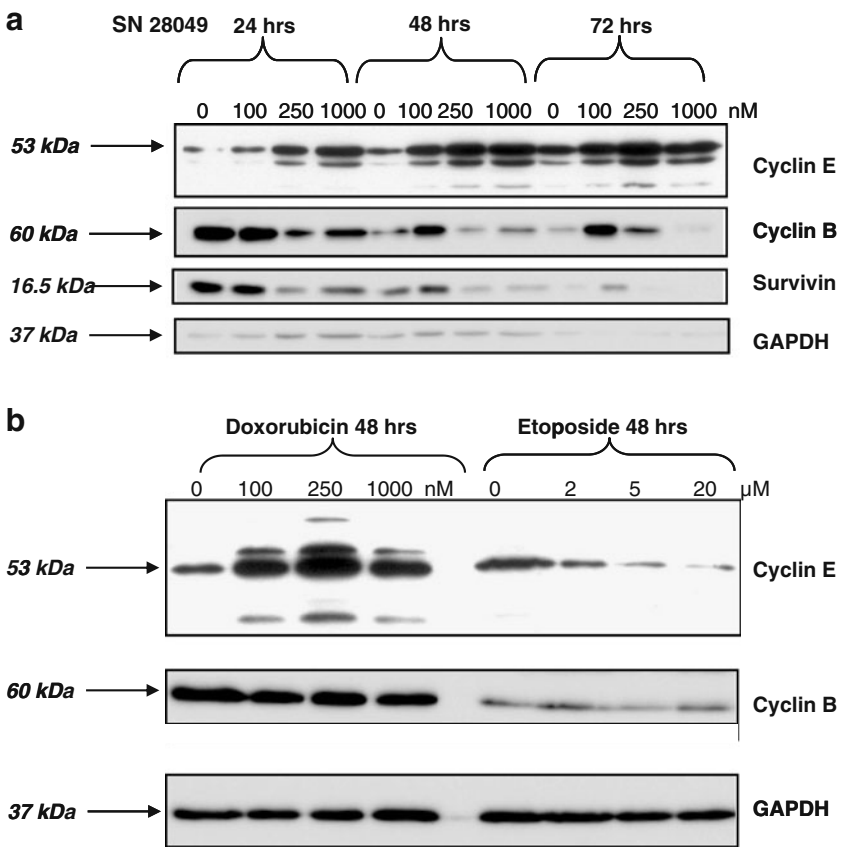


**Fig. 8** **a** Effect of transient (5 h) exposure of HCT116 cells to SN 28049 and subsequent growth for the specified time in the absence of drug on the expression of cyclin E, cyclin B and survivin. **b** Effect of transient (5 h) exposure to doxorubicin and etoposide and subsequent growth for 48 h in the absence of drug on the expression of cyclin E and cyclin B. Relative protein loading is shown by GAPDH expression

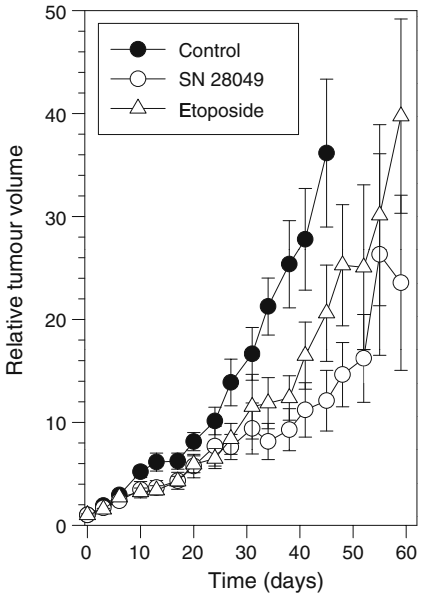


**Fig. 9** Response of NZM3 tumour xenografts untreated (●), or to SN 28049 (two doses of 5.9 mg/kg 7 days apart) (○) or to etoposide (three doses of 30 mg/kg 4 days apart) (Δ)

evidence for a mobility shift in the band shown in Fig. 2. The relatively strong DNA damage response in HCT116 cells to the topoisomerase I poison camptothecin (Fig. 3) is consistent with the report that a deficiency of Chk2 checkpoint kinase in this line leads to a larger response to topoisomerase I poisons [22].

The $IC_{50}$ value of SN 28049 for HCT116 cells was lower than that for NZM3, indicating that despite a low DNA damage response, the drug retains a strong antiproliferative effect (Table 1). Furthermore, SN 28049 induced $G_1$-phase arrest in NZM3 cells but $G_2$/M-phase arrest in HCT116 cells (Fig. 6), suggesting that its antiproliferative effect in HCT116 cells occurred later in the cell division cycle. The loss of cyclin B (Fig. 8), a cyclin that accumulates during $G_2$-phase and activates cyclin-dependent kinase-1 (cdk1) and allows progression to metaphase [23], suggested that cells had progressed beyond $G_2$-phase. Treated cells showed reduced expression of survivin, a passenger protein necessary for chromosome alignment and segregation in mitosis that also accumulates in $G_2$-phase and is degraded after mitosis [24]. The absence of mitotic cells, together with an increase in the proportion of binucleate cells (Fig. 7) suggests that cells have proceeded beyond mitosis but have not divided, and the increase in cyclin E (Fig. 8), a marker for the $G_1$-phase of the cell cycle, suggests that cells have progressed to a new $G_1$-phase without cell division. Treated cells remained in a state with $G_2$/M-phase DNA content for at least 48 h after culture in drug-free medium and also stained for the senescence marker β-galactosidase, indicating the induction of long term cycle arrest (results not shown). These observations are consistent with the activation of a postmitotic checkpoint [25, 26].

The induction of p53 and its transcription products (Figs. 4 and 5) is a feature of the action of SN 28049 on HCT116 cells and is necessary for post-mitotic arrest since HCT116 cells lacking the *TP53* gene do not arrest in response to SN 28049 and instead undergo endoreduplication, entering S-phase without dividing (results not shown). The effect of SN 28049 in this respect is similar to that caused by transient inhibition with mitotic poisons such as nocodazole and paclitaxel, where cells complete mitosis but do not divide, instead progressing to a $G_1$-tetraploid state associated with the activation of p53 [27–29]. The reduction in survivin (Fig. 8) might also be related to p53 expression since p53 is known to repress survivin expression [30].

The mechanism by which SN 28049 induces postmitotic cycle arrest is not yet clear but may involve the induction of a decatenation checkpoint that prevents completion of the last stages of mitosis [31]. Topoisomerase II cooperates with a variety of proteins including aurora kinase B [32], survivin [24] and other members of the chromosomal passenger complex to separate chromatids prior to cell division. Since topoisomerase II has an essential role in this process, SN 28049 may inhibit its decatenation activity. The low activity of topoisomerase IIα in HCT116 cells may also contribute to this effect since it is known that in topoisomerase II-depleted cells, aurora B and other proteins fail to transfer to the central spindle in late mitosis, instead remaining tightly associated with centromeres of nondisjoined sister chromatids [32].

In conclusion, the results suggest that SN 28049 exerts two actions, one mediated by topoisomerase II and leading to the induction of double-stranded DNA breaks and one mediated by the induction of a postmitotic checkpoint. Both induce the p53 pathway if it is present and the second pathway is difficult to detect in the presence of the first, since activation of the p53 pathway will induce arrest of cells in interphase and prevent them from entering mitosis. The ability of SN 28049 to induce postmitotic cycle arrest and p53 induction can be observed in HCT116 cells, which have low topoisomerase II activity and its ability to induce such arrest is much greater than that of etoposide, with doxorubicin having intermediate activity. This second action might be important for killing cells that have survived the DNA damage-induced checkpoints in interphase and might explain why SN 28049 is superior to etoposide against NZM3 xenografts (Fig. 9). The *in vivo* activity of SN 28049 against the NZM3 xenograft was not as high as that obtained for the murine Colon 38 adenocarcinoma [10] and the difference may be related to

different intrinsic sensitivities of the respective cells, since the $IC_{50}$ concentration for NZM3 cells is 25 nM (Table 1) while that for cultured Colon 38 cells is less than 1 nM (Chen YY, Finlay GJ, Baguley BC, manuscript in preparation). The distinctive antitumour effects of SN 28049, combined with its long half-life in Colon 38 tumour relative to normal tissue [11], suggests that this drug has potential activity against human cancer.

**Acknowledgements** The authors are grateful to the Auckland Cancer Society and to the Lang Foundation for financial support.

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