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SHORT REPORT

In vivo and in vitro assessment of the action of SN 28049, a benzonaphthyridine derivative targeting topoisomerase II, on the murine Colon 38 carcinoma

Ying Yi Chen · Graeme J. Finlay · James A. Kirker · Elaine S. Marshall · Emma Richardson · Bruce C. Baguley

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Summary *Aim*: SN 28049 (N-[2-(dimethylamino)ethyl]-2, 6-dimethyl-1-oxo-1,2-dihydrobenzo[b]-1,6-naphthyridine-4-carboxamide) is a new DNA binding drug that targets topoisomerase II. SN 28049 is curative against the murine Colon 38 adenocarcinoma (CT38) while etoposide, another topoisomerase II-directed drug, shows minimal activity; we investigated the basis for this difference in vivo and in vitro. *Methods*: Colon 38 tumours were grown in C57Bl mice and in immunodeficient mice. Tumour sections were examined by staining and TUNEL assays. A new cell line (Co-38P) derived from the in vivo tumour was developed and responses were analysed using flow cytometry. *Results*: Both SN 28049 and etoposide induced similar tumour histological changes, reducing mitotic index and increasing apoptotic index 8 h after administration. At later times however, SN 28049-treated tumours showed further progressive morphological changes while etoposide-treated tumours reverted to their original growth characteristics. The effects of SN 28049 on tumour growth were delayed and attenuated when Colon 38 tumours were grown in immunodeficient mice. SN 28049 and etoposide both induced dose-dependent increases of γ-phosphorylation of histone H2AX and cell cycle perturbation of the Co-38P cell line, indicative of DNA damage, although SN 28049 had 30-fold higher activity. Following 1-hour drug exposure of Co-38P cells, SN 28049 was more effective that etoposide in inducing persistent cycle arrest for the same degree of DNA damage. *Conclusion*: The superior antitumour activity of SN 28049 may result from its ability to induce long term cycle arrest. Host immune responses contribute to the curative activity of SN 28049 and this could result from the induction of cycle arrest.

Keywords Topoisomerase II · DNA damage · H2AX · Senescence · Colon

Y. Y. Chen · G. J. Finlay · J. A. Kirker · E. S. Marshall · E. Richardson · B. C. Baguley (✉)
Auckland Cancer Society Research Centre, Faculty of Medical and Health Sciences, The University of Auckland,
Private Bag 92019,
Auckland, New Zealand
e-mail: b.baguley@auckland.ac.nz

Introduction

Anticancer drugs that target the enzyme topoisomerase II [1] form an important class of anticancer drugs and DNA intercalating drugs such as doxorubicin, as well as protein binding drugs such as etoposide, are in common clinical use. These drugs induce topoisomerase II-depended DNA double-stranded breaks (DSB), mimicking in several aspects the action of ionising radiation. Clinical studies indicate that despite having the same molecular target, topoisomerase II poisons have different spectra of activity, with doxorubicin effective against breast cancer and etoposide active in lymphoma. The basis of this difference in tumour spectrum is not understood.

Amsacrine, first synthesised in this laboratory [2], is a topoisomerase II poison with clinical activity against acute leukaemia [3]. Subsequent studies in this centre have focused on the development of structurally related DNA binding topoisomerase poisons with activity against carcinomas. *N*-[2-(dimethylamino)ethyl]acridine 4-carboxamide (DACA) had superior activity to amsacrine, etoposide and doxorubicin against two murine adenocarcinomas, Lewis

lung and Colon 38 (also known as CT38) [4] and underwent clinical trial but had low dose potency, and toxicity prevented its clinical use. More recently, highly dose potent DNA binding benzonaphthyridine derivatives have been developed and the most active members are able to cause complete regression of Colon 38 tumours [5, 6]. SN 28049 (Fig. 1), the most active of these derivatives, is a topoisomerase II poison [7, 8] and its high activity against the Colon 38 tumour contrasts sharply with that of another topoisomerase II poison etoposide [4]. Here, we have addressed the question of why Colon 38 tumours respond so differently to two drugs that have the same target of action. Since the Colon 38 tumour has always been grown and passaged in vivo, we have also developed a cell line from this tumour and used it to compare responses to these drugs in vitro.

Methods

Materials

SN 28049 was synthesised at the Auckland Cancer Society Research Centre and stored as a 2 mM stock solution in 50% ethanol at −20°C. Etoposide (Bristol-Myers Squibb Pty Ltd) was obtained as clinical formulations and stored at 4°C. Drugs were diluted to working concentrations in culture medium immediately prior to use.

Cell line

Colon 38 tumours [9] were grown and passaged in female mice as previously described [4]. The development of the Co38P cell line from cultured Colon 38 tumour material will be described in a separate publication. The line was grown as substrate-attached cultures in alpha-modified MEM supplemented with 10% foetal bovine serum (FBS) and penicillin/streptomycin (60 μg/mL and 100 μg/mL respectively).

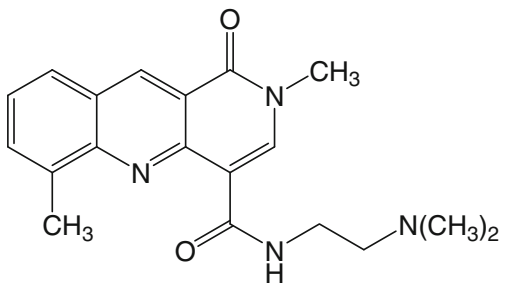

Fig. 1 Chemical structure of SN 28049

Flow cytometric analysis

Cultures of Co38P cells were set up at a density of 5×10^4 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.

Tumour growth delay experiments

C57Bl and 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. Colon 38 tumours were propagated by implanting 1 mm^3 fragments from carrier tumours subcutaneously in one flank under anaesthesia. Co38P cells grown in culture and inoculated subcutaneously (2×10^6 cells/mouse) in one flank. Tumours were allowed to grow to a diameter of 3–5 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.

In vivo TdT-mediated dUTP biotin nick end labelling (TUNEL) staining

Adjacent Colon 38 tumour sections were either stained with haematoxylin and eosin (H&E) or stained for TUNEL according to the manufacturer's guidelines (In situ Cell Death Detection kit, fluorescein; Roche). Formalin fixed paraffin embedded tumour sections (10 μm thick) were dewaxed, rehydrated, permeabilised by Proteinase K (Mebstain Apoptosis kit Direct), stained with TUNEL reaction solution and incubated in the dark at 37 ° C for 1 h. Two negative controls (labelling solution only) and one positive control (1 μg/mL DNAse—Sigma-Aldrich) accompanied each experiment. Slides were counter-stained with Hoechst 33258 (Sigma-Aldrich).

Results

Comparison of SN 28049 and etoposide effects on Colon 38 tumour histology

Mice with subcutaneous Colon 38 tumours were treated with a single dose of either SN 28049 (5.9 mg/kg) or etoposide (65 mg/kg) and killed after 8, 16 or 24 h. Sections were analysed histologically using H&E or TUNEL staining (Fig. 2). Both drugs induced similar increases of tumour apoptosis, reductions of mitotic index and histological changes after 8 h. Despite clear evidence of apoptosis following administration of either drug, many cells with normal appearance were evident. The histological changes caused by etoposide were largely abrogated by 16 h and completely so by 24 h, while those of SN 28049 were not (Fig. 3). Over these time points, tumours of mice treated with SN 28049 showed no signs of an inflammatory response; macrophages and neutrophils were present in varying numbers in treated and untreated tumours and were never abundant, with no obvious difference in the number between treated and untreated tumours. There was evidence of necrosis in both treated and untreated Colon 38 tumours and nuclear swelling was present diffusely after 24 h in the non-necrotic parts of tumours treated with. Tumour sections examined at longer times (up to 72 h) after SN 28049 treatment showed similar changes but these were accompanied by reduction in tumour size and increases in necrotic areas (80–90% of the tumour section).

Effect of growth of Colon 38 tumours in immunodeficient mice

The effects of SN 28049 (5.9 mg/kg single dose) and etoposide (65 mg/kg single dose) on Colon 38 tumour growth were compared in C57Bl mice. SN 28049 induced complete tumour regression while etoposide induced a tumour growth delay (Fig. 4). To assess the contribution of host immunity to the

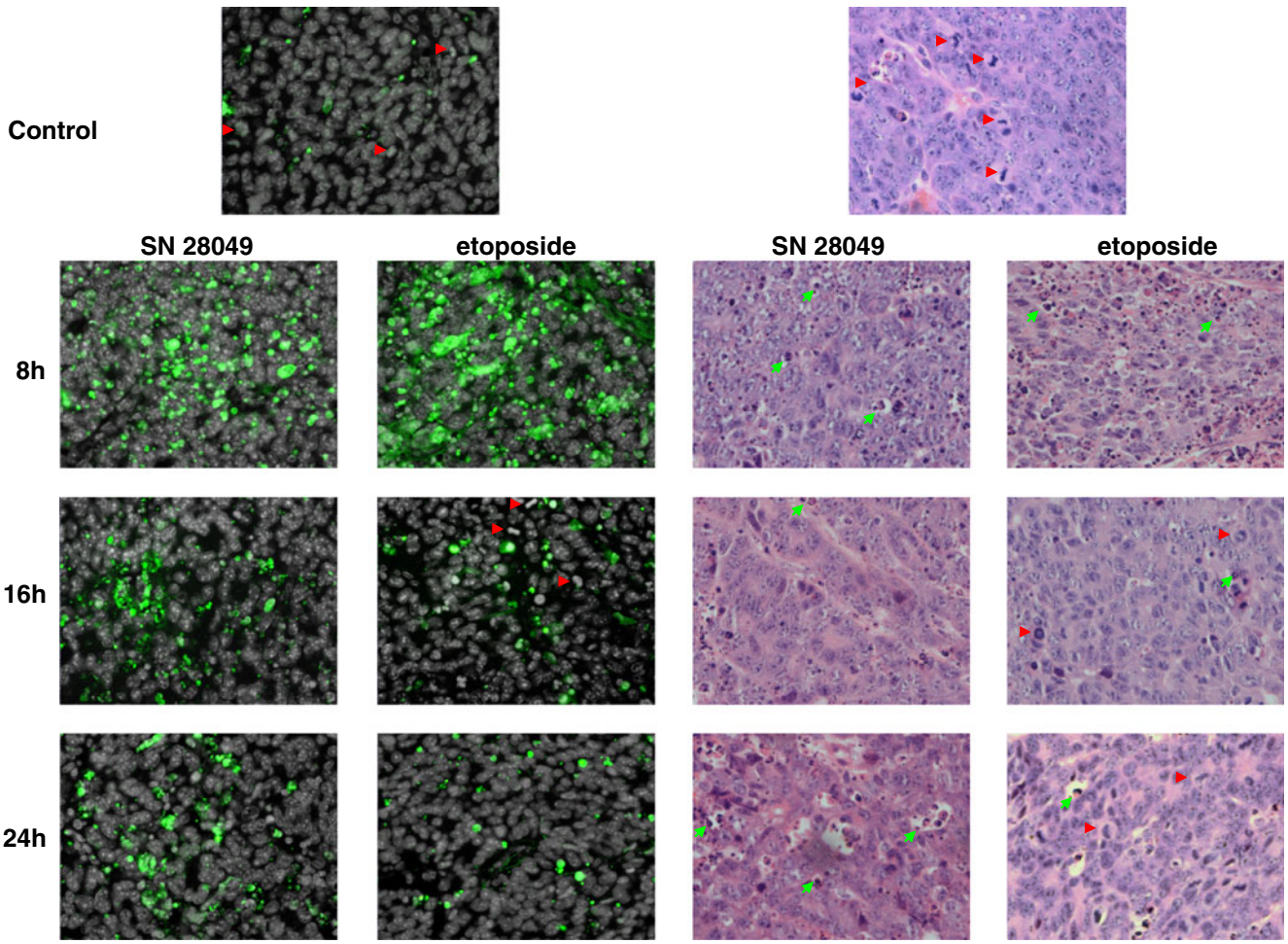

Fig. 2 Colon 38 tumour sections stained with TUNEL (green fluorescence) and Hoechst 33258 (*grey*); *left panels*, and H&E; *right panels*. Dying (apoptotic) cells were labelled with green fluorescence (TUNEL positive) or with *green arrows* on the H&E sections. Mitotic cells are labelled with *red arrowheads*

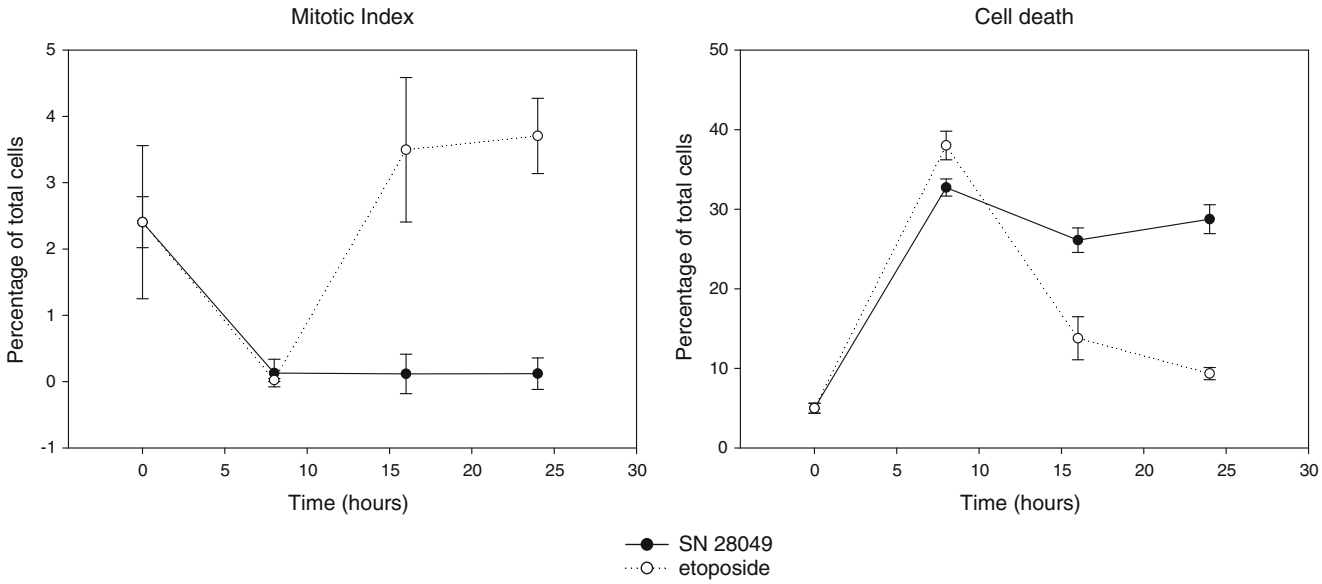

Fig. 3 Mitotic index and percentage of TUNEL positive cells induced by SN 28049 and etoposide in vivo. Calculated from H&E stained tumour sections identified by morphology, and from three different tumours per treatment. *Bars* represent standard errors of the means for each tumour group. Approximately 300 cells/events were counted from 3 different fields per section

effects of SN 28049, Colon 38 tumours were grown in Rag1 immunodeficient mice and tumour growth was assessed in response to SN 28049 (two doses of 5.9 mg/kg 7 days apart). Tumour responses did occur but tumours regrew after a delay; the initial effects of SN 28049 were also very different in the two hosts, with tumour growth continuing for the first 5 days in Rag1 mice but not in C57Bl mice.

Responses of a Colon 38 cell line to SN 28049 and etoposide

The in vivo results showed that the early antitumour effects of SN 28049 and etoposide were similar, their later effects differed, and we wished to determine whether analogous effects occurred in culture. Since the Colon 38 has always been grown and passaged in vivo in our laboratory, we developed a cell line (Co-38P) from primary cultures of Colon 38 tumour tissue; the characteristics of this cell line will be reported separately. Since the main cytotoxic effects of topoisomerase II poisons are mediated by the induction of DSB, we determined the extent of γ-phosphorylation of histone H2AX as an indicator of DNA damage. Cultured Co-38P cells were exposed to a range of concentrations of SN 28049 and etoposide for a period of 4 h, then fixed, double-stained for γ-H2AX with specific antibody and DNA content with propidium iodide, and analysed by flow cytometry. As shown in Fig. 5a, a concentration-dependent increase in γ-H2AX staining of cells at all phases of the

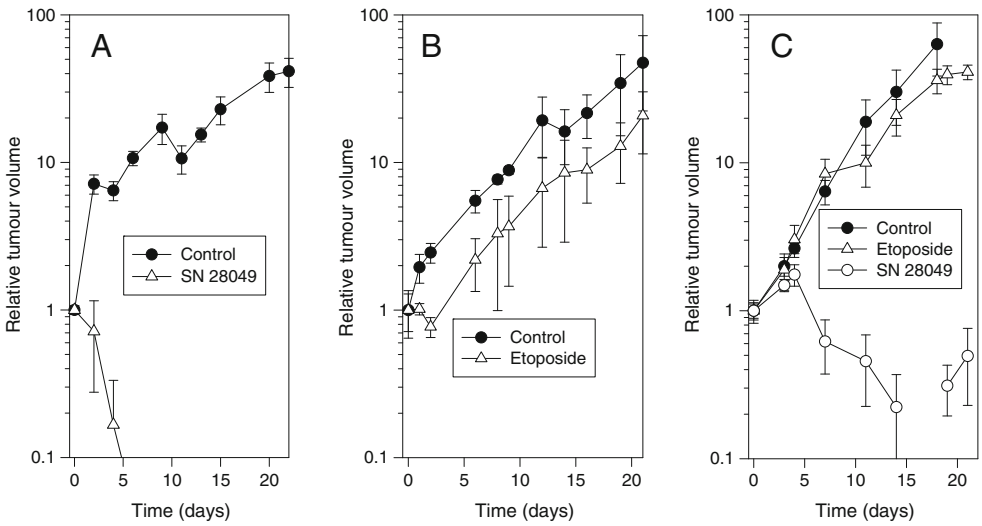

Fig. 4 Colon 38 tumour growth curves for **a** C57Bl host mice treated with SN 28049 (5.9 mg/kg single dose); **b** C57Bl host mice treated with etoposide (65 mg/kg single dose); **c** Rag1 host mice treated with SN 28049 (5.9 mg/kg every 7 days × 2) or etoposide (45 mg/kg every 4 days × 3)

cell cycle occurred with both drugs, but SN 28049 was approximately 35-fold more potent than etoposide. The concentration-dependent increase was not linear but complete saturation of the effect was not reached with either drug. A second experiment was carried out to investigate the time dependence of γ-H2AX staining. Again, the potency of SN 28049 was approximately 30-fold that of etoposide but both drugs showed signs of a saturable effect: the degree of staining observed after 4 h was approximately 1.6-fold that observed after 1 h (Fig. 5b).

The in vivo antitumour effects of SN 28049 occur against a background of an external plasma drug concentration that decreases with a half-life of 2.5 h [10]. To mimic the effects of transient drug exposure, Co-38P cultures were exposed to SN 28049 or etoposide for 1 h, washed and then cultured in drug-free medium for 4, 8, 16, 24 or 48 h before being harvested and analysed as above by flow cytometry. Flow cytometric data are shown for cells grown for 24 h after a 1-hour drug exposure (Fig. 6) but are representative of the results for the other time points. Exposure to SN 28049 (20 nM) for 1 h induced G_2/M-phase arrest and caused γ-phosphorylation of H2AX in the arrested cells (Fig. 6). A 175-fold higher concentration of etoposide was required to induce similar arrest and H2AX staining.

In vivo growth of co-38P cells

To determine whether the Co-38P cell line maintained the same in vivo sensitivity to SN 28049 as that of the original Colon 38 tumour, Co-38P cells were inoculated subcutaneously

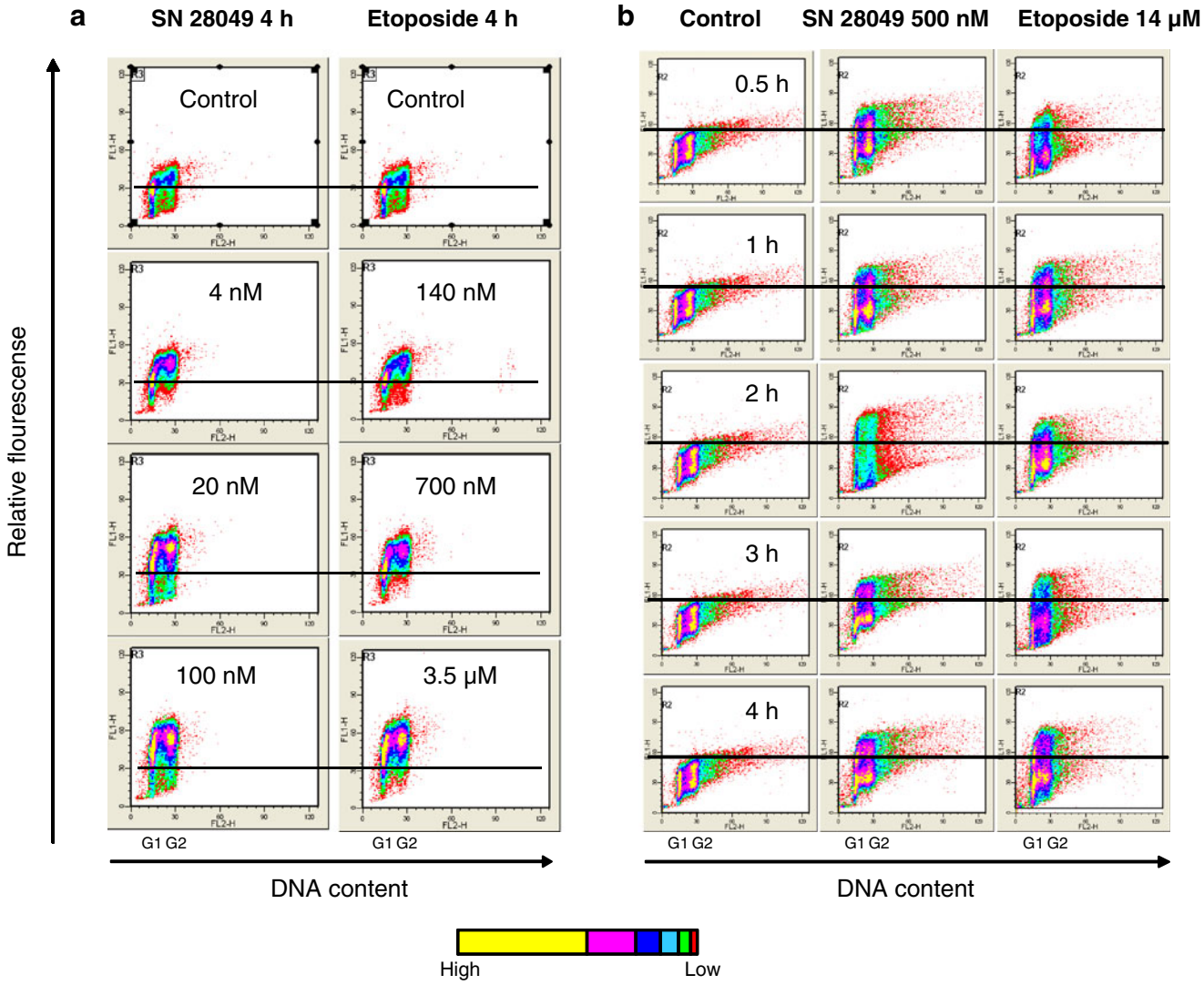

Fig. 5 Two dimensional flow cytometry profiles of Co-38P cells stained for γ-H2AX (fluorescent antibody; y-axis) and DNA (propidium iodide staining; x-axis) before and after exposure to SN 28049 and etoposide. **a** Concentration dependence of a 4-h drug exposure. **b** Time dependence for exposure to SN 28049 (500 nM) and etoposide (14 μM)

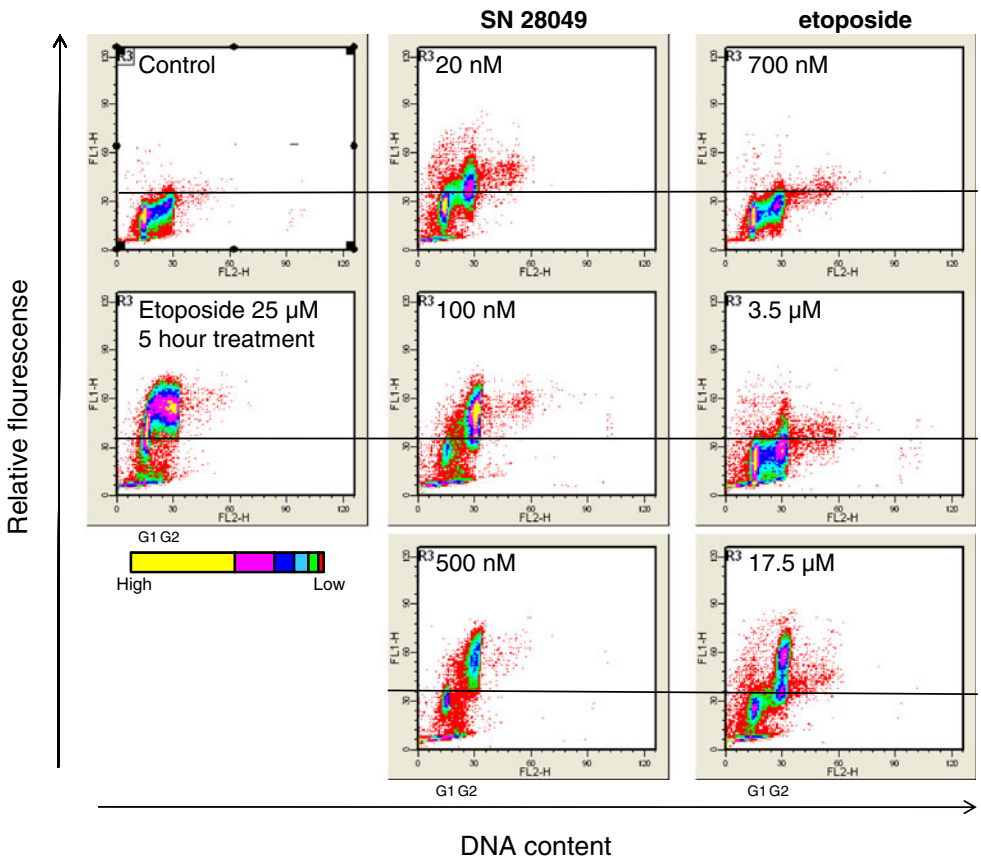

Fig. 6 Two dimensional flow cytometry profiles for Co-38P cells treated for 1 h with SN 28049 (20, 100 or 500 nM) or etoposide (0.7, 3.5 or 17.5 μM) then washed and incubated in drug-free medium for a further 24 h

into C57Bl mice. While control tumours grew initially to small tumours they spontaneously regressed, precluding assessment of drug effects and suggesting the presence of a host immune response to the cell line. Co-38P cells were therefore inoculated into Rag1 mice and control tumours were found to grow progressively. Treatment of these tumours with SN 28049 induced similar responses to those of the original tumour grown in this host (Fig. 7).

Discussion

The results show that treatment of mice with the topoisomerase II poisons SN 28049 and etoposide caused similar early responses but divergent longer term responses. As judged by conventional staining and TUNEL assays, maximal in vivo induction of apoptosis and cytopathic changes occurred after 8 h for both drugs and the changes were of similar intensity. However, at later times the histological appearance of tumours from etoposide-treated mice appeared to be normal while that from SN 28049-treated mice continued to show cytopathic changes and a decreased mitotic index (Figs. 2 and 3). In order to develop an in vitro model to explain this result, a cell line (Co-38P) was derived from the in vivo tumour and its responses to SN 28049 and etoposide were compared. Early in vitro DNA damage responses to the two drugs, as measured by γ-phosphorylation of H2AX, indicated that SN 28049 was 35-fold more potent than etoposide. When cells were treated for one hour with SN 28049 (100 nM) or etoposide (3.5 μM; a 35-fold concentration difference), washed and grown in drug-free growth medium for 24 h, SN 28049-treated cells remained in long-term growth arrest with evidence of

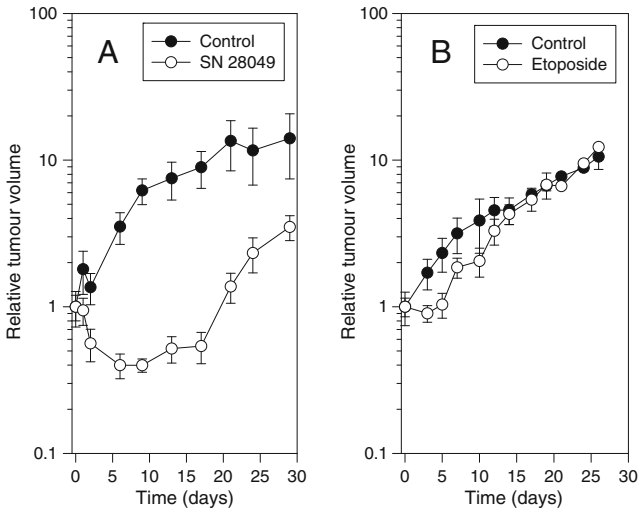

Fig. 7 Co-38P tumour growth curves for **a** Rag1 host mice treated with SN 28049 (5.9 mg/kg single dose); **b** Rag1 host mice treated with etoposide (65 mg/kg single dose)

continuing DNA damage, while the flow cytometric profile of etoposide-treated cells returned toward that of untreated cells (Fig. 6). These results suggest that the in vivo differences in the responses to these two drugs can be modelled in vitro.

It is clear from Fig. 6 that both SN 28049 at a low concentration (100 nM) and etoposide at a high concentration (17.5 μM) induce long-term cell cycle arrest, and treated cells also stain for β-galactosidase, indicative of the induction of a senescent state (results not shown). Topoisomerase II poisons resemble ionising radiation in their ability to induce DSB and it is of interest that ionising radiation induces rapidly repairable DSB at low doses but at higher doses induces persistent DNA damage that may not be repairable, leading to senescence [11]. Thus, both SN 28049 and etoposide may act to induce dose-dependent repairable and non-repairable DNA damage that is processed in a similar fashion to that of ionising radiation. The in vivo superiority of SN 28049 over etoposide may reflect differences in tumour pharmacokinetics, since SN 28049 shows a remarkable tendency to be retained selectively in Colon 38 tumour tissue [10].

Both the data on the response to SN 28049 of Colon 38 tumours growing in normal and immunodeficient mice (Fig. 4) and the data for Co-38P cells growing in immunodeficient mice (Fig. 7) suggest that host mechanisms operate to induce tumour regression in immunocompetent mice. This is supported by the observation that the Co-38P cells initially grow as small tumours in C57Bl host mice but that these tumours later spontaneously regress. It is interesting that the topoisomerase II poison doxorubicin, but not etoposide, has been reported to induce immunogenic cell death that contributes to its effect against the murine Colon 26 (CT26) tumour. The response appears is mediated by drug-induced translocation of the protein calreticulin from the endoplasmic reticulum to the tumour cell surface [12] and the effect of SN 28049 on such translocation is currently being investigated. The in vivo antitumour effect of doxorubicin on Colon 38 tumours [4] is intermediate between that of etoposide and SN 28049 [5] and it is also known that SN 28049 is more effective than etoposide in vitro in inducing G_2/M-phase arrest and senescence of the human HCT116 colon cell line, with doxorubicin having intermediate activity [8]. It will be interesting to investigate the ability of SN 28049 to induce immunogenic cell death in comparison to doxorubicin and etoposide.

In conclusion, the results provide insights into the action not only of SN 28049 but also of etoposide. Despite its relatively poor efficacy in the induction of a Colon 38 tumour growth delay (Fig. 4), etoposide is capable of inducing extensive cytopathic changes including the induction of apoptosis and cycle arrest (Figs. 2 and 3). However, these changes begin to reverse within 16 h of drug administration, leading to further tumour growth. In vitro studies (Fig. 6) suggest that both drugs induce qualitatively similar concentration-dependent responses in Co-38P cells, as measured by both DNA damage and cell cycle arrest. The in vivo superiority of SN 28049 over etoposide in treating the Colon 38 tumour may be related to its ability to be retained in tissue, where the tumour tissue half-life is more than 3-fold longer than that of normal tissues [10]. This difference makes SN 28049 an attractive candidate for further development and our current research is directed towards understanding the mechanisms underlying the ability of SN 28049 to be retained by tumour tissue.

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