Cancer Chemother Pharmacol (2009) 64:1059–1069
DOI 10.1007/s00280-009-0962-4

ORIGINAL ARTICLE

In vitro assessment of novel transcription inhibitors and topoisomerase poisons in rhabdomyosarcoma cell lines

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Received: 19 September 2008 / Accepted: 3 February 2009 / Published online: 10 March 2009

Abstract
Purpose Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children. Current chemotherapy regimes include the topoisomerase II poison etoposide and the transcription inhibitor actinomycin D. Poor clinical response necessitate identification of new agents to improve patient outcomes.
Methods We assessed the in vitro cytotoxicity (MTT assay) of DNA intercalating agents in five established human RMS cell lines. These include novel classes of transcription inhibitors and topoisomerase poisons, previously shown to have potential as anti-cancer agents.
Results Amongst the former agents, bisintercalating bis(9-aminoacridine-4-carboxamides) linked through the 9-position, and bis(phenazine-1-carboxamides) linked via their side chains, are compared with established transcription inhibitors. Amongst the latter, monofunctional acridine-4-carboxamides related to *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide, DACA, are compared with established topoisomerase poisons.
Conclusions Our findings specifically highlight the topoisomerase poison 9-amino-DACA, its 5-methylsulphone derivative, AS-DACA, and the bis(phenazine-1-carboxamide) transcription inhibitor MLN944/XR5944, currently in phase I trial, as candidates for further research into new agents for the treatment of RMS.

Keywords Rhabdomyosarcoma · Cytotoxicity · Acridine-4-carboxamides · Transcription inhibitors · Topoisomerase poisons

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Introduction

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children, there being two main histologically distinct subtypes. Embryonal RMS (ERMS), associated with a generally good prognosis, is the most frequently diagnosed variant, and presents early, with an onset around the age of 2–5 years [3, 40]. Alveolar RMS (ARMS), in contrast, is primarily diagnosed in adolescents, and is associated with poor prognosis as patients often present with metastatic disease [10, 38]. RMS chemotherapy regimes are usually based on variations of the well-established vincristine, actinomycin D and cyclophosphamide protocol, or a protocol that combines the alkylating agent ifosfamide with carboplatin and the topoisomerase II poison etoposide [48]. Importantly, patients with metastatic stage IV ERMS, and those with ARMS, continue to face a poor prognosis as a result of diminished tumour response to current chemotherapy options [10, 32]. Despite many collaborative clinical trials [7, 15, 30, 31, 36, 44] showing steady

enhancements in response rates of patients with non-metastatic ERMS, few improvements have been made in the treatment of metastatic ERMS or ARMS.

Efforts to improve the outcome in poor prognosis patient groups focus largely on trials involving new combinations of existing clinically active compounds. Potential therapeutic benefits are usually outweighed by adverse clinical effects, and this has continued to hamper progress in the development of more effective treatment regimes [1, 2, 23]. Some of the most commonly used agents in RMS protocols exploit the fragility of DNA transcription, and chromosome integrity, by physically interfering with these processes and structures. For example, actinomycin D inhibits transcription by intercalating into DNA and impeding the progression of DNA-dependant RNA polymerases [45]. Etoposide, along with the anthracyclines, camptothecin and its analogues, trap topoisomerases in their DNA cleavable complexes, resulting in the accumulation of DNA double strand breaks, fragmented chromosomes and cell death at mitosis [8, 19, 22, 39, 41]. Given the apparent importance of these biochemical targets in RMS therapy, here, we have investigated the efficacy of a number of novel DNA binding transcription inhibitors and topoisomerase poisons in five RMS cell lines that represent both ERMS (RD and JR1) and ARMS (RH30, RH3 and RH4) tumour subtypes. We have also compared their activity with that of the established transcription inhibitors actinomycin D, chromomycin and nogalamycin, and the topoisomerase poisons etoposide, amsacrine, doxorubicin, mitoxantrone and topotecan.

Each of the novel agents used comprises an intercalating tricyclic carboxamide moiety [51] (Fig. 1), and have previously been shown to be cytotoxic in leukaemia and/or solid tumour cell lines [4, 6, 50]. Some have demonstrated antileukaemic and solid tumour activity in mouse tumour models [5], DACA has been in clinical trial [17], and MLN944/XR5944 is currently under clinical investigation [49]. We studied two classes of transcription inhibitor, each of which is a bisintercalating dimer whose DNA complexes sandwich two base pairs. One class comprises two 9-aminoacridine-4-carboxamide chromophores joined via their 9-amino groups with linkers of various structure, and contain *N*,*N*-dimethylaminoethyl (DMAE) side chains designed to thread through the helix and form hydrogen bond interactions with guanine in the major groove [50]. Linker charge and flexibility determine DNA binding properties and cytotoxicity [50]. The threading diacridines are intended to be mimetics of actinomycin D, and microarray transcript analysis shows they powerfully perturb transcription [56]. The second class contains representatives of phenazine-1-carboxamide dimers bridged via their side chains with alkylamino linkers of various structures [46]. Within this class, the clinical candidate MLN944/XR5944 bisintercalates with its linker in the DNA major groove making hydrogen

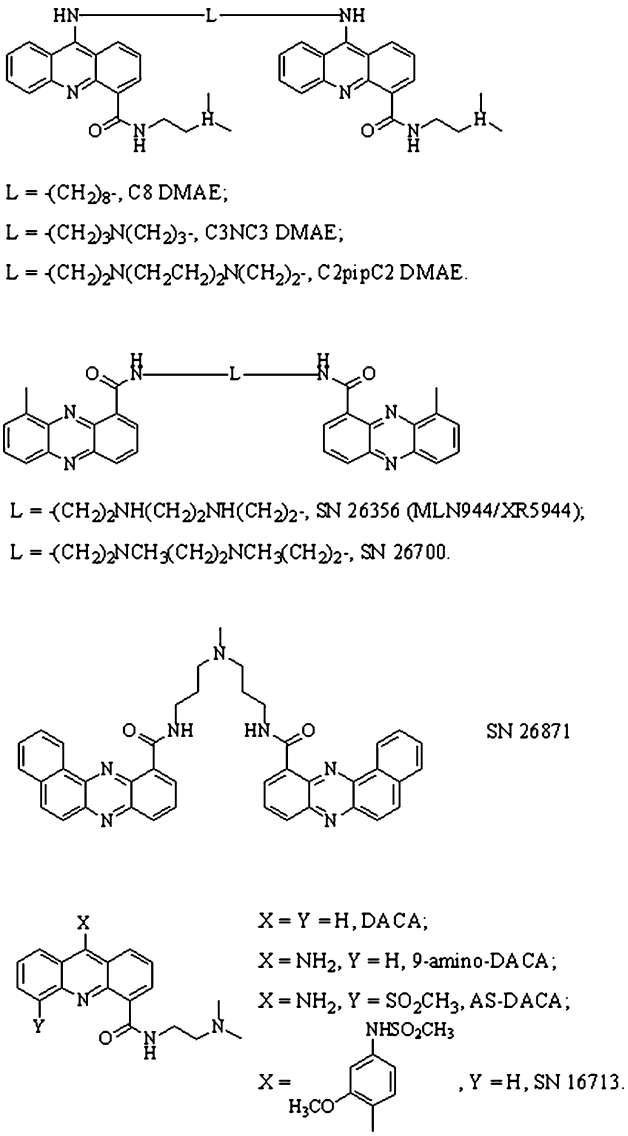

Fig. 1 Structures of the novel agents studied

bonding interactions to guanines in a sequence specific manner [16]. This compound possesses a unique mechanism of action, including the inhibition of transcription factor binding to DNA, which ultimately leads to the inhibition of transcription [12, 16]. Amongst the monofunctional agents studied, DACA, *N*-[2-(dimethyl)aminoethyl]acridine-4-carboxamide, the parent compound of the class, is a dual topoisomerase I/II poison with extensive experimental solid tumour activity, and has been evaluated in phase II trial in non-small cell lung carcinoma, advanced ovarian cancer, recurrent glioblastoma and advanced colorectal cancer [13, 17, 18, 25, 47]. Unfortunately, its low dose potency led to difficulties that impeded further clinical development. 9-amino derivatives of DACA, however, have greater cytotoxic and dose potencies, and modifications in the 5-position, such as the methyl sulphone group

in AS-DACA, promote solid tumour activity [5]. In contrast to DACA, 9-amino-DACA and AS-DACA appear to be more specific poisons for topoisomerase II [11].

Our results show that the naturally occurring transcription inhibitors, as a class, are the most toxic compounds across the five RMS cell lines, with potencies in the nM range, whereas the novel transcription inhibitors of both classes are generally several orders of magnitude less potent. The topoisomerase II poisons etoposide, amsacrine and DACA similarly have poor potencies in the μM range; in contrast to 9-amino-DACA, doxorubicin and mitoxantrone which have activity around 200 nM. Of special interest, is the finding that the topoisomerase I poison topotecan, the topoisomerase II poison AS-DACA, and the transcription inhibitor MLN944/XR5944, show highly differential responses depending on cell type. Whilst the topoisomerase I poison topotecan has recently been included in RMS clinical trial [37, 52], we believe that its strong potency in this study provides further support for its inclusion in future studies. Similarly, we conclude that the topoisomerase II poison, AS-DACA, which possesses a cytotoxicity profile like that of topotecan, and MLN944/XR5944, which complements both AS-DACA and topotecan, deserve further investigation as potential agents for clinical evaluation in childhood RMS.

Materials and methods

Drugs and antibodies

Actinomycin D, chromomycin, nogalamycin, etoposide, doxorubicin, mitoxantrone and amsacrine were purchased from Sigma Aldrich (Sydney, Australia), and topotecan hydrochloride (topotecan, 'Hycamtin®') from Glaxo Smith Klein (Sydney, Australia). The threading bis(9-aminoacridine-4-carboxamides) C8 DMAE, C3NC3 DMAE and C2pipC2 DMAE were prepared as previously described [50]. The acridine-4-carboxamides DACA, 9-amino-DACA, AS-DACA, SN 16713, and the bis(phenazine-1-carboxamides) SN 26871, SN 26700 and SN 26356 (MLN944/XR5944) were kindly provided by Professor W. A. Denny from the Auckland Cancer Society Research Center, University of Auckland, New Zealand. Drugs were dissolved in water to give 1 or 10 mM stock solutions, except for chromomycin, etoposide and amsacrine which were dissolved to 10 mM in DMSO. All stock solutions were stored at −20°C. Working solutions of concentration 0.4 mM were prepared in culture media immediately before use, except for SN 26700 and SN 26871 which were prepared in sterile distilled water as they precipitated out in PBS or culture medium. Topoisomerase I (ab28432), topoisomerase IIα (ab45175) and topoisomerase IIβ (ab15565) antibodies were purchased from Abcam (USA). Each antibody was stored at −20°C except for topoisomerase IIβ antibody which was stored at 4°C as per the manufacturer's instructions.

Cell lines and culture conditions

RD and RH30 cell lines were purchased from the ATCC (Virginia, USA). RH3, RH4 and JR1 cell lines were kindly provided by Dr. Peter Houghton, St Jude Children's Research Hospital, Memphis, USA. RH3, RH4 and RH30 are derived from ARMS tumours which was confirmed by their expression of the ARMS associated genetic translocation t(2;13)(q35;q14) by RT-PCR (data not shown). RD and JR1 were derived from ERMS tumours, and have represented this tumour subtype in several RMS in vitro studies. RD, RH3 and RH4 cell cultures were maintained in DMEM medium (Invitrogen) supplemented with 10% FBS (Sigma). RH30 and JR1 cells were maintained in RPMI medium and were also supplemented with 10% FBS (Sigma). All cell lines were grown at 37°C in humidified conditions and 5% CO_2.

Cytotoxicity assay

We assessed the cytotoxicity of 18 compounds against five human RMS cell lines using an MTT-based assay [35]. RD, RH30 and JR1 cells were grown in 96 well plates for 24 h whilst, because of their slower growth rates, RH3 and RH4 cells were grown for 48 h, prior to drug exposure to ensure treatment occurred during the cells exponential growth phase. Serial dilutions (1:10) of drug solutions were made with culture media, and transferred to the cells to make a total volume of 200 μl per well. Drugs were tested over a concentration range that produced a sigmoidal cytotoxic response (generally 2×10^{-9} to 200 μM), each plate containing triplicate assays which were repeated a minimum of three times for each cell line and each drug. After 68 h of drug exposure, 50 μl of a 0.4 mg/ml solution of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) was added to each well, and incubated for 4 h bringing total drug exposure time to 72 h. MTT and media were aspirated, and the reduced formazan dissolved in 100 μl of DMSO. The intensity of the resultant purple solution analysed using a Labsystems Multiskan Ascent multiwell plate spectrophotometer equipped with a 540 nm filter. The concentration of drug required to inhibit cell viability by 50% over a 72 h exposure (IC_{50}) was calculated from sigmoidal dose-response curves produced in GraphPad Prism 4. The degree of resistance/sensitivity to each drug was calculated relative to the median IC_{50} ($m = 600$ nM) of all the drugs tested in all five human cell lines, and termed the "Δ value" [20]. The Δ values, calculated as the $\log_{10}$ of

individual IC_{50}s divided by (*m*), were displayed graphically to illustrate the relative degree of sensitivity or resistance compared to the median IC_{50} value for all the agents used in the study.

Western blot analysis

SDS-polyacrylamide gel electrophoresis and Western blot analysis were used to establish the basal levels of expression of topoisomerase I, topoisomerase IIα and topoisomerase IIβ in each human RMS cell line. Cell pellets were harvested and snap frozen between passages 5 and 15 before being lysed in RIPA buffer + SDS (1% NP40, 10 mM Tris pH 8, 140 mM NaCl, 0.1% SDS and 1% sodium deoxycholate) supplemented with commercial protease and phosphatase inhibitor cocktails (Roche). Cell lysates were syringed with a 0.11 gauge needle and kept on ice. Protein concentration was established using a bicinchoninic acid (BCA) assay (Pierce) as per the manufacturer's instructions. 10 µg of protein was loaded into each well of a 7.5% gel and electrophoresed at 100 V for 120 min. The protein was electrophoretically transferred overnight at a current of 25 mA to a polyvinylidene fluoride (PVDF) membrane, which was later stained with 0.4% w/v Ponceau S, so as to identify any loading or transfer differences. The membranes were then blocked with 5% dry skim milk in TBS for 1 h, and incubated overnight on ice with rabbit polyclonal anti-human antibody to topoisomerase I, topoisomerase IIα and topoisomerase IIβ (Abcam) at 1:1,000, 1:500 and 1:500 dilutions, respectively. Bound primary antibody was then detected by incubating the blot with a horseradish peroxidase conjugated to anti-rabbit antibody (Molecular Probes, Invitrogen) at a 1 in 10,000 dilution, and visualized with enhanced chemiluminescence. Band intensities were measured via densitometry, quantified using ImageJ (NIH), and normalized for loading differences using Ponceau S stains.

Results

To address our main experimental aims we assessed the cytotoxicity of a range of novel and established transcription inhibitors and topoisomerase poisons against five established human RMS cell lines. MTT cytotoxicity assays were used to determine the relative activity of each drug against each cell line, and the results are shown in Table 1. In addition, the cytotoxicity of each drug in each cell line was compared to the median IC_{50} concentration (*m*) for all 18 agents in all five cell lines tested as shown in Fig. 2.

All five cell lines were highly sensitive to the naturally occurring transcription inhibitors actinomycin D, chromomycin and nogalamycin, with IC_{50} values ranging from <1 to 4.9 nM (Table 1, Fig. 2a). No cell line stands out as being noticeably more or less sensitive than the others, the average IC_{50} value across all lines being not greater than 2 nM. Within the threading bis(9-aminoacridine-4-carboxamide) series of transcription inhibitors several patterns of behaviour are notable. Firstly, with an average IC_{50} value of 844 nM for all three agents, as a class, they are approximately 420-fold less toxic than the natural products (Table 1, Fig. 2a). Secondly, there is little evidence that potency varies with linker structure, although there is a tendency for the C2pipC2 DMAE ligand to be amongst the most active across the cell panel (Table 1). Finally, with IC_{50} values of about 250 nM for all three compounds, it is evident that the RD cell line is more sensitive, by about four to fivefold, than the other cell lines to the threading agents (Table 1, Fig. 2a). Interestingly, this sensitivity is also shared by RH4 cells for the C2pipC2 DMAE dimer (Table 1, Fig. 2a).

The bis(phenazine-1-carboxamides) studied are of two structural types: SN 26356 and SN 26700 are 9-methylphenazines joined via a dicationic—$(CH_2)_2NH(CH_2)NH(CH_2)_2$—linker, and differ in that SN 26700 has the amines substituted with a methyl group, whereas SN 26871 has an *N*-methylated monocationic—$(CH_2)_3N(Me)(CH_2)_3$—linker and an 8,9-benzphenazine chromophore (Fig. 1). SN 26356 was highly effective against three of the cell lines, namely RD, RH3 and RH4, with IC_{50} values ranging from 29 to 55 nM, but showed poor potency against the remaining lines with IC_{50}s varying from 1 to 4 µM (Table 1, Fig. 2a). Methylating the linker nitrogens leaves the compound inactive against JR1 and RH30 like its parent compound, with IC_{50} values of 3 and 4 µM, and reduces activity against RD, RH3 and RH4 by a factor of about 15 on average (Table 1, Fig. 2a). Exchanging the dicationic linker for the monocationic one, and modifying the chromophore, profoundly inactivates the ligand, giving SN 26871 an average IC_{50} of 17 µM across all five cell lines. Thus, with the exception of the response of cell lines RD, RH3 and RH4 to SN 26356, the bis(phenazine-1-carboxamides) are the least cytotoxic transcription inhibitors (Table 1, Fig. 2a).

The set of established topoisomerase II poisons tested, as a class, produced highly variable responses across the RMS cell lines, with no particular cell line being noticeably more sensitive to these agents (Table 1, Fig. 2b). Etoposide is the least potent with an average IC_{50} of about 11 µM across the panel, whilst amsacrine also required a high concentration, with an average IC_{50} of 3.5 µM. In distinction, both doxorubicin and mitoxantrone are considerably more potent, having average IC_{50}s of approximately 200 and 400 nM, respectively (Table 1, Fig. 2b). Topotecan, the only established topoisomerase I poison studied, gave the greatest

Table 1 Cytotoxicity of novel and established transcription inhibitors and topoisomerase poisons in human RMS cell lines

Drug	RD IC_{50} (nM)	JR1 IC_{50} (nM)	RH30 IC_{50} (nM)	RH3 IC_{50} (nM)	RH4 IC_{50} (nM)
Established transcription inhibitors					
Actinomycin D	<1[a]	0.75 ± 0.04	2.3 ± 0.14	<1[a]	1.2 ± 0.04
Chromomycin	1.9 ± 0.085	1.5 ± 0.07	4.4 ± 0.26	<1[a]	4.9 ± 0.36
Nogalamycin	1.8 ± 0.12	1.6 ± 0.14	3.2 ± 0.11	1.5 ± 0.04	4.6 ± 0.23
Novel transcription inhibitors bis(9-aminoacridinecarboxamides)					
C8 DMAE	320 ± 15	1,200 ± 68	950 ± 54	2,200 ± 120	840 ± 40
C3NC3 DMAE	210 ± 11	1,100 ± 61	980 ± 25	1,600 ± 66	350 ± 26
C2pipC2 DMAE	260 ± 16	750 ± 67	770 ± 36	–	290 ± 12
Novel transcription inhibitors bis(phenazine-1-carboxamides)					
SN 26356 (MLN944/XR5944)	29 ± 1.6	970 ± 36	4,000 ± 250	55 ± 2.6	38 ± 2.5
SN 26700	530 ± 20	4,300 ± 220	3,100 ± 184	880 ± 57	440 ± 20
SN 26871	7,800 ± 270	18,000 ± 880	28,000 ± 1,700	11,000 ± 690	20,000 ± 1,000
Established topoisomerase poisons					
Etoposide	21,000 ± 960	2,600 ± 170	1,200 ± 47	22,000 ± 850	11,000 ± 1,000
Amsacrine	11,000 ± 540	1,400 ± 81	600 ± 22	4,300 ± 275	700 ± 51
Doxorubicin	140 ± 4	170 ± 10	150 ± 6.5	170 ± 8	280 ± 11
Mitoxantrone	260 ± 7	840 ± 32	350 ± 30	340 ± 21	280 ± 15
Topotecan	1,100 ± 48	140 ± 5.9	9.7 ± 0.43	1,300 ± 38	15,000 ± 580
Novel acridinecarboxamide topoisomerase poisons					
DACA	2,100 ± 72	1,800 ± 110	1,900 ± 77	3,800 ± 220	2,500 ± 130
9-amino-DACA	110 ± 4	400 ± 12	220 ± 7.4	510 ± 27	180 ± 8.2
AS-DACA	3,800 ± 130	440 ± 21	20 ± 0.73	1,500 ± 55	1,300 ± 53
SN 16713	3,000 ± 190	680 ± 43	820 ± 44	2,200 ± 94	330 ± 11.3

The potencies of 18 agents were assessed in two ERMS derived cell lines (RD and JR1) and three ARMS derived cell lines (RH3, RH4 and RH30)

[a] Values cannot be established as a sigmoidal response curve was not established due to toxicity evident at all concentrations, compared to untreated control

differential response across the cell lines with IC_{50}s ranging between 15 μM and 10 nM (Table 1, Fig. 2b). Both RH30 and JR1 are sensitive to topotecan, RH30 notably so, whilst RD, RH3 and RH4 responded poorly with IC_{50}s close to or greater than, *m* (Table 1, Fig. 2b).

From the class of monofunctional acridinecarboxamide topoisomerase poisons studied, DACA performed poorly across all human cell lines with an average IC_{50} of 2.4 μM (Table 1, Fig. 2b). Its dicationic derivative, 9-amino-DACA, showed improved efficacy, however, compared to both DACA and to the established topoisomerase poisons. Overall, 9-amino-DACA is consistently more potent than *m*, its average IC_{50} of 284 nM marking its toxicity as comparable to both doxorubicin and mitoxantrone (Table 1, Fig. 2b). In contrast, and in a manner strongly reminiscent of topotecan, its 5-methylsulphone derivative, AS-DACA, displayed a distinctly differential response across the panel, having highest potency in the JR1 and RH30 cell lines with an IC_{50} in the latter of 20 nM (Table 1, Fig. 2b). Its activity is poorest against RD cells where it is some 190-fold less potent than against RH30. SN 16713 marries the anilino group of amsacrine with the 4-carboxamide side chain of 9-amino-DACA (Fig. 1) to produce a topoisomerase II-poisoning threading agent [50]. Its cytotoxicity in the RMS panel mimics the response to amsacrine, rather than 9-amino-DACA, with an average IC_{50} of 1.4 μM, and no cell line showing notable sensitivity (Table 1, Fig. 2b).

Finally, to exclude the possibility that the observed variations in response to the topoisomerase poisons might be due to differences in topoisomerase expression in the human RMS cell lines, the relative abundance of the three topoisomerase I and II enzymes was measured by Western blotting with protein extracted from untreated cells. Our results show that when probed with topoisomerase I, topoisomerase IIα and topoisomerase IIβ antibodies, all five cell lines are found to express each of the topoisomerase enzyme in comparable amounts (Fig. 3).

Discussion

Optimization of existing chemotherapy protocols, and the introduction of various established cytotoxic agents into RMS clinical trial, has resulted in improved response rates

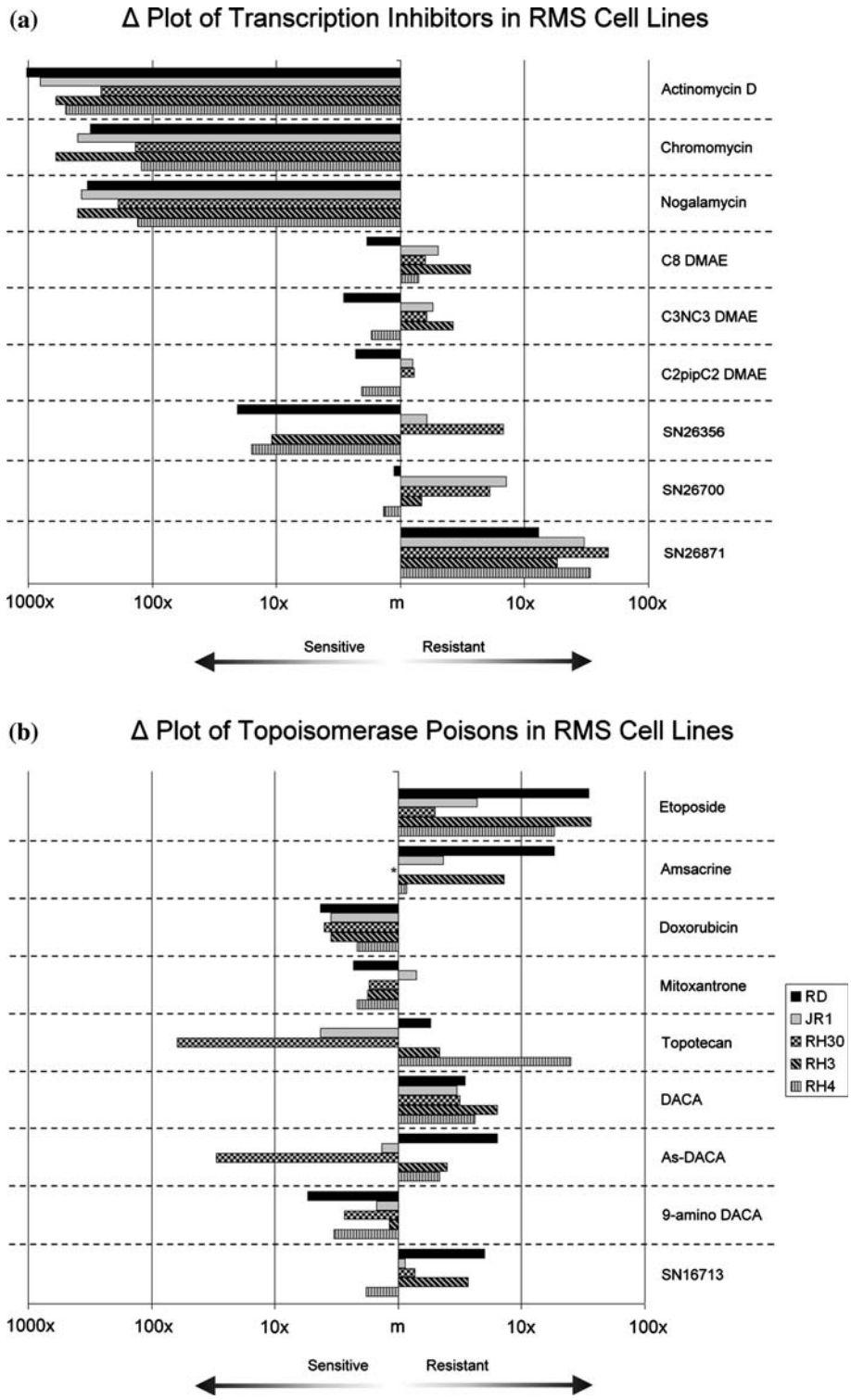

Fig. 2 Δ plots showing variations in drug potency in each of the five RMS cell lines. IC_{50}s are plotted as a $\log_{10}$ measure of sensitivity or resistance against the median (*m*) IC_{50} of all agents across all cell lines ($m = 600$ nM). This measure of potency, taken as a whole across all RMS cell lines, serves to highlight the relative differences in drug efficacy. With an IC_{50} of 600 nM, amsacrine in RH30 cells provided the median IC_{50} value of the entire data set. As such it is not observed with a *bar* in this figure

for many ERMS patients in recent decades. Despite this ARMS and metastatic ERMS, are still associated with a poor prognosis [10, 32]. Hence, there is an urgent need for the development of novel agents with activity in these difficult disease situations. Cytotoxins which specifically bind to DNA have long been used in frontline RMS treatment. Many of these agents are derived from naturally occurring compounds and some, including actinomycin D, the anthracycline antibiotics and camptothecin, have provided structural and mechanistic templates for the design of new anti-cancer drugs [54]. In this study, we present results from an in vitro assessment of the cytotoxicity in human

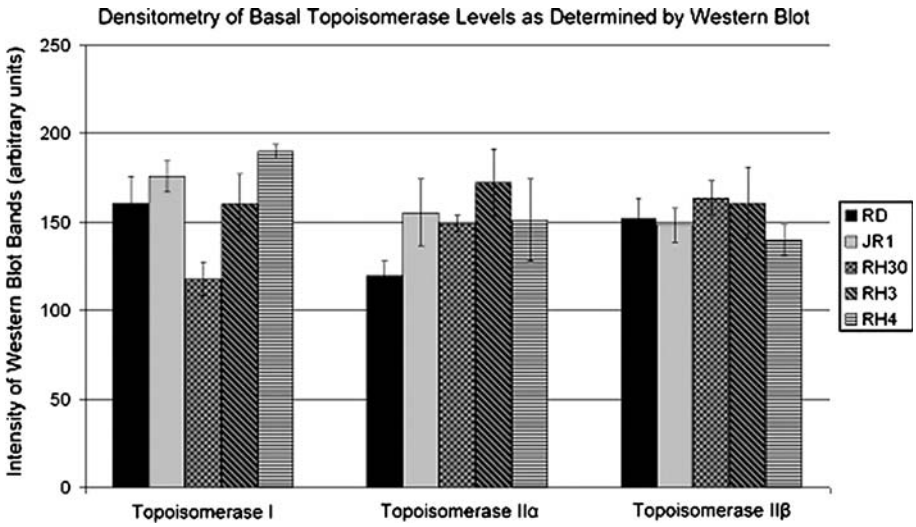

Fig. 3 Basal expression levels of topoisomerase I, topoisomerase IIα and topoisomerase IIβ were determined by Western blot analysis of protein extracted from untreated cells. Densitometry of triplicate blots were performed and highlights an adequate expression of the drug targets for many of the agents used in this study

embryonal and ARMS cell lines of established and novel agents which target transcription and topoisomerases I and II, with the purpose of identifying new agents, or new lead compounds, for potential clinical development. Whilst this in vitro approach is universally the initial assessment in cytotoxic cancer drug discovery, it is prudent to remember that these measurements reflect cytotoxic potency and not intrinsic anti-tumour activity, which can only be established in animal tumour models. Here we take cytotoxic potency as an indication of potential anti-tumour activity, notwithstanding the appreciation that in the clinical setting, potency may be a secondary consideration compared to tumour selectivity. Also, with respect to the classification of DNA binding drug classes, it is as well to be aware of the complexities in their mechanisms of action, since, although such agents are generally classified according to their most evident biochemical outcome, there is the potential for cross-talk between topoisomerase poisoning and transcription inhibition. For example, it is clear that actinomycin has the capacity to affect topoisomerase activity [53], and that topoisomerase II poisons, including the acridinecarboxamides, may also perturb transcription [56]. Nonetheless, the division between topoisomerase poisons and transcription template inhibitors is a fruitful one, that has guided much drug development [4–6, 26, 46, 50].

The anti-tumour antibiotics actinomycin D, chromomycin and nogalamycin are amongst the classical template inhibitors of transcription, each binding to DNA reversibly, but dissociating slowly so as to present a long-lived block to the passage of RNA polymerases. Actinomycin D is a monofunctional intercalating agent which places bulky cyclic peptides in the DNA minor groove, chromomycin is a minor groove binding agent [55], and nogalamycin is a monofunctional threading agent which intercalates with its nogalose sugar lying in the minor groove and its bicyclic amino sugar spearing the duplex making hydrogen bonding interactions with guanines in the major groove [28]. All are known to bind selectively to GC-rich sequences and block RNA polymerase progression by placing a bulky group in the DNA minor groove. Furthermore, all cause similar profound perturbation to transcription profiles [56]. Here, we find that all three agents are equally the most potent agents studied in the five RMS cell with activity in the nM range (Table 1, Fig. 2a). Seemingly, the fine details of how they interact with DNA to block RNA polymerase do not affect their cytotoxicity and with actinomycin D routinely used in RMS protocols, this observation suggests that chromomycin and nogalamycin are worthy of consideration for inclusion in clinical studies. Chromomycin has previously been in clinical trial against advanced breast, colorectal and GI-tract tumours [33, 42, 43] but we can find no reference to clinical studies with nogalamycin.

The development of the bisintercalating bis(9-aminoacridine-4-carboxamide) transcription template inhibitors was inspired by the threading mechanism of nogalamycin [26, 50]. Their threading design, in which the carboxamide side chains spear the DNA helix to make bonding interactions with guanine bases in the major groove, promotes transcription inhibition by enhancing DNA residence time without increasing binding affinity, a desirable characteristic for activity in solid tumours where tumour penetration correlates inversely with DNA binding affinity [26, 42, 50]. The three examples studied here, C8 DMAE, C3NC3 DMAE and C2pipC2 DMAE, despite having IC_{50} values in human leukaemia CCRF-CEM cells of 35, 50 and 63 nM, respectively [50], and similar potencies in a range of cell lines including K562 (human CML, C8 DMAE 90 nM, C3NC3 DMAE 40 nM), A2780 (human ovarian, C8 DMAE 30 nM, C3NC3 DMAE 20 nM) and KB (human cervical, C8 DMAE 80 nM) (L. P. G. Wakelin, unpublished data), are found to be about 4- to 40-fold less potent in the RMS cells, which is some 100- to 1,000-fold less active

than the naturally occurring transcription inhibitors (Table 1). The only RMS cell line that could be considered sensitive is RD as this is the only one in which all three threading dimers produced IC_{50}s marginally lower than *m* (Fig. 2a). The origins of the intrinsic resistance of the RMS cell lines to these agents is unclear, but it is unlikely to be due to membrane transport difficulties, given the activity of the natural products.

This generalized resistance to the bisacridines also extends to the bis(phenazinecarboxamide) dimers, with one important exception. These compounds were designed as bisintercalating topoisomerase I and II poisons [46], but their actual mechanism of action is complex and appears to involve both transcription inhibition, along with topoisomerase I poisoning [12]. The three compounds studied here are potently cytotoxic in mouse leukaemia P388, mouse Lewis lung and Jurkat human leukaemia cells with IC_{50}s ranging from 0.2 to 33 nM and an average value of 13 nM [24], and activity at comparable levels was maintained across the NCI tumour cell line panel [34]. The toxicity of SN 26700 and SN 26871, however, is diminished some 35- to 2,200-fold in the RMS panel (Table 1), with their IC_{50}s clustering around *m* or greatly exceeding it (Fig. 2a). The exceptional response is found with SN 26356 which is currently in clinical trial as MLN944/XR5944 [49]. Its potency in previous studies is maintained in three RMS cell lines, namely RD, RH3 and RH4, with an average IC_{50} of about 40 nM, although the remaining two lines are some 25- to 100-fold less responsive (Table 1): a distinction clearly shown in Fig. 2a. The origins of this selectivity are unknown, but our findings point to the importance of considering SN 26356 as a clinical trial candidate in RMS.

The trapping of topoisomerases in a cleavable complex with DNA is a well-established mechanism of action of many DNA binding drugs [29]. Representative topoisomerase poisons, both established and novel, were examined in this study, and produced wide ranging results. For example, amongst the clinically used topoisomerase II poisons, etoposide and amsacrine had uniformly high IC_{50}'s across the RMS cell line panel, with IC_{50}s all greater than *m*, ranging from 600 nM to 22 μM (Table 1, Fig. 2b). Such a finding sits oddly with the inclusion of etoposide in clinical RMS protocols [48]. In contrast to this, doxorubicin and mitoxantrone are uniformly active in the RMS cells with average IC_{50}s of about 200 and 400 nM, respectively (Table 1, Fig. 2b), a finding that would tend to support their inclusion in clinical studies. The only clinical topoisomerase I poison studied, topotecan, produced a differential response with activity of 10 and 140 nM in RH30 and JR1 cells, but IC_{50}s of 1–15 μM in the remaining three RMS lines (Table 1, Fig. 2b). Interestingly, this is the inverse selectivity of SN 26356, which is inactive in RH30 and JR1, and raises the intriguing question of the potential clinical activity of their use in combination. In measuring the basal expression levels of topoisomerases I, IIα and IIβ (Fig. 3), we confirm that comparable amounts of all three enzymes are available in the five RMS cell lines. Hence, the reason for differences in cytotoxic effect of each topoisomerase poison is more complex than the availability of the drug targets.

The novel topoisomerase poisons evaluated are structurally based on the acridine-4-carboxamide chromophore, the parent compound of which, DACA (Fig. 1), has been identified as a dual topoisomerase I/II poison [21]. Despite its wide solid tumour activity and its clinical evaluation [13, 17, 18, 25, 47], it shows poor potency in all RMS cell lines with IC_{50}s about 2–4 μM. In contrast, its dicationic derivative, 9-amino-DACA (Fig. 1), which binds to DNA sixfold more tightly than DACA and is only weakly active as a topoisomerase I poison [21, 27], is some tenfold more potent in all RMS cell lines, making its activity comparable to that of doxorubicin and mitoxantrone (Table 1, Fig. 2b). Although the extra charge on the chromophore of 9-amino-DACA enhances cytotoxic potency and anti-leukaemic activity in mouse tumour models [5], it diminishes solid tumour activity apparently as a consequence of poor tumour penetration due to its elevated DNA affinity [14, 27]. Electron withdrawing substituents in the acridine 5-position lowers the chromophore pK, and AS-DACA, bearing a 5-methylsulphone, has a neutral chromophore at physiological pH, binds DNA with an affinity between that of DACA and 9-amino-DACA, and is intermediate between these two agents with respect to topoisomerase selectivity [21]. These characteristics make it generally more cytotoxic than DACA, and endow it with widespread solid tumour activity [5]. In the RMS panel it returns a differential response, strongly reminiscent of that of topotecan, with JR1 and RH30 cells being sensitive and the remaining three cell lines have IC_{50}s above 1 μM (Table 1, Fig. 2b). These findings suggest that 9-amino-DACA and AS-DACA are worthy of further study in RMS tumours. Lastly, within the acridinecarboxamide family, we examined the RMS activity of SN 16713, a monofunctional threading agent that superposes the structures of amsacrine and 9-amino-DACA (Fig. 1) [9]. SN 16713 selectively poisons topoisomerase II, has an IC_{50} of 120 nM in CCRF-CEM cells [56] and 7 nM in Jurkat leukaemia [21], but has considerable higher IC_{50} in RMS cells, its responses being more akin to those of amsacrine and DACA than 9-amino-DACA (Table 1, Fig. 2b).

Whilst the majority of agents studied here produced consistent levels of cytotoxicity in each cell line, a small group of drugs including AS-DACA, SN 26356 and topotecan, induced highly differential responses across the cell line panel. Many factors may be responsible for this observed variation, however, it must be assumed that drug resistance mechanisms, intrinsic only to certain RMS cell types, act in a manner dependent on the subtle structural differences

which exist between the DNA binding compounds of each class. Given the importance of in vitro studies in pre-clinical drug investigations, it is worthwhile investigating these commonly used RMS cell lines so as to identify the subtle biological mechanisms which are intrinsic to them and produce selective drug response phenotypes.

Conclusion

We have investigated the cytotoxicity of a variety of compounds with the capacity to inhibit transcription and to poison topoisomerases I and II in a panel of five RMS cell lines in order to seek new agents for potential inclusion in clinical studies of childhood RMS. Our findings enable classification of these agents into three classes; those that are potent in all five cell lines; those that show differential responses across the panel; and those that require higher concentrations to be toxic in all cell lines. The first class includes the naturally occurring transcription inhibitors actinomycin D, chromomycin and nogalamycin, which are the most potent amongst the agents studied, the topoisomerase II poisons doxorubicin and mitoxantrone, and the experimental acridine-4-carboxamide topoisomerase II poison 9-amino-DACA. Class two includes the bis(phenazine-1-carboxamide) SN 26356, otherwise known as MLN944/XR5944, identified as a transcription inhibitor and topoisomerase I poison, the topoisomerase I poison topotecan, and the acridine-4-carboxamide topoisomerase poison AS-DACA. AS-DACA and topotecan have the same spectrum of cytotoxic activity, which is complementary to that of SN 26356. Although all members of classes one and two are worthy of consideration for inclusion in experimental RMS clinical protocols, agents such as those described in group 2 may offer alternative treatment options for tumours unresponsive to the traditional chemotherapy protocols. As such, defining mechanisms that govern the responses observed in this study is of paramount importance for the continued development of these novel compounds.

Acknowledgments We thank Professor Bill Denny from the Auckland Cancer Society Research Centre, University of Auckland, New Zealand, for providing several of the agents used. This work was supported by funding from the Children's Oncology Foundation (DRC), an Australian Postgraduate Research Award (SJW), and the Australian National Health and Medical Research Council (LPGW and BWS).

Conflict of interest statement None.

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