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Considerations for Treatment Development in Rhabdomyosarcoma: *In Vitro* Assessment of Novel DNA Binding Drugs

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1. Introduction

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children and is believed to originate from mesenchymal cells that resemble undifferentiated striated muscle cells (Wexler and Helman, 1997). It is a relatively rare tumour type with approximately 350 patients below the age of 20 diagnosed each year in the USA (Gurney et al, 1999). Incidence in Australia is also low with only 31 RMS cases out of the total 1,003 childhood cancers diagnosed between 2001 and 2005 in the state of NSW (Tracey et al 2007). Histological staining of tumour samples led to the classification of two distinct forms of tumour types: embryonal (ERMS) and alveolar (ARMS). ERMS is the most common histologically diagnosed variant of the disease and is associated with an earlier onset, most commonly around the age of 2 to 5 years (Qualman et al, 1998). Diagnosis of ERMS is made when the cells fit the criteria of appearing as stroma-rich spindle cells which are not densely packed and show no alveolar pattern of growth which characterises ARMS. Variant forms of ERMS, including botryoid and spindle cell types, have been described as being histologically similar to standard ERMS (Wexler and Helman, 1997).

Treatment of rhabdomyosarcoma employs a multimodal approach that utilizes surgical, radiological and chemotherapeutic protocols. Unlike in the treatment of adult sarcomas, surgical removal of the tumour mass in paediatric RMS patients is usually only attempted if complete resection can be guaranteed without causing cosmetic or developmental damage to the child. For this reason chemotherapy is the frontline option in the treatment of paediatric RMS both as a means of local tumour mass control and for the prevention of residual and micrometastatic disease (Stevens, 2005). Over 70% of patients with non-metastatic RMS will respond well to chemotherapy and reach a 5 year event free survival milestone. Patients with metastatic or stage IV ERMS however, and those with ARMS who generally present at diagnosis with an advanced metastatic form of the cancer, continue to face a poor prognosis as a result of diminished tumour response to the current chemotherapy options. Currently, less than 30% of patients with metastatic disease survive

without relapse and despite this drastic difference in tumour response, chemotherapy protocols continue to utilize the same compounds regardless of tumour subtype, progression or stage (Wexler and Helman, 1997).

Without agents to target specific molecular pathways and proteins of RMS, such as the PAX3-FKHR chimeric protein, chemotherapy protocols continue to utilize general cytotoxic compounds that rely on the rapid proliferation of tumour cells for selectivity and optimal efficacy. Most of these agents bind to DNA and disrupt key molecular processes involved in DNA transcription and replication. Treatment usually involves the vinca alkaloid vincristine, the transcription inhibitor actinomycin D and the alkylating prodrug cyclophosphamide (Breitfeld et al, 2005). Several other general cytotoxic agents, including the topoisomerase poisons etoposide, doxorubicin, epirubicin, topotecan and irinotecan as well as the alkylating agents ifosfamide and carboplatin have also been used in alternative treatment protocols and large scale clinical trials (Table 1). Many agents included in RMS clinical trials and standard treatment protocols can be broadly classified as general cytotoxic agents, a large proportion, including etoposide, doxorubicin and topotecan, specifically target and poison the function of the topoisomerase enzymes, whilst actinomycin D is a transcription inhibitor that has been successful in the treatment of a wide variety of tumours, including RMS.

2. DNA binding agents underpin RMS therapy – a review of clinical trials

Prior to the 1970s the prognosis for RMS patients was extremely poor regardless of tumour subtype. The earliest large scale collaboration to be established was the Intergroup Rhabdomyosarcoma Study Group (IRSG), a joint effort between US and Canadian researchers. Five trials were carried out by this group between 1972 and 2000 at which point the group merged into the Children's Oncology Group (COG) under which more recent trials have been carried out. Patients enrolled in IRSG or COG clinical trials were grouped based on various prognostic factors before an appropriate treatment schedule was assigned. The second collaboration to be established was the European based group 'International Society for Paediatric Oncology' (SIOP) which launched several large cohort trials in 1975, 1984, 1989 and 1995 from which many findings were reported. A selection of key findings from IRSG, COG and SIOP clinical trials are presented in summarized form in Table 1. With 5 year event-free survival rates (EFS) reaching 70%, patients with gross residual tumour were believed to have benefited the most in early studies. It was clear however, that patients with stage III or IV RMS required more intense chemotherapy than those in stage I and II and it was concluded that despite the successes of the VAC combinational therapies, introducing additional agents, such as topoisomerase I poisons, would help subdue the onset of local and distant failures. The prognosis for patients with non-metastatic RMS continued to improve after the fourth and fifth IRSG studies were completed, yet despite years of large cohort clinical trials and the subsequent retrospective analysis of data, response rates in patients with metastatic ERMS and ARMS remained considerably low. This has been attributed to many factors including combination chemotherapy leading to additive and overlapping adverse side effects which limit the dosages used as well as intrinsic or acquired drug resistance mechanisms.

3. Therapeutic advancement in RMS requires new agents

It is clear from this review of the chemotherapeutic treatment options available for RMS that novel agents are desperately required to improve the prognosis for patients with metastatic

Study	RMS Classification	Protocol Tested	Results	References
IRSG Study I	Group I	VAC + R	No benefit from additional R	Maurer et al, 1988
	Group II	VC + R + A	No benefit from additional A	
	Group III + IV	VAC + R + D	No benefit from additional D	
IRSG Study II	Group III	Intense repetitive pulse VAC + radiation or VDC + radiation	Improvement over IRSG 1: (SR) increased -50% to 66% (CRR) increased - 56% to 73%.	Maurer et al, 1993
	Groups I - IV	VDC	No improvement vs. VAC. Fatal side effects	
	Groups I – II	VA + C	No improvement from additional C	
	Groups I – II	Repetitive pulse VAC	Improvement over IRSG 1	
IRSG Study IV	Groups I – II	VAC, with either VAI or VIE	3yr EFS: 75% VAC, 77% VAI, 77% VIE Overall EFS of 83%. Surgery + VAI + VIE was equally effective as VAC only	Crist et al, 2001
IRSG Study IV	Intermediate risk ERMS	3yr FFS improved due to doubling of alkylating agent dosage compared to the treatment protocol used in IRSG study III. Cyclophosphamide or ifosfamide had same effect.		Baker et al, 2000
	High Risk / Stage IV	VAI or VIE every 3 wks / 12 wks + VAC every 3 wks for 36 wks.	63% OR (12 weeks)	Sandler et al, 2001
SIOP MMT89	Group III, Stage III	Novel treatment which combined 6 drugs (IVA) + (CbEV) + (IVE).	60% OS (5yr) versus 42% OS (5yr) MMT84	Stevens et al, 2005
Independent Phase I	Recurring solid tumours	varying doses of Cb + fixed doses of I + E	33% OR (4% increased)	Marina et al, 1993
Independent Phase I/II	Refractory STS sarcomas	ICbE	32% CR 63% OR	Kung et al, 1995
CCG Study I	27 RMS patients in a total cohort of	ICbE	78% 1yCR, 33% 2yCR, 66% OR ERMS ARMS	Van Winkle et al, 2005

Study	RMS Classification	Protocol Tested	Results	References
	97 STS patients		82% 1 yr OS 40% 1 yr OS 46% 2 yr OS 20% 2 yr OS	
SIOP MMT89	Untreated Stage IV RMS	Single Course C, Epi + V	53% Total OR ERMS ARMS 46% OR 58% OR	Frascella et al, 1996
IRSG V	Stage IV RMS	T or T + VAC	46% Total OR ERMS ARMS 28% OR 65% OR	Pappo et al, 2001
Independent Trial	Intermediate risk RMS	VDC + EI at 3 week intervals over a total 10 cycle course.	91% OS 85% EFS	Arndt et al, 1998

Table 1. Results from Selected RMS clinical trials involving general cytotoxic compounds. A=actinomycin D, C=cyclophosphamide, D=Doxorubicin, E=etoposide, Epi=Epirubicin, I=ifosfamide, R=radiotherapy, V=vincristine, Cb=Carboplatin; CR=Complete Response, CRR=Complete Response Rate, EFS=Event Free Survival, OR=Overall Response, OS=Overall Survival, SR=Survival Rate.

or stage IV ERMS and ARMS. To date, the only genetic abnormality consistently associated with ARMS is the t(2:13)/t(1:13) translocations that produce the oncogenic PAX3/7-FKHR chimeric proteins. One day these may be targeted by small molecules or genetic based therapies, however, the immediate future of RMS treatment remains highly dependant on general cytotoxic agents. Unfortunately, all of the available general cytotoxic agents are associated with adverse side effects that place severe limitations on the concentrations of drug that can be administered to children with the disease. To minimize these side effects each agent is used in low doses both in combination with other general cytotoxic agents and over an extended period of time. Such treatment protocols rarely guarantee full recovery and often promote the development of drug resistance mechanisms within the cancer cells that manifest themselves either during initial rounds of therapy, or more commonly, following tumour relapse.

Optimization of existing chemotherapy protocols, and the introduction of established cytotoxic agents into RMS clinical trial, has resulted in improved response rates for ERMS patients in recent decades. Despite this, ARMS and metastatic ERMS, are still associated with a poor prognosis (Breitfeld and Meyer, 2005). With such a high dependency on general cytotoxic agents for the treatment of RMS, novel compounds with improved efficacy and fewer side effects must be developed. Efforts to improve the outcome in poor prognosis patient groups focus largely on trials involving new combinations of existing clinically-active compounds. 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. 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 (Pommier Y,

2006). 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 5 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 new agent has been designed with altered DNA association/dissociation kinetics, improved tumour penetration compared to the established agents and with this in mind, their efficacy and vulnerability to common mechanisms of resistance are examined.

3.1 Novel DNA binding cytotoxic agents

With a range of novel cytotoxic compounds available to us through colleagues at the University of New South Wales and the Auckland Cancer Society Research Centre, we aimed to assess the efficacy of selected agents from various classes in an in vitro RMS cell line model that best represented both subtypes of the disease. In doing so it was our intention to identify agents with the potential to expand treatment options for RMS patients and further improve the efficacy of chemotherapy protocols that utilize general cytotoxic agents. Each of the novel compounds assessed in this study contain tricyclic carboxamide moieties that act as DNA intercalating chromophores and have previously been shown to be cytotoxic in leukaemia and/or solid tumour cell lines (Wakelin et al, 2003; Baguley et al, 1995; Atwell et al, 1984).

One group of novel transcription inhibitors (Figure 1A) contain dual intercalating chromophores that are joined via their 9-amino groups by linker chains of various structures and contain N,N-dimethylaminoethyl (DMAE) active side chains. These agents bind to DNA in a bisintercalating threading fashion inspired by the binding mechanism of nogalamycin (Wakelin et al, 2003). In this design the carboxamide sidechains spear the DNA helix and make bonding interactions with guanine bases in the major groove to promote transcription inhibition by enhancing DNA residence time without increasing binding affinity. This is a desirable characteristic for activity in solid tumours where tumour penetration correlates inversely with DNA binding affinity (Wakelin et al, 2003). Differences in these compounds are found in their linker chains with flexibility, charge and length all varying. With the linker chains laying in the minor groove of the DNA helix they play a crucial role in the overall activity of the compound by placing a physical block in the path of DNA tracking enzymes (Wakelin et al, 2003).

The second class of transcription inhibitors (Figure 1B,C) contain representatives of phenazine-1-carboxamide dimers bridged via their side chains with alkylamino linkers of various structures (Spicer et al, 2000). Within this class, the clinical candidate MLN944/XR5944 bisintercalates with its linker in the DNA major groove making hydrogen bonding interactions to guanines in a sequence specific manner. 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 (Byers et al, 2005). The bis(phenazine-1-carboxamides) studied are of two structural types: SN26356 (MLN944/XR5944) and SN26700 are 9-methylphenazines joined via a dicationic -(CH2)2NH(CH2)NH(CH2)2- linker, and differ in that SN26700 has the amines substituted with a methyl group (Figure 1B). SN26871 has an N-methylated monocationic -(CH2)3N(Me)(CH2)3- linker and an 8,9-benzphenazine chromophore (Figure 1C). ¶
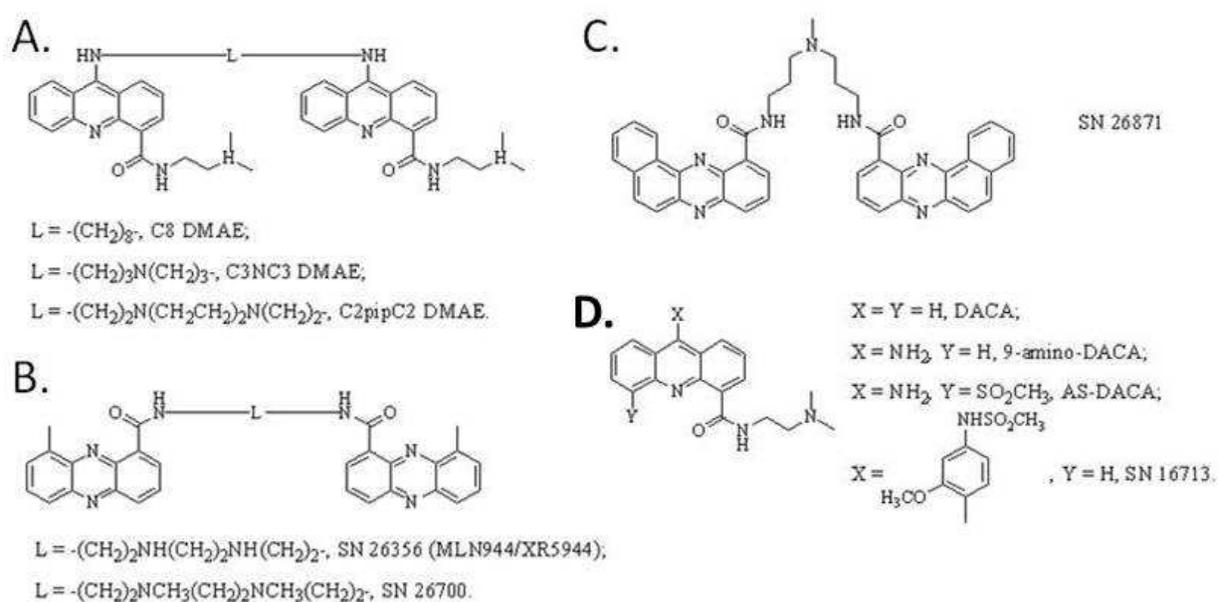
Fig. 1. *Molecular structure* of **(A)** novel transcription inhibitors Bis(9-aminoacridinecarboxamides), C8 DMAE, C3NC3 DMAE and C2pipC2 DMAE, **(B-C)** novel transcription inhibitors Bis(phenazine-1-carboxamides), 26356 (MLN944/XR5944), 26700 and 26871 **(D)** novel topoisomerase poisoning, monointercalating acridine-4-carboxamides, DACA, 9-amino-DACA, AS-DACA and SN16713.

A third class of novel compounds, also structurally based around the acridine-4-carboxamide intercalating chromophore, have previously been identified as topoisomerase poisons (Finlay et al., 1996) and act as monointercalating agents that feature electron-withdrawing moieties in place of a single active side chain (Figure 1D). N-[2-(dimethyl)aminoethyl]-acridine-4-carboxamide (DACA), a dual topoisomerase I/II poison and the parent compound from this class of agents, was unsuccessfully taken into phase II clinical trial in patients with non-small cell lung carcinoma, advanced ovarian cancer, recurrent glioblastoma and advanced colorectal cancer (Twelves et al, 2002; Caponigro et al, 2002). 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 (Atwell et al, 1987). In contrast to DACA, 9-amino-DACA and AS-DACA appear to be more specific poisons for topoisomerase II (Bridewell et al, 2001) with AS-DACA, a less lipophillic derivative (Haldane et al,1999) also known to have a wide spectrum of activity in solid tumours (Atwell et al,1987).

4. Screening novel agents indicates differential response

A panel of 5 RMS cell lines were selected for in vitro assessment of cytotoxicity of novel and established transcription inhibitors and topoisomerase poisons. RD and JR1 were selected to represent the ERMS subtype whilst RH30, RH3 and RH4 were selected to represent the ARMS subtype. We assessed the cytotoxicity of a range of novel and established transcription inhibitors and topoisomerase poisons against 5 established RMS cell lines. MTT cell viability assays were used to determine cell survival after a 72 hour exposure to each compound. Published IC_{50} values (Wolf et al, 2009) are plotted as 'Δ Plots' which

graphically represent the differences in efficacy of each drug in each cell line relative to the median (m) IC_{50} of all drugs in all cell lines (Figure 2).
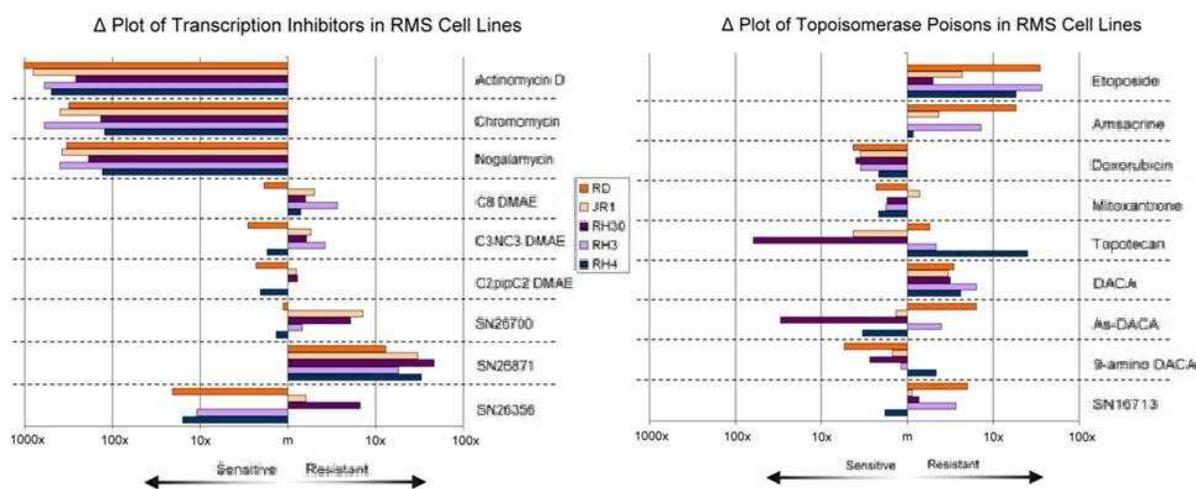

Fig. 2. *Δ plots showing variations in drug potency in 5 RMS cell lines.* IC_{50}s are plotted as a log_{10} measure of sensitivity or resistance against the median (m) IC_{50} of all agents across all cell lines (m = 600 nM). This measure of potency, taken as a whole across all RMS cell lines, serves to highlight the relative differences in drug efficacy. (Wolf, 2009)

Our findings enable classification of these agents into 3 classes; those that are potent in all 5 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. Agents such as those described in group 2 may offer alternative treatment options for RMS tumours unresponsive to the traditional chemotherapy protocols.

4.1 Cytotoxicity of novel and established transcription inhibitors in RMS cells

The antitumour 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 (Yang et al, 1999) 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 (Li and Krueger, 1991). 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 (Zilhif et al, 2006). We have found that all three agents have indistinguishable activity in the 5 RMS cell lines and that they are the most potent agents studied, with activity in the nM range (Figure 2). Seemingly, the fine details of how they interact with DNA to block RNA polymerase do not affect their cytotoxicity. With actinomycin D routinely used in RMS protocols (Table 1), this observation suggests that chromomycin and nogalamycin are worthy of consideration for inclusion in clinical studies.

The development of the bisintercalating bis(9-aminoacridine-4-carboxamide) transcription template inhibitors was inspired by the threading mechanism of nogalamycin (Wakelin et al, 2003). Their threading design, in which the carboxamide sidechains 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. 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 (Wakelin et al, 2003), and similar potencies (nM) in a range of human cancer cell lines (Wakelin unpublished), are found to be about 4 to 40 times less potent in the rhabdomyosarcoma cells, which is some 100 to 1000 times less active than the naturally occurring transcription inhibitors. RD is the only RMS cell line that could be considered sensitive and is the only one in which all three threading dimers produced IC_{50}s marginally lower than m (Figure 2). The origins of the intrinsic resistance of the RMS cell lines to these agents are unclear.

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 (Spicer et al, 2000), but their actual mechanism of action is complex and appears to involve both transcription inhibition, along with topoisomerase I poisoning (Byers et al, 2005). The three compounds studied here are potently cytotoxic in mouse leukemia P388, mouse Lewis lung and Jurkat human leukemia cells (Gamage et al, 2001). The toxicity of SN26700 and SN26871 however, is diminished some 35 to 2200 times in the RMS panel, with their IC_{50}s clustering around m or greatly exceeding it (Figure 2). The exceptional response is found with SN26356 which was used in clinical trial as MLN944/XR5944 (Verborg et al, 2007). Its potent activity in previous studies is maintained in the RD, RH3 and RH4 cell lines, with an average IC_{50} of about 40 nM. The origins of this selectivity are unknown, but our findings point to the importance of considering SN26356 as a possible clinical trial candidate in RMS.

4.2 Cytotoxicity of novel and established topoisomerase poisons in RMS cells

The trapping of topoisomerases in a cleavable complex with DNA is a well established mechanism of action of many DNA binding drugs (Li and Liu, 2001). Representative topoisomerase poisons, both established and novel, were examined in this study, and produced widely ranging results. For example, amongst the clinically used topoisomerase II poisons, etoposide and amsacrine were uniformly, poorly active across the RMS cell line panel, with IC_{50}s all greater than m, ranging from 600nM to 22mM (Figure 2). Such a finding sits oddly with the inclusion of etoposide in clinical RMS protocols (Van Winkle et al, 2005). In contrast, doxorubicin and mitoxantrone are uniformly active in the RMS cells with average IC_{50}s of about 200nM and 400nM respectively, a finding that supports their

inclusion in clinical studies. The only clinical topoisomerase I poison studied, topotecan, produced a differential response with activity of 10nM and 140nM in RH30 and JR1 cells, but IC_{50}s of 1mM to 15mM in the remaining 3 RMS lines. Interestingly, this is the inverse selectivity of SN26356, which is inactive in RH30 and JR1, and raises the intriguing question of the potential clinical activity of their use in combination.

The novel topoisomerase poisons evaluated are structurally based on the acridine-4-carboxamide chromophore, the parent compound of which, DACA (Figure 1), has been identified as a dual topoisomerase I/II poison (Finlay et al., 1996). Despite its wide solid tumour activity and its clinical evaluation (Twelves et al, 2002; Caponigro et al, 2002; Haldane et al, 1993), it shows poor potency in all RMS cell lines with IC_{50}s about 2 to 4 mM. In contrast, its dicationic derivative, 9-amino-DACA, which binds to DNA 6-fold more tightly than DACA and is only weakly active as a topoisomerase I poison (Finlay et al., 1996), is 10 times more potent in all RMS cell lines, making its activity comparable to that of doxorubicin and mitoxantrone (Figure 2). Although the extra charge on the chromophore of 9-amino-DACA enhances cytotoxic potency and antileukaemic activity in mouse tumour models (Atwell et al, 1987), it diminishes solid tumour activity as a consequence of poor tumour penetration due to its elevated DNA affinity. Electron withdrawing substituents in the acridine 5-position lower 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 (Finlay et al, 1996). These characteristics make it generally more cytotoxic than DACA, and endow it with widespread solid tumour activity (Atwell et al, 1987). In the RMS panel it returns a differential response, strongly reminiscent of topotecan, with JR1 and RH30 cells being sensitive, but the remaining three cell lines have IC_{50}s above 1mM (Figure 2). Lastly, within the acridinecarboxamide family, we examined the activity of SN16713, a monofunctional threading agent that superposes the structures of amsacrine and 9-amino-DACA, selectively poisons topoisomerase II which has an IC_{50} of 120nM in CCRF-CEM cells (Zihlif et al, 2006) and 7nM in Jurkat leukaemia (Finlay et al, 1996), is poorly active in RMS cells (Figure 2).

Several novel agents displayed comparable or improved efficacy over their established counterparts in our in vitro drug cytotoxicity study in RMS cell lines. Despite the resistance of some cell lines to these agents their overall efficacy necessitates further preclinical development for possible inclusion in RMS clinical trials. Of particular interest were the novel agents AS-DACA and 9-amino-DACA. 9-amino-DACA showed efficacy across all cell lines comparable to the established topoisomerase poisons flagging its potential as a candidate for future RMS clinical trials. By contrast AS-DACA produced a variable cytotoxic response across the cell line panel. Many factors may be responsible for this observed variation, in particular the 190x fold difference observed between two archetypal RMS cell lines, RD and RH30 (Wolf et al, 2011). The remainder of this discussion will explore our study of AS-DACA cytotoxicity in two RMS cell lines; RD and Rh30, along with AS-DACA-resistant cell line we derived from RH30, named Res30 (Wolf et al, 2011), as an illustration of the complexities of developing new treatment strategies for RMS.

5. Causes for differential drug cytotoxicity in RMS cells

Drug "resistance" is a phenomenon that impedes the efficacy of every compound used in the treatment of cancer at some stage. Mechanisms governing cellular resistance to

chemotherapy may be intrinsic, however in most cases they are acquired following repeated or extended exposure to chemotherapy. Although "acquired" drug resistance is a term that is used to describe the development of drug resistance within cells that were originally chemosensitive, it may in fact result from a clonal proliferation of a subpopulation of intrinsically resistant cells within the original tumour or cell culture. This has been noted to occur within RMS with resistant, differentiated cells making up the majority of tumour remaining after chemotherapy treatment (Klunder et al, 2003). The complexity and number of mechanisms that contribute to drug resistant phenotypes makes identifying and circumventing the source of the problem a challenge for researchers and clinicians alike. Some well established mechanisms of resistance include alterations in drug target levels and function, enhanced drug efflux via membrane bound transport proteins and drug sequestration/altered intracellular drug distribution. Further, 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 used. Given the importance of in vitro studies in pre-clinical drug investigations, it is worthwhile investigating commonly used RMS cell lines to identify the subtle biological mechanisms which are intrinsic to them and produce these selective drug resistance phenotypes. Consequently, in the remaining sections of this review, the impact of different mechanisms of resistance will be explored, with a specific focus on the differential response of AS-DACA in RD and RH30 as a paradigm of this complexity.

5.1 'Classical' drug resistance involving transport proteins

One of the most described mechanisms of drug resistance in RMS cell lines, is ATP-Binding Cassette (ABC) transport protein mediated drug efflux. ABC transport proteins span the plasma membranes of almost all cells and are responsible for active transport of many compounds, including a number of agents used in cancer therapy (Klein et al, 1999). In total 49 human genes have been described that encode various ABC transport pumps (Chang, 2007). Whilst each protein is structurally and functionally distinct, all members of the ABC transport protein family share three conserved sequence motifs within nucleotide binding domains and are common to many proteins that bind ATP (Leslie et al, 1999). For many years it was believed that the MDR1 gene, also known as ABCB1, which encodes P-glycoprotein (P-gp), was the prime contributor to drug efflux (Leslie et al, 1999). Subsequent studies however led to the identification of several related proteins which have also been linked to the multidrug resistance phenotype and include multidrug resistance-associated proteins MRP1 to MRP5 and Breast Cancer Related Protein (BCRP) (Komdeur et al 2003).

5.1.1 Multidrug Resistance-Associated Protein 1 (MRP1)

MRP1 (ABCC1) is a 170kDa protein (190kDa in its glycosylated form), that belongs to the ABC family of membrane bound transport proteins. MRP1 is comprised of 17 transmembrane segments that are grouped into three transmembrane domains (TMDs), two cytoplasmic linker regions and two cytoplasmic nucleotide binding domains. This structure is common to most members of ABCC subfamily. Although the cytoplasmic linker region, which lies between TMD0 and TMD1, has been shown to be vital for drug transport, loss of TMD0 does not greatly affect drug transport (Chang, 2007). MRP1 is understood to transport a greater number of substrates than P-gp, despite being an anion transporter. The anthracycline antibiotics, vinca alkaloids, folate based antimetabolites, antiandrogens,

organic anions and heavy metals are just some of the known substrates for MRP1 (Munoz et al, 2007). This phenomenon has been attributed to the presence of glutathione (GSH) with several studies indicating that without physiological concentrations of GSH present, MRP1 has no ability to transport unmodified anti-cancer drugs. Hence it is considered that MRP1 may co-transport GSH together with anticancer drugs, or GSH may bind to MRP1 and enhance the transport of hydrophobic molecules (Chang, 2007).

MRP1 is overexpressed in many tumours including RMS and other soft tissue sarcomas. In 2005 a study that assessed the expression levels of various ABC transport proteins detected MRP1 in 43% of the surgically resected STS samples examined and found that its expression correlated to a larger tumour size and age of the patient (>20 years) (Oda et al, 2005). Similarly, an earlier study reported MRP1 expression in 11 out of 13 paraffin-embedded primary tumour RMS samples before chemotherapy. In follow up assessments it was found that a metastasis of a tumour which had previously not expressed the protein did so after chemotherapy, and showed increased expression in three other primary tumour samples also following chemotherapy. All other samples however showed equal or decreased levels of expression following drug exposure (Klunder et al, 2003). In a separate study of 29 paediatric and 16 adult RMS cases it reported that MRP1 was expressed in 56% of cases however this expression did not contribute to the poorer response to therapy in older RMS patients (Komdeur et al, 2003).

5.1.2 P-Glycoprotein (P-gp)

P-gp is a 170 kDa protein that predominantly transports cationic or uncharged molecules and is known to efflux many of the compounds used in RMS therapy including the anthracycline antibiotics, actinomycin D, etoposide and the vinca alkaloid vincristine (Larsen et al, 2000). The extent to which P-gp contributes to the poor drug response associated with metastatic RMS has seen much debate with many studies presenting conflicting evidence on the matter. In 2003 a study that screened P-gp levels in 13 pairs of paraffin-embedded RMS samples from patients before and after treatment could not identify any consistent pattern of change in the expression levels of the protein. Of the 13 samples tested, 4 cases saw a decrease in expression of P-gp, 5 cases showed no change and only 4 cases showed an increase in expression post treatment (Klunder et al, 2003). Similarly, in 1996 it was reported that high P-gp expression was not correlated to poor drug response in RMS patients following therapy (Kuttesch et al, 1996). This study, which used immunohistochemistry to detect and measure P-gp levels from 71 patients that had been treated between 1969 and 1991 found no association between the expression levels at diagnosis and patient outcome following treatment. Instead it was suggested that multidrug resistance is a consequence of combining agents from several drug classes that subsequently induce a range of resistance mechanisms within a single population of cells. Another separate study found that despite a poorer prognosis in older RMS patients, age at diagnosis has no effect on expression levels of the protein (Komdeur et al, 2003). Whilst these studies suggested P-gp contributed little to the poor drug response associated with metastatic RMS, several papers had previously presented a strong relationship between patient prognosis and P-gp expression level. One such example correlated P-gp levels with relapse in 30 biopsy samples from RMS and STS patients, and found that of the 9 patients with detectable P-gp levels, all relapsed. Of the 21 patients without detectable P-gp levels, only 1 patient relapsed (Chan et al, 1990).

5.1.3 MRP1 transport of AS-DACA in RMS cells

The monointercalating acridine-4-carboxamide compounds used in this study are vulnerable to transport via these efflux pumps. AS-DACA is a substrate for P-gp whilst DACA, the parent compound from this class, is not (Haldane et al, 1999). Using western blot analysis the influence of AS-DACA on the expression levels of MRP1 was investigated in both resistant and sensitive cell lines. Western blot analysis of MRP1 expression levels showed substantial change in RD and RH30 cells following a 16 hour exposure to double the IC_{50} of AS-DACA for each cell line (Figure 3A). This protein was also expressed in Res-30
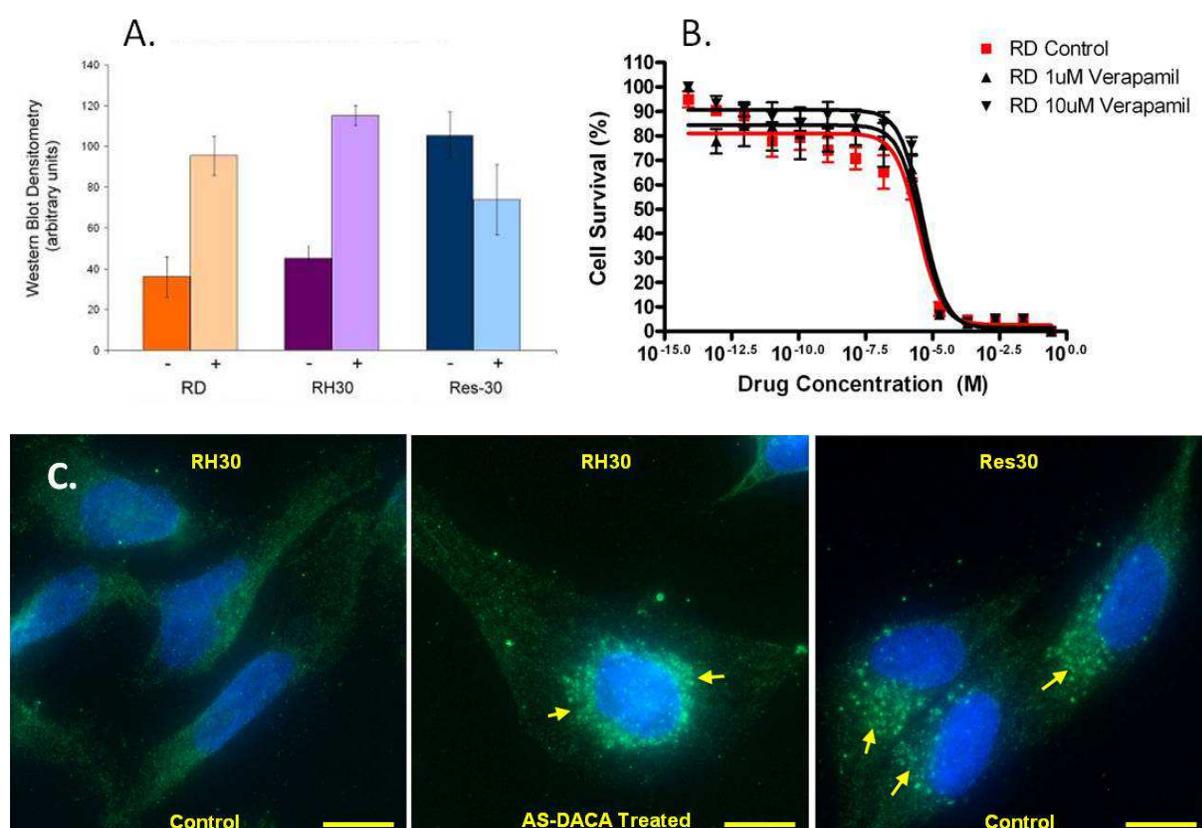
Fig. 3. *The influence of MRP1 expression on AS-DACA activity in RMS cells.* **(A)** Expression levels of MRP1 (170kDa) were determined by western blot before (-) and after (+) exposure to AS-DACA ($2xIC_{50}$ for 16 hours). Densitometry was performed on triplicate blots with band intensity normalized for both background noise and total protein loading differences as determined by Ponceau S staining. **(B)** The influence (R)-verapamil has on AS-DACA efficacy was established using MTT cell viability assays. RD and Res-30 cells were treated with 1μM and 10μM of verapamil whilst being exposed to various concentrations of AS-DACA for 72 hours. Error bars represent the SEM of three independent experiments in each case. **(C)** Intracellular localization of MRP1 in RH30 cells before and after AS-DACA exposure as determined by immunofluorescence microscopy. MRP1 appears green. Highlighted with yellow arrows is a bright staining vesicular structure in the perinuclear region following exposure to AS-DACA. All cells are co-stained with the nuclear stain DAPI (blue) and displayed as merged artificially coloured monochromatic images. Untreated Res30 show the same structures indicating it has acquired this feature during selection. Scale bars = 10μm.

cells without an additional exposure to AS-DACA. Exposure to the MRP1 inhibitor (R)-verapamil at concentrations of 1 and 10 mM failed to alter AS-DACA-induced cytotoxicity (Figure 3B), indicating the differential toxicity of AS-DACA in the RMS cells is not simply explained by understanding classical drug resistance involving MRP1 (Wolf et al 2011). So whilst the higher levels of the ABC transport protein MRP1 following treatment with AS-DACA was detected, typical of a 'classical' multidrug resistance phenotype in RMS cells, this treatment-induced increased expression of MRP1 in cell lines does not explain the differential in sensitivity to AS-DACA induced cell death. Firstly, both RD and RH30 cell lines demonstrated an equivalent increase in MRP1 expression (Figure 3A). The absence of any effects of the MRP1 inhibitor verapamil (Figure 3B), indicate that despite the changes in protein levels, impedance of MRP1 activity did not alter AS-DACA toxicity in any RMS cells.

Interestingly, MRP1 protein was shown to localize to vesicular membranes in all three cell lines (Wolf et al, 2011) this being most prominent in cells directly after exposure to AS-DACA (Figure 3C). Active mechanisms that drive sequestration of weakly basic compounds into vesicles of the membrane trafficking system are not uncommon. Several of the ABC transport pumps have been implicated in such mechanisms and alternative pathways involving the trans-golgi network (TGN), lung-resistance-related protein 1 and major vault proteins have been proposed to modify intracellular drug distributions by altering transport between the nucleus, endosomal vesicles, lysosomes and the cytoplasm. Rajagopal *et al* (2003) used HeLa cells to demonstrate MRP1, P-gp and BCRP were localized to membranes of intracellular vesicles that they contributed to drug resistance phenotypes via sequestration-based mechanisms at these sites. With this growing evidence suggesting that various ABC transport proteins, including MRP1, are localized to vesicles of the membrane trafficking system where they actively transport drug from the cytoplasm into lysosomes (Rajagopal et al, 2003) these experiments further implicated MRP1 with drug sequestration, rather than drug efflux from the cell. It is clear that understanding this impact is vital for the future development of AS-DACA and other members of the class of monointercalating acridine-4-carboxamides and hence, deserves further attention in future studies.

5.2 Alterations in drug target: Reduced topoisomerase levels

The consequence of trapping topoisomerase enzymes in cleavable complexes with DNA is an accumulation of double strand breaks in the DNA helix. There is strong evidence that AS-DACA induced cell death results from an accumulation of double stranded DNA breaks following the trapping of topoisomerase II in a cleavable complex with DNA (Bridewell et al, 2001). Hence, we determined that the expression level of each topoisomerase isoform was a contributing factor in the variation of RMS cell response to AS-DACA exposure. We first establish the level of DNA damage induced by the compound correlated with the degree of sensitivity or resistance exhibited by each cell line. Using an enzyme linked immunosorbent assay (ELISA) for phosphorylated histone 2A (γH2AX) (Burma et al, 2001), DNA damage was assessed in both sensitive and resistant cell lines following low dose exposures equivalent to the IC_{50} in RH30 cells (20nM) of AS-DACA over a time course of 48 hours. The results are displayed in Wolf et al 2011. DNA damage was substantially reduced in Res-30 cells when compared to the parental RH30 cell line even after 48 hours exposure to the drug. Whilst DNA damage was evident after just 1 hour in the sensitive RH30 cells, it does not

appear until 24 hours later in the resistant Res-30 cells. Interestingly, DNA damage is detected in RD cells after just 4 hours despite the drug exposure being approximately 450 times lower than its IC_{50} in this cell line. After 48 hours the level of DNA damage has reached 40% of what would be reached when the cells are exposed to their IC_{50} dose for the same length of time. In Res-30 cells only 10% of the DNA damage induced with a 4µM dose of AS-DACA was observed after a 48 hour exposure to 20nM of AS-DACA. From these findings it is clear that the level of DNA damage induced in each cell line correlates to the IC_{50} of AS-DACA in each cell line. Not surprisingly, the levels of DNA damage increase as the time of exposure to AS-DACA increases and this is most obvious in the sensitive cell line RH30.

The immediate interpretation of this result is that AS-DACA is being prevented from intercalating with the DNA and forming cleavable complexes. With DNA damage having been confirmed as a primary consequence of AS-DACA exposure (Wolf et al, 2011), it was essential to assess the relationship between the expression levels of each topoisomerase isoform and the levels of DNA damage induced in each cell line following exposure to the drug. Since the trapping of topoisomerase in its cleavable complex with DNA is the primary mechanism by which AS-DACA induces DNA damage (Finlay et al, 1996), we hypothesized that the differences in response between the RD and RH30 cell lines to AS-DACA was due, in part, to intrinsic differences in the levels of the primary drug target, topoisomerases. Mechanisms are known to reduce the efficacy of topoisomerase poisons through decreased expression of the target enzyme, topoisomerase. It is well established, for example, that the topoisomerase I enzyme is reduced following cell exposure to the topoisomerase I poison camptothecin and the subsequent activation of the ubiquitin/26S proteasome pathway. As described in Desai et al 1997, topoisomerase I-ubiquitin conjugates were discovered to form within minutes of camptothecin treatment in Chinese hamster ovary (CHO) cells decreasing levels of topoisomerase I by up to 80%, 2 hours after treatment. The expected reduction of topoisomerase I following camptothecin treatment was reversed using the 26S proteosome inhibitors MG-132 and lactacystin demonstrating that the ubiquitin/26S pathway was involved in the camptothecin induced down regulation of topoisomerase I (Desai et al, 1997). This correlation was further highlighted using a panel of breast cancer cell lines that had previously been shown to exhibit a high variability in their response to the drug, which was shown to result from the down regulation of topoisomerase I (Li and Liu, 2001).

This phenomenon is not exclusive to the topoisomerase I isoform however with published data suggesting that both topoisomerase IIα and topoisomerase IIβ may also be down regulated following exposure to various topoisomerase II poisons. One study highlighted that in a panel of cell lines representing various tumour types, both isoforms of topoisomerase II were degraded following activation of a proteasome pathway that specifically targeted the enzyme when it was trapped to DNA in complex with etoposide (Fan et al, 2008). The down regulation of topoisomerase IIα as a means to circumvent drug cytotoxicity is however controversial as this enzyme has previously been shown to be essential for cell survival with topoisomerase IIβ unable to compensate in its absence (Austin et al, 1998). On the other hand it has been shown that topoisomerase IIα is overexpressed in several tumour types (Murphy et al, 2007) and hence may permit a down regulation of the enzyme to a level that does not compromise cell viability yet limits the efficacy of topoisomerase IIα poisons.
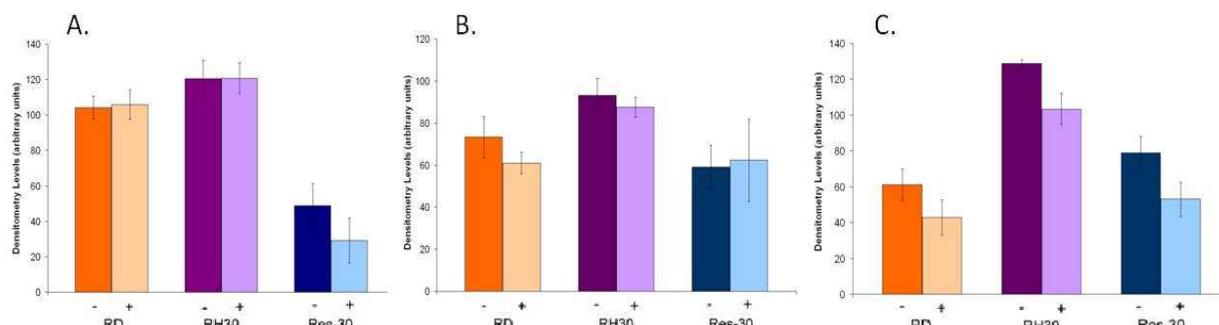
Fig. 4. (A) Topoisomerase I (90kDa), (B) Topoisomerase IIa (175kDa) and (C) Topoisomerase IIβ (180kDa) levels were determined by western blot before (-) and after (+) exposure to AS-DACA ($2x$ IC_{50} for 16 hours). Densitometry was performed on triplicate blots with band intensity normalized for both background noise and total protein loading differences as determined by Ponceau S staining. Error bars represent the SEM of intensity for each band in three independent experiments.

As topoisomerase levels are known to be reduced in many cell lines in response to topoisomerase poison exposure, we explored using western blotting whether one or more isoforms of the enzyme were altered in our RMS cell lines following AS-DACA exposure. The expression levels of each topoisomerase isoform were assessed in both treated and untreated cells following an overnight exposure to AS-DACA (16 hours) at a concentration double the IC_{50} for each individual cell line. From this analysis of topoisomerase I and II western blots (Figure 4), it was evident that the basal levels of each isoform varied in the cell lines assessed. Basal levels of topoisomerase I appeared marginally higher in the RH30 cell line compared to RD and were substantially reduced in Res-30 cells – to at least 50% of the level observed in the parental RH30 cell line. Levels of topoisomerase I were unaffected by a 16 hour exposure to AS-DACA in both RD and RH30 cell lines whilst a modest reduction was observed in the Res-30 cell line. No change in topoisomerase IIα levels were observed following an overnight exposure to AS-DACA in any cell line, however RH30 cells again appeared to possess modestly higher basal levels of this protein compared to both RD and Res-30 cells. Although Res-30 cells possessed the least amount of this protein, its expression was similar to that observed in the RD cells. Topoisomerase IIβ was the only isoform to be reduced, albeit modestly, in each cell line following overnight exposures to AS-DACA. Again RH30 appeared to possess an increased basal expression of this enzyme in comparison to both RD and Res-30 cell lines and unlike the former two isoforms, the expression of topoisomerase IIβ was at least double that of RD.

Upon examination of the expression levels of each topoisomerase isoform in RD and RH30 cells (Figure 4) (Wolf et al, 2009), it became clear that intrinsic differences in their basal levels were present as hypothesized. As all isoforms are clearly expressed in RD, and DNA damage is induced with even small concentrations of drug (Wolf et al, 2011), it is unlikely that the levels of each isoform exclusively promote the resistant phenotype, although it may be a contributing factor. In comparison, the modest overexpression of topoisomerase I and topoisomerase IIα isoforms as well as the substantial overexpression of topoisomerase IIβ in RH30 cells may promote an enhanced drug efficacy. A higher expression of the target enzyme could translate to an increased frequency in the formation of cleavable complexes

which are subsequently trapped by AS-DACA, resulting in the induction of permanent DNA damage. Furthermore, the expression levels of each isoform may also contribute to the rate at which DNA damage is induced with higher levels of topoisomerase resulting in a more rapid formation of trapped cleavable complexes. Importantly, the difference in efficacy of AS-DACA in each cell line is unlikely to be linked to an ability of the cells to decrease protein levels during an overnight exposure to the drug. A modest decrease in topoisomerase IIβ levels was evident in both sensitive and resistant cell lines and hence is unlikely to influence drug efficacy(Figure 4). The AS-DACA resistant Res-30 which was not cross resistant to known topoisomerase poisons (Wolf et al 2011) suggests a resistance mechanism peculiar to AS-DACA. The absence of double strand breaks in the Res-30 line (Wolf et al, 2011) indicates extensive impedance of the agent from its primary cytotoxic target.

5.3 Drug sequestration through the endosomal pathways

It was not long after the discovery of the lysosome by Nobel laureate Christian de Duve in the 1960's that a mechanism of drug sequestration involving acidic vesicles had been proposed (Kaufmann and Krise, 2007). In early studies of lysosomal sequestration correlation was drawn between the pKa of compounds and their ability to "induce vacuolization" (Yang et al, 1965). It was well known that weak acids and bases are capable of readily diffusing lipid bilayer membranes providing pH gradients exist on either side of the membrane partition.

The membrane trafficking system, illustrated in Figure 5A, is responsible for the transport and processing of proteins, cellular waste and foreign materials. It is comprised of several vesicle types that traffic along the cytoskeletal network of microtubules to various locations throughout the cell. Some of these vesicles include lysosomes, late stage endosomes (LSE), early endosomes (EE), multi-vesicular bodies (MVB) and macropinosomes. Each vesicle differs not only in their function but also in their intravesicular pH and in the receptors they express. Early endosomes, which originate from clathrin coated pits (CCP) in the cell's plasma membrane, are an integral component in the endocytic uptake of extracellular material (Saraste et al, 2007). These vesicles, with an approximate pH of 6.2, express SNARE proteins, the GTPases Rab4 and Rab5 and the early endosome antigen 1 (EEA1) protein. During the fusion of vesicles and formation of late stage endosomes, the vesicle pH drops to around 5.5 which not only aids in the degradation of intravesicular material but promotes recycling of the various membrane bound proteins back to the plasma membrane in recycling vesicles (Luzio et al 2001). Late stage endosomes, which express the membrane bound proteins Rab7, Lysosome Associated Membrane Protein-1 (LAMP-1) and mannose-6-phosphate receptor, receive and process material from both early endosomes and the TGN (Luzio et al 2001). The TGN is the end point in many endocytic pathways, internalizing a range of extracellular materials which are destined for endosomes or lysosomes And are trafficked in transport vesicles through a process regulated by the mannose-6-phosphate receptor. Material contained within the late stage endosomes is generally destined for degradation and when this cannot be achieved by the endosomes themselves, fusion of the vesicles with lysosomes will take place. Lysosomes, which continue to express the membrane bound protein LAMP1, may have a pH as low as 4.7 and contain acid hydrolases that degrade cellular waste including proteins and lipids (Luzio et al 2001).
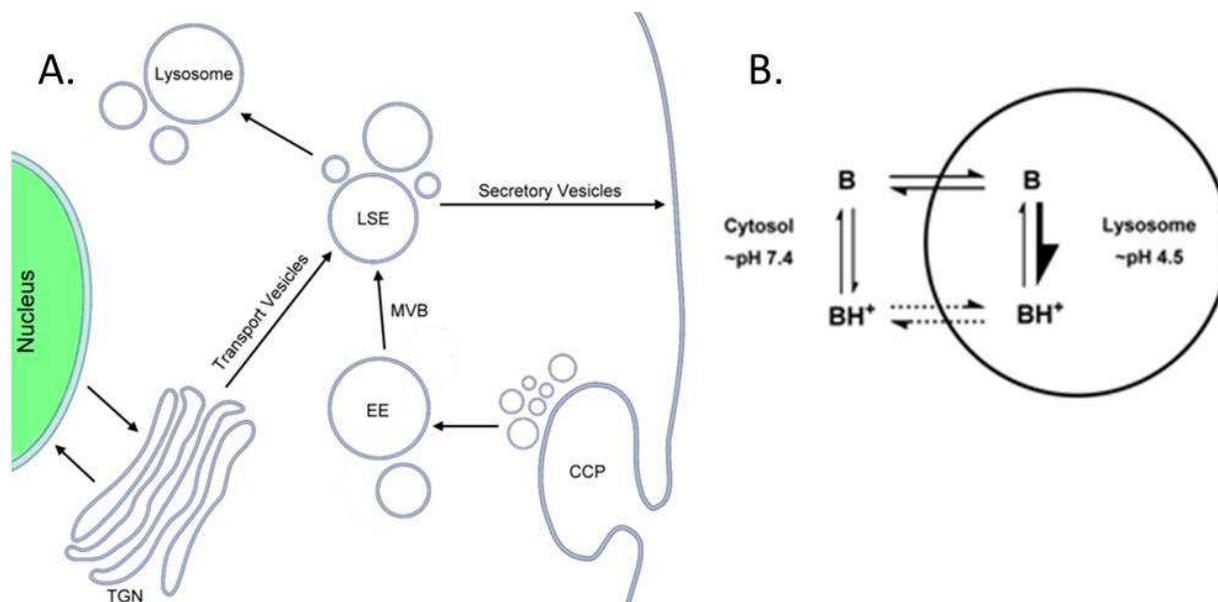
Fig. 5. **(A)** *Schematic Diagram of the Membrane Trafficking System*. This system is responsible for the distribution and processing of various proteins, lipids, foreign material and cellular waste. In this diagram CCP stands for Clathrin Coated Pit, EE for early endosome, LSE for Late Stage Endosome and MVB for multi-vesicular body, TGN for trans-golgi-network. **(B)** *Partitioning Theory* - Schematic diagram of the pH partition that is believed to be the predominant driving force in the sequestration of weakly basic in lysosomes. In the neutral conditions of the cytosol, compounds with pKa's near neutral will exist mainly in the free base form, maintaining its membrane permeability. In the lower pH of the lysosomes weak bases will exist predominately in the ionized or protonated form rendering the molecule impermeable to lipid bilayers. This figure has been reproduced from Kaufmann et al, 2007.

Classically, vesicle sequestration of the anthracycline antibiotics appears to be dictated by the "partitioning theory". The partitioning theory (Figure 5B) of drug sequestration, which was proposed not long after the discovery of the lysosome in the 1960's, is still believed to contribute significantly to drug resistance. This basic biochemical property formed the foundation of the partitioning theory of drug sequestration that was described by Christian de Duve is still accepted today (De Duve et al, 1974). The partitioning theory is based upon the principle that weak acids and bases are capable of readily diffusing lipid bilayer membranes down pH gradients, rendering these compounds able to accumulate in acidic vesicles due to protonation by free H+ ions present in the vesicular lumen. Such ionization renders the drug impermeable to the membrane and accumulation will continue whilst vesicular H$^+$ ion concentrations are maintained. So long as H$^+$ ion concentrations are maintained by V-type H$^+$-ATPase vacuolar proton pumps, drug accumulation will continue (Kaufmann and Krise, 2007). It has also recently been noted that this theory may be extended to explain the rapid drug efflux that occurs from tumour cells growing in hypoxic conditions. This occurs as a result of acidification of the extracellular space from anaerobic glycolysis and the subsequent exaggeration of the cytosol-extracellular fluid pH gradient (Chen et al, 2006).

Without an ability to view the intracellular distribution of weakly basic compounds, this sequestration may go completely unnoticed. As a research tool, the anthracycline antibiotics

are commonly used because their fluorescence properties allow the intracellular distribution of the drugs to be explored using fluorescence techniques. In 1986 it was first reported that doxorubicin accumulated in lipid based vesicles as a result of its distribution being determined by pH gradients (Mayer et al, 1986). In another study it was shown, using microspectrofluorometry, that the anthracycline antibiotic pirarubicin accumulates in the Golgi body of drug resistant K562, CEM and LR73 cells. It was proposed that this accumulation occurred as a result of the Golgi body being more acidic in MDR cells and hence possessing a stronger pH gradient across its membrane that influenced the passive diffusion of the compound (Belhoussine et al, 1998). The importance of pH gradients in the vesicular sequestration of the anthracycline antibiotics was again reported in 2003 by Ouar et al. In this study pH gradients were abolished in resistant cells using concanamycin A, an inhibitor of the vacuolar proton pumps that are responsible for the maintenance of pH. Inhibition of the proton pumps restored sensitivity to daunorubicin, doxorubicin and epirubicin and prevented the redistribution of daunorubicin from the nucleus into lysosomes.

Although vesicular sequestration of weakly basic compounds is still believed to be facilitated by passive diffusion down pH gradients, several recent studies have indicated that other mechanisms may influence this process. In 2007 it was shown that knockdown of the major vault protein (MVP) using siRNA disrupted lysosomal uptake of not only doxorubicin, but also the intracellular pH probe LysoSensor and the lysosomal specific antigen LAMP1. This study indicated that MVP was integral in redistributing the drug away from the nucleus to the lysosomes (Herlevsen et al, 2007). In another study, cells were exposed to various weak bases before lysosomes were isolated and their contents quantified using high performance liquid chromotography. It was found that the lysosomes sequestered 3 to 15 times the volume of compound that would be expected if passive diffusion was the only mechanism facilitating this process. Hence this study also arrived at the conclusion that alternative mechanisms were active in the lysosomal sequestration of weakly basic compounds (Duvvuri and Krise, 2005).

Despite strong evidence implicating acidic vesicles of the membrane trafficking system in the sequestration of weakly basic compounds in in vitro settings, there is poor understanding of the influence this mechanism plays in the overall efficacy of compounds in a clinical setting. It was recently reported that disruption of vesicular sequestration following cellular exposure to the proton pump inhibitor omeprazole increases not only the potency of doxorubicin but also its penetration through an in vitro multicell layer culture (Lee and Tannock, 2006). This article hypothesized that it is in the effective treatment of solid tumours that vesicular sequestration of anti-cancer drugs may be truly relevant. As an effective treatment is dependant on the compound's ability to penetrate the tumour mass, in situations where drug is captured in the outer layers of a tumour, penetration is limited and treatment will be rendered ineffective. Furthermore, it is important to note that other agents used in the clinic in combination with weakly basic compounds may inadvertently promote their vesicular sequestration. In 2007 it was revealed that vincristine treatment produced not only a larger number of acidic vesicles retained in the cell but also the presence of unusually large lysosomal compartments. It was believed that this occurs following destabilization of the microtubule network, which is required to direct and maintain the movement of the lysosomes, endosomes and autophagosomes (Groth-Pedersen et al, 2007).

5.3.1 AS-DACA fluorescence allows monitoring of its distribution in RMS cells

AS-DACA is a unique compound that has dynamic fluorescence properties. These had never been characterized nor had they been exploited for experimental purposes. Clearly, such a property provides an invaluable tool in the assessment of intracellular drug distribution and for this reason an experimental protocol was optimized that allowed the visualization of the drug in our RMS cell lines. Initial examination of the intracellular distribution of AS-DACA revealed that it was being localized to areas other than the nucleus, but also that each compound fluoresced different colours depending on the area to which they had localized. This latter finding was unexpected as all other established cytotoxic agents that are associated with fluorescence, emit only single colours, in all intracellular situations. The findings presented in Figures 6 highlight that AS-DACA emits different colours when excited at a single wavelength. This suggests that depending on the microenvironment to which the compounds had localized, a change in drug structure may alter the emission wavelength.
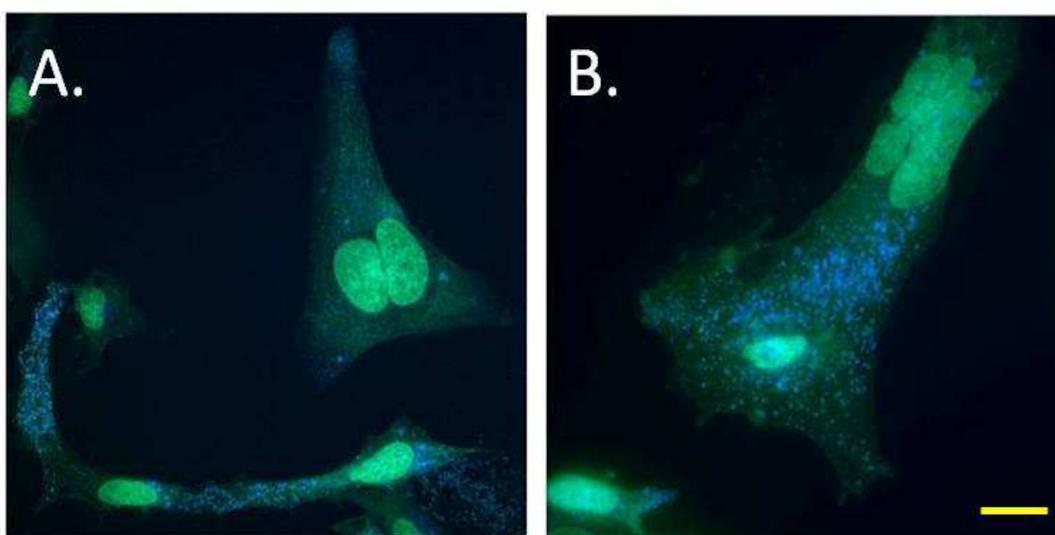
Fig. 6. *Images of the intracellular distribution of AS-DACA* in (A) RD and (B) RH30 cells. Images were captured using digital colour photography after a 4 hour exposure to 2μM of the drug. The agent was excited between 330nm - 385nm. Images were captured at 600x magnification. Scale Bar = 10mm (Wolf et al, 2011 for experimental details)

As we had hypothesized that AS-DACA would be vulnerable to protonation in low pH environments such as those found in vesicles of the membrane trafficking system, we reasoned that this shift in colour emission may correlate directly with the pH of the microenvironment in which the drugs had localized. Furthermore, AS-DACA is known to be lipophilic (Haldane et al, 1999) and thus vulnerable to sequestration in acidic vesicles. AS-DACA not only retains the ability to passively diffuse membranes but also contains a site in its structure that may be protonated at a lower pH making it subject to the partitioning theory. These factors together make the drug vulnerable to such a mechanism of sequestration. To determine if this hypothesis was correct, the influence of pH on the fluorescence properties of each compound was assessed. A luminescence spectrophotometer was used to measure the entire spectrum of emission from both compounds following excitation at 262nm in a range of solutions of differing pH. The results presented by Wolf et

al 2011 clearly highlight that pH influences AS-DACA's fluorescence profile substantially. Although green emission does not appear to change greatly, blue emission, particularly at around 440nm, decreases significantly as pH rises, indicates that between pH 6.2 and pH 6.8, the intensity of blue fluorescence becomes lower than the intensity of green fluorescence. Above pH 6.6 the intensity of green fluorescence is stronger than that of blue (Wolf et al 2011).
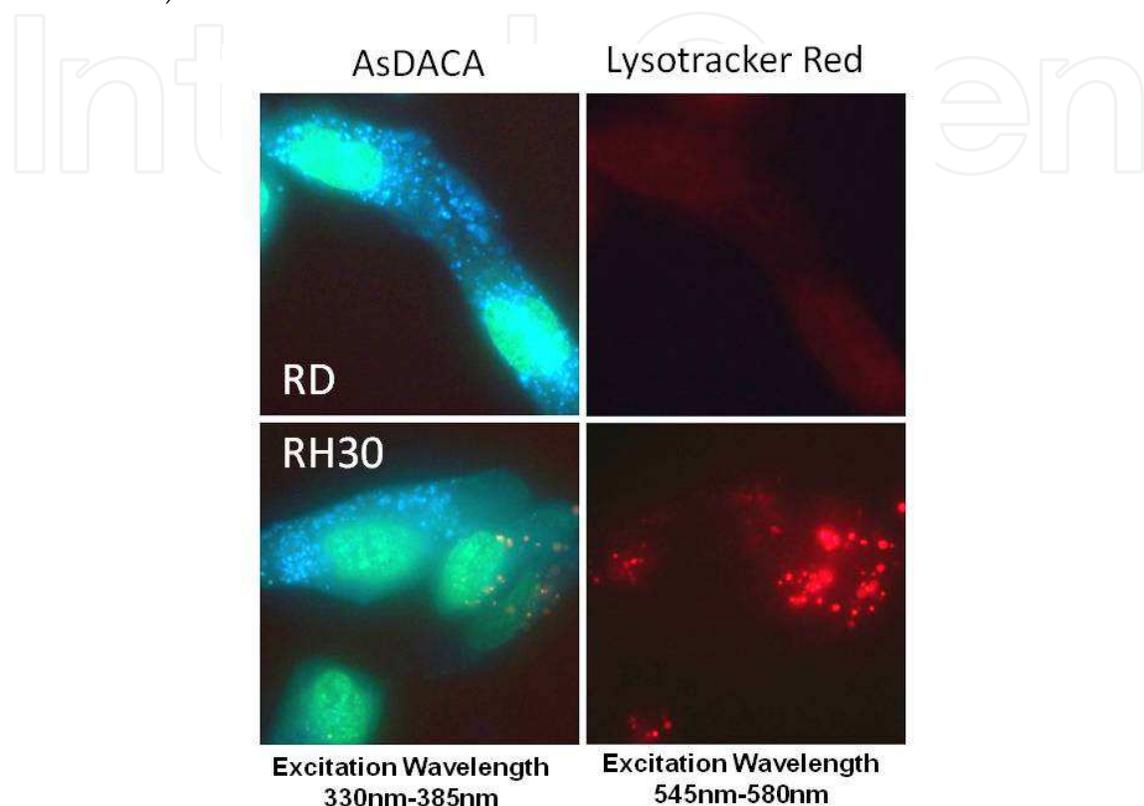

Fig. 7. *Intracellular Distribution of AS-DACA and LysoTracker™ Red in RD and RH30 Cells.* RD and RH30 cells were exposed to 2µM of AS-DACA for 4 hours and 100nM of LysoTracker™ Red for 45 minutes before being viewed on an epifluorescent microscope. Images were captured at 600x magnification. Scale bars represent 10µm.

In defining the influence pH has on the fluorescent properties of AS-DACA, it was shown that the compound fluoresced blue in the pH range below ~6.2. This provided evidence that the membrane trafficking system plays a role in AS-DACA's intracellular distribution. However, as this was only correlative, a more direct approach was taken to assess this potential link. Figure 7 highlights that following simultaneous short incubation with AS-DACA and LysoTracker™ Red, an intracellular probe that stains vesicles of low pH, RH30 cells consistently failed to stain with LysoTracker™ Red yet showed a strong sequestration of AS-DACA. In the RD cell line, whilst LysoTracker™ Red was observed within acidic vesicles co-localization was rare with most cells displaying vesicular accumulation of LysoTracker™ Red or AS-DACA individually. In rare instances however co-localization of AS-DACA and LysoTracker™ Red was observed in RD cells. Careful deconvolution of images following epifluorescence microscopy indicated that in RD cells exposed to LysoTracker™ Red, simultaneously with AS-DACA, some incomplete co-localization was observed, and limited to only a proportion of cellular vesicles (Wolf et al, 2011).

5.3.2 Determining which vesicles sequester AS-DACA

With all the evidence thus far suggesting that the observed sequestration of AS-DACA was a specific redistribution mechanism involving acidic vesicles of the membrane trafficking system, it became important to establish which vesicles were involved, and to determine how this redistribution of AS-DACA was taking place. As each intracellular vesicle functions as part of a larger network. Early endosomes traffic material to late stage endosomes which in turn traffic material to lysosomes. AS-DACA was shown to fluoresce blue when in environments below pH 6.2 (Figure 7), this confirmed that sequestration of the drug was occurring in acidic vesicles. However, this did not provide any insight into which vesicle types were responsible. To help determine which vesicles were involved in AS-DACA's sequestration, expression levels of markers specific for the various vesicle types were assessed using Western blot analysis (Figure 8). Sensitive (RH30) and resistant (RD and Res-30) cells were exposed to double the IC$_{50}$ of AS-DACA overnight and the expression levels of early endosome antigen 1 (EEA1), Rab5, LAMP1 and Rab7 were assessed. As detailed above, the two former markers are localized to the membranes of early endosomes whilst the latter two are found on late stage endosomes and lysosomes. We suspected that in order to assist in the drug's redistribution away from the nucleus, an increase in vesicle number may occur in resistant cells following drug exposure. This would be reflected by an increase in the expression levels of the marker proteins localized to the vesicle involved.
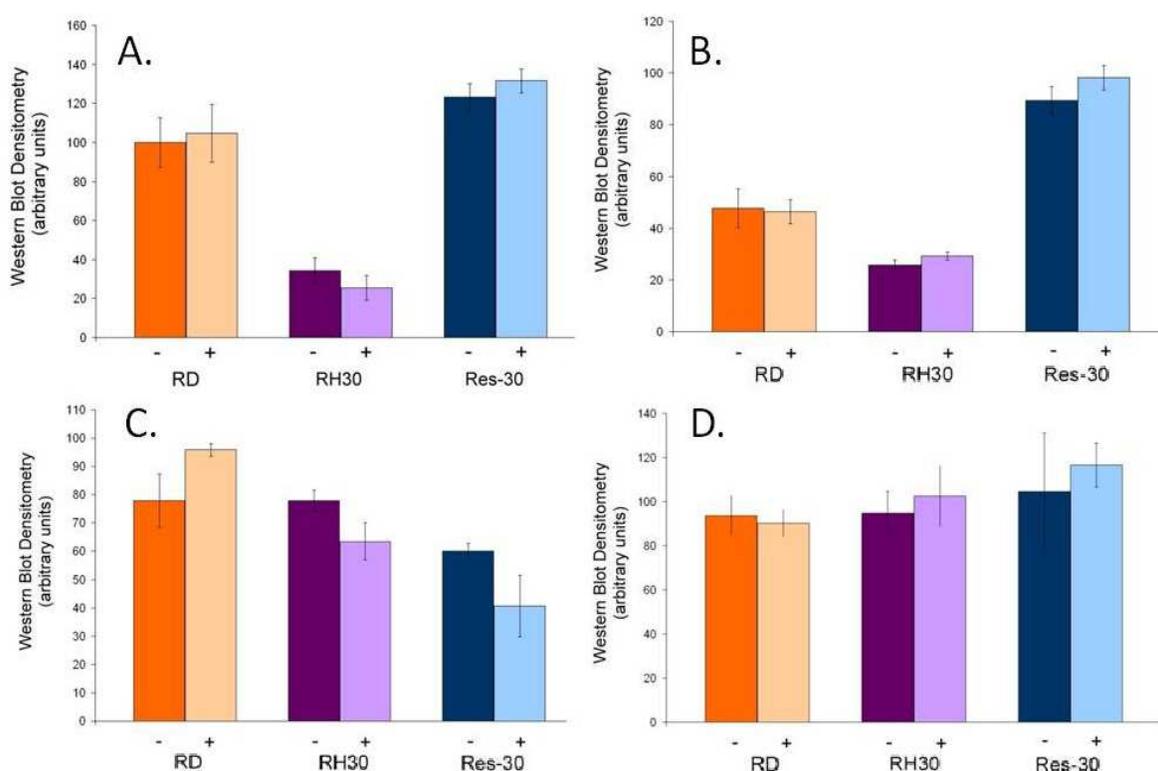

Fig. 8. *Expression levels of (A) EEA1, (B) Rab5, (C) LAMP-1 and (D) Rab7 in RMS cells.* Cells were processed by western blot for each protein before (-) and after (+) exposure to AS-DACA (2x IC$_{50}$ for 16 hours). Densitometry was performed on triplicate blots with band intensity normalized for both background noise and total protein loading differences as determined by Ponceau S staining. Error bars represent the SEM of intensity for each band in three independent experiments.

Shown in Figure 8A, expression of EEA1, a marker of early endosomes, was not altered in any cell line following a 16 hour exposure to double the IC_{50} of AS-DACA. Expression of the protein was however, substantially higher in the resistant RD and Res-30 cell lines and the clear increase observed in Res-30 cells relative to RH30 cells was independent of an additional overnight treatment of drug. Similarly, Rab5 which is also associated with early endosomes, showed no change in expression level in any cell line following a 16 hour exposure to AS-DACA (Figure 8B). A marked increase however was again observed in Res-30 cells relative to RH30 which supported the overexpression of EEA1 in Res-30 cells. LAMP-1, which is associated with both lysosomes and late stage endosomes, showed minimal change in expression levels in all cell lines following a 16 hour exposure to AS-DACA (Figure 8C) This was supported by the expression levels of Rab7, a protein associated with late stage endosomes, which also showed minimal change in expression levels in all cell lines following a 16 hour exposure to AS-DACA (Figure 8D). This opposed our original hypothesis and implicated early endosomes, or unidentified transport vesicles with similar markers, in the sequestration and redistribution of AS-DACA.

5.3.3 Disrupting the membrane trafficking system: Influence of Brefeldin A

To further assess the hypothesis that AS-DACA is selectively redistributed away from the nucleus into vesicles of the membrane trafficking system, drug efficacy and intracellular distribution were assessed in resistant cell lines following pretreatment with Brefeldin A (BFA). BFA, a potent disrupter of Golgi, functions due to its inhibition of Arf1, which is vital for formation of transport vesicles originating at the TGN (Klausner et al, 1992).

It has previously been shown that small molecules may be taken up into Golgi apparatus (Belhoussine et al, 1998) and it is well established that nuclear waste is often removed via the ER and sorted in the TGN before being transported to vesicles of the membrane trafficking system for degradation. With this in mind Golgi function was disrupted by BFA in order to establish the influence this organelle plays in AS-DACA's sequestration by the membrane trafficking system. Importantly, BFA is known to be inherently cytotoxic and could therefore only be administered at doses that did not lead to a reduction in cell growth. Using an MTT based cell viability assay it was established that BFA was cytotoxic at concentrations above 15nM in both cell lines and hence this was defined as the maximum tolerated dose (Figure 9A). The cytotoxic effect of Brefeldin A pretreatment on AS-DACA efficacy in the resistant RD cell line increased sensitivity to AS-DACA modestly with a two fold reduction in IC_{50} (Figure 9B). This suggested that disruption of the trafficking system at the TGN may influence drug resistance of RD. These changes to drug efficacy appear to correlate to the intracellular distribution of AS-DACA in both cell lines with little to no vesicular accumulation was observed in RD cells (Figure 9 C,D). To ensure however that the BFA pretreatment impacted only on vesicle formation from the Golgi body and not on the integrity or function of preexisting vesicles, cells were exposed to 100nM of LysoTracker™ Red for 1 hour. This probe provides an indication of both vesicular pH and functionality after BFA exposure and it is clear (Figure 9 C,D) that BFA exposure does not alter the frequency, integrity or functionality of vesicles in the RD RMS cell line.

Following pretreatment of these cells with agents that disrupt various stages of the membrane trafficking pathway, such as brefeldin A, provides further evidence to suggest resistance to AS-DACA is determined by sequestration into the endosomal trafficking pathways. Together, these results highlight that AS-DACA is sequestered by acidic vesicles

of the membrane trafficking system through a range of mechanisms. It appears that the TGN contributes to the redistribution of drug in RD cells whilst MRP1, or other membrane bound transport pumps, localize to vesicles in RH30 and Res-30 where they contribute to drug uptake into acidic vesicles. The results presented highlight not only that AS-DACA is vulnerable to sequestration in vesicles of the membrane trafficking system in RMS cell lines. Although the exact mechanisms that drive sequestration has not been fully defined, it appears that several pathways contribute to the removal of drug from the nucleus and subsequent accumulation in vesicles of low pH. Whether AS-DACA is merely redistributed away from the nucleus or whether it is effluxed from the cells remains unknown.
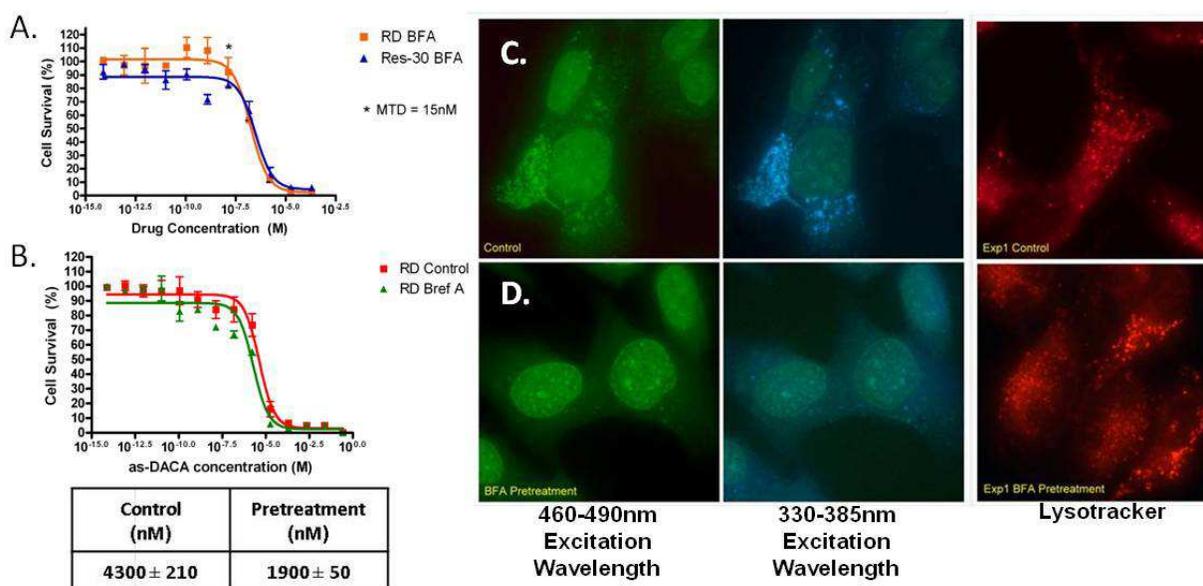
Fig. 9. *The influence BFA has on AS-DACA efficacy in RMS cells.* **(A)** MTT cell viability assay for BFA to determine maximum tolerated dose (MTD). **(B)** RD and Res-30 cells were pretreated for 16 hours with 15nM of BFA before being exposed to various concentrations of AS-DACA for 72 hours. A two fold difference in drug efficacy was seen in the RD cell line. **(C-D)** Images of AS-DACA distribution in RD cells with **(D)** and without **(C)** 16 hour exposure to 15nM of BFA and subsequent 4 hour exposure to 2μM of AS-DACA. For comparison, RD cells were pretreated with 15nM of BFA for 16 hours followed by 100nM of the acidicc vesicular probe LysoTracker™ Red for 1 hour. Images were captured using epifluorescence microscopy at 600x magnification with excitation of the probe between 545nm and 580nm.

6. Conclusion

With RMS being one of the most common paediatric solid tumours associated with poor drug response, this mechanism deserves further attention. Despite significant improvements in the treatment of non-metastatic ERMS in the past 30 years, patients with metastatic ERMS and ARMS continue to face a poor prognosis. This is due, in part, to a lack of response of the tumours to current the chemotherapy options. In this chapter, the cytotoxic effect of novel topoisomerase poisons and transcription inhibitors assessed in a panel of human RMS cell lines have been described. Findings were presented that reveal variable cell responses following exposure to a novel topoisomerase poison, AS-DACA. With this series of

experimenst we have illustrated some of the cell biology which needs to be considered when exploring the therapeutic potential of new agents. Complexities include "non-classical" resistance mechanisms that are driven by the reduction of key target proteins (eg topoisomerases), drug sequestration mechanism which result in the accumulation of AS-DACA in acidic vesicles of the membrane trafficking system. This complex suite of cellular responses to general cytotoxics raises the consideration that such patient-specific differences in RMS tumours may lead to these agents contributing to personalised therapeutic strategies.

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8. References

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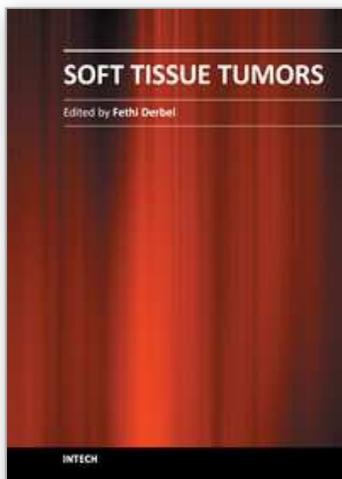
Soft Tissue Tumors

Edited by Prof. Fethi Derbel

Soft tissue tumors include a heterogeneous group of diagnostic entities, most of them benign in nature and behavior. Malignant entities, soft tissue sarcomas, are rare tumors that account for1% of all malignancies. These are predominantly tumors of adults, but 15% arise in children and adolescents. The wide biological diversity of soft tissue tumors, combined with their high incidence and potential morbidity and mortality represent challenges to contemporary researches, both at the level of basic and clinical science. Determining whether a soft tissue mass is benign or malignant is vital for appropriate management. This book is the result of collaboration between several authors, experts in their fields; they succeeded in translating the complexity of soft tissue tumors and the diversity in the diagnosis and management of these tumors.

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