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Influence of P-glycoprotein expression on *in vitro* cytotoxicity and *in vivo* antitumour activity of the novel pyrrolobenzodiazepine dimer SJG-136

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Abstract

SJG-136 is a novel pyrrolobenzodiazepine dimer analogue that acts as a minor-groove interstrand DNA cross-linking agent. The present study investigated the impact of ABCB1 (mdr-1) expression on the activity of SJG-136 using both *in vitro* and *in vivo* systems. SJG-136 was highly potent in the colon cancer cell lines HCT-116, HT-29 and SW620 (IC_{50} 0.1–0.3 nM). However, HCT-8 and HCT-15 cells expressing significant levels of mdr-1 were less sensitive (IC_{50} 2.3 and 3.7 nM, respectively) using a SRB assay. The cytotoxicity was increased in HCT-15 and $A2780^{AD}$ in presence of 5 μg/ml verapamil. Mdr-1 mRNA expression was determined by qRT-PCR and correlated to SJG-136 IC_{50}s ($r^2 = 0.86$, $P = 0.0001$). Isogenic 3T3 cells expressing mdr-1 cDNA (3T3 pHamdr-1) were less sensitive to SJG-136 than the parental 3T3 cells (IC_{50} 208 and 6.3 nM, respectively). Finally, SJG-136 (120 μg/kg/d dx5) was highly active against A2780 xenografts (SGD = 275) but not $A2780^{AD}$ xenografts (SGD = 67).

Keywords: SJG-136; Pyrrolobenzodiazepine; Interstrand cross-linking agent; DNA-binding; Minor-groove; ABCB1; mdr-1; Drug resistance; Colon cancer; Ovarian cancer

1. Introduction

SJG-136 is a new pyrrolobenzodiazepine dimer analogue. It acts as an interstrand cross-linking agent that alkylates DNA in a sequence specific manner within the 6 bp sequence: Pu–GATC–Py (Pu = purine; Py = pyrimidine) [1,2]. Pre-clinical studies have highlighted the potency of this agent in a number of tumour types [3,4]. COMPARE analysis using the NCI 60 panel identified mdr-1 as a potential factor correlated with the cytotoxicity of SJG-136.

The product of the *ABCB1* or *mdr-1* gene is a membrane protein called P-glycoprotein. P-gp belongs to the family of ABC (ATP-Binding Cassette) transporters that is the largest transporter gene family. P-gp is composed of intracellular, extracellular and membrane-spanning domains and can translocate a variety of substrates such as sugars, amino acids, and hydrophobic compounds across the membrane [5]. P-gp is expressed in a number of normal tissues, *i.e.*, intestine, blood–brain barrier, hepatocytes, kidneys, placenta, ovaries and testes [6]. Its physiological role is to prevent accumulation of toxic substances in the body by either preventing their absorption and diffusion or by increasing their excretion. P-gp is formed of two homologous halves, each containing 6 transmembrane domains and

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an ATP-binding domain. It functions as a pump, moving molecules from the intracellular to the extracellular compartment, the hydrolysis of ATP providing the energy to function against concentration gradients. The overall activity of the protein depends on its level of expression and also its functionality which is linked to both substrate specificity and transport effectiveness [7].

The *ABCB1* (*mdr-1*) gene has been studied extensively since it was recognised many years ago that its overexpression in cancer cells leads to resistance to anthracyclines [8]. Multidrug resistant tumour cells have been shown to have a highly active drug efflux, preventing the accumulation of drug in the cell cytoplasm. Since then, a broader spectrum of hydrophobic compounds have been identified as substrates for P-gp including vincristine, paclitaxel and VP16 [5]. Overexpression of P-gp has been reported in tumours that originate from tissues that physiologically express high levels of the protein such as colon, kidney and liver [9]. P-gp overexpression has been linked in colon cancers to the tumour grade and level of differentiation [10]. Moreover, P-gp can be up-regulated in tumours exposed to chemotherapeutic agents as reported in bladder cancers [11]. Finally, P-gp has recently been linked to the tumourigenesis of colon cancer. Yamada and colleagues [12] have shown that inactivation of APC or β-catenin results in the down-regulation of P-gp, the promoter of which contains several T Cell Factor binding sequences.

In the development of new anticancer drugs such as SJG-136, it is important to evaluate whether cytotoxicity is influenced by P-gp expression since it may influence the spectrum of antitumour activity.

2. Materials and methods

2.1. Materials

SJG-136 was obtained from the National Cancer Institute (Bethesda, MA). Aliquots (2 mM) in dimethylsulfoxide (DMSO) were stored at −80 °C. For *in vivo* studies, the drug was dissolved in 1% v/v DMSO in saline. Other chemicals were obtained from SIGMA. Primers were provided by Cancer Research UK research services. Mdr-1 rabbit polyclonal antibody, and secondary antibody Anti Rabbit IgG-horseradish peroxidase were from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA). Actin monoclonal antibody and anti-mouse IgM were from Calbiochem (Merck Biosciences, Nottingham, UK).

2.2. Cell culture

HT-29, HCT-8, SW620, HCT-116, HCT-15, COLO205 cell lines were obtained from the American Type Cell Culture Collection (Rockville, MD) and European Collection of Cell Cultures (Salisbury, UK). A2780 and A2780^{AD} cells were provided by the National Cancer Institute (NCI, Bethesda, USA). 3T3 GP + E-86 and pHamdr-1 were kindly provided by Dr. E. Schuetz from St. Jude's Children Research Hospital, Memphis TN. Cells were grown in monolayer in RPMI 1640 medium supplemented with 5% v/v fetal calf serum and 1% v/v L-glutamine at 37 °C in a humidified atmosphere containing 5% CO_2. A2780^{AD} cells were maintained in the presence of 10^{-7} M doxorubicin. Cells were tested regularly for mycoplasma contamination and were mycoplasma-free for the period of the study.

2.3. Cytotoxicity assay in vitro

Drug concentrations that inhibited 50% of cell growth (IC_{50}) were determined using the sulforhodamine B (SRB) technique [13]. Cells were plated on day 1 in 96-well plates. The cell density was 1500 cells/well for 3T3 GP + E-86, 1000 cells/well for pHamdr-1, 2500 cells/well for HCT-116, 3500 cells/well for HCT-8 and HT-29, 5000 cells/well for HCT-15 and A2780^{AD}, 6000 cells/well for SW620, and 7000 cells/well for A2780 and COLO205 in a volume of 150 μl/well. All cell lines were treated on day 2 except A2780 and A2780^{AD} which were treated on day 3. After drug exposure, cells were washed once with cold phosphate buffer saline (PBS) and placed in 200 μl of drug-free medium for 72 h after the end of drug exposure. The cells were then fixed with trichloracetic acid and stained with sulforhodamine B. Optical densities were measured at 540 nm with a Biohit BP-800 (Bio-Hit, Helsinki, Finland). Growth inhibition curves were plotted as percentage of control cells and IC_{50}s were determined by Graphpad Prism 3 Software (Graphpad Software, San Diego, CA) using a sigmoidal curve fitting with variable slope. The goodness of fit was determined by $r^2 > 0.9$ and the Hill coefficient <-1. The results were based on 3–4 independent experiments performed in triplicate and are presented as mean and 95% confidence intervals. The time-dependency index (TDI) is the mean of individual ratios between IC_{50} (1 h exposure) and IC_{50} (24 h exposure).

2.4. Drug treatment

Colon cell lines were treated on day 2 with SJG-136 for 1 or 24 h with a concentration range centred on the IC_{50} in a volume of 50 μl. A2780 and A2780^{AD} cells were treated on day 3 with SJG-136 for 24 h (range: 0.25 pM–2.5 nM) in a volume of 50 μl. When cells were pre-treated with verapamil, they were seeded in 100 μl medium and treated with 5 μg/ml verapamil starting 1 h before SJG-136 exposure in a final volume of 50 μl.

2.5. Expression of P-gp by immunoblotting

Cells (2×10^6) were lysed with 400 μl lysis buffer (62.5 mM Tris pH 6.8, 6 M urea, 10% v/v glycerol, 2% w/v SDS, 0.003% w/v Bromophenol Blue) and sonicated on ice for 20 s. Protein concentrations were determined using the bicinchonidic acid assay. Fifty μg of protein was denatured at 45 °C for 30 min in the presence of 5% v/v 2-mercaptoethanol, and 40 μg protein was separated by 7.5% SDS–PAGE and transferred to an Immobilon-P Transfer membrane (Millipore). The membrane was blocked in 5% w/v Marvel in Tris buffered saline pH 7.5 with 0.1% v/v Tween (TBS-T) for 1 h at room temperature, probed with Mdr-1 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1/400 in 5% Marvel in TBS-T for 1 hour at RT and washed with TBS-T and TBS. After incubation with the secondary antibody Anti Rabbit IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA) at 1/4000 in 2.5% Marvel in TBS-T for 1 h at RT and further washing with TBS-T and TBS, blots were visualised by chemiluminescence using Western blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA). The same procedure was performed with actin antibody (1/120 000) and anti-mouse IgM secondary antibody (1/4000).

2.6. Expression of P-gp mRNA by real-time PCR

Total RNA was prepared using Tri-Reagent. The RT-PCR reaction was carried out using the QIAgen SYBR Green RT-PCR kit (Qiagen, Crawley, UK) using 150 ng total RNA (0.15 ng for pHamdr-1). Primers for amplification of exon 23 in the *mdr-1* gene were used: mdr-1E23 forward primer 5′aggccaacatacatgccttc and mdr-1E23 reverse primer 5′ccttctctggctttgtccag. 22.5 pmol of each primer was used per reaction. Beta actin was used as internal standard to normalise the results using the following primers: BAF: 5′ctacgtcgccctggacttcgagc and BAR: 5′gatggagccgccgatccacacgg. Quantification was performed using a standard curve for both mdr-1 and BA from $A2780^{AD}$ total RNA (150–0.15 ng). The validation criteria were a correlation coefficient >0.99 and variation from the standard slope of <20%. Each standard was performed in duplicate and samples in triplicates. Results are mean ± SD of three independent experiments.

2.7. Antitumour activity of SJG-136

Animal experiments were carried out under a project license issued by the UK Home Office, and UKCCCR guidelines [14] were followed rigorously. A2780 and $A2780^{AD}$ cells were injected subcutaneously in 50% v/v Matrigel® (Becton Dickinson) into C57/Bl6 Nu/Nu mice. Treatment started when xenografts reached 50–150 mm^3. Animals received SJG-136 intravenously (i.v.) at the dose of 300 μg/kg as single injection or 120 μg/kg/d dx5 according to Alley and colleagues [15]. Weights were monitored daily from start of treatment to the end of the study. Toxicity was evaluated by body weight loss. Xenografts were measured 3 times a week. Tumour volumes were calculated from caliper measurements as $\text{width}^2 \times \text{length}/2$. Results are mean ± SE of 7–15 animals.

2.8. Statistical methods

Comparisons between mean values were performed using a two-sided *t* test after verification of the homogeneity of variances. Confidence intervals at 95% confidence were used for IC_{50} data. Correlations were established using Prism Software and coefficient correlation significance tested with a linearity test. In the *in vivo* experiments, a comparison of growth rate on day 7 was performed using an Anova test and *t* test ($P = 0.05$).

3. Results

3.1. Cytotoxicity of SJG-136 on colon cell lines

The cytotoxic effect of SJG-136 in a panel of colon cell lines (HCT-116, HT-29, SW620, HCT-8, HCT-15 and COLO205) after 1 and 24 h exposure is presented in Table 1. HT-29, SW620 and COLO205 cells are the most sensitive to 1 h exposure of SJG-136 with similar IC_{50}s. The least sensitive colon cell line was HCT-15 with an IC_{50} after 1 h exposure to 120 nM (95% CI = 100–140 nM).

The cytotoxic effect of SJG-136 was markedly increased in COLO205 when increasing the time exposure. Other cell lines like HCT-116, HT-29 and SW620 also showed evidence of time dependent cytotoxicity with time-dependency index (TDI) ranging from 60 to 85. However, HCT-8 and HCT-15 cells were less affected by duration of drug exposure but they were also the two least sensitive cell lines (means TDI of 35 and 38, respectively).

3.2. Impact of verapamil on the cytotoxicity of SJG-136 towards HCT-15 cells

Verapamil is a P-gp blocker that is widely used to study the impact of P-gp overexpression on drug uptake. We preincubated the least sensitive cell line of our panel, HCT-15, with 5 μg/ml of verapamil 1 h prior to the start of the exposure to SJG-136. The concentration of verapamil was chosen according to the recommendations of the Developmental Therapeutics Program of the NCI/NIH and was confirmed as inducing no significant cytotoxicity in control cells (data not shown). The survival curves, with and without verapamil pretreatment after

Table 1
SJG-136 IC_{50}s for the panel of colon cell lines determined by SRB assay

	1 h		24 h		TDI	
	Mean (nM)	95% CI	Mean (nM)	95% CI	Mean	Range
SW620	13	10–16	0.11	0.09–0.14	85	64–95
COLO205	14	12–16	0.005	0.004–0.009	3100	1300–4300
HT-29	19	15–24	0.3	0.2–0.4	82	39–110
HCT-116	21	18–25	0.35	0.3–0.45	60	52–67
HCT-8	39	26–58	2.3	2–2.8	35	20–66
HCT-15	120	100–140	3.7	3–4	38	17–69

Results are means and 95% confidence intervals of three independent experiments performed in triplicate.

1 and 24 h exposure to SJG-136, are presented in Fig. 1. The IC_{50} of 1 h exposure to SJG-136 decreased 4-fold when HCT-15 cells were pretreated with verapamil (from 126 nM for SJG-136 alone to 29 nM in the presence of 5 μg/ml verapamil). The difference was even greater after 24 h exposure with a 10-fold decrease in IC_{50} in the presence of verapamil.

3.3. Cytotoxicity of SJG-136 on A2780 and A2780^{AD} cells

A2780^{AD} cells are derived from the parental A2780 cell line and are resistant to doxorubicin. Several studies have shown that the resistance to doxorubicin is mediated by overexpression of the *mdr-1* gene. Results of the cytotoxic effect of SJG-136 using 24 h exposure for both A2780 and A2780^{AD} are presented in Fig. 2. A2780 cells were highly sensitive to SJG-136 ($IC_{50} = 0.27$ pM [0.2–0.4 pM] after 24 h exposure). The pretreatment of A2780 cells with verapamil induced a marginal increase in cytotoxicity ($IC_{50} = 0.13$ pM [0.06–0.2 pM]). Using the same time exposure, SJG-136 induced a much lower cytotoxic effect on A2780^{AD} ($IC_{50} = 13$ nM, [10–15 nM]), but it was significantly increased by the presence of verapamil ($IC_{50} = 0.7$ nM [0.4–1 nM]) representing a 20-fold increase in activity.

3.4. P-gp expression in colon and ovarian cell lines and correlation with cytotoxicity

The expression of mdr-1 protein in the panel of colon cell lines and in the two ovarian cell lines A2780 and A2780^{AD} are presented in Fig. 3(a). As expected, A2780 cells do not express P-gp while A2780^{AD} cells display a significant overexpression of P-gp. Only two colon cell lines, HCT-8 and HCT-15, expressed detectable levels of mdr-1 by immunoblotting. The

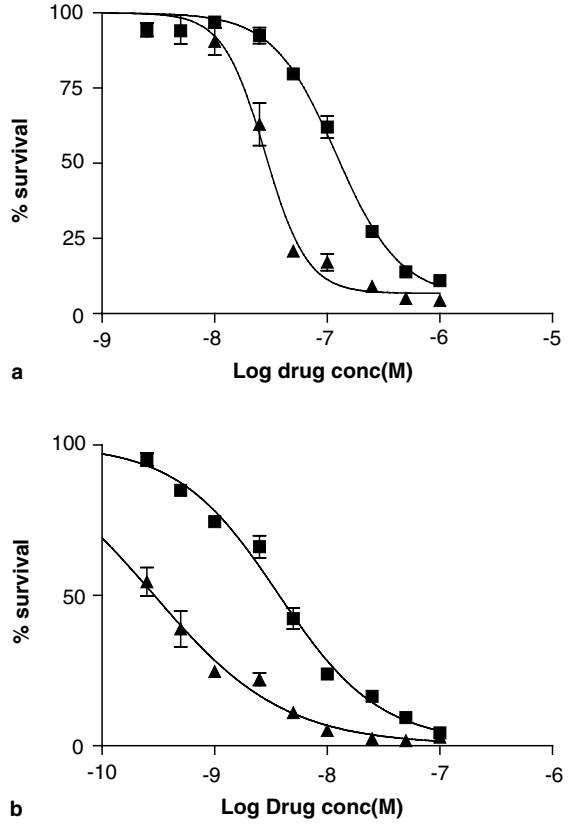

Fig. 1. Impact of P-gp inhibition on the cytotoxic effect of SJG-136 as determined by SRB assay in HCT-15 cells. Cells were treated with SJG-136 for: (a) 1 h or (b) 24 h with (▲) or without (■) pretreatment with 5 μg/ml of verapamil.

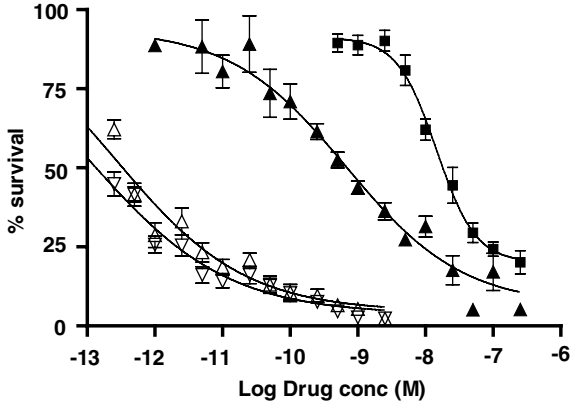

Fig. 2. Impact of P-gp inhibition on the cytotoxic effect of SJG-136 as determined by SRB assay on A2780 (open symbols) and A2780^{AD} (closed symbols) cells. Cells were treated with SJG-136 for 24 h with (▽, ▲) or without (△, ■) pretreatment with 5 μg/ml of verapamil. Results are means ± SEM.

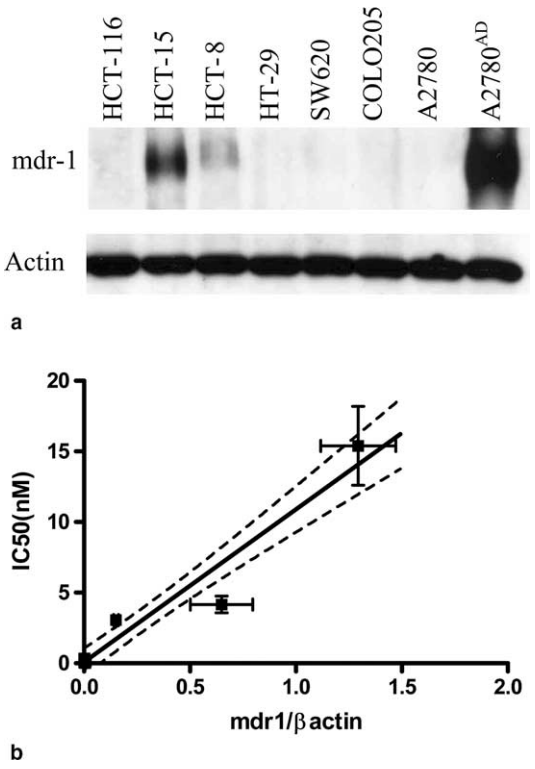

Fig. 3. (a) P-gp expression determined by immunoblotting in the panel of colon cell lines. A2780 and A2780^{AD} cells were used as negative and positive controls, respectively. (b) Correlation between ABCB1 (mdr-1) mRNA expression of the colon and ovarian cell lines determined by real-time PCR and IC_{50}s of SJG-136 after 24 h exposure. Means ± SD are presented. The dotted lines represent the 95% confidence intervals of the correlation.

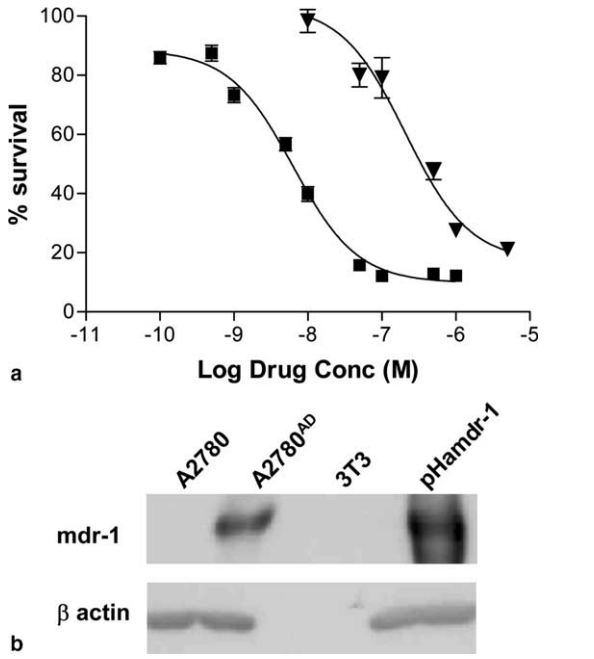

Fig. 4. (a) Cytotoxicity of 24 h exposure to SJG-136 in 3T3 GPE + E86 (■) and 3T3 pHamdr-1 (▼). Results are mean ± SEM. (b) Expression of P-gp in 3T3 GPE86 and pHamdr-1 compared to A2780 and A2780^{AD}.

quantitation of mdr-1 mRNA by real-time PCR confirmed that mRNA levels were consistent with protein expression, with HCT-8 and HCT-15 colon cell lines being the only two expressing significant levels of mdr-1 mRNA. The correlation between mdr-1 mRNA levels and the cytotoxicity of SJG-136 (24 h exposure) in both the colon and the two ovarian cell lines is presented in Fig. 3(b). The coefficient of correlation $r^2 = 0.86$ was highly significant ($P < 0.0001$, d$f = 22$). The correlation remained significant when either the colon cell lines alone or the cytotoxicity after 1 h exposure were considered.

3.5. Cytotoxicity of SJG-136 on 3T3 GP + E-86 and pHamdr-1

3T3 fibroblasts retro-transduced by either empty vector (3T3 GP + E-86) or vector expressing wild type mdr-1 (pHamdr-1) were exposed to SJG-136 for 24 h. The growth inhibition curves for both cell lines are presented in Fig. 4(a) along with P-gp expression in both cell lines (Fig. 4(b)). SJG-136 was cytotoxic against the 3T3 parental cell line with an IC_{50} of 6.3 nM [5.2–7.6]. pHa-mdr-1 cells were markedly less sensitive to SJG-136 with an average IC_{50} of 208 nM [138–312 nM].

3.6. In vivo antitumour activity of SJG-136

To confirm whether the impact of mdr-1 expression on the cytotoxicity of SJG-136 was relevant *in vivo*, SJG-136 was evaluated in A2780 and A2780^{AD} xenografts. The drug was very well tolerated with a maximum body weight loss of 7% in animals receiving 120 μg/kg/d dx5. No toxic deaths were reported.

A2780 treated with SJG-136 at 120 μg/kg/d dx5 or 300 μg/kg dx1 did respond significantly to the treatment (Fig. 5). On day 7, $V_t/V_0 = 0.9 \pm 0.07$ after 120 μg/kg/d dx5 and 1.3 ± 0.2 after 300 μg/kg dx1 compared to control animals treated with vehicle ($V_t/V_0 = 2.9 \pm 0.4$, $P = 5 \times 10^{-5}$, d$f = 21$ and $P = 8 \times 10^{-3}$, d$f = 17$, respectively). The specific growth delay (SGD) of 187 for animals treated with 300 μg/kg and 275 for animals treated with 120 μg/kg/d confirmed the efficacy of SJG-136 in this model.

However, A2780^{AD} xenografts did not respond significantly to SJG-136 treatment (Fig. 5), whatever the dose or schedule of administration. On day 7, $V_t/V_0 = 2.8 \pm 0.2$ after 120 μg/kg/d dx5 and 2.7 ± 0.2 after 300 μg/kg dx1 compared to control animals ($V_t/V_0 = 2.9 \pm 0.2$). The specific growth delays (SGD) after 300 μg/kg dx1 and 120 μg/kg/day of SJG-136 were equivalent at 67.

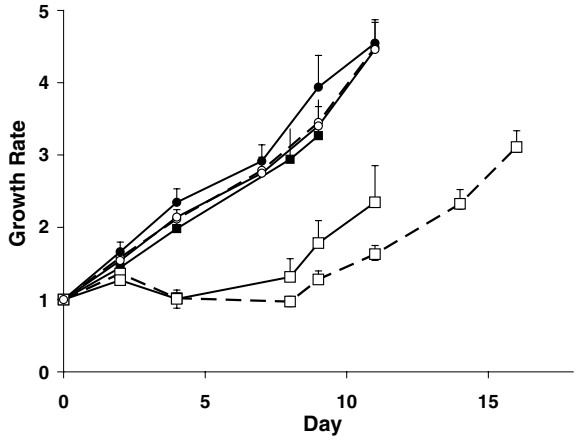

Fig. 5. *In vivo* efficacy studies of SJG-136 in A2780 (squares) and A2780^{AD} (circles). Animals were treated intravenously with vehicle (closed symbols), 300 μg/kg dx1 (plain line, open symbols) or 120 μg/kg/d dx5 (dotted lines, open symbols) of SJG-136. Results presented are mean ± SE of the growth rate (V_t/V_0).

4. Discussion

SJG-136 is a new pyrrolobenzodiazepine dimer that has shown a broad spectrum of cytotoxicity across the NCI 60 cell line panel and is also active in cisplatin-resistant cell lines A2780cis^{R} and CH11/cisR [1]. Phase I studies are ongoing to evaluate the safety profile of the drug and to identify the dose and schedule of administration for phase II studies. Therefore, it is of interest to identify biological parameters that may influence the pharmacology of the drug and its spectrum of activity, in order to guide phase II study design.

The cytotoxic effect of SJG-136 has been determined in a panel of six colon cell lines using two different time exposures of 1 and 24 h. All cell lines showed a marked enhancement in cytotoxicity with increased duration of drug exposure, except for the two least sensitive cell lines, HCT-8 and HCT-15. Matsushima and colleagues have classified anticancer agents as being either time-dependent or time-independent. Compounds described as time-dependent [*i.e.*, displaying high time-dependency indices (TDIs)] include antimetabolites such as 5FU and methotrexate. Doxorubicin, nimustine and melphalan have low TDIs (<10) [16]. High TDIs may be observed if uptake or DNA binding and adduct formation are slow. In the case of SJG-136, it is known from DNA thermal denaturation studies on calf thymus DNA that although there is significant adduct formation immediately upon exposure of the DNA to the agent, further significant adduct formation occurs for as long as 18 hours and possibly beyond (*i.e.*, ΔT_m values of 25.7, 31.9, 33.6 °C at 0, 4 and 18 h, respectively, for a 1:5 molar ratio of ligand–DNA) [1]. It is also likely that the rate of DNA binding of SJG-136 may be different between naked DNA *in vitro* and DNA within chromosomes in the nucleus.

The cytotoxicity of SJG-136 was improved in HCT-15 cells by pretreatment with 5 μg/ml of verapamil. The IC_{50} decreased from a mean of 3.7 to 0.3 nM after 24 h exposure, thus achieving an IC_{50} that is in the range of the other colon cell lines. Verapamil is a P-gp reversing agent (substrate of P-gp) that inhibits the transport function in a competitive manner. Its ability to reduce P-gp related efflux varies with P-gp substrate. For example, verapamil reverses daunorubicin and paclitaxel resistance efficiently compared to colchicine resistance which is best reversed by cyclosporin A [17]. In order to provide stronger evidence for the role of P-gp in SJG-136 cytotoxicity, the activity of SJG-136 in A2780 and A2780^{AD} ovarian cells was evaluated. The latter cell line was selected for resistance to doxorubicin by stepwise incubation with the drug, leading to a 100-fold resistance as compared to the parental A2780 cells [18]. Therefore, the overexpression of P-gp is one of the main features of A2780^{AD} cells.

A remarkable sensitivity of A2780 cells to SJG-136 was observed which was consistent with the previous report from Gregson and colleagues [1]. These authors showed that A2780CisR cells which are resistant to cisplatin (another DNA adduct forming drug), were not cross-resistant to SJG-136. However, the current study shows that A2780^{AD} cells are significantly less sensitive to SJG-136 than their parental counterparts ($IC_{50} = 13$ nM after a 24 h exposure). Preincubation of A2780^{AD} cells with verapamil improves the cytotoxicity 20-fold, but the IC_{50} remains significantly higher than in parental cells. This could be due to the selection of a specific clone from the A2780 population to obtain the A2780^{AD} cell line. It is also possible that several members of the ABC family are amplified in A2780^{AD} cells. As verapamil activity is restricted to P-gp [19], it would be necessary to study more specific inhibitors to investigate this further. Finally, the overexpression of mdr-1 may not be the only factor that is up regulated in these cells that could contribute to the difference in cytotoxicity.

Studies of mutations occurring in the mdr-1 gene have shown that they can influence both the level of expression of the protein or its substrate specificity [7]. Therefore, in this study it was important to investigate whether mdr-1 expression as determined by real-time PCR correlated with the IC_{50} of SJG-136 in the panel of colon cell lines and the two ovarian cell lines, A2780 and A2780^{AD}. A positive correlation was found between these two parameters implying that, at least in these cell lines, the level of expression of mdr-1 is an important determinant of the cytotoxicity of SJG-136. Furthermore, evidence for this was obtained by testing the drug on an isogenic system of 3T3 fibroblasts expressing wild type mdr-1 (pHamdr-1) [20]. PHamdr-1 cells were found to be 33-fold less sensitive to SJG-136 than the parental 3T3 cell line. The level of expression of mdr-1 determined by qRT-PCR was 10 000 fold

higher in pHamdr-1 than in HCT-15 cells (data not shown). Therefore, taken together, these results highlight the impact of mdr-1 on the biological effect of SJG-136, although results from the latter model are probably less relevant clinically.

In order to verify whether the impact of mdr-1 overexpression on the cytotoxic effect of SJG-136 may be relevant *in vivo*, immunodeficient mice bearing A2780 and $A2780^{AD}$ xenografts were treated with SJG-136 at its maximum tolerated dose of 300 μg/kg dx1 and 120 μg/kg/d dx5. SJG-136 showed significant antitumour activity in A2780 xenografts and the efficacy was greater when a dx5 schedule was used. This supports the recent report from Alley and colleagues [15] and our own *in vitro* data that the cytotoxicity of SJG-136 is time-dependent both *in vitro* and *in vivo*. Moreover, $A2780^{AD}$ xenografts did not respond to SJG-136 using identical doses and schedules, confirming the *in vitro* data.

In summary, these results show that the cytotoxicity of SJG-136 is dependent on the expression of P-gp in the cell lines studied, with overexpression of P-gp reducing the activity of the drug both *in vitro* and *in vivo*. Although more studies are required to confirm the clinical relevance of these findings in colorectal cancer cell lines, a rationale has now been provided to include mdr-1 expression as a potential predictive marker in further clinical evaluations of SJG-136. Polymorphisms of the mdr-1 gene have also been reported to influence the disposition of P-gp substrates [7,20], and may thus affect the pharmacokinetics of SJG-136. This will be assessed in the ongoing clinical evaluation of SJG-136.

Conflict of interest statement

David E. Thurston is a shareholder in Spirogen Ltd. No other authors have potential personal conflicts of interest. The Edinburgh Cancer Research Centre is in receipt of clinical trial costs for a Phase I trial of SJG-136.

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