Cancer Chemother Pharmacol (1994) 35: 169–173

ORIGINAL ARTICLE

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Preclinical pharmacology of 1069C85, a novel tubulin binder

Received: 11 February 1994 / Accepted: 1 June 1994

Abstract The compound 1069C85, methyl *N*-[6-(3,4,5-trimethoxybenzyloxy)imidazo(1,2b)-pyridazin-2-yl] carbamate, is a novel synthetic tubulin binder currently undergoing phase I clinical trial. It was developed with a view to overcoming multidrug resistance and is given orally. Cytotoxicity studies in vitro against human ovarian carcinoma cell lines showed a lack of cross-resistance with cisplatin and no cross-resistance in two doxorubicin-resistant cell lines that exhibit high levels of resistance to both paclitaxel and vinblastine. Pharmacokinetic studies in BALB/c mice showed the oral bioavailability to be 20%, with 35% of the drug being excreted unchanged in the faeces over the first 24 h. Maximal plasma concentrations (C_{max}) were achieved within 2 h of oral administration as compared with 7.5 min following i.v. injection. At a dose of 20 mg/kg, the tumour drug concentration exceeded the plasma C_{max} by a factor of 1.5 and was within the in vitro cytotoxic concentration range. The drug showed a linear relationship between the dose and the area under the plasma concentration versus time curve (AUC) for doses of up to 20 mg/kg, above which no further increase in AUC was observed, possibly due to saturable absorption. 1069C85 is highly protein-bound (85%–99%) and appears to be subject to metabolism. The demonstration of cytotoxic activity against multidrug-resistant human tumour cell lines and the detection of potentially cytotoxic levels in an experimental tumour following oral administration support the choice of 1069C85 for clinical development.

Key words 1069C85 · Tubulin · Pharmacokinetics

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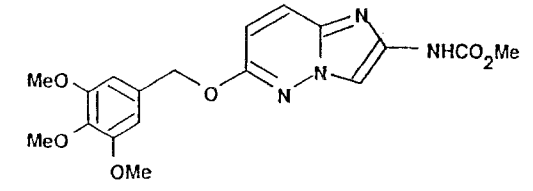
Introduction

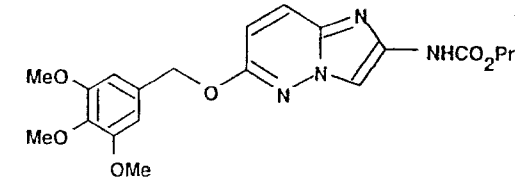
Mitotic spindle poisons include several natural antineoplastic agents used extensively in cancer chemotherapy. These compounds include tubulin-binding agents such as the vinca alkaloids, which are particularly active against haematological malignancies [5], and the microtubule depolymerization inhibitors paclitaxel and docletaxel, which also show promising activity against certain solid tumours [3, 4, 10, 12, 16, 20]. Recently, the taxanes have shown significant cytotoxicity against human ovarian carcinoma cell lines sensitive and resistant to cisplatin [1, 14].

1069C85 (Fig. 1) is a synthetic tubulin binder that binds tubulin near the colchicine site [11]. The compound shows cytotoxic activity in P388 cell lines similar to that of other spindle poison agents [11]. Previous preclinical studies have shown activity in vivo in P388 tumours made resistant

Fig. 1 A, B Chemical structures of **A** 1069C85 and **B** 27C87



A



B

to a variety of antitumour agents, including doxorubicin. This finding suggests that unlike the other spindle poisons, 1069C85 may be active in multidrug-resistant cell lines [11].

The aim of this study was to compare the cytotoxic properties of 1069C85 and other tubulin-interacting agents against a panel of human ovarian carcinoma cell lines and to evaluate its activity against cell lines exhibiting both intrinsic and acquired platinum and doxorubicin resistance. 1069C85 is undergoing phase I clinical trial under the auspices of the Cancer Research Campaign. To assist with the forthcoming trial, we performed pharmacokinetics studies in mice. The study focused on evaluating whether 1069C85 reaches cytotoxic levels in tumours following oral administration. The bioavailability of 1069C85 following oral administration was determined. The linearity of the pharmacokinetics with increasing doses, the level accumulating in EMT6 implanted tumours and the protein binding capacity, metabolism and excretion of 1069C85 were investigated.

Materials and methods

Cell lines

Four established human ovarian carcinoma cell lines were used in this study (SKOV3, 41M, A2780, CH1). Their biological properties and sensitivity to the clinically used platinum compounds have been described elsewhere [14, 15].

Cell culture

All lines were grown as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Imperial Laboratories, Andover, UK), 2 m*M* glutamine, 10 μg insulin/ml, 0.5 μg hydrocortisone/ml and 2.5 μg amphotericin B/ml in an atmosphere containing 10% CO_2/90% air. Cells were routinely checked for the presence of mycoplasma and were found to be negative throughout the course of these experiments. Lines were used within a defined range of 20 passages and the consistency of morphology and tumour-doubling time were checked during the period of the study.

Drugs

Doxorubicin was obtained from Farmitalia Carlo Erba (Herts, UK); paclitaxel, from Bristol Myers Squibb (Hounslow, UK); and 1069C85 and 27C87, from Wellcome Foundation Ltd. (Beckenham, UK).

Cytotoxicity assay

Cytotoxicity was assessed using the sulforhodamine B assay as described previously [13]. In brief, 5×10^3–10^4 cells were seeded into 96-well microplates in 200 μl growth medium. Serial dilutions of drugs were added to wells in quadruplicate and left to incubate for 96 h. Drug solutions of 10^{-6}, 2.5 10^{-7}, 10^{-7}, 2.5 10^{-8}, 10^{-9}, 2.5 10^{-10} and 10^{-10} *M* were prepared in growth medium as previously described [14]. 1069C85 was initially dissolved in dimethylsulphoxide (DMSO) at 3 mg/ml and then subsequent dilutions were made with growth medium.

Pharmacological experiment

Mice and tumour

Adult female BALB/c mice (20–30 g) were obtained from Olac (Southern Ltd., Bicester, UK). They were kept under 12-h light/12-h dark conditions at 20° ±1° C and were allowed laboratory chow and water ad libitum. They were fasted for 12 h before the experiment. For the tumour-distribution experiments, EMT6 tumour cells were grown i.m. in the gastronemius muscle of the hind leg as previously described [22]. The experiment started when the tumours measured 10–12 mm in two orthodiagonal diameters, and the tumours never exceeded 10% of the body weight.

Drug administration and sample preparation

1069C85 was given orally in a saline suspension containing 0.1% Tween 80; the i.v. vehicle was DMSO:saline (50:50, v/v). The injection volume was 10 ml/kg. At appropriate times the animals were asphyxiated under CO_2 and the blood was taken by cardiac puncture into heparinized syringes. Blood samples were centrifuged for 15 min at 3000 *g* and the plasma was recovered and frozen at –20° C until analysed. The tumours were excised rapidly, snap-frozen at –70° C in dry ice and kept frozen at –20° C until analysed.

Analytical method

Quantification of 1069C85 in plasma, brains and tumours was performed by high-performance liquid chromatography (HPLC) with fluorimetric detection. The method and its validation have been described elsewhere [17]. Briefly, 1069C85 elutes with methanol:water (75:25, v/v) from a reverse-phase column (25 cm × 4.5 mm × 5 μ*M* Hypersphere; Shandon, UK). The fluorimeter was set at 340 nm excitation and 406 nm emission. Data collection and quantitation was done with an MT2 data system (Kontron, UK) by an internally standardized method, the internal standard 27C87 (an analogue of 1069C85 also synthesised by Wellcome Foundation Ltd., Fig. 1) being added to the samples in constant concentrations (50 ng/tube). Standard curves were made in duplicate in plasma (0.625, 1.25, 2.5, 5, 10, 20, 40, 80, 160 ng/ml) and extracted in chloroform following sonication. Calibration curves were obtained by plotting the peak area ratio of 1069C85 to 27C87 as a function of the spiked 1069C85, and equations were derived from the unweighted linear least-squares fit of the curves defined by these calibration standards. Quantitation was performed using the peak area ratios on the previously defined equations. Under these conditions the lower limit of quantification was 0.25 ng/ml and the coefficient of variation for the assay was less than 5%. Tumours were homogenized in methanol (100%), the internal standard was added and quantification was performed against a standard curve in methanol.

For the protein-binding measurements, mouse plasma from previous experiments was pooled together (same levels of drugs and same time points) and filtered through Amicron protein-exclusion membranes at 25° C. The resulting filtrate was analysed by HPLC together with the initial plasma. For metabolism experiments, 100 μl urine was incubated with 2000 IU bovine liver glucuronidase (Sigma, UK) and 1000 IU *Helix ponatia* sulphatase (Sigma, UK) at 37° C and the mixture was then extracted into chloroform and analysed by HPLC with a linear gradient of methanol 0–75% water over a period of 15 min.

Bioavailability determination

The maximum tolerated i.v. dose, 1 mg/kg 1069C85, was given both i.v. and orally to animals (n = 4 per time point) and the plasma was collected at different time points over a 24-h period. The bioavailability was determined as the ratio of the areas under the plasma concentration-time curve (AUC) for oral versus i.v. administration.

Linearity of the AUC with increasing oral doses

The animals were given 1, 10, 20 and 40 mg/kg orally and the plasma was collected over a period of 72 h ($n = 3$ animals per time point).

Tumour distribution

1069C85 was given orally at 20 mg/kg to mice with EMT6 tumours implanted as previously described. Plasma and tumour samples were then collected over 72 h and the samples were assayed for 1069C85.

Data analysis

Concentrations lethal to 50% of the cells (IC_{50} values) were evaluated with INPLOT software using sigmoid curves. The difference in the cytotoxicity of the drugs was compared with the two-tailed t-test for unpaired samples (EXCEL software). Chromatographic data were recorded and quantified on an MT2 data-acquisition system. Pharmacokinetic parameters were evaluated with compartmental analysis on the PC NONLIN (version 4) program. The percentage of protein binding was calculated at different time points by dividing the levels of 1069C85 detected in mouse-plasma ultrafiltrates by the levels measured in total plasma.

Results

The cytotoxicity of 1069C85, doxorubicin, paclitaxel and vinblastine as determined in the four ovarian carcinoma cell lines and their platinum- and doxorubicin-resistant counterparts is shown in Table 1. 1069C85 was significantly more toxic than doxorubicin ($P < 0.05$) and significantly less toxic than vinblastine ($P < 0.05$) in the three parental cell lines, with cytotoxicity being observed in the nanomolar range. No significant difference in toxicity was seen with paclitaxel as compared with 1069C85 in any of the parent lines studied. Unlike doxorubicin, the compounds 1069C85, vinblastine and paclitaxel showed no cross-resistance with cisplatin. 1069C85 showed no cross-resistance with doxorubicin in any of the cell lines in which high levels of cross-resistance [resistance factor (RF), >100] were observed with both paclitaxel and vinblastine.

Figure 2 shows the pharmacokinetics of 1069C85 following i.v. administration of 1 mg/kg 1069C85 and oral administration of 1, 10 and 20 mg/kg. Following i.v. administration of 1 mg/kg 1069C85, the pharmacokinetic profile fitted a two-compartment model, the maximal plasma level being 2.75±0.8 μ*M*. After oral administration of the same dose, peak levels of 0.09 μ*M* occurred within the 1st h, and the compound was eliminated slowly with a half-life of about 8 h. The pharmacokinetic parameters are shown in Table 2. The overall bioavailability was 20%.

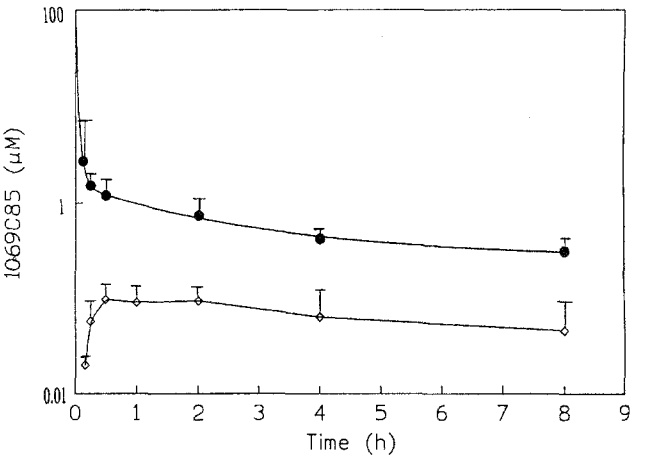


Fig. 2 1069C85 plasma concentrations measured following oral (◇) and i.v. (●) administration of 1 mg/kg to mice. Data are given as mean values ± SEM for 3 experiments

Figure 3 shows the plasma profiles obtained after increasing oral doses of 1069C85. Maximal plasma levels (C_{max}) were reached at 1–4 h post-treatment, increased with increasing concentrations and always occurred at early time points. The overall AUC value showed linearity with increasing doses of up to 20 mg/kg ($r = 0.97$) (Fig. 4), above which no further increase in the AUC was observed (Table 2). The drug was found to be highly protein-bound (85%–99%, data not shown).

The tumour levels detected following dosing of mice with 20 mg/kg 1069C85 are shown in Fig. 5. 1069C85 was detectable in all samples over a 24-h period. The plasma-to-tumour ratio was approximately 1.2:1.8. In all, 20%–35% of the drug was excreted unchanged in the faeces following administration of 20 mg/kg, 95% of which occurred during the first 24 h. No 1069C85 was detected in the urine, but two metabolites were observed following glucuronidase and sulphatase treatment (data not shown). Their identification will be performed by mass spectrometry.

Table 1 Cytotoxicity of various agents in four parental human ovarian carcinoma cell lines as determined following continuous drug exposure. Data are given as mean values ± SEM for triplicate determinations

	1069C85		Doxorubicin		Paclitaxel		Vinblastine	
	IC_{50} (n*M*)	RF[a]	IC_{50} (n*M*)	RF[a]	IC_{50} (n*M*)	RF[a]	IC_{50} (n*M*)	RF[a]
SKOV3	6.2±2		84 ± 7		3.2± 1.5		0.2± 0.1	
41M	5.5±1.7		45 ± 30		1.4± 1.5		0.5± 0.4	
41M AR	7.2±1	1.3	363 ±150	8	532 ± 14	380	83 ±45	166
A2780	4.9±2		36 ± 30		8.8± 7		0.4± 0.3	
A2780CR	4.7±3	1	40 ± 30	1	4.5± 2	1	0.6± 0.3	1.5
CH1	4.4±2		6 ± 2		3.6± 1.7		0.3± 0.2	
CH1CR	3.3±2	1	18.6± 10	3	2.9± 0.1	1	0.5± 0.1	1.6
CH1AR	9 ±3.5	2	502 ± 76	83	428 ±300	142	70.9±25	263

[a] Resistance factor, calculated as the IC_{50} in the resistant cell line divided by the IC_{50} in the sensitive cell line

Table 2 Pharmacokinetic parameters calculated by PC NONLIN following oral administration of 1, 10, 20 and 40 mg/kg 1069C85 and i.v. administration of 1 mg/kg to BALB/c mice

Medium	Dose (mg/kg)	AUC (μM h)	Kol (h^{-1})	t½ (h)	T_{max} (h)	C_{max} (nM)
Plasma	1 (i.v.)	5.51	–	7.3± 2.1	0.25±0.03	2762
Plasma	1	1.01	0.15±0.05	6.3± 1.7	0.3 ±0.03	92
Plasma	10	4.63	0.74±0.45	4.5± 1.3	2.07±0.8	368
Plasma	20	13.7	0.53±0.39	17.9± 5	2.6 ±0.8	481
Plasma	40	12.9	0.95±0.62	12.7± 3	5 ±3.0	515
Tumour	20	19.9	0.5 ±0.3	35 ±17	3.5 ±1.2	979

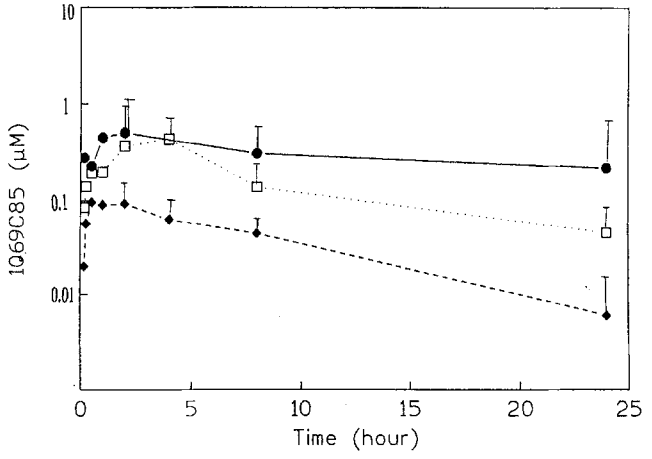


Fig. 3 1069C85 concentrations measured in mouse plasma following oral administration of 1 (◆), 10 (□) and 20 (●) mg/kg to female BALB/c mice. Data are given as mean values ± SEM for 3 experiments

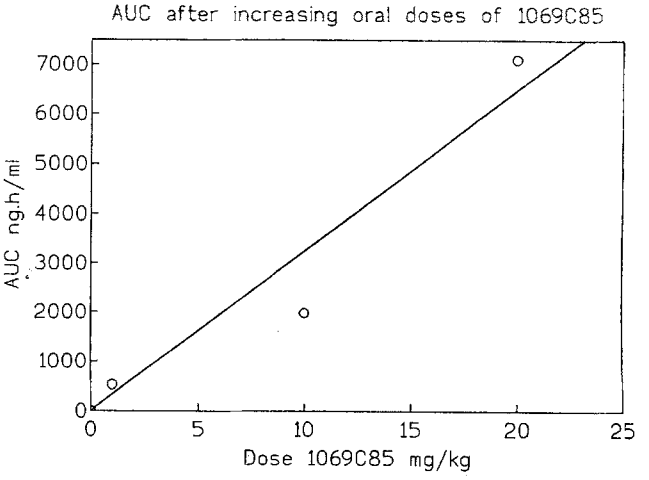


Fig. 4 Plot of AUC versus dose following oral administration of 1, 10 and 20 mg/kg 1069C85 to female BALB/c mice ($r = 0.87$)

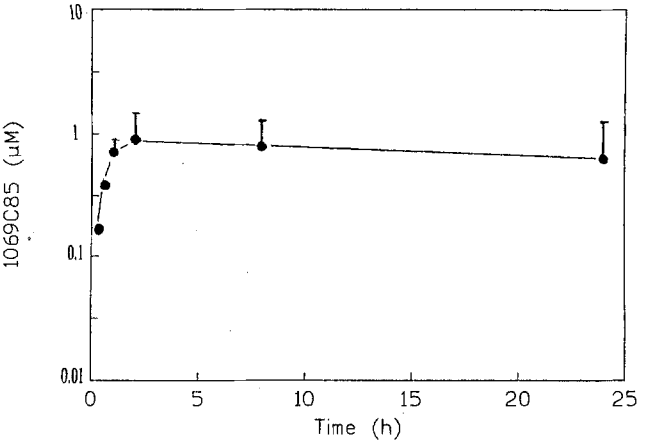


Fig. 5 Concentrations of 1069C85 detected in EMT6 tumors following oral administration of 20 mg/kg. Data are given as mean values ± SEM

Discussion

The present study demonstrates the in vitro potency of 1069C85 in four ovarian carcinoma cell lines, the observed IC_{50} being in the nanomolar range. The spectrum of activity of 1069C85 included cisplatin-resistant cell lines, where the resistance factor was less than 1.5. Paclitaxel and vinblastine also retained activity in our cisplatin-resistant lines. The ability to overcome cisplatin resistance is now a well-established feature of tubulin-interacting agents such as paclitaxel, doxetaxel and vinblastine. Previous studies have shown that the resistance to cisplatin in SKOV3 and A2780 CR cells is predominantly due to an increase in glutathione levels, whereas the resistance in CH1CR cells is due to enhanced DNA repair of platinum adducts [14, 15]. In contrast, mechanisms involving P-glycoprotein hyperexpression and tubulin-related processes have been associated with resistance to tubulin-interacting agents [12, 19]. It is therefore not surprising that 1069C85 is active in platinum-resistant cell lines. In our study, 1069C85 showed no cross-resistance with doxorubicin in either the CH1 or the 41M cell line.

These results confirm the activity of 1069C85 in multidrug-resistant cell lines. Multidrug resistance (MDR) is a well-described experimental phenomenon and has been shown, in some cases, to correlate with clinical resistance [7]. The mechanisms associated with MDR are mainly P-glycoprotein hyperexpression, although atypical MDR has been described with topoisomerase II modification and elevated glutathione S-transferase levels [2, 6, 8, 9, 18, 21]. Some mixed-MDR phenotypes have been observed. The doxorubicin-resistant cell lines described in this study have recently been shown to overexpress P-glycoprotein (data not shown). The encouraging observation that 1069C85 retains activity in these lines whilst other tubulin-interacting agents show cross-resistance with doxorubicin is difficult to interpret. More studies are needed to determine the mechanism by which 1069C85 retains activity in these cell lines. Notwithstanding these findings, our results are promising and suggest that 1069C85 could be a useful drug against ovarian carcinomas.

To evaluate the feasibility of oral administration of 1069C85 to patients in the clinical setting, we performed pharmacokinetic studies of 1069C85 in the mouse. Analysis

of the drug concentration obtained following oral administration of 1 mg/kg showed relatively rapid absorption within the 1st h and a relatively slow clearance. Only 20% of the drug was bioavailable, which is consistent with the observation that 25%–35% of the drug was found unchanged in the faeces over the first 24 h. This finding suggests that the drug is not well absorbed and may also explain the high drug doses (10 mg/kg) required to delay tumour growth in the animal models (unpublished results) as compared with the cytotoxic potency of the drug in vitro (IC_{50}, 1–10 n*M*).

When the dose was increased to 20 mg/kg, absorption was delayed, with the C_{max} occurring at 4 h as compared with 1 h for the 1-mg/kg dose. Relatively large variability was observed in the 1069C85 plasma concentrations we measured in different experiments and between different animals, which is likely to be the result of a differential oral absorption as the assay has a precision of less than 5% and a bias of less than 5% [17]. Nevertheless, the observation that there was a linear relationship between AUC and dose for oral doses of up to 20 mg/kg is encouraging as it should provide a framework for pharmacokinetically guided dose escalation in the clinical setting. Doubling the oral dose from 20 to 40 mg/kg did not significantly alter the plasma concentrations of 1069C85. This finding could be the consequence of either saturation of drug absorption and/or induction of its metabolism or excretion. The increase in half-life observed following increasing doses of 1069C85 suggests that absorption is probably the limiting factor.

Our data clearly show that 1069C85 reaches therapeutic levels in tumours after the administration of 20 mg/kg p.o. These concentrations are maintained over a period of 8–24 h. To date, two urinary metabolites have been observed (data not shown). However, there might be more metabolites that do not fluoresce or do not extract into chloroform. Further studies will focus on identifying these metabolites as well as investigating tissue metabolism. In conclusion, 1069C85 is active in multidrug-resistant ovarian carcinoma cell lines. The pharmacokinetic profiles obtained for the drug following oral administration to mice are compatible with therapeutic efficacy. 1069C85 has recently entered phase I clinical trials.

Acknowledgements This work was supported by the Cancer Research Campaign and the Wellcome Foundation Ltd.

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