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Reversal of P-gp mediated multidrug resistance in-vitro and in-vivo by FG020318

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Abstract

Overexpression of P-glycoprotein (P-gp) by tumours results in multidrug resistance (MDR) to structurally and functionally unrelated chemotherapeutic drugs. Combined therapy with MDR-related cytotoxins and MDR modulators is a promising strategy to overcome clinical MDR. This study was performed to explore the MDR reversal activity of a novel compound 2-[4-(2-pyridin-2-yl-vinyl) phenyl]-4,5-bis-(4-N,N-diethylaminophenyl)-1(H)-imidazole (FG020318) in-vitro and in-vivo. Tetrazolium (MTT) assay was used to evaluate the ability of FG020318 to reverse drug resistance in two P-gp-expressing tumour cell lines, KBv200 and MCF-7/adr. Intracellular doxorubicin accumulation was determined by fluorescence spectrophotometry in MCF-7/adr cell line. The effect of FG020318 on P-gp function was demonstrated by rhodamine 123 (Rh123) accumulation in KBv200 cells. KBv200 cell xenograft models were established to study the in-vivo effect of FG020318 on reversing MDR. FG020318 was not cytotoxic by itself against P-gp expressing KBv200 cells and MCF-7/adr cells and their parental drug-sensitive KB cells and MCF-7 cells. FG020318 could significantly increase the sensitivity of MDR cells to antitumour drugs including doxorubicin and vincristine in MCF-7/adr cells and KBv200 cells, respectively. It was much stronger than the positive control verapamil in reversal of MDR. FG020318 also increased the intracellular accumulation of doxorubicin in a concentration-dependent manner in MCF-7/adr cells, but did not affect the accumulation of doxorubicin in drug-sensitive MCF-7 cells. The Rh123 accumulation in resistant KBv200 cells was also increased by the addition of FG020318, but Rh123 accumulation was not affected by FG020318 in drug-sensitive KB cells. FG020318 potentiated the antitumour activity of vincristine to KBv200 xenografts and was an efficacious modulator in-vivo. Our results suggested that FG020318 was a highly potent, efficacious MDR modulator not only in-vitro but also in-vivo. The reversal of drug resistance by FG020318 was probably related to the increased anticancer drug accumulation and its inhibition of P-gp function of MDR tumour cells.

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Introduction

Multidrug resistance (MDR) in tumour cells is a significant obstacle to the success of chemotherapy in many cancers. MDR is characterized by a decreased sensitivity of tumour cells not only to the drug employed for chemotherapy but also to a broad spectrum of natural anticancer drugs with neither obvious structural homology nor common targets. Classic MDR is associated with the overexpression of a 170 kDa P-glycoprotein (P-gp) (Ling 1997). The P-gp is a plasma membrane protein encoded by mdr1 genes and acts as an ATP-dependent multidrug efflux pump that can prevent the accumulation of drugs by expelling them from the cell membrane before they are able to interact with their cellular targets. Other mechanisms contributing to MDR have been described, including overexpression of MDR-associated protein (MRP) (Deng et al 2002), lung resistant protein (LRP) (Laurencot et al 1997) and mutation of DNA topoisomerase II (Hochhauser & Harris 1993), protein kinase C (PKC) (Fine et al 1996) and Bcl-2, p53 (Bahr et al 2001).

To date, the strategies aimed at reversing MDR have principally focused on the inhibition or modulation of P-gp activity. Many agents that modulate the P-gp transporter, including verapamil, ciclosporin, quinidine and several calmodulin

antagonists, were identified in the 1980s (Krishna & Mayer 2000) and were often called first-generation MDR inhibitors. These agents required high concentrations to reverse MDR and their clinical usefulness was limited by unacceptable toxicity, such as cardiovascular toxicity with verapamil and immunosuppression with ciclosporin (Thomas & Coley 2003). To overcome these limitations, second-generation MDR inhibitors with less toxicity and greater potency were tested and developed, including PSC833 and VX-710. However, these modulators were confounded by unpredictable pharmacokinetic interactions with co-administered cytotoxic agents that necessitated chemotherapy dose reductions in clinical trials (Rowinsky et al 1998; Krishna & Mayer 2000). Recent investigations suggested that the third-generation MDR inhibitors possessed more advantages such as lack of nonspecific cytotoxicity, P-gp specificity, relatively long duration of action with reversibility, and lack of pharmacokinetic interaction with antitumour drugs. They offered new hope for clinical reversal of multidrug resistance. These modulators included S9788 (Tranchand et al 1998), OC144-093 (Newman et al 2000), LY335979 (Shepard et al 2003), XR9576 (Mistry et al 2001), and ONT-093 (Mistry & Folkes 2002).

Here, we report a new inhibitor of P-gp with third-generation characteristics, 2-[4-(2-pyridin-2-yl-vinyl) phenyl]-4,5-bis-(4-N,N-diethylaminophenyl)-1(H)-imidazole (FG020318), which is a novel compound designed to reverse multidrug resistance associated with cancer chemotherapy. We have explored the ability of FG020318 to reverse MDR in-vitro and in-vivo.

Materials and Methods

Drugs and materials

Doxorubicin was purchased from HISUN Pharmaceutical Co. (Guangdong province, China). Vincristine was purchased from ShenZhen Main Luck Pharmaceuticals Inc. (Guangdong province, China). MTT (tetrazolium) was purchased from Sigma Chemical Co. Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 were purchased from Gibco BRL. The powder of FG020318, with a purity of $>98\%$, was synthesized and obtained by chromatography (Figure 1).

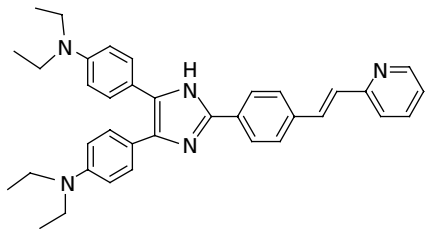

Figure 1 Chemical structure of FG020318.

Cell lines and cell culture

Drug sensitive MCF-7 human breast carcinoma and the KB human epidermoid carcinoma cell lines and their parental MDR MCF-7/adr and KBv200 sublines, displaying characteristics of MDR attributable to overexpression of P-gp (Fairchild et al 1987; Zhang et al 1994), were used. MCF-7/adr cells were established by stepwise exposure to increasing doses of doxorubicin. The MCF-7/adr and the MCF-7 cell lines were generously provided by Professor Liu Xy (from the National Cancer Institute, USA) and KB and KBv200 cell lines were obtained from the Chinese Academy of Medical Sciences (Beijing, China). KBv200 cells were cloned from parental drug-sensitive KB cells by stepwise exposure to increasing doses of vincristine and ethylmethane sulfonate (EMS) mutagenesis (Zhang et al 1994). All the cells were grown as adherent monolayers in flasks and were cultured in medium with 10% foetal bovine serum, benzylpenicillin ($50\,kU\,L^{-1}$), and streptomycin ($50\,mg\,L^{-1}$) at 37°C in a humidified atmosphere of 5% $CO_2 + 95\%$ air. The culture medium for MCF-7/adr cells and MCF-7 cells was DMEM and for KBv200 cells and KB cells it was RPMI 1640. MCF-7/adr cells were approximately 99-fold resistant to ADM and KBv200 cells were approximately 50-fold resistant to vincristine compared with KB cells according to our results.

MTT cytotoxicity assay

Cells were harvested and seeded in 96-well plates at 3.0×10^3 cells/well for MCF-7/adr cells and MCF-7 cells, and 1.0×10^3 cells/well for KB and KBv200 in a final volume of $180\,\mu L$. After a 24-h incubation, $10\,\mu L$ FG020318 and $10\,\mu L$ cytotoxic agents, or compound vehicles were added to duplicate plates. After 68 h, $10\,\mu L$ MTT solution was added to each well. DMSO ($100\,\mu L$) was added to each well 4 h later. The concentrations required to inhibit growth by 50% (IC50) were calculated from the cytotoxicity curves (Bliss's software). The degree of resistance was calculated by dividing the IC50 for the MDR cells by that for the parental sensitive cells. The fold-reversal was the ratio of the IC50 in the absence and presence of modulators.

Doxorubicin accumulation

This assay was evaluated basically as described by Fu et al (2002). MCF-7 cells and MCF-7/adr cells were harvested and resuspended at a concentration of 10^6 cells mL^{-1}. The cells were treated with FG020318 (1.25, 2.5, 5.0 or $10.0\,\mu mol\,L^{-1}$) or vehicle at 37°C for 2 h in the DMEM medium. Doxorubicin ($10\,\mu mol\,L^{-1}$) was then added to the medium and the incubation continued for another 3 h. The cells were then collected, centrifuged ($900\,g$ for 5 min) and washed three times with cold phosphate-buffered saline (PBS). The cells were resuspended in HCl $0.3\,mol\,L^{-1}$ in 60% ethanol. Following centrifugation, the supernatant was removed and assayed spectrofluorometrically at λ_{ex} 470 nm and λ_{em} 590 nm. FG020318 did not affect the absorbance or emission spectra of doxorubicin. The

accumulation of doxorubicin was calculated by the standard curve of doxorubicin. The fold accumulation of doxorubicin was calculated by dividing the value in the presence of FG020318 by that without FG020318.

Rhodamine (Rh123) efflux studies

KBv200 cells or KB cells were exposed to FG020318 at 2.5, 5 or 10 μmol L^{-1} for 2 h. The cells were collected and washed once and then resuspended in 1 mL RPMI 1640 at the concentration of 1×10^5 cells mL^{-1}. The cells were loaded with 5 ng mL^{-1} Rh123 for 30 min at 37°C. After one wash with RPMI 1640, cells were allowed to efflux the dye for 10 min in dye-free RPMI 1640 at 37°C. The cells were then washed out and suspended in 1 mL RPMI 1640, after which fluorescence analysis was carried out on a FACScan flow cytometer.

Animals

Female athymic nude mice (5–6-weeks old, 18–22 g) were used for the KBv200 xenografts. Mice were obtained from the Center of Experimental Animals, Sun Yat-sen University of Medical Sciences. The animals were maintained and reared there, being provided with sterilized food and water.

MDR human carcinoma xenografts

The KBv200 cell xenograft model was established as described by Liang et al (2000). Briefly, the KBv200 cells were harvested and implanted subcutaneously (s.c.) under the shoulder in nude mice. When the tumours had reached a mean diameter of 0.5 cm, the animals were randomized into groups of four and treated with various regimens. These included a saline control and groups receiving vincristine alone (0.2 mg kg^{-1}), intraperitoneally (i.p.), every two days (q2d × 6), FG020318 alone (200 mg kg^{-1}), p.o., q2d × 6, or vincristine (0.2 mg kg^{-1}, i.p., q2d × 6) plus FG020318 (200 mg kg^{-1}, p.o., q2d × 6).

The body weights of the animals were measured every two days for modulation of the drug dosage. The two perpendicular diameters (A and B) were recorded every two days and tumour volume (V) was estimated according to the formula:

$$V = \pi/6\ ((A + B)/2)^3$$

The curve of tumour growth was drawn according to tumour volume and time of implantation. The mice were anaesthetized and killed when the mean tumour weight was over 1 g in the control group. Tumour tissue was excised from the mice and its weight was measured. The rate of inhibition (IR) was calculated according to the formula:

$$IR = 1 - (\text{Mean tumour weight of experimental group} / \text{Mean tumour weight of control group}) \times 100\%$$

Statistical analysis

All data were repeated at least three times in independent experiments and differences were determined using the Student's *t*-test or Kruskal–Wallis test. Significance was determined at $P < 0.05$.

Results

Reversal of MDR in-vitro by FG020318

MCF-7/adr cells were approximately 99-fold resistant to doxorubicin compared with MCF-7 cells. KBv200 cells were approximately 50-fold resistant to vincristine in comparison with KB cells. The IC50 of FG020318 to KBv200, KB, MCF-7/adr and MCF-7 were all more than 50 μmol L^{-1}, respectively. FG020318 did not inhibit the growth of tumour cells under the concentrations used to reverse drug sensitivity. FG020318 enhanced the cytotoxicity of doxorubicin and vincristine in MDR resistant MCF-7/adr and KBv200 cells, but had little effect on the enhancement of drug cytotoxicity in drug sensitive MCF-7 and KB cells (Table 1).

Table 1 Modulation by FG020318 of the sensitivity to cytotoxic drugs of MDR and drug-sensitive tumour cells

Modulator	Concn (μmol L^{-1})	IC50 of vincristine ± s.d. (μmol L^{-1})		IC50 of doxorubicin ± s.d. (μmol L^{-1})		Fold-reversal of MDR			
		KBv200	KB	MCF-7/adr	MCF-7	KBv200	KB	MCF-7/adr	MCF-7
Control	0	1.302 ± 0.125	0.026 ± 0.006	21.784 ± 1.817	0.220 ± 0.006				
Verapamil	5	0.089 ± 0.008**	0.023 ± 0.004	3.603 ± 0.414**	0.223 ± 0.055	14.63	1.13	6.05	1.01
	10	0.054 ± 0.006**	0.020 ± 0.003	2.207 ± 0.173**	0.225 ± 0.050	24.11	1.23	9.87	1.02
FG020318	0.625	0.395 ± 0.133**	0.021 ± 0.005	7.838 ± 0.582**	0.204 ± 0.015	3.30	1.24	2.78	1.08
	1.25	0.061 ± 0.015**	0.025 ± 0.004	5.614 ± 1.415**	0.233 ± 0.031	21.34	1.04	3.88	0.94
	2.5	0.041 ± 0.018**	0.028 ± 0.007	2.338 ± 0.246**	0.223 ± 0.046	31.76	0.93	9.32	0.99
	5	0.028 ± 0.007**	0.025 ± 0.003	1.997 ± 0.237**	0.237 ± 0.039	46.50	1.04	10.91	0.93
	10	0.026 ± 0.010**	0.024 ± 0.004	1.810 ± 0.296**	0.220 ± 0.019	50.08	1.08	12.04	1.00

IC50 is defined as the concentration required to inhibit tumour cell growth by 50%. The IC50 of doxorubicin and vincristine was determined in the presence of various concentrations of FG020318. Each value represents the mean ± s.d. of at least three independent experiments. The fold-reversal of MDR is defined as the ratio of the IC50 for doxorubicin or vincristine alone vs the IC50 for doxorubicin or vincristine in the presence of FG020318. **$P < 0.01$ compared with the control group.

Doxorubicin accumulation

The intracellular accumulation of doxorubicin in MCF-7/adr cells was only approximately one-quarter of that in MCF-7 cells. After the MCF-7/adr cells and MCF-7 cells were treated with 0.625, 1.25, 2.5 or 5 μmol L^{-1} FG020318, the intracellular accumulation of doxorubicin was significantly increased in MCF-7/adr cells. However, the doxorubicin accumulation in MCF-7 cells was not affected by FG020318. The enhancement of doxorubicin accumulation in MCF-7/adr cells was approximately 1.4-, 2.4- and 2.5-fold for 1.25, 2.5 and 5 μmol L^{-1} FG020318, respectively (Figure 2).

Rh123 accumulation assay

Treatment with 2.5, 5 or 10 μmol L^{-1} FG020318 for up to 2 h was able to increase Rh123 accumulation by different degrees. However, the Rh123 accumulation of the drug sensitive KB cells was not affected by the addition of FG020318 (Table 2).

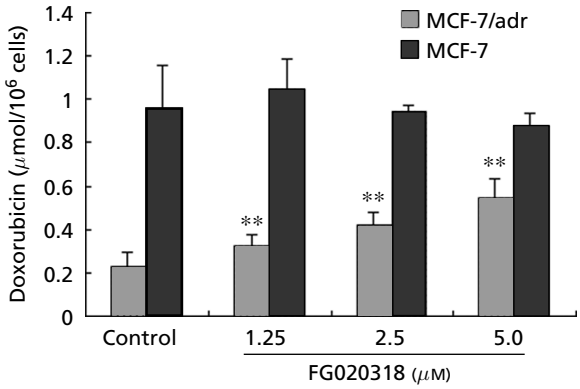

Figure 2 Effect of FG020318 on doxorubicin accumulation in MCF-7 cells and MCF-7/adr cells. Bars represent mean ± s.d. of triplicate determinations. $^{**}P < 0.01$ compared with the control group in the MCF-7/adr cells.

Table 2 Enhancement of intracellular rhodamine123 accumulation by FG020318 in KBv200 cells

FG020318 (μmol L^{-1})	Fluorescent intensity	
	KBv200	KB
0	4.55 ± 0.29	60.27 ± 7.06
2.5	9.05 ± 1.10**	63.52 ± 2.81
5	17.88 ± 4.92**	56.77 ± 5.15
10	23.21 ± 2.16**	62.37 ± 5.20

Data represents the mean ± s.d. for at least three independent experiments. $^{**}P < 0.01$ compared with the control group.

Reversal of MDR by FG020318 in KBv200 cell xenografts

Tumour weights were 1.57 ± 0.42, 1.64 ± 0.41, 1.37 ± 0.41, and 0.93 ± 0.29 g in the control group, vincristine alone, FG020318 alone and co-administration groups, respectively, at the end of the experiment. The inhibitory ratios of tumour growth were −4.9%, 12.5% and 41.0% for vincristine alone, FG020318 alone and co-administration groups, respectively (Table 3, Figure 3).

Discussion

A lot of compounds that interact with P-gp and block drug efflux have been reported to reverse MDR in-vitro (Thomas & Coley 2003). Combined therapy with MDR-related cytotoxins and some P-gp inhibitors even shrink tumours and prolongs the life span in some animal models (Sikic et al 1997). Recently, it was reported that the third generation of P-gp inhibitors had been developed, which showed no significant enhancement of pharmacokinetics and the toxicity of cytotoxins (Krishna & Mayer 2000; Thomas & Coley 2003). The continued development of these agents may hold great promise for the success of clinical reversal of P-gp-mediated MDR.

Table 3 Effect of FG020318 on the reversal of multidrug resistance in a xenograft model of KBv200 cells in nude mice

Group	Pre-experiment		Post-experiment		Tumour weight (g)	Inhibited rate (%)
	n	Weight (g)	n	Weight (g)		
Saline control	9	22.8 ± 1.2	9	25.4 ± 1.6	1.57 ± 0.42	
FG020318	7	22.7 ± 1.2	7	24.9 ± 0.8	1.37 ± 0.41	12.5
Vincristine	7	23.2 ± 1.1	7	26.3 ± 1.6	1.64 ± 0.41	−4.9
FG020318 + vincristine	8	23.3 ± 1.1	8	25.0 ± 1.9	0.93 ± 0.29[a,b]	41.0

Data represents the mean for each group ± s.d. [a]$P < 0.05$ compared with the control group. [b]$P < 0.05$ compared with those in the vincristine group.

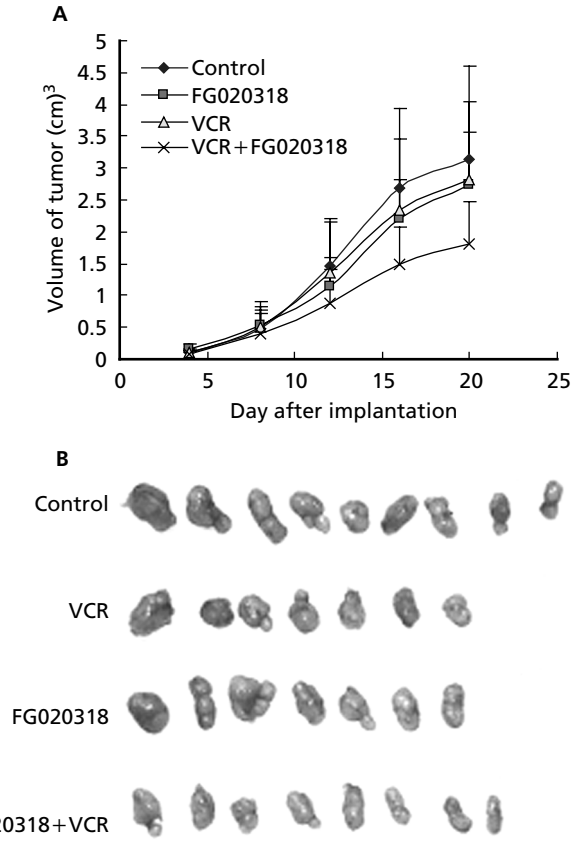

Figure 3 Effect of FG020318 on the reversal of MDR in KBv200 xenograft in nude mice. The experiment was carried out using athymic mice implanted subcutaneously with 10^6 KBv200 cells. The treatments were administered as described in Materials and Methods. A. Data represent the mean tumour volume size for each group and standard deviation in 7–9 experimental animals. B. The pictures were taken 20 days after implantation. VCR, vincristine.

In this study, the novel compound FG020318 was explored for its ability to reverse MDR in-vitro and in-vivo. The in-vitro results showed that FG020318 reversed the MDR phenotype with high potential. In drug-resistant KBv200 cells, co-incubation of vincristine with 5 μmol L^{-1} FG020318 resulted in a 46.5-fold increase in the cytotoxicity of vincristine. This was 3-fold as strong as the positive control verapamil, which only led to a 14.6-fold increase in restoring the drug sensitivity at the same concentration. The drug cytotoxicity was not affected by FG020318 in drug-sensitive MCF-7 and KB cell lines. These results demonstrated that FG020318 could potently reverse MDR in-vitro.

The promising activity of FG020318 demonstrated in-vitro was also confirmed in mice bearing MDR human KBv200 cell xenografts. The results showed that neither FG020318 alone nor vincristine alone had any antitumour action ($P > 0.05$) compared with the saline control group in xenograft models of KBv200 cells. The KBv200 cell xenografts in nude mice were established by Liang et al (2000) and Fu et al (2004) and were proved to be extremely resistant to vincristine and retain the characteristics of the MDR phenotype. However, the combination of FG020318 and the anticancer drug vincristine exhibited a significantly inhibitory effect on the growth of the xenografts and the inhibition (vs the control weight of tumour) was 41.0% ($P < 0.05$) compared with the control group. During the experiment, no animal death and decrease in body weight were observed. Therefore, there was no evidence of an increase in vincristine-associated toxicity induced by FG020318.

The studies of P-gp-positive MDR cells demonstrated clearly that the emergence of MDR in these cells was linked to a marked decrease in the intracellular accumulation of the various cytotoxic drugs. Most MDR modulators, such as verapamil or OC144-093, could increase drug accumulation in MDR tumour cells. In this study, FG020318 was tested for its effect on intracellular drug accumulation. FG020318 caused a concentration-dependent increase in doxorubicin accumulation in MDR MCF-7/adr cells, but had no effect on the doxorubicin accumulation in drug-sensitive MCF-7 cells. It could be concluded that FG020318 could restore the drug accumulation in MDR cells and increase the sensitivity of MDR cells to cytotoxic drugs.

P-gp functions as a drug transporter and mediates drug efflux. Many modulators, such as verapamil and ciclosporin, inhibit the function of P-gp and thereby reverse MDR (Tan et al 2000). Rhodamine 123 (Rh123) accumulation is a classical assay used to test function of P-gp. Rh123 is a fluorescent compound and is a good substrate for P-gp. Intracellular Rh 123 accumulation could be a good indicator of P-gp function. The MDR KBv200 cells accumulated less Rh123 than equivalent sensitive KB cells. In P-gp expressing KBv200 cells, Rh123 accumulation was increased by FG020318 in a concentration-dependent manner, but accumulation in sensitive KB cells was not affected. These results suggested that FG020318 inhibited function of P-gp in MDR KBv200 cells, which was at least partly responsible for the reversal activity of FG020318.

In conclusion, FG020318 is a highly potent, efficacious modulator not only in-vitro but also in-vivo. It may have no effect on the pharmacokinetics of anticancer drugs and may become a member of the third generation of P-gp inhibitors. FG020318 may be an attractive new agent for reversing MDR.

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