

Chemioresistance of an Adriamycin-Selective Human Small-Cell Lung Carcinoma Cell Line

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ABSTRACT

The resistance to cytotoxic anticancer drugs of two human small-cell lung cancer cell lines was determined for the parental GLC₄ cell line and the adriamycin-selective GLC₄/Adr cell line. In comparison with their parental counterparts, the GLC₄/Adr cells displayed higher resistance to all test compounds, including doxorubicin, vincristine and vinblastine, with respective IC₅₀ values of 3.553 ± 0.247, 9.630 ± 0.134 and 0.858 ± 0.072 nM leading to estimates of resistance factors of 310 ± 29, 32 ± 5 and 2.7 ± 0.3, respectively. These findings indicated that drug-resistant cells generated by *in vitro* selection in the presence of step-wise concentration of a single cytotoxic drug could display some of the characteristics associated with the overexpression of the membrane transporter protein responsible for cellular multispecific detoxification.

Key words: multidrug resistance, doxorubicin, vincristine, vinblastine, adriamycin-selective cells

INTRODUCTION

Cellular resistance of cancer cells to the lethal effect of a broad spectrum of chemotherapeutic drugs, known as the multidrug resistance (MDR) mechanism, is a major obstacle to the success of chemotherapy for patients suffering from malignant tumors, due to the capability of cancer cells to detoxify drugs. Among various resistant mechanisms of cancer cells, the multidrug-resistant phenomenon is one of the most studied. This mechanism is characterized by a decrease in cellular drug accumulation resulting from over-expression and associated with acquired multidrug resistance in cancer cells of the 170 kDa plasma membrane transporter protein, P-glycoprotein (Pgp) encoded by *MDR1*, that is also

referred to as *ABCC1* (Juliono and Ling, 1976; Ambudkar *et al.*, 1999) and the 190 kDa plasma membrane transporter protein, multidrug resistance-associated protein (MRP1/*ABCC1*) encoded by the *ABCC1* gene (Cole *et al.*, 1992). Both transporters are members of the ATP-binding cassette (ABC) transporter superfamily of membrane transporter proteins (Higgins, 1992; Huang and Sadée, 2006). The available evidence strongly suggests that Pgp1 and MRP1 are the efflux-pumps that catalyze the clearance of drugs from the cells, leading to the reduction of drug accumulation and the access of cytotoxic drugs to their targets.

When the primary protein structure is considered, the similarity of Pgp and MRP1 is lower than 15%. However, when overexpressed,

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they convert the energy produced by ATP hydrolysis to transport a number of amphiphilic drug molecules of very different chemical structures and cellular targets out of the cell against the drug concentration gradient and the cellular drug concentration becomes lower than the lethal threshold (Gottesman and Pastan, 1993; Loe *et al.*, 1996). The cells confer simultaneous resistance to a variety of chemically unrelated cytotoxic drugs widely used in cancer chemotherapy (Cole *et al.*, 1992; Higgins, 1992; Gottesman and Ambudkar, 2001). The common transport substrates of both transporters comprise natural products based anticancer drugs, such as anthracyclines (doxorubicine and daunorubicine), vinca alkaloids (vincristine), epipodophyllotoxins (etoposide) and taxanes (paclitaxel), as well as the synthetic molecules (Gottesman and Pastan, 1993; Ambudkar *et al.*, 1999).

Regarding the multidrug resistance mechanism, single cytotoxic drug selective cells may confer cross-resistance to a wide range of drugs, without sharing common structures and targets. The present study aimed to use the simplified tetrazolium cytotoxic (MTT) assay to evaluate the chemioresistance of an anthacycline antitumor antibiotic, doxorubicin, on the adriamycin-selective human small-cell lung cancer GLC₄/Adr cell line and its parental GLC₄ cell line, as well as the plausible cross resistance of the GLC₄/Adr cell line to two *Vinca* alkaloid chemotherapeutic agents, vincristine and vinblastine.

MATERIALS AND METHODS

Chemical reagents

Doxorubicin, vinblastine, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO, USA). Vincristine was purchased from Calbiochem (Damstradt, Germany). RPMI 1640

with glutamax culture medium, heat inactivated fetal bovine serum, sodium pyruvate, antibiotics (streptomycin and penicillin) and phosphate buffer saline (PBS) were purchased from Gibco (Grand Island, NY, USA).

Cell lines and cell culture conditions

Two human small-cell lung carcinoma cell lines were used as biological material, consisting of the parental cell line (GLC₄) and the adriamycin-selective cell line (GLC₄/Adr) derived from the parental cell line cultured in the presence of step-wise concentration of adriamycin (doxorubicin) up to 1.2 μ M as described in Zijlstra *et al.* (1987) and Meijer *et al.* (1991). These cell lines were kindly provided by Dr M. GARRIGOS (Laboratoire des protéine membranaires, iBiTecS, SB²SM, CEA Saclay, France).

Cells were maintained in an exponential growth in RPMI 1640 culture medium with glutamax, supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin G (100 units/mL), streptomycin (100 μ g/mL) and 1% sodium pyruvate at 37°C in a humidified atmosphere containing 5% CO₂. The adriamycin-selective GLC₄/Adr cell line was maintained in the presence of 1 μ M doxorubicin and was cultured in a doxorubicin-free culture medium for 11 d before use in all experiments.

Cell survival assay

The cellular chemioresistance of cancer cells was investigated using a simplified cytotoxic assay based upon the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to blue formazan product by mitochondrial dehydrogenase of viable cells as illustrated in Figure 1. In this assay, the quantity of resulting blue formazan product corresponds to the viability of cells after exposure to anticancer agents and was determined spectrophotometrically, as described in Carmichael *et al.* (1987), with slight modification.

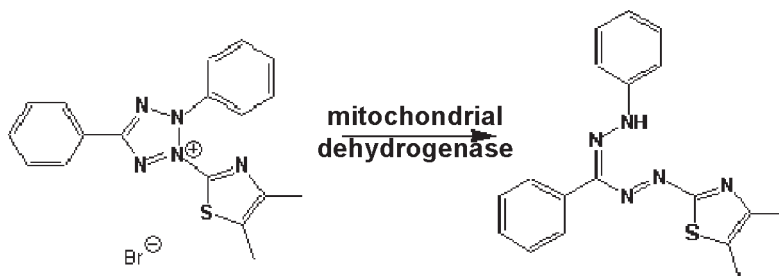


Figure 1 Reduction of tetrazolium bromide to formazan product by mitochondrial dehydrogenase.

For each cell line, cells in exponential growth were counted and were seeded into new culture media with a density of 4,000 and 25,000 cells/mL for GLC₄ and GLC₄/Adr, respectively. After 24 h of incubation at 37°C in a humidified atmosphere with 5% CO₂, a stock solution of anti-cancer drug dissolved in sterile DMSO was diluted in the culture media and added to obtain the indicated concentrations and incubated for a further 72 h. At the end of drug exposure, sterile 5 mg/mL MTT solution was added and the mixture was incubated for a further 4 h. Samples were subsequently prepared for determination of formazan product. Culture medium was carefully removed and DMSO was added to dissolve the resulting formazan product and was subjected to shaking and agitation at a speed of 100 rpm at room temperature for 1 h. The optical density was determined by spectrophotometer at 550 nm (NOVA SPEC, GENESIS II).

Statistical analysis and mathematical estimation

Descriptive statistics, (mean and standard error), were determined to demonstrate the viability of cells after exposure to cytotoxic agents. The IC₅₀ was defined as the concentration of cytotoxic anti-cancer agent that reduced the viability of the cell to 50% of control values and was estimated from a dose-response curve of cell survival. The resistance factor (RF) of each cytotoxic drug was calculated by dividing the IC₅₀ value of the GLC₄/Adr cells by that of the GLC₄ cells.

RESULTS

The cell viability of both cultured human small-cell lung carcinoma cell lines, GLC₄ and GLC₄/Adr, after exposure to varying concentrations of different anticancer drugs (doxorubicin, vinblastine and vincristine) was evaluated by MTT assay, in which the viable rates of cells was deduced from the absorbance values of the resulting formazan product of the sample relative to the control, as illustrated in Figure 2. All test anticancer drugs effectively triggered the inhibition of cell proliferation in a dose-dependent manner after 72 h of exposure for both GLC₄ and GLC₄/Adr and the adriamycin-selective cells were obviously less sensitive than the parental cells for all test compounds. Interestingly, all dose-response curves obtained in this study displayed, more or less, a biphasic dose-response. This implies toxin-mediated hormesis that, in turn, indicates an adaptive beneficial or stimulation effect of cell proliferation after exposure to a low concentration of each anticancer drug with a different range of non-cytotoxic concentrations, e.g., between 0-0.3 µM of doxorubicin for GLC₄/Adr cells. Thereafter, when cells were exposed to higher doses of drugs, the dose inhibitory effects were observed, e.g., more than 0.3-100 µM of doxorubicin for GLC₄/Adr cells.

In order to compare sensitivity to different cytotoxic agents, the IC₅₀ of each compound for each cell line (defined as the concentration of each compound that inhibits the proliferation of each cell line to 50% of control

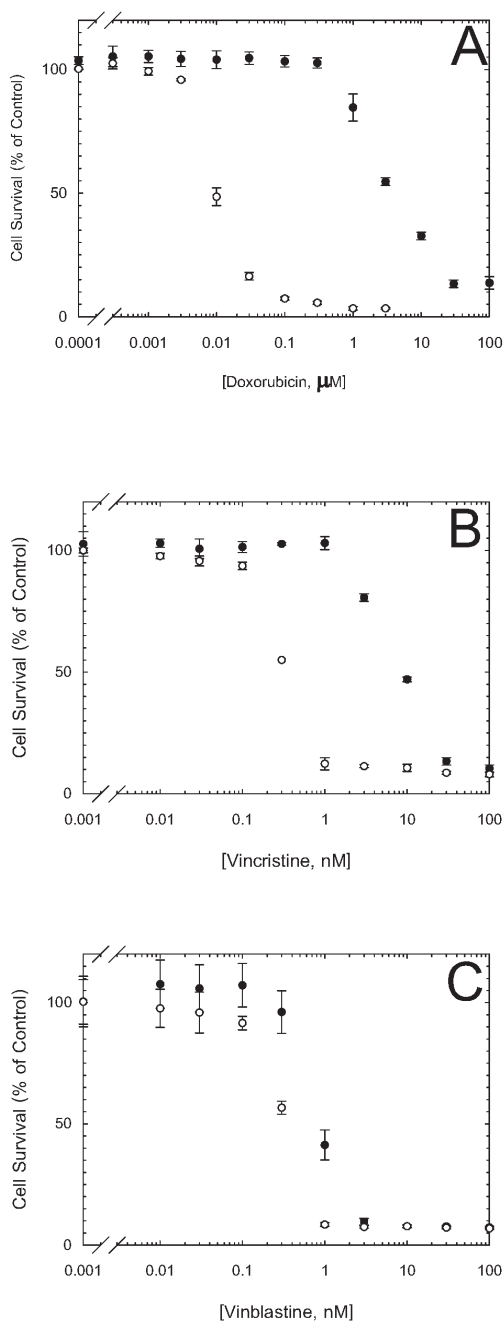


Figure 2 Survival of GLC₄ (○) and GLC₄/Adr (●) cells after 72 h of exposure to: (A) doxorubicin; (B) vincristine; and (C) vinblastine. Data points and bars represent the average and standard error of at least three independent experiments, each was conducted in sextuplet.

without drug exposure) was estimated from the dose-response curve. The GLC₄/Adr cells resistance to doxorubicin was the highest with an IC₅₀ value of 3.553 ± 0.247 μM (Table 1), followed by vincristine and vinblastine with IC₅₀ values of 9.630 ± 0.134 and 0.858 ± 0.072 nM, respectively. The GLC₄ cells were more resistant to doxorubicin than vincristine and vinblastine. The IC₅₀ for doxorubicin was estimated at 12 ± 2 nM, while for vincristine and vinblastine the IC₅₀ was approximately 25 times lower than for doxorubicin, with a value of 0.302 ± 0.022 and 0.316 ± 0.005 nM, respectively.

The IC₅₀ for all test agents was used to calculate the resistance factor (RF) of GLC₄/Adr to each cytotoxic agent by dividing the IC₅₀ value of the GLC₄/Adr cells by that of the GLC₄ cells (Table 1). The resistance of the adriamycin-selective GLC₄/Adr cells to doxorubicin was 310 ± 29 higher than the parental cells, whereas for the two *Vinca* alkaloids, vincristine and vinblastine, the value was 32 ± 5 and 2.7 ± 0.3 , respectively.

The results observed in this study underline the fact that the GLC₄/Adr cell line (selected by culture for the presence of the cellular stress-oxidative in the culture media produced by a step-wise concentration of adriamycin or doxorubicin) could induce the cellular circumstances that lead the exposed cells to acquire a higher resistance to the toxic agent than the parental cell line. Moreover, the examination of plausible cross-resistance of the GLC₄/Adr cells to other drugs, using two *Vinca* alkaloids as a model, suggested that the association with the overexpression of the membrane transporter responsible for cellular detoxification conferred the multidrug-resistant mechanism.

DISCUSSION

Cellular detoxification pathways displayed by adriamycin-selective human small-

cell lung carcinoma cells remain largely to be characterized. In this study, the resistance of the adriamycin-selective GLC₄/Adr cells to doxorubicin was investigated, as well as the cross-resistance to *Vinca* alkaloids based anticancer drugs, vinblastine and vincristine. The cytotoxic effects of all test compounds were found in a dose-dependent fashion and GLC₄/Adr cells exhibited obviously higher resistance to all compounds in comparison with the parental counterparts. This finding indicated a reduction in the intracellular concentration of toxic agents due to the enhancement of cellular drug-efflux.

The resistance factors of GLC₄/Adr to cytotoxic anticancer drugs tested here were different, in particular, the difference in resistance to the two *Vinca* alkaloids derived from the periwinkle plant, vincristine-inhibited proliferation of GLC₄/Adr cells was tenfold that displayed by

vinblastine. A similar cytotoxic activity to the parental GLC₄ cells was observed for both agents. The cytotoxic activity of these two anticancer agents is based on the inhibition of the assembly of tubulin into microtubules resulting in arresting of cell proliferation kinetics during metaphase (Himes *et al.*, 1976; Lobert *et al.*, 2000). The subtle dissimilarities in the chemical structure, wherewith a formyl group was found in vincristine instead of the methyl group in vinblastine (Figure 3) resulted in a significant reduction of the partition coefficient from vinblastine to vincristine (Lobert *et al.*, 2000). The difference in chemical structure and the lipophilicities of both agents may play important roles in their different cytotoxic effects, due to the higher cellular efflux rate of vinblastine than vincristine. Indeed, a higher *in vivo* clearance rate of vinblastine to that of vinblastine has been reported by Rahmani *et al.* (1986).

Table 1 Resistance to cytotoxic anticancer drugs of the parental human small-cell lung carcinoma GLC₄ and the adriamycin-selective GLC₄/Adr cell lines and the resistance factor of GLC₄/Adr cells.

Drugs	IC ₅₀ ^{1/}		Resistance Factor ^{2/}
	GLC ₄	GLC ₄ /Adr	
Doxorubicin	0.012 ± 0.002 µM	3.553 ± 0.247 µM	310 ± 29
Vincristine	0.302 ± 0.022 nM	9.630 ± 0.134 nM	32 ± 5
Vinblastine	0.316 ± 0.005 nM	0.858 ± 0.072 nM	2.7 ± 0.3

^{1/} IC₅₀ of each cytotoxic agent was determined using an MTT assay and estimated mathematically based upon the dose-response curve. Data are the mean ± standard error of at least three independent experiments conducted in sextuplet.

^{2/} The resistance factor of GLC₄/Adr to each cytotoxic agent was calculated by dividing the IC₅₀ value of the GLC₄/Adr cells by that of the GLC₄ cells.

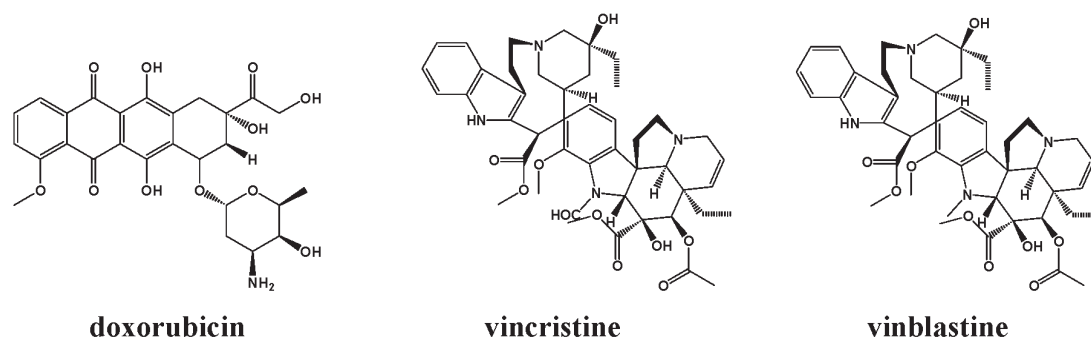


Figure 3 Chemical structure of anticancer drugs used in this study.

The observed drug resistance patterns of the GLC4/Adr cells fluctuated considerably and could be classified into three categories; high (>300 times) level of resistance to doxorubicin, moderate (>30 times) level of resistance to vincristine and low (<3 times) level of resistance to vinblastine. The manner of resistance to doxorubicin, vincristine and vinblastine found in this work was similar, but, with greatly different values, to the work of Cole *et al.* (1994) on HeLa cells transfected with two different eukaryotic expression vectors containing MRP complementarity. They reported a moderate level (5 to 15 times) for doxorubicin and vincristine and a low level (<3 times) for vinblastine. Furthermore, the estimated resistance factor for doxorubicin in this work was similar to previous work reported by Mavel *et al.* (2006).

CONCLUSION

In the present study, the adriamycin-selective GLC4/Adr cells displayed an acquired multidrug resistance phenotype of their parental counterparts with different resistance levels to doxorubicin, vincristine and vinblastine. This finding suggests that drug resistance cells generated by *in vitro* chronic exposure to the step-wise concentration of a single anticancer drug could become cross-resistant to other drugs and display some of the characteristics associated with the overexpression of the membrane transporter protein responsible for cellular detoxification at a sufficient level to confer multidrug resistance on previously sensitive cells.

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