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Cellular Chemo-Resistance and Radiosensitivity of Parental and Adriamycin-Selective Human Small-Cell Lung Cancer Cell Lines

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ABSTRACT

The radiosensitivity of two small-cell, lung cancer cell lines comprising the adriamycin-selective cell line (GLC_4/Adr) and its parental counter cell line (GLC_4) was investigated in an aqueous soluble system. A simplified resazurin assay was optimized and used in the cell viability investigation. Cell survival was evaluated after being exposed to cytotoxic agents, deduced from the resazurin reduction using the optical density of a spectrophotometer instead of a fluorescent signal. Compared with their parental counterparts, the GLC_4/Adr cells displayed a high resistance capability to doxorubicin with a resistant factor of about 320. No significant resistance to gamma radiation was demonstrated after exposure to gamma radiation from a Cs-137 source. The GLC_4/Adr cell line responded to radiation in the same manner as its parental counterpart. This finding suggested that a drug resistance phenotype occurred in the GLC_4/Adr cells (plausibly acquired after being cultured in the presence of oxidative stress produced by the step-wise concentration of adriamycin) and it is not responsible for the radioresistance of the cells.

Keywords: cellular radiosensivity, doxorubicin, adriamycin-selective cells, resazurin assay

INTRODUCTION

The resistance of cancer cells to anticancer drugs and radiation, both intrinsic resistance and acquired resistance developed during chemotherapy or radiotherapy, remains a major reason for chemotherapy and radiotherapy failure. Various mechanisms of cancer cells have been characterized regarding drug resistance. One of the most common studies has been on the multidrug resistance phenomenon. This mechanism is characterized by a decrease in intracellular drug accumulation resulting from over-expression and is associated with acquired multidrug resistance in the cancer cells of plasma membrane transporter proteins—namely, P-glycoprotein (Pgp) encoded by the *MDR1* gene that is also referred to as *ABCC1* (Juliono and Ling, 1976; Ambudkar *et al*., 1999; Lespine *et al*., 2012) and the multidrug resistance-associated protein (MRP1/ABCC1) encoded by the *ABCC1* gene (Cole *et al*., 1992; Kuo and Lu, 2012). Both transporters are members of the ATP-binding cassette (ABC) transporter superfamily of membrane transporter proteins (Higgins, 1992; Huanga and Sadée, 2006). The available evidence strongly suggests that MDR1 and MRP1 are the efflux-pumps that catalyze the clearance of drugs from the cells leading to the

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reduction of drug accumulation and the access of cytotoxic drugs to their targets, and the cells confer simultaneous resistance to a variety of chemically unrelated cytotoxic drugs widely used in cancer chemotherapy without any sharing of structure and target (Cole *et al*., 1992; Higgins, 1992; Gottesman and Ambudkar, 2001; Palakas *et al*., 2009; Budulac *et al*., 2012).

Radiotherapy is often given as an adjuvant to chemotherapy for patients with lung cancer that is generally divided into small-cell lung cancer and non-small-cell lung cancer. The small-cell lung cancer cells are generally more sensitive to radiotherapy than the non-small-cell lung cancer cells (Carmichael *et al*., 1989). However, both types of cells frequently develop a resistance to both chemotherapy and radiotherapy.

Regarding the multidrug resistance mechanism, single cytotoxic drug selective cells may confer cross-resistance to a wide range of drugs without any sharing of common structures and targets. The adriamycin-selective, human, small-cell lung cancer cell line, GLC_4/Adr, can be established by culture of the parental cell line (GLC_4) in step-wise increasing concentrations of adriamycin. This small-cell lung cancer cell line has been characterized and identified as a multidrug-resistant cell line due to the over-expression of the MRP1 membrane transporter protein (Ziljstra *et al*., 1987; Meijer *et al*., 1991). The GLC_4/Adr cells are less sensitive to doxorubicin than the parental GLC_4 cells with a resistance factor of more than 300 times (Mavel *et al*., 2006; Palakas *et al*., 2009). Since radiation is often used together with chemotherapy, it is therefore of interest to evaluate the cellular radiosensitivity of GLC_4/Adr, the MRP1 overexpression cell line.

The present work aimed to investigate the radiosensitivity of the adriamycin-selective, human, small-cell lung cancer cell line (GLC_4/Adr) in comparison with its parental counterpart cell line (GLC_4). However, in order to facilitate the assessment of cell survival in an aqueous soluble system, the simplified resazurin assay was optimized. Consequently, cell survival was evaluated after being exposed to an anticancer drug and deduced from the resazurin reduction using the optical density at two wavelengths of a spectrophotometer instead of the fluorescent signal from fluorescence spectrometry. Finally, the cellular sensitivity to gamma radiation from a Cs-137 source was investigated.

MATERIALS AND METHODS

Chemical reagents

RPMI 1640 with glutamax culture medium, heat-inactivated fetal bovine serum, sodium pyruvate, mixed antibiotic of penicillin and streptomycin and phosphate buffer saline were purchased from Gibco (Grand Island, NY, USA). Resazurin sodium salt, dimethyl sulfoxides (DMSO) and doxorubicin were obtained from Sigma Aldrich (St. Louis, MO, USA).

Cell lines and cell culture conditions

Two human, small-cell lung carcinoma cell lines consisting of the parental cell line (GLC_4) and the adriamycin-selective cell line (GLC_4/Adr) derived from the parental cell line were cultured in the presence of step-wise concentrations of adriamycin (doxorubicin) up to 1.2 µM as described by Ziljstra *et al*. (1987) and Meijer *et al*. (1991). Both of the cell lines were kindly provided by Dr. M. Garrigos (Laboratoire des protéine membranaires, iBiTecS, SB^2SM, CEA Saclay, France). Their resistance to anticancer drugs was evaluated using an MTT assay procedure (Palakas *et al*., 2009)

Cells were maintained in exponential growth in RPMI 1640 culture medium with glutamax supplemented with 10% heat-inactivated fetal bovine serum, penicillin G (100 units.mL^{-1}), streptomycin (100 µg.mL^{-1}) and 1% sodium pyruvate at 37 °C in a humidified atmosphere containing 5% CO_2. The adriamycin-selective GLC_4/Adr cell line was maintained in the presence of 1.2 µM doxorubicin and was cultured in a

doxorubicin-free culture medium for 11 d before use in all experiments.

Cell survival assay

The chemo-resistance and radiosensitivity of cancer cells were investigated using a simplified cytotoxic assay based upon the reduction of resazurin to resorufin by the mitochondrial reductase of viable cells as described in McBride *et al.* (2005) with slight modification. The change in the optical density indicated the amount of viable cells after exposure to an anticancer agent, and the gamma radiation was determined spectrophotometrically at respective wavelengths of 570 and 600 nm using a Microplate Reader (Expert Plus Microplate Reader, Biochrome Ltd.; Cambridge, UK). Resazurin reduction was subsequently used for cell survival estimation.

Toxicity of doxorubicin

Cells in exponential growth of each cell line were harvested, counted and seeded into new culture media with respective densities of 2,000 cells.mL^{-1} and 5,000 cells.mL^{-1} for GLC_4 and GLC_4/Adr. After 24 h of incubation at 37 °C in a humidified atmosphere with 5% CO_2, stock solution of doxorubicin dissolved in sterile DMSO was diluted in culture media and was added to obtain the designed concentrations and incubated for a further 72 h. At the end of drug exposure, sterile resazurin solution was added and the mixture was incubated for another 4 h. The optical density was determined at 570 and 600 nm. The survival fraction of the cells was calculated based on the reduction of resazurin.

Cellular radiosensitivity

Similar to the test for doxorubicin toxicity, cells in the exponential growth phase of each cell line were harvested, counted and resuspended in new culture media with the same initial plating cell density as mentioned in the toxicity of doxorubicin experiment. Cells were exposed to gamma radiation delivered from Cs-137 using a MARK I Irradiator (J.L. Shepherd & Associates; San Fernando, CA, USA, Canada) at varying doses from 0 to 4 Gy with a dose rate of 4.01 Gy.min^{-1}. The radiation dose was evaluated using a Fricke dosimeter (de Austerlitz *et al*., 2006). In the control experiment, cells were introduced to the sample chamber of the irradiator for an equivalent time of exposure for the highest dose without being exposed to any radiation. Cells were transferred to 96-well plates and were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 96 h. Sterile resazurin was added and the mixture was incubated for another 4 h; thereafter, the optical density of each sample was determined and the survival fraction of cells was calculated based on the reduction of resazurin as mentioned previously.

Statistical analysis and mathematical estimation

Descriptive statistics—the mean and standard error—were determined to demonstrate the viability of cells after exposure to cytotoxic agents—namely, doxorubicin and gamma radiation. The half maximal proliferative inhibitory concentration (IC_{50}) and the median lethal dose (LD_{50}) values were defined as respective concentrations of doxorubicin and radiation doses that reduced the cell viability to 50% of the control. Both values were estimated from a dose-response curve of cell survival. The resistance factor (RF) of each cytotoxic agent was calculated by dividing the IC_{50} or LD_{50} values of the GLC_4/Adr cells by that of the GLC_4 cells.

RESULTS

Optimal condition of resazurin assay

In using the resazurin assay for the determination of human cancer cell viability after exposure to cytotoxic agents, two key determinant factors were optimized—initial cell density and incubation time in the presence of resazurin. As illustrated in Figure 1A and 1B, resazurin reduction

by the GLC_4 and GLC_4/Adr cells increased as a function of their initial plating cell densities. Each cell line produced a slightly different pattern of resazurin reduction that might have been due to differences in the cell proliferation rate. The optimal initial plating cell density that still gave an initial rate of resazurin reduction was 1,000–4,000 cells.mL^{-1} and 500–5,000 cells.mL^{-1}/ for GLC_4 and GLC_4/Adras demonstrated in Figure 1C and 1D, respectively. Regarding the optimization of incubation time in the presence of resazurin, GLC_4 and GLC_4/Adr cells with respective initial plating cell density of 2,000 and 5,000 cell.mL^{-1} were incubated for 96 h before addition of resazurin solution. The reduction of resazurin increased as a linear function of time for at least 8 h as shown in Figure 2.

Cytotoxicity of doxorubicin

The cell viability of cultured, human, small-cell lung carcinoma parental cell line (GLC_4) and its adriamycin-selective cell line (GLC_4/Adr) after exposure to varying concentrations of doxorubicin was evaluated by resazurin assay. The viable rate of cells was deduced from the relative absorbance of resazurin and its reducing product, resorufin, compared to that of the control as illustrated in Figure 3. After 72 h of drug exposure, doxorubicin reduced the cell viability of GLC_4 and GLC_4/Adr cells in a dose-dependent

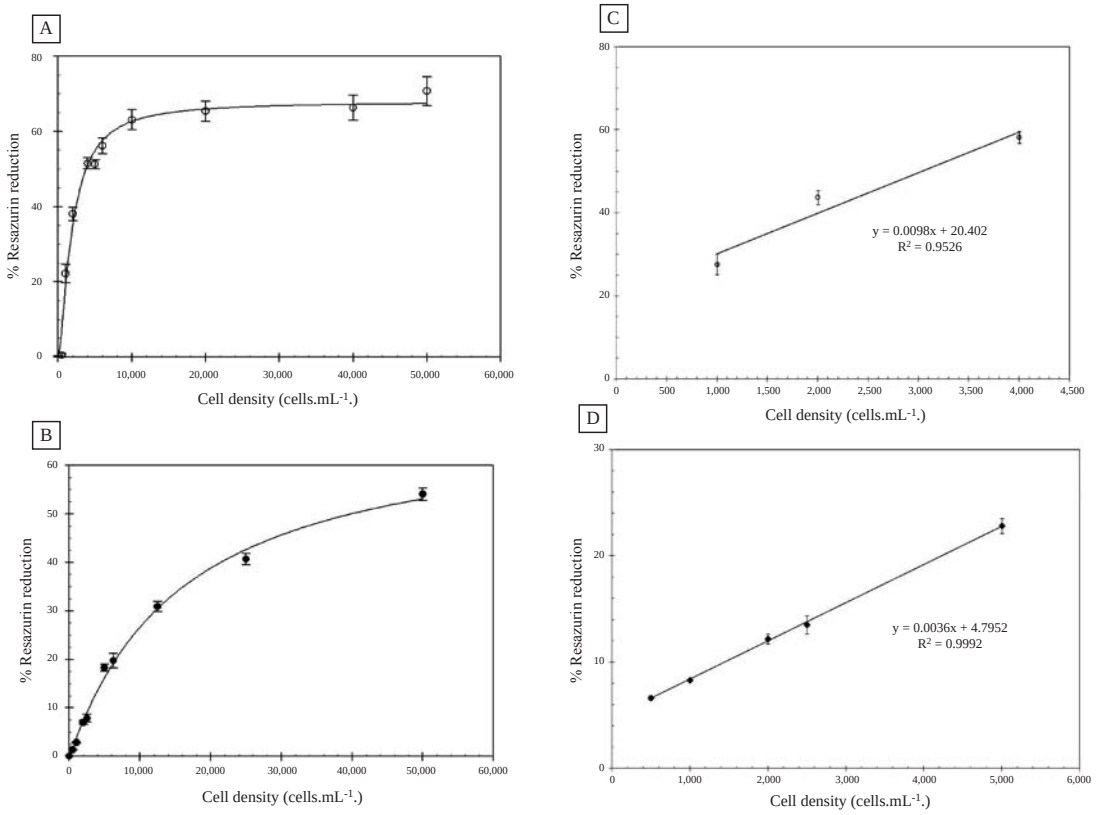

Figure 1 Resazurin reduction by GLC_4 (A) and GLC_4/Adr (B) cells after 96 h of culture with different initial plating cell densities and 4 h of incubation in the presence of resazurin. And the initial rate of resazurin reduction corresponding to initial plating cell density of GLC_4 (C) and GLC_4/Adr (D) cells. Data points and bars represent the average and standard error of at least six independent experiments, each experiment was conducted in triplicate. R^2 = correlation coefficient.

manner. The results revealed that the GLC_4 cells were more sensitive to doxorubicin than were the GLC_4/Adr cells with an IC_{50} value of 0.012 ± 0.001 µM and 3.751 ± 0.889 µM, respectively. The resistant factor (RF) deduced from the IC_{50} values of GLC_4 and GLC_4/Adr obtained from the resazurin assay was 320. The estimated IC_{50} values of doxorubicin for both cell lines as well as the RF to doxorubicin obtained in this study were in accordance with those of a previous evaluation using the MTT assay (Palakas *et al*., 2009) as shown in Table 1. These findings of the IC_{50} and RF values for doxorubicin underline the feasibility of using the resazurin assay in the determination of cell survival. Moreover, the optical densities corresponded to an innate cellular metabolic activity of viable cells that reduced the resazurin dye and led to a change in its color instead of a fluorescent signal that could be used effectively for assessment of *in vitro* cancer cell survival after being exposed to a cytotoxic agent.

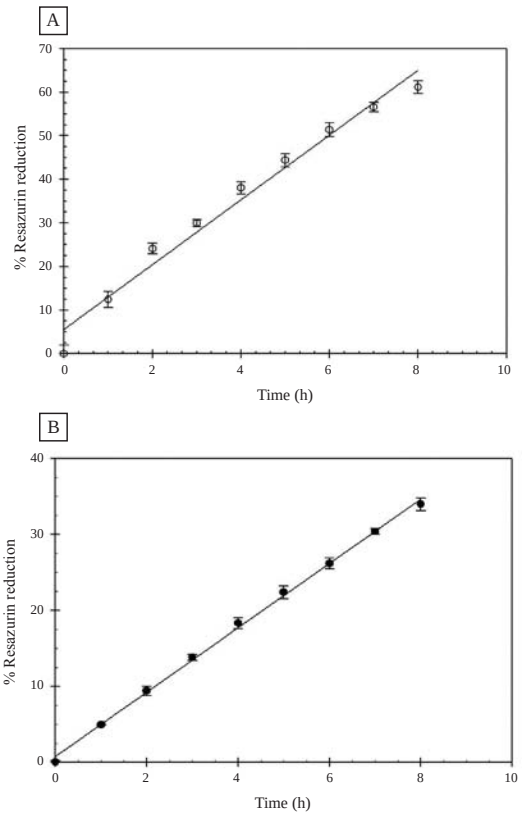

Figure 2 Resazurin reduction as a function of incubation time in the presence of resazurin of GLC_4 (A) and GLC_4/Adr (B) cells after 96 h of culture with respective initial plating cell density of 2,000 and 5,000 cells. mL^{-1}. Data points and bars represent the average and standard error of at least six independent experiments, each experiment was conducted in triplicate.

Biphasic dose-responses of cell survival after drug exposure were observed in the dose-response curves of both cancer cell lines. These implied toxin-mediated hormesis (Mattson, 2008). When exposed to a low concentration of doxorubicin, the survival curves indicated an adaptive stimulation effect of cell proliferation instead of cell proliferation inhibition. The range of non-cytotoxic concentration of doxorubicin for both cells cell lines was 0–0.005 µM. When cells were exposed to further higher doses of drugs, the obvious inhibitory effects for both cell lines were observed but with distinguishable differences for each cell line.

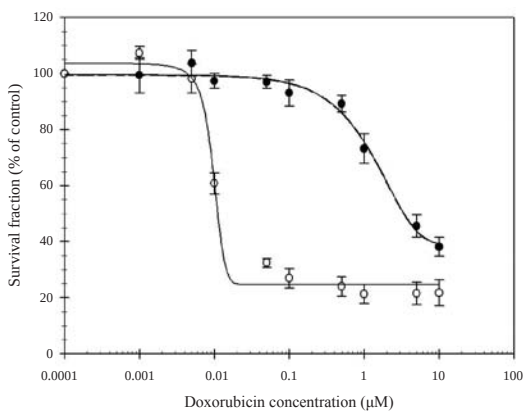

Figure 3 Survival fraction of GLC_4 (○) and GLC_4/Adr (●) cells after 72 h exposure to doxorubicin. Data points and bars represent the average and standard error of at least three independent experiments.

Table 1 Resistance to doxorubicin of GLC_4 and GLC_4/adr cells investigated by resazurin assay and MTT assay.

Drug	IC_{50} [1/]		Resistance Factor [2/]
	GLC_4	GLC_4/Adr	
Resazurin assay	0.012 ± 0.001 µM	3.751 ± 0.889 µM	320
MTT assay [3/]	0.012 ± 0.002 µM	3.553 ± 0.247 µM	310

1/ The half maximal inhibitory concentration (IC_{50}) of doxorubicin was determined using an indicated method of cell survival assay and estimated mathematically based upon the dose-response curve. Data are the mean ± standard error of at least three independent experiments.

2/ The resistance factor of GLC_4/Adr exposed to doxorubicin was calculated by dividing the IC_{50} value of the GLC_4/Adr cells by that of the GLC_4 cells.

3/ Sourced from: Palakas *et al.* (2009)

Cellular radiosensitivity of GLC_4 and GLC_4/Adr

To evaluate the cytotoxic effects of gamma radiation from a Cs-137 source and to obtain the LD_{50} values to judge the radiosensitivity of GLC_4 and GLC_4/Adr cells, the viabilities of cells exposed to different doses of radiation ranging from 0 to 4 Gy were determined using resazurin assay. The results revealed that gamma radiation reduced the cell survival in a dose-dependent manner as illustrated in Figure 4. Similar to the cell survival after exposure to doxorubicin, the exposure to low doses of gamma radiation exhibited a slightly adaptive response in cell proliferation. The maximum non-cytotoxic dose of gamma radiation was 0.4 Gy for both cell lines.

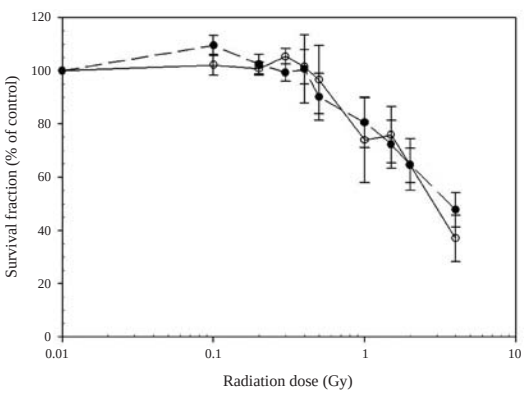

Figure 4 Survival of GLC_4 (○) and GLC_4/Adr (●) cells after exposure to gamma radiation and being cultured for 96 h. Data points and bars represent the average and standard error of at least three independent experiments.

The LD_{50} values estimated from the cell survival curves after exposure to gamma radiation and further culturing for 96 h were 2.97 ± 0.42 Gy and 3.34 ± 0.84 Gy for GLC_4 and GLC_4/Adr cells, respectively. In contrast with the chemo-sensitivity to doxorubicin, the radiosensitivity of the GLC_4 and GLC_4/Adr cells based on the LD_{50} values of each cell line were not significantly different. This finding indicated that the drug resistance phenotype found in GLC_4/Adr after the step-wise selection of increasing concentration and culture in the presence of adriamycin did not mediate the resistance of cells to radiation.

DISCUSSION

The cellular defense mechanism displayed by the MRP1 over-expression, human, small-cell lung carcinoma, GLC_4/Adr cells, remains largely to be explored. In order to overcome the complicated processes and the reduction of resource consumption in the cell viability determination after being exposed to toxic agents, a simplified resazurin assay based upon the use of optical absorbance from a spectrophotometer

instead of using fluorescent spectrometer was optimized and used to determine the survival of cells after exposure to doxorubicin as well as to gamma radiation.

Compared to the MTT assay, the optimized conditions of the resazurin assay could be used effectively for the determination of cell survival after exposure to toxic agents. Resazurin and its reducing product are water soluble and non-toxic for the test cells. Therefore, this assay is more convenient for assaying the viability of non-adherent cells compared to the MTT assay that produces formazan as a non-water soluble product. In the MTT assay, separation and dissolving of the formazan product with an organic solvent are needed but no further process is needed for the resazurin assay.

The cytotoxic effects of doxorubicin and gamma radiation displayed a dose-dependent response. The results allowed the estimation of the IC_{50} and LD_{50} values of the drug and the gamma radiation. The GLC_4/Adr cells exhibited obviously higher resistance to doxorubicin compared to their parental counterparts. The observed drug resistance pattern of GLC_4/Adr with the RF was 320 times higher than for GLC_4, indicating the reduction of intracellular concentration of toxic agents due to the enhancement of the cellular drug efflux. The estimated RF to doxorubicin of the GLC4/Adr cells in this work was in accordance with the reports by Mavel *et al*. (2006) and Palakas *et al*. (2009). However, the obtained values were greatly different when compared with Cole *et al*. (1994) who conducted their work on HeLa cells transfected with two different eukaryotic expression vectors of MRP complementarity. They reported a moderate resistance level of 5 times for doxorubicin.

Similar to the resistance of doxorubicin, gamma radiation reduced the cell proliferation of GLC_4 and GLC_4/Adr cells in a dose-dependent manner. This finding was in accordance with other studies conducted on several types of cancer cell lines, for example, cervix cancer cells (Zhang *et al*., 2006), human lung cancer cells (Watanabe *et al*., 2007), squamous cell carcinoma and human hypopharyngeal cells (Ulla *et al*., 2009), and human glioblastoma cells (Chu *et al*., 2011).

However, when the viability after exposure to gamma radiation of each cell line was compared, a distinguishable difference from that of drug resistance was observed. Similar LD_{50} values estimated from the cell survival curves after being exposed to gamma radiation of the GLC_4 and GLC_4/Adr cells were found. This indicated that the GLC_4/Adr cell line responded to radiation in the same manner as its parental counterpart.

The difference between cellular responsiveness to radiation and an anticancer drug of the GLC_4/Adr cells is of interest. This finding suggested that a drug resistance phenotype occurred in the GLC_4/Adr cells, plausibly acquired after being cultured in the presence of the step-wise concentration of adriamycin. This phenotype is associated with the over-expression of the multidrug resistance associated protein 1 (Ziljstra *et al*., 1987; Meijer *et al*., 1991; Mavel *et al*., 2006) and led to cross resistance to the unrelated molecular structure of the drug (Palakas *et al*., 2009). However, this cellular detoxification phenotype, exhibited only by the cellular drug efflux, is not responsible for the reduction of cell damage after exposure to gamma radiation. Therefore, the radiosensitivity of the adriamycin-selective cells was still the same as its parental counterpart.

After being exposed to the anticancer drug and radiation, the death of cells could be observed due to several reasons; for example, apoptosis, cell cycle arrest and the reduction of cellular anti-oxidant capacity. The approximate 300-fold resistance to doxorubicin with non-significant resistance to gamma radiation exhibited by the GLC_4/Adr cells reflects the treatment of small-cell lung cancer. Since combined radiotherapy and chemotherapy are commonly used for lung cancer

treatment, the current results suggest interesting further *vice versa* studies, for example, to develop a new cell line under radiation-induced oxidative stress conditions instead of using an anticancer drug that could be used as a new biological model for the exploration of stable and a resistant pathway to cytotoxic drugs and radiation. The fractionated radiation treatment that caused the increased radiation resistance has been reported in several cancer cell types such as breast cancer (Wazer *et al*., 1993), leukemia (Harvie *et al*., 1997) and pancreatic cancer (Lee *et al*., 1999).

CONCLUSION

The adriamycin-selective GLC4/Adr cells displayed an acquired multidrug resistance phenotype due to the exposure to *in vitro* chronic oxidative stress induced by a single anticancer drug. After exposure to doxorubicin, the GLC4/Adr cells demonstrated an approximate resistance factor of 320 compared with their parental counterparts. This result displayed some characteristics associated with the over-expression of the membrane transporter protein responsible for cellular detoxification at a sufficient level to confer multidrug resistance on previously sensitive cells. However, in a comparison with their parental cells, no significant change in radiosensitivity of the GLC4/Adr cells was demonstrated. This result indicated that the over-expression of MRP1 on the GLC4/Adr cells which mediated the resistance to the anticancer drug was not responsible for the resistance to radiation. Some further investigation should be conducted to evaluate the implication of resistance to radiation and the anticancer drug, for example, the development of a new cell line under radiation-induced oxidative stress conditions instead of using an anticancer drug and this could be used as a new biological model for the exploration of a stable and a resistant pathway to cytotoxic drugs and radiation.

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