Multidrug resistance in tumour cells: characterisation of the multidrug resistant cell line K562-Lucena 1[*]

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ABSTRACT

Multidrug resistance to chemotherapy is a major obstacle in the treatment of cancer patients. The best characterised mechanism responsible for multidrug resistance involves the expression of the MDR-1 gene product, P-glycoprotein. However, the resistance process is multifactorial. Studies of multidrug resistance mechanisms have relied on the analysis of cancer cell lines that have been selected and present cross-reactivity to a broad range of anticancer agents. This work characterises a multidrug resistant cell line, originally selected for resistance to the Vinca alkaloid vincristine and derived from the human erythroleukaemia cell K562. This cell line, named Lucena 1, overexpresses P-glycoprotein and have its resistance reversed by the chemosensitisers verapamil, trifluoperazine and cyclosporins A, D and G. Furthermore, we demonstrated that methylene blue was capable of partially reversing the resistance in this cell line. On the contrary, the use of 5-fluorouracil increased the resistance of Lucena 1. In addition to chemotherapics, Lucena 1 cells were resistant to ultraviolet A radiation and hydrogen peroxide and failed to mobilise intracellular calcium when thapsigargin was used. Changes in the cytoskeleton of this cell line were also observed.

Key words: multidrug resistance, leukaemia, P-glycoprotein, chemosensitisers.

INTRODUCTION

Resistance to chemotherapy is a major obstacle in the treatment of cancer patients. Multiple mechanisms contribute to the overall clinical resistance to cancer chemotherapy and, although investigators have examined the putative major site of drug action as the most likely site to be involved in drug resistance, it became clear that a number of patients could exhibit cross-resistance to structurally unrelated drugs that did not share a common target. This phenomenon of multidrug resistance (MDR) may also result from a variety of cellular mechanisms such as: defects in the regulation of genes controlling apoptosis, enhanced intracellular drug detoxification, alterations in DNA repair, activation or overexpression of drug export proteins.

Among the various mechanisms identified with the MDR phenotype, the best characterised involves the expression of the MDR-1 gene product, P-glycoprotein (Pgp) (Gottesman & Pastan 1993, Juranka et

al. 1989). Transfection studies indicated that MDR-1 gene overexpression is sufficient to confer multidrug resistance (Gros et al. 1986). Pgp as well as the MDR-related protein (MRP) (Cole et al. 1992, Cole et al. 1994) and the transporter of antigenic peptides (TAP) (Neefjes et al. 1993), all involved in the MDR phenotype, are members of the adenosine triphosphate (ATP)-binding cassette (ABC) supergene family that encodes ATP-dependent transmembrane transporter proteins (Higgins 1992). Cells overexpressing Pgp display a reduced intracellular concentration of the drugs that are substrates for this transporter. The Pgp drug efflux pump is capable of extruding a wide variety of structurally unrelated drugs taken up by cells through passive diffusion and, as a result, the drug intracellular level does not reach a toxic concentration (Juliano & Ling 1976). Not only chemotherapics but a number of substances may be inhibited of accumulating inside the cell, including certain fluorescent dyes and this property is used to ascertain the functional activity of the pump (Neyfakh 1988, Pétriz & Garcia-López 1997). Similar to Pgp, MRP is also capable of extruding various substances but in this case there is a preferential transport of substrates conjugated with glutathione (Cole et al. 1994, Muller et al. 1994). Another transporter associated with the MDR phenotype is the lung resistance-protein (LRP) that is implicated in nuclear-cytoplasmic trafficking of several substrates. This protein is capable of redistribute drugs from the nucleus to the cytoplasm and in this way confers resistance to DNA-binding drugs (Scheffer et al. 1995). Unlike MDR-1 and MRP genes, transfection of the LRP gene alone is not sufficient to confer a MDR phenotype (Scheffer et al. 1995).

Most studies attempting to circumvent MDR focused on the possibility of modulating Pgp activity. This can be achieved and a number of drugs known as chemosensitisers, Pgp modulators or reversors, have been described (Ford & Hait 1990, Boer & Gekeler 1995). MDR reversal through inhibition of Pgp was first obtained *in vitro* using verapamil (Tsuruo et al. 1981). A variety of other totally unrelated substances such as cyclosporins,

phenothiazines, antimalarials, antibiotics etc. where also shown to exert a modulatory effect. To avoid toxicity resulting from the use of high doses, due to the lack of binding specificity of these drugs to Pgp, analogues of these substances, as well as new compounds, were produced and selected for their MDR reversal capabilities. A number of second- and third-generation Pgp modulators are being evaluated, but despite the efforts neither their binding sites on Pgp nor their mode of action as modulators are clearly comprehended. Recent clinical trials have evidenced that elimination of Pgp^{+} cells through the combined use of chemotherapics and Pgp-modulators may lead to the selection of cells presenting alternate MDR mechanisms (List et al. 1993).

Approaches to study MDR mechanisms have relied on the analysis of cancer cell lines that have been selected for resistance to specific anticancer drugs and presented, at the end of the selective process, cross-reactivity to a broad range of unrelated anticancer agents (Dalton 1997). However, despite the fact that these cell lines present a MDR phenotype, these types of studies have shown that the mechanism of acquired drug resistance in cultured cell lines is dependent on the cytotoxic drug used to select the cells and the stage of the selective process they are in (Yanovich et al. 1989, Matsumoto et al. 1997, Modrak et al. 1997, Denis-Gay et al. 1998). The present work describes the characteristics of a MDR cell line derived from the human erythroleukaemia cell K562 and selected for resistance to the Vinca alkaloid vincristine (VCR).

CHARACTERISATION OF THE MDR PHENOTYPE

An approach to improving our understanding of the MDR mechanisms involved the establishment of a human MDR cell lineage based on the technique described by Tsuruo et al. (1983). Basically K562 cells were exposed by us to gradually increasing doses of VCR, starting at 3nM and augmenting the doses every two weeks by increments of 3nM until cells were resistant to 60nM VCR (Rumjanek et
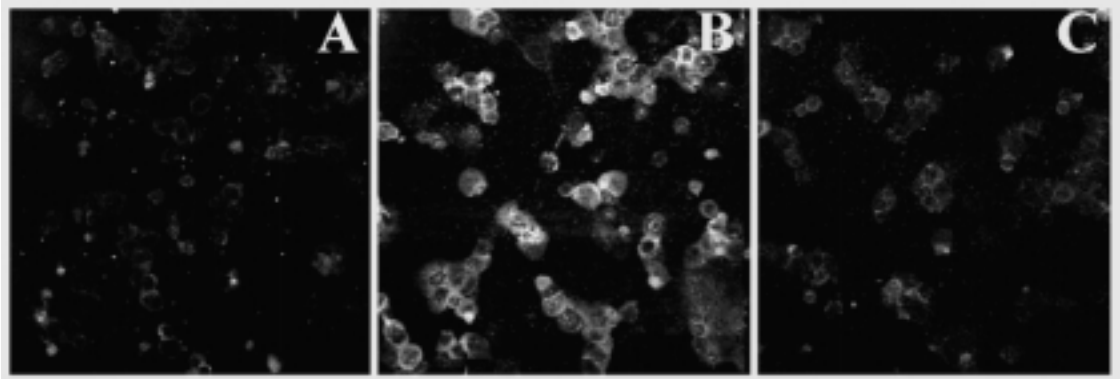
Fig. 1 – Expression of Pgp and MRP1 in Lucena 1 cells. Cells were fixed with methanol/acetone, permeabilised with 0.1% triton X-100 and incubated overnight with anti-Pgp or anti-MRP1 antibodies. After washing, cells were incubated for 2h with secondary antibody labelled with FITC and the fluorescence observed under a confocal microscope (Zeiss, Germany). **A** – control (secondary Ab); **B** – cells labelled with mAb anti-Pgp; **C** – cells labelled with mAb anti-MRP1.

al. 1994). These VCR resistant cells, known as K562-Lucena 1, can now be cultured equally well in medium containing 60nM VCR as in drug-free medium.

The MDR-1 gene is amplified in Lucena 1 cells that present five times more copies of this gene when compared to the parental cell line K562 (Brindeiro 1999). Similarly, there is an overexpression of this gene as detected by RT-PCR (Brindeiro 1999). This is accompanied by the expression of Pgp on the plasma membrane of Lucena 1 cells as evidenced by immunofluorescence (Fig. 1), immunocytochemistry, or western blot using the JSB-1 anti-Pgp antibody. Furthermore, the Pgp present in these cells is functionally active, being capable of extruding the fluorescent dye rhodamine 123 (Rho 123) as assessed by flow cytometry (Fig. 2A and Fig. 2D). Because Rho 123, albeit to a lesser extent, is a substrate for MRP, the expression of this protein in Lucena 1 cells was investigated by indirect immunofluorescence using the MRPr1 anti-MRP antibody and was found absent (Fig. 1). Furthermore, no increase in glutathione S transferase was observed in these cells.

As should be expected in a cell line expressing functionally active Pgp, Lucena 1 cells were resis-tant to chemically unrelated chemotherapics such as anthracyclines (Maia et al. 1996a). Moreover, their resistance could be reversed by the "classical" MDR-modulators: cyclosporin A (CSA), verapamil (VP) and trifluoperazine (TFP) (Fig. 3) (Rumjanek et al. 1994). Similarly, the CSA analogues CSD and CSG, already described as having chemosensi-tiser properties (Mizuno et al. 1992, Tanaka et al. 1996), were also capable of reversing resistance in Lucena 1 cells (Fig. 4). It was also possible to demonstrate that methylene blue, another phenoti-azine, can also function as a modulatory agent on these cells (Trindade et al. 2000). The sulphated polysaccharide heparin was also capable of inhibit-ing the functional activity of Pgp in Lucena 1 cells and samples from leukaemic patients (Maia et al. 1996b). However, it is our experience now that not all batches of heparin are capable of such an effect and heparin activity as a modulatory agent merits further studies.

Because resistance in Lucena 1 cells could be reversed by modulators leading to chemotherapic-induced apoptosis, it seemed improbable that the resistance mechanism in these cells involved over-expression of Bcl-2, an anti-apoptotic molecule. In agreement with this supposition flow cytometry
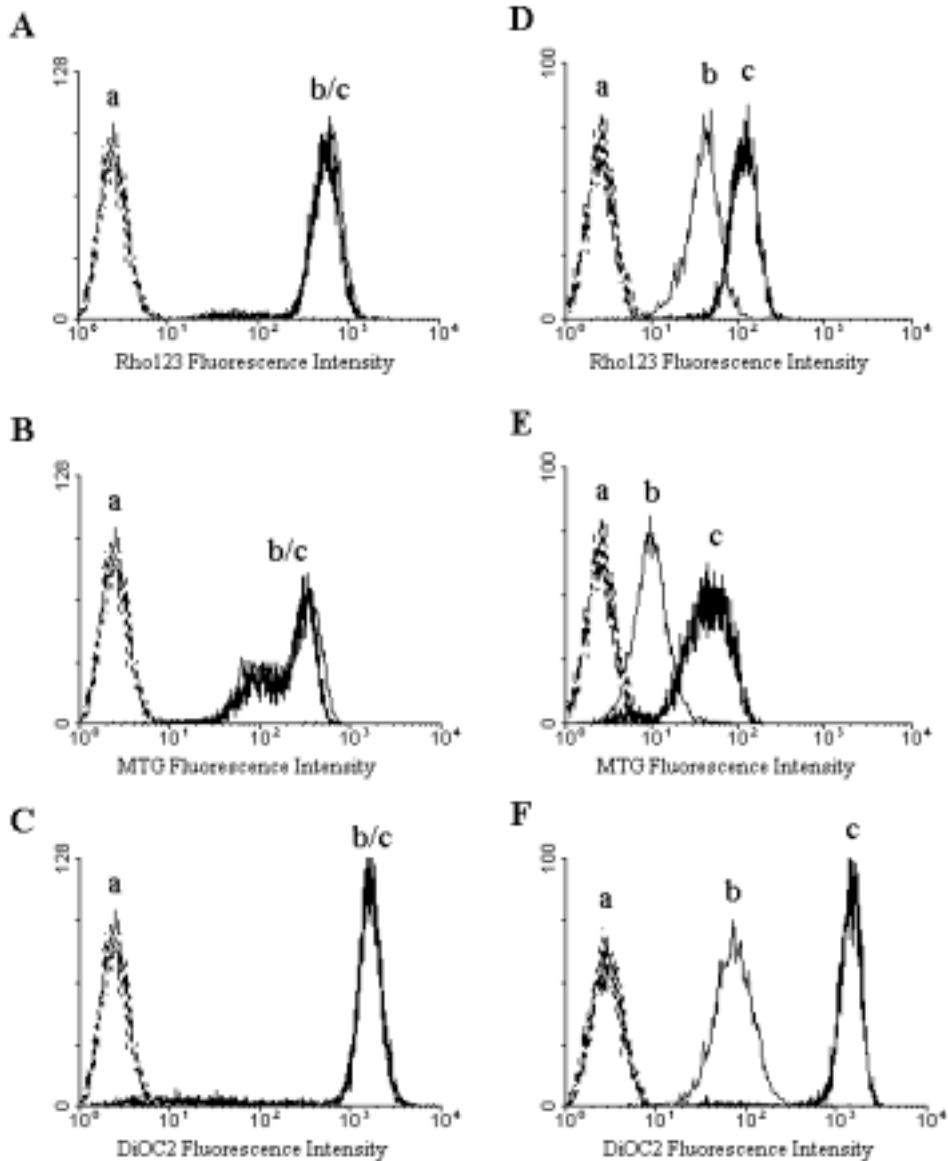
Fig. 2 – Rhodamine 123, Mitotracker Green and DiOC$_2$ are P-Glycoprotein Substrates. K562 (**A**, **B** and **C**) and Lucena 1 cell lines (**D**, **E** and **F**) were incubated in the presence of Rho 123 (200 ng/ml – **A** and **D**), MTG (50nM – **B** and **E**) or DiOC$_2$ (60 ng/ml – **C** and **F**) for 30 minutes. After this period, cells were washed with cold saline and analysed in a Flow Cytometer. Co-incubation with cyclosporin A (CSA 200 ng/ml) increase Rho 123 (**D**), MTG (**E**) and DiOC$_2$ (**F**) fluorescence in Lucena 1 cells. The same results were obtained when cells were incubated in the presence of trifluoperazyne (TFP 5μM) or verapamil (VP 5μM) instead of CSA. **a** – autofluorescence; **b** – fluorochrome; **c** – fluorochrome plus CSA. Data are representative of at least 5 experiments.
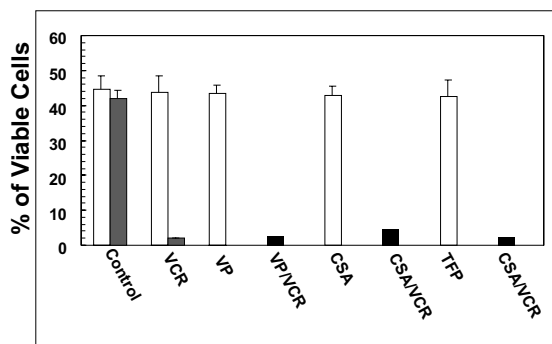
Fig. 3 – Reversal of the resistance to vincristine by MDR modulators. Lucena 1 cells were incubated for 7 days with vincristine (VCR 60 nM) in the presence of verapamil (VP 5μM), cyclosporin A (CSA 200 ng/ml) or trifluoperazyne (TFP 5μM) and the number of remaining viable cells was counted under phase microscopy. For comparison, in black are the number of viable cells of K562 control or treated by the same time with VCR.

analysis of these cells using the anti-human Bcl-2 antibody (Pharmingen) indicated that there is no difference between the parental cell line K562 and the resistant Lucena 1 cells (Wagner-Souza, K. and Echevarria-Lima, J. unpublished observations).

RESISTANCE TO THAPSIGARGIN

Calcium mobilisation from intracellular stores is a determinant step in cell signalling, including induction of apoptosis. The drug thapsigargin is capable of inhibiting the endoplasmic reticulum calcium ATPase (Sagara et al. 1992) leading to enhanced levels of cytosolic calcium as a combined result of depletion from intracellular stores and extra cellular calcium entry (Thastrup et al. 1990, Putney 1990). It has been reported that a cell line made resistant to thapsigargin was incapable of mobilising calcium and presented a MDR phenotype (Gutheil et al. 1994, Rishi et al. 1998). Conversely, MDR cell lines were also thapsigargin resistant (Gutheil et al. 1994). Lucena 1 cells did not display an increase in intracellular calcium levels following thapsigargin treatment (Orind et al.1997). Various possibilities have been advanced to explain a lack of response to thapsigargin: the diminished response to thapsigar-

gin is proportional to Bcl-2 levels (Lam et al. 1994, Kuo et al. 1998, Foyouzi-Youssefi et al. 2000), but as discussed above no increased levels of Bcl-2 were observed in Lucena 1 cells; Pgp activity is capable of extruding thapsigargin, however the use of modulatory agents could not reverse the lack of response to thapsigargin in our cells (Orind et al. 1997) nor in other MDR cell lines (Gutheil et al. 1994); the diminished response to thapsigargin results from the overexpression of calcium ATPases (Hussain et al. 1995, Rishi et al. 1998), including SERCAs not normally expressed by these cells (Gutheil et al. 1994) and not capable of being inhibited by thapsigargin, this possibility, regarding Lucena 1 cells is now under investigation.

RESISTANCE TO OXIDATIVE STRESS

A number of MDR cells lines were shown to be more resistant to lipid peroxidation than their parental lines and different mechanisms have been attributed to explain this increased resistance (Benchekroun et al. 1990, Hoban et al. 1992, Hill et al. 1995, Lenehan et al. 1995, Mazzanti et al. 1995, Kühl et al. 1997). Bcl-2 and members of its family are powerful antioxidant agents and may be augmented in MDR cell lines. Similarly, an increased expression of antioxidant enzymes such as glutathione peroxidase or high levels of scavengers such as a-tocopherol present in the cellular membrane have been described (Benchekroun et al. 1990, Mazzanti et al. 1995). Lucena 1 cells are more resistant to H_2O_2 and to ultraviolet-A radiation (UVA) than the parental K562 cell line. This resistance to UV is confined to the UVA spectrum and is not extensive to UVB or UVC (Trindade et al. 1999). As the preferential target of UVA is the cell membrane as opposed to the nucleus and as this radiation tends to induce lipid peroxidation via the formation of H_2O_2, it is understandable that only this radiation is ineffective on Lucena cells, which as said above, do not show increased levels of Bcl-2. On the other hand, a high catalase activity could be detected in these cells which could explain the resistance to hy-
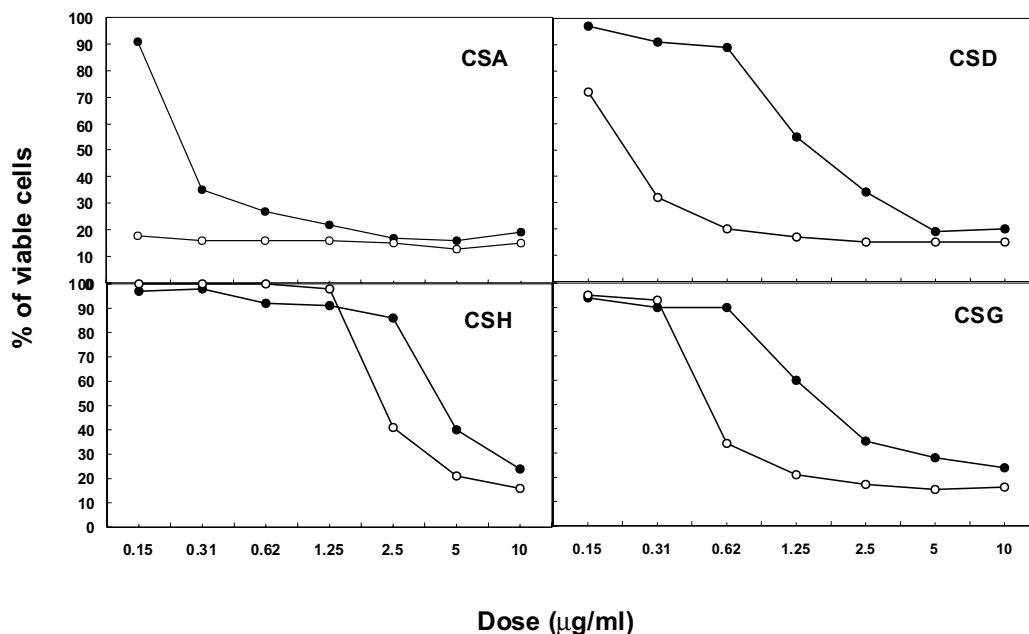
Fig. 4 – Reversal of the resistance to vincristine by cyclosporin analogues. Lucena 1 cells were incubated for 7 days with vincristine (VCR 60 nM) in the presence of cyclosporin A (CSA), cyclosporin D (CSD), cyclosporin H (CSH), cyclosporin G (CSG) and the number of remaining viable cells was counted under phase microscopy. Empty circles represent the effect of CSA or analogues in the resistance of cells to VCR. Black circles represent the cellular toxicity of CSA or analogues alone.

drogen peroxide (Trindade et al. 1999). However, Lucena 1 cells were shown to be susceptible to the photodynamic action of methylene blue indicating their sensitivity to the superoxide anion (Trindade et al. 2000), This results are in agreement with those of Dellinger et al. (1992) that could not find resistance to the photodynamic action of photofrin on an adriamycin induced MDR line.

SENSITIVITY TO CERAMIDE

The induction of apoptosis following oxidative stress, UVC, ionising radiation, heat shock, TNF, Fas/APO1, and chemotherapeutic agents such as daunorubicin, has been associated to the generation of ceramide. Ceramide belongs to the group of sphingosine-based lipid second messenger molecules involved in the regulation of diverse cellular responses to exogenous stimuli including apoptosis (Kolesnick & Krönke 1998). Ceramide may result from the catabolic pathway of sphingomyelin in the plasma membrane, being found within seconds or minutes after the stimulus, or result from *de novo* synthesis in the endoplasmic reticulum and mitochondria, a process occurring within hours (Merrill & Jones 1990). The hydrophobic nature of ceramide advanced the hypothesis that this product could be transported by Pgp becoming inaccessible for the deflagration of the apoptotic process. In this case, the lack of specificity of drug resistance and mechanisms not involving drugs could result from the extrusion of a toxic intermediary such as ceramide common to both pathways. Analysis of MDR cells do not support the idea of enhanced ceramide efflux by Pgp in these cells (Come et al. 1999, Lucci et al. 1999), and in agreement with

these results both K562 and the resistant cell line Lucena 1 were equally sensitive to the effect of exogenous C2-ceramide in doses varying from 2mM to 2.5μM (Pinto Silva, F.E. and Meletti-de-Oliveira, M.C. unpublished observations). Nevertheless, despite the lack of increased extrusion, an enhanced conversion of ceramide to glycosylceramide can be observed in MDR cells (Lucci et al. 1999).

ALTERATIONS OF THE CYTOSKELETON

Not only the plasma membrane but also the cytoskeleton may be impaired following UVA and H_2O_2 (Milzani et al. 1997, Rafferty et al. 1997). Despite presenting a high catalase activity, the resistance of Lucena 1 cells to these treatments allied to the fact that the MDR phenotype had been induced by VCR, a drug that blocks tubulin polymerisation (Himes et al. 1976), suggested the possibility that these cells presented adaptations in their cytoskeletons. Many authors have suggested that the acquisition of the MDR phenotype is accompanied by modifications of the cytoskeleton such as increased expression of actin and/or tubulin and alterations on the organisation of stress fibres, microtubules and the Golgi complex (Lee et al. 1993, Lee 1995, Seidel et al. 1995, Erokhina et al. 1994). In agreement with these observations, Lucena 1 cells were shown to express higher levels of actin m-RNA and of the proteic subunits a and b- tubulin, when compared to the parental K562 (Trindade et al. 1998). It is still unclear the role played by the modifications observed in the cytoskeleton on the MDR phenotype.

INDUCERS OF DIFFERENTIATION AND THE MDR PHENOTYPE

Pgp is transiently expressed in bone marrow cells and its expression occurs in less differentiated cells (Uchida et al. 1996). The increased use of differentiation induction therapy for a number of tumours, specially myeloid leukaemic cells, led to the evaluation of the impact of tumour cell differentiation on the MDR phenotype (Okabe-Kado et al. 1986, Okabe-Kado et al. 1991, Patterson et al. 1996). The parental cell line used in the present study K562 cells

as well as Lucena 1 cells are characterised by carrying foetal haemoglobin as defined by benzidine negative staining (Marques-Silva 1996). The use of the differentiating agents retinoic acid and phorbol ester, known to differentiate a number of leukaemic cells (Marques-Silva et al. 1990), were incapable of leading these cells lines, K562 and Lucena 1, to switch to the synthesis of adult haemoglobin whereas the chemotherapic 5-fluorouracil not only produced the haemoglobin switch but induced the extrusion of Rho 123 from K562 cells and augmented the one seen in Lucena 1 cells (Fig. 5) (Marques-Silva 1996). Work is in progress to verify if the increased extrusion observed is due to increased Pgp expression or activity, as a dissociation of these two features has been described during K562 cells differentiation (Marks et al. 1995). Alternatively it could result from the expression and activity of other pumps.

INFLUENCE OF THE MDR PHENOTYPE ON THE ASSESSMENT OF MITOCHONDRIAL MASS AND TRANSMEMBRANE POTENTIAL

Changes in mitochondrial transmembrane potential may regulate a number of cellular processes. The process of differentiation of a murine erythroleukaemia cell line involves mitochondria depolarisation (Levenson et al. 1982) and a pivotal role has been assigned to this organelle in the process of cell death (Zamzami et al. 1995, Kroemer et al. 1998). However, the relationship between the MDR phenotype and mitochondrial environment is still poorly understood. Jia et al. (1999) demonstrated that mitochondrial DNA content (mitochondrial mass) is significantly increased in a resistant cell line compared to its parental cells and proposed that this could be induced by pump expression and requirements. In this work, the authors also suggested an increased ATP generation in the resistant cell line. Many fluorescent dyes have been developed to be used as probes for the investigation of the mitochondrial membrane potential (Rho 123, JC-1, $DiOC_6$ and CMXRos) or mitochondrial mass (Nonyl Acridine Orange or Mitotracker Green). However, all these probes are lipophilic cations what make them
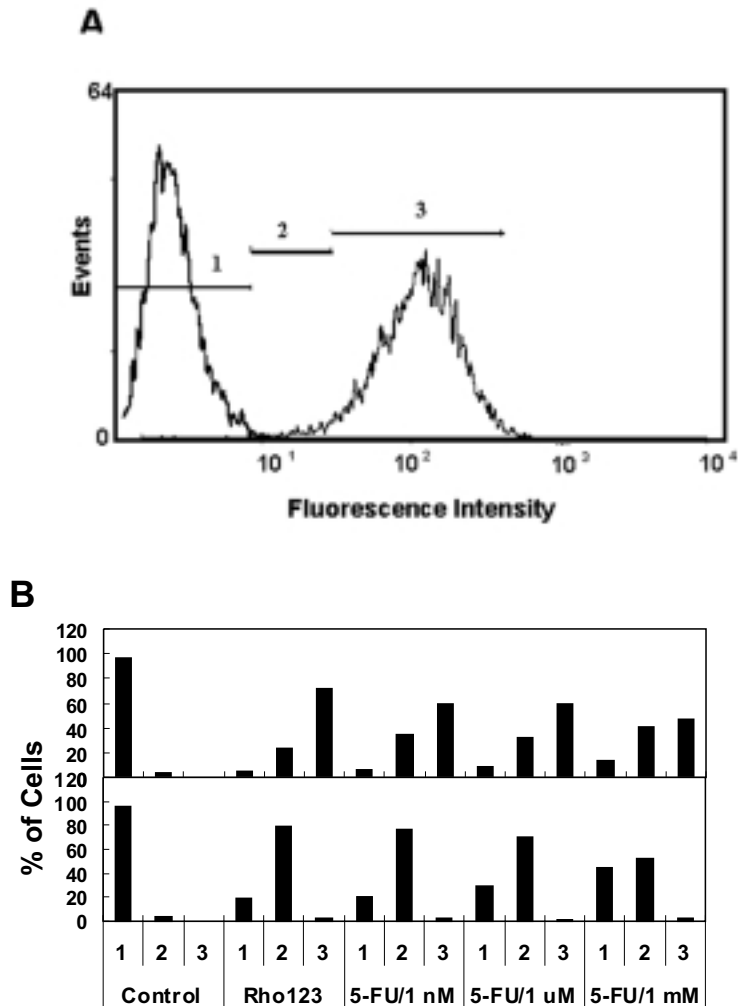
A

B

Fig. 5 – Increased extrusion of Rho 123 following 5-fluorouracil treatment. Cells were cultured with 5-fluorouracil (1nM – 1mM) for 72h. After this period cells were washed and incubated with 200 ng/ml Rho 123 for 30 min at 37°C, washed with cold saline and left to extrude the dye for a further 30 min. The cells were then washed and analysed in a flow cytometer. **A** – Fluorescence profile of K562 cells exposed to Rho 123; region 1 represents autofluorescence of cells not exposed to Rho 123, region 3 represents the region containing the fluorescence peak of these cells exposed to Rho 123 and region 2 corresponds to the area in between. **B** – Upper panel: K562 cells; Lower panel: Lucena 1 cells. Region 1 represents autofluorescence in control cells, region 2 the peak of fluorescence of Lucena 1 cells and region 3 the peak of fluorescence of K562 cells.

potential candidates to be Pgp substrates. Using Lucena 1 cells it was possible to demonstrate that Mitotracker Green, a new probe for the evaluation

of mitochondrial mass, is a P-gp substrate (Fig. 2). This result implies that an especial attention must be given when using fluorescent dyes to evaluate mi-

tochondrial membrane potential and mitochondrial mass not only in MDR tumour cells but also in normal cells such as lymphocytes, thymocytes, renal cells, or any other cell type with a known Pgp activity.

CONCLUDING REMARKS

The development and characterisation of a MDR cell line not only helps in the understanding of the resistance process in tumour cells but also may contribute to the understanding of normal cells carrying the MDR phenotype. Additionally, such a cell line may be used as a tool, functioning as positive controls in systems investigating the MDR phenomenon or in systems testing new chemosensitisers.

ACKNOWLEDGMENTS

RESUMO

A resistência a múltiplos fármacos é o principal obstáculo no tratamento de pacientes com câncer. O mecanismo responsável pela resistência múltipla mais bem caracterizado envolve a expressão do produto do gene MDR-1, a glicoproteína P. Entretanto, o processo de resistência tem fatores múltiplos. Estudos de mecanismos de resistência múltipla a fármacos têm dependido da análise de linhagens celulares tumorais que foram selecionadas e apresentam reatividade cruzada a uma ampla faixa de agentes antitumorais. Este trabalho caracteriza uma linhagem celular com múltipla resistência a fármacos, selecionada originalmente pela resistência ao alcalóide de Vinca vincristina e derivado da linhagem eritro-leucêmica K562. Esta linhagem celular, denominada Lucena 1, super-expressa a glicoproteína P e tem sua resistência revertida pelos quimio-sensibilizantes verapamil, trifluoperazina e ciclosporinas A, D e G. Ademais, demonstramos que o azul de metileno era capaz de reverter parcialmente a resistência nesta linhagem celular. Em contraste, o uso de 5-flúoruracil aumentava a resistência de Lucena 1. Adicionalmente aos quimioterápicos, células Lucena 1 eram resistentes radiação ultra-violeta A e peróxido de hidrogênio e deixavam de mobilizar o cálcio intra-celular quando se usava tapsigargina. Mudanças no cito-esqueleto desta linhagem foram também observadas.

Palavras-chave: resistência múltipla a fármacos, leucemia, glicoproteína P, quimiossensibilizantes.

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