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Circumvention of the multidrug-resistance protein (MRP-1) by an antitumor drug through specific inhibition of gene transcription in breast tumor cells

Sylvia Mansilla [a], *Marta Rojas* [a], *Marc Bataller* [a], *Waldemar Priebe* [b], *José Portugal* [a,*]

[a] *Instituto de Biología Molecular de Barcelona, CSIC, Parc Cientific de Barcelona, Josep Samitier, 1-5, E-08028 Barcelona, Spain*
[b] *The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, USA*

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ABSTRACT

Multidrug-resistance protein 1 (MRP-1) confers resistance to a number of clinically important chemotherapeutic agents. The promoter of the *mrp-1* gene contains an Sp1-binding site, which we targeted using the antitumor bis-anthracycline WP631. When MCF-7/VP breast cancer cells, which overexpress MRP-1 protein, were incubated with WP631 the expression of the multidrug-resistance protein gene decreased. Conversely, doxorubicin did not alter *mrp-1* gene expression. The inhibition of gene expression was followed by a decrease in the activity of the MRP-1 protein. The IC_{75} for WP631 (drug concentration required to inhibit cell growth by 75%) circumvented the drug-efflux pump, without addition of resistant modifiers. After treatment with WP631, MCF-7/VP cells were committed to die after entering mitosis (mitotic catastrophe), while treatment with doxorubicin did not affect cell growth. This is the first report on an antitumor drug molecule inhibiting the *mrp-1* gene directly, rather than being simply a poor substrate for the transporter-mediated efflux. However, both situations appeared to coexist, thereby a superior cytotoxic effect was attained. Ours results suggest that WP631 offers great potential for the clinical treatment of tumors displaying a multidrug-resistance phenotype.

1. Introduction

Multidrug resistance is a major impediment to the successful treatment of cancer. Although the cellular bases underlying drug resistance are not fully understood, several factors that contribute to its development have been identified [1,2]. Tumor cells often gain drug resistance through the overexpression of membrane transport proteins that effectively efflux anticancer drugs [1,3]. Increased transmembrane efflux of antitumor drugs is one of the best-characterized mechanisms of MDR and is mediated through the overexpression of ATP-binding cassette (ABC) transporter superfamily members, for example P-glycoprotein (MDR-1) and the multidrug resistant-associated protein (MRP-1) [1,4,5]. The *mrp-1* gene encodes a transmembrane protein [6], originally isolated from a doxorubicin-selected lung cancer cell line [7], and confers the ability to reduce drug accumulation in cells [1]. MRP-1 mediates resistance to a broad range of anticancer drugs

* *Corresponding author*. Tel.: +34 93 403 4959; fax: +34 93 403 4979.
E-mail address: jpmbmc@ibmb.csic.es (J. Portugal).

Abbreviations: CFSE, carboxyfluorescein diacetate succinimidyl ester; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MDR, multidrug resistance; MRP-1, multidrug-resistance associate protein-1; PCR, polymerase chain reaction; qRT-PCR, quantitative real-time PCR

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[2,3,5,8], and it is expressed in patients suffering invasive breast cancer, offering useful prognostic information [9].

MCF-7/VP breast cancer cells were originally derived from MCF-7 cells after selection with etoposide (VP-16), and they have been shown to overexpress the *mrp-1* gene [10]. MCF-7/VP cells have often been used to evaluate the effects of several drugs on multidrug resistance [10–12]. Parental MCF-7 cells are more susceptible to doxorubicin than to bisanthracycline WP631, which appears to be more cytotoxic in MCF-7/VP cells [13,14].

There is convincing evidence that highly lipophilic molecules are poor substrates for the MRP-1 efflux pump [2,3,11]. Some anthracyclines [3,11], including the novel bisanthracycline WP631 [13], circumvent to some extent the MRP-1 efflux pump in MCF-7/VP cells, thus enhancing the cytotoxic effect at nanomolar concentrations. We sought to gain insight into the mechanisms used by WP631 to exert its cytotoxicity in MRP-1-expressing breast carcinoma cells. We, along with others, have documented that WP631 is a potent inhibitor of basal and Sp1-activated transcription [15–19]. The *mrp-1* gene promoter contains an Sp1-binding site, which is involved in the regulation of its transcription [20]. Therefore, we aimed to determine whether WP631 directly inhibits the expression of *mrp-1*. Bisanthracycline WP631-binding to DNA would thus reduce the MRP-1 mRNA produced by the cells and thereby decrease, in turn, the MRP-1 efflux pump.

2. Materials and methods

2.1. Doxorubicin and WP631

WP631 was synthesized as described elsewhere [13]. Solutions containing 500 μM doxorubicin (Sigma, St. Louis, MO) or WP631 were prepared with sterile 150 mM NaCl, maintained at −20 °C, and brought to the final concentration with RPMI 1640 medium (Gibco, Life Technologies, Prat de Llobregat, Spain) just before use.

2.2. Cell line, culture conditions and cell cycle distribution

MCF-7/VP breast tumor cells were a gift of Dr. K.H. Cowan (University of Nebraska Medical Center). Cells were maintained in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (Gibco), 2 mM sodium pyruvate (Gibco) and 2 mM HEPES (pH 7.4), at 37 °C in a humidified atmosphere with 5% CO_2.

Exponentially growing cells subcultured at a density of 5×10^4 cells/ml were incubated with various concentrations of doxorubicin or WP631 at 37 °C for times ranging from 24 to 96 h.

After treatment with doxorubicin or WP631 for various periods of time, the cells were routinely harvested and stained with propidium iodide (Sigma) as described elsewhere [21]. Nuclei were analyzed with a Coulter Epics-XL flow cytometer (Coulter Corporation, Hialeah, FL) using the 488 nm line of an argon laser and standard optical emission filters. The percentages of cells at each phase of the cell cycle were estimated from their DNA content histograms after drug treatment.

2.3. Assessment of multidrug-resistance gene expression

Total RNA was isolated from control cells (those to which no drug was added) and cells treated with either the IC_{50} for doxorubicin, the IC_{50} or the IC_{75} for WP631 using the Ultraspec RNA isolation reagent (Biotecx, Houston, TX) following the vendor's guidelines. RNA samples were treated with RNAse-free DNAse I (Roche Diagnostics, Mannheim, Germany) and phenol-extracted. The occurrence of transcripts of both *mdr-1* and *mrp-1* genes in MCF-7/VP cells was assayed by semi-quantitative RT-PCR, together with the housekeeping *GAPDH* gene as an internal normalization standard, in the absence of any drug, using the primers indicated in the legend to Fig. 1. PCR samples were electrophoresed in a 2% agarose gel and stained with ethidium bromide.

QRT-PCR was used to analyze changes in *mrp-1* gene expression produced by 122 nM (IC_{50}) WP631, 307 nM (IC_{50}) doxorubicin or 594 nM (IC_{75}) WP631, after 24 and 96 h treatments [14]. The housekeeping *GAPDH* gene was used as an internal normalization standard. The rather high concentration of doxorubicin required to inhibit cell proliferation by 75% (over 3 μM) was not used in our experiments because it represents a supraclinical concentration [14]. Total RNA (70 ng, determined spectrophotometrically) was copied to cDNA using the Omniscript RT kit (Qiagen, Servicios Hospitalarios, Barcelona, Spain). The appearance of amplification bands in real time was monitored by the SYBR Green method by using the SYBR green PCR Master Mix (Applied Biosystems, Worrington, UK) in an Abi-Prism 7000 Sequence Detection System (Applied Biosystems, Tres Cantos, Spain), using the primers indicated in the legend to Table 1. QRT-PCR was run for 40 cycles with an annealing temperature of 60 °C. Amplification of the housekeeping *GAPDH* gene was performed to allow normalization among samples. The relative amounts of cDNA present in the sample were calculated from

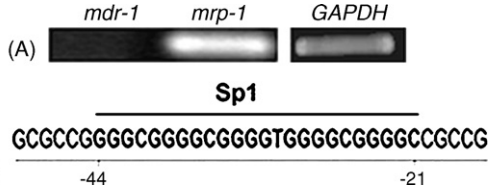

Fig. 1 – Expression of multidrug-resistance genes in the MCF-7/VP breast cancer cells. (A) RT-PCR analysis of the expression of *mdr-1* and *mrp-1* genes in MCF-7/VP cells. The specific primers used for RT-PCR amplification were: MDRdir, 5′-CCCATCATTGCAATAGCAGG-3′; MDRrev, 5′-GTTCAAACTTCTGCTCCTGA-3′; MRPdir, ACGGTCGGGGAGATTGTCAAC; MRPrev, GCCCAGATTCAGCCACAGGAG-3′. The housekeeping *GAPDH* gene was co-amplified as internal control using the primers: GAPDHdir, 5′-TCAGCCGCATTCTTCTTTTG-3′ and GAPDHrev, 5′-TGATGGCATGGACTGTGGT-3′. There was no *mdr-1* expression in MCF-7/VP cells. (B) Partial sequence of the proximal promoter of the human *mrp-1* gene (adapted from [20]; Ensembl gene ENSG00000103222) in which the presence of a multiple Sp1-binding site is highlighted. This CG-rich sequence represents potential binding sites for bisanthracycline WP631 [15,17].

Table 1 – Changes in the expression of the *mrp*-1 gene (RNA levels) in MCF-7/VP breast cancer cells induced by treatment with the IC_{50} for doxorubicin or WP631 and the IC_{75} for WP631, measured by qRT-PCR[a]

	Control	IC_{50} doxorubicin		IC_{50} WP631		IC_{75} WP631	
	Untreated	+Doxorubicin	% Inhibition[c]	+WP631	% Inhibition	+WP631	% Inhibition
24 h	225 ± 14[b]	225 ± 23[b]	0	138 ± 29	38.7	121 ± 9	46.2
96 h	443 ± 56	–	n.d.	199 ± 27	55.1	133 ± 16	69.9

[a] Primers used in the experiments: MRP1mfor, 5′-GCGCTGGCTTCCAACTATTG-3′; MRP1mrev, 5′-AGGGCTCCATAGACGCTCAG-3′; GAPDHmfor, 5′-TGGGCTACACTGAGCACCAG-3′; GAPDHmrev, 5′-GGGTGTCGCTGTTGAAGTCA-3′.
[b] Number of copies of the MRP-1 mRNA per 10 000 copies of the housekeeping GAPDH mRNA. Values represent the means ± S.D. of three replicates.
[c] Inhibition was measured from the changes in gene expression: % inhibition = 100 − [(+drug/untreated) × 100].

the CT values (the number of cycles required for each pair of primers to reach fluorescence above the threshold level), corrected for the housekeeping *GAPDH* expression, according to the equation:

$$\frac{\text{smRNA}_{(\text{mrp-1})}}{\text{mRNA}_{(\text{GAPDH})}} = 10000 \times 2^{\text{CT}_{(\text{GAPDH})} - \text{CT}_{(\text{mrp-1})}}$$

where $\text{mRNA}_{(\text{mrp-1})}$ corresponds to the expression of *mrp*-1, determined in presence and in absence of WP631 or doxorubicin after both 24 and 96 h. Results are given as copies of MRP-1 mRNA per 10 000 copies of GAPDH mRNA, and the ratio between drug-treated and untreated cells was used to calculate the percentage of *mrp*-1 gene inhibition after treatment with doxorubicin or WP631.

2.4. Western blot analysis

Protein was extracted from WP631-treated and control MCF-7/VP cells after 24, 48 and 98 h, using a lysis buffer consisting of 50 mM Tris–HCl (pH 8), 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40 and 0.1 mM phenylmethylsulphonyl fluoride, containing 2 μl/ml aprotinin and 1 μg/ml leupeptin. Total protein was quantified by the Bradford assay (Bio-Rad, Hercules, CA). About 30 μg of denatured proteins was subjected to electrophoresis on 8% SDS-polyacrylamide gels, blotted onto Optitran BA-S85 membranes (Schleicher & Schuell, Dassel, Germany), probed with monoclonal anti-MRP-1 (Sigma) and anti-β-actin (Sigma) antibodies, detected by chemiluminescence using Luminol (Sigma) and quantified using GeneTools 3.0 software (SynGene, Cambridge, USA).

2.5. Measurement of drug transport activity mediated by MRP-1 protein

Drug transport activities of the multidrug-resistance protein MRP-1 was assayed using the MultiDrugQuant Assay kit (Chemicon International, Pacisa-Giralt, Barcelona, Spain), which is based on the measurement of the intracellular fluorescence of a free acid derivative of the calcein dye, using the green channel in a Coulter Epics-XL flow cytometer. Calcein was added to the cell culture as a non-fluorescent compound that is hydrolyzed by endogenous esterases rapidly. Changes in the intracellular green fluorescence of calcein were measured in proliferating, propidium iodide-negative (living) cells, using the equipment sets described in the kit instructions. A selective inhibitor, provided within the kit, as 'inhibitor 2', allowed us to measure MRP-1 multidrug activity. Measurements were made on cells treated with the IC_{50} or IC_{75} for WP631 or the IC_{50} for doxorubicin after 24 and 96 h of continuous treatment with the drug [14], and compared with untreated cells. MRP-1-related activity and its inhibition were calculated following the manufacturer's instructions.

2.6. Quantification of intracellular drug accumulation

Accumulation of doxorubicin or WP631 in MCF-7/VP cells was quantified spectrofluorimetrically as described elsewhere [22], both the drug accumulated inside the cell and in the membranes were determined together [23]. In brief, cells were incubated with various concentrations of either doxorubicin or WP631 for the times indicated in the legend to Fig. 3. They were then rinsed three times with ice-cold RPMI 1640 medium, and the drugs were extracted from the cells using 2 ml of 80 mM HCl in 2-propanol for 16 h at 4 °C. The concentrations of the two drugs were measured using a Shimadzu RF-1501 spectrofluorophotometer (Shimadzu, Columbia, MD) with an excitation wavelength of 480 nm and an emission wavelength of 555 nm. The fluorescence intensity emitted was translated into concentrations of drug using a doxorubicin or WP631 standard curve, and expressed as ng drug/10^7 viable cells, assessed before and after treatment by exclusion of trypan blue dye.

2.7. Proliferation assays

After 96-h continuous treatment with either the IC_{50} for doxorubicin or the IC_{50} for WP631, MCF-7/VP cells were allowed to grow in drug-free fresh medium for 16 days. Cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) and plated at 10^6 cells to analyze cell division and cell cycle distribution. For comparison, treated cells were also labeled with CFSE. After withdrawal of the drug, treated cells were allowed to recover in drug-free medium for up to 3 days, and cytoplasmatic fluorescence was quantified using a Coulter Epics-XL flow cytometer. Covalently bound CFSE is evenly distributed into daughter cells, allowing the discrimination of successive rounds of division [24].

3. Results

Preliminary studies showed that WP631 is much more cytotoxic against the breast tumor cell line MCF-7/VP than

doxorubicin, while doxorubicin is more cytotoxic against the parental MCF-7 cells. This indicates that WP631 can circumvent multidrug resistance in MCF-7/VP cells [13,14].

3.1. *Inhibition of mrp-1 gene expression by WP631 in MCF-7/VP cells*

Semi-quantitative RT-PCR analysis of the expression of *mdr-1* and *mrp-1* genes in exponentially growing MCF-7/VP breast cancer cells showed that these cells expressed *mrp-1*, but not the *mdr-1* gene (Fig. 1A), which is consistent with previous reports of scarce MDR-1 activity in MCF-7/VP cells [10]. Fig. 1B shows a partial sequence of the *mrp-1* promoter, which contains a multiple Sp1-binding site [20]. This CG-rich sequence may be targeted by WP631, and thus the drug would compete with Sp1 protein, since WP631 is a potent inhibitor of Sp1-transactivated gene expression [15,17].

Quantitative RT-PCR (qRT-PCR) was used to quantify the inhibition of *mrp-1* gene transcription by WP631. MCF-7/VP cells were incubated with the IC_{50} doses for doxorubicin (307 nM) or WP631 (122 nM), and the IC_{75} dose (594 nM) for WP631 [14]. The supraclinical IC_{75} for doxorubicin (over 3 μM [14]) was not used. *mrp-1* gene expression was inhibited by about 45% after 24-h treatment at the IC_{75} dose of WP631, and

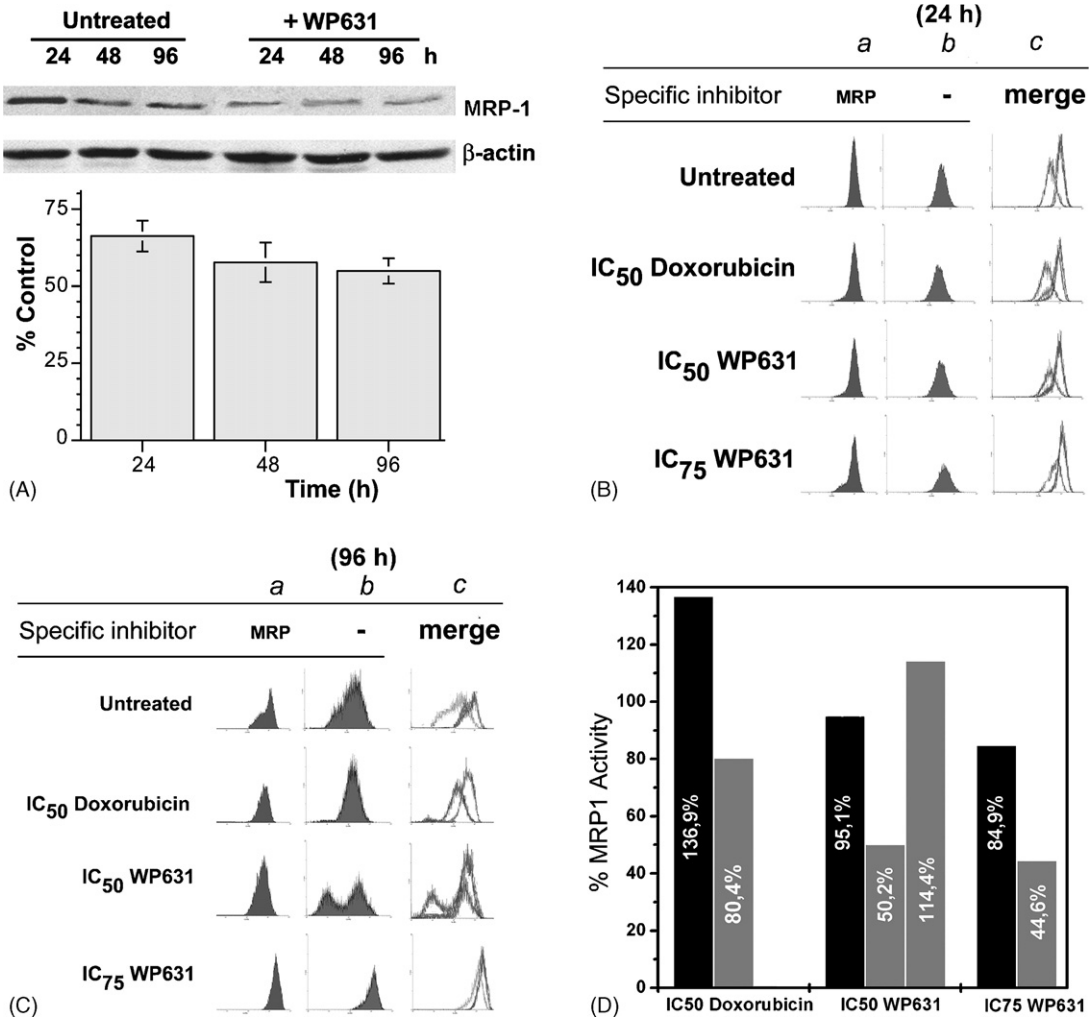

Fig. 2 – MRP-1 protein levels and multidrug-resistance activities. (A) A representative Western blot analysis of the MRP-1 protein levels in MCF-7/VP cells after treatment with 594 nM (IC_{75}) WP631, for the times indicated in the figure, and in untreated (control) cells. In treated cells, the protein levels were reduced as shown in the lower panel (means ± S.D. for three experiments). (B) Flow cytometry analysis of the fluorescence of intracellular calcein after 24-h treatment with doxorubicin or WP631, as indicated to the left margin. Histograms show intracellular green fluorescence, in a logarithmic scale, *vs.* counts (number of cells). Column *a* corresponds to experiments performed in the presence of 'inhibitor 2' (Chemicon International), which is known to inhibit the MRP-1-mediated resistance pump efflux (see main text for details). Column *b* corresponds to experiments performed in the presence of WP631 or doxorubicin alone. Column *c* shows a merge of the cytometric profiles in panels (A) and (B) allowing us to determine whether doxorubicin and WP631 influence intracellular calcein accumulation. (C) Flow cytometry analysis of the fluorescence of intracellular calcein after 96-h treatment with doxorubicin or WP631, as indicated to the left margin; other details as for panel (B). (D) Quantification of relative MRP-1 activity, obtained from the cytometric profiles shown in (B) and (C), after treatments for 24 h (black bars) or 96 h (gray bars).

by about 70% after 96 h of treatment (Table 1). In contrast, the IC_{50} produced less than 40% down-regulation after 24 h, and about 55% after 96 h. Doxorubicin had no effect on *mrp-1* expression (Table 1).

3.2. Analysis of the MRP-1 protein level, and the efflux pump activity, after treatment of MCF-7/VP cells with WP631

We sought to typify the effects of WP631 on the MRP-1 protein levels following the decline in gene expression, described above, and on the multidrug-efflux pump (i.e. the resistance activity within MCF-7/VP cells and the intracellular accumulation of the drug). The effects of WP631 on transcription resulted in a decrease in the MRP-1 protein levels (Fig. 2A), and in the activity of the multidrug-resistance-associated MRP-1 protein, which may explain the capacity of WP631 to overtake the pump efflux effect. We took advantage of the fluorescence of added-intracellular calcein – produced by living cells from a non-fluorescent precursor, see Section 2 – to determine, by flow cytometry, the accumulation of this molecule in the presence of an inhibitor of multidrug resistance and of doxorubicin or WP631. Fig. 2 (panels B–D) shows the effects of a specific MRP-1 inhibitor provided within the kit (which is sold as 'inhibitor 2', see Section 2) on the pump efflux by MRP-1. The experiments were undertaken in the absence/presence of doxorubicin or WP631. After 24-h treatment, with the IC_{50} for both drugs, changes in the effect on intracellular fluorescence (calcein content) were negligible (column *b* in Fig. 2B). All cytometric fluorescence profiles analyzed are merged in column *c*. In the presence of doxorubicin, the MRP-1 protein activity was slightly enhanced, while with the IC_{75} for WP631 it decreased by about 15% (Fig. 2B and D). Fig. 2C shows the effects of doxorubicin and WP631 on intracellular calcein fluorescence after 96-h treatment. While doxorubicin slightly affected the transporter-mediated efflux, the two concentrations of WP631 reduced MRP-1 activity differently (column *b* in Fig. 2C). Treatment with the IC_{50} for WP631 split the cell population into two parts, some cells showing little pump efflux and others showing MRP-1-mediated efflux (column *c* in Fig. 2C and D). The presence of a sub-population of cells resistant to WP631 after treatment at the IC_{50}, but not at the IC_{75}, would support this interpretation (see below). It is remarkable that the IC_{75} for WP631 reduced MRP-1 activity by more than 50% after 96 h of treatment, as depicted by the merging of fluorescence profiles around a single maximum in column *c* of Fig. 2C. These results agree with the approx. 45% *mrp-1* inhibition after 24 h, see above, but they fall short of the 70% gene inhibition observed at 96 h (Table 1). Given that changes in transcription are translated into a reduction in active MRP-1 protein, it is feasible that, as observed, the inhibition of the efflux pump was somewhat delayed. All the effects produced by WP631 were unequivocally ascribed to MRP-1, since the efflux inhibition caused by WP631 and 'inhibitor 2' together, and that caused by WP631 alone merged in the same cytometric maximum of intracellular calcein fluorescence.

3.3. Accumulation of WP631 and doxorubicin inside MCF-7/VP breast cancer cells

It is noteworthy that the experiments shown in Fig. 2B and C measured the intracellular calcein content rather than the uptake and/or release of WP631. Therefore, the lack of changes in calcein content after treatment with WP631 may also be due to direct saturation by WP631 of the MRP-1 transporter-mediated efflux that might eject both molecules. As a consequence, the levels of calcein would remain almost unaltered because not enough molecules of transporter were available. However, this does not appear to be the case because the transporter-mediated efflux prevented the accumulation of doxorubicin, while there was enough MRP-1 protein to eject some of both the intracellular calcein and doxorubicin (Fig. 2). We previously demonstrated, using Confocal laser microscopy, that WP631 accumulates in the nuclei of susceptible cells [22].

To confirm that the effect on multidrug resistance was mediated by the presence of WP631 inside the cell, we directly measured WP631 accumulation in MCF-7/VP cells (Fig. 3). The time-dependent uptake of doxorubicin and WP631 by MCF-7/VP cells (Fig. 3) was correlated with time-dependent changes in MRP-1 activity (Fig. 2D). It was evident from the very beginning of the incubation with WP631 that this drug circumvented the MRP-1 pump efflux. WP631 accumulation inside cells was equivalent after 48 h, in spite of the drug concentration in the culture medium (Fig. 3). The IC_{75} for WP631 enhanced uptake, which reached a maximum after 96-h treatment, consistent with the inhibition of MRP-1. After about 48-h treatment at the IC_{50}, the drug was progressively ejected from the cells (Fig. 3), and therefore the cells accumulated about 10% of WP631 after 96 h compared to 100% in cell treated at the IC_{75}. This would represent an underestimation of the uptake of some cells in which *mrp-1* expression was partially inhibited. This behavior can be

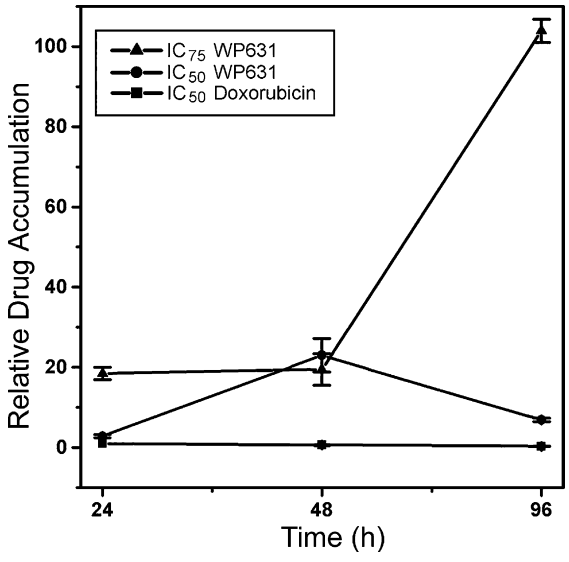

Fig. 3 – Quantitative determination of the uptake of WP631 and doxorubicin in MCF-7/VP cells. Cells were continuously treated with either 307 nM (IC_{50}) doxorubicin, 122 nM (IC_{50}) WP631 or 594 nM (IC_{75}) WP631 for 24, 48 and 96 h. Accumulation was calculated as ng drug/10^7 viable cells. For the easy of plotting, data are displayed as relative uptake values (means ± S.D. for three independent experiments) using the uptake of IC_{75} WP631 as 100%.

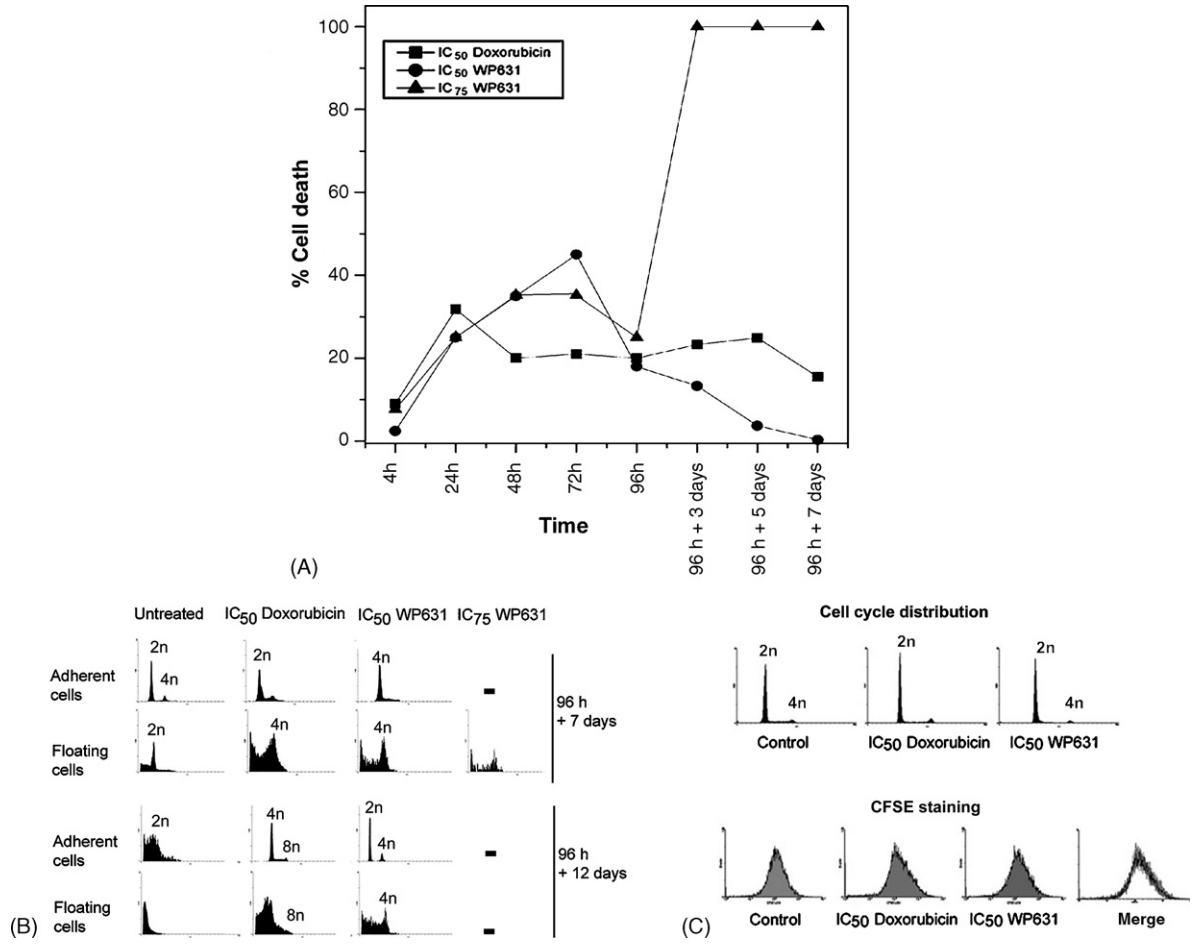

Fig. 4 – Cell death and proliferation in drug-treated MCF-7/VP breast carcinoma cells. (A) Plot showing the percentage of cell death, as determined by trypan blue staining of MCF7/VP cells treated with the IC_{50} for doxorubicin, or the IC_{50} or IC_{75} for WP631 (average value of two independent experiments). Cells, which had been treated for 96 h, were maintained, after withdrawal of the drugs, in fresh drug-free medium for the times indicated in the x-axis. Only the IC_{75} for WP631 produced time-dependent 100% cell death. (B) Cytometric comparison of the cell cycle traverse of cells treated with doxorubicin or WP631 for 264 h (96 h of continuous treatment followed by 7 days in fresh drug-free medium) or for 384 h (96 h of continuous treatment followed by 16 days in fresh drug-free medium). Flow cytometry profiles for both adherent and floating cells are displayed. With the IC_{75} dose of WP631, all the dying cells floated in the cell culture, while with the other treatments some 'resistant cells' remained attached. Black-large bars indicate the absence of adherent cells under the depicted experimental condition (C) Analysis of proliferating and growth-arrested MCF-7/VP cells. The upper panel shows flow cytometry profiles of cells treated with the IC_{50} for either doxorubicin or WP631. These profiles were obtained 16 days after incubating previously drug-treated cells (for 96 h) in free-drug medium, which a change to new fresh medium every 2–3 days. Adherent cells, which survived to drug treatment, showed a synchronized profile with cells predominantly in G1 phase (upper panels). Adherent cells incubated with CFSE (Molecular Probes) after drug treatments were allowed to grow in fresh medium. Proliferating cells divided the cytoplasmatic fluorescence evenly (lower panel). The profiles shown in this panel (fluorescence *vs.* counts (number of cells)) demonstrate that cells treated with moderate doses of doxorubicin or WP631 proliferated normally in fresh drug-free medium, since the CFSE fluorescence inside cells merged with control, untreated, cells.

accounted for by postulating that, at the IC_{50}, WP631 accumulated in some cells (roughly 50% of the cell population), while it was ejected by the others, in agreement with the rise of two populations with different pump efflux capacities (Fig. 2C). This experimental observation agreed with that 'sufficient' intracellular drug accumulation is required to inhibit Sp1-driven transcription of *mrp-1* (cf. Table 1 and Figs. 2 and 3). MCF-7/VP cells accumulated rather low quantities of doxorubicin, owing to the MRP-1-mediated efflux, regardless of the incubation time (Fig. 3).

3.4. *A comparison of the effects of different doses of WP631 on cell proliferation and death*

Given that the IC_{75} for WP631 inhibited *mrp-1* gene transcription, which resulted in lower MRP-1 protein levels, and

circumvented the resistant phenotype (Table 1 and Fig. 2), we explored whether MCF-7/VP cells underwent rapid cell death after the drug had accumulated in sufficient amounts. The IC_{50} and IC_{75} for WP631, and the IC_{50} for doxorubicin, used in our experiments correspond to a measurement of cell proliferation based in the MTT dye assay [14], but this assay fails to distinguish between growth arrest and a reduction in cell number due to cell death. Nevertheless, trypan blue exclusion measurements together with flow cytometry analyses revealed that the whole cell population treated with the high concentration of WP631 was committed to die following the withdrawal of the drug after 96-h treatment (Fig. 4A and B). The time-dependent cytotoxic effects correlated with the changes in drug accumulation described above, which suggests that a more direct circumvention of the MRP-1 pump efflux, other than the effect on *mrp-1* transcription, was also taking place. Fig. 4B shows the flow cytometry analysis of both adherent and floating cells after longer periods of incubation in fresh, drug-free medium. The IC_{50} of both WP631 and doxorubicin committed floating cells to die predominantly from G2, as deemed under the microscope after staining with trypan blue. In the intervening time the behavior of adherent cells was similar to control, suggesting that MCF-7/VP cells were split into two populations (essentially, sensitive and resistant cells) by the IC_{50} for WP631 (Fig. 4B).

We labeled MCF-7/VP cells with the fluorescent compound CFSE (see Section 2) to corroborate whether the cells that remained attached for 7 or 12 days after withdrawal of the IC_{50} of any drug, were still proliferating in fresh drug-free medium. CFSE incorporated stably into the cell cytoplasm, and it was evenly divided between daughter cells upon division. Therefore, cell division was easily followed cytometrically by the changes in fluorescence (Fig. 4C). Unlike in MCF-7/VP cells treated with the IC_{75} for WP631, which have been observed to die after entering mitotic catastrophe [14], the analysis of cell division in the attached cells, using CFSE, showed that cells treated with the IC_{50} for either drug were proliferating. Adherent cells predominantly in G1 phase underwent several divisions as shown by the decreasing amount of CFSE fluorescence, which made control, untreated cells, and those treated merge at the same fluorescence maximum (lower panel in Fig. 4C). These cells resistant to doxorubicin, and those selected by the low dose treatment with WP631, showed almost 100% clonogenic capacity when they were allowed to recover in fresh drug-free medium (not shown).

4. Discussion

In this paper, we show that the antitumor drug WP631 inhibits the expression of the *mrp-1* gene. The ability of WP631 to circumvent MRP-1 resistance *via* direct inhibition of its gene transcription represents the first case, to our knowledge, in which an antitumor drug reaches modulation of the MRP-1 phenotype through direct gene inhibition, rather than because the drug is a poor substrate for the transporter-mediated efflux, and without requiring the presence of resistant modifiers. MRP-1 inhibition appears to be dose-dependent, reaching more than 50% when MCF-7/VP breast cancer cells were treated with 594 nM (IC_{75}) WP631 (Table 1). The ability of WP631 to both inhibit gene transcription and circumvent multidrug resistance is substantiated by a concomitant decline in both the MRP-1 protein levels and the efflux pump activity (Fig. 2). Therefore, cells treated with the IC_{75} dose for WP631 were committed to die within a few days following the withdrawal of the drug (Fig. 4A), though MCF-7/VP breast cancer cells do not express caspase-3 [25]. WP631-treated cells undergo mitotic catastrophe, as we have described in detail elsewhere [14]. The results obtained with doxorubicin (Figs. 2–4) were in line with that cells surviving to an insult might sometimes undergo somatic reduction, to return to the diploid state and release mitotic descendents [26]. Some cells treated with the IC_{50} for WP631 or doxorubicin proliferated after treatment, when they were incubated in fresh drug-free medium, and showed clonogenic capacity for more than 30 days (not shown). The re-incorporation of some surviving cells into the cell cycle, after treatment with moderate (IC_{50}) doses, may contribute to carcinogenesis, as cells still proliferated (Fig. 4B and C). Since cells that survived to the insult may harbor repair capacity, our results emphasize the need to reach sufficient intracellular drug accumulation in order to obtain the desired therapeutic effect, even for a potent cytotoxic drug like WP631.

WP631 accumulates in MCF-7/VP cells and circumvents the multidrug-resistance efflux pump, acting as a potent cytotoxic, without the requirement of additional molecules to modify multidrug resistance. This finding may be of potential clinical interest in the search for alternative treatment of carcinomas-expressing MRP-1, since clinical trials conducted to evaluate the efficacy of inhibitors of multidrug resistance in tumors have often shown that the serum levels required to reverse or block the antitumor drug-efflux may be hard to achieve [3]. Only a small number of compounds have been shown to reverse multidrug resistance mediated by MRP-1 [8,27]. There is evidence that lipophilic molecules are poor substrates for both MRP-1 and MDR-1 [11,28] owing to the preferential partition into lipidic structures, thus avoiding drug interaction with the efflux pump [3,11]. This chemical property has been exploited in the design of improved anthracyclines. WP631 is a bisanthracycline that bears two chromophores [13,29], and it is known to bind to DNA with high affinity, which is largely driven by hydrophobic interactions [29]. Therefore, part of the WP631 cytotoxicity against cells-expressing MRP-1 may arise from partial circumvention of the MRP-1 efflux pump given its lipophilic structure, which would make it a much poorer substrate for the MRP-1 protein, thus explaining its lower resistance compared to doxorubicin. Indeed, both IC_{50} and IC_{75} doses produced comparable accumulation of WP631 after 48 h, and MCF-7/VP cells were similarly committed to die by both concentrations (Figs. 3 and 4A). Since WP631 acts as a potent inhibitor of Sp1-activated transcription [15,17,19], we cannot ignore the chance that other genes, related with diverse aspects of drug resistance [30], may be also altered by the presence of WP631 (since some of them present putative Sp1-binding sites in their promoters), thus contributing to the overwhelming of drug resistance observed with this anthracycline.

In summary, WP631 circumvents MRP-1 through its ability to avoid the efflux pump, but the direct effect of WP631 on

gene transcription produces its superior cytotoxicity. In agreement with our findings on the importance of inhibiting *mrp-1* gene transcription, it has been reported that the expression of MRP-1 in glioma cells can be reduced by an antisense oligonucleotide, thus increasing the sensitivity of tumor cells to chemotherapy [31]. Since both MRP and MDR proteins are the product of genes regulated by Sp1 [20,32], and Sp1-binding to DNA is strongly inhibited by WP631 (Fig. 1 and [15,17]), we foresee WP631 as a drug with potential clinical interest in the treatment of certain malignancies displaying multidrug-resistance phenotypes.

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