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The antiprogestatin drug RU 486 potentiates doxorubicin cytotoxicity in multidrug resistant cells through inhibition of P-glycoprotein function

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Abstract The antiprogestatin drug RU 486 was examined for its effect on doxorubicin cellular retention and cytotoxicity in multidrug resistant cells overexpressing P-glycoprotein (P-gp). RU 486 was shown to strongly enhance intracellular accumulation of doxorubicin in both rat hepatoma RHC1 and human leukemia K562 R7 drug-resistant cells but had no action in SDVI drug-sensitive liver cells. The antiprogestatin drug when used at 10 μM, a concentration close to plasma concentrations achievable in humans, was able to hugely increase the sensitivity of RHC1 cells to doxorubicin. RU 486 appeared to prevent the P-gp-mediated doxorubicin efflux out of RHC1 cells and was demonstrated to interfere directly with P-gp drug binding sites since it blocked P-gp labelling by the photoactivable P-gp ligand azidopine. These results thus demonstrate that RU 486 can downmodulate anticancer drug resistance through inhibition of P-gp function.

Key words: Multidrug-resistance; P-glycoprotein; Reversion; Tumor cell; RU 486; Doxorubicin

1. Introduction

Resistance to chemotherapy is a major cause of failure in the treatment of many human cancers. A major mechanism involved in this drug resistance is linked to the overexpression of a plasma membrane glycoprotein termed P-glycoprotein (P-gp) [1–3]. P-gp, encoded by mdr genes, is thought to act as an energy-dependent drug efflux pump with broad specificity; it transports various structurally and functionally unrelated antitumor compounds such as doxorubicin and *Vinca* alkaloids out of the cell, thus conferring a multidrug resistance phenotype (MDR).

A wide range of drugs termed chemosensitizers or modulator agents including verapamil, cyclosporin and quinidine, have been used to block P-gp function and thus to restore sensitivity to cytotoxic drugs [4]. Some of these reversing compounds have been demonstrated to inhibit the labelling of P-gp by MDR-related drugs, thereby suggesting that they act through competition for drug binding sites on P-gp [5]. Some endogenous compounds such as steroid hormones also interact with P-gp [6]. Corticosteroids and mineralocorticoids are substrates for P-gp transport [7] while progesterone can directly label human P-gp [8]. Some steroid antagonists also interfere with P-gp function. Indeed, P-gp-mediated drug resistance has been demonstrated to be overcome by antiestrogen compounds such as tamoxifen and toremifen [9], which display structural features usually described in modulator agents, particularly hydrophobicity and presence of phenyl rings [10]. Since these characteristics are also shared by RU 486, an antihormone compound with antiprogestatin property [11], we have questioned as to whether RU 486 could modulate anticancer drug accumulation and activity in P-gp overexpressing cells.

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Abbreviations: MDR, multidrug resistance; P-gp, P-glycoprotein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

2. Materials and methods

2.1. Chemicals

Doxorubicin was obtained from Roger Bellon laboratories (Neuilly, France), [^{3}H]vinblastine and [^{3}H]azidopine were purchased from Amersham (Buck, UK). RU 486 was a gift from Roussel-Uclaf (Romainville, France). Verapamil and progesterone were supplied by Biosedra laboratories (Levallois-Perret, France) and Sigma Chemicals Co. (St. Louis, MO, USA), respectively.

2.2. Cell culture

RHC1 rat hepatoma cells were obtained from a chemically induced liver tumor [12] and the normal rat liver epithelial cell SDVI was established in our laboratory from the liver of 10-day-old Sprague–Dawley rats according to the procedure of Williams et al. [13]. RHC1 and SDVI cells were grown in Williams' medium supplemented with 10% fetal calf serum. RHC1 cells displayed a constitutive overexpression of functional P-gp [12] while SDVI cells had not detectable P-gp; they were used as drug-resistant and drug-sensitive cells, respectively.

Multidrug-resistant human leukemia cells K562 R7 (kindly provided by Dr J.P. Marie, Hôtel Dieu, Paris) were cultured in RPMI medium supplemented with 10% fetal calf serum.

Normal rat hepatocytes were prepared by the two-step collagenase perfusion method and then maintained in conventional primary culture in a medium containing 75% minimal essential medium and 25% medium 199 [14]. Hepatocytes were used after two days of culture, at which time they displayed high levels of P-gp [15].

2.3. Intracellular doxorubicin accumulation

The intracellular concentration of doxorubicin was estimated by a fluorimetric method as described by Schott et al. [16]. Cells were exposed to doxorubicin (10 μg/ml) for 2 h with or without reversing agents and then washed three times with ice-cold phosphate buffer saline, harvested and ultrasonicated. These steps were performed quickly in order to avoid any drug efflux. Cellular proteins were precipitated with 20% trichloroacetic acid. The acid-soluble fraction was used to evaluate the intracellular concentration of doxorubicin by fluorimetry, using excitation and emission wavelengths of 485 nm and 590 nm, respectively. Preliminary studies showed that doxorubicin, verapamil and RU 486 were not cytotoxic at the concentrations used over the incubation period. An aliquot of cell lysate was used in parallel to determine cellular protein content by the Bio-Rad protein assay [17].

2.4. Evaluation of doxorubicin efflux

Cells were incubated with doxorubicin (10 μg/ml) for 2 h, washed three times with ice-cold phosphate buffer and reincubated in drug-free medium for 30 min or 60 min in the absence or presence of either

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RU 486 or verapamil (10 μM). The intracellular doxorubicin concentration was further determined as described above. Intracellular drug retention was expressed as a percentage of the initial intracellular drug accumulation.

2.5. Drug-sensitivity assay

The effect of doxorubicin on cell proliferation was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye assay [18]. Briefly, RHC1 and SDVI cells were seeded at 60,000 cells/ml in 96-well microplates and cultured with various concentrations of doxorubicin in the absence or presence of RU 486 or verapamil (10 μM). After 96 h of incubation, cells were incubated with 0.05% MTT (Sigma) for 2 h at 37°C and the blue formozan product formed was further quantified by its absorbance at 540 nm using a Titertek Multiscan MCC/340. Growth inhibition was evaluated as IC_{50}, i.e. the drug concentration providing a 50% reduction of cell number as compared to controls cultured in parallel without RU 486 or verapamil.

2.6. Photoaffinity radiolabeling of P-gp with [^{3}H]azidopine

Photoaffinity labelling of P-gp with [^{3}H]azidopine was performed as previously reported [19]. Briefly, P-gp containing membrane fractions were prepared from two days cultured rat hepatocytes by differential centrifugation as described by Germann et al. [20]. Fifty μg of membranes protein per assay were incubated in 40 mM phosphate potassium buffer, 10 μM $CaCl_2$, 4% dimethylsulfoxide with 5 μCi [^{3}H]azidopine in a final volume of 50 μl. The mixture was preincubated 1 h at 25°C in the dark in the absence or presence of nonradioactive competing ligand and then irradiated on ice for 10 min with a UV lamp at 254 nm (200,000 μJ/cm^2) at a distance of 10 cm. Membrane proteins were then separated by molecular weight following electrophoresis on a SDS-polyacrylamide (7%) gel. The gel was fixed in 25% ethanol/10% acetic acid, treated with the fluorographic reagent Amplify (Amersham) for 30 min, dried, and then exposed to X-ray film at −80°C.

For Western blot identification of P-gp, 50 μg crude membrane proteins were separated on SDS-polyacrylamide (7%) gel and transferred onto a nitrocellulose sheet. After incubation with C219 monoclonal antibody [21] (Centocor Inc. Malvern, PA) and [^{125}I]protein A (1 μCi) (Amersham, Buck, UK), the blot was washed, dried and autoradiographed at −80°C.

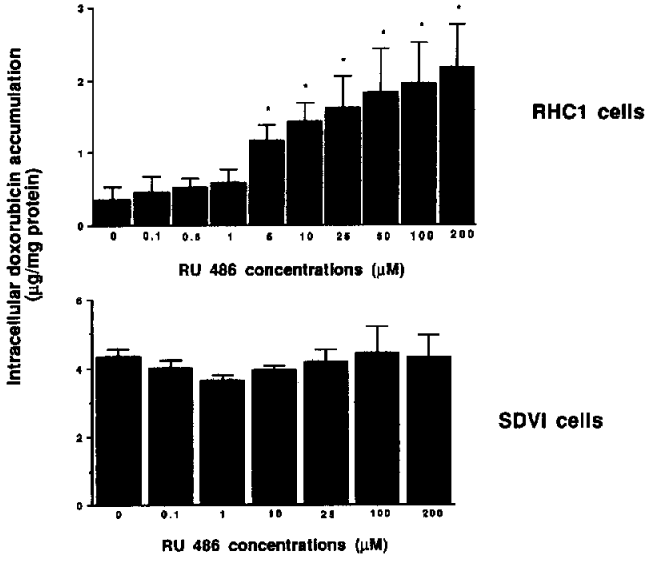

Fig. 1. Effect of various concentrations of RU 486 on doxorubicin accumulation in RHC1 and SDVI cells. Drug-resistant RHC1 cells and drug-sensitive SDVI cells were incubated for 2 h with 10 μg/ml doxorubicin in the presence of various concentrations of RU 486. The intracellular doxorubicin concentration was determined using the fluorimetric method as described in section 2. The values are the mean ± S.D. of three independent experiments in triplicate. *$P < 0.05$.

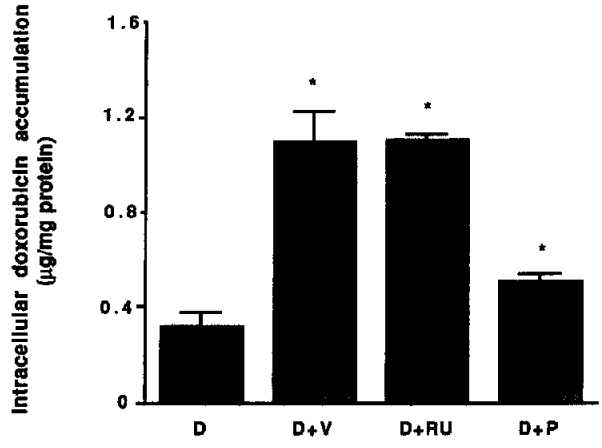

Fig. 2. Effect of RU 486, verapamil and progesterone on doxorubicin accumulation in RHC1 cells. Drug-resistant RHC1 cells were incubated for 2 h with 10 μg/ml doxorubicin either alone (D) or in the presence of 10 μM verapamil (D+V), or 10 μM RU 486 (D+RU) or 10 μM progesterone (D+P). The intracellular doxorubicin concentration was determined using the fluorimetric method as described in section 2. The values are the mean ± S.D. of three independent experiments in triplicate. *$P < 0.05$.

2.7. Statistical analysis

The results of doxorubicin accumulation and efflux studies were analyzed by the Student's *t*-test. The criterion of significance of the differences between the means (± S.D.) was *$P < 0.05$.

3. Results

In order to determine whether RU 486 was able to affect anticancer drug accumulation in cells overexpressing P-gp, intracellular levels of doxorubicin were estimated in rat drug-resistant RHC1 cells in the presence or absence of various concentrations of RU 486. Results indicated that the antiprogestatin agent strongly increased cellular doxorubicin retention. This effect was dose-dependent and began at 5 μM (Fig. 1). The concentration of 10 μM, which allowed to enhance cellular doxorubicin accumulation by approximately 4-fold, was retained in further experiments since it is in the range of RU 486 plasma concentrations usually observed in humans after oral administration of the drug [22]. In contrast to RHC1 cells, SDVI drug-sensitive cells did not display any change in intracellular doxorubicin levels in the presence of RU 486 whatever the concentration used (Fig. 1). The increase in RHC1 cell doxorubicin accumulation obtained with 10 μM RU 486 was similar to that observed with 10 μM verapamil (Fig. 2). By contrast progesterone used at the same concentration of 10 μM had a weaker effect on drug retention (Fig. 2).

Action of RU 486 on doxorubicin export out of RHC1 cells was next determined by drug efflux experiments. RU 486 was found to strongly inhibit the loss of cellular doxorubicin occurring during post-incubation in drug-free medium (Fig. 3). Similar results were observed with verapamil.

The effect of RU 486 on doxorubicin sensitivity of RHC1 cells was then monitored using the MTT dye assay. The antiprogestatin drug used at a non-toxic concentration of 10 μM was thus evidenced to strongly decrease RHC1 cell resistance to doxorubicin (Table 1). Indeed, addition of RU 486 led to a reduction by 59-fold of doxorubicin IC_{50} values and this chemo-

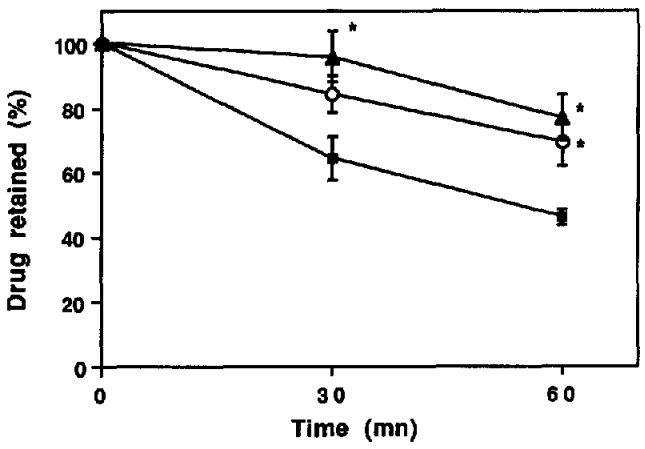

Fig. 3. Effect of RU 486 on doxorubicin efflux from RHC1 cells. Drug-resistant RHC1 cells were incubated for 2 h with 10 μg/ml doxorubicin, washed three times with ice-cold phosphate buffer and incubated again in drug-free medium for either 30 min or 60 min. These studies were performed in the absence of modulator agent (—■—) or in the presence of either verapamil (—○—) or RU 486 (—▲—) at 10 μM. Intracellular doxorubicin concentration was then determined using the fluorimetric method as described in section 2. Intracellular retained drug values were expressed relative to the initial drug accumulation values. The values are the mean ± S.D. of three independent experiments in triplicate. $^*P < 0.05$.

sensitization was similar to that observed with 10 μM verapamil. By contrast, RU 486 had no major effect on SDVI cell sensitivity to doxorubicin.

In order to establish whether RU 486 was also active in multidrug resistant cells other than RHC1 cells, its effect on cellular doxorubicin accumulation was investigated in human leukemia K562 R7 cells and in two-day-old primary cultures of normal rat hepatocytes, which both markedly overexpressed P-gp [12]. Results demonstrated that 10 μM RU 486, like verapamil, strongly enhanced doxorubicin retention in both leukemia K562 R7 cells and cultured normal rat hepatocytes (Fig. 4).

The effect of RU 486 on drug-binding to P-gp was further analysed with [^{3}H]azidopine. This compound is a photoaffinity ligand for P-gp [19] and was found to react with a band at about 150 kDa in cultured rat hepatocytes which was identified as P-gp by Western blot (Fig. 5B). RU 486 was shown to inhibit radiolabelling in a dose-dependent manner (Fig. 5A) indicating that the antiprogestatin agent competed with azidopine.

Table 1
Effects of RU 486 and verapamil on doxorubicin cytotoxicity to RHC1 and SDVI cell lines

Drugs	Doxorubicin IC_{50} (ng/ml)	
	RHC1 cells	SDVI cells
None	1039 ± 193	2.50 ± 0.40
RU 486	17.6 ± 3.4 (59)	1.38 ± 0.33 (1.8)
Verapamil	19.2 ± 2.4 (54)	2.09 ± 0.24 (1.2)

Cells were cultured for 96 h with various concentrations of doxorubicin in the presence or absence of RU 486 or verapamil (10 μM). Drug effects on cell proliferation were determined using the MTT assay as described in section 2. Growth inhibition is expressed as IC_{50}, i.e. the drug concentration providing a 50% reduction of cell numbers as compared to control cultured in parallel without drugs. Each value represents the mean (± S.D.) of at least two independent experiments in quadruplicate. Numbers in parentheses indicate the *x*-fold reversion.

4. Discussion

The results reported in the present study strongly evidence that RU 486, a compound known for its antiprogestatin properties, can also modulate anticancer drug accumulation and activity in multidrug resistant cells overexpressing P-gp. Indeed, RU 486 enhanced cellular retention and antiproliferative effect of doxorubicin in rat RHC1 resistant cells. It also augmented doxorubicin accumulation in human K562 resistant cells, therefore indicating that it is active in both human and rodent multidrug resistant cells. Moreover, vinblastine retention levels in RHC1 cells were found to be enhanced by 17-fold in the presence of RU 486 (data not shown), thus demonstrating that the effect of the antiprogestatin drug is not limited to doxorubicin. By contrast, RU 486 had no major effect on doxorubicin sensitivity and accumulation in drug-sensitive SDVI cells. These results therefore indicate that the action of RU 486 is restricted to multidrug resistant cells and is thus not a general chemosensitization of cells toward anticancer drug regardless of their level of resistance.

The enhancement in doxorubicin retention in RHC1 cells by

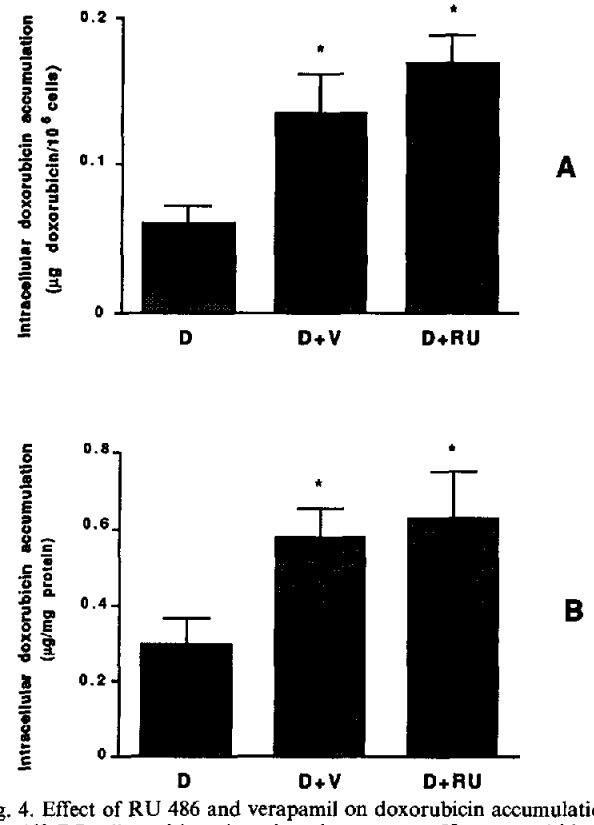

Fig. 4. Effect of RU 486 and verapamil on doxorubicin accumulation in K562 R7 cells and in cultured rat hepatocytes. Human multidrug-resistant leukemia K562 R7 cells (A) and two-days cultured rat hepatocytes (B) were incubated with 10 μg/ml doxorubicin for 2 h either alone (D) or in the presence of 10 μM verapamil (D + V), or 10 μM RU 486 (D + RU). The intracellular doxorubicin concentration was then determined using the fluorimetric method as described in section 2. The values are the mean ± S.D. of three independent experiments in triplicate. $^*P < 0.05$.

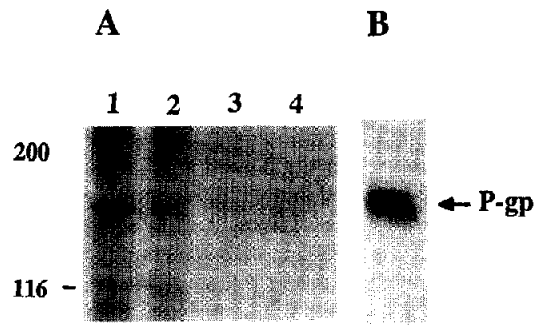

Fig. 5. Effect of RU 486 on [^{3}H]azidopine labeling of P-glycoprotein (P-gp) protein. Crude membrane proteins (50 μg/lane) prepared from two-day-old cultured rat hepatocytes were (A) photolabeled with [^{3}H]azidopine in the absence (lane 1) or presence of RU 486 at 10 μM, 100 μM or 200 μM (lanes 2, 3 and 4, respectively) and (B) used for P-gp detection by Western blot analysis with monoclonal antibody C219. The size of molecular standards (in kDa) is indicated on the left of (A).

RU 486 was associated with a decrease in doxorubicin export out of cells as assessed by drug efflux experiments. These data indicate that the antiprogestatin agent seems to act by inhibiting P-gp-mediated drug transport. This conclusion is also supported by a recent report by Gruol et al. [23] who demonstrated that the antiprogestatin agent blocked the efflux of the P-gp substrate dye rhodamine 123 in murine resistant cells. Moreover, RU 486 was shown to compete with the P-gp photoaffinity ligand azidopine, thereby suggesting that inhibitory effect on P-gp function is probably related to direct interactions with drug binding sites on P-gp. Such a mechanism of action has already been reported for many chemosensitizer agents, including verapamil and cyclosporin [4].

At 10 μM RU 486 was as effective as verapamil in increasing doxorubicin concentration and activity in RHC1 cells as demonstrated by drug-sensitivity and -accumulation experiments. By contrast, the antiprogestatin agent had a much stronger effect on doxorubicin retention than progesterone. This discrepancy could reflect particular structural features displayed by RU 486. Indeed, RU 486 differs from progesterone by a 11β-(dimethyl amino phenyl) substitution thought to be involved in its antihormone properties and which constitutes a tertiary amine side group [11]. Such a structure has been previously demonstrated to be crucial for anti-P-gp activity of many chemosensitizers, including phenothiazines and dipyridamole analogs [10] and thus could probably account for the potent reversing effect of RU 486.

RU 486 was also found to strongly enhance doxorubicin accumulation in cultured rat hepatocytes, thus suggesting that it could inhibit P-gp function in normal cells. Other chemosensitizer compounds such as verapamil or cyclosporin, have already been shown to interact with P-gp in normal tissues [24]. Since the physiological substrates for P-gp remain to be determined, the exact consequences of this inhibition of P-gp function in normal tissues by chemosensitizer agents are difficult to predict. However, recent studies have demonstrated that coadministration of verapamil or cyclosporin with chemotherapeutic drugs led to pharmacokinetic interactions, particularly a marked increase in anticancer drug plasma half-life [25] and this situation could reflect a decrease in the clearance of the anticancer drugs linked, at least partly, to an inhibition of liver and kidney P-gp.

A major obstacle to the clinical use of many chemosensitizer agents is linked to the fact that they act at elevated concentrations which are difficult to obtain in vivo partly owing to adverse effects. Indeed the use of verapamil at relevant anti-MDR concentrations is limited by the appearance of indesirable cardiac toxicity [26]. It is noteworthy that RU 486 downmodulated drug resistance at a 10 μM concentration which is in the range of the plasma concentrations observed in humans after a single oral dose [22]. These data suggest that RU 486 could have an actual effect in vivo on drug-resistant cells without major toxicity. This potential clinical relevance of the use of RU 486 as a chemosensitizer agent should be determined by further studies.

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