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Inhibition of P-glycoprotein activity in human leukemic cells by mifepristone

Olivier Fardel,[1,2] Arnaud Courtois,[1] Bernard Drenou,[2] Thierry Lamy,[3] Valérie Lecureur,[1] Pierre-Yves le Prisé[3] and Renée Fauchet[2]

[1]Groupe Détoxication et Réparation Tissulaire, Faculté de Pharmacie, 35043 Rennes, France. [2]Laboratoire d'Hématologie-Immunologie, Hôpital Pontchaillou, 35033 Rennes, France. Tel: (+33) 99 33 62 25; Fax: (+33) 99 33 68 88. [3]Service d'Hématologie Clinique, Hôpital Pontchaillou, 35033 Rennes, France.

The antiprogestatin drug mifepristone has previously been shown to potentiate anti-cancer drug activity in rodent multidrug-resistant cell lines through inhibition of P-glycoprotein (P-gp) function. In order to characterize P-gp–mifepristone interactions in human tumoral cells, we have studied the effect of the antiprogestatin agent on P-gp activity in human CD34$^+$ leukemic cells known to display high levels of P-gp-related drug efflux. P-gp-mediated transport of the fluorescent dye rhodamine 123 occurring in the CD34$^+$ KG1a myeloid leukemia cell line was found to be strongly inhibited by mifepristone in a dose-dependent manner. Similarly to verapamil, a well-known chemosensitizer agent, the antiprogestatin drug increased doxorubicin cytotoxicity in KG1a cells. Mifepristone, when used at a 10 μM concentration thought to be achievable *in vivo* without major toxicity, was also able to markedly decrease cellular rhodamine 123 efflux occurring in CD34$^+$ blast cells isolated from six patients suffering from myeloid acute leukemias. These results thus indicate that mifepristone can strongly inhibit P-gp activity in human cells, including tumoral cells freshly isolated from patients, therefore suggesting that the clinical use of this compound may contribute to down-modulate P-gp-mediated drug resistance.

Key words: Anti-cancer drugs, leukemic cells, mifepristone, multidrug resistance, P-glycoprotein.

Introduction

Drug resistance is a major obstacle to the successful chemotherapy of many human cancers. Among the mechanisms involved, a major one is linked to the overexpression of a plasma membrane glycoprotein termed P-glycoprotein (P-gp).[1] P-gp, encoded by the *MDR1* gene, is thought to act as an ATP-dependent efflux pump with broad specificity, therefore reducing the intracellular accumulation of various anti-tumor compounds such as anthracyclins, Vinca alkaloids, epipodophyllotoxins and taxol, and thus conferring a multidrug resistance (MDR) phenotype.[2,3]

Correspondence to O Fardel

P-gp overexpression has initially been described in rodent drug-resistant cell lines obtained by treatment with increased amounts of a single anti-cancer compound.[4] High levels of P-gp have next been detected in a wide variety of human tumors.[5] In particular, P-gp expression has been demonstrated to be usually high and constitutive in some solid tumors such as carcinomas of the colon, kidney, adrenal gland, pancreas and liver.[6] Elevated levels of P-gp have also been found in some hematological malignancies, including CD34$^+$ acute myeloid leukemias, some acute lymphoblastic leukemias and some lymphomas,[7–9] and have been correlated with treatment failure and poor prognosis in several types of cancer, thus indicating that P-gp may really contribute to clinical drug resistance.[8,10]

P-gp-mediated transport can be inhibited by a wide variety of structurally unrelated drugs including calcium channel blockers such as verapamil, calmodulin antagonists, steroids, antimalaric drugs such as quinine, some antibiotics and immunosuppressive agents.[11] These compounds, termed modulators or chemosensitizers, are thought to function through competition with anti-cancer drugs for drug-binding sites on P-gp and thus restore active intracellular levels of anti-tumor agents and reverse resistance. Unfortunately, the clinical use of many chemosensitizers is strongly limited by the fact that the concentrations required to inhibit P-gp function are usually high and cannot be reached *in vivo* without the appearance of undesirable side effects.[12] Indeed, administration of elevated doses of verapamil or cyclosporin A in order to *in vivo* modulate P-gp activity has led to considerable cardiovascular and neurologic toxicities.[13,14] Interestingly, we and others have recently demonstrated that the antiprogestatin drug mifepristone (also termed RU 38486), which has been found not to have serious side effects, can down-modulate MDR through inhibition of P-gp function.[15,16] Mifepristone was thus shown

to strongly potentiate anti-cancer drug cytotoxicity in hepatoma and thymoma multidrug-resistant cells when used at low concentrations thought to be readily achievable *in vivo* without major toxicity. However, these studies have been largely performed using rodent multidrug-resistant cells; the present study was therefore designed to more completely characterize mifepristone–P-gp interactions in human cells. For this purpose, we have analyzed the effect of the antiprogestatin drug on P-gp function in human $CD34^+$ leukemia cells known to constitutively display high P-gp activity.[7,17]

Material and methods

Chemicals

Rhodamine 123, doxorubicin and verapamil were obtained from Sigma (St Louis, MO). Mifepristone was a gift from Roussel UCLAF (Romainville, France).

Cell culture

KG1a is a $CD34^+$ human leukemia cell line established from an acute myelogenous leukemia;[18] K562 is a $CD34^-$ leukemia cell line established from a chronic myelogenous leukemia in blast crisis.[19] Both KG1a and K562 cells were grown in suspension in RPMI medium supplemented with 10% fetal calf serum as previously described.[20]

Patient characteristics

Mononuclear cells were isolated from blood or bone marrow of six patients suffering from acute myeloid leukemias by ficoll sedimentation. According to the French–American–British (FAB) recommendations, three leukemias were classified as M1, two as M2 and one as M5; the median age was 41 years (range 22–64 years). For each patient, the sample was collected at diagnosis before treatment. All the leukemias included in the study were $CD34^+$ as revealed by immunophenotyping of the blast cells using the anti-CD34 monoclonal antibody HPCA2 (Beckton Dickinson, Mountain View, CA).

Analysis of P-gp activity

P-gp-mediated drug transport was determined using a rhodamine 123 efflux assay.[21] Indeed, the fluorescent dye rhodamine 123 is a substrate for P-gp and its transport out of the cell has been demonstrated to reflect P-gp function.[21] Leukemic cells were incubated with rhodamine 123 (500 ng/ml) for 30 min at 37°C, washed and then kept at 4°C before analysis or re-incubated in rhodamine 123-free medium at 37°C in the absence or presence of verapamil or mifepristone. Cellular rhodamine 123 fluorescence was then determined by fluorometry using a Titertek Fluoroscan spectroflorometer (Flow Laboratories, Puteaux, France) or by flow cytometry using a Cytoron Absolute flow cytometer (Ortho Diagnostic Systems, Raritan, NJ); this analysis allowed us to evidence the loss of rhodamine 123 staining occurring during the efflux period at 37°C that is thought to directly reflect P-gp activity.[21] In flow cytometry experiments, the immunophenotype of cells displaying P-gp activity was determined by dual fluorescence analysis; cells stained with rhodamine 123 were incubated with a phycoerythrin-labeled anti-CD34 HPCA2 antibody and phycoerythrin fluorescence was then analyzed after electronic compensation of the overlapping emission spectrum between rhodamine 123 and phycoerythrin.

Analysis of *MDR1* gene expression by reverse transcriptase-polymerase chain reaction (RT-PCR)

Expression of *MDR1* mRNAs was detected by the RT-PCR method as previously described.[22] Total cellular RNAs were isolated by guanidinium thiocyanate/cesium chloride method of Chirgwin *et al.*[23] Then, 0.5 μg RNA was reverse transcribed using Moloney murine leukemia virus transcriptase and random hexanucleotide primers. cDNA representing 25 ng RNA was next subjected to PCR for 30–32 cycles in a final volume of 50 μl. Following an initial denaturation of 2 min, each cycle consisted of 30 s at 94°C, 30 s at 55°C and 60 s at 72°C. The primers used for *MDR1* and β_2-microglobulin detection were exactly as described by Noonan *et al.*[22] PCR products were separated on a 2% agarose gel and visualized by ethidium bromide.

Drug-sensitivity assay

The effects of mifepristone and verapamil on doxorubicin sensitivity of KG1a cells were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye assay.[24] Briefly, KG1a cells

cultured with doxorubicin (2 and 10 ng/ml) in the absence or presence of mifepristone or verapamil for 4 days were incubated with 0.5 mg/ml MTT for 2 h; cell growth was then evaluated by spectrophotometric determination of the blue formozan products formed using a Titertek Multiscan MCC/340 (Flow Laboratories).

Results

The effect of mifepristone on P-gp function was first analyzed in human KG1a cells. To assure that these $CD34^+$ leukemic cells display P-gp activity, both RT-PCR experiments and rhodamine 123 efflux assays were carried out. As revealed by RT-PCR analysis, *MDR1* gene expression was evidenced in KG1a cells, but not in human leukemic drug-sensitive K562 cells used as negative controls for P-gp expression (Figure 1); by contrast, similar levels of β_2-microglobulin mRNAs were found in both KG1a and K562 cells. Using the rhodamine 123 assay, KG1a cells were evidenced to rapidly lose rhodamine 123 staining during the post-incubation in fluorescent dye-free medium (Figure 2); in particular, after a 1 h efflux period, residual cellular rhodamine 123 staining was less than 5% of initial rhodamine 123 fluorescence whereas, by contrast, K562 cells only poorly effluxed rhodamine 123 (Figure 2). This P-gp-mediated rhodamine 123 efflux of the KG1a cells was found to be strongly inhibited by mifepristone in a dose-dependent manner (Figure 3). Indeed, the addition of the antiprogestatin drug during a 1 h post-incubation in rhodamine 123-free medium markedly enhanced intracellular retention of the fluorescent dye; this effect began for a mifepristone concentration of 0.5 μM and was maximum for concentrations ranging from 10 to 40 μM. Light microscopic examination of the cultures and Trypan blue dye exclusion revealed no alteration in KG1a cell viability by the various tested concentrations of the antiprogestatin drug. The use of 10 μM verapamil instead of mifepristone also led to a similar increase in cellular rhodamine 123 fluorescence (Figure 3). By contrast,

Figure 1. *MDR1* gene expression in KG1a and K562 cells. Total RNAs (0.5 μg) isolated from KG1a and K562 cells were analyzed by RT-PCR using *MDR1* and β_2-microglobulin (β-μG) gene-specific primers as indicated in Materials and methods.

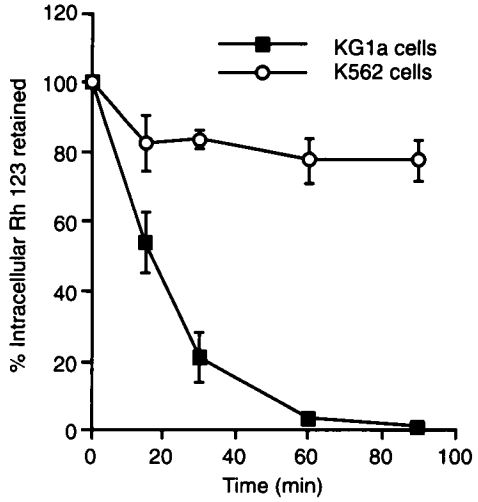

Figure 2. Cellular rhodamine 123 efflux KG1a and K562 cells. Cells were incubated with rhodamine 123 (500 ng/ml) for 30 min at 37°C, washed and reincubated in rhodamine 123-free medium for 15–90 min. Cellular rhodamine 123 retention was then analyzed by spectrofluorimetry. The values are the mean ± SD of three independent experiments.

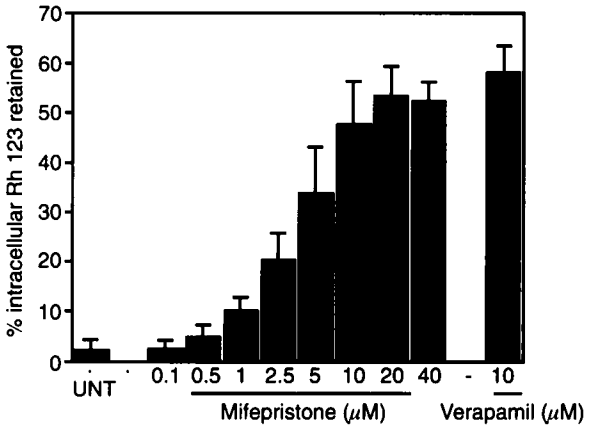

Figure 3. Effect of mifepristone on rhodamine 123 retention in KG1a cells. Cells were incubated with rhodamine 123 (500 ng/ml) for 30 min at 37°C, washed and reincubated in rhodamine 123-free medium for 60 min in the absence (UNT) or presence of mifepristone (0.1–40 μM) or verapamil (10 μM). Cellular rhodamine 123 retention was then analyzed by spectrofluorimetry. The values are the mean ± SD of three independent experiments.

both mifepristone and verapamil failed to alter rhodamine 123 retention in K562 cells (data non shown).

The effect of mifepristone on doxorubicin sensitivity of KG1a cells was then monitored using the MTT dye assay. As shown in Figure 4, the addition of 10 μM mifepristone strongly potentiated the cytotoxicity of the anti-cancer drug; indeed KG1a cell proliferation was clearly decreased when doxorubicin used at two different concentrations (2 and 10 ng/ml) was combined with the antiprogestatin compound. Similarly, the addition of 10 μM verapamil was also found to increase doxorubicin cytotoxic effects (Figure 4).

Modulation of P-gp activity in response to mifepristone was further evaluated using mononuclear cells isolated from the blood or the bone marrow of six patients suffering from $CD34^+$ acute myeloid leukemias. P-gp activity of the leukemic cells was checked by dual fluorescence analysis, which allowed us to specifically analyze $CD34^+$ blast cells. Thus, flow cytometric rhodamine 123 assay combined with anti-CD34 immunolabeling indicated that $CD34^+$ leukemic cells of patient no. 2 displayed a marked rhodamine 123 efflux which was almost completely abolished by 10 μM verapamil, thus indicating that it was largely related to P-gp activity (Figure 5). Such rhodamine 123 transport occurring in $CD34^+$ blast cells was also observed in the five other cases studied, even if intracellular rhodamine 123 retention values after the efflux period greatly differed according to the patients (Table 1). As shown in Figure 5, P-gp activity present in the $CD34^+$ blast cells of patient no. 2 was found to be strongly inhibited in the presence of 10 μM mifepristone; similarly, mifepristone also enhanced rhodamine 123 levels in the leukemic cells from the other patients analyzed (Table 1). Intracellular rhodamine 123 retention values in the presence of the antiprogestatin drug thus ranged from 68 to 91% of initial staining levels and were similar to those observed after addition of verapamil.

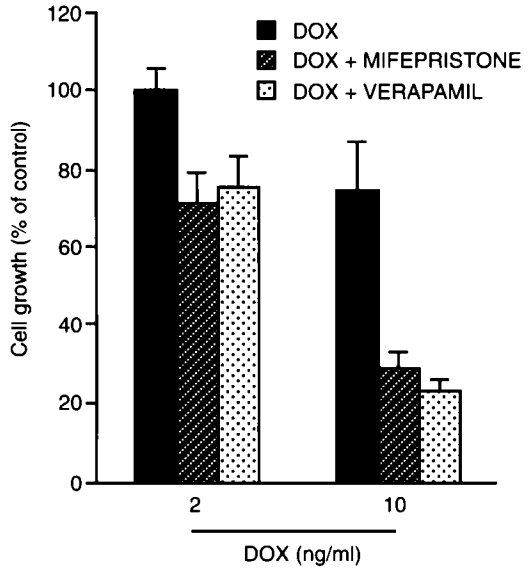

Figure 4. Effect of mifepristone on doxorubicin-induced cytotoxicity in KG1a cells. KG1a cells were treated for 4 days with doxorubicin (2 and 10 ng/ml) in the absence or presence of mifepristone (10 μM) or verapamil (10 μM). Cell growth was then analyzed as described in Materials and methods. Results are expressed as percent of untreated cells and are the mean ± SD of three independent experiments in triplicate.

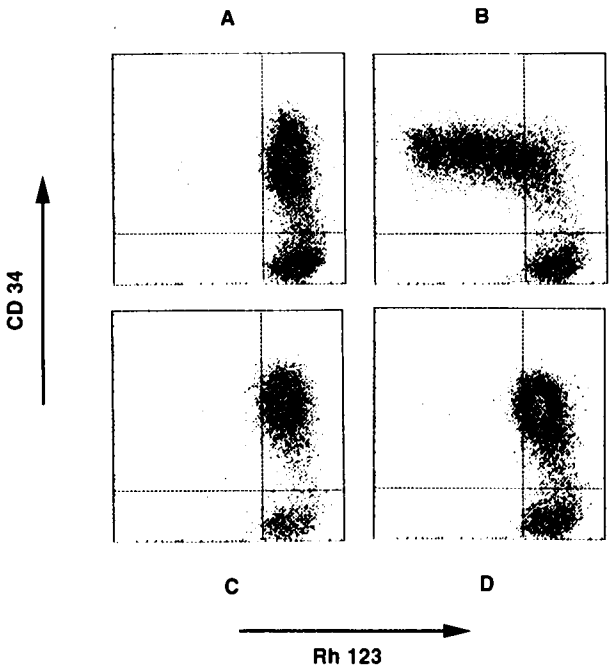

Figure 5. Example of the inhibition of cellular rhodamine 123 efflux occurring in $CD34^+$ leukemic cells from patients by mifepristone. Mononuclear cells, isolated from the bone marrow of patient no. 2, were incubated with rhodamine 123 (500 ng/ml) for 30 min at 37°C, washed and re-incubated in rhodamine 123-free medium for 60 min in the absence or presence of mifepristone (10 μM) or verapamil (10 μM). Cells were then immunolabeled with anti-CD34 antibody and analyzed by flow cytometry. Cells are characterized by rhodamine 123 fluorescence (horizontal axis) and CD34 expression (vertical axis). Graph A shows initial rhodamine 123 staining before efflux. Graphs B, C and D show rhodamine 123 retention in $CD34^+$ leukemic cells after the efflux period in the absence (B) or presence of verapamil (C) or mifepristone (D).

Table 1. Effect of mifepristone on cellular rhodamine 123 retention in CD34$^+$ leukemic cells from patients

Patients	FAB sub-type	Rhodamine 123 retained (% initial staining) with modulator		
		None	Verapamil	Mifepristone
1	M5	25	83	82
2	M1	10	86	78
3	M1	36	90	75
4	M2	14	75	68
5	M2	74	93	91
6	M1	61	83	87

Mononuclear cells isolated from the blood or the bone marrow of six patients suffering from acute myeloid leukemia were incubated with rhodamine 123 (500 ng/ml) for 30 min at 37°C, washed and re-incubated in rhodamine 123-free medium for 60 min in the absence or presence of 10 μM mifepristone or 10 μM verapamil. Cells were then immunolabeled with a phycoerythrin-conjugated anti-CD34 antibody and cellular rhodamine 123 retention in CD34$^+$ leukemic cells was determined by dual fluorescence analysis.

Discussion

Previous studies have demonstrated that the antiprogestatin drug mifepristone was able to down-modulate P-gp-mediated multidrug resistance in rodent thymoma and hepatoma cells.[15,16] The results reported here showed that mifepristone also strongly inhibits P-gp activity in human tumoral cells. Indeed, we have used CD34$^+$ leukemic cells displaying P-gp-mediated transport to demonstrate that the antiprogestatin compound markedly decreased the cellular export of the fluorescent dye rhodamine 123, a well-known substrate for P-gp.[25] In particular, the marked cellular rhodamine 123 efflux occurring in the CD34$^+$ myeloid leukemia KG1a cell line, that was evidenced to display *MDR1* gene expression by RT-PCR analysis, was strongly inhibited by mifepristone. This effect was dose dependent and maximal inhibition of P-gp activity was thus obtained with concentrations of the antiprogestatin drug ranging from 10 to 40 μM. These results agree with previous studies demonstrating that the use of mifepristone at 10 μM allowed us to strongly increase doxorubicin accumulation in rat multidrug-resistant hepatoma cells[15] and to decrease rhodamine 123 efflux in murine thymoma cells overexpressing the mouse *mdr1* gene.[16] Mifepristone at 10 μM was also evidenced to potentiate doxorubicin cytotoxicity in KG1a cells as indicated by cell growth assay, thus indicating that the antiprogestatin drug allowed us to down-modulate drug resistance in P-gp$^+$ human tumoral cells as it did in rodent multidrug-resistant cells.[15] Moreover, the effects of 10 μM mifepristone on rhodamine 123 retention and doxorubicin cytotoxicity in KG1a cells were similar to those obtained with 10 μM verapamil, thus suggesting that the two chemosensitizer agents were equally active at this 10 μM concentration.

In contrast to KG1a cells, intracellular levels of rhodamine 123 in human *MDR1*$^-$ K562 cells were not affected in response to mifepristone; similarly, the antiprogestatin drug did not alter doxorubicin accumulation in rat drug-sensitive liver cells.[15] Taken together, these results suggest that the action of mifepristone is restricted to multidrug-resistant cells and is probably related to direct inhibition of P-gp function and not to unspecific alteration of drug membrane permeability. Such a conclusion is moreover supported by the fact that mifepristone has been shown to compete with the P-gp photoaffinity ligand [^{3}H]azidopine in P-gp labeling experiments, thus suggesting that the antiprogestatin drug, like many chemosensitizer agents such as verapamil, can directly interact with drug binding sites on P-gp.[15]

The inhibitory effect of mifepristone on P-gp activity was also fully supported by rhodamine 123 efflux assays using leukemic cells freshly obtained from six patients suffering from CD34$^+$ acute myeloid leukemias. As indicated by dual fluorescence analysis, the leukemic CD34 cells analyzed displayed P-gp activity, which agrees with previous studies demonstrating a high correlation between CD34 and P-gp expression in acute myeloid leukemias.[10,17,26] However, rhodamine 123 retention values in the leukemic cells after the efflux period greatly differed according to the patients, which may be at least partly related to differences between patients in the contingent of P-gp$^+$ blast cells or in the P-gp ability to expel cytotoxic drugs. Such an heterogeneity in the proportions of CD34$^+$ acute myeloid leukemic cells displaying P-gp expression and activity has already been reported in various studies.[7,27] The use of mifepristone, like that of verapamil, allowed us to strongly reduce cellular rhodamine 123 efflux occurring in the blast cells of the six patients studied. Therefore mifepristone was active on P-gp function not only in multidrug-resistant cell lines, but also in P-gp$^+$ cells freshly isolated from patients. This last point is likely important since multidrug-resistant cell lines usually obtained by *in vitro* drug selection often display high levels of P-gp that are not necessary relevant to clinical drug resistance. Therefore, the use of P-gp$^+$ cells freshly isolated from patients seems to be required for pre-clinical investigations of chemosensitizers and the rhodamine 123 efflux assay appears

to be suitable for such studies. Moreover, it is noteworthy that the 10 μM concentration of the antiprogestatin drug used to inhibit P-gp activity in human blast cells is close to plasma concentrations thought to be achievable in humans without major toxicity,[28] whereas many potent chemosensitizer agents act on P-gp at elevated concentrations which are difficult to obtain *in vivo* without the appearance of adverse effects.[12] These data therefore suggest that the antiprogestatin drug may have a real effect on P-gp activity *in vivo* without undesirable side effects in contrast to other reversing agents. Further studies, including clinical investigations, are required to better specify the potential clinical relevance of the use of mifepristone as a chemosensitizer compound.

In conclusion, the antiprogestatin drug mifepristone was evidenced to inhibit P-gp-mediated rhodamine 123 transport in human $CD34^+$ leukemic cells. In particular, dual fluorescence analyses have demonstrated that mifepristone allowed us to strongly decrease P-gp activity in leukemic cells isolated from six patients. This effect was obtained with a 10 μM concentration that is thought to be achievable *in vivo* without major toxicity, thus suggesting that the clinical use of mifepristone may contribute to overcome P-gp-mediated multidrug resistance.

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