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Rifampicin and verapamil induce the expression of P-glycoprotein in vivo in Ehrlich ascites tumor cells

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Abstract

The effect of an in vivo treatment with two commonly employed drugs that are P-glycoprotein substrates, verapamil and rifampicin, on Ehrlich ascites carcinoma cells, was evaluated.

Ehrlich ascites carcinoma cells were inoculated i.p. in CD-1 mice and animals were orally treated for 10 days with rifampicin (60 mg/kg/day) or verapamil (6 mg/kg/day). In the harvested cells the transcripts for *mdr1a* and *mrp1*, but not those for *mdr1b*, *mrp2* and *CYP3A*, were detected, and treatment with verapamil or rifampicin did not modify the levels of the transcripts. On the contrary, an increased expression of P-glycoprotein was observed at the protein level with Western blot. The intracellular uptake of doxorubicin, a P-glycoprotein and MRP substrate, was significantly lower in cells obtained from treated animals in comparison with cells obtained from controls; in addition, the uptake was increased by a pretreatment with verapamil. The survival time of control animals implanted with untreated cells was similar to that of animals inoculated with cells obtained from rifampicin treated animals, however, the antineoplastic effect of doxorubicin was significanly higher in control animals.

A treatment with rifampicin or verapamil in Ehrlich ascites tumor confers resistance to the antineoplastic drug doxorubicin, probably through an increased expression of P-glycoprotein.

Keywords: Ehrlich ascites carcinoma; P-glycoprotein; Multidrug resistance-related proteins; Verapamil; Rifampicin; Doxorubicin

1. Introduction

One of the major obstacles to the successful treatment of many human cancers is the development of multidrug resistance. The most important mechanism of resistance is linked to a reduced drug concentration at the target site, due to enhanced cellular efflux of antitumor compounds. This pheno menon is usually related to the expression of ATP-driven efflux pumps, among which P-glycoprotein and Multidrug Resistance-related Proteins (MRPs). P-glycoprotein is a 170 kDa protein, whose substrates include a huge number of lipophylic, naturally occurring substances [1]; the protein is encoded by the *MDR1* gene in humans and by the *mdr1a* and *mdr1b* genes in rodents. The MRP family

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comprises at least nine members that transport drugs conjugated with glutathione, glucuronide, sulfate or unconjugated amphiphilic anions [2]; MRP1 and MRP2 confer resistance to a wide variety of chemotherapeutic agents [2]. The overexpression of these proteins in tumors after chemotherapy is usually attributed to induction or to selection of preexisting multidrug resistant cells. Various factors can influence the expression of P-glycoprotein and MRP, such as environmental stresses [3], physiological inducers [4], hormones [5] and drugs among which anticancer agents [6,7] as well as drugs employed in non neoplastic diseases [7–9].

It is now widely recognized that P-glycoprotein or MRP overexpression is associated with clinical evidence of drug resistance and treatment failure [10]. Considerable effort has therefore been made to apply chemotherapeutic protocols that could prevent the development of P-glycoprotein or MRP expression by combining multiple drugs and delivering them at optimal doses and intervals. On the contrary, little attention has been played to the possibility that the expression of these proteins may be affected by drugs that are administered to cancer patients for other pathologies.

Among the substances that induce P-glycoprotein and MRP in normal and tumor cells, are two commonly employed pharmacological agents such as verapamil [8], widely used in cardiovascular therapy, and rifampicin [9,11–13], a first line agent in the chemotherapy of tuberculosis. It is not unlikely that cancer patients are affected by cardiovascular diseases, and they are often immunocompromised as a consequence of the disease or of the antineoplastic therapy; therefore these drugs can be employed in cancer patients and also in subjects in which a tumor is growing but is not yet clinically evident.

It may be hypothesized that these compounds, positively tested in tumor cell lines in vitro, may also exhert their induction potential in vivo. The aim of the present study was therefore to investigate whether a chronic treatment with rifampicin or verapamil could induce the expression of P-glycoprotein, MRP1 or MRP2 in Ehrlich ascites tumor cells in vivo, and could consequently modify the sensitivity of this tumor to doxorubicin.

2. Materials and methods

2.1. Chemicals

RPMI 1640 culture medium, fetal bovine serum, L-glutamine, D-PBS (Dulbecco's modified Phosphate Buffered Saline), doxorubicin, rifampicin, verapamil, alkaline phosphatase conjugated secondary antibodies, RedTaq™ DNA polymerase and 100 bp DNA ladder were purchased from Sigma Aldrich Srl, Italy. SV Total RNA Isolation System was from Promega, Italy; SuperScript™ II RNase H^- reverse transcriptase and synthetic oligonucleotide primers were from Invitrogen life technologies, Italy; C219 monoclonal and A23 polyclonal antibodies were obtained from Alexis Vinci Biochem, Italy. All other chemicals were of analytical grade.

2.2. Animals

Adult (18–22 g) CD1 female mice, obtained from a local conventional breeding colony, were housed in standard cages at 20 °C on a 12 h light/dark cycle and had free access to both food and water. Animal experiments were performed in accordance with Italian laws (Ministry of Health-registration number 62/2000-B, 06.10.00).

2.3. Tumor cells

The murine Ehrlich ascites carcinoma (EAC) cell line, originally obtained from the American Type Cell Culture Collection, Rockville, USA, was used in the experiments. The cell line was maintained as ascites tumors in female CD1 mice by serial intraperitoneal transplantation every 10 days. Tumor cells from the donor mouse were obtained by peritoneal lavage with 5 ml of D-PBS, washed twice by centrifugation, and 5×10^6 cells in 0.1 ml of D-PBS were i.p. injected in each mouse.

2.4. In vivo treatment

In vivo induction of proteins was studied by treating animals (two animals per group) with rifampicin 60 mg/kg/day or verapamil 6 mg/kg/day orally by gavage. Control animals were similarly treated with the solvent alone. Treatment was started

on day 0 and continued till day 11; on day 1, animals were inoculated i.p. with 5×10^6 EAC cells in 0.1 ml of D-PBS. Animals were sacrificed on day 11 and ascites fluid was drained and collected. Cells obtained from control animals (EAC), from animals treated with rifampicin (EAC/RIF) or verapamil (EAC/VER) were washed twice with ice cold D-PBS, counted, and cell viability was determined by trypan blue dye exclusion test. Cell viability was always higher than 90%.

2.5. Doxorubicin uptake

To evaluate doxorubicin uptake, 1×10^6 EAC, EAC/RIF or EAC/VER viable cells were incubated in triplicate in 1 ml of RPMI 1640 medium containing doxorubicin 25 μM at 37 °C for different experimental times. In some experiments, the cells were pretreated for 1 h with verapamil 100 μM, doxorubicin (final concentration 25 μM) was added, and the incubation was then continued in the presence of both doxorubicin and verapamil. After incubation, the cells were washed twice with ice cold D-PBS, resuspended in 2 ml of 0.3 N HCl in 50% ethanol and centrifuged at 700 xg. Doxorubicin content in the supernatant fraction was determined fluorimetrically with the method of Bachur et al. [14]. Standard curves of doxorubicin dissolved in 0.3 N HCl/50% ethanol were used for computation of doxorubicin content. Protein concentration of samples was determined by the method of Lowry et al. [15].

2.6. RT-PCR

EAC, EAC/RIF and EAC/VER cells were washed twice in D-PBS and total RNA was extracted from 3×10^6 cells using the SV Total RNA Isolation System kit. Five micrograms of total RNA were reverse transcribed using the SuperScript™ II RNase H^- reverse transcriptase. Serial dilutions of the cDNA were used in PCR reaction in a final volume of 10 μl, according to Bates et al. [16]. Synthetic oligonucleotide primers were used to investigate the presence of *mdr1a*, *mdr1b, mrp1, mrp2, CYP3A* and of the household gene *β-actin* mRNA transcripts. *mdr1a* sense: 5′-TTGTGCAAACGTGCCACC-3′, antisense 5′-GGAACAACTGATAAGAGCAG-3′ (for primer designing mouse sequence Accession number M33541 was used, 532 bp fragment); *mdr1b* sense: 5′-GACGGACAGGACATCAGGACC-3′, antisense 5′-GCAAACACTGGTTGTATGCAC-3′ (mouse sequence Accession number M14757, 823 bp); *mrp1* sense, 5′-TTCCCATTTCAACGAGACCTTGCT-3′, antisense 5′-TCCGCCCCACGATGCCGACCT-3′; (mouse sequence Accession number AF022908, 533 bp); *mrp2* sense 5′-TGGCTGAGATTGGAGAG-3′, antisense 5′-TTTGTCCTTTCACTAGTTC-3′ (mouse sequence Accession number AF227274, 596 bp); *CYP3A* sense 5′-GAAGCATTGAGGAGGATCAC-3′, antisense 5′-GGGTTGTTGAGGGAATCCAC-3′ (mouse sequence Accession number X60452, 600 bp); β-*actin* sense 5′-CACCAAAGCTGAGAGGGAAATCGTCGTGA-3′, antisense 5′-AATTGCGGTGCACGATGGAGGGGCCGGACT-3′ (mouse sequence Accession number X03765, 500 bp). The reaction was performed using RedTaq™ DNA polymerase and a DNA thermal cycler Gene Amp® 9700 (PE Applied Biosystems). After denaturing at 94 °C for 5 min, the amplification was obtained by 30 (*mdr1a, mdr1b, mrp1* and *CYP3A*) or 20 (*β-actin*) cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min. Only for *mrp2,* 30 amplification cycles were performed at 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min. A final step at 72 °C for 10 min stopped the reactions. PCR products were subjected to electrophoresis on 1.5% agarose gel and visualized by ethidium bromide staining. PCR products, obtained by amplification of serially diluted aliquots of cDNA were densitometrically quantified by Gel Compare II (Applied Math, Belgium). To normalize the data, the ratio between the densitometric quantification obtained with specific primers and with β-actin primers was calculated [16].

2.7. Western blot analysis

EAC, EAC/RIF and EAC/VER cells were dissolved in a lysis buffer containing NaCl 100 mM, EDTA 1 mM, Tris–HCl 10 mM pH 7.5, and added with protease inhibitors phenylmethylsulphonyl fluoride 1 mM, aprotinin 2 μg/ml, leupeptin 2 μg/ml, pepstatin A 1 μg/ml. After centrifugation at 4 °C for 10 min at 1600 xg, the supernatant was centrifuged at 28000 xg for 1 h at 4 °C. Membranes were kept at − 20 °C till use. Samples containing 100 μg of proteins, determined by the D_C Protein assay (Biorad) were resuspended

in loading buffer [17], boiled for 5 min, loaded on 8% SDS-PAGE and transferred to nitrocellulose membrane by electroblotting for 2 h at 2 mA/cm^2 with the semy-dry protein blotter system (Pharmacia LKB Nova Blot).

The membrane was stained with red Ponceau to verify the homogeneity of samples loaded on gel, then blocked for 2 h in TTBS (Tris buffer saline: 150 mM NaCl, 50 mM Tris–HCl, pH 7.5, containing 0.05% v/v Tween − 20) added with 3% skim milk, and incubated overnight at 4 °C with the primary antibodies diluted in blocking solution: C219 monoclonal antibody (specific for P-glycoprotein, dilution 1:200) and A23 polyclonal antibody (specific for MRP1, dilution 1:500) [18]. Blots were washed three times with TTBS, and incubated with alkaline phosphatase conjugated secondary antibody (goat anti-mouse IgG, dilution 1:30.000 for C219; goat anti-rabbit IgG, dilution 1:20.000 for A23) for 1 h at room temperature. The membrane was extensively washed and finally stained by addition of bromocloroindolyl phosphate and nitroblue tetrazolium.

2.8. In vivo antitumor activity of doxorubicin

EAC cells were intraperitoneally transplanted (5×10^6 viable cells/animal) onto female CD1 mice (day 0). Cells obtained from control animals (EAC) or from animals chronically treated with rifampicin as described above (EAC/RIF) were inoculated in two groups of 40 animals each. Doxorubicin (0.125 mg/kg) was intraperitoneally administered on day 1 after tumor inoculation in 20 animals per group. Animals were weighed twice a week and inspected daily for survival and general toxicity.

2.9. Statistical analysis

For uptake data, averages ± S.E. of the means were calculated and statistical analysis was carried out using Student's *t* test for independent samples. For survival, Kaplan Meier analysis and log rank test were performed.

3. Results

3.1. Doxorubicin uptake

The intracellular uptake of doxorubicin, evaluated at different experimental times, was significantly lower in cells obtained from animals in vivo treated with rifampicin (EAC/RIF) or verapamil (EAC/VER) in comparison with that observed in EAC cells obtained from untreated animals (Fig. 1).

When EAC/RIF cells were preincubated in vitro for 1 h with verapamil 100 μM, the uptake of the anthracycline was significantly increased; on the contrary verapamil did not modify the uptake of doxorubicin in control EAC cells (Fig. 2).

3.2. RT-PCR

The β-actin PCR products in untreated or treated cells were comparable, giving a rough estimate that the same amounts of RNA were used in the three cell lines. With our primers, *mdr1b* and *mrp1*, but not *mdr1a*, *mrp2* and *CYP3A* transcripts were detected in control cells an in cells treated with verapamil or rifampicin. No difference in transcript levels was evident between control and treated cells (Fig. 3).

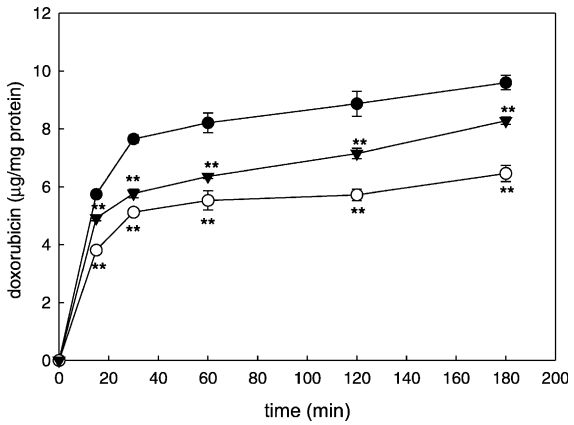

Fig. 1. Doxorubicin time dependent uptake in EAC cells (●), in cells obtained from animals in vivo treated with rifampicin (EAC/RIF) (▼) or verapamil (EAC/VER) (○). Cells were incubated in complete culture medium with 25 μM doxorubicin for up to 180 min. Each point represents the mean ± S.E. of data from three to six samples. ** : $P < 0.01$, Student's *t* test for independent data.

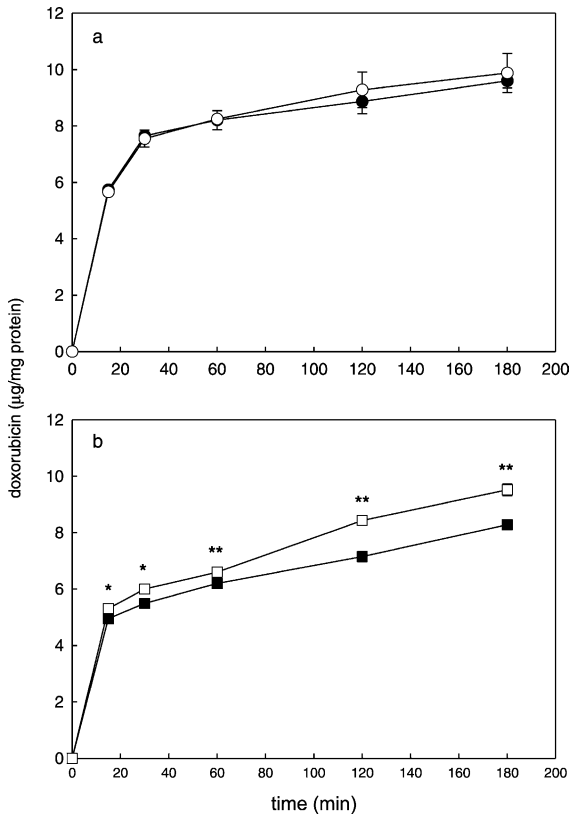

Fig. 2. Effect of verapamil on doxorubicin uptake in EAC cells (a) and in cells obtained from animals in vivo treated with rifampicin (EAC/RIF) (b). The cells were preincubated in complete culture medium with (open symbols) or without (closed symbols) verapamil 100 μM, and the doxorubicin 25 μM (final concentration) was added and the incubation continued for 180 min. Each point represents the mean ± S.E. of data from three to six samples. *: $P < 0.05$, ** : $P < 0.01$, Student's t test for independent data.

3.3. Western blot analysis

P-glycoprotein and MRP1 expression was determined: indeed, only for these proteins a transcript was revealed by RT-PCR. Western blot analysis was performed on EAC cells obtained from control animals or from animals chronically treated with rifampicin (EAC/RIF) or verapamil (EAC/VER). An increased expression of P-glycoprotein was observed in cells obtained from treated animals in comparison with controls; the analysis was repeated on cells from four different experiments and an illustrative result is represented in Fig. 4. To verify that the quantity of loaded protein was the same for each treatment, 25 and 50 μg of proteins were loaded across different lanes of the gel and the homogeneity of loading was verified by red Ponceau staining.

On the contrary, MRP1 levels were not modified by the treatment (data not shown).

3.4. In vivo study

The survival time of animals i.p. injected with EAC cells and treated with doxorubicin 0.125 mg/kg i.p. was evaluated. All animals carrying the tumor died before day 50, with a mean survival of 32.5 days. Doxorubicin treatment was extremely effective, with 75% of animals cured. The mortality of animals inoculated with EAC/RIF cells, obtained from animals treated with rifampicin, was similar to that of controls (mean survival 33.5 days). Doxorubicin was still effective, with 35% cured animals (mean survival 45 days), but a significant difference in survival time was observed between the two groups treated with the antineoplastic agent ($P < 0.05$; log rank test) (Fig. 5).

4. Discussion

In vivo and in vitro treatment of tumor cells with different antineoplastic drugs often results in the occurrence of resistance. The most important mechanism of resistance is linked to overexpression of the products of *MDR* and *MRP* genes, the 170 kDa P-glycoprotein, and the 190 kDa MRPs. A number of

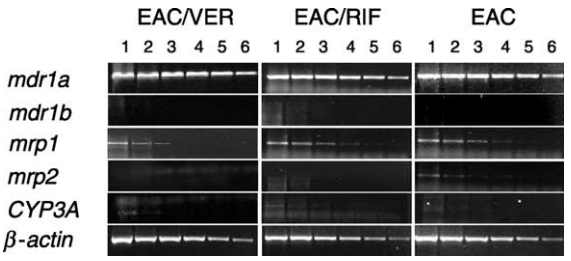

Fig. 3. RT-PCR analysis of *mdr1a, mdr1b, mrp1, mrp2, CYP3A* and *β-actin* expression in EAC cells and in cells obtained from animals treated for 10 days with verapamil (EAC/VER) or rifampicin (EAC/RIF). Representative electrophoretic runs of PCR products, obtained by amplification of serially diluted aliquots of cDNA (1 = 1, 2 = 1 : 2, 3 = 1 : 4, 4 = 1 : 8, 5 = 1 : 16, 6 = 1 : 32), are depicted.

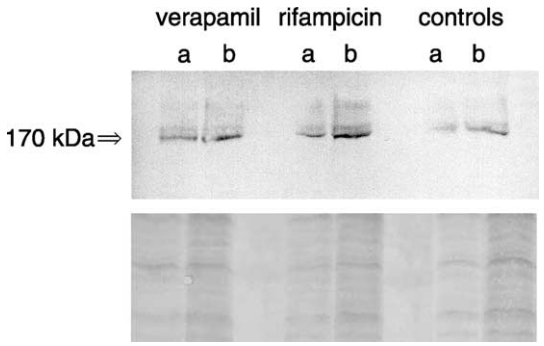

Fig. 4. Western blot analysis of P-glycoprotein expression in EAC cells. A representative immunoblot is reported. Proteins extracted from EAC cells or from cells obtained from animals in vivo treated with rifampicin (EAC/RIF) or verapamil (EAC/VER) were loaded on each lane (a: 25 μg, b: 50 μg). C219 antibody was incubated as described in the text. Molecular weight is expressed in kDa.

chemosensitizers have been developed to overcome multidrug resistance mediated by P-glycoprotein as well as by MRP. It has been however reported that many inhibitors not only inhibit the multidrug resistance phenotype, but can also increase *MDR* and *MRP* gene expression [7,8,19–22].

Among the chemosensitizers for P-glycoprotein and MRP are many commonly employed drugs that have their own pharmacological effects. These drugs are often administered to cancer patients for reasons different from multidrug resistance reversion. For example, verapamil is used to treat cardiovascular diseases and rifampicin is a first line agent in the chemotherapy of tuberculosis. These pathologies are quite frequent in cancer patiens, that are often aged and frequently immunodepressed due to the disease or to the antineoplastic therapy. Moreover, these substances can be used even before the neoplastic disease becomes evident; indeed, the mass of cells considered to be clinically detectable is around 1 billion cells, and many common tumors, such as breast, ovarian and colon carcinomas, express P-glycoprotein at diagnosis. A chronic treatment with P-glycoprotein or MRP substrates could therefore increase the expression levels of these proteins, thereby conferring resistance to cancer cells in these situations, and promoting therapeutic failure [23].

In this study we used an in vivo approach to test the hypothesis that transport proteins could be induced in tumor cells by two commonly used non antineoplastic drugs. Animals bearing EAC cells were treated in vivo with a chronic protocol with rifampicin or verapamil. The Ehrlich ascites carcinoma was chosen as it is a slowly growing tumor and hence it was possible to treat animals for 10 days with rifampicin or verapamil before the appearance of life threatening symptoms.

Many studies have been performed to evaluate the induction of transport proteins, but most deal with the evaluation of this phenomenon in tumor cells in vitro. In vivo studies have been conducted only in normal tissues, and in particular in the bowel, and, to our knowledge, no data exist on the induction of tumor cells in vivo by non antineoplastic agents.

Data obtained in different studies are often contrasting. In particular, Herzog et al. [8] have shown that verapamil and other P-glycoprotein inhibitors increased both the levels of *MDR1* mRNA and of its product P-glycoprotein in a human colon carcinoma cell line. Rifampicin is able to induce P-glycoprotein expression in the human intestine [12], in the human colon carcinoma cell line LS180 [9] and in rat liver [24], but not in human lymphocytes [25] or in primary cultures of human hepatocytes [26]. Rifampicin and other MRP inhibitors probenecid, ofloxacin and erythromycin increase the MRP levels to various degrees in AML2/WT and HL-60/WT cells [27]. The antibiotic induces MRP2 but not MRP1 expression in human duodenum [11]

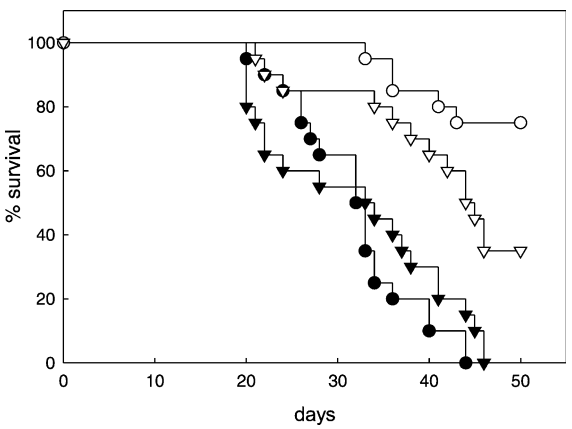

Fig. 5. Percent survival of CD1 mice i.p. transplanted with 5×10^6 EAC (●, ○) or EAC/RIF cells (▼, ▽). Doxorubicin (0.125 mg/kg) was i.p. injected on day 1 (○, ▽).

and monkey liver [13] and MRP2, MRP3 and MRP5 expression in human HepG2 cells in culture [7,28].

In rodents, P-glycoprotein is encoded by *mdr1a* and *mdr1b* genes; *mdr1a* transcript was not detected in our study, and *mdr1b* showed no increase in treated cells. On the contrary, an increased P-glycoprotein expression was evident in Western blot performed in cells obtained from animals orally treated for 10 days with rifampicin or verapamil. The analysis showed the presence of several bands; it should indeed be noted that the C219 monoclonal antibody we employed recognizes an internal, highly conserved aminoacid sequence of P-glycoprotein, but crossreacts with other proteins [29,30]. However, this is the only commercially available monoclonal antibody to P-glycoprotein that is not species specific and can therefore be used also with rodent cells. Therefore, only the band corresponding to 170 kDa was used to compare protein levels. The effect was relatively small: MDR1, such as CYP3A4, contains a PXR responsive element, and PXR is expressed in a tissue and species selective pattern. Interspecies variability in the inducing properties of rifampicin have indeed been reported for cytochrome P450 enzymes, and mice, rabbits and humans are more sensitive than rats and guinea pig [31].

Similar results were obtained by Nielsen et al. [32] in Ehrlich ascites cells an by Hill et al. [33] in human tumor cells. These Authors showed an increased expression of P-glycoprotein with no increase in *mdr1b* mRNA after fractionated irradiation, and ascribed this phenomenon to an increased half life of the protein. It is often believed that transcriptional activation is the main mechanism of rifampicin induction. However, Westphal et al. [34] also observed a reduced P-glycoprotein message, with an increased protein level, and suggested a post translational effect of rifampicin.

The functionality of P-glycoprotein was analyzed evaluating the intracellular accumulation of doxorubicin, a well known P-glycoprotein substrate. In treated cells, a reduced uptake was apparent, and this response could be reversed by the chemosensitizing agent verapamil, confirming the role of P-glycoprotein induction.

Finally, and most important, a reduced antitumor activity of doxorubicin was evident in mice i.p. implanted with EAC/RIF cells, obtained from animals treated for 10 days with rifampicin 60 mg/kg. Doxorubicin was administered one day after transplantation of tumor cells, indeed the induction of transport proteins will dissipate after a relative short time when the inducing pressure is removed, but should still be present after one day. Clonal selection by rifampicin or verapamil treatment is on the contrary extremely unlikely, as the two drugs are not cytotoxic and the number and viability of harvested tumor cells was the same in control and treated animals.

A possible cause of the reduced antitumor efficacy of doxorubicin in vivo could be ascribed to an increased metabolism of the drug by P450 enzymes. We found that CYP3A mRNA was not detectable at baseline in EAC, nor the expression could be increased in these cells by rifampicin or verapamil. In addition it should be noted that animals employed for studying the antitumor effect of doxorubicin were not treated with rifampicin or verapamil, hence the induction of hepatic cytochromes could be ruled out.

In conclusion, our study shows for the first time that an in vivo treatment with two commonly employed drugs, rifampicin and verapamil, in a model tumor system, the EAC cells, confers resistance to the anticancer drug doxorubicin, probably through induction of P-glycoprotein. Further studies are needed to verify if this warrying phenomenon occurs with other drugs and other tumors in vivo.

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